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# Subcritical water and carbonated water extraction of anthocyanins from grape pomace

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## ABSTRACT

Grape pomace, a by-product of juice and wine processing, is a rich source of anthocyanins, antioxidant compounds that may afford protection against cancer and coronary heart disease. Unfortunately, traditional extraction of these antioxidants involves use of organic solvents, which pose serious safety and disposal problems for industry. Clearly a need exists for "green" extraction technologies—such as use of subcritical water—that eliminate or reduce the amount of organic solvents. In this study, we determined the efficacy of subcritical and carbonated water in extraction of anthocyanins from red grape pomace. Extraction variables including particle size, pomace mass, and temperature were optimized, and results were compared with those obtained using a traditional solvent-extraction method. According to the total anthocyanin assay, optimum conditions for extraction consisted of the smaller particle size (400 μm) and temperature of 100°C. Under these conditions, subcritical water and carbonated water extracted about 70% of anthocyanins obtained using the traditional organic solvent method. The highest antioxidant-capacity value measured by the ORAC assay was obtained at 140°C, suggesting that Maillard browning products were produced when grape pomace was exposed to increasing temperatures. Subcritical water appears to be a promising, environmentally benign technology to recover health-promoting compounds from grape-processing waste.

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[†] L.R. Howard, faculty mentor, is a professor in the Department of Food Science.

## MEET THE STUDENT-AUTHOR

I graduated from Joplin High School in 2004 and enrolled at the University of Arkansas in the fall as a food science and Spanish major. I plan to complete my B.S. in food science and my B.A. in Spanish in May 2008.

During my time here at the University of Arkansas, I have been awarded an Honors College Fellowship, Honors College Undergraduate Research Grant, and two Honors College Study Abroad grants. I have attended the University of Valencia in Valencia, Spain, and the Copenhagen Business School in Copenhagen, Denmark. As a junior, I began working in Dr. Howard's functional foods laboratory because of my interest in nutritional science and antioxidants. Shortly thereafter, I began my own project focusing on the subcritical-water extraction of anthocyanins from grape pomace.

I have thoroughly enjoyed my time at the University of Arkansas Food Science Department, and I plan to pursue my graduate studies here. The University of Arkansas recently awarded me a Distinguished Doctoral Fellowship that will enable me to begin my Ph.D. in fall 2008. I hope to eventually become a food science professor at a university.

*Lydia Rice*

## INTRODUCTION

Dark-pigmented red grapes are a rich source of anthocyanins, antioxidant compounds with an array of health benefits (Bravo 1998; Hou 2003). These antioxidants have been shown to exhibit anticarcinogenic (Bomser 1996), antimutagenic (Gasiorowski et al., 1997), anti-inflammatory, and antioxidative properties (Wang et al., 1999). Moreover, anthocyanins can promote better eyesight (Timberlake et al., 1988), protect against declines in age-related brain function (Joseph et al., 1999), and prevent lipid oxidation that can lead to clogged arteries (Acquaviva et al., 2002; Folts 1998).

When grapes are used for wine-making, only 30% of these beneficial antioxidants are extracted, while a large amount remains in the pomace (Mazza 1995). Therefore, there is much interest in recovering phenolics from pomace waste material for utilization in value-added products such as nutraceutical ingredients or natural food colorants (Barbagallo et al. 2003). Such products would satisfy the growing consumer demand for natural foods that promote general health. Natural anthocyanin-rich extracts isolated from grape pomace are an excellent candidate to fulfill this demand.

Anthocyanins are water-soluble compounds that give fruits, vegetables, and flowers their blue, purple, red, and orange colors. Anthocyanins are flavonoids, a type of phenolic compound that fall within the class of secondary plant metabolites. Phenolics are grouped by the presence of one or more aromatic rings and one or more hydroxyl groups (Cacace and Mazza, 2007). There are six main types of anthocyanins including cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin. The predominant anthocyanin in wine grapes is malvidin-3-glucoside (Passamonti et al., 2003).

