Fatty Acid Profiles of Main Lipid Classes in Adult *Chrysomela vigintipunctata* (Scopoli) (Coleoptera:Chrysomelidae)

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The fatty acid composition of the willow leaf beetle *Chrysomela vigintipunctata* (Scopoli) (Coleoptera: Chrysomelidae) is presented in this paper. Fatty acids in the total lipid extract, triacylglycerols, free fatty acids and polar lipids were compared. One hundred and fifteen fatty acids were identified in the total lipids. The mixture comprised compounds with normal and branched-chains of 12–30 carbon atoms and zero to six double bonds in different positions in the carbon chain. Substantial amounts of unsaturated eicosanoic fatty acids known as important precursor of eicosanoids in insects were detected in the lipids as were biologically significant positionally isomeric dienes, trienes and tetraenes of the series (n-3) and (n-6) of C16, C18, and C22 fatty acids. Also present was a mixture of hydroxy-FA. Triacylglycerols contained mostly saturated and monounsaturated fatty acids. Polyunsaturated fatty acids were found mostly in free fatty acids and especially in polar lipids.

Introduction

It is well recognized at present that the examination of insect lipids leads to a better understanding of their metabolism and their biological significance for these organisms (Mittler and Dadd, 1983; Stanley-Samuelson and Nelson, 1993). Fatty acid composition is a subject of increasing interest. It has been shown that in addition to the conventional roles as energy resource and structural element of bio-membranes, fatty acids have some specific role in insects as elements of the defense system, and as precursors in the biosynthesis of waxes, pheromones and eicosanoids (Stanley-Samuelson *et al.,* 1988). Little is known about the lipids of leaf beetles or Chrysomelidae (Coleoptera). The main interest so far has been concentrated on the chemical substances that play role in the selection of host plant, in mating and, particularly, in defense (Craveiro and Bao, 1995; Gross and Hilker, 1994; Hilker and Schulz, 1994; Mitchell, 1988; Pasteels *et al.,* 1984; Pasteels *at al.*, 1988). In contrast, few papers only concern lipids in general and fatty acids in particular (Dubis *et al.,* 1986; Ogg *et al*, 1993) in these insects.

Many families and subfamilies of leaf beetles are widely spread in Southeast Europe (Gruev and Tomov, 1986) and some cause serious damage to the host plants. As a part of a broader program on examination of chrysomelids of Bulgarian origin we examined the fatty acids of the leaf beetle *Chrysomela vigintipunctata* (Scopoli) (Coleoptera: Chrysomelidae) which feeds on willow trees (*Salix* sp).

The objective of the present work was to determine the fatty acid composition of the total lipids isolated from adult insects rared in laboratory environment and fed their usual diet, and trace the distribution of fatty acids between the main lipid classes. To the best of our knowledge, no data dealing with lipids of the beetle *Chrysomela vigintipunctata* (Scopoli) have been reported as yet.

Material and Methods

Larvae of last instar and pupae of *C. vigintipunctata* (Scopoli) were collected in June 1995 near Sofia and reared to eclosion in laboratory environment by P.Kalushkov, Institute of Zoology, Bulgarian Academy of Sciences.

Chemicals and materials

All solvents and reagents were either analytical grade or HPLC grade. Light petroleum and acetone were used after preliminary distillation.

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Extraction of total lipids

The adult insects (1368 species) were first soaked for 2 min in 10 ml of ethanol to isolate the surface lipids (Nikolova *et al.*, 1999). Than, the insects were homogenized in a blender for 2 min in 10 ml of ethanol and transferred to a round bottom flask. The total lipids were extracted by refluxing the homogenate for 5–6 min with 20 ml dichloroethane-ethanol (1:1, v/v). The mixture was filtered (Filtrak 389) while still hot and the residue was suspended in fresh 20 ml of the same solvent mixture and stirred for 24 h (magnetic stirrer). After filtration the procedure was repeated for another 24 h under the same conditions. The extracts were combined and the solvents were evaporated to dryness under nitrogen and redissolved in dichloroethane.

