Asian Journal of Chemistry; Vol. 25, No. 3 (2013), 1324-1330

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.12973

Extraction of Polyphenols from Barley (*Hordeum vulgare* L.) Grain Using Ultrasound-Assisted Extraction Technology

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(*Received*: 31 October 2011; *Accepted*: 8 September 2012) AJC-12107

The optimized ultrasound-assisted extraction (UAE) were developted for extracting polyphenols from barley grain in this work. The process parameters were optimized using response surface methodology (RSM) to maximizing total polyphenol yield (TPY) and total phenolic acid yield (TPAY). Four parameters were included, *viz*., ethanol concentration (EC), solvent to material ratio (SMR), extraction time (ET) and extraction temperature (ETP). Results indicated that EC was the most significant factor ($p < 0.01$) both for TPY and TPAY, while ET insignificant ($p > 0.05$). The practicable optimal conditions for TPY were: EC, 97 %; SMR, 54 mL/g; ET, 20 min; and ETP, 50 ºC, while for TPAY were: EC, 100 %; SMR, 60 mL/g; ET, 18 min and ETP, 50 ºC. Under the respective optimal conditions, TPY could reach to 25.56 mg of tannic acid equivalent/g barley powder (BP), while TPAY could reach to 19.68 mg of gallic acid equivalent/g BP. The experimental values were significantly agreed with the predicted values calculated from the second-order models both for TPY and TPAY, thus verifying that the methods established were applicable and reliable in practice. The use of sonication can enhance the yield efficiently in barley grain polyphenol extraction.

Key Words: Polyphenol, Barley grain, Ultrasound-assisted extraction.

INTRODUCTION

Polyphenols are compounds which have more than one phenolic hydroxyl group attached to one or more benzene rings[1]. They are common constituents of plant-derived foods and major antioxidants of our diet. Current evidences strongly support a contribution of polyphenols to the prevention of cardiovascular diseases, cancers and osteoporosis and suggest a role of preventing neurodegenerative diseases and diabetes mellitus[2-4]. Therefore, they could be a great potential source of natural antioxidants used in food and pharmaceutical industries. Additionally, as one of the main types of polyphenols, phenolic acids occur widely in plants and is second only to the flavonoids as secondary metabolites[5].

Polyphenols are abundant in cereal grain, especially in barley, which includes phenolic acids, flavones, leucoanthocyanidins, catechins and coumarins[6]. The total polyphenol content in cultivated barley ranged from 0.19-0.75 mg of (+)-catechin equivalent/g barley flour[7] and total amount of phenolic acids ranged from 604-1346 μg/g of fresh weight of barley flour[8]. Considering the worldwide supply and cheap price, barley would be the potential crop for exploiting polyphenols.

Traditional methods, such as mechanical shaking extraction[9] and solvent extraction without other assisted processing[10], were used in barley polyphenol extraction. However, these methods are time-consuming and require relatively large quantities of solvents[11]. The ultrasound-assisted extraction (UAE) could be an alternative and previous studies have reported the effective extraction of phenolic compounds from plant materials by UAE[12-14]. Compared with traditional methods, the main benefits of UAE include the increase of extraction yield and faster kinetics[15]. UAE enhancing extraction yield mainly dues to the mechanical effects of ultrasound inducing a greater penetration of solvent into cellular materials (even disrupting biological cell walls to facilitate the release of components) and improving mass transfer[16].

Liu *et al*.[17,18] studied the method of extracting total polyphenols from barley by UAE. However, the effect of temperature on total polyphenol extraction was not investigated in the researches. In fact, it should be included, because higher temperatures in UAE can increase the number of cavitation bubbles formed and may increase the efficiency of extraction process[12].

This paper presents optimized UAE methods that use aqueous ethanol to extract total polyphenols (TP) and total phenolic acids (TPA) from barley grain rapidly and efficiently. The methods were developed by use of response surface methodology (RSM). RSM is the most popular optimization method used in recent years[19]. Its advantage is using lesser number of experiments to optimize the operating conditions and evaluate the effects of factors and their interactions on responses. It has been used to optimize the process of phenolic compounds extraction in many studies[20-26]. In the present study, four process parameters were included during the method development, *viz*., ethanol concentration (EC), solvent to material ratio (SMR), extraction time (ET) and extraction temperature (ETP). Moreover, their effects on total polyphenol yield (TPY) and total phenolic acid yield (TPAY) were investigated.

