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ORIGINAL RESEARCH

Extraction of Bioactive Compounds from Haskap Leaves (*Lonicera caerulea*) Using Salt/Ethanol Aqueous Two-Phase Flotation

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Abstract

Aqueous two-phase flotation (ATPF) was used for the extraction and partial purification of bioactive compounds from haskap leaves. Two ATPF systems, ammonium sulfate/ethanol and sodium phosphate/ethanol, were compared. A Box-Behnken approach was used to investigate the operating parameters which included sample loading (5.0, 52.5, and 100.0 mg), flotation time (5, 62.5, 120 min), and air flow rate (11.4, 20.0, 28.6 mL/min). Response surface optimization was performed to maximize the yields of chlorogenic acid (CGA), flavonoids, and total phenolics (total phenolic content, TPC). At the optimized parameters for the ammonium sulfate/ethanol ATPF system (i.e., 5-mg leaves, 120-min flotation time, and air flow rate of 28.6 mL/min), the extraction efficiencies were 97.1%, 98.7%, and 99.6% for CGA, flavonoids, and TPC, respectively. The corresponding partition coefficients were 29.6 for CGA, 62.4 for flavonoids, and 231.4 for TPC. Quantitation of individual compounds using high-performance liquid chromatography showed that ATPF with ammonium sulfate/ethanol achieved yields of 0.031 mg CGA/mg dry mass leaves, 0.027-mg rutin/mg dry mass leaves, 0.006-mg luteolin-7-*O*-glucoside/mg dry mass leaves, and 0.007-mg diosmin/mg dry mass leaves. Based on the overall extraction performance, ammonium sulfate/ethanol ATPF is the preferred system in comparison to sodium phosphate/ethanol ATPF, for the extraction of bioactive compounds from haskap leaves.

Keywords Aqueous biphasic flotation · Bubble-assisted extraction · Blue honeysuckle (haskap) · Salting-out

Introduction

Haskap plants or blue honeysuckle, *Lonicera caerulea*, are known for their berries which are rich in antioxidants due to the presence of various bioactive compounds such as anthocyanins and flavonoids (Celli et al. 2014; Khattab et al. 2016). They have recently gained interest as a commercial crop in North America, particularly Canada, due to their ability to withstand severe cold climates, such as in the northern prairie region (Hummer et al. 2012). These deciduous shrubs shed their leaves annually, resulting in an abundant source of leaves. Studies have shown that haskap leaves contain a range of metabolites such as chlorogenic acid, flavonoids, loganin, secologanin, and triterpenoids (Becker et al. 2017; Bonarska-Kujawa et al. 2014; Dawson 2017; Machida et al. 1995; Oszmiański et al. 2011). Chlorogenic acid for instance can be used to treat inflammation and hypertension (Naveed et al. 2018), and quercetin, which is a major flavonoid in haskap leaves, has demonstrated anticancer properties from in vitro studies and prevented cardiovascular diseases (Wang et al. 2016). The polyphenol content in the leaves can also contribute to antioxidative properties which can lower oxidative stress, DNA mutations, and other forms of cell damage (Pisoschi and Pop 2015). These health-promoting properties make the leaves from haskap plants a valuable by-product stream that could have applications in many industries that produce health supplements, functional foods, and consumer products such as cosmetics and personal health products.

Conventional extraction methods such as maceration (Radojković et al. 2016) and distillation (Donelian et al.

