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The new "Cytolase 0" enzyme processing aid improves quality and yields of virgin olive oil

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Abstract

Three olive varieties (*Leccino*, *Coratina* and *Dritta*) were processed using the percolation–centrifugation system with a new olive enzyme processing aid, "Cytolase 0". The enzyme adjuvant led to oils characterised (with respect to reference oils) mostly by: (i) higher content of pleasant volatiles, total phenols, *o*-diphenols, hydroxytyrosol-aglycones, α-tocopherol, chlorophylls and carotenoids, triterpene dialcohols, triterpene and aliphatic alcohols, and waxes; (ii) higher ratios of 1,2-diglycerides/1,3-diglycerides, campesterol/stigmasterol, *trans*-2-hexenal/hexanal, and *trans*-2-hexenal/total aroma; (iv) higher values of colour indices; (v) higher sensory score; (vi) lower values of turbidity; (vii) similar values of acidity, peroxide index, carbonyl index, UV (ultraviolet) spectrophotometric indices, and alcoholic index, and (viii) similar contents of saturated and unsaturated fatty acids, triacylglycerols, diacylglycerols, and sterols. No (or only traces of) *trans*-isomer C_{18} fatty acids and stigmastadienes were found in the two oil types. Therefore, the enzyme aid produced better qualitative and quantitative results.

1. Introduction

Olive oil, a gourmet oil, is claimed to have high nutritional value and even healing properties (Guezmir, 1997). It contains chiefly monounsaturated fatty acids (oleic acid) and is characterised by an optimal percentage of polyunsaturated fatty acids (~10%), among which an essential one (linoleic acid) is present (Guezmir, 1997).

Due to a high ratio of monounsaturated fatty acids to polyunsaturated fatty acids and to a high content of natural antioxidants (phenols and tocopherols), it is very resistant to peroxidation, and few free radicals (which are highly toxic and detrimental to health) originate from it (Litridon, Linssen, Schols, Bergmans, Posthumus & Borkon, 1997). Also, because of a higher ratio of unsaturated fatty acids to saturated fatty acids, and a content of only minute amounts of cholesterol, this foodstuff does not affect the total blood cholesterol level. Due to the oleic acid effect, even an increase in high density lipoprotein (HDL)-cholesterol has been noticed (Mattson & Grundy, 1985; Reaven, Parthasarathy & Grasse, 1991; Ulbricht & Southgate, 1991).

It has been demonstrated that diets which include virgin olive oil as an ingredient reduce atherogenesis risk and coronary heart disease, and prevent the "oxidative stress", which is the cause of ageing and of some kidney and liver diseases, as well as of pulmonary emphysema and rheumatoid arthritis (Mensik & Katan, 1987).

Further, olive oil intake seems to prevent atonic constipation, gastrointestinal diseases, essential hyperlipemia, hyperurecemia, and hyperthyroidism, and might even have a role in the control of biological membrane activity, prostaglandin synthesis, blood viscosity, and child growth, and the prevention of some malignant tumours (Kannel, Castelli & Gordon, 1979; Baudet, Esteva, Laserre & Jacot, 1986; Esteva, Baudet & Jacot, 1986).

An increase in bile secretion has also been observed and the taking of a spoon of olive oil before meal consumption is recommended for liver complaints (Willet, 1994). Finally, this oil is the most digestible one as it moves rapidly to the duodenum from the stomach (Mattson & Grundy, 1985).

To optimise the qualitative olive oil standard and the quantitative extraction outputs, we are conducting some researches aimed at verifying the possibility using enzyme aids in the extraction process. Liquid enzyme preparations have proved to be the best adjuvants (Ranalli & De Mattia, 1992; Ranalli & Ferrante, 1996; Ranalli & Lazzari, 1996), and we are now studying a new liquid complex enzyme aid, "Cytolase 0".

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This paper reports on the results achieved by employing the enzyme in the continuous combined percolation-centrifugation process, which is applied in all olive countries. The percolation extractor is called "Sinolea", which is made up of 6000 steel blades; the centrifugal extractor was a new two-phase decanter with special head plates.

In a previous paper (Ranalli & De Mattia, 1997), the results achieved by using the "Cytolase 0" enzyme aid in the direct centrifugation process are reported; we have not yet tested the enzyme adjuvant in the traditional pressing process.

The research was approved by the National Committee of Agricultural Experimentation, Ministry for the Agricultural Policies, Rome, Italy.

The "Cytolase 0" enzyme preparation (Gist-Brocades, Seclin City, France) is a complex formulation essentially containing pectinase and cellulolitic and hemicellulolitic enzymes, plus some minor enzymes. These are all present in olive fruit, but as there is significant loss during the oil extraction process, addition of "Cytolase 0" to the olive paste replaces, and even enhances, the natural enzymes of olive paste. Its activity is not less than 2000 units ml^{-1}. One unit of activity is defined as the amount of enzyme complex that liberates 1 μmol of reducing sugars per minute.

The enzyme formulation degrades the walls of the oil-bearing cells that elude crushing and also has similar effects on the colloidal system in olive paste (pectins, hemicelluloses, proteins, etc.) that retain the droplets of oil (Ranalli & De Mattia, 1997). In this way, the oil droplets are released by phase inversion and gradually merge into larger droplets until they form a mass of free oil, which is extracted mechanically. The enzyme breaks up, not only the liquid–solid emulsions, but also the liquid–liquid emulsions mainly caused by crushing and centrifuging the paste and through its endopolygalacturonase effect. It also has a positive effect on the rheological characteristics of the paste, as a result of which the phases (liquids and solids) are separated more thoroughly (Ranalli & De Mattia).

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 (Ranalli & De Mattia). Consequently, oil composition is not modified. Moreover, it is harmless to consumer health (Ranalli & De Mattia, 1997).

Industrial processing "Novoil" EDJ processing equipment (Rapanelli Company, Foligno, Italy), whose first extractor was a "Sinolea", was used. The extraction process steps are depicted in Fig. 1. Two oil types were obtained: (i) percolation oil (first extraction) and (ii) centrifugation oil (second extraction). Only the centrifugation oils (produced with or without enzyme) are compared in this paper. The characterisation of percolation oils will be done in another olive oil season, as a specific study is required. However, with most olive varieties, these are quantitatively much less meaningful. To crush the non-pitted olives, a mobile hammer crusher was used. The kneading of the resulting paste lasted 1 h and was conducted at 30°C. The plant was equipped with two kneaders which allowed a continuous processing. The first extraction (percolation) lasted 50 min, whereas the second extraction (centrifugation) lasted 20–25 min (for each paste lot) depending on the olive variety processed. The amount of paste centrifuged per h was 0.6 tonnes, and lukewarm (28°C) tap water volume employed to fluidise it was 300 litre h^{-1}. The enzyme preparation was added to the olive paste at the beginning of the kneading step using a dose of 0.3 ml (600 units) kg^{-1} olives (0.03%, v/w), which was diluted previously with lukewarm water (1:9, v/v).

