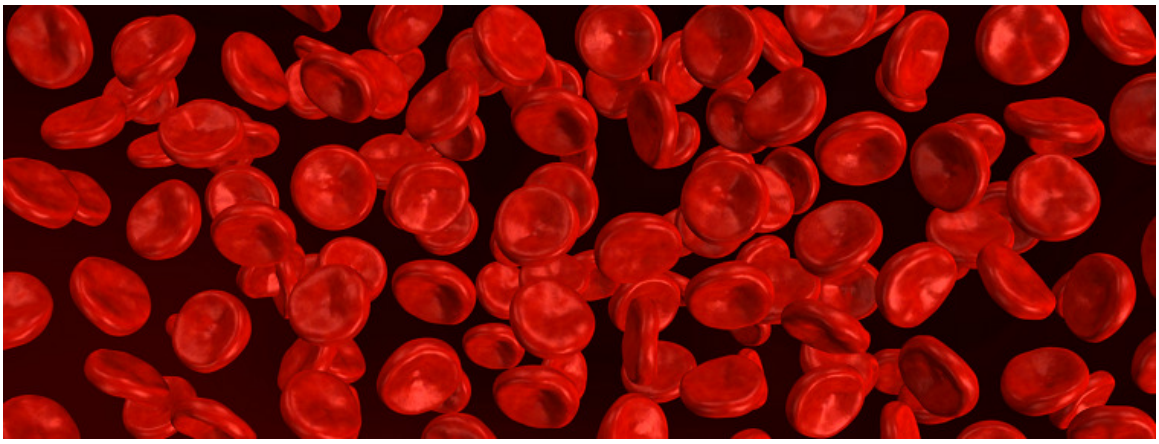
CAP-e

A white paper on the Cellular Antioxidant Protection in erythrocytes (CAP-e) assay and its use in the natural products industry



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Definitions

Free radical:

An atom or group of atoms with at least one unpaired electron. Unpaired electrons are highly reactive and can damage living cells.

Antioxidant:

A compound capable of donating an electron and thus neutralizing the electrical charge that may otherwise lead to cascading cellular damage.

Overview

Antioxidants continue to be a topic of growing interest in the nutraceutical industry. In order to understand the increasing need for documenting antioxidant capacity in nutritional products, it is important to put this in context of what antioxidants are.

Oxygen is essential for our life. As such, oxygen is used in our bodies in chemical reactions that lead to energy production. Some of these reactions produce harmful free radicals.

Free radicals are generated during normal metabolism, as well as during immune defense reactions. They are also produced by environmental factors including pollution, smoke, and sunlight. These harmful, electrically charged ions can potentially damage cells and tissues.

Antioxidants present in blood, cells, and tissue fluids play an important role in neutralizing oxidative damage caused by free radicals. These antioxidants can come from food, supplements, or can be produced by the body itself.

When chronic inflammatory conditions are combined with a lack of sufficient dietary antioxidants, oxidative stress is accelerated, and contributes to degenerative diseases and aging. Such chronic inflammatory conditions have been associated with obesity, immune dysfunction, cardiovascular disease, declining cognitive function, and cancer.

Testing for antioxidants in food and natural products is increasingly used as a way to document the potential benefits of consuming such foods.

This white paper describes the role of the CAP-e cellular antioxidant protection assay, and its role in the natural products industry.

