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Optimization of pressurized liquid extraction for *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *Angelica sinensis*

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Abstract

Pressurized liquid extraction, one of the most promising and recent sample preparation techniques, offers the advantages of reducing solvent consumption and allowing for automated sample handling. It is being exploited in diverse areas because of its distinct advantages. However, because the extraction is performed at elevated temperatures using PLE, thermal degradation could be a concern. *Z*-ligustilide, one of the biologically active components in *Angelica sinensis,* is an unstable compound, which decomposes rapidly at high temperature. In this study, we carried out a comparative study to evaluate PLE as a possible alternative to current extraction methods like Soxhlet and sonication for simultaneous extraction of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis*. The operating parameters for PLE including extraction solvent, particle size, pressure, temperature, static extraction time, flush volume and numbers of extraction were optimized by using univariate approach coupled with central composite design (CCD) in order to obtain the highest extraction efficiency. Determination of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid were carried out by means of high performance liquid chromatography with diode-array detector. The results showed that PLE was a simple, high efficient and automated method with lower solvent consumption compared to conventional extraction methods such as Soxhlet and sonication. PLE could be used for simultaneous extraction of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis*.

Keywords: Pressurized liquid extraction; Central composite design; Ferulic acid; *Z*-Ligustilide; *Z*-Butylidenephthalide; *Angelica sinensis*

1. Introduction

The extraction step has often proved to be the bottleneck of most analytical procedures, as it is one of the least evolved parts of the whole method. During the past few years, one of the most promising and recent sample preparation techniques is the pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction), which offers the advantages of reducing solvent consumption and allowing for automated sample handling [1]. Since the introduction of the first commercial PLE instrument a few years ago, the application of this technique has been focused on the extraction of environmental pollutants present in soil matrix, sewage sludge, sediments and fly ash [2,3]. However, more recently, the distinct advantages of PLE, such as significantly reduced extraction time and low solvent volume requirement, are being exploited in diverse areas, including biology, pharmaceuticals and foodstuffs [4]. An interesting and important new application area of PLE is in the extraction of chemical constituents from plants or herbal materials [4–14]. However, because extractions are performed at elevated temperatures using PLE, thermal degradation could be a concern.

The rhizome of *Angelica sinensis* (Oliv.) Diels (Umbelliferae), known as Danggui in Chinese, is one of the most important traditional Chinese medicines, which is used for tonifying the blood and treating female irregular menstruation and amenorrhoea. It is also used for treatment of anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases [15–17]. Among over 70 compounds isolated and identified in Danggui [18], the main essential ingredients, *Z*-ligustilide, butylidenephthalide and ferulic acid (Fig. 1) are thought to be the biologically active components [19–22]. *Z*-Ligustilide is a volatile and unstable compound, which can be changed to other phthalides through oxidation, isomerization, dimerization, etc. [23,24]. It decomposes rapidly at high temperature.

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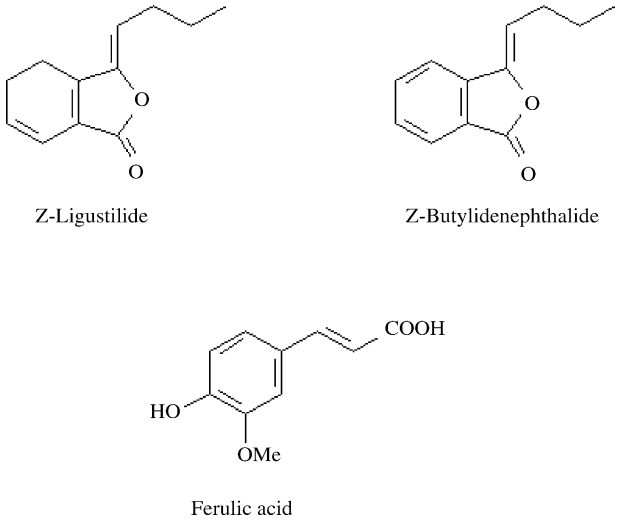

Fig. 1. The structure of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid.

