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MINOR LIPOPHILIC COMPOUNDS IN EDIBLE INSECTS

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ABSTRACT

Contemporary society is faced with the question how to ensure suffiecient nutrition (quantity and quality) for rapidly growing population. One solution can be consumption of edible insect, which can have very good nutritional value (dietary energy, protein, fatty acids, fibers, dietary minerals and vitamins composition). Some edible insects species, which contains a relatively large amount of fat, can have a potential to be a „good" (interesting, new) source of minor lipophilic compounds such as sterols (cholesterol and phytosterols) and tocopherols in our diet. For this reason, the objective of this work was to characterize the sterols and tocopherols composition of fat from larvae of edible insect *Zophobas morio* L. and *Tenebrio mollitor* L. Cholesterol and three phytosterols (campesterol, stigmasterol and β-sitosterol) were reliably identified and quantified after hot saponification and derivatization by GC-MS. Other steroid compounds, including 5,6-trans-cholecalciferol were identified only according to the NIST library. Cholesterol was the predominant sterol in all analysed samples. Both types of larvae also contained high amount of phytosterols. Different region of origin had a no significant impact on sterols composition, while the effect of beetle genus was crucial. Tocopherols were analysed by reverse phase HPLC coupled with amperometric detection. Tocopherols content in mealworm larvae was lower than content in edible oils, but important from the nutritional point of view. Change of tocopherols composition was not observed during the storage under different conditions. Larvae of edible insect can be a potential good dietary source of cholesterol, but also vitamin D_3 isomers, phytosterols and tocopherols.

Keywords: sterol; tocopherol; *Tenebrio mollitor*; *Zophobas morio*

INTRODUCTION

The large group of minor lipophylic compounds include higher hydrocarbons, alcohols, ketones and diketones, steroids, lipophilic vitamins, pigments and other compounds. Steroids and lipophilic vitamins are most important compounds from this group.

Several steroid compounds are usually present in plants and organisms, but the major steroids are phytosterols and cholesterol. Steroids are synthesised in organisms via complex mechanisms from isoprene units, isopentenyl diphosphate and dimethylallyl diphosphate. Reaction gives an important intermediate, farnesyl diphosphate. Two molecules of farnesyl phosphate give rise to triterpenic hydrocarbon squalene, which in the body of animals yields triterpenic alcohol lanosterol and the triterpenic alcohol cycloartenol in plants. Lanosterol in animals is a precursor for the biosynthesis of the most important zoosterol cholesterol. An intermediate in the biosynthesis of cholesterol is 7-dehydrocholesterol, which is a precursor of vitamin D_3. Cholesterol in the body is used for the biosynthesis of steroid hormones and bile acids **(Velíšek, 2014)**.

Insects also need cholesterol for the synthesis of vitamin D_3 and also for the synthesis of insect steroid hormones called as ecdysteroids (e.g. ecdysone, 20-hydroxy-ecdysone, makisterone A etc.). But, insects are not able to synthesize cholesterol *de novo* and they have to use plant phytosterols (β-sitosterol, campesterol, stigmasterol) for cholesterol synthesis by the side dealkylation on the C-24 alkyl group of dietary phytosterols. Cholesterol and 7-dehydrocholesterol are formed during synthetic pathway. For example, the beetle *Tenebrio molitor* produces about 17% of 7-dehydrocholesterol and about 67% of cholesterol from sitosterol **(Leclerq, 1948; Svoboda and Feldlaufer, 1991; Ikekawa et al., 1993; Svoboda and Lusby, 1994; Ikekawa et al., 2013)**.

