Characterization of Olive Oil Produced with a New Enzyme Processing Aid

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ABSTRACT: By carrying out olive oil extraction experiments with three olive varieties (Dritta, Coratina, and Leccino), a new processing cytolase enzyme aid was tested. The oils, obtained with the enzyme adjuvant upon extraction, were characterized (with respect to reference oils) by: (i) relatively higher content of natural antioxidants (free and linked phenols, *ortho*-diphenols, tocopherols), *trans*-2-hexenal, total aromatic substances, chlorophyllic pigments, and steroid hydrocarbons; (ii) slightly lower content of aliphatic alcohols, triterpene alcohols, triterpene dialcohols, β-sitosterol, and total sterols; (iii) slightly higher values of integral color index, resistance to autoxidation, and global quality indices; (iv) lower values of carotenoid color index, alcoholic index and some qualitative ratios, such as *trans*-2-hexenal/hexanal, *trans*-2-hexenal/total aroma, campesterol/stigmasterol; and (v) a higher sensory score. Hence, they exhibited better overall qualitative characteristics. The enzyme adjuvant, in addition, led to higher oil extraction outputs.
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KEY WORDS: Biotechnology, new processing, olive oil, organoleptic and chemical-physical characteristics, *ortho*-diphenols, other antioxidants, overall quality indices, pectolytic enzyme adjuvant, pigment content, resistance to autoxidation.

Enzymes are widely used in almost all food industry sectors, but they have not yet been legally recognized for use in olive processing, although they are frequently used by oil millers. In earlier times, studies on aids for olive processing mainly aimed at enhancing extraction yields, but at present, research is primarily conducted to improve product quality.

The composition and effectiveness of enzyme adjuvants, currently produced to process olives, appear considerably improved, so that authoritative proposals have been put forward to sanction their use. However, first it is necessary to modify the official norm that controls the modality of olive processing for production of virgin or extra-virgin oils. This will certainly be accomplished in the future.

The new enzyme adjuvant tested by us is a liquid preparation (obtained from Gist-Brocades, Seclin City, France) and has a prevalent pectolytic action, as generally all do that at present are studied for the biochemical treatment of olive pastes; the enzymatic complex in question is essentially constituted by pectinase and cellulolytic hemicellulolytic enzymes plus some minor enzymes. Its activity is not less than 2000 units/mL. One unit of activity is defined as the amount of enzyme complex that liberates 1 µmol of reducing sugars per minute from the galacturonic acid of olive pectins.

The enzymes contained in it are also present in olive fruit, but they are significantly destroyed during the oil extraction process. Therefore, addition of such an enzyme system to the olive pastes replaces (and even enhances) the natural enzymes of olive drupes.

This enzyme formulation degrades the walls of the oil-bearing cells that elude crushing and also has similar effects on the colloidal systems in olive paste (pectins, hemicelluloses, proteins, etc.) that retain the droplets of oil. In this way, the droplets of oil are released (by undergoing phase inversion) and gradually merge into larger droplets until they form a mass of free oil, which is extracted by mechanical means.

The interaction between the oil droplets is called coalescence, which is also induced by mixing. Slow stirring of the paste brings the colloid-bound oil droplets into contact with each other. Through interaction, they combine into larger droplets, the weight of which enables them to overcome the force that binds them to the colloid systems. The enzyme formulation exerts a complementary effect upon that of mixing, which results in heightened coalescence.

The oil inside the oil-bearing cells is partly located in the vacuole (~76%), where it is free, and partly in the cytoplasm (~24%), where it is dispersed in the form of minute droplets bound to colloids (through their lipoprotein membranes). These tiny droplets form microgels that are mainly distributed in pockets and channels in the olive mash. A large portion of the oil fraction in the cytoplasm, which forms an emulsion with the colloids, is released by the action of the enzyme, which thereby increases the percentage of oil that is recovered. As oil processing techniques stand at present, a large amount of this oil is not extracted and is removed with the byproducts. For it to be extracted, it is necessary to modify the state of colloidal dispersion. The enzyme breaks up not only the liquid–solid emulsions (interactions) but also the liquid–liquid emulsions (mainly caused by crushing and centrifuging the paste) through its endopolygalacturonasic effect.

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It also has a positive effect on the rheological characteristics of the paste; after this biochemical treatment, the oil is more readily extracted from the paste, as a result of which the phases are separated more thoroughly (oil, water, solids).

Likewise, the oil that is emulsified with the water cannot be recovered by employing normal technology; centrifugal force is not able to separate the two. Consequently, if the emulsion is not removed, this oil is lost in the vegetable water (effluent). The phenomena of oil–solids interaction then end up involving also part of the vacuolar oil when it has been released from the vacuole. Lastly, the enzyme preparation has yet another positive property: it is water-soluble. This means that, at the end of the extraction process, when it has exerted all its effects, it all comes out in the vegetable water (olive juice) and leaves no residue in the oil. Consequently, oil composition is not modified. Moreover, it is harmless to consumer health.

The oil, which is concentrated in the central cavity of the oil-bearing cell, is synthesized in the mitochondria. It can be considered a waste product of cell metabolism. All other waste substances generated by activity of the mitochondria also accumulate in the vacuole. All substances discharged into the vacuole gather around the oil droplets (which measure a few microns) to form a kind of membrane. This membrane bursts during extraction, only to reform in a more complex manner because of the involvement of other substances contained in the cell juice with which the drops of oil have come into contact. Each substance is spread through the two phases, oil and water, depending on its coefficient of distribution. This is the point at which the final composition of the oil is decided, and hence its chemical and organoleptic quality. As the fruit ripens, the central cavity of the oil-bearing cell becomes larger, owing to the gradual buildup of oil. Toward the end of the ripening process, the vacuole takes up about 90% of the cell volume.

As we will later point out, the effect of the enzyme complex on fruit tissues also results in release of a greater quantity of some important constituents, which dissolve in oil and induce a direct and indirect improvement of product quality.

