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Six Pentacyclic Triterpenes in Mature Olive Fruits “Picual”

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ABSTRACT

This study developed a procedure for extraction and for isolation through “Partition” of a mixture of six pentacyclic triterpenes from mature green fruit of “Egyptian” olive cultivar “Picual”. The fruit contains valuable amounts of pure triterpenic mixture. The percentage of triterpenes, calculated on the gross dried product, is about 0.5 g in 100 g dry weight. Thin-layer analysis for the acetylated triterpenic mixture, gave two acetate groups. One group, as a precipitate, represents the monoacetates of four triterpenic acids identified as oleanolic, ursolic, betulinic and maslinic. The other acetate group, is soluble in the mother liquor, was identified as the diacetates of two dihydroxy triterpenes, erythrodiol and uvaol. Qualitative and quantitative gas chromatographic analysis, for the two acetylated products, successfully separates the six pentacyclic triterpenes as identified above (TLC).

Key words: Olive fruits, pentacyclic triterpenes, isolation through partition, acetylation, GLC

INTRODUCTION

Since ancient times olive tree (*Olea europaea* L.) found in the Mediterranean shores. Production of olive oil is a growing industry in Egypt. Olive oil has become increasingly popular due to the advantageous nutritional and medicinal properties (Ostlund, 2002). The beneficial effects of olive oil, monounsaturated fatty acid and phenolic compounds are well recognized. However, in the last decade more attention has been paid to research on the minor components, non-steroidal pentacyclic triterpenoids, as plant bioactive metabolites. Those bioactive compounds are the main constituents almost exclusively for olive tree, leaf, fruit and oil of olives minor components (Stiti *et al.*, 2007; Guinda *et al.*, 2010; Lukic *et al.*, 2013). They could be used in new functional foods, drugs or cosmetics (Kuno and Shinohara, 2003). Also, there is evidence of the antiviral (including HIV), antibacterial, anticarcinogenic, antiallergic, antiinflammatory, hepatoprotector, gastroprotector, hypolipidimia, antiatherosclerosis and antidiabetic effects (Sotiroudis and Kyrtopoulos, 2008; Jager *et al.*, 2009; Rodriguez-Rodriguez and Simonsen, 2012).

Kotakis (1965) found that ethyl alcohol is the best solvent for extraction of triterpenes from industrial olive oil cakes which have extracted the lipid. He claimed an average of about 5% of the oil dry cake weight was triterpenes. In his study, Fedeli (1977), collected the reviewed data of the previous literature and came to the conclusion that: “There are six pentacyclic triterpenes, both, in mature fruits and olive oil”, without referring to their quantities. The six nonsteroidal pentacyclic triterpenes (Fig. 1) belong mainly to: (1) Oleanane type (from β -amyrin pool), they are; erythrodiol, oleanolic and maslinic acids, (2) Ursane type (from α -amyrin pool), they are; uvaol and ursolic acid and (3) Lupane type (from lupeol pool), it is betulinic acid (Fedeli, 1977). According to Kiritsakis (1991), the calculated dry weight residue obtained is 8.9% of fresh mature olive fruit.

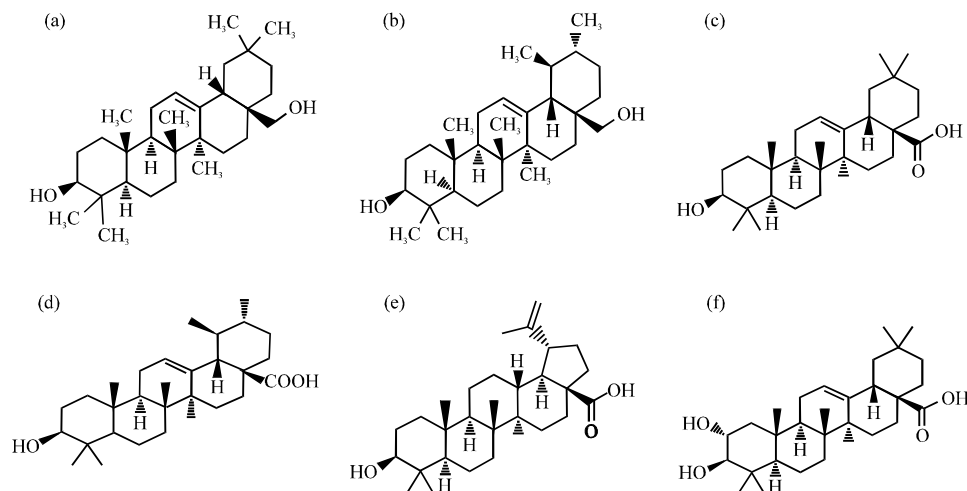


Fig. 1(a-f): Chemical structures of pentacyclic triterpenes found in mature olive fruit, (a) Erythrodiol, (b) Uvaol, (c) Oleanolic acid, (d) Ursolic acid, (e) Betulinic acid and (f) Maslinic acid (Fedeli, 1977)

