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Spectral analysis allows using the DPPH* UV–Vis assay to estimate antioxidant activity of colored compounds

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Abstract

An accurate measurement of antiradical activity to samples, such as herbal extract and natural or synthetic pure compounds, is essential to estimate its scope as antioxidant, for example, in functional foods. The 2,2-diphenyl-1-picrylhydrazyl (DPPH*) UV–Vis assay is the most widely used method for this aim. Nevertheless, it can drive to erroneous results if the spectroscopic properties of each substance, in the reaction medium, are not considered. To overcome this limitation, we present a new methodology based on spectral analysis which, instead of reading at fixed wavelength, uses the UV–Vis spectra of selected compounds, as well as simple algebraic operations and fitting by residual sum of squares. The proposed methodology was assayed successfully to determine the antiradical activity of three compounds: naringin, which does not present absorbance in the absorption band of DPPH*, naringin-Cu(II) complex, with moderate absorbance at this range, and naringin-Fe(III) complex, which shows intense absorption at the studied range. The proposed methodology is simple with a broad scope, since it allows to study the free radical scavenging activity on samples with high color that could not be analyzed, by UV–Vis spectrophotometry, up to the moment.

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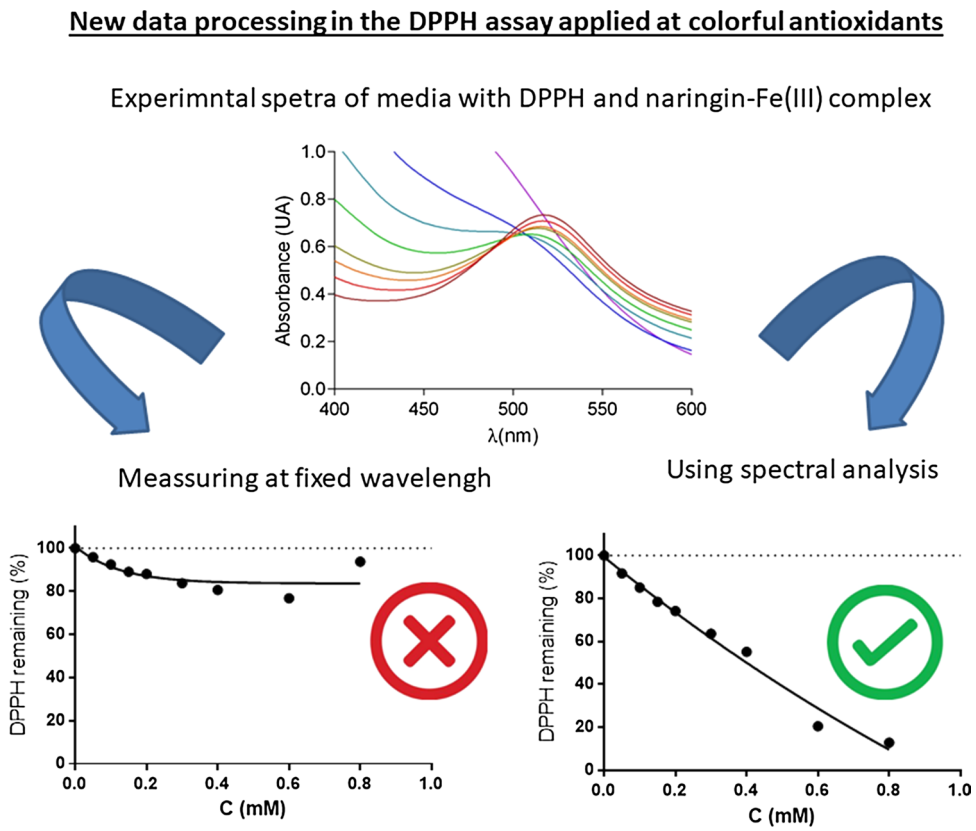
Graphic abstract



Keywords DPPH* assay · Free radical scavenging activity · Antioxidant activity · Naringin · *Citrus* flavonoids · Metal flavonoid complex

