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Phenolic Compounds in Hawthorn (*Crataegus grayana*) Fruits and Leaves and Changes during Fruit Ripening

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ABSTRACT: Phenolics in the fruits and leaves of *Crataegus grayana* were identified by HPLC-UV-ESI-MS. The contents of these compounds and their changes during autumn were also analyzed. Epicatechin [1−7 mg/g dry mass (DM) in fruits and 1−10 mg/g DM in leaves), procyanidins B2 (2−4 and 1−8 mg/g DM) and C1 (2−4 and 1−8 mg/g DM), hyperoside (0.5−1 and 2−11 mg/g DM), and a quercetin-pentoside (0.3−0.5 and 2−6 mg/g DM) were the major phenolics in both fruits and leaves. *C*-Glycosyl flavones were present in leaves (2−5 mg/g DM), whereas only trace levels were found in fruits. Ideain and 5-*O*-caffeoylquinic acid were found only in fruits. An additional 11 phenolics were identified/tentatively identified. Total phenolic contents reached highest levels by the end of August in fruits and by the end of September in leaves. The compositional profiles of phenolics in fruits and leaves of *C. grayana* were different from those of other *Crataegus* species.

KEYWORDS: *Crataegus grayana*, hawthorn, phenolics, procyanidin

INTRODUCTION

Leaves, flowers, and fruits of hawthorn (*Crataegus* spp., Rosaceae) have been widely used as medicinal and food materials in China and European countries. *Crataegus monogyna* and *Crataegus laevigata* are the major hawthorn species in Middle Europe, *Crataegus pentagyna*, *Crataegus nigra*, and *Crataegus azarolus* in southern and southeastern Europe, and *Crataegus pinnatifida* and *Crataegus scabrifolia* in China.[1−3] Scientific evidence has suggested beneficial effects of the extracts and other products of leaves, flowers, and fruits of hawthorn on immune functions, sugar and lipid metabolism, cardiovascular health, and inflammation.[4−8]

Phenolics are among the major bioactive compounds in hawthorn fruits. Compositional investigations of *C. monogyna*, *C. laevigata*, *C. pentagyna*, and *C. pinnatifida* have been carried out earlier. Flavonol glycosides, phenolic acids, and B-type procyanidins (PCs) are the main phenolic compounds in the flowers, leaves, and fruits of the European hawthorn species *C. monogyna*, *C. laevigata*, and *C. pentagyna*[3,9−12] and the Chinese species *C. pinnatifida*.[13−15] Differences in the content and composition of phenolic compounds between species and different parts of the plants have been observed.

Crataegus grayana originated from the North America but commonly cultivated in Finland and other countries in northern Europe as an ornamental plant. The fruits and leaves of *C. grayana* may have similar health effects as its European and Asian relatives. However, information on the content and composition of bioactive compounds in the fruits and leaves of this species is still very limited.

In the current study, we investigated the compositional profile of the major phenolics in the fruits and leaves of *C. grayana* by high-performance liquid chromatography combined with a diode array detection system (HPLC-DAD) and electrospray ionization mass spectrometry (HPLC-ESI-MS). Changes in the content of the phenolics during the ripening of the fruits were determined by HPLC-ESI-MS with selected ion recording (SIR) function.

MATERIALS AND METHODS

Materials. Hawthorn (*C. grayana*) fruits and leaves were picked from Turku, Finland, from August 11 to October 5, 2009, at roughly 1-week intervals. Three fruit samples and three leaf samples were collected at each harvesting date. Each sample was picked from three trees at five randomly selected collection points from different sides of each tree. Immediately after harvesting, the fruits and leaves were frozen and stored at −18 °C until analysis.

Hyperoside (quercetin-3-*O*-galactoside), ideain chloride (cyanidin-3-*O*-galactoside chloride), epicatechin, procyanidin (PC) B2 [epicatechin-(4β→8)-epicatechin], vitexin (apigenin-8-*C*-glucoside), and isovitexin (apigenin-6-*C*-glucoside) were purchased from Extrasynthese (Genay, France). Chlorogenic acid (3-*O*-caffeoylquinic acid) was purchased from Sigma-Aldrich Co. (St. Louis, MO), methanol (HPLC grade) and formic acid were from J. T. Baker (Deventer, The Netherlands), and acetone (HPLC grade) and acetonitrile (HPLC grade) were from VWR International Oy (Espoo, Finland). A PC trimer (tentatively identified as PC C1) was isolated from *C. pinnatifida* var. *major* in our laboratory by preparative HPLC and used for quantification of all procyanidin trimers. The purity of the PC trimer was >95% on the basis of HPLC-MS analysis.

Sample Preparations. *Fruits.* A sample of 20.0 g of frozen hawthorn fruits was cut manually into small pieces, and the seeds were removed. A portion of 4.0 g of flesh was transferred into a 50 mL centrifuge tube, and 20 mL of methanol was added. The mixture was homogenized by a T25 digital Ultra-Turrax high-performance disperser

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(IKA Werke GmbH & Co. KG, Staufen, Gemany) at a speed of 10 × 1000 rpm for 2 min. After homogenization, the mixture was placed in an ultrasonicator bath for 15 min, followed by centrifugation (4420*g*) for 10 min and collection of the supernatant after centrifugation. After that, the extractions with sonication (15 min) and centrifugations (4420*g*) were repeated twice. The supernatants obtained from three extractions were combined. After the removal of methanol with a vacuum rotary evaporator at 35 °C, the extract was dissolved in methanol and quantitatively transferred into a 10 mL volumetric flask to reach an accurate volume. After this, the sample was filtered through a 0.45 μm filter and analyzed by HPLC-DAD and HPLC-ESI-MS.

Another sample of 10.0 g of fruit flesh was taken for determination of moisture.

Leaves. A sample of 5.0 g of frozen hawthorn leaves was cut into small pieces and milled into a fine powder in a mortar with the aid of liquid nitrogen. A portion of 0.5 g of powder was transferred into a 50 mL centrifuge tube and extracted with 20 mL of methanol in an ultrasonicator bath for 15 min, followed by centrifugation (4420*g*) for 10 min. The supernatant was collected after centrifugation. Extraction was repeated three times, and the extracts were combined. After removal of methanol with a vacuum rotary evaporator at 35 °C, the extract was dissolved in methanol and quantitatively transferred into a 10 mL volumetric flask to reach an accurate volume. After this, the sample was filtered through a 0.45 μm filter and analyzed by HPLC-DAD and HPLC-ESI-MS.

Another sample of 3.0 g of leaf powder was taken for determination of moisture.

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 in combination with a Waters Quattro Premier mass spectrometer equipped with an ion-spray interface (Waters Corp., Milford, MA). A Phenomenex Prodigy RP-18 ODS (3) column (5 μm, 250 × 4.60 mm, Torrance, CA) combined with a Phenomenex Prodigy guard column (5 μm, 30 × 4.60 mm) 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 was 10 μL for qualitative analysis and 5 μL for quantitative analysis. The peaks were recorded at 280 and 360 nm with PDA detection.

A split joint was used after the PDA detector, directing a flow of 0.4 mL/min into the mass spectrometer and the rest into a waste bottle. The mass spectrometer was operated in the 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 desolvation temperature, 300 °C. The HPLC-ESI-MS system was operated using MassLynx 4.1 software.

