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Methods for determining in vitro antioxidant capacity

Métodos para determinação de atividade antioxidante in vitro

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RESUMO

Os métodos para medir a atividade antioxidante, in vitro, são cruciais na avaliação do potencial de substâncias antioxidantes em alimentos e compostos isolados, devido a simplicidade, custo relativamente baixo e rapidez, o que permite a triagem eficiente de inúmeras amostras. Os métodos amplamente utilizados são ORAC, ABTS, DPPH, FRAP e Folin Ciocalteu. A padronização é um desafio, pois diferentes ensaios avaliam propriedades antioxidantes específicas. Os métodos in vitro, no entanto, têm limitações. Eles não replicam totalmente o complexo sistema biológico humano onde ocorrem interações dinâmicas entre antioxidantes, enzimas e radicais, mas a correlação entre resultados in vitro e in vivo nem sempre é possível. Contudo os métodos in vitro ofereçam triagem rápida e insights preliminares sobre o potencial antioxidante, e devem ser complementados por estudos in vivo. A seleção de métodos apropriados depende dos objetivos da avaliação, e a combinação de múltiplas técnicas pode fornecer informações mais abrangentes sobre as atividades antioxidantes para a promoção da saúde através dos alimentos.

Palavras-chave: Atividade antioxidante; radicais livres; métodos in vitro; alimentos; potencial antioxidante.

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ABSTRACT

Methods for measuring antioxidant activity, *in vitro*, are crucial in evaluating the potential of antioxidant substances in foods and isolated compounds, due to their simplicity, relatively low cost and speed, which allows efficient screening of numerous samples. The widely used methods are ORAC, ABTS, DPPH, FRAP and Folin Ciocalteu. Standardization is a challenge, as different assays evaluate specific antioxidant properties. *In vitro* methods, however, have limitations. They do not fully replicate the complex human biological system where dynamic interactions between antioxidants, enzymes and radicals occur, but correlation between *in vitro* and *in vivo* results is not always possible. However, *in vitro* methods offer rapid screening and preliminary insights into antioxidant potential, and should be complemented by *in vivo* studies. The selection of appropriate methods depends on the objectives of the assessment, and the combination of multiple techniques can provide more comprehensive information on the health-promoting antioxidant activities of foods.

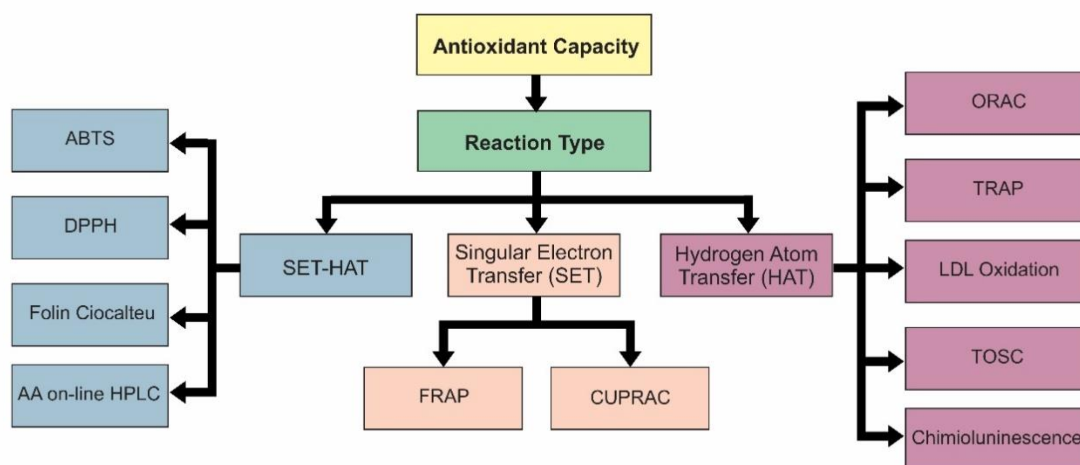
Keywords: Antioxidant activity; free radicals; *in vitro* methods; food; antioxidant potential.

INTRODUÇÃO

Measurements of antioxidant activity in vitro are important sources of information on free radical scavenging by antioxidant substances present in a food or even substances isolated from a separation method. Figure 1 shows some methods currently used to determine this property and their respective mechanisms of action. However, there is still a lack of standardization in the experimental procedures and in the expression of the results, and no method has yet achieved unanimous acceptance. Thus, one of the main challenges in the evaluation of antioxidant potential is to know which methods are most suitable for a specific application (Apak et al., 2016).