Isolation of the main lipid classes

Each extract (10 mg solution in dichloroethane) was applied on a 20x20 cm home-made preparative glass plate (1.0 mm thickness of the silica gel layer, pre-washed by two-fold development with chloroform-methanol). A reference mixture of lipid classes (eicosan, steroyl oleate, methyl oleate, triolein, oleyl alcohol, diolein, oleic acid and dioleoylphosphatidyl cholin) was applied alongside. The plate was developed with light petroleum-acetone, 100:8: (v/v) to a solvent front of 19 cm. Bands of triacylglycerols (TAG, R_f=0.7), free fatty acids (FFA, R_f=0.3 and polar lipids (PL, R_f=0.1) were detected by spraying the plate edges with 1% solution of 2,7-dichlorofluorescein in ethanol. They were scrapped, transferred to small glass columns, and eluted with diethyl ether.

Preparation of fatty acids derivatives

Methyl esters of TAG were prepared by acid-catalyzed transesterification with methanolic sulfuric acid according to Christie (Christie, 1989), except that the solvent was dichloromethane instead of toluene. FFA and PL residues were reacted directly with the reagent. The methyl esters were purified by preparative TLC on pre-washed plates (chloroform-methanol, 2:1) with mobile phase light petroleum ether-acetone 100:6 (v:v), the bands were scraped and the substances were eluted with diethyl ether. Finally, the solvent was evaporated under nitrogen and the residues redissolved in hexane to give a 2% solution.

Total non-hydroxy- and hydroxy fatty acids were separated on a semi-preparative column (RP-C18, 250 x 10 mm, 5 μm particle size) by elution with a gradient of methanol-tetrahydrofurane from 3:2 to 2:3 (v:v) over 25 min (flow 0.9 ml/min). The oxazolines were prepared by modification of the reference methods (Tulloch, 1985; Yu *et al.*, 1988).

Gas chromatographic mass-spectrometric analyses of fatty acids

Gas chromatography-mass spectrometry of the corresponding oxazolines and methyl esters of FA was done using a Finnigan 1020 B single-state quadrupole gas chromatography-mass spectrometry instrument in the electron ionization mode. A fused silica capillary column (60 m x 0.32 mm ID, SPB-1 Supelco), using splitless injection and hydrogen as the carrier gas (a linear velocity of 60 cm s^{-1}) was used both for oxazolines and methyl esters. Ionization energy was 70 eV, and electron multiplier voltage was 2.5 kV. All spectra were scanned within the range m/z 50–600 as described previously (Dembitsky *et al.*, 1993 a,b; Rezanka *et al.*, 1987; Rezanka and Mares, 1987; Kren *et al.*, 1985). The oxazolines were eluted at following temperature program: 100 °C for 1 min with a subsequent increase to 150 °C at 20 °C/min and to 300 °C at 2 °C/min. This temperature was maintained for 1 min. For methyl esters was used programmed oven temperature from 100 to 320 °C, at 5 °C/min.

Results and Discussion

Three main lipid classes were identified in *C. vigintipunctata* triacylglycerols (TAG, 16%), free fatty acids (FFA, 18%) and polar lipids (PL, 7%). The total extract contained also hydrocarbons (Nikolova *et al.*, 1999) and other non-identified lipoidal compounds. One hundred and fifteen FA were identified in the total lipids of the insect. Components with normal and branched-chains (carbon numbers in the interval C12–C30), and zero to six double bonds in different positions in the carbon chain were detected. Also present were up to 2% of hydroxy-FA; the highest quantity being found in TAG (2.3%). The composition of these acids is

under study at present. The FA composition of the total extract, TAG, FFA and PL is given in Tables I to IV.

Table I. Normal-chain saturated fatty acids in *C. vigintipunctata*[a].