In this study, we therefore carried out a comparative study to evaluate PLE as a possible alternative to current extraction methods like Soxhlet and sonication for simultaneous extraction of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis*. The operating parameters for PLE including extraction solvent, particle size, pressure, temperature, extraction time, flush volume and numbers of extraction were optimized in order to obtain the highest extraction efficiency.

2. Experimental

2.1. Materials and chemicals

Angelica. sinensis was obtained from Minxian County of Gansu Province, China. The identity was confirmed by Dr. Shaoping Li. The voucher specimen was deposited at Institute of Chinese Medical Sciences, University of Macau. The rhizome of *A. sinensis* was dried in an universal oven with forced convection (FD115, Tuttlingen, Germany) at 40 °C for 6 h. The dried sample was ground using Knifetec^{TM} 1095 Sample Mill (FOSS TECTOR, Sweden), and the powder was sieved. Particles with the size between 10 and 120 mesh (0.125–2 mm, i.d.) were collected for the study. Ferulic acid and 3-butylidenephthalide (the *Z*-isomer is 86.85%) were purchased from Sigma (St. Louis, MO, USA). *Z*-Ligustilide was purchased from Chroma-Dex (St. Santa Ana, CA, USA). Methanol, ethanol, ethyl acetate and acetonitrile for LC and petroleum ether (analytical-reagent, 60–90 °C) were purchased from Merck (Darmstadt, Germany). Acetic 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).

2.2. Pressurized liquid extraction

Pressurized liquid extractions were carried out using a Dionex ASE 200 (Sunnyvale, CA, USA) system equipped with a 24-sample carousel. Dried powder of *A. sinensis* (1.0 g) were mixed with diatomaceous earth in a proportion (1:2) and placed into an 11 ml stainless steel extraction cell, respectively. Here, diatomaceous was used for preventing the aggregation of sample particles and the blockage of extraction cell outlet [25]. The extraction cells were placed into the carousel and the samples were extracted under the extraction conditions. The extract was transferred to a 25 ml volumetric flask which was brought up to its volume with extraction solvent and filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the HPLC system.

2.3. Soxhlet extraction

Soxhlet extraction was performed as described by Xin et al. [26]. In brief, 1.0 g of *A. sinensis* powder (0.125–0.2 mm, i.d.) was transferred into a paper thimble and 60 ml mixture solution consist of methanol/formic acid (95:5) was used for extraction. The extraction was terminated until the extract turned to colorless. The extract was concentrated by rotary evaporation (Heidolph, Schwabach, Germany) and transferred to a 25 ml volumetric flask which was brought up to its volume with extraction solvent and filtered through a 0.45 μm Econofilter prior to injection into the HPLC system.

2.4. Sonication extraction

Sonication extraction was performed using an ultrasonic cleaning bath (model 9310-1, Melrose Park, IL, USA) as described by Liu et al. with modification [27]. One gram of *A. sinensis* powder (0.125–0.2 mm, i.d.) was transferred into a 100 ml flask and extracted with 50 ml methanol/formic acid mixture solution (95:5) for 30 min at room temperature. The extract was centrifuged at 4000 rpm for 10 min and the supernatant liquid was reduced in volume less than 25 ml by rotary evaporation. Then, the extract was transferred to a 25 ml volumetric flask which was brought up to its volume with extraction solvent and filtered through a 0.45 μm Econofilter prior to injection into the HPLC system.

2.5. Quantitative analysis

The quantitative analysis were performed on a Agilent Series 1100 liquid chromatography, equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD detector, connected to a Agilent ChemStation software. A ZORBAX ODS C_{18} column (4.6 mm × 250 mm i.d., 5 μm) and a ZORBAX ODS C_{18} guard column (4.6 mm × 12.5 mm i.d., 5 μm) were used. Solvents that constituted the mobile phase were A (1% aqueous acetic acid) and B (acetonitrile). The elution conditions applied were: 0–10 min, linear gradient 5–35% B; 10–30 min, linear gradient 35–50% B; 30–35 min, linear gradient 50–70% B; and finally, reconditioning steps of the column was 5% B isocratic for 15 min. Flow rate was 1.0 ml/min and the injection volume was 20 μl. The system operated at 25 °C. Peaks were detected at 284 nm. The standard curve of ferulic acid, *Z*-ligustilide and *Z*-butylidenephthalide was calibrated by using the linear least squares regression equation derived from the peak area. The concentrations of these three compounds in the samples were

calculated according to the regression parameters derived from the standard curve.

