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RESEARCH ARTICLE

Improving the Analysis of Anthocyanidins from Blueberries Using Response Surface Methodology

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Abstract:

Background:

Recent interest in the health promoting potential of anthocyanins points to the need for robust and reliable analytical methods. It is essential to know that the health promoting chemicals are present in juices and other products processed from whole fruit. Many different methods have been published using a wide variety of conditions for the hydrolysis of anthocyanins to anthocyanidins.

Objective:

To investigate the factors influencing the hydrolytic conversion efficiency. The optimum set of conditions will maximize the recovery of anthocyanidins.

Method:

Extraction procedure (freeze drying *vs*. direct liquid extraction), heating method (reflux *vs*. sealed vial), nitrogen purging and acid type were investigated. Response surface methodology was then used to find the optimum combination of incubation time, acid concentration and incubation temperature.

Results:

Anthocyanidin recovery can be maximized using this procedure: Freeze-dry homogenized fruit and extract with methanol:water:TFA, place 1 mL extract or juice in a test tube and add 440 µL 37% HCl, purge the tube with N_2, seal with a PTFE lined cap, vortex, then heat at 99°C for 6.4 minutes. Filter the hydrolysate into an autosampler vial and analyze by UPLC immediately.

Conclusion:

Maximizing the recovery of anthocyanidins (by manipulating conditions in order to maximize peak areas) leads to a more accurate measure of the anthocyanidins present in blueberries.

Keywords: Anthocyanidins, Anthocyanin, Blueberries, Extraction, Hydrolysis, Response Surface Methodology, UPLC.

INTRODUCTION

The health benefits of anthocyanins have been investigated by numerous authors. Basu, Rhone and Lyons [1] wrote an extensive review on the role of anthocyanins in preventing cardiovascular disease. Anthocyanins may also play a

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role in preventing the spread of cancer [2]. A large number of beverages made from the so-called superfruits such as blueberry, pomegranate, cranberry, acai, blackberry, grape, *etc*. are being marketed to capitalize on the health benefits of anthocyanins. How much of the anthocyanins present in the fruit are still available in the juice? Accurate, sensitive and robust methods are needed to verify product claims and improve product quality.

The analysis of anthocyanins can be challenging. Anthocyanins consist of a flavylium cation (also known as an anthocyanidin) with one or more sugar molecules attached. Almost 600 different anthocyanins have been reported [3]. They are responsible for the purple, blue and red colors in fruits and flowers. At least 27 have been identified in blueberries [4]. Separating and quantifying such a large number of compounds would be problematic. In addition, analytical standards are very expensive and are not available for most of the anthocyanins. Finally, similar masses and conjugations make identification by mass spectroscopy very difficult when using quadropole or ion-trap spectrometers. The analysis can be simplified by removing the sugar molecule using acid hydrolysis, resulting in six common anthocyanidins: delphinidin (del), cyanidin (cya), petunidin (pet), pelargonidin (pel), peonidin (peo) and malvidin (mal). (Pelargonidin is not found in blueberries.) Standards are available for all six and ultra performance liquid chromatography (UPLC) with ultraviolet (UV) detection can be used to separate and quantify them.

Several variables affect anthocyanidin recovery and reported hydrolysis methods differ in a number of details. Table **1** summarizes the wide variety of conditions reported in 25 papers investigating the hydrolysis of anthocyanidins in various fruits, vegetables and beverages. Some researchers use reflux heating, while others heat the mixture in a sealed vial. Hydrolysis temperatures range from 23°C to 166°C. Heating times range from 8 min to 5 hours. Acid concentrations used range from 1.1N to 3.0N with two reports using alkaline hydrolysis. Most reports use hydrochloric acid (HCl). Merken *et al*. [5] report that aqueous and methanolic triflouroacetic (TFA), glucosidases, other hydrolytic enzymes and dilute sulfuric acids were unsuccessfully tested. Some workers purge the mixture with N_2 or He while others do not. Pinho *et al*. [6] compared acid concentration, temperature and time using response surface methodology (RSM) to optimize the hydrolysis of anthocyanidins by reflux in red wine.

Table 1. A summary of conditions used by various researchers for the hydrolysis of anthocyanins.

References	Heating Method	Acid or base	(°C)	Minutes	Matrix
Harnly *et al*. [11]	reflux	1.2 N HCl	75	300	60 fruits, vegetables and nuts
Franke *et al*. [12]	reflux	1.2 N HCl	100	60 or 120	45 fruits and vegetables consumed in Hawaii
Hertog *et al*. [13]	reflux	1.2 N HCl	90	120	Fresh lettuce, leek, celery, onion, endive, cranberries
Merken *et al*. [5]	reflux	1.8 N HCl	75	300	Blueberries, blackberries, strawberries, onion, parsley
Pinho *et al*. [6]	reflux	2.87 N HCl	166.2	46.6	Red wine
Burdulis *et al*. [10]	reflux	2.95 N HCl	100	120	Billberry
Queiroz *et al*. [14]	reflux	2.95 N HCl	95	120	Raw and cooked blueberries
Burdulis *et al*. [8]	reflux	2.95 N HCl	100	120	Billberry
Chun *et al*. [15]	reflux	1.2 N HCl	90	120	Plum
Kosar *et al*. [16]	reflux	1% TFA	?	60	Strawberry
Takeoka *et al*. [17]	sealed vial	1 N HCl+5% formic	100	60	Concord grape puree and black bean extracts
Watson *et al*. [18]	sealed vial	1% HCl	100	40	Cranberries
Hynes and Aubin [7]	sealed vial	1.1 N HCl	150	30	Blueberries
Uddin *et al*. [19]	sealed vial	1.3 N HCl	100	120	Flowers
Lee *et al*. [20]	sealed vial	10% KOH	23	8	Blueberry fruit, juice and presscake
Nyman and Kumpulainen [21]	sealed vial	2 N HCl	90	50	Bilberry, black currant, strawberry, red wine
Zhang *et al*. [22]	sealed vial	2 N HCl	100	60	Bilberry extract powder
Wilkinson *et al*. [23]	sealed vial	2 N HCl	100	30	Muscadine grape skins, calyces of roselle
Gao and Mazza [24]	sealed vial	2 N HCl	100	60	Blueberries
Wang *et al*. [25]	sealed vial	2 N HCl	100	30	Muscadine grape pomace extract
Hong and Wrolstad [26]	sealed vial	2 N HCl	100	30	Cranberries
Rodriguez-Mateos *et al*. [27]	sealed vial	2.5 N HCl	90	60	Blueberries
Nielsen *et al*. [28]	sealed vial	3 N HCl	30	100	Flowers
Gao and Mazza [29]	sealed vial	2 N HCl	100	60	Pure anthocyanins, flower petals, grape skins
Fan-Chiang and Wrolstad [30]	sealed vial	10% KOH	23	8	Blackberries
Fan-Chiang and Wrolstad [30]	sealed vial	2 N HCl	100	45	Blackberries