EXPERIMENTAL

A two-rowed hulled barley variety Favorit harvested in the agricultural experiment station of Shihezi university in 2008 was selected as the raw material. The kernels were blast-dried for 24 h at 80 ºC, then ground using an Udy cyclone sample mill (Seedburo equipment Co., Chicago, USA). The ground powder was degreased according to the method of Macritchie and Gras[27] with some modifications, chloroform was mixed with the barley powder in a glass beaker by the ratio of 1:2 (w/v) under room temperature and magnetically stirred for 5 min, then vacuum-filtrated using a buchner funnel. The same procedure was done for extra 3 times for the residual. The degreased powder was air-dried until no chloroform smelt and then stored in sealed polyethene pouches at -20 ºC prior to further analysis. The standards of tannic acid and gallic acid were purchased from Sigma (Sigma-Aldrich (Shanghai) Trading Co. Ltd., Shanghai, China), purity is 97 %. Other reagents not specially mentioned were all analytically pure.

Polyphenol extraction: The degreased barley powder (2 g) was put into a 150 mL conical flask and extracted under the designed conditions in an ultrasonic cleaning bath (UP5200H type, 40 kHz, 200 W, Nanjing Leijunda ultrasonic electronic equipment Co. Ltd., Jiangsu, China). The extraction solvent (aqueous ethanol) contained 0.20 g ascorbic acid as antioxidants and the flask was sealed by polyethylene film to avoid the contact of solution with air during the extraction process. After the extraction, the mixture of barley powder and aqueous ethanol was transferred to a plastic centrifuge tube (250 mL), sealed and cooled in tap water for 10 min, then centrifuged for 15 min at 4000 g. The supernatant was concentrated using a rotary vacuum evaporator (R203B type, Shanghai SENCO Technology Co. Ltd., Shanghai, China). The residual was dissolved in ethanol and then centrifuged again at 4000 g for 15 min. The collected supernatant was added to 50 mL in a brown volumetric flask with ethanol and stored in dark prior to further analysis.

Determination of polyphenol contents: Total phenolic (TP) content was determined according to the prussian blue method[28] and calculations were based on a calibration curve obtained with tannic acid. Total phenolic yield (TPY) was expressed as mg of tannic acid equivalent (TAE)/g barley powder (BP). The content of TPAY was determined by the visible spectrophotometry[29] and calculations were based on a calibration curve obtained with gallic acid. TPAY was expressed as mg of gallic acid equivalent (GAE)/g BP. All measurements were carried out in triplicate and data were given as mean ± SD.

Experimental design and statistical analysis: To obtain suitable extraction conditions, a series of experiments were conducted. Firstly, a 24 factorial design (FD) containing 16 sets of experiments and 4 centre points was carried out to screening the process factors. TPY and TPAY were chosen as responses and factors of EC, SMR, ET and ETP were independent variables. The first-order model with interactions to approximate the response function in FD was given as:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ij} x_i x_j \quad (1)$$

where Y is response variable, x_i and x_j are independent variables, β_0, β_i and β_{ij} are the regression coefficients for intercept, linear and interaction terms, respectively. The analysis of variance (ANOVA) of this model was used to find significant factors. Secondly, a steepest ascent experiment was done to search the factor levels near the optimal which would be arranged as the center points of the followed Box-Behnken design (BBD) experiment approximating the true response function. The second-order model fitted by BBD was generally represented by a form of:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^{\,2} + \sum_{i=1}^{k} \beta_{ij} x_i x_j \quad (2)$$

where Y is response variable, x_i and x_j are independent variables, β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. The adequacy of the fitted model was checked by the coefficient of determination (R^2) and lack-of-fit test. A model if adequate could be used to delineate the response surface plots and identify the optimal UAE conditions.

Microsoft Office Excel (Version 2003) and the SAS System for Windows (Version 9.00, SAS Institute Inc., Cary, North Carolina, USA) were used for data analysis. The SAS system for Windows was also used for experimental designs. The three-dimensional (3D) response surface plots were generated by Design-Expert (Version 7.1.3, Stat-Ease, Inc., Minneapolis, USA). The order of the experimental treatments was randomized to minimize the effects of unexplained variability included by extraneous factors on the observed response and each treatment was done in duplicate.

RESULTS AND DISCUSSION

Screening of UAE factors for polyphenol extraction using factorial design: At fixed ultrasonic frequency and power, EC (X_1), SMR (X_2), ET (X_3) and ETP (X_4) would be the possible influencing factors of polyphenol extraction by UAE. The designs and results of FD were shown in Table-1.

In sequence, the first-order models obtained from FD to approximate the function of TPY and TPAY were (in terms of coded levels (-1, 1) and eliminating the insignificant terms):

$$Y_1 = 18.5105 + 2.899375x_1 + 2.125625x_2 + 1.108125x_3 + 0.768125x_2x_3 \quad (3)$$

TABLE-1
EXPERIMENTAL DESIGNS AND RESULTS OF FACTORIAL DESIGN EXPERIMENT[a]