FA	Total	TG	FFA	PL
12:0	0.10(0.02)[b]	0.09(0.01)	0.17(0.02)	0.09(0.01)
13:0	0.06(0.01)	0.07(0.01)	0.07(0.01)	0.05(0.01)
14:0	0.98(0.09)	1.30(0.11)	0.64(0.05)	0.52(0.04)
15:0	0.31(0.03)	0.49(0.03)	0.12(0.01)	0.33(0.03)
16:0	16.14(0.97)	20.66(1.24)	14.35(1.08)	9.32(0.78)
17:0	1.25(0.05)	0.31(0.02)	0.09(0.01)	2.12(0.18)
18:0	3.83(0.34)	3.82(0.45)	4.68(0.38)	3.29(0.26)
19:0	1.54(0.14)	0.29(0.02)	3.22(0.29)	0.28(0.02)
20:0	0.62(0.05)	1.06(0.08)	0.41(0.04)	0.66(0.07)
21:0	0.04(0.01)	0.11(0.01)	0.00	0.07(0.01)
22:0	0.31(0.04)	0.36(0.02)	0.11(0.01)	0.34(0.03)
24:0	0.33(0.04)	0.69(0.04)	0.03(0.01)	0.61(0.05)
25:0	0.02(0.01)	0.03(0.01)	0.02(0.01)	0.03(0.01)
26:0	0.16(0.02)	0.49(0.05)	0.05(0.01)	0.27(0.02)
28:0	0.01(0.01)	0.02(0.01)	0.01(0.01)	0.02(0.01)

[a] Notation of fatty acids, number of carbon atoms: number of double bonds. [b] Each value represents the mean (S.D) from five independent chromatographic analyses.

Table II. Branched-chain saturated fatty acids in *C. vigintipunctata* [a].

FA	Total	TAG	FFA	PL
i-12:0	0.03(0.01)	0.03(0.01)	0.02(0.01)	0.03(0.01)
i-13:0	0.05(0.01)	0.05(0.01)	0.01(0.01)	0.03(0.01)
ai-13:0	0.01(0.01)	0.02(0.01)	0.00	0.00
i-14:0	0.06(0.01)	0.07(0.01)	0.04(0.01)	0.05(0.01)
4,8,12-triMe-13:0	0.24(0.02)	0.23(0.02)	0.28(0.02)	0.21(0.02)
i-15:0	0.11(0.01)	0.12(0.01)	0.07(0.01)	0.08(0.01)
2-Me-14:0	0.07(0.01)	0.00	0.06(0.01)	0.08(0.01)
3-Me-14:0	0.44(0.04)	0.00	0.21(0.02)	0.33(0.03)
ai-15:0	0.09(0.01)	0.11(0.01)	0.08(0.01)	0.12(0.01)
2-Me-15:0	0.03(0.01)	0.00	0.03(0.01)	0.06(0.01)
i-16:0	0.26(0.01)	0.68(0.07)	0.02(0.01)	0.48(0.05)
Pristanic acid	0.10(0.01)	0.18(0.02)	0.01(0.01)	0.18(0.02)
7-Me-16:0	0.16(0.01)	0.00	0.09(0.01)	0.18(0.02)
5-Me-16:0	0.12(0.01)	0.00	0.14(0.01)	0.00
2-Me-16:0	0.24(0.02)	0.00	0.11(0.01)	0.16(0.01)
3-Me-16:0	0.03(0.01)	0.00	0.07(0.01)	0.00
i-17:0	0.06(0.01)	0.12(0.01)	0.03(0.01)	0.08(0.01)
ai-17:0	0.09(0.01)	0.22(0.02)	0.06(0.01)	0.16(0.01)
Phytanic acid	0.19(0.01)	0.38(0.04)	0.16(0.01)	0.33(0.03)
2-Me-17:0	0.12(0.01)	0.00	0.00	0.22(0.02)
3-Me-17:0	0.21(0.02)	0.00	0.00	0.38(0.03)
9-Me-18:0	0.13(0.01)	0.00	0.07(0.01)	0.15(0.01)
7-Me-18:0	0.11(0.01)	0.00	0.05(0.01)	0.05(0.01)
5-Me-18:0	0.22(0.02)	0.00	0.25(0.02)	0.11(0.01)
2-Me-18:0	0.05(0.01)	0.00	0.19(0.02)	0.09(0.01)
3-Me-18:0	0.24(0.02)	0.00	0.17(0.01)	0.30(0.03)
ai-19:0	0.09(0.01)	0.15(0.01)	0.17(0.01)	0.11(0.01)

[a] Notation of fatty acids and presentation of results as in Table I; i-,iso-; ai-, anteiso; Me, methyl; pristanic acid, 2,6,10,14-tetramethyl 15:0; phytanic acid, 3,7,11,15-tetramethyl 16:0.

