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Analytical Methods

Quantitative analysis of phenolic compounds in Chinese hawthorn (*Crataegus* spp.) fruits by high performance liquid chromatography–electrospray ionisation mass spectrometry

Pengzhan Liu [a], Heikki Kallio [a,b], Deguo Lü [c], Chuansheng Zhou [c], Baoru Yang [a,*]

[a] Department of Biochemistry and Food Chemistry, University of Turku, FI-20014 Turku, Finland
[b] Department of Food Science and Engineering, Jinan University, 510632 Guangzhou, China
[c] Faculty of Horticulture, Shenyang Agricultural University, Dongling Road 120, 110161 Shenyang, China

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ABSTRACT

Eleven major phenolic compounds (hyperoside, isoquercitrin, chlorogenic acid, ideain, epicatechin, two procyanidin (PA) dimers, three PA trimers and a PA dimer-hexoside) were quantified in the fruits of 22 cultivars/origins of three species of the Chinese hawthorn (*Crataegus* spp.) by HPLC–ESI-MS-SIR. Hyperoside (0.1–0.8 mg/g dry mass [DM]), isoquercitrin (0.1–0.3 mg/g DM), chlorogenic acid (0.2–1.6 mg/g DM), epicatechin (0.9–11.7 mg/g DM), PA B2 (0.7–12.4 mg/g DM), PA dimer II (0.1–1.5 mg/g DM), PA trimer I (0.1–2.7 mg/g DM), PA trimer II (0.7–6.9 mg/g DM), PA trimer III (0.01–1.2 mg/g DM) and a PA dimer-hexoside (trace–1.1 mg/g DM) were detected in all the samples. Ideain (0.0–0.7 mg/g DM) was found in all the samples except *Crataegus scabrifolia*. Significant correlations between the contents of individual PA aglycons were observed ($r > 0.9$, $P < 0.01$). A strong correlation between flavonols was also shown ($r = 0.71$, $P < 0.01$). Fruits of *Crataegus pinnatifida* var. *major* had higher contents of PAs but lower contents of flavonols compared with *Crataegus brettschneideri*. The fruits of *C. scabrifolia* contained the highest level of PA dimer-hexoside, which was present in trace amounts in the fruits of *C. pinnatifida*.

1. Introduction

Phenolic compounds and foodstuffs rich in phenolics have attracted increasing attention from both researchers and industry because of their potential effects on maintaining human health. Hawthorn berries (genus *Crataegus*, family Rosaceae) rich in phenolic compounds have been widely used as both a medical and food raw material in China and Europe (Fong & Bauman, 2002). Many studies have demonstrated the beneficial effect of extracts of hawthorn fruits on the heart and blood circulation system including cardiovascular protecting and endothelium-dependent vasorelaxing effects (Kim, Kang, Kim, & Kim, 2000). Hawthorn fruit extracts also improve coronary circulation and possess hypolipidemic effects (Pittler, Schmidt, & Ernst, 2003; Quettier-Deleu et al., 2003; Schwinger, Pietsch, Frank, & Brixius, 2000; Tadić et al., 2008). The safety of the extracts of hawthorn fruits as food ingredients has been verified (Daniele, Mazzanti, Pittler, & Ernst, 2006). A standardised extract of the European hawthorn (*Crataegus monogyna* or *Crataegus laevigata*) has been used for the treatment of patients with heart failures (Holubarsch et al., 2008; Tauchert, 2002; Zapfe Jun, 2001).

