# Standard Potential and Acidic Constants of Oleuropein

**Emilia Furia, Monica Nardi, and Giovanni Sindona***

Dipartimento di Chimica, Università della Calabria, Via P. Bucci Cubo 12/C, I-87036 Arcavacata di Rende (CS), Italy

The standard potential of oleuropein, $OleO_2H_2$, has been determined by a potentiometric titration method in 0.1 mol·dm$^{-3}$ $NaClO_4$ aqueous solutions employing a platinum wire-net working electrode. Oleuropein, the active ingredient present in food integrators extracted from olive leaves, in table olives, and in extra virgin olive oils, is a nutraceutical compound whose health benefits have been widely documented. For this reason, in our continuing investigation of metals with biological ligands, the acidic constants of oleuropein $K_1$ ($OleO_2H_2 \rightleftarrows H^+ + OleO_2H^-$) and $K_2$ ($Ole(O_2H)^- \rightleftarrows H^+ + OleO_2^{2-}$) were determined for use in further studies of the complexing power of this ligand toward metals of biological interest.

## Introduction

In the traditional Mediterranean diet, olive oil is the most usually employed lipid. There is much evidence relating the intake of olive oil to protection from cardiovascular diseases and the risk of breast cancer. The beneficial effect is attributed to a favorable fatty acid profile and to the presence of some minor components that are also responsible for its unique flavor and taste.[1] The minor component fraction includes tocopherols, carotenoids, phospholipids, and phenolic compounds. The phenols are responsible for the particular resistance of this oil to oxidative rancidity. Various studies[2,3] hypothesize the possibility that these phenols might also act as antioxidants. Among them, a phenolic compound present throughout the olive tree and in the oil and isolated from olive leaves is called oleuropein.[4] Oleuropein, $OleO_2H_2$, a secondary metabolite of terpenoid origin, is the main iridoid of *Olea europaea* and represents one of the 600 species of the *Oleaceae* family.[5] This secoiridoid glucoside is responsible for the bitter taste of olive drupes and leaves, and it is associated with many healing effects in humans and animals.[6−10]

Oleuropein, whose structure is reported in Figure 1, is an ester of 2′-(3′,4′-dihydroxyphenyl)ethanol(hydroxytyrosol) and the oleosidic skeleton common to the secoiridoid glucosides of *Oleaceae*.

This secoiridoid glucoside is known to possess several biological properties, many of which may be attributed to its antioxidant and free-radical-scavenging activities.[11] Numerous studies have shown that phenols, extracted from olives, have a wide range of pharmacological effects that include hypoglycaemia, antihypertensive agents, cytostatic, antiviral, antibacterial, and anti-inflammatory activity.[12] Many of these activities are due to the antioxidant properties of these compounds.

The presence of oleuropein in virgin olive oil and in olive leaves has been ascertained by mass spectrometric methods;[13] numerous synthetic transformations were conducted on oleuropein[14] whose derivatives show significant pharmacological properties.[15] For a variety of natural and synthetic compounds, good correlations were observed between antioxidant activities and oxidation potentials.[16,17] For this reason, to elucidate mechanisms of biological activity, the aim of this work was to determine, by potentiometric measurements employing a platinum wire-net working electrode, the standard potential of oleuropein. Moreover, to evaluate the complexing power of oleuropein toward metals of biological interest, the purpose of this work was to determine the acidic constants of oleuropein in 0.1 mol·dm$^{-3}$ $NaClO_4$ aqueous solutions. The evaluation of the dissociation constants is also important for drug design and optimization in biomedical and environmental analysis.

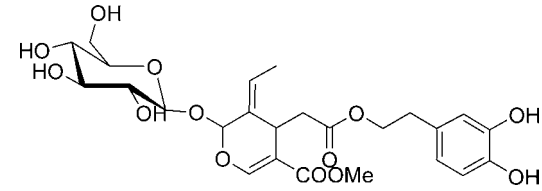


**Figure 1.** Structure of oleuropein, $OleO_2H_2$.

Taking into account our interest in the formation of metal−oleuropein equilibria, in this work $NaClO_4$ was used as the medium because it is the most inactive toward complexation. The adoption of the constant ionic medium method was necessary to minimize activity coefficient variation in spite of the change of the reagent concentration. In this way, it was possible to substitute activities with concentrations in the calculations and to minimize the liquid junction potential due to the hydrogen ion.

