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Original Article

Characterization of nine polyphenols in fruits of *Malus pumila* Mill by high-performance liquid chromatography

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ABSTRACT

Polyphenols are important bioactive substances in apple. To explore the profiles of the nine representative polyphenols in this fruit, a high-performance liquid chromatography method has been established and validated. The validated method was successfully applied for the simultaneous characterization and quantification of these nine apple polyphenols in 11 apple extracts, which were obtained from six cultivars from Shaanxi Province, China. The results showed that only abscission of the Fuji apple sample was rich in the nine apple polyphenols, and the polyphenol contents of other samples varied. Although all the samples were collected in the same region, the contents of nine polyphenols were different. The proposed method could serve as a prerequisite for quality control of *Malus* products.

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1. Introduction

Apple (*Malus pumila* Mill) is the fruit of malus plant. It belongs to the family Rosaceae and has been widely cultivated in various parts of the world for centuries. At present, the annual global apple production is about 70 million tons, thus making apple the third largest consumed fruit next to bananas and oranges. Among the numerous areas producing apples globally, China has the largest acreage of apple cultivation and is also the leading producer, with Shaanxi being the main apple production base in China.

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Results of phytochemical studies have shown that apple contains various constituents, including polysaccharides, triterpenoids, phytosterols, phenols, and other components such as protein [1], vitamins (A, C, and E), β-carotene, metal elements (e.g., iron, magnesium, calcium, zinc, manganese, sulfur, copper, potassium), and essential trace elements that humans need [2]. Apple also contains different polyphenols in different tissues. In particular, the types and contents of polyphenols in apple peel are relatively high [3]. Although there are different polyphenols in apple, phenolic acid and flavonoids were reported to be the most important constituents [4].

In 1997, Lu and Foo [5] studied apple polyphenols extracted from apple residue. Their results showed that the main functional components are catechin, caffeic acid, chlorogenic acid, phloretin-2′-xyloglucoside, hyperoside, phloridzin, quercetin-3-arabinoside, epicatechin, 3-hydroxyphloridzin, quercetin-3-xyloside, isoquercitrin, and quercetin-3-rhamnoside. Alonso et al [6] detected as many as 30 kinds of polyphenols in apple using high-performance liquid chromatography (HPLC)–diode array detector–mass spectrometer in 2004.

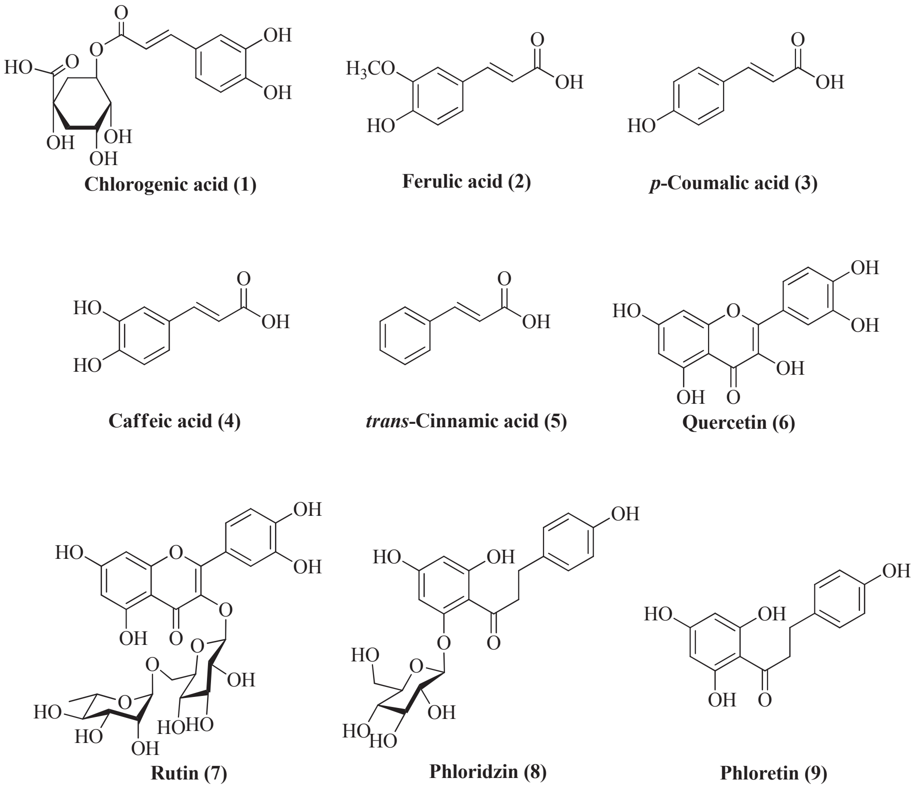
Phloridzin is the most common polyphenolic compound in apple, and is mainly found in apple plant's roots, bark, stems, leaves, and fruit. It is one of the most important compounds for the quality control of apple juice and other products, and for the establishment of fingerprint profile. Phloridzin could also efficiently trap reactive methylglyoxal (MGO) or glyoxal (GO) to form mono- and di-MGO or GO adducts under physiological conditions and inhibit the formation of advanced glycation end products (AGEs) [7]. At present, phloridzin is used in medicines, foods, cosmetics, and tissue culture of plant [8].

