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Full Length Research Paper

Optimization of ultrasonic-assisted extraction procedure of capsaicinoids from Chili peppers using orthogonal array experimental design

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In this present study, the optimal conditions of ultrasonic-assisted extraction (UAE) of capsaicinoids from hot Chili peppers were determined for large scale preparation. First, single factor experiments were performed to optimize the extraction procedure of capsaicinoids, and initial optimized results were: ratio of solvent to mass of 6 to 10 ml/g, extraction temperature of 25 to 35°C, and extraction time of 0 to 30 min. Then, an orthogonal array experimental design ($L_9(3^4)$) was used to further optimize the extraction procedure. The results of *F*-test and P-value indicated that the effect order on extraction yield of capsaicinoids from high to low was ratio of solvent to mass, extraction time, and extraction temperature. The maximum extraction yield of capsaicinoids was obtained at ratio of solvent to mass of 10 ml/g, extraction time of 40 min, and extraction temperature of 25°C. Under these conditions, the extraction yields of capsaicinoids were 2.35 ± 0.042 and 3.92 ± 0.089 mg/g for conventional and UAE methods, respectively.

Key words: Chili pepper, ultrasonic-assisted extraction, capsaicinoids, orthogonal array design, single factor experiments.

INTRODUCTION

Chili peppers (*Capsicum frutescens*), appreciated for their pungency, taste, and aroma, have been extensively used over the years as food additives, pigments, and physiological and pharmaceutical products. Capsaicinoids, a given name of pungent compounds, are the principal pungent and irritating constituents in most capsicum fruits (Xing et al., 2006), and their structures are acid amides of vanillylamine and C_9–C_{11} branched chain fatty acids (Constant et al., 1996). Although, excessive exposure to capsaicinoids would be toxic causing irritation on the contact area or respiratory problems as well as some types of cancers due to ingestion of high quantities, capsaicinoids present many biological activities, such as powerful antioxidant (Henderson et al., 1999), anti-mutagenic and anti-tumoral properties (Long and Medeiros, 2001; Rosa et al., 2002). A common use of capsaicinoids is in topical analgesics against pain, anti-arthritic and anti-inflammatory ointments (Morris et al., 1995; Sancho et al., 2002; Govindarajan and Sathyanarayana, 1991). Moreover, capsaicinoids are also utilized as natural inhibitor of pathogenic microorganisms in food industry due to their antimicrobial properties (Kurita et al., 2002, Xing et al., 2006, Jones et al., 1997). In view of wide biologically activities, the preparation of capsaicinoids requires development of efficient extraction process from select Chili peppers.

Up to now, many different extraction methods have been performed to prepare capsaicinoids from peppers, such as maceration (Kirschbaum-Titze et al., 2002), magnetic stirring (Contreras-Padilla and Yahia, 1998), enzymatic extraction(Salgado-Roman et al., 2008), ultrasonic-assisted extraction (UAE) (Barbero et al., 2008), Soxhlet (Korel et al., 2002), supercritical fluids extraction (Sato et al., 1999), pressurized liquids (Barbero et al., 2006b), and microwave-assisted

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extraction (Barbero et al., 2006a). Among these extraction methods, UAE method is particularly commended for its simplicity and low equipment cost. Thus, it has been employed to extract various organic compounds from different matrices, such as phenolics in cosmetic creams (Padilla et al., 2005), chlorinated pesticides in bird livers (Lambropoulou et al., 2006), organic acids in grapes (Palma and Barroso, 2002), phenolic compounds from strawberries (Herrera and Luque de Castro, 2005) or isoflavones from soybeans (Rostagno et al., 2003).In addition, orthogonal array design (OAD), a fractional factorial design and a series of trials assigned by orthogonal array, is often used in design experiments with multiple level factors (Oles, 1993). The results of OAD experiments can be treated by range analysis and analysis of variance (ANOVA). The main advantages of OAD include that it may minimize assay numbers and time to keep the experimental cost at a minimum level, and the optimum parameters obtained from laboratory can be utilized in larger scale of production (Yesilyurt, 2004).

In this study, UAE and OAD methods were employed to extract capsaicinoids from Chili peppers, and the extraction variables, including appropriate solvent, ratio of solvent to mass, extraction temperature, and extraction time, were optimized to obtain a systematic process for capsaicinoids extraction.