For identification of the phenolic compounds, a full scan from *m*/*z* 100 to 1200 was applied. For quantitative analysis, HPLC-ESI-MS with SIR function was used. The method was modified from our previous one for the quantification of phenolics in extracts of *C. pinnatifida* var. *major*.[16] The ions with *m*/*z* 291 (nominal mass 291.3), 303 (303.2), 355 (355.3), 433 (433.4), 449 (449.4), 463 (463.4), 579 (579.5), and 867 (867.8) were monitored. The ions presented the base peaks in the mass spectra of epicatechin (291), hyperoside (303), quercetin-pentoside (303), quercetin-hexoside acetate (303), chlorogenic acid (355), vitexin (433), ideain (449), luteolin-*C*-hexoside (449), methyl luteolin-*C*-hexoside (463), PC dimers (579), and PC trimers (867).

Qualitative and Quantitative Analysis of Phenolic Compounds. Phenolics were identified by comparing the retention times, UV spectra, and mass spectra of the analytes with those of the reference compounds and on the basis of previous reports of those compounds in the literature.

Quantitative analysis of phenolic compounds in the extracts was carried out using an external standard method. The calibration curves were constructed by HPLC-ESI-MS-SIR analysis of standard solutions of chlorogenic acid, ideain chloride, vitexin, hyperoside, epicatechin, PC B2, and PC C1 in methanol in the concentration range of 0.01−0.3 mg/mL. The injection value was 5 μL for each solution. The calibration curve of every compound was constructed using five different concentrations by plotting the peak areas versus the concentrations. The calibration curve of PC C1 isolated and purified in our laboratory was used for the quantification of all procyanidin trimers. The calibration curve of PC B2 was used for the quantification of all PC dimers, hyperoside for all quercetin glycosides, chlorogenic acid for all isomers of chlorogenic acid, and vitexin for *C*-glycosyl flavones.

Determination of Moisture. The water content was determined gravimetrically by heating the samples in an oven at 105 °C until a constant weight was reached.

RESULTS AND DISCUSSION

Identification of Phenolics in Fruits and Leaves of *C. grayana*. Figure 1 shows the HPLC-DAD chromatograms of methanol extracts of hawthorn fruits and leaves recorded at 280 and 360 nm. Twenty-two compounds (peaks) belonging to five groups of phenolic compounds (hydroxycinnamic acids, anthocyanins, procyanidins, flavonol glycosides, and *C*-glycosyl flavones) were detected and identified or tentatively identified. The typical UV spectra of example compounds of the groups are also presented in Figure 1. Structures of some selected compounds are shown in Figure 2. Table 1 summarizes the detailed information and the identification/tentative identification of the peaks.

Hydroxycinnamic Acid Derivatives and Ideain. Peaks 1, 3, and 7 in the chromatograms of Figure 1 were identified as hydroxycinnamic acid derivatives on the basis of the UV spectra.[13] Peak 3 was determined as chlorogenic acid (3-*O*-caffeoylquinic acid) with the aid of the reference compound. Compound **1** had the same mass spectrum and was identified as 5-*O*-caffeoylquinic acid, an isomer of chlorogenic acid according to the retention data reported in the literature.[17] Peak 7 overlapped with other peaks, and it was impossible to obtain a clear mass spectrum. Compound **2** was identified as ideain by comparison with the reference compound. This is the only anthocyanin found in *C. grayana* fruits.

Procyanidins. Identification of B-type procyanidins and their typical mass spectra have been described in our previous study of phenolic compounds in Chinese hawthorn (*C. pinnatifida* var. *major*) fruits.[13]

The UV spectra of compounds **4**, **5**, **6**, **8**, **9**, **10**, **11**, and **13** in the chromatogram showed clear absorption maxima at 240 and 280 nm, and these are known features of the UV spectra of procyanidins. The mass spectra of the compounds are published in our previous paper.[13]

Compound **6** was identified as epicatechin by reference compound.[18] Compounds **5** and **13** showed mass spectra typical for B-type procyanidin dimers (PC dimers I and II) with the $[M + H]^+$ ion at *m*/*z* 579 as the base peak and an ion at *m*/*z* 289 derived from the cleavage of the C−C bond between the two procyanidin monomers through QM fragmentation.[19] Compound **5** (PC dimer I) was identified as PC B2 with the aid of a reference compound. Compound **13** (PC dimer II) was tentatively identified as PC B5 [epicatechin-(4β→6)-epicatechin]

