

Identification of tetrahydrogeranylgeraniol and dihydrogeranylgeraniol in extra virgin olive oil

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SUMMARY: Olive oil contains many different compounds which are responsible for its nutritional and sensorial value. However, some compounds present in olive oil at very low amounts have not yet been identified. Here, the detection of tetrahydrogeranylgeraniol and dihydrogeranylgeraniol, in both the total aliphatic alcohol and waxy fractions of extra virgin olive oil, is reported for the first time using GC and GC-MS methodologies. It was suggested that tetrahydrogeranylgeraniol and dihydrogeranylgeraniol do not originate from the hydrolysis of the chlorophyll but are present as diterpenic esters.

KEYWORDS: *Dihydrogeranylgeraniol; Diterpenic esters; GC; GC-MS; Olive oils; Tetrahydrogeranylgeraniol*

RESUMEN: *Identificación de tetrahidrogeranilgeraniol y dihidrogeranilgeraniol en aceites de oliva virgen extra.* Los aceites de oliva contienen muchos compuestos diferentes responsables de su valor nutricional y sensorial. Sin embargo, algunos compuestos presentes en los aceites de oliva en cantidades muy bajas aún no se han identificado. En este trabajo la detección de tetrahidrogeranilgeraniol y dihidrogeranilgeraniol, en las fracciones de alcoholes alifáticos totales y en la de ceras de los aceites de oliva virgen extra, es reportado por primera vez utilizando metodologías de GC y GC-MS. Se sugiere que el tetrahidrogeranilgeraniol y el dihidrogeranilgeraniol no se originan por hidrólisis de la clorofila, sino que están presentes como ésteres diterpénicos.

PALABRAS CLAVE: *Aceites de oliva; Dihidrogeranilgeraniol; Ésteres diterpénicos; GC; GC-MS; Tetrahidrogeranilgeraniol*

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1. INTRODUCTION

Olive oil is a food matrix which is widely investigated due to its peculiar nutritional and sensorial properties. However, some minor components and/or compounds related to the oil, such as esters and glucosides have not yet been fully identified. Such is the case of sterols and tococromanols, which are still of great interest, although their study began about 60 years ago (Mariani and Bellan, 1996; Butinar *et al.*, 2011; Gasparoli and Mariani, 2016; Mariani, submitted, Nestola and Schmidtn, 2016). Tococromanols are present in olive oil not only as tocopherols and tocotrienols, but also in monounsaturated and diunsaturated forms (Mariani and Bellan, 1997; Biedermann, Haase-Aschoff and Grob, 2008; Mariani, 2017a). In some seed oils, such as Pumpkin oil, the content of γ -tocomonoenol can reach 150 mg/kg, whereas in olive, sunflower, and palm oils, the monounsaturated form of γ -tocopherol is tococromanol and it is the most abundant after α -tocopherol (Biedermann, Haase-Aschoff and Grob, 2008; Mariani and Bellan, 1996).

Olive, palm and soybean oils (Mariani and Bellan, 1996) show the presence of tocopherol esters, which do not show antioxidant or vitamin properties, but probably recover such nutritional properties after returning to their free form. The esters of tocopherols can be useful to detect fraudulent mixtures since they are not removed by refining processes due to their high molecular weight.

In the nonpolar fraction of olive oil, phytol and geranylgeraniol, and two diterpene alcohols esterified with fatty acids are present in relevant amounts. In olive oil from the Arbequina variety, these compounds are the main constituents of the nonpolar fraction (Mariani, 2017b). Note that in olive oil, the phytol derivatives are the most abundant diterpenic esters, whereas in seed oils, such as sunflower oils, the geranylgeraniol derivatives are predominant. Geranylgeraniol esters are particularly useful to reveal the presence of sunflower oil in the highly valuable cartamo oil. These two oils have an almost identical chemical composition except for being profoundly different in the composition of geranylgeraniol diterpenic esters (Biedermann, Haase-Aschoff and Grob, 2008; Mariani, 2017a).

