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Luminescence-based assays to evaluate the total antioxidant capacity of foods

Stefano GIROTTI*[a], Elida N. FERRI[a], Luca BOLELLI[a], Giuseppe ARFELLI[b], Alessandra BENDINI[b] and Lorenzo CERRETANI[b]

[a]*Department of Metallurgic Science, Electrochemistry and Chemical Techniques, University of Bologna, Via S. Donato, 15 – 40127 Bologna, Italy.*
[b]*Department of Food Science, University of Bologna, Piazza Goidanich, 60 – 47023 Cesena, Italy*

Abstract The diet is the main source of low molecular weight antioxidants which contribute to maintain a good redox balance inside the organism. The differences among species, varieties, maturation degree, and culture conditions, as well as the processing to obtain the final consumed products influence the content of the plants metabolites acting as antioxidants. Among the several assays evolved to determine this potential protective activity of food components we tested some chemiluminescent methods on wine, tea, beer, honey, and extra virgin olive oil. The Luminol/H_2O_2/Peroxidase luminescent system was applied to analyze wines at five different steps of winemaking and as final products. The same method has been applied to test the TAC values of tea infusions, different kinds of beer and unifloral honeys of different origin. In case of olive oil the hydrophilic and lipophilic components have been separated and luminescent assays different from the Luminol one have been carried out on the separated phases.

Keywords: Food antioxidants, wine, beer, honey, tea, virgin olive oil, chemiluminescence.

1. Introduction

Free radicals are continuously generated *in vivo* by the aerobic metabolic processes playing physiological roles at low concentrations or producing the deleterious process named "oxidative stress" when their levels results increased [1-3]. Various enzymatic systems have been evolved by cells to limit the levels of free reactive species and prevent serious damages to their structures. In case free-radicals production becomes excessive or the protective systems are someway impaired an imbalance can occur creating an oxidative stress condition linked to changes that accompany aging and diseases [2, 4-6]. The enzymatic defense activities are supported by low molecular weight antioxidant compounds generally displaying chain-breaking or metals-chelating activities and introduced by the diet. Foods having potential antioxidant capacities are fruits and beverages (wine, beer, tea, coffee, chocolate), vegetables and other derivatives such as honey and olive oil; they supply protective compounds like vitamin C and E, carotenoids, chlorophylls, simple phenols, flavonoid glycosides, and polyphenols [7-12].

Wine is a rich source of antioxidant substances such as flavonoids (anthocyanins, flavonols, catechins and leucoanthocyanins) and resveratrol [13-16]. The beneficial effects of tea derive primarily from its content of antioxidant substances: polyphenols, flavonoids, xanthines (mainly caffeine) [17-19]. Beer contains tannins, phenolic acids, as well as carotenoids and tocopherols capable of reducing the oxidation of low density lipoproteins (LDL) and neutralizing the "pro-oxidizing" effects of ethanol. Hops contribute to the polyphenols content in beer, which are about 2-5 % of the total compounds [20-22]. The content of potentially antioxidant compounds like phenolic acid, flavonoids, enzymes and vitamins in honey has been assessed and their beneficial effect repeatedly evaluated [23-27]. The potential health benefits of olive oil consumption have been widely recognized and partially ascribed to its high content in tocopherols and polyphenols. Especially the extra virgin type contains at least 30 hydro and lipo

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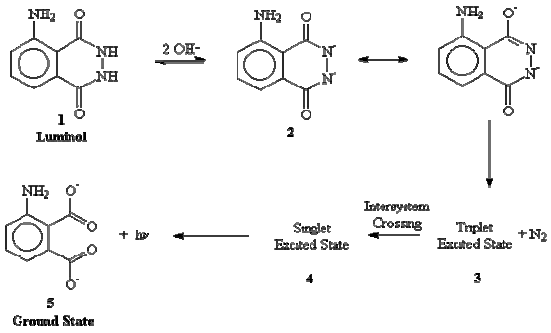
soluble phenolic compounds with high antioxidant potential [28-34].

