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Short communication

Optimization of pressurized liquid extraction of five major flavanoids from *Lysimachia clethroide*

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Abstract

As an alternative of traditional extraction method, pressurized liquid extraction (PLE) was applied for five flavanoids extraction from *Lysimachia clethroide*. The operational parameters of PLE, such as extraction solvent, temperature, pressure, static extraction time, flush volume and cycles were optimized by univariate approach coupled with central composite design (CCD) in order to obtain the highest extraction efficiency. The optimized result employed 50% acetonitrile aqueous as extraction solvent, 100 °C of extraction temperature, 1500 psi of extraction pressure, 25 min of static time, 70% flush volume, and only one cycle to extract the target compounds completely. Finally, the contents of five major flavanoids in *L. clethroides* from different sources were determined simultaneously by the combination of the presented PLE and HPLC method.

Keywords: *Lysimachia clethroide*; PLE; Optimization; CCD; HPLC-DAD

1. Introduction

Lysimachia clethroides Duby, one of the species of genus *Lysimachia*, is a traditional folk Chinese medicine, distributed widely in many provinces of China. This plant has been used widely for treatment of throat ache, edema, and menoschesis, etc. [1]. Chemical study showed that flavonoids and saponins were present in this plant [2–4], and the flavonoids were proved to be the main biological constituents, with the activities of anti-tumor, anti-bacterial and anti-platelet aggregation [5–8]. However, up to now, there was only one paper to describe the quantification of flavonoids in *L. clethroide* by UV method [9], and the references on the analyses of the other *Lysimachia* medicinal plants were very limited [10–12].

In the previous analytical process, sonication, Soxhlet and reflux methods have been investigated for the extraction of this genus plants [9–12]. These methods were time-consuming and solvent-intensive and a filtration step was often required. As an alternative of traditional extraction method, pressurized liquid extraction (PLE), an extraction technique under elevated temperatures and pressures, has been recently used in the extraction of biologically active constituents from herbal medicines for its small amount of solvent consumption, automated sample handling, rapid extraction process and high extractive efficiency [13–17].

Optimization of the PLE parameters was normally accomplished using the classical univariate approach (one-variable-at-a-time) [18]. But, this method cannot determine the interactions between parameters and find the most suitable PLE condition, so some "experimental design" was adopted to detect the influencing factors while the number of trials can be kept to a minimum [19,20]. In this paper, central composite design (CCD) [21,22] was used for optimization of PLE parameters for flavanoids from *L. clethroides*, and overall desirability (OD) [21,22], the geometric mean of the contents of five major flavanoids, namely, rutin (1), isoquercitrin (2), kaemperol-3-*O*-rutinoside (3), isorhamnetin-3-*O*-rutinoside (4) and prunin (5) were used as marker to evaluate the extraction efficiency. Finally, the analysis results of these five flavanoids in *L. clethroides* from different sources were given to show the applicability of the methods presented.

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2. Experimental

2.1. Materials and chemicals

The crude drugs of *L. clethroides* Duby were purchased from several medicinal plant markets. The identities of these plants were confirmed by Prof. Pengfei Tu, and the voucher specimen was deposited at the Center of Traditional Chinese Medicine, Peking University. The samples were dried at 60 °C in vacuo, and ground to fine powder. The standards of rutin, isoquercitrin, kaemperol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside and prunin were isolated from *L. clethroides* and identified by our group, and the purities of these standards were all above 98%. Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Phosphoric acid of analytical-reagent grade was purchased from Riedel-de Haën (Seelze, Germany). Pure water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.2. Pressurized liquid extraction

An ASE 200 System (Dionex, Sunnyvale, CA, USA) with 11 ml stainless steel ASE vessels was used for the pressurized liquid extraction. About 0.5 g of *L. clethroide* were mixed homogeneously with double weight of diatomaceous earth and placed into an extraction cell. The extract was transferred to a 25 ml volumetric flask and diluted to its volume with 50% aqueous acetonitrile. The sample solutions were filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the HPLC system, and 20 μl solution was injected.

