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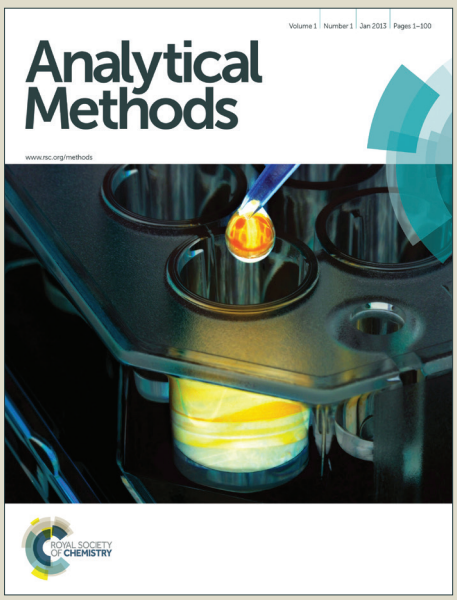

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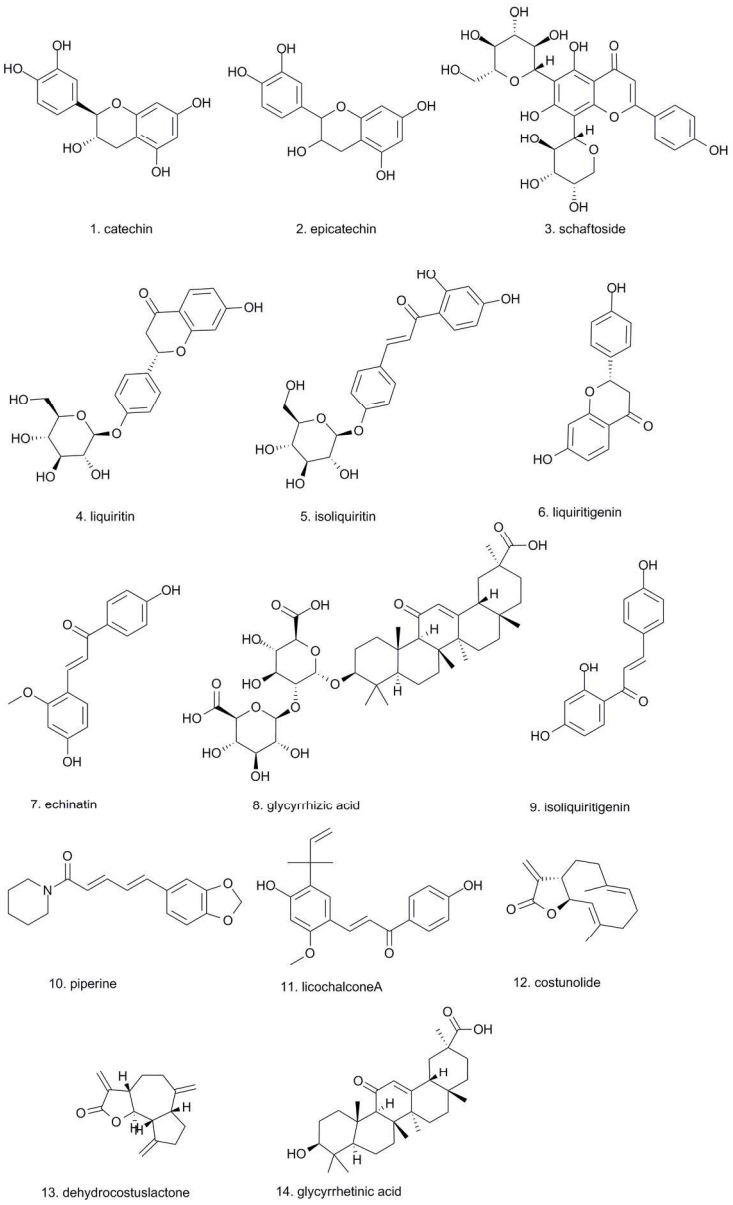

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Simultaneous determination of 14 major components in Longhu Rendan pills by ultra-high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry

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ABSTRACT: A novel method based on ultra-high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry was developed for the simultaneous determination of the 14 major active constituents of Longhu Rendan pills. These 14 compounds were separated within 20 min in a C18 column (2.1 mm i.d. × 100 mm, 3 μm), and good linearity was achieved (r > 0.9980). Gradient elution was applied using a mobile phase of 0.01% formic acid containing 0.2 mM ammonium formate/acetonitrile. The analytes were quantified on an LCQ ion trap mass spectrometer in electrospray ionisation full-scan mode. Variations in the intra- and inter-day precisions of all analytes were below 4.60%, and the accuracy was evaluated by a recovery test within 94.41% to 103.39%. The method successfully quantified the 14 compounds in three sample batches of Longhu Rendan pills. Therefore, our method enables the highly accurate, sensitive and reliable determination of 14 major active constituents, which can aid the quality control investigation of Longhu Rendan pills.

Keywords: Longhu Rendan pill; ultra-high-performance liquid chromatography-mass spectrometry; quality control

1 Introduction

Heatstroke and motion sickness are common illnesses. In China, traditional Chinese medicine (TCM) has been used for thousands of years to prevent and treat heatstroke and motion sickness. Longhu Rendan pills (LRP), which consist of *Mentholum*, *Borneolum Synthcticum*, *Flos Caryophylli*, *Fructus Anisi Stellati*, *Radix Aucklandiae*, *Fructus Amomi*, *Cortex Cinnamomi*, *Fructus Piperis*, *Rhizoma Zingiberis*, *Catechu* and *Radix Glycytthizae*. LRP are one of the widely used traditional Chinese over-the-counter medicines and are authorised for sale by the state food and drug administration (SFDA) of China (NO.Z20025168) for the prevention and treatment of heat stroke and motion sickness. Experimental study has proven that LRP produce significant anti-heat stroke, anti-motion sickness and peripheral antiemetic effects in rats.[1] The annual sales volume of LRP has exceeded one hundred million RMB (~$16 million U.S.) since 2011.

