Labrox Application Note #011

Labrox® multimode plate reader:

Determination of antioxidant capacity with the Labrox reader using the Oxygen Radical Absorbance Capacity (ORAC) Assay

Susana Laakso

Introduction

Metabolism, stress, and environmental pollutants cause cells in the body to produce Reactive Oxygen Species (ROS) molecules. ROS can, themselves, be oxygen free radicals. They can also form free radicals through interaction with biological molecules (proteins, DNA/RNA, and lipids). ROS and free radicals are natural and necessary intermediates in the body. However, too high free radicals concentration lead to oxidative damage, thought to play a role in several diseases (cancer, cardiovascular, Alzheimer's, and Parkinson's) as well as in aging (1-5).

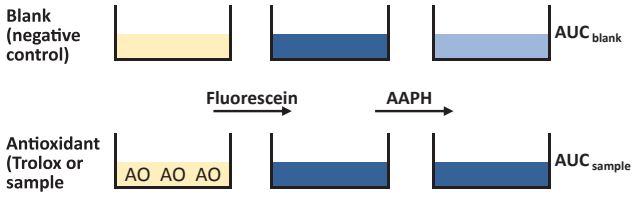
Oxygen free radicals and ROS are eliminated from the human body through enzymes (catalase, glutathione peroxidase, etc.). Their interaction with biological antioxidants (transferrin, ceruloplasmin, urate, etc.) also helps elimination. Nevertheless, these methods do not eliminate ALL these reactive intermediates from the body. The rate at which damage is caused is determined by how fast the reactive oxygen species are generated and then inactivated by endogenous defence agents called antioxidants. The rate at which damage is removed is dependent on the level of repair enzymes. (6).

Unfortunately, under the present day life-style conditions many people run an abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions. It is believed that the consumption of antioxidant rich foods is one way to help eliminate excess oxygen free radicals and ROS from the body: an inverse relation has been shown between the consumption of fruits and vegetables (antioxidant rich foods) and diseases (7-9). Drawbacks to these studies were determining the exact antioxidant capacity of the food as well as determining which food components (flavonoids, vitamins, etc.) act as antioxidants (10-12).

A method was needed that could measure the antioxidant capacity of a substance: the Oxygen Radical Absorbance Capacity (ORAC) Assay is such a method. This assay was first developed by Dr. Guohua Cao in 1993 (13) and was based upon the work of Glazer (14).

The ORAC Assay:

This assay measures the fluorescent signal from a probe (fluorescein) that decreases (is quenched) in the presence of an ROS generator: AAPH (2,2'-azo-bis-2-methylpropionamidine; dihydrochloride). The addition of an antioxidant antagonises the generated ROS, resulting in the maintenance of the fluorescent signal. The inhibition of oxidative damage to the probe can be correlated with the antioxidant capacity of the added free radical scavenger. Trolox® (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is a vitamin E analogue and a known antioxidant. It is used in the ORAC assay as a standard by which all unknown antioxidants are compared.



Integration: Net AUC (ORAC capacity) = AUC sample – AUC blank

Figure 1. ORAC assay principle

In this application note an ORAC assay is performed using the Fluorescence Intensity (FI) technology of a Labrox multimode reader.

Figure 2. Labrox plate reader with a dispenser.

Materials

- Fluorescein sodium
  (Fisher Scientific, cat no 10090160)
- Trolox® (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid)
  (Fisher Scientific, cat. no 218940010)
- AAPH (2,2'-azobis-2-methyl-propionamidine; dihydrochloride)
  (Fisher Scientific, cat. no 401560250)
- 5 different fruit beverages purchased from a local supermarket (all were of the same brand but some were "whole juice"- referred as juice from now on - and some were "fruit drinks", more diluted - referred as drink from now on)
- One red Port wine sample.
- Lonza AccuGENE™ Molecular Biology Water cat. BE51200
- PBS buffer (Lonza, cat. number BE17-516F)
- Pipettes and tips
- Black 96 wells plates
  (PerkinElmer, OptiPlate-F™ 96, 6005270)
- Microplate seal (Biotop, 4titude, prod. code 4ti-0510)
- Labrox multimode plate reader

Methods

1. A series of dilutions of Trolox from 200 to 12,5 µM was prepared (200; 100; 50, 25, 12,5) in phosphate buffer.

2. Series of dilutions of the 6 different fruit juices and the Port wine were also prepared. (1; 1:10; 1:100; 1:1000) in phosphate buffer.

3. 150 µl of a 10 nM Fluorescein Sodium solution were added to all the wells of the 96 wells plate. Then 25 µl of the standards, samples and PBS (blank) were placed into the plate.

