Analytical Methods

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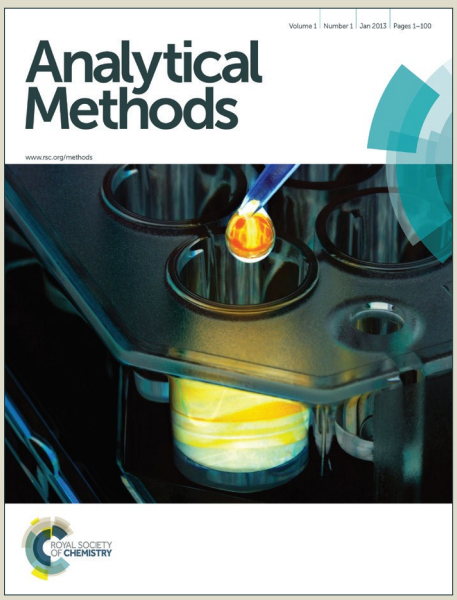

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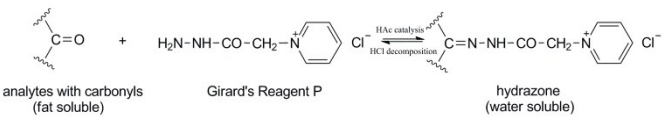

In this study, there were three stages including purification, microextraction and determination of the analytes. First stage was that these analytes were subjected to reversible reaction with GR in the special acid environment. Next stage was the course of microextraction: HF-SLME was an improved technique of hollow fiber liquid-phase microextraction with a stainless-steel stirring bars inserted into the hollow of the hollow fiber. By the way of rapid and automatic agitation, the analytes could entirely transfer into organic solvent located in the pores of hollow fiber in a very short time.[29] Finally, we developed a suitable scheme using HPLC-DAD for simultaneous determination of five main naphthoquinones active constituents of QLY, namely regiolone(1), juglone(2), 3-ethoxy-juglone(3), 2-ethoxy-juglone(4) and engelharquinone(5) (Fig.1). The results showed that the novel preconcentration technique played an important role to improve the resolution of chromatographic peak and reduced the interferences from background components.

2 Experimental

2.1 Chemicals and materials

Five reference compounds, including regiolone, juglone, 3-ethoxy-juglone, 2-ethoxy-juglone and engelharquinone were purchased from Mansite Pharmaceutical (Chengdu, China). Acetonitrile of chromatographic grade was purchased from Fisher Chemicals (Pittsburgh, PA, USA), HPLC-grade water was purified by Milli-Q system (Milford, MA, USA), 1100V-WD rotary evaporator (Tokyo Rikakikai Co.,Ltd., Japan), Glacial acetic acid (98%-100% pure), hydrochloric acid (36%-38% pure), anhydrous ethanol and other analytical reagents were purchased from Beijing Chemical Factory (Beijing, China). Q 3/2 Accurel polypropylene hollow fiber (600 μm inner diameter, 200μm wall thickness, 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). Girard's Reagent P was obtained from Meilun biology technology CO., LTD. (Dalian, China). Stirring paddle was homemade which was combined with the handheld electric mixer which was purchased from Ouke electrical device company (Jiangxi, China). Water membrane filter was purchased from Xingya purification materials Factory (Shanghai, China).

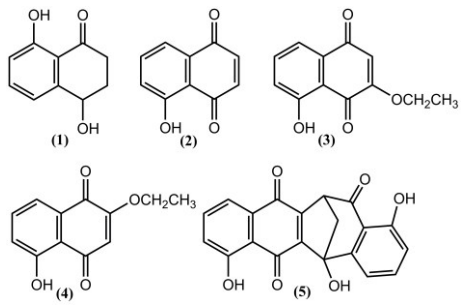

Fig.1 Structures of five active naphthoquinones of QLY: regiolone(1), juglone (2), 3-ethoxy-juglone (3), 2-ethoxy-juglone(4), engelharquinone(5).

