Pectinesterase and Polygalacturonase in Changes of Pectic Matter in Olives (cv. Hojiblanca) Intended for Milling

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ABSTRACT: Virgin olive oil is a highly valued product, and it is important to optimize extraction yield. The pectic composition and the related enzymatic activities, present in the raw material, are variables that may affect that process. The pectinolytic activities producing modifications in the pectic matter of olive fruits (variety Hojiblanca) during ripening and the associated changes in texture were studied. Pectinesterase (PE) activity increased with ripeness until reaching a peak when anthocyanin synthesis in the fruit became marked (turning color stage). From then on, it decreased. In contrast, polygalacturonase (PG) activity in the ripe-green fruit decreased sharply when anthocyanin formation began (small reddish spots stage) and then increased, reaching a maximum in the ripe-black fruit. Parallel changes were noted in the texture and pectic content of the olives, related to endo- and exo-PG enzymatic activities, together with a decrease in the degree of esterification of the pectic matter, which could be associated with PE action. The distribution of the pectic fractions in the raw material and the changes in them during the olive oil extraction process showed the role of PE and PG in the fruits that was related to the yield of extracted oil.

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KEY WORDS: Oil extraction, olive, pectin, pectinesterase, polygalacaturonase, texture.

Virgin olive oil is a natural product obtained exclusively by mechanical extraction. The olives are milled, and the resulting paste is beaten and then centrifuged to separate the oil from the solid residue, which includes the plant water, and is known as alperujo. Much research has been devoted to maximizing the oil olive extraction yield in industry and searching for coadjutants that alter the surface tension without causing changes in the chemical composition of the oil. The works of Alba-Mendoza *et al.* (1,2) contain a list of approved technological coadjutants and additives for the preparation of edible plant oils. It is currently very common to add talc during the industrial extraction of olive oil as a coadjutant to increase process yields. Other studies with the same aim can be found on the use of enzymes during olive oil extraction processes (2,3) enabling increased extraction yields and obtaining oils with composition and characteristics similar to those from untreated materials (4) or even of higher quality (3). The enzymatic preparations used in these studies have been mainly pectinolytic, hemicellulolytic, and cellulolytic activities. These are appropriate for degrading plant cell walls, composed mainly of polysaccharides. In order to determine the best enzyme mixture to increase olive oil extraction yields, Huisman *et al.* (5) characterized the cell wall polysaccharides of olives (variety Frantoio) in three states of ripeness. They found that the changes in cell wall composition during olive ripening are mainly due to pectins.

The pectins are natural polymers based on polymerized galacturonic acid partially esterified with methanol. These polymers may also be considered copolymers, due to the existence of regions linked to neutral sugars. Chemical changes in the pectic matter cause the greatest alterations in cell structure, and hence in texture, during ripening and processing of numerous fruits. In the final softening of papaya, the most important factor is pectin hydrolysis, although alteration of hemicellulose is also involved (6). Softening of mango flesh during ripening is the result of cell wall disintegration caused by degradation of structurally linked pectin (7). During tomato ripening, the pectins are the most altered polysaccharides (8).

The cell wall enzymes mainly attributed to the changes taking place in the pectic composition of fruits during ripening and processing are pectinesterase (PE) and polygalacturonase (PG) (7,9,10). PE catalyzes the hydrolytic de-esterification of pectins causing pectic chains with a lower degree of esterification. The results of PE action are varied and important in plant development, above all during ripening because of its involvement in changes of fruit texture and in plant–pathogen interaction. *In vivo*, de-esterified pectins can bond with calcium ions to form gels, as happens in peaches. This causes thickening of the cell wall and reduces the amount of juice that can be extracted (11). Taylor *et al.* (12) observed the same phenomenon in plums and established a correlation between fruit texture and PE activity. In frozen cherries, Alonso *et al.* (9) related PE activity with an increase in texture (= firmness), as a result of forming calcium bridges between adjacent chains of de-esterified pectins.

PG hydrolyzes of the α-1,4-glycosidic bonds of the galacturonic acid units forming the pectins. The involvement of PG in the softening process usually associated with fruit ripening has been widely discussed, and much evidence has been reported. Many factors could explain such varied results (13): different methods of measuring texture; the presence of different isoenzymes in the fruits; a great variety of assay conditions; and, above all, the use of PG extraction methods unable to completely separate the enzyme linked to the cell wall. Endo-PG is responsible for the softening that takes place during fruit

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ripening, by depolymerization of the middle lamella (14,15). Many researchers have found correlations between fruit softening and increased PG activity, such as in carambola fruit (10), persimmon and jujube fruits (16), and kiwi (17).