Other authors analyzed pentacyclic triterpenes either through their concentration in the unsaponifiable matter or directly in the extracted oils. The first group got from the oil unsaponifiable matter two dihydroxy triterpenes: erythrodiol (about 83%) and uvaol (Itoh *et al.*, 1981). GLC analysis of the relative content of triterpene dialcohols in the total sterolic constituents was trace-1% for virgin oils, 2% for refined oils and 8-10% for β -residue oils (Itoh *et al.*, 1981). Amelio *et al.* (1992), tried to activate separations of the nonsaponifiable matter: Sterols, erythrodiol, uvaol and alkanols, by means of solid phase extraction prior to HPLC analysis. Also he measured the recovery of erythrodiol + uvaol at different saponification times. The total of the two dialcohols represents 3% of the nonsaponifiable matter (Amelio *et al.*, 1992). The second group of researchers determined the pentacyclic triterpenes from olive by direct analysis of the extracted oil without the need for prior saponification. Bianchi *et al.* (1994) studied the pentacyclic triterpenes present in the skin of olive fruit. The chloroform extract (mainly surface waxes) was in the range of 0.08-0.16% of total olive weight. Further extraction with methanol gives large quantities of pure pentacyclic triterpene acids (0.08-0.13%). Those components were mainly oleanolic acid (31-44%) and maslinic acid (55-68%) (Bianchi *et al.*, 1994). On the other hand Gil *et al.* (1997), macerated solid wastes resulting from olive oil production in ethanol and extracted the oil with hexane. Fractionation of oil on silica gel gave three fractions: Fraction 1 (major) is mainly triglycerides. Spectral analysis of the minor identified fractions 2 and 3, showed two oxy-oleanane derivatives and together represents 250 mg from 5 kg solid waste (Gil *et al.*, 1997). Blanch *et al.* (1998), developed a method for rapid direct analysis of erythrodiol and uvaol in olive oils, without the need for prior enrichment of the dialcohols via saponification. The results ensured what has been established already that absolute concentrations of erythrodiol and uvaol in pressed oils are clearly lower than those corresponding to the raw solvent extracted oils (Itoh *et al.*, 1981). For this reason, analysis of dialcohols is used for distinguishing among olive oils of different quantities such as cold pressed (extra virgin) and either solvent extracted or pressed residues oils (Blanch *et al.*, 1998).

Recently, in his study, Stiti *et al.* (2007), stated that triterpene content is closely dependent on state of developing and that a regulation process takes place through the cyclization of the common precursor oxidosqualene that constitute a branch point between primary sterol pathway and secondary triterpenoid pathway. Direct analysis of olive oil has been achieved first by fractionation on TLC. The different triterpenes separated was subjected to acetylation before GLC analysis. Results showed that the green mature fruit has 0.276 g in 100 g dry weight. The main triterpenoids were as follows: erythrodiol 401, maslinic 830, oleanolic 1257 and uvaol 245 $\mu\text{g g}^{-1}$, ursolic and betulinic acids were 6 and 21 $\mu\text{g g}^{-1}$ dry weight, respectively (Stiti *et al.*, 2007). On the other hand, Guinda *et al.* (2010) in their work on pentacyclic triterpenes from Picual olive fruit, first dried the fruit and after maceration subjected to a flash extraction in hexane. The hexane extracted olives were twice extracted in absolute ethanol. The collected ethanol was evaporated and the obtained residue was subjected for silylation and the derivative was analyzed by GLC. Analysis of the triterpenes gave 0.2% of dry weight and the green mature Picual gave only 0.06 $\mu\text{g g}^{-1}$ oleanolic and 0.15 $\mu\text{g g}^{-1}$ maslinic acids (Guinda *et al.*, 2010).

The aim of this study is to develop a physical method for the extraction and isolation of a maximum quantity of the pentacyclic triterpene mixture in green mature fresh olive fruit. This triterpene mixture is almost free from any other steroidal components. Traditionally, pentacyclic triterpene mixture is obtained via alkaline hydrolysis of olive oil, "i.e., via saponification" and then concentration of the triterpenes as a part of the nonsaponifiable matter (EEC., 1991; IOC., 2011). Probably, saponification leads to hydrolysis of the triterpene acids to the alcohol form. The present simple method for the isolation of pentacyclic triterpene mixture is reliable because it is completely dependent on physical separations, with no need for any chemical process. Two steps were involved: (1) The choice of 80% ethyl alcohol-water as an extractive solvent for the pentacyclic triterpenes. (2) Using the phenomenon of "Partition" for isolation of the triterpenes. The analysis of this mixture is usually carried out by derivatization of the triterpene mixture prior to their analysis by TLC, GLC or HPLC etc. Acetylation of the triterpene mixture in this work, leads to another physical separation (TLC) of two acetate groups: monoacetate and diacetate-triterpenes. This last achievement helps much in their fractionation on TLC and also in their GLC analysis.

MATERIALS AND METHODS

Materials: Fresh mature fruits of "Picual" variety with an intense green color epidermis were purchased from local market. Fruits were washed with distilled water. All solvents used for triterpene extraction and analysis were of analytical grade. Authentics: oleanolic acid (>97%) and ursolic acid (>90%) were purchased from Sigma (Sigma Chemical Company, St. Louis, MO, USA).

Extraction of triterpenes: One kilogram of olive flesh (mesocarp) including skin was twice macerated with 1.5 L of ethyl alcohol-water (80:20, v/v) for 3 h with occasional shaking. The combined extracts were filtered and evaporated in vacuo to dryness. A dark brownish heavy resinous residue was obtained (Harborne, 1984).