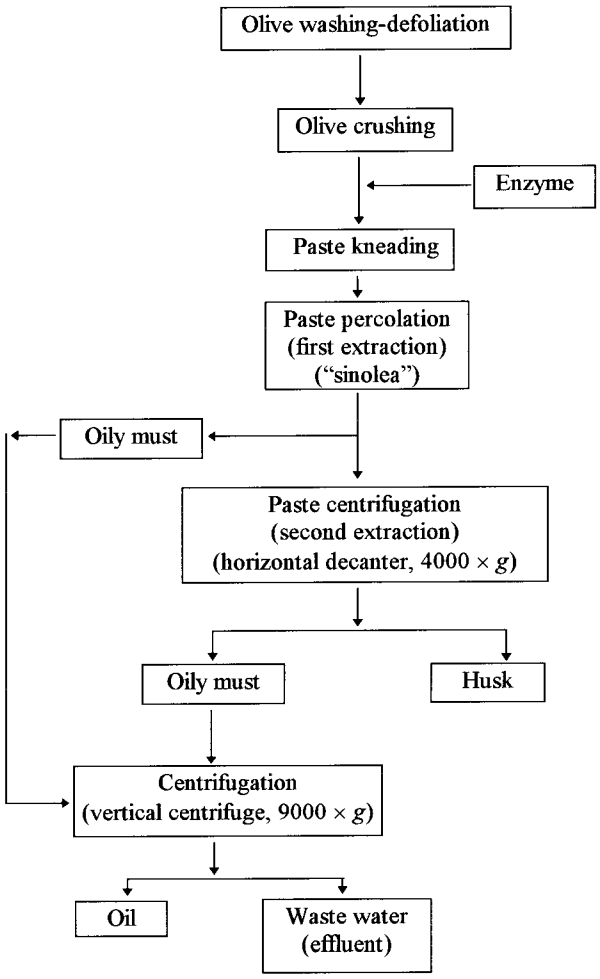

Fig. 1. Flowsheet for the percolation–centrifugation olive processing.

Three Italian olive varieties (*Coratina*, *Dritta* and *Leccino*) produced on the farm of the local Institute (Olive and Olive Oil Research Institute, Pescara, Italy), were processed in the oil mill. The varieties are in the order very popular in Apulia, Abruzzo and Tuscany

regions, and were selected since they have different technological characteristics, the *Dritta* variety being less difficult to process than the *Leccino* and the *Coratina*. Such characteristics appeared to be related to the relative content of solids, moisture and oil of the fruits (Table 1).The values are means of eight replicates and all have variation coefficients (CV) $<9\%$. To grind the olive samples for analyses, a laboratory mini oil mill (Valpesana Company, S. Casciano Val di Pesa, Italy) was used.

2. Materials and methods

2.1. Experimental design and sampling

For each variety, a homogeneous sample of 2.4 tonnes of olives was processed, 1.2 tonnes with the enzyme aid and 1.2 tonnes according to the reference procedure without employing the enzyme adjuvant (control).

Each half was divided into four equal 300 kg parts which were processed and tested as replicate batches. Thus, a 2×3 factorial design (two extraction techniques $\times$ three olive varieties) was adopted. For each test, a sample of olives (5 kg), one of oil (2 litres), one of husk (5 kg) and one of waste water (1 litre) were drawn for analyses.

2.2. Olive and by-product sample analyses

The percentage of olive paste and husk moisture and of dry waste water matter was determined by the oven method keeping at 105°C a 50 g (or 50 ml) sample until constant weight. The residue was utilised for determination of the oil percentage which was carried out by using a soxhlet apparatus and petroleum ether (b.p. 40–70°) as the solvent (Ranalli, 1992b). The olive paste solid percentage was evaluated by the formula: 100-(oil% + moisture%).

For waste water, other analytical measurements were carried out by using mostly the methods detailed by Bianucci and Bianucci (1980) with some modifications (Ranalli, 1992b). The COD (chemical oxigen demand) was determined by oxidising 0.1 to 0.2 ml of waste with 15 to 20 ml 0.04 M $K_2Cr_2O_7$ and titrating the excess of bichromate with 0.04 M Mohr's salt, using *o*-phenanthroline as the indicator. Phenols and *o*-diphenols were quantified colorimetrically using the Folin Ciocalteus's reagent and the Arnow's one, respectively; the absorbance data were obtained at 725 and 450 nm wavelengths.

Conductivity and salinity were measured on a Model LF-537 conductometer, Vittadini Company, Milan, Italy, using a TetraCon 96 conductivity cell. For the pH measurements, a Model SA 520 pH-meter was used. Turbidity (as for oil samples) was measured on a Model ratio turbidimeter (HACH Company, Loveland, CO, USA).

Reducing sugars were determined gravimetrically by the Fehling's method giving a cuprous oxide precipitate, which was weighed and the value converted to reducing sugar content with the Meissl's table.

Total nitrogen was quantified by the Kjeldahl method based on titration (in the presence of specific mixed indicator) of the ammonia solution obtained upon distillation, following mineralisation of 20–25 ml waste water.

Total phosphorus was quantified colorimetrically taking the absorbance readings at 720 nm wavelength; 0.2–0.3 ml waste water were previously mineralised using both sulphuric and nitric acid; colour was developed using ascorbic acid as a reagent.

Almost all reagents used for the chemical analyses of olive and by-product samples were of analytical grade and were obtained from Farmitalia C. Erba (Milan, Italy).

2.3. Oil sample analyses

Volatiles were analysed by HRGC (high-resolution gas chromatography) (Ranalli & Angerosa, 1996) using a carbowax 20 M capillary column (25 m, 0.32 mm i.d., 0.20 μm film thickness, from Nordion Ltd., Helsinki, Finland). The volatiles were extracted by stripping at 37°C in a nitrogen stream, and entrapping by activated charcoal; they were then eluted with diethyl ether. The internal standard was nonan-1-ol ($>99\%$ pure). The chromatogram (aromagram) of this fraction showed many peaks but, as yet, only 21 components have been identified (Ranalli & Angerosa), such as: *n*-octane, ethyl acetate, 2-methyl-butyraldehyde, 3-methyl-butyraldehyde, ethanol, 3-pentanone, 1-penten-3-one, hexanal, isobutyl alcohol, *trans*-2-pentenal, 1-penten-3-ol, isoamyl alcohol, *trans*-2-hexenal, *n*-amyl alcohol, 2-penten-1-ol, 1-hexanol, *cis*-3-hexen-1-ol, *trans*-2-hexenol, acetic acid, 1-octanol, and 2-butanone.

Tocopherols were analysed by HPLC (high-performance liquid chromatography) (Ranalli & De Mattia, 1997) with a direct-phase M-porasil column (3.9×300 mm, 10 μm, from Waters Corporation, Milford, MA, USA), using a hexane-propan-2-ol (98.5:1.5, v/v) eluent and a UV detector at 292 nm wavelength. α-, β-, γ-, and δ-tocopherol were detected.

Table 1
Compositional characteristics of the three processed olive varieties[a]

Olive variety	Oil (%)	Moisture (%)	Solids (%)
Coratina	22.0	44.8	33.2
Dritta	25.1	48.4	26.5
Leccino	19.3	46.1	34.6

[a] Values are means of eight replicates; CVs all $<9\%$.

Phenolics were analysed by HRGC (Angerosa, D'Alessandro, Konstantinov, & Di Giacinto, 1995), using a 25 m long capillary column (0.32 mm i.d., 0.1 μm film thickness, SE-54 stationary phase, from Lab. Service Analitica Ltd., Anzola Emilia, BO, Italy). After extraction with methanol, the resulting extract was concentrated to dryness by a rotary evaporator and dissolved in 10 ml of acetonitrile. This solution, after three washings with hexane, was evaporated in a vacuum, at $<35°C$. The residue was dissolved in acetone and to 1 ml of this solution were added 150 μl of BSTFA (*bis*-trimethylsilyltrifluoroacetamide). After 1 h the prepared mixture was injected (0.5 μl) into a gas chromatograph. The internal standard was resorcinol (>99% pure). Free tyrosol and hydroxytyrosol and tyrosol- and hydroxytyrosol-aglycones (dialdehydic forms of elenolic acid linked to tyrosol or hydroxytyrosol) have been identified in the HRGC chromatogram of the phenolic fraction (Angerosa et al., 1995). Total polyphenols and *o*-diphenols were determined colorimetrically following the procedure adopted for waste water.

