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Increasing anthocyanin content in black carrot juice by an enzyme assisted process: Optimization using response surface methodology

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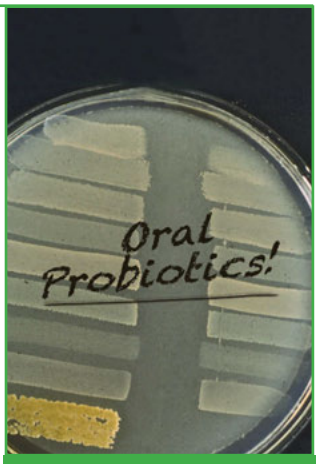

Keywords:
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Abstract

A three-level, three-factor, Box-Behnken response surface methodology (RSM) was used to optimize enzyme- assisted processing (EAP), for enhancing the concentration of anthocyanins extracted (ACNs) from black carrots. The black carrot mash was subjected to pre-treatments with different concentrations of Viscozyme L (0.05–0.25% v/w), extraction temperatures (50–70°C) and incubation time (30–90 min). Overall, a two-fold increase in ACN recovery was observed compared to untreated mash. Response surface analysis of the data,- was used to develop a three-degree polynomial equation which provided the following optimal extraction conditions: enzyme concentration = 0.23% v/w, temperature = 60°C and extraction time = 78 min. Under the optimal conditions, black carrot juice extracted via EAP had a high juice yield (86.31%) and high total monomeric ACNs (1252.15 mg/l). Results demonstrate that Viscozyme L is a potential enzyme combination for enhancing juice yields and ACN content from black carrots.

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Introduction

Anthocyanins (ACNs) have recently emerged as promising nutraceutical ingredients for developing functional foods and dietary supplements [1]. The health-enhancing properties of ACNs are associated with their ability to act as antioxidants and scavengers of reactive oxygen species and hydroxyl radicals. ACNs possess numerous biological functions such as anti hypertensive, anti mutagenic, anti-carcinogenic and anti-diabetic properties [2]. ACN extracts of red cabbage, *Hibiscus rosasinensis* petals, and the flesh of sweet potato have been shown to decrease the expression of inflammatory mediators, reduce oxidative stress and exerts a strong hepatoprotective effect. Overwhelming epidemiological evidence suggests that these pigments may help to reduce blood glucose, glucosuria and Hb A1c (glycosylated haemoglobin) levels by increasing insulin secretion and improving insulin resistance. Thus they are emerging as strong bioactive compounds for managing the global challenge of type- 2 diabetes. ACNs from purple corn (*Zea mays* L.), black soybean (*Glycine max* L. Merr.), blood orange (*Citrus sinensis* L. Osbeck), cornelian cherries (*Cornus mas*), blueberries (*Vaccinium angustifolium*) , strawberries (*Fragaria ananassa*) and blackberries have been shown to have a positive role in the prevention of Alzheimer's disease, and attenuation of liver injury and hyperglycaemia -in-

