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Improving Virgin Olive Oil Quality by Means of Innovative Extracting Biotechnologies

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Three major virgin olive oil varieties (Dritta, Leccino, and Coratina) extracted by a modern centrifugation system aided with a new plant enzyme preparation (having prevalently pectolytic activity) were characterized. These oils showed a clearly enhanced quality standard, owing to higher levels of some important minor components (phenolics, volatiles, tocopherols, carotenes, and chlorophylls) and to frequently lower concentrations of oxidized triglycerides and diglycerides. The oils were therefore characterized by lower susceptibility to oxidation and longer shelf life, and their flavor, aroma, and color features appeared to be significantly improved. The saponifiable fraction was practically not affected as the enzymatic effects involved only the membranes of the oil droplets, where the nonglyceridic compounds are essentially located.

KEYWORDS: Virgin olive oil; pectolytic enzymes; sensory evaluation; chemometrics; oil composition; typicality

INTRODUCTION

Virgin olive oil is a natural product claimed to have biological, antioxidizing, and health properties. Therefore, its commercial value is high due also to its high production costs (*1*). At present, olive processing is carried out using industrial discontinuous (pressing) or continuous (centrifugation) systems. Both quality and yields have not been optimized yet. In fact, these mechanical systems are capable of extracting no more than 80−90% of the oil contained in the fruit. The overall content of residual oil in the byproducts (olive pomace and vegetable water) reaches values up to 40 kg/ton of olives processed. This means a great monetary loss for the olive oil sector. In addition, the quality level of the recovered oil is frequently not satisfactory, especially when the pressing method is used. In this case organoleptic defects ("rinsed with wine", "fustiness", and others) can characterize the oil (*2*). Such problems are not solvable using a double extraction cycle. This, in fact, increases the production costs and induces positive effects only on the yields, without improving the oil quality. Some researchers are studying the use of natural enzyme preparations (vegetable extracts) to aid the above mechanical extraction systems (*2*, *3*). These preparations are obtained from organisms not genetically modified and contain enzyme species that are also present in the olive fruit. Such enzymes are claimed to have a role in determining the level of both oil quality and yield. Unfortunately, the fruit enzymes (endogenous enzymes) are largely inactivated during the critical crushing step, and this inactivation could likely be due to oxidized phenols that could bind to their prosthetic group (*3*). To replace them, active exogenous enzymes need to be added to the olive paste during processing.

In this work a new complex enzyme preparation degrading the uncrushed vegetable cell walls and promoting the release of functional components was tested, using olives of different nature and investigating thoroughly the composition of the resulting oils. The objective was to set up an innovative environmentally friendly extracting technology yielding high-quality oils and worth proposing to the European Commission to draft a new CE regulation recognizing the biological processing techniques.

MATERIALS AND METHODS

Chemicals and Equipment. Most of the solvents, reagents, and equipment used for the analytical characterization of the oil samples have been given in earlier works (*4*, *5*). Chemicals were mostly of chromatographic grade and were commercially available from Carlo Erba (Milan, Italy), Fluka (Buchs, Switzerland), and Sigma-Aldrich Chemical (St. Louis, MO).

Features of the Enzyme Preparation Used. The enzyme complex tested, *Rapidase adex* D (Gist-Brocades, Seclin City, France), is essentially made up of pectolytic, cellulolytic, and hemicellulolytic enzyme species. Its activity is not less than 2000 units/mL, 1 unit of activity being defined as the amount of enzyme complex that liberates 1 μmol of reducing sugars per minute from pectins. It degrades the vegetable colloids (pectins, hemicelluloses, proteins, etc.) emulsifying the minute oil droplets. The oil−water emulsions are likewise removed

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as it also contains an endopolygalacturonase enzyme. The rheological characteristics of the olive paste are in addition improved. The enzyme preparation is water-soluble and comes out in the liquid effluent (wastewater) during the final step (oily must centrifugation) of the extraction cycle.

Olive Varieties Employed. Dritta, Leccino, and Coratina olive varieties (*Olea europaea* L.), yielding typical Italian oils bearing a European trademark, were processed. They were produced according to the organic agriculture rules, using neither pesticides nor inorganic fertilizers (*6*), and were harvested mechanically from trees grown in central Italy in October−November 2001, when their removal strength, measured by a dynamometer, reached a value of 470−480 g. Their contents of oil were 21.5, 18.5, and 17.6% (mean of four replicates; CVs all <7%), respectively.

Preparation of Oil Samples. Virgin olive oil samples were obtained by a Novoil EDJ/1 enzyme-assisted dual phase centrifuging processing system (Rapanelli, Foligno, Italy). The industrial processing cycle included the following steps: (i) washing and defoliation of the olive batches; (ii) grinding by a mobile hammer crusher with a sieve size of 6 mm; (iii) malaxation (beating) of the resulting paste for 1 h at 30 °C; (iv) dilution of the paste with 100 L/h tap water at 30 °C and subsequent extraction by a horizontal centrifuge (decanter) operated at 4000 rpm (no water was added with the Dritta variety); and (v) separation of the extracted oily must into oil and water by means of a vertical automated discharge centrifuge. The liquid enzyme formulation, after dilution with lukewarm water (1:4, v/v), was added to the oily paste at the beginning of the malaxation step, using an inoculum of 600 units/kg of olives. For each variety, a homogeneous sample of 1.6 tons of olives was processed, 0.8 ton with the enzyme complex and 0.8 ton without the enzyme complex. Each half was divided into four equal 200 kg parts, which were processed as replicate batches. For each experiment, samples of olives, oils, vegetable water, and husk were drawn for analyses.