Consumer demand often drives technological advances in industry in that while consumers want antioxidant-rich products, they also want products to be processed in an environmentally friendly manner. Traditional antioxidant extractions have been performed with organic solvents. These solvents work on cells near the plant surface where anthocyanins are located. Commonly used solvents include aqueous mixtures of methanol, ethanol, and acetone, which are typically acid-

ified to improve anthocyanin stability. A comparative study of methanol, ethanol, and water acidified with various organic acids or HCl showed that methanol was the most effective solvent for extraction of anthocyanins from grape pomace followed by ethanol and water (Ju and Howard, 2003; Metivier et al., 1980). Organic acids and low concentrations of mineral acids improve the efficacy of organic solvents through the denaturization of cellular membranes of anthocyanin-containing cells. However, the amount of acid added must be well-controlled to prevent hydrolysis of sugar residues and acyl groups during subsequent concentration procedures (Jackman and Smith, 1996).

Research has shown that elevated extraction temperatures can improve solubility of analytes in solvents and speed diffusion rates (Ju and Howard, 2003). However, anthocyanins can degrade when exposed to elevated extraction temperatures. Thus, anthocyanin extraction is typically done at temperatures ranging from 20 to 50°C because temperatures greater than 70°C rapidly degrade anthocyanins and the expression of their pigments. Time and temperature determine rate of anthocyanin degradation. Therefore, extraction conditions consisting of high temperatures and short times are most successful in slowing anthocyanin degradation in fruits. Besides temperature, factors such as oxygen presence, metals, sugars, and light have been shown to affect stability of anthocyanins (Jackman and Smith, 1996).

Traditional organic solvents are expensive and potentially toxic, and thus present serious waste-disposal issues for industry (Ju and Howard, 2005). Therefore, industry retains an invested interest in subcritical-water extractions, where manipulations in temperature (>100°C) and pressure (>10 mPa) can be used to provide an environmentally sound and effective extraction procedure (Bakker et al., 1998). This method has been shown to be effective in extraction of biologically active compounds from a wide range of biological species such as antioxidants from rosemary and yams, essential oil from oregano, lignans from flaxseed, and ginsenosides from American ginseng (Cacace and Mazza, 2007). The basic concept of this method is that under the right conditions, an increase in the pressure of water can maintain water in its liquid state even after its boiling point is surpassed. The chemical properties of water are lowered in such a way that its dissociation constant, surface tension, viscosity, and polarity are closer to those of organic solvents than those of water at ambient -pressure conditions and temperature (Cacace and Mazza, 2007). Indeed, the presence of pressure enables a fast and efficient extraction of heat-sensitive compounds such as anthocyanins (Ju and Howard, 2005) that are normally degraded when held at high temperatures (>50°C) for extended periods of time (Jackman and Smith, 1996).

In order to confirm efficacy of subcritical water in extraction of anthocyanins from grape pomace, many extraction variables need to be tested and optimized. In this study, effects of pomace particle size and mass as well as solvent type and temperature on the extraction of antioxidant-rich anthocyanins from red grape pomace were studied.

## MATERIALS AND METHODS

Red grape pomace (variety Sunbelt) obtained from the University of Arkansas wine-processing laboratory was lyophilized and ground in a Wiley mill (Thomas Scientific, Swedesboro, N.J., USA) to two particle sizes (ca 400 and 840 μm) by passing the material through 40- and 20-mesh screens. Powdered samples were stored in sealed brown vials at –20°C prior to extraction.