2.3. HPLC analysis

The analysis was performed on an Agilent 1100 series chromatograph equipped with a DAD detector (Waldbronn, Germany). The columns used were a Kromasil 100 C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and an Agilent C_{18} guard column (12.5 mm × 4.6 mm i.d., 5 μm) maintained at 35 °C. The mobile phase was acetonitrile–0.1% phosphoric acid (17:83) at a flow-rate of 1.0 ml/min. Simultaneous monitoring was performed at 350 nm (for rutin, isoquercitrin, kaemperol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside) and 275 nm (for prunin). The standard stock solutions were prepared in 50% acetonitrile aqueous at a concentration of 0.25 mg/ml, and stored at 4 °C in the dark for not longer than one month.

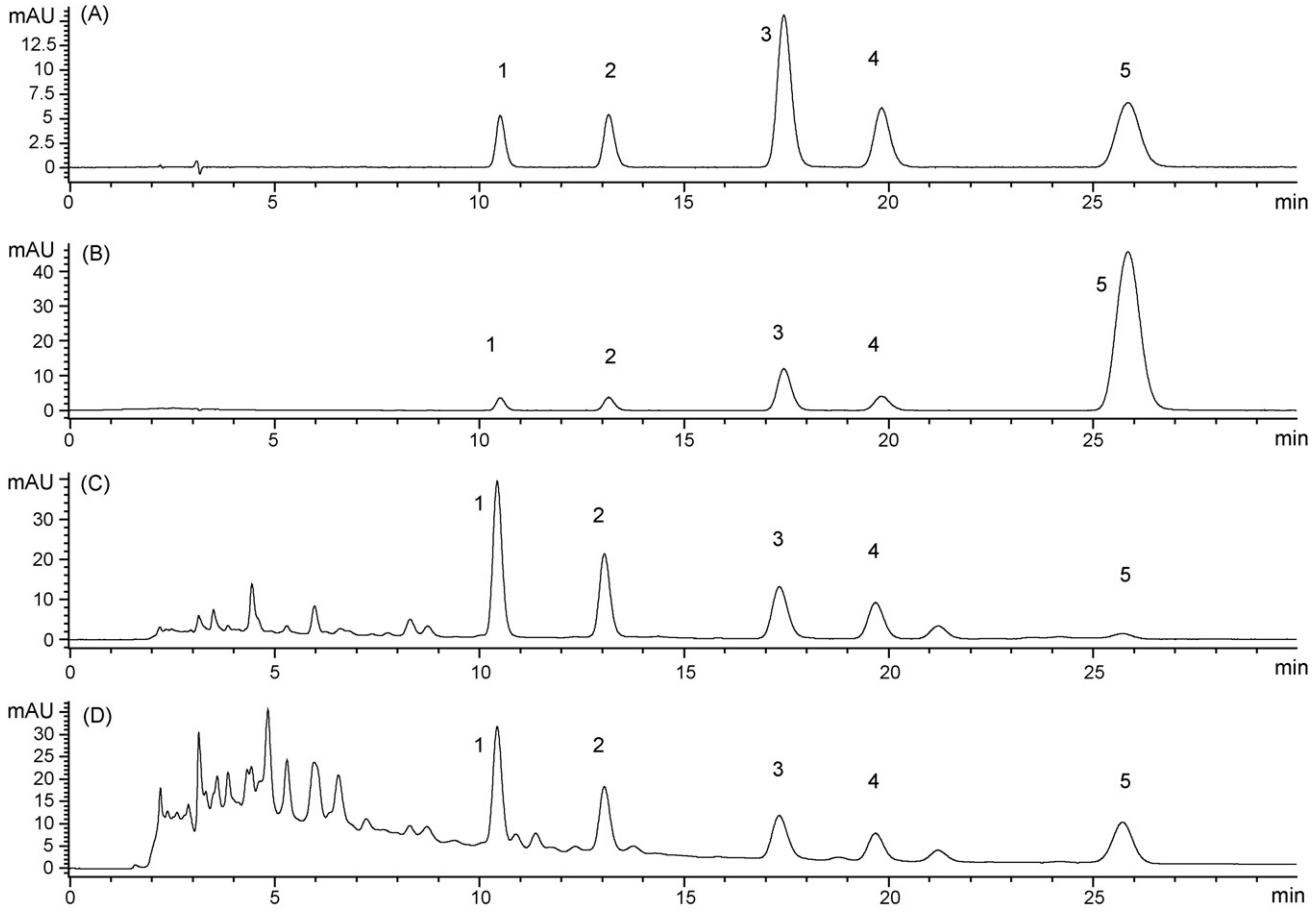

Fig. 1. HPLC chromatograms of mixed standards and PLE extract of *L. clethroide*. (A) Mixed standards detected at 350 nm; (B) mixed standards detected at 275 nm; (C) sample 1 detected at 350 nm; (D) sample 1 detected at 275 nm; 1: rutin; 2: isoquercitrin; 3: kaempferol-3-*O*-rutinoside; 4: isorhamnetin-3-*O*-rutinoside; 5: prunin.

Table 1
Calibration curves, limits of detection and quantification, and recoveries for five compounds in *L. clethroide*

Compound[a]	$y = ax + b$		r^2	Range (μg/ml)	Detection limit (μg/ml)	Quantity limit (μg/ml)	Recovery (%)
	Slope (a)	Intercept (b)					
1	17.47	−3.3094	0.9999	2.38–47.62	0.24	0.71	100.6
2	23.958	−5.6503	0.9999	3.33–66.67	0.20	0.60	92.8
3	17.098	1.6027	1	3.81–121.91	0.38	0.95	102.2
4	19.285	−3.7977	1	2.86–57.14	0.35	1.06	100.9
5	18.224	−6.1428	0.9997	2.86–42.86	0.29	0.92	103.2

[a] 1: Rutin; 2: isoquercitrin; 3: kaempferol-3-*O*-rutinoside; 4: isorhamnetin-3-*O*-rutinoside; 5: prunin.

3. Results and discussion

3.1. Method validation of HPLC

