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Original article

Blue CrO_5 assay: A novel spectrophotometric method for the evaluation of the antioxidant and oxidant capacity of various biological substances

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ABSTRACT

Oxidative stress plays a pivotal role in the ageing process and in the pathogenesis of numerable diseases. The quantification of the phenomenon is of paramount importance. In the present study, we introduce a novel and simple assay, the Blue CrO_5 assay, for the evaluation of the oxidant and antioxidant capacity of various biological samples and known antioxidants. Chromium peroxide (CrO_5) is produced by ammonium dichromate in an acidic environment in the presence of H_2O_2. It is a deep blue potent oxidant compound, miscible and relatively stable in polar organic solvents, that can be easily measured by spectrophotometry. Its reduction by known antioxidants, both water- and lipid-soluble (ascorbate and α-tocopherol, respectively, in this study), detected spectrophotometrically as a decrease in the absorption and depicted in EPR spectra, can act as a measure of the antioxidant capacity of a certain compound. The assay displays significant sensitivity, stability, linearity, specificity and repeatability.

1. Introduction

Oxidative stress is the resultant of intracellular and extracellular conditions which in turn lead to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1,2]. These compounds constitute a by-product of the evolutionary selection of oxygen molecule as the terminal in the electron transport chain, during the intracellular energy production. Also, there are external factors that contribute to their production (e.g. diet, food additives, toxic and carcinogenic compounds, radiation, ozone, air pollution, smoking, psychological stress, etc.) [3–6]. Their biological role is of paramount importance because under the presence of transition metal ions (e.g. Fe, Cu, Mn, and Cr), free radicals are easily generated. For example, H_2O_2 through Fenton-type reactions generates hydroxyl radical ($H_2O_2 + Fe^{+2} \rightarrow Fe^{+3} + HO^- + HO\bullet$), one of the most reactive radical in damaging biological systems. Their role is pivotal in the ageing process and in the pathogenesis of numerable diseases, such as cancer, atherosclerosis, diabetes mellitus, Alzheimer's disease, cataract and the list is continuously growing [2,7–9].

Of course, the living organisms have developed, in the course of evolution, defence mechanisms against these damaging reactions, and their interaction constitutes a dynamic balance. These mechanisms include a variety of substances and enzyme systems which we refer to as antioxidants in general [10]. The cell is fully equipped with both intracellular and extracellular antioxidant systems. The intracellular defence systems include enzymes [11–15], and also sequestration of transition metal ions (such as iron, copper, etc.) that can act as catalysts for the production of extremely reactive free radicals, mainly by binding proteins such as transferrin, lactoferrin, ferritin, ceruloplasmin and metallothioneins [16–20].

The extracellular defence against free radical formation is equally important. It consists mainly of low molecular weight antioxidants and fewer enzyme systems than in the intracellular environment. Some are produced *in vivo* and a large number is absorbed from the daily food intake [21]. These defence systems include small concentrations of enzymes and molecules such as albumin, bilirubin, ceruloplasmin, transferrin, lactoferrin, uric acid, haptoglobin, hemopexin, glucose, lipoic acid, ubiquinone, α-tocopherols and ascorbic acid to name but a few [10,22–27].

The quantification of this phenomenon is of paramount importance. A standard antioxidant is compared to the antioxidant properties of the sample under examination, thus producing a measure of its antioxidant capacity. This is, in general, the principle of the existing methods [11,28–30]. These methods are divided in two major categories: (1) the inhibition assays, where the lag phase or the suppression of an oxidation reaction, in the presence of an

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antioxidant, is being measured, and (2) the reduction assays, which are based on the reductive properties of antioxidants, where the capacity of a certain sample to induce the reduction of marker-molecule is a measure of its content in antioxidants.

The first category includes: (a) the ABTS compound oxidation inhibition, known as TAC-assay kit, (b) the TRAP-assay which is based on the measurement of the oxygen consumption time of blood plasma in dilution with linoleic acid, and (c) the ORAC-assay, which measures the produced fluorescence by the AAPH compound and its non-linear degradation. The second category includes: (a) the FRAP-assay, which measures the reduction of coloured trivalent iron complex, and (b) the $ABTS^{+}$ decolorization assay, where it acts as a reduced substrate. Recently, efforts were made to evaluate the total reductive capacity of various samples by means of embedded electrodes and cyclic voltametry [31,32]. The main drawback of these assays is their lack of specificity, because of various factors that affect free radical formation and can alter the readings, and the variety in the speed of chemical reactions that take place in complex biological samples, such as blood, and occasionally exceed the measurement time suggested by the assay.

