Food Chemistry 113 (2009) 331–335

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Ascorbic acid contribution to ORAC values in berry extracts: An evaluation by the ORAC-pyrogallol red methodology

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ARTICLE INFO

Article history:
Received 10 April 2008
Received in revised form 10 July 2008
Accepted 22 July 2008

Keywords:
Antioxidant capacity
ORAC
Fruit extracts
Ascorbic acid
Pyrogallol red

ABSTRACT

An oxygen radical absorbance capacity (ORAC) method based on pyrogallol red bleaching (ORAC-PGR) was used to evaluate the scavenging activity of berry extracts (blackberry, blueberry, and raspberry). Among berry extracts, only raspberry protected pyrogallol red through a clear induction time, related exclusively to ascorbic acid. The lag time allowed an estimation of the ascorbic acid concentration and its contribution to the total ORAC value, estimating that 66% of the ORAC-PGR value of raspberry is related to ascorbic acid. Also, from the induction time, an ascorbic acid concentration of 36 mg per 100 g of fresh weight was estimated for raspberry samples. The ORAC-PGR procedure could be considered as a fast and specific methodology for an estimation of ascorbic acid concentrations in complex samples.

1. Introduction

Epidemiological evidence suggests an association between consumption of diets rich in fruits and vegetables and a decreased risk of cardiovascular diseases, hypertension, certain forms of cancer, type II diabetes, and other degenerative or age-related diseases (Ames, Shigena, & Hegen, 1993; Boyer & Liu, 2004; Gillman et al., 1995; Halliwell & Gutteridge, 2000; Kohlmeier, Simonsen, & Mohus, 1995; Liu, 2003, 2004; Rissanen et al., 2003; Steinmetz & Potter, 1996). These associations can be explained, at least partially, in terms of the presence in fruits and vegetables of free radical scavengers, such as polyphenols and ascorbic acid. In particular, the health-benefits of berries could be mostly attributed to their particularly high concentration of antioxidants. In fact, berries rank as one of the most concentrated sources of antioxidants amongst the commonly consumed fruits and vegetables (Kähkönen, Hopia, & Heinonen, 2001).

Several methodologies have been developed to evaluate the total charge of antioxidants in complex mixtures, and applied to their evaluation in fruits and beverages (Perez, Leighton, Aspée, Aliaga, & Lissi, 2000; Prior & Cao, 1999). An advantage of these procedures is that they allow a simple evaluation of the total amount of antioxidants. However, its main drawback is that they do not discriminate among the different antioxidants, titrating together polyphenols and other antioxidants, such as ascorbic acid.

Among the methods more employed to evaluate total charge of antioxidants are the ORAC methodologies, particularly that employing fluorescein as target molecule. This methodology has been applied to the evaluation of the antioxidant capacity of several fruits, including different berries (Ou, Hampsch-Woodill, & Prior, 2001; Wu et al., 2004). Recently, we have proposed a modified ORAC-like methodology that employs pyrogallol red as target molecule (López-Alarcón & Lissi, 2006). This methodology has the advantage that the obtained ORAC values are more influenced by the reactivity of the tested compounds. Pyrogallol red does not produce induction times, even with very reactive polyphenols. In particular, ascorbic acid was the only antioxidant, among the tested compounds, that generated a clear induction time (López-Alarcón & Lissi, 2005). This methodology has been applied to several teas and beverages, such as wines. In all cases, protection by the additives takes place without generating observable induction times (López-Alarcón & Lissi, 2006; Alarcón, Campos, Edwards, Lissi, & López-Alarcón, 2008). In the present communication we show that induction times appear in the evaluation of ascorbic acid rich samples and that an analysis of the shape of the pyrogallol red consumption trace allows independent evaluation of ascorbic acid and polyphenolic antioxidants. From this observation, we propose that it can constitute a fast and specific

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doi:10.1016/j.foodchem.2008.07.063

methodology for an estimation of ascorbic acid concentrations in complex samples.

2. Materials and methods

2.1. Chemicals

2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), was used as peroxyl radical source. Pyrogallol red, Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), fluorescein disodium salt, AAPH, ascorbic acid, and ascorbate oxidase were purchased from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteau reactive and sodium carbonate were supplied by Merck (Darmstadt, Germany). All compounds were employed as received.

2.2. Berry extracts

Three species of berries were evaluated. These included blueberries (cultivar Duke), blackberries (cultivar Cherokee), and raspberries (cultivar Heritage). Samples of berries were harvested at commercial ripe stage from an established growing field located at a south Region of Chile (latitude 40–44°). Immediately after arrival to the laboratory, samples were frozen and stored at −20 °C until their analysis (less than 3 months). Extracts of berries (blackberry, blueberry and raspberry) were prepared from frozen fruits. Briefly, 10 g of each berry sample were homogenized using a commercial household blender, and 100 mL of extraction solvent (75/25 v/v, acetone/water) were added. Extracts were shaken in a water-bath at 20 °C for 90 min, and centrifuged at 950g for 15 min using a Labofuge 200 Heraeus centrifuge (Germany). The supernatants were analyzed immediately. In some experiments juices of berries were evaluated. Briefly, 10 g of frozen fruits were homogenized and 100 mL of water (3% oxalic acid) were added. The juices were centrifuged at 950g for 15 min, and the supernatants were analyzed immediately.

