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

Ka-Yan Kwok[1]*, Jun Xu[1]*, Hing-Man Ho[1], Hu-Biao Chen[1], Min Li[1], Yan Lang[2]**, Quan-Bin Han[1]**

[1]*School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China;*

[2] *First Affiliated Hospital, Zhengzhou University, Zhengzhou 450001, China*

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

Abstract:

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

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

1. Introduction

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

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

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

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

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

2. Experimental

2.1. Chemicals and materials

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

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

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

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

2.2. Sample preparation

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

2.3. HPLC analysis.

All analyses were performed on an Agilent Series 1100 (Agilent Technologies, USA) system, equipped with a vacuum degasser, a quaternary pump, an auto-sampler, a column compartment, a diode-array detector, controlled by Agilent 1100 LC software. The separation of fourteen analytes was achieved with a Diamonsil C18 column (100 mm×4.6 mm i.d., 2.6 μm) at 50 °C. The mobile phase consisted of (A) 0.1% formic acid aqueous solution and (B) methanol containing 0.1% formic acid. The gradient elution was optimized as 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 and sample injection volume was 2 μL. The fourteen analytes were simultaneously monitored at 254 nm (geniposide, genipin-1-β-D-gentiobioside), 275 nm (berberine, palmatine, jatrorrhizine, coptisine, epiberberine, magnoflorine, baicalin, baicalein, wogonoside and wogonin), and 440 nm (crocin I).

2.4. Method validation

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

3. Results and discussion

3.1. Method optimization

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

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

3.2. Method validation

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

3.3. Quantification of fourteen analytes in commercial HLJDT samples

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

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

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

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

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

4. Conclusion

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

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

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Figure Legends

Fig. 1 Chemical structures of fourteen investigated compounds

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

Tables:

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

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

Table(s)

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

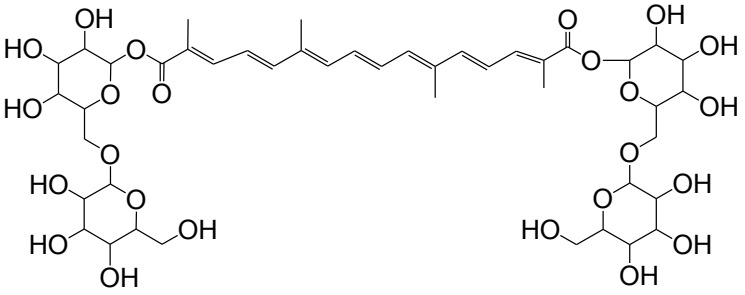
[a] The compound numbers are the same as in Fig. 2;
[b] The data was present as average of triplicate determinations;
[c] The RSD value of triplicate quantitative results (%);
[d] Under the limit of detection (LOD);
[e] LOD value of the corresponding analyte (mg/g);
[f] Under the limit of quantification (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

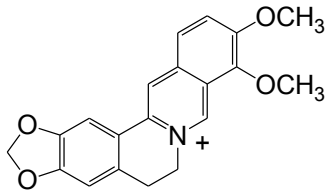
Analyte	Linearity			LOQ	LOD	Repeatability (RSD, %, n=6)		Spike recovery (RSD, %, n=3)			Stability
	Range (μg/mL)	Equation	R^2	(μg/mL)	(μg/mL)	Intra-day	Inter-day	High	Middle	Low	(RSD, %, n=6)
1 [a]	0.36~39.90	y=6.6673x-3.4768	0.9996	0.30	0.09	2.02	4.35	101.23% (1.41)	101.26% (2.71)	99.57% (2.39)	1.54
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

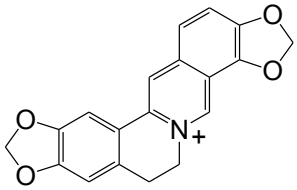
Figure(s)



Crocin I



Berberine



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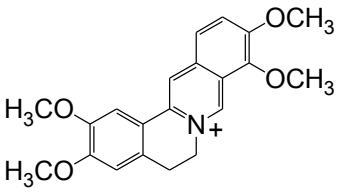
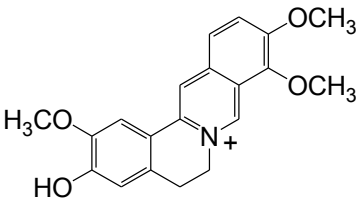
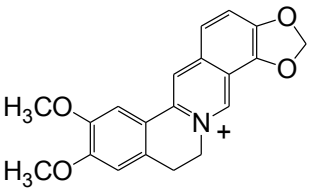


Palmatine



Jatrorrihizine



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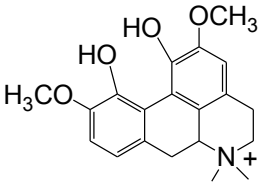

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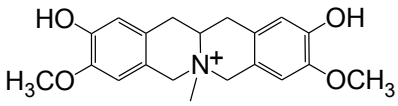
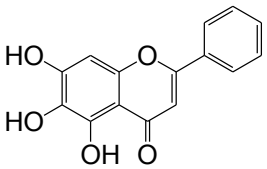
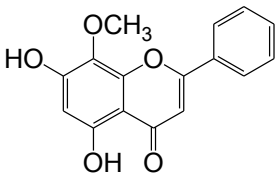


