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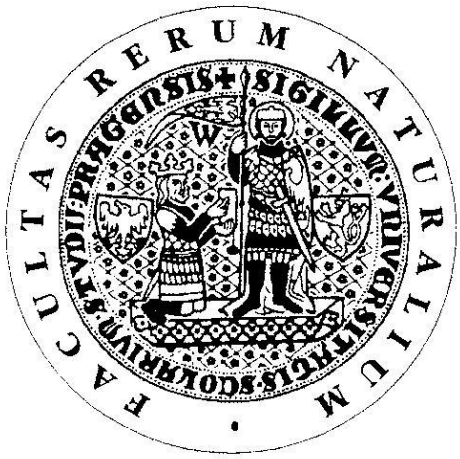

Mgr. Petr Žáček

USE OF THE MODERN SEPARATION TECHNIQUES FOR THE ANALYSIS OF INSECT PHEROMONES

Využití moderních separačních technik pro analýzu hmyzích feromonů

Ph.D. Thesis

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Declaration

I hereby declare that I have worked out this Thesis independently and that it has not been submitted elsewhere with intention to obtain any other academic degree.

I am aware that any use of the results obtained in this work, beyond the Charles University in Prague and the Institute of Organic Chemistry and Biochemistry AS CR, is possible only with a written consent of these institutions.

In Prague, 10/01/2014

…….

Signature

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I would never have been able to finish my Thesis without a number of people who helped me with my work or encouraged me in difficult times during the Ph.D. study:

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Abstrakt (ČJ)

Komunikace zprostředkovaná chemickými sloučeninami (semiochemikáliemi) je hlavní způsob dorozumívání se u hmyzu, zvláště pak hmyzu společenského. Metody plynové chromatografie v jednorozměrném, dvourozměrném komprehensivním a preparativním uspořádání spojené s hmotnostním a/nebo elektroantenografickým detektorem byly použity ke studiu těkavých nebo středně těkavých semiochemikálií u různých druhů hmyzu.

V rámci této práce byla zkoumána biosyntéza pohlavního feromonu u samců čmeláků druhů *Bombus terrestris*, *B. lucorum* a *B. lapidarius* pomocí aplikace předpokládaných biosyntetických prekurzorů feromonů (octan sodný, vyšší mastné kyseliny) izotopicky značených atomy ^{2}H a ^{14}C. Pro tento účel bylo zkoumáno chromatografické chování a možnosti detekce izotopicky značených sloučenin v jednorozměrném a dvourozměrném komprehensivním systému (^{2}H, ^{13}C). U všech testovaných kolon byl pro sloučeniny značené oběma izotopy potvrzen "inverzní izotopický efekt".

Vyvinuté analytické postupy byly aplikovány na reálné vzorky čmeláků. Analýza extraktů tkání čmeláků druhů *B. terrestris* a *B. lucorum* po *in vitro* inkubaci se značeným octanem sodným dokázala přítomnost značených metabolitů a potvrdila tak možnost *de novo* biosyntézy feromonu přímo v labiální žláze. Značené metabolity byly nalezeny i v případě *in vivo* inkubace s deuterovanými mastnými kyselinami u samců druhu *B. lucorum* a *lapidarius*. Tento experiment podpořil hypotézu, že vyšší mastné kyseliny, uložené ve formě triacylglycerolů v tukovém tělese v abdomenu, mohou být prekurzory pro biosyntézu alifatických sloučeniny pohlavního feromonu.

Dále byla zkoumána produkce feromonu v labiální žláze samců u druhů *B. terrestris* a *lucorum* během jejich života. Mezi oběma druhy byly nalezeny výrazné rozdíly. U samců čmeláků druhu *B. terrestris* je největší množství feromonu mezi pátým a desátým dnem života. V případě samců druhu *B. lucorum* je maximálního množství dosaženo pátý den a pak zůstává stejné až do konce života. Tyto výsledky, získané pomocí plynové chromatografie, byly podpořeny studiem ultrastruktury labiálních žláz.

Byla objevena a charakterizována nová exokrinní žláza u čmeláků druhu *B. terrestris*. Nachází se v nohou jedinců všech tří kast – královny, dělnic a samců. Chemické složení její sekrece bylo analyzováno metodou dvourozměrné plynové chromatografie s hmotnostní detekcí. Bylo zjištěno, že se skládá převážně z lineárních uhlovodíků a voskových esterů s alifatickou nebo terpenickou alkoholovou částí. Byly nalezeny rozdíly v chemickém složení této sekrece mezi jednotlivými kastami. V případě samců byl rovněž studován vývoj ve složení sekrece během života. Behaviorální experimenty nepotvrdily roli chemických sloučenin obsažených ve žláze při sexuálním chování, takže její biologická funkce je zatím nejasná.

Byly analyzovány těkavé složky obranné sekrece evropského druhu ploštice *Graphosoma lineatum* kombinací metod mikroextrakce na pevné fázi (SPME) s dvourozměrnou plynovou chromatografií s hmotnostní detekcí a preparativní plynové chromatografie. Bylo identifikováno 57 látek, z nichž 39 bylo u tohoto druhu nalezeno nově.

Abstract (EN)

Communication mediated by chemical compounds (semiochemicals) is the most important way of information transfer in insects, especially in the social species. Gas chromatographic methods (one-dimensional, two-dimensional comprehensive, and preparative arrangement) coupled with mass spectrometric and/or electroantennographic detector were used for volatile or semivolatile semiochemicals analysis in various insect species.

In this Thesis, biosynthesis of the bumblebee male sex pheromone in species *Bombus terrestris*, *B. lucorum,* and *B. lapidarius* was studied using putative biosynthetic precursors (sodium acetate, fatty acids) labeled with ^{2}H and ^{14}C. For the purpose of labeled volatile metabolites analysis, a method of separation and detection of isotopically labeled compounds in two-dimensional comprehensive gas chromatography was studied (^{2}H, ^{13}C). An “inverse isotope effects” was confirmed for compounds labeled with both isotopes in all examined types of columns. Concerning the biological samples, analysis of *in vitro* incubated tissues with labeled sodium acetate showed that pheromone components are synthesized *de novo* in the labial gland in *B. terrestris* and *B. lucorum*. Nevertheless, experiments of *in vivo* incubation of deuterated fatty acids confirmed biotransformation of the precursors into main pheromone components in *B. lucorum* and *B. lapidarius*. These results supported the hypothesis that fatty acids, stored in fat body as triacylglycerols, are precursors for the aliphatic pheromonal components biosynthesis.

Changes of pheromone production during life were studied in *B. terre*stris and *B. lucorum*. Significant differences were found between these two species. Pheromone production reaches a maximum between the day 5 and 10 of life in *B. terrestris* males and then rapidly decreases. In the case of *B. lucorum,* the maximum is achieved on the day 3 and stays constant over the rest of life. The gas chromatographic results were confirmed by an ultrastructural study of the pheromone gland tissue.

A new exocrine gland located in legs of bumblebee males, queens, and workers was discovered in *B. terrestris* species. Components of the glandular secretion were analyzed by means of comprehensive two-dimensional gas chromatography coupled with mass detection. The secretion consists mostly of linear hydrocarbons and wax esters with aliphatic or terpenic alcohols. Differences in the secretion composition among castes were found. Furthermore, dynamics of the secretion production was studied in males. Its function in mating behavior was suggested, but this has not been confirmed by behavioral experiments. Thus, the biological function remains unclear.

Volatile components of a defensive secretion of the European stink bug *Graphosoma lineatum* were studied. A method combining the headspace solid-phase microextraction (SPME) sampling procedure and comprehensive two-dimensional gas chromatography coupled with mass detector as well as preparative gas chromatography was used

for chemical analyses. There were 57 compounds identified, 39 of them have not been described in the *G. lineatum* secretion before.

Key words

Two-dimensional comprehensive gas chromatography
Gas chromatography
Pheromone biosynthesis
Labial gland
Leg tendon gland
Defense secretion
Bombus terrestris
Bombus lapidarius
Bombus lucorum
Graphosoma lineatum

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1 List of abbreviations

APCI……	Atmospheric pressure chemical ionization
API……..	Atmospheric pressure ionization
APPI……	Atmospheric pressure photo ionization
CAR……	Carboxen
CD……	Circular dichroism
CI…….	Chemical ionization
CIS……..	Cooled injection system
d_f……..	Stationary phase film thickness
DHB…….	Dihydroxybenzoic acid
DHF…….	(*S*)-2,3-dihydro-6-*trans*-farnesol
DMDS…….	Dimethyl disulfide
DVB…….....	Divinylbenzene
EAD…….	Electroantennographic detector
EAG…….	Electroantennography
ECD…….	Electron capture detector
EI…….	Electron ionization
ESI……...	Electrospray ionization
FAS…….	Fatty acid synthase
FB……	Fat body
FFA…….....	Free fatty acid
FID…….……..	Flame ionization detector
FPD…….……	Flame photometric detector
FTIR…….	Fourier transform infrared spectrometer
GC……...	Gas chromatography
GC-MS…….……...	Gas chromatography coupled with mass spectrometry
GC×GC…….……..	Comprehensive two-dimensional gas chromatography
GC×GC-MS…….……....	Comprehensive two-dimensional gas chromatography coupled with mass detection
GLC…….……	Gas-liquid chromatography
GSC…….	Gas-solid chromatography
HPLC……..	High performance liquid chromatography
ID…….	Internal diameter
IIE……	Inverse isotopic effect
IR…….	Infrared spectrometry
KBr.....	Potassium bromide
LC……...	Liquid chromatography

LG……	Labial gland
LMCS……..	Longitudinal modulating cryogenic system
MALDI……	Matrix assisted laser desorption ionization
MCP……..……...	Micro-channel plate
mRNA…….……	Messenger ribonucleic acid
MS……...	Mass spectrometer
MSD……	Mass selective detector
NPD……..	Nitrogen-phosphorous detector
ORD……	Optical rotary dispersion
PDMS……..	Polydimethylsiloxane
PFC…….	Preparative fraction collector
PTFE……..……..	Polytetrafluoroethylene
PTV…….	Programmable temperature ionization
SIM……..	Single ion monitoring
SPME……..	Solid phase micro-extraction
SRC…….	Single cell recording
TCD…….....	Thermal conductivity detector
TIC……..……..	Total ion current
TLC……..	Thin layer chromatography
ToF……..…….	Time of flight
UV……	Ultraviolet
vdW…….	van der Waals

2 Introduction

One of the fundamental features of all living creatures is their ability to exchange information between individuals – to communicate. As we, the humans, communicate through speech, sign and body language, or written language, other species have their own ways. Insects are considered one of the most successful classes of animals on the Earth due to their ability to adapt to various types of environmental conditions. The number of existing insects species can be estimated between eight to ten millions [1], which represents over 90 % of all animal forms existing on our planet [2]. It is clear that their evolutional success would not have been possible without using an efficient system of information transfer. Insects have evolved complex and sophisticated systems of chemical communication that became more important than other cues. If we want to understand their life and draw inspiration from it, we have to learn their chemical language.

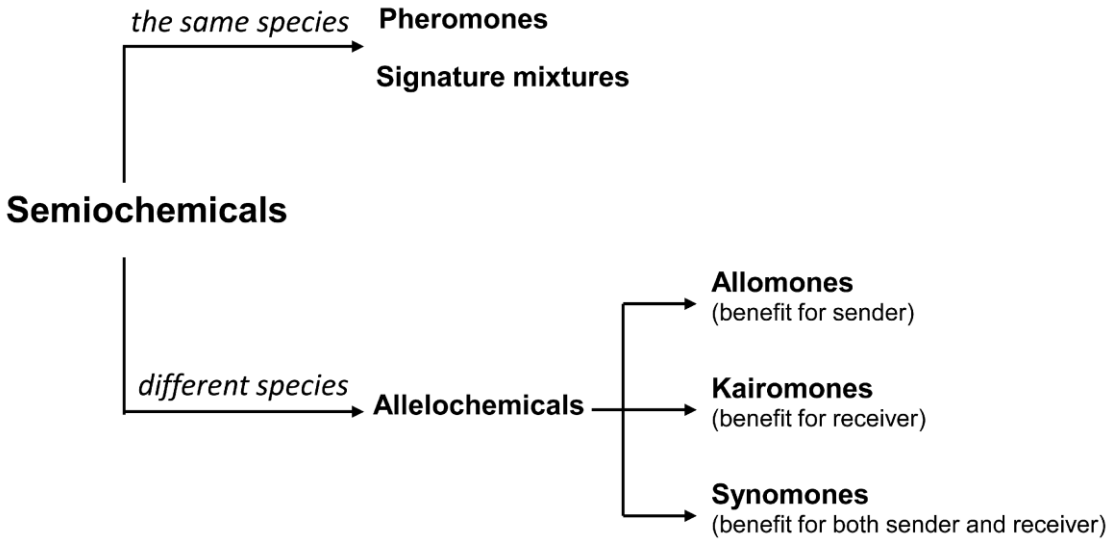
Study of the structure, function, origin, and significance of naturally occurring compounds that mediate inter- and intraspecific interactions between organisms is the main objective of the scientific discipline of chemical ecology. From the year 1959, when the first insect communication compound was identified [3] until now, an enormous expansion of the knowledge about nature was achieved. All of this would not be possible without extensive development of methods and instrumentations in analytical chemistry and other experimental techniques. In the case of the research of chemical communication in insects, invention and further development of gas chromatography (GC) was essential.

This Thesis deals with development and application of gas chromatographic methods in their classical one-dimensional arrangement as well as preparative and comprehensive two-dimensional setups in the research of chemical communication in bumblebees and stink-bugs.

3 Chemical communication

Communication can be defined as any behavior of one organism that affects the current or future behavior of another organism. Insects, similarly to other animals, use five senses to acquire information from their environment. Contact senses are taste and touch - the information exchange can occur only when the individuals are touching one another. On the other hand olfaction, vision and hearing are remote senses - the information is propagated over a considerable distance.

In the case of the olfactory sense the information transfer is mediated by volatile or semi-volatile compounds often called semiochemicals or infochemicals **(Scheme 1)**.



Scheme 1: Classification of semiochemicals.

Semiochemicals can be classified into two main groups based on who is the “sender” and who is the “receiver” of the information. Chemical signals that mediate information within the same species are called pheromones. The pheromones can be further divided into few groups according to their biological functions: **sex pheromones** – attracting of conspecific sexual partner for mating and stimulation of sexual behavior; **trail-following pheromones** – marking of the way to the food sources; **alarm pheromones** – warning of other colony members; **marking (territorial) pheromones** – marking of the territory or food source; **aggregation pheromone** – calling of other colony members (for example to break the host defense); **primer pheromone** – changing of developmental events. Recently, Wyatt proposed a new insight into the functional mechanism of chemical compounds in intraspecific communication by defining the term **signature mixtures** [4]. In contrast to the pheromones, that are species-wide signals which elicit innate responses, the signature mixtures are variable subsets of molecules of an animal’s chemical profile which are learnt by other

animals, allowing them to distinguish particular individuals or colonies. However, more results are needed to confirm if the proposed concept of the signature mixtures really applies in nature.

Chemicals that carry information between individuals of different species are called allelochemicals. They can be divided into three subgroups depending on who has the advantage from the signalization. Allomones benefit the sender (e.g repellent compounds that deter predators). Kairomones benefit the receiver (e.g. odor compounds are detected by a parasite and used to find its host; predator stalks prey). Synomones benefit both sender and receiver (e.g. plant volatiles that attracts insect pollinators).

From the chemical point of view, semiochemicals involve compounds of various structural types (e.g. aliphatic saturated/unsaturated compounds, terpenes, macrocycles, aromatic compounds...). The communication compounds may further contain various types of functional groups (e.g. hydroxy-, carboxylic-, esteric-, etheric-, aldehydic- ...) [5].

4 Analytical methods of semiochemicals analysis

4.1 Volatile compounds sampling methods

To obtain a valuable and representative sample is a first step in the analytical process. It is a critical point especially in the case of biological and environmental samples, where complex matrices can be expected (blood, tissues, soil). It is desirable to use sample methods that maximize recovery while minimizing coextractants. The sampling methods should be simple, reproducible, sensitive to unstable compounds [6] and compatible with the analytical methods that follow. Therefore, it is necessary to have at least basic knowledge of features of the sampled material (polarity, volatility e.g.).

Compounds used by insect for communication purposes are usually volatile or semi-volatile. These are perceived by insects on short or long distances. Non-volatile compounds mediate information transfer only when touched by the insects perceiving organs (chemoreceptors). Polarity of the communication chemicals varies between non-polar hydrocarbons to polar organic acids [5].

4.1.1 Headspace techniques

Headspace techniques are appropriate for compounds which are volatile or semivolatile at ambient conditions. The technical arrangement may be either static or dynamic. The static method involves the gain of the atmosphere above a sampled material by e.g. gas-tight syringe. This method is practical for compounds whose amount and vapor pressure is sufficient for analysis in the sample aliquot. The dynamic arrangement means that volatiles are entrained by pre-cleaned air and pass through a sorbent where are accumulated **(Fig. 1-A)**. This method enables sampling of the volatile profile that represents true proportions of compounds in the headspace. Another advantage of this approach is the possibility of concentrating of the analytes which increases the sensitivity of the analysis. The disadvantage is a need of sophisticated apparatus. Furthermore, an inappropriately selected solvent may coelute with highly volatile analytes during the separation by GC. The most used sorbent materials are Porapak Q (copolymer of ethylvinyl and divinylbenzene), Tenax (polymer of 2,6-diphenyl-*p*-phenyloxide) and activated charcoal. It is also possible to trap the analytes by cooling the air stream using liquid nitrogen or solid carbon dioxide. However, moisture may condense and form an ice plug, which may prevent the air flow. It could also be difficult to avoid the water from the sample.

In 1992 a new technique called Solid Phase Micro-Extraction (SPME) was introduced by a Pawliszyn research group [7,8]. SPME is a sampling technique that involves trapping volatiles on adsorbent-coated fibers followed by thermal desorption of the compounds by insertion of the fiber directly into a GC injector **(Fig. 1-B).** It was originally developed for

environmental analysis of organic compounds in water. Later, it was revealed that the method is suitable also for headspace sampling. The operation procedure is simple. A sample (live animals, plants, aqueous solutions...) is held in a vial with a septum closure. The septum is penetrated by the needle cover and the adsorbent fiber is then extended by means of plunger into a headspace **(Fig. 1-B)**, or directly into the aqueous solution.

There is also a possibility to sample by touching the surface or the glandular duct of the animal directly with the fiber (contact SPME) [9]. A big advantage of this modification is the selective sampling of particular body-parts. However, the sampling procedure may cause a considerable contamination of the coating adsorbent by non-volatile compounds or may lead to the breakage of the fragile fiber **(Fig. 1-C)**.

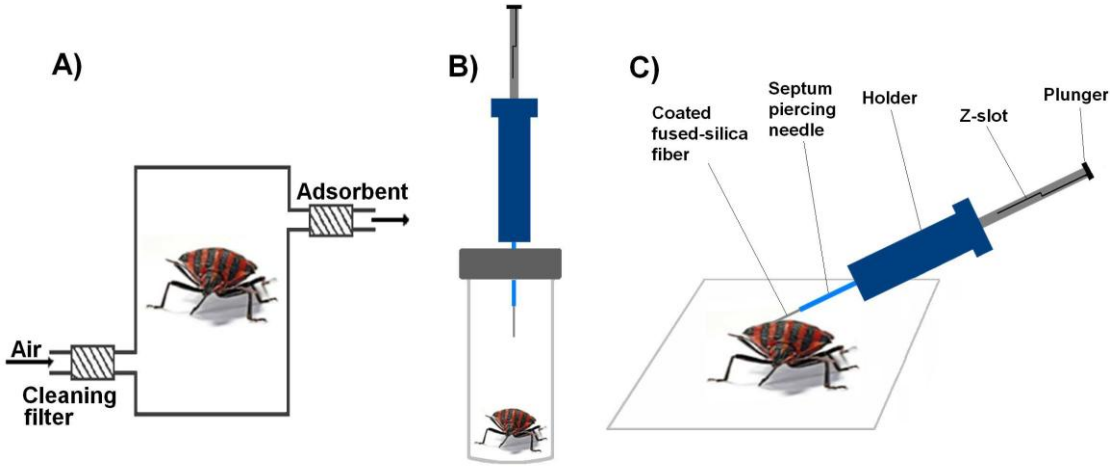


Fig. 1: A) Dynamic headspace sampling; B) Headspace SPME; C) Sampling of insect's surface by means of SPME fiber.

The SPME devices are available with interchangeable fibers coated with different thickness (7-100 μm) of several adsorbents (Carbowax/divinylbenzene, Carboxen™-polydimethylsiloxane, polydimethylsiloxane (PDMS), polyacrylate). The materials are similar to the GC stationary phases. The adsorbents differ in the selection of various classes of compounds, which enables efficient sampling of compounds of different polarities and volatilities.

To obtain reproducible and quantitative results the fiber must be exposed to the headspace volatiles long enough to reach equilibrium [10,11,12]. The adsorption is inversely dependent on the temperature. It is also very important to realize that the profile of adsorbed volatiles may not represent true proportions of compounds in the headspace, due to the adsorption material selectivity towards compounds of particular size or polarity. Therefore, a calibration is necessary. Despite its limitations, the SPME has several important advantages: it is simple, rapid, enables sampling of virtually any odor source and the samples are free of solvents. Furthermore, the SPME method is fully compatible with GC.

4.1.2 Tissue extractions by solvents

Extraction of materials by means of liquid solvents is a traditional technique, used for sampling of various types of compounds, not only volatiles. The major disadvantage of the method is its low selectivity resulting usually in a complex mixture of volatile and nonvolatile compounds. Therefore, numerous fractionation steps may be required for isolation of pure compounds. It often results in a substantial volume of solvent, which must be removed, and the subsequent concentration process may lead to the loss of volatiles or concentration of possible solvent impurities. Furthermore, the solvent may cover the highly volatile components, thus preventing their analysis. Nevertheless, a proper solvent selection may solve some of the problems. For example, by using nonpolar solvents (hexane, pentane) only nonpolar compounds are extracted selectively. An extract composition can be also influenced by the manner design. For instance a particular part of the insect body instead of the entire body can by extracted. Samples may be extracted by dipping, soaking, or homogenizing in solvent. The insects' tissues are mostly extracted fresh or frozen, because chemical changes may occur when the tissue dies or dries. The solvent must be chosen with respect to the analytical methods that follow. For example, water is not compatible with GC, but suitable for liquid chromatography (LC). Similarly to the SPME, the profile of volatiles obtained is usually not representative of the blend released by the intact living organisms.

4.1.3 Solid sample injection

The technique of solid sample injection is used in connection with GC. It is a solvent-free technique suitable when a small amount of material is available (e.g. insect's pheromone glands). The technique requires a modified GC injector **(Fig. 2)**.

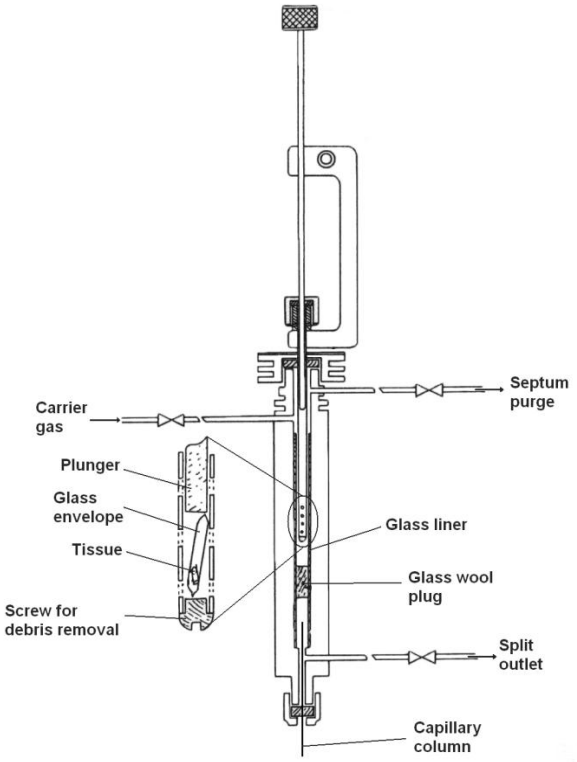


Fig. 2: GC injector for solid sample injection [13,14]

The sample is sealed in a capillary and placed in the cold-liner. The injection starts by crushing the capillary with a metal plunger and rapid heating of the injector. All volatiles are subsequently sampled on the separation column without a solvent. The main disadvantages are that only one analysis per sample is possible, and a contamination of the injector may complicate the next analysis [14].

4.1.4 Preliminary sample fractionation

Extracts are usually complex mixtures of chemicals and only a minor part have a biological relevance with respect to communication purposes. Despite the extract is introduced to some powerful separation techniques, there might be some components non-compatible with the analytical methodology (for example peptides or sugars are undesirable for GC). Therefore, sometimes it is necessary to pre-clean sample. There are few basic principles that can be used. Extracts can be fractionized based on molecular size (dialysis, size exclusion chromatography), volatility (distillation, steam distillation), polarity (solvent partitioning, acid-base properties, column chromatography), or reactivity (derivatization).

4.2 Instrumental analytical methods

4.2.1 Methods of gas chromatography

Most of the insect semiochemicals are volatile or semi-volatile compounds; therefore the most extended analytical method is GC. Today's instrumentation allows analysis of compounds up to forty-fifty carbons, which exhibit very low volatility [15]. Of course, the GC method can be used only if the low volatile analytes survive temperatures higher that 350 ^{o}C. However, there are plenty of derivatization techniques that may increase volatility or stability of analytes, which extends the range of measurable compounds (see below). An integral part of the GC is a detector. The most useful is mass detector allowing an identification of the unknown compounds according their mass spectra.

4.2.1.1 Detectors for gas chromatography

4.2.1.1.1 Mass spectrometric detectors (MSD)

Mass spectrometry (MS) is a destructive analytical method based on a simple idea: a compound or mixture of compounds is ionized in an ion source and broken into fragments. The ions are then analyzed in the mass analyzer on the basis of mass/charge ratio (*m/z*) and the relative abundance of each ion is recorded as a spectrum. The sensitivity of the detection depends on compounds and the instrumentation employed.

MS is usually used for two main purposes in the chemical ecology research. First, if the studied compounds have been characterized and their mass spectra are known, then the mass spectrometer becomes an analytical tool whose sensitivity and specificity are unsurpassed. In this case, the coupling of mass spectrometer to chromatographic instruments (gas and liquid chromatographs) as detectors is routine and commonplace.

The other use of MS is in structure elucidation of either completely unknown compounds or compounds whose structures are unknown but related to known compounds. In some cases, the structure can be determined outright according to the mass spectrum, but more often, the MS data complement information obtained by other spectrometric methods such as nuclear magnetic resonance (NMR) or infrared spectrometry (IR).

The field of mass spectrometry is covered by instrument types rather then by specific compounds types to be analyzed. The type of instrument is determined by ionization methods and ion-separation methods.

Ionization methods

The most widely used and historically first method for generating ions for mass spectrometry is ionization by means of electrons **(Fig. 3)**. Vapor phase sample molecules are bombarded

with high energy electrons (70 eV) that eject an electron from a sample molecule to produce a radical cation – a molecular ion. The energy of typical organic compounds is less than 15 eV, so the bombarding electrons impart the excess of energy to the newly created molecular ion. This energy is then dissipated in part by breaking of covalent bonds, whose bond strengths is between 3 and 10 eV. The bond breaking process is usually extensive and also highly reproducible and characteristic for a particular compound.

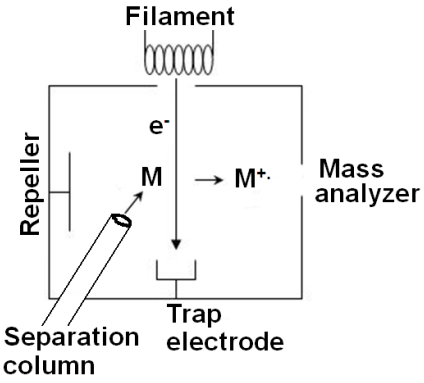


Fig. 3: Scheme of an electron ionization ion source
(based on http://en.wikipedia.org/wiki/File:Schematic_diagram_of_an_EI_ion_source.jpg).

Electron ionization often results in such extensive fragmentation that no molecular ion is observed (e.g. primary alcohols). On the contrary, a method of chemical ionization (CI) belongs to "soft" ionization techniques and the resulting mass spectrum exhibits very low fragmentation [16]. In CI, a reagent gas (methane, isobutane or ammonia) is introduced into the ion source an ionized. Sample molecules (M) collide with the reagent gas ions (CH_5^+, $C_4H_9^+$, etc.) and undergo secondary ionization, usually by proton transfer from the reagent gas ions, producing $[MH]^+$ ($[M+1]^+$) ions. Nevertheless, hydride abstraction (producing $M\text{-}H^+$ ions) and charge transfer are also common, so that a cluster of ions around the molecular ion rather than a single ion is observed. Furthermore, electrophilic addition of reagent gas ions to the parent compound usually produces ions at $[M+15]^+$ and/or $[M+29]^+$ for methane, $[M+43]^+$ for isobutane and $[M+18]^+$ for ammonia. The excess of energy transferred to the analyte molecules is much lower than in the EI (usually less than 5 eV). Therefore, $[M+1]^+$ ion is highly abundant and much less fragmentation than in EI takes place, which results in greater sensitivity because the total ion current (TIC) is concentrated into a few ions. For this reason the CI-MS is suitable for molecular mass determination. However, it is not useful for the structure elucidation due to the low fragmentation. Today's instrumentation allows the CI directly in the EI ion sources **(Fig. 3)**.

Mass analyzers

Quadrupole analyzer

A quadrupole analyzer is the most common mass analyzer used in the GC-MS instruments. A quadrupole is comprised of four rods, 10-20 cm long and hyperbolic in cross section, mounted in parallel to each other at the corners of a square **(Fig. 4-A)**.

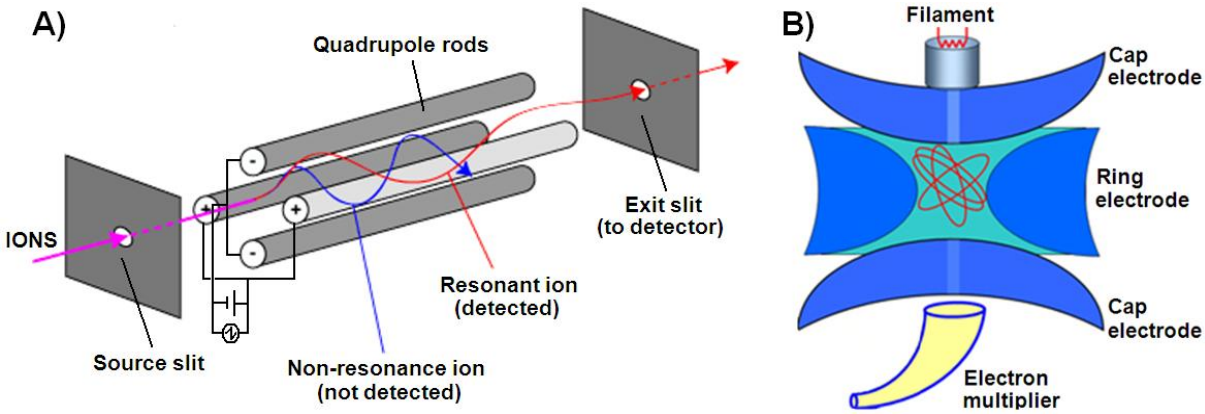


Fig. 4: A) Scheme of a quadrupole mass analyzer (http://www.bris.ac.uk/nerclsmsf/images/quadrupole.gif); B) Scheme of an ion trap mass analyzer (https://encrypted-tbn0.gstatic.com/images?q=tbn:ANd9GcTyzS3SC3P-ReUA32bggK4sdL4mCKhtbpyZMawSpQ3IDwVZ1IJA.)

A constant direct current (DC) voltage modified by a radio frequency voltage is applied to the rods. Ions are introduced at one end into the centre of the four rods and follow the spiral trajectory down to the middle of the axis **(Fig. 4-A)**. For any given combination of direct (DC) voltage and modifying voltage applied at the appropriate frequency, only the ions with a given *m/z* value have a stable trajectory and are able to spiral all the way down through the quadrupole and reach the detector. The other ions with different *m/z* have unstable or erratic paths and collide with one of the rods or pass outside the quadrupole. The quadrupole analyzer can be considered as a scanning mass filter. The analyzer can be operated in a single ion monitoring (SIM) mode, where only one ion is allowed to reach the detector. Therefore, the sensitivity can be considerably increased due to the increased selectivity of the target analyte, but the other structural information is lost. The nowadays instrumentation allows scanning of the whole mass range (e.g. *m/z* 30-700) in less than one second. The scanning rate in the SIM mode can be considerably higher. The current upper mass limit is around *m/z* 5000. Quadrupole analyzer operates more efficiently with ions of low velocity. Therefore, it is ideal for interfacing also to liquid chromatography systems for atmospheric pressure ionization (API) or electrospray ionization (EI) techniques (See High Performance Liquid Chromatography (HPLC)).

To increase the effectiveness of the analysis, it is possible to build an instrument where two or more mass analyzers are linked in series. This instrument is capable of selecting a single ion which is further fragmented and analyzed by next mass analyzer.

For example, three quadrupoles (tripple quad) can be linked to produce a tandem mass spectrometry. In this arrangement the first quadrupole selects a particular ion that continues to the second quadrupole, which serves as a collision cell where the selected ion is fragmented. The function of the third quadrupol is to analyze the fragments.

Ion trap analyzer

Ion trap can be considered as a variant of quadrupole because the physical principle and operation of the two are related **(Fig. 4-B)**. However, the ion trap can be more versatile and, unlike the quadrupole, it is able to "trap" ions for relatively long periods of time. The ion trap analyzer consists of three electrodes: one ring electrode with a hyperbolic inner surface and two hyperbolic endcap electrodes at either end **(Fig. 4-B)**. The ring electrode is operated with a sinusoidal radio frequency field while the endcap electrodes are operated in one of three modes: at ground potential or with either a DC or AC (alternating current) voltage. The mathematics describing the motion of ions in three-dimensional space is given by Mathieu equation [17].

There are three basic modes in which the ion trap can be operated. The first and the simplest is a sequential ejecting of the trapped ions to a detector, producing conventional spectrum comparable with databases. In the second mode, one or more ions can be selected and allowed to reach the detector, similarly to the SIM mode. The third mode of operation is similar to the second, with the application of an additional oscillatory field between the endcap electrodes, which results in adding kinetic energy selectively to a particular ion. With a small amplitude auxiliary field, the selected ions gain kinetic energy slowly, during which time they usually undergo a fragmentation collision (MS-MS experiment), similarly to the triple quad mass analyzers.

Time of flight mass analyzer (ToF)

The ToF analyzer determines ions mass by measuring their travel times through a field-free region [18,19]. After ionization in the ion source, the ions are orthogonally accelerated as discrete packages into a flight tube by applying a pulse generated by electrical field **(Fig.19)**. The flight time is proportional to the square root of the *m/z*. In mostly used instruments the ion's flight is in the order of microseconds. Together with a fast electronics, this type of mass analyzer allows fast scanning of the effluent. Therefore, it is suitable as a detector for fast GC or comprehensive two-dimensional GC (GC×GC)) [see Chapter 4.2.1.5: Two-dimensional comprehensive gas chromatography (GC×GC)].

Today's instruments allow mass determination with precision up to 25000 ppm, which is fully sufficient for high precision mass determination of molecular masses of small

molecules. These types of instruments need an additional ion focusing part such as ion reflector or ion mirror.

The ToF is widely used in connection with pulsed ionization techniques including plasma and laser desorption (e.g., Matrix-Assisted Laser Desorption Ionization (MALDI)).

Magnetic sector mass analyzer

The magnetic sector was the first commercially available type of mass spectrometric detector coupled with GC. The magnetic sector uses a magnetic field to deflect moving ions into a curved path. Ions are separated based on their *m/z* ratios – the lower mass ions are deflected more than higher mass ions with the same charge. The magnetic sector analyzer allows a very high mass resolution (<100000 ppm).

Ions detection

The charged particles leaving mass analyzer are subsequently detected in detector. For the purpose some types of electron multipliers such as micro-chanel plate (MCP) [20], Faraday cup [21] or ion-to-photon detector [22] are used.

4.2.1.1.2 Other detectors for gas chromatography

Flame ionization detector (FID)

The most extended non-selective detector for GC is FID. The detector consists of a hydrogen-air diffusion flame burning at the tip of a jet **(Fig. 5-A)**. When organic compounds, leaving the separation column, are introduced into the flame, electrically charged species are formed. Subsequently, these particles are collected at an electrode which leads to an increase of current that is proportional to the amount of carbon-hydrogen bonds in the flame. FID is a destructive detector exhibiting a high sensitivity to virtually all organic compounds and a wide linear range – about 10^7 **(Table 1)**.

Thermal conductivity detector (TCD)

The operation of the TCD detector is based on a measurement of the changes in the thermal conductivity of the carrier gas caused in the presence of eluted substances **(Fig. 5-B)**. TCD is a universal nondestructive detector, which is approximately three orders of magnitude less sensitive to hydrocarbons in comparison to FID. It is a useful detector for preparative applications or for inorganic gas detection where FID cannot be used.

Other detectors

A nitrogen-phosphorous detector (NPD), electron capture detector (ECD), and flame photometric detector (FPD) are selective to particular types of compounds. Therefore, they are used only for specific purposes [23].

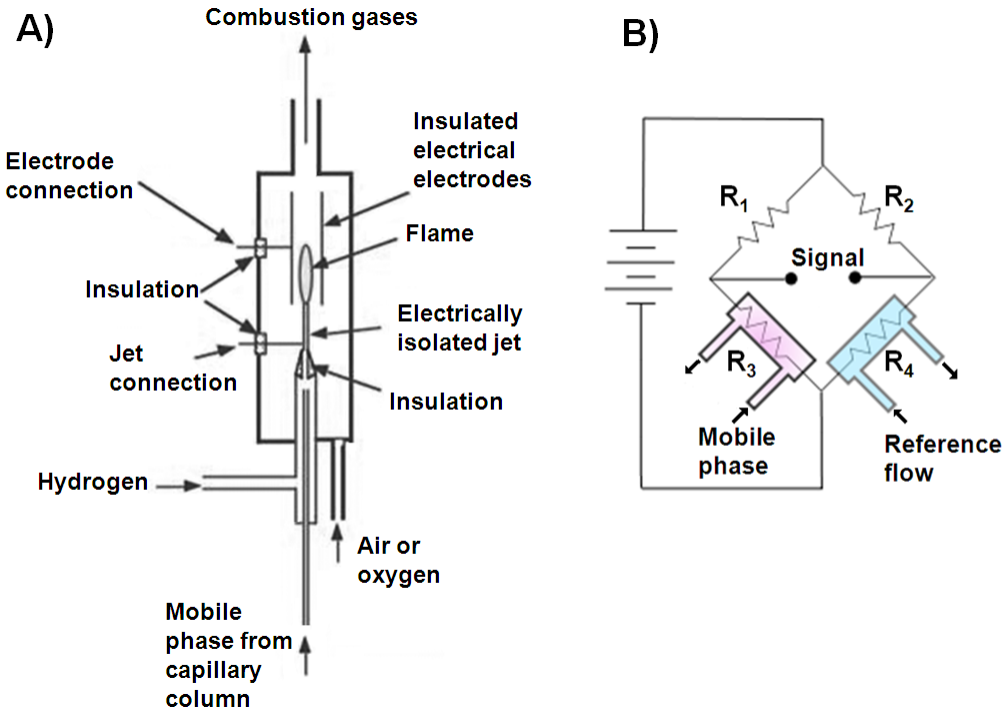


Fig. 5: A) Schematic diagram of FID (http://www.chromatography-online.org/Chrial-GC/images/image135.jpg); B) Scheme of a two-cells TCD detector. The reference cell R_4 compensates for the drift in the analytical cell R_3 due to flow temperature fluctuation (http://upload.wikimedia.org/wikipedia/commons/thumb/c/cb/Thermal_Conductivity_Detector_1.svg/340px-Thermal_Conductivity_Detector_1.svg.png).

Table 1: Basic characteristics of common GC detectors [24].

Detector name	Selectivity	Detector limit [g]	Linear dynamic range
Flame ionization (FID)	almost universal	$< 10^{-12}$	10^7
Thermal conductivity (TCD)	universal	10^{-7}-10^{-6}	10^6
Mass selective detector (MSD)	universal	10^{-12}-10^{-9a}	10^5
Nitrogen-phosphorous (NPD)	N,P, heteroatoms	$\sim 10^{-12}$	10^4
Electron capture (ECD)	halogens, nitrates	10^{-15}-10^{-12b}	10^4
Flame photometric (FPD)	P,S	$\sim 10^{-12}$	10^3 - 10^4

[a]Sensitivity in a full scan range is ~1 ng. With a SIM mode, this range is increased to the low picograms.

[b]Sensitivity depends on the number and type of halogen atoms present.

4.2.1.2 Preparative GC

Gas chromatography is essentially a separation technique that can be utilized for analytical purposes – identification and quantification of analytes. However, gas chromatography can also be used for isolation and purification of compounds. The use of GC for this purpose is referred to as preparative GC.

Preparation of small quantities of substances (tens or hundreds of micrograms) can readily be performed with analytic GC system by repetitive injections and collections. Nevertheless, if milligrams or grams of pure compounds are required it is necessary to modify some parts of the instrumentation.

Basically, the preparative gas chromatographic system consists of the same parts as used for analytical applications, but in the case of preparative GC, the effluent is usually splitted in two parts after leaving the separation column: smaller part leading to a detector, usually FID or TCD, and the larger part continues to a fraction collector where the isolated analytes condensate. Detector response and the collecting device are synchronized, similarly to the GC-EAD system [see chapter 4.2.1.3: Gas chromatography coupled with electroantennographic detector (GC-EAD)]. This ensures a proper heart-cutting of the trapped analyte from the chromatogram.

4.2.1.2.1 Separation columns

First attempts to use GC for isolation of volatile compounds, which are difficult to separate by means of distillation, were performed in the mid-fifties. Evans and Tatlow separated fluorocarbons in gram quantities using packed column of a large size - 4.8 meters long and 3 cm of an internal diameter [25]. However, its separation efficiency was poor. Later, Ambrose and Collerson considered using an automatic repetition of the analytical cycle rather than increasing sizes of chromatographic columns for large amounts of materials processing [26]. In the mid-eighties, fused silica capillary column with large internal diameters were introduced. Many applications previously performed on a packed column could have been done with megabore columns – capillary columns of 0.53 mm with a fairly thick film of stationary phase. These types of columns combine the attributes of fused silica columns (high separation efficiency) and packed columns (high loading capacity). Their loading capacity is approximately ten or twenty times higher in comparison to the widely used analytical column of the inner diameter 0.25 mm **(Table 2)**.

Despite the fact that the capillary columns practically replaced the packed column nowadays, they still have been used for the preparative purposes.

Table 2: Column capacity as a function of the inner diameter and the film thickness. Data were abstracted from J&W Scientific catalog 1994 – 1995.

Inner diameter [mm]	Film-thickness [μm]	Capacity[1] [ng / component]
0.25	0.15	60-70
	0.25	100-150
	0.50	200-250
	1.0	350-400
0.32	0.25	150-200
	0.5	250-300
	1.0	400-450
	3.0	1200-1500
0.53	1.0	1000-1200
	1.5	1400-1600
	3.0	3000-3500
	5.0	5000-6000

[1]…Capacity is defined as the amount of component where peak asymmetry occurs at 10% at half-height.

4.2.1.2.2 Preparative GC inlet

In principle it is desirable to maximize concentration of the analyte in the injected volume, or increase the volume of the sample introduced to the system. If concentration of the analyte is possible, then there is no need to use any special instrumental equipment or setting. However, highly volatile compounds can be lost during sample concentration; therefore it is necessary to increase the total injected volume in this case. Basically, there are two techniques allowing large volume injections.

During the on-column injection, the solvent is generally vaporized in a few meters of uncoated deactivated capillary (retention gap) and vented via an early vapor exit valve. The on-column large-volume injection systems consists of a GC equipped with an on-column injector, a retention gap connected with a retaining precolumn, a separation column, and a heated early solvent vapor exit located between the precolumn and the separation column. An injection rate has to be carefully optimized with respect to the length of the retention gap and the evaporation. The oven temperature during the injection should be set slightly below boiling point of the used solvent [27]. There should also be properly optimized closing of the vapor exit [28]. Early closing would result in exceeding of the capacity of the separation column by the solvent, while the delayed closing would result in loosing highly volatile components. This method is also suitable for thermolabile compounds [29]. Nevertheless,

frequent analysis of samples containing non-volatile parts of matrix contaminates the column inlet and decreases the column efficiency [30].

Vogt and co-workers (1979) described an injector that allowed the injection of up to 250 μl [31,32] – using Programmable Temperature Vaporization (PTV) inlet. The PTV inlet can be cooled or heated rapidly, and the split valve is large enough to be able to vent the solvent vapor produced during injection **(Fig. 6)**. The cooling is usually mediated by liquid nitrogen or carbon dioxide. The sample is injected at a controlled rate to the inlet where the temperature is reduced to bellow boiling point of the solvent, similarly to the on-column technique. During the sample introduction, the solvent is selectively eliminated by venting the vapor via split valve. When the solvent elimination is finished, less volatile components retained in the liner are introduced to the separation column by rapid heating of the inlet **(Fig. 7)**. In order to achieve a quantitative analysis, there are few parameters to be carefully optimized: sample injection speed, liner packing material (glass, quartz or PTFE wool, Tenax TA), solvent venting temperature, solvent venting time, and inlet pressure. Unfortunately, these parameters are not independent of each other; which makes the optimization more complicated [33,34].

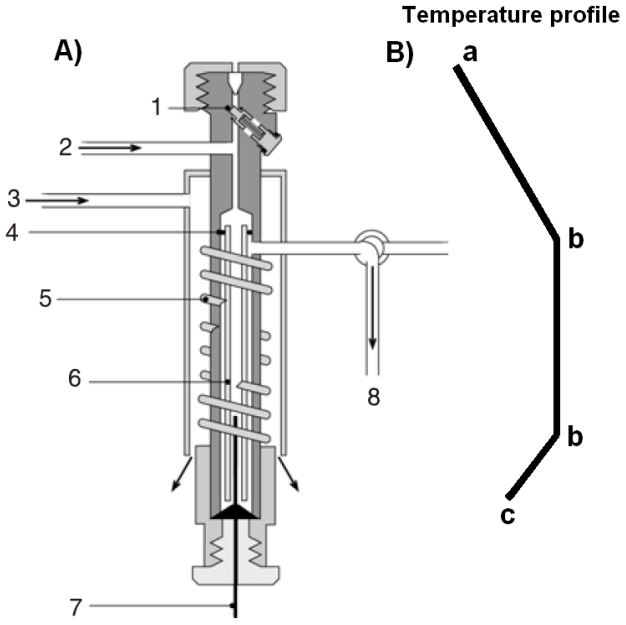


Fig. 6: A) PTV inlet (Cooled injection system CIS 4 (Gerstel, Mülheim, Germany): 1-septumless sampling head, 2-carrier gas line, 3-cooling line, 4-liner seal, 5-heating coil, 6-glass inlet liner, 7-capillary column, 8-split vent. B) Inlet temperature profile: a- <150^{o}C (depends on the setpoint, b-setpoint temperature, c-10% below the setpoint temperature.

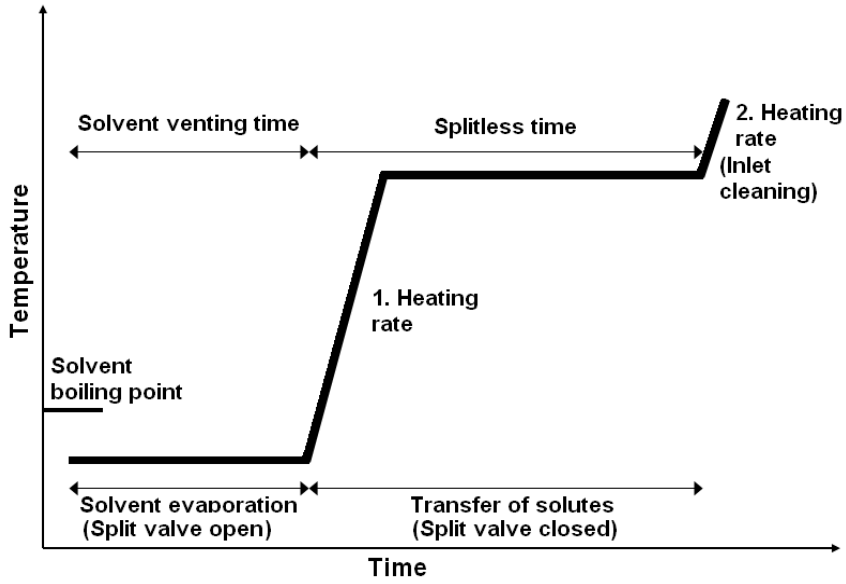


Fig. 7: Scheme of programmed-temperature vaporization [35].

4.2.1.2.3 Fraction collector

Fraction collector is a device allowing automatic collection and accumulation of compounds previously separated in the GC. The fraction collector used in this Thesis was constructed by Gerstel (Preparative Fraction collector PFC, Mülheim, Germany) **(Fig. 8-C)**. The effluent leaving the GC continues to the switching device via heated transferline **(Fig. 8-A)**, from which the effluent continues to the one of seventh trap capillaries **(Fig. 8-B)**. The trapping capillaries are made of glass and are located in the movable trap cooling vessel, which can be cooled (by means of liquid nitrogen) or heated. Analytes condense on the wall of the trapping capillary. The analytes can be washed out of the capillary with a small amount of a suitable solvent when the analysis is finished. The device allows collection up to six compounds or parts of the GC effluent. There is an additional trap available to collect all compounds not collected in the main traps that can be saved for further analysis or, for example, “subtraction studies”. The mobile phase then leaves the cooled capillary and continues to the solenoid valve. Each of the traps is controlled by its own on/off solenoid valve. Only one of the valves can be opened at a time, therefore the effluent cannot be directed to more than one trap at a time. Location of the controlling valves at the end of the effluent line prevents possible contamination of the valve mechanism by the effluent components. The whole collection device contains several junctions, which must be checked thoroughly for possible leaks before preparation.

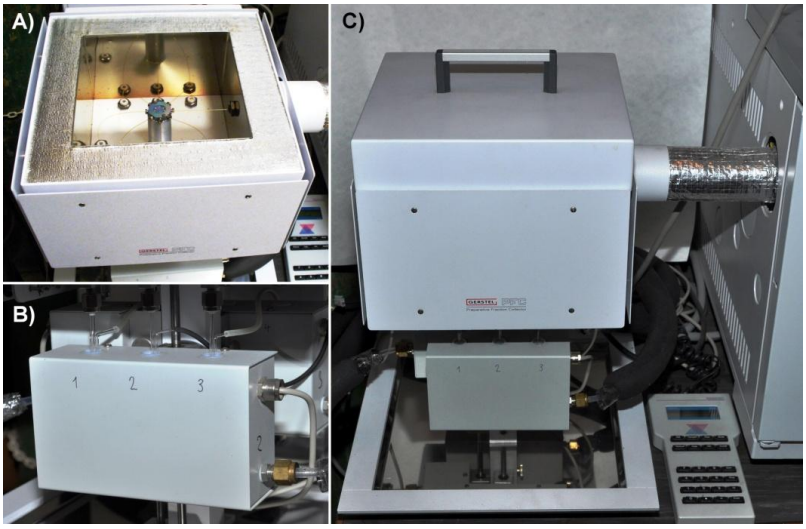


Fig. 8: Photo of the fraction collector (PFC, Gerstel, Mülheim, Germany); A) Detail of the preparative switching device; B) Detail of the trap cooling vessel; C) General view of the device.

4.2.1.3 Gas chromatography coupled with electroantennographic detector (GC-EAD)

Electroantennography (EAG) is a neurophysiological technique that records an insect's perception of a tested semiochemical. The methodology is based on the discovery published by Schneider in 1957 [36]. He measured voltage fluctuations when connected electrodes to tip and base of an insect antenna while stimulated with pheromones. The precise mechanism behind the signal of the EAG is not clear, but it was assumed that depolarization of many olfactory neurons across the antenna generates an electric potential difference in the electrodes. The signal can be amplified and recorded. The amplitude of an EAG response increases with increasing of a stimulating compounds concentration until a saturation level is reached. Despite the EAD recording is technically relatively easy and does not require highly sophisticated instrumentation, for a given species and sex, the signal depends on many not well defined factors [37]. Most insect species require a flexible attitude and a certain sense for improvisation of the operator.

The insect antenna is covered with a large number of sensilla **(Fig. 9)**. There are various types of the olfactory receptor (sensillum) on the antenna and each of them is assumed to be sensitive to a particular compound.

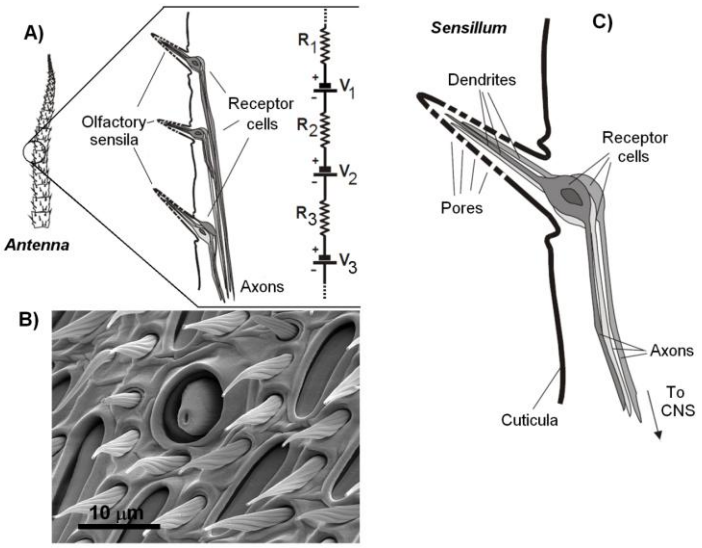


Fig. 9: A) Scheme of an insect antennae and the electrical equivalent of the receptor cells [37]; B) Electron micrograph of antenna surface detail of a wasp *Vespula vulgaris* (http://en.wikipedia.org/wiki/File:Vespula_vulgaris_SEM_Antenna_03.jpg); C) Scheme of an antennae sensilla [37].

From the electrical point of view, each sensillum can be considered to be a combination of a voltage source and resistor **(Fig. 9-A)**. Therefore, the whole antenna consisting of a number of sensilla forms an array of voltage sources and antennal resistance connected in serial. The total antennal voltage source is rather weak. The amplitude of the potential fluctuation may vary from microvolts to milivolts. The tip of the antennae usually becomes negative with respect to the base. The total resistance of the whole antennae can be in order of mega ohms and further increases when the antenna dries during a measurement [37]. The typical setting of the electroantennographic experiment consists of a pair of Ag/AgCl (argentchloride) electrodes filled with a saline solution. The electrode tips are connected via a conductive gel to the tip and to the base of the insect antenna, or head - if the antenna is too tiny. The electrodes are grounded via an aluminum foil wrapped around; if this is not sufficient enough, a Faraday cage is needed. Coaxial cables connect the electrodes to the amplifier and then to the recording device.

The technique can stand on itself or be coupled with a separation step. First connection of the EAG to a gas chromatograph was made in 1969 by Moorhouse [38]. He sampled the effluent leaving the column every fifteen seconds and then allowed the fraction to be tested on the electroantennograph. Finally, it was Arn who succeeded in direct coupling a capillary GC to an electroantennograph and introduced the term electroantennographic detection (EAD) [39].

In the most common experimental set-up, the effluent from the GC column is diluted with nitrogen (make-up gas) and divided in two, usually equal parts. One goes to the FID and the second continues via a heated transferline to the EAD. The effluent stream leading to the

EAD is further diluted by a humidified air, which prevents the antenna from quick drying **(Fig. 10)**. The signal from antennae is amplified and sent to the recording device. The signals from FID and EAD are synchronized; therefore it is possible to assign the EAD signal to the corresponding peak in the FID chromatogram.

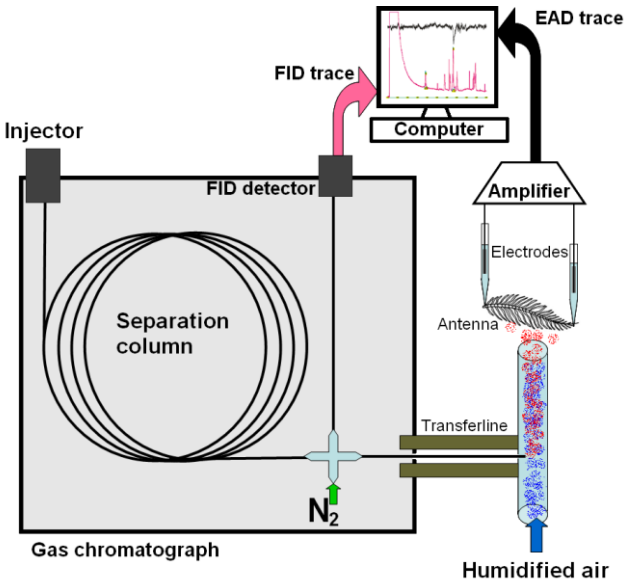


Fig. 10: Scheme of GC-EAD system.

The GC-EAD-FID system has been used in the pheromone research, because it allows determination of the biologically active compounds – the compounds of which the insect antenna is able to perceive **(Fig. 11)**. Nevertheless, this system is not able to indicate what form the activity takes (trail pheromone, sex attractant, prime pheromone or repellent etc.). Solving this task requires a behavioral bioassay.

The electroantennographic detector can be considered as a full chromatographic detector exhibiting approximately linear response with the logarithm of the amount of stimulant [36,39]. Important limiting factor of this detector is the life time of an excised antenna of most insect species that varies from few minutes to one or two hours.

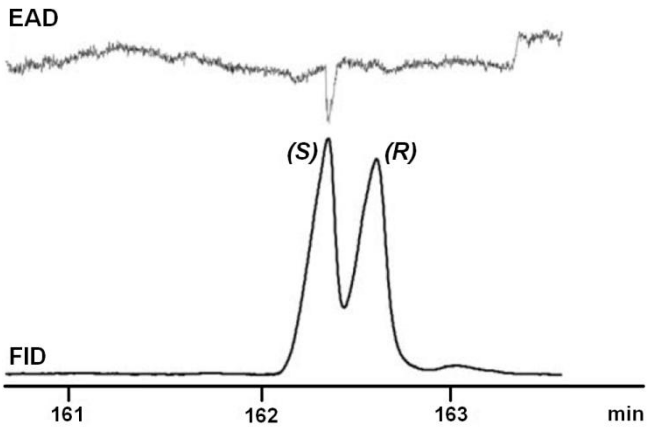


Fig. 11: An example of GC-EAD-FID response of *B. terrestris* queens the two enantiomers of 2,3-dihydrofarnesol [40].

All types of sensilla, perceiving various types of molecules, are spread evenly across the surface of the antennae. The registered EAD signal consists of contributions of many sensilla responses. A much more detailed understanding of the perception of odor molecules by insects can be obtained by recording the response of individual olfactory cells. When connected with GC, Single Cell Recording (GC-SCR) provides a highly specific method for determination of the neurophysiological activity to pheromone components [41]. The experimental design is very similar to that used for GC-EAD, except that recordings of the action potentials generated by the receptor cells associated with individual olfactory sensilla are done using tungsten microelectrodes [42]. The output of the examined cells is then measured by an oscilloscope and a loudspeaker.

4.2.1.4 GC coupled with Fourier transform infrared spectrometer (GC-FTIR)

The infrared absorption spectroscopy measures the bending, twisting, rocking, and vibrational motions of atoms in molecules. During the interaction with broad spectrum infrared radiation (between 10 and 12800 cm^{-1}), portions of the incident radiation are absorbed when the energy of the incident radiation coincides with the energy required to excite a particular vibrational mode. For the majority of the analytical applications of compound structure determination the spectral region lies between 670 and 4000 cm^{-1}. The multiplicity of simultaneous vibrations results in a complex absorption spectrum that is uniquely characteristic of both the functional groups in the molecule and the overall configuration of the atoms.