Taking into account the different molecular structures, the not completely clarified mechanisms of action, and the still discussed actual *in vivo* activity of food antioxidants [35-37] it is clear that there cannot be a short-cut approach to determining the antioxidant potential. Several are the methods which should be used to obtain chemical information that can be related directly to this kind of property of the investigated sample and there is a lot of debate about this measurement [38].

Most of the methods developed to obtain a realistic picture of the Total Antioxidant Capacity (TAC) of food samples are based on the measurable effects of the free radicals scavenging by the molecules present in the sample; these methods differ in the choice of the source of radicals, of their target, and in the way how to detect the oxidized products [39-41]. An inherent uncertainty characterises the TAC values because of the presence of a great number of different compounds acting through different mechanisms and undergoing complex interactions [42].

Chemiluminescence (CL) reactions have considerable analytical potential because the numerous advantages that underlies the success of this technique: high sensitivity and selectivity, wide linear range, simplicity, and the use of inexpensive instrumentation to record the light emission [43]. CL techniques have been conveniently used in the determination of many organic and inorganic compounds in food samples, including the antioxidant ones [44] and different CL methods to determine antioxidant capacity were repeatedly developed and applied [45-51]. Most of them were based on the scavenging principle, i.e. the sample inhibited the radical induced light emission in proportion to its content of chain-breaking antioxidants.

In our studies the CL method suggested by Whitehead *et al*. [45] was slightly modified to optimize its application to the specific food matrices. The method was simple to perform, widely applicable, and used stable, low-cost and easily handled reagents. The light emission occurred when Luminol, the CL substrate, was oxidized by the radicals produced from the hydrogen peroxide in a reaction catalyzed by a peroxidase, usually the Horseradish enzyme. The excited intermediate formed by this oxidation reached the ground state emitting light at 425nm.



Scheme 1. Free radicals triggering of Luminol light emission.

Radical scavenging (chain-breaking) molecules in the sample inhibit this chemiluminescence for a time that is directly proportional to the total antioxidant capacity (TAC) of the sample itself: the light emission was restored when all the added antioxidants have been consumed in the reaction. If the generation of radical intermediates was constant, then the length of time of light suppression was directly related to the amount of antioxidant present [45] and the constant production of oxidized Luminol intermediates resulted in a continuous light emission (plateau emission).

The study of the properties of samples like the olive oil, containing both hydrophilic and lipophilic components, all with influence on the antioxidant capacity of this food, requested the application also of analysis different from the Luminol assay to investigate the whole samples or their lipophilic components.

2. Experimental

2.1. Reagents

Luminol (5 amino-2,3-dihydro -1,4-phthalazine dione) and Horseradish Peroxidase (HRP, E.C. 1.11.1.7) were from Sigma). Hydrogen Peroxide 30 % was from Merck (Milan, Italy). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, pure > 98 %), a water soluble vitamin E analogue used as reference antioxidant, was from Fluka (Milan, Italy). All other reagents and compounds were of analytical-reagent grade. All of the solutions were

prepared with pyrogen-free reagent-grade water using a Milli-Q system (Millipore, Milan Italy).

2.2. Instrumentation

A manual, single sample LKB-Wallac 1250 luminometer (Turku, Finland) was used to measure light emission produced by the Luminol assay. The kinetics of light emission was recorded on a LKB 2210 potentiometric recorder, displaying the light intensity in mV. An automated microplate luminometer ("Luminoscan Ascent", Labsystems, Helsinki, Finland) allowed the measurement of several samples by using black 96 wells microplates (Thermo Labsystems, Helsinki, Finland). The measurement of the low-level spontaneous light emission was performed by using a more sensitive Biolumat LB 9500 C (Berthold, Bad Wildbad, Germany) luminometer.

2.3. Samples

Wine samples included bottled red and white wines from different cultivars supplied by local producers. When the effects of different vinification techniques, clarifying additives or ageing and storage conditions were under study the samples were supplied by the Department of Food Sciences, Bologna University. The wine samples must be diluted by buffer (0.1 M potassium phosphate buffer, pH 7.4) prior to be analyzed because the high content of scavenging molecules, especially in case of the red ones which were diluted at the 1:1000 up to the 1:3000 ratio. The dilution ratio for white wines was about one order of magnitude lower [52-55].

