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Automated and manual luminescent assay of antioxidant capacity: analytical features by comparison

S. Girotti [a,*], E. Ferri [a], F. Fini [a], L. Bolelli [a], A.G. Sabatini [b], R. Budini [a], D. Sichertova [c]

[a] *Institute of Chemical Science, U.C. Scienze Chimiche Radiochimiche e Metallurgiche, University of Bologna, Via S. Donato 15, I-40127 Bologna, Italy*
[b] *Istituto Nazionale di Apicoltura, Via Saliceto 80, I-40128 Bologna, Italy*
[c] *Department of Inorganic and Physical Chemistry, Palacky University, Tř. Svobody 26, CZ-771 46 Olomouc, Czech Republic*

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Abstract

The analytical performances of a manual and a partially automated chemiluminescent (CL) assay, of total antioxidant capacity (TAC) were assessed. In both cases the light emitting reaction involved luminol, horseradish peroxidase and hydrogen peroxyde, but the emission kinetics and the parameters taken into account to calculate TAC values were completely different. The major characteristics expressing the quality of the two analytical methods, i.e. inaccuracy, repeteability and reproducibility, sensitivity, time required for the analysis and detection limit, were estimated by using standard solutions of Trolox. The reliability of the automated method, in comparison with the more validated manual one, was demonstrated testing food samples such as honey, wine and dietary supplements and performing a statistical analysis of the results. The comparison of the two series of data by *t*-test resulted in p values in the range 0.1–0.01. The time required for the analysis of each sample was reduced to one third using the automated method.

Keywords: Chemiluminescence; Antioxidant capacity; Microplate luminometer; Foods

1. Introduction

The role of the antioxidant strategy developed by living organisms to counteract the deleterious effects of the "oxidative stress" has been progressively emphatised in parallel with the increasing number of diseases ascribed to cell free radicals injury [1,2].

The protective effect against oxidative stress of the dietary intake of low molecular weight antioxidant compounds has been widely recognized [3–13], stimulating the interest about the antioxidants content of foods and the consumption of dietary supplements containing mixture of antioxidant molecules. Then, great importance has been attributed to analytical methods able to assess the total antioxidant capacity (TAC) in vivo as well as in foods. Most of them measure the inhibition of an artificially-generated oxidative process by scavenging molecules present in the sample, they differ in the choice of the oxidation source and target and in the detection of the oxidized products [14–16]. Among others, different CL methods were repeatedly developed and applied [17–24]. Generally, the sample inhibits the radical-induced light emission in proportion to its content of chain-breaking antioxidants.

Chemiluminescent (CL) reactions, thanks to advantages such as high sensitivity and selectivity, wide linear range, simplicity and the use of inexpensive instrumentation for monitoring emission, have considerable analytical potential in a great variety of applications [25] and whenever a CL assay was automated even better results were achieved in terms of reproducibility of the data, number of tested samples and easiness of employment [26–30].

Previously we applied a TAC enhanced CL manual assay, modified with respect to that suggested by Whitehead et al. [22], to serum samples, as well as to beverages like wine, beer, tea [31–33]. We obtained interesting results but we

* Corresponding author. Tel.: +39-051-2095660; fax: +39-051-2095652.
E-mail address: girotti@biocfarm.unibo.it (S. Girotti).
URL: http://biocfarm.unibo.it/~girotti/.

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experienced the typical disadvantage of this method: the long time required to obtain a single measurement. The aim of this work was to compare, in term of analytical performances, the results obtained by the usual manual method and by an automated one, developed on a multiplate luminometer with computerised data processing.

Standard solutions of Trolox were used to define the analytical quality of the two methods; food samples like wine, honey and dietary supplements were analysed in order to compare the two sets of results.