Importantly, most authors do not distinguish the terms antioxidant capacity from antioxidant activity, but some authors classify capacity measures as endpoint assays, which measure the inactivation of reactive species in a fixed time (thermodynamic methods) and activity measures those kinetics-based assays expressed as reaction rates or elimination percentages per unit time. In the present work we treat both terms interchangeably (Apak et al., 2013; Apak et al., 2016).

Figure 1: Some methods for antioxidant assay and their mechanisms.



Source: Authors (2023).

There are several methods and techniques for determining AA such as those that look for activity against specific radicals like radical sequestering assays: superoxide, nitric oxide, hydroxyl, and hydrogen peroxide (Fernando and Soysa, 2015; Lalhminghlui

and Jagetia, 2018) Chemiluminescence methods, which use luminol and fluorescence measurement with the excitation lamp turned off (Pogačnik and Ulrih, 2012). Total oxyradical elimination capacity (TOSC) method, which measures the decrease in ethylene gas production caused by inhibition of thermal hydrolysis by two reagents and in the presence of antioxidant compounds (Garrett et al., 2010). Lipid peroxidation method that uses fluorescence and thiobarbituric acid (Chang and Kim, 2018).

In addition, online HPLC methods available measure the AA of each species eluted from the column through a T-type connection through which the reagent (DPPH) enters and a second UV-VIS detector is coupled (Pedan et al., 2016).

There are also in vitro methods with biological particularities, like deoxyribose degradation assay. This method is widely used to evaluate the hydroxyl radical and can simultaneously measure pro-antioxidant and antioxidant activities depending on the reaction medium employed. Furthermore, the anti-hemolytic activity assay measures the ability to inhibit hemolysis in red blood cells exposed to oxidative stress by ROS in the presence of antioxidant substances (Chobot, 2010; Karim, et al., 2020).

Despite all the variety and even specificity of some methods available, the most widely used in the scientific literature today are still: ORAC, ABTS, DPPH, FRAP, and Folin Ciocalteu, which will be further detailed (Mazumder et al., 2020).

The SET and HAT mechanisms

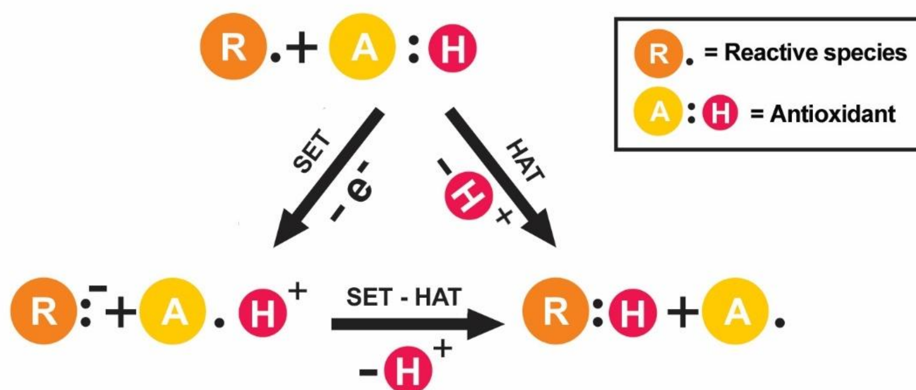
As mentioned above, in methods for measuring AA in vitro antioxidant substances react with free radicals generated in the medium by two different mechanisms: SET or HAT or a combination of both. The HAT-type reaction occurs in a single step (Figure 2), where the free radical removes a hydrogen atom from the antioxidant substance and thus the antioxidant itself becomes a radical (undergoes oxidation). The SET mechanism occurs by the transfer of a single electron from the antioxidant substrate to the free radical, producing a radical intermediate and itself becoming oxidized to a cation. Depending on properties such as ionization potential, solubility and partition coefficient of the radical formed, a second step can occur, in this case a HAT step, where the hydrogen from the cation is captured giving rise to a stable molecule and a radical of the antioxidant substance (Kumar et al., 2017). The HAT and SET mechanisms provide conceptually different information:

- HAT is a kinetic measurement, while SET is a thermodynamic measurement based on the redox potential of the reactants.