Tea samples included both teabags and loose tea leaves. The content of one teabag or 1.5 g of tea leaves were poured in 100 mL of distilled water, which had been brought to boiling. Part of the solution was filtered after 3, 5, 7 and 10 minutes to allow caffeine-tannins complexation. The cooled extracts were diluted 1:1000 by phosphate buffer before analysis. Sweetened tea samples were prepared in the same way with the addition of 1 (4 g), 2, and 3 teaspoonfuls of sugar to 150 mL of tea. [53].

Honey samples from different floral sources like: acacia, thistle, basswood, citrus fruits, honeydew, sunflower, eucalyptus, fir, chestnut, heather, thyme, linden, strawberry-tree, dandelion were supplied by Istituto Nazionale di Apicoltura, Bologna, Italy. The honey samples were diluted 0.1 or 0.01 g/mL in 0.1 M potassium phosphate buffer, pH 7.4 [54].

Samples of different kind of *beer* (Lager, caramel-coloured lager "Adelscott", Dark "Guinness", and HefeWeisse) were diluted 1:100 with 0.1 M potassium phosphate buffer (pH 7.4) without de-aeration and stored in the dark for 10 min before the analysis. [53].

The virgin *olive oil* samples were supplied and characterized, concerning polyphenols content and other parameters, by the Department of Food Science of the University of Bologna. They included samples from olives of different cultivars, geographical origin, and different degree of maturation. The lipophilic and hydrophilic components of these samples were separated by a liquid phase extraction: 4 g of virgin olive oil were diluted in 4 ml of hexane and extracted by 2.8 ml by a methanol:water solution (2:3, v/v), repeated three times [56].

2.4. Luminol chemiluminescent assay

The CL mixture (CLM) was prepared at the time of analysis by mixing 1 mL of 2 mM Luminol solution to 9 mL of 10 mM hydrogen peroxide solution. For each measure 100 µL of the CLM were supplemented with 20 µL of the working HRP solution, obtained by diluting the stock solution (1 mg/mL) with 0.1 M potassium phosphate buffer (pH 7.4) in order to obtain a constant light emission (plateau) for a suitable period of time. This mixture constituted the reference system, representing 100 % of the light emission in the absence of inhibition by the sample or standard antioxidant solutions.

To evaluate the total antioxidant capacity 10 µL of the sample or the standard solutions were injected into the cuvette once the emission had reached the maximum. By recording the data on paper, the kinetics of the emission could be followed to detect the time when the reaction was at a maximum. As an analytical parameter, the time required to reach 30 % of the initial light emission was measured, and the antioxidant capacity was expressed by comparing the inhibition time of the samples with those of the various points on the calibration curve, expressed as mM of Trolox. To obtain calibration curves a stock solution of 10 µM Trolox (2.5 mg/L) was diluted in 0.1 M potassium phosphate buffer (pH 7.4), and the calibration curves were drawn in a suitable concentrations interval.

Standard solutions for the calibration curve of sweetened tea were made by adding 4, 8 and 12 g of sugar to three containers each containing 150 mL of black tea infusion.

2.5. Evaluation of the spontaneous luminescence

The emission of fluorescent compounds like the fatty acids hydroperoxides contained in the olive oil samples was determined both simply diluting the whole sample in hexane and by adding to the samples a solution of Luminol in hexane.

2.6. Cytochrome C induced luminescencnce

To 50 µL of the lipophilic fractions or of the whole olive oil samples were added 20 µL of 0.1 M Luminol and 20 µL of 0.01 mg/mL Cytochrome C solution. Both solutions were prepared by using the 0.1 M phosphate buffer, pH 7.4, or the 0.2 M borate buffer, pH 8.5. The assay was performed according to [57].

