Subcritical Water Extraction of Anthocyanins from Fruit Berry Substrates

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ABSTRACT

The extraction of anthocyanin-based pigments from fruit berries and grapes is normally accomplished using ethanol or aqueous-based ethanol solutions, and occasionally ethyl acetate, acetone, or methylene chloride. Although ethanol is classified as a GRAS (Generally-Recognized-As-Safe) solvent, its utilization must be strictly accounted for under legal statues. Subcritical water under modest compression above the boiling point of normal water is an alternative medium to ethanol due to its temperature-dependent dielectric constant and cohesional energy density. In this study, subcritical water under pressure, and held at temperatures between 110-160°C was utilized for the extraction of anthocyanin-based pigments from fruit berries (both wet and dry), such as elderberry, raspberry, bilberry, chokeberry; and their associated stems, skins, and pomaces.

Extraction and screening experiments were executed using a home-built, flow through extraction system in which water and acidified water solutions were fed at a high velocity with the aid of liquid booster pump in an attached Spe-ed unit module. Additional screening experiments were conducted utilizing a Dionex Model 300 ASE (Accelerated Solvent Extraction) instrument in which the default values for normal operation of the ASE were changed via the microprocessor controller to facilitate extractions under the above conditions. Samples of various fruit berries and their by-products (pomaces) were placed in the extraction cell and the oven heated to temperatures between 120-160'C. Both deionized and Milli-Q-purified neat water as well as acidified water (0.01% HCl, pH ~ 2.3) were fed typically at a rate of 24 mL/min against a constant pressure of 40 bar (580 psi). Similarly, rapid extractions were conducted on the ASE system using both pure water, water-ethanol mixtures, and acidified water. Extractions were monitored visually, spectrophotometrically, and via HPLC analysis. The efficiency of the sub-H_2O extractions were compared to results obtained using a 70% ethanolic extract.

Results from the flow extractor experiments indicate that the volume of subcritical water required to carry out an equivalent anthocyanin extraction to that obtained with ethanol is less, and particularly so if only 90% of the available anthocyanins are extracted. Reductions in solvent use range between two- and four-fold, resulting in a more concentrated extract and

accelerated extractions. These results are partially due to the high superficial fluid velocity (~ 0.1 cm/sec) through the extractor bed. ASE results indicate extracts of higher tinctorial strength occur when acidified water is used, and when the extraction process is optimized at 120°C where pigment degradation is minimized.

A multi-fold interpretation of the above results is afforded by looking at both the thermodynamic and mass transfer factors which impact on subcritical water-based extractions. Calculations using the empirical method of Clifford for estimating solute (anthocyanin) mole fractions in subcritical water confirm increased solubilization of the anthocyanin moieties, as do trends in the solvent dielectric constant and solubility parameter. The dielectric constant for subcritical water at temperatures over 100°C is less than 50, approaching values typically associated with polar organic solvents at ambient temperature. Likewise the loss of tertiary structure in water at the above temperatures, and its associated lower solubility parameter, support the trends in reduction of its dielectric constant. Solute diffusivities are estimated to be less than 10^{-10} m^2/s and allow calculation of the rate of extraction.

In summary subcritical water extraction is a highly efficient method for recovering anthocyanins from berry substrates and compliments both mechanical expression and SC-CO_2 extraction for juice and oil recovery from fruit berries. The derived extracts appear equivalent or better than those obtained via ethanol-based extractions with respect to their composition, nutritional value, and antioxidant activity. In addition, the use of water above its normal boiling point facilitates in-situ sterilization of the extract, similar to that experienced using thermal retorting. A patent covering the above process has recently been filed.

INTRODUCTION

Natural foods and dietary supplements accounted for $13.9 billion of an approximately $480 billion in U.S. foods sales in 1998. This is an increase of 23% from the previous year. An estimated 16 million Americans consume dietary supplements in the belief these will improve overall health and prevent disease. Many supplements are on the market with little regulation or scientific evaluation due to the Dietary Supplement Health and Education Act of 1994. Many supplements derived from plant sources contain mixtures of phytochemicals that have not been quantified or even identified. Other segments of the dietary supplement industry are also isolating polyphenolic compounds from fruits and vegetables.

