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Antithrombotic Lipid Minor Constituents from Vegetable Oils. Comparison between Olive Oils and Others

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Many epidemiological studies suggest that vegetable oils and especially olive oil present a protective effect against atherosclerosis. In this study, total lipids (TL) of Greek olive oils and seed oils of four kinds, namely, soybean, corn, sunflower, and sesame oil, were separated into total polar lipids (TPL) and total neutral lipids (TNL) via a novel extraction procedure. TPL and TNL of olive oil were fractionated by HPLC for further study. Each lipid fraction from HPLC separation along with TL, TPL, and TNL lipid samples from oils were tested in vitro for their capacity to induce or to inhibit washed rabbit platelet aggregation. Comparison between olive and seed oils supports the superiority of olive oil as high levels of platelet activating factor (PAF) antagonists have been detected, mainly in TPL. In addition, the structure of the most active fraction from olive oil was elucidated, as a glycerol−glycolipid. Because it has already been reported that PAF plays a pivotal role in atherogenesis, the existence of PAF agonists and antagonists in vegetable oils may explain their protective role against atherosclerosis.

KEYWORDS: Olive oil; sesame oil; corn oil; soybean oil; sunflower oil; bioactive lipids; high-performance liquid chromatography; Mediterranean diet; platelet activating factor; atherogenesis

INTRODUCTION

Vegetable oils and especially olive oil are traditionally considered to be basic components of the Mediterranean diet, which exhibits a protective effect against coronary heart disease. Regional cardiovascular morbidity and mortality data of the Western hemisphere show a particularly low prevalence in the Mediterranean region (*1*). Furthermore, epidemiological studies have shown a correlation between diet and the incidence of coronary heart disease (CHD). A lower incidence of CHD in Mediterranean countries has been correlated with the so-called Mediterranean diet, which is rich in vegetables, grains, legumes, fruits, and vegetable oils, mainly olive oil (*2*, *3*). Results from many clinical, experimental, and epidemiological studies are in accordance with this correlation (*4*). Thus, the Mediterranean diet has been suggested to reduce the incidence of CHD. The role of dietary lipids is being intensely studied by many authors, with regard to not only the amount but also the quality of the dietary fat. Thus, vegetable oils are considered to have anti-atherogenic effects (*5*, *6*).

Furthermore, it is proved that oxidatively modified low-density lipoproteins (ox-LDLs) become cytotoxic to endothelial cells and chemotactic for monocytes and exhibit an increased affinity for their scavenger receptor, thus stimulating the formation of foam cells loaded with cholesterol esters (*7*). These processes affect the structure and function of the intima and lead to the formation of atherosclerotic plaques. On the other hand, the response to injury hypothesis explains atherosclerosis as a chronic inflammatory response to injury of the endothelium, where complex cellular and molecular interactions are taking place among blood cells and cells derived from the endothelium (*8*, *9*).

As we previously described, we propose a mechanism by which platelet activating factor (PAF) (*10*) is implicated in atheromatosis generation (*11*) on the basis of the previous detection of PAF, which is produced during LDL oxidation (*12*) and causes in situ inflammation. This mechanism is further supported because PAF not only is the strongest inflammatory lipid mediator and is present as a constituent of atheromatic plaque but also is essential for the activation of leukocytes and their binding in the endothelial cells, which are critical events in atherogenesis (*13*). Furthermore, PAF antagonists exhibit protective action against atheromatosis generation (*14*, *15*) and PAF−acetylhydrolase, the main enzyme responsible for the degradation of PAF, exhibits reduced activity in ox-LDL relative to unmodified LDL (*12*).

Therefore, the detection of lipid compounds in Mediterranean foods, which inhibit PAF action, could explain their protective

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effect against atheromatosis generation. Until now the reported studies, although in general exhaustive, have focused mostly on fatty acids of vegetable oils (*16*). Other compounds that exist as microconstituents in vegetable oils are tocopherols, flavonoids, anthocyanins, hydroxy- and dihydroxyterpenic acids, sterols, phenolic constituents, and phospholipids. The amount of these compounds depends on various conditions such as climate, degree of ripeness of the fruit, and its handling (*17*). These compounds are also present in lower concentrations in low-quality oils, characterized by a high degree of acidity, that are subjected to further refining (*18*). Although there are a number of studies concerning some of these constituents (*19*−*21*), there are no studies concerning the bioactivity of phospholipids and glycolipids of vegetable oils. Moreover, studies have been generally restricted to changes in lipids and lipoproteins, without examining factors related to the thrombotic risks such as platelet aggregation. There is, however, still no adequate basis to explain a direct influence of any nutrient intake on the low cardiovascular mortality in this region.

The effect of dietary fat on plasma lipid concentration, especially on the cholesterol level, has also been extensively studied (*22*−*24*). One of the most important reasons for all of these studies is that hypercholesterolemia is thought to be an important cause of coronary heart disease (*25*). However, high cholesterol levels seem to play a secondary role because even the same cholesterol level can result in an important variation in the clinical expression of the disease (*26*). Furthermore, although there is a similar distribution of other coronary risk factors such as high blood pressure, serum cholesterol, body mass index, and cigarette smoking, the mortality due to CHD is lower in Crete, where the population follows a traditionally Cretan Mediterranean diet (*27*). Previous studies in our laboratory demonstrated the existence of compounds that inhibit PAF aggregatory action in olive oil (*28*), honey (*29*), milk and yogurt (*30*), fish (*31*), and red wine (*32*).

The interest in research on the biological effects of lipid extracts from vegetable oils, such as their ability to antagonize factors such as PAF and thrombin on platelet aggregation and the importance of these dietary compounds in the prevention of CHD, prompted us to investigate the effects of total, neutral, and polar lipids from vegetable oils, before and after separation and purification with HPLC, on platelet aggregation. It is a real challenge and absolutely necessary to determine the biologically active lipid microconstituents in order to understand not only their bioactivities and possible side effects but also mechanisms of pathophysiological conditions such as atherogenesis, which remain an unresolved controversy.

This work aims to evaluate the bioactivity of lipid fractions of various kinds of vegetable oils and to elucidate the structure of bioactive lipids. The results of this study raise the question of whether total lipid bioactivity could serve as a nutritional index.

MATERIALS AND METHODS

Instrumentation. The separation of lipids was performed at room temperature on an HP HPLC aeries 1100 liquid chromatograph model (Hewlett-Packard, Waldbronn, Germany) equipped with a 100 μL loop Rheodyne (7725 i) loop valve injector, a degasser G1322A, a quat gradient pump G1311A, and an HP UV spectrophotometer G1314A as a detection system. The spectrophotometer was connected to a Hewlett- Packard model HP-3395 integrator−plotter. Neutral lipids were separated on a Nucleosil-300 7 μm C-18 reverse column (250 × 4.6 mm i.d.) from Analysentechnik (Wöehlerstrasse, Mainz, Germany) with a C-18 ODS (20 × 4.0 mm i.d.) precolumn cartridge. For the separation of the polar lipids was used either a Partisil 10 μm SCX column (250 × 4.6 mm i.d) from Analysentechnick with an SCX (20 × 4.0 mm i.d.) precolumn cartridge or a Sphereclone 5u NH_2 normal phase column (250 × 4.6 mm i.d.) from Phenomenex (Hurdsfield, Cheshire, U.K.) with an NH_2 silica (20 × 4.0 mm i.d.) precolumn cartridge and a Kromasil 100 5 μm silica column (250 × 4.6 mm) from Analysentechnick with a silica precolumn cartridge (20 × 4.0 mm i.d.). Radioactivity was measured in a 1209 RackBeta-Flexivial β-counter (LKB-Pharmacia, Turku, Finland). PAF-induced aggregation was measured in a Chrono-Log (Havertown, PA) aggregometer coupled to a Chrono-Log recorder. Electrospray ionization (ESI) mass spectrometry experiments were performed on a Q-Tof (Micromass U.K. Ltd., Manchester, U.K.) orthogonal acceleration quadrupole time-of-flight mass spectrometer equipped with nano-electrospray ionization and purchased with funding from the BBSRC, the University of Leeds, and Micromass U.K. Ltd.