Finally, the existing methods deal, in majority, with the water-soluble antioxidants, whereas lipid-soluble antioxidants, such as vitamin-A, α-tocopherol, etc., remain undetected. But biological samples are mainly bi-phasic solutions with a large hydrophilic compartment that surrounds a lipophilic core. Multiple interactions take place between the two compartments, while various substances such as α-tocopherol with its chroman head group and phytyl side chain are active in both compartments. In this direction, efforts have been made, with the introduction of new solvents, to measure lipid-soluble compounds with the Improved TEAC assay. However, the assay isolates the two phases, whereas *in vivo* they co-exist and interact displaying synergistic effects in the level of antioxidant defence systems [11]. Therefore, the measurement of the total antioxidant capacity of various biological samples remains as an elusive target.

This article contributes to this effort for a better evaluation of the redox state and the antioxidant and oxidant capacity of various biological samples by introducing the novel and relatively simple spectrophotometric assay of the blue chromium peroxide (CrO_5). Chromium peroxide is a deep blue product and a powerful oxidant. It is generated in an aqueous phase by the interaction of an inorganic salt with hydrogen peroxide in an acidic environment. Since it is mainly miscible in organic solvents, after its production it moves through the aqueous phase to the organic phase of a bi-phasic solution. Thus it exerts its oxidative properties in both phases of the solution but also it is liable to the antioxidant action of various antioxidants, both hydrophilic and lipophilic. In this way, it is a very promising marker-molecule for the development of assays measuring both the oxidative and the antioxidant capacity of various samples.

2. Materials and methods

2.1. Chemical reagents and materials

Isoamyl alcohol, propylene carbonate, ammonium dichromate, α-tocopherol and hydrogen peroxide of high purity were purchased

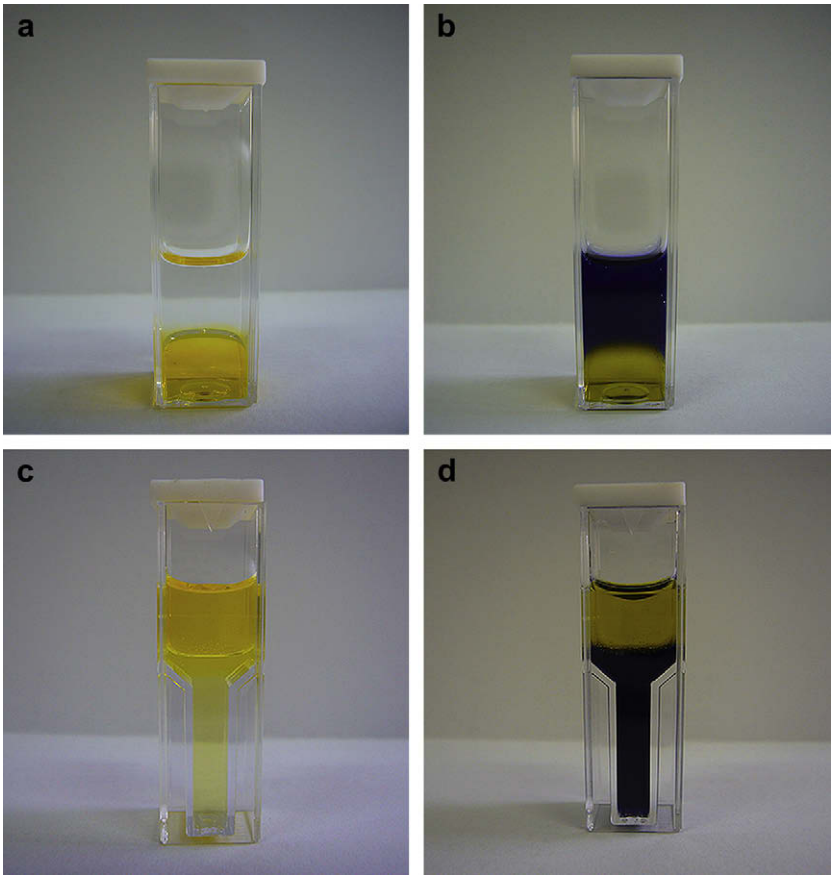

Photo 1. (a) The colourless organic phase above (isoamyl alcohol) and the yellow aqueous phase below (due to ammonium dichromate), before the addition of H_2O_2. (b) The blue organic phase above (due to the formation of chromium peroxide) and the yellow aqueous phase below (due to ammonium dichromate), after the addition of H_2O_2. (c) The yellow aqueous phase above (due to ammonium dichromate) and the colourless organic phase below (due to propylene carbonate), before the addition of H_2O_2. (d) The yellow aqueous phase above (due to ammonium dichromate) and the blue organic phase below (due to formation of chromium peroxide), after the addition of H_2O_2.