Cholesterol is mainly found in animal fats and in human tissues. In lower animals other sterols, known collectively as zoosterols may also be present. Cholesterol and its esters are present in all membranes and in blood lipids, but particularly rich sources are nervous tissues, especially the brain. Egg yolk is other very important source of dietary cholesterol. Other sources include meat, milk and cheeses, but also animal fats, lard and butter to a greater extent **(Velíšek, 2014)**. Sterols are essential components of lipoproteins and lipid membranes in animals. They are particularly important in nerve tissues and in the transport of lipids, which are bound in lipoproteins. In humans, dietary cholesterol intake is lower than the daily requirement, therefore the body synthesises (in the liver) the majority of cholesterol that is needed. Cholesterol in the diet is easily absorbed, but problems may occur during transport of cholesterol from the intestinal wall during lymph and blood circulation. Excessive cholesterol transport in low-density lipoproteins may cause cardiovascular diseases. It is therefore recommended that the intake of dietary cholesterol should not exceed 300 mg per day **(Dinh et al.,**

2011; Golebiowski et al., 2014; Velíšek, 2014).

Vitamin D_3 is formed from 7-dehydrocholesterol after UV irradiation (wavelength 280 – 320 nm) through the intermediate precholecalciferol. Calcitriol (1α,25-dihydroxycholecalciferol) is the active form of vitamin D_3 that is created in oxidation in the liver and subsequently in the kidney. Along with two other hormones, calcitonin (from thyroid gland) and parathormone (parathyroid gland) act in the resorption, metabolism and excretion of calcium and phosphorus **(Lawson, 1971; Velíšek, 2014; Finke, 2015)**. Two geometrical isomers of the vitamin D_3 and its 25-hydroxyderivative can be formed during synthesis from 7-dehydrocholesterol and consecutive oxidation in the liver. 5,6-*cis* isomer, after oxidation in the kidney, stimulates intestinal calcium transport. Vitamin activity of 5,6-*trans* isomer is significantly lower, but this compound shows another biological activity – mainly antiproliferative activity **(Holick et al., 1972; Chen et al., 2000; Filip et al., 2010)**.

Plants synthesise a number of steroid substances from cycloartenol, mainly demethylsterols (campesterol and other phytosterols) and 4,4-dimethylsterols (mainly as saponins – betulinic acid etc.) or 4-methylsterols as minor compounds **(Kuksis, 2001; Velíšek, 2014)**.

Vitamin E, formerly also known as antisterile vitamin, has eight basic structurally-related derivatives of chroman-6-ol (2H-1-benzopyran-6-ol). Structural bases common to all compounds with the reported activity of vitamin E are tocol and tocotrienol, which contain a hydrophobic chromane ring with a saturated or unsaturated isoprenoid side chain of 16 carbon atoms **(Velíšek, 2014)**. Vitamin E, especially α-tocopherol is the most important lipophilic antioxidant that acts in eucaryotic cells to protect (poly)unsaturated lipids against free radical damage. Tocopherols show the antioxidant activity *in vivo* and also *in vitro*. It protects the structure and integrity of biomembranes, such as the cytoplasmic cell membrane (or plasmolema) and intracellular membranes of organelles (nucleus, mitochondria, lysosome and endoplasmic reticulum). It is also employed in the protection of lipoproteins present in plasma. It is transported in the bloodstream by association with the lipid phase of low density lipoprotein (LDL) particles. Each LDL particle contains six molecules of vitamin E **(Li et al., 1996; Munné-Bosch and Alegre, 2002; Hofius and Sonnewald, 2003; Velíšek, 2014)**.

The aim of this study was to characterize the profile of minor lipophilic (unsaponifiable) compounds (mainly steroids and vitamin E) in three types of the edible insect.

MATERIAL AND METHODOLOGY

Analyzed samples

Insect species used for analysis were larvae of mealworm (*Zophobas morio* L.) and larvae of superworm (*Tenebrio mollitor* L.). The stage of development of analyzed insect was suitable for culinary preparation. *Zophobas morio* and *Tenebrio mollitor* were purchased lyophilized in local market in Sumatra. *Zophobas morio* sample were also purchased in Radek Frýžela Company, Brno, Czech Republic. Insects were starved for 48 hours, killed with boiling water (100 °C) and dried at 105 °C to constant weight. These samples were homogenized and stored at 4 – 7 °C until analysis. Sampels of *Zophobas morio* (from Czech Republic) used for tocopherols analysis were stored at two different conditions: at 5 – 6 °C and at 25 °C (room temperature).

