PRE-QUALIFICATION OF FLAVONOID - DIHYDROQUERCETIN (DHQ) DIETARY ANTIOXIDANT & SIGNALING MOLECULE

Introduction

Plants synthesize a range of flavonoids which may vary in respect of the degree of hydroxylation and subsequent modifications to the basic flavonoid skeleton. Flavonoids are the most widespread group of secondary metabolites found in plants, occurring in species ranging from mosses to angiosperms (Harborne, 1988). The generic term "flavonoid" describes a number of different polyphenolic compounds, all of which contain the same basic carbon skeleton.

Dihydroflavonols and namely dihydroquercetin belongs to “flavonoid family”. The dihydroflavonols, (2R, 3R)-dihydrokaempferol, -**dihydroquercetin** and -dihydromyricetin, can be reduced to their respective leucoanthocyanidins, 3,4-cis- leucopelargonidin, **-leucocyanidin** and -leucodelphinidin by the action of dihydroflavonol 4-reductase (Heller et al., 1985a, b).*** The leucoanthocyanidins are converted to the anthocyanidins, pelargonidin, **cyanidin** and delphinidin by the action of a single enzyme, the anthocyanidin synthase.*** Anthocyanidins are unstable compounds which are stabilized within the cell by further modification e.g. glycosylation (reviewed in Heller and Forkmann, 1988), the conjugation with different sugar moieties.

Dihydroflavonols are also substrates for the enzyme flavonol synthase (Holton et al., 1993). (2R, 3R)-dihydrokaempferol, - **dihydroquercetin**, -dihydromyricetin are converted to the flavonols: kaempferol, **quercetin** and myricetin respectively.***

Dihydroquercetin is a molecule mainly found in species of the genus *Larix*, *Douglas Fir*, *Cedrus* and *Pseudotsuga*. Evergreen trees, especially those from the family of *Pinaceae* are considered rich sources of dihydroquercetin.

During the last decades, a flavonoid dihydroquercetin has been extensively studied due to its wide spectrum of unique biological properties. On the role of heartwood extractive dihydroquercetin in high plants the attention was paid quite long time ago due the properties to extend duralibility of trees where dihydroquercetin was found. The significance of heartwood extractives for natural durability was demonstrated early (Hawley et al. 1924), and has been repeatedly discussed in the literature (e.g. Rudman 1963, Reyes-Chilpa et al. 1998, Celimene et al. 1999, DeBell et al. 1997, Schultz et al. 1990). As major phenolic compounds in larch wood the flavonoid dihydroquercetin is reported, beside minors quercetin, kaempferol and lignans (Babkin et al. 2001, Hegnauer 1962, Giwa and Swan 1975, Sasaya 1987). The flavonoid taxifolin (3,3',4',5,7-Pentahydroxyflavanone) or dihydroquercetin is known to be a major phenolic compound in larch heartwood (Hegnauer 1962).

Heartwood extractives play a prominent role in natural durability, the decay resistance against fungi (Eaton and Hale 1993). Free hydroxyl groups are essential if phenolic compound such as dihydroquercetin acts as uncoupling agent that inhibits oxidative phosphorylation, the main source of energy in decay fungi (Hart 1989). However, based on fungicidal activities found in extractives of highly durable heartwood (Schultz et al. 1995, Rudman 1963) and the fact that brown-rot fungi are believed to use some type of free radicals in order to initially disrupt cell walls (Backa et al. 1993), a dual defense function of the extractives was proposed: extractives possess fungicidal activity as well as being excellent free radical scavengers (antioxidants) (Schultz and Nicholas 2000).

Dihydroquercetin directly relates to flavonoids, which are in general were studied for more than 80 years *in vivo* and *in vitro* systems. They have been shown to exert potent anti-oxidant capacities in some instances stronger than antioxidant vitamins. They have been also shown to exhibit beneficial effects on capillary permeability and fragility, to have anti-platelet, hypolipidemic, anti-hypertensive, anti-microbial, anti-viral, anti-allergenic, anti-ulcerogenic, cytotoxic, anti-neoplastic, anti-inflammatory, anti-atherogenic, and anti-hepatotoxic activities.

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Assaying Larch Tree Extract – Dihydroquercetin (DHQ)

An important criterion when sourcing raw materials is the purity of the material or the active ingredient content of the product. For many products this is an easy and straightforward undertaking but for larch tree extract such as Dihydroquercetin (DHQ) in particular this is a particularly challenging effort.

Regardless of the method used, one always needs a measuring stick. For many analytical determinations, establishing this reference is easy: reference material with a known composition and purity is used. Unfortunately, this is not such a trivial pursuit when dealing with dihydroquercetin.

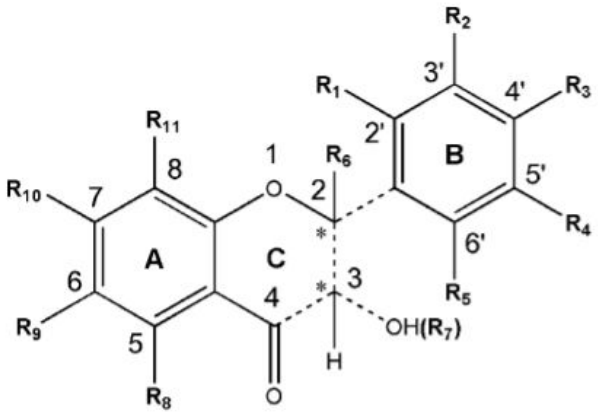
Besides establishing a reference, one also needs to have a protocol or method that is fast, reliable and cheap in terms of reagents and equipment. Again, this is not an easy task when dealing with dihydroquercetin, hereinafter referred to a short name DHQ.

