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# Evaluation of antioxidant activity of hydrophilic and lipophilic compounds in edible oils by a novel fluorimetric method

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ABSTRACT

A novel fluorimetric method is described for the evaluation of the antioxidant activity of hydrophilic and lipophilic compounds and complex natural products such as edible oils. The method is based on the measurement of fluorescence emission intensity of *N*-methylacridone produced during the reaction of lucigenin with hydrogen peroxide. The presence of antioxidants in the sample inhibits the concentration of *N*-methylacridone and reduces the fluorescence intensity. The method was fully validated and applied to a variety of hydrophilic and lipophilic compounds as well as to various types of edible oils and their corresponding hydrophilic and lipophilic extracts. Results were compared to those derived from a lucigenin based chemiluminescent method and the Folin–Ciocalteau method for total phenols. The differences in total antioxidant activity of edible oils of various origins and the effect of heating on total antioxidant activity was further studied and discussed.



## 1. Introduction

Antioxidant activity (AA) is one of the important properties of natural products, and closely related to their nutrition value and quality since health benefits of the consumption of natural products, rich in antioxidants, have been extensively reported [1–3]. Several methods have been developed for the estimation of AA using different approaches [4–7] and many of them have been applied to edible oils. However, due to the lipophilicity of edible oils, the majority of published methods for the determination of antioxidant activity are usually restricted to extracts of oils [8–13]. A limited number of methods have been developed for the determination of AA of edible oils without prior treatment [14–19].

Analytical fluorescence offers simplicity, sensitivity and low limits of detection and has been used for food analysis and characterisation of edible oils [20–23]. However, the application of this technique to the determination of antioxidant activity is relatively limited. The ORAC assay is widely used for several radical generators and fluorescent probes and the kinetics of oxidation is studied in order to determine the antioxidant activity of the sample. The ORAC assay has been also used for the determination of antioxidant activity of edible oils extract [24–26] as well as for total antioxidant activity of oils [18].

The oxidation of lucigenin (*N*,*N*′-dimethyl-9,9′-biacridinium dinitrate) by hydrogen peroxide to *N*-methylacridone (NMA) is a well known chemiluminogenic reaction and the mechanism of this reaction has been studied extensively by several research groups in many solvents including 2-propanol [19]. This reaction has been used for the determination of the antioxidant activity of compounds and natural products using the inhibition on the CL intensity by the presence of antioxidants [13,19,27]. NMA emits intense fluorescence within the region 400–450 nm when excited at 390 nm but this property has not been exploited yet for the determination of antioxidant activity. The fluorimetric determination of antioxidant activity using the inhibition of NMA production would have many advantages compared to the CL determination of AA using the same reaction. In the CL assays, the reactions which emit radiation are usually very fast and last only few seconds [19] and, therefore, it is obvious that kinetic factors influence the analytical results. In the fluorimetric determination, the influence of the activity of all antioxidants is evaluated in the results since there is enough time for all antioxidant to react.

The main purpose of this work is to show that the fluorescence emission intensity of NMA produced during the reaction of lucigenin with hydrogen peroxide can be exploited for the evaluation of antioxidant activities of various compounds as well as for complex natural products, such edible oils. 2-Propanol was chosen as

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solvent due to its ability to dissolve all compounds participating in the above-mentioned reaction [19].

## 2. Experimental

### 2.1. Apparatus

Fluorescence measurements were performed on a JASCO spectrofluorimeter FP-777. The excitation and emission wavelengths were 390 and 425 nm, respectively. Chemiluminescence measurements were performed on a 1250 Bio-Orbit luminometer equipped with a Hamamatsu 105-21 side window photomultiplier tube (spectral range 300–620 nm) controlled by a homemade software program which allows continuous monitoring of the output signal. Absorption measurements were performed on a JASCO V-500 spectrophotometer.

### 2.2. Reagents and solutions

All chemicals were of analytical purity and were used without further purification. 2-Propanol, hexane, methanol and hydrogen peroxide solution (30% w/v) were purchased from Panreac. Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Jansen Chimica. Caffeic acid, ascorbic acid and catechin were obtained from Fluka-Riedel de Haen. Oleuropein was purchased from Extrasynthese. Lucigenin, α-tocopherol, gallic acid, chlorogenic acid, *p*-coumaric acid, oleic acid, linoleic acid, glycerin trioleate, glycerin trilinolate and *N*-methylacridone (NMA) were purchased from Sigma–Aldrich. Butylated hydroxylanisol (BHA) and butylated hydroxyltoluene (BHT) were obtained from Across Organics.

All commercial edible oils, such as extra virgin olive oils, refined olive oils, sunflower oil, soybean oils, sesame oil and corn oil were purchased from local supermarkets.

Stock solution of lucigenin (0.00100 M) was prepared and stored at 4 °C. The solution was stable for at least 3 weeks. Stock solution of sodium hydroxide (1.00 M) was prepared in deionised water.

