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Quantification of the Polyphenols and Triterpene Acids in Chinese Hawthorn Fruit by High-Performance Liquid Chromatography

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The levels of seven polyphenols (epicatechin, procyanidin B2, procyanidin B5, procyanidin C1, hyperoside, isoquercitrin, and chlorogenic acid) and two triterpene acids (oleanolic acid and ursolic acid) in the matured fruits of Chinese hawthorn (*Crataegus pinnatifida* Bge. var. *major* N.E.Br.) were determined by high-performance liquid chromatography methods. The average contents of those constituents in 37 representative cultivars were 1405, 1505, 339, 684, 56, 41, 234, 952, and 147 μg/g fresh weight (FW), respectively. A significant inverse correlation between the procyanidin contents and the latitude of the geographical origin of the cultivars was observed ($r = 0.3851$, $P < 0.02$). Correlation analysis of the levels of the nine compounds in the 37 cultivars yielded a strong correlation ($P < 0.001$) between the individual levels of the four procyanidins and the sum of the procyanidins level ($r = 0.7413-0.9898$) and between the flavonoids and the chlorogenic acid ($r = 0.5383-0.9212$). The changes in level of the nine compounds in the hawthorn fruit were evaluated during maturation using the Hebei Dajinxing cultivar. Sixty-one days after blossom, the polyphenol level reached the highest point and the sum of the contents was 1.36 g/100 g FW.

KEYWORDS: HPLC; hawthorn; *Crataegus pinnatifida* Bge. var. *major* N. E. Br.; procyanidin, flavonoid; triterpene acid; chlorogenic acid

INTRODUCTION

In Europe, standard extracts of the leaves and flowers of *Crataegus oxyacantha* or *Crataegus monogyna* have been used for treating mild to moderate congestive heart failure (NYHA I−III) (*1*−*3*), and many clinical tests have shown that these preparations are beneficial, improving heart-related symptom complexes with less poisonous side effects (*4*, 5). Generally, flavonoids and procyanidins are considered to be the two main groups of active constituents in hawthorn extracts, and in many state pharmacopoeias, these two groups are used for standardization and quality control (*6*, 7). Some reports have shown that hawthorn extracts possess endothelium-dependent vasorelaxation effects (*8*, 9) and endothelin-1 inhibitory action (*10*). It is interesting that the procyanidin fraction isolated from a standard hawthorn extract presented remarkable vasorelaxation effects but that the flavonoid fraction did not show any such observable effects (*9*).

There are 18 species and six varieties of Chinese hawthorn, and its presence in the edible and medicinal history of China can be dated back to before 300 A.D. (*11*). During its long period of cultivation and domestication, Chinese hawthorn has come to be represented by the predominant species today, *Crataegus pinnatifida* Bge. var. *major* N.E.Br. Unlike European hawthorn, Chinese hawthorn is planted for its edible fruit, which is bigger and tastes better than its European relative. This popular fruit is utilized as a fresh, dried fruit, is utilized in jams, juices, and tinned foods, and is a basic ingredient for making wines and for various sweet foods. The largest planting areas in China are considered to be the most important hawthorn fruit sources in the world (*11*). The fruits of *C. pinnatifida* Bge. and *C. pinnatifida* Bge. var. *major* N.E.Br. are two of the major ingredients in both Chinese herbal medicinal products and functional foods (*12*). Despite its long history as an edible and medicinal fruit, reports on the active constituents of Chinese hawthorn fruit are limited in number (*1*). Inspired by the research results on European hawthorn, several researchers have analyzed the polyphenol (*13*−*16*) and triterpene acid (*17*, *18*) contents in Chinese hawthorn fruit and concluded that the flavonoids and triterpene acids may be the active constituents that determine its quality (*13*, *19*). However, details of polyphenols and triterpene acids in Chinese hawthorn, especially the information on the concentration of procyanidins and another important group of active constituents, as well as on the concentration

