Food Chemistry 157 (2014) 290–295

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Tannin analysis of chestnut bark samples (*Castanea sativa* Mill.) by HPLC-DAD–MS

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ARTICLE INFO

Article history:
Received 22 April 2012
Received in revised form 11 December 2013
Accepted 3 February 2014
Available online 12 February 2014

Keywords:
Chestnut bark
Ellagitannins
HPLC-DAD–MS

ABSTRACT

In the present investigation, an HPLC-DAD/ESI-MS method for the complete analysis of tannins and other phenolic compounds of different commercial chestnut bark samples was developed. A total of seven compounds (vescalin, castalin, gallic acid, vescalagin, 1-*O*-galloyl castalagin, castalagin and ellagic acid) were separated and quantified, being 1-*O*-galloyl castalagin tentatively identified and found for the first time in chestnut bark samples. Thus, this method provided information regarding the composition and quality of chestnut bark samples, which is required since these samples are commercialised due to their biochemical properties as ingredients of food supplements.

1. Introduction

Tannins are complex polyphenols synthesized by a wide range of plants and trees (Mueller-Harvey, 2001); thanks to their ability to precipitate gelatin and other proteins from solutions (Mehansho, Butler, & Carlson, 1987), they are proposed to play key roles in the chemical defences of the plant species against biological decay and to deter herbivores. These properties influence other tannin characteristics such as taste and toxicity, as well as some pharmacological effects (Vivas, Bourgeois, Vitry, & Glories, 1996).

Based on their structure, tannins are conventionally divided into condensed and hydrolysable tannin molecules. Condensed tannins have a flavonoid core as a basic skeleton, and hydrolysable tannins are esters of a polyol (most often β-D-glucose) with either gallic acid (gallotannins) or hexahydroxydiphenic acid (HHDP, ellagitannins) (Mämmelä, Savolainen, Lindroos, Kangas, & Vartiainen, 2000; Salminen, Ossipov, Loponen, Haukioja, & Pihlaja, 1999). Several species, such as *Acacia*, *Acer*, *Quercus* and *Castanea* sp. are well known for having both condensed and hydrolysable tannins (Mueller-Harvey, 2001; Živković et al., 2009).

Castanea sativa Mill. belongs to the Fagaceae family and it is one of the most spread chestnut species (De Vasconcelos, Bennett, Rosa, & Ferreira Cardoso, 2007).

The composition of tannins obtained from *C. sativa* wood (Pasch & Pizzi, 2002), bark (Garro-Gálvez, Riedl, & Conner, 1997) and nut (Hwang, Hwang, & Park, 2001; Tsujita, Yamada, Takaku, Shintani, Teramoto, & Sato, 2011) has been determined and it was found that the main components belong to the group of hydrolysable tannins (Vázquez, González-Alvarez, Santos, Freire, & Antorrena, 2009). Based on their hydrolysis products, hydrolysable tannins include gallotannins and ellagitannins. In particular, sweet chestnut contains high amounts of ellagitannins, which form hexahydroxydiphenic (HHDP) acid. The name ellagitannins is derived from ellagic acid, which is created spontaneously in aqueous solution via an *intra*-molecular esterification reaction of HHDP acid (Vermerris & Nicholson, 2006, chap. 1).

The main ellagitannins found in *C. sativa* are castalin and vescalin (Peng, Scalbert, & Monties, 1991), castalagin and vescalagin (Viriot, Scalbert, Hervé du Penhoat, & Moutounet, 1994), kurigalin, 5-*O*-galloylhamamelose, (3′,5′-dimethoxy-4′-hydroxyphenol)-1-*O*-β-D-(6-*O*-galloyl)glucose, chestanin and acutissimin A (Lampire et al., 1998; Peng et al., 1991).

Various chestnut plant materials (leaves, fruit, galls, bark and wood) are used to produce tannin extracts and several thousand tons of sweet chestnut tannins are sold every year in Europe (Vivas et al., 1996). These commercial tannin extracts are used for animal feed (Mueller-Harvey, 2001), by the leather (Scalbert, Monties, &

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http://dx.doi.org/10.1016/j.foodchem.2014.02.003
0308-8146/

Janin, 1989) and by the food industry, especially in wine and spirit production (Sanz et al., 2010; Vivas et al., 1996).

The qualitative composition and the structure of commercial chestnut tanning agents was first studied by Tang, Hancock, and Covington (1992). In particular, castalagin and vescalagin were isolated by thin layer and column chromatography; then their structure was established by means of nuclear magnetic resonance and fast atom bombardment mass spectroscopy. Some other compounds were also found, but their structures were not defined.

The botanical origin of commercial tannin extracts was determined by the analysis of specific species-markers (Vivas, Chauvet, Glories, & Sudraud, 1993; Vivas, Chauvet, Sudraud, & Glories, 1993), but no information on the structure of tannins found in commercial preparations was reported. Next, the qualitative composition of commercial tannin extracts by liquid secondary ion mass spectrometry was investigated by Vivas et al. (1996). Finally, hydrolysable and condensed tannins in commercial vegetable tanning agents and in tannery wastewaters were investigated by reversed-phase liquid chromatography–electrospray ionisation-tandem mass spectrometry (Zywicki, Reemtsma, & Jekel, 2002).

Although chestnut bark extracts are widely used by industry, at the best of our knowledge there is only limited information on the qualitative and quantitative characterisation of the phenolic fraction.

