Biochemical Changes in Phenolic Compounds during Spanish-Style Green Olive Processing

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Phenolic compounds have been determined by HPLC in the flesh of olives and brines of two cultivars (Manzanilla and Hojiblanca) during Spanish-style green olive processing. The NaOH treatment hydrolyzed oleuropein into hydroxytyrosol (3,4-dihydroxyphenylethanol) and elenolic acid glucoside. After a rapid diffusion of hydroxytyrosol from the flesh of fruits into the brines, the concentration of the *o*-diphenol remained practically constant throughout the lactic acid fermentation step. In contrast, elenolic acid glucoside increased in concentration in the olive brines during the first few days of brining but disappeared after 100–200 days. Other minor phenolic compounds also diffused rapidly into the olive brines during the first few days of brining. Caffeic acid, oleuropein, and *p*-coumaric acid diminished in concentration throughout the fermentation period, whereas the concentration of tyrosol (4-hydroxyphenylethanol) remained constant.

Keywords: *Green olives; polyphenols; fermentation*

INTRODUCTION

Within the types of commercial table olives, Spanish-style green olives account for ca. 40–50% of the world production. The method of processing consists of a treatment with dilute NaOH solution (lye) to debitter the olives, followed by one to three rinse cycles to eliminate the excess alkali. Finally, fruits are placed into sodium chloride brine in which a lactic acid fermentation takes place (Fernández, 1991).

The fermentation rate is influenced by the presence of inhibitors in the brines. These inhibitors are phenolic compounds, mainly oleuropein and products of its hydrolysis (Juven et al., 1972; Fleming et al., 1973; Ruiz et al., 1993). The antimicrobial activities of these compounds have been shown to affect other microorganisms as well as lactobacilli (Tassou and Nychas, 1994).

In the olive fruit, the main phenolic compound is oleuropein, a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol (Amiot et al., 1986). Other phenolic compounds isolated in the olive fruit are demethyloleuropein, rutin, luteolin 7-glucoside, verbascoside, ligstroside, and elenolic acid glucoside (Vázquez et al., 1974; Kubo and Matsumoto, 1984; Amiot et al., 1989).

These compounds and products of their hydrolysis are important in Spanish-style green olive processing not only due to their antimicrobial action but also due to their effects on the color of brines and olives. Thus, *o*-diphenols may oxidize in alkaline conditions and darken fruits and brines (Brenes et al., 1988, 1992).

Moreover, there is great interest in these compounds due to their antioxidative properties (Chimi et al., 1991; Tutour and Guedon, 1992) and their repellent effects on the fly *Dacus oleae* (Scalzo et al., 1994).

However, limited data are available on the biochemical changes in these polyphenols during Spanish-style green olive processing (Gaviña et al., 1973; Vázquez and Janer del Valle, 1977). It has been reported that 3,4-dihydroxyphenylethanol (hydroxytyrosol) is formed during the NaOH treatment (Vázquez and Janer del Valle, 1977; Amiot et al., 1990), but there are no data on other products of the alkaline hydrolysis of oleuropein.

The aim of the present study was to investigate the biochemical changes that occur in the phenolic compounds, particularly oleuropein and products of its hydrolysis, during Spanish-style green olive processing.

MATERIALS AND METHODS

Olives. Experiments were carried out with fruits (*Olea europaea* L.) of the Manzanilla and Hojiblanca cultivars. Olives with a green-yellow surface color were picked during the 1993/1994 and 1994/1995 seasons and graded to eliminate leaves and small fruits.

As is customary in the industrial procedure, olives were stored for 24 h (21 ± 2 °C) before processing to avoid sloughing of fruits when treated with NaOH.

Thirty kilograms of olives was placed in cylindrical PVC vessels and covered with 20 L of 0.48 M NaOH solution. The alkaline treatment lasted for 7–9 h at room temperature (21 ± 2 °C) until the lye penetrated two-thirds of the distance to the pit, tested with phenolphthalein solution. The NaOH solution was then poured off, and the olives were washed in running water for 12–17 h.