On the other hand defining a standard DHQ is due to the complexity of the extract also not possible: the relative composition from one batch to another is fairly constant but is nevertheless fluctuating, thus defining a standard DHQ is difficult if not impossible. Furthermore, nobody will accept a standard imposed by a competitor and availability thus becomes an issue. Consequently, standardized extracts are only of value as in-house standards.

It is noteworthy that a "standard" material such as DHQ sold by lab chemical vendors is also fluctuating. These companies source their materials from multiple suppliers and the grade is not always the same. Furthermore, the DHQ sold by lab chemical vendors is seldom the highest quality grade available on the market.

Specific DHQ features

DHQ presents a unique structural feature known as **chirality**, which distinguishes ingredient from all other classes of flavonoids. The vast majority of chiral dihydroflavonols (dihydroquercetin) can be purchased from chemical companies, but they are mainly available only as racemates (equivalent proportions of both enantiomers or epimers).



(2R,3R)-Dihydroquercetin (DHQ)
enantiomerically pure native molecule form

* Denotes chiral center

The term **chiral** in general is used to describe an object that is non-superposable on its mirror image. **Achiral** (not chiral) objects are objects that are identical to their mirror image. Chiral compounds rotate plane polarized light. Each enantiomer will rotate the light in a different sense, clockwise or counterclockwise. Molecules that do this are said to be **optically active**. The native optically active and enantiomerically pure molecule of DHQ presents: (2R, 3R) Dihydroquercetin.

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Chirality can effect plant growth regulation. In the case of DHQ it had been found a unique example of a chiral ingredient that is a plant growth regulator, an insect semiochemical that has pheromone activity in certain species and induces plant stress volatiles in plants for defense against herbivores and has been found to possess strong antioxidant activity against oxidative phosphorylation.

It is important to consider that some chiral forms of dihydroflavonols (dihydroquercetin) are stereochemically unstable depending on the substitution pattern of various functional groups around the stereogenic center. For compounds with more than one stereogenic center such as DHQ, a process called epimerization occurs when there is a change of configuration at a single chiral center (D. Wistuba *at al*. 2006, Anal. Chem. 78, 3424).

The racemization process, which is characterized by a process reaching equilibrium between the two enantiomers is facilitated by temperature, moisture, solvent, pH, among other factors (N.R. Srinivas 2004, Biomed. Chromatogr. 18, 207). Therefore, non-stereospecific assay methods cannot interpret the time-course development of an individual enantiomer and the results of using achiral assays could be misleading in determining concentration dependence of each enantiomer of a racemic flavonoid in terms of efficacy or toxicity. There are no studies that have examined the pharmacokinetics, anti-cancer, or anti-inflammatory activity of the individual enantiomers of chiral forms of dihydroflavonols (dihydroquercetin). Furthermore, due to their possible therapeutic uses scientists and nutraceutical companies are now employing dihydroflavonols as potential lead compounds and synthesizing a variety of derivatives, such as chiral dihydrofuroflavones (A.W. Lantz *at al*. 2004, Electrophoresis 25, 2727).

DHQ undergoes nonenzymatic interconversion of one stereoisomeric form into another. When isomerization occurs causing the formation of a racemate it is termed racemization, racemization is the process of an enantioenriched substance becoming a mixture of enantiomeric forms and thus the formation of a racemate from a pure enantiomer. Racemization is normally associated with the loss of optical activity over a period of time since 50:50 mixtures of enantiomers are optically inactive, while enantiomerization is the reversible interconversion of enantiomers. The importance of temperature and pH dependent epimerization or enantiomerization barriers of all dihydroflavonols such as Dihydroquercetin (DHQ) should be taken into consideration. Non-enzymatic inversion of DHQ is important in the nutraceutical manufacturing processes and has implications for the shelf-life of an ingredient and the economic feasibility of the stereomer solution. Non-enzymatic inversion can also occur during the stereospecific chromatographic procedures. Racemization may also occur in physiological fluids, such as the acidic environment of the stomach.

Demonstration of racemization or epimerization may have profound consequences for the development of stereochemically pure Dihydroquercetin (DHQ) as a nutraceutical entity. A better understanding of the factors facilitating such interconversions may greatly aid its development by identifying this feature at an early stage and thereby reducing nutraceutical and bioanalytical workload. Regulatory agencies are increasingly asking for evidence regarding this phenomena following administration of racemates or single enantiomer ingredient candidates (FDA's Policy statement for the development of new stereoisomeric drugs, 1992, Chirality 4, 338).

Furthermore, it is prudent for the analyst to avoid any environment that may epimerize or racemize the chiral center of DHQ. This would be for example the use of extreme alkaline or acidic conditions or elevated temperatures. Finally, the lack of availability of optically pure enantiomers and epimers renders evaluation of configurational stability of chromatographic methods complicated.