Introduction

Free radicals of oxygen and nitrogen species (ROS and RNS, respectively) are present in the atmosphere and in living organisms, since they are generated from stable molecules by solar radiation and by respiration. Unpaired electrons in free radicals give them a great reactivity, which may cause beneficial and harmful effects in living systems. At low concentrations, the beneficial effects involve responses in several cellular signaling systems such as defense against infectious agents (Pham-Huy et al. 2008). Conversely, at high level they may damage lipids, proteins and nucleic acids and thus the cell structures (Kedare and Singh 2011). High levels of free radicals are diminished by the antioxidant action of enzymatic systems or by antioxidant substances; if they are not, they can lead to the development of diseases such as arteriosclerosis, arthritis, Parkinson's disease, heart disease and cancer (Klein and Ackerman 2003). It is necessary to consume food, medicines, nutraceuticals or dietary supplements rich in antioxidants so as to minimize the harmful effects of the free radicals in the organism. To address this issue properly, it is essential to count with reliable methodologies to compare the effectiveness of different substances in reducing the generation of radicals or to increase the passivation of the previously formed free radicals.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH*) assay is the most widespread method to quantify the radical scavenging activity of pure substances, mixtures or extracts. By searching for "DPPH" just in www.sciencedirect.com, it can be verified that since 1995 up to the present there was an exponential increase in published papers concerning the DPPH* assay (Supplementary Fig. 1). It was used to determine antioxidant activities of pure substances or mixtures; to study the reaction itself in theoretical and/or experimental form; to develop new methodologies; and to find it new uses, such as following the loss of antioxidant substances in various matrices after drying them (Branisa et al. 2017).

The DPPH* free radical, in alcoholic solution, has a deep violet color resulting from the molecular planarity. When antiradicals with phenolic group are present in the medium,

the main reaction is the transfer of a hydrogen atom from an –OH group to the nitrogen atom that supports the unpaired electron (Fig. 1). There are others mechanisms through an antioxidant can reacts with the DPPH* radical, and the extention of each one depends of the antioxidant molecule and the medium in which the reaction is carried out (Foti 2015). For example, a polyhydroxylated flavonoid such as quercetin in methanol/water reacts through a complex mechanism with secondary reactions that ends up obeying a kinetics of a fractional order with respect to the antioxidant (Foti et al. 2011). Despite the mechanism by which the reaction is carried out, it is caused by the loss of DPPH* planarity and the consequent disappearance of its violet color. Therefore, the consumption of the radical can be followed spectrophotometrically by measuring the absorbance of the remnant DPPH* (λ_{max} around 517–520 nm). The different procedures to determine the antiradical activity measure the initial DPPH* concentration and the remaining DPPH* in the reaction medium after an incubation period (% $DPPH_R$). If Eq. 1 is applied, the percentage of remaining DPPH* can be determined.

$$\%\mathrm{DPPH_R} = \frac{[\mathrm{DPPH}*]_t}{[\mathrm{DPPH}*]_0}.100 = \frac{A_t/\varepsilon_{\mathrm{DPPH}*}b}{A_0/\varepsilon_{\mathrm{DPPH}*}b}.100 = \frac{A_t}{A_0}.100 \quad (1)$$

In Eq. 1, A_t is the absorbance of the sample after incubation, A_0 is the absorbance of the control with solvent instead of antioxidant at the same incubation time, ε is the molar absorptivity, and b is the optical path length. This equation does not take into account the spectroscopic properties of all the compounds in the system, and thus, it may yield incorrect results, since the reduced analog (DPPH-H), the antioxidant and the product of the antioxidant might have non-negligible absorptivities and therefore absorb part of the radiation. The limitation of the traditional methodology, which uses reading at fixed wavelength, was clearly demonstrated in a recent publication (Olszowy and Dawidowicz 2018). Different methodological alternatives have been proposed to overcome this interference. However, most of these alternatives require specialized and expensive instruments such as HPLC with DAD and/or MS/MS detectors (Kedare