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2009) have many drawbacks. These methods usually rely on toxic organic solvents in large volumes, have low extraction efficiencies, require multiple steps, and use high temperatures which may cause degradation to thermolabile bioactive compounds. In the recent years, there are emerging extraction technologies such as ultrasound-assisted extraction (Horžić et al. 2012; Jiang et al. 2019a), microwave-assisted extraction (Krishnaswamy et al. 2013; Périno-Issartier et al. 2011; Routray and Orsat 2012), pulsed ohmic heating (El Darra et al. 2013), and supercritical extraction (Bimakr et al. 2012; Leal et al. 2007; Pereira and Meireles 2010). These techniques require sophisticated equipment such as ultrasound transducer, magnetron, electrodes, and high-pressure pumps, respectively. Aqueous two-phase flotation (ATPF) is explored as an emerging mild extraction technique for recovering biomolecules, and integrates both aqueous two-phase extraction (ATPE) and solvent sublation. ATPE uses an aqueous two phase system (ATPS) comprised of two incompatible aqueous components that partition into two distinct phases (Hatti-Kaul 2000). ATPE occurs when solutes from a solid or liquid are selectively partitioned in an ATPS. Solvent sublation occurs in the ATPF system when the biomolecules adsorb onto the surface of rising gas bubbles and concentrate in the top phase after some time (Bi et al. 2010a). ATPF promotes both the mass transfer of the biomolecules via solvent sublation and simultaneous selective partitioning between two aqueous phases. As ATPF can be designed to use less toxic phase-forming components such as alcohols and salts, it is an environmentally friendly technique for separation, concentration, and partial purification of biomolecules. The high water content of an ATPS and its ability to occur at lower temperatures provides a mild extraction environment for the leaves. ATPF has been used to extract baicalin, a flavone glycoside (Bi et al. 2013), betacyanin (Leong et al. 2018), puerarin, an isoflavone (Bi et al. 2010b), polyphenols (de Araújo Padilha et al. 2018), bromelain (Pakhale et al. 2013), lincomycin, an antibiotic (Li and Dong 2010), and various proteins (Lakshmi et al. 2012; Nandini and Rastogi 2011; Ng et al. 2020; Platis and Labrou 2006). As ATPF has a simple set-up, requiring only a flotation column and an air source, it is easy to scale-up and much cheaper than emerging extraction technologies, as mentioned previously (Lee et al. 2016). ATPF can be optimized by the classical one factor-at-a-time method or by the design of experiments approach. The former method can be time-consuming and involves numerous experimental runs. With the design of experiments approach, response surface methodology (RSM) allows users to examine the interaction effects between different variables and to minimize the number of experimental runs required. RSM has been successfully applied in other ATPE work (Cheng et al. 2017; Jiang et al. 2019b; Ma et al. 2013) and ATPF work (Han et al. 2014; de Araújo Padilha et al. 2017).

The objective of this research was to compare the performance of ATPF in two salt/ethanol systems, namely ammonium sulfate $(NH_4)_2SO_4$/ethanol and sodium dihydrogen phosphate NaH_2PO_4/ethanol, for the extraction of bioactive compounds from haskap leaves. Both are categorized as generally recognized as safe (GRAS) substances, where $(NH_4)_2SO_4$ is used as a dough strengthener and firming agent while NaH_2PO_4 is used as a thickening agent and emulsifier in animal feed (US Food and Drug Administration 2020). These ATPS were the focus of this work, as they have shown promising results in a recent ATPE study (Chong et al. 2020), and the extracts have the benefit of being compatible in food and consumer care products. Three factors, sample loading, flotation time, and air flow rate, were optimized based on bioactive extraction yields using response surface methodology (RSM). Specifically, analyses of chlorogenic acid (CGA), flavonoids, and total phenolic content (TPC) were conducted using UV-Vis spectrophotometric assays. The optimized extracts were then analyzed using high-performance liquid chromatography (HPLC), and the ATPF technique was compared to ATPE and conventional extraction. The novelty of this work is that it is the first study that investigates and optimizes the extraction of bioactive compounds from haskap leaves using ATPF. This work will explore the feasibility of ATPF as an assisted "green" extraction technology that uses leaves as a sustainable byproduct obtained from haskap cultivation and produces natural extracts that will have wide application in food and consumer care products.