EXPERIMENTAL PROCEDURES

To execute the experiment, three varieties of olives (Dritta, Coratina and Leccino), produced on the farm of our Institute (Olive and Olive Oil Research Institute, Percana, Italy) were mechanically processed at industrial level in our oil mill by using two-phase continuous equipment. The steps of the technological process were as follows: (i) removal of leaves from olive lots; (ii) milling of drupes by a hammer crusher; (iii) kneading of the resultant paste for 60 min at 30˚C; (iv) centrifugation of paste by a two-phase decanter; and (v) separation of the oily must into oil and water by means of an automated discharge centrifuge. The paste during centrifugation was fluidized by adding ~100 L/h of drinking water that was heated at 30˚C. The quantity of paste centrifuged per hour was 0.6 MT. The enzyme preparation was added to the paste at the beginning of the kneading step by using a dose corresponding to 30 mL per 0.1 MT of olives (0.03% vol/wt) after its dilution with lukewarm water (1:9, vol/vol).

For each variety, a homogeneous sample of 1.8 MT of olives was processed, 0.9 MT with the processing aid and 0.9 MT according to the reference procedure without employing the enzyme adjuvant (control). Each half was divided into three equal parts, which were processed and tested. The compositional characteristics of the olive varieties processed were the following: Dritta, oil 19%, moisture 58.5%, solids 22.5%; Coratina, oil 12.1%, moisture 49.8%, solids 38.1%; Leccino, oil 9.5%, moisture 57.1%, solids 33.4%. The ripening index, determined according to the method developed by INRA of Jaen-Spain, was 2.45, 1.59 and 4.06, respectively, for the three olive varieties.

During testing, samples of the olives were taken, along with byproducts and oils, by applying the same sampling techniques briefly described in previous reports (1–5). The methods applied to perform the analytical assessments on the olives, husk, and vegetable water also are summarized in these references. On the oils, the same analytical evaluations outlined in a previous paper (1) were performed; however, in addition, other instrumental determinations were made, such as: (i) High-resolution gas-chromatographic (HRGC) analysis of the phenolic fraction (6) by means of a 25-m capillary column (internal diameter 0.32-mm, film thickness 0.1-μm, internally covered with SE-54 liquid), after extraction with pure methyl alcohol. The resulting methanolic extract was concentrated to dryness by rotating evaporator, and the residue was subsequently recovered with 10 mL CH_3CN. This solution, after three washings with hexane, was evaporated *in vacuo* at a temperature below 35˚C. The residue was dissolved into acetone, and to 1 mL of this solution, 150 μL of bis(trimethylsilyl)-trifluoroacetamide were added. After 1 h, the injection into the gas chromatograph was effected. The internal standard was resorcinol. This method is a modified version of the one described earlier (1). (ii) High-pressure liquid chromatographic (HPLC) analysis of the phenolic fraction (4) by means of a reversed-phase column, after dissolution of oil in hexane and extraction of minor polar components three times by a 3:2 (vol/vol) methanol/water mixture, and subsequent washing of each extract with hexane, followed by centrifugation, reunion of extracts, evaporation to dryness, and recovery of residue with methanol. The calculation of peaks was made by considering their areas to the total area of the chromatogram colorimetrically quantitated (and expressed as caffeic acid). (iii) ^{13}C Nuclear magnetic resonance (NMR) analysis of the phenolic fraction (7). From 20 g of oil, the minor polar components were extracted, which were then dissolved into 500 μL of deuterated methanol. The resulting solution was placed into a 500-mL NMR tube. The ^{13}C NMR spectrum was recorded from this solution at 100 MHz. (iv) ^{13}C NMR analysis of triglycerides and diglycerides (8): 300 mg of oil was dissolved into 300 μL of deuterated chloroform ($CDCl_3$), and tetramethylsilane was used as an internal reference. From this solution, the ^{13}C NMR spectrum was recorded. (v) Pyrolysis at Curie point (530˚C) in a nitro-

gen inert gas atmosphere or *in vacuo*. By this procedure (9–14), organic molecules are thermally degraded to produce volatile fragments, which, by means of a mass spectrometer coupled with the pyrolysis apparatus, are ionized and separated on the basis of their mass/charge ratio (*m/z*); inputs deriving from the formed ions are recorded into the pyrolysis–mass spectrum of the oil sample (pyrogram or fragmentogram), which represents a picture of the chemical profile or fingerprint of the oil. Normalized data from the pyrogram are statistically processed by applying both conventional (principal component analysis, PCA; canonical variate analysis, CVA; principal component canonical variate analysis, PCCVA; hierarchical cluster analysis, HCA) and neural (artificial neural networks; kohonen artificial neural networks; and others) statistical multivariate analysis methods, to see if the oil samples can be discriminated, i.e., if they are different. These latter chemometric analysis methods are applied by using an artificial neural network (artificial intelligence), which is interfaced with a computer (PCA, CVA, PCCVA, and HCA methods do not give a higher reliability of results). The oil (1 μL) is pyrolyzed for a duration of 3 s by placing it upon a small metal sheet (alloy composed of iron and nickel, 1:1 w/w) and introducing this inside a glass tube, which is fitted in the pyrolysis apparatus, where the oil is irradiated by a radio frequency of 425 MHz. The radiation is absorbed by the magnetic dipoles of the alloy elements, which develop a considerable quantity of heat, up to the Curie temperature. At this point, the sheet stops to exhibit ferromagnetic properties and absorb the radio frequency, with the consequent interruption of heating (electromagnetic thermostasis step). The pyrolysis fragments spread and arrive into the ionization chamber of the mass spectrometer, where they are irradiated by a low-energy electron beam. From a few microliters of oil, an enormous number of ions ($0.5–2.0 \times 10^6$) is formed, whose mass for our samples ranges from 51 to 200. A Rapyd-400 instrument was used, which was provided by Horizon Instruments Ltd. (Sussex, Great Britain). To process the data relating to the pyrolysis–mass spectra, the statistically fitted software Genstat was used, along with Excel 4.0 for Windows. The sophisticated combined pyrolysis–mass techniques, which are recent and innovative, were also used by us to achieve other objectives, i.e., to certify oil controlled origin denomination, protected origin denomination, geographical indication (see Italian law No. 169/92 and EEC regulation No. 2081/92), and furthermore to assess typicality or determine purity or authenticity of oils.

