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## Measuring the antioxidant activity of olive oil mill wastewater using chemiluminescence

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### Abstract

A sensitive and simple procedure is described for determining the total phenolic/antioxidant levels of olive oil mill wastewater (OMW), using for the first time Co(II)/ethylenediaminetetracetic acid (EDTA)-induced luminol chemiluminescence. Olive oil wastewater samples were tested for their composition in simple phenolic compounds as a function of the extraction system (two- and three-phase centrifugation systems). The results revealed that the three-phase system had a stronger antioxidant activity and a higher total phenolic content than the two-phase system. The relationship between antioxidant values and total phenolic content is also discussed.

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*Keywords:* Phenolic content; Olive mill residues; Caffeic acid; Antioxidant activity

### 1. Introduction

A major environmental concern in the Mediterranean countries is the disposal and/or treatment of the large quantities of olive oil mill wastewater (OMW) produced during olive oil processing. The high-polluting power of OMW is generally associated with the high biochemical oxygen demand (BOD), chemical oxygen demand (COD), total solids, organic carbon and the slightly acidic character of OMW (Cabrera et al., 1996). These undesirable aspects of OMW make the direct waste disposal or the reutilization of the rich organic and inorganic content difficult. Regarding the organic load of OMW, phenolics and related compounds were reported to be present in considerable concentrations. Previous reports concluded that the presence of phenolic compounds in OMW inhibit the growth of certain microorganisms, particularly bacteria, and is the major cause, together with fatty acids, for the methanogenic toxicity of OMW (Pérez et al., 1992). In addition, many authors have observed the phytotoxic effects of OMW,

which have also been attributed to its phenolic content (Capasso et al., 1992; Flouri et al., 1996).

Researchers have identified several phenolic compounds in OMW, including cinnamic acid derivatives (such as caffeic, coumaric and ferulic acid), benzoic acid derivatives (such as protocatechuic, hydrobenzoic, vanillic and gallic acid) and  $\beta$ -3,4-dihydroxyphenyl ethanol derivatives (such as *p*-tyrosol and hydroxytyrosol; Sayadi et al., 2000; Lesage-Meesen et al., 2001).

In general, phenolic compounds possess ideal structural chemistry for free radical-scavenging and metal-chelating properties, and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis (Rice-Evans et al., 1997). The electron-donating properties of the phenolic moieties of a compound, such as quercetin, and its ability to delocalise the resulting radical have been shown to be directly proportional to the antioxidant strength of the species (Costin et al., 2003). The interest in natural antioxidants is increasing due to the evidence for the involvement of oxygen-derived free radicals in several pathological processes (Tapiero et al., 2002). Epidemiological studies have suggested a connection between the consumption of polyphenol-rich food and the prevention of diseases associated with oxidative stress, such as cancers,

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cardiovascular diseases, inflammations and other. The strong antioxidant properties of OMW may turn the olive oil residues into a cheap source of natural antioxidants. It is therefore essential to enable fast, simple screening of the antioxidant capacity of OMW residues, not only for assessing their environmental toxicity and the possibility of treating or disposing the waste produced, but also for determining the amount of natural antioxidants present. Antolovich et al. (2002) reviewed the major methodologies for the determination of antioxidant activity used by the food industry, with the diphenylpicrylhydrazyl (DPPH) radical assay being one of the more utilised due to its relative simplicity; it is, however, a lengthy procedure. A more rapid and robust procedure for the estimation of total phenolic content or antioxidant activity would be of great benefit.

Chemiluminescence is an alternative detection technique used for the determination of antioxidant activity, having the advantages of low detection limits, wide linear dynamic ranges and the speed of response (Costin et al., 2003). Luminol and lucigenin have been widely used for the determination of reactive oxygen species in a variety of biological systems and have been used indirectly to evaluate antioxidant activities. These chemiluminescence reactions provide a more rapid approach for measuring antioxidant activities when compared with standard methods (Parejo et al., 2000a,b).

The objective of this work was to investigate the effects of the classical three-phase centrifugation system and the two-phase centrifugation system on the phenolic composition of olive oil residues. Simple phenolic compounds were quantified and studied with regard to their antioxidant activity. For the first time, a rapid, simple and sensitive method, Co(II)/ethylenediaminetetraacetic acid (EDTA) enzyme-free and stable signal (plateau) chemiluminescence detection method, is used for the estimation of total phenolic/antioxidant levels in OMW samples. The results are compared, and the relationship between the total phenolic content and antioxidant activity is also discussed.