- HAT-based assays (ORAC, for example) allow you to estimate the capture capacity of most reactive compounds.

- SET-based assays (FRAP, for example) provide an overall picture of the oxidation/reduction efficiency of all antioxidants present in the sample, including the "slow" ones, which are not detected by kinetic methods (Ferreira and Avaca, 2008; Sousa, 2013; Liang and Kitts, 2014).

Figure 2: General scheme of the antioxidant reaction steps of SET and HAT mechanisms.



Source: Authors (2023).

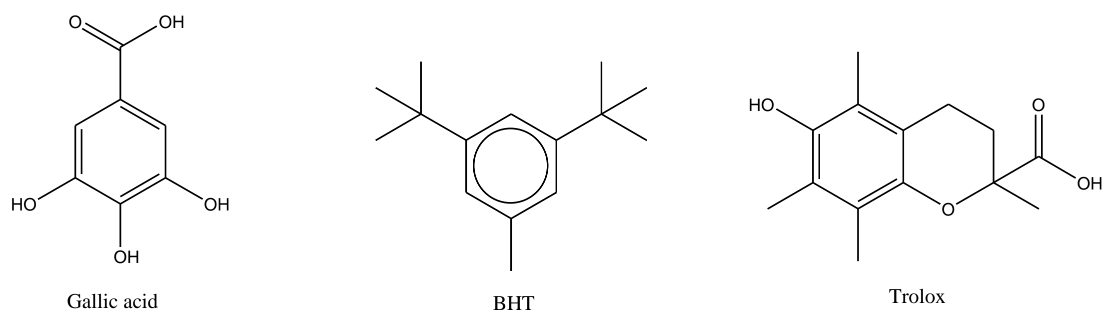
Data supplementation with biological assays nowadays is important since the antioxidant capacity estimated from HAT and SET mechanisms does not have a correlation with a live organism process. There are several methods for assessing antioxidant capacity, especially in foods, using vivo methods, like specific cell cultures and biological activity methods (Zhang et al., 2017; Furger, 2021).

Equivalence methods

The obtained AA measurements can be compared with the response of known antioxidants by using linear regression curves for known antioxidant substances (Figure 3). This is an attempt to normalize the response of the method by comparing it to the response of another antioxidant. According to Litescu et al., 2014, the most commonly used antioxidant substances in this attempt at response standardization are: Trolox (6-hydroxy-2-5-7-8-tetramethylchromo2-carboxylic acid - an analog of vitamin E), vitamin

C, gallic acid, quercetin and BHT. Therefore, the TEAC (Trolox Equivalent Antioxidant Capacity) term refers to a normalization made in method results from a calibration curve made with Trolox data (Branina et al., 2019).

Figure 3: Some chemical structures of standard antioxidant molecules.



Source: Authors (2023).

Most used in vitro methods

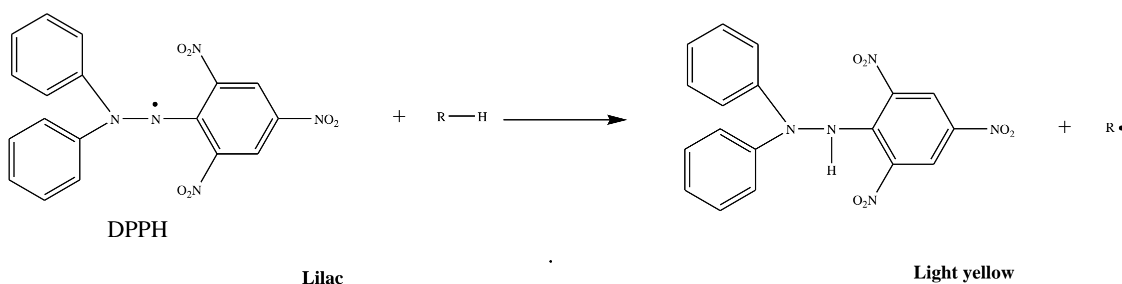
DPPH

This method is based on measuring the antioxidant ability of a substance to sequester the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical by reducing it to hydrazine (DPPH-H) (Figure 4). DPPH is a stable, violet-colored, organic nitrogen radical that has absorption in the range 515-520 nm. The reduction of the DPPH radical is monitored by the decrease in absorbance after reacting with the antioxidant substance(s) in the sample to a light-yellow color. The color change is stoichiometric and depends on the number of electrons and hydrogens captured in the SET-HAT sequence, so the color change can be from decreasing in intensity from purple to producing a light yellow. The absorbance reading should occur exactly 30 minutes after the start of the reaction in a light-free environment (Blois, 1958; Akar et al., 2017).