In the present work, a rapid HPLC-DAD/ESI-MS method for the investigation of tannins and other phenolic compounds of chestnut bark samples was developed. In particular, qualitative and quantitative analysis of the tannin fraction of four commercial chestnut bark samples was studied and discussed. Moreover, the extraction yields of tannins and other phenols and the total phenol content of chestnut bark samples were evaluated through the Folin–Ciocalteau test.

2. Experimental

2.1. Chemicals and samples

Methanol (p.a.), monohydrate gallic acid (assay 99.1%) and ellagic acid (assay ⩾96%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (gradient grade, for HPLC) was from VWR (Milano, Italy), formic acid (assay 98–100%) was from Merck (Darmstadt, Germany). Deionized water was obtained from an Elix 10 water purification system from Millipore (Bedford, MA, USA). Sodium molybdate dihydrate was from Carlo Erba (Rodano, Milano, Italy). Na_2CO_3 was from BDH AnalaR (Poole, U.K.). Folin–Ciocalteu reagent was purchased from Merck.

A total of four commercial chestnut bark samples (TAN 1 to TAN 4), containing oligosaccharides, vegetable resins and gums as excipients, were provided from a local distributor (Emila-Romagna region, Italy), and analysed in this work. TAN 1 and TAN 4 samples were in powder, while TAN 2 and TAN 3 were in granular and coated forms, respectively.

2.2. Tannin and other phenol extraction from chestnut bark samples

According to the methods described by Vekiari, Gordon, García-Macías, and Labrinea (2008) and Bianco, Handaji, and Savolainen (1999), 350 mg of chestnut bark sample was dissolved in 20 mL of methanol. The mixture was vortexed for 1 min, kept at ambient temperature for 30 min and then sonicated for 30 min in an ultrasonic bath operating at a frequency of 35 kHz. All samples were filtered on cellulose acetate filters (0.45 μm), diluted 1:2 with water and stored at −18 °C until analysis.

2.3. Spectrophotometric determination of tannin and other phenol extraction yield and total phenol (TP) content

The extraction yield of tannins and other phenolic compounds and the total phenol (TP) content of chestnut bark samples were determined by the Folin–Ciocalteau method at 750 nm (Singleton & Rossi, 1965), using a Shimadzu Spectrophotometer UV–VIS 1204 (Kyoto, Japan).

The yield of tannin and other phenol extraction obtained with different solvents and solvent mixtures was expressed as the unitary net absorbance, which was calculated by using the following formula:

$$A_{un} = (As - Ae)/W$$

where A_{un} is the unitary net absorbance (AU/g); *As* is the sample absorbance at 750 nm (AU); *Ae* is the extraction solvent absorbance at 750 nm (AU); *W* is the chestnut bark sample weight (g).

TP content was calculated as gallic acid equivalent (GAE) from the calibration curve of gallic acid standard solutions ($r^2 = 0.9998$) and they were expressed as g GAE/100 g of extract. The analyses were done in triplicate and mean values and standard deviations were calculated.

2.4. HPLC-DAD–MS analysis

HPLC analysis were carried out on an HP 1100 Series (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system, a degasser, an autosampler, a HP diode-array UV–Vis detector and a HP mass spectrometer. A C18 Luna column 5-μm particle size, 25 cm × 3.00 mm I.D. (Phenomenex, Torrance, CA, USA) was used. All solvents were filtered through a 0.45-μm filter disk (Millipore Co., Bedford MA, USA). A mobile phase composed by water–formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile (solvent B) was used. The following gradient elution (according to Sandhu and Gu (2010)) was applied: from 0 to 2 min, 5% B; from 2 to 10 min, 5–20% B; from 10 to 15 min, 20–30% B; from 15 to 20 min, 30–35% B; from 20 to 60 min, 35–80% B; from 60 to 65 min, 80–85% B; from 65 to 70 min, 85–5% B, followed by a re-equilibration of the column for 5 min in the initial conditions. The flow-rate was 0.5 mL/min. The injection volumes were 10 μL. All the analyses were carried out at room temperature.

On the other hand, MS analyses were carried out using an electrospray (ESI) interface operating both in positive and in negative mode. The following conditions of ESI interface were used: drying gas flow, 9.0 L/min; nebulizer pressure, 35 psig; gas drying temperature, 350 °C; capillary voltage, 3000 V; fragmentor voltage, 60 V.

Tannins and other phenols extracted from chestnut bark samples were identified by comparing retention times, UV and MS spectra of the detected peaks with those of commercial standards (gallic and ellagic acid); if reference compounds were not available a tentative identification was made by analysing and comparing elution order, spectroscopic and spectrometric information with literature data. The quantification of each compound was performed using eight-point regression curves obtained using gallic ($r^2 = 0.9993$) or ellagic acid ($r^2 = 0.9992$). Gallic acid amount was calculated at 280 nm with gallic acid as reference standard; ellagic acid and ellagitannins (vescalin, castalin, vescalagin, castalagin and 1-*O*-galloyl castalagin) amounts were quantified at 254 nm using the ellagic acid calibration curve. For vescalin and castalin, a correction of molecular weight with a multiplication factor of 632/302 was applied; for vescalagin and castalagin a multiplication factor of 934/302 was used; finally for 1-*O*-galloyl castalagin the correction of molecular weight with a multiplicative factor of 1086/302 was calculated. These correction factors were applied to take into account the different molecular weight of tannins and external standards used for quantification (Mulinacci et al., 2001). Both