## Materials and Methods

***Instrumentation.*** The cell arrangement was similar to the one described by Forsling et al.[18] Ag/AgCl electrodes were prepared according to Brown.[19] Glass electrodes, manufactured by Metrohm, were of the 6.0133.100 type. They acquired a constant potential within 10 min after the addition of the reagents and remained unchanged to within ± 0.1 mV for several hours. A platinum wire-net working electrode was used for the measurements of the oleuropein concentrations. The titrations were carried out with a programmable computer controlled data acquisition switch unit 34970 A supplied by Hewlett and Packard. The EMF values were measured with a precision of ± $10^{-5}$ V using an OPA 111 low-noise precision DIFET operational amplifier. A slow stream of nitrogen gas was passed through five bottles containing 1 mol·dm$^{-3}$ NaOH, 1 mol·dm$^{-3}$ $H_2SO_4$, a Cr(II) solution, twice distilled water, and 0.1 mol·dm$^{-3}$

* Corresponding author. Phone: +39-0984-492046. Fax: +39-0984-493307. E-mail: sindona@unical.it.

10.1021/je901005x 
Published on Web 06/22/2010

$NaClO_4$, respectively, and then into the test solutions through the gas inlet tube. During the EMF measurements, the cell assembly was placed in a thermostat kept at (25.00 ± 0.05) °C. The spectrophotometric measurements have been conducted with a Varian Cary 50 Scan UV−visible spectrophotometer. The temperature of the cell-holder was kept at (25.0 ± 0.3) °C by a Grant circulating water bath. Matched quartz cells of thickness 1 cm were employed. The absorbance, $A_\lambda$, was recorded to ± 0.0001 units. The formulations of the parameters and the acquisition of the data have been managed with the aid of a computer connected to the tool.

$^1H$ NMR spectra were recorded on a Bruker WM 300 instrument at 300 MHz on samples dissolved in $CDCl_3$. The probe temperature was 23 °C. Field-frequency stabilization was established by means of a capillary tube filled with $D_2O$ inserted coaxially in the NMR tube.

The elemental analysis has been conducted through an Elemental Analyzer System 2400 Series II CHNS/O manufactured by Perkin-Elmer.

***Reagents and Analysis.*** Perchloric acid stock solutions were prepared and standardized as described previously.[20] Sodium perchlorate stock solutions were prepared and standardized according to Biedermann.[21] All solutions were prepared with twice distilled water.

The oleuropein was obtained from 100 g of olive leaves, dried in an oven at 50 °C for 48 h, to which was added 1 L of a MeOH/$H_2O$ (8:2) solution in the presence of catalytic amounts of hydroquinone, and then was allowed to flow for about 8 h. The solution was separated from the leaves, filtered, and evaporated to dryness under reduced pressure, which gives a rough sticky material containing various derivatives including oleuropein. The crude material was filtered and then washed with acetone to remove hydrophilic impurities. The resulting crude product was purified by flash chromatography on silica gel (mixture 8:2 v/v $CH_2Cl_2$/MeOH as eluent in the presence of a few drops of triethylamine). This produced about 1.25 g of oleuropein, whose appearance is a yellow spongy solid. The identity of the product was confirmed by $^1H$ NMR spectroscopy as well as by mass spectrometric methods.[13] The salt purity was determined by elemental analysis: anal. calcd for $C_{25}H_{32}O_{13}$ (540.52 g·mol$^{-1}$) C, 55.56; H, 5.97; found C, 55.42; H, 5.95.

***Solubility Measurements.*** To evaluate the maximum concentration of ligand that could be used, the solubility ($S$, mol·dm$^{-3}$) of oleuropein was evaluated, at 25 °C, in $NaClO_4$ ionic media by spectrophotometric measurements in the UV region. The ionic strength values were chosen to be (0.10, 1.00, and 3.00) mol·dm$^{-3}$ in $NaClO_4$ aqueous solutions. Saturated oleuropein solutions were prepared with a leaching apparatus suitable to prevent solid particles from coming into contact with the magnetic stirrer. As a matter of fact, preliminary measurements showed an increase of solubility over periods of weeks when the solid was in mechanical contact with the stirrer because the solid was transformed into a dispersed phase. To avoid grinding by the stirrer, solid oleuropein was wrapped up in a highly retentive filter paper (Whatman 42) bag. This in turn was kept in a glass cylinder containing sodium perchlorate solution at pre-established ionic strength values ($I$ = (0.1, 1, and 3) mol·dm$^{-3}$), while continuously stirring with a magnetic bar. The experimental apparatus is reported in Figure 2.

