Arabian Journal of Chemistry (2015) **8**, 400–406

King Saud University

Arabian Journal of Chemistry

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Response surface modeling of betulinic acid pre-concentration from medicinal plant samples by miniaturized homogenous liquid–liquid extraction and its determination by high performance liquid chromatography

Mostafa Khajeh [a,*], Mansour Ghaffari Moghaddam [a], Atousa Zafar Danesh [a], Behrouz Khajeh [b]

[a] *Department of Chemistry, University of Zabol, Zabol P.O. Box 98615-538, Iran*
[b] *School of Pharmacy, Zabol Medical Science University, Zabol, Iran*

Available online 16 March 2013

KEYWORDS

Homogenous liquid–liquid extraction;
Betulinic acid;
Response surface methodology;
Box–Behnken experimental design;
Medicinal plant samples

Abstract This work describes a miniaturized homogenous liquid–liquid extraction (HLLE), combined with high performance liquid chromatography (HPLC) procedure to determine betulinic acid in the medicinal plant samples. The method was based on the rapid extraction of betulinic acid from a methanol solution of sample into 67 μL chloroform, as an extraction solvent. After addition of water into the mixture, the extracting solvent phase immediately formed a distinct water-immiscible phase under the vial, which could easily be separated, evaporated and re-dissolved in 0.5 mL of acetonitrile for further HPLC analysis. The effects of various experimental parameters in the extraction step were also studied using response surface methodology. Three independent variables were volume of extracting solvent (*A*: 30–90 μL), time (*B*: 1–10 min) and volume of water (*C*: 1–10 mL). The statistical analysis showed that the independent variable *A*, the quadratic term (A^2) and the interaction between *B* and *C* have significant effects on the peak area of betulinic acid ($p < 0.05$). The optimized conditions were found to be 67 μL of extracting solvent volume, an extraction time of 4.3 min and 5.2 mL of water volume. Under these conditions, the detection limit (LOD) was obtained as 1.6 ng/g. Furthermore, the relative standard deviation of the 10 replicate was less than

* Corresponding author. Fax: +98 542 2226765.
E-mail address: m_khajeh@uoz.ac.ir (M. Khajeh).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2013.03.001

2.7%. The developed procedure was applied to the extraction and determination of betulinic acid in medicinal plant samples.

1. Introduction

Betulinic acid (Fig. 1) is a pentacyclic triterpenoid of natural origin isolated from various plants (Suhaj, 2006; Gbaguidi et al., 2005; Razborsek et al., 2008). Recently this compound has gained considerable interest due to their multiple pharmacological influences including anti-HIV and anti-inflammatory activities (Suhaj, 2006; Gbaguidi et al., 2005; Razborsek et al., 2008). Considering the diversity of analytical techniques available, HPLC with reverse phase column and UV–Vis or diode array detection are the analytical techniques that have dominated the separation and determination of pentacyclic triterpenoids such as betulinic acid (Gbaguidi et al., 2005; Claude et al., 2004).

The sample preparation is crucial for the development and application of an analytical procedure. In general, liquid–liquid extraction is the most useful sample preparation procedure. However, this method uses a large amount of toxic organic solvents, and it is time consuming. Due to the use of harmful organic solvents, this procedure causes environmental pollution and health hazards to laboratory personnel. It was felt that a special attention is focused on the procedures that are characterized by a considerable reduction of organic solvents. These procedures protect the environment against additional quantities of solvents and also reduce the cost of analysis (Farajzadeh et al., 2009; Khajeh and Musavi Zadeh, 2012).

Homogenous liquid–liquid extraction (HLLE) is a simple and powerful pre-concentration procedure that reduces the extraction time, disposal costs, exposure and consumption of organic solvents. In this procedure, the solute sample can be extracted efficiently from a homogeneous aqueous solution into a very small immiscible sedimented phase formed by the phase separation phenomenon (Alizadeh et al., 2010). Therefore, the initial sample solution is completely homogeneous without any interface between the water-miscible organic solvent phase and the water phase, so that the surface area of the interface between the aqueous and organic phases will become infinitely large (Alizadeh et al., 2010; Shamsipur and Hassan, 2010; Wang et al., 2008).

