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IDENTIFICATION OF CYCLOPROPYL FATTY ACIDS IN WALNUT (*JUGLANS REGIA* L.) OIL

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Aim: Identification of cyclopropyl fatty acids in walnut oil.

Method: GC/MS method was developed for the determination of eight cyclopropyl fatty acids in walnut (*Juglans regia*) oil.

Results: Monocyclopropane acids: methyl 9-cyclopropyl-nonanoate, 6,7-methylene-, 8,9-methylene-, 9,10-methylene-, 11,12-methylene octadecanoates, and dicyclic acid – methyl 9,10,12,13-dimethylene octadecanoate, tricyclic acid – methyl 9,10,12,13,15,16-trimethylene octadecanoate, and unsaturated - methyl 2-octylcyclopropene-1-octanoate were detected in walnut oil by GC-MS and their mass spectra studied. Four cyclic fatty acids were identified for the fist time in seed oils.

Conclusions: Eight cyclopropyl fatty acids were detected in the Mediterranean nuts for the first time.

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) are found in at least two phylogenetically distant groups of plants belonging to families: Bombacaceae, Malvaceae, Sterculiaceae[1-4], Leguminosae[5], and Rannunculaceae[6]. Most of the seed oils consist predominantly of triacylglycerols. Fatty acids in the plant kingdom are mainly saturated or olefinic unsaturated straight-chain C_{16} or C_{18} compounds with a terminal carboxyl group. In the diene and triene derivatives, respectively, the double bonds are interrupted by a methylene group. Typical members incorporating these structure types are palmitic, stearic, palmitoleic, oleic, linoleic, and linolenic acid. Although they are best known as components of seed oils, CPFA do occur in vegetative plant parts[6-10].

CPFA have been the subject of many investigations due to their carcinogenic[11, 12], cocarcinogenic[13-15] and other biological, medical and mitogenic effects on animals[16-18]. The presence of CPFA in food is dangerous to human health[19-23].

It is known that many chemical reactions such as oxidation, polymerization, hydrolysis, isomerization and cyclization occur during deep fat frying.[19] These reactions lead to the formation of fatty acid geometrical isomers[24], dimers[25, 26], CPFA[27-31] and polymeric triglycerides[32-34] as well as some oxidative components[24, 35]. It has been found that seed oils containing mono-, di- and tri- unsaturated fatty acids when heated to over 200 °C in the presence of air form a number of cyclic fatty acids, including CPFA[36-41].

This work is a continuation of our previous investigation of Mediterranean nut oils[42] and presents new data about the CPFA formed from unsaturated fatty acids in nut oils rich particularly in linolenic and linoleic acids.

EXPERIMENTAL

Samples

Walnuts (*Juglans regia*) were purchased in a local market in Jerusalem in February 2005.

Analytical procedure

The walnuts (500 g) were shelled, and the kernels were homogenized in a high-speed unit. They were then spread as a layer (0.2 см) in a special glass box with an open upper cover and exposed to sunlight for 100 days. After 30, 70 and 100 days, samples were used for GC/MS analysis. The lipids were extracted with a mixture of chloroform-methanol (2:1, v/v), from control and three samples (30, 70 and 100 days) according to an established procedure[43], followed by addition of a saturated solution of sodium chloride in water. The mixture was vigorously shaken for 30 s. After phase separation the lower layer was removed, dried over sodium sulphate, and filtered. The oil residue was dissolved in CH_2Cl_2 and stored at –20 °C prior to GC/MS analysis.

GC/MS analysis of the methyl esters of fatty acids was performed on a Hewlett Packard 6890 series II chromatograph linked to a HP 5973 Mass detector equipped with HP automatic injector and a 30 m long, 0.25 mm ID, 0.25 μm film thickness HP-5MS capillary column. The ionization energy was 70 eV. The carrier gas was He (flow 1.0 ml/min). The temperature of the injection block was 250 °C. The GC oven temperature was programmed as

Table 1. Distribution of cyclopropyl fatty acids in walnut oil.