Materials and Methods

Materials

Ethyl alcohol (95% volume) was obtained from Greenfield Specialty Alcohols Inc. (Toronto, Ontario, Canada). Methanol (≥ 99.9%) was supplied by VWR Analytical (Mississauga, Ontario, Canada). Sodium dihydrogen phosphate 96% and diosmin were purchased from Alfa Aesar (Ward Hill, MA, USA). The following chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada): 1-propanol (≥ 99%, food grade), ammonium sulfate (ACS, ≥ 99%), sodium nitrite (≥ 97%, ACS), aluminum nitrate nonahydrate (ACS, ≥ 97%), sodium carbonate (ACS, 99.95–100.05% dry basis), Folin and Ciocalteu's phenol reagent (2 N), gallic acid monohydrate (ACS, ≥ 98%), chlorogenic acid (≥ 95%, titration), and rutin hydrate (≥ 94%, HPLC grade). In addition, luteolin-7-*O*-glucoside (95.1%) was purchased from ChromaDex, and deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Fresh leaves were harvested in July 2018 from haskap plants of the Aurora variety which were in their first growth year in Nova Scotia, Canada. The geographical coordinates of the harvest location are 44° 28′ 10.8″ N, 64° 39′ 25.6″ W. The fully developed leaves were picked after the berries had been harvested. At this stage of the plant growth cycle, there were signs of natural senescence (leaf browning) starting to occur on some parts of the plant, however the majority of the leaves on the haskap plants were still green. For this study, leaves that appeared green without any browning were selected for harvesting. The samples were frozen at − 20 °C and subsequently freeze-dried in a Labconco FreeZone 2.5 Plus freeze dryer (Labconco, Kansas City, MO, USA). The dried samples were sealed in vacuum pouches and stored at − 20 °C in the dark. Before each independent experiment, samples were ground and sieved through a 500-μm mesh opening. Validation studies were performed at the end of the second independent experiment.

Methods

Extraction

Conventional Extraction Conventional extraction was performed using maceration in an incubator shaker (G25 Controlled Environment Incubator Shaker, New Brunswick Scientific, NJ, USA) at 200 rpm. Ground leaves were agitated with 80% (v/v) methanol for 24 h at 25 °C (Safdar et al. 2017). The extract was centrifuged (Sorvall T1 centrifuge, Thermo Scientific, USA) at 1500*g* for 1 min, and the supernatant was analyzed using UV-Vis spectroscopy.

Aqueous Two-Phase Flotation A schematic diagram of the ATPF setup at 25 °C is shown in Fig. 1. A glass chromatography column with a length of 28.5 cm from the porous disc to top of the column and internal diameter of 1.5 cm was used as the flotation column. A porous stainless-steel disc with pore size of 10 μm and 1.27 cm diameter was fitted at the bottom end of the column to generate bubbles. A variable-area flowmeter (Cole-Parmer Instruments Co., Chicago, IL) was connected to the column to regulate the air flow rate. The surface-active compounds would then attach to the air bubbles and rise to the top phase.

Ammonium sulfate/ethanol and sodium phosphate/ethanol ATPSs were used in this study. Salt/ethanol ATPSs were used as they have been proven to extract high yields of bioactive compounds from an earlier work (Chong et al. 2020). The ATPS composition was selected based the same earlier work which maximized the yields of bioactive compounds in haskap leaves. The composition for $(NH_4)_2SO_4$/ethanol ATPS was 20 wt% $(NH_4)_2SO_4$, 27.5 wt% ethanol, and 52.5 wt% water while the composition for NaH_2PO_4/ethanol ATPS was 10 wt% NaH_2PO_4, 37.0 wt% ethanol, and 53 wt% water. The pH of $(NH_4)_2SO_4$/ethanol ATPS top phase was 5.54 while the pH for NaH_2PO_4/ethanol ATPS top phase was 3.81. The experiments were performed at 25 °C. An ATPS of 20 g was added into the column with ground leaves which were weighed using an analytical balance with a precision of ± 0.1 mg (Denver Instrument PI-314, Bohemia, NY, USA). The solution and ground leaves were mixed vigorously by switching on the air flow at 28.6 mL/min for 30 s. The corresponding superficial air velocity was 0.0027 m/s. This mixing step was consistently performed for all ATPF experiments to ensure a homogenous phase prior to the partitioning mechanism. Then the experiment was conducted according to a specific set of operating parameters, as described in the "Design of experiment, optimization, and statistical analysis" section. At the end of the reaction time, the air supply was turned off to equilibrate the system for 1 min. The top and bottom phases were gently removed from the column, ensuring that the leaves at the interphase remained in the column, and their respective volumes recorded. The top phase from each experiment was then analyzed using the UV-Vis spectrophotometric methods described in the "UV-Vis spectrophotometric analysis of bioactive compounds" section. Solid phase extraction (SPE) was used to purify the bottom phase prior to analysis. Briefly, Hypersep SPE 500 mg/2.8 mL C18 cartridges (Thermo Scientific, Rockwood, TN, USA) were preconditioned and equilibrated with water prior to sample loading. The cartridges were then washed with acidified water containing 0.1% (v/v) formic acid, to remove the salt components and the organic fractions were eluted with 75% (v/v) and 100% ethanol. These fractions were dried under nitrogen and dissolved in 80% (v/v) methanol prior to analysis using UV-Vis spectroscopy.