Polyphenolic extracts of cranberry, elderberry, bilberry, blueberry, grape and soy can be purchased commercially. Prices for polyphenolic extracts cover a wide range depending on the concentration of active components, some costing into the hundreds of dollars per kilogram. Novasoy™ (isoflavones) concentrated from ethanolic washes of soybeans [1] and Grape Max™ (procyanidins, catechins and anthocyanidin), isolated and concentrated using ethanol and ethyl acetate, are but two commercially-available products whose compositions are somewhat known. As an example of the complexity of these extracts, Table 1 shows the polyphenolic composition of some fruit berry extracts that are currently on the market.

Isolation of polyphenolic mixtures can be expensive. Large quantities of raw materials, which generally contain very small amounts of active compounds, are required to isolate a limited

amount of material. The anthocyanin (ANC) contents in chokeberries were found at levels from 3.1-6.3 mg/g (dry wt.) with the levels varying between variety and geographical location [2]. Blueberry varieties have been reported to have highly variable levels of ANCs ranging from 0.83-3.7 mg/g (dry wt.) [3]. Ellagic acid levels in different varieties of strawberries ranged from 0.83-4.64 mg/g (dry wt.) [4]. Other components such as procyanidins and flavanols are found at similarly low levels in fruits depending on growing conditions and cultivars [5]. Most of the harvested fruits go into processing juice products (whole or partial). Therefore, the most readily available source of these compounds comes from pomace (spent skins, stems and seeds) after the juicing process, which accounts for a large percentage of the mass processed.

With the availability of these nutrient-rich by-products (pomaces), an effective means of removing at least a portion of these compounds is warranted. With prices at $50-200/ kg or higher for fruit extracts, their potential commercial value is in the millions of dollars. Subcritical water extraction (SWE) offers a potential cheap, efficient and consumer-friendly means to isolate these valuable polyphenolic nutrients. Polyphenolics and more specifically ANCs are frequently extracted with ethanol or aqueous ethanolic solvents, and this must be done with care due to light-, heat-, and air-sensitivity of ANCs. Extraction using SWE is largely dependent on altering the extraction temperature of the fluid above its normal boiling point under pressure, thereby changing the dielectric constant of water and the solvation power of the fluid [6]. The use of SWE for the extraction of natural products have been documented for kava-kava [7], rosemary [8], and savory/peppermint [9], St. Johns wort [10], and has been nicely summarized recently by Clifford and Hawthorne [11]. As noted by King [12], SWE complements SFE using SC-CO_2 for the "green" processing of nutraceutical ingredients. In this study, we extend the applicability of SWE to the removal of polyphenolics from fruit berries and their residual by-products.

MATERIALS AND METHODS

The experimental apparatus used to conduct the SWEs is shown in Fig. 1. It consists of a modified Applied Separations Inc. (Allentown, PA) Spe-ed pumping unit feeding water from a reservoir into an extraction vessel (cell) contained in a thermo-regulated oven (Model 3710A, ATS, Inc., Butler, PA). The extraction cell was a 316 SS, 1" o.d., 9/16"i.d., approximately 55 mL in volume. As shown in Fig. 1, the water is pumped through an equilibration coil contained in the oven to bring it into its subcritical state at temperatures above its normal boiling point under pressure, and then passed through the extraction cell before exiting the oven into a cooling bath reservoir (Model 801, Polyscience, Inc., Nile, IL). Back pressure was maintained on the system with the aid of a micro-metering valve which also allowed adjustment of the water flow rate. Aqueous extracts were collected after exiting the micro-metering valve. The first thermocouple in Fig. 1 was connected to the temperature controller (Part No. CN4800, Omega Engineering, Stanford, CT) which regulated the oven temperature, while the other thermocouples were connected to a digital meter to obtain an accurate reading of the water temperature, both before and after the extraction cell.

