Effect of Enzyme Treatment during Mechanical Extraction of Olive Oil on Phenolic Compounds and Polysaccharides

Esther Vierhuis,[†] Maurizio Servili,[‡] Maura Baldioli,[§] Henk A. Schols,[†] Alphons G. J. Voragen,[†] and GianFrancesco Montedoro*[,§]

Department of Agrotechnology and Food Sciences, Laboratory of Food Chemistry, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands; Dipartimento di Scienze e Tecnologie Agro-alimentari, Ambientali e Microbiologiche, University of Molise, Via De Sanctis, 86100 Campobasso, Italy; and Dipartimento di Scienze degli Alimenti, University of Perugia, Via S. Costanzo, 06126 Perugia, Italy

The effect of the use of cell-wall-degrading-enzyme preparations during the mechanical extraction process of virgin olive oil on the phenolic compounds and polysaccharides was investigated. The use of the enzyme preparations increased the concentration of phenolic compounds in the paste, oil, and byproducts. Especially, the contents of secoiridiod derivatives such as the dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol (3,4-DHPEA-EDA) and an isomer of oleuropein aglycon (3,4-DHPEA-EA), which have high antioxidant activities, increased significantly in the olive oil. Furthermore, the use of an N_2 flush during processing strongly increased the phenolic concentration. Analyses of the pectic polymers present in the paste showed that the use of pectinolytic enzyme preparations increased the yield of the buffer soluble pectins and the proportion of molecules with a lower molecular mass. Also, the content of uronic acids in the buffer soluble extract increased considerably due to the use of the enzyme preparations. Analysis of the polymeric carbohydrates in the vegetation waters showed the presence of mainly pectic polymers. The addition of commercial enzyme preparations increased the uronic acid content of the polysaccharides in the vegetation water substantially compared to the blank. This study showed that the addition of cell-wall-degrading enzymes did improve the olive oil quality; however, mechanisms remained unclear.

Keywords: *Phenols; secoiridoids; polysaccharides; olive oil; enzyme preparations; oxidation*

INTRODUCTION

Secoiridoids such as oleuropein and demethyloleuropein are the predominant phenolic compounds of olive fruit (*1*−*3*). In addition, olive fruit also contains verbascoside and low amounts of other classes of phenolic compounds including phenyl alcohols such as 3,4-dihydroxyphenylethanol (3,4-DHPEA) and *p*-hydroxyphenylethanol (*p*-HPEA), phenyl acids, and flavonoids (*4*−*7*). The phenol alcohols 3,4-DHPEA and *p*-HPEA are also present in virgin olive oil. However, the prevalent phenolic compounds in the oil are secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to 3,4-DHPEA or *p*-HPEA (3,4-DHPEA-EDA or *p*-HPEA-EDA, respectively) and an isomer of oleuropein aglycon (3,4-DHPEA-EA) (*8*).

The concentration of phenolic compounds present in virgin olive oil is strongly affected by the extraction conditions used during processing (*9*−*11*). In this ambit, the loss of secoiridoids and phenyl alcohols in the oil during malaxation is well-known (*4*, *12*). So far, however, the mechanism that explains the quantitative modification of secoiridoids in the oil during malaxation is unknown. Enzymatic oxidation by endogenous oxidoreductases such as polyphenol oxidase (PPO) and peroxidase (POD) may promote oxidation of phenolic compounds (*12*, *13*), but nonenzymatic oxidation processes are also involved (*10*).

Interactions between polysaccharides and phenolic compounds present in the olive pastes may also be involved in the loss of phenols during processing. Previous work has shown that polyphenols are able to complex with certain types of polysaccharides, which most probably results from the ability of the polysaccharide to form a structure that encapsulates the polyphenol (*14*−*17*). The interaction of the phenolic compounds with polysaccharides may reduce their release in the oil during crushing and malaxation. In fact, it is shown that the use of technical preparations containing cell-wall-degrading-enzyme activities during processing can improve the phenolic concentration in the oil (*18*−*23*).

Although the changes in the composition of the phenolic compounds due to the use of enzyme preparations have been studied before, very little is known about the effect of these enzyme preparations on the cell wall structure. This paper reports both elements and also the effect of malaxation under N_2 flush to reduce the oxidative degradation of phenolic compounds in pastes during malaxation.

MATERIALS AND METHODS

Materials. Olives from the cultivar Moraiolo were used. Olivex is an enzyme preparation rich in pectolytic enzymes and in addition hemicellulolytic and cellulolytic side activities

* Author to whom correspondence should be addressed (fax +39 75 585 7916; e-mail montegia@unipg.it).
[†] Wageningen University.
[‡] University of Molise.
[§] University of Perugia.

