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Optimisation of triterpenoids extraction from Anli pears (*Pyrus ussuriensis* Maxim) by pressurised liquid extraction

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RESEARCH ARTICLE

Abstract

The extraction of triterpenoids from Anli pears (*P. ussuriensis* Maxim) by pressurised liquid extraction (PLE) has been studied. The effect of extraction temperature, time, number of cycles and ethanol concentration were investigated by an orthogonal design experiment. The orthogonal experiment results showed that at a temperature of 85 °C, extraction time of 4 min, number of cycles of 3, ethanol concentration of 55%, maximum triterpenoids extraction was achieved (0.862%). The results are comparable to that of traditional methods of heat reflux extraction and microwave-assisted extraction. Overall, the current study highlighted PLE as a useful method for triterpenoid extraction from Anli pears.

Keywords: Anli pears (*Pyrus ussuriensis* Maxim), orthogonal experiment, pressurised liquid extraction, triterpenoids

1. Introduction

Pear (*Pyrus* spp.), which is one of the most widely consumed fruits through the whole world, has also been used as a traditional folk remedy in China for more than 2,000 years (Li *et al.*, 2014). There is a great diversity of pear varieties in China due to its widespread consumption. Anli pears (*Pyrus ussuriensis* Maxim), a cold temperate species and the most frost resistant of the *Pyrus* species, is indigenous to northeastern China (Lim, 2012). The fruits can be edible and have nutritive and medicinal properties. The cultivars have a smaller fruit weight and a higher level of sugars (Pan *et al.*, 2002). Arbutin and chlorogenic acid were found to be the main phenolic constituents (Cui *et al.*, 2005), and also malic and citric acid were found to be dominant organic acids in *P. ussuriensis* Maxim fruits (Sha *et al.*, 2011). In recent years, growing attention has been paid to triterpenoids (Wang *et al.*, 2013; Xu *et al.*, 2013; Zuo *et al.*, 2012). Triterpenoids, which display a wide range of pharmacological activities, including anti-tumor, hemolysis, anti-inflammatory, anti-bacterial, anti-viral, immune modulatory, hepatoprotective (Wu, 2007) and anti-asthma effects (Park *et al.*, 2002), were considered as one kind of the effective components in pears. It is of considerable interest to find a suitable method to extract and determine the triterpenoids from Anli pears.

In terms of triterpenoids extraction, a great variety of approaches based on different principles have been developed. Conventional organic solvent extraction, microwave-assisted extraction (MAE), and reflux extraction (Lin *et al.*, 2012), ultra-sonic-assisted extraction (He *et al.*, 2012), and supercritical fluid extraction (Domingues *et al.*, 2012) have been applied to extract triterpenoids. However, there is no report, to the best of our knowledge, on triterpenoids extraction in Anli pear with the help of a pressurised liquid extraction (PLE) technique. PLE is a sample preparation technique that combines elevated temperature and pressure with liquid solvents to achieve fast and efficient extraction of the analytes from the solid matrix (Du *et al.*, 2014). Compared with heat reflux extraction (HRE) and MAE, PLE has significant advantages over competing techniques with regard to time saving, solvent use, automation and efficiency. For example, HRE may be sometimes inefficient and slow, and the sample preparation time involved in clean-up of the extracts can give rise to high labour costs. PLE has also an advantage over MAE in that no additional filtration step is required,

since the matrix components that are not dissolved in the extraction solvent may be retained inside the sample extraction cell. This is very convenient for the purposes of automation and on-line coupling of the extraction and separation techniques (Carabias-Martínez *et al.*, 2005).

The purpose of this study was to investigate the optimal conditions of PLE of triterpenoids from Anli pears by an orthogonal design experiment, and compare the results with heat reflux and MAE.

2. Materials and methods

Materials

Anli pears were collected from Qianan City, Hebei Province, China, in mid-October 2012. The fruits were divided into three parts: the peel, pulp, and core. The pulp was cut into thin slices and then oven dried at 50 °C for 24 h. The dried samples were pulverised in a high-speed mixer-grinder and sieved by 40-mesh screen. The sample was then stored in a desiccator until use.

Chemicals

Ursolic acid was purchased from National Institute of the Control of Pharmaceutical and Biological Products, Ministry of Health (Beijing, China P.R.), vanillin was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China P.R.), ethanol and glacial acetic acid were purchased from Tianjin Fengchuan Chemical Technology Co., Ltd. (Tianjin, China P.R.), perchloric acid was purchased from Beijing Nan Shang Le Chemical Factory (Beijing, China P.R.). All the other reagents were analytical grade.

