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Analysis of triacylglycerols in fat body of bumblebees by chromatographic methods

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Abstract

Triacylglycerols (TAGs) from the fat body of several bumblebee species (*Bombus lucorum*, *B. terrestris*, *B. lapidarius*, *B. hypnorum*, *B. hortorum*, and *B. confusus*) were studied using chromatographic techniques. Semi-preparative thin-layer chromatography was used to isolate the TAGs from the tissue extract. Gas chromatography (GC) enabled us to identify the fatty acids (FAs) that form bumblebee TAGs and to quantify their relative proportions. The TAGs were subsequently analysed by high-performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry. Two chromatographic systems, including non-aqueous reversed-phase chromatography and silver ion chromatography on cation exchange resin in silver (I) ionic form, were optimised and their performance compared. The most abundant fatty acids in bumblebees TAGs contained 18 or 16 carbon atoms; oleic acid predominated in most samples. TAGs were found to be a complex mixture of isomers; some of them, e.g. OLnO, PLnO, PoPoO, PoPoP, POO, or OOO (where Po is palmitoleic, P is palmitic, Ln is linolenic, and O is oleic acid) were abundant in particular species. The composition of both FAs and TAGs was found to be species-specific. Only minor differences were found among specimens of the same species.

Keywords: Atmospheric pressure chemical ionisation; Bumblebees; *Bombus*; Fat body; Silver ion chromatography; Triacylglycerols; Triglycerides

1. Introduction

Insect fat body is an important metabolic tissue. It serves as a storage site for food reserves, as the major tissue for intermediary metabolism, and as a source for the hemolymph proteins, carbohydrates and lipids. Lipids supply energy for flight, reproduction, and during starvation and stress. The major lipid components are triacylglycerols (TAGs) stored in intracellular droplets. TAG fatty acids (FAs) originate both from diet and de novo synthesis from excess carbohydrates. Newly-formed FAs are bonded to glycerol backbones by acyl transferases; the exact composition of TAGs depends both on acyl transferase specificities and a pool of available FAs [1].

Bumblebees (Hymenoptera: Apidae) are social insects that are important pollinators of many wild plants, fodder crops (e.g. clover, alfalfa) or vegetables (e.g. tomatoes and peppers in greenhouses). During mid-to-late summer the colony switches from producing workers to reproductive individuals. The males leave the nest soon and seek for new queens. Males of most species deposit a queen-attracting scent in suitable places and patrol them later on [2–5]; the scent attracts conspecific females for mating [6]. The males' marking secretion (pheromone) is strictly species-specific. Its composition is known for many species [4,7–13]; however, little is known about the pathways involved in their biosynthesis. Our preliminary studies [14,15] showed similarities in the chain lengths and double bond positions of the main components of the pheromone secretions and fat body FAs. Therefore, we focused our research on characterizing the TAGs in bumblebee fat bodies. Surprisingly little information about intact TAGs from insects exists in the literature. The studies are usually limited to determining the relative proportions of FAs [16–22]; intact insect TAGs have rarely been analysed [23].

The analysis of TAGs using various analytical techniques is a subject of many books [24–26], reviews [27–35], and web sites [36,37]. TAGs are usually analysed by gas chromatography (GC) or high performance liquid chromatography (HPLC). Although intact TAGs are directly amenable to GC [38], the

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most common analytical approaches [25] use various procedures to convert TAGs to volatile derivatives of FAs prior to analysis, mostly to methyl esters (FAMEs). FAMEs differing in chain length, double bond position or geometry can be distinguished and identified based on their retention parameters, e.g. equivalent chain length (ECL) values [39,40]. Electron ionisation (EI) mass spectrometry is also valuable tool for FAMEs identification; several methods for determination of double bond position have been published [41–43]. Analysis of FAMEs using GC is well established, however, information how the FAs were bonded to glycerol backbone is lost. From this point of view, high performance liquid chromatography (HPLC) offers advantages over GC.

