Food Chemistry 119 (2010) 765–769

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Influence of olive paste preparation conditions on virgin olive oil triterpenic compounds at laboratory-scale

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ARTICLE INFO

Article history:
Received 27 April 2009
Received in revised form 22 June 2009
Accepted 15 July 2009

Keywords:
Crushing level
Malaxation temperature
Malaxation time
Virgin olive oil
Triterpenes

ABSTRACT

The influence of olive paste preparation conditions on the triterpenic content of virgin olive oils from Arbequina and Picual cultivars was investigated. For this purpose, three sieve diameters of the hammer mill (4, 5, and 6 mm), two malaxation temperatures (20 and 30 °C), and two malaxation times (20 and 40 min) were tested. Results obtained showed that for Arbequina oils, a finer crushing level resulted in higher maslinic acid and erythrodiol content. Increasing malaxing temperature and time lead to a rise in both oleanolic and maslinic acid concentration, whereas erythrodiol content increased only for the longer malaxation time. For Picual oils, higher concentrations of oleanolic acid, maslinic acid, and uvaol were obtained by prolonging the paste malaxation time. A finer crushing level resulted also in an increase of maslinic acid content. These findings suggest that virgin olive oil triterpenic composition can be improved by regulating olive paste preparation conditions.

1. Introduction

Virgin olive oil represents one of the most important components of the Mediterranean diet, and it is highly appreciated by consumers thanks to its health benefits and pleasant, peculiar flavour. This product is obtained from the fruit of olive trees (*Olea europaea*, L.) using only mechanical techniques including crushing of the olive fruits, malaxing of the resulting paste and separation of the oily phase. All of these operations can affect the quality, chemical composition and sensory characteristics of the final product, and therefore optimisation of these extraction steps is necessary (Kiritsakis, 1998). The malaxing process has been widely investigated and several works have reported the effect of the malaxation variables: temperature (Parenti, Spugnoli, Masella, & Calamai, 2008; Ranalli, Contento, Schiavone, & Simone, 2001), time (Di Giovacchino, Costantini, Ferrante, & Serraiocco, 2002; Ranalli, Pollastri, Contento, Iannucci, & Lucera, 2003) and even both (Gómez-Rico, Inarejos-García, Salvador, & Fregapane, 2009; Jiménez, Hermoso, & Uceda, 1995; Kalua, Bedgoog, Bishop, & Prenzler, 2006) on the composition and quality of virgin olive oil. The influence of the separation process has also been largely described (Salvador, Aranda, Gómez-Alonso, & Fregapane, 2003; Torres & Maestri, 2006). Conversely, the crushing step was less studied, and in general published papers were oriented to compare the quality and composition of olive oils obtained from stone mills, used in the past, and those produced using metallic crushers equipped either with hammers, blades or disks (Caponio, Alloggio, & Gomes, 1999; Caponio, Gomes, Summo, & Pasqualone, 2003; Servili, Piacquadio, De Stefano, Taticchi, & Sciancalepore, 2002). Moreover, to our knowledge, there is little data on the effect of the crushing level although its regulation is essential for obtaining good process yields (Uceda, Jiménez, & Beltrán, 2006) and may have significant influence on the transfer of components to the oil (Cert et al., 1999). On the other hand, most of the olive oil processing studies are mainly focussing on volatile and phenolic compounds (Gómez-Rico et al., 2009; Kalua et al., 2006; Parenti et al., 2008), hence neglecting other minor compounds like pigments, sterols, squalene, and triterpenes amongst others.