The cylinder was then placed in a thermostatic water bath at (25.00 ± 0.05) °C, and the oleuropein concentration was monitored over time, until it reached a constant value, which usually occurred in about 7 days. Finally, the absorption spectra of the oleuropein solutions in the UV region were recorded.

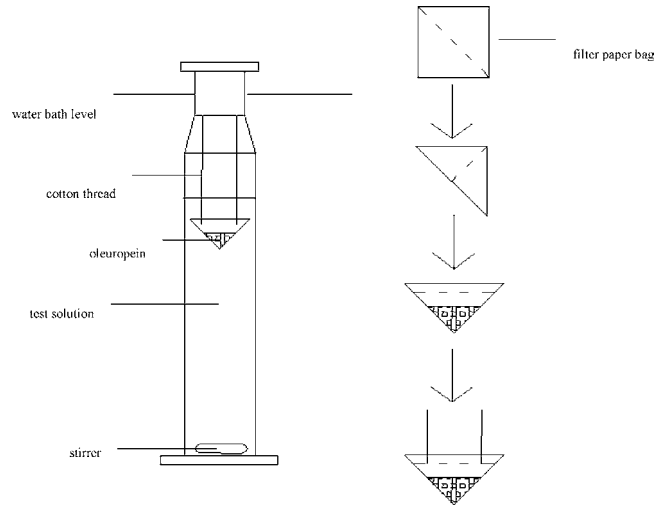


**Figure 2.** Apparatus for preparation of saturated oleuropein solutions.

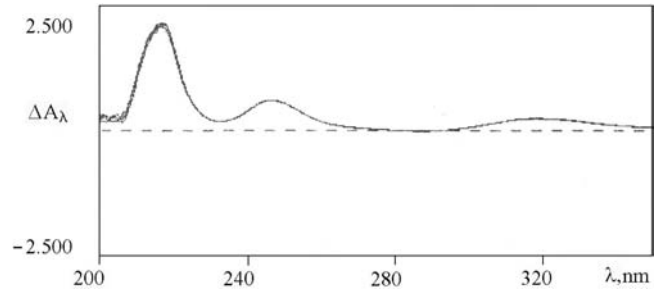


**Figure 3.** Absorption spectrum for oleuropein solution.

**Table 1. Solubility of $OleO_2H_2$ in $I$ (mol·dm$^{-3}$) $NaClO_4$**

| $I$, mol·dm$^{-3}$ | $S\cdot10^3$, mol·dm$^{-3}$ |
|---|---|
| 0.10 | 1.97 ± 0.02 |
| 1.00 | 2.42 ± 0.02 |
| 3.00 | 3.84 ± 0.04 |

Taking as a blank the ionic medium, the absorbance, $A_\lambda$, may be expressed as eq 1

$$A_\lambda = l\varepsilon[OleO_2H_2] \quad (1)$$

where $l$ is the optical path and $\varepsilon$ is the molar absorbivity. $A_\lambda$ values were measured between (200 and 350) nm to find suitable conditions for determining the solubility, $S$, of oleuropein. Three replicates were run for each point. A typical spectrum of oleuropein recorded is reported in Figure 3.

As can be seen, the absorption spectrum of oleuropein shows three bands centered at (220, 246, and 326) nm. The most intense peak at 220 nm seems the most suitable; however, its reproducibility is very poor. On the contrary, the peak at 246 nm was considered more adequate for accurate determinations. The absorbance at 326 nm was not used since it is less intense than that at 246 nm. The solubility, $S$, was deduced by interpolation on a calibration curve, based on standard solutions. The reproducibility of the solubility data was 1 %. The results are summarized in Table 1.

As can be seen from Table 1, the salt effect on oleuropein solubility is low, and therefore it was preferred to perform the potentiometric measurements in 0.1 mol·dm$^{-3}$ $NaClO_4$.