After the washing step, olives were covered with a 1.9 M NaCl solution and left for 7 months at 25 ± 3 °C to undergo the characteristic lactic fermentation process.

Polyphenol Analysis. Approximately 2–5 g of olive flesh from 10 olives was mixed in duplicates in a blender with 100 mL of 80% (v/v) ethanol, containing 400 ppm of sodium metabisulfite. The mixture was agitated for 20 min and filtered. This extraction was repeated successively three times with the same volume of ethanol and acetone at −30 °C. The extracts were collected and the organic liquids evaporated under vacuum. Four successive hexane extractions allowed removal of pigments and most of the lipids. The phenolic compounds were extracted with ethyl acetate. After six successive extractions, the ethyl acetate was evaporated under vacuum and the dry residue dissolved in 2–10 mL of methanol/water (1:1).

Samples of the brines were diluted (1:8) with distilled water, and after centrifugation, they were injected into the chromatograph.

Detection and quantification of phenols was carried out by HPLC in a Waters 600 E (Millipore, Inc.) apparatus equipped with a diode array detector (Waters 994). The chromatographic conditions have been described elsewhere (Brenes et al., 1993).

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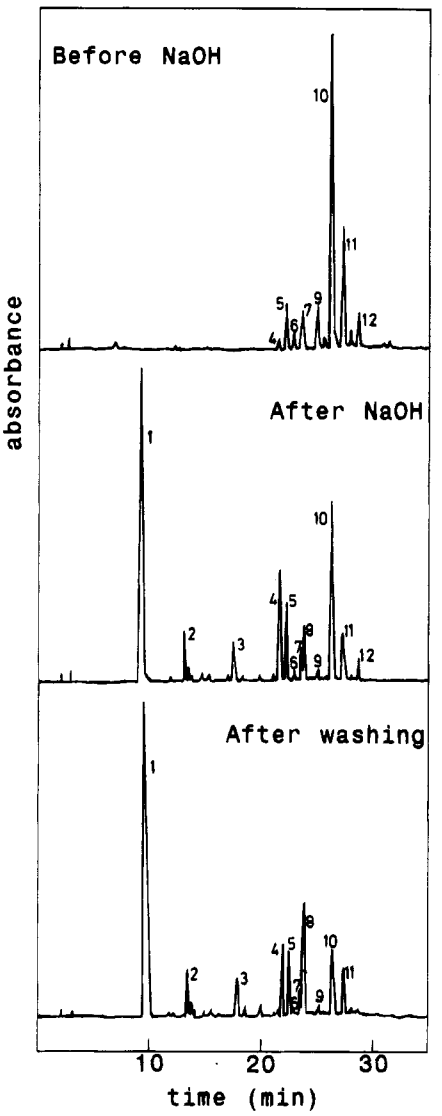

Figure 1. HPLC chromatograms of the phenolic extracts from olive flesh before and after NaOH treatment and after washing. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, caffeic acid; 4, *p*-coumaric acid; 5, verbascoside; 6, rutin; 7, luteolin 7-glucoside; 8, unidentified; 9, unidentified; 10, oleuropein; 11, unidentified; 12, unidentified. Detection was at 280 nm. Fruits were of the Manzanilla cultivar picked during the 1994/1995 season.

Phenolic compounds were identified by their retention times and absorption spectra in the 200–380 nm range. The structure of elenolic acid glucoside was determined by NMR and MS.

Quantification of elenolic acid glucoside was made using pure compound isolated with a semipreparative HPLC column (Spherisorb ODS-2, 5 μm, 25 cm × 1 cm i.d.).

Free Acidity. Free acidity was determined by titration with a Metrohm 670 Titroprocessor up to pH 8.3 with 0.2 M NaOH and expressed as percent (w/v) of lactic acid.