Pentacyclic triterpenes

Isolation of pentacyclic triterpenes partition: Part of the above residue (40 g) was redissolved in about 100 mL warm 80% ethyl alcohol-water. The homogeneous room temperature solution was extracted with n-hexane three times. The first addition of hexane precipitates immediately white matter at the interface. However, continuous extraction with hexane gave two turbid hexane layers

and the third layer was clear. The pentacyclic terpenes may be obtained either by: (1) Direct cooling of the large given volume of the combined hexane extracts for e.g., an overnight in refrigerator, where the white triterpene residue precipitated, (2) Extraction of the total given volume of the combined hexane with 80% ethanol, then distillation under vacuo for the combined alcohol extract with frequent addition of dry alcohol to get the triterpene residue and (3) Simply by concentration the large volume of the combined hexane extract, where it turns turbid, followed by addition of anhydrous sodium sulfate, as if for drying, will cause immediately the precipitation of the triterpene mixture over the sulfate crystals. Filtration of hexane gave the triterpene precipitate on filter paper together with the anhydrous sodium sulfate. Washing with hexane first, then with water will give the pure pentacyclic triterpene on the filter paper. For us this last method was the most convenient. The experiment was repeated three times to ensure the reproducibility of the method (Galanos and Kapoulas, 1962; Christie, 1982).

Derivatization of pentacyclic triterpenes: Because of low volatility and high molecular weight of pentacyclic triterpenes, derivatization prior to gas chromatography and even for fractionation on thin-layer, is required. The acetylating reagent is prepared by mixing 2 mL of anhydrous pyridine and 2 mL of acetic anhydride. To 100 mg of each of triterpenoid mixture, oleanolic and ursolic acids the acetylating reagent was added and left overnight at room temperature. Then, 30 mL of methanol-water (1:1, v/v) was added slowly till complete precipitation. Filtration on a Buchner funnel gave white matter. The later residue represents four acetate derivatives of pentacyclic triterpenic acids. The mother liquor was evaporated to dryness in vacuo, with occasional addition of small portion of absolute ethyl alcohol. The residue obtained represents the diacetates of two dihydroxy triterpenes (Christie, 1982; Harborne, 1984). If the acetylation reagents are excess or room temperature is high, precipitation of the monoacetate may not be achieved. In such case, extraction of the whole reaction mixture with diethyl ether and the residue obtained from diethyl ether layer will represent the four acetate derivatives. The diacetates residue is obtained by the distillation of the alcohol-water layer with frequent addition of dry ethanol.

Thin-layer chromatography (TLC)

Lipid classes: Samples of: (1) Crude olive oil, (2, 3) Oil extracted with hexane before and after partition, (4) Reference mixture of oleanolic and ursolic acids, (5) Precipitate obtained by partition and (6) β -sitosterol as reference; all were subjected to chromatography on silica gel plates. The developing system used: n-hexane-diethyl ether-acetic acid (80:20:1, v/v/v). The spray reagent used to visualize the separated spots, specific for lipid classes is 0.5% α -naphthol in methanol-water (1:1, v/v). The air dried plate is sprayed till wet, air dried again and sprayed lightly with sulfuric acid-ethanol (1:1, v/v). The plate is heated at 120°C. Lipid components appear as different shades of brown spots, while triterpenes appear as purple spots. Identification and R_f values for all the separated lipid classes were determined (Stahl, 1969; Perkins, 1975; Christie, 1982; Harborne, 1984).

Acetate products: Samples of each of the pentacyclic triterpenic mixture as well as their two types of acetates alongside with authentic samples, oleanolic and ursolic acids and also their acetates, all were subjected to chromatography on silica gel layers. The developing system used was chloroform-diethyl ether (9:1, v/v). The spray reagent, to visualize the separated fractions, was 5% solution of concentrated sulfuric acid in ethyl alcohol specific for triterpenes. Identification and R_f values were determined for all separated spots (Stahl, 1969).

Gas Liquid Chromatography (GLC): Identification and quantification of acetates of the different pentacyclic triterpenes were carried out by gas liquid chromatographic analysis. One microliter of sample dissolved in chloroform was injected in a Hewlett Packard HP 6890 Series GC, equipped with HP-INNOWAX Polyethylene Glycol (30 m×530 μ m) 1 μ m (Film thickness). Carrier gas: N₂: 30 mL min⁻¹, H₂: 30 mL min⁻¹, Air: 300 mL min⁻¹. The temperature program included a fast rise from 60-230°C (30°C min⁻¹), a slow rise from 230-280°C (2°C min⁻¹) and a plateau at 280°C for 10 min for monoacetates. For triterpene diacetates, the final temperature at 280°C was held for 25 min instead of 10 min (plateau) (Stiti *et al.*, 2007). Back inlet: (Splitless), injection temperature 275°C, flow 16.1 mL min⁻¹ and pressure 12.28 psi. Flame Ionization Detector (FID), temperature 300°C. The samples were analyzed in triplicate.

Statistical analysis: Experimental process was performed in triplicates. Means of the three replicates were calculated. All the data are presented as the Means±SD (n = 3) of the weight % for the six pentacyclic triterpenes analyzed in triplicate by GLC in the tested mature olive fruit. ANOVA two-way analysis (ANOVA SPSS-Version 13) was used to reach the conclusion with respect to the variability of the different component percentages and their stability through replication of the chemical analysis on more than one single sample, at a 5% significance level.