Lucigenin ($1.00 \times 10^{-4}$ M) and sodium hydroxide (0.0100 M) working solutions were prepared immediately before use by appropriate dilution of the stock solution in 2-propanol. Hydrogen peroxide working solution (0.100 M) was prepared daily by appropriate dilution of the 30% (w/v) hydrogen peroxide solution in 2-propanol. Antioxidant standard solutions for fluorescence measurements were daily prepared by accurately weighing and diluting with 2-propanol.

### 2.3. Sample preparation

For the estimation of the total antioxidant activity (TAA) of edible oils, stock solutions of 5.0% v/v of olive oils or 10.0% v/v of seed oils in 2-propanol were prepared. For the estimation of the contribution of the corresponding hydrophilic or lipophilic extracts to TAA of edible oils, solutions of hydrophilic and lipophilic extracts were prepared as following:

*Hydrophilic extract*: 10.00 mL of the oil sample were diluted in 50 mL hexane, extracted three times with 50 mL methanol/water (60/40, v/v) and the solvents were removed in a rotary evaporator under vacuum at temperatures of about 50 °C. To the solid residue of the hydrophilic extract, 50.00 mL of 2-propanol were added and the mixture was stirred vigorously. Then, 1.250 mL of olive oil or 2.500 mL of seed oil extract solutions were diluted to 5.00 mL with 2-propanol in order to obtain 5.0% and 10.0% v/v hydrophilic extract stock solutions of olive oils and seed oils, respectively.

*Lipophilic extract*: for the preparation of 5.0% or 10.0% v/v stock solutions of the corresponding lipophilic phases of olive or seed oil, 0.250 mL of olive oil or 0.500 mL of seed oil extracts were diluted to 5.00 mL with 2-propanol.

Working solutions of 0.825% v/v of olive oil or 1.65% v/v of seed oils in 2-propanol were prepared from the above stock solutions and used for the determination of total antioxidant activity of oil (TAA), Antioxidant activity of hydrophilic extract ($AA_{hydro}$) and antioxidant activity of lipophilic extract ($AA_{lipo}$).

The effect of heating on total antioxidant activity of edible oils was studied by heating the sample to 100 °C in oil bath under stirring for 1 and 3 h before measuring AA by the proposed fluorimetric method.

### 2.4. Fluorescence measurements

Under the optimized conditions, 500 μL sodium hydroxide working solution (0.0100 M) was transferred into a cuvette containing 500 μL lucigenin working solution ($1.00 \times 10^{-4}$ M), 500 μL hydrogen peroxide working solution (0.100 M) and 1000 μL of the antioxidant solution. The final solution was left for 1 h before measurement of the fluorescence emission intensity ($\lambda_{exc}$ = 390 nm, $\lambda_{em}$ = 425 nm) ($I$). The blank emission intensity ($I_0$) is recorded by introducing 1000 μL of 2-propanol instead of antioxidant solution into the reaction cell. The final concentrations of olive oils and seed oils were 0.33 and 0.66% v/v, respectively. For a given concentration of antioxidant, the antioxidant activity AA (%) is calculated by Eq. (1):

$$\text{AA} \quad (\%) = \frac{I_0 - I}{I_0} \times 100 \tag{1}$$

Not less than five measurements were taken for each test solution.

### 2.5. Chemiluminescence measurements

The reaction conditions used for the fluorescent measurements were also used for the chemiluminogenic evaluation of antioxidant activity. 100 μL of lucigenin working solution ($1.00 \times 10^{-4}$ M), 200 μL of antioxidant solution and 100 μL of hydrogen peroxide working solution (0.100 M) were transferred into the reaction cell. The CL reaction was initiated by injecting 100 μL of sodium hydroxide working solution (0.0100 M) and the emission intensity ($I$) was recorded. The blank emission intensity ($I_0$) was recorded by introducing 200 μL of 2-propanol instead of antioxidant solution into the reaction cell. Not less than five measurements were taken for each test solution. The antioxidant activity AA (%) was calculated using Eq. (1).

### 2.6. Spectrophotometric determination of total phenols in hydrophilic extracts of edible oils

A modified Folin–Ciocalteau method was used for the determination of total phenols content [28]. An aliquot of 10.00 mL of the hydrophilic oil extract was diluted with 10.00 mL distilled water, mixed with 2.00 mL of Folin–Ciocalteau reagent (2 N) and stirred. 5.00 mL of sodium carbonate solution (7.5%, w/v) were then added and the mixture was stirred vigorously. The mixture was diluted to 50.00 mL with distilled water, incubated to 45 °C into a water bath for 15 min and the absorbance at 750 nm was recorded. The results were expressed as mg gallic acid equivalents per liter of edible oil (mg GAE/L).