Samples of various fruit berries and their by-products (pomaces) were placed in the extraction cell and the oven heated to temperatures between 110-160^{o}C. Both de-ionized and Milli-Q-purified neat water as well as acidified water (0.01% HCl, pH ~ 2.3) were fed at a rate of 24 mL/min at a constant pressure of 4.0 MPa. This pressure was well in excess of that

required to prevent the formation of steam within the extraction cell. Incremental samples were obtained every 60-80g of aqueous solution expelled from the extractor over a 40 min time interval, however extracts were not taken until the cell was at the desired extraction temperature and pressure. Extract color was monitored visually to an approximate equivalent of 20 ppm of cyanin-3-glucoside (a specific model ANC). Extract samples were analyzed by the HPLC procedure described by Skrede et al. [13]. The efficiency of the SWE extraction was compared to results obtained using a 70% ethanolic extract. The control sample was extracted with 70% ethanol in water for 40 min using sonication and washed with excess ethanol to remove any remaining color from the berry substrate. Because of the extreme sensitivity of ANCs to light, heat, and oxygen; all samples were immediately prepared after extraction for injection into the HPLC as described above.

Fig. 2 shows the ASE Model 300 system (Dionex Corp., Sunnyvale, CA) that was used for rapidly ascertaining the effect of SWE on elderberry pomace, using both pure degassed water, acidified water, and water/ethanol mixtures. The extraction temperature was varied between 120 – 160^{o}C. All total, 13 different extraction conditions were utilized to allow the optimization of the SWE. Although the ASE (Accelerated Solvent Extraction) unit employs a N_2 back pressure to prevent boiling of the extraction solvent, this is very similar to the effect of using a back pressure regulating device on the home-built, continuous flow extractor described previously. Utilization of the ASE system permits a "combinatorial" experimental approach to study SWE of the polyphenolics from berry-derived substrates. This permitted after statistical analysis, choice of the extraction protocol that yielded the highest ANC concentration/gram of extract, for testing on the larger scale continuous flow SWE system.

RESULTS AND DISCUSSION

Table 2 presents the results using SWE on the home-built extraction apparatus for dried elderberries, dried elderberry seeds and stems, and a black raspberry pomace. Each of these substrates were extracted under the conditions described in the Experimental section with ethanol, subcritical water, and with subcritical water - at times and conditions adequate to extract approximately 90% of the available ANCs as determined against ethanolic extraction. yields. Yields in terms of mg ANC/g – substrate on both a "as is" and dry basis are tabulated for each type of extraction and substrate in Table 2. Based on the dry weight figures, the % ANC extracted relative to ethanolic extraction was calculated and presented in Column 5 in Table 2. In most cases, irrespective of substrate, 90% or greater yields were obtained for the ANCs using SWE. SWE ANC yields were slightly greater than 100% for the dried elderberry extractions however this may be due to either obtaining a better extraction of ANCs with SWE, or perhaps experimental error. Somewhat inferior results were obtained for SWE in the case for wet black raspberry pomace; but even in this case an 80% ANC yield was obtained via SWE.

The concentration of ANC in :g/g – solvent are given in Column 6, and are interesting. In every case in Table 2, SWE gave an equivalent or better result than that obtained with ethanol extraction. Evaluating the :g ANC/g – solvent for an approximately 90% removal of ANCs from the respective matrices relative to that obtained using ethanolic extraction, indicates that in most cases, results in an extract having a greater concentration of ANC, and consequently having a higher tinctorial strength. This is due to the 2 – 4 fold difference in the solvent/substrate ratio (Column 7 – Table 2) which exists between the 90%SWE water

extracts and the more exhaustive SWE or ethanol-based extractions. This indicates that a minimal quantity of residual ANC is extracted from the substrate as the SWE is extended for too long and supports truncating the extraction at the 90% ANC yield point. Only in the case of the black raspberry results, did we fail to extract over 90% of the available ANC.

