Journal of Chromatography A, 1216 (2009) 106–112

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Simultaneous analysis of anthocyanins and flavonols in petals of lotus (*Nelumbo*) cultivars by high-performance liquid chromatography-photodiode array detection/electrospray ionization mass spectrometry

Rui-Zhen Yang[a,b], Xiao-Lei Wei[a,b], Fen-Fang Gao[a,b], Liang-Sheng Wang[a,*], Hui-Jin Zhang[a], Yan-Jun Xu[c], Chong-Hui Li[a,b], Yu-Xuan Ge[a,b], Jing-Jing Zhang[a,b], Jie Zhang[a,d]

[a] *Beijing Botanical Garden, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China*
[b] *Graduate University of Chinese Academy of Sciences, Beijing 100049, China*
[c] *Department of Applied Chemistry, China Agricultural University, Beijing 100094, China*
[d] *College of Science, Northwest A&F University, Yangling, Shaanxi 712100, China*

ARTICLE INFO

Article history:
Received 18 September 2008
Received in revised form 9 November 2008
Accepted 14 November 2008
Available online 25 November 2008

Keywords:
Lotus
Anthocyanin
Flavonol
HPLC
Mass spectrometry

ABSTRACT

A fast and reliable HPLC method for the simultaneous separation of anthocyanins and flavonols in lotus petals was developed based on the study of four candidate solvent systems. Fifteen flavonoids were identified by high-performance liquid chromatography with photodiode array detection/mass spectrometry. Among them, two anthocyanins and nine flavonols were discovered in lotus petals for the first time. This work is valuable for both the hybrid breeding on lotus oriented to flower color and the utilization of lotus petals as functional food materials.

1. Introduction

Lotus, also known as *Nelumbo*, is a perennial aquatic herb, which consists of two species, *N. nucifera* Gaertn. and *N. lutea* (Willd.) Pers. based on the morphological characters. It is one of the most important ornamental and economic plants grown widely in Asia, Australia and North America. By 2005, the number of lotus cultivars had exceeded 600 [1,2]. Lotus flowers could be made into liquor and tea in folk diet [3,4]. The petals have also been found useful in the therapies of hematemesis, eczema, weak spleen and stomach trouble [5].

Anthocyanins and flavonols are two major subclasses of flavonoid compounds existing widely in flowers, fruits and vegetables. Many reports have explored the relationship between flower colors and pigments composition [6–9]. Apart from their contribution in pigmentation, they have been receiving considerable attention due to their wide range of biological activities, including antioxidant, anti-inflammatory, antiallergic, antiulcer, antibiotic and anticarcinogenic properties [10–13]. The structure–activity relationship suggested that the *ortho*-dihydroxyphenyl structure on the B-ring of anthocyanins was essential to the anticarcinogenic action in mouse JB6 cells and lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells, which was only discovered in delphinidin and cyanidin [14,15]. It was reported that malvidin exhibited the highest inhibitory activity against human cancer cell lines, AGS (stomach), HCT-116 (colon), MCF-7 (breast), NCI H460 (lung), and SF-268 (central nervous system, CNS) included [16]. According to Dugas et al. [17], quercetin was the best scavenger of peroxyl radical out of seven common flavonoids. In view of the mass consumption of medicinal and functional food, it is of great importance to select lotus species or cultivars rich in these active components.

To our knowledge, there was no other detail information about the anthocyanins and flavonols composition in petals of lotus except the studies carried out by Rahman et al. [18] and Masato et al. [19]. The former work was a preliminary study performed by adsorption chromatography on magnesium trisilicate and just deduced one flavonol. The other one revealed five anthocyanins, but it lacked MS data to validate them. Thus, there is a solid need to use more advanced equipments to investigate the anthocyanins and flavonols composition and distribution in lotus petals. Considering the tremendous survey task on hundreds of cultivars, it is an urgent

* Corresponding author. Tel.: +86 10 62836654; fax: +86 10 62590348.
E-mail address: wanglsh@ibcas.ac.cn (L.-S. Wang).

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.11.046

requirement to establish a method by which anthocyanins and flavonols could be analyzed simultaneously with higher peak resolution and shorter time span. The two aims of the present work are to develop a fast and reliable HPLC method for the flavonoid analysis of lotus petals in large quantities and to illustrate the anthocyanins and flavonols composition by investigating several representative cultivars.

2. Experimental

2.1. Standards and solvents

Malvidin-3, 5-di-*O*-glucoside chloride (Mv3G5G) was purchased from Extrasynthese (Genay, France). Quercetin 3-*O*-rutinoside (rutin) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Kaempferol was purchased from Shanghai Tauto Biotech (Shanghai, China). Authentic samples of anthocyanins comprised cyanidin-3-*O*-glucoside (Cy3G), peonidin-3-*O*-glucoside (Pn3G), delphinidin 3-*O*-glucoside (Dp3G), petunidin 3-*O*-glucoside (Pt3G) and malvidin 3-*O*-glucoside (Mv3G), in which Cy3G and Pn3G were used as a raw mixture obtained from tree peony cultivars 'Qing Long Wo Mo Chi' and 'Hohki' [6,9], the others were extracted from Crape myrtle cultivar 'Mei Gui Hong' [7]. In the same way, the authentic sample of quercetin 3-*O*-galactoside (Qu3Gal) was obtained from a blueberry cultivar 'Bluecrop'. Standards of quercetin, isorhamnetin and kaempferol 3-*O*-glucoside were generously offered by Professor Xiao Wang (Shandong Analysis and Test Center, Shandong Academy of Sciences, Shandong, China). Methanol and acetonitrile used for high-performance liquid chromatography-photodiode array detection/electrospray ionization multistage mass spectrometry (HPLC-DAD/ESI-MS^n) analysis were of chromatographic grade and purchased from Alltech Scientific (Beijing, China). Trifluoroacetic acid (TFA; ≥99%) was obtained from Merck (Darmstadt, Germany). Methanol and formic acid were of analytical grade and purchased from Beijing Chemical Works (Beijing, China). HPLC-grade water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA).