Treat	$x_1(X_1)$ (EC, %)	$x_2(X_2)$ (SMR, mL/g)	$x_3(X_3)$ (ET, min)	$x_4(X_4)$ (ETP, ºC)	Y_1:TPY (mg of TAE/g BP)	Y_2:TPAY (mg of GAE/g BP)
1	-1(45)	-1(15)	-1(5)	-1(40)	11.43 ± 0.02	10.64 ± 0.02
2	1(85)	-1(15)	-1(5)	-1(40)	20.93 ± 0.05	14.20 ± 0.01
3	-1(45)	1(35)	-1(5)	-1(40)	18.07 ± 0.11	15.68 ± 0.13
4	1(85)	1(35)	-1(5)	-1(40)	21.37 ± 0.13	19.01 ± 0.01
5	-1(45)	-1(15)	1(15)	-1(40)	15.67 ± 0.12	9.76 ± 0.04
6	1(85)	-1(15)	1(15)	-1(40)	18.32 ± 0.25	13.86 ± 0.06
7	-1(45)	1(35)	1(15)	-1(40)	21.12 ± 0.13	10.37 ± 0.04
8	1(85)	1(35)	1(15)	-1(40)	24.91 ± 0.19	16.94 ± 0.05
9	-1(45)	-1(15)	-1(5)	1(60)	11.39 ± 0.18	14.34 ± 0.00
10	1(85)	-1(15)	-1(5)	1(60)	19.52 ± 0.20	14.78 ± 0.12
11	-1(45)	1(35)	-1(5)	1(60)	13.50 ± 0.14	12.76 ± 0.06
12	1(85)	1(35)	-1(5)	1(60)	21.19 ± 0.06	15.20 ± 0.09
13	-1(45)	-1(15)	1(15)	1(60)	13.42 ± 0.20	11.11 ± 0.04
14	1(85)	-1(15)	1(15)	1(60)	18.58 ± 0.19	13.61 ± 0.03
15	-1(45)	1(35)	1(15)	1(60)	18.47 ± 0.08	13.03 ± 0.01
16	1(85)	1(35)	1(15)	1(60)	24.64 ± 0.24	17.45 ± 0.24
17	0(65)	0(25)	0(10)	0(50)	19.91 ± 0.01	13.87 ± 0.04
18	0(65)	0(25)	0(10)	0(50)	18.18 ± 0.04	14.01 ± 0.07
19	0(65)	0(25)	0(10)	0(50)	20.11 ± 0.06	13.17 ± 0.01
20	0(65)	0(25)	0(10)	0(50)	19.48 ± 0.13	14.99 ± 0.01

[a]x_i (i = 1, 2, 3, 4) are coded levels and X_i (i = 1, 2, 3, 4) are actual levels: $x_1 = (X_1-65)/20$, $x_2 = (X_2-25)/10$, $x_3 = (X_3-10)/5$ and $x_4 = (X_4-50)/10$, respectively. EC, SMR, ET, ETP, TPY, TAE, BP, TPAY, GAE: As in text.

$$Y_2 = 13.939 + 1.71x_1 + 1.13375x_2 \quad (4)$$

R^2 of eqns. 3 and 4 had been 0.94 and 0.87, respectively, while Joglekar and May[30] suggested that for a good fit of a model, R^2 should be at least 0.80, thus, both of the models could adequately represented the real relationship between the responses and factors. Furthermore, ANOVA (Table-2) showed both of the models were statistically significant ($p < 0.01$) and lack-of-fits were insignificant ($p > 0.05$), indicating the models could appropriately explain the actual process within the experimental ranges.

ANOVA results for FD (Table-2) revealed that the effects of EC, SMR, ET and the interaction between SMR and ET were significant for TPY ($p < 0.01$ or $p < 0.05$) and ETP was found insignificant ($p > 0.05$). Thus, EC, SMR and ET were selected for further optimization in the following experiments. The effects of EC and SMR were significant for TPAY ($p < 0.01$), while ET, ETP and all of the interactions were insignificant ($p > 0.05$). Thus, EC and SMR should be selected to involve in the following experiments. The stability of polyphenols may be declined and the oxidation may be accelated at too high temperature[31] and within the range of 40-60 ºC, the effect of ETP was insignificant for polyphenol extraction, thus ETP was kept at 50 ºC (zero level) for all of the following experiments.

Searching for the factor levels near the optimum by steepest ascent experiment: All of the interactions and quadratic effects on TPY and TPAY were insignificant ($p > 0.05$) (Table-2). The curvatures of the two response surfaces showed by the analysis of FD by Design-Expert were insignificant ($p > 0.05$). These indicated that the optimal points of the two responses located outside of the regions of FD. Therefore, searching for the factor levels which would result in a

TABLE-2
ANOVA RESULTS OF THE FIRST-ORDER MODELS FOR PREDICTING THE YIELD OF POLYPHENOL EXTRACTION[a]