## 2. Experimental and analytical

### 2.1. Materials

EDTA, luminol (3-aminophthalhydrazine; >97% pure) caffeic acid (>99% pure) and boric acid (>99.5% pure) were obtained from Sigma-Aldrich (Germany). Cobalt (II) chloride hexahydrate, perhydrol 30% H<sub>2</sub>O<sub>2</sub>, sodium hydroxide and sodium chloride (>99.5% pure) were from Merck (Germany). All chemicals were used without further purification. All solutions used for the experiments were prepared with water purified by an EASY-pureRF water purification system supplied by Barnstead/Thermolyne.

### 2.2. Olive oil mill wastewater (OMW) samples

Olive oil residues were collected during the 2003–2004 harvest season in the Chania region from olive oil mills, using either the classical three-phase centrifugation system or the new centrifugation system with a horizontal centrifugal two-phase decanter. In the classical three-phase centrifugation system, olive oil was extracted using a classical three-phase centrifugation system: leafless and washed olives were crushed, kneaded and then extracted with a horizontal centrifugal three-phase decanter. This extraction process requires 60–70 L fresh water/100 kg of olives processed to separate the oil from the other two phases, wastewaters and olive husk. In the new centrifugation system with a horizontal centrifugal two-phase decanter, a single wet solid residue is produced, composed of olive pulp and husk. In all cases, fresh olive oil residues from both systems were filtered and kept at 4 °C in the dark. The average COD values for the two-phase and three-phase OMW were 2930 and 33300 mg/L, respectively. COD values were measured by using the closed reflux colorimetric method. Each time, the appropriate amount of sample was introduced into commercially available digestion solution (Hach Europe, Belgium), and the mixture was then incubated for 120 min at 150 °C in a COD reactor (Model 45600-Hach, USA). COD concentrations were measured colorimetrically using a DR/2010 spectrophotometer (Hach, USA).

### 2.3. Solutions

- (1) Boric acid buffer (0.05 M): The pH of the solution was adjusted to 9, using a NaOH solution (1 M).
- (2) Luminol solution: Luminol 100 µg/L ( $5.6 \times 10^{-4}$  M) in the above borate buffer (pH 9).
- (3) Co(II)/EDTA in borate buffer: 10 mg of CoCl<sub>2</sub> · 6H<sub>2</sub>O ( $8.4 \times 10^{-4}$  M) were weighed and mixed with 50 mg of EDTA ( $2.63 \times 10^{-3}$  M) and were then dissolved into 50 mL of borate buffer.
- (4) Hydrogen peroxide ( $5.4 \times 10^{-3}$  M) was prepared by diluting the 30% stock solution accordingly. The concentration of the final solution was measured using the reflectometric PEROXIDE TEST, MERCK code 16974.

The experiments were performed in freshly prepared solutions given that Co(II) can be easily oxidised to Co(III) in basic solutions (Vogel, 1987).

### 2.4. Chemiluminescence method

Chemiluminescence measurements were carried out on a JENWAY™ 6200 fluorimeter (Jenway Gransmore Green Felsted, Dunmow, Essex, UK), keeping the light off and using only the photomultiplier of the apparatus.

Each day the fluorimeter signal assigned as 100 was measured. This was done by mixing 2 mL of the Co(II)/EDTA solution with 200  $\mu$ L of luminol solution in a test tube and vortexed for 15 s. Then, 50  $\mu$ L of the H<sub>2</sub>O<sub>2</sub> solution was added, and the mixture was vortexed for 10 s in order to initiate the chemiluminescent reaction in situ. The mixture was then rapidly transferred into a glass cuvette, and the intensity of the emission was measured. When the chemiluminescent signal reached the plateau (which lasts up to 1 min), the intensity of the signal was calibrated as 100 ( $I_0$ ). The 0 signal was adjusted with no chemiluminescent solution in the cuvette.

Sample analysis: 2 mL of Co(II)/EDTA were first mixed with 200 mL of luminol solution in a test tube and vortexed for 15 s. Fifty microliter of H<sub>2</sub>O<sub>2</sub> aqueous solution were mixed with 50  $\mu$ L of the sample (for all calibration experiments, caffeic acid solutions were used instead) in a second test tube and vortexed for 10 s. The luminol buffer cocktail was then added into the second test tube, using precision pipettes and was mixed thoroughly. Then the mixture was rapidly transferred into a glass cuvette, and the chemiluminescent measurement was taken, the intensity of which was assigned as ( $I$ ).

