Evidence for the Presence of Neutral Glycerylether Derivatives in Pollen Lipids of Pine Tree *Pinus halepensis*

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The separation and identification of glyceryl ether (GE) derivatives from pollen neutral lipids of Pine tree *Pinus halepensis* (Miller) is described.

The separation and purification of GE derivatives from the other lipid classes, especially from monoglycerides and diglycerides, was done by thin layer chromatography.

The purified GE derivatives were examined by infrared spectrometry analyzed by gas liquid chromatography, and the glycerylether backbones were identified by gas chromatography-mass spectrometry of its isopropylidene derivatives.

Fatty acids were detected as esterified constituents after mild alkaline hydrolysis of the GE fraction.

The GE derivatives are characterized as 1-O-(di)hydroxyalke(die)nyl-glycerols, the side hydroxyl group(s) of the O-carbonchain being esterified mainly with saturated fatty acids.

Introduction

Glyceryl ethers (1-O-alkyl glycerols) were first isolated in 1922 from the unsaponifiable fraction of fish liver oils [1] and two years later, in 1924, their alk-1-enyl analogs were discovered in the form of glycerophosphatides (plasmalogens) in histochemical preparations [2]. Since then it was progressively recognized that both, alkyl and alk-1-enyl glycerols are much more widespread in nature than originally thought; their chemistry and biochemistry were intensively studied and comprehensive reviews were edited in 1972 by Snyder [3] and recently by Mangold and Paltauf [4].

In contrast to most other living organisms (animals, protozoa, bacteria), the presence of ether lipids in plants has been questionable. Reported evidence for the occurrence of plasmalogen in plant phospholipids was not unequivocally documented [5], since it was based only on the plasmal reaction, except one case, reported by Kaufman *et al.* in 1971, in which the identification of plasmalogens was based on the GLC analysis of the aldehydes derived from the polar lipids of dormant green peas and soybeans as well as pea and soybean seedlings, after $LiAlH_4$ treatment followed by acid catalyzed hydrolysis of the separated rather polar neutral fraction [6]. Similarly, experimental evidence reported in 1975, for the presence of alkyl glycerols in the lipids of a unicellular plant was limited in the periodate-Schiff visualizing reagent on TLC after the Vitride reaction [7], and in a recent review [8] Mangold concluded that ether lipids must be present in plants only in traces, if at all.

In this paper, the presence of glyceryl ether derivatives in the pollen of a pine tree is unequivocally documented by a combination of chemical, IR, GLC and GC-MS analysis.

Abbreviations: c(9:0) e.t.c., carbon chain:number of double bounds; DG, diglycerides; FA, fatty acids; F.AL., fatty alcohols; GC, gas chromatograph(y); GC-MS, gas chromatography-mass spectrometry; GE, glycerylether; GE(pollen) fraction/content, lipid material comigrating with GE authentic standard on TLC; GLC, gas-liquid chromatography; IR, infrared; MG, monoglycerids; MW, molecular weight; RT, retention time; SF, solvent front; ST, sterols; STD, standards; T, traces; TL, total lipids; TLC, thin layer chromatography; yes, detectable amount comigrating on SF.

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Experimental

Materials

Pollen (free from microbial contamination) was collected by hand from Pine trees at Chalandri of Attica, Greece.

All reagents and chemicals were of analytical grade supplied by Merk (Darmstadt, FRG).

Standard substances were obtained from Supelco (Bellefonte, Pennsylvania, USA) and the diacyl GE esters and GE isopropylidene derivatives were synthesized in our laboratories [9].

The water was deionized and distilled.

Instrumentation

GLC analyses were performed with a Perkin-Elmer (Norwalk, Connecticut, USA), model Sigma 3 GC, fitted with flame ionization detector and coupled to a model 2 integrator-calculator printer and to a model 550 recorder.

Glass GC columns, 1.80 m × 2.2 mm I.D., were packed in our laboratories with the appropriate column supports, purchased from Supelco and Varian (Palo Alto, California, USA).

GC-MS was carried out in a Hewlett-Packard (Palo Alto, California, USA) model 5980 A, data system, using a stainless-steel, 3.5 m × 2.0 mm I.D., prepacked column (Varian).