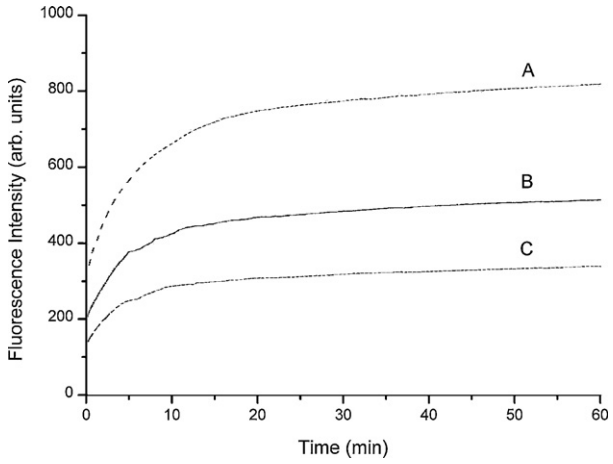


**Fig. 1.** Reaction profiles of fluorescence intensity vs. time of NMA produced during the reaction of $1.00 \times 10^{-4}$ M of lucigenin with 0.100 M of hydrogen peroxide in the presence of 0.0100 M of sodium hydroxide without antioxidant (A) and in the presence of $8.0 \times 10^{-7}$ (B) and $1.6 \times 10^{-6}$ M (C) of oleuropein, respectively.

## 3. Results and discussion

### 3.1. Method development

In designing the fluorimetric analytical procedure, all the reaction conditions have been controlled in order to attain a direct proportionality between reduction of fluorescence signals and concentration of antioxidants. Reproducible results are obtained after the reaction of lucigenin to NMA is almost complete (Fig. 1). Hence, the measurement was taken 1 h after mixing the reagents.

The optimum concentrations of lucigenin, hydrogen peroxide and sodium hydroxide solutions were found to be equal to $1.00 \times 10^{-4}$ M, 0.100 M and 0.0100 M, respectively, and were used for all further studies.

### 3.2. Application to antioxidant compounds

The method developed was applied to the determination of antioxidant activity of 15 selected lipophilic and hydrophilic compounds, some of which are constituents of edible oils with important nutritional value. The regression line for each antioxidant was calculated using Eq. (2):

$$\text{AA} \quad (\%) = a(\pm s_a) + b(\pm sb) \times \log \quad C_{\text{antioxidant}} \tag{2}$$

where AA (%) = antioxidant activity calculated using Eq. (1); $a$, $s_a$ = intercept and corresponding standard deviation; $b$, $s_b$ = slope and corresponding standard deviation; $C_{\text{antioxidant}}$ = concentration of antioxidant in mol/L. AA (%) was also linearly proportional to the concentration of antioxidant compound but logarithm of concentration has been used for extending the linear range.

The regression lines for all antioxidants are shown in Table 1. However, comparable antioxidant activities can be obtained using the $IC_{50}$-value which corresponds to the concentration of a given antioxidant which reduces the blank signal by 50%. Furthermore, since $IC_{50}$ values depend strongly on reaction parameters and conditions used, the antioxidant activity expressed in $IC_{50}$ values is also compared to that of a reference compound measured under the same conditions. Gallic acid, caffeic acid and trolox are usually used as reference compounds. In this work, trolox was chosen as reference compound and the TEAC (trolox equivalent antioxidant capacity) values of the antioxidant activity of compounds were also calculated by Eq. (3):

$$\text{TEAC} = \frac{IC_{50}(\text{antioxidant})}{IC_{50}(\text{trolox})} \tag{3}$$

The derived $IC_{50}$ and TEAC values of the antioxidant compounds studied are shown in Table 2.

In Table 2, $IC_{50}$ and TEAC values of antioxidant compounds, measured by a chemiluminescent method using the same reaction [19], are also presented for comparison reasons. The ranking of the antioxidant compounds according to their antioxidant activity evaluated by the fluorimetric method was the following: caffeic acid > (±)-catechin > oleuropein > gallic acid > chlorogenic acid > ascorbic acid > trolox > α-tocopherol > glycerin trioleate > glycerin trilinolate > BHT > *p*-coumaric acid > BHA > oleic acid > linoleic acid. In the case of the chemiluminescent method the ranking was: (±)-catechin > caffeic acid > gallic acid > oleuropein > ascorbic acid > trolox > chlorogenic acid > α-tocopherol > BHT > *p*-coumaric acid > BHA > oleic acid > linoleic acid. As it was ascertained, both methods of measurement exhibit similar trends but some differences are observed. For example in the FL method, trolox and chlorogenic acid exhibit stronger antioxidant activity compared to that estimated by the CL method. Also, since glycerin trioleate and glycerin trilinolate are the corresponding tri-esters of oleic and linoleic acids, respectively, it was expected that the antioxidant activity would be almost three times higher than that of the free acids. Nevertheless, the antioxidant activities measured by the FL method were found to be 40 times higher than the expected ones.

The differences observed in antioxidant activity of some compounds by the FL and CL methods can be attributed to the different measurement principles. CL is a kinetic method of analysis and the measurement, which lasts only few seconds, is restricted to fast reacting antioxidant compounds and, therefore, is affected by many parameters associated with the reaction medium. In the FL method, there are no kinetics restrictions since the measurement is taken after completion of the reaction with all antioxidants.

### 3.3. Application to edible oils

The method developed was applied for the evaluation of total antioxidant activity (TAA), Antioxidant activity of hydrophilic ($AA_{hydro}$) and lipophilic ($AA_{lipo}$) extract of a selection of edible oils.