Fat extraction

Fat from analysed samples was extracted by Soxtherm® apparatus (Gerhardt, Königswinter, Germany). Approximately 5 g of homogenized sample was weight into extraction cartridge and extracted by petroleum ether 120 min at 70 °C. Fat content was measured gravimetrically after drying to the constant weight at 103 °C. Extracted fat was used for sterols and tocopherols analysis.

Sterols analysis

Sterols content was determined according to AOCS Official Method Ch 6-91, American Oil Chemists´ Society, USA, 1997. Approximately 0.5 g of fat was boiled by 50 ml ethanolic KOH (2 $mol.L^{-1}$) for 1 hour. The unsaponifiable fraction was extracted with diethylether. The solvent was evaporated using rotary vacuum evaporator. Dried samples were silylated by pyridine and BSTFA (Bis(trimethylsilyl)-trifluor-acetamide; Merck, Czech Republic). Sterols were determined by GC Agilent 7820A coupled with mass detector Agilent 5975 Series MSD (Agilent Technologies, Palo Alto, CA, USA). Analytes were separated by capillary column Supelco (SACTM5, 22 m x 025 mm I.D. x 0.25 µm film). High purity helium was used as carrier gas at a flow rate of 20 $mL.min^{-1}$. Column temperature program started at

245 °C for 1 min, than heated at a rate 10 °C/min to 290 °C for 33 min, than increased by 5 °C/min to 310 °C for 15 min. The injector temperature was set to 280 °C. Samples were injected (1µL) in a split mode (20:1). 5α-cholestane was used as internal standard for quantification of cholesterol, campesterol, stigmasterol and ß-sitosterol in SCAN mode. Peaks were identified by their retention time compared with pure standard, comparison of their mass spectra with the NIST library spectra and also by comparison with literature.

Tocopherols analysis

Tocopherols were determined by reverse phase HPLC with amperometric detection The analysis was performed under the following conditions: a mobile phase - mixture of acetonitrile/methanol (1:1, v/v) with $LiClO_4$ (0.02 $mol.L^{-1}$) and NaCl (0.005 $mol.L^{-1}$); a flow rate 1 ml/min (LCP 4020.31 nonmetallic pump Ecom, Prague, Czech Republic); injected volume 20 µL. Separation was performed by steel column (4 x 250 mm, a particle size 5 µm, Tessek, Prague, Czech Republic); column temperature 28 °C (LCO 101 column heater, Ecom, Prague, Czech Republic); detection potential - +0.7 V (HP 1049A amperometric detector with a glassy – carbon working and solid state Ag/AgCl reference electrode (Agilent Technologies, St. Clara, USA). The quantification of tocopherols was provide by external calibration. For the determination of tocopherols insect fat samples were prepared as follows. Approximately 0.25 g of fat extracted from insect was weighed into 25 mL volumetric flask and filled to the mark with acetone.

RESULTS AND DISCUSSION

Amounts of sterols in samples of edible insects are shown in Table 1.

Table 1 Sterol composition of edible insect species.

Insect	Sterol			
	Cholesterol (mg.kg^{-1}±SD)	**Campesterol (mg.kg^{-1}±SD)**	**Stigmasterol (mg.kg^{-1}±SD)**	**ß–sitosterol (mg.kg^{-1}±SD)**
Zophobas morio	1784.1 ±30.4	227.6 ±19.9	79.3 ±9.4	344.1 ±35.8
Zophobas morio*	1594.9 ±164.1	169.2 ±8.45	unquantified	260.2 ±12.3
Tenebrio mollitor	669.4 ±34.7	350.5 ±56.0	71.9 ±2.5	244.7 ±12.0