2.3. Total phenolics

Total phenol content in extracts of berries was determined according to the Folin–Ciocalteau colorimetric method (Singleton & Rossi, 1965), using Trolox as standard. Briefly, appropriate dilutions of the samples (1 mL) were added to Folin-Ciocalteau reagent (5 mL, 0.2 Normal). After 5 min, sodium carbonate (75 g/L) was added. The mixtures were incubated for 2 hours and the absorbance of the resulting blue colour was measured at 740 nm using an Agilent 8453 spectrophotometer (Palo Alto, CA, USA). Quantification was carried out on the basis of the standard curve of Trolox, and the results were expressed as Trolox equivalents (μM).

2.4. ORAC determinations

Stock solutions of pyrogallol red (1×10^{-4} M) or fluorescein (1×10^{-5} M) were prepared daily in phosphate buffer 75 mM, pH 7.4. A reaction mixture containing AAPH (10 mM), pyrogallol red (5 μM) with or without the tested berry sample was incubated in phosphate buffer (75 mM, pH 7.4) at 37 °C. Pyrogallol red consumption was evaluated from the progressive absorbance decrease measured at 540 nm in the thermostatized cuvette of either an Agilent 8453 (Palo Alto, CA, USA) or an Unicam Helios-α (Cambridge, England) UV–visible spectrophotometer. A similar procedure was carried out employing fluorescein (70 nM), but its consumption was assessed from the decrease in the sample fluorescence intensity (excitation: 493 nm; emission: 515 nm). Fluorescence measurements were carried out in a Perkin Elmer LS-55 spectrofluorimeter (Beaconstield, U.K). The consumption of the probe molecules, fluorescein and pyrogallol red, associated to its incubation in presence of AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively. Values of (F/F_0) or (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 (F/F_0) or (A/A_0) reached a value of 0.2. These areas were employed to obtain ORAC values, according to Eq. (1). All experiments were carried out in triplicate.

$$\mathrm{ORAC} = \frac{(\mathrm{AUC} - \mathrm{AUC}^0)}{(\mathrm{AUC}_{\mathrm{Trolox}-\mathrm{AUC}^0)}} f[\mathrm{Trolox}] \quad (1)$$

where: AUC = Area under curve in presence of the tested berry extract, integrated between time zero and that corresponding to 80% of the probe consumption; AUC = Area under curve for the control. $\mathrm{AUC}_{\mathrm{Trolox}}$ = Area under curve for Trolox. f = Dilution factor, equal to the ratio between the total volume of the working solution (target molecule plus AAPH, plus berry extract) and the added berry extract volume. [Trolox] = Trolox molar concentration.

2.5. Ascorbic acid determination

Stock solutions of ascorbic acid at 1 mM concentration were prepared daily in ethanol. Working solutions of ascorbic acid were prepared diluting stock solution in phosphate buffer, 75 mM, pH 7.4. The final concentration was estimated using a molar absorptivity of 13,800 $\mathrm{M^{-1}\,cm^{-1}}$ at 270 nm (Karayannis, Samios & Gousetis,1977). Ascorbic acid was directly added to solutions containing pyrogallol red plus AAPH with or without an aliquot of berry extract. In some experiments, solutions containing an aliquot of berry extract or ascorbic acid were preincubated with ascorbate oxidase. Briefly, ascorbate oxidase was reconstituted with phosphate buffer 4 mM, pH 6.5 (2 mL). The activity was estimated by both UV–visible spectrophotometry, and reflectometry (RQflex 10, Merck). A mixture of ascorbate oxidase (final concentration = 0.09 U/mL), berry extract and/or ascorbic acid in buffer phosphate was incubated at room temperature during 40 min. After this preincubation, the solution was thermostatized at 37 °C, and pyrogallol red (or fluorescein), and AAPH were added. The course of the reaction was followed by UV–visible spectroscopy at 540 nm or fluorescence (excitation: 493 nm; emission 515 nm) for pyrogallol or fluorescein studies, respectively. In addition, ascorbic acid concentration in berry juices was estimated by high perfomance liquid chromatography (HPLC), according to Vrhovsek et al. (Vrhovsek, Rigo, Tonon, & Mattivi, 2004). Briefly, 20 g of each berry sample were homogenized using a commercial household blender, and 200 mL of a solution of metaphosphoric acid (6%) in water containing sodium meta-bisulfite (1 g/L) were added. Homogenizate was filtered through 0.2 μm, 10 mm PTFE (Whatman), and centrifuged at 950g for 15 min using a Labofuge 200 Heraeus centrifuge (Germany). The supernatants were diluted ten times with mobile phase and analyzed immediately. An ion-pairing RP–HPLC with electrochemical detection was used. The liquid chromatography system consisted of a Merck–Hitachi (Tokyo, Japan) pump, model L-6000, a Gemini (Phenomenex, Torrance, CA) 250 mm × 4.6 mm 5 μm, C-18 column, and a BAS (West Lafayette, IN) electrochemical detector, model LC-4 C, equipped with a glassy carbon electrode. The detector potential was set at +0.65 V, with a sensitivity setting of 50 nA vs an Ag/AgCl reference electrode. The mobile phase was 40 mM sodium acetate, 0.54 mM Na_2EDTA, 0.75 mM dodecyltriethylammonium bromide, and 15% methanol, taken to pH 4.75 with glacial acetic acid. Elution was isocratic at a flow rate of 0.8 mL/min and at ambient temperature.

Ascorbic acid in orange and lemon juices was estimated by reflectometry using an RQflex 10 device (Merck).