2.6. Statistical methods

Statistical analysis was carried out by SAS system for windows release version 6.12 (SAS Institute, Cary, NC, USA) which comprises a number of "procedures"—graphical, statistical, reporting, processing and tabulating procedures—that enable simple and rapid data evaluation.

3. Results and discussion

3.1. System precision, linearity, limit of detection and limit of quantitation of HPLC

Z-Ligustilide, *Z*-butylidenephthalide and ferulic acid were used as the markers for evaluation of extraction efficiency. HPLC profiles of a PLE extract from *A. sinensis* were shown in Fig. 2. The relative standard deviation (R.S.D., $n = 6$) of peak areas (retention time) for *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid were 1.02% (0.26%), 1.58% (0.18%) and 1.34% (0.13%), respectively. Linearity range was 40–400 μg/ml, 9.3–173.7 μg/ml and 10.2–163.2 μg/ml for *Z*-ligustilide ($r = 0.9999$), *Z*-butylidenephthalide ($r = 0.9998$) and ferulic acid ($r = 0.9994$), respectively. Limit of detection (LOD) was defined as the signal-to-noise ratio (S/N) of 3. And limit of quantitation (LOQ) was defined as the signal-to-noise ratio (S/N) of 10. The LOD (LOQ) values of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid were 1.32 (5.86) μg/ml, 0.58 (1.86) μg/ml and 0.24 (1.52) μg/ml, respectively.

3.2. Effect of extraction solvent

In PLE, solvent is a key factor affecting the recovery of analytes. Here, water, methanol, ethanol, ethyl acetate and petroleum ether were chosen for test because the polarity of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid were significantly different. The experiments were performed at the default conditions (temperature, 100 °C; pressure, 1500 psi; static extraction time, 5 min; flush volume, 60% and one extraction cycle). As shown in Fig. 3A, the extraction efficiency of