There are numerous of methods for sample preparations. For example, liquid samples can be applied between two places of sodium chloride or other salt transparent in infrared region (IR). Solid or liquid samples can be diluted, for example, in methylene chloride and analyzed in the cuvette made of salt (sodium chloride). Solid samples can be mixed with KBr powder and then compressed into pellets, or dispersed in hydrocarbon oil (Nujol).

In connection with the GC there are basically two techniques. In the first, the effluent passes through a heated flow cell, which consists of a glass tube coated internally with gold (“light pipe”). The vapor-phase spectra are obtained as the compounds travel through the cell [43]. The other techniques [44,45] are based on the cryo-condensation of the GC effluent. There are two main advantages of the cryo-technique: the analytes are focused and concentrated in a small space and, furthermore, can be repeatedly scanned even when the separation is finished, which results in a higher sensitivity than in the first method. Additional advantage of both trapping systems is that the used cold temperatures minimize temperature-related band-broadening effects, therefore the (IR) absorbance is sharper than in standard IR spectra. Nevertheless, the IR spectra in solid and gas phase may qualitatively

differ. For example, signals corresponding to the hydrogen bridges are visible during measurements in the solid phase.

4.2.1.5 Two-dimensional comprehensive gas chromatography (GC×GC)

GC×GC can be defined as one of the gas chromatographic techniques, where all mixture components injected to the gas chromatograph are separated by two columns with different selectivity. These columns are connected in serial and analytes are periodically transferred from the first to the second column by means of a modulator. Therefore, the separation power of each column is fully applied to each analyte independently.

First comprehensive two-dimensional separations were performed already in 1944 [46]. It was an analysis of amino acids on thin layer with cellulose stationary phase. Various pairs of solvents were tested. In the case of gas chromatography, fully comprehensive separation was theoretically suggested by Giddings in 1984 [47]. Nevertheless, first two-dimensional comprehensive analysis was performed in 1991 by Liu and Phillips who succeeded in constructing an on-column thermal modulator interface [48] – a device for transferring analytes between first and second separation column.

The next twenty years brought a plenty of new modulation techniques, fast and sensitive types of detectors and introduced computation technique capable of handling the fast data collection.

4.2.1.5.1 System scheme

System consists of an injector, primary (1st dimension) column, modulator, secondary (2nd dimension) column and detector. In most of the systems, each column is located in separated oven to enable more flexible temperature control **(Fig. 12)**.

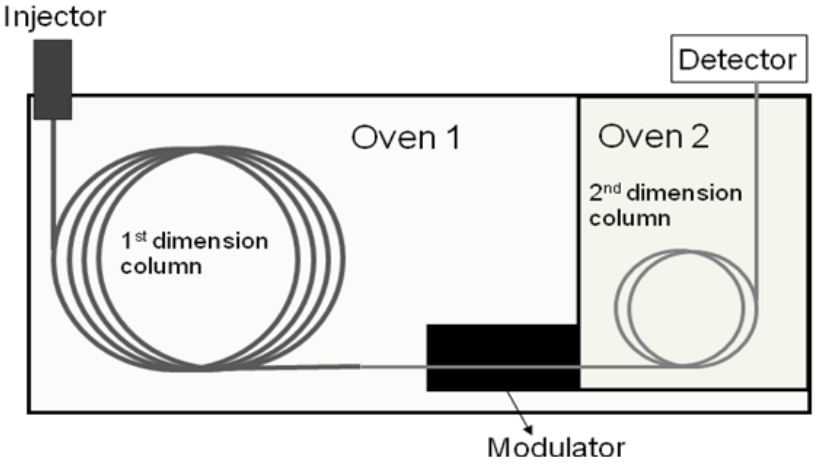


Fig. 12: The two-oven GC×GC system.

4.2.1.5.2 Modulation

The interface between the two GC columns, modulator, has a few main functions: it should accumulate and trap, refocus, and rapidly release fractions leaving the primary column to the

secondary column. In the case of one dimensional gas chromatography, the “heart” of the system was considered the separation column, while the modulator is the system “heart” in the comprehensive two-dimensional gas chromatography [49].

The first types of modulators were so-called dual-stage thermal desorption modulators, which used electrical heating, introduced by Liu and Phillips [48]. Nevertheless, these types of modulators are no longer used because of their fragility and manufacturing problems. They were replaced by modulators based on a moving heater technique [50], but also this modulation technique is no longer commercially available. Modulators based on cooling of the effluent leaving the primary column are used almost exclusively nowadays. The first cryo-modulator was introduced by Marriott and co-workers [51]. They constructed longitudinal modulating cryogenic system (LMCS), which uses expanding liquid CO_2 for trapping and focusing of the analytes at the beginning of the secondary column. After a certain adjustable time period, the trap (moved by a motor-piston) is quickly moved to an upstream position and exposes the focused zone of compounds to the GC oven air temperature. The analytes, condensed in the narrow zone, are rapidly re-volatilized and re-injected to the secondary column.

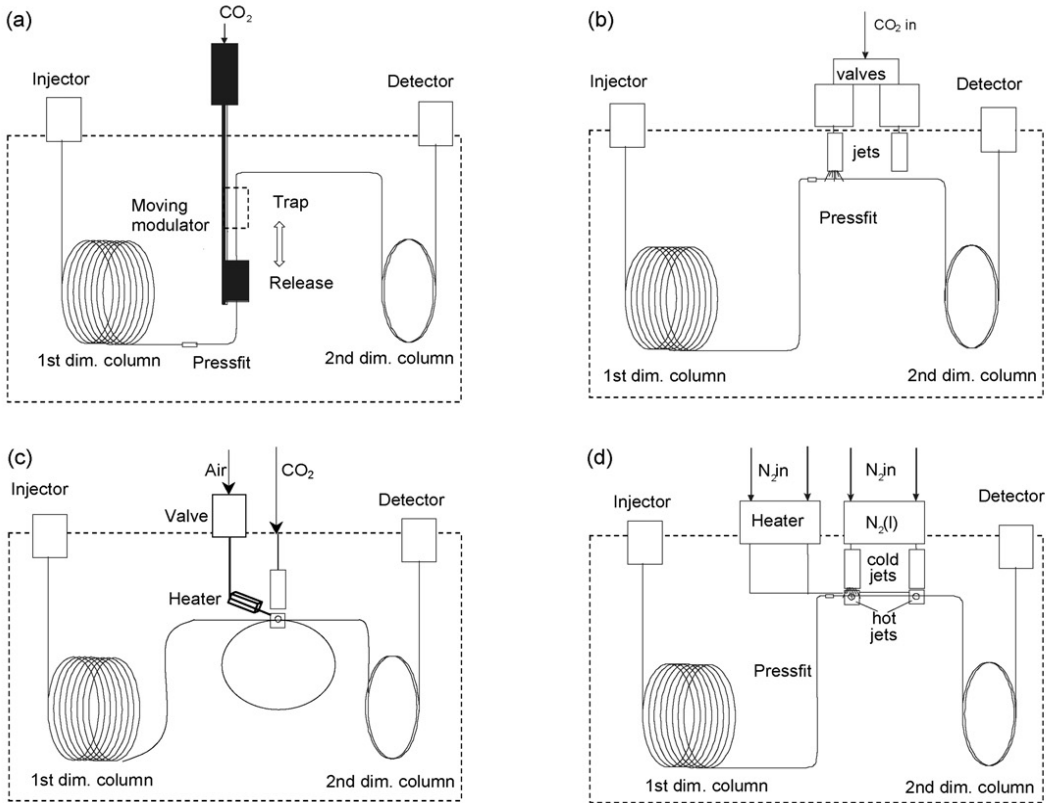


Fig. 13: Schemes of four types of modulators: a) LMCS; b) dual-jet CO_2 modulator; c) dual-jet N_2 and d) four-jet N_2 modulators [52].

Recently, a several types of jet-based modulator have begun to dominate the field **(Fig. 13 b-d)**. Their main advantage is absence of moving parts. As a cooling medium can be

used liquid nitrogen or carbon dioxide. For most of the applications modulators with liquid carbon dioxide for cooling is fully satisfactory, nevertheless, for the very volatile compounds (boiling points lower than decane) focusing liquid nitrogen as a cooler is necessary [53].

The modulation in the GC×GC system (Pegasus III, Leco Corporation) used in the Thesis is accomplished via a dual-stage, quad-jet thermal modulator using a liquid nitrogen as a cooler. **(Fig. 14)**. The effluent leaving the primary column is introduced into the secondary column by means of the time synchronized system of hot and cold pulses between both parts (stages 1 and 2) of the modulator. The control software (ChromaTOF 4.3) allows setting the duration of the hot pulse and the modulation period. The cold pulse duration is calculated from the set data automatically.

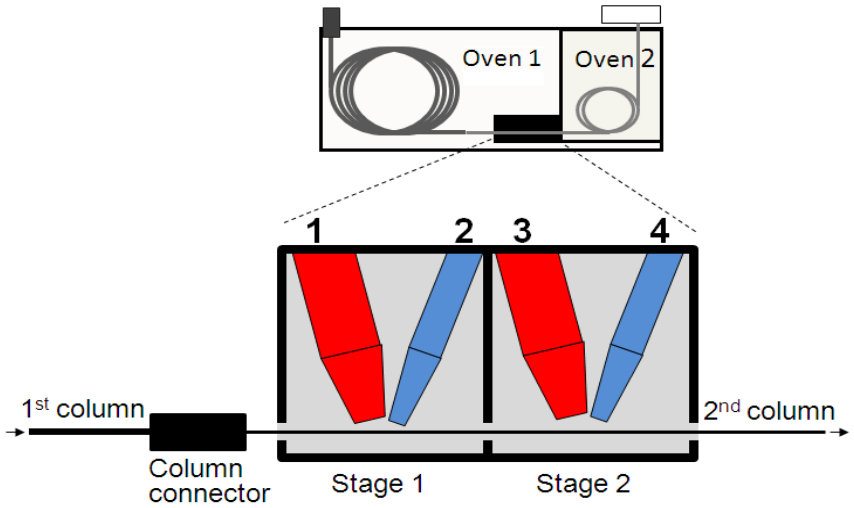


Fig. 14: Four-jet N_2 cryo modulator (Pegasus III, Leco Corp.). 1, 3: hot jets; 2, 4: cold jets.

The operation of the GC×GC equipped with the modulation systems using liquid nitrogen as the coolant is quite expensive due to the cost of the considerable amount of the liquid N_2 which is consumed during the analysis. However, modulators using freezers with carbon dioxide as the cooling medium are not suitable for analysis of the very volatile compounds. The possible solution could be systems with valve-based modulation using synchronized pressure pulses for focusing and subsequent releasing of the effluent. Nevertheless, optimization of these systems requires more attention and the instrumentation is rather complex [54,55,56].

The modulated fractions should not be wider than about one-third or one-quarter of the peak widths in the first dimension; otherwise the gain of the first dimension separation (resolution) is decreased considerably. It is because the first dimension chromatogram is reconstructed from the second dimension chromatograms **(Fig. 15)**. The more modulations per peak - the more precise is the first dimension reconstruction. However, adjustment of too short modulation period (duration between two modulations) may cause that analytes with longer retention in the second dimension do not manage to leave the column before

beginning of the next modulation. The peaks then show up in later modulation than in which they were injected. This phenomenon is called wrap-around. The modulation period should be longer than the elution time of the most detained analyte in the second column. The modulation period usually varies between 2 to 8 seconds.

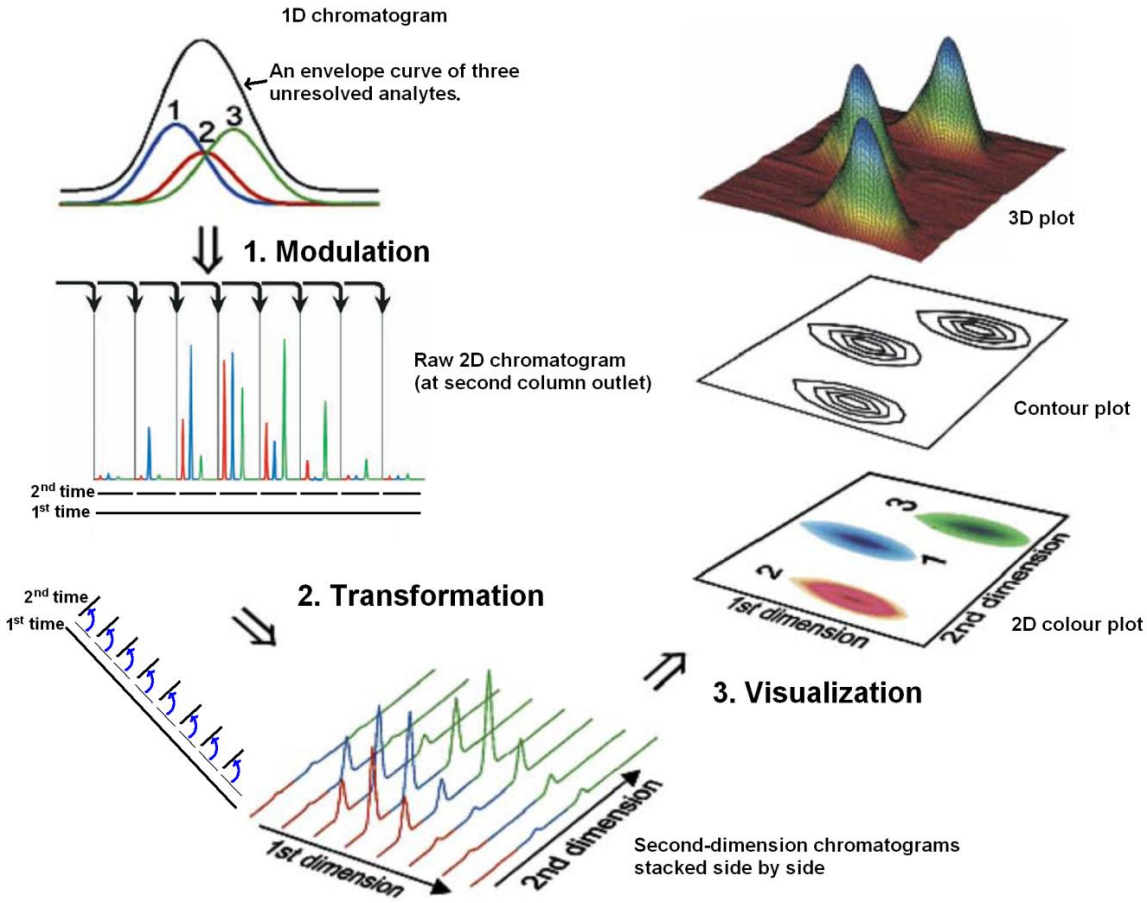


Fig. 15: Generation and the visualization of the 2D GC×GC chromatogram [49].

After the GC×GC analysis, a "raw 2D chromatogram" is obtained. It consists of a sequence of second dimension chromatograms sorted behind. Chromatographic software then cuts the 2D chromatograms into pieces of length of time corresponding to the modulation period. The 2D and 3D visualization is obtained by mathematic transformation of the data including flipping the second dimension chromatograms by ninety degrees **(Fig. 15–2. Transformation)**. The quantity of the signal can be displayed by means of the contour plot, or as a gradient of colors in 2D or 3D plot **(Fig. 15-3. Visualization)**.

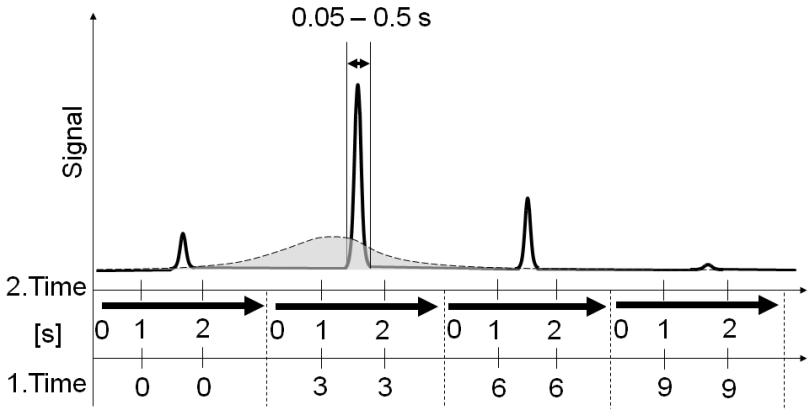


Fig. 16: Comparison of the peak thickness of the conventional 1D-GC (dashed line) and the corresponding second dimension peaks produced by the comprehensive GC×GC.

The analysis in the secondary column produces very narrow peaks, usually ten or twenty times narrower than peaks occurring in the corresponding one dimensional GC **(Fig. 16)**. This is due to the focusing of the effluent in the modulator and subsequent very fast analysis in the secondary column. Furthermore, a peak area measured by 1D GC should be the same as the sum of secondary peaks areas. Therefore, signal-to-noise ratio in the comprehensive GC×GC is considerably higher than in the 1D GC, which makes the analysis substantially sensitive **(Fig. 16)**.

4.2.1.5.3 Columns combinations

In the most applications, samples are separated in the first dimension on a 15-30 m of the length × 0.25-0.32 mm of internal diameter (ID) × 0.1-1 μm of stationary phase film thickness (d_f) column with a non-polar stationary phase. The secondary column is usually much shorter, narrower – with dimensions 0.5-2 m × 0.1-0.18 mm ID × 0.1 μm d_f containing medium-polar or shape-selective (e.g. chiral) stationary phase. In the case of using non-polar column in the first dimension, volatility is the only parameter of interest, so boiling-point separation is obtained. If a polar column in a secondary dimension is used, analytes are separated according specific interactions but to some degree also by volatility. Nevertheless, the separation in the second dimension takes place rapidly in 2-8 s so the analysis can be considered as isothermal. The analytes introduced from the first column to the second one, by means of the modulator, are of the same volatility. Therefore, there will be significantly reduced dispersive interactions (separation according boiling points) in the second dimension. Thus, only specific interactions (polarity, chirality, shape selectivity) with the stationary phase will govern the retention [52].

4.2.1.5.4 Separation system orthogonality

The two-dimensional comprehensive system can be considered as orthogonal when the separation mechanisms are statistically independent. To achieve a full orthogonality is important for two reasons: a) orthogonal separation uses efficiently the separation space and so has greater peak capacity than non-orthogonal separations; b) retention in two dimensions of an orthogonal chromatogram is determined by two independent mechanisms and thus provides two independent analyses. For example, a high degree of retention correlation in both columns can reduce a multidimensional separation to, in fact, a one-dimensional separation with peaks distributed along a diagonal **(Fig. 17-A)**. Orthogonality in the two-dimensional comprehensive chromatography can be influenced by a proper combination of the primary and secondary columns and by applying of a suitable temperature program **(Fig. 17)**. Usually, the most important task is the elimination of the retention mechanisms that are in common for the two columns. In most cases it is separation according volatility. Some examples of how the temperature program applied on the columns can affect the orthogonality of the separation and distribution of the peaks on the chromatographic plane are shown in the **Fig. 17** [57]. The best result is obtained when the same temperature program is applied on both columns, and the actual temperature on second one is incremented **(Fig. 17-D)**.

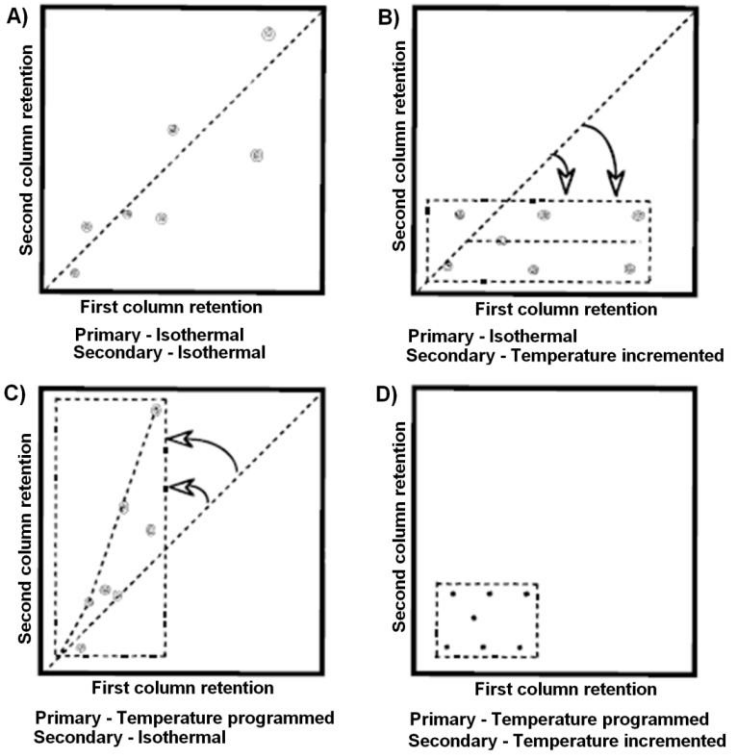


Fig. 17: Simulated comprehensive two-dimensional gas chromatogram showing the influence of the temperature on the sample components retention. The dashed rectangle delimits the accessible area of the retention plane [57].

An important benefit of the orthogonal GC×GC separation is the ordered structures of the separated compounds **(Fig. 18)**. Analytes of the similar nature (structurally related homologues, congeners and isomers) form continuous bands or clusters. Structured chromatograms are a valuable tool for unknown compounds or group-types identification [52,58,59]

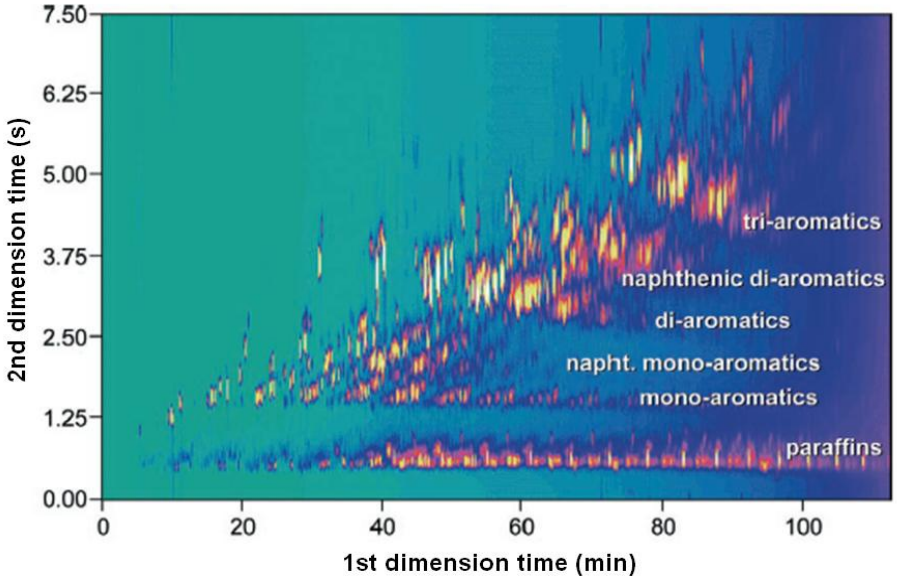


Fig. 18: GC×GC–FID color plot of a light cycle oil using a 25 m x ID 0.25 mm DB-1 x 1.5 m, ID 0.1 mm OV-1701 column combination [49].

4.2.1.5.5 Detectors for two-dimensional gas chromatography

The very fast separation occurring in the second-dimension column produces narrow peaks. The actual peak widths depend on the type of modulator used, gas flow, length of the second dimension column, and, since the analysis is isothermal, on the retention time in the second dimension. The peak width varies, in most cases, between 50 and 500 ms at the baseline **(Fig. 16)** [49,60,61]. Peaks of these parameters require suitable detectors with a fast electronics, small internal volume and a high data acquisition rate to ensure a proper sampling of the chromatogram. The acceptable sampling frequency should not be lower than 50 Hz; however for a proper quantification no less than 100 Hz is needed.

The first detector which met the requirements was FID. Today, a modern FID can acquire data at frequencies of 50-300 Hz. It has been commonly used mainly for the petrochemical analysis, because its response is proportional to the number of C-H bonds [62]. Later, an increased number of applications to the trace level analysis of various classes of organohalogens have led to the modification of the current ECD (Electron Capture Detector). In the late 1990s, a micro-ECD (μECD) suitable for fast GC was marketed [53]. Nevertheless, the μECD detector needs a high operating temperature (320-350 ^{o}C) and high auxiliary gas flows (200-450 ml/min).

The previous detectors do not provide structural information. For this purpose, only mass detector is suitable. Mass spectrometers with ToF (Time of Flight) analyzers are fast enough to meet the requirements for GC×GC. The ToF analyzer can operate at very high repetition rates; typically 5000-30000 raw mass spectra per second can be taken. The raw mass spectra are accumulated or averaged, and about 10-500 spectra per second are saved in the computer. The ToF analyzer can produce also high-mass resolution, but commercially available GCxGC-ToF MS instrument Pegasus III (Leco Corporation) can work in a unit-mass resolution. Nevertheless, the next-generation instruments are expected to be equipped with the high-resolution ToF mass analyzer.

In the recent time, a fast quadrupole mass analyzer was introduced by a Shimadzu company, achieving scanning rate of 10 000 *m/z* per second. This corresponds to the 25 Hz when full mass spectrum (*m/z* 30 – 400) is scanned, which is not sufficient for quantification purposes. Nevertheless, this analyzer can be used in a limited mass range scanning mode (or even SIM mode).

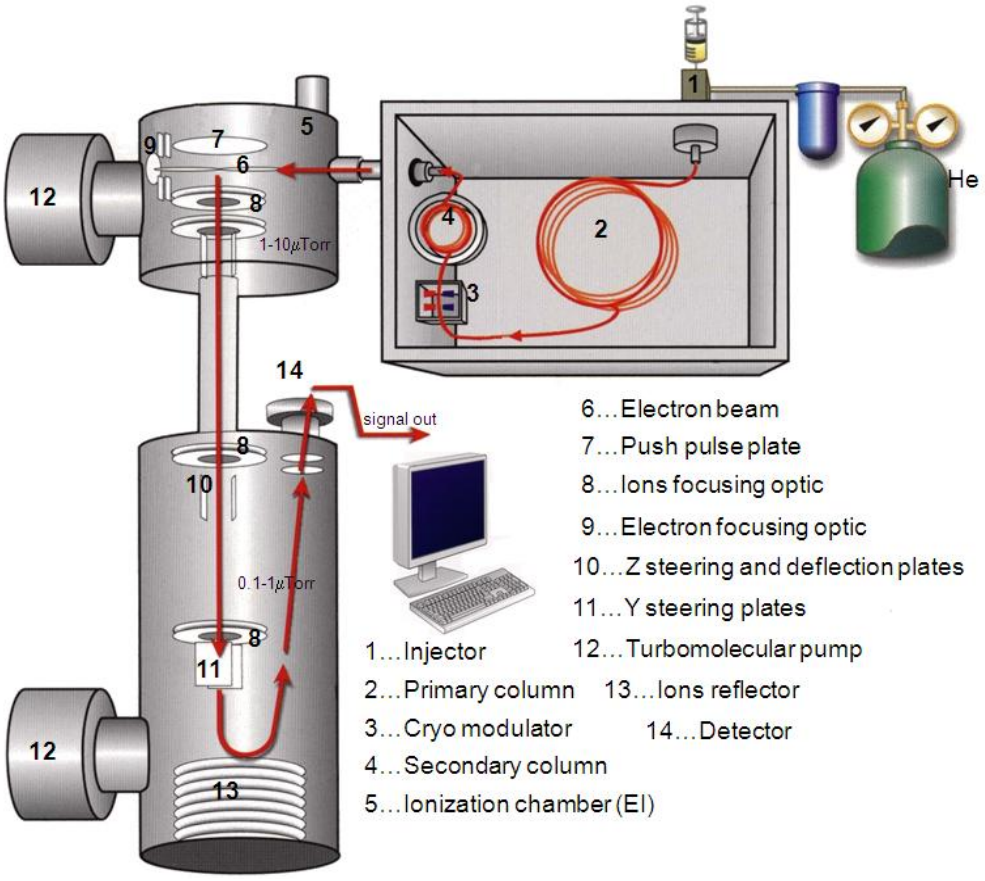


Fig. 19: Scheme of the GCxGC-MS ToF system (ChromaTOF Software Help).

4.2.2 Other analytical techniques

Despite the gas chromatography coupled with many types of detectors is the most widespread method in the insects chemical ecology research, there are many other analytical techniques that can be used for solving of some specific analytical problems.

4.2.2.1 High performance liquid chromatography (HPLC)

HPLC is a very powerful separation technique, which can be used for non-volatile, very polar, or thermally unstable compounds. The HPLC technique offers a wider range of parameters that can be used for optimization of the separation selectivity (e.g. number of solvents for mobile phase, or stationary phase material) than GC. Nevertheless, in general, separation power and sensitivity is lower in comparison with GC. HPLC can be coupled with various types of detectors; however the technique has no detector comparable in versatility as FID for GC. The most extended is a spectroscopic detector working in the ultraviolet region of radiation (UV detector). Similarly to GC, most useful and powerful detector is mass spectrometer. However, different ionization techniques must be used due to the presence of liquid solvents. The most used are Atmospheric Pressure Chemical Ionization (APCI) [63] Atmospheric Pressure Photo Ionization (APPI) [64] or Electro Spray Ionization (ESI) [65].

4.2.2.2 Matrix assisted laser desorption – time of flight mass spectrometer (MALDI-TOF)

MALDI is a soft ionization technique used in mass spectrometry, originally developed for large biomolecules analysis [66]. The analyzed sample is diluted in a solution of a special composition (matrix) and applied on a metal plate. Then the solvent is allowed to evaporate. The plate with the sample is put into the instrument that is subsequently evacuated. Then a UV laser beam is applied. The matrix material absorbs the UV laser light, which results in ablation of upper layer (micrometers of the matrix material). The hot plume produced by the laser contains a mixture of ionized and neutral molecules of matrix and the sample. The molecules of analytes are ionized by protonation or deprotonation in the hot plume. The mechanism of the ionization process is still unclear. The matrix consists of crystallized salts of small organic acids. The most used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxicinnamic acid and 2,5-dihydroxybenzoic acid (DHB). The ToF mass spectrometer is the most common detector for MALDI, mainly due to its large mass range.

Despite the MALDI technique is usually used for ionization of the large biomolecules, it has already been used for small organic molecules, too. For example analysis of hydrocarbons or other neutral lipids produced by termites [67], plant leaves, fruit flies [68], or male bumblebees [69].

4.2.2.3 Nuclear magnetic resonance (NMR)

Using of NMR method in the chemical communication research is rather limited because it is often impossible to ensure the necessary amount of analyte, or the studied compounds are present in the complex mixtures. Nevertheless, these problems are possible to solve by applying some separation and/or concentration techniques (Thin Layer Chromatography, TLC) [70], column chromatography or preparative gas chromatography in connection with more sensitive variants of NMR (cryo-probe, microprobe [71]). Recently, some publications where NMR was used as an HPLC detector were published [72].

4.2.2.4 Methods for detection of radioactivity

The following radioanalytical methods were used for the detection of the ^{14}C in the metabolites within the bumblebee male's pheromones biosynthesis research.

4.2.2.4.1 Scintillation spectrometer

The principle of the operation of the radioactivity detection in the scintillation spectrometers is the conversion of the absorbed energy of the ionizing radiation (^{14}C emits β-radiation) to the energy of photons that belong energetically to the visible or near-ultraviolet spectral region. Radioactive samples are usually dissolved in the liquid scintillation cocktail (toluene-based). The photons, emitted by scintillation cocktail components, are subsequently detected by two photomultipliers. The number of counts is proportional to the number of radioactive nuclei.

4.2.2.4.2 Position-sensitive radioactivity detector

The method was used for direct detection of the radioactivity from the TLC plates. The detection of an ionizing radiation is directly produced by an electron-avalanche multiplication in gas mixture (90% Ar, 10% CH_4). The detector consists of so-called multiwire chamber in which the amplification of the ionization electrons, generated by the ionizing radiation in the gaseous medium, is produced by virtue of an avalanche around a thin anode wire. The electric signal spreads the multiwire cathode by an electric induction process. The position sensing of the radioactive compounds on the TLC is based on the time delay of the electric impulse. The TLC spots of the compounds containing radioactive isotopes are displayed as peaks **(Fig. 20)**. The spatial resolution of the radioactive spot depends on the energy of the emitted radiation, approximately 1 mm for the ^{14}C.

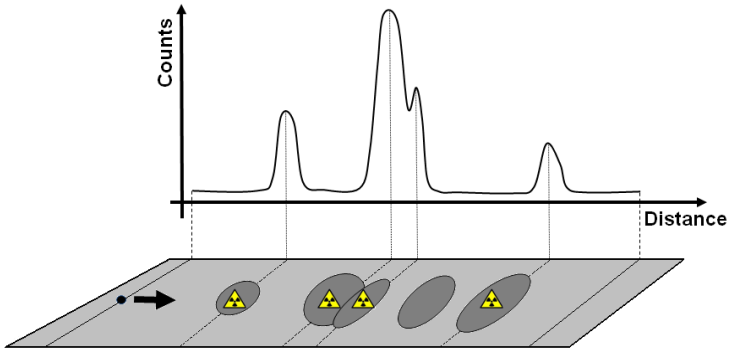


Fig. 20: Conversion of the radioactive TLC spots into the peaks by a position sensitive detector.

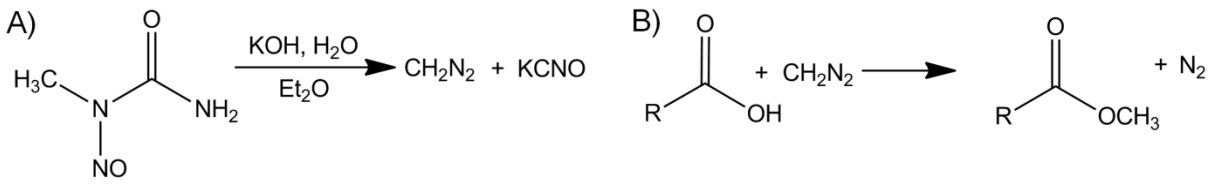
4.3 Structure determination using gas chromatography

There is a number of possibilities of the identification of unknown compounds. The basic parameter that characterizes a compound is its retention behavior on a chromatographic column of a well-defined stationary phase. This parameter became even more important beginning of eighties when high-resolution fused silica capillary columns were introduced [73], where the retention time can be determined in order of seconds. The retention behavior is usually expressed by means of Kovats indices [74]. An unknown compound can be considered as identified when its retention indices (determined on two columns of different polarity) matches with indices of a standard compound of the known structure. The main disadvantage of this method is the need of a large number of standards. This problem can be avoided by using a selective detector; the most desirable is a mass spectrometer.

The mass spectrometer with an electron ionization produces a mass spectrum that is unique for a given structure and can by compared with spectra of other compounds present in some of the mass spectra databases [75]. Nevertheless, differences in the mass spectra between structurally similar compounds can by negligible, or even none in the case of enantiomers. For these reasons, number of derivatization techniques that considerably extends a number of compounds that can be analysable by GC, or make their mass spectrum interpretation much easier, have been developed. The following techniques were used in the Thesis.

4.3.1 Free fatty acids derivatization

Fatty acids were analyzed as their corresponding methyl esters due to their inappropriate chromatographic behavior on nonpolar columns. Derivatization with diazomethane was used. The diazomethane can by prepared from nitrosomethylurea in diethyl ether **(Scheme 21-A)** and subsequently purified by distillation. The derivatization occurs by a simple addition of the diethyl ether diazomethane solution to the extract containing free fatty acids. The reaction proceeds both rapidly **(Scheme 21-B)** and quantitatively [76].



Scheme 2: A) A synthesis of diazomethane from nitrosomethylurea; B) An esterification using diazomethane.

4.3.2 Double bond position determination

Double bonds can be determined by an addition of dimethyl disulfide (DMDS) to unsaturated alkenes, acetates, fatty acid esters, aldehydes and alcohols. The DMDS adducts cleave in the mass spectrometer with electron ionization preferentially across the C-C bond between the CH_3S- substituents, leading to two major fragment ions **(Fig. 21)**. The reaction is nearly quantitative [76]. The method can be used also for multiple unsaturated compounds; however, introducing more CH_3S- groups to the structure decreases volatility of the adducts considerably [77].

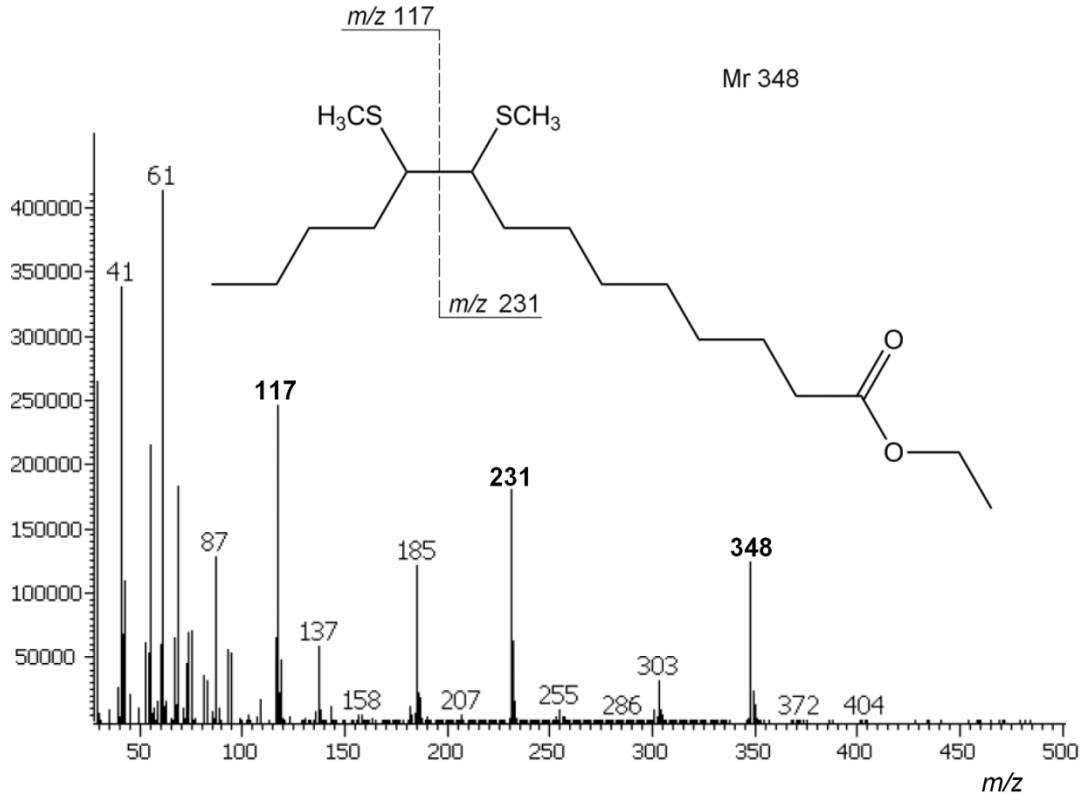


Fig. 21: EI mass spectrum and a structure of methylthiolated ethyl tetradec-9-enoate.

4.3.3 Double bonds configuration determination

Using the mass spectrometry for the double bond configuration produces unreliable results. Much more convincing is the use of FTIR method. The analytically significant spectral region for double bonds configuration determination are stretching vibrations of the =C-H bond in the region above 3000 cm^{-1} **(Fig. 22-A)** and out-of-plane bending vibrations between 960 and 980 cm^{-1} **(Fig. 22-B)** [76].

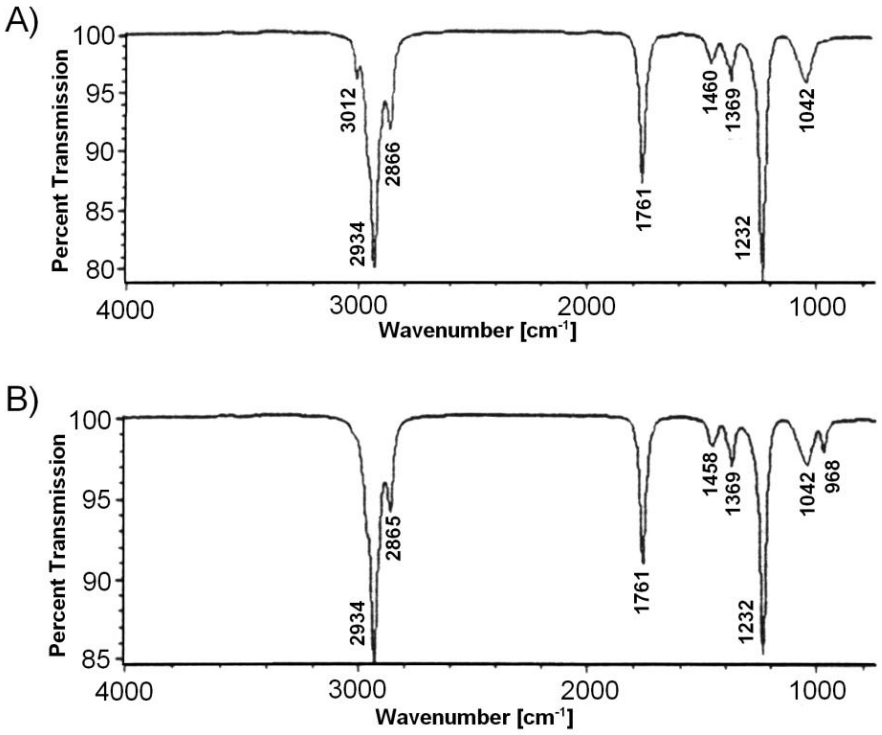


Fig. 22: Gas-phase infrared of A) (*Z*)-tetradec-5-enyl acetate and (*E*)-tetradec-5-enyl acetate [76].

4.3.4 Absolute configuration determination

There are several methods that can be used for the absolute configuration determination, for example Optical Rotary Dispersion (ORD) [78] or Circular Dichroism (CD) [79]. The method of NMR can be applied using either chiral shifts reagents [80] or diastereomeric derivatives [81]. However, these techniques require a relatively high amount of pure analytes.

Another possibility can be formation of diastereomeric derivatives and their subsequent analysis by conventional separation techniques, such as HPLC or GC [80,82]. Nevertheless, derivatization of the chiral compounds in the complicated mixture can make the analysis difficult (e.g. possible lowering of the volatility or the sample contamination by the derivatization procedure). The most convenient method is a direct analysis of the sample using chiral stationary phases in GC or LC. The chiral stationary phases work by providing a chiral environment interacting differently with each of an enantiomeric pair. In GC, the first chiral stationary phases were based on amino acids modifications. In the late eighties chiral stationary phases based on modified cyclodextrines were developed and they are the most common types of chiral stationary phases in GC nowadays [83] **(Fig. 23)**. The cyclodextrines α, β and γ, are made up of 6, 7 and 8 D-glucopyranoside units, respectively **(Fig. 23-A)**. They form a toroid shape in the space **(Fig. 23-B)**. The separation mechanism is based on comparative “fit” of each enantiomer into the cavity. Nevertheless, there is no general rule

which of the enantiomers will be separated and which will not. The exact order of the individual isomers (*R, S*) is also essentially unpredictable; therefore standards of both enantiomers are always needed.

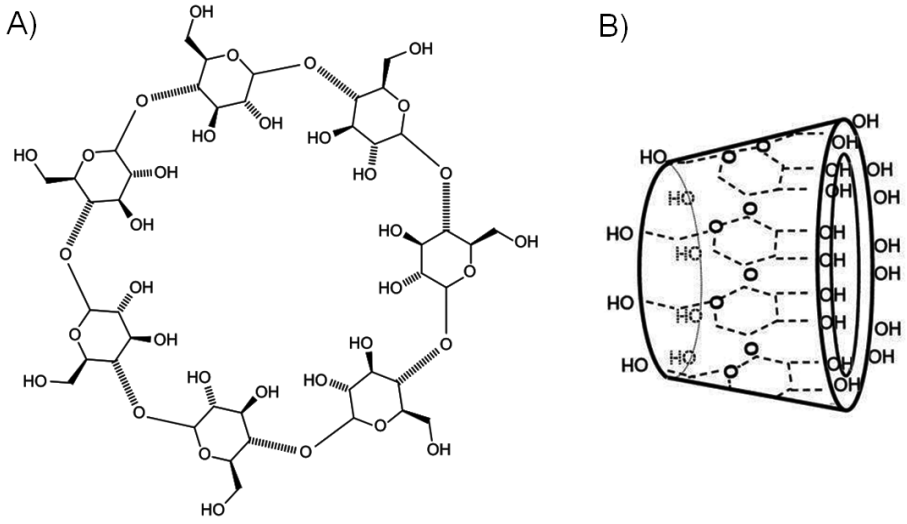


Fig. 23: A) Structure of an unmodified β-cyclodextrine (http://upload.wikimedia.org/wikipedia/commons/thumb/5/51/Cyclodextrin.svg/2000px-Cyclodextrin.svg.png); B) An unmodified γ-cyclodextrine toroid structure showing a spatial arrangement (http://en.wikipedia.org/wiki/File:Gamma_CD_cone_shape.jpg).

4.4 Separation of the isotopically modified compounds in the GC

Separations of labeled (isotopically modified) compounds from their unlabeled analogs have always been a big challenge for analytical chemists since the beginning of the gas chromatography. First experiments were performed in the late nineteen fifties and sixties [84,85,86,87]. It is important to note that, despite the poor separation efficiency of the used GC systems, examiners achieved remarkable results. Nevertheless, the experimental separations worked mostly at low temperatures that allowed separations only permanent gasses [86,88] or liquids with low boiling points [85]. Since the last fifty years the labeled compounds have begun to be used as a tool for tracing the transformation of compounds of interests. For example, research of organic reaction mechanisms or transformation of biologically active compounds in the living organisms. Therefore, a more comprehensive analytical tool was needed. Gas-liquid chromatography (GLC) with fused-silica capillary columns with thermally stable stationary phases is nowadays used in the majority of applications. Thus, a mechanisms and theory of an isotope effect in the GLC, of labeled compounds was extensively studied [89].

The isotope effect can be assessed by the retention order of a labeled compound and its unlabeled analog. The situation when unlabeled isotopomers are eluted earlier is called a “normal isotope effect” (NIE). The opposite behavior is called the “inverse isotope effect” (IIE). The NIE usually occurs in gas-solid chromatography (GSC) at lower temperatures [84,85]. The IIE is mostly found in the GLC [89,90,91]. For the purpose of the biosynthesis research of bumblebee’s sexual pheromone, the isotopes of hydrogen (deuterium- ^{2}H) and carbon (^{13}C, ^{14}C) were used. Therefore, labeled compounds were heavier than the unlabeled ones.

The different retention behavior of the isotopomers results from their mass differences of the atomic nuclei in various functional groups of the molecule [92,93]. Heavier isotopic molecules have a slightly lower mobility. It results from the relation: $kT = 0.5mv^2$ (k = Bolzmann constant, T = absolute temperature, m = molecular mass, v = average molecular velocity). The heavier molecules have a lower average molecular speed; therefore they have a lower diffusion velocity and a smaller collision frequency with other molecules. Also, the heavier isotopic molecules generally have higher binding energies. For better illustration of the competing forces between molecules, a model describing chemical bond can be used [94] **(Fig. 24)**.

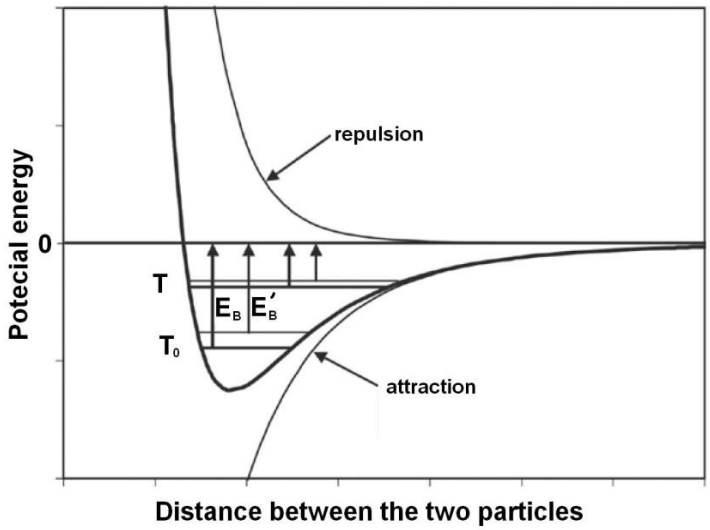


Fig. 24: Scheme of the potential energy distribution caused by the repulsive and attractive forces. Thin and heavy lines represent energy systems of the heavy and light isotopic particle. Arrows indicate the respective binding energies. At higher temperatures, the difference between the binding energies of the isotopic particle is smaller. This results in a smaller isotope effect [94].

There are two forces involved in the chemical bond formation as well as in a condensation of molecules: attractive and repulsive. The attractive force decreases less rapidly with the distance than the repulsive one. As the result of these forces, the two particles will be located in a certain distance from each other. A higher binding energy for heavier isotopic molecules results in lower vapor pressure – this is called normal vapor pressure isotopic effect. Nevertheless, under certain conditions, the potential energy of the heavier isotopic molecule can be higher [95]. In this case, the labeled compounds elute first and the calculated boiling point is lower than in the unlabeled analog [96]. The retention behavior (elution order) of the labeled-unlabeled pair will depend on the nature of the prevailing interactions that occur between the solute and stationary phase during the separation process.

The analytical aspects of the isotopes separation in the GLC were clearly outlined by Schmarr and his co-workers [93]: There are two main processes involved in the separation mechanisms. One is the mixing of the analyte with the partitioning liquid stationary phase. This is considered to be an isotope insensitive process. The second process is the condensation and the evaporation. This process is considerably more isotope sensitive, because the dispersive van der Waals (vdW) interactions dominate between analyte and stationary phase. In this case the inverse isotope effect is usually observed [89]. The vdW-forces (or interactions) are long-distance forces from the electric field produced by a neutral molecule. These can be divided into three groups: inductive, orientation, and dispersive. The first ones are dipole-induced interactions (Debye interaction); the second interactions are dipole-dipole related (Keesom). The dispersive (London) interactions are the most comprehensive among the others an also additive. In general, the vdW-forces are

operative in the condensed phase, which results in the shift of the molecular potential in the potential well. Since a heavier isotope lies lower in the potential well, it has a lower vibrational frequency and smaller vibrational amplitude. Also bond lengths and bond angles involving a heavier isotope are different from the lighter one. For example it was observed that C-D bond is shorter by 0.005 Å than C-H bond [97]. Therefore, deuterated compounds are smaller than non-deuterated, so the molar volume exhibits a negative isotope effect [91]. As a consequence of a lower molar volume (can be outlined as van der Waals volume [98]) lower London dispersion interactions are expected due to a more compact electron distribution and a decrease in the electronic polarizability [99]. Some of the London disperse interactions appear due to the oscillation of the atomic nuclei in the molecular bond that creates an electromagnetic field inducing a field of opposite charge in the neighboring molecules.

For example the C-D bond has a lower oscillation frequency (2334 cm^{-1}) than a C-H bond (3300 cm^{-1}) [93]. This induces lower forces that attract solute to the stationary phase, which causes a quicker elution from the column. In general, the more are vdW interactions suppressed, the higher is the IIE and the better separation between isotopomers in GLC is achieved. The reduction of the molar volume is considerably more pronounced for deuterated isotopes than with, e.g. ^{13}C-isotopes (due to the lower ratio of the atomic weights of $^{13}C/^{12}C$ than $^{2}H/^{1}H$), thus the inverse isotopic effect is stronger [100].

It was observed that the retention behavior of the labeled compounds is influenced by the position of the isotope within the structure [93]. This effect was traced to hindered translations and rotations with the internal vibrations in the condensed phase, which results in the isotope-dependent shifts of the internal molecular frequencies [101,102].

The GLC columns are nowadays used in gas chromatography much more than columns allowing the separation based on gas-solid interactions. Therefore the IIE is usually observed. However, a new material for stationary phases was introduced recently – ionic liquids [103]. Surprisingly, isotope separations on the columns with this type of stationary phase showed a NIE [93].

5 Preface to the articles

5.1 Optimization of the analysis of the labeled aliphatic esters

5.1.1 Separation of the labeled aliphatic esters

The main object of this Thesis was to study biosynthesis of sex pheromone components of bumblebee males using compounds (expected biosynthetic precursors) labeled with deuterium or radioactive ^{14}C. Research strategies were based on *in vivo* and *in vitro* incubations of various body parts or tissues with isotopically modified compounds and a subsequent analysis of resulting labeled metabolites using gas chromatography, or radioanalytical techniques in the case of metabolites containing ^{14}C. It proved necessary to study the retention behavior and other analytically important properties (EI mass spectra) of the labeled compounds. The sex pheromone of the most studied species (*B. lucorum* and *B. terrestris*) contains ethyl esters as the most abundant aliphatic components [104,105], thus we studied chromatographic behavior of labeled methyl and ethyl esters.

It has already been reported that labeled compounds usually exhibit an inverse isotopic effect in the gas-liquid chromatography [89,90,100]. Nevertheless, the magnitude of the isotopic effect may depend on properties of the stationary phase, thus on the prevailing interactions participating in the analyte retention in the GC column (see chapter: 4.4 Separation of the isotopically modified compounds in the GC).

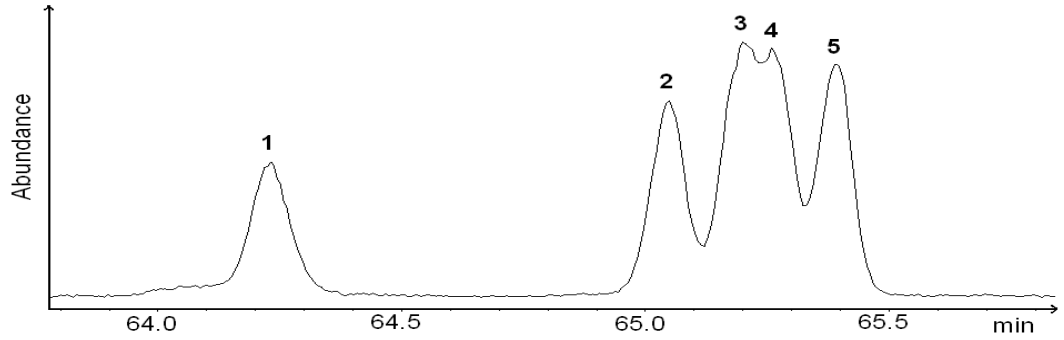


Fig. 25: Chromatogram of methyl hexadecanoates containing various number of deuterium atoms in the carbon chain. 1:d_{31}-per-deuterated; 2:(13,13,14,14,15,15,16,16,16)-d_9; 3:(15,15,16,16,16)-d_5; 4:(16,16,16)-d_3; 5:d_0-unlabeled. [Separation column: ZB-5MS; Oven temperature program: 50 ^oC (held for 1 min)-rate 2 ^oC/min-to 320 ^oC; mobile phase: He (1 ml/min).]

We confirmed the inverse isotopic effect on all types of the examined chromatographic columns **(Table 3** and **4)**. The magnitude of the effect (retention of the unlabeled compound with respect to its labeled analog) was linearly proportional to the

number of deuterium atoms present in the carbon chain **(Fig. 25** and **26)**. The linearity was observed for both isothermal (data not shown) and temperature programmed analysis **(Fig. 26)**. The same results were obtained for both methyl and ethyl esters (data not shown for ethyl esters). If we draw slopes of the linear dependency of the number of deuterium atoms on Kovats indices against the carbon chain length of the examined methyl esters, we get a sigmoid dependency **(Fig. 27)**.

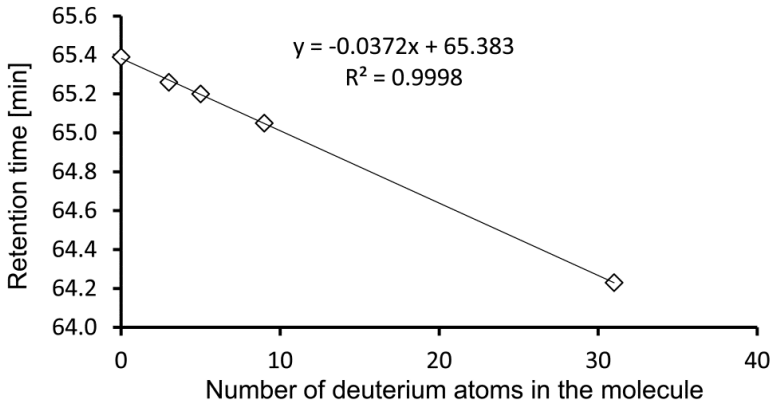


Fig. 26: Linear dependence of the retention time of the deuterated methyl hexadecanoates on the number of deuterium atoms present in the carbon chain. 1:d_{31}-per-deuterated; 2:(13,13,14,14,15,15,16,16,16)-d_9; 3:(15,15,16,16,16)-d_5; 4:(16,16,16)-d_3; 5:d_0-unlabeled. [Oven temperature program (ZB-5MS): 50 ^{o}C (held for 1 min)-rate 2 ^{o}C/min-to 320 ^{o}C; mobile phase: He (1 ml/min).]

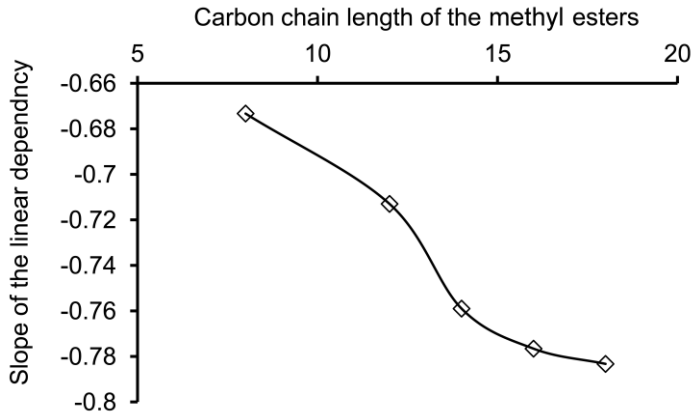


Fig. 27: Sigmoid dependence of the slopes, taken from the linear regression of the dependency of the Kovats indices on the number of deuterium atoms in the methyl ester of the same carbon chain length, on the length of the carbon chain of the analyzed methyl esters. [Oven temperature (ZB-5MS): isothermal, held on 170 ^{o}C; mobile phase: He (1 ml/min).]

In the GLC, the vdW interactions are isotopically sensitive and responsible for the separation of the labeled and non-labeled compounds. These interactions are also called non-specific and are dominant for retention on non-polar columns (separation according to a boiling

temperature). We observed that DB-WAX and ZB-5MS columns, differing considerably in polarity (determined according McReynold's constants [106]), showed a similar selectivity to the methyl tetradecanoate and methyl tetradecanoate-d_{27} (fully deuterated) **(Table 3)**. It proved that the non-specific interactions are dominant for the separation of the deuterated and non-deuterated compounds on both types of stationary phases.

Table 3: Parameters of the used gas chromatographic columns.

Column	Selectivity[a]	Resolution[b]	Number of theoretical plates[c]
ZB-5MS[d]	1.099	6.67	99000
DB-WAX[e]	1.112	4.95	62000

[a]...Selectivity was determined for methyl tetradecanoate (non-deuterated) and methyl tetradecanoate-d_{27} (fully deuterated).
[b]...Resolution was determined for methyl tetradecanoate (non-deuterated) and methyl tetradecanoate-d_{27} (fully deuterated).
[c]...Column efficiency expressed as a number of theoretical plates was determined for methyl tetradecanoate-d_{27} (fully deuterated).
[d]...ZB-5MS (Phenomenex), (30m, ID 0.25mm, PT 0.25μm). Oven temperature: isothermal, held on 170 ^{o}C.
[e]...DB-WAX (J&W Scientific), (30m, ID 0.25mm, PT 0.25μm); Oven temperature: isothermal, held on 170 ^{o}C.

The retention behavior of the same compounds containing equal number of deuterium atoms located on various positions within the molecule could be different (see chapter: 4.4 Separation of the isotopically modified compounds in the GC). However, we did not observe any retention differences between two ethyl esters, first labeled with three deuterium atoms on the terminate carbon in the ethanol part and the second with the same number of deuterium on the terminate carbon in the fatty acid chain **(Fig. 28)**. Nevertheless, it is possible that the effect would be observable if more deuterium atoms were present in the molecule, or a column of lower polarity and/or higher efficiency was used.

