Analytical Methods

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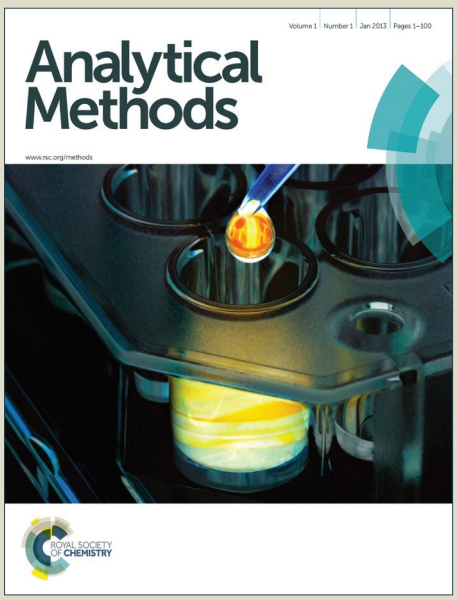

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ARTICLE

Electrolysis induced fast activation of the ABTS reagent for antioxidant capacity assay

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An improved TEAC (Trolox Equivalent Antioxidant Capacity) analytical procedure to evaluate the *in vitro* antioxidant capacity of some radical scavengers including copper proteins (i.e. Hemocyanin, Ceruloplasmin or Diamine oxidase) and some copper complexes (i.e. serine$_2$-copper), is described. The TEAC largely used method consists in measuring the reduction of the 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical monocation ($ABTS^{\bullet+}$) with a decoloration induced by the scavenger to be analyzed. Several disadvantages, such a particularly long time (about 16 h) for the activation of ABTS with potassium persulfate (free radical initiator) justify the need to improve this operatory mode. Consequently, we propose a new procedure consisting in a rapid activation of ABTS by electrolysis in about 20 sec instead of 16 hours as required by the activation with potassium persulfate. Furthermore, the ABTS activation by electrolysis provides an improved reagent stability for 7-8 days whereas the stability in the case of the classical method is limited to 3 days. The fast assay was compared with the classical activation with potassium persulfate as method of reference to activate ABTS and the results were expressed in equivalents of Trolox (standard reference scavenger). A good linearity of the answer in terms of Trolox amounts has been obtained with the fast assay, as with the classical method. Therefore, the fast assay seems compatible with the classical method, with the only difference and advantage that is much faster and easier to operate.

Keywords: Antioxidant properties; Copper-proteins; ABTS electrolysis fast activation; Radical scavengers; Reactive oxygen species (ROS); TEAC improved method.

1. Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($\bullet O_2^-$) and the hydroxyl radical ($\bullet OH$), are highly reactive towards DNA, lipids and proteins, often causing damage of membranes.[1-3] These biochemical changes are frequently involved in cardiovascular dysfunctions, neurodegenerative diseases associated with aging (Alzheimer's, Parkinson's diseases), inflammatory diseases, diabetes, tumorigenesis and cancer.[4-6] The ROS can destroy the integrity of DNA, forcing the organisms to protect themselves and triggering repair systems.[7] Organisms possess several antioxidant molecules to reduce or counteract these ROS. Some components in the antioxidant defence system are specific including the superoxide dismutase (EC 1.15.1.1) scavenging superoxide as well as catalase (EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9) acting against hydrogen peroxide. Others are non-specific antioxidants such as albumin, transferrin and copper proteins [8,9] which among other functions can contribute to the body's defence systems. They are involved in protecting the body against reactive oxygen species (ROS) caused by oxidative stress phenomena preventing thus various pathologies. Certain copper proteins are involved in several metabolic reactions and are present in vertebrate (Ceruloplasmin, CP; serum amine oxidase), in invertebrate (Hemocyanin, HC) animals and plants (Diamine oxidase, DAO). The CP is a copper protein (6 copper atoms) present in the blood plasma. Its antioxidant activity has been showed *in vitro* and *ex vivo* [8,10-14] with a marked capacity to scavenge several ROS.[15] The HC is a copper protein with two copper atoms, responsible for oxygen transport in the tissues of molluscs and arthropods. [16-18] The DAO, also known as Histaminase (due to its ability to degrade the histamine) is a copper protein (2 copper atoms) encountered in both plants (peas, chickpeas, lentils) and animals (placenta, kidney and intestinal mucosa vesicular structures). The DAO has a protective effect in post-ischemia reperfusion at cardiac or intestinal level.[19,20] The interest for the antioxidant biomolecules is continuously growing due to their therapeutic or protective potential benefits in the pharmaceutics, cosmetics and food for the prevention and treatment of several diseases.[13,21,22] Within the last decades, several methods to quantify antioxidant capacities were developed, each of them for certain fields of applicability and in function of their scavenging specificity. One of the largely used methods to evaluate antioxidant capacities, the ORAC (Oxygen Radical Antioxidant Capacity) assay [23-27] was recently removed from the list of recommended *in vitro* methods by the USDA (United States Department of Agriculture). This was due to erroneous values of the antioxidant capacity of bioactive components.[28] Consequently the TEAC (Trolox Equivalent Antioxidant Capacity) method or $ABTS^{\bullet+}$ (azinobis- (3-ethylbenozthiazoline-6-sulfonic acid) decolorization assay remained the *in vitro* method recommend for antioxidant capacity determination. In this assay, first reported by Miller and Rice-Evans [29], $ABTS^{\bullet+}$ radicals are generated through the peroxidase activity of metmyoglobin in the presence of hydrogen peroxide and can easily