Source	DF	Y_1:TPY				Y_2:TPAY			
		SS	MS	F	Pr > F	SS	MS	F	Pr > F
x_1	1	134.5020	134.5020	75.9361	0.0001	46.7856	46.7856	31.1988	0.0003
x_2	1	72.2925	72.2925	40.8143	0.0001	20.5662	20.5662	13.7145	0.0049
x_3	1	19.6471	19.6471	11.0922	0.0088	6.8644	6.8644	4.5775	0.0611
x_4	1	7.7145	7.7145	4.3554	0.0665	0.2070	0.2070	0.1381	0.7188
x_1x_2	1	1.2600	1.2600	0.7114	0.4208	2.3716	2.3716	1.5815	0.2402
x_1x_3	1	7.3577	7.3577	4.1539	0.0720	3.8220	3.8220	2.5487	0.1448
x_1x_4	1	3.9105	3.9105	2.2078	0.1715	3.7636	3.7636	2.5097	0.1476
x_2x_3	1	9.4403	9.4403	5.3297	0.0463	0.0361	0.0361	0.0241	0.8801
x_2x_4	1	1.1183	1.1183	0.6314	0.4473	4.9952	4.9952	3.3310	0.1013
x_3x_4	1	0.1040	0.1040	0.0587	0.8140	2.8224	2.8224	1.8821	0.2033
Model	10	257.3468	25.7347	14.5291	0.0002	92.2342	9.2234	6.1506	0.0058
Error	9	15.9413	1.7713	–	–	13.4964	1.4996	–	–
(Lack of fit)	6	13.6839	2.2806	3.0309	0.1955	11.8108	1.9685	3.5034	0.1655
(Pure Error)	3	2.2574	0.7525	–	–	1.6856	0.5619	–	0.0003
Total	19	273.2881	–	–	–	105.7306	–	–	0.0049

[a]DF: Degree of freedom; SS: Sum of square; MS: Mean square; TPY, TPAY: As in text.

response value near the optimum should be conducted. EC, SMR and ET were positive on TPY. EC and SMR were also positive on TPAY. ET, which was not contained in eqn. 4, was negative (the regression coefficient is -0.655), however insignificant ($p > 0.05$), on TPAY. Therefore, a steepest ascent experiment towards increasing levels of the factors was carried out to simultaneously search the factor levels near the optimum for both TPY and TPAY. The designs and results were showed in Table-3. The starting points of the steepest ascent experiment were just the center points of FD. However, the step change values were empirical rather than calculated from the first-order model equations described by Grum and Slabe[32], because the levels of the calculated values were inconsistent and impractical for the two responses. Table-3 showed both of TPY and TPAY reached to maximum at step 4 with the actual factor levels: EC, 93 %; SMR, 53 mL/g and ET, 18 min. Considering the range of EC, when the above-mentioned factor levels used for center points, a narrow region would allow little necessary to do further experiments to check the adequacy of the first-order model. Hence, step 4 could be regarded near the level leading to the optimal response and making the above-mentioned factor levels as the center points of the following BBD experiment, the conditions of TP and TPA extractions could be further optimized simultaneously.

Box-Behnken design (BBD) and response surface analysis for polyphenol extraction: BBD[33] is a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs, which had been demonstrated slightly more efficient than the central composite design but much more efficient than the three-level full factorial designs[34]. In the present study, a 3^3 BBD consisting of 12 trails and 3 center points was applied to finally optimizing the three selected factors of UAE. The coded levels, actual levels and design matrix of the three independent variables, *viz.*, EC (X_1), SMR (X_2) and ET (X_3), were presented in Table-4 along with the experimental and predicted values of the two responses.

The experimental data of BBD (Table-4) were analyzed by multiple regression analysis and the equation expressing the function of the predicted response TPY (Y_1) to the three factors could be obtained and given as (in terms of coded levels (-1, 1)):

$$Y_1 = 25.06333 + 1.49875x_1 + 0.835x_2 + 0.03625x_3 - 1.479167x_1^2 - 2.366667x_2^2 - 0.304167x_3^2 - 0.4325x_1x_2 + 0.47x_1x_3 + 0.1525x_2x_3 \quad (5)$$

Because of the probobility of the model < 0.05 and probobility of the lack-of-fit > 0.05 (Table-5), this second-order model could fit the experiment significantly. The coefficient of determination ($R^2 = 0.9373$) also indicated that the general availability of this model is adequate.

Table-5 indicated the main and quadratic effects of EC and SMR were significant for TPY ($p < 0.01$ or $p < 0.05$). However, the main effect of ET was not significant ($p > 0.05$). Perhaps within the ET range arranged in BBD, polyphenols in the material had been extracted completely and further prolonging ET had not been able to increase the yield. All of the interaction effects were not significant ($p > 0.05$). Perhaps

TABLE-3
EXPERIMENTAL DESIGNS AND RESULTS OF THE STEEPEST ASCENT DESIGN[a]

Treat	ΔX_i	0	1	2	3	4	5
X_1 (EC, %)	7	65	72	79	86	93	100
X_2 (SMR, mL/g)	7	25	32	39	46	53	60
X_3 (ET, min)	2	10	12	14	16	18	20
Y_1 (TPY, mg of TAE/g BP)		18.95 ± 0.08	14.33 ± 0.39	15.76 ± 0.97	18.34 ± 0.31	19.73 ± 0.52	18.72 ± 1.03
Y_2 (TPAY, mg of GAE/g BP)		13.87 ± 0.15	14.16 ± 0.79	15.55 ± 0.94	17.33 ± 0.18	18.44 ± 0.76	17.73 ± 0.54

[a]ΔX_i (i = 1,2, 3) are the step changes. EC, SMR, ET, TPY, TAE, BP, TPAY, GAE: As in text.