It is difficult to choose optimum conditions from the literature. A number of variables effect recovery. Some (reflux *vs*. sealed vial, purging with inert gas, acid type) are independent of each other. Three variables are interdependent: acid concentration, incubation time and incubation temperature. Changing one at a time fails to take into account interactions. RSM is an efficient statistical tool for discovering the optimum values for all three variables.

Our goal is to find the best conditions for the analysis of blueberry juice and extracts. The optimal conditions will result in the highest area counts generated by the chromatographic analysis. The first objective of this work was to investigate the effects of extraction procedure (freeze drying *vs*. direct liquid extraction), heating method (reflux *vs*. sealed vial), nitrogen purging and acid type. RSM was then used to find the optimum combination of incubation time, acid concentration and incubation temperature. Maximizing the recovery of anthocyanidins (by manipulating conditions in order to maximize peak areas) leads to a more accurate measure of the anthocyanidins present in blueberries.

MATERIALS AND METHODS

Fruit

Commercially ripe 2012 rabbiteye blueberries (*Vaccinium ashei* cv. 'Tifblue') were harvested by Blue River Farms, LLC (Mt. Olive, MS), sorted, graded, cleaned, washed and boxed for commercial freezing (blast frozen with forced air at about -23 to -29°C for 72 hr) and stored at -20°C in the Nordic Cold Storage facility (Hattiesburg, MS). Fruit were shipped on dry ice to the Southern Regional Research Center and stored at -20°C until processed.

Chemicals and Consumables

Deionized water was obtained from a Millipore Gradient A-10 (EMD-Millipore, Billerica, MA). Solvents were purchased from Spectrum Chemicals (New Brunswick, NJ) and acids from Sigma-Aldrich (St. Louis, MO). Autosampler vials were from Agilent (2 mL size with Polytetrafluoroethylene (PTFE) lined screwcaps, Santa Clara, CA). Amber glass vials with PTFE lined caps were obtained from Qorpak (15 x 45mm, Bridgeville, PA) and test tubes with PTFE lined screw caps came from Fisher Scientific (Houston, TX). All samples were filtered through 0.22 µm syringe filters (Restek, State College, PA) prior to analysis.

UPLC

An Acquity UPLC equipped with a BEH (Bridged Ethylene Hybrid) C_{18} guard column (5mm X 2.1 mm X 1.7 µm), a BEH C_{18} analytical column (50mm X 2.1 mm X 1.7 µm) and a tunable ultra-violet (TUV) detector, controlled by Empower 2 software (Waters Corporation, Milford, MA) was used to analyze the hydrolysates. Conditions were slightly modified from Hynes and Aubin [7]. The aqueous phase was 3% phosphoric acid and the organic phase was 100% acetonitrile. A flow rate of 1.0 mL/min started at 10% organic, changed by linear gradient to 20% at 2 min., then to 100% at 2.1, held at 100% until 2.5, then returned to 10% at 2.8 min. Absorbance was recorded at 525 nm.

Extraction

Two extraction methods were compared: (1) freeze drying followed by extraction and (2) direct liquid extraction. The freeze-drying method was modified by Barnes *et al*. [3]. Frozen blueberries were thawed, homogenized in a blender (Waring, South Shelton, CT), then poured into tared watchglasses. After recording the total weight, homogenates were freeze-dried (Virtis Genesis model 25ES, SP Industries, Warminster, PA). Dry samples were removed, weighed, and wet and dry weights calculated. Dried berries were ground to a powder in a mortar and pestle. Dried powders (2.50 ± 0.01 g) were mixed with 25 mL extraction solvent (methanol:water:TFA, 70:30:1, v:v:v) in 50 mL centrifuge tubes. Each tube was vortexed for 15 seconds, sonicated for 20 min, left undisturbed for 60 min and centrifuged for 20 min at 4600 x g (IEC, Needham Heights, MA).

For direct liquid extraction, methods developed by Burdulis *et al*. [8] and Garcia-Viguera *et al*. [9] were modified. Frozen blueberries (60 g) were thawed and homogenized with 200 mL of acidified methanol (methanol:TFA, 100:1 v:v) with a blender (Waring, South Shelton, CT). Water was not needed in this solvent system because the blueberries were not dried. The homogenate was poured into centrifuge tubes, sonicated for 20 min, allowed to sit for 60 min undisturbed and spun down as above.