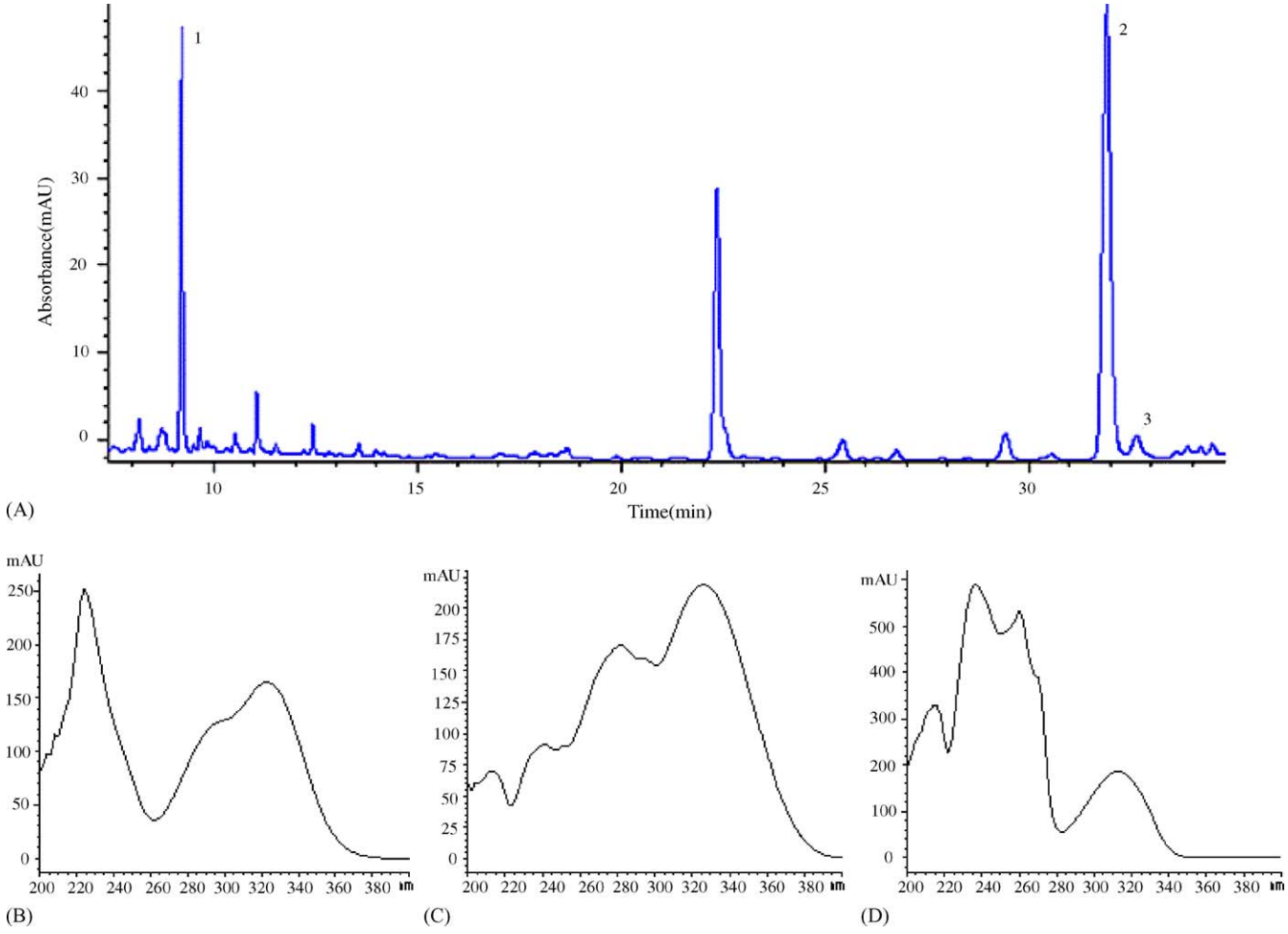

Fig. 2. HPLC chromatograms of PLE extract from *A. sinensis* (A) and the UV-spectra of ferulic acid (B), *Z*-ligustilide (C) and *Z*-butylidenephthalide (D). (1) Ferulic acid; (2) *Z*-ligustilide; (3) *Z*-butylidenephthalide.