***Potentiometric Measurements.*** The determination of the standard potential as well as of the acidic constants of oleuropein was conducted by potentiometric measurements, performed as titrations, at 25 °C in 0.1 mol·dm$^{-3}$ $NaClO_4$. Oleuropein is particularly sensitive to even small traces of oxygen, and

therefore all measurements were performed under a slow stream of nitrogen gas, previously purified through the passage in five bottles containing 1 M NaOH, 1 M $H_2SO_4$, a Cr(II) solution, double-distilled water, and 0.1 M $NaClO_4$, respectively. This procedure was essential to prevent any protolytic impurities and oxidizing substances. The gas line was built using conical glass to avoid any contact with atmospheric oxygen. The attainment of accurate data has been facilitated by the possibility to add the reagents in a totally closed system by the coulometric generation in situ of known quantities of pure reagents. In particular, for the preparation of solutions to different acidities, the generation of $OH^-$ ions, according to reaction 2

$$H_2O + 2e^- \rightarrow 2OH^- + H_2(g) \quad (2)$$

was carried out by a coulometric circuit represented below

$$(-)Pt/TS/AE(+) \quad (C)$$

in which AE stands for the auxiliary electrode

$$AE = 0.1\ mol\cdot dm^{-3}\ NaCl/Hg_2Cl_2/Hg$$

and TS were the test solutions whose general compositions are shown in detail below. To minimize activity coefficient changes, test solutions were made to contain 0.1 $mol\cdot dm^{-3}$ $NaClO_4$.

The potentiometric measurements were carried out with cell (G)

$$RE/TS/GE \quad (G)$$

using an alkaline glass electrode, GE, as the working electrode for measurements of the hydrogen ion concentrations, and with cell (H)

$$RE/TS/Pt \quad (H)$$

using a platinum wire-net working electrode for the measurements of the oleuropein concentrations. For both cells, RE is the silver reference electrode = Ag/AgCl/0.01 $mol\cdot dm^{-3}$ $AgClO_4$ and 0.09 $mol\cdot dm^{-3}$ $NaClO_4$/0.1 $mol\cdot dm^{-3}$ $NaClO_4$, and TS is the test solution = $C_L$ $mol\cdot dm^{-3}$ $OleO_2H_2$, $C_A$ $mol\cdot dm^{-3}$ $HClO_4$, $\mu$eq $e^-$ removed, $(0.1 - C_A)$ $mol\cdot dm^{-3}$ $NaClO_4$, for the determination of standard potential of oleuropein, and = $C_L$ $mol\cdot dm^{-3}$ $OleO_2H_2$, $C_A$ $mol\cdot dm^{-3}$ $HClO_4$, $C_B$ $mol\cdot dm^{-3}$ NaOH, $(0.1 - C_A - C_B)$ $mol\cdot dm^{-3}$ $NaClO_4$, for the study of the protolytic equilibria of oleuropein. $C_L$ and $C_A$ are the analytical concentrations of oleuropein and perchloric acid, respectively, which were kept constant during each titration but were varied between the following range

$$1\cdot10^{-3}\ mol\cdot dm^{-3} \le C_L \le 2\cdot10^{-3}\ mol\cdot dm^{-3}$$

$$3\cdot10^{-3}\ mol\cdot dm^{-3} \le C_A \le 5\cdot10^{-3}\ mol\cdot dm^{-3}$$

The higher $C_L$ value is imposed by the limited solubility of the ligand.

The EMF of cell (G) can be written, in millivolts, at the temperature of 25 °C, as

$$E = E^\circ + 59.16 \log[H^+] + E_j \quad (3)$$

where $E^\circ$ is constant in each series of measurements and $E_j$ is the liquid junction potential which is a function of $[H^+]$.[22] In each run, $E^\circ$ was determined in the absence of $OleO_2H_2$ from measurements in solutions of $(2\cdot10^{-3} \ge [H^+] \ge 1\cdot10^{-4})$ $mol\cdot dm^{-3}$. In this range, $E^\circ$ values are constant to within 0.1 mV or better. $[H^+]$ was decreased stepwise by coulometric generation of $OH^-$ ions with the circuit (C).

