Ultrasound-Assisted Extraction of Anthocyanins from Haskap Berries 
*(Lonicera caerulea L.)* Using a Deep Eutectic Solvent (DES)

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**SUMMARY**

*Research background.* Haskap berries are one of the richest natural sources of anthocyanins and their extracts can be used for nutraceuticals and functional food ingredients. Deep eutectic solvents (DES) comprised of food-grade or generally recognized as safe (GRAS) components show promise as natural solvents, but have not been applied to haskap berries. Thus, the aim of this study was to investigate the extraction of anthocyanins from haskap berries using a DES of citric acid and d-(+)-maltose.

*Experimental approach.* The experimental approach used ultrasound-assisted extraction with a DES of citric acid and d-(+)-maltose as the solvent to achieve a sustainable green extraction process. Response Surface Methodology (RSM) using a Box-Behnken (BB) experimental design was used to study the effect of varying the extraction temperature/°C, t/min, solvent/sample ratio (V/mL)/(mL/g) and the water content in the DES, φ(water)/% (V/V) on the total anthocyanin content (TAC) in the haskap berry extracts.

*Results and conclusions.* The optimal extraction conditions (75 °C, 10 min, 50.4 mL/g and 90 % water) had a predicted TAC extraction of 21.2 mg/g dry mass, which was experimentally validated with only 7.2 % error. The TAC yield and anthocyanin profiles were similar to those obtained with conventional organic solvents.

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Novelty and scientific contribution. This is the first study investigating the use of a food-grade DES comprised of GRAS components for the extraction of anthocyanins from haskap berries. These results indicate that the DES studied (citric acid and d-(+)-maltose) is a suitable alternative solvent for extracting anthocyanins for food-grade applications.

Key words: anthocyanins, Box-Behnken design, deep eutectic solvent, green extraction, haskap

INTRODUCTION

Haskap berries (*Lonicera caerulea* L.), a fruit native to Siberia and northeastern Asia, have recently entered the North American market (1) and are known for their high anthocyanin content (2). Anthocyanins are flavonoids associated with anti-oxidative, anti-inflammatory, and anti-carcinogenic properties (3), and are highly desirable for their application as dietary supplements and natural colourants (4). Currently, they are extracted from natural sources using traditional solvents, such as methanol and ethanol (5).

The extraction of bioactives from natural sources can be made more environmentally friendly with assisting technologies such as ultrasound to improve extraction efficiency (6), and the development of green solvents. Green solvents are alternatives to organic solvents, and may be non-petroleum derived, biodegradable, with low toxicity (7). Deep eutectic solvents (DES) can be produced by mixing two or more natural components capable of hydrogen bond interactions (8). DES have been used for extraction of several flavonoids, such as asgenistin, genistein and apigenin (9), icariin, catechin, (+)-catechin, quercetin, kaempferol, myricetin, quercetin-O-rhamnoside (10), rutin, α-mangostin, and cryptotanshinone (11). Recently, the extraction of anthocyanins using various DES has been reported for grape skin (12,13), wine lees (14) and *Lycium ruthenicum* Murr. fruit (15).

The objective of this study was to investigate the ultrasound-assisted extraction (UAE) of anthocyanins from haskap berries using a DES made of citric acid and d-(+)-maltose as an alternative, sustainable, food-grade solvent. The extraction parameters studies were: Temperature/°C, t/min, water content in the DES, φ(water)/% (V/V), and solvent to sample ratio, (V/m)/(mL/g). A Box-Behken design (BBD) of experiments and Response Surface Methodology (RSM) were used to maximize anthocyanin extraction with DES, and the anthocyanin profile with DES extract compared with a conventional methanol extract.

MATERIALS AND METHODS

Chemicals

All chemicals used were analytical and High Performance Liquid Chromatography (HPLC) grade, and were purchased from Sigma Aldrich (Oakville, Ontario, Canada). The anthocyanin HPLC
standards included cyanidin-3,5-di-glucoside (C3,5GL), cyanidin-3-glucoside (C3GL), cyanidin-3-galactoside (C3GA), pelargonidin-3-glucoside (PL3GL) cyanidin-3-rutinoside (C3RT), and peonidin-3-O-glucoside (P3GL).