2.2 Instruments and chromatographic parameters

An Agilent Technologies 1260 infinity HPLC system (Agilent Technologies, Germany) was equipped with a G1311C quaternary pump, G1329B automatic liquid sampler, G1316C column oven and G1315D diode array detector. The data was acquired using the LC 1260 workstation. For chromatographic analysis, a Diamonsil $C1_8$ column (4.6 mm × 250 mm, 5 μm) was used. The mobile phase was consisted of acetonitrile (A) and water containing 0.2% acetic acid (B) using a gradient program: 0-28 min, 25-41% A; 28-30 min, 41-41% A; 30-33 min, 41-46% A; 33-35 min, 46-46% A; 35-38 min, 46-48% A; 38-60 min, 48-72% A. The flow rate was 1.0 mL min-1 and column temperature was maintained at 35 °C. The detection wavelength was set at 275 nm for acquiring chromatograms.

2.3 Sample preparation

Three samples(samples 1-3) cultivated in different areas were collected from Changbai Mountains (Jilin, China), Maoer Mountain (Heilongjiang, China) and Phoenix Mountain (Heilongjiang, China) in late July, respectively. The dried samples were grounded into fine powder (60 mesh), dried thoroughly in an oven at 40 °C for 3 days. Each of sample powder was accurately weighed and used in further experiment.

2.4 Extraction procedure

2.4.1 RE

A 60 mg of sample powder was placed in a 10.0 mL round bottomed flask and 5.0 mL of chloroform was added into the flask. The extraction was carried out at 65 °C for 1.0 h and repeated 3 times. The extracting solution was merged by rotatory evaporation at 45 °C and diluted with acetonitrile to 5.0 mL. The resulting solution was filtered through a 0.22 μm membrane filter as the sample solution. A 10.0 μL of filtrate was injected into the HPLC-DAD system.

2.4.2 SE

A 60 mg of sample powder was extracted by UE in 5.0 mL of chloroform. UE was performed at 400W for 30 min and then the chloroform solution was filtered. The above process was repeated three times. The merged filtrates were evaporated to give a dry residue. This residue was recovered in 5.0 mL of acetonitrile. The resulting solution was filtered through a 0.22 μm membrane filter as the sample solution. A 10.0 μL of filtrate was injected into the HPLC-DAD system.

2.4.3 GR treating followed HF-SLME

Firstly, a 10 mg of sample powder was extracted with 3.0 mL chloroform by way of SE. The extraction process was the same as 2.4.2. The dry residue was dissolved in 1 mL of anhydrous ethanol and mixed with 100.0 μL glacial acetic acid to reach 12% (v/v). The mixed solution was injected into the reaction vessel by syringe. Reaction vessel was a polypropylene column similar to the SPE device, which loaded with 3.0 g GR. After the end of reaction, 5.0 mL deionized water was injected into the reaction vessel to flush the vessel.

Then the rest of water layer was placed in a 10 mL extraction beaker and acidized to pH 1.5 with hydrochloric acid. 300.0 μL acetonitrile was added into the beaker to form saturated solution. Cross stainless steel stirring bars were fixed