Concerning the determination of the acidic constants of oleuropein, after the introduction of a known quantity of ligand, the acidity was decreased stepwise, between $(1\cdot10^{-7.1}$ and $1\cdot10^{-9.7})$ $mol\cdot dm^{-3}$, by coulometric generation of $OH^-$ ions with the circuit (C).

The results on the standard potential and on acid−base properties are presented in two different sections.

## Results and Discussion

***Standard Potential of Oleuropein.*** The experimental results were explained according to the following equilibrium

$$OleO_2H_2 \rightleftarrows OleO_2 + 2H^+ + 2e^- \quad (4)$$

The primary $C_L$, $C_A$, $[OleO_2]$, $[OleO_2H_2]$, and $[H^+]$ data form the basis of the treatment to obtain the standard potential of oleuropein, $E^\circ_R$.

Since the effects of composition changes on activity coefficients can be considered negligible, the EMF of the $OleO_2$/$OleO_2H_2$ system can be written in millivolts and at the temperature of 25 °C according to Nernst's law.

Taking into account our experimental conditions, preliminary tests have been carried out for verifying the stability of the oleuropein. A carefully weighed quantity of oleuropein was left at a pH ~ 2.3 to 2.5 and at a temperature of 25 °C for 24 h. During this time, monitoring by HPLC[13] indicated no degradation processes were detected and the oleuropein was the only organic molecule present in our solution.

Preliminary experiments have been carried out for verifying the reproducibility of the potentiometric measurements also. As a result of the oxidation current, it was observed that the redox potential, $E_R$, was different over time. A reasonable explanation of this behavior was the transformation of the oxidized form, active to the electrode, into another form which is inactive to the platinum wire-net electrode.

For this reason, the potentiometric data must be processed according to the following sketch: $V$ = volume of test solution; $\mu_0$ = microequivalents of reduced form; $\mu_i$ = micro-Faradays generated during each generic point $i$; $V[red]_i = \mu_0 - \Sigma\mu_i$ are microequivalents of the reduced form at each generic point $i$. In the course of every evaluation and as a result of the generation of $\mu_i$ it was observed that the oxidized form, contrary to the reduced form, diminished in time. Therefore, for a generic point the scale of time can be fixed between $t = 0$, time to the term of the coulometric generation, and $t = \tau$, time of the last reading prior to the successive generation; in the course of the measurements, $\tau$ has been set to 10 min.

The equivalent concentration $[ox]_i$ was variable in time between $[ox(t = 0)]_i$ and $[ox(t = \tau)]_i$. The evaluation of the oleuropein standard potential has been made for each $(E_{Ri}(t), [ox(t)]_i)$ data pairs. To consider the redox potential value to the time $\tau$, it was necessary to estimate the concentration $[ox(t = \tau)]$ for each point of the titration. For each of the generic $(E_{Ri}(t), [ox(t)]_i)$, the quantities reported in equations 5 to 8 were calculated

$$E_{Ri}(t = 0) = E^\circ_R + 29.58 \log([ox(t = 0)]_i/[red]_i) \quad (5)$$

$$E_{Ri}(t = \tau) = E^\circ_R + 29.58 \log([ox(t = \tau)]_i/[red]_i) \quad (6)$$

$$[ox(t = \tau)]_i = [ox(t = 0)]_i 10 \exp((E_{Ri}(t = \tau) - E_{Ri}(t = 0))/29.58) \quad (7)$$

$$[ox(t = 0)]_i = [ox(t = \tau)]_{i-1} + \mu_i \quad (8)$$

The standard potential, $E^\circ_R$, of oleuropein is constant in each series of measurements, and it was calculated according to Nernst's equation.

**Table 2. Summary of the Relevant Data Taken in Five Titrations for Acidic Constants $K_1$ and $K_2$**

| $C_L$ / mol·dm$^{-3}$ | pH range | $Z_B$ range |
|---|---|---|
| $1.37 \cdot 10^{-3}$ | 7.68 to 8.64 | 0.037 to 0.28 |
| $2.33 \cdot 10^{-3}$ | 7.94 to 8.80 | 0.062 to 0.38 |
| $1.87 \cdot 10^{-3}$ | 7.07 to 9.72 | 0.009 to 1.20 |
| $1.13 \cdot 10^{-3}$ | 7.87 to 9.73 | 0.057 to 1.22 |
| $1.10 \cdot 10^{-3}$ | 7.23 to 8.95 | 0.013 to 0.48 |

To verify the reversibility of the equilibrium, every titration was carried out as an oxidation process and successively by a process of reduction, which was easily achievable by inverting the polarity of the wire-net working electrode. The oleuropein standard potential, mediated on the different titrations, versus the Standard Hydrogen Electrode, SHE, is $E°_R = (0.77_5 \pm 0.03)$ V.