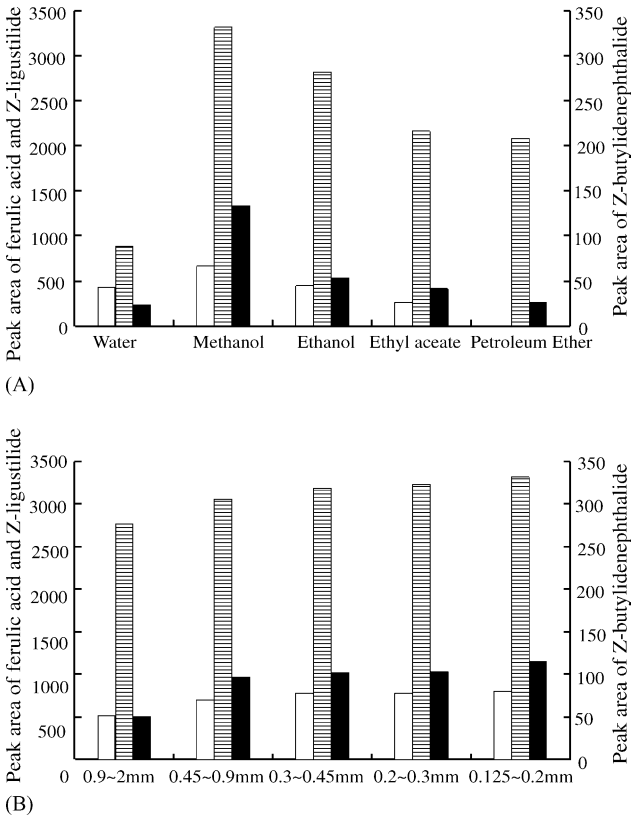

Fig. 3. Effects of solvent (A) and particle size (B) on the PLE of *Z*-ligustilide (▤), *Z*-butylidenephthalide (■) and ferulic acid (□) in *A. sinensis*. Condition: Particle size, 0.125–0.2 mm (A), or solvent, methanol (B); temperature, 100 °C; static extraction time, 5 min; pressure, 1500 psi; flush volume, 60%; extraction cycle, 1; and numbers of extraction, 1. The mean values of three determinations are presented. The variation is less than 3% of the mean.

methanol was the highest. Especially, ferulic acid cannot be extracted by using petroleum ether. Therefore, methanol was used as solvent for the further investigations.

3.3. Effect of particle size

Particle size is another variable to be considered in undertaking PLE. Generally, extraction efficiency increase with the particle size reduced (Fig. 3B). In this study, particle size at 0.125–0.2 mm was preferred so as to avoid compaction of the sample in the extraction cell, which tends to build up at the cell outlet and can clog the system.

3.4. Experimental design and optimization by central composite design

Central composite design (CCD) was used for optimization of PLE parameters: temperature, pressure, static extraction time and flush volume. Before specific limits for individual CCD factors were selected, pilot experiments had to be carried out in which the effects of temperature, pressure, static extraction time and flush volume were studied (Fig. 4).

Out of the four factors, three were selected, which displayed the most pronounced effect on the extraction efficiency of PLE, the factors being: temperature, static extraction time and flush volume. Based on the effect of pressure within the range permitted, 1500 psi as the default level was selected (Fig. 4B). For evaluation of extraction efficiency, overall desirability (OD), the geometric mean of peak area for *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid were used as markers [28].

Table 1
Experimental range and levels of the independent test variables

Variables	Range and levels				
	−1.668	−1	0	+1	+1.668
X_1: temperature (°C)	40	60	90	120	140
X_2: static extraction time (min)	5	9	15	21	25
X_3: flush volume (%)	10	25	50	75	90

The ranges and the levels of the variables (temperature, static extraction time and flush volume) investigated in this study were given in Table 1. Each factor in the design was studied at five different levels (−1.668, −1, 0, 1, 1.668). All variables were taken at a central coded value considered as zero.

In general, CCD is constructed in such a way that $2f + 2f + 1$ experiments are required where f represents the number of factors to be studied. Therefore, a three-factor CCD requires 15 experimental points, each of which being a result of different experimental conditions. Five additional experiments were carried out at the centre point to estimate the overall error, the total number of experiments thus amounted to 20. The experimental conditions for the CCD and OD were presented in Table 2. 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 polynomial equation. Thus, a mathematical regression model for total peak area fitted in the coded factors was given as following:

$$Y = 0.0864 + 0.0121X_1 + 0.0078\,X_2 + 0.0062X_3 - 0.00005X_1^2 + 0.00034X_2^2 + 0.00003X_3^2 - 0.00003X_1X_2 - 0.00006X_1X_3 - 0.00023X_2X_3$$

where Y was the response, that was the total peak area of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid, 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, which were listed in Table 3. The larger the magnitude of the *t*-value and smaller the *P*-value, the more significant is the corresponding coefficient. This implies that the first-order main effect of temperature (i.e. X_1) was highly significant as was evident from its *P*-values ($P = 0.005$) and with its second-order main effects ($X_1^2 = 0.013$ and $X_1X_3 = 0.009$). These suggest that temperature have a direct relationship with the extraction efficiency of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis*.

