Characterization of Seven New Hydrocarbon Compounds Present in the Aroma of Virgin Olive Oils

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Seven isomeric hydrocarbons were detected in the volatile fraction of three samples of virgin olive oil. Chemical ionization mass spectrometry was used to assign the molecular formulas. Their structures were confirmed by comparison of retention time and mass spectral data with those of a synthetic sample obtained by pentene radical coupling. A final characterization of each chromatographic peak was done by means of a chiral capillary column to distinguish the optically active compounds from the isomers without chiral centers. For the quantitation the recovery factor in the oily matrix during the extraction of the volatile fraction was obtained using a related chemical, the commercially available β-citronellene. On the basis of the previous literature and the present experiments a tentative rationalization of the involved biochemical pathway is proposed.

Keywords: *Virgin olive oil; flavor; lipoxygenase; pentene dimers; radical coupling*

INTRODUCTION

Fresh and good quality virgin olive oil is highly appreciated by consumers for its delicious taste and aroma. These are due to both some nonvolatile compounds, i.e. complex phenols (bitter taste) (Angerosa et al., 1995), and a number of volatile chemical compounds generated during the crushing−malaxation steps of oil production (Morales et al., 1994).

The volatiles present in the virgin olive oil aroma belong to different chemical classes. Some of them, such as carbonyl compounds and alcohols, have been studied by, in addition to the usual direct gas chromatography of the whole aroma extract, HPLC methods after suitable derivatization reactions (Solinas et al., 1987a,b). Chemicals pertaining to other classes, e.g. hydrocarbons, are difficult to derivatize, and therefore they can be characterized only by direct gas chromatographic analysis of the total aroma fraction. The semivolatile hydrocarbon fraction has been partially investigated by several researchers. In 1980 Olías et al. pointed out its complexity; they provided evidence for three hydrocarbons with the same molecular formula ($C_{10}H_{18}$) which were attributed to monoterpenes on the basis of mass spectra. Camera and Solinas (1990) characterized olive oil volatile compounds by GC/MS and detected five different compounds showing identical mass fragmentations. They suggested that these compounds were isomers and that a rearrangement occurred in the ionization source, giving rise to the same spectrum. We feel despite the above evidence the topic is still unclear and a full characterization of such compounds is required.

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EXPERIMENTAL PROCEDURES

Materials. Three samples of virgin olive oil, extracted respectively from olives of Coratina (Italian oil), Koroneiki (Greek oil), and Picual (Spanish oil) varieties cultivated in Abruzzo, Italy, Crete, Greece, and Sevilla, Spain, were used for the investigation. All solvents, for organic residual analysis, were purchased from J. T. Baker (Deventer, Holland). 3-(*E*)-Hexenoic acid was purchased from Aldrich (Steinheim, Germany) and 3,7-dimethyl-1,6-octadiene (β-citronellene) from Fluka Co. (Buchs, Switzerland). NaOH, $AgNO_3$, and $K_2S_2O_8$, all analytical grade reagents, were purchased from Carlo Erba (Milano, Italy). Activated charcoal (0.5−0.85 mm; 20−35 mesh ASTM) was obtained from E. Merck (Schuchardt, Germany). Charcoal was cleaned by a treatment of 2 h in a Soxhlet apparatus with diethyl ether and tested before the analyses.

Volatile Compound Extraction. Fifty grams of oil was put into a 120-mL Drechsel gas washing bottle with a porous distributor. Volatiles were stripped with nitrogen (1.2 dm^3 min^{-1}, 37 °C) for 2 h, trapped on 50 mg of activated charcoal, and eluted with 1 mL of diethyl ether (Figure 1). A purification−enrichment sequence was necessary for a better characterization of the isomeric hydrocarbons. Four charcoal traps obtained from four further extraction steps were collected together in a 4-mL screw-cap vial, with 1 mL of pentane added and left for 30 min. Separation of the hydrocarbon fraction from the total aroma extract was done by use of a 1-mL "isolute" SPE cartridge filled with 200 mg of silica supplied by IST International (Hengoed-Mid-Glamorgan, U.K.). Pentane was the solvent adopted in the elution. Seven 1-mL fractions were collected, and the compound distribution in the eluate was followed by GC. The hydrocarbon compounds were detected in fraction 2 (~70%), 3 (20%), and 4 (the remaining).