4. The microplate was sealed and incubated for 30 min. at 37°C in the Labrox reader.

5. After incubation, FI measurements (ex. 485 nm; em. 520 nm) were performed every 90 secs to determine the background signal. Three measurements were taken.

6. After this, 25 µl of 240 mM AAPH were dispensed into all the wells using the Labrox reader dispenser and FI measurements were performed every 90 seconds up to 2 hours.

Results

The obtained results are shown in figures 3-5 and Table 1.

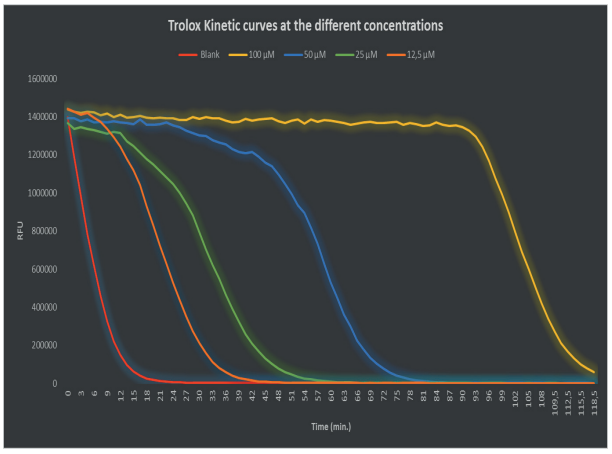


Figure 3. Kinetic curves for the different Trolox® concentrations recorded on the Labrox multimode reader.

The Trolox measurements showed the typical expected results.

AUC (antioxidant capacity) was determined for all Trolox concentrations as well as for the drinks. The following formula was used:

AUC = (R1/R1) + (R2/R1) + (R3/R1) + ...(Rn/R1), where R1 is the fluorescence reading at the beginning of the measurement and Rn the last measurement value.

Then the Net AUC was calculated:

Net AUC = AUC_{sample} – AUC_{blank}

With this data, a Trolox standard curve was obtained:

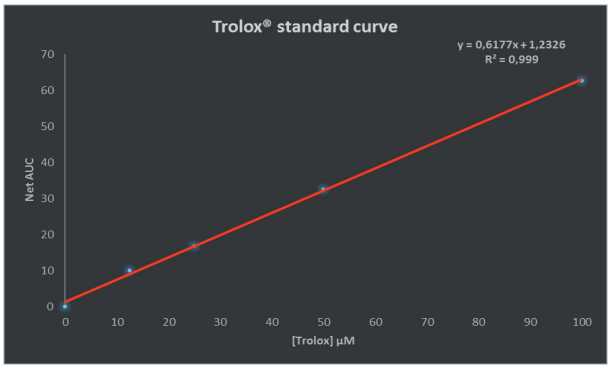


Figure 4. Trolox standard curve.

The Trolox standard curve can be interpolated for determination of the unknown antioxidant capacity of the analyzed drinks and reported as Trolox equivalents (TE). To determine TE of each sample range the ratio of the slope (m) of the linear regression analysis of the drink (Fig. 5) to the slope of the linear regression of Trolox (Fig. 4) was used: TE (range of concentrations) = m compound / m Trolox.

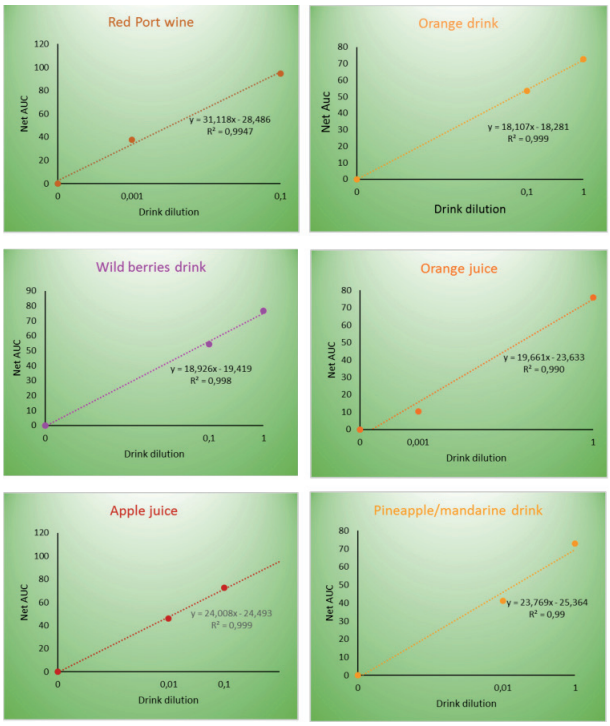


Figure 5. Antioxidant Dose Response Curves for the 6 tested drinks.