RESULTS AND DISCUSSION

The results obtained, relating to the analytical characteristics of the oils, are given in Tables 1 through 5.

Total polyphenols, ortho-*diphenols, tyrosol, hydroxytyrosol, hydrolyzable phenols, tocopherols.* For all three olive varieties processed, the oils produced by adding the pectolytic enzyme to the oily pastes constantly exhibited higher total polyphenol and *ortho*-diphenol contents (in general, averaging 18.8 and 18.9%). Similar results were observed for tyrosol and hydroxytyrosol (which were the major free phenols present in the oils) and for tyrosol-aglycons and hydroxytyrosol-aglycons. These linked phenols, which were found in oils in quantities decidedly higher than free phenols, have recently been studied and characterized by Montedoro and coworkers (15–18); their chemical structures have revealed that they are linked with dialdehydic forms of elenolic acid, which are esterified with tyrosol or hydroxytyrosol. The resistance to oxidation and organoleptic characteristics of oils is influenced by the most phenolic substances (both free and conjugated phenols). Some aglycons that contain tyrosol and probably an aglycon that contains hydroxytyrosol are responsible for bitter and hot notes of the oil (7). The higher content of phenolic substances (quantitated by HRGC, HPLC, NMR, and colorimetric crossed analyses), recorded for oils extracted by treating the oily pastes with the enzymatic system, suggests that this phenomenon might be ascribed to the biochemical action that the enzyme preparation is able to exert on the complex molecular structures of olive fruit (in which phenol components are plentiful, mainly as glucosides and esters), from which consequently a higher quantity of phenol constituents could be freed and dissolved in the oil. The values for the tocopherols were also higher in the oils produced with the enzyme adjuvant. These substances, in agreement with literature data, were represented almost exclusively by α-tocopherol, while β-tocopherol and others (γ- and δ-tocopherols) were absent or present in low amounts. α-Tocopherol has a marked vitamin action, which is much higher than that of other tocopherols. The latter, on the other hand, exert a higher antioxidant action (δ-tocopherol is the strongest, followed in order by γ- and β-tocopherol).

Panel test, Rancimat stability, global quality indices (GQI_1, GQI_2). The values of resistance to autoxidation of the oils (hours taken by peroxidizing reactions to reach the induction step, at 120°C and an air flow rate of 20 L/h) were always higher (in agreement with the higher phenol substance content) in the oils obtained with the enzyme processing aid. Because of higher phenol content, these oils also received a higher sensory score (even if these components are not the only substances responsible for flavor and "fruity taste" of the product). The oils produced (with or without enzyme aid) were all without defects, and therefore qualitative scores were attributed to them only on the basis of their positive organoleptic attributes. Also, the influence of the phenolic fraction on oxidative stability and upon "fruity taste" of the oils depends on its qualitative composition (19). In effect, some phenolic compounds exert low antioxidant action or are even slight prooxidants, such as tyrosol, vanillic acid, syringic acid, *para*-hydroxybenzoic acid, and *para*-coumaric acid, while other phenolic components are able to produce an antioxygen action (e.g., caffeic acid, protocatechuic acid, hydroxytyrosol). However, the anti- or prooxidant activity degree of each phenolic component has not yet been elucidated well. Almost all phenolic substances contribute to the oil's taste, which is influenced either by their chemical structure or by their quantity (absolute or relative) in the oil.

TABLE 1
Analytical and Compositional Characteristics of Olive Oils Extracted by Using a New Processing Enzyme Aid, Compared to Reference Oils

	Dritta		Coratina		Leccino	
	Aid	Control	Aid	Control	Aid	Control
Acidity (% oleic acid)	0.48	0.44	0.20	0.22	0.17	0.17
Peroxide index (meq O_2/kg)	6.9	6.8	7.2	7.1	5.5	5.8
Carbonyl index—MWI[a]	1.66	3.87	7.19	6.14	2.13	4.97
Total polyphenols (as caffeic acid) (mg/L)[b]	214	178	257	229	117	88
O-diphenols (as caffeic acid) (mg/L)[b]	141	116	175	156	81	62
Tyrosol (as resorcinol) (mg/L)[c]	6.1	4.6	1.7	1.1	2.2	2.1
Hydroxytyrosol (as resorcinol) (mg/L)[c]	3.5	3.0	2.0	1.1	0.9	0.2
Hydroxytyrosol/tyrosol	0.6	0.7	1.2	1.0	0.4	0.1
Tyrosol-aglycons (as resorcinol) (mg/L)[c]	17.8	13.8	30.8	15.3	2.2	0.2
Hydroxytyrosol-aglycons (as resorcinol) (mg/L)[c]	37.2	24.9	46.0	36.0	17.9	8.0
Free phenols/linked phenols	0.17	0.20	0.05	0.04	0.15	0.28
Rancimat stability (h)	12.1	9.8	15.0	15.0	17.1	11.7
Turbidity (NTU)[d]	200	157	390	300	169	36
K 232	1.599	1.566	1.576	1.536	1.420	1.478
K 270	0.158	0.133	0.162	0.141	0.106	0.110
$\Delta K \times 10^3$	−4	−4	−11	−10	−4	−5
R (K_{232}/K_{270})[e]	10.1	11.8	9.7	10.9	13.4	13.4
Panel test (score)	7.6	7.2	8.2	7.8	7.0	6.7
Global quality index (GQI1)	7.5	7.4	8.2	8.0	7.7	7.3
Global quality index (GQI2)	35.9	35.8	37.3	37.1	36.4	35.5
Chlorophylls and pheophytins (mg/kg)	7.2	6.8	29.2	22.2	5.2	5.0
Chlorophyllic color index (%)	28.5	27.3	159.6	120.8	25.5	25.6
Carotenoid color index (%)	104.0	111.8	255.0	244.1	116.1	132.7
Brightness (%)	69.2	71.6	51.4	56.0	72.2	73.7
Chroma (%)	81.2	83.9	97.3	97.3	86.6	86.6
Hue (nm)	577	577	578	578	578	578
Color ratio (A_{446}/A_{668})	3.9	4.4	1.6	2.1	4.5	5.0
Integral color index	11.1	12.2	28.1	24.5	12.2	11.5

[a]MWI, Watts and Major's index.
[b]As determined by colorimetric method.
[c]As determined by high-resolution gas-chromatographic method.
[d]NTU, nephelometric turbidity units.
[e]Wolff's ratio.