For juice analysis, thawed blueberries were hand-pressed through muslin cloth and the juice collected. The extracts and juice were frozen at -80° C until analyzed.

To compare the effectiveness of the alternative extraction methods, 5 reps of 1 mL of each extract (freeze-dried and direct) were mixed with 100 µL 37% HCl in tubes. They were purged with nitrogen, capped with PTFE lined caps, vortex mixed and heated for 30 min at 95 °C in a GC oven (HP5890II, Agilent, Santa Clara, CA).

Heating Method

For in-tube heating, 1 mL blueberry extract was pipetted into a tube and 100 µL 37% HCl was added. The vial was purged with N_2, capped, vortexed for 5 seconds and baked at 100°C for 30 min. After cooling, its contents were filtered into an autosampler vial. Each analysis was replicated 5 times.

For reflux heating, 25 mL of extract was poured into a round-bottom flask and 2.5 mL 37% HCl was added. The flask was purged with N_2, fitted with a condenser, placed in a water bath at 100°C and refluxed for 30 min. After cooling, 1 mL was filtered into an autosampler vial. Five replicates were analyzed.

Nitrogen Purging

In order to determine whether or not tubes should be purged before hydrolysis, 1 mL blueberry juice and 100 µL 37% HCl were placed in each of 10 tubes. Five tubes were purged with N_2. This was repeated with direct liquid extract. All tubes were capped, vortexed and heated at 95°C for 30 minutes. The contents of each tube were filtered into autosampler vials.

Acid Comparison

To study the effects of acids with lower acid dissociation constants, 1 mL blueberry direct liquid extract was placed into each of 100 tubes. Each tube also received 100 µL of one of 4 acids: 37% HCl, 88% formic, 85% phosphoric and 100% acetic. All tubes were purged with N_2, capped, vortexed and heated at 95°C. Five tubes for each acid were removed at each of 5 time points: 20, 40, 60, 90 and 120 min. The contents of each tube were filtered into autosampler vials.

Response Surface Methodology

Experiments with three independent variables were conducted using a full factorial central composite design [6]. A center point (coded 0), 2 levels (coded + and -) and 2 axial levels (calculated by multiplying the difference between the 0 and + levels by 1.682), coded A and a were chosen. Each combination was repeated twice except the central combination, which was repeated 4 times. This resulted in a total of 32 combinations. The entire experiment was repeated twice. The codes and levels are listed in Table **2**. For each combination, 1 mL direct liquid extract was placed into a tube. Water and 37% HCl were added to produce the acid concentration. The tube was then purged with N_2, capped, vortex mixed and heated. The contents of each tube were filtered and analyzed.

Table 2. Composite Central Design and actual and predicted results for hydrolysis of anthocyaninins from blueberry extract. Area is the sum of areas for all 5 anthocyanidins.

Experiment	Pattern	Temp.	Time	Acid	Measured	Predicted
		°C	min.	M	Total Area	Total Area
1	+++	120	60	3	621,179	583,652
1	++-	120	60	1	127,475	134,706
1	+-+	120	20	3	857,717	873,198
1	+--	120	20	1	249,367	424,252
1	-++	80	60	3	1,162,381	959,480
1	-+-	80	60	1	341,132	227,372
1	--+	80	20	3	1,159,693	915,796
1	---	80	20	1	127,312	183,689
1	000	100	40	2	750,005	701,231
1	00A	100	40	3.7	1,060,562	1,047,023
1	00a	100	40	0.32	75,292	52,686
1	0A0	100	74	2	516,119	596,739
1	0a0	100	6.4	2	790,250	804,493
1	A00	134	40	2	420,084	327,492

(Table 2) contd.....

Experiment	Pattern	Temp.	Time	Acid	Measured	Predicted
		°C	min.	M	Total Area	Total Area
1	a00	66	40	2	354,381	442,467
2	+++	120	60	3	555,793	583,652
2	++-	120	60	1	165,965	134,706
2	+-+	120	20	3	965,528	873,198
2	+--	120	20	1	424,648	424,252
2	-++	80	60	3	877,115	959,480
2	-+-	80	60	1	256,990	227,372
2	--+	80	20	3	868,097	915,796
2	---	80	20	1	176,446	183,689
2	000	100	40	2	707,169	701,231
2	00A	100	40	3.7	837,036	1,047,023
2	00a	100	40	0.32	113,409	52,686
2	0A0	100	74	2	520,581	596,739
2	0a0	100	6.4	2	816,442	804,493
2	A00	134	40	2	312,790	327,492
2	a00	66	40	2	337,595	442,467

The areas of each anthocyanidin were summed for each treatment. After the optimum combination of conditions was determined, 5 repeated samples of blueberry extract were run at those conditions to compare actual and predicted recovery.

Statistical Methods

Analysis of variance was performed on each experiment described in sections 2.4 through 2.8 with PROC MIXED. Multiple mean comparisons using least square differences with Tukey's adjustment were accomplished with Enterprise Guide v. 5.1 (SAS Inc. Cary, NC, USA). Mean comparisons are noted on figures ($\alpha \leq 0.05$).

Response Surface Analysis was performed using JMP 11.0 Statistical software (SAS, Cary, NC, USA). Analysis was performed on two replications of the experiment. Then the means for each treatment combination were calculated and the analysis was performed on the means for each experiment with experiment included as block replication effect.

