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Analytical Methods

Gas chromatographic–mass spectrometric characterisation of triterpene alcohols and monomethylsterols in developing *Olea europaea L.* fruits

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ABSTRACT

Five triterpene alcohols and four 4-monomethylsterols were identified by GC–MS during the ripening of *Picholine* olive. The quantitative characterisation of these compounds was performed using GC–FID. The results showed that the maximum level of total triterpene alcohols (263.68 mg/100 g oil) was reached at 26th week after the flowering date (WAF) of olive; whilst the highest level of total 4-monomethylsterols (234 mg/100 g oil) was attained at 24th WAF of fruit. The percentage of these two classes represented 20–33% of total phytosterols during olive maturity. 24-Methylene cycloartenol (12–207 mg/100 g oil) and cycloartenol (27–198 mg/100 g oil) were the predominant triterpene alcohols during the ripening of *Picholine* olive; whereas citrostadienol (30–161 mg/100 g oil) and cycloeucalenol (11–74 mg/100 g oil) were the main 4-monomethylsterol compounds followed by obtusifoliol and gramisterol. β-Amyrin, δ-amyrin and traroxerol were less present in *Picholine* olive and they accounted for 14% of total triterpene alcohols at complete maturity of fruit. The level of these methylsterols was overwhelmed by the amount of 4-desmethylsterols at each stage of *Picholine* olive maturity.

1. Introduction

Triterpene alcohols and 4-monomethylsterols are a group of natural products present in vegetable oils and are the important constituents of phytosterols. These compounds have been widely studied for their health properties. The average dietary consumption of phytosterols is approximately 250 mg/day (Nair, Kanfer, & Hoogramartens, 2006). Clinical research has shown that phytosterols reduce biliary cholesterol absorption in the intestine (Conchillo et al., 2005), thereby increasing faecal excretion of cholesterol (Hicks & Moreau, 2001). Decreasing plasma cholesterol levels is important for the prevention of cardiovascular disease, which is the main cause of death in Europe (Santos et al., 2007). Additionally, phytosterols have anti-inflammatory (Safayhi & Saler, 1997; Ovesna, Vachalkova, & Horvathova, 2004) and anti-carcinogenic (Awad, Downie, Fink, & Kim, 2000) effects. In vegetable oils, phytosterols represent the major fraction of unsaponifiable matter and are divided into three main classes, names 4-desmethylsterols (sterols), 4-monomethylsterols and 4,4′-dimethylsterols (triterpene alcohols) (Benveniste, 2002). Methylsterols are generally considered to be intermediates in the biosynthesis of 4-desmethylsterols (Grunnwald, 1975). These compounds have been used as makers to characterise (Cert, Moreda, & Carcía-Moreno, 1997) and to detect admixture of vegetable oils (Jiménez & del Valle, 1996). Triterpene alcohols were found to be minor components of the unsaponifiable matter compared to 4-desmethylsterols. The major triterpene alcohols compounds, at complete maturity of olive, are 24-methylene cycloartenol and cycloartenol (Azadmard-Damirchi & Dutta, 2006). Cycloartenol is formed as the first cyclic triterpenoid precursor of sterols and is the substrate for the first methylation reaction, resulting in 24-methylene cycloartenol. β-Amyrin and cycloartenol have 2,3(S)-oxidosqualenes as the same biosynthetic precursor molecule (Benveniste, 2002). 4-Monomethylsterols are less abundant in olive oils than the triterpene alcohols; whilst they are considered as the key in the biosynthetic pathway of 4-desmethylsterols. Citrostadieol and cycloeucalenol are the predominant compounds of 4-monomethylsterols in olive oils (Azadmard-Damirchi & Dutta, 2006). The change of methylsterols level during the olive ripening process is closely dependent on the stage of fruit development and the regulation of oxidosqualene (OS) cyclisation step (Stiti, Triki, & Hartman, 2007). Cyclisation of OS is a branch point between triterpenoids and steroids biosynthesis pathway (Benveniste, 2002).