Table 1. Samples of Vegetable Oils

olive oil	extraction procedure	source (location)
1	pressing	Belika Messinis
2	centrifugal	Maurolia
3	centrifugal	St. Sotiria Messinis
4	pressing	Batsikes
5	two phases	Kalamata
6	pressing	Kalamata
7	two phases	Kalamata
8	centrifugal	St Pelagia
9	centrifugal	Trifillias town
10	centrifugal	Kalamata
11	centrifugal	Kalamata
12	centrifugal	Koukounara

seed oil	type	source
1	soybean	Minerva
2	corn	Elais
3	corn	Minerva
4	sunflower	Minerva
5	sunflower	Elais
6	sesame	Limnos Area (Axiladelis)

Materials. All reagents and chemicals were of analytical grade supplied from Merck (Darmstadt, Germany). The solvents used for high-performance liquid chromatography (HPLC) were purchased from Ruthburn (Walkerburn, Peebleshire, U.K.). All standards were obtained from Sigma (St. Louis, MO). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (*10*). [^{3}H]PAF (NET 910) was purchased from NEN (DuPont, Boston, MA). PAF−acetylhydrolase from human serum was purified according to the method of Stafforini et al. (*33*). Bovine serum albumin (BSA), creatine phosphate (CP)/creatine phosphate kinase (CPK), BN 52021, and indomethacin were obtained from Sigma. Olive oil samples extracted via different procedures were all of the Koroneiki variety from Kalamata and its surrounding area, whereas seed oils were from eponymic oil industries with the exception of sesame oil, which was from Limnos Island (**Table 1**).

Separation of Total Lipids to Polar and Neutral Lipids. The vegetable oils in the form just as they were purchased are considered as total lipids. Their separation to polar and neutral lipids (**Figure 1**) was achieved with a modification of the countercurrent distribution extraction procedure of Galanos and Kapoulas (*34*). Polar lipids contained mainly phospholipids and some glycolipids. The distribution is carried out in three separatory funnels by the single withdrawal procedure (*35*). That is, an amount of vegetable oil is diluted in a quadruple volume of petroleum ether (bp 40−70 °C) pre-equilibrated with ethanol. This constitutes the initial total volume. The solution is transferred to a separatory funnel and shaken with one-fifth of the total initial volume, ethanol 87% (lower phase), pre-equilibrated with petroleum ether. After 5 min, the two phases are separated, and in the equilibrated upper phase of petroleum ether (oil), ethanol in an amount of one-fifth of the total initial volume is added. Both the equilibrated lower phase and ethanol are transferred to a second funnel, and petroleum ether in an amount of two-fifths of the total initial volume

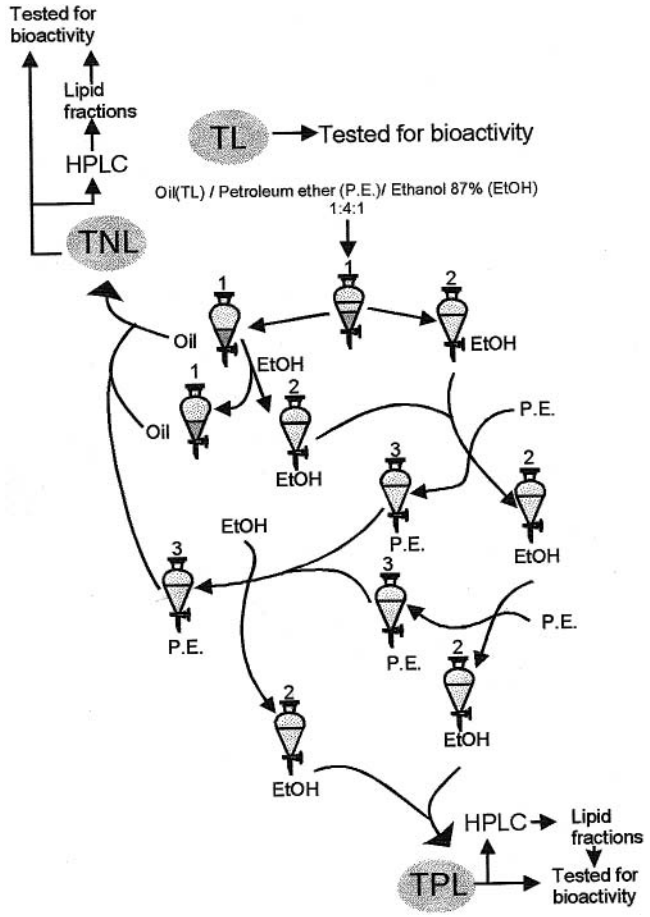

Figure 1. Separation of total lipids from vegetable oils to total neutral and total polar lipids as well as the general experimental procedure.

is added, two times successively. The upper phases of petroleum ether, from the above procedures, are pooled together in a third funnel, and ethanol in an amount of two-fifths is added. The ethanol phases at the second funnel yield 96% of the polar lipids as evaluated with [^{3}H]-PAF, whereas the combined petroleum ether phases contain the neutral lipids. The petroleum ether phase is transferred in a glass-stoppered flask, evaporated at 35 °C in a vacuum on the rotary evaporator, dissolved with chloroform/methanol (2:1), and stored at −20 °C until further analysis. The ethanol phase is evaporated at 35 °C in a vacuum on the rotary evaporator, transferred in a test tube, evaporated to dryness at 35 °C under a stream of nitrogen, dissolved with chloroform/methanol (2:1), and stored at −20 °C until further analysis.

HPLC Separation of Lipid Classes from Olive Oil. *Polar Lipid Separation.* Separation on HPLC was performed on an SCX column using an isocratic elution system consisting of 60% acetonitrile and 40% methanol/water 4:1 v/v (*36*).

Separation of polar lipids was also performed on a normal phase NH_2 column with a gradient elution system (*37*). The following solvents and elution profiles were used: solvent A, acetonitrile/methanol (70:30 v/v); solvent B, methanol (100%); solvent C, water (100%); elution profile, 0−35 min, 100% A (isocratic elution); 35−40 min 0−100% B (linear gradient); 40−45 min, 100% B; 45−50 min, 100% C (linear gradient); 50−60 min, 100% C.