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be detected spectrophotometrically at 734 nm. However, certain interferences lead to overestimation of antioxidant capacity [30] due to compounds interfering with the formation of the $ABTS^{\bullet+}$. Several modifications of the method to generate $ABTS^{\bullet+}$ were proposed using, for instance, chemical reagents such as manganese dioxide [31], 2,2'-azobis- (2-amidinopropane)HCl (ABAP) [32] or potassium persulfate [33] or enzyme reactions with horseradish peroxidase [34]. In general, chemical activation (i.e. with potassium persulfate) requires a long time (up to 16 h with potassium persulfate) or high temperatures (60 ºC for ABAP generation), whereas enzyme activation is faster, but the reaction mechanism may shift with pH: for example, electron transfer is facilitated at acid pH.[35]
An electrochemically generated ABTS radical cation was reported by Ivekovic et *al* [36] using a Flow Injection Analysis System with an electrolysis process for each sample in a flow-through electrolysis cell.
This report was aimed to evaluate the assay of the antioxidant capacity *in vitro* of copper bioactive agents such as Hemocyanin, Ceruloplasmin, Diamine oxidase and serine$_2$-copper complex by the TEAC assay based on a new and fast way to activate ABTS by electrolysis and to compare the new rapid assay with the ABTS activation to $ABTS^{\bullet+}$ radical cation generated by potassium persulfate. The novel activation procedure markedly simplifies the assay reducing the time of operation from about 16 hours to less than one minute and offering a higher stability of $ABTS^{\bullet+}$ activated reagent.

2. Results

2.1. ABTS activation by electrolysis

2.1.1. Color of the reagent depending on the electrolysis time for ABTS activation

Native ABTS in saline solution is colorless. Electrolysis induces a blue-green color with different absorbency intensities associated to the activation as $ABTS^{\bullet+}$ and depending on the electrolysis time (Figure 1). This color changes first from colorless to blue-green (A_{max} 734 nm) and then to yellow-green and orange when electrolysis time is extended (Figure 1a and 1b).

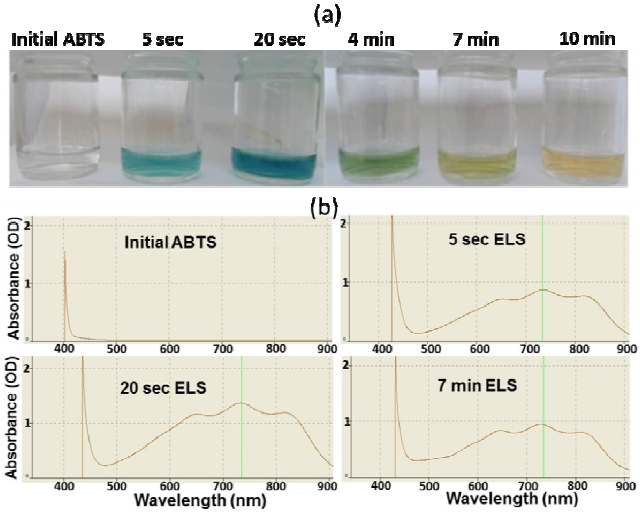


Figure 1: Influence of the electrolysis time on the color intensity (a) and the visible spectra (b) of the activated $ABTS^{\bullet+}$

2.1.2. Optimized ABTS activation time

Maximal absorbency at 734 nm, corresponding to radical cation $ABTS^{\bullet+}$ solution was obtained in the range of 15 to 35 sec of electrolysis. After this interval, there is a decrease in the absorbance intensities at 734 nm (Figure 2) and the characteristic blue-green colour diminishes (Figure 1a and 2).
Since the maximal absorbency ascribed to ABTS activation ($ABTS^{\bullet+}$) was obtained at 15-35 sec, the optimal operation time for ELS for the Fast TEAC was selected as 20 sec, enough for the formation of radical monocation, and obviously advantageous for the rapidity of the assay. Furthermore, it appears that the fast assay fits well with the classical ABTS method based on activation by potassium persulfate, but requiring 16 h for $ABTS^{\bullet+}$ generation.