A redox potential value gives information about the real oxidation/reduction ability of a molecule and its prevalent form (oxidized or reduced) in the system. Because of its thermodynamic nature, a redox potential value does not give any information about the rate of the reaction which may occur. Nevertheless, in a system where at least two redox couples are present, its measurement may be useful to predict whether, and in which direction, an oxidation/reduction reaction will take place. For this reason, the assessment of the redox potential of oleuropein may represent an interesting way for assessing the antioxidant capacity and efficiency of a food product.

***Acidic Constants of Oleuropein.*** The experimental data are explained according to equilibria 9 and 10

$$Ole(OH)_2 \rightleftarrows H^+ + OleO_2H^- \qquad K_1 \qquad (9)$$

$$OleO_2H^- \rightleftarrows H^+ + OleO_2^{2-} \qquad K_2 \qquad (10)$$

The primary $C_L$, $C_A$, $C_B$, and $[H^+]$ data form the basis of the treatment to obtain the acidic constants of oleuropein, $K_1$ and $K_2$.

The data used to calculate the acidic constants, $K_1$ and $K_2$, were acquired performing five titrations; in particular, the resulting data set used for calculations was 80 experimental points.

A summary of the relevant data taken in all titrations to evaluate the acidic constants is reported in Table 2.

The experimental data ($C_L$, $C_A$, $C_B$, and $[H^+]$) were processed by graphical as well as by numerical procedures. The graphical methods consist essentially in the comparison of model functions[23] with the experimental function $Z_B$ ($-\log[H^+]$) for

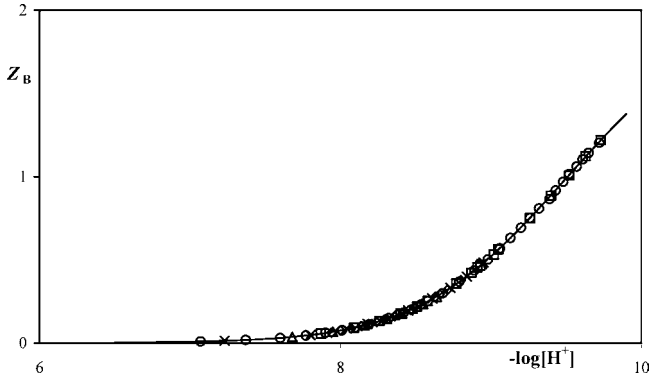


**Figure 4.** $Z_B$ as a function of $-\log [H^+]$. The curve was calculated with the values of the constants reported in Table 3. The symbols refer to $C_L$ mol·dm$^{-3}$: triangles, $1.37 \cdot 10^{-3}$ mol·dm$^{-3}$; diamonds, $2.33 \cdot 10^{-3}$ mol·dm$^{-3}$; circles, $1.87 \cdot 10^{-3}$ mol·dm$^{-3}$; squares, $1.13 \cdot 10^{-3}$ mol·dm$^{-3}$; and crosses, $1.10 \cdot 10^{-3}$ mol·dm$^{-3}$.

**Table 3. Survey of the $-\log K_n$ Values, Molar Scale, By Graphical and Numerical Methods**

| $-\log K_1$ | | $-\log K_2$ | |
|---|---|---|---|
| graphical | numerical ($3\sigma$) | graphical | numerical ($3\sigma$) |
| $9.10 \pm 0.08$ | $9.07 \pm 0.02$ | $9.92 \pm 0.08$ | $9.98 \pm 0.06$ |

equilibrium 3, where $Z_B$ represents the average number of released protons per ligand and is equal to $\{[H^+] - C_A - (K_w/[H^+])/C_L\}$. A plot of $Z_B$ ($-\log [H^+]$) is shown in Figure 4. Values of the ion product of water, in 0.1 mol·dm$^{-3}$ $NaClO_4$, were taken from Baes and Mesmer.[24]

For different $C_L$ values, the points coincide within the limit of experimental error. Comparison with the normalized function[24] indicates that the data are fully explained with the equilibria $OleO_2H_2 \rightleftarrows H^+ + Ole(O_2H)^-$ and $Ole(O_2H)^- \rightleftarrows H^+ + OleO_2^{2-}$. From the best fit, we calculate the values of the constants given in Table 3. The error was evaluated taking into account the shift along the $x$ axes that still gave an acceptable fit. The agreement between experimental points and the model curves is shown in Figure 4.