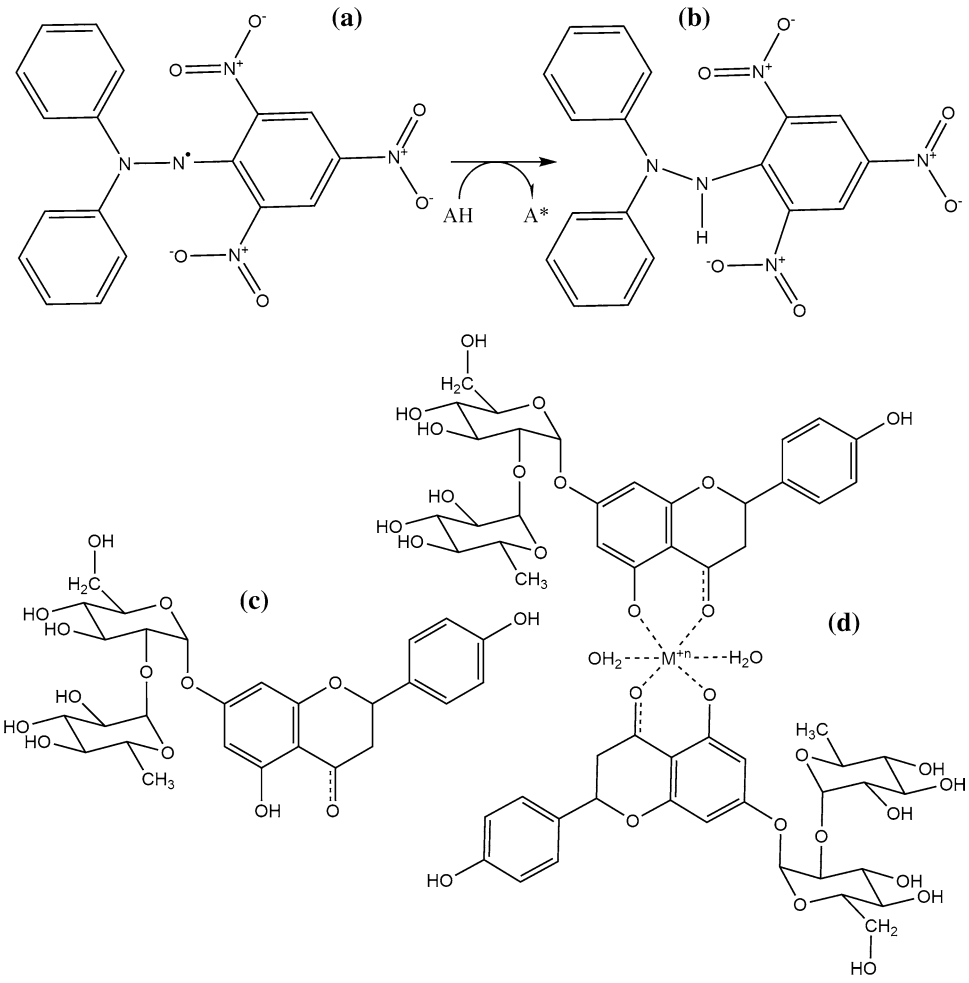


Fig. 1 Structures of **a** 2,2-diphenyl-1-picrylhydrazyl (DPPH*), **b** its reduced analog (DPPH-H), **c** naringin and **d** naringin complexes (M^{+n} can be Cu^{+2} or Fe^{+3})

and Singh 2011) or electron paramagnetic resonance (EPR) spectroscopy (Yeo and Shahidi 2019).

In this work, we propose a variant of the DPPH* assay that corrects this underestimation, based on UV–Vis spectral analysis. The method involves minimizing the residual sum of squares using software such as Excel datasheet or similar. The developed methodology was applied to measure the antioxidant activity of three compounds whose spectral features present different degrees of complexity: naringin that does not present absorbance at DPPH* band wavelength, and two naringin complexes that show absorption at this range: naringin-Cu(II) with moderate absorbance and naringin-Fe(III) with intense absorbance.

The goal of the experimental methodology and the data processing presented is to determine the antiradical activity of samples that present high absorbance in the same range of the DPPH* absorption band.

Experimental

Reagents

Naringin was produced from agro-industrial grapefruit waste by a simple and economic process (Geronazzo et al. 2000). It briefly consists in grinding the solid discarded parts to an average size of 2–4 mm and then performing an extraction in a fixed bed column with distilled water at 80 °C. The obtained extract is cooled, resulting in crystallization of the flavanone. The precipitate is filtered, washed and, finally, dried at 50 °C. This naringin is re-crystallized in water. The naringin obtained had a purity by HPLC (UV detection at 280 nm) over 98%.

Naringin-copper (II) complex was obtained by putting 25 mL of 25 mM copper (II) acetate in water (Anedra, Argentina) in a glass and adding dropwise 50 mL of 25 mM naringin in water/ ethanol (1:1, v/v) solution. The pH was adjusted to 7, and the reaction medium was kept under stirring at 60 °C for 1 h. Next, the reaction medium was cooled at 4 °C overnight. The solid was recovered by centrifugation. Then, it was washed with acetone to eliminate any naringin rest (naringin is soluble and the complex not). Afterward, it was dried in an oven at 50 °C. The complex obtained was a light green color.

Naringin-Fe(III) complex: It was obtained similarly to the naringin-copper (II) complex replacing Cu (II) salt by Fe(III) chloride (Biopack, Argentina). This complex did not precipitate in the reaction medium, and thus, the solvent was evaporated. The resulting solid was purified as the Cu (II) complex. The complex obtained was burgundy color.

Butylhydroxyanisole (BHA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH*) were purchased from Sigma (USA and Germany, respectively). All other reagents were of analytical grade.

Experimental for the proposed DPPH* assay

The foundation of the presented method comes from Brand-Williams et al. (1995). An DPPH* stock solution was prepared by dissolving the radical in methanol at a concentration of 6.3×10^{-5} M. A methanol solution of reduced DPPH* (DPPH-H) at the same concentration (6.3×10^{-5} M) was obtained from the previous one by the addition of a small amount of BHA which does not absorb at the spectral range studied. The antioxidants tested were dissolved at a concentration of 20 mM in dimethyl sulfoxide (DMSO).