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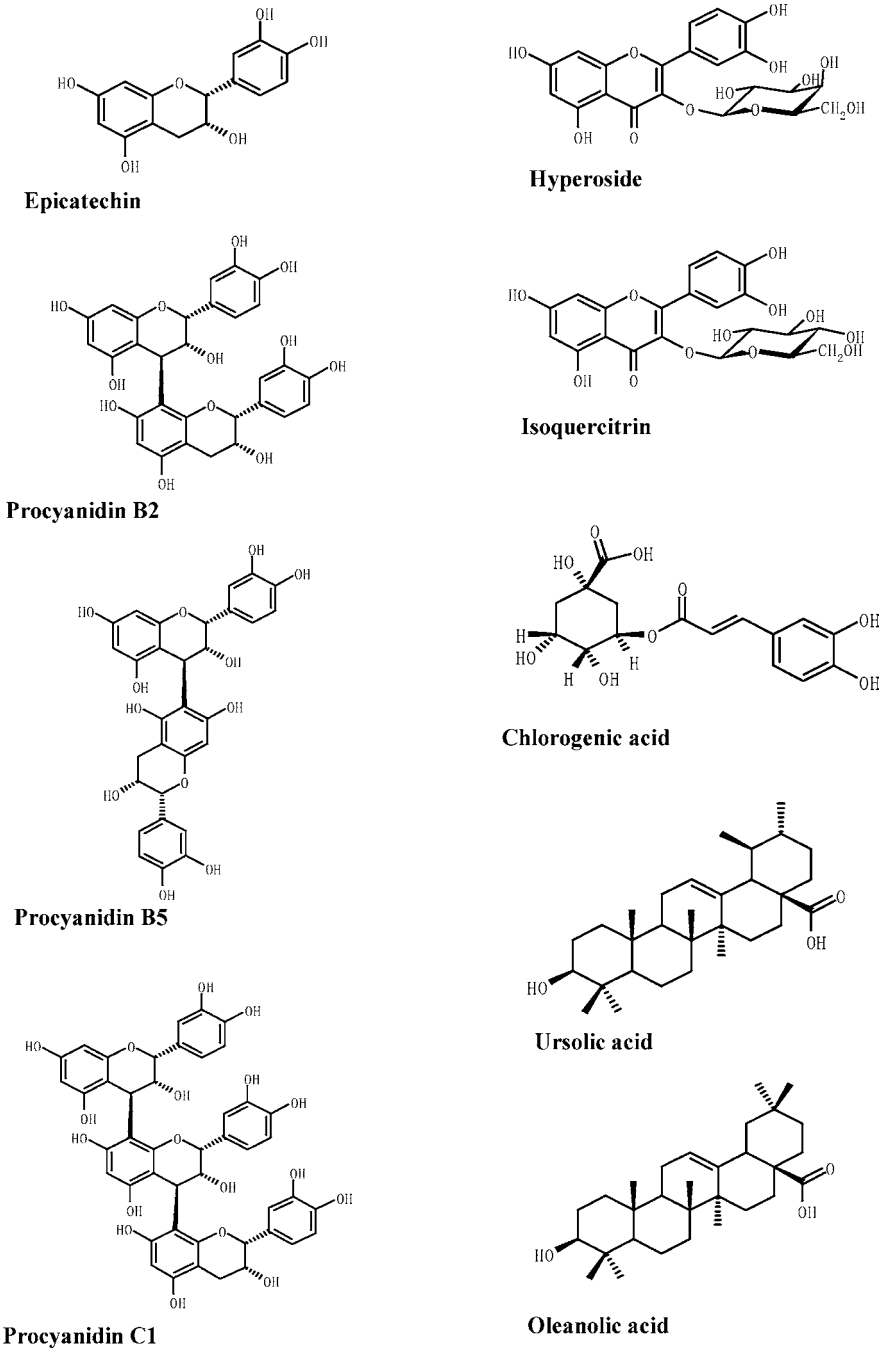

Figure 1. Chemical structures of the active constituents in Chinese hawthorn.

changes during fruit maturation, have not been obtained thus far. In the present work, we evaluated the levels of epicatechin, procyanidin B2, procyanidin B5, procyanidin C1, hyperoside, isoquercitrin, chlorogenic acid (3′-caffeoylquinic acid), ursolic acid, and oleanolic acid (**Figure 1**) in the matured fruits of 37 representative planted cultivars of Chinese hawthorn and determined the content changes of the polyphenols, the active components, during the fruit maturation using high-performance liquid chromatography (HPLC) methods.

MATERIALS AND METHODS

Plants. Thirty-seven representative cultivars of Chinese hawthorn (*C. pinnatifida* Bge. var. *major* N.E.Br.) were chosen for our comparative study of the contents of the active constituents. With the exception of the Hebei Dajinxing cultivar, all 37 cultivars were planted at the hawthorn nursery of the State Center of Fruit Tree Species in Shenyang City (Liaoning Province, China) and the sample fruits were picked on October 10, 2003. The Hebei Dajinxing cultivar was planted at the experimental farm of Hebei Agricultural University in Baoding (Hebei Province, China), and its fruits were picked on October 16, 2003. The cultivar names and their Chinese provinces of origin are listed in **Table 1**. For the analysis of the variations in the composition contents at different stages of fruit growth, the fruits of the cultivar Hebei Dajinxing were picked from the first day of full bloom (May 4) to the first day of maturity (October 16) at 3 week intervals.

Chemicals. Epicatechin, chlorogenic acid, oleanolic acid, and ursolic acid were purchased from Sigma Chemical Co. (St. Louis, MO); procyanidin B2, hyperoside, and isoquercitrin were purchased from Funakoshi Co. Ltd. (Tokyo, Japan); procyanidins B5 and C1 were isolated and purified from the fruits of *C. pinnatifida* Bge. var. *major* N.E.Br using the methods previously reported (*20*−*22*) and were identified by electrospray ionization mass spectrometry (ESI-MS) and the thiolysis method (*20*). Procyanidins B5 generated the MS peaks with the *m*/*z* as 579 [$(M + H)^+$] and 601 [$(M + Na)^+$]. By thiolysis,

Table 1. Contents[a] of Procyanidins in Chinese Hawthorn Fruit (μg/g FW)

