A method was developed for the determination of simple phenolic compounds (PCs) in waste waters from olive oil production plants by liquid chromatography (LC). The sample under examination was acidified to pH 2 to precipitate proteins, acetone was added to eliminate the colloidal fraction, and hexane was used for extraction to eliminate lipidic substances. The solution obtained was filtered and injected into the LC system; the wavelength selected for the spectrophotometric detection was specific for PCs, so that carbohydrates, organic acids, and short-chain free fatty acids did not interfere. Recoveries of 9 PCs spiked to a real sample were 90–100% for concentrations ranging from 20 to 2000 mg/L for each analyte.

The countries that produce the greatest amounts of virgin olive oils belong to the Mediterranean region, specifically, Southern Europe (Spain, Italy, Greece, Turkey, and Portugal) and Northern Africa (Tunisia, Morocco, and Algeria). Waste water is the main by-product obtained from oil production and is called vegetative water (VW). VWs are composed of water originally contained in the olives and water eventually added during the production process. The 3-phase olive oil extraction systems provide for addition of water; in the 2-phase systems, which do not provide for addition of water, the amount of VWs obtained is lower. VWs are an acidic (pH 4–5.5), highly complex matrix of dark (from red to black) color; they are strongly bad-smelling and subjected to putrefaction. VWs consist of water (83–96%), sugars (1.0–8.0%), nitrogenous substances (N × 6.25 = 0.5–2.4%), organic acids (0.5–1.5%), pectins, mucilages, and tannins (1.0–1.5%), and inorganic substances (0.5–2.0%; 1–4). Their poor biodegradability and high phytotoxicity are due to the presence of phenolic compounds (PCs), free fatty acids, and inorganic (in particular potassium) salts (2, 3, 5–14), as well as the presence of large numbers of bacteria, yeasts, and fungi (15). For these reasons, VWs show rather high biochemical (BOD) and chemical oxygen demand (COD) values that make it difficult to correctly dispose of them according to current regulations (1–3, 5, 6, 9, 16). Therefore, VWs represent a considerable environmental problem for the countries involved.

Several possibilities have been evaluated to lower the polluting load of VWs, including chemical, physical, physicochemical, and biological treatments, or their combination (1–6, 8–10, 13, 16, 17). During biological treatments, simple PCs strongly interfere with the purification processes, owing to their toxic effect on the microorganisms used. A method for determination of PCs in VWs, therefore, should relate the various treatments adopted with the effectiveness of lowering of toxic substances and consequently with the possibility of an easier disposal or a profitable use of VWs.

The classical method for determination of PCs is the colorimetric procedure, which uses the Folin-Ciocalteu reagent. However, this method is characterized by poor specificity, as other compounds present in the matrix may contribute to the absorbance (14, 18–20). Moreover, it is not possible to quantify the individual phenols because the Folin-Ciocalteu procedure evaluates total phenols. Procedures that provide for the separation and quantitative determination of individual PCs by either gas chromatography (GC) or liquid chromatography (LC) are much more satisfactory, as each phenol may show a different toxicity (8, 11, 12). However, in the case of complex matrixes the key to the determination of PCs lies in the preliminary sample processing, which requires a separation of the phenolic fraction from the other constituents pres-
ent. This reduces to a minimum the possibility of interferences during subsequent GC or LC determination, and lengthens the life of the analytical column. Solvent extraction is frequently used; methanol was used for preliminary separation of PCs from apples (21), maize (22), and fruit puree (23), methanol–water from leaf material (24), hexane and acetonitrile from apples (21), and ethyl acetate from aqueous matrixes and from VWs (7, 25–29). However, rather low extraction recoveries of some phenols into organic solvents from aqueous systems were reported (25, 30–32). Recently, solid-phase extraction cartridges were used for separation of PCs from wine (33), fruit puree (23), and natural and waste waters (34–36).

The method described here for the determination of simple PCs in VWs is based on preliminary sample processing to eliminate most interfering components present in the matrix, and subsequent determination of the analytes of interest by LC.

**Experimental**

**Standards and Reagents**

(a) **Vanillin, homogentisic, protocatechuic, homovanillic, vanillic, caffeic, chlorogenic, syringic, p-coumaric, ferulic, sinapic, and cinnamic acids.**—Fluka (Buchs, Switzerland).

(b) **Tyrosol, shikimic, veratric, and p-hydroxybenzoic acids.**—Aldrich Chemical Co. (Steinheim, Germany).

(c) **Oleuropeine.**—Extrasynthèse (Genay, France).