As phytosterols play a key role in lowering the level of plasma cholesterol, recently food products are enriched with soluble phytosterols. Nowadays, the objective of industry is to identify plant matrices rich in those high value-added compounds. Moreover, these bio-molecules were used to check oils authenticity. Although triterpene alcohols and 4-monomethylsterols composition have been studied at the ripeness stage of olives (Azadmard-Damirchi, Savage, & Dutta, 2005; Ranalli et al., 2002), very little information is available on triterpene alcohols and 4-monomethylsterols

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accumulation during the maturity of olive. Therefore, the goal of our investigation is to conduct both the qualitative and the quantitative characterisation of triterpene alcohols and 4-monomethylsterols during the ripening of *Picholine* olive (Tunisia), and then to determine exactly the time when the olive accumulates maximally those compounds.

2. Materials and methods

2.1. Samples

The variety of *Picholine* olive (*Olea europea L.*) was grown on the Agronomy farm of the O.T.D.G (Office Terres Domaniales Ghzala, Bizerte) in the north of Tunisia. Olives were hand-harvested from the same tree at intervals of one week from the formation of the olive (21st week after the flowering date (WAF) of *Picholine* olive) until there complete maturity (38th WAF). Only healthy fruits, without any sign of infection or physical damage, were selected.

2.2. Reagents and standard

Acetone, chloroform, diethyl ether and petroleum ether were purchased from Fisher Scientific SA. (Loughborough, Spain). Ethanol was from Scientific Limited (Northampton, UK). Pure methylsterols, lanosterol, 5-α-cholestanol and *N*,*O*-bistrimethylsilyltrifluoroacetamide (BSTFA) were acquired from Sigma (St. Louis, MO, USA). TLC silica plates (silica gel 60 G F254, 20 × 20 cm, 0.25 thickness), potassium hydroxide pellets, pyridine and anhydrous sodium sulphate were from Merck (Darmstadt, Germany). Pyridine was purchased from Fluka (Neu-Ulm, Germany).

2.3. Determination of oil content

Oil content was determined by extracting dry material of olives (olives were dried at 20 °C in dry air sterilizers) with petroleum ether using a Soxhlet apparatus (AOCS, 1989). The extraction remained 4 h at 42 °C and was repeated three times for each sample. The extract was dried in a rotary evaporator at 32 °C. Oil was weighed and stored at −10 °C.

2.4. Saponification

Oil samples were saponified following the method described by Cunha, Fernandes, and Oliveira (2006). In brief, unsaponifable fraction of lipids were determined by saponifying 5 g of oil mixed with both 200 μl lanosterol solution (internal standard of methylsterols (triterpene alcohols and 4-monomethylsterols); 0.2% (w/v)) and an ethanolic KOH 12% (w/v) solution; the mixture was heated at 60 °C for 1.30 h. After cooling, 50 ml of H_2O were added. The unsaponifiable matter was extracted four times with 50 ml of petroleum ether. The combined ether extract was washed with 50 ml of ethanol/water (1:1, v/v). The extracted ether was dried over anhydrous Na_2SO_4 and evaporated to dryness using N_2. The dry residue was dissolved in chloroform for TLC analysis.

2.5. Thin layer chromatography

Separation of unsaponifiable fraction by TLC was carried out according to Azadmard-Damirchi et al. (2005) after slight modification by Sakouhi et al. (2009). Briefly, the unsaponifiable matter was separated into subfractions on preparative silica gel thin-layer plates (silica gel 60 G F254) using 1-dimensional TLC with hexane/diethyl ether (6:4, v/v) as the developing solvent. The unsaponifiable fraction diluted in chloroform was applied on the silica gel plates. So as to correctly identify the sterols band, a reference sample of purified methylsterol, lanosterol, was applied on the left and the right sides of the TLC plates. After development, the plate was sprayed with 2,7-dichlorofluorescein and viewed under UV light. The band corresponding to triterpene alcohols and 4-monomethylsterols was scraped, extracted three times with chloroform/diethyl ether (1:1, v/v), filtered to remove the residual silica, dried in a rotary evaporator and stored at −10 °C for GC–MS/GC–FID analyses.

