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Authors

Ka-Yan Kwok, Jun Xu, Hing-Man Ho, Hubiao Chen, Min Li, Yan Lang, and Quan-Bin Han

1 **Quality evaluation of commercial Huang-Lian-Jie-Du-Tang based**
2 **on simultaneous determination of fourteen major chemical**
3 **constituents using high performance liquid chromatography**

4
5 Ka-Yan Kwok^{1*}, Jun Xu^{1*}, Hing-Man Ho¹, Hu-Biao Chen¹, Min Li¹, Yan Lang^{2**}, Quan-Bin
6 Han^{1**}

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8
9 ¹*School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China;*

10 ²*First Affiliated Hospital, Zhengzhou University, Zhengzhou 450001, China*

11
12 *Equal contribution; **Corresponding authors: Q.B. Han, e-mail: simonhan@hkbu.edu.hk; Y.
13 Lang, email: graduatelly@163.com.

14

15 **Abstract:**

16 Huang-Lian-Jie-Du-Tang (HLJDT), comprising *Coptidis Rhizoma*, *Scutellariae Radix*, *Phellodendri Cortex*
17 and *Gardeniae Fructus*, is one of the commonly used Chinese medicine formulas for clearing heat and
18 detoxifying. Quality control of the herbal complex like Chinese medicine formulas still remains a challenge.
19 The successful approval of botanical drug Veregen by FDA indicated the importance of quantitative analysis in
20 quality control of herbal medicines. In this study, an effective quantitative method based on conventional
21 HPLC-DAD was developed for simultaneous determination of fourteen major ingredients (seven alkaloids,
22 four flavonoids, three terpenes) in HLJDT. The established method was well validated in terms of linearity,
23 sensitivity, precision, accuracy and stability and then successfully applied to quality evaluation of commercial
24 HLJDT samples. The developed method can quantitatively determine up to 70% of the chemicals of
25 commercial HLJDT sample and effectively revealed the significant variation in the quality of the commercial
26 HLJDT samples collected from different locations.

27

28 **Keywords:** Huang-Lian-Jie-Du-Tang, Chinese medicine formula, Quantitative analysis, Quality evaluation

29

30 **1. Introduction**

31 Huang-Lian-Jie-Du-Tang (HLJDT), one of often used Chinese medicinal prescriptions, which is composed
32 of four commonly used medicinal herbs, namely *Coptidis Rhizoma*, *Scutellariae Radix*, *Phellodendri Cortex*
33 and *Gardeniae Fructus* in 3:2:2:3 proportions, is historically employed for clearing heat and detoxifying [1].
34 Current studies manifested that alkaloids (mostly isoquinoline alkaloids) from *Coptidis Rhizoma* and
35 *Phellodendri Cortex*, flavonoids from *Scutellariae Radix* and terpenes (largely iridoid glycosides) from
36 *Gardeniae Fructus* are the major active components in HLJDT and therefore are normally regarded as markers
37 for quality control of HLJDT [2-7].

38 In view of the popular use of HLJDT in Asia countries and the lack of agreed quality standard for the
39 commercial HLJDT, quality control is crucial for ensuring its safety and efficacy. Additionally, because of the
40 complicated chemical properties of HLJDT, if possible, the method for quality evaluation of HLJDT should be

41 furthest comprehensive to take account of all kinds of active components. In recent years, seldom analytical
42 methods have been proposed and developed for quality evaluation of HLJDT. Even in these reported methods,
43 only eight or less compounds were used as chemical markers for quality evaluation of HLJDT [8-10]. These
44 methods are far powerful and reasonable since there are still many undetermined peaks (compounds) in the
45 chromatograms given in these studies. Furthermore, these methods were only applied to self-made but not any
46 commercial samples.

47 As known, it is very difficult for botanical drugs to get approved by FDA (Food and Drug Administration,
48 USA) due to their obscure and complicated chemical components. FDA asserted that comprehensive quality
49 control methods must be issued if the active ingredients of botanical drugs are not definitely confirmed [11].
50 Up to now, only two botanical drugs derived from herbal extracts with complex chemical profiles, namely
51 Fulyzaq and Veregen, were approved by FDA in 2013 and 2006 respectively, in which up to 90% components
52 were controllable [11, 12] . On the other hands, however, the quality control of herbal medicines are
53 commonly based on several chemical components, which only account for about 10% (even lower) total herbal
54 materials[13, 14]. It is obviously unreasonable and could not far meet the requirements of FDA. Thus,
55 developing more powerful quantitative methods is urgent for quality evaluation of herbal medicines, especially
56 traditional herbal formulas.

