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An approach about *in vitro* antioxidant capacity of plant foods and beverages

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Abstract

Oxidative stress leads to the formation of potentially toxic and harmful compounds to our body. These compounds can cause harm to our health by increasing the risk of heart diseases and degenerative diseases, and they also lead to aging. However, several epidemiological studies conducted in recent decades suggest that the consumption of vegetables and beverages rich in natural antioxidants may reduce the incidence of diseases related to oxidative stress. As a result, several studies have evaluated the *in vitro* antioxidant capacity of different plant foods and beverages. Yet, several factors may influence the *in vitro* antioxidant capacity of a sample, particularly in complex matrices such as wine, fruits and other vegetables, and lead to significant differences in the results. In this work, the main factors that may influence the *in vitro* antioxidant capacity of a sample were addressed. It can be concluded that different tests to different mechanisms still may and should be employed when determining *in vitro* antioxidant capacity. Moreover, the kinetic curve of the antioxidants of the studied sample should also be drawn, as it allows to explain how antioxidants work. In addition, the curve enables the prediction of the antioxidant behavior in living organisms.

Keywords: Natural Antioxidants. Antioxidant Capacity. Plant Foods. Beverages. Analytical Methods.

Introduction

It is known that oxidative stress leads to the formation of compounds that are potentially toxic and harmful to our body, such as free radicals. The latter act on vital cellular components such as lipids, proteins and DNA, and they can harm human health, increasing the risk of heart diseases and degenerative diseases as well as leading to aging.[1,2,3] However, several epidemiological studies conducted in recent decades suggest that the consumption of vegetables and beverages rich in natural antioxidants may reduce the incidence of diseases related to oxidative stress.[4]

The optimal activity of natural antioxidants is due to the chemical structure of these substances. In flavonoids, for example, antioxidant activity is associated with the presence of hydroxyl groups at positions 3 'and 4' of the B-ring, which give high stability to the radical formed by participating in the displacement of the electron, and also with a double bond between carbons C_2 and C_3 of the C-ring together with the carbonyl group at the C_4 position, which enables the displacement of an electron of the B-ring. In addition, the free hydroxyl groups at position 3 of the C-ring and at position 5 of the A-ring, together with the carbonyl group at position 4, are also important for the antioxidant activity of these compounds (Figure 1). Quercetin, a flavonoid found in high quantities in onions, tea and apple, combines all these features and is one of the most powerful natural antioxidants known.[5]

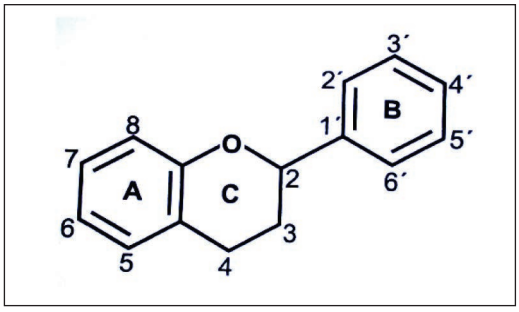

Figure 1. General chemical structure and numbering pattern for flavonoids.

As for their mechanism of action, several natural antioxidants can act both as primary antioxidants or free radical scavengers, and as secondary or preventive antioxidants.[6] Primary antioxidants remove free radicals from the human body by interrupting free radical chain reactions by donating a hydrogen atom to the radical (Figure 2). Due to their molecular conformation, the phenoxy radicals formed are quite stable intermediates and do not easily start a new chain reaction. These radicals react with other free radicals, blocking the propagation reactions of oxidation.[7]

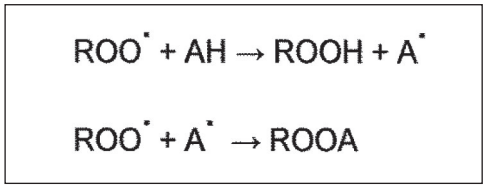

Figure 2. Mechanism of action of primary antioxidants or free radical scavengers.