RESULTS AND DISCUSSION

Polyphenol Transformations. The main phenolic compound found in the flesh of Manzanilla and Hojiblanca cultivar olives was oleuropein (Figure 1), in agreement with previous studies on other cultivars (Vázquez et al., 1974; Amiot et al., 1986). Rutin, luteolin 7-glucoside, *p*-coumaric acid, and verbascoside (a heterosidic ester of caffeic acid and hydroxytyrosol) were also present. Some of the peaks, numbers 8, 9, 11, and 12, were not consistently found in both cultivars or in the two seasons. These were not, therefore, identified during this work, although some of their characteristics could be of interest for other purposes. Thus, peak 11 was detected only in the Manzanilla cultivar, but further research is needed in this field.

Sodium hydroxide treatment causes the hydrolysis of glucosides, but in Spanish-style green olive processing the NaOH is permitted to reach only about two-thirds of the distance from the skin to pit. After the immersion of fruits in the NaOH solution, an abundant formation of hydroxytyrosol was detected in both cultivars owing to oleuropein and verbascoside hydrolysis. In fact, the hydroxytyrosol content is strongly related to the extent to which NaOH penetrates into the olive flesh, and its determination has been proposed as a method for monitoring the debittering step (Amiot et al., 1990).

Caffeic acid from verbascoside hydrolysis was detected in the flesh of both cultivars after the NaOH treatment. Tyrosol (4-hydroxyphenylethanol) was also found in both cultivars. This compound may have arisen from the hydrolysis of ligstroside, a heterosidic ester of tyrosol and elenolic acid, identified in olive flesh and seeds (Kubo and Matsumoto, 1984; Vázquez et al., 1974). The rutin and luteolin 7-glucoside content in the olive flesh diminished with the alkaline treatment, and the former compound had practically disappeared after the washing step. Peaks 9, 11, and 12 followed a similar trend. In contrast, the concentration of *p*-coumaric acid increased during these alkaline steps. The residual level of oleuropein after the NaOH treatment was high since this solution penetrated only two-thirds of the way into the olive flesh.

Phenolic chromatograms from the NaOH solution and rinse waters (data not shown) had a pattern similar to those of the olive flesh. Rinsing caused diffusion of polyphenols from the flesh into the rinse waters and some of the chemical reactions continued in these alkaline media. Thus, longer washing periods produced lower oleuropein concentrations.

Hypothesis of Alkaline Oleuropein Hydrolysis during Spanish-Style Green Olive Processing. When polyphenol detection was carried out at 240 nm, the chromatogram at day 1 of brining showed a major peak with a retention time of 17 min (Figure 2). The absorbance spectrum of this peak between 200 and 380 nm was similar to that observed by other authors for elenolic acid, with a maximum absorbance at 240 nm (Montedoro et al., 1993). The substance was isolated on a semipreparative HPLC column, and its identification as elenolic acid glucoside was carried out by NMR and MS; these data were in agreement with those reported in the literature (Amiot et al., 1989; Damtoft et al., 1992). A compound with a rather similar structure named oleoside has been identified in the leaves of *O. europaea* (Gariboldi et al., 1986). This compound differs from the elenolic acid glucoside in that the two carboxylic groups of the molecule are esterified. The presence of elenolic acid glucoside in olives has also been reported by Amiot et al. (1989), who found that its content increased as maturation progressed. However, we have not found the elenolic acid glucoside in fresh Manzanilla or Hojiblanca cultivar olives used in the experiments reported here. This substance is either absent from these cultivars altogether or absent from fruits at the maturation stage used here. The appearance of elenolic acid glucoside after the NaOH treatment was therefore a consequence of the alkali treatment. The alkaline hydrolysis of oleuropein can, therefore, be

