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Original Research Article

Optimisation of assay conditions for the determination of antioxidant capacity and polyphenols in cereal food components

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ABSTRACT

The measurement of the antioxidant activity of foods, food ingredients and plant extracts is an important parameter for determining their quality. Whole grain wheat flour, which is a key component of the human diet, was chosen as a food model to optimise 2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods by using different solvent extractions or assay conditions. The ABTS method was found to have a wide pH range of stability (at least pH 1–8), while for DPPH method, a pH range between 4 and 8 was needed. The reagent dilution (resulting from the addition of the sample to the reaction mixture) was the most relevant limiting factor of the ABTS assay with a sample/reagent volume ratio that should be at most 1/10. The direct ABTS assay for solid samples was optimised and compared with previously reported solvent extractions demonstrating that the various solvents have different antioxidant compound extraction capacities and that the residual bound molecules can be assayed by the direct method. Total polyphenol and flavonoid assays showed no evident pH-dependence. General guidelines regarding the optimal conditions for the extraction and measurement of the total antioxidant activity of foods and food extracts, can be recommended from the present results.

1. Introduction

Cereal-based foods, especially those based on wheat, rice and maize, are an essential component of the daily human diet. Nutritionally, they are an important source of carbohydrates, proteins, dietary fibre and vitamins as well as many non-nutrient compounds (such as phenolics, flavonoids, carotenoids and fructans). Phytochemicals and antioxidants in whole grains have not received as much attention as those present in fruits and vegetables, although the increased consumption of whole grains and their derived food products has been associated with a protective role against several Western diseases, such as cardiovascular disorders, type 2 diabetes and some cancers (Liu, 2007). The health benefits of whole grains are attributed in part to their unique phytochemical composition and antioxidant activity (Adom and Liu, 2002; Fardet, 2010; Liu, 2007; Serpen et al., 2008), but relatively little is known about the inherent varietal differences in metabolic profiles, total phenolic and carotenoid contents, or total antioxidant activities of different wheat varieties, which ultimately influence the associated nutritional and health benefits of wheat and derived products (Adom et al., 2003). The concentration of phenolics in whole grains is influenced by grain type, variety and by the grain sampling (Adom and Liu, 2002; Adom et al., 2003; Fardet, 2010; Liu, 2007). The most common polyphenols found in cereals are phenolic acids and flavonoids. Phenolic acids (e.g. hydroxybenzoic and hydroxycinnamic acid derivatives) are commonly present in bound forms, as components of complex structures such as lignins and hydrolysable tannins, or are linked through ester bonds to cell wall structural components (Liu, 2007). Flavonoids are ubiquitous in plants and represent the most abundant antioxidants in human diet. In whole-grain wheat they are 2–3 times less abundant than phenolic acids. Both flavonoids and phenolic acids can act on animal cell signalling pathways modifying gene regulation and/or cell redox status and exerting final positive effects on health (Fardet, 2010).

The phytochemical content in cereals has been commonly underestimated in the literature, due to the fact that bound phytochemicals were not usually taken into consideration although they include the major portion of phenolics in grains

Abbreviations: AA, ascorbic acid; Abs, absorbance; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; CAT, (+)-catechin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; FW, fresh weight; GA, gallic acid; M1–M8, methanol:Tris–HCl at pH 1–8; M–H_2O, methanol:water; M–HCl, methanol:12 N HCl.

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(e.g. 85% in maize, 75–76% in oats and wheat, and 62% in rice) (Adom and Liu, 2002; Liu, 2007), and are the major contributors to the total antioxidant activity (90% of the total activity in wheat) (Adom and Liu, 2002). Bound phytochemicals can survive stomach and intestinal digestion to reach the colon, where, after microbiota transformation (Crozier et al., 2010) and absorption by the mucosa, enter the blood stream and provide important healthy effects (Adom and Liu, 2002).