GC Analysis. Gas chromatography was carried out with a Carlo Erba (Rodano, Italy) Mega series 5160 fitted with a Carbowax 20 M silica capillary column (Nordion, Helsinki, Finland) (50 m length; 0.32 mm i.d.; 0.5 μm film thickness), and equipped with an on-column injection system, a CO_2 cryogenic accessory to hold the oven at 25 °C and a flame ionization detector (FID). The oven temperature program was run at 25 °C for 7 min, raised at 0.8 °C min^{-1} to 33 °C (no hold), then at 2.4 °C min^{-1} to 80 °C (no hold) and at 3.7 °C min^{-1} to 155 °C, and held there for 20 min. The temperature of the detector was held at 240 °C, and H_2 was used as carrier gas at 30 kPa. Injection volume was 0.5 μL. Quantitation

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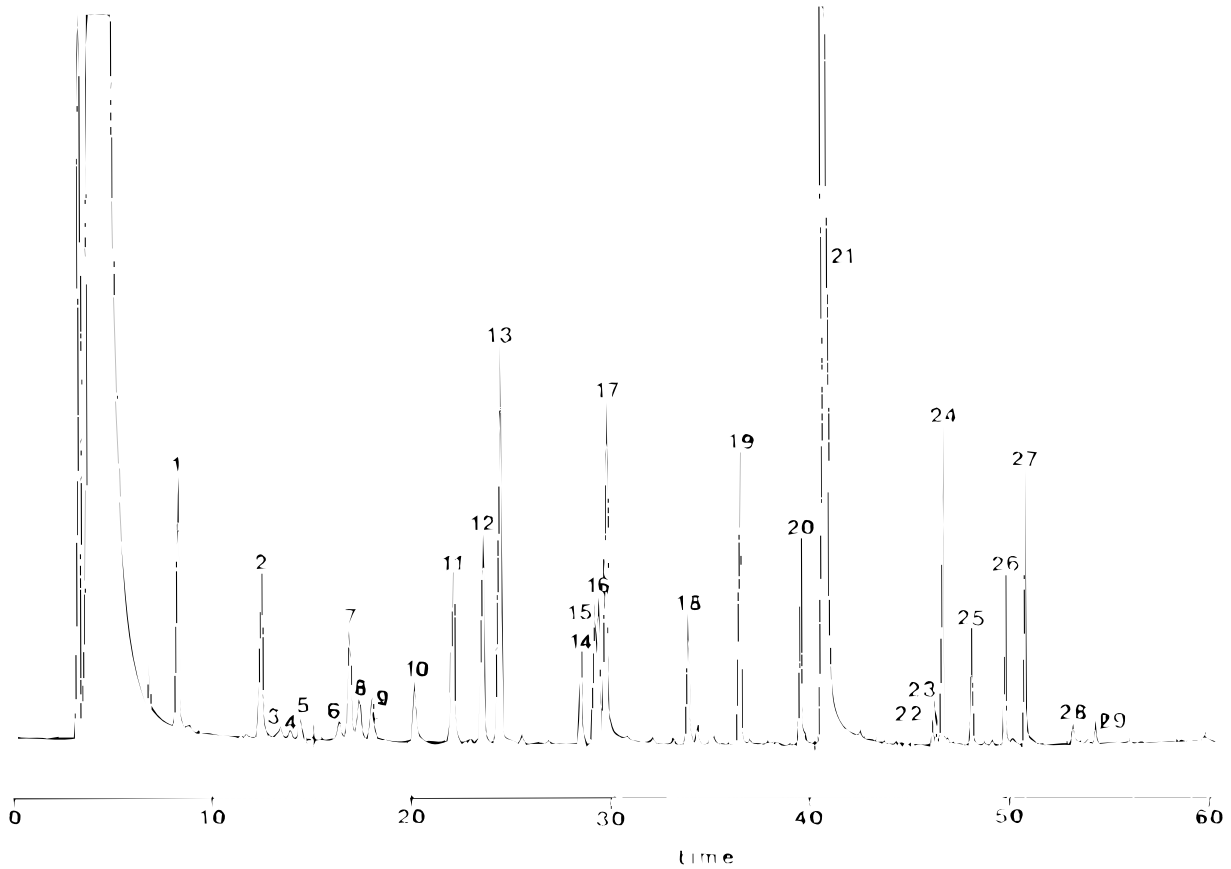