RESULTS AND DISCUSSION

Extraction

Freeze drying followed by extraction resulted in significantly higher recoveries for all anthocyanidins (except malvidin) than extracting the homogenized whole berries in solvent (Fig. **1**). Barnes *et al*. [3] reported that precision suffers when using direct liquid extraction which they attributed to the small sample size. We observed good reproducibility with RSDs of 2% or less. Bulk sample homogenization is necessary because extracting one or two individual berries would undoubtedly decrease precision due to berry to berry variation. Direct liquid extraction is less time consuming and less costly than freeze drying followed by extraction, but results in decreased recoveries (ranging from 0.5 to 11%, (Fig. **1**).

Heating Method

The recoveries of all anthocyanidins were significantly higher (ranging from 7 to 41%) when hydrolysis was carried out in sealed vials rather than by reflux (Fig. **2**). Relative standard deviations ranged from 3 to 6% for refluxing and were less than 2% for in-vial hydrolysis. Since flasks and reflux condensers must be washed between analyses, performing the hydrolysis in vials is less costly and time consuming then refluxing. Refluxing also requires a larger sample volume compared with performing the hydrolysis in a sealed vial.

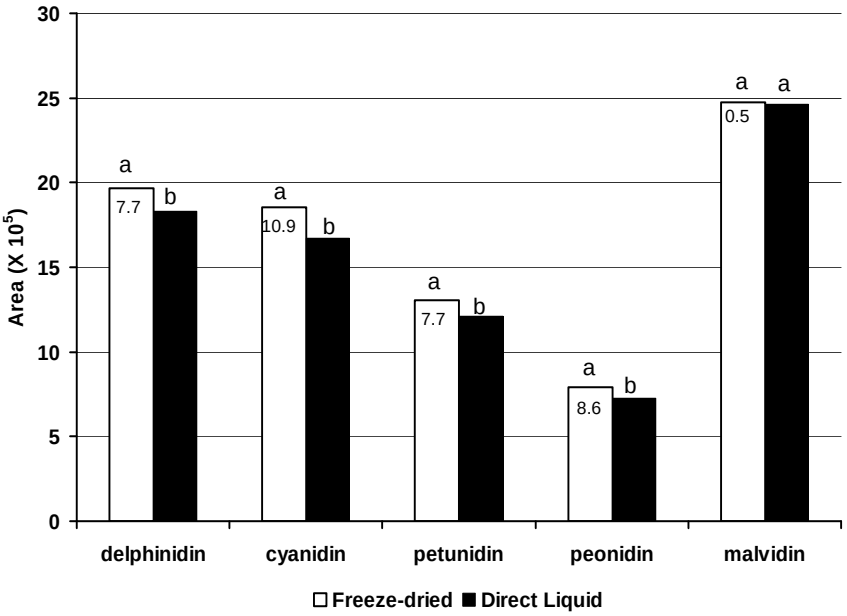

Fig. (1). Concentration of anthocyanidins in freeze-dried extracted and direct liquid extracted blueberries. Means across an anthocyanidin with the same letter are not statistically different at the 5% probability level. The numbers indicate the percent difference between the 2 extraction procedures for each anthocyanidin.

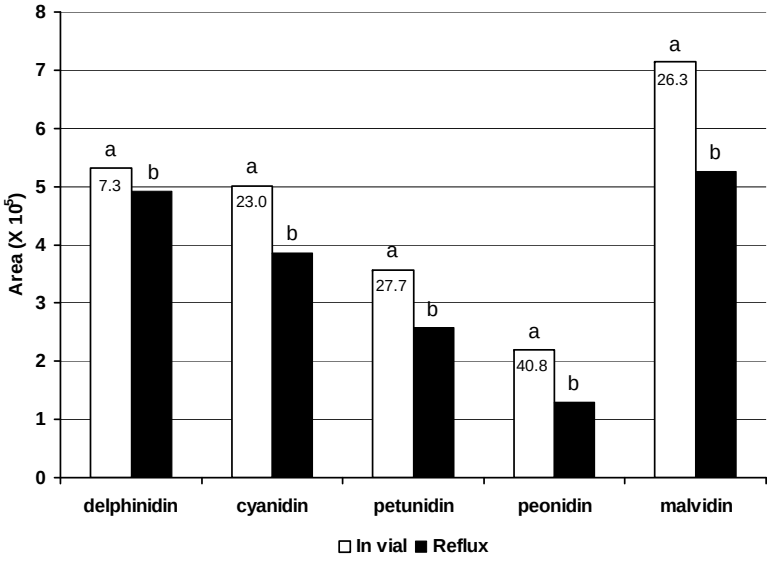

Fig. (2). Recoveries of anthocyanidins from blueberry juice hydrolyzed in sealed vials or by reflux. Means across an anthocyanidin with the same letter are not statistically different at the 5% probability level. The numbers indicate the percent difference between the 2 hydrolyzation procedures for each anthocyanidin.

Nitrogen Purging

Recoveries of all anthocyanidins from hydrolyzed extracts were slightly and significantly higher when the vials were purged with nitrogen (Fig. **3**). However, when juice was hydrolyzed, only malvidin showed a significant increase in recovery when purged (data not shown). Nitrogen displaces oxygen from the vials, preventing losses of anthocyanidins due to oxidation. We routinely purged all vials with nitrogen.

