Relationship between Antioxidant Capacity and Food Chemistry

Caroline Gaucher, Pierre Leroy, Ariane Boudier*

Université de Lorraine, CITHÉFOR, EA 3452, Faculty of Pharmacy, BP 80403, F-54001 Nancy Cedex, France
*Corresponding author: ariane.boudier@univ-lorraine.fr

Abstract  A practical experiment devoted to study the properties of common foods (lemon, tea and garlic) containing antioxidant molecules is presently described. The experimental part was based on four main steps: (i) realization of calibration curves with standard antioxidants reacting with the 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH•), (ii) quantitation of antioxidant molecules in food extracts, and (iii) determination of food extract antioxidant capacity vs. DPPH•, which (iv) was linked to the content of antioxidant molecules in each food extract. The educational approach was based on both conceptual thermodynamic processes and on practical knowledge on food chemistry and also diet related to healthcare. As a result, in addition to gain experience in simple spectroscopic methods, students went deeper in the understanding of the redox mechanisms devoted to oxidative stress basic learning.

Keywords:  1,1-diphenyl-2-picryl-hydrazyl radical, food extract, redox, UV-visible spectrophotometry, spectrofluorimetry, antioxidant content


1. Introduction

Oxidative stress is extensively studied in many fields related to human health: ageing [1], cancer, infections, cardiovascular and neuronal diseases [2], life environment, and food chemistry [3]. It has been defined as an imbalance between reactive oxygen/nitrogen species (ROS/RNS) production and efficiency of antioxidant (AO) systems, in favor of ROS/RNS [4,5]. This may activate adverse cell signaling pathways leading to apoptosis, and cell, tissue or organ damages [6]. Nutritional source of AOs is an important strategy to fight against deleterious effects resulting from oxidative stress. However, it is of main importance to chemically define a global AO capacity of consumed foods in relation to structurally defined compounds [3].

Teaching oxidative stress to first-year undergraduate students implies numerous academic disciplines (chemistry, biochemistry, molecular and cell biology, and pharmacology [7]) and it represents a real educational challenge. Nevertheless, basic learning in the field of oxidative stress has to start with the understanding of chemical processes involved between AOs and free radicals [8,9,10,11,12], which are the main deleterious components of ROS/RNS. Four main mechanisms can illustrate the reaction between a free radical (FR•) and an AO. However, in aqueous media, the main resulting products are the reduced form of FR (i.e. FR-H) by hydrogen atom transfer (HAT) coming from the AO (under its oxidized form, AO•):

\[ \text{FR}^{\bullet} + \text{AOH} \rightarrow \text{FR-H} + \text{AO}^{\bullet} \]

For example, in the case of lipid peroxidation, HAT involves a radical generation leading to the formation of a hydroperoxide derivative.

This course was organized around four educational objectives as illustrated in Figure 1.

Figure 1. General organization of the presented practical course showing the educational and experimental objectives

Herein, AO properties in food extracts were presently studied through the reaction of a synthetic stable radical (1,1-diphenyl-2-picryl-hydrazyl (DPPH•)) with active compounds extracted from three different foods. Then, a correlation was established between AO properties of food extracts and their AO content. This methodology represents an originality compared to previously reported literature (study of only one synthesized AO compound or...
only one food extract [13,14,15]). However, due to the diversity of natural products presently used, leading to a diversity of AO compounds, which content was quantified, this study presents an original approach to understand nutritional source of AOs.

2. Experimental

Three foods widely consumed all over the world were proposed for experiments: lemon (Citrus limonum), green tea (Camellia sinensis), and garlic (Allium sativum). A targeted literature was supplied to each group of three students in order to help them to identify the main compound(s) responsible of the AO activity in the selected food extract (one per group) [14,16,17]. For each food, the AO properties were mainly associated with either a defined compound such as ascorbic acid for lemon, or a class of substances such as polyphenols for tea and sulphydryl compounds (thiols) for garlic. Then, the practical course was organized around experimental objectives in two 3-h periods as shown in Fig. 1. To help students, supervisors had previously provided students a handout material (see the supporting information). Depending on students’ skills, additional personal work might be required to write the report.