Apple polyphenols have been demonstrated to have potential beneficial effects on health, such as exhibiting antioxidant activity [9,10], antibacterial effect [11], anti-inflammatory effect [12], antiradiation effect [13], antitumor [14,15], and antiobesity effect [16]. Because of these multiple benefits for human health, apple polyphenols attracted significant attention in the scientific field. In this regard, nine polyphenols, namely, chlorogenic acid (**1**), ferulic acid (**2**), *p*-coumalic acid (**3**), caffeic acid (**4**), *trans*-cinnamic acid (**5**), quercetin (**6**), rutin (**7**), phloridzin (**8**), and phloretin (**9**) from 11 apple extracts, which were obtained from six cultivars from Luochuan, were used for simultaneous quantitative analysis using the developed HPLC–diode array detector method. The established method could serve as a prerequisite for quality control of *Malus* products.

2. Materials and methods

2.1. *Chemicals and reagents*

The methanol (MeOH) used was of HPLC grade (Merck, Darmstadt, Germany). Other reagent solutions, such as acetic acid and water, were of analytical grade (Hengxing Chemical Reagent Co., Ltd., Tianjin, China). Chemical standards of chlorogenic acid (**1**), ferulic acid (**2**), *p*-coumalic acid (**3**), caffeic acid (**4**), and *trans*-cinnamic acid (**5**) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Reference compounds of quercetin (**6**), rutin (**7**), phloridzin (**8**), and phloretin (**9**) were prepared in our laboratory. The purity of these self-produced standards was all above 98%, as determined by HPLC analysis. The chemical structures of these reference compounds are shown in Fig. 1.



Chlorogenic acid (1)

Ferulic acid (2)

***p*-Coumalic acid (3)**

Caffeic acid (4)

***trans*-Cinnamic acid (5)**

Quercetin (6)

Rutin (7)

Phloridzin (8)

Phloretin (9)

Fig. 1 – Chemical structures of the identified polyphenols in apple.

Table 1 – Samples numbers of *Malus*. fruits ($N = 11$).

Samples no.	*Malus* cultivar	Samples no.	*Malus* cultivar
A	EtOH extract of Gala	B	H_2O extract of Gala
C	EtOH extract of Fuji	D	H_2O extract of Fuji
E	EtOH extract of Starking	F	H_2O extract of Starking
G	EtOH extract of Huasheng	H	H_2O extract of Huasheng
I	EtOH extract of abscission of Fuji	J	H_2O extract of abscission of Fuji
K	H_2O extract of Cider		

EtOH = ethanol.

2.2. *Chromatographic conditions and instrumentation*

HPLC analysis was performed on an Agilent 1260 Infinity LC separation module (Agilent Technologies, Santa Clara, CA, USA), which consisted of a degasser, a quaternary pump, an autosampler, and a diode array detector. A Luna C-18 column (5 μm, 4.6 mm i.d. × 250, Phenomenex Inc., Torrance, CA, USA) was used in chromatographic analysis. The reaction conditions are as follows: flow rate was 1.0 mL/min; column temperature was maintained at 20°C. The mobile phase was composed of A [1.0% (v/v) acetic acid–water solution] and B (MeOH) with a gradient elution: 0 minutes, 95% A; 0–10 minutes, 95–70% A; 10–25 minutes, 70–50% A; 25–35 minutes, 50–30% A; 35–40 minutes, 30–95% A. The chromatogram was monitored at a wavelength of 320 nm for Compounds **1**–**5**, **8**, and **9**, and at 280 nm for Compounds **6** and **7** during the experiment.

