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Biologically Active Lipids with Antiatherogenic Properties from White Wine and Must

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Wine can be considered an integral part of the Mediterranean diet. Evidence from epidemiological and experimental studies suggests a protective effect against the development of coronary heart disease with moderate consumption of wine and especially red wine. The exact nature of the protective effect remains to be established. However, mechanisms including low-density lipoproteins oxidation, inhibition of platelet aggregation, modification of eicosanoid metabolism, and endothelium-dependent relaxation of blood vessels are recognized as contributory. In this study, a new approach has been examined, based on previous reports that platelet-activating factor (PAF) is involved in atherogenesis. An attempt was made to detect key components in wine/must that through inhibition of PAF action may contribute to the protective role of white wine/must against atherosclerosis. More specifically, polar lipids from four wines and three musts were fractionated by thin-layer chromatography, and fractions were tested in vitro for their ability to inhibit PAF and thrombin-induced washed rabbit platelet aggregation and/or to cause platelet aggregation. On the basis of the above results, a white wine from the principle Greek grape Rompola and its respective must with and without extra yeast were chosen for further high-performance liquid chromatography separation. A significant number of biological active lipids were detected, and structural data for the most active lipids are provided.

KEYWORDS: Wine; must; biologically active lipids; platelet-activating factor; PAF; atherosclerosis; coronary heart disease

INTRODUCTION

A number of epidemiology studies have demonstrated that a moderate consumption of alcohol is associated with reduced mortality and coronary heart disease (*1*−*3*). In most countries, although not in some regions of France, a high intake of saturated fats is strongly correlated with high mortality from coronary heart disease. The Southern French have a very low incidence of coronary heart disease despite having a high-fat diet and smoking habits (*4*−*6*). This epidemiological evidence is referred to as the "French paradox", and it has been attributed to the regular drinking of wine, which is an important component in Mediterranean dietary traditions.

Several mechanisms have been proposed to explain the beneficial effects of wine in the prevention of coronary heart disease. Initially, the alcohol was proposed as having this cardioprotective effect. Approximately half of the alcohol effect may be attributed to an increase of HDL (high-density lipoproteins) serum levels, as well as to the reduction of platelet aggregation and blood clotting (*7*, *8*). Nevertheless, investigations demonstrated that wine is the most potent alcoholic beverage conferring protection against atherosclerosis. Because wine, especially red wine, differs most notably from all other forms of beverage alcohol in the content of phenolic substances, attention has been directed to these compounds to define their potential role as antiatherogenic agents. A large number of studies, which examined the inhibition of LDL (low-density lipoproteins) oxidation by phenolic components of wines, including gallic, caffeic, and *p*-coumaric acids, catechin, quercetin, and myricetin, have been done (*9*). Inhibition of platelet aggregation, modification in the eicosanoid metabolism, and endothelium-dependent relaxation of blood vessels are other possible mechanisms that have been proposed to explain the beneficial effects of wine in the prevention of coronary heart disease. Quercetin blocks the ADP (adenosine diphosphate)-induced aggregation of washed human platelets in vitro and also potentiates the antiaggregatory effects of prostacyclin (*10*). More recently, *trans*-resveratrol and quercetin were found to inhibit ADP and thrombin-induced human platelet aggregation (*11*). Quercetin also seems to inhibit phospholipase A_2, a key enzyme

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in eicosanoid metabolism (*12*). It is important to notice that the effect of phenolic substances on platelet aggregation induced by platelet-activating factor (PAF) has not yet been studied.

White wines are usually made with free-running juices, which have no contact with the grape skins. This is the main reason that the concentration of phenolic compounds in white wines is lower than that of red wines. However, a number of studies demonstrate that white wine phenols have a comparable or higher antioxidant capacity than red wine phenols (*13*). Although efforts have been made to identify the structures of the phenolic constituents of red or white wines, little information exists on the lipid composition of both wines, which is reported to be influenced by the lipid components in yeast (*14*−*16*).

The response-to-injury hypothesis presents atherosclerosis as a chronic inflammatory response to injury of the endothelium, which leads to complex cellular and molecular interactions among endothelial cells, smooth muscle cells, and several blood cell components (*17*). As we have previously described, a mechanism by which PAF (*18*) is implicated in atherogenesis has been proposed (*19*), demonstrating that PAF is produced during LDL oxidation (*20*) and causes in situ inflammation. Moreover, studies show that PAF, which is known as the strongest inflammatory mediator, is a compound of atheromatic plaque and is essential for the activation of leukocytes and their binding in the endothelial cells (*21*). Additional evidence of the implication of PAF in atherogenesis is provided by studies in animals, which indicated that PAF antagonists have protective action against atherosclerosis (*22*, *23*). On the other hand, acetylhydrolase, the main enzyme responsible for the degradation of PAF, is active in native LDL but is converted inactive in ox-LDL, which leads to higher PAF levels (*20*).

The above evidence shows that the existence of PAF inhibitors in wine/must may contribute to the protective effect of wine against coronary heart disease. We have already reported the existence of such compounds in Cabernet Sauvignon red wine (*24*) and other foods (*25*−*29*). From our previous work (*30*), it was concluded that the inhibition of PAF-induced platelet aggregation from total lipid extracts of wine and must is attributed mainly to total polar lipids. In the present study, total polar lipids, which include phospholipids, glycolipids, and phenolics of wines and musts, were further separated by thin-layer chromatography (TLC), and the biological activity of the fractions was tested. In addition, identification of active components from a white wine and its respective must, with and without extra yeast, after further separation on high-performance liquid chromatography (HPLC), is reported.

MATERIALS AND METHODS

Instrumentation. HPLC was performed on a Hewlett-Packard (Avondale, PA) series 1100, supplied with a 100 μL loop Rheodyne (i 7725) injector. A 1100 HP UV spectrometer was used as the detector. The spectrophotometer was connected to a Hewlett-Packard model HP-3396A integrator-plotter.

Separation of lipids was carried out on a 250 mm × 4.6 mm i.d. reverse phase C18 Nucleosil-300 column (Analyzentechnik) at room temperature.

The PAF-induced aggregation was measured in a Crono-Log (Havertown, PA) aggregometer coupled to a Crono-Log recorder. A Varian Cary 3E UV−vis spectrometer was used.

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-ESI. Samples were dissolved at a concentration of ca. 10 ng/μL in methanol/water (70:30, v/v) 0.01 M in ammonium acetate. 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. 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 were processed using the MassLynx software supplied with the mass spectrometer.

Reagents. All reagents and chemicals were analytical grade supplied by Merck (Darmstadt, Germany). Resveratrol, quercetin, naringin, hesperidin, rutin, and gallic acid were purchased from Sigma (St. Louis, MO). HPLC solvents were from Rathburn (Walkerburn, Peebleshire, U.K.) Lipid standards were obtained from Sigma. Semisynthetic PAF (80% C-16 PAF and 20% C-18 PAF) was synthesized in our laboratory as previously described (*18*). Chromatographic material used for TLC was silica gel H-60 (Merck). PAF−acetylhydrolase was from human serum, purified according to the method of Stafforini (*31*). Bovine serum albumin (BSA), creatine phosphate (CP)/creatine phosphate kinase (CPK), BN 52021 (ginkgolide B), and indomethacin were obtained from Sigma. Domaine Hatzimichalis kindly supplied the following wines and musts: Ambelon (white wine with principle grape Rompola, 1998); Chardonnay (white wine with principle grape Chardonnay, 1997); Cuvee Maison (red wine with principle grapes Cabernet Sauvignon, Xinomauro, Limnio, 1997); Erythros (red wine with principle grapes Carignan, Grenache, Syrah, Cabernet Sauvignon, 1998); Rompola (white must); Rompola with extra yeast (UVAFERM 228) (white must); and Cabernet Sauvignon (red must).