MATERIALS AND METHODS

Chemical and reagents

Ethanol, ethylether, n-hexane, dimethyl sulfoxide, petroleum ether (60 to 90°C), and acetone were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). The capsaicin standard (97%) was obtained from Sigma-Aldrich (Steinheim, Germany).

Peppers and preparation

Chili peppers (*C. frutescens*) were produced in Sichuan province (China) and purchased from local markets. The peppers were dissected, and sepatated into peduncle, seeds, and pericarp. The pericarp was employed to optimize extraction parameters of UAE method, which was dried in a 65°C oven (Baixin Instrument and Equipment Factory, Shanghai, China) until the constant weight was achieved; and then triturated with a conventional pulverizer (Qingzhou Sanyang Co. Ltd, Shandong, China), to form homogeneous samples.

Extraction procedure

Oven-dried preparations (0.5 g dry weight) were used to extract capsaicinoids by conventional (non ultrasonic-assisted) and UAE methods. The preparation and solvent mixtures were homogenized in 10 ml conical glass tubes and placed in an ultrasonic water bath of 360 W (Time Ultrasonic Factory, Shanghai, China) coupled with a temperature controller. And different extraction parameters were evaluated, including solvents (ethanol, ethylether, n-hexane, petroleum ether (60 to 90°C), and acetone), ratio of solvent to mass (ml/g) (4 to 12), extraction temperature (20 to 50°C), and extraction time (0 to 120 min).

Analytical methods

After extration, concentration of capsaicinoids was determined by measuring OD_{280} (optical density at 280 nm). According to our previous study, the relationship between capsaicinoids concentration (mg/L) and OD_{280} is shown as follows:

$Y = 49.567x + 0.73$, $R = 0.9977$

Where, x is OD_{280} value; y (mg/L) is concentration of capsaicinoids. The yield of capsaicinoids was calculated using the following equation:

$$Y = \frac{y \times n}{W}$$

Where, Y (mg/g) is extraction yield of capsaicinoids; n is dilution ratio; W (g) is quantity of oven-dried preparations.

Orthogonal array experimental design

An OAD ($L_9(3^4)$) was performed to investigate the optimal ultrasonic-assisted extraction procedure of capsaicinoids from Chili peppers. As seen in Tables 1 and 2, extraction experiments were carried out with four factors and three levels, namely ratio of solvent to mass (factor *A*), extraction time (factor *B*), extraction temperature (factor *C*), and extra column (factor *E*).

The range of each factor level was based on the results of single factor tests, which were identified to have larger effects on extraction yield of capsaicinoids. The OAD tests were operated following the method described in extraction procedure. Data analysis was carried out through the range analysis and analysis of variance (ANOVA) to reflect the optimal reaction conditions and their magnitudes. Optimal conditions were obtained via OAD experiments and subsequent data analysis. Finally, experiments were repeated under the optimal conditions to verify the data.

RESULTS AND DISCUSSION

Results of single factor experiments

Selection of solvents for capsaicinoids extraction

As we know, the effectiveness of UAE depends on extraction solvent's capacity for absorbing and transmitting the energy of ultrasounds (Barbero et al., 2008). Thus, selecting an appropriate solvent for extracting interested analytes from matrix of samples is an improtant step in the development of UAE method.

To select the most suitable one, five different organic solvents, including ethanol, ethylether, acetone, petroleum ether (60 to 90°C), and n-hexane, were investigated in this work. The tests were performed with 0.5 g triturated sample, in 5 ml of solvent, at a temperature of 20°C for an extraction period of 30 min. All assays were performed in triplicate, and results are represented in Figure 1.

Table 1. Factors and levels for orthogonal array experimental design.

Level	Factor		
	Ratio of solvent to mass *A* (ml/g)	Extraction time *B* (min)	Extraction temperature *C* (°C)
1	6	20	25
2	8	30	30
3	10	40	35

Table 2. Extraction yield of capsaicinoids in an orthogonal array experimental design ($L_9(3^4)$).

