Journal of Food Composition and Analysis 46 (2016) 15–21

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

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

Original Research Article

Determination of free and bound phenolics using HPLC-DAD, antioxidant activity and *in vitro* digestibility of *Eragrostis tef*

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ARTICLE INFO

Article history:
Received 17 August 2015
Received in revised form 30 October 2015
Accepted 3 November 2015
Available online 5 November 2015

Keywords:
Antioxidant activity
Gluten-free cereals
Eragrostis tef
Flavonoids
In vitro digestibility
Phenolics
Teff
Food analysis
Food composition.

ABSTRACT

White and brown *Eragrostis tef* were assessed for total flavonoid and phenolic content, HPLC profile of the most common phenolics and antioxidant activity including both free and bound phenolics. Antioxidant activity was evaluated in correlation with free and bound phenolics and *in vitro* digestibility was determined. Content of flavonoids (0.52–1.02 mg RE/g) and phenolics (0.90–1.42 mg GAE/g) as well as antioxidant activity (1.70–4.37 μmol TEAC/g using ABTS method) was higher in free phenolic fraction. Correlation showed that bound flavonoids were not significant contributors to antioxidant activity (R^2 = 0.4513 and 0.4893, respectively). The main free phenolics in brown teff were *trans-p*-coumaric, protocatechuic, ferulic and gallic acids, while the major free phenolics in white teff were rutin, protocatechuic and ferulic acids. The main bound phenolics in brown teff were ferulic and gallic acids, quercetin and catechin, in white teff ferulic acid, rutin, catechin and quercetin. Cooked teff showed very high level of *in vitro* organic matter digestibility (80.5–85.1%), whereas brown teff was significantly more digestible than white teff ($P < 0.05$).

1. Introduction

Dietary consumption of whole-grain cereals has been shown to contribute to reduce risk of cardiovascular ailments, type II diabetes, ischaemic stroke, obesity and cancers (Deng et al., 2012; Wang et al., 2014). These health benefits are probably the result of a combination of fibre, phenolics and other bioactive components. Phenolics include hydroxybenzoic and hydroxycinnamic acids, flavonoids, stilbenes and lignans (Chandrasekara and Shahidi, 2012) and their positive attributes include modulating cellular oxidative status and preventing DNA molecules from oxidative damage (Wang et al., 2014). Phenolics in cereal grains occur in both free or free/conjugated and bound forms (Wang et al., 2014). In cereals such as wheat and barley, for example, 80–90% of the total amount is bound phenolic acids. They are mostly ester linked to cell wall polymers and consist mainly of ferulic, vanillic, cinnamic, coumaric and protocatechuic acids (Serpen et al., 2008). On the contrary, in finger millet (*Elusine coracana*) the phenolics have been reported mainly in their free form (Wang et al., 2014) found in the outer layers of the kernel (pericarp, testa and aleurone) (Dykes and Rooney, 2006). Ferulic, *p*-coumaric, and cinnamic acids are reported as the major phenolic acids in millets (Dykes and Rooney, 2006; Subba Rao and Muralikrishna, 2002), Wang et al. (2014) stated that *p*-hydroxybenzoic and gallic acids are the most abundant in barley, oat, rice and millet.

Due to their high nutritional value, antioxidant and radical scavenging activity, alternative grains such as *Eragrostis tef* (Zuccagni) Trotter are getting more and more attention as an ingredient for the production of cereal based functional foods. Their importance increases strongly as an ingredient in multigrain and gluten-free cereal products (Chandrasekara and Shahidi, 2011; Hager et al., 2012; Taylor et al., 2014). Teff comes from Ethiopia where teff flour is used for making several types of leavened flat bread (Forsido et al., 2013; Hager et al., 2012). There is an effort to grow the crop outside Ethiopia for specialty food markets. Teff can be grown in the temperate climates of northwest Europe, such as in the Netherlands, but this was not economically beneficial until now (van Delden et al., 2012). Nowadays, teff imported to Europe comes mainly from Bolivia. There are two main types distinguished by the colour of seeds: white teff and brown/red teff (Winch, 2007), both belonging to the group of millets (Abebe and Ronda, 2014; Alaunyte et al., 2012; Collar and Angioloni, 2014).

