Lipid Analysis of Greek Walnut Oil (*Juglans regia* L.) [§]

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The walnut oil (*Juglans regia* L.) total lipids (TL) were extracted by the Bligh-Dyer method and the lipid classes have been isolated by chromatographic techniques and they were analyzed by high performance thin layer chromatography (HPTLC) /FID and GC-MS. The oil was found to be rich in neutral lipids (96.9% of total lipids) and low in polar lipids (3.1% of total lipids). The neutral lipid fraction consisted mainly of triacylglycerides whereas the polar lipids mainly consisted of sphingolipids. GC-MS data showed that the main fatty acid was linoleic acid. Unsaturated fatty acids were found as high as 85%, while the percentage of the saturated fatty acids was found 15%. Two types of liposomes were prepared from the isolated walnut oil phospholipids and characterized as new formulations. These formulations may have future applications for encapsulation and delivery of drugs and cosmetic active ingredients.

Introduction

The walnut tree (*Juglans regia* L, Juglandaceae) is native in southeastern Europe, Asia Minor, India and China. Some species of the walnut tree is now cultivated throughout Europe, North America, North Africa and East Asia. The beneficial action of walnut oil on skin is known for centuries and it is widely used in cosmetic manufacturing industry. The walnut oil is a component of dry skin creams, antiwrinkle and antiaging products, because it presents moisturizing properties as well as free radical scavenging capacity (Espin *et al*., 2000). Apparently the use of the walnut oil for cosmetic purposes may be due to its high content in essential fatty acids and in particular linoleic and linolenic acids (Karleskind, 1996). These acids are referred as crucial agents of the most important function of the skin, which is the regulation of the transepidermal water loss (Qiang *et al*., 1993). Liposomes are hollow spheres of lipid bilayers, which are formed mainly of phospholipid molecules. Liposome formulations are widely used as media for active ingredients and lipid transfer to the skin (Hatziantoniou *et al*., 2000). The objective of this study was the detailed chemical characterization and identification of the lipid fractions of the walnut oil, and the preparation of liposomes from isolated lipids, with a high content of linoleic and linolenic acid.

Experimental

Materials

Walnut seeds (*Junglans regia* L., fam. Junglandaceae*)* were collected in Evia island (Greece). Dr D. Perdetzoglou (Lab. of Pharmacognosy, University of Athens) identified the plant material and a voucher specimen has been deposited in the Herbarium of the Laboratory of Pharmacognosy, University of Athens (Greece). TLC standards such as cholesteryl ester, trioleylglycerol, oleic acid, cholesterol (CH), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phophatidic acid (PA), cardiolipine (CL), sphingomyeline (SM), ceramides (type III and type IV), galactosylceramides (type 1 and type 2) were purchased from Sigma Chemical Co. (St. Louis, MO). Lipids for liposome preparation i.e. cholesterol and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids, In. (Alabaster, Alabama USA). Hexane, chloroform, methanol, diethy-

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lether and acetic acid were analytical grade and purchased from Labscan Ltd. Ireland. Salts and buffers were purchased from Sigma Chemical Co.

Total lipid extraction

After peeling, the walnut seeds were weighed and the total lipids extracted according to Bligh and Dyer (1959). The solvent was removed by a rotary evaporator at 30 °C and the remainder (a yellow oil) was weighed and stored in the dark at −18 °C diluted with $CHCl_3$ /CH_3OH 9:1 v/v, to 100 ml.

Isolation of lipid classes

The separation of neutral and polar lipids of the extracted walnut oil was performed by vacuum liquid chromatography (VLC) using silica gel 60 (230–400 mesh Merck). The solvent mixtures for the isolation of the lipid classes were: $CHCl_3$ 100% for the neutral lipids, $CHCl_3$ /CH_3OH 99:1 to 95:5 v/v for the sphingolipids and CH_3OH 100% for the phospholipids (Karleskind, 1996). The isolated lipid classes were monitored by TLC and classified as sample I, sample II and sample III, using standards for their identification.