The measurement was performed in 0.33% and 0.66% v/v olive oils and seed oils solutions, respectively, and the estimated AA (%) are expressed as trolox equivalent concentration, calculated by equation (4). The antioxidant activity of oils is also expressed in mmol TE/L oil.

$$\text{AA} \quad (\%) = 366(\pm 15) + 65.2(\pm 2.5) \times \log C_{\text{trolox}} \tag{4}$$

Results are shown in Table 3.

### 3.4. Method validation

The fluorimetric method was validated for linearity, precision, intra-laboratory variations (robustness), accuracy and additivity. The method uncertainty was also estimated.

Method linearity was established by measuring antioxidant activity of at least four solutions of each antioxidant compound at known concentrations. Each measurement was repeated five times and the mean value was used for calculation of the regression lines (Table 1). All compounds examined exhibit antioxidant activity and can be measured at the sub-millimolar concentration with correlation coefficients within the range 0.97–0.999. For all compounds, the linear range was at least one order of magnitude.

In order to establish the appropriate dilution of oil for the preparation of working solutions which exhibit AA (%) within the linear

**Table 1**
Regression lines[a] of 15 antioxidants by the fluorimetric method developed.

| Antioxidant compound | $a\ (\pm s_a)$ | $b\ (\pm s_b)$ | $S_{y/x}$ | $R(N)$[b] |
|---|---|---|---|---|
| *Hydrophilic antioxidants* | | | | |
| Trolox | 366 ± 1 | 65 ± 2 | 2.9 | 0.994 (5) |
| Gallic acid | 295 ± 3 | 45 ± 2 | 2.9 | 0.993 (5) |
| Caffeic acid | 277 ± 10 | 39 ± 1 | 3.8 | 0.995 (5) |
| Oleuropein | 270 ± 8 | 38 ± 1 | 2.5 | 0.998 (4) |
| Ascorbic acid | 404 ± 18 | 67 ± 3 | 5.2 | 0.99 (5) |
| Catechin | 284 ± 9 | 40 ± 1 | 4.5 | 0.993 (5) |
| BHT | 221 ± 6 | 50 ± 2 | 2.3 | 0.996 (5) |
| BHA | 241 ± 5 | 75 ± 2 | 3.1 | 0.997 (4) |
| Chlorogenic acid | 375 ± 7 | 61 ± 2 | 3.1 | 0.998 (5) |
| *p*-Coumaric acid | 216 ± 4 | 61 ± 2 | 7.2 | 0.97 (4) |
| *Hydrophobic antioxidants* | | | | |
| α-Tocopherol | 251 ± 3 | 45 ± 1 | 1.3 | 0.9993 (5) |
| Oleic acid | 220 ± 5 | 76 ± 1 | 3.5 | 0.994 (4) |
| Glycerin Trioleate | 271 ± 5 | 51 ± 1 | 3.5 | 0.996 (4) |
| Linoleic acid | 107 ± 2 | 26.2 ± 0.3 | 3.3 | 0.994 (4) |
| Glycerin trilinoleate | 228 ± 3 | 41.9 ± 0.8 | 6.5 | 0.993 (4) |

[a] Regression equation: $AA\ (\%) = a\ (\pm s_a) + b\ (\pm s_b) \times \log C_{antioxidant}$.
[b] Correlation coefficient (number of measurements).

**Table 2**
Antioxidant activities expressed as $IC_{50}$ and TEAC of hydrophilic and hydrophobic compounds by the proposed fluorimetric method (FL) and a chemiluminescent (CL) method.

| Antioxidant compounds | $IC_{50}$ (μM) ($n = 5$) | | TEAC | |
|---|---|---|---|---|
| | FL | CL | FL | CL |
| *Hydrophilic antioxidants* | | | | |
| Trolox | 14 ± 2 | 1.4 ± 0.1 | 1 | 1 |
| Gallic acid | 3.5 ± 0.1 | 0.73 ± 0.04 | 0.25 | 0.52 |
| Caffeic acid | 1.4 ± 0.1 | 0.57 ± 0.01 | 0.10 | 0.40 |
| Oleuropein | 1.6 ± 0.1 | 0.81 ± 0.03 | 0.12 | 0.58 |
| Ascorbic acid | 5.7 ± 0.4 | 0.96 ± 0.08 | 0.40 | 0.68 |
| Catechin | 1.5 ± 0.1 | 0.44 ± 0.03 | 0.11 | 0.31 |
| BHT | 412 ± 19 | 60 ± 2 | 30 | 43 |
| BHA | 2900 ± 97 | 342 ± 14 | 209 | 244 |
| Chlorogenic acid | 4.9 ± 0.2 | 1.9 ± 0.1 | 0.35 | 1.4 |
| *p*-Coumaric acid | 1862 ± 54 | 297 ± 15 | 134 | 212 |
| *Hydrophobic antioxidants* | | | | |
| α-Tocopherol | 38 ± 1 | 6.7 ± 0.5 | 2.7 | 4.8 |
| Oleic acid | 5991 ± 256 | 395 ± 45 | 431 | 282 |
| Glycerin trioleate | 48 ± 1 | [a] | 3.4 | [a] |
| Linoleic acid | 6733 ± 234 | 473 ± 30 | 484 | 338 |
| Glycerin trilinolate | 57 ± 1 | [a] | 4.1 | [a] |

[a] Not measured by CL.