Trial number	Factor				Results of extraction yield, Y_i (mg/g)
	Ratio of solvent to mass, *A* (ml/g)	Extraction time, *B* (min)	Extraction temperature, *C* (°C)	Experimental error, *E*	
1	6	20	25	1	3.512
2	6	30	30	2	3.462
3	6	40	35	3	3.582
4	8	20	30	3	3.566
5	8	30	35	1	3.492
6	8	40	25	2	3.726
7	10	20	35	2	3.702
8	10	30	25	3	3.762
9	10	40	30	1	3.786
K_{j1}	10.556	10.78	11	10.79	
K_{j2}	10.784	10.716	10.814	10.89	$Y_T = \sum_{i=1}^{9} Y_i$ =32.59
K_{j3}	11.25	11.094	10.776	10.91	
$\overline{K_{j1}}$	3.519	3.593	3.667	3.597	
$\overline{K_{j2}}$	3.595	3.572	3.605	3.63	
$\overline{K_{j3}}$	3.75	3.698	3.592	3.637	
R_j	0.231	0.126	0.075	0.04	
Order of significant factors			*A*>*B*>*C*		
Optimal levels			$A_3B_3C_1$		

In the light of Figure 1, it can be observed that acetone is the most appropriate solvent for extracting capsai-cinoids from hot peppers, and gives a maximum extraction yield of 3.92 mg/g. Ethyl ether is a fairly efficacious solvent, which gives extraction yield of only about 91% of that obtained with acetone. Furthermore, similar quantities of capsaicinoids were obtained with ethanol, petroleum ether and n-hexane; and no significant differences (p>0.05) were observed in the extraction yield obtained with these three solvents. Thus, it was decided to employ acetone as solvent for the development of UAE method.

Effect of ratio of solvent to mass on extraction yield of capsaicinoids

The ratio of volume of solvent (ml) to mass (g) is an important factor that must be optimized to increase the efficacy of extraction of capsaicinoids. In conventional techniques of liquid–solid extraction, the tendency is to change volume of solvent, while holding quantity of sample for studying the effect of ratio of solvent to mass on extraction yield.

To evaluate the effect of ratio of solvent to mass on extraction yield of capsaicinoids, a series of ratios (4, 6, 8, 10, and 12 ml/g) were carried out with different volumes of solvent while maintaining a constant quantity of triturated hot Chili peppers of about 0.5 g. The rest extraction conditions were: temperature at 20°C, 30 min of extraction and acetone as solvent. All assays were performed in triplicate, and the results are given in Figure 2.

In Figure 2, it can be observed that extraction yield of capsaicinoids was enhanced to the critical value (3.89 ± 0.05 mg/g) at the ratio of 10 ml/g, and then increased in a mild slope with ratio of solvent to mass increasing. Thus,

