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The OxiSelect Cellular Antioxidant Assay (CAA) on the FLUOstar Omega

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- **Determination of leaf and oil extracts**
- **Plant flavonoid quercetin used as standard substance**
- **MARS Data Analysis software offers one-click feature for whole data processing**

Introduction

Reactive Oxygen Species (ROS) are generated in the body as part of the normal metabolism process when we eat or breathe. Accumulation of abnormal levels of ROS in the body has been implicated in several diseases including diabetes, renal ischemia, atherosclerosis, cancer and ageing in general.
There is a possibility that eating foods with high levels of antioxidants can substantially contribute to an individual's health and wellbeing. In order to lend support to this theory it is necessary to be able to quantify the antioxidant potential of foods.
To this end, a suite of assays have been developed in recent years for *in vitro* antioxidant analysis of foods. These include assays such as; TEAC, FRAP, TRAP, Folin, DPPH, CUPRAC and ORAC.[1] Although these assays are able to quantitate the amount of anti-oxidants in raw and processed foods they do not give any information on the bioavailability of antioxidants when ingested.
In order to partly address this limitation a Cellular Antioxidant Assay (CAA) utilising human hepatocar-cinoma (HEPG2) cells was first developed by Wolfe and Liu.[2] Recently the assay has also been adapted for many different cell lines.[3] In addition, a Cellular Antioxidant Assay kit (OxiSelect) is now commercially available. This application note shows results obtained with the OxiSelect kit for an anonymous plant essential oil and leaf extract.

Assay Principle

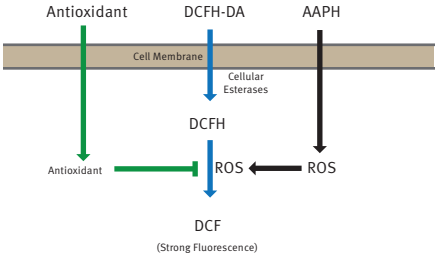

Fig. 1: Principle of the OxiSelect Cellular Antioxidant Assay

This assay relies on the ability of live cells to allow the non-fluorescent esterified dye precursor 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) to diffuse across the cell membrane. Once inside the cell, the dye is de-esterified to 2', 7'-Dichlorodihydro-fluorescein (DCFH) by cellular esterases and remains trapped inside. The cells are washed and upon addition of the a radical initiator (AAPH) the non-fluorescent dye (DCFH) is transformed to the highly fluorescent 2', 7'-Dichlorofluorescein (DCF) (Fig. 1).

When the dye-containing cells are also incubated with an antioxidant, the free radical induced reaction leading to DCF can be prevented to a greater or lesser extent. In order to quantify the ability of an antioxidant to prevent the free radical induced reaction in the live cells, a standard curve of fluorescence vs time with varying amounts of a standard (quercetin, a strong antioxidant) is firstly constructed and the ability of various food extracts to inhibit the free radical induced reaction are compared to this standard.

Materials & Methods

- OxiSelect™ Cellular Antioxidant Activity Assay Kit (#STA-349) from Cell Biolabs, Inc.
- FLUOstar Omega multidetection microplate reader from BMG LABTECH

The kit contains a 96-well tissue culture treated clear bottom black microplate, DCFH-DA dye, the free radical initiator (AAPH) as well as quercetin standard.

Quercetin standard curve
Preparation of quercetin standards, 2.8% radical initiator solution, and 2x dilution of the DCFH-DA stock solution was performed as described in the assay manual.

Sample Preparation
Essential oil stock concentration was 20 µl/ml of cell culture medium, from which four dilutions were prepared: 10 µl/ml, 5 µl/ml, 2.5 µl/ml, and 1.25 µl/ml. Leaf extract stock was made at 20 mg/ml cell culture medium from which 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml solutions were prepared.