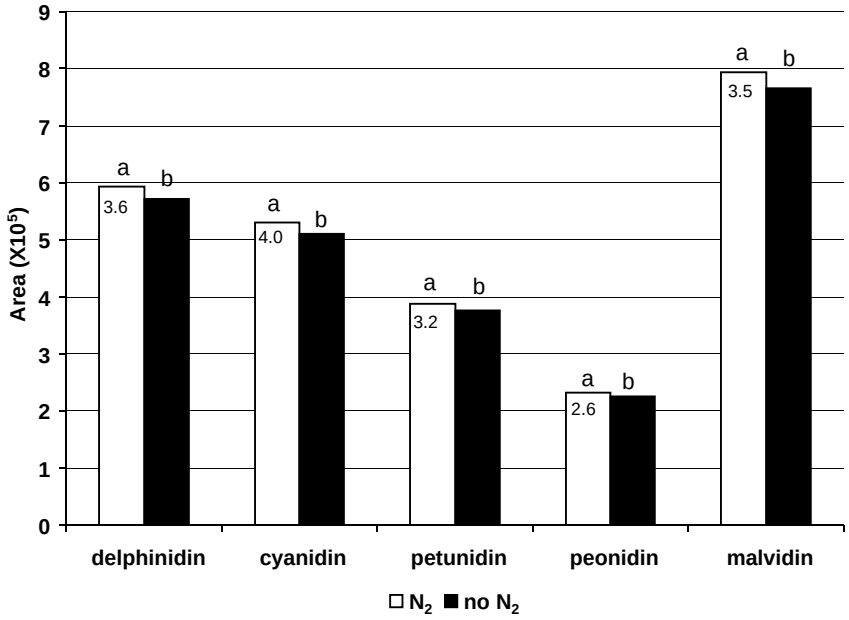

Fig. (3). Recoveries of anthocyanidins from direct extracted blueberries hydrolyzed with and without nitrogen purging. Means within an anthocyanidin with the same letter are not statistically different at the 5% probability level. The numbers indicate the percent difference between the 2 procedures for each anthocyanidin.

Acid Comparison

Juice hydrolyzed with hydrochloric acid for 20 minutes yielded significantly higher anthocyanidin recoveries than the other three acids (Fig. **4**). Hydrolysis was incomplete with the weaker acids even with longer incubation times (Fig. **5**) displaying data for malvidin). Of the acids analyzed, 37% HCl resulted in the highest yields at all time points. Extended exposure to acids (especially HCl) seems to degrade anthocyanidins.

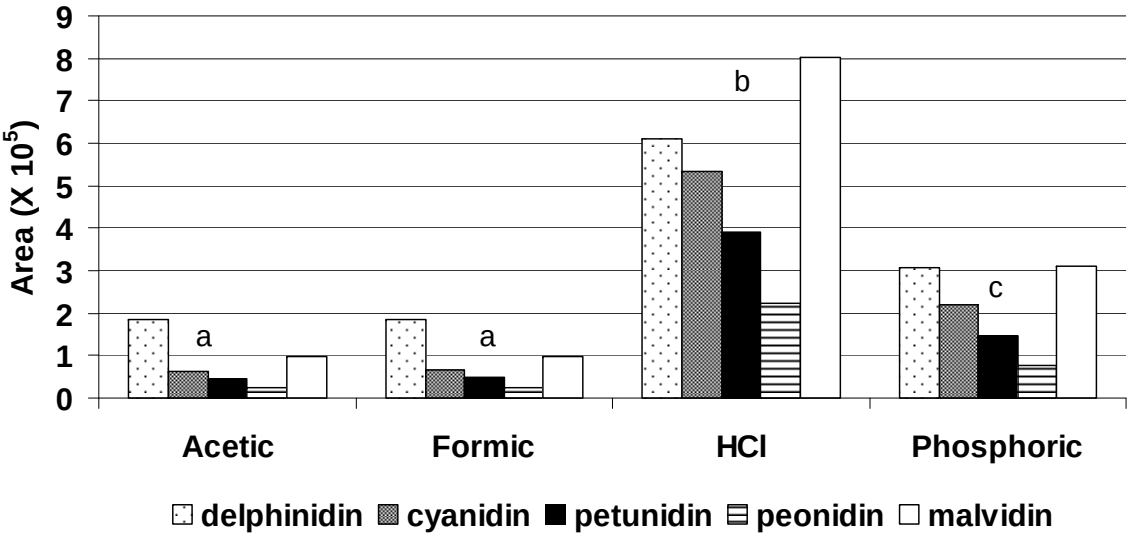

Fig. (4). Recoveries of anthocyanidins from blueberry juice hydrolyzed with 4 acids. Means across the acids for all anthocyanidins with different letters are statistically different at the 5% probability level.

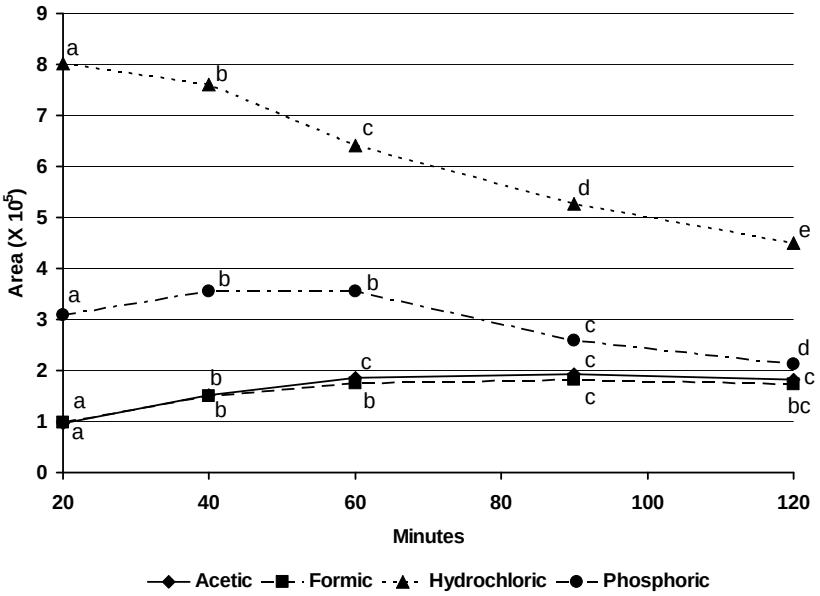

Fig. (5). Recovery of malvidin from blueberry juice hydrolyzed with 4 acids for times ranging from 20 to 120 minutes. Means across an acid with the same letter are not statistically different at the 5% probability level. Note that acetic and formic generated almost identical recoveries.