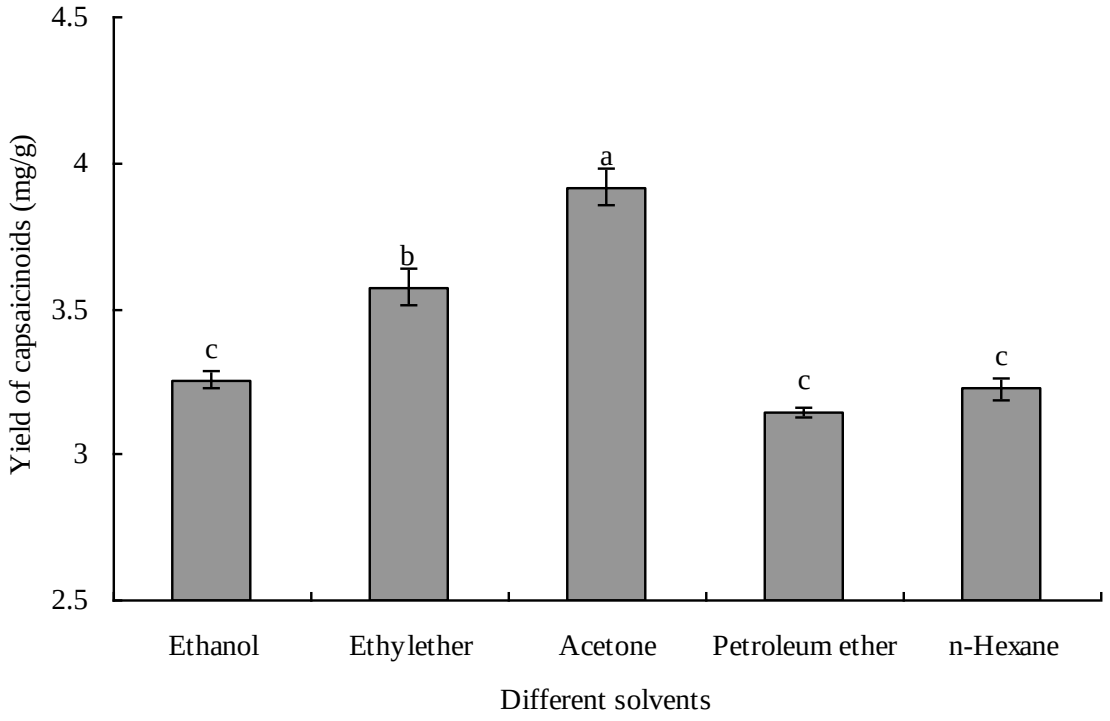

Figure 1. Effect of different solvents on extraction yield of capsaicinoids from hot Chili peppers. *Vertical bars* represent the mean ± SE (n=3). Data sets significantly different from each other are identified by different letters (P<0.05).

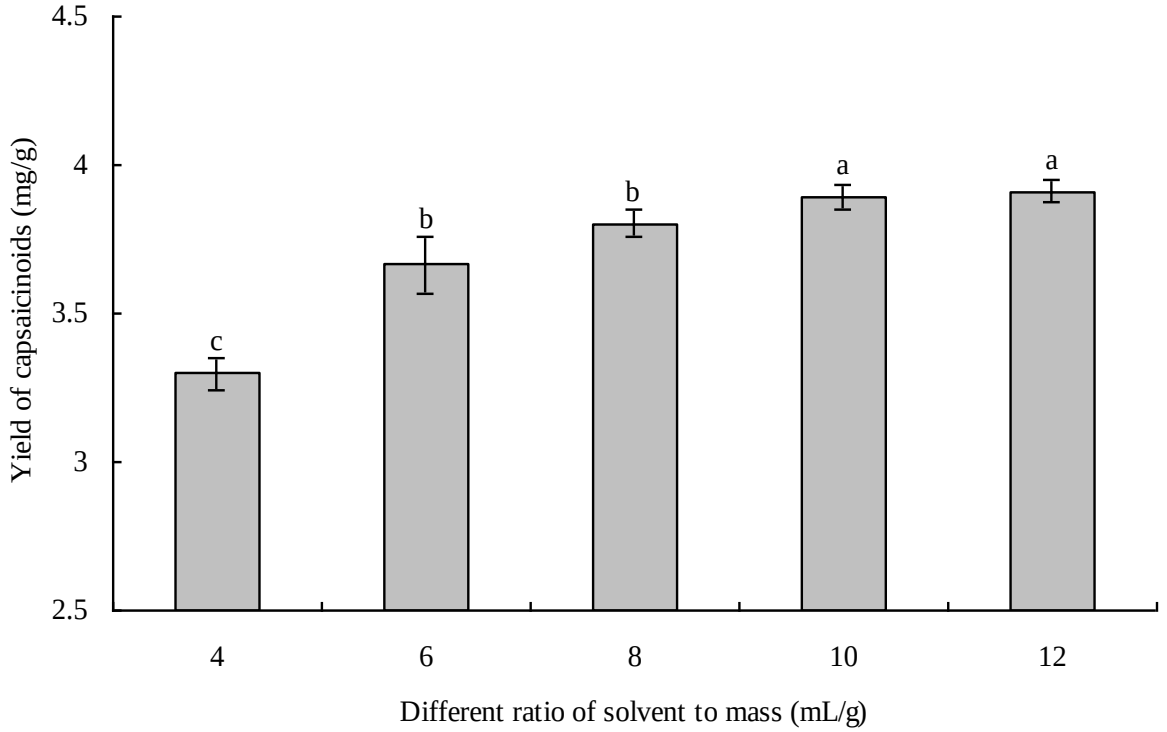

Figure 2. Effect of different ratios of solvent to mass on extraction yield of capsaicinoids from hot Chili peppers. Vertical bars represent the mean±SE (n=3). Data sets significantly different from each other are identified by different letters (P<0.05).

