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Review

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Natural Phenolic Antioxidants in Human Fluids: Analytical Approaches and Antioxidant Capacity Studies

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Abstract

Phenolic compounds are the most abundant natural antioxidants in our diet. Epidemiological studies have shown the possible prevention effects of consumption of fruits and vegetables rich in phenolic compounds on degenerative diseases, such as cardiovascular diseases and cancers. However, there is a serious lack of fundamental knowledge on the uptake and metabolism of phenolic compounds in humans. It is clear that phenolic molecules, only absorbed by humans, can exert biological effects. This review presents a current knowledge on the analytical methods, antioxidant capacity measurements, as well as research strategies related to natural phenolic antioxidants on human health. Both GC-MS and LC-MS have proved to be very useful analytical techniques that can be employed to identify and quantitate targeted phenolic antioxidants and their metabolites in biofluids. Free radical quenching tests provide a direct measurement of antioxidant capacity but lack specificity and may oversimplify the *in vivo* human physiological environment. Research strategies are diverse and mainly focused on positive health effect of antioxidants. In the future studies, multiple potential bioactivities, both positive and negative, should be considered.

Keywords: *Natural phenolic antioxidants, flavonoids, antioxidant capacity, free radicals, HPLC, GC-MS, LC-MS, plasma, urine.*

Introduction

All aerobic cells can produce free radicals and reactive oxygen species (ROS), which are believed to participate in many bioactivities via redox reactions. For example, *in vivo*, one or two electron reduction of O_2 generates O_2^- radical and H_2O_2, respectively, both of which can convert into OH radical in the presence of transitional metal ions (Fe^{3+} or Cu^{2+}) [1-4]. Hydroxyl radical is extremely reactive and can initiate oxidative modification of biomolecules. Both endogenous and exogenous sources lead to the production of intracellular free radicals. Normally, mitochondrial electron transport, peroxisomal fatty acid metabolism, cytosolic enzyme systems, and phagocytic cells are the major endogenous contributors to the formation of free radicals and ROS. Exogenously, UV irradiation, exposure to environmental toxins, smoking, and even dietary can significantly affect the free radical concentration in human intracellular environment [3-7]. Studies have shown that free radicals are essential for normal cellular metabolism. Free radicals and ROS are extensively involved with gene expression and signal transduction pathways, as well as regulation of cell growth [7-10].

On the other hand, although the related mechanisms have not been well documented, it is conclusive that free radicals can cause harm in human body [11-14]. It is speculated that aging, heart disease, cancer, Parkinson's disease, Alzheimer's disease are all free radical related since free radicals can damage DNA,

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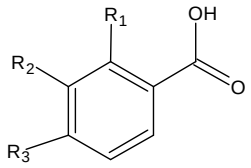
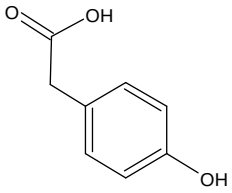
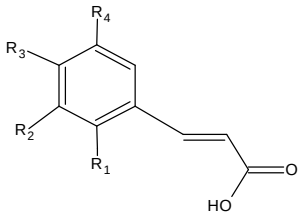
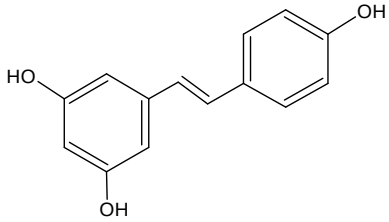