Analysis of Olive and Byproduct Samples. These were analyzed for the content of oil, using a Soxhlet apparatus and petroleum ether (40−70 °C) as the solvent (*7*).

Analyses of Oil Samples. These were fully characterized by determining both glyceridic and nonglyceridic components, including those related to quality, flavor, aroma defects, shelf life, color, and genuineness. Most of the analytical methods used have been outlined in previous works (*4*, *5*, *7*).

Triglyceride and diglyceride contents were determined by ^{13}C nuclear magnetic resonance (NMR) using a Bruker 300 spectrometer (Bruker Instruments, Karlsruhe, Germany) operated at 30.0 MHz. The oil spectra were run in $CDCl_3$ (deuteriochloroform) (250 mg of oil/0.5 mL of $CDCl_3$). Chemical shifts were relative to the signal of Me_4Si (tetramethylsilane) (*8*). The contents of individual triglycerides, expressed as percent of the total triglycerides, were determined by high-performance liquid chromatography (HPLC), using an LDC_4 100 Ms model system equipped with a Shodex RF Se-61 differential refractometer and a chromoject integrator (Thermo Separation Products, Schaumburg, IL). Separation was carried out by a 250 mm × 4.5 mm i.d., 5 μm reversed-phase column, coated with Supelcosil LC-18 (Supelco Inc., Bellefonte Park, PA). The mobile phase consisted of an acetone/acetonitrile (60:40, v/v) mixture, which was pumped at 1 mL/min at room temperature (*9*). The levels of oxidized triglycerides and triglyceride oligopolymers were determined by high-performance size exclusion chromatography (HPSEC). The chromatographic system consisted of a Perkin-Elmer pump series 10, a 7125 S sample injector (Rheodyne), a 50 μL injector loop, and a series of three 30 mm × 7.5 mm PL-gel columns (Perkin-Elmer, Beaconsfield, U.K.). The detector was a differential refractometer connected to an integrator. CH_2Cl_2 for HPLC was the elution solvent at a flow rate of 1.0 mL/min (*10*). Peak identification in the chromatograms and quantitative determinations of these compounds were performed as described previously (*11*). A chromatographic silica gel column as described in the IUPAC method was used (*12*). This procedure also gave the content of diglycerides and other polar compounds.

Fatty acid composition, calculated as the percent of the total fatty acids, was determined by capillary gas chromatography (cGC), after conversion to methyl esters (FAMEs). Separation was done on a 25 m × 0.35 mm i.d., 0.25 μm fused-silica capillary column coated with biscyanopropyl-cyanopropylphenylsiloxane (Nordion Ltd., Helsinki, Finland), using hydrogen as the carrier gas (column pressure = 50 kPa) (*9*). The content of total saturated fatty acids in position 2 of triglycerides was also determined (*9*).

Tocopherols were analyzed by HPLC with a 300 mm × 3.9 mm i.d., 10 μm direct-phase M-porasil column (Waters Corp., Milford, MA), using a hexane/propan-2-ol (98.5:1.5, v/v) eluent and a UV detector at 292 nm (*2*).

Volatiles were quantified by a dynamic headspace (DHS)−cGC method, using a 25 m × 0.32 mm i.d., 0.20 μm Carbowax 20 M capillary column coated with ethylene glycol (Nordion Ltd.). These substances, prior to analysis, were stripped for 2 h at 37 °C in a nitrogen stream, entrapped by activated charcoal, and eluted with diethyl ether. The internal standard was nonan-1-ol (>99% pure) (*5*).

Secoiridoid derivatives (tyrosol, hydroxytyrosol, and their aglycons), after extraction with methanol, were analyzed by cGC using a 25 m × 0.32 mm i.d., 0.20/μm capillary column coated with dimethylpolysiloxane (Lab. Service Analitica Ltd., Anzola Emilia, Bologna, Italy). The internal standard was resorcinol (>99% pure) (*4*). Total polyphenols and *o*-diphenols were quantified colorimetrically (*4*).

Sterols, diterpene dialcohols, long-chain aliphatic alcohols, and triterpene alcohols were determined by cGC, using a 25 m × 0.30 mm i.d., 0.20 μm capillary glass column coated with phenyldimethylpolysiloxane (Supelco Inc.). The internal standard was α-cholestanol or arachidilic alcohol (*4*, *9*). The alcoholic index (ratio of major aliphatic alcohols to major triterpene alcohols) × (ratio of geranylgeraniol to C_{22}) was also assessed (*5*). Carotenes and chlorophylls were determined colorimetrically (*13*). The chromatic parameters, from which Naudet's integral color index was assessed, were determined by transmittance measurements (*2*). Oxidative stability was evaluated by Swift's accelerated test (120 °C; air flow rate of 20 L/h), assuming as an oxidation mark the induction time of the peroxidizing reactions (*4*). The UV indices (specific extinctions), k_{232} and k_{270}, were determined on an oil sample dissolved in isooctane (*9*). The carbonyl index was determined colorimetrically with phloroglucin reagent obtaining the absorbance values at 540 nm (*5*). Free acid content and peroxide value were evaluated by titrimetric methods (*9*).