**Plant material**

Frozen haskap berries (*Lonicera caerulea* L.) that were previously harvested and frozen at Northern Light Orchards (Saskatchewan, Canada) were used. Berries were freeze dried in a 4.5 L bench-top freeze-dryer (FreeZone, Labconco, Kansas City, MO, USA) until constant weight, with final moisture content of 4.95 % (fresh mass). Freeze-dried samples were wrapped in aluminium foil and kept in a desiccator at –18°C. Immediately prior to extraction the samples were ground (Smartgrind, Black & Decker, Mississauga, ON, Canada) and sieved through a 0.5 mm (32 mesh) sieve.

**Solvent preparation**

The DES was prepared according to Jeong *et al.* (13), with modifications. Briefly, citric acid and d-(+)-maltose monohydrate were combined at a 4:1 molar ratio in powdered form, and then dissolved in Milli-Q water. The solution was placed on a rotary evaporator (HiTEC RE-51, Yamato Scientific America, Santa Clara, CA, USA) at 4.2 kPa and 50°C for elimination of excess water (16). Prior to extraction, the appropriate volume of DES was diluted with Milli-Q water (Table 1). The solvent was always prepared and used for extraction in the same day.

**Experimental design**

A Box-Behnken design (BBD) for four factors (17) was used to determine levels of the experimental parameters: extraction temperature/°C, t/min, φ(water)/% (V/V), and (V/m)/(mL/g) (Table 1). DES molar ratio was held constant at the optimized 4:1 citric acid:maltose ratio according to Jeong *et al.* (13).

**Ultrasound-assisted extraction (UAE) of anthocyanins**

UAE was performed in an ultrasound water bath (Branson 2510R-DTH, Branson Ultrasonics Corp., Danbury, CT, USA), at 40 kHz and 100 W. Extractions were conducted in 5 mL glass tubes, where the appropriate mass of sample and volume of solvent was added according to the BBD (Table 1). The tubes were covered with aluminium foil, vortexed for 10 seconds, and placed in the ultrasound bath. Following extraction, samples were centrifuged (Sorvall RT1, Thermo Scientific, Madison, WI, USA) at 4°C and 4,000 rpm for 10 min. The supernatant was then filtered through a 0.45 µm syringe filter and immediately used for spectrophotometric analysis (18). For comparison with DES, methanol extraction was conducted using the following optimal parameters based on a similar study a
conventional organic solvent by Celli et al. (18): solvent:sample ratio 25:1 (mL/g), solvent composition of 80 % methanol solution, 0.5 % formic acid, extraction temperature of 35°C for 20 min.

**Determination of total monomeric anthocyanin content (TAC)**

TAC was determined using the pH-differential method (19) conducted in duplicate using a UV-Visible spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, Madison, WI, USA). The TAC of the extract was calculated using Eqs 1 and 2:

\[
A = (A_{1.0-510} - A_{1.0-700}) - (A_{4.5-510} - A_{4.5-700}) \quad /1/
\]

\[
\text{TAC (mg/L)} = (A \cdot MW \cdot DF \cdot 10^3)/\varepsilon \quad /2/
\]

where \(A_{1.0-510}\) and \(A_{1.0-700}\) are the absorbances of the pH 1.0 (KCl) solution and \(A_{4.5-510}\) and \(A_{4.5-700}\) are the absorbances of the pH 4.5 (NaAc) solution each read at 510 and 700 nm, respectively; \(A\) = absorbance value from Eq. 1; \(MW = 449.38\ g/mol;\ DF = \text{dilution factor} = 25;\) and \(\varepsilon = 26,900\ M^{-1}cm^{-1}\) (3). Results are expressed as mg of C3GL equivalents per g dry mass (dm) of haskap berries.

**RSM analysis and optimization**

Minitab® (v. 17.3.1) was used for analysis (20). A polynomial model was fitted to the experimental results followed by backwards elimination to reduce the model to significant factors. The model was then analysed using analysis of variance (ANOVA) with a 0.05 level of significance. RSM was used to obtain optimized extraction conditions, and experiments were run to validate these results.

**Anthocyanin profiling by HPLC**

Both DES and methanol extracts were injected on a Synergi 4 μm Max-RP C12 column, 80 A, 250 × 4.6 mm (Phenomenex, Canada) reversed-phase column at 30°C using diode array detection (DAD) at 520 nm (Agilent 1100 Series, Agilent Technologies, Hewlett-Packard, Waldbronn, Germany) (21). The elution was carried out at 0.8 mL/min flow rate using A) water/methanol (90:10) with 1.0 % formic acid and B) pure methanol with 0.1 % formic acid started at 10 % B, increased to 20 % B in 7 min, then 45 % B in 13 min, up to 70 % B in 5 min, ended to 100 % B in 3 min and held for 3 min, and then went back to 10 % B in 9 min. Chromatograms were acquired using ChemStation A.10.02 software (22). Peaks were identified and quantified by comparing their retention times obtained with those of the authentic standards.
TAC yield using DES varied between 7.75 mg/g and 19.62 mg/g (Table 1). The final reduced model is shown in Eq. 3, and ANOVA results summarized in Table 2. It is evident that the lack-of-fit was not significant (p > 0.05), indicating a good fit to the experimental data.