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duced hepatic oxidative damage [3,4]. ACN Extraction and stability is an important issue for the nutraceutical industry since it is governed by several factors (temperature, pH, co-pigments, etc.) which limit, their use in food applications. However, acylated ACNs, from black carrots and red cabbage exhibit remarkable stability and are resistant to colour fading over their unacylated analogs [5]. Black carrot (*Daucus carota* ssp. *Sativus* var. *atrorubens Alef.*) ACNs are acylated with p-coumaric, ferulic, p-hydroxybenzoic and sinapic acids and are thus more resistant to hydration, light and food pH [6]. The two major acylated ACNs in black carrot are, vinylcatechol adducts of cyaniding3-*O*-(6-*O*-feruloyl-β-D-glucopyranosyl)-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-galactopyranoside and cyanidin 3-O-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside [7]. In addition, they provide an excellent bright strawberry red shade at acidic pH values; contain low amounts of non-ACN phenolics and possess high antioxidant activity [8,10]. ACNs pigment has no off flavours and does not impart an objectionable taste. Altogether, it is an attractive functional ingredient for food applications in the pigment industry. Recent legislation by the FDA (Food and Drug Administration) has approved black carrot concentrate as a replacement for carmoisine in foods. ACNs from black carrots thus have great commercial value so there is an urgent need to optimize the extraction process. In recent years, enzyme-assisted processing (EAP) for extracting bioactive compounds from plants, has been widely investigated and recommended for its higher efficiency, ease of operation and environmentally friendly nature. In addition, the enzymes are non-toxic and an excellent application of green technology. The carbohydrate-hydrolysing enzymes, such as pectinase, cellulase, hemicellulase and glucanase have been reported to improve polyphenol extraction efficiency in various fruit matrices [11,14]. A combination of pectinolytic and cellulolytic enzymes showed the highest increase in phenolic yield from grape pomace [15,16]. Pectinex Smash and Biopectinase considerably improved total ACN recovery from bilberry and blackcurrant juices [17].

There are numerous reports on the optimization of conditions for enhanced extraction phenolics from black currant, berry fruits, purple potato and purple corn using response surface methodology (RSM) [18,19]. However, to best of our knowledge there has been no systematic study on the EAP of black carrots. Consequently, the main objective of this investigation was to optimize an enzyme assisted process for extracting enhanced ACNs from black carrots using RSM.

Materials and Methods

Up-stream and downstream process of enzymatic liquefaction of black carrots

Freshly harvested, medium sized roots of the black carrot variety 'Pusa Asita' were generously supplied by the Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi, India. The roots were thoroughly washed under running water, peeled and pulped in a domestic blender (Philips, India) for 2 min. The mash was then heated to 90°C for 1 min in a water bath to inactivate endogenous polyphenol oxidase activity. Enzymatic liquefaction was performed as described by Kammerer et al.[9] with slight modifications. Briefly, the crushed mash (approx. 500 g) was poured to 1 litre wide- mouth glass bottles (tightly secured with a lid) to prevent evaporation during the incubation period. The mash was mixed thoroughly with the enzyme mixture Viscozyme L (100 FBG/g) (optimal conditions of pH 4.5 and temperature 50°C) [20] from *Aspergillus aculeatus* (Novozyme) and added at an enzyme/mash ratio of 0.05–0.25 % (v/w) as mentioned in the experimental design section. The glass jars were then placed in a thermostatically controlled incubator with a shaker (Innova 42, New Brunswick scientific) and incubated at different temperatures (50–70°C) for 30–90 min according to the planned RSM (Table 1).The treatments were replicated three times and carried out at the intrinsic pH of black carrots (5.4), which falls within the optimum activity range of the (3.3–5.5) [21]. After liquefaction the macerate was placed in nylon filter bags and pressed in a hydraulic press (Johnston automation, India) at a pressure of 1,800 lb/cm^2. The extracted juice was heat processed at 90°C for 1 min to inactivate the enzymes and analysed immediately for total monomeric ACNs.

Independent variables	Units	Code levels		
		-1	0	+1
Enzyme Concentration (A)	%	0.05	0.15	0.25
Temperature (B)	°C	50	60	70
Time (C)	minutes	30	60	90

Table 1 - Independent variables with coded levels and actual values for fitting response surface model

Control juice was extracted in a similar way but without enzyme treatment. Juice extracted (under optimized conditions (best response) using EAP was clarified using bentonite (0.71 g/l, 50°C for 2h) and analysed for colour values and polymeric colour.