Glycolipid Separation. Separation was performed on an adsorption silica column and a gradient elution system (*38*) with the following solvents and elution profiles: solvent A, acetonitrile (100%); solvent B, methanol (100%); elution profile, 0−5 min, 100% A; 5−30 min, 0−100% B (linear gradient); 30−37 min, 100% B.

Neutral Lipid Separation. Separation was performed on a Nucleosil-300 C-18 column and a stepped gradient elution system, with the following solvents and elution profiles: solvent A, methanol/water (80:20 v/v); solvent B, acetonitrile/methanol (70:50); solvent C, acetonitrile/tetrahydrofuran (99.5:0.5 v/v); solvent D, 2-propanol/acetonitrile (99:1 v/v) (*39*); elution profile, 0−10 min, 100% A−100% B; 10−15 min, 100% B; 15−25 min, 100% C, 25−40 min, 100% D, 40−64 min, 100% D.

The flow rate for the separation of all lipid classes was 1 mL/min, and the UV detector was operating at 208 nm.

Biological Assay. Total lipids (TL), total polar lipids (TPL), and total neutral lipids (TNL), as well as purified fractions of each lipid class by the above HPLC separations, were tested for their biological activity against washed rabbit platelets according to the method of ref *10*. PAF and the examined samples were dissolved in 2.5 mg of bovine serum albumin (BSA)/mL of saline. Thrombin was dissolved in saline. The platelet aggregation induced by PAF (1×10^{-11} M, final concentration) was measured as PAF-induced aggregation, in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various concentrations of the examined sample. Consequently, the plot of percent inhibition (ranging from 20 to 80%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample, which inhibited 50% PAF-induced aggregation, is calculated. This value is defined as IC_{50}, namely, inhibitory concentration for 50% inhibition. The IC_{50} values are expressed as microliters of initial volume of vegetable oil required for 50% inhibition against PAF (1×10^{-11} M, final concentration). IC_{50} values of the various fractions were tested against thrombin (0.025 unit/cuvette) activity, and the percent inhibition was determined. Desensitization experiments (*40*) with PAF and thrombin and experiments with specific inhibitors [0.7 mM creatine phosphate(CP)/creatine phosphate kinase (CPK), 10 μM indomethacin, and 0.1 μM BN 52021] were also performed for fractions that exhibited aggregatory action.

PAF—Acetylhydrolase Assay. A volume of 50 μL of Tris buffer solution (50 mM, pH 7.5) and 50 μL of the examined sample in BSA (2.5 mg/mL of saline), corresponding to a quantity as much as 5 times the quantity capable of inducing platelet aggregation, are added and mixed vigorously to a prewarmed at 37 °C test tube. From this mixture the aggregatory activity of the 1/5 mixture is tested and defined as the activity at zero time. Afterward, human serum PAF−acetylhydrolase is added, and the whole mixture is incubated at 37 °C. At different time intervals, aliquots are taken to test the ability of the possibly partially catabolized lipid sample to induce washed rabbit platelet aggregation. As a control, the same procedure is carried out with PAF—in an amount that induces platelet aggregation equivalent to that of the examined sample—instead of the lipid sample.

Mild Alkaline Hydrolysis and Acetylation. This procedure was carried out according to the method of Demopoulos et al. (*10*). The sample, in a quantity as much as twice the quantity inducing platelet aggregation, was dissolved in 1 mL of chloroform/methanol (1:4 v/v), and then 0.1 mL of 1.2 N NaOH in methanol/water (1:1 v/v) was added and allowed to stand for 20 min at 60 °C. The mixture was neutralized with 0.2 mL of 1 N acetic acid and 2 mL of chlorofom/methanol (9:1 v/v). Afterward, 1 mL of methanol and 2 mL water were added, and two phases resulted, which were then separated. The chloroform phase was washed with 1 mL of methanol/water (1:2 v/v), whereas the water phase was washed with 1 mL of chloroform. Each of the two phases was separated in two equal fractions. Half was tested for its bioactivity against platelets, and the other half was evaporated to dryness under a stream of nitrogen and subjected to reacetylation by the addition of 1 mL of acetic anhydrite and incubation at 60 °C for 45 min. After that, the reaction mixture is evaporated and extracted according to the Bligh−Dyer method (*41*) and tested for its ability to induce washed rabbit platelet aggregation. Acetylation of the initial compound was also performed using the same procedure as above for reacetylation.

Chemical Determinations. Phosphorus determination was carried out according to the method of Bartlett (*42*).

Sugar determination was carried out according to the method of Galanos and Kapoulas (*43*).

Mass Spectrometry. Samples were dissolved at a concentration of ~10 ng/μL in 1:1 v/v aqueous methanol. An aliquot of this solution (2−3 μL) was used to fill a gold-plated, borosilicate nanospray vial, which was placed inside the ionization source of the mass spectrometer. For both positive and negative ionization experiments, a capillary voltage of 900 V and a sampling cone voltage of 40 V were used.

Table 2. IC_{50} Values of TL and TNL from Olive Oils and Seed Oils

sample	IC_{50TL}[a] (μL)	IC_{50TNL} (μL)	initial vol (μL) for shape change (TL act.)
		Olive Oils	
1	0.175	0.120	nd[b]
2	0.118	0.141	0.200
3	0.250	0.207	0.200
4	0.172	0.114	0.150
5	0.185	0.214	0.150
6	0.202	0.162	0.150
7	0.278	0.136	0.150
8	0.180	0.153	0.200
9	0.185	0.086	nd
10	0.178	0.140	nd
11	0.140	0.123	nd
12	0.210	0.147	nd
		Seed Oils	
1	0.832	0.096	nd
2	0.470	0.088	nd
3	0.165	0.112	nd
4	0.440	0.147	nd
5	0.128	0.080	0.300
6	0.168	0.108	nd

[a] Values are expressed in microliters of initial volume of oil which induces 50% inhibition against 1×10^{-11} M PAF, final concentration. [b] Not detected.

Nitrogen was employed as the drying gas. The microchannel plate detector was set at 2850 V.

Tandem mass spectrometric (MS/MS) studies were performed by selecting a precursor ion with the first (quadrupole) analyzer. These ions were transmitted to a collision cell, where they were bombarded with argon gas at an energy of 10−50 eV. The resulting fragment ions were *m*/*z* analyzed by the second (time-of-flight) analyzer.

Data were acquired over the appropriate *m*/*z* range and spectra processed using the MassLynx software supplied with the mass spectrometer.

RESULTS AND DISCUSSION

Separation and Biological Activity of Total Lipid Fractions. The procedure for the separation of TL to TPL and TNL as well as the general experimental strategy is shown in **Figure 1**. An amount of total lipids (TL) was kept for testing biological activity; the rest was separated in neutral and polar lipids according to the modified countercurrent distribution extraction procedure. By this procedure the total polar lipid fraction contained glyco- and phospholipids. The recovery of the method was estimated to be 96% by the addition of [^{3}H]PAF and the measurement of radioactivity at the polar lipid fraction after the extraction procedure.