The epicarp of the olive fruit (*O. europaea*, L.) has been known for a long time to contain a variety of triterpenes (Bianchi, Murelli, & Vlahov, 1992; Frega & Lercker, 1986), including oleanolic acid, maslinic acid, uvaol and erythrodiol (Fig. 1). Interest on the pharmacological properties of these compounds is constantly increasing and many biological activities have been attributed such as antioxidant (Andrikopoulos, Kaliora, Assimopoulou, & Papageorgiou, 2002), antiinflammatory (Márquez-Martín, De la Puerta, Fernández-Arche, Ruiz-Gutiérrez, & Yaqoob, 2007), and antitumoral (Juan, Planas, Ruiz-Gutiérrez, Daniel, & Wenzel, 2008; Juan, Wenzel, Daniel, & Planas, 2008). However, there are little data

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doi:10.1016/j.foodchem.2009.07.031

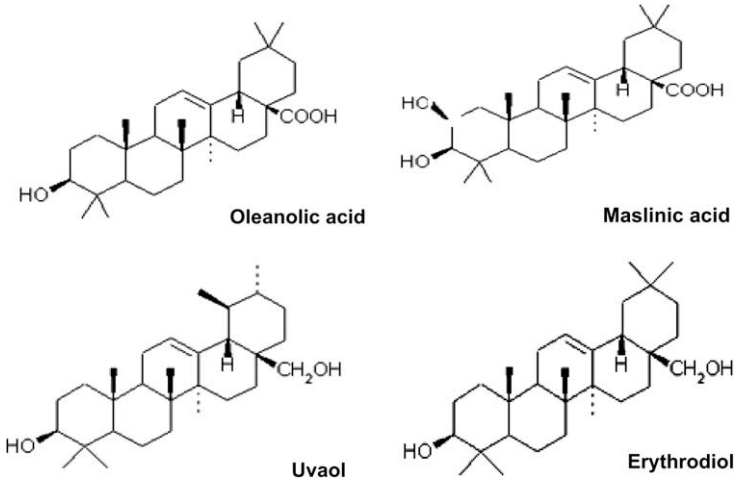

Fig. 1. Chemical structure of oleanolic acid, maslinic acid, uvaol, and erythrodiol.

available regarding their concentration in virgin olive oils (Allouche et al., 2009; Pérez-Camino & Cert, 1999) as well as their behaviour related to different processing conditions during virgin olive oil extraction (Cert et al., 1999; Pérez-Camino & Cert, 1999). Thus the objective of the present work was to investigate, under controlled laboratory-scale conditions, the influence of the crushing level, as well as the malaxation temperature and time on the content of oleanolic acid, maslinic acid, uvaol, and erythrodiol. For this purpose, three level combinations of sieve size of the hammer mill (4, 5, and 6 mm) and two level factorial combinations of malaxing temperature (20 and 30 °C) and time (20 and 40 min) were considered as variable factors. Change in triterpenic content was evaluated on virgin olive oils obtained from Arbequina and Picual, the most extensively grown cultivars for oil production in Spain.

2. Materials and methods

2.1. Plant material

For this work, 25 years old olive trees (*O. europaea*, L.) of Picual and Arbequina cultivars with uniform characteristics were selected, five trees per cultivar. The trees were spaced 7 × 7 m and grown in the experimental orchard of Centro IFAPA 'Venta Del Llano'-Mengibar, Jaen (Spain) using standard growing techniques. The study was carried out during the 2007/08 crop year. Fruit samples were harvested by hand at the ripening index 3, according to the fruit classification based on skin and flesh colour described in the ripening index method (Uceda & Frías, 1975). From each cultivar, 25 kg of olives were harvested from the pool of trees.

2.2. Solvents, reagents, and standards

All solvents and reagents, analytical or HPLC grade, were obtained from Merck (Germany). Standards of oleanolic acid, erythrodiol, uvaol, betulinic acid, betulin, and silylating reagent (Sil-A) were purchased from Sigma (St. Louis, MO, USA). Maslinic acid was provided by Dr. A. Garcia-Granados, Department of Organic Chemistry, University of Granada, Spain.

2.3. Olive oil extraction

For each cultivar, 24 samples (12 treatments × 2 replicates) were processed using Abencor extraction unit (Abencor series 100, MC2 Ingeniería y Sistemas, S.L. Seville, Spain). Olive samples were crushed in a hammer mill at a velocity of 3850 rpm, using three sieve diameters (4, 5, and 6 mm). The olive paste was then malaxed in a thermo-beater at two different temperatures (20 and 30 °C) for two times (20 and 40 min). The oil was separated after centrifugation for 1 min at 3500 rpm. The oily must was left for decantation and then filtered. Oils were stored at −20 °C until analysis.

