Evaluation of Antioxidative Activity of Some Antioxidants by Means of a Combined Optothermal Window and a DPPH* Free Radical Colorimetry

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Antioxidative activities of vitamin C, BHA, BHQ and BHT oxidants were determined at ambient temperature by means of a DPPH* colorimetry (in methanol) combined with the optothermal window (OW) detection scheme at 514 nm. The results of OW-DPPH* experiments suggest differences in the reaction kinetics for various compounds; these were also found to be concentration dependent. Vitamin C reacted with DPPH* more rapidly than BHA, BHQ and BHT. Although OW-DPPH* colorimetry exhibits a dynamic range that is wider than that typically encountered in conventional spectrophotometry (SPM), its sensitivity is inferior to that of SPM. Search for new colorimetric reactions resulting in a more intense color yield is encouraged, if the unique advantage of OW-DPPH* method (to handle optically opaque samples) is to be fully realized.

Keywords: antioxidative activity, optothermal window spectroscopy

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Antioxidants are often used to delay auto-oxidation in food lipids, a process responsible for off-flavors and formation of constituents that might pose a potential health risk. For this reason it is of interest to assess antioxidative activity (AOA) of specific antioxidant. The AOA is conventionally used to indicate ability of antioxidant to scavenge some radicals.

One among tests proposed for assessment of AOA is a free radical colorimetry [1,2] that relies on the reaction (DPPH*+AH → DPPH-H+A*) of specific antioxidant (AH) with a stable free radical 2,2-diphenyl-1-picryl-hydrazyl DPPH*, ($C_{18}H_{12}N_5O_6$, M= 394,33 g) dissolved in methanol. As a result of a reduction of DPPH* by antioxidant, the optical absorbance at 514 nm of this purple-blue colored solution of DPPH* in MeOH decreases; this change is normally detected by means of a traditional transmission spectrophotometry (SPM).

The main objective behind the experiment described in this paper, was to explore potential of a combined use of the optothermal window (OW), the low cost, non-traditional detector of absorbance, and a DPPH* test colorimetry at 514 nm, to assess AOA of antioxidants such as vitamin C, BHA, BHQ and BHT. Prospects for feasibility of a OW-DPPH* approach for assessing AOA, are based on a direct proportionality between magnitude of the OW signal and the optical absorbances. The performance of the newly proposed OW-DPPH* was compared to that of SPM.

Experimental

The operational principle of OW was discussed in our previous papers[3,4]. Here, the 514 nm radiation (10 mW) provided by LEXEL 85 argon ion laser is reflected at two gold coated front surface mirrors before passing through Isomet 1205-603F acousto-optical modulator and reaching the OW. Basically, the OW is a 300 μm thick sapphire (Al_2O_3) disc (high transparency at 514 nm) with a piezoelectric (PZT) annular ring glued to its rear face. Fluid test sample is pipetted directly into a hollow PTFE cylinder (height 30 mm, diameter 10 mm) that itself was mounted (silicon glue Rhodorsil CAF-4) on upper side of sapphire disc. To eliminate the undesirable effects of fluid movement caused by surrounding laboratory environment, the opening at a top of cylinder was covered by a black, removable plastic cap. The output from OW was processed by a SR830 two phase lock-in amplifier (Stanford Research Systems) and data stored on a PC.

Antioxidants studied here included vitamin C [L-ascorbic acid $C_6H_8O_6$ M=176.1 g], BHQ (tert-butylhydroxy quinone) [$C_{10}H_{14}O_2$ M=166.2 g], BHT (butylated hydroxy toluene) [$C_{15}H_{24}O$ M=220.4 g] and BHA (butylated hydroxy anisole or 2-tertbutyl-4-methoxy-phenol) [M=180.2 g]; all the chemicals were obtained from Fluka. Methanol and ethanol (99.5 % purity and spectrophotometric grade) were products from Merck Chemical Co. (Darmstadt, Germany) while DPPH (1,1-diphenyl-2-picryl-hydrazyl) was purchased from Sigma Chemical Co. (St. Louis, MO).