57 Hereby, a comprehensive analytical approach using multiple wavelengths HPLC-DAD on simultaneously
58 determination of fourteen components, including seven isoquinoline alkaloids (berberine, palmatine,
59 jatrorrhizine, coptisine, phellodendrine, epiberberine and magnoflorine), four flavonoids (baicalin, wogonin,
60 baicalein and wogonoside) and three terpenes (two iridoid glycosides: geniposide and genipin-1- β -D-
61 gentiobioside; one diterpene: crocin I) was established and validated, and then successfully applied to quality
62 evaluation of commercial HLJDT samples.

63

64 **2. Experimental**

65 *2.1. Chemicals and materials*

66 The commercial HLJDT samples (HLJDT-01 to HLJDT-09) were purchased from different pharmacy shops
67 in Zhejiang, Sichuan, and Taiwan provinces, China and sample HLJDT-10 was purchased from Tokyo, Japan

68 (Table 1). The voucher specimens were deposited at School of Chinese Medicine, Hong Kong Baptist
69 University, Hong Kong, China.

70 Methanol (HPLC grade) from RCI Labscan Ltd. (Bangkok, Thailand) and formic acid (analytical grade)
71 from Guangdong Chemical reagent Co. (Guangdong, China) were purchased. Deionized water was prepared
72 by Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

73 The reference compounds (Fig. 1), berberine, palmatine, jatrorrhizine, coptisine, epiberberine, magnoflorine,
74 geniposide, baicalin, baicalein, wogonoside, wogonin and crocin I were purchased from Shanghai Yuanye Bio-
75 Technology Co., Ltd. (Shanghai, China), and the other two reference compounds, phellodendrine and genipin-
76 1- β -D-gentiobioside were bought from Shanghai Shifong Bio-Technology Co., Ltd. (Shanghai, China). All of
77 them were confirmed by their MS spectra before use.

78 **2.2. Sample preparation**

79 The sample powder was homogeneously pulverized (60-80 mesh), accurately weighed (approximately
80 0.0200 g) and then ultrasonic extracted with 10 mL 70% methanol for 30 min. After the powder was well
81 dissolved by mechanical vibration (no any obvious megascopic particles could be found in the solution), the
82 solution were filtered through a 0.22 μ m nylon-membrane filter (Millipore, Barcelone) prior to injection into
83 the HPLC system.

84 **2.3. HPLC analysis.**

85 All analyses were performed on an Agilent Series 1100 (Agilent Technologies, USA) system, equipped with
86 a vacuum degasser, a quaternary pump, an auto-sampler, a column compartment, a diode-array detector,
87 controlled by Agilent 1100 LC software. The separation of fourteen analytes was achieved with a Diamonsil
88 C18 column (100 mm \times 4.6 mm i.d., 2.6 μ m) at 50 $^{\circ}$ C. The mobile phase consisted of (A) 0.1% formic acid
89 aqueous solution and (B) methanol containing 0.1% formic acid. The gradient elution was optimized as
90 follows: 15-38% B (0-15 min), 38-72% B (15-25 min), 72-15% B (25-30 min). The flow rate was 1.0 mL/min
91 and sample injection volume was 2 μ L. The fourteen analytes were simultaneously monitored at 254 nm
92 (geniposide, genipin-1- β -D-gentiobioside), 275 nm (berberine, palmatine, jatrorrhizine, coptisine, epiberberine,
93 magnoflorine, baicalin, baicalein, wogonoside and wogonin), and 440 nm (crocin I).