Finally, the oils obtained by employing the enzyme adjuvant stood out for the higher values of global quality indices because of higher sensory scores and higher polyphenol content; this suggests that these oils might be considered superior to the reference oils on the basis of overall evaluation.

Ultraviolet spectrophotometric indices, peroxide index, acidity, Watts-Majors's index (MWI). These qualitative indices of the oils were not significantly influenced by biochemical treatment of the olive pastes. The small or insignificant variations observed between the analytical data relating to the two oil types, obtained by the two technological procedures, can probably be attributed to experimental error. However, the carbonyl index (MWI) and R ($K_{232}/K_{270,}$ Wolff's ratio) value tended to be higher in the reference oils.

Turbidity, chromatic characteristics, lipochrome contents of oils, and spectrophotometric curves in the visible light zone between 400 and 700 nm. Turbidity and color are not considered by the EEC method (panel test) for the evaluation of sensory characteristics of olive oil, but they actually influence acceptability of the product by the consumer. The values recorded for turbidity were always lower in the reference oils than in the oils obtained with the enzyme adjuvant. However, this parameter seemed also to depend on the variety of olives and ripening index. The oils produced by the biological pro-

TABLE 2
Other Compositional Characteristics of Olive Oils Extracted by Using a New Processing Enzyme Aid, Compared to Reference Oils

	Dritta		Coratina		Leccino	
	Aid	Control	Aid	Control	Aid	Control
Tocopherols (mg/kg)	76.2	67.2	197.1	195.1	292.4	257.4
α-Tocopherol (mg/kg)	76.1	67.1	196.7	194.7	291.5	256.6
γ-Tocopherol (mg/kg)	0.1	0.1	0.4	0.4	0.9	0.8
Steroid hydrocarbons (mg/kg)	0.092	0.088	0.181	0.284	0.295	0.048
Campestadiene (mg/kg)	0.050	0.018	0.120	0.050	0.128	0.011
Stigmastatriene (mg/kg)	0.029	0.007	0.061	0.132	0.167	0.016
Stigmastadiene (mg/kg)	0.013	trace	trace	0.102	trace	0.021
Total waxes (mg/kg)	453	434	199	226	737	756
Waxes C_{34} (mg/kg)	16	13	5	6	11	10
Waxes C_{36} (mg/kg)	191	178	47	62	379	370
Waxes C_{38} (mg/kg)	138	125	57	61	175	180
Waxes C_{40} (mg/kg)	40	43	22	22	63	74
Waxes C_{42} (mg/kg)	30	45	30	28	46	49
Waxes C_{44} (mg/kg)	12	12	12	12	22	22
Waxes C_{46} (mg/kg)	26	18	26	35	41	51
Waxes $C_{40} + C_{42} + C_{44} + C_{46}$ (mg/kg)	108	118	90	97	172	196
Alcoholic index	0.19	0.22	0.22	0.04	0.32	0.36
Aliphatic alcohols (mg/100 g)	11.2	9.9	10.0	11.8	14.9	17.5
1-Docosanol (C_{22}) (%)	15.8	16.0	11.5	12.3	21.2	21.7
1-Tetracosanol (C_{24}) (%)	22.4	24.2	23.0	19.2	34.2	34.1
1-Hexacosanol (C_{26}) (%)	41.1	42.4	42.8	45.5	27.6	27.6
1-Octacosanol (C_{28}) (%)	20.7	17.4	22.7	23.0	17.0	16.7
Triterpene alcohols (mg/100 g)	51.3	49.6	75.7	84.5	45.4	51.5
β-Amyrin + butyrospermol (%)	20.3	19.1	21.3	19.2	23.7	21.9
Cycloartenol (%)	17.4	18.5	13.6	15.7	18.8	17.5
24-Methylenecyclo artanol (%)	62.3	62.4	65.1	65.1	57.5	60.7

cedure were slightly richer in chlorophyllic pigments (*a* and *b* chlorophyll, *a* and *b* pheophytin) and poorer in carotenoid components; their integral color index frequently was relatively higher. These results were confirmed by information given by visible-light spectra.

Aromatic volatile fraction. The oils obtained by employing the adjuvant in olive processing more often exhibited higher contents of total aromatic substances. However, the richness in volatile substances of the oils mainly appeared to depend on genetic factors, i.e., on the olive variety processed. Total value of these compounds is an excellent indicator of oil quality when produced from qualitatively good olives and when rational conditions of processing are adopted. Furthermore, the enzyme aid, with two out of three varieties, enhanced the *trans*-2-hexenal content of the oils; this substance is the major volatile constituent of fresh and qualitatively good oils. It is also the component mainly responsible for flavor, along with other volatile compounds and phenolic substances; this aldehyde is responsible for the green-fruity taste notes of oils, along with *cis*-3-hexenyl acetate. In contrast, the values of *trans*-2-hexenal/hexanal and *trans*-2-hexenal/total aroma ratios seemed to be more favorable for the reference oils. These ratios are believed to be important quality indices, the former because hexanal, unlike *trans*-2-hexenal (which has a pleasant odor of fresh cut grass), has a disagreeable smell and is present in elevated quantity in defective oils and only in small amounts in oil of good quality. Besides hexanal, among the volatile components identified in oil aromagrams, other substances with nasty smells (*n*-octane, acetic acid, ethyl acetate, ethanol, *trans*-2-pentenal, isoamyl alcohol, isobutyl alcohol, etc.) were found in the oils produced (see Table 4), even though these were of good quality. This means that the presence of small quantities of these components in virgin or extra-virgin oils is to be considered normal. Their total content, or the concentration of a part of them, considerably increases in defective oils. Some volatile bad substances are responsible for specific defects (20–23). Oils without defects but obtained from olives of less than excellent quality or overripe olives, or that were stored poorly or for a long time, are also rich in such bad substances. These also increase during aging of oil. Their effect on aroma in qualitatively good oils is avoided by that of compounds with a pleasant odor. These latter normally diminish in oil when the bad volatile compounds rise. The good compounds, during olive storage,