*Czech Republic

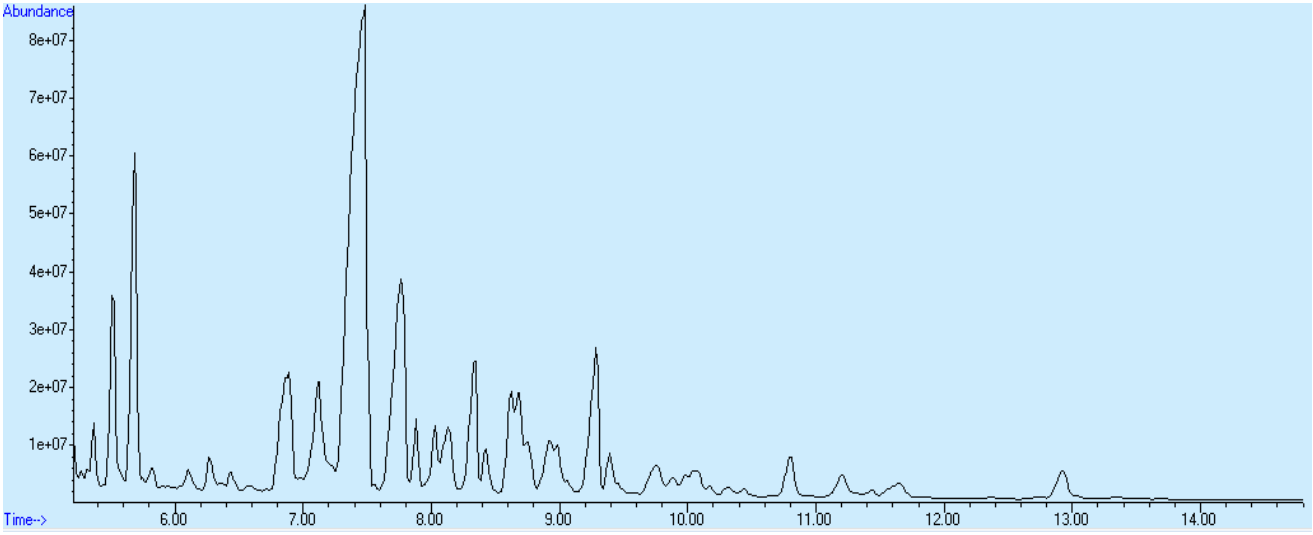
Table 2 Tocopherols composition of *Zophobas morio* (Czech Republic) stored at different conditions.

Conditions (°C)	α – tocopherol mg.kg^{-1} ±SD	ß + γ – tocopherol mg.kg^{-1} ±SD	δ – tocopherol mg.kg^{-1} ±SD
5 – 6	75.7 ±3.2	5.3 ±0.1	LOQ
25	77.2 ±0.1	5.3 ±0.3	LOQ

Cholesterol, typical animal sterol, was the most abundant sterol found in analysed samples. There are only minimum amount of cholesterol in food source of plant origin. Negative health impact (metabolic syndrome) of cholesterol is well known for a long time **(WHO, 2004).** On the other hand, cholesterol lowering effect of phytosterols is well known as well **(Peterson, 1951)**. Phytosterols reduce intestinal cholesterol absorbtion **(Normén et al., 2000)** and plasma LDL-cholesterol **(Piironen et al., 2000)**. Phytosterols (namely ß-sitosterol, campesterol and stigmasterol), typical plant origin sterols, were also determined in insects' samples. Identity of these compounds was confirmed by comparison with standards.

Cholesterol content was quite similar for the same insect species which came from different regions *Z. morio* from the Czech Republic (1594.9 mg.kg^{-1}) and *Z. morio* from Sumatra (1784.1 mg.kg^{-1}). The results indicate, that the extent of cholesterol synthesis is probably determined genetically and the influence of climate or type of feed is negligible. But larger set of samples would be needed to confirm these conclusions. However, sterols composition differences were much bigger between various insect species. *Z. morio* contained more cholesterol (1594.9 – 1784.1 mg.kg^{-1}) than *T. mollitor* (669.4 mg.kg^{-1}). An interesting trend can be seen in the content of phytosterols, which is the opposite content ratios of campesterol and β-sitosterol of mealworm larvae (228 and 344 mg.kg^{-1}) and superworm larvae (350 and 246 mg.kg^{-1}). This could indicate a different mechanism of cholesterol biosynthesis. Mealworm probably favors a demethylation of β-sitosterol (C_{29}-C_{28}), superworm favors easier demethylation of campesterol (C_{28}). The cholesterol content of the *T. mollitor* was comparable with content in meat of common livestock. Larvae of *Z. morio* synthesized much larger amount of cholesterol than there is in eggs – cholesterol content in the whole eggs is 2000 – 3500 mg per kg **(Velíšek, 2014)**. High cholesterol level could mean a nutritional problem.