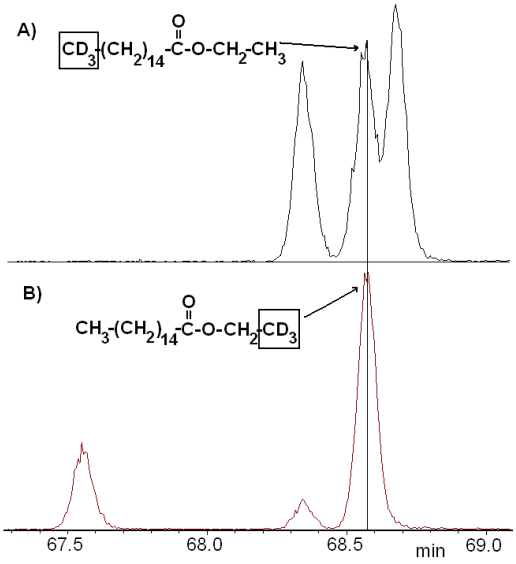


Fig. 28: Comparison of the retention behavior of the ethyl esters containing three deuterium atoms located in the different parts of the molecule. [Oven temperature program (ZB-5MS): 50 °C (held for 1 min)-rate 2 °C/min-to 320 °C; mobile phase: He (1 ml/min).]

We also studied chromatographic behavior of the compounds labeled with ^{13}C isotope for comparative purposes of the isotope effect with deuterium. Only a partial separation was reported so far for carbon isotope-containing compounds in the GLC [100]. We observed a minor separation between methyl hexadecanoate-$^{12}C_{16}$ and methyl hexadecanoate-$^{13}C_{16}$ **(Fig. 29)**.

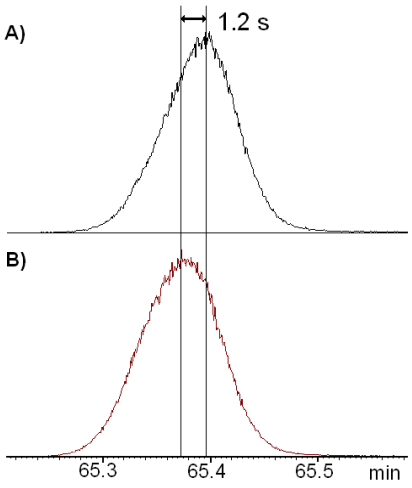


Fig. 29: Comparison of the retention behavior of the A) methyl hexadecanoate-$^{12}C_{16}$ and B) methyl hexadecanoate-$^{13}C_{16}$. [Oven temperature program (ZB-5MS): 50 °C (held for 1 min)-rate 2 °C/min-to 320 °C; mobile phase: He (1 ml/min).]

According to our measurements, the isotopic effect of the deuterium is approximately thirty times higher (with respect to the retention behavior) than ^{13}C in methyl esters. This number was calculated as follows: [($Rt_{methyl\ hexadecanoate\text{-}d0}$ - $Rt_{methyl\ hexadecanoate\text{-}d9}$)/9] /

[($Rt_{methyl\ hexadecanoate\text{-}^{12}C16}$ - $Rt_{methyl\ hexadecanoate\text{-}^{13}C16}$)/16], where Rt is a retention time in seconds [Oven temperature program (ZB-5MS): 50 °C (held for 1 min)-rate 2 °C/min-to 320 °C.].

5.1.1.1 Separation of the labeled compounds in the GC×GC

Virtually all knowledge about the retention behavior of the isotopically modified compounds achieved in one dimensional GC can be applicable for comprehensive two-dimensional GC for a simple reason - the separation in the GC×GC is performed using the same types of capillary columns. The GC×GC system is very powerful for complex samples separation, because two separation mechanisms are applied to the analytes in one analysis. So, the more different types of structures are present in the sample, the more we can gain from the comprehensive system. However, if we consider the separation of the unlabeled compounds from their labeled analogs, the GC×GC will not help. The decisive role of the separation process is performed on the primary column **(Table 4)** where even lower separation efficiency is achieved in comparison with the same column used in one dimensional GC arrangements. The explanation is in the chapter: 4.2.1.5 Two-dimensional comprehensive gas chromatography (GC×GC). The main benefit of the GC×GC for the labeled compounds analysis is an easy and more accurate identification of the isotopically modified compounds according to their retention behavior with respect to the unmodified analog in the 2D chromatogram **(Fig. 30, 36** and **43).** It is very similar to the analysis of enantiomers in the GC×GC [107,108].

Table 4: Parameters of the used gas chromatographic columns.

	Column	Number of theoretical plates[a]
Primary column	ZB-5MS[b]	90000
Secondary column 1	BPX-50[c]	15300
Secondary column 2	RTX-50[d]	9600

[a]...Column efficiency expressed as a number of theoretical plates was determined for methyl tetradecanoate-d_{27} (fully deuterated).
[b]...ZB-5MS (Zebron), (29 m, ID 0.25 mm, PT 0.25 μm). Oven temperature: isothermal, held on 170 °C.
[c]...BPX-50 (SGE), (2.10 m, ID 0.1 mm, PT 0.1 μm). Oven temperature: isothermal, held on 231 °C.
[d]...RTX-50 (Restec), (1.93 m, ID 0.18 mm, PT 0.2 μm). Oven temperature: isothermal, held on 203 °C.

We used a non-polar column (ZB-5MS) in the first dimension and a middle-polar column (DB-50 or RTX-50) in the second dimension **(Table 4)**. Therefore, we expected and also confirmed IIE in both columns **(Fig. 30)**. However, the isotopic effect in the second dimension is lower for a couple of reasons: 1) The disperse interactions occurring in the secondary column are suppressed by a temperature setting – the actual temperature of the

secondary column is 5 to 10 ^{o}C higher then the actual temperature of the primary column (according to the setting). The disperse interactions are then suppressed and compounds are predominantly separated by specific interactions (separation according polarity), which is isotopically less sensitive process; 2) The higher is the polarity of the column the lower are the disperse interactions (the BPX-50 and RTX-50 columns are considered to be middle-polar, while ZB-5MS is non-polar) [106].

For a given combination of primary and secondary columns a few parameters that could influence the separation were examined: temperature program (range: 2-20 ^{o}C/min), secondary oven temperature increment (range: 5-30 ^{o}C) and hot pulse duration (range: 0.6-1.2 s). The last two parameters (secondary oven temperature increment and hot pulse duration) may only affect separation in the secondary oven. However, it appeared that separation of the isotopically modified compounds in both dimensions can be significantly changed by a temperature program adjustment only: the slower temperature gradient was applied – the better total separation (in both columns) was achieved. Per-deuterated methyl esters were fully separated from the non-deuterated using the fastest temperature program that was examined (20 ^{o}C/min) (data not shown). On the other hand, the slowest program (2 ^{o}C/min) was not sufficient for separation of the methyl esters containing three deuterium atoms from the non-labeled, but it was enough to separate methyl esters with five deuteriums **(Fig. 30)**. The rate 8 ^{o}C/min was sufficient to fully separate compound with nine deuterium atoms (data not shown).

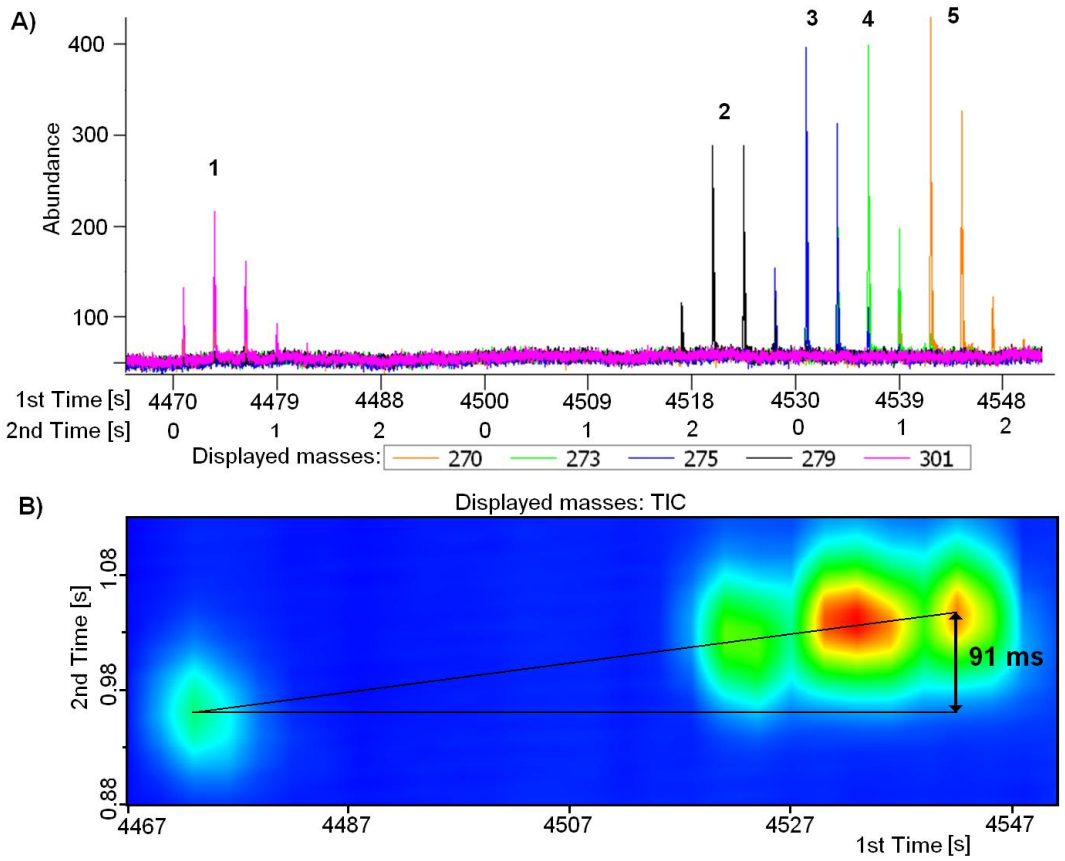


Fig. 30: Chromatograms of the two-dimensional comprehensive separation of the methyl hexadecanoates containing various number of deuterium atoms in the carbon chain. A) Linear visualization of the GC×GC chromatogram, 1:d_{31}-per-deuterated; 2:(13,13,14,14,15,15,16,16,16)-d_9; 3:(15,15,16,16,16)-d_5; 4:(16,16,16)-d_3; 5:d_0-unlabeled. B) Contour plot visualization of the GCxGC chromatogram. The peaks displayed in part A) correspond with the spots in the contour plot chromatogram in part B). [Primary oven (ZB-5MS): 50 ^{o}C (held for 1 min)-rate 2 ^{o}C/min-to 320 ^{o}C; secondary oven (RTX-50): 5 ^{o}C incremented with respect to the primary oven; modulation period: 3s; mobile phase: He (1 ml/min).]

The separation of the methyl esters labeled with $^{13}C_{16}$ atoms from the non-labeled analogs was unsuccessful in both dimensions as was expected based on the previous experiments performed for one dimensional separation **(Fig. 29)**. However, modulations at the beginning of the peak were enriched with the labeled compound **(Fig. 31)** and the modulations at the end were slightly enriched with the non-labeled analog. Only a minor separation was observed in the second dimension.

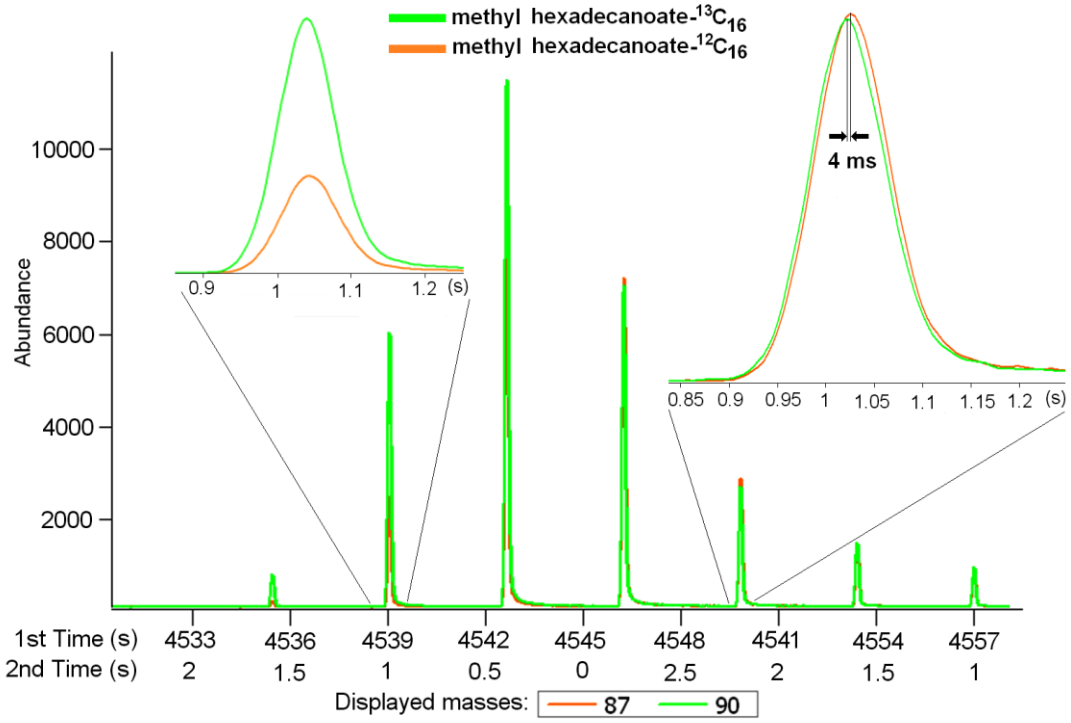


Fig. 31: Comparison of the retention behavior of the methyl hexadecanoate-$^{12}C_{16}$ and methyl hexadecanoate-$^{13}C_{16}$ in the two dimensional comprehensive separation – linear visualization. [Primary oven (ZB-5, 29 m, ID 0.25 mm): 50 ^{o}C (held for 1 min)-rate 2 ^{o}C/min-to 320 ^{o}C; secondary oven (RTX-50): 5 ^{o}C incremented with respect to the primary oven; Modulation period: 3 s; mobile phase: He (1 ml/min).]

We performed the analysis on two types of secondary columns **(Table 4)**. The chromatographic columns contained the same stationary phase but differed in the inner diameter and phase thickness **(Table 4)**. The He flow (mobile phase) was set to 1 ml/min for the comparative experiments, which was optimal for the first dimension column and also for the secondary column RTX-50 (our GC×GC instrumentation did not allow independent adjustment of the mobile phase flow for both primary and secondary columns). The secondary column BPX-50 showed a better separation power even if the He flow was faster than optimum (data not shown). Therefore, we used secondary column BPX-50 for the labeled metabolites analysis in the bumblebee pheromone research.

The experiments showed that the separation of the labeled compounds depends mostly on the separation efficiency of the used columns in the first and the second dimension, because all examined columns (in both dimensions) exhibited a similar selectivity.

5.1.2 Mass detection of the labeled compounds

The detection of labeled compounds had to be carefully optimized as well as the separation process. The biggest problem was the fact that the labeled metabolites have been expected in a substantially lower concentration in comparison to the non-labeled (native) compounds.

Preliminary results showed that the ration conc.(native)/conc.(labeled) could take values between 100 to 1000. Concerning the identification, the tremendous excess of the native (non-labeled) compound would cover the mass spectrum of the labeled metabolite without an efficient separation and make the detection and identification impossible. Based on this finding, we did not use compounds labeled with carbon isotopes for GC×GC analysis in the biosynthetic study.

The fragmentation of the deuterated compounds is analogical to the non-deuterated **(Fig. 32)**, however the observed proportionality between separation and number of deuterium atoms in the structure can be applied also for the selectivity and sensitivity of the detection: the more deuterium atoms are incorporated in the molecule, the higher number of specific mass fragments is formed, and, therefore, the mass detection is more sensitive **(Fig. 32)**. In the case of esters (both methyl and ethyl esters), a substantial increase in selectivity and sensitivity is obtained when the label is in the vicinity of the ester group, because the label is then incorporated to the abundant fragments (**Fig. 32-A** and **E:** *m/z* 77 and 91, *m/z* 74 and 87 respectively). The mechanism of the fragments *m/z* 74 (77), and *m/z* 87 (91) formation is called McLafferty rearrangement and γ-cleavage, respectively [109]. The other mass fragments highly specific for all deuterated compounds with aliphatic carbon chain are *m/z* 46 and 50 ($[C_3D_5]^+$, $[C_3D_7]^+$, respectively) **(Fig. 32)**. Their non-labeled analogs are fragments *m/z* 41 and 43. Specific fragments for deuterated aliphatic alcohols, except the *m/z* 46 and 50 are 34 $[C_2HD_4]^+$ and 62 ($[CD_3\text{-}CD{=}CD\text{-}C^+D_2]$. Examples of the mass spectra of the identified metabolites are shown in the figures: **Fig. 33, 34** and **35**.

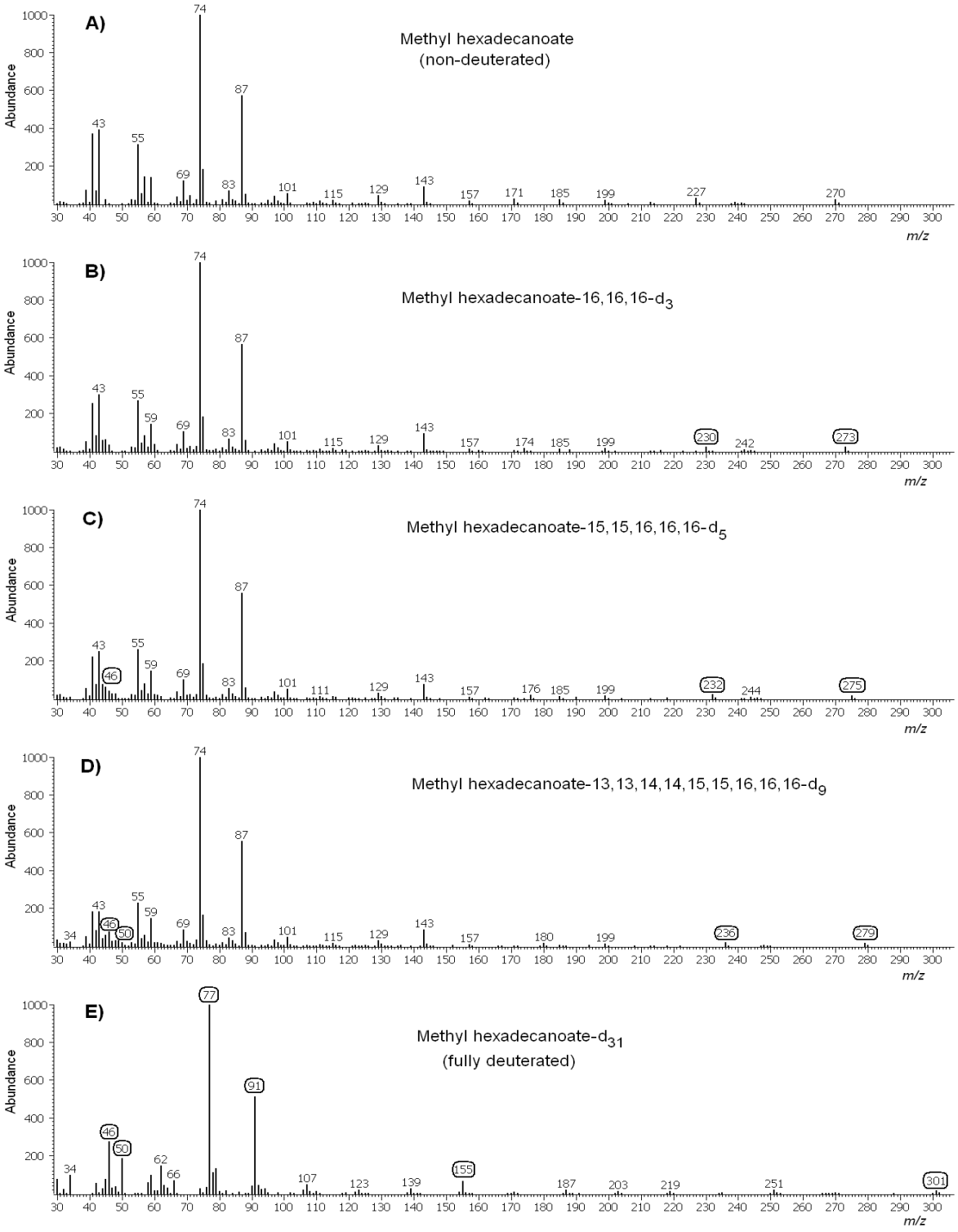


Fig. 32: Mass spectra of the methyl hexadecanoates containing various number of deuterium atoms in the carbon chain. The circled numbers are analytically important for selective detection of the particular compound in the chromatogram.

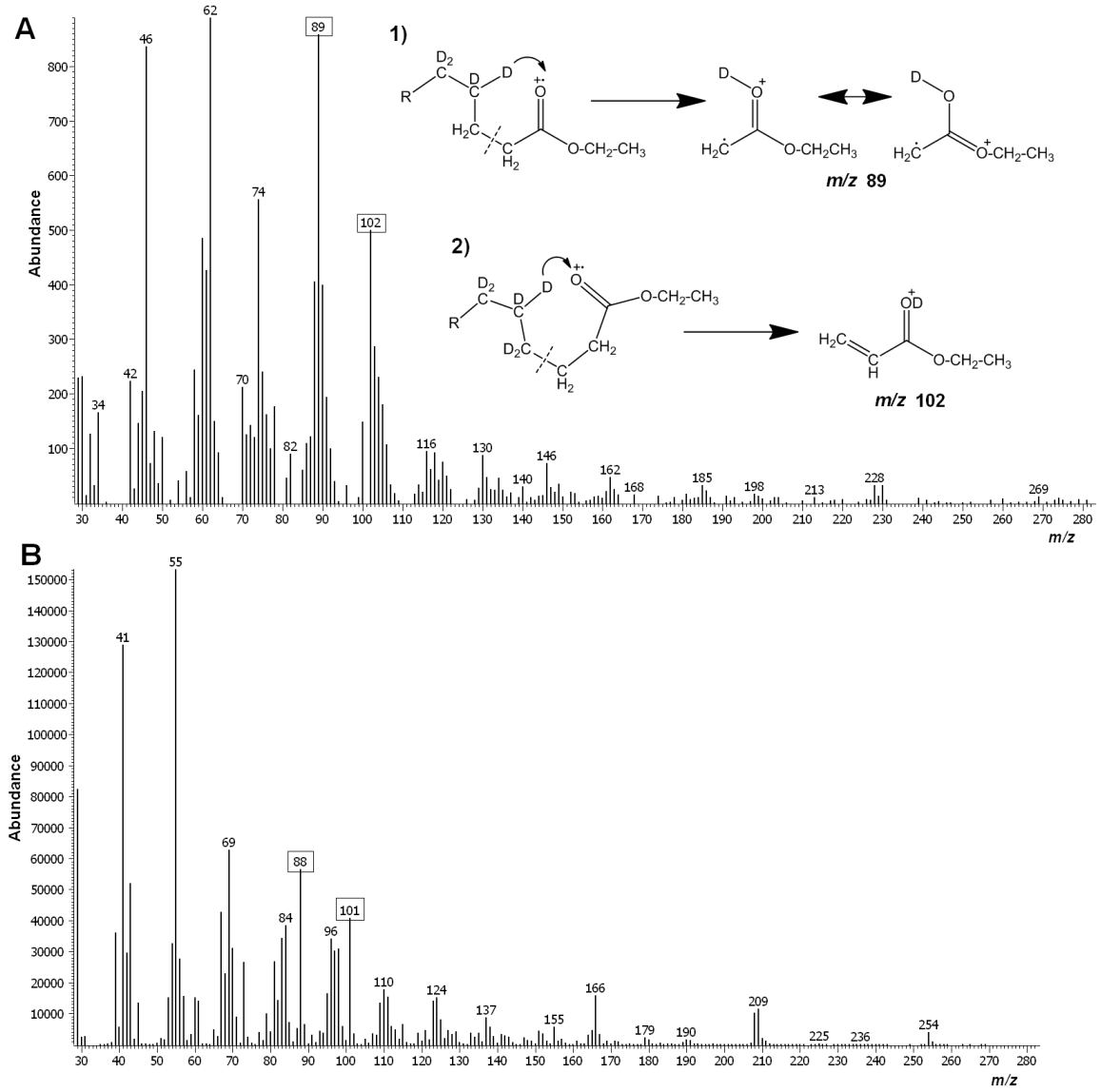


Fig. 33: Mass spectra of ethyl tetradec-9-enoates. A) Mass spectrum of ethyl tetradec-9-enoate-d_{21} that was biosynthesized from the per-deuterated dodecanoic acid-d_{23}. The schemes 1 and 2 presents the mechanisms of the formation of the masses *m/z* 89 (γ-cleavage) and 102 (McLafferty rearrangement [109]). These masses can be used for the confirmation of the structure of the esters with prolonged carbon chain. The molecular mass was not observed due to the low abundance. B) Mass spectrum of the non-deuterated ethyl tetradec-9-enoate. Highlighted masses *m/z* 88 and 101 are analogical with those in the spectrum A.

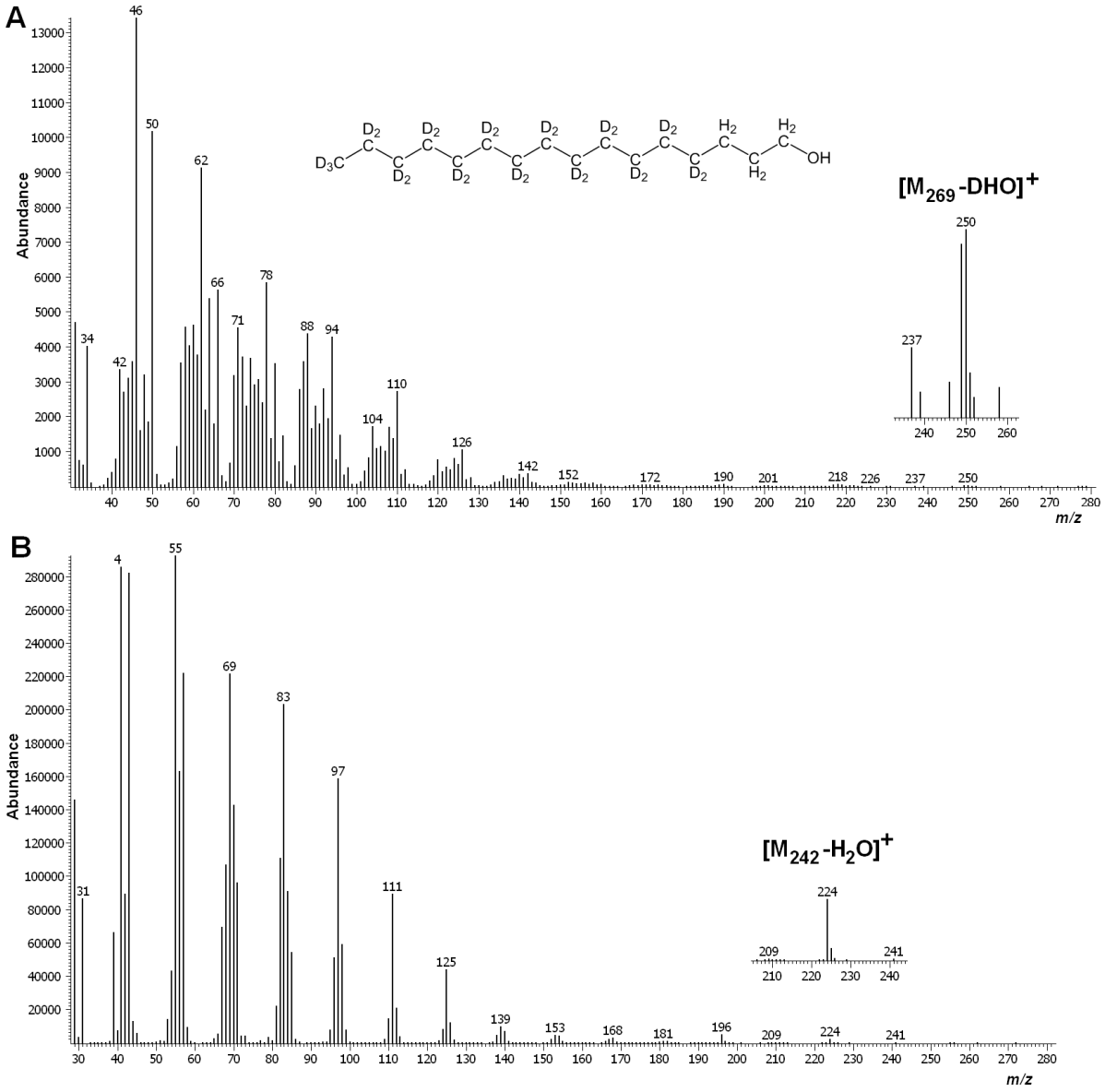


Fig. 34: Mass spectra of tricosanes. A) Mass spectrum of tricosane-d_{27}. This compound was formed by extending of the carbon chain of the per-deuterated tetradecanoic acid-d_{27}. Mass spectrum contains both masses from the deuterated (*m/z* 46, 50, 62, 78…) and non-deuterated (*m/z* 43, 57, 71, 85…) part. Compare with mass spectrum in B-section; B) Spectrum of the non-deuterated tricosane.

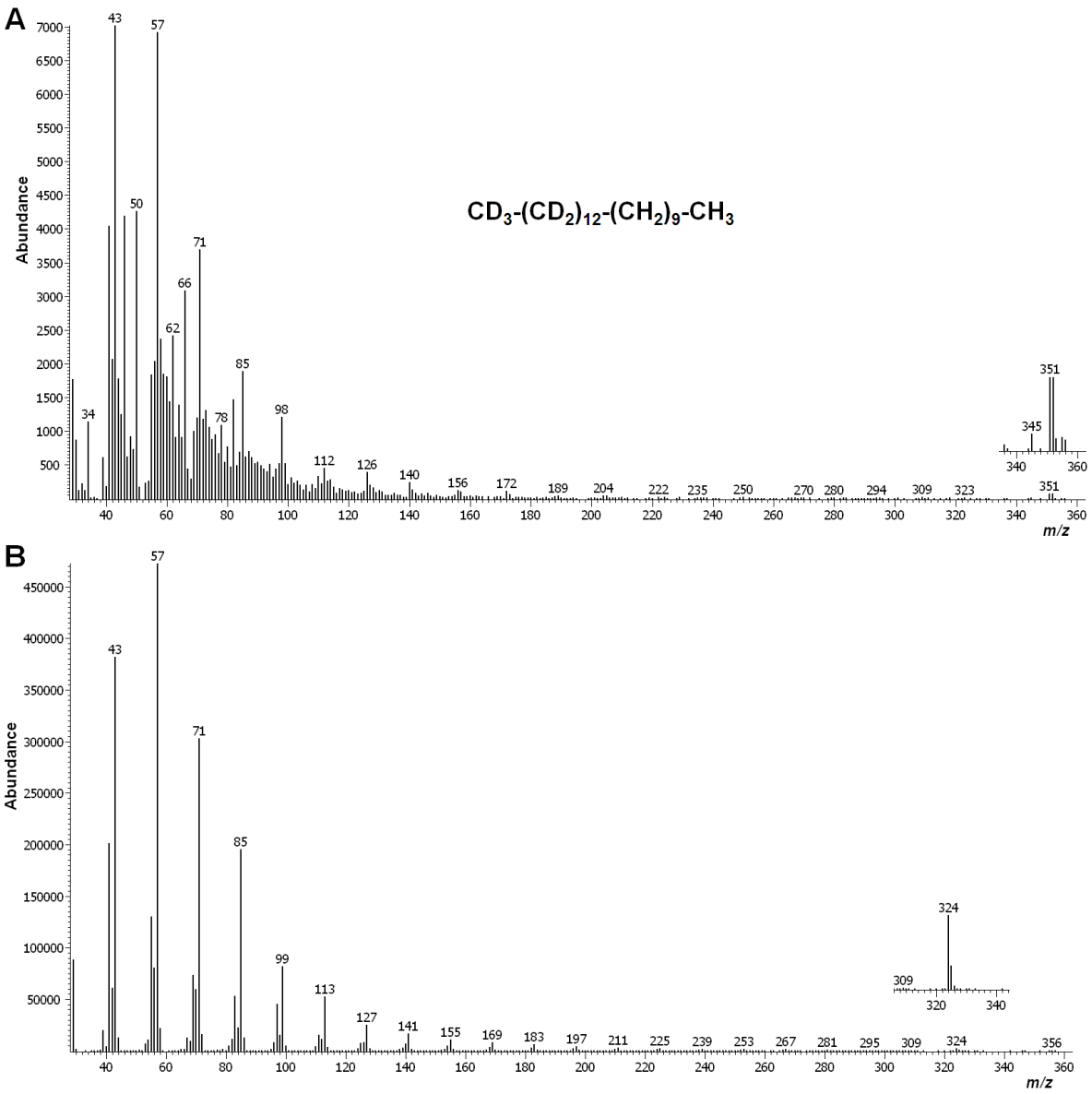


Fig. 35: Mass spectra of hexadecanols. A) Mass spectrum of hexadecanol-d_{27}. This compound was formed by extending of the carbon chain of the per-deuterated tetradecanoic acid-d_{27}. The highest molecular mass observable in the mass spectrum is *m/z* 250, which is molecular mass lowered by mass of water – in this case partly deuterated [109]. The analogical mass is observed in the mass spectrum in the B-section; B) Mass spectrum of the non-deuterated hexadecanol.

In many cases, the abundance of the deuterated metabolites was not high enough to obtain a high-quality mass spectrum that could alone be used for an unambiguous identification of the structure. Nevertheless, the characteristic retention behavior in the GC×GC chromatogram together with the appearance of the characteristic fragments was sufficient for the deuterated metabolite structure elucidation **(Fig. 36** and **43)**.

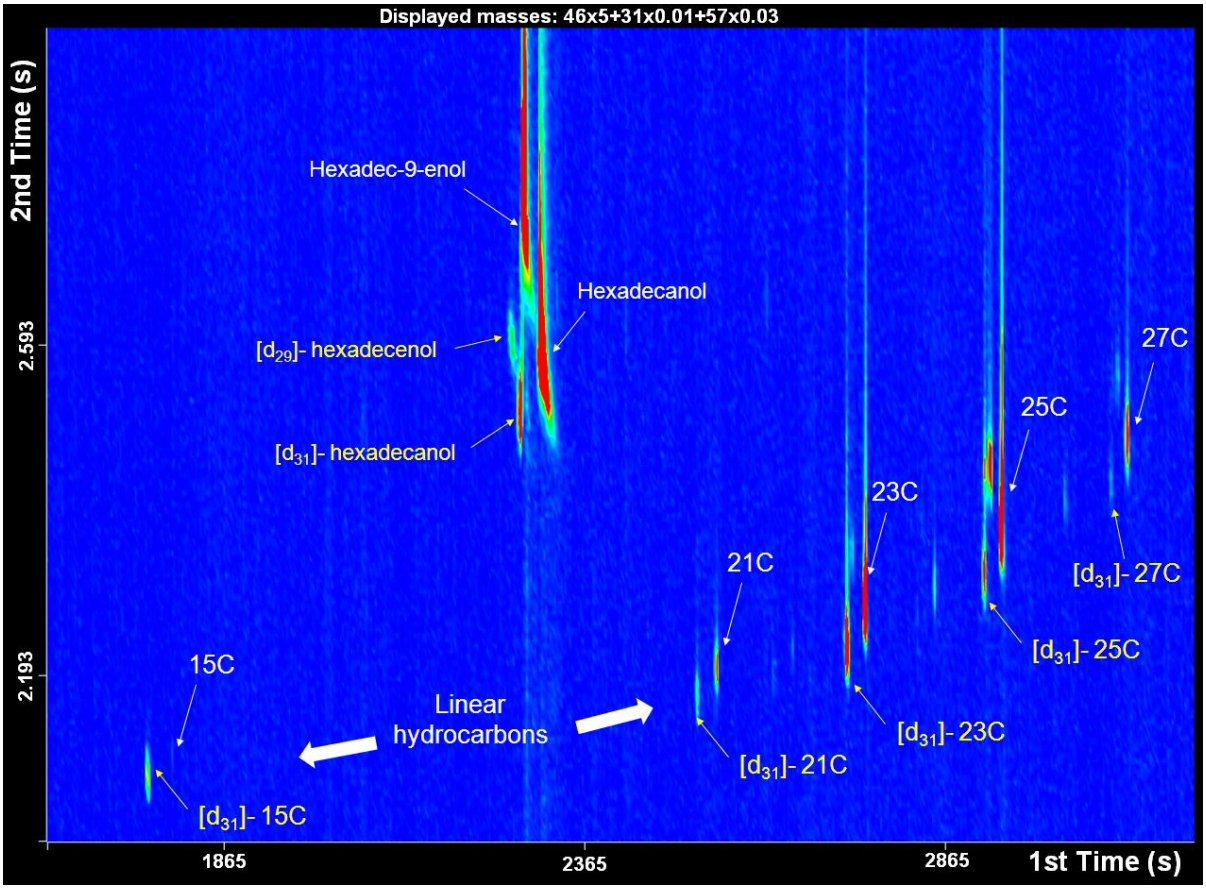


Fig. 36: An example of the analysis of the hexane extract of the labial gland of the *Bombus lapidarius* male that was incubated *in vivo* with hexadecanoic acid-d_{31}. [Primary oven (DB-5): 40 ^{o}C (held for 2 min)-rate 5 ^{o}C/min-to 320 ^{o}C (held for 5 min); secondary oven (BPX-50): 5 ^{o}C incremented with respect to the primary oven; modulation period: 3 s; mobile phase: He (1 ml/min).]

A combination of a specific fragment detection and GC×GC separation creates a powerful analytical tool for the analysis of the deuterated compounds in complex mixtures.

5.1.3 Biology vs. analytics

It was shown in the previous chapters that the higher number of labeled atoms is incorporated in the structure, the higher is the isotopic effect, thus bigger differences in chemical and physical properties. The living organisms use highly efficient enzymatic systems, which are able to recognize specific structures. Therefore, the labeling of the substrate can affect the ability of the labeled compound to be used for enzymatic transformation or the results could lead to the misinterpretation of the studied biochemical processes. Nevertheless, we decided to use fully deuterated compounds for the bumblebee male's sexual pheromone biosynthesis study because a much higher sensitivity of the detection and a more confident structure identification of the metabolites could be achieved. However, we are aware, that some of the biological interpretations could be affected by the isotopic effect of the used labeled compounds.

Fig. 37: Bumblebee queen and male of *Bombus lucorum* species during copulation.

Fig. 38: Bumblebee male of *Bombus terrestris* species.

Fig. 39: Bumblebee male of *Bombus lapidarius* species.

5.2 Biosynthesis study of the bumblebee males species *Bombus terrestris*, *Bombus lucorum,* and *Bombus lapidarius*

Males of the most bumblebee species (genus *Bombus*) exhibit a mating strategy unique among other social bees. They patrol regular circuits and pause on some prominent objects, such as tree trunks, flowers or stones. This behavior has been observed already in 1851 by Newman and later by Darwin [110,111]. It was proposed by Sladen in 1912 that the males deposit odors at the sites and attract queens [112]. He observed that the sites acquired the same odor as the males themselves, and proposed that the secretion emanates from the male´s head. This suggestion was supported by Hass [113]. First chemical analysis was performed by Stein in 1963 on species *B. terrestris* [114]. He separated pentane head extracts of males by means of thin layer chromatography and used infrared and UV spectroscopy for the most abundant compound structure determination. He identified the main component as farnesol. However, subsequent GC-MS analysis showed that the main component was (*S*)-2,3-dihydro-6-*trans*-farnesol (2,3-dihydrofarnesol), not farnesol [115,116]. Later on, Kullenberg found that the secretion is released from the cephalic part of the labial gland [117]. This secretory tissue consists of numerous acini, formed by 20-30 associated cells, and connecting ducts **(Fig. 40)** [118,119].

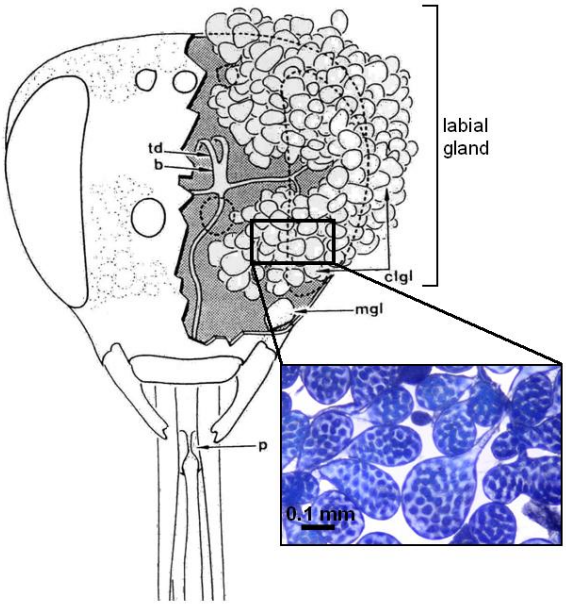


Fig. 40: Semidiagrammatic drawing of a bumblebee male head (frontal view) and optical microscope micrograph of the labial gland acinus with the secretory cells. (Figure based on Ágren, 1979 [118])

The male's sex pheromone consists mostly of aliphatic fatty acid derivatives (esters, aldehydes and alcohols) and/or terpenic alcohols. The chemical composition is species specific [105,115,117,120-130]

Biosynthesis of the pheromone components remains unclear, but Lanne proposed that the aliphatic components may be produced from saturated fatty acids by the action of appropriate enzymes (desaturases, esterases, reductases, decarboxylases...) [126,131]. First experimental results suggested that the fatty acids, as the precursors, are stored in a form of triacylglycerols in the fat body in the abdomen, released subsequently as diacylglycerols and transported to the labial gland via haemolymph when needed [131,132]. An alternative hypothesis suggests *de novo* biosynthesis of the fatty acids in the labial gland [131]. No data about the terpenic compounds biosynthesis in bumblebees are available so far.

The aim of this Thesis was to study biosynthesis of the main components of the sex pheromone of *Bombus terrestris* **(Fig. 38)**, *Bombus lucorum* **(Fig. 37)**, and *Bombus lapidarius* **(Fig. 39)** males. The labial gland of *B. terrestris* males contains a mixture of aliphatic and terpenic compounds, where 2,3-dihydrofarnesol and ethyl dodecanoate are the most abundant [104]. In the case of *B. lucorum* the labial gland consists almost entirely of aliphatic compounds, with ethyl (*Z*)-tetradec-9-enoate (53%) being the most abundant [105]. *B. lapidarius* contains only aliphatic compounds, too. Unlike the *B. lucorum* species, the most abundant components in *B. lapidarius* are alcohols (Z)-hexadec-9-en-1-ol (52%) and hexadecan-1-ol (31%) [119].

All animals of the studied species originated from the laboratory breeding. It was necessary to work with individuals that grew under defined conditions, such as temperature, sufficient food supply, and were of defined age. The establishment and maintenance of laboratory breeding of bumblebees is not a simple matter and every species needs its own strategy [133]. For this reason, only *B. terrestris* was available throughout the year in a sufficient number. Therefore, the most experiments were carried out with this species.

5.2.1 Pheromone components determination

Despite the fact that compositions of male's labial gland secretions of the most of the bumblebee species have been studied in details by many authors, contribution of the individual components of the secretion to the pheromone is not clear. Basically, only compounds that can be perceived by queen's antennae are the possible sex pheromone components. Thus, we used a method of GC-EAD to determine the antennally-active compounds. The experiment was performed with *B. terrestris* and *B. lucorum* species. The hexane extracts of the male labial glands were separated on GC and the individual components were transferred via a transferline to the EAD consisting of an antenna of a conspecific queen **(Fig. 41)**.

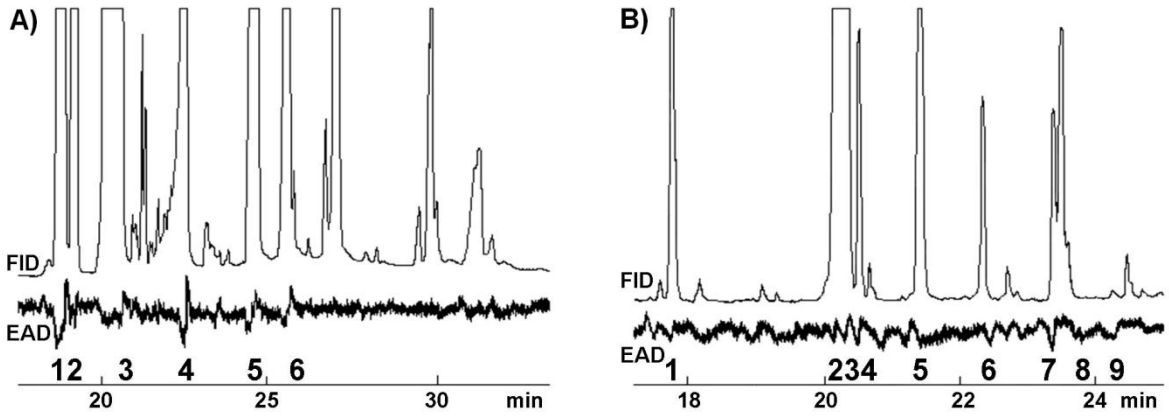


Fig. 41: GC-EAD record of the labial gland extract of 7 days old male. A) *B. terrestris*: **1** ethyl dodecanoate; **2** 2,3-dihydrofarnesal; **3** 2,3-dihydrofarnesol; **4** hexadecanol; **5** octadeca-9,12,15-trienol; **6** geranylcitronellol; B) *B. lucorum*: **1** ethyl dodecanoate, **2** ethyl tetradec-7-enoate, **3** ethyl tetradec-9-enoate, **4** hexadec-7-enal; **5** ethyl hexadec-9-enoate; **6** hexadecanol; **7** octadeca-9,12-dienol; **8** octadeca-9,12,15-trienol; **9** octadecanol. [Oven temperature program (DB-5): 50 ^{o}C (held for 2 min)-rate 30 ^{o}C/min-to 270 ^{o}C; mobile phase: He (1 ml/min).]

The response of the queen's antenna to the particular compounds was poor and unstable. However, repeated measurements revealed six and nine active compounds in *B. lucorum* and *B. terrestris* species, respectively **(Fig. 41)**. These compounds could be considered as the putative components of the sexual pheromone. However, the "pheromone" that triggers the biological behavior probably concerns a synergic effect of all of the active compounds that should be perceived at the same time. These EAD recordings are the first reported on bumblebees, thus a deeper investigation is needed.

5.2.2 Dynamics of the pheromone components production

The pheromone components were quantified in the males of different ages. The experiment was performed with *B. terrestris* and *B. lucorum* species. Labial glands of males of defined ages varying between 0 (shortly after eclosion) and 33 days were analyzed by means of GC-MS and the antennally-active compounds were quantified **(Fig. 42)**.

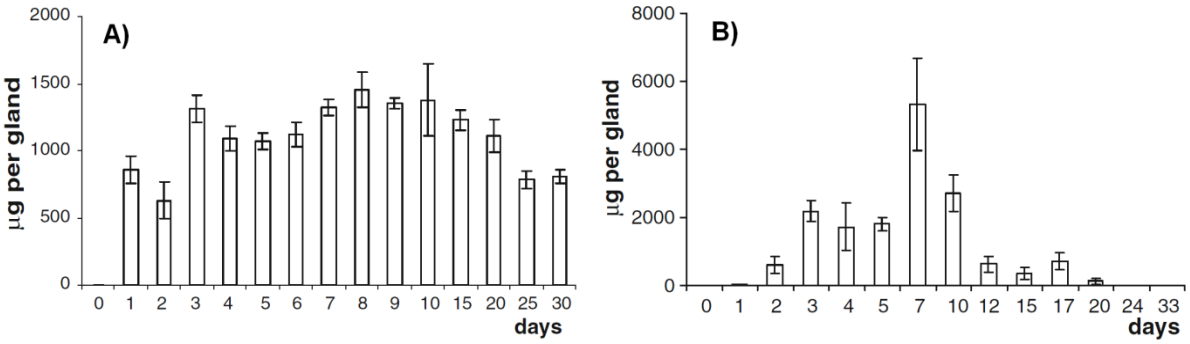


Fig. 42: Concentration changes with regard to age of adult male of the most abundant antennally-active compounds in the labial gland; (mean ± standard error). A) *B. lucorum*: ethyl (*Z*)-tetradec-9-enoate + ethyl (*Z*)-tetradec-7-enoate; B) 2,3-dihydrofarnesol.

The quantification of the particular compounds within the male's age showed significant differences in the dynamics of the pheromone quantity in the labial gland between both examined species. The pheromone production in *B. lucorum* starts shortly after eclosion and reaches a maximum within three days. The labial gland content remains relatively constant over the adult life of the males. On the other hand, *B. terrestris* starts the production between the second and the third day and reaches maximum between fifth and tenth days. Then the labial gland content decreases sharply. The concentration profile of the displayed compounds in the **Fig. 42** corresponds to the profile of the rest of the antennally-active compounds for both species. A microscopy of the labial glands confirmed the results from the GC-MS analysis. *B. lucorum* secretory cells of the labial gland remain active throughout the male's lifespan, while in *B. terrestris* the cells die after several days of secretory activity (between the 5th and 10th day). A possible explanation could be a toxic effect of the terpenic alcohols on the cells of the labial glands in *B. terrestris*, or a different biosynthetic strategy of both species. This will be a subject of a further research.

5.2.3 *In vivo* incubation of the bumblebee males with labeled fatty acids

Results from quantification experiments of the labial gland contents were used for selection of the males of the suitable age (2 days) for the following *in vivo* incubation. The aim of the experiment was to support Lanne's hypothesis that the aliphatic components of the pheromone are synthesized from fatty acids [126]. The experiment was performed with *B. lucorum* and *B. lapidarius* species, because their sex pheromone contains exclusively aliphatic compounds [105,120]. The experiment consisted in application of per-deuterated fatty acids of various carbon chain lengths into the head capsule or abdomen of the living insects and, after a certain time of incubation (2 – 4 days), the analysis of the labeled metabolites in the particular tissues' extracts. The application procedure (application solution composition, concentration of the deuterated substrate in the application solution, total

volume injected to the males' body) has been developed as a compromise between the acceptable level of mortality of the animals and the sufficient amount of the injected deuterated fatty acid. Mixture of dimethylsulfoxide:ethanol:water (7:2:1) was found to be the most suitable. The application dose per animal was 2 μl of the application solution containing 80 μg of perdeuteated fatty acid of a particular chain length. In the case of applications to the head, the average mortality was about 20 % while in abdomen-applications, the mortality was lower – approximately 10 %.

A new analytical tool was used for the analysis of the deuterated metabolites: two-dimensional comprehensive gas chromatography coupled with mass spectrometric detector. The study involved an analytical method development including a study of the retention behavior of the labeled compounds in one dimensional and two-dimensional comprehensive gas chromatography systems, and their detection and identification (see chapter: 5.1 Optimization of the analysis of the labeled aliphatic esters).

The chemical analysis of the extracts of the dissected labial glands and selected fat bodies of the incubated animals (one animal per sample) showed a presence of various types of deuterated metabolites in both examined species **(Table 5** and **Table 6)**. The examples of the chromatograms with selectively displayed deuterated metabolites and their non-labeled analogs are given in **Fig. 36** (*B. lapidarius*) and **Fig. 43** (*B. lucorum*).

The abundance of the deuterated metabolites found in the tissues was very low. Less than 5 % of the applied amount of the deuterated fatty acids was transformed into the pheromone components according to our estimation. The likely explanation is that the substantial amount of the pheromone is synthesized from the male's own sources. It was found by Jiroš that bumblebee male's hatch with fully developed fat body [134], which could serve as a source of fatty acids.

No qualitative differences of the deuterated metabolites were observed between animals with applications of the precursors to the head capsule or abdomen. In general, the deuterated fatty acids were transformed into the most abundant aliphatic compounds that are naturally present in the labial glands of both species. The deuterated metabolites were of the same carbon chain length as the applied fatty acid or longer. No shortening of the deuterated carbon chain was observed (β-oxidation process). The most probable explanation is an appearance of the isotopic effect of the deuterium. In the case of hydrocarbons there were found one carbon shorter compounds than the applied precursor. However, this was probably caused by an action of an appropriate decarboxylase resulting in a cleavage of carboxylic group and, therefore, shortening of the precursor by one carbon atom. The β-oxidation process would shorten the carbon chain by two carbons.

Table 5: List of the deuterated metabolites found in *B. lucorum* [135].

B. lucorum	**Deuterated metabolites**		
	Labial gland	Fat body	
		Analyzed as methyl esters	
Applied acid		MAG + DAG	TAG
dodecadecanoic acid-d_{23} CD_3-$(CD_2)_{10}$-COOH 8 animals: 4 head, 4 abdomen	Ethyl dodecanoate, CD_3-$(CD_2)_{10}$-COO-CH_2-CH_3 Ethyl tetradecanoate + Ethyl tetradec-9-enoate CD_3-$(CD_2)_{10}$-$(CH_2)_2$-COO-CH_2-CH_3 Ethyl hexadecenoate + Ethyl hexadec-9-enoate CD_3-$(CD_2)_{10}$-$(CH_2)_4$-COO-CH_2-CH_3 Hexadecanol CD_3-$(CD_2)_{10}$-$(CH_2)_5$-OH Hexadecanal CD_3-$(CD_2)_{10}$-$(CH_2)_4$-COH Hexadecyl acetate CD_3-$(CD_2)_{10}$-$(CH_2)_4$-OOC-CH_3 Hydrocarbons (n = 9,11,13) CD_3-$(CD_2)_{10}$-(CH_2)n-CH_3	Not analyzed	Not analyzed
tetradecanoic acid-d_{27} CD_3-$(CD_2)_{12}$-COOH 18 animals: 6 head, 12 abdomen	Ethyl tetradecanoate + Ethyl tetradec-9-enoate, Ethyl tetradec-7-enoate CD_3-$(CD_2)_{12}$-COO-CH_2-CH_3 Ethyl hexadecanoate + Ethyl hexadec-9-enoate + Ethyl hexadec-7-enoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_2-CH_3 Hexadecyl acetate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-OOC-CH_3 Hexadecanol CD_3-$(CD_2)_{12}$-$(CH_2)_3$-OH Hexadecanal CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COH Hydrocarbons (n= 0[a], 7, 9, 11[b], 13) CD_3-$(CD_2)_{12}$-(CH_2)n-CH_3	Methyl tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 Methyl hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3 Methyl octadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_4$-COO-CH_3	Methyl tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 Methyl hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3 Methyl octadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_4$-COO-CH_3
hexadecanoic acid-d_{31} CD_3-$(CD_2)_{14}$-COOH 2 animals: 1 head, 1 abdomen	Ethyl hexadecanoate + Ethyl hexadec-9-enoate CD_3-$(CD_2)_{14}$-COO-CH_2-CH_3 Hexadecyl acetate CD_3-$(CD_2)_{14}$-OOC-CH_3 Hexadecanol CD_3-$(CD_2)_{14}$-CH_2-OH Hexadecanal CD_3-$(CD_2)_{14}$-COH Ethyl octadecanoate CD_3-$(CD_2)_{14}$-$(CH_2)_2$-COO-CH_2-CH_3 Hydrocarbons (n=0[a], 5, 7, 9) CD_3-$(CD_2)_{14}$-(CH_2)n-CH_3	Not analyzed	Not analyzed
octadecanoic acid-d_{35} CD_3-$(CD_2)_{16}$-COOH 10 animals: 5 head, 5 abdomen	Ethyl octadecanoate + Ethyl octadec-9-enoate CD_3-$(CD_2)_{16}$-COO-CH_2-CH_3 Icosanol CD_3-$(CD_2)_{16}$-$(CH_2)_3$-OH Hydrocarbons (n = 0[a], 3, 5, 7, 9) CD_3-$(CD_2)_{16}$-(CH_2)n-CH_3	Not analyzed	Not analyzed

a...hydrocarbon is one carbon shorter than the applied acid;

b…unsaturated analog was observed (double bond position was not determined).

Table 6: List of the deuterated metabolites found in *B. lapidarius*.

B. lapidarius	**Deuterated metabolites**		
	Labial gland	Fat body	
		Analyzed as methyl esters	
Applied acid		MAG + DAG	TAG
tetradecanoic acid-d_{27} CD_3-$(CD_2)_{12}$-COOH 16 animals: 7 head, 9 abdomen	Hexadecanol + Hexadecenol[a] CD_3-$(CD_2)_{12}$-$(CH_2)_3$-OH Hydrocarbons[b] CD_3-$(CD_2)_{12}$-$(CH_2)_n$-CH_3	Methyl tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 Methyl hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3	Methyl tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 Methyl hexadecanoate + Methyl hexadecenoate[a] CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3
hexadecanoic acid-d_{31} CD_3-$(CD_2)_{14}$-COOH 19 animals: 6 head, 13 abdomen	Hexadecanol + Hexadecenol[a] CD_3-$(CD_2)_{14}$-CH_2-OH Hydrocarbons[b] CD_3-$(CD_2)_{14}$-$(CH_2)_n$-CH_3	Methyl hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3	Methyl hexadecanoate + Methyl hexadecenoate CD_3-$(CD_2)_{14}$-COO-CH_3
octadecanoic acid-d_{35} CD_3-$(CD_2)_{16}$-COOH 30 animals: 6 head, 24 abdomen	Hydrocarbons[b] CD_3-$(CD_2)_{16}$-$(CH_2)_n$-CH_3	Methyl octadecanoate, CD_3-$(CD_2)_{16}$-COO-CH_3	Methyl octadecanoate + Methyl octadecenoate[a] CD_3-$(CD_2)_{16}$-COO-CH_3

a...double bond position was not determined due to the low abundance of the deuterated metabolite;
b...n is an odd number, the shortest hydrocarbon is one carbon shorter than applied acid.

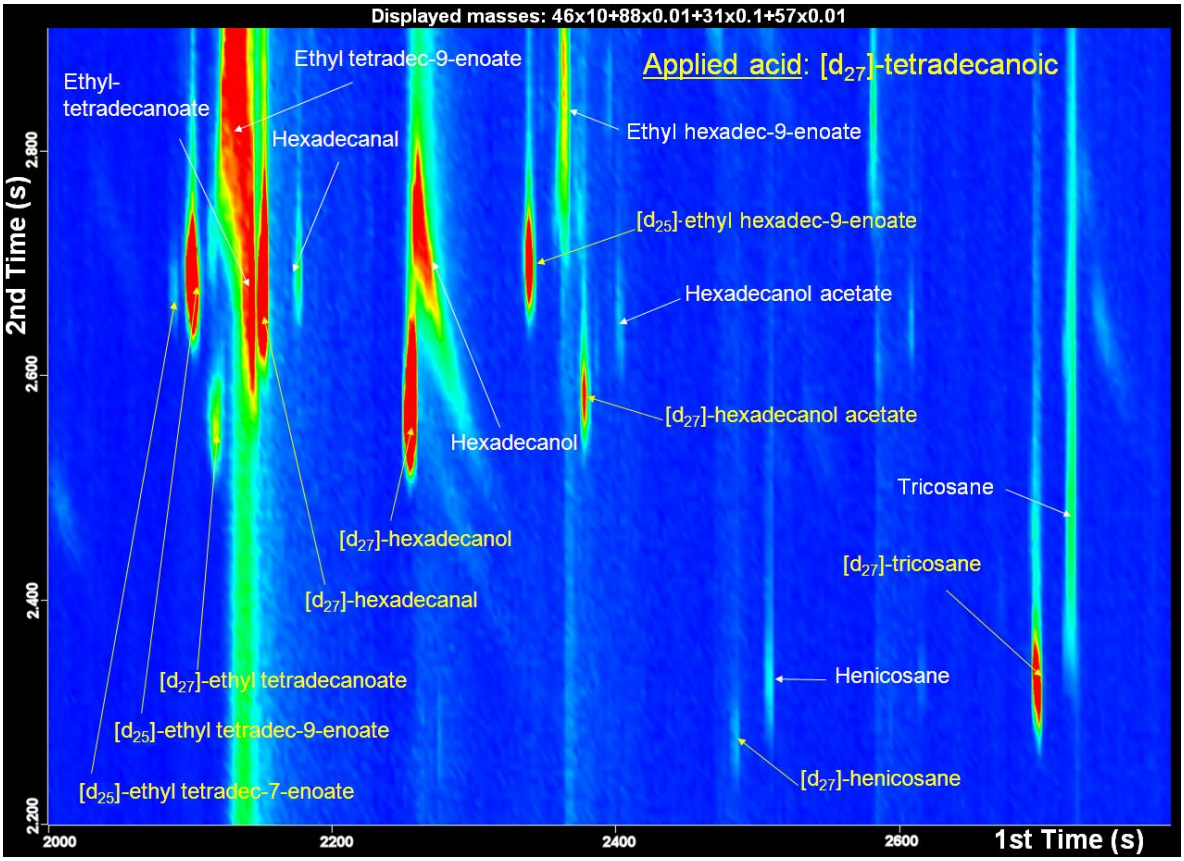


Fig. 43: Chromatogram of the labial gland extract of a *B. lucorum* male that was incubated *in vivo* with tetradecanoic acid-d_{27}. [Primary oven (DB-5): 40 °C (held for 2 min)-rate 5 °C/min-to 320 °C (held for 5 min); secondary oven (BPX-50): 5 °C incremented with respect to the primary oven; modulation period: 3 s; mobile phase: He (1 ml/min).]