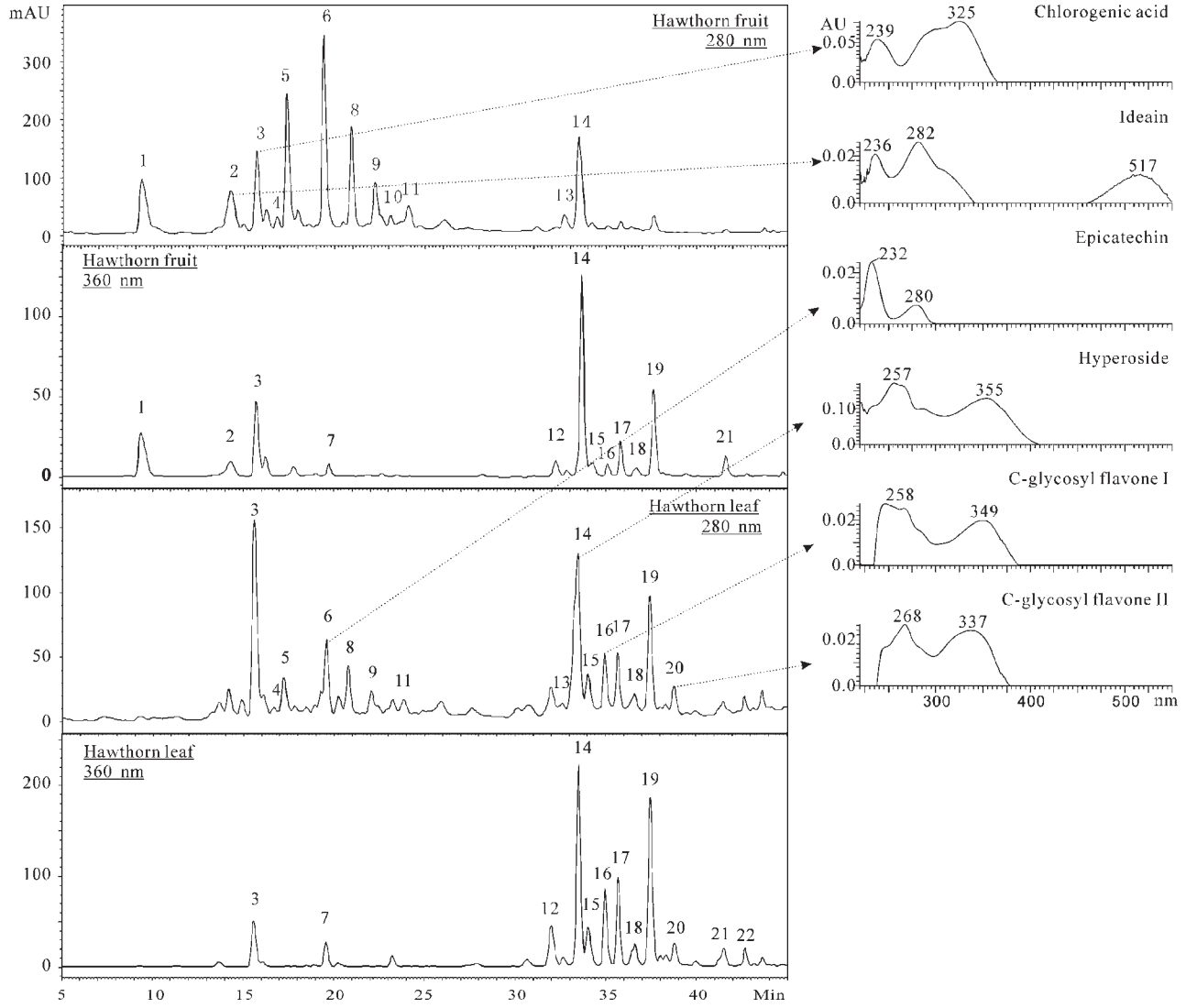

Figure 1. HPLC-DAD chromatograms of methanol extracts of fruits and leaves of *C. grayana* at 280 and 360 nm and typical UV spectra of major compounds. The UV spectrum of chlorogenic acid represents spectra of hydroxycinnamic acid derivatives, epicatechin procyanidins, ideain anthocyanidins, hyperoside flavonol glycosides, *C*-glycosyl flavone I *C*-glycosyl derivatives of luteolin, and *C*-glycosyl flavone II derivatives of apigenin.

by comparing the HPLC chromatograms with those in the literature.[9,15,20] Compounds **4**, **8**, and **10** showed $[M + H]^+$ ions at *m/z* 867 in the mass spectra and were identified as B-type procyanidin trimers (PC trimers I, II, and III). Compound **8** was preliminarily identified as PC C1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] on the basis of an earlier report.[9,15,20] Compounds **9** and **11** were identified as B-type procyanidin tetramers.[13,21]

Flavonol Glycosides. In the current study, seven compounds were identified as flavonol glycosides according to their UV spectra. The mass spectra suggested that compounds **12**, **14**, **17**, **19**, and **21** were derivatives of quercetin and compounds **18** and **22** were derivatives of kaempferol. Their mass spectra are presented in Figure 3.

Flavonol glycosides commonly exhibit strong peaks corresponding to the protonated aglycon moieties in ESI-MS spectra in the positive ion mode.[13,22] In the mass spectra of compounds **12**, **14**, **17**, **19**, and **21**, strong peaks at *m/z* 303 were detected. The ions were evidently derived from the aglycon quercetin.

The mass spectrum of compound **12** showed a $[M + H]^+$ ion at *m/z* 611. This compound was found also in *C. pinnatifida* var. *major* and was identified as a diglycoside (with a hexose and a methylpentose, 162 + 146 amu) of quercetin on the basis of its fragment ions (Figure 3A).[13] The compound had the same molecular weight as rutin (quercetin-3-*O*-rhamnosylglucoside) but a different retention time in HPLC analysis. In a previous study, both rutin and its isomer quercetin-3-*O*-rhamnosylgalactoside have been found in various hawthorn species.[23] There is, however, a lack of reference compounds, and compound **12** was tentatively identified as quercetin-rhamnosylhexoside.

Compound **14** was identified as hyperoside based on the reference compound (Figure 3B).

Compounds **17** and **19** showed similar mass spectra. The difference of 132 amu between the $[M + H]^+$ ion and the protonated aglycon moieties suggested the compounds to be quercetin-pentosides (Figure 3C). A compound with the structure of quercetin-3′-*O*-arabinoside has been reported in hawthorn,[23] but there is no commercial reference compound available; yet there

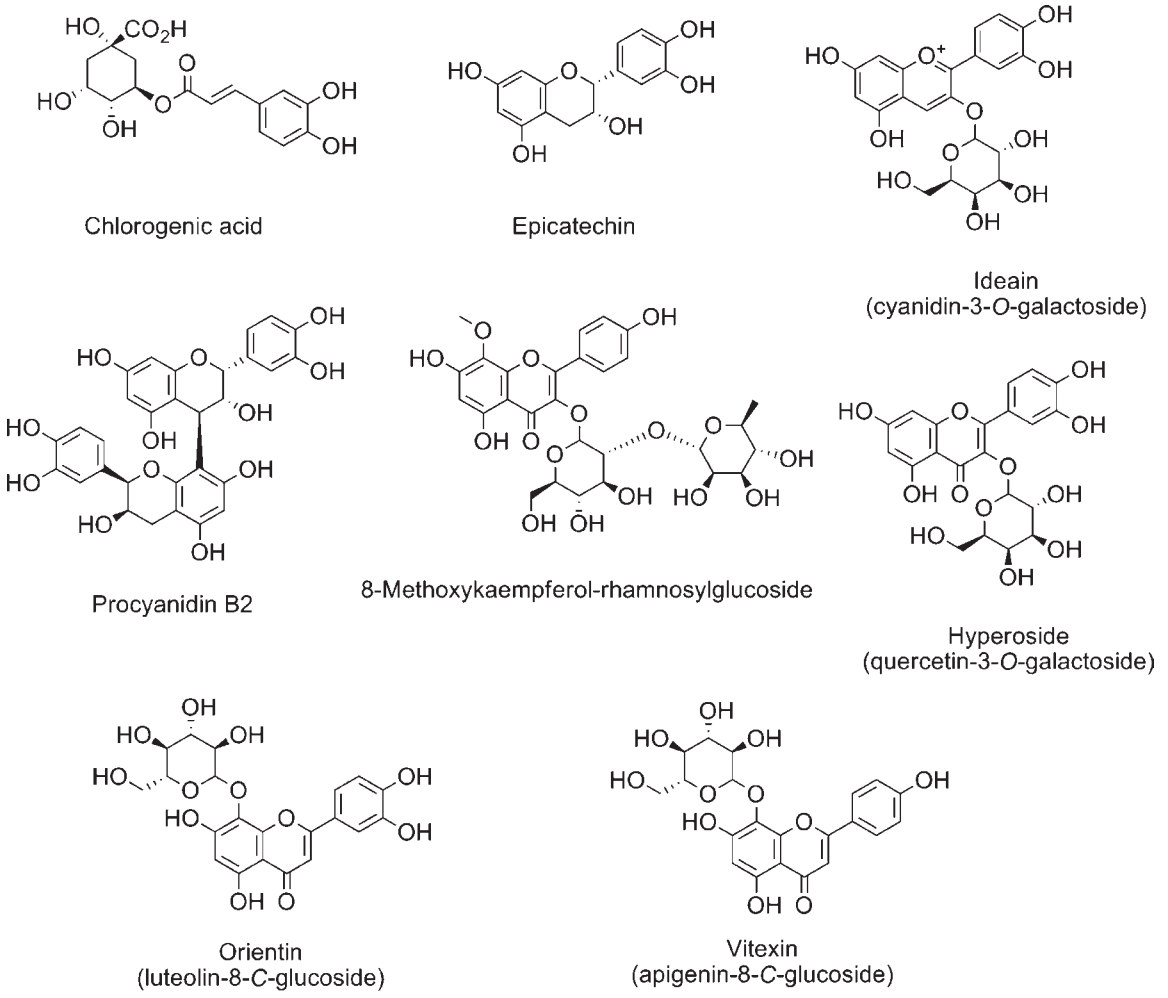

Figure 2. Representative structures of different groups of phenolic compounds found in hawthorn.

are also two quercetin-pentosides detected in fruits and leaves of *C. grayana*. Thus, it is not possible to decide the more specific structure.