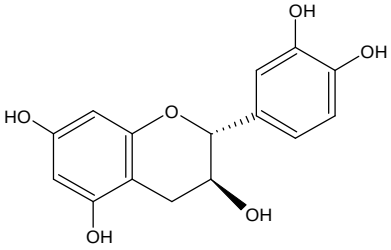


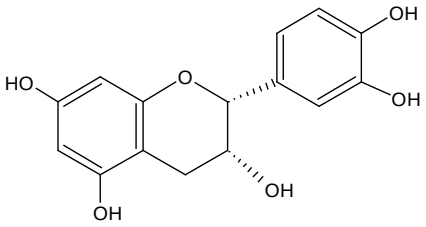
Benzoic acid $R_1 = R_2 = R_3 = H$ *p*-Hydroxyphenylacetic Acid
o-Hydroxybenzoic Acid $R_1 = OH$, $R_2 = R_3 = H$
m-Hydroxybenzoic Acid $R_1 = R_3 = H$, $R_2 = OH$
p-Hydroxybenzoic Acid $R_1 = R_2 = H$, $R_3 = OH$
o-Phthalic Acid $R_1 = COOH$, $R_2 = R_3 = H$
2,3-Dihydroxybenzoic Acid $R_1 = R_2 = OH$, $R_3 = H$
Vanillic Acid $R_1 = H$, $R_2 = OCH_3$, $R_3 = OH$
2,4-Dihydroxybenzoic Acid $R_1 = R_3 = OH$, $R_2 = H$



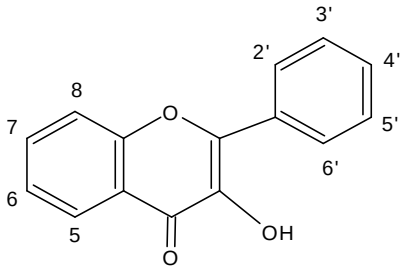


trans-Cinnamic Acid $R_1 = R_2 = R_3 = R_4 = H$ *trans*-Resveratrol
o-Hydroxycinnamic Acid $R_1 = OH$ $R_2 = R_3 = R_4 = H$
p-Coumaric Acid $R_1 = R_2 = R_4 = H$, $R_3 = OH$
Ferulic Acid $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = OH$
Caffeic Acid $R_1 = R_4 = H$, $R_2 = R_3 = OH$
Sinapic Acid $R_1 = H$, $R_2 = R_4 = OCH_3$, $R_3 = OH$





Catechin (-)-Epicatechin



Flavanols

Quercetin 5=7=3'=4'=OH
Myricetin 5=7=3'=4'=5'=OH
Kaempferol 5=7=4'= OH
Galangin 5=7=OH
Fisetin 7=3'=4'= OH

Figure 1. Chemical Structures of Common Natural Antioxidants.

Oxidize cell membrane lipids, and modify enzymes via oxidation if the free radicals and ROS overwhelm the antioxidant systems. Human antioxidant defense system consists of three groups: (i) a variety of antioxidant enzymes such as catalase, and glutathione peroxidase; (ii) a special protein family-peroxiredoxins; and (iii) various small organic compounds such as vitamin A, C, and E, glutathione, and phenolic substances, most of which can be supplied via daily dietary [15-22]. Corresponding antioxidant mechanisms and their sources vary by these species. Antioxidant enzymes and peroxiredoxins are regulated by gene expression and biomessengers. The antioxidant organic compounds are absorbed from ingested food and beverage. The main natural antioxidant components are phenolic compounds derived from vegetables, fruits and other plants, which can be categorized as simple phenolic acids, such as hydroxybenzoic acids, phenylpropanoids (hydroxycinnamic acids), flavonoids (hydroxylated polyphenols), and complex derivatives of these compounds Fig. 1 presents the structures of some common natural phenolic antioxidants. Animal or human cells cannot break down the phenol ring so the consumption of these substances via fruits or vegetables will not produce energy but in the past decade people have never ceased pursuing the possible health benefits associated with the consumption of polyphenol-rich foods, which is due to the fact that phenolic compounds can neutralize free radicals that would adversely affect human health [23-27].