2.4. Determination of quality parameters

Regulated physicochemical quality parameters (free acidity, peroxide values and UV absorption characteristics at 232 and 270 nm (K_{232} and K_{270})) were determined following the analytical methods described by Regulation EEC 2568/91 of the Commission of the European Union (1991) and the later modifications (European Union Commission Regulation, 1991). Free acidity was expressed as % of oleic acid, peroxide values were expressed as milli-equivalents of active oxygen per kilogram of oil (mEq O_2/kg), K_{232} and K_{270} extinction coefficients were calculated from absorption at 232 and 270 nm, respectively.

2.5. Determination of triterpenic dialcohols

The analysis of uvaol and erythrodiol was performed according to the EU Regulation 2568/91 (European Union Commission Regulation, 1991) for the determination of sterols in olive oil. The oil sample was saponified with ethanolic potassium hydroxide solution. The unsaponifiable fraction was removed with ethyl ether and the sterol fraction was separated by Silica gel plate chromatography. Then, the silylating reagent was added to the sterol fraction and allowed to stand for at least 15 min at room temperature. Separation and quantification of the silylated sterol was performed on a Helwett Packard instrument model 6890 gas chromatograph, equipped with a HP-5 capillary column (25 m, 0.25 mm i.d., 0.25 μm of thickness). The working conditions were: oven temperature 260 °C, injector at 305 °C split/splittless, FID detector 330 °C. The injected volume was 1 μl using helium as carrier gas. For quantification, betulin was used as an internal standard. The same response factor was considered for both triterpenic dialcohols uvaol and erythrodiol. For each oil sample, analyses were performed in duplicate and results were expressed as mg/kg.

2.6. Determination of triterpenic acids

The acidic fraction was isolated by solid phase extraction using bonded aminopropyl cartridges and betulinic acid was added as

internal standard, as previously described by Pérez-Camino and Cert (1999). The extract was then evaporated, silylated, and left at room temperature for at least 10 min before gas chromatographic analysis. The chromatographic analysis was carried out using a Perkin–Elmer gas chromatograph, Autosystem model, fitted with a flame ionisation detector and a split injection system (split ratio 1:0.25). Separation was performed on a HP-5 capillary column (30 m, 0.32 mm i.d., 0.25 μm of thickness). The operating conditions were: oven temperature 260 °C for 5 min and then increased at 4 °C/min up to 320 °C; injector and detector at 320 °C. Helium was used as carrier gas at a column head pressure of 25 Ψ. Triterpenic acids were quantified assuming the same response factor for all triterpenes. For each oil sample, analyses were done in duplicate and results were expressed as mg/kg of betulinic acid.

2.7. Statistical analysis

Results are expressed as mean values ± standard error (SE). Analysis of variance (ANOVA) was applied to data obtained according to a $3 \times 2 \times 2 \times 2$ factorial design (three sieve size diameters × two malaxing temperatures × two malaxing times × two olive cultivars) to evaluate the influence of these parameters on the triterpenic content of Arbequina and Picual oils. When a significant F value was found, Tukey's HSD test was used to determine significant differences between means ($P \leqslant 0.01$). These determinations were carried out using software Statistix, Version 8.0.