Finally, the quantitative descriptive sensory profiling (QDSP) was performed according to the procedure described in Annex XII of CE Regulation 2568/91 (*9*) and subsequent modifications (*14*). Olfactory−gustatory−tactile evaluations were made. All oil samples were thermostated at 30 °C prior to analysis. The perceptions were assessed for each sensory attribute on a nonstructured scale. A standard profile sheet and an official glossary were used. A fully trained analytical taste panel recognized by the International Olive Oil Council (IOOC), Madrid, Spain, and made up of 12 assessors performed these appraisements, using isolated air-conditioned booths. Next, an overall evaluation of the magnitudes of positive and negative (off-flavors) attributes was made, and the sensory score was obtained on a grading nine-point scale from 1 (lowest quality) to 9 (optimal quality).

The oil samples ($n = 24$) were stored frozen (at −20 °C) until the moment of the chemical and sensory analyses, using hermetically sealed green screw-capped glass bottles (250 mL) without headspace.

Statistics. A 2 × 3 factorial design (two extraction techniques × three olive varieties) was adopted. To test for the enzyme effects on the qualitative and quantitative results, the two-sided variance analysis (ANOVA) with replications was used. When a significant *F* value was found, means were separated using Tukey's post hoc pairwise test (*15*). Multivariate parametric techniques, such as principal component analysis (PCA) and hierarchical cluster analysis (HCA), as well as the nonparametric *K* nearest neighbor classification (KNN) method, were also used (*15*, *16*). The statistical software packages Statistica release 6.0 (Statsoft Inc., Tulsa, OK) and Minitab release 13.0 (Minitab Inc., State College, PA) were used.

RESULTS AND DISCUSSION

Free Acid Content, Peroxide Value, UV Indices, and Sensory Scoring. These are the analytical parameters considered by the in-force regulations to assess virgin olive oil quality (*9*).

Table 1. Values of the Official Qualitative Parameters in Three Enzyme-Treated Virgin Olive Oils As Compared to the Controls[a]

	Dritta		Leccino		Coratina	
analytical oil parameter	enzyme	control	enzyme	control	enzyme	control
acidity (as oleic acid, g/kg)	8.0 (0.4)	7.1 (0.3)	16.1 (0.7)*	19.2 (0.4)	8.1 (0.3)	7.2 (2.8)
peroxide value (mequiv of O_2/kg)	14.3 (0.7)	14.1 (0.8)	12.5 (0.8)	14.0 (0.7)	19.2 (0.7)	19.2 (0.8)
k_{232}	1.80 (0.10)	1.71 (0.10)	1.70 (0.11)	1.82 (0.11)	1.90 (0.10)	2.01 (0.11)
k_{270}	0.10 (0.00)	0.11 (0.00)	0.11 (0.00)	0.10 (0.00)	0.20 (0.00)	0.21 (0.00)
sensory scoring (panel test)	7.5 (0.3)**	7.0 (0.3	7.0 (0.5)**	6.5 (0.4)	7.6 (0.4)*	7.2 (0.3)

[a] Data are means of four replicates. Standard deviations are shown in parentheses. Within each row, values with one or two asterisks are significantly different from the corresponding controls (Tukey's HSD range test; *, $p < 0.05$; **, $p \leq 0.01$).

Table 2. Values of Major Analytical Parameters Related to Flavor, Aroma, or Shelf Life in Three Enzyme-Treated Virgin Olive Oils As Compared to the Controls[a]

	Dritta		Leccino		Coratina	
analytical oil parameter	enzyme	control	enzyme	control	enzyme	control
pleasant volatiles[b] (as nonan-1-ol, mg/kg)	305 (28)**	218 (19)	440 (35)**	283 (23)	969 (75)**	858 (74)
phenols (as caffeic acid, mg/kg)	92 (7)*	64 (4)	89 (5)*	60 (4)	128 (9)**	93 (7)
o-diphenols (as caffeic acid, mg/kg)	49 (4)*	36 (3)	52 (4)*	31 (2)	73 (6)**	37 (3)
secoiridoid derivatives[c] (as resorcinol, mg/kg)	36 (3)*	24 (2)	33 (2)**	20 (1)	66 (4)**	46 (3)
phenolic antioxidants/polyunsaturated fatty acids	8.4 (0.6)**	5.8 (0.3)	8.8 (0.5)**	6.1 (0.3)	13.9 (1.0)**	10.3 (0.5)
tocopherols (a + y, mg/kg)	100 (8)*	86 (6)	242 (14)**	185 (15)	210 (16)**	185 (14)
Swift's test (h)	8.0 (0.2)**	6.6 (0.2)	6.8 (0.1)*	5.6 (0.1)	8.0 (0.2)**	5.3 (0.1)
carbonyl index	5.0 (0.2)	5.0 (0.2)	5.5 (0.1)	4.9 (0.1)	5.3 (0.2)	5.3 (0.2)
triacylglycerol oligopolymers (g/kg)	nd[d]	nd	0.2 (0.0)	0.2 (0.0)	0.3 (0.0)	0.4 (0.0)
oxidized triacylglycerols (g/kg)	6.8 (0.3)*	6.2 (0.2)	6.9 (0.1)**	7.6 (0.2)	10.7 (0.3)**	11.6 (0.2)
diacylglycerols[e] (g/kg)	22.0 (0.9)*	20.6 (0.7)	28.1 (0.9)*	30.3 (1.0)	23.4 (0.6)	24.2 (0.4)
1,2-diacylglycerols/total diacylglycerols ratio	1.0 (0.0)**	0.5 (0.0)	0.6 (0.0)*	0.4 (0.0)	1.0 (0.1)*	0.6 (0.0)