2.7. KO_2 induced luminescence

Following the procedure suggested in [58] the olive oil samples were diluted 1:2 in dimethoxyethylene (DME) from Sigma; the lipophilic extracts were dried under nitrogen flux and then re-suspended in the same volume of DME. To 200 µL of the samples were added 50 µL of saturated KO_2 solution by direct injection into the cuvette already placed inside the LKB luminometer, in order to immediately record the light burst, produced by the KO_2 addition.

2.8. Fluorescent determination of the H_2O_2 scavenging ability

The H_2O_2 scavenging ability of olive oil samples and of both their fractions was determined by applying a peroxyoxalate chemiluminescent assay, using the 9,10 diphenylantracene as fluorophore. The samples and the solutions were prepared in an acetonitrile/ethylacetate mixture (1:9) and employed according to the procedure reported in [59].

2.9. Polyphenol analysis

The determination was based on the reaction of the Folin Ciocalteau reagent with the phenolic –OH groups in an alkaline medium by adding sodium carbonate, according to Singleton and Rossi [60]. Measurements were made after two hours by reading the absorbance at 750 nm on a SHIMADZU PC 1204 spectrophotometer (Shimadzu, Kyoto, Japan) and calculating the concentration (g/L) according to a calibration curve using as standard a gallic acid solution in the range 12.5–500 mg/L.

3. Results and discussion

The data concerning the analytical quality of the assays performed on the manual or the automatic luminometers were collected working with standard solutions and then compared.

In **Table 1** the mean values of the parameters determining the analytical quality of the two methods are summarized.

The comparison of the TAC values obtained for standard solutions and real samples suggested that the quality of the two procedures was the same, with the advantage of shorter time of measurement and lower costs for automated one.

Table 1. The main analytical parameters evaluated to compare the performance of manual and automated luminescent methods.

Parameters	Manual	Automated
Inaccuracy	13.7 %	10.1 %
Recovery	97 %	96 %
Repeatability	4.2 %	3.1 %
Reproducibility	16.7 %	16.3 %
Sensitivity	61.5 µM/s	71.6 µM/s
1 sample assay (triplicate)	43 min	13 min
Detection limit	1 µM Trolox	5 µM Trolox

To identify a possible antioxidant or pro-oxidant effect caused by the ethanol contained in the *wine samples*, ethanol solutions were prepared in buffer at the same concentration (12 %) present in the various wines. The inhibition of the signal from such solutions was negligible, similar to the dilution effect produced by the addition of buffer. No inhibition time was possible to detect, only a very slight reduction of the maximum emitted light.

It is known that the total content of polyphenols, to which the TAC is usually correlated, depends on the type of cultigen, the vinification techniques and the storage conditions [61]. In this

respect, it was interesting to look at the influence on the luminescence determined TAC values of the different cultivars, productive areas and winemaking techniques used on the same cultivar.

For example, the antioxidant capacity was very low in the Sangiovese wine "Novello" type (produced by 100 % carbonic maceration and shorter fermentation time) compared to the wine produced in the traditional manner from the same red grapes: only the 10-20 % of the traditional Sangiovese TAC (**Fig. 1**, sample no. 7).

The differences in TAC values among the different red wines resulted in some case definitely significant but the greater difference was detected, as expected, between the red samples (**Fig. 1**, samples 1-7) and the white wines (**Fig. 1**, samples 8 and 9). The inhibition effect was noticeably different: the signal did not fall to values close to zero after injection as with the red wine samples, and in addition it rose again very rapidly, though it did not regain the maximum emission level. This is due to the low level of total phenols, depending from the lack of the maceration step in the white wine making process and to the grape composition [62].

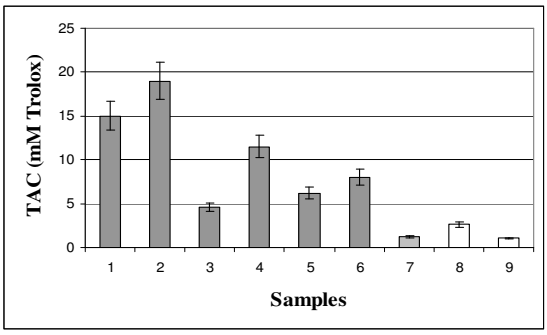


Fig. 1. Antioxidant capacity of different red and white wines (1: Cabernet Sauvignon, 2: Montepulciano, 3: Lambrusco di Sorbara, 4: Sangiovese A, 5: Sangiovese B, 6: Sangiovese C, 7: Sangiovese "Novello", 8: Trebbiano, 9: Chardonnay).