94 **2.4. Method validation**

95 The method for quantitative analysis was validated in terms of linearity, sensitivity, precision, accuracy and
96 stability. 70% methanol stock solutions of fourteen reference compounds were diluted to appropriate
97 concentrations for the construction of calibration curves. At least eight concentrations of the solution were
98 analyzed in duplicate, and then the calibration curves were constructed by plotting the peak areas versus the
99 concentration of each analyte. The stock solutions were diluted to a series of appropriate concentrations with
100 70% methanol, and an aliquot of the diluted solutions were injected into HPLC for analysis. The limits of
101 detection (LODs) and limits of quantification (LOQs) under the present chromatographic conditions were
102 determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. Intra- and inter-day variations were
103 chosen to determine the precision of the developed assay. For intra-day variability test, the sample HLJDT-10
104 was extracted and analyzed for six replicates within one day, while for inter-day variability test, the same
105 sample was examined in duplicates for consecutive three days. Variations were expressed by the RSDs of the
106 data. The spiked recovery test was used to evaluate the accuracy of the method. The recovery was performed
107 by adding a known amount of individual standards into a certain amount (0.0100 g) of sample HLJDT-10.
108 Three replicated were performed for the test. The spike recoveries were calculated by following equation:
109 Spike recovery (%) = (total amount detected – amount original)/amount spiked x 100%. The stability test was
110 performed by analyzing the sample (HLJDT-10) extract over period of 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, the RSDs
111 of the peak areas of each analyte were taken as the measures of stability.

112

113 **3. Results and discussion**

114 ***3.1. Method optimization***

115 In sample preparation procedure, multifarious solvents, such as different concentrations (10%, 30% 50%,
116 70% and 90%) of ethanol and methanol, were tested and 70% methanol solution was selected because of its
117 excellent dissolving capacity for HLJDT sample. For HPLC analysis, two mobile phase systems, including
118 acetonitrile-water and methanol-water, in various proportions were compared and different mobile phase
119 additive, such as phosphate buffer, formic acid and acetic acid were also investigated, and finally, 0.1% formic
120 acid aqueous solution and methanol containing 0.1% formic acid were used as mobile phases which could
121 provide satisfactory separation and peak shapes of all fourteen investigated compounds. In addition, determine

122 wavelengths for these different kinds of analytes were also optimized for improving sensitivity. Finally, in
123 consideration of corresponding maximum ultraviolet absorptions of the analytes, three ultraviolet wavelengths,
124 254 nm, 275 nm and 440 nm, were respectively chosen for detection.

125 **3.2. Method validation**

126 The linearity, regression and linear ranges of fourteen analytes were summarized in Table 2. The data
127 indicated good relationship between concentrations and peak areas of the analytes within the test ranges
128 ($R^2 \geq 0.9990$). The LOQs and LODs of all analytes were less than 1.06 and 0.41 $\mu\text{g/mL}$ on column, respectively.
129 It is worth mentioning that the LOD and LOD values in this study were much lower than those in previous
130 publications [8-10], which demonstrated that the newly developed method is more sensitive. The overall RSDs
131 of intra- and inter-day variations for fourteen analytes were not more than 4.49 % and 6.54 %, respectively.
132 The established method also had acceptable accuracy with spike recovery of 95.50-104.92 % for all analytes.
133 As to stability test, the RSDs of the peak areas for fourteen analytes detected within 24 h were lower than
134 4.25 %. All these results demonstrated that the developed HPLC method was sufficiently reliable and accurate
135 for simultaneous quantification of the fourteen investigated compounds in HLJDT.

136 **3.3. Quantification of fourteen analytes in commercial HLJDT samples**

137 The developed HPLC method was successfully employed for simultaneous determination of the fourteen
138 major active components in ten batches of commercial HLJDT samples collected from different localities.
139 Typical chromatograms of reference compounds (A) and HLJDT samples (B and C) were shown in Fig. 2. The
140 analytical time was greatly shortened compared with the previous publications while more analytes with ideal
141 resolution were quantified in this study [8-10]. The identification of the investigated compounds was carried
142 out by comparison of their retention time and UV spectra with reference chemicals. The contents of fourteen
143 investigated compounds in ten commercial HLJDT samples were summarized in Table 1.

144 As shown in Table 1, the contents of fourteen analytes varied greatly in different HLJDT samples. In the
145 sample HLJDT-10, all investigated compounds could be detected and baicalin was the most abundant. More
146 impressively, the total contents of fourteen analytes in this sample reached to 70.83% (of the sample material
147 weight), which inspired that the developed method might be quite suitable and reasonable for quality control of
148 commercial HLJDT since it could cover the majority of the components. As to the samples HLJDT 01-08,

149 however, the situation is quite different. Only part of the investigated compounds could be determined in these
150 samples with extremely low contents. The total contents of fourteen analytes in these samples existed in a
151 narrow range of about 0.03-1.78%. HLJDT-09 showed a significant improvement with the total content of
152 9.94%, which is still much lower than that of the HLJDT-10.

