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INORGANIC AND PHYSICAL-CHEMICAL ASPECTS ON THE SPECTROPHOTOMETRIC DETERMINATION OF THE 1,1-DIPHENYL-2-PICRYLHYDRAZYL RADICAL

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

INORGANIC AND PHYSICAL-CHEMICAL ASPECTS ON THE SPECTROPHOTOMETRIC DETERMINATION OF THE 1,1-DIPHENYL-2-PICRYLHYDRAZYL RADICAL

Por

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Universidade Federal de Santa Maria
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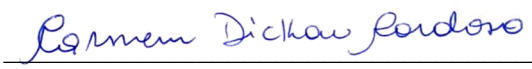

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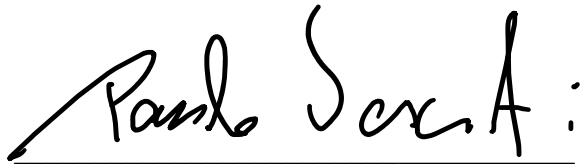

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Santa Maria, 06 de novembro de 2020.

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RESUMO

Título: Aspectos inorgânicos e físico-químicos na determinação espectrofotométrica do radical 1,1-difenil-2-picrilhidrazil.

Autor: Bryan Brummelhaus de Menezes

Orientador: Prof. Dr. Marcelo Barcellos da Rosa

O método DPPH tem sido reportado de forma equivocada em um grande número de estudos, de modo a impossibilitar uma comparação direta dos resultados. Chama-se a atenção para um erro básico na unidade usada para expressar o valor de IC_{50} do ácido ascórbico e outras substâncias antioxidantes. A concentração absoluta do antioxidante é amplamente mal utilizada, desconsiderando a concentração inicial de $DPPH^{\bullet}$, enquanto a escolha correta é a razão molar de antioxidante/$DPPH^{\bullet}$. Dados de 25 estudos apresentado grande variação nos valores de IC_{50} foram renormalizados de acordo com a estequiometria de reação, resultando em dados mais coerentes e mais próximos ao valor ideal de 0,25 para pelo menos 15 deles. Além disso, o modelo atualmente utilizado para calcular a concentração de $DPPH^{\bullet}$ pode levar a uma superestimativa em torno de 7%, pois não leva em consideração a pequena contribuição do produto da reação. Diante disso, apresentamos um modelo matemático para corrigir a superestimativa da concentração de $DPPH^{\bullet}$.

Palavras-chave: DPPH, correção de IC_{50}, estequiometria, modelo matemático

ABSTRACT

Title: Inorganic and physical-chemical aspects on the spectrophotometric determination of the 1,1-diphenyl-2-picrylhydrazyl radical.

Author: Bryan Brummelhaus de Menezes

Academic Advisor: Prof. Dr. Marcelo Barcellos da Rosa

The DPPH method has been reported with misconceptions in a large number of studies, precluding the direct comparison of results. Attention is drawn to a common mistake in the unit used to express the IC_{50} value of ascorbic acid and other antioxidant substances. Concentration of the antioxidant is widely misused disregarding the $DPPH^{\bullet}$ concentration, while the molar ratio of antioxidant/$DPPH^{\bullet}$ is de correct choice. Data from 25 studies with widely varying IC_{50} values were renormalized according to reaction stoichiometry, resulting in values more coherent and closer to the ideal one of 0.25 for at least 15 of them. In addition, the model currently used to calculate the $DPPH^{\bullet}$ concentration can lead to an overestimation of around 7%, as it does not take into account the small contribution of the reaction product. In view of that, we present a mathematical model to correct the overestimation of the $DPPH^{\bullet}$ concentration.

Keywords: DPPH, IC_{50} correction, stoichiometry, mathematical model

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MOTIVATION AND OBJECTIVES

The motivation of this work came from the observation of a serious lack in the literature respect to DPPH assay, at least 25 studies, published in journals with high impact factor in the last decade, have been reporting their results with incoherent units. Besides, the L-ascorbic acid, one of the principals "standard antioxidant compound" used as reference to evaluate the "antioxidant activity" of any interest analyte, have a complex redox chemistry and is very unstable and sensible to contaminants in solution. The combination of this two main points leads to a compendium of incoherent reported values across literature, precluding the reproducibility and the direct comparison of the reported values side by side, two of the most important criteria of the scientific method.

The expected goals for this work are:

- To draw attention to the use of appropriate units by the scientific community, when reporting the results of their work.
- To investigate the influence of both the DPPH radical and its reduction product, in the total photon absorption, and to establish an adequate way for its spectrophotometric determination.
- To investigate redox behavior of the L-ascorbic acid and the effect of its metal complexes on the observed antioxidant activity.

Chapter I – Literature Background

DPPH assay:

This method was developed in 1958 by Blois[1] to easily determine the antioxidant activity using a stable free radical. The assay is based on the measurement of the scavenging capacity of antioxidants towards the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants.[2]

The stability of the free radical DPPH is due the delocalization of the spare electron over the molecule as a whole (Figure 1.1), avoiding the dimerization of the molecule, like most other free radicals. The unpaired electron also gives a deep violet color to the molecule, with a strong absorption in methanol solution at around 515 nm, while its reduced form loss the strong absorption band at 515 nm, resulting in a pale-yellow color. The Lambert-Beer law is obeyed over the useful range of absorption.[1]

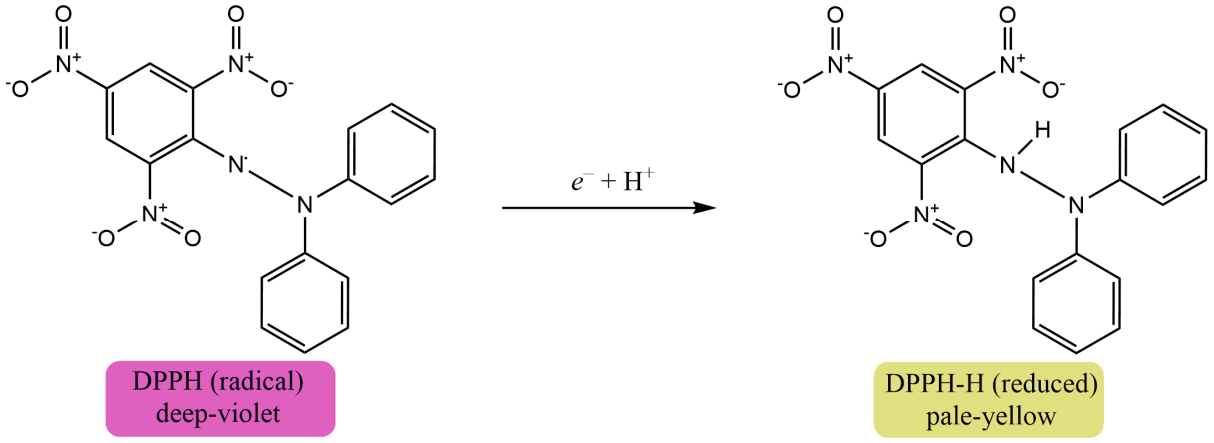

Figure 1.1. Representation of the DPPH radical reduction, by adding one hydrogen at the odd electron, leading to change the color from deep-violet to pale-yellow.

It is a rapid, simple, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It can also be used to quantify antioxidants in complex biological systems, for solid or liquid samples.[3] It is a convenient method for the antioxidant assay of cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds,[4] for olive oil, fruits, juices and wines.[5]

DPPH method may be utilized in both aqueous and organic solvents and can be used to examine both hydrophilic and lipophilic antioxidants, while antioxidant analysis by other methods may be limited to those compounds soluble in the selected solvents.[6] DPPH assay is considered a valid accurate, easy and economic method to evaluate radical scavenging activity of antioxidants, since the radical compound is stable and need not be generated.[7]

When introduced Blois, the antioxidant used as standard model was the cysteine, with one available hydrogen for reduction, resulting in an observed 1:1 stoichiometry (DPPH:cysteine). However, if the molecule has two adjacent internally connected sites for hydrogen abstraction, like the ascorbic acid, then there may be a further hydrogen abstraction reaction after the first one, given a 2:1 stoichiometry (DPPH:ascorbic acid). Similar pattern is shown with hydroquinone (1,4- dihydoxybenzene) forming quinine (1,4-benzoquinone) by a similar two-step mechanism. Other compounds actively participating in this reaction are glutathione, aromatic amines, α-tocopherol and polyhydroxy aromatic compounds. Inorganic ions in lower valence states (particularly Fe^{2+}) may interfere in this reaction.[1]

Brand-Williams et al. added modifications to the method in 1995,[2] since then it has been extensively used.[8–11] This suggested the oversimplification of the interpretation by Blois and that because of the complexity of the reactions, the overall stoichiometry need not necessarily be a whole integer.[12]. Furthermore, the initial step Eq. 1 may be reversible, as can be demonstrated by adding the reduced form DPPH-H at the end of the reaction.[7,13] Brand-

Williams et al. and Bondet et al. used the term "EC_{50}" (efficient concentration) or "IC_{50}" (inhibition concentration) for the interpretation of the results from DPPH method. This is defined as the concentration of substrate that causes 50% reduction in the DPPH color. This parameter was subsequently used by several groups for presenting their results.[6,14,15]

Lambert-Beer Law derived from Electromagnetism:

Complex refraction index:

When light passes through a medium, some part of it will always be attenuated. This can be conveniently taken into account by defining a complex refractive index

$$n = n' - i\kappa'' \tag{1.1}$$

Here, the real part n is the refractive index and indicates the phase velocity, while the imaginary part κ is called the extinction coefficient.

Complex relative permittivity:

In electromagnetism, the absolute permittivity, often simply called permittivity and denoted by the Greek letter ε (epsilon), is a measure of the electric polarizability of a dielectric.

the electric displacement field **D** resulting from an applied electric field **E** is

$$\mathbf{D} = \varepsilon\mathbf{E} \tag{1.2}$$

As opposed to the response of a vacuum, the response of normal materials to external fields generally depends on the frequency of the field. This frequency dependence reflects the fact that a material's polarization does not change instantaneously when an electric field is applied. The response must always be causal, which can be represented by a phase difference. For this reason, permittivity is often treated as a complex function of the angular frequency ω of the applied field (since complex numbers allow specification of magnitude and phase). The definition of permittivity therefore becomes:

$$\mathbf{D_0}e^{-i\omega t} = \bar{\varepsilon}(\omega)\mathbf{E_0}e^{-i\omega t} \tag{1.3}$$

Since the permittivity of a wave can be split into an real and an imaginary part, the following relations arrive:

$$\bar{\varepsilon}_r(\omega) = \varepsilon_r'(\omega) - i\varepsilon_r''(\omega) \tag{1.4}$$

$$\varepsilon_r''(\omega) = 2nk \tag{1.5}$$

$$n = \sqrt{\varepsilon_r \mu_r} \approx \sqrt{\varepsilon_r} \tag{1.6}$$

Expansion from dispersion model:

Including electromagnetic theory into the dispersion formulas, dispersion is seen as the consequence of a vibration of opposing charges under the influence of an electric field. Accordingly, the basic equation of this type of motion is given by:

$$\mu \frac{d^2x}{dt^2} + \mu\gamma \frac{dx}{dt} + \omega_0^2 x = qE_0 \exp(-i\omega t) \tag{1.7}$$

wherein μ is the reduced mass of the charges and *x* their displacement, and we have assumed that the restoring force is proportional to this displacement (Hook's law).

The solution for the displacement in dependence of time derived from eq. (1.7) is well-known:

$$x(t) = \frac{q}{\mu(\omega_0^2 - \omega^2 - i\omega\gamma)} E(t) \tag{1.8}$$

If this displacement is multiplied with the charge, we obtain the dipole moment *p* which is linked to the macroscopic polarization *P* via:

$$P = qxN_A C \tag{1.9}$$

And also:

$$P = \varepsilon_0(\bar{\varepsilon}_r - 1)E \tag{1.10}$$

With combination of equations (1.8), (1.9) and (1.10), we have:

$$\bar{\varepsilon}_r = 1 + \frac{N_A C}{\mu\varepsilon_0} \frac{q^2}{\omega_0^2 - \omega^2 - i\omega\gamma} \tag{1.11}$$

To obtain the final form of the dispersion relation, we define a molar oscillator strength *S** via:

$$S^{*2} = \frac{q^2 N_A}{\mu \varepsilon_0} \tag{1.12}$$

Given, from equations (1.4), (1.11) and (1.12):

$$\varepsilon_r'(\omega) - i\varepsilon_r''(\omega) = 1 + \frac{CS^{*2}}{\omega_0^2 - \omega^2 - i\omega\gamma} \tag{1.13}$$

Rationalizing and separating the real ($\Re$) and imaginary ($\Im$) parts we have:

$$\Re\ \varepsilon_r'(\omega) = 1 + \frac{CS^{*2}(\omega_0^2 - \omega^2)}{(\omega_0^2 - \omega^2)^2 + \omega^2\gamma^2} \tag{1.14}$$

$$\Im\ \varepsilon_r''(\omega) = \frac{CS^{*2}\omega\gamma}{(\omega_0^2 - \omega^2)^2 + \omega^2\gamma^2} \tag{1.15}$$

Based on the relations (1.5) and (1.6), we can obtain *k* and *n*:

$$k(\omega) = \frac{1}{2n} \times \frac{CS^{*2}\omega\gamma}{(\omega_0^2 - \omega^2)^2 + \omega^2\gamma^2} \tag{1.16}$$

$$n(\omega) = \sqrt{1 + \frac{CS^{*2}(\omega_0^2 - \omega^2)}{(\omega_0^2 - \omega^2)^2 + \omega^2\gamma^2}} \tag{1.17}$$

Considering that for $x \ll 1;\ \sqrt{1+x} \approx 1 + \frac{x}{2}$:

$$n(\omega) \approx 1 + \frac{1}{2} \times \frac{CS^{*2}(\omega_0^2 - \omega^2)}{(\omega_0^2 - \omega^2)^2 + \omega^2\gamma^2} \tag{1.18}$$

Switching to wavenumbers, the absorbance is defined as:

$$A(\tilde{\nu}) = \frac{4\pi\tilde{\nu}}{\ln 10} k(\tilde{\nu}) d \tag{1.19}$$

where *d* is the thickness and $\tilde{\nu}$ the wavenumber. From eqs. (1.18) and (1.19) we obtain:

$$A(\tilde{\nu}) = \frac{2\pi\tilde{\nu}}{n \times \ln 10} \times \frac{CdS^{*2}\tilde{\nu}\gamma}{(\tilde{\nu}_0^2 - \tilde{\nu}^2)^2 + \tilde{\nu}^2\gamma^2} \tag{1.20}$$

L-ascorbic acid as a ligand:

In the nearly 90 years since its discovery (1928), L-ascorbic acid (**$AscH_2$**) has become "the most famous but yet least understood of the vitamins". The crystal structure of L-ascorbic acid (Figure 1.2) was reported in 1964 by Hvoslef.[16] Despite the simplicity of the molecule, its biochemistry is poorly understood due to a very complicated redox chemistry which makes the molecule both an interesting and intriguing reducing agent in inorganic systems. Many solution studies have since been carried out on reactions between ascorbic acid and metal ions. The important work of Martell in this field established the catalytic role of metals in the oxidation of **$AscH_2$**.[17]

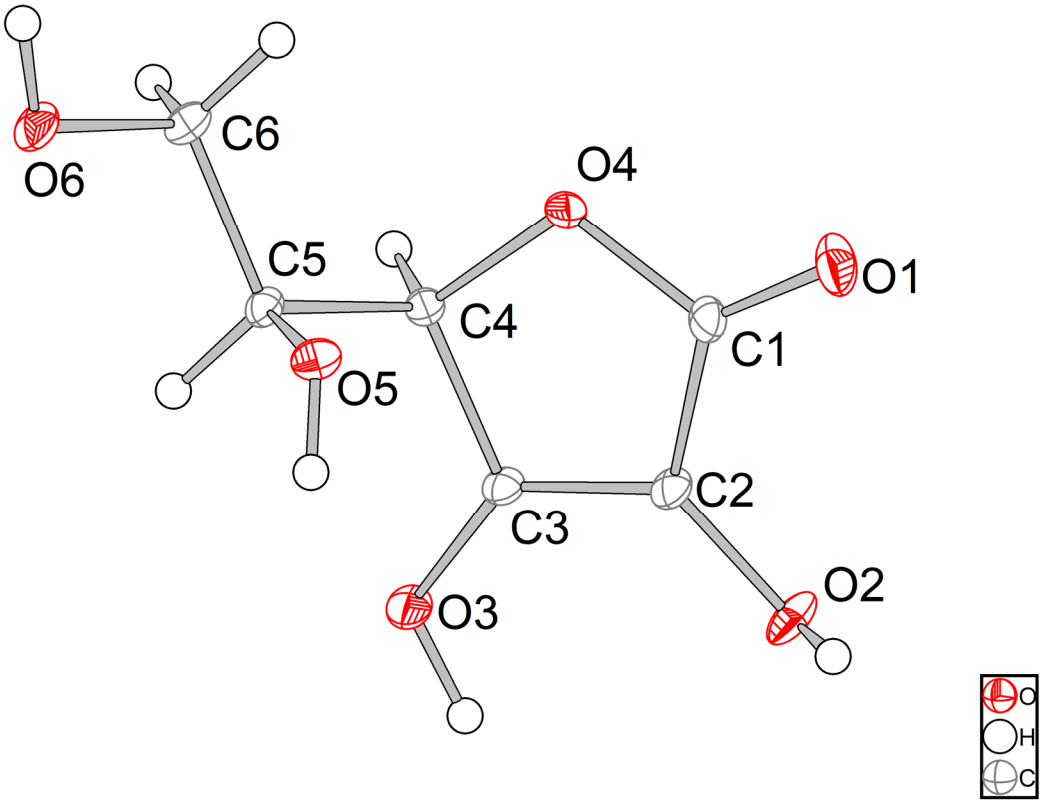

Figure 1.2. Crystal structure of L-ascorbic acid.

As a weak diprotic acid (pK_{a1} = 4.25 and pK_{a2} = 11.79), the monoanion (**AscH**$^{-}$) forms at pH 4–5 with deprotonation of O(3)–H and the dianion (**Asc**$^{2-}$) forms at pH 11–12 with deprotonation of the O(2)–H. The mono-anionic form is more stable due to the delocalization of the negative charge between the oxygen atoms at the 1 and 3 positions. Although **AscH$_2$** has several donor atoms capable of metal complex formation, the interaction of **AscH**$^{-}$ with metals mainly occurs monodentately through the O(3) atom, or by chelation via O(3) and O(2), depending on the nature of the metal cation and the pH of the solution. In the solid state, several other bonding modes have been proposed including the participation of the carbonyl oxygen and side chain OH groups.

The stabilities of the complexes are generally less than might have been expected. The formation constants of the 1:1 complexes are in the range of 10 to 103.6.[17] The values are quite small possibly because of the low negative charge on the ligand anion. Jabs and Gaube determined the ligand field parameters of the **AscH**$^{-}$ ligand and suggested that ascorbate should take an intermediate position in the spectroscopic series around H_2O and ox^{2-} and just before the fluoride ligand in the nephelauxetic series.[18] Later, Cieslak-Golonka et al. calculated crystal field parameters of some chromium ascorbate complexes for octahedral and tetragonal symmetries from diffuse reflectance spectra.[19] They found that the Dq values are in the region 1600–1800 cm^{-1} and larger in solution, typical of oxygen ligands.

Nearly all the work on transition metal pure ascorbate complexes has been performed with the first-row metals and on powdered samples. Since no single crystal data are available, the structural assignments have been generally deduced from UV-vis, NMR, IR and magnetic measurements. Due to the unstable nature of the molecule and hydrolytic instabilities of the complexes there have not been many reports on the isolation of solid complexes of ascorbic acid. The proposed structures of the pure ascorbate complexes have generally been the subject of controversy in the absence of X-ray crystal data.

The first systematic synthesis and isolation of binary ascorbate complexes with redox-inert transition metals were reported by Jabs and Gaube.[20] Complexes of the type $M(\mathbf{AscH})_n \cdot xH_2O$ (M = TiO^{2+}, Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}) were obtained through the reaction:

$$MSO_4 + Ba(OH)_2 + 2\mathbf{AscH}_2 \rightarrow M(\mathbf{AscH})_n \cdot xH_2O + BaSO_4 + 2H_2O \quad (1.21)$$

Since then, complexes with high oxidized transition metals have been investigated by other authors. In those cases, the metal center is generally reduced by ascorbic acid via an inner-sphere reaction. Ferrer et al.[21] demonstrated that the primary complexes generated by the interaction of **dhAsc** with metal ions are not stable and irreversibly hydrolyze to diketogulonic acid complexes of the related metal.

