LWT - Food Science and Technology 58 (2014) 609–613

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Determination of triterpenic acids in natural and alkaline-treated Greek table olives throughout the fermentation process

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ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form
16 August 2013
Accepted 3 April 2014
Available online 18 April 2014

Keywords:
Maslinic acid
Oleanolic acid
Table olives
Spanish-style
Greek-style

ABSTRACT

Maslinic and oleanolic acids are among the most abundant triterpenic acids found in olive fruits. Both acids are considered to be important bioactive compounds which can confer multiple beneficial health effects to the consumer. In the present work, we have monitored and quantified maslinic and oleanolic acids throughout processing in alkaline-treated green olives (Spanish-style) and in natural green olives of the Conservolea variety that is particularly popular in Greece. Our findings clearly demonstrate that the fast de-bittering process with NaOH treatment in Spanish-style olives has a profound negative effect on the concentration of both acids. This decrease of concentration was more prominent regarding maslinic acid when compared to oleanolic acid. In contrast, the slow de-bittering during natural fermentation of green olives had no effect on the content of maslinic or oleanolic acid. To verify the broad applicability of our observation we have also looked into the natural processing of the Kalamon variety (Greek-style). Our findings were consistent, since once again, natural fermentation did not influence the concentration of both acids.

1. Introduction

For thousands of years, olive oil and table olives have been basic components of the Mediterranean diet. The two most important commercial types of table olives worldwide are the green Spanish-style olives and the black Greek-style olives (Gomez, Garcia, & Navarro, 2006). Processing of Spanish-style green olives includes an initial step of alkaline treatment as a fast de-bittering procedure, followed by rinsing with water and fermentation in brine. Natural black (Greek-style) olives and natural green olives, on the other hand, are directly put in brine for natural fermentation, after having been first rinsed with water.

Table olives are known for their high nutritional value. They contain several nutritional components, which largely depend on the olive variety, the maturation stage of the olive fruit, the cultivating conditions and the processing method (López, García, & Garrido, 2008; López-López, Montaño, Cortés-Delgado, & Garrido-Fernández, 2008; López-López, Rodríguez-Gómez, Ruíz-Méndez, Cortés-Delgado, & Garrido-Fernández, 2009; Romero et al., 2004). Processed table olives are rich in vitamins, they contain reasonable amounts of minerals, while they are well-known sources of phenolic compounds, with the major ones being hydroxytyrosol and tyrosol (Kailis & Harris, 2007, chap. 2; Charoenprasert & Mitchell, 2012). Moreover, the consumption of table olives has been associated with a variety of health benefits which have sometimes been attributed to specific nutritional or bioactive components (Kailis & Harris, 2007, chap. 2).

Pentacyclic triterpenes are found in abundance in a variety of plants (Jäger, Trojan, Kopp, Laszczyk, & Scheffler, 2009) and in the case of olive fruits represent 90–95% of their cuticle lipids (Bianchi, Pozzi, & Vlahov, 1994). More specifically, olive fruits are remarkably rich in maslinic and oleanolic acids throughout their development (Stiti, Triki, & Hartmann, 2007). These triterpenic acids are located in the epicarp of the olive fruit and they constitute the main

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http://dx.doi.org/10.1016/j.lwt.2014.04.005
0023-6438/

substances of the surface waxes (Bianchi, 2003; Bianchi et al., 1994; Guinda, Rada, Delgado, Gutiérrez-Adánez, & Castellano, 2010). Triterpenic acids have been reported to have multiple biological effects both *in vitro* and *in vivo*. There are several studies indicating the anti-inflammatory, antioxidant (Ismaili et al., 2004; Liu, 1995; Tsai & Yin, 2008), antimicrobial (Horiuchi et al., 2007), antiviral (Parra et al., 2009), cardioprotective (Allouche, Beltrán, Gaforio, Uceda, & Mesa, 2010), anti-hypertensive (Rodriguez-Rodriguez, Perona, Herrera, & Ruiz-Gutierrez, 2006), anti-hyperlipidemic (Liu, Sun, Wang, Mu, Liao, & Zhang, 2007), anti-diabetic (Liu, Sun, Duan, Mu, & Zhang, 2007; Tang et al., 2008), and even anti-tumour activities (Hsum et al., 2011; Juan & Planas, 2010; Li et al., 2010; Reyes, Centelles, Lupianez, & Cascante, 2006; Reyes-Zurita, Rufino-Palomares, Lupianez, & Cascante, 2009) of maslinic and oleanolic acids.

In recent years, there is a significant shift in the market for functional foods. Taking into consideration the potential importance of maslinic and oleanolic acids for the consumers' health and consequently the possible role of table olives with high triterpenic acid content as a functional food, it is of particular interest to assess whether processing has an adverse effect on the availability of these acids in the final product. In the present study, we report the influence of Spanish-style and natural processing of green table olives on the concentration of the above triterpenic acids in the Conservolea variety. In order to validate our findings, we also examined the natural black olives (Greek-style) of the Kalamon variety. Our research also aims to shed further light on the differences between Spanish-style and natural processing of table olives.