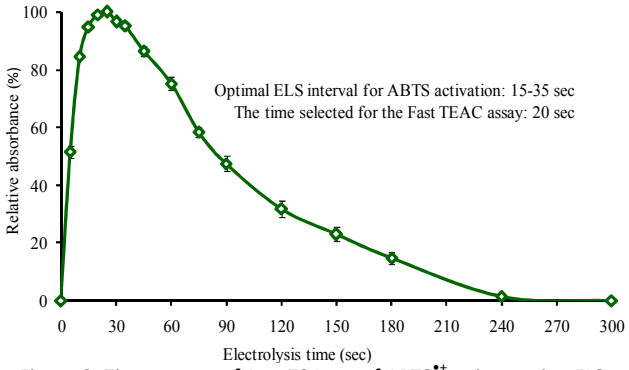


Figure 2: Time course of A_{max} 734 nm of $ABTS^{\bullet+}$ at increasing ELS time (each point is the mean of n=5 measurements ±SD).

2.2. Stability of activated $ABTS^{\bullet+}$

The ABTS activated as $ABTS^{\bullet+}$ with potassium persulfate (standard activation) is a reagent that can be stable for three days in dark at 25 ºC. When activated by electrolysis, the stability of $ABTS^{\bullet+}$ was prolonged to eight days (Figure 3).

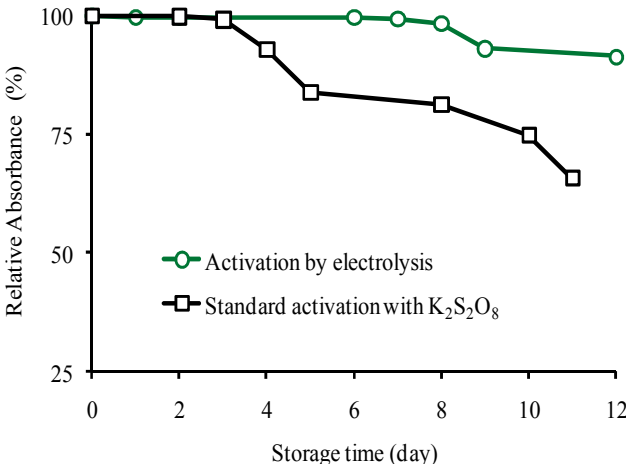


Figure 3: Stability of the $ABTS^{\bullet+}$ reagent obtained by ABTS activation with potassium persulfate (classical assay) and via ELS (20 sec) for the Fast TEAC assay, as showed by A_{max} 734 nm

2.3. The reactivity of activated ABTS$^{\bullet+}$ reagent versus different concentrations of Trolox

In the presence of Trolox (standard antioxidant) at various concentrations, the absorbance of ABTS$^{\bullet+}$ activated by classical method with potassium persulfate was rapidly decreased in response to the Trolox antioxidant scavenging capacity, following by a stabilization of the intensity for the next 1 to 5 min and slowly decreasing after up to 30 min and more (Figure 4a). The same results were obtained with ABTS$^{\bullet+}$ activated by electrolysis (Figure 4b). The absorbency decrease was linearly proportional to Trolox concentration.

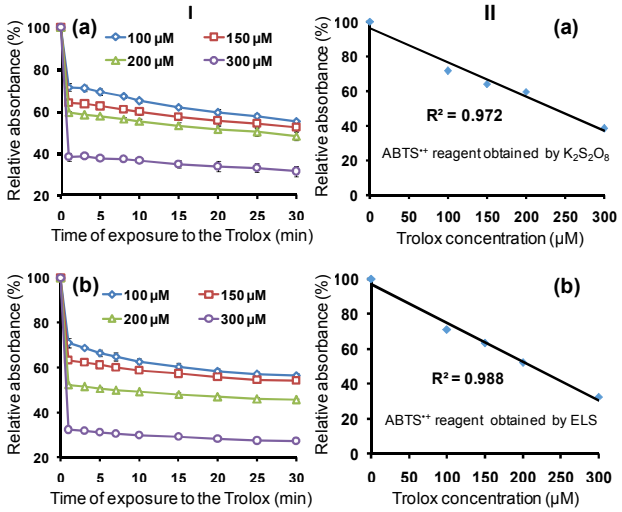


Figure 4: Time course (I) and absorbance dependency (II) of the ABTS$^{\bullet+}$ reagent activated by potassium persulfate (a) classical method and by electrolysis (b) fast method, at different concentrations of Trolox

