CHARLES UNIVERSITY IN PRAGUE
Faculty of Science
Department of Analytical Chemistry

ANALYSIS OF BUMBLEBEE LIPIDS USING CHROMATOGRAPHIC METHODS

Studium a stanovení lipidů čmeláků chromatografickými metodami

Diploma thesis
Study program Clinical and Toxicological Analysis

Prague 2010

Michaela Kudzejová

This diploma thesis was done in scope of the research plan MSM0021620857, grant of the Ministry of Education of the Czech Republic, No. 2B06007 and grant of Czech Science Foundation, No. 502/10/1734.

Proclamation

I confirm that this Diploma thesis is based on the work which I have done by myself under the supervision of Doc. RNDr. Irena Valterová, CSc. (Guarantee: Doc. RNDr. Zuzana Bosáková, CSc.) at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic and I have properly quoted all references used in this work.

I am conscious 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 in Prague, is possible only with a written consent of these institutions.

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Acknowledgements

I am deeply grateful to my supervisor Doc. RNDr. Irena Valterová, CSc for her invaluable support throughout this work and for her detailed and constructive advice and comments.

I wish to express my wish and sincere acknowledgement to Doc. RNDr. Zuzana Bosáková, CSc. for her significant help and support throughout all my studies.

I wish to extend my warmest thanks to all who have helped me with my work at the Institute of Organic Chemistry and Biochemistry AS CR in Prague, especially Mgr. Pavel Jiroš, Mgr. Robert Hanus, Ph.D. and Mgr. Aleš Tomčala, Ph.D. Big thanks belongs to all colleagues from the laboratory for their advice and creation of excellent working environment.

I owe my thanks to my parents, my partner and my closest friends for their loving support during my study, their encouragement and appreciation.

ABSTRACT

Lipids from the fat body of queens of *Bombus terrestris* species in different life stages were studied using chromatographic methods. High-performance liquid chromatography - mass spectrometry with electrospray ionization was used to analyze all lipids from the tissue. Semi-preparative thin-layer chromatography was used to isolate triacylglycerols (TGs) from the fat body tissue. The TGs were subsequently analyzed by high-performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry. Quantitative differences between different life stages have been found. Qualitative composition has not been changing significantly. Only minor differences have been found in the substances, which were present in amount less than 1%.

Key words:

Atmospheric pressure chemical ionization
LC-MS
Lipids
Triglycerides
Triacylglycerols
Bombus terrestris
Bumblebee queens
Fat body

ABSTRAKT

Lipidy z tukového tělesa matek čmeláka zemního (*Bombus terrestris*) v různých stádiích života byly studovány pomocí chromatografických metod. Vysokoúčinná kapalinová chromatografie s hmotnostním spektrometrem (LC-MS) s elektrosprejovou ionizací byla použita k analýze všech lipidů z tkáně. Semipreparativní tenkovrstvá chromatografie byla použita k izolaci triglyceridů (TG) z tkáně tukového tělesa. TG byly následně analyzovány pomocí LC-MS s chemickou ionizací za atmosférického tlaku. Byly nalezeny výrazné kvantitativní rozdíly ve složení lipidů matek v různých fázích života. Kvalitativní složení se výrazně neměnilo. Pouze malé rozdíly byly nalezeny u látek, které byly ve vzorcích přítomny v zastoupení menším než 1 %.

Klíčová slova:

Chemická ionizace za amosferického tlaku
LC-MS
Lipidy
Triacylglyceroly
Triglyceridy
Čmelák zemní
Bombus terrestris
Čmeláčí matky
Tukové těleso

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LIST OF ABBREVIATIONS

AKH	Adipokinetic hormone
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
B.	Bombus
c.	*circa*, approximately
cAMP	Cyclic adenosine monophosphate
CA	*corpora allata*
CN	Total number of carbons
corpora allata	Paired or fused ganglion-like bodies in the head of insects
DGs	Diacylglycerols
DG-BP	Diacylglycerol binding protein
DB	Total number of double bonds
de novo	Rising from the basic biosynthetic units
ECL	Equivalent chain length
ECN	Equivalent carbon number
e.g.	*exempli gratiā*, for example
ESI	Electrospray ionization
FA	Fatty acid
FAME	Fatty acid methyl ester
GC	Gas chromatography
HDLp	High-density lipophorin
HPLC	High-performance liquid chromatography
i.e.	*Id est,* that is
MALDI	Matrix assisted laser desorption/ionization
MS	Mass spectrometry
M	Mass
m/z	Mass to charge ratio
LDLp	Low density lipophorin
LSD	Least significant difference
PC	Phosphatidylcholine

PE	Phosphatidylethanolamine
PLs	Phospholipids
PS	Phosphatidylserine
RSD	Relative standard deviation
SD	Standard deviation
sn-	Stereospecific numbering
TGs	Triacylglycerols
TLC	Thin layer chromatography
UV	Ultra violet
VHDLp	Very high-density lipophorin

1. INTRODUCTION

The importance of bumblebees for the pollination of agricultural plants is well known. The seed production of some plants (e.g. clover) is fully dependent on bumblebees as specific pollinators. The role of bee pollinators in agriculture became more important with the development of the greenhouse plant growing. The presence of pollinators in greenhouses, where crops are grown more and more extensively, is crucial.

It was shown that mutual behavior between bumblebees is influenced by small, species-specific group of chemical compounds. The so called “semiochemicals” are responsible for information transfer between organisms; in the case of intraspecific interactions these substances are called “pheromones”. These are secreted into the environment by an individual of particular species and another individual of the same species responds to them by a change in behavior or physiological response[1]. A lot of research has been already focusing on finding out the chemical nature and mechanisms of action of pheromones in different organisms. Chemical structure is a heterogeneous and diverse set of compounds from different types of alkaloids and terpenoids *via* modified dipeptides, to the heterocycles or aliphatic unsaturated hydrocarbons[2]. On the contrary, much less explored area is the clarification of the biosynthesis of these compounds in a bumblebee body.

Research of the pheromone biogenesis rapidly increased only in the past few years. The first results showed a similarity between the composition of the fat body fatty acids and the structure of male bumblebee pheromones, from where the hypotheses that pool lipids are precursors of male marking pheromones. Similar hypothesis could be assumed about the queen and worker pheromones and hence originate the need of a more extended study of the composition of the fat in bumblebees.

1.1 Aims of thesis

The aim of this thesis was to analyze the quantitative and qualitative composition of triacylglycerols in different stages of bumblebees' queens of *Bombus terrestris*.

The qualitative study should tell us more about the biochemistry in bumblebee queens, involvement of lipids in the metabolism in this species and their use in biosynthesis of infochemicals such as hormones or pheromones.

The quantitative study will be performed in order to understand the mechanism of nutritional storage – which compounds are most involved in nutrition during different stages of bumblebee life cycle and the diapause.

Results of this research should give us a compact image of the fate of triacylglycerols in queens' body in this species.

2. THEORETICAL PART

2.1 Bumblebees (*Hymenoptera: Apidae: Bombini*)

Bumblebees are middle-sized (9 mm) to very large (30 mm) hairy bees. Their hairs are often arranged in color bands. There are known about 250 species in the world. Bumblebees for the most part occur in cool climates and are most abundant in Holarctic region, with many more species in Eurasia than in North America[3].

Bumblebees nest commonly in old rodent nests, left bird nests, cavities or under bunch of grass or other vegetation. Except for the social parasites, bumblebees form eusocial colonies[4].

2.2 Colony cycle

Colonies of bumblebees are usually annual. In temperate and arctic climate the start of the colony occurs in the spring[5]. Each colony is started by a single queen - solitary phase.

Her early progeny are all workers, usually much smaller than the queen, especially those of the first batch of eggs. They are morphologically very similar to the queen and they gradually adopt all the nest tasks as nest construction, food collecting, nursing and feeding of the brood and the queen reduces her duties to eggs laying and warming of the brood - social phase of the colony.

The males and young queens are produced later. They mate, the colony fades away and the young mated queens hibernate until the following spring, when the cycle restarts.

2.2.1. Solitary phase

Queens searching for nest site can easily be recognized because they alternate short, slow flight in small heights with periods of crawling into vegetation or cavities. When

she is successful, she starts to prepare wax pots for food storage (pollen and honey) and wax cells to lay eggs. The queen collects pollen and nectar from flowers and feed first batch of larvae – the first batch of workers. Larvae are fed progressively, either by food introduced through the tops of the cells (pollen storers) or by food pressed in through pockets at the base of the cells and forming the cell floors (pocket makers)[6]. Both methods may occur in the same species, usually at different seasons or for different castes. When mature, the larvae spin cocoons and the wax of the cell is removed for reuse, leaving the cocoons in clumps, each of that represents the young that originated in a single cell. The entire process from an egg to an adult bee can take up to five weeks, depending on the environmental conditions and the particular species. The cells do not form neat combs, nonetheless they tend to form irregular horizontal layers, for new cells are usually established on the tops of the old cocoons. The pots for both honey and pollen are often made of old cocoons.

2.2.2. Social phase

The social phase of the colony is characterized by two distinct phases of regulation of reproduction. During the first phase, the queen is the only reproductive active female laying diploid eggs and producing workers, while the following competition phase is characterized by reproductive competition between the queen and dominant egg laying workers[7]. At least before the competition phase, worker reproduction is inhibited by the presence of the queen[8]. The mechanism for inhibiting worker reproduction is to slow their ovarian development by suppressing the production of juvenile hormone, which is apparently involved in the regulation of ovarian development[8]. There is a tight temporal link between the onset of worker reproduction and queen and male production (the queen switches to laying haploid eggs); with the completion point predictably occurring c. 10 days after the queen lays the first diploid eggs that develop as new queens. This moment depends on different conditions: species, environmental factors, ratio workers / larvae and resource availability[9].

Males have just one function in their life: to mate. When the adult males emerge, they spend a few days in the nest, but they do not work. After 4-5 days, they leave the nest for good and forage for themselves. New queens emerge one week or so after

males. When a young queen is ready to mate, she flies to places where chemical attractants have been deposited by a male and waits for a suitable mate[10].

Mated young queens drink lots of nectar to build up their fat body and fill their honey stomach. This will enable them to survive the winter diapause. When fed enough, they find a suitable place to hibernate. As with many other animals that hibernate, it appears that bumblebees must reach a certain weight in order to survive the winter. For the largest bumblebee in the United Kingdom, *Bombus terrestris,* the queens must weigh at least 0.6 g to hibernate successfully and emerge next spring[5]. If the temperature drops below a certain point during the diapause, glycerol is automatically produced in the queen's body. This is a form of anti-freeze and prevents formation of ice crystals which would cause the fluids inside her body to expand and the body to burst.

The queen is the longest living caste in bumblebees, about 1 year, including 7-9 month of hibernation. This caste undergoes different phases of life - maturation and production of sexual pheromones, flight for mating and copulation, preparing for hibernation e.g. making of fat and sugar reserves, the own hibernation, the starting of her colony – nourishment of the first batch of workers, then inhibition of the sexual maturation of workers e.g. production of supposed inhibitory compounds and other compounds necessary for nest functioning, facing to workers (daughters) aggression. All that events require a lot of energy and different metabolic pathways which can be directly or indirectly connected with fat body and its metabolism.

2.3 Bumblebee fat body

The fat body is an organ analogous to vertebrate adipose tissue and liver. This organ develops especially in bumblebee queens who prepare themselves for the survival of the period of hibernation, since during the winter sleep it is the only source of storage nutrients[11]. Hemolymph proteins are synthesized there and glycogen is stored there as well[12]. In most of the insects, bumblebees included, the fat body constitutes of two different layers: a wall layer that adheres to walls of the body and visceral layer coating the alimentary tract and other internal organs[13]. Before diapause, queens eat as much as they can to enlarge their fat body and to accumulate food reserves. During the hibernation period, those reserves are used almost completely.