An amount of the total lipid fractions (TL, TPL, TNL), of the 12 olive oils and the 6 seed oils was tested for its ability either to induce or to inhibit PAF-induced washed rabbit platelet aggregation. All TL and TNL caused inhibition of PAF-induced washed rabbit platelet aggregation, in a dose-dependent manner. TL but not TNL of the majority of olive oil samples as well as TL of sunflower oil (Elais) caused, in specific amounts, shape change of platelets (**Table 2**). Because TNL had effects on washed rabbit platelets similar to those of TL, the results indicate that the shape change was caused by TPL, which may have significant biological activity. Indeed, TPL from all olive oil samples exhibited inhibitory activity against PAF action, in a dose-dependent manner, up to a certain amount, whereas larger amounts caused aggregation of platelets (**Table 3**). TPL from seed oil samples also caused dose-dependent inhibition of PAF action. Larger amounts caused total inhibition except soybean oil, which caused shape change of platelets, and sesame oil, which exhibited aggregatory activity on platelets (**Table 3**). These results indicated the existence of microconstituents in TPL of vegetable oils, especially of olive oils that act as PAF antagonists. Although all vegetable oils tested presented biological activity, the above results showed that olive oil samples were more bioactive than seed oils, with the exception of sesame oil. This superiority seems to be due to TPL. Furthermore, these findings raised the question of whether TL bioactivity could be used as a nutritional index.

Table 3. IC_{50} and C_{PAF} Values of TPL from Olive and Seed Oils

olive oil	IC_{50}[a]	initial vol (μL) that induces aggregation	$C_{PAF} \times 10^{-11}$ M[b]
1	16.2	75	8.2
2	162	312	14
3	12.5	162	7.3
4	9.38	138	34
5	12.2	375	11
6	8.12	250	10
7	11.8	250	15
8	7.00	150	3
9	17.5	462	11
10	11.1	375	6.5
11	6.25	88	11
12	6.25	150	15

seed oil	IC_{50}	initial vol (μL) that exerts bioactivity	bioactivity
1	138	1125	shape change
2	255	1125	*I*[c]: 100%
3	362	1125	*I*: 100%
4	125	1125	*I*: 100%
5	100	1125	*I*: 100%
6	7.5	625	*A*[d]: $C_{PAF} = 6.2 \times 10^{-11}$ M

[a] Values are expressed in microliters of initial volume of oil that induces 50% inhibition against 1×10^{-11} M PAF, final concentration. [b] Concentration of PAF that exhibits the same aggregatory activity as indicated in the table amount (in microliters) of initial volume of oil. [c] Inhibition of platelet aggregation induced by 1×10^{-11} M PAF, final concentration. [d] Aggregation of washed rabbit platelets.

Although statistical data cannot be presented because of the small number of samples that pertain to the same category, results pointed out the above significant conclusions since the scope of the present work was to investigate the bioactivity of certain fractions of vegetable oils and to elucidate structures of unidentified lipid microconstituents. The identification of such compounds may be of help to the field of nonsteroidal anti-inflammatory drugs as well as to the understanding of various metabolic paths concerning serious pathophysiological conditions. The greater bioactivity of olive oils in total polar lipids (TPL) prompted us to further investigate an olive oil sample.

Separation and Biological Activity of Olive Oil Polar Lipid on an SCX Column. The amount of total polar lipid fraction from 10 mL of olive oil, after evaporation in a vacuum on a rotary evaporator and dilution in chloroform/methanol (1:1), was fractionated (*36*) in five sequential separations of 100 μL each (**Figure 2**). Fractions of the same retention times from each separation were pooled together to give nine fractions, which were tested for their biological activities.

Seven of the nine fractions caused inhibition of platelet aggregation against 1×10^{-11} M PAF. The results are shown in **Table 4**. Fractions 3−5, which were the most potent PAF inhibitors, were also tested for their inhibitory activity against thrombin-induced washed rabbit platelet aggregation in the IC_{50} amount expressed as initial volume of olive oil. These fractions

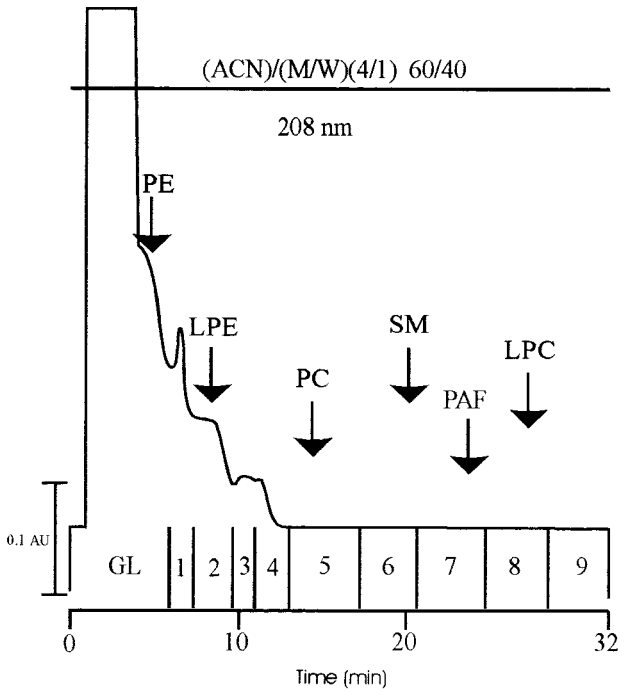

Figure 2. Representative HPLC chromatogram of total polar lipids (TPL) of olive oil on an SCX column. Conditions and elution of phospholipid standards were as indicated. PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PAF, platelet activating factor; LPC, lysophosphatidylcholine; GL, glycolipids; numbers 1–9 above the *x*-axis refer to the collected fractions.

Table 4. IC_{50} and C_{PAF} Values of Polar Lipid Fractions from Olive Oil after Separation on an SCX Column

polar lipid fraction	IC_{50}[a]	standards eluted in the same time
1	2499	between PE[b] and LPE[c]
2	3123	same with LPE
3	437.5	between LPE and PC[d]
4	162.5	between LPE and PC
5	375.0	between PC and SM[e]
6	637.5	between PC and SM
9	1000	beyond LPC[f]

polar lipid fraction	μL	$C_{PAF} \times 10^{-11}$ M[g]	standards eluted in the same time
7	375	3.5	same with PAF[h]
	562	5.8	
8	1125	3.2	same with LPC

[a] Values are expressed in microliters of initial volume of oil that induces 50% inhibition against 1×10^{-11} M PAF, final concentration. [b] Phosphatidylethanolamine. [c] Lysophosphatidylethanolamine. [d] Phosphatidylcholine. [e] Sphingomyelin. [f] Lysophosphatidylcholine. [g] Concentration of PAF that exhibits the same aggregatory activity as indicated in table amount (in microliters) of initial volume of oil. [h] Platelet activating factor.

were more active inhibitors of thrombin; they caused 100, 80, and 100% inhibition of thrombin activity, respectively.

Two of the above fractions (fractions 7 and 8) caused aggregation of the platelets. The aggregatory activity of fraction 7, which was the most active, was dose-dependent. This fraction desensitized 1×10^{-11} M PAF (final concentration) at 100% in an amount of 375 μL (initial volume of oil), and it also desensitized thrombin (0.025 unit/cuvette) at 100% in an amount of 562 μL.

Separation and Biological Activity of Olive Oil Polar Lipids on a Normal Phase NH_2 Column. Even though the separation of polar lipids on an SCX column results in excellent purification of the majority of phospholipids, some of them such as phosphatidylinositol (PI) and phosphatidylserine (PS) as well as phosphoglycolipids are not well separated.