Pressurised liquid extraction

PLE was performed with APLE-3000 system (Beijing Titan Instruments Co., Ltd, Beijing, China P.R.) where the schematic diagram was shown in Figure 1. Accurately weighted portion of 20 g of sample was mixed with 20 g of diatomite which acts as the dispersion agent. Then the mixture was placed into a 100 ml stainless steel extraction cell, the two endings of which were padded with filter paper to make the extract filtered. After predetermined temperature was approached, the nitrogen was delivered passing upwards through the extraction cell at a pressure of around 10 MPa and compressed to the predetermined extraction pressure by an air driven booster pump. The sample was subjected to dynamic extraction for 2-10 min according to a special solvent rate, and then the extracts were collected into a collection vial and analysed by UV for the triterpenoids yield in the extracts (Pitipanapong *et al.*, 2007).

Heat reflux extraction

Method of Wu and Huang (2009) was followed as the reference. Accurately weighted portion of 20 g of sample, and 500 ml 65% aqueous ethanol was introduced into an airtight Erlenmeyer flask. The flask was put in a constant temperature water-bath (Ronghua Instrument Manufacturing Co., Ltd., Jiangsu, China P.R.) at 85 °C for 2.5 h. Extracts were filtered and then analysed.

Microwave-assisted extraction

Method of Bai *et al.* (2006) was followed for microwave-assisted extraction. 500 ml 65% aqueous ethanol was added into 20 g sample. The solution was subjected to microwave power of 160 Watt (G80W23YCSL-Q3; Guangdong Galanz Group Co., Ltd., Foshan, China P.R.) each time for 3 min. Extracts were filtered and then analysed.

Determination of triterpenoids

1 ml extract was accurately pipetted into a 25 ml flask, and the flask was put to a constant temperature water-bath at 100 °C until ethanol evaporated entirely, and then 0.40 ml 5% vanillin-glacial acetic acid solution (w/v) and 1.60 ml perchloric acid were added. The flask was put to

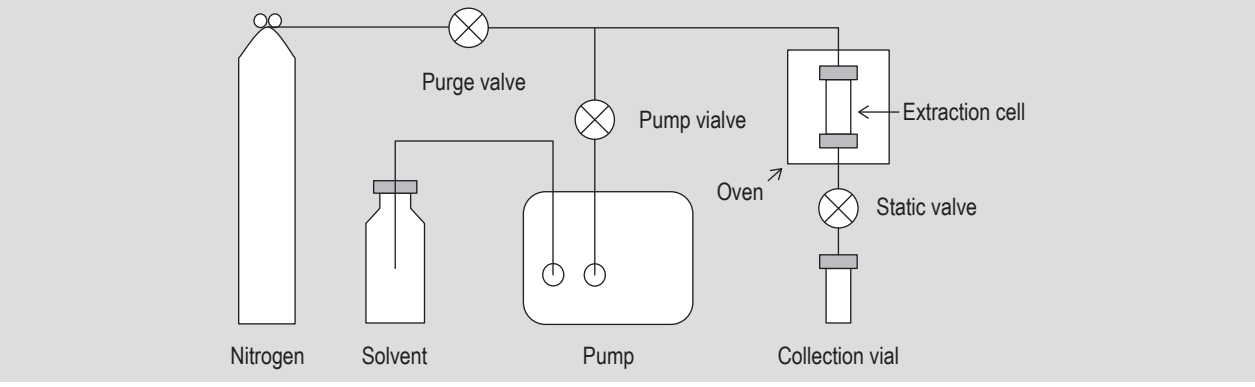

Figure 1. Schematic diagram of pressurised liquid extraction system.

a constant temperature water-bath at 65 °C for 15 min and then shifted into ice-water bath, followed by addition of 5.00 ml glacial acetic acid. After mixing, the solution was allowed to stand for 15 min at room temperature, and the absorbance at 548 nm was determined with a visible spectrophotometer (Model 722S; Shanghai Optical Instrument Co., Ltd., Shanghai, China P.R.). Triterpenoids content was calculated using ursolic acid as the calibration standard. A good linear relationship was obtained over the range of 0.0054-0.064 mg/ml, and the regression equation was found as y = 0.0293x + 0.0016, with a correlation coefficient of R^2=0.9968, where y denoted triterpenoids content (mg/ml) and x denoted the absorbance at 548 nm (Kou *et al.*, 2011).

Statistical analysis

Analyses were performed in triplicate. Statistical analysis was performed using SPSS 11.0 software package (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was conducted, and Ducan's multiple range tests were used to determine the significant differences between group means using the probabilities of 0.05.