The $[M + H]^+$ ion at m/z 507 was found in the mass spectrum of compound **21**. The molecular weight suggested that it may be a quercetin-hexoside acetate, and the ion at m/z 477 probably represents the moiety produced by loss of the acetate group (Figure 3D).

In the mass spectra of compounds **18** and **22**, peaks at m/z 287 and 317 suggested the compounds to be derivatives of methoxykaempferol. The ions at m/z 287 are likely derived from the moieties of kaempferol and those at m/z 317 from methoxykaempferol. The difference of 146 amu between the $[M + H]^+$ ion peak of compound **18** and the fragment ion at m/z 479 suggested the existence of a methylpentose and the difference of 162 amu, between m/z 479 and 317 suggested the existence of a hexose. Thus, compound **18** was preliminarily identified as a methoxykaempferol-methylpentosylhexoside (Figure 3E). One 8-methoxykaempferol-rhamnosylglucoside has been identified in hawthorn earlier,[23] and the molecular weight and the mass spectrum match those of compound **18** very well. Therefore, compound **18** may be the same compound as reported. The $[M + H]^+$ peak of compound **22** is 132 amu higher than m/z 317. This suggests that compound **22** might be a pentoside of methoxykaempferol (Figure 3F).

C-Glycosyl Flavones. Three compounds were identified as *C*-glycosyl flavones in chromatograms of methanol extracts of both fruits and leaves of *C. grayana*. Unlike in the mass spectra of flavonols, in the mass spectra of *C*-glycosyl flavones, $[M + H]^+$ ions are the typically base peaks, and there is normally a lack of fragment ions.

Compound **15** was tentatively identified as luteolin-*C*-hexoside. Its UV and mass spectra were similar to those of orientin (luteolin-8-*C*-glucoside) and isoorientin (luteolin-6-*C*-glucoside) reported earlier.[24] The $[M + H]^+$ ion at m/z 449 is also in agreement with the molecular weight (Figure 3G). Both orientin and isoorientin have been reported in other hawthorn species such as *C. pentagyna*.[23,25] Because of the lack of reference compounds in our study, it is impossible to decide the extract structure of compound **15**. Compound **16** had a UV spectrum quite similar to that of compound **15**. This suggests that compound **16** may be a derivative of luteolin-*C*-hexoside (Figure 3H). The m/z value of the $[M + H]^+$ ion 463 was 14 amu higher than those of orientin/isoorientin, indicating the presence of an additional methyl group in the molecule.

Compound **20** had UV and mass spectra similar to those of the reference compounds vitexin (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside) (Figure 3J). This suggests compound **20** to be a *C*-hexoside of apigenin. The retention time was, however, different from that of both vitexin and isovitexin. The compound is identified as an apigenin-*C*-hexoside.

Contents of Phenolic Compounds in Fruits and Leaves and Changes during Fruit Ripening. In the current study,

Table 1. Phenolics Determined by HPLC-DAD and Positive Ion HPLC-ESI-MS in the Methanol Extracts of Hawthorn Fruits and Leaves

peak[a]	plant part	t_R(min)	λ_{max}[b](nm)	$[M + H]^+(m/z)$	other ions in mass spectra(m/z)	identification/tentative identification [c]	method of identification[d]
1	fruits	10.30	231, 324, 288sh	355	377, 711	5-*O*-caffeoylquinic acid	UV, MS, L
2	fruits	13.76	236, 282, 517	449	287, 433, 435	ideain	R, UV, MS
3	fruits and leaves	15.44	239, 325, 288sh	355	377, 711	chlorogenic acid (3-*O*-caffeoylquinic acid)	R, UV, MS
4	fruits and leaves	16.57	242, 276	867	425, 649, 805	B-type PC trimer I	UV, MS
5	fruits and leaves	17.03	239, 277	579	289, 601	B-type PC dimer I (PC B2)	R, UV, MS
6	fruits and leaves	18.91	237, 279	291	581, 871	epicatechin	R, UV, MS
7	fruits and leaves	19.28	243, 328			unknown hydroxycinnamic acid derivative	UV
8	fruits and leaves	20.50	243, 277	867	476, 579	B-type PC trimer II (PC C1)	UV, MS, L
9	fruits and leaves	22.28	246, 277	1156	1178	B-type PC tetramer	UV, MS
10	fruits and leaves	23.21	242, 273	867	476, 579	B-PC trimer III	UV, MS
11	fruits and leaves	24.52	245, 276	1156	1178	B-type PC tetramer	UV, MS
12	fruits and leaves	32.10	257, 355, 266sh	611	303, 465, 633	quercetin-rhamnosylhexoside	UV, MS
13	fruits and leaves	32.29	239, 277	579	289, 601	B-type PC dimer II (PC B5)	UV, MS, L
14	fruits and leaves	33.04	257, 354, 266sh	465	303, 487	hyperoside	R, UV, MS
15	fruits and leaves	33.32	258, 349	449		luteolin-*C*-hexoside	UV, MS
16	fruits and leaves	34.07	253, 349	463		methyl luteolin-*C*-hexoside	UV, MS
17	fruits and leaves	34.91	256, 355, 266sh	435	303, 457	quercetin-pentoside I	UV, MS
18	fruits and leaves	36.00	250, 353, 266sh	625	287, 317, 479, 647	methoxykaempferol-methylpentosylhexoside	UV, MS
19	fruits and leaves	37.06	256, 354, 266sh	435	303, 457	quercetin-pentoside II	UV, MS
20	leaves	38.37	268, 337	433		apigenin-*C*-hexoside	UV, MS
21	fruits and leaves	40.81	254, 353, 266sh	507	303, 477, 507, 529	quercetin-hexoside acetate	UV, MS
22	leaves	42.05	266, 353, 254sh	449	287, 317, 419	methoxykaempferol-pentoside	UV, MS

[a] The peak numbers correspond to the peak labels in Figure 1. [b] sh, shoulder. [c] Compounds identified without the aid of reference compounds are tentatively identified. [d] R, reference compounds; UV, UV spectrum; MS, mass spectrum; L, literature.

samples of fruits and leaves of *C. grayana* were picked during the fruit ripening season (August 11−October 5) at 1-week intervals. Thirteen major phenolic compounds in fruits and 15 in leaves were analyzed quantitatively by the HPLC-MS-ESI-SIR method. Changing trends in the contents of the compounds are described below. Some minor phenolics were also found in the samples, but the contents were too low to be quantified.

Fruits. Procyanidins were the major phenolic compounds in the fruits of *C. grayana*. The contents of total procyanidins varied from 6 to 17 mg/g DM during fruit ripening. Epicatechin, PC B2, and PC trimer II (tentatively identified as PC C1) were the most abundant procyanidins. Compared with the contents of procyanidins, those of flavonol glycosides were quite low (1−1.5 mg/g DM). Hyperoside and quercetin-pentoside II were the major flavonol glyosides. Chlorogenic acid was present at levels of 0.5−1 mg/g DM and its isomer 5-*O*-caffeoylquinic acid at 0.2−0.5 mg/g DM. The level of ideain in the fruits increased from 0.2 to 3 mg/g DM during the period. The contents of *C*-glycosyl flavones in the fruits were too low to be quantified.

Figure 4A presents the changing pattern of procyanidin levels in fruits of *C. grayana*. All of these compounds showed quite similar changing trends in the fruit ripening period. The highest contents were typically found in the fruits collected on August 19 for the procyanidin compounds. From mid August to early September, the content of procyanidins was at a high level (15−17 mg/g DM in total). From the beginning to the end of September, the levels of procyanidins decreased dramatically. By the beginning of October, the total content of procyanidins was up to 10 mg/g DM again.