Analytical results showed some details regarding of the metabolism of the applied deuterated precursors. For example: abundant components in the *B. lucorum* labial gland are ethyl (*Z*)-tetradec-9-enoate and also ethyl (*Z*)-hexadec-9-enoate. The deuterated analog of the compound was formed from the applied dodecadecanoic acid-d_{23} and also tetradecanoic acid-d_{27}. The fact that the double bond in the deuterated analogs with prolonged carbon chain was located in the 9th position too, suggests that the applied precursors were prolonged first and then desaturated (dehydrogenated) **(Fig. 44)**.

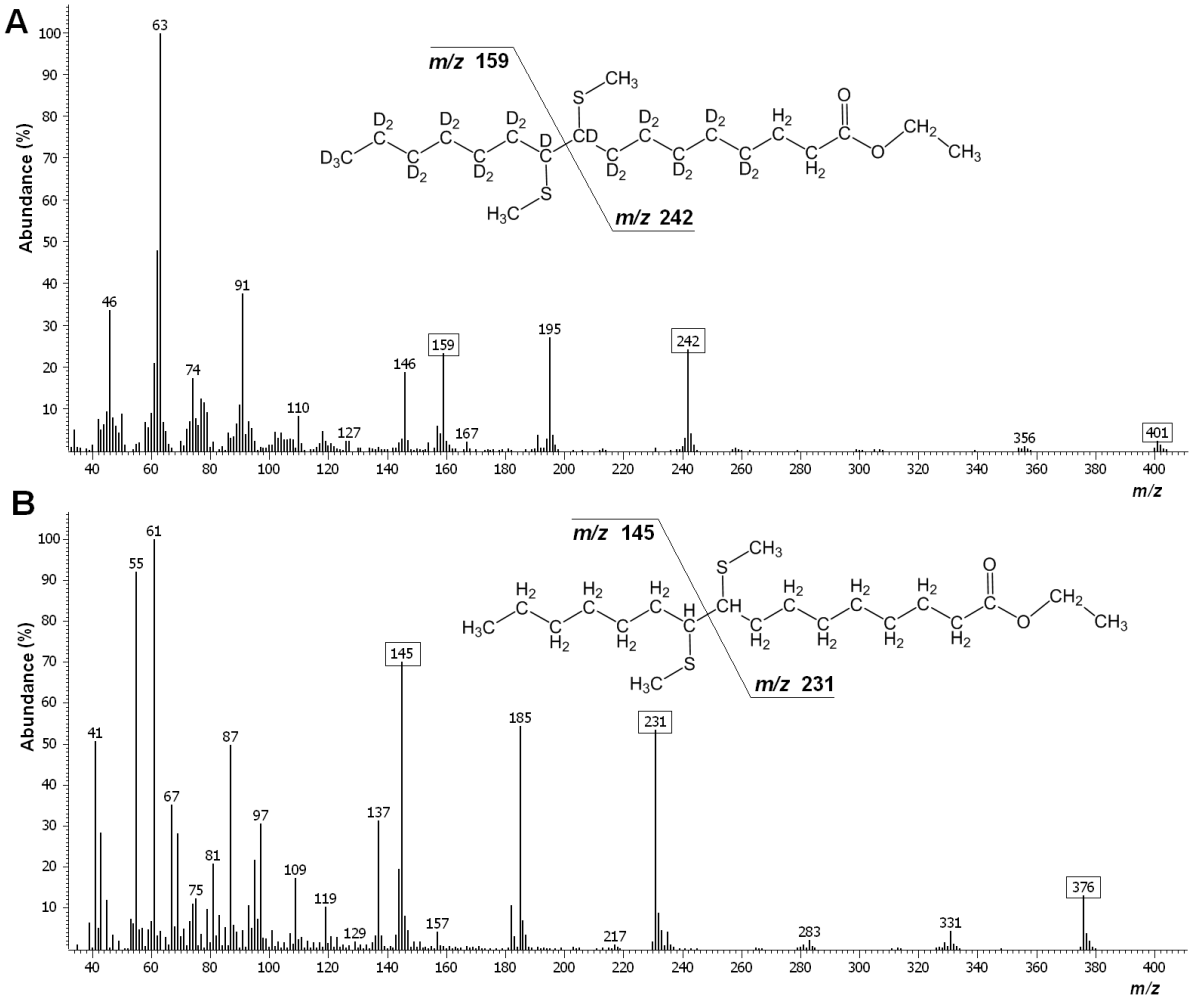


Fig. 44: Mass spectrum of ethyl hexadecenoate after the derivatization of the *B. lucorum* labial gland extract (applied per-deuterated tetradecanoic acid) by means of DMDS for double bond position determination. A) Mass spectrum of derivatized ethyl hexadec-9-enoate-d_{25}. The masses *m/z* 199, 242 and 401 clearly show that the biosynthetic precursor of this compound was per-deuterated tetradecanoic acid; B) Mass spectrum of the analogical derivatized non-deuterated ethyl hexadec-9-enoate.

The analysis of fat bodies confirmed that the deuterated fatty acids were incorporated into the triacylglycerols and also to the transport form of the fatty acids – diacylglycerols. In the case of *B. lucorum* species, the analysis of the fat body was performed for the application of per-deuterated tetradecanoic acid only **(Table 5)**. We expected a presence of

tetradec-9-enoic acid, which would correspond with the most abundant component in the labial gland – ethyl tetradec-9-enoate [105]. However, no unsaturated deuterated fatty acids were found in fat body of *B. lucorum*. In the case of *B. lapidarius*, the experiment was extended and a fat body was analyzed for all applied deuterated fatty acids. Similarly to *B. lucorum,* we expected a presence of deuterated hexadec-9-enoate – one of the most abundant labial gland components [120]. The hexadecenoate was finally observed in the triacylglycerol fraction (also as a result of prolongation of the per-deuterated tetradecanoic acid), but the position of the double bond could not have been determined due to the low abundance of the compound in the sample.

These *in vivo* incubation experiments were not evaluated quantitatively, due to the observed influence of the isotopic effect that could affect the results, but the qualitative results of these experiments confirmed that fatty acids can serve as precursors of the aliphatic pheromone components. However, the experimental setting could not give a clear answer about the origin of the fatty acids, because in both types of applications (to the head capsule and to the abdomen), the free fatty acids were injected to the haemolymph (insect's "blood"), which could distribute them throughout the body in a short time. For this reason, a new type of experiment – feeding of the males with labeled fatty acids and analysis of the incorporation of the precursors into the fat body, haemolymph and labial gland - will be performed as the next step of the research. The results should reveal which fatty acids are mobilized from the fat body to the haemolymph and if any further selection of the particular fatty acids exists between the haemolymph and the labial gland. However, it will be necessary to both quantitatively and qualitatively determine the influence of the deuterium (for example by simultaneous using of the fatty acids with partly deuterated carbon chain or using a different label – ^{13}C, ^{14}C; nevertheless, this would probably need a different analytical approaches).

5.2.4 *In vitro* incubation of the bumblebee males tissues

The *in vivo* experiments with the labeled fatty acids showed that the labial gland is able to transform the fatty acids into the aliphatic pheromonal components, however did not exclude the second hypothesis that the synthesis of the fatty acids can also proceed in the labial gland *de novo*. To support the hypothesis of *de novo* formation of the pheromonal components, the labial glands and fat bodies were incubated *in vitro* with sodium acetate as the biosynthetic precursor, labeled with radioactive carbon (^{14}C) or deuterium (^{2}H), under optimized incubation conditions (incubation solution composition, incubation duration and temperature). The experiment was performed with males of *B. terrestris* and *B. lucorum*. Various methods of chemical analysis were optimized and used for sampling and analysis of the labeled metabolites.

The radioactive metabolites were analyzed in the labial gland and fat bodies extracts by means of TLC and position sensitive radioactivity detector **(Fig. 45)**. The analysis showed that different types of compounds were formed in fat bodies and labial glands in both species. While the radioactive products in fat bodies were similar in both species, the radioactive metabolites found in the labial glands differed. There was found a substantial amount of terpenic alcohols in *B. terrestris*. However, the TLC method could not determine the particular structure of the radioactive metabolites. For this reason a method of preparative gas chromatography was used. In the case of *B. terrestris* the radioactive spot containing fatty acids **(Fig. 45 A-3)** was scraped off and the fatty acids were fractionized according to their carbon chain lengths by means of preparative gas chromatography. The subsequent analysis of the radioactivity distribution showed that hexadecanoic acid was synthesized predominantly.

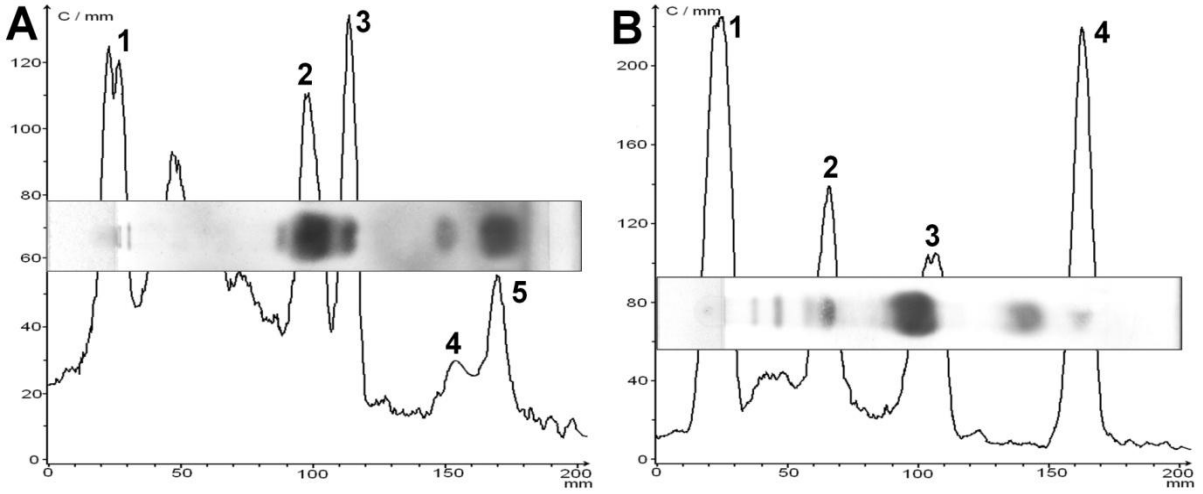


Fig. 45: Chromatograms of TLC radioactive signals (*B. terrestris*). A) LG chloroform extract and non-selective visualization of the corresponding TLC plate. Signal identification: 1-polar lipids, 2-terpenic alcohols, 3-free fatty acids (FFA), 4-esters, 5-hydrocarbons; B) FB chloroform extract and non-selective visualization of the corresponding TLC plate. Signal identification: 1-polar lipids, 2- fatty acids, 3-triacylglycerols, 4-hydrocarbons.

This result was confirmed by incubation with deuterated sodium acetate and a following GC×GC analysis. The metabolites formed *de novo* from the deuterated acetate were recognized and identified according to typical features in their mass spectrum **(Fig. 46-A)**, **(Fig. 47-A)**. The spectra reflect ion clusters containing deuterium randomly distributed within the structure. The ion clusters follow the same pattern as the mass spectra of non-deuterated analogues and the apex mass in the clusters is always higher than the corresponding mass in the spectra of native analogues due to the presence of deuterium atoms. This difference in mass corresponds to the most probable number of deuterium atoms present in the ion. The average number of deuterium atoms incorporated in the molecule could be estimated according to the most abundant mass in the molecular mass

cluster. There were found compounds that could be unambiguously assigned to the particular structures - deuterated ethyl esters (containing deuterium in the alcoholic or in the acid part **(Fig. 47)**) and terpenic compounds (2,3-dihydrofarnesol **(Fig. 46)** and geranylcitronellol). Furthermore, the analysis found the same concentration proportion between labeled and native 2,3-dihydrofarnesol and geranylcitronellol (data not shown).

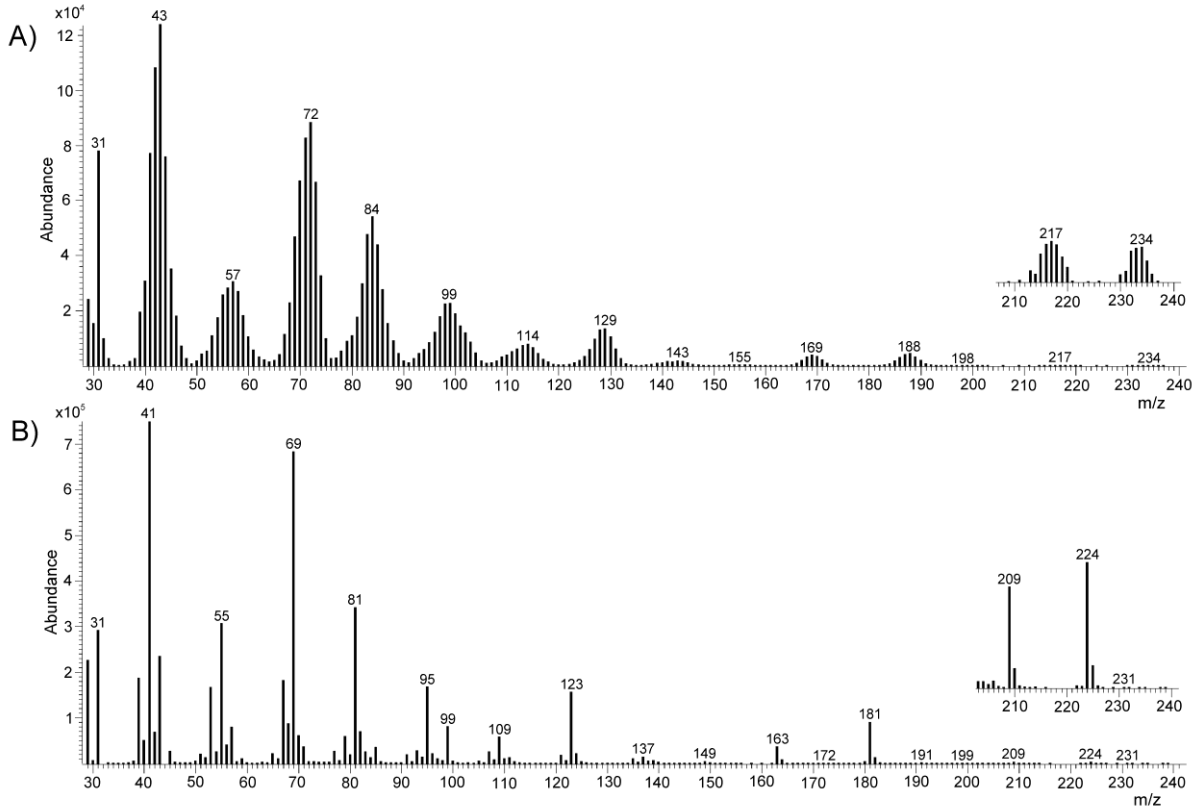


Fig. 46: Mass spectra of 2,3-dihydrofarnesol (DHF) found in the *B. terrestris* LG extracts. The LG was incubated with acetate-d_3. A) Mass spectrum of DHF containing deuterium randomly distributed within the molecule; B) Mass spectrum of native DHF. See the molecular masses of both types of DHF.

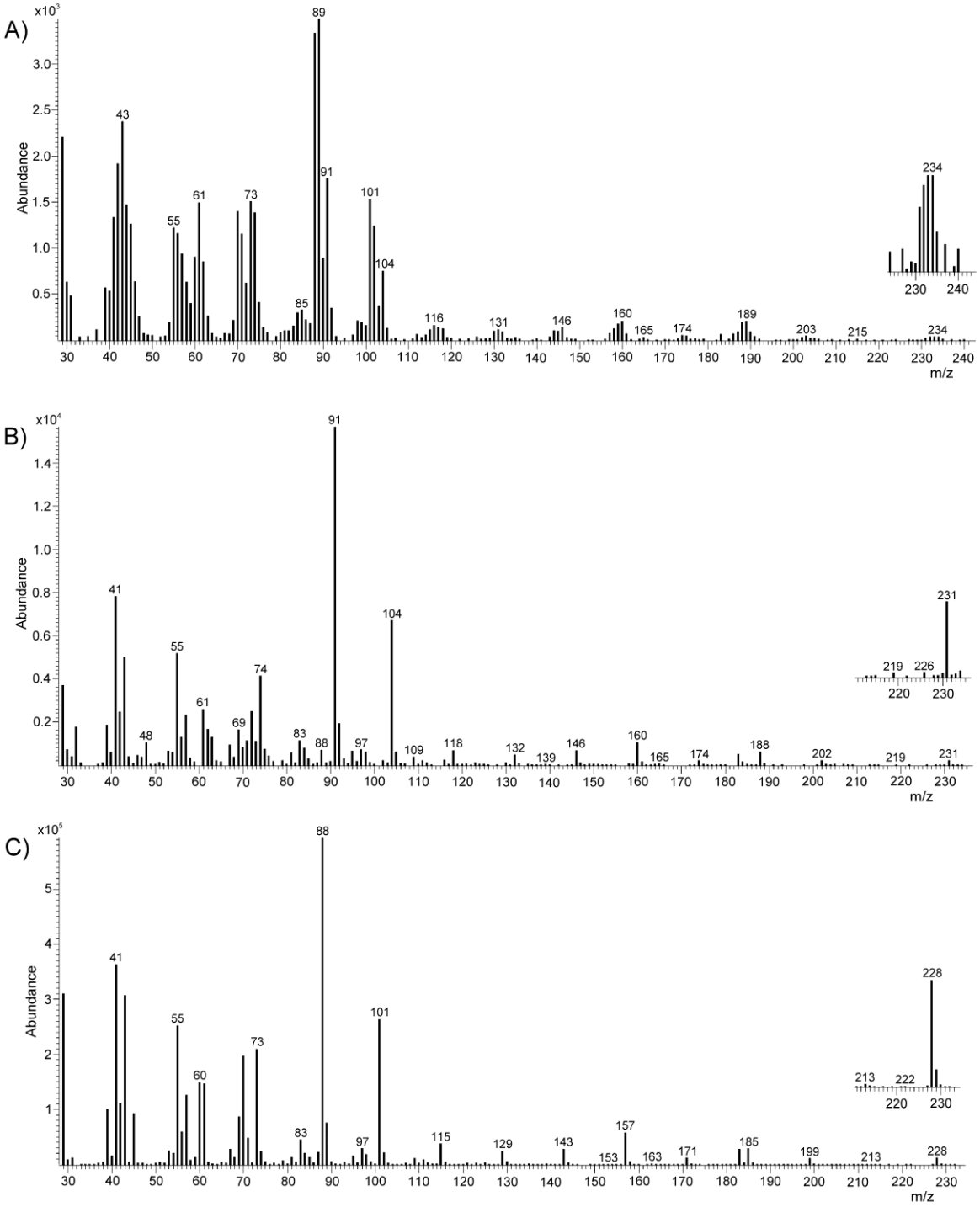


Fig. 47: Mass spectra of ethyl laurate (Et-C12) found in the LG of *B. terrestris* after incubation with acetate-d_3. A) Mass spectrum of Et-C12 containing deuterium randomly distributed within the acidic part of the molecule. The spectrum is partly contaminated by the masses of ethyl-2,2,2-d_3 laurate (see *m/z* 91 and 104 in spectrum B) due to poor chromatographic separation; B) Mass spectrum of ethyl-2,2,2-d_3 laurate; C) Mass spectrum of native Et-C12.

It was confirmed by the sequence of the incubation experiments and measurements that also the alcoholic part of the ethyl esters can be biosynthesized from the acetate precursor via acetaldehyde **(Fig. 47-B)**.

Incubation of the *B. lucorum* labial glands with deuterated acetate was not successful and no deuterated metabolites were found in the labial glands extracts. However, a possible explanation might be the use of incompatible incubation conditions, because all incubation condition optimizations were performed with *B. terrestris* due to the availability of the biological material.

The results from the *in vitro* incubation experiments were supported by a comparison of the relative Fatty Acid Synthase (FAS) mRNA gene transcription level in FB and LGs of *B. terrestris* and *B. lucorum* males that proved a high biosynthetic activity in the LGs of both species. Altogether, these results indicate that pheromone components are synthesized *de novo* in the labial glands of bumblebee males of *B. terrestris* and *B. lucorum*.

5.2.5 Conclusion

Despite the fact that the *de novo* hypothesis was clearly proved, the hypothesis concerning fatty acids, stored in a form of triacylglycerols in the fat body, as the aliphatic pheromonal components precursors cannot be excluded. Therefore, an alternative hypothesis should be considered - *de novo* biosynthesis of the fatty acids in the labial gland and their transport from the fat body may proceed together, based on the biological factors. The clarification of the hypothesis will be the next direction of the research.

5.3 Leg tendon glands in male bumblebees (*Bombus terrestris*): structure and secretion chemistry

Most of the chemical compounds that are involved in the chemical communication and serve as an information mediator are released from exocrine glands that could be located virtually everywhere in insect bodies [136,137]. For example, only in the ants' leg were discovered 20 glandular structures or gland complexes so far [138]. The number of leg glands discovered in bees is smaller [137,139]. The main function in chemical communication was assigned to the tarsal gland located within the fifth tarsomere [140,141]. However, the studies of the tarsal gland structure showed that it has no opening; therefore the glandular secretion cannot be used for chemical communication as it was believed. Its probable function is to inflate the arolium in order to increase the adhesion when walking on smooth surfaces [142]. Recently, a tendon gland was discovered in stingless bees (*Melipona seminigra*) whose opening is located in the vicinity of the tarsal gland [69]. A detailed study of the glandular structure and the chemical composition of the secretion confirmed that the tendon gland is the source of the footprint compounds [69]. Our observation confirmed a presence of similar glandular structures in the legs of bumblebees (*Bombus terrestris*). The aim of this work was to characterize the structure of the gland and chemical composition of the glandular secretion, including composition changes within the age, and propose a biological function of the secretion.

Based on the preliminary chemical analysis of the secretion, performed on GC-FID, which showed a presence of wax esters found in males only, the research focused mainly on the males' tendon gland. Nevertheless, the chemical analysis of the tendon glands secretion, tarsal glands content and cuticular hydrocarbons (represented by wings hexane extracts) were performed also in queens and workers for comparative purposes.

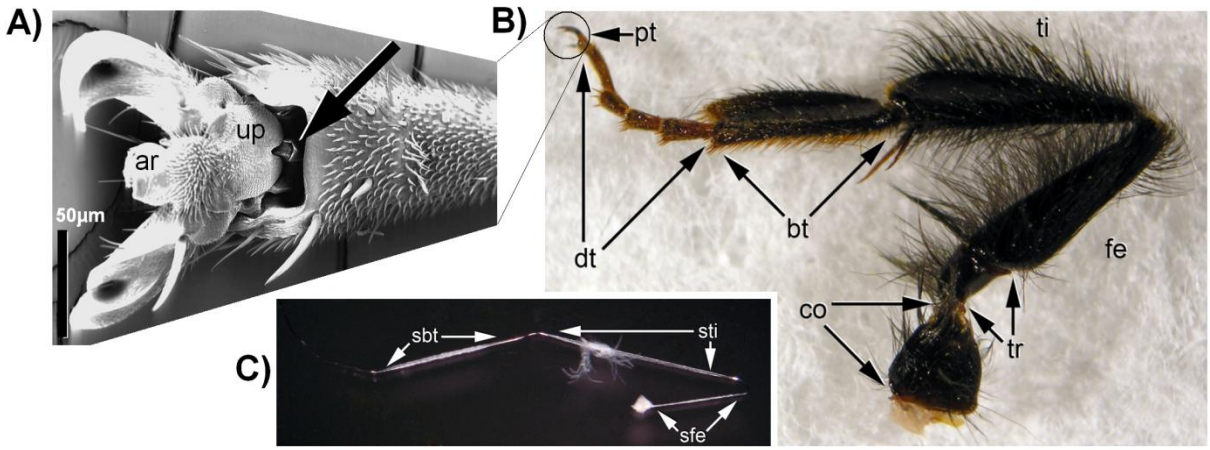


Fig. 48: Localization of the tendon gland in the leg of *B. terrestris*. A) Ventral view of the fifth tarsomere and pretarsus of a mid-leg. The arrow indicates the gland opening. Abbreviation: *ar* arolium, *up* unquitractor plate. B) Photo of the hind leg. Abbreviation: *bt* basitarsus, *co* coxa, *dt* distitarsus, *fe* femur, *pt* pretarsus, *ti* tibia, trochanter. C) Disected hind leg tendon. Abbreviation: sbt secretory epithelium within basitarsus, sfe secretory.

The ultrastructure of the males' tendon gland showed a similarity with the tendon gland already described in the stingless bees [69]. The secretory cells are located in three parts (femur, tibia and basitarsus) of each of six legs in all three castes (queens, workers and males) **(Fig. 48)**. The secretion is accumulated in the hollow tendon and released from an opening at the base of the unquitractor plate **(Fig. 48-A)**.

A method of the GC×GC-MS was used for volatile and semivolatile compounds analysis. The resolving power of the two-dimensional GC was helpful primarily for separation of the wax esters containing the same number of carbons in the molecule. For the double bond position determination and configuration, microderivatization using DMDS reagent and FTIR spectroscopy were used, respectively. The non-volatile components of tendon and tarsal glands were analyzed by MALDI-TOF technique, optimized for small non polar molecules [68].

Chemical analysis showed differences among castes in the composition of the secretion of the tendon gland. The tendon gland secretion of workers and queens contained hydrocarbons only **(Fig. 49)**, while the males' secretion contained also wax esters. The hydrocarbon profile of the tendon gland differed among all castes **(Fig. 49)**. In comparison to the males, hydrocarbons with a longer carbon chain were present in queens and workers.

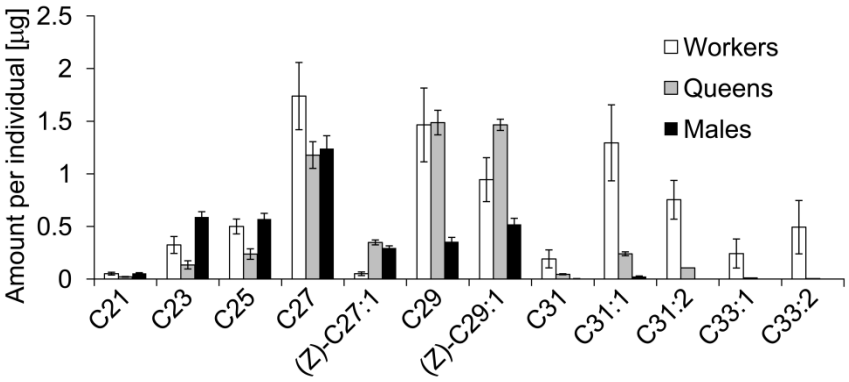


Fig. 49: Total amounts of the most abundant hydrocarbons present in the extracts of leg tendon gland in workers, queens and males. Bars represent means±SE of five bees each of age 7 days. Hydrocarbons nomenclature: C21 henicosane, (*Z*)-C27:1 heptacosene with one double bond in *cis* configuration, C31:1 hentriacontene with one double bond of an unknown configuration.

The analysis of the double bond position in the most abundant unsaturated hydrocarbons (heptacosene, nonacosene and hentriacontene) showed surprising results **(Fig. 50)**. The double bonds in males' hydrocarbons are located almost exclusively in the 9th position, while in workers and queens the double bond was located in more positions with a different distribution between the two castes. Further study showed that the total amount of the tendon gland secretion in males increases within the males' age. These observations suggest that the males' tendon gland secretion is involved in the chemical communication related to the mating behavior - for example, together with the labial gland secretion as additional information about males' fitness for conspecific females. However, this hypothesis was not confirmed by our behavioral tests.

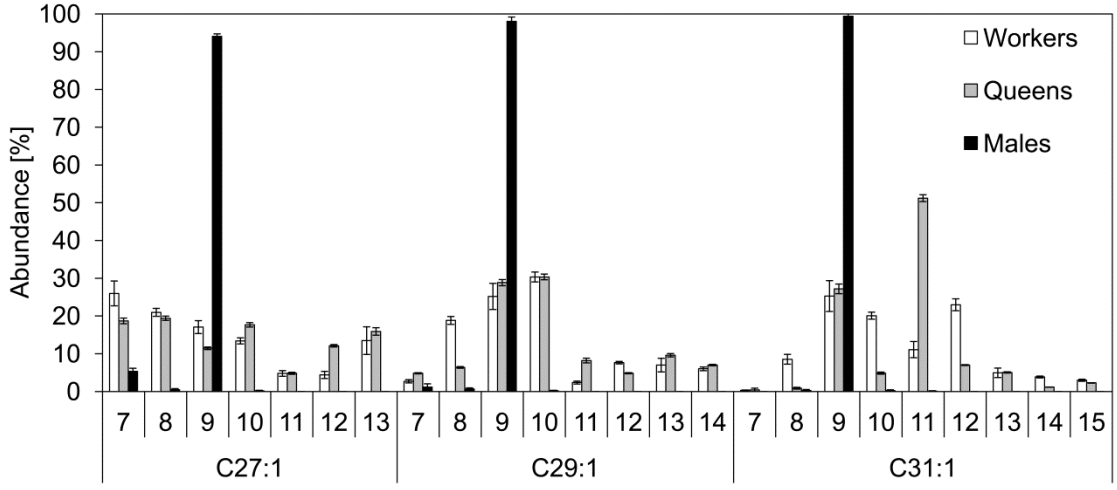


Fig. 50: Double bond positions in unsaturated hydrocarbons (C27–C31) present in leg tendon glands of workers, queens, and males. The abundance is relative to the TIC signal distribution per unsaturated hydrocarbon of the same number of carbons. Bars represent means±SE. For the hydrocarbons nomenclature – see **Fig. 49**.

The overall comparison of the hydrocarbons in the tendon gland, a tarsal gland and a cuticle among castes was performed. It was found that the hydrocarbon profile in the tarsal gland and the cuticle is identical for each caste but different among the castes.

The chemical composition of the tendon and tarsal glands in males were studied in detail including the identification of the observed compounds. There were found wax esters up to 50 carbons found by MALDI-TOF technique, but no substantial differences were confirmed.

Based on the chemical analysis we can suggest a communicational function (probably pheromone) of the tendon gland secretion. Its role in sexual behavior in males was not proved. However, the gland can take over the functions previously attributed to the tarsal gland (e.g. footprints).

Fig. 51: Stink bug of *Graphosoma lineatum* species (undefined sex).

5.4 Profiling and characterization of volatile secretions from the European stink bug *Graphosoma lineatum*

The stink bug species *Graphosoma lineatum* **(Fig. 51)** belong to the largest families of Heteroptera [143]. Its evolutionary success is largely associated with the effective defensive system against predators (birds, lizards, other insects...). In the event of an attack the stink bugs release a foul odor from the metathoracic glands [144].

Various analytical approaches were used for collecting of the released compounds and their identification. The volatiles were trapped either using the whole body washes [145,146] or SPME [147,148] followed by GC-MS analysis. The analysis confirmed the presence of (*E*)-alk-2-enals with (*E*)-hex-2-enal and (*E*)-dec-2-enal to be the most abundant, linear hydrocarbons, and a relatively large amount of furanones [147]. Nevertheless, previous works suffered from irreproducibility that might be attributed to variable rearing conditions, the age of the examined individuals, gender, food, or a geographical origin. Furthermore, the limiting factor of the one-dimensional gas chromatography, especially a low peak capacity, would prevent the identification of some biologically important compounds in the defensive secretion that are present in low concentrations.

The aim of this work was to analyze volatiles of the defensive secretion of a stink bug *Graphosoma lineatum* using headspace SPME sampling method followed by the two-dimensional comprehensive gas chromatography GC×GC-MS and the preparative GC.

Concerning the sampling part, the most important factor was the selection of the solid phase trapping material and the duration of the trapping. The commercially available mixture of polymers Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) was selected as the most suitable solid-phase for SPME. The trapping duration was optimized using the presumed most abundant compounds in the secretion. It was observed that the trapped amount of some of the tested standards declined within the extraction duration **(Fig. 52)**, probably due to degradation by action of the air oxygen. Therefore, the extraction time 15 minutes was chosen as the compromise between sensitivity and the analysis time.

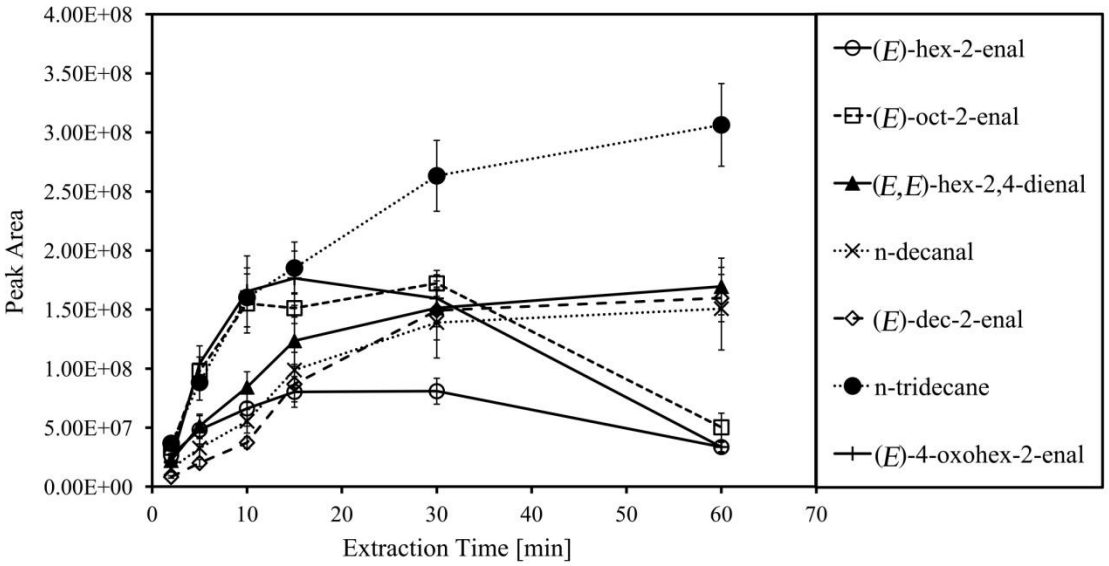


Fig. 52: The effect of an extraction time on the headspace amount of the selected standards.

In the subsequent chemical analysis of the headspace of the disturbed stink bugs, 57 volatiles were identified in the defensive mixture - 39 have not been described before in the *G. lineatum* species [147,148].

This work clearly shows advantages of the GC×GC-MS because some of the compounds could not be identified or properly quantified using conventional one-dimensional GC [**Fig. 53-A** (e.g. compounds 4 and 18; 23 and 24; or 6 and 41)].

A structure of 5-ethylfuran-2(5*H*)-one was described in the previous work [147] in the *G. lineatum* defensive secretion **(Fig. 53-C)**. Nevertheless, the retention behavior of the identified identified in the secretion differed from the synthesized standard. Thus, a preparative gas chromatography was used for an isolation of the compound for the structure determination by means of NMR. The compound was identified as (*E*)-4-oxohex-2-enal **(Fig. 53-B)**. Both compounds when subjected to the electron ionization provide practically identical mass spectra. In this way we corrected the earlier described literature date.

The comparison of relative proportions of the secretion components revealed no differences between sexes.

The headspace SPME sampling method in the connection with GC×GC-MS showed a great potential for characterization of new minor components of defensive secretions in other stink-bug species. However, a special attention has to be paid to the use of SPME for quantification purposes.

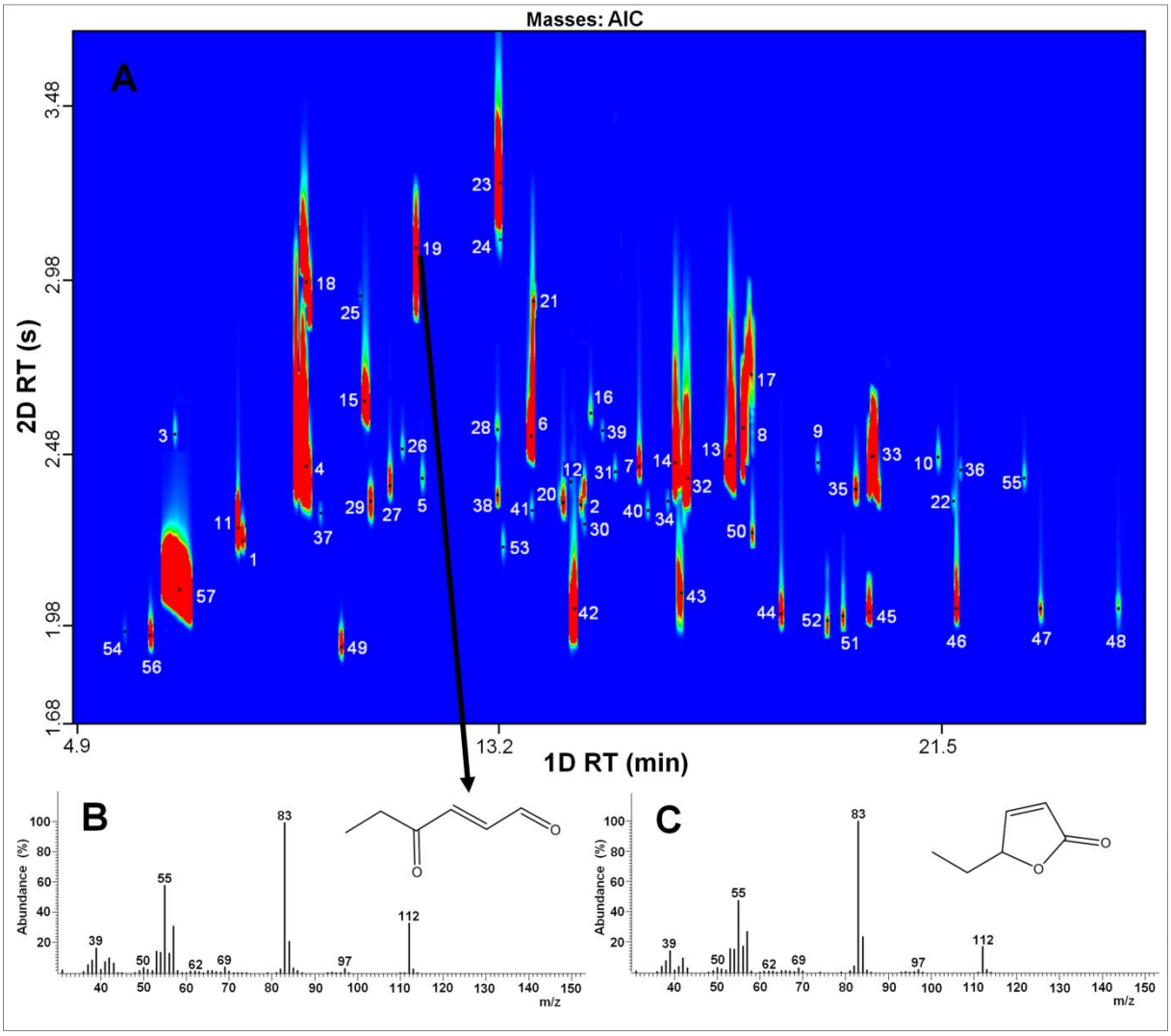


Fig. 53: A) An analytical ion chromatogram contour of the secretion of an adult *G. lineatum* male; B) Mass spectrum of the isolated (*E*)-4-oxohex-2-enal; C) Mass spectrum of the synthesized 5-ethylfuran-2(5H)-one; the numbered peaks relate to the ID numbers of the compounds listed in the paper - Table 1. [Primary oven (DB-5, 30 m, ID 0.25 mm): 50 ^{o}C (held for 2 min)-rate 10 ^{o}C/min-to 300 ^{o}C (held for 10 min); secondary oven (BPX-50, 2.0 m, ID 0.1 mm): 5 ^{o}C incremented with respect to the primary oven; modulation period: 3 s; mobile phase: He (1 ml/min).]

6 Conclusions

Methods of labeled metabolite analysis using GCxGC-MS, preparative GC, and radioanalytical techniques were developed and applied for sex pheromone biosynthesis of bumblebee males' species *Bombus lucorum*, *B. lapidarius* and *B. terrestris* research in this work. The results supported the hypothesis that fatty acids may be precursors for the aliphatic components biosynthesis in *B. lucorum* and *B. lapidarius*. *De novo* biosynthesis of the sex pheromone in the labial gland was confirmed for *B. terrestris* species and supported for *B. lucorum*.

A new exocrine gland was characterized in *B. terrestris* species. Chemical composition of the glandular secretion in queens, workers and males was determined using GCxGC-MS.

Defensive secretion of stink bug *Graphosoma lineatum* was analyzed using a new approach: SPME sampling method followed by analysis using GCxGC-MS. Moreover, a structure of a previously described compound was corrected using preparative GC and NMR.

7 Literature

[1] A.D. Chapman, Numbers of living species in Australia and the World, Australian Biological Resources Study, Canberra, 2006, p. 60.

[2] T.L. Erwin: Tropical forests: their richness in Coleoptera and other arthropod species, Coleopt. Bull. 36 (2000) 74-75.

[3] A. Butenandt, R. Beckmann, D. Stamm, E. Hecker, Zeitschrift Fur Naturforschung Part B-Chemie Biochemie Biophysik Biologie Und Verwandten Gebiete 14 (1959) 283.

[4] T.D. Wyatt, Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology 196 (2010) 685.

[5] W. Francke, S. Schulz, Comprehensive Natural Products 8, Elsevier Science Ltd., Oxford, 1999, p. 191.

[6] G. Gries, K.N. Slessor, R. Gries, G. Khaskin, P.D.C. Wimalaratne, T.G. Gray, G.G. Grant, A.S. Tracey, M. Hulme, Journal of Chemical Ecology 23 (1997) 19.

[7] C.L. Arthur, L.M. Killam, K.D. Buchholz, J. Pawliszyn, J.R. Berg, Analytical Chemistry 64 (1992) 1960.

[8] C.L. Arthur, K. Pratt, S. Motlagh, J. Pawliszyn, R.P. Belardi, Hrc-Journal of High Resolution Chromatography 15 (1992) 741.

[9] A. Peppuy, A. Robert, E. Semon, C. Ginies, M. Lettere, O. Bonnard, C. Bordereau, Journal of Insect Physiology 47 (2001) 445.

[10] R.J. Bartelt, Analytical Chemistry 69 (1997) 364.

[11] A.J. Matich, D.D. Rowan, N.H. Banks, Analytical Chemistry 68 (1996) 4114.

[12] B. Schafer, P. Hennig, W. Engewald, Hrc-Journal of High Resolution Chromatography 18 (1995) 587.

[13] E.D. Morgan, L.J. Wadhams, Journal of Chromatographic Science 10 (1972) 528.

[14] A.B. Attygalle, E.D. Morgan, Angewandte Chemie-International Edition in English 27 (1988) 460.

[15] K. Stránský, M. Zarevúcka, I. Valterová, Z. Wimmer, Journal of Chromatography A 1128 (2006) 208.

[16] A.G. Harrison, Chemical Ionization Mass Spectrometry, CRC Press, Inc., Boca Raton, FL, 1983.

[17] B.D. Nourse, R.G. Cooks, Analytica Chimica Acta 228 (1990) 1.

[18] W.E. Stephens, Physical Review 69 (1946) 691.

[19] R.J. Cotter, Time-of-Flight Mass Spectrometry, American Chemical Society, Washington DC, 1994.

[20] F. Dubois, R. Knochenmuss, R. Zenobi, A. Brunelle, C. Deprun, Y. Le Beyec, Rapid Communications in Mass Spectrometry 13 (1999) 786.

[21] K.L. Brown, G.W. Tautfest, Review of Scientific Instruments 27 (1956) 696.

[22] F. Dubois, R. Knochenmuss, R. Zenobi, International Journal of Mass Spectrometry 169 (1997) 89.

[23] R.L. Grob, Moder Practice of Gas Chromatography, Wiley Interscience, New York, 3rd ed., 1995.

[24] J.G. Millar, K.F. Haynes (Eds.), Methods in Chemical Ecology, vol. 1, Kluwer Acad. Publishers, London, 1998 (Chapter 3) p. 111.

[25] D.E.M. Evans, J.C. Tatlow, Journal of the Chemical Society (1955) 1184.

[26] D. Ambrose, R.R. Collerson, Nature 177 (1956) 84.

[27] H.G.J. Mol, H.G.M. Janssen, C.A. Cramers, J.J. Vreuls, U.A.T. Brinkman, Journal of Chromatography A 703 (1995) 277.

[28] K. Grob, Journal of Chromatography A 703 (1995) 265.

[29] J.C. Bosboom, H.G. Janssen, H.G.J. Mol, C.A. Cramers, Journal of Chromatography A 724 (1996) 384.

[30] H.M. Muller, H.J. Stan, Hrc-Journal of High Resolution Chromatography 13 (1990) 697.

[31] W. Vogt, K. Jacob, H.W. Obwexer, Journal of Chromatography 174 (1979) 437.

[32] W. Vogt, K. Jacob, A.B. Ohnesorge, H.W. Obwexer, Journal of Chromatography 186 (1979) 197.

[33] F.J. Senorans, J. Tabera, J. Villen, M. Herraiz, G. Reglero, Journal of Chromatography 648 (1993) 407.

[34] D. Stajnbaher, L. Zupancic-Kralj, Journal of Chromatography A 1190 (2008) 316.

[35] H.J. Stan, M. Linkerhagner, Journal of Chromatography A 727 (1996) 275.

[36] D. Schneider, Zeitschrift Für Vergleichende Physiologie 40 (1957) 8.

[37] J. van der Pers, Electroantenography a Practical Introduction, Syntech Ltd., Hilversum, The Netherlands, 2004.

[38] J.E. Moorhouse, R. Yeadon, P.S. Beevor, B.F. Nesbitt, Nature 223 (1969) 1174.

[39] H. Arn, E. Stadler, S. Rauscher, Zeitschrift Fur Naturforschung C-a Journal of Biosciences 30 (1975) 722.

[40] I. Valterová, J. Kunze, A. Gumbert, A. Luxová, I. Liblikas, B. Kalinová, A.K. Borg-Karlson, Arthropod-Plant Interactions 1 (2007) 137.

[41] L.J. Wadhams, Zeitschrift Fur Naturforschung C-a Journal of Biosciences 37 (1982) 947.

[42] J. Boeckh, Zeitschrift Für Vergleichende Physiologie 46 (1962) 212.

[43] P.R. Griffiths, S.L. Pentoney, A. Giorgetti, K.H. Shafer, Analytical Chemistry 58 (1986) 1349.

[44] G.T. Reedy, D.G. Ettinger, J.F. Schneider, S. Bourne, Analytical Chemistry 57 (1985) 1602.

[45] A.M. Haefner, K.L. Norton, P.R. Griffiths, S. Bourne, R. Curbelo, Analytical Chemistry 60 (1988) 2441.

[46] R. Consden, A.H. Gordon, A.J.P. Martin, Biochemical Journal 38 (1944) 224.

[47] J.C. Giddings, Analytical Chemistry 56 (1984) 1258.

[48] Z.Y. Liu, J.B. Phillips, Journal of Chromatographic Science 29 (1991) 227.

[49] J. Dallüge, J. Beens, U.A.T. Brinkman, Journal of Chromatography A 1000 (2003) 69.

[50] J.B. Phillips, J. Beens, Journal of Chromatography A 856 (1999) 331.

[51] R.M. Kinghorn, P.J. Marriott, Hrc-Journal of High Resolution Chromatography 22 (1999) 235.

[52] M. Adahchour, J. Beens, U.A.T. Brinkman, Journal of Chromatography A 1186 (2008) 67.

[53] E.M. Kristenson, P. Korytar, C. Danielsson, M. Kallio, M. Brandt, J. Makela, Journal of Chromatography A 1019 (2003) 65.

[54] P.A. Bueno, J.V. Seeley, Journal of Chromatography A 1027 (2004) 3.

[55] J. Harynuk, T. Gorecki, Journal of Separation Science 27 (2004) 431.

[56] H.M. Cai, S.D. Stearns, Analytical Chemistry 76 (2004) 6064.

[57] C.J. Venkatramani, J.Z. Xu, J.B. Phillips, Analytical Chemistry 68 (1996) 1486.

[58] J.C. Giddings, Journal of Chromatography A 703 (1995) 3.

[59] H.J. de Geus, I. Aidos, J. de Boer, J.B. Luten, U.A.T. Brinkman, Journal of Chromatography A 910 (2001) 95.

[60] A.L. Lee, A.C. Lewis, K.D. Bartle, J.B. McQuaid, P.J. Marriott, Journal of Microcolumn Separations 12 (2000) 187.

[61] J. Beens, M. Adahchour, R.J.J. Vreuls, K. van Altena, U.A.T. Brinkman, Journal of Chromatography A 919 (2001) 127.

[62] U. Baier, Lc Gc Europe 16 (2003) 67.

[63] V.G. Zaikin, J.M. Halket, European Journal of Mass Spectrometry 12 (2006) 79.

[64] J.A. Syage, Journal of the American Society for Mass Spectrometry 15 (2004) 1521.

[65] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.

[66] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yohida, Rapid Communications in Mass Spectrometry 2 (1988) 151.

[67] R. Hanus, V. Vrkoslav, I. Hrdý, J. Cvačka, J. Šobotník, Proceedings of the Royal Society B-Biological Sciences 277 (2010) 995.

[68] V. Vrkoslav, A. Muck, J. Cvačka, A. Svatoš, Journal of the American Society for Mass Spectrometry 21 (2010) 220.

[69] S. Jarau, M. Hrncir, M. Ayasse, C. Schulz, W. Francke, R. Zucchi, F.G. Barth, Journal of Chemical Ecology 30 (2004) 793.

[70] B. Kalinová, J. Kindl, P. Jiroš, P. Žáček, S. Vašíčková, M. Buděšínský, I. Valterová, Journal of Natural Products 72 (2009) 8.

[71] S.M. Abernathy, R.R. Gil, A. Akinyemi, Atlas Journal of Chemistry & Biochemistry 1. 1 (2011) 10.

[72] J. Sýkora, P. Bernášek, M. Zarevúcka, M. Kurfürst, H. Sovová, J. Schraml, Journal of Chromatography A 1139 (2007) 152.

[73] R.D. Dandeneau, E.H. Zerenner, Journal of High Resolution Chromatography 2 (1979) 351.

[74] E. Kovats, Helvetica Chimica Acta 41 (1958) 1915.

[75] NIST Mass Spec Data Center SES, Retention Indices, 6th ed., NationalInstitute of Standards and Technology, Gaithersburg, MD, 2008.

[76] A.B. Attygalle, A. Svatoš, C. Wilcox, S. Voerman, Analytical Chemistry 66 (1994) 1696.

[77] D.A. Carlson, C.S. Roan, R.A. Yost, J. Hector, Analytical Chemistry 61 (1989) 1564.

[78] K. Mori, M. Ikunaka, Optical rotary dispersion of spiroacetals. Liebigs Annalen der Chemie (1987) 333.

[79] E.L. Eliel, S.H. Wilen, L.N. Mander, Stereochemistry of organic compounds. John Wiley & Sons, New York 1994.

[80] K. Mori, The significance of chirality: methods for determining absolute configuration and optical purity of pheromone and related compounds, in:Techniques in Pheromone research, eds. H.E. Hummel, T.A. Miller, Springer-Verlag, New York. 1984, p. 323.

[81] I. Ohtani, T. Kusumi, Y. Kashman, H. Kakisawa, Journal of the American Chemical Society 113 (1991) 4092.

[82] S.G Allenmark, Chromatographic Enantioseparation: Methods and Applications. John Wiley & Sons, New York, 1988.

[83] W.A. König, Gas chromatographic Enantiomer Separation with Modified Cyclodextrins. Hüthig, Heidelberg, 1992.

[84] K.E. Wilzbach, P. Riesz, Science 126 (1957) 748.

[85] W.E. Falconer, R.J. Cvetanovič, Analytical Chemistry 34 (1962) 1064.

[86] W.A. Vanhook, M.E. Kelly, Analytical Chemistry 37 (1965) 508.

[87] F. Bruner, G.P. Cartoni, A. Liberti, Analytical Chemistry 38 (1966) 298.

[88] F. Bruner, G.P. Cartoni, Possanzi.M, Analytical Chemistry 41 (1969) 1122.

[89] M. Matucha, W. Jockisch, P. Verner, G. Anders, Journal of Chromatography 588 (1991) 251.

[90] M. Matucha, E. Smolková, Journal of Chromatography 127 (1976) 163.

[91] J. Bermejo, C.G. Blanco, M.D. Guillen, Journal of Chromatography 351 (1986) 425.

[92] W.A. VanHook, Isotope Separation by Gas Chromatography, American Chemical Society, Knoxville, 1969 (Chapter 7) p. 99.

[93] H.G. Schmarr, P. Slabizki, S. Muntnich, C. Metzger, E. Gracia-Moreno, Journal of Chromatography A 1270 (2012) 310.

[94] W.G. Mook, Environmental Isotopes in the Hydrological Cycle: Principles and Applications, IAEA.org – International Atomic Energy, Groningen, 2000, p. 23.

[95] G. Jancso, W.A. Vanhook, Chemical Reviews 74 (1974) 689.

[96] R.T. Davis, R.W. Schiessler, Journal of the American Chemical Society 75 (1953) 2763.

[97] L.S. Bartell, H.K. Higginbo, Journal of Chemical Physics 42 (1965) 851.

[98] A. Bondi, Journal of Physical Chemistry 68 (1964) 441.

[99] R. Yaris, J.R. Sams, Journal of Chemical Physics 37 (1962) 571.

[100] M. Matucha, Chromatographia 27 (1989) 552.

[101] J. Bigeleisen, S.V. Ribnikar, M. Stern, W.A. Vanhook, Journal De Chimie Physique Et De Physico-Chimie Biologique 60 (1963) 60.

[102] J. Bigeleisen, W.A. Vanhook, S.V. Ribnikar, Journal of Chemical Physics 38 (1963) 489.

[103] M. Koel, Ionic Liquid in Chemical Analysis, CRC Press, Boca Raton, 2008.

[104] B. Kullenberg, G. Bergström, S. Ställberg-Stenhagen, Acta Chemica Scandinavica 24 (1970) 1481.

[105] K. Urbanová, I. Valterová, O. Hovorka, J. Kindl, European Journal of Entomology 98 (2001) 111.

[106] W.O. McReynolds, Journal of Chromatographic Science 8 (1970) 685.

[107] L.R. Bordajandi, L. Ramos, M.J. Gonzalez, Journal of Chromatography A 1125 (2006) 220.

[108] M. Junge, H. Huegel, P.J. Marriott, Chirality 19 (2007) 228.

[109] F. W. McLafferty, F. Tureček, Interpretation of Mass Spectra, 4th ed., University Science Books, Mill Valley, 1994 (Chapters 8 and 9).

[110] H. W. Newman (1851) Habits of the Bombinatrices. Proceedings of the Entomological Society, London, 1, 86.

[111] C. Darwin, Über die Wege der Hummelmännchen. Cited by: R.B. Freeman (1968) Bulletin of the British Museum of Natural History, (Historical Series) 3 (1885) 177.

[112] F.W.L. Sladen, Nature 88 (1912) 586.

[113] A. Hass, Zeitschrift für Naturforschung, 11(1946) 596-600.

[114] G. Stein, Naturwissenschaften 50 (1963) 305.

[115] G. Bergström, B. Kullenberg, S. Ställberg-Stenhagen, Arkiv for Kemi 28 (1968) 453.

[116] S. Ställberg-Stenhagen, Acta Chemica Scandinavica 24 (1970) 358.

[117] B. Kullenberg, G. Bergström, B. Bringer, B. Carlberg and B. Cederberg, Zoon Supplement 1 (1973) 23.

[118] L. Ágren, B. Cederberg, B.G. Svensson, Zoon 7 (1979) 1.

[119] J. Šobotník, B. Kalinová, L. Cáhlíková, F. Weyda, V. Ptáček, I. Valterová, Journal of Insect Physiology 54 (2008) 204.

[120] D.H. Calam, Nature 221 (1969) 856.

[121] B.G. Svensson, G. Bergström, Insectes Sociaux 24 (1977) 213.

[122] G. Bergström, B.G. Svennson, M. Appelgren and I. Groth, The Systematics Association Special Volume No. 19: Biosystematics of Social Insects (eds P.E. Howse and J.L. Clément) (1981) 175.

[123] G. Bergström, P. Bergman, M. Appelgren, J.O. Schmidt, Bioorganic & Medicinal Chemistry 4 (1996) 515.

[124] B. Cederberg, B.G. Svensson, G. Bergström, M. Appelgreen and I. Groth, Nova Acta Regiae Societatis Scientiarium Upsaliensis 3 (1983) 161.

[125] B.G. Svensson, M. Appelgren and G. Bergstrom, Nova Acta regiae Societatis Scientiarum Upsalla, Ser. V:C 3 (1984) 145.

[126] B.S. Lanne, G. Bergström, A.B. Wassgren, B. Tornback, Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 88 (1987) 631.

[127] M. Appelgren, G. Bergström, B.G. Svensson, B. Cederberg, Acta Chemica Scandinavica 45 (1991) 972.

[128] P. Bergman, G. Bergström, M. Appelgren, Chemoecology 7 (1996) 140.

[129] P. Bergman, G. Bergström, Journal of Chemical Ecology 23 (1997) 1235.

[130] I. Valterová, K. Urbanová, O. Hovorka, J. Kindl, Zeitschrift Für Naturforschung C-a Journal of Biosciences 56 (2001) 430.

[131] A. Luxová, I. Valterová, K. Stránský, O. Hovorka, A. Svatoš, Chemoecology 13 (2003) 81.

[132] P. Matoušková, A. Luxová, J. Matoušková, P. Jiroš, A. Svatoš, I. Valterová, I. Pichová, Chembiochem 9 (2008) 2534.

[133] V. Ptáček, R. Borovec, E. Pernová, Pszczelnicze Zeszyty Naukowe 44 (2000) 305.

[134] P. Jiroš, J. Cvačka, R. Hanus, J. Kindl, E. Kofroňová, I. Valterová, Lipids 46 (2011) 863.

[135] K. Frišonsová, Diploma Thesis, Charles University in Prague, 2009.

[136] J. Billen, E.D. Morgan, Pheromone communication in socialinsects: sources and secretions. In: Vander Meer RK, Breed MD, Winston ML, Espelie KE (eds), Pheromone communication in social insects: ants, wasps, bees, and termites. Westview, Boulder, 1998, p. 3.

[137] C. Cruz-Landim, F.C. Abdalla, M.A. Cruz-Hofling, Brazilian Journal of Biology 65 (2005) 241.

[138] J. Billen, Arthropod Structure & Development 38 (2009) 2.

[139] C.D. Cruz-Landim, R.L.M.S.D. Moraes, H.C. Salles, R.D. Reginato, Revista Brasileira de Zoologia 15 (1998) 159.