TABLE-4
EXPERIMENTAL DESIGNS AND RESULTS OF BOX-BEHNKEN DESIGN AND COMPARISON BETWEEN EXPERIMENTAL AND PREDICTED VALUES BASED ON THE FINAL MODELS[a]

Treat	x_1 (X_1) (EC, %)	x_2(X_2) (SMR, mL/g)	x_3(X_3) (ET, min)	Y_1: TPY(mg of TAE/g BP)		Y_2: TPAY(mg of GAE/g BP)	
				Y_0	Y_i	Y_0	Y_i
1	-1(86)	-1(46)	0(18)	17.93 ± 0.38	18.45	17.33 ± 0.04	17.30
2	-1(86)	1(60)	0(18)	20.70 ± 1.24	20.99	17.76 ± 0.21	17.79
3	1(100)	-1(46)	0(18)	22.60 ± 0.32	22.31	19.19 ± 0.05	19.16
4	1(100)	1(60)	0(18)	23.64 ± 0.12	23.12	19.65 ± 0.09	19.68
5	0(93)	-1(46)	-1(14)	22.04 ± 0.65	21.67	18.36 ± 0.20	18.22
6	0(93)	-1(46)	1(22)	21.31 ± 0.32	21.44	17.38 ± 0.30	17.58
7	0(93)	1(60)	-1(14)	23.17 ± 0.50	23.04	19.07 ± 0.09	18.87
8	0(93)	1(60)	1(22)	23.05 ± 0.55	23.42	17.81 ± 0.04	17.95
9	-1(86)	0(53)	-1(14)	22.37 ± 0.45	22.21	17.45 ± 0.21	17.62
10	1(100)	0(53)	-1(14)	23.62 ± 0.42	24.27	18.62 ± 0.30	18.79
11	-1(86)	0(53)	1(22)	22.00 ± 0.44	21.35	16.31 ± 0.13	16.14
12	1(100)	0(53)	1(22)	25.13 ± 0.11	25.28	18.90 ± 0.39	18.73
13	0(93)	0(53)	0(18)	25.76 ± 0.18	25.06	18.40 ± 0.04	18.19
14	0(93)	0(53)	0(18)	24.06 ± 0.07	25.06	18.07 ± 0.00	18.19
15	0(93)	0(53)	0(18)	25.37 ± 0.10	25.06	18.11 ± 0.05	18.19

[a]x_i (i = 1, 2, 3) are Coded levels and X_i (i = 1, 2, 3) are Actual levels: $x_1 = (X_1-93)/7$, $x_2 = (X_2-53)/7$ and $x_3 = (X_3-18)/4$, respectively. EC, SMR, ET, TPY, TAE, BP, TPAY, GAE: As in text; Y_0: Experimental value; Y_i: Predicted value.

TABLE-5
ANOVA RESULTS OF THE SECOND-ORDER MODELS FOR PREDICTING THE YIELD OF POLYPHENOL EXTRACTION[a]

Source	DF	Y_1: TPY				Y_2: TPAY			
		SS	MS	F	Pr > F	SS	MS	F	Pr > F
x_1	1	17.9700	17.9700	25.7035	0.0039	7.0500	7.0500	113.4385	0.0001
x_2	1	5.5778	5.5778	7.9780	0.0369	0.5151	0.5151	8.2884	0.0346
x_3	1	0.0105	0.0105	0.0150	0.9072	1.2013	1.2013	19.3288	0.0070
x_1^2	1	8.0785	8.0785	11.5551	0.0193	0.0019	0.0019	0.0312	0.8667
x_1x_2	1	0.7482	0.7482	1.0702	0.3483	0.0002	0.0002	0.0036	0.9544
x_1x_3	1	0.8836	0.8836	1.2639	0.3120	0.5041	0.5041	8.1112	0.0359
x_2^2	1	20.6810	20.6810	29.5812	0.0029	0.3596	0.3596	5.7864	0.0612
x_2x_3	1	0.0930	0.0930	0.1331	0.7302	0.0196	0.0196	0.3154	0.5986
x_3^2	1	0.3416	0.3416	0.4886	0.5157	0.4534	0.4534	7.2952	0.0427
Model	9	52.2168	5.8019	8.2987	0.0157	10.1722	1.1302	18.1862	0.0026
(Linear)	3	23.5583	7.8528	11.2322	0.0116	8.7664	2.9221	47.0186	0.0004
(Quadratic)	3	26.9336	8.9779	12.8415	0.0087	0.8819	0.2940	4.7298	0.0637
(Cross product)	3	1.7249	0.5750	0.8224	0.5351	0.5239	0.1746	2.8101	0.1474
Error	5	3.4956	0.6991	–	–	0.3107	0.0621	–	–
(Lack of fit)	3	1.9096	0.6365	0.8026	0.5962	0.2459	0.0820	2.5270	0.2962
(Pure error)	2	1.5861	0.7930	–	–	0.0649	0.0324	–	–
Total	14	55.7124	–	–	0.0039	10.4829	–	–	–