RESULTS AND DISCUSSION

Isolation of pentacyclic triterpenes: Results showed that the alcohol-water extract of the olive fruit mesocarp (1 kg) gave a dark brownish heavy resinous residue, 80 g (8%). Part of the resinous residue (40 g) dissolved in alcohol-water (80%) and extracted with hexane. The reduced hexane extracts (turbid), was dried over anhydrous sodium sulfate, where precipitation of the pentacyclic triterpene mixture occurs over the sodium sulfate. Filtration retained the triterpene mixture with the sulfate. Hexane washes, followed by water washes left the clean terpene only on the filter paper. The triplicate average of the dried terpene isolated was about 0.5 g of 100 g dry weight. The dry weight of olive mesocarp was calculated according to Kiritsakis (1991) (Table 1). The pentacyclic triterpene mixture obtained was pure with no steroidal contaminants. However, almost all previous work dealt only with the estimation of pentacyclic triterpenes and not with its isolation.

Study of the literature review emphasizes that it would be preferable if lipids could be separated into individual classes without being chemically modified. The method we adopted for obtaining the relatively polar pentacyclic triterpene is their physical isolation through "Partition" i.e., the distribution of substances between two immiscible liquids. The method involved the presence of water that together with alcohol (80% alcohol-water), increase the polarity of the media and also both helps rupturing the tissues of olive fruit; hence increase the availability of the triterpenes. The choice of n-hexane as a solvent-in the presence of water-constitutes with alcohol two phases, an

Table 1: Components of green mature olive fruit (Kiritsakis, 1991)

Components	Percentage
Water	50.0
Oil	22.0
Sugar	19.1
Cellulose*	5.8
Protein*	1.6
Ash*	1.5

*DW: Dry weight = 8.9%

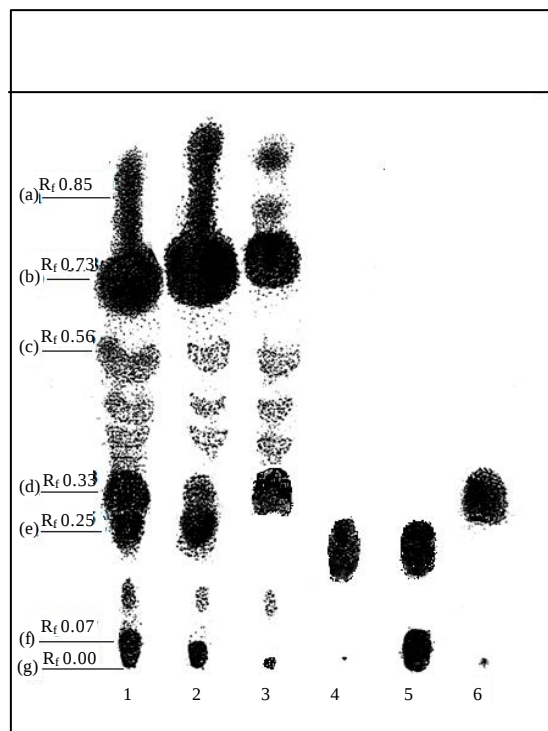


Fig. 2: TLC of lipid classes in olive oil. Solvent system: n-hexane-diethyl ether-acetic acid (80:20:1, v/v/v). Spray reagent: α -naphthol-sulfuric acid, 1: Crude olive oil, 2: Oil from hexane before partition, 3: Oil from hexane after partition, 4: Reference mixture of oleanolic and ursolic; 5- precipitate separated by partition; 6: β -sitosterol reference. Lipid classes, a: Hydrocarbons and steryl acetates, b: Triacylglycerols, c: Free fatty acids, d: β -sitosterol, e: Pentacyclic triterpenes, f: Polar pentacyclic triterpenes and g: Complex polar lipids

upper hexane, getting more polar, where it is saturated with alcohol-water and a lower alcohol-water phase i.e., two immiscible liquids. Those two phases helps much in increasing the affinity of the relatively polar pentacyclic triterpenes to be incorporated with the lipophilic matter (oil) and thus extracted in the becoming relatively polar hexane layer. Complex polar lipids are retained in the lower polar alcohol-water phase (Galanos and Kapoulas, 1962; Christie, 1982).

Identification of pentacyclic triterpenes by TLC: Thin-Layer Chromatography (TLC) has proven to be an extremely useful tool in the analysis of lipid classes (Perkins, 1975; Christie, 1982). Through the literature (Fedeli, 1977; Itoh *et al.*, 1981; Stiti *et al.*, 2007), identification and purification of the pentacyclic terpene in the olive fruit mesocarp were done mainly via TLC. Most frequently mixture of n-hexane, diethyl ether and acetic acid are used in different proportions e.g., 80:20:1 (v/v/v), separates almost all lipid classes in vegetable oils (Perkins, 1975; Christie, 1982). Accordingly, TLC results (Fig. 2), showed that the isolated precipitate (sample 5), obtained by "Partition" (above) gave two spots (both are purple with α -naphthol, or red with sulfuric acid-acetic acid) with R_f values of 0.25 and 0.07. Both spots were also traced, as the same R_f values and the same color testes, in the lipid classes of crude olive oil (sample 1) and that of hexane oil before