IR spectra were obtained with a Perkin-Elmer, model 397, spectrophotometer.

Chromatographic conditions

Preparative TLC was performed on glass plates, 20 × 20 cm, with silica gel G (Merk), 0.50 mm thickness and the separated lipid fractions were visualized by exposure to iodine vapors. The developing solvent systems are given in the text.

Isothermal GLC analysis of FA was performed by directly injecting 1 μl of chlorophorm solution into a GP-5% DEGS-PS on Supelcoport 100/120 mesh column (Supelco). The column temperature was 200 °C and the temperatures of inzector and detector 220 °C. The flow of the carrier gas (N_2) was 20 ml/min and the sensitivity R 10 × 16 (1 mv).

Other chromatographic and spectrophotometric conditions are given in the figure legends.

Isolation of pollen GE fraction

Extraction of pollen lipids, their fractionation by preparative TLC and the qualitative and quantitative analysis of the major lipid classes are reported separately [10]. Briefly, pollen total lipids were extracted by a modified Bligh and Dyer procedure [10] after breakage of the cell-walls in a pressure pump (Yeda Press, Rehovot, Israel).

The individual lipid classes were isolated by preparative TLC (40 mg TL on each chromatoplate) in the solvent system diethyl ether/formic acid, 100/6, (v/v) up to the R_f = 0.4, followed by petroleum ether (b.p. 40–60 °C)/diethyl ether/formic acid, 80/20/3 (v/v/v). The wide bands migrating to the MG and GE area (R_f 0.4) were scrapped off the chromatoplates and extracted with chloroform/methanol, 2/1 (v/v). The pooled extracts were evaporated to dryness and re-chromatographed in the same system. One half on this mixed (MG + GE) fraction was submitted to acetolysis and saponification [11] and the unsaponifiable material was chromatographed by the TLC technique mentioned above. The band comigrating with an authentic GE standard (R_f 0.4) was extracted as above and appropriate aliquots were used for colorimetric GE determination [12]. Another part of this free GE solution was used for the preparation of isopropylidene derivatives [13]. The products of the reaction comigrated with the isopropylidene derivatives of an authentic GE standard (R_f 0.45) by TLC in the solvent system petroleum ether (b.p. 40–60 °C)/diethyl ether/formic acid, 90/10/1 (v/v/v). They were isolated by extraction of the silica gel and submitted to analysis by GLC (Figs. 4 and 5) and GC-MS (Fig. 6).

The other half of the mixed (MG + GE) fraction was resolved into its components by preparative TLC using a multiple-development technique with solvent

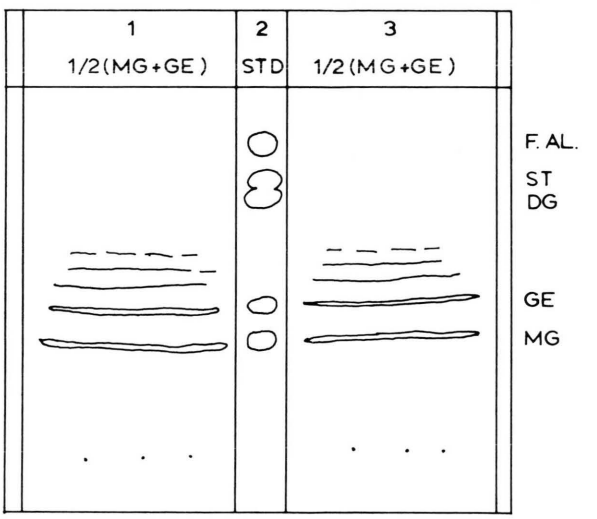


Fig. 1. TLC separation of pollen monoglycerides and glycerylethers. Developing solvents: petroleum ether (b.p. 40–60 °C)/ether/formic acid used on three developments, 1st and 2nd by the ratios 60/40/3 (v/v/v) and the 3rd by 50/50/3 (v/v/v). Lanes: 1 and 3, one half of the pollen lipid (MG + GE) fraction, respectively; 2, authentic standards (Supelco). Visualization by exposure to iodine vapors.