Under the optimized chromatographic conditions (Fig. 1), all the calibration curves for the five target compounds were linear in a relatively wide concentration range. Limit of detection (LOD, S/N = 3), limit of quantification (LOQ, S/N = 10), and recoveries are given in Table 1. The overall precision of the analysis was satisfactory. The intra- and inter-day precision of the five analytes varied 97.85–102.6% and 98.11–103.8%, respectively, and the relative standard deviations (R.S.Ds) failed between 0.20–1.15% and 0.27–1.56%, respectively.

3.2. Optimization of the pressurized liquid extraction conditions

In order to achieve the most efficient extraction for the target compounds, the extraction conditions, such as solvent, temperature, pressure, static time, flush volume and cycle were optimized by a univariate approach coupled with central composite design. In order to obtain a comprehensive evaluated result, overall desirability [21,22], the geometric mean of the contents of rutin, isoquercitrin, kaemperol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside and prunin detected at 350 nm wavelength were used for evaluation of the extraction efficiency.

3.2.1. Choice of the extraction solvent

Methanol, 50% aqueous methanol, 50% aqueous acetonitrile and 20% aqueous acetonitrile were chosen for their similar polarities to the target compounds, and the result showed that the extraction efficiency of 50% aqueous methanol and 50% aqueous acetonitrile were higher than the other two solvent. Considering that aqueous acetonitrile was the mobile phase, 50% aqueous acetonitrile was chosen as solvent for the further investigation.

3.2.2. Experimental design and optimization by CCD

Before specific limits for individual CCD factors were selected, the effects of temperature, pressure, static extraction time and flush volume were studied using the classical univariate approach. The result showed that the pressure displayed the smallest effect on the extraction efficiency of PLE. Thus, the other three factors, temperature, static extraction time and flush volume were selected as CCD factors, and the pressure was set at the default level, 1500 psi.