Fatty acids (wt % of total ME FAs)	Control	30 days	70 days	100 days
Total Saturated	4.81	5.28	5.99	5.30
16:0	3.62	3.79	4.04	3.63
18:0	1.19	1.49	1.95	1.67
Total unsaturated	92.85	88.03	83.18	76.01
(Z)-9-18:1	21.34	18.86	15.88	13.25
(Z,Z)-9,12-18:2	65.40	64.21	63.96	60.51
(Z,Z,Z)-9,12,15-18:3	6.11	4.96	3.34	2.25
Total CPFA	0.16	1.73	2.96	5.57
1. 9-cyclopropyl-nonanoate	n.d.	n.d.	0.21	0.43
2. 6,7-methylene octadecanoate	n.d.	0.12	0.18	0.28
3. 8,9-methylene octadecanoate	n.d.	0.22	0.24	0.31
4. 9,10-methylene octadecanoate	0.16	0.97	1.18	2.29
5. 2-octylcyclopropene-1-octanoate	n.d.	0.11	0.45	0.67
6. 11,12-methylene octadecanoate	n.d.	0.31	0.42	0.62
7. 9,10,12,13-dimethylene octadecanoate	n.d.	n.d.	0.11	0.38
8. 9,10,12,13,15,16-trimethylene octodecanoate	n.d.	n.d.	0.17	0.59
Total dioic, keto, oxygenated, other	2.18	4.96	7.87	13.12

Structures CPFA refer to Fig. 1.
Abbreviations: n.d., not detected.

follows: initial temperature 60 °C (2 min) followed by a temperature increase of 3 °C/min up to 260 °C (10 min) and second rate of 10 °C/min to the final temperature of 290 °C (10 min). Cyclopropyl FA were identified using Wiley 7th Edition Library. Mass spectra and comparion with mass spectra published by other researchers.

RESULTS AND DISCUSSION

GC-MS analyses of all studied samples are presented in Table 1. Control samples contained only cyclopropane acid-9,10-methylene octadecanoate (4, 0.16 % of total fatty acids). It was found that after sunlight exposure some other CPFA were produced (Fig. 1). During our experimental work, the air temperature in Jerusalem ranged from 36 to 46 °C (June – August, 2005). The concentration of CPFA increased during the experiment, and reached 5.57 % of total fatty acids (Table 1). Many other oxygenated lipid compounds as well as cyclopropyl fatty acids were detected in walnut oil: dioic (dicarboxylic), keto, hydroxy, and epoxy fatty acids (not discussed in this paper). Their concentration was found up to 13 %. Mass spectra of the four cyclopropyl fatty acids are shown in Fig. 2.

Cyclopropane fatty acids, the most prevalent of the cyclic fatty acids, are widely distributed among both Gram-positive and Gram-negative bacteria, including *Brucellaceae, Clostridia, Enterobacteria, Lactobacilli*, and *Streptococci*[4, 44], *cis*-11,12-Methyleneoctanoic (lactobacillic) acid 6, and *cis*-9,10-methylenehexadecanoic acid are the most commonly found cyclopropanoid acids in bacteria. An unusual fatty acid containing six cyclopropane rings was recently isolated from *Streptomyces* sp.[45, 46].

Previously, dihydrosterculic acid (4) as well as sterculic acid (5) were found in *Pachira aquatica* seed oils[47]. The biosynthesis of cyclopropane and cyclopropene fatty acids has been discussed in some reviews[48, 49].

CPFAs have also been found in the slime mold *Polysphobdylium pallisum*[50], the freshwater amphipod *Acanthogammarus grewingkii*[51], and in some marine invertebrates[52]. CPFA has also been isolated from some pathogenic fungi[53, 54].