153 The greatly varied contents of the analytes in the investigated commercial HLJDT samples might be
154 attributed to several reasons. First, confused originals of the compositional herbal materials used in the HLJDT
155 sample preparation with different purposes might make their quality discrepancy. Herbal materials from GAP
156 (Good Agricultural Practices) farms are preferred for quality control purpose. It is also suggested that the
157 quality analysis method itself should not be tested only with self-made sample, validation with representative
158 real sample based on large number of sample batches is more necessary. Second, the inconsistent
159 manufacturing processes followed by different manufacturers, e.g. with/without excipients, could directly
160 affect the contents of individual ingredients. And the existence excipients could also influence the dissolubility
161 and detection of the investigated compounds in the samples. Furthermore, when we check back the instruction
162 of these commercial samples, it is found that all the samples (HLJDT 01-08) collected in China were
163 veterinary medicine while only those from Taiwan and Japan (HLJDT-09 and 10) were human use. The
164 different quality standards of HLJDT samples for veterinary and human use might directly lead to their distinct
165 quality, suggesting that more attention to the quality control of veterinary medicines is also burn-desired.

166 Compared to the reported methods, the current method unprecedentedly reach a high level quantitation of
167 the active ingredients beyond 70% of HLJDT commercial sample, which make it reasonable that Chinese
168 medicine formula, even much more complicated than a single botanical drug, could be brought under desired
169 quantitative control, if the manufacturing process is well designed and the quantitation method is considerate
170 enough.

171

172 **4. Conclusion**

173 In this study, an efficient HPLC-DAD analytical method using multiple UV wavelengths was established
174 and validated for simultaneous determination of fourteen major components, including seven isoquinoline
175 alkaloids, four flavonoids and three terpenes, and then successfully applied to quality evaluation of commercial

176 HLJDT products. The experimental results demonstrated that the developed method was very well validated
177 and effective, and therefore could make a contribution to the quality control of HLJDT products.

178

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181 13/006, and RC-start up grant).

182

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224

225

226

227 **Figure Legends**

228 Fig. 1 Chemical structures of fourteen investigated compounds

229 Fig. 2 Typical HPLC chromatograms of reference standards (A) and HLJDT samples (HLJDT-10 and HLJDT-
230 02) (B and C) with DAD detection under different wavelengths. 1, berberine; 2, coptisine; 3, palmatine; 4,
231 jatrorrhizine; 5, epiberberine; 6, magnoflorine; 7, phellodendrine; 8, baicalein; 9, wogonin; 10,
232 wogonoside; 11, baicalin; 12, geniposide; 13, genipin-1- β -D-gentiobioside; 14, crocin I

233 Tables:

234 Table 1 Contents of fourteen investigated compounds in ten batches of commercial HLJDT samples (mg/g).

235

236 Table 2 Calibration curves, LODs, LOQs, repeatability, accuracy and stability of the HPLC assay of fourteen
237 compounds.

Table 1 Contents of fourteen investigated compounds in ten batches of commercial HLJDT samples (mg/g)