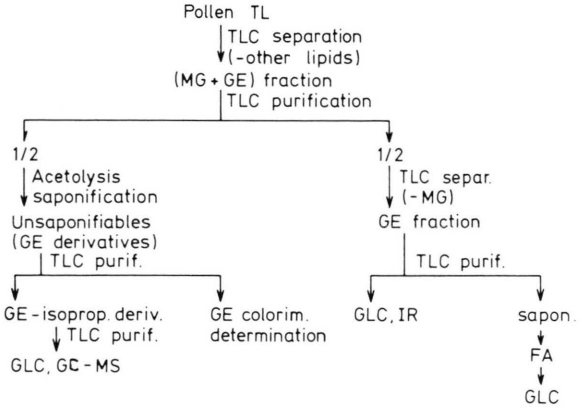
Table I. Distribution of the esterified fatty acids in the MG and GE pollen lipid fraction from GLC analysis.

FA	MG fraction [%]	GE fraction [%]
c(9:0)	yes	yes
c(9:0)	T	T
c(10:0)	0.39	0.26
c(11:0)	0.79	0.78
c(11:1)	1.51	2.56
c(12:0)	3.73	3.87
c(14:0)	11.11	9.39
c(16:0)	52.07	33.84
c(18:0)	7.07	31.21
c(18:1)	T	4.45
c(18:2)	T	5.13
c(20:0)	23.33	8.58
Total	100.00	100.00
Total in TL	1.261	0.191
Total mmol in TL	4.861	0.733

systems petroleum ether (b.p. 40–60 °C)/diethyl ether/formic acid, 60/40/3 (twice) and 50/50/3 (once). As it is shown in Fig. 1, the mixture was resolved into two main band comigrating with authentic MG and GE standards respectively. The components of the GE band were re-chromatographed by the same multiple-development technique. The purified GE fraction was re-dissolved in 800 µl of chloroform and appropriate aliquots were used for GLC (Fig. 3) and IR (Fig. 2) analysis. In addition, the free fatty acids obtained by saponification [14] of 400 µl of the above solution were submitted to quantitative analysis by GLC using pentadecanoic acid as internal standard (Table I).

The separated MG fraction was also treated as above.

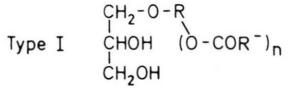
The treatment of pollen lipids is represented by the following flow diagram.



Results and Discussion

Our first series of experiments indicated that pollen lipids contain a peculiar GE species, bearing on their molecules esterified fatty acids together with the two free hydroxyls of the glycerol moiety. The latter argument was supported by comparison of the GLC behavior of the pollen GE species, chimyl or batyl alcohols, their mono- and diacetyl derivatives and monomyristoyl glycerol (Fig. 3). These data conform with formulas of the type I, where

Type I



R and R′ are saturated or unsaturated fatty chains and n = integral ≥ 1.

To test this hypothesis we repeated the entire study, starting with two 80-mg lots of pollen TL, with every possible caution during the purification steps (by re-chromatography), as described in the previous section. The combined experimental data may be summarized as follows:

The total lipid content was found 2.5 ± 0.2% of pollen and the GE content of pollen neutral lipids was found 0.45 ± 0.03 µmol/100 mg TL. The amount of free fatty acids liberated by saponification of the re-purified intact GE fraction was found 0.73 ± 0.04 µmol/100 mg TL, corresponding to a molar ratio of acyl groups to ether bonds approx. 2:1. As shown in Table I, GLC analysis of the fatty acids liberated from the purified MG and GE fractions by the TLC of Fig. 1 indicated significant differences of composition, thus suggesting that the GE fraction was not contaminated by MG.

As it is shown in Fig. 2, the IR spectrum of the purified GE fraction indicated the presence of hydroxyl, methyl and methylene groups, double bonds, ester carbonyl and C-O, and ether C-O bonds.

The GLC patterns of non-derivatized pollen GE fraction and a mixture of chimyl and batyl alcohols, their mono- and diacetyl derivatives and monomyristoyl glycerol, Fig. 3, indicated that the pollen GE fraction contains three main components in percentages 72.6, 8.8 and 18.6 (1st, 2nd and 3rd components respectively). The RTs of first and third components were similar (but not identical) to these of chimyl and batyl alcohols respectively and the second component had an intermediate RT.