2.6. Silylation of triterpene alcohols and 4-monomethylsterols fraction

An amount of 2 mg of methylsterols residue were mixed with 125 μl of BSTFA (with 1% TMCS), 125 μl of pyridine and 450 μl of acetone, the mixture vortexed for about 10 s and heated at 70 °C for 20 min. After silylation reaction, 1.5 ml of chloroform were added to the mixture and 1 μl of the solution were directly injected to gas chromatograph.

2.7. Gas chromatography–mass spectrometry detection

GC–MS analyses were performed using a DB-5MS fused silica capillary column (30 m × 0.25 mm I.D., 0.25 m film thickness; J&W Scientific, Folsom, CA, USA) in an Varian SAR 3400Cx gas chromatograph coupled directly to the mass detector (MS Varian SATURN). Helium was used as carrier gas, with a constant flow rate of 1 ml min^{-1}. The injector and detector temperatures were 250 °C. The oven temperature was programmed from 150 to 300 °C at 4 °C min^{-1}. The final temperature was held constant for 10 mn and the transfer line temperature was 250 °C. Electron impact mass spectra were measured at acceleration energy of 70 eV. Manual injection of 1 μl of the solution of sterol was performed in the split mode at a 60:1 split ratio. The triterpene alcohols and 4-monomethylsterols were identified by comparing their retention times and mass spectra with those of their pure molecules. The peaks were also confirmed with NIST Mass Spectral Library. The retention time and mass spectrometric data of trimethyl derivatives triterpene alcohols and 4-monomethylsterols identifying by GC–MS were provided in Table 1.

2.8. Gas chromatography–flame ionisation detection (GC–FID)

Due to the higher sensibility of GC–FID detector compared to GC–MS one (Azadmard-Damirchi & Dutta, 2006; Cunha et al., 2006), the quantification of 4-desmetylsterols and phytostanols was performed using GC–FID. The GC system used was a HP 4890A gas chromatograph (Agilent Technologies, Palo Alto, USA) equipped with a split-splitless injector, a FID and a DB-5MS (30 m × 0.25 mm I.D., 0.25 m film thickness; J&W Scientific, Folsom, CA, USA) column was used. The initial column temperature was 150 °C and programmed to increase at a rate of 4 °C min^{-1} to 300 °C and then held for 25 min. The injector and detector temperatures were 280 °C and 310 °C, respectively, and helium was used as carrier gas at an inlet pressure of 12 psi, giving a column flow of 1 ml/min. Quantification of phytosterols (4-monomethylsterols, triterpene alcohols and 4-desmethylsterols) was achieved by addition of lanosterol and 5-α-cholestanol as internal standards, respectively, for methylsterols and 4-desmethylsterols. The level of each phytosterol was calculated as milligrams per 100 g of oil using the following formula: amount = $100-(PA_s)(m_{is})/(PA_{is})(m)$, where PA_s = phytosterol peak area, PA_{is} = internal standard area, m_{is} = weight (mg) of the internal standard and m = weight (g) of oil taken for analysis. The phytosterols composition was determined for three independent replicates at each stage of olive maturity.

Table 1
Retention time and mass spectrometric data for trimethylsilyl derivatives of triterpene alcohols and 4-monomethylsterols identified by GC–MS.

	Retention time (mn)	Compounds	Mains fragmentation ions (M/Z)
Triterpene alcohols	46.09	δ-Amyrin	399, 275, 261, 250, 83, 47
	46.34	Taraxerol	395, 272, 251, 196, 83, 43
	46.63	β-Amyrin	367, 275, 254, 189, 83, 47
	47.05	Cycloartenol	393, 365, 251, 215, 69, 41
	48.21	24-Methylene cycloartenol	398, 261, 250, 135, 83, 47
4-Monomethylsterols	45.78	Obtusifoliol	394, 255, 175, 129, 83, 73
	47.43	Gramisterol	393, 265, 254,206, 83, 41
	47.85	Cycloeucalenol	399, 362, 250, 83, 47, 35
	49.52	Citrostadienol	399, 264, 250, 83, 47, 35

Table 2
Change in total triterpene alcohols, 4-monomethylsterols and 4-desmethylsterols contents (expressed in mg/100 g oil) during ripening of *Picholine* olive.