[a] Data are means of four replicates. Standard deviations are shown in parentheses. Within each row, values with one or two asterisks are significantly different from the corresponding controls (Tukey's HSD range test; *, $p \leq 0.05$; **, $p \leq 0.01$). [b] Includes *trans*-2-hexenal, *trans*-2-hexen-1-ol, *cis*-3-hexenyl acetate, *cis*-3-hexen-1-ol, pent-1-en-3-one, *cis*-2-pentenal, *trans*-2-pentenal, and others. [c] Free tyrosol and hydroxytyrosol and their aglycons. [d] nd, not detectable (<0.1 g/kg). [e] As determined by HPSEC method.

They were generally not meaningfully affected by the enzymatic treatment of olive paste (**Table 1**). Only the sensory scoring, a major quality parameter, increased significantly (**Table 1**), and this was due to the higher levels of flavor and aroma constituents (**Table 2**). Both treated and untreated oils showed no sensorial defects, so they were scored only on the basis of their positive attributes. Their most remarkable sensory descriptors were fruitiness, cut green lawn, green leaf of twig, green olives, wild flowers, green banana, green tomato, almond, artichoke, apple, walnut husk, green hay, bitter, and pungent. Nuances for each of them were often perceived by the assessors.

Phenols, Volatile Compounds, Tocopherols, Stability to Oxidation, Carbonyl Index, Polar Compounds, Oxidized Triglycerides, Triglyceride Oligopolymers, and Diglycerides. These analytical parameters are related either to flavor and aroma or to shelf life, oxidation state, and hydrolytic degradation (*2*, *10*, *11*). The treated oils showed an increase in the contents of total phenols, *o*-diphenols, and secoiridoid derivatives, such as major free phenols (tyrosol and hydroxytyrosol) and major hydrolyzable phenols (dialdehydic forms of elenolic acid containing either tyrosol or hydroxytyrosol) (**Table 2**). An increase concerning the phenols/polyunsaturated fatty acids ratio, which is claimed to have great importance in predicting shelf life (*2*, *4*), was also observed. These biologically active compounds are claimed to be flavor components and to have antioxidant and health properties (*2*). They are major contributors to the bitterness, pepper-like, astringency, and fruitiness flavors (*4*, *17*, *18*). Evidently, the enzyme effect on the parenchyma tissue of the fruit increased the release of such constituents, thus increasing their dissolution into the oil phase. According to some authors, the phenol increase could also be partly due to a reduced complexation of phenolics with polysaccharides (*3*).

Olive fruit contains only complex phenol species, such as oleuropein, ligustroside, verbascoside, rutin, and luteolin 7-glucoside (*19*). During extraction less complex phenol compounds or simple phenols form, owing to enzymatic or chemical hydrolytic reactions (*2*, *4*, *5*). In addition, the hydrosoluble catecholmelanin pigment forms because of polymerization processes involving *o*-diphenol compounds, catalyzed by the polyphenol oxidase (PPO) and peroxidase (POD) enzymes. This brown pigment has been found to be the most polluting component of the liquid effluent (*5*).

Similarly, the treated oils exhibited higher contents of volatiles (aldehydes, alcohols, esters, and carbonyls) (**Table 2**). These compounds were mainly represented by the unsaturated *trans*-2-hexenal aldehyde together with other C_6 metabolites arising from the lipoxygenase (LOX) pathway, the precursors of which are the 13-hydroperoxides of linoleic or linolenic acid, polyunsaturated compounds containing a *cis*,*cis*-1,4 pentadiene system (*20*). The C_6 metabolites as well as the C_5 volatiles, probably originating from another LOX pathway, are considered to be major contributors to the green notes and the fruity sensation. Frequently, they have relationships with typicality (*21*). Among such pleasant volatiles, hexyl acetate seems to contribute to the fruity and sweet notes; hexanal to the apple sensation; *trans*-2-hexenal to the almond, banana, lawn, and bitter attributes; *trans*-2-hexen-1-ol to the flower and tomato perceptions; *cis*-3-hexenyl acetate to the leaf odor; *cis*-3-hexen-1-ol to the grassy smell; pent-1-en-3-one to the tomato, bitter, and sharp flavors; *cis*-2-pentenal to the almond taste; and finally *trans*-2-pentenal to the green fruit flavor, which is reminiscent