Absorbance spectra were obtained from the individual species dissolved in DMSO/methanol (1:19, v/v): DPPH*; DPPH-H; and antioxidants (from 0.025 to 1.00 mM). From these spectra, the molar absorptions for each compound were determined.

The reaction time needed to reach a state where the concentration does not change against time (steady state) was determined as follows: solutions at a concentration of 1 mM of the antioxidants were prepared in DMSO. Then 1900 µL of the DPPH* solution was mixed with 100 µL of antioxidant solution (final concentration 0.05 mM) in several capped tubes. The solutions were kept under dark conditions at 20 °C. At every hour, one tube was removed and its absorbance was read at 515 nm.

To determine the antiradical activity of the tested compounds, 1900 µL of DPPH* solution and 100 µL of antioxidant solution in DMSO at different concentrations (0.5–20 mM) were mixed. When the steady state was reached (8 h), the spectra of the solutions between 400 and 600 nm were recorded in a spectrophotometer Cintra 101 (GBC, Australia).

Calculation methodology

When DPPH* reacts with an antioxidant, one must consider that DPPH*, DPPH-H, the antioxidant and the product (or products) derived from the antioxidant can be present in the medium. Therefore, it was suggested that each experimental spectrum could be constructed by a linear combination of the individual spectra of the species involved. This means that the total absorbance (measured in unities of absorbance, UA) at each wavelength will be given by the following expression:

$$A(\mathrm{UA}) = A_{\mathrm{DPPH*}} + A_{\mathrm{DPPHH}} + A_{\mathrm{antioxidant}} + A_{\mathrm{product}} \quad (2)$$

where, according to the Lambert–Beer law,

$$A_{\mathrm{DPPH*}} = b * \varepsilon_{\mathrm{DPPH*}} * C_{\mathrm{DPPH*}} = b * \varepsilon_{\mathrm{DPPH*}} * x_1 * C^0_{\mathrm{DPPH*}} \quad (2a)$$

$$A_{\mathrm{DPPHH}} = b * \varepsilon_{\mathrm{DPPHH}} * C_{\mathrm{DPPHH}} = b * \varepsilon_{\mathrm{DPPHH}} * (1 - x_1) * C^0_{\mathrm{DPPH*}} \quad (2b)$$

$$A_{\mathrm{atiox}} = b * \varepsilon_{\mathrm{antiox}} * C_{\mathrm{antiox}} = b * \varepsilon_{\mathrm{antiox}} * x_2 * C^0_{\mathrm{antiox}} \quad (2c)$$

$$A_{\mathrm{prod}} = b * \varepsilon_{\mathrm{prod}} * C_{\mathrm{prod}} = b * \varepsilon_{\mathrm{prod}} * (1 - x_2) * C^0_{\mathrm{antiox}} \quad (2d)$$

In these equations, A represents the absorbance of each species, b the optical path length, ε the molar absorptivities, C and C^0 (initial) molar concentrations and X_1 and X_2 the molar fractions of DPPH* and antioxidant, respectively, which still presents in the solutions.

Therefore, the general equation is:

$$A(\mathrm{UA}) = b * \varepsilon_{\mathrm{DPPH*}} * C_{\mathrm{DPPH*}} + b * \varepsilon_{\mathrm{DPPHH}} * C_{\mathrm{DPPHH}} + b * \varepsilon_{\mathrm{antiox}} * C_{\mathrm{antiox}} + b * \varepsilon_{\mathrm{prod}} * C_{\mathrm{prod}} \quad (3a)$$

or:

$$A(UA) = bC^0_{DPPH*}\left[\varepsilon_{DPPH*} * x_1 + \varepsilon_{DPPHH} * (1 - x_1)\right] + bC^0_{antiox}\left[\varepsilon_{antiox} x_2 + \varepsilon_{prod} * \left(1 - x_2\right)\right] \quad (3b)$$

Fitting X_1

If the parameters in Eq. 3b (b, C^0_{DPPH} and C^0_{antiox}) are fixed, the variables (X_1 and X_2) can be fitted so that Eq. 3b represents the experimental spectra, once the reaction between DPPH* and antioxidant is carried out. In the second term of Eq. 3a can be seen the variable X_2. In the studied cases, considerations could be made by simplifying the analysis in order to only optimize X_1. However, it could not be the more general case and both values (X_1 and X_2) should be determined. Two types of reaction media were presented:

Reaction media with antioxidants spectrophotometrically non-active In this case, the third term of Eq. 3 may be ignored. To know whether the fourth term can also be ignored, the reaction should proceed until $C_{\mathrm{DPPH*}} = 0$; therefore, $C_{\mathrm{DPPH\text{-}H}} = C^0_{\mathrm{DPPH*}}$, and the first term is also canceled. Then, the contribution of DPPH-H is subtracted from the experimental spectrum. Therefore,

$$A\,(\mathrm{UA}) - b * \varepsilon_{\mathrm{DPPHH}} * C^0_{\mathrm{DPPH*}} = b * \varepsilon_{\mathrm{prod}} * C_{\mathrm{prod}} \quad (4)$$

The difference $A\,(\mathrm{UA}) - b * \varepsilon_{\mathrm{DPPHH}} * C^0_{\mathrm{DPPH*}}$ represents the contribution made by the product, derived from the antioxidant, to the total absorbance of the medium.

Reaction media with antioxidants spectrophotometrically active If the antioxidant presents absorbance in the spectral region of study, the term associated with it cannot be eliminated from Eq. 3. Once the contribution of DPPH-H is subtracted, the spectrum of the reaction medium with DPPH* and antioxidant at t∞ should be compared with the spectrum of the antioxidant, at the same concentration, to estimate if the product derived from the antioxidant provides absorbance. If the differences between these spectra are small, it means that ε antiox and ε prod are similar. So, for these cases:

$$A_{\mathrm{atiox}} + A_{\mathrm{prod}} = bC^0_{\mathrm{antiox}}\left[\varepsilon_{\mathrm{antiox}} x_2 + \varepsilon_{\mathrm{prod}} * \left(1 - x_2\right)\right] = bC^0_{\mathrm{antiox}}\varepsilon_{\mathrm{antiox}}, \quad (5a)$$

and therefore, Eq. 3 is as follows:

$$A(\mathrm{UA}) = bC^0_{\mathrm{DPPH*}}\left[\varepsilon_{\mathrm{DPPH*}} * x_1 + \varepsilon_{\mathrm{DPPHH}} * (1 - x_1)\right] + bC^0_{\mathrm{antiox}}\varepsilon_{\mathrm{antiox}}. \quad (5b)$$

The best values for X_1 were determined by minimizing the residual sum of squares (RSS) using the complement solver included in Excel datasheet. To do this, a seven-column table was assembled distributed, from first to sixth column, as follows: (1) wavelengths; (2) the molar absorptivities of DPPH*; (3) the molar absorptivities DPPH-H; (4) the molar absorptivity multiplied by the corresponding antioxidant concentration; (5) the spectrum of each experimental reaction medium; (6) an absorption spectrum generated using both Eq. 3 (or its parents) and a tentative value of the molar fraction X_1. In the seventh column, the residual between calculated and experimental absorbance, at each wavelength (sixth column and fifth column), was determined. At the end of seventh column, the RSS was calculated. Finally, the complement was requested to minimize the RSS by adjusting the initial value of X_1, with the restriction that it takes positive values between 0 and 1. This process is iterative, and, in each cycle, a new X_1 is assayed until RSS no longer decreases. The molar fractions X_1 obtained by this methodology were converted into % $\mathrm{DPPH*_R}$ multiplying it by 100 and then compared with the values that were obtained by directly applying Eq. 1 evaluated at 515 nm.

Special case of reaction media with antioxidants partially insoluble The media with the naringin-Fe(III) complex at the two highest concentrations presented a small amount of precipitate at the end of the incubation. In these cases, the simulation with the equations proposed was not adequate, since the concentration of dissolved antioxidant decreased with the incubation time. In order to adequately reproduce these experimental curves, it was necessary to apply a minor modification to the methodology to consider this effect. It consisted in placing in different tubes 1900 uL of DPPH* solution and 100 uL of complex solution of the desired concentration. Then, a complex blank was prepared for each medium by replacing the volume of DPPH* solution with the same volume of methanol. After incubation, UV spectra were run from both: the reaction media and the blanks.

For the calculation, only the column of the molar absorptivity multiplied by the concentration of the complex (4th columns) was replaced by the spectrum of the complex solution in methanol at the same concentration after incubation. The data processing in all case was the same.

Plots and fitting

Plots and fitting of data were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results and discussion

Spectra of the compounds studied

The spectra of DPPH*, its reduced derivative (DPPH-H) and the tested antioxidants are presented in Fig. 2. Naringin has practically no absorbance between 400 and 600 nm (Fig. 2b). Its Cu (II) complex has moderate absorbance (Fig. 2c), and its Fe(III) complex absorbs strongly (Fig. 2d). We can see in Fig. 2a that the absorbance of DPPH-H is not at all negligible. It is known that the absorbance of DPPH-H increases as pH increases, reaching absorptivities close to those of DPPH* (Friaa and Brault 2006). This result, despite being reported in the literature, is usually not considered to calculate antiradical activities using the DPPH* essays. The molar absorptivities of the used compounds were obtained from these graphs (Supplementary Fig. 2).