For the numerical treatment, we employed the least-squares computer program SUPERQUAD[25] to seek the minimum of the function

$$U = \sum (E_i^{obs} - E_i^{cal})^2 \qquad (11)$$

where $E_{obs} = E° + 59.16 \log[H^+]$, and $E_{cal}$ is a value calculated for a given set of parameters. In the numerical treatment, the ion product of water has been maintained invariant. Results are given in Table 3. The agreement with the graphical treatment is satisfactory. It was not possible to compare our results to any other because no data were available in the literature.

Anyway, as expected, the first and the second protolysis equilibrium of oleuropein, studied at 25 °C and in 0.1 mol·dm$^{-3}$ $NaClO_4$ aqueous solutions, take place in the alkaline range ($-\log [H^+] \geq 7$) and involve the two hydroxylic groups of the aromatic ring.

The acidic constants of oleuropein were evaluated for further studies of the complexing power of this ligand toward metals of biological interest.

## Abbreviations Used

$OleO_2H_2$ stands for Oleuropein.

$E°_R$, standard potential, stands for $OleO_2 + 2H^+ + 2e^- \rightleftarrows OleO_2H_2$.

$K_1$, equilibrium constant for $OleO_2H_2 \rightleftarrows H^+ + Ole(O_2H)^-$.

$C_L$, $C_B$, and $C_A$ stand for molarity (mol·dm$^{-3}$) of Oleuropein, NaOH, and $HClO_4$, respectively.

$Z_B = \{[H^+] - C_A - (K_w/[H^+])/C_L\}$ represents the average number of released protons per ligand.

## Literature Cited

(1) Soler-Rivas, C.; Espín, J. C.; Wichers, H. J. Review Oleuropein and related compounds. *J. Sci. Food Agric.* **2000**, *80*, 1013–1023.
(2) Visioli, F.; Galli, C. Natural antioxidants and prevention of coronary heart disease: the potential role of olive oil and its minor constituents. *Nutr., Metab. Cardiovasc. Dis.* **1995**, *5*, 306–314.
(3) Le Tutour, B.; Guedon, D. Antioxidative activities of Olea europaea leaves and related phenolic compounds. *Phytochemistry* **1992**, *31*, 1173–1178.
(4) Panizzi, L.; Scarpeti, M. L.; Oriente, E. G. Structure of the bitter glucoside oleuropein. Note II. *Gazz. Chim. Ital.* **1960**, *90*, 1449–1485.
(5) De Nino, A.; Di Donna, L.; Mazzotti, F.; Muzzalupo, E.; Perri, E.; Sindona, G.; Tagarelli, A. Absolute Method for the Assay of Oleuropein in Olive Oils by Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry. *Anal. Chem.* **2005**, *77*, 5961–5964.

(6) Manna, C.; Galletti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. The Protective Effect of the Olive Oil Polyphenol (3,4-Dihydroxyphenyl)- ethanol Counteracts Reactive Oxygen Metabolite-Induced Cytotoxicity in Caco-2 Cells. *J. Nutr.* **1997**, *127*, 286–292.

(7) Evangelista, C. M. W.; Antunes, L. M. G.; Francescato, H. D. C.; Bianchi, M. L. P. Effects of the olive, extra virgin olive and canola oils on cisplatin-induced clastogenesis in Wistar rats. *Food Chem. Toxicol.* **2004**, *42*, 1291–1297.

(8) Somova, L. I.; Shode, F. O.; Ramnanan, P.; Nadar, A. Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea*, subspecies *africana* leaves. *J. Ethnopharmacol.* **2003**, *84*, 299–305.

(9) Markin, D.; Duek, L.; Berdicevsky, I. *In vitro* antimicrobial activity of olive leaves. *Mycoses* **2003**, *46*, 132–136.

