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ORIGINAL ARTICLE

Optimization of ultrasound assisted extraction of anthocyanins from red cabbage using Taguchi design method

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Abstract There is a growing demand for developing suitable and more efficient extraction of active compounds from the plants and ultrasound is one of these novel methodologies. Moreover, the experimental set up to reach an appropriate condition for an optimum yield is demanding and time consuming. In the present study, Taguchi L_9 orthogonal design was applied to optimize the process parameters (output power, time, temperature and pulse mode) for ultrasound assisted extraction of anthocyanins from red cabbage and the concluding yield of anthocyanin was measured by pH differential method. The statistical analysis revealed that the most important factors contributing to the extraction efficiency were time, temperature and power, respectively and the optimum condition was at 30 min, 15 °C and 100 W which could result the maximum anthocyanin yield of about 20.9 mg/L. The theoretical result was confirmed experimentally by carrying out the trials at the optimum condition and evaluating the actual yield.

Keywords Red cabbage · Anthocyanin · Ultrasound assisted extraction · Taguchi design

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Introduction

Anthocyanins are widely used as natural colorants in the food industry. They have a wide spectrum of colour tones, ranging from orange through red, to purple and blue, depending on the molecular structure and pH value. The interest in anthocyanins is not only because of their colouring effect but also is the result of their beneficial properties; examples ranging from inhibition of DNA damage in cancer cells in vitro (Hour 2003), inhibition of digestive enzymes (McDougall and Stewart 2006), induction of insulin production in isolated pancreatic cells (Jayaprakasam et al. 2005), reduction in inflammatory responses (Tall et al. 2004) to protection against age related decline in brain function (Lau et al. 2006), improvement in the tightness of capillary blood vessels and prevention of thrombocyte aggregation, all of which reduce the risk of circulatory diseases (Degenhardt et al. 2000; Giusti and Wrolstad 2003; Tsai et al. 2002). Thus Red cabbage dye has been used as a pH indicator in pharmaceutical formulations (Chigurupati et al. 2002) and as a colorant in food systems (Giusti and Wrolstad 2003). Due to growing environmental concern with regard to synthetic dyes, natural dyes offer scope for ecofriendly way of food and drug coloration.

To date, several conventional extraction techniques have been reported for the extraction of phenolic compounds, e.g. solvent extraction (Anagnostopoulou et al. 2006; Jeong et al. 2004), hot water extraction (Xu et al. 2008), alkaline extraction (Bocco et al. 1998), resin based extraction (Kim et al. 2007), enzyme assisted extraction (Li et al. 2006), electron beam and gamma irradiation based extractions (Kim et al. 2008) and supercritical fluid extraction (Giannuzzo et al. 2003). Both conventional and more innovative extraction techniques may either cause the degradation of the targeted compounds due to high temperature and long extraction times as in solvent extractions, or pose some health related risks due

Table 1 Factors and levels for the Taguchi L_9 orthogonal design

Factor	Unit	Type	L1	L2	L3
Temperature	°C	Factor	15	30	45
Time	Min	Factor	30	60	90
Power	Watt	Factor	50	75	100
Pulse Mode	–	Factor	0.3	0.65	1
Anthocyanin Content	mg/ L	Response	4.387		15.628

to the unawareness of safety criteria during irradiation. Furthermore, enzyme assisted extraction is limited due to problems of enzyme denaturation. With the development of the "Green Chemistry" concept during the last few years, environment friendly techniques are becoming more and more attractive. The extraction of bioactive compounds under ultrasound irradiation at frequency of 20–100 kHz is one of the newest extraction techniques that may offer high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Chemat et al. 2008). Optimisation of ultrasound assisted extraction has been described recently to extract hesperidin from Penggan (*Citrus reticulata*) peel (Ma et al. 2008a), phenolic acids and flavanone glycosides from Satsuma Mandarin (*Citrus unshiu Marc*) peel (Ma et al. 2009; Ma et al. 2008b) and total phenolic content from Penggan peel (Ma et al. 2008a).