Heat stroke is a life-threatening illness that is clinically characterised by severe hyperthermia and multiple organ damage, such as cardiovascular and central nervous system dysfunction, caused by a complex interplay among heat cytotoxicity, coagulopathies and the systemic inflammatory response syndrome induced by intestinal endotoxins. The combination of rapid cooling, supportive care, anti-inflammatory, anti-endotoxin, antioxidant, antipyretic, anti-coagulation and anti-shock treatments with naloxone can be utilised in clinical settings to alleviate the symptoms of heat stroke with neuroprotection.[2] Motion sickness is a common disturbance of the inner ear that is caused by acceleration motions in cars, trains, airplanes and boats. The main symptoms of motion sickness are headache, dizziness, fatigue, nausea and vomiting triggered by changes in neurotransmitter levels, such as increased histamine and acetylcholine and decreased norepinephrine, within the brain. In clinical settings, anticholinergic, antihistamine, central inhibition and promotion of gastrointestinal motility effects can help

alleviate motion sickness.[3, 4] LRP contain diverse classes of compounds, such as the polyphenols catechin (**1**) and epicatechin (**2**); the flavonoids schaftoside (**3**), liquiritin (**4**), isoliquiritin (**5**), liquiritigenin (**6**), echinatin (**7**), isoliquiritigenin (**9**) and licochalcone A (**11**); the triterpenoids glycyrrhizic acid (**8**) and glycyrrhetinic acid (**14**); the amide piperine (**10**); and the sesquiterpenoids costunolide (**12**) and dehydrocostuslactone (**13**). Catechin,[5] schaftoside,[6] isoliquiritigenin,[7] licochalcone A,[8] glycyrrhizic acid,[9] glycyrrhetinic acid[10] and dehydrocostuslactone[11] have anti-inflammatory effects; catechin,[12] epicatechin,[13] echinatin,[14] isoliquiritigenin[15] and glycyrrhizic acid[16] have anti-oxidation effects; catechin,[17] liquiritin,[18] isoliquiritigenin,[19] glycyrrhizic acid,[20] piperine[21] and costunolide[22] have neuroprotective effects; piperine has antipyretic effects;[23] and costunolide has antispasmodic effects.[24] Therefore, these compounds possibly contribute to the preventive and therapeutic effects of LRP on heat stroke and motion sickness.

According to the regulatory documents published by SFDA and reported literature, catechin and epicatechin, which were detected by high-performance liquid chromatography (HPLC), and menthol and borneol, which were detected by gas chromatography (GC), were chosen as "marked compounds" for the quality control of LRP. However, quantitative analysis of only a limited number of components in herbal medicine formulas may not be adequate. Thus, determining the other components of LRP is necessary to ensure the reliability and repeatability of quality assessments.

In the present study, we developed an accurate, sensitive and reliable method of ultra-high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry (UHPLC-ESI-MS) to determine the active compounds of LRP. These compounds include catechin, epicatechin, schaftoside, liquiritin, isoliquiritin, liquiritigenin, echinatin,

glycyrrhizic acid, isoliquiritigenin, piperine, licochalcone A, costunolide, dehydrocostuslactone and glycyrrhetinic acid (Figure 1). The method successfully detected these 14 compounds in three batches of LRP samples.

2 Experimental

2.1 Reagents and materials

Catechin, epicatechin, liquiritin, glycyrrhizic acid, piperine and glycyrrhetinic acid were purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Schaftoside, echinatin, isoliquiritigenin and licochalcone A were obtained from Shanghai Yuanye Bio-Technology Company (Shanghai, China). Isoliquiritin and liquiritigenin were purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). Costunolide and dehydrocostuslactone were obtained from Nanjing Spring and Autumn Biological Engineering Co., Ltd. (Nanjing, China). The purities of all reference compounds were greater than 98% according to HPLC analysis. LRP were provided by Shanghai Zhonghua Pharmaceutical Co., Ltd. (Shanghai, China).

Acetonitrile and methanol were of HPLC grade from Burdick and Jackson Company (Ulsan, Korea). Formic acid (HPLC grade) was purchased from CNW Technologies GmbH Company (Düsseldorf, Germany). Ammonium formate (HPLC grade) was obtained from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Acetic acid (HPLC grade) was purchased from Tedia Company (Fairfield, USA). Ultra-pure water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

2.2 Chromatography and MS conditions

Chromatographic analysis was performed on a Shimadzu UHPLC-XR system (Shimadzu, Japan) equipped with a binary solvent delivery system, a vacuum degasser, an autosampler and a column compartment. Chromatographic separation was performed on a Thermo BDS Hypersil C18 column (2.1 mm i.d. × 100 mm, 3 μm) maintained at 30 °C. The mobile phase consisted of 0.01% formic acid containing 0.2 mM ammonium formate (A) and acetonitrile (B) with a gradient elution of 10% B from 0 min to 1.5 min, 10% to 80% B from 1.5 min to 15 min, 80% B from 15 min to 16 min and 10% B from 16 min to 20 min with a flow rate of 0.25 mL/min. Mass spectra were acquired using an LCQ ion trap mass spectrometer (Thermo Fisher Scientific, USA) equipped with an electrospray ionisation (ESI) source that switches between positive and negative polarity mode in a single run. The mass spectrometer parameters were ion spray voltage at 5000 V (+) or 4500 V (–), capillary voltage at 26 V (+) or 37 V (–), capillary temperature at 300 °C, sheath gas flow rate at 40 psi and auxiliary gas flow rate at 5 psi.