cultivar	origin	epicatechin	procyanidin B2	procyanidin B5	procyanidin C1	TPC[b]
725	Jilin	1030 ± 148	1173 ± 60	289 ± 19	469 ± 25	2961 ± 223
Benxi-2	Liaoning	1564 ± 87	1628 ± 286	409 ± 69	633 ± 27	4234 ± 381
Shanchengzi-2	Liaoning	1461 ± 248	1530 ± 363	466 ± 146	640 ± 81	4097 ± 837
Sishanling	Liaoning	1775 ± 167	1971 ± 158	490 ± 13	720 ± 110	4957 ± 331
Benxi-7	Liaoning	1108 ± 106	1117 ± 215	234 ± 44	459 ± 50	2918 ± 403
Benxi-4	Liaoning	1234 ± 159	1470 ± 119	421 ± 10	642 ± 99	3763 ± 62
Shanchengzi-1	Liaoning	1236 ± 69	1584 ± 207	456 ± 21	648 ± 41	3924 ± 231
Niuxintai-1	Liaoning	1421 ± 23	1523 ± 81	312 ± 64	639 ± 16	3895 ± 112
Xihong	Liaoning	1086 ± 24	1133 ± 25	230 ± 14	482 ± 41	2929 ± 16
Liaohong	Liaoning	1539 ± 37	1420 ± 206	339 ± 70	679 ± 55	3977 ± 321
Anshan Dajinxing	Liaoning	695 ± 40	712 ± 57	204 ± 63	339 ± 56	1950 ± 56
Mopanshanzha	Liaoning	1037 ± 230	1189 ± 257	245 ± 36	581 ± 125	3051 ± 568
Yinyeling-7	Liaoning	1313 ± 207	1519 ± 273	344 ± 16	754 ± 119	3929 ± 576
Shen-78213	Liaoning	779 ± 143	829 ± 111	319 ± 61	421 ± 69	2347 ± 315
Tianshuishanzha	Liaoning	1555 ± 97	1636 ± 239	387 ± 16	823 ± 144	4402 ± 333
Fushun Shangzhuanbaizha	Liaoning	872 ± 19	1039 ± 33	118 ± 3	353 ± 40	2382 ± 69
Huangbaoyu-2	Beijing	1008 ± 217	1178 ± 107	267 ± 13	439 ± 55	2892 ± 368
Luanhong	Hebei	1444 ± 64	1399 ± 238	314 ± 61	711 ± 152	3866 ± 209
Longhuafenrou	Hebei	1478 ± 583	1478 ± 465	308 ± 62	806 ± 446	4070 ± 1554
Hebei Dajinxing	Hebei	1622 ± 14	1681 ± 12	479 ± 10	766 ± 28	4548 ± 415
Hongmianzha	Shandong	741 ± 153	794 ± 80	161 ± 14	335 ± 16	2031 ± 234
Yidohong	Shandong	965 ± 117	770 ± 29	175 ± 16	312 ± 36	2222 ± 128
Yidu-78738	Shandong	1818 ± 570	2164 ± 460	639 ± 169	1152 ± 304	5641 ± 1607
Linfen-1	Shanxi	1269 ± 353	1289 ± 369	343 ± 56	575 ± 237	3475 ± 100
Tianshengshanzha	Shanxi	958 ± 29	943 ± 113	215 ± 12	499 ± 91	2614 ± 163
Jinxian Dashanzha-1045	Shanxi	2305 ± 504	2300 ± 421	363 ± 33	1206 ± 244	6075 ± 1369
Jiangxian-798202	Shanxi	1503 ± 191	1715 ± 281	309 ± 56	792 ± 162	4319 ± 614
Jiangxianshanzha	Shanxi	1350 ± 108	1404 ± 318	249 ± 39	590 ± 56	3591 ± 507
Jiangxian-798203	Shanxi	1943 ± 106	2421 ± 47	363 ± 10	1070 ± 70	5797 ± 173
Jiangxian-798201	Shanxi	1231 ± 345	1187 ± 462	324 ± 119	601 ± 169	3343 ± 1083
Yubeihong	Henan	1828 ± 153	1809 ± 370	323 ± 34	845 ± 131	4805 ± 559
Chengoudahong	Henan	1689 ± 140	1747 ± 84	260 ± 27	762 ± 36	4458 ± 271
Yu-8002	Henan	2469 ± 254	2463 ± 261	577 ± 58	993 ± 76	6502 ± 589
Linxian Shangkou	Henan	1826 ± 288	2094 ± 378	372 ± 62	962 ± 177	5253 ± 807
Yu 8001	Henan	1176 ± 409	1318 ± 456	258 ± 61	626 ± 188	3311 ± 1009
Ganyu-2	Jiangsu	1256 ± 177	1440 ± 70	272 ± 23	628 ± 58	3595 ± 280
Xuzhoudahou	Jiangsu	2387 ± 553	2615 ± 514	706 ± 155	1363 ± 313	7071 ± 1530
average of 37 cultivars[c]		1405 ± 432	1505 ± 476	339 ± 125	684 ± 244	3933 ± 1232

[a] Values are expressed as means ± SD of three results. [b] TPC is the total of the four procyanidin contents. [c] Values are expressed as means ± SD ($n = 37$).

it generated epicatechin and 4-benzylthioepicatechin. Procyanidins C1 gave a MS peak with $m/z = 889$ [$(M + Na)^+$]. The thiolysis productions included epicatechin, procyanidin B2, 4-benzylthioepicatechin, and 4′-benzylthioprocyanidin B2. The methanol, acetonitrile, and water used for the HPLC mobile phase were HPLC grade. Stock standard solutions for the determination of the seven phenols and the two triterpene acids were prepared at a concentration of 1 mg/mL in methanol. Working solutions (0−200 μg/mL) were prepared by diluting the standard solutions with purified water.

Sample Preparation. The sample fruits were collected from several individual trees randomly. Using three independent groups of the 10 fruits for every cultivar, the sample solutions for HPLC quantification were prepared using the following procedure. The seeds from the 10 hawthorn fruits of each cultivar were removed, and the seedless fruits were cut into pieces. Each 5 g sample was mashed into a slurry with 5 mL of cold methanol (0 °C) and 2 drops of 50% (v/v) phosphoric acid in a cold mortar at 0 °C. The cream slurry was transferred into a 50 mL volumetric flask using 35 mL of precooled methanol (0 °C) and was subjected to extraction in a US C-202 model ultrasonic bath (Polong Co., Shanghai, China) for 10 min before being brought to the volume using methanol. After the methanol extract was filtered through a 0.45 μm membrane, a portion of the filtrate was directly used for the determination of the triterpene acids by HPLC, and 1 mL of the filtrate was evaporated to dryness under vacuum and the residue was dissolved in 1 mL of 25% methanol (v/v) for the determination of the polyphenols by HPLC.