Chapter II – A critical approach of DPPH method: mistakes and inconsistencies in stoichiometry and IC_{50} determination by UV/VIS spectroscopy

Chapter II comprises the first article of this work, focused on the DPPH method, reviewing the correct methodology for its spectrophotometric determination, including a new mathematical model to consider the species formed in solution, and recalculating IC_{50} values for ascorbic acid, inappropriately reported in several works in the literature.

A critical approach of DPPH method: mistakes and inconsistencies in stoichiometry and IC_{50} determination by UV/VIS spectroscopy

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Abstract

The DPPH method has been reported with misconceptions in a large number of studies, precluding the comparison of results. Attention is drawn to a common mistake in the unit used to express the IC_{50} of ascorbic acid ($AscH_2$) and other antioxidant substances. Concentration of the antioxidant is widely misused disregarding the $DPPH^{\bullet}$ concentration, while the molar ratio of antioxidant/$DPPH^{\bullet}$ is de correct choice. Data from 25 studies with widely varying IC_{50} values were renormalized according to reaction stoichiometry, resulting in values more coherent and closer to the ideal one of 0.25 for at least 15 of them. In addition, the model currently used to calculate the $DPPH^{\bullet}$ concentration can lead to an overestimation of around 7%, as it does not take into account the small contribution of the reaction product. In view of that, we present a mathematical model to correct the overestimation of the $DPPH^{\bullet}$ concentration.

Keywords: DPPH, IC_{50} correction, stoichiometry, mathematical model

Chemical compounds studied in this article

1,1-Diphenyl-2-picrylhydrazine (PubChem CID: 74358); L-ascorbic acid (PubChem CID: 54670067); 2,6-Di-tert-butyl-4-methylphenol (PubChem CID: 31404)

2.1. Introduction

The DPPH assay is a well-known method which is frequently employed as it is simple, has a low cost, requires little operator skills, and uses a simple spectrophotometer.[22] It has been applied to quantify antioxidant activity in food, plant extracts, and beverages[23,24] using antioxidant standards as ascorbic acid ($AscH_2$), butylated hydroxyl toluene (BHT), α-tocopherol, butylated hydroxyl anisole (BHA), gallic acid and Trolox.[15] Nonetheless, determination of the antioxidant potential of a substrate through the DPPH assay draws attention from the literature because it often presents inconsistencies.[15,25] The two main problems observed are: first, the absolute determination of the fifty percent inhibitory concentration (IC_{50}) despite the initial concentration of DPPH used, when a relative value would be more coherent and could be compared with findings of other studies;[26,27] and second, the mistake in the determination of the percentage of scavenged DPPH radical ($DPPH^{\bullet}$), due to the missing measure of the absorbance of the molecular DPPH (DPPH-H) formed in the reaction.[28,29]

Regarding the first problem, an increase in the initial $DPPH^{\bullet}$ concentration also increases its consumption, even when the ratio of antioxidant to $DPPH^{\bullet}$ is maintained, thus affecting IC_{50} determination.[26] Studies have shown that DPPH activity and antioxidant concentration are not linear; so, each sample would need its own calibration curve, something that is not feasible and does not have a good "fit".[27,30] Therefore, presentation of IC_{50} results always requires comparison with a standard to determine the DPPH concentration obtained from the calibration curve due to uncertainties in direct determination.[31] Besides, IC_{50} values calculated as "oxidizing power" do not take into account the reaction time to reach equilibrium. This time is required to complete the redox reaction and the equilibrium on the reversibility of the reaction.[12]

With respect to the second problem mentioned above, the originally proposed DPPH method[1] and the protocol later suggested[2] undergo changes in the measurement parameters (e.g., concentration, reaction time, pH, solvent and presence of metal ions or inorganic salts) as needed.[23] The non-standardization of the DPPH method for antioxidant activity evaluation generated discrepant data in several studies, even for samples from the same matrix. These parameters must be carefully monitored for they affect the reaction kinetics and equilibrium, thus producing a variation in IC_{50} values. One of the main problems in the incompatibility of results between studies is not considering the standardization of a solvent for the proper proposal.[28] Solvent nature can interfere with the number of exchanged electrons as it influences the degree of secondary reactions of partially oxidized antioxidants and the rate of initial oxidation steps.[29] In addition, several spectrometric and non-spectrometric techniques have been used for the determination of antioxidant activity, and this further complicates comparisons.[32] Nevertheless, due to the colorimetric mechanism, a UV-Vis spectrophotometer is one of the simplest and cheapest techniques to use; it has proven to be a practical alternative for universities and companies for it is quick, does not have extra costs with solvents or inert gas flows, and does not require intense training for operation.

Although quite simple, expressing results in IC_{50} values hampers comparison between studies. Thus, the method parameters are not the main hindrance, but the data-interpretation errors; they cause a distortion that prevents comparisons and the reliability of the measurements themselves. Another burden is the expression of the antioxidant activity using an incorrect unit.[33]

These issues have already been critically addressed in studies showing a large difference in IC_{50} results for various reference standards. For L-ascorbic acid ($AscH_2$), for instance, a range from 10.2 to 746.5 μM has been described. The main problem reported is the great variation in the DPPH assay protocol. Therefore, literature values can be misleading, making it difficult to

correctly estimate the antiradical parameters of new molecules and precluding comparison with others.[15,25,34] We intend to continue assessing this matter by analytically approaching the mathematics and stoichiometry and also reevaluating some literature data.

In view of such considerations, we present a reminder of how to properly determine IC_{50} by reviewing the literature and collecting data from articles that have mistakenly expressed IC_{50} values; moreover, we propose a mathematical model to correct the overestimation of the $DPPH^{\bullet}$ concentration caused by an overlap of the maximum lambda by the DPPH-H signal, which generates overestimated IC_{50} values.

2.2. Materials and methods

1,1-Diphenyl-2-picrylhydrazyl (min. 95%), reference L-ascorbic acid (99.7-100.5%), 2,6-Di-tert-butyl-4-methylphenol (99%) and UV/HPLC grade methanol were purchased from Sigma Aldrich Chemical Co. Fresh stock solutions were prepared before each analysis. The spectrophotometric measurements were performed in a Varian Cary 50 spectrophotometer with 1 cm quartz cuvettes.

2.2.1. DPPH assay

Calibration curves were measured for the $DPPH^{\bullet}$ and the DPPH-H solutions. For $DPPH^{\bullet}$, a 200 μmol L^{-1} methanolic solution was prepared, from which three dilutions until 25 μmol L^{-1} were made. For DPPH-H, the methanolic solution was prepared with $DPPH^{\bullet}$ and $AscH_2$, both at 200 μmol L^{-1} (100% excess of $AscH_2$), protecting the reaction from light for 1 h; dilutions were performed as in the previous step.

The DPPH assay was performed adding constant aliquots of 30 μL of an $AscH_2$ methanolic solution (50 mg L^{-1} or 280 μmol L^{-1}) to 3 mL of a $DPPH^{\bullet}$ solution (25 μmol L^{-1} in methanol). The same procedure was applied to other three $DPPH^{\bullet}$ solutions (50, 100 and 200 μmol L^{-1}), using 560, 1120 and 2240 μmol L^{-1} of $AscH_2$, respectively. The negative (NC) and positive (PC) controls were taken from calibration curves measurements. All the reactions were kept in the dark for 30 min until measurements.

For the kinetic analysis, 1.5 mL of a methanolic BHT solution (50, 100, 200 and 400 μmol L^{-1}) was added to 1.5 mL of a 200 μmol L^{-1} DPPH solution and the absorbance at 515 nm was measured each 10 minutes, during 100 minutes. Further investigation of kinetic in a large excess of BHT (600, 800 and 1000 μmol L^{-1}) was performed collecting absorbance data each 10 s, during 3600 s. The reaction with $AscH_2$ cannot be monitored properly due to the fast speed of the reaction.

2.2.2. Literature data

Data used in item 3.1 were gathered by reviewing articles that determined the antioxidant activity through the DPPH method employing $AscH_2$ as the standard. Twenty-five research papers containing sufficient information for the analyses (initial DPPH concentration and volume of both analyte and DPPH) were selected from the totality of reviewed articles, with a greater appreciation for papers published in journals with the highest impact factors.

2.2.3. Computational methods

The initial structure of the DPPH$^{\bullet}$ was taken from the X-ray crystallographic structure.[35] The DPPH-H was constructed by manually adding one hydrogen to the radical structure. Geometry optimizations were performed at the CAM-B3LYP/6-31+G(d,p) level of theory, and confirmed to be at the minimum energy analyzing the normal modes. The singlet ground states were calculated with the spin-restricted DFT (Density Functional Theory), whereas the spin-unrestricted DFT was used for the doublet ground states. The electronic transition energies were calculated at the same levels of theory using TD-DFT (Time-Dependent DFT), implemented in the Gaussian09 program.[36] All calculations were carried out in the PCM (Polarizable Continuum Model) using methanol as a solvent.

2.3. Results and discussion

The DPPH• assay addresses two main points. Point one deals with a serious error that has perpetuated in the literature regarding the unit chosen to report the antioxidant/antiradical capacity of $AscH_2$ or other substances. Data from 26 scientific articles published in different journals in the last decades are presented here; some of them were recalculated according to the method proposed by Brand-Williams and co-workers in 1995 to demonstrate the implications of this misunderstanding.[2] Point two is the possibility of overestimating DPPH• concentrations with the simple UV-Vis measurement. This hypothesis is based on experimental UV-Vis measurements of the reaction between $AscH_2$ and DPPH•. The antioxidant analyzed for both topics was $AscH_2$, as it is a commonly used standard. However, the same analogy applies to any substance evaluated as an antioxidant in a DPPH test.

2.3.1. Point 1: The appropriate units for IC_{50} representation

The use of IC_{50} to represent the antioxidant potential of a given substance comes precisely from the idea of seeking a standard value that is useful for comparing different studies. The most appropriate way to report IC_{50} was presented by Brand-Williams and co-workers in 1995 as the ratio of antioxidant moles per mole of DPPH.[2] Unfortunately, inconsistencies have been recently inserted in the methodology due to subsequent citations that do not refer to the original research document. Studies with DPPH still express their results using the IC_{50} term; however, authors refer to IC_{50} as “the antioxidant concentration needed to reduce DPPH• by 50%”. But an essential question is neglected: 50% of how much?

For example, if an "X" amount of antiradical is required to eliminate 50% of DPPH• from a 100 μmol L^{-1} concentration solution, it is expected that a "2X" amount of antiradical will be required to eliminate 50% of DPPH• from a 200 μmol L^{-1} concentration solution and so on. So, reporting absolute antiradical concentration with a total disregard of the number of

related DPPH$^{\bullet}$ moles, without referring to these values, makes it impossible to compare results that were not obtained by the exact same procedures. Reporting antiradical concentration relative to DPPH concentration is essential to be methodologically scientific. To avoid confusion, we will henceforward refer to "Absolute" and "Relative" IC_{50} as AIC_{50} and RIC_{50}, respectively.

Therefore, we propose to renormalize AIC_{50} data from the literature as a function of the initial DPPH concentration reported in each study, and to verify how the new RIC_{50} values fit the expected stoichiometry of the reaction. Results from 25 studies with the reaction $AscH_2$ and DPPH$^{\bullet}$ are presented in Table 2.1. Only two of these works,[37 and 38] have correctly reported the RIC_{50}.

The antioxidant potential of $AscH_2$ comes from its ability to be converted into dehydroascorbate through the abstraction of two protons and two electrons (Figure 2.1a). This redox reaction provides the electrons needed to stabilize radicals like DPPH, as well as the proton to balance charges (Figure 2.1b).[39,40]

Table 2.1. Compilation of ascorbic acid (**$AscH_2$**) Absolute Inhibitory Concentration (AIC_{50}) measurements for DPPH radicals disclosed in 25 studies. The reported values are compared with the new calculated Relative Inhibitory Concentration (RIC_{50}) based on the initial DPPH concentration.

AIC_{50} µmol L^{-1}		AIC_{50} mg L^{-1}		Initial [DPPH]	Stoic	RIC_{50} $AscH_2$/DPPH		Ref
Rep	Calc	Rep	Calc			mol/mol	g/mol	
10.2	12.5	1.8	2.2	50	0.40	0.20	35.2	[15]
12.8	12.5	2.3	2.2	50	0.52	0.26	45.8	[41]
13.4	15.9	2.4	2.8	63	0.42	0.21	37.0	[6]
18.7	20.0	3.3	3.5	80	0.46	0.23	40.5	[42]
20.1	25.0	3.5	4.4	100	0.40	0.20	35.2	[43]
21.6	25.0	3.8	4.4	100	0.44	0.22	38.7	[38]
22.5	20.8	4.0	3.7	83	0.54	0.27	47.6	[37]
27.6	25.0	4.9	4.4	100	0.56	0.28	49.3	[44]
30.7	37.5	5.4	6.6	150	0.40	0.20	35.2	[45]
33.2	41.7	5.9	7.3	167	0.40	0.20	35.2	[46]
34.6	42.9	6.1	7.5	171	0.40	0.20	35.2	[47]
35.0	36.3	6.2	6.4	145	0.48	0.24	42.3	[48]
50.0	62.5	8.8	11.0	250	0.40	0.20	35.2	[49]
91.0	75.0	16.0	13.2	300	0.60	0.30	52.8	[3]
131.7	111.1	23.2	19.6	444	0.60	0.30	52.8	[50]
Not matching stoichiometry								
21.0	10.4	3.7	1.8	42	**1.00**	0.50	88.1	[8]
23.0	89.3	4.1	15.7	357	**0.12**	0.06	10.6	[51]
28.4	16.7	5.0	2.9	67	**0.86**	0.43	75.7	[52]
34.1	12.1	6.0	2.1	48	**1.42**	0.71	125.0	[53]
34.6	12.0	6.1	2.1	48	**1.44**	0.72	126.8	[54]
47.7	16.9	8.4	3.0	68	**1.42**	0.71	125.0	[10]
55.9	25.0	9.8	4.4	100	**1.12**	0.56	98.6	[55]
61.3	125.0	10.8	22.0	500	**0.24**	0.12	21.1	[56]
354.3	12.5	62.4	2.2	50	**14.18**	7.09	1248.7	[41]
746.5	15.3	131.5	2.7	61	**24.34**	12.17	2143.4	[57]

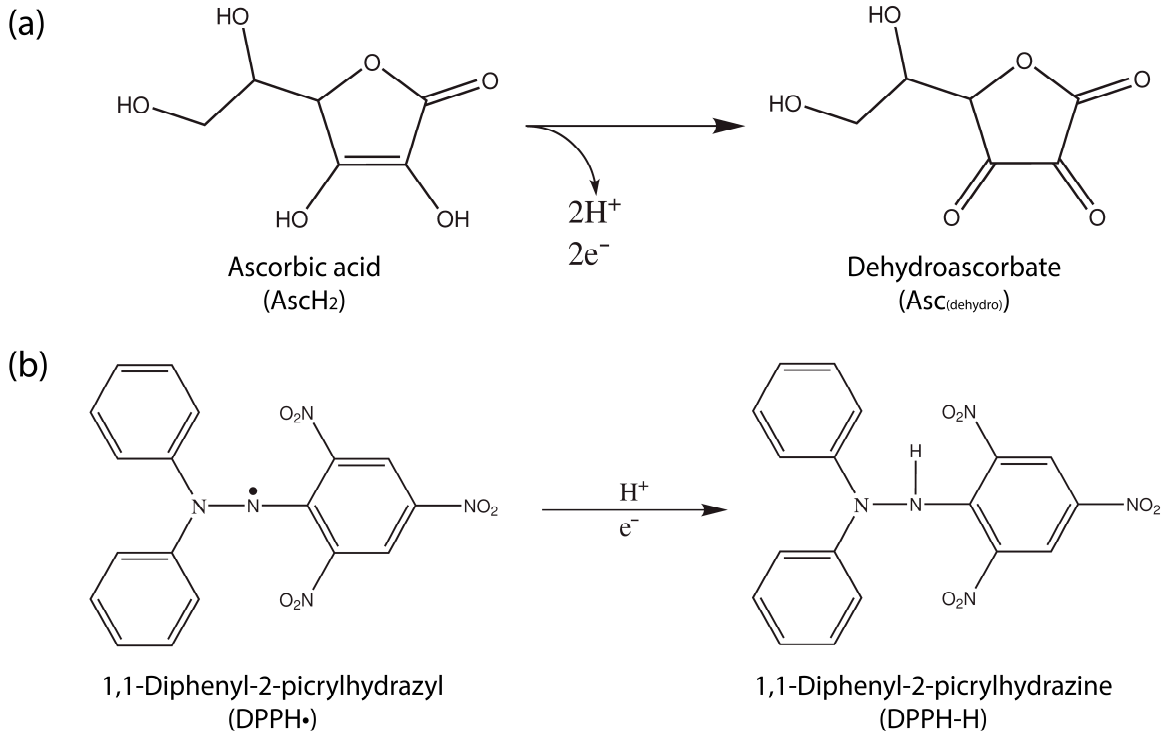

Figure 2.1. Semi-reactions of DPPH radical scavenging by ascorbic acid ($AscH_2$). (a) Conversion of $AscH_2$ into dehydroascorbate, producing two protons and two electrons. (b) DPPH radical neutralization with one electron and one proton.

According to the semi-reactions, it is possible to define the reaction stoichiometry, where each mole of the DPPH radical needs 0.5 mol $AscH_2$ to be completely scavenged, that is, the theoretical RIC_{50} is 0.25 mol $AscH_2$ per 1.0 mol DPPH, as seen in the following global reaction:

$$2\ \mathrm{DPPH^{\bullet}} + \mathrm{AscH_2} \rightarrow 2\ \mathrm{DPPH\text{-}H} + \mathrm{Asc_{dehydro}}$$

Widely varying "AIC_{50}" values have been reported for $AscH_2$, giving the impression that the results cannot be compared.[15,25] Values from 10.2 to 131.7 μmol L^{-1} (1.8 to 19.6 μg mL^{-1}) are presented in 15 studies (Figure 2.2); nonetheless, when expressed in RIC_{50}, they are all in the range of 0.2 to 0.3 mol mol^{-1}, which is closer to the ideal value of 0.25 mol mol^{-1}. The remaining 10 studies indicate values which are not compatible with the reaction stoichiometry (0.5 **$AscH_2$**/$DPPH^{\bullet}$). Two samples have stoichiometries of 0.12 and 0.24. It is

known that some contaminants, such as mineral acids, can greatly enhance the antioxidant capacity through the regeneration of $AscH_2$.[58] The other eight studies showed stoichiometry above 0.5. Attention should be drawn to two studies that presented stoichiometries of 14.18 and 24.34; these experiments had an efficacy much lower than expected, indicating the presence of some agent inhibiting the reaction. We cannot disregard the possibility of typos or miscalculations in such findings.

The inconsistent results found in some papers regarding $AscH_2$ lead us to another observation about its use as a standard. It has advantages as a fast and well-known reaction; however, it has the drawback of being very sensitive to the presence of contaminants, especially at such low working concentrations. We also faced this problem when a reduction in $AscH_2$ concentration was detected in the stock solution after a few hours of downtime. Fortunately, this problem was overcome by using a fresh new bottle of MeOH. Even though it can be considered troublesome, it may be a positive fact: checking for inconsistencies in the expected value of a reference compound, such as $AscH_2$, allows to investigate and eliminate potential interferents from the remaining assays.