2.3 Sample preparation

Powdered LRP (30 mg) were extracted with 10 mL of 30% acetonitrile in an ultrasonic bath for 20 min. The extracted solution was centrifuged at 12,000 rpm for 10 min. A 10 μL aliquot of the supernatant was injected into the UHPLC-MS system for analysis.

2.4 Preparation of standard solutions

The reference standards were accurately weighed and dissolved in acetonitrile to prepare stock solutions. All standards were completely dissolved in the mixed standard working solution. Mixed standard working solutions containing 14 compounds were obtained by diluting the stock solutions with acetonitrile–water (30:70, v/v) to a series of appropriate concentration ranges for

the construction of calibration curves. A mixed standard working solution was prepared before use. The stock and working solutions were stored in a refrigerator at 4 °C.

2.5 Method validation

2.5.1 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

A mixed standard working solution containing the 14 analytes was diluted to the appropriate concentration range to establish calibration curves. The calibration curve of each compound was constructed with at least six concentrations. The LOD and LOQ were determined as signal-to-noise ratios of 3 and 10, respectively.

2.5.2 Precision and accuracy

Intra-day variations at three times within 1 day and inter-day variations for 3 consecutive days were chosen to determine the precision of the developed method. Recovery was used to further evaluate the accuracy of the method. Three levels (low, middle and high) of the 14 standards were added to known amounts of samples. The amount of each analyte was calculated using the corresponding calibration curve; the recovery of each analyte was calculated as Recovery (%) = $(\text{Amount}_{\text{detected}} - \text{Amount}_{\text{original}})/\text{Amount}_{\text{spiked}} \times 100)$.

2.5.3 Repeatability and stability

To investigate the repeatability, five solutions of LRP were analysed, and the RSD was considered as a measure of reproducibility. The same sample solution was stored at 4 °C and was analysed at 0, 2, 4, 6, 8, 12 and 24 h to investigate the stability of the solution. Moreover, the 14 standard stock solutions were determined weekly to detect possible degradation during storage.

3 Results and discussion

3.1 Method validation

3.1.1 Calibration curves, LOD and LOQ

Table 1 shows the regression equation for the 14 analytes, as well as the LOD and LOQ values and mass spectrometry parameters. All calibration curves showed satisfactory linearity ($r > 0.9980$).

3.1.2 Precision and accuracy

The precision of the results is shown in Table 2. The precision of the intra- and inter-day variation for the detected levels of the investigated compounds was less than 4.60%. Table 3 lists the mean recoveries of the 14 analytes, which ranged from 94.41% to 103.39%, with RSD values < 6.05%.

3.1.3 Repeatability and stability

The RSD of the repeatability test was not over 5% for all analyses. When the solution was stored at 4 °C, the 14 compounds stabilised within 24 h (RSD < 4.69%).

These results indicate that the developed method was accurate, sensitive and reliable enough for the quantification of the major constituents in LRP.

3.2 Optimisation of the LC-MS conditions

Several chromatographic columns and mobile phases were tested to achieve better separation and higher response signals for the 14 compounds. Three reversed-phase columns were used for the separation analysis, including an Agilent Zorbax SB C18 (4.6 mm i.d. × 250 mm, 5 μm), a

Thermo BDS Hypersil C18 (2.1 mm i.d. × 100 mm, 3 μm) and a Thermo BDS Hypersil C18 (2.1 mm i.d. × 50 mm, 3 μm). The Thermo BDS Hypersil C18 (2.1 mm i.d. × 100 mm, 3 μm) column was the most suitable for this application. Acetonitrile separated 14 compounds more effectively than methanol and was thus selected as the organic phase. Formic acid, acetic acid, ammonium acetate and ammonium formate were added to the mobile phase to achieve high sensitivity and restrain peak tailing. Adding appropriate concentrations of formic acid and ammonium acetate enhanced the signal intensity of the compounds and improved the peak shape. As a result, an optimum mobile phase was achieved using 0.01% formic acid containing 0.2 mM ammonium formate and acetonitrile in a gradient elution mode.

The mass spectral conditions were optimised in full scan mode using the reference compounds. Protonated [M+H]+ molecular ions of schaftoside, liquiritin, isoliquiritin, echinatin, glycyrrhizic acid, isoliquiritigenin, piperine, licochalcone A, costunolide, dehydrocostuslactone and glycyrrhetinic acid were generated in the positive ionisation mode, whereas deprotonated [M−H]− molecular ions of catechin, epicatechin, schaftoside, liquiritin, isoliquiritin, liquiritigenin, echinatin, glycyrrhizic acid, isoliquiritigenin, licochalcone A and glycyrrhetinic acid were generated in the negative ionisation mode. Some reference compounds exhibited strong signals in both recording modes. The reference compounds were also used to optimise these parameters to meet the demands of the quantitative analysis based on the lowest interference and the highest signal intensity. We detected 10 compounds in positive ionisation mode and 11 compounds in negative ionisation mode. Therefore, a full scan mode with the polarity of the electrospray ion source alternating between positive and negative modes was used for quantification in a single run. The total ion chromatograms are shown in Figure 2, and the extracted ion chromatograms of these reference compounds are shown in Figure 3.

In the LRP extract, catechin and epicatechin, liquiritin and isoliquiritin, liquiritigenin and isoliquiritigenin are isomers. However, the retention time of these isomers differed after each standard was separately injected to analysis. The exact retention time and mass spectra were compared between the peaks of the LRP sample and the standards to ensure that the same components were detected and to avoid the effect of isomers on the identification of components.