[a]DF, SS, MS: As in Table-2; TPY, TPAY: As in text.

within these level ranges arranged, the roles of the factors could be well played and the mass transfer and penetration of solvent into cellular materials were performed well, thus the factors were never restricted by each other. For any terms in the model, a larger regression coefficient and a smaller *p*-value would indicate a more significant effect on the response[25]. Thus Table-5 and eqn. 5 showed the main effect of EC had the most significant effect on TPY, following the main effects of SMR and ET and quadratic effects of SMR and EC.

The 3D response surface plots and two-dimensional contour plots are the graphical representations of the regression equation. The relationship between independent variables and response can be visualized in the 3D plots generated by varying two of the variables within the experimental range and holding another one constant at the center point. Fig. 1a showed that at a fixed SMR value, TPY increased sharply when EC increased from 86.00 % to *ca.* 96.50 %. However, it decreased gradually with EC further increased. Maybe when EC was at lower levels, the hydrogen bond and hydrophobic force between polyphenols and proteins and polysaccharides could't be completely destroied[35]. When EC increased, the hydrogen bond and hydrophobic force gradually faded away and TPY increased. However, when EC kept on increasing, the polarities of the solvent and barley grain polyphenols would differ more and more and TPY decreased instead[31]. Similar character could be found in the effect of SMR on TPY, both of them performing a significant quadratic effect. Nevertheless, TPY decreased more evident while in higher SMR levels. The interaction between EC and ET at the fixed SMR (53 mL/g) was shown in Fig. 1b. TPY slightly decreased when ET increased to the highest level, indicating that maybe the oxidation of some extracted polyphenols had been beginning in the solvent. The effect of EC on TPY also displayed a very obvious quadric mode. Fig. 1c showed the effect of the interaction between SMR and ET on TPY. There was only a little change of TPY when ET increasing within the experimental range. However, the effect of SMR was arc-shaped and TPY declined sharply when SMR increased further from about 56.50 mL/g

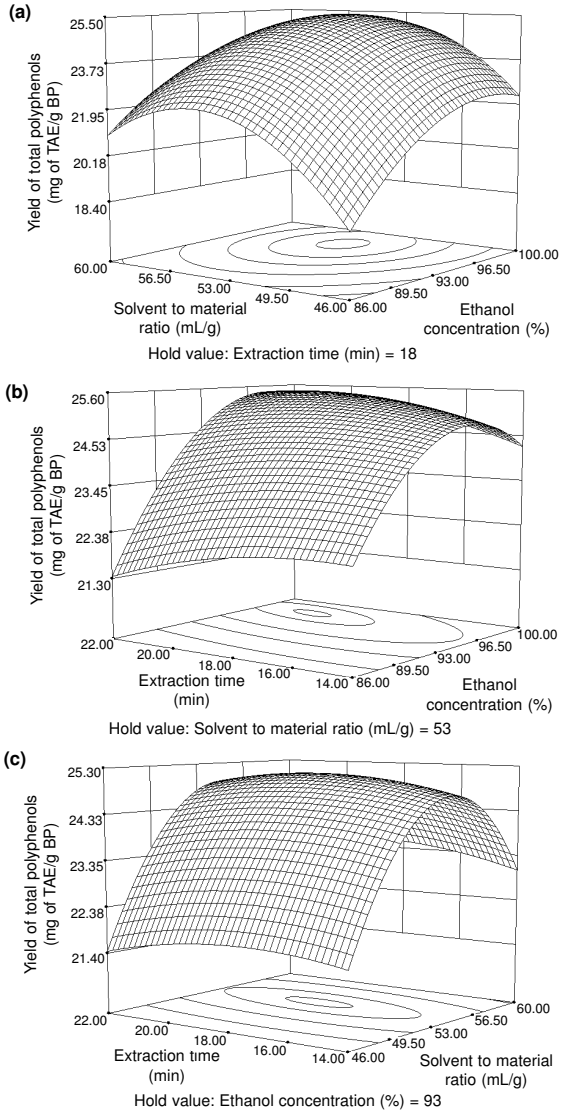

Fig. 1. Response surface plots of TPY as a function of: (a) Ethanol concentration and solvent to material ratio; (b) Ethanol concentration and extraction time; (c) Solvent to material ratio and extraction time

to the final level. Perhaps too high SMR prolonged the following concentration procedure, which increased the oxidation of extracted polyphenols. The three plots indicated that a change of EC or SMR could bring a significant change of TPY in UAE.