In fruit tissues, adjacent cells are linked by the pectin-rich middle lamella. Tissue weakening implies cell separation and rupture and the release of the cell contents, giving the fruits juiciness (18). An important potential factor in increasing or reducing the ease of oil recovery from olive paste is the pectic composition, mediated by the presence of pectinolytic enzymes. The aim of our work was to study the involvement of PE and PG during olive ripening and to determine its relationship with the pectic composition of the fruits during the fruit-picking period of olives intended for milling and the yield of virgin olive oil obtained from them. Our interest in these enzymes lies in their direct relationship with the increase in soluble pectins and the consequent softening that accompanies fruit ripening. An attempt was made to establish whether the level of intrinsic pectinolytic activity of the olives, depending on degree of ripeness, on the protopectins forming part of the cell wall of the fruits is directly related to higher oil recovery.

MATERIALS AND METHODS

Raw material. The study was carried out on olives (*Olea europaea arolensis;* variety Hojiblanca). The fruits were picked from trees of the Servicio de Extensión Agraria in Cabra (Córdoba, Spain) (field experiment) or were supplied by an olive oil extraction company (industrial experiment).

Sampling. (i) Field experiment. The fruits were picked from all around the tree, until a sample of approximately 1 kg had been collected. On each date, 100 olive fruits were selected at random, and the most representative color at that moment was evaluated according to the ripeness index (19). The sequence of color change is as follows: ripe-green, small reddish spots, turning color, purple, black, and ripe-black. Sampling was done at 7-d intervals from the end of November to the end of January. The samples were monitored for pectinolytic activity of the fruits, together with the modifications produced in their pectic matter by the enzymes and the associated changes in texture.

(ii) Industrial experiment. The studies were carried out on samples supplied weekly from the middle of December to the end of January by the olive mill Cooperativa Sor Angela de la Cruz of Estepa (Seville, Spain). Both the olives received by the mill and the alperujos left after oil extraction from the fruits were analyzed. During processing, the fruits were milled and then beaten. In the latter phase, talc was added as coadjutant. Lastly, the resulting mass was centrifuged, giving two products: oil and alperujos (a paste comprised of mainly the solid and aqueous parts of the fruit).

The fat and pectic fraction contents were determined in both olives and alperujo. The ripeness states of the olives supplied from the mill ranged between turning color and black, the latter predominating as the date of picking became later. The percentage of fruits is displayed in each ripeness state in the samples analyzed (Table 1).

Preparation of enzymatic extracts. Enzymatic extracts were obtained following the method described by Mínguez-Mosquera (20) and based on those proposed by Rouse and Atkins (21) for PE and Bell *et al*. (22) for PG. Destoned fruit (50 g) was sliced, and 100 mL of 0.15 M Na-acetate buffer (pH 4.5), NaCl (2 or 10% for PG and PE, respectively) was added. The mixture was homogenized in a polytron (Ultra-Turrax, Model T-25; Janke & Kunkel, Ika-Labortechnik, Staufen, Germany) for 1 min, stirred continuously for 2 h at 5°C, and then filtered through gauze. The filtrate was the active extract. The presence of polyphenols, inhibitors of PG, in the crude enzymatic extract necessitated a partial purification by dialysis. The active enzymatic extract was placed in a semipermeable cellophane tube (12,000 Da cutoff, D-9527; Sigma, St. Louis, MO) and dialyzed against distilled water for 72 h, replacing the external aqueous phase four times. The extract was then centrifuged at 13,000 × *g* for 20 min. The initial salt concentration was restored to the supernatant, which was used as the enzymatic extract for the measurement of activity. The enzymatic extracts were obtained in duplicates for all samples, and the enzymatic activities were determined three times for each extract.

Determination of PE activity. PE activity was detected at constant pH (pH = 7.6) with 0.05 N NaOH (Merck, Darmstadt, Germany), using an automatic titration apparatus. Measurement was carried out using 50 mL of substrate/1% pectin solution (Sigma; P-9561) in 0.2 M NaCl, previously adjusted with 0.5 N NaOH solution to a pH close to that for measuring activity. Enzyme solution (5 mL) was added, and the pH was rapidly adjusted to that for measurement. The reaction time was 30 min at 30ºC. Controls were prepared by adding the same volume of inactivated enzyme solution (heated at 100ºC for 5 min) to the substrate. The units of PE activity were defined as the number of microequivalents of carboxyl groups hydrolyzed per minute and per gram of destoned fruit.