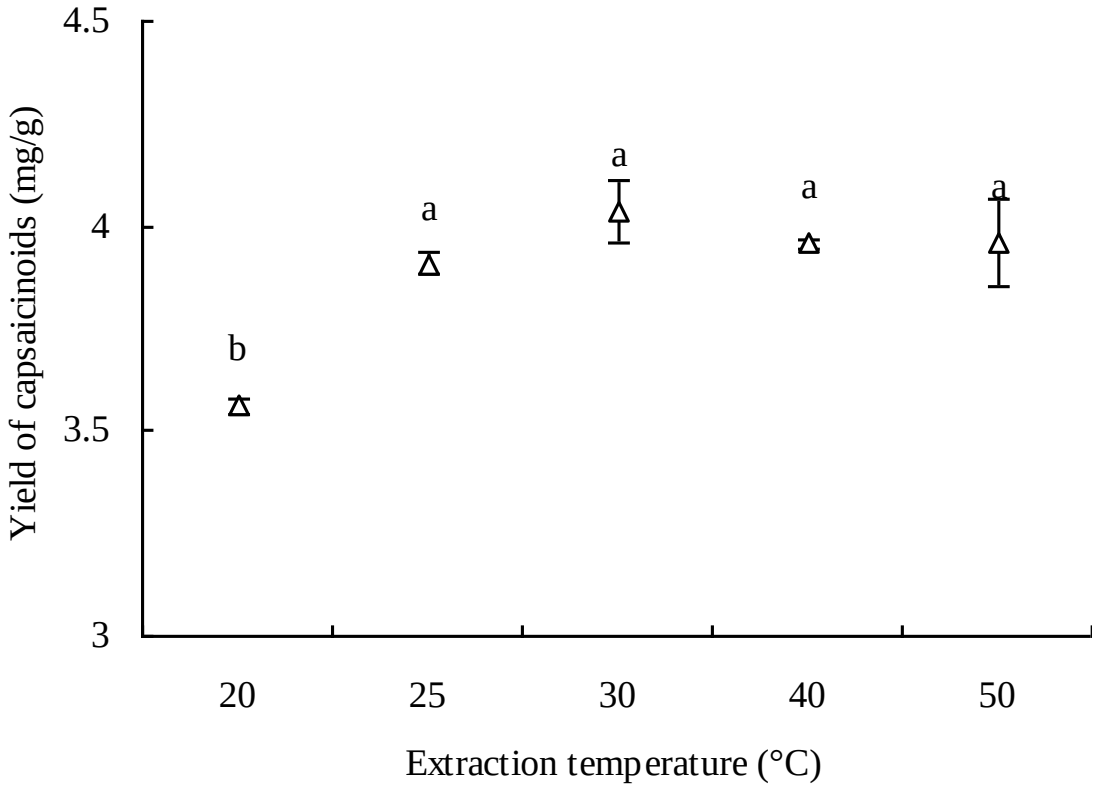

Figure 3. Effect of different extraction temperatures on extraction yield of capsaicinoids from hot Chili peppers. *Vertical bars* represent the mean±SE (n = 3). Data sets significantly different from each other are identified by different letters (P<0.05).

it was decided to employ ratio of volume of solvent (ml) to mass (g) at 10 ml/g for further study.

Effect of extraction temperature on extraction yield of capsaicinoids

Generally speaking, the higher the extraction temperature, the higher velocity of solvent molecules could accelerate mass transfer rate; and the larger solubility would enhance yield of target compounds. However, solvent volatilization and some degradation processes may occur at high temperature, which would lead to the lower extraction yield.

In this study, one aim was to evaluate the effects of extraction temperatures ranging from 20 to 50°C on extraction yield of capsaicinoids. It was not proposed to perform extractions at higher temperatures because boiling point of acetone is 56.5 °C. Other extraction parameters were given as following: extraction period of 30 min, 5 ml acetone as extraction solvent, and 0.5 g triturated hot Chili peppers. All assays were carried out in triplicate. The effect of extraction temperature on extraction yield of capsaicinoids is listed in Figure 3.

As shown in Figure 3, a maximum extraction yield of capsaicinoids (4.04 mg/g) was obtained at 30°C, although the differences were not significant ($p > 0.05$) between 25 and 50°C. At temperatures lower than 30°C, the extraction yield of capsaicinoids was increased with increasing extraction temperature. It may be due to the slower extraction kinetics at lower temperature. Therefore, 30°C was used as extraction temperature for later experiments.