**Table 3**
Total antioxidant activity (TAA), antioxidant activity of hydrophilic ($AA_{hydro}$) and lipophilic ($AA_{lipo}$) extracts of commercial edible oils estimated by the proposed fluorimetric method.

| Edible oils | Antioxidant activity (mmol TE/L oil ± sd, $n = 5$) | | | | Difference (%) |
|---|---|---|---|---|---|
| | TAA | $AA_{hydro}$ | $AA_{lipo}$ | $AA_{hydro} + AA_{lipo}$ | |
| *Extra virgin olive oils* | | | | | |
| Altis Paradosiako | 8.3 ± 0.3 | 4.8 ± 0.2 | 3.3 ± 0.1 | 8.1 | −2.0 |
| Minerva Horio | 8.1 ± 0.7 | 4.3 ± 0.1 | 3.9 ± 0.2 | 8.2 | +1.3 |
| Arhaiko | 7.6 ± 0.4 | 5.5 ± 0.1 | 2.4 ± 0.1 | 7.9 | +3.2 |
| *Refined olive oils* | | | | | |
| Klassiko Altis Elais | 3.2 ± 0.3 | 0.44 ± 0.03 | 3.2 ± 0.2 | 3.64 | +11 |
| Klassiko Minerva | 4.7 ± 0.2 | 1.01 ± 0.05 | 3.5 ± 0.1 | 4.51 | −3.8 |
| *Seed oils* | | | | | |
| Corn oil Flora | 2.4 ± 0.1 | ND[a] | 2.3 ± 0.2 | 2.3 | −3.4 |
| Sesame oil Helios | 2.4 ± 0.2 | 0.670 ± 0.03 | 2.0 ± 0.1 | 2.67 | +15 |
| Sunflower oil SOL | 1.5 ± 0.1 | ND[a] | 1.4 ± 0.1 | 1.4 | −11 |
| Soybean oil Best Price | 2.5 ± 0.2 | ND[a] | 2.2 ± 0.1 | 2.22 | −11 |
| Soybean oil Sogiola | 2.3 ± 0.1 | ND[a] | 2.2 ± 0.1 | 2.2 | −5.1 |

[a] ND: not detectable AA.

range of the method, different dilutions of oil were examined and it was concluded that the final solution for measurement should be within the range 0.01–0.80% v/v.

The precision of the method was evaluated by estimating the method repeatability and reproducibility. The method repeatability was investigated by measuring the antioxidant ability of each antioxidant compound at three different concentrations. The mean value of the relative standard deviation of the blank solution was 3.7% while the mean value of the relative standard deviation of the tested solutions covering all the working range was 4.6%. Method

reproducibility was calculated by measuring the antioxidant ability of each edible oil sample solution at three different days and using trolox solutions, measured at different concentrations during five different days. The mean value of relative standard deviation was 5.8% and the mean value of relative standard deviation of trolox solutions measurements was 7.3%. The %RSD of the slope and the intercept of trolox linear regression during five different days are equal to 4.2% and 3.8%, respectively.

The method robustness was examined by studying the effect of varying the optimum concentrations of lucigenin, hydrogen peroxide and sodium hydroxide up to ±10% on 0.2% v/v extra virgin olive oil solution. Analysis of variance (ANOVA) showed that no significant change in the antioxidant activity of the test solution occurs ($p < 0.05$) so the method is robust for variation of ±10% of the reagents concentrations.

Due to the lack of a reference material or a standard method for the estimation of TAA, the accuracy of the present method was estimated using recovery assays and by comparing the results by the proposed method with those obtained by the CL and the Folin–Ciocalteau method. Recovery experiments were carried out by measuring TAA of 0.022% v/v olive or 0.028% v/v seed oil solutions before and after spiking with 15 μM of trolox. Recovery was calculated using Eq. (5):

$$\%\ \text{recovery} = \frac{\text{antioxidant activity of spiked samples}}{\text{expected antioxidant activity}} \times 100 \quad (5)$$

The mean recovery was estimated to be equal to 102.3 ± 5.5% ($n = 10$) which was satisfactory as verified by Student test at a confidence level of 95% ($t_{theor} = 2.262 > t_{exp} = 1.610$) and, therefore, the method does not exhibit systematic error.

The TAA of edible oils estimated by the fluorimetric (FL) method developed was compared to those obtained by the CL method and the total phenol content determined by the Folin–Ciocalteau (F–C) method (Table 4). Regression analysis between the results of the FL method with the CL and F–C methods revealed the following equations:

$$(\text{mmol TE/L})_{FL} = 0.64(\pm 0.22) + 2.14\,(\pm 0.11) \times (\text{mmol TE/L})\text{CL},$$
$$R = 0.98 \quad (n = 10)$$

and

$$(\text{mmol TE/L})\text{FL} = 0.72(\pm 0.66) + 9.9(\pm 1.2) \times (\text{mmol GAE/L})_{F-C},$$
$$R = 0.97 \quad (n = 6)$$

In both cases, significant correlation between the FL and CL and between the FL and F–C methods has been established.