HPLC Analysis. An improved method previously reported (*23*) was employed for the polyphenol analysis. The chromatograph (Elite Analytical Instruments Co., Dalian, China) was equipped with two P200II solvent delivery units, a UV200 detector, an Elite system controller, and an Echrom 98 chromatography workstation. Chromatographic analysis was performed on a 250 mm × 4.6 mm i.d., Hypersil BDS C_{18} column (Elite Analytical Instruments Co., Dalian, China). The flow rate was 0.8 mL/min, and the injection volume was 10 μL. An amount of 0.05% formic acid was employed as mobile phase A, and methanol:acetonitrile (23:77, v/v) was employed as mobile phase B. The gradient procedure was 0−8 min with 14% B, 8−15 min with 14−18% B, 15−25 min with 18% B, 25−28 min with 18−50% B, 28−33 min with 50% B, 33−35 min with 50−14% B, and 35−50 min with 14% B. The column temperature was 25 °C, and detection was carried out at 280 nm. For the triterpene acid analysis, isocratic elution was carried out; the mobile phase was 92% methanol in water containing 0.03% phosphoric acid, the column temperature was set at 18 °C, and detection was carried out at 210 nm. The external standard methods were used for the quantification of both polyphenol and triterpene acid.

Liquid Chromatography−Mass Spectrometry (LC-MS) Analysis. LC-MS analysis was performed using an Agilent 1100 Series LC/MSD Trap System (Agilent Technologies, Palo Alto, CA). The HPLC analysis was performed on a 250 mm × 4.6 mm i. d., 5 μm, Mightysil RP-18 GP Aqua column (Kanto Chemical Co., Inc., Tokyo, Japan.). The flow rate was 0.8 mL/min, and the injection volume was 10 μL. Formic acid (0.04% v/v) was employed as mobile phase A, and methanol: acetonitrile:tetrahydrofuran (52:37:11, v/v/v) was employed as mobile phase B. The gradient procedure was 0−6 min with 16% B, 6−18 min with 16−18% B, 18−28 min with 18−24% B, 28−31 min with 24% B, 31−36 min with 24−50% B, 36−43 min with 50% B, 43−46 min with 50−16% B, and 46−55 min with 16% B. The column temperature was 40 °C, and detection was carried out at 280 nm. Mass spectra were acquired in ESI mode using nitrogen gas at a temperature

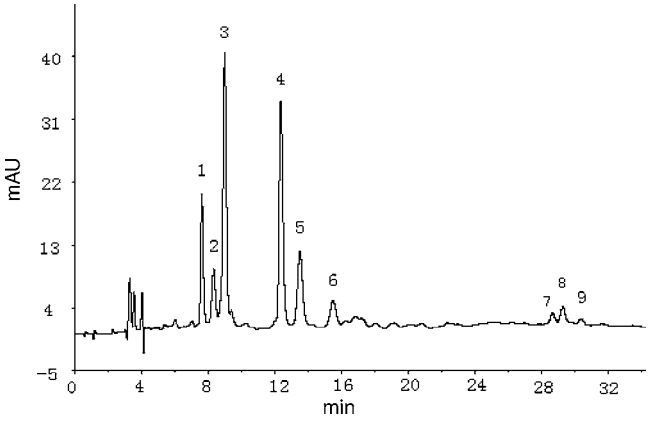

Figure 2. HPLC profile of the polyphenols in Chinese hawthorn fruit (Hebei Dajinxing) detection at 280 nm. Peak numbers: 1, chlorogenic acid; 2, unknown procyanidin trimer; 3, procyanidin B2; 4, epicatechin; 5, procyanidin C1; 6, procyanidin D1; 7, hyperoside; 8, procyanidin B5; and 9, isoquercitrin.

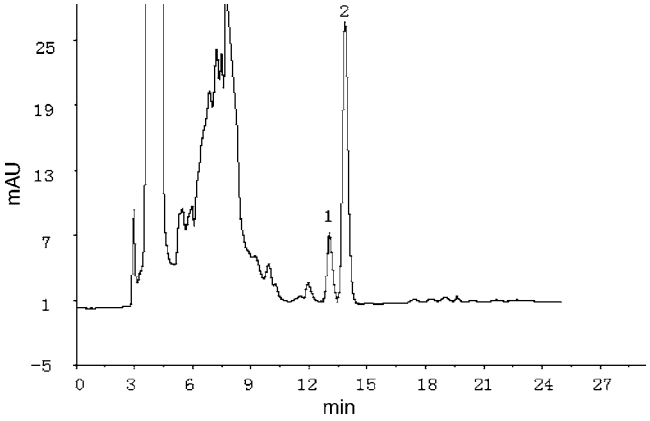

Figure 3. HPLC profile of the triterpene acids in Chinese hawthorn fruit (Hebei Dajinxing) detection at 210 nm. Peak numbers: 1, oleanolic acid; and 2, ursolic acid.

of 300 °C, a flow rate of 10 L/min, a nebulizer pressure of 50 psi, a quadrupole temperature of 30 °C, and a capillary voltage of 3500 V. The mass spectrometer was operated in positive mode at 80 eV; the target was *m*/*z* 1200; the scanning range was *m*/*z* 100−2000.