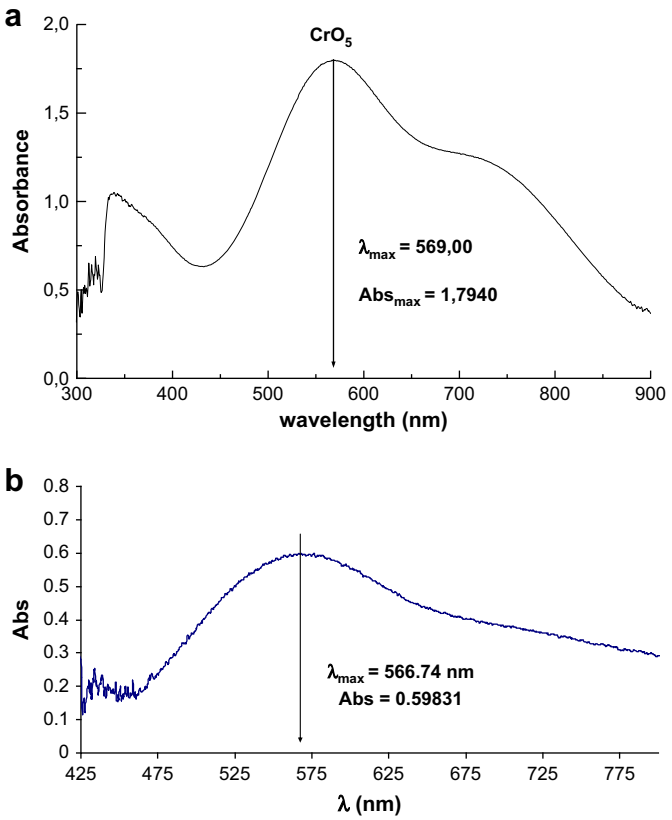

Fig. 1. (a) Absorption spectrum of CrO_5 in isoamyl alcohol (37 °C); and (b) absorption spectrum of CrO_5 in propylene carbonate (37 °C).

from Sigma–Aldrich Co. Sulfuric acid and perchloric acid of analytical grade were also purchased from Sigma–Aldrich Co. For the preparation of aqueous solutions double distilled water was used, whereas in organic solutions isoamyl alcohol or propylene carbonate were used. Measurements were made with a Shimadzu 1601 UV–vis spectrophotometer using disposable polysterene square cuvettes of 10 mm optical pathway purchased from Sarsterdt Co. EPR spectra were performed in a Bruker ER200D-SRC.

2.2. Assay principle

Chromium peroxide ($Cr(O_2)_2 \cdot H_2O$ or briefly CrO_5) is an extremely potent oxidant. This compound is a product of the following reaction:

$$(NH_4)_2Cr_2O_7 + 4H_2O_2 + 2H^+ \rightarrow 2Cr(O_2)_2 \cdot H_2O + 4H_2O + \text{ammoniumsalt} \quad (1)$$

Chromium peroxide is a deep blue product, relatively stable in polar organic solvents and can be easily measured via spectrophotometry [33]. The acidic environment for the reaction was provided by acids such as H_2SO_4 and $HClO_4$ (forming ammonium sulfate and perchlorate, respectively). When the products of the reaction (1) are mixed with an organic solvent (e.g. isoamyl alcohol and propylene carbonate), the formed CrO_5 moves and dissolves in the organic phase (*solution 1*) of the bi-phasic solution, the aqueous phase of the solution containing the reagents (Photo 1a–d). The antioxidant compounds of a certain substance or biological sample, added to the above solution 1, inhibit the formation of the deep blue CrO_5. The extent of the inhibition of the colour formation in solution 1, measured by means of a spectrophotometer, represents

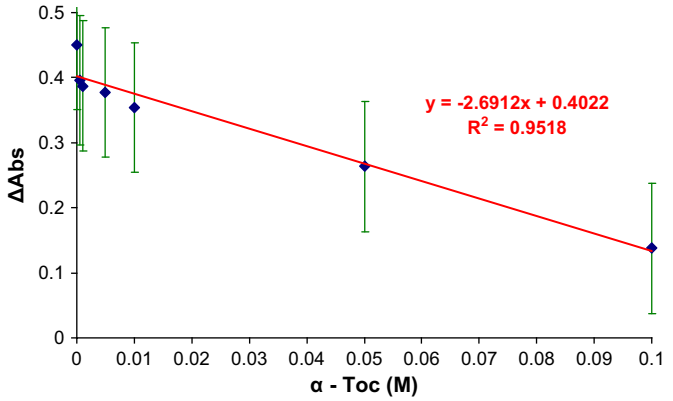

Fig. 2. Area of α-tocopherol solutions of intermediate concentration, in isoamyl alcohol (37 °C).

the *antioxidant capacity* (or the oxidative status) of the sample under measurement.

