Analytica Chimica Acta 724 (2012) 127–135

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

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

Simultaneous qualitative assessment and quantitative analysis of flavonoids in various tissues of lotus (*Nelumbo nucifera*) using high performance liquid chromatography coupled with triple quad mass spectrometry

Sha Chen[a,b,c], Linchuan Fang[a], Huifen Xi[c], Le Guan[c], Jinbao Fang[d], Yanling Liu[a], Benhong Wu[c,*], Shaohua Li[a,*,1]

[a] Key Laboratory of Plant Germplasm Enhancement and Speciality Agriculture, Wuhan Botanical Garden, The Chinese Academy of Sciences, Wuhan 430074, PR China
[b] Graduate School of Chinese Academy of Sciences, Beijing, 100049, PR China
[c] Beijing Key Laboratory of Grape Science and Enology, and Key Laboratory of Plant Resource, Institute of Botany, Chinese Academy of Sciences, Beijing, 100093, PR China
[d] Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou 450009, PR China

ARTICLE INFO

Article history:
Received 23 October 2011
Received in revised form 20 February 2012
Accepted 25 February 2012
Available online 10 March 2012

Keywords:
HPLC
Mass spectrometry
Flavonoids
Lotus
Tissues

ABSTRACT

Flavonoid composition and concentration were investigated in 12 different tissues of 'Ti-1' lotus (*Nelumbo nucifera*) by high performance liquid chromatography equipped with photodiode array detection tandem electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS^n). A total of 20 flavonoids belonging to six groups (myricetin, quercetin, kaempferol, isohamnetin, diosmetin derivatives) were separated and identified. Myricetin 3-*O*-galactoside, myricetin 3-*O*-glucuronide, isorhamnetin 3-*O*-glucuronide and free aglycone diometin (3′,5,7-trihydroxy-4′-methoxyflavone) were first reported in lotus. Flavonoid composition varied largely with tissue type, and diverse compounds (5–15) were found in leaf and flower stalks, flower pistils, seed coats and embryos. Flower tissues including flower petals, stamens, pistils, and, especially, reproductive tissue fruit coats had more flavonoid compounds (15–17) than leaves (12), while no flavonoids were detectable in seed kernels. The flavonoid content of seed embryos was high, 730.95 mg 100 g^{-1} DW (dry weight). As regards the other tissues, mature leaf pulp (771.79 mg 100 g^{-1} FW (fresh weight)) and young leaves (650.67 mg 100 g^{-1} FW) had higher total flavonoid amount than flower stamens (359.45 mg 100 g^{-1} FW) and flower petals (342.97 mg 100 g^{-1} FW), while leaf stalks, flower stalks and seed coats had much less total flavonoid (less than 40 mg 100 g^{-1} FW).

1. Introduction

Lotus (*Nelumbo nucifera* Gaertn.), a traditional Chinese medicinal herb, is a flavonoid-rich plant. It has been cultivated for more than 2000 years in China and consumed around the world [1]. Almost all the tissues of lotus, including leaves, leaf stalks, flower stalks, flower petals, flower stamens, flower pistils, seeds and rhizomes, are used as vegetables or traditional Chinese medicinal herbs [2]. Lotus seed kernels and rhizomes are usually used as a healthful cooked food, and they are often considered as human health immunomodulators [3,4]. Moreover, leaves and embryos have been evaluated as important Chinese herbal drugs [5]. Petals and stamens containing natural pigment and flavonols are made into healthy tea and functional food additions, and they also have ornamental value [6–8].

Lotus leaves and embryos are rich in flavonoids and other secondary metabolites, and they have been extensively studied for their antioxidant, antibacterial, anti-HIV and anti-obesity functions [9–14]. Eight flavonoids in lotus stamens were isolated and identified by nuclear magnetic resonance spectroscopy (NMR), and their antioxidant properties were revealed by some free radical ion scavenging activity tests [15]. Recently, twelve flavonoids were identified in lotus petals, and five of these flavonoids were also found in lotus fruit coats by HPLC-MS^n [16,17]. The successful identification and quantification of flavonoids has greatly helped the study of antioxidant and other healthy-protective properties in lotus. In addition, leaves and stamens of lotus have been identified as enriched sources of natural flavonoids [12,15]. However, flavonoid composition and accumulation varies with tissue, which may be largely modulated by genetic regulation [18]. Tissue-dependent assessment may not only be essential for quality control of the medicinal herb, but also effective as traceable markers for genetic and metabolic research [18–20].