10.1021/jf000578s CCC: $20.00
Published on Web 02/22/2001

produced from *Aspergillus aculeatus* and especially suitable for application in the extraction of vegetable oils. Novoferm 12 is a pectolytic enzyme preparation of *Aspergillus niger* origin, which in addition contains β-glucosidase activity. Both commercial enzyme preparations were kindly provided by Novo Nordisk Ferment Ltd. (Dittingen, Switzerland).

Sample Preparation. Olives (90 kg) were crushed with a hammer mill (α-Laval, Lund, Sweden) and slowly mixed (malaxed) for 60 min at 30 °C. Extraction of the oil was performed with an α-Laval decanter UVNX-414 with a working capacity of 1.00 ton/h [paste/water ratio 1:0.15 (w/v)]. Samples of the pastes and vegetation waters were taken and immediately frozen in liquid nitrogen to inhibit enzymatic activity, freeze-dried, and stored at −30 °C before analysis. The enzyme preparations were added at the beginning of malaxation (500 mg/kg of pastes). To study the effect of O_2 during processing, malaxation was performed with and without an N_2 flush. The oxygen value in the pastes was measured, during malaxation, using a Mettler Toledo oxygen sensor model 4100 (Greifensee, Switzerland).

Extraction, Purification, and Separation of Phenolic Compounds from Pastes, Virgin Olive Oil, and Vegetation Waters. *Pastes.* Freeze-dried paste (10 g) was mixed with 50 mL of 80% methanol containing 20 mg/L sodium diethyldithiocarbamate (DIECA) at −25 °C to inhibit PPO. The mixture was homogenized in an Omni-mixer (Sorvall) for 30 s at 16000 rpm and filtered using a Büchner funnel apparatus. The procedure was repeated six times, and the extracts were collected. The methanol was evaporated in a vacuum at 35 °C under a flow of nitrogen. The water extract (2 mL) was loaded on a 5 g/20 mL Extract-Clean high-load C_{18} cartridge (Alltech Associates Inc., Deerfield, IL) and eluted with methanol (600 mL). The eluate was collected and the organic solvent evaporated in a vacuum at 35 °C under a flow of nitrogen. The residue was dissolved in methanol (1 mL) and analyzed by HPLC (*3*).

Vegetation Waters. Freeze-dried vegetation water was rehydrated with water containing DIECA (20 mg/L) and loaded (2 mL) on a 5 g/20 mL Extract-Clean high-load C_{18} cartridge (Alltech Associates Inc.) and eluted with ethyl ether (600 mL). The concentration and analysis on HPLC were carried out as reported previously (*3*).

Virgin Olive Oil. Phenols were extracted with 80% methanol from the virgin olive oil, the crude oil from the mechanical extraction, and evaluated by HPLC according to the procedure performed by Montedoro et al. (*24*).

Reference Compounds. 3,4-DHPEA was synthesized in the laboratory according to the procedure of Baraldi et al. (*25*). 3,4-DHPEA-EDA, 3,4-DHPEA-EA, and verbascoside were extracted from virgin olive oil and olive fruit, respectively, and the chemical structures were verified by NMR according to the method of Montedoro et al. (*21*). Oleuropein glucoside was obtained from Extrasynthèse Co. (Genay, France). *p*-HPEA was obtained from Janssen Chemical Co. (Beerse, Belgium), luteolin 7-glycoside was obtained from Roth Co. (Karlsruhe, Germany), and rutin was obtained from BDH Co. (Poole, U.K.).

Isolation of Alcohol Insoluble Solids (AIS). The freeze-dried pastes were defatted by (Soxhlet) extraction with petroleum ether (bp 40-60 °C). A sieve of 425 μm was used to separate the stones from the pulp. Subsequently, the lipid-free material was extracted with 70% (v/v) ethanol at 40 °C for 1 h and centrifuged. The extraction with 70% (v/v) ethanol was repeated until the extracts were free of sugars as monitored by the phenol−sulfuric acid test (*26*). The residue (AIS) was dried by solvent exchange [96% (v/v) ethanol and acetone] at room temperature. The freeze-dried vegetation waters were defatted by extraction with petroleum ether (bp 40-60 °C) and extracted with 70% (v/v) ethanol as described above to isolate the AIS.

Extraction of AIS. The AISs prepared from olive pulp (75 mg) were extracted with 2.25 mL of 0.05 M NaOAc buffer, pH 5.2, at 70 °C for 1 h (hot buffer soluble solids). The solubilized polymers were separated from the insoluble residue by centrifugation. The extracts were analyzed by high-performance size exclusion chromatography (HPSEC), and the sugar composition of the polysaccharides present in the extracts was determined.