2.5. Antioxidant capacity evaluation

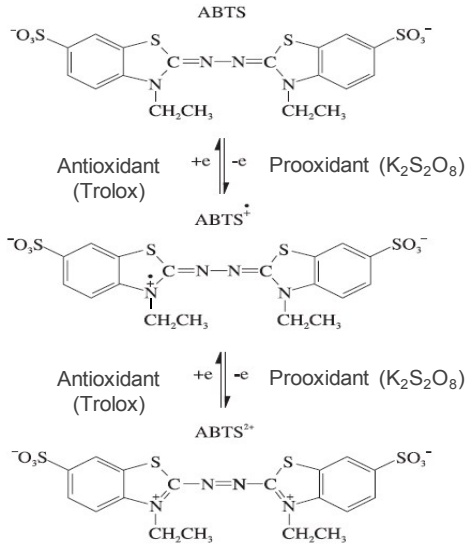
The antioxidant capacities of various scavengers were evaluated by the original method and by the Fast TEAC assay and were expressed in Trolox Equivalent units/ g of sample. Different scavenging capacities of various agents with known or possible antioxidant properties as Hemocyanin (HC), Ceruloplasmin (CP), Diamine oxidase (DAO), Bovine Serum Albumin (BSA) and Serine$_2$-copper are presented in Table 1. For every tested agent, the scavenging values determined by the classical procedure and by the fast assay, were close each other.

Table 1: Antioxidant capacity of samples

Samples		HC	CP	DAO	BSA	Serine$_2$ copper
Antioxidant capacity (Trolox U/g)	Classical method	18.18 ±5.9	131.17 ±8.7	51.1 ±3.9	56.0 ±3.1	6.1 ±1.5
	Fast method	25.3 ±4.1	132.9 ±4.5	52.4 ±2.4	56.9 ±7.1	4.1 ±1.0

3. DISCUSSION

Oxidation of ABTS with potassium persulfate ($K_2S_2O_8$) to generate ABTS$^{\bullet+}$ radical cation requires the transfer of one electron. Then, at prolonged oxidative conditions, this radical can be transformed into a dication ABTS^{2+} (Scheme 1).[42,43]



Scheme 1: Hypothetical structures and mechanisms involved in ABTS activation in pro-oxidant conditions and antioxidant scavenging action of Trolox standard, according to Venkatasubramanian and Maruthamuthu [42] and Branchi et *al*. [43].

This change can also be obtained by prolonged ELS and is associated to a decoloration and a shift of the maximal absorbency from 734 nm to 514 nm wavelength (Figure 1a and Figure 5 annex). The ABTS$^{\bullet+}$ radical cation for classical method may be formed either by chemical oxidation of ABTS with $K_2S_2O_8$ for about 16 h [41,44] or enzymatically with laccase (EC 1.10.3.2) or horseradish peroxidase (EC 1.11.1.7) [35,45-47] and this must be done several hours prior to analysis. By electrolysis, which generates free radical species [48,49], the ABTS was rapidly converted by oxidation into ABTS$^{\bullet+}$ radical cation with blue-green colour development after only a few sec with a maximal oxidation of ABTS achieved in 15 to 35 sec of electrolysis (Figure 2). After thirty min of electrolysis, the solution became colorless. The decreasing intensity of green-blue coloration with reduced absorbency at 734 nm is probably due to the degradation of ABTS$^{\bullet+}$. To support the hypothesis that the ABTS$^{\bullet+}$ was degraded, the absorbance of the ABTS electrolyzed solution (ABTS ELS) was read at 280 nm in order to evaluate if the aromatic rings have been affected. The absorbencies were determined at 280 nm to check the status (degradation or not) of ABTS$^{\bullet+}$ aromatic rings by electrolysis. No differences were detected when compared with ABTS solution without electrolysis suggesting that the aromatic rings of ABTS$^{\bullet+}$ evaluated at 280 nm are still preserved (Figure 7 annex). Therefore, they have been apparently stable following electrolysis (400 V, 10 mA), with no degradation. The variation of absorbency between 15 sec and 60 sec at ELS could be due to the ABTS$^{\bullet+}$ double bond delocalization, followed by a stabilization (Figure 7 annex).