However, despite the numerous papers on diterpenic esters (Mariani *et al.*, 1992; Grob *et al.*, 1993; Grob and Mariani, 1994; Marini and Venturini 2002; Reiter and Lorbeer, 2001; Mariani and Venturini, 2003), the fraction of the diterpenic esters is not yet completely understood. In different works (Soulier and Farines, 1992; Bonaga and Cappella, 1997; Boskou, Blekas and Tsimidou, 2006; León-Camacho *et al.*, 2013), phytols and geranylgeraniol detected in the unsaponifiable fraction of olive oil are reported as products deriving from

the hydrolysis of chlorophyll. Chlorophyll, which is present at levels of about 10 mg/kg in extra virgin olive oil, can provide a maximum of 3–5 mg/kg of phytols. Therefore, it is possible to suggest that, when the amount of phytols reaches a high level in the olive oil's unsaponifiable fraction, such as in the case of olive oil from the Arbequina variety (250 mg/kg), phytols must be derived from another source or from the esterified terpene fraction (Artho, Grob and Mariani, 1993). Moreover, it is well known that all refined oils from olives and seeds contain the esters of phytols and geranylgeraniol although they do not contain chlorophyll, which is eliminated by bleaching earth.

It is also important to consider some aspects of the biosynthesis of tocopherol esters and diterpenic esters present in the nonpolar fraction of olive oil. With regards to the composition of tocopherol esters in olive oil, the main component is α -tocopherol esterified with palmitic acid, although palmitic acid represents on average only 10–15% of the triglyceride acids. The same trend is observed in palm oils, where the main tocopherol ester is α -tocopherol esterified with myristic acid present in a low amount (Mariani and Bellan, 1996; Mariani and Bellan, 1997; Biedermann, Haase-Aschoff and Grob, 2008; Mariani and Venturini, 2003). Diterpenic esters follow the same trend. In extra virgin oil the most abundant ester of phytols esterified with arachic acid is present at about 0.5% (Biedermann, Haase-Aschoff and Grob, 2008). It is also important to note that sterols are often present as esters, such as in sunflower oils, where the 7Δ stigmastanol, typical of this oil, is mainly present in the ester fraction (Johansson, 1979; Mariani, Fedeli and Grob, 1991). These observations suggest that the esterification of the minor components is not a simple esterification but follows a specific biosynthetic path.

In this paper, two compounds present as unknown peaks in the chromatograms of both the total aliphatic alcohol and the waxy fractions were identified by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS).

2. MATERIALS AND METHODS

2.1. Materials

Sodium dodecyl sulfate and heptadecanoyl stearate were purchased from Nu Check (Elysian Mn USA). Hexane and isooctane for pesticide residue analysis were purchased from Fluka (Milano I) and Merck (Darmstadt Germany), respectively. Diethyl ether P.A. and ethanol were purchased from Carlo Erba (Rodano I). Potassium hydroxyde and silica gel were purchased from Merck (Darmstadt Germany) and Sylon BFT from Supelco (Bellefonte Ca. USA).

2.2. Waxes and aliphatic alcohol analyses

Minor components, both free and esterified, were analyzed according to the method reported by Mariani, Fedeli and Grob 1991. Waxes were obtained according to the method reported in COI/T20/Doc.N°18 Rev.2-2003. The nonpolar fraction, obtained in accordance with COI/T20/Doc.N°28 February-2004, was saponified and treated according to the COI/T20/N°26-2003 aliphatic alcohol procedure, as shown in Figure 1.