Initially, 0.1 ml aliquots of 1.81 x10^{-3} M (in MeOH) of each antioxidant was added (one at the time) to 0.9 ml of 6.15 x10^{-3} M methanol solution of DPPH* in a reagent tube. After shaking tube gently, 1 ml of solution was quickly pipetted directly on the OW. The overall time interval elapsed between the onset of reaction and actual beginning of the measurement was 30 seconds. The magnitude of OW signal was on-line monitored until the steady state was reached. The plots obtained show reaction kinetics and provide information about AOA of various antioxidants. Additional OW-DPPH* studies using higher concentrations of antioxidant have also been performed. Likewise, parallel experiments were carried out on UV-Vis spectrophotometers (Perkin Elmer Lamda 18 and Varian-Carry 1E) both using 1 cm long standard quartz cuvette.

Results

Figure 1 exhibits reaction kinetics (amplitude of lock-in signals versus time acquired at 1 Hz) relevant to vitamin C, BHT, BHQ and BHA. The plot obtained from DPPH* solution in MeOH but without antioxidant added is also shown. As stated above, concentration of each antioxidant added to 6.15 x10^{-3} M of DPPH* was 1.81 x10^{-3} M. The 1 Hz modulation frequency was chosen because of the favorable signal to noise ratio. At 10 mW laser power and 1 Hz, the magnitude of the background signal (i.e. signal from pure MeOH) was about 50 times lower than that obtained typically from samples containing DPPH*.

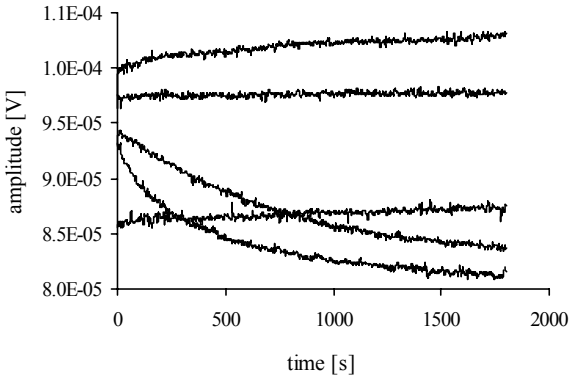

Fig. 1 The amplitude of the OW (lock-in voltage at 1 Hz) signal obtained when adding 1.81 x10^{-3} M (of each of four different antioxidants) to a 6.15x10^{3} M solution of DPPH* (uppermost trace) in methanol. To what it concerns final amplitude value (at 1800 sec), vitamin C (second trace from above) and BHQ were found to react more rapidly with DPPH* than BHT and BHA (bottom trace).

The OW signal (and hence also the absorbance) of the solution decreases depending on the intrinsic AOA of antioxidant as well as on the speed of the reaction between DPPH* and the same antioxidant. In case of a rapid kinetic behaviour (second trace from above in Fig. 1), practically all antioxidant (Vit C) material reacted within a very short time, and a steady state is reached practicaly immediately. On the other hand, slow kinetic behaviour (see lowest trace in Fig. 1 for BHA) implies longer periods before steady state is reached. Although vitamin C shows a rapid kinetic behaviour, it is not a strong reducer of a DPPH* radical, i.e. it has low AOA. Unlike this, BHA exhibits slow kinetic behaviour but relatively strong reducing capacities, thus high AOA. BHQ on the other hand features rapid kinetic behaviour with a relatively large AOA. The results are in the agreement with data reported in the literature[1]. During the next research experiments with higher (5.70 x10^{-2} M to 2.28 x10^{-3} M) concentration of BHT in MeOH were carried out. Figure 2 illustrates kinetics between 0 and 1800 seconds. Clearly, the reaction kinetics depends on the concentration of BHT. For example, samples containing lower concentrations of BHT reacted slowly (steady state condition was not reached within 1800 sec) with DPPH*, while at a highest concentration level of BHT (5.70 x10^{-2} M) steady state is apparently reached after approximately 1000 seconds.