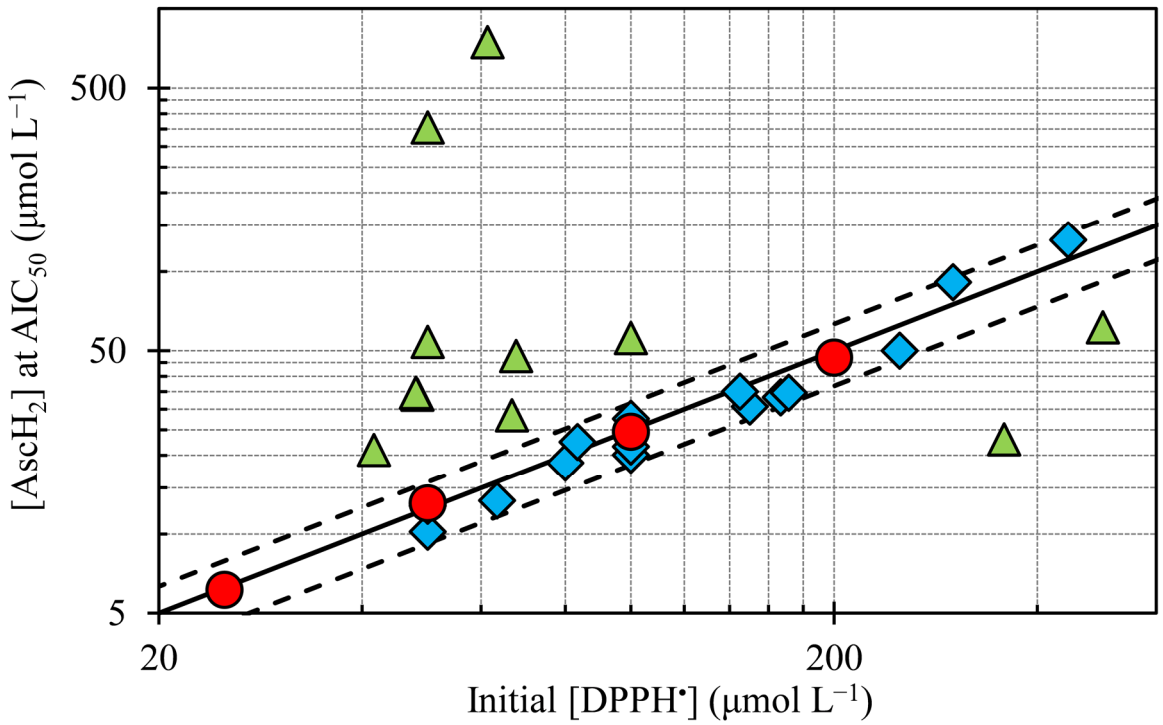

Figure 2.2. Concentration of ascorbic acid ($AscH_2$) at Absolute Inhibitory Concentration (AIC_{50}) vs. the initial concentration of DPPH radical reported in 25 different studies and four assays tested in this work. The solid line represents the ideal values based on a 0.5 stoichiometry. The dashed lines represent a tolerance area of 1.96σ (95% confidence interval; stoichiometry between 0.37 and 0.63). Blue diamonds are the studies that fit into the expected range, and green triangles are the studies with anomalous results. Red circles are measurements of this work.

Two mechanisms dominate the DPPH• – antioxidant reaction.[59] The first one is the proton-coupled electron transfer mechanism (PC-ET), which primarily occurs in nonpolar solvents of low dielectric constant and basicity, thus making feed reaction kinetics slower. The second one is the sequential proton loss electron transfer (SPLET), which is characteristic of solvents of high dielectric constants and basicity, thus increasing the reaction kinetics.[60] The literature does not report the use of a standard solvent; our choice for methanol is because its dielectric constant is slightly higher than that of ethanol, 33.10 and 25.10 at 20 °C respectively.[61] This small difference results in a higher molar absorptivity and, consequently,

the sensitivity of the spectrophotometric method to determine lower analyte concentrations is increased.[4]

Metal ions, water and hydrogen have important mechanisms in the process of free radicals;[58,62] therefore, the presence of these ions in samples with antioxidant potential becomes an essential research parameter. Flavonoids, for example, can form complexes with Fe (III) and Cu (II), which, in many cases, present a greater activity against free radicals and thus have a stronger reaction with DPPH when compared to compounds without metal ions.[63] Dawidowicz and co-workers (2012) evaluated the effects of pH and the presence of water on the $DPPH^{\bullet}$ – antioxidant reaction and observed the increase in the kinetics of the reaction in the presence of water concerning systems without water. The amount of water present may be associated with impurities in the extraction solvents or the humidity of the extracted plant material.[59,62] Regarding the concentration of hydrogen (pH) it is important to say that there is no consensus on this effect. However, previous studies point out the influence of pH on the antioxidant properties of compounds.[25,38,64] and the increase in the concentration of hydrogen ions results in a decrease in reaction kinetics. The acid pH of the extracts is associated with the presence of natural acids from plant extracts.[62]

Furthermore, in order to recalculate the RIC_{50} from published data, we also tested the reaction with four different DPPH initial concentrations so that linearity of the response in terms of AIC_{50} and RIC_{50} could be tested. Results are shown in Figure 2.2 and Table 2.S1. Assays showed good linearity between AIC_{50} and the initial DPPH concentration, thus validating RIC_{50} calculations from AIC_{50} results, as presented above.

It is important to remember that, although this work has focused only on data regarding ascorbic acid, the importance of reporting the IC_{50} properly is much broader as it applies to all substances that have been tested against DPPH radical.[65–71]

2.3.2. Point 2: Determination of the percentage of scavenged DPPH radical

Since the main misconceptions about how to report DPPH analysis results have been discussed, we can now focus on a minor but still important correction. There is a small difference between the expected and calculated values from equation 2.1 commonly used to determine the percentage of scavenged DPPH•.

$$\%\ DPPH_{Sca} = \left[\frac{(Abs_{NC} - Abs_{samp})}{Abs_{NC}}\right] \times 100 \quad (2.1)$$

Where $\%DPPH_{Sca}$ is the percentage of inhibited DPPH•, and Abs_{NC} and Abs_{samp} are the NC and sample absorbance, respectively.

With eq. 1, the percentage of scavenged DPPH• is considered as the percentage of discoloration from the initial concentration of DPPH•; the NC absorbance corresponds to the initial DPPH• solution, without the addition of the antioxidant compound, and the sample absorbance corresponds to DPPH• with the evaluated antioxidant compound. This percentage is measured with three different antioxidant concentrations and then a calibration curve is plotted. However, this oversimplified method does not take into account the absorbance of the reaction products. Thus, the apparent percentage of discoloration at the specific wavelength is not the real percentage of scavenged DPPH•, since the product of the reaction, DPPH-H, has a wide absorption band, with a "tail" that also absorbs in the 515 nm region. Figure 2.3 shows how absorption at 515 nm decreases with the addition of $AscH_2$; however, it never reaches zero, even with a stoichiometric excess. Other authors have also noted that the absorbance of the DPPH-H should be considered in order to have a more accurate analysis.[72] The reaction of DPPH• with $AscH_2$ should not form any product that absorbs at 515 nm. However, tests carried out with different antioxidants can form by-products that also absorb at 515 nm, further increasing the error.

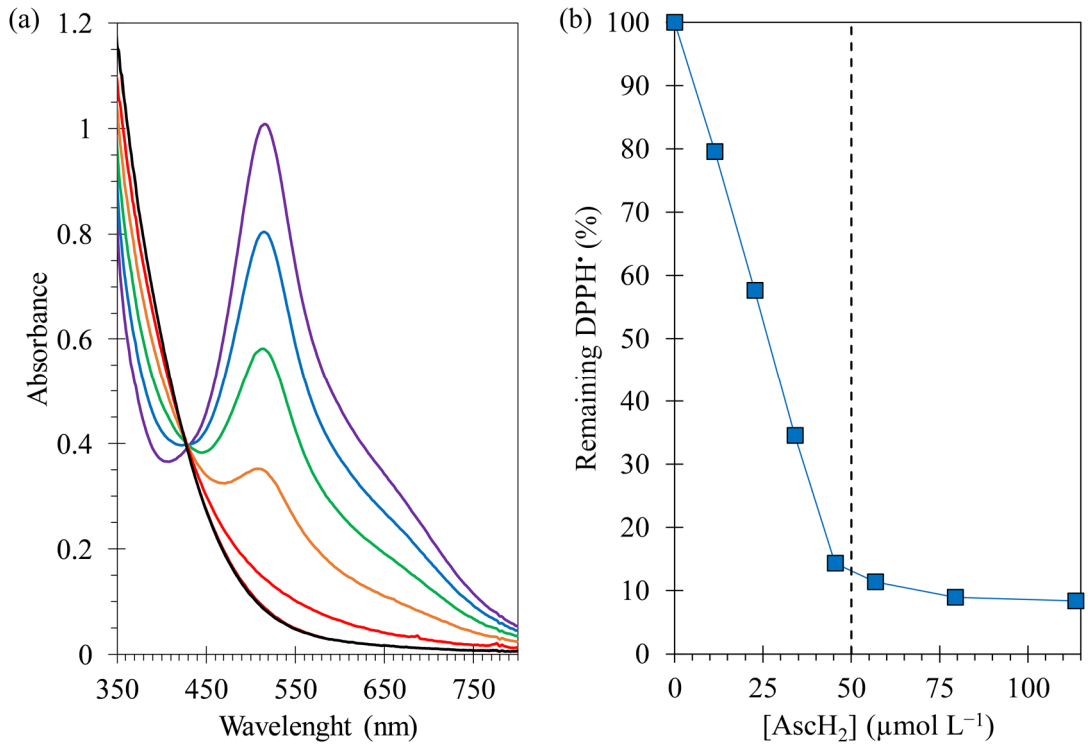

Figure 2.3. (a) UV-Vis spectra of 100 µmol L^{-1} DPPH radical (DPPH $^{\bullet}$) with addition of ascorbic acid ($AscH_2$) from 0 to 20 mg L^{-1} (0 to 113.6 µmol L^{-1}). (b) Remaining DPPH$^{\bullet}$ concentration vs. added $AscH_2$ calculated by eq. 1 (dashed line represents the theoretical stoichiometric point.

2.3.2.1. TD-DFT calculations

The UV-Vis spectra were compared with TD-DFT calculations in order to investigate the remaining absorption in the 515 nm region for DPPH-H. The experimental spectra were deconvoluted with Gaussian line shape (Figure 2.4 a and b) to obtain the information of the individual peaks, mainly the full width at half maximum (FWHM) of each spectrum, which allows to relate the oscillator strength to the molar absorptivity.

presents a summary of the calculated electronic transitions and comparisons with the experimental results. Detailed information and molecular orbital diagrams of DPPH$^{\bullet}$ and DPPH-H molecules are available in the supplementary information. The CAM-B3LYP functional was used to obtain the long-range corrections for the very delocalized electron in the DPPH radical, to better calculate the intramolecular charge transfer excitations of $\pi \rightarrow \pi^*$.[73]

The Polarizable Continuum Model (PCM) provides a suitable solvation correction without a high computational cost.[74] All calculated excitation energies (λ_{max}) are slightly blueshifted, but the errors are close to the expected in comparison with the previously reported mean error of 0.25 eV for the 6-31+G(d,p) basis set.[74,75] The experimental *vs* calculated energies plot showed that the calculated energies are related to the experimental ones by a factor of 0.912 (Figure 2.S6), which can be considered a small correction factor.[76] When the correction factor is applied to the calculated energies (eV), the peaks have a good fit with the experimental ones (Figure 2.4c), and in good agreement with reported calculation with large basis set (6-311++G(2d,p)) and global hybrid (B3LYP) functional.[77] Figure 2.4d shows also how the TD-DFT line shapes are very similar to the experimental spectra. DPPH-H has wider peaks than DPPH$^{\bullet}$ (FWHM 0.80 and 0.45 eV, respectively), which gives peak areas in good agreement with the oscillator strengths (*f*) calculated with TD-DFT. The DPPH-H/DPPH ratio for the absorbance at experimental λ_{max} (515 nm) and calculated λ_{max} (537 nm) is 0.077 and 0.119, respectively, considering the FWHM values are equal to the experimental ones.

Table 2.2. Results of TD-DFT calculations.

	Major contributions	Excitation energies (eV)		(nm)		Oscillator Strength (*f*)	Peak area Exp.*
		calc.†	exp.	calc.†	exp.		
DPPH$^{\bullet}$	SOMO(α) → LUMO(α) (43%)	2.10	1.94	592	640	0.029	0.082
	SOMO(β) → LUMO(β) (48%)	2.31	2.41	537	515	0.242	0.242
	SOMO(α) → LUMO+2(α) (30%) SOMO−4(β) → LUMO(β) (28%)	2.79	2.89	444	430	0.029	0.077
	SOMO(β) → LUMO+1(β) (52%)	3.40	3.33	364	372	0.043	0.088
	SOMO−3(β) → LUMO(β) (67%)	3.71	3.81	333	326	0.115	0.373
DPPH-H	HOMO → LUMO (66%)	3.22	3.01	385	412	0.087	0.099
	HOMO−1 → LUMO (52%)	3.46	3.31	359	375	0.093	0.120
	HOMO → LUMO+1 (50%)	3.72	3.84	333	323	0.080	0.076
	HOMO−1 → LUMO+1 (47%)	3.81	3.92	326	316	0.425	0.522

* Deconvoluted peak areas, normalized relative to peak at 515 nm = 0.242.
† TD-DFT calculated energies corrected by a factor of 0.912, from linear regression with the experimental ones.

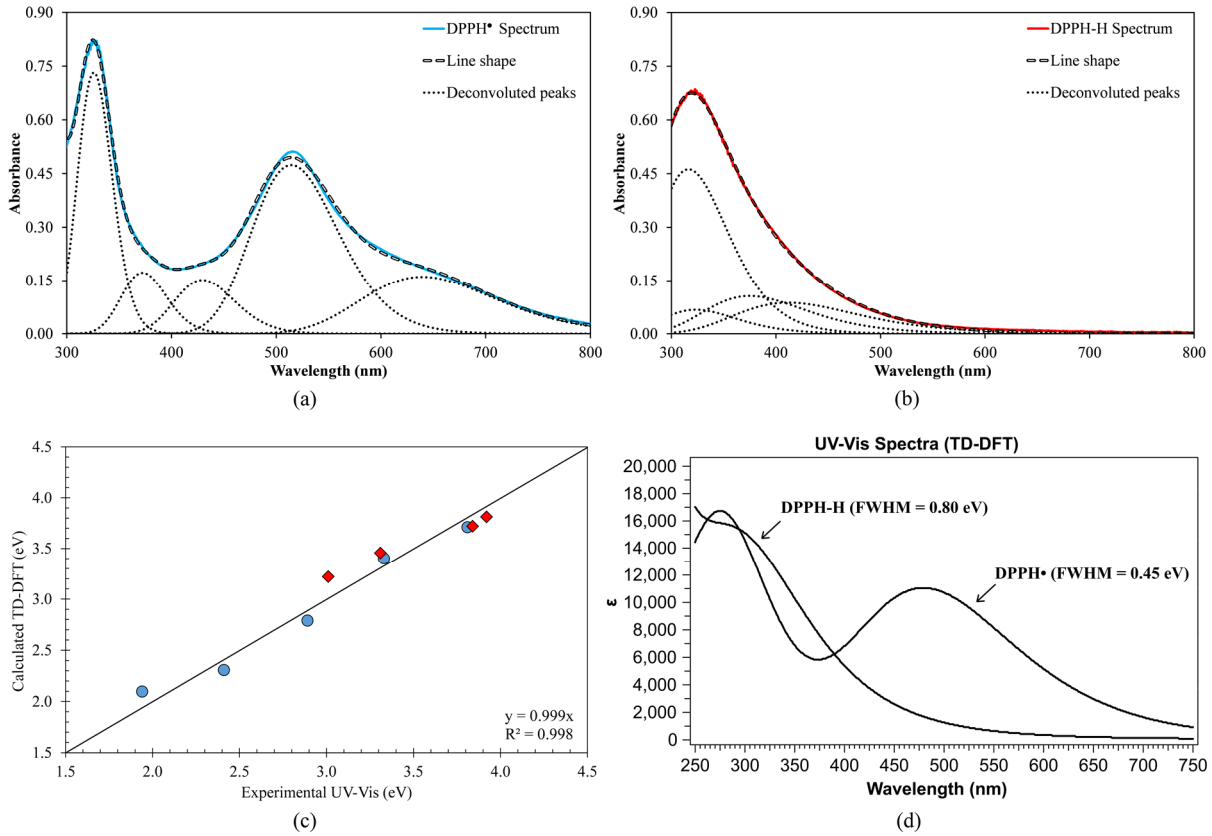

Figure 2.4. Gaussian line shape deconvolution with five peaks for DPPH• (a) and four peaks for DPPH-H (b). Calculated vs. Experimental UV-Vis transition energies, in eV (c). Simulated UV-Vis spectra with Gaussian09 (d).

2.3.2.2. Equations for the correct determination of DPPH• scavenged percentage

One way to determine the composition of each substance in the sample is to establish its molar absorptivity. This is more intuitive with DPPH•: it is necessary to use a stoichiometric excess of $AscH_2$ in order to determine the molar absorptivity of the reaction product so that the DPPH• is fully converted into its molecular form.

Before we begin to work on IC_{50} determination, we need to establish the following relationships:

$$c_R + c_H = c_0 \tag{2.2}$$

$$c_R = c_0 - c_H \tag{2.3}$$

$$\%\ DPPH_{Sca} = \frac{c_H}{c_0} \times 100 \tag{2.4}$$

Where c_R, c_H and c_0 are the concentrations of DPPH in their radical form, molecular form and initial concentration, respectively. One must also consider the Lambert-Beer law:

$$Abs = \varepsilon \times c \times l \tag{2.5}$$

Where Abs, ε and c are absorbance, molar absorptivity and concentration of the substance, respectively, and l is the optical path.

Assuming that the absorbance of the sample results from the sum of the absorbances of DPPH in the molecular and radical forms, we have:

$$Abs_{samp} = Abs_{DPPH\text{-}H} + Abs_{DPPH^{\bullet}} \tag{2.6}$$

When applying the Lambert-Beer law (eq. 2.5) and considering the optical path (l) equal to 1 cm, we have:

$$Abs_{samp} = (\varepsilon_H \times c_H) + (\varepsilon_R \times c_R) \tag{2.7}$$

It is possible to eliminate the c_R term using the relations (eq. 2.2) and (eq. 2.3):

$$Abs_{samp} = (\varepsilon_H \times c_H) + [\varepsilon_R \times (c_0 - c_H)] \quad (2.8)$$

$$Abs_{samp} = [(\varepsilon_H - \varepsilon_R) \times c_H] + (\varepsilon_R \times c_0) \quad (2.9)$$

Rewriting eq. 2.9 in terms of DPPH concentration in its molecular form (c_H):

$$c_H = \frac{[Abs_{samp} - (\varepsilon_R \times c_0)]}{(\varepsilon_H - \varepsilon_R)} \quad (2.10)$$

Considering the concentration of DPPH-H (c_H) (eq. 2.10) and knowing the initial concentration of DPPH$^{\bullet}$ (c_0), we can obtain the percentage of DPPH$^{\bullet}$ removal:

$$\%\, \boldsymbol{DPPH_{Sca}} = \frac{[\boldsymbol{Abs_{samp} - (\varepsilon_R \times c_0)}]}{(\boldsymbol{\varepsilon_H - \varepsilon_R})} \times \frac{\boldsymbol{100}}{\boldsymbol{c_0}} \quad (2.11)$$

With eq. 2.11, we can determine the concentration of both DPPH forms in the solution, and follow the reaction accurately.

The use of molar absorptivity is the ideal choice; nevertheless, this method requires a greater number of analyses. Aiming at a more accurate and still quite practical determination technique, alternative approximation methods are desired. A positive control (PC) can be used together with the previously described NC to contemplate the effect of reaction products. The PC consists of a sample with the same DPPH$^{\bullet}$ concentration as the NC and with a stoichiometric excess of $AscH_2$ in order to have only DPPH-H molecules contributing to the spectrum. Thus, we can measure the contribution of reaction products to sample absorbance.

Considering:

$$X_R + X_H = 1 \quad (2.12)$$

$$X_R = 1 - X_H \quad (2.13)$$

Where X_R and X_H are the DPPH fractions in their radical and molecular forms, respectively. By applying eq. 2.13 together with eq. 2.6, we have:

$$Abs_{samp} = Abs_{NC} \times (1 - X_H) + Abs_{PC} \times X_H \quad (2.14)$$

$$Abs_{samp} = Abs_{NC} + (Abs_{PC} - Abs_{NC}) \times X_H \quad (2.15)$$

$$X_H = \left[\frac{(Abs_{NC} - Abs_{samp})}{(Abs_{NC} - Abs_{PC})}\right] \quad (2.16)$$

Or, in terms of the percentage of removed DPPH:

$$\%\, \boldsymbol{DPPH_{Sca}} = \left[\frac{(\boldsymbol{Abs_{NC} - Abs_{samp}})}{(\boldsymbol{Abs_{NC} - Abs_{PC}})}\right] \times \mathbf{100} \quad (2.17)$$

Equation 2.17 is a good approximation to equation 2.11 and efficiently transposes the problem of the inaccurate determination of inhibition, based on the apparent discoloration of the sample.