Table III. Monoenoic fatty acids in *C. vigintipunctata*[a].

Fatty acids	Total lipids	TG	FFA	Polar
12:1n5	0.06(0.01)	0.06(0.01)	0.06(0.01)	0.06(0.01)
13:1n5	0.01(0.01)	0.01(0.01)	0.00	0.01(0.01)
14:1n7	0.16(0.01)	0.18(0.02)	0.06(0.01)	0.12(0.01)
14:1n5	0.03(0.01)	0.02(0.01)	0.02(0.01)	0.04(0.01)
15:1n9	0.40(0.04)	0.05(0.01)	0.15(0.01)	0.06(0.01)
15:1n7	0.21(0.02)	0.25(0.02)	0.26(0.02)	0.21(0.02)
16:1n9	4.87(0.37)	2.68(0.27)	7.69(0.78)	6.37(0.58)
16:1n7	0.93(0.08)	1.12(0.14)	0.47(0.04)	0.60(0.05)
t-16:1n13	0.38(0.05)	0.12(0.01)	0.39(0.04)	0.59(0.05)
16:1n5	0.21(0.02)	0.18(0.01)	0.15(0.01)	0.20(0.02)
i-17:1n9	0.23(0.02)	0.05(0.01)	0.36(0.03)	0.05(0.01)
ai-17:1n9	0.07(0.01)	0.01(0.01)	0.14(0.01)	0.00
17:1br	0.05(0.01)	0.13(0.01)	0.05(0.01)	0.10(0.01)
17:1n9	0.03(0.01)	0.06(0.01)	0.08(0.01)	0.06(0.01)
17:1n7	0.01(0.01)	0.03(0.01)	0.00	0.01(0.01)
18:1n13	0.14(0.01)	0.13(0.01)	0.16(0.01)	0.13(0.01)
18:1n11	0.24(0.02)	0.22(0.02)	0.78(0.08)	0.22(0.02)
18:1n9	25.15(1.83)	31.38(2.99)	17.69(1.55)	15.34(1.72)
18:1n7	0.47(0.04)	0.29(0.03)	0.44(0.04)	0.58(0.06)
18:1n5	0.24(0.02)	0.23(0.02)	0.31(0.03)	0.23(0.02)
19:1br	0.63(0.05)	0.15(0.01)	0.58(0.06)	0.11(0.01)
19:1br	0.10(0.01)	0.18(0.02)	0.19(0.02)	0.09(0.01)
19:1n8	0.26(0.03)	0.05(0.01)	0.25(0.02)	0.06(0.01)
20:1n15	0.05(0.01)	0.09(0.01)	0.11(0.01)	0.10(0.01)
20:1n13	0.21(0.02)	0.43(0.04)	0.14(0.01)	0.39(0.03)
20:1n11	0.25(0.02)	0.52(0.05)	0.25(0.02)	0.46(0.05)
20:1n9	0.64(0.07)	1.08(0.11)	0.32(0.03)	1.19(0.11)
20:1n7	0.16(0.02)	0.49(0.05)	0.00	0.30(0.03)
21:1n8	0.17(0.02)	0.01(0.01)	0.02(0.01)	0.00
22:1n15	0.13(0.01)	0.31(0.03)	0.04(0.01)	0.23(0.02)
22:1n13	0.15(0.01)	0.56(0.05)	0.02(0.01)	0.28(0.03)
22:1n11	0.28(0.02)	0.64(0.06)	0.03(0.01)	0.51(0.04)
22:1n9	0.16(0.01)	0.74(0.07)	0.02(0.01)	0.29(0.03)
22:1n7	0.03(0.01)	0.24(0.02)	0.02(0.01)	0.05(0.01)
23:1n9	0.01(0.01)	0.02(0.01)	0.01(0.01)	0.02(0.01)
24:1n11	0.14(0.01)	0.27(0.02)	0.03(0.01)	0.25(0.03)
24:1n9	0.31(0.03)	0.53(0.06)	0.06(0.01)	0.51(0.04)
25:1n9	0.01(0.01)	0.01(0.01)	0.02(0.01)	0.01(0.01)
26:1n11	0.02(0.01)	0.01(0.01)	0.01(0.01)	0.03(0.01)
26:1n9	0.05(0.01)	0.06(0.01)	0.01(0.01)	0.07(0.01)
27:1	0.01(0.01)	0.01(0.01)	0.01(0.01)	0.00
28:1	0.01(0.01)	0.01(0.01)	0.01(0.01)	0.01(0.01)
30:1	0.01(0.01)	0.02(0.01)	0.00	0.02(0.01)