Chromatograms from sterols analysis in fat from different edible insect are shown in Figure 1. According to mass spectra, the following sterols and stanol (saturated sterol) were identified by the NIST library: lanosterol (RT 7.9), ergosterol (RT 8.1) and stigmastanol (RT 9.4). Ergosterol was found as pro-vitamin D_2 in food from plant and microbial origin **(Gorman et al., 1987; Tsiaras et al., 2011)**. All analyzed samples contained significant amount of the compound, that was identified as cholecalciferol (vitamin D_3). The main food sources of cholecalciferol are fish (mainly fatty fish such as herring, mackerel and salmon), meat, eggs, milk and dairy products **(Schmid and Walther, 2013; Velíšek, 2014)**. The compound with retention time 7.7 was identified as cholecalciferol according to the NIST library. However, retention time of cholecalciferol standard was different (6.1 to 7.1). Comparison of standard, real sample and the NIST library mass spectra of cholecalciferol is shown in Figure 2. As shown in this figure, the spectra are practically identical. Similarity of mass spectra and differences in retention times of these compounds suggests that these compounds are probably different cholecalciferol isomers. This compound is probably 5,6-*trans*-cholecalciferol, which is described in literature. This isomer was identified with high probability only at *Z. morio* samples. This isomer was identified with high probability only at *Z. morio* samples. 5,6-t*rans* vitamin D_3 does not have the same biological activity as 5,6-*cis* vitamin D_3, but it has other biological activities **(Borsje et al., 1977; Chen et al., 2000; Filip et al., 2010)**. 5,6-*cis* vitamin D_3 plays the crucial role in calcium and phosphorus metabolism (bone development and maintenance) and is also important for cell differentiation process and immune system **(Ovesen et al., 2003; Velíšek, 2014)**. Concerning other peaks of analytes, they were not identified with sufficient reliability.



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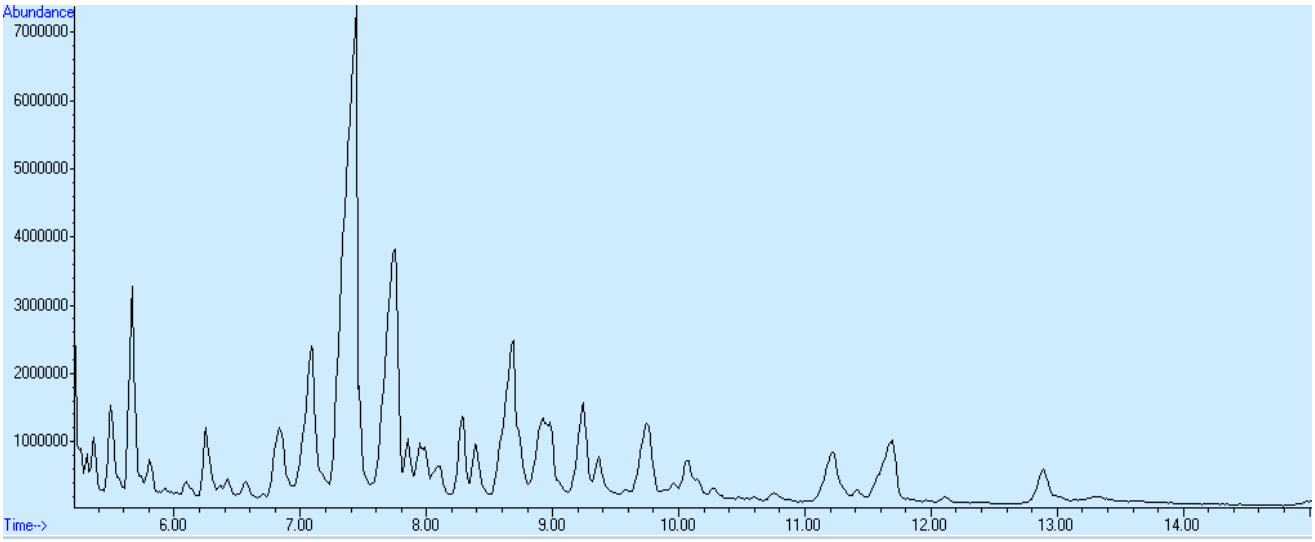