Figure 1. Dynamic headspace gas chromatogram of volatile components of oil extracted from Picual olives. Peaks: 1, *n*-octane; 2, ethyl acetate; 3, methanol; 4, 2-methylbutanal; 5, 3-methylbutanal; 6, unknown; 7, ethanol; 8, pentene dimer; 9, pentene dimer; 10, pentan-3-one; 11, pentene dimer; 12, pentene dimer; 13, 1-penten-3-one; 14, pentene dimer; 15, pentene dimer; 16, pentene dimer; 17, hexanal; 18, 2(*E*)-pentenal; 19, 1-penten-3-ol; 20, 2-methylbutan-1-ol + 3-methylbutan-1-ol; 21, 2(*E*)-hexenal; 22, 2-penten-1-ol; 23, 3(*Z*)-hexenyl acetate; 24, 2-penten-1-ol; 25, hexan-1-ol; 26, 3(*Z*)-hexen-1-ol; 27, 2(*E*)-hexen-1-ol; 28, acetic acid; 29, copaene.

was achieved by peak area integration with a Carlo Erba (Rodano, Italy) Mega series integrator. Suitable separation of enantiomeric forms was carried out with a Perkin-Elmer (Nieuwerkerk a/d Ijssel, The Netherlands) series 8310 fitted with a Mega (Legnano, Italy) silica capillary Megadex 5 column (25 m length; 0.25 mm i.d.; 0.25 μm film thickness), and equipped with a split/splitless injection system and a FID. The oven temperature program was run at 30 °C for 0.5 min, raised at 1.5 °C min^{-1} to 80 °C (no hold), then at 10 °C min^{-1} to 200 °C, and held there for 2 min. The temperature of the detector was held at 250 °C, and He was used as carrier gas at 200 kPa. Injection volume was 1 μL, and the GC injector temperature was 250 °C. The injection was achieved in splitless mode, and the split valve was opened after 0.5 min (split ratio of 1:20). Quantitation was achieved by peak area integration with a Perkin-Elmer LCI-100 laboratory computing integrator.

GC/MS Analysis. GC/MS and chemical ionization GC analyses were performed using a Finnigan Mat 8222 (San Jose, CA) mass spectrometer coupled to a Varian (Walnut Creek, CA) 3400 gas chromatograph. Suitable separation of the analytes was achieved using a DB-Wax capillary column (J&W Scientific, Folsom, CA; 50 m length, 0.32 mm i.d., 1 μm film thickness). Helium was employed as the carrier gas with a linear velocity of 26 cm/s. Sample aliquots of 1 μL were injected. The oven temperature program was as follows: 2 min at 40 °C, from 40 to 100 °C at 5 °C/min, 0 min at 100 °C, from 100 to 230 °C at 8 °C/min, 5 min at 230 °C. The GC injector temperature was 200 °C with a split ratio of 1:50; the transfer line temperature was held at 230 °C. Chemical ionizations were performed with high-purity methane (Matheson, East Rutherford, NJ; res. pur. > 99.9995%). The pressure inside the ionization source, as indicated by a remotely positioned ionization gauge, was ~60 Pa, and the temperatures were 200 °C. Acquisition mass ranges were 27−820 amu for 70 eV EI and 100−820 amu for CH_4 chemical ionization; scan time was 1 s.