The calculated TEs (µmol/ml) are shown in Table 1.

Table 1. Antioxidant capacity of the 6 analyzed drinks.

Antioxidant Capacity	µmol TE/ml
Red Port wine5	0,38
wild berries drink	30,64
Orange drink	29,31
Orange juice	31,83
Apple juice3	8,87
pineapple/mandarine drink	38,48

Discussion

The obtained antioxidant capacities of the five fruit beverages were closer to each other (variation between 29,31 and 38,87) than we were expecting (Table 1). The fact that all the measured juices and drinks were manufactured by the same company could explain these results, being the beverages composition probably quite similar: as highly processed drinks, their composition is very likely made mainly of other substances than real fruit. As expected, the ORAC value for the orange juice (31,83) was higher than for the orange drink (29,31), which is more diluted. A higher value would be expected for the wild berries drink, knowing the berries high antioxidants concentration, but since it is a diluted beverage, that probably results in a lower than expected ORAC value. The obtained results clearly indicate that the concentration of real fruits used in these commercial, highly processed drinks is indeed very low.

The red Port wine showed (Table 1) a much higher antioxidant capacity than the juice drinks (30- 60 % more). These results are in total agreement with the expected, knowing the high levels of antioxidants that are present in red wines (15).

Conclusions

The presented results show that the ORAC assay can be easily and accurately performed with the Labrox multimode reader. In addition, the reader´s onboard dispenser and heating function allow the performance of all the assay`s steps in sequence using only the reader, with no need for any further equipment.

References

1. Ames, B.N., Sgigenaga, M.K and Hagen, T.M (1993). Oxidants, Antioxidants and the Degenerative Diseases of Aging. Proc. Natl. Acad. Sci. USA 90: 7915-7922.

2. Ames, B. N.; Gold, L. S.; Willet, W. C. The causes and prevention of cancer. Proc. Natl. Acad. Sci. U.S.A. 1995, 92, 5258-5265.

3. Christen, Y. Oxidative stress and Alzheimer's disease. Am. J. Clin. Nutr. 2000, 71, 621S-629S.

4. Diaz, M. N.; Frei, B.; Keaney, J. F., Jr. Antioxidants and atherosclerotic heart disease. New Engl. J. Med. 1997, 337, 408-416.

5. Lang, A. E.; Lozano, A. M. Parkinson's disease. First of two parts. N. Engl. J. Med. 1998, 339, 111-114.

6. Davies, K. J. A. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 2000, 50, 279-289.

7. Block, G.; Patterson, B.; Suber, A. Fruits, vegetables and cancer pervention: a review of the epidemiological evidence. Nutr. Cancer 1992, 18, 1-29.

8. World Cancer Research Fund, American Institute for Cancer Research. Food, Nutrition and the PreVention of Cancer: A Global PerspectiVe; American Institute for Cancer Research: Washington, DC, 1997.

9. Joshipura, K.J., Ascherio, A., Manson, J.E., Stampfer, M.J., Rimm, E.B., Speizer, F.E., Hennekens, C.H., Spiegelman, D., Willett, W. Fruit and vegetable intake in relation to risk of ischemic stroke. Journal of American Medical Association 1999,282, 1233–1239.

10. Cooper, D. A.; Eldridge A. L.; Peters, J. C. Dietary carotenoids and certain cancer, heart diseases, and age-related macular degeneration: a review of recent research. Nutr. ReV. 1999, 57, 210-214.

11. Emmert, D.H., Kirchner, J.T. The role of vitamin E in the prevention of heart disease. Archives of Family Medicine 1999, 8, 537–542.

12. Hercberg, S., Galan, P., Preziosi, P. Antioxidant vitamins and cardiovascular disease: Dr. Jekyll or Mr. Hyde? American Journal of Public Health 1999, 89, 289–291.

13. Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. Free Radical Biol. Med. 1993, 14, 303-311.

14. Glazer, A. N. Phycoerythrin Flurorescence-Based Assay for Reactive Oxygen Species. Methods Enzymol. 1990, 186, 161-168.

15. Diego Fernando Rocha-Parra, María Cecilia Lanari, María Clara Zamora, Jorge Chirife. Influence of storage conditions on phenolic compounds stability, antioxidant capacity and colour of freeze-dried encapsulated red wine In: Elsevier LWT- Food Science and Technology, July 2016.

Labrox

Rautakatu 5
FIN-20520 Turku, Finland
Tel. +358 (0)50 372 3080

sales@labrox.fi
Twitter: @LabroxCo
www.labrox.fi

AN#011 Ver. 02
11. 2016