Calculation of Juice yield

The extracted juice was weighed and the yield of juice was calculated based on the fresh weight of mass:

Juice yield (%) = (Weight of expressed juice/ Weight of mash) ×100

Determination of total monomeric (ACN) content

Total monomeric ACN content was determined using the pH differential method described by Wrolstad et al [22]., which relies on the structural transformation of the ACN chromophore as a function of pH. The expressed juice was centrifuged at 10,000 g for 15 min, and the supernatant was taken for analysis. Aliquots of juice were diluted with pH 1.0 and 4.5 buffers, respectively. The absorbance of each solution was measured after equilibration using a spectrophotometer (Varian 50; Agilent Technologies) at wavelengths of maximum absorption of 520 nm and 700 nm versus a blank cell filled with distilled water. Absorbance was calculated as Abs = $(A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$, with molar absorbance of 26,900 for cyanidin-3-glucoside. The total monomeric ACN content was calculated and expressed as cyanidin-3-glucoside equivalents mg/l using the following equation:

$$\text{Anthocyanin content (mg l}^{-}) = \text{Abs} \times MW \times DF \times 1000/(\varepsilon \times L)$$

where Abs is the absorbance, ε is the cyanidin-3-glucoside molar absorbance (26,900 $M^{-1} \cdot cm^{-1}$), L is the cell path length (1 cm), MW is the molecular weight of cyanidin-3-glucoside (448.37 g/mol); and DF is the dilution factor. All measurements were conducted in triplicate.

Determination of colour, colour density, turbidity and polymeric colour

A colorimeter (Minolta Chroma Meter CR-100) was used to measure lightness and chromaticity coordinates in the $L^*a^*b^*$ colour space (CIELab). L^* indicates lightness, and a^* and b^* are the chromaticity coordinates. h^* (hue) values and E (colour change) were calculated from a^* and b^* values. Colour density and polymeric content were determined using the bisulphite bleaching method [23]. Juice turbidity was measured as NTU (Nephelometric Turbidity Unit),using Loveland, CO, USA).

Identification of anthocyanins from black carrot by HPLC

Black carrot anthocyanin concentrations obtained by enzymatic extraction were analysed using an HPLC instrument fitted with a 600 quaternary pump, with an auto injector (20µL loop), a 2998 photodiode array detector (Waters Corp., Milford, MA., U. S. A.) and a 250 × 4.6 mm diameter and 5- µm C_{18} column (ODS Hypersil column; (Thermo Electron Corporation). Aliquots (2ml) of each sample solution were filtered through a 0.45-µm nylon filter before injection into the HPLC system. The mobile phase consisted of a gradient flow of Solvent A: (water, (0.1% TFA) and Solvent B (water: ACN:- TFA, (53:46:1). Anthocyanins were monitored at a wavelength of 520 nm. A computer using "Empower 2" software was used to integrate the peak areas.

Experimental design

In the present study, RSM was used to optimize and study the effect of independent variables such as enzyme concentration, incubation temperature and extraction time on juice yield and the recovery of ACNs from black carrot mash. The experiment and statistical analysis were performed using Stat-Ease software (Design-Expert 8.0 Trial; Stat-Ease Inc., Minneapolis, MN,USA). A three-level, three-factor, Box–Behnken design was chosen to evaluate the combined effect of the three independent

variables enzyme concentration, temperature and time on the response. The variables and their levels were chosen, based on the results of preliminary experiments. The minimum and maximum values for enzyme concentration were 0.05 and 0.25%, temperature was set between 50 and 70°C and extraction time was between 30 and 90 min (Table 1). The response values were juice yield (%) and ACN (mg/l). The design consisted of 17 combinations, including five replicates of the centre point used to determine experimental error (Table 2).

Analysis of variance (ANOVA) was applied to ACN and juice yield; the results are shown in Tables 3 and 4. Three experiments for each condition were carried out and the observed response were stated as mean values .

The model used for response surface was in the form:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + e$$

where Y is the response variable.A, B and C represent the three independent factors under study: A is Viscozyme concentration (mg/l), B is Temperature (°C) and C is Incubation time (min). β_0, β_i, β_{ii} and β_{ij} [i < j = 1 and 2] are the parameters of the model to be estimated. e is the error term which is assumed to be independently and identically distributed following normal distribution with zero mean and constant variance. The model was fitted using stepwise regression, to identify the significant parameter estimates and the model fitness was assessed using adjusted R^2. The graph of the response surface curve was used to visualize the change in response, at different levels of the input variables, and cube diagrams were used to flag the maximum response at optimum input points.