TABLE 3
Fatty Acid Composition and Diacylglycerol and Triacylglycerol Contents of Olive Oils (expressed in %) Extracted by Using a New Processing Enzyme Aid, Compared to Reference Oils

	Dritta		Coratina		Leccino	
	Aid	Control	Aid	Control	Aid	Control
Myristic acid (C_{14},0)	trace	trace	trace	trace	trace	trace
Palmitic acid (C_{16},0)	14.3	13.5	10.4	10.2	14.6	13.2
Palmitoleic acid (C_{16},1)	0.9	0.9	0.4	0.4	1.2	1.0
Heptadecanoic acid (C_{17},0)	0.1	0.1	0.1	0.1	0.1	0.1
Heptadecenoic acid (C_{17},1)	0.1	0.1	0.1	0.1	0.1	0.1
Stearic acid (C_{18},0)	3.2	3.3	2.9	3.1	2.1	2.4
Oleic acid (C_{18},1)	70.6	71.0	77.3	77.0	73.0	73.4
Linoleic acid (C_{18},2)	9.6	9.8	7.2	7.4	7.6	8.4
Linolenic acid (C_{18},3)	0.5	0.5	0.8	0.8	0.7	0.8
Arachidonic acid (C_{20},0)	0.4	0.5	0.4	0.5	0.3	0.3
Eicosenoic acid (C_{20},1)	0.2	0.2	0.3	0.3	0.2	0.2
Behenic acid (C_{22},0)	0.1	0.1	0.1	0.1	0.1	0.1
Lignoceric acid (C_{24},0)	trace	trace	trace	trace	trace	trace
trans-Oleic isomers	0.01	trace	0.01	trace	0.02	0.01
trans-Linoleic isomers + *trans*-Linolenic isomers	trace	trace	trace	trace	trace	trace
Saturated acids/ unsaturated acids	0.22	0.21	0.16	0.16	0.21	0.19
Oleic acid/linoleic acid	7.3	7.2	10.7	10.4	9.6	8.7
Total diglycerides	2.16	2.54	1.33	1.41	1.15	1.28
1,2-diglycerides	1.35	1.50	1.11	1.15	1.04	1.16
1,3-diglycerides	0.81	1.04	0.22	0.26	0.11	0.12
1,2-diglycerides/1,3-diglycerides	1.67	1.44	5.05	4.42	9.45	9.67
Total diglycerides × 1,3-diglycerides	1.75	2.64	0.29	0.37	0.13	0.15
Total diglycerides/1,3-diglycerides	2.67	2.44	6.05	5.42	10.45	10.67
Triglycerides	97.8	97.0	98.7	98.6	99.0	98.7

undergo a gradual natural quantitative reduction, even if the drupes were wholesome and undamaged; such reduction does not always result in worsening of other qualitative indices. So, these good volatile compounds as a whole should be considered an individual (and even nonsecondary) quality parameter of oil. Isobutyl alcohol, isoamyl alcohol, lactic acid, and *n*-octane (which forms by decomposition of hydroperoxide in position 10 of oleic acid) seem to be responsible for the fustiness defect when they are present in oil in elevated quantity (but this did not occur for our oils). Amyl and isoamyl alcohols are afforded by the metabolism of proteins. Acetic acid, ethyl acetate, and ethanol are afforded by fermentative processes (alcoholic fermentation of sugars) and cause the "washed with wine" defect. In overripe or damaged drupes, either before or after harvesting but especially during storage, an acceleration of processes occurs that causes a reduction or disappearance of good volatile components and at the same time an enhancement of those that are bad, especially because it facilitates invading of fruit tissues by bacteria and molds. In such cases, exogenous enzymes interact with endogenous ones and cause the decay and evolution of the oil flavor. Enzymatic, fermentative, and oxidative processes occur, and deleterious effects on the quality of oil are produced. Final aroma of oil depends on its absolute or relative content of various volatile components and is also influenced by interactions among them (24–29). The sensory threshold of each volatile compound is another factor that affects the aroma of oil. While *trans*-2-hexenal formed by enzymatic biosynthesis from the 13-L-hydroperoxide of linolenic acid, other good aromatic substances, such as 2-penten-1-ol and 1-penten-3-ol, were mostly present in more elevated quantities in the oils obtained by using the enzyme adjuvant. However, some volatile compounds with unpleasant aroma (isobutyl alcohol, isoamyl alcohol, acetic acid, 1-penten-3-one, hexanal) also increased in the oils because of the enzyme effect. Evidently, action of the enzyme, which was added to the olive paste prior to beginning of malaxation, raised the effect by enhancing the content of total aromatic substances in the oil (30,31).