Nomenclature to Distinguish Between Stereisomers of DHQ (dihydroquercetin) products:

2,3 – *trans*	2,3 – *cis*	2,3 – *trans*	2,3 – *cis*
(+)	-	-	(+)
2*R*, 3*R*	2*R*, 3*S*	2*S*, 3*S*	2*S*, 3*S*
DHQ	epiDHQ	*ent*-DHQ*	*ent*-epiDHQ*
Natural	Artifact	Artifact	Artifact

2R,3R (+)- Dihydroquercetin
2S,2S (-)- Dihydroquercetin
2S,3R (+)- Epidihydroquercetin
2R, 3S (-)- Epidihydroquercetin

*Considered preparative artifacts by Lundgren and Theander [1] , but natural products by Nonaka et al.[2]

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What does it mean purity for Dihydroquercetin (DHQ) ?

For instance in Russia, where DHQ was commercialized in 1990s, there are two applicable state standards on a purity of ingredient. The state standard of DHQ # 42-2399-94 (1996) assaying DHQ, where the mixture of natural (2R,3R) and artifacts (mainly 2S,3S) molecule forms represents DHQ. This standard does not consider monomer or polymer forms of DHQ as well degraded forms of DHQ molecule (due to alkaline and acidic conditions of extraction and elevated temperatures causing molecule thermohydrolisis). The state standard of DHQ # 9308-2009 (2009), which was additionally applied in Russia, verifies active content e.g. natural (2R, 3R) molecule form in total amount of mixture with racemic artifacts and polymerized molecule forms, but does not verify monomer content by comparing to short and long molecule polymer forms.

Additionally, both standards of DHQ relates to main raw material sources represent by Larix Sibirica and Larix Dahurica, however different parameters of manufacturing process of ingredient isolation were used for obtaining standards i.e. solvents, pH, temperatures. The common feature between both standards outlines only raw material and the assay of total DHQ.

Recently in Russia two methods for the detection of long polymers of dihydroquercetin (DHQ) has been developed.[3] First method is based on UV spectrophotometry. It was shown that the quantity of long polymers in aqueous solutions can be estimated by the ratio of the absorption bands at 340 and 290 nm, since the 340-nm band was attributed to the monomeric form of DHQ, whereas the 290-nm band was attributed to both the monomeric and polymeric forms. The second method is based on the high-sensitive measurement of lightscattering intensity in aqueous solutions of diluted DHQ preparations using a spectrofluorometer with crossed monochromators. It has been shown that the filtration of DHQ solutions through Millipore filters with a pore diameter of 0.05–0.45 microns makes it possible to nearly completely eliminate long polymers and their aggregates. Long polymers at high concentrations can aggregate. The longest polymers and their aggregates may be 0.1 mm in length, which leads to fluctuations in the light-scattering intensity on the second and minute time scale.

Purity (Assay) and ORAC value (antioxidant capacity *in vitro*)

There is significant experience in the extraction and isolation of secondary metabolites from plants; for example, as part of biosynthetic, biochemical, chemotaxonomic, ecological, phytochemical, pharmacological, and plant tissue culture studies. The effective extraction of a plant compound of interest from a natural source depends largely on solubility, stability, and functional-group considerations. All these parameters directly correlate with the ORAC test method in order to show antioxidant activity of dihydroquercetin (DHQ) *in vitro*.

Assaying total content DHQ is the initial step to pre-qualify ingredient taking into consideration above mentioned specific features of DHQ. Assaying monomer part and active part (2R,3R) of DHQ is the second step of pre-qualification, which can partially show estimated antioxidant activity of ingredient *in vitro* (i.e. in test tube). The final stage of pre-qualification, which can clearly answer how much active DHQ is contained in commodity DHQ by comparing to other vendors, can be considered the ORAC test method, the test tube method which is comparatively fast, reliable and cheap in terms of reagents and equipment.

Oxygen Radical Absorbance Capacity (ORAC) is a standardized test adopted by the U.S. Department of Agriculture to measure the Total Antioxidant Potency of foods and nutritional supplements. This standardized test was developed by Dr. Guohua Cao, a physician and chemist at the National Institute on Aging in Baltimore, Maryland. It provides us with a very precise way of determining the Free Radical destroying or neutralizing power of a particular food, supplement or compound. USDA Human Nutrition Research Center on Aging at Tufts University, the ORAC test is one of the most sensitive and reliable methods for measuring the ability of antioxidants to absorb free radicals. It is the only test to combine both time and degree of inhibition of free radicals.

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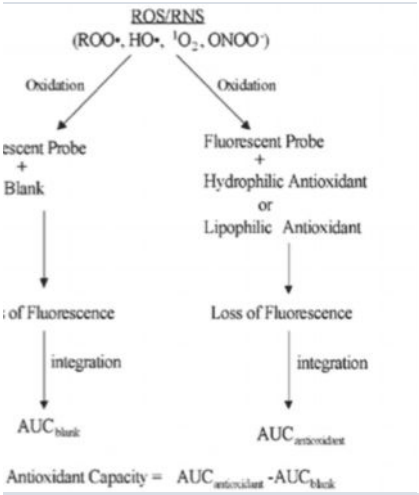
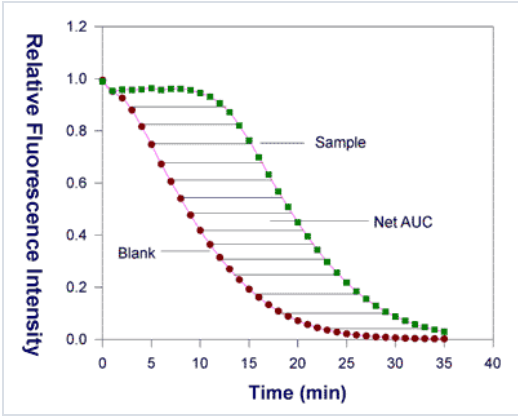
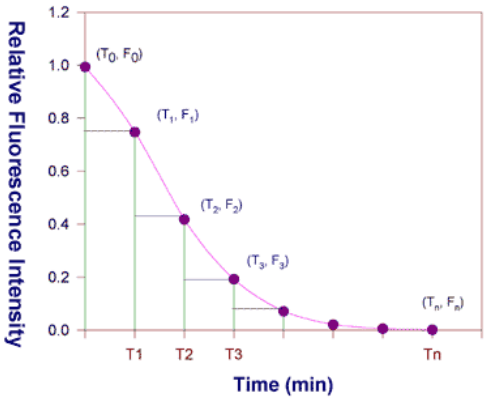