Epidemiological evidence such as studies focused on "French Paradox" makes dietary supplementation a very promising and practical approach to improve the antioxidant defense, which has been supported by amounting *in vitro* and *in vivo* studies. Red wine [28-30], olive oil [31], tea [17, 32], coffee [33, 34], cocoa [35], cranberry [19, 36-39], tomato, and other phenol-rich or flavonoid-rich food and beverage have been examined for their antioxidant potential. Successful application of this approach requires a thorough understanding about the metabolism of antioxidants, mechanism of *in vivo* antioxidant capacity, optimal dosage, and appropriate bioenvironment in which natural antioxidants can affect human antioxidant system. Nevertheless, biospecificity of an antioxidant also should be investigated. For example, vitamin E showed protective effects in Alzheimer's disease but not in early Parkinson's disease [40,41]. Indeed, this approach is much more complicate than it seems to be. This paper provides a minireview of analytical approaches for phenolic antioxidants involved in human health studies.

Analytical Techniques

After ingestion of food and beverage, natural phenolic antioxidants enter human intracellular environment via the circulation. Therefore, a great deal of efforts has been invested on the examination for antioxidants and their metabolites or corresponding biomarkers in human fluids such as urine, serum, or plasma. Various analytical methods have been developed to monitor the bioavailability and bioconcentration of natural antioxidants.

Sample Preparation

Although sample preparation is the first critical step in the characterization and quantitation of phenolic compounds in human fluids, definitive procedures for collection, storage and pretreatment have not been established. Zhang and Zuo [19] have reported that bloods are usually collected into Vacutainer K_2EDTA (potassium ethylenediaminet-etraacetate) tubes to provide anticoagulation. After centrifugation to sediment the cells, the clear platelet-poor plasma was collected and stored at -80^{o}C. In contrast to plasma samples, collection of urine is relatively easier.

Phenolic compounds are present predominantly as glucuronide and sulfate conjugates in food products and human fluids [19, 20, 37-39, -44]. A hydrolysis step is usually involved prior to extraction and analysis of phenolics. There are two main procedures to cleave the glycoside and ester bonds reported in the literature, acid- and enzyme- catalyzed hydrolysis [19, 44]. Hydrochloric acid (HCl) in aqueous or methanol solvent is commonly used. To determine specifically the amount of glucuronides or sulfates, β-glucuronidase or sulfatase is used. A mixture of β-glucuronidase and sulfatase can be employed for the determination of total phenolics. These enzymes are commercially available. The released

phenolic compounds are then extracted using an organic solvent, most commonly ethyl acetate or methylene chlorid, or a C_{18} solid-phase extraction (SPE) [45]. It is important to control pH values for both SPE and classic liquid-liquid extraction processes. Ascorbic acid is commonly added into human fluid samples to prevent oxidation of phenolic compounds. When GC or GC-MS is used for the analysis, extraction is often followed by conversion of phenolic molecules into their trimethylsilyl derivatives. The recent application of advanced LC-MS allows simultaneous determination of free and conjugated phenolic compounds in human fluids without a hydrolysis step.

Spectrophotometric methods

The total phenol content of foods, beverages and human fluids is traditional determined as gallic acid equivalents using Folin Ciocalteu reagent [27,46] or a modified method by Swain and Hillis [47] which avoids interference from proteins in biological fluids [48,49]. The blue color formed after reaction of phenolic compounds with Folin-Ciocalteu reagent is measured at 725-735 nm. A disadvantage is that reducing substances, such as ascorbic acid, and transition metals, such as Fe and Cu, may interfere the measurement. All phenolic compounds absorb radiation in the UV region and could be determined by their characteristic absorbance at 280 nm [37]. To determine individual phenolic compounds based on UV spectrometric characteristics, a UV absorption or a photodiode array detector (PAD) is coupled with high performance liquid chromatography (HPLC) or capillary electrophoresis (CE).