Fatty acid composition: triglycerides and diglycerides. Fatty acid composition of the oils was not significantly affected by enzyme treatment of the olive pastes. These components seem to be influenced by the olive variety from which the oil was produced. Total triacylglycerol content of the oils also was hardly affected by the processing adjuvant. Analytical data showed that the enzymatic complex had no influence on total diglycerides, 1,2- and 1,3-diglycerides and other glyceridic indices, such as 1,2-diglycerides/1,3-diglycerides, total diglycerides × 1,3-diglycerides and total diglycerides/1,3-

TABLE 4
Aromatic Volatile Substances (expressed as 1-nonanol, mg/kg) Identified in Aromagram of Olive Oils Extracted by Using a New Processing Enzyme Aid, Compared to Reference Oils

	Dritta		Coratina		Leccino	
	Aid	Control	Aid	Control	Aid	Control
n-Octane	9.8	9.0	1.3	1.2	3.1	3.3
Ethyl acetate	10.1	1.7	7.9	1.7	14.7	14.2
2-Methyl-butyraldehyde	3.4	2.7	5.8	2.1	6.1	2.7
3-Methyl-butyraldehyde	3.4	1.7	6.5	2.3	7.7	4.5
Ethanol	25.0	19.4	17.8	10.1	3.5	25.3
3-Pentanone	28.0	23.1	12.5	10.9	9.8	6.5
1-Penten-3-one	8.2	8.2	26.8	15.5	12.4	12.9
Hexanal	27.6	25.5	32.5	23.7	21.6	20.6
Isobutyl alcohol	13.4	8.5	2.5	1.1	2.5	1.9
trans-2-Pentenal	2.5	2.3	5.0	3.1	2.0	2.0
1-Penten-3-ol	21.7	19.2	60.9	35.2	11.4	9.8
Isoamyl alcohol	22.3	17.0	8.9	3.7	9.4	7.5
trans-2-Hexenal	364.8	354.1	1081.6	773.4	320.0	416.8
n-Amyl alcohol	1.8	1.7	1.0	0.8	0.9	0.7
2-Penten-1-ol	12.0	10.5	30.8	18.2	6.8	5.9
1-Hexanol	25.5	22.2	18.6	18.0	13.4	11.1
cis-3-Hexen-1-ol	4.7	4.3	7.3	8.2	3.5	3.3
trans-2-Hexenol	43.0	50.0	80.9	38.6	44.1	26.1
Acetic acid	5.1	6.0	2.5	1.1	3.7	1.4
1-Octanol	trace	0.2	trace	trace	trace	trace
2-Butanone	1.0	trace	1.0	trace	1.3	0.8
Total volatile substances	723.3	664.1	1530.9	1036.4	800.4	652.4
trans-2-Hexenal/total aroma (%)	50.4	53.3	70.7	74.6	40.0	63.9
trans-2-Hexenal/hexanal	13.2	13.9	33.3	32.6	14.8	20.2

TABLE 5
Sterol and Triterpene Dialcohol Composition (expressed in %) in Olive Oils Extracted by Using a New Processing Enzyme Aid, Compared to Reference Oils

	Dritta		Coratina		Leccino	
	Aid	Control	Aid	Control	Aid	Control
Total sterols (mg/100 g)	127.6	120.1	110.8	117.5	197.7	205.0
Campesterol/stigmasterol	2.9	3.2	3.0	6.4	3.1	3.9
Cholesterol	0.2	0.4	0.2	0.3	0.2	0.2
Brassicasterol	trace	trace	trace	trace	trace	trace
24-Methylene-cholesterol	0.3	0.3	0.3	0.3	0.1	0.1
Campesterol	3.2	3.2	3.4	3.5	3.0	3.1
Campestanol	0.4	0.4	0.3	0.3	0.3	0.3
Stigmasterol	1.1	1.0	1.1	0.5	1.0	0.8
Δ^7-Campesterol	trace	trace	trace	trace	trace	0.2
Δ^5-23-Stigmastadienol	0.1	trace	trace	trace	trace	trace
Chlerosterol	1.0	0.9	0.8	1.1	0.8	0.9
β-Sitosterol	75.7	75.6	79.5	80.6	83.1	81.7
Sitostanol	1.0	1.0	0.8	1.7	0.9	1.3
Δ^5-Avenasterol	15.3	15.7	12.0	10.1	8.4	9.1
Δ^5-24-Stigmastadienol	0.9	0.8	0.7	0.7	0.6	0.7
Δ^7-Stigmastenol	0.5	0.4	0.5	0.4	0.5	0.5
Δ^7-Avenasterol	0.4	0.5	0.5	0.3	1.0	1.1
Total β-sitosterol[a]	94.0	93.9	93.7	94.3	93.9	93.6
Triterpene dialcohols	3.0	3.5	2.8	3.1	1.6	1.6
Erythrodiol	2.4	3.0	2.4	2.4	1.0	1.1
Uvaol	0.6	0.5	0.4	0.6	0.6	0.5

[a]Δ^5-23-Stigmastadienol + chlerosterol + β-sitosterol + sitostanol + Δ^5-avenasterol + Δ^5-24-stigmastadienol.

diglycerides. The 1,2-diglycerides/1,3-diglycerides ratio was proposed (32) as an index of quality freshness of oils. The other five indices, cited above, involving diglycerides seem also to be related to oil quality, so that, as integration of the EEC regulation No. 2568/91 was proposed (33), for extra-virgin oils the values of at least two of them should not be higher than certain well-studied and defined limits. If the values of all five indices involving diglyceride compounds are not higher than the proposed limits, extra-virgin oil may be considered to be of high quality. In effect, our oils, which all exhibited good qualitative levels, had values of all five indices involving diglyceride compounds that were not higher than the proposed limits, except for some oils, which were almost all produced from the Dritta variety. However, we used an NMR method for the determination of partial glycerides, while the authors who proposed the above indices adopted an HRGC method. The good qualitative standards of oils obtained by us were confirmed by the presence of small quantities or traces of *trans*-oleic, *trans*-linoleic, and *trans*-linolenic fatty acids.

Long-chain aliphatic and triterpene alcohols; alcohol index. The values of the alcoholic index (which is inversely related to the quality of oil) were frequently slightly lower in oil produced by using the enzyme aid. Furthermore, the oil exhibited relatively lower contents of total aliphatic and triterpene alcohols.