Taguchi's optimization technique is a unique and powerful optimization discipline that allows optimization with minimum number of experiments. The Taguchi experimental design reduces cost, improves quality, and provides robust design solutions. This method has evolved into an established approach for analyzing interaction effects when ranking and screening various controllable factors. Moreover, Taguchi method is applicable to solving a variety of problems involving continuous, discrete and qualitative design variables (Tan and Tang 2001; Khoei et al. 2002).

A literature search did not yield any reference about earlier reports on the ultrasound assisted extraction of anthocyanin from red cabbage. The objective of this work is to outline the potential of ultrasonic waves in the fast preparation of extracts

Table 2 The L9 $(3)^4$ Taguchi design matrix for ultrasound assisted extraction of anthocyanin from red cabbage

Standard	Run	Temperature	Time	Power	Mode	Anthocyanin Content
18	7	45	60	50	0.30	14.257
20	22	45	60	50	0.30	14.394
19	23	45	60	50	0.30	14.12
14	5	30	30	75	0.30	9.87
23	24	30	30	75	0.30	9.733
24	25	30	30	75	0.30	10.007
11	2	15	90	100	0.30	11.515
25	26	15	90	100	0.30	11.515
26	27	15	90	100	0.30	11.378
16	4	30	90	50	0.65	13.023
13	18	30	90	50	0.65	12.338
12	19	30	90	50	0.65	13.846
6	6	15	60	75	0.65	11.104
9	16	15	60	75	0.65	10.83
8	17	15	60	75	0.65	11.241
17	8	45	30	100	0.65	12.475
22	20	45	30	100	0.65	12.612
21	21	45	30	100	0.65	12.338
3	3	15	30	50	1.00	4.387
2	10	15	30	50	1.00	4.798
1	11	15	30	50	1.00	5.895
27	9	45	90	75	1.00	12.749
5	12	45	90	75	1.00	13.161
4	13	45	90	75	1.00	12.201
15	1	30	60	100	1.00	15.354
10	14	30	60	100	1.00	15.628
7	15	30	60	100	1.00	15.354

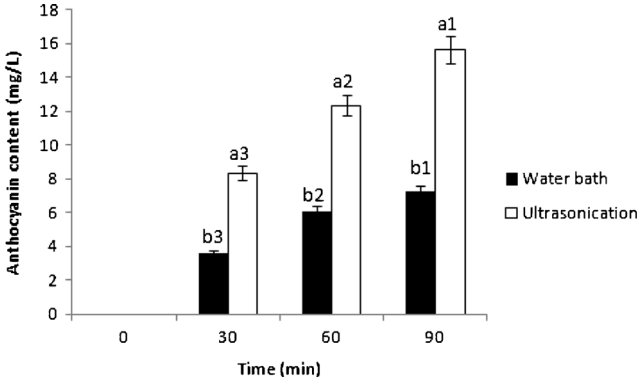

Fig. 1 The effect of ultrasound on the extraction of anthocyanin from red cabbage (100 W, 45 °C, 90 min). Data are the means±S.D, ($p<0.05$), (alphabet = constant time, numbers = varied time)

rich in anthocyanins from red cabbage. Several parameters that could potentially affect the extraction efficiency were evaluated and optimised using Taguchi design.

Materials and methods

Experimental setup

Ultrasonic extraction experiments were carried out using ultrasonic system (Hielscher, UP100H, 30 kHz, 100 W). The sonotrode (10 mm head diameter) was inserted in centre of a temperature controlled jacketed glass beaker. The whole system was placed in a sound proof vessel. Control experiments were performed in a water bath (Fater Electronic, W600S, Iran).

Fresh red cabbage (*Brassica oleracea* L. Var. Capitata f. Rubra) was purchased from vegetable market and kept refrigerated at 4±1 °C until used in the experiment.