Additionally, an increase of absorbance intensity was observed when the time of ELS was extended (Figure 8 annex). The A_{max} 734

nm was ascribed to $ABTS^{\bullet+}$ whereas the shift of maximal absorbency from 734 nm to 514 nm with maintaining of the absorbency at 280 nm was attributed to the $ABTS^{2+}$ dication formation [50] at prolonged ELS time. After more than 35 sec of ELS, the radical cation $ABTS^{\bullet+}$ was further oxidized to an $ABTS^{2+}$ dication with change of the colour intensity and with the shift of maximum absorption wavelength following by a loss of intensity (Figure 1). The purple-red colour identified at a longer ELS time was attributed to the $ABTS^{2+}$ dication. As example, this colour was obtained after ten minutes of electrolysis (Figure 1) and was ascribed to the dication $ABTS^{2+}$ as shown in absorbency curve (Figure 8 annex).

The $ABTS^{\bullet+}$ radical cation obtained by ELS appeared stable for 7-8 days as showed by the absorbency at 734 nm, whereas the $ABTS^{\bullet+}$ radical cation obtained by classical assay with potassium persulfate was stable only for 3 days (Figure 3).

The presence of potassium persulfate residues in $ABTS^{\bullet+}$ radical cation solution could favor the recovery of the electron which had been ceded to ABTS, making the conversion slowly reversible for the classical method and explaining thus the stability limited for only three days. For activation of ABTS by the present method, there are probably other oxidant species (i.e. hypochlorous acid) generated during electrolysis which may contribute to maintain the $ABTS^{\bullet+}$ radical level. When the electrolysis process is stopped, the generation of electrons is arrested and no other accumulated agents can influence the stability of the resulting $ABTS^{\bullet+}$ radical. Differently, in the case of classical method the remaining persulfate can influence the $ABTS^{\bullet+}$ radical stability. This can explain the fact that although the same compound ($ABTS^{\bullet+}$) is formed, the stability of the $ABTS^{\bullet+}$ radical cation generated by the classical method is only for 3 days in comparing with the proposed method by electrolysis, about of 8 days.

At the same initial concentration of ABTS, the level of $ABTS^{\bullet+}$ radical cation obtained by the two methods (electrolysis and potassium persulfate) measured by the absorbance at 734 nm showed no significant differences. The absorbency value was maintained for 8 days when ABTS was activated by ELS whereas the value was moderately decreased (with about 20 %) after 3 days when ABTS was activated by potassium persulfate at 734 nm showing a better stability of ABTS activated by ELS, as mentioned at the section 2.2.

For the evaluation of scavenging capacity of various antioxidants, we have followed the quenching of the absorbency at 734 nm as a measure of the scavenging activity of the antioxidant sample against $ABTS^{\bullet+}$ radical cation. In the presence of different concentrations of Trolox (100 µM, 150 µM, 200 µM and 300 µM), the reactivity of $ABTS^{\bullet+}$ radical cation solution obtained by ELS showed a similar behavior as that of the reactive radical ($ABTS^{\bullet+}$ cation) reagent generated by the classical method. The absorbance decrease, equivalent of quenching induced by reference antioxidant (Trolox), can be followed during an interval of 1-5 min stability (Figure 4 and Figure 6 annex). Thereby, an end point value read at any moment of the interval between 1-5 min can be effective for evaluation of the antioxidant sample. Absorbency decrease was more pronounced with the radical $ABTS^{\bullet+}$ reagent when it was produced by ELS. This method offers then more sensibility for the absorbance quenching of $ABTS^{\bullet+}$ solution and more rapidity for the assay.

Trolox Equivalent Antioxidant Capacities of different samples (Hemocyanin, Ceruloplasmin, Diamine Oxidase, Bovine Serum Albumin and $Serine_2$-copper were each of them evaluated separately with a solution of $ABTS^{\bullet+}$ radical cation obtained by activation with potassium persulfate and by ELS and compared with antioxidant capacities of different concentrations of Trolox. No marked differences between the TEAC values with $ABTS^{\bullet+}$ obtained by the classical activation (with potassium persulfate) and by the ELS were found (Table 1). The fast assay was also compared with the classical activation with potassium persulfate as method of reference to activate ABTS. A good linearity of the answer in terms of Trolox amounts has been obtained. Therefore, the fast method seems compatible with the classical method, with the only difference and advantage that is much faster and easier to operate with a larger stability of the $ABTS^{\bullet+}$ reagent obtained by the fast assay.