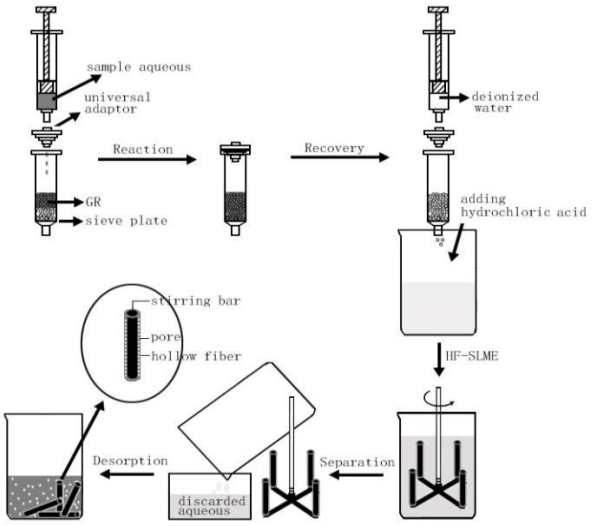

Fig.2 Preconcentration and extraction procedure

on the handheld electric mixer, the end of which were vertical upward and covered with hollow fiber. The hollow fiber was cut into 1.0 cm pieces and rinsed with acetone for purpose of removing impurities, and then dried in the air. The processed fiber piece was immersed in toluene for 3min in order to impregnate the fiber pores, and rinsed with water in order to remove the excess of organic solvent from the surface of fiber. After that, the resulting hollow fiber fixed on the extraction bars were inserted into sample aqueous which could be rotated accompany with mixer at 600 rpm. After 10 min later, the hollow fiber fixed on the extraction bar was detached and water-washed until neutral (Fig.2). The organic solvent loaded in the pores was desorbed with 1 mL acetonitrile by UE for 3 min and then dried by nitrogen stream. The residue was dissolved and diluted with acetonitrile to 600.0 μL. The resulting solution was filtered through a 0.22 μm membrane filter. A 10 μL of filtrate was injected into the HPLC-DAD system.

3 Results and discussion

3.1 Optimization of the procedure

To obtain optimal results, the influences of different parameters, such as reaction time, elution volume, extraction temperature and time, the type and volume of extractant and salt addition were investigated and optimized.

3.1.1 Reaction time

Reaction time referred to the time it spent that naphthoquinones turned into hydrazone in the reaction vessel. Generally, the reaction time decreased with the increase of the temperature. However, the reaction temperature was greater than 50 °C, some naphthoquinones were apt to volatilize. Thus, the reaction time was tested at 50 °C. At first the yield of each constituent increased gradually between 1 h and 3 h, and then remained about the same when the time is longer than 3h. After the reaction, the residues of reaction vessel were extracted by chloroform and then carried out HPLC analysis. There were no any signals of analytes in residues, which indicated that the reaction of structural transformation had been completely finished within 3 h (Fig. 3A). Therefore, the reaction period of 3 h was selected for experiments.

3.1.2 Amount of the catalyst

The addition of suitable amount of glacial acetic acid into the sample extracts usually accelerate reaction, and consequently enhance extraction efficiency on account of catalyst effect. The amount of catalyst was not the more the better. Structures of these analytes might be damaged due to the excessive use of acid. The effect of the glacial acetic acid amount was evaluated by varying glacial acetic acid concentration from 6% to 14% (v/v) in proportion to the extracting concentrate. As shown in Fig. 3B, the extraction yields of the three analytes were the highest when amount of the catalyst was 12% (v/v). Thus, it was adopted for further experiments.

3.1.3 Selection of the extraction solvent

Organic solvents used in microextraction affects the recovery and the selectivity of the method. There are a few requirements for choice of organic solvents. First, it should be strongly extraction. And then, organic solvent should be immiscible in water and should have high solubility for the analytes. It should have low volatility to protecting solvent against loss during the experiment. Based on these factors, five solvents immobilized in the hollow fiber pores including n-hexane, benzene, toluene, diethyl ether and ethyl acetate were tested. Toluene provided the best extraction efficiency, probably because it could reduce the potential solvent loss with the low volatility and increase extraction yield due to the similar polarity to the analytes (Fig.4A). Thus, it was chosen as the organic solvent of the micoextraction.

3.1.4 Effect of pH in extraction course

In order that the analytes could be restored in prototype form efficiently, the pH of the donor phase should be adjusted to decompose hydrazone by means of acidifying the samples aqueous. To study the effect of pH of the donor phase, the pH values were varied from 0.5 to 3.5 (Fig.4B). In most cases, it was found that the lower the pH of the extraction solution was, the higher the yield of target product was. However, it would cause some object analytes dehydrated in more acidic environment such as regiolone. The results showed that the extraction efficiency was the highest when the pH value was 1.5. Therefore, pH 1.5 was selected as the optimum value for the further experiments.

3.1.5 Effect of extraction time

HF-SLME is a mass-transfer process，so it is a time-dependent process. Therefore, in this experiment the effect of extraction time was examined in the range of 5-35 min (Fig.4C). Based on the results obtained, an extraction time of 10 min provided the maximum extraction efficiency. Equilibrium was reached at this time, and then the extraction efficiency reduced when the extraction time was increased. Generally, a long extraction time could result in the loss of the organic solvent from the fiber and reduce the extraction efficiency. Thus, the extraction time of 10 min was selected for subsequent studies.