[140] U. Schmitt, G. Lubke, W. Francke, Chemoecology 2 (1991) 35.

[141] D. Goulson, J.C. Stout, J. Langley, W.O.H. Hughes, Journal of Chemical Ecology 26 (2000) 2897.

[142] W. Federle, E.L. Brainerd, T.A. McMahon, B. Holldobler, Proceedings of the National Academy of Sciences of the United States of America 98 (2001) 6215.

[143] J.E. McPherson, R.M. McPherson, Stink Bugs of Economic Importance in America. North of Mexico, CRC Press LLC, Boca Raton, 2000.

[144] J.R. Aldrich, Annual Review of Entomology 33 (1988) 211.

[145] T. Tsuyuki, Y. Ogata, I. Yamamoto, K. Shimi, Agricultural and Biological Chemistry 29 (1965) 419.

[146] D. Durak, Y. Kalender, Comptes Rendus Biologies 332 (2009) 34.

[147] K. Stránský, I. Valterová, K. Ubik, J. Čejka, J. Křeček, Hrc-Journal of High Resolution Chromatography 21 (1998) 475.

[148] D. Djozan, T. Baheri, R. Farshbaf, S. Azhari, Analytica Chimica Acta 554 (2005) 197.

8 Publications and presentations

8.1 Publications in impacted journals

A. Buček, H. Vogel, P. Matoušková, D. Prchalová, **P. Žáček**, V. Vrkoslav, P. Šebesta, A. Svatoš, U. Jahn, I. Valterová, I. Pichová: The role of desaturases in the biosynthesis of marking pheromones in bumblebee males. Insect Biochem. Mol. Biol. 43 (2013) 724.

P. Žáček, D. Prchalová-Horňáková, R. Tykva, J. Kindl, H. Vogel, A. Svatoš, I. Pichová, I. Valterová: *De novo* biosynthesis of sexual pheromone in the labial gland of bumblebee males. ChemBioChem 14 (2013) 361.

S. Jarau, **P. Žáček**, J. Šobotník, V. Vrkoslav, R. Hadravová, A. Coppee, S. Vašíčková, P. Jiroš, I. Valterová: Leg tendon glands in male bumblebees (*Bombus terrestris*): structure, secretion chemistry, and possible functions. Naturwissenschaften 99 (2012) 1039.

J. Kindl, P. Jiroš, B. Kalinová, **P. Žáček**, I. Valterová: Females of the bumblebee parasite, *Aphomia sociella*, excite males using a courtship pheromone. Journal of Chemical Ecology 38 (2012) 400.

M. Šanda, **P. Žáček**, L. Streinz, M. Dračínský, B. Koutek: 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. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 881-82 (2012) 69.

R. Hanus, J. Šobotník, J. Krasulová, P. Jiroš, **P. Žáček**, B. Kalinová, K. Dolejšová, J. Cvačka, T. Bourguignon, Y. Roisin, M.J. Lacey, D. Sillam-Dusses: Nonadecadienone, a new termite trail-following pheromone identified in *Glossotermes oculatus* (Serritermitidae). Chemical Senses 37 (2012) 55.

M.J. Lacey, E. Semon, J. Krasulová, D. Sillam-Dusses, A. Robert, R. Cornette, M. Hoskovec, **P. Žáček**, I. Valterová, C. Bordereau: Chemical communication in termites: *syn*-4,6-dimethylundecan-1-ol as trail-following pheromone, *syn*-4,6-dimethylundecanal and (5*E*)-2,6,10-trimethylundeca-5,9-dienal as the respective male and female sex pheromones in *Hodotermopsis sjoestedti* (Isoptera, Archotermopsidae). Journal of Insect Physiology 57 (2011) 1585.

P. Žáček, B. Kalinová, J. Šobotník, O. Hovorka, V. Ptáček, A. Coppee, F. Verheggen, I. Valterová: Comparison of age-dependent quantitative changes in the male labial gland secretion of *Bombus terrestris* and *Bombus lucorum*. Journal of Chemical Ecology 35 (2009) 698.

B. Kalinová, J. Kindl, P. Jiroš, **P. Žáček**, S. Vašíčková, M. Buděšínský, I. Valterová: Composition and electrophysiological activity of constituents identified in male wing gland secretion of the bumblebee parasite *Aphomia sociella*. Journal of Natural Products 72 (2009) 8.

P. Žáček, A. Dransfeld, O. Exner, J. Schraml: ^{15}N NMR chemical shifts of ring substituted benzonitriles. Magnetic Resonance in Chemistry 44 (2006) 1073.

8.2 Proceedings

J. Krasulová, R. Hanus, D. Sillam-Dusses, P. *Žáček, P.* Jiroš, J. Šobotník, B. Kalinová, Z. Bosáková, I. Valterová: Analytical methods Used in the Studies on Chemical Ecology of Termites. Book of proceedings, 8th ISC "Modern analytical chemistry", (ISBN 978-80-7444-017-5) (2012) 1.

L. Kaluža, J. Sýkora, J. Karban, **P. Žáček**, Z. Vít, M. Zdražil: Hydrodesulfurization of Model Feed Containing Olefins over Al2O3, TiO2, and ZrO2 Supported Transition Metal Sulfides. European Congress of Chemical Engineering /8./, Berlin (Germany), 25.-29.9. 2011.

L. Kaluža, J. Sýkora, J. Karban, **P. Žáček**, Z. Vít, M. Zdražil: Effect of Support on Activity of Transition Metal Sulfides in Hydrogenation of 1-Methylcyclohexene Parallel with Hydrodesulfurization of 1-Benzothiophene. 42. Symposium Catalysis, Prague (Czech Republic) 1.-2.11. 2010.

P. Žáček, R. Tykva, J. Kindl, I. Valterová: *In vitro* incubation of the labial gland and fat body of the bumblebee males *Bombus terrestris* with [1,2-^{14}C]acetate and analysis of the metabolites. Book of proceedings, 6th ISC "Modern analytical chemistry", (ISBN 978-80-7444-005-2) (2010) 92.

P. Žáček, A. Jirošová, J. Kindl, I. Valterová: Two dimensional gas chromatography as a tool for biosynthesis study of the components of the marking pheromones of the males species *Bombus lucorum* and *Bombus lapidarius*. Book of proceedings, 4th ISC "Modern analytical

chemistry", (ISBN 978-80-903103-2-2) (2008) 36.

L. Kaluža, J. Sýkora, J. Karban, **P. Žáček**, Z. Vít, M. Zdražil: Parallel Hydrogenation of 1-Methylcyclohexene and Hydrodesulphurization of 1-Benzothiophene over Transition Metal Sulphides Supported on Al2O3, TiO2 and ZrO2. International Symposium on the Molecular Aspects of Catalysis by Sulfides /5./, Copenhagen (Denmark), 30.5.-3.6. 2010.

8.3 Posters

D. Prchalová, **P. Žáček**, I. Valterová, I. Pichová: Biosynthesis of terpenic compounds in marking pheromone blend of *Bombus terrestris*. 28th ISCE Annual Meeting, Vilnius, (Lithuania) 22.-26.7. 2012.

I. Valterová, B. Kalinová, J. Kindl, P. Jiroš, **P. Žáček**: Chemical and acoustic communication in pre-mating behavior of *Aphomia sociella.* 26th ISCE Annual Meeting, Tours, (France) 31.7.-4.8. 2010.

P. Žáček, R. Tykva, Jiří Kindl, I. Valterová: De novo biosynthesis study of the sexual pheromone of the bumblebees males *Bombus lucorum* and *Bombus terrestris*. 6th International Symposium on Molecular Insect Science, Amsterdam (Nederland) 2.-5.10 2011.

P. Žáček, R. Tykva, Jiří Kindl, I. Valterová: De novo biosynthesis study of the components of marking pheromone of bumblebee males (*Bombus lucorum* and *Bombus terrestris*) by means of in vitro incubation with [1,2-^{14}C]acetate uptake. 27th ISCE Annual Meeting, Tours, (France) 31.7.-4.8. 2010.

P. Žáček, R. Tykva, Jiří Kindl, I. Valterová: Biosynthetic studies: In vitro incubation of the labiál gland and fat body of the bumblebee males (*Bombus lucorum* and *Bombus terrestris*) with [1,2-^{14}C]acetate uptake. 26th ISCE Annual Meeting, Neuchâtel, (Switzerland) 23.-27.8. 2009

P. Jiroš, B. Kalinová, J. Kindl, **P. Žáček**, I. Valterová: Bee moth *Aphomia sociella* and scent of host bumblebee nest. 26th ISCE Annual Meeting, Neuchâtel, (Switzerland) 23.-27.8. 2009

P. Žáček, A. Luxová, J. Kindl, I. Valterová: Biosynthetic studies of the male marking

pheromone of bumblebees species *Bombus lucorum* and *B. lapidarius*. 4th IUSSI European Meeting, La Roche-en-Ardenne, (Belgium), 30.8.-4.9. 2008

P. Žáček, A. Luxová, J. Kindl, I. Valterová: Biosynthetic studies of the male marking pheromone of bumblebees species *Bombus lucorum* and *B. lapidarius*. 24th Anniversary Meeting of ISCE, State college, Pennsylvania (USA) 17.-22.08 2008

P. Jiroš, B. Kalinová, J. Kindl, **P. Žáček**, J. Cvačka, S. Vašíčková, I. Valterová: Wing glands secretion of the bee moth *Aphomia sociella*. 24th Anniversary Meeting of ISCE, State college, Pennsylvania (USA) 17.-22.08 2008

P. Žáček, A. Luxová, J. Kindl, O. Hovorka, I. Valterová: Výzkum biosyntézy složek samčích značkovacích feromonů čmeláků druhů *Bombus lucorum* a *Bombus lapidarius*. VII. Mezioborové setkání mladých biologů, biochemiků a chemiků (Sigma-Aldrich). Žďárské Vrchy (Czech Republic) 10.-13.06. 2008.
Poster was among top 5 posters.

P. Žáček, I. Valterová, B. Kalinová, J. Šobotník, O. Hovorka, V. Ptáček: Changes in the composition of the labiál gland secretion dutiny the life of *Bombus lucorum*. 23rd ISCE Annual Meeting, Jena (Germany), 22.-26.7. 2007

8.4 Oral presentations

P. Žáček, J. Kindl, D.Horňáková-Prchalová, O. Hovorka, I. Valterová: Application of the labeled precursors and analysis of their metabolites by means of GCxGC-TOFMS – tool for biosynthesis of the bumblebee male's sexual pheromone research. 3rd European GCxGC Symposium, Nice (France), 17.-18.9. 2013.

P. Žáček, S. Jarau, J. Šobotník, V. Vrkoslav, R. Hadravová, A. Coppée, S. Vašíčková, P. Jiroš, I. Valterová: Tendon gland – a new member of exocrine organ set in bumblebee: Structure, secretion chemistry and possible functions. 28rd ISCE Annual Meeting, Vilnius (Lithuania), 22.-26.7. 2012.

P. Žáček, M. Šanda, L. Streinz, M. Dračínský, B. Koutek: Analýza obranného sekretu ploštice kněžice páskované (*Graphosoma lineatum*). Ovarová hlava 2011 (competition of PhD students organised by Czech University of Life Sciences, Prague (Czech Republic), 20.3. 2012.
The presentation was awarded 1. place.

P. Žáček, R. Tykva, J. Kindl, I. Valterová: Biosyntéza sexuálních feromonů u samců čmeláků druhů *Bombus lucorum* a *Bombus terrestris*. XI. Mezioborové setkání mladých biologů, biochemiků a chemiků (Sigma-Aldrich). Žďárské Vrchy (Czech Republic) 24.-26.5. 2011.

P. Žáček, R. Tykva, J. Kindl, I. Valterová: *In vitro* incubation of the labial gland and fat body of the bumblebee males *Bombus terrestris* with [1,2-^{14}C]acetate and analysis of the metabolites. 6th ISC "Modern analytical chemistry", Prague (Czech Republic), 23.-24.9 2010.

P. Žáček: Biosynthesis of Bumblebee Pheromones: from Chemistry to Molecular biology. IOCB AV CR internal conference, Frymburk (Czech Republic), 4.-7.5. 2010.

P. Žáček, R. Tykva, J. Kindl, I. Valterová: Biosyntetická studie*: In vitro* inkubace labiální žlázy a tukového tělesa samců čmeláků (*Bombus lucorum* a *Bombus terrestris*) s [1,2-^{14}C]octanem sodným. Pokroky v organické, bioorganické a farmaceutické chemii: 44. konference odborné skupiny organické, bioorganické a farmaceutické chemie České společnosti chemické. Nymburk (Czech Republic) 27.-29.11. 2009.

P. Žáček, A. Jirošová, J. Kindl, I. Valterová: Two dimensional gas chromatography as a tool for biosynthesis study of the components of the marking pheromones of the males species *Bombus lucorum* and *Bombus lapidarius*. Book of proceedings, 4th ISC "Modern analytical chemistry", Prague (Czech Republic), 29.-30.1. 2008.

9 Papers

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Comparison of Age-dependent Quantitative Changes in the Male Labial Gland Secretion of *Bombus Terrestris* and *Bombus Lucorum*

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Abstract Age-related changes of antennal-active components of male labial gland extracts were studied in two closely related bumblebee species, *Bombus terrestris* and *B. lucorum*. In *B. terrestris,* compounds eliciting electroantennogram (EAG) responses of virgin queens were ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecan-1-ol, octadeca-9,12,15-trien-1-ol, and geranylcitronellol. Compounds that elicited EAG responses from queens of *B. lucorum* were ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecan-1-ol, hexadec-7-enal, octadeca-9,12-dien-1-ol, octadeca-9,12,15-trien-1-ol, and octadecan-1-ol. Quantities of these compounds in the labial glands changed significantly over the lifetime of the respective males of the two species. In both species, concentrations of the respective compounds reached their maximum within seven days after eclosion. Subsequently, a rapid decrease in the amount of EAG-active compounds occurred in *B. terrestris*, whereas in *B. lucorum* the amount of active compounds stayed approximately constant or decreased at a slow rate. Microscopy showed that in *B. terrestris* secretory cells of the labial glands undergo apoptosis from the fifth to the tenth day of life, whilst in *B. lucorum* labial gland cells remain unchanged throughout the life of the males.

Keywords Bumblebee · *Bombus terrestris* · *Bombus lucorum* · Labial gland · Male marking pheromone · Sex pheromone · GC-EAD

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Introduction

The most common European bumblebee species, *Bombus terrestris* (Linnaeus 1758) and *Bombus lucorum* (Linnaeus 1761), both belong to the subgenus *Bombus sensu stricto*. As observed for other bumblebee species during premating behavior, *B. terrestris* and *B. lucorum* males scent-mark prominent objects on their flight routes with a species-specific sex pheromone ('patrolling behavior'; Calam 1969; Schremmer 1972; Svensson 1980; Lloyd 1981; Morse 1982; Villalobos and Shelly 1987) that attracts conspecific virgin queens to the marked spots for mating (Kullenberg et al. 1970; Bergström et al. 1981; Bergman 1997).

The male-produced pheromones of these bumblebees are produced in the cephalic part of the labial gland (Kullenberg et al. 1973; Bergman and Bergström 1997). Volatile components of the gland secretion are deposited on prominent objects on the flight routes or on perches (Bergman and Bergström 1997; Kindl et al. 1999). The secretion is a complex mixture, comprised of many compounds, usually with one or two major components (Valterová and Urbanová 1997; Terzo et al. 2003). In closely related bumblebee species, the secretions may be similar but usually differ in dominant components and/or compound proportions. Although the main components of the secretion are presumed to comprise the sex pheromone, behavioral roles of specific components in female attraction have yet to be demonstrated. Thus, the actual male-produced pheromone components in secretions of bumblebee species are unknown.

The composition of bumblebee labial gland secretions is used as a tool for taxonomic identification and species and subspecies discrimination (Paterson 1993; Terzo et al. 2005; Rasmont et al. 2005; Bertsch et al. 2005; Coppée et al. 2008). However, there is great inter-individual variability in the composition of secretion components within a single species (Svensson and Bergström 1977; Ågren et al. 1979; Šobotník et al. 2008), which can make differentiation difficult, especially among related species. Recently, changes of cephalic labial gland ultrastructure in relation to age of males were reported for *B. terrestris* (Šobotník et al. 2008). It was found that the secretory activity of the gland cells is high in newly emerged males, but drops as males age. Five days after eclosion of the adult male bumblebee, the biosynthetic activity within the gland stops, and the secretory cells degenerate (Šobotník et al. 2008). Maximal gland content occurred in 2–7 day-old males, and decreased in older males. This age-related change in gland content was paralleled by the ability of gland extracts from different age males to elicit electroantennogram (EAG) responses from queen antennae. Although the study by Šobotník et al. (2008) demonstrated changes in the volume of gland content with age, there was no detailed chemical analysis of changes in the quantities of components of the secretion. They did, however, perform coupled gas chromatogram-electroantennogram detection (GC-EAD) experiments, using male labial gland secretions and queen antennae, and found at least six antennal-active compounds, ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadecatrienol, and geranylcitronellol, that could potentially play a role in male sex pheromone signaling (Šobotník et al. 2008; Valterová et al. 2007).

Qualitative analysis of the labial gland extract from *B. lucorum* has previously been performed (Bergström et al. 1973; Urbanová et al. 2001). However, no attention has been paid to age of males. There are a few reports on seasonal variation in the secretion composition of other bumblebee species (Kullenberg et al. 1970; Svensson and Bergström 1977; Ågren et al. 1979), but thus far the phenomenon of age-related changes in composition has not been studied systematically.

In the present study, we report age-dependent quantitative changes of major EAG-active components of the labial gland of two related bumblebee species, *B. lucorum* and *B. terrestris*. This allowed us to determine whether the relatively time-limited labial gland secretion activity observed in *B. terrestris* also occurs in another species in the subgenus *Bombus sensu stricto.*

Methods and Materials

Insects Colonies of *B. lucorum* and *B. terrestris terrestris* (L.) were established by the two-queens cascade method (Ptáček et al. 2000). All mother queens were taken from their natural habitats during the nest-searching period in order to minimize the possible negative influence of artificial conditions on the progeny. Bumblebee colonies were kept in plastic boxes of 0.6–1 L volume and fed with honeybee pollen pellets and concentrated sugar solution (sucrose:fructose 1:1). When colonies started to produce males, male cocoons were removed from the parental hives and left to mature separately under the care of several workers supplied with food (Ptáček 1999). Freshly emerged males were removed and kept according to age. Animals of the following age were studied. *Bombus terrestris*: shortly after eclosion, 1, 2, 3, 4, 5, 7, 10, 12, 17, 20, 24, and 33 days after eclosion (five individuals of each age); *B. lucorum*: shortly after eclosion, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30 days after eclosion (5–6 individuals of each age). Males were killed by freezing (−18°C) and were kept frozen prior to dissection. Labial glands were dissected and extracted with hexane (100 µl per gland) containing 1-bromodecane as internal standard (1.79 mg/ml for *B. terrestris* and 2.13 mg/ml for *B. lucorum*). Glands, in solvent, were shaken for 30 min after which the extracts were transferred to clean vials and kept at −18°C prior to analysis.

Identification of Compounds Extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) using a splitless injector (200°C), mass detector (200°C, Fisons MD 800) and autosampler AI3000 (Thermo). A DB-5 ms column (30 m×0.25 mm, film thickness 0.25 µm, Agilent Technologies) and helium gas (constant flow of 1 ml min^{-1}) were used for separations. The temperature programs differed for samples of the different species: for *B. terrestris*, 70°C (2 min. delay) to 320°C at 10°C min^{-1}; for *B. lucorum*, 70°C (2 min. delay) to 140°C at 40°C

min^{-1}, to 240°C at 2°C min^{-1}, and then to 320°C at 4°C min^{-1}. Compounds were identified based on their mass spectra and on co-chromatography with synthetic or commercially available standards.

Gas chromatography-electroantennogram detection GC-EAD experiments were performed with a 5890A Hewlett-Packard gas chromatograph (GC) equipped with a DB-5 column (30 m×0.25 mm, film thickness 0.25 µm, J & W Scientific). The column was split by a Graphpack 3D/2 four-arm splitter. The splitter led the eluate to flame ionization (FID) and EAD detectors. N_2 make-up gas at 20 ml min^{-1} was introduced via one arm of the splitter. Labial gland extracts (1–5 µl) were injected in the splitless mode. The GC was temperature programmed from 50°C (2 min. delay) to 270°C at 30°C min^{-1}. The temperature of the injector and FID were set to 230 and 260°C, respectively. The EAD detector consisted of a queen antenna connected via two glass Ag/AgCl electrodes to a universal AC/DC 10XProbe (Syntech, Hilversum, The Netherlands). The EAD and FID signals were fed to a computer via the serial IDAC interface box (Syntech) and analyzed by using GC-EAD software (Syntech). The antenna was exposed to compounds that eluted from the GC via an Effluent Conditioner Tube (Syntech) heated to 180°C. Virgin queens (N=4) used for GC-EAD recording were kept at low temperature (5°C) and high humidity until use. Isolated antennae, with the distal tips excised, were used for recordings. Each antenna was used only once.

Chemicals The following standards were used for the quantification of EAG-active components of the labial gland secretions: (*E*)-farnesol (Firmenich, Geneva, Switzerland), geranylgeraniol (ICN, Irvine, CA, USA), ethyl tetradec-9-enoate (Nu-Check-Prep, Elysian, MN, USA). (*Z,Z,Z*)-Octadeca-9,12,15-trien-1-ol, ethyl dodecanoate, hexadecan-1-ol, octadecan-1-ol, (*Z,Z*)-octadeca-9,12-dien-1-ol, and ethyl hexadec-9-enoate were purchased from Sigma (St Louis, MO, USA). (*Z*)-Hexadec-9-enal was prepared earlier in our laboratory.

Quantitative Analyses Only EAD-active compounds were quantified. For *B. terrestris*, 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, geranylgeraniol, geranylcitronellol, (*Z,Z,Z*)-octadeca-9,12,15-trien-1-ol, and ethyl dodecanoate were quantified; *B. lucorum:* ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadecan-9,12-dienol, octadecan-9,12,15-trienol, and octadecanol were quantified. Quantification was carried out in Total Ion Current mode of the mass detector and based on peak areas. Because the different compounds were present in samples at widely differing concentrations, we had to avoid overloading the mass detector with the most abundant component. Separation of ethyl tetradec-7-enoate and ethyl tetradec-9-enoate under the conditions used was poor. Therefore, these two isomers were quantified together according to the external calibration for ethyl tetradec-9-enoate. Previous studies (Urbanová et al. 2001) showed that the concentration ratio of these isomers in the labial gland extracts was: $\mathrm{area}_{\mathrm{ethyltetradec-9-enoate}} / \mathrm{area}_{\mathrm{ethyltetradec-7-enoate}} = 187.5$. We assumed that the difference in responses of these isomers in the mass detector was less than 5%, and the concentration ratio remained constant over a bumblebee's life.

The calibration curve for (*Z*)-hexadec-7-enal was constructed by using (*Z*)-hexadec-9-enal, as the correct isomer was not available. 2,3-Dihydrofarnesol and 2,3-dihydrofarnesal quantification was calibrated by using farnesol, and geranylcitronellol was calbrated by using geranylgeraniol, due to unavailability of standards. Considering the close similarities in structures between calibrants and compounds, we assumed the quantification error to be low. Octadeca-9,12-dienol and octadeca-9,12,15-trienol could not be separated, but the same approach and assumption as used for ethyl tetradec-7-enoate and ethyl tetradec-9-enoate were used for the samples and quantitative calibration.

The same internal standard (IS; 1-bromodecane, ~2 mg/ml) was used for both calibration and sample sets. Calibration curves were constructed from peak area (ratios of standard/IS). Second degree polynomial equations were used to fit the obtained data. Unknown sample concentrations were calculated by using the calibration curve equations and expressed in µg per gland for each component. Mean values and standard errors were calculated for each male age group.

Microscopy Fixed cephalic parts of labial glands of *B. lucorum* originated from males of ages, pharate imago (darkly colored males enclosed in pupal cuticle shortly before eclosion), <1 d, 1, 2, 3, 4, 5, 6, 9, and 13 days. Two to four samples of each age were studied using optical microscopy and, as only slight differences were observed over the adult life of a male, only a single sample of each age was studied by transmission electron microscopy. Dissection took place in a droplet of fixative (2% glutaraldehyde and 2.5% formaldehyde in 0.1 M phosphate buffer), in which the tissue stayed for 1 day at ambient temperature. After post-fixation in 1.5% OsO_4 in 0.1 M phosphate buffer, the samples were dehydrated by passing through a series of ethanol-water mixtures (50, 75, and finally 100%). Tissues were then embedded into standard Spurr resin. Semithin sections (1 µm) were stained with Azure II and observed under Amplival (Zeiss) optical microscope (equipped with Canon EOS 300D camera). Ultrathin sections were studied with a Jeol 1011 transmission electron microscope.

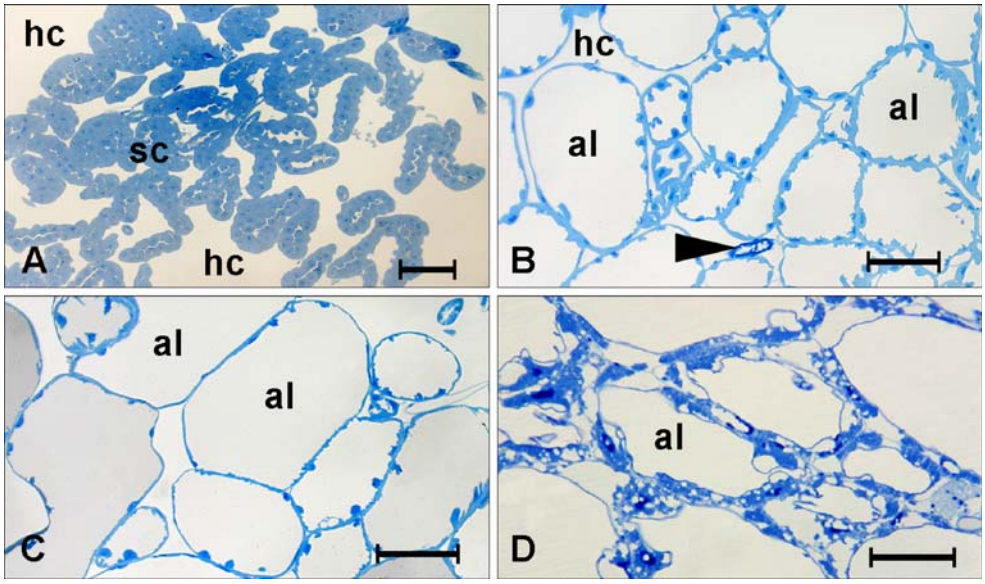

Fig. 1 Development of acini in the cephalic part of the labial gland of *Bombus lucorum* (A–C) and *B. terrestris* (D) (optical microscopy); **a** development of acini in a 1-day-old adult male of *B. lucorum*; **b** development of acini in a 3-day-old male of *B. lucorum*. The arrowhead marks an excretory duct of the labial gland; **c** development of acini in a 13-day-old male of *B. lucorum*; **d** development of acini in a 13-day-old male of *B. terrestris* (all cells are already dead, but the acini are still full of secretion). Bar represents 100 µm in all figures. Abbreviations: al, acinar lumen; hc, haemocoel; sc, secretory cells

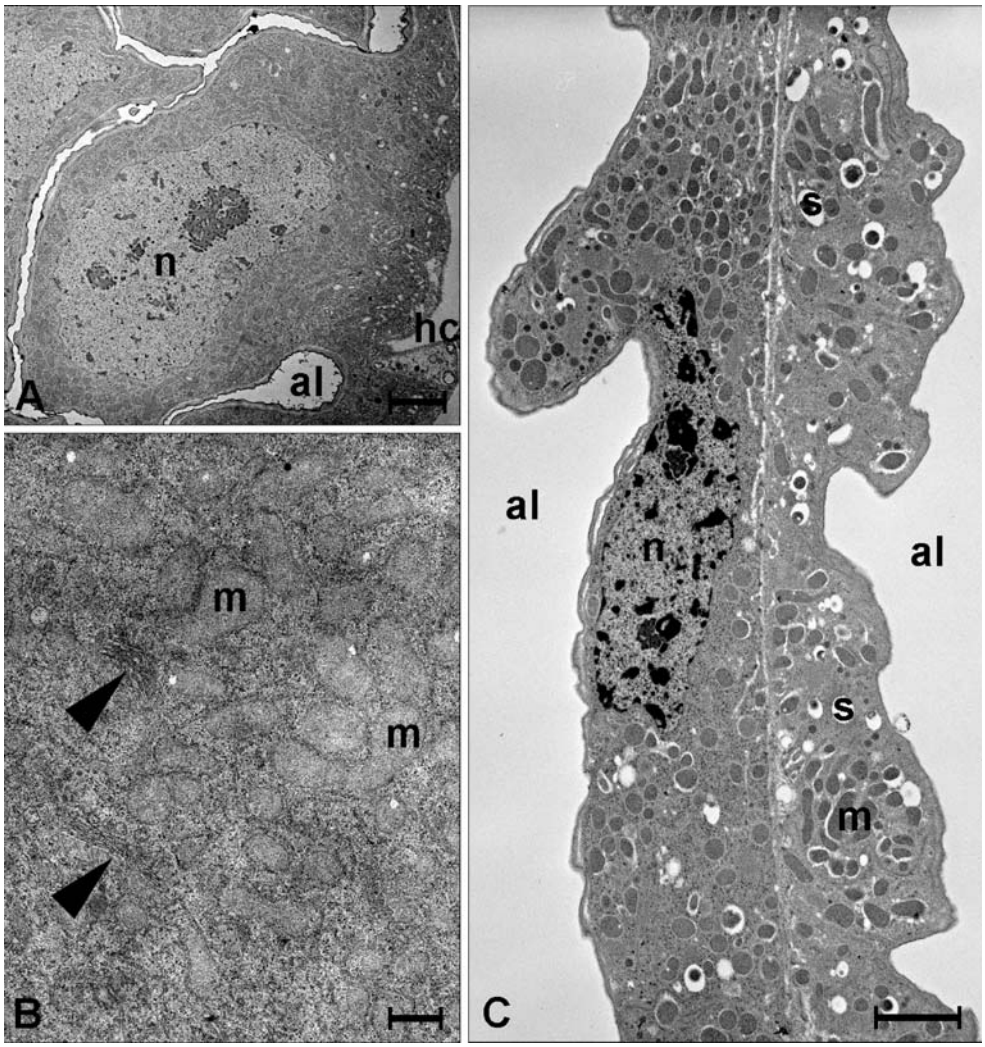

Fig. 2 Transmission electron micrographs of acinar secretory cells in *Bombus lucorum*; **a** whole secretory cell in pharate male imago. Note the active transport at the cell base occurring as pinocytotic vesicles. Bar represents 2 µm; **b** detail of cytoplasm in <1d adult male. Arrowheads mark smooth endoplasmic reticulum producing a secretion. Bar represents 500 nm; **c** walls of two neighbouring acini in a 13-day old male. Bar represents 2 µm. Abbreviations: al, acinar lumen; hc, hemocoel; m, mitochondria; n, nucleus; s, secretion within the cells

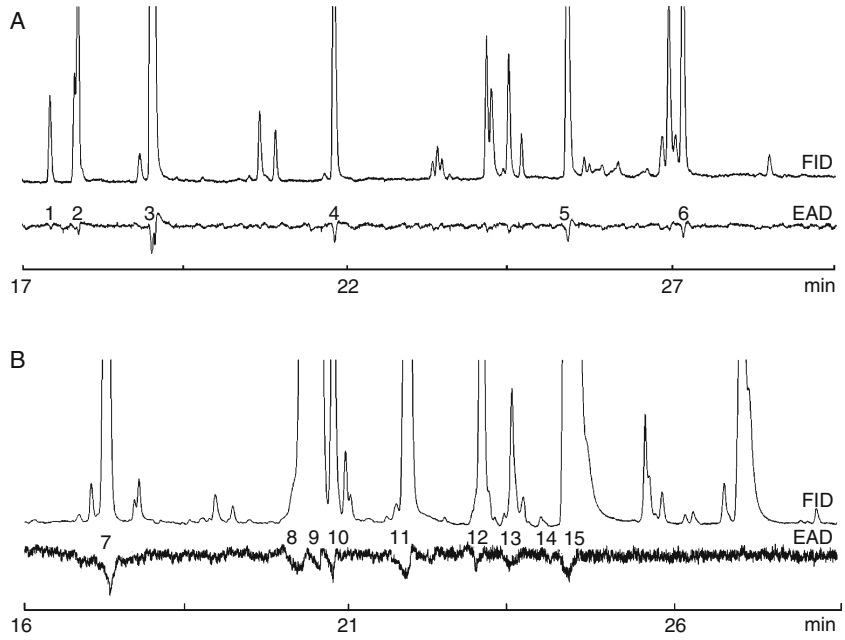

Fig. 3 Coupled gas chromatograph-electroantennogram detection recording of the labial gland extract of 7-day-old bumblebee males. **a** *Bombus terrestris*; active compounds: **1** = ethyl dodecanoate; **2** = 2,3-dihydrofarnesal; **3** = 2,3-dihydrofarnesol; **4** = hexadecan-1-ol; **5** = octadeca-9,12,15-trien-1-ol; **6** = geranylcitronellol. **b** *B. lucorum*; active compounds: **7** = ethyl dodecanoate, **8** = ethyl tetradec-7-enoate, **9** = ethyl tetradec-9-enoate, **10** = hexadec-7-enal; **11** = ethyl hexadec-9-enoate; **12** = hexadecan-1-ol; **13** = octadeca-9,12-dien-1-ol; **14** = octadeca-9,12,15-trien-1-ol; **15** = octadecan-1-ol. FID (upper trace) = flame ionixation detection, EAD (lower trace) = electroantennogram detection

Results

Gland Microscopy Microscopy of the cephalic labial gland showed differences between the two species. Unlike *B. terrestris*, the labial gland of *B. lucorum* remained functional throughout the life of the adult male. Additionally, in *B. lucorum*, the production of lipid secretion by the smooth endoplasmic reticulum (SER) started earlier (at time of emergence) (c.f., in *B. terrestris* production started on the 2nd day after emergence). The lipids were continuously secreted at the cell apices and, as their volume increased, were accompanied by swelling of the lumen and a decrease in cell layer thickness (Fig. 1a–c; optical microscopy).

The active transport of precursors from the hemolymph was observed in young males of both species, with the transport stopping during the third day after eclosion in *B. terrestris* (Šobotník et al. 2008), but continuing in males of *B. lucorum* up to the 13th day after eclosion. This

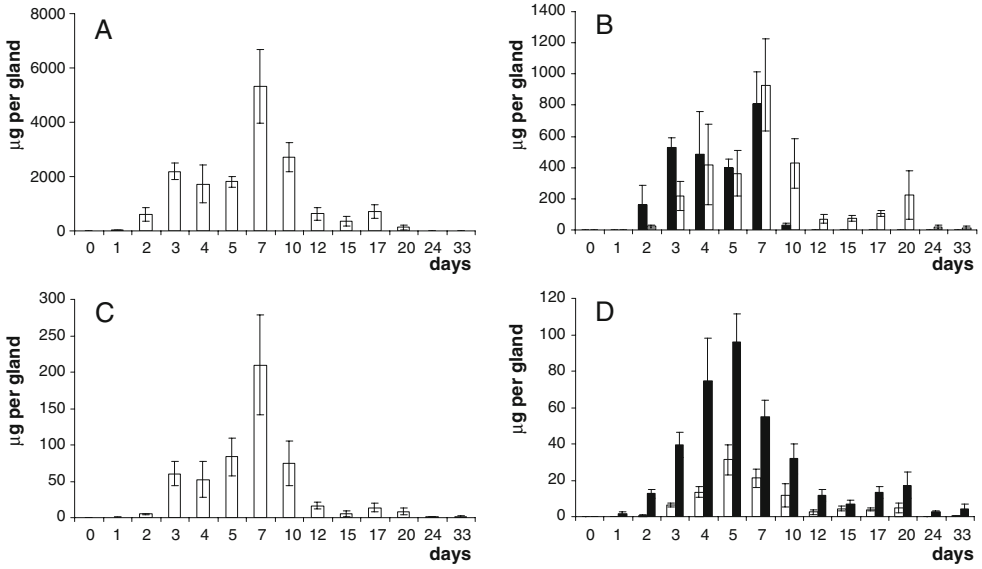

Fig. 4 Concentration changes, with regard to age of adult male, of electroantennogram-active compounds in the cephalic labial gland of *Bombus terrestris* males (mean ± standard error). **a** 2,3-dihydrofarnesol; **b** geranylcitronellol (white bars) and ethyl dodecanoate (black bars); **c** hexadecan-1-ol; **d** 2,3-dihydrofarnesal (white bars) and octadeca-9,12,15-trien-1-ol (black bars)

difference is associated with the different fates of the secretory cells in both species. The cells died after several days of secretory activity in *B. terrestris* (between the 5th and 10th day, Fig. 1d), whereas they conitnued to produce secretion throughout the life of adult *B. lucorum* males. However, even in older *B. lucorum* males, flattening of cells, lower volume of SER, and fewer droplets of secretion in the cell cytoplasm were observed (Fig. 2a,b; electron microscopy) indicating a decreasing rate of secretion production.

GC-MS, GC-EAD, and quantitative analyses In the labial gland of *B. terrestris,* the EAD-active compounds were identified as ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadeca-9,12,15-trienol, and geranylcitronellol (Fig. 3a). In *B. lucorum*, ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadeca-9,12-dienol, octadeca-9,12,15-trienol, and octadecanol elicited antennal responses in conspecific queens (Fig. 3b).

The EAD-active compounds of both species were quantified. In *B. terrestris*, the most abundant EAD-active component of the extract was 2,3-dihydrofarnesol (Fig. 4a). Males produced small quantities (ca. 20 µg/gland) of this compound on the day of eclosion. The quantity of this compound increased up to 2 mg/gland over the next two days, and remained roughly constant at this level for three more days. On the 6th and 7th days following eclosion, the concentration increased almost three times to ca. 6 mg/gland. Subsequent to this, the amount of 2,3-dihydrofarnesol decreased dramatically, such that at 10 days after eclosion, it fell to ca. 0.5 mg/gland. By day 20, 2,3-dihydrofarnesol was present in the gland at a very low amount (2 µg/gland). A similar pattern was observed for geranylcitronellol (1 µg–1 mg/gland, Fig. 4b) and hexadecanol (0.5 µg–200 µg/gland, Fig. 4c). A different age-dependent pattern was observed for ethyl dodecanoate, which appeared in the secretion later than the terpenes (at 2 days, 0.5 µg/gland), increased to its maximum concentration (800 µg/gland) on day 7, and then dropped to 0.7 µg/gland by day 20 (Fig. 4b). The remaining EAD-active compounds, octadecatrienol and 2,3-dihydrofarnesal, reached a maximum concentration around day five (100 µg/gland and 30 µg/gland, respectively, Fig. 4d).

The concentration profiles of the most abundant EAD-active compounds (i.e., ethyl tetradec-7-enoate and ethyl tetradec-9-enoate) present in the gland of *B. lucorum*, (Fig. 5a), differed from those in *B. terrestris.* In *B. lucorum*, no significant maxima were observed in the profile of the most abudnant EAD-active compounds, with the quantities remaining relatively constant over the adult life of the males. Ethyl tetradec-7-enoate and ethyl tetradec-9-enoate were both present (ca 0.9 mg/gland) in the gland on the day after eclosion, with maximum quantities (total of 1.5 mg/gland) occurring between days 7–20. The quantities of these two compounds declined significantly by day 25. Other EAD-active compounds, ethyl dodecanoate and hexadecanol (Fig. 5b), were present at roughly one tenth the amount (113 and 91 µg/gland, respectively) of the main component, but their concentration profiles exhibited a similar pattern with no significant maximum or changes between days 4–10. However, by day 15, the amount of hexadecanol increased, especially in comparison to ethyl dodecanoate, and reached a maximum in 25-day old males (185 µg/gland). Patterns in concentration changes similar to that of hexadecanol were observed for ethyl hexadec-9-enoate and hexadec-7-enal (Fig. 5c). The alcohols octadeca-9,12-dienol, octadeca-9,12,15-trienol (Fig. 5d),

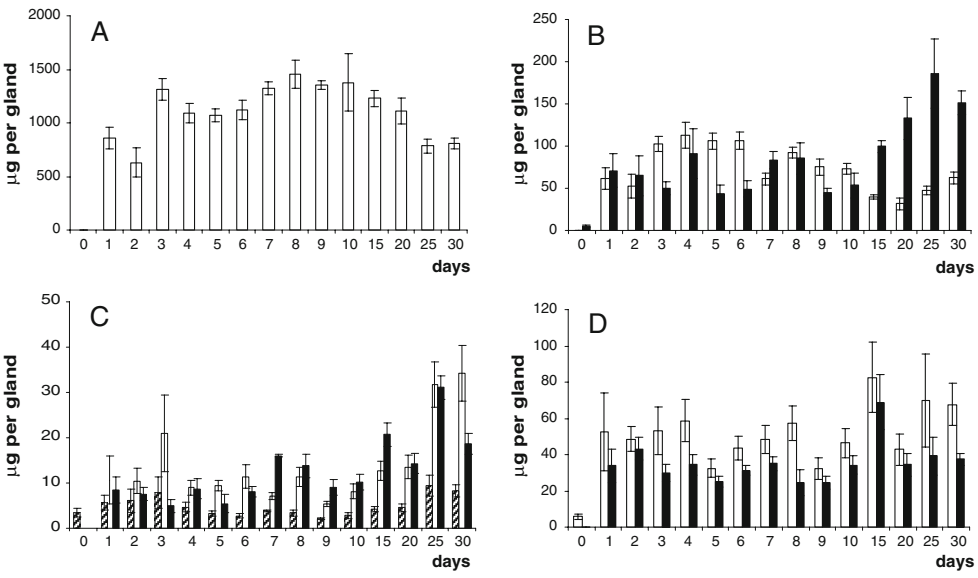

Fig. 5 Concentration changes, with regard to age of adult male, of electroantennogram-active compounds in the cephalic labial gland of *Bombus lucorum* males (mean ± standard error). **a** total ethyl tetradec-7-enoate and ethyl tetradec-9-enoate; **b** ethyl dodecanoate (white bars) and hexadecan-1-ol (black bars); **c** octadecan-1-ol (hatched bars), ethyl hexadec-9-enoate (white bars), and hexadec-7-enal (black bars); **d** octadeca-9,12-dien-1-ol (white bars) and octadeca-9,12,15-trien-1-ol (black bars)

and octadecanol (Fig. 5c) remained at a similar concentration (30–70 µg/gland) throughout the life of the male.

Discussion

The patterns in the quantitative profiles of the various EAD-active compounds corresponded well with the physiological and morphological changes observed in the labial glands for both species. While in *B. lucorum*, the secretory activity of the acinar cells continued over the lifetime of adult males, in *B. terrestris*, production stopped in males older than 10 days. The reasons behind the profound differences in labial gland morphlogy and chemical production between the closely related species are not clear and cannot be explained by lifetime of males, since laboratory observations show no difference in longevity (~50 days) of both species.

We speculate that the age-dependent changes in gland content may play a role in mate selection. Maximal labial gland content (and activity of secretory cells) was found in 3–5 day-old males of *B. terrestris*, which corresponds well with the time when males usually leave their natal nests (fifth day after eclosion) and start to mark and patrol. Additionally, maximal content in the gland matches maximal sperm content (6th day post-emergence; Tasei et al. 1998). In the laboratory, *B. terrestris* males mate between day 6–27 with the probability of successful mating dropping dramatically after day 11 (Tasei et al. 1998). Information about optimal reproductive behavior and physiology in *B. lucorum* is not available, but it would be interesting to know whether it matches the greater duration of labial gland content of EAD-active compounds found in this study.

It has been hypothesized that virgin queen bumblebees searching for a mate are likely to be more attracted to locations where higher amounts of secretion have been deposited by patrolling males (Ågren et al. 1979). Thus, given our results, it is possible that younger males of *B. terrestris* may have an advantage, over older males, in attracting queens, because their scent marks are likely to contain higher amounts of labial gland secretions than those of older males. Such an advantage would not be expectecd in *B. lucorum*, since young and old males have similar quantities of labial gland secretions.

Earlier reports on the variation in composition of the labial gland secretions of bumblebees are scarce. Kullenberg et al. (1970) described considerable seasonal variation in the diterpene content in the glands of *B. hortorum* and *B. hypnorum*. Svensson and Bergström (1977) found that diterpenic components in the labial gland secretion of *B. pratorum* males appear later in the season. Ågren et al. (1979) studied changes in labial gland secretion of various bumblebees species. No labial gland secretion compounds were observed in pupae of *B. hypnorum*. The compounds appeared from day 1 after emergence, and their amounts increased rapidly over the next 4–7 days. For *B. lapidarius*, measurements of gland content were not made until 7 days after eclosion. Secretion content had dropped 14 days after eclosion and further declined to undetectable by 19 –29 days. A similar declining trend was observed for *B. hortorum* males (Ågren et al. 1979). Although this study utilized an analytical technique (TLC) that did not allow precise quantifications of compounds to be made, the results are consistent with our observations for *B. terrestris*.

Although attractiveness of the male labial gland secretion for young queens has been demonstrated by Bergman (1997), roles of individual components in the blend have not been studied. Our study on the quantitative changes of EAG-active chemicals has identified a number of potential pheromone components, which should be tested in bioassays.

Finally, our results show that the production and composition of the labial gland secretion of male bumblebees vary considerably with age. Thus, chemotaxonomic studies that use labial gland secretions to distinguish between closely related species should take this variation into account, perhaps by analyzing solely mature and sexually active males. Such chemotaxonomic studies are best combined with other techniques such as morphology and genetic analysis, for differentiating clsoely related species (Bertsch et al. 2005).

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References

ÅGREN, L., CEDERBERG, G., and SVENSSON, G. 1979. Changes with age in ultrastructure and pheromone content of male labial glands in some bumblebee species (Hymenoptera, Apidae). *Zoon* 7:1–14.

BERGMAN, P., 1997. Chemical communication in bumblebee premating behaviour. Ph.D. Thesis, Göteborg University, Sweden.

BERGMAN, P., and BERGSTRÖM, G. 1997. Scent marking, scent origin, specifity in male premating behavior of two Scandinavian bumblebees. *J. Chem. Ecol.* 23:1235–1251.

BERGSTRÖM, G., KULLENBERG, B., and STÄLLBERG-STENHAGEN, S. 1973. Studies on natural odoriferous compounds: VII. Recognition of two forms of *Bombus lucorum* L. (Hymenoptera, Apidae) by analysis of the volatile marking secretion from individual males. *Chem. Scr.* 4:174–182.

BERGSTRÖM, G., SVENSSON, B.G., APPELGREN M., and GROTH I. 1981. Complexity of bumble bee marking pheromones: biochemical , ecological and systematical interpretations, pp. 175–183, in P. J. Howse and J.-L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London, New York.

BERTSCH, A., SCHWEER, H., TITZE, A., and TANAKA, H. 2005. Male labial gland secretions and mitochondrial DNA markers support species status of *Bombus cryptarum* and *B. magnus* (*Hymenoptera*, *Apidae*). *Insectes Soc.* 52:45–54.

CALAM, D. H. 1969. Species and sex-specific compounds from the heads of male bumblebees (*Bombus* spp.). *Nature* 221:856–857.

COPPÉE, A., TERZO, M., VALTEROVÁ, I., and RASMONT, P. 2008. Intraspecific variation of the cephalic labial gland secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Chem. Biodiversity* 5:2654–2661.

KINDL, J., HOVORKA, O., URBANOVÁ, K., and VALTEROVÁ, I. 1999. Scent marking in male premating behavior of *Bombus confusus*. *J. Chem. Ecol*. 25:1489–1500.

KULLENBERG, B., BERGSTRÖM, G., and STÄLLBERG-STENHAGEN, S. 1970. Volatile components of the cephalic marking secretion of male bumblebees. *Acta Chem. Scand.* 24:1481–1483.

KULLENBERG, B., BERGSTRÖM, G., BRINGER, B., CARLBERG, B., and CEDERBERG, B. 1973. Observations on scent marking by *Bombus* and *Psithyrus* males and location of site of production of the secretion. *Zoon Suppl.* 1:25.

LLOYD, J. E. 1981. Sexual selection: Individuality, identification, and recognition in a bumblebee and other insects. *Fla. Entomologist* 64:89–107.

MORSE, D. H. 1982. Behavior and ecology of bumble bees, pp. 245–322, in H. R. Hermann (ed.). Social Insects, vol. III. Academic Press, New York.

PATERSON, H.E.H. 1993. Evolution and the Recognition Concept of Species. Collected writings. Johns Hopkins University Press, Baltimore.

PTÁČEK, V. 1999. Obtaining and Overwintering Young Bumble Bee (Hymenoptera Bombinae) Queens. - Insect Pollination in Greenhouses. APIMONDIA, ICPBR, Soesterberg, The Netherlands, 55–57.

PTÁČEK, V., PERNOVÁ, E., and BOROVEC, R. 2000. The two-queen cascade method as an alternative technique for starting bumble bee (*Bombus,* Hymenoptera, Apidae) colonies in laboratory (Preliminary study). *Pszczel. Zesz. Nauk*. 44:305–309.

RASMONT, P., TERZO, M., AYTEKIN, M., HINES, H., URBANOVÁ, K., CAHLÍKOVÁ, L., and VALTEROVÁ, I. 2005. Cephalic secretions of the bumblebee subgenus *Sibiricobombus* Vogt suggests *Bombus niveatus* Kriechbaumer and B*ombus vorticosus* Gerstaecker are conspecific (Hymenoptera, Apidae, *Bombus*). *Apidologie* 36:571–584.

SCHREMMER, F. 1972. Beobachtungen zum Paarungsverhalten der Männchen von *Bombus confusus* Schenk. *Z. Tierpsychol*. 31:503–512.

ŠOBOTNÍK, J., KALINOVÁ, B., CAHLÍKOVÁ, L., WEYDA, F., PTÁČEK, V., and VALTEROVÁ, I. 2008: Age-dependent changes in structure and function of the male labial gland in *Bombus terrestris*. *J. Insect Physiol.* 54:204–214.

SVENSSON, B.G. 1980. Species-isolating mechanisms in male bumble bees (Hymenoptera, Apidae). *Abstr. Upps. Diss. Fac. Sci.* 549:1–42.

Svensson, B.G., and BERGSTRÖM, G. 1977. Volatile marking secretions from the labial gland of North European *Pyrobombus* D. T. males (Hymenoptera, Apidae). *Insectes Sociaux* 24:213–224.

TASEI, J.-N., MOINARD, C., MOREAU, L., HIMPENS, B., and GUYONNAUD, S. 1998. Relationship between aging, mating and sperm production in captive *Bombus terrestris. J. Apicultural Res.* 37:107–113.

TERZO, M., VALTEROVÁ, I., URBANOVÁ, K., and RASMONT, P., 2003. De la nécessité de redécrire les phéromones sexuelles des mâles de bourdons [Hymenoptera: Apidae, Bombini] publiées avant 1996 pour leur utilisation en analyse phylogénétique. *Phytoprotection* 83:39–49.

TERZO, M., URBANOVA, K., VALTEROVA, I., and RASMONT, P. 2005. Intra and interspecific variability of the cephalic labial glands' secretions in male bumblebees: the case of *Bombus (Thoracobombus) ruderarius* and *B. (Thoracobombus) sylvarum* [Hymenoptera, Apidae]. *Apidologie* 36:85–96.

URBANOVÁ, K., VALTEROVÁ, I., HOVORKA, O. and KINDL, J. 2001. Chemotaxonomical characterisation of males of *Bombus lucorum* collected in the Czech Republic. *Eur. J. Entomol.* 98:111–115.

VALTEROVÁ, I. and URBANOVÁ, K. 1997. Chemical signals of bumblebees. *Chem. Listy* 91:846–857 (in Czech).

VALTEROVÁ, I., KUNZE, J., GUMBERT, A., LUXOVÁ, A., LIBLIKAS, I., KALINOVÁ, B., and BORG-KARLSON, A.-K. 2007. Male bumble bee pheromonal components in the scent of deceit pollinated orchids; unrecognized pollinator cues? *Arthropod-Plant Interactions* 1:137–145.

VILLALOBOS, E.M., and SHELLY, T.E. 1987. Observations on the behavior of male *Bombus sonorus* (Hymenoptera: Apidae). *J. Kansas Ent. Soc.* 60:541–548.

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COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY AS A TOOL FOR BIOSYNTHESIS STUDY OF THE COMPONENTS OF THE MARKING PHEROMONES OF THE MALES SPECIES *Bombus lucorum* AND *Bombus lapidarius*.

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Abstract

Biosynthesis of the bumblebee's male marking pheromone of species *Bombus lucorum* and *Bombus lapidarius* was studied. Deuterated fatty acids were applied to the abdomen of the bumblebee's body. After a few days of incubation, extracts of labial glands and fat bodies were analyzed. Comprehensive two dimensional gas chromatography separation with mass detection technique has been optimised and used for the analysis of the tissue. Decarboxylation, reduction, and esterification of applied labeled fatty acids have been found. Prolongation of deuterated precursor's carbon chain has been observed too, but no shortening.

Keywords

Comprehensive two dimensional gas chromatography; labial gland; fat body; Bombus lucorum, Bombus lapidarius; marking pheromone

1. Introduction

Bumblebees belong to social species of insect similar to bees, ants, or termites. The communication by means of chemical compounds coordinates all aspects of their life in a colony.

This study is focused on the biosynthesis of the components of the male marking pheromones of species *Bombus lucorum* and *Bombus lapidarius*. A premating behavior of bumblebee males has been studied for long time mainly by Swedish authors[1,2]. The majority of bumblebee species exhibit a patrolling behaviour. They fly around the nest or maximally few hundred meters away from it and mark prominent objects in their territories (stones, leaves) with a pheromone secretion from the labial gland[3,4]. The marked places attract conspecific females for mating[5]. The composition of the secretion is species-specific[1]. The gland secretion of species *Bombus lucorum* contains mainly ethyl esters of fatty acids, chiefly ethyl (Z)-tetradec-9-enoate (53%)[6] while main components of the labial gland secretion of *Bombus lapidarius* are hexadecan-1-ol (31%) and (Z)-tetradec-9-en-1-ol (52%). Biosynthesis of these components has been discussed[7]. It was suggested, that these compounds are produced from saturated fatty acids by means of specific enzymes. Some experimental data[8] are available from a recent time which show us the first look on the biosynthetic pathways of the pheromone formation. Authors applied labeled [$^2H_{31}$]-hexadecanoic acid into the head capsule and the abdomen of the bumblebee's body. After a certain time of incubation in living animals, hexane extracts of dissected labial glands and fat bodies were analysed by GC/MS. In labial glands of species *B. lucorum* two metabolites of the applied compounds have been found: ethyl [$^2H_{31}$]-hexadecanoate and ethyl [$^2H_{29}$]-hexadec-9-enoate. The analysis of the fat body showed that labeled compounds were incorporated into the triacylglycerols (TAG) in acidic part. In the case of species *B. lapidarius* there have also been found two metabolites in the labial gland: [$^2H_{31}$]-hexadecan-1-ol and [$^2H_{29}$]-hexadec-9-en-1-ol. Similary to *B. lucorum* precursors incorporated into TAG. No prolongation or shortening of deuterated carbon chain was found. This study follows the previous work, but it uses a new analytical tool for separation and identification of labeled compounds. Comprehensive two-dimensional gas chromatography with mass spectrometric detector (GCxGC-MS) is a new method used for biosynthetic studies for the first time.

2. Experimental

2.1 Animals

The males of bumblebee species *B. lucorum* and *B. lapidarius* were obtained from laboratory colonies from cooperating laboratory in Faculty of Science of Masaryk University in Brno.

2.2 Chemicals

Compounds labeled by deuterium have been used: [$^2H_{27}$]-tetradecanoic acid (Aldrich), [$^2H_{31}$]-hexadecanoic acid (Aldrich), [$^2H_{35}$]-octadecanoic acid (Aldrich). For extractions of the tissue redistillated hexane purchased from Merck was used. The same hexane was used as a blank. Application mixture: 4 μg of labeled compound was dissolved in 100 μl of mixture of solvents: dimethylsulfoxide:ethanol:water 7:2:1. No mixtures of labeled precursors were done in this study.

2.3 Application/Dissection

All animals were approximately 2 days old when labeled compounds were applied. An application dose was 2 μl of the application mixture per animal and the solution was injected into the abdomen. Labial glands and the peripheral fat bodies (fat body) were dissected after 2 or 4 days of incubation. The dissected labial glands were extracted with hexane: 100μl per gland. All samples were stored in freezer (-30^{o}C) until their analyses. The dissected fat body was used for a further treatment (see Preparation of derivatives).

2.4 Preparation of Derivatives

The dissected fat body was dissolved in 100 μl of mixture (methanol:chloroform 1:1 + BHT (5 mg / 100 ml) (butylated hydroxytoluene) as an antioxidant, then homogenized and next 100 μl of chloroform was added. Mixture was separated on precleaned glass thin layer chromatography plates (TLC) (75 x 59 mm, coated with Adsorbosil-Plus (Applied Science Labs), layer thickness 0.2 mm, added gypsum (12%)) using the mixture hexane:diethylether:formic acid 80:20:1 as a mobile phase. Spots were visualized by spraying Rhodamine 6G solution (0.05% in ethanol). Three fractions were obtained: 1: hydrocarbons (R_f = 0.91), 2: triacylglycerols (TAG) (R_f = 0.59), 3: mixture of mono and diacylglycerols (R_f = 0.24). Each of the fractions was extracted with approximately 0.5 ml of ether and cleaned on silica gel column. The cleaned extract was put over to the ampoule of the known weight. Then the extract was dried by argon flow until there was no solvent. After that the ampoule was dried under vacuum until constant weight. Fractions of TAG and MAG + DAG were transesterified[9] and analysed as fatty acids methyl esters by GCxGC-MS.

2.5 Chromatographic Analyses

All analyses were performed using GCxGC-MS Pegasus 4D from LECO, Co. (USA). This system consists of gas chromatograph Agilent 6890N with split/splitless injector. Inside the GC oven, a dual-stage jet modulator and the secondary oven were mounted. Resistively heated air was used as a medium for hot jets, while cold jets were supplied by gaseous nitrogen, secondary cooled by liquid nitrogen. A second part of this system was the mass spectrometer with Time Of Flight (TOF) mass analyser (LECO Pegasus III).

Separation parameters: Split (1/20); constant He column flow 1 ml/min column, inlet temperature 250 ^{o}C, injection volume 2 μl (1 μl sample + 1 μl hexane), modulation time: 3 s (hot pulse 0.6 s); modulation temperature offset: 30 ^{o}C.

First dimension column: length 30 m, ID 250 μm, film thickness 0.25 μm, phase DB-5 (J & W Scientific, USA). Applied temperature gradient: 40 ^{o}C (2 min), then 5 ^{o}C/min to 320 ^{o}C (5 min).

Second dimension column: length 1.79 m, ID 100 μm, film thickness 0.1 μm, phase BPX-50 (SGE, Australia). applied temperature gradient: 45 ^{o}C (2 min), then 5 ^{o}C/min to 325 ^{o}C (5 min).

MS parameters: electron ionisation mode (-70 eV), acquisition rate 150 Hz, mass range 29-500 amu, ion source temperature 220 ^{o}C, transfer line temperature 250 ^{o}C, detector voltage −1750 V. ChromaTOF software (LECO, Co.) was used for the processing of collected data.

2.6 Comprehensive Two-dimensional Gas Chromatography

The separation in this system is provided by two different separation mechanisms. First, the sample goes to the first column, which is usually an ordinary nonpolar column of the same parameters as for one dimensional GC. Then the sample, already separated in the first column, continues to the modulator. Zones of compounds are focused by means of timed cold and hot pulses in the modulator and injected to the second column (usually polar). This column is only a few meters long, more than two times thinner and located in a second oven, which can be heated independently. This column is connected with MS detector with TOF mass analyser (**Fig. 1**). The software reconstructs first dimension of separation by means of chromatographic data resulted from separation from secondary column and visualises the whole chromatogram as a contour plot or 3D visualisation (**Fig. 2**). Main advantages of this system: 1) separation according to two different mechanisms, 2) focusation

of the chromatographic zones in cryogenic modulator – increasing sensitivity, 3) obtaining true baseline – more accurate quantification.