(d) **Hydroxytyrosol.**—Synthesized according to the method described by Capasso et al. (37).
Table 1. Parameters of regression lines for calibration of phenolic compounds considered

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxytyrosol</td>
<td>51229</td>
<td>648</td>
<td>-349246</td>
<td>131984</td>
<td>0.9989</td>
<td>3</td>
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<tr>
<td>Protocatechuic acid</td>
<td>76491</td>
<td>287</td>
<td>317162</td>
<td>57714</td>
<td>0.9999</td>
<td>3</td>
</tr>
<tr>
<td>Tyrosol</td>
<td>28786</td>
<td>293</td>
<td>15958</td>
<td>59231</td>
<td>0.9993</td>
<td>3</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>76705</td>
<td>791</td>
<td>-266905</td>
<td>161552</td>
<td>0.9993</td>
<td>3</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>89549</td>
<td>859</td>
<td>373250</td>
<td>174223</td>
<td>0.9994</td>
<td>3</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>163493</td>
<td>777</td>
<td>91269</td>
<td>155363</td>
<td>0.9998</td>
<td>3</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>144154</td>
<td>421</td>
<td>175867</td>
<td>83629</td>
<td>0.9999</td>
<td>3</td>
</tr>
<tr>
<td>Vanillin</td>
<td>198551</td>
<td>263</td>
<td>445897</td>
<td>54082</td>
<td>1.0000</td>
<td>3</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>254673</td>
<td>920</td>
<td>17532</td>
<td>185225</td>
<td>0.9999</td>
<td>3</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>118896</td>
<td>2704</td>
<td>792574</td>
<td>557497</td>
<td>0.9964</td>
<td>3</td>
</tr>
<tr>
<td>Veratic acid</td>
<td>64502</td>
<td>1674</td>
<td>397062</td>
<td>346607</td>
<td>0.9953</td>
<td>3</td>
</tr>
<tr>
<td>Oleuropeine</td>
<td>9689</td>
<td>301</td>
<td>4633</td>
<td>61539</td>
<td>0.9933</td>
<td>3</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>395582</td>
<td>2407</td>
<td>951490</td>
<td>489455</td>
<td>0.9997</td>
<td>3</td>
</tr>
</tbody>
</table>

* a, slope; b, standard error of the slope; c, intercept; d, standard error of the intercept; e, R square; f, number of points (3 replicates of each point).

(e) Oxalic, citric, maleic, phthalic, ascorbic, and mesotartaric acids.—Carlo Erba (Milan, Italy).

(f) Acetic, propionic, and butyric acids.—Merck (Darmstadt, Germany).

(g) Methanol.—J.T. Baker (Deventer, The Netherlands).

(h) Acetone, n-hexane, and 37% hydrochloric acid.—Merck.

Standard Solutions of PCs

About 15 mg homogentisic acid, hydroxytyrosol, protocatechuic acid, tyrosol, p-hydroxybenzoic acid, homovanillic acid, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, vanillin, p-coumaric acid, ferulic acid, veratic acid, sinapic acid, oleuropeine, and cinnamic acid were accurately weighed and dissolved in 3 mL methanol each (caution: methanol is very toxic). A 100 μL volume of each solution was transferred to a 5 mL volumetric flask and a solution of 5% acetic acid and 5% methanol in Milli-Q water (Millipore, Milford, MA) was added to the mark. The standard solution obtained, containing 100 mg/L of each PC, was filtered through a 0.2 μm PTFE filter (Lida, Kenosha, WI).

About 200 mg each of protocatechuic acid, vanillic acid, caffeic acid, syringic acid, vanillin, p-coumaric acid, ferulic

Table 2. Percent recoveries of phenolic compounds from vegetative water sample, as function of amount spiked and of temperature of procedure

<table>
<thead>
<tr>
<th>Temperature</th>
<th>30°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic compound</td>
<td>2000 mg/L</td>
<td>200 mg/L</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>99 ± 8</td>
<td>103 ± 6</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>100 ± 7</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>102 ± 8</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>97 ± 7</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Vanillin</td>
<td>92 ± 4</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>101 ± 7</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>93 ± 6</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>Veratic acid</td>
<td>91 ± 6</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>Oleuropeine</td>
<td>ND b</td>
<td>ND</td>
</tr>
</tbody>
</table>

* a, average percent recovery ± standard deviation. Recovery tests were performed in duplicate; both solutions were injected 3 times.

b CL = completely lost; ND = not determined.
acid, veratric acid, and oleuropeine was accurately weighed and dissolved in 50 mL methanol. The standard solution obtained, containing 4000 mg/L of each PC, was filtered through a 0.2 µm PTFE filter.

About 100 mg each of hydroxytyrosol, protocatechuic acid, tyrosol, p-hydroxybenzoic acid, vanillin, caffeic acid, syringic acid, vanilin, p-coumaric acid, ferulic acid, veratric acid, oleuropeine, and cinnamic acid was accurately weighed and dissolved in 20 mL methanol. The stock standard solution obtained, containing 5000 mg/L of each PC, was filtered through a 0.2 µm PTFE filter. By successive dilutions with the 5% acetic acid and 5% methanol in Milli-Q water solution, working standard solutions containing 5 mg/L of each PC were prepared.