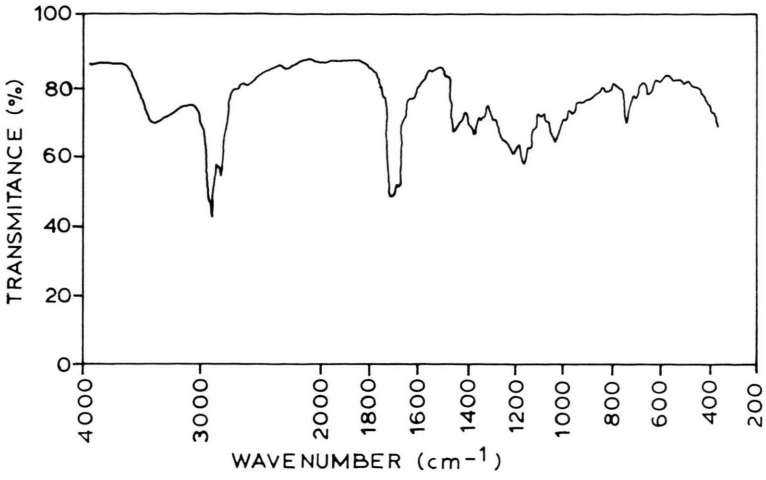


Fig. 2. IR spectra of pollen glyceryl-ether derivatives. Conditions: Scan, 4 min; slit, 2; sample, film technique on KBr "windows" from chloroform solution.

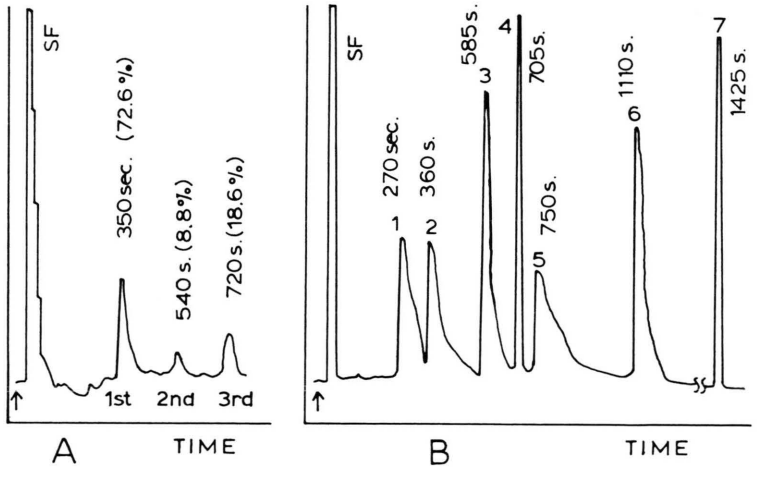


Fig. 3. GLC analysis of glycerylether derivatives. A. Pollen glycerylether components. B. Standard mixture (0.25% each). Peaks: 1, monomyristine; 2, chimyl alcohol; 3, 2-acetyl-chimyl alcohol; 4, 2-3-diacetyl-chimyl alcohol; 5, batyl alcohol; 6, 2-acetyl-batyl alcohol; 7, 2-3-diacetyl-batyl alcohol. Chromatographic conditions: Column packing, 3% SE-30 on Gas Chrom Q 80/100 mesh (Varian); oven, 200 °C (isothermal); injector and detector (FID), 220 °C; carrier gas, nitrogen (80 ml/min); sensitivity R 10 × 4 (1 mV). Injections: 2 µl of chloroform solution.

The noticable higher RTs of the mono- and di-esterified chimyl and batyl alcohol, with the low MW acetic acid (Fig. 3B) from the first and third pollen components (Fig. 3A) are also indicative in addition to the IR evidence, of the presence of free hydroxyl groups in the glycerol moiety.

GLC analysis in two columns of different polarity (3% SE-30 and 15% DEGS) was also performed on the isopropyliden derivatives prepared from the pollen free GE components obtained after acetolysis and saponification of the (MG + GE) fraction. As it is shown in Figs. 4 and 5, in both systems the pollen derivatives were resolved again into three main components.