3.1.6 Effect of the stirring speed

The stirring speed was a very important parameter as it reduced the time to reach the mass transfer equilibrium. The effect of

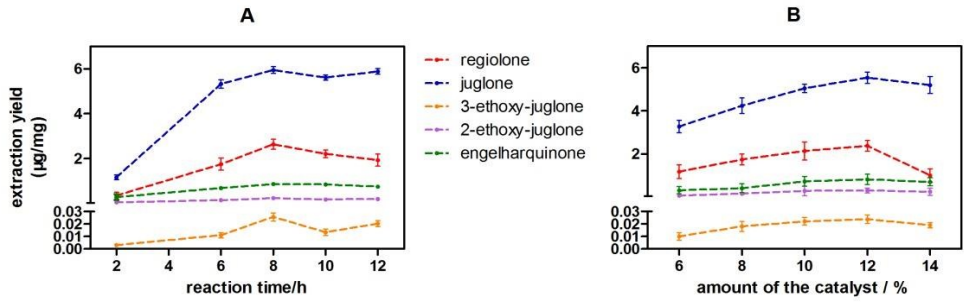

Fig.3 3(A) Effect of reaction time on extraction yields. Conditions: sample amount, 10 mg; sample volume, 1 mL; glacial acetic acid volume, 100.0 μL; extraction temperature, 50 °C; extraction solvent, toluene; pH value, 1.5; extraction time, 10min; stirring speed, 600 rpm; and 3(B) Effect of catalyst on extraction yields. Conditions: reaction time, 3h; other conditions as Fig.3(A) except for glacial acetic acid volume.

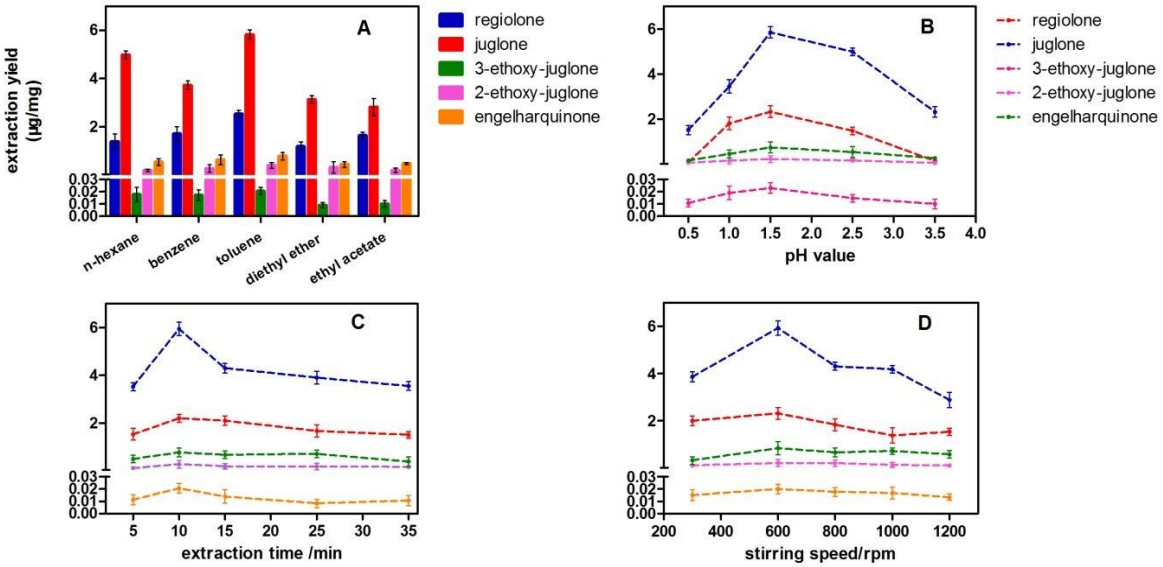

Fig.4 4(A) Effect of extraction solvent on extraction yields. Conditions: sample amount, 10 mg; sample volume, 1 mL; glacial acetic acid volume, 100.0 μL; reaction time, 3h; extraction temperature, 50 °C; pH value, 1.5; extraction time, 10min; stirring speed, 600 rpm; and 4(B) Effect of pH on extraction yields. Conditions: extraction solvent, toluene; other conditions as Fig.4(A) except for glacial acetic acid volume; and 4(C) Effect of extraction time on extraction yields. Conditions: extraction solvent, toluene; other conditions as Fig.5(A) except for extraction time; and 4(D) Effect of stirring speed on extraction yields. Conditions: extraction solvent, toluene; other conditions as Fig.4(A) except for stirring speed.