2.3. *Plant materials*

Six batches of apple, namely, *Malus domestica* "Gala," *M. domestica* Borkh. CV. Red Fuji, *M. domestica* "Starking," *M. domestica* "Huasheng," and abscission of Fuji and Cider, were collected in September 2014 from Luochuan County, Shaanxi Province, China. Their botanical origins were identified by the corresponding author (NB), and voucher specimens were deposited in the herbarium of Northwest University, China. The sample numbers are listed in Table 1.

2.4. *Preparation of sample solutions*

After the cores had been removed, the fruits were mashed into a paste. The wet paste (20 g) was weighed accurately and divided into two portions. These portions (10 g each) were separately added into two 50-mL conical flasks with a stopper. To these flasks, 40 mL of water and ethyl alcohol, respectively, were added. After accurate weighing, ultrasonication (40 kHz) was performed at room temperature for 2 hours, and then the same solvent was added to compensate for the weight lost during the extraction. After centrifugation (13,000 rpm, 15 minutes), the supernatant was concentrated under vacuum to 10 mL and stored at 4°C. We then took out 1 mL of this solution and filtered it through a 0.22-μm membrane filter prior to performing HPLC analysis.

2.5. *Preparation of standard solutions*

A mixed standard stock solution containing the Reference Compounds **1**–**9** was prepared in MeOH and stored at 4°C in darkness for further analysis. Working standard solutions for calibration curves were prepared by diluting the mixed standard stock solution with 10% MeOH at different concentrations, and the concentration ranges for these nine analytes were as follows: **1**, 0.90–90.50 μg/mL; **2**, 0.041–4.06 μg/mL; **3**, 0.045–4.40 μg/mL; **4**, 0.062–6.22 μg/mL; **5**, 0.24–25.40 μg/mL; **6**, 0.14–14.06 μg/mL; **7**, 1.07–100.20 μg/mL; **8**, 3.11–314.50 μg/mL; and **9**, 3.00–300.50 μg/mL. The standard solutions were filtered through a 0.22-μm membrane prior to injection.

Table 2 – Calibration curves and LOD and LOQ data of compounds investigated by HPLC–diode array detector.

Compounds	Calibration curves [a]	R^2	Linear range (μg/mL)	LOD [b] (μg/mL)	LOQ [b] (μg/mL)
1	$Y = 45.51373X - 172.06528$	0.99929	0.90–90.50	0.181	0.660
2	$Y = 75.36489X + 37.47940$	0.99989	0.041–4.06	0.008	0.026
3	$Y = 68.44006X - 146.3940$	0.99909	0.045–4.40	0.009	0.031
4	$Y = 64.26195X - 279.36425$	0.99985	0.062–6.22	0.039	0.012
5	$Y = 0.67462X + 1.66240$	0.99960	0.24–25.40	0.160	0.048
6	$Y = 44.63414X + 133.55077$	0.99965	0.14–14.06	0.088	0.026
7	$Y = 29.13537X - 91.48764$	0.99914	1.07–100.20	0.216	0.721
8	$Y = 4.95179X + 6.86960$	0.99963	3.11–314.50	0.624	2.079
9	$Y = 9.28496X + 1.64971$	0.99958	3.00–300.50	0.626	2.085

HPLC = high-performance liquid chromatography; LOD = limit of detection; LOQ = limit of quantification.

[a] Y is the value of peak area, and X is the value of the reference compound's concentration (μg/mL).

[b] LOD and LOQ were determined at signal-to-noise ratio of about 3 and 10, respectively.

Table 3 – Precision, repeatability, stability, and recovery of the nine analytes.