When the correct %DPPH$_{Sca}$ values for different [AscH$_2$] are known, it is possible to determine the RIC$_{50}$ value by linear regression. Four different [DPPH$^{\bullet}$]$_0$ were tested (25, 50, 100 and 200 μmol L^{-1}) and all of them had similar RIC$_{50}$, showing that the assay scales well with the concentration. However, it is interesting to note that the RIC$_{50}$ values for the 200 μmol L^{-1} solution proved to be slightly lower than the others (Figure 2.5a and Table 2.S2), which may be due to the fact that it is a solution with an absorbance close to 2. The most suitable is to work with concentrations equal to or below 100 μmol L^{-1}. The results from equations 2.1, 2.11 and 2.17 are shown in Table 2.3. The linear regressions plots and equations are in the supplementary information (Figures 2.S3, 2.S4 and 2.S5). The molar absorptivity in methanol at 515 nm for both DPPH$^{\bullet}$ and DPPH-H were determined by calibration curve (Figures 2.S1 and 2.S2) as 9936 and 764 L mol^{-1} cm^{-1}, respectively (Abs Total$_{515nm}$ = 0.930 × DPPH$^{\bullet}$ + 0.070 × DPPH-H). These values are in agreement with literature data.[28]

Most works using DPPH assays are focused on plant extracts, food samples, or other complex matrices that do not have a well-defined composition.[11] A representation in number of moles is not possible in such cases, making it common to represent concentrations in μg mL^{-1} or ppm. In this context, it is interesting to calculate the reference $AscH_2$ RIC_{50} in mass units as well. If the molar RIC_{50} for $AscH_2$ is 0.25 mol per $DPPH^{\bullet}$ mol, the mass RIC_{50} should be determined by multiplying the molar mass of $AscH_2$ (176.12 g mol^{-1}). The theoretical RIC_{50} mass is: 44.03 g of $AscH_2$/1.0 mol of $DPPH^{\bullet}$.

Table 2.3. Comparison between the theoretical values of percentage of scavenged DPPH and IC_{50}, with the values calculated with equations 2.1, 2.11 and 2.17.

Model	**$AscH_2$/DPPH (RIC_{50})†**				**Stoichiometry**
	mol/mol	**(SD)**	**g/mol**	**(SD)**	
Equation 2.1	0.265	(0.010)	46.635	(1.779)	0.530
Equation 2.11	0.247	(0.011)	43.525	(1.976)	0.494
Equation 2.17	0.242	(0.008)	42.595	(1.408)	0.484
Theoretical*	**0.250**		**44.030**		**0.500**

***** Theoretical values based on the 1:2 reaction stoichiometry.
† Mean values from four initial DPPH concentrations (25, 50, 100 and 200 μmol L^{-1}).

Equations 2.11 and 2.17 shows an 6% and 9% increase, respectively, in the calculated amount of scavenged $DPPH^{\bullet}$ when compared to the results calculated by eq. 2.1. These values are close to the 7% of contribution from DPPH-H on the molar absorptivity, and reflects an underestimated antioxidant potential from eq. 2.1 (Figure 2.5).

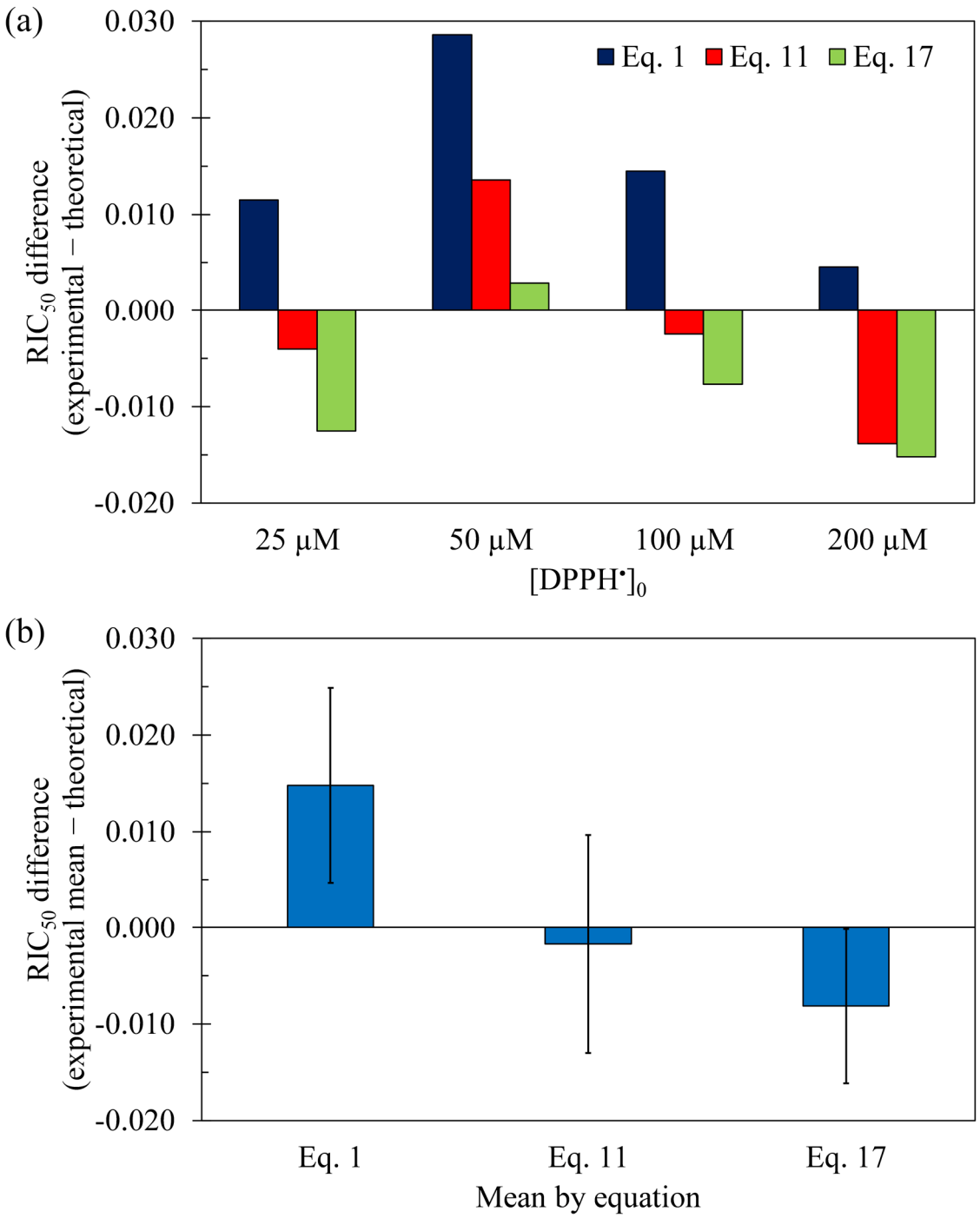

Figure 2.5. (a) Difference from theoretical RIC_{50} (0.250) calculated using equations 2.1, 2.11 and 2.17 for all tested initial $DPPH^{\bullet}$ concentration. (b) Mean of the RIC_{50} in four initial $DPPH^{\bullet}$ concentrations.

Even the determination of RIC_{50} by linear regression is questionable if we consider two criteria:

a) If there is a linear relationship between the antioxidant concentration and the percentage of inhibited $DPPH^{\bullet}$, then the concentration for 50% inhibition can be determined by linear extrapolation from a single measured point;
b) If there is no linear relationship, a linear regression model is not applicable for the determination of the 50% concentration either.

It is evident that the use of three or more distinct points rather than a single one reduces the estimation error, but it is important to note the criterion expressed in (b). Results presented in Table 2.S3 demonstrate how single-point RIC_{50} determination works very well for $AscH_2$ in the range of 44% to 92%, with deviations occurring in values close to 0% or 100%, where Relative experimental error is considerable.

$$Single\ point\ IC_{50} = \frac{(\%DPPH_{Sca} \times 50)}{[\mathbf{AscH_2}]} \times \frac{1}{[DPPH^{\bullet}]_0} \quad (2.18)$$

2.3.2.3. Complementary tests with BHT (slow kinetics and complex mechanism)

The wide use, the well-defined mechanism and the fast kinetics favor the study of reactions with $AscH_2$. However, plant extracts and food samples do not present simple mechanisms, so it is interesting to test the behavior of an antioxidant molecule with slower kinetics and more complex mechanism. 2,6-Di-tert-butyl-4-methylphenol (BHT) is a good alternative study because it is more similar to the reality of most substrates against $DPPH^{\bullet}$.

The rate constant of $DPPH^{\bullet}$ with $AscH_2$ is very high, and we are unable to properly measure kinetics with a UV-Vis spectrophotometer (details in the supplementary information). With the method used, it is possible to observe that the consumption of $DPPH^{\bullet}$ occurs mainly

in the first 20 s, and the absorbance remains stable after 120 s (Figure 2.S11). On the other hand, the kinetics for the slow reaction with BHT could be studied. It was noticed that the reaction kinetic, consequently the time needed to reach the plateau, depends on the concentration of DPPH$^{\bullet}$ and BHT. However, the reaction does not exactly follow a second order kinetic model, but rather a fractional order kinetic, typical of consecutive reactions.[78,79] The ability of each mol of BHT to remove multiple moles of DPPH$^{\bullet}$, which is consistent with a mechanism of multiple consecutive reactions, was also observed by Bondet and co-workers.[7] In order to work with the effective concentration of antioxidant available for the reaction, it is necessary to apply a stoichiometric factor (σ) to BHT:

$$\mathrm{DPPH}^{\bullet} + \sigma\mathrm{BHT} \rightarrow \mathrm{DPPH\text{-}H} + \text{by-products}$$

To determine the stoichiometry of the reaction a fractional kinetic model was used:

$$\frac{\mathrm{d[DPPH]}}{\mathrm{dt}} = k \times [\mathrm{DPPH}]^{m} \times \left(\frac{1}{\sigma}[\mathrm{BHT}]\right)^{n} \quad (2.19)$$

Where k is the kinetic constant, m and n represent the order of reaction related to each reagent, and σ is the factor that adjusts the stoichiometry of the reaction concerning the BHT. The m (1.39) and n (0.69) indexes are close to the found by Bondet and co-workers (1.5 and 0.4, respectively).[7] Indexes m and n and the factor σ (0.42) were determined numerically with the solver package for Excel from the line fitting of equation 2.19 with the experimental curves (Figure 2.6c). The kinetic constant (k) found for this reaction is 12.9 $\mathrm{L}^{1.08}\,\mathrm{mol}^{-1.08}$ s.

The kinetics of reduction of DPPH$^{\bullet}$ against BHT is considerably slow compared to the kinetics of reaction with $AscH_2$. In the BHT:DPPH$^{\bullet}$ reaction with a 25:100 μM ratio, 60% of DPPH was removed in 100 minutes (Figure 2.6a), evidencing the capacity of each mol of BHT to remove several moles of DPPH$^{\bullet}$. However, even with the addition of excess BHT (from 200 μM to 500 μM), the absorbance at 515 nm stabilizes on a plateau with approximately 13.3% of the original absorbance (Figure 2.6b), which rules out the hypothesis of an equilibrium

mechanism involved, and indicates that all DPPH• must have reacted at this point. Bondet and co-workers also observed the non-reversibility of the reaction.[7] The stabilization of the absorbance at 13.3% of the original value proves the hypothesis that all the consumption of the DPPH• occurs between the absorbance values of the NC and PC (Figure 2.6b).

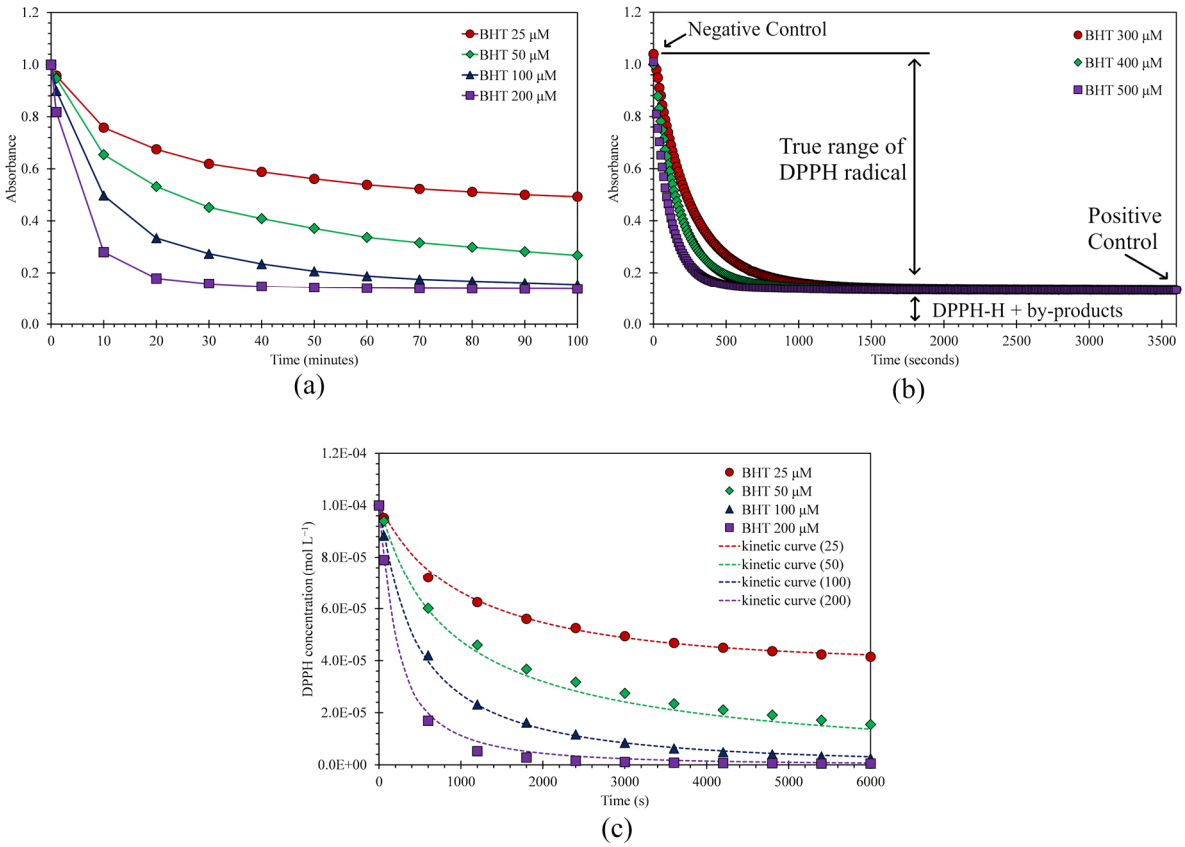

Figure 2.6. Kinetic evaluation for the reaction of 100 uM of DPPH with different concentrations of BHT. (a) Reaction progress with four different concentrations of BHT (25, 50, 100 and 200 µmol L^{-1}) over 100 minutes. (b) Reaction progress with a large excess of BHT (300, 400 and 500 µmol L^{-1}) over 60 minutes, showing the consistency of the absorbance plateau, regardless of BHT concentration. (c) Line fitting of experimental data with numerically calculated values from eq. 19.

It is important to observe that the "residual absorbance" at 515 nm is higher in the reaction with BHT compared to the reaction with $AscH_2$, even with a large excess of antioxidant in both reactions. Considering the complete consumption of DPPH• in the reaction, it is possible to infer that not only the DPPH-H absorbs in this wavelength, but also eventual dimers and

organic peroxides formed during the reaction, depending on the substrate used to reduce the DPPH•. Therefore, it is even more important to use equation 2.17. The use of "positive control" (PC) corrects the effect of the absorbance of the products formed in the reaction, giving the correct percentage of DPPH• scavenged. To obtain the PC it is necessary to add the antioxidant/antiradical substrate of interest in excess until the absorbance on the plateau remains stable with the addition of more substrate.

Some works point out several inconsistencies in the results of the DPPH tests and suggesting that the method is not suitable for use.[4,15,28,80,81] However, the practicality, the relatively low cost of the tests and the possibility of being performed in a simple spectrophotometer, keeps the interest of the scientific community. Studying the data, we observed that a large part of these inconsistencies is not inherent in the method, but are due to errors or lack of knowledge about the chemistry behind the method. More in-depth studies on kinetics and mechanisms are still needed to better define the capabilities of the DPPH assay.

2.4. Conclusions

- Most works have reported the results of the DPPH test simply as the concentration of antioxidant/antiradical substrate; this unit is inadequate to compare studies performed under distinct conditions. The IC_{50} value must be expressed as a function of the DPPH mol number used in the reaction in order to have consistency among the diverse results.
- When UV-Vis spectrophotometry is used, there is an evident underestimation of the $DPPH^{\bullet}$ concentration when DPPH-H is present in the medium and its absorption is neglected. We propose a simple method to correct the calculated DPPH concentration, as presented in equation 2.17.
- Measurement of a standard such as $AscH_2$ has been seen as a simple qualitative comparison with the analyte of interest. Measuring the antioxidant/antiradical capacity of a molecule with well-known mechanism and stoichiometry is also important to verify that the procedure is being well executed, with no interference or involvement of unknown factors. In the case of $AscH_2$, if the IC_{50} or the reaction stoichiometry greatly differ from 0.25 or 0.50, respectively, then the assays are not valid because something is preventing the reaction from proceeding correctly.
- The most critical and essential part to be highlighted is the appropriate unit to report DPPH IC_{50} results, as discussed in section 2.3.1. If researchers work with different units, obscured inside the "IC_{50}" tag, it is not possible to carry out an effective data evaluation for the DPPH assay.

- The proper correction presented in section 2.3.2 can solve an apparent overestimation that varies depending on the antioxidant used and the byproducts formed during the DPPH$^{\bullet}$ reduction. We found an error of approximately 7% for $AscH_2$ and 13% for BHT. However, complex matrices can present a series of products that absorb in the 515 nm region, being extremely necessary to know the absorbance of the sample after the complete consumption of DPPH$^{\bullet}$, instead of attributing this absorbance to a nonexistent DPPH$^{\bullet}$ concentration.

Conflict of Interest

Authors declare existence of no competing financial interest.

Financial support

This study was supported by Coordination for the Improvement of Higher Education Personnel (**CAPES**).

Supplementary Information

Table 2.S1. Determination of the percentage of scavenged DPPH with four initial DPPH$^{\bullet}$ concentrations (25, 50, 100 and 200 μmol L^{-1}), obtained from equations 1, 11 and 17, and determination of negative (NC) and positive (PC) controls.