[a] Notation of fatty acids and presentation of results as in Table I; n#, position of double bond.

The normal chain saturated FA (25.7% of the total, Table I) were represented by a homologue series of C12–C28 with even and odd number of carbon atoms. 23:0 was not detected, probably because of the very low amount. Logically, TAG were more saturated than FFA and PL. 16:0 was the main component in all lipid fractions, followed by 18:0.

Table II presents the complex mixture of twenty-eight iso-, anteiso- and methyl-branched saturated FA with even and odd number of carbon atoms. These FA comprised only 3.5% of the total extract with PL containing the highest proportion and the most complex mixture while few such FA

Table IV. Polyenoic fatty acids in *C. vigintipunctata*[a].

Fatty acids	Total lipids	TG	FFA	Polar
14:2n5	0.09(0.01)	0.05(0.01)	0.11(0.01)	0.05(0.01)
16:2n4	0.26(0.02)	0.28(0.03)	0.14(0.01)	0.43(0.04)
18:2n9	0.51(0.04)	0.56(0.06)	0.46(0.05)	0.38(0.04)
18:2n6	11.38(1.08)	5.73(0.61)	15.88(1.61)	15.40(1.52)
20:2n9	0.59(0.06)	0.00	0.66(0.07)	0.99(0.10)
20:2n6	0.34(0.03)	0.00	0.46(0.05)	0.64(0.05)
20:2n3	0.41(0.04)	0.00	0.31(0.03)	0.76(0.08)
22:2n6	0.04(0.01)	0.01(0.01)	0.04(0.01)	0.08(0.01)
26:2n6	0.03(0.01)	0.00	0.09(0.01)	0.01(0.01)
16:3n6	0.39(0.04)	0.02(0.01)	0.39(0.04)	0.43(0.04)
16:3n3	0.66(0.07)	0.20(0.02)	0.55(0.05)	1.22(0.024)
18:3n6	1.82(0.17)	0.38(0.03)	5.86(0.49)	3.00(0.34)
18:3n3	4.77(0.45)	14.58(1.66)	6.50(0.57)	8.34(0.91)
20:3n9	0.36(0.03)	0.00	0.49(0.04)	0.66(0.05)
20:3n6	0.42(0.05)	0.00	0.56(0.05)	0.78(0.08)
20:3n3	0.94(0.10)	0.00	0.36(0.04)	1.19(0.12)
22:3n6	0.04(0.01)	0.00	0.03(0.01)	0.07(0.01)
22:3n3	0.16(0.01)	0.00	0.19(0.02)	0.30(0.03)
16:4n3	0.72(0.07)	0.00	0.38(0.04)	1.35(0.12)
16:4n1	0.31(0.03)	0.00	0.17(0.02)	0.58(0.07)
18:4n3	0.94(0.09)	0.00	1.23(0.13)	0.66(0.08)
20:4n6	2.05(0.21)	0.00	2.41(0.24)	3.44(0.42)
20:4n3	0.73(0.05)	0.00	0.46(0.05)	0.71(0.09)
20:5n3	0.68(0.06)	0.00	0.64(0.06)	0.52(0.06)
22:4n6	0.67(0.06)	0.00	0.86(0.09)	0.56(0.05)
22:5n6	0.41(0.05)	0.00	0.34(0.04)	0.49(0.06)
22:4n3	0.60(0.06)	0.00	0.45(0.04)	0.59(0.06)
22:5n3	0.26(0.03)	0.00	0.16(0.01)	0.45(0.06)
22:6n3	1.40(0.17)	0.00	0.87(0.09)	2.10(0.22)

[a] Notation of fatty acids and presentation of the results as in Table I; n# position of the first double bond.

were detected in TAG (Table II). Iso-16:0 was the main component in TAG and PL with 0.68% and 0.48%, respectively. 4,8,12-trimethyl-13:0 (0.28%) was the main component in FFA fraction. Most of the these FA were presented in trace amounts only.