3. Results and discussion

Various parameters potentially could affect the extraction process, so the optimisation of the experimental conditions is a critical step in the development of a PLE method to extract triterpenoids from Anli pears. Orthogonal experiments is an appropriate method to examine the factors, such as temperature, time, number of cycles and ethanol concentration. Therefore, $L_9(3^4)$ orthogonal experiment was chosen as the experimental design. Table 1 shows the factors and levels for the extraction of triterpenoids, and the results are shown in Table 2.

Extraction results

The results shown in Table 2 indicate that there are great differences of yield among each set of PLE conditions. The triterpenoids yield was expressed as control indexes through intuitive analysis, the influence of the factors on PLE of triterpenoids from Anli pears were as follows: number of cycles > temperature > ethanol concentration > time. Variance analysis on the data from the orthogonal experiments was applied in Table 3. From Table 3, it is seen

Table 1. Factors and levels of orthogonal experiment.

Levels	Factors			
	A: temperature (°C)	B: time (min)	C: number of cycles	D: ethanol concentration (%)
1	75	2	1	55
2	85	4	2	65
3	95	6	3	75

Table 2. The results of orthogonal experiment.

Number	Temperature (°C)	Time (min)	Number of cycles	Ethanol concentration (%)	Yield of triterpenoids (%)
1	75	2	1	55	0.746
2	75	4	2	65	0.740
3	75	6	3	75	0.781
4	85	2	2	75	0.778
5	85	4	3	55	0.862
6	85	6	1	65	0.858
7	95	2	3	65	0.827
8	95	4	1	75	0.806
9	95	6	2	55	0.702
K1	22.670	23.510	24.100	23.100	
K2	24.980	24.080	22.200	24.250	
K3	23.350	23.410	24.700	23.650	
R	0.770	0.223	0.833	0.383	

K1 = the sum of yield for level 1; K2 = the sum of yield for level 2; K3 = the sum of yield for level 2; R = range.

Table 3. ANOVA table for the pressurised liquid extraction of triterpenoids from Anli pears.

Source of variance	Sum of squares	df	Mean square	F[1]
Temperature	0.029	2	0.014	607.364[a]
Time	0.003	2	0.002	63.920[a]
Number of cycles	0.032	2	0.016	679.123[a]
Ethanol concentration	0.006	2	0.003	127.724[a]

[1] P=0.01 (99% confidence level); $F(2,18)$=6.01.

that for triterpenoids extraction yield, number of cycles is the most significant at P<0.01 factor among other conditions.

Effect of temperature

It was observed that extraction yield increased as temperature increased, however, the yield decreased after the temperature reached 75 °C (as shown in Figure 2). Temperature during the extraction is one of the critical factors that could affect the efficiency and selectivity in PLE. High temperatures can improve the efficiency of the extraction as it helps the disruption of analyte-sample matrix interactions caused by Van der Waals forces, hydrogen bonding and dipole attraction (Richter *et al.*, 1996). In addition, high temperature can improve diffusion rate, mass transfer of the molecule in the solvent (Mustafa and Turner, 2011). But higher temperature causes intermolecular interactions (dipole-dipole and hydrogen bonding) within the solvent to decrease, causing higher molecular motion, and making the solute to be more easily dissolved in the solvent (Pitipanapong *et al.*, 2007). Therefore, the extraction temperature of 75 °C was found appropriate.