*Subcritical water extraction of anthocyanins.* A Dionex accelerated solvent extractor (ASE) Model 200 (Dionex Corp., Sunnyvale, Calif., USA) outfitted with a solvent controller was used for the study. Variables tested included mass of grape pomace (0.25, 0.5, and 1 g) and particle size (400 and 840 μm). Each sample was mixed with 29 g of sea sand and then placed into a 22 mL extraction cell containing a cellulose paper filter at the bottom of the cell. After addition of solvent, the extraction cell was pressurized and then heated. The ASE system was operated at zero extraction time and one extraction cycle. Approximately 5 to 7 min were needed to heat the sample/solvent from ambient temperature to the desired temperature, and then a short 40-sec static extraction was performed. Next, the cell was rinsed with 15.4 mL of fresh extraction solvent and purged from the extraction cell with a flow of nitrogen for 90 sec. To determine effects of extraction temperature on the recovery of anthocyanins, temperatures of 100, 110, 120, 130, and 140°C were tested using the basic ASE conditions described above, using Milli-Q-grade water (pH~7.3) and Milli-Q-grade carbonated water (sparged with $CO_2$ gas for fifteen min prior to use, pH ~3.6.) To prevent anthocyanin oxidation during extraction, solvents were sparged with nitrogen for two h prior to use. Samples were rapidly cooled following ASE extraction, adjusted to 50 mL with water, and stored at -20°C until analysis. Preliminary experiments of five extraction cycles showed that over 90% of anthocyanins were extracted within the first two extraction cycles and over 85% of anthocyanins were extracted within the first extraction cycle. All extractions were performed in triplicate.

*Conventional solvent extraction of anthocyanins.* For comparison with water and carbonated-water extractions, grape pomace samples (400 μm particle size) were

extracted with a solution consisting of methanol/water/formic acid at a ratio of 60:37:3 (v/v.) Samples (0.5 g) were homogenized for 1 min in 20 mL of extraction solvent, and then homogenates were filtered through Miracloth (CalBiochem, LaJolla, Calif., USA.) Filtrates were collected and centrifuged for 10 min at 2739 x *g*. Following centrifugation, supernatants of extracts were collected and adjusted to 50 mL with water. Extracts were stored at -20°C until analysis. All extractions were performed in triplicate.

*Determination of total anthocyanins*. Total anthocyanins were measured using the pH differential assay described by Giusti and Wrolstad (2001) using a Hewlett Packard 8425A photodiode array spectrophotometer (Palo Alto, CA, USA). The spectrophotometer was zeroed with distilled water at 510 and 700 nm. Two prepared dilutions, one with sodium acetate buffer at pH 4.5 and one with potassium chloride buffer at pH 1.0, were allowed to equilibrate for 15 min prior to the measurement of absorbance of each diluted sample consisting of 0.5 mL of sample and 4.5 mL of each buffer at 510 and 700 nm. Absorbance of the diluted sample was calculated by $A= (A_{510}-A_{700})_{pH\ 1.0}-(A_{510}-A_{700})_{pH\ 4.5}$ and monomeric pigment concentration in the original sample was calculated using the following formula: Monomeric anthocyanin pigment (mg/liter)=(A x molecular weight x dilution factor x 1000)/ (Molar absorptivity x 1). Molecular weight and molar absorptivity of malvidin-3-glucoside (m3g) were 492 and 28000, respectively. Results were expressed as mg of m3g equivalents per $kg^{-1}$ dry weight.