Response Surface Methodology

The acid concentrations, hydrolysis times and temperatures used in the central composite design, along with the observed and predicted values from blueberry extracts are listed in Table **2**. Statistics associated with the analysis of variance of the RSM model are listed in Table **3**. The model was significant ($\alpha<0.01$) and the lack of fit was not significant ($\alpha = 0.197$), showing that this model is valid for blueberry extract. Fig. (**6**) shows a good relationship between the experimental and predicted values, indicating a good fit of this model with an $R^2 = 0.93$. Two equations were used to calculate the predicted values in Table **2**. The variables were coded using equation 1.

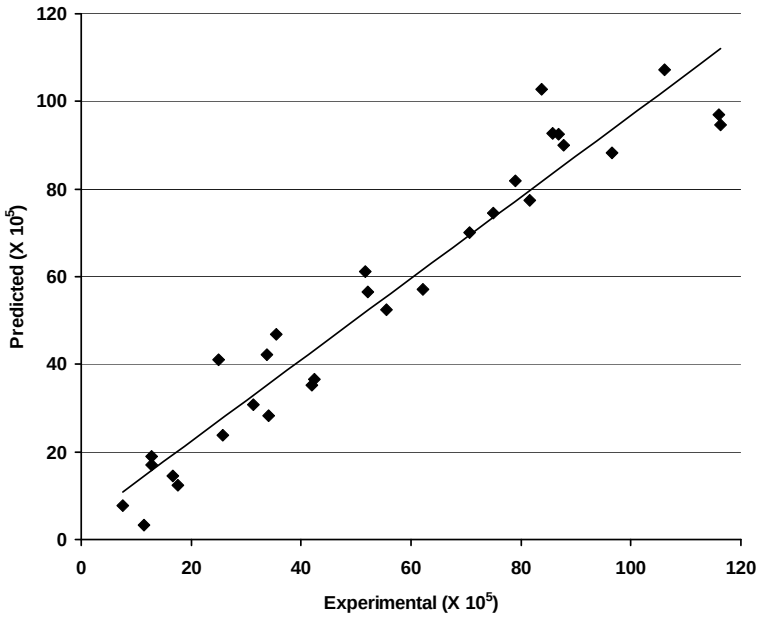

Fig. (6). Experimental values plotted against the predicted values of peak area of total anthocyanididns. $R^2 = 0.93$.

Table 3. Analysis of variance for response surface quadratic model for the sum of peak areas for all 5 anthocyanidins.

Source	DF	Sum of Squares	F Ratio	Prob>F
Model	8	2.95E+12	35.6959	<0.0001[a]
Temp	1	3.44E+10	2.91	0.1021
Time	1	1.04E+11	8.7533	0.0073[a]
Acid	1	2.37E+12	200.4893	<0.0001[a]
Temp*Time	1	1.11E+11	9.3811	0.0057[a]
Temp*Acid	1	8.02E+10	6.7738	0.0162[a]
Temp*Temp	1	2.46E+11	20.7415	0.0002[a]
Acid*Acid	1	5.85E+10	4.9461	0.0367[a]
Lack of fit	6	1.16E+11	1.6693	0.1967
Pure error	15	1.45E+11		
Total error	21	2.60E+11		
Total	29	3.22E+12		

[a] Indicates significance at the 5% probability level.

$$X_i = (x_i - X_0)/\Delta X_i \quad \textbf{(1)}$$

X_i is the coded value of the variable i, x_i is the real value of the independent variable, X_0 is the value of x_i at the center point and ΔX_i is the difference between the center point and the maximum value. The coded variables are X_{te} (temperature), X_{ti} (time), and X_a (acid).

The predicted value of the sum of anthocyanidin peak areas (Y) is calculated using equation 2.

$$Y = 703563 - 60106\,(X_{te}) - 103877\,(X_{ti}) + 497168\,(X_a) - 239342\,(X_{te}X_{ti}) - 203380\,(X_{te}X_a) - 316251\,(X_{te}X_{te}) - 154324\,(X_aX_a). \quad \textbf{(2)}$$

As can be seen in Table **3**, temperature alone was not significant. The combinations temperature*time, temperature*acid and temperature*temperature, however, were all significant. Acid concentration had a greater impact on hydrolysis than time or temperature. This can be seen graphically in the response surface plots shown in Fig. (**7**), demonstrating the effects of different variables on the sum of anthocyanidin peak areas. Part a shows the combined effect of time and temperature, part b the effect of acid and temperature and part c shows the effect of acid and time. In each case, the third variable is held constant.

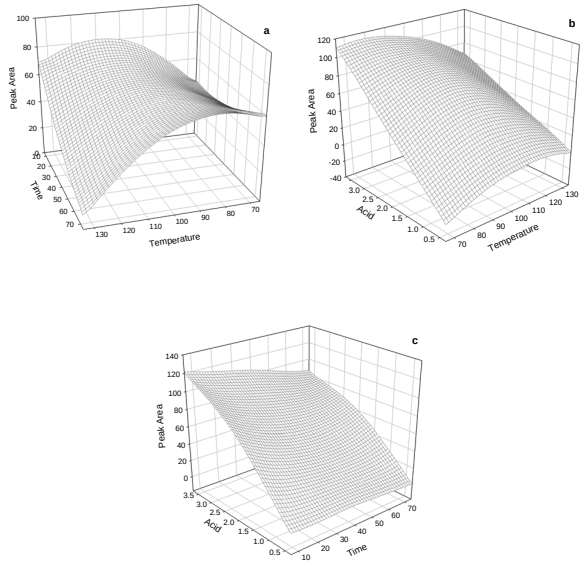

Fig. (7). Response surface plots on the sum of peak area of anthocyanidins in blueberry exrtracts as affected by extraction temperature, extraction time and acid concentration: **(a)** Time and temperature at constant acid concentration; **(b)** Acid concentration and temperature and constant time; **(c)** Acid concentration and time at constant temperature.