$[DPPH^{\bullet}]_0$	$[AscH_2]$		Abs	Calculated scavenged DPPH$^{\bullet}$ (%)			
μmol L^{-1}	mg L^{-1}	μmol L^{-1}		Eq. 1	Eq. 11	Eq. 17	Theoretical
25	0.5	2.84	0.199	21.8	21.4	24	22.7
25	1	5.68	0.145	43.1	45.1	47.5	45.4
25	1.5	8.52	0.088	65.6	70.1	72.2	68.1
25	2	11.36	0.034	86.7	93.5	95.4	90.8
50	1	5.68	0.415	18.9	17.8	20.8	22.7
50	2	11.36	0.306	40.2	41.6	44.3	45.4
50	3	17.03	0.197	61.5	65.3	67.7	68.1
50	4	22.71	0.093	81.9	88.2	90.2	90.8
100	2	11.36	0.804	20.4	20.7	22.3	22.7
100	4	22.71	0.582	42.4	44.9	46.2	45.4
100	6	34.07	0.349	65.5	70.3	71.4	68.1
100	8	45.42	0.145	85.6	92.5	93.5	90.8
200	4	22.71	1.520	23.6	25.4	25.6	22.7
200	8	45.42	1.065	46.5	50.3	50.4	45.4
200	12	68.14	0.618	68.9	74.6	74.7	68.1
200	16	90.85	0.269	86.5	93.7	93.8	90.8
Negative Control (NC)							
25	—	—	0.255	—	—	—	
50	—	—	0.512	—	—	—	
100	—	—	1.010	—	—	—	
200	—	—	1.990	—	—	—	
Positive Control (PC)							
25	5	28.4	0.023	90.8	98.1	100.0	
50	10	56.8	0.047	90.8	98.0	100.0	
100	20	113.6	0.085	91.6	99.1	100.0	
200	40	227.1	0.155	92.2	99.9	100.0	

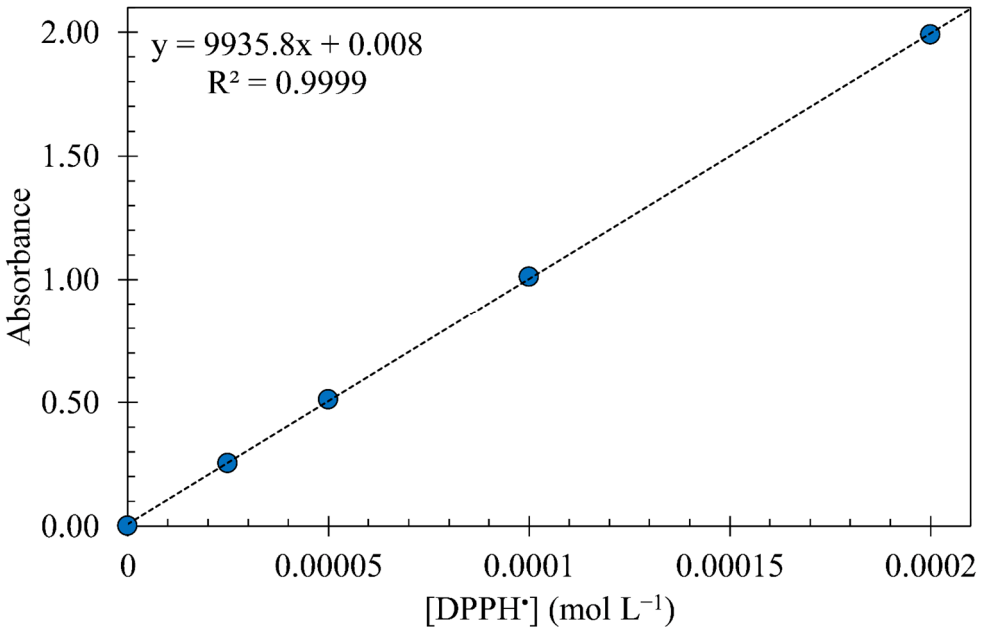

Figure 2.S1. Calibration curve for the DPPH• in methanol (Negative Control), measuring absorbance at 515 nm.

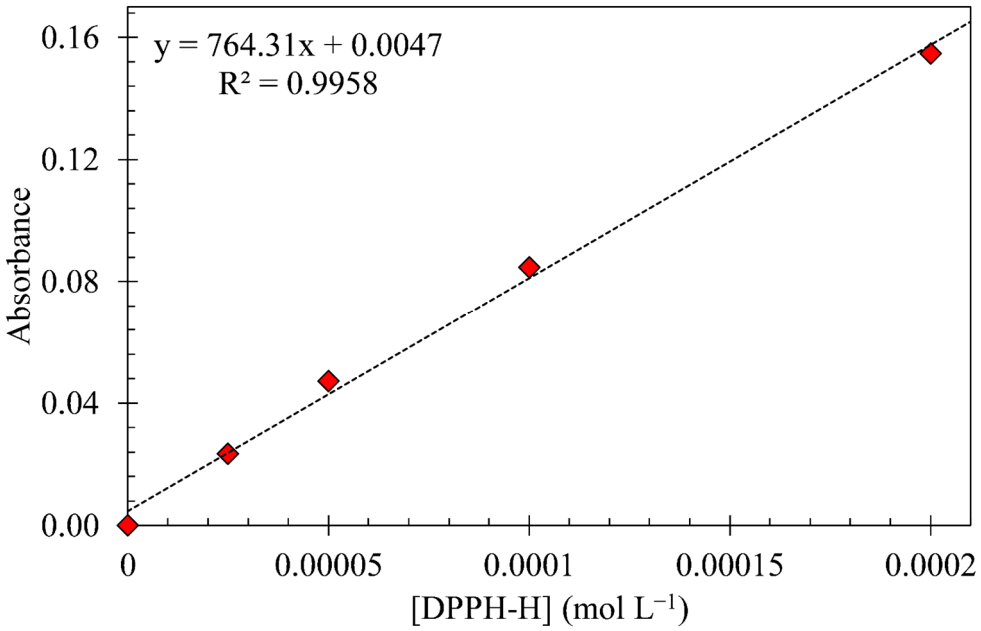

Figure 2.S2. Calibration curve for the DPPH-H in methanol (Positive Control), measuring absorbance at 515 nm.

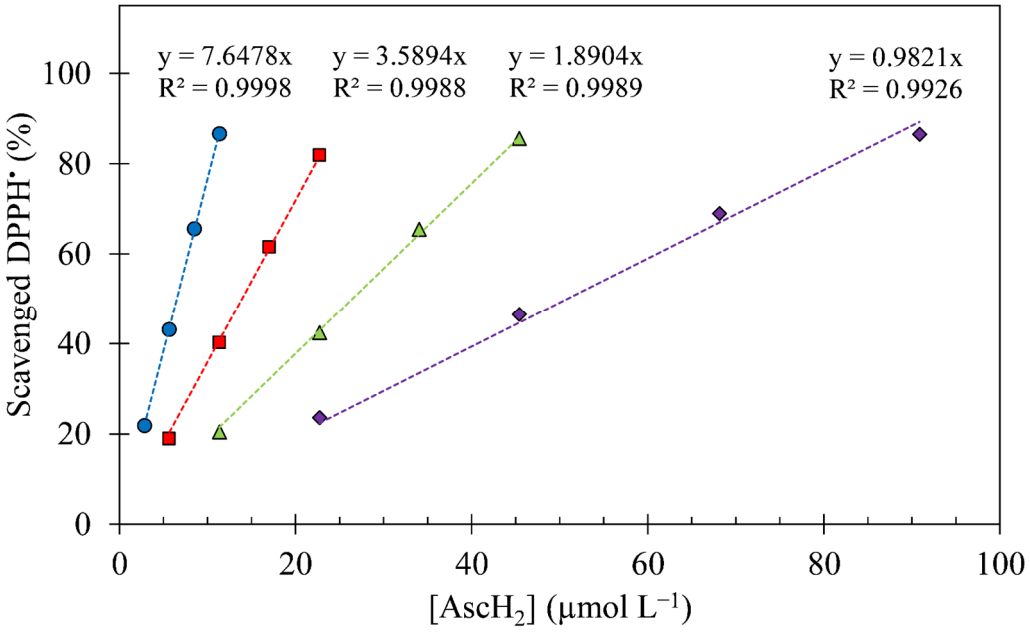

Figure 2.S3. Linear regression plots and equations for AIC_{50} determination, based on eq. 1, with four $[DPPH^{\bullet}]_0$: 25 (blue circles), 50 (red squares), 100 (green triangles) and 200 (purple diamonds) µmol L^{-1}.

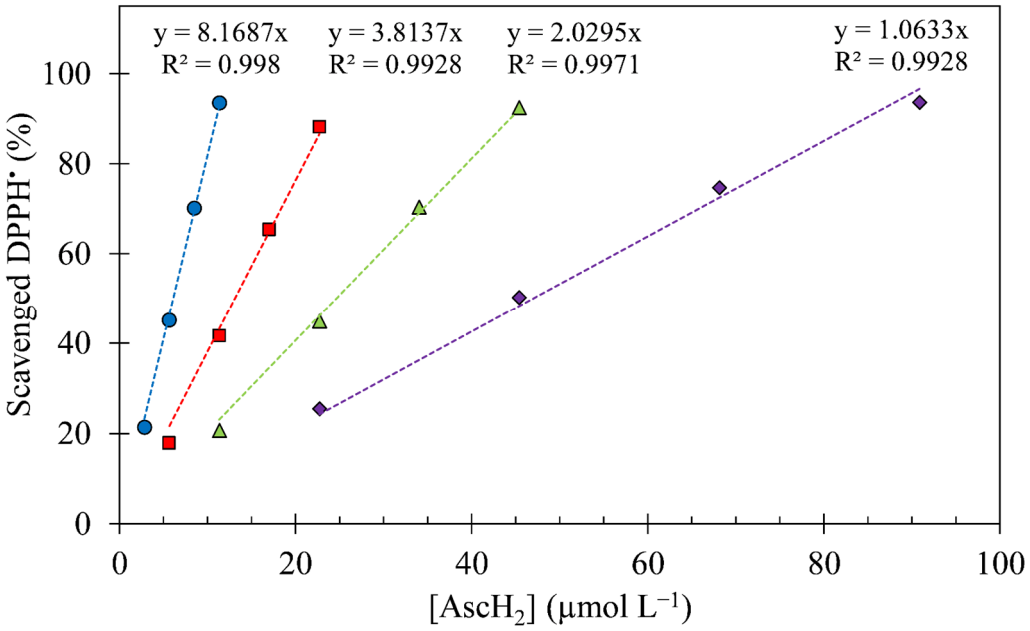

Figure 2.S4. Linear regression plots and equations for AIC_{50} determination, based on eq. 11, with four $[DPPH^{\bullet}]_0$: 25 (blue circles), 50 (red squares), 100 (green triangles) and 200 (purple diamonds) µmol L^{-1}.

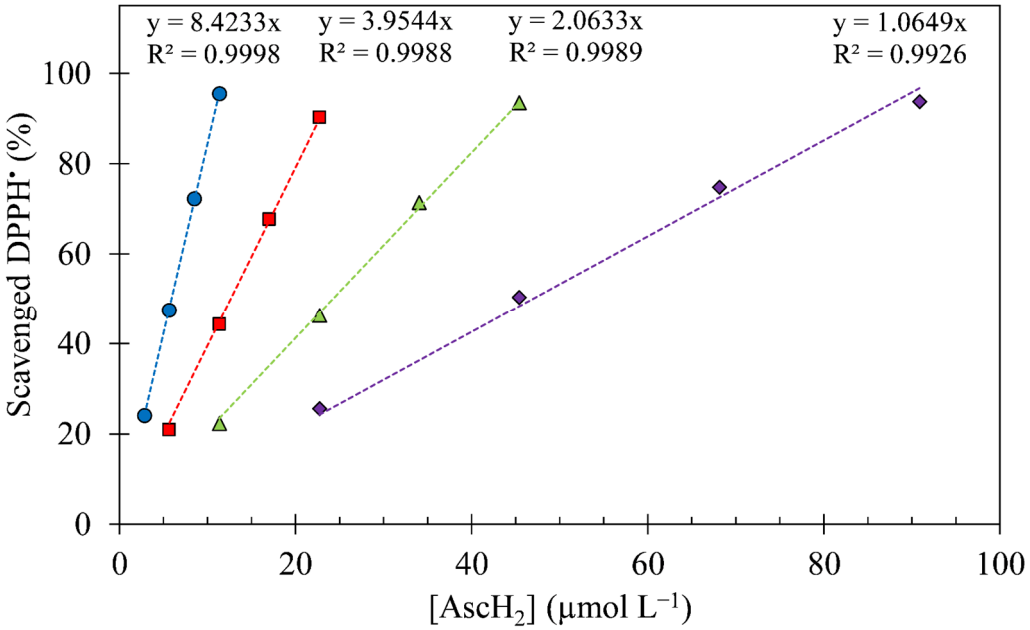

Figure 2.S5. Linear regression plots and equations for AIC_{50} determination, based on eq. 17, with four $[DPPH^{\bullet}]_0$: 25 (blue circles), 50 (red squares), 100 (green triangles) and 200 (purple diamonds) µmol L^{-1}.

Table 2.S2. Determination of absolute and relative IC_{50} with four initial $DPPH^{\bullet}$ concentrations (25, 50, 100 and 200 µmol L^{-1}), obtained from equations 1, 11 and 17.

$[DPPH^{\bullet}]_0$ (µmol L^{-1})	AIC_{50} (µmol L^{-1})			RIC_{50} ($AscH_2$/DPPH)			Theoretical*	
	Eq. 1	Eq. 11	Eq. 17	Eq. 1	Eq. 11	Eq. 17	AIC_{50}	RIC_{50}
25	6.54	6.12	5.94	0.262	0.245	0.237	6.25	0.250
50	13.93	13.11	12.64	0.279	0.262	0.253	12.50	0.250
100	26.45	24.64	24.23	0.264	0.246	0.242	25.00	0.250
200	50.91	47.02	46.95	0.255	0.235	0.235	50.00	0.250

* Theoretical values based on the 1:2 reaction stoichiometry.

Table 2.S3. Data obtained from the reaction of DPPH radical ($DPPH^{\bullet}$) at 100 µmol L^{-1} with different concentrations of ascorbic acid ($AscH_2$).

$[AscH_2]$		UV-Vis	% scavenged DPPH			Single point RIC_{50}*
(mg L^{-1})	(µmol L^{-1})	Abs	Eq. 1	Eq. 11	Eq. 17	mol/mol
0	0	1.010	0	0	0	—
2	11.4	0.804	20.4	20.3	22.3	0.280
4	22.7	0.582	42.4	44.5	46.2	**0.255**
6	34.1	0.349	65.5	70.0	71.4	**0.243**
8	45.4	0.145	85.6	92.2	93.5	**0.246**
10	56.8	0.115	88.6	95.5	96.7	0.297
14	79.5	0.091	91.0	98.2	99.4	—
20	113.6	0.085	91.6	98.8	100.0	—

* Based on the % scavenged from eq. 11 (IC_{50} from linear regression: 0.248 mol/mol).

Table 2.S4. Results of TD-DFT calculations without correction factor.

	Major contributions	Excitation energies (eV)		Excitation energies (nm)		Oscillator Strength (*f*)	Peak area Exp.*
		calc.	exp.	calc.	exp.		
DPPH$^{\bullet}$	SOMO(α) → LUMO(α) (43%)	2.30	1.94	540	640	0.029	0.082
	SOMO(β) → LUMO(β) (48%)	2.53	2.41	490	515	0.242	0.242
	SOMO(α) → LUMO+2(α) (30%) SOMO−4(β) → LUMO(β) (28%)	3.06	2.89	405	430	0.029	0.077
	SOMO(β) → LUMO+1(β) (52%)	3.73	3.33	332	372	0.043	0.088
	SOMO−3(β) → LUMO(β) (67%)	4.07	3.81	304	326	0.115	0.373
DPPH-H	HOMO → LUMO (66%)	3.53	3.01	351	412	0.087	0.099
	HOMO−1 → LUMO (52%)	3.79	3.31	327	375	0.093	0.120
	HOMO → LUMO+1 (50%)	4.08	3.84	304	323	0.080	0.076
	HOMO−1 → LUMO+1 (47%)	4.18	3.92	297	316	0.425	0.522

* Deconvoluted peak areas, normalized relative to peak at 515 nm = 0.242.

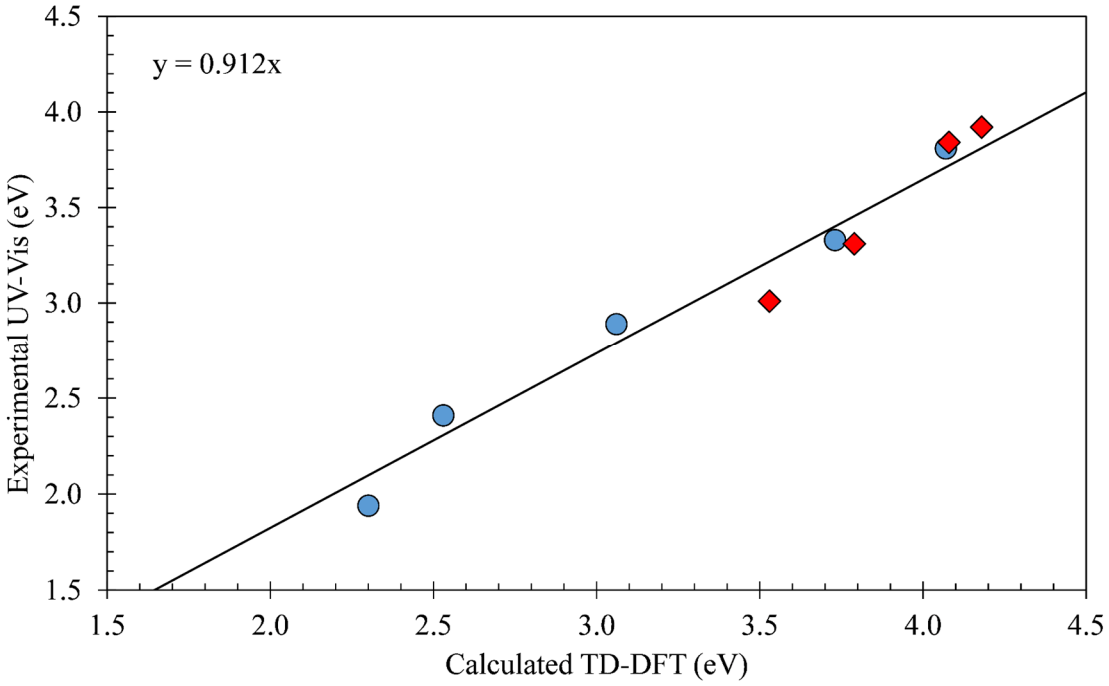

Figure 2.S6. Experimental vs. calculated electronic transition energies plot (data from Table 2.S4), demonstrating the relation of the values by a factor of 0.912 (obtained from linear regression).

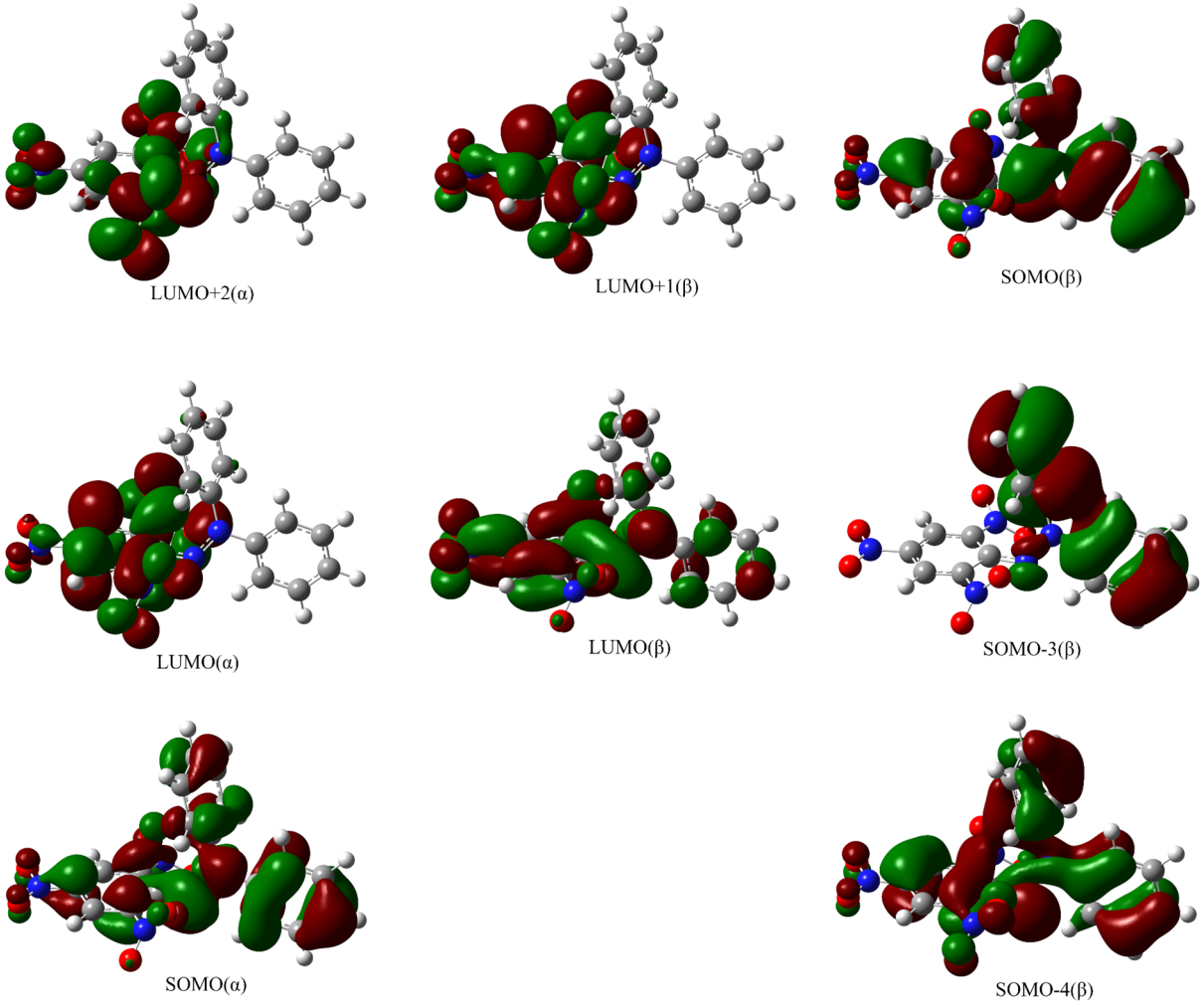

Figure 2.S7. Diagrams of molecular orbitals involved in the calculated electronic transitions for the DPPH$^{\bullet}$ molecule.