CAP-e – a biological model

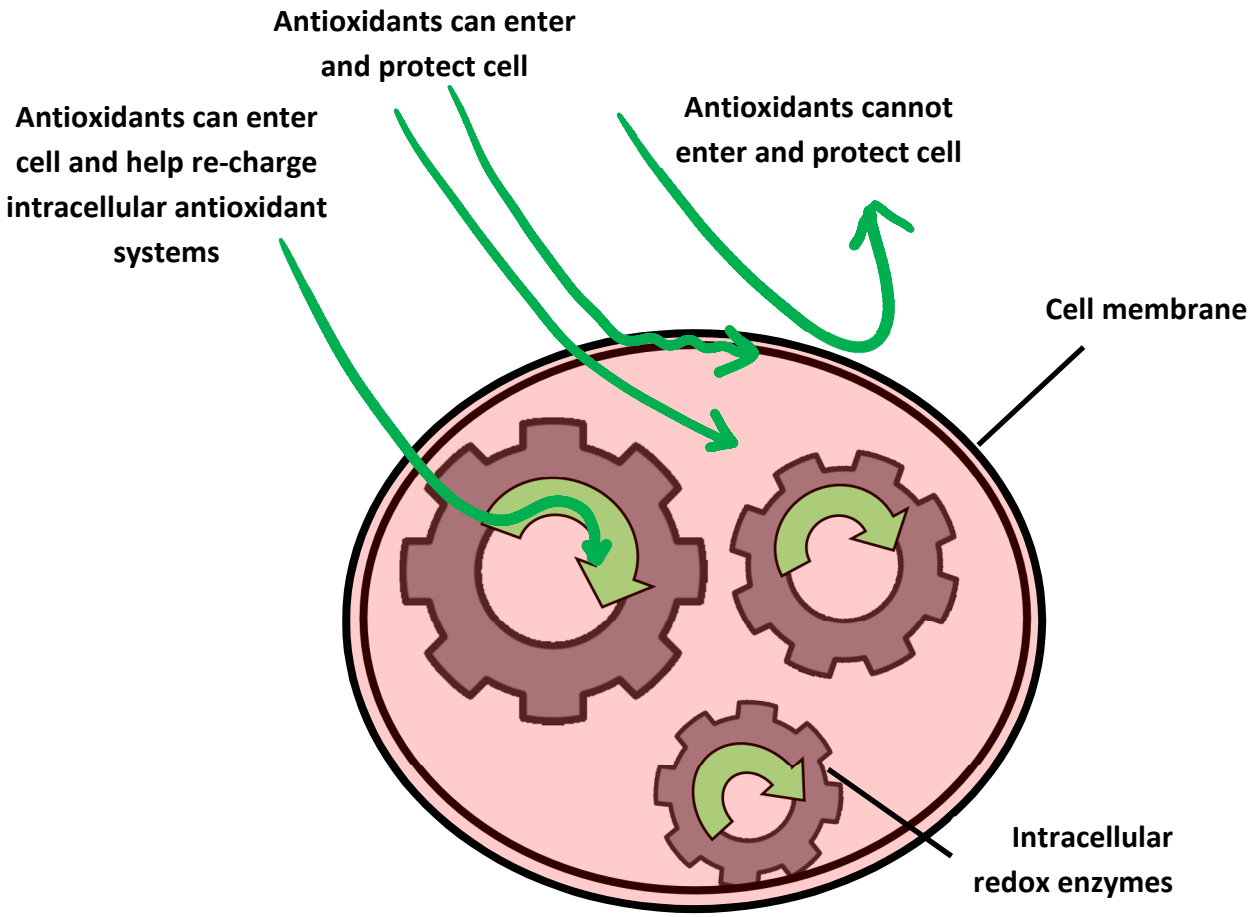
The invention and development of the CAP-e cellular antioxidant protection assay arose as an opportunity to address the question of whether compounds can function as protective antioxidants in biological systems. The validation, through statistical analysis, of methods to reduce inter-assay variation has been a focus of ongoing work at NIS Labs.

An accelerated microplate-based assay was developed during 2007-2008 [1] [2]. A provisional patent application for the protocol used for the CAP-e assay was filed in October 2008, and has since been converted to a full application. The CAP-e assay has been useful for evaluation of polyphenol-rich natural products and extracts, in vitro [3] and in clinical studies [4].

The CAP-e assay was used, in collaboration with researchers at the USDA, to evaluate 7 isolated flavonoid compounds from Acai [5]. The CAP-e assay was also used by the team of Dr. Wu at the USDA to document the much higher antioxidant protection in a lesser known variant of Acai (*Euterpe precatoria)* [6].

Why CAP-e

The CAP-e bioassay detects evidence of biologically relevant antioxidant protection in situations where antioxidant compounds in a test product are able to enter into a cell. The cellular antioxidant absorption can involve the layers of the cell membrane, as well as the interior of the cell. It may also involve the capacity of antioxidants to help replenish already existing intracellular antioxidants after they have engaged in redox reactions to quench free radicals.



The “e” in CAP-e

The ‘e’ in CAP-e stands for “erythrocytes” (red blood cells). These cells are highly specialized in delivering oxygen to peripheral tissue, where oxygen is exchanged for carbon dioxide, which gets transported to the lungs and exhaled.

Produced from stem cells in the bone marrow, the final stages of red blood cell maturation before entering the blood circulation involve packing of the cytoplasm with:

- hemoglobin for transport of oxygen and carbon dioxide
- redox enzymes, involved in the oxidant/anti-oxidant balance.

In humans and other mammals, but not in birds or reptiles, the final maturation of red blood cells in the bone marrow also involves expelling the nucleus and organelles, such as mitochondria [7] [8]. Despite the lack of mitochondria in the mature mammalian red blood cell, the cells produce energy [9] and interact with the vascular environment.

Mature red blood cells survive in the blood circulation for approximately 120 days, after which time they undergo erythroptosis and are cleared in the spleen [10].