2.1. DPPH• Interaction with Standard AOs

The radical DPPH•, which is stable and colored (purple), was reacted with the standard AOs (stdAO), i.e. ascorbic acid, phloroglucinol (a polyphenolic compound), or N-acetylcysteine (a thiol) (Figure 2 and Figure 3), corresponding to model compounds able to mimic AO molecule(s) contained in food extracts.

As DPPH• presents a maximum of absorbance at a wavelength of 515 nm (A515) and its reduced form (i.e. 1,1-diphenyl-2-picryl-hydrazine (DPPH-H)) is colorless (yellow), the reaction was monitored by spectrophotometry in the visible range (see supporting information for detailed protocols).

Students had to dilute a DPPH•stock solution. Its exact concentration is calculated by Eq (1), using the measured absorbance value and the given molar absorbance of DPPH• (ε = 12.5 mM−1 cm−1 [18]) at 515 nm within Beer Lambert’s law [19] (c being the molar concentration of the chromophoric species, and b the optical path length, 1 cm):

\[ A = \varepsilon \times c \times b \]  

Figure 2. Chemical structure of standard antioxidants

The DPPH• solution was then incubated with successive dilutions of either ascorbic acid, or phloroglucinol or N-acetylcysteine solutions, as a function of the selected food. This allowed students to obtain a relationship between A515 of DPPH• and the stdAO molar concentration (CstdAO).

2.2. AO Content in Food Extracts

In a second step, spectroscopic techniques were applied to determine AO concentration in each food after extraction (CextAO) (squeezed lemon juice, infused tea and crushed garlic solutions) by specific assays: ortho-phenylenediamine (OPDA) [20], Folin-Ciocalteu’s [21] and Ellman’s [22] methods for ascorbic acid, polyphenols and sulphydryl compounds, respectively (see supporting information for detailed protocols). As far as Folin-Ciocaltéu’s reaction is concerned, students have to be aware of the fact that it is not totally specific to polyphenol quantitation and it may react with other reducing agents. Only OPDA assay required spectrofluorimetric measurements to avoid the interference between ascorbic acid-OPDA adduct and natural compounds present in infused tea.

2.3. DPPH• Reaction with Food Extracts and Their AO Properties

At last, the global AO properties of each food extract were evaluated by reaction with DPPH•. Dilutions of food extracts were incubated with DPPH• solution and, again, the radical bleaching was monitored by visible spectrophotometry (see supporting information for detailed protocols).

3. Hazard

Eye protection, gloves and coats have to be worn all the time while in the laboratory, and care was taken when handling laboratory glassware and boiling water. Some solutions were prepared by supervisors to save time and/or because of their possible associated risks (see Table S1 of supporting information for the complete list of hazards associated to each chemical product). All solutions and single use plastic were thrown away into hazardous waste containers to avoid environmental hazard.

4. Results and Discussion

Apart the insights in oxidative stress operated by
students during this practical course, several objectives in physical and analytical chemistry teaching were achieved. Taking into account the theoretical thermodynamic courses taught before, students could assume that the main mechanism occurring in an aqueous medium was the reaction between DPPH• and AOs which led to DPPH• reduction into DPPH-H.

4.1. Thermodynamic Concepts

The study of stdAO allowed a better understanding of the reaction mechanisms. From a thermodynamic point-of-view, following points had to be addressed:
• redox couples were defined as DPPH•/DPPH-H and A•/AO;
• \( \Delta G^\circ \), the free enthalpy or Gibbs energy of the reaction, is given by Eq (2):
\[
\Delta G^\circ = -nF\Delta E^\circ \tag{2}
\]
With \( \Delta G^\circ \) the free enthalpy or Gibbs energy of the reaction, n, number of exchanged electrons, F, Faraday constant (96,500 C.mol\(^{-1}\)) and \( \Delta E^\circ \), difference of standard redox potential values between both redox couples;
• to ensure a spontaneous reaction \( \Delta G^\circ \) must be negative.