Hawthorn fruits have long been eaten in China. Commonly, it has been considered that the Chinese hawthorn comprises 18 species, of which *Crataegus pinnatifida* Bge. and its variety Shanlihong (*C. p.* Bge. var. *major* N.E.Br.) are the most important (Zhao & Tian, 1996). In addition, the fruits of other species such as *Crataegus brettschneideri* (Fu hawthorn) and *Crataegus scabrifolia* (Yun'nan hawthorn) are also commonly used as medicinal or food materials (Gao, Feng, & Qin, 1995; Zhao & Tian, 1996). Phenolics are considered among the most important bioactive compounds in Chinese hawthorn fruits (Chang, Zuo, Harrison, & Chow, 2002). Our previous study revealed the presence of more than 40 phenolic compounds in Chinese hawthorn fruits (*C. p.* Bge. var. *major* N.E.Br.). Most of these compounds belonged to B-type procyanidins (PAs) and the rest were flavonol glycosides, anthocyanins or phenolic acids (Liu, Yang, & Kallio, 2010). The phenolic composition in hawthorn fruits varies among species and cultivars (Gao et al., 1995) but information of the contents of phenolics in different species of Chinese hawthorn fruits is still limited.

Quantitative analysis of phenolic compounds, especially of PAs in plant materials, is a challenging task. This is largely because of a lack of commercial reference compounds and deficient separation between PAs using high performance liquid chromatography (HPLC). The total contents of PAs are commonly determined by colorimetric methods without detailed information on the profiles

* Corresponding author. Tel.: +358 2 3336844; fax: +358 2 3336860.
E-mail address: baoru.yang@utu.fi (B. Yang).

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and contents of individual PAs (Hiermann, Kartnig, & Azzam, 1986; Rohr, Meier, & Sticher, 2000). In addition, the results are often affected by the presence of other colour compounds in the sample. Quantitative analyses of PAs have been carried out by normal phase or reversed phase HPLC with limited separation of the analytes. Normal phase HPLC separates PAs based on the degree of polymerisation (DP), but isomers with the same DP values are not separated (Gu et al., 2002; Karonen et al., 2006). In reversed phase HPLC, PA isomers with equal DP numbers can be separated, but some PAs differing in DP values can overlap (Hellstrom & Mattila, 2008; Hellstrom, Sinkkonen, Karonen, & Mattila, 2007; Karonen et al., 2007). To achieve satisfactory results, one or several solid phase extraction (SPE) columns have to be employed to fractionate the samples before HPLC analysis. Polyamide columns and Sephadex LH-20 columns are commonly used for fractionating PAs from different sources (Hellstrom & Mattila, 2008; Svedström, Vuorela, Kostiainen, Laakso, & Hiltunen, 2006; Svedström et al., 2002). The fractionation steps, though improving the separation of PAs in the subsequent HPLC analysis, make the whole quantification procedure complex and tedious.

In the current study, the single ion recording function of HPLC-electrospray ionisation mass spectrometry (HPLC–ESI-MS-SIR) was applied to quantify the phenolic compounds in the aqueous ethanol extract of hawthorn fruits without prefractionation steps. Phenolic compounds in 22 hawthorn samples belonging to four species/varieties were determined with this significantly simplified quantitative analysis.

2. Materials and methods

2.1. Plant materials

Hawthorn fruits of 22 cultivars and origins belonging to four species/varieties were collected in China. Among these, 21 samples were collected in the Chinese National Fruit Germplasm Repository, Shenyang Hawthorn Garden (Shenyang, Liaoning Province), including 10 cultivars of *C. pinnatifida* Bge. var. *major* N.E.Br. harvested in October 2007, eight cultivars of *C. brettschneideri* harvested in August 2008 and three natural origins of *C. pinnatifida* Bge. harvested in September 2008. One sample of *C. scabrifolia* was harvested in Kunming, Yun'nan Province, China in September 2007. For each cultivar/origin, 500 g optimally ripe fruits were collected from 2 to 4 trees, from five randomly selected collection points from different sides of each tree. All the samples were sliced and dried in a cool and shady place after harvesting.

2.2. Reference compounds

Hyperoside (quercetin-3-*O*-galactoside), isoquercitrin (quercetin-3-*O*-glucoside), ideain chloride (cyanidin-3-*O*-galactoside chloride), epicatechin and PA B2 (epicatechin-(4β → 8)-epicatechin) were purchased from Extrasynthese (Genay, France). Chlorogenic acid was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Reference compounds of PA dimer II, PA trimer II and PA dimer-hexoside were isolated from *C. pinnatifida* Bge. var. *major* N.E.Br. in our laboratory by preparative HPLC, and the purity of each sample was tested with HPLC-MS. The purity of the PA dimer II and PA trimer II was more than 95%. The purity of the PA dimer-hexoside was about 80%.