Investigating the effect of ultrasound on the anthocyanin extraction from red cabbage

Freshly cut red cabbage pieces of 5 mm dimension (cubic in shape) and 92.11±0.45 % moisture content were used for this experiment. The extraction jacketed glass beaker (volume: 200 ml) was charged with 100 ml of distilled water and 2 g of red cabbage pieces. The beaker was covered with aluminium foil to prevent loss of solvent (water) by evaporation during the process. In all experiments the temperature in the beaker was maintained using thermostatic controller. Samples were finally collected, filtered and centrifuged at 4000 rpm and supernatants were utilized to determine the anthocyanin yield. Extraction in water bath was carried out as control experiment. All tests were carried out in triplicates and the standard error was calculated for the data.

Quantification of anthocyanins

The spectrophotometric pH differential method (Rodriguez-Saona et al. 2001) was used to quantify anthocyanins in the extracts. Two dilutions of the same sample were prepared using 0.025 M potassium chloride solution and 0.4 M sodium acetate solution adjusted to pH 1.0 and 4.5 with HCl, respectively. The absorbance (A_{519}) of each dilution was measured at 519 nm against a distilled water blank using the UV–visible spectrophotometer (Perkin- Elmer, Junior Model 35 Spectrophotometer). Anthocyanin content was calculated by the following Eq. 1,

$$Anthocyanin\ content\left(\frac{mg}{L}\right) = \frac{A*Mw*DF*1000}{\varepsilon*L} \quad (1)$$

Where $A = A_{519}\ (pH_{1.0}) - A_{519}\ (pH_{4.5})$, $A_{519}\ (pH_{1.0}) = A_{519}$ at pH 1.0 buffer, $A_{519}\ (pH_{4.5}) = A_{519}$ at $pH_{4.5}$ buffer, Mw is the molecular weight of anthocyanin (433.2 g/mol), DF = the dilution factor (10), e = the extinction coefficient (31, 600 L cm^{-1} mol^{-1}) and L = the path length (1 cm).

Taguchi Method

The use of Taguchi L_9 orthogonal design helps to determine the minimum number of experiments needed which may produce the most favourable information for a given set of factors. The influence of the process parameters such as ultrasonic output power (50, 75, 100 W), extraction time (30, 60, 90 min), cycle of pulsation (0.3, 0.65, 1) and temperature (15, 30, 45 °C) were investigated. The anthocyanin content (mg/L) was evaluated at the end of each experiment. The orthogonal array L_9 was used to study the influence of the four factors. Each factor was considered at three levels. The factors involved and their levels are

Table 3 Model fitting results for ultrasound- assisted extraction of anthocyanins from red cabbage

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* Value	*p* Value Prob>F
Mean vs Total	3701.59	1	3701.59		
Linear vs Mean	112.40	4	28.10	8.11	0.0004
2FI vs Linear	70.70	3	23.57	204.68	<0.0001
Residual	2.07	18	0.12		
Total	3886.76	26	149.49		

Table 4 ANOVA for Response Surface 2FI Model

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*- Value	Prob>F
Model	183.10	7	26.16	227.17	<0.0001
A	1.64	1	1.64	14.28	0.0014
B	7.58	1	7.58	65.82	<0.0001
C	1.84	1	1.84	15.97	0.0008
D	24.22	1	24.22	210.33	<0.0001
AB	2.62	1	2.62	22.75	0.0002
AC	59.32	1	59.32	515.20	<0.0001
BC	32.24	1	32.24	279.98	<0.0001
Residual	2.07	18	0.12		
Lack of Fit	0.12	1	0.12	1.06	0.3184

shown in Table 1. The Taguchi orthogonal array L_9 optimized the experimental set up and reduced the number of trials to 9 simple and effective experiments (Table 2). All experiments were performed in triplicate. Moreover, the interaction between factors was investigated and considered significant. Finally, anthocyanin extraction process was optimized to achieve the highest anthocyanin yield.

Statistical analysis

The results of anthocyanin yield were investigated by analysis of variance (ANOVA) for different models of main effects (linear) or 2 factor interaction (2FI) using Design Expert statistical software (version 8.0). The significant model terms were determined and their coefficients were estimated. The simultaneous effects of each pair of parameters were shown in contour plots. The optimum condition to obtain the highest extraction yield was introduced by the software. The validity of the modelling was tested at optimum condition by calculating bias of the predicted response from the actual data. The experiment was performed in triplicate. P values less than 0.05 were considered as significant.