To improve the extraction of betulinic acid, response surface methodology (RSM) was applied to analyze the effects of extraction parameters and their interaction on the peak area of betulinic acid from plants. RSM is an effective statistical experimental design technique for optimizing complexing processes, and it used widely in optimizing the extraction variables (Chen et al., 2012). Box–Behnken experimental design based on a 3 level and 3 variable central composite, was used to obtain the best possible combination of extraction time, volume of extracting solvent and water for extraction of betulinic acid (Chen et al., 2012; Box et al., 1997; Khajeh, 2009, 2011).

To our knowledge, there is no report to use HLLE method for the separation and determination of betulinic acid in medicinal plant samples. Therefore, the aim of this work is to develop a HLLE method followed by HPLC with UV detection for separation and determination of betulinic acid in medicinal plant samples. In order to obtain optimum conditions, and also to investigate the interactions between the factors, the Box–Behnken experimental design is also used.

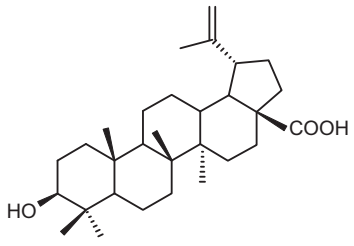

Figure 1 Structure of betulinic acid.

2. Materials and methods

2.1. Apparatus

Chromatographic analysis was performed on an HPLC system (Jasco, Tokyo, Japan) consisted of a model Pu1580 pump, a UV detector and a model As1550 autosampler. The analytical column was a C18 (Finepak SIL) reversed-phase column (250 mm × 4.6 mm i.d.; 5 μm particle size). Mobile phase was filtered through a Millipore 0.45 μm membrane filter before use. The elution was performed using the mixture of acetonitrile:water (9:1) at pH 3.0 (with phosphoric acid) at a flow-rate of 1 mL min^{-1} and the eluate was monitored at 210 nm (Kumar et al., 2010). All experiments were performed in triplicate and the means of values were used for optimization in this study. Quantification was performed by the integration of the peak using an external standard method. Each of the sample solution and the standard were injected into the chromatograph and peak areas were recorded. From the peak area of betulinic acid, the amount of it in the extract was computed by an external standard method.

2.2. Reagents

HPLC grade organic solvents including acetonitrile, chloroform, methanol, dichloromethane and acetone (Merck, Darmstadt, Germany) were used for the HPLC analysis. Double deionized water was filtered before use. Betulinic acid was isolated from Malaysian *Callistemon speciosus* according to the procedure described by Ahmad et al. (1999). A stock standard solution of betulinic acid (100 mg/L) was prepared in acetonitrile.

2.3. Procedure

The medicinal plant samples fortified with betulinic acid at the concentration level of 0.5 mg/L, were extracted as follows: 1 g of sample was exactly weighed into a 50 mL centrifuge tube,

and then 10 mL of methanol was added. The flask was mixed ultrasonically exposed under ultrasound for 20 min. the mixture was centrifuged for 5 min at 4500 rpm. Then an aliquot of 5 mL supernatant solution of sample was placed in a 20 mL screw-cap glass test tube with a conical bottom. Different volumes of chloroform (as extraction solvent: 30–90 μL) were rapidly injected into the above test tube. The different volumes of water (2–10 mL) were then added. The mixture was shaken for different times (1–10 min) and was then centrifuged for 5 min at 4500 rpm, the extracting solvent was immediately separated from the sample and transferred to another test tube and was evaporated to dryness and re-dissolved in 0.5 mL of acetonitrile for further HPLC analysis. All experiments were performed at room temperature.

2.4. Experimental design

Single factor was used to evaluate the preliminary range of the extraction variables. Then a three-level, three-factorial Box–Behnken experimental design was used to determine the best combination of extraction variables for the recovery of betulinic acid. Table 1 represents the non-coded values of the experimental variables and 15 experimental points. Three central points (13–15) were used to determine the pure error. The general form of the quadratic polynomial was as follows:

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_i^2 + \sum \sum \beta_{ij} \chi_i \chi_j + \varepsilon \quad (1)$$

where Y is the process response or output (dependent variable), β_0 is the constant, χ_i and χ_j are the coded independent variables, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interaction effect, and ε is the random error or allows for description or uncertainties between predicted and measured values. F and p-values were employed to check the significances of the regression coefficient.