The amounts of reserves vary amount species, but in average, lipids are major components representing about 50% of dry weight of these reserves[14]. More than 90% of the pool lipids are triacylglycerols (TGs) [15]. They can be used for energy production through β-oxidation[16]. There are two ways how TGs appear in the bumblebee body: they can originate from dietary fats absorbed by midgut epithelium during feeding or they can be synthesized in fat body *de novo* from carbohydrates[17]. Beside the source of lipids, the key role of the fat body is in the basic metabolism. Fatty acids are known to be important precursors for many important substances such as eicosanoids, cuticular hydrocarbons, and pheromones[18]. The composition of bumblebee pheromones is known for many species, however, very little is known about metabolic pathway of their biosynthesis. Luxova and coworkers[19] showed a similarity in the chain length and position of double bond of the main components of male pheromones and fat body fatty acids hypothesizing that pool lipids may serve as precursors of pheromones. Palmitic, palmitoleic, stearic, and α-linoleic acids were found as most common FAs in bumblebees' male fat bodies[20]. The composition is strictly species-dependent, while the individual variation within a species is quite low. Fatty acids are also needed in substantial amounts for the synthesis of phospholipid waxes[21].

In addition to the role of storage and utilization of nutrients, fat body is an endocrine organ that produces several antimicrobial peptides and participates in detoxification of nitrogen metabolism[22].

2.4 Fat metabolism in insects

There are very few literary reports about fat metabolism in bumblebees, but the knowledge about the metabolism of insects in general can be sufficient to understand processes in bumblebees. Insects digest and absorb lipids similarly to vertebrates, but with some important differences. In many ways, fat metabolism in insects is less complex than in vertebrates. Thus, insects can serve us as a good model to understand basic principles of fat metabolism.

2.4.1. Absorption

Insects do not use bile salts to facilitate lipid solubilization. They convert the absorbed fatty acids and partial acylglycerols into intestinal DGs, TGs, and phospholipids. There are two possible ways how the synthesis can be effectuated: 1) acylation of 2-monoacylglycerol or 2) *de novo* pathway which includes acylation of *sn*-glycerol-3-phosphate.

The absorption of phospholipids and glycolipids has been much less studied. Glycolipids are hydrolyzed to form sugars and DGs, which are absorbed[23]. DGs can be consequently exported to the hemolymph or converted to TGs.

2.4.2. Transport

Lipids are exported from enterocytes by special lipoproteins named lipophorins, which are not produced *de novo* in the body[24]. They function as a reusable, non-internalized shuttle[25]. They circulate between midgut and fat body to pick up and deliver lipids to the target cells. The name of lipophorins is related to their density: low-density lipophorin (LDLp), high-density lipophorin (HDLp), and very high-density lipophorin (VHDLp). Very little is known about the mechanism of the transfer of lipophorin from the midgut to the hemolymph.

2.4.3. Storage in fat body

Males do not enter to diapause, contrary to females, who accumulate twice the lipid reserves of their non-diapausing counterparts. The storage in the fat body is done during the feeding period by collecting plant juices rich in carbohydrates, while the dietary fat is transferred from the midgut to the fat body. Moreover, TG storage can be a result of *de novo* biosynthesis in the fat body from carbohydrates[13].

2.4.4. Mobilization of lipids from fat body

In case of need, TGs can be used as a source of energy. It has been demonstrated[26] and confirmed[13] that fat body mobilizes lipids as DGs in insects. Mobilization of lipids

is induced by two types of hormones: adipokinetic hormone (AKH) and octopamine[13]. Octopamine has a role of a neurotransmitter that modulates the release of AKH from the *corpus cardiacum*[27]. If AKH binds to its receptor, it can result in the induction of several events that activate key enzymes in lipid and carbohydrate mobilization in fat body. It activates adenylate cyclase and mediates an increase of Ca^{2+} influx, giving rise to two intracellular messengers, Ca^{2+} and cAMP[28]. Lipase is activated through A-kinase cascade. This enzyme can transform TGs into 1, 2-diaclylglycerols (DGs) by hydrolysis. DG-binding protein (DG-BP) can transport DGs to the plasma membrane, where DGs leaves the cell and is added to high-density lipophorin (HDLp). This binding is catalyzed by the lipid transfer particle (LTP) that plays a similar role to microsomal triglyceride transfer protein in humans[29]. Low density lipoprotein (LDLp) is afterwards produced. This complex moves to the target cells; here DGs are unbound, hydrolyzed by lipoprotein lipase and FAs are liberated, so they can be used as a source of the energy. After delipidation, LDLp are converted back to HDLp so they can be reused in the cycle. The schema of this process is shown in Figure 1.

This system seems a bit complicated when comparing to vertebrates, who use free fatty acids as energy source. The reason is an open circulatory system of insects: Free fatty acids cannot be carried away by the blood flow, so they are rapidly taken back into the fat body and converted into TGs[30].

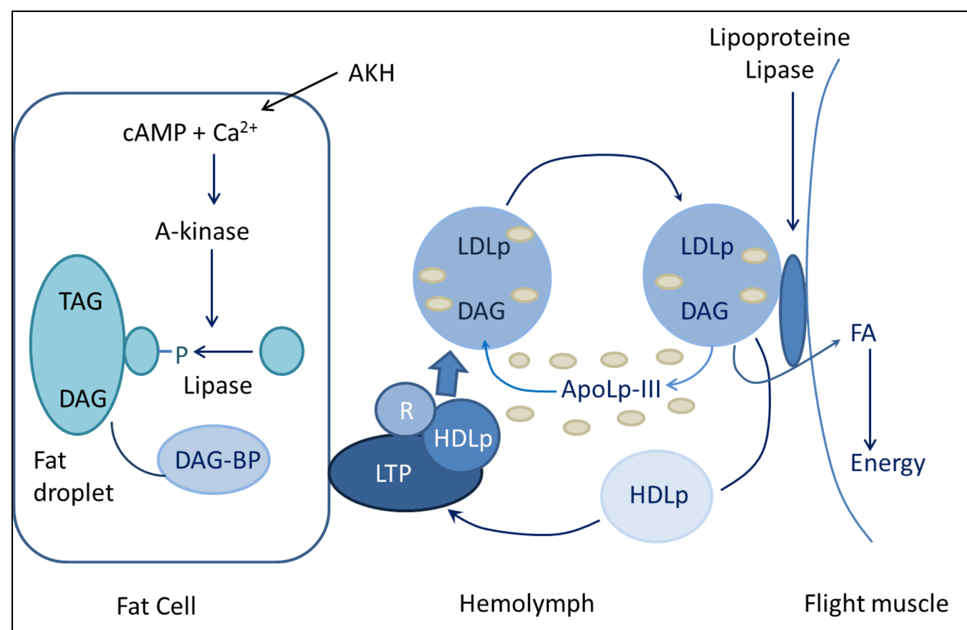

Figure 1 in accordance with reference 13: A model of mobilization of TG from fat body in insects

Bumblebee pheromones

Bumblebee pheromones can be classified by function into several groups.

The most important are the sexual pheromones and the queen's pheromone. The queen has many exocrine glands on her body that produce pheromones with different composition and function. Mandibular gland is the source of sexual pheromone of virgin females, which work on shorter distances. This pheromone serves to attract the males to mate and causes the copulatory behavior in males[31].

Mandibular gland was also reported as a source of queen's pheromone, which regulates the caste differentiation in colonies with complicated mechanism[32]. If the status of a bumblebee queen is dominant enough, queens produce a signal that inhibits the activity of workers' *corpora allata* (CA), resulting in a low juvenile hormone titers and thus in restricted ovarian development and inhibition of egg laying[33]. Once the queen loses control over her workers or when the queen dies, the CA of a few dominant workers are activated and this allows them to lay eggs and to inhibit the CA of other workers[32]. About 500 substances were identified in the secretion of exocrine gland of *Bombus terrestris*[34]. Those were mainly aliphatic substances, major components were (3*S*)-hydroxydecanoic acid, (*E*)-octadec-9-enoic acid and octadecanoic acid. A later study reevaluated the role of the mandibular gland in workers' ovary inhibition and the location of the signal source in the mandibular gland has not been proven[35].

Defense substances are produced by all castes of bumblebees in case of danger. These substances are produced by mandibular gland and their aim is to chase away the enemy[36].

The caste pheromone serves to distinguish between workers and queens. In *Bombus terrestris* species, the queen secretes a pheromone, which, if present within the first 5 days of larval life, determines that the larva will develop into a worker. If the pheromone is absent, and if the larva receives sufficient food (more than a larva predetermined to become a worker) during her final instar, she will develop into a queen. This pheromone is produced by Dufour gland of workers[37].

Trail pheromone serves to workers to mark the nest in the land cavities and they are specific for a species and a particular colony[38]. They are secreted by Dufour gland in the abdomen of workers.

The marking pheromone of bumblebee male is produced by the cephalic part of the labial gland[39]. Chemical compositions of these pheromones have been examined extensively in the past[40]. These pheromones serve to males to mark their territory. Chemical composition of the marking secretion is species specific and it helps to avoid hybridization between species[41].

2.5 *Bombus terrestris*

Bombus terrestris or buff-tailed bumblebee is one of the most frequent species of bumblebees in Europe. The queen is 2–2.7 cm long, while the workers are 1.5–2 cm (Figure 2). The latter are characterized by their white-ended abdomens. The life cycle of this species is very similar to the general life cycle described in the part above.

B. terrestris is thought to be a mainly singly-mating species. This is unusual for social insect queens where mating with several males (polyandry) has been shown to have several benefits. The lack of multiple mating by *B. terrestris* queens may be caused by male interference in the process. *B. terrestris* males plug the female's sexual tract with a sticky secretion during mating which appears to temporarily reduce the female's ability to successfully mate with other males for several days[42].

Figure 2: *Bombus terrestris* worker (left) and queen (right) with cocoons.

Triacylglycerols

Triacylglycerol (triglyceride, triacylglyceride, or TG) is an ester composed of glycerol and bound to three fatty acids[43]. They are the most abundant lipids in nature and they are known as a main component of animal fats and vegetable oils. Their main role is in serving an energy depot; they play an important role in metabolism and in biosynthesis of different substances[44]. Each TG consists of three fatty acids (FAs) esterified to glycerol; each of the position in glycerol may be occupied by a different FA (Figure 3).

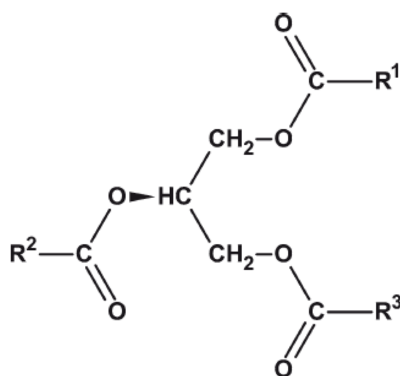

Figure 3: Structure of TG.

The molecular composition of triacylglycerol mixtures is typically very complex due to a combination of variety of fatty acids, differing in their chain-length, degree of unsaturation and distribution between the *sn*-1 (stereospecific numbering), *sn*-2, and *sn*-3 positions of the glycerol backbone.

The most common fatty acids residues are listed in Table 1 (next page).

Table 1: Most common fatty acids in natural compounds, CN represents total number of carbon atoms, DB is total number of double bonds.