Since the quadratic response surface is calculated in $(f + 1)$ dimensions, where f is the number of factors in the CCD, the quadratic response surface for the three factors involved gener-

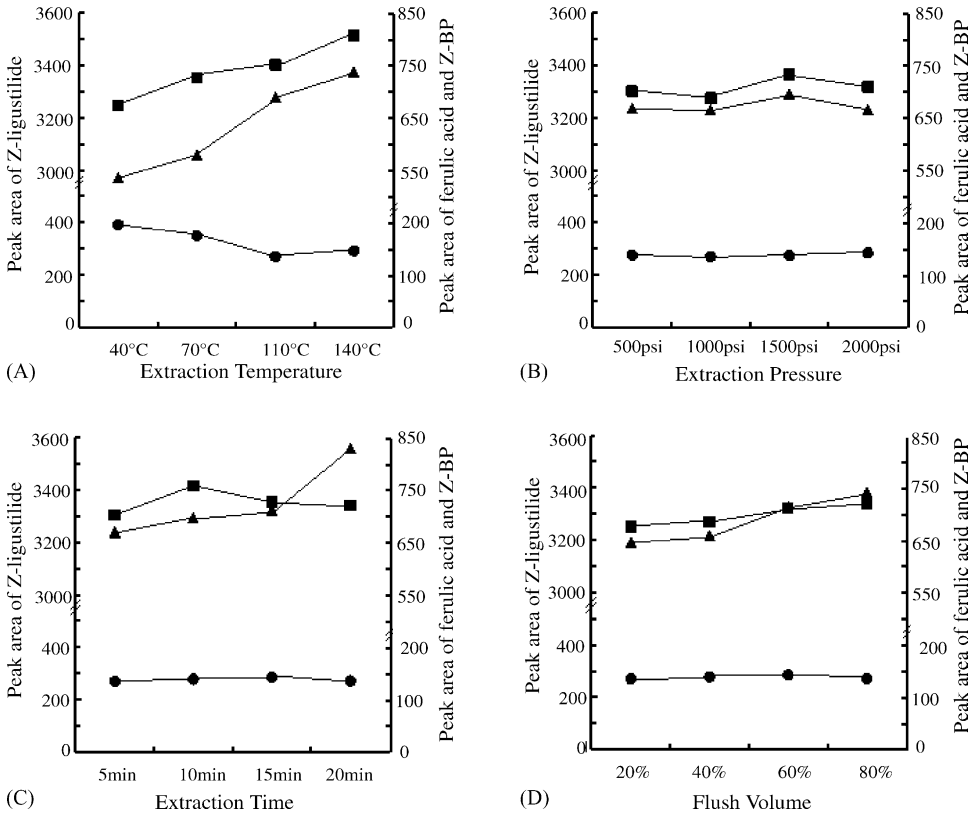

Fig. 4. Influence of selected factors including temperature (A), pressure (B), static extraction time (C) and flush volume (D) on the PLE extraction of *Z*-ligustilide (■), *Z*-butylidenephthalide (●) and ferulic acid (▲) in *A. sinensis*. Condition: To determine one of the parameters including temperature, pressure, static extraction time and flush volume, the others were set at the system default value (temperature, 100 °C; pressure, 1500 psi; static extraction time, 5 min; flush volume, 60%; and extraction cycle, 1). Solvent, methanol; particle size, 0.125–0.2 mm. The mean values of three determinations are presented. And R.S.D. is not more than 3%.

ates a four-dimensional response surface, which can be readily visualized in a three-dimensional (3D) response surface. The response model was mapped against two experimental factors while the third was held constant at its optimum. That way, 3D response function was depicted in Fig. 5.