### 2.5. Phenol content determination

The total phenol content was determined colorimetrically using the Folin–Ciocalteu reagent (Folin and Ciocalteu, 1927). A diluted sample or phenolic standard was mixed with the Folin–Ciocalteu reagent and 1 ml of a sodium carbonate saturated solution. The final solution was left in the dark for 1 h, after which the absorbance of the solution was measured at 725 nm and compared against a blank prepared following the same protocol but without any sample. The standard curve was prepared using 0, 20, 40, 80, 120, 160 and 200 mg/l solution of caffeic acid in methanol/water. Total phenol values were expressed as caffeic acid equivalents ( $\mu$ g/ml).

## 3. Results and discussion

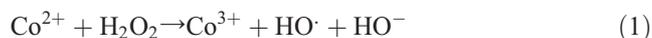
In general, the antioxidant effectiveness is measured by monitoring the inhibition of a suitable substrate. After the substrate is oxidized under standard conditions, the extent of oxidation is measured by chemical, instrumental or sensory methods. Hence, the essential features of any test are a suitable substrate, an oxidation initiator and an appropriate measure of the end point (Robards et al., 1999).

Over the past decade, chemiluminescence has been established as a valuable detection technique. The analytical advantages of chemiluminescence over common spectrophotometric procedures include speed of response, high sensitivity and reproducibility as well as wide linear dynamic ranges, all of which can be generally achieved using simple and cost-effective instru-

mentation. Basically, the analytical applications of chemiluminescence can be divided into those procedures involving well-known chemiluminescent reactions [luminol, acridinium esters peroxyoxylates, dioxetanes and tris(2,2'-bipyridyl) ruthenium(II)], which are responsible for the vast majority of applications and those procedures involving chemiluminescent emission from the analyte which is the substrate and are usually identified as “direct chemiluminescent determinations” (Gómez-Taylor Corominas et al., 2003).

In the case of luminol-induced chemiluminescence, light emission was found to be markedly amplified due to its ability of reacting with hydroxyperoxide functions. In the literature, there are many publications demonstrating the wide use of luminol chemiluminescence as a sensitive assay for monitoring free radicals and reactive metabolites from cell-free, enzyme, cell or organ systems, and for screening antioxidant activity (Parejo et al., 2000a,b). This method allows the evaluation of total antioxidant potential and total antioxidant activity, with luminol being an index of radical reactions.

In the literature, reported sources of free radicals include hydrogen peroxide, organic peroxides or even 2,2'-azobis(2-amidinopropane) (Parejo et al., 2000a). Furthermore, in several reports, it was demonstrated that the presence of a catalyst is paramount to the chemiluminescence method. Many transition metal cations catalyze the decomposition of hydrogen peroxide into hydroxyl radicals via the superoxide-driven Fenton reaction (reactions 1 and 2). In a recent work (Parejo et al., 2000a), Co(II), Cu(I), Cu(II), Fe(II) and Fe(III) with EDTA and citric acid as chelators were used in order to decrease the speed of the reaction and stabilise the chemiluminescence signal. Co(II) and EDTA were found to be the most effective combination. Upon initiation of the reaction, the signal reaches a plateau in 2 min and remains constant for many seconds (up to 1 min), depending on peroxide concentration and pH.



Furthermore, the plateau intensity was linearly proportional to peroxide concentration. When antioxidant activity needed to be measured, addition of the antioxidant into the cuvette, during the plateau, induced a reduction of the stable signal, which served as an index for antioxidant activity. The simplicity of the method lied in the fact that no enzymes and cheap chemicals are used and no complicated instrumentation or signal integration procedures are involved.

For the purpose of the present experiments, caffeic acid was used as the standard to evaluate the antioxidant capacity of OMW. In general, phenolic compounds such as caffeic acid, ferulic and *p*-coumaric acids were previously identified in OMW samples (Sayadi et al., 2000; Lesage-Meesen et al., 2001). From these, caffeic acid is considered to be a