Table 3. Values of Analytical Parameters Related to Color in Three Enzyme-Treated Virgin Olive Oils As Compared to the Controls[a]

	Dritta		Leccino		Coratina	
analytical oil parameter	enzyme	control	enzyme	control	enzyme	control
chlorophylls (mg/kg)	18.3 (1.0)*	15.4 (1.0)	21.5 (1.4)*	18.7 (1.3)	20.4 (1.3)*	16.0 (0.9)
carotenes (mg/kg)	30.7 (2.1)	29.6 (1.7)	27.7 (1.2)*	23.6 (1.1)	38.3 (2.0)*	34.4 (1.8)
chroma (%)	99.7 (4.3)*	98.3 (5.0)	97.7 (4.8)**	93.7 (6.8)	98.7 (5.3)	98.4 (5.8)
brightness (%)	64.5 (4.1)*	66.5 (3.9)	65.6 (3.0)*	66.2 (2.6)	51.6 (2.6)**	58.8 (3.1)
hue (nm)	578 (6)	578 (7)	578 (6)	577 (5)	579 (8)	579 (8)
Naudet's color index	19.0 (0.7)**	16.9 (0.6)	17.9 (0.7)*	16.8 (0.6)	28.4 (1.0)**	22.7 (0.9)

[a] Data are means of four replicates. Standard deviations are shown in parentheses. Within each row, values with one or two asterisks are significantly different from the corresponding controls (Tukey's HSD range test; *, $p \leq 0.05$; **, $p \leq 0.01$).

of healthy, fresh olive fruits harvested at the proper ripening degree (*20*, *21*).

By contrast, the treated oils showed lower contents of total unpleasant volatiles, including *n*-octane, ethyl acetate, isobutyl alcohol, *n*-amyl alcohol, acetic acid, and others (data not shown). This may be attributed to the olive paste rheology improvement, which resulted in greater processing speed. Consequently, the unavoidable and undesirable fermentation processes, involving reducing sugars (fructose and glucose) or amino acids (valine, leucine, and isoleucine), as well as the peroxidation processes, involving mainly the polyunsaturated fatty acids, were to a large extent prevented (*5*, *20*). The oils in question were also characterized by higher levels of two major tocopherols, α- and γ-tocopherol, which showed a trend similar to the contents of volatiles or phenols (**Table 2**). The tocopherol constituents are claimed to have both vitamin and antioxidant properties (*2*).

Values obtained by Swift's accelerated oxidation test, referring to the induction time parameter and related to shelf life (*2*, *5*), were higher when the enzyme formulation was used, thus confirming that the resulting oils had higher contents of natural antioxidants (**Table 2**). These oils, in general, showed lower contents of diglycerides and oxidized triglycerides, suggesting that they had a better starting oxidation state. The overall content of diglycerides and free fatty acids is indicative of the hydrolytic degradation degree (*10*, *11*). The 1,2-diglycerides/total diglycerides ratio (glyceridic index), which has been considered to be a quality marker (*5*, *7*), seemed to be higher in the above treated oils (**Table 2**), probably due to a selective enzymatic mechanism of triacylglycerol hydrolysis during extraction. Finally, these oils showed frequently lower contents of total polar compounds, which include free fatty acids, diglycerides, and products of triglyceride oxidation and polymerization (**Table 2** and **Figure 1**). The ^{13}C NMR and HPSEC methods used for determination of the diglycerides were both accurate and reproducible and therefore gave strongly correlated results ($r = 0.9451$***; $p \leq 0.001$).

Chloroplast Pigments and Color. The enzyme preparation exerted a significant effect on the color of the oils. In fact, greater amounts of chlorophylls and carotenes were released from the hypoderm tissue of the fruit (**Table 3**), where these chloroplast pigments are mostly located (*2*). These findings were confirmed by values of the integral color index (Naudet's index) and the parameters chroma and brightness relating, respectively, positively and negatively with color (*5*). For both treated and untreated oils, the values of hue fell steadily in the range 577−579 nm, indicating that the yellow color prevailed over green. Quality is closely associated with the color features (*2*, *13*). The above natural colorings (lipochromes) are largely degraded during olive ripening and oil storage, and the level of their breakdown products is considered to be a reliable index of the oil freshness (*5*). According to recent studies, these constituents would have important nutritional, physiological, and pharmaceutical properties (*22*).

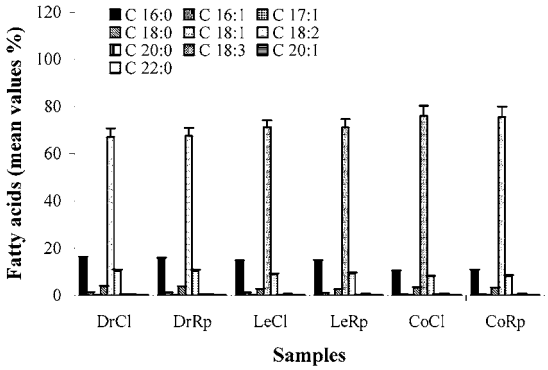

Figure 1. Fatty acid composition (as percent of total fatty acids) of enzyme-treated (Rp) or untreated (Cl) virgin olive oils from Dritta (Dr), Leccino (Le), and Coratina (Co) cultivars. Data are means of four replicates. Error bars indicate standard deviations. Within each variety, the statistical differences between homologous fatty acids are not significant at $p \leq 0.05$ (Tukey's HSD range test). C14:0 = myristic acid; C16:0 = palmitic acid; C16:1 = palmitoleic acid; C17:0 = heptadecanoic acid; C17:1 = heptadecenoic acid; C18:0 = stearic acid; C18:1 = oleic acid; C18:2 = linoleic acid; C18:3 = linolenic acid; C20:0 arachidic acid; C20:1 = eicosenoic acid; C22:0 = behenic acid; C24:0 = lignoceric acid.