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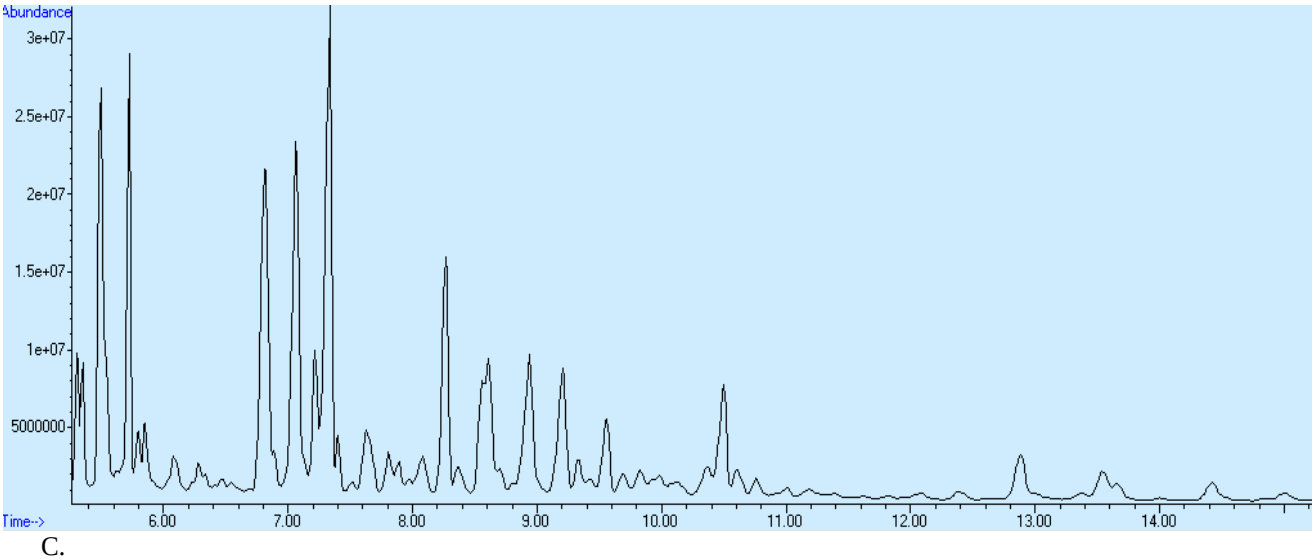
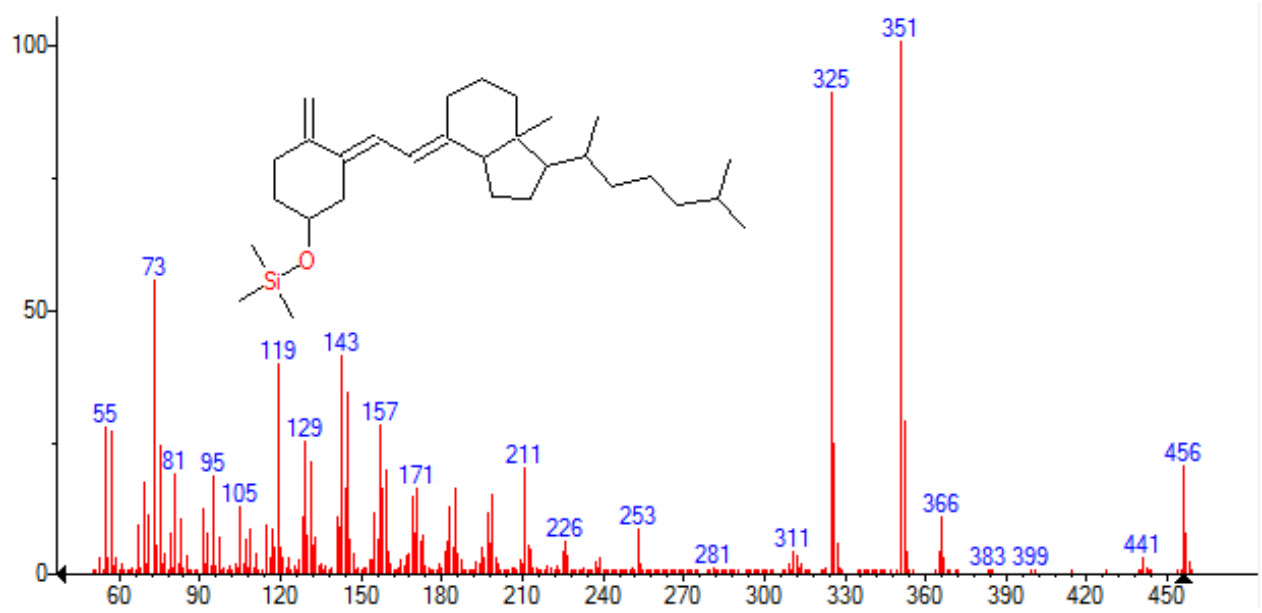