2. Materials and methods

2.1. Reagents

Luminol (5 amino-2,3-dihydro-1,4-phthalazinedione) and Horseradish Peroxidase (HRP, E.C. 1.11.1.7, Grade II) were obtained from Boehringer Mannheim (Germany). Hydrogen Peroxide 30% was from Merck (Milan, Italy). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2carboxylic acid, pure >98%), was from Fluka (Milan, Italy). All other reagents and compounds were of analytical-grade. All solutions were prepared with pyrogen-free reagent-grade water using a Milli-Q system (Millipore, Milan, Italy).

2.2. Instrumentation

An LKB-Wallac 1250 luminometer (Turku, Finland) was used for the manual assay. The signal, expressed as mV, was displayed on paper by means of an LKB 2210 potentiometric recorder.

A Luminoscan Ascent luminometer (Labsystems, Helsinki, Finland) was used to perform the automated assays. The data were recorded by computer employing the Ascent software for kinetics measurements. The 96-wells black microplates employed in the automated assay were from Thermo Labsystems, Helsinki, Finland.

2.3. Samples

- Red (Sangiovese, Novello di Sangiovese) and white (Trebbiano, Chardonnay) wines were supplied by Cooperativa Le Rocche Malatestiane (Rimini, Italy). The red wines Montepulciano d'Abruzzo, Merlot, Schioppettino and Clinto were obtained from local producers.

  Red wine samples were diluted 1:1000 and 1:1500, white wines 1:100 and 1:300, in 0.1 M potassium phosphate buffer pH 7.4 and stored in the dark for about 10 min before analysis.
- Honey samples from different floral sources: acacia, thistle, basswood, citrus fruits, honeydew, sunflower, eucalyptus, fir and chestnut were supplied by Istituto Nazionale di Apicoltura, Bologna, Italy. The honey samples were diluted 0.1 or 0.01 g ml^{-1} in 0.1 M potassium phosphate buffer pH 7.4.
- Dietary supplements: Natura Mix Aboca™ (Aboca, Italy), Carovit™ (Biochimici PSN, Italy), Polase™ (Wyeth Lederle, USA) Biokromaton Mineral Vit™ (Menarini, Italy), were bought at local pharmacies.

The dietary supplement preparations were diluted, in order to fit with the calibration curve range, in 0.1 M potassium phosphate buffer pH 7.4, alone or added with 3% of ethanol 96° when the preparation contained lipophylic antioxidants. When necessary the samples were centrifuged at 2100 rpm for 3 min, to avoid the presence of suspended particles.

All samples, in both procedures, were tested as triplicate.

2.4. The manual TAC assay

The reagent solutions used in this assay were prepared, stored and employed as it was already published for the CL antioxidants assay [32]. During routine analysis the calibration curve, 1–10 μM Trolox, was measured one time per week and any time when the stable reagents (luminol and HRP solutions) were newly prepared. Otherwise, at each experimental session was measured only one standard concentration that, compared with the calibration curve, was used to calculate a "correction factor" of the TAC values. When this value was lower than 0.8 or higher than 1.2 a new calibration curve was prepared.

2.4.1. Procedure

Twenty microliter of the peroxidase solution were added to 200 μl of CLM. The parameter that allowed to calculate the TAC values was the time elapsed between the sample addition (10 μl) and the return to a light emission 30% of the maximum reached prior to the addition (see Fig. 1b). This time interval is correlated to the sample content of chain-breaking antioxidants. The antioxidant capacity was expressed, fitting the times obtained on the calibration curve, as μM of Trolox.

2.5. The automated assay

The reagent solutions were the same than in the manual assay. The range of standard concentrations was different, 5–33.4 μM, when the routine procedure, i.e. simultaneous assay of several samples, was followed because of the lower detection limit of this procedure. The correction factor was used also in this procedure to calculate the antioxidant activity of the samples.