3. Results and discussion

3.1. Preliminary studies

Preliminary studies were carried out in order to determine the required type and volume of extraction solvent, extraction time and volume of water for the extraction of betulinic acid from medicinal plant samples. Dichloromethane, *n*-hexane, toluene, benzene and chloroform were tested as extraction solvents. The experimental results showed that among the solvents examined, chloroform had a higher peak area. Therefore, chloroform was selected as the extraction solvent. To study the effect of the volume of chloroform on the peak area of betulinic acid, the experiments were performed by adding different volumes of chloroform (20–90 μL) as the extraction solvent to 5.0 mL methanolic solution containing betulinic acid, while other extraction parameters were as follows: extraction time 5 min and volume of water 5 mL. The peak area of betulinic acid was increased with the increasing volume of chloroform to 70 μL and then was slightly decreased. Therefore, volume of 30–90 μL was favorable for the extraction of betulinic acid.

Extraction time is another factor that would affect the extraction of betulinic acid. Extraction process was performed using the time (1–10 min), while other extraction parameters were as follows: volume of chloroform 70 μL and volume of water 5 mL. A plateau in the peak area was reached at the extraction time of 5 min. Thus, extraction time 1–10 min was favorable for this study.

To study the effect of different volumes of water on the peak area of betulinic acid, extraction process was performed using different volumes of water (2–15 mL), while other extraction condition was as follows: volume of chloroform 70 μL and extraction time 5 min. The peak area of betulinic acid was nearly constant to 10 mL and then decreased. Therefore, volume of water 2–10 mL was favorable for the extraction of betulinic acid.

3.2. Response surface methodology

The design matrix and the corresponding results of RSM experiments to determine the influences of the three independent variables including volume of chloroform (A: 30–90 μL), extraction time (B: 1–10 min) and volume of water (C: 2–10 mL) are shown in Table 1. An empirical relationship between the response and the independent variables has been expressed by the following quadratic polynomial equation model:

Table 1 Design matrix in the Box–Behnken model, observed response and predicted values.

Trial No.	V^a (μL)	t (min)	V^b (mL)	Observed, $Y_o{}^c$ (%)	Predicted, Y_p (%)	Error (%)
1	30	1	6	22892	23715	−3.60
2	90	1	6	24486	25253	−3.13
3	30	10	6	30955	30189	2.48
4	90	10	6	34903	34080	2.36
5	30	5.5	2	22868	23113	−1.07
6	90	5.5	2	27173	27475	−1.11
7	30	5.5	10	26133	25831	1.15
8	90	5.5	10	27144	26899	0.90
9	60	1	2	29585	28517	3.61
10	60	10	2	29124	29645	−1.79
11	60	1	10	23587	23066	2.21
12	60	10	10	36170	37238	−2.95
13	60	5.5	6	29340	29849	−1.74

[a] Volume of chloroform.
[b] Volume of water.
[c] Average of triplicate extraction.

$$Y = 14574.9 + 417.6(A) - 1108.7(B) + 566.3(C) - 3.0(A)^2 + 55.5(B)^2 - 84.8(C)^2 + 4.4(A)(B) - 6.9(A)(C) + 181.2(B)(C) \quad (2)$$

The critical point in the surface response is found by solving these equation systems for the condition of $\frac{\delta(Y)}{\delta(A)} = 0$, $\frac{\delta(Y)}{\delta(B)} = 0$ and $\frac{\delta(Y)}{\delta(C)} = 0$. The calculated values for the critical point are as follows: $A = 67.0\ \mu L$, $B = 4.3$ min and $C = 5.2$ mL.

Statistical testing of this model was carried out in the form of analysis of variance (ANOVA). The ANOVA for the fitted quadratic polynomial model of extraction of betulinic acid is shown in Table 2. The quadratic regression model indicated that the determination coefficient (R^2) value was 0.971, which suggested that 97.1% of the total variations could be illustrated by the fitted quadratic polynomial model. The value of adjusted R^2 was 0.918, which implied that only less than 9.0% of the total variations were not illustrated by this model. It also showed a high correlation degree between the predicted and observed values. A low value of the coefficient of variation (C.V. = 4.08%), explained a good deal of the reliability of the conducted experiment values. These variables could be more significant if the F-value becomes higher and the p-value becomes smaller (Yetilmezsoy et al., 2009). The p-value less than 0.05 indicated that quadratic model terms were significant. Therefore, the F-value ($F = 18.31$) and p-value ($p = 0.003$) suggested that quadratic model was significant. Also, significance of the quadratic model was represented by lack-of-fit test. The result is shown in Table 2, F-value and p-value of the lack-of-fit were 3.79 and 0.216, respectively, which suggested that it was not significant and a 21.6% chance could occur because of noise (Chen et al., 2012). As shown in Table 3, independent variable A, quadratic term A^2 and interaction between B and C were significant ($p < 0.05$).