C.

Figure 1 Chromatograms – sterols composition of A - *Zophobas morio* (Sumatra), B - *Zophobas morio* (Czech Republic), C - *Tenebrio mollitor* (Sumatra); retention times: 5α-cholestane 5.4, cholesterol 7.4, 5,6-*trans*-cholecalciferol 7.7, lanosterol 7.9, ergosterol 8.1, campesterol 8.3, stigmasterol 8.6, ß-sitosterol 9.2, stigmastanol 9.4.



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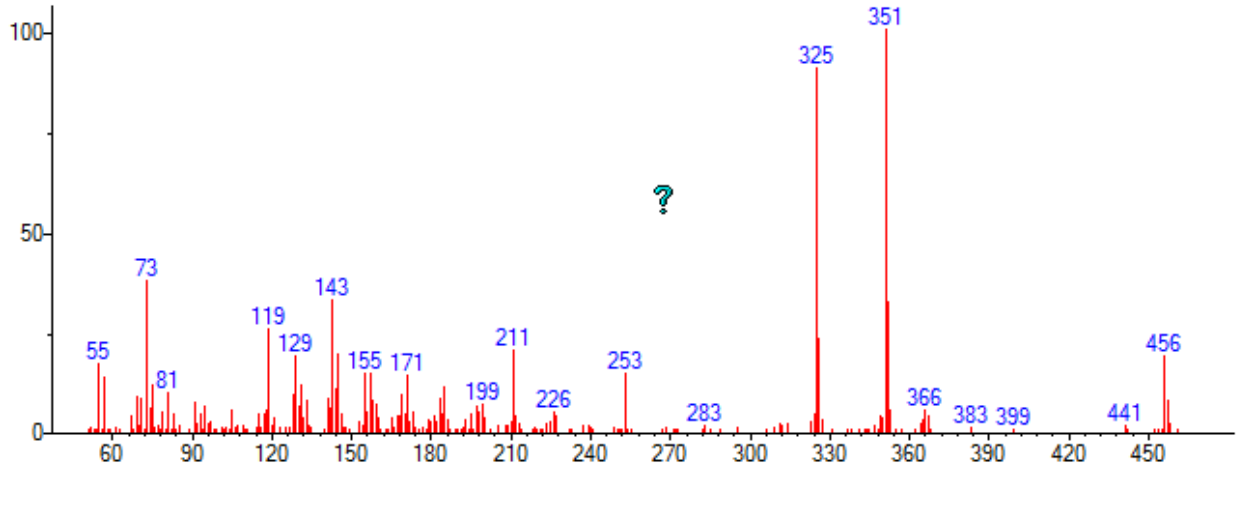