Enzyme Incubation of AIS with Olivex. The AISs prepared from vegetation water were dissolved (2.5 mg/mL) in 0.05 M NaOAc buffer, pH 5.0, and incubated with the commercial enzyme preparation Olivex [0.1% (w/v)] at 40 °C for 24 h. After incubation, the samples were heated for 15 min at 100 °C to inactivate the enzymes. The change in molecular weight distribution of the samples was studied by HPSEC.

Analytical Methods. *Total Uronic Acid Content.* Total uronic acid content was determined colorometrically by using the automated *m*-hydroxydiphenyl assay (*27*). Galacturonic acid was used as a standard. Corrections were made for the interference of neutral sugars in the samples. Analysis of the uronic acid content was performed in triplicate. The mean values are reported in Tables 4 and 5 and have a standard deviation ≤1.

Neutral Sugar Composition. The neutral sugar composition of the AISs and the polysaccharides present in the hot buffer extracts was determined by gas chromatography according to the method of Englyst and Cummings (*28*) using inositol as internal standard. The samples were treated with 72% (w/w) sulfuric acid for 1 h at 30 °C prior to hydrolyses with 1 M sulfuric acid for 3 h at 100 °C. The released constituent sugars were converted into their alditol acetates and analyzed. Cellulosic glucose in the AISs was calculated as the difference between the glucose contents determined with and without pretreatment with 72% (w/w) sulfuric acid. Analysis of the neutral sugar composition was performed in triplicate. The mean values are reported in Tables 4 and 5 and have a standard deviation ≤1.

HPSEC. HPSEC was performed as described previously (*29*). Calibration was performed using dextrans (M_w = 4000−500000 Da).

Statistical Analysis. Experiments to determine the content of phenolic compounds were performed in triplicate, and the means ± standard deviations are reported in Tables 1−3. One-way analysis of variance (ANOVA) using the Tukey test was performed to evaluate the significance of differences between mean values among three or more different experimental groups. Statgraphics version 6.1 (Statistical Graphics Corp., 1992, Manugistics, Inc., Rockville, MD) was used to perform the statistical analyses.

RESULTS AND DISCUSSION

The crushed paste showed a high concentration of the secoiridoid derivative 3,4-DHPEA-EDA, whereas oleuropein, demethyloleuropein, and verbascoside were present in small amounts (Table 1). The high concentration of 3,4-DHPEA-EDA is due to the hydrolysis of oleuropein and demethyloleuropein catalyzed by endogenous glycosidases during the crushing of olive fruit (*12*). The virgin olive oil obtained from the crushed paste contained a high concentration of the aglycon derivatives 3,4-DHPEA-EDA and 3,4-DHPEA-EA (Table 2). Oleuropein and demethyloleuropein were not present in the olive oil. The phenolic composition of vegetation waters was very different from the composition of the oil as the vegetation waters contained secoiridoid glucosides and other polar compounds such as luteolin 7-glucoside and rutin that were not detected in virgin olive oil. The secoiridoid derivative 3,4-DHPEA-EDA was the predominant phenolic compound of vegetation waters (Table 3).

Malaxation reduced the concentration of phenolic compounds in the pastes, oil, and vegetation waters (Tables 1−3) and is the most critical point in the mechanical extraction process of oil. In fact, the nutritional value of oil is directly related to the concentration of its natural antioxidants such as 3,4-DHPEA, 3,4-

Table 1. Phenolic Composition of Olive Pastes with and without Enzymatic Treatment during Processing (Malaxation Was Performed in the Absence and Presence of N_2)

	crushed paste blank	malaxed paste blank	malaxed paste + NF12/Olivex	malaxed paste under N_2 flush	malaxed paste under N_2 flush + NF12/Olivex
O_2[a] (ppm)		6.9 ± 0.7^{a}	6.6 ± 0.5^{a}	0.16 ± 0.07^{b}	0.18 ± 0.08^{b}
phenolic compounds[b] (mg/g of dry wt)					
3,4-DHPEA	0.54 ± 0.01^{a}	0.04 ± 0.01^{b}	0.03 ± 0.01^{b}	0.04 ± 0.01^{b}	0.03 ± 0.01^{b}
p-HPEA	0.32 ± 0.02^{a}	0.04 ± 0.01^{b}	0.05 ± 0.01^{b}	0.03 ± 0.01^{b}	0.09 ± 0.01^{c}
demethyloleuropein	1.54 ± 0.02^{a}	0.30 ± 0.01^{b}	0.51 ± 0.04^{c}	0.71 ± 0.04^{d}	0.90 ± 0.02^{e}
verbascoside	3.3 ± 0.2^{a}	1.3 ± 0.1^{b}	1.2 ± 0.2^{b}	2.4 ± 0.2^{c}	2.8 ± 0.4^{ac}
3,4-DHPEA-EDA	20.8 ± 0.9^{a}	5.7 ± 0.3^{b}	7.5 ± 0.5^{c}	10.6 ± 0.5^{d}	13.9 ± 0.1^{e}
oleuropein	2.3 ± 0.1^{a}	0.75 ± 0.05^{b}	0.75 ± 0.07^{b}	1.19 ± 0.03^{c}	1.89 ± 0.02^{d}
luteolin 7-glucoside	0.22 ± 0.02^{a}	0.13 ± 0.01^{b}	0.12 ± 0.01^{b}	0.25 ± 0.01^{a}	0.18 ± 0.01^{c}
rutin	0.16 ± 0.01^{a}	0.11 ± 0.01^{bc}	0.09 ± 0.01^{b}	0.17 ± 0.01^{a}	0.12 ± 0.01^{c}