Genuineness Parameters. The contents of waxes, sterols, triterpene dialcohols, long-chain aliphatic alcohols, superior triterpene alcohols, glycerol triesters, and esterified fatty acids in the oils did not seem to be modified by the olive paste enzyme treatment (**Table 4** and **Figures 1** and **2**). The content of total saturated fatty acids in position 2 of the triglycerides was not affected either (data not shown). With such classes of components the variance in the experimental data was essentially attributable to the olive variety factor. Sterols, triterpene dialcohols, fatty acids, saturated fatty acids in position 2 of triglycerides, trilinolein, and waxes are official genuineness markers and are used in disclosing commercial frauds (*9*). These parameters showed values falling always within the limits set by the official standard (*9*).

Results of Multivariate Analyses. The nonparametric technique KNN, based on the analytical data concerning the nonglyceridic oil components, proved to be effective in grouping the oil samples by variety (**Figure 3**). In fact, both error rate and error risk tended to zero.

Inspection of the biplot (score and loading plot) in **Figure 4** revealed that the PCA technique, based on the fatty acid composition data, was likewise capable of characterizing the oil variety. In fact, along the first dimension (accounting for 71.5% of the total variance) were differentiated the Coratina (negative half) and Dritta (positive half) varieties, whereas along the second dimension (accounting for 15.1% of the total

Table 4. Values of Analytical Parameters Related to Genuineness in Three Enzyme-Treated Virgin Olive Oils As Compared to the Controls[a]

	Dritta		Leccino		Coratina	
analytical oil parameter	enzyme	control	enzyme	control	enzyme	control
waxes (mg/kg)	265 (20)	261 (18)	571 (41)	591 (49)	139 (12)*	117 (9)
sterols (g/kg)	1.1 (0.1)	1.1 (0.1)	1.4 (0.1)	1.5 (0.1)	1.0 (0.1)	1.0 (0.1)
triterpene dialcohols (mg/kg)	32.3 (2.2)	31.1 (2.2)	31.0 (2.4)	39.8 (3.0)	59.2 (4.9)	55.9 (4.9)
aliphatic alcohols (mg/kg)	297 (23)	272 (20)	319 (22)*	280 (20)	149 (12)	131 (11)
triterpene alcohols (g/kg)	1.8 (0.2)	1.5 (0.1)	1.4 (0.1)	1.4 (0.1)	2.2 (0.2)*	1.9 (0.1)
alcoholic index	0.5 (0.0)	0.5 (0.0)	0.7 (0.0)	0.7 (0.1)	0.1 (0.0)	0.1 (0.0)
triacylglycerols[b] (%)	98.2 (5.9)	98.2 (5.4)	98.7 (4.7)	98.5 (5.0)	98.9 (4.6)	98.7 (4.8)

[a] Data are means of four replicates. Standard deviations are shown in parentheses. Within each row, values with one or two asterisks are significantly different from the corresponding controls (Tukey's HSD range test; *, $p \leq 0.05$; *, $p \leq 0.01$). [b] As determined by ^{13}C NMR method.

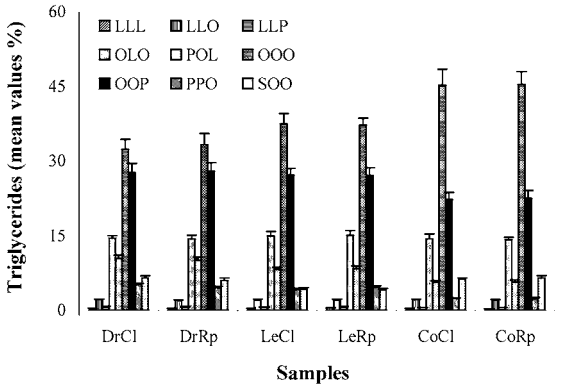

Figure 2. Triglyceride composition (as percent of total triglycerides) of enzyme-treated (Rp) or untreated (Cl) virgin olive oils from Dritta (Dr), Leccino (Le), and Coratina (Co) cultivars. Data are means of four replicates. Error bars indicate standard deviations. Within each variety, the statistical differences between homologous triglycerides are not significant at $p \leq 0.05$ (Tukey's HSD range test). LLL = trilinolein; LLO = 1,2-dilinoleoyl-3-oleoylglycerol; LLP = 1,2-dilinoleoyl-3-palmitoylglycerol; OLO = 1,3-dioleoyl-2-linoleoylglycerol; POL = 1-palmitoyl-2-oleoyl-3-linoleoylglycerol; OOO = triolein; OOP = 1,2-dioleoyl-3-palmitoylglycerol; PPO = 1,2-dipalmitoyl-3-oleoylglycerol; SOO = 1-stearoyl-2,3-dioleoylglycerol.