For this purpose, a separation on a normal phase NH_2 column was also performed. Polar lipids from 100 mL of olive oil were also separated in a single step (**Figure 3**) on a normal phase NH_2 column. The amount of polar lipid fraction was fractionated (*37*) in 21 sequential separations of 40 μL each. Fractions of the same retention times from each separation were pooled together to give 26 fractions, which tested for their biological activities.

Six of the 26 fractions did not exhibit any biological activity on platelets. Seventeen caused inhibition of platelet aggregation against 1×10^{-11} M PAF and 0.025 unit/cuvette thrombin. Three fractions (fractions 23, 25, and 26) caused aggregation of the platelets. The results are shown in **Table 5**. Fraction 23, which was the most active one exhibiting aggregatory activity, in the amount of 1789 μL (initial volume of oil), desensitized 1×10^{-11} M PAF at 98%. The aggregatory activity is inhibited 96% by BN (specific inhibitor of PAF), 18% by indomethacin (specific inhibitor of thrombin), and 44% by the enzymatic system of creatine phosphate/creatine phosphate kinase (CP/CPK), which inhibits the aggregation caused by the possible ADP secretion.

Separation and Biological Activity of Olive Oil Glycolipids. To separate the glycolipids from olive oil, the solvent front from the HPLC separation of polar lipids (**Figure 2**) was further fractionated by HPLC (*38*) (**Figure 4**). The amount of glycolipid fraction was evaporated in a vacuum on a rotary evaporator and diluted in chloroform/methanol (1:1). It was then fractionated in seven sequential separations of 18 μL each. Fractions of the same retention times from each separation were pooled together to give 14 fractions, which were tested for their biological activities.

Twelve of the 14 fractions caused inhibition of platelet aggregation against 1×10^{-11} M PAF (final concentration). The results are shown in **Table 6**.

Fractions 9 and 12, which caused aggregation of the platelets in an amount of 900 μL (initial volume of oil), desensitized 1×10^{-11} M PAF (final concentration) at 100 and 92%, respectively.

Separation and Biological Activity of Olive Oil Neutral Lipids. To separate neutral lipids from olive oil, the total neutral lipid extract from the modified countercurrent distribution was combined with the solvent front from the HPLC separation of glycolipids (**Figure 4**). This fraction was evaporated in a vacuum rotary evaporator and diluted up to 13 mL with chloroform/methanol (1:1). An amount of 1 mL of this fraction was diluted up to 3 mL, and 81 μL from this fraction was subjected to nine sequential separations by HPLC (*39*) (**Figure 5**). Fractions of the same retention times from each separation were pooled together to give 29 fractions, which tested for their biological activities.

Five of the 29 fractions (fractions 1, 2, 7, 8, and 13) did not exhibit any biological activity. The other fractions caused inhibition of platelet aggregation against 1×10^{-11} M PAF. The results are shown in **Table 7**. Seven fractions (fractions 10, 14−16, 24, 27, and 28) that were the most potent PAF inhibitors were also tested for their inhibitory activity against thrombin-induced washed rabbit platelet aggregation. The results

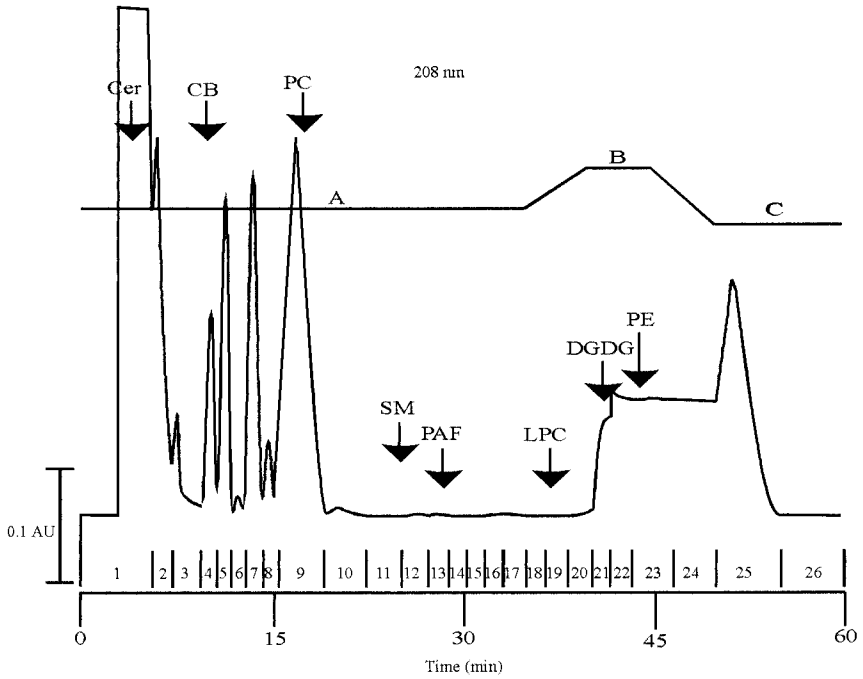

Figure 3. Representative HPLC chromatogram of total polar lipids (TPL) from olive oil on a normal phase NH_2 column. Conditions and elution of polar lipid standards as well as the solvent gradient used were as indicated. A, acetonitrile/methanol (70:30 v/v); B, methanol (100%); C, water (100%); Cer, ceramides; CB, cerebrosides; PC, phospatidylocholine; SM, sphingomyelin; PAF, platelet activating factor; LPC, lysophosphatidylcholine; DGDG, digalactosyldiglycerides; PE, phosphatidylethanolimine; numbers 1–26 refer to the collected fractions.

Table 5. IC_{50} and C_{PAF} Values of Total Polar Lipid Fractions from Olive Oil after Separation on a Normal Phase NH_2 Column

polar lipid fraction	IC_{50PAF}[a]	IC_{50Thr}[b]	standards eluted in the same time
1	493	287	same as Cer[c]
2	5211	1270	same as Cer
3	6632	6632	same as CB[d]
4	19326	8202	same as CB
5	6158	11748	same as CB
6	1895	18950	same as CB
8	3432	6021	before PC[e]
12	4263	4058	same as PAF[f]
13	1253	4922	same as PAF
14	4168	5262	after PAF
15	1680	10400	before LPC[g]
17	12347	nd[h]	before LPC
18	5368	8678	same as LPC
19	10442	nd	same as LPC
20	3541	nd	same as LPC
21	2779	4969	same as DGDG[i]
22	5495	4264	after DGDG

polar lipid fraction	μL	C_{PAF}[j]	standards eluted in the same time
23	1789	13.1	same as PE[k]
25	2368	25.0	beyond PE
26	3789	3.20	beyond PE

[a] Values are expressed in microliters of initial volume of oil that induces 50% inhibition against 1×10^{-11} M PAF, final concentration. [b] Values are expressed in microliters of initial volume of oil that induces 50% inhibition against thrombin 0.025 unit/cuvette, final concentration. [c] Ceramides. [d] Cerebrosides. [e] Phosphatidylcholine. [f] Platelet activating factor. [g] Lysophosphatidylcholine. [h] Not detected. [i] Digalactosyldiglycerides. [j] Concentration of PAF that exhibits the same aggregatory activity as indicated in table (in microliters) of initial volume of oil. [k] Phosphatidylethanolamine.

are shown in **Table 7**. Most of these fractions are also inhibitors of thrombin.