The recoveries of ANCs at an extraction temperature of 120^{o}C might seem somewhat surprising considering their inherent thermal instability, however calculations of the superficial velocity of subcritical water through the extraction cell (~0.1 cm/sec) indicate rapid longitudinal transport of the ANCs out of the extraction cell. This factor coupled with the rapid mass transport of the ANCs from the substrate using subcritical water facilitates a very fast and effective extraction process. An additional benefit of the "hot" water extraction process is the potential in-situ sterilization of resultant product, which has the potential of avoiding thermal retorting of the final product.

The results of the ASE-based extractions for elderberry pomace can be seen Fig. 3. Elderberry pomace because of it inherently high moisture content (65% versus 7.4 – 9.3% for the other moist elderberry substrates) was mixed with a diatomaceous earth dispersant to affect the ASE extractions. Fig. 3a shows the ASE collection vials in the sequence that they were collected for SWEs done at 120, 140, and 160^{o}C using pure water. These may be compared with a similar extraction scheme utilizing acidified water as the extractant as shown in Fig. 3b. It would appear at 120^{o}C, that the acidified water facilitates extraction of additional colored material, but this trend appears to be reversed for extractions conducted at 160^{o}C. This result may be due to some degradation of chromaphoric material under acidified extraction conditions. It was found that increasing the amount of ethanol in water from 10 to 40% for an identical extraction sequence on the elderberry pomace sample seemed to result in a slightly higher color in the fourth collection vial. For the ASE experiments, the acidified water extraction at 120^{o}C yielded the highest quantity of anthocyanins extracted per gram of pomace, 0.724 mg ANC/g – pomace (dry basis). However it was also found that pure water at 120^{o}C resulted in a slightly lower quantity of ANC/g – pomace, and a higher ratio of ANC to overall extracted material (10.65g – ANC/100 g – extract on a dry basis).

Dried extracts contained an ANC content well within the range of what is currently sold in the nutraceutical marketplace. Average percentages of ANCs in the final aqueous extract ranged from 8-10% for the extraction of berry seeds/stems to 2.5-4.5% from the pomaces. Although the tintorial strength of such extracts is high, it would be desirable to further concentrate these extracts for applications in the nutraceutical or functional food areas. This potentially could be accomplished by coupling a membrane process step after SWE. It should be noted that SFE with SC-CO_2 (neat and with cosolvents) has been reported in the literature for extracting both oil and enriched polyphenolic fractions from grapes [14-16]. Such results suggest that by combing sequential extractions using SC-CO_2 and subcritical water, that an array of useful natural product extracts could be obtained, as noted previously by this author [12].

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Table 1. Polyphenolics Present in Common Berries

Group Classification	Specific Compounds
Phenolic acids (hydroxybenzoic and hydroxycinnamic)	
Caffeic acid	Coumaric acid
Chlorogenic acid	Ferulic acid
Cinnamic acid	Gallic acid
Hydrolyzable tannins	
Ellagic acid	
Flavan-3-ols	
(+)-Ccatechin	(-)-epicatechin
Stilbene	
Resveratrol	
Flavonols (aglycons and their glycosides)	
Kaempferol	Quercetin
Myricetin	
Anthocyanins (aglycones, their glycosides and their acylated glycosides)	
Cyanidin-3-glucoside	Peonidin-3-glucoside
Cyanidin-3-galactoside	Peonidin-3-galactoside
Cyanidin-3-arabinoside	Peonidin-3-arabinoside
Cyanidin-3-sambubioside	Petunidin-3-glucoside
Cyanidin-3,5-diglucoside	Petunidin-3-galacotside
Cyanidin-3-sambubioside-5-glucoside	Petunidin-3-arabinoside
Cyanidin-3-xyloside	Malvidin-3-glucoside
Delphinidin-3-glucoside	Malvidin-3-galacotside
Delphinidin-3-galactoside	Malvidin-3-arabinoside
Delphinidin-3-arabinoside	