Figure 4B shows the changing trends in the contents of the four derivatives of quercetin in the fruits of *C. grayana*. The changing patterns were all analogous, increasing slowly from August 11 to 27 and then decreasing for the rest of the period. The highest contents were detected in fruits collected on August 27.

Changes in the two caffeoylquinic acids in fruits of *C. grayana* are presented in Figure 4C. Chlorogenic acid (3-*O*-caffeoylquinic acid) increased from August to early September and then slightly decreased during the following month. In the end, it reached a level above 1 mg/g DM by October 5. The content of its isomer (5-*O*-caffeoylquinic acid) decreased during the whole period.

The content of ideain in the fruits increased continuously from 0.1 to 3 mg/g DM during the whole period (Figure 4D).

On the basis of the color and texture, the berries of *C. grayana* used in the current study were optimally ripe at mid September. The contents of phenolic compounds of *C. grayana* except ideain reached highest levels by the end of August in the fruits. Similar phenomena were reported earlier with Chinese hawthorn and other fruits.[26,27] Procyanidins and flavonols are typically at highest levels in unripe fruits and decrease during fruit ripening. The presence of phenolics in unripe fruits could provide protection against premature feeding due to the astringent taste of phenolics.[26] The enzymes involved in flavonoid biosynthesis include phenylalanine ammonia-lyase (PAL), dihydroflavonol 4-reductase (DFR), chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), flavonol synthase (FLS), flavonoid 7-*O*-glucosyltransferase (F7GT), and flavonoid 3-*O*-glucosyltransferase (F3GT). PAL and CHS/CHI are the rate-limiting enzymes in the formation of C6−C3−C6 structure.

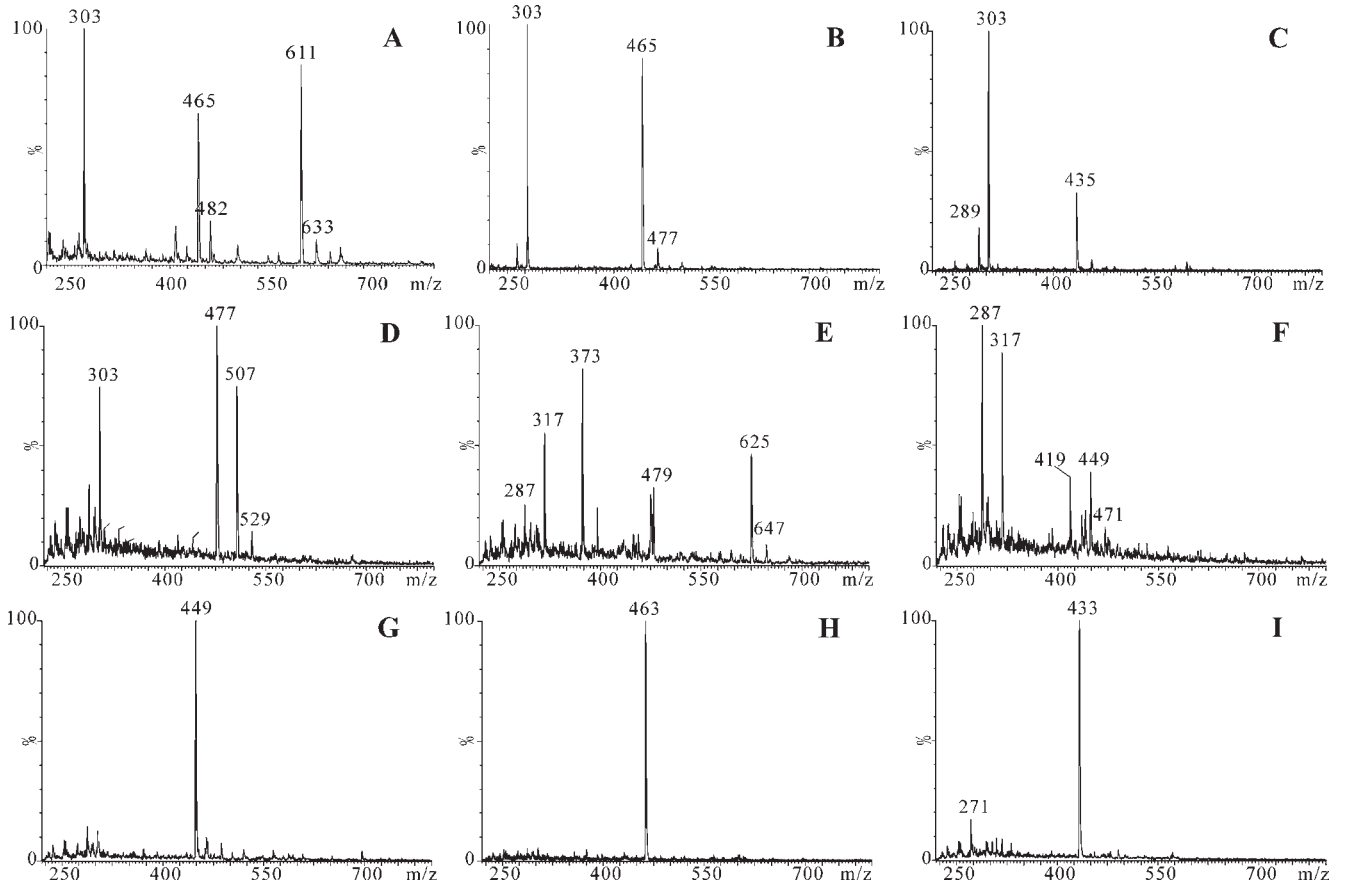

Figure 3. Mass spectra of flavonol glycosides and *C*-glycosyl flavones in methanol extracts of hawthorn fruits and leaves: (A) peak 12, quercetin-rhamnosylhexoside; (B) peak 14, hyperoside; (C) peaks 17 and 19, quercetin-pentoside; (D) peak 21, quercetin-hexoside acetate; (E) peak 18, methoxykaempferol-methylpentosylhexoside; (F) peak 22, methoxykaempferol-pentoside; (G) peak 15, luteolin-*C*-hexoside; (H) peak 16, methyl luteolin-*C*-hexoside; (I) peak 20, apigenin-*C*-hexoside. The isotopic peaks were combined by MassLynx 4.1. The peaks are numbered as in Figure 1.

PAL is a key enzyme located at the interface between primary and secondary metabolism. It catalyzes the conversion of the phenylalanine into cinnamic acid. After that, the cinnamic acid is converted to naringenin (a chalcone) by the catalysis of CHS/CHI. In strawberry, there are two distinct activity peaks of the key flavonoid enzymes at early and late fruit ripening, respectively.[28] This two-phase flavonoid biosynthesis has also been observed in grapes.[29] Changes in the contents of phenolics in fruits of *C. grayana* can also be explained by this two-phase mechanism. The increase in the content of procyanidins and chlorogenic acid in October may have been caused by a second activity peak of the enzymes.

Leaves. The total procyanidin content in the leaves ranged from 5.4 to 30 mg/g DM. As in the fruits, epicatechin, PC B2, and PC trimer II were the major procyanidins. The contents of total flavonol glycosides varied in the range of 7−21 mg/g DM during autumn. Hyperoside was the most abundant flavonol glycoside. The total *C*-glycosyl flavone contents varied from 2 to 5 mg/g DM. The contents of chlorogenic acid in the leaves varied from 3 to 11 mg/g DM.