Method additivity was evaluated by measuring $AA_{hydro}$ and $AA_{lipo}$ and comparing with TAA (Table 3). The results show that the difference between the sum ($AA_{hydro} + AA_{lipo}$) and TAA is within ±15%. The mean difference was found equal to 0.54 ± 8.5 which is satisfactory as verified by the Student test at a confidence level of 95% ($t_{theor} = 2.20 > t_{exp} = 0.18$). Hence, there is no significant difference in TAA and the sum ($AA_{hydro} + AA_{lipo}$).

The uncertainty of the results in the present method derives from (a) the uncertainty associated with the preparation of trolox standard solutions ($u_{std}$), (b) the uncertainty associated with the regression line of trolox ($u_c$), (c) the uncertainty associated with precision ($u_{prec}$), and (d) the uncertainty associated with accuracy ($u_{Rec}$). Total combined relative standard uncertainty, $u_c$, for oil of antioxidant activity approximately equal to 8.0 mmol trolox equivalents per oil liter was estimated using Eq. (6):

$$\frac{u_c}{C} = \sqrt{\left(\frac{u_{sdr}}{C_{std}}\right)^2 + \left(\frac{u_c}{C}\right)^2 + u_{prec}^2 + \left(\frac{u_{Rec}}{Rec}\right)^2} \quad (6)$$

and was found equal to 0.378. The relative expanded uncertainty $U = ku_c$ ($k = 2$ in a confidence interval of about 95%) is equal to 0.756.

### 3.5. Comparison of antioxidant activity of different kinds of oils and the influence of heating in oils antioxidant activity

Useful information about the distribution of TAA in the hydrophilic and the lipophilic part of oil can be extracted from the results of Table 3. Finally the TAA of oils was compared to that determined after heating oils at 100 °C for 1 and 3 h (Table 5).

For the above tables it can be concluded that:

1. the antioxidant activity of extra virgin olive oils is almost four times higher than that of seed oils;
2. the antioxidant activity of extra virgin olive oils is almost two times higher than that of virgin olive oils;
3. the contribution of the lipophilic extract to the total antioxidant activity of extra virgin olive oils is as significant as that of the hydrophilic extract. This is probably the only method available which proves that the high antioxidant activity of extra virgin olive oils is attributed to hydrophilic phenolic compounds [29];
4. the antioxidant activity of hydrophilic extracts of virgin olive oils is significantly reduced while that of lipophilic extract is stable comparing to that of extra virgin olive oil. This can be explained to the harsh production conditions of virgin olive oils where parts of hydrophilic compounds are spoiled or removed [30];
5. with the exception of sesame oil, the contribution of the hydrophilic extract to the total antioxidant activity of seed oils is insignificant compared to that of the lipophilic extract. More precisely, the contribution of the hydrophilic extract to the total antioxidant activity of seed oils is almost nil;
6. the effect of heating is more severe in seed oils than olive oils since TAA of seed oils was reduced by 25% compared to 15% for olive oils.

Generally, the antioxidant activity of edible oils depends on the concentration of antioxidants present which, in turn, depend on the type, location, degree of maturation as well as on the conditions of preparation and storage of oil [28].

The antioxidant activity of the hydrophilic and lipophilic extracts of different types of oils can be attributed to different antioxidants. The hydrophilic extract of olive oils contains antioxidants such as phenolic derivatives of benzoic acid (gallic acid) and cinnamic acid (caffeic acid, chlorogenic acid) or alcoholic derivatives of them (hydroxyl-isochromanes, flavonoids, secoiroids, lignans) [29]. On the other hand, in the lipophilic extract of olive oil, the major contributors to the antioxidant activity are hydrophobic compounds such carotenoids (β-carotene, lutein), tocopherols (mainly α-tocopherol), chlorophylls, pheophytins, polymeric proanthocyanidines and high molecular weight tannins such as hydroxytyrosyl malate and tyrosyl oleates [31–34]. Seed oils, with the exception of sesame oil, contain negligible amounts of phenolic compounds and this explains the very low values of antioxidant activity of the hydrophilic extract. On the contrary, seed oils contain high amounts of tocopherols. It has been reported that the content of tocopherols in soybean oil is equal to 1800 mg/kg and in sunflower to 630 mg/kg which is many times higher than those in olive oils (220 mg/kg) [11]. The tocopherol content in sesame oil is relatively lower but it contains other unique phenolic compounds, such as sesamin, sesamol and sesamolin which contribute to the antioxidant activity of hydrophilic extracts [35]. Finally, part of the antioxidant activity of lipophilic extract of both olive and seed oils should be attributed to polyunsaturated triglycerides, such as glycerin trioleate and glycerin trilinolate which in the present method has shown antioxidant activities comparable to that of α-tocopherol. This seems contradictory to the

**Table 4**
Comparison of TAA of edible oils by the proposed fluorimetric (FL) method with results from chemiluminescence (CL) method and total phenolic content by the Folin–Ciocalteau (F–C) method.