Analyte	HLJDT-01	HLJDT-02	HLJDT-03	HLJDT-04	HLJDT-05	HLJDT-06	HLJDT-07	HLJDT-08	HLJDT-09	HLJDT-10
1 ^a	0.46 ^b (0.30) ^c	- ^d (0.05) ^e	- (0.05)	+ ^f (0.15) ^g	- (0.05)	- (0.05)	1.18 (1.40)	- (0.05)	- (0.05)	10.01 (0.30)
2	- (0.11)	- (0.11)	1.96 (3.11)	- (0.11)	0.57 (3.33)	- (0.11)	- (0.11)	- (0.11)	- (0.11)	16.49 (1.32)
3	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	13.34 (4.10)
4	- (0.05)	- (0.05)	- (0.05)	- (0.05)	- (0.05)	- (0.05)	- (0.05)	- (0.05)	- (0.05)	9.86 (1.03)
5	+ (0.15)	- (0.08)	1.90 (3.01)	- (0.08)	- (0.08)	- (0.08)	- (0.08)	- (0.08)	2.28 (2.34)	7.10 (1.15)
6	0.57 (0.37)	- (0.13)	1.46 (4.24)	- (0.13)	- (0.13)	- (0.13)	+ (0.35)	- (0.13)	0.95 (1.35)	2.36 (0.25)
7	- (0.15)	- (0.15)	- (0.15)	- (0.15)	- (0.15)	- (0.15)	+ (0.51)	- (0.15)	9.62 (0.75)	3.88 (1.01)
8	+ (0.53)	- (0.18)	1.58 (0.13)	+ (0.53)	- (0.18)	- (0.18)	2.85 (1.86)	+ (0.53)	2.01 (3.48)	57.26 (0.58)
9	4.30 (4.03)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	7.54 (0.71)
10	- (0.03)	- (0.03)	- (0.03)	- (0.03)	- (0.03)	- (0.03)	2.81 (2.20)	- (0.03)	28.80 (0.35)	69.13 (3.18)
11	1.99 (1.55)	0.29 (3.57)	8.10 (2.73)	- (0.06)	- (0.06)	0.91 (2.35)	1.28 (0.36)	1.43 (2.55)	47.28 (1.15)	414.17 (0.91)
12	1.15 (1.34)	- (0.05)	1.95 (0.25)	- (0.05)	1.76 (0.44)	+ (0.17)	2.59 (3.71)	1.04 (0.52)	7.87 (0.29)	62.46 (1.69)
13	- (0.21)	- (0.21)	0.85 (0.83)	- (0.21)	- (0.21)	- (0.21)	1.10 (0.81)	- (0.21)	+ (0.51)	13.12 (0.15)
14	- (0.02)	- (0.02)	- (0.02)	0.26 (1.24)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	- (0.02)	21.55 (1.42)
Total	8.47	0.29	17.80	0.26	2.33	0.91	11.81	2.47	98.81	708.27

^a The compound numbers are the same as in Fig. 2;

^b The data was present as average of triplicate determinations;

^c The RSD value of triplicate quantitative results (%);

^d Under the limit of detection (LOD);

^e LOD value of the corresponding analyte (mg/g);

^f Under the limit of quantification (LOQ);

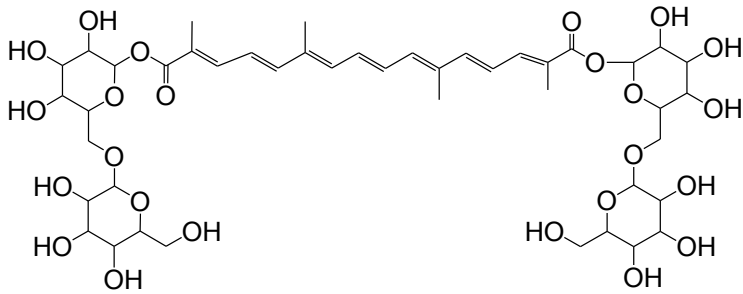
^g LOQ value of the corresponding analyte (mg/g)

Table 2 Calibration curves, LODs, LOQs, repeatability, accuracy and stability of the HPLC assay of fourteen compounds