The IC 50 is a way to express the Capture Index (CI) of the reductant (antioxidant substance) by the DPPH radical. The IC50 expresses the sample concentration capable of reducing the radical by 50%. the higher the calculated IC50 value, the lower the antioxidant activity, because a high sample concentration is required to halve the DPPH radical. To calculate the IC50, it is necessary that multiple dilutions of the sample be made and a linear concentration regression curve be constructed. The use of sample

concentration curves is one more criterion that can be added to all methods to ensure linearity over the concentration range used (Shimamura et al., 2014).

Figure 4: Chemical reaction of DPPH with an antioxidant.



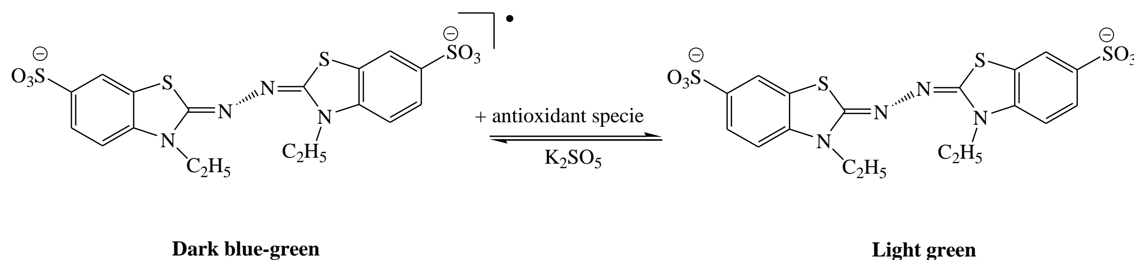
Source: Authors (2023).

ABTS (TEAC: Trolox Equivalent Antioxidant Capacity)

This method is based on the generation of $ABTS^{\bullet+}$, which has a blue-green (dark) color, through reaction with potassium persulfate (Figure 5). With the addition of an antioxidant contained in the sample, the reduction of $ABTS^{\bullet+}$ to ABTS occurs, promoting the loss of the coloration of the reaction medium to a greenish-yellow (light green) tone. the method is based on the ability of the substances present in the sample to inactivate the $ABTS^{\bullet+}$ radical in a given time (6 minutes). The $ABTS^{\bullet+}$ solution should be stored in a dark place at room temperature for 12 to 16 hours before use. The absorbance reading is taken at 734 nm. The percentage of $ABTS^{\bullet+}$ inhibition is calculated and transformed to Trolox Equivalent using trolox, a standard correlated to vitamin E, which is subjected to the same conditions as the antioxidant analysis by means of a calibration curve. The results are expressed as TEAC (Trolox Equivalent Antioxidant Capacity). The final TEAC unit is expressed as µmol of Trolox /g of sample (Miller and Rice-Evans, 1997).

It is important to check the absorbance of the $ABTS^{\bullet+}$ solution throughout all readings due to the instability of the radical. If necessary, the concentration should be adjusted to absorbance values of up to 0.8 ± 0.02. Concentration adjustment is done by dilution with 95% ethanol or by concentrating from the solution of the ABTS+ radical until the reading is in the original solution range. This check should be repeated at every third reading of samples, blank, and at the curve reading (all prepared in triplicate) (Olszowy and Dawidowicz, 2018).

Figure 5: Chemical reaction of stabilization of the $ABTS^{\bullet+}$ radical.