GC analyses were carried out using a Carlo Erba Mega 8560 gas chromatograph (Rodano-Milan,

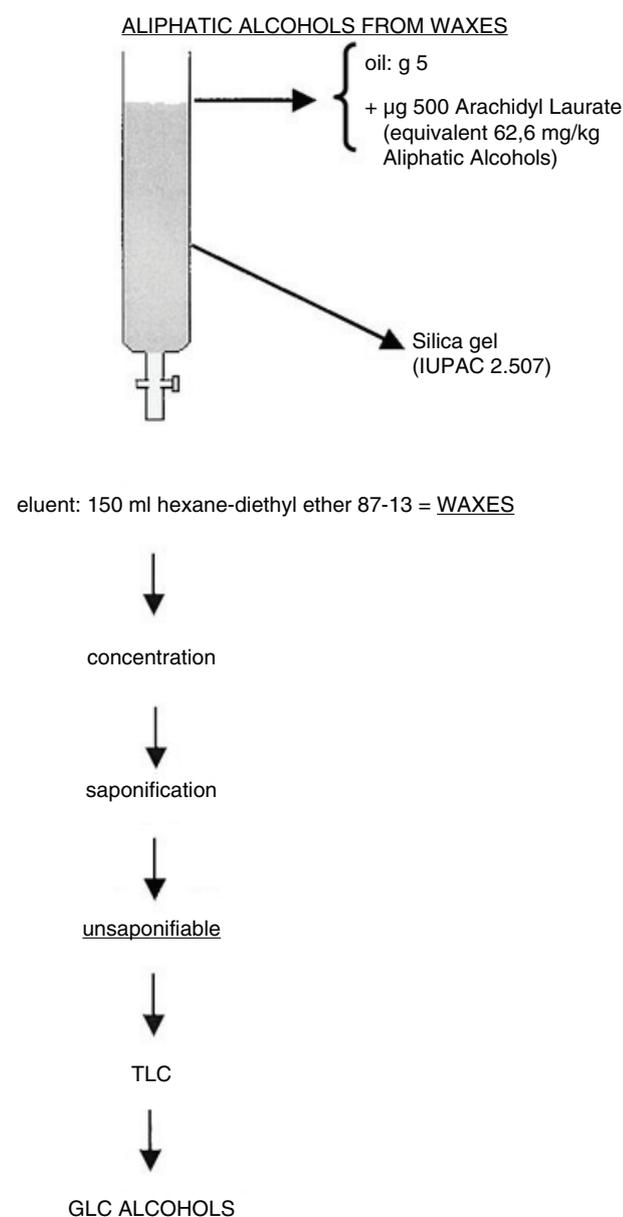


FIGURE 1. Method of aliphatic alcohol preparation from the nonpolar fraction.

Italy) equipped with a cold on-column injector and a flame ionization detector (FID) and a PS 255 fused silica capillary column (10 m × 0.25 mm id, 0.1 mm film thickness). The FID gases (air and hydrogen) and the make-up gas (nitrogen) flow rates were 330, 35, and 30 mL/min, respectively. Hydrogen was used as the carrier gas (2.0 mL/min). After the injection of samples, the oven temperature was programmed as follows: from 80 to 200 °C with a ramp rate of 20 °C/min followed by a 5-min hold at 330 °C for wax analyses and from 80 to 160 °C with a ramp rate of 20 °C/min followed by 5-min hold at 250 °C for alcohol analyses. The detector temperature was 350 °C and the injector temperature was 80 °C.

GC-MS analyses were performed on a Thermo Finnigan Voyager Rodano – Mi (I) gas chromatograph in the same experimental condition as the wax analyses. Helium was the carrier gas at a flow rate of 1.8 mL/min. The temperature of the ion source and the transfer line were 220 and 320 °C, respectively. Electron impact mass spectra were recorded at an ionization energy of 70 eV.

3. RESULTS AND DISCUSSION

As widely reported in the literature (International Olive Oil council, 2015), the chromatogram of the olive oil aliphatic alcohol fraction shows several identified peaks but also unknown peaks such as two peaks that elute between the phytols and the geranylgeraniol. These two unknown peaks are generally present in higher amounts in pressure olive oil than in extraction oil (Biedermann, Haase-Aschoff and Grob, 2008; Aparicio and Aparicio-Ruiz, 2000) and are particularly concentrated in the waxy fraction rather than in the total alcohol fraction (Mariani *et al.*, 1991).

