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Relationship between Antioxidant Capacity and Food Chemistry

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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*

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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•):

$$FR^{\bullet}\ (aq) + AOH\ (aq) \rightarrow FR\text{-}H\ (aq) + AO^{\bullet}\ (aq)$$

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.

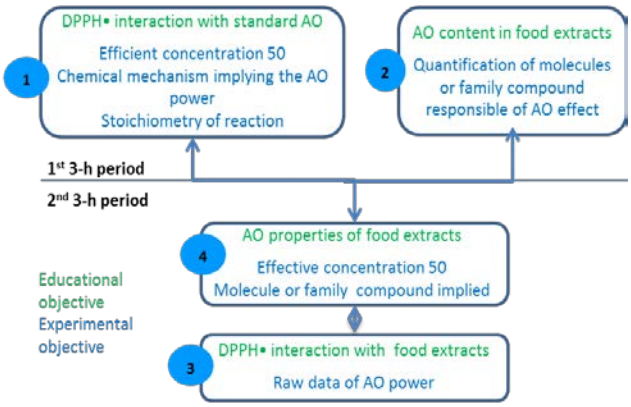

Figure 1. General organization of the presented practical course showing the educational and experimental objectives

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 sulfhydryl 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.

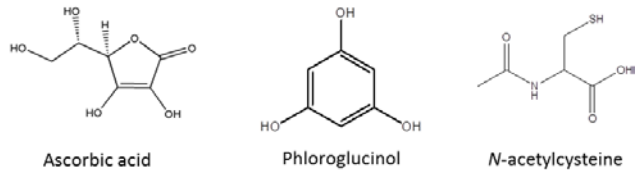

Figure 2. Chemical structure of standard antioxidants

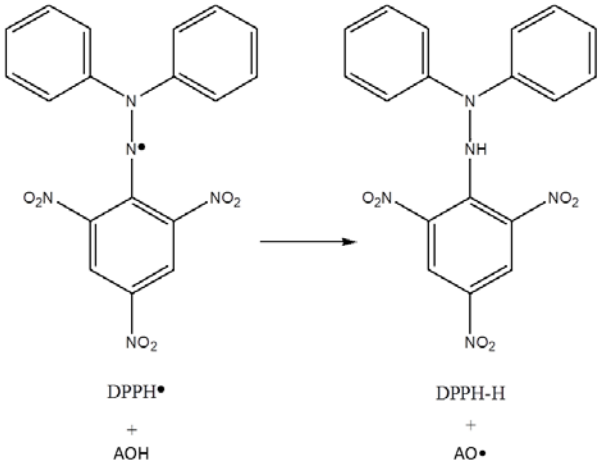

Figure 3. Reaction scheme between the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) radical and an antioxidant (AOH) through hydrogen atom transfer mechanism. Products are 1,1-diphenyl-2-picryl-hydrazine (DPPH-H) and the oxidized form of AO (AO•)

As DPPH• presents a maximum of absorbance at a wavelength of 515 nm (A_{515}) 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):

$$A = \varepsilon \times c \times b \quad (1)$$

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 A_{515} of DPPH• and the stdAO molar concentration (C_{stdAO}).

2.2. AO Content in Food Extracts

In a second step, spectroscopic techniques were applied to determine AO concentration in each food after extraction (C_{extAO}) (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 sulfhydryl compounds, respectively (see supporting information for detailed protocols). As far as Folin-Ciocalteu'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;
- $\Delta_rG°$, the free enthalpy or Gibbs energy of the reaction, is given by Eq (2):

$$\Delta_r G° = -n \times F \times \Delta E° \quad (2)$$

With $\Delta_rG°$ the free enthalpy or Gibbs energy of the reaction, n, number of exchanged electrons, F, Faraday constant (96,500 C.mol^{-1}) and $\Delta E°$, difference of standard redox potential values between both redox couples;

- to ensure a spontaneous reaction $\Delta_rG°$ must be negative.

This suggested that DPPH• was more oxidant than A•, E°(DPPH•/DPPH-H) was higher (+ 0.54 V *vs.* standard hydrogen electrode [18]) than E°(A•/AO), and the reaction was:

$$\text{DPPH•} + \text{AO} \rightarrow \text{DPPH-H} + \text{A•},\ K$$

K being the equilibrium constant linked to $\Delta_rG°$ by Eq (3):

$$\Delta_r G° = -R \times T \times LnK \quad (3)$$

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; $K > 10^4$ 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):

$$A_{515} = a \times C_{sdtAO} + b \quad (4)$$

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).