Initially, if hydrogen peroxide (H_2O_2) is not added to the solution 1, then chromium peroxide is not produced (see reaction (1)) and the organic solution is not coloured deep blue (*solution 2*). If in this colourless solution 2 is added a substance or a biological sample containing hydrogen peroxide or other peroxides, then it becomes deep blue, due to the formation of chromium peroxide by the reaction of ammonium dichromate with the sample peroxide, in the presence of H_2SO_4 (see reaction (1)). The intensity of the formed blue colour, spectrophotometrically measured, reflects the sample content in peroxide compounds and its *oxidant capacity*.

2.3. Sample preparation and conditions

All measurements were taken at a temperature of 37 °C and the spectrophotometer was set at $\lambda = 569$ nm (for isoamyl alcohol) or $\lambda = 566$ nm (for propylene carbonate) and calibrated against air. In a 4 mL plastic capped cuvette, 1 cm light path, 1200 μL of organic solvent, 400 μL of 0.02 M ammonium dichromate, 400 μL of 0.025 M sulfuric acid solution and 20 μL of the sample under measurement were pipetted. The cuvette was then inserted into the spectrophotometer and was incubated for a period of 3 min. The first absorbance reading was then taken. The cuvette was then removed from the photometer and 20 μL of 1.6 M hydrogen peroxide solution was pipetted. After a mild stirring the cuvette was inserted again at the photometer and an absorbance reading was taken at 3 min time. The increase in the absorption was then calculated (ΔA). For each sample there was a triplet of measurements. Mean value and standard deviation were then calculated.

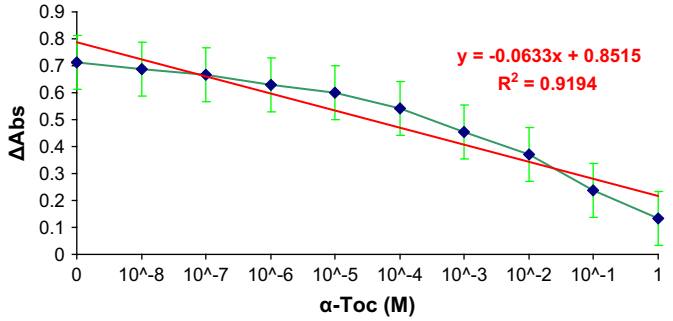

Fig. 3. Grading of the assay using α-tocopherol solutions of various molarities, in propylene carbonate (37 °C).

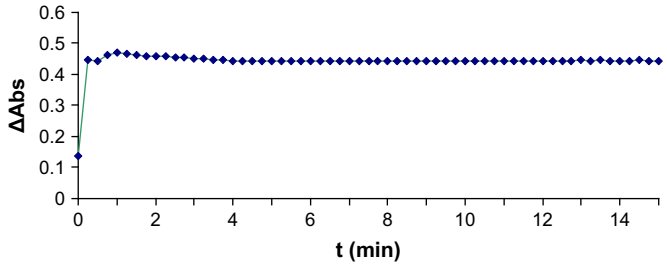

Fig. 4. Stability of the assay using the blank solution.

The same procedure was followed for blank measurements but instead of using 20 μL of a sample we added 20 μL of the organic solvent. For measurements of our standard antioxidant we used 20 μL of 0.1 M α-tocopherol solution in our organic solvent instead of a sample. The calculation ΔA of blank and standard solutions took place before each set of measurements in order to avoid daily fluctuations in the stock solutions used.

The calculated fraction:

$$[\alpha\text{-tocopherol}] \times \frac{\Delta A_{\text{blank}} - \Delta A_{\text{sample}}}{\Delta A_{\text{blank}} - \Delta A_{\text{standard}}}$$

is a measure of the antioxidant capacity of this sample as compared to that of 0.1 M α-tocopherol solution, and is measured in mol/l of α-tocopherol.

3. Results

Chromium peroxide is a deep blue product easily miscible in organic solvents, with a maximum absorption at $\lambda = 569$ nm for isoamyl alcohol (Fig. 1a) and at $\lambda = 566$ nm for propylene carbonate (Fig. 1b). The assay was graded using samples of 20 μL of α-tocopherol solutions of various molarities in isoamyl alcohol, which was the main solvent we used in our measurements. The assay displayed exceptional linearity in the area of intermediate α-tocopherol concentrations, which was the area of the absorbance readings of the samples in our measurements, thus contributing to the accuracy of the assay (Fig. 2). The assay was also graded with propylene carbonate displaying similar linearity (Fig. 3).