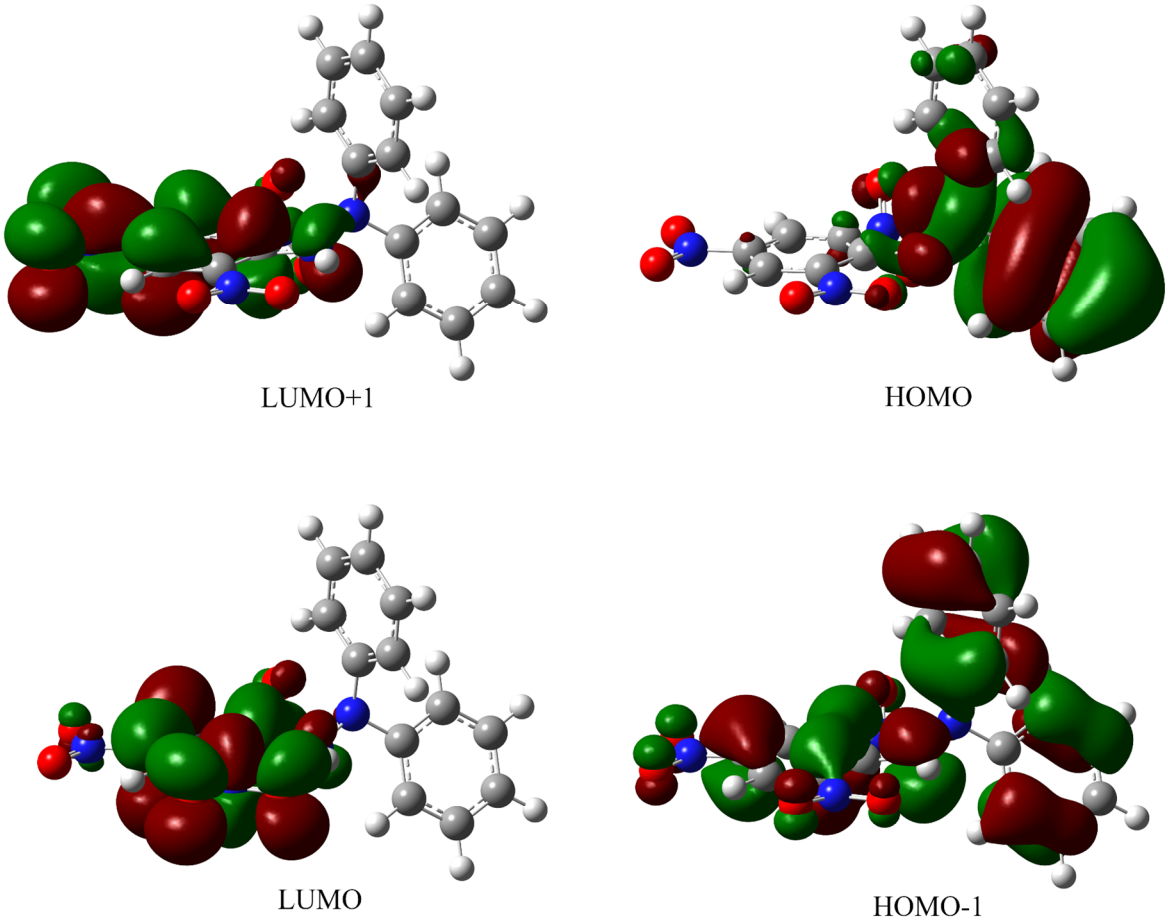

Figure 2.S8. Diagrams of molecular orbitals involved in the calculated electronic transitions for the DPPH-H molecule.

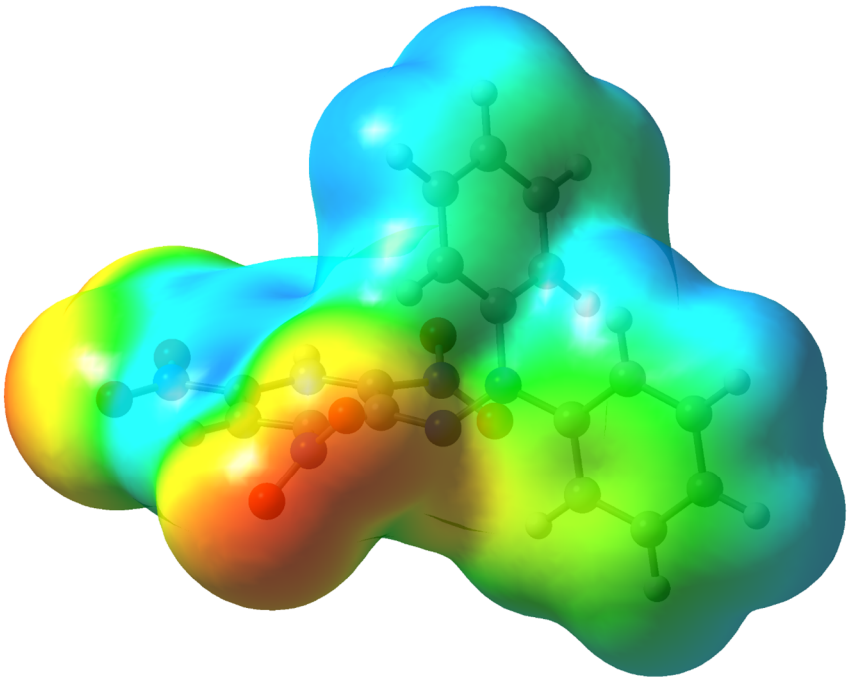

Figure 2.S9. Electrostatic Potential (ESP) surface calculated for DPPH radical, using CAM-B3LYP/6-31+G(d,p) level of theory, in Gaussian09.

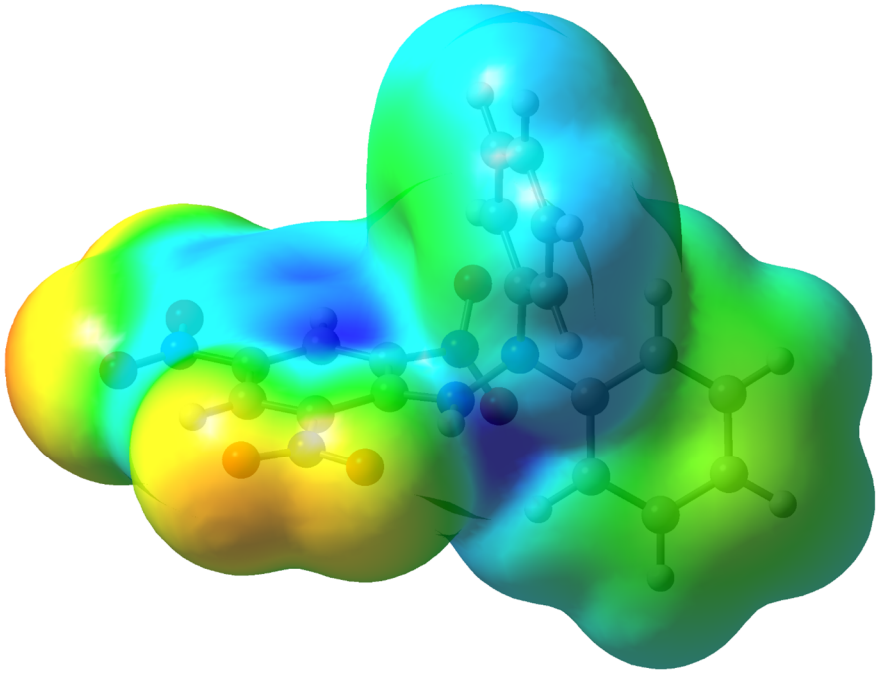

Figure 2.S10. ESP surface calculated for DPPH-H, using CAM-B3LYP/6-31+G(d,p) level of theory, in Gaussian09.

In order to observe the kinetic of the reaction of $AscH_2$ with DPPH$^{\bullet}$, aliquots of a $AscH_2$ methanolic solution (3.0 mmol L^{-1}) were added directly to the cuvette containing 3.0 mL of a DPPH$^{\bullet}$ methanolic solution (100 µmol L^{-1}) at 180 s intervals, and the decay of the absorbance of the solution at 515 nm was monitored with intervals of 5 s (details in the Table 2.S5).

Table 2.S5. Sequential addition of $AscH_2$ aliquots to the cuvette containing 3.0 mL of 100 µmol L^{-1} DPPH$^{\bullet}$ methanolic solution.

Time (s)	Vol. of aliquot added (µL)	Total vol. added (µL)	$AscH_2$ Conc. (mmol L^{-1})	$AscH_2$ Conc. in the cuvette (µmol L^{-1})
0	10	10	3.0	10
180	10	20	3.0	20
360	30	50	3.0	50
540	30	80	3.0	80
720	30	120	3.0	120

The kinetics of the reaction showed too fast to be determined by this method, however, it is possible to observe that the consumption of DPPH$^{\bullet}$ occurs mainly in the first 20 s, and the absorbance remains stable after 120 s (Figure 2.S11).

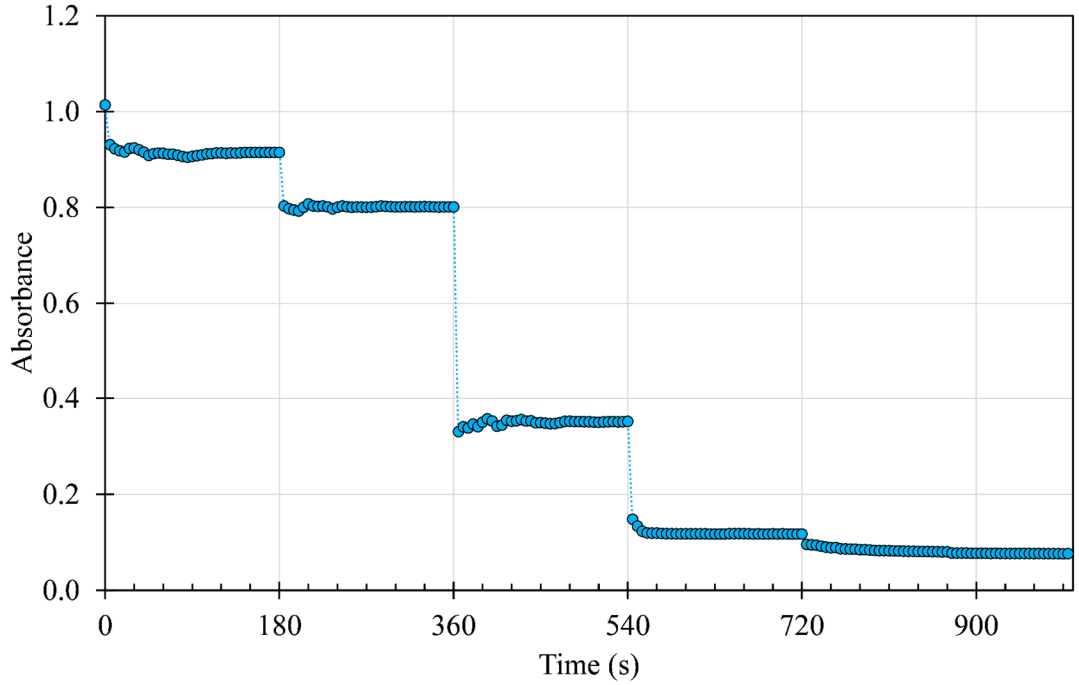

Figure 2.S11. Monitoring of DPPH$^{\bullet}$ consumption with the addition of $AscH_2$ aliquots at 180 s intervals.

Chapter III – Reviewing the effect of metal complexes formation in the antioxidant/antiradical proprieties of the L-ascorbic acid

So far, the discussion has turned to DPPH and how to correctly determine the reduction in the concentration of radicals in solution, using spectrophotometry. However, when it comes to assessing the antioxidant potential of a substance against DPPH, i.e., determining the relationship between the amount of antioxidant substance added and the reduction in the concentration of DPPH radicals, it is necessary to look at the behavior of the antioxidant at the reaction medium.

The antioxidant potential of a substance can be easily underestimated, or overestimated if the reaction is experiencing unnecessary interference. For the case of ascorbic acid, the p*H*, and the complexes formation with metals in solution, were evaluated on the reaction with radical DPPH.

In chapter III is brought a proposed article, based on the ability of ascorbic acid to complex with metals in solution, and how the metal complexes formed can affect the antioxidant behavior expected for ascorbic acid.

Reviewing the effect of metal complexes formation in the antioxidant/antiradical proprieties of the L-ascorbic acid

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3.1. Introduction

Ascorbic acid is among the most widely cited forms of water-soluble biological antioxidants. The ability to scavenge free radical reactions appears, in part, to involve one-electron oxidations where ascorbate serve as reductant towards radical species.[82] Reaction of ascorbic acid with peroxyl or hydroxyl radicals typically yields radical intermediate species, which can be subsequently quenched as part of an overall antioxidant effect. On the basis of the redox potentials of ascorbic acid,[82] it appears that radical intermediates may form with nearly equal facility during radical scavenging reactions.[83] However, ascorbic acid may also act as a prooxidant in fats and especially in aqueous fat systems.[84]

Metal ions appear to be involved in the prooxidative activity of ascorbic acid, as shown by the inhibition of this activity by metal chelating compounds such as ethylenediaminetetraacetic acid (EDTA) or polyphosphates. Indeed, Fe^{3+} and Cu^{2+} were reported to accelerate the prooxidant activity of ascorbic acid toward lipids.

Ascorbic acid has the first standard reduction potential ($E°$) around 0.72 V, and only the second is around −0.17 V, this means that even though it is a great anti-radical agent, it is not necessarily a reducing agent. We have to remember that free radicals are unstable and have high $E°$, allowing the transfer of electrons by **AscH**$_2$. Its antioxidant character is linked to the availability of electrons to reduce strong oxidizing agents, such as free radicals, while **AscH**$_2$ is not intended to act as a strong reducing agent in the biological environment.[83]

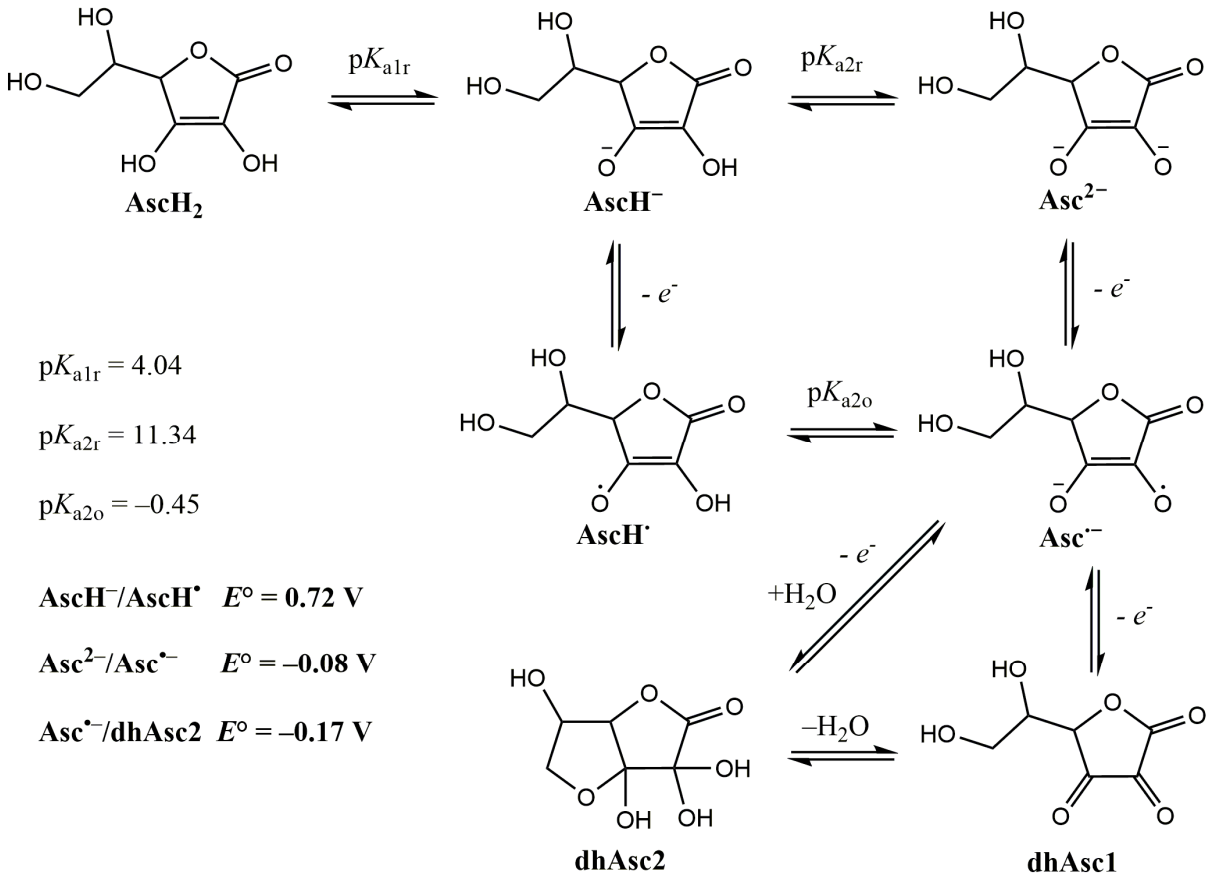

Figure 3.1. Possible mechanisms for the oxidation of $AscH_2$ to dhAsc.

It is very interesting to note how **AscH**$_2$ is an extremely efficient molecule and adapted to function in the physiological environment. Figure 3.1 shows that at pH close to 7.0, **AscH**$_2$ appears almost entirely in its monoprotic **AscH**$^-$ form (pK_{a1r} = 4.04), while the pK_{a2r} value is 11.34. If **AscH**$^-$ (**AscH**$^-$/**AscH**$^\bullet$ $E°$ = 0.72 V) comes into contact with an oxidizing agent strong enough, it undergoes oxidation by transferring 1 electron, forming the radical **AscH**$^\bullet$.

Once in radical form, it becomes a strong base, promptly losing the second proton (pK_{a2o} = −0.45 against pK_{a2r} = 11.34). The **Asc**$^{\bullet-}$/**dhAsc** pair is a much more efficient reducer ($E°$ = −0.174 V) transferring the second electron easily to the substrate.

When deprotonated, **AscH**$_2$ acts as an O-donor base with low polarizability, a hard base according to hard and soft acids and bases (HSAB) theory, easily complexing with several metal

ions present in solution, mainly transition metals with high state of oxidation (e.g. Fe^{3+}, Co^{3+}, Cu^{2+}, Cr^{6+}, V^{5+}). The complexation is due to the ability that the **AscH**$^-$ and **Asc**$^{2-}$ species have to donate electronic density to the metal ions, forming a stable complex. The interaction with the metallic center totally changes the expected redox behavior for **AscH**$_2$.

3.2. Effect of transition metals over the redox chemistry of AscH$_2$

According to what being studied in the literature, regarding the effect of metal complexation on **AscH**$_2$, we can divide metals into three most common categories:

3.2.1. High oxidation state metals: Co(III), Cr(VI) and V(V)

The complexation of the **AscH**$_2$ with metal ions that has $E°$ greater than 0.72 V, e.g Co (III), Cr (VI) and V(V) (Table 3. 1) promotes the electron transfer of from **AscH**$_2$ to the metal, leading to formation of the **dhAsc**. Usually **dhAsc** is irreversibly hydrolyzed to diketogulonic acid.[21] In this scenario, happen the reduction in concentration of **AscH**$_2$ in the medium, causing, for example, the measured antioxidant activity to be underestimated in a test with radical DPPH. Some known redox complexation reactions for **AscH**$_2$ are:

$$3\mathrm{AscH^-} + 2\mathrm{Cr(VI)} \rightarrow 3\mathrm{dhAsc} + 2\mathrm{Cr(III)} + 3\mathrm{H^+}$$

$$\mathrm{AscH^-} + \mathrm{Co(III)} \rightarrow \mathrm{dhAsc} + \mathrm{Co(II)} + \mathrm{H^+}$$

$$\mathrm{AscH^-} + \mathrm{V(V)} \rightarrow \mathrm{dhAsc} + \mathrm{V(IV)} + \mathrm{H^+}$$

3.2.2. Redox inert transition metals: Zn(II), Cd(II), Mn(II) and Ni(II)

The interaction of these metals with **AscH**$_2$ has been investigated in the solution and solid phases.[85] The complexes with general formula $M^{n+}(\mathbf{AscH}^-)_n \cdot 2H_2O$ show good stability without any electron transfer (ET) from **AscH$_2$** to the metal center. However, we still not found studies about the effect of these complexation in the antioxidant activity of **AscH$_2$**.