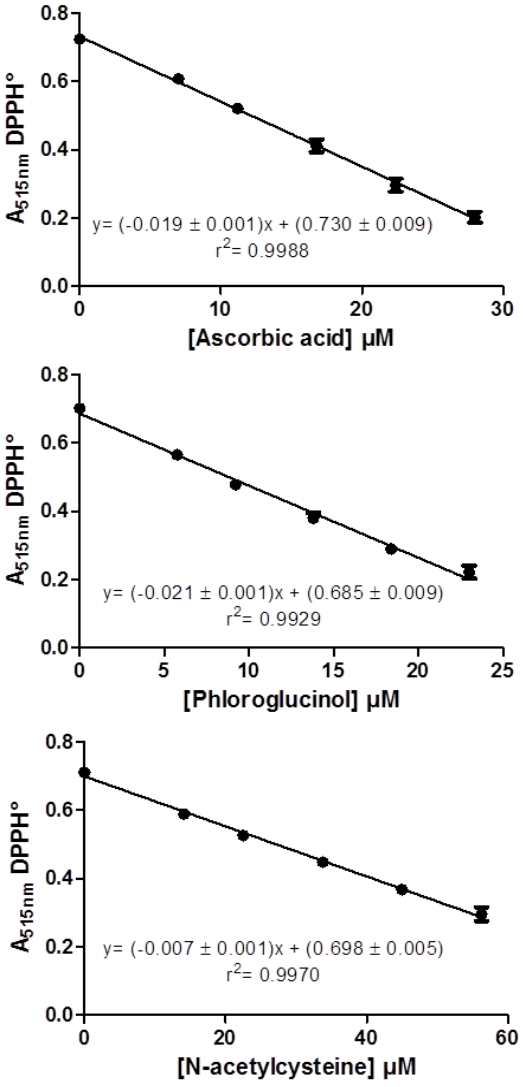

Figure 4. Calibration curves of DPPH• reduction by the three studied stdAO. Results were presented as mean ± SD (n = 5)

Table 1. Results of DPPH• reaction with stdAO (mean ± SD, n = 5).

Antioxidant	EC50 (µM)	Stoichiometry of reaction (ratio DPPH•/AO)	Redox chemical center(s) (oxidized/reduced forms)
Ascorbic acid	19.4 ± 0.8	1/2	Carbonyl/Hydroxyl(=O/-OH)
Phloroglucinol	15.9 ± 0.7	1/2	Carbonyl/Hydroxyl(=O/-OH)
N-acetylcysteine	47.1 ± 0.7	1/1	Disulfide/Thiol(-S-S-/-SH)

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 $C_{ascorbic\_acid}$ being the concentration of ascorbic acid, respectively):

$$I_F = a \times C_{ascorbic\_acid} + b. \quad (5)$$

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_{extAO} expressed as either ascorbic acid or phloroglucinol or *N*-acetylcysteine equivalent as expressed in Eq (6):

$$A_{515} = a \times C_{extAO} + b \quad (6)$$

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.

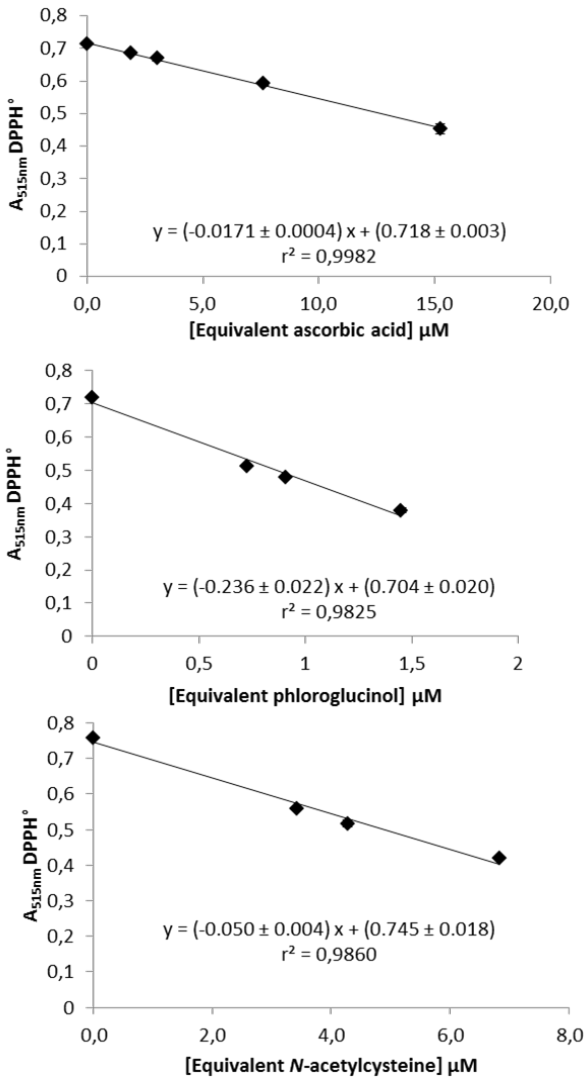

Figure 5. Antioxidant activity in food extracts expressed as equivalent of stdAO concentration measured by the reduction of the radical DPPH•. Equivalent concentrations of ascorbic acid, phloroglucinol and *N*-acetylcysteine in lemon, tea and garlic, respectively were calculated from results shown in Table 2. Results were presented as mean ± SD (n = 3).