With respect to the optimization of TPA extraction by BBD, the second-order model was also fitted and analyzed, which was same as TP extraction and given as (in terms of coded levels (-1, 1)):

$$Y_2 = 18.19333 + 0.93875x_1 + 0.25375x_2 - 0.3875x_3 - 0.022917x_1^2 + 0.312083x_2^2 - 0.350417x_3^2 + 0.0075x_1x_2 + 0.355x_1x_3 - 0.07x_2x_3 \quad (6)$$

Because this model was significant ($p < 0.01$) and the lack-of-fit was insignificant ($p > 0.05$) (Table-5), it could be valid to predict the response and optimize the conditions for TPA extraction. The coefficient of determination ($R^2 = 0.9704$) indicated only less than 3 % variations of the TPA extraction process could not be explained by this model.

ANOVA (Table-5) suggested that the main effects of three selected factors, the quadratic effect of ET and the interaction between EC and ET were significant for TPAY ($p < 0.01$ or $p < 0.05$). The main effect of ET was significant ($p < 0.01$) in BBD but insignificant in FD ($p > 0.05$). Perhaps within the factor level ranges in FD, EC and SMR were greatly restricted so that the change of ET would never be able to lead a increase or decrease of TPAY. However, the levels of EC and SMR must be more suitable for TPA extraction in BBD experiment, then the effect of ET on TPAY had become significant. For TPAY, the main effect of EC was still most significant, followed by main effects of ET and SMR, interaction between EC and ET and the quadratic effect of ET.

Fig. 2a depicted the interaction between EC and SMR while ET kept constant at 18 min. When SMR was fixed, TPAY showed a rather rapidly linear increase with EC increasing from 86-100 %. When EC was fixed, TPAY decreased with SMR increased from 46.00 mL/g to *ca.* 53.00 mL/g. When SMR surpassed *ca.* 53.00 mL/g, TPAY increased. Perhaps when SMR was below 53.00 mL/g, the penetration of solvent into solid materials was not sufficient enough, therefore bound phenolic acids could not be released well and the extracted TPA was mainly free phenolic acid type. With SMR increasing, the oxidation of the completely extracted free phenolic acids would increase because of the prolonged concentration and TPAY decreased. However, when SMR above 53.00 mL/g, bound phenolic acids begined to be released a lot, then TPAY increased again. The interaction between EC and ET at the fixed SMR (53 mL/g) was shown in Fig. 2b. At a fixed ET level, TPAY also linearly increased with EC increase within the experimental range. However it would gradually decrease with ET increased at a fixed EC level. Fig. 2c showed that the interaction between SMR and ET was an obvious saddle effect.

After fitting the second-order model and checking its adequacy, a canonical analysis could be conducted to investigate the mathematic characteristics of the stationary point of the response surface, whether it is a maximum, minimum, saddle, rising ridge, or stationary ridge in RSM[36]. According