Fatty acids, after transformation to methyl esters, were analysed by HRGC using a 25 m long capillary column (0.35 mm i.d., 0.25 μm film thickness, polyglycol type stationary phase, from Nordion Ltd., Helsinki, Finland). These components were represented by myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), heptadecanoic acid ($C_{17:0}$), heptadecenoic acid ($C_{17:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidonic acid ($C_{20:0}$), eicosenoic acid ($C_{20:1}$), behenic acid ($C_{22:0}$), and lignoceric acid ($C_{24:0}$).

Sterols and triterpene dialcohols were analysed by HRGC (Ranalli, De Mattia & Ferrante, 1996) using a 25 m long glass capillary column (0.30 mm i.d., 0.20 μm film thickness, SE-52 stationary phase, from Supelco, Inc., Bellofonte, PA, USA). The oil sample was first saponified with an ethanolic KOH solution, the unsaponifiable matter extracted with diethyl ether, and the components separated on a basic silica-gel TLC (thin-layer chromatography) plate using a benzene/acetone (95:5, v/v) mixture as the eluent. The recovered sterols and dialcohols were transformed into trimethylsilyl (TMS) ethers for analysis. Under UV light, a mildly basic alcoholic 2,7-dichlorofluoroscein solution was used to reveal the component bands for analysis. A pyridine/hexamethyldisilazane/trimethyl-chlorosilane (9:3:1, v/v/v) mixture was used as a silanizing reagent. Internal standard was α-cholestanol (2 g kg^{-1}, in $CHCl_3$). This fraction included cholesterol (traces), brassicasterol, 24-methylenecholesterol, campesterol, campestanol, stigmasterol, Δ^7-campesterol, Δ^5-23-stigmastadienol, chlerosterol, β-sitosterol, sitostanol, Δ^5-avenasterol, Δ^5-24-stigmastadienol, Δ^7-stigmastenol, and Δ^7-avenasterol. Triterpene dialcohols were represented by erythrodiol and uvaol.

Aliphatic and triterpene alcohols were analysed by HRGC (Ranalli, De Mattia, & Ferrante, 1996). The analytical procedure was similar to that for sterols except that alcohol bands, instead of sterols, are recovered by the TLC step. Internal standard was arachidilic alcohol. From the data obtained, the alcoholic index was evaluated (Ranalli & De Mattia, 1997). The aliphatic alcohols identified were 1-docosanol (C_{22}), 1-tetracosanol (C_{24}), 1-hexacosanol (C_{26}), and 1-octacosanol (C_{28}). The triterpene alcohols identified included β-amyrin, butyrospermol, cycloartenol, and 24-methylenecycloartanol.

Triglycerides and diglycerides were analysed by ^{13}C-NMR (nuclear magnetic resonance) (Howarth & Vlahov, 1996; Vlahov, 1996). The oil sample spectra were run in $CDCl_3$ (deuterochloroform) (250 mg olive oil/0.5 ml $CDCl_3$). Free induction decays (FIDs) were acquired at 25°C using a spectral width of 13,000 H_Z, 131 K acquisition points zero filled to 256 K points. A 45° excitation pulse and a 20 s relaxation delay were employed to collect 256 scans. FIDs were processed before Fourier transformation by a Gaussian filter of 0.1 H_Z Lorentzian narrowing and 0.15 Gaussian broadening. Chemical shifts were relative to the signal of Me_4Si (tetramethylsilane).

Waxes were analysed by HRGC (Ranalli & Lazzari, 1996) using a SPBTH-5 capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness, from Supelco Inc., Bellofonte, PA, USA) after their separation with a 70 to 230 mesh hydrated silica gel column and *n*-hexane/diethyl ether (99:1, v/v) eluant. The first eluted fraction (~140 ml), with a polarity lower than the triglycerides, was evaporated to dryness, then recovered with *n*-heptane and finally analysed. Internal standard was lauryl arachidate. This fraction included waxes (C_{34}), (C_{36}), (C_{38}), (C_{40}), (C_{42}), (C_{44}), and (C_{46}).

Stigmastadienes were analysed by HRGC (Ranalli & Ferrante, 1996) using a NB-54 capillary column (25 m, 0.32 mm i.d., 0.10 μm film thickness, from Nordion Ltd., Helsinki, Finland) after their separation with a chromatographic column filled with activated silica 60 and tamponed at the bottom with glass wool, and subsequent recovery with hexane eluant. Internal standard was cholestadiene.

Oxidative stability (induction time of the peroxidising reactions) was determined using a Model 67 "Rancimat" apparatus (Metrohm Co., Basel, Switzerland), which automatically applied the accelerated Swift's test (120°C; air flow rate of 20 litre/h) (Läubly & Bruttel, 1986).

Carotenoids (yellow pigments), such as lutein, β-carotene, violaxanthin, and neoxanthin, were determined colorimetrically, after separation by TLC, according to Mosquera and Garrido Fernandez (1989), with some modifications (Ranalli, 1992a).

The other analytical measurements performed were: spectrophotometric determination of chlorophylls and

pheophytins (green pigments) according to Wolff (1968), spectrophotometric determination of chlorophyll and carotenoid colour indices according to Papaseit Totosaus (1986), determination of colour ratio (A^{446}/A^{668}) directly on oil, determination of brightness (h%), chroma (σ%), and hue (λd), through transmittance measurements, and calculation of integral colour index ($= \sigma\%$ log 100/h%) according to Ranalli (1992c), titrimetric determination of free acidity and peroxide index, and determination of UV spectrophotometric indices according to E.C. Regulation no. 2568 (1991), spectrophotometric determination of carbonyl index and nephelometric determination of turbidity according to Ranalli and Costantini (1994).

The overall quality index (OQI_1) was evaluated by the algorithm developed by International Olive Oil Council [International Olive Oil Council (I.O.O.C.), 1990], based on the acid values, peroxide index and k_{270} (UV specific extinction at 270 nm wavelength), as well as the sensory score; the other overall quality index (OQI_2) involved also the oil polyphenol content (Solinas, Bandino, & Orrù, 1992).

To perform the quantitative descriptive sensory analyses, the I.O.O.C. test included in the E.C. Regulation no. 2568 (1991) was applied by using a standard profile sheet. The gustatory–olfactory–tactile perceptions were evaluated for each sensory attribute using an intensity scale from 0 to 5. A fully trained analytical taste panel recognised by I.O.O.C. and made up of 12 assessors and a sensory laboratory were used. All panellists had more than 8 years of experience in evaluating any olive oil types (extra-virgin, virgin, lampant, and refined). Oil samples were heated at 30°C by a thermostat before sensory analyses and were presented fully randomised to the tasters. Dark-blue glasses were used as no colour evaluation was to be made. The magnitude of several sensory attributes, such as ''olive fruity'' (ripe or green), apple, other ripe fruits, bitter, pungent, sweet, other allowable, sour/winey/vinegary/acid, rough, metallic, mustiness/humidity, muddy sediment, fusty, rancid, and other unallowable, was assessed. Next, an overall evaluation of the magnitudes of positive and negative (off-flavours) attributes was made and, by means of a grading structured scale ranging from 1 to 9, the sensory score was obtained.

The oil samples (as the olive and by-product samples) were stored frozen until the moment of the analyses. They were also pyrolysed at Curie-point (Goodacre, Kell, & Bianchi, 1992) and from the pyrolysed materials, by means of a mass-spectrometer, the pyrograms or fragmentograms (fingerprints of the samples) were obtained. A Rapyd-400 instrument (coupled to a mass-spectrometer), which was provided by Horizon Instruments Ltd., Sussex, UK, was used.