Name of acid	Chemical Structure	CN:DB	Abbreviation
Unsaturated Fatty Acids			
Myristoleic acid	$CH_3(CH2)_3CH=CH(CH_2)_7COOH$	14:1	Mo
Palmitoleic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	16:1	Po
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	18:1	O
Linoleic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	18:2	L
α-Linolenic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	18:3	Ln
Saturated Fatty Acids			
Lauric acid	$CH_3(CH_2)_{10}COOH$	12:0	La
Myristic acid	$CH_3(CH_2)_{12}COOH$	14:0	M
Palmitic acid	$CH_3(CH_2)_{14}COOH$	16:0	P
Stearic acid	$CH_3(CH_2)_{16}COOH$	18:0	S
Arachidic acid	$CH_3(CH_2)_{18}COOH$	20:0	A

2.6 TGs analysis

Many methods to study TGs have been developed. Gas chromatography (GC) is one of the TGs analysis methods spread mostly among biologists. When using this method, the intact TGs can be directly applied to GC[45]or they can be converted to volatile derivatives of FAs, most frequently to methyl esters (FAMEs)[20, 55]. FAMEs differing in chain length, double bond position, and configuration can be distinguished and identified based on their parameters, e.g., equivalent chain length (ECL) values[43, 46]. This method is well established. Methods for determination of the double bond position have been published[47].

Although this method is still very popular within the community of biologists, it fails in determination of how the FA is bound to glycerol backbone. This problem can be solved by using high-performance liquid chromatography.

2.7 High-performance liquid chromatography-mass spectrometry

High-performance liquid chromatography (HPLC) is used nowadays as one of the most efficient methods to study TGs, especially when connected with mass spectrometry. Two HPLC systems were shown to separate TGs quite efficiently: 1) non aqueous reversed-phase chromatography on octadecyl modified silica, 2) argentation chromatography using stationary phases with silver ions.

Reversed-phase HPLC is the most commonly used chromatographic technique for TGs analysis[48]. The separation is based on both molecular size and the degree of unsaturation of molecules. The molecules elute in ascending order of equivalent carbon number ECN (ECN=CN-2DB), CN is a total number of carbon atoms, DB represents total number of double bonds; the retention on the stationary phase is reduced by each of the double bonds.

Non-aqueous reversed-phase HPLC on octadecyl modified silica has been used to study TGs of natural origin[49]. Acetonitrile is mostly used as a mobile phase, usually mixed with other solvent, which can modify the solubility of TGs and improves the selectivity and efficiency of the separation. The retention behavior of TGs depends on the total number of carbon atoms (CN) and double bonds in fatty acids. The retention order obeys usually the ECN, nevertheless, minor exceptions exist.

The retention mechanism of argentation (or silver ion) chromatography is completely different[50]. Silver ion chromatography is based on the distinctive property of unsaturated organic compounds to complex with transition metals in general and silver in particular. Silver ions react with π-electrons of double bond in FA residues and reversibly form polar complexes. The lipids are separated according to the number, configuration, and position of the double bonds within FA residues and to the regiospecific distribution of FAs chains.

There are many possibilities how to detect TGs: flame ionization, UV[51], evaporative light-scattering[52], or mass spectrometric detections. The last mentioned seems to be the best to use because it provides conclusive information about identity of TGs.

2.8 HPLC ionization techniques

The ionization of TGs can be achieved using ion sources: e.g., electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) [53,54,55] and matrix-assisted laser desorption/ionization MS[56,57]. All of those techniques produce protonated molecules $[M+H]^+$ or other molecular adducts, for example $[M+NH_4]^+$. APCI, APPI and MALDI technique produce also molecular fragments. APCI technique does not yield $[M + H]^+$ for saturated TGs.

2.8.1. Electrospray ionization

ESI is a method based on ion formation in solution. The liquid containing the analyte(s) of interest is dispersed by electrospray into a fine aerosol. Usually, ionic species such as ammonium or sodium ions are added to the eluent system to facilitate ionization, because non-polar triacylglycerols are not in ionic form in typical HPLC conditions. Typically, ESI of TGs yields $[M+NH_4]^+$ and $[M+Na]^+$ ions without fragmentation[46]. Some types of analyzers (e.g. triple quadrupole instruments with applying collision-induced-dissociation of $[M+NH_4]^+$ iont or ion trap) can give us additional information about the lipid structure.

2.8.2. Matrix assisted laser desorption /ionization

MALDI is a soft ionization technique that employs the use of UV lasers. The laser is fired at the crystals in the MALDI spot, where the energy is absorbed by the matrix and causes the primary ionization of the matrix. Part of this charge is consequently transferred to the molecule of the analyte. Together, they form a protonated molecule, for example $[M+H]^+$ in the case of an added proton, $[M+Na]^+$ in the case of an added sodium ion, or $[M-H]^-$ in the case of a removed proton. The study[58] confirms that MALDI-MS is suitable for cases when only a pattern of the TGs composition is

important in large set of samples, when there's no need to analyze the detailed structure of TGs. The advantage of this method is short time of analysis and data-evaluation.

2.9 APCI ionization

APCI is a gas-phase ionization process also suitable for the ionization of non-polar molecules. It was shown[59] that HPLC method provides excellent chromatographic resolution and allows the analysis of high-molecular weight TGs. This technique can represent a useful tool for studying insect lipids.

In APCI, the HPLC effluent is nebulized into a tube heated to 300 to 400 °C in order to volatilize the solvent. The droplets and flowing gases that leave the heated tube are exposed to a corona discharge. This is generated from a sharp needle point held at high potential. Plasma of protons, ionic species or mobile electrons is present in the corona discharge plume, which effect ionization of lipid by an ion-molecule reaction. Ions are then swept into mass spectrometer by way of a skimmer-nozzle or a heated capillary. This method can tolerate relatively high HPLC flow rates up to 1 ml/min[60]; in some cases even 2 ml/min

HPLC/APCI-MS combines the properties of separation power of chromatography and mass spectrometry, which allows obtaining extended information about the composition of TG mixture[56]. The disadvantage of this method lies in time-consuming data-interpretation, especially when a large number of samples is to be analyzed.

To summarize, the HPLC/APCI-MS is a method of choice when conclusions from exact composition of TGs are to be drawn[56] (chemotaxonomy, the connections of lipids and secondary metabolites etc.).

3. EXPERIMENTAL PART

3.1 Materials

Chloroform, p.a	Penta (Chrudim, Czech republic)
Methanol, p.a	Penta (Chrudim, Czech republic)
Diethylether, p.a	Penta (Chrudim, Czech republic)
Hexane for residue analysis	Merck (Darmstadt, Germany)
Formic acid, 98%	Lachema (Brno, Czech republic)
Rhodamine 6G solution 0.05% in ethanol	Lachema (Brno, Czech republic)
4-hydroxy-3-methoxybenzaldehyde (vanilin) 98%	Sigma-Aldrich (St. Louis, MO, USA)
Acetonitrile-Chromasolv® for HPLC, gradient grade	Sigma-Aldrich (St. Louis, MO, USA)
Propan-2-ol for HPLC	Lab-Scan (Gliwice, Poland)
Ammonium acetate, p.a	Fluka Chemie AG (Buchs, Switzerland)
Standard Triolein	Sigma-Aldrich (St. Louis, MO, USA)
Silica Gel for TLC 60G	Merck (Darmstadt, Germany)

Solvents used for chromatography were distilled prior analyses.

3.2 Biological material

Bumblebees of *Bombus terrestris* species were obtained from the laboratory rearing of Faculty of Science of Masaryk University in Brno. Colonies were established using a cascade method of two queens to simulate oviposition in laboratory conditions[61].

Queens in the following life stages were studied: pharate queens, queens after eclosion, before hibernation, after hibernation, egg-laying queens with first cell, and old queens. In this work, the term “pharate queen” stands for the queen that is still in cocoon, before she chews out of it. The term “queen after eclosion” represents queen that has already left the cocoon.

3.3 Instruments and methods for TG analysis

3.3.1. High-performance liquid chromatograph with mass spectrometer detection

Separation of TGs was performed on liquid chromatograph system consisting of an SMC 1000 vacuum membrane degasser, a P 4000 gradient pump, an SN 4000 control unit (all instruments from Thermo Separation Product, San Jose, CA, USA). Manual injection was accomplished with a Rheodyne-type model D injection valve (Ecorn, Prague, Czech Republic) equipped with 5 µl internal injection loop. TGs were separated on two stainless steel columns Nova-Pak® C18 (300×3.9 mm and 150×3.9 mm, 4 µm, Waters, Milford, MA, USA) connected in series. The columns were tempered in a thermostat, RT04 (Labio, Prague, Czech Republic). Mass spectrometer LCQ Fleet (Thermo Fisher Scientific, USA) with 3D ion trap and APCI was used for the detection. Data collection and evaluation of chromatograms were performed in the Xcalibur program, version 2.0.7 (Thermo Fisher Scientific, USA) and TriglyAPCI program, version 1.5.1 (Josef Cvačka, Ph.D., reference 66).

3.3.2. Sample preparation

Queen bumblebees of *Bombus terrestris* species of different stages were used in this study: 17 pharate queens, 27 queens after eclosion, 20 queens before hibernation, 19 queens after hibernation, 19 queens that have laid first egg and 12 old queens.

Bumblebees were immobilized in cold and their fat bodies were dissected. The fat body tissue was transferred into a glass vial, crushed using a glass stick and suspended in $CHCl_3/CH_3OH$ (1:1 v/v; 7 ml). The sample was sonicated for 5 minutes, then filtered over glass wool and washed with 4×200 µl ether. The solvent was evaporated to dry and the weight of total lipids was determined.

3.3.3. Separation of TGs

The crude extract was separated on pre-cleaned TLC glass plates of different sizes coated with silica gel (Silica Gel 60G, Merck) with gypsum (12%) using hexane/diethyl ether/formic acid (80:20:1, v/v/v) mobile phase. TLC zones were made visible by spraying Rhodamine 6G solution (0.05% in ethanol) and UV light (366 nm). TGs were the most intensive zone. The position of TGs on the TLC plate was also determined experimentally using a plate with standard of TGs (R_F = 0.6) – triolein. The visualization of standard on the analytical plate has been performed in destructing manner by spraying 0.01% vanillin in sulfuric acid (w/v) and subsequent incineration.

The band containing TGs was scraped off the plate and extracted with diethyl ether (7 ml). The solvent was evaporated under argon flow and the residue was transferred into an ampoule by washing with 4×200 µl $CHCl_3$. The solvent was then dried and the weight of the pure TG of each sample was determined. The TGs were reconstituted in $CHCl_3$ to concentration of 1% and stored in sealed glass ampoules in argon at -18 °C until analyzed.

3.3.4. High performance liquid chromatography with mass spectrometry

Table 2 shows parameters of the analytic program used for TGs analysis.

Table 2: HPLC program used for TG analysis.

Mobile phase:		Acetonitrile (A) and propan-2-ol (B)	
Volume of injected sample		5 µl	
Temperature of columns:		30 °C	
Temperature of APCI source		400 °C	
Temperature of heated capillary		200 °C	
Corona discharge current		4.5 µA	
Time	Flow (ml/min)	A (%)	B (%)
0	1	100	0
108	1	30	70
122	0.7	20	80
128	0.7	100	0
130	1	100	0

The mobile phase was mixed post column in a low-dead volume T-piece with 100 mM ammonium acetate prepared in propan-2-ol/water 1:1 (v/v), flow rate 10 µL min^{-1}. The full scan mass spectra were recorded in the range of *m/z* 75-1300. TGs were quantified from reconstructed chromatograms calculated for $[M+H]^+$ and $[M+MN_4]^+$.

3.4 Instruments and methods for analysis of all lipids

HPLC combined with electrospray ionization mass-spectrometry (ESI-MS) was used to analyze the composition of all lipids isolated from fat body[62]. 1 sample of each stage has been randomly chosen for this purpose.