Compounds	Precision (RSD, %)		Repeatability (RSD, %, $n = 6$)	Stability (RSD, %, $n = 6$)	Recovery (%, $n = 3$)	
	Intraday ($n = 6$)	Interday ($n = 6$)			Mean	RSD, %
1	0.19	1.02	0.59	1.05	94.86	2.31
2	0.23	0.88	1.02	1.72	99.41	2.05
3	0.29	1.64	1.64	1.12	97.24	2.24
4	0.37	0.87	1.23	1.68	95.63	1.98
5	0.20	0.94	1.35	1.37	96.61	1.54
6	0.15	0.57	0.75	0.84	98.75	1.79
7	0.31	0.68	0.92	2.31	96.79	2.45
8	0.36	1.04	1.32	1.10	101.34	1.39
9	0.43	1.38	1.44	2.52	95.21	1.47

RSD = relative standard deviation.

2.6. *HPLC method validation*

The standard curve was obtained by plotting the peak area versus the corresponding concentration of each compound and was fitted to a linear equation $y = ax + b$, where x and y represent concentration and peak area, respectively. The lowest concentration of working solution for calibration use was diluted with water or ethanol (EtOH) to a series of appropriate concentrations. They were then analyzed until the signal-to-noise ratio (S/N) for each compound was about 3 for the limit of detection (LOD) and 10 for the limit of quantification (LOQ). The precision of the method was evaluated by analyzing the standard solutions containing the nine standard compounds. The experiment was repeated six times on the same day and additionally on 3 consecutive days to determine intraday precision and interday precision, respectively. Then, the relative standard deviation (RSD) of peak area for each of the marker compounds was calculated, respectively [17]. To confirm the repeatability, six different sample solutions prepared from the same sample (Sample 1) were analyzed and variations expressed by RSD. To evaluate the stability of the solution, one of the aforementioned sample solutions was stored at 25°C and analyzed at 0 hours, 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours, respectively. A recovery test was performed to evaluate the accuracy of this method. The test was conducted by adding known quantities of one standard into a certain amount (80 μg) of *M. pumila* Mill fruits (Sample 1). The spiked samples were then extracted, processed, and quantified in accordance with the aforementioned methods. Three replicates were performed for the test. The detected amounts (actual) were calculated by subtracting the total amount of each compound before spiking from the total amount after spiking. The ratio of detected amount (actual) to spiked amount (theoretical) was used to calculate the recovery percentage. The percent recovery rates for the analytes were presented as mean.

2.7. *Identification and quantification*

Identification of the nine polyphenols was carried out by comparing the HPLC retention time and UV spectra of target peaks with those of the standards. Quantification was performed based on linear calibration plots of the peak areas versus the concentration.

3. Results and Discussion

3.1. *HPLC method validation*

The quantitative analysis method was validated in terms of the linearity, LOD, LOQ, precision, repeatability, stability, and accuracy. The results demonstrated that all calibration curves exhibited excellent linear regressions with the determination coefficients (r^2) ranging from 0.9991 to 0.9999, and the calibration ranges adequately covered variations in the amounts of the compounds investigated in the samples. The overall LODs and LOQs were less than 0.700 μg/mL and 2.100 μg/mL, respectively (Table 2). Among the tested samples, the sample of EtOH extracts of abscission of Fuji was rich in the nine apple polyphenols, which demonstrated that this sample was suitable for the repeatability, stability, and recovery tests. Therefore, we chose it for the experiments. The intraday and interday variations, repeatability, and stability RSD values of the nine compounds were all less than 2.60% (Table 3). The overall recoveries lay between 94.86% and 101.34% for the nine reference compounds, with RSDs less than 2.45%, which indicated that the established method was accurate enough for the determination of the nine polyphenols in apple (Table 3).

3.2. *Identification of the nine polyphenols*

By comparing their HPLC retention times and UV spectra with those of reference compounds, the nine constituents in apple were unequivocally identified as chlorogenic acid, ferulic acid, *p*-coumalic acid, caffeic acid, cinnamic acid, quercetin, rutin, phloridzin, and phloretin. The results are presented in Table 4 and Fig. 2.

