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Original article

Quality assessment of traditional herbal formula, Hyeonggaeyeongyo-tang through simultaneous determination of twenty marker components by HPLC–PDA and LC–MS/MS

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ABSTRACT

Simultaneous analysis of 20 marker components (gallic acid, cimifugin, geniposide, paeoniflorin, ferulic acid, nodakenin, narirutin, naringin, neohesperidin, arctiin, baicalin, oxypeucedanin hydrate, wogonoside, baicalein, arctigenin, glycyrrhizin, wogonin, pulegone, decursin, and decursinol angelate) for quality assessment of the traditional herbal formula, Hyeonggaeyeongyo-tang (HYT) was carried out by using high-performance liquid chromatography (HPLC) with photodiode array detection (PDA) and liquid chromatography–mass spectrometry with tandem mass spectrometry (LC–MS/MS). The coefficient of determination showed excellent linearity of more than 0.9999 for all analytes. The recovery of 20 marker components was 93.92 to 102.66% with relative standard deviation (RSD) < 3.00% and RSD value of precision was ≤ 3.44%. The amounts of 20 marker components using HPLC–PDA and LC–MS/MS were determined to be 0.18–14.60 and 0.01–1.76 mg/freeze-dried g, respectively.

1. Introduction

Generally, herbal medicines or herbal medicine prescriptions, which consist of two or more herbal medicines, contain many bioactive ingredients that can be effective in various diseases. Despite the increasing interest in herbal medicine prescriptions around the world, many quality assessment methods are still inadequate. Standardization is necessary to ensure constant efficacy and to assess the scientific evidence of today's herbs or formulations. Hyeonggaeyeongyo-tang (HYT), also known as Jing Jie Lian Qiao Tang in Chinese and Keigai-rengyo-to in Japanese, consists of 13 medicinal herbs, Schizonepetae Spica, Forsythiae Fructus, Saposhnikoviae Radix, Angelicae Gigantis Radix, Cnidii Rhizoma, Bupleuri Radix, Aurantii Fructus Immaturus, Scutellariae Radix, Angelicae Dahuricae Radix, Platycodonis Radix, Paeoniae Radix, Gardeniae Fructus, and Glycyrrhizae Radix et Rhizoma, in a 1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.4:1.0 ratio based on dry weight (Heo, 2007). HYT has been recorded in the Joseon Dynasty's *Dongeuibogam* since it was first recorded in *Wanbinghuichun* in the Mying Dynasty (Heo, 2007; Park and Hong, 2012). It has various reported biological effects on, for example, allergic rhinitis, as well as antioxidant, anti-inflammatory, and antimicrobial properties (Oh et al., 2003; Park et al., 2011; Park and Hong, 2012; Hong et al., 2014; Kim et al., 2016). The main components of each of the individual herbs forming HYT are: monoterpenoids (e.g., pulegone) from Schizonepetae Spica (Chun et al., 2010), lignans (e.g., arctigenin) from Forsythiae Fructus (Kang et al., 2008), flavonoids (e.g., cimifugin) and polyphenols (e.g., *prim*-*O*-glucosylcimifugin) from Saposhnikoviae Radix (Kim et al., 2011), coumarins (e.g., nodakenin, decursin, and decursinol angelate) from Angelicae Gigantis (Ahn et al., 2008), phenylpropanoids (e.g., ferulic acid) from Cnidii Rhizoma (Lu et al., 2005), triterpenoids (e.g., saikosaponin A) from Bupleuri Radix (Han and Lee, 1985), flavonoids (e.g., narirutin, naringin, and neohesperidin) from Aurantii Fructus Immaturus (Wang et al., 2010), flavonoids (e.g., baicalin and wogonoside) from Scutellariae Radix (Tong et al., 2012), coumarins (e.g., imperatorin and oxypeucedanin) from Angelicae Dahuricae Radix (Youn et al., 2010), triterpenoids (e.g., platycodin D) from Platycodonis Radix (Ha and Kim, 2009), monoterpenoids (e.g., paeoniflorin) and phenols (e.g.,

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gallic acid) from Paeoniae Radix (Xu et al., 2009), iridoids (e.g., geniposide) from Gardeniae Fructus (Lee et al., 2014), and triterpenoids (e.g., glycyrrhizin) and flavonoids (e.g., liquiritin apioside and liquiritin) from Glycyrrhizae Radix et Rhizoma (Wu et al., 2013). Recently, Matsumoto et al. (2018) reported a pharmacokinetic study of the bioactive flavonoids (apigenin, baicaein, genistein, hesperetin, liquiritigenin, luteolin, nagrigenin, and wogonin) of HYT that showed antibacterial effects on *Staphylococcus aureus* based on analysis by liquid chromatography–mass spectrometry with tandem mass spectrometry (LC–MS/MS). However, this study alone did not provide sufficient data for standardization of the traditional herbal formula, HYT. Therefore, we conducted simultaneous determination of 20 marker components for quality control of HYT based on analysis by high-performance liquid chromatography with photodiode array detection (HPLC–PDA) and by LC–MS/MS; the compounds examined were pulegone (Schizonepetae Spica), arctiin and arctigenin (Forsythiae Fructus), cimifugin (Saposhnikoviae Radix), nodakenin, decursin, and decursinol angelate (Angelicae Gigantis), ferulic acid (Cnidii Rhizoma), narirutin, naringin, and neohesperidin (Aurantii Fructus Immaturus), baicalin, baicalein, wogonin, and wogonoside (Scutellariae Radix), oxypeucedanin hydrate (Angelicae Dahuricae Radix), gallic acid and paeoniflorin (Paeoniae Radix), geniposide (Gardeniae Fructus), and glycyrrhizin (Glycyrrhizae Radix et Rhizoma).

2. Materials and methods

2.1. Plant materials

The 13 raw medicinal herbs constituting HYT (Table 1) were purchased from Kwangmyungdang Medicinal Herbs (KMH, Ulsan, Korea) in November 2017. The origin of each herb was confirmed by Dr. Seung-Yeol Oh, president of KMH, based on "The Dispensatory on the Visual and Organoleptic Examination of Herbal Medicine" (Lee, 2013). Voucher specimens (2017KE60–1 to 2017KE60–13) have been deposited at the Herbal Medicine Research Division, Korea Institute of Oriental Medicine.

2.2. Chemicals and reagents

The reference standard components, gallic acid (97.5–102.5%), naringin (95.0%), and baicalein (98.0%) were purchased from Merck KGaA (Darmstadt, Germany); cimifugin (98.0%), nodakenin (99.5%), oxypeucedanin hydrate (98.0%), and wogonin (98.9%) were purchased from ChemFaces Biochemical Co., Ltd. (Wuhan, China); narirutin (99.5%), glycyrrhizin (99.1%), and pulegone (99.3%) were purchased from Biopurify Phytochemicals (Chengdu, China); geniposide (≥98.0%) and baicalin (98.0%) were purchased from Wako Chemicals (Osaka, Japan); paeoniflorin (99.4%), ferulic acid (98.0%), neohesperidin (98.4%), arctiin (98.1%), wogonoside (98.9%), and arctigenin (99.4%) were purchased from Shanghai Sunny Biotech (Shanghai, China); and decursin (98.0%) and decursinol angelate (98.0%) were purchased from NPC Bio Technology (Yeongi, Korea). The chemical structures of these reference compounds are shown Fig. 1. HPLC-grade solvents (methanol, acetonitrile, and water) and ACS reagent-grade formic acid (≥98.0%) for test solution preparation and simultaneous analysis were purchased from J. T. Baker (Phillipsburg, NJ, USA) and Merck KGaA (Darmstadt, Germany), respectively.