RESULTS AND DISCUSSION

Conditions of HPLC Analysis. The physiologically active constituents in hawthorn fruit include polyphenols and triterpene acids. We speculated that the separation of the triterpene acids and the polyphenols might require quite different chromatographic conditions, so we estimated that it would be difficult to separate all of the components in one sampling. The polyphenols in hawthorn fruit include procyanidins, phenolic acids, and flavonoids. The procyanidins in the chromatogram eluted faster when a lower ratio of acetonitrile to methanol was used in the mobile phase; as the proportion of acetonitrile in mobile phase B was increased, those peaks gradually eluted more slowly. The higher the polymerization degree of the procyanidins, the more distinct was this trend. Meanwhile, the chromatographic behavior of phenolic acids such as chlorogenic acid was the opposite. The phenolic acids gradually eluted faster and preceded the peak of procyanidin B2 when the proportion of acetonitrile in mobile phase B was increased. Therefore, by adjusting the ratio of acetonitrile to methanol in mobile phase B, the nine main phenolic components were separated and determined simultaneously in 50 min including the column reequilibration. Under these conditions, procyanidin C1 and some other procyanidins followed the epicatechin, which was better than the previous (*23*). A typical chromatogram for the separated polyphenols in the hawthorn fruit samples (Hebei Dajinxing) is shown in **Figure 2**.

The triterpene acids in hawthorn fruit are mainly a pair of isomers, oleanolic acid and ursolic acid, and their separation on a reversed phase column requires a mobile phase of methanol−water, a particular acidity, and a lower column temperature (<20 °C). Our HPLC conditions were able to ensure a baseline separation for the two triterpene acids in not more than 15 min. A typical chromatogram for the separated triterpene acids in the hawthorn fruit samples (Hebei Dajinxing) is shown in **Figure 3**.

Procyanidin Levels in the Fruit of Different Hawthorn Cultivars. The polyphenol and triterpene acid contents in the fruit of 37 representative cultivars of Chinese hawthorn were determined by the HPLC methods described. **Table 1** lists their origins and shows the levels of the procyanidins. The average concentration of sum of the four procyanidins in the 37 samples was 3933 μg/g fresh weight (FW), which is equal to or even more than that of European hawthorn fruits (*24*−*26*), and was far higher than that reported in popular fruits such as grape (10−1600 μg/g FW), apple (trace−150 μg/g FW), red raspberry (20−480 μg/g FW), and strawberry (20−500 μg/g FW) (*27*). This result agreed with the previous reported data, according to which Chinese hawthorn fruits have much higher antioxidative properties than other popular fruits (*28*). Among the analyzed procyanidins, the average level of the monomer epicatechin (1405 μg/g FW) was near to that of a dimer, procyanidin B2 (1505 μg/g FW), which was the highest. The level of the trimer, procyanidin C1, was moderate (684 μg/g FW), and that of another dimer, procyanidin B5 (339 μg/g FW), in which the C4 of an epicatechin unit is connected to the sterically hindered C6 position of another epicatechin unit, was far less than that of dimer, procyanidin B2. According to the levels of the four major kinds of procyanidins in the 37 samples, the mean degree of polymerization (mDP) was 1.54. There were some additional peaks, as shown in **Figure 2**, including a procyanidin trimer (retention time, 8.5 min; peak 2), a procyanidin tetramer (retention time, 15.5 min; peak 6), and some unknown small peaks mainly distributed between 16 and 23 min. By LC-MS analysis (ESI), compositions of unknown small peaks generated MS peaks with the *m*/*z* 887, 1155, and 1443, respectively, and it was indicated that the peaks were consistent with oligomeric procyanidins containing 3−5 epicatechin units. Taking into account these peaks, it could be supposed that the mDP of the procyanidins in the Chinese hawthorn fruit is around 2. This value is lower than that for European hawthorn fruit (*24*) and far lower than that for the leaves and flowers of hawthorn (*24*). This might be related to the high level of organic acids in the Chinese hawthorn fruit (*13*) and the decomposition of oligomeric procyanidins into smaller molecular fragments in acidic conditions (*22*). Smaller procyanidins, monomer to trimer, have been reported to be absorbed in the human intestine directly in vitro (*29*−*31*). As for the significance of these results, the fruits of Chinese hawthorn may become a preferred resource of edible oligomeric procyanidins.

As shown in **Table 1**, despite the fact that the samples were derived from the same species of hawthorn (*C. pinnatifida* Bge. var. *major* N.E.Br.), the trees of the 36 cultivars (Hebei Dajinxing excluded) are planted on the same farm and the cultivation conditions are quite similar; the content divergence of the four procyanidins among the 37 cultivars was quite large, and the relative standard deviations (RSDs) were 30.7, 31.6,

Table 2. Contents[a] of Flavonoids, Chlorogenic Acid, and Triterpene Acids in Chinese Hawthorn Fruit (μg/g FW)

