The CAP-e assay: Testing antioxidant bioavailability at the cellular level, and creating a foundation for further biological testing of natural products.

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Objective

To present recent advances using the CAP-e cell-based antioxidant protection assay, with regards to its role in natural products testing, its application as a QC tool, and the importance of controlling for inter-assay variability between batches of cells used for the assay.

Summary

We have developed a novel accelerated method for evaluation of cell-based antioxidant protection using erythrocytes, the CAP-e assay [1, 2]. The CAP-e assay was designed to assist the natural products industry when moving from analytical chemistry testing towards more complex biological testing. The method uses assay principles comparable to the Oxygen Radical Absorbance Capacity (ORAC) assay, applies natural products to intact erythrocytes, and specifically measures antioxidants capable of penetrating into and protecting the cells from free radical damage. The assay can be applied to testing of antioxidant availability to live cells *in vitro* [2] and documenting antioxidant uptake *in vivo* [3]. During the past year, we have made progress in several areas, particularly with respect to quality control of the cells used for this assay, including controlling for inter-assay variability.

CAP-e testing of foods–solids versus liquids

Data on antioxidant activity in foods can be presented in the scientific way, per gram, or in the more practical manner, per serving. The graph to the left in Figure 1 below shows the scientific way of looking at the data, which is per gram. When we compare 1 gram of blueberries to 1 gram of oregano, oregano had more antioxidants available to live cells. Oregano as a nutritional supplement would provide a concentrated source of antioxidants available to cells. However, this does not translate in proportion to where we are most likely to get antioxidants from in our diet.

A portion of fresh berries is 1 cup, which is approximately 120 grams. Therefore, a serving of fresh berries would provide approximately 42 CAP-e units. A cup of hot chocolate usually contains 1 tablespoon of cocoa (5 grams), thus a cup of cocoa would equate to 20 CAP-e units. The amount of dried oregano on 1 small pizza could reasonably be 1/4 teaspoon, equivalent to 0.2 gram. Therefore, the oregano on the pizza would contribute 2 CAP-e units.

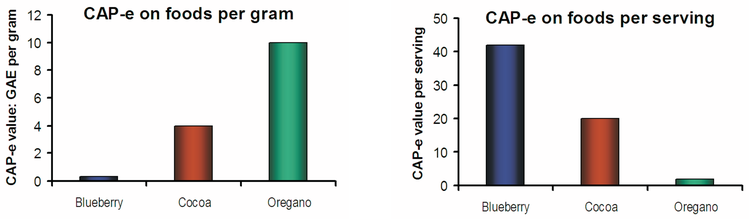


Figure 1. CAP-e data on selected common off-the-shelf foods per weight (left graph) and per serving (right graph). When performing the CAP-e assay on solid products, the calculation of the antioxidant protection capacity, the CAP-e value, is generated as the proportional difference between the IC50** dose of product versus the dose of the standard antioxidant Gallic Acid. Since both product and GA IC50 is measured in grams/Liter, the resulting number must be reported in GAE (Gallic Acid Equivalent).*

CAP-e antioxidant protection units are calculated differently for dry powders and fresh foods, versus liquids. When testing a solid food or a dry powder, the data are reported in GAE (Gallic Acid Equivalents). When testing liquids, the data are reported in Mol GA/liter. There is no simple direct conversion factor from juices to solids – it depends on how thick a juice is, how much pulp it has, whether extracts were added, how those extracts were made, etc.

If the CAP-e value of a liquid product needs to be compared to the CAP-e value of a solid product, several things would have to be taken into account. For example, if liquid green tea is tested in the CAP-e assay, one would need to take into account how many grams dried tea leaves went into making the green tea, and how much water was used. The green tea liquid itself represents a hot water extract of a certain amount of dry leaves into a certain amount of liquid. If the green tea liquid was subsequently freeze-dried, a powder of the hot water extract would be produced. The CAP-e assay on the liquid can either be relative to weight of the starting material that went into producing the tea, or it can be relative to the amount of dissolved material in the extract.

This is similar to the ORAC. This may go unnoticed by many people, and lead to confusion when comparing different products. ORAC on powders is reported in Trolox Equivalents per gram product. Liquids are reported in Mol Trolox/L. There is no simple and direct conversion factor from liquids to solids.