3.3 Sample extraction optimisation

The extraction methods, solvents, solvent volume and extraction time were investigated to identify the optimum extraction efficiency. The results demonstrated that ultrasonic bath extraction was more effective than refluxing for the 14 components. The acetonitrile concentration (30%, 50%, 70%, 90% and 100%, v/v), solvent volume (5, 10 and 20 mL) and extraction time (10, 20, 40 and 80 min) were also investigated. The optimal extraction was achieved with 30 mg of sample in powder form, extracted with 10 mL of 30% acetonitrile in an ultrasonic bath for 20 min.

3.4 Sample analysis

The developed method was used to detect the 14 analytes in three batches of LRP samples. Each batch was analysed in five replicates. The analytes were identified by comparing their exact retention time and mass spectra with those of the reference materials. As shown in Table 4, the analytes had similar levels among the three batches. Among the 14 analytes, catechin (**1**) and glycyrrhizic acid (**8**) exhibited the highest concentrations, followed by epicatechin (**2**). The sum of the remaining compounds was approximately 10% of the total. The combined level of catechin and epicatechin was 29.2 ± 2.7 mg/g, which meets the quality standard of no less than 20 mg/g in

LRP per SFDA. However, in the traditional quality standard, the remaining components, which account for 50% of the total, were not considered even through some of them are known to exhibit pharmacological effects.[5–24] The capability of this method to analyse more ingredients could improve the quality assessment of LRP. Other components, such as borneol, in the LRP have important pharmacological effects, including neuroprotection[25] and inhibition of acetylcholine-mediated effects.[26] Borneol can easily penetrate the blood–brain barrier and enhance the oral bioavailability and the distribution of drugs in brain tissue.[27, 28] These effects may contribute to the anti-heat stroke and anti-motion sickness effects of LRP. However, this study did not include volatile components, such as borneol, because of the limitation of LC-MS. These ingredients will be detected by GC-MS/MS in a separate investigation.

4 Conclusion

In this study, a simple, rapid and accurate UHPLC-MS method was established to determine 14 major components of LRP. The method was used to successfully quantify the 14 components in three batches of LRP samples. The results showed that the analytes had similar levels among the three batches of LRP. The validation results demonstrated that the proposed method is reliable and sensitive.

References

1. X. H. Li, W. Pan, J. H. Jin, J. L. Du, J. Li and S. D.F., *Pharmacol. Clin. Chin. Mat. Med.*, 2009, 25, 61-62.
2. L. R. Leon and B. G. Helwig, *J. Appl. Physiol.*, 2010, 109, 1980-1988.
3. A. Shupak and C. R. Gordon, *Aviat. Space. Environ. Med.,* 2006, 77, 1213-1223.
4. T. P. Zajonc and P. S. Roland, *Ear. Nose. Throat. J.*, 2006, 85, 25-35.
5. L. Q. Tang, W. Wei and X. Y. Wang, *Adv. Ther.*, 2007, 24, 679-690.
6. G. O. De Melo, M. F. Muzitano, A. Legora-Machado, T. A. Almeida, D. B. De Oliveira, C. R. Kaiser, V. L. Koatz and S. S. Costa, *Planta. Med.*, 2005, 71, 362-363.
7. S. Kumar, A. Sharma, B. Madan, V. Singhal and B. Ghosh, *Biochem. Pharmacol.*, 2007, 73, 1602-1612.
8. J. Furusawa, M. Funakoshi-Tago, T. Mashino, K. Tago, H. Inoue, Y. Sonoda and T. Kasahara, *Int. Immunopharmacol.*, 2009, 9, 499-507.
9. H. Takei, Y. Baba, A. Hisatsune, H. Katsuki, T. Miyata, K. Yokomizo and Y. Isohama, *J. Pharmacol. Sci.,* 2008, 106, 460-468.
10. M. Tsukahara, T. Nishino, I. Furuhashi, H. Inoue, T. Sato and H. Matsumoto, *Chem. Pharm. Bull.*, 2005, 53, 1103-1110.
11. M. Jin, H. J. Lee, J. H. Ryu and K. S. Chung, *Arch. Pharm. Res.*, 2000, 23, 54-58.
12. A. I. Haza and P. Morales, *J. Appl. Toxicol.*, 2011, 31, 53-62.
13. E. Cuevas, D. Limon, F. Perez-Severiano, A. Diaz, L. Ortega, E. Zenteno and J. Guevara, *Eur. J. Pharmacol.*, 2009, 616, 122-127.
14. H. Haraguchi, H. Ishikawa, K. Mizutani, Y. Tamura and T. Kinoshita, *Bioorg. Med. Chem.*, 1998, 6, 339-347.
15. Y. W. Chin, H. A. Jung, Y. Liu, B. N. Su, J. A. Castoro, W. J. Keller, M. A. Pereira and A. D. Kinghorn, *J. Agric. Food. Chem.*, 2007, 55, 4691-4697.
16. S. Rahman and S. Sultana, *Chem. Biol. Interact.*, 2006, 160, 61-69.
17. S. L. Li, Z. W. Liu, P. Q. Lu, Y. Zhang, J. D. Zhang, D. H. Jia and Z. B. Cao, *Chin. Pharm. Bull.*, 2010, 2, 255-257.
18. Y. X. Sun, Y. Tang, A. L. Wu, T. Liu, X. L. Dai, Q. S. Zheng and Z. B. Wang, *J. Asian. Nat. Prod. Res.*, 2010, 12, 1051-1060.
19. E. J. Yang, J. S. Min, H. Y. Ku, H. S. Choi, M. K. Park, M. K. Kim, K. S. Song and D. S. Lee, *Biochem. Biophys. Res. Commun.*, 2012, 421, 658-664.
20. S. W. Kim, Y. Jin, J. H. Shin, I. D. Kim, H. K. Lee, S. Park, P. L. Han and J. K. Lee, *Neurobiol. Dis.*, 2012, 46, 147-156.
21. Q. Q. Mao, Z. Huang, S. P. Ip, Y. F. Xian and C. T. Che, *Cell. Mol. Neurobiol.*, 2012, 32, 531-537.
22. N. A. Rayan, N. Baby, D. Pitchai, F. Indraswari, E. A. Ling, J. Lu and T. Dheen, *Front. Biosci. (Elite Ed).*, 2011, 3, 1079-1091.
23. X. P. Li, Y. T. Zhao, Y. Deng, L. Lei and W. L. Deng, *Pharmacol. Clin. Chin. Mat. Med.*, 2013, 29, 63-66.
24. O. P. Gupta and B. J. Ghatak, *Indian. J. Med. Res.*, 1967, 55, 1078-1083.
25. R. Liu, L. Zhang, X. Lan, L. Li, T. T. Zhang, J. H. Sun and G. H. Du, *Neuroscience.*, 2011, 176, 408-419.
26. T. J. Park, Y. S. Park, T. G. Lee, H. Ha and K. T. Kim, *Biochem. Pharmacol.*, 2003, 65, 83-90.
27. Z. Cai, S. Hou, Y. Li, B. Zhao, Z. Yang, S. Xu and J. Pu, *J. Drug. Target.*, 2008, 16, 178-184.
28. B. Yu, M. Ruan, Y. Sun, X. B. Cui, Y. Yu, L. L. Wang and T. H. Fang, *Neural. Regen. Res.*, 2011, 6, 1876-1882.