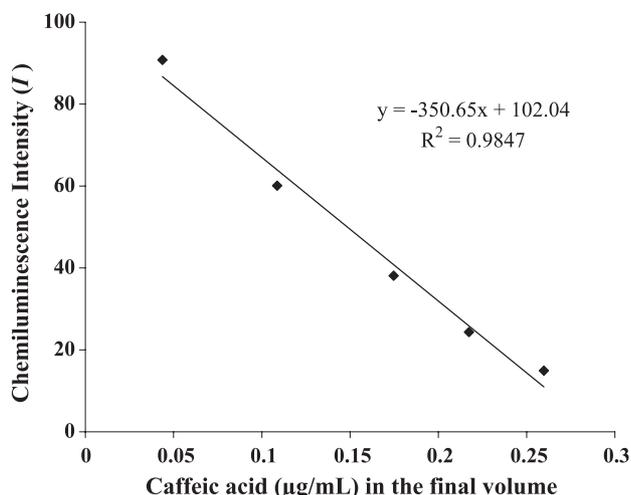


Fig. 1. Reduction on the chemiluminescence intensity ( $I$ ) as a function of the amount of caffeic acid added. The linear relationship as well as the correlation factor ( $R^2$ ) is also given.

more effective antioxidant against low-density lipoproteins (LDL) oxidation, with the order of efficacy being caffeic acid > ferulic acid > *p*-coumaric acid (Laranjinha et al., 1995).

For caffeic acid, a calibration curve was calculated using five spiking levels in the concentration range 0.04–0.26 µg/mL (in the final volume). For each spiking level, three replicate analyses were performed. The instantaneous reduction in luminol intensity elicited by the addition of the standard can be considered as a measure of its antioxidant activity. The light intensity in the absence of standard ( $I_0$ ) was decreased by addition of the standard antioxidant concentration to a value ( $I$ ). As shown in Fig. 1, the relationship between the light intensity and caffeic acid concentration was found to be linear, yielding a correlation coefficient ( $R^2$ ) equal to 0.9847 ( $y = -350.65x + 102.04$ ).

The repeatability of the method, expressed as relative standard deviation (RSD), was evaluated by extracting six consecutive aqueous samples spiked at 16 µg/mL with caffeic acid and was found to be equal to 2.2%. Furthermore, the reproducibility of the method representing the interday precision was determined by extracting every day three replicate samples spiked at 16 µg/L over a period of five working days. The RSD value for this measurement was equal to 3.0%. Overall, the proposed method proved to be a linear, sensitive and reproducible analytical method for evaluating the antioxidant activity of standard solutions.

The proposed detection method was then used to evaluate the antioxidant activity of samples originating from two- and three-phase olive oil mill systems. It should be mentioned here that all samples originating from both the two- and the three-phase systems were diluted (1:10 and 1:100 ratios, respectively) with deionised water. In all measurements, the dilution ratios were taken into consideration for calculating the concentrations in the original samples. The results revealed that the

three-phase olive oil mill samples exhibited the highest antiradical activity than those of the two-phase olive oil mill systems, yielding concentrations 1158 and 103.6 µg/mL in caffeic acid equivalents, respectively. The total phenolic loads of the same samples were also evaluated by the classic Folin–Ciocalteu test. As expected, the three-phase OMW had a higher level of phenolic compounds than the two-phase OMW (1645 and 166.9 µg/mL caffeic acid equivalents, respectively). This indicates that there might be a relationship between the total phenolic content and the antioxidant activity. Many studies have been aimed at exploring such possible relationship. Papadopoulos et al. (2003) used a chemiluminescent detection system with lucigenin and alkaline hydrogen peroxide to determine the total polyphenolic content in oils. The authors showed that the phenol content results determined by the chemiluminescent method are similar with those measured and calculated from the Folin–Ciocalteu experiments. Owen et al. (2000) observed that the antioxidant capacity of the olive oil was significantly correlated with the total phenolic content. Other studies have also concluded that much of the total antioxidant activity of different samples is related to their phenolic content (McDonald et al., 2001; Borbalan et al., 2003; Fernández-Pachón et al., 2004). Recently, Velioglu et al. (1998) and Deighton et al. (2000) have demonstrated a linear relationship between antioxidant capacity and total phenolics in *Rubus* sp. However, other studies indicate that there is not enough evidence for the existence of such a relationship. According to Atoui et al. (2005), the Folin–Ciocalteu index is not always in high correlation with antioxidant activity because the response of phenolics in the Folin–Ciocalteu assay