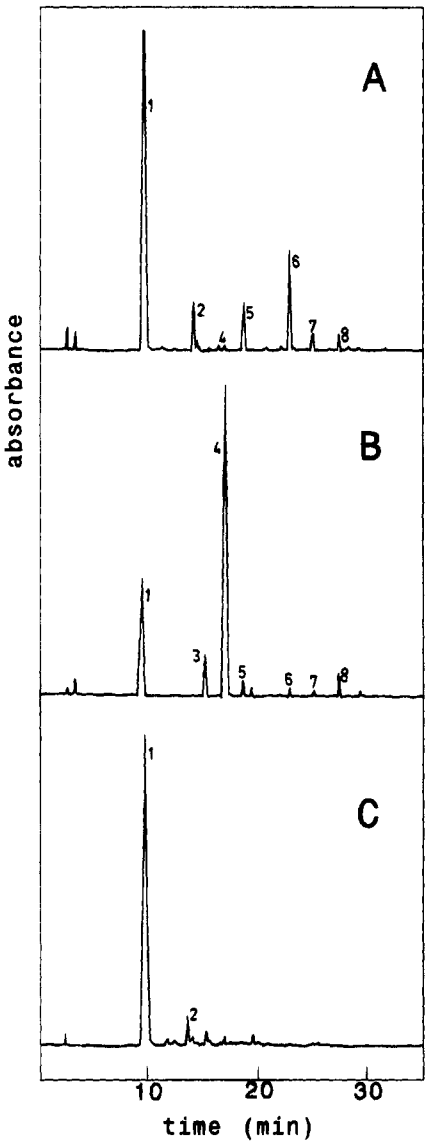

Figure 2. HPLC chromatograms of phenolic compounds in olive brines: (A) day 1 of brining, detection at 280 nm; (B) day 1 of brining, detection at 240 nm; (C) day 80 of brining, detection at 280 nm. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, unidentified; 4, elenolic acid glucoside; 5, caffeic acid; 6, *p*-coumaric acid; 7, luteolin 7-glucoside; 8, oleuropein. Fruits were of the Manzanilla cultivar picked during the 1994/1995 season.

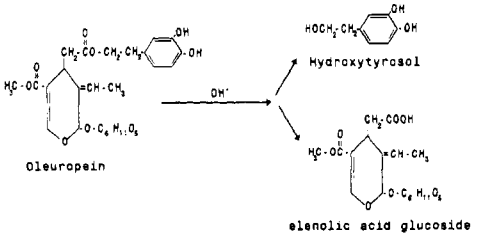

Figure 3. Structures of oleuropein and its alkaline hydrolysis products.

described according to the scheme shown in Figure 3. The ester bond between elenolic acid glucoside and hydroxytyrosol was the only one broken in the oleuropein molecule during the NaOH treatment.

Olives after NaOH treatment have no bitter taste. It follows, therefore, that the elenolic acid glucoside also has a nonbitter taste, as do other oleuropein hydrolysis products, hydroxytyrosol and elenolic acid (Walter et al., 1978).

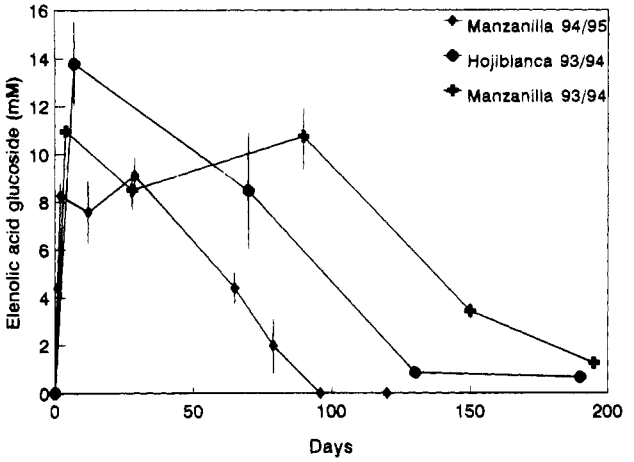

Figure 4. Evolution of elenolic acid glucoside concentration in olive brines during fermentation. Fruits were of the Manzanilla cultivar picked during the 1993/1994 and 1994/1995 seasons and the Hojiblanca cultivar picked during the 1993/1994 season.