The chosen parameters of electrolysis voltage of 400 V with current 10 mA were justified by previous works. In fact, similar parameters have been used previously (Dumoulin et al, 1996; Jackson et al, 986) to generate oxidative damage simulating post-ischemia-reperfusion on isolated rat heart. In our case, at higher than 400 V electrolysis voltage or higher current the amount of electric charges per unit time increases, so the ABTS will be oxidized faster (less than 20 seconds, possibly with undesired dication formation, but is difficult to make a statement on stability). The optimal electrolysis time may depend on concentration of ABTS used. However, for this TEAC method, the chromophore reagent ($ABTS^{\bullet+}$ radical cation solution) should be diluted such as to have an absorbency of 0.7 (±0.02) before use. A moderate-low concentration (0.14 mM) of native ABTS allows to reach this intensity. The concentration of ABTS used for Figures 1 and 2 was the same.

4. Conclusion

Commonly used to evaluate the Trolox Equivalent Antioxidant Capacity (TEAC), the $ABTS^{\bullet+}$ radical cation evanescence classical assay was improved by a fast ABTS activation using electrolysis in only about 20 sec versus about 16 hours for the classical method. This activation procedure by electrolysis gives similar results in term of antioxidant capacity values, with the advantage of a faster and easier procedure to apply. In addition, our procedure involves an optimized time of activation and same batch of activated $ABTS^{+}$ reagent,which is stable several days, can be used for serial sampling.

Conflict of Interest

The authors declare that they have no competing interests.

5. Experimental

5.1 Materials

The ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (purity 98 %) and Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid) 97 % were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trolox antioxidant stock solution (2.5 mM) was prepared in ethanol 50 %. Potassium persulfate (99.5 %) and sodium chloride (99 %) were from Anachemia (Montreal, QC, Canada). The bovine serum was obtained from blood collected at the Slaughterhouse Tarte (Marieville, QC, Canada).

All other reagents used were of analytical grade.

5.2 Ceruloplasmin purification

Ceruloplasmin (CP) was purified from bovine plasma by ammonium sulphate fractionations (35 and 55 % saturation) followed by a chromatographic single-step separation on aminoethyl-agarose

[36], obtained by agarose (Superose 6B, Pharmacia LKB, Uppsala, Sweden) treatment with chloroethylamine.[37] The retained pool of purified CP obtained by elution with 200 mM potassium phosphate pH 7.2 was electrophoretically homogeneous, with an A_{610}/A_{280} ratio of ≥ 0.04 (a value corresponding to the standard criteria for the purified enzyme, where A_{610} is given by the blue copper site of CP and $A_{280\ nm}$ by the protein content). It was stored at -20 °C, until use.

5.3 Enzymatic activities

Ceruloplasmin oxidase activity was determined spectrophotometrically at 540 nm and room temperature (25 °C), according to Osaki's method [39] based on the oxidation of 1 mL p-Phenylenediamine (pPDA) reagent (10 mM pPDA in 0.25M sodium acetate buffer pH 5.2, containing 5 mM EDTA) in the presence of 50 µL of CP sample. One enzyme unit was the amount of enzyme (CP) able to oxidize 1.0 µmole of substrate (pPDA) per min at pH 7.0 and 25 °C.

The activity of DAO was determined using a peroxidase coupled reaction to quantify the released H_2O_2, as previously described. [39] The assay mixture containing 3.2 mL of 50 mM potassium phosphate buffer (pH 7.2), 50 µL of peroxidase solution (0.1 mg/mL), 250 µL of 30 mM N,N-diethyl para-phenylenediamine solution and 1 mL of 30 mM putrescine solution was incubated for 5 min at 37 °C. The assay started with addition of DAO for the dosage. After incubation for 10 min at 37 °C, the absorbency was read at 515 nm using an Ocean Optic© fiber optic spectrometer (Winter Park, FL, USA). The standard curve was prepared using various concentration of H_2O_2 ranging from 0 to 0.4 mM. One enzyme unit (EU) of DAO was considered as the amount of enzyme required to catalyze the oxidation of 1.0 µmol of putrescine per min at pH 7.2 at 37 °C.