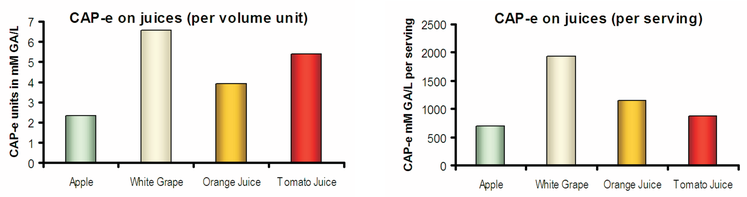


Figure 2. CAP-e data on selected generic brand-name juices per volume (left graph) and per serving (right graph). When performing the CAP-e assay on liquid products, the calculation of the antioxidant protection capacity, the CAP-e value, is generated as the proportional difference between the IC50 dose of product versus the dose of the standard antioxidant Gallic Acid. Since product IC50 is measured in Liter/Liter, and GA IC50 is measured in Mol/Liter, the resulting number must be reported in Mol GA/Liter product.*

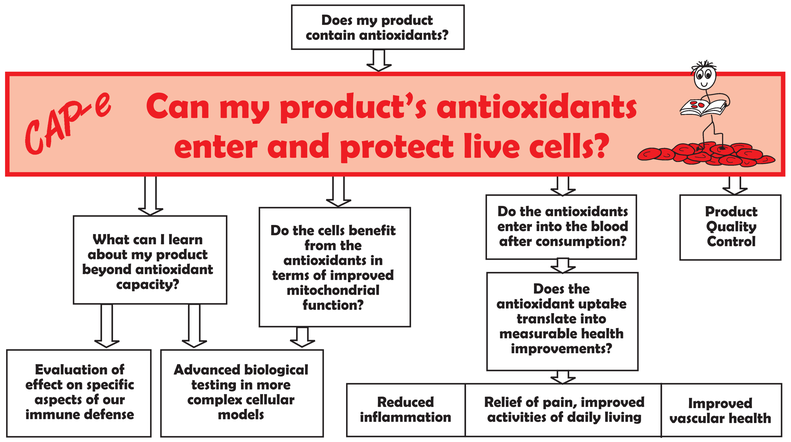
**Serving sizes are as recommended by the USDA.*
***IC50: Concentration of compound leading to a 50% inhibition.*

The advantages of a sequential testing strategy for complex natural products

The CAP-e cell-based antioxidant protection assay serves as a bridge when moving from analytical to biological testing of natural products [2]. The antioxidant capacity is only one limited aspect of most natural products. For example, juices can be made from fruits and berries, but the complex biological activities differ from fruit to fruit, independent of their antioxidant capacity.

The CAP-e assay generates data on antioxidant uptake at the cellular level. The CAP-e data are necessary for making proper interpretations of data from more complex biological assays. Many natural products will induce formation of reactive oxygen species (ROS) in more complex cell-based assays. Such assays are necessary for documenting other biological effects of a product, but cannot be used to document antioxidant capacity in a biological system.

On the other hand, many natural products possess potent anti-inflammatory properties, even at doses where the antioxidant capacity is undetectable. An example is the Amazonian Palm Berry Açai. Freeze-dried Açai has a very high antioxidant capacity in the ORAC assay [2], and performs well in the CAP-e assay. Açai also has potent anti-inflammatory effects, which are not detectable at doses where antioxidant capacity is best demonstrated. The anti-inflammatory property is clearly independent of the antioxidant content of the product (see also Figure 4).



Creating a foundation for understanding complex mechanisms of action

Applications of the CAP-e assay

The CAP-e assay has three distinct uses:

1) Testing of natural products for antioxidants available to enter and protect live cells;

2) Testing of serum samples obtained from human subjects before and after consuming antioxidant-rich products (see our parallel poster presentation: *"Pain reduction and improvement of range of motion by consumption of MonaVie Active™, a fruit-and berry-blend."* by Jensen et al.);

3) Testing of red blood cells obtained from human subjects before and after consuming antioxidant-rich products.

References

1. Jensen GS. Cell-based antioxidant protection assay. US patent application No. 60/985,166.
2. Honzel D, Carter SG, Redman KA, Schauss AG, Endres JR, Jensen GS. Comparison of Chemical and Cell-Based Antioxidant Methods for Evaluation of Foods and Natural Products: Generating Multifaceted Data by Parallel Testing Using Erythrocytes and Polymorphonuclear Cells. J Agric Food Chem. 2008 Sep 24;56(18):8319-25.
3. Jensen GS, Wu X, Patterson KM, Barnes J, Carter SG, Scherwitz L, Beaman R, Endres JR, Schauss AG. In Vitro and in Vivo Antioxidant and Anti-inflammatory Capacities of an Antioxidant-Rich Fruit and Berry Juice Blend. Results of a Pilot and Randomized, Double-Blinded, Placebo-Controlled, Cross-over Study. J Agric Food Chem. 2008 Sep 24;56(18):8326-33.

Quality of cells, inter-assay quality control

Consistency in the CAP-e test can be obtained when the source of cells is tightly controlled. When erythrocytes are removed from the blood they contain varying amounts of redox enzymes and antioxidants obtained from the donor's diet. Even within the same donor, day-to-day variations exist as a result of diet and inflammatory conditions. In female donors, the menstrual cycle will affect the antioxidant capacity of freshly isolated erythrocytes. This makes fresh erythrocytes a variable cell source for the testing of antioxidants in foods, beverages, and natural products/extracts.