[a] The O_2 concentration is the mean value of six independent determinations evaluated during malaxation ± standard deviation. [b] The phenolic content is the mean value of three independent experiments ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another.

Table 2. Phenolic Composition of Virgin Olive Oil (Milligrams per Kilogram) with and without Enzymatic Treatment during Processing (Malaxation Was Performed in the Absence and Presence of N_2)

	crushed paste blank	malaxed paste blank	malaxed paste + NF12/Olivex	malaxed paste under N_2 flush	malaxed paste under N_2 flush + NF12/Olivex
3,4-DHPEA[a]	2.7 ± 0.3^{a}	0.7 ± 0.1^{b}	1.9 ± 0.1^{c}	2.0 ± 0.2^{ac}	1.7 ± 0.3^{c}
p-HPEA	2.3 ± 0.4^{a}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	2.6 ± 0.3^{a}	0.81 ± 0.03^{b}
3,4-DHPEA-EDA	515 ± 23^{ab}	317 ± 16^{c}	439 ± 16^{d}	504 ± 6^{a}	556 ± 13^{b}
p-HPEA-EDA	24.8 ± 1.9^{a}	25.8 ± 1.4^{ab}	29.4 ± 0.8^{c}	28.4 ± 1.4^{bc}	31.4 ± 1.0^{c}
p-HPEA derivative	32.5 ± 1.4^{a}	24.2 ± 0.8^{b}	28.5 ± 0.9^{c}	21.6 ± 1.3^{b}	22.4 ± 0.9^{b}
3,4-DHPEA-EA	357 ± 13^{a}	177 ± 8^{b}	218 ± 8^{c}	242 ± 5^{c}	299 ± 10^{d}

[a] The phenolic content is the mean value of three independent experiments ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another.

Table 3. Phenolic Composition of Vegetation Waters (Milligrams per Gram of Dry Weight) with and without Enzymatic Treatment during Processing (Malaxation Was Performed in the Absence and Presence of N_2)

	crushed paste blank	malaxed paste blank	malaxed paste + NF12/Olivex	malaxed paste under N_2 flush	malaxed paste under N_2 flush + NF12/Olivex
3,4-DHPEA[a]	1.52 ± 0.06^{a}	0.37 ± 0.03^{b}	0.44 ± 0.01^{b}	0.37 ± 0.02^{b}	0.55 ± 0.04^{c}
p-HPEA	0.14 ± 0.01^{a}	nd[b]	0.04 ± 0.01^{c}	0.04 ± 0.01^{c}	0.07 ± 0.01^{d}
demethyloleuropein	1.39 ± 0.06^{a}	0.47 ± 0.06^{b}	0.82 ± 0.01^{c}	0.95 ± 0.10^{c}	1.8 ± 0.1^{d}
verbascoside	nd	nd	nd	nd	nd
3,4-DHPEA-EDA	98.6 ± 8.6^{a}	17.5 ± 1.1^{b}	31.9 ± 2.6^{c}	35.4 ± 0.7^{cd}	44.2 ± 1.2^{d}
oleuropein	2.8 ± 0.3^{a}	0.60 ± 0.04^{b}	1.1 ± 0.1^{c}	1.1 ± 0.1^{c}	1.2 ± 0.2^{c}
luteolin 7-glucoside	0.20 ± 0.01^{a}	0.11 ± 0.01^{b}	0.11 ± 0.01^{b}	0.14 ± 0.01^{c}	0.17 ± 0.01^{d}
rutin	0.19 ± 0.01^{a}	0.06 ± 0.01^{b}	0.10 ± 0.01^{c}	0.08 ± 0.01^{bc}	0.18 ± 0.01^{a}

[a] The phenolic content is the mean value of three independent experiments ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another. [b] nd, not detected.