Teff grains are an important source of minerals (Fe, Ca, Mg and Zn). They also contain polyphenols (produced as secondary plant metabolites) which affect the nutritional properties (Collar and Angioloni, 2014; Hager et al., 2012; van Delden et al., 2012).

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http://dx.doi.org/10.1016/j.jfca.2015.11.001
0889-1575/

However, very little detailed research exists that would address the composition with respect to the content of phenolics. There is a very few available literature which deals with the relation of the free and bound phenolics, as well as antioxidant activity and digestibility.

For this reason the objectives of this study were to prepare free and bound phenolic extracts of teff, to determine total flavonoid and phenolic content, to determine individual phenolics in free and bound extracts using HPLC, to investigate antioxidant activity using ABTS and DPPH with correlation to determine the contribution of the free and bound flavonoids and phenolics to antioxidant activity and to determine organic matter (OMD) and dry matter (DMD) *in vitro* digestibility.

2. Materials and methods

2.1. Grain samples and preparations

Five commercial samples of *Eragrostis tef* were purchased in local markets in Zlín region (Czech Republic) in amount of five packages of 400 g: brown teff flour (country of origin: Bolivia), brown teff grains (Bolivia) and white teff grains (Bolivia). Next, brown and white teff grain samples were purchased in local markets in Idaho (USA) in amount of five packages of 450 g. The grains of both countries were harvested in 2013. Each pack of teff sample was divided into equal parts; representative sub-samples (50 g) were stored in intact packing in an air-conditioned laboratory out of sunlight. Samples were not stored for more than 1 month before grinding. Teff samples were prepared shortly before analysis by using a Combi-Star mill grinder (Waldner Biotech, Lienz, Austria). Each commercial pack was analyzed three times.

2.2. Chemicals and reagents

Methanol, ethanol, ACN, $AlCl_3{\cdot}6H_2O$ and CH_3COOH were obtained from Fluka Analytical (Hannover, Germany). Acetone, NaOH, H_2SO_4, HCl, $NaCO_3$, $NaNO_2$, CH_3COONa, $K_2S_2O_8$, KH_2PO_4 and $Na_2HPO_4{\cdot}12\ H_2O$ were obtained from Penta (Prague, Czech Republic). Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, purity ≥97%) and 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) (all Sigma Aldrich, Prague, Czech Republic) were applied for phenolics and antioxidant potential evaluation.

Phenolic standards were as follows: gallic acid, catechin, vanillic acid, caffeic acid and protocatechuic acid (all Sigma Aldrich, Prague, Czech Republic), rutin trihydrate (Carl Roth, Karlsruhe, Germany), ferulic acid, cinnamic acid, resveratrol and quercetin (all Merck, Darmstadt, Germany), syringic acid, *trans-p*-coumaric acid, *m*-coumaric acid (all Tokyo Chemical Industry, Tokyo, Japan) were used for the total flavonoid and phenolic content determination and HPLC analysis. All phenolic standards and solvents used in the study were of HPLC-grade (purity ≥98.5–99.0%). Redistilled water was purified by an Aqua osmotic system (Aquaosmotic, Tišnov, Czech Republic).

Pepsin (E.C. 3.4.23.1) with an activity of 0.7 FIG-U/mg for biochemistry and a mixture of pancreatin enzymes with an activity of 350 FIG-U/g protease, 6000 FIG-U/g lipase and 7500 FIG-U/g amylase were purchased from Merck (Darmstadt, Germany). Enzyme activity was declared by Merck (Darmstadt, Germany), it was not determined in this study.

