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Biochemical aspects of olive freezing-damage: Impact on the phenolic and volatile profiles of virgin olive oil

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Tyrosol (PubChem CID: 10393)
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(E)-hex-2-enol (PubChem CID: 54670067)
hexyl acetate (PubChem CID: 8908)
(E)-hex-2-en-1-yl acetate (PubChem CID: 5363374)

ABSTRACT

In this study, the biochemical changes induced by severe freezing of olives of the Picual and Arbequina cultivars were evaluated, paying particular attention to the phenolic and volatile profiles of the oils obtained from them. The content of the main phenolic glycosides, oleuropein and demethyloleuropein, and the activity levels of β-glucosidase and polyphenol oxidase in the olive tissue were severely reduced by freezing. On the contrary, unusually high levels of hydrolytic secoiridoid derivatives were found in freeze-damaged fruits. In both cultivars, the oils obtained from freeze-damaged fruits contained significantly lower levels of secoiridoid compounds, with a 21% and 42% decrease in Picual and Arbequina, respectively. The volatile composition of the oils obtained from Arbequina freeze-damaged fruits were also more strongly affected than that of Picual oils, with a higher reduction in the content of C6-aldehydes, 85% and 65% respectively. The changes observed in the main volatile components were closely related to the changes provoked by freezing in the activity level of the enzymes of the lipoxygenase pathway.

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1. Introduction

Virgin olive oil (VOO) is extracted exclusively from fresh undamaged olive fruit by mechanical or other physical means, and under conditions that do not alter the oil, particularly thermal conditions. Due to this peculiar extraction process, VOO retains a significant amount of biologically active metabolites (e.g., phenolic compounds, tocopherols, sterols and pigments) which enhance its nutritional value (Visioli & Bernardini, 2013), also contributing to its extraordinary flavor.

Phenolic and volatile compounds undoubtedly exert the main influence on the sensory quality of VOO. The biosynthesis of these flavor related compounds takes place mostly during the milling of olive fruits, being the biosynthetic activity strongly limited during the malaxation process (Clodoveo, Hachicha-Hbaieb, Kotti, Mugnozza, & Gargouri 2014). In this sense, despite the fact that processing technology may severely affect VOO quality (Cevik, Ozkan, & Kiralam, 2016) it is quite clear that the composition and biochemical status of the olive fruit are the most important variables defining the quality of the oil. Oleuropein, ligstroside and demethyloleuropein are the main phenolic glycosides in olive fruit, and experimental evidences point to β-glucosidase, polyphenol

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oxidase (PPO) and peroxidase (POX) as the main enzymes involved in the biosynthesis and degradation of phenolic compounds during the extraction of VOO (Romero-Segura, García-Rodríguez, Sanz, & Pérez, 2011). Thus, the hydrolytic derivatives identified as the dialdehydic forms of decarboxymethyloleuropein and ligstroside aglycones (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively), and the aldehyde forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively), are the most abundant phenolic components in most VOOs. In a similar way, it is well stablished that the biosynthesis of VOO aroma occurs through the lipoxygenase (LOX) pathway, comprising mainly the actuation of LOX, hydroperoxide lyase (HPL), alcohol dehydrogenase (ADH), and alcohol acyltransferase (AAT) enzymes, which gives rise to the straight-chain six-carbons (C6) aldehydes, alcohols, and their respective esters identified as the key volatile components of VOO (Olías, Perez, Rios & Sanz., 1993).

Agronomic factors may induce some changes to the biochemical pathways that determine the phenolic and volatile composition of VOOs (Romero & Motilva, 2010, pp. 43–50; Romero, Saavedra, Tapia, Sepúlveda, & Aparicio, 2016b). Thus, severe freezing damage due to an abrupt drop in temperature can induce intracellular freezing in the fruit, affecting the protoplasmic structure, while only indirect damage occurs when ice forms outside of the cells (i.e.: extracellular freezing) (Ruiz-Baena, Lorite-Torres, Gavilan-Zafra, Navarro-Garcia, & Estevez- Gualda, 2007). However, there is little information as to the metabolic changes induced in the olive fruit damaged by freezing, particularly in reference to markers of aroma in VOO (Inarejos-García, Santacatterina, Salvador, Fregapane, & Sanchez-Alonso, 2010; Zhu,H., Wang, & Shoemaker, 2014). Likewise, the phenolic profile of the oils obtained from freeze-damaged olives has not been well characterized. In addition to contributing to a deeper understanding of the biochemistry of plant foods, the biochemical characterization of damaged olive fruits could be a useful complement to the chemical determination of VOO defects and to the sensory evaluation of such defects.