All other cell types are more complex, and they can produce free radicals as part of the following processes:

- mitochondrial function,
- during cellular signaling,
- immune defense,
- programmed cell death (apoptosis),

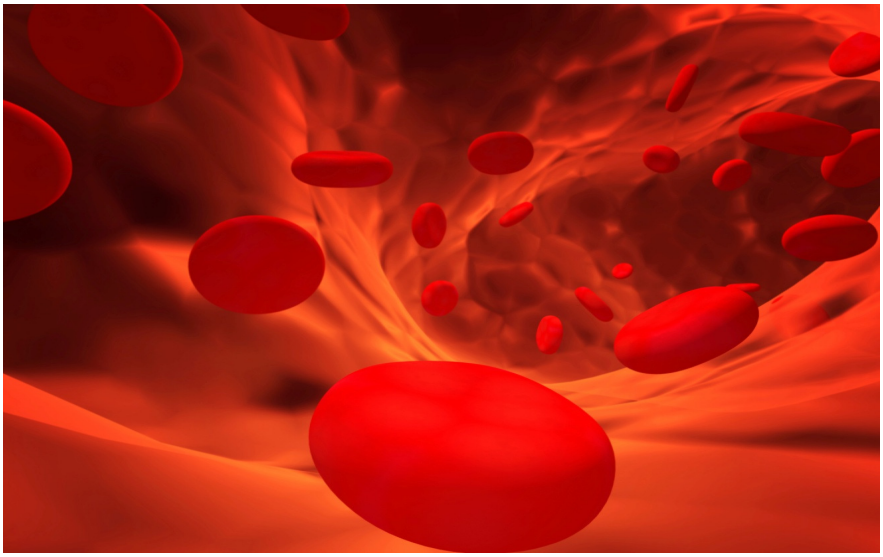
This complexity makes specific data interpretation regarding antioxidant uptake inconclusive [1].

The importance of red blood cells for antioxidant protection

The choice of red blood cell as a model for evaluation of intracellular antioxidant protection is important [11].

The role of red blood cells in the body goes above and beyond the oxygen-transporting function. During their life in the blood circulation the cells provide antioxidant protection for white blood cells. They help inhibit inflammation by de-activating white blood cells by preventing endothelial adhesion [12].

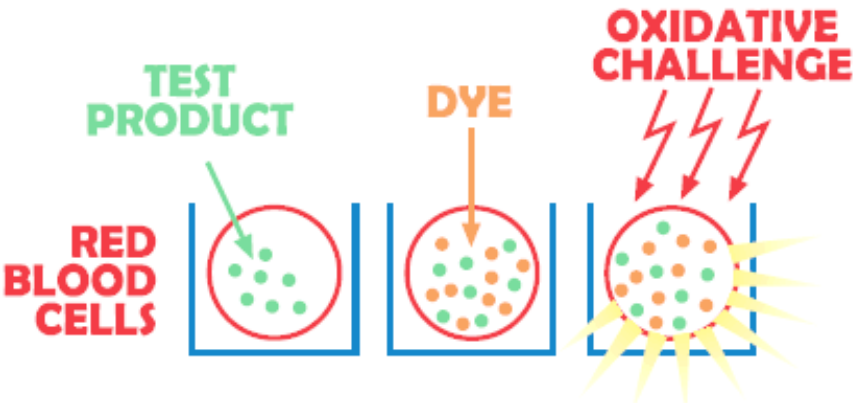
Healthy red blood cells scavenge reactive oxygen and nitrogen species. This is a direct antioxidant and anti-inflammatory protection of the body. In contrast, red blood cells that have been damaged by oxidative stress and inflammation are more prone to clump and adhere to the vessel walls, thus contributing to poor circulation and vascular damage [13] [14].



Many types of laboratory methods involve red blood cells to evaluate oxidative damage as a result of inflammation, and to evaluate antioxidant protection by natural products [15] [16] [17] [18] [19] [20] [21].

In the CAP-e bioassay for the evaluation of cell-based antioxidant protection using erythrocytes [5] [6], the oxidative challenge is performed under gentler conditions than in those methods relying on RBC lysis (or rupture) as a measurement of damage [22] [23] [24].

In addition, the CAP-e method specifically measures antioxidants capable of absorption into and protecting the cells from free radical damage.