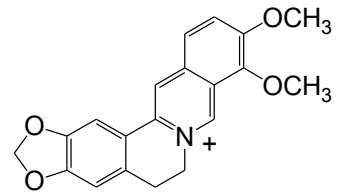
Analyte	Linearity		R ²	LOQ (µg/mL)	LOD (µg/mL)	Repeatability (RSD, %, n=6)		Spike recovery (RSD, %, n=3)			Stability (RSD, %, n=6)
	Range (µg/mL)	Equation				Intra-day	Inter-day	High	Middle	Low	
1 ^a	0.36~39.90	y=6.6673x-3.4768	0.9996	0.30	0.09	2.02	4.35	101.23% (1.41)	101.26% (2.71)	99.57% (2.39)	1.54
2	0.50~80.17	y=4.7851x-4.12	0.9995	0.45	0.22	2.97	4.37	101.45% (2.63)	104.11% (0.74)	100.12%(2.17)	2.68
3	1.00~71.40	y=12.03x-8.0755	0.9997	0.12	0.04	2.39	3.89	101.13% (3.23)	104.48% (4.07)	102.31%(3.02)	4.25
4	0.85~21.80	y=8.5564x-1.8856	0.9998	0.31	0.10	1.99	3.46	102.70% (3.92)	97.93% (1.13)	100.03% (3.89)	1.85
5	2.66~42.60	y=3.8716x-1.5643	1.0000	0.30	0.15	2.93	4.40	95.91% (1.54)	98.67% (0.83)	97.68% (0.64)	2.25
6	0.70~82.20	y=2.6718x-0.3366	1.0000	0.70	0.25	4.04	4.24	100.04% (1.32)	96.91% (1.92)	97.13% (1.63)	3.21
7	7.35~117.00	y=1.6177x+0.1395	1.0000	1.01	0.30	3.75	3.13	99.85% (3.61)	102.78% (3.83)	102.89%(4.22)	3.63
8	1.27~163.30	y=6.2962x-1.5128	0.9990	1.06	0.35	4.49	6.01	101.41% (2.78)	100.07% (3.41)	99.99% (1.63)	2.89
9	1.18~18.90	y=18.189x-6.1544	0.9997	0.14	0.04	4.36	4.69	96.81% (1.68)	102.95% (0.70)	102.29%(1.40)	1.89
10	1.02~178.50	y=7.5572x-1.0339	1.0000	0.19	0.05	2.71	4.25	100.78% (2.52)	103.20% (1.32)	97.18% (3.26)	4.21
11	0.47~1048.50	y=4.8022x-10.469	0.9993	0.39	0.12	2.32	3.97	102.33% (4.64)	104.83% (0.52)	104.92%(0.32)	3.20
12	0.62~187.00	y=2.0377x-2.8414	0.9993	0.34	0.09	2.93	4.26	97.47%(1.98)	102.98% (1.67)	99.11% (3.76)	2.96
13	1.50~75.00	y=2.2663x-0.8219	0.9999	1.02	0.41	4.36	6.54	96.99% (0.67)	99.91% (4.03)	98.56% (0.83)	2.21
14	0.30~177.70	y=11.583x+3.8005	0.9999	0.08	0.03	2.36	2.58	96.25% (1.19)	95.50% (3.25)	95.62% (2.23)	3.25

^a The compound numbers are the same as in Fig. 2

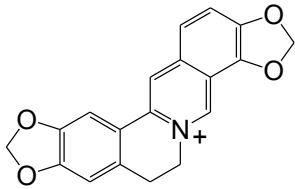
Figure(s)



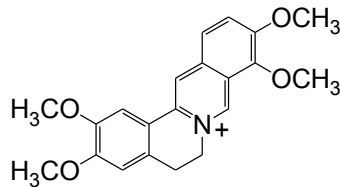
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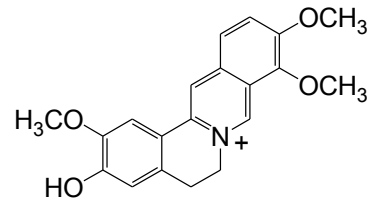
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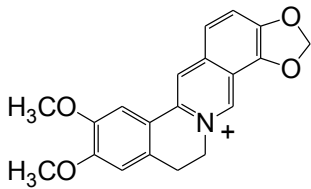
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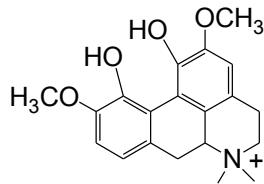
Palmatine



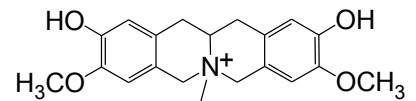
Jatrorrhizine



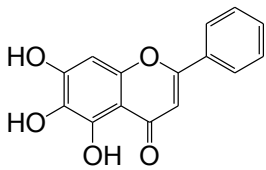
Epiberberine



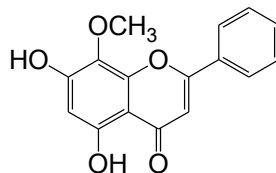
Magnoflorine



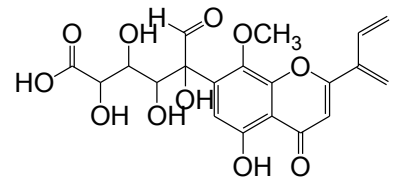
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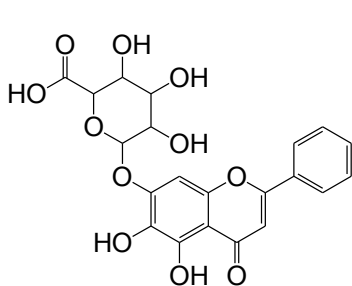
Baicalein