Secondary antioxidants can act through numerous mechanisms, reducing or slowing down the oxidation initiation rate by blocking the decomposition of peroxides and hydroperoxides. As singlet oxygen suppressors, secondary antioxidants capture energy from singlet oxygen ($^1O2$), which returns to the ground state (O_2). As metal ion chelates, these antioxidants are able to interact and disable metal ions that catalyze lipid peroxidation reactions.[8]

As a result, several studies have evaluated the *in vitro* antioxidant capacity of different plant foods and beverages.[9,10,11] However, several factors can influence the *in vitro* antioxidant capacity of the sample, leading to significant differences in the findings.

Main factors that can influence *in vitro* antioxidant capacity

Procedure for isolating and purifying the substance

Due to the large existing chemical diversity, antioxidants are difficult to separate and quantify individually in biological matrices.[12] On the other hand, it is known that synergistic antioxidant combination in food is more effective than the sum of the individual effects. As a result, several studies have evaluated the total antioxidant capacity of different biological samples, particularly in complex matrices such as wine, fruits and other vegetables. However, amino acids and other non-antioxidant constituents of these matrices, such as uronic acids, may overestimate the results.[13]

Type and polarity of extraction solvent

To separate nonpolar from polar substances, which exhibit activity, it is necessary to make the correct choice of solvents. However, the most important natural antioxidants are vitamin C as well as most of the phenolic compounds (flavonoids, tannins, phenolic acids and coumarins) classified as hydrophilic antioxidants, in addition to carotenoids and vitamin E, classified as lipophilic

antioxidants. Thus, the solubility of these substances is also directly associated with the nature of the solvent used in the extraction.[14] For this reason, the *in vitro* antioxidant capacity of a sample can differ significantly by the same method in different solvents because its antioxidants may not behave the same way in all the solvents used, interacting differently with them.[15]

Thus, in any test of antioxidant capacity, the results should be measured in extracts or solutions and associated with a calibration curve in the same solvent. Depending on the characteristics of the sample and the natural antioxidants under study, our studies used sequential extraction with solvents of different polarities [16] (Figure 3).