Procedure. Total lipids were extracted according to the Bligh−Dyer method (*32*). The total lipids fraction was separated into total neutral and total polar lipids by counter-current distribution (*33*). From previous data, it was concluded that the inhibition of PAF-induced platelet aggregation is attributed mainly to total polar lipids. Total polar lipids were further separated by preparative TLC, with the elution system chloroform:acetone:methanol:acetic acid:water 50:20:17:5:5, and the biological activity of the fractions was examined. On the basis of the above results, the white wine, Ambelon, and its must, Rompola, with and without extra yeast, were chosen for further examination. Briefly, the TLC fractions exhibiting the strongest biological activity were separated on a reverse phase HPLC column using a stepped gradient elution with the following solvents: A, 20% aqueous methanol; B, 10% aqueous methanol; C, acetonitrile. The elution system consisted of a gradient from solvent A to solvent B in 25 min, a gradient from solvent B to solvent C in 10 min, and finally a hold for 35 min in solvent C. The flow rate was 1 mL/min. The purified fractions from the above separations were separately collected and tested for biological activity.

Biological Assay. PAF and the examined samples were dissolved in 2.5 mg of BSA per 1 mL of saline. Thrombin was dissolved in saline. Various concentrations of the examined sample were added into the aggregometer cuvette, and the aggregation induced by the sample was studied in a Chronolog aggregometer (*18*). Experiments with specific inhibitors, 0.7 mM CP/CPK 13 units per mL saline, 10 μM indomethacin (10% aqueous ethanol), and 0.1 mM BN 52021 (0.3% aqueous dimethyl sulfoxide), were also performed. These inhibitors were added to washed rabbit platelets 1 min prior to the addition of the examined sample into the aggregometer cuvette. This experiment was carried out according to Lazanas et. al (*34*). In cross-desensitization experiments, platelets were desensitized by the addition of the examined lipid to the platelet suspension at a concentration that caused reversible aggregation. Second stimulation with PAF or thrombin was performed immediately after complete disaggregation. The platelet aggregation induced by PAF (2.5×10^{-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 (*18*). Consequently, the plot of percent inhibition

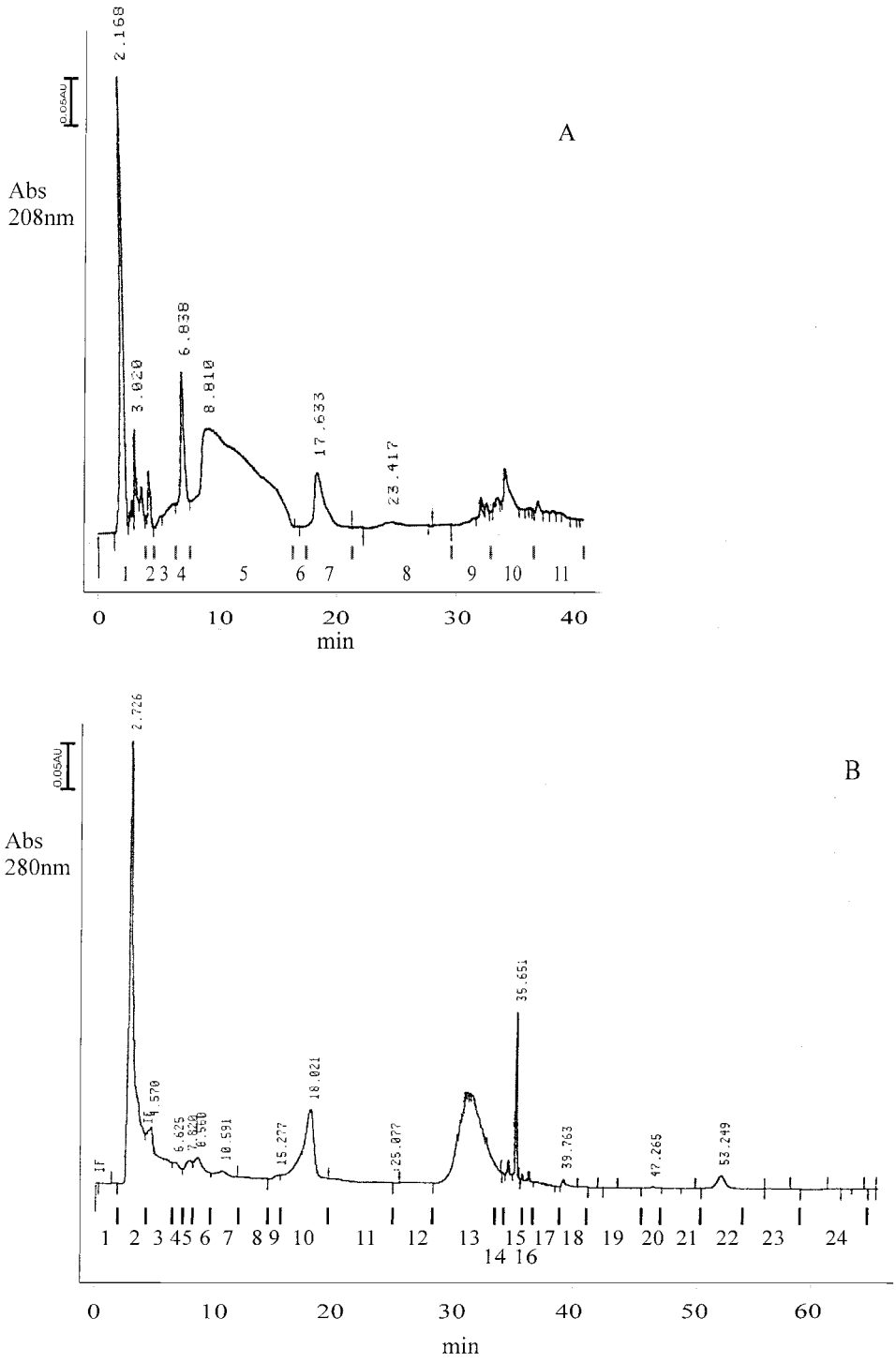

Figure 1. (**A**) Typical reverse phase HPLC separation of fraction TLC2 of wine Ambelon. (**B**) Typical reverse phase HPLC separation of fraction TLC9 of wine Ambelon.

(ranging from 0 to 100%) vs different concentrations of the sample is linear. From this curve, the concentration of the sample that inhibited 50% PAF-induced aggregation was calculated, and this value was defined as IC_{50}. The IC_{50} values are expressed as milliliters of red wine required for 50% inhibition against PAF. This experiment was also performed with thrombin (0.125 Units/cuvette) in order to assess the inhibition of thrombin-induced aggregation.

Treatment with Acetylhydrolase. The effect of PAF human serum acetylhydrolase, an enzyme specific to short or intermediate length *sn*-2 chains, on the ability of fractions to induce platelet aggregation was examined. Briefly, Tris buffer (pH 7.4), human serum acetylhydrolase, and the examined sample in BSA at 2.5 mg/mL saline were added to a prewarmed (37 °C) test tube. The enzymatic system was incubated at 37 °C, and at different time intervals, aliquots were taken to test their ability to induce washed rabbit platelet aggregation.