3.2.3. Special cases, formation of catalytical systems: Cu(II) and Fe(III)

The redox pair $Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$ own $E°$ equal to 0.771 V, which is relatively high and indicates that the reduction of Fe^{3+} is thermodynamically favorable, however, it is unusual to find Fe^{2+} ions in oxidizing environment, like in the presence of O_2. The Fe^{3+} has a semi-filled d orbital ($3d^5$), ensuring greater stability compared to Fe^{2+} ($3d^6$), especially when complexed with intermediate or weak field ligands, which promote an unfolding of the crystalline field smaller, stabilizing the high spin specie. That easiness that Fe^{2+} has to be quickly oxidized to Fe^{3+}, in the presence of O_2, afford a catalytic system that promotes the oxidation of **AscH$_2$**, even with trace amounts of Fe^{3+} in the medium.

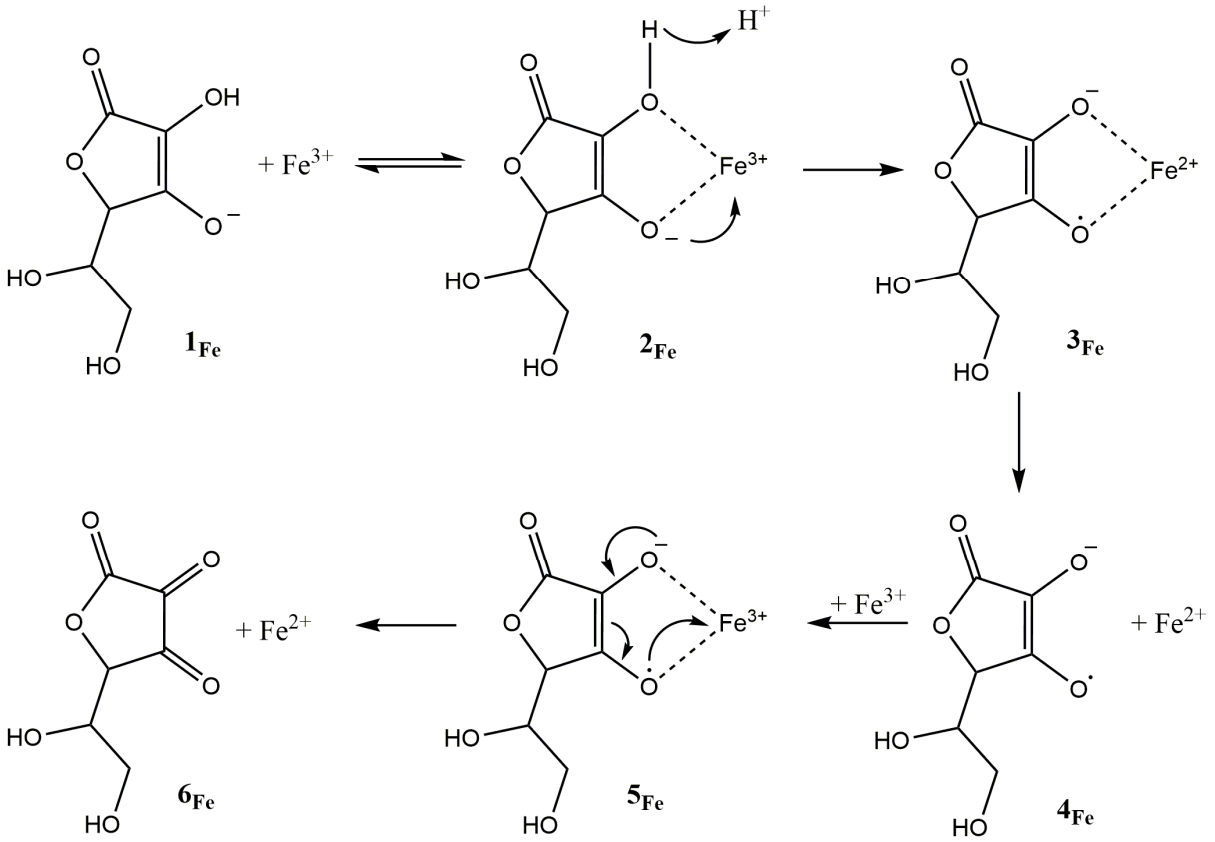

Figure 3.2. Proposed mechanism for the catalytic oxidation of the $AscH_2$ by the Fe^{3+}.

The Cu^{2+} cation is a classic case of the Jahn-Teller effect with a greater stabilization of the d_{Z^2} orbital in the $3d^9$ configuration. Cu^+ in turn, have a full filled subshell ($3d^{10}$) which can also provide stability to the monovalent cation. However, since it is a soft acid (HSAB theory), the Cu^+ needs an equally soft base, little polarizable, as an S-donor base in order to form a stable complex. In an aqueous medium, the enthalpy of hydration of the Cu^+ is so high that the cation oxidates quickly to the divalent state.

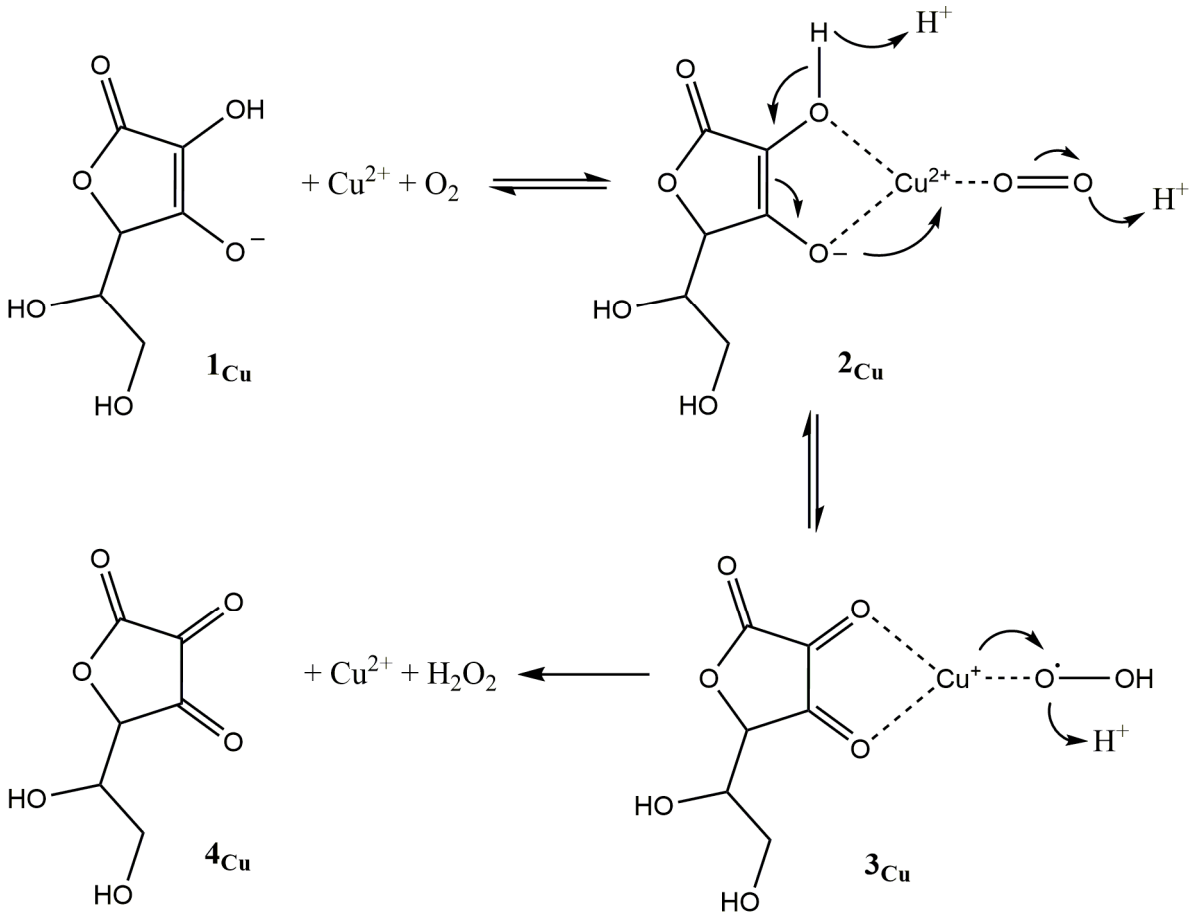

Figure 3.3. Proposed mechanism for the catalytic oxidation of the $AscH_2$ by the Cu^{2+}, with formation of hydrogen peroxide.

Table 3. 1. Selected standard reduction potentials for some transition metals.

Redox reaction	$E°$	Redox reaction	$E°$
$Cu^{2+} + e^- \rightleftharpoons Cu^+$	0.15	$Co^{3+} + e^- \rightleftharpoons Co^{2+}$	1.92
$Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$	0.77	$Co^{2+} + e^- \rightleftharpoons Co$	−0.28
$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightleftharpoons 2Cr^{3+} + 7H_2O$	1.36	$Ni^{2+} + 2e^- \rightleftharpoons Ni$	−0.26
$Cr^{3+} + e^- \rightleftharpoons Cr^{2+}$	−0.41	$O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O_2$	0.69
$Al^{3+} + 3e^- \rightleftharpoons Al$	−1.62	$VO_2^+ + 2H^+ + e^- \rightleftharpoons VO^{2+} + H_2O$	0.99

3.3. Perspectives for this work

As described in Chapter II, the presence of metal ions has a great influence on the reaction mechanism in the study of antioxidant potentials. L-ascorbic acid, one of the most studied antioxidant agents in the literature, suffers a strong influence when placed in front of metal ions forming stable metal complexes, i.e., it has its antioxidant activity altered.

Based on this logic, for further work it is interesting to have more experimental tests evaluating, mainly, the complexation chemistry for $AscH_2$ which, despite being studied for a long time, lacks clear information in the literature such as characterization of crystals, for example. In addition, there are no reports of studies evaluating the antioxidant activity of $AscH_2$ complexes mainly, of complexes with inert oxidation number metals mentioned in item 2 of this chapter.

Therefore, in order to understand the behavior of L-ascorbic acid and the effect of metal ions on its antioxidant potential, specific experimental tests must be carried out to complete the existing literature and assist future work on antioxidant evaluation, especially of Zn(II), Cd(II), Mn(II) and Ni(II) metals. Which already have known complexes but their antiradical potentials have not yet been evaluated.

Chapter IV – General Conclusions

Despite many studies pointing the DPPH method as inconsistent, and even not recommending its use, this method is still widely used due to its relative ease and low cost. Studying the data, we observed that a large part of these inconsistencies is not inherent in the method, but are due to errors or lack of knowledge about the chemistry behind the method. More in-depth studies on kinetics and mechanisms are still needed to better define the capabilities of the DPPH assay.

It was observed that most of the apparent inconsistencies in the results of the tests with DPPH accrue from an error in the choice of units, since the IC_{50} value — commonly used — does not take into account the initial concentration of the DPPH radical. In a preliminary comparison of literature data from 25 studies with IC_{50} measurements for ascorbic acid (standard for DPPH assays), all reported values appear random and inconsistent with each other. However, the normalization of the IC_{50} reported by the initial concentration of DPPH, makes it evident that for 15 of these studies, the results are within the expected range, and the only problem is the lack of care with the units.

This work also showed that both DPPH-H and other by-products - depending on the antiradical compound used in the reaction - absorb at 515 nm, which is the region of the maximum absorbance of DPPH$^{\bullet}$. Nevertheless, the method commonly used to calculate the DPPH$^{\bullet}$ scavenging, considers that only DPPH$^{\bullet}$ contributes to the absorbance of the solution, this leads to an error in determining the percentage of DPPH$^{\bullet}$ scavenged. Chapter II showed how this error can be easily corrected, with the simple addition of one more element in the equation: the "Positive Control", where an excess of antioxidant is added, to determine the residual absorbance of the sample (when all DPPH$^{\bullet}$ was consumed).

Regarding the use of ascorbic acid as a standard for the DPPH assay, much remains to be studied, but it is already possible to affirm that despite its apparently simple antiradical mechanism, the results can suffer great deviations due to impurities, mainly metals. Working with ascorbic acid in low concentrations is especially complicated and demands attention, as even impurities in the solvent used can lead to the oxidation of ascorbic acid to dehydroascorbate, so that in the results of DPPH assay, the antioxidant potential of ascorbic acid appears much lower than the real.

The interference of traces of metals is also very important, since the ability to act as a reducing agent, can lead ascorbic acid to, for example, promote the reduction of Fe^{3+} to Fe^{2+}, catalyzing Fenton reactions, resulting in an apparent pro-oxidant activity.

References

(1) BLOIS, M. S. Antioxidant Determinations by the Use of a Stable Free Radical. *Nature* **1958**, *181* (4617), 1199–1200. https://doi.org/10.1038/1811199a0.

(2) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT - Food Sci. Technol.* **1995**, *28* (1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5.

(3) Mohamad H, Abas F, Permana D, L. N. A. A. et. al. DPPH Free Radical Scavenger Components from the Fruits. *J. Biosci.* **2004**, *59 (11-12)*, 811–815. https://doi.org/10.1038/oby.2010.258.

(4) Kedare, S. B.; Singh, R. P. Genesis and Development of DPPH Method of Antioxidant Assay. *J. Food Sci. Technol.* **2011**, *48* (4), 412–422. https://doi.org/10.1007/s13197-011-0251-1.

(5) Sanchez-Moreno, C. Review: Methods Used to Evaluate the Free Radical Scavenging Activity in Foods and Biological Systems. *Food Sci. Technol. Int.* **2002**, *8* (3), 121–137. https://doi.org/10.1106/108201302026770.

(6) Fidrianny, I.; Elviana, D.; Ruslan, K. In Vitro Antioxidant Activities in Various Beans Extracts of Five Legumes from West of Java-Indonesia Using DPPH and ABTS Methods. *Available online www.ijppr.com Int. J. Pharmacogn. Phytochem. Res.* **2016**, *8* (3), 470–476.

(7) Bondet, V.; Brand-Williams, W.; Berset, C. Kinetics and Mechanisms of Antioxidant Activity Using the DPPH• Free Radical Method. *LWT - Food Sci. Technol.* **1997**, *30* (6), 609–615. https://doi.org/10.1006/fstl.1997.0240.

(8) Srirama, R.; Deepak, H. B.; Senthilkumar, U.; Ravikanth, G.; Gurumurthy, B. R.; Shivanna, M. B.; Chandrasekaran, C. V.; Agarwal, A.; Shaanker, R. U. Hepatoprotective Activity of Indian *Phyllanthus*. *Pharm. Biol.* **2012**, *50* (8), 948–953. https://doi.org/10.3109/13880209.2011.649858.

(9) Mimica-Dukic, N.; Bozin, B.; Sokovic, M.; Simin, N. Antimicrobial and Antioxidant Activities of Melissa Officinalis L. (Lamiaceae) Essential Oil. *J. Agric. Food Chem.* **2004**, *52* (9), 2485–2489. https://doi.org/10.1021/jf030698a.

(10) Brighente, I. M. C. M. C.; Dias, M.; Verdi, L. G. G.; Pizzolatti, M. G. G. Antioxidant

Activity and Total Phenolic Content of Some Brazilian Species. *Pharm. Biol.* **2007**, *45* (2), 156–161. https://doi.org/10.1080/13880200601113131.

(11) Pyrzynska, K.; Pękal, A. Application of Free Radical Diphenylpicrylhydrazyl (DPPH) to Estimate the Antioxidant Capacity of Food Samples. *Anal. Methods* **2013**, *5* (17), 4288. https://doi.org/10.1039/c3ay40367j.

(12) Molyneux, P. The Use of the Stable Free Radical Diphenylpicryl- Hydrazyl (DPPH) for Estimating Antioxidant Activity. *J. Sci. Tecnol.* **2004**, *26* (2), 211–219. https://doi.org/10.1287/isre.6.2.144.

(13) Ayscough, P. B.; Russell, K. E. SPECTROSCOPIC STUDIES OF THE REVERSIBLE REACTION BETWEEN 2,2-DIPHENYL-1-PICRYLHYDRAZYL AND 2,4,6-TRI- t - BUTYLPHENOL. *Can. J. Chem.* **1965**, *43* (11), 3039–3044. https://doi.org/10.1139/v65-418.

(14) lkay Koca, Ali Keleş, İ.; Gençcelep, H. Antioxidant Properties of Wild Edible Mushrooms. *J. Food Process. Technol.* **2011**, *02* (06). https://doi.org/10.4172/2157-7110.1000130.

(15) Mishra, K.; Ojha, H.; Chaudhury, N. K. Estimation of Antiradical Properties of Antioxidants Using DPPH Assay: A Critical Review and Results. *Food Chem.* **2012**, *130* (4), 1036–1043. https://doi.org/10.1016/j.foodchem.2011.07.127.

(16) Hvoslef, J.; Bergson, G.; Thorkilsen, B.; Halvarson, H.; Nilsson, L. The Crystal Structure of L-Ascorbic Acid, "Vitamin C". *Acta Chem. Scand.* **1964**, *18*, 841–842. https://doi.org/10.3891/acta.chem.scand.18-0841.

(17) MARTELL, A. E. Chelates of Ascorbic Acid; 1982; pp 153–178. https://doi.org/10.1021/ba-1982-0200.ch007.

(18) Jabs, W.; Gaube, W. Verbindungen Der L-Ascorbinsäure Mit Metallen. IV. Ligandeigenschaften Des Monoanions Der L-Ascorbinsäure, C6H7O6−. *ZAAC - J. Inorg. Gen. Chem.* **1986**, *538* (7), 166–176. https://doi.org/10.1002/zaac.19865380717.

(19) Adach, A.; Janyst, J.; Cieślak-Golonka, M. Interaction of Carcinogenic Chromium(vi) Oxide, CrO3, with Main Nonenzymatic Cellular Reductants at Physiological Conditions. Electronic Spectra and Magnetic Studies. *Spectrosc. Lett.* **1995**, *28* (8), 1259–1273. https://doi.org/10.1080/00387019508009463.

(20) Jabs, W.; Gaube, W. Verbindungen Der L-Ascorbinsäure Mit Metallen. I. Zur Darstellung von Ascorbatkomplexen Einiger 3d-Elemente. *ZAAC - J. Inorg. Gen. Chem.* **1984**, *514* (7), 179–184. https://doi.org/10.1002/zaac.19845140722.

(21) Ferrer, E. G.; Williams, P. A. M.; Baran, E. J. Interaction of the Vanadyl(IV) Cation with L-Ascorbic Acid and Related Systems. *Zeitschrift fur Naturforsch. - Sect. B J. Chem. Sci.* **1998**, *53* (2), 256–262. https://doi.org/10.1515/znb-1998-0220.

(22) Arnao, M. B. Some Methodological Problems in the Determination of Antioxidant Activity Using Chromogen Radicals: A Practical Case. *Trends in Food Science and Technology*. Elsevier Ltd November 1, 2000, pp 419–421. https://doi.org/10.1016/S0924-2244(01)00027-9.

(23) Pyrzynska, K.; Pękal, A. Application of Free Radical Diphenylpicrylhydrazyl (DPPH) to Estimate the Antioxidant Capacity of Food Samples. *Anal. Methods* **2013**, *5* (17), 4288–4295. https://doi.org/10.1039/c3ay40367j.

(24) Wrona, M.; Blasco, S.; Becerril, R.; Nerin, C.; Sales, E.; Asensio, E. Antioxidant and Antimicrobial Markers by UPLC®–ESI-Q-TOF-MSE of a New Multilayer Active Packaging Based on Arctostaphylos Uva-Ursi. *Talanta* **2019**, *196*, 498–509. https://doi.org/10.1016/j.talanta.2018.12.057.

(25) Sharma, O. P.; Bhat, T. K. DPPH Antioxidant Assay Revisited. *Food Chem.* **2009**, *113* (4), 1202–1205. https://doi.org/10.1016/j.foodchem.2008.08.008.