The Durbin–Watson (DW) statistic (Eq. (3)) is another value that shows whether autocorrelation, or correlation between errors, is present in a model. The DW statistic is used to examine the linear association between adjacent residuals and the range of it is between 0 and 4. The values of DW below and above 2 can indicate a positive and negative autocorrelation, respectively (Yetilmezsoy et al., 2009). If the value of DW is around 2, this shows a good fit of this model.

$$DW = \sum_{i=2}^{n}(e_i - e_{i-1})^2 / \sum_{i=1}^{n} e_i^2 \quad (3)$$

In this work, the DW statistic (DW = 1.8) was determined to be very close to 2, showing the goodness of fit of the model, as similarly reported by Yetilmezsoy et al., 2009.

The relationship between dependent and independent variables was explained by the three dimensional representation of the response surface generated by the quadratic model (Fig. 2). Since the regression quadratic model has three independent variables, one variable was kept at zero level for each plot, therefore, a total of three response 3D plots were produced for responses. Fig. 3 shows the predicted and the actual peak area plot. Actual values were the measured peak area data for a particular run and the predicted values were determined by approximating functions employed for the models.

The percentage of contributions (PC: Eq. (4)) for each term is shown in Table 3.

$$PC\% = \frac{SS}{\sum SS} \times 100 \quad (4)$$

As similarly done by others (Yetilmezsoy et al., 2009), the final part of the ANOVA was completed in the same way to obtain the total PC values for the first-order (TPC_i), quadratic (TPC_{ii}) and interaction (TPC_{ij}) terms according to the following equations, respectively:

$$TPC_i = \frac{\sum_{i=1}^{n} SS_i}{\sum_{i=1}^{n}\sum_{i=1}^{n} SS_i + SS_{ii} + SS_{ij}} \times 100 \quad (5)$$

$$TPC_{ii} = \frac{\sum_{i=1}^{n} SS_{ii}}{\sum_{i=1}^{n}\sum_{j=1}^{n} SS_i + SS_{ii} + SS_{ij}} \times 100 \quad (6)$$

$$TPC_{ij} = \frac{\sum_{i=1}^{n}\sum_{i=1}^{n} SS_{ij}}{\sum_{i=1}^{n}\sum_{j=1}^{n} SS_i + SS_{ii} + SS_{ij}} \times 100 \quad (7)$$

SS_i, SS_{ii} and SS_{ij} are the computed sum of squares for first-order, quadratic and interaction terms, respectively (Yetilmezsoy et al., 2009). A detailed schematic indicating the PC of terms is depicted in Fig. 4.

3.3. Analytical figures of merit

To validate this method, linearity, correlation coefficient, limit of detection (LOD), enrichment factor and repeatability using spiked solution of sample were tested under the optimum condition of the above method. Linear relation for betulinic acid (with HLLE) was obtained in the concentration range of 0.015–1.2 ng/g, with the correlation coefficient (r^2) = 0.992.

Table 2 ANOVA analysis for extraction of betulinic acid.

Source	Sum of squares (*SS*)	Degree of freedom	Mean square (MSS)	*F*-value	*P*
Regression	2.2×10^8	9	2.4×10^7	18.31	0.003
Linear	4.4×10^7	3	1.4×10^7	11.08	0.012
Square	3.8×10^7	3	1.3×10^7	9.66	0.016
Interaction	4.6×10^7	3	1.6×10^7	11.68	0.011
Residual	6.6×10^6	5	1.3×10^6		
Lack-of-fit	5.6×10^6	3	9.9×10^6	3.79	0.216
Pure error	9.9×10^5	2	5.0×10^5		
R^2	0.971				
Adj-R^2	0.918				

Table 3 Multiple regression results and significance of the components for the quadratic model.