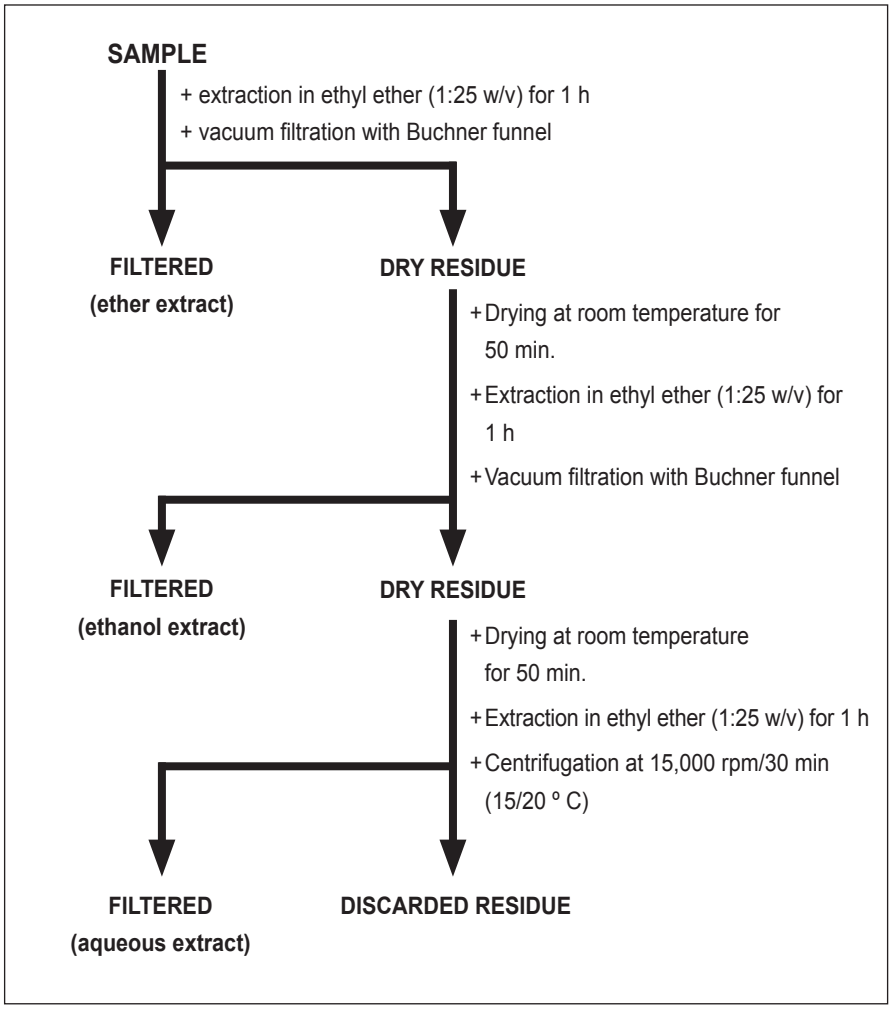

Figure 3. Sequential extraction with solvents of different polarities.

In this technique, extraction is performed first with ethyl ether (lower polarity solvent). Then, the residue of the first extraction is dried and extracted in ethyl alcohol (intermediate polarity solvent). Finally, the residue of the second extraction is also dried and extracted in water (higher polarity solvent). Our results showed a higher content of dry residue in the ether extract, which is indicative of increased presence of low polarity compounds in this extract: more specifically, lipids present in high amounts in the samples.

Antioxidant Test System

To date, there are still no approved or standardized methods for determination of antioxidant capacity. The methods used for determining *in vitro* antioxidant capacity are assays which involve different mechanisms of the antioxidant defense system of our body, and they can be divided into two groups: methods based on removal of free radicals from the environment and methods that employ lipid as a substrate. Methods based on removal of free radical from the environment involve the generation of different radicals through a variety of mechanisms, as well as a range of final products, at a fixed time or over a time interval.[17]

Among the methods that employ lipids as substrate, the great diversity of existing analytical methods (chemical, physical and/or physico-chemical) also poses some difficulties in selecting the most suitable method for a particular evaluation study of antioxidant capacity.[18] Depending on the system in which the antioxidants were evaluated (aqueous or lipid), model compounds were shown to be strongly antioxidant in one method and pro-oxidant in another.[19] The so-called "polar paradox" was also observed, i.e., hydrophilic antioxidants were more effective than lipophilic ones in high volumes of oil, while lipophilic antioxidants were more active in emulsions.[20]

Substrate to be protected

According to the method selected, different substrates are used to measure the protective action of antioxidants against lipid oxidation. Oils from sunflower, soybean, olive and palm are commonly used as substrates in lipid systems. Similarly, liposomes and microsomes are used in systems similar to the practical conditions *in vivo*, because the lipid membrane and the biological membranes have a similar composition. Human LDL is also used as a substrate; it simulates the inhibition of oxidation of low density lipoproteins in blood plasma after oral administration of antioxidants.[21] According to the nature of the substrate to be protected by antioxidants, significant differences can be found in the *in vitro* antioxidant capacity of the sample.

Main antioxidant methods

Among the methods based on removal of free radicals from the environment, the most frequently used in determining the *in vitro* antioxidant capacity are the following assays: ferric reducing antioxidant power (FRAP), radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS radical cation decolorization, and oxygen radical absorbance capacity (ORAC). The main methods that employ lipids as substrate are the β-carotene linoleic acid system, the thiobarbituric acid reactive substances (TBARS) assay and human LDL.