**Calibration Graphs**

A 10 µL volume of each standard solution containing 5–350 mg/L PC was directly injected into the LC system.

**Sample Processing**

A 50 mL volume of the VW under examination was transferred to a separatory funnel, acidified with 37% HCl to pH 2; 50 mL acetone was added to precipitate the colloids. After ca 1 h, the sample was centrifuged at 5500 rpm for 15 min; the clarified sample was extracted 3 times with 50 mL n-hexane to remove the lipidic substances. The water–acetone layer was concentrated in a rotary vacuum evaporator at 30°C to ca 1–2 mL, and then quantitatively transferred to a 10 mL volumetric flask and diluted to the mark with methanol. The water–methanol solution was filtered through a 0.2 µm PTFE filter; a 10 µL volume was then injected into the LC system.

**Gas Chromatography**

A sample of VW was processed as described above to remove the lipidic substances. A 2 mL volume of the solution obtained was applied to a Buechner filter previously filled with 2 g Celite 560 and 1 g Polyclar AT (BDH, Poole, Dorset, UK). The PCs retained were then eluted with 100 mL methanol under suction. The methanol solution was evaporated to dryness in a rotary vacuum evaporator at 30°C; the residue was dissolved in 10 mL anhydrous acetone. The volume was reduced to 1 mL under a nitrogen stream to eliminate possible traces of moisture. The acetone solution was derivatized with 150 µL bis(trimethylsilyl)trifluoroacetamide (Merck) at room temperature for 1 h, and 0.1 µL of the obtained solution was injected into the GC system. A Mega series 5300 gas chromatograph (Carlo Erba), equipped with a cold on-column injector and a flame ionization detector, and an SPB-5 fused-silica capillary column, 30 m x 0.32 mm id, 0.10 µm film thickness (Supelco, Bellefonte, PA) were used. The oven temperature was programmed from 70 to 135°C at 2°C/min, held for 10 min at 135°C; then from 135 to 220°C at 4°C/min, held for 10 min at 220°C; then from 220 to 270°C at 4°C/min and maintained for 20 min; detector temperature was 290°C; carrier gas (helium) flow rate, 2.0 mL/min. Peak areas were determined by a Carlo Erba Mega integrator.

**Liquid Chromatography**

A Perkin Elmer liquid chromatograph equipped with binary LC pump 250, diode array detector 235, and 20 µL loop was used (Perkin Elmer, Norwalk, CT). A Spherisorb ODS-1 classic column (250 x 4.6 mm, 5 µm particle size) and a Guard Cartridge ODS-1 precolumn (7.5 x 4.6 mm, 5 µm particle size) were used (Alltech Associates, Deerfield, IL). Analyses were performed at room temperature. The mobile phase was 5% acetic acid and 90% methanol in Milli-Q water (A) vs 5% acetic acid and 5% methanol in Milli-Q water (B), and the gradient was changed as follows: 100% B for 15 min; from 100 to 50% B between 15 and 60 min; 50% B between 60 and 65 min; from 50 to 100% A between 65 and 70 min; 100% A between 70 and 80 min; from 100% A to 100% B between 80 and 85 min. The flow rate was 1.0 mL/min and the equilibration time was 10 min with 100% B. The eluates were detected at both 280 and 365 nm. Peak areas were determined by a Perkin Elmer 1020 LC Plus computer system.

**Determination of Recoveries**

A 2 L volume of a VW was kept for 6 months in an open flask at room temperature to oxidize the PCs originally present. Aliquots of 0.25, 2.5, and 25 mL of the standard solution of PCs containing 4000 mg/L of each component were transferred into a 50 mL volumetric flask, and the VW was added to the mark to obtain concentrations of PCs of 20, 200, and 2000 mg/L. The sample obtained was subjected to the procedure described under Sample Processing (above); the only difference was that the final water–methanol solution was obtained in a 50 mL volumetric flask.

**Statistical Analysis**

A Student’s t-test was used to determine if significant differences existed between results obtained under different experimental conditions.

**Results and Discussion**

**Sample Processing**

Although ethyl acetate extraction was widely used by others in previous studies for preliminary recovery of PCs from
For VWs, we obtained poor and variable recoveries (20–40%) of some PCs (particularly caffeic and vanillic acids) by this technique. The wide variability in chemical nature and characteristics of the PCs present in VWs caused some analytes of interest to be retained in the aqueous phase and consequently could not be extracted into organic solvents with satisfactory yields. Therefore, we set up a procedure of preliminary sample processing to eliminate most interfering compounds from VWs, rather than separating PCs from the matrix. The final water–methanol phase obtained was then used for separation and quantitative determination of individual PCs. LC was chosen as the separation technique because carbohydrates, whose separation with respect to phenols is the critical point of the procedure (2, 25), do not interfere in the final LC determination; the wavelengths selected for the detection are specific for PCs, not for carbohydrates. The wavelength of 365 nm was selected only for confirmation purposes, because some PCs (caffeic, ferulic, and synapic acids) also absorb at this wavelength; all analyses were routinely performed at 280 nm.