By analysing the samples collected at five different steps during the winemaking process sampling it was possible to observe a continuous decrease of the antioxidant capacity, the highest reduction produced by centrifugation or filtration on a perlite support, while the total phenolic value remained practically unchanged. This effect was ascribed to the oxidation of the phenolic compounds with high antioxidant potential (epicatechin gallate, epigallocatechin gallate, quercetin, delphinidin etc.) but present in low amounts which do not affect markedly the total phenolic index.

One of the winemaking steps, i.e. the clarification by addition of compounds like PVPP (polyvynilpolypyrrolidone) or gelatine, was studied more carefully, determining the TAC of different types of red wines treated or not by clarifying agents, immediately and some period after the first analysis. It has been possible to observe that a complex dependence between the specific phenolic composition of each wine and the different clarifying agents effect on TAC exist and that these effects can appear definitely different when evaluated immediately or after some period of time: the untreated samples showed higher TAC when measured immediately, but their values decreased, during time, more than the treated ones.

This finding confirms in some way the observed changes in TAC of bottled wines immediately after opening and measured some time later. In fact, values obtained from the specimens measured in the same day of the opening were notably higher than values obtained even one day later, while they remained quite stable in the following period: the decrease in the antioxidant capacity of red wines was in the range 40-70 % independently if the measurement was done one day after opening or two weeks later. This behaviour was probably to ascribe to the effect of oxygen that, after opening, starts to react with some, extremely sensitive antioxidant components.

The change in antioxidant capacity during aging of the red wine Sangiovese in oak barrels was also followed and always compared with the total polyphenol content. This content was practically constant for 200 days, then increases between day 200 and day 300 and again reaches a new plateau. In contrast, the antioxidant capacity varies throughout the one-year period, alternatively increasing and decreasing. Thus, there is no obvious correlation between the total polyphenol content and the antioxidant capacity, but both methods respect the trend in total polyphenol content, increasing during ageing in the oak casks which leash such compounds into the wine.

The analysis on the *tea infusions* showed that the antioxidant capacity generally remained constant with regard to infusion time, although maximum antioxidant activity took place for 5 minutes infusion time. The antioxidant capacity of tea in teabags was lower (cca. 10-30 %) than that of loose leaf teas. Among them the green teas reached values slightly higher than the black teas. The antioxidant capacity of other commercial tea formulations like the soluble teas as well as the bottled tea was far lower (between 1.4 and 1.6 mM Trolox equivalent) compared with tea infusions, which reached values higher than 5 mM Trolox equivalent. An interesting effect was observed after the addition of various amounts of sugar, equivalent to 1, 2 or 3 teaspoonfuls, which reduced the antioxidant capacity and the greater the amount of added sugar was, the more reduced was the antioxidant capacity. For 12 g addition of sugar it was impossible to detect any antioxidant capacity, because no light inhibition occurred (**Fig. 2**). The addition of an aspartame-based sweetener also reduced the antioxidant activity to a similar degree as adding one teaspoon of sugar (4 g).

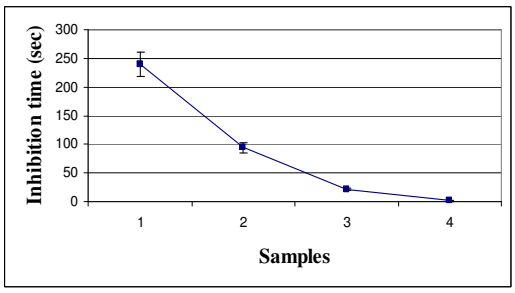


Fig. 2: Decrease of the inhibition time (i.e. of the TAC) of tea samples in dependence of the amount of added sugar (1: unsweetened tea, 2. plus 4 g of sugar, 3: plus 8 g, 4: plus 12 g).