Stage of ripening	21st WAF	24th WAF	26th WAF	30th WAF	35th WAFD	38th WAF
4-Desmethylsterols (mg/100 g oil)	991.25 (13.7)	739.72 (13.4)	1321.23 (15.6)	998.22 (13..8)	850.43 (13.6)	659.72 (13.1)
Triterpene alcohols (mg /100 g oil)	186.60 (8.9)	107.34(7.3)	262.86 (11.8)	209.27 (11.6)	237.45 (12.2)	221.89 (11.4)
4-Monomethysterols (mg/100 g oil)	111.14 (8.8)	200.34 (9.5)	28.31 (3.2)	108.26 (8.5)	65.98 (4.7)	63.82 (2.8)
Total phytosterols (mg/100 g oil)	1296.99	1047.44	1612.22	1315.75	1153.86	945.43

3. Results and discussion

3.1. Change in total phytosterols content

During the ripening of *Picholine* olive, the total levels of both triterpene alcohols and 4-monomethylsterols (expressed in mg/100 g of oil) were lower compared to 4-desmethylsterols (Table 2). Their percentages moved from 18 to 30% of total phytosterols during ripening of *Picholine* olive. The maximum level of both 4-desmethylsterols (1321 mg/100 g oil) and triterpene alcohols (263 mg/100 g oil) were reached at 26th WAF; whilst the maximum level of 4-monomethylsterols (200 mg/100 g oil) was attained early at 24th WAF of fruit. These results indicate that the 4-monomethylsterols biosynthesis occurred early in the young olive fruit that will be used as a precursor for the biosynthesis of both 4-desmethylsterols and triterpene alcohols compounds. During the last WAFs the levels of the three classes of phytosterols decreased, which indicates the end of their biosynthesis. In fact, during the end of olive maturity the phytosterols were not denovo synthesized but, instead it is a result of conversion of existing phytosterols to other sterol forms (stanols and steryl esters) due to some dehydratation, hydrogenation and dehydrogenation reactions (Venkatramesh, Karunanandaa, Gunter, Boddupalli, & Kishore, 2002). The quantitative characterisation of phytosterol classes differs from one vegetable oil to another. Harrabi et al. (2007) reported that maize oil contained 14% of triterpene alcohols, 4% of 4-monometylsterosls and 82% of 4-desmethylsterols, whilst the corresponding figures in hazelnut oil were 5–8, 4–5 and 88–91%, respectively (Azadmard-Damirchi & Dutta, 2006). However, our results show that *Picholine* olive oil contained 24% of triterpene alcohols, 8% of 4-monomethylsterols and 67% of 4-desmethylsterols. Thus, the combined characterisation of these three phytosterol classes may be used as a marker to check the authenticity and to detect the admixture of vegetable oils. The study of the phytosterols accumulation during the ripening of olives has a great importance, in fact, in *Picholine* fruit the highest amount of total phytosterols was reached at 26th WAF. Therefore, at this date, the *Picholine* olive may be a potential source of phytosterols which have great benefits for human health. The total methylsterols contents did not give a clear figure about the level of each individual triterpene alcohol and 4-monomethylsterol components, so a qualitative and a quantitative characterisation of individual methylsterol should be of great value.

3.2. Change in triterpene alcohols contents

In *Picholine* olive oil, triterpene alcohols contents were minor compared to the 4-desmethylsterols (Table 2). At complete maturity of fruit, 24-methylene cycloartenol (141 mg/100 g oil), cycloartenol (49 mg/100 g oil), β-amyrin (17 mg/100 g oil) were the major triterpene alcohols that accounting for over 93%. However, δ-amyrin (10 mg/100 g oil) and traroxerol (5 mg/100 g oil) were less present in *Picholine* olive oil. These results are in agreement with those listed in literature (Azadmard-Damirchi & Dutta, 2006; Itoh, Yoshida, Yatsu, Tamura, & Matsumoto, 1981). The small difference in the quantitative characterisation may be explained by the fact that the content of phytosterols is affected by geographical growing area and difference in olive varieties (Casas, Bueno, García, & Cano, 2004; Koutsaftakis, Kotsifaki, Stefanoudaki, & Cert, 2000; Stefanoudaki, Chartzoulakis, Koutsaftakis, & Kotsifaki, 2001). Results from the quantitative characterisation of samples examined here showed that from the 21st WAF the concentration of cycloartenol started to decrease gradually and reached its minimum (27 mg/100 g oil) at 26th WAF of fruit (Fig. 1). In contrast, the level of 24-methylene cycloartenol increased to attain its maximum