The CAP-e value

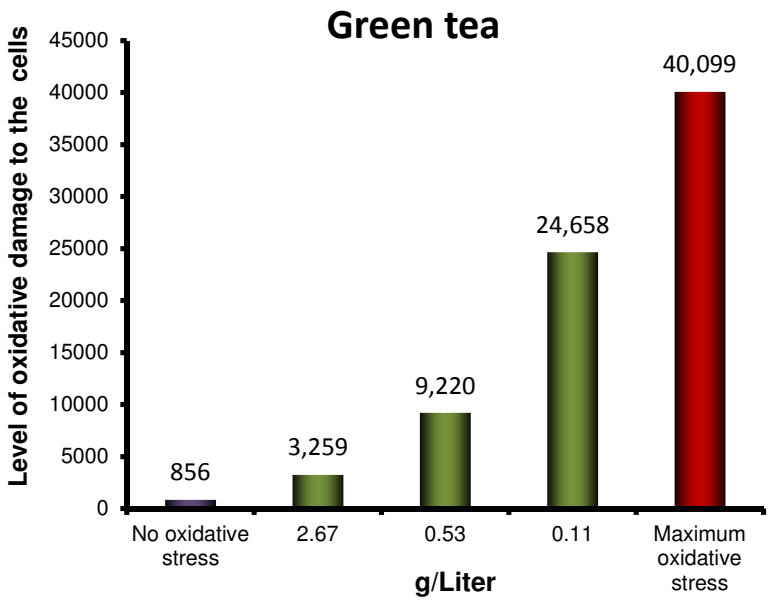
The CAP-e value is a relative measurement, in Gallic Acid equivalent units, of the ability of a product's antioxidants to prevent oxidation within a living cell.

This measurement provides a strong indication of how much antioxidant protection a particular compound provides to the cell.

CAP-e values

When testing products in the CAP-e assay, some cells are left untreated and not exposed to oxidative stress. These cells serve as a reference point (or negative control) for healthy cells. Other cells are exposed to oxidative damage, without any antioxidant protection; these cells serve as a reference point (or positive control) for maximum oxidative damage.

Test products are added to cells at various doses and exposed to oxidative damage to evaluate at what doses a protective effect may be documented. Oxidative damage is recorded by a reporter dye that becomes fluorescent when damaged by free radicals.



The dose of test product that achieves 50% inhibition of oxidative damage is compared to the reference standard compound, Gallic Acid. Thus the CAP-e value, in Gallic Acid Equivalent (GAE) units, is a relative measurement of the ability of a product's antioxidants to prevent oxidation within the cell and show cellular bioavailability in vitro.

Chemical versus biological assays

Testing of purified antioxidant compounds, isolated from the food matrix, can help shed light on which chemical structures can enter and protect live cells, and which ones cannot [5]. Some isolated compounds have shown very high CAP-e values, and likely contribute to a portion of the antioxidant protection in foods, supplements, and extracts that contain these compounds. Other purified compounds may show high antioxidant values in chemical assays, but may not have good bioavailability to enter and protect cells.

A sequential testing strategy often involves initial testing of chemical antioxidant capacity, followed by one or more biological (functional) tests, such as the CAP-e cellular assay.

The antioxidant compounds responsible for giving a high value in a chemical assay may or may not be readily available to enter into and protect live cells from oxidative damage. The chemical and biological antioxidant values are not always related.

We specifically developed the CAP-e assay to help bridge the transition from chemical antioxidant assays (such as ORAC and total phenolics testing) to biological testing in more complex systems.

Comparing ORAC and CAP-e values

Work done by scientists at the USDA on isolated flavonoid compounds from açai showed that purified antioxidant compounds with high ORAC scores could have either extremely high CAP-e values or no CAP-e value at all. This data suggested that flavonoids with simpler chemical structures are better able to enter living cells and provide protection [5].

This has been confirmed by researchers at the Swedish Agricultural University using mass spectrometry on red blood cells from CAP-e assays on specific polyphenols [25].

As an example, as part of screening and choosing ingredients for a new blend of natural products, two different grape seed extracts were compared. The ORAC and CAP-e values are listed below in Table 1. It can be seen that Extract 1 had a lower ORAC value than Extract 2, but was almost 20 times more capable at protecting cells from oxidative damage. This could be related to the content of catechins.

Comparison of ORAC and CAP-e values of two different nutritional products.

	Extract 1	*Extract 2*
Total Catechins	*9.7%*	*Not detected*
$ORAC_{peroxyl}$ µM TE/gram	*652*	*10,233*
CAP-e GAE/gram	*960*	*52*

Teas and extracts

Camellia sinensis is the plant from which many common and specialty teas are produced, including black, green, white, and pu'erh teas.

Type of Tea	
white	unopened leaf buds
green	mature leaves
black	oxidized leaves
pu'erh	microbially fermented

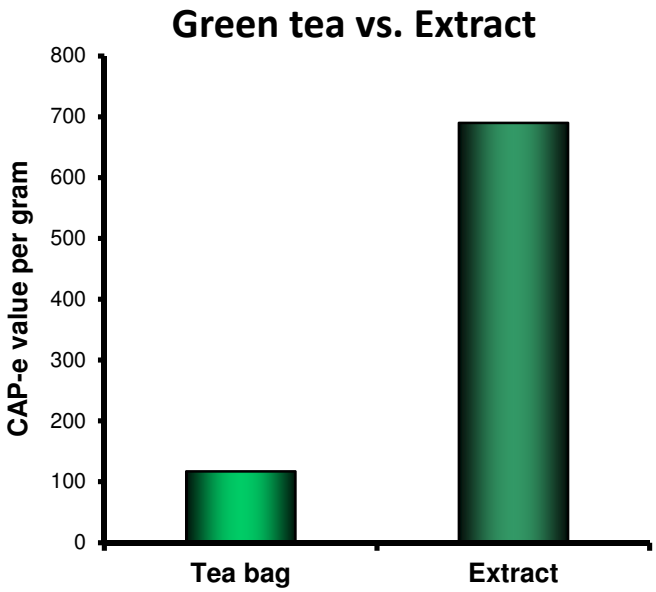
Tea is widely consumed as a beverage. In addition to this traditional use, there is also an expanding interest in the use of high-potency extracts of the different teas, allowing intake of higher levels of tea polyphenols and other bioactive compounds.