*Antioxidant capacity evaluation*. Oxygen radical absorbing capacity ($ORAC_{FL}$) of extracts was measured using a FluoStar Optima microplate reader (Biomedical Solutions, Inc., Stafford, Texas, USA) using the method of Prior et al. (2003). Extracts from the first ASE extraction cycle were used for this assay. Grape pomace extracts were diluted 200-fold with phosphate buffer (75mM, pH 7) prior to ORAC analysis. The phosphate buffer prepared by combining 0.75M $K_2HP_{O4}$ and 0.75M $NaH_2PO_4$ in a ratio of 61.6:38.9 (v/v) was diluted 1:9 (v/v) with DI water. A stock solution of fluorescein was prepared by dissolving 0.0225 g fluorescein (Sigma-Aldrich, St. Louis, Mo., USA) in 50 mL of 0.075M phosphate buffer. A second stock solution was made by diluting 50 µL of previously made stock solution with 10 mL phosphate buffer. A 1 mM Trolox (Sigma-Aldrich, St. Louis, Mo., USA) solution was prepared with phosphate buffer and stored at –20°C. The Trolox working solution was prepared by dilution of the Trolox stock solution to a final concentration of 50 µM with phosphate buffer. The Trolox working solution was diluted in phosphate buffer to obtain standard concentrations of 6.25, 12.50, 25.00, and 50.00 µM. Thereafter, 40 µL of diluted samples, trolox standards, and blanks (phosphate buffer) were manually pipetted into appropriate wells on 48-well microplates. A 320 mM solution of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; Wako Chemicals USA, Inc., Richmond, Va., USA) was prepared immediately prior to running the assay. Fluorescein solution was diluted (320 µL into 20 mL phosphate buffer) for the working solution. The FLUOstar Optima instrument equipped with two automated injectors was then programmed to add 400 µL of fluorescein followed by 150 µL of AAPH to each well. Fluorescence readings (excitation 485 nm, emission 520 nm) were recorded after addition of fluorescein, after the addition of AAPH, and every 192 sec thereafter for 112 min to reach 95% loss of fluorescence. Final fluorescence measurements were expressed relative to initial readings. Results were calculated based upon differences in areas under the fluorescein decay curves between blanks, samples, and standards. The standard curve was obtained by plotting four concentrations of trolox equivalents (TE) against net area under the curve (AUC) of each standard. Final ORAC values were calculated using the regression equation between TE concentration and AUC and were expressed as µmol TE per g dry weight.

*Statistical analysis*. Effects of pomace mass, particle size, and extraction temperature on the total anthocyanin content and antioxidant capacity of water, carbonated water, and conventional solvent extracts were analyzed by analysis of variance (ANOVA) using JMP® software (SAD Inst. Inc., Cary, N.C., USA). The Pearson correlation test was used to determine the correlation among total anthocyanins and ORAC values in water and carbonated-water extracts.

## RESULTS AND DISCUSSION

Total anthocyanins (Fig. 1) generally decreased as extraction temperature increased. Subcritical water extracted the highest level of total anthocyanins over the 100 to 110°C temperature band, while subcritical carbonated water extracted the highest level at 100°C. At the optimal extraction temperature band of 100 to 110°C, water extracted 70% of the anthocyanins compared to the conventional methanol extraction method (1254.4±20.8 mg/kg DW), while carbonated water extracted 71% of anthocyanins compared to conventional methanol extraction.

The smaller particle size allowed for greater extraction of anthocyanins when solvent temperature was 100°C-130°C. At 140°C, there was no significant differ-

ence between the small and large particle size (Fig. 2). Maximum extraction of anthocyanins occurred at 100°C for the smaller particle size, while larger particle size achieved comparable extractions across the 100-110°C temperature band. Smaller particle size coupled with an extraction temperature of 100°C extracted 80% of anthocyanins compared to conventional methanol extraction. Presumably, smaller particle size allowed for greater contact with the solvent, which facilitated extraction of anthocyanins.

Carbonated water was more effective than water in extraction of anthocyanins for the small particle size (Fig. 3). However, when large particle size was used, both solvents achieved comparable extraction.

Use of the small particle size resulted in an approximate 64% extraction of anthocyanins compared to the conventional methanol extraction method for all sample masses tested (Fig. 4). However, extracts obtained with the larger particle size had higher amounts of anthocyanins with decreasing sample mass. Small particle size offered enough surface area to achieve the best extraction regardless of sample mass. The larger particle size with less surface area benefited from increased mass transfer obtained by a decrease in sample mass.