The chemistry of CPFA [55] including their mass spectra has been discussed in several papers[56-58]. Both in these publications and as well as from our own data, the characteristic peaks for monounsaturated methyl esters contain *m/z* M-32 (loss of methanol from the ester function), *m/z* M-74 (loss of the ester group plus one carbon from the chain) and *m/z* M-116 (loss of the ester group plus four carbons from the chain) (see Fig. 2). Peaks which might be expected by fragmentation on either side of the cyclopropane rings at *m/z* 113 and 197 or 153 and

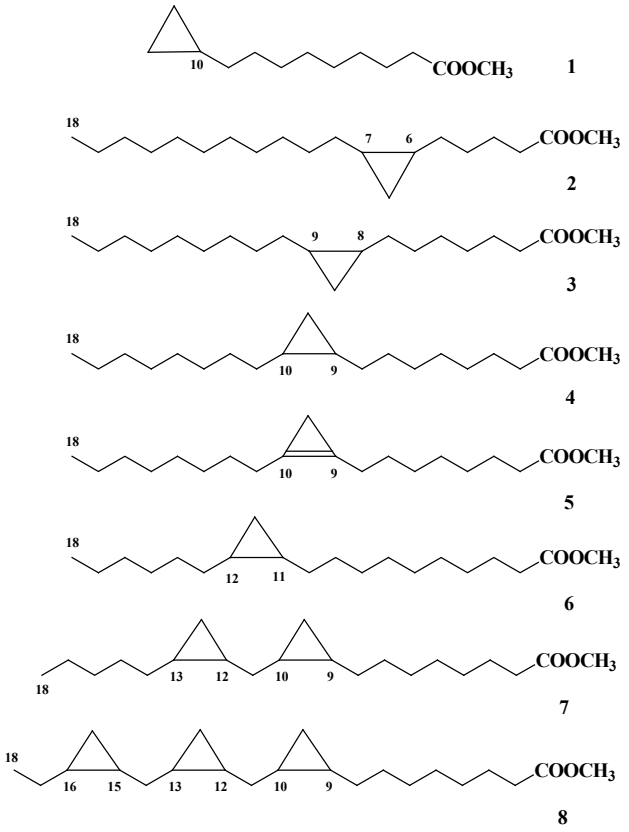

Fig. 1. Structural formulae of cyclopropyl fatty acids identified from walnut oil.
Names of the acids referred to in Table 1.

157 are more prominent in the mass spectrum of the cyclopropane fatty acid than in that of the parent ester.

GC/MS has been so far the method of choice for identifying the cyclopropane fatty acids in fats and oils formed during heating or similar treatment. However, the interpretation of the mass spectra is often difficult. According to[30, 36-41] the methyl ester of octadecanoic acids that contain cyclopropyl ring(s) give four characteristic fragments A, B, C and D, which are found in the mass spectra (see Fig. 2 and Fig. 3).

Electron impact mass spectrometry of methyl ester C_{18} fatty acids give fragments indicated in Fig. 3. Additional ion fragments are D-32 (loss of methanol and D-32-18 (further loss of water). A (B + 1) fragment is present in most spectra due to a protonation of fragment B. All these fragments are present in mass spectra of identified CPFA in the present work.

The results of this study show for the first time the presence of eight cyclopropyl fatty acids in the Mediterranean walnut. We do not know the origin of CPFA in walnut oil. However, it is possible that the CPFA originates from the intensively growing microflora promoted by sunlight exposure.

Other reasons why these compounds are present in walnuts oil include: the fact that CPFAs are formed from unsaturated fatty acids due to transformations such as oxidation and cyclization during heating fats and oils[36-41].