Non-aqueous reversed-phase HPLC on octadecyl-modified silica has been widely used for analysing TAGs of natural origin [44–50]. The mobile phases usually consist of acetonitrile mixed with a modifier solvent that improves the solubility of TAGs and affects the selectivity and efficiency of the separation. The retention behaviour of TAGs in these separation systems depends both on the total number of carbon atoms (CN) and double bonds (DB) in the fatty acyl moieties. The retention order usually follows the equivalent carbon number, ECN (ECN = CN − 2DB), however, minor exceptions exist. Separation of TAGs with the same ECN is possible for some isomers. An extensive list of TAG retention characteristics was published by Perrin and Naudet [51]. Silver ion (or argentation) chromatography [28,52–55] utilizes an entirely different retention mechanism. Silver ions react with π-electrons of double bonds in FA residues to reversibly form polar complexes. Such systems separate lipids according to the number, geometry and position of the double bonds within FA residues and to the regiospecific distribution of FAs chains. TAGs can be detected using a refractive index [56,57], flame ionisation [52,58], UV [59,60], evaporative light-scattering detectors [54,61] or mass spectrometric detectors, which can provide conclusive information on the identity of TAGs. Both atmospheric pressure chemical ionisation (APCI) [45–47,49,62,63] and electrospray ionisation (ESI) [53,64–68] mass spectrometry have been used. APCI spectra yield protonated molecules $(M + H)^+$ (or other molecular adduct ions) as well as fragments. The main fragment ions are of three types: diacylglycerol ions $(MH–R_iCOOH)^+$, monoacylglycerol ions $(MH–R_iCOO–R_i'CO)^+$ and acyl ions $(R_iCO)^+$ [62,63]. The most intense fragments are diacylglycerol ions. Because of different probabilities of FA loss during fragmentation, the relative intensities of diacylglycerol ions provide regiospecific distribution information of FA on the glycerol backbone. Losses from the *sn*-1 and *sn*-3 positions are more likely and equally favoured. Therefore, one can distinguish FAs bonded to the *sn*-2 position from those attached to the *sn*-1/*sn*-3 positions. A TAG pair of AAB/ABA type can thus be distinguished by comparing diacylglycerol ion intensity ratios with a theoretical value of 0.5. However, the intensity ratio depends also on the number and position of double bonds in particular FAs. Therefore, if TAG standards are not available, the positions of the FAs on the glycerol backbone might be interpreted incorrectly [33]. Diacylglycerol fragments can be used to quantify the positional isomers providing that the standard regioisomers are measured [48,49]. The relative proportion of molecular adducts and diacylglycerol ions in APCI spectra depend on the degree of TAG unsaturation; molecular adduct ions are usually intense in the spectra of unsaturated TAGs and weak or missing in the spectra of saturated ones. ESI spectra do not show protonated molecules; cationisation reagents such as ammonium (I) or sodium (I) ions are added to the mobile phase to induce formation of molecular adduct ions. Fragment ions are usually of low intensity or missing in ESI spectra [33].

Our paper focuses on TAGs of fat bodies of selected bumblebee species. Three separation methods were used in this study: thin-layer chromatography (TLC) to isolate TAGs from a mixture of lipids in fat bodies, GC to characterize individual FAs, and HPLC both in non-aqueous reversed-phase and silver ion systems to identify TAGs. The objectives of this work were (i) to optimise the chromatographic conditions for analysing TAGs in the fat body of bumblebees and (ii) to analyse selected bumblebee samples and report on their composition. To the best of our knowledge this is the first report on TAG isomers in bumblebee fat bodies and perhaps the first report on TAG isomer composition of the fat body in insects.

2. Experimental

2.1. Materials

Both acetonitrile gradient grade and methanol gradient grade were obtained from Riedel-de Haën (Seelze, Germany). The other solvents (acetone, chloroform, diethyl ether, hexane, 2-propanol) used for chromatography and for sample preparations were distilled in glass from analytical grade solvents (Lachema, Brno, Czech Republic). Ammonium acetate (Fluka, Buchs, Switzerland), formic acid (Lachema), and 2,6-di-*tert*-butyl-4-methylphenol, i.e. butylated hydroxytoluene, or BHT (Fluka) were of reagent grade and used as purchased. The bumblebee males of *Bombus* (*Pyrobombus*) *hypnorum* (Linnaeus, 1758), *Bombus* (*Megabombus*) *hortorum* (Linnaeus, 1761), and *Bombus* (*Confusibombus*) *confusus* (Schenck, 1859) were collected during the summers of 2003 and 2004 in the central region of the Czech Republic. The males of *Bombus lucorum* (Linnaeus, 1761), *Bombus terrestris* (Linnaeus, 1758), and *Bombus* (*Pyrobombus*) *lapidarius* (Linnaeus, 1758) were obtained from the laboratory colonies established using a mated queen from the previous year. Two to four bumblebees of each species were used for the analysis; the results for two of them are presented here.

2.2. Sample preparation

Bumblebee males were immobilized in cold and their peripheral fat bodies were carefully dissected. The fat body tissue from each individual was transferred into a glass vial with 100 μL of $CHCl_3/CH_3OH$ (1:1, v/v) containing 25 μg/ml BHT (antioxidant), the sample was sonicated for 15 min and stored at −18 °C when needed. The tissue was further crushed using a glass stick and extracted three times with 100 μL of $CHCl_3/CH_3OH$ (1:1, v/v). The crude extract was separated on pre-cleaned glass