Fig. 5A showed the response surface function developed by the model for static extraction time and flush volume; the response showed that the longer static extraction time and the less flush volume, the more pronounced the response in the maximum direction. Fig. 5B showed the response surface function developed by the model for temperature and flush volume, the response showed a maximum at 110 °C and 10%, respectively. Fig. 5C showed the function for temperature and static extraction time, giving a maximum for temperature of 110 °C

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	*Z*-Ligustilide	*Z*-Butylidenephthalide	Ferulic acid[a]	OD[b]
1	−1	−1	−1	3247.3	79.0	589	0.732
2	1	−1	−1	3294.3	98.3	695.1	0.836
3	−1	1	−1	3243.6	145.7	569	0.887
4	1	1	−1	3351.4	142.6	692.4	0.951
5	−1	−1	1	3299.6	143.3	596.8	0.902
6	1	−1	1	3356	86.6	725.7	0.818
7	−1	1	1	3293.5	139.4	611.2	0.900
8	1	1	1	3322.2	79.4	783.8	0.813
9	−1.668	0	0	3176.8	93.8	573.3	0.763
10	1.668	0	0	3297.3	76.8	718.6	0.779
11	0	−1.668	0	3319	136.6	647.1	0.914
12	0	1.668	0	3357.7	136.2	696.9	0.939
13	0	0	−1.668	3307.8	138	668.5	0.926
14	0	0	1.668	3369.8	139.1	686	0.942
15–20	0	0	0	3334	117.7	650.8	0.872

[a] Peak area.
[b] Overall desirability.

Table 3
Regression results from the data of CCD experiments

Model term	Parameter estimate	Standard error	*t*-Value	*P*-value
Intercept	0.086379	0.204526	0.422	0.690
X_1	0.012104	0.002575	4.700	0.005
X_2	0.007877	0.011515	0.684	0.524
X_3	0.006204	0.002569	2.415	0.061
X_1X_1	−0.000049	0.000013	−3.780	0.013
X_1X_2	−0.000030	0.000057	−0.528	0.620
X_1X_3	−0.000057	0.000014	−4.164	0.009
X_2X_2	0.000337	0.000322	1.044	0.344
X_2X_3	−0.000231	0.000068	−3.402	0.019
X_3X_3	0.000025	0.000020	1.256	0.265

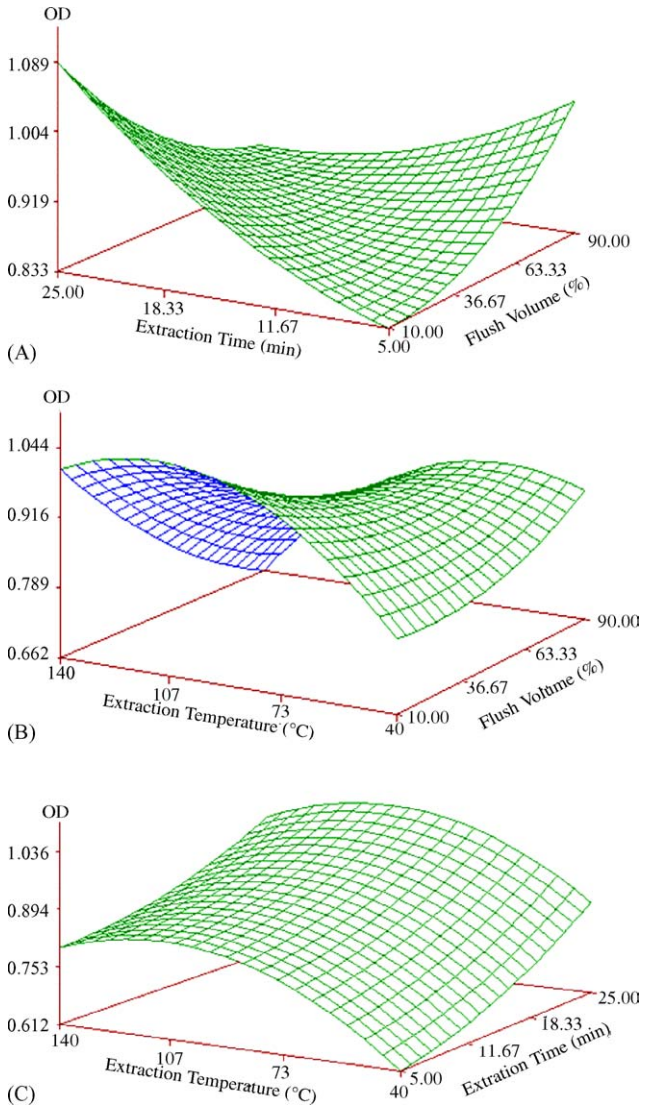

Fig. 5. Response surface for overall desirability (OD) response function of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid. (A) Static extraction time (min, X_2) vs. flush volume (%, X_3). Temperature (X_1) is held at its optimum. (B) Temperature (°C, X_1) vs. flush volume (%, X_3). Static extraction time (X_2) is held at its optimum. (C) Temperature (°C, X_1) vs. static extraction time (min, X_2). X_3 is held at its optimum.