ORAC is an assay that determines total antioxidant capacity of samples, not solely the antioxidant capacity of anthocyanins. Total antioxidant capacity of subcritical-water and carbonated-water extracts increased as temperature increased (Fig. 5). According to statistical analysis, there was a significant inverse correlation between ORAC and Total Anthocyanins ($r_{xy}$=-0.29). This was most likely due to formation of antioxidant-rich Maillard reaction products when grape pomace was exposed to increasing temperatures. Compounds with antioxidant properties are formed during development of the Maillard reaction (Nicoli et al., 1997), and an increase in antioxidant capacity of foods typically occurs when they are heated at elevated temperatures due to the formation of brown melanoidin pigments formed during advanced stages of the Maillard reaction (Anese et al., 1999).

The general trend for particle size and temperature is that antioxidant capacity increased with increasing temperature for the large particle size. For the small particle size, there was no significant difference among temperature treatments (Fig. 6). Extracts obtained using smaller particle size had higher antioxidant capacities than extracts obtained using larger particle size. As with total anthocyanin results, increased surface area due to smaller particle size achieved greater antioxidant capacity than the large particle size.

Extracts obtained from samples with masses of 0.5 g and 0.25 g had similar ORAC values, while extracts obtained using a sample mass of 1.0 g had greater ORAC values at elevated extraction temperatures. This data group reinforces the trend that the greatest antioxidant capacity occurred at 140°C (Fig. 7).

Extracts obtained using small particle size had the highest ORAC value with a sample mass of 0.25 g, but antioxidant capacity decreased with increased sample mass (Fig. 8). Highest antioxidant capacity obtained with a sample mass of 0.25 g was 86% of the value obtained using conventional methanol extraction method (1191.3±18.1 μmol TE/g DW). Conversely, extracts obtained using large particle size showed a trend of increasing antioxidant capacity with increase in sample mass. Highest antioxidant capacity obtained using a sample mass of 1.0 g was 75% of the value obtained using conventional methanol extraction.

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## LITERATURE CITED

Acquaviva, R., F. Bonina, A. Russo, A. Campisi, F. Galvano, N. Ragusa, and A. Vanella. 2002. Red orange extract: result on DNA cleavage. J. Food Sci. 67:2814-2818.

Anese, M., L. Manzacco., M.C. Nicoli, and C.R. Lerici. 1999. Antioxidant properties of tomato juice as affected by heating. J. Sci. Food Agric. 79:750-754.

Bakker, J., P. Bridle, S.J. Bellworthy, C. Garcia-Viguera, H.P. Reader, and S.J. Watkins. 1998. Effect of Sulphur dioxide and must extraction on color, phenolic composition and sensory quality of table wine. J. Sci. Food Agric. 78:297-307.

Barbagallo, R., M. Durante, P. Pifferi, G. Spagna, and A. Todaro. 2003. A method for anthocyanin extraction from fresh grape skin. Ital. J. Food Sci. 15:337-345.

Bomser, J. 1996. In vitro anticancer activity of fruit extracts from *Vaccinium* species. Planta Med. 62: 212-216.

Bravo, L. 1998. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutr. Rev. 56:317-333.

Cacace, J.E. and G. Mazza. Pressurized low polarity water extraction of biologically active compounds from plant products. In Functional Food Ingredients and Nutraceuticals: Processing Technologies, J. Shi, Ed., Taylor and Francis, Boca Raton, Fla., 2007, pp. 135-155.

Folts, J. 1998. Antithrombotic potential of grape juice and red wine for preventing heart attacks. Pharmaceutical Biol. 36:21-28.

Gasiorowski, K., B. Szyba, B. Brokos, B. Kolaczynska, M. Jankowiak-Wlodarczyk, and J. Oszmianski. 1997. Antimutagenic activity of anthocyanins isolated from *Aronia melanocarpa* fruits. Cancer Lett. 119:37-46.

Giusti, M.M. and R.E. Wrolstad. 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In Current Protocols in Food Analytical Chemistry 1st ed., R.E. Wrolstad, T.E. Acree and H. An. (eds.) John Wiley & Sons, Hoboken, N.J., pp. F12.1-F1.2.13.