Tea is basically a hot water extraction process. When the hot water extract is consumed as a liquid, a typical dose may be to use a single tea bag of 2 grams powder into a cup of hot water. The insoluble compounds, resins, fibers and other plant parts are left behind in the tea bag or other filtering device.

If the hot water extract is dried to a powder, this is now a simple form of a tea extract. This powder is 100% water-soluble and can be consumed at high doses in capsules, or in blends with other supplements, or it may be used as an ingredient in foods and beverages.

The liquid tea represents an intermediate stage between the crude plant powder and the powdered extract. The antioxidant capacity of a liquid must be evaluated in relation to the volume and the concentration, not in units of weight of either the starting crude powder or the resulting extract.

Comparison of crude powders from the tea plant versus high-potency extracts in the CAP-e assay has confirmed that the tea antioxidants available to protect living cells are enriched in a tea extract.



The antioxidant capacity obtained by ingestion of either tea or a tea extract must also be evaluated in light of the portion size. Using the example above, a person drinking 6 cups of green tea per day would ingest the same level of antioxidants as a person consuming 1 gram of a crude green tea extract.

Grapes and grape extracts

Consuming red grapes will provide a person with a complex blend of polyphenol antioxidants from both the flesh and the skin. The simpler water-soluble compounds may rapidly be absorbed, and other antioxidants can be released during the digestive process (discussed in more detail below).

Red wine will contain many of the compounds from the original whole grape, but

a) Some compounds are left behind in the pulp;

b) Other compounds are generated during the yeast-based fermentation process.

Therefore, consuming red wine provides a complex polyphenol profile that differs from whole grapes or grape juice.

There is much interest around Resveratrol, which is one of the predominant polyphenol antioxidants in red grapes and other fruits. The gene for Resveratrol has been expressed in microbial organisms so the pure compound may be produced economically, allowing consumption of 100-fold higher doses than if either the fruit or the wine was the only source.

There is also much interest in complex blends of polyphenol-rich natural products, with suggested biological synergy between the different antioxidant compounds.

When antioxidants are difficult… Solvents!

Many potent antioxidants are not water-soluble. The curcuminoids in the spice Turmeric (*Curcuma longa*) is an example of such hydrophobic antioxidants. Low bioavailability is often an issue, and much effort is being placed on developing modified compounds with higher bioavailability [26] [27].

The hydrophobic, low-bioavailable antioxidants often depend on delivery in the food matrix during digestion [28]. Therefore, a cell-based antioxidant assay may not demonstrate certain properties of the native product. The digestive process may produce bioavailable metabolites not previously present in the native product.

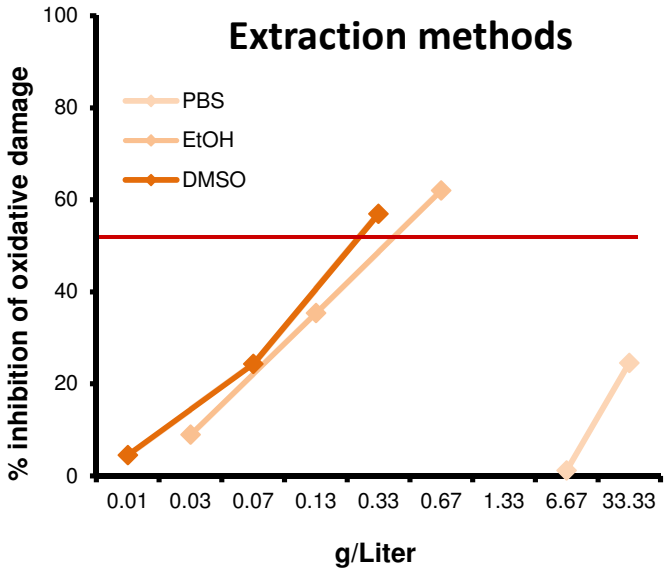
The whole Turmeric spice does, however, contain other antioxidants that are water-soluble, as demonstrated below. Such antioxidants may be lost in an extraction process so an extract may be more potent and contain higher levels of the compounds of interest, but the extract does not necessarily reflect the original diversity of compounds.