The ORAC assay depends on the free radical damage to a fluorescent probe through the change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free radical damage. In the presence of antioxidant, the inhibition of free radical damage by an antioxidant, which is reflected in the protection against the change of probe fluorescence in the ORAC assay, is a measure of its antioxidant capacity against the free radical (Figure 1).



The uniqueness of the ORAC assay is that the reaction is driven to completion and the quantitation is achieved using "area under the curve" (AUC).

Figure 2. Antioxidant activity of tested sample expressed as the net area under the curve (AUC).



In particular, the AUC technique allows ORAC to combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity.

Figure 3. Calculation of ORAC values. AUC=0.5 + F1/F0 +F2/F0+F3/F0+.....+Fn/F0;
Relative ORAC value = [(AUCSample-AUCBlank)/(AUCstandard- AUCBlank) x (molarity of standard/molarity of sample)

According to the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays. The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. ET-based assays measure an antioxidant's reducing capacity, and the HAT-based assays measure the hydrogen atom donating capacity. The hydrogen atom transfer is a key step in the radical chain reaction. Therefore, the HAT-based assays are more relevant to the radical chain-breaking antioxidant capacity. Among all the HAT-based assays, ORAC adopted an AUC (Area Under Curve) technique to quantify antioxidant capacity. The advantage of the AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phases. This approach unifies the lag time method and initial rate method, and it is particularly useful for food samples, which often contain multiple ingredients and have complex reaction kinetics.

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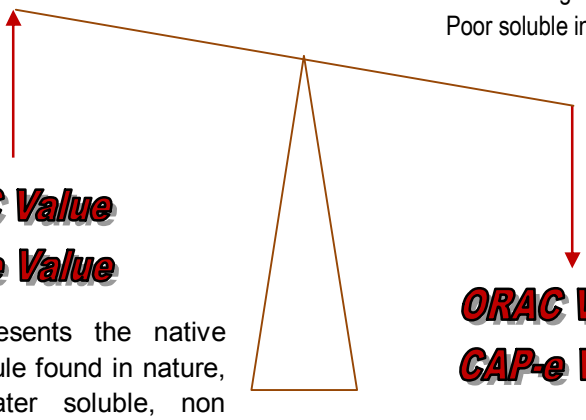
Therefore, the ORAC assay has been broadly applied in academics and the food and supplement industry as the method of choice to quantify antioxidant level e.g. capacity in the test tube. In fact, an antioxidant database has been generated applying the ORAC assay in combination with the total phenols assay.

Even pre-qualifying DHQ on initial step, the ORAC value for ingredient can be different with the same DHQ assay from different vendors. However, there are many factors that contribute to the final ORAC value of an actual product DHQ – primarily, the way it has been processed and concentrated. ORAC tests also to be done often, because processing can affect DHQ antioxidant levels. Frequent testing must to be done because antioxidant levels can vary from vendor to vendor.

High monomer optically active molecules content
Minimum or nothing impurities
Minimum lipophilic flavonoid relatives
Stable moisture content
Water solubility

Impurities: resins, oils, saponins – less active
Artifacts/Racemic molecule structure – less active
Low & High polymer structure – less active, fast degradation
Overloading with lipophilic flavonoid relatives – less active
High moisture content – less active
Poor soluble in water – less active, poor bioavailability



FlavitPURE™ represents the native form of DHQ molecule found in nature, non-conjugated, water soluble, non racemic, non polymer, safe, bioactive and bioavailable, supported by important initial quality control tests *in vitro*.

Maximum ORAC value of DHQ from different vendors contributes **20,000 µM TE/g.**

The **$ORAC_{hydro}$** value of FlavitPURE™ Dihydroquercetin is over **28,000 µM TE/g.***

** The $ORAC_{hydro}$ reflects water-soluble antioxidant capacity. Trolox, a water-soluble Vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micro mole Trolox equivalent (TE) per gram.*

ORAC is also useful for indicating early on whether DHQ has a high antioxidant capacity, so that a vendor can decide whether to then pursue biological testing.

In order to prove how effective an antioxidant will be once in the body, biological tests need to be run to determine its actual biological effects.

Larchwood saw logs become the main source for FlavitPURE™ - Dihydroquercetin (DHQ) extraction by pure organic solvents water and grain alcohol, making "vodka" mixture available for extraction without any chemical compounds, which may alter the native molecule form of DHQ. Such essential wastes serve an excellent friendly environmental base to obtain commercial volumes of FlavitPURE™ - Dihydroquercetin (DHQ).