The gas chromatographic analyses were carried out using Mega Series Model 5160 apparatus (Farmitalia C. Erba, Milan, Italy); for the HPLC analyses, a Model Series 3 apparatus (Perkin–Elmer, Norwalk, USA), was used; the spectroscopic observations were obtained by an UV/VIS (visible) Perkin–Elmer Model Lambda 2 spectrophotometer; the ^{13}C-NMR spectra were obtained using a Bruker Model AC 300 spectrometer (Bruker Instruments, Inc., Karlsruhe, Germany).

Chemicals were mostly of chromatographic grade and were almost all supplied by Farmitalia C. Erba (Milan, Italy). *Bis*-trimethylsilyltrifluoroacetamide and resorcinol (for HRGC analysis of phenols), Folin Ciocalteus's reagent (for colorimetric analysis of phenols) and silicagel 60 (for HRGC analysis of waxes) were provided by Merk (Darmstadt, Germany); hexane and chloroform (for HRGC analysis of sterols and waxes) were obtained from Baker (Deventer, Holland); pyridine (for HRGC analysis of sterols) was from Fluka (Buchs, Switzerland); finally, nonan-1-ol (for HRGC analysis of volatiles) and deuterated chloroform and tetramethylsilane (for ^{13}C-NMR analysis of triglycerides and diglycerides) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

2.4. Statistic analyses

Average data concerning both yields and analytical variables were statistically tested with the two-sided variance analysis (ANOVA) with replications. If the null hypothesis was rejected, Tukey's studentised range test was applied to separate the means (Lison, 1982). Probabilities higher than $p = 0.05$ were considered non-significant. Data concerning analytical oil and waste water variables were also processed by Principal Components Analyses (PCA) (Pigott & Jardine, 1986). Barlett's sphericity and Kevin–Meyer–Olkin tests were used to check that PCA might be applied to the data set. PCA was applied under the following conditions: Kaiser's normalisation, varimax rotation, tolerance limits for matrix inversion (0.0001). The cross-validation procedure was used to determine the maximum number of significant dimensions to avoid data over-fitting. The package Unscrambler II Version 5.52 (CAMO A/S, Trondheim, Norway) was used. The softwares Genstat and Excel 5.0 for Windows were also utilised.

3. Results and discussion

The mean data and standard deviations for the major analytical parameters and indices determined on the oils are given in Tables 2 and 3.

3.1. Volatiles, phenolics and tocopherols

Quality was defined by Kramer and Twigg (1966) as being composed of those chemical and physical

Table 2
Main analytical and compositional characteristics of oils from three olive varieties processed with a new enzyme aid, compared to reference oils[a]

Codes	Analytical oil parameters	*Coratina*		*Dritta*		*Leccino*	
		Aid	Control	Aid	Control	Aid	Control
		CoE	CoC	DrE	DrC	LeE	LeC
1	Phenolics (as caffeic acid, mg kg^{-1})[b]	265 (28)**	165 (15)	108 (12)*	86 (9)	135 (15)*	104 (11)
2	*o*-Diphenols (as caffeic acid, mg kg^{-1})[b]	183 (18)**	116 (11)	65 (6)*	51 (4)	72 (6)*	59 (5)
3	Tyrosol (as resorcinol, mg kg^{-1})[c]	23.7 (2.5)**	11.1 (1.3)	4.1 (0.5)	3.2 (0.3)	6.4 (0.6)	4.9 (0.4)
4	Hydroxytyrosol (as resorcinol, mg kg^{-1})[c]	2.6 (0.3)	3.1 (0.4)	1.5 (0.1)	1.4 (0.1)	1.3 (0.1)	1.2 (0.1)
5	Tyrosol-aglycones (as resorcinol, mg kg^{-1})[c]	8.4 (0.9)*	11.5 (1.1)	1.9 (0.2)	1.2 (0.1)	1.1 (0.1)**	4.1 (0.4)
6	Hydroxytyrosol-aglycones (as resorcinol, mg kg^{-1})[c]	21.5 (2.0)**	10.2 (0.9)	15.5 (1.7)*	9.3 (1.1)	6.8 (0.8)*	3.7 (0.4)
7	Oleic acid (%)[d]	82.8 (6.3)	82.7 (5.4)	76.1 (5.6)	76.3 (5.3)	77.0 (6.0)	76.9 (5.7)
8	Linoleic acid (%)[d]	5.7 (0.3)	6.1 (0.4)	8.2 (0.5)	7.7 (0.6)	7.1 (0.6)	7.3 (0.7)
9	Linolenic acid (%)[d]	0.4 (0.0)	0.5 (0.1)	0.4 (0.0)	0.4 (0.0)	0.5 (0.0)	0.5 (0.0)
10	Palmitic acid (%)[d]	8.3 (0.8)	8.6 (0.7)	10.7 (0.7)	11.0 (0.9)	11.8 (1.0)	11.8 (1.1)
11	Stearic acid (%)[d]	2.0 (0.1)	2.0 (0.1)	3.5 (0.3)	3.5 (0.4)	2.3 (0.1)	2.3 (0.1)
12	Triacylglycerols (%)[e]	98.2 (6.6)	98.3 (7.0)	98.0 (6.5)	98.1 (7.4)	98.2 (9.1)	98.3 (9.9)
13	Diacylglycerols (%)[e]	1.8 (0.2)	1.7 (0.2)	2.0 (0.2)	1.9 (0.1)	1.8 (0.2)	1.7 (0.2)
14	1,2-Dyglycerides/1,3-diglycerides	4.1 (0.3)*	3.3 (0.3)	2.3 (0.2)*	1.8 (0.1)	6.7 (0.7)*	6.0 (0.4)
15	Waxes ($C_{40}+C_{42}+C_{44}+C_{46}$, mg kg^{-1})	34 (3)*	24 (2)	85 (9)*	62 (7)	91 (10)	91 (9)
16	Total waxes (mg kg^{-1})	119 (12)	79 (8)	277 (26)	212 (24)	394 (38)	354 (42)
17	Total sterols (mg 100 g^{-1})	123 (12)	122 (12)	115 (12)	117 (13)	158 (14)	156 (12)
18	Aliphatic alcohols ($C_{22}+C_{24}+C_{26}+C_{28}$, mg 100 g^{-1})	7.4 (0.9)*	6.3 (0.7)	10.6 (1.1)*	6.8 (0.7)	11.7 (1.1)*	9.6 (0.8)
19	Triterpene alcohols (mg kg^{-1})	93 (9)**	78 (9)	103 (10)*	94 (11)	117 (12)**	97 (10)
20	Alcoholic index	0.03 (0.00)	0.04 (0.00)	0.16 (0.01)	0.18 (0.01)	0.17 (0.02)	0.15 (0.01)
21	Erythrodiol (mg kg^{-1})	21.1 (1.8)*	19.2 (1.8)	22.4 (2.4)	23.3 (2.8)	17.0 (1.5)*	10.3 (0.7)
22	Uvaol (mg kg^{-1})	5.2 (0.5)*	3.3 (0.3)	6.0 (0.6)*	4.1 (0.4)	11.1 (1.0)*	6.6 (0.7)

[a] Data are means of four replicates. Standard deviations are shown in brackets. Values with one or two asterisks are significantly different from corresponding controls (Tukey's studentised range test; $*p \leqslant 0.05$, $**p \leqslant 0.01$).
[b] As determined by colorimetric method.
[c] As determined by HRGC method.
[d] Related to the total area of chromatogram.
[e] Related to total glyceridic classes.

characteristics that give a product consumer appeal and acceptability. Volatile and phenol components are basically responsible for the flavour and "fruity taste" of olive oil and therefore are key factors which determine high-quality and typicality of this product (Ranalli & Costantini, 1994). Phenols and noticeably *o*-diphenols are also natural antioxidants and are largely responsible for the shelf-life of olive oil (Ranalli & Ferrante, 1996). Only some phenol components (e.g. tyrosol and oleuropein) have no (or negligible) antioxidising activity (Ranalli & Lazzari, 1996). Recently, we have shown (Ranalli, De Mattia, & Ferrante, 1997), using a HRGC determination method, that some complex phenols are major components of the phenol fraction of virgin olive oil. Similar results had been obtained by Montedoro, Servili, Selvaggini, Miniati, and Macchioni (1993) by using a spectroscopic determination method.