Hou, D. 2003. Potential mechanisms of cancer chemoprevention by anthocyanins. Curr. Molec. Med. 3:149-159.

Jackman R.L. and J.L. Smith. 1996. Anthocyanins and betalains. In Natural Food Colorants,Second Edition. G.A.F. Henry and J.D. Houghton (eds). Blacklie Academic and Professional, Glasgow, U.K.pp. 244-309.

Joseph, J.A., B. Shukitt-Hale, N.A. Denisova, D. Bielinski, A. Martin, J.J. McEwen, and P.C. Bickford. 1999. Reversals of age-related declines in neuronal signal transduction, cognitive and motor behavorial deficits with blueberry, spinach or strawberry dietary supplementation. J. Neurosci. 19:8114-8121.

Ju, Z.Y. and L.R. Howard. 2003. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. J. Agric. Food Chem. 51:5207-5213.

Ju, Z.Y. and L.R. Howard. 2005. Subcritical water and sulfured water extraction of anthocyanins and other phenolics from dried red grape skin. J. Food Sci. 70:270-276.

Mazza, G. 1995. Anthocyanins in grape and grape products. Crit. Rev. Food Sci. Nutr. 35:341-371.

Metivier, R.P., F.J. Francis, F.M. Clydesdale. 1980. Solvent extraction of anthocyanins from wine pomace. J. Food Sci. 45:1099-1100.

Nicoli, M.C., M. Anese, M.T. Parpinel., S. Franceschi, and C.R. Lerici. 1997. Loss and/or formation of antioxidants during food processing and storage. Cancer Lett 114:71-74.

Passamonti, S., U. Vrhovsek, A. Vanzo, and F. Mattivi. 2003. The stomach as a site for anthocyanins absorption from food. FEBS Lett. 544:210-214.

Prior R.L., H. Hoang, L. Gu, X. Wu, M. Bacchiocca, L. Howard, M. Hampsch-Woodill, D. Huang, B. Ou, and R. Jacob. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. J. Agric. Food Chem. 51:3273-3279.

Timberlake, C.F. and B.S. Henry. 1988. Anthocyanins as natural food colorants. Prog. Clin. Biol. Res. 280:107-121.

Wang, H. M.G. Nair, G.M. Strasberg, Y.C. Chang, A.M. Booren, J.I. Gray, and D.L.Dewitt. 1999. Antioxidant and anti-inflammatory activities of anthocyanins and their aglycone cyanidin from tart cherries. J. Nat. Prod. 62:294-296.

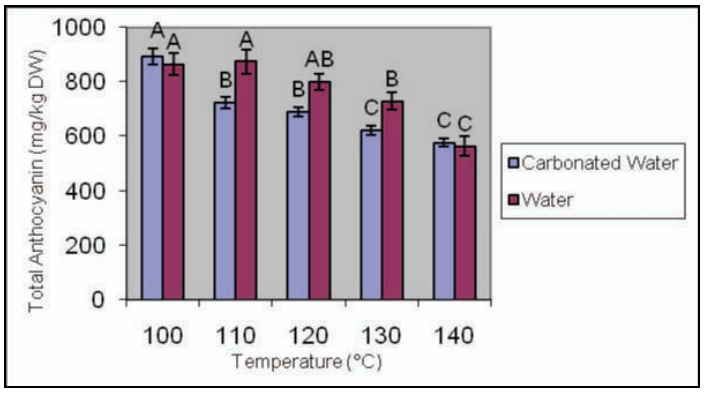


**Fig. 1.** Effects of temperature and solvent on total anthocyanins. Bars represent ± standard error of the mean (n=3). Bars with similar letters within each solvent are not significantly different (P>0.05).