Factor (coded)	Parameter	Coefficient	SE[a]	*t* Ratio	*p* Value	SS[b]	(PC%)[c]
Intercept	β_0	14574.9					
A	β_1	417.6	89.3	4.677	0.005	2.91×10^7	24
B	B_2	−1108.7	466.03	−2.379	0.063	7.53×10^6	6
C	B_3	566.3	572.1	0.990	0.368	1.30×10^6	1
A^2	B_{11}	−3.0	0.67	−4.438	0.007	2.62×10^7	21
B^2	B_{22}	55.5	29.64	1.871	0.120	4.66×10^6	4
C^2	β_{33}	−84.8	37.52	−2.259	0.073	6.79×10^6	6
(*A*)(*B*)	β_{12}	4.4	4.27	1.020	0.354	1.39×10^6	1
(*A*)(*C*)	β_{13}	−6.9	4.81	−1.428	0.213	2.71×10^6	2
(*B*)(*C*)	β_{23}	181.2	32.04	5.654	0.002	4.25×10^7	35

[a] Standard error.
[b] Sum of squares.
[c] Percentage contribution (%).

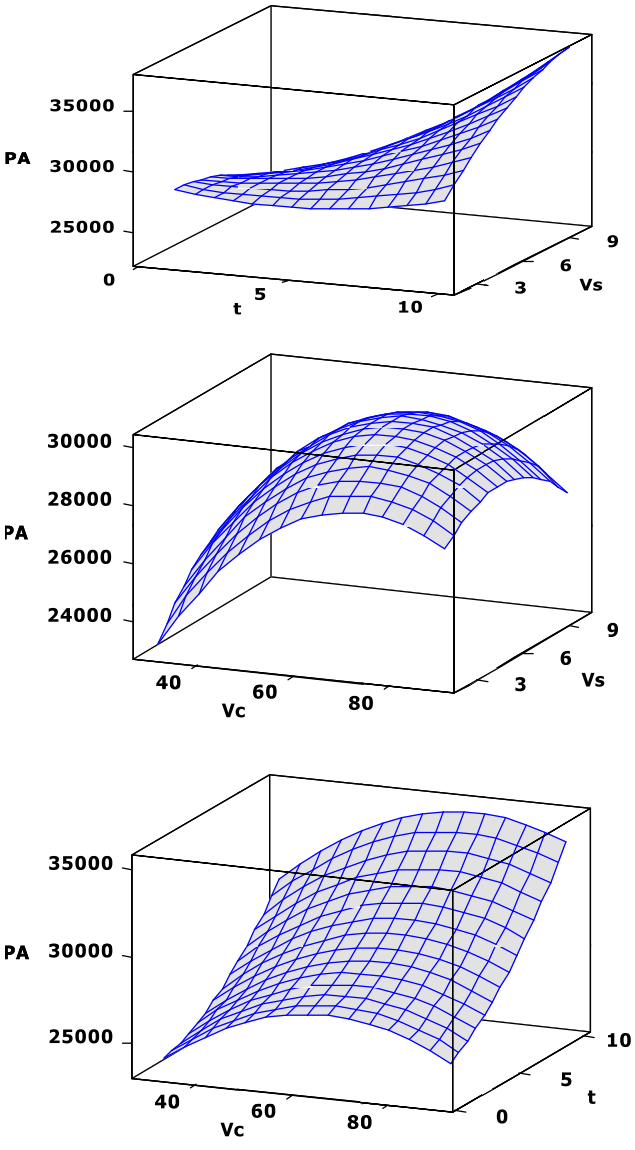

Figure 2 Response surface obtained from Box–Behnken design for extraction efficiency of betulinic acid. (Vc: volume of chloroform (μL), t: time (min) and Vs: volume of water (mL))

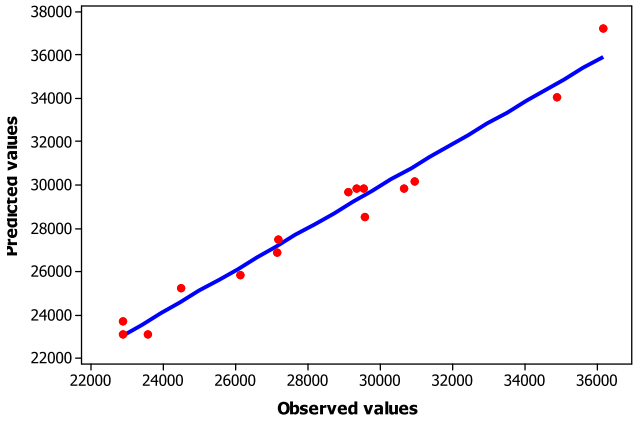

Figure 3 Parity plot shows the correlation between the observed and predicted values.