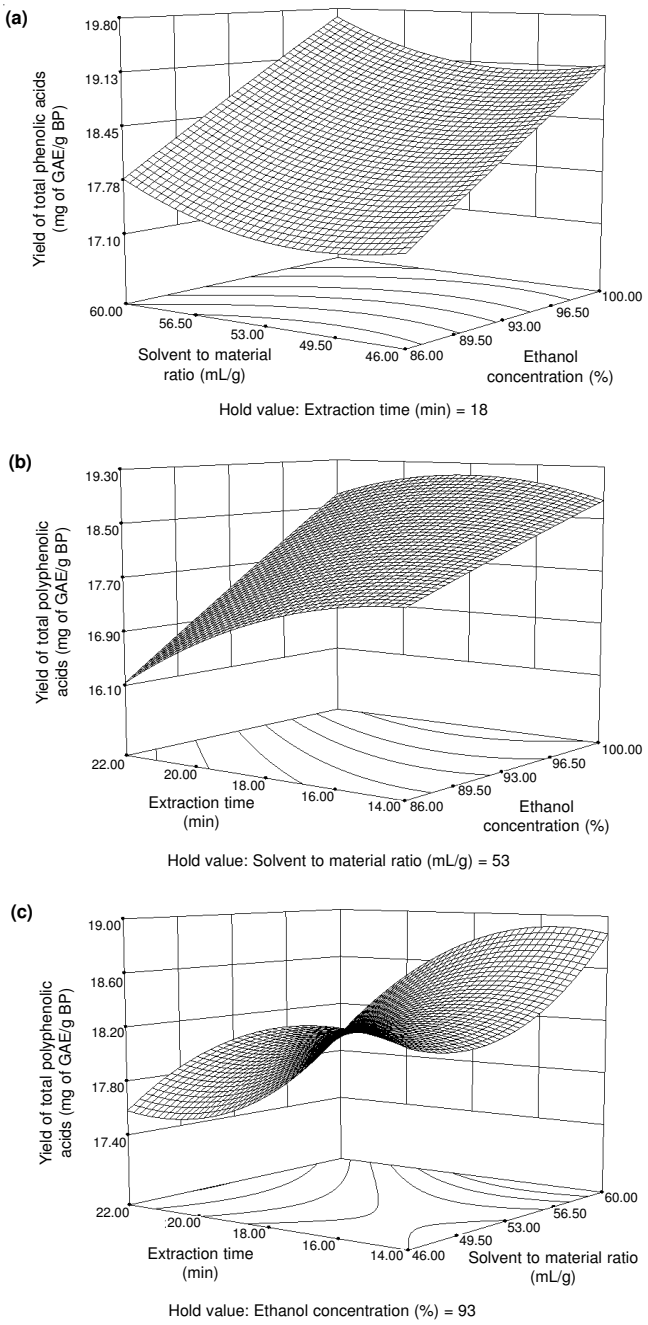

Fig. 2. Response surface plots of TPAY as a function of: (a) Ethanol concentration and solvent to material ratio; (b) Ethanol concentration and extraction time; (c) Solvent to material ratio and extraction time

to the results of canonical analysis, the eigenvalues of the three factors (ET, SMR and ET) on TPY were -0.25835, -1.46731 and -2.42434, respectively. Because of their uniform negative signs, the stationary point of the TPY response surface was a maximum. The predicted maximum response value was 25.56 mg of TAE/g BP and the corresponding optimal conditions for TP extraction by UAE in terms of actual levels were: EC, 97.00 %; SMR, 53.99 mL/g and ET, 20.15 min, respectively.

Quite different from TPY, the canonical analysis demonstrated the eigenvalues of the three factors (ET, SMR and ET) on TPAY were 0.314035, 0.054397 and -0.42968, respectively. Because the eigenvalues were of mixed sign, the stationary point of the TPAY surface was a saddle point, which indicated

that the maximum response was not at the stationary point. Thus, ridge analysis need be done to search for it[36]. Within the experimental range, TPAY reached its maximum when ridge radius was 1.40, where the predicted response value was 19.67 mg of GAE/g BP and the corresponding optimal conditions for TPA extraction by UAE in terms of actual levels were: EC, 99.88 %; SMR, 59.97 mL/g; and ET, 17.73 min.

Verification of the final models: Both of the optimal conditions for TP and TPA extraction were experimentalized to verify the suitability of the models for predicting the optimal responses. Considering the practicality, the optimal conditions for TP extraction were adjusted to: EC, 97 %; SMR, 54 mL/g; ET, 20 min and ETP, 50 ºC. Under these conditions, the predicted value of TPY was 25.56 mg of TAE/g BP and the experimental value was 24.76 ± 0.15 mg of TAE/g BP (n = 3). The optimal conditions for TPA extraction were adjusted to: EC, 100 %; SMR, 60 mL/g; ET, 18 min and ETP, 50 ºC, which happened to be a treatment of the BBD experiment. The differences between the experimental values from the verification experiment and BBD and the corresponding predicted values (Table-4) were checked by T-test. The results indicated a close agreement between experimental values and predicted values both of TPY and TPAY, which would verify the adequate fitness of the two response equations for predicting TPY and TPAY.

Control experiment to verify the effect of ultrasound: A control experiment was carried out at the practicable optimal conditions without sonication both for TP and TPA extraction. An amount of 8.03 ± 0.30 mg of TAE/g BP (n = 3) of TPY and 4.94 ± 0.16 mg of GAE/g BP (n = 3) of TPAY were obtained. The results showed that the use of sonication enhanced TPY by 3.09 folds and enhanced TPAY by 3.98 folds. The enhancement effect of sonication on the extraction yield was well varified.

Conclusion

Based on the present study, the levels of TPY and TPAY could be significantly enhanced by increasing EC within the suitable range. Under the practicable optimal conditions of TP and TPA extraction, the experimental values were significantly consistent with the predicted values, which verified the fitness of the models. The present study also demonstrated UAE is an efficient and repeatable method for polyphenol extraction from barley grain. On the other hand, an enhancement of TPY or TPAY could not mean the inevitable increase of all the components of TP or TPA from barley, neither the bioactivity. Thus, further researches should be focused on the improvement of the extraction of desired components or components tightly related with TP or TPA bioactivity.

ACKNOWLEDGEMENTS

The authors are grateful to the Natural Science Foundation of China (30760112) and the earmarked fund for Modern Agro-industry Technology Research System for their financial support. The authors also express their gratitude to Mr. Shi Guoliang and Ren Yuzhong for skilled technical assistance.

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