The oils produced using the enzyme processing aid were steadily richer in total phenols, *o*-diphenols, and hydroxytyrosol-aglycones (Table 2). The data suggest that the 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 glycosides and esters) from which, consequently, a higher quantity of phenol constituents could be freed and dissolved in the oil.

The enzyme effect also resulted in an increase in the oil of the total identified pleasant volatiles (1-penten-3-one, 1-penten-3-ol, *trans*-2-hexenal, 2-penten-1-ol, 1-hexanol, *cis*-3-hexen-1-ol, and *trans*-2-hexenol) (Table 3); also the characteristic ratios of *trans*-2-hexenal to hexanal and that of *trans*-2-hexenal to total aroma of the oils were higher (data not shown).

Trans-2-hexenal is the most important volatile present in good virgin olive oils (Ranalli & Lazzari, 1996). According to some authors (Solinas, Marsilio, & Angerosa, 1987), this component (which forms by enzymic degradation from 13-L-hydroperoxide of linoleic acid) gives a very pleasant odour of fresh cut grass, and is responsible for the sensory green-fruity notes of the product, along with *cis*-3-hexenyl acetate. According to other authors (Morales, Alonso, Rios, & Aparicio, 1995), *trans*-2-hexenal is responsible for the bitter almond flavour. In contrast, hexanal gives a very disagreeable smell and is present in small amounts (as the other undesirable volatiles) in good virgin olive oils (Solinas et al., 1987).

Table 3
Other important analytical and compositional characteristics of oils from three olive varieties processed with a new enzyme aid, compared to reference oils[a]

Codes	Analytical oil parameters	*Coratina*		*Dritta*		*Leccino*	
		Aid	Control	Aid	Control	Aid	Control
		CoE	CoC	DrE	DrC	LeE	LeC
23	Lutein (mg l^{-1})	3.95 (0.40)**	3.02 (0.34)	2.76 (0.27)	2.58 (0.22)	3.78 (0.37)**	2.86 (0.25)
24	β-Carotene (mg l^{-1})	1.08 (0.10)*	0.81 (0.08)	0.65 (0.05)	0.60 (0.07)	0.96 (0.10)*	0.77 (0.07)
25	Violaxanthin (mg l^{-1})	0.55 (0.06)*	0.40 (0.04)	0.31 (0.03)	0.29 (0.03)	0.47 (0.04)*	0.33 (0.03)
26	Neoxanthin (mg l^{-1})	0.51 (0.05)*	0.38 (0.03)	0.29 (0.02)	0.29 (0.02)	0.42 (0.04)*	0.30 (0.03)
27	Chlorophylls and pheophytins (mg kg^{-1})	16.2 (1.8)**	10.9 (1.1)	7.7 (0.8)**	5.6 (0.5)	8.3 (0.7)**	6.2 (0.5)
28	Hue (nm)	578 (32)	578 (38)	578 (40)	578 (36)	578 (38)	577 (40)
29	Saturation (%)	96.4 (10.1)*	93.1 (10.0)	92.5 (8.9)	92.3 (9.1)	96.7 (8.4)	94.6 (7.1)
30	Brightness (%)	66.6 (4.4)**	70.6 (6.0)	73.5 (6.8)	74.5 (6.6)	69.5 (6.7)*	71.1 (6.4)
31	Turbidity (NTU)[b]	360 (20)*	410 (26)	36 (2)*	65 (4)	520 (39)	510 (24)
32	Free acidity (as oleic acid, %)	0.31 (0.01)	0.33 (0.02)	0.45 (0.03)	0.42 (0.03)	0.40 (0.02)	0.39 (0.02)
33	Peroxide index (meq O_2 kg^{-1})	3.60 (0.26)	3.70 (0.30)	7.62 (0.56)	7.58 (0.49)	4.01 (0.23)	4.40 (0.19)
34	k_{232}	1.36 (0.09)	1.39 (0.07)	1.38 (0.06)	1.44 (0.08)	1.47 (0.09)	1.29 (0.09)
35	k_{270}	0.13 (0.01)	0.14 (0.01)	0.09 (0.01)	0.10 (0.01)	0.09 (0.01)	0.11 (0.01)
36	Carbonyl index-MWI	3.4 (0.30)	3.8 (0.31)	3.3 (0.30)	3.0 (0.14)	5.0 (0.28)	5.5 (0.35)
37	Pleasant volatiles (as nonan-1-ol, mg kg^{-1})	711 (69)*	603 (52)	210 (22)*	145 (17)	355 (36)**	253 (23)
38	Unpleasant volatiles (as nonan-1-ol, mg kg^{-1})	138 (15)*	86 (10)	47 (4)*	80 (8)	54 (5)*	93 (8)
39	*Trans*-2-hexenal	552 (48)*	477 (47)	165 (19)*	129 (14)	268 (29)**	177 (20)
40	α-Tocopherol (mg kg^{-1})	112 (10)*	89 (7)	76 (6)*	58 (6)	221 (20)*	175 (18)
41	γ-Tocopherol (mg kg^{-1})	0.4 (0.0)	0.3 (0.0)	0.2 (0.0)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
42	Swift's test (h)	10.9 (0.9)**	8.7 (0.7)	5.6 (0.4)*	4.8 (0.4)	8.7 (0.6)*	7.7 (0.4)
43	Panel test (score)	7.7 (0.3)*	7.3 (0.4)	7.2 (0.2)*	6.9 (0.4)	6.8 (0.3)*	6.5 (0.3)

[a] Data are means of four replicates. Standard deviations are shown in parentheses. Values with one or two asterisks are significantly different from corresponding controls (Tukey's studentised range test; $*p \leqslant 0.05$, $**p \leqslant 0.01$).
[b] Nephelometric turbidity units.

The total value of volatile components is an excellent indicator of oil quality when produced from qualitatively good olives and when rational conditions of processing are adopted (Ranalli & Serraiocco, 1996). There is, for these oils, a high logarithmic correlation between total phenol content and total volatile content (Fig. 2).

Tocopherols consisted essentially of α-tocopherol, while only minute amounts of β-, γ- and δ-tocopherol were present in the two oil kinds. The oil produced with the enzyme was significantly richer in α-tocopherol (Table 3).

3.2. Autoxidation rate, sensory score, overall quality indices

The enzyme processing aid led to oils regularly having a higher oxidative stability (Swift's test) (Table 3), and this was consistent with the higher phenol and tocopherol content. The oils, in agreement with the higher volatile and phenol content, also received a higher sensory score (Table 3). They showed a slightly more pronounced bitter and pungent note, possibly related to a greater content of some phenol components. There was a good linear relationship ($r = 0.8057$*) between phenol content and sensory score, and between volatile content and sensory score ($r = 0.7345$).

The oils produced (with or without enzyme aid) were all without defects, and therefore, qualitative scores were based only on their positive organoleptic attributes. The values (not shown) of overall quality indices were slightly higher in the oil obtained with the enzyme as this slightly affected the analytical parameters involved to calculate the indices (phenol content excepted).