Aqueous Two-Phase Extraction ATPE was conducted as a comparison by using the same sample loading and extraction time from Table 1. Ground leaves were mixed for 30 s in the flotation column as described above, however air was not introduced for ATPE. Analysis was conducted as described previously for ATPF extracts.

Water Extraction For comparison with ATPF, the ground leaves were extracted with water in the flotation column at the optimized operating parameters obtained from ATPF. The same parameters, i.e., sample loading, flotation time, and air flow rate in Table 1, were used. The experiment was also conducted without air (i.e., no flotation) using water at the same operating parameters for comparison with ATPE.

UV-Vis Spectrophotometric Analysis of Bioactive Compounds

Individual calibration curves were plotted for the two ATPS used to minimize salt interference (Golunski et al. 2016). Samples were diluted when necessary using the corresponding top phase to ensure that the measurements were in the range of the calibration curve.

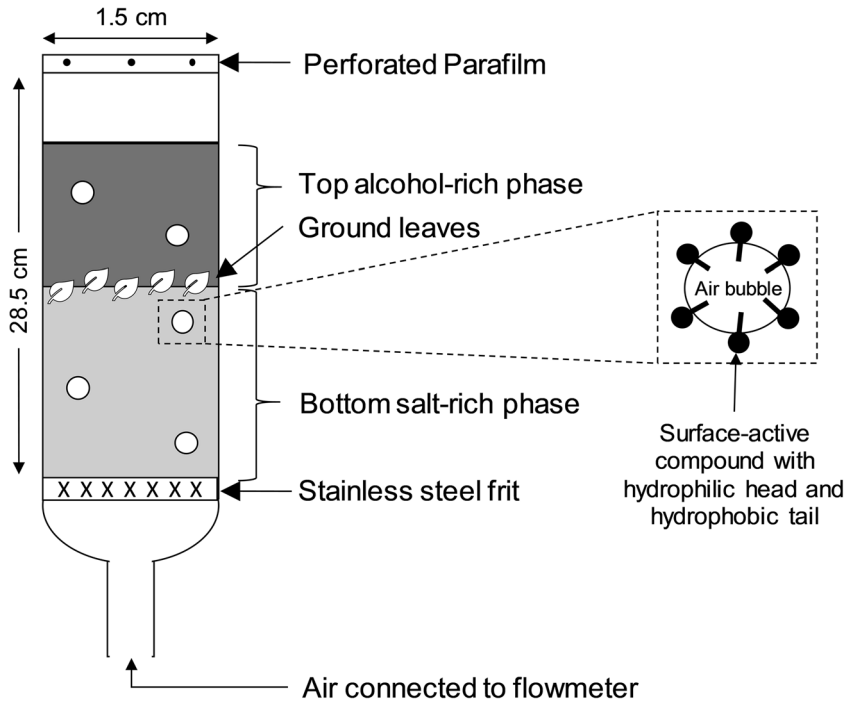

Fig. 1 Experimental setup of ATPF

Chlorogenic Acid Dilutions of the extract were performed using its corresponding ATPS top phase when required. The absorbance of the extract was determined at 328 nm, and chlorogenic acid was used as the standard for the calibration curve (Tan et al. 2014).