2.5.1. Procedure

In each well of the microplate were manually injected: 10 μl of the sample or standard solution and 20 μl of HRP solution. The reaction started when 200 μl of CLM were injected automatically in each well. In this case, as shown in Fig. 1a, the scavenger molecules present in the sample inhibit the peak appearance and it is the delay to reach the

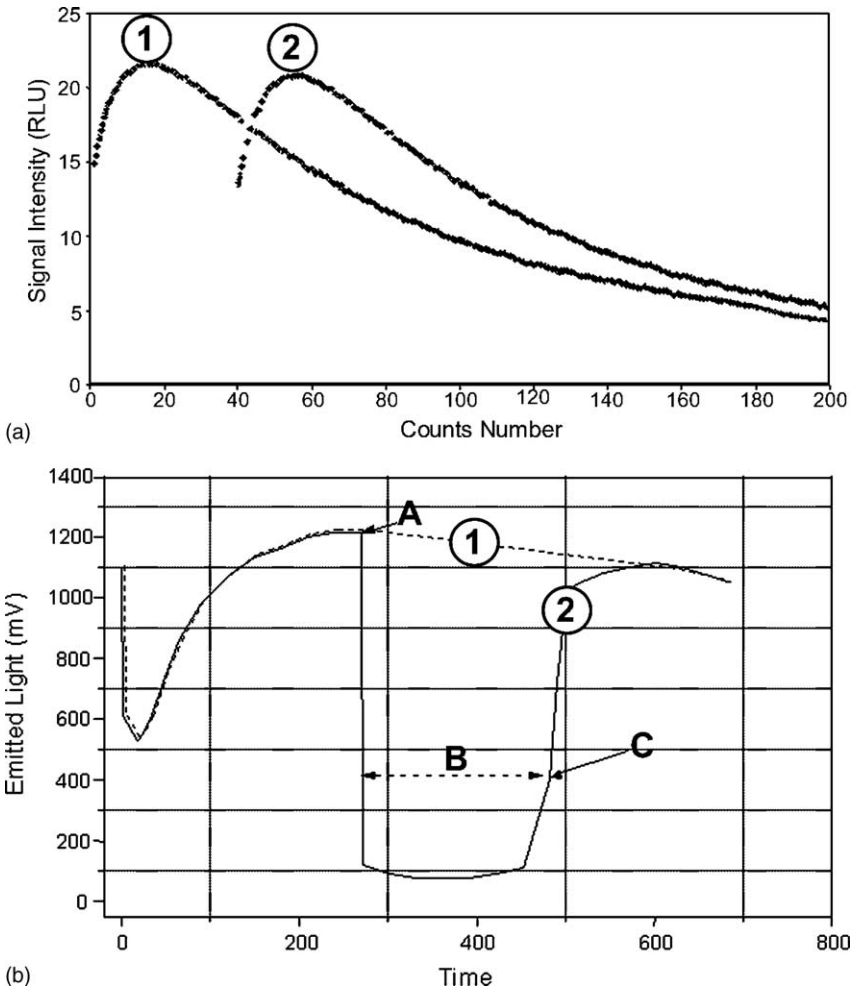

Fig. 1. Kinetics of the light emission in the automated (a) and manual (b) CL assay. In Fig. 1a (1) indicates the blank light emission and (2) the kinetics when a sample is added. The delay in the appearance of the maximum is the parameter taken into account to calculate the TAC. In Fig. 1b (1) and (2) mean the same: A indicates the sample addition, B the time required to reach the 30% (C) of the previous maximum of emission. B is the parameter take into account in this case.

maximum light emission to be correlated to the antioxidants content.

The following criteria: inaccuracy, imprecision (repeteability and reproducibility), sensitivity, time required for the analysis and detection limit were used to compare the quality of the analytical results. Statistical comparison of the two sets of data was performed using the *t*-test [34].

3. Results and discussion

The chemiluminescent reaction on which both methods were based involved the horseradish peroxidase catalysed oxidation of luminol by hydrogen peroxide, the light output was due to the production of free radical intermediates and the compounds affecting the emission belonged to the chain-breaking antioxidants [27,28]. Although the reagents were the same in both methods, two completely different kinetics of the light emission were recorded, as shown in Fig. 1. A possible explanation of this experimental finding can be that found in the different mixing sequences that can influence the extremely complex and till now fairly understood mechanism of the luminol-HRP reaction.