Table 4. Sugar Composition Expressed as Mole Percent of Cell Wall Material (AIS) from Pastes and Vegetation Waters

sample	rhamnose	arabinose	xylose	mannose	galactose	glucose	uronic acid	w/w%
crushed paste blank	1	13	31	1	4	36	14	45
malaxed paste blank	2	13	30	1	4	36	14	46
malaxed paste + NF12/Olivex	2	12	30	2	3	37	15	42
vegetation water blank	3	24	3	1	21	8	39	32
vegetation water + NF12/Olivex	3	20	1	1	11	2	63	47

DHPEA-EA, and 3,4-DHPEA-EDA (*30*, *31*). The use of commercial enzyme preparations with activity toward polysaccharides during processing increased significantly the concentration of 3,4-DHPEA-EDA in the malaxed paste. Also, the release of secoiridoid derivatives, such as 3,4-DHPEA-EDA and 3,4-DHPEA-EA, into the oil improved greatly. The vegetation waters showed a significant increase of 3,4-DHPEA-EDA, oleuropein, and demethyloleuropein. The use of an N_2 flush during malaxation reduced the O_2 level in the pastes and greatly increased the phenolic concentration in the oil, paste, and vegetation waters (Tables 1−3).

To study the effect of the commercial enzyme preparations on the polysaccharides present in the cell wall, cell wall material was isolated from the olive pastes before and after malaxation and analyzed for sugar composition (Table 4). Glucose, xylose, and arabinose were the major neutral sugars in the olive pastes. About 94% of the glucose in the paste represented cellulose. The uronic acid content was 14-15 mol %. The results revealed that the sugar composition of the paste hardly changed during processing without commercial enzyme preparations added. However, the addition of commercial enzyme preparations during processing also hardly changed the sugar composition of the paste. This may indicate that the degradation of the polysaccharides present in the cell wall by endogenous enzymes or the addition of exogenous enzymes was very limited, despite the positive effect of the latter on the level of phenolic compounds.

The enzyme preparations used during processing were rich in pectolytic enzymes. Therefore, an extraction with hot buffer was performed to study whether processing or the addition of enzyme preparations had an

Table 5. Yield and Sugar Composition (Mole Percent) of Polysaccharides Solubilized with Hot Buffer from Crushed and Malaxed Pastes

sample	yield[a]	rhamnose	arabinose	xylose	mannose	galactose	glucose	uronic acid
crushed paste blank	7.0	3	23	2	1	14	4	53
malaxed paste blank	7.0	3	22	3	1	14	4	53
malaxed paste + NF12/Olivex	9.5	4	24	1	1	8	2	60

[a] Percent of total polysaccharides solubilized.

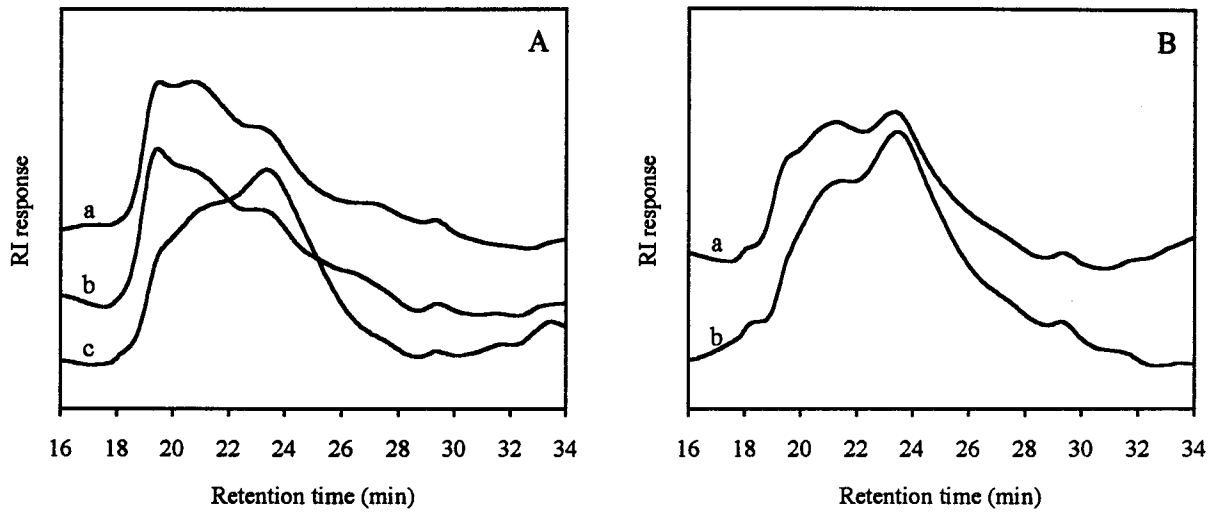

Figure 1. HPSEC patterns of polysaccharides extracted with hot buffer from crushed and malaxed pastes (A) and polysaccharides present in vegetation waters (B): (A) crushed paste blank (a), malaxed paste blank (b), malaxed paste plus NF12/Olivex (c); (B) vegetation water blank (a), vegetation water plus NF12/Olivex (b).