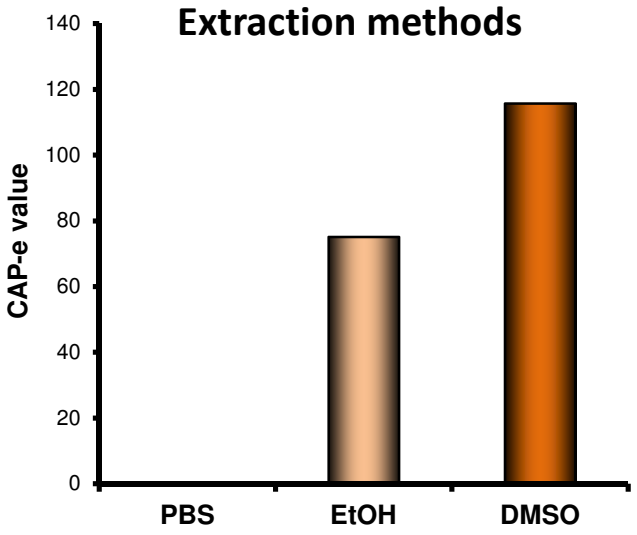
In order to get some preliminary ideas of the chemical and biological properties of hydrophobic compounds, solvents are necessary. The use of certain solvents, for extraction or purification, allows the compounds to be tested in the laboratory and suggests/predicts effects that may play a role after consumption.

Working with living cells does require that a test product be presented to the cells in a liquid medium that the cells can tolerate. Some solvents are not well tolerated by cells.

As an example of a crude natural product rich in hydrophobic antioxidants, we tested off-the-shelf turmeric in the CAP-e assay. The crude spice powder was dissolved

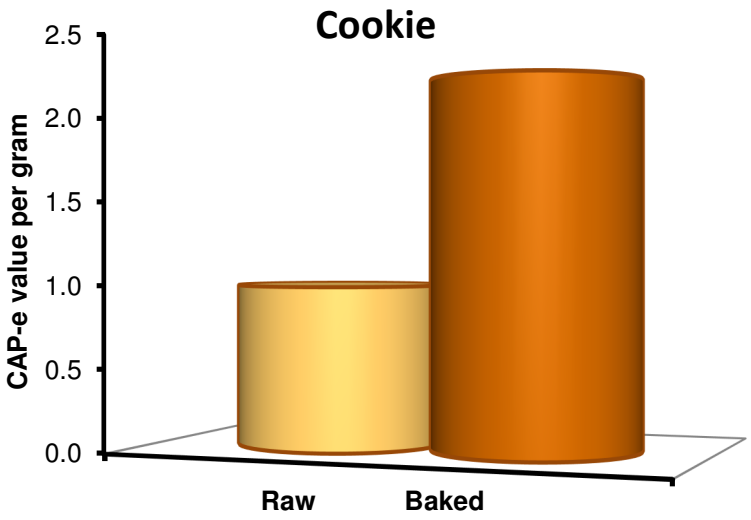
in either physiological saline (PBS), 95% ethanol (EtOH) or dimethyl sulfoxide (DMSO).





Foods and food preparation

Cooking can help break down certain food matrices and can liberate or convert antioxidants to be more biologically available.



An antioxidant-rich cookie recipe containing acai, ginger, and other spices was used to test this principle. The raw cookie dough was compared to the baked cookie in the CAP-e assay.

Antioxidants available to protect living cells were measurable in the raw dough, but the level more than doubled after baking.

Digestion and its effect on antioxidant bioavailability

The standard ways of preparing natural products for testing is to make a suspension of the product in either water or a solvent such as ethanol. As a meaningful alternative to solvents, it is technically possible to mimic the human digestive process. Products rich in polyphenols that are bound in the food matrix may be liberated during the digestive process. Further, microbial fermentation may generate yet other antioxidant compounds that are more bioavailable.

The digestive process leads to breakdown of the food matrix, such as fibers and protein, and liberates bound antioxidants such as carotenoids, lycopene, and other phytonutrients [29]. Compounds with low solubility in water can be carried on lipid particles in the blood for delivery to cells and tissues [30].