The assay displayed stability in the absorbance readings over an incubation period of time of several minutes, with no significant changes taking place after the 3-min time period we used in our measurements, both in the blank solution (Fig. 4) and in the standard solution (Fig. 5).

The specificity of the assay was also examined with no interactions taking place between the produced chromium peroxide and the various polar organic solvents we used. The production of blue chromium peroxide also displayed a H_2O_2 concentration dependence, with the aqueous phase of the bi-phasic solution receiving the excess of CrO_5 after the saturation of the organic phase (Fig. 6).

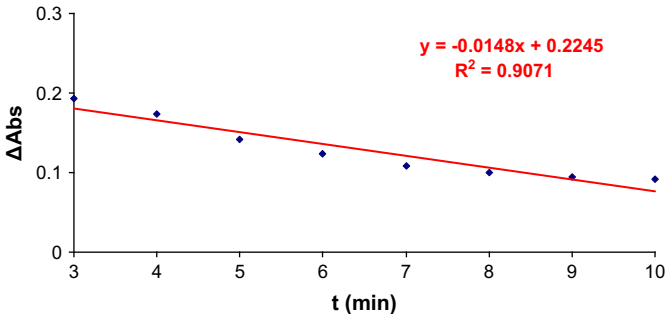

Fig. 5. Stability of the assay using the standard solution of 0.1 M α-tocopherol after $t = 3$ min.

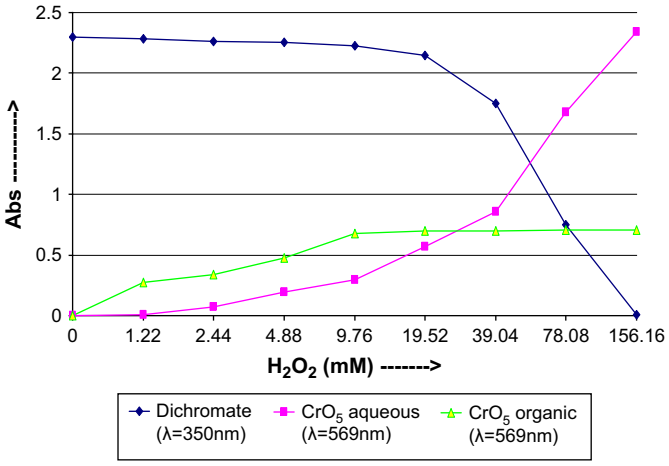

Fig. 6. H_2O_2 concentration dependence of CrO_5 formation (absorption at $t = 10$ s with various H_2O_2 concentrations, isoamyl alcohol as organic solvent). Note that the aqueous phase of the bi-phasic solution is receiving the excess of CrO_5 after the saturation of the organic phase.

In our measurements, the acidic environment was in a slight excess to the molarity of the ammonium salt in order to shift the chemical balance of the reaction (1) to the right, thus producing more blue CrO_5 and increasing the sensitivity of the assay. As shown in Figs. 2 and 3 the assay was capable in detecting α-tocopherol at final concentrations of 10 nM (overall sensitivity).

We also studied the chemical system using ascorbate (a prominent water-soluble antioxidant) as a potential standard antioxidant

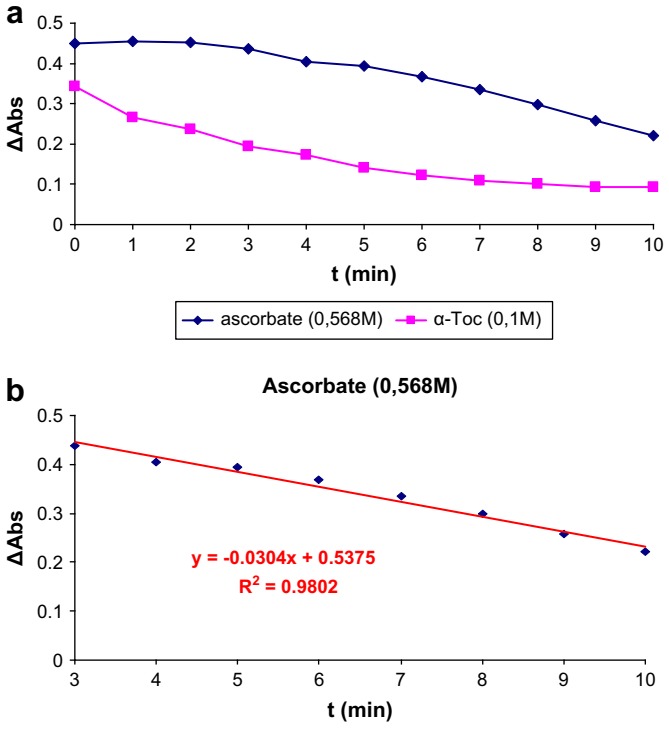

Fig. 7. (a) Absorption kinetics of the assay using 0.568 M ascorbate or 0.1 M α-tocopherol. (b) Absorption kinetics of the assay using 0.568 M ascorbate after $t = 3$ min.