Determination of PG activity. The reaction mixture was obtained by mixing 5 mL of dialyzed extract and 25 mL of 2% pectin (Sigma; P-9135) in acetic-acetate buffer (Panreac, Barcelona, Spain) (pH = 4.5). A few drops of toluene were added to inhibit microbial growth. The mixture was incubated at 30ºC for 72 h. A control was prepared by adding 5 mL of the inactivated enzyme extract (heated at 80ºC for 20 min) to 25 mL of pectin. PG activity was expressed as the percentage of the decrease in viscosity of the active extract (A) compared with the control (C), according to Equation 1:

TABLE 1
Ripeness Stage of the Olives Supplied from the Mill

		Ripeness stage (%)			
Sample	Harvest date	Ripe-green	Turning color	Purple	Black
1	12/21/99	1.0	13.4	61.5	24.1
2	12/28/99	6.9	5.9	74.3	12.9
3	1/4/00	6.7	20.0	62.7	10.7
4	1/11/00	0.0	7.1	67.0	25.9
5	1/18/00	0.0	0.0	59.8	40.2
6	1/25/00	0.0	0.0	55.0	45.0

$$\%\ \text{decrease of viscosity}\ (\eta) = \frac{\eta(C) - \eta(A)}{\eta(C)} \times 100 \quad [1]$$

where $\eta(A)$ = viscosity of active extract; $\eta(C)$ = viscosity of control extract.

Determining the degree of esterification of the pectic matter. The alcohol-insoluble solids (AIS) were prepared according to the method of Gee *et al.* (23). The AIS were obtained from 400 g of destoned olives milled in a polytron with 1.2 L of 95% ethanol, followed by extraction with 800 mL of ethanol acidified to inactivate the enzymes. The residue was mixed with 800 mL of 70% ethanol until the chlorides were eliminated and then with acetone until the washings were colorless. The residue was left to dry at room temperature.

The degree of esterification was measured as a percentage, assaying the methanol liberated from the pectic matter (23). This consisted of an initial evaluation of the free carboxyl groups in the AIS using 0.1 N NaOH, and then, after saponifying the ester groups of the resulting suspension and neutralizing, the evaluation with 0.1 N NaOH was repeated. The presence of acetyl groups was determined by colorimetry directly on the AIS according to the method of McComb and McCready (24).

Pectin fractionation and analysis. For pectin fractionation and analysis, the method previously described by Levi *et al.* (25) was used. The AIS were prepared by homogenizing 250 g of destoned olives or alperujo in a polytron four times in succession with 300 mL of 70% ethanol each time, followed by two extractions with 300 mL of acetone. The residue was dried at room temperature. AIS (200 mg) were used for extracting all pectic fractions. For extracting soluble pectins (SP), samples were vigorously stirred for 10 min with 20 mL of distilled water and then centrifuged at $27{,}000 \times g$ for 15 min. This procedure was repeated four times. The supernatants with the SP fraction were pooled, and the pellet was subjected to calcium pectate (CaP) extraction following the same procedure with a solution containing 0.1 M buffer Tris/HCl and 0.2% EDTA at pH 6.2. The supernatants were collected for CaP determination. The final pellet was extracted once for protopectin (PP) with 50 mL of 0.05 M NaOH. The amount of each pectic fraction was assessed colorimetrically (26), with a spectrophotometer, for its galacturonic acid (GA) content. The extraction of AIS was carried out in duplicate for all samples, and each pectic fraction was assayed three times for its GA content.

Texture measurement. The fruit texture was measured using a texturometer (Model 1011; Instron, Canton, MA), fitted with a Kramer shear-press cell. The shear test was performed on 10 destoned olives from each harvested sample. The operating speed was set at 200 mm/min and the force scale was 0 to 500 N. The results given were the means of 10 repetitions.

Fat content (FC) and oil extraction yield of olives. FC of olives and alperujos were determined according to a standard method (27). Dry and ground samples (10 g) were extracted with hexane in a Soxhlet extractor for 5 h, and the results were expressed as percentage of dry weight. The oil extraction yield (OEY) was calculated according to Equation 2:

$$\text{OEY}(\%) = \frac{\text{FC(O)} - \text{FC(A)}}{\text{FC(O)}} \times 100 \quad [2]$$

where FC(O) = fat content of the whole olive; FC(A) = fat content of the alperujo.