Monounsaturated FA were most abundant in *C. vigintipunctata* with 37.7% of the total content. Forty three monoenoic components were identified in total. The highest proportion was found in TAG (Table III). Chain-lengths varied in the interval of C12 to C30 and components with both even and odd number of carbon atoms were detected. All double bonds were in *cis*-configuration with one exception: *trans*-16:1(n-13). Most of the monoenes, especially 18:1, 20:1 and 22:1, were represented by more than one positional isomer although some species were detected in trace amounts only. Also present were some branched-chain 17:1 and 19:1. *Cis* 18:1(n-9) was by far the most abundant component in all lipid classes followed by 16:1(n-9). The other monoenes presented were detected in amounts lower than 1%.

The composition of the dienoic FA (13.6% of the total) is given in Table IV. Nine *cis*-dienes with even number of carbon atoms were determined comprising a homologue series of chain-lengths in the interval C14–C22; present also was 26:2. Positional isomers of 18:2 and 20:2 were identified. The proportion of dienes in FFA and PL was high while few components only were detected in TAG. 18:2(n-6) was the major component in all three lipid classes.

Nine positional isomers of 16:3, 18:3, 20:3 and 22:3 were detected (9.6% of the total, Table IV). 18:3(n-3) was the dominating component in all lipids classes. The proportion of the trienes in TAG, FFA and PL was almost the same but while FFA and PL contained all nine trienes, only four trienoic FA were identified in TAG and of these 18:3(n-3) comprised 96%.

Table IV presents also the eleven FA with more than three double bonds, denoted in this work as polyenoic fatty acids (PUFA). These FA comprised 8.8% of the total FA. TAG contained no PUFA, while positionally isomeric C16, C18, C20 and C22 tetraenes, C20 and C22 pentaenes and 22:6(n-3) were detected in FFA and PL.

Summarizing the results, firstly, they revealed that:the lipids of *C. vigintipunctata* contained relatively low but measurable quantities of iso-, anteiso-, and branched- chain saturated FA with odd and even number of carbon atoms, concentrated predominately in PL and FFA. The occurrence of such FA were expected in insects but representatives were seldom identified (Nelson, 1993). Secondly, series of positional isomeric mono-, di-, tri-tetra- and penta-unsaturated FA were detected including practically all biologically significant positional isomers of the series (n-3) and (n-6). So far, higher amounts of PUFA were reported for aquatic insects only (Hanson *et al.*, 1985; Chioni *et al.*, 1996). Thirdly, the lipids of *C. vigintipunctata* contained a low but clearly measurable amounts of unsaturated eicosanoic fatty acids. These acids are considered as important precursor of eicosanoids in insects (Stanley-Samuelson, 1993), but so far traces only were found in chrysomelids (Ogg *et al.*, 1993). Fourthly, to the best of our knowledge to date the presence of hydroxo fatty acids has been reported only in cuticular lipids of aphids (Stransky *et al.*, 1972).

Although, no data about the fatty acid composition of the diet were available at this stage, it is

generally known that plants do not synthesize longer chain (beyond C20) polyunsaturated fatty acids and positionally isomeric unsaturated FA are relatively rare (Gunstone, 1994; Lie Ken Jie and Khysar Pasha, 1998). Thus, it can be assumed that most of the 115 FA determined in the lipids of the *C. vingintipunctata* are a product of the insect own metabolism. So far, ascribed to the diet was only the single *trans* FA, 9–16:1, which is a typical plant FA (Padley *et al.,* 1994).

It is evident from these data that the fatty acid composition of leaf beetles is a very complex mixture of components. We trust that these data will be of help for better understanding of lipid metabolism in chrysomelids.

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