Reaction media's spectra

Figure 3 presents the spectra of the reaction media with each antioxidant as a function of the final concentration at the steady state (8 h).

In naringin and naringin-Cu (II) spectra (Fig. 3a, b), there was a clear concentration response effect. In the set of naringin, an isosbestic point at 435 nm was observed. This indicates the coexistence of 2 spectroscopically active species linked by a chemical reaction. As naringin presents low absorbance at this range, this point is due to the conversion of DPPH* to DPPH-H. It is worth noting that the existence of this isosbestic point ensures that the only two forms are present in the system, DPPH* and DPPH-H, ruling out the possibility of a subsequent conversion of any of them by secondary reactions. For the set of naringin-Cu (II), the isosbestic point was displaced due to the absorbance of the complex. The group of curves of the naringin-Fe(III) complex did not present a defined isosbestic point due to the strong absorbance of the complex. The absorbance of the complex was so significant that even the maximum at 515 nm disappeared.

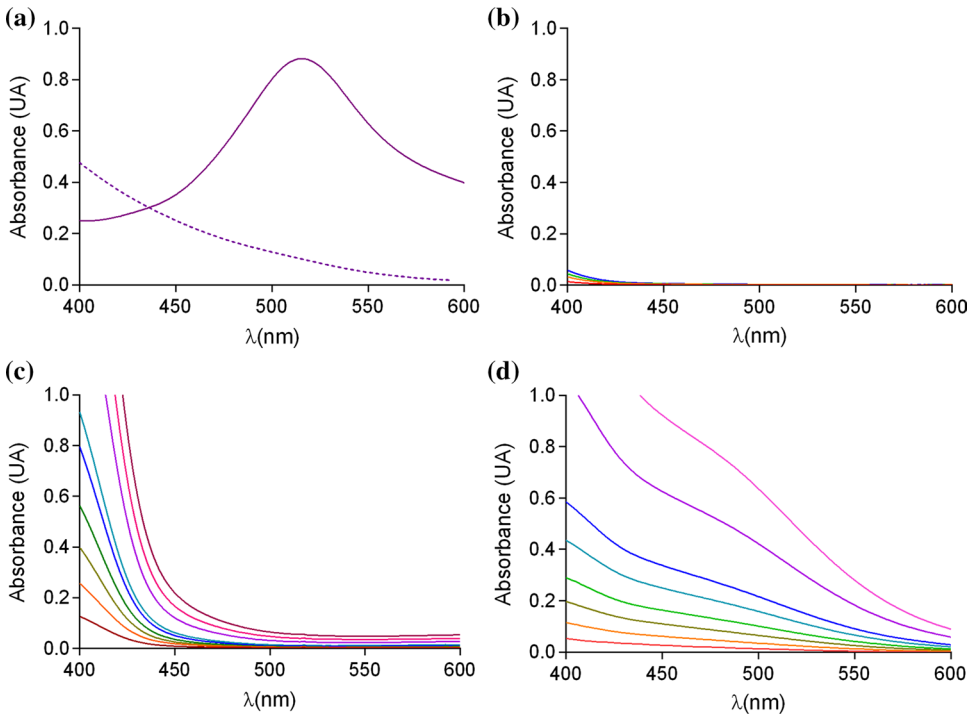


Fig. 2 Absorption spectra (400–600 nm) in DMSO/methanol 1:19 (v/v) of **a** DPPH* 6.0×10^{-5} M (continued line) and DPPH-H 6.0×10^{-5} M (dashed line); **b** naringin (0.05–1.00 mM); **c** naringin-Cu(II) (0.025–0.50 mM); **d** naringin-Fe(III) (0.05–0.80 mM)

