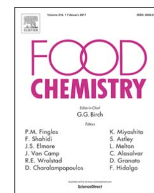
Food Chemistry 245 (2018) 829–837

Contents lists available at ScienceDirect

Food Chemistry

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



Development of extraction method for characterization of free and bonded polyphenols in barley (*Hordeum vulgare* L.) grown in Czech Republic using liquid chromatography-tandem mass spectrometry

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A R T I C L E I N F O

Keywords:
Barley
Free and bonded polyphenols
Hydrolysis
RP-HPLC-MS/MS, design-of-experiment

A B S T R A C T

Complete characterizations of free and bonded phenolic compounds, presented in four cultivars of barley from two regions of Czech Republic, were achieved, using optimized solvent extraction and liquid chromatography coupled with tandem mass spectrometry. The optimization of extraction of free polyphenols was performed using Box–Behnken design and response surface methodology. The intra-day and extra-day precision of developed method were below 6% and 12%, respectively. The isolation of polyphenols bonded to the cell wall structure was carried out by a hydrolysis process. In all cultivars, *p*-hydroxybenzoic, *p*-coumaric and ferulic acids were the most abundant compounds. Their average amounts in barley samples were 17.6, 15.2 and 54.4% (m/m), respectively. The highest amount of these compounds was found in the bonded form, proving the importance of this procedure for the correct characterization of total polyphenols in food matrices.

1. Introduction

In recent years, the market of functional foods and dietary supplements is rapidly growing; consequently, the characterization of nutraceuticals, *i.e.* natural products used for prevention of diseases, is a topic of great interest. Barley (*Hordeum vulgare* L.) is an ancient and important cereal crop. Although it is used mainly for beer production, there is renewed interest in barley food, because of its nutritional value. Barley grain provides low fat, complex carbohydrates (mainly starch), proteins, insoluble and soluble fibres with general (rapid food passage in colon) and specific health benefits from minerals, vitamins, especially vitamin E, and other antioxidants, mostly phenolic compounds (Baik & Ullrich, 2008).

The phenolic compounds identified in barley hull are mainly benzoic, gallic, ferulic and coumaric acids, with lower amounts of 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, vanillic acid and vanillin (Garrote, Cruz, Domínguez, & Parajó, 2008). Moreover, more than 50 proanthocyanidins were reported in barley, including oligomeric and polymeric flavan-3-ol, catechin (c) and gallocatechin (gc). The most abundant proanthocyanidins are dimeric proanthocyanin B3 and procyanidin B3; major trimers include T1 (gc-gc-c), T2 (gc-c-c), T3 (c-gc-c), and T4 or procyanidins *C*2 (c-c-c) (Friedrich, Eberhardt, & Galensa, 2000).

Due to their well-known antioxidant and anticancer properties and other positive effects on human health (Rios-Hoyol et al., 2014; Zhang & Tsao, 2016; Zhou et al., 2016), phenolic compounds have been thoroughly investigated for a long time; however, there is still lack of a widely applicable method for their isolation and determination. In recent years, various methods have been developed for the extraction of bioactive compounds from cereals (Gangopadhyay, Hossain, Rai, & Brunton, 2015) and plants in general (Wang & Weller, 2006). The methods utilized ultrasound-assisted extraction (Wang, Sun, Cao, Tian, & Li, 2008; Wang, Qi, Wang, & Cao, 2013), microwave-assisted extraction (Li, Chen, Nie, & Yao, 2004; Rostagno, Palma, & Barroso, 2007) and extraction using supercritical fluid (Kuk & Dowd, 1998); however, solvent extraction is still among the simplest and the most common approaches used for the isolation of phenolic compounds (Ignat, Volf, & Popa, 2011). Methanol, ethanol, acetone, water, ethyl acetate, diethyl ether and, to a lesser extent, propanol, dimethylformamide and their combinations, are frequently applied for the extraction of phenolic compounds, with an extraction time varying from 1 min to 24 h. The recovery of polyphenols from food products is also influenced by the sample-to-solvent ratio, the particle size and the temperature (Naczk & Shahidi, 2004). These extraction methods can be used for isolation of soluble components from plant matrices.