2.3. Preparation of HYT water extract

The 13 crude herbs, Schizonepetae Spica, Forsythiae Fructus, Saposhnikoviae Radix, Angelicae Gigantis Radix, Cnidii Rhizoma, Bupleuri Radix, Aurantii Fructus Immaturus, Scutellariae Radix, Angelicae Dahuricae Radix, Platycodonis Radix, Paeoniae Radix, Gardeniae Fructus (393.24 g each), and Glycyrrhizae Radix et Rhizoma (281.12 g) constituting HYT were mixed and extracted in water for 2 h at 100 °C under pressure (98 kPa) using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The extracted solution was filtered and freeze-dried to give a powder sample. The amount of extracted HYT was 1296.4 g (25.9%).

2.4. Preparation of samples and standard solutions

To simultaneously analyse the 20 biomarker components (Fig. 1) in HYT extract, 100.0 mg of freeze-dried HYT sample was added to a 10 mL volumetric flask and 70% methanol was added. Ultrasonic extraction was then performed for 60 min at room temperature with a Branson 8510 ultrasonicator (Denbury, CT, USA). After extraction, the solution was filtered through a 0.2 μm GHP membrane filter (PALL Life Sciences, Ann Arbor, MI, USA) before HPLC injection. Standard solutions of the 20 reference standard analytes were prepared at a concentration of 1000.0 μg/mL using methanol, and each prepared stock solution was stored in a refrigerator until use.

2.5. HPLC–PDA equipment and conditions used for analysis of the 20 marker components

The HPLC system used for simultaneous analysis of the 20 biomarker constituents in HYT was a Shimadzu Prominence LC-20A series HPLC system (Kyoto, Japan) with a two pumps (LC-20AT),

Table 1
Composition of HYT.

Herbal medicine	Scientific name	Family	Part used	Origin	Composition ratio (%)
Schizonepetae Spica	*Schizonepeta tenuifolia* Briquet	Labiatae	Spike	Yeongcheon, Korea	7.86
Forsythiae Fructus	*Forsythia viridissima* Lindley	Oleaceae	Fruit	Uiseong, Korea	7.86
Saposhnikoviae Radix	*Saposhnikovia divaricate* Schischkin	Umbelliferae	Root	China	7.86
Angelicae Gigantis Radix	*Angelica gigas* Nakai	Umbelliferae	Root	Pyeongchang, Korea	7.86
Cnidii Rhizoma	*Cnidium officinale* Makino	Umbelliferae	Rhizome	Yeongyang, Korea	7.86
Bupleuri Radix	*Bupleurum falcatum* Linné	Umbelliferae	Root	Cheongsong, Korea	7.86
Aurantii Fructus Immaturus	*Citrus aurantium* Linné	Rutaceae	Fruit	China	7.86
Scutellariae Radix	*Scutellaria baicalensis* Georgi	Labiatae	Root	Yeosu, Korea	7.86
Angelicae Dahuricae Radix	*Angelica dahurica* Bentham et Hooker F.	Umbelliferae	Root	Yeongyang, Korea	7.86
Platycodonis Radix	*Platycodon grandiflorum* A. De Candolle	Campanulaceae	Root	Yeongju, Korea	7.86
Paeoniae Radix	*Paeonia lactiflora* Pallas	Paeoniaceae	Root	Uiseong, Korea	7.86
Gardeniae Fructus	*Gardenia jasminoides Ellis*	Rubiaceae	Fruit	Imsil, Korea	7.86
Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis Fischer*	Leguminosae	Root and Rhizome	China	5.68
				Total (%)	100.00

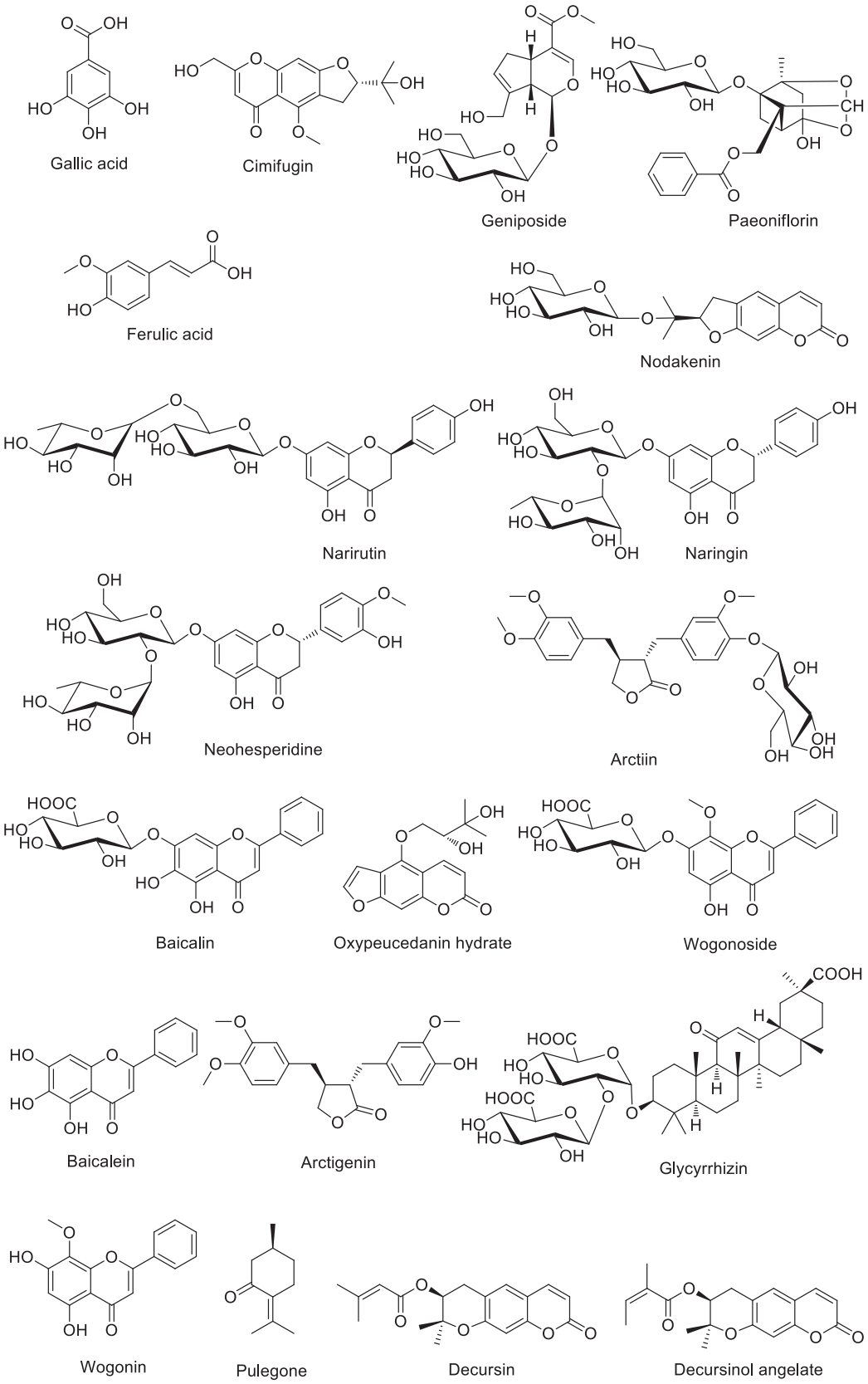

Fig. 1. Chemical structures of the 20 biomarker components in HYT.

Table 2
Linear range, calibration curve, r^2, LODs, and LOQs for marker compounds ($n = 3$) obtained by HPLC analysis.