Table 2: Pentacyclic triterpene acetates (TLC and GLC) in mature olive fruit

Pentacyclic triterpenes	R _f (TLC)	GLC		
		R _T (GLC)	Weight % (average)	Mean±SD
Oleanolic acid	0.43	21.43	8.94	8.94±0.23
Erythrodiol	0.38	23.70	8.29	8.29±0.28
Ursolic acid	0.28	25.87	8.57	8.57±0.25
Uvaol	0.21	29.80	9.27	9.27±0.06
Betulinic acid	0.14	34.90	19.86	19.86±0.47
Maslinic acid	0.03	40.74	45.05	45.05±0.23

R_f: Retardation factor, R_T: Retention time, SD: Standard deviation, Data is expressed as Means±SD of the three replicated experimental measurements, p<0.05

“Partition” (sample 2), while both spots almost disappeared in hexane oil sample after “Partition” (sample 3). However, the authentic reference of pentacyclic triterpene mixture of oleanolic and ursolic acids (sample 4) gave one spot with R_f and color tests similar to that of the first (less polar) spot isolated from the precipitate at R_f 0.25 (sample 5). It is worth mentioning that the results i.e., R_f values and the color tests prove the preliminary “Identity” of the precipitate (sample 5) as pentacyclic triterpene(s) mixture. Also the resolution of this precipitate as two spots on TLC, without any contamination so ever from above i.e., with β -sitosterol (R_f 0.33) or from below i.e. with complex polar lipids on the start line (phospholipids or glycolipids)-proves the preliminary “Purity” of the isolated precipitate (sample 5) as pentacyclic triterpene mixture(s).

Identification of acetylated pentacyclic triterpenes by TLC: On acetylation of the triterpene mixture, two products were obtained. One as a precipitate of a mixture of four acetates of triterpenic acids. The second product as two diacetates for the two dihydroxy triterpene assessed in the mother liquor. Thin-layer chromatography was a convenient method not only as a preliminary tool for triterpene fractionation but also as a reliable method for their identification especially after their derivatization and, if needed, for their quantification. A sample of each of the following: the six triterpene mixture, the first acetate group of four triterpene acids, the second diacetates of the two dihydroxy triterpenes, alongside with oleanolic and ursolic acids as references before and after acetylation, were chromatographed on silica gel layers. The developing system was chloroform-diethyl ether (9:1, v/v). The spots were observed as red spots with sulfuric acid-ethyl alcohol reagent. The triterpene mixture gave two spots, one heavy dark spot near the start line and the other spot is less dense and less polar at R_f 0.13. The display of four spots from the first acetate group were identified-against references of acetates of oleanolic and ursolic acids according to increase polarity-as acetates of oleanolic, ursolic, betulinic and maslinic acids. The other less polar group gave two acetates, identified as erythrodiol and uvaol (Table 2, Fig. 3). Literature data and different polarities of the different triterpene acetates helped much in their identification. The results and the R_f values are well represented in Fig. 3 and Table 2. Interpretation of the results showed that the choice of acetylation as a derivatization method was suitable because it separates the mixture of the six pentacyclic triterpenes into two acetate groups. One group as a precipitate represents four triterpenic acids identified as oleanolic, ursolic, betulinic and maslinic. The other acetate group is soluble in the mother liquor, was identified as the diacetates of two dihydroxy triterpenes, erythrodiol and uvaol. This separation helped much in acquiring six different R_f values and hence facilitates identification. Also one can recover pure samples for the six triterpenes, if

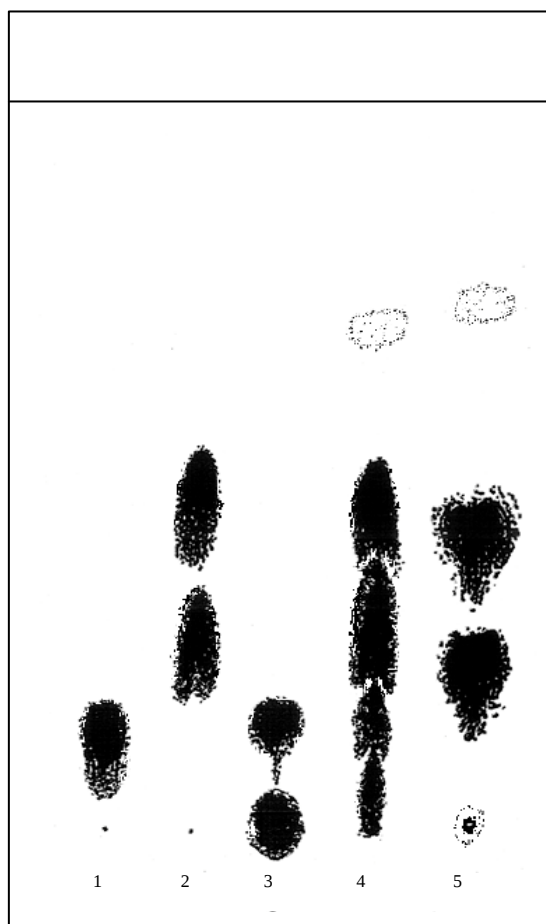


Fig. 3: TLC of pentacyclic triterpenic compounds in mature olive fruit. Solvent system: Chloroform-diethyl ether (9:1, v/v). Spray reagent: Sulfuric acid-ethyl alcohol, 1: Reference mixture of oleanolic and ursolic, 2: Acetylated mixture of oleanolic and ursolic, 3: Pentacyclic triterpene mixture of olive fruit, 4: Monoacetates of oleanolic acid, ursolic acid, betulinic acid and maslinic acid and 5: Diacetates of erythrodiol and uvaol