Quantitation. A seven-point gas chromatographic calibration plot of β-citronellene was performed by dissolving a known quantity in diethyl ether. The range of concentration covered was 10−100 mg/L. For the evaluation of recoveries from oily matrix, a known quantity of β-citronellene was added to a fresh refined olive oil (~40 ppm). By dilution of this mother oily solution with refined olive oil, seven daughter solutions were derived. The range of concentration covered was 0.2−4 ppm.

Synthesis of Reference Compounds. A modification of a literature method was used for the synthesis of the pentene dimers (Fristad and Klang, 1983). A solution of 3(*E*)-hexenoic acid (294 mg), NaOH (103 mg), and $AgNO_3$ (8 mg) in 30 mL of water was brought to reflux. A solution of 1.394 g of potassium persulfate in 20 mL of water was added after 30 min, and refluxing was pursued for a further 30 min. Extraction with dichloromethane, washing with 5% NaOH, drying, and evaporation of the solvent afforded 150 mg of the hydrocarbon mixture. GC showed the presence of seven pentene dimers. The relative abundance of each isomer matches almost perfectly that found in virgin olive oil samples.

Catalytic Hydrogenation. A purified hydrocarbon fraction deriving from 200 g of virgin olive oil was submitted to a N_2 current until a volume of 0.5 mL was reached. Two milliliters of MeOH and 10 mg of Pd adsorbed on charcoal were added, and the resulting suspension was submitted to an H_2 atmosphere for 1 h. Direct GC/MS analysis of the filtered solution showed the presence of three compounds recognized as the three corresponding decane isomers (MW = 142).

RESULTS AND DISCUSSION

The quality of virgin olive oils strictly depends on the presence of C-6 components. However, other com-