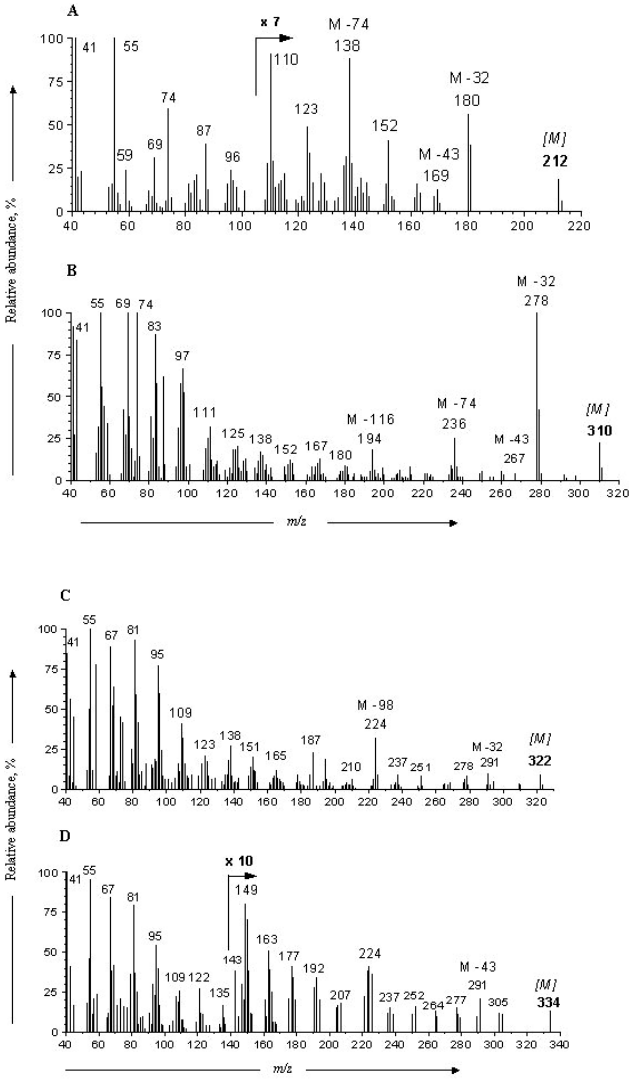

Fig. 2. Mass spectra of methyl esters of cyclopropyl fatty acids identified from walnut oil.
A. Cyclopropanenonanoic acid (**1**),
B. 2-Hexylcyclopropanedecanoic acid (**6**),
C. 2-[(2-pentylcyclopropyl)methyl]cyclopropane octanoic acid (**7**),
D. 2-[[2-ethylcyclopropyl)methyl]cyclopropyl]methyl]cycpropaneoctanoic acid (**8**).
Structures refer in Fig. 1.

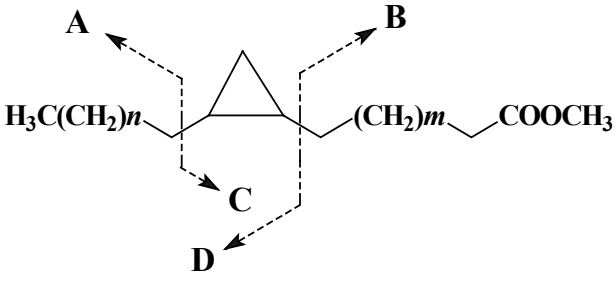

Fig. 3. Common EI mass spectral fragmentations of the cyclopropyl fatty acids.

Another possibility is that these compounds represent metabolites that could be formed by the microflora found in walnuts. The microorganisms containing CPFA[4, 44, 59] isolated from walnuts include: *Pseudomonas flavescens* sp. nov.[60], *Penicillium crustosum*[61], *Aspergillus glaucus*, *A. niger, A. flavus, A. candidus, Penicillium cyclopium*, *P. viridicatum*[62], and *A. parasiticus*[63, 64]. Isolates of the genera *Alternaria, Cladosporium, Fusarium*, and *Helminthosporium* have also been found in walnuts[62]. *Humicola grisea* var. *themoidae* and *Thermoascus aurantiacus* are rarely encountered in walnuts[63, 64]. Those microorganisms living at 45 °C and over[63, 64] may also produce and accumulate mycotoxins, CPFA[53, 54] and other metabolic products are degradation products formed by these microorganisms[54]. Which of these hypotheses is right, is difficult to say. In our opinion, it is the combination of the above hypotheses that can best explain the phenomenon.

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