Results and discussion

The results indicate that there is a significant enhancement in anthocyanin yield and extraction efficiency when complementing the extraction process with the ultrasound (Fig. 1). The anthocyanin yield was almost doubled in ultrasonic assisted process in comparison with water bath extraction method. This increase could be because of rupturing the membrane of anthocyanoplasts by ultrasonic waves and simple release of anthocyanins, while with regard to water bath method, anthocyanins might be discharged through the concentration gradient. Sivakumar et al (2009) demonstrated a significant increase in betalains (red coloured cyanins) extraction from beetroot in comparison with the magnet stirrer extraction method.

According to Table 2, the minimum response i.e. the lowest yield of anthocyanin in extraction medium was determined to be 4.387 mg/L at 15 °C, 50 W, 0.3 pulse cycle and 30 min processing time. The highest yield of anthocyanin (15.628 mg/L) was obtained at 30 °C, 60 min, 100 W, and 1 pulse cycle (continuous sonication).

To analyze the results of the experimental design, polynomial linear model was considered first to correlate the response and significant factors. The model was significant ($p<0.0001$, Table 3); however, adjusted and predicted R-Squares (0.53 and 0.37 respectively) were not close i.e. the linar model could fit to only 53 % of actual experimental results. A very high significant ($p<0.0001$) lack of fit of the model showed that some effects might not be considered for the statistical modeling. The data was then evaluated by a two factor intraction (2FI) model ($p<0.0001$). Unlike the linear model, 2FI model demonestrated a strong correlation

Table 5 Equation coefficient estimate for 2FI model

Factor	Coefficient Estimate	Degree of Freedom	Standard Error	95 % CI Low	95 % CI High
Intercept	11.66	1	0.067	11.52	11.80
A	0.46	1	0.12	0.20	0.71
B	−1.23	1	0.15	−1.54	−0.91
C	0.63	1	0.16	0.30	0.96
D	3.09	1	0.21	2.64	3.53
AB	−1.24	1	0.26	−1.78	−0.69
AC	−5.89	1	0.26	−6.44	−5.35
BC	−3.19	1	0.19	−3.59	−2.79

(respective adjusted and predicted R-Squares of 0.98 and 0.97) and a nonsignficant lack of fitness (p=0.3184). Based on these findings, the 2FI model was used to fit the data in the present study. The differences between adjusted and predicted data which are called residuals were normally distributed confirmimg application of the parametric test (ANOVA) for the modeling (Table 4).

According to Table 4, main effects of A (temperature), B (time), C (power), D (pulse mode) and their interaction effects (AB, AC, and BC) were determined significant in the 2FI model. The coefficients of main and interaction effects were estimated and provided in Table 5. As a constraint for this type of modeling, interactions of the parameter D (pulse mode) were not considered since they aliased by other effects. Responses predicted form the mathematical model were highly correlated with the actual data (Fig. 2).

Figure 3a shows a contour of anthocyanin yield against time and temperature when the experiments were performed at ultrasound lower power levels. At low temperature, the anthocyanin yield increased by extraction time, but this effect was not prominent at high temperature. It means that when the temperature was low, the effect of time was more pronounced, but when the time was short, the effect of temperature was more apparent. Effects of extracting temperature on the yield of oxymatrine were investigated by Xia et al (2012). The yield gradually increased when the temperature increased from 25 to 50 °C, and then it decreased from 50 to 70 °C (Xia et al. 2012). Increasing anthocyanin yield with the increase of extracting temperature might result from the increased diffusivity of the water into cells and enhanced desorption and solubility of anthocyanins from the cells. When the extracting temperature increased, there was a markedly decrease in the yield, which might be due to the degradation of anthocyanins at high temperature under ultrasonic irradiation. Thus, the extracting temperature had a profound influence on the ultrasound assisted extraction of bioactive compounds from plant materials particularly when the time of extraction is short.