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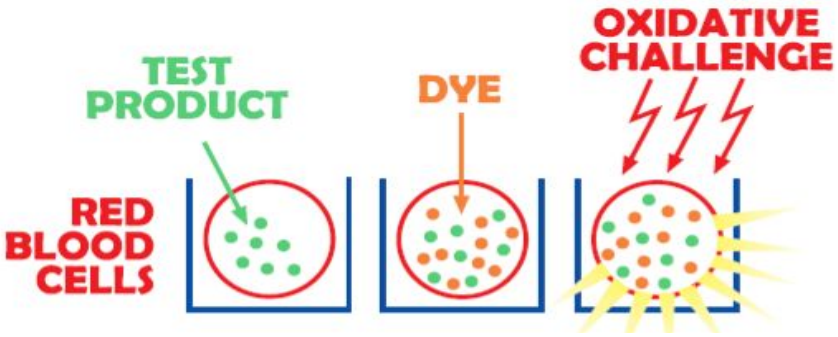
FlavitPURE™ is water soluble and immediately available to protect living cells, which is confirmed by biological tests *ex vivo* (on living cells).

Seeking faster and economical assyas as an addition to existing tests such as the ORAC causes a turn towards the cell-based laboratory testing. CAP-e, the cell-based test developed by NIS Labs, answers the question – Can a specific antioxidant enter and protect a living cell? In this assay, the cells are exposed to test products in physiological saline. The cells are allowed time to absorb compounds from the test product. Any compounds not absorbed by the cells during that period are removed by centrifugation and subsequent washing.

The cells are exposed to a precursor dye that becomes fluorescent when exposed to oxidative damage. Subsequently, the cells are subject to an oxidative challenge such as radicals H_2O_2 or AAPH. The fluorescence intensity reflects the amount of oxidative damage. As a positive control, cells are exposed to oxidative challenge without any antioxidant protection, and serve as the measure of maximum oxidative damage. Any reduction of oxidative damage to the cells pre-treated with the test product reflects antioxidant protection. The assay is qualitative in principal.

The assay can be applied to testing of antioxidant availability to live cells *in vitro*[4] and documenting antioxidant uptake *in vivo*[5].



The red blood cell is a convenient test model for examination of antioxidants that are able to enter living cells.

FlavitPURE™ Dihydroquercetin (85% monomer form) CAP-e assay as a cell-based antioxidant protection assay using erythrocytes to address the question of whether antioxidants in complex natural products enter the cytosol and contribute to the reduction of oxidative damage within the cell.

CAP-e antioxidant capacity:

Sample	NIS code	Lot/Batch#	Type of product	CAP-e units per gram
Flavit Dihydroquercetin (85%)	LV01	D89603	powder	9.9
Flavit Dihydroquercetin (85%)	LV02	D89426	powder	10.5

Protocol reference: NIS/CAPe/AAPH/20081124.

The red blood cells were mixed into a solution of DHQ, then the excess DHQ was washed away from the cells. After being subjected to the oxidative stress, it became apparent that the cells resisted the oxidative damage from the inside out. Thus, whatever was protecting the red blood cells from damage didn't merely coat the outside of the cell but was actively taken up by the cell and persisted after the DHQ was washed away.

The CAP-e value reflects the IC_{50} dose of the test product, i.e. the dose that provided 50% inhibition of oxidative damage. This is then compared to the IC_{50} dose of the known antioxidant Gallic Acid.

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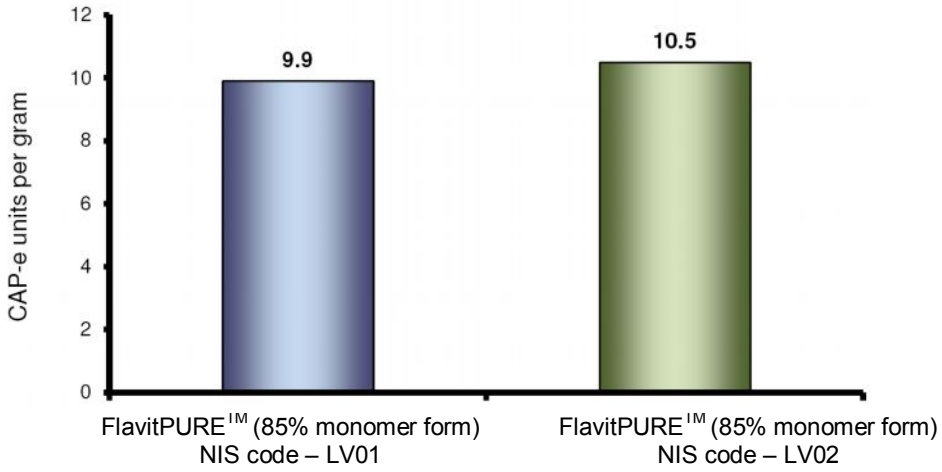
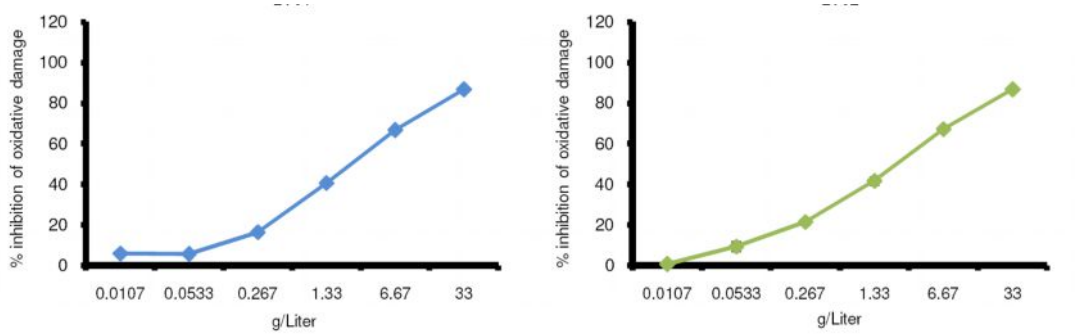