The data concerning the analytical quality of the two assays were collected working with standard solutions and assuming as detection limit 1 μM of Trolox in both cases. The imprecision of the measurements, i.e. repeatability and reproducibility, was determined calculating the coefficient of variation of repeated determinations on the same sample in an experimental session and in subsequent days. The assays were also performed by different operators in the laboratory. To value the inaccuracy of the methods recovery values were determined. In Table 1 the mean values of the parameters determining the analytical quality of the two methods are summarised.

The manual assay gave CV values in the range 6–15% concerning repeatability and in the range 13.9–19.6% concerning reproducibility. In the automated assay the ranges were 10–14% and 13.9–18.8%, respectively. It is possible to affirm that the imprecision is the same in both methods.

Table 1

Analytical parameters	Manual method	Automated method
Inaccuracy (%)	13.7	10.1
Recovery (%)	97	96
Repeatability (%)	4.2	3.1
Reproducibility (%)	16.7	16.3
Sensitivity ($\mu M\,s^{-1}$)	61.5	71.6
Time per sample (triplicate) (min)	43	13
Detection limit (μM Trolox)	1	5
Cost per sample (eurocents)	0.25	0.12
Instrument cost (euro)	∼5000	∼15000

The recovery values were similar in the manual and automated assay, 90–118 and 90–111%, respectively, though the latter assay included one automated injection, that should have reduced the manipulation errors.

The sensitivity of the two assays, determined as the slope of the calibration curves prepared and measured in different days, indicated that the automated assay had slightly better sensitivity. The slope values were in the range 50.8–73.4 $\mu M\,s^{-1}$ for the manual assay and in the range 51.1–96.9 $\mu M\,s^{-1}$ for the automated one.

A very important difference between the two procedures concerned the time required for the analysis of each sample. In the manual assay the time required to obtain a single kinetics curve was about 12–14 min. Since each sample was measured as triplicate, the data concerning a single sample were collected in about 43 min. The measurement of the three wells concerning a sample could be obtained, in the automated instrument, within 13 min since they were done simultaneously.

The detection limit of the automated assay can be different according to the number of wells interested in the measurement. When only few standard concentrations were measured the detection limit was the same, 1 μM, in both cases. When a routine procedure was employed, i.e. in case that several samples were measured in the same microplate with standard solutions, the detection limit was only 5 μM. This was due to the time required for the automatic injection of the CLM, that did not allow to record the very short inhibition time of the lower concentrations. To be detectable the sample must give an inhibition time longer than 10 s (three times the background value).

Taking into account the costs concerning the two methods, the price of the automated instrument is three times greater that for the manual one, whereas the cost per sample is double in the manual assay.

3.1. Analysis of honey samples

The analysis of these samples gave the data reported in Fig. 2 and looking to the *P*-values it was possible to confirm the equivalence of the two assays.

The data concerning two samples, fir and chestnut honey, were not reported in Fig. 2 because of their extremely high values. These were dark colour honeys and they showed TAC values corresponding to 8.2 and 6.0 μM of Trolox, respectively. These data confirmed the correlation of the antioxidant capacity of honeys to their colour [13].

3.2. Dietary supplements

The data reported in Fig. 3 showed that the two methods gave similar TAC values for the tested dietary supplements, distributed in a very wide range (1–240 μM of Trolox). The *P*-values, higher than usual, obtained for these samples could be ascribed to the complex composition of these preparations, that are mainly a mixture of natural products, fruits and vegetables extracts, and a variable combination of vitamins and minerals. At least some of these components can have a different influence on the luminol-HRP reaction, that follows probably different pathways in the two methods.