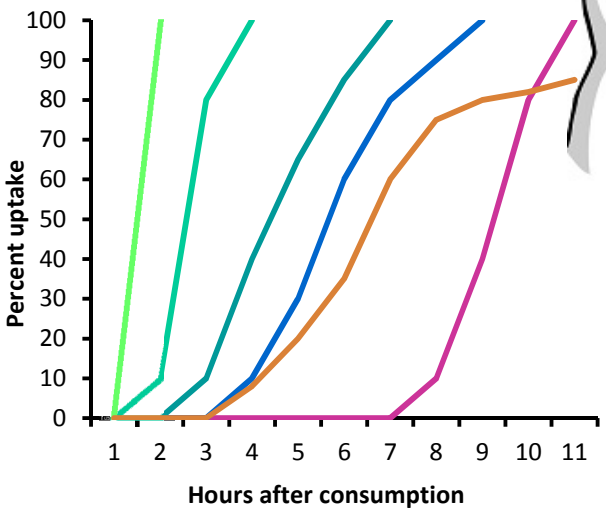
Clinical use of the CAP-e assay

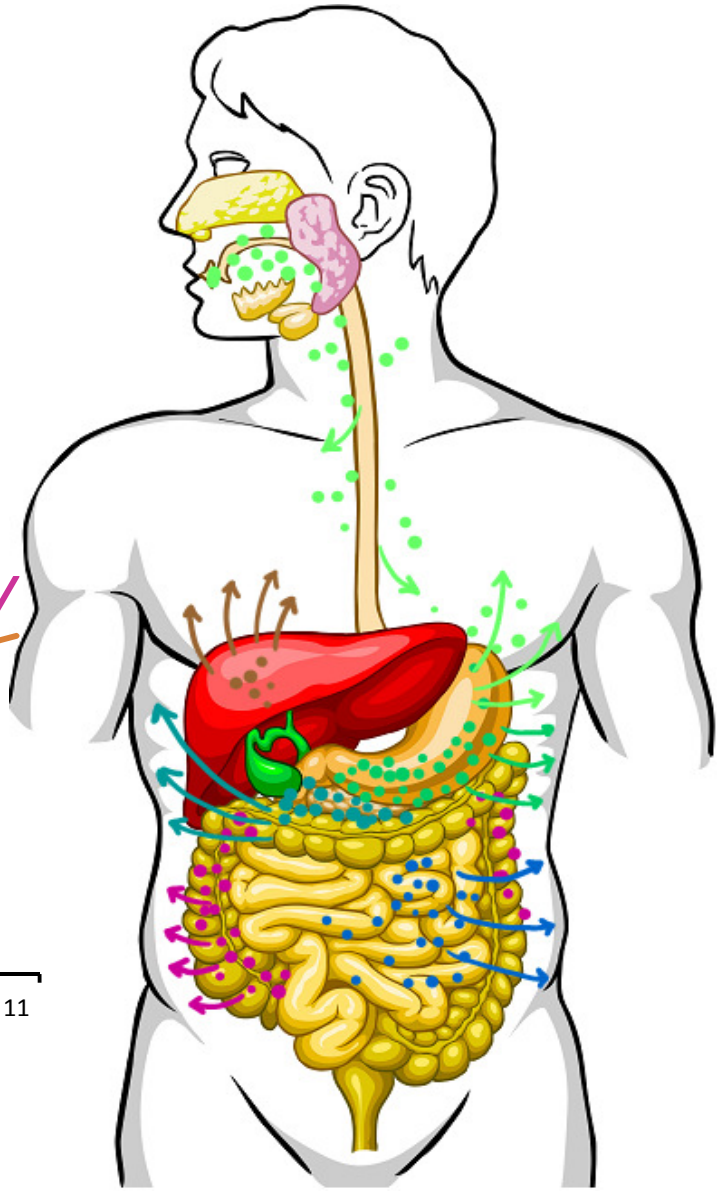
The ultimate proof of antioxidant bioavailability comes from human clinical studies. Study designs may either focus on acute uptake of antioxidants, or use long-term study designs to test cumulative changes in the antioxidant status of a study population. In terms of antioxidant uptake, an acute cross-over study is more conclusive than a long-term study. If an increase in serum antioxidant capacity is seen in a long-term study, this may be a direct effect of antioxidant uptake, but may also be partly caused by anti-inflammatory effects, leading to reduced production of free radicals by the body.

Regardless whether an acute cross-over study or a long-term parallel arms study is being performed, blood samples are taken and the serum is used to measure cellular antioxidant protection in the modified CAP-e assay. The compounds that contribute to the serum antioxidant protection may include:

1. Freely bioavailable water-soluble antioxidants
2. Antioxidants liberated from the food matrix or generated by chemical alteration by digestive processes in the stomach (acid, peptin)
3. Antioxidants liberated from the food matrix, or generated by chemical alteration by digestive processes initiated in the duodenum (pancreatic enzymes, bile salts)
4. Antioxidants liberated from the food matrix, or generated by chemical alteration by digestive processes during the passage through the small intestine
5. Non-bioavailable and/or non-antioxidant compounds altered to become bioavailable antioxidants by microbial fermentation in the gut
6. Compounds that were absorbed into the blood stream from either small or large intestine and transported to the liver, then metabolized by liver enzymes.