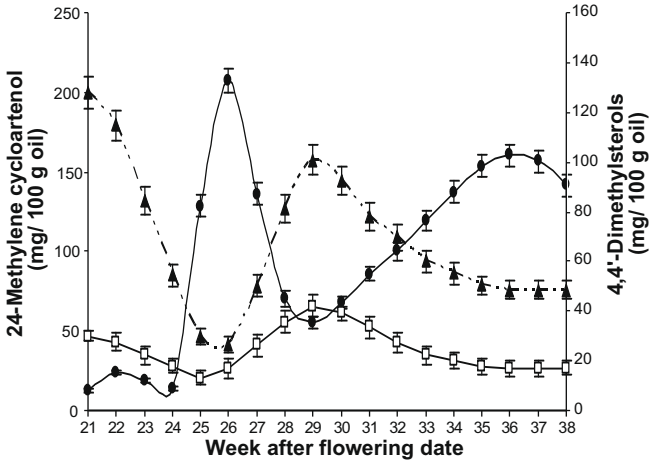
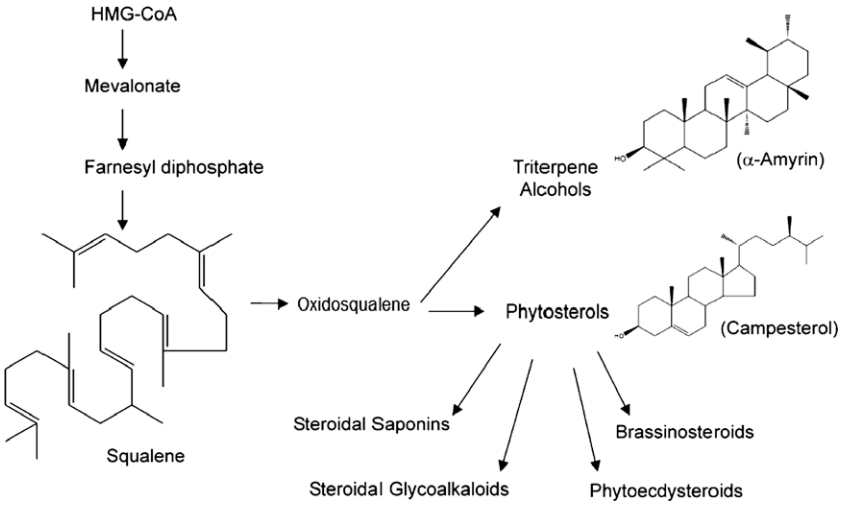


Fig. 1. Change in 24-methylene cycloartenol (●), cycloartenol (▲) and β-amyrin (□) amounts (expressed in mg/100 g of oil) during ripening of *Picholine* olives. Mean of three measurements (vertical line).



Scheme 1. Biosynthesis of sterols and triterpenoids compounds via the acetate/mevalonate pathway.