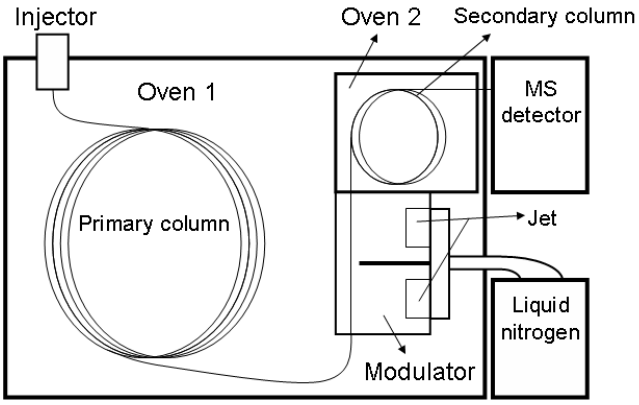

Fig. 1 – Schema GCxGC-MS.

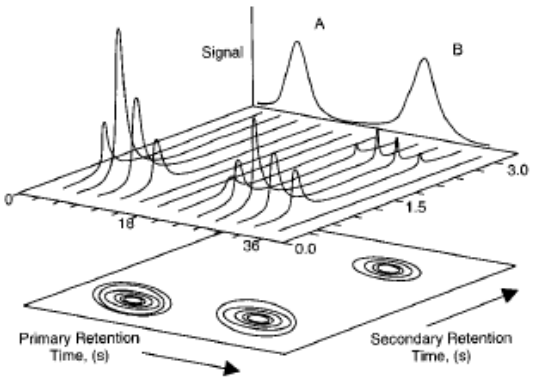

Fig. 2 – Data visualization.

3. Results and Discussion

Biological aspects of this study will not be discussed in this paper.

3.1 Identification of the Labeled Compounds

In previous study[8] only metabolites which were of the same carbon chain length originated from the labeled acid were found in LG and fat body. Analysis by means of GCxGC-MS found much more isotopically labeled compounds (**Tables 1** and **2**). However, only some of them could have been sprightly identified according to their MS spectra due to the abundance of the metabolites. Nevertheless, the identification by combination of two retention indexes and mass spectra appeared to be sufficient. Deuterated compounds are eluted from the first dimensional column (non-polar) slightly sooner then their non-deuterated (native) analogs. The same behaviour is observed in the second dimension (used polar column) (**Fig. 3**).

Hydrocarbons: Deuterated hydrocarbons were found in both examined species: CD_3-$(CD_2)_n$-$(CH_2)_m$-CH_3 where n = 12 (precursor was [$^2H_{27}$]-tetradecanoic acid), n = 14 (precursor was [$^2H_{31}$]-hexadecanoic acid) or n = 16 (precursor was [$^2H_{35}$]-octadecanoic acid) and m = 1,3,5,7,9,11,..-this number depends on the incorporation of deuterium to the bumblebee organism and concentration of the extract. The most abundant are C23 and C25 hydrocarbons in both species. Molecular ion could have been observed very rarely. Typical fragments

for deuterated compounds in MS spectrum are m/z = 46 and 50 ($[C_3D_5]^+$, $[C_3D_7]^+$, respectively. These fragments are analogs of m/z = 41 and 43 in native hydrocarbons. The pattern of the spectrum is similar but in deuterated hydrocarbons abundance of fragment 46 is higher or comparable to 50. The same situation is in case of unsaturated hydrocarbons. Branched carbon chains do not appear in the examined tissues. The mass spectrum became quite difficult when the hydrocarbons were partly deuterated and partly non-deuterated. Then the final spectrum was a mixture of both and the identification by means in comparison of retention behaviour of native and partly deuterated compounds is sufficient. All compounds appeared in the analysed material consisted of long carbon chain (10-30C) with a functional group in position 1. It means that in the mass spectrum fragments similar to hydrocarbons, especially fragments *m/z* 46 and 50 arise. If they both appeared in the spectrum it was highly probable that the compound originated from applied deuterated compounds.

Alcohols: An acid group in a labeled acid is reduced to alcohol group, which means that the methyl groups located next to the hydroxyl contain hydrogens: CD_3-$(CD_2)_m$-$(CH_2)_n$-OH, (m = 12 and n = 1) if [$^2H_{27}$]-tetradecanoic acid is applied and (m = 14 and n = 2) if [$^2H_{31}$]-hexadecanoic acid is applied. Molecular ion was not observed because alcohols loose DOH by deuterium rearrangement, so mass $[M-19]^+$ was observed. Typical fragment of native primary deuterated alcohols is *m/z* 34. Secondary and tertiary alcohols do not appear in the analyzed material. The next typical fragment in deuterated alcohols (saturated and unsaturated) was *m/z* 62 [CD_3-CD=CD-C^+D_2], which originates from *m/z* 55. Fragments *m/z* 46 and 50 are also very abundant.

Esters: The general formula of esters found in the analysed samples was as follows: CD_3-$(CD_2)_l$-$(CH_2)_m$-COO-$(CH_2)_n$-CH_3 (**Tables 1** and **2**). In the case of (MAG + DAG) and TAG the methyl group arises from transesterification of the proper glycerides. In LG extracts the ethyl group was connected by enzymatic system of the animal.

The identification of aliphatic esters is very easy. There are two important fragments in their mass spectrum: one originates from McLafferty rearrangement of γ hydrogen: $[CX_2=C(O^+H)-OR]$, X = D if the ester came from labeled acid which was prolonged by two or more carbons R can be methyl or ethyl group, and the second fragment arises from γ bond splitting. The exact mechanism is still unknown. The fragment is formally represented by formula $[CX_2.CX_2=C(O)-OR]^+$. If the ester was saturated intensities of these two fragments were far the most intensive in the spectrum. In unsaturated ester their intensities were comparable with abundances of fragments 46 and 50. Molecular ion was usually observed. The deuterated metabolites found in the tissue are listed in the **Tables 1** and **2**.

Table 1 – Deuterated compounds found in *B. lucorum* species.

B. lucorum	**Deuterated metabolites**		
	Labial gland	Fat body	
Applied acid		MAG + DAG	TAG
$[^2H_{27}]$-tetradecanoic acid CD_3-$(CD_2)_{12}$-COOH 8 animals	E.[a] tetradecanoate, E.[a] tetradecenoate[b,c] CD_3-$(CD_2)_{12}$-COO-CH_2-CH_3 Hexadecanol CD_3-$(CD_2)_{12}$-$(CH_2)_3$-OH Hydrocarbons[b,d] CD_3-$(CD_2)_{12}$-$(CH_2)_n$-CH_3	M.[e] tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 M.[e] hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3 M.[e] octadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_4$-COO-CH_3	M.[e] tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 M.[e] hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3 M.[e] octadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_4$-COO-CH_3

[a]Ethyl, [b]Position of the double bond was not determined, [c]Two isomers with different position of double bond were observed, [d]Odd carbon number only (C23 and C25 were most abundant), [e]Methyl.

Table 2 – Deuterated compounds found in *B. lapidarius* species.

B. lapidarius	**Deuterated metabolites**		
	Labial gland	Fat body	
Applied acid		MAG + DAG	TAG
$[^2H_{27}]$-tetradecanoic acid CD_3-$(CD_2)_{12}$-COOH 12 animals	Hexadecanol, Hexadecenol[a] CD_3-$(CD_2)_{12}$-$(CH_2)_3$-OH Hydrocarbons[a,b] CD_3-$(CD_2)_{12}$-$(CH_2)_n$-CH_3	M.[c] tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 M.[c] hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3	M.[c] tetradecanoate CD_3-$(CD_2)_{12}$-COO-CH_3 M.[c]hexadecanoate, M.[c] hexadecenoate[a] CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3
$[^2H_{31}]$-hexadecanoic acid CD_3-$(CD_2)_{14}$-COOH 11 animals	Hexadecanol, Hexadecenol[a] CD_3-$(CD_2)_{14}$-CH_2-OH Hydrocarbons[a,b] CD_3-$(CD_2)_{14}$-$(CH_2)_n$-CH_3	M.[c] hexadecanoate CD_3-$(CD_2)_{12}$-$(CH_2)_2$-COO-CH_3	M.[c] hexadecanoate, M.[c] hexadecenoate[a] CD_3-$(CD_2)_{14}$-COO-CH_3
$[^2H_{35}]$-octadecanoic acid CD_3-$(CD_2)_{16}$-COOH 26 animals	Hydrocarbons[a,b] CD_3-$(CD_2)_{16}$-$(CH_2)_n$-CH_3	M.[c] octadecanoate, CD_3-$(CD_2)_{16}$-COO-CH_3	M.[c] octadecanoate, M.[c] octadecenoate[a] CD_3-$(CD_2)_{16}$-COO-CH_3

[a]Position of the double bond was not determined, [b]Odd carbon number only (C23 and C25 were most abundant), [c]Methyl.

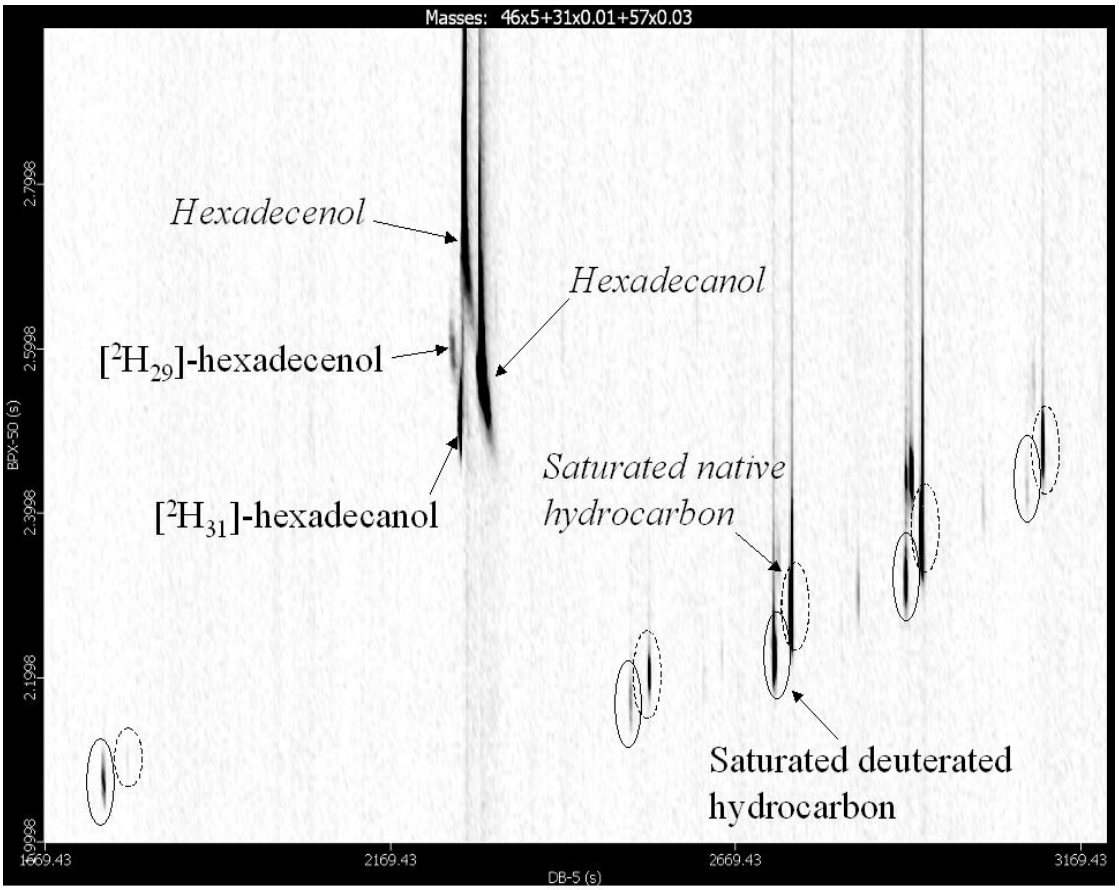

Fig. 3 – Chromatogram of the hexan extract of the labial gland species *B. lapidarius* ([^{2}H$_{31}$]-hexadecanoic acid was applicated).

4. Conclusions

Deuterated compounds were found in the all types of tissues except for the hydrocarbon fraction from the fat body (**Tables 1** and **2**). Quantification has not been done yet, but correlation between abundance of native compounds and their deuterated analogs was observed. Prolongation of deuterated precursor's carbon chain has been observed, however, deuterated metabolites with shorter carbon chain than the applied precursor have not been found.

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References

[1] Bergström G., Svensson B. G., Appelgren M., Groth I.: *Academic Press, London and New York Vol.* ***19*** *(1981), p. 175*.

[2] Kullenberg B., Bergström G., Ställberg-Stenhagen S.: *Acta Chem. Scand.* ***24*** *(1970), 1481*.

[3] Morse D. H.: *Social Insect (H. R. Hermann, Ed.), Academic Press, New York.* ***Vol. III*** *(1982), p. 245*.

[4] Bergström G., Kullenberg B., Bringer B., Carlberg B., Cederberg B.: *Zoon Suppl.* ***1*** *(1973), 25*.

[5] Kullenberg B.: *Zoon Suppl.* ***1*** *(1973), 31*.

[6] Urbanová K., Valterová I., Hovorka O., Kindl J.: *Eur J Entomol* ***98*** *(2001), 111-115*.

[7] Lanne B.S., Bergström G., Wassgren A.-B., Törnbäck B.: *Comp Biochem Physiol* ***88B*** *(1987), 631-636*.

[8] Luxová A., Valterová I., Stránský K., Hovorka O., Svatoš A.: *Chemecol* ***13*** *(2003), 81-87*.

[9] Stránský K., Jursík T.: *Fett/Lipid* ***98*** *(1996) 65-67*.

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De Novo Biosynthesis of Sexual Pheromone in the Labial Gland of Bumblebee Males

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De novo biosynthesis of male sex pheromone from two bumblebee species (*Bombus terrestris* and *Bombus lucorum*) was studied by using in vitro incubations of labial glands (LGs) with radioactive [1,2-^{14}C]acetate and deuterated [D_3]acetate. The labeled substrate was incorporated into several types of compounds, such as terpenic alcohols, fatty acids, esters, and hydrocarbons. A similar incubation of [1,2-^{14}C]acetate with fat bodies (FB) led to the formation of fatty acids, triacylglycerols (TAG), and hydrocarbons. To support the results from in vitro incubations, PCR analysis of fatty acid synthase (FAS) transcripts in LG and FB was performed. Relative quantification of FAS transcription levels revealed that the abundance of mRNA from the FAS gene is a function of the age of *B. terrestris* males. A comparison of the relative FAS mRNA gene transcription level in FB and LGs of *B. terrestris* and *B. lucorum* males proved that high biosynthetic activity takes place in the LGs of both species. Together, these results indicate that pheromone components are synthesized de novo in the LG.

Introduction

Communication in social insects is, to a large extent, chemically mediated. Pheromones control practically all aspects of the insects' lives, such as finding their way to food sources, recognition of the social status of colony members, and mating. Males of most bumblebee species have unique mating strategies, which differ from other social insect species. They patrol along regular routes and mark prominent objects (stones, fence posts, or leaves) with a mixture of chemicals. These secretions serve as a sexual/marking pheromones and attract conspecific females for mating.[1–5] Sexual pheromones are released from the cephalic part of the labial gland (LG), and their composition is highly species-specific.[6]

Bombus terrestris and *Bombus lucorum* were selected as model bumblebee species for this study. LGs of *B. terrestris* males contain a mixture of aliphatic and terpenic compounds, with ethyl dodecanoate and 2,3-dihydrofarnesol being the most abundant.[2] *B. lucorum* produces aliphatic esters and alcohols.[4,7,8] Very little is known about the synthesis of these pheromone components. The first biosynthetic studies were performed with *B. lucorum*[9,10] and *Bombus lapidarius*,[9] whose LG secretions consist almost entirely of aliphatic compounds, with the most abundant being ethyl (*Z*)-tetradec-9-enoate (53%) in *B. lucorum*,[7] and (*Z*)-hexadec-9-enol (52%) and hexadecanol (31%) in *B. lapidarius*.[5,9] For both species the authors confirmed the formation of pheromonal components from fatty acids applied in vivo and labeled with deuterium. Nevertheless, the biosynthetic origin of the fatty acids (subsequently transformed into the pheromonal components) remains unclear.

Two potential pathways are proposed for the biosynthesis of fatty acid pheromone precursors. The first model involves fatty acids that are biosynthesized and stored in the form of triacylglycerols (TAGs) in fat-body (FB) adipocytes.[11] From TAG, fatty acids are mobilized by the action of appropriate lipases and released as diacylglycerols (DAGs). They are bound to lipophorin, the hemolymphatic lipoprotein that carries and distributes DAG to target tissues.[12] Lipophorin works as a reusable shuttle, and selectively delivers DAG to "flight" muscles, hydrocarbons to the cuticle, and pheromones from the site of their production to the pheromonal gland in moths.[13,14] Lipids move from the lipophorin complex to cells by passive diffusion or by the action of active transporters. Alternatively, lipophorin itself may be taken up by the cells, and the bound lipids are released once inside.[12] Inside the cell, DAG is hydrolyzed to fatty acids by a membrane-bound lipophorin lipase.[15] Relevant fatty acids are then transformed into pheromone components by the action of various types of enzymes (desaturases, decarboxylases, ethyl esterases, and reductases).[16]

The second model for the biosynthesis of fatty acid pheromone precursors is de novo biosynthesis from acetate units,

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directly in the LG.[17] This type of pathway for the production of pheromone components has been demonstrated in other insect species, for example, moths,[18] bark beetles,[19–21] and honey bees.[22,23] For de novo biosynthesis of pheromone blend components in the LG of *B. terrestris*, fatty acid synthase (FAS) would putatively be the key enzyme in the production of aliphatic components (e.g., ethyl dodecanoate). In general, FAS catalyzes de novo synthesis of saturated fatty acids from acetyl-CoA, malonyl-CoA, and NADPH. Thus far, only one enzyme (a Δ^9 desaturase) has been identified and characterized in the LG and FB of male *B. lucorum*.[10] Moreover, the relative proportion of fatty acids in the FB TAGs that would correlate with the structure of pheromone components are quite low for both species, *B. terrestris* and *B. lucorum*.[24] Thus, results reported to date do not clearly support either of the two hypotheses, and the origin of fatty acids in pheromone biosynthesis remains unclear.

In the case of the terpenic pheromone components (e.g., 2,3-dihydrofarnesol and geranylcitronellol), no literature is available on their biosynthesis in bumblebees. We assume variants of de novo biosynthesis or sequestration from food, as this has already been described for other insect species.[25,26] However, species-specific composition of pheromonal blends found in specimens from different localities, as well as comparison of wild and laboratory colonies, strongly supports the de novo hypothesis. For de novo biosynthesis of terpenes, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase would be the key putative enzyme. HMG-CoA reductase (converting HMG-CoA to mevalonic acid) is the rate-controlling enzyme of the mevalonate pathway.

In this study, we performed in vitro incubations with [1,2-^{14}C]acetate and deuterated [D_3]acetate with LG and FB collected from *B. terrestris* and *B. lucorum* males. We analyzed LB and FB metabolites to test our hypothesis that aliphatic and terpenic pheromonal components are formed de novo in the LG. The results from the incubation experiments are supported by relative quantification of the transcription level of the fatty acid synthase gene.

Results and Discussion

Optimization of incubation conditions

Incubation experiments were carried out with sodium [1,2-^{14}C]acetate and both cephalic LG and peripheral FB dissected from *B. terrestris* and *B. lucorum* male abdomens. LG function has been associated with accumulation and release of sexual pheromones,[27] but de novo biosynthesis of pheromones in the LG has not previously been confirmed. In contrast, high biosynthetic and metabolic activities have been associated with the FBs of various insect species.[28–30] For this reason, we chose FB as a comparative tissue for our biosynthetic experiments with the LG.

The basic composition of the incubation solutions were taken from Lee et al.,[28] who studied the activity of adipokinetic peptides based on their inhibition of lipid synthesis in the FB of *Locusta migratoria*. We adapted some of the parameters to match the natural conditions of the examined bumblebee tissues: incubation time, pH, temperature, and the concentration of unlabeled acetate (Table 1). The parameters were optimized for LG and FB independently. Incubation efficiency was evaluated based on the overall radioactivity of extracts (without identification of biosynthetic products). We assumed that radioactivity in tissue extracts is proportional to biosynthetic activity.[28] All optimizations were carried out with *B. terrestris* males, as they are easier than *B. lucorum* to rear in the laboratory.

Table 1. Optimal conditions for in vitro incubations of labial glands (LG) and fat bodies (FB) of *B. terrestris* males. Radioactivity was measured in tissue extracts (see the Experimental Section).

	t [h][a,b,c]	Incubation Solution pH[b,d,e]	*T* [°C][b,e,f]	Non-radioactive acetate concentration [mM][e,g,h]
	5	**6.3**	**30**	**0**
FB	431 ± 61***	588 ± 119	578 ± 35	0.54 ± 0.06***
LG	57 ± 36**	228 ± 40	217 ± 64	0.13 ± 0.04***
	9	**6.8**[i]	**35**[i]	**5**
FB	1336 ± 174*	724 ± 116	745 ± 62	68 ± 3**
LG	230 ± 59**	230 ± 42	271 ± 94	12 ± 6**
	16[i]	**7.0**	**40**	**10**
FB	2612 ± 172	672 ± 75	810 ± 77	125 ± 33
LG	1455 ± 278	220 ± 170	130 ± 46*	31 ± 9
	24	**7.3**	**45**	**20**[i]
FB	2525 ± 112	782 ± 80	203 ± 17***	213 ± 21
LG	1652 ± 278	157 ± 55	46 ± 23*	58 ± 8
		7.8		**40**
FB		481 ± 33		395 ± 71
LG		123 ± 8*		128 ± 26*
				60
FB				454 ± 46*
LG				131 ± 15**

[a] Constant parameters: pH 6.8, incubation temperature 35 °C, non-radioactive acetate concentration 0 mM. [b] Results are expressed in Bq per tissue sample. [c] Radioactivity measured in the entire disrupted tissue. [d] Constant parameters: incubation duration 16 h, incubation temperature 35 °C, nonradioactive acetate concentration 0 mM. [e] Radioactivity measured in chloroform extract of the tissue. [f] Constant parameters: incubation duration 16 h, pH 6.8, nonradioactive acetate concentration 0 mM. [g] Constant parameters: incubation duration 16 h, pH 6.8, incubation temperature 35 °C. [h] Results are expressed in nmol(acetate)/16 h/tissue. [i] Conditions identified as optimal for both tissues. Asterisks indicate where the treatment was significantly different from the selected optimal conditions. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Student's *t* distribution). Data are mean ± SEM of 4 or 5 determinations.

The time-dependence of [1,2-^{14}C]acetate absorption was determined at the beginning of the optimization. Incubations of both tissue types were terminated after 5, 9, 16, and 24 h (Table 1). Radioactivity levels in tissue were measured after disruption by a tissue solubilizer. This approach provided information about the total amount of [1,2-^{14}C]acetate that penetrated into the tissue (unlike chloroform extraction, in which only nonpolar compounds are dissolved). The time courses for both tissue types were similar, with a significant increase between 9 and 16 h of incubation. However, the dynamics of [1,2-^{14}C]acetate uptake differed between LG and FB: a sigmoid dependency was obtained for LG, whereas for FB the highest rate of [1,2-^{14}C]acetate absorption was at the beginning (Table 1). This indicates a difference in the kinetics of the pro-

cess of acetate penetration into tissue. A probable explanation is a difference in the structures of adipocytes and LG secretory cells.[27,29] An incubation time of 16 h was selected as a compromise suitable for both tissue types.

For all subsequent optimizations, radioactivity was measured in chloroform extracts of the tissues. In this way, the nonpolar biosynthetic products of interest (such as pheromone components and free fatty acids) could be separated from residual sodium [1,2-^{14}C]acetate.

The pH of the incubation solution was tested over the range 6.3–7.8 (Table 1). The pH curve exhibited a similar shape for the two tissue types, with large deviations in measured radioactivity and no clear maximum for either type. A significant decrease was observed at pH 7.8 for the FB. pH 6.8 was selected for further use, as this was the pH of the freshly prepared incubation solution (without pH adjustment). This selected pH is consistent with previously published work with FB.[28,30]

The influence of incubation temperature was tested at 30, 35, 40, and 45 °C. The observed maxima of incubation efficiency differed slightly between FB and LG. While FB performed best at 40 °C, LG showed the highest efficiency at 35 °C. It seems that the FB enzymatic system can operate at higher temperatures than that of LG. This may be associated with the different locations of the two organs in the bumblebee body.[31] A temperature of 35 °C was selected for both tissue types, to enable parallel incubations (in the same water bath).

The optimal concentration of acetate for the incubation experiments was determined by using a mixture containing a constant concentration of labeled [1,2-^{14}C]acetate and an increasing concentration of [1,2-^{12}C]acetate (unlabeled). The reaction rate increased significantly with increasing concentrations of unlabeled sodium acetate, and reached a maximum at 60 mM acetate per incubation column for both tissue types. However, such a high concentration of unlabeled acetate would substantially dilute the radioactivity, lower the sensitivity of detection, and not allow identification of radioactive metabolites. Therefore, 20 mM was selected as a compromise between [1,2-^{14}C]acetate incorporation and the sensitivity of detection.

The examined tissue types differed in the amount of radioactivity present in their chloroform extracts: radioactivity was, on average, three times lower in the LG than in the FB. This might be attributable to the difference in the weight of the tissue samples taken for each incubation experiment (LG (4.1 ± 0.1) mg; FB (11.1 ± 0.3) mg). After adjusting for this difference, the incorporation rates of acetate into the two tissue types were comparable.

Radioactive metabolites in the LG and FB of *B. terrestris*

Incubation experiments were carried out with the optimized parameters, and tissue extracts were fractionated by means of thin layer chromatography (TLC). Radioactive signals were visualized by a position sensitive radioactivity detector, and the radioactive signals on the TLC plates were transformed into chromatographic peaks. LG extracts produced five signals from radioactive metabolites (Figure 1 A). At first, peak 1 could not

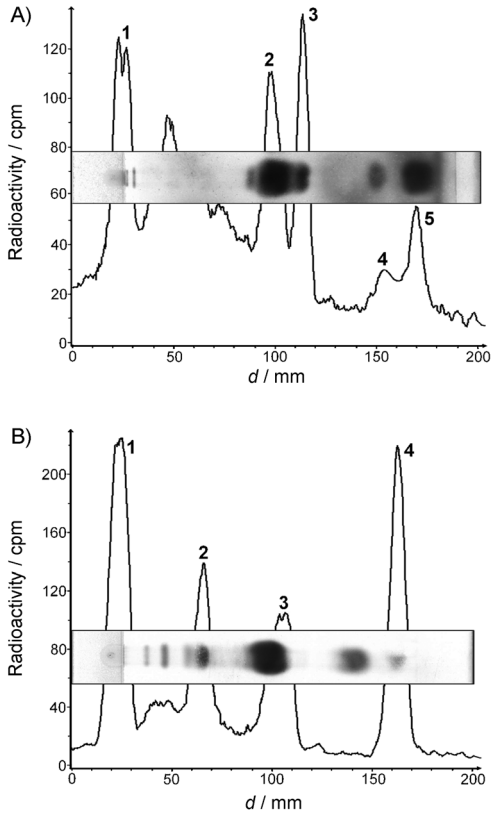


Figure 1. Chromatograms of TLC radioactive signals (*B. terrestris*). A) LG chloroform extract and nonselective visualization of the corresponding TLC plate: 1) polar lipids, 2) terpenic alcohols, 3) free fatty acids, 4) esters, and 5) hydrocarbons; B) FB chloroform extract and nonselective visualization of the corresponding TLC plate: 1) polar lipids, 2) fatty acids, 3) triacylglycerols, and 4) hydrocarbons.

be precisely assigned to any compound or group of compounds. Given the chromatographic conditions (nonpolar mobile phase and polar stationary phase), we assume that peak 1 arose from polar lipids such as phospho- or glycolipids. These compounds originate from cell membranes rather than from pheromones, and the signal was thus not further analyzed. The second fraction, with a relative-to-front factor (R_F) of 48, was assigned to terpenic alcohols by comparison with standards. In *B. terrestris*, this group of compounds is represented mainly by 2,3-dihydrofarnesol and geranylcitronellol. As the retention behavior of these compounds could not reveal if the ^{14}C isotope was incorporated into them, our proof relies on an analogous experiment with ^{2}H-labeled acetate (see below). The third signal ($R_F = 54$) belonged to free fatty acids. The fourth and fifth radioactive TLC fractions were identified as esters ($R_F = 80$) and hydrocarbons ($R_F = 93$), respectively. All the chemical species containing the ^{14}C label are naturally present in the LG.[8,27,32]

Most of the fatty acids formed from [^{14}C]acetate were present in the incubation solution. A chloroform extract of this solution contained one radioactive TLC signal, and this was assigned to free fatty acids (see Figure S1 in the Supporting Information). For quantification purposes, the amounts of free fatty acids in the LG extract and in the incubation solution

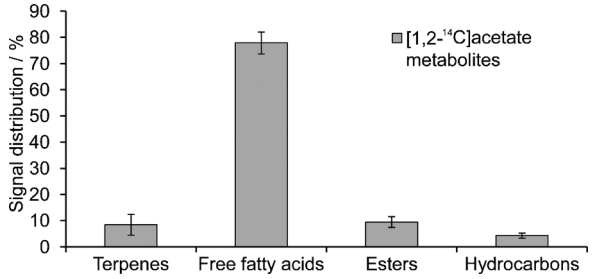


Figure 2. Distribution of compounds in the radioactive signals found in *B. terrestris* LG extracts (excluding polar lipids, see Figure 1). The fatty acids fraction was corrected according to the amount found in the incubation solution extract (see the Experimental Section and Figure S1). Bars represent mean ± SEM of five determinations.

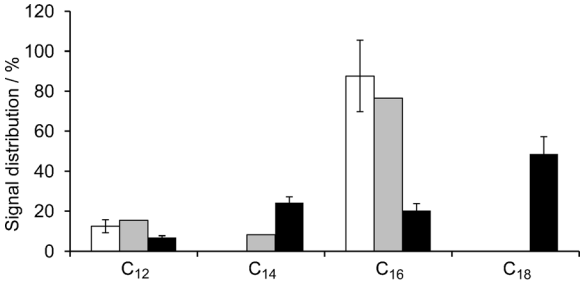


Figure 3. Distribution of free fatty acids of different lengths found in *B. terrestris* LG extracts, analyzed as fatty acid methyl esters (FAME): A) LG after incubation with deuterated [D_3]acetate ($N=5$, □); B) LG after incubation with radioactive [1,2-^{14}C]acetate ($N=1$, ■); C) freshly dissected LG of three-day-old males ($N=5$, ■). Data are mean ± SEM.

were combined. Thus, free fatty acids dominated in the radiolabeled products (Figure 2).

In comparison to LG, a different composition of radioactive products was observed in FB (Figure 1 B). Signal 1 likely reflects similar types of polar lipids to those in LG. Signal 2 was assigned to free fatty acids ($R_F=21$), and signals 3 and 4 were identified as TAGs ($R_F=44$) and hydrocarbons ($R_F=76$), respectively. As expected, no evidence of ^{14}C incorporation into terpenic compounds was found. A considerably high fraction of radioactivity was observed in hydrocarbons (Figure 1 B). However, hydrocarbons probably do not originate from adipocyte biosynthetic activity. Rather, they are produced by oenocytes (small cells distributed among adipocytes).[11] Unlike FB, which is of mesodermal origin, oenocytes are derived from the ectoderm. They are specialized cells that can be associated with either epidermal or FB cells. Their function is to synthesize hydrocarbons, but they are also implicated in the synthesis of cuticular lipids and proteins.[11]

Based on the results of the in vitro experiments, we conclude that the selected incubation conditions were appropriate to sustain basic biosynthetic processes in both LG and FB cells. Fatty acids and their derivatives (hydrocarbons, TAG, esters) were found in both tissue types. Together with the presence of terpenic alcohols exclusively in the LG, this suggests that pheromonal components are synthesized de novo in the LG.

Distribution of radioactivity within fatty acids of different carbon chain length (LG of *B. terrestris*)

Further experiments were carried out to elucidate the details of aliphatic component biosynthesis. The TLC fraction corresponding to fatty acids was scraped off, extracted with diethyl ether, and the fatty acids were transformed into fatty acid methyl esters (FAME). FAME with C_{12}, C_{14}, C_{16}, and C_{18} carbon chains were obtained by preparative gas chromatography. Saturated and unsaturated Me-esters of each carbon chain length were collected together. The fractions were transferred to scintillation vials, and radioactivity was measured. The analysis showed a clear prevalence of radioactivity in the fraction containing C_{16} FAME (Figure 3 B). A considerably lower proportion of radioactivity was detected in the C_{12} and C_{14} fractions. No radioactivity was found in the C_{18} fraction. Based on these observations, we conclude that FAS in the LG biosynthesizes fatty acids with chain lengths of up to 16 carbons.[33,34]

In vitro incubation with deuterated sodium [D_3]acetate (LG of *B. terrestris* males)

The LG from *B. terrestris* males was incubated with deuterated sodium [D_3]acetate to obtain mass spectra of metabolites and to enable their identification. With the exception of acetate concentration, the incubation conditions were the same as those used for the [1,2-^{14}C]acetate uptake experiments. We utilized 60 mM deuterated [D_3]acetate, which was the highest substrate concentration examined in the optimization procedures (Table 1). Chloroform extracts of the LG were analyzed by comprehensive two-dimensional GC×GC MS.

Metabolites containing ^{2}H label were identified according to their specific retention behavior[9,35] and mass spectra. Deuterated compounds eluted earlier than their non-deuterated analogues on capillary gas-liquid chromatography (Figure S2). On GC×GC MS, deuterated compounds eluted first in both dimensions. Two deuterated terpenic compounds were found in the LG extracts: 2,3-dihydrofarnesol (d-DHF; Figures 4 A and S2) and geranylcitronellol (d-GC; Figure S3 A). The mass spectra of the two compounds showed similar patterns. The spectra reflect ion clusters containing deuterium randomly distributed within the structure. The ion clusters follow the same pattern as the mass spectra of non-deuterated analogues. Because of the presence of deuterium atoms, the apex mass in the clusters is always higher than the corresponding mass in the spectra of native analogues. This difference in mass corresponds to the most probable number of deuterium atoms present in the ion. The average number of deuterium atoms incorporated in the molecule could be estimated according to the most abundant mass in the molecular mass cluster. In the case of d-DHF, the average number of deuterium atoms was nine or ten (13 for d-GC). These data suggest that more than half of the acetate units used for biosynthesis originated from the deuterated precursor.

In the case of the aliphatic compounds, deuterium atoms were detected in free fatty acids that were analyzed as FAME and ethyl esters (FAEt). The mass spectra of the methyl esters

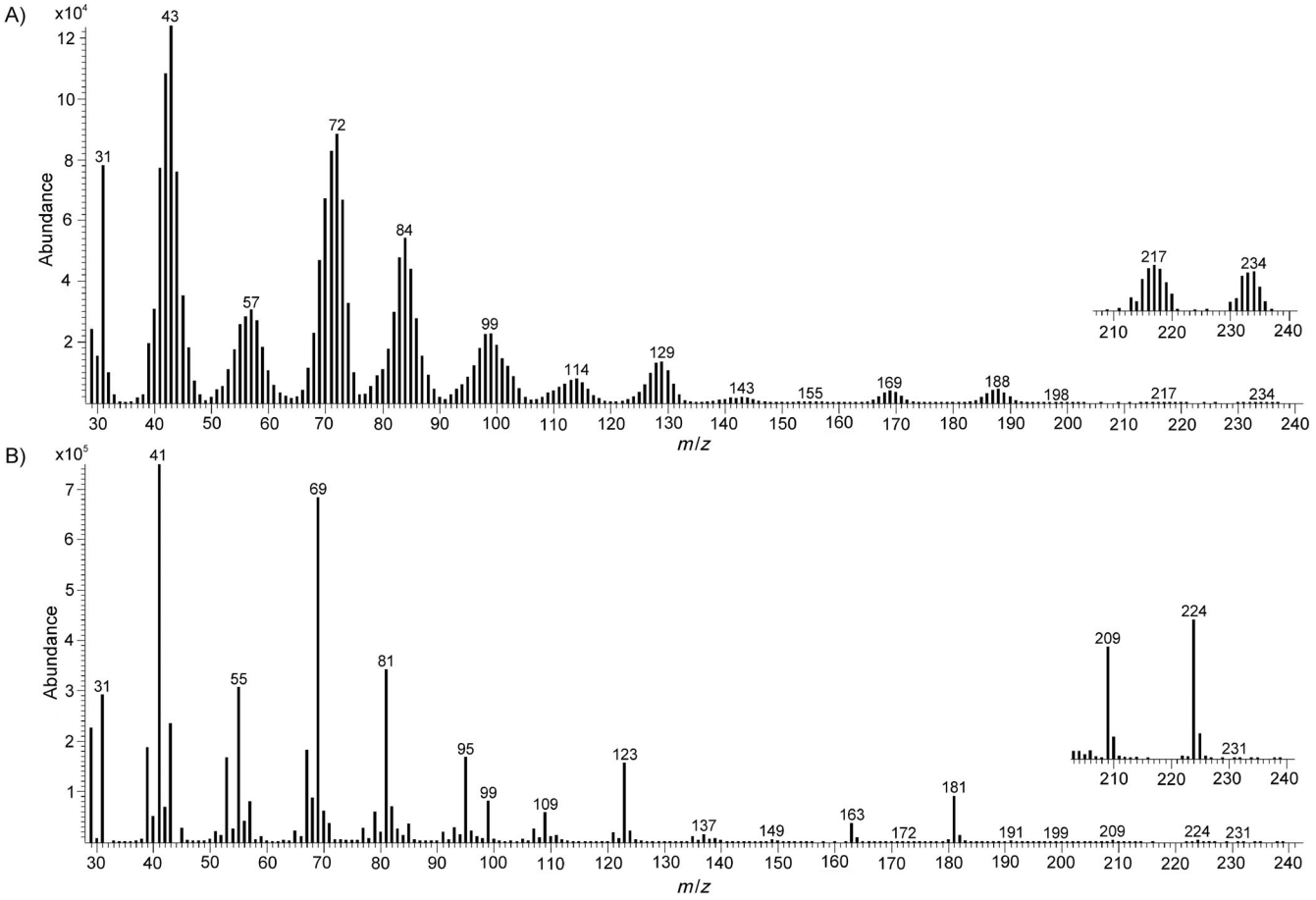


Figure 4. Mass spectra of 2,3-dihydrofarnesol (DHF) found in extracts from *B. terrestris* LG incubated with [D_3]acetate: A) DHF containing deuterium randomly distributed within the molecule (d-DHF); B) native DHF. Note the molecular masses of both types of DHF.

exhibited similar ion clustering as described for deuterated terpenes (Figures 5 and S4). This suggests that free fatty acids also contain randomly distributed deuterium in the carbon chain. The most intense ions in the non-deuterated saturated methyl esters were *m/z* 74 and 87 (88 and 101 for ethyl esters). These ions are formed by a series of hydrogen (or deuterium) rearrangements involving an ester group, an alcohol part, and the first four or five carbons of the acid chain, respectively[36] (Figure S5). Relative quantification of the deuterated FAME data validated the results from ^{14}C-acetate incubations (Figure 3A). The most abundant was deuterated methyl palmitate. Deuterated methyl laurate was present in a substantially lower quantity, and only a trace of deuterated methyl myristate was detected. There was a discrepancy between free fatty acid carbon-chain distribution found in the in vitro experiments (labeled acetates) and the native distribution (Figure 3). Biosynthesis of fatty acids and their derivatives[8,27,32] probably proceeds via palmitic acid (synthesized by FAS) and subsequent reactions (chain shortening or elongation) that are subjected to further regulation.

Two different types of deuterated ethyl esters were identified by their mass spectra (Figures 6A and B). The first type contained deuterium randomly distributed within the molecule (Figure 6A). This was observed only in ethyl laurate and not in other ethyl esters. It can be explained by the high abundance of ethyl laurate relative to other ethyl esters in the native LG.[8,27,32] The deuterated analogues of the other ethyl esters might have been present in the LG at concentrations below the detection limit. The second type of deuterated ethyl esters contained deuterium in a fixed position in the alcohol moiety. These esters were identified by comparison with synthesized standards as [2,2,2-D_3]ethyl esters (Figure 6B). [2,2,2-D_3]Ethyl was observed in the ethyl esters of acids of various carbon chain lengths. Relative quantification revealed [2,2,2-D_3]ethyl laurate to be the most abundant compound. [2,2,2-D_3]Ethyl myristate was present in a substantially lower quantity, and only trace amounts of the palmitate homologue were found (Figures S6 and S7). Further study, including solid-phase micro extraction (SPME) headspace extraction of the incubation solution, confirmed that the [2,2,2-D_3]ethanol originated from [D_3]acetate. A series of experiments showed that [2,2,2-D_3]ethanol occurred only in the presence of LG in the incubation solution (Figures S8 and S9). The same results were obtained for [2,2,2-D_3]acetaldehyde (Figures S10 and S11), also found in the incubation solution. The identity of both structures was confirmed by comparison of their mass spectra and retention behaviors with synthetic standards. We therefore hypothesize that formation of [2,2,2-D_3]ethanol in the LG takes

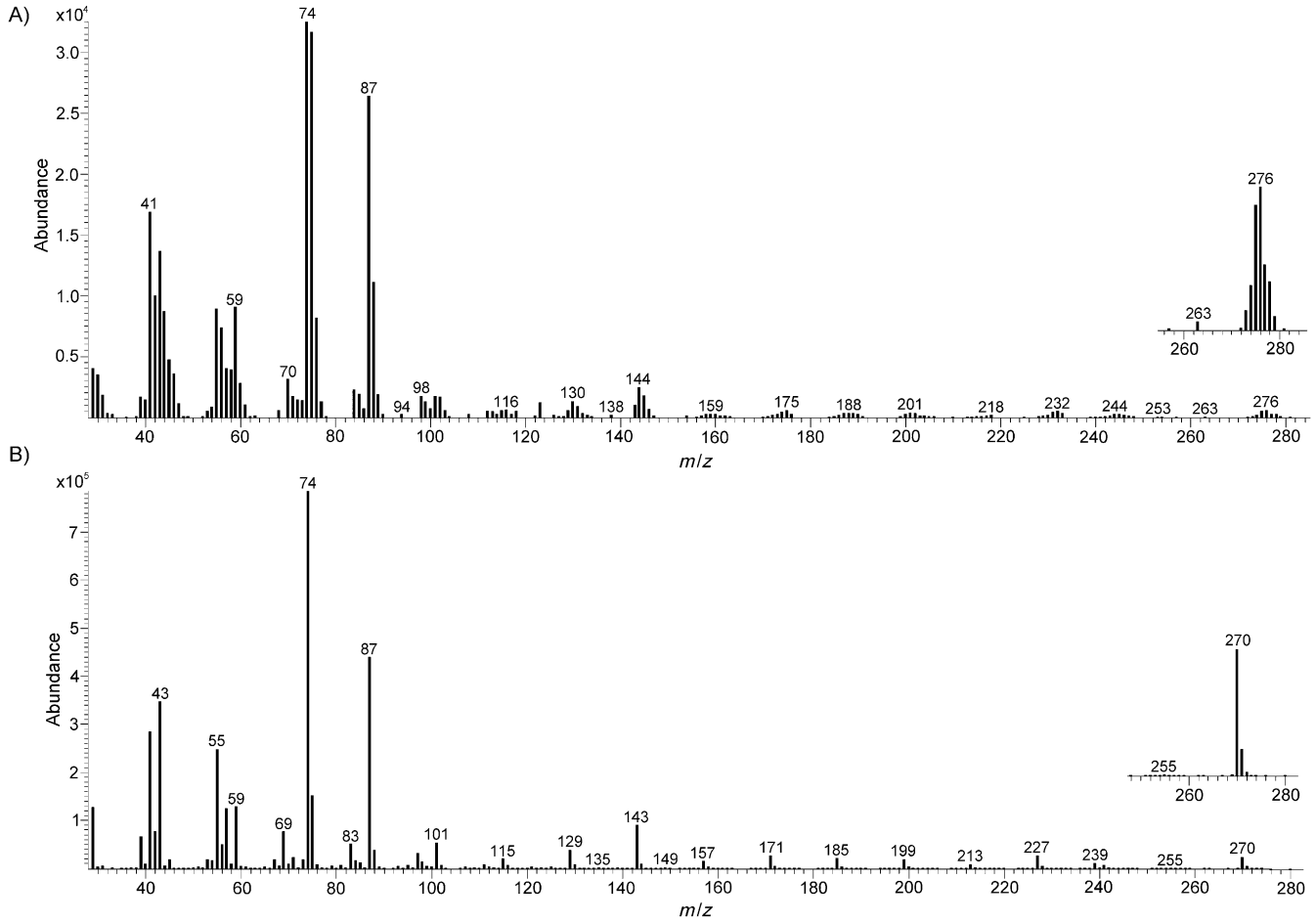
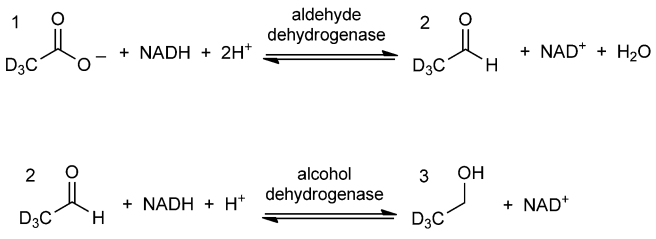


Figure 5. Mass spectra of methyl palmitate (Me-C_{16}) found in the LG of *B. terrestris* after free fatty acid esterification with diazomethane. LG was incubated with [D_3]acetate. A) Me-C_{16} containing deuterium randomly distributed within the acid part of the molecule; B) native Me-C_{16}. Note the molecular masses of both types of Me-C_{16}.

place via [2,2,2-D_3]acetaldehyde according to Scheme 1. It has been proven that fermented nectar is the source of ethanol for the synthesis of ethyl oleate (primer pheromone) in the honey bee (*Apis mellifera* L.).[37] However, acetate reduction could be a source of ethanol in the LG of male bumblebees. The expected enzymes for this, such as alcohol dehydrogenase and aldehyde dehydrogenase, have previously been found in fruit fly (*Drosophila melanogaster*)[38,39] to catalyze the oxidation of ethanol to acetate. This mechanism of ethanol detoxification is also known in mammals.[40]



Scheme 1. Proposed transformation of [D_3]acetate (**1**) to [2,2,2-D_3]ethanol (**3**) via [2,2,2-D_3]acetaldehyde (**2**).

In vitro incubation of LG from male *B. lucorum*

In vitro experiments with [1,2-^{14}C]acetate were also carried out with LGs from *B. lucorum* males. As only a limited number of insect specimens were available for this species, we used slightly modified incubation conditions, optimized for *B. terrestris* (no unlabeled acetate addition to the incubation medium). The incubations resulted in the formation of radioactive metabolites, identified as free fatty acids and aldehydes (Figure 7). Similarly to *B. terrestris,* fatty acids were the most abundant radioactive metabolites. High radioactivity was observed at R_F 40–60, which is a retention area where acetates and ethyl esters elute. However, poor chromatographic separation prevented more-accurate assignment of radioactive signals to particular compounds.

Relative quantification of fatty acid synthase transcript levels

To support the results from the in vitro incubations, we used qPCR to determine relative transcript levels of FAS in LG from *B. terrestris* individuals of different ages. FAS is the key enzyme in the fatty acid biosynthetic pathway. The FAS sequence was

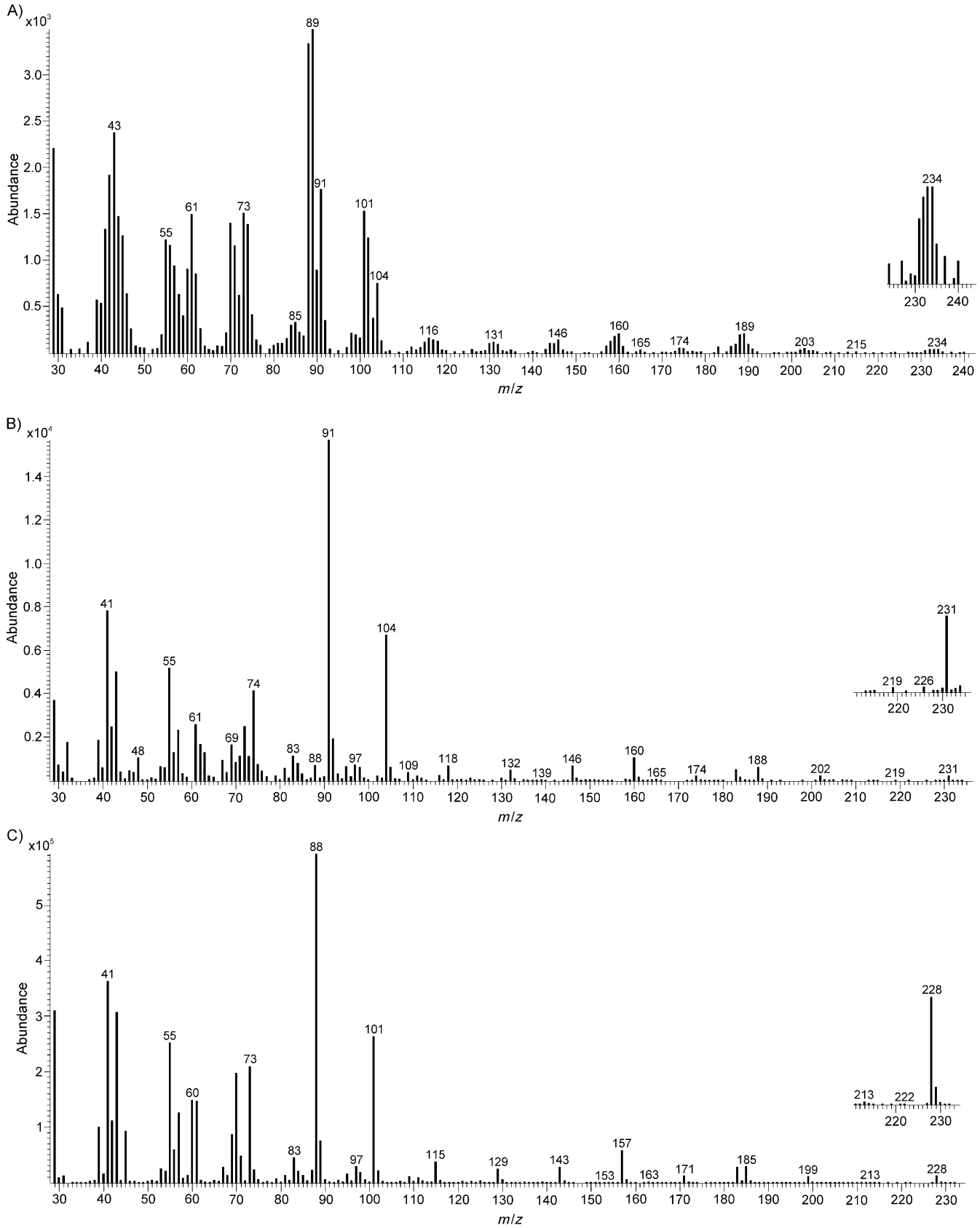


Figure 6. Mass spectra of ethyl laurate (Et-C_{12}) found in the LG of *B. terrestris* after incubation with [D_3]acetate. A) Et-C_{12} containing deuterium randomly distributed within the acid part of the molecule. The spectrum is partly contaminated by the masses of [2,2,2-D_3]ethyl laurate (*m/z* 91 and 104 in spectrum B) due to poor chromatographic separation; B) [2,2,2-D_3]ethyl laurate; C) native Et-C_{12}.

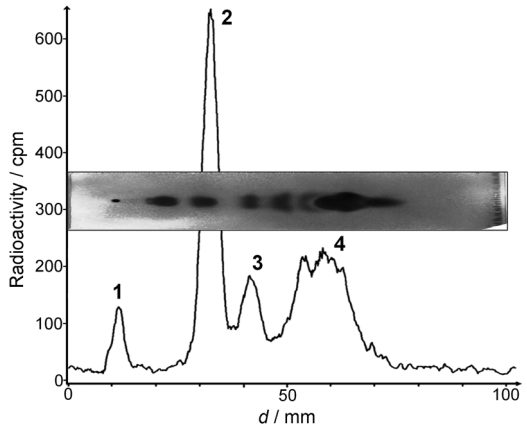


Figure 7. Chromatogram of TLC radioactive signals (*B. lucorum*). Chloroform extract of LG and nonselective visualization of the TLC plate: 1) polar lipids, 2) fatty acids, 3) aldehydes, 4) esters.

obtained by using RNA-seq technology from non-normalized cDNA libraries of *B. terrestris* LG and FB. *B. terrestris* FAS (GenBank accession number: JN982132) shares 96% sequence identity with the predicted FAS from *Bombus impatiens*, 75% identity with the predicted FAS from *A. mellifera*, and 55% identity with a previously characterized FAS from *Aedes aegypti*.[41] We detected an increasing level of *B. terrestris* FAS transcripts in LG from pharate imago up to three days old. There was a modest decrease in LG FAS transcript level in five-day-old bumblebees. Relatively constant FAS mRNA levels were found in LG from older *B. terrestris* individuals (Figure 8). We also performed relative quantification of FAS transcript levels in FB from pharate imago, and from 1-, 3-, and 12-day-old *B. terrestris*. mRNA level was significantly higher in the LG than in the FB, with the exception of the pharate imago (Figure 8), where the FAS transcript levels were identical.

We further analyzed the normalized counts of reads (reads per kilobase of exon model per million mapped reads; RPKM)[42] in RNA-seq for FAS mRNA from LG and FB. We found 15-fold higher FAS expression in the LG. The expression pattern of the FAS gene obtained by qPCR correlated well with the analytical measurements of age-dependent concentration changes in fatty acids and their derivatives in the LG.[8] We observed accumulation of fatty acid pheromones in the LG from *B. terrestris* between the third and seventh day of its life, and a significant decrease in fatty acid products in older bumblebees.

From the RNA-seq data, we also obtained the FAS gene sequence of *B. lucorum* (GenBank accession number: JQ177158). *B. lucorum* FAS shares 99% sequence identity with *B. terrestris* FAS. Similarly to *B. terrestris,* we detected approximately sixfold higher levels of FAS mRNA in *B. lucorum* LGs than in FB. This was based on the ratio between normalized counts of reads in each tissue. These results suggest that biosynthesis of the fatty acids (the precursors of pheromones) also proceeds de novo in the LG of *B. lucorum*.

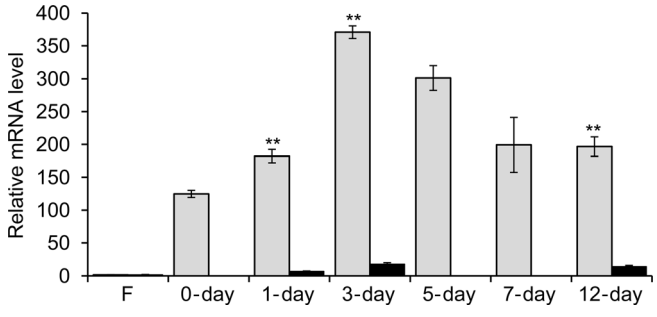


Figure 8. Relative gene expression levels of the FAS gene in the LGs (■) from pharate (F), and 0-, 1-, 3-, 5-, 7-, and 12-day-old *B. terrestris*, and in the FB (■) from pharate, and 1-, 3-, and 12-day-old *B. terrestris*. Data are mean ± SEM of three determinations (**$P < 0.01$).

Conclusions

The biosynthesis products formed in the incubation experiments of LG from *B. terrestris* and *B. lucorum* correspond to compounds naturally present in these tissues. Our data thus prove that de novo biosynthesis of both aliphatic and terpenic pheromone components can take place in the labial gland. Incubation results for aliphatic components are further supported by the quantification of relative FAS transcript levels. Maximal gene expression in three-day-old males correlated with maximal secretory activity of the LG, as described in previous work.[27] The abundance of mRNA from the FAS gene is approximately 15 times higher in the LG than in the fat body of *B. terrestris* (six times higher for *B. lucorum*). This means that significant biosynthetic activity can be assigned to the LG. Together with the incubation results, this is strong evidence for de novo biosynthesis of aliphatic pheromone components.

The hypothesis that aliphatic pheromone components are formed from lipid precursors still cannot be excluded.[9,10] Further research is needed to clarify whether either biosynthetic pathway dominates under particular conditions or physiological state of the insect.

Experimental Section

Insects: Male *B. terrestris* and *B. lucorum* bumblebees were obtained from laboratory colonies (Masaryk University, Brno, Czech Republic).[43,44] Tissue samples (the cephalic part of LG and peripheral FB) used in all incubation experiments were dissected from two-day-old bumblebees. The average weights of the FB and LG taken for one incubation experiment were (11.1 ± 0.3) mg (mean ± SEM; $N = 137$) and (4.1 ± 0.1) mg ($N = 137$), respectively.

The cephalic parts of *B. terrestris* LG and FB used for qPCR were carefully dissected (LG from pharate imago and from 0-, 1-, 3-, 5-, 7-, and 12-day-old male bumblebees; FB from pharate imago and from 1-, 3-, and 12-day-old males), frozen in liquid nitrogen, and stored at −80 °C for further use. LG and FB for RNA sequencing were from zero- to five-day-old *B. terrestris* and three-day-old *B. lucorum* males.

Chemicals: Aqueous sodium [1,2-^{14}C]acetate was prepared from commercially available ethanol solution (specific activity 3.7 kBq μL^{-1}; PerkinElmer). The ethanol was evaporated under

argon, and the radioactive salt was diluted in the same volume of distilled sterilized water. The specific activity remained the same.

Sodium acetate labeled with three deuterium atoms (sodium [D_3]acetate) was purchased from Sigma–Aldrich. A solution in sterile distilled water was prepared (31 μg in 100 μL).

[2,2,2-D_3]Ethyl laurate (C_{12}) was prepared from commercially available [2,2,2-D_3]ethanol (Sigma–Aldrich) and lauric acid as follows. [2,2,2-D_3]Ethanol (100 μL) and dried sodium sulfate (100 mg) were added to chloroform (1 mL) containing lauric acid (10 mg). The reaction mixture was stirred and kept overnight at 60 °C. The chloroform phase was then fractionated on TLC plates (75×59 mm, coated with Adsorbosil-Plus (0.2 mm, supplemented with 12% gypsum); Applied Science Labs, State College, PA). A mixture of hexane/ethyl acetate (9:1) was used as the mobile phase. From its chromatographic signal, ethyl laurate was scraped off the plate and extracted with ether. The extract was subsequently evaporated under argon and reconstituted in hexane.

[2,2,2-D_3]Acetaldehyde was prepared from [2,2,2-D_3]ethanol as follows. Deuterated ethanol (50 μL) was diluted in water (200 μL), and concentrated sulfuric acid (one drop) and potassium dichromate (50 mg) were added. The reaction mixture was carefully heated to approximately 50 °C and maintained at this temperature for a few seconds. Nonane (300 μL) was then added, and the mixture was shaken by hand. Lastly, the organic phase was removed with a Pasteur pipette and transferred to a new vial. Nonane was used for its chromatographic behavior with respect to [2,2,2-D_3]acetaldehyde.

Incubation conditions: Acetate incubations were carried out for 16 h in filtration columns (Zymo-Spin IIN; Zymo Research, Irvine, CA) in a medium[28] consisting of a mixture (1:1) of Schneider's Insect Medium (Lonza, Basel, Switzerland) and Eagle's Minimum Essential Medium (Lonza), with HEPES (20 mM, Sigma–Aldrich), BSA (1%, Sigma–Aldrich), sucrose (5%, Sigma–Aldrich), and (unlabeled) sodium [1,2-^{12}C]acetate (20 mM), pH 6.8. The incubation columns were kept in a water bath at 35 °C. The medium was filtered through a membrane filter (0.22 μm) prior to use. Each tissue sample (FB or cephalic part of LG) was incubated separately in five parallel experiments. The incubation process began with the addition of aqueous sodium [1,2-^{14}C]acetate (5 μL). Control incubations (without the addition of radioactive acetate) were run in parallel and used for GC×GC MS analysis.