This suggested that DPPH• was more oxidant than A•, \( E^\circ(DPPH•/DPPH-H) \) was higher (+ 0.54 V vs. standard hydrogen electrode [18]) than \( E^\circ(A•/AO) \), and the reaction was:
\[
\text{DPPH•} + \text{AO} \rightarrow \text{DPPH-H} + \text{A•} \tag{3}
\]
\( K \) being the equilibrium constant linked to \( \Delta G^\circ \) by Eq (3):
\[
\Delta G^\circ = -R \times T \times \ln K \tag{3}
\]
R being the ideal gas constant, and T the temperature.

\( K \) value had to be positive and the higher it was, the higher the product yield of DPPH• to DPPH-H was; \( K > 10^6 \) meant nearly 100 % (full reaction accomplishment).

Taking these concepts into account, students were able to interpret their results.

4.2. Results Obtained from stdAO

Graphics representing calibration curves (\( A_{515} = f(C_{stdAO}) \)) were built (Figure 4).

Students obtained a linear decreasing relationship between \( A_{515} \) of DPPH• and the stdAO molar concentration (\( C_{stdAO} \)) (\( a \) and \( b \) being the slope and intercept, respectively) calculated by a mean square regression bearing a determination coefficient, \( r^2 \), higher than 0.995 as expressed by Eq (4):
\[
A_{515} = a \times C_{stdAO} + b \tag{4}
\]

For each stdAO, students graphically determined the effective concentration 50 (EC50), corresponding to the concentration of stdAO reducing 50% of DPPH• (initial concentration of ca. 71 µM) in the present experimental conditions [8]. They could further deduce the stoichiometry of the reaction by comparing the molar concentrations of EC50 value and the corresponding DPPH• concentration (35.5 µM, which is half of DPPH• initial concentration, ca. 71 µM).

Results were presented in Table 1 and were in accordance with already published data [8,18].

Lastly, based on the stdAO chemical formulae, students could select the chemical functional group responsible of AO redox properties and propose an oxidation mechanism (see the oxidized form of AO redox chemical center, Table 1).

![Figure 4. Calibration curves of DPPH• reduction by the three studied stdAO. Results were presented as mean ± SD (n = 5)](image)

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>EC50 (µM)</th>
<th>Stoichiometry of reaction (ratio DPPH•/AO)</th>
<th>Redox chemical center(s) (oxidized/reduced forms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>19.4 ± 0.8</td>
<td>1/2</td>
<td>Carbonyl/Hydroxyl(=O/-OH)</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>15.9 ± 0.7</td>
<td>1/2</td>
<td>Carbonyl/Hydroxyl(=O/-OH)</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>47.1 ± 0.7</td>
<td>1/1</td>
<td>Disulfide/Thiol(S-S/-/SH)</td>
</tr>
</tbody>
</table>
4.3. Results Obtained from Food Extracts

The study performed on food extracts demonstrated in a practical way how plants could bring AO portion in our diet. Again, the three stdAO were selected to build the corresponding calibration curves. The equations of calibration curves were calculated with the mean square regression and obey to Beer Lambert’s law for Ellman’s and Folin-Ciocalteu’s assays, and to the following relationship for OPDA assay as mentioned in Eq (5) (\(I_F\) being the emitted fluorescence intensity, \(a\) and \(b\) being the slope and the intercept, and \(C_{\text{ascorbic acid}}\) being the concentration of ascorbic acid, respectively):

\[
I_F = a \times C_{\text{ascorbic acid}} + b.
\] (5)

For these reactions, the relevant compensation liquid to suppress any measurement interference from both reagents and food extracts was discussed with students.

Table 2 summarized the results obtained after the quantitation of ascorbic acid, polyphenols and thiols in the three food extracts (squeezed lemon juice, infused tea and crushed garlic solution).

For each quantitation, students used the previously obtained calibration curve to calculate the AO content. The result (expressed as an equivalent of stdAO) had to be expressed referred to a mass (e.g. the volumetric mass of lemon juice (i.e. 1.0324 g/mL), mass of tea leaves and mass of garlic used for the extraction). Depending on the dedicated time, students could either perform all the three reactions on their food extract or only choose a reaction according to the information in the supplied publications [14,16,17]. Based on these results and even though students had generally quantified only one compound in each extract (i.e. ascorbic acid in squeezed lemon juice, polyphenols in infused tea and thiols in crushed garlic solution), it was important to highlight that these matrices were generally composed of several types of AOs. Even though the polyphenol amount in garlic was important, the study could be based on thiol content to widen the AO variety.