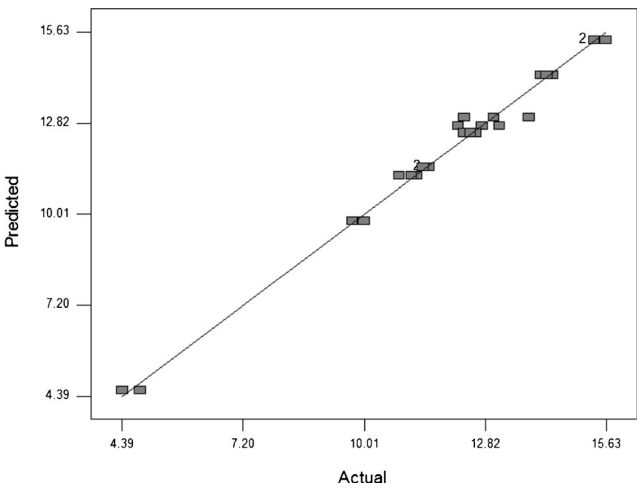

Fig. 2 Predicted data versus actual data

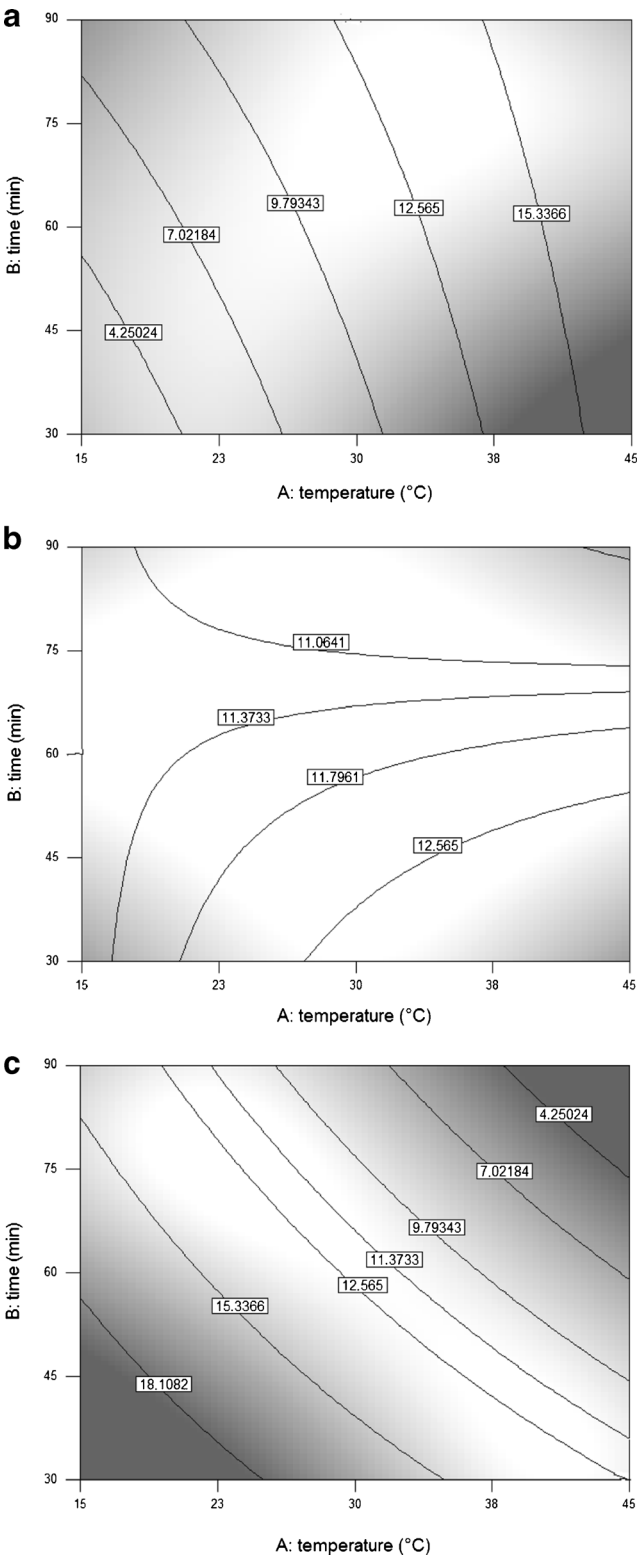

Fig. 3 Contour plot of anthocyanin yield at (**a**) low power (50 W), (**b**) moderate power (75 W) and (**c**) high power (100 W)