HPLC/CE with UV absorption, PAD or electrochemical detection

HPLC combined with UV absorption or PAD has been used in the determination of phenolic antioxidants in foods and human fluids. Specific highly purified deconjugating enzymes in combination with HPLC-PAD has also been employed for analysis of plasma samples, containing conjugated phenolic antioxidants and their metabolites [45, 50]. But the identification based on UV spectra has been a major problem. Not only are the *in vivo* phenolic concentrations near the detection limits, but the resolution of HPLC is not usually sufficient to separate analytes from sample matrices clearly and the retention times can shift due to residual protein present in biological samples [38,51]. Obviously, retention time and UV-absorbance are useful but inadequate as sole identification and quantification means. HPLC with coulometric-array detection can provide fingerprint-type information on the nature of a compound [52]. However, this technique only allows the identification of previously known substances [53]. The information and availability of the metabolic standards are required for targeted antioxidants. Capillary electrophoresis (CE) is an efficient separation technique, especially when coupled with an on-line photodiode array detector (PAD), for the analysis of phenolics [54, 55]. Unfortunately, CE does not have a good reproducibility, and PAD sensitivity and identification power is not sufficient for characterization of metabolites of phenolic antioxidant [56, 57].

GC-MS methods

Since the first studies of metabolites in the late 1960s by Horning's [58] research group, GC-MS has become one of the most popular analytical techniques for the analysis of complex biological samples due to its extremely high separation and identification power [19, 30, 39, 59]. However, all phenolic antioxidants contain polar functional groups, have a relatively low volatility and are not suitable for direct capillary GC analysis. Derivatisation steps aimed to produce more volatile products thus are required to improve the stability and sensitivity of subsequent GC determination. Zhang and Zuo [19] developed a derivatization procedure using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) reagent to identify phenolic and benzoic compounds in human plasma that originate from cranberry juice. Because the previous derivatization protocol did not work well for the flavonoids in plasma samples, the researchers increased the derivatization temperature and time. With the GC-MS method developed, Zhang and Zuo identified 15 flavonoids and phenolic and benzoic acids in cranberry juice, and 7 in human plasma 4.5 hours after consumption of the cranberry juice. The abundant

information derived from GC-MS chromatogram (retention time, peak height and area) and Mass spectra (molecular ion and characteristic fragments) provide an excellent means for identification, quantification, and characterization of phenolic antioxidants and their unknown metabolites in human fluids. But the advantages of GC-MS analysis of phenolic compounds are somehow offset by loss of sample during the additional manipulation.

LC-MS methods

Although GC-MS techniques are widely used in bioavailability studies of phenolic antioxidants, in the recent years, LC-MS has been increasingly employed for the phenolic determination in human fluids and other biological samples [60-65]. Unlike GC-MS, LC-MS technique does not require a tedious derivatization process. LC-MS, especially equipped with an electrospray ionization (ESI) interface between HPLC and MS units, can be applied directly to the analysis of thermally unstable or involatile phenolic antioxidants in plasma samples. Meanwhile, this tandem technique also provides rich information related to the molecular weight and structure of a compound since ESI-MS produces mainly molecular ions $[MH]^+$, which is critical to the structure identification [60-63]. However, LC-MS suffers from matrix effects in which other ions present may influence the determination of the desired phenolic compounds, and LC cannot handle the large number of similar molecules that may exist in human fluids. Table 1 summarized some published chromatographic methods and the targeted phenolic analytes.

Table 1. List of some published chromatographic methods and the targeted phenolic analytes

Technique	Human Fluid	Source of antioxidants	Targeted Antioxidant	Ref.
HPLC	serum	virgin olive oil	vitamin/uric acid	[66]
HPLC	plasma	foods	quervetin	[43]
HPLC	plasma	olive oil	oleuropein	[67]
HPLC	plasma	foods	flavonoids/phenolic acids	[52]
HPLC	plasma	beer	phenolic acids	[44]
HPLC	plasma	wine	phenolic compounds	[42]
HPLC	plasma	Concord grap juice	flavonoids/-tocopherol	[68]
HPLC	plasma	coffee/black tea	homocysteine	[69]
HPLC	plasma/urine	mycophenolate mofetil	mycophenolic acid	[55]
HPLC	plasma/urine	acidum gallicum tablets	galiic acid	[70]
LC-MS	plasma/urine	conventional/organic food	flavonoids	[60]
LC-MS	plasma/urine	prunes	hydroxycinnamates	[61]
LC-MS	plasma	walnut	polyphenolics	[62]
LC-MS	plasma	tomato	flavonol glycosides	[63]
LC-MS	plasma	virgin olive oil	phenolics	[64]
LC/GC-MS	plasma	blachcurrant juice	polyphenols	[65]
GC-MS	urine	red wine	phenolic aicds	[30]
GC-MS	plasma	red wine	catechin	[59]
GC-MS	plasma	cranberry juice	phenolic compounds	[19]