Compound	Linear range (μg/mL)	Slope	Intercept	r^2	LOD[a] (μg/mL)	LOQ[b] (μg/mL)
Gallic acid	0.78–50.00	35948.04	−6660.81	0.9999	0.04	0.13
Cimifugin	0.78–50.00	39219.86	619.91	1.0000	0.10	0.32
Geniposide	1.56–100.00	24983.56	12795.82	0.9999	0.33	1.01
Paeoniflorin	1.56–100.00	16644.46	−471.60	1.0000	0.13	0.40
Ferulic acid	0.78–50.00	80306.61	17771.76	0.9999	0.18	0.55
Nodakenin	0.78–50.00	34392.55	7850.45	0.9999	0.20	0.61
Narirutin	0.78–50.00	17712.02	2844.50	0.9999	0.17	0.51
Naringin	3.13–200.00	18401.39	12604.61	0.9999	0.43	1.29
Neohesperidin	1.56–100.00	22196.83	7453.75	0.9999	0.28	0.86
Arctiin	0.78–50.00	6160.87	895.99	0.9999	0.11	0.33
Baicalin	3.13–200.00	40000.35	16407.75	0.9999	0.69	2.09
Oxypeucedanin hydrate	0.78–50.00	32886.81	8226.87	0.9999	0.18	0.53
Wogonoside	3.13–200.00	49151.40	28419.52	0.9999	0.58	1.77
Baicalein	0.78–50.00	61272.42	−5827.91	1.0000	0.09	0.28
Arctigenin	0.78–50.00	10745.95	1493.81	0.9999	0.14	0.41
Glycyrrhizin	3.13–200.00	9021.58	7572.14	0.9999	0.60	1.81
Wogonin	0.78–50.00	89117.36	10775.13	0.9999	0.14	0.42
Pulegone	0.78–50.00	20965.36	4642.95	0.9999	0.19	0.57
Decursin	0.78–50.00	40716.07	13179.77	0.9999	0.17	0.52
Decursinol angelate	0.78–50.00	26680.95	7563.47	0.9999	0.17	0.52

[a] $LOD = 3.3 \times \sigma/S$.
[b] $LOQ = 10 \times \sigma/S$.

Table 3
Recovery test for the assay of the 20 components in HYT using HPLC.

Compound	Original conc. (μg/mL)	Spiked conc. (μg/mL)	Found conc. (μg/mL)	Recovery (%)[a]	SD	RSD (%)
Gallic acid	6.59	1.00	7.58	99.00	1.64	1.66
		2.00	8.53	96.97	0.75	0.78
		4.00	10.53	98.45	1.41	1.43
Cimifugin	7.60	2.00	9.50	94.79	0.46	0.48
		4.00	11.54	98.35	1.00	1.01
		8.00	15.38	97.21	0.49	0.51
Geniposide	29.34	6.00	35.32	99.63	1.21	1.22
		15.00	44.25	99.35	0.46	0.46
		30.00	58.41	96.89	0.14	0.15
Paeoniflorin	27.82	6.00	33.75	98.88	2.04	2.07
		15.00	42.74	99.49	1.30	1.31
		30.00	58.32	101.69	0.34	0.34
Ferulic acid	3.35	1.00	4.33	97.90	1.24	1.26
		2.00	5.25	95.10	1.11	1.16
		4.00	7.21	96.41	0.51	0.53
Nodakenin	10.05	2.00	11.98	96.11	1.07	1.11
		5.00	14.97	98.22	0.40	0.41
		10.00	19.52	94.67	0.97	1.02
Narirutin	9.80	2.00	11.75	97.43	1.41	1.45
		5.00	14.75	98.98	1.58	1.60
		10.00	19.78	99.74	1.08	1.08
Naringin	41.24	8.00	49.24	99.99	0.95	0.95
		20.00	60.76	97.59	0.39	0.40
		40.00	81.88	101.60	0.84	0.83
Neohesperidin	28.67	6.00	34.45	96.25	0.84	0.87
		15.00	42.81	94.23	0.39	0.42
		30.00	57.09	94.73	0.38	0.40
Arctiin	10.77	2.00	12.76	99.49	2.15	2.17
		5.00	15.83	101.20	1.04	1.02
		10.00	20.65	98.72	1.22	1.23
Baicalin	70.64	14.00	84.79	101.05	0.57	0.56
		35.00	104.64	97.13	0.42	0.43
		70.00	137.82	95.96	0.81	0.84
Oxypeucedanin hydrate	4.43	1.00	5.38	94.70	0.79	0.83
		2.00	6.35	95.74	1.31	1.37
		4.00	8.19	93.92	0.67	0.72
Wogonoside	39.82	8.00	47.68	98.25	1.65	1.68
		20.00	58.96	95.72	0.76	0.80
		40.00	77.72	94.76	0.75	0.80

Table 3 (*continued*)

Compound	Original conc. (μg/mL)	Spiked conc. (μg/mL)	Found conc. (μg/mL)	Recovery (%)[a]	SD	RSD (%)
Baicalein	12.89	2.00	14.88	99.42	2.23	2.24
		4.00	16.82	98.14	2.45	2.49
		8.00	20.87	99.69	1.64	1.64
Arctigenin	8.36	2.00	10.34	98.87	1.32	1.34
		4.00	12.36	100.01	1.23	1.23
		8.00	16.16	97.57	1.78	1.83
Glycyrrhizin	37.22	8.00	45.07	98.24	2.80	2.85
		20.00	57.27	100.28	2.33	2.33
		40.00	76.16	97.37	0.64	0.65
Wogonin	1.76	1.00	2.73	96.97	1.23	1.27
		2.00	3.74	98.68	2.42	2.45
		4.00	5.65	97.23	1.27	1.31
Pulegone	2.82	1.00	3.80	98.15	2.82	2.87
		2.00	4.80	98.68	1.63	1.65
		4.00	6.63	95.28	1.59	1.67
Decursin	2.54	1.00	3.54	100.05	0.91	0.91
		2.00	4.48	96.67	0.86	0.89
		4.00	6.46	97.82	0.23	0.24
Decursinol angelate	2.13	1.00	3.12	98.78	0.62	0.62
		2.00	4.19	102.66	1.68	1.64
		4.00	6.13	99.81	0.50	0.50

[a] Recovery (%) = (Found conc. – Original conc.) / Spiked conc. × 100.

Table 4
Precision assay for the 20 marker compounds in HYT by HPLC.