Figure 5A shows the variation in the levels of procyanidins in hawthorn leaves. The contents of the compounds remained stable in August and increased progressively in September until reaching the highest levels by the end of the month (30 mg/g DM) and decreased after that.

Panels B and C of Figure 5 present the changing patterns in the content of quercetin derivatives and *C*-glycosyl flavones. The curves are quite similar, remaining constant in August, increasing sharply by 2−3 times in early September, and then increasing slowly. The changing trend in the content of chlorogenic acid in leaves (Figure 5D) is close to that of procyanidins.

The accumulation of phenolics in leaves is commonly suggested to be sensitive to the environment. A negative correlation has been found between contents of quercetin derivatives in tomato leaves and growth temperature.[30] The increase in the contents of phenolic compounds in hawthorn leaves during September may be a response to a drop in temperature.

Comparison of Phenolic Compounds between Fruits and Leaves of *C. grayana*. In the current study, 22 major phenolics were detected and identified, some of them tentatively. The compositions of the major procyanidins of the fruits and leaves of *C. grayana* were almost identical. Epicatechin, PC B2, PC B5, PC trimer I, PC trimer II (tentatively identified as PC C1), PC trimer III, and two PC pentamers were the major procyanidins in both fruits and leaves.

Hyperoside, a quercetin-3-*O*-rhamnosylhexoside, two quercetin-pentosides, a quercetin-hexoside acetate, a methoxykaempferol-methylpentosylhexoside, a luteolin-*C*-hexoside, and a methyl luteolin-*C*-hexoside were found in both fruits and leaves, but the contents of luteolin-*C*-hexoside and the methyl luteolin-*C*-hexoside were very low in the fruits. The apigenin-*C*-hexoside and methoxykaempferol-pentoside were found in the leaves, but not in the fruits.

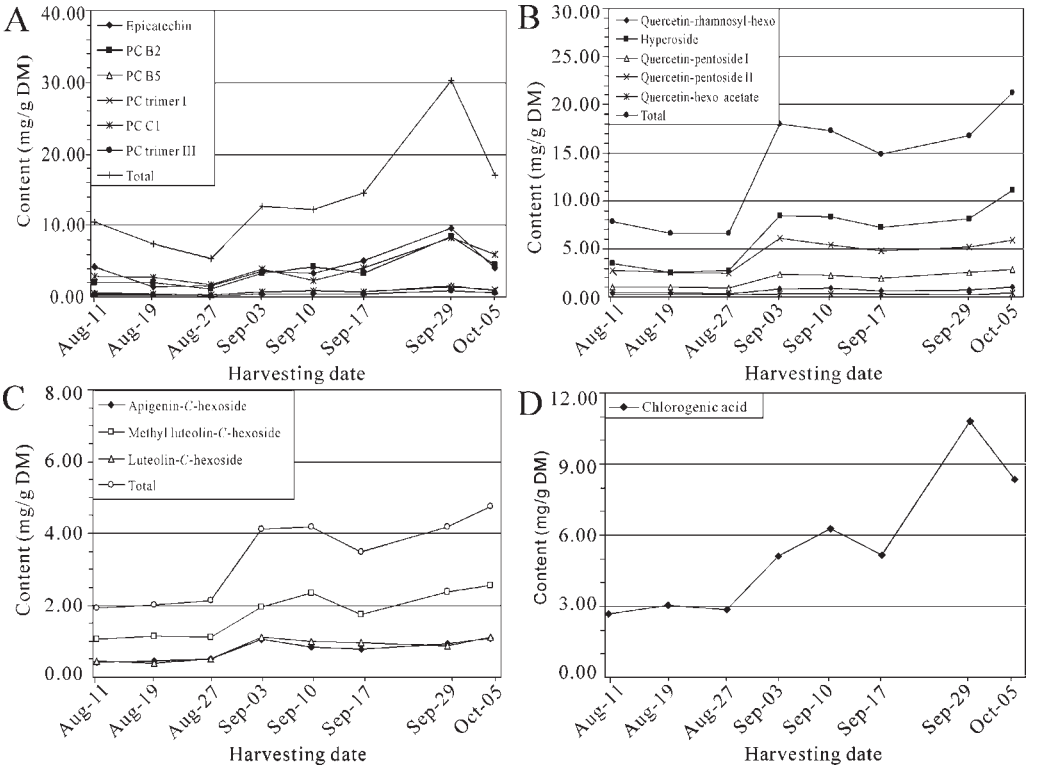

Figure 4. Changes in the content of phenolics in fruits of *C. grayana* during fruit ripening season: (A) procyanidins; (B) derivatives of quercetin; (C) caffeoylquinic acids; (D) ideain. hexo, hexoside.

Ideain was found in the fruits only, being the main red pigment of the peel and flesh of hawthorn fruits. 5-*O*-Caffeoylquinic acid was also found only in the fruits. Its isomer, chlorogenic acid (3-*O*-caffeoylquinic acid), was present in both fruits and leaves.

The contents of phenolics of *C. grayana* reached highest levels between the middle of August and the beginning of September in the fruits but between the end of September and the beginning of October in the leaves.

Difference in Phenolics of *C. grayana* and Other Hawthorn Species. Systematic analysis of phenolic compounds in the 80% aqueous ethanol extracts of Chinese hawthorn fruits has been carried out in our previous studies.[13] Forty-two phenolic compounds were identified by HPLC-DAD and HPLC-ESI-MS in the fruits of *C. pinnatifida* var. *major*. The major phenolic compounds in the fruits of three species (*C. pinnatifida*, *C. brettschneideri*, and *C. scabrifolia*) and a variety of *C. pinnatifida* (var. *major*) of Chinese hawthorn were quantified by HPLC-ESI-MS-SIR.[16]

Epicatechin and B-type procyanidins with degrees of polymerizations (DP) of <4 have been found to be the major procyanidins in Chinese hawthorns. The profile of procyanidins in the fruits of *C. grayana* is very similar to that of the Chinese species. The total content of procyanidins in the fruits of Chinese hawthorn varied from 3 to 37 mg/g DM. The level of procyanidins in optimally ripe fruits of *C. grayana* (9 mg/g DM) was close to the levels in *C. pinnatifida* (9 mg/g DM) and *C. brettschneideri* (8 mg/g DM) but lower than those in *C. pinnatifida* var. *major* (18 mg/g DM) and *C. scabrifolia* (20 mg/g DM).[16]

Procyanidin glycosides were detected in the fruits of some Chinese hawthorn species. Especially in the fruits of *C. scabrifolia* and *C. pinnatifida* var. *major*, the content of a procyanidin dimer hexoside was even higher than 1 mg/g DM. The content was very low in *C. brettschneideri* (0.04 mg/g DM).[16] In the extract of fruits of *C. grayana*, no procyanidin glycosides were found. Also, procyanidin glycosides were absent in the aqueous ethanol extract of fruits of *C. pinnatifida*.[16] The contents of procyanidin glycosides in fruits may be used as chemotaxonomic markers for distinguishing the species/varieties of hawthorn.