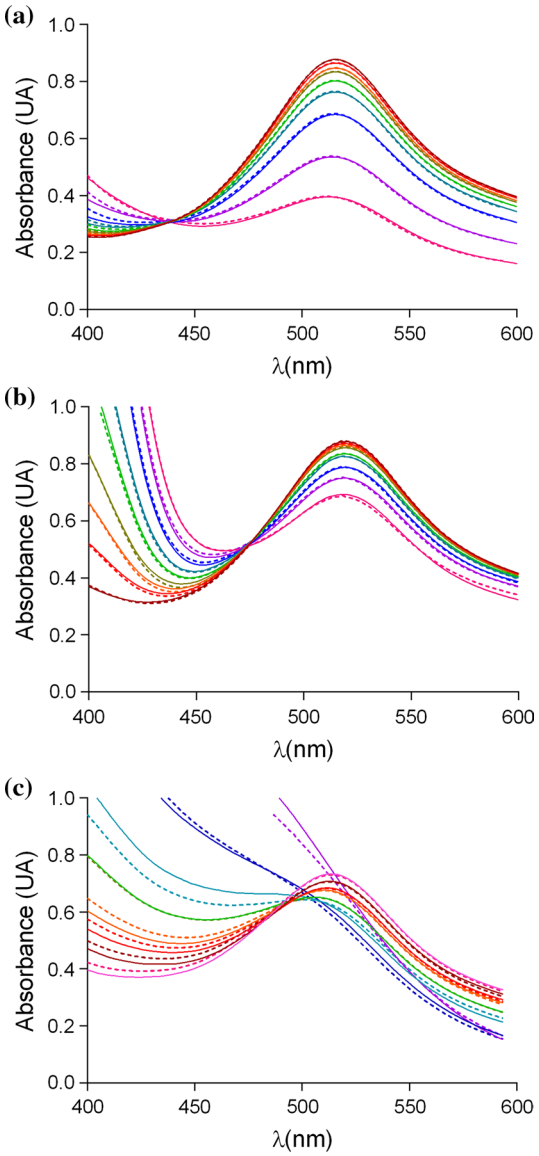


Fig. 3 Experimental (solid line) and constructed (with Eqs. 5b and 6b, dashed line) spectra of reaction media: **a** naringin (0.05–1.0 mM), **b** naringin-Cu (II) (0.025–0.5 mM) and **c** naringin-Fe(III) (0.05–0.80 mM)

Determination of the antiradical activity

To determine the antiradical activity, simplifications in the calculation were introduced according to the spectral properties of the antioxidants.

Solutions with antioxidant spectrophotometrically non-active

Naringin is a spectrophotometrically non-active antioxidant between 400 and 600 nm (Fig. 2b). The subtraction $A\,(\mathrm{UA}) - b * \varepsilon_{\mathrm{DPPHH}} * C^{0}_{\mathrm{DPPH*}}$ gave virtually zero across the spectrum, and thus, the product derived from naringin does not provide significant absorbance ($b * \varepsilon_{\mathrm{prod}} * C_{\mathrm{prod}} = 0$).

The results obtained for the optimized X_1 parameters are presented in Table 1. It can be observed that the residual sum of squares was in the order of deviations less than 0.06% with respect to solutions spectra, which means an almost perfect fitting (Fig. 3a).

Solutions with antioxidant spectrophotometrically active

Both the naringin-Cu (II) complex and the naringin-Fe(III) complex showed absorbance at the wavelengths studied (Fig. 3c, d, respectively). This indicates that in Eq. 4 the term associated with the antioxidant cannot be eliminated. Once the contribution of DPPH-H was subtracted, the reaction between DPPH* and the complex at t∞ was compared with the spectra's complexes, at the same concentration, in order to estimate the spectral profiles of the products derived from the antioxidants (Supplementary Fig. 3b and 3c). The differences found were minimal, which indicates that $\varepsilon_{\mathrm{antiox}}$ and $\varepsilon_{\mathrm{prod}}$ are similar. So, for these cases:

$$A_{\mathrm{atiox}} + A_{\mathrm{prod}} = bC^{0}_{\mathrm{antiox}}\left[\varepsilon_{\mathrm{antiox}}x_2 + \varepsilon_{\mathrm{prod}} * \left(1 - x_2\right)\right] = bC^{0}_{\mathrm{antiox}}\varepsilon_{\mathrm{antiox}} \tag{6a}$$

So, Eq. 4 is reduced to Eq. 6b:

$$A\,(\mathrm{UA}) = bC^{0}_{\mathrm{DPPH*}}\left[\varepsilon_{\mathrm{DPPH*}} * x_1 + \varepsilon_{\mathrm{DPPHH}} * (1 - x_1)\right] + bC^{0}_{\mathrm{antiox}}\varepsilon_{\mathrm{antiox}} \tag{6b}$$

The best values for X_1 parameters were obtained by minimizing the RSS, between the experimental curves and the curves constructed with Eqs. 5b and 6b, according to the antioxidant spectrophotometric properties, starting the iteration with an arbitrary coefficient X_1. Figure 3 presents the experimental spectra and those constructed with the optimized X_1 parameters. Table 1 summarizes the calculated X_1 parameters with their respective RSS, expressed as a percentage of the deviation of each simulated curve with respect to the corresponding experimental spectrum.

The X_1 parameter was converted to remaining DPPH* (%) in the steady state multiplied by 100 and after that compared with the antiradical activity of the antioxidants at different concentrations obtained by applying Eq. 1 (Fig. 4).