Result and discussion

Juice yield and Total Anthocyanin

EAP is a complex process, influenced by multiple factors such as enzyme type, concentration, temperature and incubation time, which cumulatively determine a variable response. In recent years, RSM has become increasingly popular among researcher as a powerful statistical and mathematical tool for developing, improving and optimizing complex processes [24] with a minimal number of experiments.

In the present paper, EAP of black carrots was optimized using a multicomponent, Viscozyme L (100 FBG/g) enzyme preparation, produced from *Aspergillus aculeatus* (Table 1). The enzyme is a multi-complex containing a wide range of carbohydrases, including arabanase, cellulase, hemicellulase, xylanase and pectinase activity. Significant (p<0.05) variations in juice yield were found in response to different enzymatic maceration treatments (Table 2). The obtained juice yield ranged from 74.00 to 87.00% in comparison to 49.50 to 54.45%, in untreated mash (50–70°C, 60 min).

The juice yield data were consistent with the interpretation that increased juice is a result of enzyme catalyzed degradation of the pectin in the plant cell wall matrix and the middle lamella that acts as putty between the cells and binds water. Significant pectin degrading activity, due to the presence of pectin esterase, pectin lyase, and polygalacturonase, catalyses the degradation of the smooth regions of the pectic substance. Further, the additional activities due to cellulase and hemicellulase, present in viscozyme L facilitate in the breakdown of insoluble matrix leading to complete disintegration and release of the bound water. The overall synergistic action of various enzyme activities, account for enhanced juice yields. The results are in agreement with the reports of previous researchers:- Viscozyme L significantly increased juice yield, and recovery of quercetin, protein and oil content in oats [21,25].

ACNs are potentially the most bioactive compounds in black carrots, and thus optimization of EAP is crucial. The values for total anthocyanin content in black carrot juice at various experimental conditions are presented in Table 2. As expected, ACN increased with enzyme dose, extraction temperature and incubation time. Maximum ACN concentration (1,290.99 mg/l) was observed at a 0.25% enzyme dose, temperature of 70°C and incubation time of 60 min. Both enzyme dose and temperature are crucial factors as excessive dosage can lead to breakdown of ACN structure, accompanied by colour loss. The median enzyme dose was determined based on preliminary experi-

ments. Temperature plays a vital role as it disrupts the cell wall and opens up the structure there by favouring the penetration of enzymes and facilitating the release of phenolics [16]. Degradation of fibrous matrix, under the optimum enzyme dosage, coupled with high temperature enables the release of bound water and concomitant release of water soluble ACNs. Heat also enhances solubility and the diffusion coefficient, thus facilitating higher ACN release. Similar improvements in ACN extraction from blueberry and strawberry juices have been confirmed by previous researchers [26]. In addition, other factors such as the initial raw material and processing conditions also affect recovery. Reports on black carrot processing are few [11]. However, ACN content was recently reported as ranging from 451 to 589 mg/l with a juice yield of 36% for black carrot juice[27]. Both these values are very low, compared to the corresponding values obtained in the present study, apparently because the conventional straight pressing method employed (without enzymes), prevent incomplete liquefaction resulting in low juice yields and low recovery. The results obtained here are in close agreement with previous work. Enhanced CAN recovery from elderberry, purple corn, black currant and grape wines using pectinase enzyme has been reported [26]. An increase in temperature and a longer period of maceration pulsed electric fields resulted in higher concentrations of the different ACNs [28] in grape wines. The predominant ACNs found in wines were