Qualitative and quantitative analysis of the lipid classes

The analysis of the walnut oil lipid classes was carried out with a High Performance Thin Layer Chromatography /FID analyzer (Iatroscan TH-10, Iatron Lab. Inc., Tokyo, Japan). Hydrogen flow rate was 160 ml/min, air flow rate 1900 ml/min, scan speed 23 sec/scan, silica gel, 75 μ precoated thin-layer rods (Chromarods-SII, Iatron Lab. Inc.) in set of 10 were used as stationary face.

Chromarod development and scanning

Three samples (1 μl each) were analyzed on the Chromarods: Walnut oil's total lipids (sample I), polar lipid fraction (sphingolipids and phospholipids mixture) (sample II) and pure phospholipid fraction (sample III). Sample I was developed by n-hexane / diethyl ether / acetic acid (80:20:2 v/v/v), sample II (sphingolipids and phospholipids mixture) was developed by chloroform / methanol / water (90:10:1 v/v/v) and sample III was developed by chloroform / methanol / water (65:25:4 v/v/v) (Karleskind, 1996). After the development the Chromarods were dried at 100 °C for 1 min and scanned. Each sample was analyzed on four Chromarods and the average calculated.

Isolation and identification of fatty acids in total lipids and in polar lipid fractions

In order to examine in detail the fatty acid content of each lipid class, the sphingolipids as well as the phospholipids were further fractionated using preparative TLC. Chloroform / methanol / water (90:10:1 v/v/v) was used for fractionalizing of sphingolipid mixture while chloroform / methanol / water (65:25:4 v/v/v) was used to fractionalize the phospholipid mixture. The free fatty acids along with those esterified were converted to the corresponding methyl esters by 2 N KOH in methanol and vortexing for 2 min. The fatty acid methyl esters were then extracted by n-hexane and analyzed using GC-MS. The unsaponificable sphingolipid residue was subjected to further saponification to examine the amide-linked fatty acids adding 0.5 ml HCl 1 N in methanol (HCl 1 N, H_2O 10 M) and heating at 100 °C for five h. The fatty acid methyl esters were extracted by n-hexane.

The fatty acid methyl esters were analyzed by a Hewlett Packard (HP) 6890 gas chromatograph equipped with a 30 m HP-5 fused silica capillary of 0.25 mm film thickness and coupled by a Hewlett Packard 5973 mass spectrometer. The column was temperature programmed as follows: 170 °C for 15 min, temperature increase 2 °C/min until 220 °C were it stayed for 30 min. Injection port temperature 250 °C, detector port temperature 250 °C, Helium gas carrier pressure 53.1 kPa, split ratio 1:20. The injection volume was 1 μl and the fatty acids were identified using the Wiley 275k MS Database (Coudrec, 1995).

Isolation and analysis of sterols

The neutral lipid fraction isolated by vacuum liquid chromatography (VLC) was saponificated using KOH solution in ethanol. The non-saponifiable components were extracted by diethyl ether and the sterols obtained after purification using preparative TLC (n-hexane / diethyl ether / acetic acid (70:30:1 v/v/v)). The sterols were converted to volatile trimethylsilyl-derivatives suitable for GC

analysis. The gas chromatograph was equipped with a 30 m SGE fused silica capillary column (0.32 mm I. D.) packed with HPx5 (non-polar). The column temperature was 265 °C were it stays for 41 min. Injection port temperature 270 °C, detector port temperature 285 °C, Helium gas carrier pressure 125 kPa, split ratio 1:55. The injection volume was 1 μl and the sterols were identified by comparing retention times to standards purchased from Sigma (Hamilton and Hamilton 1993).

Differential scanning calorimetry (DSC)

The transition temperature (Tm) of the phospholipid fraction of the walnut oil was determined by a Perkin Elmer 7 series thermal analysis system (DSC 7). 7 mg of phospholipids were weighed into appropriate pans and sealed after adding 7 μl water. The heating rate was 10 °C/min and the curve was recorded from −10 to 50 °C.