Mild Alkaline Hydrolysis and Acetylation. This procedure was carried out according to the method of Demopoulos et al. (*18*). Briefly, the sample was dissolved in 1 mL of chloroform:methanol (1:4 v/v), and then, 0.1 mL of 1.2 N NaOH in 50% methanol was added and kept for 20 min at 60 °C. The mixture was neutralized with 0.15 mL of 1 N acetic acid and 2 mL of chloroform:methanol (9:1 v/v), and then, 1 mL of methanol and 2 mL of water were added. The two phases were separated, and the chloroform phase was washed with 1 mL of 33% aqueous methanol. Both phases were separately evaporated to dryness and subjected to reacetylation by the addition of 1 mL of acetic anhydride and warming at 60 °C for 45 min. Each reaction mixture

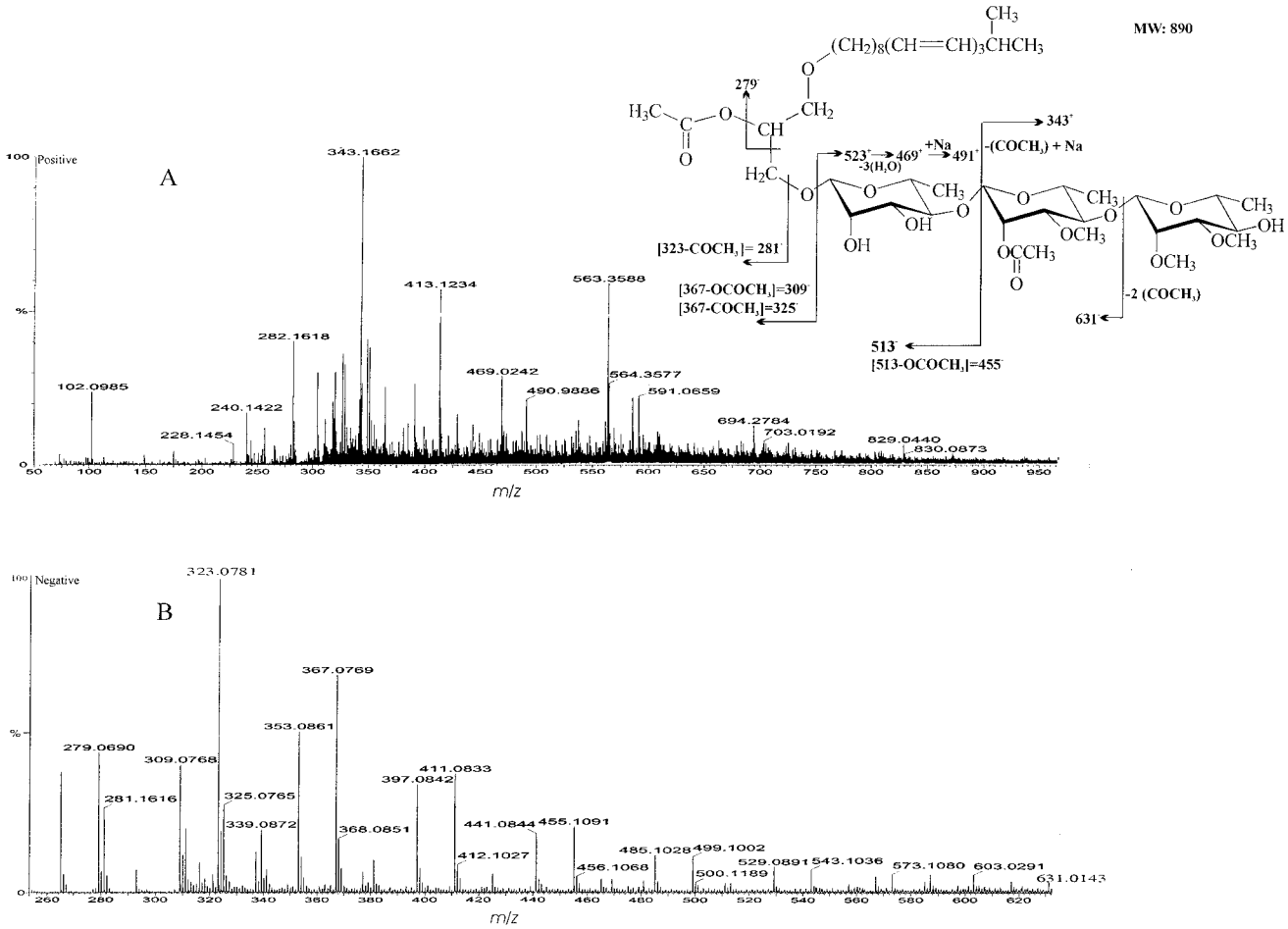

Figure 2. Positive (**A**) and negative (**B**) ion ES/MS of fraction Ambelon/15 and its proposed structure with the most important identified fragments.

from the above phase was evaporated and extracted by the Bligh−Dyer method (*32*). All samples from this procedure were tested for their ability to induce washed rabbit platelet aggregation.

Chemical Determinations. Phosphorus determination was carried out according to the method of Bartlett (*35*). Sugar determination was carried out according to the method of Galanos and Kapoulas (*36*). The phenol content was determined using a modified method of Singleton and Rossi (*37*). Samples were dried under a stream of nitrogen and dissolved in 3.5 mL of water. A 0.1 mL amount of Folin−Ciocalteu reagent was added, followed after 3 min by 0.4 mL of 35% aqueous Na_2CO_3. The reaction mixture was kept for 1 h, and the intensity of the blue color was measured at 725 nm. Standards of gallic acid were prepared similarly. Ester determination was carried out according to the method of Renkonen (*38*).

RESULTS AND DISCUSSION

TLC Separation and Biological Activity of Total Polar Lipids Fractions of Wines and Musts. The extraction and the purification procedure of the lipids of wines and musts were as follows. Total lipids were separated into neutral and polar lipids by counter-current distribution. Total polar lipids from four wines and three musts were then first separated by TLC. A small amount of wine or must along with standards was run on a preparative TLC plate, and after they were stained with iodine, every spot or every space between two spots was collected and lipids were extracted with cloroform:methanol:water 1:2:0.8. The exact R_f of standards is as follows: ceramides, 0.93; quercetin, 0.91; resveratrol, 0.82; cerebrosides, 0.76; digalactosyldiglycerides, 0.72; phosphatidylethanolamine, 0.56; sulfatides, 0.42 and 0.37; hesperidin, 0.32; phosphatidylcholine, 0.28; naringin, 0.28; rutin, 0.20; sphingomyelin, 0.17; and lyso-phosphatidylcholine, 0.11. All lipid fractions extracted were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF or thrombin-induced washed rabbit platelet aggregation. It appeared that white wines (Ambelon, Chardonnay) contained more TLC fractions that induced washed platelet aggregation than red wines (Cuvee Maison, Erythros). All

Table 1. Biological Activity of Each TLC Fraction of Wine Ambelon and Its Musts[a]

TLC	Ambelon		Rompola		Rompola extra yeast	
1	I	1.5/1.3	I	0.80/0.57	I	2.9/10
2	I	0.78/0.15	I	3.3/1.8	A	9.3/6.2
3	I	1.3/0.72			I	4.6/1.9
4	I	5.2/2.9	A	2.0/12	I	7.9/0.0
5	I	1.0/4.8	A	2.0/10	I	7.6/7.0
6	A	27/1.5	A	2.0/8.1	A	11/1.2
7	A	27/1.5	A	1.0/10	A	11/2.9
8	A	14/6.7	A	1.0/4.3	I	0.87/1.2
9	A	17/16	A	0.72/4.5	A	11/2.9
10	A	14/4.9	A	1.0/8.5	A	5.9/2.5
11	I	1.0/-	A	1.0/5.9	A	2.3/2.3
12	A	27/1.5	I	11/3.3	I	6.7/17
13			A	2.0/8.3	A	11/0.4
14			A	6.2/0.5	I	5.9/9.6
15			A	6.2/3.4	A	11/2.0
16					A	3.1/4.2
17					A	3.1/7.4
18					A	3.1/3.2

[a] I, inhibition. IC_{50} PAF/IC_{50} thrombin: IC_{50} value is expressed as milliliters wine per must that inhibits 50% of PAF or thrombin aggregation. A, aggregation. Milliliters of wine or must per centimeter of aggregation induced by fraction.