Flavonoid Content The aluminium complex colorimetric method was used (Hou et al. 2018). A sample of 450 μL was mixed with 75 μL of 5% (v/v) sodium nitrite solution, and this solution was incubated at room temperature for 6 min. Then 75 μL of 10% (v/v) aluminium nitrate solution was added and vortexed thoroughly. This solution was further incubated for 6 min, and 750 μL of 4% (w/w) sodium hydroxide solution was added. An additional 15 min of incubation was required for the reaction to develop. The solution was then centrifuged at 1500*g* for 1 min, and the absorbance of the supernatant was measured at 510 nm. The absorbance was compared against the standard curve using rutin hydrate as a standard and the flavonoids concentration was expressed in terms of rutin equivalents (RE).

Total Phenolic Content This analysis was based on the Folin-Ciocalteu method (Singleton et al. 1999). Here, 100 μL of the sample was mixed with 200 μL Folin-Ciocalteu reagent (diluted 1:10 by volume with water). After incubation for 5 min, 800 μL of 7.5% (w/v) aqueous sodium carbonate solution was added. The solution was vortexed and incubated in the dark for 2 h at room temperature. The solution was then centrifuged at 1500*g* for 1 min and absorbance was measured at 665 nm. Gallic acid monohydrate was used as the standard, and the results were expressed in terms of gallic acid equivalents (GAE).

Performance Indicators

Three performance indicators, yield, partition coefficient (k), and extraction efficiency (EE), were used to assess the effectiveness of the ATPF and ATPE experiments. Yield was used as the response variable to optimize extraction conditions and it was calculated using Eq. 1 (Fu et al. 2019):

$$\text{Top phase yield} = \frac{C_T \times V_T}{\text{Dry weight of leaves}} \tag{1}$$

Here, C_T and V_T are the concentration and volume of top phase, respectively.

The partition coefficient and extraction efficiency of the optimized extracts were calculated using Equations 2 to 4.

$$\text{Partition coefficient}, k = \frac{C_T}{C_B} \tag{2}$$

$$\text{Volume ratio}, R_V = \frac{V_T}{V_B} \tag{3}$$

$$\text{Extraction efficiency, EE}\ (\%) = \left(\frac{KR_V}{1+KR_V}\right) \times 100 = \left(\frac{M_{\text{top}}}{M_{\text{total}}}\right) \times 100 \tag{4}$$

Here, C_B is the concentration in the bottom phase; V_T and V_B are volumes of top and bottom phase at equilibrium; M_{top} is the mass of target compounds in the top phase, and M_{total} is the total mass of target compound in both phases.

Design of Experiment, Optimization, and Statistical Analysis

Table 1 shows the RSM design using a Box-Behnken approach with the factors and their levels in parentheses: sample loading (5.0, 52.5, and 100.00 mg), flotation time (5, 62.5, 120 min), and air flow rate (11.4, 20.0, 28.6 mL/min). A Box-Behnken design allows efficient estimation of the first and second-order coefficients of mathematical models (Bezerra et al. 2008).

Since the total mass of the ATPS was 20 g, the sample loading can be expressed as 0.025%, 0.263%, and 0.5% (w/w) for 5.0-, 52.5-, and 100.0-mg leaves, respectively. The maximum sample loading of 100 mg was selected so that the top and bottom phases could be identified (Chong et al. 2020), and the maximum flotation time of 120 min was based on other ATPF studies (Bi et al. 2010a; Lin et al. 2015). Preliminary tests showed that the maximum air flow rate that could be used with the solution and column was 28.6 mL/min, as higher air flow rates resulted in the column overflowing due to increased gas holdup (Besagni and Inzoli 2017). Two independent experiments comprising of 15 base runs were performed in randomized order for each type of salt/ethanol system and the yields for CGA, flavonoids, and TPC were determined from triplicate analyses.