The analysis has been performed on LCQ Fleet (Thermo Fisher Scientific, USA) mass spectrometer with 3D ion trap, coupled to a HPLC system, equipped with autosampler SpectraSYSTEM® AS3000 (Thermo Fischer Scientific, USA) and thermostat, RT04 (Labio, Prague, Czech Republic). Lipids were separated on Gemini HPLC column (150×2.0 mm, 3 µm, Phenomenex, Torrance, CA, USA). The mobile phase was composed of (A) 5 mmol l^{-1} ammonium acetate in methanol, (B) water and (C) propan-2-ol. A linear gradient of A:B:C changing from 92:8:0 to 50:0:50 within 80 min was used with a flow rate of 150 µl min^{-1}. The column temperature was maintained at 30 °C. The mass spectrometer was operated in the positive ion detection mode at +4 kV with capillary temperature at 220 °C. Nitrogen was used as both the shielding and the auxiliary gas. Mass range 440 – 1100 Da were scanned every 0.5 s to obtain the ESI mass spectra of the respective DGs and TGs. For the investigation of the DG and TG structures the CID (collisional - induced decomposition) multi-stage ion trap tandem spectra MS^2 were recorded with respective 5 and 3 Da isolation windows. Maximum ion injection time was 100 ms and collision energies were 30% (MS^2).

3.5 Statistical analysis

The analysis of variance (ANOVA) has been used to compare the mean abundances of TGs relative to total lipid content in various life stages of the queen. To test the prerequisite for the use of ANOVA, i.e. the equal variances of all sample categories (life stages), the Levene's test for homogeneity of variances has been used.

To evaluate the contribution of particular categories to this inequality of means, the Post hoc comparison using the Fisher's least significant difference test (LSD) has been used.

The main idea of the LSD is to compute the smallest insignificant difference between two means as if the means had been the only means to be compared and to declare significant any difference larger than the LSD[63]. The condition to use LSD technique is that the value of the *t* statistics evaluating the differences between Groups *a* and *a'* is equal to equation 4.1 and follows a student's *t* distribution with $N - 1$ degrees of freedom.

$$t = \frac{M_{a+} - M_{a'+}}{\sqrt{MS_{S(A)}\left(\frac{1}{S_a} + \frac{1}{S_{a'}}\right)}} \tag{4.1}$$

where *a* is a given group, S_a number of observation of the *a*-th group, $S_{a'}$ number of observation of the *a'*-th group, *N*, total number of observation, M_{a+} the mean of a group *a*, $M_{a'+}$ the mean of a group *a'*, $MS_{S(A)}$ the mean square of error from the ANOVA.

The ratio *t* would therefore be declared significant at a given α level if the value of *t* is larger than at the critical value for the α level obtained from the *t* distribution and denoted $t_{\alpha\nu}$ (where $\nu = N - A$ is the number of degrees of freedom of the error).

If this ratio is rewritten, a difference between the means of Group *a* and *a'* will be significant if

$$\left|M_{a+} - M_{a'+}\right| > LSD = t_{\nu,\alpha}\sqrt{MS_{S(A)}\left(\frac{1}{S_a} + \frac{1}{S_{a'}}\right)} \tag{4.2}$$

where M_{a+} is the mean of a group *a*, $M_{a'+}$ the mean of a group *a'*, *t* student's distribution, $MS_{S(A)}$ the mean square of error from the ANOVA, S_a number of observation of the *a*-th group, $S_{a'}$ number of observation of the *a'*-th group.

In order to evaluate the difference between the means of Groups a and a', the absolute value of the difference between the means is taken and compared to the value of LSD. If

$$\left| M_{i+} + M_{j+} \right| \geq LSD \tag{4.3}$$

then the comparison is chosen significant at the chosen α level. The procedure is repeated for all $\frac{A(A-1)}{2}$ comparisons[63].

3.6 Principal component analysis

To visualize the chemical diversity in the TG composition of the fat body within and among particular life stages of *B. terrestris* queens, the principal component analysis (PCA) has been used. PCA is a mathematical procedure that serves to decorrelate the data. It is based on the transformation of a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components[64].

4. RESULTS AND DISCUSSION

4.1 Lipid extraction from biological material

All lipids were isolated from the fat body according to the procedure described in the part 3.4.1. The weight of all lipids was determined for each sample. Table 3 shows the means of weights of all lipids of each life stage and standard deviations. The results prove that the amount of total lipids is highest before hibernation, when bumblebee queens build up lipid stores for winter hibernation, and lowest amount is after winter, when the lipids stores have been consumed.

Table 3: Comparison of means of weights of total lipids in different life stages of *Bombus terrestris* queens.

Stage	*N* [number of samples]	Total lipids weight, mean [mg]	Standard deviation [mg]
Pharate queens	17	20.5	4.7
Queens after eclosion	24	11.1	1.9
Queens before hibernation	20	28.8	5.1
Queens after hibernation	19	4.8	4.0
Egg-laying queens with first cell	19	7.1	2.0
Old queens	12	11.3	4.9

4.2 Separation of TGs

Five samples of each stage with the highest weight of all lipids were chosen for the consecutive work. Thin layer chromatography was used to separate the TGs from other lipids (applied method has been described in part 3.4.2). Figure 4 shows the scan of two analytical plates used for determination of position of TGs. As there were used different sizes of wide separation plates, two sizes of analytical plates had to be used as well (Figure 4). The size of the wide plate was chosen with regard to the weight of the all lipids isolated from the fat body. The position of the TGs on the analytical plate is at the same place as the spot of the standard to the right of the plate. The position and the boarders of TGs part on the wide plate were reconfirmed by spraying Rhodamine 6G solution 0.05% in ethanol and consequent visualization in UV light (Figure 5).

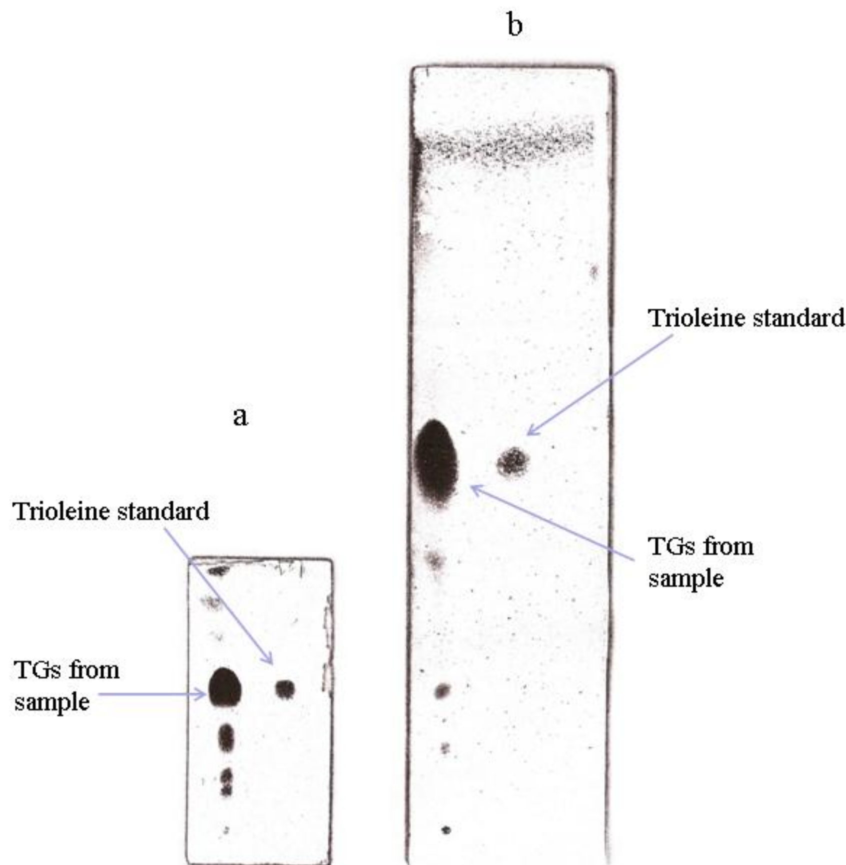

Figure 4: TLC plates after separation of TGs of the sample and the standard of trioleine; mobile phase: hexane:diethyl ether:formic acid (80:20:1, v/v/v), visualization: 0.01% vanillin in sulfuric acid (w/v), incineration. Small plates (a) were used for samples with small weight (e.g., queens after hibernation), large ones (b) for samples with higher weight (e.g., queens before hibernation).

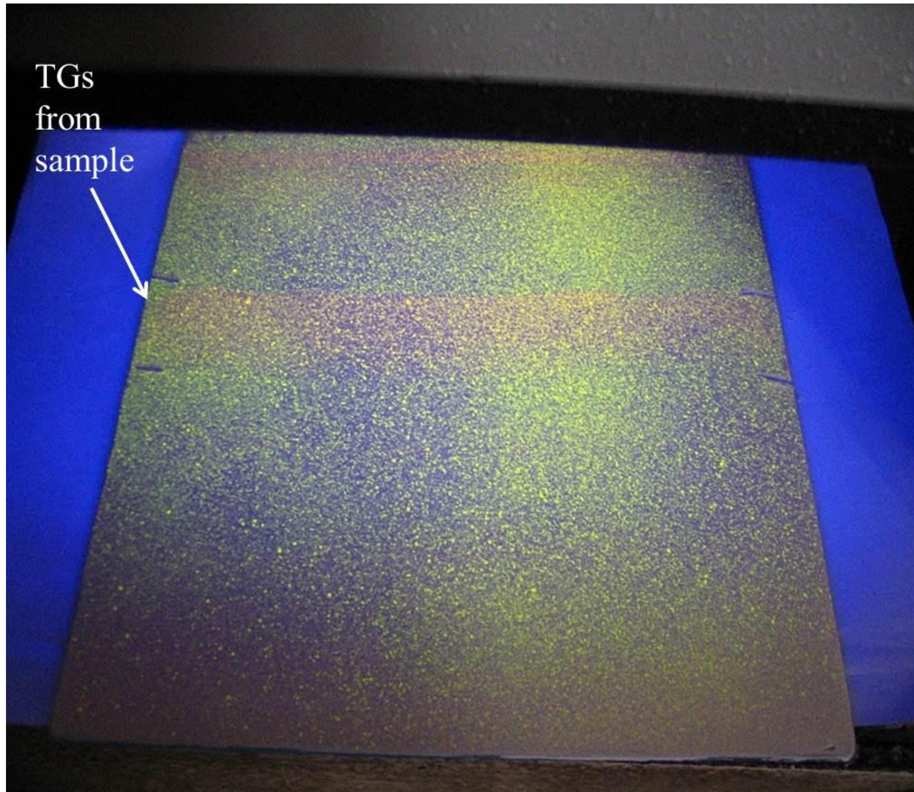

Figure 5: TLC wide plate after separation of TGs of sample. Mobile phase: hexane:diethyl ether:formic acid (80:20:1, v/v/v), visualization: Rhodamine 6G solution 0.05% in ethanol; observation under UV lamp at 366 nm.

TGs from each sample were isolated and weighed. Table 4 shows the comparison of the means of weight of TGs of different stages and Figure 6 shows the comparison of the means of weights of both, total lipids and TGs, in various life stages of *B. terrestris* queens and their standard deviations.

Table 4: Comparison of the TGs weights in different life stages of *B. terrestris* queens.