Phellodendrine



Baicalein



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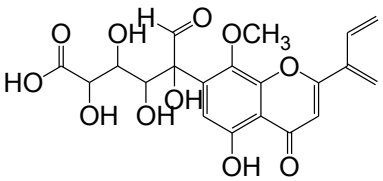

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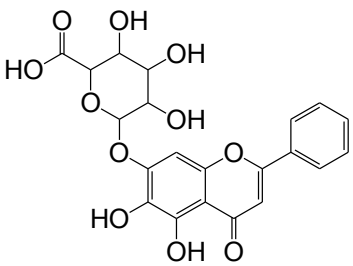

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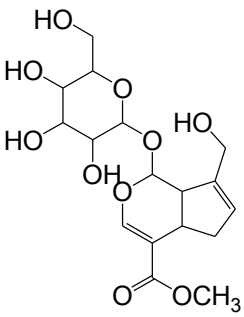
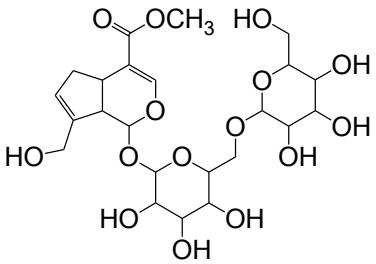
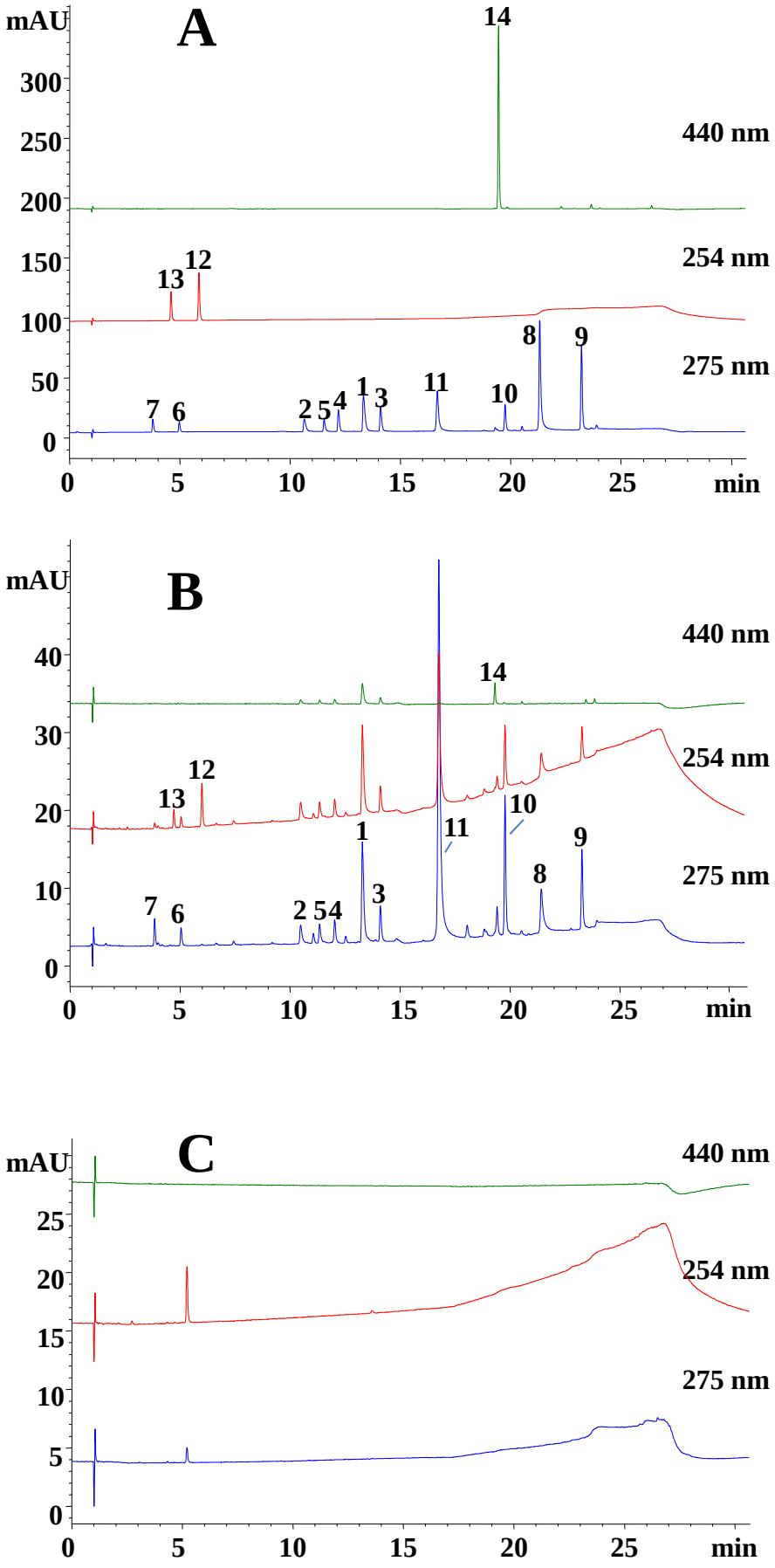


Geniposide



Genipin-1-ß-D-gentiobioside

Fig. 1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4
1
3
11
10
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B
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Fig. 2