stirring speed on the extraction efficiency was studied by varying the stirring speed from 300 to 1200 rpm and the results were shown in Fig.4D. The extraction yields of the analytes were increased by increasing the stirring speed up to 600 rpm. When the stirring speed was increased over 600 rpm, the extraction efficiency was decreased on account of promotion of solvent evaporation or production of air bubbles on the surface of the hollow fiber. Therefore, 600 rpm was selected for the rest of the experiments.

3.2 Validation of the method

Method validation of quantitative analysis was performed by parameters such as linearity, precision, accuracy, stability, LOD and LOQ.

The stock solution containing five analytes was diluted (5, 10, 20, 25, 50 folds) with acetonitrile to appropriate concentrations for the establishment of calibration curves. Each calibration curve was made up of five concentrations and was created by plotting peak area versus concentration. An aliquot of the diluted solutions were injected into HPLC for analysis. The LOD and LOQ were determined at S/N of 3 and 10, respectively. The results were shown in Table 1. All calibration curves showed good linearity ($r > 0.9995$) between the peak area and concentration.

Table 1 Linearity, sensitivity, and recovery data for the five analytes (n=3)

Analytes	Linear range (μg/mL)	Regression equationa	r	LOD[b] (μg/mL)	LOQ[c] (μg/mL)	Recovery			
						Original (μg/mg)	Spiked (μg/mg)	Recovery (%)	RSD (%)
regiolone	0.10-120.00	y=8.9821x+32.377	0.9995	0.039	0.124	2.28	1.80	85.03	1.58
						2.28	2.20	97.20	2.62
						2.28	2.70	96.41	0.94
juglone	0.15-550.00	y=7.6439x+18.502	0.9997	0.028	0.092	5.91	4.70	93.84	2.04
						5.91	5.90	92.19	1.03
						5.91	7.10	85.16	3.46
3-ethoxy -juglone	0.01-280.00	y=20.464x+42.920	0.9996	0.0043	0.0142	0.02	0.01	98.61	2.06
						0.02	0.02	101.62	2.92
						0.02	0.03	88.17	2.06
2-ethoxy -juglone	0.08-500.00	y=16.778x+17.838	0.9998	0.035	0.117	0.19	0.15	93.04	1.12
						0.19	0.20	79.19	1.92
						0.19	0.23	102.02	1.36
engelharquinone	0.10-720.00	y=9.876x+21.076	0.9998	0.031	0.105	0.82	0.72	98.56	1.13
						0.82	0.82	91.32	1.57
						0.82	1.00	86.13	1.44

[a] y=peak area;x=theoretical concentration of naphthoquinone; [b] Signal/noise ratio=3:1; [c] Signal/noise ratio=10:1

Table 2 Stability, intra- and inter-day precision of the analytes

Analytes	Repeatability (RSD%, n=6)		Stability (RSD%, n=3)
	Intra-day	Inter-day	
regiolone	1.45	1.73	0.73
juglone	1.43	1.20	3.40
3-ethoxy-juglone	2.84	3.52	2.79
2-ethoxy-juglone	2.03	2.81	2.34
engelharquinone	1.55	2.74	0.88

The precision was evaluated by measuring intra- and interday RSDs.Then intraday variability was determined by analyzing sample 1 six times within 24 h, and the inter-day precision test was determined on six consecutive days. The results were listed in Table 2.

The stability test was performed by analyzing the sample 1 extract over period of 0h, 4h, 8h, 12h, 24h, 48h, the RSDs of the peak areas of each reference compound were taken as the measures of stability. The results were listed in Table 2.