| Edible oil | TAA (mmol TE/L oil ± sd, $n = 5$) | | Total phenolic content by F–C (mmol GAE/L oil ± sd, $n = 3$) |
|---|---|---|---|
| | FL | CL | |
| *Extra virgin olive oil* | | | |
| Altis Paradosiako | 8.3 ± 0.3 | 3.3 ± 0.1 | 0.79 ± 0.04 |
| Minerva Horio | 8.1 ± 0.7 | 3.6 ± 0.1 | 0.76 ± 0.03 |
| Arhaiko | 7.6 ± 0.4 | 3.2 ± 0.1 | 0.59 ± 0.02 |
| *Refined olive oil* | | | |
| Klassiko Altis Elais | 3.2 ± 0.3 | 1.22 ± 0.09 | 0.29 ± 0.02 |
| Klassiko, Minerva | 4.7 ± 0.2 | 2.5 ± 0.2 | 0.47 ± 0.04 |
| *Seed oils* | | | |
| Corn oil Flora | 2.4 ± 0.1 | 6.59 ± 0.07 | N.D |
| Sesame oil Ilios & Gh | 2.4 ± 0.2 | 0.72 ± 0.05 | 0.13 ± 0.02 |
| Sunflower oil Sol | 1.5 ± 0.1 | 0.59 ± 0.04 | N.D |
| Soybean oil Best Price | 2.5 ± 0.2 | 0.76 ± 0.04 | N.D |
| Soybean oil Sogiola | 2.3 ± 0.1 | 0.84 ± 0.03 | N.D |

**Table 5**
Effect of heating at 100 °C on the TAA of edible oils.

| Edible oil | TAA (mmol TE/L oil ± sd, $n = 5$) (% decrease of TAA) | | |
|---|---|---|---|
| | At room temperature | After heating at 100 °C for | |
| | | 1 h | 3 h |
| *Extra virgin olive oil* | | | |
| Altis Paradosiako | 8.3 ± 0.3 | 7.9 ± 0.4 (4) | 7.3 ± 0.4 (12) |
| Minerva Horio | 8.1 ± 0.7 | 8.1 ± 0.6 (0.6) | 7.7 ± 0.3 (5) |
| Arhaiko | 7.6 ± 0.4 | 6.9 ± 0.3 (9) | 6.7 ± 0.4 (12) |
| *Refined olive oil* | | | |
| Klassiko Altis Elais | 3.2 ± 0.2 | 2.7 ± 0.4 (16) | 2.5 ± 0.2 (22) |
| Klassiko Minerva | 4.7 ± 0.1 | 4.6 ± 0.2 (2) | 4.0 ± 0.2 (14) |
| *Seed oils* | | | |
| Corn oil Flora | 2.4 ± 0.1 | 1.8 ± 0.1 (25) | 1.8 ± 0.1 (25) |
| Sesame oil Ilios & Gh | 2.3 ± 0.2 | 1.9 ± 0.2 (16) | 1.8 ± 0.2 (22) |
| Sunflower oil Sol | 1.5 ± 0.1 | 1.3 ± 0.2 (16) | 1.1 ± 0.2 (25) |
| Soybean oil Best Price | 2.5 ± 0.2 | 2.3 ± 0.2 (7) | 1.7 ± 0.2 (30) |
| Soybean oil Sogiola | 2.3 ± 0.1 | 2.2 ± 0.3 (6) | 1.9 ± 0.2 (20) |

common knowledge that polyunsaturated fatty acids are oxidation substrates. However, whichever compound stabilizes free radicals, acts as antioxidant and contributes to the estimated total antioxidant activity.

The stability of extra virgin olive oils to temperature is mainly related to the presence of polyphenols which are very effective stabilizers [16]. The decrease of antioxidant activity of seed oils after heating is attributed to the instability of polyunsaturated fats and tocopherols which are the main contributors on total antioxidant activity of oil. In same cases and upon heating at 170 °C, these polyunsaturated fats are also responsible for the formation of harmful trans compounds upon [17].

## 4. Conclusions

In this work, the fluorescence of NMA produced during the reaction of lucigenin with hydrogen peroxide has been investigated for the determination of antioxidant activity. The analytical method developed can be used for the estimation of antioxidant activities of lipophilic and hydrophilic compounds as well as for more complex samples such as edible oils and their corresponding hydrophilic or lipophilic extracts. It was found that the antioxidant activities of olive oils are higher than those of seed oils (up to 4-fold) and that the contribution of antioxidant activities of their corresponding hydrophilic and lipophilic extracts to the total antioxidant activity of the whole oil is different for olive oils than in seed oils. The hydrophilic extracts of seed oils show negligible AA. Fluorescence measurements also certify that seed oils are more vulnerable to higher temperatures than olive oils. The fluorescence method proved to be simple, fast, low cost, sensitive and accurate and rendered unnecessary the employment of other techniques employing more specialized equipment.