3. Results and discussion

Virgin olive oils obtained from Arbequina and Picual fruits were used to determine significant changes in the triterpenic fraction due to the different operative conditions performed for oil extraction. Preliminary, the commercial quality of all olive oil samples was checked to ensure high oil quality during the experiment. Based on the values obtained, Arbequina oils were defined as belonging to the category 'extra virgin olive oil' as established by the EU regulation (European Union Commission Regulation, 1991) (Free acidity < 0.8%; peroxide values < 20 mEq O_2/kg; $K_{232} < 2.50$; $K_{270} < 0.20$). Picual oils were classified into the category 'virgin olive oil' ($K_{270} > 0.20$) probably because of the pigment interference (European Union Commission Regulation, 1991). These results are expected since olive fruits were fresh, healthy, and harvested at the optimal ripening stage. Moreover, the sum of erythrodiol and uvaol, expressed as sum of percent of total sterols, was checked. All of the olive oil samples studied showed erythrodiol + uvaol values below to the limit 4.5% set by the official normal standard (European Union Commission Regulation, 1991) for the 'extra virgin olive oil' category. The values obtained are not reported since no substantial differences were detected either for the olive paste preparation conditions or for both cultivars.

However, virgin olive oil triterpene content varied according to cultivar and to olive paste conditioning variables. Tables 1 and 2 summarise the quantitative data of oleanolic acid, maslinic acid, uvaol, and erythrodiol for the different crushing level, malaxation temperature and time combinations, respectively, for Arbequina and Picual oils. Results of variance analysis applied to these data are presented in Tables 3 and 4.

The crushing level affected significantly to maslinic acid and erythrodiol content in Arbequina oils (Table 3) obtaining higher values when olive fruits were crushed with the smallest sieve diameter (4 mm). No differences were observed between the other sieve diameters (5 and 6 mm) (Table 1). In contrast, for Picual oils only maslinic acid was significantly influenced (Table 4), showing a higher content when the 4 mm sieve diameter was used. Our results are consistent with a previous paper (Cert et al., 1999) on the limited influence of the sieve diameter on the triterpenic compounds. By comparing sieve diameter 5 and 6 mm, these authors did not find significant differences for the triterpenic dialcohols and acid fraction in oils obtained from the Picual cultivar. In the present work, a smallest sieve size diameter was tested and significant differences were found ($P \leqslant 0.01$). Triterpenic compounds are mainly located in the epicarp of the olive fruit (Bianchi et al., 1992; Frega & Lercker, 1986). Therefore, a finer milling would help to obtain a more complete breakage of olive skin, hence increasing their concentration in the oil.

By evaluating the effect of malaxation temperature, it could be observed that for Arbequina and Picual oils, both oleanolic and maslinic acids were significantly influenced (Tables 3 and 4) showing higher contents when olive paste was malaxed at the highest

Table 1
Quantitative data[a] (mg/kg) of triterpenic compounds in Arbequina oils for different crushing level, malaxation temperature and time combinations during oil extraction.

Processing conditions	Oleanolic acid	Maslinic acid	Uvaol	Erythrodiol
4 mm/20 °C/20 min	6.17 ± 0.67	10.55 ± 1.20	1.99 ± 0.59	24.31 ± 0.26
5 mm/20 °C/20 min	4.97 ± 0.12	6.55 ± 0.23	0.90 ± 0.17	13.39 ± 1.11
6 mm/20 °C/20 min	3.46 ± 0.33	5.91 ± 1.13	1.93 ± 0.92	16.46 ± 1.24
4 mm/20 °C/40 min	8.63 ± 0.48	13.97 ± 1.02	1.25 ± 0.18	27.43 ± 0.80
5 mm/20 °C/40 min	6.04 ± 0.39	11.51 ± 2.50	2.42 ± 1.10	17.00 ± 1.54
6 mm/20 °C/40 min	5.23 ± 0.24	6.33 ± 0.38	3.14 ± 0.99	19.13 ± 0.80
4 mm/30 °C/20 min	6.87 ± 0.72	11.48 ± 0.96	1.31 ± 0.35	24.26 ± 1.70
5 mm/30 °C/20 min	7.02 ± 1.12	7.24 ± 0.32	1.15 ± 0.34	15.64 ± 0.24
6 mm/30 °C/20 min	4.71 ± 0.33	5.87 ± 0.24	4.33 ± 0.48	15.98 ± 0.46
4 mm/30 °C/40 min	9.74 ± 0.98	14.17 ± 1.05	2.20 ± 0.53	25.49 ± 2.20
5 mm/30 °C/40 min	12.40 ± 0.46	14.27 ± 0.30	2.27 ± 0.52	21.66 ± 3.57
6 mm/30 °C/40 min	14.20 ± 2.10	16.05 ± 2.10	1.79 ± 0.98	20.39 ± 1.42

[a] Data are expressed as means ± standard error (SE).