Sound analytical methods can provide accurate and rich information, which can help researchers to better understand antioxidants. A better understanding about antioxidants will, in turn, help to design better analytical methods. With the development in computer science and engineering, people will be able to better monitor the metabolism of antioxidants of interest; improve the sensitivity; minimize the matrix interference of plasma, urine or serum samples; and select the priority of analysis – antioxidants themselves, their metabolites or corresponding biomarkers.

Major antioxidant capacity measurements

Except identification and quantitation of phenolic antioxidants, researchers are also interested in examining their antioxidant potential under normal bioenvironment. It is useful to quantify the antioxidant potential of a compound, a food, or a beverage, which will make comparison possible since there are so many foods that contain antioxidants and so many compounds that have antioxidant capacity.

Oxygen-radical absorbing capacity (ORAC) assay [71] of antioxidants in serum or plasma uses beta-phycoerythrin as an indicator protein and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator. Under appropriate conditions, the loss of phycoerythrin fluorescence in the presence of reactive species is an index of oxidative damage. The inhibition by an antioxidant, which is reflected in protection against the loss of phycoerythrin fluorescence is a measure of its antioxidant capacity. O'Byrne *et al* [68] measured the antioxidant potential of serum by the ORAC assay after the supplementation of Concord grape juice. Natella *et al* [34] used a similar method, TRAP (total radical trapping antioxidant parameter, which is expressed as the amount of peroxyl radicals trapped by 1L of plasma.) to evaluate the antioxidant potential of human plasma after consumption of coffee and tea. The Trolox equivalent antioxidant capacity (TEAC) is defined as the concentration of Trolox with the same antioxidant capacity as a 1 mM concentration of the antioxidant under investigation. The assay is designed to test the ability of an antioxidant to scavenge a preformed radical, cation chromophore of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+ radical), in relation to that of6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), an aqueous soluble vitamin E analogue [60,72,73]. Similarly, a method using 1,1-diphenyl-2-picrylhydrazyl (DPPH.) as a reactive free radical has been used to test the antioxidant activity of naturally occurring phenolic compounds resulted from their electronic structure.

The resistance of LDL to oxidative species can also reflect the antioxidant capacity of a compound since after intestinal absorption some suspected antioxidants or their metabolites would bind LDL, which can affect the oxidation of LDL. Princen *et al* [74] reported a method, in which LDL was oxidized via exposure to copper ions and oxidation kinetics were determined by measuring the formation of conjugated dienes using UV. Caccetta *et al*. [30] used the method to examine whether ingestion of red wine could affect *ex vivo* lipoprotein oxidizability.

These assays cannot simulate the real free radical environment in human biosystems because of the simplified design, in which there is only one free radical generator. The result is only valid if antioxidants would only behave as a scavenger of free radicals. Indeed, there are various sources of free radical in humans and antioxidant mechanism varies by different antioxidants, which has not been well understood. At least, according to published studies an antioxidant in biofluid can prevent oxidation damage by scavenging radicals or inhibiting the free radical formation [2, 75] so the antioxidant capacity measured by these assays are useful but incomplete. They are just an estimation of the antioxidant capacity of a targeted compound under manipulated conditions. In some studies the antioxidant capacity could not explain biospecificity of some antioxidants [5]. Under difference circumstances, the free radical attack could be universal or specific. Aging has long been related with oxidative stress but studies found that not all the proteins or cells were oxidized at same rate [76]. Would natural antioxidants provide same protection against free radicals or ROS under these conditions? Nevertheless, antioxidant capacity measurements focus on LDL may ignore a very important fact that natural antioxidants not only bind LDL but also other proteins in plasma. Therefore it is not uncommon that some phenols show antioxidant capacity *in vitro* but not *in vivo* [66, 77, 78].