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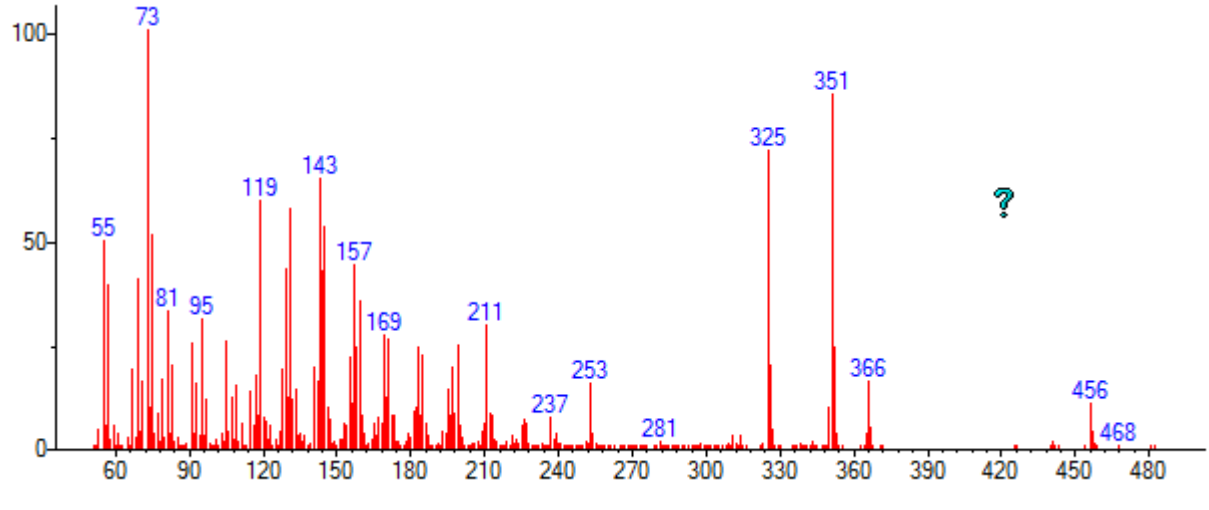

C.

Figure 2 Mass spectra of cholecalciferol: A – NIST libary, B – standard, C – analyzed sample.

Tocopherols content in edible insect samples is shown in Table 2. The α-tocopherol was the most abundant tocopherol analyzed in larvae of mealworm *Zophobas morio*, followed by β- and γ-tocopherols. Δ-tocopherol content was under the limit of quantification (LOQ). The content of α-tocopherol can be interesting from the nutritional point of view. The requirement of α-tocopherol for adults is about 15 mg of tocopherol equivalent per day. In this case, the consumption of about 50 g of edible insect brings approximately 25% of recommended daily intake. The storage temperature had no impact to the tocopherols content in analyzed insect samples.

The content of α-tocopherol was 75.7 mg per kg and 77.2 mg per kg in sample stored at 5 – 6 °C and 25 °C, respectively.

CONCLUSION

Larvae of beetle *Zophobas morio* L. and *Tenebrio mollitor* L. had a relatively high content of minor lipophilic compounds, which can be interesting from a nutritional point of view. The cholesterol content at superworm larvae was comparable with its content in meat, mealworm larvae had its content significantly higher. Both types of larvae contain approximately the same amount of phytosterols, their total content was comparable with cholesterol in superworm larvae, and in case of mealworm larvae. Mealworm larvae contained significant amounts of α-tocopherol, other tocopherols content was insignificant.

Relatively high level of phytosterols and α-tocopherol may be one of the major benefits of this new food commodity.

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