Table 2
Quantitative data[a] (mg/kg) of triterpenic compounds in Picual oils for different crushing level, malaxation temperature, and time combinations during oil extraction.

Processing conditions	Oleanolic acid	Maslinic acid	Uvaol	Erythrodiol
4 mm/20 °C/20 min	4.82 ± 0.16	8.19 ± 0.38	1.97 ± 0.25	17.09 ± 2.07
5 mm/20 °C/20 min	3.71 ± 0.51	7.52 ± 0.97	1.49 ± 0.14	13.66 ± 0.59
6 mm/20 °C/20 min	2.80 ± 0.16	3.83 ± 0.08	1.05 ± 0.28	12.40 ± 0.99
4 mm/20 °C/40 min	4.76 ± 0.13	7.32 ± 0.40	2.64 ± 0.57	16.47 ± 2.08
5 mm/20 °C/40 min	3.89 ± 0.24	5.14 ± 0.61	0.92 ± 0.06	13.81 ± 0.42
6 mm/20 °C/40 min	5.59 ± 0.50	5.85 ± 0.61	2.85 ± 0.65	17.68 ± 2.37
4 mm/30 °C/20 min	5.68 ± 0.32	9.98 ± 0.58	5.23 ± 1.51	18.00 ± 1.86
5 mm/30 °C/20 min	6.18 ± 0.45	6.75 ± 0.73	4.11 ± 1.56	17.64 ± 1.16
6 mm/30 °C/20 min	7.41 ± 0.71	7.58 ± 0.23	4.28 ± 0.87	16.47 ± 0.86
4 mm/30 °C/40 min	6.54 ± 0.80	10.66 ± 1.22	3.44 ± 0.44	17.86 ± 0.59
5 mm/30 °C/40 min	7.24 ± 0.90	7.75 ± 0.62	3.15 ± 1.81	15.55 ± 1.17
6 mm/30 °C/40 min	5.95 ± 0.40	7.01 ± 0.48	5.83 ± 1.64	15.85 ± 3.38

[a] Data are expressed as means ± standard error (SE).

Table 3
Partial mean squares[a] from analysis of variance for the effect of olive paste conditioning variables on Arbequina oils triterpenic compounds.

	Oleanolic acid	Maslinic acid	Uvaol	Erythrodiol
Sieve diameter (Ø) (mm)	1.38	15.88*	12.52	53.97*
Temperature (T) (°C)	24.77*	8.12*	0.64	0.86
Time (t) (min)	31.44*	32.80*	0.68	11.79*
Ø (mm) × T (°C)	6.96*	4.67	0.48	3.31
Ø (mm) × t (min)	3.52	2.26	7.64	1.11
T (°C) × t (min)	9.19*	4.92	2.02	0.14
Ø (mm) × T (°C) × t (min)	4.74	7.06	14.00*	0.85
Error	18.00	24.29	62.03	27.97

[a] Partial mean squares for each variable, expressed as percentage of total corrected sum of squares.
* Significance $P \leqslant 0.01$.

Table 4
Partial mean squares[a] from analysis of variance for the effect of olive paste conditioning variables on Picual oils triterpenic compounds.