3.3. *Quantification of the nine polyphenols*

In this study, the established HPLC method was subsequently applied to a simultaneous determination of the nine markers in 11 apple extracts, which comprised six cultivars from Shaanxi province, China. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis. The results (Table 5) showed that the abscission of Fuji sample was rich in the nine apple polyphenols, although their contents were obviously

Table 4 – Chromatographic and spectrometric data of nine polyphenols found in *Malus*. fruits.

Compounds	t_R (min)	UV data (nm)
1	14.49	320
2	22.05	320
3	21.29	320
4	17.75	320
5	30.80	320
6	31.83	280
7	23.69	280
8	27.90	320
9	34.00	320

different. In the water extracts of abscission of Fuji, the total content of these investigated compounds reached as high as 1857.116 μg/g, whereas it was only 23.137 μg/g in the EtOH extracts of red Fuji. As for the individual compounds determined in the experiments, remarkable differences were also observed. For example, the highest content of rutin was 419.368 μg/g in EtOH extracts of abscission of Fuji, whereas it was only 0.719 μg/g in EtOH extracts of Gala. When it comes to ferulic acid and phloretin, these two components could not be detected in many samples. They were found to be present in some samples, but the content was only a trace amount. Although all the samples were

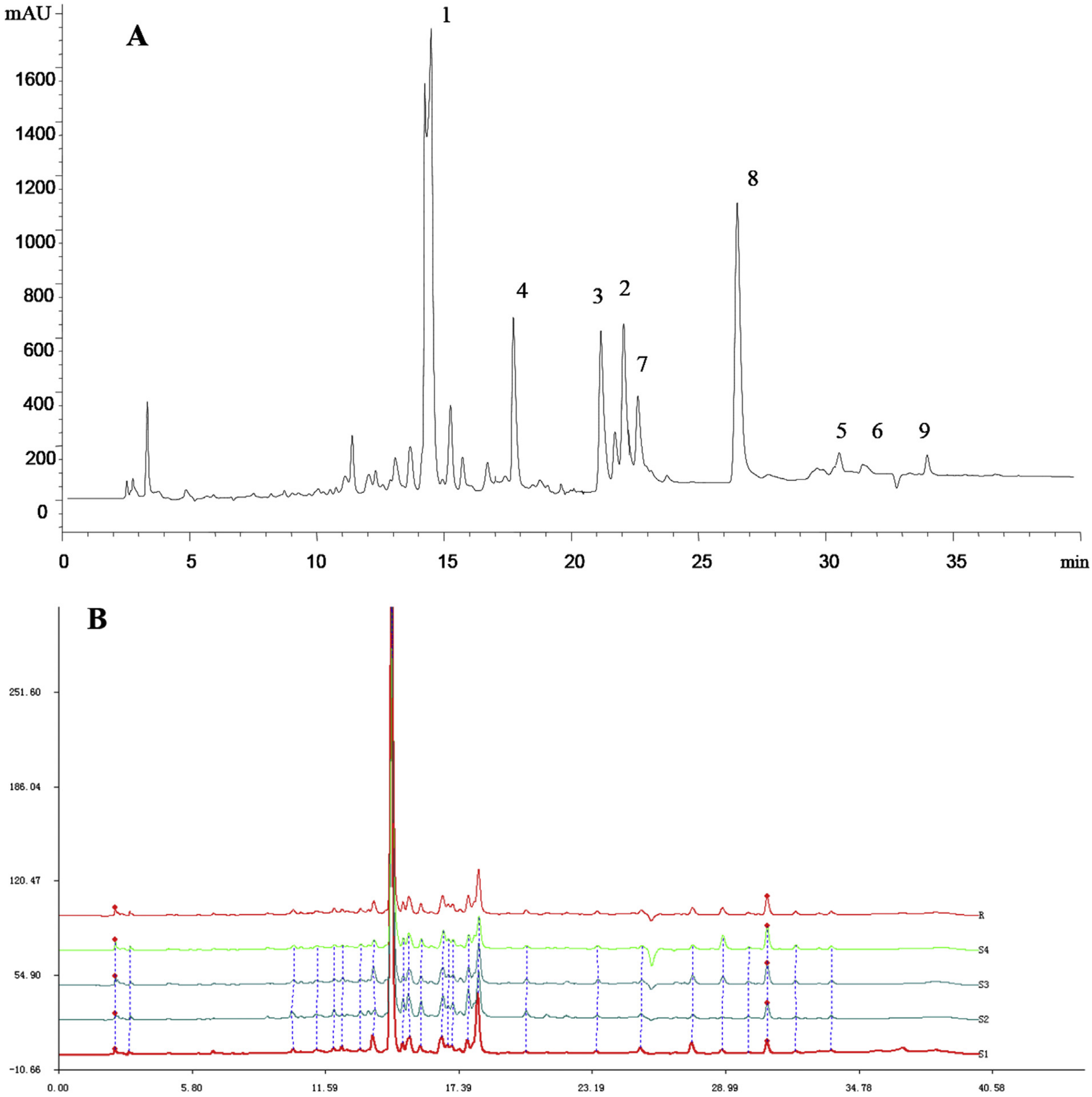


Fig. 2 – (A) High-performance liquid chromatography (HPLC) chromatograms of solution of samples (H_2O extract of abscission of Fuji) and (B) HPLC chromatographic fingerprints of four apple samples. Peaks: 1, chlorogenic acid (16.67 μg/mL); 2, ferulic acid (15.56 μg/mL); 3, *p*-coumalic acid (12.37 μg/mL); 4, caffeic acid (13.89 μg/mL); 5, cinnamic acid (13.89 μg/mL); 6, quercetin (14.47 μg/mL); 7, rutin (15.56 μg/mL); 8, phloridzin (15.80 μg/mL); 9, phloretin (8.35 μg/mL). S1 (H_2O extract of Gala); S2 (H_2O extract of Fuji); S3 (H_2O extract of Starking); and S4 (H_2O extract of Huasheng).

Table 5 – Contents of nine polyphenols in *Malus* fruits.