The graphs below show the average of each duplicate set of data points for the serial dilutions of the product. For each data point, vertical bars show the standard deviation for each duplicate data set. When duplicate values are almost identical, the standard deviation bars may not be visible.



BIOAVALABILITY, EFFICIENCE and SAFETY

The effective extraction of DHQ depends largely on solubility, stability and functional – group considerations. The type of safety evaluation would depend on the nature of the DHQ molecule (pure compound) and on the proposed use potentially leading to a significant increase in exposure. **FlavitPURE™** - DHQ is the soluble, stable native molecule form with the confirmed molecule functional – group efficiency by ORAC and CAP-e tests.

Given the physiological levels expected to be reached upon many flavonoids intake of lipophilic nature, the results point at possible flavonoid–xenobiotic interactions affecting the toxicokinetic behavior of these xenobiotics, especially at the level of some important transport processes. **FlavitPURE™** - DHQ hydrophilic or "water-lover" molecule's nature possesses minor xenobiotic recognition being able to work friendly in both aqueous and lipid environments with minor toxycokinetics by comparing to lipophilicity.

Researchers suggest that the stereo-chemical structure of native dihydroquercetin molecule form (2R,3R) facilitates its penetration into a lipid phase enable the flavonoid to act as a chain-breaking radical inhibitor in protein-lipid and lipid-lipid cellular environments (Ratty A.K., Sunamoto J., Das N.P. 1988). The effectiveness of antioxidant protection by flavonoids like in nature is related to their ability to interact and penetrate biomembranes causing changes in membrane structure and fluidity (Saija et al., 1995). At the water-lipid interface, the tridimensional structure and the number and distribution of flavonoid hydroxyl groups of more hydrophilic groups determine the formation of hydrogen bonds with the polar headgroups of lipids, therefore protecting the membrane from external damage (Erlejman et al., 2004; Oteiza et al., 2005; Saija et al., 1995).

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A proper assessment of absorption/bioavailability/efficacy ought therefore to take this portion into account, also because in a complex system such as blood the distribution between free and bound populations may not necessarily be constant as the total load varies. The oral bioavailability of molecules depends on their physicochemical properties which have been described as the "rule of five". Absorption is most probable if a compound such as monomer DHQ form has less than 5 hydrogen bond donors, less than 10 hydrogen acceptors, a relative molecular mass below 500 and a logP smaller than 5. Exceptions from this rule are known for many orally active ingredients which often share structural similarity with substrates of specific transporters which enable enhanced absorption. Plasma content is not in any case directly related to activity, since DHQ is well known to bind avidly to albumin and other proteins, and is therefore "sequestered" in plasma as well. A major metabolic fate of DHQ in monomer form *in vivo* is via glucuronidation. In animal and human studies, DHQ was exposed to intensive biotransformation with occurrence of glucuronides due to the DHQ native optically active molecule form' high polarity (hydrophilic nature). Direct measurements reveal that flavonoid classes differ in their ability to cross the blood-brain barrier,[10] and these differences appear to be dependent in part on the particular flavonoid's lipophilicity and polarity. As a consequence, O-methylation and glucuronidation during phase II metabolism may have a significant impact on flavonoid bioavailability, including the brain. Evidence exists from in situ research demonstrating that P-glycoprotein transporters play a role in the flux of flavonoids into the brain.[11]

It is well known that flavonoids have an affinity for protein and DNA[6] and that complex formation is favored in neutral and basic pH conditions.[7] Many flavonoids including DHQ have been demonstrated to bind to lipoproteins,[8] and were shown to bind to DNA via both intercalation and external binding.[9]

The extent of this occurrence *in vivo* is not known, nor is it known whether binding of DHQ monomer is comparable to that of DHQ polymer; however, the notion of tight binding of DHQ to proteins implies that tissue extraction methods could have a significant impact on levels of DHQ recovered. It was established that the recovery ratio of DHQ monomer form from blood plasma averagely amounted to 94.5 ± 2.0%.[14]

The extraction ratio of DHQ (**FlavitPURE™**) from blood plasma[14]

Taken (µg/ml)	Detected (%)			*x*	+ SD
0.05	95.7	91.4	97.3	94.7	3.0
0.10	92.7	93.4	95.6	93.9	1.5
0.25	94.6	96.5	93.5	94.9	1.5
				94.5	2.0

During procedure of incubation of organ homogenates (liver, spleen, lungs), containing glucoronic conjugates of DHQ with p-glucuronidase, in water bath in 37°C, the activation of other ferments occurred.