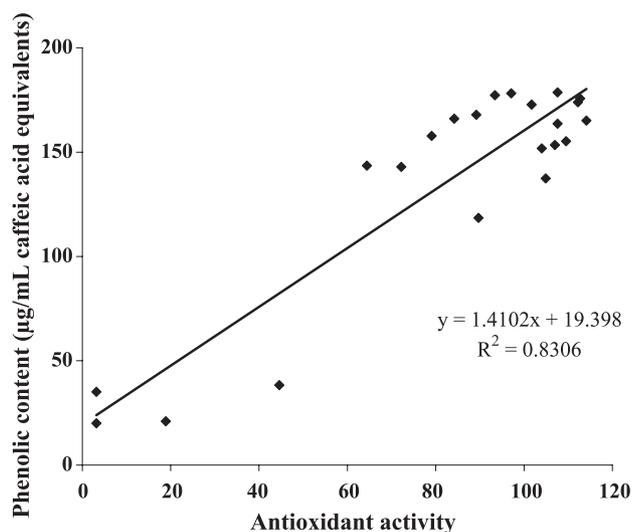


Fig. 2. Total phenolic content measured using the Folin–Ciocalteu method (in µg/mL caffeic acid equivalents) plotted against the results from the chemiluminescent method for OMW samples originating from two-phase decanters.

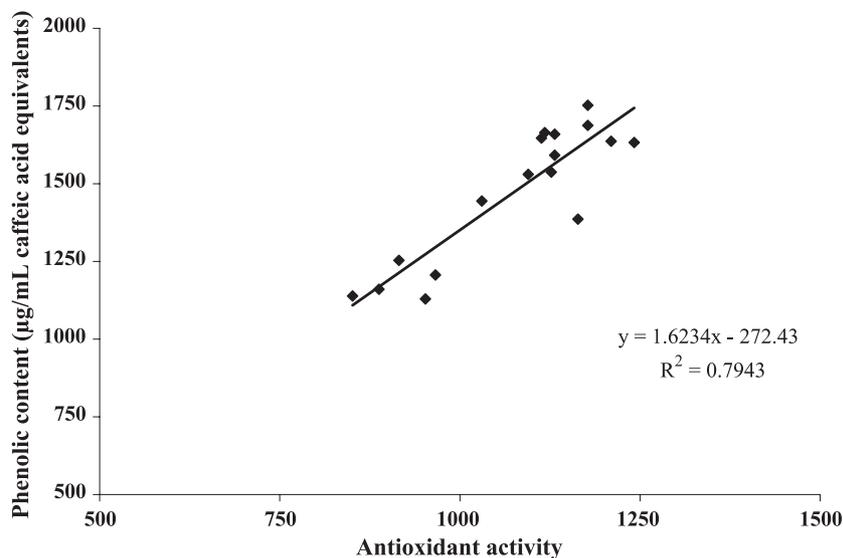


Fig. 3. Total phenolic content measured using the Folin–Ciocalteu method (in µg/mL caffeic acid equivalents) plotted against the results from the chemiluminescent method for OMW samples originating from three-phase decanters.

depends on their chemical structure. Furthermore, the antioxidant activity may derive from synergistic phenomena among individual polyphenols (Psarra et al., 2002). Results of other publications as well, do support the second argument (Arnous et al., 2001; Kefalas et al., 2003).

In order to investigate the relationship between the total phenolic content and the antioxidant activity, 39 samples of OMW and of OMW exposed to various treatments for the reduction of their total phenolic load were evaluated by the classic Folin–Ciocalteu test and the luminol Co(II)/EDTA-enhanced chemiluminescence method, and the results for the two- and three-phase olive oil mill systems were plotted (Figs. 2 and 3, respectively). As can be seen, a fair linear relationship between the total phenolic content and the antioxidant activity was observed for the OMW samples originating from two- and three-phase decanters ( $R^2=0.8303$  and  $0.7943$ , respectively). If we consider the antioxidant activity of the OMW as a suggestive index of toxicity for the environment, we have seen that it is not directly linked to the total phenols. Therefore, we suggest the luminol Co(II)/EDTA enhanced chemiluminescence technique as an eventual tool for the monitoring of the phenolic content of OMW, inasmuch as it relates the total phenols (expressed as caffeic acid) directly to the environmental toxicity (measured as antioxidant activity).

#### 4. Conclusions

The luminol Co(II)/EDTA-enhanced chemiluminescence method proved to be a powerful alternative tool for monitoring the antioxidant activity of OMW samples. Samples were tested for their composition in simple phenolic compounds as a function of extraction systems,

with the three-phase centrifugation system exhibiting stronger antioxidant activity and a higher phenolic content when compared to the two-phase system. The proposed method enabled fast and simple monitoring of the environmental toxicity of these wastewaters. This work also confirms the interest in olive oil residues, especially those issued from the three-phase olive oil extraction system, as a cheap source of natural antioxidant phenolic compounds, in concentrations considerably higher than those commonly found in olive oil.

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