Table 2. Biological Activity of Each HPLC Fraction, from the Further Separation of Fractions TLC9 and TLC2 of Ambelon Wine

		aggregation				inhibition IC_{50}[a]	
TLC9 fraction	biological action	[PAF][b] × 10^{-11}	mL wine	TLC2 fraction	biological action	PAF	thrombin
1	A[c]	0.31	20	1	A		
2	A	1.68	20	2	I	N[e]	5.1
3	A	0.65	20	3	I	11	N
4	A	1.61	20	4	I	7.7	N
5	A	1.16	20	5	I	6.1	5.5
6	A	1.88	20	6	I	5.0	2.7
7	A	1.49	20	7	I	8.3	N
8	I[d]			8	I	7.9	2.5
9	A	1.15	20	9	I	1.2	N
10	A	2.12	15	10	A		
11	A	1.51	10	11	A		
12	A	1.51	3.0				
13	A	1.87	3.0				
14	A	0.86	12				
15	A	1.60	12				
16	A	1.14	12				
17	A	1.11	12				
18	N						
19	A	0.58	20				
20	A	0.74	20				
21	A	1.64	20				
22	A	1.86	20				
23	A	2.11	20				
24	A	1.74	20				

[a] IC_{50} value is expressed as milliliters wine per must that inhibit 50% of PAF or thrombin induced aggregation. [b] [PAF], equivalent concentration of PAF that exerts in platelets the same biological activity as the amount (milliliters of wine) of fraction. [c] A, aggregation. [d] I, inhibition. [e] N, no biological activity.

Table 3. Biological Activity of Each HPLC Fraction, from the Further Separation of Fractions TLC9,10 and TLC16 of Musts Rompola with and without Extra Yeast, Respectively

		aggregation				aggregation	
TLC9,10 fraction	biological action	[PAF][a] × 10^{-11}	mL wine	TLC16 fraction	biological action	[PAF][a] × 10^{-11}	mL wine
1	A[b]		4.6	1	A	3.8	6.4
2	N[c]	0	4.6	2	A	3.6	6.4
3	A	2.58	4.6	3	A	3.2	6.4
4	A	1.26	4.6	4	A	3.2	6.4
5	A	3.25	4.6	5	A	3.2	3.2
6	A	2.14	0.69	6	A	3.2	6.4
7	A		4.6	7	A		0.64
8	A	1.61	0.92	8	A	1.81	0.0022
9	A	1.94	4.6	9	A	3.2	6.4
10	A	2.11	1.2	10	A	2.17	0.64
11	A	1.61	4.6	11	A	1.67	0.064
12	A	1.51	6.9	12	A	1.36	7.2
13	A		1.2	13	A	0.75	8.0
14	A		1.2	14	A	0.53	6.4
15	A		1.2	15	A	1.21	6.4
16	A	1.84	1.2	16	A	1.71	0.0096
17	A	2.30	1.2	17	A		6.4
18	N	0	1.2	18	A	0.61	6.4
19	N	0	1.2	19	A	0.46	6.4
20	N	0	4.6	20	A		6.4
21	N	0	1.2	21	A	1.54	6.4
22	N	0	1.2	22	A	1.62	0.96
23	N	0	1.2	23	A	1.75	1.3
24	A	0.23	4.6				
25	A	0.38	4.6				
26	A	0.18	4.6				
27	N	0	4.6				
28	N	0	4.6				
29	A	0.41	4.6				
30	N	0	4.6				
31	N	0	4.6				
32	N	0	4.6				
33	N	0	4.6				

[a] [PAF], equivalent concentration of PAF that exerts in platelets the same biological activity as the amount (milliliters of wine) of fraction. [b] A, aggregation. [c] N, no biological activity.

fractions that induced platelet aggregation have common chromatographic characteristics (low R_f values) suggesting that substances of the same class may exist in both red and white wines and are responsible for the aggregation. Musts have fractions that induced platelet aggregation with a variety of R_f values. On the basis of the above results, the wine, Ambelon, and its must, Rompola, with and without extra yeast were chosen for further examination. The biological activity of each TLC fraction of wine Ambelon and its musts is summarized in **Table 1**.

HPLC Separation, Biological Activity, and Characterization of Fractions of Wine Ambelon. Two TLC fractions from Ambelon wine were chosen, one with the most potent inhibitory action with R_f 0.80−0.91 (TLC2) and the other, which induced the most potent platelet aggregation, with R_f 0.19−0.28 (TLC9).

The HPLC separation of fractions TLC2 and TLC9 is shown in **Figure 1A**,**B** with detection at 208 and 280 nm, respectively. All fractions of the HPLC separation of TLC2 and TLC9 were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF or thrombin-induced washed rabbit platelet aggregation. The majority of fractions, from the separation of TLC2, inhibits PAF or thrombin-induced aggregation with IC_{50} values presented in **Table 2**. All of the fractions from the separation of TLC9, with the exception of fractions 8 and 18, induced platelet aggregation in a dose-dependent manner, with fractions 2, 6, 10, 13, and 15 as the most potent. Cross-desensitization experiments and experiments with specific inhibitors were performed on the latter fractions. Fractions 2, 10, 13, and 15 seemed to act through PAF and arachidonic pathways since they desensitized platelets against PAF and thrombin and their aggregation was fully inhibited by the specific inhibitor of PAF, namely, BN 52021, and partly inhibited (approximately 50%) by the specific inhibitor of arachidonic acid, namely, indomethacin. Fraction 6 acted through the PAF pathway only, since its aggregation was not affected by indomethacin and CP/CPK and was inhibited by BN 52021. In addition, this fraction desensitized platelets only against PAF.

Fractions 2, 6, 10, and 13 are still under investigation and will be published elsewhere. Consequently, an attempt was made to elucidate the structure of fraction 15. The UV spectrum of this fraction, with a large peak at 203 nm and two small shoulders at 228 and 286 nm, did not show the obvious presence of phenolic components, and this was confirmed by the negative phenolic determination. Determinations of fatty acid esters (0.375 μmol ester/total fraction) and sugar (0.453 μmol glucose/total fraction) were positive while determination of phosphorus was negative. Alkaline hydrolysis rendered it inactive, while acetylation of the chloroform soluble fraction from the hydrolysis resulted in a molecule with significantly lower activity. Acetylation of the initial fraction also significantly reduced the biological activity. In addition, acetylation of the water soluble fraction did not give any activity, suggesting that an ether bond is present on its backbone. These data suggest the presence of free hydroxyl group acetylation, which is responsible for the reduction of the activity. Incubation of fraction 15 with acetylhydrolase resulted in its time-dependent inactivation,