As an example, the GC profiles of both the total aliphatic alcohols and the aliphatic alcohols of the waxy fraction of extra virgin olive oil from the Arbequina cultivar are reported in Figure 2 and Figure 3A, respectively. The two unknown peaks (peaks 2 and 3) between the phytols (peak 1) and geranylgeraniol (peak 4) were detected in both chromatograms together with peaks belonging to the classic aliphatic alcohols from 22 (peak 6) to 28 carbon atoms (peak 9), and a peak due to C20 alcohol (peak 5).

In order to identify the two unknown peaks, the olive oil waxy fraction was investigated by GC-MS, (see Figure 3B). The mass spectra of phytols, geranylgeraniol and the two unknown compounds are depicted in Figure 3B. All MS spectra showed a peak at 143 *m/z* due to the presence of silylate phytols. The MS spectrum of phytols, (Figure 3B1), showed a molecular ion peak at 368 *m/z* and a fragment at 278 *m/z*, whereas the MS spectra of the two unknown compounds, (see Figures 3B2 and 3B3), showed molecular ion peaks at 366 *m/z* and 364 *m/z* and the fragments at 276 and 274 *m/z*, respectively.

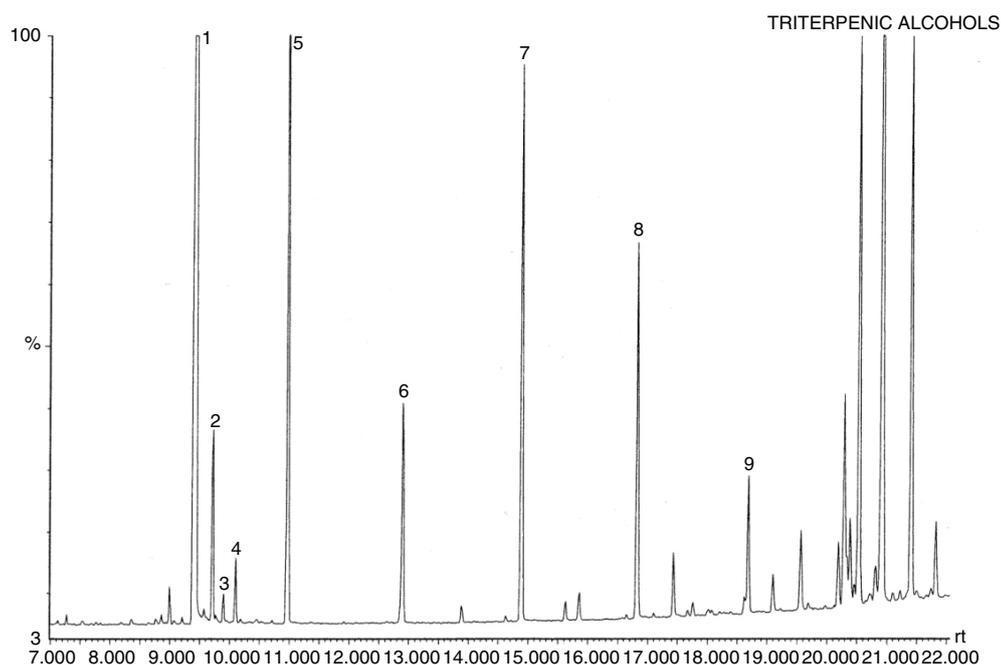


FIGURE 2. GC trace of the total aliphatic alcohol fraction of an Extra Virgin Olive Oil (Arbequina variety): 1: Phytols, 2: unknown, 3: unknown, 4: Geranylgeraniol, 5: Alcohol C20 I.S., 6: Alcohol C22, 7: Alcohol C24, 8: Alcohol C26, 9: Alcohol C28.