Liposome preparation

Liposomes were prepared from walnut oil phospholipids and mixture of walnut oil phospholipids with DPPG (3:1 molar ratio) using the "thin lipid film hydration" method (Lawrence *et al.*, 1990). The lipid film was prepared by dissolving the lipid mixture in chloroform, which was slowly evaporated in a flash evaporator. Multilamellar vesicles (MLVs), were prepared by adding 2 ml aqueous solution of ammonium sulfate 150 nM (p H = 5.5, 535 mOsm), and vigorous shaking in a water bath at 50–60 °C to achieve a final concentration of 5% lipids (w/v). The samples were then maintained above the phase transition temperature for one hour to allow the equilibration of the water across the lipid bilayers. The size of the vesicles was reduced by freeze and thaw for 10 times. The resultant large oligolamellar vesicles were extruded 10 times through an extruder device, equilibrated at the same temperature as that of the lipids phase transitional temperature. The extruder was fitted with two polycarbonate filters with a pore diameter 200 and 100 nm respectively. The liposomes were visualized by transmission electron microscopy after negative staining. The liposome size was evaluated by the diameter of the resultant vesicles.

Results and Discussion

Lipid classes of walnut oil

Walnut oil showed the presence of all the lipid classes. Neutral lipids were in high amounts (96.9% of total lipids) and the predominant lipid class were the triacylglycerides and steryl esters (94.6%), while the polar lipids represented only 3.1% of total lipids. The polar lipids were found to be consisted of 73.4% sphingolipids and 26.6% phospholipids. The percentage of sphingolipids and phospholipids in walnut oil was 2.3% and 0.8% respectively. Further analysis of sphingolipids presented four distinctive groups of which type III and type IV ceramides were 64.7% and type 1 and type 2 galactosylceramides were 35.3% of total sphingolipids. In the phospholipid class the predominant phospholipid was phosphatidylethanolamine (48.5% of phospholipids).

Fatty acid composition of neutral lipids

Table I. The fatty acid (F. A.) composition (as methyl esters) of walnut oil.

F. A. as methyl esters		% of total F. A.
Tetradecanoic	(C14:0)	0.1
Pentadecanoic	(C15:0)	0.1
9-Hexadecenoic	(C16:1)	0.4
Hexadecanoic	(C16:0)	10.4
Heptadecanoic	(C17:0)	0.1
9,12-Octadecadienoic	(C18:2)	74.0
9,12,15-Octadecatrienoic	(C18:3)	10.0
Octadecenoic	(C18:1)	tr*
Octadecanoic	(C18:0)	3.9
11,14-Eicosadienoic	(C20:2)	tr
Nonadecanoic	(C19:0)	tr
11-Eicosenoic	(C20:1)	0.6
Eicosanoic	(C20:0)	0.3
Heneicosanoic	(C21:0)	tr
Docosanoic	(C22:0)	0.1
total		100.0
Saturated		15.0
Unsaturated		85.0
Unsat/Sat ratio		*5.7*
[a] **MUFA's**		1.0
[b] **PUFA's**		84.0
PUFA's/MUFA's		*84.0*

* *tr : trace*
[a] **MUFA's :** Monounsaturated fatty acids
[b] **PUFA's :** Polyunsaturated fatty acids

The most abundant fatty acid was linoleic acid (9,12-octadecadienoic acid), representing 74.0% of fatty acids in walnut oil (Table I). The amount of the unsaturated fatty acids is very high (85.0%) while the percentage of the saturated fatty acids was found 15.0%.

Fatty acid composition of sphingolipids

The sphingolipids were further fractionalized by preparative TLC, as described previously yielding four sub-fractions. The composition of ester- and amide-linked fatty acids of total sphingolipids as well as that of each sample was determined by GC-MS after hydrolysis. Three fatty acids were predominant in the sphingolipids and its subfractions i.e. linoleic acid (9,12-octadecadienoic acid) (40.1–55.6%), linolenic (9,12,15-octadecatrienoic acid) (15.5–29.5%) and palmitic acid (hexadecanoic acid) (11.6–28.7%). The unsaturated / saturated ratio varied from 3.4 to 4.8 in all fractions except for fraction 3 in which it was 1.5. The PUFAs / MUFAs ratio was varied from 12.8 to 29.6 for the four subfractions (1–4) (Table II).