2.3. Other chemical agents

Ethanol was purchased from Primalco Oy (Rajamäki, Finland), methanol (HPLC grade) and formic acid from J.T. Baker (Deventer, Holland) and acetone (HPLC grade) and acetonitrile (HPLC grade) from VWR International Oy (Espoo, Finland).

2.4. Sample preparation

Dried, seedless hawthorn fruits were milled into a fine powder with the aid of liquid nitrogen in a mortar and kept in a desiccator overnight before extraction. A sample of 1.0 g of hawthorn fruit powder was transferred into a 25 ml volumetric flask with 20 ml of 80% aqueous ethanol. The mixture was ultrasonicated for 30 min to enhance the dissolution of the phenolics into the solvent. Eighty percent aqueous ethanol was added to bring the volume to the scale and mixed thoroughly. A sample of 1.0 ml was taken and filtered through a 0.45 μm filter and analysed by HPLC.

2.5. HPLC-DAD–ESI-MS analysis

HPLC-DAD–ESI-MS analysis was performed using a Waters Acquity Ultra Performance LC system equipped with a Waters Acquity 2996 PDA detector and in combination with a Waters Quattro Premier mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an ion-spray interface. A Phenomenex Prodigy RP-18 ODS (3) column (5 μm, 250 × 4.60 mm, Torrance, CA, USA) combined with a Phenomenex Prodigy guard column (5 μm, 30 × 4.60 mm, Torrance, CA, USA) was used. A binary solvent system was employed consisting of formic acid/water (0.5:99.5, v/v) as solvent A and acetonitrile/methanol (80:20, v/v) as solvent B. The gradient program was 0–5 min with 10% solvent B, 5–15 min with 10–18% B, 15–25 min with 18% B, 25–30 min with 18–25% B, 30–35 min with 25% B, 35–40 min with 25–35% B, 40–45 min with 35–60% B, 45–50 min with 60–10% B and 50–55 min with 10% B. The flow rate of the mobile phase was 1 ml/min, and the injection volume 5 μl. The peaks were monitored at 280 nm with PDA detection.

A split joint was used after the PDA detector, directing a flow of 0.3 ml/min to the mass spectrometer and the rest to a waste bottle. The mass spectrometer was operated in a positive ion mode.

The capillary voltage was set to 4.0 kV, the cone voltage to 22 V and the extractor voltage to 3 V. The source temperature was 150 °C and the dissolution temperature 300 °C. The HPLC–ESI-MS system was operated using MassLynx 4.1 software. Selected ion recording (SIR) was used for the quantitative analysis compared with the full scan method used before (Liu et al., 2010). The ions with *m*/*z* 291 (nominal mass 291.3), 303 (303.2), 355 (355.3), 449 (449.4), 579 (579.5), 741 (741.7) and 867 (867.8) were monitored. The ions presented the base peaks in the mass spectra of epicatechin (291), hyperoside (303), isoquercitrin (303), chlorogenic acid (355), ideain (449), PA dimers (579), PA dimer-hexoside (741) and PA trimers (867).

2.6. Calibration

Standard solutions of chlorogenic acid, ideain chloride, hyperoside, isoquercitrin, epicatechin, PA B2, PA dimer II, PA dimer-hexoside and PA trimer II were prepared in methanol in the concentration range of 0.01–0.3 mg/ml and analysed by HPLC. Five microlitres of each solution was injected into HPLC. The calibration curve of every compound was constructed using five different concentrations by plotting the peak areas versus the concentrations. The calibration curve of the PA trimer II isolated and purified in our laboratory was used for the quantification of all the PA trimers. The purities of the compounds were taken into account when preparing the standard solution of PA dimer II, PA timer II and PA dimer-hexoside.