Sample 1 was selected to carry out the recovery test in order to validate the accuracy of the developed method and the results were obtained and summarized in Table 1. The recoveries varied from 79.19-102.02% with RSDs ranging from 0.94-3.46%. The results showed that this method is suitable for determination of regiolone, juglone, 3-ethoxy-juglone, 2-ethoxy-juglone and engelharquinone.

3.3 Real sample analysis

Table 3 Contents of five naphthoquinones quantified in QLY samples from different habitats

Analyte	Content (μg/mg)[a]		
	1	2	3
regiolone	2.28±0.02	2.11±0.01	1.96±0.03
juglone	5.91±0.15	6.18±0.32	6.02±0.26
3-ethoxy-juglone	0.02 [b]	0.14±0.01	0.06 [b]
2-ethoxy-juglone	0.19±0.02	0.26±0.01	0.08±0.03
engelharquinone	0.82±0.03	0.75±0.02	0.59±0.02

QLY samples were subjected to the proposed approach to evaluate the concentrations of five naphthoquinone compounds. The samples were analyzed by HPLC-DAD, after the performance of the GR purity and HF-SLME procedures. The results of determination were shown in Table 3. Through the comparison of three different habitats samples, we've found that they had similar chemical constitutions, but the content of the available compositions were not the same. Juglone with both toxicity and activity, which was isolated from the husk part, was proved to be the most abundant constituent. Chromatograms of extract of sample 1-3 by GR treating followed HF-SLME were shown in Fig.5A.

3.4 Comparison of the present method with RE and SE

To evaluate the present method, UE and RE were investigated. Sample 1 was used. The results were listed in Table 4. The yields of five analytes obtained by the proposed method and UE are similar, both of which were higher than by RE. Naphthoquinones with the characteristic of small molecular

Table 4 Yield of five analytes by three extraction methods (mean±SDa, μg/mg, n=3)

Analytes	GR treating followed HF-SLME	SE	RE
regiolone	2.28±0.02	2.32±0.04	1.84±0.03
juglone	5.91±0.15	5.88±0.08	1.52±0.01
3-ethoxy-juglone	0.02 [b]	0.02 [b]	— [c]
2-ethoxy-juglone	0.19±0.02	0.22±0.01	— [c]
engelharquinone	0.82±0.03	0.79±0.02	— [c]

[a] Standard deviation (n=3);
[b] Standard deviation is less than 0.01;
[c] This analyte was not detected.

structure were easily destroyed at higher temperatures. So RE was not suitable for extracting these unstable substances. In addition, the results indicated that the optimized method had no obvious advantages to UE in the respects of extraction time and solvent consumption. But the proposed method in this study dramatically reduced impurity interferences and facilitated the simultaneous determination of multicomponent without tedious optimization of detection condition. Chromatograms of extract of sample 1 by SE and RE were shown in Fig. 5B, 5C, respectively.

4. Conclusion

As a complex system, OLY contains numerous ingredients and it is difficult to achieve efficient chromatographic separation. Therefore, it is crucial to improve the sensitivity and reduce or remove these impurity interferences. In general, the main solution is to optimize the detection condition. Obviously, it is tedious work mainly based on repeated test on-line. In this study, a novel combined preconcentration technique was established for simultaneous determination of five main naphthoquinones active constituents of QLY. In comparison to traditional extraction method such as RE and SE, GR purification joined with HF-SLME technique could selectively obtain and monitor almost all the components with carbonyl such as naphthoquinone analytes. Thus, it effectively reduced the heavy workload of optimization of detection condition by excluding interferences from impurity peaks. The experimental results demonstrated that the developed method was validated and effective. At the same time, the method of GR purification also can be potentially used in other herbal medicine containing components with carbonyl even if it owns complex composition system.