3.3. UV spectrophotometric indices, peroxide index, carbonyl index, free acidity degree

There is no statistically significant effect exerted by the enzyme aid on these analytical parameters (Table 3). Usually, technological treatments have little or no effect on them (Ranalli & De Mattia, 1997). Such parameters (carbonyl index excepted), together with sensory score, are the only ones at present considered by the E. C. Regulation no. 2568 (1991) to assess the olive oil quality. The specific extinction at 232 nm wavelength (k_{232}) (Table 3) is related to the primary oxidation rate of olive oil, while Δk (triene peak) and k_{270} (Table 3) are related to the secondary oxidation rate (Ranalli & Costantini, 1994). The spectrophotometric indices also are useful for detecting fraudulent additions of refined olive oil to virgin olive oil (E.C. Regulation no. 2568, 1991).

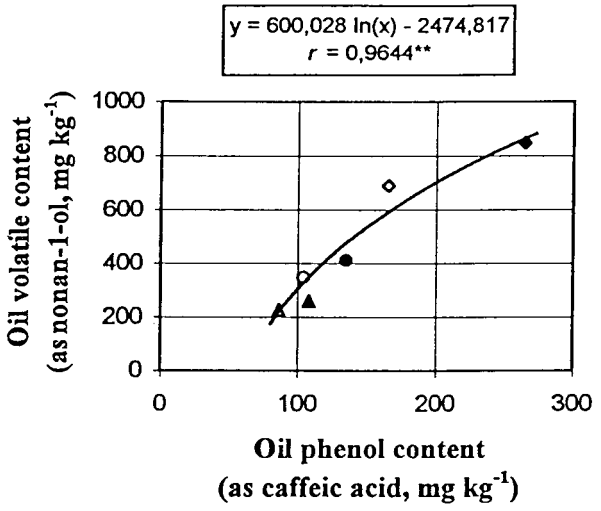

Fig. 2. Logarithmic relationship between total phenols (determined colorimetrically) and total volatiles (determined by HRGC) for the oils extracted from *Coratina* (◆ ◇), *Dritta* (▲ △) and *Leccino* (● ○) olive varieties; values are means of four replicates. Closed symbols = aid; open symbols = control; $^{**}p \leqslant 0.01$.

3.4. Lipochromes, chromatic parameters, colour indices, turbidity

The contents of green and yellow pigments and the values of σ% (saturation) were frequently higher in the treated oil (Table 3); in contrast, the values of h% (brightness) were frequently lower (Table 3). The values of λd (hue) did not differ from the reference oil (Table 3), indicating that both oil types were characterised by a yellow colour, which clearly prevailed over green; nor did the colour ratio (between carotenoid absorbance and chlorophyll absorbance). The values (not shown) of integral and of carotenoid and chlorophyll colour indices were frequently higher and agreed with those of the above colour parameters. The enzyme seemed to influence the release of yellow and green lipochromes from vegetable tissues and their solubilization in the oil. The values of turbidity were frequently higher. Both turbidity and colour are not considered by the panel test method (E.C. Regulation no. 2568, 1991) for the evaluation of sensory characteristics of olive oil, but they actually influence acceptability of the product by the consumer (Ranalli & Ferrante, 1996).

3.5. Sterols and triterpene dialcohols

The enzyme processing aid did not seem to influence the content of individual and total sterols of the oils, whereas a significant positive influence was frequently exerted on the content of triterpene dialcohols (Table 2). The values (not shown) of the campesterol/stigmasterol ratio, a parameter which is directly related to quality (the stigmasterol value being higher in defective oil) (Ranalli & De Mattia, 1997), appeared frequently positively affected ($p \leqslant 0.05$). The values of total sterols and of β-sitosterol, campesterol and stigmasterol (major sterols), and of brassicasterol, cholesterol, and Δ^7-stigmastenol (minor sterols), as well as of total triterpene dialcohols (all considered genuineness parameters), were in both oil types within the limits set by the official normal standard (E.C. Regulation no. 2568, 1991).

3.6. Long-chain aliphatic and triterpene alcohols

The values of these components were higher for the oils resulting from the enzyme-aided processing system (Table 2). However, the values (not shown) of the alcoholic index (Ranalli et al., 1997), a parameter which correlates negatively with quality (Ranalli & De Mattia, 1997), were comparable with those recorded for the reference oils. Such a phenomenon suggests that the enzyme increased the solubilization of alcoholic compounds in the oil without affecting the relative alcoholic composition and consequently the values of the alcoholic index.

3.7. Waxes and stigmastadienes

The oils produced with the enzyme aid were higher in both $C_{40}+C_{42}+C_{44}+C_{46}$ waxes and total waxes (Table 2), perhaps by dissolution of these components in the oils during the extraction process. There were good linear relationships between total waxes and total alkanols, triterpenic alcohols and dialcohols, substances which are all present in the external cuticle of olive fruit (Bianchi, Murelli, & Vlahov, 1992). The surface lipid layer, which is also present in the olive leaves (Bianchi, Vlahov, Anglani, & Murelli, 1993), is believed to be important for chemotaxonomical purposes (Bianchi et al., 1992). It also seems to have a role in determining the resistance of olive tree to biotic or abiotic stresses (Bianchi et al., 1993).

Analytical data showed that no (or only traces of) stigmastadienes (isomeric steroid hydrocarbons which are not resolved by capillary column) were present in the two genuine oil types. Such compounds are produced by dehydroxylation of the β-sitosterol (Tiscornia, 1992) and are useful in detecting additions of non-virgin olive oil or de-sterolised vegetable oils to virgin olive oil (E.C. Regulation no. 2568, 1991).

3.8. Triacylglycerols, diacylglycerols, fatty acid composition

Total triglycerides, total diglycerides and fatty acid composition of the oils were not affected by the enzyme processing aid, but the 1,2-diglycerides/1,3-diglycerides ratio, a parameter directly related to quality-freshness

of product (Ranalli et al., 1997), was positively influenced (Table 2).

The other qualitative glyceridic indices (Catalano, De Felice, Caponio, & De Leonardis, 1995), such as total diglycerides × 1,3 diglycerides product and total diglycerides/1,3 diglycerides ratio, showed variations which were not statistically significant (figures omitted). Our data confirmed that the *trans*-isomer C_{18} fatty acids (*trans*-oleic, *trans*-linoleic, and *trans*-linolenic acid isomers) are practically absent in genuine olive oil. The determination of these compounds are considered by the E.C. Regulation no. 2568 (1991) to detect fraudulent additions of refined olive oil or de-sterolised seed oil to virgin olive oil, and to ascertain whether non-edible olive oil with low acidity has been transformed to edible olive oil by bland de-acidification treatment. Some fatty acids (myristic, linolenic, arachidonic, eicosenoic, behenic and lignoceric acid), as well as trilinolein and sterols, are considered important (E.C. Regulation no. 2568, 1991) for revealing the presence of seed oil in virgin olive oil. The determination of saturated fatty acids in position 2 of triglycerides is considered by the same Regulation to detect additions of esterified oil to virgin olive oil.

Additions of husk oil to pressed oil are detected by determining, in the mixture, the content of waxes ($C_{40}+C_{42}+C_{44}+C_{46}$) or that of triterpene dialcohols or alkanols (above discussed), as husk oil is rich in these substances (Dionisi, Amelotti, & Cert, 1992). Triterpene dialcohols, however, can be removed from the husk oil by strong oxidative treatments, and alkanols can as well, but by other treatments (Dionisi et al.; Tiscornia, 1992).