The data concerning the *honey samples* confirmed the already demonstrated dependence of the antioxidant capacity of honey from its colour [23]. Fir, chestnut, and strawberry-tree honeys, dark colour honeys, showed extremely high values, so high that it was usually impossible to report their results in the same graph together with all other samples. It was observed, by analyzing the same kind of honey collected in different years, that the ageing of the samples did not influenced significantly their TAC value. On the contrary, the site of production greatly influenced the antioxidant capacity of the same kind of honey (**Fig. 3**).

In all cases a good correlation between the TAC and the total phenols content was obtained, especially when the darker samples, showing extremely high antioxidant capacity were excluded. In this case R^2 values equal to 0.9 or higher were obtained.

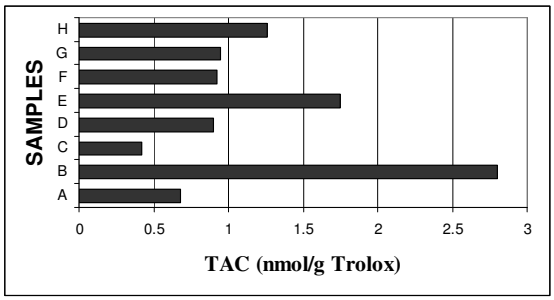


Fig. 3: Differences in the total antioxidant capacity of samples of the same kind of honey (Citrus) collected in different geographical sites.

Fig. 4 shows the differences in antioxidant capacity of various types of tested *beers*. Also for these samples the TAC values were found in accordance with the content of polyphenol, which derived from the raw materials (malt, maize, hops) and in particular from the different degrees of barley toasting.

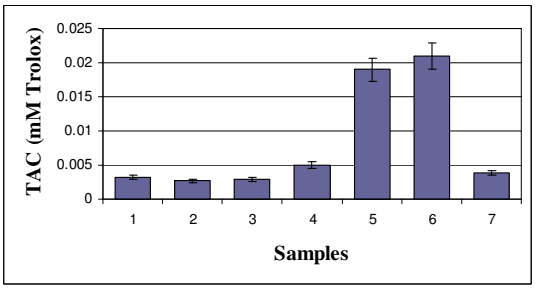


Fig. 4. Antioxidant capacity of different beer samples (1-4: Lager (different trademarks); 5: Dark Guinness; 6: HefeWeisse; 7: Adelscott (caramel-coloured lager).

The higher antioxidant capacity of Dark Guinness beer could be due to the greater number of

Maillard reaction products and the greater amount of hops added, while in the case of HefeWeisse beer the influence of the different raw material (grain instead of barley) and of the milder chemical and physical stabilization treatments must be taken into account to explain this property.

Various luminescent based assays, able to work in aqueous or in lipophilic environment were applied with the aim to collect information useful to evaluate *olive oil* stability and quality, in terms of antioxidant capacity. Firstly, the spontaneous light emission of olive oil, the fluorescent emission of compounds like the oxidized fatty acids (hydroperoxides), was evaluated. All samples were of virgin olive oil and no significant differences in their weak emissions were recorded.

The hydrophilic fractions were tested for their TAC by the Luminol/H_2O_2/HRP inhibition assay. In this case the differences among the samples were clear and important, but it was not possible to establish a relevant correspondence between the polyphenols content and the respective TAC values.

Carrying out these measurements it was observed, after the expected light inhibiting antioxidant effect, an unusual light emission stimulating effect, which was partially related, and proportional, to the TAC of each sample (**Fig.5**).

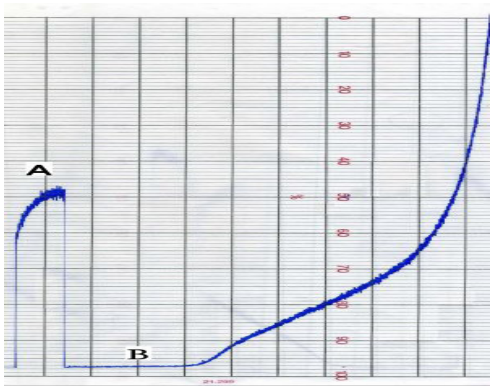


Fig. 5. Light emission kinetics during the evaluation of the TAC of an olive oil sample (the record is from left to right). A: maximum light emission of the MCL mixture; B: inhibition produced by the sample addition, followed by the recovery phase and a subsequent light emission higher than the previous maximum.