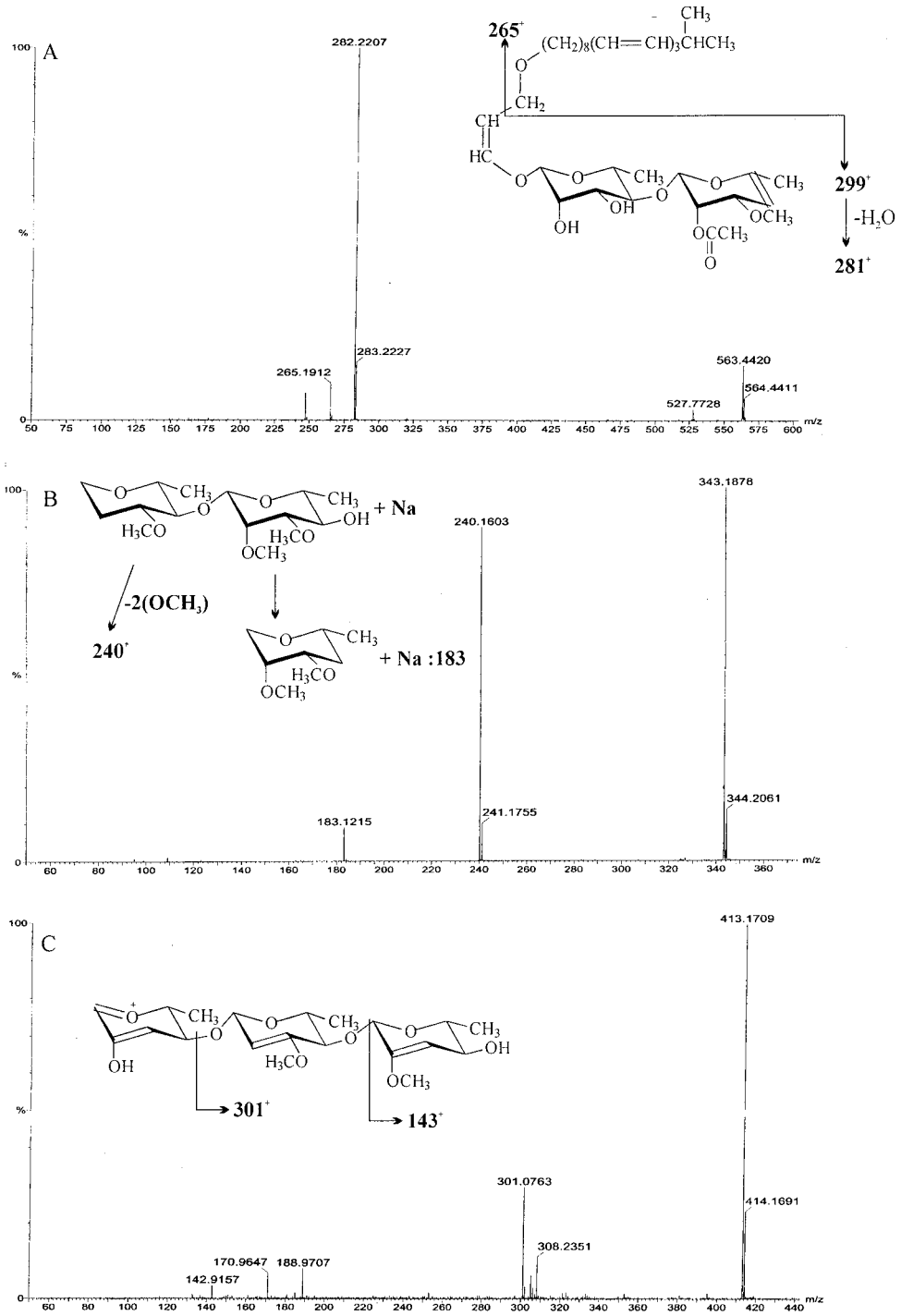

Figure 3. ES/MS-MS of positive ions *m/z* 563 (**A**), *m/z* 413 (**B**), and *m/z* 343 (**C**) of fraction Ambelon/15.

similar to that with PAF, suggesting that at least one acetyl group is present at the *sn*-2 position of a glycerol backbone. The above data and the ES/MS of this fraction support the structure of the glycolipid shown in **Figure 2**. The proposed molecular weight of this fraction is 890, and the positive ion at *m/z* 829 corresponds to the deacetylated molecule plus sodium. The most characteristic fragments in the negative ion MS appear after the cleavage of glucoside bond(s) and acetyl group(s), such as those at *m/z* 309, 325, 367, 455, 513, and 631. Positive ion fragments at *m/z* 469 and 491 also appeared from the cleavage of glycosidic bonds. ES/MS-MS was obtained for positive ion peaks at *m/z* 343, 413, and 563 (**Figure 3**). The MS-MS of *m/z* 343 showed a fragment with *m/z* 240, which is believed to appear after the loss of a methanol fragment from each sugar moiety (*39*) and a fragment with *m/z* 183, corresponding to [2methoxy-rhamnose-CH_3O-H + Na]. The MS-MS of the *m/z* 413 ion showed a fragment with *m/z* 301, which is believed to result from the loss of a sugar moiety and a fragment with *m/z* 143, which may represent the terminal sugar moiety (**Figure 3**). The fragments resulting from the MS-MS of ion *m/z* 563 are represented in **Figure 3**.

HPLC Separation, Biological Activity, and Characterization of Fractions of Rompola Musts with and without Extra Yeast. From each of the two Rompola musts, a TLC fraction was chosen, namely, fractions TLC9 and TLC10 pooled together, resulting in fraction TLC9,10 from Rompola without