Compound	Conc. (μg/mL)	Intraday (n = 5)			Interday (n = 5)			Repeatability (n = 6)	
		Observed conc. (μg/mL)	Precision (%)[a]	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)	RSD (%) of retention time	RSD (%) of peak area
Gallic acid	12.5	12.19	0.04	0.31	97.52	12.18	0.17	0.04	0.21
	25.0	24.28	0.10	0.40	97.13	24.35	0.46		
	50.0	49.00	0.18	0.37	97.99	49.15	0.43		
Cimifugin	12.5	12.47	0.15	1.22	99.78	12.58	0.38	0.02	0.38
	25.0	24.97	0.25	1.02	99.88	24.95	0.35		
	50.0	50.52	0.82	1.62	101.04	50.43	0.88		
Geniposide	25.0	24.20	0.42	1.75	96.82	24.51	0.81	0.01	0.22
	50.0	48.41	0.67	1.38	96.81	48.21	0.89		
	100.0	97.09	2.06	2.12	97.09	96.79	2.05		
Paeoniflorin	25.0	24.33	0.40	1.63	97.33	24.34	0.56	0.01	0.51
	50.0	50.01	0.74	1.48	100.01	49.86	1.15		
	100.0	100.48	0.35	0.35	100.48	100.34	1.36		
Ferulic acid	12.5	12.06	0.20	1.67	96.48	12.20	0.39	0.02	0.25
	25.0	24.12	0.33	1.37	96.49	24.04	0.43		
	50.0	48.54	1.01	2.08	97.09	48.43	1.04		
Nodakenin	12.5	12.06	0.21	1.74	96.44	12.21	0.42	0.02	0.24
	25.0	24.12	0.33	1.37	96.48	24.03	0.45		
	50.0	48.51	1.03	2.12	97.03	48.37	1.04		
Narirutin	12.5	12.05	0.07	0.55	96.39	12.01	0.08	0.02	0.24
	25.0	23.98	0.15	0.65	95.93	24.10	0.56		
	50.0	47.71	0.20	0.41	95.43	47.78	0.31		
Naringin	50.0	48.25	0.29	0.60	96.51	48.10	0.31	0.02	0.26
	100.0	96.07	0.61	0.64	96.07	96.38	1.64		
	200.0	191.14	0.82	0.43	95.57	191.39	1.29		
Neohesperidin	25.0	24.12	0.14	0.59	96.47	24.04	0.15	0.02	0.29
	50.0	48.00	0.28	0.58	96.00	47.94	0.40		
	100.0	95.51	0.36	0.38	95.51	95.64	0.61		
Arctiin	12.5	12.12	0.08	0.65	96.93	12.12	0.08	0.02	0.28
	25.0	24.13	0.13	0.52	96.53	24.13	0.22		
	50.0	47.98	0.16	0.34	95.95	48.11	0.26		
Baicalin	50.0	48.25	0.25	0.52	96.51	48.10	0.27	0.02	0.29
	100.0	96.12	0.48	0.50	96.12	95.99	0.83		
	200.0	191.54	0.63	0.33	95.77	191.80	1.23		
Oxypeucedanin hydrate	12.5	12.03	0.17	1.38	96.27	12.14	0.29	0.01	0.27
	25.0	24.16	0.34	1.41	96.63	24.07	0.44		
	50.0	48.48	1.03	2.12	96.96	48.38	1.04		

(*continued on next page*)

Table 4 (*continued*)

Compound	Conc. (μg/mL)	Intraday (n = 5)			Interday (n = 5)			Repeatability (n = 6)	
		Observed conc. (μg/mL)	Precision (%)[a]	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)	RSD (%) of retention time	RSD (%) of peak area
Wogonoside	50.0	48.22	0.31	0.65	96.43	48.07	0.29	0.01	0.33
	100.0	96.29	0.54	0.56	96.29	96.14	0.83		
	200.0	191.38	0.68	0.36	95.69	191.65	1.27		
Baicalein	12.5	12.12	0.07	0.58	96.97	12.10	0.12	0.01	0.24
	25.0	24.23	0.08	0.33	96.93	24.19	0.28		
	50.0	48.39	0.14	0.28	96.78	48.47	0.36		
Arctigenin	12.5	12.11	0.10	0.85	96.90	12.07	0.13	0.01	0.29
	25.0	24.05	0.19	0.79	96.21	24.02	0.25		
	50.0	47.85	0.22	0.45	95.71	47.88	0.34		
Glycyrrhizin	50.0	48.38	0.88	1.82	96.76	48.97	1.61	0.01	0.29
	100.0	96.64	1.44	1.49	96.64	96.32	1.76		
	200.0	194.31	4.22	2.17	97.16	193.51	4.16		
Wogonin	12.5	12.03	0.08	0.65	96.27	12.00	0.08	0.01	0.45
	25.0	23.99	0.14	0.59	95.97	23.93	0.23		
	50.0	47.60	0.21	0.44	95.20	47.78	0.35		
Pulegone	12.5	12.14	0.18	1.45	97.14	12.16	0.19	0.01	0.22
	25.0	24.29	0.30	1.24	97.17	24.15	0.41		
	50.0	48.38	0.71	1.46	96.77	48.32	0.81		
Decursin	12.5	12.07	0.20	1.66	96.55	12.17	0.29	0.01	0.31
	25.0	24.13	0.33	1.36	96.51	24.05	0.43		
	50.0	48.43	0.94	1.95	96.86	48.33	0.97		
Decursinol angelate	12.5	12.06	0.21	1.72	96.50	12.15	0.28	0.01	0.28
	25.0	24.11	0.34	1.41	96.44	24.05	0.42		
	50.0	48.41	0.97	2.01	96.83	48.32	0.97		

[a] Precision is expressed as RSD (%) = (SD / mean) × 100.

online degasser (DGU-20A_3), a forced air circulation type column oven (CTO-20A), automatic sample injector (SIL-20A), and PDA detector (SPD-M20A). All chromatographic signals were acquired and processed with LabSolution software (Version 5.53, SP3, Kyoto, Japan). A reverse-phase SunFire™ C_{18} analytical column (4.6 × 250 mm, 5 μm; Waters, Torrance, CA, USA) maintained at 40 °C was used for chromatographic separation. For efficient separation of all marker components, a gradient of 0.1% (v/v) aqueous formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B), was used: 0–50 min, 5–60% B; 50–60 min, 60–100% B; 60–70 min, 100% B; 70–80 min, 100–5% B. The flow rate of the mobile phase was 1.0 mL/min, and 10 μL of standard or test solution was injected.

2.6. LC–MS/MS apparatus and conditions for analysis of the 20 marker components

LC–MS/MS analysis of the 20 marker components in HYT was conducted with a Waters ACQUITY UPLC H-Class system (Milford, MA, USA) with a Xevo TQ-S micro triple quadrupole mass spectrometer (Milford) and ESI source. LC–MS/MS analysis of the 20 analytes was conducted with a Waters ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm) (Milford) maintained at 40 °C. Water with 0.1% (v/v) formic acid (A) and acetonitrile (B) were used as mobile phases with a gradient of 10% B (initial–1.0 min), 10–40% B (1.0–6.0 min), 40–95% B (6.0–10.0 min), 95% B (100–15.0 min), 95–10% B (15.0–15.1 min), and 10% B (15.1–18.0 min). The flow rate was 0.3 mL/min and injection volume of 2.0 μL were used. Samples were maintained at 5 °C. MS detection was conducted with a positive and negative ion ESI–MS/MS in the multiple reaction monitoring (MRM) mode. The optimized analytical parameters, collision energy, cone voltage, and transition, for MS/MS MRM analysis are summarized in Table 1S. Other MS parameters were as follows: capillary voltage, 1.2 kV; source temperature, 150 °C; desolvation temperature, 450 °C; desolvation gas flow, 800 L/h; and cone gas flow, 50 L/h. All data were acquired and processed with Waters MassLynx software version 4.2 (Milford).

2.7. Analytical method validation

The analytical method was validated with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, intraday and interday precision, and repeatability according to the International Conference on Harmonisation (ICH) guidance for Q2B validation of analytical procedures (International Conference on Harmonisation, 1996). Namely, linearity was evaluated by the coefficient of determination (r^2) of the calibration curve in the tested linear range of each compound. LOD and LOQ values were calculated as LOD = 3.3 × σ/S and LOQ = 10 × σ/S, based on the standard deviation of the *y*-intercept in each calibration curve (σ) and the slope of the calibration curve (S). Accuracy, tested as percentage recovery, was determined by using the standard addition method and calculated as Recovery (%) = (founded concentration – original concentration) / spiked concentration × 100. Intraday precision was determined by analyzing a single sample five times within a day and interday precision was determined by measuring the sample on three consecutive days. Repeatability was measured six times using a standard solution. These parameters, intraday and interday precision, and repeatability, were evaluated by calculating the relative standard deviation (RSD) and calculated by the following equation. RSD (%) = standard deviation (SD) / mean × 100. To assess the effectiveness of the HPLC analytical method, the system suitability parameters capacity factor (k'), selectivity factor (α), resolution (Rs), number of theoretical plates (N), and tailing factor (Tf) were calculated (Center for Drug Evaluation and Research, 1994).