needed, by using preparative TLC (PTLC), for their acetates, then with back hydrolysis of each acetate to the alcohol form. In this aspect, the acetylation method is superior to silylation (Guinda *et al.*, 2010) where the acetate derivative we get (PTLC) is stable and thus recoverable through back hydrolysis of acetate layer. While the silylated derivative is unstable and have to be measured by GLC within 30 min. However, the silylation method could be helpful in fast estimation of compounds.

Identification of acetylated products by GLC: Identification and quantification of the components of the two acetylated products of the pentacyclic triterpenes were carried out by GLC (Fig. 4, 5) through traditional conditions of sterol acetates (in the experimental section) (Stiti *et al.*, 2007; Guinda *et al.*, 2010). For triterpene diacetates, the final temperature at 280°C was held for

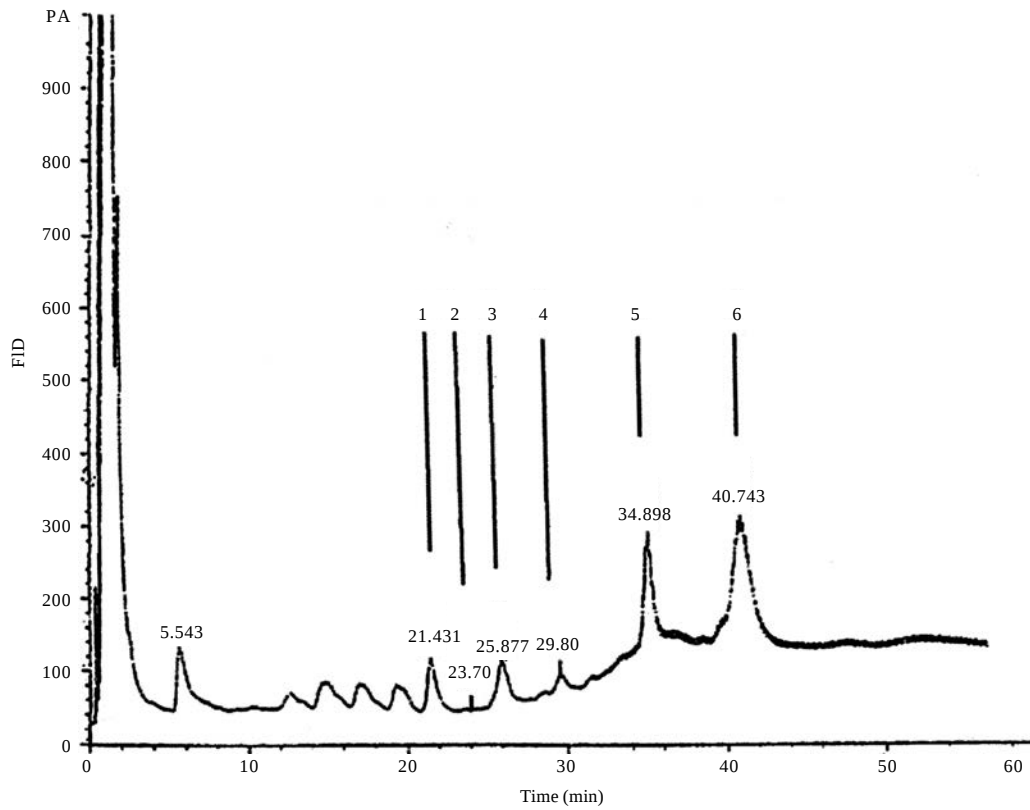


Fig. 4: GLC of monoacetates of pentacyclic triterpenic compounds in mature olive fruit, (1) Oleanolic acid, (2) The expected R_T for erythrodiol, (3) Ursolic acid, (4) Expected R_T for uvaol, (5) Betulinic acid and (6) Maslinic acid