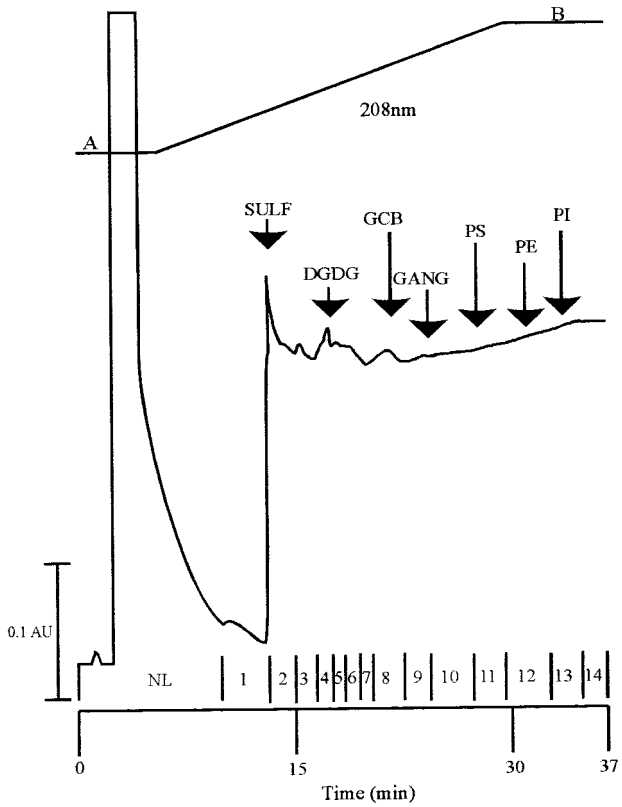

Figure 4. Representative HPLC chromatogram of glycolipids of olive oil on a silica column. Conditions and elution of glyco- and some phospholipid standards as well as the solvent gradient used were as indicated. A, acetonitrile (100%); B, methanol (100%); SULF, sulfatides; DGDG, digalactosyldiglycerides; GCER, galactocerebrosides; GANG, gangliosides; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; NL, neutral lipids; numbers 1–14 refer to the collected fractions.

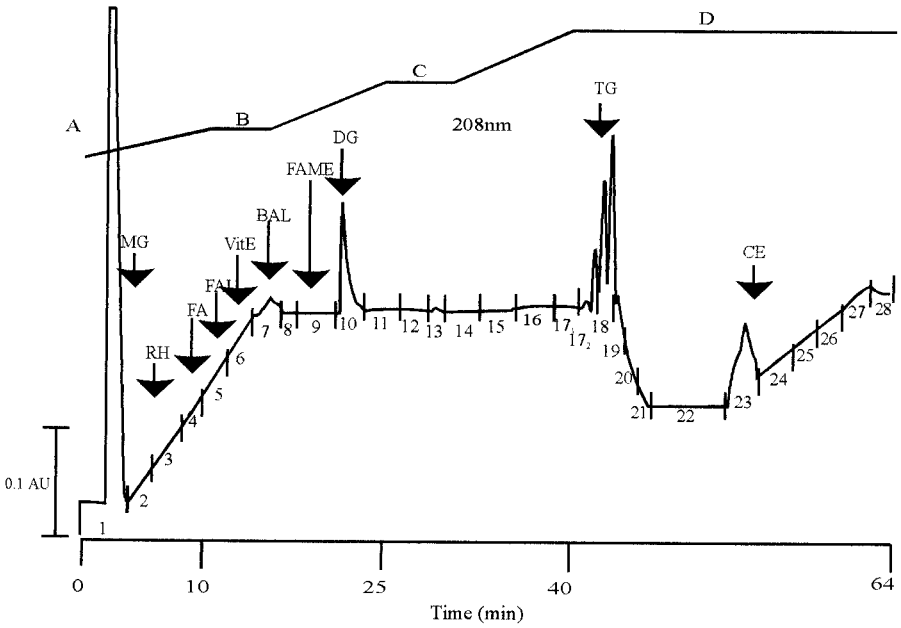

Figure 5. Representative HPLC chromatogram of total neutral lipids (TNL) of olive oil on a C-18 column. Conditions and elution of neutral lipid standards as well as the solvent gradient used were as indicated. A, methanol/water (80:20 v/v); B, acetonitrile/methanol (70:50); C, acetonitrile/tetrahydrofuran (99.5:0.5 v/v); D, 2-propanol/acetonitrile (99:1 v/v); MG, monoglycerides; RH, hydrocarbons; FA, fatty acids; FAL, fatty alcohols; VitE, vitamin E; BAL, batyl alcohol; FAME, fatty acid methyl esters; DG, diglycerides; TG, triglycerides; CE, cholesterol esters; numbers 1–28 refer to the collected fractions.

Table 6. IC_{50} and C_{PAF} Values of Glycolipid Fractions from Olive Oil

glycolipid fraction	IC_{50}[a]	standards eluted in the same time
1	670	before SULF[b]
2	403	same as SULF
3	448	after SULF
4	174	between SULF and DGDG[c]
5	374	between SULF and DGDG
6	426	before DGDG
7	576	same as DGDG
8	517	between DGDG and GCB[d]
10	100	between GANG[e] and PS[f]
11	590	between PS and PE[g]
13	830	same as PI[h]
14	270	beyond PI

glycolipid fraction	initial vol (μL)	$C_{PAF} \times 10^{-11}$ M[i]	standards eluted in the same time
9	900	2	same as GCB
12	900	2	same as PE

[a] Values are expressed in μL of initial volume of oil which induces 50% inhibition against 1×10^{-11} M PAF, final concentration. [b] Sulfatides. [c] Digalactosyldiglycerides. [d] Galactocerebrosides. [e] Gangliosides. [f] Phosphatidylserine. [g] Phosphatidylethanolamine. [h] Phosphatidylinositol. [i] Concentration of PAF that exhibits the same aggregatory activity as indicated in table (in microliters) of initial volume of oil.

Fraction 4, which was coeluted with fatty acids, was the only one that caused platelet aggregation. This fraction, in an amount of 1.35 μL (initial volume of oil), exhibited the same aggregatory activity with 6.8×10^{-11} M PAF (final concentration). The same quantity desensitized 1×10^{-11} M PAF at 52%. The aggregatory activity is inhibited 100% by BN (specific inhibitor of PAF) and 44% by the enzymatic system of CP/CPK, which inhibits aggregation caused by the possibly secreted ADP.

Structural Studies. An attempt to elucidate the structure of fraction 23 from the separation of total polar lipids from olive oil was made using chemical determinations, biological tests, and electrospray mass spectrometry (ES-MS). Sugar determination was positive (0.540 μmol of glucose/total fraction), whereas phosphorus determination was negative. To gain additional information about the structure of this bioactive lipid fraction, we examined the effect of human serum PAF−acetylhydrolase on the ability of the fraction to induce platelet aggregation. PAF−acetylhydrolase affected the biological activity of this fraction in an unusual way as the activity disappeared in the first 3 min while increasing gradually after a period of 6 min of treatment. It should also be noted that the final aggregation pattern after 12 min of treatment with PAF−acetylhydrolase was different from the original one. These results indicated the existence of two types of esterified acetic groups (*44*), on this molecule, that play a significant but controversial role in the biological activity.