1. Oral
2. Stomach
3. Duodenal
4. Small intestine
5. Large intestine
6. Liver metabolism





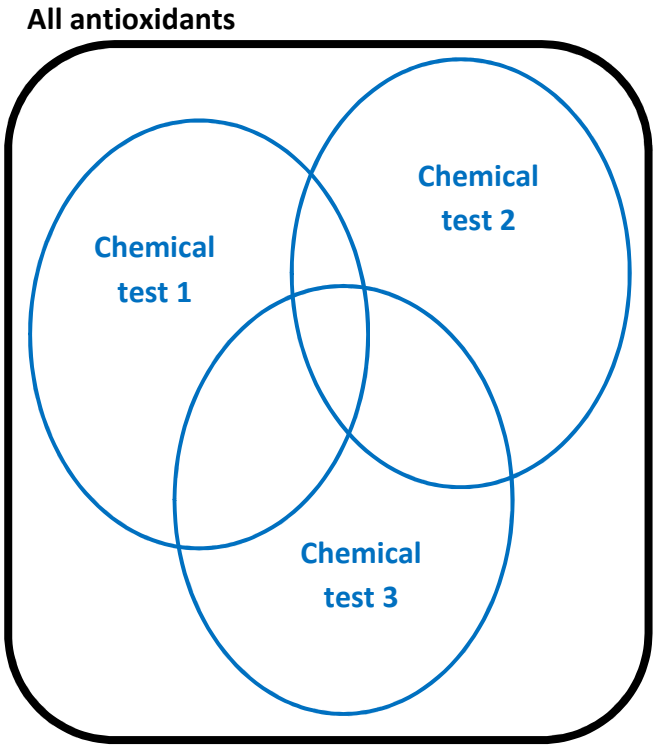
Data from recent human clinical trials have shown that even products with CAP-e values below 10 GAE/gram have shown statistically significant antioxidant protection in clinical studies.

Study design for acute uptake must be placebo-controlled. In cases where a placebo is not easily created for a given product, the study participants must still be tested in a similar fashion on a day where they do not consume active product. The physiological parameters we endeavor to evaluate can be dynamic, even over a few hours, because each individual's physiology is impacted by varying metabolism, circadian rhythms, and other factors. Without such a controlled study design, changes cannot be interpreted as related to product intake, but could simply reflect normal variations in a given person.

Summary

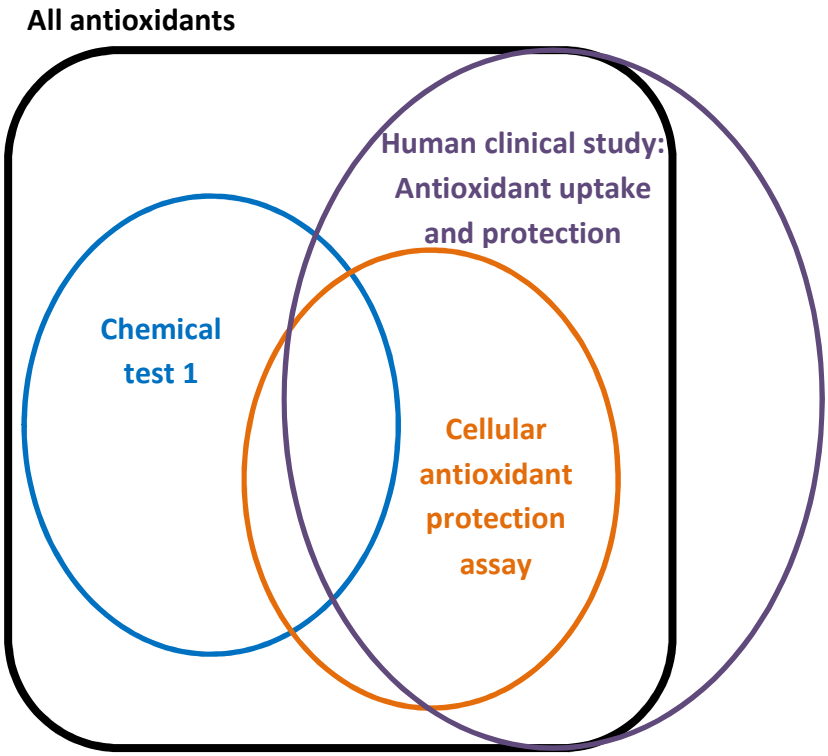
Data from the CAP-e assay takes a step beyond chemical assessment of antioxidant properties.

Different chemical assays are able to demonstrate antioxidant properties in various ways, dependent on the chemical reactions involved in a given assay.



In contrast, the CAP-e bioassay demonstrates antioxidant capacity in the context of cellular compounds and redox enzymes. Some antioxidants may be measurable in both chemical assays and the CAP-e bioassay, whereas the cellular assay only documents the presence of biologically active antioxidants. A human clinical study may demonstrate effects of antioxidants native to a food or

nutritional product, as well as antioxidants generated as a result of metabolic processes, i.e. not initially present in the product.



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