\[ \text{TAC (mg/g dm)} = -22.12 + 0.638 \cdot X_1 + 0.319 \cdot X_2 + 0.0748 \cdot X_3 + 0.4554 \cdot X_4 - 0.00378 \cdot X_1^2 - 0.00452 \cdot X_4^2 - 0.00605 \cdot X_1 \cdot X_2 \]

Surface plots are shown in Fig. 1. Here, the interactions on TAC response are shown for \( \phi \text{(water)/% (V/V) × (V/m)/(mL/g)} \) (Fig. 1a), \( t/\text{min} \times \phi \text{(water)/% (V/V)} \) (Fig. 1b), Temperature/°C × \( \phi \text{(water)/% (V/V)} \) (Fig. 1c), \( t/\text{min} \times (V/m)/(mL/g) \) (Fig. 1d), Temperature/°C × \( (V/m)/(mL/g) \) (Fig. 1e) and Temperature/°C × \( t/\text{min} \) (Fig. 1f). Fig. 1f shows that high yields are obtained at either a combination of high temperature for short time or at lower temperature for longer extraction time. The optimized extraction conditions are \( X_1 = 75 \text{°C}, X_2 = 10 \text{ min}, X_3 = 90 \% \text{ water in DES and } X_4 = 50.4 \text{ mL/g, with a predicted TAC response of 21.3 mg/g dm. Validation experiments at these conditions resulted in TAC of 19.8 mg/g dm. Although anthocyanin degradation is reported at high temperatures (23), the optimal conditions were at the highest temperature (75°C). Temperature generally contributes to higher extraction yields due to enhanced mass transfer mechanisms (24). In the present study, the chemical environment of the DES system may also have had a positive influence in the thermal stability of the compounds extracted. The main degradation mechanisms related to thermal processing of anthocyanins include oxidation, cleavage of covalent bonds or enhanced oxidation reactions (25), and these processes are influenced by several factors, such as chemical structure of anthocyanins, acylation of the molecule, pH, light, oxygen, temperature, exposure time, presence/activity of enzyme polyphenol oxidase, and presence of other substances (26-28). During thermal processing, these factors may act synergistically, contributing to a higher stability of the bioactive compounds. In the present study, factors such as thermal inactivation of the enzyme polyphenol oxidase and the pH of the DES system may have contributed to higher stability at high temperature. Furthermore, it is possible that the DES could have caused acylation of the anthocyanin molecule, another factor that improves anthocyanin thermal stability (25,28). Bubalo et al. (29) reported higher extraction of anthocyanins in organic acid-based DES compared to DES of lower acidity, achieving optimal UAE yield at 65°C for 50 min. The short extraction time needed to achieve optimal yield (10 min) is a positive aspect of the process, showing that the DES system can easily access the intracellular structure to promote extraction. Furthermore, it could also have contributed to
the application of high temperatures for optimal extraction, avoiding thermal degradation due to longer exposure to high temperature.

(Fig. 1)

The optimal water content in DES (% V/V) was at its maximum of 90 %. To ensure that pure water was not more efficient, extraction was performed under optimal conditions using 100 % water as the solvent, resulting in a TAC of 17.3 mg/g dm, significantly lower than optimal DES yield. The increased water content likely increased extraction by decreasing the viscosity of the pure DES, allowing greater access to the plant intracellular structure (24) and greater mass transfer rates (30). This may also indicate a high polarity of anthocyanins extracted, as more polar anthocyanins are extracted better with DES containing higher water content (16,29). The high water content in the solvent is also a positive economic benefit, as it decreases the overall cost of the solvent.