The aggregation curve pattern from this fraction was different (long-standing with slight slope) from the one of PAF (short-standing with sharp slope). After alkaline hydrolysis, the chloroform phase exhibited a different aggregatory activity because its pattern was similar to the one of PAF. On the other hand, the water phase was not active. The reacetylation of the water phase was also not active. These results, which are in accordance with the ones from PAF−acetylhydrolase treatment, indicated the existence of ester bonds because alkaline hydrolysis results in a fat-soluble molecule with significant biological activity, even though different from the initial. Furthermore, reacetylation of the chloroform phase resulted in a molecule with a smaller activity. Also, acetylation of the initial intact compound resulted in a compound that presented decreased biological activity. This activity was identical to that of the reacetylated chloroform phase. Both data show that the existence of free hydroxyl groups plays a pivotal biological role. These findings suggest also that an ether bond as well as free hydroxyl group(s) exists in the molecule. The above shows that the ratio of acetyl to hydroxyl groups is important for the exhibition of the biological activity of the molecule and also that the esterified acid is the acetic acid.

The above data along with ES-MS for sample 23 of olive oil polar lipids (**Figure 6**) support the structure of the glycerol

Table 7. IC_{50} Values of Total Neutral Lipid Fractions from Olive Oil and Their Percent Inhibition against Thrombin 0.025 Unit/Cuvette

neutral lipid fraction	$IC_{50\ PAF}$[a]	% inhibition of thrombin[b]	standards eluted in the same time	neutral lipid fraction	$IC_{50\ PAF}$	% inhibition of thrombin[a]	standards eluted in the same time
3	3.61		same as RH[c]	18	1.15		same as TG[d]
5	5.87		same as FAL[e]	19	6.21		same as TG
6	4.52		same as VitE[f]	20	2.84		after TG
9	3.10		same as DG[g]	21	2.80		after TG
10	0.36	57	same as DG	22	2.19		between TG and CE[h]
11	3.27		after DG	23	1.49		same as CE
12	2.90		between DG and TG	24	0.84	0	after CE
14	0.20	61	between DG and TG	25	3.04		beyond CE
15	0.95	51	between DG and TG	26	4.63		beyond CE
16	0.20	74	between DG and TG	27	0.74	26	beyond CE
17_1	1.53		before TG	28	0.27	79	beyond CE
17_2	1.75		same as TG				

[a] Values are expressed in microliters of initial volume of oil that induces 50% inhibition against 1×10^{-11} M PAF, final concentration. [b] Percent inhibition of thrombin was tested for the fractions with the most potent inhibitory effect of PAF action against washed rabbit platelets in an amount equal to C_{50} of PAF. [c] Hydrocarbons. [d] Triglycerides. [e] Fatty alcohols. [f] Vitamin E. [g] Diglycerides. [h] Cholesterol esters.

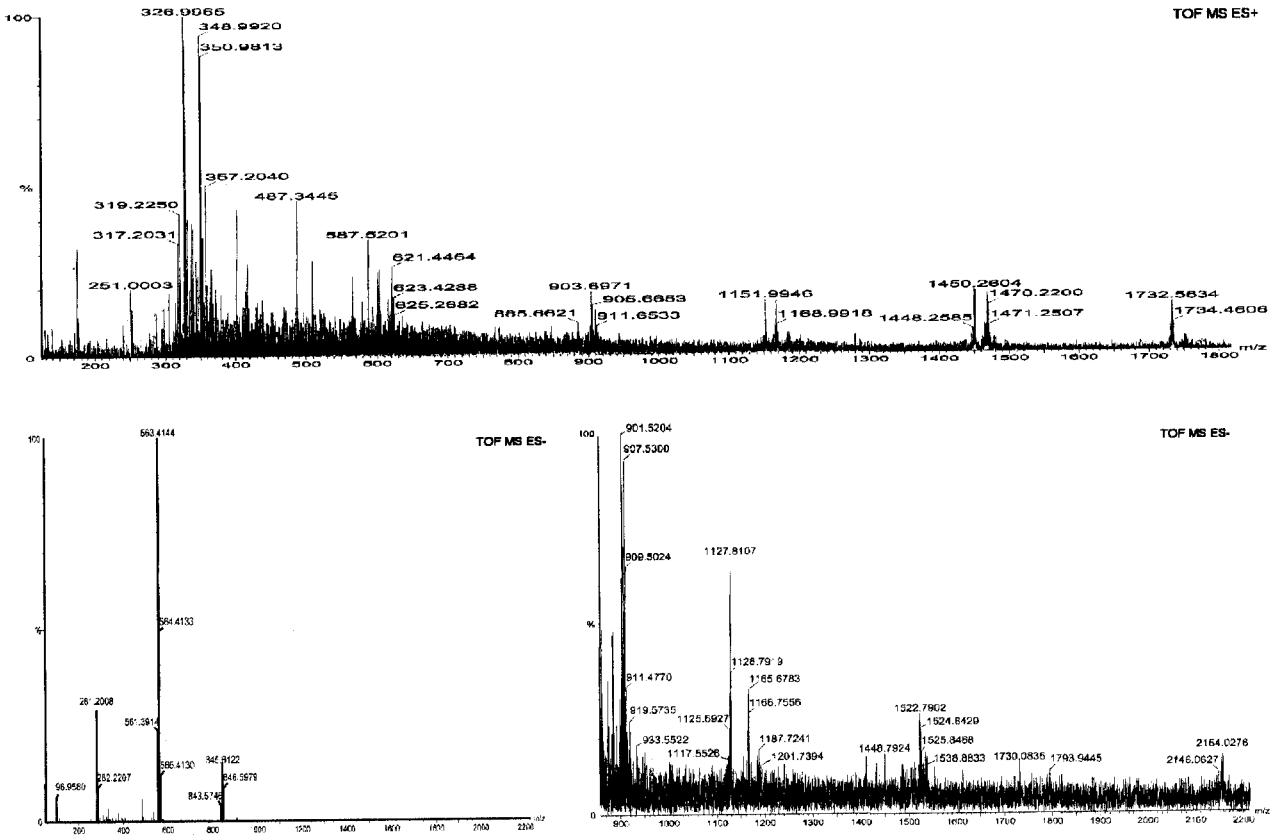

Figure 6. Positive and negative ES-MS of sample 23 from olive oil polar lipids.

glycolipid compound shown in **Figure 8** with a molecular weight equal to *m*/*z* 1789, along with its MS fragmentation.