On the contrary, the anthocyanin yield did not changed significantly with time and temperature at moderate sonication power (Fig. 3b). The results were even revervsed at high power i.e. the anthocyanin yield decreased by extraction time and this effect was more intense at high temperature (Fig. 3c).

It may be indicative of destroying effect of ultrasound waves at high temperatures or during the course of extraction. Other studies proved that the contribution of ultrasonic waves

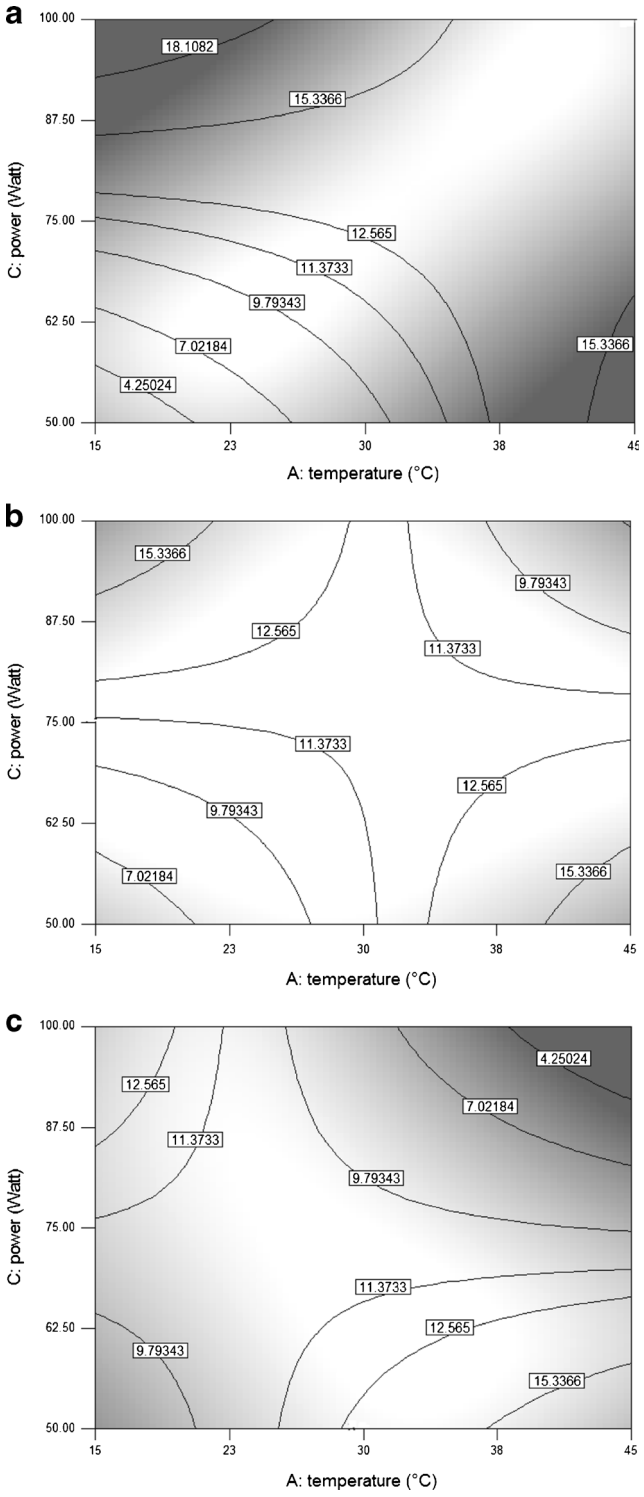

Fig. 4 Contour plot of anthocyanin yield at (**a**) short extraction time (30 min), (**b**) moderate extraction time (60 min) and (**c**) long extraction time (90 min)