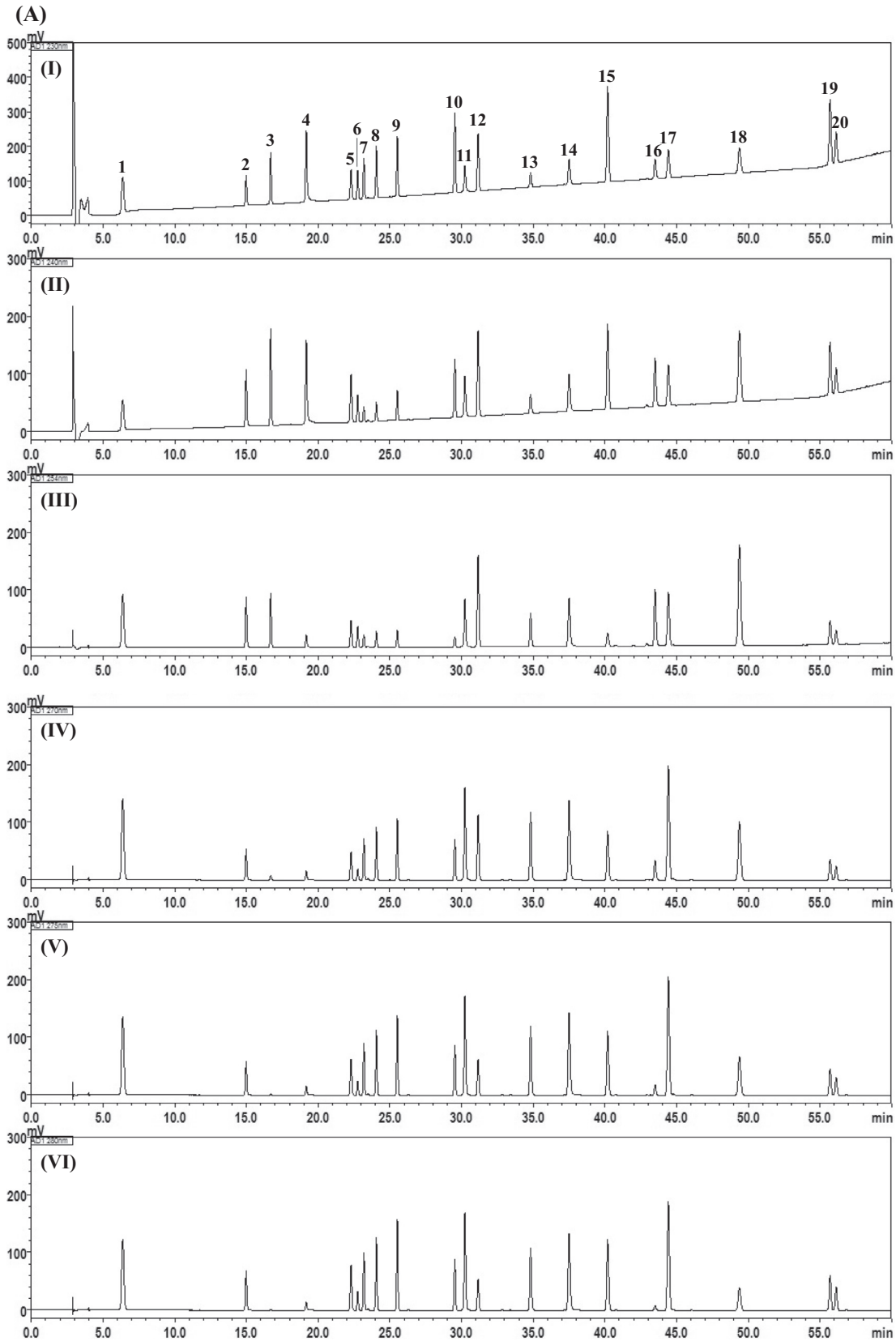

Fig. 2. HPLC chromatograms of the standard solutions (A) and HYT sample (B) at 230 nm (I), 240 nm (II), 254 nm (III), 270 nm (IV), 275 nm (V), 280 nm (VI), 310 nm (VII), 320 nm (VIII), 325 nm (IX), 330 nm (X), and 335 nm (XI). Gallic acid (**1**), cimifugin (**2**), geniposide (**3**), paeoniflorin (**4**), ferulic acid (**5**), nodakenin (**6**), narirutin (**7**), naringin (**8**), neohesperidin (**9**), arctiin (**10**), baicalin (**11**), oxypeucedanin hydrate (**12**), wogonoside (**13**), baicalein (**14**), arctigenin (**15**), glycyrrhizin (**16**), wogonin (**17**), pulegone (**18**), decursin (**19**), and decursinol angelate (**20**).

3. Results and discussion

3.1. Optimization of chromatographic conditions

A range of HPLC analysis conditions including column types (e.g., SunFire C_{18}, Gemini C_{18}, Capcellpak UG120 C_{18}, and OptimaPak C_{18} columns), column temperatures (e.g., 30, 35, 40, and 45 °C), and mobile phases (e.g., formic acid with distilled water and acetonitrile) were applied to separate the 20 marker compounds (Fig. 1) from a standard solution of HYT, and the separation efficiency of the conditions were assessed. Optimum HPLC analytical conditions were established in which all 20 marker components were efficiently separated with a resolution greater than 1.5 within 60 min using a SunFire C_{18} column (250 mm × 4.6 mm, 5 μm), a column temperature of 40 °C, and mobile phase system of distilled water–acetonitrile, both containing formic acid, as described in Section 2.5.