Fatty acid composition of phospholipids

In order to analyze the phospholipid class, further fractionation was achieved by preparative TLC. The fractionation resulted in 10 phospholipid subfractions. The analysis of the fatty acids (as methyl esters) of the total phospholipids as well as each subfraction was performed by GC-MS after hydrolysis of the phospholipids and trans-esterification of the resultant free fatty acids. The predominant fatty acid in total phospholipids and in subfraction 10 was linoleic acid (9,12-octadecadienoic acid) (50.9% and 54.1% respectively). Palmitic acid (hexadecanoic acid) varied from 20.7% to 54.6% in all the subfractions. Linolenic acid (9,12,15-octadecatrienoic acid) was present only in subfraction 10 (16.1%) and in total phospholipids in similar percentages (17.2%). Subfraction 6 contained only two fatty acids: pentadecenoic (62.2%) and stearic (octadecanoic acid) (37.8%). The unsaturated / saturated ratio varied from 2.4 to2.6 for the total phospholipids and for the subfraction 10. For the subfractions 1,2, 4, 5 and 6, varied from 0.8 to 1.6, and in the subfrac-

Table II. Fatty acid (F. A.) composition (as methyl esters) of walnut oil sphingolipid subfractions.

Fatty acid methyl ester		Total Sphingolipids		**1**		**2**		**3**		**4**	
		esteric	amidic	esteric	amidic	esteric	amidic	esteric	amidic	esteric	amidic
Tetradecanoic	(C14:0)	0.3	5.6	0.7	4.0	0.8	5.9	–	1.9	1.2	4.2
Hexadecanoic	(C16.0)	16.2	28.7	16.6	19.9	14.3	12.8	26.1	31.9	11.6	23.5
9,12-Octadecadienoic	(C18:2)	55.6	24.5	48.9	6.6	47.4	8.8	40.1	28.9	54.1	18.2
9,12,15-Octadecatrienoic	(C18:3)	19.4	18.3	25.5	29.5	25.1	39.6	15.5	15.9	25.9	24.9
9-Octadecenoic	(C18:1)	2.5	9.2	3.1	20.8	5.6	22.8	4.0	3.9	2.7	12.7
Octadecanoic	(C18:0)	4.5	13.7	3.6	19.2	5.1	10.0	9.2	13.5	3.6	16.5
Eicosanoic	(C20:0)	0.5	–	0.4	–	–	–	1.3	2.7	0.2	
Docosanoic	(C22:0)	1.1	–	1.2	–	1.7	–	3.7	1.2	0.5	
Total (%)		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Saturated		22.5	48.0	22.6	43.0	21.9	28.7	40.4	51.0	17.2	44.1
Unsaturated		77.5	52.0	77.4	56.9	78.1	71.2	59.6	48.9	82.8	55.8
Unsat/Sat ratio		*3.4*	*1.1*	*3.4*	*1.3*	*3.6*	*2.5*	*1.5*	*0.9*	*4.8*	*1.3*
[a] **MUFA′s**		2.5	9.2	3.1	20.8	5.6	22.8	4.0	3.9	2.7	12.7
[b] **PUFA′s**		75.0	42.8	74.3	36.1	72.4	48.4	55.6	44.9	80.1	43.2
PUFA′sMUFA′s ratio		*30*	*4.6*	*23.7*	*1.7*	*12.8*	*2.1*	*13.9*	*11.5*	*29.6*	*3.4*

Subfractions **1**–**4** represented on the above Table, correspond to: **1**:ceramide type III; **2**: ceramide type IV; **3**: glycosylceramide type 2; **4**: glycosylceramide type 1.
[a] **MUFA′s :** Monounsaturated fatty acids.
[b] **PUFA′s :** Polyunsaturated fatty acids.

tions 3, 7, 8 and 9 was 0.2. The polyunsaturated fatty acids were the most abundant of the unsaturated fatty acids of total phospholipids, and of the subfraction 1 and 10 (Table III). The PUFAs / MUFAs ratio was 29.2 in total phospholipids and varied from 0.0 to 32.1 for the ten subfractions (1–10).

Sterol composition

The predominant sterol is β-sitosterol (84.6% of sterols), while Δ5-avenasterol, campesterol and cholesterol are present in much lower persentages (7.3%, 4.6% and 1.1% respectively).

DSC analysis of the phospholipid fraction

In order to prepare liposomes walnut phospholipids, the thermotropic behaviour of this lipid fraction was studied. DSC analysis has shown that the onset temperature was 23 °C but it did not present a specific peak. This result was explained by the fact that the fraction is a mixture of many constituents. The liposome preparation was performed well above 36 °C, which was the maximum phase transition temperature of phospholipid fraction.