Incubations with deuterated acetate were performed in the same incubation solution as described above, but with sodium [2,2,2-D_3]acetate instead of radioactive [1,2-^{14}C]acetate. Only LGs of *B. terrestris* were used for these experiments. The incubation process was initiated by injecting aqueous sodium [D_3]acetate (5 μL) into the incubation column (final concentration 60 mM).

Sample preparation for subsequent analysis: All LG and FB incubations were terminated by separation of the tissues from the medium by centrifugation (2000 *g*, 20 s for LG; 4000 *g*, 20 s for FB). The incubation medium was collected in a separate tube and used for radioactivity measurements (when incubated with radioactive acetate). Any remaining tissue on the column was washed three times with incubation medium (300 μL) free of sodium acetate. The same centrifugation parameters were used to separate the medium after each wash.

Both types of incubated tissue were removed from the filtration columns and placed into vials containing chloroform (400 μL). The tissues were then disrupted by freezing in liquid nitrogen. After thawing, the vials were sonicated in a water bath (15 min). Vials containing tissue and chloroform extracts were then centrifuged (3 min), and the chloroform phase was removed for TLC separation. LG extracts incubated with deuterated sodium acetate were analyzed by GC×GC MS without prior TLC fractionation.

The incubation medium after incubation of both tissues with sodium [1,2-^{14}C]acetate was also extracted with chloroform (400 μL). These extracts were subjected to TLC separation. An NNCS 502 tissue solubilizer (Amersham Biosciences) was used to study the time-dependence of acetate absorption. LG and FB samples were washed and disrupted by the tissue solubilizer, and transferred directly into scintillation vials.

Radioanalysis of liquid samples: Chloroform extracts (50 μL) of LG or FB were mixed with liquid scintillation cocktail (5 mL; Ultima Gold, PerkinElmer) and analyzed in a Tri-Carb 2900TR spectrometer (PerkinElmer).

TLC analysis: Thin layer chromatography separation of extract components was performed on TLC silica 60 F_{254} gel plates (10× 20 cm, with a 2.5 cm concentrating zone; Merck Millipore) with 75 or 100 μL of extract. The mobile phase consisted of hexane/ethyl acetate/formic acid (70:30:1) for LG of *B. terrestris* samples and hexane/ethyl acetate/formic acid (90:10:1) for the FB of *B. terrestris* and LG or FB of *B. lucorum* samples. The TLC plates were analyzed by nondestructive detection of ^{14}C distribution with a RITA Star position-sensitive detector (Raytest, Straubenhardt, Germany).

Identification of biosynthesis products: Biosynthesis products of LG and FB incubated with radioactive acetate were identified by comparison with standards and by GC×GC MS analysis of tissues extracts from control incubations. Three samples were simultaneously fractionated on each TLC plate (lane A: standards, a mixture of compounds known to be present in the tissue sample;[8,32,45] lane B: chloroform extract of tissue incubated with radioactive sodium [1,2-^{14}C]acetate; lane C: chloroform extract of the same tissue incubated with nonradioactive sodium [1,2-^{12}C]acetate). Radioactive signals in lane B were visualized by a position-sensitive radioactivity detector, and the signals' R_F values were compared against the standards in the lane A. TLC zones in lane C corresponding to the radioactive zones in lane B were scraped off, extracted with diethyl ether, and analyzed by GC×GC MS. Components were identified by comparing the obtained mass spectra with those found in the NIST/Wiley mass spectra database libraries.[46]

Biosynthesis products of LG incubated with deuterated [D_3]acetate were identified by comparing their retention times and mass spectra to those of non-deuterated analogues.

GC/MS analysis: Structural types of incubation products were assigned by comparing their R_F values to those of standards and control samples. Gaseous iodine was used to visualize unlabeled components. The iodine evaporated from the TLC plate within 1 h. Detected signals were scraped off the plates and extracted with ether (2×150 μL). The ether was subsequently removed under argon. The remaining residue was dissolved in hexane (50 μL), and an internal standard (1-bromodecane, 1 μL, 1.9 mg mL^{-1}) was added. Signals containing fatty acids were methylated by diazomethane and analyzed in the form of methyl esters. LG incubated with unlabeled sodium acetate or deuterated sodium [D_3]acetate were analyzed by comprehensive two-dimensional gas chromatography with mass detection (Pegasus III GC×GC MS; LECO Corp., St. Joseph, MI).

GC×GC MS separation parameters (extracts of tissues incubated with unlabeled sodium acetate): splitless injection, inlet temperature 250 °C, injection volume 1 μL, constant He column flow rate

1 mL min^{-1}, modulation time 4 s (hot pulse 0.5 s), modulation temperature offset 30 °C. First dimension column: DB-5 (30 m × 250 μm × 0.25 μm; J & W Scientific/Agilent Technologies, Wilmington, DE); temperature program: 50 °C (1 min), then increase (10 °C min^{-1}) to 320 °C, and hold (1 min). Second dimension column: BPX-50 (1.86 m × 100 μm × 0.1 μm; SGE Analytical Science, Ringwood, Australia); temperature program: 60 °C (1 min), increase (10 °C min^{-1}) to 330 °C, and hold (1 min). Separation parameters for GC × GC MS (extracts of tissues incubated with deuterated sodium [D_3]acetate) were as follows. First dimension column temperature program: 60 °C (1 min), increase (4 °C min^{-1}) to 320 °C, and hold (15 min); second dimension: 70 °C (1 min), increase (4 °C min^{-1}) to 330 °C, and hold (15 min).

Solid-phase micro extraction (SPME fiber, 75 μm, Carboxen–polymethylsiloxane (PDMS); Supelco/Sigma–Aldrich) headspace method was used for sampling the [2,2,2-D_3]ethanol and [2,2,2-D_3]acetaldehyde biosynthesized in the incubation solution. Extraction time was 2 min. Compounds captured by the SPME fiber were analyzed by GC × GC MS as above but with the following parameters. Inlet temperature 220 °C, split 50; first dimension column temperature program: 40 °C (5 min), increase (20 °C min^{-1}) to 200 °C; second dimension: 50 °C (5 min), increase (20 °C min^{-1}) to 210 °C.

Preparative GC: Samples obtained from incubations containing ^{14}C-labeled acetate were first separated by TLC. Spots containing labeled fatty acids were scraped off the plates and extracted with ether (2 × 200 μL). Fatty acids were methylated by using diazomethane.

Preparation was performed by using a 6890N gas chromatographer (Agilent) equipped with a liquid nitrogen cooled EPC PTV inlet (Gerstel, Mühlheim, Germany) and an FID. Samples were injected with a 7683B autosampler (Agilent). The gas chromatographer was linked to a preparative fraction collector (Gerstel) equipped with a liquid nitrogen cooling system. Hexane solution (5 μL) was injected to the PTV inlet (cooled to −20 °C). After 25 s, the inlet was flash-heated (12 °C s^{-1}) to 350 °C and held (2 min). An HP-5 fused silica capillary column (30 m × 0.53 mm, i.d. 0.88 μm, coated with a diphenyl/dimethylpolysiloxane (5:95) stationary phase; Hewlett–Packard) was used with helium as carrier gas (constant flow mode, 6.4 mL min^{-1}). The column temperature was held at 70 °C (1 min), then increased (15 °C min^{-1}) to 300 °C, and held (1 min). The effluent was split into two parts, one leading to the detector (25%) and the other to the fraction collector (75%). Transfer line temperature was maintained at 270 °C. Analytes were captured in a trapping capillary by cooling the effluent with liquid nitrogen to −80 °C. The fraction collector enabled the collection of six fractions in a single GC run. Two collection capillaries were used for determining background radioactivity, and the other four were used for trapping methyl esters.

Quantitative real-time PCR: Sequences of fatty acid synthase genes were obtained with RNA-seq technology from non-normalized cDNA libraries of *B. terrestris* and *B. lucorum* LG and FB. The sequences were deposited in the GenBank database (NCBI) under the accession numbers JN982132 (*B. terrestris* FAS) and JQ177158 (*B. lucorum* FAS).

Real-time PCR was carried out with cDNA prepared from total RNA isolated from three biological replicates of each age. RNA was isolated by using Trizol (Invitrogen/Life Technologies) according to the manufacturer's instructions. Possible genomic DNA contamination was eliminated by digestion with TURBO DNase (Life Technologies) for one hour at 37 °C. RNA was then purified by using an RNeasy Mini Kit (Qiagen) according to the manufacturer's procedure (RNA Cleanup protocol). RNA quantity was assessed with a NanoDrop ND-1000 UV/Vis spectrophotometer, and RNA integrity was verified by using a Bioanalyzer 2100 with RNA Nano chips (Agilent Technologies). cDNA was synthesized from 0.25 μg of total RNA by using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamer primers according to the manufacturer's instructions.

Primers for qPCR (FAS forward: CAAGCA TTAGGT CATATC CCAGAA; FAS reverse: GATTGT TTGCAT GACCTC GTTTAT) were designed by using PrimerSelect software (DNASTAR, Madison, WI) and first tested with standard PCR. Real-time PCR was performed in a LightCycler 480 real-time PCR System (Roche) with SYBR green fluorescent labeling. PCR reactions were carried out in a total volume of 20 μL containing DyNAmo HS SYBR Green qPCR Master Mix (Finnzymes/Thermo Scientific), primers (0.625 μM each), and the appropriate cDNA (1 μL). The cycling conditions were as follows: 95 °C for 15 min; then 45 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. A melting curve analysis was performed at the end of each amplification reaction, and measurements were recorded between 55 and 95 °C. All samples were examined in two replicates. Data were exported from the LightCycler 480 (software version 1.5) into Microsoft Excel, and analyzed with GenEx software (http://www.multid.se). Relative gene expression was normalized to phospholipase A2 (PLA2) and elongation factor 1α (EEF1A). Primers for PLA2 were PLA2 forward (GGTCAC ACCGAA ACCAGA TT) and PLA2 reverse (TCGCAA CACTTC GTCATT TC); primers for EEF1A were EEF1A forward (AGAATG GACAAA CCCGTG AG) and EEF1A reverse: (CACAAA TGCTAC CGCAAC AG).[47]

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Keywords: *Bombus lucorum* · *Bombus terrestris* · fat bodies · fatty acids · gene expression · isotopic labeling

[1] G. Bergström, B. G. Svensson, M. Appelgren, I. Groth in *Biosystematics of Social Insects* (Eds.: P. E. Howse, J.-L. Clément), Academic Press, London, **1981**, pp. 175 – 183.
[2] B. Kullenberg, G. Bergström, S. Ställberg-Stenhagen, *Acta Chem. Scand.* **1970**, *24*, 1481 – 1483.
[3] D. H. Morse in *Social Insect, Vol. 3* (Ed.: H. R. Hermann), Academic Press, New York, **1982**, pp. 245 – 322.
[4] B. Kullenberg, *Zoon Suppl.* **1973**, *1*, 31 – 42.
[5] D. H. Calam, *Nature* **1969**, *221*, 856 – 857.
[6] B. Kullenberg, G. Bergström, B. Bringer, B. Calberg, B. Cederberg, *Zoon Suppl.* **1973**, *1*, 23 – 29.
[7] K. Urbanová, I. Valterová, O. Hovorka, J. Kindl, *Eur. J. Entomol.* **2001**, *98*, 111 – 115.
[8] P. Žáček, B. Kalinová, J. Šobotník, O. Hovorka, V. Ptáček, A. Coppée, F. Verheggen, I. Valterová, *J. Chem. Ecol.* **2009**, *35*, 698 – 705.
[9] A. Luxová, I. Valterová, K. Stránský, O. Hovorka, A. Svatoš, *Chemoecology* **2003**, *13*, 81 – 87.
[10] P. Matoušková, A. Luxová, J. Matoušková, P. Jiroš, A. Svatoš, I. Valterová, I. Pichová, *ChemBioChem* **2008**, *9*, 2534 – 2541.
[11] E. L. Arrese, J. L. Soulages, *Annu. Rev. Entomol.* **2010**, *55*, 207 – 225.
[12] E. L. Arrese, L. E. Canavoso, Z. E. Jouni, J. E. Pennington, K. Tsuchida, M. A. Wells, *Insect Biochem. Mol. Biol.* **2001**, *31*, 7 – 17.
[13] C. Schal, V. Sevala, R. T. Cardé, *Naturwissenschaften* **1998**, *85*, 339 – 342.

[14] K. Matsuoka, H. Tabunoki, T. Kawai, S. Ishikawa, M. Yamamoto, R. Sato, T. Ando, *Insect Biochem. Mol. Biol.* **2006**, *36*, 576 – 583.
[15] R. Van Antwerpen, J. H. Law, *Arch. Insect Biochem. Physiol.* **1992**, *20*, 1 – 12.
[16] B. S. Lanne, G. Bergström, A.-B. Wassgren, B. Törnbäck, *Comp. Biochem. Physiol. Part B* **1987**, *88*, 631 – 636.
[17] J. Mann, *Chemical Aspects of Biosynthesis*, Oxford University Press, Oxford, **1994**.
[18] L. B. Bjostad, W. L. Roelofs, *Science* **1983**, *220*, 1387 – 1389.
[19] S. J. Seybold, D. R. Quilici, J. A. Tillman, D. Vanderwel, D. L. Wood, G. J. Bloomquist, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8393 – 8397.
[20] B. S. Lanne, P. Ivarrson, P. Johnsson, G. Bergström, A.-B. Wasgren, *Insect Biochem.* **1989**, *19*, 163 – 168.
[21] G. J. Blomquist, R. Figueroa-Teran, M. Aw, M. Song, A. Gorzalski, N. L. Abbott, E. Chang, C. Tittiger, *Insect Biochem. Mol. Biol.* **2010**, *40*, 699 – 712.
[22] E. Plettner, K. N. Slessor, M. L. Winston, J. E. Oliver, *Science* **1996**, *271*, 1851 – 1853.
[23] E. Plettner, K. N. Slessor, M. L. Winston, *Insect Biochem. Mol. Biol.* **1998**, *28*, 31 – 42.
[24] A. Luxová, *PhD thesis*, Institute of Chemical Technology, Prague (Czech Republic), **2004**.
[25] A. Burse, S. Frick, A. Schmidt, R. Buechler, M. Kunert, J. Gerschenzon, W. Brandt, W. Boland, *Insect Biochem. Mol. Biol.* **2008**, *38*, 76 – 88.
[26] R. Nishida, H. Enomoto, T. E. Shelly, T. Ishida, *Entomol. Exp. Appl.* **2009**, *131*, 85 – 92.
[27] J. Šobotník, B. Kalinová, L. Cahlíková, F. Weyda, V. Ptáček, I. Valterová, *J. Insect Physiol.* **2008**, *54*, 204 – 214.
[28] M. J. Lee, G. J. Goldsworthy, *J. Insect Physiol.* **1995**, *41*, 163 – 170.
[29] G. C. Roma, O. C. Bueno, M. I. Camargo-Mathias, *Micron* **2010**, *41*, 395 – 401.
[30] M. W. Lorenz, *Arch. Insect Biochem. Physiol.* **2001**, *47*, 198 – 214.
[31] B. Heinrich, *Bumblebee Economics*, Harvard University Press, Cambridge, **2004**, Chapter 6.
[32] A. Coppée, M. Terzo, I. Valterova, P. Rasmont, *Chem. Biodiversity* **2008**, *5*, 2654 – 2661.
[33] C. F. Semenkovich, *Prog. Lipid Res.* **1997**, *36*, 43 – 53.
[34] S. J. Wakil, *Biochemistry* **1989**, *28*, 4523 – 4530.
[35] M. Matucha, W. Jockisch, P. Verner, G. Anders, *J. Chromatogr. A.* **1991**, *588*, 251 – 258.
[36] F. W. McLafferty, F. Tureček, *Interpretation of Mass Spectra*, 4th ed., University Science Books, Mill Valley, **1994**, Chapters 8 and 9.
[37] C. Castillo, H. Chen, C. Graves, A. Maisonnasse, Y. Le Conte, E. Plettner, *Insect Biochem. Mol. Biol.* **2012**, *42*, 404 – 416.
[38] J. D. Fry, C. M. Bahnck, M. Mikucki, N. Phadnis, W. C. Slattery, *Integr. Comp. Biol.* **2004**, *44*, 275 – 283.
[39] B. W. Geer, S. W. McKechnie, M. M. Bentley, J. G. Oakeshott, E. M. Quinn, M. L. Langevin, *J. Nutr.* **1988**, *118*, 398 – 407.
[40] C. S. Lieber, *Acta Med. Scand. Suppl.* **1985**, *703*, 11 – 55.
[41] A. Alabaster, J. Isoe, G. Zhou, A. Lee, A. Murphy, W. A. Day, R. L. Miesfeld, *Insect Biochem. Mol. Biol.* **2011**, *41*, 946 – 955.
[42] A. Mortazavi, B. A. Williams, K. McCue, L. Schaeffer, B. Wold, *Nat. Methods* **2008**, *5*, 621 – 628.
[43] V. Ptáček in *Proceedings: Apimondia ICPBR*, Soesterberg, The Netherlands, **1999**, p. 55 – 57.
[44] V. Ptáček, E. Pernová, R. Borovec, *Pszczelnicze Zesz. Nauk.* **2000**, *44*, 305 – 309.
[45] J. Cvačka, E. Kofroňová, S. Vašíčková, K. Stránský, P. Jiroš, O. Hovorka, J. Kindl, I. Valterová, *Lipids* **2008**, *43*, 441 – 450.
[46] NIST Mass Spec Data Center SES, *Mass Spectra*, 6th ed., National Institute of Standards and Technology, Gaithersburg MD, **2005**.
[47] D. Horňáková, P. Matoušková, J. Kindl, I. Valterová, I. Pichová, *Anal. Biochem.* **2010**, *397*, 118 – 120.

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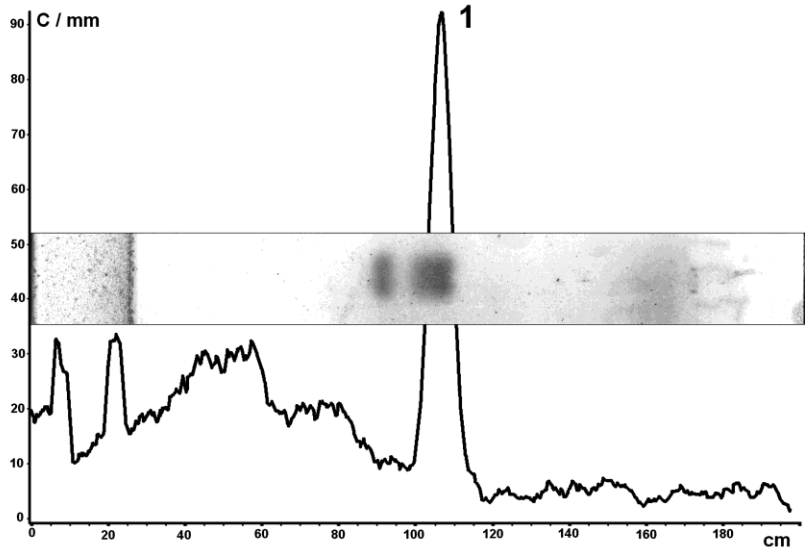
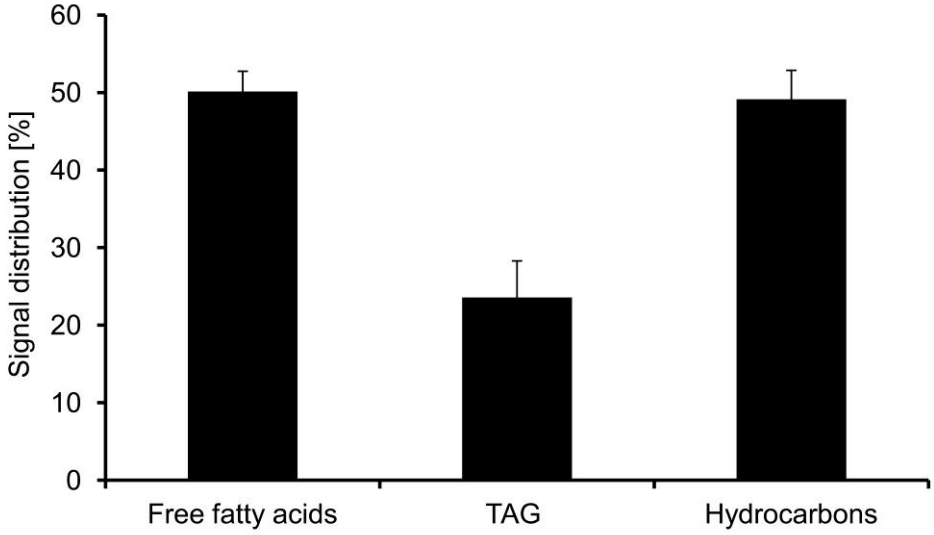
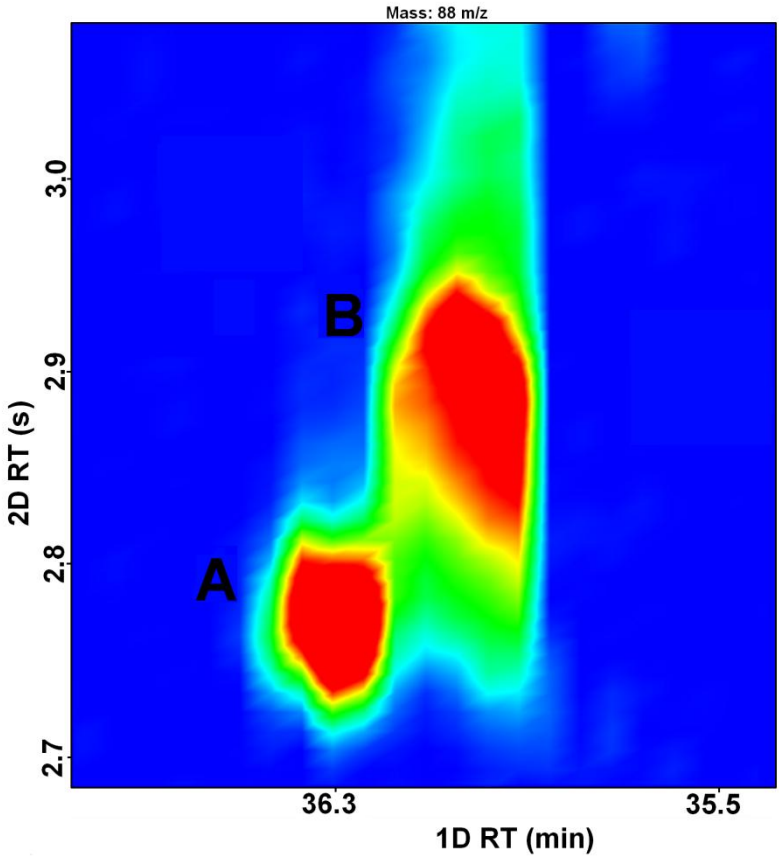


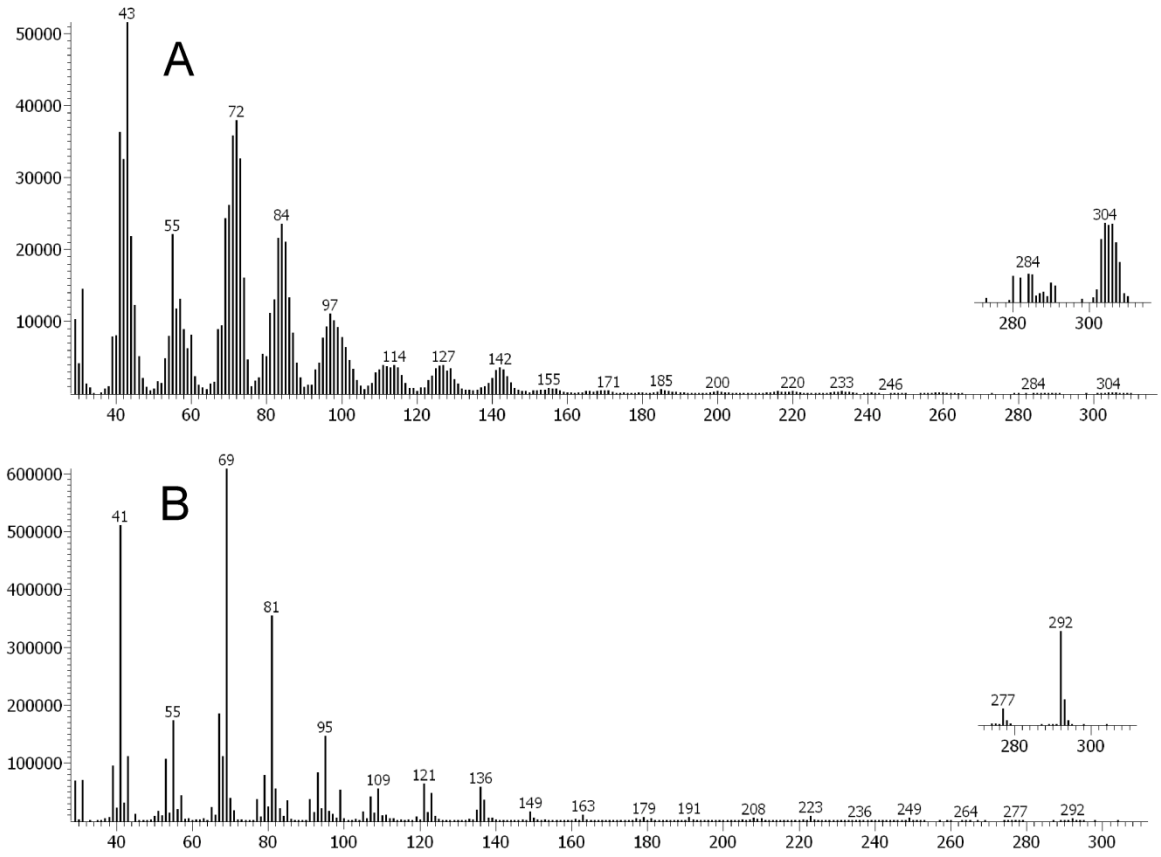
Sup. 1: Chromatogram of the TLC radioactive signals (*B. terrestris*) from the incubation solution chloroform extract after incubation and the non-selective visualization of the corresponding TLC plate. The radioactive signal 1 was assigned to free fatty acids (FA). Area of the FA signal was added to the FA signal found in the corresponding LG extract (see Fig. 1A) for quantification purposes (see Fig. 2).



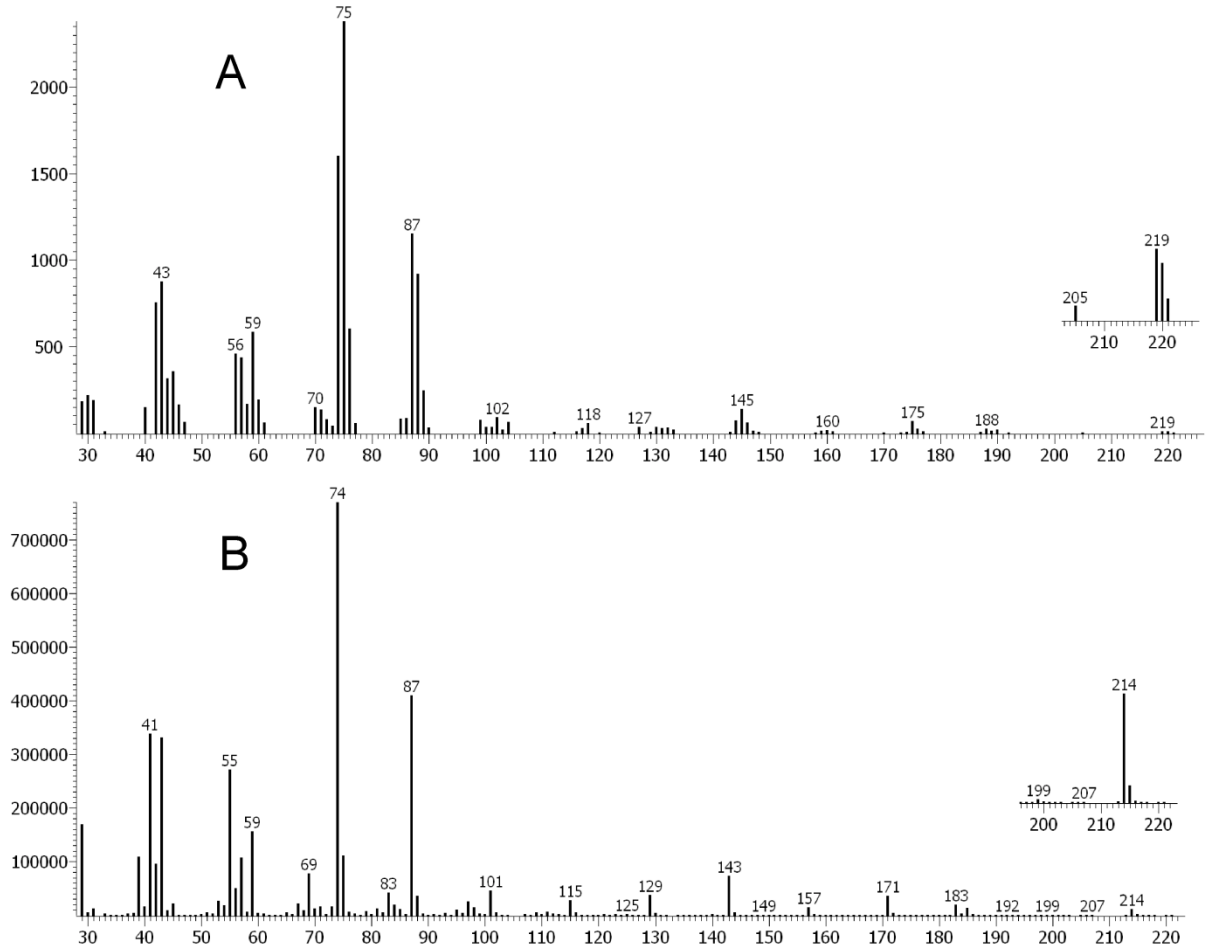
Sup. 2: Compounds species distribution of the radioactive signals found in the *B. terrestris* fat body extract. Signal from the polar lipids (Fig.1) is not included. Proportion of fatty acids was corrected according the amount found in the incubation solution extract (see Experimental and Sup. 1). Bars represent means ± S.E. of 5 determinations.



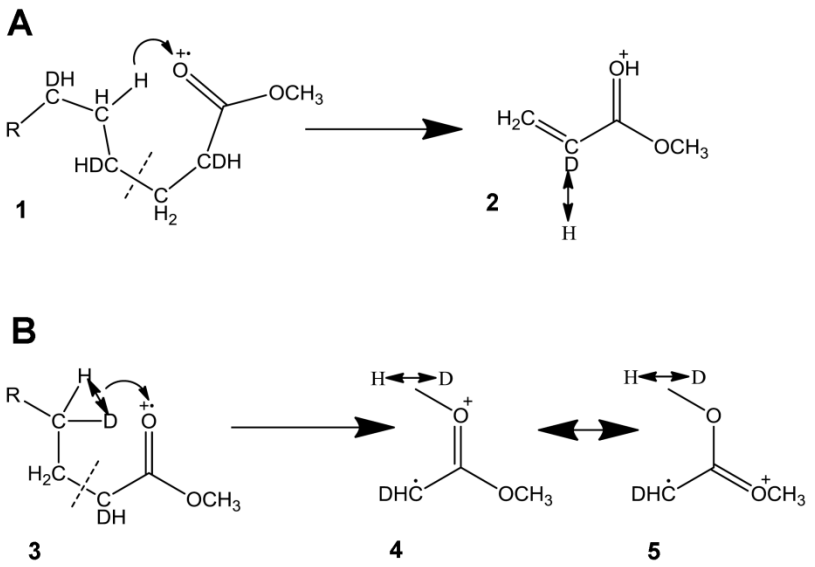
Sup. 3: Zoomed part of contour plot visualization of the two-dimensional chromatogram of the LG chloroform extract including A) deuterated DHF (d-DHF) and B) non-deuterated DHF (H-DHF). LG was incubated with acetate-d_3. Mass *m/z* 88 was chosen for visualization to obtain comparable intensities of the signals for both DHF (see Fig. 4). Notice the retention behavior of the compounds: d-DHF (A) elutes slightly earlier to the H-DHF (B) in both chromatographic dimensions.



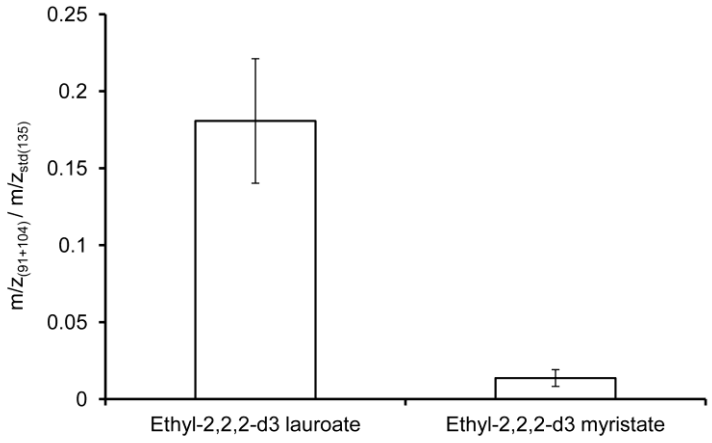
Sup. 4: Mass spectra of geranylcitronellol (GC) found in the LG of *B. terrestris*. A) GC containing deuterium randomly distributed within the molecule (d-GC); B) native GC. See the molecular masses of both types of GC.



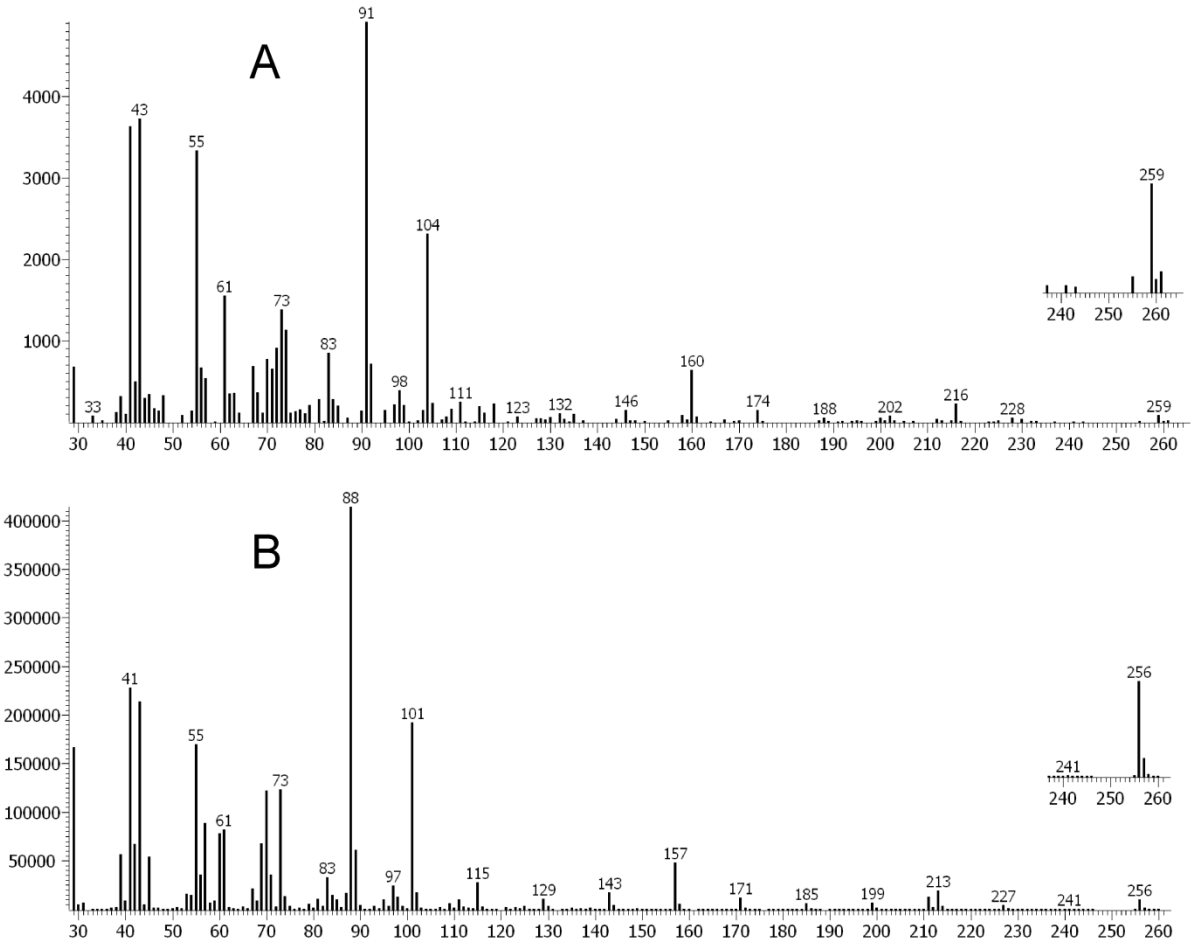
Sup. 5: Mass spectra of methyl laurate (Me-C12) found in the LG of *B. terrestris* after esterification of free fatty acids by means of diazomethane. LG gland was incubated with acetate-d_3. A) Mass spectrum of Me-C12 containing deuterium randomly distributed within the molecule in the acid part. The clustering of the masses is not as clear as in the case of deuterated Me-C16 (see Fig. 5A) due to low abundance of the compound in the extract (see Fig. 3); B) Mass spectrum of native Me-C12. See the molecular masses of both types of Me-C16.



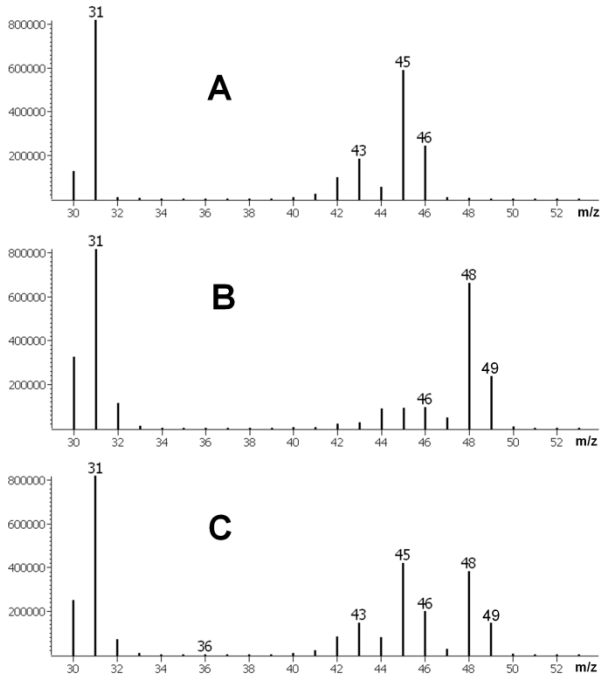
Sup. 6: Mechanisms of the formation of the most abundant masses in the aliphatic methyl esters according McLafferty [36]. These mechanisms can also be applied to ethyl esters. Scheme A) shows formation of mass *m/z* 87 in the case of non-labeled methyl esters (*m/z* 101 for non-labeled ethyl esters) – structure 2. Scheme B) shows formation of mass *m/z* 74 in the case of non-labeled methyl esters (*m/z* 88 for non-labeled ethyl esters) – resonance structures 4 and 5. Structures 1 and 3 represents methyl esters hypothetically synthesized from acetate-d_3 only – every second carbon in the acid chain contains one deuterium [34].



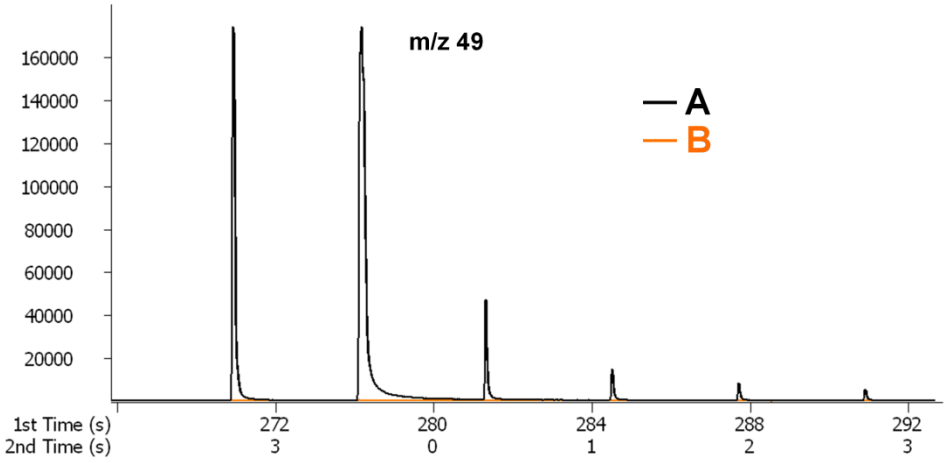
Sup. 7: Comparison of the relative abundance of ethyl-2,2,2-d_3 laurate and ethyl-2,2,2-d_3 myristate found in the *B. terrestris* LG after incubation with acetate-d_3. Bars represent means ± S.E. of 5 determinations.



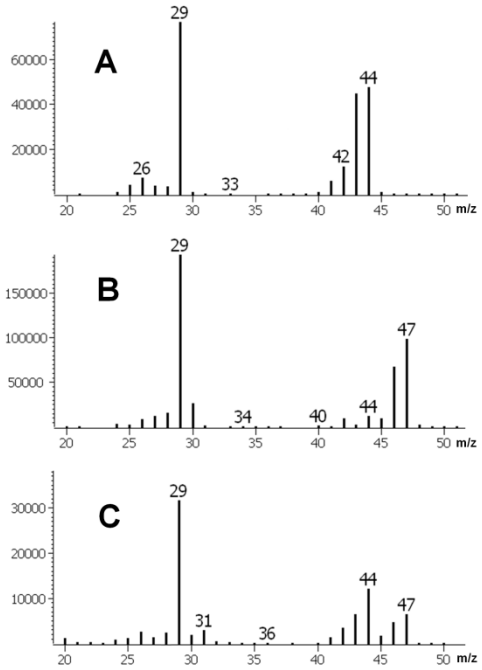
Sup. 8: Mass spectra of ethyl myristate found in the *B. terrestris* LG after incubation with acetate-d_3. A) Mass spectrum of ethyl-2,2,2-d_3 myristate; B) Mass spectrum of native ethyl myristate.



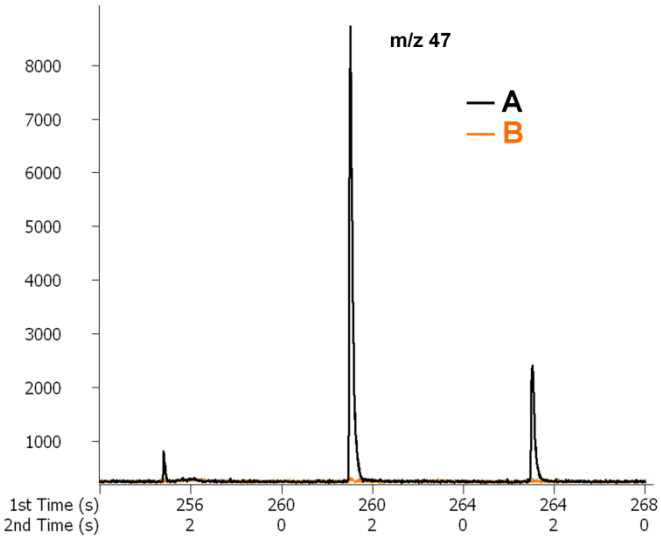
Sup. 9: Mass spectra of ethanol. A) Mass spectrum of non-labeled ethanol (standard); B) Mass spectrum of deuterated ethanol-2,2,2-d_3 (standard); C) Mixed mass spectrum (poor chromatographic separation) of non-labeled ethanol and ethanol-2,2,2-d_3 found in the *B. terrestris* LG after incubation with acetate-d_3.



Sup. 10: Modulations of two overlapping two-dimensional chromatographic signals of ethanol-2,2,2-d_3 (visualized by means of mass *m/z* 49; see Sup. 9). Chromatogram A (black): Headspace of the incubation solutions after incubations of the *B. terrestris* LG with acetate-d_3. Chromatogram B (orange): Headspace of the incubation solutions after incubations with acetate-d_3 without *B. terrestris* LG (LG was not present in the incubation solution during incubation).



Sup. 11: Mass spectra of acetaldehyde. A) Mass spectrum of non-labeled acetaldehyde - standard; B) Mass spectrum of deuterated acetaldehyde-2,2,2-d_3 - synthesized standard (see Experimental); C) Mixed mass spectrum (poor chromatographic separation) of non-labeled acetaldehyde and acetaldehyde-2,2,2-d_3 found in the *B. terrestris* LG after incubation with acetate-d_3.



Sup. 12: Modulations of two overlapping two-dimensional chromatographic signals of acetaldehyd-2,2,2-d_3 (visualized by means of mass *m/z* 47; see Sup. 11). Chromatogram A (black): Headspace of the incubation solutions after incubations of the *B. terrestris* LG with acetate-d_3. Chromatogram B (orange): Headspace of the incubation solutions after incubations with acetate-d_3 without *B. terrestris* LG (LG was not present in the incubation solution during incubation).

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ORIGINAL PAPER

Leg tendon glands in male bumblebees (*Bombus terrestris*): structure, secretion chemistry, and possible functions

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Abstract Among the large number of exocrine glands described in bees, the tarsal glands were thought to be the source of footprint scent marks. However, recent studies showed that the compounds used for marking by stingless bees are secreted by leg tendon instead of tarsal glands. Here, we report on the structure of leg tendon glands in males of *Bombus terrestris*, together with a description of the chemical composition of their secretions and respective changes of both during the males' lives. The ultrastructure of leg tendon glands shows that the secretory cells are located in three independent regions, separated from each other by unmodified epidermal cells: in the femur, tibia, and basitarsus. Due to the common site of secretion release, the organ is considered a single secretory gland. The secretion of the leg tendon glands of *B. terrestris* males differs in its composition from those of workers and queens, in particular by (1) having larger proportions of compounds with longer chain lengths, which we identified as wax esters; and (2) by the lack of certain hydrocarbons (especially long chain dienes). Other differences consist in the distribution of double bond positions in the unsaturated hydrocarbons that are predominantly located at position 9 in males but distributed at seven to nine different positions in the female castes. Double bond positions may change chemical and physical properties of a molecule, which can be recognized by the insects and, thus, may serve to convey specific information. The function of male-specific compounds identified from their tendon glands remains elusive, but several possibilities are discussed.

Keywords Bumblebee · Hydrocarbons · Leg tendon glands · Sex specific secretion · Wax esters

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Introduction

The daily life of social insects largely relies upon chemical communication between the members of a colony, between the sexes for the purpose of mating, between individuals from different nests, or for orientation, e.g., towards food sources or the nests (Free 1987; Hölldobler and Wilson 1990; Wilson 1990; Giglio et al. 2005; Detrain and Deneubourg 2009; Goulson 2009; Jarau 2009; Reinhard and Srinivasan 2009; Slaa and Hughes 2009). The vast majority of the involved chemical compounds are secreted from exocrine glands located virtually everywhere in insect bodies, from antennae to posterior abdomen (e.g., Hölldobler and Wilson 1990; Billen and Morgan 1998; Cruz-Landim et al. 2005; Billen 2009). Regarding exocrine glands, the best studied social insects undoubtedly are ants. For example, a recent review focusing just on the ants' leg glands revealed a stunning diversity of 20 different glandular structures or gland complexes (Billen 2009). The number

of different leg glands in bees seems to be smaller, but glandular structures have been described from all segments of all three pairs of legs (Cruz-Landim et al. 1998, 2005; Cruz-Landim and Franco 2000, 2001). However, a function of the secretions from leg glands of bees in chemical communication was mainly assigned to the tarsal glands (Schmitt et al. 1991; Stout et al. 1998; Goulson et al. 2000). These glands are sac-like structures, formed by class 1 secretory cells (sensu Noirot and Quennedey 1974), located within the fifth tarsomeres (Dahl 1885; Arnhart 1923). Ironically, several studies, in which the structure of tarsal glands was examined, unequivocally showed that they do not possess openings to release their secretions to the outside (honey bees: Arnhart 1923; Lensky et al. 1985; Federle et al. 2001; bumblebees: Pouvreau 1991; stingless bees: Cruz-Landim et al. 1998; Jarau et al. 2005). Instead, tarsal gland secretions are used to inflate the arolium in order to increase adhesion to smooth surfaces during walking (Federle et al. 2001). The seeming contradiction between the use of footprint secretions in chemical communication and the lack of openings of tarsal glands was resolved in the stingless bee *Melipona seminigra* Friese 1903. Jarau et al. (2004, 2005) discovered that the footprint hydrocarbons, which are left behind wherever a bee walks, are secreted from glandular systems associated with the claw retractor tendon of each leg (henceforth called leg tendon glands). The secretion produced by glandular epithelia in the femur and tibia of a leg accumulates in the hollow tendon and is released from an opening at the base of the unguitractor plate, i.e., at the leg tip (Jarau et al. 2004). Preliminary observations revealed the presence of similar glandular structures in the legs of the bumblebees *Bombus terrestris* (L. 1758), along with differences in the chemical composition of the gland secretions between males and workers (Baliet and Jarau, unpublished). These chemical differences indicate a special function of the male-specific compounds that is most likely linked to bumblebee mating behavior.

In order to better understand the function of male-specific leg tendon gland secretions, we describe the structure of these glands in males of *B. terrestris*, as well as the chemical composition of their secretions. Furthermore, we report on age-dependent changes in the structure of the glands and in the composition of their secretions, which was previously also shown for the labial glands of *B. terrestris* males (Šobotník et al. 2008; Žáček et al. 2009).

Material and methods

Material origin

All *B. terrestris* individuals used for this study originated from two laboratory breeds (Institute for Experimental Ecology, University of Ulm, Germany; Faculty of Science, Masaryk University, Brno, Czech Republic). This work focuses on males because preliminary data had shown the presence of male-specific chemical compounds in leg tendon glands (Baliet and Jarau, unpublished). The structure of the glands and the composition of their secretion were investigated in males that differed in age (1, 7, and 15 days, respectively) and originated from the same nest (colony A). For a comparison of the chemical composition of leg tendon and tarsal gland secretions with the cuticular hydrocarbons represented by wing washes (see Martin et al. 2010) in males, workers, and queens, we used individuals of the same age (7 days) originating from a single colony (colony B).

Light microscopy

Five males and workers from a *B. terrestris* colony were killed by freezing at −40 °C and their legs were cut off (Fig. 1). The femora, tibiae, and tarsi were separated and fixed in separate vials using Duboscq–Brasil fixative for 5 days, subsequently washed in 80 % ethanol, dehydrated in a series of ethanol with increasing concentration, and finally embedded in Spurr's resin (Serva Electrophoresis GmbH, Heidelberg, Germany) using the suppliers' standard protocol for hard resin. The resin was polymerized for 20 h at 62 °C.

Longitudinal and cross sections 7–8 μm thick were cut with a steel knife on a rotation microtome (model 1130, R. Jung, Heidelberg, Germany). Sections were mounted on slides, stained with 0.1 % toluidine blue in 2.5 % borax solution for 6 min at 40 °C, and embedded in DPX mountant.

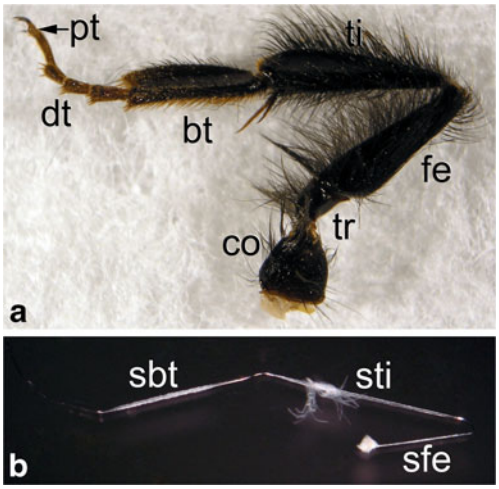

Fig. 1 The hind leg of a *Bombus terrestris* male (**a**) and the dissected hind leg tendon (**b**). *bt* basitarsus, *co* coxa, *dt* distitarsus, *fe* femur, *pt* pretarsus, *sbt* secretory epithelium within basitarsus, *sfe* secretory epithelium within femur, *sti* secretory epithelium within tibia, *ti* tibia, *tr* trochanter

Scanning electron microscopy

To observe the openings of leg tendon glands at the leg tips of bumblebees, the last tarsomeres of three freshly killed males were cut off, dehydrated in a series of ethanol mixtures with increasing concentration (starting with 70 %), placed in acetone for 3 days, and finally dried chemically using hexamethyldisilazane. The preparations were mounted on aluminum stubs, sputter-coated with platinum (20 nm), and examined in a Zeiss DSM962 scanning electron microscope.

Ultrastructural study

Legs were cut off from living males, put into a droplet of fixative (mixture of 2 % glutaraldehyde and 2.5 % formaldehyde in 0.1 M phosphate buffer, pH 7.2), and separated into the following parts with a razor blade: the fifth tarsomere together with pretarsus, remaining tarsomeres, tibia, and femur. The respective parts from all three leg pairs were fixed at ambient temperature for 1 day, washed in 0.1 M phosphate buffer, and subsequently postfixed in 1.5 % osmium tetroxide in the same buffer for 2 h. After fixation, the leg parts were washed in ultrapure (HPLC) water, dehydrated in a series of ethanol mixtures with increasing concentration, and embedded in Araldite resin. Ultrathin (about 50 nm) cross sections of all leg parts were prepared with a Reichert Ultracut ultramicrotome, stained with uranyl acetate and lead citrate (standard recipe after Reynolds 1963), and examined with a Jeol 1011 transmission electron microscope.

Sample preparation for chemical analysis

Pooled extracts of the leg tendon glands dissected from 30 legs of five 1-, 7-, and 15-day-old males each were prepared for the elucidation of the chemical structures of their secretion's components. The entire claw retractor tendons with their glandular tissues were dissected in ultrapure (HPLC) water using a dissecting microscope. Prior to dissection, the fifth tarsomere containing the whole tarsal gland (Pouvreau 1991) was removed in order to prevent possible contamination of the leg tendon gland extracts with tarsal gland compounds. All tendons and their gland epithelia were carefully separated from other tissues, except for a few muscle fibers that were tightly attached to them (Fig. 1). The 30 leg tendon glands of each age class were extracted in hexane (400 μl), ultrasonicated for 6 min, left in the solvent for 20 h, and evaporated under an argon flow to 50 μl. Extracts from the tarsal glands located within the fifth tarsomeres were prepared by cutting of the entire tarsomeres and pretarsi and extracting the whole structures in hexane in the same way as the tendons.

The compositions of the leg tendon and tarsal gland secretions and of cuticular hydrocarbons were compared between 7-day-old males, queens, and workers (N=5 each, for material origin see above). The composition of the compounds present on the cuticle surface was studied using right fore- and hindwing extracts (see Martin et al. 2010). The glands and wings of an individual were placed separately into 400 μl of hexane, ultrasonicated for 6 min, and left for 20 h in a freezer (−30 °C). Prior to analyses, all extracts were evaporated to a volume of 50 μl under an argon flow.

TLC fractionation

This approach was only used for samples studied by means of MALDI-TOF (see below). The leg tendon and tarsal gland extracts of 15-day-old males were fractionated on pre-cleaned glass thin layer chromatography plates (coated with Adsorbosil-Plus purchased from Applied Science Labs), using a mixture of hexane/diethylether=93:7 as mobile phase. Spots were visualized by spraying Rhodamine 6 G solution (0.05 % in ethanol). Two fractions were obtained: (1) hydrocarbons (R_f=0.94) and (2) wax esters (R_f=0.68). Each fraction was extracted with 1.5 ml of diethylether and cleaned on silica gel columns. The extracts were subsequently dried under an argon stream and the remains dissolved in 10 μl of chloroform.

MALDI mass spectrometry

MALDI-TOF experiments were performed on Reflex IV (Bruker Daltonik GmbH, Bremen, Germany) operated in the reflectron mode with an acceleration voltage of 20 kV and 200 ns extraction pulse. Desorption and ionization was achieved using a nitrogen UV laser (337.1 nm). Matrix ions were suppressed below m/z 200. Data were collected and analyzed using FlexAnalysis 3.0 software (Bruker Daltonik GmbH, Bremen, Germany). Spectra were averaged from 500 laser shots. Samples dissolved in chloroform were spotted on the target using thin layer technique: Matrix (^{6}LiDHB, 10 mg/ml in acetone/chloroform=2:1, 0.8 μl) was spotted on the MALDI plate and the matrix solvent was dried at ambient lab temperature. Each sample (0.8 μl) was then applied on top of the matrix and the solvent (chloroform) dried in the fume box at ambient lab temperature. The mass spectra were externally calibrated using a mixture of PEG 400, 600, and 1000.

GC×GC–MS analysis

We added 1 μl of internal standard (1-bromoicosane; 1.1 mg/ml of hexane) to each sample prior to injection. The quantification was carried out by external calibration

of pentacosane. The GC×GC–MS analyses were performed on Pegasus III (LECO Corporation, St. Joseph, MI, USA). The first dimension column was a 30-m×0.25-mm×0.25-μm Rxi-5 ms (Restek, Bellefonte, PA, USA), and the second dimension column was a 2.5-m×0.10-mm×0.10-μm BPX-50 (SGE, Ringwood, Australia). The columns were connected by a Presstight Connector (Restek, Bellefonte, PA, USA). The operating conditions were as follows: gas chromatography, primary oven temperature 50 °C (2 min), 10 °C/min to 320 °C (15 min); secondary oven temperature 10 °C above the primary oven temperature; modulator temperature 30 °C above the primary oven temperature; modulation period 4 s; the carrier gas was helium (corrected constant flow 1 ml/min); injection mode splitless for 30 s; injection temperature 320 °C, injection volume 1 μl; ToF-MS, electron ionization (−70 eV); ion source temperature 220 °C; acquired mass range 30–600 *m*/*z*; acquisition rate 100 spectra/s; solvent delay 420 s; and transfer line temperature 280 °C. ChromaToF software version 2.32 (LECO Corporation, St. Joseph, MI, USA) was used for system control, data acquisition, and processing. The NIST/EPA/NIH Mass Spectral Library was used for identification of compounds (NIST 2008).

Double bond position and configuration determination

To identify the double bond positions in alkenes found in the leg tendon glands of all castes and tarsal glands of males, derivatization with dimethyl disulfide (DMDS) was done according to a standard procedure (Carlson et al. 1989). Positions of the double bonds in alkadienes (C31 and C33) and wax esters could not be determined because the masses of their DMDS derivatives were too large to be transferred into the gas phase of the GC equipment.

A Fourier transform infrared instrument (Bruker model 55) and the KBr pellet (1.5 mm) technique were used to determine double bond configurations in the most abundant hydrocarbons. The *Z*/*E* configuration could not be determined in unsaturated compounds if their relative abundance in the extracts was below 4 %.

Nomenclature of hydrocarbons and wax esters

For unambiguous denomination of the identified compounds, we have used abbreviated nomenclature. In the presented bar graphs, for example, the abbreviation (*Z*)-C29:1 represents a hydrocarbon with straight carbon chain containing 29 carbons with one double bond in the *Z* configuration. For designation of wax esters, 16:0–16:1 denotes hexadecyl hexadecenoate.

Statistics

To test for significance in dissimilarities between the hydrocarbon profiles extracted from leg tendon glands, tarsal glands, and from the cuticle surface (wings) within each sex or caste, we conducted one-way analysis of similarities (ANOSIM) with 10,000 permutations based on Bray–Curtis distances using the program PAST version 2.15 (Hammer et al. 2001). Significance values (*p*) were adjusted according to the Bonferroni method.