For naringin, which does not absorb in the work zone at low concentrations of antioxidant, the amount of DPPH-H produced is low and there are no relevant differences between the two methods of calculation. However, at higher concentrations, the amount of DPPH-H produced is significant and contributes to the total absorbance at 515 nm, so the values calculated by Eq. 1 underestimate the actual antiradical activity of naringin.

For antioxidants with relevant absorption in the spectral region of the DPPH* band, the difference between both methodologies increased with the increase in both the molar

Table 1 X_1 parameters calculated with their respective RSS

c (mM)	Naringin		Naringin-Cu (II)		Naringin-Fe (III)	
	x_1	RRS*	x_1	RRS*	x_1	RRS*
0.025	–	–	0.99	0.09	–	–
0.050	0.99	0.02	0.98	0.05	0.92	2.5
0.075	–	–	0.97	0.05	–	–
0.100	0.98	0.02	0.96	0.08	0.85	2.7
0.150	0.96	0.04	0.92	1	0.78	2.6
0.200	0.94	0.03	0.90	0.7	0.74	3.2
0.300	0.90	0.7	0.84	1	0.64	0.3
0.400	0.85	0.7	0.78	1	0.55	7
0.500	–	–	0.69	1	–	–
0.600	0.75	2	–	–	0.20	3
0.800	0.56	2	–	–	0.13	4
1.000	0.37	2	–	–	–	–

*RSS is expressed as percentage of the deviation of each simulated curve with respect to the corresponding experimental spectrum

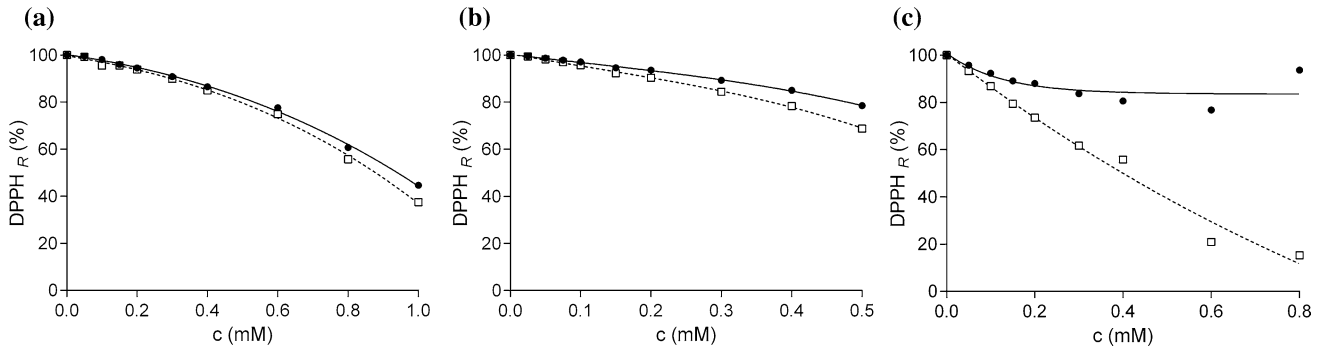


Fig. 4 Antiradical activity of the antioxidants at different concentrations expressed as remaining DPPH* (%) at the steady state, calculated by the proposed methodology (dashed line) and by Eq. 1 (solid line). **a** Naringin, **b** naringin-Cu (II) and **c** naringin-Fe(III)

absorptivity and the antioxidant concentration. The highest difference found in these experiments was for naringin-Fe(III) complex at 0.8 mM concentration, where, according to Eq. 1, the remaining DPPH* (%) was 93.7%, whereas it was 15.3% when using Eq. 5.

Conclusions

The DPPH* assay is the most widely used method to estimate the antiradical capacity of extract or pure substances. However, for its correct application it is necessary to consider the spectroscopic properties of all the components in the reaction media; otherwise, it may yield incorrect results.

In this work, a new methodology, which employed the spectra of DPPH*, DPPH-H and antioxidants, simple algebraic operations and minimization of the residual sum of squares by an iterative process, was successfully used to determine the molar fraction of DPPH* that remains in the reaction medium after the incubation (X_1). The developed method uses spectra for the calculation, instead of measures of absorbance at fixed wavelengths. The methodology presented corrects the underestimation that results when more simple calculation methods are used, since it does not take into account the contribution of neither DPPH-H nor antioxidants to the absorbance.

The proposed methodology is simple, using only one datasheet, and has the advantage of giving reliable results for both spectrophotometrically non-active antioxidants and for samples with high absorbance over the DPPH* band absorption. It was also proved that a slight modification to the method allows analyzing antioxidants, which modify their effective concentration in solution, caused by its low solubility, during incubation.

Although pure substances were used for the study, the proposed methodology could likewise be used to determine the antiradical activity of any extract, since the mathematics used allows it.

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