We obtained a very complex chromatographic trace when GC was chosen as the separation technique; in fact, carbohydrates underwent derivatization together with the PCs of interest and appeared in the chromatogram as heavily interfering peaks. Therefore, we set up a procedure that schematically consists of the following steps: acidification of vegetative waters to pH 2; addition of acetone; centrifugation; hexane extraction; concentration of the water–acetone solution by rotary vacuum evaporation; addition of methanol; and injection of the water–methanol solution into the LC system. The key steps of the method are precipitation of the protein fraction by acidification to pH 2; elimination of the colloidal fraction by centrifugation; hexane extraction; and elimination of the lipidic fraction by hexane extraction.

Specificity

The preliminary sample processing chosen eliminated several interfering components from the matrix; however, other compounds (in particular organic acids and short-chain free fatty acids) were not eliminated and were injected into the LC system.

Several organic acids (oxalic, citric, maleic, phthalic, ascorbic, and mesotartaric), known to be present in VWs, were checked; shikimic acid, which was reported to be present in olive oils (19) and is therefore possibly present in VWs, was checked as well. A sample of VW, previously subjected to the procedure described to give blank values, was spiked with aqueous solutions of the organic acids and free fatty acids tested; the matrix obtained was subjected to the whole procedure. Neither the organic acids tested nor the short-chain free fatty acids absorbed at the wavelengths selected, and neither of the acids interfered in the LC trace.

Figure 1 shows a typical separation obtained on a standard mixture containing 100 mg/L of the PCs frequently found in VWs. Figure 2 shows a typical separation obtained on a VW from a production plant of the Friuli-Venezia Giulia region (Italy). The identity of the peaks was established by co-injection with the corresponding pure standards.

Calibration

A straight line was obtained for all the PCs considered for concentrations ranging from 5 to 350 mg/L, which represented values typically found in VWs in previous papers (10, 14, 25, 28, 29, 38, 39). The parameters of the linear equations relative to the calibration obtained for the PCs considered are presented in Table 1.

Recoveries

Recoveries were determined by adding appropriate volumes of a standard solution of PCs to a VW. The standard solution consisted of 9 PCs that are known to be present in VWs (protocatechuic acid, vanillic acid, caffeic acid, syringic acid, vanillin, p-coumaric acid, ferulic acid, veratric acid, and oleuropeine). The matrix selected was kept for 6 months at room temperature to oxidize most phenols originally present; consequently, a preliminary blank test showed very low background levels of PCs in the chromatogram. The volumes of the standard solution added to the matrix were selected to obtain final concentrations of each PC of 2000, 200, and 20 mg/L, respectively. The amount found with respect to the sum of the amount added and that originally present in the matrix represents the recovery:

\[
\text{Rec.} \%, \quad \frac{\text{amount found (mg)}}{\text{amount added (mg)} + \text{originally present (mg)}} \times 100
\]

The results obtained are presented in Table 2. Recoveries for all PCs examined were quantitative, independently of their concentrations, when the rotary vacuum evaporation was performed at 30°C. On the contrary, recoveries of some PCs were significantly lower at the 0.05 level when the rotary vacuum evaporation was performed at 50°C, and some others were completely lost.

Application

A project supported by the European Community was conducted to investigate the possibility of setting up an integrated process for VW treatment with energy recovery. Aerobic cultures of bacteria able to remove PCs, yeast cultures able to produce ethanol, and an anaerobic consortium able to produce methane were preliminarily selected. In the subsequent phase, these cultures were used to inoculate a pilot plant located in an olive mill of the Friuli-Venezia Giulia region, where an integrated biological process was performed. The pilot plant consisted of 4 modules: the first for an equalization step, in which the VW pH was buffered at 7; the second for VW dilution to reduce the toxicity characteristics toward microorganisms; the third for PC removal under aerobic conditions; and the fourth for anaerobic treatment with methane production. The procedure described was applied to the determination of PCs in waste waters from the 4 modules; the results obtained are shown in Table 3. Only hydroxytyrosol, tyrosol, and caffeic acid were detected in fresh VWs of this plant. After dilution and PC removal, the waste waters from the anaerobic step showed no detectable levels of PCs.
References

(15) Gonzales Cancho, F. (1960) Grasas Aceites (Seville) 11, 9–14