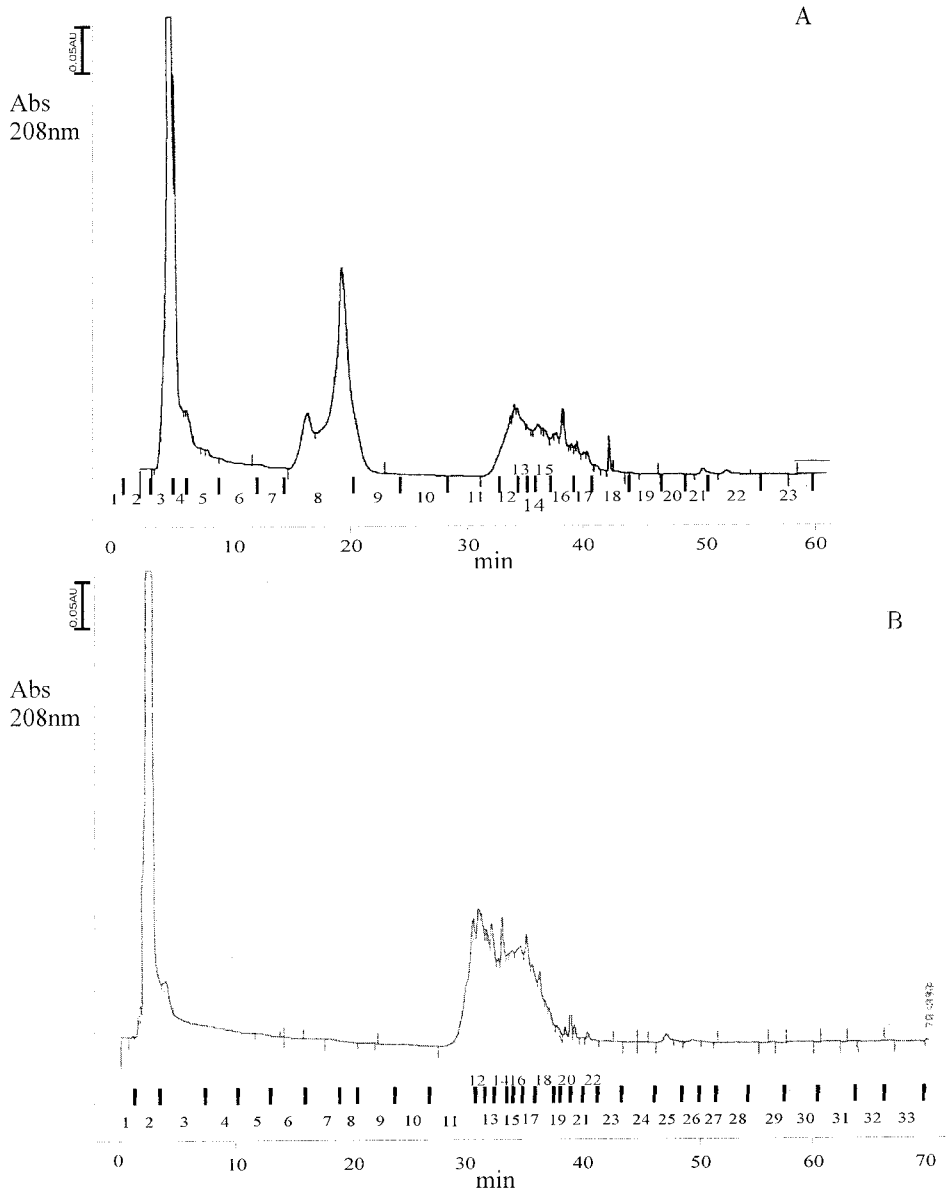

Figure 4. (**A**) Typical reverse phase HPLC separation of fraction TLC9,10 of must Rompola. (**B**) Typical reverse phase HPLC separation of fraction TLC16 of must Rompola with extra yeast.

extra yeast, while fraction TLC16 was chosen from Rompola with extra yeast. These fractions had a significant ability to induce platelet aggregation and shared similar R_f values with fraction TLC9 from Ambelon wine (**Table 1**). These two fractions were further separated on a reverse phase HPLC with the same elution system as the wine and detection at 208 nm. Typical profiles from the separations are present in **Figure 4A**,**B**, respectively. All of the fractions resulting from the above separations were tested for their biological activity.

The results from the biological experiments of the two musts are summarized in **Table 3**. All of the biological active fractions induced platelet aggregation in a dose-dependent manner, while the rest of them did not exert any biological activity. The ability of fractions to induce platelet aggregation seemed to fluctuate, with a maximum at fractions 3, 6, 8, 10, 16, and 17 for Rompola must and fractions 8, 11, 16, and 22 for Rompola with extra yeast must. These fractions, which are the most potent ones, were selected in order to perform cross-desensitization experiments and experiments with specific inhibitors. Fractions 3 and 10 from Rompola as well as fraction 22 from Rompola with extra yeast did not seem to act through a specific pathway since they desensitized platelets against PAF and thrombin and their aggregation was almost fully inhibited by BN52021, indomethacin, and CP/CPK. Fractions 6 and 8 from Rompola, as well as fraction 8 from Rompola with extra yeast, acted through PAF and arachidonic acid pathways since they desensitized platelets against PAF and thrombin but their aggregation was inhibited by BN52021 and indomethacin. Fractions 16 and 17 from Rompola, as well as fractions 11 and 16 from Rompola with extra yeast, acted only through the PAF pathway since their aggregation was fully inhibited only by the specific inhibitor of PAF BN52021, and also, they did not desensitize platelets against thrombin. Because fractions 16 from both musts are potent agonists, which seem to act through the PAF pathway only, they were selected for further study in order to elucidate their structures.

Fraction Rompola/16. The UV spectrum of this fraction, with a large peak at 202 nm and two small shoulders at 225 and 278 nm, did not show the obvious presence of phenolic components, which was also confirmed with the negative phenolic determi-

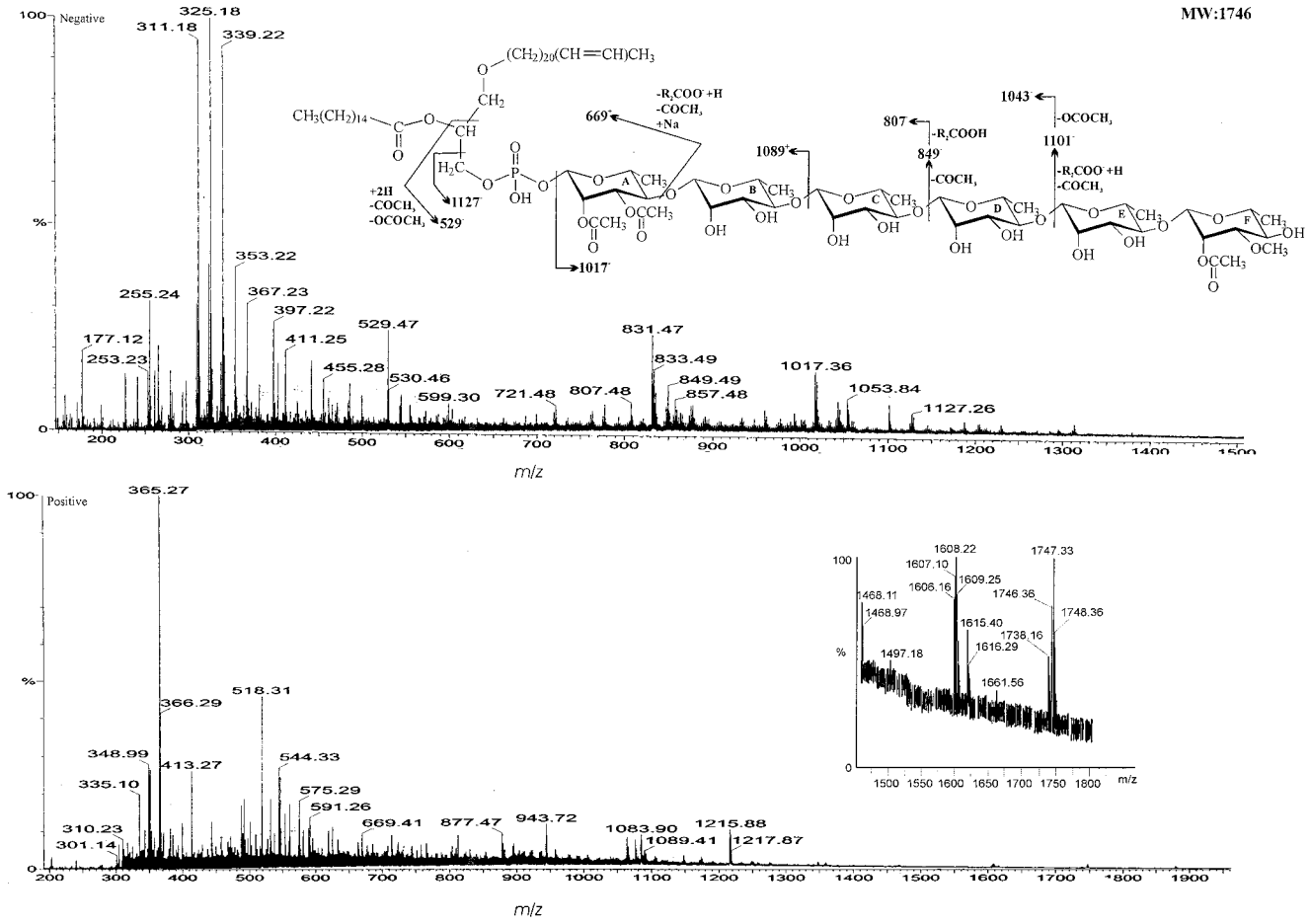

Figure 5. Positive and negative ion ES/MS of fraction Rompola/16 and its proposed structure with the most important identified fragments.

nation. Determinations of fatty acid esters (0.427 μmol ester/total fraction), sugar (0.271 μmol glucose/total fraction), and phosphorus (0.102 μmol phosphorus/total fraction) were all positive. Acetylation of the fraction decreased its ability to induce platelet aggregation, indicating that at least one free hydroxyl group is present. Alkaline hydrolysis rendered it inactive, while acetylation of both chloroform and water soluble fractions from the hydrolysis resulted in a molecule with increased activity only in the case of chloroform soluble fraction. These data suggest the presence of ester bond(s) and indicate the presence of an ether bond on its backbone. Treatment with acetylhydrolase did not affect its action, suggesting that there is not an acetyl group present in the glyceryl moiety of the molecule.