Effect of extraction time on extraction yield of capsaicinoids

Until saturation, by increasing extraction time, the quantity of analytes extracted increased. However, the longer the extraction time, the more extraction solvent will be volatile. To determine the effect of extraction time on extraction yield of capsaicinoids from hot Chili peppers, extraction time was set at different lengths (0, 30, 60, 90, and 120 min), and other extraction parameters were given as following: extraction temperature at 30°C, 5 ml acetone as extraction solvent and 0.5 g triturated hot Chili peppers. All assays were performed in triplicate and the results are shown in Figure 4, in which it can be found that with increasing extraction time from 0 to 30 min, the extraction yield of capsaicinoids increased from low to high till at 30 min to maximum, and then increased slightly

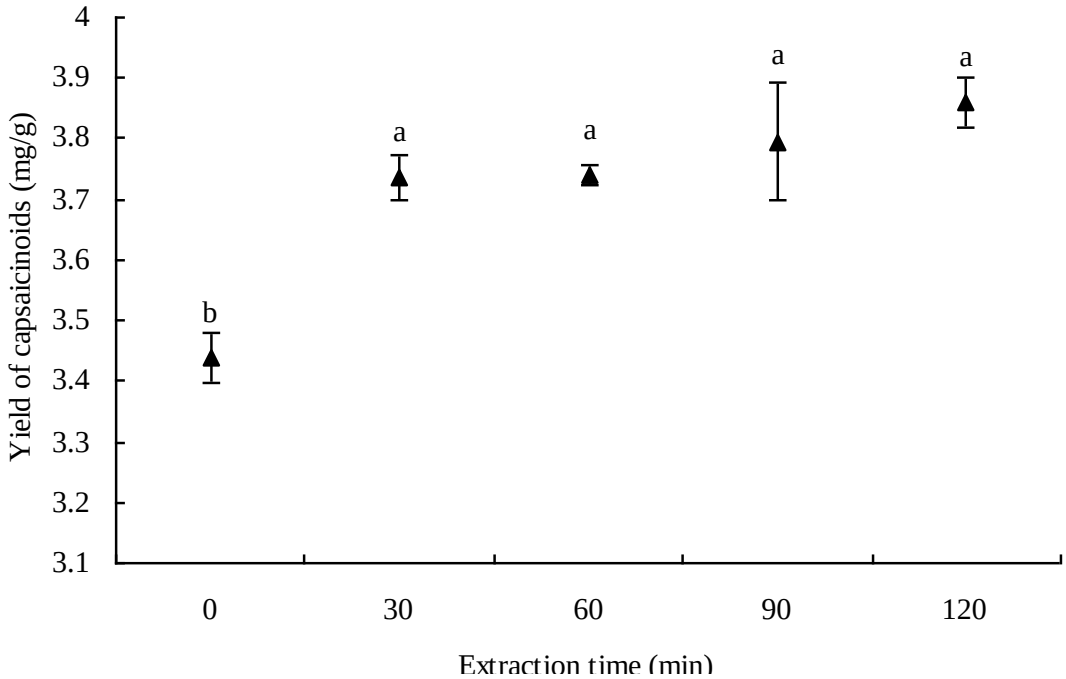

Figure 4. Effect of different extraction time on extraction yield of capsaicinoids from hot Chili peppers. *Vertical bars* represent the mean±SE (n=3). Data sets significantly different from each other are identified by different letters (P<0.05).

after 30 min in these extraction conditions (Figure 4).

Results of orthogonal array design experiments

Range analysis

Orthogonal array design was employed to optimize the extraction procedure of capsaicinoids from hot Chili peppers as described above. Nine extraction trials were carried out based on the $L_9(3^4)$ matrix. The results of OAD are listed in Table 2, which showed that the range of extraction yield of capsaicinoids varied from 3.462 mg/g to 3.786 mg/g. These data were taken as original data and used for range analysis and ANOVA.

In the range analysis, $\overline{K_{ji}}$ is often used to determine the optimal level and the optimal combination of factors. The optimal level for each factor could be obtained when $\overline{K_{ji}}$ is the largest (Wu and Leung, 2011). Here, *j* is the number of factor (*j*=*A*, *B*, *C*); *i* is the level of factor (*i*=1, 2, 3). Accorrding to $\overline{K_{ji}}$ (Table 2), it is inferred that the third level is best for ratio of solvent to mass (factor *A*) and extraction time (factor *B*), whereas the first level is suited for extraction temperature (factor *C*). Therefore, the optimal extraction condition combination for capsaicinoids is A_3-B_3-C_1.