Then, the reaction of successive extract dilutions with DPPH• was performed. Students were able to build a linear relationship (Figure 5) between the absorbance of DPPH• (\(A_{515}\)) and \(C_{\text{extAO}}\) expressed as either ascorbic acid or phloroglucinol or \(N\)-acetylcysteine equivalent as expressed in Eq (6):

\[
A_{515} = a \times C_{\text{extAO}} + b
\] (6)

Again, from these data, students were required to calculate the EC50 value for each type of extract expressed in reference to the dedicated stdAO (Table 2).

From all the collected results, as far as the stdAO are considered, a classification of the compounds, regarding their AO efficiency (EC50), can be given as follows: phloroglucinol > ascorbic acid > \(N\)-acetylcysteine. In parallel, while examining the data obtained on food extracts, the classification is the following: tea > garlic > lemon. This can be explained by the relatively high content of polyphenols measured in garlic extract, which AO efficiency may be synergic to the effects induced by thiols. However, the limitation of our study has to be mentioned, since only one AO test was performed, which is not enough to state a solid conclusion.

Table 2. Antioxidant concentrations measured in the different food extracts (mean ± SD, (n = 5); limit of detection (LOD) was calculated based on the standard deviation of the response and the slope according to the formula given in [23]; LOD for OPDA assay and for Ellman’s reaction were 0.05 µM and 1 µM, respectively)

<table>
<thead>
<tr>
<th>Food extract</th>
<th>Ascorbic acid (µmol/g)</th>
<th>Polyphenols (µmol/g)</th>
<th>Thiols (µmol/g)</th>
<th>EC50 (µM) (expressed as antioxidant equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon</td>
<td>1.6 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>21.2 ± 2.2 (ascorbic acid)</td>
</tr>
<tr>
<td>Tea</td>
<td>&lt; LOD</td>
<td>558 ± 24</td>
<td>&lt; LOD</td>
<td>1.5 ± 0.6 (phloroglucinol)</td>
</tr>
<tr>
<td>Garlic</td>
<td>&lt; LOD</td>
<td>9.5 ± 0.5</td>
<td>3.4 ± 0.4</td>
<td>7.3 ± 0.5 (N-acetylcysteine)</td>
</tr>
</tbody>
</table>
All the results, calculations and comments were compiled into a report, written either during the experimental period or afterwards. Supervisors gave it back to students with comments. Most of the time, report contents showed that the following concepts were well understood:

- food is a source of a chemical variety of AOs;
- the AO effect can be measured using a simple assay;
- AO effect can be linked to the content of at least either one component or a family compound.

Then, a meeting was organized with the whole class to summarize the collected information for each food extract.

5. Conclusion

This practical course was devoted to the analysis of foods in terms of AO properties. Through simple experimental procedures associated to spectroscopic techniques, students could go from theoretical aspects to experimental procedures associated to spectroscopic techniques, students could go from theoretical aspects to experimental procedures associated to spectroscopic techniques, students could go from theoretical aspects to experimental procedures associated to spectroscopic techniques, students could go from theoretical aspects to experimental procedures associated to spectroscopic techniques, students could go from theoretical aspects to experimental procedures associated to spectroscopic techniques, students could go from theoretical aspects to experimental procedures associated to spectroscopic techniques.

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Supporting Information

Included are the instructor’s notes, the required reagents, apparatus and equipment, the student experiment procedures, and report content.

References


Supporting Information

Instructor’s Notes

Lectures related to oxidative stress and antioxidants must be given before this practical teaching. Relevant information to design the lectures can be found in the literature. Moreover, information concerning DPPH• reaction with AOs can be read in references [8,18].
A 30-student group is divided for this practical teaching in working groups of 2 or 3 students. Usually, one day is enough to complete the experimental part as it is organized as follows:

- First 3-hour period: DPPH• reaction with std AOs and AO content in food extracts;
- Second 3-hour period: AO properties of food extract and DPPH• reaction with food extracts.

Some publications [14,16,17] have to be distributed at the beginning of the practical course to help students to find the main family compound or the main substance that they have chosen.