Data on reaction kinetics (Fig. 2) provide possibility to assess the extent of AOA for BHT. To do so, it is necessary to convert diagrams shown in Fig. 2 into new plots displaying the fraction of residual DPPH* in solution as a function of time. The fraction of residual DPPH* can be calculated from:

$$1 - [(c_{DPPH*(t=0)} - c_{DPPH*(t=t)})/c_{DPPH*(t=0)}]$$

where $c_{DPPH*(t=0)}$ and $c_{DPPH*(t=t)}$ are concentrations of DPPH* at t=0, and t=t respectively. The $c_{DPPH*(t=0)}$ was determined using the absorption coefficient per unit length β (obtained via above mentioned amplitude measurements of OW signal), absorbance A, thermal diffusion length μ and a molar extinction coefficient ε[5-7]; the relevant expression is $A=0.4343\beta\mu = \varepsilon c_{DPPH*(t=o)} \mu$. Likewise, $c_{DPPH*(t=t)}$ is calculated in the very same manner.

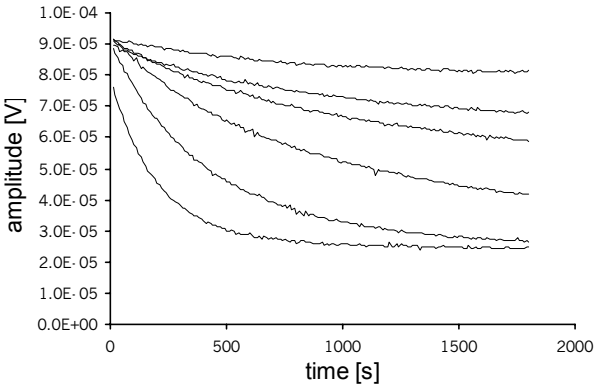

Fig. 2 Time evolution (0 to 1800 seconds) of the lock in signal obtained from BHT dissolved in 6.15x10^{3} M methanol solution of DPPH*. The concentration of BHT varies from 5.7x10^{-2} M (lowest trace) to 2.28x10^{-3} M (uppermost trace). Only for sample containing highest concentration, was the steady state within 1800 seconds

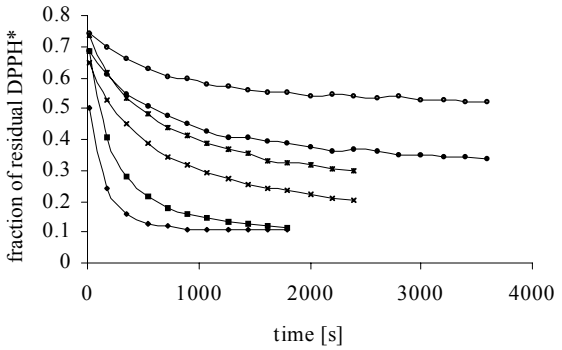

Fig. 3 Fraction of the residual DPPH* plotted versus time for concentration of BHT varying from 5.7x10^{-2} M (lowest trace) to a 2.28x10^{-3} M (uppermost trace).

Figure 3 illustrates time dependent plots of residual DPPH* for various BHT concentrations (see Fig.2). Using data from Fig. 3 one can construct a new plot that displays a fraction of residual DPPH* left in solution at a *steady state condition*, as a function of BHT concentration (expressed as [mole BHT/mole DPPH*]

ratio) as shown in Fig. 4. At this stage it is useful to introduce a concept of "efficient concentration" EC_{50} defined as the amount of BHT needed to reduce initial concentration $C_{DPPH*(t=0)}$ by a factor of two (at steady state). The parameter EC_{50} is a direct quantitative measure for AOA; highly effective (high AOA) antioxidant is characterized by low EC_{50} value, and vice versa. The inspection of Fig. 4 gives EC_{50}= 0.08 (moles BHT/mole DPPH*).