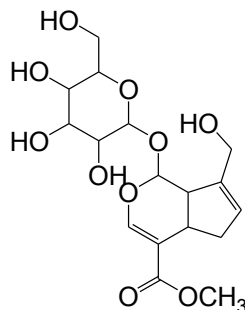
Wogonin



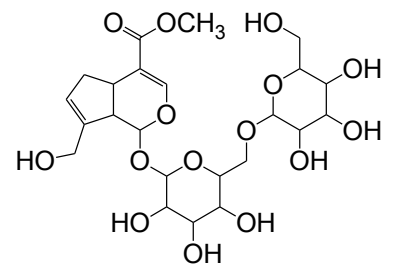
Wogonoside



Baicalin



Geniposide



Genipin-1-β-D-gentiobioside

Fig. 1

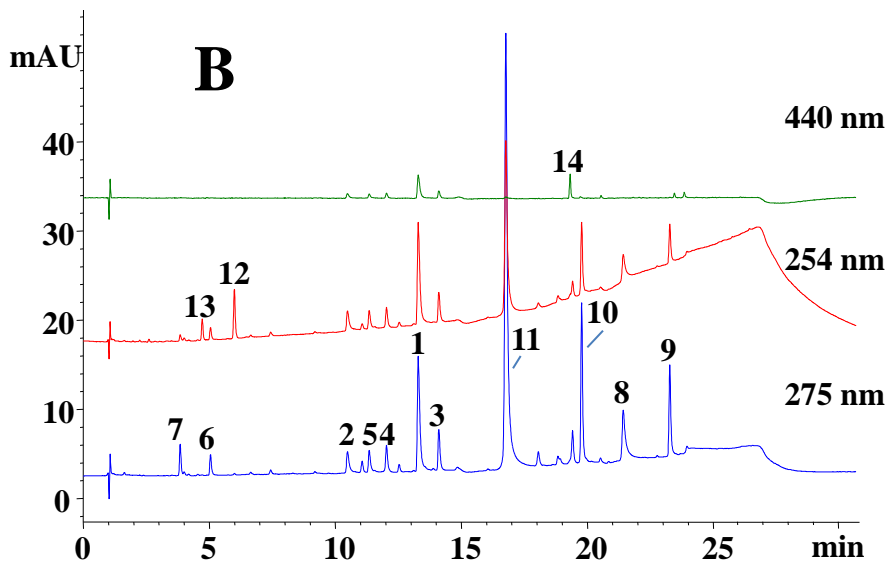
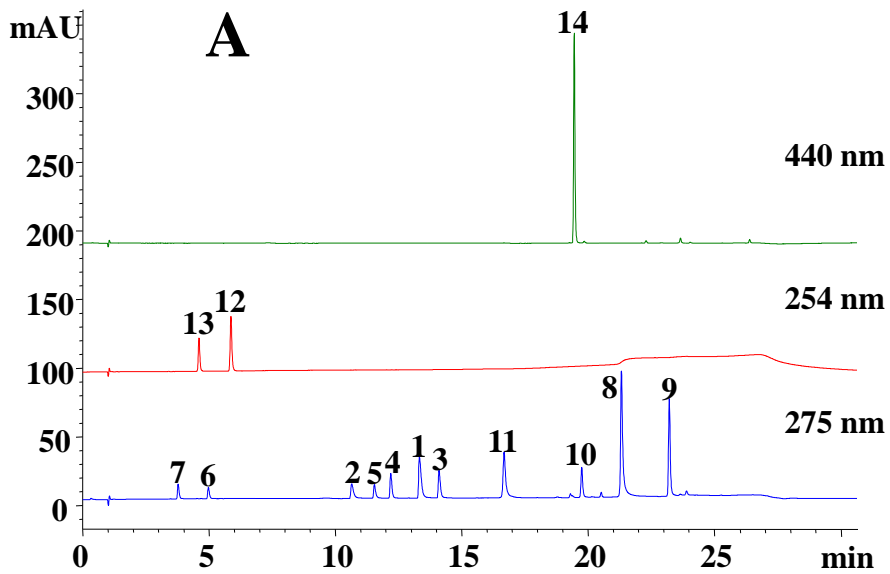


Fig. 2