Sterol composition; campesterol/stigmasterol ratio; triterpene dialcohols. The processing adjuvant led to oils with lower contents of β-sitosterol and total sterols. These oils were also characterized by relatively lower values of the campesterol/stigmasterol ratio (which is positively related to quality) and lower contents of triterpene dialcohols. These latter are present in elevated quantities in husk oil; a high value of them is a negative index of quality. Furthermore, these components are considered important for revealing the presence of other vegetable oils in virgin olive oil. In the oils produced with or without the enzyme aid, the values of total triterpene dialcohols and main sterols (listed in enclosure No. 1 of EEC regulation No. 2568/91) were all lower than the limits set by the official norm.

Steroid hydrocarbons and waxes. Determinations of waxes and of stigmastadienes (isomeric hydrocarbon compounds not resolved by capillary column) are respectively considered by the official norm to detect fraudulent additions of husk oil to pressed oil and to disclose the presence of refined oil in virgin oil. Therefore, it is important to know the variation limits of these components in nonadulterated virgin oils by investigating the influence exerted on their values by the various factors (including technological treatments). Analytical data

TABLE 6
Oil Extraction Outputs and Analytical Characteristics of By-Products Obtained by Using a New Processing Enzyme Aid, Compared to Reference Process

	Dritta		Coratina		Leccino	
	Aid	Control	Aid	Control	Aid	Control
Olives						
Oil output (% fruit weight)	16.5	15.7	10.6	9.9	8.1	7.2
Oil output (% fruit oil)	86.8	82.6	87.6	81.8	85.3	75.8
Husk						
Moisture (%)	60.1	69.0	49.9	55.0	49.7	56.9
Residual oil (% as is)	4.98	4.91	3.15	3.40	4.47	4.00
Residual oil (% DM)[a]	12.48	15.84	6.29	7.56	8.89	9.28
Pulp/stone ratio (%)	66.4	63.8	85.2	81.4	73.3	75.6
Effluent						
Dry matter (% wt/vol)	11.1	10.4	10.2	9.1	10.1	10.8
Oil (g/L)	8.40	12.07	5.73	7.20	9.00	14.47
Oil (% DM)	7.57	11.61	5.62	7.91	8.91	13.40
Phenols (as caffeic acid) (g/L)	5.2	5.0	6.6	5.9	3.8	3.1
ortho-Diphenols (as caffeic acid) (g/L)	3.0	2.9	4.3	4.0	2.7	2.2
COD (g/L)[b]	115	145	150	150	136	144
Turbidity (NTU $\times 10^{-3}$)[c]	25	43	9	23	53	62

[a]DM, dry matter.
[b]COD, chemical oxygen demand.
[c]Abbreviation as in Table 1.

showed that the enzyme adjuvant had no significant influence on total wax content of the oils. However, the qualitative composition of waxes was significantly influenced by the olive variety. The enzyme adjuvant also had little influence on stigmastadiene content of the oils, even if total steroid hydrocarbons tended to be higher.

Processing the data relating to the pyrograms by applying both classic and neural statistical multivariate techniques showed little difference between the oils produced with the enzyme and the reference oil in oil quality.

Finally, as far as the technological quantitative results are concerned, we observed (Table 6) that the enzyme adjuvant led to higher outputs (generally 0.8% average). This increase in yield, in industrial processing, is significant. It could still be higher if the olives processed had not been characterized by low oil content. The effluent produced by using the processing aid was relatively richer in polyphenols (as was the corresponding oil), but in spite of that, the value of chemical oxygen demand was slightly lower. The husk was characterized by a lower content of liquid components, both residual oil and water. Lastly, the enzyme complex led to a higher quantity of husk (as dry matter) and effluent. In fact, it induced a more effective separation of phases (oil, water, and solids) from the olive paste; consequently, a higher quantity of vegetable water was separated from the husk.

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REFERENCES

1. Ranalli, A., and F. Angerosa, Integral Centrifuges for Olive Oil Extraction. The Qualitative Characteristics of Products, *J. Am. Oil Chem. Soc. 73*:417–422 (1996).
2. Ranalli, A., and A. Serraiocco, Effect Induced by a Pectolytic Adjuvant in Olive Oil Extraction by the Present Technological Systems. Pluriannual Research Results, *Riv. Ital. Sostanze Grasse 72*:355–364 (1995).
3. Ranalli, A., and N. Martinelli, Integral Centrifuges for Olive Oil Extraction, at the Third Millenium Threshold. Transformation Yields, *Grasas Aceites 46*:255–263 (1995).
4. Ranalli, A., and A. Serraiocco, Quantitative and Qualitative Effects of a Pectolytic Enzyme in Olive Oil Production, *Ibid. 47:* 227–236 (1996).
5. Ranalli, A., Carotenoid in Virgin Olive Oils. Effect of Technology, *Italian J. Food Sci. 4*:53–57 (1992).
6. Angerosa, F., N. D'Alessandro, P. Konstantinov, and L. Di Giacinto, GC–MS Evaluation of Phenolic Compounds in Virgin Olive Oil, *J. Agric. Food Chem. 43*:1082–1087 (1995).
7. Sacchi, R., F. Addeo, I. Giudicianni, L. Paolillo, L. Mannina, and A.L. Secre, High-Resolution NMR Analysis of Olive Oil, *Proceedings of the 2nd Italian Congress of Food Chemistry*, Giardini-Naxos, Messina, Italy, May 1995, p. 173.
8. Vlahov, G., The Structure of Triglycerides of Monovarietal Olive Oils: a ^{13}C NMR Comparative Study, *Lipid 98*:203–205 (1996).
9. Goodacre, R., D.B. Kell, and G. Bianchi, Neural Networks and Olive Oil, *Nature 27*:359–364 (1992).
10. Goodacre, R., D.B. Kell, and G. Bianchi, Rapid Assessment of the Adulteration of Virgin Olive Oils by Other Seed Oils Using Pyrolysis Mass Spectrometry and Artificial Neural Networks, *J. Sci. Food Agric. 63*:297–307 (1993).
11. Goodacre, R., D.B. Kell, and G. Bianchi, Quality Control of Olive Oil by Pyrolysis–Mass Spectrometry Coupled to an "Artificial Neural Networks," *Olivae 7*:36–38 (1993).
12. Goodacre, R., D.B. Kell, and G. Bianchi, Food Adulteration Exposed by Neural Networks, *Analysis Europa 16*:35–37 (1995).
13. Salter, J.G., R. Goodacre, D.B. Kell, M. Lazzari, and G. Bianchi, Assessment of Food Authenticity Using Pyrolysis Mass Spectrometry and Artificial Neural Networks; Application to Olive Oils, *Food Authenticity,* in press.
14. Bianchi, G., L. Giansante, and M. Lazzari, Innovative Analytical Method to Protect Genuineness and Origin from Geographical Zone or Variety of Vegetable Oils, *L'infor. Agrario 52:* 45–48 (1996).
15. Montedoro, G.F., M. Servili, M. Baldioli, and E. Minati, Simple and Hydrolyzable Phenolic Compounds in Virgin Olive Oil. Note 1. Their Extraction, Separation, and Quantitative and Semiquantitative Evaluation by HPLC, *J. Agric. Food Chem. 40*:1571–1576 (1992).
16. Montedoro, G.F., M. Servili, M. Baldioli, and E. Minati, Simple and Hydrolyzable Phenolic Compounds in Virgin Olive Oil.