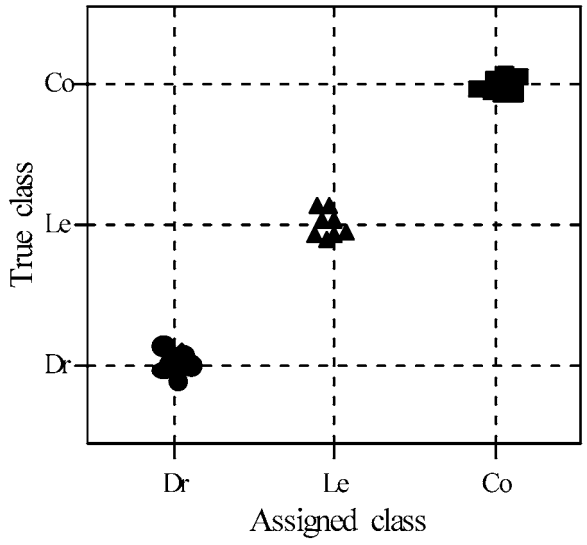

Figure 3. Classification of virgin olive oils from Dritta (●), Leccino (▲), and Coratina (■) cultivars based on values of the nonglyceridic components, using the nonparametric KNN method.

variance) was discriminated the Leccino variety (negative half). Moreover, with this method, within each variety, the treated oils appeared to be differentiated from the untreated oils.

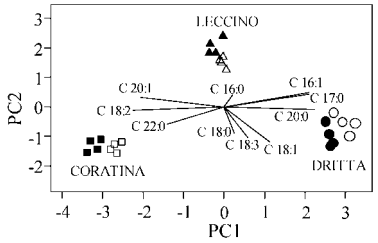

Figure 4. Score and loading plot (based on the fatty acid composition data set) by dimensions 1 and 2 from PCA, showing groupings (varieties) and subgroupings, such as enzyme-treated (solid symbols) and untreated (open symbols) virgin olive oils.

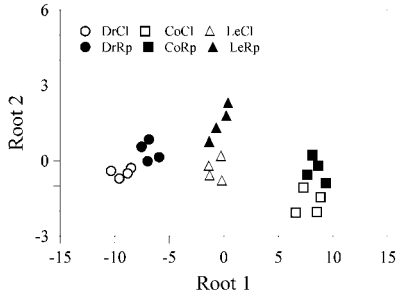

Figure 5. Plot by the roots (canonical functions) 1 and 2 from CDA (based on the triglyceride composition data set), showing groupings, such as Dritta (Dr), Leccino (Le), and Coratina (Co) varieties, and subgroupings, such as enzyme-treated (Rp = solid symbols) and untreated (Cl = open symbols) virgin olive oils.

Comparison between the two PCA plots suggests that palmitoleic (C16:1), heptadecanoic (C17:0), arachidic (C20:0), and oleic (C18:1) acids were mainly responsible for discrimination of the Dritta variety, whereas linoleic (C18:2), eicosenoic (C20:1), and behenic (C22:0) acids were the major contributors to separation of the Coratina variety; finally, palmitic acid was the sole contributor to differentiation of the Leccino variety.

The CDA multivariate technique, based on the triglyceride composition data, generated a plot (**Figure 5**) showing groupings (oil varieties) with subgroupings (treated or untreated oils).

On the basis of the above results of multivariate analyses, it can be concluded that the use of the enzyme preparation in olive processing does not raise any problems regarding traceability of the typical oils bearing a European protected origin denomination (POD) trademark or a European protected geographical indication (PGI) trademark. In fact, most of the variance in the analytical data is accounted for by the olive variety factor (genetic store).

Thus, the recognition of the use of such enzyme preparation and other effective ones in olive processing will lead to enhanced quality levels of virgin olive oil and reduced environmental

impact of the liquid effluent, especially when such biological extracting techniques are combined with biological olive production techniques. On the other hand, enzyme preparations have already successfully been introduced in several agrofood industries, such as enological (Klerzyme and AR 2000), fruit juice (Clarex and Pearex), dairy (Maxdren and Deluoren), beer (Brewers and Filtrase), and other industries.

Oil Yields. The enzyme preparation effects also resulted in substantial increase of the oil yields, which ranged from 11.3 to 16.7 kg/ton of olives, regardless of the variety processed. Evidently, greater amounts of oil were freed from the vegetable tissue, and in addition the coalescence phenomena involving the minute oil droplets were more marked (*2*, *5*). The minimum and maximum mode class limits, between which fell nearly 84% of the observations, of the frequency distribution of the yield increase variable were 12.5 and 14.6 kg of oil/ton of olives. These quantitative findings were corroborated by the lower contents of overall residual oil found in the byproducts, which ranged from 1.3 to 2.2% of dry matter. In addition, the enzyme preparation produced a more environmentally friendly liquid waste, with a probable reduction of the polluting potential of ~30%, owing to lower contents of oil and suspended solids as the separation, during centrifugation, of the oil and water phases from the solid phase was more effective.