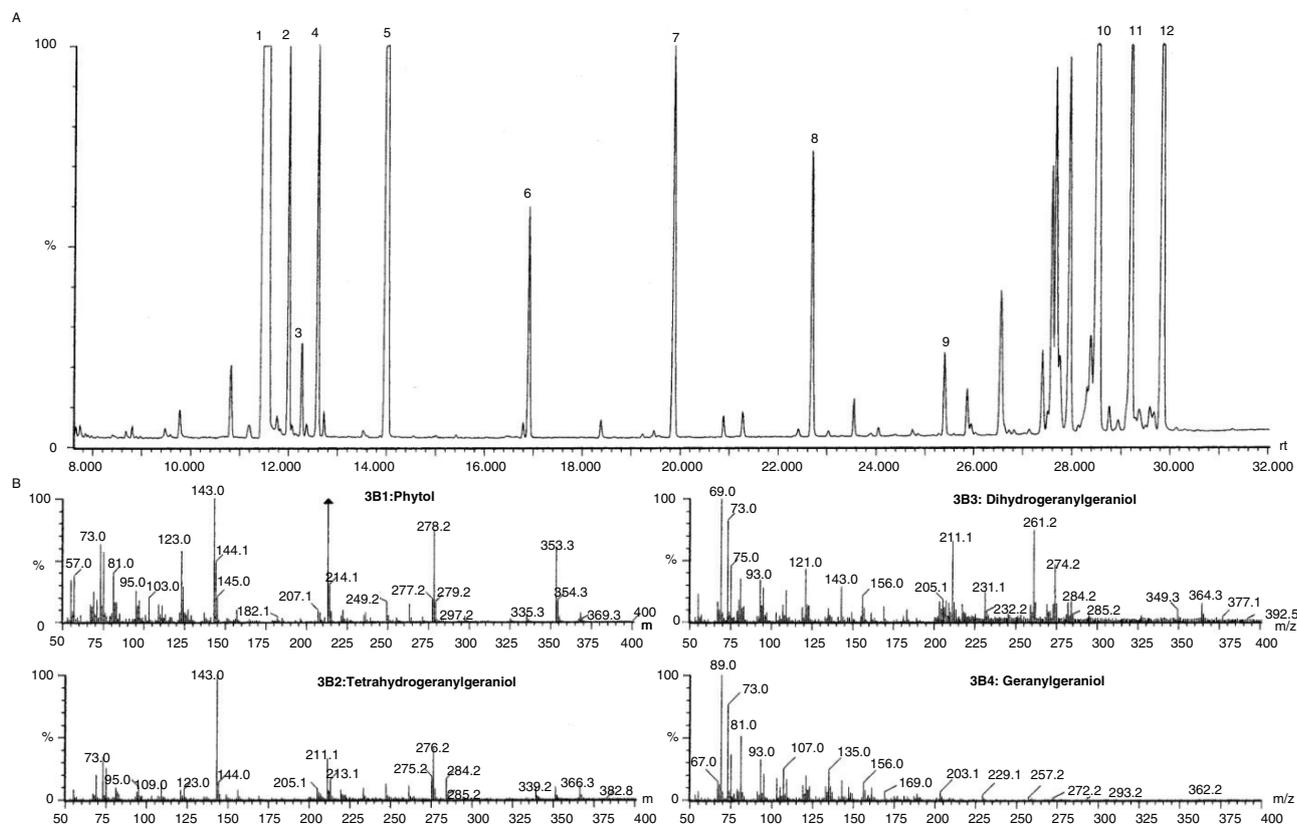


FIGURE 3. (A) GC-MS trace of the aliphatic alcohols obtained by saponification of the nonpolar fraction of an extra virgin olive oil from the Arbequina variety produced in Argentina. 1: Phytols, 2: Tetrahydrogeranylgeraniol, 3: Dihydrogeranylgeraniol, 4: Geranylgeraniol, 5: Alcohol C20 I.S., 6: Alcohol C22, 7: Alcohol C24, 8: Alcohol C26, 9: Alcohol C28, 10: Cycloartenol, 11: 24Methylcycloartenol, 12: Citrostadienol. (B) MS spectra expansions (between 50 m/z and 400 m/z) of peaks 1, 2, 3, and 4.