(26) Fadda, A.; Serra, M.; Molinu, M. G.; Azara, E.; Barberis, A.; Sanna, D. Reaction Time and DPPH Concentration Influence Antioxidant Activity and Kinetic Parameters of Bioactive Molecules and Plant Extracts in the Reaction with the DPPH Radical. *J. Food Compos. Anal.* **2014**, *35* (2), 112–119. https://doi.org/10.1016/j.jfca.2014.06.006.

(27) Chen, Z.; Bertin, R.; Froldi, G. EC50 Estimation of Antioxidant Activity in DPPH Assay Using Several Statistical Programs. *Food Chem.* **2013**, *138* (1), 414–420. https://doi.org/10.1016/j.foodchem.2012.11.001.

(28) Foti, M. C. Use and Abuse of the DPPH • Radical. *J. Agric. Food Chem.* **2015**, *63* (40), 8765–8776. https://doi.org/10.1021/acs.jafc.5b03839.

(29) Abramovič, H.; Grobin, B.; Ulrih, N. P.; Cigić, B. Relevance and Standardization of in Vitro Antioxidant Assays: ABTS, DPPH, and Folin–Ciocalteu. *J. Chem.* **2018**, *2018*, 1–

9. https://doi.org/10.1155/2018/4608405.

(30) Carmona-Jiménez, Y.; García-Moreno, M. V.; Igartuburu, J. M.; Garcia Barroso, C. Simplification of the DPPH Assay for Estimating the Antioxidant Activity of Wine and Wine By-Products. *Food Chem.* **2014**, *165*, 198–204. https://doi.org/10.1016/j.foodchem.2014.05.106.

(31) Milardović, S.; Iveković, D.; Grabarić, B. S. A Novel Amperometric Method for Antioxidant Activity Determination Using DPPH Free Radical. *Bioelectrochemistry* **2006**, *68* (2), 175–180. https://doi.org/10.1016/j.bioelechem.2005.06.005.

(32) Kandi, S.; Charles, A. L. Statistical Comparative Study between the Conventional DPPH Spectrophotometric and Dropping DPPH Analytical Method without Spectrophotometer: Evaluation for the Advancement of Antioxidant Activity Analysis. *Food Chem.* **2019**, *287*, 338–345. https://doi.org/10.1016/j.foodchem.2019.02.110.

(33) Abderrahim, F.; Arribas, S. M. M.; Carmen Gonzalez, M.; Condezo-Hoyos, L. Rapid High-Throughput Assay to Assess Scavenging Capacity Index Using DPPH. *Food Chem.* **2013**. https://doi.org/10.1016/j.foodchem.2013.04.055.

(34) Goujot, D.; Cuvelier, M. E.; Soto, P.; Courtois, F. A Stoichio-Kinetic Model for a DPPH·-Ferulic Acid Reaction. *Talanta* **2019**, *196*, 284–292. https://doi.org/10.1016/j.talanta.2018.12.056.

(35) Kiers, C. T.; de Boer, J. L.; Olthof, R.; Spek, A. L. The Crystal Structure of a 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Modification. *Acta Crystallogr. Sect. B Struct. Crystallogr. Cryst. Chem.* **1976**, *32* (8), 2297–2305. https://doi.org/10.1107/S0567740876007632.

(36) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. a.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. a.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, a. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, a. F.; Sonnenberg, J. L.; Williams; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr., J. a.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. a.; Kobayashi, R.;

Normand, J.; Raghavachari, K.; Rendell, a. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J. Gaussian 09, Revision D.01. Gaussian, Inc.: Wallingford CT, CT, USA 2013.

(37) Ley, J. P.; Engelhart, K.; Bernhardt, J.; Bertram, H.-J. 3,4-Dihydroxymandelic Acid, a Noradrenalin Metabolite with Powerful Antioxidative Potential. *J. Agric. Food Chem.* **2002**, *50* (21), 5897–5902. https://doi.org/10.1021/jf025667e.

(38) Lo Scalzo, R. Organic Acids Influence on DPPH Scavenging by Ascorbic Acid. *Food Chem.* **2008**, *107* (1), 40–43. https://doi.org/10.1016/j.foodchem.2007.07.070.

(39) Pehlivan, F. E. Vitamin C: An Antioxidant Agent. In *Vitamin C*; InTech, 2017. https://doi.org/10.5772/intechopen.69660.

(40) Nimse, S. B.; Pal, D. Free Radicals, Natural Antioxidants, and Their Reaction Mechanisms. *RSC Adv.* **2015**, *5* (35), 27986–28006. https://doi.org/10.1039/C4RA13315C.

(41) Shirwaikar, A. A.; Shirwaikar, A. A.; Rajendran, K.; Punitha, I. S. R. *In Vitro* Antioxidant Studies on the Benzyl Tetra Isoquinoline Alkaloid Berberine. *Biol. Pharm. Bull.* **2006**, *29* (9), 1906–1910. https://doi.org/10.1248/bpb.29.1906.

(42) Eklund, P. C.; Långvik, O. K.; Wärnå, J. P.; Salmi, T. O.; Willför, S. M.; Sjöholm, R. E. Chemical Studies on Antioxidant Mechanisms and Free Radical Scavenging Properties of Lignans. *Org. Biomol. Chem.* **2005**, *3* (18), 3336–3347. https://doi.org/10.1039/b506739a.

(43) Sârbu, C.; Casoni, D. Comprehensive Evaluation of Biogenic Amines and Related Drugs' Antiradical Activity Using Reactive 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical. *Cent. Eur. J. Chem.* **2013**, *11* (5), 679–688. https://doi.org/10.2478/s11532-013-0210-y.

(44) Wang, K.-J. J.; Li, N. Norlignan Derivatives from Curculigo Crassifolia and Their DPPH Radical Scavenging Activity. *Arch. Pharm. Res.* **2008**, *31* (10), 1313–1316. https://doi.org/10.1007/s12272-001-2111-4.

(45) Mamat, N.; Jamal, J. A.; Jantan, I.; Husain, K. Xanthine Oxidase Inhibitory and DPPH Radical Scavenging Activities of Some Primulaceae Species. *Sains Malaysiana* **2014**,

43 (12), 1827–1833. https://doi.org/10.17576/jsm-2014-4312-03.

(46) Kanimozhi, P.; Prasad, N. R. Antioxidant Potential of Sesamol and Its Role on Radiation-Induced DNA Damage in Whole-Body Irradiated Swiss Albino Mice. *Environ. Toxicol. Pharmacol.* **2009**, *28* (2), 192–197. https://doi.org/10.1016/j.etap.2009.04.003.

(47) Ramaswamy, V.; Varghese, N.; Simon, A. An Investigation on Cytotoxic and Antioxidant Properties of Clitoria Ternatea L. *Int. J. Drug Discov.* **2011**, *3* (1), 74–77. https://doi.org/10.9735/0975-4423.3.1.74-77.

(48) Assanga, S. I.; Luján, L. L.; Rivera-Castañeda, E.; Gil-Salido, A. A. A.; Acosta-Silva, A.; Rubio-Pino, J. *Effect of Maturity and Harvest Season on Antioxidant Activity, Phenolic Compounds and Ascorbic Acid of Morinda Citrifolia L. (Noni) Grown in Mexico (with Track Change)*; Academic Journals, 2013; Vol. 12, pp 4630–4639. https://doi.org/10.4314/ajb.v12i29.

(49) Yang, Z.; Tu, Y.; Baldermann, S.; Dong, F.; Xu, Y.; Watanabe, N. Isolation and Identification of Compounds from the Ethanolic Extract of Flowers of the Tea (Camellia Sinensis) Plant and Their Contribution to the Antioxidant Capacity. *LWT - Food Sci. Technol.* **2009**, *42* (8), 1439–1443. https://doi.org/10.1016/j.lwt.2009.03.017.

(50) Ding, H.-Y. Y. Extracts and Constituents of Rubus Chingii with 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Free Radical Scavenging Activity. *Int. J. Mol. Sci.* **2011**, *12* (6), 3941–3949. https://doi.org/10.3390/ijms12063941.

(51) Abdulmajed, K.; McGuigan, C.; Heard, C. M. Topical Delivery of Retinyl Ascorbate: 4. Comparative Anti-Oxidant Activity towards DPPH. *Free Radic. Res.* **2005**, *39* (5), 491–498. https://doi.org/10.1080/10715760500072263.

(52) Ko, R. K.; Kim, G.-O.; Hyun, C.-G.; Jung, D. S.; Lee, N. H. Compounds with Tyrosinase Inhibition, Elastase Inhibition and DPPH Radical Scavenging Activities from the Branches of Distylium Racemosum Sieb. et Zucc. **2011**. https://doi.org/10.1002/ptr.3439.

(53) J. Munasinghe, T. C.; Seneviratne, C. K.; Thabrew, M. I.; Abeysekera, A. M. Antiradical and Antilipoperoxidative Effects of Some Plant Extracts Used by Sri Lankan Traditional Medical Practitioners for Cardioprotection. *Phyther. Res.* **2001**, *15* (6), 519–523. https://doi.org/10.1002/ptr.994.

(54) Kane, N. F.; Kyama, M. C.; Nganga, J. K.; Hassanali, A.; Diallo, M.; Kimani, F. T. Comparison of Phytochemical Profiles and Antimalarial Activities of Artemisia Afra Plant Collected from Five Countries in Africa. *South African J. Bot.* **2019**, *125*, 126–133. https://doi.org/10.1016/j.sajb.2019.07.001.

(55) Kano, M.; Takayanagi, T.; Harada, K.; Makino, K.; Ishikawa, F. Antioxidative Activity of Anthocyanins from Purple Sweet Potato, Ipomoera Batatas Cultivar Ayamurasaki. *Biosci. Biotechnol. Biochem.* **2005**, *69* (5), 979–988. https://doi.org/10.1271/bbb.69.979.

(56) Tang, D.; Li, H.-J.; Chen, J.; Guo, C.-W.; Li, P. Rapid and Simple Method for Screening of Natural Antioxidants from Chinese Herb Flos Lonicerae Japonicae by DPPH-HPLC-DAD-TOF/MS. **2008**, *31* (20). https://doi.org/10.1002/jssc.200800173.

(57) Gong, C.-F.; Wang, Y.-X.; Wang, M.-L.; Su, W.-C.; Wang, Q.; Chen, Q.-X.; Shi, Y. Evaluation of the Structure and Biological Activities of Condensed Tannins from Acanthus Ilicifolius Linn and Their Effect on Fresh-Cut Fuji Apples. *Appl. Biochem. Biotechnol.* **2019**, 1–16. https://doi.org/10.1007/s12010-019-03038-6.

(58) Vrchovská, V.; Sousa, C.; Valentão, P.; Ferreres, F.; Pereira, J. A.; Seabra, R. M.; Andrade, P. B. Antioxidative Properties of Tronchuda Cabbage (Brassica Oleracea L. Var. Costata DC) External Leaves against DPPH, Superoxide Radical, Hydroxyl Radical and Hypochlorous Acid. *Food Chem.* **2006**, *98* (3), 416–425. https://doi.org/10.1016/j.foodchem.2005.06.019.

(59) Musialik, M.; Litwinienko, G. Scavenging of Dpph • Radicals by Vitamin E Is Accelerated by Its Partial Ionization: The Role of Sequential Proton Loss Electron Transfer. *Org. Lett.* **2005**, *7* (22), 4951–4954. https://doi.org/10.1021/ol051962j.

(60) Litwinienko, G.; Beckwith, A. L. J.; Ingold, K. U. The Frequently Overlooked Importance of Solvent in Free Radical Syntheses. *Chem. Soc. Rev.* **2011**, *40* (5), 2157. https://doi.org/10.1039/c1cs15007c.

(61) Mohsen-Nia, M.; Amiri, H.; Jazi, B. Dielectric Constants of Water, Methanol, Ethanol, Butanol and Acetone: Measurement and Computational Study. *J. Solution Chem.* **2010**, *39* (5), 701–708. https://doi.org/10.1007/s10953-010-9538-5.

(62) Dawidowicz, A. L.; Wianowska, D.; Olszowy, M. On Practical Problems in Estimation of Antioxidant Activity of Compounds by DPPH Method (Problems in Estimation of Antioxidant Activity). *Food Chem.* **2012**, *131* (3), 1037–1043.

https://doi.org/10.1016/j.foodchem.2011.09.067.

(63) Grazul, M.; Budzisz, E. Biological Activity of Metal Ions Complexes of Chromones, Coumarins and Flavones. *Coord. Chem. Rev.* **2009**, *253* (21–22), 2588–2598. https://doi.org/10.1016/j.ccr.2009.06.015.

(64) Amorati, R.; Pedulli, G. F.; Cabrini, L.; Zambonin, L.; Landi, L. Solvent and PH Effects on the Antioxidant Activity of Caffeic and Other Phenolic Acids. *J. Agric. Food Chem.* **2006**, *54* (8), 2932–2937. https://doi.org/10.1021/jf053159+.

(65) Sateanchok, S.; Wangkarn, S.; Saenjum, C.; Grudpan, K. A Cost-Effective Assay for Antioxidant Using Simple Cotton Thread Combining Paper Based Device with Mobile Phone Detection. *Talanta* **2018**, *177*, 171–175. https://doi.org/10.1016/j.talanta.2017.08.073.

(66) Olajide, T. M.; Liu, T.; Liu, H.; Weng, X. Antioxidant Properties of Two Novel Lipophilic Derivatives of Hydroxytyrosol. *Food Chem.* **2020**, *315*. https://doi.org/10.1016/j.foodchem.2020.126197.

(67) Annunziata, G.; Barrea, L.; Ciampaglia, R.; Cicala, C.; Arnone, A.; Savastano, S.; Nabavi, S. M.; Tenore, G. C.; Novellino, E. <scp> *Arctium Lappa* </Scp> Contributes to the Management of Type 2 Diabetes Mellitus by Regulating Glucose Homeostasis and Improving Oxidative Stress: A Critical Review of in Vitro and in Vivo Animal-based Studies. *Phyther. Res.* **2019**, *33* (9), 2213–2220. https://doi.org/10.1002/ptr.6416.

(68) Ait Lahcen, S.; El Hattabi, L.; Benkaddour, R.; Chahboun, N.; Ghanmi, M.; Satrani, B.; Tabyaoui, M.; Zarrouk, A. Chemical Composition, Antioxidant, Antimicrobial and Antifungal Activity of Moroccan Cistus Creticus Leaves. *Chem. Data Collect.* **2020**, *26*, 100346. https://doi.org/10.1016/j.cdc.2020.100346.

(69) Değirmenci, H.; Erkurt, H. Relationship between Volatile Components, Antimicrobial and Antioxidant Properties of the Essential Oil, Hydrosol and Extracts of Citrus Aurantium L. Flowers. *J. Infect. Public Health* **2020**, *13* (1), 58–67. https://doi.org/10.1016/j.jiph.2019.06.017.

(70) Almeida, A. da R.; Maciel, M. V. de O. B.; Machado, M. H.; Bazzo, G. C.; Armas, R. D.; Vitorino, V. B.; Vitali, L.; Block, J. M.; Barreto, P. L. M. Bioactive Compounds and Antioxidant Activities of Brazilian Hop (Humulus Lupulus L.) Extracts. *Int. J. Food Sci. Technol.* **2020**, *55* (1), 340–347. https://doi.org/10.1111/ijfs.14311.

(71) Fernández-Agulló, A.; Freire, M. S.; Ramírez-López, C.; Fernández-Moya, J.; González-Álvarez, J. Valorization of Residual Walnut Biomass from Forest Management and Wood Processing for the Production of Bioactive Compounds. *Biomass Convers. Biorefinery* **2020**. https://doi.org/10.1007/s13399-019-00598-9.

(72) Szabo, M.; Idiţoiu, C.; Chambre, D.; Lupea, A. Improved DPPH Determination for Antioxidant Activity Spectrophotometric Assay. *Chem. Pap.* **2007**, *61* (3), 214–216. https://doi.org/10.2478/s11696-007-0022-7.

(73) Yanai, T.; Tew, D. P.; Handy, N. C. A New Hybrid Exchange-Correlation Functional Using the Coulomb-Attenuating Method (CAM-B3LYP). *Chem. Phys. Lett.* **2004**, *393* (1–3), 51–57. https://doi.org/10.1016/j.cplett.2004.06.011.

(74) Escudero, D.; Laurent, A. D.; Jacquemin, D. Handbook of Computational Chemistry. In *Handbook of Computational Chemistry*; Leszczynski, J., Kaczmarek-Kedziera, A., Puzyn, T., G. Papadopoulos, M., Reis, H., K. Shukla, M., Eds.; Springer International Publishing: Cham, 2017; pp 1–2381. https://doi.org/10.1007/978-3-319-27282-5.

(75) Ciofini, I.; Adamo, C. Accurate Evaluation of Valence and Low-Lying Rydberg States with Standard Time-Dependent Density Functional Theory. *J. Phys. Chem. A* **2007**, *111* (25), 5549–5556. https://doi.org/10.1021/jp0722152.

(76) Suendo, V.; Viridi, S. Ab Initio Calculation of UV-Vis Absorption Spectra of a Single Chlorophyll a Molecule: Comparison Study between RHF/CIS, TDDFT, and Semi-Empirical Methods. *ITB J. Sci.* **2012**, *44 A* (2), 93–112. https://doi.org/10.5614/itbj.sci.2012.44.2.1.

(77) Zamani, M.; Moradi Delfani, A.; Jabbari, M. Scavenging Performance and Antioxidant Activity of γ-Alumina Nanoparticles towards DPPH Free Radical: Spectroscopic and DFT-D Studies. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2018**, *201*, 288–299. https://doi.org/10.1016/j.saa.2018.05.004.

(78) Ozawa, T. Non-Isothermal Kinetics of Consecutive Reactions. *J. Therm. Anal. Calorim.* **2000**, *60* (3), 887–894. https://doi.org/10.1023/A:1010111825431.

(79) Bahram, M. Mean Centering of Ratio Spectra as a New Method for Determination of Rate Constants of Consecutive Reactions. *Anal. Chim. Acta* **2007**, *603* (1), 13–19. https://doi.org/10.1016/j.aca.2007.09.041.

(80) GIRALDO, L. J. L.; LAGUERRE, M.; LECOMTE, J.; FIGUEROA-ESPINOZA, MARIA-CRUZ, B. B.; WEISS, J.; DECKER, E. A.; VILLENEUVE, § AND PIERRE. Kinetic and Stoichiometry of the Reaction of Chlorogenic Acid and Its Alkyl Esters against the DPPH Radical. *J. Agric. Food Chem.* **2009**, *57*, 863–870. https://doi.org/https://doi.org/10.1021/jf803148z.

(81) Campos, A. N. A. M.; Duran, N.; Lopez-alarcón, C.; Lissi, E. KINETIC AND STOICHIOMETRIC EVALUATION OF FREE RADICALS SCAVENGERS ACTIVITIES BASED ON DIPHENYL-PICRYL HYDRAZYYL (DPPH) CONSUMPTION. *J. Chil. Chem. Soc.* **2012**, *57* (4), 1381–1384. https://doi.org/http://dx.doi.org/10.4067/S0717-97072012000400010.

(82) Crans, D. C.; Baruah, B.; Gaidamauskas, E.; Lemons, B. G.; Lorenz, B. B.; Johnson, M. D. Impairment of Ascorbic Acid's Anti-Oxidant Properties in Confined Media: Inter and Intramolecular Reactions with Air and Vanadate at Acidic PH. *J. Inorg. Biochem.* **2008**, *102* (5–6), 1334–1347. https://doi.org/10.1016/j.jinorgbio.2008.01.015.

(83) Tu, Y. J.; Njus, D.; Schlegel, H. B. A Theoretical Study of Ascorbic Acid Oxidation and HOO / O 2- Radical Scavenging. *Org. Biomol. Chem.* **2017**, *15* (20), 4417–4431. https://doi.org/10.1039/c7ob00791d.

(84) KANNER, J.; MENDEL, H.; BUDOWSKI, P. PROOXIDANT AND ANTIOXIDANT EFFECTS OF ASCORBIC ACID AND METAL SALTS IN A ?-CAROTENE-LINOLEATE MODEL SYSTEM. *J. Food Sci.* **1977**, *42* (1), 60–64. https://doi.org/10.1111/j.1365-2621.1977.tb01218.x.

(85) Zümreoglu-Karan, B. *The Coordination Chemistry of Vitamin C: An Overview*; Elsevier, 2006; Vol. 250, pp 2295–2307. https://doi.org/10.1016/j.ccr.2006.03.002.