Furthermore, the more abundant ion at *m*/*z* 326 (**Figure 8**) represents the terminal sugar moiety, whereas the sodiated molecule yields the ion at *m*/*z* 348. The deacylation of the terminal sugar moiety yields the negative ion at *m*/*z* 281. Loss of the terminal sugar moiety gives rise to the positive ion at *m*/*z* 1450. The *m*/*z* values of the positive ions at *m*/*z* 623, 603, and 587 along with the abundant negative ion at *m*/*z* 563 correspond to the fragment that contains the two terminal sugar moieties. The tandem mass spectrum (**Figure 7**) after collisionally activated dissociation of the parent ion at *m*/*z* 563 contains only the ion at *m*/*z* 281, which represents the deacetylated terminal sugar moiety as well as the other sugar moiety. Even more, loss of this fragment gives rise to the positive and negative ions at *m*/*z* 1169 and 1167, respectively. The structure of the fragment containing the three terminal sugar moieties is supported by positive ions at *m*/*z* 911, 905, and 885 as well as by the negative ion at *m*/*z* 845. Loss of the three terminal sugar moieties results in the positive and negative ions at *m*/*z* 903 and 901, respectively. The fragment containing all four sugar moieties gives rise to the positive and negative ions at *m*/*z* 1152 and 1128, respectively. Finally, the positive and negative ions at *m*/*z* 1732 and 1730, respectively, correspond to the loss of the *sn-2* acetic acid, $[M + H - 58]^+$ and $[M - H - 58]^-$.

In this work we developed a modified countercurrent distribution for the separation of total lipids (TL) of vegetable oils to total polar lipids (TPL) and total neutral lipids (TNL). Biological activities of TL, TNL, and TPL lipids of several olive and seed oils were evaluated. The results showed that the

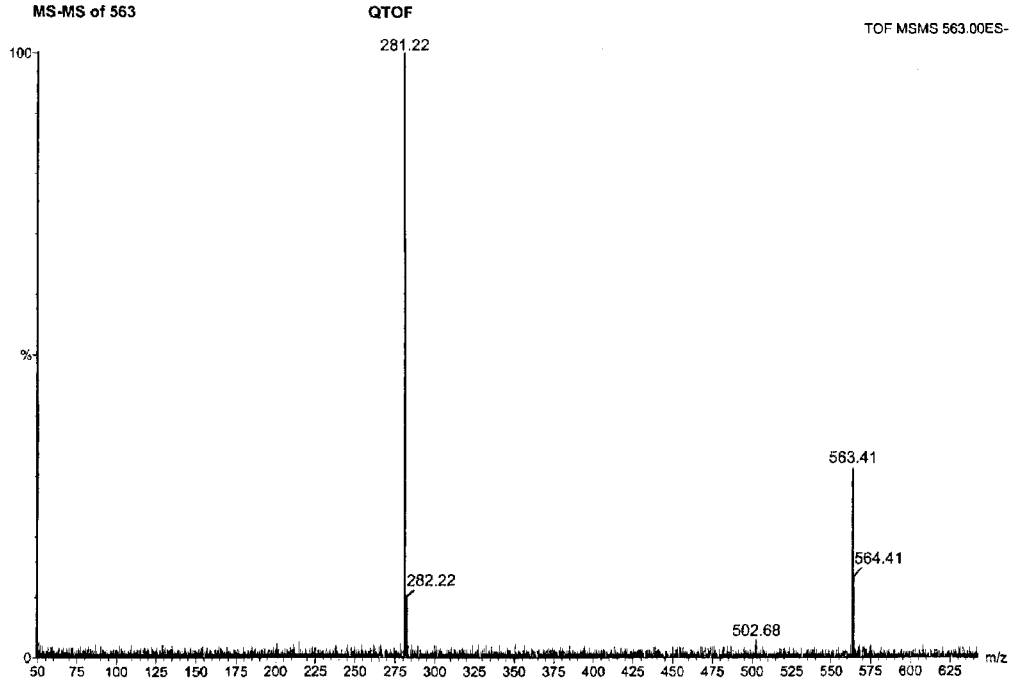

Figure 7. Tandem mass spectrometry (MS/MS) of the negative ion at *m/z* 563.

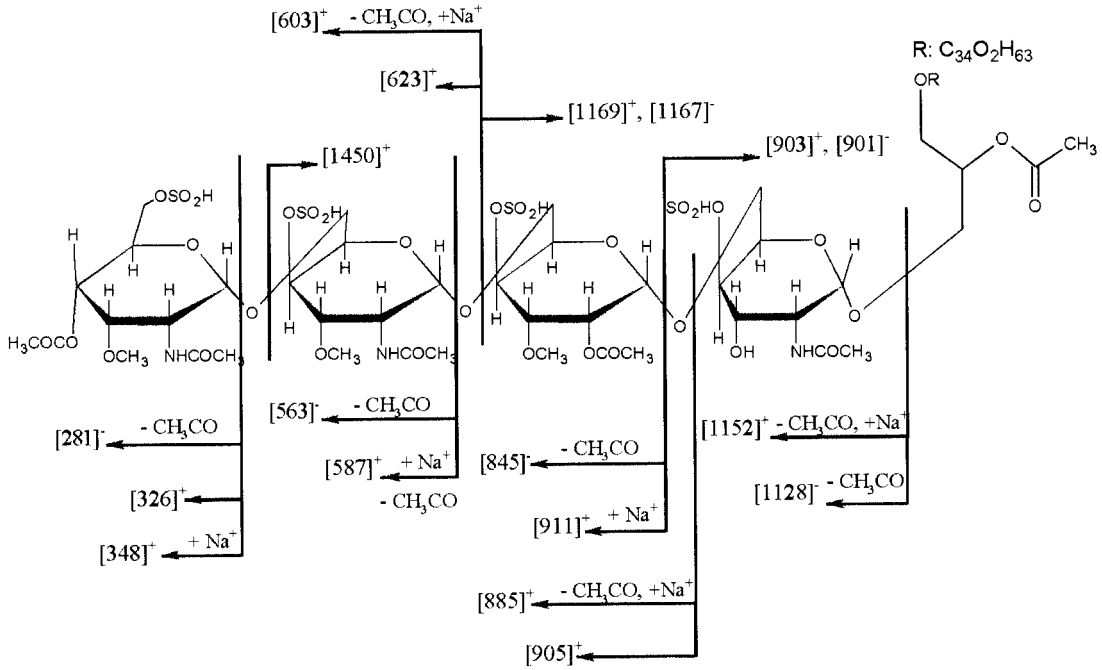

Figure 8. Proposed structure of sample 23 from olive oil polar lipids and its MS fragmentation.

biological activity is attributed mainly to TPL, whereas the contribution of TNL is less significant. Olive oils seem to be more active than seed oils, except sesame oil, which presents biological activity that resembles that of the olive oils. The existence of a variety of PAF and thrombin inhibitors along with weak PAF-like agonists has been demonstrated in olive oil. Biological activities based on sugar determination showed that the most active agonist is almost 9 orders of magnitude less active than PAF, which means that action of such a compound in several cells and/or tissues, through PAF receptors, would minimize the biological effects of PAF and finally acts as a PAF inhibitor.

Our findings enhance the beneficial effects of the consumption of vegetable oils and especially olive oil as they contain a significant number of lipid-like components with antithrombotic and antiatherogenic actions in vitro. These compounds could be helpful in the field of nonsteroidal anti-inflammatory drugs as well as to the understanding of biochemical mechanisms concerning pathophysiological conditions and metabolic pathways. The above data in correlation with our proposed mechanism of atheromatosis generation may offer a new approach for the explanation of the beneficial effect of the Mediterranean diet on a biochemical basis.

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