RESULTS AND DISCUSSION

Field experiment. Figure 1 shows the changes in enzymatic activity of PE and PG present in the olives during the ripening period. PE was present throughout ripening, with its activity in the fruits increasing progressively to a maximum between the turning color and purple states. PE activity then fell sharply to approximately 50% and subsequently disappeared. PG followed a different pattern. The PG activity of the ripe-green olive decreased sharply on reaching the turning color state, and then increased slightly, although the level of activity was lower than that measured in the green fruit. Later, there was a marked increase of enzymatic activity in the ripe-black fruit that reached a maximum, around 95% reduction in viscosity. At the same time, during the different stages of ripening, the fruits lost texture, accompanied by a decrease in pectin content and degree of esterification. These changes were not uniform with time (Table 2).

In a first stage, the step from ripe-green to small reddish spots, the loss of texture was appreciable (16.1%), and coincided with the sharpest decrease in fruit pectin content (39.7%). These changes could be related to the PG activity detected in the ripe-green fruit. The implications of PG in the depolymerization of the pectic chain of a fruit not yet fully ripe are relatively recent. In 1982, Brady *et al.* (28) detected three isoforms of endo-PG during ripening in the tomato and estab-

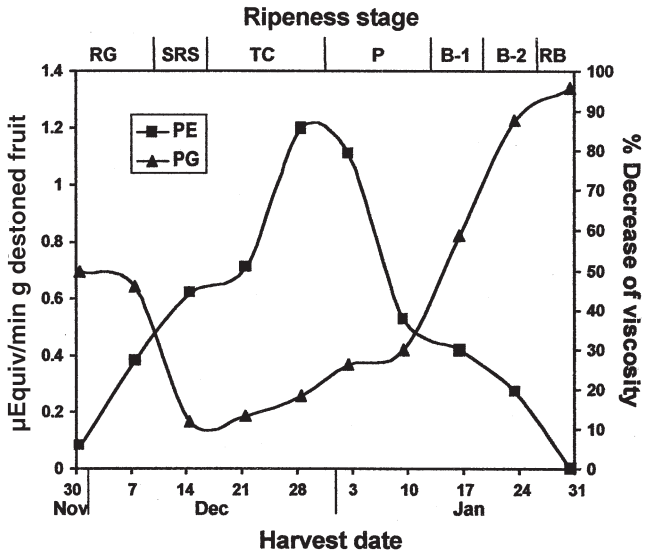

FIG. 1. Changes in PE and PG activity in fruits during ripening of olives. Abbreviations: RG, ripe-green; SRS, small reddish spots; TC, turning color; P, purple; B, black; RB, ripe-black; B-1, fruits with black surface and white pulp; B-2, fruits with black surface and purple pulp; µEquiv, microequivalents; PE, pectin esterase; PG, polygalacturonase.

TABLE 2
Changes in Texture, Total Pectins (TP), and Pectin Esterification Degree in Fruits During Ripening of Olives[a]

Ripeness stage	Harvest date	Texture (N/100 g of fruits)	TP (mg GA/100 g dry wt of fruits)	Degree of esterification (%)
Ripe-green	11/30/98	3889.6 ± 155.3	1678.6 ± 72.2	63.30
	12/7/98	3023.5 ± 140.7	1464.3 ± 60.0	65.34
Small reddish spots	12/14/98	2537.2 ± 108.8	882.4 ± 41.5	44.12
Turning color	12/21/98	2428.4 ± 112.4	852.9 ± 38.4	42.42
	12/28/98	2394.7 ± 98.2	823.5 ± 41.1	40.88
Purple	1/4/99	2253.6 ± 112.9	789.5 ± 31.3	27.39
	1/11/99	2260.5 ± 90.4	763.2 ± 32.2	27.59
Black-1	1/18/99	2119.7 ± 97.5	680.5 ± 30.6	23.39
Black-2	1/25/99	1358.3 ± 57.8	580.0 ± 25.0	24.21
Ripe-black	1/2/99	1027.6 ± 52.5	510.6 ± 21.4	12.03

[a]Black-1, fruits with black surface and white pulp; Black-2, fruits with black surface and purple pulp; GA, galacturonic acid.