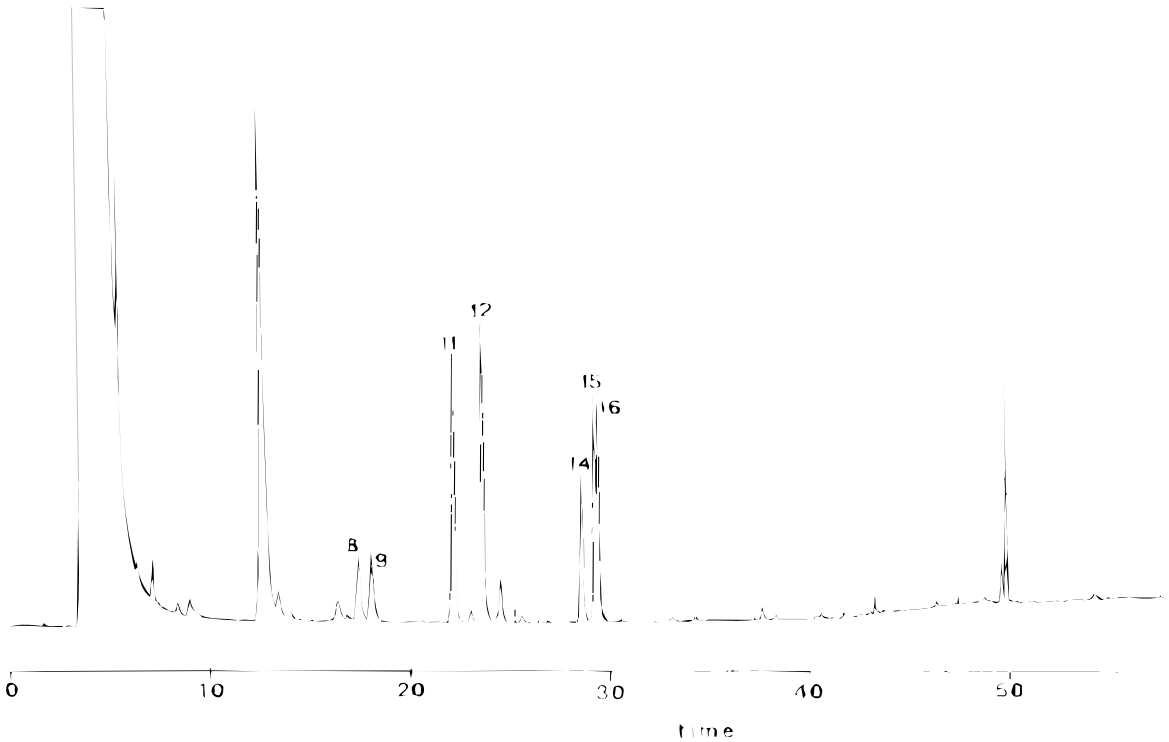


Figure 2. Chromatogram of a silica gel cartridge purified fraction of virgin olive oil aroma extract.

Table 1. CH_4 Chemical Ionization of the Seven Isomeric Hydrocarbons

peak no.	*m/z* 139 [MW + 1]	*m/z* 137 [MW − 1]	*m/z* 125 [MW − 13]	*m/z* 123 [MW − 15]	*m/z* 111 [MW − 27]	*m/z* 109 [MW − 29]
8	62	100	12	17	25	42
9	33	100		21	25	29
11	80	100	7	34	26	66
12	100	96	7	37	27	85
14	24	100	6	18	22	23
15	37	100	5	34	15	31
16	31	100	6	26	24	24

pounds, present in minor amounts, can also represent useful markers. The aroma fractions of the analyzed oils contain C-6 aldehydes, alcohols, and esters in the range of 60−80%. Other products detected are C-5 carbonyl compounds, pentenols, hydrocarbons, and other minor products not deriving from the transformation of the fatty acids (Figure 1).

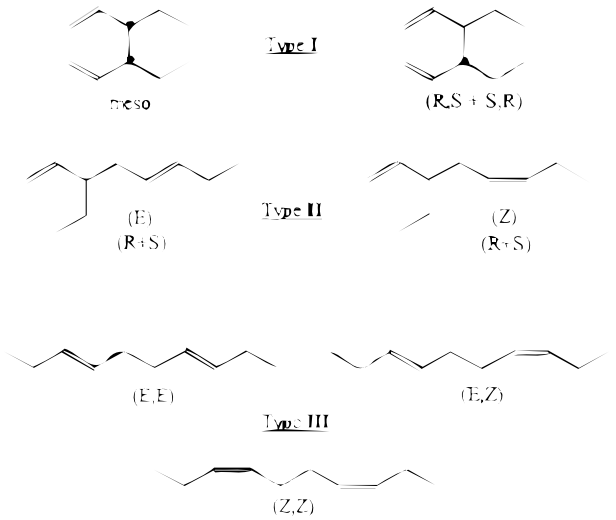
Hydrocarbons are less polar than the others, and for this reason they are easily isolable by means of a chromatography process using silica gel as stationary phase and pentane as eluent solvent (see Volatile Compound Extraction).

In all of the extra virgin olive oils analyzed we found the presence of seven components, with retention times ranging between 15 and 30 min (Figure 2), showing exactly the same mass spectrum (Figure 3, in the Supporting Information). This finding prompted us to solve the problem of the full characterization of cited compounds. The typical fragment ions of EI mass spectra were *m/z* 39 (28−32), *m/z* 41 (100), *m/z* 53 (10−12), *m/z* 67 (26−34), *m/z* 68 (26−28), *m/z* 69 (68−78), *m/z* 95 (10−11), and *m/z* 109 (17−18). Information about molecular ions was not satisfactory. For this reason we adopted a soft ionization method, methane chemical ionization mass spectrometry (CH_4-CI-MS), to obtain further data to assign the correct chemical structure to the compounds. CI showed that (i) the seven compounds had again almost the same fragmentation pathways in the related mass spectra, indicating that they are isomers, and (ii) the molecular weight was, in all cases, *m/z* 138. The molecular formula proposed from these data is $C_{10}H_{18}$, since oxygenated compounds should not be present because of the initial chromatographic fractionation. The spectra recorded in CI mode show always two peaks at *m/z* 137 ($[MW - 1]^+$) and *m/z* 139 ($[MW + 1]^+$) with different ratios between their relative abundances. Table 1 notes that peaks 14−16 showed a ratio between the abundances of the fragments $[MW - 1]^+$ and $[MW + 1]^+$ markedly lower than the other. It is well-known that the abundance of the $[MW - 1]^+$ peak is related to the number of allylic hydrogen atoms in these molecules, since these are relatively easy to remove (Field, 1968).