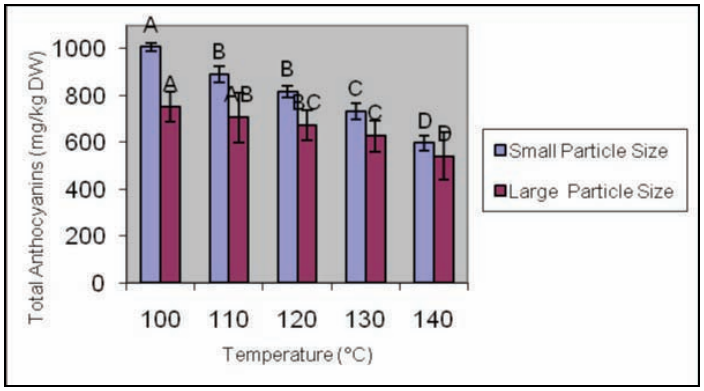


**Fig. 2.** Effects of temperature and particle size on total anthocyanins. Bars represent ± standard error of the mean (n=3). Bars with similar letters within each particle size are not significantly different (P>0.05).

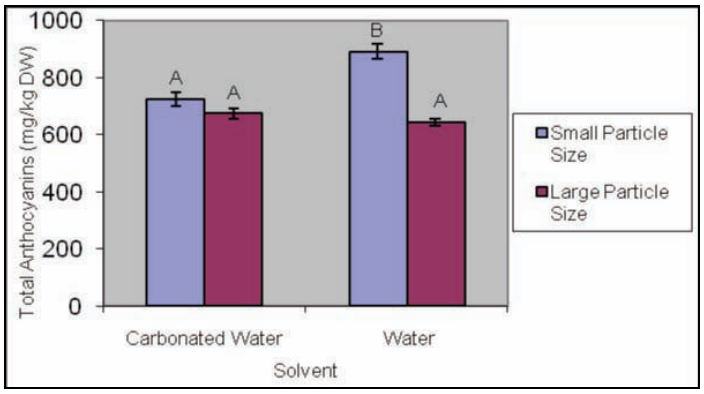


**Fig. 3.** Effects of particle size and solvent on total anthocyanins. Bars represent ± standard error of the mean (n=3). Bars with similar letters within each solvent are not significantly different (P>0.05).

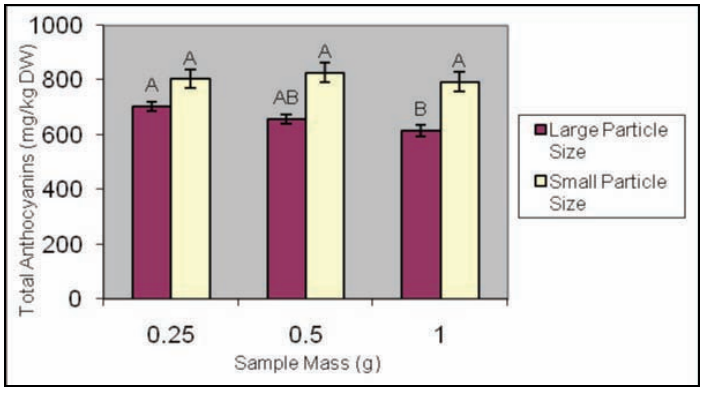


**Fig. 4.** Effects of particle size and sample mass on total anthocyanins. Bars represent ± standard error of the mean (n=3). Bars with similar letters within particle size are not significantly different (P>0.05).

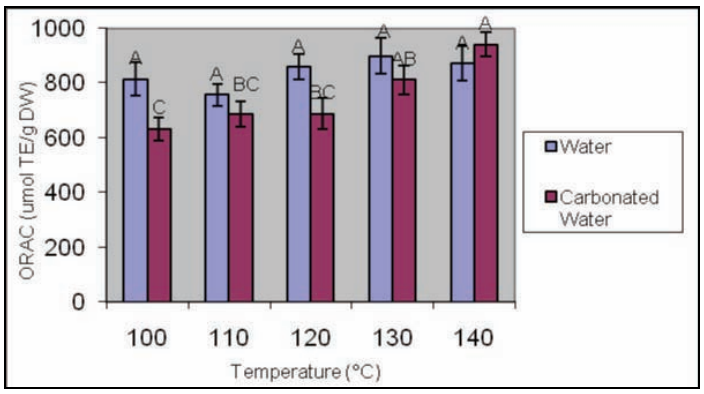


**Fig. 5.** Effects of temperature and solvent on ORAC. Bars ± standard error of the mean (n=3). Bars with similar letters within each solvent are not significantly different (P>0.05).