Therefore, the aim of this paper was to evaluate the influence of fruit freezing on the biochemical events related to the synthesis of VOO flavor. For this purpose, the biochemical status of severely freeze-damaged olive fruits was studied paying particular attention to the key biochemical factors involved in phenolic and volatile biosynthesis.

2. Materials and methods

2.1. Plant material

Olive fruits (*Olea europaea* cv. Picual and Arbequina) were cultivated at the experimental fields of Instituto de la Grasa and collected at an average maturity index of 2.5 (turning stage). Olive fruits (6 kg) were immediately transported to the lab, randomly distributed and placed in perforated plastic boxes. To evaluate the biochemical changes induced by intracellular ice crystal formation caused by a rapid freezing (Levitt, 1980), fresh harvested olives were subjected to a simulated fast freezing process in the lab. Fruits (3 kg) were placed in a laboratory freezer (−18 °C) for three days and then subjected to a gradual thawing process (24 h at 5 °C plus 5 h at room temperature) prior to fruit analysis and oil extraction. Control fruits (3 kg) were kept 24 h at 5 °C and 5 h at room temperature.

2.2. Chemicals

Reagents to assess enzyme activity, for enzyme extraction and other measurements were supplied by Sigma-Aldrich (St. Louis, MO, USA). Phenolic compounds were purchased from Extrasynthese (Genay, France).

2.3. Olive oil extraction

Olive oil was extracted using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates the industrial process of VOO production on a laboratory scale (Martínez, Muñoz, Alba, & Lanzón, 1975). As previously mentioned control and freeze-damaged fruits were processed after 5 h conditioning to room temperature. Two batches of 1 kg olive fruits were processed per each treatment. Abencor processing parameters have been precisely described in a previous study (Sánchez-Ortiz, Romero-Segura, Sanz, & Pérez, 2012b).

2.4. Enzyme activities

The activity levels of different enzymes related to the synthesis of phenolic and volatile compounds that characterize VOO were measured three times in each of the two extracts prepared for each of them as described below. All enzyme activities were expressed as nano katal per gram of fruit (*nkat*/g FW).

LOX activity was extracted from fresh olive pulp as described previously (Luaces, Pérez, García, & Sanz, 2005). LOX activity was determined at 25 °C, using linolenic acid as the substrate, and monitoring the increase in absorbance at 234 nm ($\varepsilon = 2.5 \times 10^4$ mol^{-1} L cm^{-1}) due to the conjugated double bonds formed by hydroperoxidation of this acid.

HPL activity was extracted from fresh olive pulp as described elsewhere (Sánchez-Ortiz, Pérez, & Sanz, 2013). HPL activity was determined spectrophotometrically at 25 °C, using 13-hydroperoxylinolenic acid as the substrate and quantifying its degradation by measuring the decrease in absorbance at 234 nm as described for LOX.

ADH was extracted from acetone powders prepared from fresh olive pulp as described by Sánchez-Ortiz et al. (2012a) and its activity was measured by monitoring the reduced nicotinamide adenine dinucleotide (NADH) oxidation at 338 nm using acetaldehyde as the co-substrate ($\varepsilon = 6160$ mol^{-1} L cm^{-1}).

AAT enzyme extracts were prepared from 0.1 g of acetone powders obtained from fresh olive seeds, diluted in 3 mL of extracting buffer (50 mmol/L Tris-HCl, pH 8.0, 7 mmol/L DTT, 100 g/L glycerol and 2 g/L Triton X-100). AAT activity was assayed as described previously (Olías, Pérez, & Sanz, 2002) with minor modifications, using 5,5′-dithiobis nitrobenzoic acid (DTNB) as the substrate.

POX activity was extracted from olive seeds (0.5 g) and determined spectrophotometrically by following guaiacol oxidation at 470 nm ($\varepsilon = 26600$ mol^{-1} L cm^{-1}) through the method described elsewhere (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011).

PPO extracts were prepared from acetone powders as described by García-Rodríguez et al. (2011). PPO activity was determined by constantly monitoring the increase in absorbance at 400 nm due to the oxidation of *tert*-butylcatechol (TBC).

β-Glucosidase activity was extracted from acetone powders and determined spectrophotometrically by following the hydrolysis of the synthetic glucoside *p*-nitrophenyl-β,D-glucopiranoside (pNPG) at 405 nm (Romero-Segura, García-Rodríguez, Sánchez Ortiz, Sanz, & Pérez, 2012).

2.5. Extraction and analysis of fruit and VOO phenolic compounds

Fruit phenolic compounds were extracted according to a previously developed protocol (García-Rodríguez et al., 2011). Representative fruits samples (1 g) were kept at 4 °C for 24 h in dimethyl