Aqueous Extraction of Limonin from *Citrus reticulate* Blanco

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Abstract

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The replacement of organic solutions in the extraction of limonin from citrus seeds with an alkaline solution was investigated. This method was based on the reversible conversion of limonin to limonoate A-ring lactone via ring-opening of D-ring lactone at different pH values. The extraction conditions, optimised using Taguchi experimental design, were as follows: pH 11, temperature 70°C, alkaline solution/seeds ratio 20:1 (v/w), ultrasonic power 800 W for 30 minutes. A yield of 7.5 mg/g (limonin/citrus seeds) of 98% pure limonin was obtained.

Keywords: optimisation; citrus seeds; conversion; Taguchi approach; ultrasonic extraction

Limonin is a highly oxygenated triterpenoid dilactone which occurs naturally in plants from the *Rutaceae* and *Meliaceae* families, and which is particularly abundant in the seeds (Roy & Saraf 2006). It exhibits a significant biological activity, including anticancer, cholesterol-lowering, antioxidant, and antiviral activities (Kurowska *et al.* 2000a,b; Battinelli *et al.* 2003; Miller *et al.* 2004; Manners 2007). Limonin is therefore a compound important for human health.

Since limonin is a medium polarity compound and insoluble in water, the traditional extraction methods for limonin involve the use of organic solvents, such as dichloromethane, ethyl acetate, and acetone under refluxing extraction conditions. However, the use of large quantities of organic solvents results in both serious environmental and food security problems.

In recent years, several new extraction methods have been investigated, for instance supercritical carbon dioxide (SC-CO_2) and hydrotropic extraction (Yu *et al.* 2007; Dandekar *et al.* 2008). SC-CO_2 extraction reduces the organic solvent use dramatically, resulting in a more environmentally-friendly process. Nevertheless, the high pressure needed and costs involved limit its application when scaling up the process. The introduction of large quantities of hydrotrope can increase the solubility of limonin in water, however, significant amounts of sodium salt residues remain in the limonin extract and cause problems in limonin purification. Currently, there is no method for the extraction of limonin which is appropriate for the mass production.

It has been reported that liminon (water-insoluble) converts to limonoate A-ring lactone (water-soluble) in an alkaline environment, while limonoate A-ring lactone can be reconverted into limonin in an acidic environment (Fisher 1978; Kola *et al.* 2010), via the reversible ring-opening of

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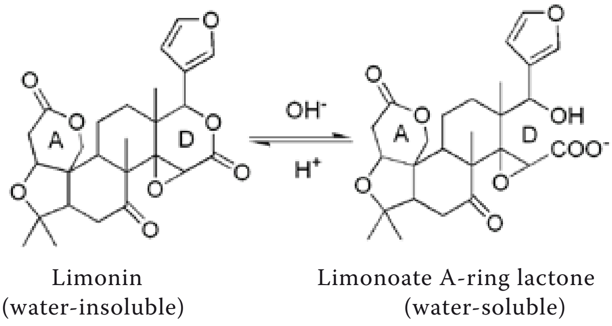

Figure 1. Reversible conversion of ring D at different pH

limonin D ring at different pHs (Figure 1) (Emerson 1952; Maier & Grant 1970; Hasegawa *et al.* 1980). Based on these characteristics, we have designed a novel method for the limonin extraction with alkaline solution.

MATERIAL AND METHODS

Materials. Defatted citrus (*Citrus reticulate* Blanco) seeds were obtained from Jiangmen Haolun Co. (Jiangmen, China); HPLC-grade acetonitrile was purchased from J & K Chemical Co. (Beijing, China); the standard limonin was from Sigma-Aldrich Co. (St. Louis, USA); and the Amberlite XAD-16 resin was purchased from Rohm & Haas Co. (Philadelphia, USA). All the other reagents were of analytical grade.

Experiment design. The yield of limonin was mainly dependent on the extraction conditions used, such as pH, temperature, and solution/seeds ratio. In order to optimise the extraction conditions, Taguchi's $L_{16}(4^3)$ orthogonal array was used to investigate the effects of the process parameters on the limonin yield. The actual levels of the corresponding coded values are listed in Table 1. All the experiments were carried out in triplicate and the data were analysed by SPSS for Windows software, version 11.5 (SPSS Inc, Chicago, USA).

Extraction. The citrus seeds were mixed with alkaline solution, and placed in an ultrasonic extractor (Hongxianglong Co., Beijing, China). The extraction was performed using 800 W ultrasonic power for 30 minutes. Parameters such as pH value, temperature, and solution/seeds ratio were adjusted according to the experimental design.