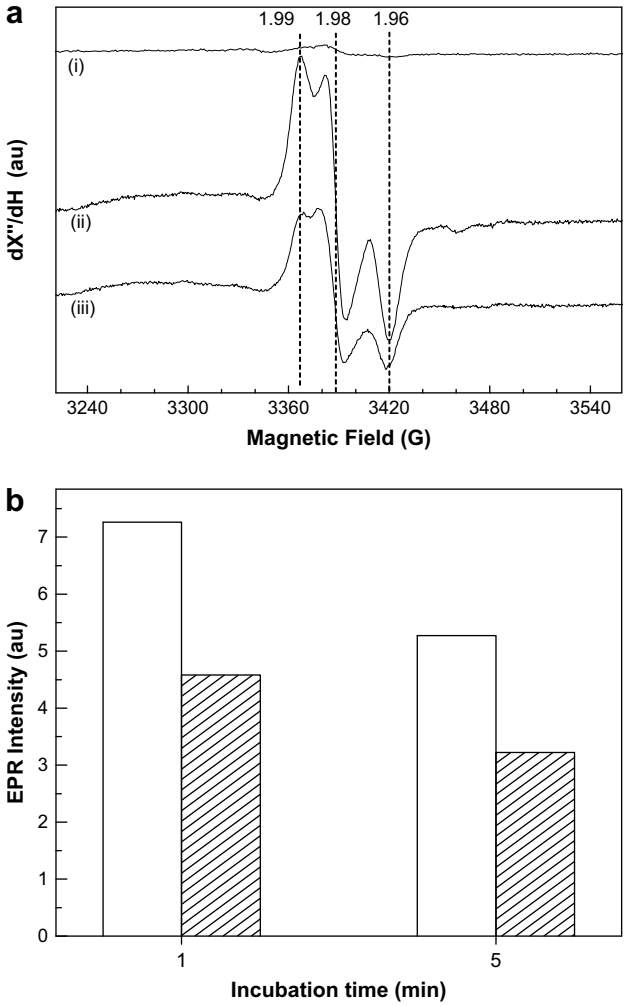

Fig. 8. (a) EPR spectra of the chemical system of the assay in isoamyl alcohol (experimental conditions: $T = 77$ K, modulation frequency 100 kHz, modulation amplitude 5 G, microwave power 10 dB). (i) Absence of signal before the addition of H_2O_2. (ii) After the addition of H_2O_2 the characteristic double spike of the formation of a radical chromium paramagnetic body. (iii) The spike is diminished by the antioxidant action of α-tocopherol. (b) Effect of incubation time on the intensity of the EPR signal at g 1.98 in the absence (empty columns) and in the presence (filled columns) of α-Toc.

with the results shown in Fig. 7a and b. By comparing the absorption kinetics of the two antioxidants (ascorbate and α-tocopherol) in Figs. 5 and 7b, respectively, we can see clearly that with α-tocopherol we have more stable absorption readings after a period of 3 min whereas with ascorbate the absorption continues to decrease rapidly beyond the 3-min time period, making it unsuitable for standard antioxidant in the assay.

EPR spectra of the chemical system of the assay were performed in isoamyl alcohol, as shown in Fig. 8a. There we can see in spectrum (i) the absence of signal before the addition of H_2O_2 and the formation of a radical paramagnetic body. In spectrum (ii), after the addition of H_2O_2, we can see the characteristic double spike of the formation of a radical chromium paramagnetic body, and in spectrum (iii) the spike is diminished by the antioxidant action of α-tocopherol. The effect of incubation time in the EPR signal, both in the presence and in the absence of α-tocopherol is shown in Fig. 8b, where we can see the very gradual diminishing of the signal.