Hemocyanin phenoloxidase (catecholase) activity has been evaluated at 25 °C using 10 mM catechol in 100 mM phosphate buffer pH 7 as substrate. Reaction medium contained 0.5 mL of Hemocyanin and 2.5 mL of 10 mM catechol. The absorbance was measured every 30 sec for 5 min. One enzyme unit was defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per min at pH 7 and 25°C**.**

5.4 The TEAC method based on $ABTS^{\bullet+}$ generated by potassium persulfate

Trolox Equivalent Antioxidant Capacity (TEAC) original method is based on the scavenging of the blue-green $ABTS^{\bullet+}$ radical cation by electron-donating antioxidants.[29] The ability of the tested sample to scavenge the $ABTS^{\bullet+}$ radical cation is compared to that of Trolox (structural hydrosoluble analogue of vitamin E) used to establish the standard curve. In standard procedure, the assay is based on the capacity of the antioxidant to scavenge the blue-green $ABTS^{\bullet+}$ radical cation into colorless ABTS [31,33,41]. Practically, for the classical assay, the $ABTS^{\bullet+}$ was produced by reacting 4 mL of 7 mM ABTS solution with 1mL of 12.25 mM potassium persulfate ($K_2S_2O_8$) and maintaining the mixture in the dark at 37 °C for 16 h before use. The $ABTS^{\bullet+}$ solution was then diluted to adjust its absorbance to 0.700 (± 0.020) at 734 nm. To determine the scavenging activity of an antioxidant agent, 950 µL of diluted $ABTS^{\bullet+}$ solution was mixed with 50 µL of the antioxidant sample to be evaluated or of Trolox standard solutions (concentration 0-360 mM) and the absorbance was measured at 734 nm during 1 min after the initial mixing. The Trolox equivalent antioxidant capacities of the samples were estimated by measuring the antioxidant activity of solutions of these compounds and expressed versus that of standard solutions of Trolox**.** The Trolox equivalence corresponds to the Trolox concentration having the same activity as the tested sample at a given concentration. Results are expressed in µM or mM of Trolox equivalence per gram (g) of product or per mL for solution. The percentage of decrease of absorbance at 734 nm is plotted against the concentration of Trolox as standard**.**

5.5 Modified TEAC assay with ABTS fast activation by electrolysis

The improved antioxidant capacity assay with ABTS activated by electrolysis (ELS) drastically reduces the time of procedure. The principle of the modified method consists in the activation of ABTS by electrolysis, which is very fast, instead activation by potassium persulfate requiring 16 h. An amount of ABTS was dissolved in saline (0.85 % NaCl) aqueous solution in order to obtain a final ABTS concentration of 140 µM. The NaCl electrolyte solution allows the movement of electrons by a continuous current. The activation of ABTS was carried out by electrolysis (400 volts, 10 mA) for different times (0-240 sec). The two electrodes were in platinum, with 2.12 mm diameter 25.33 mm long and space inter-electrodes of 26.36 mm measured with an Electronic Digital Caliper (Docap Corp., St-Laurent, Quebec, Canada) for the range 0-200 mm with readings at a precision of tens of microns.

Absorbency at 734 nm was measured and plotted as a function of electrolysis time of ABTS. Electrolysis time was chosen such as to have the maximal absorbance. This electrolysis optimized time of 20 sec generated a maximal oxidation of ABTS amount.

5.5.1 Fast TEAC assays

The improved TEAC rapid assay was hereto shortly called Fast TEAC assay and is based on the same principle as the TEAC classic assay. The major difference is that ABTS should be activated by electrolysis for some 20 sec instead 16 h of activation by potassium persulfate. The ABTS solution prepared as above was electrolyzed without antioxidant, generating the cation radical ($ABTS^{\bullet+}$) which is in fact the reagent for the antioxidant assay. To establish the standard curve, Trolox was used in various concentrations and its antioxidant capacity was determined by monitoring the decrease of absorbance at 734 nm in the electrolyzed $ABTS^{\bullet+}$ solution.

Practically, a volume of 6.5 mL (140 µM) ABTS was electrolyzed during 20 sec to generate the $ABTS^{\bullet+}$ radical (according the needs of the test, a stock volume of reagent can be produced). For the assay, the $ABTS^{\bullet+}$ solution (950 µL) was then mixed with 50 µL of 0-200 µM Trolox or of the antioxidant sample. The absorbance at 734 nm was measured for 1 min after the initial mixing with the electrolysis induced $ABTS^{\bullet+}$. By measuring -ΔA/min, the analyzed antioxidant was compared with Trolox in terms of capacity to scavenge $ABTS^{\bullet+}$ as is currently done for the TEAC classic assay. The decrease of absorbency due to the antioxidant capacity reflects the scavenging of the free radical ($ABTS^{\bullet+}$).