Moreover, the range value of factors in each line, $R_j = \max(\overline{K_{Ai}}) - \min(\overline{K_{Ai}})$, indicates the significance of factor's effect; and a larger R_j means the factor has a bigger impact on extraction yield (Cui et al., 2009). Comparing the range values (R_j) of different factors (Table 2 and Fig. 5), the factors' levels of significance are as follows: ratio of solvent to mass (0.231) > extraction time (0.126) > extraction temperature (0.075). That is, ratio of solvent to mass had the greatest impact on extraction yield of capsaicinoids, whereas extraction time had a comparatively lesser impact, and the last one was extraction temperature.

Analysis of variance (ANOVA)

Although, the range analysis can easily determine the optimal value of different factors, it does not use all the data information. Thus, ANOVA was utilized to assess the OAD results, which can reflect the volatility of data, that is dispersion of the data (Deng, 1995) and the formulas of ANOVA are as follows: Firstly, sum of square deviation for each factor (SS_j) reflects differences of experimental results caused by change in every level of factor *j*, which shows the influence of factor *j* on experimental results. SS_j can be calculated by the following formula:

$$SS_j = \frac{1}{3}\sum_{i=1}^{3} K_{ji}^2 - \frac{(\sum_{i=1}^{9} Y_i)^2}{9} \quad (j = A, B, C, E)$$

Where, Y_i is the value of No. *i*. The meaning of other

Table 3. Analysis of variance (ANOVA) of the orthogonal array experimental design ($L_9(3^4)$).

Source	SS	df	V	*F*	$F_{0.05}(2, 2)=19$	P(%)
A	0.083	2	0.042	30.273	>	67.82
B	0.027	2	0.014	9.902	<	22.18
C	0.010	2	0.005	3.476	<	7.79
e	0.003	2	0.001			2.24
T	0.123	8				100

SS, sum of square deviation; df, degree of freedom; V, variance; *F*, *F*-ratio; *e*, experimental error.

symbol is the same as above.

Secondly, the freedom degree of each factor equals the levels of each factor minus 1, such that freedom degree of *A*: df_A=3-1=2. Total freedom degree of the experiments (df_T) equals total numbers of experiments minus 1 (df_T=9-1=8 for the present study).

Thirdly, the variance for each factor (V_j) can be calculated:

$$V_j = \frac{SS_j}{df_j} = \frac{SS_j}{2} (j = A, B, C, E)$$

Fourthly, the *F* value of each factor (F_j) is $F_j = \frac{V_j}{V_e}$, which indicates that the ratio of sum of the square of each factor's mean deviations to that of the experimental error (Cui et al., 2009, Ross, 1988). In addition, F_α is a constant and defined as the critical value of *F*-value for different inspection levels, and can be found from the distribution table of *F*-values (Ross, 1988). As for inspection level, α=0.05, the critical *F*-value can be found out ($F_{0.05}(2,2)$=19). The factor effect for results is prominent when F_j is larger than F_α, otherwise on the contrary.

Finally, the percentage contribution of each factor (P_j) can also indicate the relative power of a factor to reflect each factor's influence (Wu and Lee, 2005, Ghambarian et al., 2009), which can be calculated as follows:

$$P_j = \frac{SS_j}{SS_A + SS_B + SS_C + SS_e} \times 100\% (j = A, B, C)$$

The results of *F*-value for different variables are shown in Table 3. Variance of extraction temperature (V_C = 0.005) is only fivefold that of experimental error (V_e = 0.001), which means the extraction temperature effect (factor *C*) is small. When comparing F_j and F_α, it is clear that factor *A* is statistically significant for capsaicinoids extraction ($F_A > F_\alpha$,), while factor *B* and *C* have no significant effect ($F_B < F_\alpha$, $F_C < F_\alpha$). Furthermore, from the percentage contribution (Table 3), it can be also deduced that the most important factor contributing to the extraction yield of capsaicinoids is factor *A* (ratio of solvent to mass, 67.7%), followed by factor *B* (extraction time, 22.18%), and lastly, factor *C* (extraction temperature, 7.79%). Therefore, ANOVA verified the consistent results with Figure 5.