 FIGURE 4. Molecular structure of the 69 *m/z* fragment.

The fragments at 278, 276 and 274 *m/z* were generated from the loss of 3-trimethylsilyl-2-oxazolidinone (TMSO) group. It is noteworthy that all MS spectra showed the 69 *m/z* fragment, characteristic of a double bond on the last carbon atom in the alkyl side chain, (see the molecular structure depicted in Figure 4). These results suggest that peak 2 and peak 3 could be attributed to phytol derivatives, namely, tetrahydrogeranylgeraniol (THGG) with two double bonds and dihydrogeranylgeraniol (DHGG) with three double bonds, respectively. The GC-MS trace also showed the presence of minor peaks near peaks 2 and 3, possibly due to a DHGG isomer characterized by double bonds in different positions. DHGGs with double bonds in Δ -2-10-14 have been previously identified during a study regarding the biosynthesis of chlorophyll (Schoch and

Schafer, 1978). Whereas DHGG with a double bond in Δ -2-6-14 has been reported to be an intermediate of the bacteriochlorophyll (BChl)-B biosynthesis (Mizouguchi *et al.*, 2009).

The generation of possible isomers of DHGG has been explained by the biosynthetic pathways proposed by Schoch and Schafer (1978) and Mizouguchi *et al.*, (2009), (see Schemes depicted in Figures 5 and 6). Figure 6 suggests the presence of Δ -2-6-14 Dihydrogeranylgeraniol.

It can be of interest to investigate the origin of THGG and DHGG, which have never been identified in olive oil to best of our knowledge. Contrary to the hypothesis that THGG and DHGG in the olive oil derive from chlorophyll (Schoch and Schafer, 1978; Mizouguchi *et al.*, 2009), it can be considered that the pigments present in the waxy fraction were removed through saponification and purification on Silica gel. Therefore, it can be hypothesized that THGG and DHGG are present in the nonpolar fraction as diterpene esters, normally present in all olive oil categories especially in extra virgin olive oil. This evidence has also been reported in the case of phytols present in olive oil, for the most part as fatty

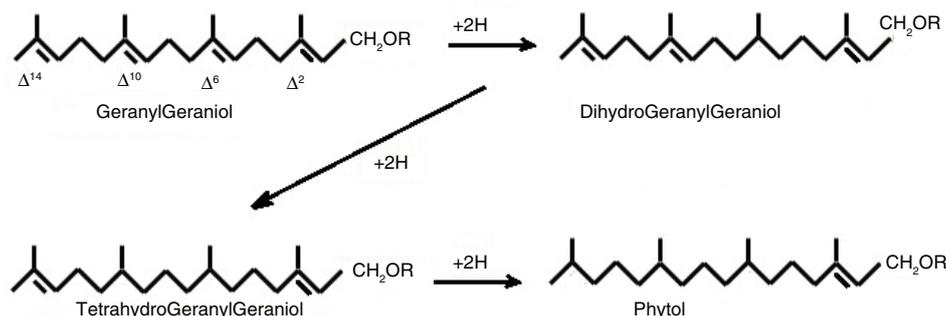
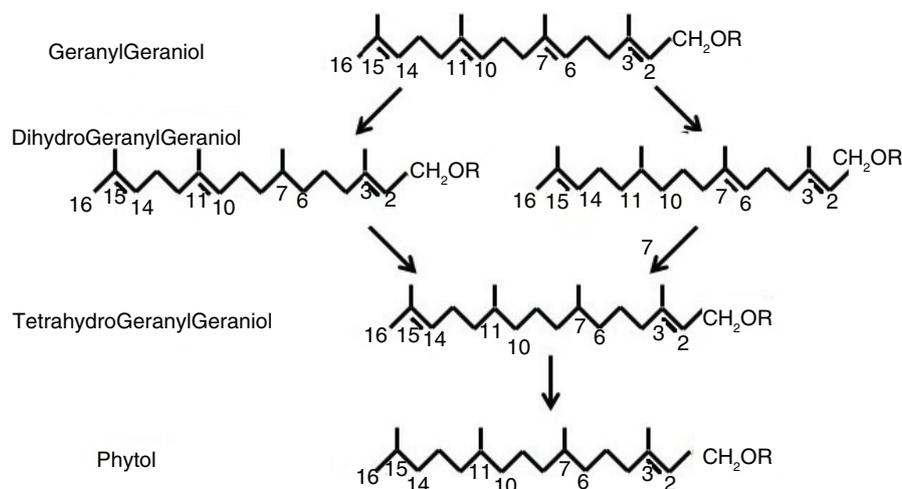


