Determination of antioxidant capacity in human serum using the Agilent Cary Eclipse for the ORAC assay

Application Note

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Introduction

The exposure of living organisms to reactive oxygen species (ROS) is unavoidable in aerobic life since the generation of ATP from molecular oxygen demands electrons. ROS fall into two groups; those that contain unpaired electrons (O_2-, OH-), or those that have the ability to extract electrons from other molecules (H_2O_2, HOCl). These species may damage biomolecules directly, or initiate chain reactions in which ROS are passed from one molecule to another, resulting in extensive damage to cell structures such as membranes and proteins.

This paradoxical need for a toxic fuel molecule is central to the Life Sciences since organisms must continually work to maintain an appropriate balance of pro-oxidants and antioxidants. If the balance sways in the direction of pro-oxidants, 'oxidative stress' can arise which, under normal circumstances, is controlled by a broad range of antioxidant enzymes, proteins and antioxidants provided by the diet. Breakdown or deficiency of these defenses against ROS can lead to damage which has been strongly associated with a wide variety of chronic diseases including Alzheimers, autoimmune disease, cancer, cardiovascular disease, diabetes, multiple sclerosis and arthritis[1]. In contrast, levels of ROS must not become too low given their important roles in the immune system. Therefore, there is a need for constant monitoring and regulation of the redox potential of the blood. One strategy to determine the efficacy of these controls is to measure levels of ROS indirectly, by measuring total levels of antioxidants (which are more stable than ROS) in serum. Antioxidants in plasma are not generally measured individually because of difficulties arising due to sensitivity and interactions between different antioxidants[2].

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Introduction

There are several existing methods to measure total antioxidant capacity, but most of the literature refers to three methods (or derivatives thereof):

1. FRAP (ferric reducing ability of plasma[3])
2. TEAC (trolox equivalent antioxidant capacity[4])
3. ORAC (oxygen radical absorbance capacity[5]).

The ORAC methodology is arguably the most accepted and accurate indicator of antioxidant status, mainly because it is based on measurements of fluorescence rather than absorbance[2]. This increases sensitivity and so permits a much lower molar ratio of antioxidant sample: reagents, thus minimizing the likelihood of cross-reactions between sample and reagents. In addition, the ORAC methodology measures 'total radical scavenging ability', since it is unique in that it takes reactions to completion, permitting a calculation of 'total area under the curve'.

The ORAC assay works by the following principle. A sample is added to a free radical-generating system, the inhibition of the free radical action is measured and the results calculated are related to the antioxidant capacity of the sample. AAPH (2,2'-azobis(2-aminopropane) dihydrochloride) is used as the free radical generator and R-PE (R-phycoerythrin) is used as a target for free radical attack. Free radicals cause conformational changes in the protein structure of R-PE leading to fluorescence quenching in a dose and time-dependent manner.

The present study aimed to demonstrate that the ORAC assay could be easily performed using the Agilent Cary Eclipse fitted with a temperature-controlled multicell accessory.

Materials and methods

Equipment

- Agilent Cary Eclipse fluorescence spectrophotometer
- Peltier-thermostatted multicell holder (with integral electronic stirrer)
- Temperature controller
- Temperature probes
- Magnetic stirrer bars
- Quartz cuvettes

Reagents

Phosphate buffered saline (PBS, 75mM, pH 7.0) was prepared by first mixing 0.75 M K_2HPO_4 with 0.75M Na_2HPO_4 in the respective ratio 61.1:38.9 (v/v). This mixture was diluted 1:9 (v/v) with MilliQ water and pH adjusted to 7.0.

Human serum. Blood (20 ml) was taken from a healthy volunteer (20-24 years; non-fasted) into serum clot tubes (Greiner Laboratories). These were centrifuged at 2500 x g for 10 min. Serum was removed and dilited further (1:100 v/v) in PBS before being used in the assay.

Phycoerythrin (R-PE; Sigma Laboratories). A stock solution of 0.17 mg/ml was prepared by dissolving 1 mg PE in 5.9 ml PBS. This was diluted further to give a working solution of 3.38 mg/l.

AAPH (2,2'-azobis(2-aminopropane) dihydrochloride; Molecular Probes). A working solution of 320 mM was prepared fresh by adding 10 ml PBS to 868 mg AAPH in a Universal container. This was stored on ice until used for analyses.

Trolox (6-Hydroxy-2,5,7,8-tetramethethylchroman-2-carboxylic acid; Sigma Laboratories). A stock solution (100 μM) was prepared by dissolving 5.0 mg trolox in 200 ml PBS. This was further diluted 1:4 v/v in PBS to give a working solution of 20 μM.

Protocol

Methods were adapted from those described by Cao and Prior 2[2]. Briefly, the multicell holder (with temperature probes and magnetic stirring accessories) was fitted into the sample chamber of the Eclipse. Peltier temperature was set to 37 °C.

Using the 'Scan' application, excitation and emission spectra of R-PE were obtained using 3 ml working solution in a quartz cuvette in order to determine the optimal excitation and emission wavelengths of R-PE.

Into each of three quartz cuvettes the following were added: 2738 µl R-PE working solution, 37 µl PBS and 150 µl trolox standard, blank (PBS) or serum working solution. Cuvettes were placed into three of the four positions in the multicell holder. Small (3 mm length) magnetic stirrer bars were placed into each cuvette, and the solution was left stirring to equilibrate for 10 min.

The 'Kinetics' application was used to record the rate of change of fluorescence intensity in each sample. Optimal excitation and emission wavelengths determined in step 1 were used during analyses. The operating parameters are illustrated in Figure 1.