Comparison between conventional and UAE methods

To compare the effect of different methods on extraction yield of capsaicinoids from hot Chili peppers, experiments were performed using conventional and UAE methods, and extraction parameters were given as following: the ratio of solvent to mass of 10 ml/g, the extraction time of 40 min, and extraction temperature of 25 °C. Under these conditions, the extraction yields of capsaicinoids were 2.35 ± 0.042 and 3.92 ± 0.089 mg/g for conventional and UAE methods, respectively. Thus, UAE method is better than conventional method for capsaicinoids extraction from hot Chili peppers.

Conclusion

In this study, detailed effects of UAE for capsaicinoids from hot Chili peppers were investigated using single factor and orthogonal array design experiments. Based on the results of single factor experiments, a preliminary quantitative range of three factors was: ratio of solvent to mass of 6 to 10 ml/g, extraction temperature of 25 to 35°C, and extraction time of 0 to 30 min.

Furthermore, a four-factor three-level orthogonal array experimental design ($L_9(3^4)$) was performed to effectively optimize extraction procedure of capsaicinoids. The *F*-test and *P* value indicated that the effect order on extraction yield of capsaicinoids from high to low was ratio of solvent to mass, extraction time, and extraction temperature. And the optimal extraction conditions were obtained: ratio of solvent to mass of 10 ml/g, extraction time of 40 min, and extraction temperature of 25°C. Under these conditions, the extraction yields of capsaicinoids were 2.35 ± 0.042 and 3.92 ± 0.089 mg/g for conventional and UAE methods, respectively.

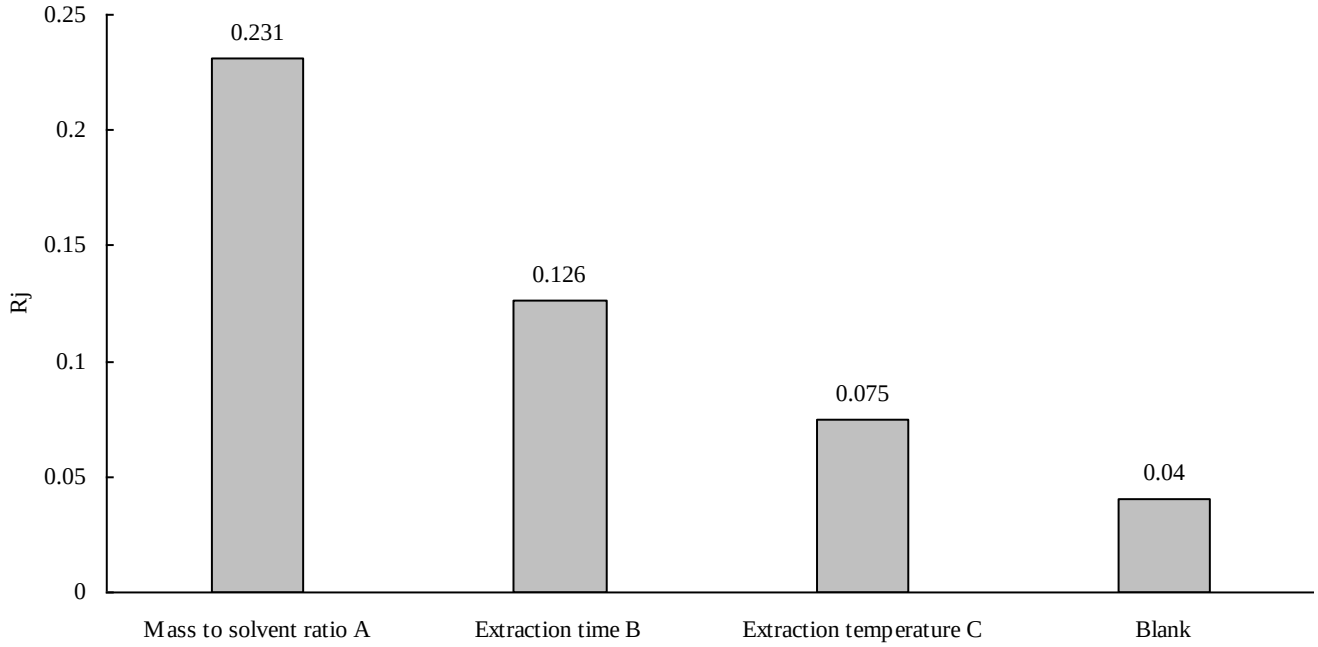

Figure 5. Range values of different factors for capsaicinoids extraction.

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