Note 2. Initial Characterization of the Hydrolyzable Fraction, *Ibid. 40*:1577–1580 (1992).
17. Montedoro, G.F., M. Servili, M. Baldioli, R. Selvaggini, E. Miniati, and A. Macchioni, Simple and Hydrolyzable Compounds in Virgin Olive Oil. Note 3. Spectroscopic Characterization of the Secoiridoid Derivatives*, Ibid. 41*:2228–2234 (1993).
18. Servili, M., M. Baldioli, E. Miniati, and G.F. Montedoro, Antioxidant Activity of New Phenolic Compounds Extracted from Virgin Olive Oil and Their Interaction with α-Tocopherol and β-Carotene, *Riv. Ital. Sostanze Grasse 73*:55–59 (1996).
19. Servili, M., and G.F. Montedoro, Recovery of Phenols from the Vegetable Water of Olives and Assessment of Their Antioxidant Capacity, *Ind. Alimentari 28*:14–18 (1989).
20. Angerosa, F., L. Di Giacinto, and M. Solinas, Influence of *Dacus oleae* Infestation on Flavor of Oils, Extracted from Attacked Olive Fruits, by HPLC and HRGC Analyses of Volatile Compounds, *Grasas Aceites 43*:134–142 (1991).
21. Solinas, M., F. Angerosa, and A. Cucurachi, Relation Between Chemical Data and Organoleptic Rancidity. Note 1, *Riv. Ital. Scienza dell'Alimentazione 14*:361–368 (1985).
22. Solinas, M., F. Angerosa, and A. Cucurachi, Relation Between the Autoxidation Products of Oils and Fats and the Rising of Rancidity in Organoletptic Evaluation. Note 2: Quantitative Determination, *Riv. Ital. Sostanze Grasse 64*:137–145 (1987).
23. Angerosa, F., L. Di Giacinto, and M. Solinas, Influence of Storage of Olives on the Aroma of Oils: Evaluation of "Fustiness" Defect by Means of HPLC and GLC Analyses of Volatile Components, *Riv. Merceologia 29*:275–294 (1990).
24. Olías Jiménez, J.M., A. Del Barrio, and R. Gutiérrez, Volatile Components in the Aroma of Virgin Olive Oil. Note 1, *Grasas Aceites 28*:107–112 (1977).
25. Angerosa, F., M. Solinas, and L. Di Giacinto, Influence of Storage of Olive Oil on Its Flavor, *Proceedings of the 1st European Congress "Chevreul" on the Fatty Substances*, Angers, France, June 1989, p. 571.
26. Solinas, M., and F. Angerosa, New Quality Indices of Virgin Olive Oil, *Ibid.*, p. 563.
27. Olías Jiménez, J.M., F. Gutiérrez Rosales, and M.C. Dobarganes García, Volatile Components in the Aroma of Virgin Olive Oil. IV. Their Evaluation and Influence on the Aroma During the Fruit Ripening Process in the "Picual" and "Hojiblanca" Varieties, *Grasas Aceites 31*:391–402 (1980).
28. Camera, L., and M. Solinas, Identification of Some Aromatic Substances in Olive Oil by GLC–MS, *Proceedings of the Workshop on Olive Oil and Table Olives, Technology and Quality*, Pescara, Italy, April, 1990, p. 153.
29. Gutiérrez Rosales, F., S. Perdiguero, R. Gutiérrez, and J.M. Olías Jiménez, Evaluation of the Bitter Taste in Virgin Olive Oil, *J. Am. Oil Chem. Soc. 69*:394–395 (1992).
30. Montedoro, G.F., and M. Servili, Olive Oil Quality Parameters in Relationship to Agronomic and Technological Aspects, *Riv. Ital. Sostanze Grasse 69*:563–573 (1992).
31. Montedoro, G.F., and L. Garofalo, The Qualitative Characteristics of Virgin Olive Oils. The Influence of Variables Such as Variety, Environment, Preservation, Extraction, Conditioning of the Finished Product, *Ibid. 61*:157–168 (1984).
32. Sacchi, R., F. Addeo, I. Giudicianni, and L. Paolillo, Quality Control of Olive Oil by ^{13}C NMR Spectroscopy, *Proceedings of the Study Day on Qualitative Problems of Olive Oil*, Sassari, Italy, November, 1990, p. 287.
33. Catalano, M., T. De Leonardis, and S. Gomes, Diacylglycerols in the Evaluation of Virgin Olive Oil Quality, *Grasas Aceites 45*:380–384 (1994).