The stabilities of $ABTS^{\bullet+}$ reagent (reactive radical species) obtained by chemical (potassium persulfate) and by ELS ways and maintained at room temperature for several days were evaluated as absorbency at 734 nm.

Each measurement consisted in at least five readings and the values represent the mean ± standard deviations.

Annexes

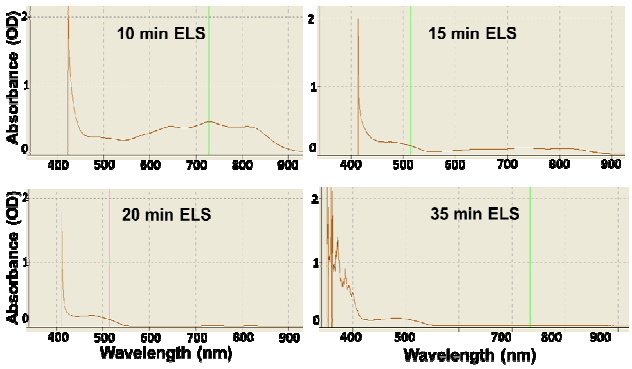


Figure 5: Maximal absorbance wavelength shifts of activated ABTS depending on the electrolysis time

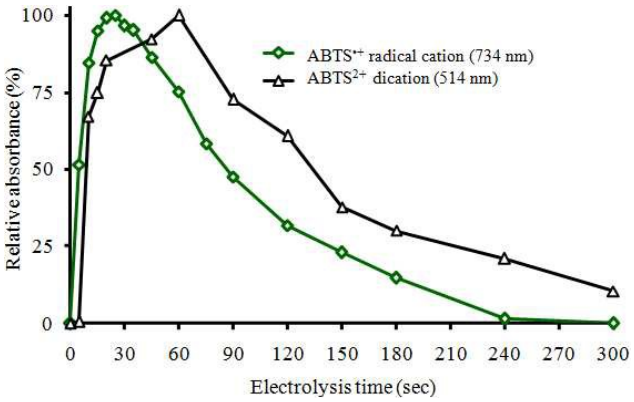


Figure 8: Relative absorbency at 514 nm ($ABTS^{2+}$dication formation) at increasing electrolysis time

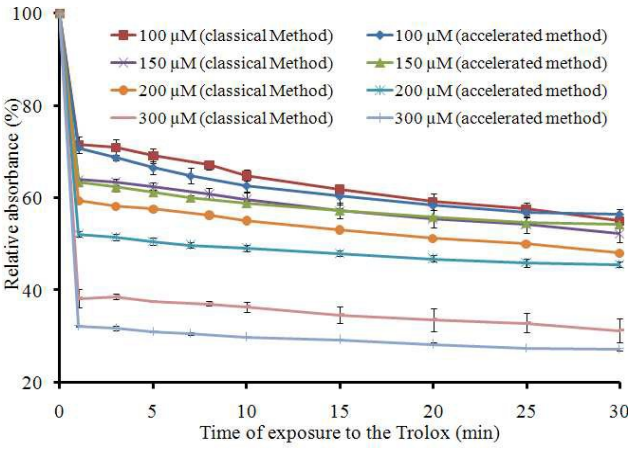


Figure 6: Comparison of time courses of absorbency of $ABTS^{\bullet+}$ reagent in the presence of different concentrations of Trolox

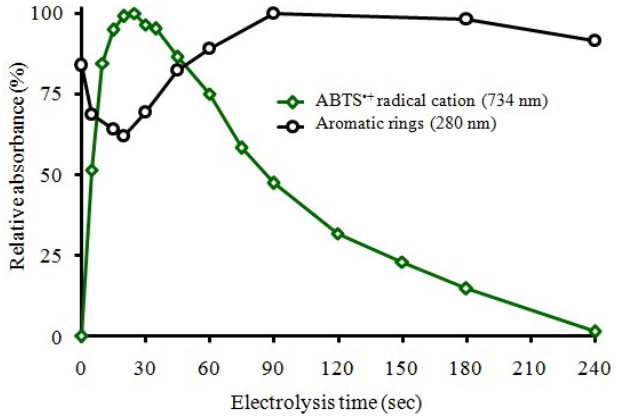


Figure 7: Relative absorbency at 280 nm (aromatic rings of ABTS) at increasing electrolysis time

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Graphical abstract