Polyphenol Changes during Fermentation. The major phenolic compounds and the products of their hydrolysis in olive brines were hydroxytyrosol, elenolic acid glucoside, and tyrosol (Figure 2). Fermentation markedly affected the changes in the polyphenol concentrations in olive brines. The initial concentration of elenolic acid glucoside was high, as indicated by the high concentration of its precursor in olives (oleuropein). After equilibrium, which was reached in a few days, its concentration reached 8–14 mM (Figure 4). However, the concentration of this compound sharply decreased as fermentation progressed and practically disappeared after 100–200 days.

The mechanism of elenolic acid glucoside degradation in brine is still unknown, but it must involve not only the glycosidic bond breaking but also other transformations of the elenolic acid ring since the acid was not detected in brines. In any case, if glucose is released during the degradation of elenolic acid glucoside, the amount of sugar produced could contribute to a high proportion of the total acidity produced during fermentation. Thus, not only were the sugars, mainly glucose, and organic acids diffused from the flesh of the olives into the brines as substrates of the lactic acid fermentation (Montaño et al., 1993), but also glucose from the elenolic acid glucoside might have played an important role in the progress of fermentation. It has been reported that in vitro β-glucosidase from *Lactobacillus plantarum* may break the glycosidic bond of oleuropein (Ciafardini et al., 1994). Acid hydrolysis of oleuropein in directly brined olives without *L. plantarum* growth has also been demonstrated (Brenes et al., 1993). Thus, the hydrolysis of the elenolic acid glucoside during Spanish-style green olive processing may occur by various mechanisms, and research is underway to elucidate its transformation.

The changes in the concentration of hydroxytyrosol during fermentation are given in Figure 5. There was a rapid accumulation of this compound during the first few days of brining due to its diffusion from the olives into the surrounding brines. Subsequently, its concentration remained practically constant throughout fermentation. This *o*-diphenol is easily oxidized in alkaline conditions (García et al., 1992). The high pH values during NaOH treatment, rinsing, and the first few days of brining can favor this polymeric reaction and give a certain color to the brines (Brenes et al., 1988). As

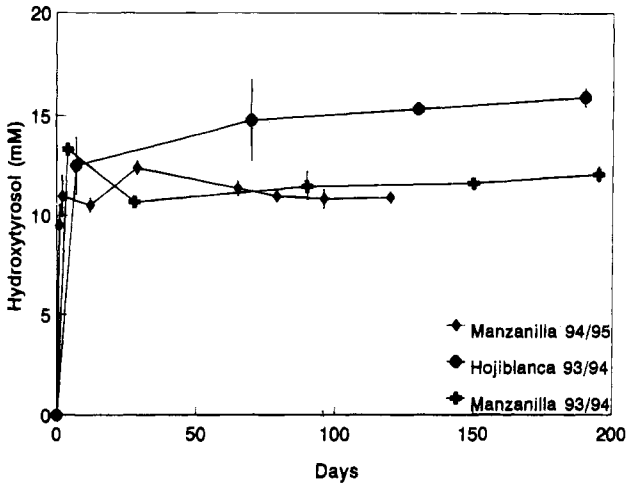

Figure 5. Evolution of hydroxytyrosol concentration in olive brines during fermentation. Fruits were of the Manzanilla cultivar picked during the 1993/1994 and 1994/1995 seasons and the Hojiblanca cultivar picked during the 1993/1994 season.