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)

Food extract	Ascorbic acid (µmol/g)	Polyphenols (µmol/g)	Thiols (µmol/g)	EC50 (µM) (expressed as antioxidant equivalent)
Lemon	1.6 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	21.2 ± 2.2 (ascorbic acid)
Tea	< LOD	558 ± 24	< LOD	1.5 ± 0.6 (phloroglucinol)
Garlic	< LOD	9.5 ± 0.5	3.4 ± 0.4	7.3 ± 0.5 (N-acetylcysteine)

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 practical applications using stdAO and natural extracts encountered in usual diet. This teaching program highlighted how solid bases in physical chemistry were required for a better understanding of complex processes such as oxidative stress.

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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.

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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 [2,3,5,7]. 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-h period: DPPH• reaction with std AOs and AO content in food extracts;
- Second 3-h 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 characterizing the antioxidant property in the food 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.

Table S1. List of chemicals used during this practical course

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

Table S2 summarizes the required equipment.

Table S2. List of the required equipment

Equipment	References
Spectrophotometer	UV-1800 Shimadzu
Spectrofluorimeter	F-2000 Hitachi

The following solutions are prepared by instructors:

1. 10 mM ammonium citrate buffer pH 7.4 (stored at 4°C)

Dissolve 2.1 g of citric acid in 1 L of water and adjust the pH value to 7.40 ± 0.05 with 25% ammonia solution.

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

Mix 600 mL of methanol with 400 mL of 10 mM ammonium citrate buffer pH 7.4.

3. Stock solution of DPPH• at a concentration of 710 µM

Dissolve 28.0 mg of DPPH• in 100 mL of methanol, using an ultrasonic bath for 3 min. The solution is stored at 4°C for a period which does not exceed one week.

4. *o*-phenylenediamine solution (OPDA solution)

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 NH_4Cl and dissolve in *ca.* 25 mL of water. Add 15.75 mL of concentrated NH_3 and fill up to 100 mL with water.

6. Na2CO3 (7.5 % m/v)

Weigh 7.5 g of Na_2CO_3 and dissolve in 100 mL water.

7. Dilution of Folin Ciocalteu's reagent

Dilute the Folin Ciocalteu's reagent 10-fold in water. This solution is extemporaneously prepared.

8. Phosphate buffer 0.1 M pH 7.5

Weigh 28.6 g Na_2HPO_4 7 H_2O, dissolve in 1 L water and adjust the pH value to 7.50 ± 0.05 using concentrated H_3PO_4.

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 extemporaneously prepared.

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-H.

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:

DPPH• stock solution at 710 µM is diluted 1/5 in solvent 1 at 142 µM in a 25.0-mL volumetric flask. The solution is stored in the dark at 4 °C.

N-acetylcysteine 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 50.0-mL volumetric flask with solvent 1.

Ascorbic acid 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•

Tube n°	1	2	3	4	5	6
AO working solution (µL)	0	250	400	600	750	1000
Solvent 1 (µL)	1000	750	600	400	250	0
DPPH• (142 µM) (µL)	1000					

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 (1/20, 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.

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
- From this solution, realize several dilutions (1/2, 1/5, 1/8, 1/10 and 1/100) for a final volume of 2000 µL in solvent 1 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 *o*-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 spectrophometry 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 S4. Dilution table of the ascorbic acid solution

Tube n°	1	2	3	4	5	6
Ascorbic acid solution (µL)	0	10	25	50	75	100
Water (µL)	1000	990	975	950	925	900

- 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 ± 1.162) × C + (432.413 ± 147.599); $r^2 = 0.999$ (n = 5).

Operate in the same way by using 1000 µL of a 1/20 or 1/50 or 1/100 dilution of the food extract in purified water.

Folin-Ciocalteu'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 S5. Dilution table of the phloroglucinol solution

Tube n°	1	2	3	4	5	6
Phloroglucinol solution (µL)	0	100	250	500	750	1000
Water (µL)	1000	900	750	500	250	0

- 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 ± 0.00057) × C + (0.0321 ± 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 1/100 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 S6. Dilution table of the *N*-acetylcysteine solution

Tube n°	1	2	3	4	5	6
N-acetylcysteine solution (µL)	0	100	200	500	750	1000
Phosphate buffer 0.1 M pH 7.5 (µL)	1000	900	800	500	250	0

- 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 (1000 µL) + 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 ± 0.00091) × C - (0.00224 ± 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 EC_{50} 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 EC_{50} value.

Answers to these items are given in the article.