Liposome preparation

The first phospholipid formulation was prepared from pure walnut phospholipids. Due to the high content of phosphatidylethanolamine (48.5% of phospholipids) the phospholipid surface is positively charged. The charged surface of the liposomes may interfere with the active ingredient encapsulation capacity of the liposomes. To prepare non-charged liposomes, a negatively charged lipid i.e. dipalmitoylphosphatidylglycerol (DPPG) was added to the lipid mixture in a molar ratio 3:1. This fact indicates that the preparation of liposomes may play an important role in delivery of drugs and cosmetic ingredients loading into lipo-

Table III. Fatty acid (F. A.) composition (as methyl esters) of walnut oil phospholipid subfractions.

F.A. as methyl esters		Total phospholipids	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Dodecanoic	(C12:0)	0.6	–	–	27.1	–	–	–	–	–	–	0.1
Tetradecanoic	(C14:0)	0.8	5.0	7.9	15.5	6.1	5.8	–	–	–	–	0.1
Pentadecenoic	(C15:1)	–	–	6.5	–	4.9	3.9	62.2	–	–	–	–
Hexadecenoic	(C16:1)	–	–	12.3	–	9.5	8.3	–	–	–	–	–
Hexadecanoic	(C16:0)	22.6	31.9	32.9	29.1	34.8	32.0	–	54.6	54.3	51.5	20.7
9,12-Octadecadienoic	(C18:2)	50.9	23.0	5.3	2.7	11.9	18.2	–	4.5	5.7	–	54.1
9,12,15-Octadecatrienoic	(C18:3)	17.2	–	–	–	–	–	–	–	–	–	16.1
Octadecenoic	(C18:1)	2.3	24.9	20.5	10.9	20.6	21.1	–	14.0	13.5	19.1	2.2
Octadecanoic	(C18:0)	4.6	15.2	14.6	14.5	12.2	10.8	37.8	26.9	26.5	29.4	4.1
Eicosanoic	(C20:0)	0.3	–	–	–	–	–	–	–	–	–	0.3
Docosanoic	(C22:0)	0.7	–	–	–	–	–	–	–	–	–	1.3
Tricosanoic	(C23:0)	–	–	–	–	–	–	–	–	–	–	0.3
Tetracosanoic	(C24:0)	–	–	–	–	–	–	–	–	–	–	0.7
Total (%)		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Saturated		29.5	52.0	55.4	86.3	53.1	48.6	37.8	81.5	80.8	80.9	27.7
Unsaturated		70.5	47.9	44.5	13.7	46.9	51.4	62.2	18.5	19.2	19.1	72.3
Unsat/Sat ratio		*2.4*	*0.9*	*0.8*	*0.2*	*0.8*	*1.1*	*1.6*	*0.2*	*0.2*	*0.2*	*2.6*
[a] **MUFA′s**		2.3	24.9	39.3	11.0	34.9	33.3	62.2	14.0	13.5	19.1	2.2
[b] **PUFA′s**		68.1	23.0	5.3	2.7	11.9	18.2	0.0	4.5	5.7	0.0	70.1
PUFA's/MUFA's ratio		*29.2*	*0.9*	*0.1*	*0.3*	*0.3*	*0.5*	*0.0*	*0.3*	*0.4*	*0.0*	*32.1*

Subfractions **1–10** represented on the above Table, correspond to: **1:** PS; **2:**Pl; **3:** PA; **4:** PC; **5:** unidentified; **6:** unidentified; **7:** CL; **8:** PE; **9:** unidentified; **10:** unidentified.
[a] **MUFA′s :** Monounsaturated fatty acids.
[b] **PUFA′s :** Polyunsaturated fatty acids.

somes, due to their high content of DPPG which has been added to equilibrate the charge of walnut oil phospholipids (Maswadeh *et al*., 2000). In either case both liposome formulations (walnut oil phospholipids 100% and walnut oil phospholipids/ DPPG, 3:1 molar ratio) contained a high percentage of unsaturated acids (linoleic and linolenic acid) (Table III) the role of which may be essential for their future applications.

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