It, therefore, is important to know the variations in non-adulterated virgin olive oil of the genuineness parameters (some of which, such as fatty acid composition and some relative sterol, alcohol and diglyceride ratios, are also quality indices) by investigating the influence exerted on the values by different factors (including technological treatments) (Tiscornia, 1992; Ranalli & Lazzari, 1996).

3.9. Multivariate analysis results

A summary of total variation of the major analytical oil parameters (Table 2 and Table 3) is presented by their factor loadings from the first two factors of the PCA (Fig. 3) However, three dimensions of the PCA model were found to be significant and explained 84% of the variance.

The first component, accounting for 39% of the total variance, was dominated by the variables 1, 2, 3, 4, 5, 7, 23, 24, 25, 26, 34, 36, 39, and 40 (positively loaded) and the variables 8, 10, 11, 15, 19, 29, 31, and 32 (negatively loaded); however, other variables also contributed to the variance this factor described, except for variables 9, 14, 16, 20, 21, 27, 33, 35, and 38.

The second dimension, accounting for 28% of the total variance, was dominated by the variables 9, 12, 14, 16, 21, 28, 30, 35, and 38 (positively loaded) and the variables 6, 13, 20, 27, 32, and 41 (negatively loaded).

Finally, a third dimension, accounting for 17% of the total variance, was dominated by the variables 6, 13, 17, 18, 21, 22, 27, 28, and 33 (positively loaded), and the variables 5, 9, 12, and 34 (negatively loaded).

The PCA scores of samples on the 1 and 2 dimensions are given in Fig. 4. The two PCA factors were most effective in discriminating between oil varieties. The *Leccino* and *Dritta* varieties are discriminated along the first component (negative half) and the Coratina variety along the second component (positive side). The genetic factor (olive variety) effect, as expected, predominated over the enzyme processing aid effect. This was confirmed by the Hierarchical Cluster Analysis (Goodacre et al., 1992). In fact, the dendrogram (Fig. 5) showed three blocks, each consisting of one variety, with a similarity percentage >70% (*Coratina*), >80% (*Leccino*) and >90% (*Dritta*).

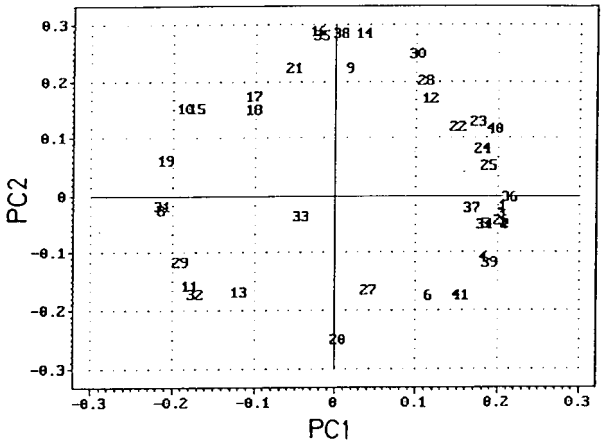

Fig. 3. PCA loadings of analytical oil variables for dimensions 1 and 2.

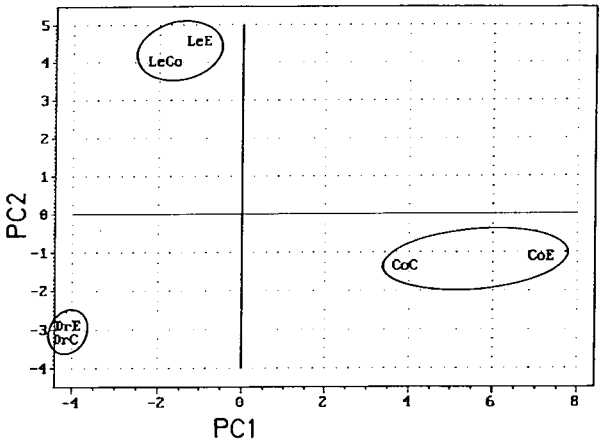

Fig. 4. Score plot, by the dimensions 1 and 2 from PCA, of the olive oils produced with or without enzyme processing aid from the *Coratina*, *Dritta*, and *Leccino* olive varieties.

The comparison between score plot and loading plot indicated that the variables 16, 21 and 35 were mainly responsible for discrimination of the *Leccino* variety, while the *Dritta* variety was mainly differentiated by the variables 11, 12, 13, and 29, and the *Coratina* variety by the variables 4, 26, 34, 37, 39.

Concerning the analytical waste water parameters, three dimensions of the PCA model were found of be significant and explained 86% of the total variance. The factor loadings (Fig. 6) from the dimensions 1 and 3 of the PCA (accounting for 30 and 33% of the total variance, respectively) showed how all the variables were loaded along the first component (negative half, except for variable A). The PCA scores of samples on the 1 and 3 dimensions are given in Fig. 7. The two PCA factors seemed to be able to discriminate between processing techniques (with or without enzyme)

3.10. Oil output and analytical features of by-products

The enzyme aid led, with all the three olive varieties processed, to higher overall yields (generally $>1\%$ w/w average, olive fruit basis) which were statistically significant ($p \leqslant 0.05$) (data not shown). This increase in output, in industrial processing, is very significant. Percolation yield increase was always higher than centrifugation yield increase. Also, percolation and overall yield increases were both higher with "difficult olives" (*Leccino* and *Coratina*).

The average value of residual oil found in the husk, regardless of the olive variety processed, was 6.04 and 7.00% d.s. with and without enzyme, respectively, while the average value of oil found in the effluent was 9.99 vs 12.69% d.r. These data were consistent with those of the yields.

Also, the amount of husk produced was lower (48.8 vs 51.2%) as its moisture content decreased, due in turn to a greater separation of the vegetable water from the

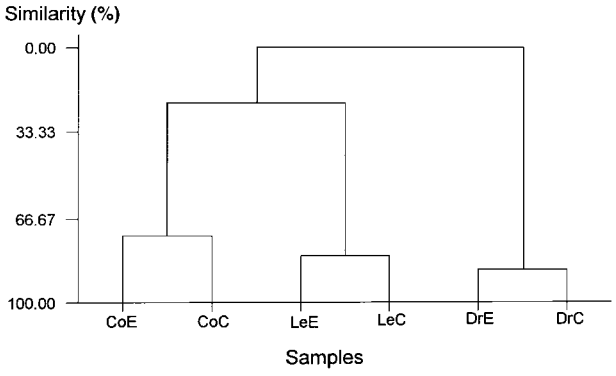

Fig. 5. Dendrogram showing the clustering of the *Coratina*, *Dritta*, and *Leccino* olive varieties independently of processing technique (with or without enzyme aid) using the analytical oil variable data set.

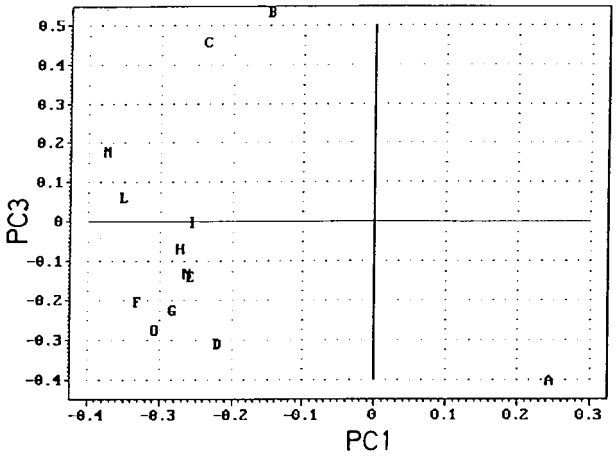

Fig. 6. PCA loadings of analytical oil mill waste water variables for dimensions 1 and 3.