The main difference between the fruits of Chinese hawthorn species and those of *C. grayana* was in the composition of flavonols and *C*-glycosyl flavones. The two major flavonol glycosides in the fruits of Chinese hawthorn were hyperoside (0.1−0.8 mg/g DM) and isoquercitrin (0.1−0.3 mg/g DM). In the extracts of fruits of *C. grayana*, hyperoside was also a predominating flavonol glycoside (0.9 mg/g DM), but isoquercitrin was not detected at all. Instead, a quercetin-pentoside (quercetin-pentoside II, 0.5 mg/g DM) was the second most abundant flavonol glycoside. This compound was not present in Chinese hawthorn. Quercetin-rhamnosylhexoside, quercetin-pentoside I, a quercetin-hexoside acetate, and a methoxykaempferol-methylpentosylhexoside
were the minor flavonol compounds in *C. grayana*. Quercetin-(dirhamnosyl)hexoside was a minor compound in Chinese hawthorns but not found in the fruits of *C. grayana*. The situation was vice versa with quercetin-pentoside I, quercetin-hexoside acetate, and methoxykaempferol-methylpentosylhexoside. The total content of flavonols in Chinese hawthorn fruits (0.2−1 mg/g DM) was lower than or close to the content in the optimally ripe fruits of *C. grayana* (1 mg/g DM).

Two *C*-glycosyl flavones, luteolin-*C*-hexoside and methyl luteolin-*C*-hexoside, were found in the fruits of *C. grayana*, whereas no *C*-glycosyl flavones were found in Chinese hawthorn species. In methanol extracts of *C. grayana* fruits, two caffeoylquinic acids (chlorogenic acid and 5-*O*-caffeoylquinic acid) and

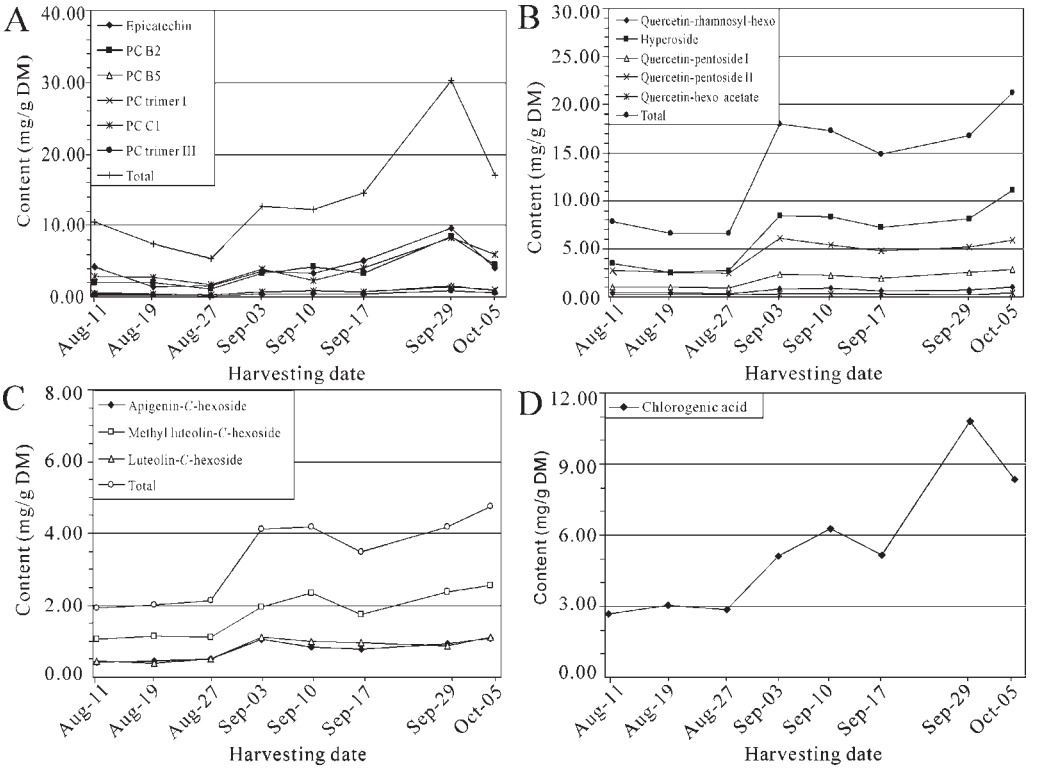

Figure 5. Changes in the content of phenolics in leaves of *C. grayana* during fruit ripening season: (A) procyanidins; (B) derivatives of quercetin; (C) *C*-glycosyl flavones; (D) chlorogenic acid. hexo, hexoside.

an unknown hydroxycinnamic acid were detected, but only chlorogenic acid was found in the aqueous ethanol extract of fruits of Chinese hawthorn species. Ideain was found in all species except *C. scabrifolia*.

Other reports of the composition profile of phenolics in European hawthorn species also suggested that the flavonoids were the major markers for the differentiation of *C. monogyna*, *C. laevigata*, and *C. pentagyna* from one another.[3] It was found that isoorientin, orientin, isoorientin-2″-*O*-rhamnoside, orientin-2″-*O*-rhamnoside, isovitexin-2″-*O*-rhamnoside, and methoxykaempferol-3-*O*-glucoside were detected only in leaves of *C. pentagyna*. 4‴-Acetylvitexin-2⁗-*O*-rhamnoside, a major *C*-glycosyl flavone in leaves of *C. monogyna*, was not found in leaves of *C. pentagyna* and *C. laevigata*.[3]

The composition of phenolics in the leaves of *C. grayana* was very different from those of other European species reported earlier.[3] In the methanol extract of leaves of *C. grayana*, vitexin-2″-*O*-rhamnoside, vitexin, isovitexin, rutin, isoquercitrin, isoorientin-2″-*O*-rhamnoside, orientin-2″-*O*-rhamnoside, isovitexin-2″-*O*-rhamnoside methoxykaempferol-3-*O*-glucoside, and 4‴-acetylvitexin-2″-*O*-rhamnoside were not detected. However, the existence of methyl luteolin-*C*-hexoside, methoxykaempferol-pentoside, and the isomer of vitexin/isovitexin in *C. monogyna*, *C. laevigata*, and *C. pentagyna* has not been reported.

This study characterized phenolics in the methanol extracts of *C. grayana* fruits and leaves. Twenty-two phenolic compounds were found. Ideain (cyanidin-3-*O*-galactoside), chlorogenic acid, PC B2 [epicatechin-(4β→8)-epicatechin], epicatechin, and hyperoside (quercetin-3-*O*-galactoside) were identified using UV spectra, mass spectra, and reference compounds. In addition, 17 compounds were tentatively identified on the basis of the UV and mass spectra. These compounds belong to B-type procyanidins, flavonol glycosides, *C*-glycosyl flavones, and hydroxycinnamic acids. It was also found that the compositional profiles of phenolic compounds of fruits and leaves were different. The relative contents of procyanidins of fruits were higher than those in leaves. The contents of phenolics reached highest levels between the middle of August and the beginning of September in the fruits and between the end of September and the beginning of October in the leaves. Compared with the Chinese species, the composition of procyanidins was similar, but those of flavonol glycoside and *C*-glycosyl flavones were different in the fruits of *C. grayana*. This is the first systematic report of the composition and content of phenolic compounds in the fruits and leaves of *C. grayana*.

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REFERENCES

(1) Fong, H. H. S.; Bauman, J. L. Hawthorn. *J. Cardiovasc. Nurs.* **2002**, *16*, 1–8.

(2) Zhao, H. C.; Tian, B. F. *China Fruit − Plant Monograph, Hawthorn Flora*; Zhongguo Linye Press: Beijing, China, 1996; pp 1−27.