A comparison of the RT values of the first and third GE components (free and isopropylidene derivatives) with those of the chimyl and batyl alcohol (free and isopropylidene derivatives) respectively suggest that these two couples are not members of the same homologous series of compounds, because an inverse relation occurs between the RT values of the isopropylidene derivatives (Figs. 4 and 5) and of the respective non derivatized components (Fig. 3). Namely, both SE-30 (Fig. 4) and DEGS (Fig. 5) columns the RT values of both the pollen isopropylidene derivatives are slightly higher than those of the chimyl and batyl alcohol respectively, but in the same SE-30 column (Fig. 3) the non-derivatized first

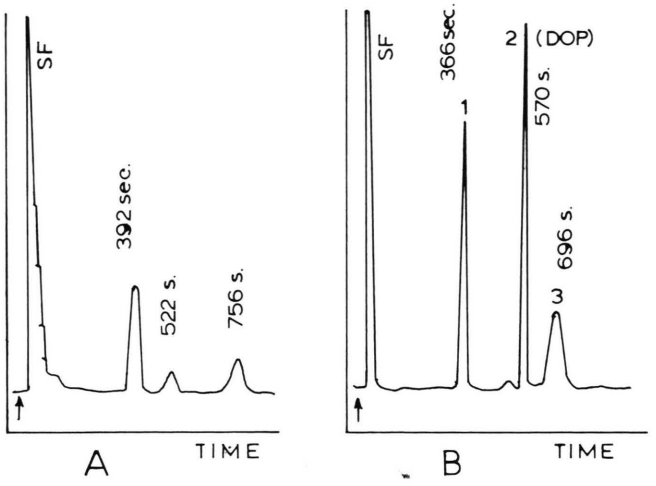


Fig. 4. GLC analysis of glycerylether isopropylidene derivatives on a polar GC column. A. Pollen glycerylether isopropylidene derivatives (after acetolysis and saponification). B. Standard mixture. Peaks: 1, chimyl alcohol isopropylidene derivative (0.01%); 2, dioctyl phtalate (0.001%); 3, batyl alcohol isopropylidene derivative (0.01%). Chromatographic conditions: as in Fig. 3. Injections: 1 µl of chloroform solution.

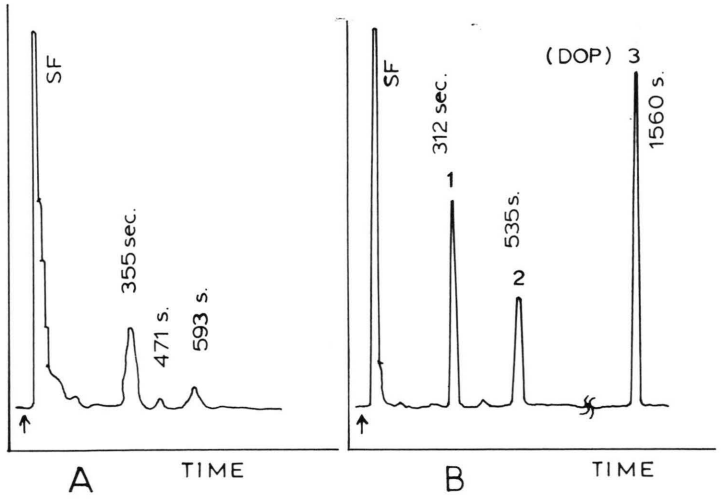
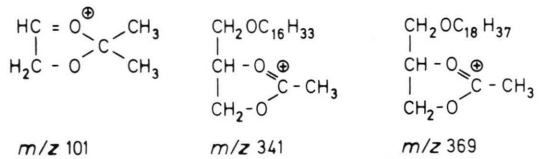


Fig. 5. GLC analysis of glycerylether isopropylidene derivatives on a non-polar GC column. A. Pollen glycerylether isopropylidene derivatives (after acetolysis and saponification). B. Standard mixture. Peaks: 1, chimyl alcohol isopropylidene derivative (0.01%); 2, batyl alcohol isopropylidene derivative (0.01%); 3, dioctyl phalate (0.001%). Chromatographic conditions: Column packing, 15% DEGS on chromosorb W, AW 80/100 mesh (Varian); other conditions as in Fig. 4. Injections: 1 µl of chloroform solution.

and third pollen components have slightly lower RT values that chimyl and batyl alcohol respectively.