	Oleanolic acid	Maslinic acid	Uvaol	Erythrodiol
Sieve diameter (Ø) (mm)	0.30	35.65*	4.15	7.62
Temperature (T) (°C)	47.61*	21.81*	29.17*	6.22
Time (t) (min)	3.04	0.00	0.06	0.22
Ø (mm) × T (°C)	4.30	3.16	0.91	1.41
Ø (mm) × t (min)	0.12	1.85	5.58	4.39
T (°C) × t (min)	1.57	0.86	1.23	3.46
Ø (mm) × T (°C) × t (min)	14.03*	8.68*	1.17	3.63
Error	23.05	27.99	57.72	73.05

[a] Partial mean squares for each variable, expressed as percentage of total corrected sum of squares.
* Significance $P \leqslant 0.01$.

temperature (30 vs. 20 °C) (Tables 1 and 2). The same trend was observed for uvaol in Picual oils (more than 2-fold-higher). Previous works have reported that only at temperatures higher than 50–60 °C, some of the substances such as waxes, aliphatic alcohols and triterpenic dialcohols can become more soluble in the oily phase increasing their concentration in the virgin olive oil (Martel & Alba Mendoza, 1981). On the other hand, Cert et al. (1999) have obtained an increase of triterpenic acid content only when olive paste was malaxed at a very high temperature (40 °C). In our study, we have found a significant effect ($P \leqslant 0.01$) for the triterpenic acid compounds and uvaol even at the lowest temperatures (20 and 30 °C). This rise can be explained by the fact that higher temperatures decrease the oil viscosity, thus favouring the extraction of these compounds from the olive paste. The percent increase was different for each triterpenic acid and cultivar. For Arbequina oils, oleanolic, and maslinic acids increased by 37.23% and 20.65% whereas for Picual oils, the rise was by 34.44% and 23.88%, respectively. It should be noted that for both cultivars, maslinic acid rise was lower than that of oleanolic acid. These differences may be attributed to the higher polarity of maslinic acid that includes an extra hydroxyl group in its molecule.

Concerning the malaxation time, a different behaviour was observed for each cultivar. Indeed for Arbequina oils, a significant increase in triterpenic acids and erythrodiol content was observed ($P \leqslant 0.01$) (Table 3). Malaxing olive paste during 40 min increased the amount of oleanolic acid, maslinic acid and erythrodiol respectively by 40.98%, 37.59%, and 16.07%. These results suggest that by prolonging the malaxing operation, these compounds are released in greater extent from the fruit epicarp and consequently, they are dissolved in higher amounts in the oil phase (Ranalli et al., 2003). However for Picual oils, prolonging the malaxation time from 20 to 40 min did not influence the triterpenic compounds concentration (Table 4). The same result was obtained by Cert et al. (1999) for the same cultivar but using more extended malaxation times (60, 75, and 90 min).

4. Conclusions

To sum up, there was evidence that the triterpenic fraction of Arbequina and Picual cultivars behaved differently against the different operative conditions applied during virgin olive extraction. In the case of Arbequina oils, the crushing level, malaxation temperature and time influenced at different degrees each one of the studied triterpenoids. We have obtained higher values of oleanolic acid at the highest malaxation temperature 30 °C and for the longest malaxation time 40 min (Table 1) independently of the sieve size diameter, in agreement with ANOVA results (Table 3). Maslinic acid, in addition to showing high concentrations at the highest malaxation temperature and time, displayed higher values when olive fruits were crushed with the smallest sieve diameter (4 mm) for all malaxation times and temperatures assayed (Table 1). Erythrodiol was clearly affected by the sieve size diameter of the hammer crusher and time malaxation, since low concentrations were obtained at a short malaxation time (20 min) and thicker grinding (5 and 6 mm) (Table 1).

Unlike Arbequina, triterpenes in Picual oils were mainly influenced by the malaxing temperature (Table 4), particularly for oleanolic acid, maslinic acid and uvaol showing greater concentrations at higher temperature (30 °C). Maslinic acid content was also influenced by the sieve diameter, being higher for the smallest sieve size (4 mm) at all malaxation times and temperatures (Table 2).

Results of the present research confirm that the genetic factor (cultivar) has a fundamental importance on the final virgin olive oil triterpenic content, as previously reported (Allouche et al., 2009), but also demonstrate that to produce oils with greater triterpenic concentration, the processing variables must be differentiated according to the olive cultivar. Further studies are therefore needed to obtain more precise information for a wide range of olive cultivars.

Acknowledgement

This work has been supported by the INIA Project 'RTA 2008-00066-C03-01', financed by FEDER.

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