Since the light emitted by the Luminol system depends strictly by the production of free radicals this can be identified as a pro-oxidant effect, that we observed by the luminescent assay only for the hydrophilic phenols extracted from olive oil. The pro-oxidant properties which can be expressed by various phenols of natural origin are known and have been repeatedly demonstrated [63-69].

The work is in progress in our laboratory to ascertain the specific components of the hydrophilic extract responsible of this effect in our samples.

On the lipophilic fractions, as well as on the whole olive oil samples, the content of hydroperoxides was evaluated by Luminol emission in presence of cytochrome c, acting as heme catalyst of hydroperoxides degradation. Also in this case the samples showed very different intensity of the light emitted, which was constantly higher in the lipophilic extract than in the respective whole sample. In the lipophilic fraction a great part of the antioxidant molecules have been removed and then the unsaturated fatty acids can be easily oxidized. By comparing the data obtained from this assay with other results, in particular the TAC values it was found that these two parameters were inversely proportional: the lower the antioxidant capacity the higher the emission produced by oxidation products.

A direct dependence, even not so strict, was observed between the light intensity and the unsaturated fatty acid content. A relationship can be established between the intensity of the emitted light and the unsaturated fatty acids/polyphenols content ratio in the samples. The addition of the strong oxidant, KO_2, to olive oil samples and lipophilic extracts, which would show the maximum level of oxidizable substrates, lead to a quite opposite result: the higher emissions were obtained from the whole samples, taking into account that phenols can be included among the oxidizable compounds.

A clear example of the unreliability of data obtained from a single assay to define the properties of a certain sample was offered by the assay, based on the inhibition of the bis(tricholophenyl)oxalate chemiluminescence, using the 9,10 diphenylantracene as fluorophore, of the H_2O_2 scavenging ability of olive oil and of both the lipophilic and hydrophilic fractions. According to our data this ability could be mainly ascribed to the lipophilic fraction, or to the whole sample, being in some cases negligible, or

very low, for the hydrophilic extracts (**Fig. 6**). Moreover, evaluating the effect of the specific solvents of each kind of extract it was revealed that the simple methanol-water mixture showed an inhibition effect of about the 50 %.

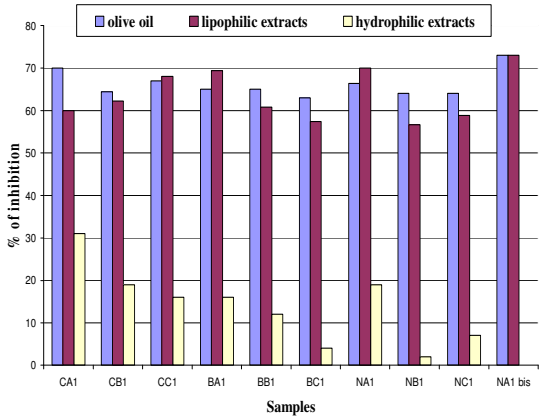


Fig. 6. Inhibition of the fluorescence emission produced by olive oil samples and by their respective hydrophilic and lipophilic fractions, separated by extraction. Samples abbreviation indicate the different cultigens (C, B, N), collected at different degree of ripening (A1, B1, C1).

4. Conclusions

The data here reported, concerning the application of just few of the several luminescent methods that can be applied to estimate the role of complex samples or compounds in presence of free radicals, are contradictory enough to illustrate the need for a very careful interpretation of the results obtained by each assay, a deep knowledge of the reaction mechanisms for each component of the system, and for the use, and comparison among them, of different techniques to obtain a realistic conclusion.

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6. References

* E-mail address: stefano.girotti@unibo.it

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