The range and the levels of the variables (temperature, static extraction time and flush volume) investigated in this study were given in Table 2 (the maximum temperature was set at 140 °C for the flavonoids degraded at high temperature [23]). The experiments were performed in random order to avoid systematic error.

By applying multiple regression analysis on the experimental data, the results of the CCD were fitted with a second-order

Table 2
The central composite design matrix of three test variables in coded and natural units along with the observed responses

No.	X_1	X_2	X_3	Temperature (°C)	Extraction time (min)	Flush volume (%)	OD[a]
1	−1	−1	−1	76	9	50	0.884
2	1	−1	−1	124	9	50	0.983
3	−1	1	−1	76	21	50	0.946
4	1	1	−1	124	21	50	0.922
5	−1	−1	1	76	9	70	0.903
6	1	−1	1	124	9	70	0.915
7	−1	1	1	76	21	70	0.918
8	1	1	1	124	21	70	0.953
9	−1.668	0	0	60	15	60	0.861
10	1.668	0	0	140	15	60	0.915
11	0	−1.668	0	100	5	60	0.946
12	0	1.668	0	100	25	60	0.988
13	0	0	−1.668	100	15	40	0.955
14	0	0	1.668	100	15	80	0.967
15–20	0	0	0	100	15	60	0.960

[a] Overall desirability.

polynomial equation. Thus, a mathematical regression model for total peak area fitted in the coded factors was given as following:

$$Y = 0.249692 + 0.012524X_1 + 0.003312\ X_2 + 0.00063X_3 - 0.000048X_1^2 + 0.000013X_2^2 - 0.000008X_3^2 - 0.000087\ X_1X_2 - 0.000015X_1X_3 + 0.000108X_2X_3$$

where Y was the response, that was the total contents of rutin, isoquercitrin, kaemperol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside and prunin and X_1, X_2 and X_3 were the coded values of the test variables temperature, static extraction time and flush volume, respectively. The significance of each coefficient was determined by Student's *t*-test and *P*-values, and the results showed that extraction temperature played the main effect on the extraction efficiency.

Following validation of the model, graphs of surface responses were drawn in Fig. 2. From Fig. 2, we could found that the effect of temperature on the extraction efficiency was most obvious. Fig. 2A showed the maximum extraction efficiency was obtained at temperature of 96–100 °C and static extraction time of 25 min. Fig. 2B showed the maximum at 100–104 °C, with the inconspicuous effect of the flush volume. So, the temperature was set at 100 °C and static extraction time was set at 25 min. Fig. 2C showed the extraction efficiency enhanced slowly accompanied with the increase of the flush volume, but the trend was not obvious. Considering from the solvent saving, the flush volume was set at 70%.

At last, the extraction time of PLE was optimized by performing consecutive pressurized liquid extractions for three times on the same sample. After one time extraction, the target compounds were almost undetectable, suggesting that the PLE with one cycle was enough.

3.3. Comparison of PLE, Soxhlet and sonication

The extraction efficiency of PLE for Cortex Dictamni was compared with those traditional extraction methods of Soxhlet and sonication. As shown in Table 3, the overall extraction efficiency of PLE was higher than those of Soxhlet and sonication, and this method had the advantages of small amount of solvent consumption and shorter extraction time.

3.4. Analyses of L. clethroide from different sources

Using the above-optimized PLE parameters and HPLC condition, the contents of five major target compounds in *L.*