Results

Leg tendon gland structure

The claw retractor tendons in all three pairs of legs of *B. terrestris* males, workers, and queens extend from the mid femur to the proximal part of the unguitractor plate, where an opening to the outside is located (Figs. 1 and 2). Two groups of muscles are attached to a tendon, the first to its proximal end within the femur and the second in the proximal tibia. The entire tendon is a hollow rod, to which three areas of glandular tissue are attached. Glandular cells are located (1) in the distal part of the femur, (2) the middle part of the tibia, and (3) within the entire basitarsus (Figs. 1 and 3). In the other parts, the tendon is either surrounded by flat, nearly organelle-free cells (proximal and distal tibia and basitarsus, articulations), by a basal lamina only (in tarsomeres other than the basitarsus), or penetrated by muscle attachments (proximal femur, small part in tibia). The cuticle overlying the secretory cells consists of chitin cuticle and epicuticle. The chitin cuticle is about 1.5 μm thick and does not show any particular layers. Pore canals through the cuticle are enlarged and contain numerous pore canal filaments. The epicuticle consists of an inner (about 30 nm thick) and an outer (about 10 nm thick) epicuticular layer, which is perforated by tiny pores. The

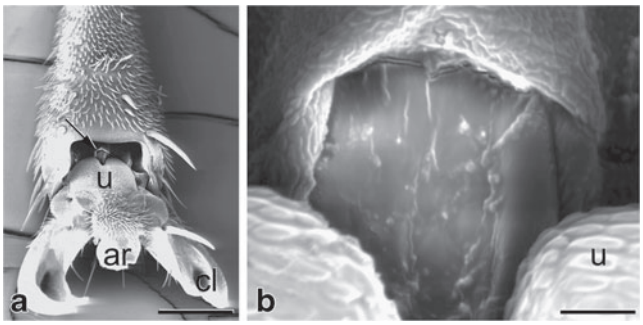

Fig. 2 **a** Ventral view of the fifth tarsomere and pretarsus of a mid-leg from a *Bombus terrestris* male. The *arrow* indicates the opening of the leg tendon gland at the base of the unguitractor plate, which is enlarged in **b**. Scale bars represent 200 μm (**a**) or 10 μm (**b**). Abbreviations: *ar* arolium, *cl* claw, *u* unguitractor plate

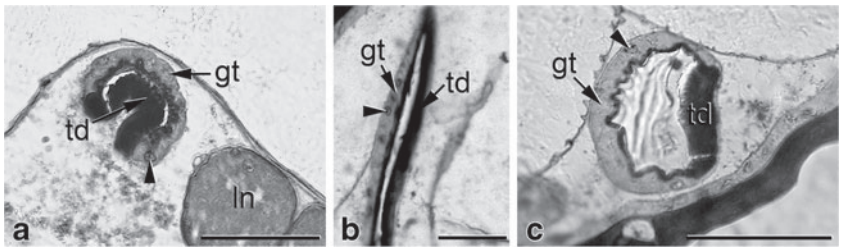

Fig. 3 Histological sections of the leg tendon gland within the mid-leg of a *B. terrestris* male. **a** Cross section in the range of distal femur. **b** Oblong section in the range of the mid tibia. **c** Cross section in the range of the basal basitarsus. Scale bars represent 50 μm. Note the difference between the strong cuticular part of the tendon (*td*) and the glandular tissue (*gt*) with its large nuclei (*arrow heads*); *ln* leg nerve

nonglandular part of the claw retractor tendon shows a similar cuticle structure, but its chitin layer is about two times thicker, lacks marked pore canals, and is devoid of epicuticle perforations.

The following description of the glandular epithelium is based on observations of 15-day-old males. The thickness of the glandular epithelium ranges from 5 to 12 μm. It consists of a single type of class 1 secretory cells (according to the classification by Noirot and Quennedey 1974) approximately cubic in shape. The apical parts form irregular projections and reach the tendon cuticle (Fig. 4). The basal parts of the cells form weakly developed invaginations. The basement membrane is formed by a single lamina, about 70 nm thick (locally two to three laminae of the same thickness were

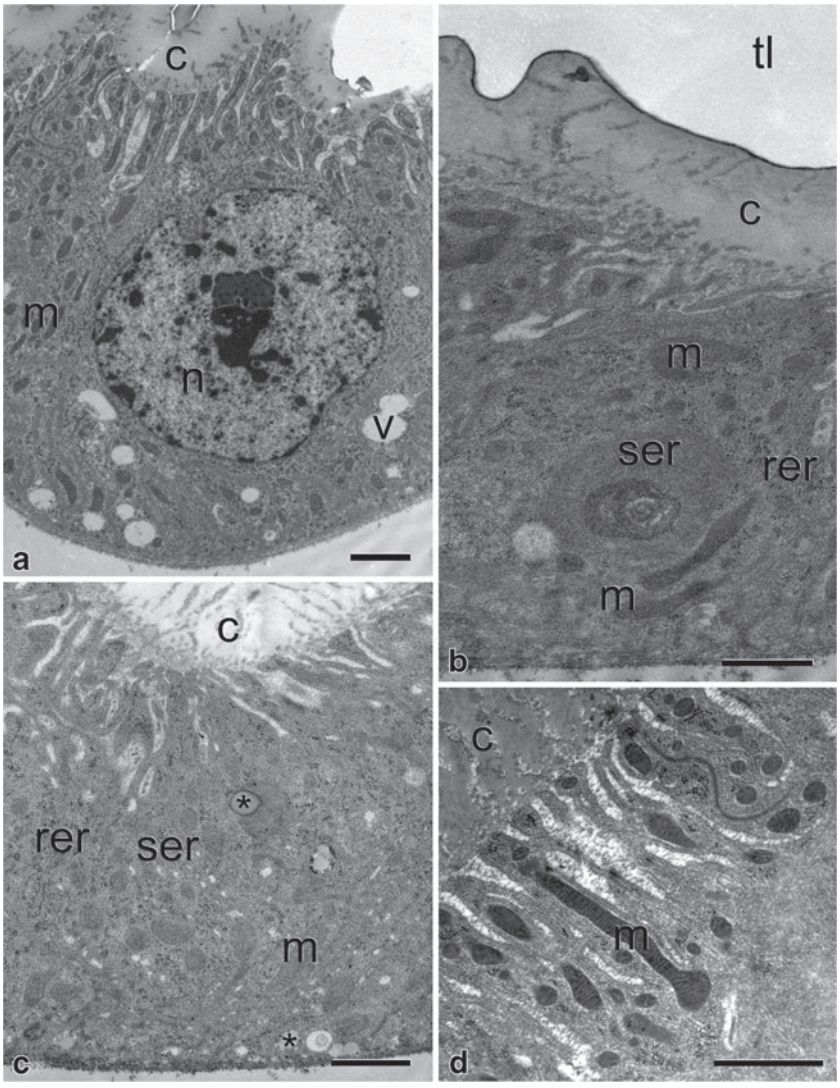

Fig. 4 Ultrastructure of leg tendon gland cells. **a** Gland cell in tibia of a 7-day-old male. Note the presence of numerous secretory vesicles. **b** Gland cell in basitarsus of a 15-day-old male. Note smooth endoplasmic reticulum oriented into a whorl. **c** Gland cell in basitarsus of a 15-day-old male. Note numerous lipid droplets (*asterisks*). **d** Detail of a secretory cell in basitarsus of a 7-day-old male. Note mitochondria inserted into apical projections and enlarged pore canals in the cuticle. Scale bars represent 1 μm. Abbreviations: *c* cuticle, *m* mitochondria, *n* nucleus, *rer* rough endoplasmic reticulum, *ser* smooth endoplasmic reticulum, *tl* tendon gland lumen, *v* electron-lucent vesicle

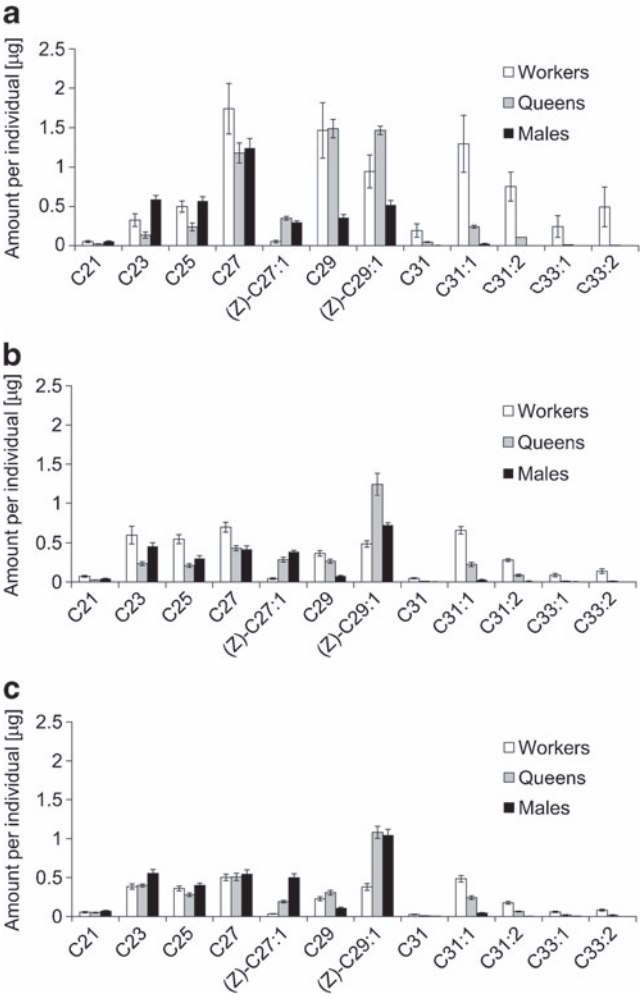

Fig. 5 Total amounts of the most abundant hydrocarbons present in the extracts of leg tendon gland (**a**), tarsal gland (**b**), and wings (**c**) of *B. terrestris* workers, queens, and males. Bars represent means±SE of five bees each of age 7 days

observed), and is connected to the secretory cells by hemidesmosomes. Intercellular junctions are formed by long apical septate junctions (Fig. 4) and rare gap junctions; substantial parts of the membranes of neighboring cells are free (approximately three quarters measured from the cells' base). Numerous microtubules, about 15 nm in diameter and oriented predominantly apico-basally, are present in the secretory cells. They are more abundant at the gland margins, close to areas where the secretory epithelium changes into nonsecretory epithelium. The nuclei of the gland cells are about 5 μm in their largest dimension, spherical or ovoid in shape, and located in the cells' center (Fig. 4). Mitochondria are moderately abundant, scattered throughout the cells, but more numerous in the apical projections (Fig. 4). The most abundant secretory organelles are smooth endoplasmic reticulum (ER) and less extensive rough ER scattered among the tubules of the smooth ER (Fig. 4). Inclusions consist of electron-lucent vesicles (0.5–1 μm in diameter), electron-dense granules (about 600 nm in diameter) delimited by a membrane, and small lipid-like droplets (150–300 nm in diameter) localized predominantly at the cell bases (Fig. 4). The electron-dense granules were frequently observed to turn into lipid-like droplets (Fig. 4).

Only slight differences in the structure of the glandular tissue were observed according to the age of males. The thickness of the secretory epithelium increases with age and is particularly low in 1-day-old males (3–8 μm). The amount of smooth ER and the abundance of lipid-like droplets are also lower in younger males. Pinocytotic activity was rarely observed at the cell bases of 1-day-old males only.

Chemical analyses

The leg tendon gland extracts are dominated by linear saturated and unsaturated hydrocarbons that are qualitatively

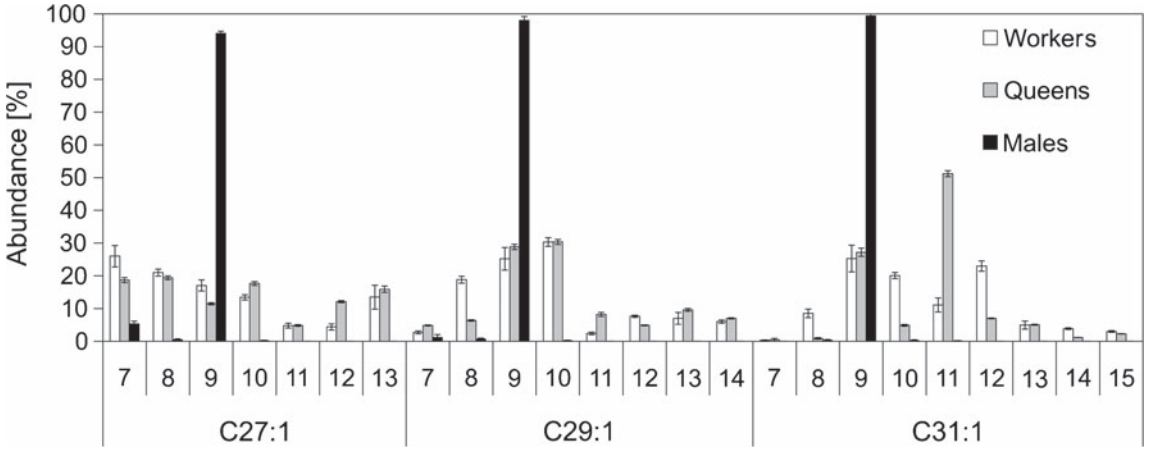

Fig. 6 Double bond positions in unsaturated hydrocarbons (C27–C31) present in the leg tendon glands of workers, queens, and males. The abundance is relative to the TIC signal distribution per unsaturated hydrocarbon of the same number of carbons. Bars represent means±SE of five bees each of age 7 days

similar in all castes but differ in their quantities and relative proportions (Fig. 5a). Differences between the castes are found in the relative abundances of the common hydrocarbons, as well as in hydrocarbons with longer chain lengths and two double bonds that are present only in workers and queens (Fig. 5a). The leg tendon glands of males and females also differ in regard to the double bond positions in the unsaturated hydrocarbons. Males produce compounds with a double bond almost exclusively in position 9 (27:1, 29:1, and 31:1), whereas the double bond position is distributed among seven to nine different positions in queens and workers (Fig. 6). All double bonds present in hydrocarbons with an abundance of more than 4 % in the crude extract were in the *Z* configuration. In addition, the leg tendon gland extracts of the males contain wax esters (Table 1, Fig. 7) that are lacking in the glands of workers and queens.

The relative proportions of the hydrocarbons in the tarsal gland extracts (Fig. 5b) and of the cuticular hydrocarbons (Fig. 5c) are similar within each caste, whereas the composition of hydrocarbons in leg tendon glands differs from these two sources (Fig. 5a). ANOSIM results of pairwise comparisons confirm significant dissimilarities between the leg tendon gland hydrocarbon composition to that of the tarsal glands or cuticular surface, respectively, for males, workers, and queens alike (all Bonferroni corrected p values<0.04). No significant dissimilarities were found by ANOSIM between tarsal gland and cuticular hydrocarbons for any sex or caste (all Bonferroni corrected p values>0.08). The composition of the males' leg tendon gland secretion shows age-dependent changes in the absolute amounts of particular hydrocarbons resulting in differences in their relative composition (Fig. 8). The majority of the compounds considerably increase in amount with the age of a male, except for the hydrocarbons (*Z*)-nonacos-9-ene and hentriacont-9-ene.

We analyzed in detail the structures of heavier nonvolatile compounds from the males' leg tendon and tarsal glands (Table 1). The most abundant wax esters were hexadecyl hexadecanoate and hexadecyl octadecenoate in both extracts. The extracts of the fifth tarsomeres also contained terpenic compounds (2,3-dihydrofarnesol and its esters) that were absent from leg tendon glands. The wax esters with the highest molecular weight identified by GC×GC–MS contained 34 carbons. However, the presence of heavier wax esters with up to 50 carbon atoms was confirmed by MALDI analysis but was not further identified.

Table 1 List of the compounds found in leg tendon and tarsal gland extracts prepared from 7-day-old bumblebee males (extracts of 30 glands from five males)

Compounds	LRI[a]	Abundance [%][b]	
		Tendon gland	Tarsal gland
2,3-Dihydrofarnesol	1694		1.0
Icosene	1995	3.7	2.2
Henicosane	2100	1.5	1.2
Tricos-9-ene	2275	0.9	1.7
Tricosane	2300	13.1	14.7
Tetracosane	2400	2.0	0.6
Pentacos-9-ene	2482	0.5	1.9
Pentacosane	2500	14.6	10.1
Hexacos-9-ene	2581	0.5	0.6
Hexacosane	2600	2.1	1.0
(*Z*)-Heptacos-9-ene	2682	4.4	11.7
Heptacosane	2700	23.8	13.2
2,3-Dihydrofarnesyl dodecanoate	2760		0.4
Octacos-9-ene	2779	0.6	1.4
Docosenamide	2790	0.5	1.0
Octacosane	2800	1.2	0.5
Squalene	2820	1.8	1.2
(*Z*)-Nonacos-9-ene	2880	7.4	20.4
Nonacosane	2900	8.9	3.1
Hexadecyl dodecanoate	2973		0.3
2,3-Dihydrofarnesyl tetradecenoate	2964		0.2
Hentriacont-9-ene	3077	0.5	1.3
Hentriacontane	3100	0.3	0.3
Hexadecyl tetradecanoate	3170	0.6	0.2
2,3-Dihydrofarnesyl hexadecanoate	3164		0.5
2,3-Dihydrofarnesyl hexadecenoate	3166		0.2
2,3-Dihydrofarnesyl octadecenoate	3350		0.7
2,3-Dihydrofarnesyl octadecatrienoate	3359		0.4
Hexadecyl hexadecanoate	3365	6.0	1.8
Hexadecyl hexadecenoate	3365	1.8	1.2
Hexadecyl octadecenoate	3537	2.2	3.2
Hexadecyl octadecadienoate	3539		0.5
Hexadecyl octadecatrienoate	3557	0.3	1.4
Hexadecyl octadecanoate	3557	0.4	
Hexadecenyl octadecenoate	3557	0.3	

[a] Linear Retention Indexes (LRI)

[b] Abundance of the compounds according to total ion chromatogram (TIC) signal distribution

Discussion

The leg tendon gland we describe here represents a new member of the exocrine gland set known from bumblebees. The general structure and location of the leg tendon glands is similar to that described for the stingless bee *Melipona seminigra* (Jarau et al. 2004), with the difference that in *B. terrestris* the secretory cells are located also in the basitarsus in addition to femur and tibia. The leg tendon gland is clearly an exocrine organ, as evidenced (1) by the increased

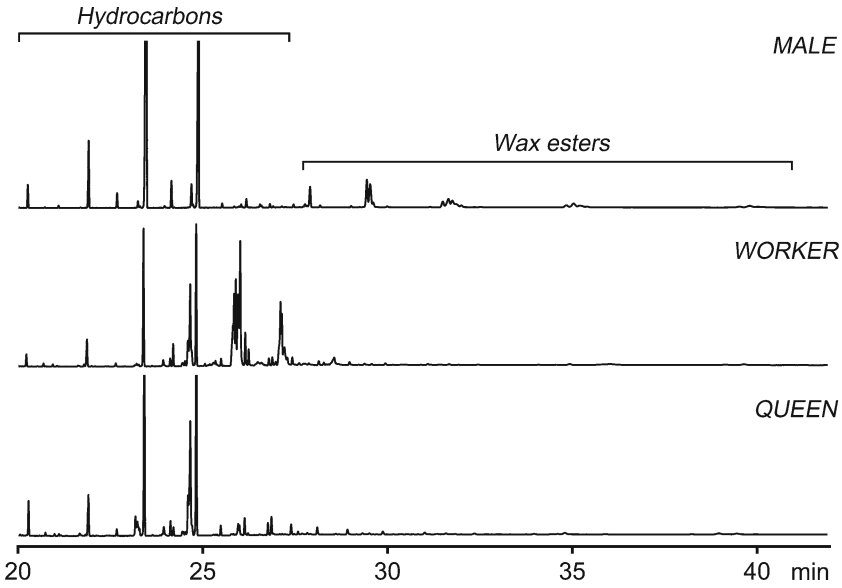

Fig. 7 Gas chromatographic separation of the compounds extracted from leg tendon glands of a *Bombus terrestris* male, worker, and queen. Wax esters are predominantly found in male secretions

volume of the secretory cells due to the presence of well-developed secretory organelles (smooth and rough ER), (2) by the presence of projections of the apical plasma membrane, and (3) by modifications of the cuticle overlying the secretory cells, having enlarged pore canals and perforations through the epicuticle allowing the transport of secretion from the cells into the gland's reservoir formed by the hollow tendon. Although the secretory cells are located in three independent and well-separated regions, we consider the gland as a single exocrine secretory organ according to the same ultrastructure of the secretory cells and the joint delivery of their secretion via the opening of the hollow tendon at the base of the unguitractor plate. Our observations suggest that the leg tendon gland secretion is primarily synthesized by smooth ER, which is the dominant secretory organelle in *B. terrestris* males' labial (Šobotník et al. 2008) and leg tendon glands, and is also known to be the most important source of nonpolar compounds including hydrocarbons and wax esters (Fawcett 1966; Percy-Cunningham and MacDonald 1987; Tillman et al. 1999).

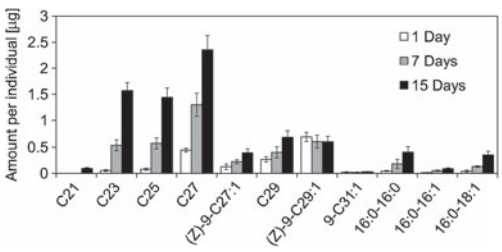

Fig. 8 Age-dependent changes of the most abundant compounds from leg tendon gland extracts of *Bombus terrestris* males. Bars represent means±SE of five bees studied per age group (1, 7, and 15 days old). Note that the quantity of all compounds is gradually increasing, except for (*Z*)-9-C29 and 9-C31

The secretion of the leg tendon glands of *B. terrestris* males differs in its composition from that of workers and queens, supporting our hypothesis that leg tendon gland secretions of males are used for specific purposes. In particular, they have larger proportions of heavier wax esters with aliphatic alcohol parts. In addition, the composition of the leg tendon gland hydrocarbons differs between the sexes, showing remarkable differences in the double bond positions in the unsaturated hydrocarbons. Interestingly, the double bond position in alkenes found in the secretion of male leg tendon glands is highly conserved, being almost exclusively at position 9, whereas females synthesize alkenes with double bonds at various positions in their glands. The position of a double bond influences the chemical and physical properties of a molecule, which can be perceived by insects and, thus, can serve to convey specific information (Martin et al. 2010). For example, the male sex pheromones from labial gland secretions of the related species *Bombus ruderarius* and *Bombus sylvarum*, seemingly similar in composition, contain components with different double bond positions (hexadec-9-enol and octadec-11-enol in *B. ruderarius* and hexadec-7-enol and octadec-9-enol in *B. sylvarum*; Terzo et al. 2005). These differences likely convey species specificity in the pheromone.

The chemical analyses had shown that the secretion of the leg tendon glands differs from both the secretion of the tarsal glands and from the cuticular hydrocarbon profile (represented by wing washes). Nevertheless, leg tendon and tarsal glands share certain compounds that may represent constituent parts of the bumblebee cuticle. We assume that compounds found in leg tendon gland extracts that increase in quantity with bumblebee age are produced by the glands, while compounds showing unchanged amounts

may have been extracted from the cuticle. For example, the amount of (*Z*)-nonacos-9-ene in the leg tendon glands of males does not change with age and also represents the most abundant hydrocarbon in wing washes. The same applies to hentriacont-9-ene that was detected only in trace amounts in all samples prepared from males. More importantly, however, the amount of the remaining compounds from leg tendon gland secretions increased with the age of males, coinciding with an increased attractiveness of their labial gland secretions to virgin queens with the males' age (Coppée et al. 2011).

The function of male-specific compounds from leg tendon gland secretions remains elusive; however, several possibilities can be assumed. Differences in the composition of the secretion as compared to queens and workers strongly suggest that these compounds serve a communication purpose related to sexual behavior. Male leg tendon gland secretions may be used to mark flight routes, to which virgin queens are attracted for mating, in addition to labial gland secretions of males known to serve this purpose (Kullenberg et al. 1973; Free 1987; Bergman and Bergström 1997; Kindl et al. 1999). The amount of leg tendon gland secretion at a marking spot may tell how many males are using this spot, or how much time has elapsed since marking, based on the higher volatility of male's labial gland secretion compared to the secretion of leg tendon glands. The compounds could then influence a queen's decision of whether to stay or to fly on looking for another marked spot. In that sense, leg tendon gland secretions could act as an "arrestment signal." The gland secretion may also act as anti-aphrodisiac deposited by the males on queens during mating. The role of anti-aphrodisiacs to render mated females unattractive has already been reported for some bee species, as well as for other insects (Gilbert 1976; Kukuk 1985; Ayasse et al. 2001). Interestingly, *B. terrestris* males regularly drum their front legs on a queen's body during copulation (Jarau and Šobotník, unpublished observation), which may facilitate the deposition of leg tendon gland secretions on her body. Furthermore, males first investigate the head and abdomen of a queen with their antennae when approaching her in a mating attempt (Djegham et al. 1994). This behavior may serve to asses a queen's mating status. Since queens of *B. terrestris* mate only with a single male (Estoup et al. 1995; Schmid-Hempel and Schmid-Hempel 2000) that transfers a mating plug to their genital tract during copulation (Duvoisin et al. 1999; Baer et al. 2001; Sauter et al. 2001), any male approaching a queen could gain advantage by quickly identifying whether she is already mated before mounting and trying to copulate. The mating plug itself, which contains palmitic, linoleic, oleic, and stearic acid, as well as the cyclic peptide cycloprolylproline (Baer et al. 2000), does not alter the mating motivation of males (Baer et al. 2001). Whether specific compounds from leg tendon glands play a role in species or kin discrimination, to arrest virgin queens at the males' marking spots, or serve as information about a queen's mating status needs to be tested in field bioassays.

Our study revealed only slight age-dependent changes in both the structure of the leg tendon gland tissue and in the chemical composition of the gland secretion in *B. terrestris* males. Although the gland ultrastructure is already well developed in 1-day-old males, the overall activity, as evidenced by increased amounts of both smooth ER and lipid droplets, increases as a male gets older. This trend corresponds to observations on the labial gland structure and changes in composition of their secretions in bumblebee males, which clearly correlates with the attractiveness of males towards virgin queens, which is highest in young males, roughly between the age of 5 and 15 days (Šobotník et al. 2008; Žáček et al. 2009; Coppée et al. 2011).

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References

Arnhart L (1923) Das Krallenglied der Honigbiene. Arch Bienenkunde 5:37–86

Ayasse M, Paxton R, Tengö J (2001) Mating behavior and chemical communication in the order Hymenoptera. Annu Rev Entomol 46:31–78

Baer B, Maile R, Schmid-Hempel P, Morgan ED, Jones GR (2000) Chemistry of a mating plug in bumblebees. J Chem Ecol 26:1869–1875

Baer B, Morgan ED, Schmid-Hempel P (2001) A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. PNAS 98:3926–3928

Bergman P, Bergström G (1997) Scent marking, scent origin, and species specificity in male premating behavior of two Scandinavian bumblebees. J Chem Ecol 23:1235–1251

Billen J (2009) Occurrence and structural organization of the exocrine glands in the legs of ants. Arthropod Struct Dev 38:2–15

Billen J, Morgan ED (1998) Pheromone communication in social insects: sources and secretions. In: Vander Meer RK, Breed MD, Winston ML, Espelie KE (eds) Pheromone communication in social insects: ants, wasps, bees, and termites. Westview, Boulder, pp 3–33

Carlson DA, Roan C-S, Yost RA, Hector J (1989) Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. Anal Chem 61:1564–1571

Coppée A, Mathy T, Cammaerts M-C, Verheggen FJ, Terzo M, Iserbyt S, Valterová I, Rasmont P (2011) Age-dependent attractivity of males' sexual pheromones in *Bombus terrestris* (L.) [Hymenoptera, Apidae]. Chemoecology 21:75–82

da Cruz-Landim C, Franco AC (2000) Epithelial bags inside the tibia and femur of males of *Centris* (Hymenoptera, Anthophoridae): localization and ultrastructure. Rev Bras Entomol 44:97–103

da Cruz-Landim C, Franco AC (2001) Light and electron microscopic aspects of glands and pseudoglandular structures in the legs of bees (Hymenoptera, Apinae, Euglossini). Braz J morphol Sci 18:81–90

da Cruz-Landim C, de Moraes RLMS, Salles HC, Reginato RD (1998) Note on glands present in Meliponinae (Hymenoptera, Apidae) bees legs. Rev Bras Zool 15:159–165

da Cruz-Landim C, Abdalla FC, Gracioli-Vitti LF (2005) Morphological and functional aspects of volatile-producing glands in bees (Hymenoptera: Apidae). Insect Sci 12:467–480

Dahl F (1885) Die Fussdrüsen der Insekten. Arch mikrosk Anat 25:236–262

Detrain C, Deneubourg J-L (2009) Social cues and adaptive foraging strategies in ants. In: Jarau S, Hrncir M (eds) Food exploitation by social insects. Ecological, behavioral, and theoretical approaches. CRC, Boca Raton, pp 29–51

Djegham Y, Verhaeghe JC, Rasmont P (1994) Copulation of *Bombus terrestris* L. (Hymenoptera: Apidae) in captivity. J Apic Res 33:15–20

Duvoisin N, Baer B, Schmid-Hempel P (1999) Sperm transfer and male competition in a bumblebee. Anim Behav 58:743–749

Estoup A, Scholl A, Pouvreau A, Solignac M (1995) Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites. Mol Ecol 4:89–94

Fawcett DW (1966) The cell. Its organelles and inclusions. Saunders, Philadelphia

Federle W, Brainerd EL, McMahon TA, Hölldobler B (2001) Biomechanics of the movable pretarsal adhesive organ in ants and bees. PNAS 98:6215–6220

Free JB (1987) Pheromones of social bees. Cornell University Press, Ithaca

Giglio A, Ferrero EA, Brandmayr TZ (2005) Ultrastructural identification of the antennal gland complement in *Siagona europaea* Dejean 1826, a myrmecophagous carabid beetle. Acta Zool 86:195–203

Gilbert LE (1976) Postmating female odor in *Heliconius* butterflies: a male contributed antiaphrodisiac? Science 193:419–420

Goulson D (2009) The use of scent marks by foraging bumble bees. In: Jarau S, Hrncir M (eds) Food exploitation by social insects. Ecological, behavioral, and theoretical approaches. CRC, Boca Raton, pp 251–260

Goulson D, Stout JC, Langley J, Hughes WOH (2000) Identity and function of scent marks deposited by foraging bumblebees. J Chem Ecol 26:2897–2911

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package or education and data analysis. Palaeontol Electron 4:9, http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Hölldobler B, Wilson EO (1990) The ants. Harvard University Press, Cambridge

Jarau S (2009) Chemical communication during food exploitation in stingless bees. In: Jarau S, Hrncir M (eds) Food exploitation by social insects. Ecological, behavioral, and theoretical approaches. CRC, Boca Raton, pp 223–249

Jarau S, Hrncir M, Ayasse M, Schulz C, Francke W, Zucchi R, Barth FG (2004) A stingless bee (*Melipona seminigra*) marks food sources with a pheromone from its claw retractor tendons. J Chem Ecol 30:793–804

Jarau S, Hrncir M, Zucchi R, Barth FG (2005) Morphology and structure of the tarsal glands of the stingless bee *Melipona seminigra*. Naturwissenschaften 92:147–150

Kindl J, Hovorka O, Urbanová K, Valterová I (1999) Scent marking in male premating behavior of *Bombus confuses*. J Chem Ecol 25:1489–1500

Kukuk P (1985) Evidence for an antiaphrodisiac in the sweat bee *Lasioglossum* (*Dialictus*) *zephyrum*. Science 227:656–657

Kullenberg B, Bergström G, Bringer B, Carlberg B, Cederberg B (1973) Observations on scent marking by *Bombus* Latr. and *Psithyrus* Lep. males (Hym., Apidae) and localization of site of production of the secretion. Zoon Suppl 1:23–30

Lensky Y, Cassier P, Finkel A, Delorme-Joulie C, Levinsohn M (1985) The fine structure of the tarsal glands of the honeybee *Apis mellifera* L. (Hymenoptera). Cell Tissue Res 240:153–158

Martin JS, Carruthers JM, Williams PH, Drijfhout FP (2010) Host specific social parasites (*Psithyrus*) indicate chemical recognition system in bumblebees. J Chem Ecol 36:855–863

NIST Mass Spec Data Center SES (2008) Mass spectra, 6th edn. National Institute of Standards and Technology, Gaithersburg, http://webbook.nist.gov/chemistry

Noirot C, Quennedey A (1974) Fine structure of insect epidermal glands. Annu Rev Entomol 19:61–80

Percy-Cunningham JE, MacDonald JA (1987) Biology and ultrastructure of sex pheromone-producing glands. In: Prestwich GD, Blomquist GJ (eds) Pheromone biochemistry. Academic, Orlando, pp 27–69

Pouvreau A (1991) Morphology and histology of tarsal glands in bumble bees of the genera *Bombus*, *Pyrobombus*, and *Megabombus*. Can J Zool 69:866–872

Reinhard J, Srinivasan MV (2009) The role of scents in honey bee foraging and recruitment. In: Jarau S, Hrncir M (eds) Food exploitation by social insects. Ecological, behavioral, and theoretical approaches. CRC, Boca Raton, pp 165–182

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212

Sauter A, Brown MJF, Baer B, Schmid-Hempel P (2001) Males of social insects can prevent queens from multiple mating. Proc R Soc Lond B 268:1449–1454

Schmid-Hempel R, Schmid-Hempel P (2000) Female mating frequencies in *Bombus* spp. from Central Europe. Insect soc 47:36–41

Schmitt U, Lübke G, Francke W (1991) Tarsal secretion marks food sources in bumblebees (Hymenoptera: Apidae). Chemoecology 2:35–40

Slaa EJ, Hughes WOH (2009) Local enhancement, local inhibition, eavesdropping, and the parasitism of social insect communication. In: Jarau S, Hrncir M (eds) Food exploitation by social insects. Ecological, behavioral, and theoretical approaches. CRC, Boca Raton, pp 147–164

Šobotník J, Kalinová B, Cahlíková L, Weyda F, Ptáček V, Valterová I (2008) Age-dependent changes in structure and function of the male labial gland in *Bombus terrestris*. J Insect Physiol 54:204–214

Stout JC, Goulson D, Allen JA (1998) Repellent scent-marking of flowers by a guild of foraging bumble bees (*Bombus* spp.). Behav Ecol Sociobiol 43:317–326

Terzo M, Urbanová K, Valterová I, Rasmont P (2005) Intra and interspecific variability of the cephalic labial glands' secretions in male bumblebees: the case of *Bombus* (*Thoracobombus*) *ruderarius* and *B.* (*Thoracobombus*) *sylvarum* [Hymenoptera, Apidae]. Apidologie 36:85–96

Tillman JA, Seybold SJ, Jurenka RA, Blomquist GJ (1999) Insect pheromones—an overview of biosynthesis and endocrine regulation. Insect Biochem Mol Biol 29:481–514

Wilson EO (1990) Success and dominance in ecosystems: the case of the social insects. In: Kinne O (ed) Excellence in ecology, book 2. Ecology Institute, Oldendorf/Luhe

Žáček P, Kalinová B, Šobotník J, Hovorka O, Ptáček V, Coppée A, Verheggen F, Valterová I (2009) Comparison of age-dependent quantitative changes in the male labial gland secretion of *Bombus terrestris* and *Bombus lucorum*. J Chem Ecol 35:698–705

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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

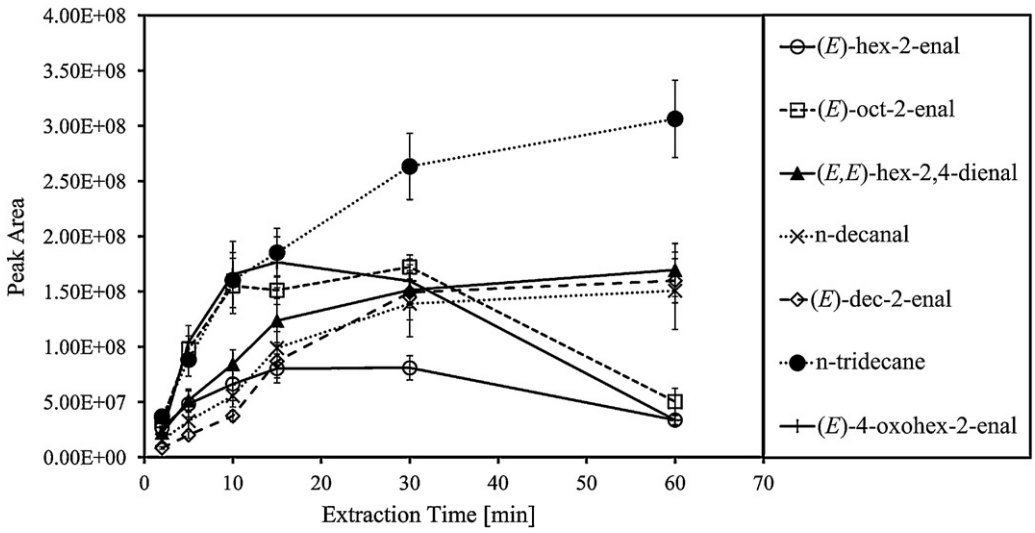

Fig. 1. The effect of extraction time on headspace amounts of selected *G. lineatum* volatiles using a DVB/CAR/PDMS fiber at 25 °C; each data point represents an average of three individual runs.

(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.

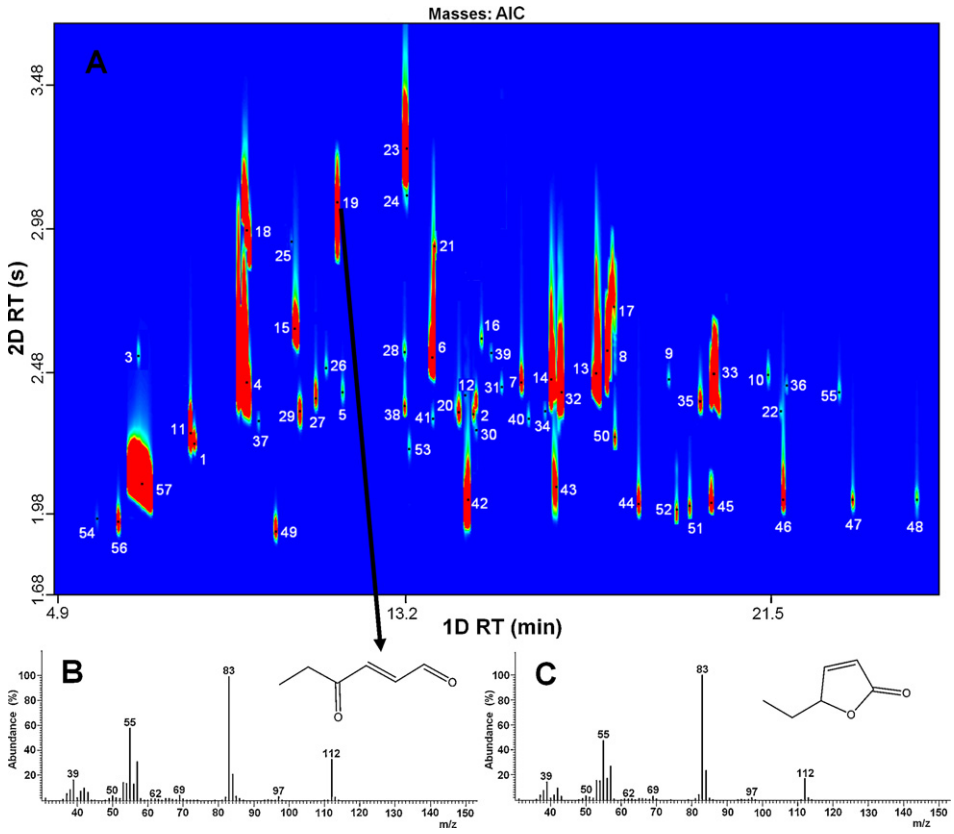

Fig. 2. An analytical ion chromatogram contour plot showing the 2D distribution of the volatile oxygenates and hydrocarbons in the secretions of an adult *G. lineatum* male (A), the mass spectra of the isolated (*E*)-4-oxohex-2-enal (B) and synthesized 5-ethylfuran-2(5*H*)-one (C); the numbered peaks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

References

[1] D.W. Whitman, M.S. Blum, D.W. Alsop, in: D.L. Evans, J.O. Schmidt (Eds.), Insect Defenses. Adaptive Mechanisms and Strategies of Prey and Predators, SUNY Press, Albany, NY, 1990 (Chapter 12).
[2] P. Laurent, J.C. Braekman, D. Daloze, Top. Curr. Chem. 240 (2005) 167.
[3] J.E. McPherson, R.M. McPherson, Stink Bugs of Economic Importance in America. North of Mexico, CRC Press LLC, Boca Raton, 2000.
[4] J.R. Aldrich, Ann. Rev. Entomol. 33 (1988) 211.
[5] H. Ho, J.G. Millar, Zool. Stud. 40 (2001) 193.
[6] J.R. Aldrich, in: R.T. Cardé, W.J. Bell (Eds.), Chemical Ecology of Insects 2, Chapman & Hall, New York, 1995, p. 318.
[7] T. Tsuyuki, Y. Ogata, I. Yamamoto, K. Shinmi, Agric. Biol. Chem. 29 (1965) 419.
[8] D. Durak, Y. Kalender, C. R. Biol. 332 (2009) 34.
[9] K. Stránský, I. Valterová, K. Ubik, J. Čejka, J. Křeček, J. High Resol. Chromatogr. 21 (1998) 475.
[10] D. Djozan, T. Baheri, R. Farshbaf, Sh. Ashari, Anal. Chim. Acta 554 (2005) 197.
[11] R. Shellie, P. Marriott, P. Morrison, Anal. Chem. 73 (2001) 1336.
[12] J. Dallüge, R.J.J. Vreuls, J. Beens, U.A.Th. Brinkman, J. Sep. Sci. 25 (2002) 201.
[13] J.J. Dallüge, L.L.P. van Stee, X. Xu, J. Williams, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 974 (2002) 169.
[14] M.T. Roberts, J.P. Dufour, A.C. Lewis, J. Sep. Sci. 27 (2004) 473.
[15] W. Welthagen, R.A. Shellie, J. Spranger, M. Ristow, R. Zimmermann, O. Fiehn, Metabolomics 1 (2005) 65.
[16] T. Čajka, J. Hajšlová, J. Cochran, K. Holadová, E. Klimánková, J. Sep. Sci. 30 (2007) 534.
[17] L. Mondello, P.Q. Tranchida, P. Dugo, G. Dugo, Mass Spectrom. Rev. 27 (2008) 101.
[18] M. Adahchour, J. Beens, U.A.Th. Brinkman, J. Chromatogr. A 1186 (2008) 67.
[19] E. van Wilgenburg, D. Ryan, P. Morrison, P.J. Marriott, M.A. Elgar, Naturwissenschaften 93 (2006) 309.
[20] B. Kalinová, P. Jiroš, J. Žďárek, X. Wen, M. Hoskovec, Talanta 69 (2006) 542.
[21] G.T. Eyres, S. Urban, P.D. Morrison, P.J. Marriott, J. Chromatogr. A 1215 (2008) 168.
[22] R.R. Heath, B.D. Dueben, in: J.G. Millar, K.F. Haynes (Eds.), Methods in Chemical Ecology, vol. 1, Kluwer Acad. Publishers, London, 1998 (Chapter 3).
[23] S. Nojima, C.S. Apperson, C. Schal, J. Chem. Ecol. 34 (2008) 418.
[24] S. Nojima, C. Schal, F.X. Webster, R.G. Santangelo, W.L. Roelofs, Science 307 (2005) 1104.
[25] M. Takita, H. Sugie, J. Tabata, S. Ishii, S. Hiradate, Appl. Entomol. Zool. 43 (2008) 11.
[26] M. D'Alessandro, V. Brunner, G. von Mérey, T.C.J. Turlings, J. Chem. Ecol. 35 (2009) 999.
[27] C.P.G. Rühle, J. Niere, P.D. Morrison, R.C. Jones, T. Caradoc-Davies, A.J. Canty, M.G. Gardiner, V.-A. Tolhurst, P.J. Marriott, Anal. Chem. 82 (2010) 4501.
[28] B.S. Krall, R.J. Bartelt, C.J. Lewis, D.W. Whitman, J. Chem. Ecol. 25 (1999) 2477.
[29] S. Risticevic, E. Carasek, J. Pawliszyn, Anal. Chim. Acta 617 (2008) 72.
[30] S. Cui, S. Tab, G. Ouyang, S. Juany, J. Pawliszyn, J. Chromatogr. B 877 (2009) 1901.
[31] M. Ndagijimana, M. Vallicelli, P.S. Cocconcelli, F. Cappa, F. Patrignani, R. Lanciotti, M.E. Guerzoni, Appl. Environ. Microbiol. (2006) 6053.
[32] A. Giglio, P. Brandmayr, R. Dalpozzo, G. Sindona, A. Tagarelli, F. Talarico, T.Z. Brandmayr, E. Ferrero, Microsc. Res. Techn. 72 (2009) 351.
[33] B. Koutek, M. Hoskovec, P. Vrkočová, L. Feltl, J. Chromatogr. A 759 (1997) 93.
[34] J.A. Moreira, J.G. Millar, J. Chem. Ecol. 31 (2005) 965.
[35] J. Ma, S.H. Wang, G.R. Tian, Syn. Commun. 36 (2006) 1229.
[36] (a) NIST Mass Spec Data Center SES, Mass Spectra, 6th ed., 2005; (b) NIST Mass Spec Data Center SES, Retention Indices, 6th ed., National Institute of Standards and Technology, Gaithersburg, MD, 2005, http://webbook.nist.gov/chemistry.
[37] R.P. Adams, Identification of Essentials Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed., Allured Publ., Carol Stream, IL, 2007.
[38] M.Z. Özel, F. Gögüs, A.C. Lewis, J. Chromatogr. A 1114 (2006) 164.
[39] J. Aitchison, The Statistical Analysis of Compositional Data, Chapman & Hall, London, 1986.
[40] S. Geiselhardt, T. Otte, M. Hilker, J. Chem. Ecol. 35 (2009) 1162.
[41] L. Setkova, S. Risticevic, J. Pawliszyn, J. Chromatogr. A 1147 (2007) 213.
[42] L. Ferreira, R. Perestrelo, J.S. Câmara, Talanta 77 (2009) 1087.
[43] D. Ryan, R. Shellie, P. Tranchida, A. Casilli, L. Mondello, P. Marriott, J. Chromatogr. A 1054 (2004) 57.
[44] F.A. Marques, E.P. Wendler, B.H.L.N. Sales Maia, M.U. Ventura, I.C. Arruda-Gatti, J. Braz. Chem. Soc. 18 (2007) 1242.
[45] M. Pareja, M. Borges, R.A. Laumann, M.C.B. Moraes, J. Insect Physiol. 53 (2007) 639.
[46] M.C.B. Moraes, M. Pareja, R.A. Laumann, M. Borges, Neotrop. Entomol. 37 (2008) 489.
[47] Z.M. Zhang, W.W. Wu, G.K. Li, J. Chromatogr. Sci. 47 (2009) 291.
[48] J.P. Farine, O. Bonnard, R. Brossut, J.L. Le Quere, J. Chem. Ecol. 18 (1992) 1673.
[49] A. Fucarino, J.G. Millar, J.S. McElfresh, S. Colazza, J. Chem. Ecol. 30 (2004) 1257.
[50] P.I. Innocenzi, D. Hall, J.V. Cross, H. Hesketh, J. Chem. Ecol. 31 (2005) 1401.
[51] H.-Y. Ho, R. Kou, H.-K. Tseng, J. Chem. Ecol. 29 (2003) 2101.
[52] M.F. Feldlaufer, M.J. Domingue, K.R. Chauhan, J.R. Aldrich, J. Med. Entomol. 47 (2010) 140.
[53] K. Kawai, K. Matsuno, H. Kasai, Mutat. Res. 603 (2006) 186.
[54] M.D. Guillén, E. Goicoechea, Crit. Rev. Food Sci. Nutr. 48 (2008) 119.
[55] K.L. Prudic, K. Noge, J.X. Becerra, J. Chem. Ecol. 34 (2008) 734.
[56] P. Wojtowicz, J. Zrostlíková, T. Kovalczuk, J. Sochůrek, T. Adam, J. Chromatogr. A 1217 (2010) 8054.
[57] M.F. Almstetter, I.J. Appel, M.A. Gruber, C. Lottaz, B. Timischl, R. Spang, K. Dettmer, P.J. Oefner, Anal. Chem. 81 (2009) 5731.

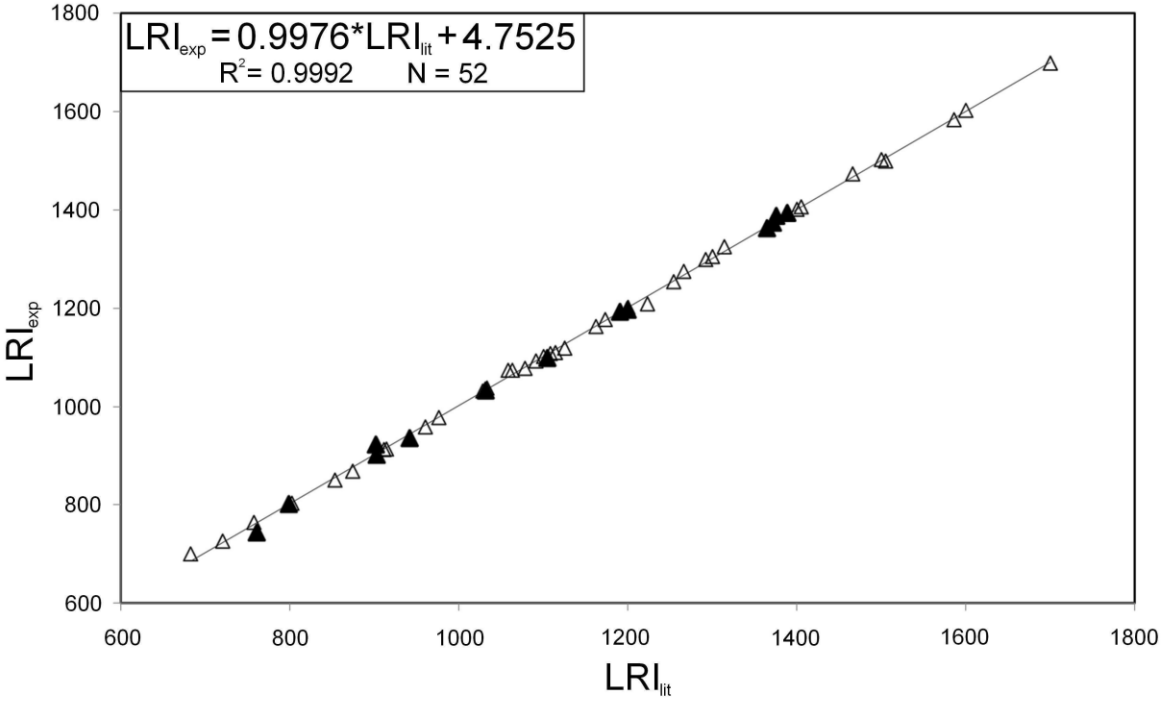

Figure S1: The relation between the linear retention indices calculated for the first dimension of the GCxGC/TOF-MS analysis and those reported in the literature for the DB-5 GC column or equivalents [36, 37]; the unfilled triangles correspond to the authentic standards.

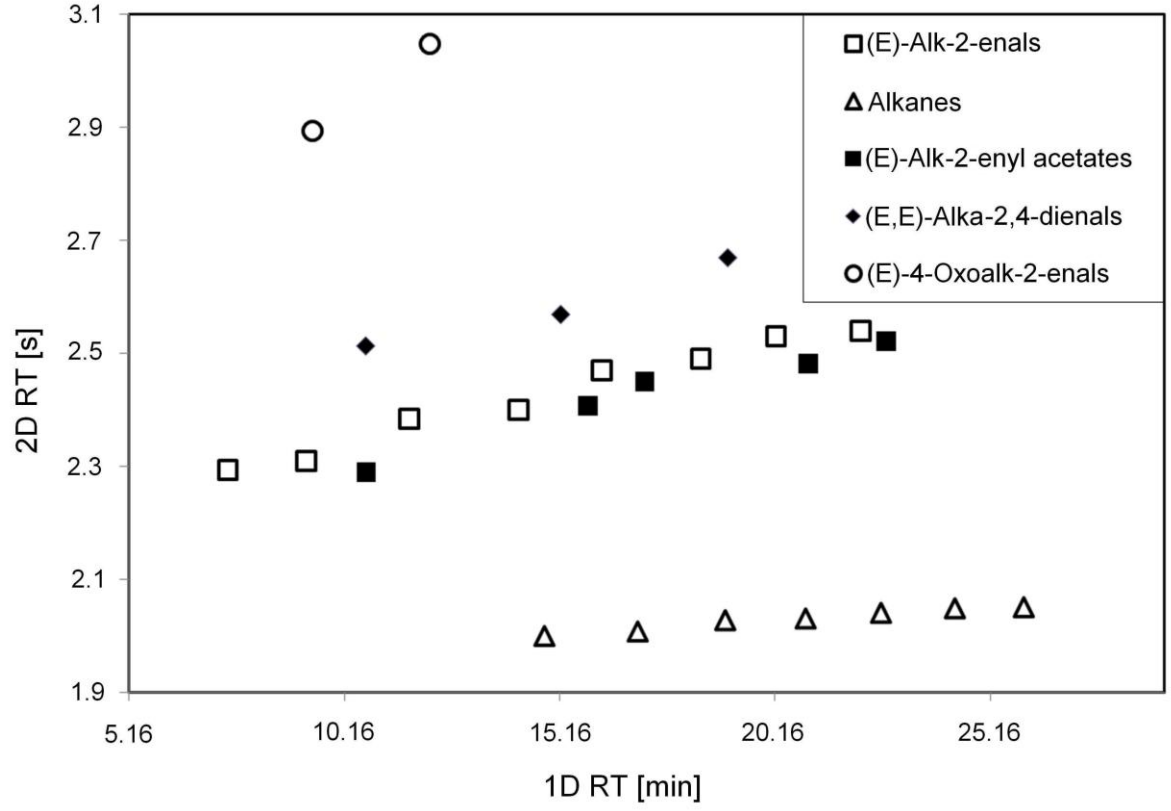

Figure S2: The apex plot of some compound types identified in the secretions of adult *G.lineatum* bugs.

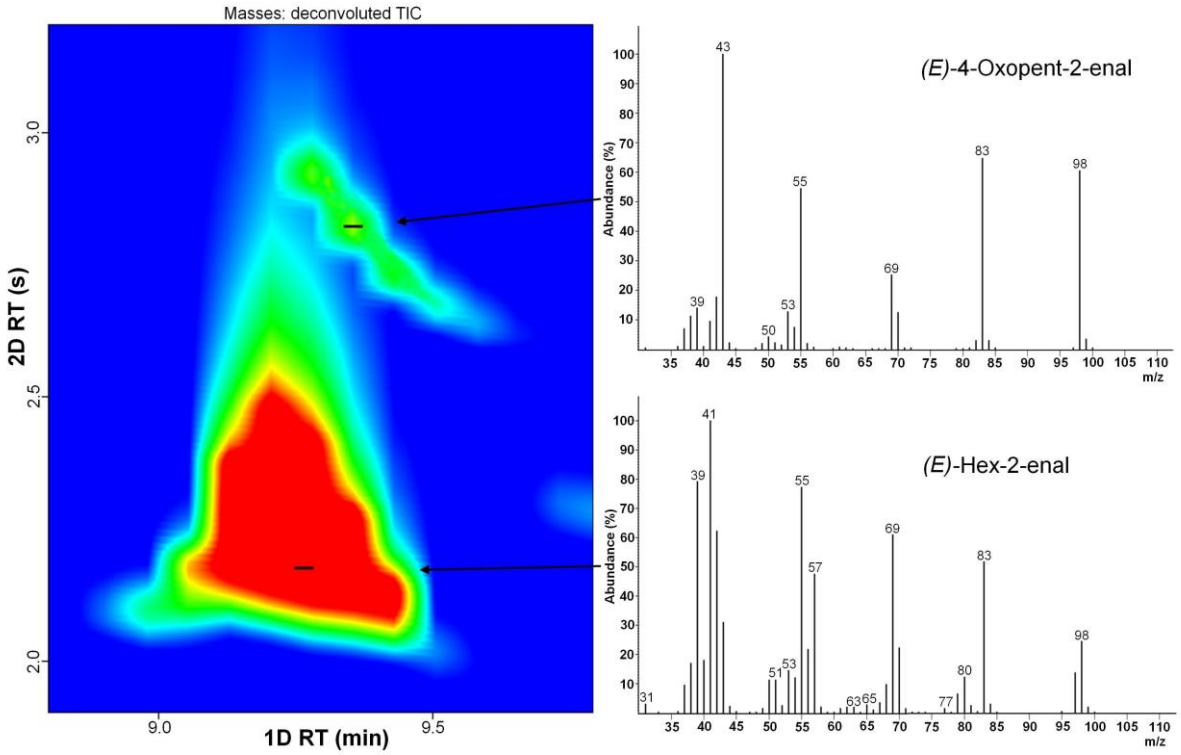

Figure S3: GCxGC/TOF-MS contour plot of the *(E)*-4-oxopent-2-enal and *(E)*-hex-2-enal mixture and corresponding mass spectra.

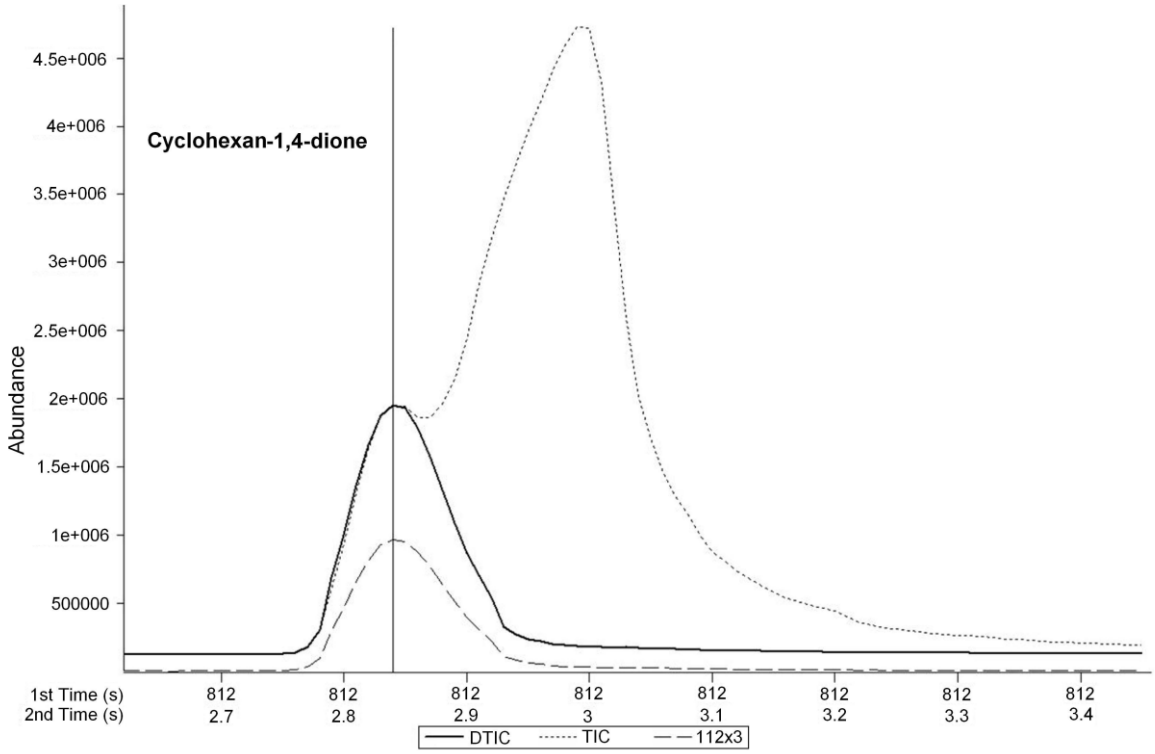

Figure S4: TIC and deconvoluted TIC (DTIC) chromatogram for cyclohexan-1,4-dione from *G. lineatum* volatile secretion; the real profile of the peak is obvious from the trace of its characteristic mass 112.