By comparison of our spectroscopic results with those reported by Salch et al. (1995) on soy seeds, it is possible to propose, as possible structures, the dimeric forms of 1,3-pentene radical (Chart 1). In the soy seed Salch found only five isomers, but all of the other spectroscopic data are similar to our own, as obtained in 70 eV EI mode. Statistic coupling would give seven isomers, as indeed we found.

Further significant data were obtained by use of a chiral capillary column in a GC apparatus (Figure 4 in the Supporting Information). The peaks of compounds **8**, **11**, and **12** were clearly split, while those of compounds **9** and **14**−**16** were not. From this last result it is possible to reach to the following conclusions. Peak 8 is attributable to the pair of enantiomers of type I,

Chart 1. Molecular Structures of the Seven Isomer Hydrocarbons



while peak 9 corresponds to its meso form. Peaks 11 and 12 are attributable to the formula of type II, whereas peaks 14−16 are the linear forms of the decadiene structure. This is in accord with CI-MS data. In fact, in formulas of type III, in which we observed the highest relative abundance of the $[M - 1]^+$ fragment, there are eight allyl hydrogens, whereas formulas of types I and II contain only two and five allyl hydrogens, respectively. On the other hand, in peak 9 we also observed a relative abundance of $[M - 1]^+$ fragment similar to the abundances observed for the straight chain. We cannot rationalize this phenomenon, but Field (1972) reported that for multiple or cyclic alkenes there is no proportionality between the number of allylic hydrogens and the relative abundance of the $[M - 1]^+$ peak. Moreover, Field observed that the latter was also dependent on the conformation of the molecule. In conclusion, the relative abundance of $[M - 1]^+$ peak principally depends on the number of allylic hydrogens when n is very high, but with lower values of n the conformation of the molecules becomes important, and in some case dominant.

To confirm the attribution, we performed two independent experiments:

(i) The 1,3-pentene radical can be generated by oxidative decarboxylation of the 3(*E*)-hexenoic acid [this is an adaptation of the method used by Fristad and Klang, (1983) for phenylacetic acids]. In an inert solvent (water) the preferential termination step of the reaction is radical coupling, leading to the same distribution of products found in the virgin olive oil volatile fraction. Furthermore, the GC analysis by means of chiral capillary column of the synthetic sample (much more abundant than those extracted from the oil) showed again a clear splitting of peaks 8, 11, and 12; the others were single peaks.