cultivar	origin	hyperoside	isoquercitrin	chlorogenic acid	oleanolic acid	ursolic acid
725	Jilin	9 ± 2	3 ± 1	119 ± 10	186 ± 30	1024 ± 134
Benxi-2	Liaoning	29 ± 15	19 ± 14	191 ± 37	106 ± 63	966 ± 63
Shanchengzi-2	Liaoning	33 ± 25	29 ± 24	201 ± 79	172 ± 22	989 ± 59
Sishanling	Liaoning	53 ± 2	50 ± 5	272 ± 25	187 ± 65	1103 ± 123
Benxi-7	Liaoning	61 ± 12	46 ± 9	187 ± 46	224 ± 98	1011 ± 41
Benxi-4	Liaoning	56 ± 12	43 ± 10	214 ± 37	188 ± 38	1091 ± 112
Shanchengzi-1	Liaoning	37 ± 1	32 ± 0.4	188 ± 19	163 ± 97	931 ± 100
Niuxintai-1	Liaoning	63 ± 11	52 ± 10	172 ± 4	148 ± 35	900 ± 89
Xihong	Liaoning	27 ± 3	18 ± 3	205 ± 16	107 ± 32	953 ± 19
Liaohong	Liaoning	34 ± 6	23 ± 5	221 ± 27	120 ± 59	899 ± 48
Anshan Dajinxing	Liaoning	79 ± 13	51 ± 10	285 ± 5	64 ± 26	711 ± 55
Mopanshanzha	Liaoning	41 ± 4	22 ± 4	201 ± 52	128 ± 37	779 ± 47
Yinyeling-7	Liaoning	46 ± 3	31 ± 7	179 ± 45	63 ± 23	813 ± 27
Shen-78213	Liaoning	152 ± 20	101 ± 18	544 ± 33	131 ± 83	1100 ± 23
Tianshuishanzha	Liaoning	54 ± 24	37 ± 12	195 ± 10	223 ± 97	1177 ± 144
Fushun Shangzhuanbaizha	Liaoning	72 ± 7	64 ± 9	171 ± 33	96 ± 6	924 ± 75
Huangbaoyu-2	Beijing	40 ± 18	37 ± 1	150 ± 32	151 ± 56	950 ± 146
Luanhong	Hebei	50 ± 23	34 ± 22	176 ± 53	166 ± 41	987 ± 57
Longhuafenrou	Hebei	68 ± 26	48 ± 15	276 ± 83	110 ± 18	895 ± 70
Hebei Dajinxing	Hebei	59 ± 1	37 ± 4	186 ± 1	35 ± 12	827 ± 44
Hongmianzha	Shandong	79 ± 18	33 ± 5	369 ± 55	48 ± 38	615 ± 19
Yidohong	Shandong	64 ± 22	43 ± 13	190 ± 18	135 ± 13	1142 ± 47
Yidu-78738	Shandong	147 ± 44	89 ± 24	307 ± 36	169 ± 47	1080 ± 99
Linfen-1	Shanxi	39 ± 10	28 ± 4	194 ± 67	192 ± 56	931 ± 147
Tianshengshanzha	Shanxi	114 ± 7	69 ± 2	198 ± 17	99 ± 23	770 ± 80
Jinxian Dashanzha-1045	Shanxi	66 ± 1	55 ± 3	324 ± 43	201 ± 79	1078 ± 189
Jiangxian-798202	Shanxi	48 ± 9	36 ± 12	273 ± 16	202 ± 48	940 ± 65
Jiangxianshanzha	Shanxi	57 ± 11	42 ± 7	228 ± 28	207 ± 33	768 ± 327
Jiangxian-798203	Shanxi	35 ± 5	26 ± 3	348 ± 27	166 ± 69	1043 ± 37
Jiangxian-798201	Shanxi	65 ± 24	44 ± 6	201 ± 39	158 ± 88	1148 ± 19
Yubeihong	Henan	28 ± 10	20 ± 8	261 ± 71	137 ± 15	958 ± 114
Chengoudahong	Henan	26 ± 4	20 ± 1	261 ± 30	146 ± 15	881 ± 105
Yu-8002	Henan	11 ± 1	28 ± 1	217 ± 20	185 ± 10	647 ± 70
Linxian Shangkou	Henan	50 ± 2	44 ± 6	264 ± 85	131 ± 40	1114 ± 45
Yu 8001	Henan	64 ± 10	61 ± 13	254 ± 92	149 ± 23	1012 ± 101
Ganyu-2	Jiangsu	52 ± 1	27 ± 1	161 ± 22	149 ± 47	921 ± 20
Xuzhoudahou	Jiangsu	78 ± 25	63 ± 22	281 ± 28	190 ± 57	1147 ± 22
average of 37 cultivars[b]		56 ± 30	41 ± 19	234 ± 76	147 ± 47	952 ± 140

[a] Values are expressed as means ± SD of three results. [b] Values are expressed as means ± SD ($n = 37$).

36.9, and 35.7%, respectively. Cultivars such as Xuzhoudahou, Yu-8002, Jinxian Dashanzha-1045, Jiangxian-798203, and Yidu-78738 had higher levels of the sum of procyanidins (7071, 6502, 6075, 5797, and 5641 μg/g FW, respectively). On the other hand, the cultivars Anshan Dajinxing, Hongmianzha, Yidohong, Shen-78213, and Fushun Shangzhuanbaizha had lower total procyanidins levels (1950, 2031, 2222, 2348, and 2382 μg/g FW, respectively).

Chinese hawthorn grows mainly in five large regions, which comprise more than 15 provinces from the north to the south of China (*11*). In the present experiment, the 37 cultivars were derived from eight provinces of origin, as listed in **Table 1**. After the data were arranged according to the latitudes of the geographic origins of these cultivars, an inverse correlation trend between the contents of the sum of procyanidins and the latitude of the geographic origins of the cultivars was observed ($r = 0.3851$, $P < 0.02$; for details, see the Supporting Information) because 36 of the 37 cultivars in fact were grown in a same place. Thus, a possible explanation for this result is that the geographic factor (latitude) has an influence on the genetic characters relating the procyanidins levels of the cultivars continuously during the long period. This primary result remains to be proved.