ABBREVIATIONS USED

POD, protected origin denomination; PGI, protected geographical indication; FAMEs, fatty acid methyl esters; cGC, capillary gas chromatography; DHS, dynamic headspace; PL, polymer laboratories; HPSEC, high-performance size exclusion chromatography; PCA, principal component analysis; HCA, hierarchical cluster analysis; KNN, *K* nearest neighbor classification; CDA, canonical discriminant analysis; ANOVA, analysis of variance; CV, coefficient of variation; CE, European Community; IOOC, International Olive Oil Council; IUPAC, International Union of Pure and Applied Chemistry; QDSP, quantitative descriptive sensory profiling; LOX, lipoxygenase; HSD, honestly significant difference.

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LITERATURE CITED

(1) Leenen, R.; Roodenburg, A. J. C.; Vissers, M. N.; Schuurbiers, J. A. E.; van Putte, K. P. A. M.; Wiseman, S. A.; van dePut, F. H. M. M. Supplementation of plasma with olive oil phenols and extracts: influence on LDL oxidation. *J. Agric. Food Chem.* **2002**, *50*, 1290−1297.

(2) Ranalli, A.; Angerosa, F. Integral centrifuges for olive oil extraction. The qualitative characteristics of products. *J. Am. Oil Chem. Soc.* **1996**, *73*, 417−422.

(3) Vierhuis, E.; Servili, M.; Baldioli, M.; Schols, H. A.; Voragen, A. G. I.; Montedoro, G. F. Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. *J. Agric. Food Chem.* **2001**, *49*, 1218−1223.

(4) Ranalli, A.; Ferrante, M. L.; De Mattia, G.; Costantini, N. Analytical evaluation of virgin olive oil of first and second extraction. *J. Agric. Food Chem.* **1999**, *47*, 417−424.

(5) Ranalli, A.; Costantini, N.; De Mattia, G.; Ferrante, M. L. Evaluating two kinds of centrifuged virgin oils arising from continuous olive processing. *J. Sci. Food Agric.* **2000**, *80*, 673−683.

(6) European Community. Regulation 2092/91. *Off J. Eur. Communities* **1991**.

(7) Ranalli, A.; Pollastri, L.; Contento, S.; Di Loreto, G.; Iannucci, E.; Lucera, L.; Russi, F. Acylglycerol and fatty acid components of pulp, seed, and whole olive fruit oils. Their use to characterize fruit variety by chemometrics. *J. Agric. Food Chem.* **2002**, *50*, 3775−3779.

(8) Vlahov, G. Improved quantitative ^{13}C nuclear magnetic resonance criteria for determination of grades of virgin olive oil. The normal ranges for diglycerides in olive oils. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1201−1203.

(9) European Community. Regulation 2568/91. *Off J. Eur. Communities* 1991.

(10) Gomes, T. Oligopolymer, diglyceride and oxidized triglyceride contents as measure of olive oil quality. *J. Am. Oil Chem. Soc.* **1992**, *69*, 1219−1223.

(11) Gomes, T.; Caponio, F. Effort to improve the quantitative determination of oxidation and hydrolysis compound classes in edible vegetable oils. *J. Chromatogr. A* **1999**, *844*, 77−86.

(12) IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th ed.; Blackwell Scientific Publications: Oxford, U.K., 1987; No. 2507.

(13) Gandul-Rojas, B.; Minguez-Mosquera, M. Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varieties. *J. Sci. Food Agric.* **1996**, *72*, 31−39.

(14) European Community. Regulation 796/02. *Off J. Eur. Communities* **2002**.

(15) Statistica. *User's Guide*, release 6.0; Statsoft: Tulsa, OK, 1999.

(16) Minitab. *User's Guide*, release 13.0; Minitab: State College, PA, 1995.

(17) Briante, R.; La Cara, F.; Tonziello, M. P.; Febbraio, F.; Nucci, R. Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by hyperthermophilic β-glycosidase. *J. Agric. Food Chem.* **2001**, *49*, 3198−3203.

(18) Gutierrez, F.; Albi, M. A.; Palma, R.; Rios, J. J.; Olias, J. M. Bitter taste of virgin olive oil: correlation of sensory evaluation and instrumental HPLC analysis. *J. Food Sci.* **2000**, *49*, 4231−4235.

(19) Briante, R.; La Cara, F.; Febbraio, F.; Patumi, M.; Nucci, R. Bioactive derivatives from oleuropein by a biotransformation on *Olea europaea* leaf extracts. *J. Biotechnol.* **2002**, *93*, 109−119.

(20) Morales, M. T.; Aparicio, R. Effect of extraction conditions on sensory quality of virgin olive oil. *J. Am. Oil Chem. Soc.* **1999**, *76*, 295−300.

(21) Aparicio, R.; Morales, M. T.; Alonso, V. Relationship between volatile compounds and sensory attributes of olive oils by the sensory wheel. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1253−1264.

(22) Britton, G. Biological actions of carotenoids: do oxidation products and metabolites play a key role? In *Proceedings of the 2nd International Congress on Pigments in Food*; Empis, J. A., Ed.; Sociedade Portuguesa de Quimica: Lisbon, Portugal, 2002; pp 33−40.

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