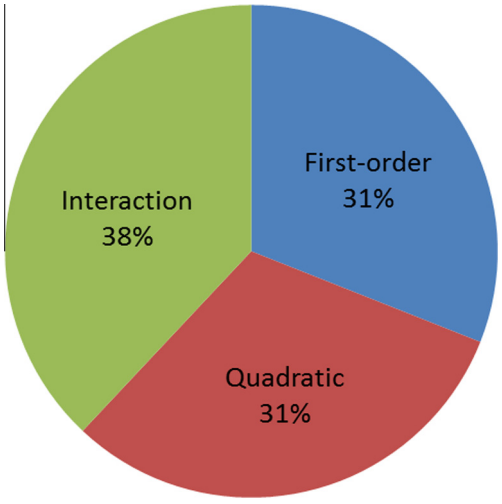

Figure 4 A detailed schematic showing the percentage contribution of components.

The LOD obtained from $C_{LOD} = 3\ (S_d)_{blan}$ was 1.6 ng/g where S_d is the standard deviation of ten consecutive measurements of the blank. The experimental enhancement factor cal-

Table 4 Determination of betulinic acid in medicinal plant samples ($N = 3$).

Samples	Betulinic acid content (ng/g)		Recovery (%)
	Added	Found (±RSD) (%)	
Carum copticum	0.0	–	–
	10.0	9.5 ± 3.1	95.0
Borage officinalis	0.0	–	–
	10.0	9.2 ± 3.5	92.0
Curcuma longa	0.0	–	–
	10.0	9.8 ± 3.9	98.0
Bunium persicu	0.0	–	–
	10.0	9.75 ± 4.2	97.5
Cuminum cyminum	0.0	–	–
	10.0	9.3 ± 4.6	93.0
Summer savoury	0.0	–	–
	10.0	9.7 ± 3.9	97.0
	0.0	–	–
Mentha piperita	10.0	9.8 ± 2.8	98.0
	0.0	–	–
Green tea	10.0	9.65 ± 4.1	96.5
	0.0	–	–
Agrostemma githago	10.0	9.8 ± 2.9	98.0
Plantago psyllium	0.0	–	–
	10.0	9.77 ± 3.1	97.7

Table 5 Comparison of the HLLE procedure with other related methods for determination of betulinic acid in plant samples.

Extraction method	LOD[a]	RSD %	Reference
Solvent extraction	6.2 (ng/g)	1.33	Zhao et al. (2007)
Soxhlet's apparatus	4.562 (mg/g)	3.16	Zhang et al. (2008)
HLLE	1.6 (ng/g)	2.7	This study

[a] Limit of detection.

culated as the ratio of the slopes of the calibration graphs with and without pre-concentration was 80.0. The relative standard deviation (RSD%) of the 10 replicate determination was less than 2.7%, which indicated that this method has a good precision for the analysis of trace analyte in the sample solution.

3.4. Analysis of real samples

The proposed method was used for the betulinic acid determination in plant samples. Additionally, the recovery experiments of different amounts of betulinic acid were carried out, and the result is shown in Table 4.

4. Conclusion

In this study, we investigated a homogenous liquid–liquid solvent extraction method to pre-concentration of betulinic acid from medicinal plant samples prior its determination by HPLC using RSM. HLLE method is a simple, powerful and efficient pre-concentration procedure that reduces the extraction time and it uses extracting solvent at a microliter level. The recommended HLLE procedure allows the separation, pre-concentration and determination of betulinic acid in medicinal plant samples in a simple way. This method was compared with other procedures (Table 5). Therefore, the results showed that the method has a very low detection limit in comparison with other methods.