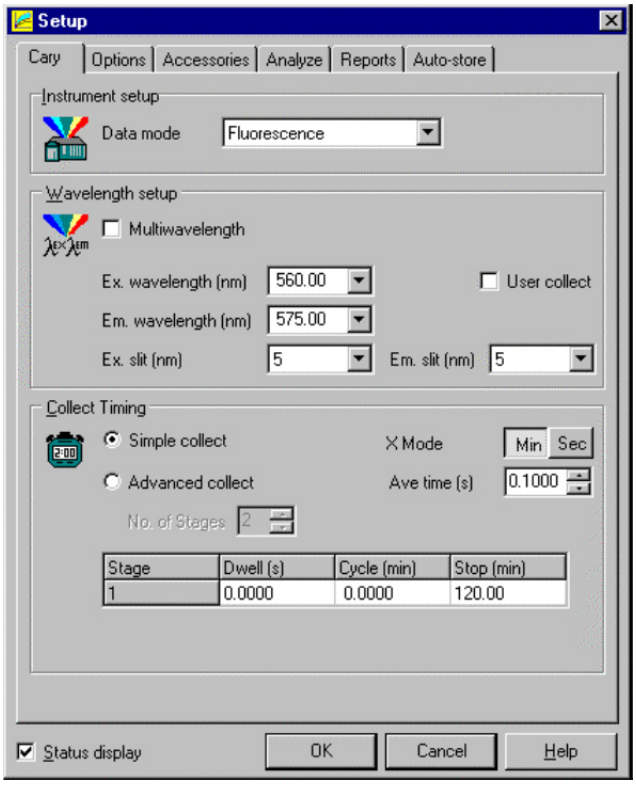

Figure 1. Operating parameters for ORAC assay

Reactions in each cuvette were started by the addition of 75 µl of a 50:50 (v/v) mixture of AAPH:PBS.

Results

Graphs of intensity vs. time for each of the three samples serum, trolox standard and blank were constructed using the Cary Eclipse software. These are shown in Figure 2.

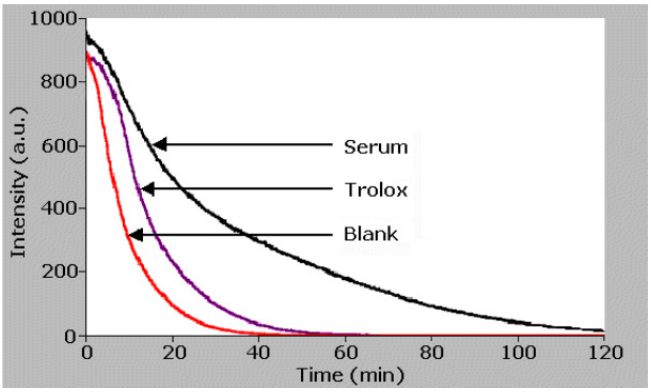

Figure 2. Fluorescence intensity vs. time for the free-radical attack of R-PE by AAPH (1) with protection by antioxidants (from human serum) or trolox and (2) without antioxidant protection (Blank)

Data analysis

The 'integrate' function was used to determine the total area under the curve (S) for each sample. Total antioxidant capacity, or ORAC values (μM) were calculated as follows:

ORAC value (μM) = 20*k* ($S_{Sample} - S_{Blank}$) / ($S_{Trolox} - S_{Blank}$)

Where *k* is the sample dilution factor.

Total areas under the curve (S) and ORAC values are given in Table 1.

Table 1. (S) and ORAC values for blank, trolox and serum samples. Derived from data in Figure 2

Sample	S (A.U.)	ORAC value (mM)
Blank	8485	n/a
Trolox	14008	1
Serum	31299	8.23

Discussion

Results demonstrated clear resolution between the three samples tested (Figure 2). Graphs for blank and serum describe exponential decay curves as the fluorescence of R-PE was progressively impaired by free radical attack from AAPH. The rate of decay of fluorescence (compared to blank) was slowed by the presence of serum and, to a lesser extent, trolox. This was probably because serum contains a battery of enzymes which act in an additive fashion to preserve one another[6]. This would explain the shoulder seen at the beginning of the trolox curve (Figure 2). It is possible that trolox molecules absorb free radicals up to a point (approximately 10 min) after which a single molecule of trolox becomes saturated with free radicals such that it cannot absorb any further free radicals or protect any other trolox molecules against attack. This begins to occur in other trolox molecules, leading to an exponential decrease in the capacity of the trolox sample to protect R-PE against free radical attack (the steep part of the curve).

The ORAC value for the human sample was calculated to be 8.23 mM. This value is considerably greater than that quoted by Cao and Prior[2] (3.1 mM). Possible explanations for this are:

The volunteers for the serum sample in the present study were 20-24 years whereas volunteers in Cao and Prior's study were 70 years on average.

Serum used in this study was not from a fasted volunteer, whereas volunteers from Cao and Prior's study fasted overnight.

Conclusion

The Agilent Cary Eclipse instrument with multicell accessory is suitable for carrying out the ORAC assay and calculating ORAC values. The assay demands precise temperature control and this is provided effectively in up to four samples (+/- 0.2 °C cell-cell temperature variation at 37 °C) using Peltier control and continuous, uniform magnetic stirring. Given the sensitivity and speed of the instrument, it would be of interest to increase the concentration of AAPH (and/or further dilute samples of sera) to increase reaction times and optimize throughput. Furthermore, the monochromator-based scanning facility of the Eclipse allows the methodology to be adapted to novel redox-sensitive probes whose spectra have not been characterized.

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