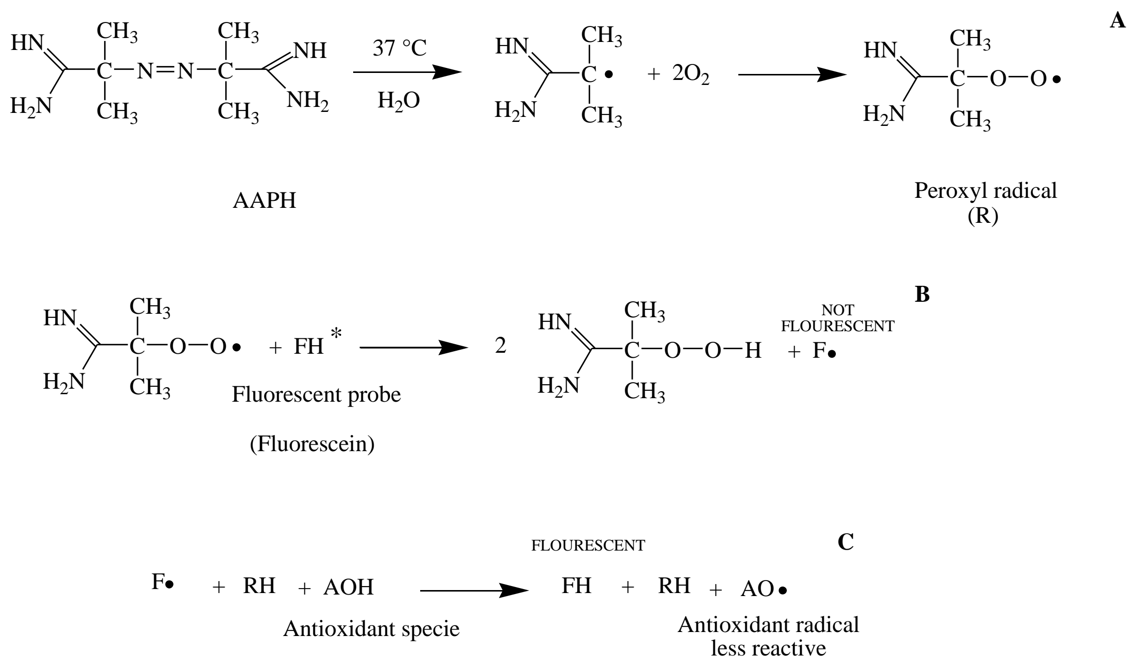
Source: Authors (2023).

ORAC (Oxygen Radical Antioxidant Capacity)

The technique for measuring antioxidant activity by the ORAC (Oxygen Radical Absorbance Capacity) method is based on the generation of a peroxyl radical (ROO.) by degradation of the reagent AAPH (2,2'-azobis-(2-methylpropaneamidine) at pH 7.4 at 37oC in the reaction medium (Figure 6A). The ORAC method has the advantage of producing a biological radical that approximates the in vitro reaction to the biological process. Without the presence of antioxidants in the medium, the generated peroxyl radical would be stabilized by the presence of a fluorescent probe (fluorescein) that would donate a proton to it and be consumed without fluorescing (Figure 6B). In the presence of an antioxidant compound or antioxidant mixture from the food, the fluorescence consumption does not occur because the oxidizing compounds are consumed in the preferential reaction with the peroxyl radical. The fluorescent probe begins to react with the radical when this oxidizable substrate no longer exists in the medium, and also becomes consumed, decreasing the fluorescence signal over time on the kinetic curve (Figure 6C). The peroxyl radical is able to react with both the oxidizable substrate and fluorescent probe, increasing the rate of fluorescence decay as the oxidizing substrate is consumed (Zhong and Shahidi, 2015).

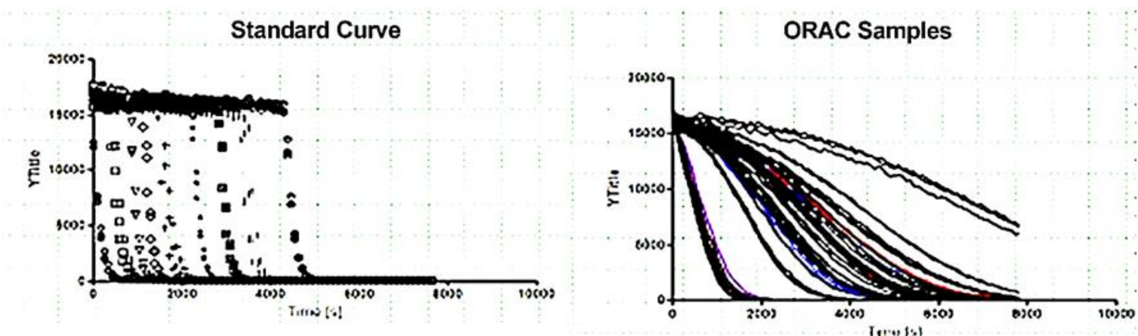
Fluorescence is measured at 485 nm (excitation) and 535 nm (emission) and a kinetic curve with 90 measurements per minute performed on a black colored microplate. It is necessary to integrate the consumption curve of the flurescein probe for both standard (trolox curve) and samples (Figure 7). Thus, the result is obtained by calculating the area under the curve (AUC) of fluorescence and is expressed as equivalent to micromoles of Trolox per mL (for beverages) or per g of sample - TEAC (Brescia, 2012).