Before this course, all students should manage UV-visible and fluorescence spectrometry both at theoretical and practical levels. They should also be familiar with general, physical, and analytical chemistry (thermodynamic concepts, redox reactions, and stoichiometry). They have to know the way to use classical laboratory glassware (volumetric flasks), automatic pipets. Plastic and glass wastes must be available in the teaching room, and chemical waste is separated in organic solvents (for solutions containing methanol) or aqueous solutions. Main difficulties are usually encountered when operating the dilutions (volumes to be pipetted and associated calculations). Supervisors can check this particular point if necessary.

For safety reasons, eye protection, gloves and coats are worn full time while in the laboratory, and care is taken when handling laboratory glassware and boiling water. For instructors, preparation of ammonium containing buffers is done under a fume hood when using concentrated ammonia.

**Reagents, Apparatus, Equipment**

Table S1 summarizes the chemicals used during this course and the corresponding suppliers.

Garlic, lemon and green tea should be bought just before the practical teaching.

Dissolve 250.0 mg of o-phenylenediamine in a 50.0 mL-volumetric flask HCl 0.1 M. The solution is stored at 4°C for a period which does not exceed two hours.

5. 25 % (v/v) ammonia / 2.5 M ammonium chloride buffer pH 9.5

Weigh 13.4 g of NH₄Cl and dissolve in ca. 25 mL of water. Add 15.75 mL of concentrated NH₃ and fill up to 100 mL with water. Dissolve 250.0 mg of o-phenylenediamine in a 50.0 mL-volumetric flask HCl 0.1 M. The solution is stored at 4°C for a period which does not exceed one week.

5. 25 % (v/v) ammonia / 2.5 M ammonium chloride buffer pH 9.5

Weigh 13.4 g of NH₄Cl and dissolve in ca. 25 mL of water. Add 15.75 mL of concentrated NH₃ and fill up to 100 mL with water.

6. Na₂CO₃ (7.5 % m/v)

Weigh 7.5 g of Na₂CO₃ and dissolve in 1 L of water and adjust the pH value to 7.40 ± 0.05 using concentrated ammonia solution.

7. Methanol – 10 mM ammonium citrate buffer pH 7.4 (60:40; V/V) (stored at 4°C)

Weigh 13.4 g of NH₄Cl and dissolve in 25 mL of methanol, 400 mL of 10 mM ammonium citrate buffer pH 7.4.

8. Phosphate buffer 0.1 M pH 7.5

Weigh 28.6 g Na₂HPO₄ and dissolve in L 1 water and adjust the pH value to 7.50 ± 0.05 using concentrated H₃PO₄.

9. Ellman’s reagent

Dissolve 10.0 mg of 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB) in 25.0 mL phosphate buffer 0.1 M pH 7.5 in a volumetric flask. This solution is temporarily prepared.

**Table S1. List of chemicals used during this practical course**

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS No</th>
<th>Supplier</th>
<th>Hazard code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,1-diphenyl-2-pieryl-hydrazyl (DPPH•)</td>
<td>1898-66-4</td>
<td>Sigma Aldrich</td>
<td>H317-H334</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>616-91-1</td>
<td>Sigma Aldrich</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>62624-30-0</td>
<td>Sigma Aldrich</td>
<td>-</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>108-73-6</td>
<td>Sigma Aldrich</td>
<td>H315-H317-H319-H335</td>
</tr>
<tr>
<td>Citric acid</td>
<td>77-92-9</td>
<td>Sigma Aldrich</td>
<td>H319</td>
</tr>
<tr>
<td>Orthophenylenediamine (OPDA)</td>
<td>95-54-5</td>
<td>Sigma Aldrich</td>
<td>H301-H312 + H332-H317-H319-H341-H351-H410</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>12125-02-9</td>
<td>Roth</td>
<td>H302-H319</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>497-19-8</td>
<td>Sigma Aldrich</td>
<td>H319</td>
</tr>
<tr>
<td>Folin-Ciocalteu’s reagent</td>
<td>-</td>
<td>Merck</td>
<td>H290,H302,H314,H335</td>
</tr>
<tr>
<td>5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB)</td>
<td>69-78-3</td>
<td>Sigma Aldrich</td>
<td>H315-H319-H335</td>
</tr>
<tr>
<td>Sodium phosphate dibasic</td>
<td>7558-79-4</td>
<td>Sigma Aldrich</td>
<td>-</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>7647-01-0</td>
<td>Fluka</td>
<td>H290-H315-H319-H335</td>
</tr>
<tr>
<td>Methanol</td>
<td>67-56-1</td>
<td>Carlo Erba</td>
<td>H225-H301-H311-H331-H370</td>
</tr>
<tr>
<td>Ammonia</td>
<td>7664-41-7</td>
<td>VWR</td>
<td>H331-H314-H400</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>7664-38-2</td>
<td>Sigma Aldrich</td>
<td>H290-H314</td>
</tr>
<tr>
<td>Ultrapure water (&gt; 18.2 MΩ.cm)</td>
<td>-</td>
<td>Teaching platform</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table S2. List of the required equipment**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrophotometer</td>
<td>UV-1800 Shimadzu</td>
</tr>
<tr>
<td>Spectrofluorimeter</td>
<td>F-2000 Hitachi</td>
</tr>
</tbody>
</table>
Introduction to the practical course