Table 2. Anthocyanin Extraction Yield Comparison for Four Berry Substrates

Sample	Extraction	mg ANC/g substrate (as is basis)	mg ANC/g substrate (dry basis)	% ANC extracted (of ethanol)	µg ANC/g solvent	Solvent to Substrate Use Ratio
Elderberry Stems (dry)	Ethanol	19.91	21.50	100.0	228	87:1
	SWE	19.26	20.08	93.4	272	71:1
	+90% SWE*	18.52	20.00	93.0	658	28:1
Elderberry Seed (dry)	Ethanol	4.76	5.25	100.0	142	33:1
	SWE	4.34	4.79	91.2	213	21:1
	+90% SWE*	4.17	4.60	87.6	1853	7:1
Dried Elderberry	Ethanol	3.81	4.13	100.0	110	34:1
	SWE	4.42	4.79	116.0	111	40:1
	+90%SWE*	4.08	4.42	107.0	277	15:1
Black Raspberry Pomace (wet)	Ethanol	4.79	13.70	100.0	141	35:1
	SWE	3.85	11.01	80.4	137	28:1
	+90%SWE*	3.50	10.01	73.1	237	15:1

*Reflects 90% of all anthocyanins extracted by the SWE process.

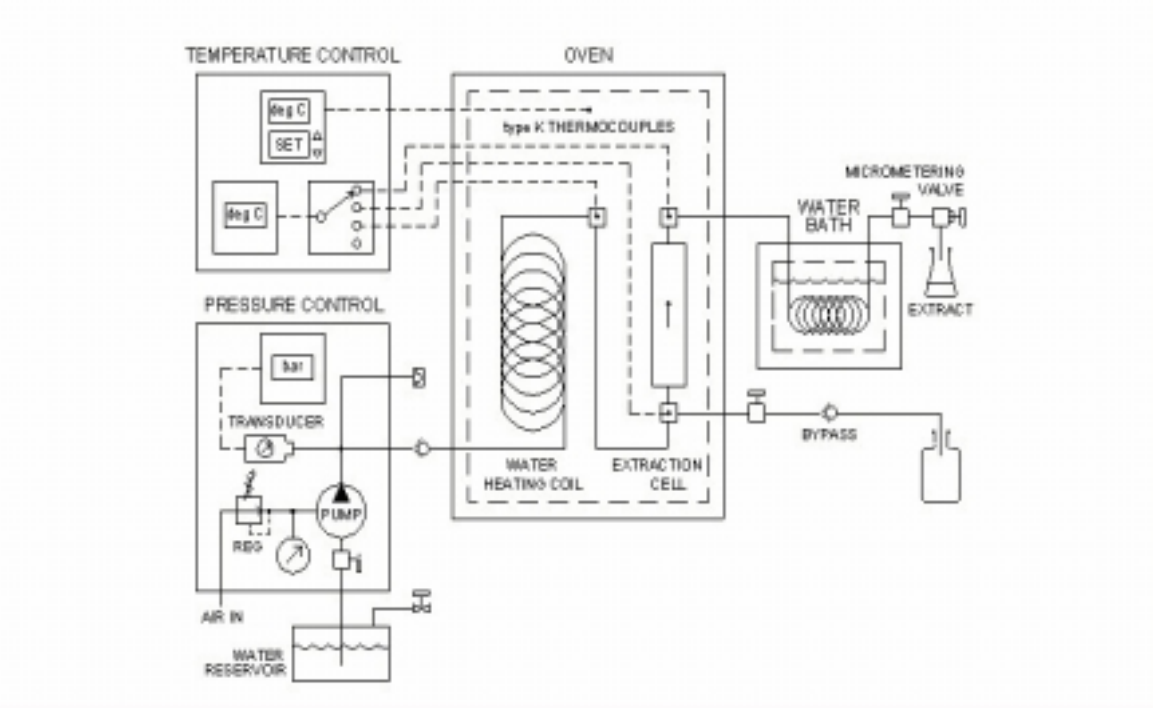

Fig. 1 Subcritical water extraction system for extracting ANCs from fruit berry substrates.

Fig. 2 Accelerated solvent extraction module (Dionex Model 300) used for the SWE of fruit berry substrates.

Fig. 3a SWE of elderberry pomaces using degassed water as a function of temperature.

Fig. 3b SWE of elderberry pomaces using acidified water as a function of temperature.