Table 1. Calibration curves, LODs and LOQ of the 14 major compounds

No	Calibration curve	Correlation coefficient	Linear range (μg/mL)	LOQ (ng/mL)	LOD (ng/mL)	M/Z	Detected ion
1	Y = -58.7323+7969.49*X	0.9996	0.11 - 2.26	11.29	3.42	289.2	[M-H]-
2	Y = -27.1152+9662.59*X	0.9998	0.10 - 1.92	9.58	2.90	289.2	[M-H]-
3	Y = 21.5606+45664.2*X	0.9997	0.04 - 0.74	0.74	0.22	564.9	[M+H]+
4	Y = 1412.54+17234.1*X	0.9988	0.44 - 8.72	2.91	0.88	417.2	[M-H]-
5	Y = 148.628+31138.9*X	0.9994	0.09 - 1.82	0.61	0.18	417.2	[M-H]-
6	Y = 506.994+26899*X	0.9992	0.09 - 1.75	4.36	1.32	255.2	[M-H]-
7	Y = 1355.87+103418*X	0.9986	0.03 - 0.53	0.53	0.16	271.0	[M+H]+
8	Y = -3837.66+68406.2*X	0.9990	0.22 - 4.40	1.47	0.44	821.8	[M-H]-
9	Y = -25.543+46162.5*X	0.9993	0.02 - 0.38	1.88	0.57	255.2	[M-H]-
10	Y = -67.6756+806193*X	0.9987	0.01 - 0.23	0.23	0.07	286.1	[M+H]+
11	Y = 2756.77+191952*X	0.9984	0.03 - 0.50	0.50	0.15	339.1	[M+H]+
12	Y = -57.0924+5792.34*X	0.9988	0.17 - 3.38	33.82	10.25	232.9	[M+H]+
13	Y = -39.382+2253.51*X	0.9983	0.43 - 8.69	43.43	13.16	230.9	[M+H]+
14	Y = -2462.4+112672*X	0.9980	0.04 - 0.84	2.11	0.64	471.2	[M+H]+

Table 2.Precision levels of the assay of the 14 components.