Levels of Flavonoids, Chlorogenic Acid, and Triterpene Acids in the Fruit of Different Chinese Hawthorn Cultivars. **Table 2** shows the levels of hyperoside, isoquercitrin, chlorogenic acid, oleanolic acid, and ursolic acid in the hawthorn fruit. The major flavonoids found in Chinese hawthorn fruits were the two quercetin glycosides, hyperoside and isoquercitrin, and other flavonoids have not been observed previously. The contained flavonoids were different from those in hawthorn leaves, where the main flavonoid constituents were *C*-glycosides, such as vitexin glycosides. From the average levels in the 37 cultivars (**Table 2**), the sum of the levels of the two flavonoids in Chinese hawthorn fruits was calculated to be 97 μg/g FW, which corresponds to the levels in European hawthorn fruit as determined by HPLC (*25*, *26*, *32*). The cultivars Shen-78213, Yidu-78738, and Tianshengshanzha had higher total flavonoid levels (253, 236, and 183 μg/g FW, respectively), whereas the cultivars 725, Yu-8002, Xihong, Yubeihong, Chengoudahong, and Benxi-2 had lower flavonoid levels (12, 39, 45, 46, 48, and 48 μg/g FW, respectively). It has been reported (*13*) that the total flavonoid level in Chinese hawthorn (dry fruit), as detected by photometric assay, exceeded 1%. However, when the flavonoids were detected by spectrophotometric assay using Al(III) as the color reagent, the other copresent phenolic components, especially procyanidins at higher levels, would seriously interfere with the determination and the results would be greatly exaggerated (*33*). Our present results for the flavonoids seem reasonable. The mean content of chlorogenic acid was 234 μg/g FW, which was distinctly higher than that of the flavonoids but was only 6% of the procyanidins (3933 μg/g FW).

Triterpene acids are a group of nonphenolic active components in Chinese hawthorn fruit. The total average content of the two triterpene acids, oleanolic acid, and ursolic acid, shown

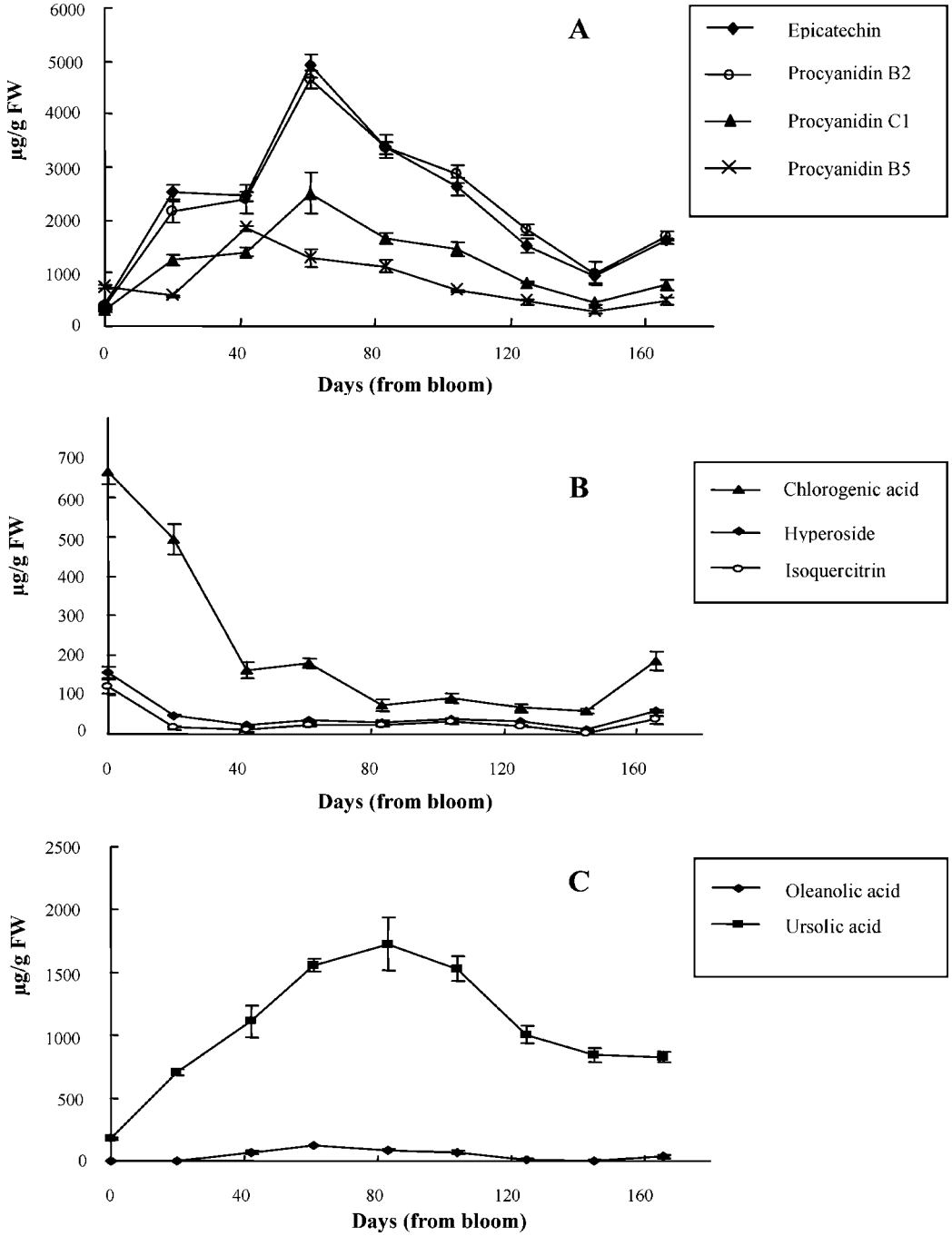

Figure 4. Content changes in the polyphenols and triterpene acids in Chinese hawthorn during fruit maturation (Hebei Dajinxing). (**A**) Procyanidins, (**B**) flavonoids and chlorogenic acid, and (**C**) triterpene acids. Values are expressed as means ± SD of three results.

in **Table 2**, was 1099 μg/g FW, which was lower than that of the procyanidins. The content of ursolic acid was higher than that of oleanolic acid, and the cultivars Tianshuishanzha, Jiangxian-798201, Xuzhoudahou, and Yidohong had the highest levels of ursolic acid content (1177, 1148, 1147, and 1142 μg/g FW, respectively). The divergence in the ursolic acid contents among the 37 cultivars was not very large, and the RSD was 15%. As a result of our analysis, we concluded that the procyanidins are the representative phenolic active components in Chinese hawthorn fruit.