The assessment of food antioxidant activity is a valuable parameter in food science research, as it combines data related to shelf life and sensorial quality with those of health and nutrition. In the past fifty years, several antioxidant activity assaying methods, have been published. In addition to the traditional methodologies based on fat oxidation monitoring, conjugate diene formation and measure of thiobarbituric reactive substances, the use of coloured radical compounds, such as ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl), has been adopted as a result of simplicity, rapidity, low cost and good reproducibility (Blois, 1958; Brand-Williams et al., 1995; Re et al., 1999). Both DPPH and ABTS assays adopt a similar chemical reaction mechanism in which a single electron transfer (SET assay) is involved in the reduction of a given compound (Huang et al., 2005; Prior et al., 2005; Schaich, 2006). The addition of antioxidant compounds reduces ABTS or DPPH coloured cations, thus causing a reagent decolourisation that is measurable spectrophotometrically, depending on the antioxidant type and concentration and on the incubation time (Brand-Williams et al., 1995; Huang et al., 2005; Prior et al., 2005; Re et al., 1999). The factors that may influence these reactions have been discussed, sometimes reaching conflicting conclusions (Molyneux, 2004; Nicolaev et al., 2008; Ozcelik et al., 2003; Ozgen et al., 2006; Re et al., 1999; Serpen et al., 2007, 2008; Singh et al., 2007; Zhao et al., 2006). The most important and controversial subjects of debate are the pH of the reaction mixture and the type of solvent used for sample extraction.

The majority of previous studies reported phenolic levels and antioxidant activity of grain extracts (obtained by different aqueous solutions of methanol, ethanol, acetone, etc.), based on the assumption that the use of finely powdered samples and/or long extraction times would ensure maximum phenolic extraction. In addition the insoluble-bound fraction of grain phenolics has been measured (Adom and Liu, 2002; Adom et al., 2003; Liu, 2007) by re-adapting a previously used alkaline extraction method (Sosulski et al., 1982). More recently, a direct procedure to measure the antioxidant activity of insoluble cereal components still bound to cell wall, was developed by Serpen et al. (2007). This involved adding ABTS or DPPH reagent solutions directly to the sample without any previous solvent extraction, procedure that may present some advantages with respect to the methods based on successive solvent extractions and hydrolysis steps because it enables: (i) evaluation of a wider range of compounds (free plus bound independently from their solubility); (ii) avoidance of the underestimation of activity due to incomplete extraction by the alkali treatment; (iii) maintenance of any synergistic effect among different antioxidants due to the fact that the total activity is measured in a single step instead of being subdivided among several sample extracts (Serpen et al., 2008).

The aim of this study was to determine the best experimental conditions (such as pH, dilution, ratio sample/solvent, solvent type) in a range of methodologies in order to optimise the measurement of total antioxidant activity in food products. Whole wheat flour was chosen as food model because of its importance as an ingredient in the human diet. The results obtained make it possible to recommend optimal procedural guidelines that are generally applicable to different foods, food ingredients and plant extracts.

2. Materials and methods

All the chemicals and solvents were purchased from Sigma–Aldrich (Milan, Italy).

2.1. Solvent and standard preparation

Different types of methanol-based solvents were used: (a) 95:5, methanol:water (M–H_2O, measured pH 6.3 ± 0.1); (b) 98:2,

Table 1
Tested extraction conditions of wheat flour. Combination between flour weight and solvent type and volume. All the extractions were repeated twice. Two biological replicate samples with two technical replicates were performed giving similar results ($n = 4$).

Solvent	Wheat flour fresh weight			Analysis	Related result figure
	10 mg	100 mg	500 mg		
M1–M8					
5 mL			X	ABTS, DPPH, total polyphenols and flavonoids, DW	3
M–H_2O					
1 mL	X			ABTS	5
5 mL			X	ABTS, DPPH, total polyphenols and flavonoids, DW	3
6 mL	X			ABTS, total polyphenols	4B
M–HCl					
1 mL	X			ABTS	5
5 mL			X	ABTS, DPPH, total polyphenols and flavonoids, DW	3
6 mL	X			ABTS, total polyphenols	4B
Water					
1 mL	X			ABTS	5
6 mL	X			ABTS, total polyphenols	4B
ABTS working solution					
1.7 mL	X	X		ABTS	4A
6 mL	X	X		ABTS	4
10 mL	X			ABTS	4B
15 mL	X			ABTS	4B
20 mL	X			ABTS	4B, 5
DPPH working solution					
1.7 mL	X	X		DPPH	4A
6 mL	X	X		DPPH	4A