Food Chemistry 127 (2011) 761–768

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Validation of a quantitative assay for the total content of lipophilic and hydrophilic antioxidants in foods

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ARTICLE INFO

Article history:
Received 18 May 2010
Received in revised form 19 October 2010
Accepted 31 December 2010
Available online 9 January 2011

Keywords:
Antioxidants
Food
FRAP
Hydrophilic
Lipophilic
Reaction rate curves
Validation

ABSTRACT

One of the main methods used to assess total antioxidant content in foods is the Ferric Reducing Ability of Plasma (FRAP) assay. The FRAP assay has previously not been extensively validated. In the present study, 39 pure compounds, such as different polyphenols, ascorbic acid, tocopherols, tocotrienols and carotenoids dissolved in water/methanol, water/2-propanol and 2-propanol, were tested. Our results demonstrate that the FRAP assay can quantitate satisfactorily most hydrophilic and lipophilic compounds with antioxidant properties. The reaction was followed for 60 min. The most extensive reaction occurred within the first 4 min for most compounds and foods. The lipophilic tocopherols and tocotrienols were easily quantitated and reached endpoint within 4 min while carotenoids were somewhat more demanding due to low solubility and slower reaction kinetics. We conclude that the FRAP assay, with 4 min reaction time, is suitable for high-throughput screening of total antioxidant content of edible items.

1. Introduction

A diet rich in fruit and vegetables has been found to protect against several chronic diseases (Dauchet, Amouyel, Hercberg, & Dallongeville, 2006; Van Horn, McCoin, Kris-Etherton, Burke, & Carson, 2008; WHO, 2003). It is often assumed that antioxidants in dietary plants are responsible for these protective effects. However, results from intervention trials with single antioxidants like vitamins E and C or β-carotene have mainly resulted in no effect or even adverse disease outcome. One possible explanation may be that the beneficial health effect of fruits and vegetables is contributed by other antioxidants. Based on results presented in Halvorsen et al. (2002) it can be estimated that the combined contribution of specific well-known antioxidants (such as vitamin E and C, and β-carotene) to the total antioxidant content of dietary plants is typically less than 10%. Thus, since most antioxidants cannot replace each other in complex biologically systems like the human body, other antioxidants may have better bioactivity than the few antioxidants that have been tested in clinical trials. Additionally, the doses used in clinical trials have also by far exceeded the doses corresponding to a plant-based diet, and might therefore have been in the toxic range. Another explanation could be that the thousands of molecules with antioxidant properties in foods operate in a network, which may be necessary for the protection against oxidative damage. Therefore, one approach to the understanding of antioxidant properties of food is total antioxidant content, a measure of the combined activity of all redox active components.

Several assays have been used to assess the antioxidant content of foods. The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) assay of Miller and RiceEvans (1996), the Ferric Reducing Ability of Plasma (FRAP) assay of Benzie and Strain (1996), and the oxygen radical absorbance capacity (ORAC) assay first presented by Glazer's laboratory (DeLange & Glazer, 1989) and further developed by others (Cao, Alessio, & Cutler, 1993; Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002) are among the methods used. Different chemical reaction mechanisms are involved in the various assays. The assays can mainly be divided into two groups: inhibition assays and reduction assays. Inhibition assays are based on antioxidants ability to react with or neutralise free radicals generated in the assay system (e.g. ORAC, TEAC), while reduction assays are based on the ability of antioxidants to reduce an oxidant which also functions as a probe that changes colour when it is reduced (e.g. FRAP). Results obtained by the various assays correlates reasonably well, implying that even though the assays produce different values of antioxidant content, the ranking of the products is often similar (Pellegrini, Serafini, Colombi, Del Rio, & Salvatore, 2003; Pellegrini, Serafini, Salvatore, Del Rio, Bianchi, & Brighenti, 2006).

During the last years our research group has measured the antioxidant content of more than 3100 different foods from around the

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doi:10.1016/j.foodchem.2010.12.142

world (Carlsen et al., 2010). For several reasons we elected the FRAP assay for our comprehensive research. The FRAP assay is a simple, fast and inexpensive method, which has the ability of quantitative determination of the amounts of antioxidants in samples. The assay has little selectivity and measures almost all reductants (i.e. antioxidants) with a reduction potential below 0.7 V, which is the reduction potential of the Fe^{3+}-TPTZ/Fe^{2+}-TPTZ couple (Prior, Wu, & Schaich, 2005). Assay conditions such as extraction solvents, that promotes detection of both water- and fat soluble antioxidants have been chosen (Halvorsen et al., 2002). The FRAP assay does not detect glutathione (GSH) or protein thiols. This is an advantage over the ORAC and TEAC assays because these thiols, which are present in high concentrations in animal and plant foods, are mainly degraded in the intestine and poorly absorbed. A disadvantage of the FRAP assay is its inability to detect other small molecular weight thiols and sulphur containing molecules of e.g. garlic, which may play a role in the antioxidant defence. Based on these considerations the FRAP assay was selected as the most appropriate assay for measurements of antioxidant content in a large number of different edible items.

In a previously published article we have included results from a preliminary validation study (Halvorsen et al., 2002). In the present paper we have extended the validation through a more thorough examination of the assay which focused on the ability of the FRAP assay to quantitate different water soluble as well as fat soluble compounds dissolved in selected solvents. Moreover, we have examined the reaction kinetics of the assay with respect to pure compounds and food extracts as well as the efficiency of the extraction procedure.