effect on the buffer soluble pectic material in olive paste. The extracts were analyzed for their sugar composition (Table 5), and the molecular weight distribution was determined by HPSEC (Figure 1A). It appeared that processing without the addition of enzyme preparations hardly changed the sugar composition or the solubility of the pectic material extracted with hot buffer. Also, the molecular weight distributions as determined by HPSEC were identical for the buffer soluble pectins from the crushed paste and from the malaxed paste. Although olive fruit contains endogenous enzymes such as glycosidases and polygalacturonase and enzymes with cellulolytic activity (*32*−*34*), the enzymes had hardly any effect on the polysaccharides during malaxation. It may be suggested that the high concentration of phenolic compounds present in olive pulp inhibited these enzymes (*3*, *4*, *35*, *36*).

The addition of commercial enzyme preparations increased the extractability of pectic material with hot buffer, and also the sugar composition of the extractable polysaccharides changed compared to the blank. The relative amount of uronic acids extracted with buffer was substantially higher, and consequently the ratio of neutral sugars to uronic acids decreased. Also, the elution profile of the extracted material on HPSEC changed due to the addition of commercial enzyme preparations. The paste obtained by malaxation with commercial enzyme preparations added during processing had a relatively larger proportion of molecules with a lower molecular mass (~100000 Da).

The polysaccharides present in the vegetation waters were also studied. The polysaccharides were isolated by ethanol precipitation, and the sugar composition was determined (Table 4). The presence of the neutral sugars arabinose and galactose and the high content of uronic acid in both vegetation waters indicated that mainly pectins were present. Pectic polymers consist of regions of linear galacturonan and regions of more highly substituted rhamnogalacturonan with complex chains of arabinose and galactose linked to the rhamnose residues (*37*). Therefore, the ratio of the sugars arabinose and galactose to uronic acid gives an indication of the degree of branching of the pectin molecules present in the vegetation water. The ratio was 1.2 for the polysaccharides present in the vegetation water of the blank and 0.5 for the polysaccharides present in the vegetation water with Novoferm 12 and Olivex added during processing. These ratios indicate that the addition of commercial enzyme preparations partly debranched the pectic material or solubilized additional pectic substances with a high galacturonic acid content.

The HPSEC elution patterns showed almost identical molecular weight distributions for the polysaccharides isolated from both vegetation waters despite the differences in the sugar composition (Figure 1B). Only a slight shift toward lower molecular weight ranges could be observed. Polymeric material was still present in the vegetation waters and was not completely degraded to oligosaccharides or their constituent monosaccharides even though enzyme preparations were added during processing. To check whether the structure of the pectins restricted the action of the enzymes, the pectic polymers isolated from the vegetation waters were incubated with the enzyme preparation Olivex, which was also used during processing. The HPSEC elution patterns revealed that the polysaccharides could be degraded to a substantial extent (not shown). About 90% of the polysaccharides were degraded to oligomers (molecular mass < 4000 Da) and monosaccharides. Therefore, the structures of the polysaccharides or the enzyme preparations used were not limiting for the degradation. Probably, the enzymes present in the commercial enzyme preparations were partially inacti-

vated during processing due to phenolic compounds present in the pastes, as was suggested before.

CONCLUDING REMARKS

It is known that the addition of cell-wall-degrading-enzyme preparations during the mechanical extraction of olive oil can improve the release of phenolic compounds in oil. However, this is the first time that the effect of the addition of enzyme preparations has been studied by analyzing both the phenolic compounds and the polysaccharides present in the pastes and vegetation waters. In fact, previous papers reported only results about quantitative and qualitative modifications on the phenolic composition of oil when enzymatic preparations were used during processing (*19*−*23*). However, although the results of this study revealed that the use of commercial enzyme preparations changed part of the cell wall structure, it is difficult to conclude how these changes affected the phenol content of the oil. The addition of commercial enzyme preparations might have reduced the complexation of the phenolic compounds with the polysaccharides, thus improving the concentration of free phenols in the pastes and their release in the oil and vegetation water during processing. Also, the addition of pectolytic enzymes could have resulted in weakening and disruption of the cell wall, thus facilitating the release of phenolic compounds from the fruit. However, the content of phenolic compounds in the oil is also influenced by enzymatic and nonenzymatic oxidation. In fact, the inhibition of the oxidation process by reducing the O_2 level in the paste during malaxation strongly improved the concentrations 3,4-DHPEA-EDA and 3,4-DHPEA-EA, which have high antioxidant activities (*38*) and are mainly affected by oxidative degradation.