FIGURE 5. Biosynthesis of phytols according to Schoch and Schafer (1978).


 FIGURE 6. Biosynthesis of phytols according to Mizouguchi *et al.*, (2009).

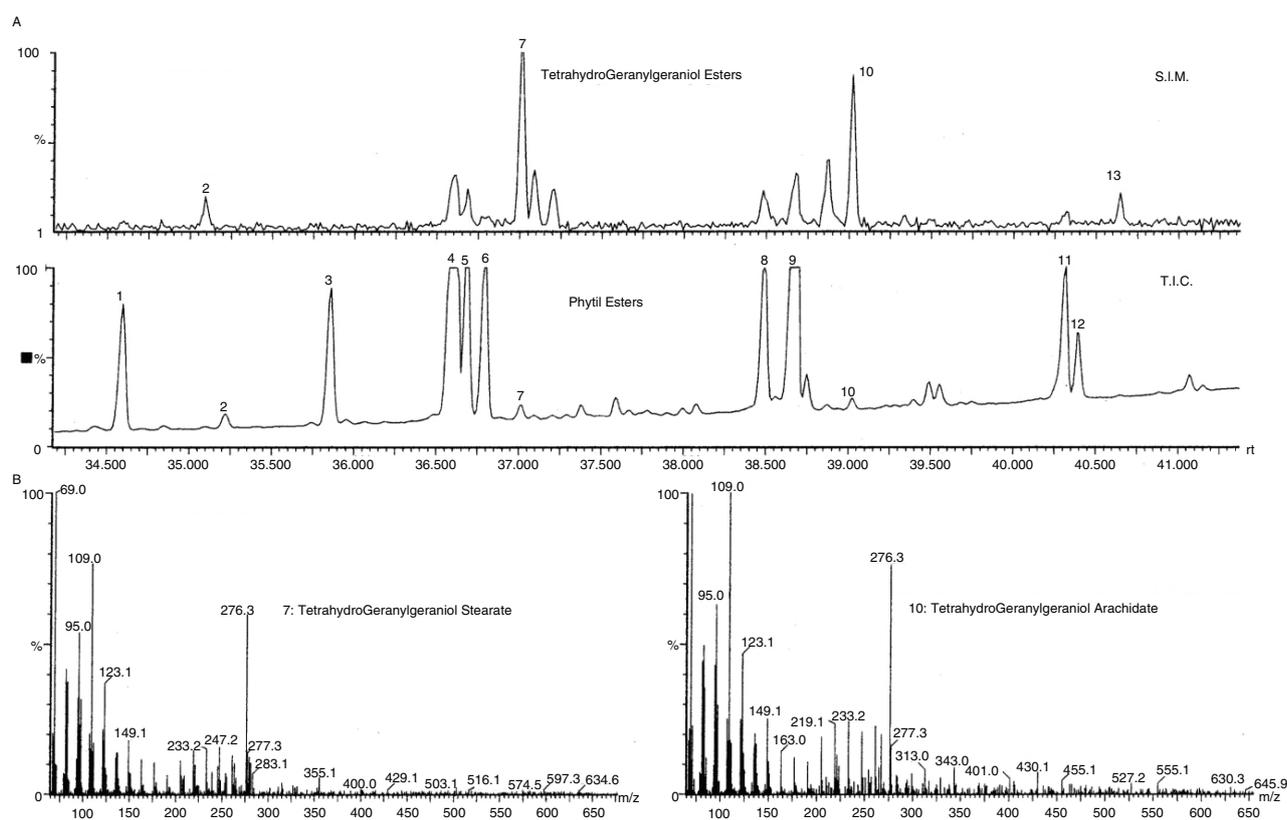


FIGURE 7. Part GC-MS trace on the zone of the diterpene esters of an extra virgin olive oil of the Arbequina variety (7A) mass spectra of compounds 7 and 10 (7B). 1: Phytol Palmitate, 2: Tetrahydrogeranylgeraniol Palmitate, 3: Heptadecanyl Stearate I.S., 4: Phytol Linoleate + Oleate, 5: Phytol Vaccenate, 6: Phytol Stearate, 7: Tetrahydrogeranylgeraniol Stearate, 8: Phytol Eicosenoate, 9: Phytol Arachidate, 10: Tetrahydrogeranylgeraniol Arachidate, 11: Phytol Behenate, 12: Lignoceryl Palmitate, 13: Tetrahydrogeranylgeraniol Behenate.

acid esters and not as chlorophyll esters (Capella *et al.*, 1997).

In order to verify this hypothesis, the fractions of both free and esterified minor components were isolated following the procedure previously described (Mariani, Fedeli and Grob, 1991) and then analyzed through GC-MS. The chromatogram in figure 7A shows a single ion recording (SIM) at m/z , typical of THGG; whereas figure 7B shows the total ion chromatogram (TIC) of the diterpene esters of an extra virgin olive oil. The mass spectra of THGG stearate (peak 7) and THGG arachidate (peak 10) are depicted in figures 7C and 7D.

A peak at 123 m/z due to phytols esterified with fatty acids was observed together with a peak at 276 m/z due to the loss of the fragment $[RCO_2H]$; whereas the molecular ion was not detected. In the spectrum, THGG stearate (peak 7) and arachidate (peak 10) were well observable; whereas the THGG palmitate (peak 2) and behenate (peak 13) as well as DHGG derivatives were not easily identified. Furthermore, unsaturated oleic acid derivatives were not detected probably due to their elution together with phytol oleate and phytol vaccenate.