lished that the one denominated PG1 is accumulated first in the ripe-green fruit, followed by PG2A and PG2B in the ripe fruit. Working along the same lines, DellaPenna *et al*. (15), studying the transgenic Rin tomato, concluded that the solubilization and depolymerization of the polyuronides take place before the appearance of the isoenzymes PG2A and PG2B. They concluded that the presence of the isoenzyme PG1 is sufficient for depolymerization *in vivo*. Later, Pathak and Sanwal (29), studying PG during ripening in the banana, separated three forms of the enzyme. The first (PG1) was detected as having endo-PG activity in the preclimacteric unripe fruit, while as ripening advanced, they found an increase in another two activities: one (PG2), an exo-PG, and the other (PG3), an endo-PG. In accordance with all these results, the sharp decrease in texture and pectin content that takes place in the Hojiblanca olive between the ripe-green and small-reddish-spot states could be due to a PG1-type endo-PG isoenzyme.

In a second stage of olive ripening, beginning in the fruits with external signs of ripening caused by the start of anthocyanin synthesis, that is, in fruits with small reddish spots, turning color fruits, and purple fruits, there was a moderate decrease in both texture (5.9%) and pectic content (4.1%), while the deesterification of the pectic matter became more marked (33%). This can be directly related to the high values of PE activity present in the fruits. From these results, we postulate that PE action on the pectic chain and the lower levels of PG found in the fruits at this stage might favor the formation of pectin-calcium bridges, and thus be responsible for the smaller losses in both texture and total pectin content (Table 2). A similar situation has been previously described in cherries (9) and mango (7).

Finally, in the last stage, as the fruit became fully ripe and black, the loss in texture of the olives (35.9%) was very marked, but not that in pectin content (14.8%). This is when PG reached its maximum activity and shows the involvement of this enzyme in the alteration of its substrate and, consequently, in the loss in fruit texture. However, its effect on this parameter must be accompanied in this stage by other glycosidase-type enzymatic activities, as noted by Heredia *et al.* (30), causing greater texture losses in the olive.

Therefore, the stage of ripeness at which olives are picked for oil extraction is important, since the predominant enzyme at the beginning, middle, or end of picking will be either PE or PG. Their different action *in vivo* contributes to the characteristics of the fruit.

Industrial experiment. With both the levels of PE and PG present during the ripening of olives recently picked from the tree and their action *in vivo* on the pectic matter established, the next step was to analyze the pectic content of the olives received by an oil-extraction mill throughout the fruit-picking period. The aim of this study was to determine whether changes took place in the pectic composition of the fruits during the oil extraction process that affected the yield. To this end, the pectic fraction was quantified both in the raw material (the fruits received by the mill) and in the solid residues (alperujos) left after extracting of the oil from the olives.

The total pectin (TP) content of the olives (Fig. 2) showed that the samples entering the mill were in ripeness states between the first and last as defined in the previous section,

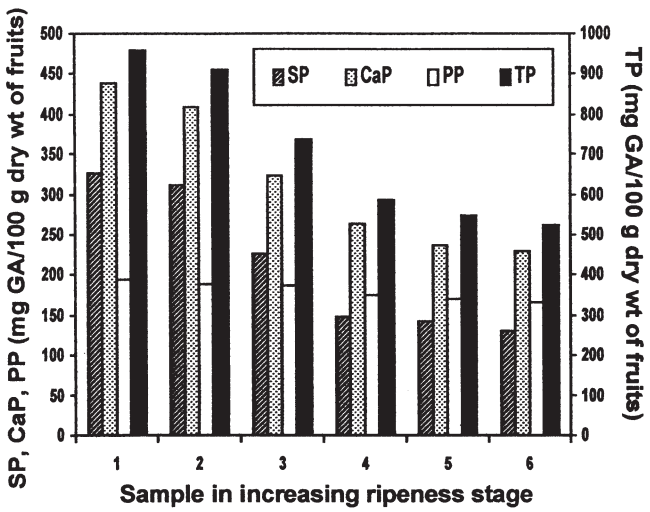

FIG. 2. Changes in pectic matter of olives throughout the fruit picking period, supplied from the mill; coefficient of variation <5% in all cases; mean of three replicates. Abbreviations: SP, soluble pectin; CaP, calcium pectate; PP, protopectin; TP, total pectin; GA, galacturonic acid.

excluding the two extreme states, in which fruits were not received at the mill. All the fractions decreased with fruit ripening, but at different rates. CaP was always the major fraction. Its formation *in vivo* should be a consequence of earlier activity of PE, since, in the first olive samples to enter the mill, the predominant fruits were those turning color and purple, states of ripening established as having preferential maximum PE activity. In the first three samples, the next major fraction was SP, while in the rest, of riper fruits, it was PP. Figure 3 plots the percentage of retention of the various pectic fractions vs. fruit ripeness state. As the former is represented as semilogarithms, the slope of the line joining two contiguous ripeness states could be considered a measurement of the relative degradation rate for the various pectic fractions, allowing them to be ranked and compared. The SP fraction was preferentially degraded, followed by CaP. The insoluble fraction (PP) was the least altered.