Furthermore, we compared our novel assay with another well-established spectrophotometric assay for the measurement of antioxidant status, the Improved TEAC [30]. We measured the same

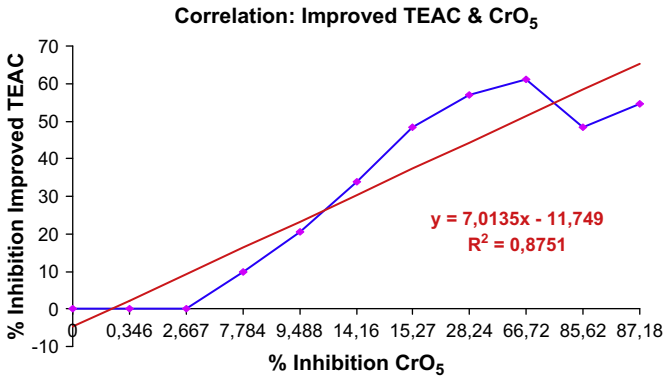

Fig. 9. Measurement of the antioxidant activity of various α-tocopherol solutions with both Improved TEAC and Blue CrO_5 (expressed in % inhibition of the absorption). Note that Blue CrO_5 assay can detect lower concentrations of α-tocopherol than Improved TEAC.

solutions of various concentrations of α-tocopherol with the two assays, the results expressed in % inhibition of the absorption are shown in Fig. 9. The greater sensitivity of the Blue CrO_5 assay over the Improved TEAC in detecting low concentrations of α-tocopherol is seen in the section of the curve where x and y axis are crossed. At low concentrations of α-tocopherol, the Blue CrO_5 assay detects an inhibition of the absorption, whereas Improved TEAC displays no inhibition of the absorption (note the section of the curve where x and y axis are crossed in Fig. 9).

Preliminary results from the measurement of blood plasma samples from patients with myocardial infarction (a classic model of ischemia – reperfusion injury) [34,35], using both assays, at 30 min, 2, 4 and 12 h time show similar fluctuations of the antioxidant status (Fig. 10). Blood samples were drawn from patients with myocardial infarction, at the above mentioned time points, in 15 mL tubes without anticoagulant and then centrifuged in a Heraeus bucket centrifuge at ×400g for 20 min. The supernatant (blood plasma) was then collected in 1.5 mL Eppendorf tubes and stored at $T = -70$ °C for future measurement. We used a non-parametric test (Spearmann's correlation) for interpreting the results. Both assays correlated significantly ($p = 0.0001$), but the Blue CrO_5 assay is far more accurate in detecting alterations in the antioxidant status early after myocardial infarction than Improved TEAC (its standard deviation is three times smaller at 30 min and at 2 h) (Fig. 11).

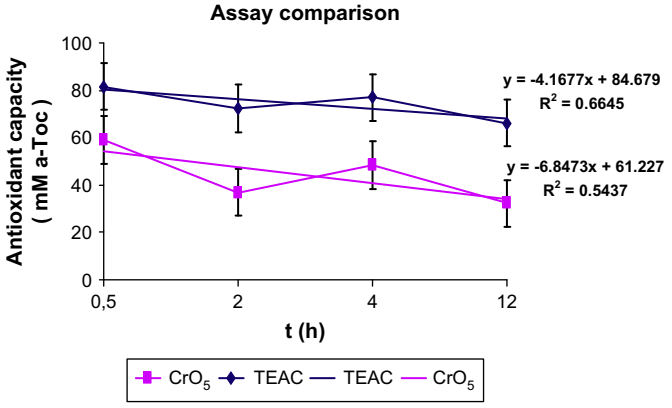

Fig. 10. Measurement of the antioxidant activity of blood plasma samples from patients with myocardial infarction using both Improved TEAC and Blue CrO_5 (expressed in mM of α-tocopherol solution).

4. Discussion

In the present study, the results of the evaluation of the redox state and the antioxidant capacity of various biological substances by a novel and relatively simple spectrophotometric assay of the blue chromium peroxide (CrO_5) are reported. The assay is based on the formation of CrO_5 by ammonium dichromate in an acidic environment in the presence of H_2O_2 (reaction (1)) as shown in the EPR spectra (Fig. 8a and b). CrO_5 is deep blue oxidative compound, reduced in the presence of antioxidants (Fig. 8a and b), that can be easily detected spectrophotometrically (Fig. 1a and b).

The already existing methods are capable of detecting only a specific antioxidant each time, but are unable of evaluating their various interactions and overall synergy. Total antioxidant capacity refers to the total amount of free radicals that can be scavenged by a certain antioxidant in a biological sample. A standard antioxidant is compared to the antioxidant properties of the sample under examination, thus producing a measure of its antioxidant capacity. This is, in general, the principle of the existing methods [28–30]. The Blue CrO_5 assay is able to detect both water-soluble and lipid-soluble antioxidants as it is evident from the detection by this assay of the antioxidant capacity of ascorbate and α-tocopherol. This ability is due to the bi-phasic nature of the chemical system of the assay (aqueous and organic, Photo 1a–d), which enables water- and lipid-soluble antioxidants to react with the substrate of the assay and thus be detected. It also simulates better the *in vivo* conditions where synergistic effects take place.