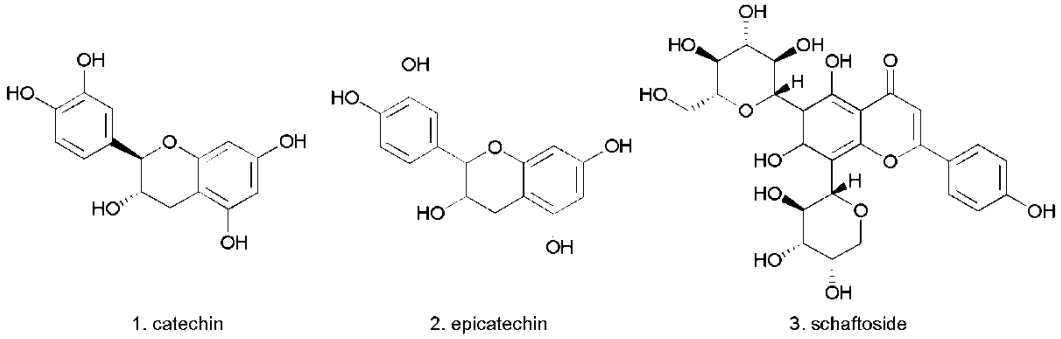
NO.	Concentration (μg/ml)	Intra-day (n=3) Mean (μg/ml)	Intra-day (n=3) RSD (%)	Inter-day (n=3) Mean (μg/ml)	Inter-day (n=3) RSD (%)
1	0.11	0.12 ± 0.00	3.43	0.12 ± 0.00	3.31
	0.45	0.45 ± 0.02	3.90	0.45 ± 0.01	2.93
	1.81	1.80 ± 0.04	1.96	1.80 ± 0.03	1.89
2	0.10	0.10 ± 0.00	4.46	0.10 ± 0.00	3.50
	0.38	0.38 ± 0.00	0.55	0.38 ± 0.01	2.30
	1.53	1.52 ± 0.01	0.58	1.52 ± 0.03	1.79
3	0.04	0.04 ± 0.00	3.87	0.04 ± 0.00	3.74
	0.15	0.15 ± 0.01	4.26	0.14 ± 0.01	3.90
	0.59	0.59 ± 0.01	1.50	0.60 ± 0.01	1.57
4	0.44	0.41 ± 0.01	2.93	0.41 ± 0.01	3.48
	1.74	1.85 ± 0.02	0.82	1.85 ± 0.03	1.49
	6.97	6.90 ± 0.05	0.74	6.88 ± 0.10	1.48
5	0.09	0.09 ± 0.00	2.38	0.09 ± 0.00	1.95
	0.36	0.37 ± 0.01	1.61	0.37 ± 0.01	1.82
	1.45	1.44 ± 0.02	1.19	1.44 ± 0.03	1.88
6	0.09	0.08 ± 0.00	0.89	0.08 ± 0.00	2.67
	0.35	0.36 ± 0.01	1.92	0.36 ± 0.01	1.57
	1.40	1.38 ± 0.01	0.76	1.38 ± 0.03	2.19
7	0.03	0.02 ± 0.00	0.89	0.02 ± 0.00	4.52
	0.11	0.11 ± 0.00	2.69	0.11 ± 0.00	2.73
	0.42	0.41 ± 0.01	3.04	0.41 ± 0.01	2.46
8	0.22	0.23 ± 0.01	3.28	0.23 ± 0.01	4.14
	0.88	0.83 ± 0.03	3.29	0.83 ± 0.03	3.20
	3.52	3.55 ± 0.05	1.38	3.57 ± 0.04	1.18
9	0.02	0.02 ± 0.00	4.01	0.02 ± 0.00	3.93
	0.08	0.08 ± 0.00	3.22	0.08 ± 0.00	2.47
	0.30	0.30 ± 0.01	2.77	0.30 ± 0.01	1.96
10	0.01	0.01 ± 0.00	2.82	0.01 ± 0.00	3.75
	0.05	0.05 ± 0.00	2.35	0.05 ± 0.00	3.66
	0.18	0.18 ± 0.00	1.20	0.18 ± 0.00	1.30
11	0.02	0.02 ± 0.00	1.59	0.02 ± 0.00	2.68
	0.10	0.11 ± 0.00	3.45	0.11 ± 0.00	3.48
	0.40	0.39 ± 0.00	0.71	0.39 ± 0.00	0.94
12	0.17	0.18 ± 0.00	2.37	0.18 ± 0.01	4.60
	0.68	0.65 ± 0.02	2.34	0.66 ± 0.02	3.23
	2.71	2.75 ± 0.07	2.46	2.68 ± 0.08	2.82
13	0.43	0.48 ± 0.01	1.55	0.47 ± 0.02	3.59
	1.74	1.57 ± 0.01	0.75	1.58 ± 0.04	2.40
	6.95	6.94 ± 0.12	1.76	6.97 ± 0.15	2.19
14	0.04	0.05 ± 0.00	2.76	0.05 ± 0.00	4.34
	0.17	0.15 ± 0.00	1.13	0.15 ± 0.00	2.74
	0.68	0.68 ± 0.01	1.31	0.68 ± 0.01	1.89

Table 3. Accuracy and reproducibility levels of the 14 analytes in LRP.

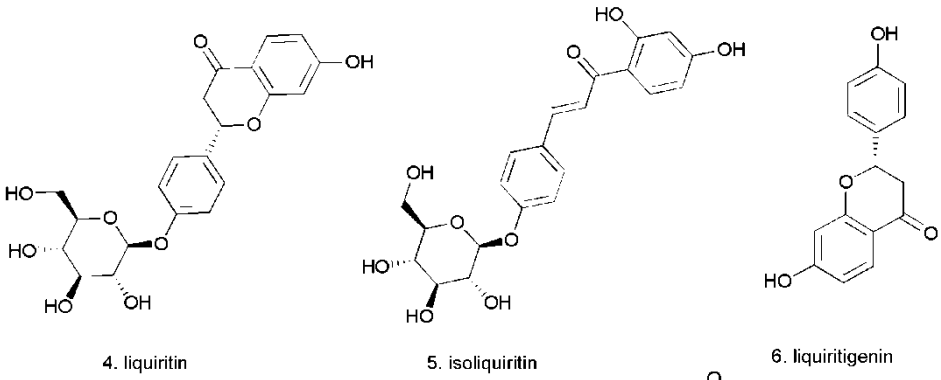
NO.	Accuracy (n=3)		Reproducibility (n=5)	
	Recovery (%)	RSD (%)	Mean (μg/ml)	RSD (%)
1	95.93 ± 2.53	2.64	1.40 ± 0.05	3.47
2	95.51 ± 3.86	4.04	0.51 ± 0.01	1.49
3	99.66 ± 5.05	5.07	0.73 ± 0.01	0.66
4	97.61 ± 5.90	6.05	5.89 ± 0.11	1.81
5	98.36 ± 5.79	5.89	0.13 ± 0.01	4.99
6	103.39 ± 2.56	2.47	0.66 ± 0.03	3.75
7	99.71 ± 5.08	5.10	0.06 ± 0.00	4.88
8	94.41 ± 2.43	2.57	1.27 ± 0.04	2.93
9	97.49 ± 5.07	5.21	0.19 ± 0.01	3.53
10	99.67 ± 3.34	3.35	0.03 ± 0.00	3.27
11	95.58 ± 4.37	4.57	0.30 ± 0.01	1.69
12	101.23 ± 3.17	3.13	0.47 ± 0.01	3.23
13	98.04 ± 4.41	4.49	1.04 ± 0.05	4.64
14	96.67 ± 3.70	3.83	0.15 ± 0.00	2.04