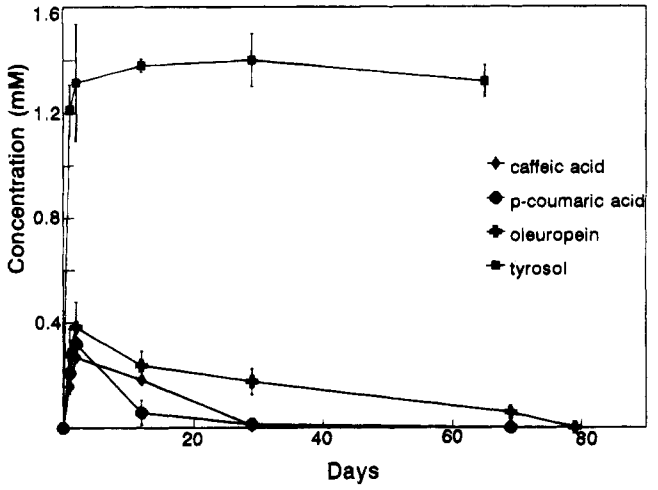

Figure 6. Evolution of tyrosol, caffeic acid, *p*-coumaric acid, and oleuropein concentrations in olive brines during fermentation. Fruits were of the Manzanilla cultivar picked during the 1994/1995 season.

fermentation progressed, anaerobic conditions and the use of oxygen by microorganisms limited the oxidation of hydroxytyrosol and, consequently, its concentration remained constant.

Luteolin 7-glucoside was detected in brines only during the first few days of the fermentation process and rapidly disappeared after 4–7 days (data not shown). The concentrations of other phenols also studied are shown in Figure 6. All of them diffused rapidly into the brines during the first few days. Caffeic acid, *p*-coumaric acid, and oleuropein reached moderate levels and slowly disappeared during fermentation. Caffeic acid was absent from the brines of both cultivars after 30–40 days; *p*-coumaric acid was detected in very low concentrations from day 30 to day 50, and oleuropein disappeared after 80 days (1994/1995 season) or 30–60 days (1993–1994 season). The mechanisms of their degradation are not clear, although the acidic conditions formed during fermentation may contribute.

Finally, another major phenolic compound found in olive brines was tyrosol (Figure 6). The concentration of this compound increased sharply after brining and then remained constant. This indicates that this compound was formed during the alkaline steps of Spanish-style green olive processing and not during fermentation, as has been suggested in wine processing by the growth of microorganisms (Castino, 1988).

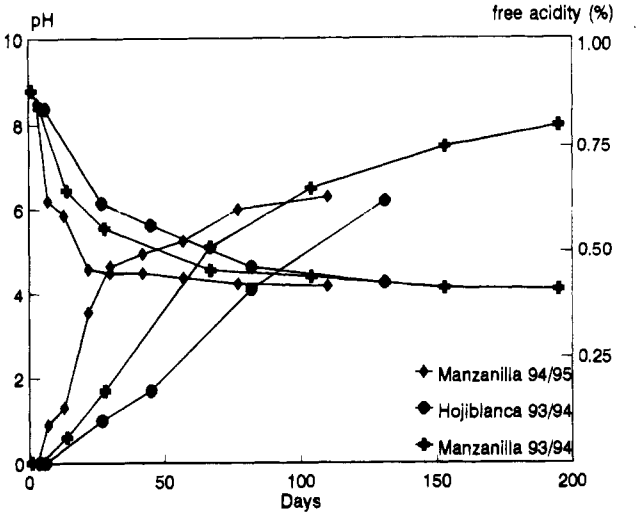

Figure 7. Evolution of pH and free acidity (percent w/v) in olive brines during fermentation. Fruits were of the Manzanilla cultivar picked during the 1993/1994 and 1994/1995 seasons and the Hojiblanca cultivar picked during the 1993/1994 season.

Changes in the pH and free acidity, expressed as percent of lactic acid, of brines during fermentation are shown in Figure 7. In general, the pH sharply decreased during the first 20–30 days of fermentation. Thereafter, the rate of decrease moderated until around day 100 of brining. Subsequent changes in pH were minimal. This decrease in the pH of the brines was due to the formation of lactic acid during fermentation in both seasons and cultivars assayed. The progressive increase in the acidic conditions of the medium could have caused the phenol transformations described previously. Thus, a certain relationship between the decrease in elenolic acid glucoside and the diminution of the pH of brines can be observed. Research is underway to elucidate the mechanism by which this compound disappears during fermentation, taking into account the high concentrations in which this compound is found in Spanish-style green olive brines.

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