(10) Visioli, F.; Galli, C.; Plasmati, E.; Viappiani, S.; Hernandez, A.; Colombo, C.; Sala, A. Olive Phenol Hydroxytyrosol Prevents Passive Smoking-Induced Oxidative Stress. *Circulation* **2000**, *102*, 2169–2171.

(11) Visioli, F.; Poli, A.; Galli, C. Antioxidant and other biological activities of phenols from olives and olive oil. *Med. Res. Rev.* **2002**, *22*, 65–75.

(12) Ranalli, A.; Contento, S.; Lucera, L.; Di Febo, M.; Marchigiani, D.; Di Fonzo, V. Factors Affecting the Contents of Iridoid Oleuropein in Olive Leaves (*Olea europaea* L.). *J. Agric. Food Chem.* **2006**, *54*, 434–440.

(13) (a) De Nino, A.; Lombardo, N.; Perri, E.; Procopio, A.; Raffaelli, A.; Sindona, G. Direct Identification of Phenolic Glucosides from Olive Leaf Extracts by Atmospheric Pressure Ionization Tandem Mass Spectrometry. *J. Mass Spectrom.* **1997**, *32*, 533–541. (b) De Nino, A.; Mazzotti, F.; Morrone, S. P.; Perri, E.; Raffaelli, A.; Sindona, G. Characterization of cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. *J. Mass Spectrom.* **1999**, *34*, 10–16. (c) De Nino, A.; Mazzotti, F.; Perri, E.; Procopio, A.; Raffaelli, A.; Sindona, G. Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from *Carolea* and *Coratina* cultivars by ionspray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2000**, *35*, 461–467.

(14) Procopio, A.; Sindona, G.; Gaspari, M.; Costa, N.; Nardi, M. Italian Patent MI2007A000903, Request International Patent. PCT/IT2008/000303.

(15) Beauchamp, G. K.; Keast, R. S. J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C. H.; Smith, A. B.; Breslin, P. A. S. Enzymes in an inflammation pathway are inhibited by oleocanthal, a component of olive oil. *Nature* **2005**, *437*, 45–46.

(16) Buettner, G. R. The Pecking Order of Free Radicals and Antioxidants: Lipid Peroxidation, α-Tocopherol, and Ascorbate. *Arch. Biochem. Biophys.* **1993**, *300*, 535–543.

(17) Van Acker, S. A. B. E.; Koymans, L. M. H.; Bast, A. Molecular pharmacology of vitamin E: Structural aspects of antioxidant activity. *Free Radical Biol. Med.* **1993**, *15*, 311–328.

(18) Forsling, W.; Hietanen, S., III. The hydrolysis of mercury (I) ion, $Hg_2^{2+}$. *Acta Chem. Scand.* **1952**, *6*, 901–909.

(19) Brown, A. S. A type of silver chloride electrode suitable for use in dilute solutions. *J. Am. Chem. Soc.* **1934**, *56*, 646–647.

(20) Furia, E.; Porto, R. The effect of ionic strength on the complexation of copper (II) with salicylate ion. *Ann. Chim. (Rome)* **2002**, *92*, 521–530.

(21) Biedermann, G. Study on the hydrolysis equilibria of cations by emf methods. *Sven. Kem. Tidskr.* **1964**, *76*, 362.

(22) Biedermann, G.; Sillèn, L. G. Studies on the hydrolysis of metal ions. IV. Liquid junction potentials and constancy of activity factors in $NaClO_4$-$HClO_4$ ionic medium. *Ark. Kemi* **1953**, *5*, 425–440.

(23) Sillén, L. G. Some Graphical Methods for Determining Equilibrium Constants. II. On "Curve-fitting" Methods for Two-variable Data. *Acta Chem. Scand.* **1956**, *10*, 186–202.

(24) Baes, C. F.; Mesmer, R. E. *The Hydrolysis of Cations*; A Wiley-Interscience Publication: New York, 1976.

(25) Gans, P.; Sabatini, A.; Vacca, A. SUPERQUAD: an improved general program for computation of formation constants from potentiometric data. *J. Chem. Soc., Dalton Trans.* **1985**, *6*, 1195–1200.

Received for review November 27, 2009. Accepted May 6, 2010.

JE901005X