Treatment Combinations	Independent variables			Dependent variables	
	A	B	C	Juice yield (%)	Anthocyanin content (mg/l)
1	0	-1	1	83	1103.89
2	0	0	0	82.33	1103.73
3	1	-1	0	84.16	1102.90
4	0	0	0	83	1169.68
5	0	1	-1	76.33	895.69
6	0	0	0	80.50	1115.56
7	-1	1	0	80.83	950.00
8	0	0	0	86.00	1148.72
9	1	1	0	77.50	1135.92
10	1	0	1	82.33	1290.99
11	0	0	0	82.00	1169.93
12	-1	0	-1	87.00	821.79
13	0	-1	-1	83.83	902.81
14	1	0	-1	82.16	1101.91
15	-1	-1	0	74.00	895.19
16	0	1	1	82.33	1185.07
17	-1	0	1	84.16	905.29

Here (A) represents Enzyme Concentration ; Temperature (B) and Time (C)

Table 2 - Box-Behnken design and experimental data for juice yield and total anthocyanin content

Source	Sum of Squares	DF	Mean Square	F Value	Significance
Model	254000.00	5	50807.15	16.17	< 0.0001
A	140100.00	1	140100.00	44.58	< 0.0001
B	12615.14	1	12615.14	4.01	0.0704
C	46203.60	1	46203.60	14.7	0.0028
A^2	13896.31	1	13896.31	4.42	0.0593
C^2	38471.74	1	38471.74	12.24	0.005
Residual	34568.00	11	3142.57		
Corrected Total	288600.00	16			

Stepwise Regression with Alpha at 10% level of significance was used to remove the non-significant parameter estimates from the model

Table 3 - ANOVA Table of ACN

Source	Sum of Squares	DF	Mean Square	F Value	Significance
Model	535.61	5	107.12	46.99	< 0.0001
A	238.38	1	238.38	104.57	< 0.0001
C	120.2	1	120.2	52.73	< 0.0001
AC	17.35	1	17.35	7.61	0.0186
A^2	103.52	1	103.52	45.41	< 0.0001
C^2	47.85	1	47.85	20.99	0.0008
Residual	25.08	11	2.28		
Corrected Total	560.69	16			

Stepwise Regression with Alpha at 10% level of significance was used to remove the non-significant parameter estimates from the model

Table 4 - ANOVA Table of Juice Yield

malvidin-3-O-glucoside (reaching values of 15.10 - 60.40 mg/l, malvidin-3-*O*-(6-*O*-acetyl) glucoside + peonidin-3-*O*-(6-*O*-acetyl) glucoside, (5.15 - 23.81 mg/l; and petunidin-3-glucoside (2.22 to 6.88 mg/l).

Another critical factor in EAP is the length of the incubation period. An extraction period that is too long, may adversely affect the detrimental for extraction of phenolic antioxidants, especially ACNs, which are sensitive and can polymerize and degrade [27]. However, a sufficient length of time is required so they can be efficiently from cellular matrix, otherwise they remain trapped and are wasted into pomace. Short extraction periods were also observed to be more favourable for the extraction of phenolics as long durations can lead to the autoxidation of phenolics. In the present study, 60 min was found to be optimum for maximum ACN recovery and juice yield from black carrots [29].

Response surface modelling

In order to verify the predictive ability of the model, optimum conditions were established by RSM and the between predicted results and the actual values were compared by rechecking the experiments using the presumed optimal conditions.

The fitted model for juice yield together with the standard error of the parameter estimates is as follows:

$$Y = 83.048 + 5.46A + 3.876C + 2.083AB - 4.95A^2 - 3.37C^2$$

(Adjusted R2 = 0.93) (p<0.001)

(0.59) (0.53) (0.53) (0.75) (0.73) (0.73)

Three-dimensional surface plots (Fig. 1a–c) were also drawn to illustrate the main and interactive effects of independent variables, on the dependent variable ones. The surface plots for juice yield were drawn by taking different values of two input factors in the range of -1 and +1 and one factor at level zero. The three surface plots correspond with the combinations AB, BC and AC of input factors with juice yield. Fig. 1a, shows the response surface curve for juice yield at a fixed incubation time of 60 min. As the temperature increases, there is an increase in juice yield followed by a small decline.