Table 4. Contents of the 14 compounds in three batches of Longhu Rendan pills

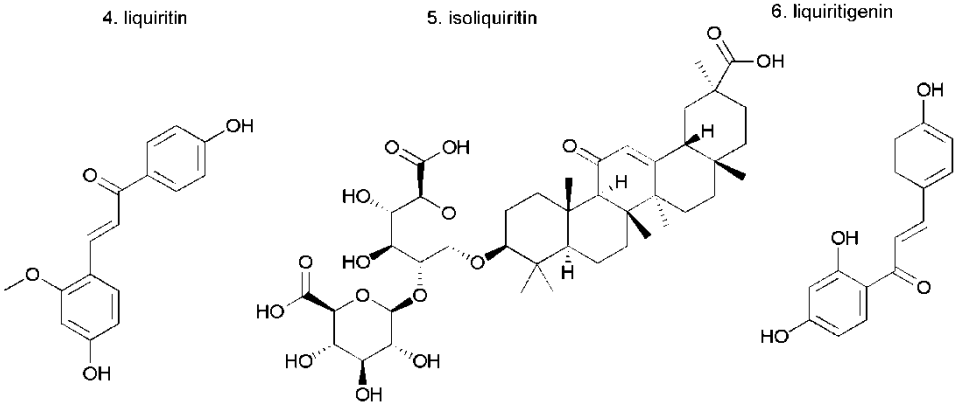
NO.	Content (mg/g)								
	Sample 1			Sample 2			Sample 3		
1	23.407	±	0.812	21.359	±	0.381	18.996	±	0.460
2	8.489	±	0.127	7.937	±	0.148	7.419	±	0.261
3	0.242	±	0.002	0.245	±	0.005	0.245	±	0.003
4	1.965	±	0.035	2.251	±	0.062	1.980	±	0.070
5	0.547	±	0.027	0.646	±	0.035	0.558	±	0.026
6	0.220	±	0.008	0.252	±	0.005	0.215	±	0.010
7	0.241	±	0.012	0.249	±	0.009	0.267	±	0.012
8	20.675	±	0.605	22.754	±	0.477	20.855	±	0.580
9	0.065	±	0.002	0.077	±	0.002	0.063	±	0.003
10	0.502	±	0.016	0.658	±	0.028	0.552	±	0.012
11	1.266	±	0.024	1.383	±	0.023	1.340	±	0.028
12	0.157	±	0.005	0.155	±	0.007	0.142	±	0.006
13	0.346	±	0.016	0.303	±	0.008	0.333	±	0.009
14	0.050	±	0.001	0.057	±	0.003	0.049	±	0.001



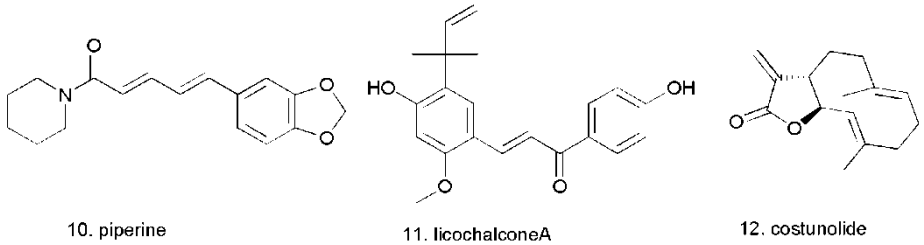
1. catechin 2. epicatechin 3. schaftoside



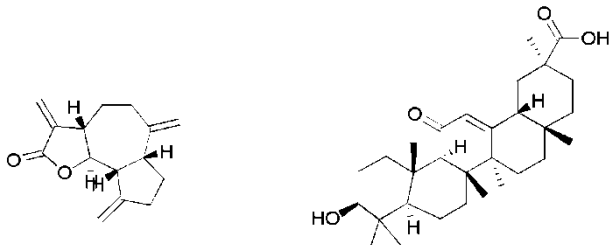
4. liquiritin 5. isoliquiritin 6. liquiritigenin



7. echinatin 8. glycyrrhizic acid 9. isoliquiritigenin



10. piperine 11. licochalconeA 12. costunolide



13. dehydrocostuslactone 14. glycyrrhetinic acid

Figure 1. Structures of the 14 analytes.