4. CONCLUSIONS

Esterified compounds, such as diterpen esters, present in minor concentrations in olive oil samples, have not yet been completely identified, although they have important properties and are responsible of fraudulent adulterations in extra virgin olive oil with minor quality oils or seed oils (Olofsson, Hultqvist and Holmdahl, 2011).

In this work, two new compounds, DHGG and THGG, intermediates of the biosynthetic pathway of phytols, were identified in both the total aliphatic alcohol and the waxy fractions of extra virgin olive oil. Furthermore, it has been demonstrated that, as in the case of phytols, the presence of these compounds in olive oils is mainly due to diterpene esters of the nonpolar fraction of extra virgin olive oil rather than to the hydrolysis of chlorophyll. Moreover, since extra virgin olive oil can have a phytol content of several hundred mg/Kg, it is clear that the presence of phytols in olive oil is not totally due to chlorophyll. In fact, the amount coming from chlorophyll is only a small part of the total.

Finally, it can be highlighted that the similarity between these classes of compounds and tocopherols and tocotrienols depends solely on the side chains of phytol and Isoprenyl (geranylgeranyl), respectively, for tocopherols and tocotrienols.

The THGG and DHGG identified in this work show a remarkable similarity with the mono and diunsaturated derivatives of tocopherols, recently identified as biosynthetic intermediates in the synthesis of tocopherols (Gasparoli and Mariani, 2016).

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