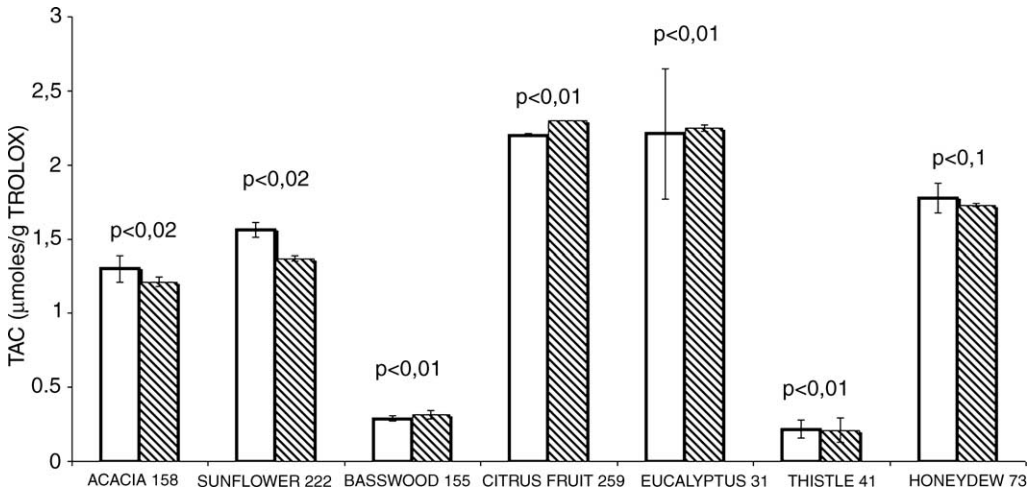

Fig. 2. TAC of honey samples determined by automated (white) and by manual (hatched) CL assay.

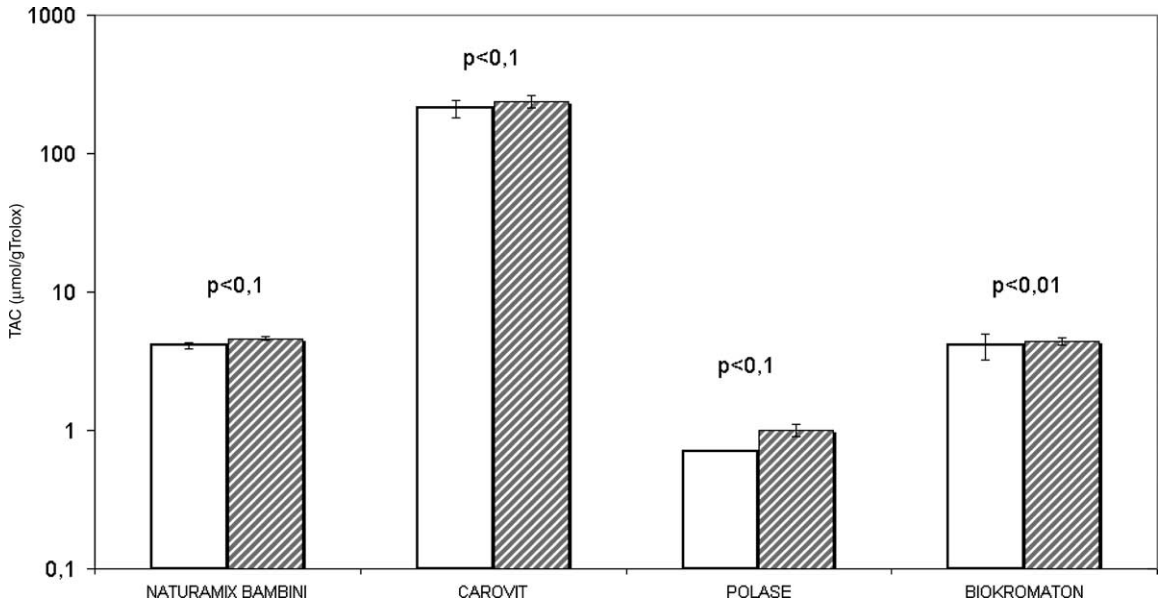

Fig. 3. Automated (white) and manual (hatched) determination of the TAC values of dietary supplements.