about 207 mg/100 g oil at 26th WAF of *Picholine* olive that explains the conversion of cycloartenol into 24-methylene cycloartenol (Goodwin, 1979). Cycloartenol is formed as the first cyclic triterpenoid precursor of phytosterols and works as a substrate for the first methylation reaction, resulting in 24-methylene cycloartenol (Benveniste, 2002). However, from 26th to 29th WAF of fruit, we observed the increase of cycloartenol (101 mg/100 g oil) precursor of steroids which explained the higher level of 4-desmethylsterols in this period (Table 2). In fact, sterols and triterpenoids classes are synthesized via the acetate/mevalonate pathway (Scheme 1) and share common oxidosqualene as precursor (Benveniste, 2002). Moreover, Fig. 1 shows that the patterns of β-amyrin and cycloartenol were nearly similar; their maximum levels were reached at the 29th WAF of *Picholine* olive. In fact, β-amyrin and cycloartenol have 2,3(S)-oxidosqualene (OS) as the same precursor (Benveniste, 2002). The 2,3(S)-oxidosqualene molecule was mainly cyclised into cycloartenol since the concentration of cycloartenol was found to be higher than that of β-amyrin during all stages of *Picholine* olive maturity. In higher plants, the biosynthesis of cycloartenol is initiated in the chair-boat-chair-like conformation of OS whilst the formation of amyrins and related pentacyclic triterpenes is initiated in the chair-chair-chair-like conformation of OS (Benveniste, 2002). In addition to β-amyrin, *Picholine* olive contained δ-amyrin isomer. Results show that starting from the 21st WAF the δ-amyrin content decreased gradually to reach a constant value (about 5 mg/100 g oil) at 25th to 29th WAF, then it increased to attain its maximum level (18 mg/100 g oil) at 32nd WAF. At the last 5 weeks of maturity, it was found that δ-amyrin reached to a constant value of about 10 mg/100 g of oil. At all stages of olive maturity, δ-amyrin was expressed at lower amount than β-amyrin compound. As previously mentioned that OS is the precursor of the different amyrins isomers; we suggested that during the ripening of *Picholine* olives OS is more converted into β-amyrin isomer. Besides these major triterpene alcohols that mentioned above, taraxerol was found in *Picholine* olive oil at the lowest concentration. The higher level of this compound (13 mg/100 g oil) was reached at 24th WAF.

3.3. Change in 4-monomethylsterols contents

In *Picholine* olive oil the total 4-monomethylsterol components were the minor proportion of phytosterols compared to 4,4′-dimethylsterols and 4-desmetylsterols classes (Table 2). In the present study, the total 4-monomethylsterol components were found at about 10% of total phytosterols. At the maturity of *Picholine* olive, the most abundant 4-monomethylsterol compounds were cycloeucalenol (39 mg/100 g oil) and citrostadienol (15 mg/100 g oil) followed by obtusifoliol (7 mg/100 g oil) and gramisterol (4 mg/100 g oil). The quantitative and qualitative characterisations of 4-monometylsterols, at complete maturity of *Picholine* olives, were in agreement with those listed in literature (Azadmard-Damirchi & Dutta, 2006). Fig. 2 shows that from the 21st to 24th WAF, citrostadienol content increased to reach its maximum level (161 mg/100 g oil at 24th WAF); whilst the concentration of cycloeucalenol decreased to attain its lowest level at 26th WAF (8 mg/100 g oil). From 26th WAF to the complete maturity of fruit, citrostadienol and cycloeucalenol had nearly the same patterns. The contents of these two compounds were 39 mg/100 g oil and 15 mg/100 g oil, respectively, at complete maturity of *Picholine* olive. Citrostadienol and cycloeucalenol together accounted about 86% of total 4-monomethylsterols. Fig. 3 shows that from 22nd WAF obtusifoliol content increased to reach its maximum level (11 mg/ 100 g oil) at the 24th WAF then, it fluctuated to attain 7 mg/100 g oil at complete maturity of fruit. Gramisterol was the minor 4-monomethylsterols component during ripening of *Picholine* olive, its higher level was attained (9 mg/100 g oil) at 24th WAF of fruit. The maximum level of these total 4-monomethylsterols components (200 mg/100 g oil) was reached at 24th WAF of *Picholine* olive. The qualitative and quantitative characterisations of 4-monomethylsterols components differed from one vegetable oil to another. Harrabi et al. (2007) suggested that corn oils

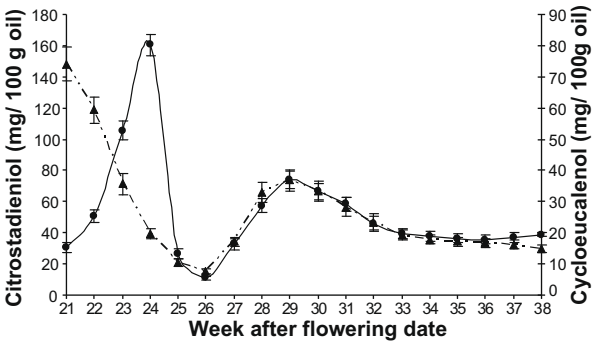


Fig. 2. Change in citrostadienol (●) and cycloeucalenol (▲) amounts (expressed in mg/100 g of oil) during ripening of *Picholine* olives. Mean of three measurements (vertical line).