The decrease of TP thus involved mainly the SP and CaP fractions, with a minimum participation of the PP fraction. As noted by Levi *et al*. (25), the pectinolytic enzymes are those responsible for pectin degradation. Exo-PG removes terminal galacturonic acid monomers from the macromolecule, thereby reducing the content of the various pectic fractions, whereas endo-PG very effectively degrades the macromolecular pectin structure to smaller fractions. The state of the pectin fractions of the fruits received at the mill (Fig. 2) enables a better interpretation of the involvement of PG (Fig. 1) in the changes in texture and total pectins of the fruits. As noted in the previous section and in accordance with previous studies (15,28,29), depolymerization of the pectin in the ripe-green olive fruit was due to the action of a PG1-type endo-PG, with the subsequent intervention of a PG2-type exo-PG, forming low-molecular-weight monomers or oligomers that are soluble in alcohol and will be eliminated during the obtaining of the AIS, directly decreasing SP content. The same enzyme may act by removing nonconstitutive fragments from the main calcium pectate chain. The small alteration in the insoluble PP fraction during this period suggested the existence of a second endo-PG (PG3), with little presence at the start of ripening but becoming greater in ripe fruits.

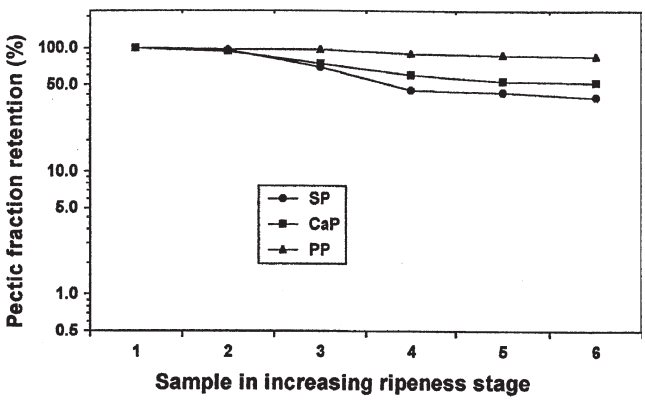

FIG. 3. Percentage of retention of the pectic fractions during ripening of Hojiblanca variety olives. See Figure 2 for abbreviations.

The data for the pectic fractions in the solid residues (alperujo) left after extraction of the oil show changes in all the pectic fractions during extraction (Fig. 4). In alperujo, the percentage of SP increased between some 10 and 15%, but for the CaP fraction, the opposite occurred, with a decrease of between 4 and 10%. This seems to indicate that in a first stage, the increase in SP was at the expense of the decrease in CaP. Such alteration would involve an exo-PG, explained by the permanence of the PP fraction in both the olive and the alperujo in the first three trials. Subsequently, this fraction decreased considerably in the alperujo compared with the content found in the fruits, suggesting the activity of an endo-PG, present in the riper olives during the oil extraction process. The PP fraction enables two groups of samples to be distinguished.

All these differences indicated that during virgin olive oil extraction, the long pectin chains were depolymerized to shorter ones, reflected as an increase in the SP fraction in the alperujo. In the samples with a lower degree of ripeness, this increase was produced at the expense of the CaP, while in the riper olives, the PP fraction contributed as a major agent to the increase in SP.