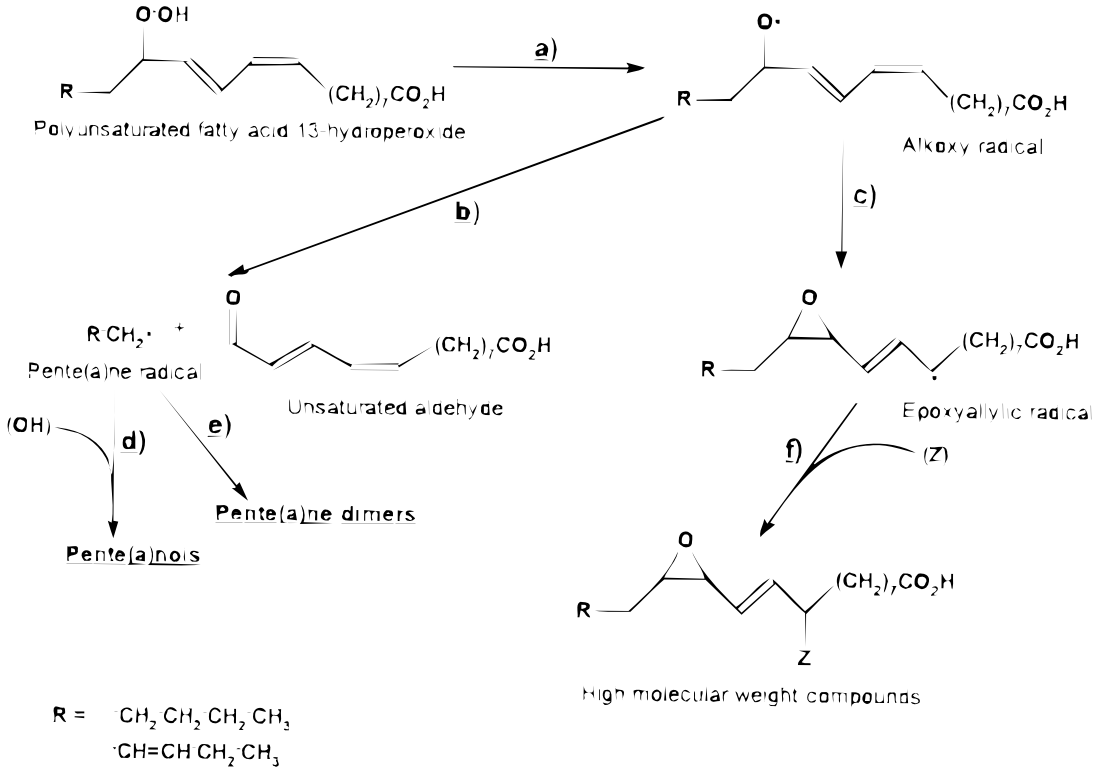
(ii) Catalytic hydrogenation of the natural hydrocarbon fraction gave three compounds recognized by GC/MS as the three corresponding decanes. The reduction was also carried out on the synthetic hydrocarbon sample, giving exactly the three compound previously detected.

Quantitation. Since the recovery of compounds in any oil sample by use of a dynamic headspace apparatus is strictly related to the functional group of each molecule (Angerosa et al., 1997), a recovery factor of a model alkadiene compound was necessary for the quantitation. This was done with a single compound rather than by the synthetic mixture. We performed by GC the calibration curves of 3,7-dimethyl-1,6-octadiene by diluting its known increasing quantity in diethyl ether (Figure 5 in the Supporting Information). To verify the recovery of compound from the oily matrix, this was added to a fresh refined olive oil in increasing quantities. The samples were submitted to the extraction of the volatile fraction and then were analyzed by GC. In the range of considered concentration, the alkadiene showed a linear correlation between its original content in the oil and the corresponding peak area (regression coefficient was 0.999; Figure 6 in the Supporting Information). The recovery can be calculated by the ratio of slopes of the regression straight of the analyte extracted from the oil and its corresponding calibration straight. The value obtained was 0.46. By use of both the recovery factor and the calibration straight it is possible to obtain the total amount of pentene dimers in the analyzed oils. We found 0.9 ppm for the Italian, 0.8 ppm for the Greek, and 1.1 ppm for the Spanish oil.

Biochemical Pathway. A rationalization of the presence of the above hydrocarbons can now be proposed. In Figure 1 is reported a chromatogram of the aroma fraction of virgin olive oil. All three oils analyzed showed the same distribution of products differing only in the relative amounts. These can be collected in four main groups: hydrocarbons, oxygenated C-5, oxygenated C-6, and other minor products not deriving from the transformation of the fatty acids. The first step of the process is the enzyme-catalyzed oxygenation (by LOX) of the 1,4-pentadienic moiety [for a review on the LOX-mediated oxidation of polyunsaturated fatty acid see Hatanaka (1993)]. It is known that olive LOX are not selective versus 9- or 13-hydroperoxide formation and both L and Ln are oxidized, yielding mixtures 65:35 (L) and 57:43 (Ln), respectively (Olías et al., 1993). The finding that C-9 components were not detected in the aromatic extract makes it possible to suppose the exclusion of 9-hydroperoxides in the formation of short-chain products. It was likewise proved that C-6 components were generated by the action of heterolytic hydroperoxide lyases on the 13-hydroperoxide (Olías et al., 1993). A mechanism for the formation of C-5 compounds and C-10 hydrocarbons can now be considered. In a recent paper on soy seeds Salch et al. (1995) reported that LOX catalyze, besides the hydroperoxide formation, also the hydroperoxide cleavage via an alkoxy radical. The final products in the above process are both C-10 hydrocarbons and C-5 alcohols. On the other hand, other researchers reported that a homolytic hydroperoxide lyase activity, catalyzing the specific cleavage of 13-hydroperoxide of linolenic acid to form 2-penten-1-ol and other nonvolatile products, was found in the enzyme extract from soybean cotyledons (Kondo et al., 1995). In this last paper it is shown that other volatile products were not detected.