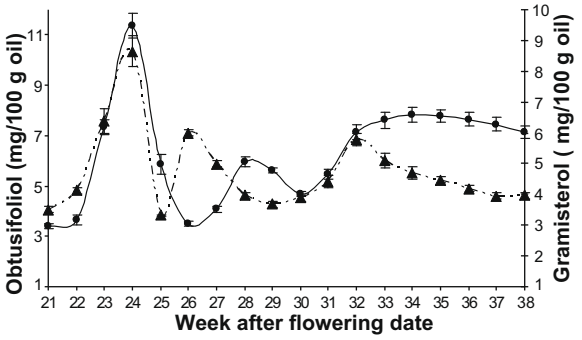


Fig. 3. Change in obtusifoliol (●) and gramisterol (▲) amounts (expressed in mg/100 g of oil) during ripening of *Picholine* olives. Mean of three measurements (vertical line).

contained 34% of obtusifoliol, 28% of gramisterol and 44% of citrostadienol. However, Malecka (2002) has identified citrostadienol at lower level in tomato seed oil; Azadmard-Damirchi et al. (2005) demonstrated also that cycloeucalenol was present at only a trace level in hazelnut oils. In the other hand, our results show that citrostadienol (60%) and cycloeucalenol (22%) were the major 4-monomethylsterols compounds in olive oils. Therefore, we propose to use 4-monomethylsterols fraction to detect the adulteration of vegetable oils.

The greatest change in triterpene alcohols (4,4′-dimethylsterols) and 4-monomethylsterols ratio (TRA/4-MMS ratio) occurred during ripening of *Picholine* olives. Triterpene alcohols were accumulated at higher levels than 4-monomethylsterols except at the 23rd and 24th WAF, the maximum level of TRA/4-MMS ratio was observed at the 26th WAF (TRA/4-MMS = 11). Latter date corresponded to the maximum level of 24-methylene cycloartenol which was the major component of triterpene alcohols. However, the 23rd and the 24th WAF corresponded to the maximum level of citrostadienol (abundant compound of 4-monomethylsterols). During ripening of *Picholine* olives TRA/4-MMS ratio was irregular. This result can be explained by the fact that during the process of ripening, olive moderated the biosynthesis of triterpene alcohols and 4-monomethylsterols according to its need for these compounds to achieve their physiologic functions. The qualitative characterisation of triterpene alcohols and 4-monomethylsterols during the ripening of *Picholine* olive was in agreement with those reported by Stiti et al. (2007). In fact, these authors have identified 24-methylene cycloartenol, cycloartenol, β-amyrin, δ-amyrin, traroxerol, cycloeucalenol, citrostadienol, obtusifoliol and gramisterol in *Chemlali* Tunisia olive oils. However, the two Tunisian varieties were in disagreement concerning the quantitative characterisation of these compounds. The maximum accumulation of the free methylsterols was reached at the 26th WAF in *Picholine* cultivar but, the highest level of these compounds in *Chemlali* olive was observed at the 30th WAF. So, the difference in the quantitative characterisation of the two cultivars is due to the geographical differences for each variety, *Picholine* is from the north of Tunisia (*Mateur*) but, *Chemlali* is from the centre (*Béni Khalled*).

4. Conclusions

Triterpene alcohols and 4-monomethylsterols compounds were present at all stages of *Picholine* olives maturity. The methylsterols contents were closely dependent on the stage of olive repening. Except the 23rd and 24th WAF, the proportions of phytosterol classes were 4-desmethylsterols followed by 4-dimethylsterols and 4-monomethylsterols. 24-Methylene cycloartenol and citrostadienol were the main triterpene alcohols and 4-monomethsterols compounds respectively, during the ripening of fruit. The maximum level of the phytosterols classes was reached at 26th WAF which is the best time to exploit at maximum these high value-added compounds of *Picholine* olive.

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