Table 1. Parameters and levels in experimental design

Parameters	Levels			
(A) pH	8	9	10	11
(B) Temperature (°C)	60	65	70	80
(C) Solution/seeds ratio (v/w)	7:1	10:1	15:1	20:1

Purification. The purification of limonin was conducted using the 3 steps described below. (a) Column chromatography: the extracted solution was centrifuged at 5000 r/min for 15 min to obtain the supernatant, which was then clarified by ultrafiltration through hollow fiber membranes (MW = 10 000) to eliminate macromolecular proteins. The adjusted filtrate (pH 4.1) was then subjected to chromatography (Amberlite XAD-16 resin, 3.0 × 40 cm) at a flow rate of 1.5 ml/minute. Finally, the column was successively eluted with 4 times the resin bed volume of 70% alcohol. (b) Acidification: the collected eluant (500 ml) was concentrated to about 40 ml by vacuum rotary evaporation at 50°C. A white precipitate appeared when the concentrated solution was acidified to pH 1.0 with 4M HCl, and it was subsequently collected through centrifugation at 5000 r/min for 15 minutes. (c) Crystallisation: the obtained precipitate was dissolved in a mixed solution of dichloromethane/isopropanol (1:3, v/v), and was then kept at 4°C for 2 h (Emerson 1948). The limonin crystals obtained were dried at 60°C, and analysed by IR and HPLC.

HPLC system. A Waters HPLC system (Waters Co., Milford, USA) coupled with a Waters Sunfire C18 column, 5 μm particle size (150 × 4.6 mm), Waters 600E HPLC pump (Waters Co., Milford, USA), and Waters 2478 dual λ absorbance detector (Waters Co., Milford, USA) were used. The elution of limonin was carried out at 1.0 ml/min flow rate using a mobile phase composed of acetonitrile/water (45:55) and 0.2% formic acid. The column temperature was set at 25°C. The injection volume was 20 μl, and limonin was detected by UV absorbance at 210 nm (Sun *et al.* 2005). The calibration curve was obtained by plotting the concentration of limonin versus the absorption peak area.

RESULT AND DISCUSSION

Optimisation of extraction conditions

The results of Taguchi experimental design are shown in Table 2, where *K*-values give the average limonin yield (Chen *et al.* 2007; Fang *et al.* 2010). The *K*-values indicate that A (pH) has the great-

Table 2. Taguchi's experimental design matrix and corresponding limonin yield

Trial No.	A	B	C	Limonin yield (mg/g)
1	1	1	1	3.65
2	1	2	2	3.81
3	1	3	3	4.33
4	1	4	4	4.52
5	2	1	2	5.88
6	2	2	1	6.13
7	2	3	4	6.82
8	2	4	3	6.71
9	3	1	3	7.08
10	3	2	4	7.42
11	3	3	1	6.78
12	3	4	2	7.19
13	4	1	4	7.21
14	4	2	3	7.18
15	4	3	2	7.37
16	4	4	1	6.83
*K*1	4.08	5.96	5.85	
*K*2	6.39	6.14	6.06	
*K*3	7.12	6.33	6.33	
*K*4	7.15	6.31	6.49	

A – pH; B – temperature; C – solution/seed ratio

est effect on the limonin yield in level 4, while B (temperature) and C (solution/seeds ratio) show their greatest effects in levels 3 and 4, respectively. The level corresponding to the maximum average yield among the four levels is chosen for the optimal set of parameters, therefore the optimal conditions for the limonin extraction are determined as $A_4B_3C_4$.

In order to identify the significance of the effect of each parameter on the yield of limonin, an analysis of variance (ANOVA) was applied onto the data (Mousavi *et al.* 2007; Lu *et al.* 2008). As shown in Table 3, with respect to the *F* value, the pH is the most important parameter for the yield of limonin, and the effects of the three parameters decrease in the following order: A (pH) > C (solution/seed ratio) > B (temperature). The model obtained from ANOVA indicates that the adjusted R^2 is 0.98, meaning that the model can explain 98% variation in the response; the coefficient of determination (R^2) is 0.99, which illustrates that the model applied is adequate.

The validation experiments were repeated 3 times under the optimised conditions. The results are shown in Table 4, and indicate that the optimal condition, $A_4B_3C_4$, is reliable for the extraction process.

Table 3. Analysis of variance (ANOVA)

Source	Sum of squares	DF	Mean square	*F* value	Prob > *F*
Corrected model	26.447	9	2.939	110.793	
(A) pH	25.110	3	8.370	315.575	< 0.001
(B) Temperature	0.365	3	0.122	4.586	0.054
(C) Solution/seed ratio	0.972	3	0.324	12.217	0.006
Error	0.159	6	0.027		
Residual	638.055	16			
Cor total	26.606	15			

Table 4. Validation experiments under optimised conditions

Number	Liomonin yield (mg/g)
1	7.64
2	7.42
3	7.59
Mean	7.55

IR and HPLC analysis

The extracted limonin was identified by comparison with a limonin standard. IR spectra analysis was done on an FT-IR spectrometer (Nicolet 750). Figure 2A illustrates the IR spectrum of the extracted sample. The IR spectrum exhibits a single broadened, high intensity, carbonyl absorption band at 1756 cm^{-1} (C = O stretching vibration for saturated ester, lactone) and 1709 cm^{-1} (C = O stretching vibration for saturated six membered ring ketone), and characteristic bands assigned to