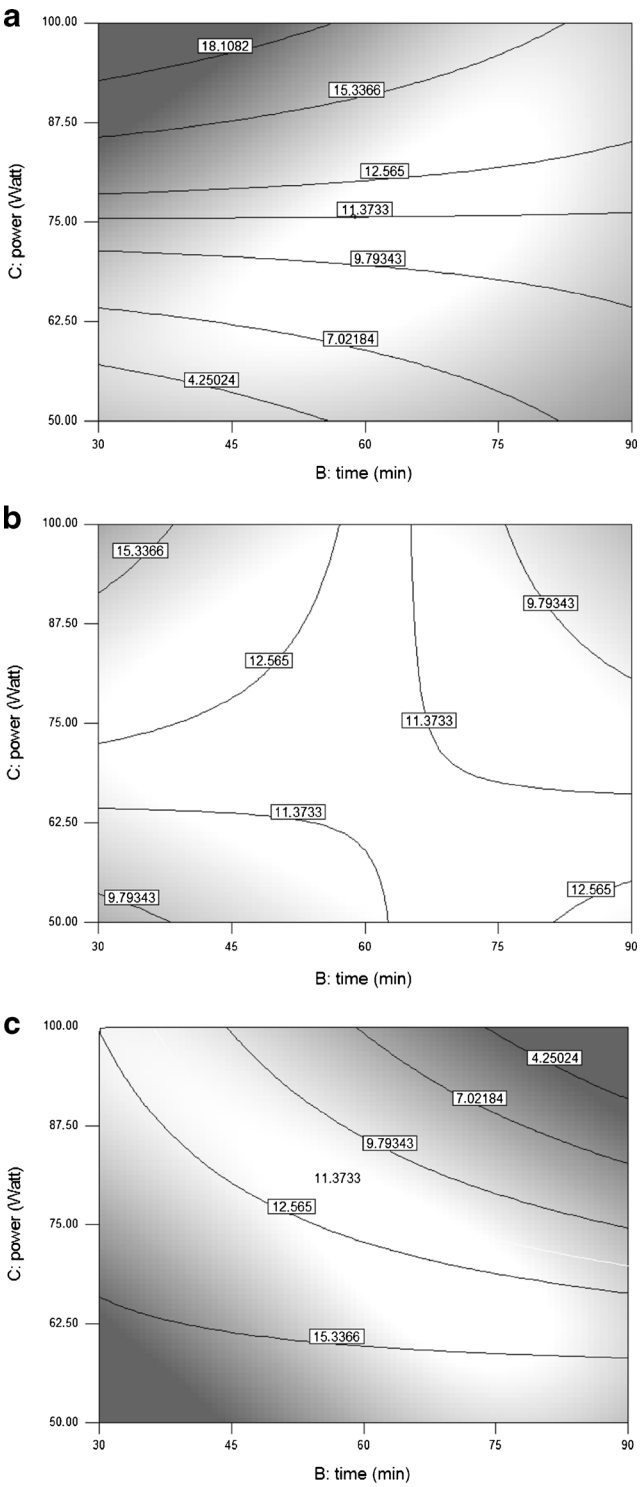

Fig. 5 Contour plot of anthocyanin yield at (**a**) low temperature (15 °C), (**b**) moderate temperature (30 °C) and (**c**) high temperature (45 °C)