The extraction ratio of DHQ (**FlavitPURE™**) from homogenates of various rat organs and tissues (the arithmetic mean of three determinations)[14]

Study object	*x*	+ SD
Kidneys	94.0	2.6
Skeletal muscles	92.8	2.4
Heart	93.4	2.3
Brain	95.8	2.1
Liver	91.6	3.2
Spleen	92.4	2.9
Lungs	90.7	3.4

At the same time the high content of DHQ in hepatic tissues could be explained by the fact that ingredient's excretion is performed though this organ.

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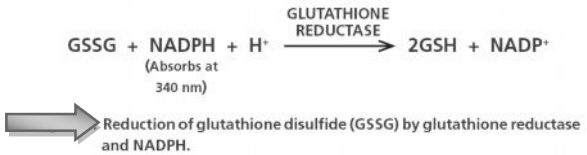
The maximal concentration of DHQ determined in blood plasma of rats (C_{max}) amounted to 6.97 μg/ml. The analysis of the main value characterizing the extent of bioavailability of the active substance from the dosage form - $AUC_{0\rightarrow\infty}$ showed that the value of the present parameter was 16.75 μg /ml-h.[14]

Pharmacokinetic parameters of DHQ (**FlavitPURE™**) in blood plasma of rats after oral administration[14]

Ingredient	Parameters						
	$C_{max}/AUC_{0\rightarrow\infty}h^{-1}$	$T_{1\backslash 2el}$ h	T_{max} h	C_{max} μg/ml×h	MRT hour	$AUC_{0\rightarrow 6}$ μg/ml×h	$AUC_{0\rightarrow\infty}$ μg/ml×h
DHQ	0.416	1.30	0.5	6.97	2.59	16.10	16.75

Considering DHQ tissue concentration relative to endogenous glutathione, it is extremely unlikely that the primary mode of health beneficial action of DHQ is via direct radical quenching. It is however possible that DHQ effect is mostly due to signal transduction pathways, gene expression, and other known and unknown mechanisms, which are responsible for DHQ observed health effects. However, the oxidized glutathione (GSSG) is regenerated by glutathione reductase (GRD) that uses NADPH as the reducing agent (Halliwell, 1994), which is commonly used by DHQ being in reduced form, showing the antioxidant biological effect.



The oxidation of NADPH to NADP+ is monitored by the decrease in absorbance at 340 nm. Glutathione reductase plays an important role in protecting hemoglobin, red cell enzymes, and biological cell membranes against oxidative damage by increasing the level of reduced glutathione (GSSGR) in the process of aerobic glycolysis. DHQ optically active monomer form is also severed as hydrogen atom donor for reduced glutathione with same decrease in absorbance at 340 nm when it is in oxidized form, being able to be regenerated by uses NADPH as the reducing agent.

According numerous investigations was suggested that poor recovery of DHQ following feeding studies is not the result of ingredient degradation during storage but rather the result of degradation *in vivo* or **during initial ingredient processing** and that the degree of B-ring hydroxylation mediated the degradation of DHQ molecule to the phenolic acid and aldehyde constituents. Also the ability of gut microflora to degrade DHQ to simple phenolic acids have been investigated and concluded that this is the predominant metabolic fate of consumed DHQ. These phenolic acids may have bioactivity and can influence and are influenced by xenobiotic metabolism and can account for some of the bioactivity derived from the consumption of DHQ mainly in **polymerized form**. Namely degradation of DHQ by GIT microbiota results to the formation of 3,4 – dihydroxyphenilacetic acid, the antioxidant ingredient, which is limited for consumption due to the acid is genotoxic in big amounts.[12] USDA Chromatographic examination of the urine of two human volunteers before and after the ingestion of 2 grams of dihydroquercetin demonstrated the conversion of the compound to 3,4-dihydroxyphenyl-acetic, *m*-hydroxyphenylacetic, and 3-methoxy-4-hydroxyphenylacetic acids.[12]

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Genotoxic properties of **FlavitPURE™** - DHQ were *in vivo* studied by the method of chromosome aberrations counting and DNA-comet assay. **FlavitPURE™** was administered repeatedly 5 times (0.15 and 1.5 mg/kg) and once in doses of 15, 150, and 2000 mg/kg respectively, induced no DNA damages in mouse bone marrow, blood, liver, and rectal cells. Single administration of **FlavitPURE™** in doses of 1.5 and 150 mg/kg and 5-fold administration in a dose of 1.5 mg/kg had no effect on the level of chromosome aberrations in mouse bone marrow cells.[13]