Spectrophotometry, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) are the methods

* Corresponding author. Tel.: +86 10 62836664; fax: +86 10 62836026.
E-mail addresses: bhwu@ibcas.ac.cn (B. Wu), shhli@wbgcas.cn (S. Li).
[1] Tel.: +86 27 87510599; fax: +86 27 87510251

0003-2670/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2012.02.051

used for quality control of flavonoids in lotus [21–23]. However, HPLC-DAD-ESI-MS has proven to be a reliable and simple method for the identification and determination of flavonoids in recent years [17,19,20,24]. Moreover, we recently reported the use of an HPLC-DAD-ESI-MS^n technique combined with UV detection of a gradient elution procedure for isolation and characterization of flavonoids in lotus leaves, and 13 flavonoids identified in the previous study [25]. However, separation efficiency and resolution of target peaks from various tissues are often bottlenecks in flavonoid analyses. They may not be comprehensive if suitable extraction and separation protocols have not been established. The latter is dependent on an appropriate HPLC column, mobile phases and elution gradients, column temperature, and flow rate. Because little information is available about the composition and content of flavonoids in different tissues of lotus, the importance of suitable sample separation and analytical methods should not be undervalued.

In this study, we analyzed flavonoids in various edible and inedible lotus tissues, including young leaves, mature leaf pulps, mature leaf veins, leaf stalks, flowers (stalks, petals, stamens, pistils), fruit coats and seed (coats, kernels), with some modified and improved procedures. The objective was to establish suitable and efficient HPLC-DAD-ESI-MS^n methods to analyze flavonoids in various lotus tissues, and to determine the characteristics of flavonoid composition and content. The methods described here may be useful for quality control of the lotus used in traditional Chinese medicines and could make valuable contributions to the clear identification of the medical values of different tissues of lotus.

2. Experimental

2.1. Plant material

Plants of the lotus cultivar 'Ti-1' were grown in a pool in Wuhan Botanical Garden, the Chinese Academy of Sciences, Wuhan. Twelve different tissues including young leaves, mature leaf pulps, mature leaf veins, leaf stalks, flower stalks, flower petals, flower stamens, flower pistils, fruit coats, seed coats, seed kernels, and seed embryos (known as plumula nelumbinis) were manually collected in mid-July from three individual plants, comprising three replicates. Seed embryos were picked when the color of seed coats turned into black.

Except for seed embryos, approximately 50 g of fresh tissues were powdered immediately in liquid nitrogen with an analytical mill (IKA A11 basic machine, German) and then stored at −40 °C for later analysis. The seed embryos were frozen and lyophilized, and then stored at room temperature in a closed desiccator for later analysis.

2.2. Extraction

One of the most important experimental variables during flavonoid extraction of fresh and dry materials is the ratio of solution to tissue. A preliminary study showed that a dry lotus sample should be extracted at a high ratio of solution to tissue. Through the comparison of different ratios, it was determined that about 0.5 g dry seed embryos should be extracted with 25 mL methanol–water (70:30, v/v), and 1 g of the other fresh tissues should be extracted with 30 mL methanol–water (70:30, v/v) at 4 °C for 36 h. The extract was centrifuged at 20,000 × *g* for 10 min, the supernatant was collected, and the tissues were re-extracted as above two additional times. The combined supernatant solutions were evaporated to dryness in a rotary vacuum (RE52AA, Yarong, Shanghai, China) at 35 °C, then re-dissolved in 2 mL 100% methanol. Aliquots of the sample solution were filtered by a solid phase extraction cartridge (Oasis HLB, Waters, USA) for clean-up. For the purification, the cartridge was first equilibrated with methanol and water. After introduction of the supernatant, the cartridge was eluted with 20% methanol 3 times so as to eliminate interferences from sugars, chlorophyll, and other strongly polar polyphenols. Finally, sample solution was reconstituted in 4 mL 100% methanol and filtered through a 0.22 μm Millipore filter (Alltech Scientific Corporation, Beijing, China) before HPLC analysis.