Figure 6 (A B, C): Steps of the chemical reactions involved in the determination of antioxidant activity by ORAC.



Source: Authors (2023).

Figure 7: Examples of AUC-type curves for standard and samples in the ORAC method.



Source: Authors (2023).

Other biological radicals in the ORAC method

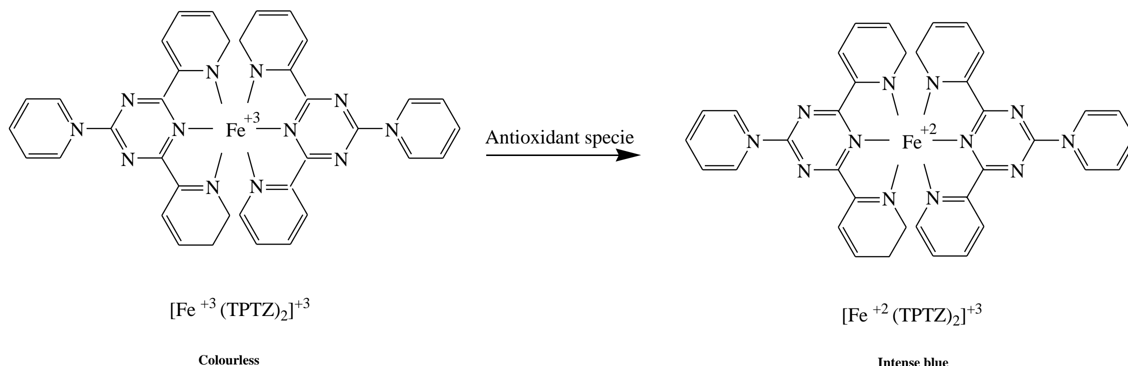
In addition to the original ORAC assay using the peroxyl radical, there are ORAC assays that have been developed using other radicals (individually or in groups) such as: ROS with superoxide anion (O2--), hydroxyl radical (OH-) and singlet oxygen (1O2). There is also RNS, peroxynitrite (ONOO-) and even a reactive chlorine species (RCS),

hypochlorite (ClO-). These multi-radical assays at the same time are called the Oxygen Radical Absorbance Capacity of Multiple Radicals (ORACMR), which is the sum of the antioxidant capacity of these six evaluated species. Another commercially available ORAC multi radical test, in this case without the chlorine radical species, is ORACMR5 (Prior, 2015).

FRAP (Ferric Reducing Ability Power)

The reagent TPTZ (2,4,6 - tripyridyl - 1,3,5 - triazine) forms a complex with iron and estimates the ability of antioxidants to reduce the complex [Fe+3(TPTZ)2] to the complex [Fe+2(TPTZ)2] at pH 3.6 (Figure 8). In the FRAP assay, the reduced complex formed in the presence of antioxidants shows blue coloration and the absorbance of the solution is measured at 595 nm, allowing monitoring of the reducing activity of the sample. Chelating agents in the sample can capture the iron ions from the complex with the TPTZ and interfere with the analysis. Therefore, it is important to use excess Fe+3 ions in the reaction medium (Benzie and Strain, 1999).

Figure 8: Chemical reaction of the FRAP method.