Sample no.	Contents of analytes [a] (μg/g, $n = 3$)									Total	Extraction rate [b] (%, $n = 3$)
	1	2	3	4	5	6	7	8	9		
A	8.380	ND	0.985	ND	18.800	0.335	0.719	3.000	ND	32.219	4.22
B	10.610	ND	1.624	0.864	21.768	0.536	1.190	2.983	ND	39.575	3.60
C	7.436	ND	0.702	0.565	10.090	0.0417	3.032	1.270	ND	23.137	4.86
D	11.107	ND	1.065	1.162	27.978	0.128	2.912	0.921	ND	45.273	4.86
E	7.153	ND	0.668	0.675	25.800	1.275	9.232	5.388	ND	50.191	5.16
F	8.014	0.00755	1.250	0.820	37.06	1.582	6.101	2.951	ND	57.786	5.38
G	4.572	ND	0.562	0.524	23.890	2.204	16.551	8.570	ND	56.873	6.20
H	8.143	0.00755	0.870	0.902	42.480	3.439	18.676	1.132	ND	75.650	6.44
I	16.742	0.396	1.213	1.234	128.280	0.204	419.368	359.748	0.541	927.726	5.72
J	177.304	1.782	2.806	18.724	326.440	33.282	38.860	1257.300	0.618	1857.116	9.96
K	183.640	0.634	22.667	9.700	ND	ND	ND	1.576	ND	218.217	ND

[a] Content $= \dfrac{X\frac{V_1\text{(injection volume of standard solution)}}{V_2\text{(injection volume of sample solution)}}\times V\text{(sample volume)}}{M\text{(sample amount)}}$

[b] Extraction rate = solids content of the extract/sample volume.

collected in the same region, the contents of nine polyphenols were different and the variation may be due to many factors, including genetic variation, plant origin, and climate.

In summary, the aforementioned results showed that the proposed method could serve as a prerequisite for quality control and standardization of *Malus* products. The simple, accurate, and reliable method could also be used for developing the HPLC fingerprint of *M. pumila* Mill and determination of nine bioactive polyphenols. In view of the advantages of reliability and sensitivity for quantitative analysis using the developed HPLC method, there is a potential for its wide application in identifying and assessing the quality of *M. pumila* Mill. Based on the analytical results that apple is rich in polyphenols, it could be a promising natural source for future industrial research of polyphenols with potential benefits for human health.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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