In order to improve health, antioxidants in food are more and more studied from a scientific point-of-view. The aim of these studies is to widen the AO sources through a better understanding of their AO quality and quantity found in common foods.

This practical teaching will focus on three foods: green tea, lemon and garlic. Their AO capacity will be evaluated via the reaction with a stable radical: 1,1-diphenyl-2-picryl-hydrazyl (DPPH•). This power will be related to the presence and amount of an active compound (ascorbic acid) or a family of compounds (polyphenols, thiols), which are all characterized by reductive properties.

The study is based on the reaction between DPPH• and antioxidants (AOs) which are encountered in food. The products of the reaction are the oxidized AO (A•) and 1,1-diphenyl-2-picryl-hydrazine DPPH•-

The reaction is studied in solvent 1 (methanol / ammonium citrate buffer pH 7.4 (60:40, v/v)) and is monitored by spectrophotometry at 515 nm (maximum wavelength of DPPH• absorbance). The experimental objectives are:

1. DPPH• reaction with standard AOs
2. AO content in food extract
3. DPPH• interaction with food extract
4. AO properties of food extracts

Based on the provided documents [14,16,17] and the chosen food (tea, lemon or garlic), working groups have to select the standard AOs (i.e. phloroglucinol for green tea, ascorbic acid for lemon and N-acetylcysteine for garlic) to start the experimental part.

Student Experimental Procedures

OPERATING CONDITIONS TO ANALYZE ANTIOXIDANT CAPACITY AND CONCENTRATION IN FOOD EXTRACTS

Ultrapure deionized water (> 18.2 MΩ·cm) is used for the preparation of all solutions. Solvent 1 is a methanol/ammonium citrate buffer (60:40, v/v). Ammonium citrate buffer is a citric acid solution (10 mM) adjusted at pH 7.4 by addition of concentrated ammonia. Solvent 1 allows at the same time a good solubility of DPPH• (use of methanol) as well as the work at a physiological pH (pH = 7.4). The ammonium citrate buffer offers also the best spectral response between the oxidized and the reduced forms of the redox couple as reported in the literature [18].

Except when indicated, all reactions take place at 20 ± 2 °C. DPPH• reaction with standard antioxidants

DPPH• working solution:

- Weigh 18.0 mg of N-acetylcysteine and dissolve in 0.01 M HCl using a 100.0-mL volumetric flask.
- Dilute this solution 1/10 in a 25.0-mL volumetric flask with solvent 1.

N-acetylcysteine working solution

- Weigh 14.0 mg of ascorbic acid and dissolve in solvent 1 using a 25.0-mL volumetric flask.
- Dilute 175 µL of the previous solution in a 10.0-mL volumetric flask with solvent 1.

Phloroglucinol working solution

- Weigh 14.0 mg of phloroglucinol and dissolve in water using a 100.0-mL volumetric flask.
- Dilute this solution 1/25 in a 25.0-mL volumetric flask with solvent 1.

DPPH• reaction with stdAOs

Dilute the AO working solution and prepare the reaction with DPPH• according to Table S3.