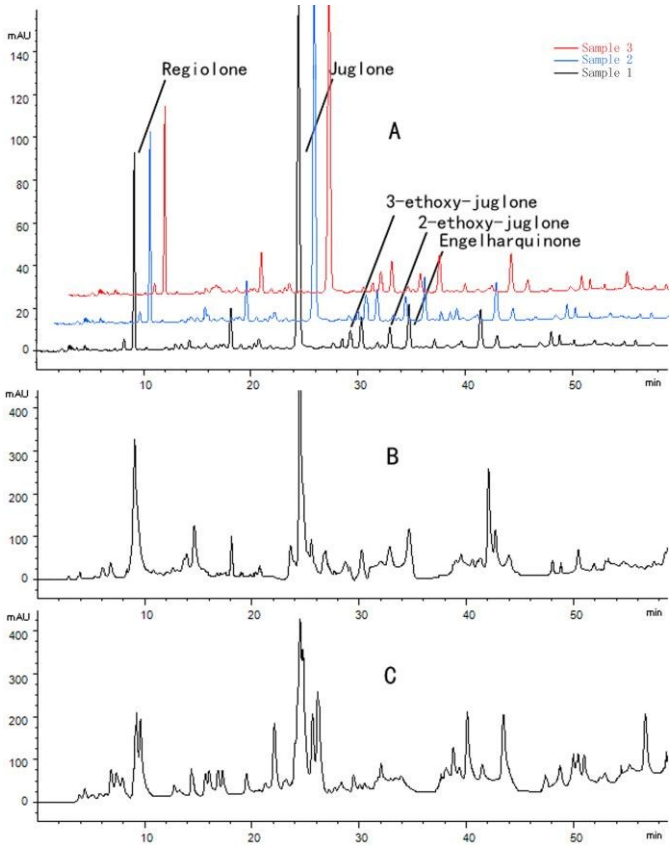

Fig.5 Chromatograms of extract of sample by multiple extraction method, GR treating followed HF-SLME (A), SE (B), RE (C).

Acknowledgements

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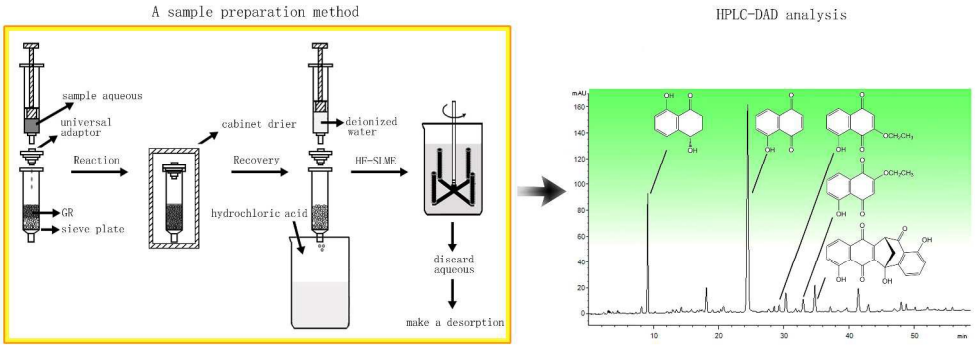
References

1 D.L. Yao, C.H. Zhang, J. Luo, M. Jin, M.S. Zheng, J.M. Cui, J.K. Son and G. Li, *Arch. Pharm. Res.*, 2015, **38**, 480-484.
2 H. Lin, Y.W. Zhang, Y. Hua, Y.L. Bao, Y. Wu, L.G. Sun, C.L. Yu, Y.X. Huang, E.B. Wang, H.Y. Jiang and Y.X. Li, *J Asian Nat Prod Res*., 2014, **16**, 819-824.
3 S.H. Kim, K.S. Lee, J.K. Son, G.H. Je, J.S. Lee, C.H. Lee and C.J. Cheong, *J. Nat. Prod.*, 1998, **61**, 643-645.
4 W.N. Bai, W.J. Liao and D.Y. Zhang, *New Phytol.*, 2010, **188**, 892-901.
5 Y.L. Zhang, Y.Q. Cui, J.Y. Zhu, H.Z. Li, J.W. Mao, X.B. Jin, X.S. Wang, Y.F. Du and J.Z. Lu, *Afr J Tradit Complement Altern Med.*, 2013, **10**, 258-269.
6 G. Park and M.S. Oh, *Exp Toxicol Pathol.*, 2014, **66**, 97-101.
7 G.H. Park, D.S. Jang and M.S. Oh, *Biochem Biophys Res Commun*., 2012, **421**, 343-348.