Stage	*N* [number of samples]	TGs weight, mean [mg]	Standard deviation [mg]
Pharate queens	5	17.6	1.7
Queens after eclosion	6	8.8	0.5
Queens before hibernation	5	20.1	3.9
Queens after hibernation	6	4.5	4.2
Egg-laying queens with first cell	5	1.3	0.6
Old queens	7	2.8	3.8

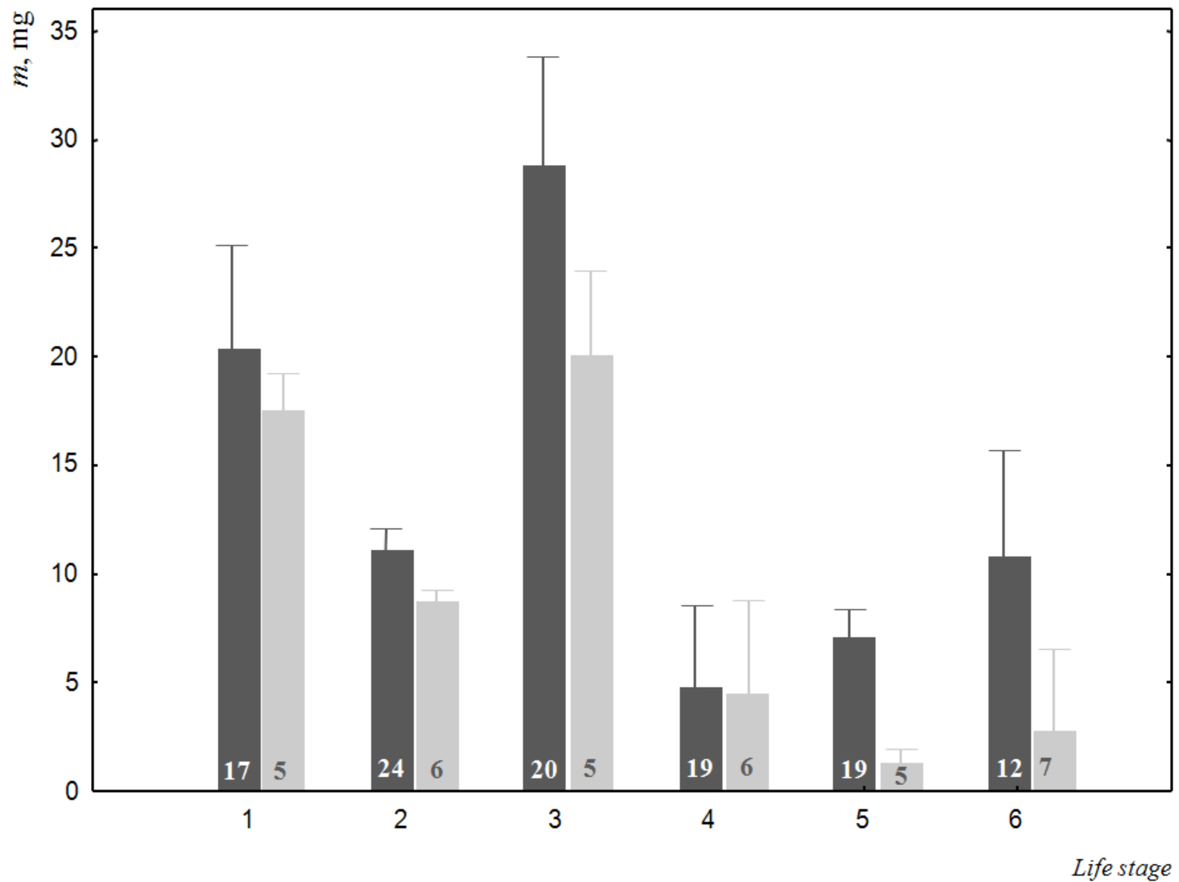

Figure 6: Comparison of means of weights of total lipids isolated from fat body and means of weights of separated TGs and their standard deviations in various life stages of *B. terrestris* queens: (1) pharate queens, (2) queens after eclosion, (3) queens before hibernation, (4) queens after hibernation, (5) egg-laying queens with first cell, (6) old queens; the number at the bottom of each column represents the number of samples (N).

The ratio of weights of separated TGs to total lipids isolated from fat body has been calculated. We can observe that in the first four stages TGs form 60 to 75% of all lipids in the fat body, contrary to the two last stages (egg-laying queens and old queens) where TGs form approximately 20% of all lipids.

4.2.1. Comparison of weights - statistical evaluation

The Levene's test for homogeneity of variances revealed to be non-significant at the level $\alpha = 0.05$, what allowed to perform the ANOVA test.

The ANOVA test proved to be significant with $F = 23.7$ and $p < 10^{-3}$, rejecting thus the null hypotheses of equal means for all categories (life stages).

In the subsequent Post Hoc Fischer LSD test, the means of the first three stages (pharate queens, queens after eclosion and queens before hibernation) were not found to be significantly different (Figure 7). First and third stages are also similar to the fourth stage (after hibernation), but the means of the stages 2 and 4 differ significantly. The differences of pairwise comparisons between the fifth (egg-laying queens) and sixth (old queens) stages to all other stages were found to be significant.

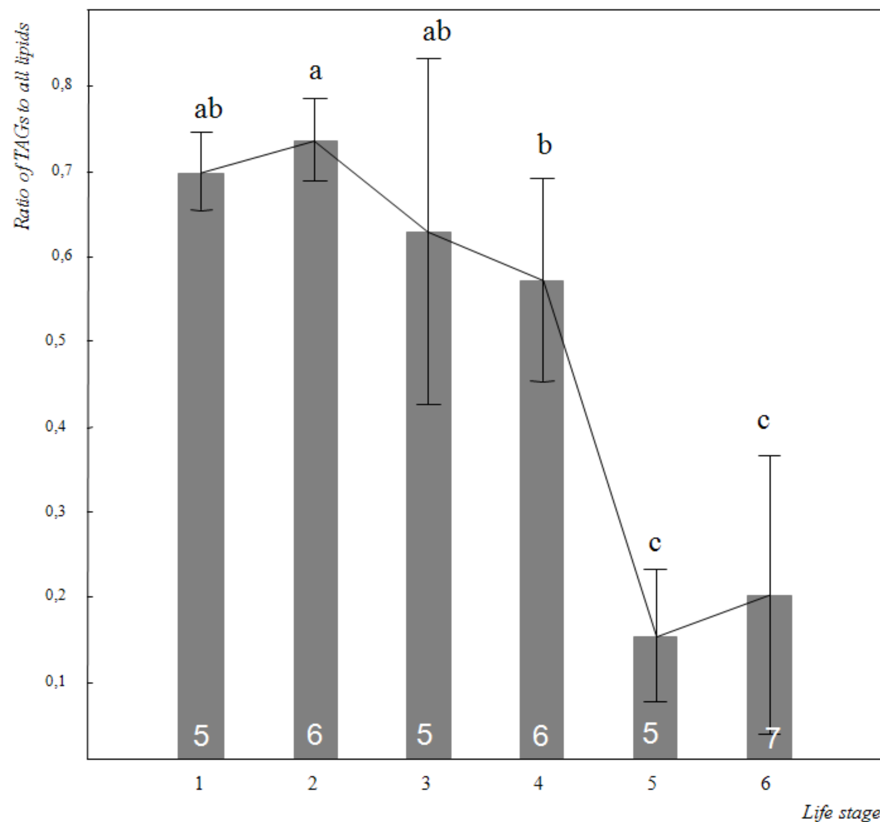

Figure 7: Comparison of ratios of TGs to total lipids, in various life stages of *B. terrestris* queens: (1) pharate queens, (2) queens after eclosion, (3) queens before hibernation, (4) queens after hibernation, (5) egg-laying queens with first cell, (6) old queens; the number at the bottom of each column represents the number of samples (*N*); The results of Post Hoc comparison by means of the Fisher Least Significant Difference test (LSD) are indicated by the letters above the columns – the means marked with different letters are significantly different from each other.

4.3 High-performance liquid chromatography with mass detection for TGs

All samples with isolated TGs have been analyzed on HPLC-MS as described in part 3.4.3. The differences in composition between particular stages were already apparent from the chromatograms (Figure 8a and 8b), where one chosen sample of each stage is shown. Each stage shows a different chromatographic profile; first and second stage are very similar, other stages differ among themselves. Base peaks are described with abbreviations explained in part 2.7. Afterwards, the qualitative analyze has been effectuated in order to know the exact composition of samples.

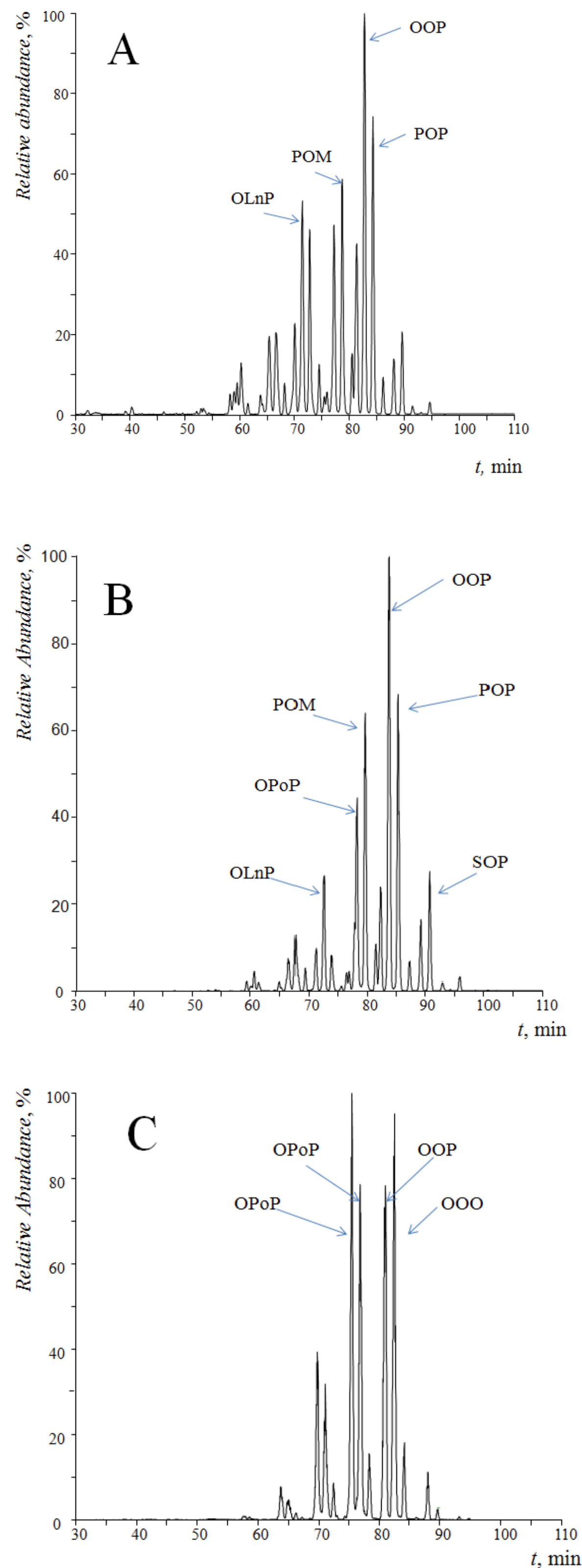

Figure 8a: Base peak chromatograms of TGs isolated from fat body of *B.terrestris* queens in various life stages: (A) pharate queen, (B) queen after eclosion, (C) queen before hibernation. TG abbreviations as well as chromatographic conditions are explained in parts 2.7 and 3.4.3.