The fitted model for TAC along with the standard error of the parameter estimates is as follows:

$$Y = 1130.687 + 132.34A + 39.71B + 75.99C - 57.37A - 95.49C^2$$

(Adjusted R2 = 0.87) (p<.001)

(22.27) (19.81) (19.81) (19.81) (27.71) (27.28)

Results show that both models are good fits for the data obtained. The surface plots corresponding to the combinations AB, BC and AC of input factors show the optimum conditions of the extraction process and high recovery of ACN (Fig. 1 d–f). The surface plots showing the optimum ACN content are drawn by taking different values of two input factors in the range of - 1 and +1 and one factor at level zero. As expected, ACN increased with enzyme concentration and temperature of the mash. The ACN content was 899.69 mg/L (0.05%, 60°C) which increased to 1148 mg/L (0.15%, 60°C) and finally to 1290 mg/L (0.25%, 60°C).

Further, we optimized the responses (i.e. juice yield, and ACN) using a multi-response optimization technique by taking both responses together and restricting the intervals of A, B and C to 0.05–0.25%, 50–70°C and 30–90 min respectively. The optimum values for A, B and C were A = 0.23 mg/L, B = 60°C and C = 78 min.

Responses at optimal extraction conditions

The best combination of process variables for the best set of response properties were an enzyme concentration of 0.233% (v/w), incubation temperature of 60°C and incubation time of 78 min. The responses calculated at optimal extraction conditions, were juice yield and ACN content of 86.31 % and 1252.15 mg/l respectively. Mean values for color density, polymeric colour and colour (L^*, a^*, b^*) of the juice were 66, 18 and (8.7, 28.7 and - 25), respectively(data not shown). The same response was confirmed through an upscale process which is schematically represented in Fig. 2.

Anthocyanin composition

Optimized Black carrot juice was found to contain a mixture of four anthocyanins which were characterized as cyanidin 3-xylosylglucosylgalac-