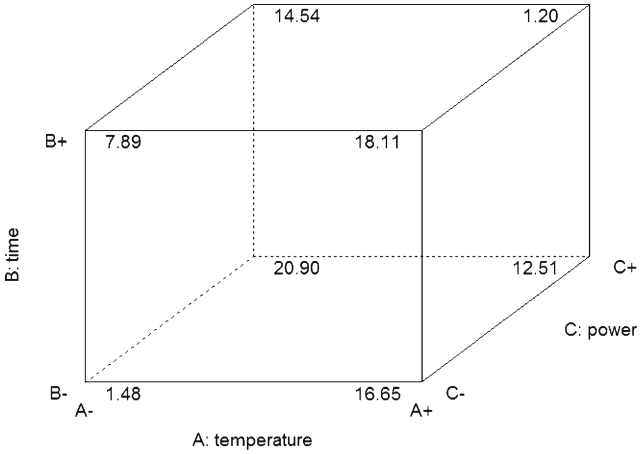

Fig. 6 The cubic graph of anthocyanin yield depending on three parameters of time, temperature and power

on the enhancement of the extraction, largely at low temperatures (Chemat et al. 2002).

Figure 4a shows the simultaneous effects of sonication power and temperature on the anthocyanin yield. In a short time at low sonication power, the increase in anthocyanin yield is influenced by both the temperature and power. However, the anthocyanin yield is less subjective to temperature and power, when both parameters are at their higher operating levels. At long or moderate time of extraction, again the influence of power or time on the anthocyanin yield were more distinct, i.e. increasing each factor resulted in increase of the yield but simultaneous increase resulted in decrease of yield (Fig. 4b and c). One of the probable reasons for this phenomenon is that phenolic compounds were thermally unstable and suffered pyrolysis due to local high temperature and high pressure produced by cavitation collapse. It has been reported previously by Herrera and Luque de Castro 2005, that ultrasonic treatment resulted in significant degradation (close to 100 %) of phenolics from strawberries, whereas degradation decreased by reducing ultrasound exposure time. Therefore, the application of ultrasound in extraction of unstable bioactive compounds should be carefully considered.

At the low and moderate temperatures, the yield was more dependent on the sonication power than the extraction time. The yield was increased by the power to the maximum yield acheivable in the study (Fig. 5a). By increasing the temperature from 15 to 30 °C, the effect of time was obvious and dependent on the sonication power (Fig. 5a and b). Conversely, at high temperature, the maximum yield was obtained at low power and short time. The destructive effect of sonication power was more obvious after the long extraction time (Fig. 5c).

The decrease in the anthocyanin yield by increasing time could be the result of destroying effect of ultrasonic waves on phenolic compounds and anthocyanin degradation. At high power, large amount of sound energy is changed to heat causing anthocyanin degradation. Therefore, exposing the materials containing anthocyanins to high temperature could decrease the yield of anthocyanins when extration time is too long. As chigurupati et al reported in 2002, anthocyanins are very sensitive to pH and temperature changes. Therefore, although the high temperature resulted in decreasing the extraction time since it expands capillary channels and cell wall pores, it may destruct anthocyanin molecules.

To take the effect of all the parameters simultaneously into account, the cubic graph was constructed. The optimum yield was determined at the power of 100 W, the time of 30 min and the temperature of 15 °C which resulted the anthocyanin yield of about 21 mg/L (Fig. 6). In this case the predicted and the observed values were in good agreement with biases well below 15 % for the evaluated model point (Table 6).

Conclusion

Our findings indicate that ultrasound may enhance the yield of anthocyanins extracted from red cabbage. The statistically significant variables that influence the ultrasonic extraction process are time, temperature and power, respectively. Here, application of pulsation during sonication does not produce any significant effects. The cavitation phenomena is responsible for enhancement in the extraction process, this can suggest that the supersonic jets from bubble implosions open spaces (capillaries) improving cell hydration. The statistical significance of the interactions between parameters reflects that enhancing the maximum anthocyanin content depends on various factors. The optimum yield was determined at the power of 100 W, the time of 30 min and the temperature of 15 °C which resulted the anthocyanin yield of about 21 mg/L.

Table 6 The predicted values and the experimental results of maximum anthocyanin yield prepared under the optimum condition

Response	Predicted value	Experimental value	Bias (%)
Anthocyanin content (mg/ L)	20.9	18.6	12.36

bias (%)=(predicted value- experimental value)/ experimental value *100

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