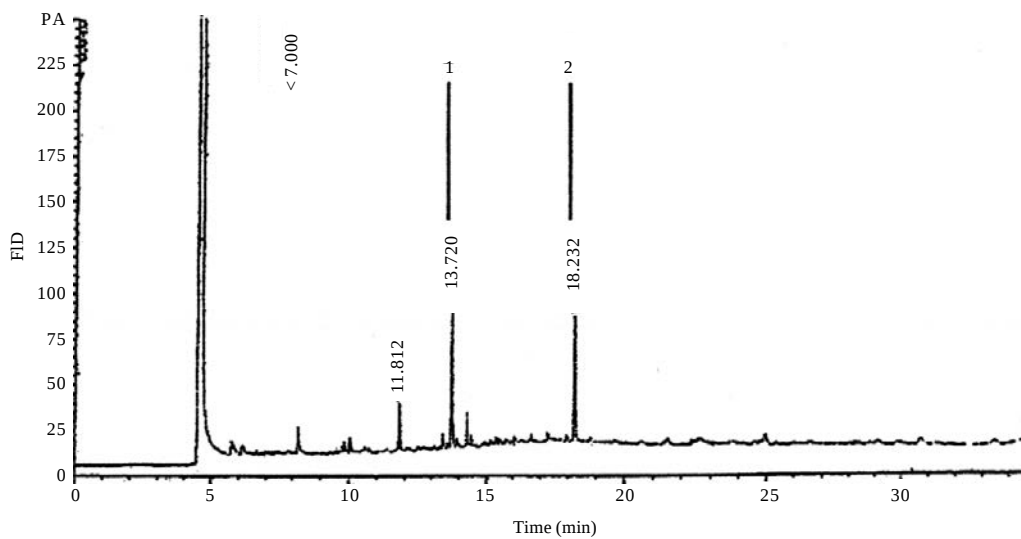


Fig. 5: GLC of the diacetates of the two dihydroxy pentacyclic triterpenes in mature olive fruit, (1) Erythrodiol and (2) Uvaol

25 min (Fig. 5) instead of 10 min for the monoacetates (Fig. 4) (Stiti *et al.*, 2007). No comparable study in the literature was available on GLC of pentacyclic triterpenes as acetates.

Comparison with the literature: Back to the literature, most authors (Fedeli, 1977; Itoh *et al.*, 1981; Amelio *et al.*, 1992) have analyzed pentacyclic triterpenes through alkali hydrolysis of olive oil usually extracted from the substrate by the weak polar solvent hexane. The isolated triterpenes as a part of the nonsaponifiable matter were, in our opinion, liable to carboxylic hydrolysis. The acidic triterpenes (-OOCH) are hydrolyzed to terpene alcohols (-OH). The results explain the increase of the dialcohols erythrodiol and uvaol on the expense of oleanolic and ursolic acids, to the extent that the last two were mostly not traced (Itoh *et al.*, 1981; Amelio *et al.*, 1992). However, up till now Casas *et al.* (2009) and Lukic *et al.* (2013) on studying the sterols, triterpene acids and diols content in olive oil of different varieties, locations and the determination of its degree of ripening, still made use of the recommended legislation regulated by the European Union (EEC., 1991) and the trade standard applying to olive oils and olive-pomace oils set by the International Olive Council (IOC., 2011). Both legislations are depending on alkali hydrolysis that almost degraded the terpene acids to the diols: erythrodiol and uvaol. Here, one has to mention that estimation of each of erythrodiol and uvaol could be intended through direct analysis (Blanch *et al.*, 1998) or as usual through nonsaponifiable matter (EEC., 1991; IOC., 2011). This is used for distinguishing whether olive oil is cold pressed (virgin) where the values of the two diols are lower than in either solvent extracted or pressed residues oils (Blanch *et al.*, 1998).

Some researchers moved to direct analysis of the oil extracted by different polar solvent(s) (Bianchi *et al.*, 1994; Blanch *et al.*, 1998). Stiti *et al.* (2007) analyzed the fractionated (TLC) acetylated pentacyclic triterpenes from olive oil of mature green fruit. The result showed that the terpenes were mainly acids of oleanolic, 1257; followed by maslinic 830 and erythrodiol 401 and uvaol 245 $\mu\text{g g}^{-1}$ dry weight. Ursolic and betulinic acids were in trace (6 and 21 $\mu\text{g g}^{-1}$ dry weight). Thus the total triterpenes estimated (as we calculated from the manuscript) was 0.27 g/100 g dry weight (Stiti *et al.*, 2007). On the other hand, Guinda *et al.* (2010) analyzed the silylated alcohol extract of the mature green olive fruit by GLC. The results showed only acids of oleanolic, 0.06 and maslinic 0.15 $\mu\text{g g}^{-1}$ DW, together give weight of total triterpenes as 0.21% DW. On the other hand, our results showed that the total pentacyclic triterpene mixture was 0.5 g in 100 g dry weight. The component terpenes were: erythrodiol, 8.29; uvaol, 9.27; oleanolic acid, 8.94; ursolic acid, 8.57; betulinic acid, 19.86 and maslinic acid, 45.05 weight percent. Differences in total pentacyclic triterpenes as well as percentages of different component terpenes depends mainly on the stage of olive fruit development (Stiti *et al.*, 2007).

Fedeli (1977) reviewed data from previous literature and came to the important conclusion that: "There are six pentacyclic triterpenes, both in mature fruits and olive oil". Those triterpenes are, according to increase polarity: two dihydroxy triterpenes, erythrodiol and uvaol and four carboxylic acids, oleanolic, ursolic, betulinic and maslinic (Fig. 1). The present results are comparable with what Fedeli (1977) collected. The novelty is that the six pentacyclic triterpenes were isolated altogether from olive fruit in one process (Fig. 2, 3; Table 2), without any steroidal contaminants or changing their physical nature.