In recent years, researchers have focussed also on the application of

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https://doi.org/10.1016/j.foodchem.2017.11.101
Received 4 July 2017; Received in revised form 9 November 2017; Accepted 28 November 2017
Available online 29 November 2017
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alkaline (Li, Pickard, & Beta, 2007; Madhujith & Shahidi, 2009) or acidic (Verardo, Bonoli, Marconi, & Caboni, 2008), or both, hydrolysis processes (Chandrasekara & Shahidi, 2011; Cheng et al., 2014), for recovery of bonded polyphenols. Phenolic compounds, in fact, occur in plants in a soluble form (free or conjugated to soluble carbohydrates by ester/ether bonds) or in an insoluble form, bound by ester/ether bonds to the cell wall constituents (cutin, lignin, suberin). Insoluble polyphenols are considered the major contributors to the total antioxidant capacity of cereals (Perez-Jimenez & Saura-Calixto, 2005; Serpen, Capuano, Fogliano, & Gokmen, 2007; Serpen, Gokmen, Pellegrini, & Fogliano, 2008). Adom and Liu (2002) reported that 74% and 69% of total phenolics present in a rice and corn, respectively, were in the insoluble-bound form; more generally, more than 90% of polyphenols in cereal grains are present in bonded form. Most of the reported studies overlook the insoluble bound phenolic compounds, greatly underestimating the content of phenolic compounds present in the samples analysed (Madhujith & Shahidi, 2009).

Hydrolysis represents an essential step of the extraction process, without which the total content of polyphenols would be underestimated; however, it could lead to the degradation of some phenolic compounds. For example, for caffeic and sinapic acids the loss has been estimated to be 67% and 36% of the initial values, respectively (Krygier, Sosulski, & Hogge, 1982). A study published by Nardini and Ghiselli (2004) suggests that the presence of ascorbic acid (AA) and ethylenediaminetetraacetic acid (EDTA), a metal chelator, completely prevents the degradation of phenolic acids during alkaline hydrolysis.

Several analytical methods were used for the determination of polyphenols, including high performance liquid chromatography (HPLC), high speed counter current chromatography, paper chromatography, thin-layer chromatography, capillary electrophoresis and gas chromatography (GC). GC methods developed for the analysis of polyphenols require a derivatisation step (*e.g.* methylation, trifluoroacetylation or silylation), yielding volatile derivatives (Ignat et al., 2011).

Reversed-phase HPLC (RP-HPLC) occupies a leading position in the analysis of phenolic compounds, frequently coupled with spectrophotometric or fluorescence detection (Verardo, Gomez-Caravaca, Marconi, & Caboni, 2011). HPLC coupled with mass spectrometry (HPLC-MS) or tandem mass spectrometry (HPLC-MS/MS) is the best analytical approach to study polyphenols from different biological sources, and for the determination of the structure of phenolic compounds (Bureau, Renard, Reich, Ginies, & Audergon, 2009; Chandrasekara & Shahidi, 2011).

Based on this knowledge, the aim of current research was to develop an extraction procedure for the complete recovery of free and bonded phenolic compounds from barley samples. The design-of-experiment approach was used for that purpose. The target polyphenols present in barley extracts were analysed, using the fast RP-HPLC-MS/MS method in multiple reaction monitoring mode. The developed methods were applied for characterization of four varieties of barley grown in two regions of Czech Republic.

2. Materials and methods

2.1. General

The study was performed using four cultivars of barley (Bojos, Sebastian, Xanadu and Malz) bred in two regions of Czech Republic (Jaroměřice and Uherský Ostroh) in the year 2013. Individual samples (one kg of each) were obtained from the Institute of Brewing and Malting in Brno, Czech Republic. Approximately 50 g of the individual samples were obtained from the bulk amount by the quartation process. This amount was ground and sieved, to obtain small particles (≤300 Mesh). Then, two grammes of the sample were taken for the analysis. The optimization of the extraction procedure was carried out with samples Bojos and Sebastian from the Jaroměřice region. The content of selected phenolic compounds was determined in all samples, using optimized conditions. The scheme of sample preparation procedure is presented in Fig. 1.