The optimum combination of factors needed to achieve maximum conversion of anthocyanins to anthocyanidins is

3.7 N HCl at 99°C for 6.4 minutes. These values were calculated by maximizing the prediction profiler in JMP-Fit Least Squares solutions. Analysis of blueberry extract under these conditions yielded an average total area of 2.58 x 10^6 with an RSD of 0.35% (n = 5). The predicted total peak area at these conditions is 1.15 x 10^6. At 95%, the lower and upper confidence intervals are 1.04x10^6 and 1.46x10^6, respectively.

CONCLUSION

The data presented here indicate that recoveries of anthocyanidins following hydrolysis of anthocyanins in blueberry juice and extracts can be maximized by using the following procedures: Freeze-dry homogenized fruit and extract with methanol:water:TFA (70:30:1 v:v:v), place 1 mL extract or juice in a test tube and add 440 µL 37% HCl, purge the tube with N_2, seal with a PTFE lined cap, vortex, then heat at 99°C for 6.4 minutes. Filter the hydrolysate into an autosampler vial and analyze by UPLC immediately.

Anthocyanins occur in many different fruits, vegetables and plants, all of which differ in matrix, water content and anthocyanin concentrations. The results reported here for blueberries are not applicable to all matrices. For example, Pinho *et al*. [6] found that the optimum combination of factors that provided the maximum sum of peak areas from refluxed red wine extracts were 46.6 minutes at 166.2°C in 2.87 N HCl. This temperature was of the heating block rather than the sample itself. Since the wine:methanol (1:1 v:v) mixture was boiling, its temperature must have been slightly lower than 100°C. As can be seen in 1, many other researchers used a wide variety of conditions for the analysis of other fruits and vegetables. Only Pinho *et al*. [6] used RSM to optimize conditions, and only Burdulis *et al*. [10] and Merken *et al*. [5] reported data showing how they optimized the hydrolysis conditions. RSM is a useful and efficient technique when faced with factors which interact with each other. We recommend that RSM should be used to determine hydrolysis conditions for each matrix analyzed.

LIST OF ABBREVIATIONS

BEH	=	Ethylene bridged hybrid
Cya	=	Cyanidin
Del	=	Delphinidin
HCl	=	Hydrochloric acid
HPLC	=	High pressure liquid chromatography
Mal	=	Malvidin
Pel	=	Pelargonidin
Peo	=	Peonidin
Pet	=	Petunidin
PTFE	=	Polytetrafluoroethylene
TFA	=	Triflouroacetic
TUV	=	Tunable ultra-violet
UPLC	=	Ultra performance liquid chromatograph

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] Basu A, Rhone M, Lyons TJ. Berries: emerging impact on cardiovascular health. Nutr Rev 2010; 68(3): 168-77. [http://dx.doi.org/10.1111/j.1753-4887.2010.00273.x] [PMID: 20384847]

[2] Welch CR, Wu Q, Simon JE. Recent advances in anthocyanin analysis and characterization. Curr Anal Chem 2008; 4(2): 75-101. [http://dx.doi.org/10.2174/157341108784587795] [PMID: 19946465]

[3] Barnes JS, Nguyen HP, Shen S, Schug KA. General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography-electrospray ionization-ion trap-time of flight-mass spectrometry. J Chromatogr A 2009; 1216(23): 4728-35. [http://dx.doi.org/10.1016/j.chroma.2009.04.032] [PMID: 19414178]

[4] Wu X, Prior RL. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. J Agric Food Chem 2005; 53(7): 2589-99.
[http://dx.doi.org/10.1021/jf048068b] [PMID: 15796599]

[5] Merken HM, Merken CD, Beecher GR. Kinetics method for the quantitation of anthocyanidins, flavonols, and flavones in foods. J Agric Food Chem 2001; 49(6): 2727-32.
[http://dx.doi.org/10.1021/jf001266s] [PMID: 11409958]

[6] Pinho C, Melo A, Mansilha C, Ferreira IM. Optimization of conditions for anthocyanin hydrolysis from red wine using response surface methodology (RSM). J Agric Food Chem 2011; 59(1): 50-5.
[http://dx.doi.org/10.1021/jf103839j] [PMID: 21158431]

[7] Hynes M, Aubin A. Acquity UPLC for the rapid analysis of anthocyanidins in berries. Milford, MA: Waters Corporation 2007; p. 6.