2.3. Extraction of free phenolics

Free phenolics were extracted using the method reported by Shao et al. (2014) with minor modification. Briefly, 0.5 g of ground teff was extracted twice with 8 mL of MeOH/H_2O (80/20, v/v) in ultrasonic bath at 35 °C for 1 h. The mixture was than centrifuged (Hettich Eba 20; Andreas Hettich, Tuttlingen, Germany) at 4321 × g for 25 min at 20 °C and supernatants were combined. The pH of supernatant was adjusted at 4.0–4.5 using 6 M HCl.

2.4. Extraction of bound phenolics

Obtained residues after free phenolics extraction were re-washed with 20 mL of redistilled water to extract bound phenolic compounds. Water was then separated off and samples were blended twice with 25 mL of 0.4 M NaOH for 2 h in ultrasonic bath. Both supernatants were combined and adjusted to pH 4.0–4.5 using 6 M HCl. After centrifugation at 3421 × g for 25 min, supernatant was used as bound phenolic extract (Shao et al., 2014; Sompong et al., 2011).

2.5. Determination of total flavonoid content

The total flavonoid content (TFC) was determined according to Dewanto et al. (2002) with a modification. Briefly, 8.5 mL of 20% ethanol were mixed with 0.85 mL of the extract and 0.375 mL of 0.5 M $NaNO_2$. After 3 min, 0.375 mL of 0.3 M $AlCl_3{\cdot}6\ H_2O$ was added and the mixture was allowed to stand for 3 min. Then, 2.5 mL of 1 M NaOH was added. The absorbance was measured after 15 min at 506 nm. Rutin (0–1200 μg/L) was used as a standard and the results were expressed as mg of rutin equivalent (RE) per g of the sample (mg RE/g sample).

2.6. Determination of total phenolic content

The total phenolic content (TPC) was determined by Folin–Ciocalteu method with a modification (Singleton et al., 1999). Briefly, 0.2 mL of the sample extract was added to 5 mL of redistilled water and 0.5 mL of Folin–Ciocalteu reagent was added. After a 5-min equilibration, the mixture was neutralized with 1.5 mL of 20% $NaCO_3$ and mixed by a vortex. After a 30-min reaction, the absorbance of the mixture was measured at 765 nm with a UV/VIS spectrophotometer (Lambda 25; Perkin Elmer, MA, USA). Gallic acid (0–1000 mg/L) was used as a reference standard, and the results were expressed as mg of gallic acid equivalents (GAE) per g of the sample (mg GAE/g sample).

2.7. Determination of phenolics profile using HPLC

The profile of common phenolic compounds was determined using a HPLC system (Thermo Scientific Dionex Ultimate 3000; MA, USA) consisting of a Thermo Scientific Dionex UltiMate 3000 Diode Array Detector type DAD-3000RS, an UltiMate 3000 rapid separation autosampler, a binary pump HPG-3x00RS and a solve selector valve HPG-3400RS. Data signals were acquired and processed on a PC running the LC Chromeleon™ 7.2 Chromatography Data System (Thermo Scientific, MA, USA). The phenolics profile was measured according to Deng et al. (2012) with small modification. Phenolic acids were separated using a Kinetex column C18 (150 × 4.6 mm; 2.6 μm) (Phenomenex, distributor Chromservis, Prague, Czech Republic). For phenolic compounds, 10 μL of sample volume were introduced onto the column and eluted under gradient conditions performed with redistilled water:acetic acid in ratio 99:1 (A) and redistilled water:ACN:acetic acid in ratio 67:32:1 (B). The solvent gradient was programmed as follows: 10% B at 0 min, increasing from 0–10 min to 20%, 10–16 min 20–40% B, 16–20 min 40–50% B, 25–26 min 50–70% B, 26–30 min 70% B, 30–40 min 70–10% B, 40–45 min 10% B. The solvent flow rate was 1 mL/min, column temperature was set at 30 °C and the chromatogram was recorded at 275 nm, DAD