(3) Prinz, S.; Ringl, A.; Huefner, A.; Pemp, E.; Kopp, B. 4‴-Acetylvitexin-2″-*O*-rhamnoside, isoorientin, orientin, and 8-methoxy-kaempferol-3-*O*-glucoside as markers for the differentiation of *Crataegus monogyna* and *Crataegus pentagyna* from *Crataegus laevigata* (Rosaceae). *Chem. Biodiversity* **2007**, *4*, 2920–2931.

(4) Elango, C.; Devaraj, S. N. Immunomodulatory effect of hawthorn extract in an experimental stroke model. *J. Neuroinflammation* **2010**, *7*, 97.

(5) Akila, M.; Devaraj, H. Synergistic effect of tincture of *Crataegus* and *Mangifera indica* L. extract on hyperlipidemic and antioxidant status in atherogenic rats. *Vasc. Pharmacol.* **2008**, *49*, 173–177.

(6) Walker, A. F.; Marakis, G.; Simpson, E.; Hope, J. L.; Robinson, P. A.; Hassanein, M.; Simpson, H. C. R. Hypotensive effects of hawthorn for patients with diabetes taking prescription drugs: a randomised controlled trial. *Br. J. Gen. Pract.* **2006**, *56*, 437–443.

(7) Pittler, M. H.; Schmidt, K.; Ernst, E. Hawthorn extract for treating chronic heart failure: meta-analysis of randomized trials. *Am. J. Med.* **2003**, *114*, 665–674.

(8) Elango, C.; Jayachandaran, K. S.; Niranjali Devaraj, S. Hawthorn extract reduces infarct volume and improves neurological score by reducing oxidative stress in rat brain following middle cerebral artery occlusion. *Int. J. Dev. Neurosci.* **2009**, *27*, 799–803.

(9) Svedström, U.; Vuorela, H.; Kostiainen, R.; Tuominen, J.; Kokkonen, J.; Rauha, J.; Laakso, I.; Hiltunen, R. Isolation and identification of oligomeric procyanidins from *Crataegus* leaves and flowers. *Phytochemistry* **2002**, *60*, 821–825.

(10) Svedström, U.; Vuorela, H.; Kostiainen, R.; Laakso, I.; Hiltunen, R. Fractionation of polyphenols in hawthorn into polymeric procyanidins, phenolic acids and flavonoids prior to high-performance liquid chromatographic analysis. *J. Chromatogr., A* **2006**, *1112*, 103–111.

(11) Barros, L.; Carvalho, A. M.; Ferreira, I. C. Comparing the composition and bioactivity of *Crataegus monogyna* flowers and fruits used in folk medicine. *Phytochem. Anal.* **2010**, *22*, 181–188.

(12) Urbonaviciūte, A.; Jakstas, V.; Kornysova, O.; Janulis, V.; Maruska, A. Capillary electrophoretic analysis of flavonoids in single-styled hawthorn (*Crataegus monogyna* Jacq.) ethanolic extracts. *J. Chromatogr., A* **2006**, *1112*, 339–344.

(13) Liu, P.; Yang, B.; Kallio, H. Characterization of phenolic compounds in Chinese hawthorn (*Crataegus pinnatifida* Bge. var. *major*) fruit by high performance liquid chromatography−electrospray ionization mass spectrometry. *Food Chem.* **2010**, *121*, 1188–1197.

(14) Liu, W.; Chen, G.; Cui, T. Determination of flavones in *Crataegus pinnatifida* by capillary zone electrophoresis. *J. Chromatogr. Sci.* **2003**, *41*, 87–91.

(15) Cui, T.; Nakamura, K.; Tian, S.; Kayahara, H.; Tian, Y. Polyphenolic content and physiological activities of Chinese hawthorn extracts. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 2948–2956.

(16) Liu, P.; Kallio, H.; Lü, D.; Zhou, C.; Yang, B. Quantitative analysis of phenolic compounds in Chinese hawthorn (*Crataegus* spp.) fruits by high performance liquid chromatography−electrospray ionisation mass spectrometry. *Food Chem.* **2011**, *127*, 1370–1377.

(17) Lin, L. Z.; Harnly, J. M. A screening method for the identification of glycosylated flavonoids and other phenolic compounds using a standard analytical approach for all plant materials. *J. Agric. Food Chem.* **2007**, *55*, 1084–1096.

(18) Salminen, J. P.; Karonen, M.; Lempa, K.; Liimatainen, J.; Sinkkonen, J.; Lukkarinen, M.; Pihlaja, K. Characterisation of proanthocyanidin aglycones and glycosides from rose hips by high-performance liquid chromatography-mass spectrometry, and their rapid quantification together with vitamin C. *J. Chromatogr., A* **2005**, *1077*, 170–180.

(19) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513–7521.

(20) Svedström, U.; Vuorela, H.; Kostiainen, R.; Huovinen, K.; Laakso, I.; Hiltunen, R. High-performance liquid chromatographic determination of oligomeric procyanidins from dimers up to the hexamer in hawthorn. *J. Chromatogr., A* **2002**, *968*, 53–60.

(21) Karonen, M.; Leikas, A.; Loponen, J.; Sinkkonen, J.; Ossipov, V.; Pihlaja, K. Reversed-phase HPLC-ESI/MS analysis of birch leaf proanthocyanidins after their acidic degradation in the presence of nucleophiles. *Phytochem. Anal.* **2007**, *18*, 378–386.

(22) Polasek, J.; Queiroz, E. F.; Hostettmann, K. On-line identification of phenolic compounds of *Trifolium* species using HPLC-UV-MS and post-column UV-derivatisation. *Phytochem. Anal.* **2007**, *18*, 13–23.

(23) Petereit, F.; Nahstedt, A. *Crataegus* from the analytical viewpoint. Official contents of hawthorn drugs (in German). *Pharm. Unserer Zeit* **2005**, *34*, 22–26.

(24) Rayyan, S.; Fossen, T; Andersen, M. Ø. Flavone *C*-glycosides from seeds of fenugreek, *Trigonella foenum-graecum* L. *J. Agric. Food Chem.* **2010**, *58*, 7211–7217.

(25) Lopez-Lazaro, M. Distribution and biological activities of the flavonoid luteolin. *Mini Rev. Med. Chem.* **2009**, *9*, 31–59.

(26) Jaakola, L.; Maatta, K.; Pirttila, A. M.; Torronen, R.; Karenlampi, S.; Hohtola, A. Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant Physiol.* **2002**, *130*, 729–739.

(27) Cui, T.; Li, J.; Kayahara, H.; Ma, L.; Wu, L.; Nakamura, K. Quantification of the polyphenols and triterpene acids in Chinese hawthorn fruit by high-performance liquid chromatography. *J. Agric. Food Chem.* **2006**, *54*, 4574–4581.

(28) Halbwirth, H.; Puhl, I.; Haas, U.; Jezik, K.; Treutter, D.; Stich, K. Two-phase flavonoid formation in developing strawberry (*Fragaria* × *ananassa*) fruit. *J. Agric. Food Chem.* **2006**, *54*, 1479–1485.

(29) Boss, P. K.; Davies, C.; Robinson, S. P. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv Shiraz grape berries and the implications for pathway regulation. *Plant Physiol.* **1996**, *111*, 1059–1066.

(30) Løvdal, T.; Olsen, K. M.; Slimestad, R.; Verheul, M.; Lillo, C. Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. *Phytochemistry* **2010**, *71*, 605–613.