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## References

[1] E. Tripoli, M. Giammanco, G. Tabacchi, D. Di Majo, S. Giammanco, M. La Guardia, Nutr. Res. Rev. 18 (2005) 98–112.
[2] C.S. Yang, J.M. Landau, M.T. Huang, H.L. Newmark, Ann. Rev. Nutr. 21 (2001) 381–406.
[3] M.I. Covas, V. Ruiz-Gutiérrez, R. de la Torre, A. Kafatos, R.M. Lamuela-Raventós, J. Osada, R.W. Owen, F. Visioli, Nutr. Rev. 64 (2006) S20–S30.
[4] M. Antolovich, P.D. Prenzler, E. Patsalides, S. McDonald, K. Robards, Analyst 127 (2002) 183–198.
[5] C. Sanchez-Moreno, Food Sci. Technol. Int. 8 (2002) 121–137.
[6] M. Laguerre, J. Lecomte, P. Villeneuve, Prog. Lipid Res. 46 (2007) 244–282.
[7] V. Roginsky, E.A. Lissi, Food Chem. 92 (2005) 235–254.
[8] S. Gorinstein, O. Martin-Belloso, E. Katrich, A. Lojek, M. Cız, N. Gligelmo-Miguel, R. Haruenkit, Y.S. Park, S.T. Jung, S. Trakhtenberg, J. Nutr. Biochem. 14 (2003) 154–159.
[9] F. Saura-Calixto, I. Goni, Food Chem. 94 (2006) 442–447.
[10] A. Szydłowska-Czerniak, C. Dianoczki, K. Recseg, G. Karlovits, E. Szłyk, Talanta 76 (2008) 899–905.
[11] C.I.G. Tuberoso, A. Kowalczyk, E. Sarritzu, P. Cabras, Food Chem. 103 (2007) 1494–1501.

[12] K.S. Minioti, C.A. Georgiou, Food Chem. 109 (2008) 455–461.
[13] K. Papadopoulos, T. Triantis, E. Yannakopoulou, A. Nikokavoura, D. Dimotikali, Anal. Chim. Acta 494 (2003) 41–47.
[14] S. Mannino, S. Buratti, M.S. Cosio, N. Pellegrini, Analyst 124 (1999) 1115–1118.
[15] J.C. Espin, C. Soler-Rivas, H.J. Wichers, J. Agric. Food Chem. 48 (2000) 648–656.
[16] N. Pellegrini, F. Visioli, S. Buratti, F. Brighenti, J. Agric. Food Chem. 49 (2001) 2532–2538.
[17] J.L. Quiles, M.C. Ramirez-Tortoza, J.A. Gomez, J.R. Huertas, J. Mataix, Food Chem. 76 (2002) 461–468.
[18] K.X. Hay, V.Y. Waisundara, M. Timmins, B. Ou, K. Pappalardo, N. Mchale, D. Huang, J. Agric. Food Chem. 54 (2006) 5299–5305.
[19] D. Christodouleas, C. Fotakis, K. Papadopoulos, E. Yannakopoulou, A.C. Calokerinos, Anal. Chim. Acta 652 (2009) 295–302.
[20] K.I. Poulli, G.A. Mousdis, C.A. Georgiou, Anal. Chim. Acta 542 (2005) 151–156.
[21] N. Dupuy, Y. Le Dréau, D. Ollivier, J. Artaud, C. Pinatel, J. Kister, J. Agric. Food Chem. 53 (2005) 9361–9368.
[22] F. Guimet, J. Ferré, R. Boqué, M. Vidal, J. Garcia, J. Agric. Food Chem. 53 (2005) 9319–9328.
[23] K.I. Poulli, G.A. Mousdis, C.A. Georgiou, Food Chem. 105 (2007) 369–375.
[24] P. Ninfali, G. Aluigi, M. Bacchiocca, M. Magnani, J. Am. Oil Chem. Soc. 78 (2001) 243–247.
[25] P. Ninfali, M. Bacchiocca, E. Biagiotti, M. Servili, G. Montedoro, J. Am. Oil Chem. Soc. 79 (2002) 977–982.
[26] L.L. Yu, K.K. Zhou, J. Parry, Food Chem. 91 (2005) 723–729.
[27] Y. Chen, P. Deuster, Chem.-Biol. Interact. 182 (1) (2009) 7–12.
[28] S. McDonald, P.D. Prenzler, M. Antolovich, K. Robarts, Food Chem. 73 (2001) 73–84.
[29] M. Servili, R. Selvaggini, S. Esposto, A. Taticchi, G. Montedoro, G. Morozzi, J. Chromatogr. A 1054 (2004) 113–127.
[30] A. Garcıa, M.V. Ruiz-Méndez, C. Romero, M. Brenes, J. Am. Oil Chem. Soc. 83 (2006) 159–164.
[31] A. Sanchez-Perez, M. Delgado-Zamarreno, M. Bustamante-Rangel, J. Hernandez-Mendez, J. Chromatogr. A 881 (2000) 229–241.
[32] O. Aust, H. Sies, W. Stahl, M.C. Polidori, J. Chromatogr. A 936 (2001) 83–93.
[33] U.M. Lanfer-Marquez, R.M.C. Barros, P. Sinnecker, Food Res. Int. 38 (2005) 885–891.
[34] A. Bianco, N. Uccella, Food Res. Int. 33 (2000) 475–485.
[35] J. Alencar, G. Gosset, M. Robin, V. Pique, M. Culcasi, J.-L. Clement, A. Mercier, S. Pietri, J. Agric. Food Chem. 57 (2009) 7311–7323.