The *in vitro* antioxidant capacity can and should be evaluated with different assays for different mechanisms. However, the diversity of test conditions and model systems used for the determination of antioxidant capacity has generated some difficulties in interpreting and comparing the results obtained through these methods. Nevertheless, TROLOX (a water-soluble vitamin E analog) can be used as a standard antioxidant in different methods.[22] Thus, for comparison purposes, the results of different methods can be expressed as TROLOX equivalents.

Importance of kinetic study

The kinetic study of antioxidants in the sample analyzed is important, since it allows to elucidate the details of the behavior of these substances. First, this study helps explain how antioxidants work. In addition, it allows to predict the behavior of these substances in living organisms.[23] Therefore, the readings obtained during the determination of *in vitro* antioxidant capacity enable the kinetic curve of the antioxidants of the sample to be traced and their effectiveness to be evaluated, as appropriate, for analysis in variable time intervals.

Figure 4 shows, respectively, the kinetic curves of the antioxidant capacity of the ethanol extract and aqueous extract of a sample, compared with Trolox and BHT as standards, in the ORAC method.

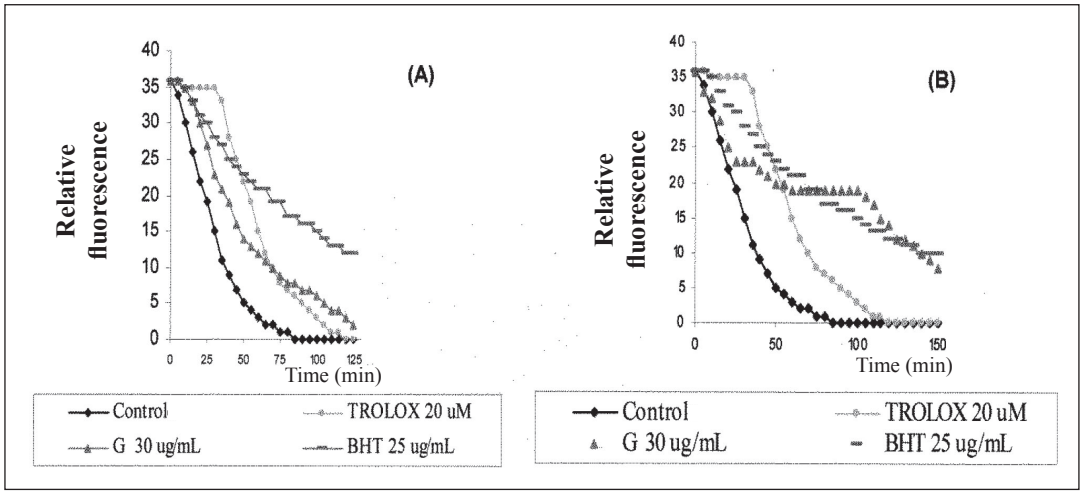

Figure 4. Kinetic curve of the antioxidant capacity of A) ethanolic extract and B) aqueous extract of a sample, compared with Trolox and BHT as standards, in the ORAC method (Control - without antioxidant, G - sample).

As can be seen in Figure 4, each sample exhibited different behavior in the OAC assay. In the study concentrations, the Trolox standard showed the highest antioxidant capacity among the samples in the first 50 minutes of the assay. However, after this time, the antioxidant capacity of Trolox decreased quickly. The aqueous extract showed strong antioxidant capacity compared with ethanol throughout the assay. However, the antioxidant capacity of the aqueous extract was below that of BHT in the first 65 minutes, remained constant between 65 and 100 minutes, and was higher than that of BHT between 85 and 100 minutes. After that time, the antioxidant capacity of the aqueous extract decreased again.

Conclusion

Considering all the factors that can influence the *in vitro* antioxidant capacity of a sample, the selection of the most appropriate method for this measure should be carefully made. Different tests for different mechanisms can and should be employed. However, for comparison of results obtained by different methods, they can be expressed as Trolox equivalents.

The kinetic curve of antioxidants in the sample must also be drawn, because it explains how antioxidants work, and it also allows the prediction of their behavior in living organisms.

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