2.3. Preparation of standard solutions and calibration curves

Calibration curves, the limit of detection (LOD) and limit of quantification (LOQ), and linearity of response were established as shown by Chen et al. [25]. Flavonoid is presented as mg 100 g^{-1} per FW (fresh weight) for all tissues, except embryo where mg 100 g^{-1} per DW (dry weight) is used.

2.4. Flavonoid aglycone hydrolysis

The fruit coat was chosen for detection of the aglycone compositions, since it contained the most flavonoid peaks during HPLC separation. A hydrolysis procedure was modified on previous study [25] and carried out as follows: first, 10 g of powdered fruit coat was dissolved in 300 mL 70% MeOH and extracted at 4 °C for 36 h. Then, the solution was centrifuged at 20, 000 × *g* for 10 min. The supernatant was purified using a solid phase cartridge as shown in Section 2.2, dried in a rotary evaporator (35 °C) and finally redissolved in 30 mL 2 M HCl in a methanol–water solution (50:50, v/v). The solution was heated in a capped tube at 105 °C for 90 min. The hydrolyte obtained was purified before HPLC analysis.

2.5. HPLC methods

All the reagents were HPLC grade from Sigma–Aldrich (St. Louis, MO, USA). Extracted and hydrolyzed solutions were analyzed by HPLC-UV (Agilent 1290 series; Agilent, Palo Alto, CA, USA). Columns of Waters Sunfire C_{18} (150 mm × 4.6 mm, 3.5 μm, Waters, USA), reverse-phase Xbrige Amide C_{18} (150 mm × 4.6 mm, 3.5 μm) and Atlantis® T 3 (150 mm × 4.6 mm; 3.5 μm, Waters, USA) were compared in this study. In the solvent system, eluent A was Milli-Q water (Millipore, Billerica, MA, USA) containing 0.5% (v:v) formic acid, and eluent B was CH_3CN containing 0.1% (v:v) formic acid.

The separation of flavonoids from seed embryos was accomplished using the Atlantis® T 3 column at 30 °C with a linear elution gradient protocol of 0–5 min, 10% B; 5–37 min, 10–20% B, 37–45 min, 20–30% B; 45–48 min, 30–60% B, and 48–53 min, 10% B, at a flow rate of 0.6 mL min^{-1}. The separation of flavonoids in all other tissues was done as described by Chen et al. [25].

For aglycone analyses from all tissues, the Waters Sunfire C_{18} column was used at 30 °C with a gradient program of 0–25 min, 20–40% B; and 25–26 min, 40–20% B, at a flow rate 1.0 mL min^{-1}. Chromatograms were acquired at 350 nm and photodiode array spectra were recorded from 210 to 600 nm.

2.6. Identification of flavonoids

Structural elucidation of flavonoids was based on mass spectral data. HPLC-UV was used for separation, and HPLC-MS was used for identification (Agilent 1290 and 6460 triple quad mass spectrometry series; Agilent, Palo Alto, CA, USA). Electrospray ionization (ESI) was performed in both positive (PI) and negative (NI) modes for MS analyses. Nitrogen auxillary gas was provided.

The identification conditions were confirmed with available standards, with the positive mode as follows: HV voltage, 3.5 kV; capillary, 7 μA; nozzle voltage, 500 V; delta emv, 300 V; 5 L min^{-1} gas flow; gas temp, 350 °C; Nebulizer, 45 psi; sheath gas temp, 350 °C; sheath gas flow, 11 L min^{-1}; and scan range, *m*/*z* 100–1000 units. In the negative mode, a delta emv was set at −300 V. Since