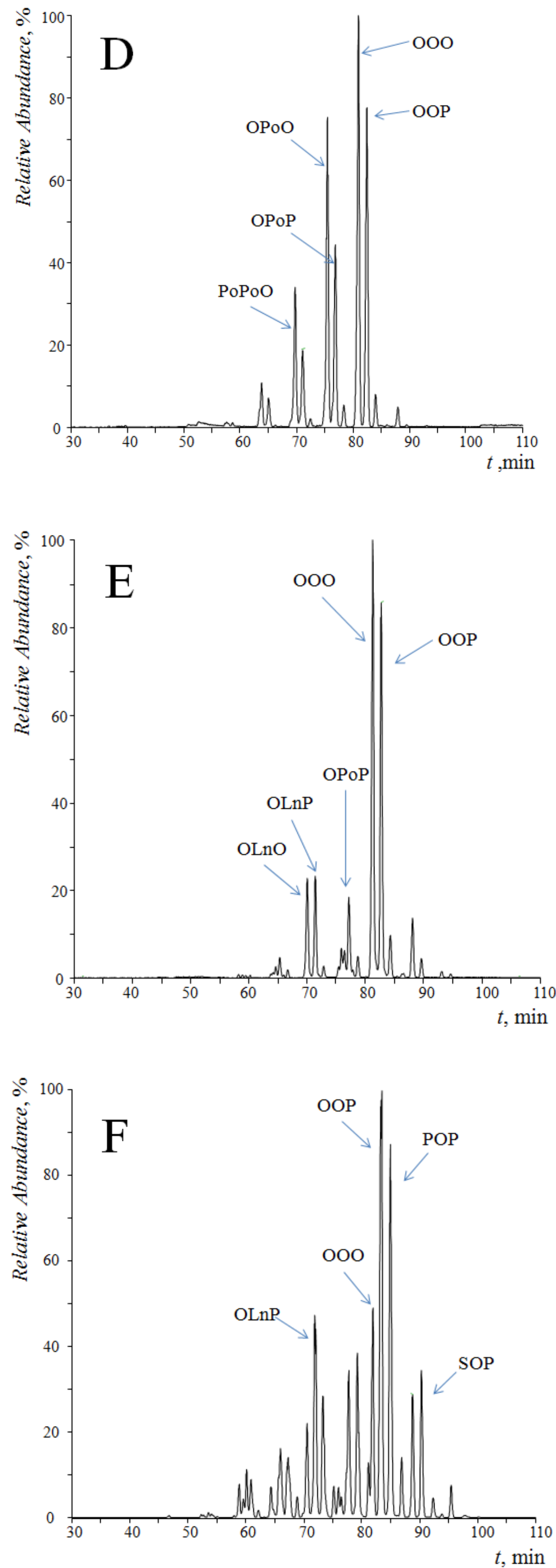

Figure 8b: Base peak chromatograms of TGs isolated from fat body of *B.terrestris* queens in various life stages: (D) queen after hibernation, (E) egg-laying queen with first cell, (F) old queen. TG abbreviations as well as chromatographic conditions are explained in parts 2.7 and 3.4.3.

4.4 APCI spectra interpretation

As saturated TGs do not show protonated molecular ions in their APCI spectra, ammonium acetate has been added to promote formation of $[M+NH_4]^+$ ions. The main ions in the APCI spectra are pairs of $[M+H]^+$ and $[M+MH_4]^+$ molecular adducts. The first mentioned is not present in the spectra of saturated TGs. There are three types of main fragment ions: diacylglycerol ions $[MH–R_iCOOH]^+$, monoacylglycerol $[MH–R_iCOO-R'_iCO]^+$, and acyl ions $[R_iCO]^+$ [reference 61]. Diacylglycerol ions give the most intense fragment.

FAs on different position have different probabilities of loss during fragmentation so the relative signal intensities of diacylglycerol ions help us to find out the regiospecific distribution on glycerol backbone. The most likely and favored losses are from the positions *sn*-1 and *sn*-3. The molecular weight was determined from the molecular adduct ions. The total number of carbons and double bonds was calculated from the molecular weight.

The examples of substance determination are shown below, where three different types of TGs spectra are shown: TG with same FAs in all positions (Figure 9), TG with one different and two identical FAs at position *sn*-1 and *sn*-2 (Figure 10) and TG with 3 different FAs (Figure 11).

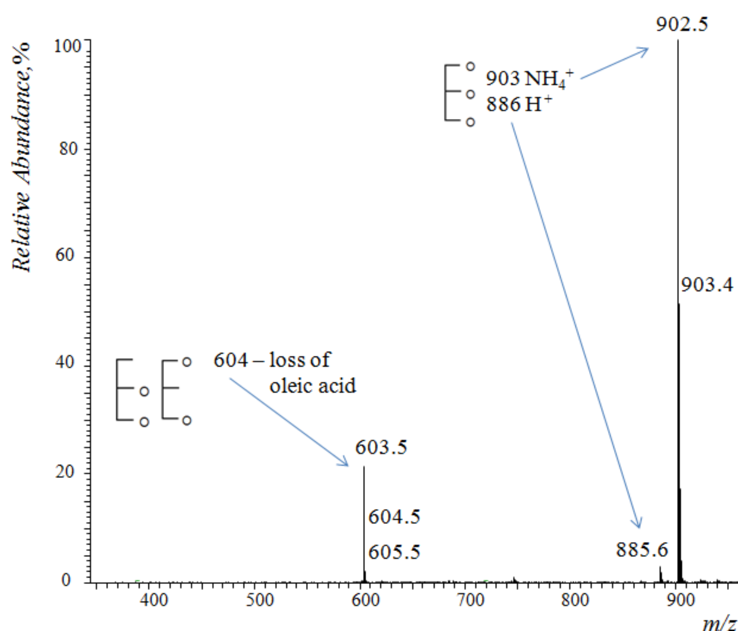

Figure 9: APCI-MS spectrum of TG (t_R = 80.8 min) from fat body of *B. terrestris* (queen before hibernation) interpreted as OOO. The diacylglycerol fragment is at *m/z* 603 (loss of oleic acid), molecular adducts are observed at *m/z* 885 for $[M+H]^+$ and at *m/z* 902 for $[M+NH_4]^+$.

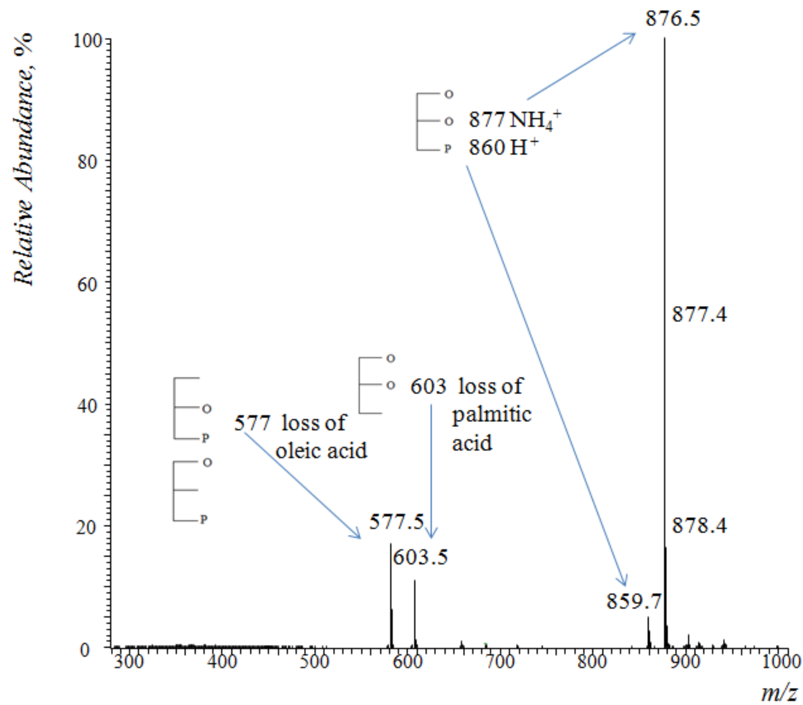

Figure 10: APCI-MS spectrum of TG (t_R = 82.3 min) from fat body of *B. terrestris* (queen after eclosion) interpreted as POO. The diacylglycerol fragments are at *m/z* 577 (loss of oleic acid) and *m/z* 603 (loss of palmitic acid)-, molecular adducts are observed at *m/z* 859 for $[M+H]^+$ and at *m/z* 876for $[M+NH_4]^+$.

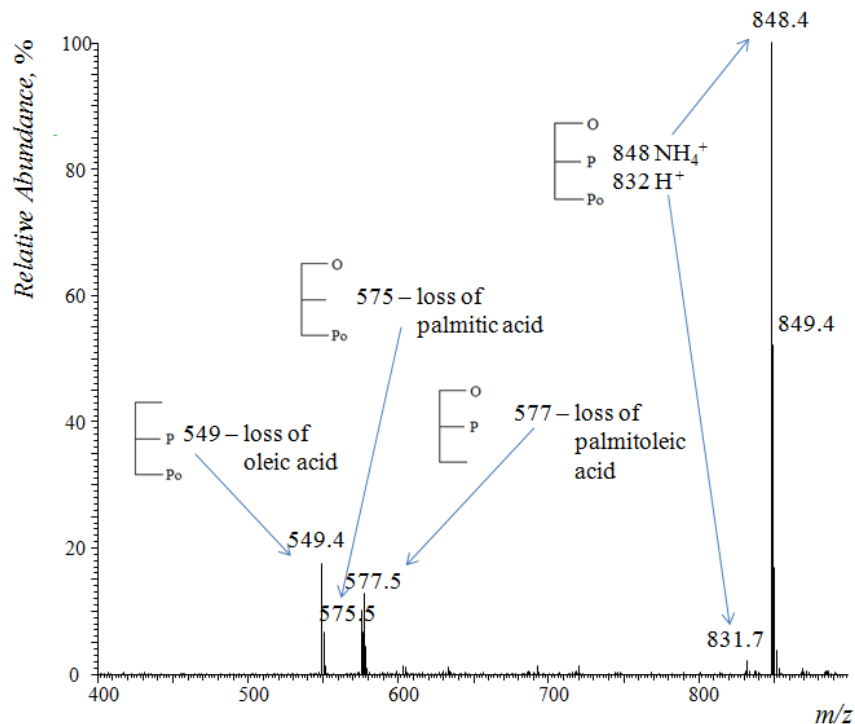

Figure 11: APCI-MS spectrum of TG (t_R = 76.7 min) from fat body of *B. terrestris* (queen after hibernation) interpreted as OPPo. The diacylglycerol fragments are at *m/z* 549 (loss of oleic acid), m/z 575 (loss of palmitic acid) and *m/z* 577 (loss of palmitoleic acid), molecular adducts are observed at *m/z* 831 for $[M+H]^+$ and at *m/z* 848 for $[M+NH_4]^+$.

4.5 APCI spectra interpretation with APCI program

An algorithm for interpreting APCI mass spectra of triacylglycerols has been developed [66]. It uses diacylglycerol fragments and molecular adducts to suggest the structures of TGs. First, fragment ions and molecular adducts are identified; the program determines whether the ion is a fragment or a molecular adduct, and they are then assigned two parameters: number of carbons and number of double bonds in the acyl group. The algorithm selects the FA in the *sn*-2 position based on the fragment ion intensities. The TriglyAPCI program based on this algorithm was used to aid identification of individual TGs present in each sample. TGs from each sample have been identified and subsequently analyzed quantitatively using the peak area percentage.

For quantification, masses of molecular adducts $[M+H]^+$ and $[M+NH_4]^+$ were calculated and corresponding reconstructed chromatograms were generated[20]. (Ex: The reconstructed chromatogram for OPPo (Figure 11) was calculated using the adducts 848.4 – $[M+NH_4]^+$ and 831.5 – $[M+H]^+$ added together). The peaks were integrated and their area was used to calculate the relative composition of TGs. The sum of the areas of all peaks was assumed as 100%, area of the peak of specific substance was calculated and the relative substitution was established.

Three to five samples of each stage have been analyzed in this way, the medians of areas for each substance and each stage have been compared. Relative standard deviation (RSD) for most peaks was 10% or less, for the small peaks (< 1%) the RSD was higher, about 25%.

4.5.1. Most abundant substances

The differences in composition between each stage have been found. The list of all TGs found in the fat body of *B. terrestris* queen in different stages and their relative composition is summarized in Appendix A. 65 substances have been identified in the fat body, their ECN range between 40 and 52. The substances with the small ECN (40, 42) were present just in small amount (up to 1%); the most abundant substances had ECN equal to 44 or 46.

The substances, which were most abundant in *B. terrestris* queens (substances present in more than 4% in at least 2 stages) are indicated in Table 5. The first two rows show a mixture of substances with ECN = 44 that could not be separated and did not give resolved peaks in chromatograms.

Table 5: Most abundant TGs of *B. terrestris* queen fat body in different life stages identified using reversed-phase HPLC/MS-APCI. The relative composition is given in peak area percent; TGs abbreviations as well as chromatographic conditions are explained in parts 2.7 and 3.4.3; ECN - equivalent carbon number, CN – total number of carbon atoms, DB – total number of double bonds.