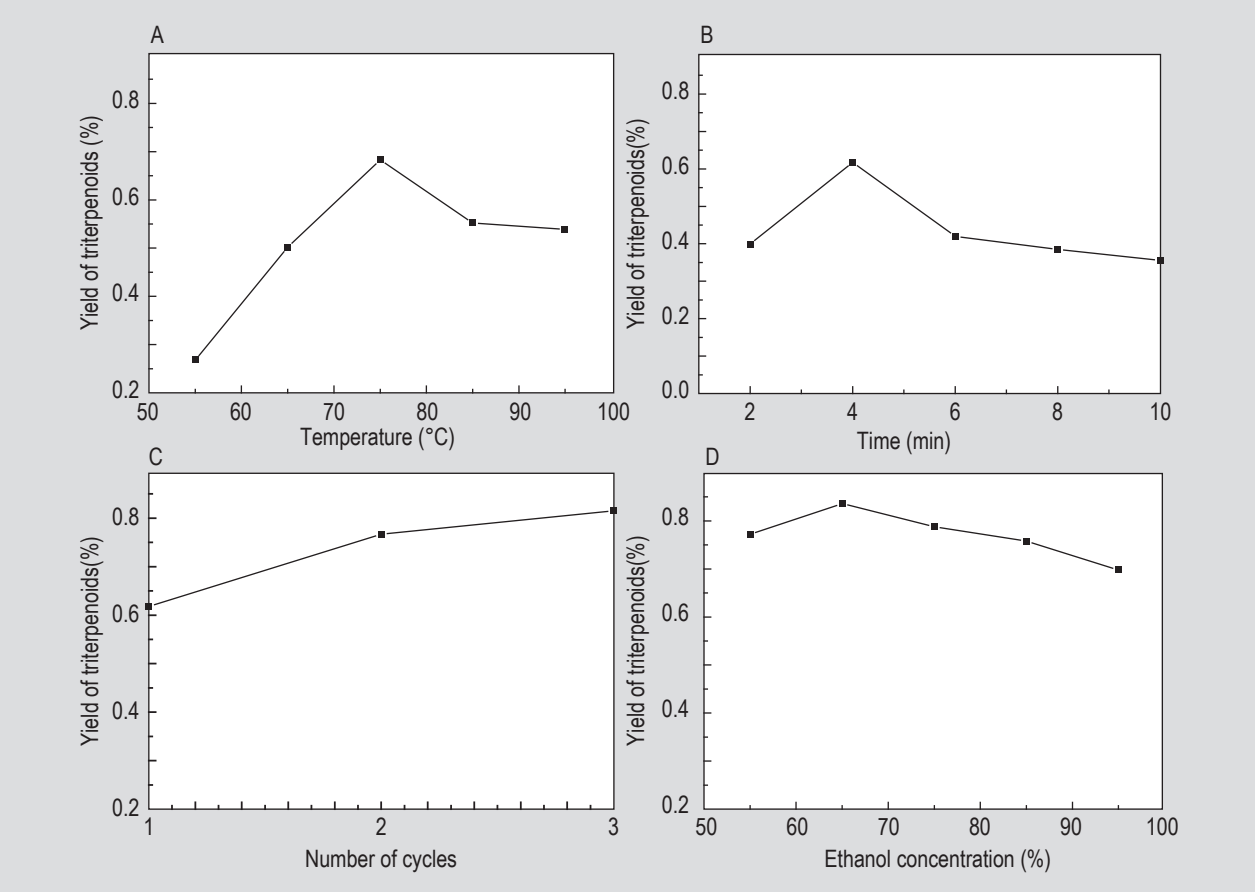

Figure 2. Effect of temperature, time, number of cycles and ethanol concentration on triterpenoids yield using pressurised liquid extraction.

Table 4. The optimal conditions of pressurised liquid extraction (PLE) compared with heat reflux extraction (HRE) and microwave-assisted extraction (MAE).

Methods	Pressure (Mpa)	Time (min)	Solvent amount (ml)	Yield (%)
HRE	atmospheric pressure	150	500	0.689
MAE	atmospheric pressure	3	500	0.675
PLE	10	4	45	0.862

Effect of extraction time

Figure 2 shows that, as time increases, the yield increased and then decreased, extraction time of 4 min results the maximum yield. Long-term high temperature state might have led to the compounds thermal decomposition, so that the yield decreased significantly. From the view of energy-saving and time-saving, the extraction time of 4 min was found appropriate.

Effect of number of cycles

As can be seen from Figure 2, with the increase of number of cycles, the yield increased. The yield increased significantly from 1 to 2 times (P<0.05) and remained steady from 2 to 3 times. It was concluded that 2 pass extraction would be sufficient, from an economic point of view.

Effect of ethanol concentration

One of the key steps of solvent extraction process is that the target compounds are dissolved in the selected solvent. According to the principle of 'like dissolves like', solvent of a polarity, which is similar to that of the target compounds, is likely to be dissolved more of the latter. These different concentrations led to different polarities of the solvent (Zhang *et al.*, 2008). Figure 2 demonstrated that an ethanol concentration of 65% resulted in the highest triterpenoids yield.

Comparison of PLE with HRE and MAE

HRE and MAE were performed as described before. The results are given in Table 4. Compared with HRE and MAE, PLE has an obvious advantage of high yield, less time and solvent consumption. It is found as the appropriate extraction method of triterpenoids.

4. Conclusions

Optimal conditions of PLE of Anli pears triterpenoids, i.e. temperature of 85 °C, time of 4 min, number of cycles of 3, ethanol concentration of 55%, were found through an orthogonal design. Under these optimal conditions, the triterpenoids yield can reach 0.862%. A comparison between PLE, HRE, MAE demonstrated the higher extraction yield of PLE. It can be concluded that PLE is a very useful technique for the extraction and isolation of triterpenoids from Anli pears.

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