We have shown that when erythrocytes are allowed to incubate at 4˚C for several weeks, the innate antioxidant capacity gets depleted, and variations between different samples are reduced. By comparing over 60 experiments performed with erythrocytes that were either fresh or had aged for various times, we have shown that using erythrocytes aged for 4-7 weeks generate highly consistent data in the standard CAP-e assay, when testing natural products, extracts, foods, and beverages in vitro.

Quality control (QC) of the source of cells used for each CAP-e assay is an important factor for reducing inter-assay variability and standardizing the CAP-e assay. As part of our erythrocyte ageing study, we monitored the ageing by a) microscopy, b) optical density (OD) measurements, c) expression of phosphadityl serine on the cell surface, and d) performance in the CAP-e assay. We have established the OD reading as a standard pre-assay QC measure in our lab. As a post-assay QC measure, the IC50 of the standard GA must be within a specific range, as determined by 60 independent experiments.

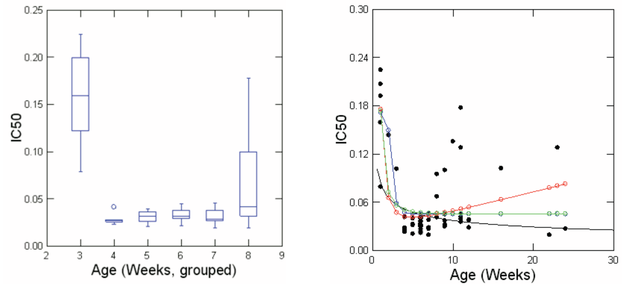
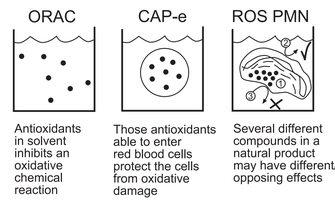


Figure 3. Regression of Gallic Acid IC50 against RBC age in weeks. The graph to the left shows a box plot of the RBC groups according to age in weeks, and the IC50 for Gallic Acid is shown. The inter-sample variability of fresh cells disappears after 4 weeks of ageing in vitro. The cells age 4-7 weeks behave similar in the CAP-e assay, and a narrow range of Gallic Acid IC50 values can be obtained. A curve fitting program (CurveExpert 1.3) was used to fit the IC50 data to RBC age. Several different models gave comparable fits (R=0.70 – 0.72). Four models are shown in the diagram to the right. [Black: Power fit to data. Blue: Weibull model (y=a-be-c x Aged; R=0.72). Red: "Heat capacity" model (y=a+b x Age+c/Age; R=0.72). Green: Logistic model (y=a/(1+b x exp(-c x Age)); R=0.697).] All four models show that cells aged 4-7 weeks provide most consistent data on the standard antioxidant compound Gallic Acid. This analysis is the basis for the implication of cellular ageing and post-assay QC monitoring of GA IC50.

Source of cells

In the CAP-e assay, erythrocytes (red blood cells) are chosen as a model because they represent a more inert type of cell in our body. Erythrocytes do not engage in complex inflammatory reactions and are not capable of producing reactive oxygen species (ROS) as a result of stress or death. Furthermore, unlike all other cells, erythrocytes do not contain mitochondria. Mitochondria engage in production of ROS. Testing the antioxidant capacity in an erythrocyte model eliminates confounding factors that in other cellular models makes data interpretation difficult. It assists in creating a baseline of information, based upon which data from more complex cellular assays can be interpreted [2].



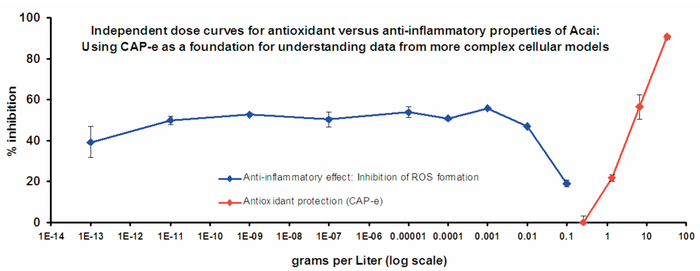


Figure 4. Freeze-dried Açai is used as an example to illustrate a natural product where the anti-inflammatory properties are clearly independent of the antioxidant capacity. The CAP-e assay and the Reactive Oxygen Species formation by Polymorphonuclear cells (ROS PMN) assay were used for this comparison. At the doses where we can measure that the antioxidants in Açai are able to enter and protect live cells from oxidative damage, we cannot measure an anti-inflammatory effect. However, at lower doses, the oxidative damage caused by ROS formation is inhibited; the data would not be conclusive without a parallel CAP-e assay. The dose comparison to the CAP-e assay clearly allows the conclusion to be made that the reduction in oxidative damage is caused by mechanisms other than a direct antioxidant effect, and provides a foundation for appreciating the anti-inflammatory capacity of freeze-dried Açai.