Compared with these assays, some indirect measurements based on biomarkers or products of free radical reaction seem more specific and accurate [50, 60, 68]. Under the attack of free radicals, there must be some intermediate products or modification on normal proteins. Information regarding these biomarkers can be directly linked to certain diseases. Monitoring these species might

be more rational way to present the antioxidant capacity of a compound. However, this approach requires the detailed knowledge of the mechanism of free radical reaction.

Research strategies

Current research strategies are very diverse but are dominated by the assumption that natural antioxidants can benefit humans. Some researchers focus on a particular antioxidant for various purposes such as an antioxidant compound with a high content in certain food, a compound exhibits significantly higher antioxidant capacity than others, or a compound with a potential pharmaceutical application to certain disease. Quercetin [50] is an antioxidant that has been investigated extensively for the above reasons. For this kind of antioxidant, their food source and analytical methods used to monitor their bioavailability, *in vitro* antioxidant capacity, and *in vivo* metabolism have been of great interest. Approach from this angle provides specific information and in-depth understanding of the metabolism, bioconcentration, health effect of a natural phenolic antioxidant. But before its antioxidant acting mechanism is well understood, researchers should not overestimate its antioxidants potential. Neither should they overlook potentials of other co-existing antioxidants in the same food.

Some researchers emphasize on certain food or beverage due to epidemiological evidence that consumption of the food or beverage has a negative correlation with certain diseases or the total phenol content is very high, which makes it a good source of antioxidant. Studies of consumption of olive oil, red wine, and cranberry fit in this category. Generally, identification and quantification of antioxidants existing in the food are the first step. Then, emphasis would be laid on the dominant compounds or special antioxidants that have never been found in other sources. Human study is very common in this category. So far, it is less challenging to find the correlation between the consumption of the food and its effect on human health than to clarify the mechanism. This is due to the complex nature of metabolism and limited understanding about the cancer, cardiovascular diseases, and other targeted diseases. In addition, results of human studies vary by selected subjects, design of studies, and some uncontrollable factors.

Based on priorities of a study, two approaches could be applied independently or together. Investigation on different foods would help to find better source for natural antioxidants. Focusing on an antioxidant would lead to a better understanding on its metabolism and health potential. Currently, the studies of natural antioxidants have been dominantly focused on their health benefits. It is known that free radicals are involved in many bioactivities such as gene expression. Too many free radicals can cause oxidative stress, while if the concentration of free radical is lower than normal level it will impair host defenses and decrease proliferative response. It is interesting to understand whether consumption of certain antioxidant-rich food would result abnormally low concentration of free radical; Furthermore, whether these natural antioxidants would affect other physiological functions or not.

Future studies of antioxidants

Epidemiologic studies show that consumption of certain food may benefit human health by improving antioxidant defense, which can be linked with various diseases such as Alzheimer's and Parkinson's diseases caused by free radicals. Molecular biologists have studied these diseases by starting with the proteins or DNA's affected by free radicals. If they can identify the impaired DNA or protein they will repair it via gene therapy. But the risk of gene therapy is that it might permanently change the genetic characteristics of a patient and the change could be passed to next generation. The alternative approach would be to design a drug that can eliminate the excess free radicals or minimize the damage caused by free radicals. However, the process of drug development is extremely time consuming and expensive. Obviously, dietary supplementary seems more practical and cost and time efficient but it lacks the specificity of gene therapy or drugs. To bridge the gap between these approaches and make them complementary to each other would fully utilize the health benefits of natural antioxidants.

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