2.2. Plant materials

Petals of six lotus cultivars, 'Yanyangtian' (red purple), 'Hongtaiyang' (red), 'Meiyuanxiuse' (purple violet), 'Qianbanlian' (pink), 'Youyimudanlian' (greenish-yellow) and 'Dabilian' (light yellow) included, were collected in 2007 at Beijing Botanical Garden, Institute of Botany, the Chinese Academy of Sciences (lat. 39°48′N long, 116°28′E, alt. 76 m). These cultivars were introduced from all over the world and have been planted in the same-sized containers (diameter, 40 cm; height, 30 cm) in Beijing Botanical Garden for more than 3 years under the same cultivated conditions regarding fertilization, irrigation and disease prevention. Full-blown petals were taken into the laboratory immediately after harvested, powdered in liquid nitrogen with mortars and pestles, and then stored at −40 °C for later analysis.

2.3. Preparation of standard solutions

Standards of Mv3G5G and rutin were accurately weighted, dissolved in 0.1% HCl–methanol and methanol, respectively, and then diluted to appropriate concentrations, containing 9.38–187.60 μg/mL for Mv3G5G and 9.17–184.30 μg/mL for rutin.

2.4. Extraction of anthocyanins and flavonols

Approximately 1 g of frozen petal powder was extracted with 5 mL 70% methanol aqueous solution containing 0.1% HCl (pH 2.08) at 4 °C in the dark for 24 h, shaken in a QL-861 vortex (Kylinbell Lab Instruments, Jiangsu, China) every 6 h. The liquid was separated from the solid matrix by filtration through sheets of qualitative filter paper (Hangzhou Special Paper Industry, Zhejiang, China). The filtrate was further passed through 0.22 μm reinforced nylon membrane filters (Shanghai ANPEL, Shanghai, China) before HPLC analysis. Three replicates were performed for each sample.

2.5. Acid hydrolysis of flavonols extraction

The filtered extract solution of 'Dabilian' was dried in a rotary evaporator (35 °C), re-dissolved in 4 mL of 1.5 M HCl in a methanol–water solution (50:50, v/v), and then heated in a capped tube at 90 °C for 3 h [20]. The hydrolyte obtained was purified through a Supelclean ENVI-18 SPE Tube (Supelco, Bellefonte, PA, USA), which was conducted according to Giusti and Wrolstad [21] and Pirisi et al. [22].

2.6. Optimization of HPLC separation for anthocyanins and flavonols

The chromatographic separation was carried out on a Dionex (Sunnyvale, CA, USA) HPLC system equipped with a P680 HPLC pump, an UltiMate 3000 autosampler, a TCC-100 thermostatted column compartment and a Dionex PDA100 photodiode array detector. The analytical column was a C18 column of ODS-80Ts QA (150 mm × 4.6 mm I.D., Tosoh, Tokyo, Japan) protected with a Transgenomic CARB Sep Coregel 87C Guard Cartridge (Transgenomic, Omaha, NE, USA). An aliquot of 10 μL solution was injected for HPLC analysis. Chromatograms were acquired at 520 and 350 nm for anthocyanins and flavonols, respectively, and photodiode array spectra were recorded from 200 to 800 nm. Four candidate solvent systems, abbreviated as S I, S II, S III and S IV, were tested to optimize the simultaneous separation of anthocyanins and flavonols. In S I, 1.5% phosphoric acid was selected as phase A and phosphoric acid–acetic acid–acetonitrile–water (1.5:20:25:53.5; v/v/v/v) as phase B [19]. S II was the same as S I except acetic acid was substituted by formic acid [6,23]. S III was much simpler with 0.1% TFA in water as phase A and acetonitrile as phase B, in contrast with the former two [24]. With regard to S IV, TFA–formic acid–water (0.1:2:97.9, v/v/v) constituted solvent A and TFA–formic acid–acetonitrile–water (0.1:2:35:62.9, v/v/v/v) constituted solvent B [25].

2.7. Identification of anthocyanins and flavonols

2.7.1. Qualitative analysis of anthocyanins

Anthocyanins were primarily identified according to their HPLC retention times, elution order, UV–vis spectra and MS fragmentation pattern and by comparison with published data and authentic samples such as petal extracts of tree peony and crape myrtle [6,7,9]. HPLC–ESI-MS^n analysis for anthocyanins was performed on an Agilent-1100 HPLC system coupled with a DAD system and a LC/MSD Trap VL electrospray ion mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation conditions were those obtained in the optimization experiment (see Section 3.1 for details). The MS conditions were as follows: positive ion mode; gas (N_2) temperature, 325 °C; flow rate, 8.0 L/min; nebulizer pressure, 241.3 kPa; HV voltage, 3.5 kV; octopole RF amplitude, 150 Vpp; skim 1 voltage, 37.5 V; skim 2 voltage, 6.0 V; capillary exit, 111.8 V; cap exit offset, 74.3 V and scan range, m/z 100–1000 units.