In conclusion, the addition of commercial enzyme preparations improved the release of phenolic compounds in the oil, but the oxidative reactions during malaxation have also an important impact on the final concentration in the oil and vegetation waters. Work is in progress to define the interaction between the oxidative degradation of phenolic compounds and the activity of endogenous oxidoreductases such as PPO and POD during the mechanical extraction of olive oil.

ACKNOWLEDGMENT

We thank Dr. Roberto Selvaggini and Roberto Santibacci for technical assistance.

LITERATURE CITED

(1) Panizzi, L.; Scarpati, M. L.; Oriente, G. Chemical Structure of Oleuropein, Bitter Glucoside of Olive with Hypotensive Activity. *Gazz. Chim. Ital.* **1960**, *90*, 1449−1485.

(2) Ragazzi, E.; Veronese, G.; Guitto, A. The Demethyloleuropein, a New Glucoside Extracted from Ripe Olives. *Ann. Chim.* **1973**, *63*, 13−20.

(3) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Montedoro, G. F. HPLC evaluation of phenols in olive fruit, virgin olive oil, vegetation water and pomace and 1D- and 2D-NMR characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873−882.

(4) Servili, M.; Baldioli, M.; Mariotti, F.; Montedoro, G. F. Phenolic composition of olive fruit and virgin olive oil: distribution in the constitutive parts of fruit and evolution during oil mechanical extraction process. *Acta Hortic.* **1999**, *474*, 609−619.

(5) Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC: Boca Raton, FL, 1990; pp 1−126.

(6) Brenes-Balbuena, M.; Garcia-Garcia, P.; Garrido-Fernandez, A. Phenolic Compounds Related to the Black Color Formed during the Processing of Ripe Olive. *J. Agric. Food Chem.* **1992**, *40*, 1192−1196.

(7) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables and Grains*; CRC: Boca Raton, FL, 1993; pp 64−67.

(8) Montedoro, G. F.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A. Simple and Hydrolyzable Compounds in Virgin Olive Oil. 3. Spectroscopic Characterization of the Secoiridoids Derivatives. *J. Agric. Food Chem.* **1993**, *41*, 2228−2234.

(9) Servili, M.; Baldioli, M.; Montedoro, G. F. Phenolic composition of virgin olive oil in relationship to some chemical and physical aspects of malaxation. *Acta Hortic.* **1994**, *1*, 331−336.

(10) Servili, M.; Baldioli, M.; Selvaggini, R.; Mariotti, F.; Federici, E.; Montedoro, G. F. Effect of malaxation under N_2 flush on phenolic and volatile compounds of virgin olive oil. *Adv. Plant Lipid Res.* **1998**, 307−310.

(11) Morales, M. T.; Angerosa, F.; Aparicio, R. Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: chemical and sensory implications. *Grasas Aceites* **1999**, *50*, 114−121.

(12) Servili, M.; Baldioli, M.; Mariotti, F.; Montedoro, G. F. Secoiridoids of virgin olive oil: modification during the oil mechanical extraction process. In *Proceedings of the World Conference and Exhibition on Oil Seed nd Edible Oils Processing*, Istanbul, Oct 6−10, 1996.

(13) Sciancalepore, V. Enzymatic browning in five olive varieties. *J. Food Sci.* **1985**, *50*, 1194−1195.

(14) McManus, J. P.; Davis, K. G.; Beart, D. J. E.; Gaffney, S. H.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1429−1438.

(15) Ozawa, T.; Lilley, T. H.; Haslam, E. Polyphenol interactions: astringency and the loss of astringency in ripening fruit. *Phytochemistry* **1987**, *26*, 2937−2942.

(16) Spencer, C. M.; Cai, Y.; Russel, M.; Gaffney, S. H.; Goulding, P.; Magnolato, D.; Lilley, T. H.; Haslam, E. Polyphenol complexation some thoughts and observation. *Phytochemistry* **1988**, *27*, 2397−2409.

(17) Haslam, E.; Lilley, T. H. Natural astringency in foodstuffs—a molecular interpretation. *Crit. Rev. Food Sci. Nutr.* **1988**, *27*, 1−40.

(18) Siniscalco, V.; Montedoro, G. F. Mechanical extraction of olive oil using technological adjuvants. Note 1: drains and enzymes. *Riv. Sostanze Grasse* **1988**, *65*, 675−678.