Table S3. Dilution table of stdAOs working solution for the reaction with DPPH•

<table>
<thead>
<tr>
<th>Tube no</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO working solution (µL)</td>
<td>0</td>
<td>250</td>
<td>400</td>
<td>600</td>
<td>750</td>
<td>1000</td>
</tr>
<tr>
<td>Solvent (µL)</td>
<td>1000</td>
<td>750</td>
<td>600</td>
<td>400</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>DPPH• (142 µM) (µL)</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each resulting solution is incubated for 30 min in the dark and the absorbance is read at 515 nm (compensation liquid is solvent 1).

FOOD EXTRACT PREPARATION AND REACTION WITH DPPH•

Green tea working solution

- Infuse one tea bag (1.3 g of green tea leaves) in ca 100 mL of boiling water in a 200 mL beaker and incubate for exactly 3 min.
- Dilute this solution at 1/100 in a 25.0-mL volumetric flask with solvent 1, after cooling at room temperature
- From this solution, realize several dilutions (1/2, 1/5, 1/8, 1/10 and 1/100) in solvent 1 for a final volume of 2000 μL in microtubes.

Lemon juice working solution

- Cut one lemon in two pieces with a knife
- Extract the juice with a juicer
- Filter the juice with a cellulose paper filter using an Erlenmeyer and a funnel
- Measure the volumetric mass with a 5.0-mL volumetric flask
- From this solution, realize several dilutions (1/2, 1/5, 1/8, 1/10 and 1/100) in solvent 1 for a final volume of 25.0 mL (use volumetric flasks)
- From the 1/100 dilution, operate other dilutions (1/2, 1/5 and 1/8) in solvent 1 in a final volume of 2000 μL using microtubes.

Working solution of garlic

- Peel garlic cloves and weigh ca 10.0 g
- Crush garlic cloves, add 100.0 mL of water and shake for 10 min under magnetic stirring
- Filter on a cellulose paper filter using an Erlenmeyer and a funnel
- Measure the volumetric mass with a 5.0-mL volumetric flask
- From this solution, realize several dilutions (1/20, 1/50 and 1/100) in solvent 1 for a final volume of 25.0 mL (use volumetric flasks)
- From the 1/100 dilution, operate other dilutions (1/2, 1/5 and 1/8) in solvent 1 in a final volume of 2000 μL using microtubes.

Reaction with DPPH•

- Pipet 1000 µL of each dilution
- Mix with 1000 µL DPPH• solution at 142 µM prepared in solvent 1
- Incubate the resulting solution for 1 h in the dark
- Read the absorbance at 515 nm (compensation liquid: solvent 1)
ANTIOXIDANT CONTENT IN FOOD EXTRACTS

Spectrofluorimetric quantitation of ascorbic acid

**Principle**

The adduct obtained after condensation of ascorbic acid with \( \alpha \)-phenylenediamine (OPDA) in alkaline medium exhibits fluorescence properties [20]. Although Folin-Ciocalteu’s reagent can react with ascorbic acid, the presently used OPDA assay including spectrofluorimetry instead of visible spectrophotometry allows a specific determination of this compound.

**Operating conditions**

- Weigh 22.0 mg of ascorbic acid and dissolve in water in a 50.0-mL volumetric flask
- Dilute according to indications in Table S4.

<table>
<thead>
<tr>
<th>Tube n°</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid solution (µL)</td>
<td>0</td>
<td>10</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Water (µL)</td>
<td>1000</td>
<td>990</td>
<td>975</td>
<td>950</td>
<td>925</td>
<td>900</td>
</tr>
</tbody>
</table>

- Add 1000 µL of the OPDA solution in each tube
- Add 1500 µL of 25% (v/v) ammonia / 2.5 M ammonium chloride buffer pH 9.5 in each tube
- Mix and incubate for 30 min in the dark
- Read the fluorescence intensity \( I_F \) at 426 nm after excitation at 328 nm (compensation liquid: 1000 µL water + 1000 µL OPDA solution + 1500 µL ammonia/ammonium buffer).