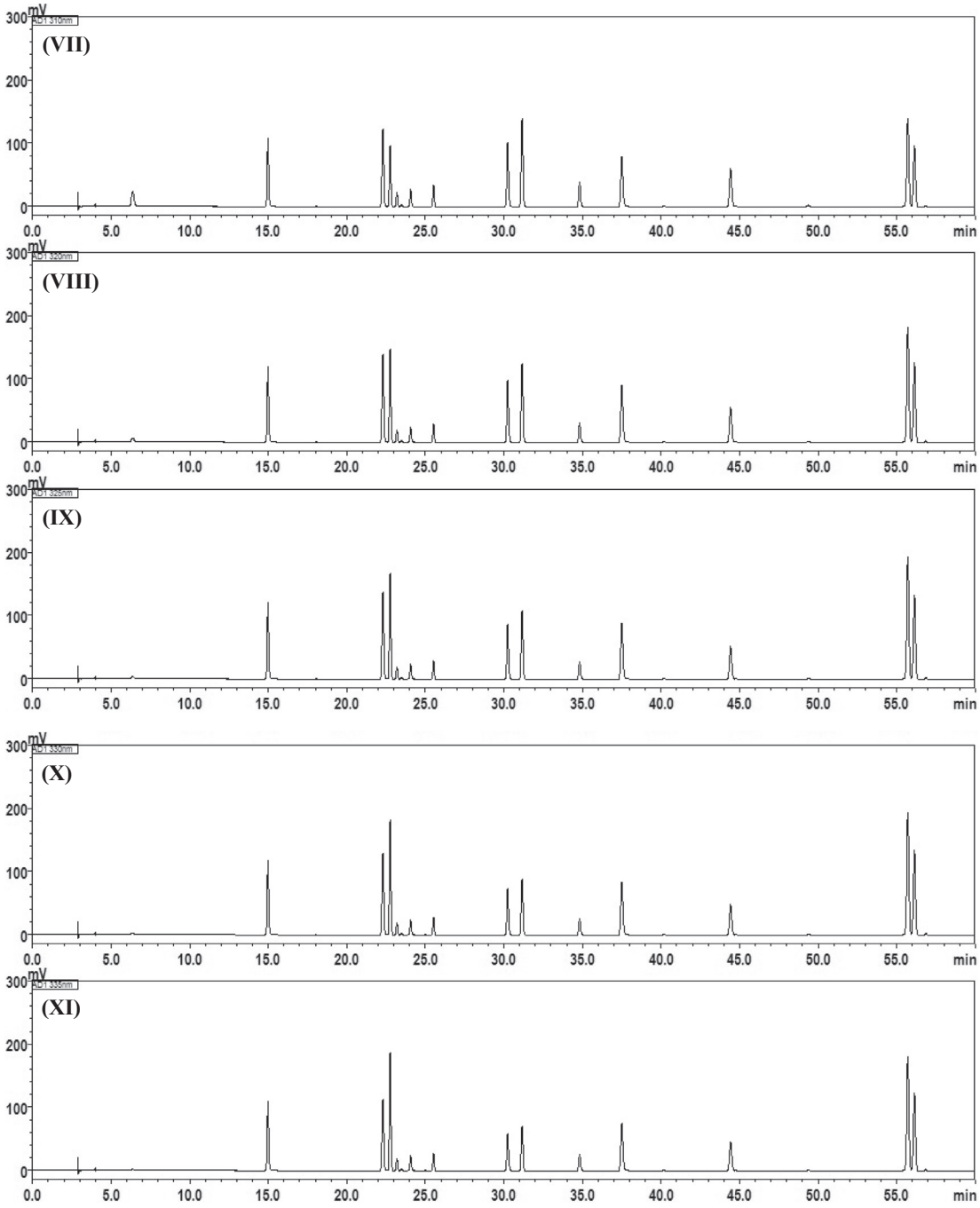

Fig. 2 (*continued*)

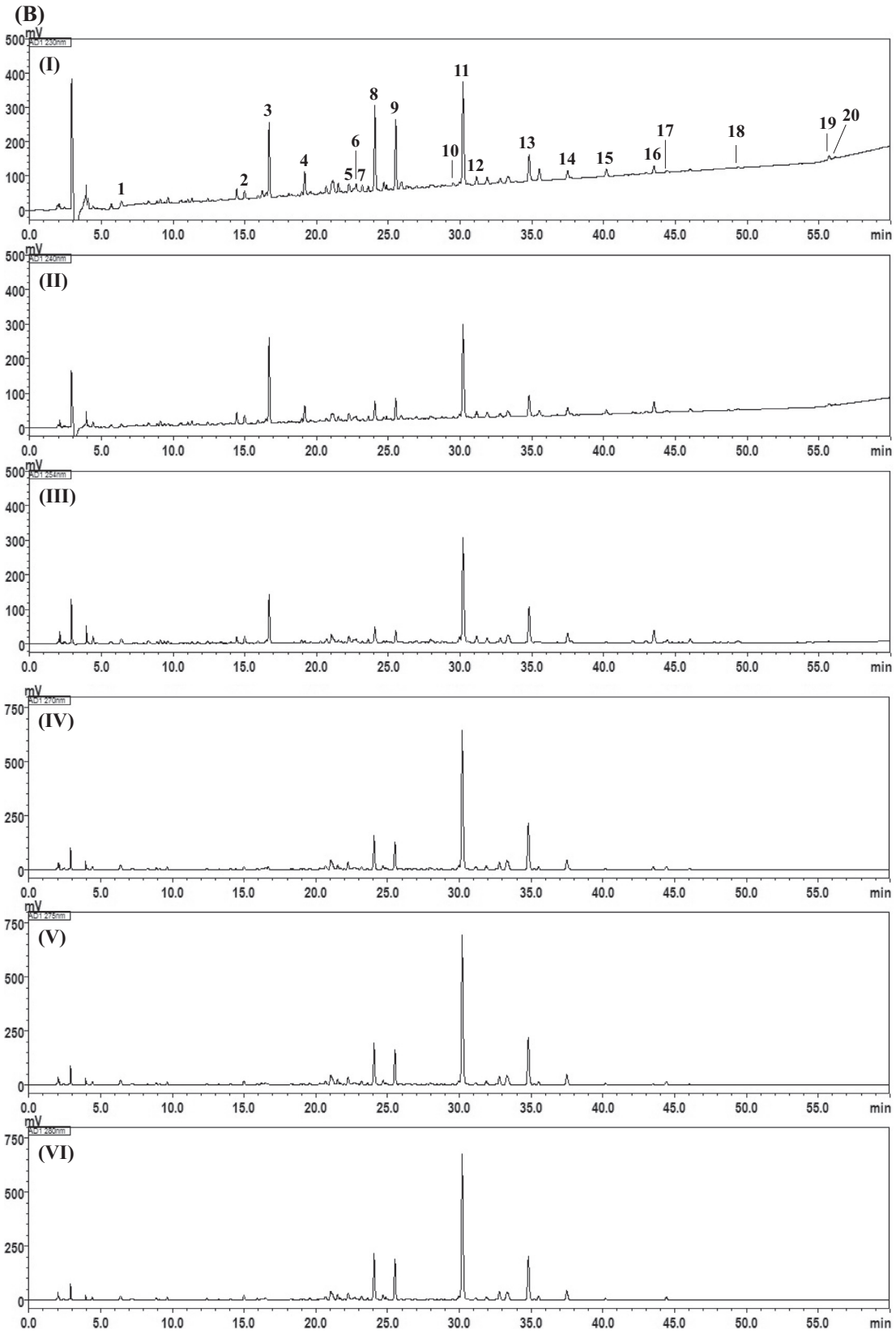

Fig. 2 (*continued*)

