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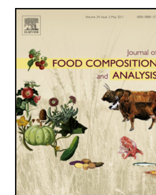

Original Research Article

Antioxidant capacity of phenolic compounds in acidic medium: A pyrogallol red-based ORAC (oxygen radical absorbance capacity) assay

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ABSTRACT

A novel ORAC (oxygen radical absorbance capacity) assay to assess antioxidant capacity of phenolic compounds in near-gastric conditions (pH 2.0) is presented. AAPH (2,2′-azo-bis(2-amidinopropane)-dihydrochloride) was used as peroxyl radicals source, and fluorescein, pyranine and pyrogallol red were employed as target molecules. Only pyrogallol red (PGR) showed a behavior compatible with an ORAC assay under acidic conditions (ORAC-PGR_a). Excepting Trolox and ascorbic acid, phenolic compounds protected PGR, giving kinetic profiles without the presence of an induction time. ORAC-PGR_a values, which reflect the reactivity of the antioxidants toward peroxyl radicals, ranged from 0.2 (caffeic acid) to 29.1 (myricetin) gallic acid equivalents. The ORAC-PGR_a method showed analytical parameters in agreement with other ORAC-like assays and was applied to wines, teas, commercial juices and herb infusions, peach juice being the sample with the highest ORAC-PGR_a value (7.1 mM gallic acid equivalents). In addition, ascorbic acid concentration in complex mixtures can be determined from kinetic profiles.

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1. Introduction

The human gastrointestinal tract is commonly exposed to substances capable of inducing oxidative stress, such as foods (mainly meats) that contain large amounts of lipids, hydroperoxides, free metals and myoglobin (Kanner, 1994). In the stomach cavity, free metals or myoglobin are known to catalyze hydroperoxides decomposition generating reactive species that are able to induce lipid peroxidation (Lapidot et al., 2005a,b). These reactions are favored by the low pH of the stomach fluid that promotes, among others, the generation of peroxyl radicals and carbonyl compounds (Lapidot et al., 2005b). These processes have been associated with vitamin oxidation and postprandial modification of low density lipoproteins and with their consequent deleterious effects on human health (Gorelik et al., 2005; Kanner et al., 2012).

On the other hand, human diets often comprise rich-phenolic foods and beverages such as fruits, vegetables, wines and teas, whose consumption is expected to lead to high concentration of phenolic compounds in the stomach fluid (Halliwell et al., 2000). For example, after the intake of 80 mL of red wine, quercetin concentration in the stomach can reach values close to 3 μM (a value estimated considering a quercetin concentration in red wine close to 30 μM and a ten time dilution factor in the stomach cavity) (Neveu et al., 2010; Burton et al., 2005). Under gastric conditions, phenolic compounds have shown to inhibit lipid peroxidation (Lapidot et al., 2005b), an effect associated with their reaction with peroxyl radicals. This phenolic activity is related to their capacity to donate a hydrogen atom or a single electron to a damaging free radical. Nonetheless, the low stomach pH could influence the ability of phenolic compounds to neutralize peroxyl radicals *via* hydrogen donation. In spite of the relevance of these processes, it has been few attempts to evaluate the antioxidant capacity of phenolic compounds in acid medium. In this context, Di Majo et al. (2011) compared throughout competitive reactions, the antioxidant activity of phenolic compounds (flavonoids and phenolic acids) toward peroxyl radicals at pH 3.5 and 7.4. The capacity of phenolic compounds to inhibit the peroxyl radical-induced consumption of a probe like crocin was strongly influenced by media pH. In fact, increasing the pH of the media implied an increase in antioxidant activity (Di Majo et al., 2011).

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The present work was designed to develop an ORAC (oxygen radical absorbance capacity) assay capable of evaluating the antioxidant capacity of single phenolic compounds and their complex mixtures (wines, fruit juices and teas) under stomach-like acidic conditions. To such purpose, the peroxyl radicals-induced oxidative consumption of fluorescein, pyranine and pyrogallol red, three probes widely used in ORAC assays run at physiological neutral pH was evaluated under acidic conditions using a simulated gastric fluid as medium.

2. Materials and methods

2.1. Chemicals

2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) was used as peroxyl radical source. Pyrogallol red (PGR) fluorescein (Fl), pyranine (Py), Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), ascorbic acid, AAPH, and all phenolic compounds employed were purchased from Sigma–Aldrich (St. Louis, MO, USA). All compounds were analytical grade and employed as received.

2.2. Solutions

Unless otherwise indicated, experiments were carried out in simulated gastric fluid without pepsin (SGF), comprising a solution of sodium chloride (17 mM) adjusted to pH 2.0 with concentrated hydrochloric acid (The United States Pharmacopoeia). Stock solutions of PGR, Fl or Py (300 μM) were prepared daily in phosphate buffer 75 mM, pH 7.4. Stock solutions of phenolic compounds (1 mM) were prepared daily in ethanol. AAPH stock solutions (0.6 M) were prepared daily in SGF.