			Life stage					
ECN	CN:DB	Substance	Pharate	After eclosion	Before hibernation	After hibernation	Egg-laying	Old
44	46:1	P O La M M O	9.1	6.6	1.4	< 1	1.1	5.5
44	48:2	O M Po Po Po P	2.7	2.5	5.8	4.2	1.6	2.1
44	50:3	O O Mo	4.8	1.7		1.3	1.3	2.9
44	52:4	O Ln P	12.8	8.3	< 1	1.7	12.4	11.6
44	54:5	O Ln O	4.7	3.0	4.2	5.6	8.6	5.0
46	48:1	P O M	9.4	9.7	2.6	0.4	2.4	6.7
46	50:2	O Po P	5.6	8.0	13.9	11.5	6.2	4.1
46	52:3	O Po O	1.7	< 1	14.3	13.8	3.8	0.8
48	52:2	O O P	9.0	17.6	17.7	13.0	18.2	15.0
48	54:3	O O O	2.3	4.7	15.6	19.8	16.6	6.1
48	50:1	P O P	6.3	10.1	2.6	< 1	3.1	7.3

These substances (Table 5) can be divided into two groups: a) TGs which relative composition did not vary within the life of bumblebee and b) TGs which relative composition varied within the life of bumblebees.

The substances belonging into the first group are for example 1-oleoyl-2-myristoyl-3-palmitoleoylglycerol (OMPo) and 1,2-dipalmitoleoyl-3-palmitoylglycerol (PoPoP) which relative composition varied only at interval 1-3% within all the stages. Another such a substance is 1,3-dioleoyl-2-linoleoylglycerol (OLnO) whose relative composition was approximately 5% at all stages (except egg-laying queens – 8.5%). In case of 1,2-dioleoyl-3-palmitoylglycerol (OOP), the differences among the life stages were bigger

(from 9 to 18%) nevertheless we can say that this substance was present in large quantities in all stages. Nomenclature is used in accordance with reference 39.

Regarding the second group, the differences in case of 1,3-dioleolyl-2-palmitoleoylglycerol (OPoO) are clearly visible. The substance was present in very small amount in the first two stages (~ 1%) in fat bodies of bumblebees, whereas before and after hibernation, it formed about 14%. The percentage decreased in the two last stages, what could mean that these substances are important during hibernation. A similar phenomenon can be observed for trioleoylglycerol (OOO), where the relative composition varied from 2.3% in pharate stage up to 19.8% before hibernation.

Opposite trend is observable for substances like 1-palmitoyl-2-olyoyl-3-laurylglycerol (POLa), 1,2-dimirystoyl-3-oleoylglycerol (MMO) and 1-olyoyl-2-linoleoyl-3-palmitoylglycerol (OLnP) – the substances were very highly abundant in early stages (pharate and after eclosion – about 10%) but very little present at the moment of hibernation (less than 1%). It is interesting that for the OLnP glycerol, the relative composition in two last stages increased from 1% up to 12%. This could mean that this substance is not at all important during the stage of diapause but important for the later stages. Other components of the samples (Appendix A) were present in the amount less than 1% or not at all detectable in some stages.

4.6 Multivariate comparison of TGs composition in various life stages

PCA has been performed with the relative proportions of the 35 most represented TGs from the HPLC/APCI-MS analysis for different life stages of *B. terrestris* queens. The multivariate exploratory analysis by means of PCA suggests that the TG composition is correlated with the life stage. Figure 12 shows that samples from one life stage are associated together according to the content of characteristic TGs. Those characteristic TGs can be assigned according to Figure 13 (next page): For example, for the life stage 1 – pharate queens (situated at the first quadrant at the Figure 12), the typical components are H, A, P, C, and E (also situated at the first quadrant at the Figure 13). These substances correspond to the TGs specified in appendix B.

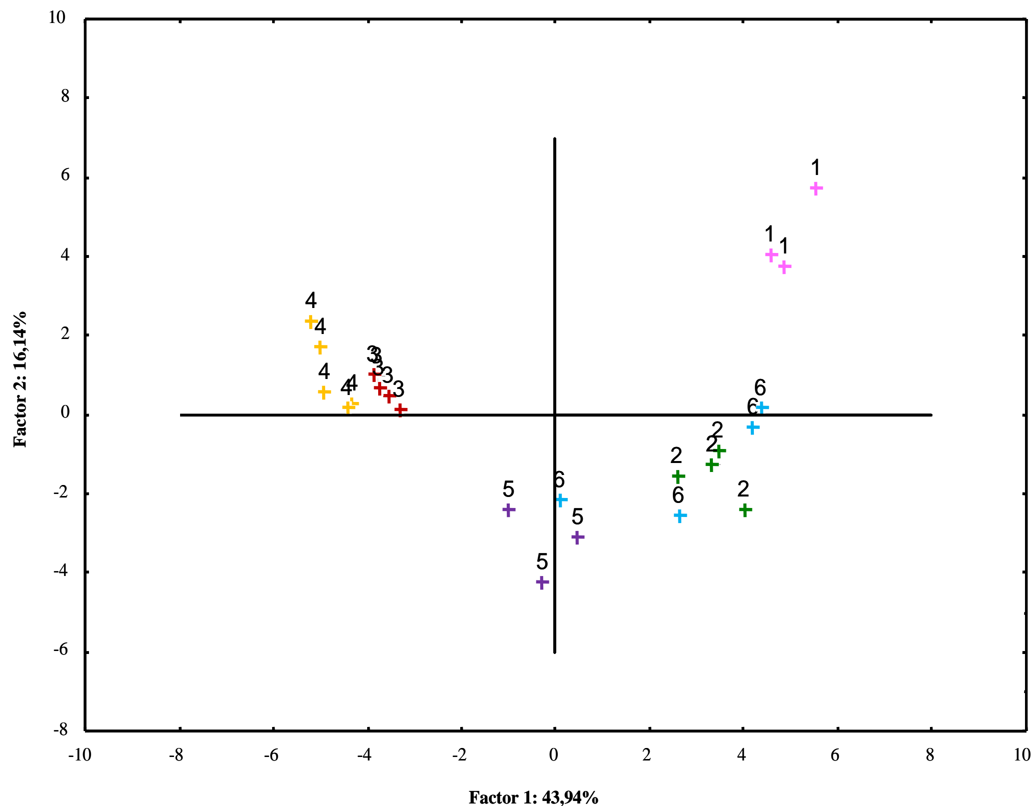

Figure 12: Graphic representation of the factor scores based on the first 2 principal components extracted from the PCA performed with the relative proportions of 35 most represented TGs from the HPLC/APCI-MS analysis for the different life stages of *B. terrestris* queens: (1) pharate queens, (2) queens after eclosion, (3) queens before hibernation, (4) queens after hibernation, (5) egg-laying queens with first cell, (6) old queens.

It is obvious that there exist some typical TGs for each life stage. Old queens make an exception – the composition of their TGs is not as specific as in the other stages – the TGs composition is heterogeneous as can be deduced from the heterogeneity of the graphic representation of the stage 6. This phenomenon can be explained by the fact that old queens form a very heterogeneous group in terms of age; in consequence, they differ in composition of TGs.

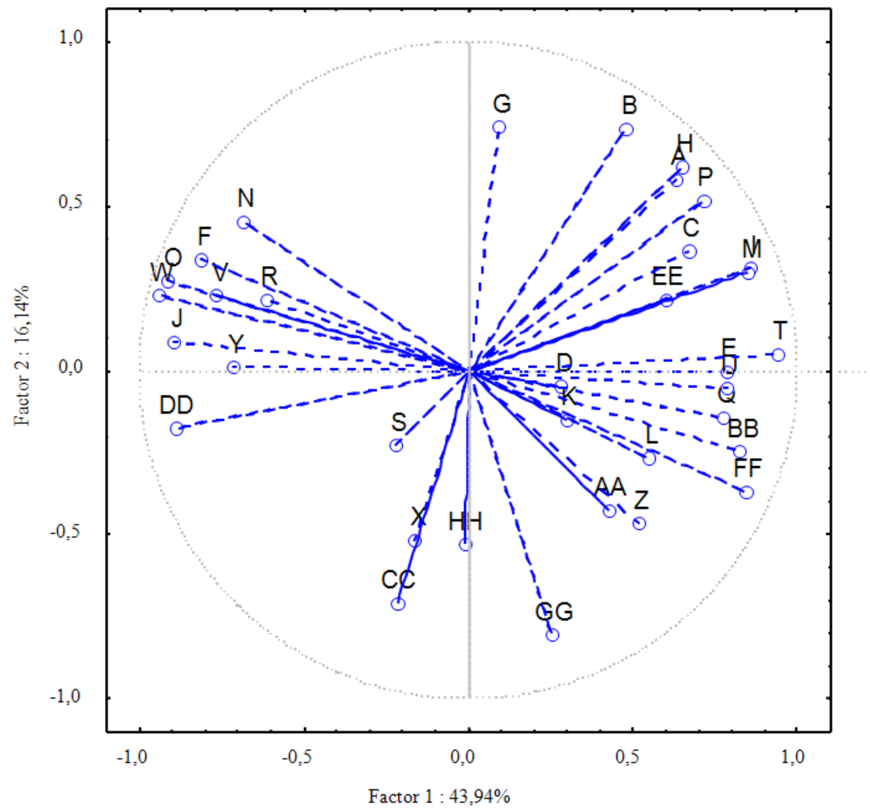

Figure 13: Principal component analysis of the TGs. The correlation of particular variables (relative abundances of TGs) with the first two factors of the PCA. The letters represent particular TGs; the list of abbreviations is specified at Table in Appendix B.

4.7 ESI spectra interpretation

ESI-MS was used to identify all lipids isolated from fat body. Each substance has been defined according to characteristic fragmentation which is made at 30 - 40% of collision energy - the *m/z* of most intensive peaks have been searched and allocated to concrete substance.

The lipids found in the fat body were, apart from TGs, mostly structural lipids: lysophosphatidylethanolamines, lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines and DGs (Figure 14).

The list of all identified substances for one of the stages, except TGs, is shown in Table 6 (next page). Qualitative differences among the stages were insignificant. Quantitative analysis has not been effectuated in this case.

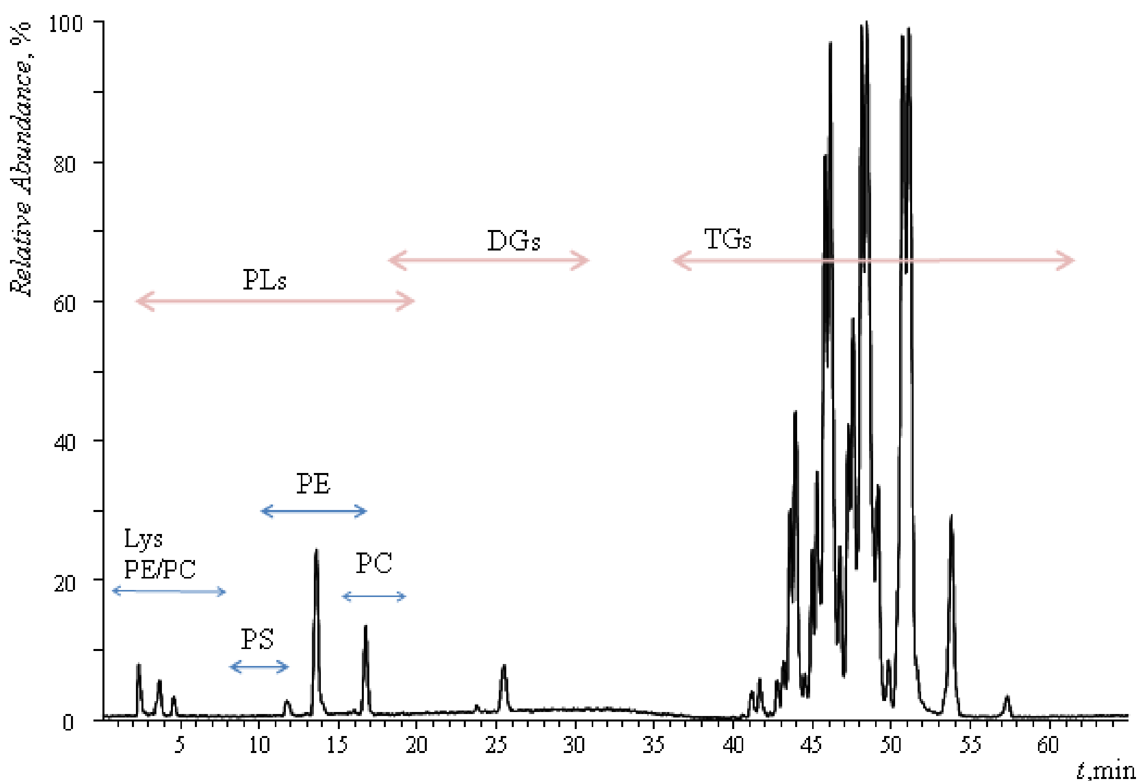

Figure 14: ESI-MS spectrum of all lipids separated from the fat body of queen *B. terrestris* (after eclosion). Bidirectional arrows indicate the expansion of each type of lipid in chromatogram: PLs – phospholipids, Lys PE/PC- lysophosphatidylethanolamines/lysophosphatidylcholines, PE – phosphatidylethanolamines, PS – phosphatidylserines, DGs – diacylglycerols, TGs- triacylglycerols Chromatographic conditions are explained in part 3.4.4.