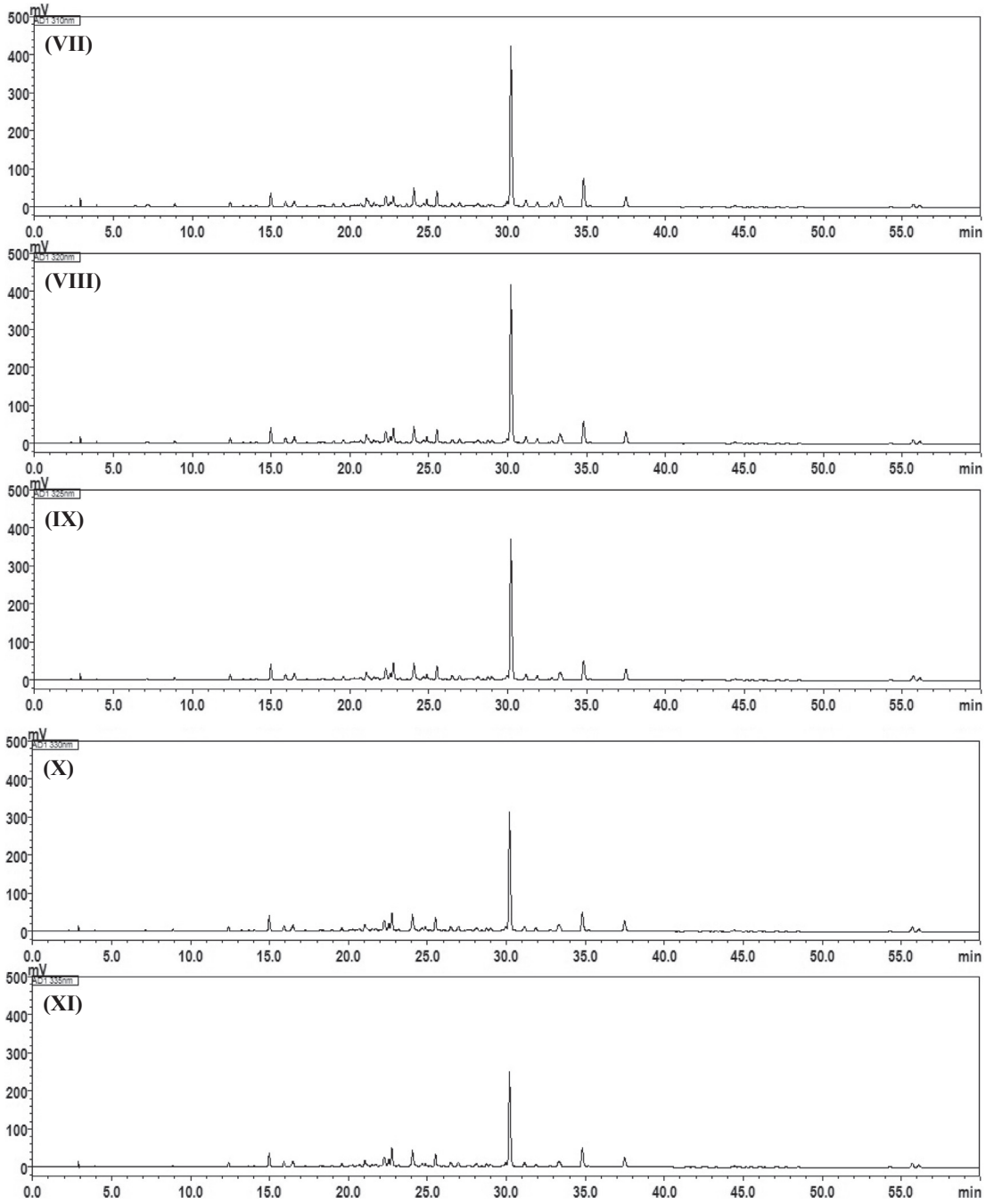

Fig. 2 (*continued*)

3.2. Method validation

3.2.1. HPLC analysis

System suitability parameters were calculated to characterize the assay for the analysis of the 20 marker components of HYT. The parameters *k′*, *α*, *N*, *Rs*, and *Tf* of the 20 marker components in the established HPLC analytical method were 1.20–18.37, 1.01–3.47, 2124.64–263946.70, 1.59–33.70, and 1.00–1.20, respectively (Table 2S). All calibration data, namely, the calibration equation, range, r^2, LOD, and LOQ are presented in Table 4 and the data showed good linearity with $r^2 \geq 0.9999$ over the tested concentration range. The LOD and LOQ values of all marker compounds were determined in the concentration range 0.09–0.69 μg/mL and 0.28–2.09 μg/mL, respectively (Table 2). The recovery measured for of all marker components at three concentration levels ranged from 93.92 to 102.66%, and RSD values were

Table 5
Amounts of the 20 marker components in the HYT using HPLC–PDA (n = 3).

Compound	Batch No.					
	1		2		3	
	Mean (mg/g) ±SD ($\times 10^{-2}$)	RSD (%)	Mean (mg/g) ±SD ($\times 10^{-2}$)	RSD (%)	Mean (mg/g) ±SD ($\times 10^{-2}$)	RSD (%)
Gallic acid	0.66 ± 1.03	1.56	0.66 ± 0.27	0.41	0.66 ± 0.33	0.50
Cimifugin	0.78 ± 1.85	2.36	0.78 ± 1.74	2.24	0.78 ± 1.13	1.45
Geniposide	6.13 ± 6.00	0.98	6.11 ± 0.74	0.12	6.10 ± 1.81	0.30
Paeoniflorin	3.17 ± 3.04	0.96	3.13 ± 6.49	2.07	3.17 ± 0.17	0.05
Ferulic acid	0.34 ± 0.43	1.29	0.34 ± 0.18	0.55	0.33 ± 0.05	0.15
Nodakenin	1.06 ± 0.94	0.89	1.04 ± 0.75	0.72	1.05 ± 0.40	0.39
Narirutin	1.00 ± 0.56	0.56	0.98 ± 0.75	0.77	0.99 ± 1.03	1.04
Naringin	8.27 ± 6.83	0.83	8.23 ± 2.41	0.29	8.22 ± 2.67	0.32
Neohesperidin	5.83 ± 4.08	0.70	5.84 ± 7.15	1.23	5.80 ± 1.76	0.30
Arctiin	1.21 ± 0.99	0.82	1.16 ± 1.45	1.25	1.21 ± 1.99	1.64
Baicalin	14.60 ± 14.64	1.00	14.56 ± 5.50	0.38	14.52 ± 4.87	0.34
Oxypeucedanin hydrate	0.43 ± 0.25	0.59	0.43 ± 0.84	1.97	0.42 ± 0.13	0.31
Wogonoside	4.04 ± 4.04	1.00	4.01 ± 0.62	0.15	4.01 ± 0.73	0.18
Baicalein	0.78 ± 1.10	1.41	0.78 ± 1.24	1.60	0.76 ± 1.22	1.60
Arctigenin	0.80 ± 0.41	0.52	0.79 ± 0.47	0.59	0.80 ± 0.42	0.53
Glycyrrhizin	3.76 ± 0.90	0.24	3.72 ± 3.81	1.03	3.76 ± 1.34	0.36
Wogonin	0.18 ± 0.09	0.48	0.18 ± 0.03	0.14	0.18 ± 0.02	0.10
Pulegone	0.28 ± 0.17	0.60	0.28 ± 0.18	0.63	0.28 ± 0.12	0.43
Decursin	0.25 ± 0.05	0.20	0.25 ± 0.19	0.76	0.25 ± 0.31	1.24
Decursinol angelate	0.21 ± 0.08	0.36	0.21 ± 0.07	0.34	0.21 ± 0.21	0.98

below 3.00% (Table 3). Furthermore, as shown in Table 4, good RSD values of $\leq$ 3.44% were measured for the intraday, interday, and repeatability measurements that established the precision. These results demonstrate that the optimized HPLC analytical method is suitable for simultaneous determination of the 20 marker components in HYT.

3.2.2. LC–MS/MS analysis

As shown in Table 1S and Fig. 1S, six components, gallic acid, geniposide, paeoniflorin, narirutin, naringin, and glycyrrhizin, were detected in the negative ion mode $[M-H]^-$ at *m/z* 169.30, 387.34, 479.17, 579.26, 579.45, and 821.56, respectively, whereas thirteen analytes, ferulic acid, cimifugin, nodakenin, neohesperidin, baicalin, oxypeucedanin hydrate, wogonoside, baicalein, arctigenin, wogonin, pulegone, decursin, and decursinol angelate, were detected in the positive ion mode $[M+H]^+$ at *m/z* 195.04, 307.19, 409.19, 611.26, 447.25, 305.23, 461.15, 271.17, 373.22, 285.14, 153.30, 329.19, and 329.19, respectively. Arctiin was detected at *m/z* 557.22 in the form of $[M+Na]^+$ in positive ion mode. The calibration curves of all the analytes had a coefficient of determination of more than 0.99, thus showing good linearity within the tested concentration range. The LOD and LOQ values of these compounds were calculated to be 0.09–0.69 μg/mL and 0.28–2.09 μg/mL based on signal-to-noise ratios of 3.3 and 10, respectively. The analytical parameters, calibration curve data, LOD, and LOQ, for quantitative analysis of each component are summarized in Table 3S.