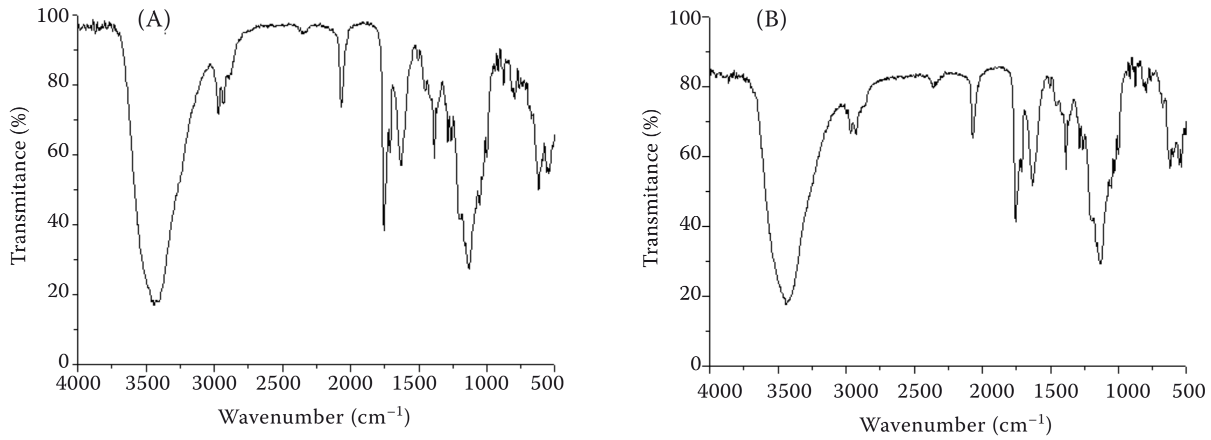

Figure 2. Infrared spectra of limonin sample (A) and limonin standard (B)

a β-substituted furan ring at 1506 and 870 cm^{-1}. The infrared spectrum of the extracted sample is identical with that of limonin standard (Figure 2B), indicating that the sample has the same structure as limonin. Figure 3 shows the chromatograms of the extracted and standard limonins, respectively. The major peak is observed at 7.2 min in Figure 3A which is identical to the retention time of the standard (Figure 3B), indicating that the major peak was limonin.

According to the linear regressive relation between the concentration and the absorption peak area, the calibration curve for limonin could be expressed using the following equation:

$$A = 16671C + 35617\ (R^2 = 0.99) \qquad (1)$$

where:
C – concentration of limonin (μg/ml)
A – corresponding absorption peak area of limonin

The limonin purity (%) was calculated using the following equation:

$$P = (C \times V/M) \times 100\% \qquad (2)$$

where:
P – purity of limonin (%)
V – total volume of sample solution (ml)
C – concentration of limonin (g/ml)
M – weight of sample (g)

The purity (98%) of the limonin was calculated using Eqs (1) and (2). From the data, it is evident that a high yield of highly pure limonin can be obtained using the new extraction method.

In this work, limonin was successfully extracted using an alkaline solution which results in a lower cost and more environmentally-friendly process than the traditional, organic solvent dependent processes. A high yield of limonin (7.85 mg/g (limonin/citrus seeds)) was obtained with a 98% purity, as judged by IR and HPLC. It is speculated that limononate A-ring lactone present in the seeds may be partly converted to limonin during the process. Further research into the mechanism and details of the conversion are in progress. In

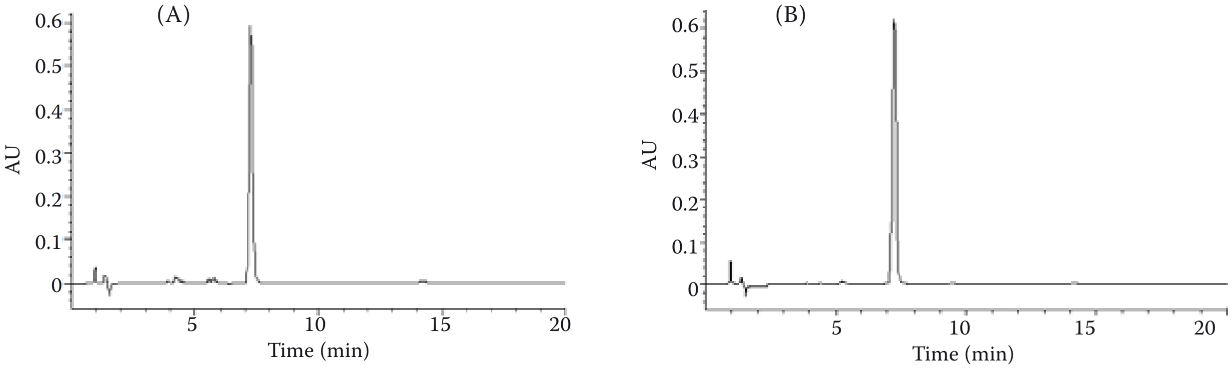

Figure 3. Liquid chromatograms of limonin sample (A) and limonin standard (B)

conclusion, the extraction method presented here is an ideal process for the mass production of limonin.

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