The use of DES to extract anthocyanins from other plant materials has been reported by other researchers. For example, Jeong et al. (13) evaluated the same DES system used in the present work and found it to be an efficient method for extracting anthocyanins from grape skins, when compared with traditional solvents including water, methanol, 80 % aqueous methanol, ethanol and 70 % aqueous ethanol. Radošević et al. (31) and Bosiljkov et al. (14) both used DES made of a combination of choline chloride with malic acid, and found this DES to be more efficient for extraction of anthocyanins compared with 70 % methanol and acidified ethanol, respectively. Sang et al. (15) used a DES of choline chloride and 1,2-propandiol and found increased anthocyanin extraction compared with acidified methanol. Given these results, DES are an effective alternative to methanol for extracting anthocyanins. Previous work optimizing UAE of TAC from haskap berries using acidified aqueous ethanol (80 %) as extraction solvent was conducted by our research group using the same haskap berries (18), where a TAC yield of 22.73 mg/g dm was obtained at optimized UAE conditions. Comparing this to the TAC of 19.8 mg/g dm from the present study indicates that this DES system is similar to the conventional solvent.

The results from HPLC-DAD analysis of DES and methanol extracts from haskap berries indicate that similar anthocyanin profiles were obtained at 520 nm (Fig. 2). The major component of both extracts was identified as C3GL which was over 80 % of the total anthocyanins, along with other small amounts of C3RT and P3GL. These data are in accordance with the anthocyanin profile of haskap berries that was reported in a previous study (3). The chromatogram in Fig. 2 suggests that DES had the same anthocyanin-extracting capacity from haskap berries compared with the conventional acidified methanol extraction.

(Fig. 2)

These results indicate that this DES system is an effective alternative to organic solvents for sustainable anthocyanin extraction. As citric acid and d-(+)-maltose are considered GRAS, this has
important implications for the application of DES solvents that are GRAS in the food industry. For example, DES extracts comprised of GRAS components could be used to extract bioactive compounds from food processing by-products to increase the sustainability of food processing. Then, the DES extracts could be used as novel functional food ingredients and incorporated directly into food products without post-extraction purification, where the DES components may confer additional benefits to the final product. For example, citric acid is an antioxidant and could add to the antioxidant activity of the bioactives in the extract and the final food product.

CONCLUSIONS

This is the first investigation of anthocyanin extraction from haskap berries using a DES system. Optimized conditions for UAE of TAC using a citric acid/d-(+)-maltose DES were 75°C, 10 min, 50.4 mL/g solvent:sample ratio and 90 % (V/V) water content in DES. The maximum TAC yield was 19.8 mg/g dm, and similar anthocyanin HPLC profiles were obtained for DES and methanol extracts. These results indicate that this DES system is an effective green alternative to organic solvents for sustainable anthocyanin extraction. The prospects for DES extraction in the food industry are promising as they could increase the sustainability of the food industry by utilizing processing by-products and replacing organic solvents with renewable GRAS components. Furthermore, DES extracts using GRAS components could be used as novel functional food ingredients. Future studies should investigate possible chemical interactions between the DES system and the anthocyanins, and the stability of the extracts.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

AUTHORS’ CONTRIBUTION
AM performed the experiments, performed the analysis, drafted the manuscript and designed the figures. YP and GJ provided guidance in the laboratory. MSB supervised the research and obtained funding for the research. AM and MB planned the research experiments. YP, GJ and MSB aided with the interpretation of data and writing the manuscript. YP and MSB edited and revised the manuscript according to the journal's requirements.

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Table 1. Box-Benken experimental design with natural levels of each factor investigated and the total anthocyanin content (TAC) obtained.

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<th>$X_1 = t$/min</th>
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\* Run number is not indicative of experimental run order

\* Data omitted

\b Predicted yield: 21.2 mg C3GL/g dm

**Table 2.** ANOVA results of significant factors in quadratic model

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<td>&lt; 0.0001</td>
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<td>20.278</td>
<td>7.82</td>
<td>0.012</td>
</tr>
<tr>
<td>Temperature/°C . t/min</td>
<td>1</td>
<td>20.278</td>
<td>20.278</td>
<td>7.82</td>
<td>0.012</td>
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<tr>
<td>Error</td>
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<td>Lack-of-Fit</td>
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<td>43.460</td>
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<td>5.796</td>
<td>1.159</td>
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<tr>
<td>Total</td>
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<td>377.255</td>
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</table>

R² = 86.94 %, R² (adj) = 82.13 %, R²(pred) = 69.34 %

\*In the final model, while time as a single factor is not significant (p > 0.05), it is significant in the 2-way interaction and is therefore included.
Fig. 1. Surface plots of TAC extraction
Fig. 2. Anthocyanin profile from HPLC-DAD analysis: A=anthocyanin standards (a=C3,5GL, b=C3GA, c=C3GL, d=C3RT, e=PL3GL, f=P3GL), B=MeOH extract, and C=DES extract.