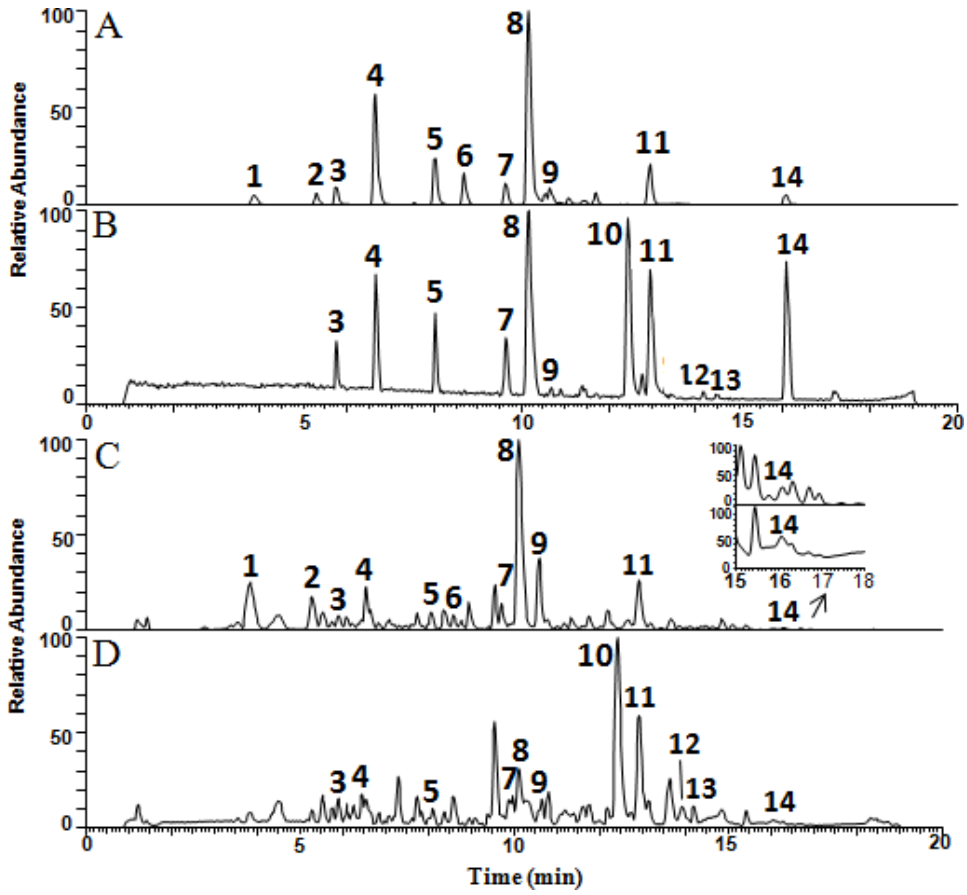


Figure 2. Total ion chromatograms of (A) reference compounds in negative mode; (B) reference compounds in positive mode; (C) Longhu Rendan pills (LRP) sample in negative mode; and (D) LRP sample in positive mode: (1) catechin, (2) epicatechin, (3) schaftoside, (4) liquiritin, (5) isoliquiritin, (6) liquiritigenin, (7) echinatin, (8) glycyrrhizic acid, (9) isoliquiritigenin, (10) piperine, (11) licochalcone A, (12) costunolide, (13) dehydrocostuslactone, (14) glycyrrhetinic acid.

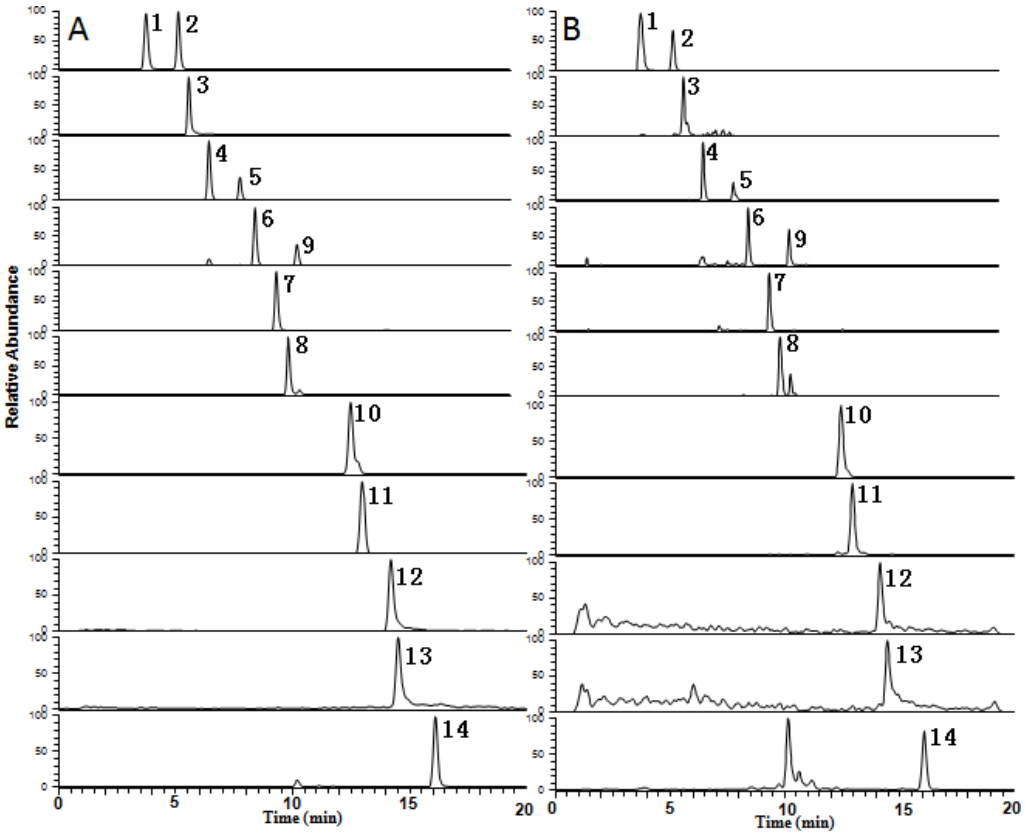


Figure 3. Extracted ion chromatograms of (A) reference standards and (B) Longhu Rendan pills sample: (1) catechin, (2) epicatechin, (3) schaftoside, (4) liquiritin, (5) isoliquiritin, (6) liquiritigenin, (7) echinatin, (8) glycyrrhizic acid, (9) isoliquiritigenin, (10) piperine, (11) licochalcone A, (12) costunolide, (13) dehydrocostuslactone, (14) glycyrrhetinic acid.