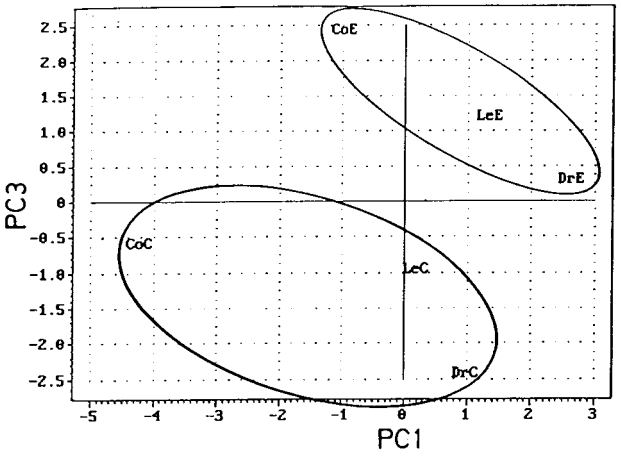

Fig. 7. Score plot, by the dimensions 1 and 3 from PCA, of the oil mill waste waters produced with or without enzyme processing aid from the *Coratina*, *Dritta*, and *Leccino* olive varieties.

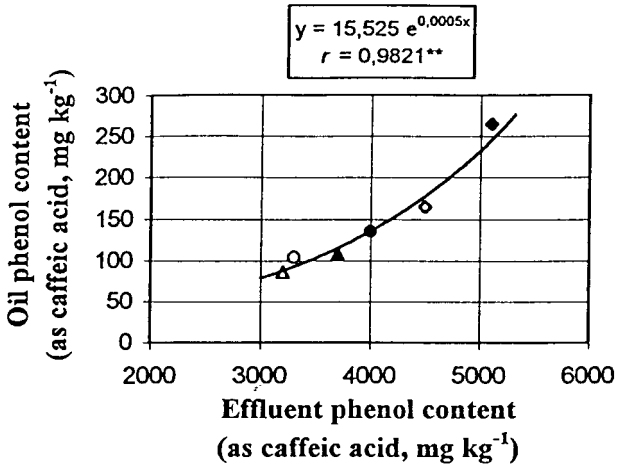

Fig. 8. Exponential relationship between phenol content of effluent and phenol content of olive oil produced by processing *Coratina* (◆ ◇), *Dritta* (▲ △) and *Leccino* (● ○) olive varieties; values are means of four replicates. Closed symbols = aid; open symbols = control; $^{**}p \leqslant 0.01$.

Table 4
Main analytical data of oil mill waste waters from three olive varieties processed with a new enzyme aid, compared to the reference waste waters[a]

Codes	Analytical effluent parameters	*Coratina*		*Dritta*		*Leccino*	
		Aid	Control	Aid	Control	Aid	Control
		CoE	CoC	DrE	DrC	LeE	LeC
A	Turbidity (NTU × 10^{-3})[b]	42.2 (4.0)*	44.6 (4.5)	48.0 (5.3)	49.8 (3.9)	48.0 (4.0)	50.5 (5.0)
B	*o*-Diphenols (as caffeic acid, g kg^{-1})	2.9 (0.3)*	2.5 (0.2)	2.3 (0.2)*	1.7 (0.1)	2.5 (0.2)*	1.9 (0.2)
C	Phenolics (as caffeic acid, g kg^{-1})	5.1 (0.4)*	4.5 (0.4)	3.7 (0.3)*	3.2 (0.3)	4.0 (0.5)**	3.3 (0.4)
D	COD (g l^{-1})[c]	98.5 (6.4)**	137.2 (13.9)	96.4 (10.9)**	135.1 (11.7)	80.1 (7.5)	80.1 (8.3)
E	Total solids (105°C) (g 100 m l^{-1})	9.7 (0.9)*	10.9 (1.2)	9.1 (0.9)*	10.2 (1.1)	8.0 (0.7)	7.9 (0.7)
F	Reducing sugars (g l^{-1})	25.8 (2.0)**	36.7 (2.9)	25.0 (2.1)	25.8 (2.5)	24.6 (2.5)*	33.2 (3.7)
G	pH	5.17 (0.4)	5.33 (0.5)	4.99 (0.4)	5.16 (0.5)	5.00 (0.4)*	5.40 (0.5)
H	Conductibility	37.6 (2.4)**	45.8 (3.4)	33.3 (3.1)*	37.3 (2.6)	43.1 (3.8)	44.1 (4.4)
I	Salinity	11.9 (0.7)**	16.1 (1.0)	8.5 (0.6)*	10.9 (0.8)	15.4 (1.3)	15.3 (1.2)
L	Nitrogen (g l^{-1})	0.61 (0.11)*	0.75 (0.10)	0.37 (0.04)*	0.46 (0.06)	0.59 (0.14)*	0.67 (0.17)
M	Phosphorus (g l^{-1})	0.45 (0.05)	0.49 (0.03)	0.31 (0.02)	0.29 (0.01)	0.33 (0.02)*	0.41 (0.03)
N	Ashes (g l^{-1})	9.4 (0.8)**	10.5 (1.0)	9.1 (0.9)*	9.8 (0.9)	8.4 (0.7)	8.1 (0.5)
O	Oil (g l^{-1})	10.27 (1.04)*	13.25 (1.47)	9.37 (1.09)*	12.06 (1.42)	9.0 (0.94)	9.3 (1.12)

[a] Data are means of four replicates. Standard deviations are shown in parentheses. Values with one or two asterisks are significantly different from corresponding controls (Tukey's studentised range test; *$p \leqslant 0.05$, **$p \leqslant 0.01$).
[b] Abbreviation as in Table 3.
[c] Chemical oxygen demand.

paste, with consequent higher production of liquid effluent; the latter was, however, less concentrated (d.r. = 8.9 vs 9.7% w/v), with a lower value for the COD (91 vs 117 kg^{-1}), even though it was richer in total phenols (4.3 vs 3.7 g kg^{-1}) as well as in *o*-diphenols (2.6 vs 2.0 g kg^{-1}) (as was the corresponding oil). There is a good exponential relationship between phenol content of effluent and phenol content of oil (Fig. 8).

Our data (Table 4) confirm that oil mill waste water is most rich in both suspended colloidal material and solubilized (or emulsified) organic substances, some of which are harmless (e.g. reducing sugars) but others (such as phenols, fat, and brown pigment) exert antimicrobial activity (Ranalli, 1991). The catecholmelanin pigment might be a major component (Ragazzi, Veronese, & Pietrogrande, 1967) and might form during olive processing by polymerisation of *o*-diphenols caused by polyphenoloxidase enzymes (Ranalli, 1987). Thus, the waste can be a very strong source of pollution, as substantiated also by its high COD value, and its filtering should be carried out before disposing of it in the environment (Ranalli, 1987). To treat it biologically (by selected bacteria), three pre-treatments have been suggested (Ranalli, 1992b), such as: (i) adjustment of the pH to an approximate value of 7; (ii) adjustment of the initial C:N:P ratio to the optimum ratio (100:10:2) by using nitrogen and phosphorus salts; and (iii) dephenolisation.

4. Conclusion

The new enzyme processing aid positively modified several analytical and compositional parameters and indices related to quality and typicalness of the oils. The shelf-life and the flavour were affected as well. This was confirmed by examination of the pyrograms. Some genuineness parameters were also modified but their values were within the variability range set by the official normal standard (E.C. Regulation no. 2568, 1991). The qualitative and quantitative results achieved make use of the enzyme economically advantageous (despite its cost) and we propose that its employment in olive processing is officially recognised in olive-producing countries throughout the world.

Acknowledgements

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