2. Materials and methods

2.1. Processing and sampling

For the Spanish-style green olives of the Conservolea variety, olive fruits (50 kg) with a green-yellow surface colour were put into high density polyethylene (HDPE) barrels, covered with 30 L of 1.5 g NaOH/100 ml H_2O and maintained in the alkaline solution until the alkali penetrated 2/3 the way to the olive pit (approximately 12 h). The penetration was checked optically using the phenolphthalein indicator. Subsequently, olives were rinsed twice with tap water for 12 h and then covered with brine (8 g NaCl/100 ml H_2O and 0.3 ml lactic acid/100 ml H_2O). Fermentation was performed under anaerobic conditions, using a tight barrel lid, for 4.5 months with addition, at regular time intervals, of NaCl until the concentration in the brine was slightly above 8 g NaCl/100 ml brine. The concentration of NaCl in the brine was determined using a conductivity metre. Initial samples corresponded to raw olive fruits. The next three samples were collected after the alkaline treatment and after each water rinsing. Afterwards, samples were withdrawn periodically until the 18th week of fermentation.

For the natural green olives in brine of the Conservolea variety, olive fruits (50 kg) with a green-yellow surface colour were put into HDPE barrels and covered with 30 L of acidified brine (3.5 g NaCl/100 ml H_2O and 0.15 ml lactic acid/100 ml H_2O). Fermentation was performed under anaerobic conditions, using a tight barrel lid, for 10 months with addition, at regular time intervals, of NaCl until the concentration in the brine was slightly above 8 g NaCl/100 ml brine. Raw olive fruit samples and samples withdrawn periodically until the 40th week of fermentation were examined.

Finally, for the natural black olives of the Kalamon variety, olive fruits with a black surface colour were processed as Greek-style in the same way as it is described above for the natural green Conservolea olives. Samples were taken periodically and examined until the 32nd week of fermentation.

All samples were processed in the Agricultural Cooperative of Rovies and supplied to us through the Central Cooperative Union of Olives and Olive Oil Producers "ELEOURGIKI".

2.2. Extraction of triterpenic acids from the olive flesh

Extraction of triterpenic acids from the olive flesh was performed as described by Romero et al. (2010) with slight modifications. Ten grams of pitted olive fruits were finely chopped, dried at 105 °C until weight stabilization and pulverized. One gram of dry powder olives was mixed with 4 ml of methanol/ethanol (1:1, v/v) in a centrifuge tube, stirred for 1 min, centrifuged at 10,000 × *g* for 5 min at 20 °C and the solvent was separated from the solid phase. This procedure was repeated six times, and the solvent extracts were evaporated under vacuum. Subsequently, the residue was dissolved in 2 ml of methanol and centrifuged at 8500 × *g* for 5 min at 20 °C. The supernatant was filtered through 0.2 μm pore size filter and an aliquot (20 μl) was used for HPLC analysis. The extraction was performed at least in triplicates for each sample.

2.3. HPLC analysis of triterpenic acids

Analysis was performed on an HPLC system consisting of a GBC LC 1150 HPLC pump and a GBC LC 1200 UV/Vis detector (GBC Scientific Equipment Pty Ltd, Dandenong, Victoria, Australia). A Spherisorb ODS-2 (5 μm, 25 × 46 mm i.d.; Waters Inc., Mildford, MA, USA) column was used. Elution was performed at 35 °C (Chemical Electronics 1270 Column Heater) with a mixture of methanol/water (92:8, v/v), acidified with phosphoric acid at pH 3.0, at a flow rate of 0.8 ml/min. The absorbance of the eluate was monitored at 210 nm. Maslinic and oleanolic acids were quantified using standard curves plotted with solutions of pure maslinic and oleanolic acid (Sigma, St. Louis, MO, USA) in a concentration range of 0–3000 mg/l.

2.4. Statistical analysis

Multiple sample comparison was performed with analysis of variance (ANOVA) for $p < 0.05$ followed by the post-hoc Tukey's HSD (Honestly Significant Difference) test as those are implemented in STATGRAPHICS Centurion XV (StatPoint Technologies, Inc., Warrenton, Virginia, VA).

3. Results and discussion

The present study aims to determine the influence of Spanish-style and natural preparations of table olives on two triterpenic acids, maslinic and oleanolic, for the first time throughout the entire process. A good separation of peaks corresponding to the retention times of maslinic and oleanolic acids was achieved by the chromatographic system used (Fig. 1).

We have initially investigated Conservolea olives prepared following the Spanish-style. Samples were withdrawn periodically until the 18th week of fermentation. The alkaline treatment used in this process accelerates the removal of olive fruits' bitterness by the chemical hydrolysis of oleuropein and the fermentation period varies from 3 to 7 months (Corsetti, Perpetuini, Schirone, Tofalo, & Suzzi, 2012; Gomez et al., 2006).

The initial concentrations of maslinic and oleanolic acids in the raw fruits were 1230 ± 108 and 541 ± 44 mg/kg olive flesh, respectively (Fig. 2). The concentrations in the final product (18 weeks of fermentation) were 552 ± 16 and 331 ± 33 mg/kg olive flesh, respectively.

The decrease in the concentration of maslinic acid occurred in three phases (Fig. 2). The first reduction was observed after the 12 h