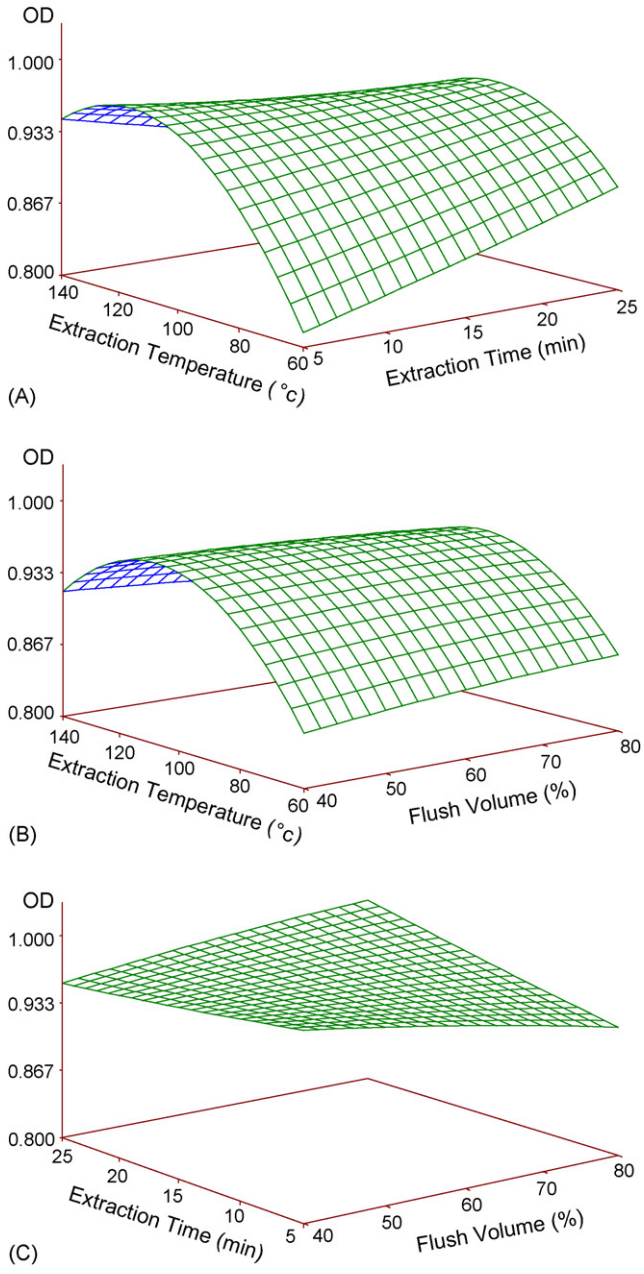

Fig. 2. Response surface for overall desirability (OD) response function of rutin, isoquercitrin, kaemperol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside and prunin. (A) Temperature (°C, X_1) vs. static extraction time (min, X_2). X_3 is held at its optimum. (B) Temperature (°C, X_1) vs. flush volume (%, X_3). Static extraction time (X_2) is held at its optimum. (C) Static extraction time (min, X_2) vs. flush volume (%, X_3). Temperature (X_1) is held at its optimum.

Table 3
Comparison of PLE, Soxhlet and sonication method for the extraction of the five flavanoids in *L. clethroide*[a]

Method	Time	Solvent volume (ml)	Recoveries (mg/g)					OD
			1	2	3	4	5	
PLE	24 min	27	1.34	0.44	0.95	0.42	0.92	0.982
Soxhlet	5 h	70	1.35	0.41	0.90	0.30	1.00	0.912
Sonication	30 min	30	1.30	0.38	0.83	0.29	0.84	0.841

[a] The mean values of three determinations are presented, and R.S.D. is less than 3%.

Table 4
Contents (mg/g) of the five flavonoids in *L. clethroide* ($n = 3$)

Sample	Habitat	Collected time	1	2	3	4	5
1	Bozhou, Anhui	July 2005	1.87 ± 0.01	0.84 ± 0.02	1.05 ± 0.02	0.69 ± 0.03	0.99 ± 0.02
2	Bozhou, Anhui	April 2001	1.34 ± 0.01	0.44 ± 0.02	0.95 ± 0.02	0.42 ± 0.02	0.92 ± 0.01
3	Bozhou, Anhui	September 2003	0.13 ± 0.03	0.08 ± 0.03	0.09 ± 0.04	0.09 ± 0.03	–[a]
4	Zhengzhou, Henan	April 2005	1.10 ± 0.03	0.44 ± 0.01	0.53 ± 0.02	0.49 ± 0.01	1.08 ± 0.03
5	Anguo, Hebei	May 2005	0.52 ± 0.01	0.15 ± 0.02	0.14 ± 0.02	0.23 ± 0.02	–[a]

[a] Undetectable.

clethroide from different sources were determined, and the results were summarized in Table 4.