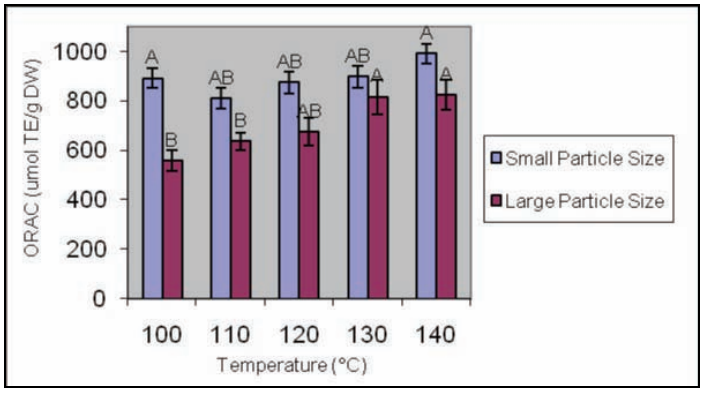


**Fig. 6.** Effects of temperature and particle size on ORAC. Bars represent ± standard error of the mean (n=3). Bars with similar letters within each particle size are not significantly different (P>0.05).

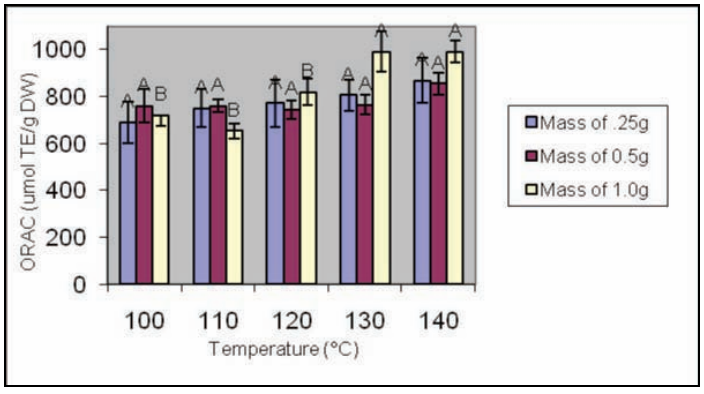


**Fig. 7.** Effects of temperature and sample mass on ORAC. Bars represent ± standard error of the mean (n=3). Bars with similar letters within each sample mass are not significantly different (P>0.05).

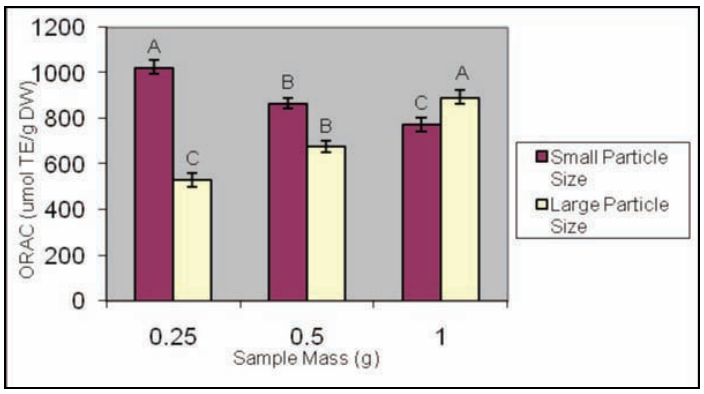


**Fig. 8.** Effects of particle size and sample mass on ORAC. Bars represent ± standard error of the mean (n=3). Bars with similar letters within each particle size are not significantly different (P>0.05).