Standards of phenolic acids (caffeic, chlorogenic, ferulic, gallic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic, gentisic, sinapic, vanillic and syringic acids), flavonoids (kaempferol, quercetin, rutin, catechin, myricetin, taxifolin, epicatechin, apigenin) and vanillin were of 98% or higher purity and were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as hexane and acetonitrile (ACN), both HPLC or HPLC/MS grade. Acetone (for HPLC), hydrochloric acid (35%), diethyl ether (DE), ethyl acetate (EA), ascorbic acid (AA), EDTA, formic acid (98%, FA), sodium hydroxide and thiourea were purchased from LachNer (Neratovice, Czech Republic). All chemicals were of analytical reagent grade. Deionized water was prepared by a Milli-Q purification system (Merck Millipore, Germany). The mixture of twenty standards (Fig. 2), used for optimization of separation, was prepared using 20 µl of stock solution for each standard (1 g/l dissolved in methanol), and by adding 20% (v/v) acetonitrile to the final volume of 2 ml. The structures of studied compounds shown in Fig. 2 were drawn in ACD/ChemSketch program (ACD/Labs, Toronto, Ontario, Canada).

2.2. Preparation of barley samples

2.2.1. Extraction of free polyphenolic compounds

The barley grains (about 50 g) were ground and sieved, to obtain small particles (≤ 300 Mesh). Then, two grammes of the individual sample were weighted in two repetitions, defatted by shaking for 15 min with 15 ml of hexane three times; after centrifugation (5000 rpm, 3 min) the residue was dried by a gentle stream of nitrogen and extracted three times by shaking with 20 ml of 70% acetone/water mixture (v/v) for 25 min and centrifuged. The supernatant was acidified to pH 2 with 35% (m/m) hydrochloric acid and extracted three times with 20 ml of EA by shaking for 15 min. The organic phase was evaporated under nitrogen to dryness and stored at −18 °C prior to analysis. This extract is referred to as sample α.

2.2.2. Alkaline hydrolysis

The aqueous phase after extraction of free polyphenols and the solid residue were subjected to alkaline hydrolysis. The sample was thus shaken for four hours with 15 ml of 4 M sodium hydroxide at laboratory temperature (25 °C). Then, EDTA (87.6 mg) and AA (300 mg) were added to prevent the degradation of phenolic acids, according to Nardini and Ghiselli (2004). The samples were then acidified to pH 2 with 35% (m/m) hydrochloric acid and extracted three times with 20 ml EA by shaking for 15 min each. The organic phases were evaporated under nitrogen to obtain sample β and sample δ, from the aqueous phase and the solid residue, respectively. Both samples were stored until their use at -18 °C.

2.2.3. Acidic hydrolysis

The aqueous phases after extraction of free polyphenols and alkaline hydrolysis were further subjected to acidic hydrolysis. Hydrolysis was carried out by adding 10 ml of 1 M hydrochloric acid solution and by placing the sample in a water bath at 90 °C for 45 min. After cooling, the samples were extracted three times with 20 ml of EA by shaking for 15 min. Obtained organic phase was evaporated under nitrogen to dryness and stored at −18 °C (sample γ).

Before the HPLC-MS/MS analysis, the extracts were dissolved in 2 ml of 20% ACN/water solution (v/v), filtered through 0.45 µm membrane polytetrafluoroethylene filters and diluted (1:1) with 20% ACN/water solution (v/v).

2.3. Instrumentation and analytical conditions

HPLC-UV analysis was performed using a HPLC system equipped with a LC-20ADXR binary gradient pump, a DGU-20 degassing unit, a

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