3.3. Simultaneous determination of the 20 marker components in HYT samples by using HPLC–PDA

The established analytical method using HPLC–PDA was successfully applied for the simultaneous quantification of the 20 marker analytes (Fig. 1) for quality assessment of HYT samples. Samples were measured in triplicate for each batch. The origin of the peak corresponding to each analytic component was confirmed by comparing the UV spectrum and the retention time with those of the reference standard. Representative HPLC–PDA chromatograms of standard solutions and a HYT sample are shown in Fig. 2; all analytes eluted within 60 min. The assays revealed that the 20 marker analytes were found in the samples at 0.18–14.60 mg/freeze-dried g (Table 5).

3.4. Simultaneous determination of the 20 marker components in HYT samples using LC–MS/MS

The 20 marker components in HYT were analyzed and quantified using the optimized LC–MS/MS assays. Quantification of all analytes was conducted by using the MRM method in both positive and negative ion modes. The 20 components, gallic acid, geniposide, paeoniflorin, ferulic acid, cimifugin, nodakenin, narirutin, naringin, neohesperidin, arctiin, baicalin, oxypeucedanin hydrate, wogonoside, baicalein, arctigenin, glycyrrhizin, wogonin, pulegone, decursin, and decursinol angelate, were detected at 1.04, 3.59, 4.22, 4.65, 5.02, 5.03, 5.12, 5.30, 5.61, 5.78, 6.45, 6.58, 6.68, 7.84, 8.10, 8.19, 8.72, 9.38, 9.91, and 9.96 min, respectively (Fig. 3), and the amounts of these analytes in the samples were determined to be 0.01–1.76 mg/-lyophilized g (Table 6).

4. Conclusion

We have described the first quantitative analysis of 20 marker components of the oriental herbal prescription HYT by using a simple and convenient HPLC–PDA method and a more sensitive and selective LC–MS/MS MRM method. As a result of the analysis of all the marker analytes under the optimized analytical conditions, baicalin, the main ingredient of Scutellariae Radix, was found to be most abundant at 14.60 and 1.76 mg/freeze-dried g based on the HPLC–PDA method and LC–MS/MS method, respec-

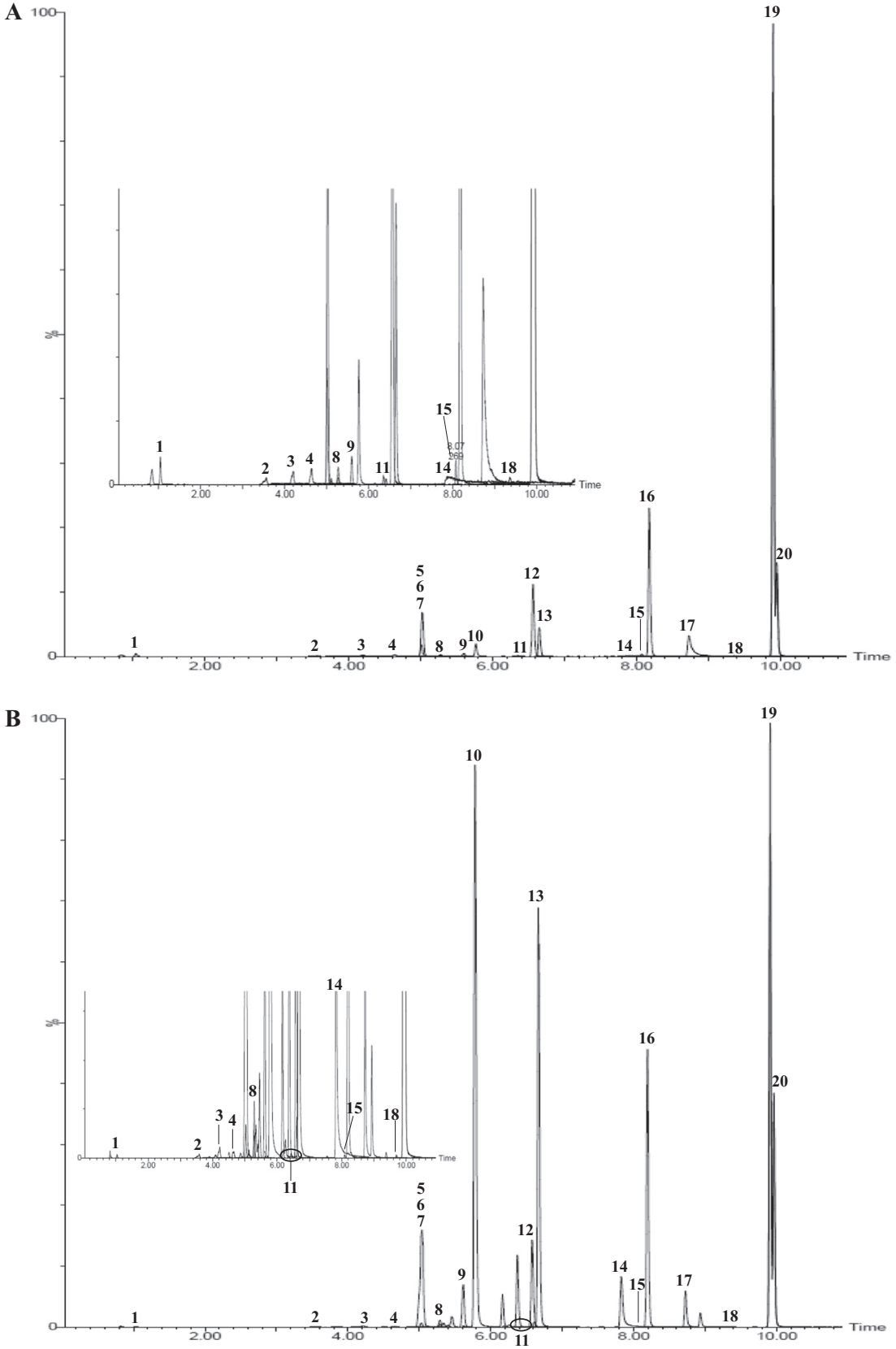

Fig. 3. Total ion chromatograms of the reference standard (A) and HYT sample (B) by LC–MS/MS MRM mode. Gallic acid (**1**), geniposide (**2**), paeoniflorin (**3**), ferulic acid (**4**), cimifugin (**5**), nodakenin (**6**), narirutin (**7**), naringin (**8**), neohesperidin (**9**), baicalin (**10**), arctiin (**11**), oxypeucedanin hydrate (**12**), wogonoside (**13**), baicalein (**14**), glycyrrhizin (**15**), arctigenin (**16**), wogonin (**17**), pulegone (**18**), decursin (**19**), and decursinol angelate (**20**).

Table 6
The amount of 20 marker components in the HYT using LC–MS/MS (n = 3).

Compound	Amount (mg/g)		
	Mean	SD ($\times 10^{-2}$)	RSD (%)
Gallic acid	0.11	0.36	3.39
Cimifugin	0.01	0.01	0.92
Geniposide	0.97	3.87	3.97
Paeoniflorin	0.47	2.20	4.65
Ferulic acid	0.03	0.09	3.25
Nodakenin	0.12	0.81	6.79
Narirutin	0.05	0.13	2.74
Naringin	0.67	0.88	1.30
Neohesperidin	0.61	4.00	6.56
Baicalin	1.76	4.71	2.67
Arctiin	0.05	0.14	2.89
Oxypeucedanin hydrate	0.04	0.19	4.28
Wogonoside	0.44	3.76	8.50
Baicalein	0.14	0.57	4.20
Arctigenin	0.10	0.37	3.81
Glycyrrhizin	0.44	0.75	1.70
Wogonin	0.03	0.11	3.23
Pulegone	0.04	0.26	6.12
Decursin	0.04	0.06	1.39
Decursinol angelate	0.11	0.62	5.71

tively. These HPLC–PDA and LC–MS methods are expected to be effective for quality evaluation of HYT and other related herbal formulas.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.02.003.

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