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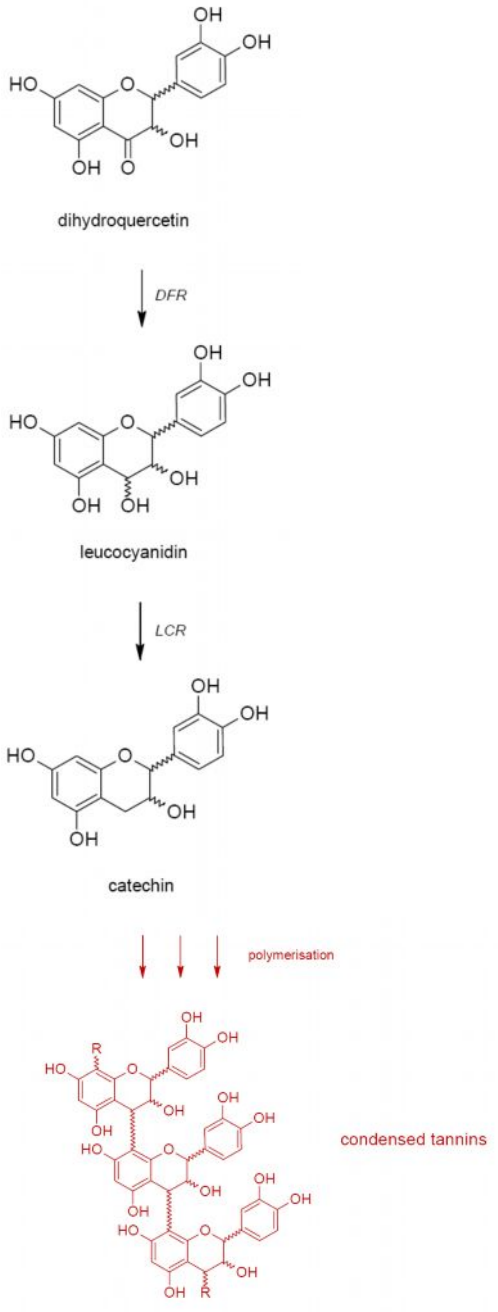
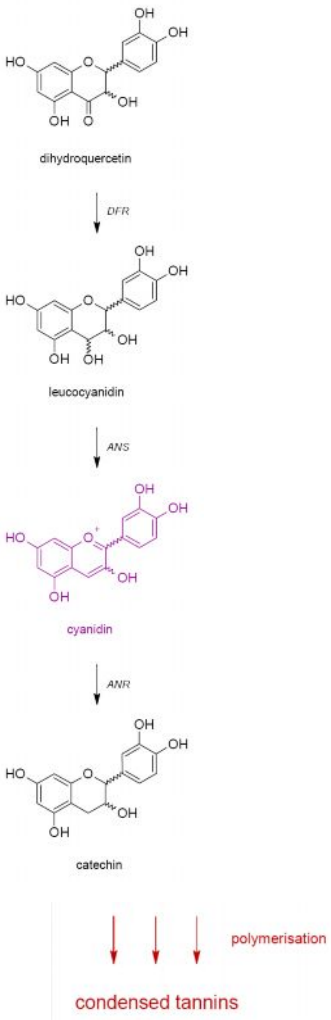
Attachment 1

Catechin biosynthesis from dihydroflavonols and polymerisation reaction to condensed tannins.
F3´H, flavonoids 3´-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *LCR* leucoanthocyanin reductase;



Catechin biosynthesis via anthocyanins and polymerisation reaction to condensed tannins.
F3´H, flavonoids 3´-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase, *ANR*, anthocyanidin reductase;



The catechins, subunits of the other important polymeric pigment class, the condensed tannins, are formed by reduction of either colourless leucoanthocyanins, e.g. leucopelargonidin or leudoanthocyanidin or the strong coloured anthocyanins. Leucoanthocyanidin reductase and anthocyanidin reductase are known as catalysts. Anthocyanins are synthesized via leucanthocyanins by the enzyme anthocyanidin synthase (WINKEL-SHIRLEY 2001, KOES et al. 2005).

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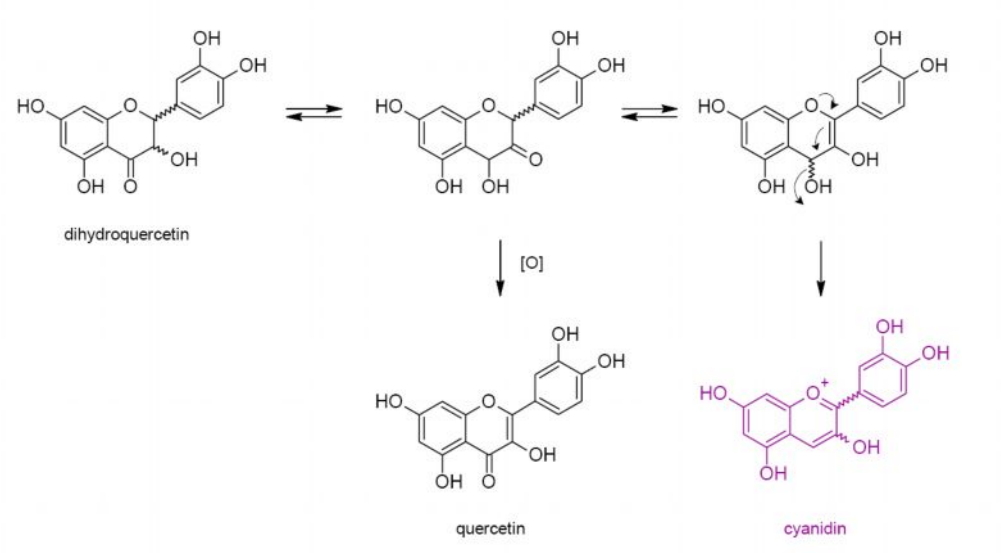
Alternative possibility for anthocyanin and flavonol biosynthesis via dihydroflavonols.



An alternative anthocyanin pathway was published by TORSSELL, 1983 mentioning a synthesis of either flavonols, e.g. quercetin, or anthocyanins such as cyanidin via dihydroquercetin, a dihydroflavonol.
TORSELL, K. (1983) *Natural Product Chemistry: A mechanistic and biosynthetic approach to secondary metabolism*. John Wiley & Sons Ltd.. Chichester [u.a.].

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