The methods in use are divided in two major categories: (1) the inhibition assays, where the lag phase or the suppression of an oxidation reaction, in the presence of an antioxidant, is being measured, and (2) the reduction assays, which are based on the reductive properties of antioxidants, and where the capacity of a certain sample to induce the reduction of marker-molecule is a measure of its content in antioxidants. Our assay belongs to the reduction assays, since it detects the inhibition of the formation of CrO_5 by reduction from the antioxidants of the sample under measurement, as seen in the EPR spectra (Fig. 8a and b). The main drawback of these assays is that they are based upon the competition between the substrate – marker and the antioxidants of the measured sample for the oxidative compound. The whole phenomenon is much more complex and secondary reactions take place between the already oxidized substrate and the sample antioxidants, which also reflect the antioxidant capacity of the sample, but affect the total absorbance and the final readings. Furthermore, many biologically important reductants are unable to reduce the oxidizable substrate of the individual assay and are thus undetectable by a certain assay. That is the major cause of inconsistency between the various assays. Finally, the existing methods deal, in majority, with the water-soluble antioxidants, whereas lipid-soluble antioxidants, such as vitamin-A, α-tocopherol, etc., remain undetected. In this direction, efforts have been made, with the introduction of new solvents, to measure lipid-soluble compounds with the ImprovedTEAC assay but the assay isolates the two phases, whereas *in vivo* they co-exist and interact displaying synergistic effects in the level of antioxidant defence systems. In contrast, the dual nature of our assay seems to overcome these obstacles, since it allows the antioxidants to react with the substrate both in the aqueous and in the organic phase, as mentioned above.

The Blue CrO_5 assay is relatively stable, since the reaction is mostly completed at a time period of 3 min and absorption readings diminish insignificantly over an additional time of 15 min, as seen in Figs. 4 and 5. The assay also displays specificity as its substrate (blue CrO_5) does not react with polar organic substances but only with antioxidants such as α-tocopherol. Our assay displays adequate linearity and sensitivity, since it detects α-tocopherol at concentrations of 10 nM, as seen in Figs. 2 and 3. Furthermore, it significantly correlates with Improved TEAC ($R^2 = 0.8751$) with greater sensitivity in detecting lower concentrations of α-tocopherol (Fig. 9).

Major interferences in the repeatability and accuracy of the assay are the photosensitivity of the standard α-tocopherol solution and the gradual hydrolysis of the hydrogen peroxide solution when stored for over a period of several months. This requires that both α-tocopherol and hydrogen peroxide solutions must be freshly prepared from stock solutions for the measurement of a number of samples and that stock solutions must not be one year older. Moreover, biological samples with rich protein content tend to form a thin layer of denaturated proteins between the two phases of the measurement solution, thus obstructing chemical interactions between them. Further centrifugation of these samples solves this problem in most cases. The use of reusable glass cuvettes is not recommended because denaturated proteins of the samples form a thin film in their walls and alter the absorbance readings in the photometer.

Variable	Obs	Mean	Std. Dev.	Min	Max
$0{,}5h_{CrO5}$	15	59.122	1.344719	57.54	60.65
$2h_{CrO5}$	15	37.244	3.191571	34.09	41.43
$4h_{CrO5}$	15	48.452	5.608917	41.74	53.92
$12h_{CrO5}$	15	33.178	5.336405	25.46	39.96
$0{,}5h_{TEAC}$	15	80.544	4.344785	75.91	85.53
$2h_{TEAC}$	15	72.222	7.907174	62.18	82.88
$4h_{TEAC}$	15	74.678	5.025984	68.34	81.7
$12h_{TEAC}$	15	64.882	4.169432	57.81	68.11

Fig. 11. Spearmann's correlation of the two assays (CrO_5 and TEAC) at 0.5, 2, 4 and 12 h after the myocardial infarction. Note the accuracy of CrO_5 at 0.5 h and 2 h (smaller standard deviation values).

The Blue CrO_5 assay is probably suitable for the detection of the oxidative capacity of a given sample, since CrO_5 is produced only in the presence of H_2O_2, even at low concentrations. Preliminary results of our own seem to confirm this ability of the assay. It is currently used in our laboratory for the estimation of the antioxidant and oxidant capacity of various biological samples with very promising results.

In conclusion, the Blue CrO_5 is a relatively simple, stable, cost-effective assay with notable specificity, linearity and reproducibility. It also has an advantage over the other existing spectrophotometric methods in estimating both water- and lipid-soluble antioxidants, thus making it very promising.

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