3.3. Red and white wines

Especially for red wines the time elapsed between the opening of the bottle and the assay is very important. 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. Although the decrease in the antioxidant capacity was significant the first day, in the range 40–70%, the values remained quite stable even 2 weeks later. This behaviour was probably to ascribe to the effect of oxygen that reacted with some, extremely sensitive, antioxidant compounds.

Sangiovese Novello, red wine that undergoes a particular wine-making procedure including carbonic maceration and a shorter fermentation time, showed low TAC values (about 6 mM l^{-1}). This result was expected because the total content of polyphenols, to which the TAC is usually correlated, depends not only on the type of cultivar, but also on the vinification techniques and the storage conditions [28]. Very low values, in the range 1.5–2 mM l^{-1}, were found for

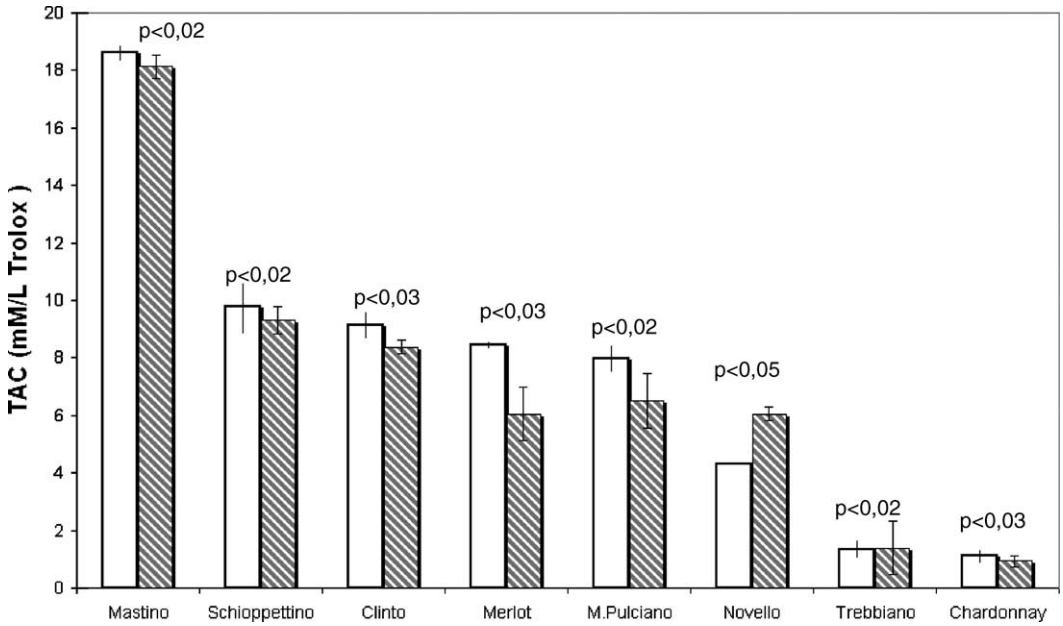

Fig. 4. TAC of red and white wines determined by automated (white) and manual (hatched) CL assay.

white wines, as it was expected according to their antioxidant compounds content (Fig. 4).

The obtained data allowed us to conclude that the automated luminescent method showed the same analytical reliability of the manual assay, together with the advantages of shorter time of measurement and lower costs. However, the most important improvement offered by this method concerns the kinetics data of light emission, that are collected and processed automatically, avoiding the long time required to calculate the kinetics parameters of interest on paper records and the inevitable errors due to the personal interpretation of the graphs. The possibility to analyse several samples in a relatively short time allows to generate easily significant estimates of the analytical precision.

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