8 H.L. Xu, X.F. Yu, S.C. Qu and D.Y. Sui, *Bioorg Med Chem Lett.*, 2013, **23**, 3631-3634.
9 K. Machida, E. Matsuoka, T. Kasahara and M. Kikuchi, *Chem. Pharm. Bull.*, 2005, **53**, 934-937.
10 G. Chen, X.M. Pi and C.Y. Yu, *Nat Prod Res.*, 2015, **29**, 174-179.
11 J. Li, J.X. Sun, H.Y. Yu, Z.Y. Chen, X.Y. Zhao and H.L. Ruan, *Chinese Chem. Lett.*, 2013, **24**, 521-523.
12 H.J. Yang, H.J. Cho, S.H. Sim, Y.K. Chung, D.D. Kim, S.H. Sung, J. Kim and Y.C. Kim, *Bioorg Med Chem Lett.*, 2012, **22**, 2079-2083.
13 K. Machida, Y. Yogiashi, S. Matsuda, A. Suzuki and M. Kikuchi, *J Nat Med.*, 2009, **63**, 220-222.
14 Y.H. Kong, L. Zhang, Z.Y. Yang, C. Han, L.H. Hu, H.L. Jiang and X. Shen, *Acta Pharmacol Sin.*, 2008, **29**, 870-876.
15 W. Zhang, A.H. Liu, Y. Li, X.Y. Zhao, S.J. Lv, W.H. Zhu and Y. Jin, *Can. J. Physiol. Pharmacol.*, 2012, **90**, 1553-1558.
16 H.L. Xu, X.F. Yu, S.C. Qu, X.R. Qu, Y.F. Jiang and D.Y. Sui, *Food Chem Toxicol.*, 2012, **50**, 590-596.
17 R.J. Wang, S. Wang, Y.J. Xia, M.W.L.J. Tu, L.J. Zhang and Y.M. Wang, *Int J Biol Macromol.*,2015, **72**, 771-775l.
18 S.H. Chao, A.L. Greenleaf and D.H. Price, *Nucleic Acids Res.*, 2001, **29**, 767-773.
19 N. Sharma, P. Ghosh, U.K. Sharma, S. Sood, A.K. Sinha and A. Gulati. *Anal. Lett.* ,2009, **42**,2592-2609
20 J.J. Inbaraj and C.F. Chignell, *Chem. Res. Toxicol.*, 2004, **17**, 55-62.
21 M. R. Kviecinski, R.C. Pedrosa, K.B. Felipe, M.S. Farias, C. Glorieux, M. Valenzuela, B. sid, J. Benites, J.A. Valderrama, J. Verrax and P. Buc Calderon. *Biochem. Bioph. Res. Co.*,2012, **421**, 268-273.
22 M. Salimi, M.H. Ardestaniyan, H. Mostafapour Kandelous, S. Saeidnia, A.R. Gohari, A. Amanzadeh, H. Sanati, Z. Sepahdar, S. Ghorbani and M. Salimi, *Cell Prolif.*, 2014, **47**, 172–179.
23 O. Lavrynenko, R. Nedielkov, H.M. Möller and A. Shevchenko, *J Lipid Res.*, 2013, **54**, 2265-2272.
24 J.W. Gouw, P.C. Burgers, M.A. Trikoupis and J.K. Terlouw, Rapid Commun. *Mass Spectrom*., 2002, **16**, 905-912.
25 A. Meljon, S. Theofilopoulos, C. H. Shackleton, G. L. Watson, N. B. Javitt, H. J. Knolker, R. Saini, E. Arenas, Y. Wang and W. J. Griffiths, *Journal of lipid research*, 2012, **53**, 2469-2483.
26 Mirzaei Mohammad and Rakh Mojgan, *J. Sep. Sci.*, 2014, **37**, 114-119.
27 S. Zorita, L. Martensson and L. Mathiasson, *J. Sep. Sci.*, 2007, **30**, 2513-2521.
28 M. Mirmoghaddam, M.Kaykhaii and H. Yahyavi. *Anal. Mehods*, 2015, **7**, 8511-8523.
29 B. Zargar and A. Hatamie. *Anal. Mehods*, 2014, **6**, 2506-2511.



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