2. Materials and methods

2.1. Reagents

TPTZ (2,4,6-tri-pyridyl-s-triazine) was obtained from Fluka (Deisenhofen, Switzerland), sodium acetate trihydrate and $FeSO_4 \times 7\ H_2O$ from Riedel-deHaën (Seelze, Germany), acetic acid and hydrochloric acid from Merck (Darmstadt, Germany), $FeCl_3 \times 6H_2O$ from BDH Laboratory Supplies (Dorset, England). MilliQ water (Millipore, Bedford, MA, USA) was used to ensure proper water quality. Methanol, 2-propanol, and tert-butyl methyl ether of HPLC grade, and benzene for analysis were obtained from Merck. Ethanol (absolute alcohol prima) was purchased from Arcus Kjemi (Norway), and dimethyl sulfoxide (DMSO) (minimum 99.5% for GC) was from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Quercetin dihydrate, apigenin, kaempferol, D,L-sulforaphane, resveratrol, genistein, curcumin, epicatechin, caffeic acid, rutin hydrate, coumarin, chlorogenic acid, luteolin, naringenin, daidzein, L-ascorbic acid, lycopene, β-carotene, lutein, 17α-estradiol, 17β-estradiol, and progesterone were obtained from Sigma. Eugenol, quinic acid, Trolox, L-glutathione (GSH), benzoic acid, and epigallocatechin gallate (EGCG) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Myricetin, zeaxanthin, and quercetin-3-β-glucose were from Fluka. α-, β-, γ-, and δ-Tocopherol and α-, β-, γ-, and δ-tocotrienol were obtained from Calbiochem (San Diego, USA).

2.2. Preparation of standard solutions for measurements of total antioxidant activity and reaction kinetics

Stock solutions of α-, β-, γ-, and δ-tocopherol, α-, β-, γ-, and δ-tocotrienol, 17α-estradiol, 17β-estradiol, progesterone, kaempferol, curcumin, chlorogenic acid, genistein, apigenin, coumarin, resveratrol, epicetachin were prepared in ethanol. Stock solutions of carotenoids (β-carotene, lycopene, lutein and zeaxanthin), were made in benzene and rutin, luteolin, daidzein, D,L-sulforaphane, naringenin and myricetin were prepared in DMSO. EGCG, eugenol, quinic acid, benzoic acid, quercetin dihydrate, caffeic acid and L-ascorbic acid were dissolved in methanol. All stock solutions were prepared daily and further diluted in three different solvents: (i) water/methanol (1 + 9, v/v), (ii) water/2-propanol (1 + 9, v/v) and (iii) 2-propanol, before analysis. The concentration of stock solutions of tocopherols, tocotrienols and carotenoids were determined spectrophotometrically.

2.3. Preparation of food samples for measurement of reaction kinetics

Apples, blueberries, broccoli, baking cocoa, carrots, coffee, green tea, hazelnuts, orange juice, soybean oil, turmeric, and walnuts were used for measurements of total antioxidant content as a function of incubation time. All foods, except blueberries, were purchased from grocery stores. Blueberries were handpicked and stored at −20 °C until analysis. All solid samples were chopped up and homogenised in a food processor. Coffee and tea were prepared as described on the packaging. After homogenising, analytical aliquots were weighed. All samples were extracted in triplicates in three different solvents: (i) 10 mL water/methanol (1 + 9, v/v), (iii) 10 mL water/2-propanol (1 + 9, v/v), and (iii) 10 mL 2-propanol. Total antioxidant content was measured in triplicates of each sample replicate (i.e. nine data points per sample/solvent combination).

2.4. Preparation of food samples for examination of the extraction procedure

Carrots, hazelnuts, broccoli, apples, walnuts, and blueberries were homogenised and extracted with water/methanol (1 + 9, v/v) for 15 min, 4 °C on an ultrasonic bath. Twelve replicates were performed for each food item. After extraction, the supernatant was removed by water suction. In order to study reextraction of the pellet, we have used 4 different solvents: (i) water/methanol (1 + 9, v/v), (ii) water/2-propanol (1 + 9, v/v), (iii) 2-propanol, or (iv) t-butyl-methyl ether. Three pellets were extracted with 2 mL of each of the above mentioned solvents for each sample. Total antioxidants were measured in triplicates (i.e. nine data point per sample/solvent combination) using the FRAP assay. The result of the second extraction is in per cent (%) of the first water/methanol extraction.

2.5. Measurement of total antioxidant activity/content

The antioxidant assay of Benzie and Strain (1996) was used with modifications that allowed quantification of most hydrophilic and lipophilic antioxidants as previously described (Dragland, Senoo, Wake, Holte, & Blomhoff, 2003; Halvorsen et al., 2006; Halvorsen et al., 2002). A Technicon RA 1000 system (Technicon instruments corporation, New York, USA) was used for the measurements of absorption changes that appear when the Fe^{3+}-TPTZ complex reduces to the Fe^{2+}-TPTZ form in the presence of antioxidants. An intense blue colour with absorption maximum at 593 nm develops. The measurements were performed at 600 nm after 4 min incubation at 37 °C. An aqueous solution of 500 μmol/L $FeSO_4 \times 7\ H_2O$ was used for calibration of the instrument. The results from measurements of pure compounds are presented as the number of reduced Fe^{3+}-TPTZ complexes. This is calculated as μmol Fe^{2+}-TPTZ/μmol compound, and is equal to the number of electrons (e^-) or protons (H^+) donated in the reaction.

A less comprehensive validation of the assay was described in a previous paper (Halvorsen et al., 2002). Briefly, the within-day repeatability measured as relative standard deviation (RSD %) in standard solutions ranged from 0.4% to 6%. The between-day repeatability was <3%. The variation in the total antioxidant