[8] Burdulis D, Ivanauskas L, Dirse V, Kazlauskas S, Razukas A. Study of diversity of anthocyanin composition in bilberry (*Vaccinium myrtillus* L.) fruits. Medicina (Kaunas) 2007; 43(12): 971-7.
[PMID: 18182842]

[9] Garcia-Viguera C, Zafrilla P, Tomás-Barberán FA. The use of acetone as an extraction solvent for anthocyanins from strawberry fruit. Phytochem Anal 1998; 9: 274-7.
[http://dx.doi.org/10.1002/(SICI)1099-1565(199811/12)9:6<274::AID-PCA416>3.0.CO;2-G]

[10] Burdulis D, Janulis V, Milašius A, Jakštas V, Ivanauska L. Method development for determination of anthocyanidin content in bilberry (*Vaccinium myrtillus* L) fruits. J Liquid Chromatogr Relat Technol 2008; 31: 850-64.
[http://dx.doi.org/10.1080/10826070801893433]

[11] Harnly JM, Doherty RF, Beecher GR, *et al.* Flavonoid content of U.S. fruits, vegetables, and nuts. J Agric Food Chem 2006; 54(26): 9966-77.
[http://dx.doi.org/10.1021/jf061478a] [PMID: 17177529]

[12] Franke AA, Custer LJ, Arakaki C, Murphy SP. Vitamin C and flavonoid levels of fruits and vegetables consumed in Hawaii. J Food Compos Anal 2004; 17: 1-35.
[http://dx.doi.org/10.1016/S0889-1575(03)00066-8]

[13] Hertog MG, Hollman PC, Venema DP. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. J Agric Food Chem 1992; 40: 1591-8.
[http://dx.doi.org/10.1021/jf00021a023]

[14] Queiroz F, Oliveira C, Pinho O, Ferreira IM. Degradation of anthocyanins and anthocyanidins in blueberry jams/stuffed fish. J Agric Food Chem 2009; 57(22): 10712-7.
[http://dx.doi.org/10.1021/jf9021948] [PMID: 19845348]

[15] Chun OK, Kim D-O, Moon HY, Kang HG, Lee CY. Contribution of individual polyphenolics to total antioxidant capacity of plums. J Agric Food Chem 2003; 51(25): 7240-5.
[http://dx.doi.org/10.1021/jf0343579] [PMID: 14640564]

[16] Kosar M, Kafkas E, Paydas S, Baser KH. Phenolic composition of strawberry genotypes at different maturation stages. J Agric Food Chem 2004; 52(6): 1586-9.
[http://dx.doi.org/10.1021/jf035093t] [PMID: 15030215]

[17] Takeoka GR, Dao LT, Full GH, *et al.* Characterization of black bean (*Phaseolus vulgaris* L.) anthocyanins. J Agric Food Chem 1997; 45: 3395-400.
[http://dx.doi.org/10.1021/jf970264d]

[18] Watson DJ, Bushway AA, Bushway RJ. Separation of peonidin and cyanidin, two anthocyanidins, in cranberries by capillary electrophoresis. J Liquid Chromatogr Relat Technol 2004; 27: 113-21.
[http://dx.doi.org/10.1081/JLC-120027089]

[19] Uddin AF, Hashimoto F, Miwa T, Ohbo K, Sakata Y. Seasonal variation in pigmentation and anthocyanidin phenetics in commercial Eustoma flowers. Sci Hortic (Amsterdam) 2004; 100: 103-15.
[http://dx.doi.org/10.1016/j.scienta.2003.07.002]

[20] Lee J, Durst RW, Wrolstad RE. Impact of juice processing on blueberry anthocyanins and polyphenolics: Comparison of two pretreatments. J Food Sci 2002; 67: 1660-7.
[http://dx.doi.org/10.1111/j.1365-2621.2002.tb08701.x]

[21] Nyman NA, Kumpulainen JT. Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography. J Agric Food Chem 2001; 49(9): 4183-7.
[http://dx.doi.org/10.1021/jf010572i] [PMID: 11559107]

[22] Zhang Z, Kou X, Fugal K, McLaughlin J. Comparison of HPLC methods for determination of anthocyanins and anthocyanidins in bilberry extracts. J Agric Food Chem 2004; 52(4): 688-91.
[http://dx.doi.org/10.1021/jf034596w] [PMID: 14969517]

[23] Wilkinson M, Sweeny JG, Iacobucci GA. High-pressure liquid chromatography of anthocyanidins. J Chromatogr A 1977; 132: 349-51.
[http://dx.doi.org/10.1016/S0021-9673(00)89311-5]

[24] Gao L, Mazza G. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. J Food Sci 1994; 59: 1057-9.
[http://dx.doi.org/10.1111/j.1365-2621.1994.tb08189.x]

[25] Wang X, Tong H, Chen F, Gangemi JD. Chemical characterization and antioxidant evaluation of muscadine grape pomace extract. Food Chem 2010; 123: 1156-62.
[http://dx.doi.org/10.1016/j.foodchem.2010.05.080]

[26] Hong V, Wrolstad RE. Cranberry juice composition. J Assoc Off Anal Chem 1986; 69: 199-207.

[27] Rodriguez-Mateos A, Cifuentes-Gomez T, Tabatabaee S, Lecras C, Spencer JP. Procyanidin, anthocyanin, and chlorogenic acid contents of highbush and lowbush blueberries. J Agric Food Chem 2012; 60(23): 5772-8.
[http://dx.doi.org/10.1021/jf203812w] [PMID: 22175691]

[28] Nielsen AH, Olsen CE, Møller BL. Flavonoids in flowers of 16 *Kalanchoë blossfeldiana* varieties. Phytochemistry 2005; 66(24): 2829-35.
[http://dx.doi.org/10.1016/j.phytochem.2005.09.041] [PMID: 16297414]

[29] Gao L, Mazza G. Rapid method for complete chemical characterization of simple and acylated anthocyanins by high-performance liquid chromatography and capillary gas-liquid chromatography. J Agric Food Chem 1994; 42: 118-25.
[http://dx.doi.org/10.1021/jf00037a020]

[30] Fan-Chiang H-J, Wrolstad RE. Anthocyanin pigment composition of blackberries. J Food Sci 2005; 70: C198-202.
[http://dx.doi.org/10.1111/j.1365-2621.2005.tb07125.x]