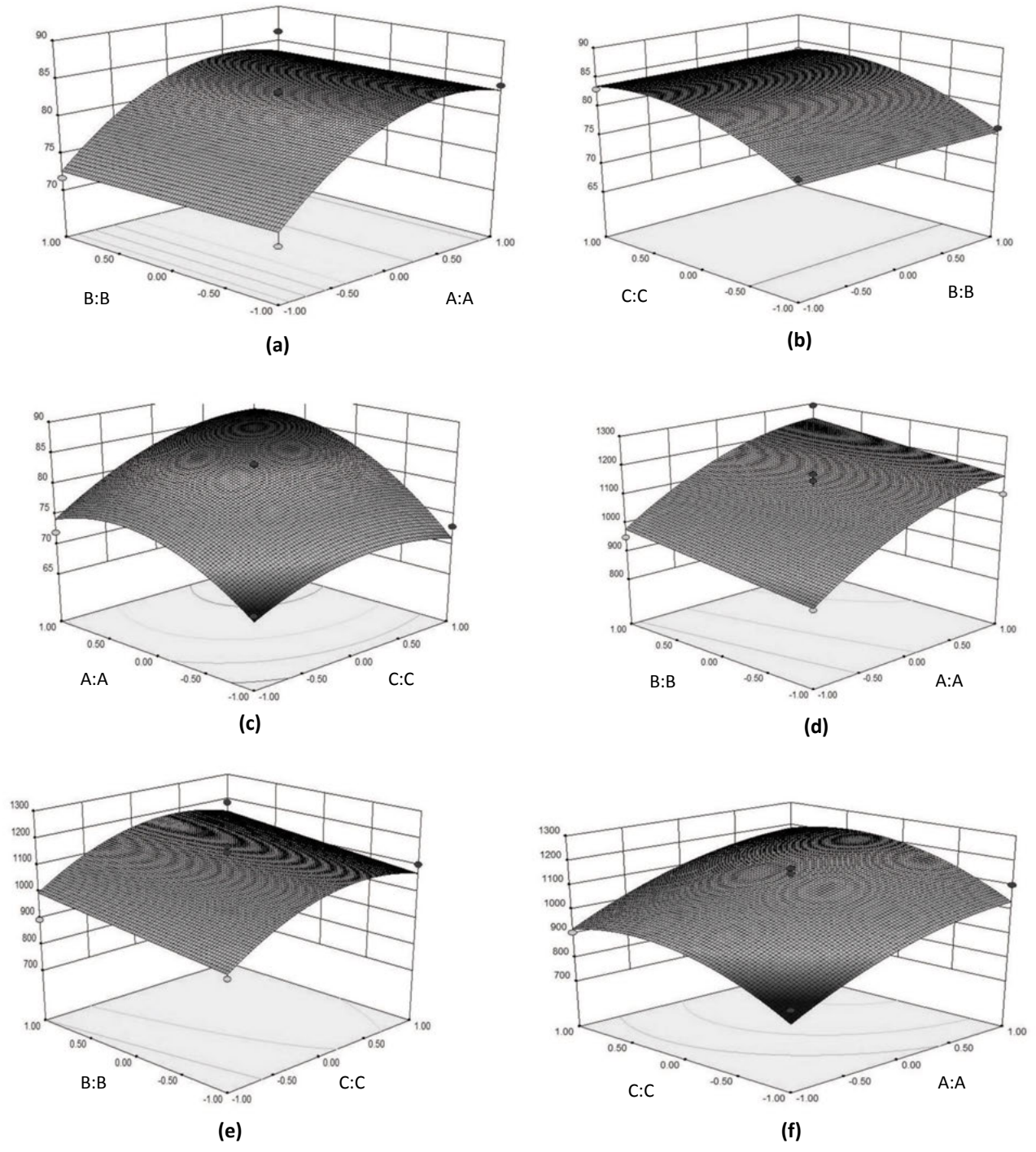

Figure 1 - **a** Response surface curve for juice yield (%) in black carrot juice. A: enzyme concentration (%); B: temperature(°C). **b** Response surface curve for juice yield (%) in black carrot juice. B: temperature (°C); C: time (min). **c** Response surface curve for juice yield (%) in black carrot juice. A: enzyme concentration (%); C: time (min). **d** Response surface curve for total anthocyanin content (mg/l) in black carrot juice. A: enzyme concentration (%); B: temperature (°C). **e** Response surface curve for total anthocyanin content (mg/L) in black carrot juice. B: temperature (°C); C: time (min). **f** Response surface curve for total anthocyanin content (mg/L) in black carrot juice. A: enzyme concentration (%); C: time (min)

toside, cyanidin 3-xylosylgalactoside, cyanidin 3-sinapoylxylosylglucosylgalactoside and cyanidin 3-feruloylxylosylglucosylgalactoside (Fig.3) based on their UV spectra and other characteristics.

This findings is in agreement with earlier reports [6]. However, only four prominent peaks were detected although researchers have reported five major anthocyanins including the coumaric derivative, but this discrepancy may be due to differences in variety and environmental conditions.