4. Conclusions

From the above results, it is possible to conclude that PLE can supply a new choice for the sample preparation method for natural medicines, and the combination of PLE with HPLC-DAD can be readily utilized as a suitable quality control method for *L. clethroide* and other *Lysimachia* plant.

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References

[1] Jiangsu Botanic Institute, Program of Xinhua Herb (Part II), Press of Shanghai Science and Technology, Shanghai, 1991, pp. 364–371.
[2] H.Y. Zou, P.F. Tu, Chin. J. Nat. Med. 2 (2004) 59–61.
[3] F.Z. Ren, J.K. Qie, H.H. Qu, X.H. Luan, Y.M. Zhao, Pharm. J. Chin. PLA 17 (2001) 178–180.
[4] K. Isao, M. Akiko, Y. Itiro, Chem. Pharm. Bull. 20 (1972) 2226–2234.
[5] X.Y. Xu, L.H. Tang, Z.Q. Liang, Z.L. Gu, Chin. Wild Plant Resour. 22 (2003) 31–34.
[6] The group for prevention and cure of cancer in Hankou Hospital of Air force, New Med. 8 (1977) 112–113.
[7] The group for prevention and cure of cancer in Hankou Hospital of Air force, New Med. 12 (1977) 293–294.
[8] Editing Committee of Chinese herbs, Chinese Herb (fasicule 6), Shanghai Science and Technological Publisher, Shanghai, 1999, pp. 92–110.
[9] L.H. Tang, X.Y. Xu, Z.D. Xue, J.P. Cao, Anti-infect. Pharm. 2 (2005) 118–120.
[10] Chinese Pharmacopoeia Committee Editing, Chinese Pharmacopoeia (Part I), Chemical and Industrial Publisher, 2005, p. 152.
[11] W.Q. Lv, J.B. Pi, Y.T. Song, C.T. Li, J. Li, Chin. Trad. Herb. Drug 35 (2004) 457–459.
[12] J.K. Tian, L.Z. Xu, Z.M. Zou, H.W. Zhang, H.M. Mu, S.L. Yang, Chin. J. Pharm. Anal. 24 (2004) 577–580.
[13] B. Benthin, H. Danz, M. Hamburger, J. Chromatogr. A 837 (1999) 211–219.
[14] J. Suomi, H. Siren, K. Hartonen, M.L. Riekkola, J. Chromatogr. A 868 (2000) 73–83.
[15] C.W. Huie, Anal. Biol. Chem. 373 (2002) 23–30.
[16] B. Kaufmann, P. Christen, Phytochem. Anal. 13 (2002) 105–113.
[17] M.A. Rostagno, M. Palma, C.G. Barroso, Anal. Chim. Acta 522 (2004) 169–177.
[18] R. Carabias-Martínez, E. Rodríguez-Gonzalo, P. Revilla-Ruiz, J. Hernández-Méndez, J. Chromatogr. A 1089 (2005) 1–17.
[19] C. Holst, A. Mueller, F. Serano, S. Sporring, E. Bjoerklund, Chromatographia 61 (2005) 391–396.
[20] L. Pallaroni, C. Von Holst, J. Chromatogr. A 993 (2003) 39–45.
[21] K. Outinen, H. Haario, P. Vuorela, M. Nyman, E. Ukkonen, H. Vuorela, Eur. J. Pharm. Sci. 6 (1998) 197–205.
[22] P. Li, S.P. Li, S.C. Lao, C.M. Fu, K.K.W. Kan, Y.T. Wang, J. Pharm. Biomed. Anal. 40 (2006) 1073–1079.
[23] Z. Piñeiro, M. Palma, C.G. Barroso, J. Chromatogr. A 1026 (2004) 19–23.