ES/MS confirmed the above data and indicated the structure of the phospho-glycolipid shown in **Figure 5** with a molecular weight of 1746. The carboxylate anion from the fatty acyl group at *sn*-2 is observed in the negative ion mass spectrum at *m*/*z* 255. The most abundant negative ions at *m*/*z* 311, 325, and 339 are thought to represent fragments of the phosphate group plus the sugar moiety (**Figure 5**). Fragments of the phosphate group plus sugar moieties A + B, A + B + C, A + B + C + D, A + B + C + D + E, and plus all of the sugar moieties yield the negative ions at *m*/*z* 455, 603, 763, 895, and 1113, respectively. Deacetylation of the above fragments gives rise to the negative ions at *m*/*z* 253 (311-58), 227 (311-2 × 42), 241 (325-2 × 42), 297 (339-42), 281 (339-58), 397 (455-58), 339 (455-2 × 58), 545 (603-58), 721 (763-42), 705 (763-58), 663 (763-42-58), and 1055 (1113-58), all supporting the existence of acetyl groups on the sugar moieties. Also, the deaceylated terminal sugar moiety yields the negative ion at *m*/*z* 177. The negative ion at *m*/*z* 411 represents the glyceryl moiety after cleavage of the fatty acid side chain from the *sn*-2 position. The fragment containing the sugar moieties A, B, C, D, and E is represented at *m*/*z* 831. Also, the fragmentations resulting in the negative ions at *m*/*z* 529, 807, 849, 857, 1017, 1043, 1101, and 1127 are represented in **Figure 5**.

The positive ion spectrum (**Figure 5**) shows the $[M + H]^+$ ion of the molecule at *m*/*z* 1747 while the ion at *m*/*z* 1217 represents the dehydration product after the loss of the three terminal sugars. The fragment corresponding to 1217 is confirmed by the ES/MS-MS analysis (**Figure 6A**), where *m*/*z* 687 is the parent molecule minus the two terminal sugars (B + C) and after the neutral loss of 256 (palmitic acid). Cleavage of the three ester bonds at fragment *m*/*z* 1217 yields the positive ion at *m*/*z* 877.

Loss of the three terminal sugars along with the neutral loss of 256 and the loss of an acetyl group gives the positive ion at *m*/*z* 943, which represents the sodiated fragment. This structure is supported by the ES/MS-MS analysis (**Figure 6B**). The positive ion at *m*/*z* 1083 is thought to represent the fragment after loss of the two terminal sugars plus the neutral loss of 256 amu together with an acetyl group. Furthermore, additional loss of the other acetyl group results in the sodiated fragment at *m*/*z* 1063.

The fragments at *m*/*z* 669 and 1089 are also represented in **Figure 5**. Finally, the terminal disaccharide moiety with and without the oxygen of the glycoside bond yields the positive ions at *m*/*z* 365 and 349, respectively.

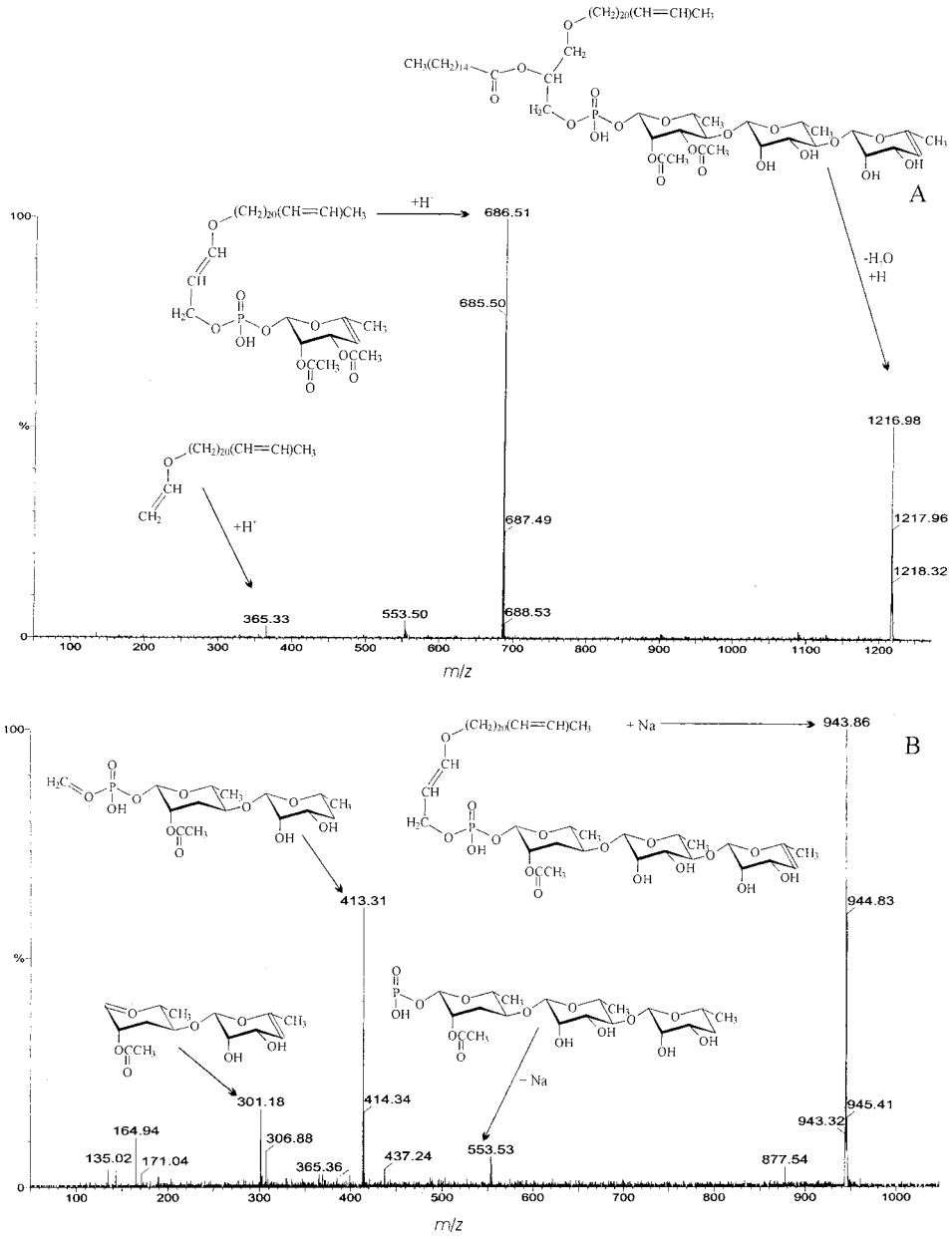

Figure 6. ES/MS-MS of positive ions *m/z* 1217 and *m/z* 943 of fraction Rompola/16.