Table 6: The list of identified substances isolated from *B. terrestris* queen (after eclosion) fat body except the TGs: Abbreviations: Lys PE- lysophosphatidylethanolamine. Lys PC- lysophosphatidylcholine, PE – phosphatidylethanolamine, PS – phosphatidylserine, DG – diacylglycerol; *m/z* - mass to charge ratio; FAs - fatty acids.

Type of substance	*m/z*	FAs	Type of substance	*m/z*	Fas
Lys PE	476	18:3	PC	786	18:1/18:1
Lys PE	478	18:2	PC	788	18:0/18:1
Lys PE	480	18:1	PS	784	18:2/18:2
Lys PC	496	16:0	PS	786	18:0/18:3
Lys PC	522	18:2	PS	788	18:1/18:1
Lys PC	688	18:1	PS	790	18:0/18:1
PE	690	16:1/16:1	DG	500	12:0/14:1
PE	690	16:0/16:1	DG	502	12:0/14:0
PE	714	16:1/18:2	DG	526	14:1/14:1
PE	714	16:0/18:3	DG	528	12:0/16:1
PE	716	16:1/18:1	DG	552	12:0/18:3
PE	718	16:0/18:1	DG	554	12:0/18:2 14:0/16:1
PE	728	17:0/18:3	DG	556	12:0/18:1
PE	730	16:1/16:1	DG	558	14:0/16:0
PE	730	17:0/18:2	DG	578	14:1/18:3
PE	730	17:1/18:1	DG	580	14:0/18:3 16:1/16:1
PE	732	17:0/18:1	DG	582	14:1/18:1 16:0/16:1
PE	736	18:3/18:3	DG	584	14:0/18:1
PE	738	18:3/18:2	DG	586	16:0/16:0
PE	740	18:3/18:1	DG	608	16:0/18:3
PE	742	18:2/18:1	DG	610	16:1/18:1
PE	742	18:3/18:0	DG	610	16:0/18:2
PE	744	18:1/18:1	DG	612	16:0/18:1
PE	744	18:2/18:0	DG	614	16:0/18:0
PE	746	18:1/18:0	DG	622	17:1/18:2
PC	754	16:1/18:3	DG	630	18:3/18:3
PC	756	16:0/18:3 16:1/18:1	DG	632	18:2/18:3
PC	758	16:0/18:2	DG	634	18:1/18:3
PC	760	16:0/18:1	DG	636	18:1/18:2
PC	778	18:3/18:3	DG	636	18:0/18:3
PC	780	18:2/18:3	DG	638	18:1/18:1
PC	782	18:1/18:3	DG	638	18:0/18:2
PC	784	18:1/18:2	DG	640	18:0/18:1
PC	784	18:0/18:3	DG	642	18:0/18:0

5. CONCLUSION

111 samples of fat bodies of *Bombus terrestris* queens in different life stages have been analyzed in order to study the composition of triacylglycerols in their fat bodies.

Lipids have been isolated from the fat body, weighted and analyzed using ESI-MS. The weight of all lipids varied during the life cycle – it culminated at queens before hibernation ($m = 28.8 \pm 5.1$ mg/individual) and was lowest at queens after hibernation ($m = 4.8 \pm 4.0$ mg/individual). The composition of lipids found in the fat body was the following: lysophosphatidylethanolamines, lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, diacylglycerols and triacylglycerols were present in the samples.

Triacylglycerols have been consecutively isolated from part of the samples, weighted and analyzed using APCI-MS. The highest weight of TGs was observed at queens before hibernation ($m = 20.1 \pm 3.9$ mg/individual), lowest amounts were determined at egg-laying queens ($m = 1.3 \pm 0.6$ mg/individual). Statistical analysis has proved that TGs form about 70% of total weight of lipids in fat body in the first four stages whereas in two last stages they form just 20% of the weight.

Qualitative HPLC-MS analysis of TGs in each stage showed qualitative differences among particular stages. There have been found 65 different triacylglycerols, most of them were present in less than 1% of relative composition, those substances were not detected in all stages. Substances with higher relative abundance – more than 2% - were detected in all life stages. Certain substances were highly abundant in early and last stages, but very few present in hibernation stages (i.e. 1-palmitoyl-2-oleoyl-3-lauroylglycerol, 1,2-dimirystoyl-3-oleoylglycerol, 1-oleoyl-2-linoleoyl-3-palmitoylglycerol), others in contrary were mostly present in stages before and after hibernation (i.e. 1,3-dioleolyl-2-palmitoleoylglycerol, trioleoylglycerol). The relative composition of the rest of the most abundant substances was similar in all life stages. The PCA analysis showed that some TGs are typical for particular life stage.

To summarize, it can be claimed that all objectives of the thesis have been executed. Nevertheless, there exists a need for a further study which could explain how particular TGs are used during the hibernation and which of them are exactly involved in formation of bumblebee pheromones.

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Appendix

Appendix A: The triacylglycerols in *Bombus terrestris* queen fat body in different life phases identified using reversed-phase HPLC/MS-APCI [a,b].

			Life stage					
ECN	CN:DB	Substance	Pharate queens	After eclosion	Before hibernatiom	After hibernation	Egglaying q. with 1st cell	Old queens
38	44:3	L 12:1 M	< 1					< 1
38	50:6	Ln Ln M	< 1					< 1
40	40:0	La P La, M M La	< 1	< 1				< 1
40	42:1	Mo P La, M M Mo	< 1	< 1	< 1		< 1	
40	46:3	M Ln M					< 1	< 1
40	46:3	P Ln La, Ln La P	2.0	< 1	< 1			
40	46:3	20:3 Mo Po				< 1		
40	46:3	Po Po Mo				< 1		
40	44:2	Ln O La, O La Ln, O Ln La			< 1	< 1		
40	48:4	Ln Ln P	1.2	< 1	< 1	< 1	< 1	< 1
40	52:6	Po Po Mo, Po Po Ln	1.1	< 1	< 1	< 1	< 1	< 1
40	50:5	L L Mo	< 1		< 1	< 1	< 1	< 1
42	42:0	M P La, M M M	< 1	< 1	< 1		< 1	< 1
42	44:1	M Po M	1.8	1.9	< 1	< 1		1.9
42	46:2	Po O 12:1;Po M Po	< 1	< 1	1.2	< 1		< 1
42	48:3	Po Po Po	3.9	< 1	1.1	1.4	< 1	1.8
42	48:3	P Po La, M M Po	4.1	1.3	< 1		< 1	< 1

			Life stage					
ECN	CN:DB	Substance	Pharate queens	After eclosion	Before hibernatiom	After hibernation	Egglaying q. with 1st cell	Old queens
42	50:4	O M Ln; O Po 16:2		< 1	< 1	< 1		2.7
42	50:4	Ln O M	4.1	1.7	< 1	1.0	1.4	
42	52:5	Po Po 20:3	< 1	< 1	1.41	1.7~~2~~		< 1
42	52:5	Ln L P	< 1	< 1	< 1	< 1	< 1	1.1
42	54:6	L L O, L L L	< 1	< 1	< 1	< 1	< 1	< 1
44	44:0	P La P , M M P	<1	1.1	< 1		< 1	< 1
44	46:1	P O La, M M O	9.1	6.6	1.4	< 1	1.1	5.5
44	48:2	O M Po, Po Po P	2.67	2.5	5.8	4.2	1.6	2.1
44	50:3	Po Po O	< 1	< 1	6.1	5.4	< 1	< 1
44	50:3	O O Mo	4.8	1.7		1.3	1.3	2.9
44	52:4	O L Po			< 1	2.5		
44	52:4	O Ln P	12.8	8.3		1.7	12.4	11.6
44	54:5	O Ln O	4.7	3.0	4.2	5.6	8.6	5.0
46	48:1	P O M	9.4	9.7	2.6	< 1	2.4	6.7
46	46:0	P M P	< 1	1.4	< 1		< 1	< 1
46	50:2	O Po P	5.6	8.0	13.9	11.5	6.2	4.1
46	50:2	P P L		< 1				
46	52:3	O Po O	1.7	< 1	14.3	13.8	3.8	< 1

			Life stage					
ECN	CN:DB	Substance	Pharate queens	After eclosion	Before hibernatiom	After hibernation	Egglaying q. with 1st cell	Old queens
46	52:3	P O M		2.1	1.0	2.3	1.4	2.7
46	54:4	O O L	< 1	< 1	1.1	4.7	1.0	1.0
46	54:4	L S L	2.4	1.7		0.3	3.8	2.2
48	48:0	P P P	< 1	< 1	< 1		< 1	< 1
48	50:1	P O P	6.3	10.1	2.6	< 1	3.1	7.3
48	52:2	O O P	9.0	17.6	17.7	13.0	18.2	15.0
48	54:3	O O O	2.3	4.7	15.6	19.8	16.6	6.1
50	50:0	P S P	1.0	< 1	< 1		< 1	< 1
50	52:1	S O P	3.7	4.4	< 1		2.2	4.2
50	54:2	O O S	1.3	2.6	1.5	1.1	4.5	3.4
50	56:3	O 20:1 O			< 1	< 1	< 1	
52	54:1	S S O, SSS	< 1	< 1	< 1		1.2	< 1

[a] The relative composition is given in peak area percent.

[b] TGs abbreviations as well as chromatographic conditions are explained in parts 2.7 and 3.4.3. In case of missing abbreviation, the total number of carbons with total number of double bond is used.

Appendix B: The list of abbreviations of substances used for PCA analysis. TGs abbreviations are explained in part 2.7. In case of missing abbreviation, the total number of carbons with total number of double bond is used.

Abbreviation used for PCA analysis	Type of TG	Abbreviation used for PCA analysis	Type of TG
A	P Ln La, Ln La P	R	O Ln P
B	Ln Ln P	S	O Ln O
C	Po Po Mo, Po Po Ln	T	P O M
D	M P La, M M M	U	P M P
E	M Po M	V	O Po P
F	Po O 12:1; Po M Po	W	O P Po
G	Po Po Po	X	P O M
H	P Po La, M M Po	Y	O O L
I	Ln O M	Z	L S L
J	Po Po 20:3	AA	P P P
K	Ln L P	BB	P O P
L	P La P , M M P	CC	O O P
M	P O La, M M O	DD	O O O
N	O M Po, Po Po P	EE	P S P
O	Po Po O	FF	S O P
P	O O Mo	GG	O O S
Q	O L Po	HH	S S O, SSS