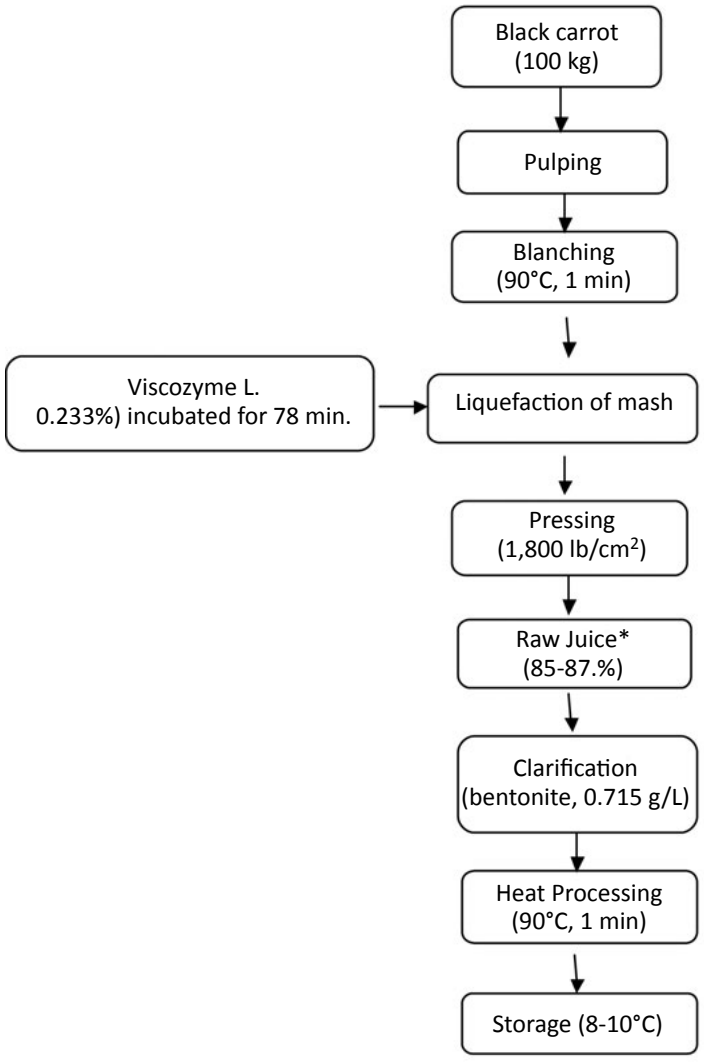

Figure 2 - Flow diagram for the processing of black carrot juice.
*Juice range indicates variations accounted for by losses during preparation

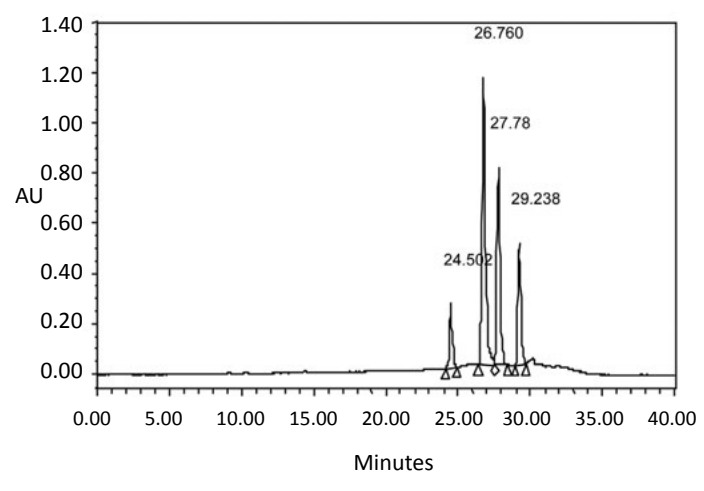

Figure 3 - HPLC chromatogram of black carrot anthocyanin

Conclusion

The results suggest that changes in Viscozyme L concentration, temperature and incubation time have a significant effect on the juice yield and ACN content of black carrots. With an increase in enzyme concentration and temperature, the juice yield and ACN increased sharply but reached a plateau when extraction was conducted beyond 60 min. Under these optimized conditions, the experimental maximum juice yield and ACN concentration were 86.31% and 1252.15 mg/l respectively. There is excellent agreement between of the experiment values and the predicted values indicating the suitability of the developed models and the success of RSM in optimizing extraction conditions.

Human and Animal rights note:
This article does not contain any studies with human or animal subjects performed by the any of the authors.

Conflict of Interest
Authors declare that they have no conflict of interest.

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