and for static extraction time of 25 min. Resulting from this study, the optimum PLE conditions to obtain the highest extraction efficiency of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis* were selected as: solvent, methanol; particle size, 0.125–0.2 mm; temperature, 110 °C; static extraction time, 25 min, and flush volume, 10%.

3.5. Recovery of PLE

The recovery of PLE for the analytes was determined by performing consecutive pressurized liquid extractions for three times on the same sample under the optimized conditions. The recovery was calculated based on the total amount of individual investigated components. As a result, no *Z*-ligustilide or *Z*-butylidenephthalide was detected in the second times extract. And the recoveries at one times extraction obtained for every analyte were higher than 99.7% (R.S.D. < 4%, $n = 5$). Thus, we concluded that PLE conditions at one times extraction would be acceptable. On the other hand, accurate amounts of three analytes were added to approximate 0.5 g of *A. sinensis*, and then extracted and analyzed as described above to evaluate the effect of PLE temperature on stability of investigated compounds. The result showed that the average recoveries of ferulic acid, *Z*-ligustilide and *Z*-butylidenephthalide were 99.1, 97.7 and 98.5% (R.S.D. < 5%, $n = 5$), respectively, which suggested the temperature was available for PLE of three investigated compounds from *A. sinensis*. That *Z*-Ligustilide was stable at 110 °C during PLE may be derived from the extraction performed under an inert atmosphere and short time.

3.6. Comparison of PLE, Soxhlet and sonication

The extraction efficiency of PLE for analytes in *A. sinensis* was compared with those of Soxhlet and sonication. As shown in Table 4, the extraction efficiency of PLE was comparable to that of Soxhlet extraction and higher than that of sonication, which suggested that PLE could be an alternative method for the extraction of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis*. The results also showed that reduced solvent consumption and shorter extraction time were the other major advantages of PLE when comparing to Soxhlet and sonication. It was very interesting that the temperature showed no obvious effect on extraction efficiency of *Z*-Ligustilide which is thermal labile compound during this study. The reasons may be that the extraction performed under an inert atmosphere and short time

Table 4
Comparison of PLE, Soxhlet and sonication method for the extraction of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis*

Method	Time	Solvent volume (ml)	Peak area[a]		
			Z-Ligustilide	*Z*-BP	FA
PLE	20 min	20	3295.6	95.1	801.3
Soxhlet	6 h	60	3021.1	53.9	893.9
Sonication	30 min	50	2284.0	71.6	596.5

BP, *Z*-butylidenephthalide; FA, ferulic acid.
[a] The mean values of three determinations are presented. The variation is less than 5% of the mean.

(PLE), or low temperature (Soxhelt extraction was performed under 70 °C).

4. Conclusion

By using univariate approach coupled with CCD, PLE parameters, such as solvent, particle size, pressure, temperature, static extraction time, flush volume and numbers of extraction, for extraction of *Z*-ligustilide, *Z*-butylidenephthalide and ferulic acid in *A. sinensis* were optimized. The results showed that PLE could be an alternative to Soxhlet and sonication for the extraction of the analytes. The optimized conditions are as follows: solvent, methanol; particle size, 0.125–0.2 mm; pressure, 1500 psi; temperature, 110 °C; static extraction time, 25 min; flush volume, 10% and numbers of extraction, one. The PLE is a simple, efficient and rapid method with lower solvent consumption compared to conventional extraction methods such as Soxhlet and sonication.

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