APPLICATION NOTE

Determination of Antioxidant Capacity on the Thermo Scientific Varioskan LUX Multimode Reader

SP&A Application Laboratory,
Thermo Fisher Scientific, Vantaa, Finland

Keywords
oxygen radical absorbance capacity (ORAC), multimode reader, Varioskan™ LUX, dispenser

Goal

This application note describes a method to measure the ORAC of different food and beverage samples with a fluorometric assay using the Thermo Scientific™ Varioskan™ LUX multimode microplate reader. Microplate reader handling and data analysis was controlled by the Thermo Scientific™ SkanIt™ software. We show that with the Varioskan LUX reader, determination of the antioxidant capacity of samples is very fast, sensitive and accurate.

Figure 1: Thermo Scientific Varioskan LUX multimode microplate reader.

Introduction

Metabolism in living cells generates reactive oxygen species (ROS) as natural byproducts that have important roles in cell signaling and homeostasis. However, in excess levels, ROS may cause damage to cell structures and chronic inflammation called oxidative stress. Oxidative stress is shown to play a significant role in aging and many diseases, including cancer, stroke, heart disease and neurodegenerative diseases.

Antioxidants inhibit the oxidation of other molecules and thus prevent cell damage caused by free radicals. Therefore, antioxidants are believed to promote health and are widely used as dietary supplements. In addition, they are used as food preservatives, including natural antioxidants such as ascorbic acid (AA, E300) and synthetic antioxidants such as propyl gallate (PG, E310), to keep guard against deteriorations of food exposed to oxygen or light. Antioxidant preservatives are also added to fat-based cosmetics, such as lipstick and moisturizers, to prevent rancidity of unsaturated fats.

ORAC is considered to be the industry standard for expressing antioxidant strength of foods, beverages and food additives. The assay is based on the measurement of free radical damage to a fluorescent probe (fluorescein) that results in fluorescent signal loss over time.
The free radical damage is induced by ROS, generated

in the assay during thermal decomposition of 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH). The presence of antioxidants in the samples inhibits the free radical damage to the fluorescent compound, which is observed as the more stable fluorescence signal. Trolox, a water-soluble analogue of vitamin E, is widely used as a standard to which the investigated antioxidant compounds are compared. The antioxidant capacity of the investigated samples is typically expressed as Trolox equivalents (TE).[1]

This application note describes the use of the Varioskan LUX multimode microplate reader for determination of ORAC of different food samples with a fluorometric ORAC assay.

Materials and Methods

Instrument

- Thermo Scientific Varioskan LUX Multimode Microplate Reader

Microwell Plates

- Thermo Scientific™ Nunc™ F96 MicroWell™ Black Polystyrene Plate (Thermo Scientific 237108)

Pipettes

- Reagents and samples were pipetted with the Thermo Scientific™ E1-ClipTip™ Electronic Equalizer 8-channel, 15–1250 µl pipette (#4672100), which features adjustable tip spacing to transfer samples from tubes to plates[2]

Reagents

- Fluorescein sodium salt (Sigma-Aldrich F6377)
- 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich 238813)
- 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (Sigma-Aldrich 440914)
- Phosphate buffer (10 mM, pH 7.4)
- Food samples to be tested

Test Setup

Different dilutions for Trolox (200 µM–3.1 µM) and food samples were prepared in phosphate buffer, immediately prior to the measurement. The tested food samples were commercial orange and blueberry juices diluted 1:100 in the assay buffer. The following reactions were pipetted on a test plate, each in triplicate:

- 25 µl of phosphate buffer as blank
- 25 µl of Trolox standards
- 25 µl of appropriately diluted samples
- 150 µl of a 1µM Fluorescein solution was added to each well

The microplates containing fluorescein plus the respective blank, standard or food sample were incubated for 30 minutes at 37°C within the Varioskan LUX instrument before starting the measurement. Fluorescence (Ex. 485 nm, Em. 518 nm) was measured in a kinetic reaction every 30 seconds for 90 minutes. The first four cycles were taken to determine the background signal. Then the oxidative reaction was triggered by dispensing 25 µl of 50 mg/ml AAPH solution to the wells by an onboard dispenser of the Varioskan LUX (Figure 1). Dispensing speed was chosen as Medium High in order to efficiently mix the reagents. Adding reagent through the onboard reagent dispenser was practical, since the ROS-generator AAPH displayed immediate activity after injection and fluorescence decreased fast.

Data Analysis

The loss in fluorescence signal is expressed as the area under the curve (AUC) of the kinetic plot of each standard and of the samples. The AUC can be automatically calculated by the Thermo Scientific SkanIt software (Figure 2). When the AUC values are plotted against the Trolox concentrations, a standard curve is fitted and used to interpolate the antioxidant capacity of unknown samples.

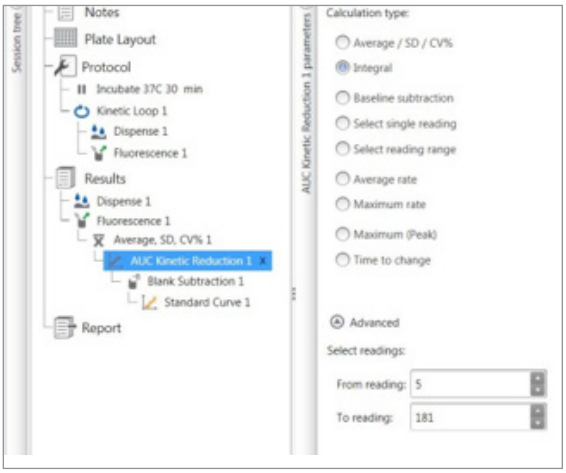

Figure 2: SkanIt software measurement protocol and calculation steps for determining the Trolox equivalents.