2.3. Samples

Herb and tea bags (green and black tea, *Rosa moschata*, *Mentha piperita*, and *Peumus boldus*) were Chilean commercial products. Infusions were prepared by adding 150 mL of distilled water (95–100 °C) to one bag (containing 2 g of dry material) and were brewed for 5 min. After withdrawing the bags, the resulting solution was cooled to 20 °C and immediately used to assess its total phenolic content and antioxidant properties (see below).

Wines (red and white) and commercial juices were centrifuged at 10,000 × g during 2 min (20 °C) and aliquots (10–400 μL) were taken and directly added to the working solutions (3 mL, final volume).

2.4. Oxygen consumption

Solutions of AAPH (10 mM) in SGF or phosphate buffer (75 mM, pH 7.4) were incubated in a thermostatized cell at 37 °C. Oxygen consumption was assessed employing an ISO-OXY-2 electrode (WPI Inc., Sarasota, FL, USA) and registered in a TBR4100, Free Radical Analyzer instrument (WPI Inc., Sarasota, FL, USA).

2.5. Working solutions

2.5.1. Reaction of probes with AAPH-derived peroxyl radicals

Reaction mixtures containing AAPH (10 mM), PGR, Fl, or Py were incubated at 37 °C in SGF in the thermostatized cuvette of an Agilent 8453 (Palo Alto, CA, USA) UV-visible spectrophotometer. Probe consumption was followed at 465, 437, and 403 nm, for PGR, Fl, and Py, respectively. In some experiments (*i.e.* for Fl or Py at concentrations lower than 5 μM) probe consumption was followed by fluorescence spectroscopy. For Fl, 437 and 515 and for Py, 403 nm and 504 nm were employed as excitation and emission wavelengths, respectively. Fluorescence measurements were carried out using a Perkin Elmer LS-55 spectrofluorimeter (Beaconsfield, U.K).

2.5.2. Effects of antioxidants on the PGR consumption induced by peroxyl radicals

Solutions containing AAPH (10 mM), PGR (5 μM) and the tested samples were incubated in SGF at 37 °C. Addition of the samples (single antioxidants or beverages) did not modify the pH of solutions (2.0). PGR consumption was evaluated from the progressive absorbance decrease measured at 465 nm in the thermostatized cuvette of either, an Agilent 8453 (Palo Alto, CA, USA) or a Unicam Helios-γ (Cambridge, U.K) UV-visible spectrophotometer.

In some cases, the addition of beverages resulted in a small contribution at 465 nm. This absorbance was subtracted from the absorbance intensity of PGR at such wavelength.

2.6. ORAC determinations

Values of the relative absorbance (A/A_0) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (A/A_0) equal to 0.2. These areas were employed to obtain ORAC values, according to Eqs. (1) and (2) for single antioxidants and beverages, respectively:

$$\mathrm{ORAC} = \frac{(\mathrm{AUC_{XH}} - \mathrm{AUC^0})}{(\mathrm{AUC_{GA}} - \mathrm{AUC^0})} \frac{[\mathrm{GA}]}{\mathrm{XH}} \tag{1}$$

$$\mathrm{ORAC} = \frac{(\mathrm{AUC} - \mathrm{AUC^0})}{(\mathrm{AUC_{GA}} - \mathrm{AUC^0})} f[\mathrm{GA}] \tag{2}$$

where $\mathrm{AUC_{(XH)}}$ is the area under curve in the presence of the tested sample, integrated between time zero and that corresponding to 80% of the probe consumption; $\mathrm{AUC^0}$ is the area under curve for control experiment (in the absence of antioxidants); $\mathrm{AUC_{GA}}$ is the area under curve obtain in the presence of gallic acid; f is the dilution factor, equal to the ratio between the total volume of the AAPH-PGR solution and the added beverage volume and [GA] is the gallic acid molar concentration.

2.7. High performance liquid chromatography (HPLC) experiments

2.7.1. Reaction of PGR with AAPH-derived peroxyl radicals

PGR solutions (10–30 μM) were incubated at 37 °C in the presence of AAPH 10 mM in SGF, under aerobic conditions. At different times, aliquots were extracted and immediately injected into the HPLC system. No concentration changes were observed in control experiments carried out in the absence of AAPH. All experiments were carried out in duplicate or triplicate.

Method A:

Chromatograms were obtained using an Agilent 1100 Series HPLC (Palo Alto, CA, USA), equipped with a Phorospher STAR RP-18e (5 μm) 4.6 mm × 250 mm HPLC column (Merck), and a diode array detector (DAD G1315A).

Method B:

Aliquots of the PGR-AAPH solution were analyzed by HPLC-DAD-MS/MS employing a Shimadzu HPLC system (Tokyo, Japan) equipped with a quaternary LC-10ADVP pump with a FCV-10ALVP elution unit, a DGU-14A degasser unit, a CTO-10AVP oven, and an UV-vis diode array detector (model SPD-M10AVP) coupled in tandem with a QTrap LC/MS/MS 3200 Applied Biosystems MDS Sciex (Foster City, CA, USA). Instrument control and data collection system were carried out using a CLASS-VP DAD Shimadzu Chromatography Data System and Analyst software (version

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