Fraction Rompola with Extra Yeast /16. The UV spectrum of this fraction, with a large peak at 202 nm and two small shoulders at 230 and 278 nm, did not show the obvious presence of phenolic components, which was also confirmed with the negative phenolic determination. Determinations of fatty acid esters (0.924 μmol ester/total fraction), sugar (0.495 μmol glucose/total fraction), and phosphorus (0.168 μmol phosphorus/total fraction) were positive. Acetylation of the fraction decreased its ability to induce platelet aggregation, indicating that at least one free hydroxyl group is present. Alkaline hydrolysis rendered it inactive, while acetylation of the chloroform and water soluble fractions resulted in loss of activity and in change of platelets shape, respectively. These data suggest that there are ester bonds esterified with aliphatic fatty acids, and their replacement with acetic acid leads to an inactive molecule. Treatment with acetylhydrolase resulted in its inactivation in a shorter time than PAF, indicating the presence of acetyl group(s) in another position than in the glyceryl backbone.

ES/MS confirmed the above data and indicated the structure of the 1,2-diacyl glycerophospho-glycolipid shown in **Figure 7**. In the negative ion spectrum, the fragments at *m/z* 127, 265, and 267 represent the fatty acid anions. The intensity distribution of these ions indicates that the glycerophospho-glycolipid is present as a mixture and the main component contains the carboxylate anions corresponding to *m/z* 127 and 265, with a molecular weight of 976, since the positive ion $[M + H]^+$ at *m/z* 977 as well as the negative ion $[M - CH_3CO]^-$ at *m/z* 933, are observed. The data showed that this molecule shares similarities with Rompola/16 but that Rompola/16 has many more sugar moieties. The most important fragments are shown in **Figure 7**. Additionally, the positive fragment at *m/z* 903 probably results from the loss of the acetyl group at 42 amu and the neutral loss of the methoxy group, while the positive fraction at *m/z* 803 appears after the loss of the three acetyl groups at 58 amu each. The losses of the fatty acyl groups from the *sn*-1 and *sn*-2 positions lead to the fragment $[MH-R_1-C{=}O+H-R_2C{=}O + H]^+$ at *m/z* 619. The negative ion fragment at *m/z* 97 corresponds to HPO_4^{-2}. ES/MS-MS was performed on negative ion fragment *m/z* 529. Fragments at *m/z* 267, 265,

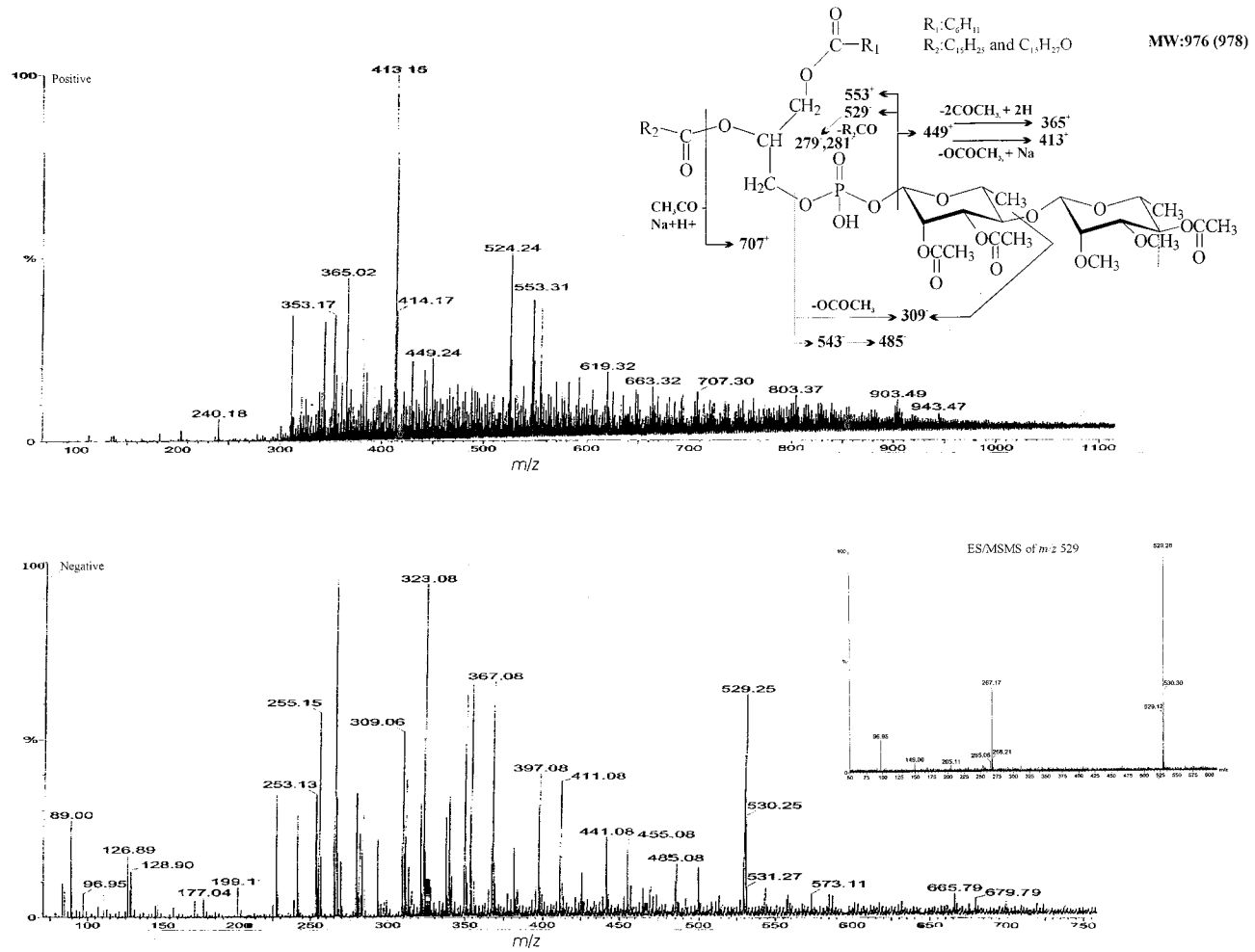

Figure 7. Positive and negative ion ES/MS of fraction Rompola with extra yeast/16 and ES/MS-MS of negative ion *m*/*z* 529 and its proposed structure with the most important identified fragments.

corresponding to $[R_1COO]^-$ and $[R_1'COO]^-$, and at *m*/*z* 97 appeared, confirming the above structure.

In this work, total polar lipids of several wines and musts were separated by preparative TLC, and the biological activity of the fractions was compared. The results showed that all fractions that induced platelet aggregation have common chromatographic characteristics of low R_f values, suggesting that substances of the same structural class exist in white and red wines and are responsible for the aggregation. Moreover, musts seem to have more fractions inducing platelet aggregation. The most potent wine and its respective must, with and without yeast, was chosen for further HPLC separation. Many biologically active compounds were found that inhibited PAF/thrombin action or as PAF agonists minimized PAF biological effects in several cells and tissues. Additionally, the structure of the most potent ones was identified. It appears that the most active lipid in must, with and without extra yeast, and the one in wine are lipids with a glycerol backbone although they have some differences. Most interesting is that the lipids in must are phospho-glycolipids, while in wine it is a glycolipid. These findings suggest that the lipids of grape or yeast are subject to chemical modification during fermentation and moreover that the biologically active lipids come from the grape, since almost the same structure was found in the must and wine. The question of whether lipids of yeast contribute to the total biological activity of the final product (wine) is under investigation. These support and enhance the France paradox since wines and musts contain a significant number of lipids with antithrombotic and antiatherogenic action in vitro.

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