HepG2 cells at a concentration of 6 x 10^4 cells in 100 µl growth media per well were incubated for 24 hours until cells were 90% to 100% confluent. The outer wells of the microplate were left empty. After seeding, the media was removed, and cells washed gently 3 times with PBS/HBSS. All wells were then treated with 2x diluted DCFH-DA probe solution (50 µl) and with either quercetin standards or prepared samples (50 µl). The microplate was incubated for 60 minutes at 37°C. After this the liquid was removed and cells washed 3 times with PBS/HBSS, then the last wash removed and discarded. Lastly, 100 µl of free radical initiator solution was injected with the on-board injectors to all wells and the plate was read on the FLUOstar Omega at 37°C for 1 hour, collecting data every 1-5 minutes.

Controls did not contain standard or sample. Blanks consisted only of cells without addition of radical initiator.

FLUOstar Omega instrument settings

Measurement method	Fluorescence intensity
Reading mode	Plate mode, top reading
Positioning delay	0.5 s
Filters	Ex 485nm, Em 520nm
No. of flashes per well	20
No. of Cycles	12
Cycle time	300 s
Temperature	37°C

Results & Discussion

In control wells, containing only cells with dye precursor, addition of AAPH leads to a strong increase in fluorescence over time (Fig. 2).

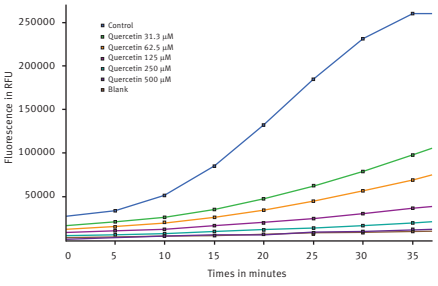

Fig. 2: Signals of controls, standards and blanks over time

The blanks, that do not contain the radical initiator, show only a very small fluorescence increase over time, caused by ROS already present in the cells. The quercetin standard prevents the dye to be oxidized. The degree of prevention depends on the antioxidant's concentration and on its ability to cross the cell membrane and survive metabolism by the many enzymes and degradation processes in the live cell.

From the signal curves the area under the curve (AUC) is determined using the 'SUM' function in the MARS Data Analysis software. The AUC for blanks was then subtracted from all wells. CAA values were calculated by the software based on the equation:

CAA = 100 - (AUC (sample) /AUC (Control) *100).

The CAA values of the quercetin standards were used to create a 4-parameter standard curve (Fig. 3).

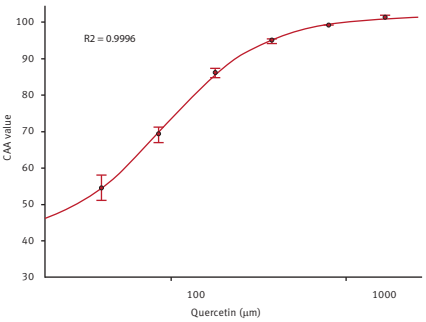

Fig. 3: CAA values dependent on quercetin concentration

To determine the QE value of a sample, a concentration of antioxidant is chosen that falls within the concentration limits of the quercetin standard curve. The dilution factor is considered by the software. The resulting values are in µmoles of quercetin (quercetin equivalents, QE) per ml for the oil and quercetin equivalents per g for the leaf extract.

A more rigorous calculation method is to find the EC_{50} value for each antioxidant used on the plate and these compared. The method is described in detail by Wolfe.[2] The QE value determined by this method takes into account all concentrations that were measured, simultaneously. Comparison of QE values obtained with both methods is shown in Table 1. From the results it can be seen that the leaf extract has 2x more antioxidant potential than the essential oil from the same plant.

Table 1: Quercetin equivalents (QE) from essential oil and leaf extract samples

QE value	4-parameter fit	Median effect plot
Essential oil	23.2 µmoles QE/ml	19.0 µmoles QE/ml
Leaf extract	40.7 µmoles QE/g	38.1 µmoles QE/g

Conclusion

Together with the MARS Data Analysis software, the FLUOstar Omega microplate reader can easily be used to measure cellular antioxidant potential of foods or food extracts via the OxiSelect Cellular Antioxidant Assay.

References

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3. Blasa M., Angelino D., Gennari L., Ninfali P. (2011). *Food Chemistry*, **125**, 685-691.