A calibration curve built with ascorbic acid in the range 25-250 µM gives rise to the equation: \( I_F \) (arbitrary units) = \((10.408 \pm 1.162) \times C + (432.413 \pm 147.599); r^2 = 0.999 \) (n = 5). Operate in the same way by using 1000 µL of a 1/20 or 1/100 dilution of the food extract in purified water.

**Ellman’s assay**

**Principle**

A mixture of phosphotungstic and phosphomolybdic acids is quantitatively reduced by phenolic compounds giving rise to colored products (maximum wavelength of absorbance at 765 nm) [21].

**Operating conditions**

- Weigh 10.0 mg of phloroglucinol and dissolve in water by using a 100.0-mL volumetric flask
- Dilute according to indications in Table S5.

<table>
<thead>
<tr>
<th>Tube n°</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloroglucinol solution (µL)</td>
<td>0</td>
<td>100</td>
<td>250</td>
<td>500</td>
<td>750</td>
<td>1000</td>
</tr>
<tr>
<td>Water (µL)</td>
<td>1000</td>
<td>900</td>
<td>800</td>
<td>500</td>
<td>250</td>
<td>0</td>
</tr>
</tbody>
</table>

- Add 4000 µL of Na2CO3 (7.5% w/v) in each tube
- Add 5000 µL of the 1/10 dilution of Folin Ciocalteu’s reagent
- Mix and incubate for 1 h in the dark
- Read the absorbance at 765 nm (compensation liquid: 1000 µL water + 4000 µL Na2CO3 + 5000 µL of the 1/10 dilution of the Folin-Ciocalteu’s reagent).

A calibration curve built with phloroglucinol in the range 8-80 µM gives rise to the equation: \( A_{765} = (0.01448 \pm 0.00057) \times C + (0.0321 \pm 0.0119); r^2 = 0.999 \) (n = 5). Operate in the same way using 1000 µL of a 1/20 or 1/10 dilution of the food extract in purified water.

**Ellman’s assay**

**Principle**

The free thiol content in food extracts is determined by Ellman’s assay. The absorbance of the released chromophoric moiety, i.e. 2-nitro-5-thiobenzoic acid (TNB), is read in the visible range [22].

**Operating conditions**

- Weigh 50.0 mg of N-acetylcysteine and dissolve in HCl 0.1 M using a 100.0-mL volumetric flask
- Dilute this solution 1/10 in a 100.0-mL volumetric flask and fill up to the volume with phosphate buffer 0.1 M pH 7.5
- Dilute according to indications of Table S6.

<table>
<thead>
<tr>
<th>Tube n°</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylcysteine solution (µL)</td>
<td>0</td>
<td>100</td>
<td>200</td>
<td>500</td>
<td>750</td>
<td>1000</td>
</tr>
<tr>
<td>Phosphate buffer 0.1 M pH 7.5 (µL)</td>
<td>1000</td>
<td>900</td>
<td>800</td>
<td>500</td>
<td>250</td>
<td>0</td>
</tr>
</tbody>
</table>

- Add 200 µL of DTNB solution in each tube
- Mix and incubate for 10 min in the dark
- Read the absorbance at 412 nm (compensation liquid: phosphate buffer 0.1 M pH 7.5 + DTNB solution (200 µL))

A calibration curve built with N-acetylcysteine in the range 3-30 µM gives rise to the equation: \( A_{412} = (0.01284 \pm 0.00091) \times C - (0.00224 \pm 0.00251); r^2 = 0.999 \) (n = 5). Operate in the same way using 1000 µL of a 1/2 or 1/4 dilution of the food extract in phosphate buffer 0.1 M pH 7.5.

**Student report content**

It includes:
- Comments on the choice of the AO used as a standard (stdAO) for the corresponding studied food according to literature data;
- Graphs related to the reaction between the stdAO and DPPH•; calculation of the stdAO EC50 value, the stoichiometry of the reaction, and DPPH• molar absorbance;
- Table indicating all dilutions realized on food extracts to perform the different assays;
- Quantitation of the AO in the appropriate dilution of the food extract (equation of the calibration curve built with stdAO + concentration value measured in the food extract);
- Graph related to the reaction between concentrations of AOs in different dilutions of the food extract and DPPH•; calculation of food extract EC50 value.

Answers to these items are given in the article.