In the olive oil volatile fraction the detection of a reasonable amount of both pentenols and unsaturated dimers allows us to suggest that the alkoxy radical is an intermediate involved during the aroma biogeneration (pathway a) in Scheme 1). We are not able to distinguish whether the reaction is catalyzed by LOX or by another kind of cleaving enzyme (homolytic hydroperoxide lyase), but, in our rationalization, it is not fundamental since the alkoxy radical intermediate

Scheme 1. Proposed Reaction Mechanism Leading to C-5 Compounds and C-10 Hydrocarbons in Olive Fruit Tissue during the Olive Oil Prodution[a]



[a] (a) Hydroperoxide cleavage; (b) β-scission; (c) cyclization; (d) hydroxylation; (e) radical coupling; (f) radical trapping [an example of this reaction using a phenol as radical trap is reported in Wilcox and Marnett (1993)].

is always involved (Gardner, 1991). Despite the preferential evolution of any 13-alkoxy radicals in the cyclization (pathway c) in Scheme 1) to give the long-lived epoxyallylic radicals (in both cases L and Ln; Wilcox and Marnett; 1993), in the case of the 13-alkoxy radical of Ln, the slow rate of equilibration of the two radicals increases the probability of the alternative pathway, viz. β-scission (pathway b in Scheme 1); this leads to the formation of stabilized 1,3-pentene radical. In fact, alkoxy radical can be generated from both L and Ln 13-hydroperoxides present during the olive oil production but their evolution strictly depends on the presence of a double bond in γ-position to the alkoxy function. This makes it possible that the β-scission mechanism, with a concomitant formation of an allyl radical, occurs. The formation of a stabilized radical is clearly the driving force of the reaction. At this point it is easy to conclude that both dimerization (pathway e in Scheme 1) and coupling between the allyl and an hydroxy radical present in the system (pathway d in Scheme 1) are the preferred termination steps of the overall reaction.

In the case of the 9-hydroperoxides, if the enzyme cleavage occurs, the alkoxy radical will rearrange quantitatively to the epoxyallylic radical which, in the presence of a radical trap such as phenols that are present in great amount in olive pulp, leads to a large distribution of high molecular weight compounds.

In conclusion, seven isomeric hydrocarbons were detected and fully characterized in three representative samples of virgin olive oil. Furthermore, on the basis of our results and available literature, a rationalization of the presence of both the above hydrocarbons and the C-5 alcohols was proposed.

ABBREVIATIONS USED

LOX, lipoxygenase; L, 9,12-(*Z*,*Z*)-octadecadienoic acid (linoleic acid); Ln, 9,12,15-(*Z*,*Z*,*Z*)-octadecatrienoic acid (linolenic acid); C-5, five carbon atom compounds; C-6, six carbon atom compounds; CI, chemical ionization; EI, electronic impact.

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Supporting Information Available: Figures showing the mass spectrum of peak 11, the chiral capillary column chromatogram of the synthetic sample of pentene dimers, the calibration straight of β-citronellene in diethyl ether, and the recovery of β-citronellene from the oily matrix (4 pages). Ordering information is given on any current masthead page.

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