References

Ahmad, F., Omar, J., Ali, A.M., 1999. Chemical examination of local plant: triterpene from leaf of Malaysian *Callistemon speciosus* D.E. Ultra Sci. 11, 357–360.

Alizadeh, K., Zohrevand, S., Ghiasvand, A.R., Hashemi, P., Shamsipur, M., Sharghi, H., Khalifeh, R., 2010. Selective homogeneous liquid–liquid extraction and preconcentration of copper(II) into a micro droplet using a benzo-substituted macrocyclic diamide, and its determination by electrothermal atomic absorption spectrometry. Microchim. Acta 168, 115–121.

Box, G.E.P., Hunter, W.G., Hunter, J.S., 1997. Statistics for Experimenters. Wiley, New York.

Chen, W., Wang, W.P., Zhang, H.S., Huang, Q., 2012. Optimization of ultrasonic-assisted extraction of water-soluble polysaccharides from *Boletus edulis* mycelia using response surface methodology. Carbohydr. Polym. 87, 614–619.

Claude, B., Morin, Ph., Lafosse, M., Andre, P., 2004. Evaluation of apparent formation constants of pentacyclic triterpene acids complexes with derivatized β- and γ-cyclodextrins by reversed phase liquid chromatography. J. Chromatogr. A 1049, 37–42.

Farajzadeh, M.A., Bahram, M., Zorita, S., Ghorbani Mehr, B., 2009. Optimization and application of homogeneous liquid–liquid extraction in preconcentration of copper(II) in a ternary solvent system. J. Hazard. Mater. 161, 1535–1543.

Gbaguidi, F., Accrombessi, G., Moudachirou, M., Quetin-Leclercq, J., 2005. HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*. J. Pharm. Biomed. Anal. 39, 990–995.

Khajeh, M., 2009. Optimization of microwave-assisted extraction procedure for zinc and copper determination in food samples by Box–Behnken design. J. Food Compos. Anal. 22, 343–346.

Khajeh, M., 2011. Response surface modelling of lead pre-concentration from food samples by miniaturised homogenous liquid–liquid solvent extraction: Box–Behnken design. Food Chem. 129, 1832–1838.

Khajeh, M., Musavi Zadeh, F., 2012. Response surface modeling of ultrasound-assisted dispersive liquid–liquid microextraction for determination of benzene, toluene and xylenes in water samples: Box–Behnken design. Bull. Environ. Contam. Toxicol. 89, 38–43.

Kumar, D., Mallick, S., Vedasiromoni, J.R., Pal, B.C., 2010. Anti-leukemic activity of *Dillenia indica* L. fruit extract and quantification of betulinic acid by HPLC. Phytomedicine 17, 431–435.

Razborsek, M.I., Voncina, D.B., Dilecek, V., Voncina, E., 2008. Determination of oleanolic, betulinic and ursolic acid in Lamiaceae and mass spectral fragmentation of their trimethylsilylated derivatives. Chromatographia 67, 433–440.

Shamsipur, M., Hassan, J., 2010. A novel miniaturized homogenous liquid–liquid solvent extraction high performance liquid chromatographic–fluorescence method for determination of ultra traces of polycyclic aromatic hydrocarbons in sediment samples. J. Chromatogr. A 1217, 4877–4882.

Suhaj, M., 2006. Spice antioxidants isolation and their antiradical activity: a review. J. Food Compos. Anal. 19, 531–537.

Wang, X., Zhao, X., Liu, X., Li, Y., Fu, L., Hu, J., Huang, C., 2008. Homogeneous liquid–liquid extraction combined with gas chromatography–electron capture detector for the determination of three pesticide residues in soils. Anal. Chim. Acta 620, 162–169.

Yetilmezsoy, K., Demirel, S., Vanderbei, R.J., 2009. Response surface modeling of Pb(II) removal from aqueous solution by Pistacia vera L.: Box-Behnken experimental design. J. Hazard. Mater. 171, 551–562.

Zhang, M., Zhang, Y., Xie, J., 2008. Simultaneous determination of jujuboside A, B and betulinic acid in semen *Ziziphi spinosae* by high performance liquid chromatography-evaporative light scattering detection. J. Pharm. Biomed. Anal. 48, 1467–1470.

Zhao, G., Yan, W., Cao, D., 2007. Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC. J. Pharm. Biomed. Anal. 43, 959–962.