The calculated concentrations of unknowns, expressed as concentration of Trolox (mmol*L^{-1}), are called Trolox equivalents (TE). All these calculations could be completed directly in the SkanIt software.

Results and Discussion

Signal curves of the Trolox standards and food samples are shown in Figure 3. AAPH was dispensed to the reactions after four cycles, which lead to a loss in fluorescence signal inversely proportional to the antioxidant power of the sample. Calculated TE for the orange juice and blueberry juice were 125,5 and 51,8 μmol*L^{-1} respectively (Figure 4). It means that the antioxidant power of these beverages is equal to that of 125,5 μM or 51,8 μM Trolox.

The data demonstrate the ability of the Varioskan LUX multimode reader to perform the ORAC assay and measure antioxidant activity of samples. Sample replicates showed highly reproducible responses. Since the ORAC assay is very sensitive to slight temperature changes, it is important that the incubator of the Varioskan LUX controls the reaction temperature in the measurement chamber with superior uniformity. The onboard dispenser of the instrument in turn allows controlled addition of reagents to the wells. The built-in calculations of the SkanIt software provide versatile tools for analyzing kinetic data.

The ORAC method estimates the “total antioxidant activity” of a sample in vitro, and care must be taken when interpreting the results. The units and composition of samples being compared should be similar (e.g. fresh, dry or frozen wet weight of food) and typically applied quantity of the investigated product should be considered (spices vs. intact whole foods). Undiluted heterogeneous food or beverage samples often contain inhibitors that disturb the assay, therefore test of dilution series of such samples is always recommended.

Of note, beneficial physiological effects of foods with “high antioxidant activity” have recently been questioned, and publication of ORAC values for common foods have been withdrawn by The United States Department of Agriculture.[3] Also, use of ORAC values to promote food and dietary supplement products is discouraged by regulatory agencies.[4,5] Nevertheless, the ORAC assay is easy to perform, inexpensive and highly reproducible. Therefore it remains a valuable research method for evaluating total antioxidant activity of many different biological samples.[6]

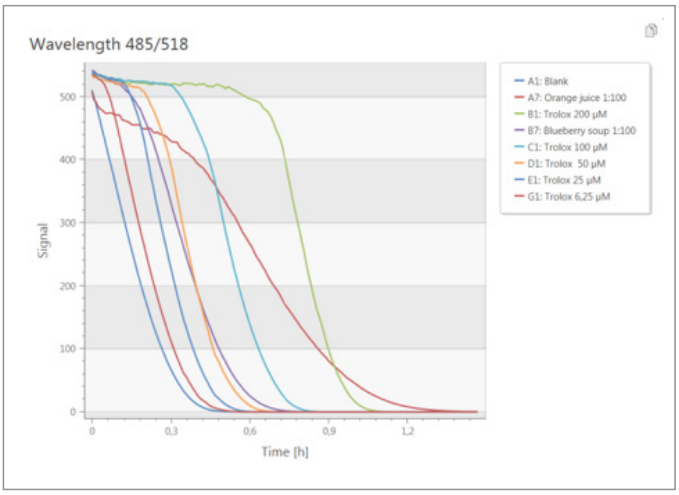

Figure 3: Signal curves (relative fluorescent units) of Trolox standards and beverage samples after adding the ROS-generator AAPH. Curves refer to average of triplicate samples.

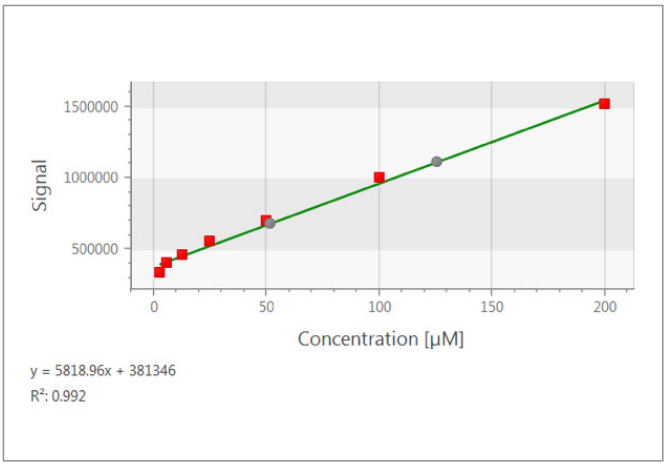

Figure 4: Standard curve, created in SkanIt software, shows a calculation of the Trolox equivalent of food samples. Red squares are Trolox standards, and grey circles are orange and blueberry juices. Signal is AUC calculated from triplicate fluorescence curves.

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Conclusions

By using the Varioskan LUX instrument, we were able to measure the total antioxidant capacities of different food samples with the popular ORAC method. The Varioskan LUX multimode reader in combination with the SkanIt software provides a range of benefits for this type of biochemical measurements, including:

- Simple protocol setup via the intuitive interface of the SkanIt software
- Onboard reagent dispenser for easy and accurate reagent addition for reaction triggering at any stage of the experiment
- Real-time kinetic curves shown during the measurement
- Easy kinetic data reduction, e.g. average of kinetic data and calculation of the net area under the curve (AUC) in the SkanIt software
- Automatic standard curve plotting and calculating of the concentration (TE) of unknown samples

Figure 5: E1-ClipTip™ Electronic Equalizer 8-channel pipette.

References

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