One last important observation from the literature is that fatty acids and triterpenes were the main energy compounds present in the hexane fraction of olive oil pressed wastes. Their isolated triterpenes were very small quantities of two degraded oleanane derivative as acetoxylean and methyl-diacetoxylean. The degradation is due to wide range of bacteria present in the oil meal

Table 3: Statistical analysis of the three replicated experiments for the pentacyclic triterpene acetates (GLC) in mature olive fruit

Pentacyclic triterpenes	(GLC) weight %			Mean	SD	SE	Max. limit	Min. limit
	1st	2nd	3rd					
Oleanolic acid	8.82	9.21	8.79	8.94	0.23	0.13	9.53	8.34
Erythrodiol	8.36	8.54	7.99	8.29	0.28	0.16	9.00	7.58
Ursolic acid	8.45	8.86	8.39	8.57	0.25	0.14	9.02	7.75
Uvaol	9.28	9.21	9.34	9.27	0.06	0.03	9.42	9.11
Betulinic acid	19.87	19.39	20.33	19.86	0.47	0.27	21.04	18.67
Maslinic acid	45.20	44.79	45.16	45.05	0.23	0.13	45.63	44.46

Table 4: ANOVA two-way analysis between the six pentacyclic triterpene acetates in columns and the three replicated experiments in rows in mature olive fruit

Source of variation	SS	DF	MS	p-value	F	F critical	Significance
Rows	3197.43	5	639.48	2.72E-17	6728.458	3.325	Significant*
Columns	4.44E-05	2	2.22E-05	0.999	0.00023	4.102	Non significant
Error	0.95	10	0.095				
Total	3198.38	17					

*Significant at 0.05 level of significance

process (Gil *et al.*, 1997), especially if the pressed wastes were stored. This deterioration criteria is also attached to our olive laboratory experimental work. However, it could be avoided even through weeks with good refrigeration. The only stable phase in this work is our product: the purified dried pentacyclic triterpene mixture.

Statistical analysis results: It is worthwhile to note that: the data of the pentacyclic triterpene acetates (GLC) in mature olive fruit in Table 2 as Means $\pm$ SD for the three replicated experiments. Observation on Table 3 showed that: it is expected at a confidence level of 95% that the average ratio of erythrodiol would fall between 9.00 (max. limit) and 7.58 (min. limit), while for maslinic acid the average ratio fall between 45.63 (max. limit) and 44.46 (min. limit). Generally, the adopted method is acceptable in stability since the consistency of estimated ratios in the samples adopted was of high consistency, as indicated by the calculated interval limit and the relatively low standard errors. Table 4 ANOVA two-way analysis showed that the rows are statistically significant at 0.05 significance level (5%) but the columns not significant, where the F calculated in the rows was 6728.458 compared to the F critical 3.32 and these leads to be significant, $p < 0.05$. As conclusion, logically percentages of the considered components vary significantly; while for each component percentages are almost constant for repeated experiments. Accordingly, the used method is reliable in providing stable conclusions with respect to component composition and their percentages.

CONCLUSION

It would be preferable if lipid could be separated into individual classes, without being chemically modified. Pentacyclic triterpenes are usually obtained from olive oil as part of its nonsaponifiable matter, i.e. after alkali hydrolysis of the oil. This hydrolysis, probably attacks the natural carboxylic group (-COOH) of the terpene acids to the alcohol form (-OH) and give the two diols: Erythrodiol and uvaol. Accordingly, we developed a method for a complete physical separation of the relatively polar pentacyclic terpenes through "Partition", i.e., the distribution of substances between two immiscible liquids. The method is well explained in the experimental and discussion

sections. The pure mixture of six pentacyclic triterpenes have been isolated for the first time without any steroidal contaminants. Also, two observations have been added to the subject. These are the easy precipitation of the triterpene mixture over the drying agent (anhydrous sodium sulfate). Also, derivatization of the triterpene mixture via acetylation gave two products in the reaction mixture. One product as a precipitate, represents the monoacetates of four triterpene acids: oleanolic, ursolic, betulinic and maslinic. The other product gave two diacetates of the diols, erythrodiol and uvaol, soluble in the mother liquor. The separation of the two acetate groups facilitates TLC and GLC analyses.

Finally, five points have to be mentioned:

- “The Addition” in the present study is evident in regard to the following sequence of unprecedented steps (to the best of our knowledge): The choice of the solvent mixture for extraction of olive fruit mesocarp; then getting the pentacyclic triterpenes from that extract through “Partition”; and the separation of a considerable amount of pure triterpene mixture through “Precipitation on anhydrous sodium sulfate”
- “The Purity” of the separated pentacyclic triterpene mixture and its components were proven through physical and chemical work. The physical behavior of the separated triterpene mixture on TLC-with the usually recommended solvent system for separation of lipid classes- i.e., its R_f values and also its response to specific color tests, were like that of the estimated authentic triterpene(s). The chemical behavior of the triterpene mixture through derivatization (acetylation) and the analysis of the acetylated products by both, TLC and GLC, have proven the expected chemical formulae of the six pentacyclic triterpenes when compared to the estimated authentic triterpene acetate(s) and also to the literature data
- The bioactive natural pure pentacyclic triterpene mixture obtained, as is, or fractionated could help much in proper drug and food industries
- “Partition” as a physical trend could be extended in the area of separations of minor natural products
- The method that was adopted could help much in developing other methods in the area of olive fruit, oil, or meal pressed cakes, such as the methods given in the two international references European and Spanish

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