Figure 5 shows the percentage of oil obtained from each olive sample as a function of total extractable oil, that is,

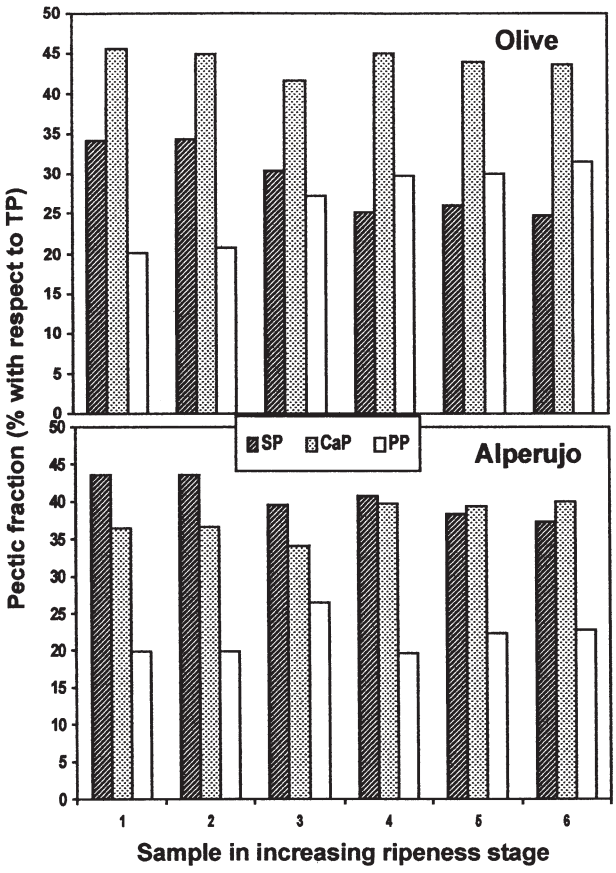

FIG. 4. Percentage changes in the pectic fractions of olives, supplied from the mill throughout the fruit picking period, and their respective alperujos; coefficient of variation <5% in all cases; mean of three replicates. See Figure 2 for abbreviations.

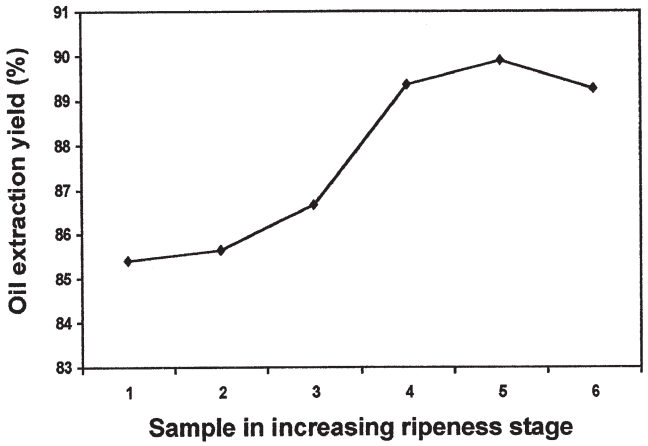

FIG. 5. Percentage of oil obtained from olives supplied from the mill throughout the fruit picking period, as a function of total extractable oil.

taking into account the fat content of the fruits, which ranged between some 15 and 22%. In all cases, as expected because of the addition of talc, the oil yields were high. There were, however, differences between the first three samples with a mean oil yield of around 86%, and the other three in which the yield increased to approximately 90%. This difference, although small, was related to the different proportion of pectic fractions in each sample, depending on degree of fruit ripeness. In the first three samples, the SP/PP ratio was greater than unity, while in the last three, the ratio was less than unity. The ratio between the CaP and PP fractions decreased with increasing degree of fruit ripeness, the differences between them being greater in the first three samples than in the other three, in which the amounts of CaP and PP were notably lower and less perceptible.

The distribution of the pectic fractions in the raw material and the changes in them during the olive oil extraction process showed a role of PE and PG in the fruits that was related with the yield of oil extracted. The development of emulsions may be related to or be the result of PE levels in the fruits, enabling the formation of calcium bridges between de-esterified chains (9). Therefore, we deduced that the extraction of virgin olive oil is easier or more difficult depending on prior action of the enzyme PE in the fruits. The resulting de-esterified chains, on which PG has not yet acted, could link by calcium bridges to form emulsions, hindering the separation and complete extraction of the oily juice of the olive. This would take place despite the preventive action of talc added during virgin olive oil extraction, which obviously greatly lessens the effect of the substrate and of the pectic enzymes in the fruits.

Although oil extraction yields were high in all the samples analyzed, there was a certain tendency for the extraction of virgin olive oil to increase as the amount of total fruit pectins decreased. This was a consequence of the decrease in CaP and SP, possibly mediated by the presence of PE and PG in the olives. During virgin olive oil extraction, there is a certain degradation of the CaP and PP fractions to SP.

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