(19) Siniscalco, V.; Montedoro, G. F. Mechanical extraction of olive oil using technological adjuvants. Note 2: extraction of integral and stoned pastes by the percolation-centrifugation system. *Riv. Sostanze Grasse* **1989**, *66*, 85−90.

(20) Servili, M.; Begliomini, A. L.; Montedoro, G. F.; Petruccioli, M.; Federici, F. Utilisation of a yeast pectinase in olive oil extraction and red wine making processes. *J. Sci. Food Agric.* **1992**, *58*, 253−260.

(21) Montedoro, G. F.; Begliomini, A. L.; Servili, M.; Petruccioli, M.; Federici, F. Pectinase production from olive vegetation waters and its use in the mechanical olive oil extraction process to increase oil yield and improve quality. *Ital. J. Food Sci.* **1993**, *5*, 355−362.

(22) Ranalli, A.; Serraiocco, A. Quantitative and qualitative effects of a pectolytic enzyme in olive oil production. *Grasas Aceites* **1996**, *47*, 227−236.

(23) Ranalli, A.; De Mattia, G. Characterization of olive oil production with a new enzyme processing aid. *J. Am. Oil Chem. Soc.* **1997**, *74*, 1105−1113.
(24) Montedoro, G. F.; Servili, M.; Baldioli, M.; Miniati, E. Simple and Hydrolyzable Compounds in Virgin Olive Oil. 1. Their Extraction, Separation and Quantitative and Semiquantitative Evaluation by HPLC. *J Agric. Food Chem*. **1992**, *40*, 1571−1576.
(25) Baraldi, P. G.; Simoni, D.; Manfredini, F.; Menziani, E. Preparation of 3,4-dihydroxy-1-benzeneethanol: a Re-investigation. *Liebigs Ann. Chem*. **1983**, 684−686.
(26) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem*. **1956**, *28*, 350−356.
(27) Thibault, J.-F. Automatisation du dosage des substances pectiques par la methode au meta-hydroxydiphenyl. *Lebensm. -Wiss. -Technol.* **1979**, *12*, 247−251.
(28) Englyst, H. N.; Cummings, J. H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* **1984**, *109*, 937−942.
(29) Vierhuis, E.; Schols, H. A.; Beldman, G.; Voragen, A. G. J. Isolation and characterisation of cell wall material from olive fruit (*Olea europaea* cv Koroneiki) at different ripening stages. *Carbohydr. Polym*. **2000**, *43*, 11−21.
(30) Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G. F.; Galli, C. Inhibition of Platelet Aggregation and Eicosanoid Production by Phenolic Component of Olive Oil. *Thromb. Res*. **1995**, *78*, 151−160.
(31) Visioli, F.; Bellomo, G.; Montedoro, G. F.; Galli, C. Low density lipoprotein oxidation is inhibited in vitro by olive oil costituents. *Atherosclerosis* **1995**, *117*, 25−32.
(32) Heredia, A.; Fernández-Bolaños, J.; Guillèn, R. Identification of endoglucanases in olives (*Olea europaea arolensis*). *Z. Lebensm. Unters. Forsch.* **1991**, *193*, 554−557.
(33) Heredia, A.; Guillèn, R.; Jiménez, A.; Fernández-Bolaños, J. Activity of glycosidases during development and ripening of olive fruit. *Z. Lebensm. Unters. Forsch.* **1993**, *196*, 25−32.
(34) Fernández-Bolaños, J.; Rodríguez, R.; Guillén, R.; Jiménéz, A.; Heredia, A. Activity of cell wall-associated enzymes in ripening olive fruit. *Physiol. Plant.* **1995**, *93*, 651−658.
(35) Heredia, A.; Fernández-Bolaños, J.; Guillèn, R. Cellulase inhibition by polyphenols in olive fruits. *Food Chem*. **1990**, *38*, 69−73.
(36) Knee, M.; Paull, R. E.; Ben Arie, R.; Hawker, J. S. Enzymes in fruit. In *Food Enzymology*; Fox, P. F., Ed.; Elsevier: London, U.K., 1991; pp 545−598.
(37) Voragen, A. G. J.; Pilnik, W.; Thibault, J.-F.; Axelos, M. A. V.; Renard, C. M. G. C. Pectins. In *Food Polysaccharides and Their Applications*; Stephen, A. M., Ed.; Dekker: New York, 1995; pp 287−339.
(38) Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G. F. Antioxidant Activity of Tocopherols and Phenolic Compounds of Virgin Olive Oil. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1589−1593.

Received for review May 12, 2000. Revised manuscript received December 19, 2000. Accepted December 26, 2000. This research work was supported by the European Economic Community, under Project AIR3-CT-93 1355.

JF000578S