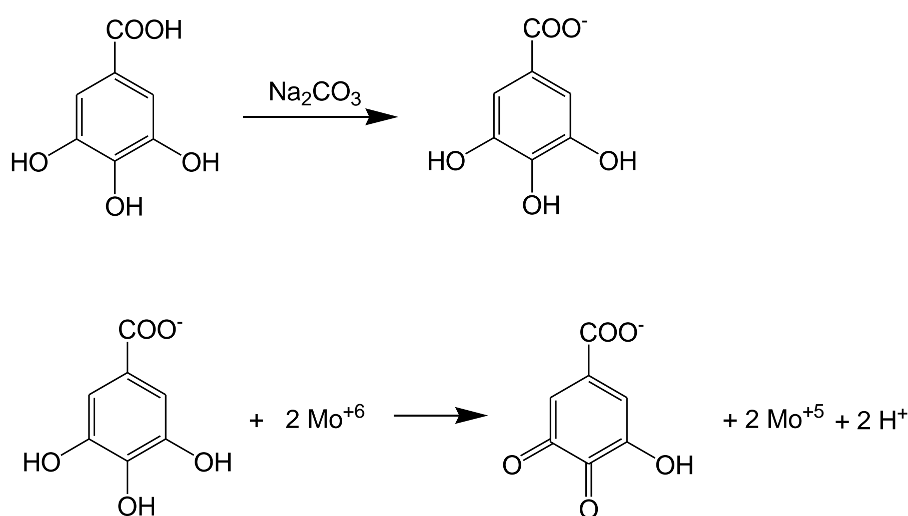
Source: Authors (2023).

Folin Ciocalteu (total phenolics)

The Folin-Ciocalteau reagent is a solution consisting of a mixture of phosphotungstic acid ($H_3PMo_{11}O_4$) and phosphomolybdic acid ($H_3PW_{11}O_4$) with a yellow color. When in the presence of the phenolate ion (from phenolic compounds), the phosphotungstic-phosphomolybdic complex is reduced to a mixture of blue colored tungsten and molybdenum oxides. The color change of the solution is proportional to the content of phenolic compounds. The concentration of phenols is correlated with the

standard curve of gallic acid, which is used as the equivalence standard. The results are expressed as gallic acid equivalents (GAG). Figure 9 shows the deprotonation of gallic acid in alkaline medium, followed by reaction with molybdenum, a constituent of the Folin-Ciocalteu reagent. This method can overestimate the values of total phenolics, because proteins, carbohydrates, and minerals can also participate in the reaction. So, some authors consider that the measure is antioxidant capacity of all of these molecules (Sánchez-Rangel et al., 2013).

Figure 9: Chemical reaction of the Folin-Ciocalteau method (total phenolics) for molybdenum salt.



Source: Authors (2023).

Electrochemistry methods

In Electrochemical antioxidant capacity methods, the samples are oxidized by applying a variable potential. The electrons are released from the antioxidant species and detected as a current at an electrode. New portable electrochemical devices are now used, making the sample preparation and operating system very easy, like BRS® from BQC Redox Technologies (Pisoschi et al., 2015, Al-Surhanee and Ameena, 2022).

FINAL CONSIDERATIONS

In conclusion, the evaluation of antioxidant activity through in vitro methods presents both advantages and challenges. These methods serve as valuable tools for assessing the potential health benefits of antioxidants in various food sources and ingredients, besides, biological fluids. Today the selection of methods for determining

antioxidant/oxidant activity in foods is based on trying to produce as much information as possible, using SET and HAT techniques.

The difficulty of access to specific instrumentation such as fluorimeters, UV/Visible spectrophotometers, and adapted liquid chromatographs, among others, is the bottleneck to the choice of techniques; as well as the unambiguous interpretation of results and a more economical and less laborious methods.

The techniques that produce biological radicals have also become an advantage and the antioxidant properties in cell culture may be an in vivo approach, complementary to the in vitro data obtained. However, the lack of standardization poses a significant hurdle. The multitude of available assays with differing mechanisms and outcomes complicates the comparison of results across studies. This lack of uniformity diminishes the clarity and comparability of measurement outcomes, which in turn hampers the formation of consistent conclusions.

Furthermore, in vitro, methods are inherently limited in their ability to replicate the complex interactions that occur within the human body. The absence of dynamic enzymatic and biological processes that influence antioxidant behavior in vivo means that these assays might not accurately predict real-world health outcomes. Despite these limitations, in vitro, methods remain invaluable tools in the initial assessment of antioxidant potential. They provide researchers with a preliminary understanding of the capacity of substances to neutralize harmful radicals. To enhance the reliability and applicability of in vitro assays, efforts toward standardization and the integration of multiple techniques should be pursued.

In the broader context of research, the combined use of in vitro, in vivo, and even clinical studies offers a more comprehensive and reliable approach to understanding the true impact of antioxidants on health. It is expected that the combination of the antioxidant assays may, in the future, result in more consolidated information to produce precise data about the balance of antioxidants for health promotion, ingested from food.

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