Divergences among the Cultivars and Correlations among the Constituents. As shown in **Tables 1** and **2**, the content divergence for the nine active constituents among the 37 cultivars was quite large and the RSD ranged from 31 to 54%, with the exception of that for ursolic acid (15%). The number of samples was sufficiently enough for statistical estimation, so the results showed a reliable disparity among the levels of those constituents in the different cultivars of Chinese hawthorn.

To investigate the correlations between the nine active constituents, the data in **Tables 1** and **2** were reexamined and the results can be found in the Supporting Information. It showed that all of the compounds from the same class of active constituents had a significant correlation ($P < 0.01$). Between the four procyanidins and the sum of the procyanidins, the correlation coefficients ranged form 0.7413 to 0.9898, which is quite high ($P < 0.001$). The correlation between the two flavonoids was 0.9212 ($P < 0.001$), and that between the two triterpene acids was 0.5088 ($P < 0.01$). The procyanidins at higher levels, such as epicatechin, procyanidin B2, and the sum of the procyanidins, especially showed stronger correlations.

However, the correlation coefficients were lower between different classes of compounds, such as procyanidins and flavonoids or procyanidins and chlorogenic acid. This indicates that the generation of flavonoids and that of chlorogenic acid are independent of that of procyanidins and triterpene acids in Chinese hawthorn fruit. Thus, we consider that it is difficult to estimate the levels of active constituents in hawthorn by only determining the flavonoid levels (*13*). The significant correlation between the chlorogenic acid and flavonoids ($P < 0.001$, $r =$ 0.5678 and 0.5383, respectively) suggests that the generation of these two polyphenols may be related in Chinese hawthorn fruit.

Changes in Levels of Polyphenol and Triterpene Acid during Fruit Maturation. Figure 4 shows the changes in levels of procyanidins, flavonoids, chlorogenic acid, and the triterpene acids in Chinese hawthorn fruit over the entire growth period. In the primary stage, the four procyanidins were synthesized rapidly and became the preponderant polyphenols (**Figure 4A**). In conjunction with the accumulation of these procyanidins, the sum of the polyphenol contents also rose rapidly. By 61 days after bloom (July 4, an average weight of sample fruits was 4.37 g), it reached its highest level, 1.36% of the fresh fruit, and then decreased gradually until just before maturity (an average weight of sample fruits was 11.64 g). This fluctuation of the polyphenol content in the hawthorn fruit was similar to that in apple and other fruits of the family Rosaceae (*34*). This indicates that the period in which the fruit has the highest polyphenol content is not maturity, putting into doubt the listing of the "dry mature fruit" of hawthorn as a raw medicinal product in the Chinese pharmacopoeia (*12*). In terms of the exploitation of resources containing active components, the collection of hawthorn material should be carried out in summer to improve the quality of the medicine and to reduce the cost of hawthorn extraction.

In the early fruit-bearing period, the chlorogenic acid and flavonoid levels were relatively higher (**Figure 4B**). These levels were similar to those in the leaves. As the fruits grew from this point onward, the levels of these constituents declined rapidly and remained low until fruit maturation. The two triterpene acids shown in (**Figure 4C**), especially the ursolic acid, have a similar pattern as the procyanidins. At summer (82 days after bloom, July 25), the sum content of the two triterpene acids reached the highest (1812 μg/g FW).

Although many reports have suggested that flavonoids possess many beneficial physiological effects (*32*, *35*, *36*), in the examined samples of the 37 cultivars of Chinese hawthorn, the total content of the two major flavonoids was equivalent to 1/40 of that of the four procyanidin components. This analytical result suggests that the procyanidins are the major active constituents in the fruit of Chinese hawthorn and not the flavonoids. Although the content of chlorogenic acid was higher than that of the flavonoids, it was not remarkable as compared with that in many other popular fruits, such as the pear (*37*) and the apple (*38*, *39*). Triterpene acids have been reported to have several important physiological effects (*19*, *40*−*42*). Our results showed that the content of triterpene acids in the Chinese hawthorn fruit was considerable.

Procyanidins are a class of proanthocyanidins consisting of flavan-3-ol units, epicatechin, and/or catechin. It has been reported that procyanidins protect the skin from UV irradiation (*43*) and human lymphocytes from γ-ray irradiation (*44*). Another report has shown that procyanidins are selective protein kinase C inhibitors, so the hair growth is distinctly promoted by them (*45*); the efficacy of procyanidin B2 is the most obvious (*46*, *47*). Oligomeric procyanidins have been reported to relax endothelium-dependent blood vessels in remarkably low concentrations as a special inhibitor of endothelin-1 release (*10*, *48*) and are considered to be the main active constituents of hawthorn in the treatment of congestive heart failure (*9*). Our results revealed higher procyanidin contents in the Chinese hawthorn fruit, and the mDP of these procyanidins is suitable for absorption by the human intestine (*27*). Therefore, we conclude that Chinese hawthorn fruit is an excellent source of edible procyanidins in addition to grapes, apples (*27*), and cocoa (*48*). In terms of the content change in the polyphenols during growth, the Chinese hawthorn fruit had the highest procyanidin content at the middle stages of growth, and the immature fruit was excellent for preparing hawthorn fruit extracts with high procyanidin contents.

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Supporting Information Available: Correlation between the sum of the procyanidin content and the latitude of the geographical origin of the cultivars and results of the correlation analysis of the nine main active compositions in the fruits of the 37 cultivars of Chinese hawthorn. This material is available free of charge via the Internet at http://pubs.acs.org.

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