The dramatic change of the relative RT of the DOP internal standard in the two columns of different polarity (Figs. 4 and 5) is an instructive example of the influence of the nature of compounds on their relative RT values.

Conclusive evidence was obtained by GC-MS analysis of the isopropylidene derivatives mentioned above. As it is shown in Fig. 6, the RT values of the three pollen derivatives were 610, 720 and 1050 sec. The isopropylidene derivatives of chimyl and batyl alcohol under the same conditions gave again slightly lower RT values of 590 and 1020 sec respectively, a common ion *m/z* 101 accompanied by a second ion, *m/z* 341 from the chimyl alcohol derivative and *m/z* 369 from the batyl alcohol derivative:



The ion species *m/z* 101 was also common in the mass spectra of all the three pollen isopropylidene derivatives, each accompanied by a second ion species with *m/z* 341, 355 and 369 (first, second and

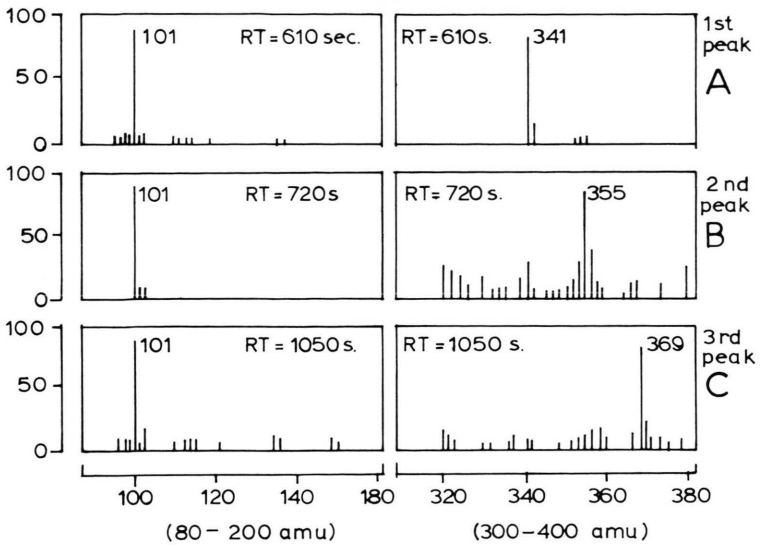
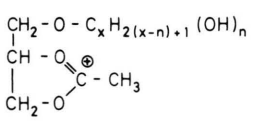


Fig. 6. GC-MS spectra of pollen glyceryl-ether isopropylidene derivatives (after acetolysis and saponification). A, B and C: first, second and third components respectively. GC conditions: Column packing Carbowax 20M/chromosorb W, AW, DMCS 80/100 mesh (Supelco); oven, 230 °C (isothermal); injector 150 °C; carrier gas, helium (33 ml/min). MS conditions: electron impact; ion source, 175 °C; electron energy 70 eV. Injections: 0.1 µl of ethanol solution.

third pollen component respectively). The latter ions correspond to chimyl, 1-O-heptadecyl and batyl alcohol respectively, but these structures cannot comply with our experimental data already discussed which indicate the presence of additional hydroxyl groups (originally esterified in the native compounds), as well as of double bonds.

On another hand, by introducing one double bond plus one hydroxyl group in a fatty chain its *m/z* number increases by 14, that is as much as by an additional methylene group. Therefore, the following ion species of type II, derived from compounds of type I, are alternatives to the saturated species mentioned above:

Type II



m/z = 341 for x = 15, n = 1, or x = 14, n = 2
m/z = 355 for x = 16, n = 1, or x = 15, n = 2
m/z = 369 for x = 17, n = 1, or x = 16, n = 2

In conclusion, taking into consideration that the molar ratio of acyl to ether groups in the native pollen components was found 2:1 (see above), the latter seem to be glyceryl ether derivatives of type I (with n = 2), *i.e.* diacyl derivatives of 1-O-dihydroxytetradecadienyl, 1-O-dihydroxypentadecadienyl and 1-O-dihydroxy-hexadecadienyl-glycerol (first, second and third component respectively).

Acknowledgements

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