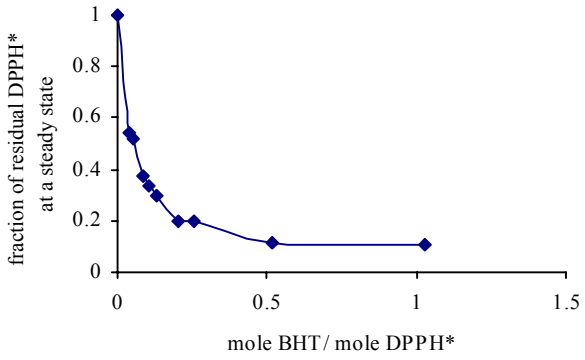

Fig. 4 Fraction of the residual DPPH* left at a steady state plotted, versus the ratio (mole BHT/mole DPPH*). From the graph one obtains 0.08 mole BHT/ mole DPPH* as efficient concentration EC_{50}.

It was of interest to find out how does the performance of OW-DPPH* compare (in terms of parameters such as dynamic range and the sensitivity) to that of conventional spectrophotometry (SPM).
The absorbance measured (by means of the SPM) at 514 nm from a $6x10^{-5}$ M solution of DPPH* in a cuvette 1 cm long, is 0.283. However, the sample containing $6x10^{-3}$ M of BHT is opaque and cannot be studied by SPM. The dynamic range (i.e. the ratio of absorbance measured at 514 nm when no BHT is added to solution of DPPH*, and the absorbance measured when no DPPH* left) is 2. However, when a $6x10^{-3}$ M solution of DPPH* is studied by OW method, the dynamic range, defined as the amplitude of OW signal (here 1 mV) obtained from a DPPH* solution, divided by that acquired from a methanol (here 0.03 mV), is as high as 33. Intensifying the coloring reaction (and hence the opacity) by two orders of magnitude (i.e. from $6x10^{-5}$ to $6x10^{-3}$ M) results in a sixteenfold (33/2) expansion of a dynamic range.

Discussion

The AOA of vitamin C, BHA, BHQ and BHT were determined by means of DPPH* colorimetry combined with OW detection. The test performed in methanol as a polar medium, at ambient temperature enables one to assess EC_{50} as a quantitative measure of AOA. The results of OW-DPPH* experiments suggest differences in reaction kinetics for various compounds; these were also found to be concentration dependent. As stated already, vitamin C reacted more rapidly with DPPH* than BHA, BHQ and BHT [1].
At 1 Hz modulation frequency, the effective sampling pathlength in the OW experiment is short (one thermal diffusion length is about 176 μm). The quantity of material needed for the analysis by OW-DPPH* is rather small, and a good thermal contact between test sample and the sapphire disk is essential. Furthermore, depositing and removing of the sample is rapid and easy. Minimum detectable concentration for conventional SPM, is nearly two orders of magnitude superior to that of OW-DPPH* (sampling length (1 cm) is much longer), but the dynamic range is 16 times narrower than that obtained for OW-DPPH*.

Practically all colorometric reactions reported in the literature were developed for SPM that operates in transmission mode; this imposes restrictions to what it concerns the absorbance that can still be measured reliably. However, in order to fully realize the advantages of OW-DPPH* method, it is worthwhile exploring possibility for new colorimetric reactions that would result in a more intense color yield. It is the unique potential of OW to handle optically opaque samples, otherwise not amendable for studies by SPM, that makes this approach interesting for use in practice. One anticipated application is the assessment of AOA for antioxidants added to lipids subjected to oxidation[8]. Since the OW-DPPH * test seems sensitive and cheap, this approach could be an interesting tool for on-line monitoring changes in AOA of foods (i.e. fruit and vegetables) that contain natural antioxidants. This might eventually enable one to optimise technological processes by minimizing the loss of natural antioxidants during processing.

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