The behavior of the response surface was modelled with a second-order polynomial, as shown by Equation 5.

$$Y_i = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_{AA} A^2 + \beta_{BB} B^2 + \beta_{CC} C^2 + \beta_{AB} AB + \beta_{AC} AC + \beta_{BC} BC \quad (5)$$

Here, Y_i is the yield and β_0 is the estimated constant coefficient; β_A, β_B, and β_C are the estimated coefficients for linear effect terms; β_{AA}, β_{BB}, and β_{CC} are the estimated coefficients for quadratic terms; and β_{AB}, β_{AC}, and β_{BC} are the estimated coefficients for interaction terms. A, B, and C are the uncoded independent variables for sample loading, flotation time, and air flow rate, respectively. Final models were obtained for each yield by manually removing insignificant variables ($P > 0.01$ for the ammonium sulfate/ethanol ATPF and $P > 0.05$ for the sodium phosphate/ethanol ATPF) while following the hierarchical model convention and retaining all lower order terms for significant interactions.

Analysis of variance (ANOVA) and response surface optimization were performed using Minitab statistical software (Version 18, Minitab Inc. USA). One-way ANOVA was used to test for significant differences between means using Tukey's multiple comparison test, and error bars included in figures and tables represent the standard error of means (SEM). Multiresponse optimization was used to predict the conditions that would result in maximized yields for CGA,

Table 1 Box Behnken experimental design matrix and yields (mg/mg leaves) from two independent experiments

Run	Sample loading (mg)	Flotation time (min)	Air flow rate (mL/min)	Ammonium sulfate/ethanol ATPF			Sodium phosphate/ethanol ATPF		
				CGA yield	Flavonoids yield	TPC yield	CGA yield	Flavonoids yield	TPC yield
1[a]	52.5	62.5	20	0.041 ± 0.001	0.174 ± 0.002	0.061 ± 0.004	0.039 ± 0.006	0.213 ± 0.006	0.090 ± 0.002
2	52.5	120	11.4	0.039 ± 0.003	0.162 ± 0.015	0.058 ± 0.003	0.046 ± 0.001	0.219 ± 0.005	0.090 ± 0.004
3	5	5	20	0.018 ± 0.006	0.195 ± 0.022	0.073 ± 0.011	0.038 ± 0.003	0.227 ± 0.031	0.105 ± 0.008
4[a]	52.5	62.5	20	0.040 ± 0.001	0.171 ± 0.004	0.060 ± 0.001	0.040 ± 0.05	0.212 ± 0.005	0.086 ± 0.003
5	5	62.5	28.6	0.044 ± 0.001	0.192 ± 0.004	0.074 ± 0.003	0.017[b]	0.197[b]	0.099[b]
6	52.5	120	28.6	0.049 ± 0.001	0.203 ± 0.006	0.070 ± 0.000	0.049 ± 0.002	0.235 ± 0.021	0.091 ± 0.007
7	100	62.5	11.4	0.033 ± 0.001	0.135 ± 0.004	0.047 ± 0.002	0.044 ± 0.000	0.257 ± 0.003	0.079 ± 0.001
8	100	5	20	0.032 ± 0.002	0.149 ± 0.004	0.050 ± 0.001	0.039 ± 0.001	0.223 ± 0.005	0.072 ± 0.002
9	52.5	5	11.4	0.036 ± 0.001	0.158 ± 0.007	0.050 ± 0.000	0.039 ± 0.003	0.178 ± 0.022	0.082 ± 0.002
10	100	120	20	0.041 ± 0.001	0.166 ± 0.005	0.059 ± 0.002	0.047 ± 0.000	0.303 ± 0.016	0.084 ± 0.001
11	5	120	20	0.058 ± 0.072	0.229 ± 0.029	0.083 ± 0.007	0.040 ± 0.002	0.240 ± 0.026	0.107 ± 0.008
12[a]	52.5	62.5	20	0.038 ± 0.000	0.164 ± 0.000	0.060 ± 0.000	0.048 ± 0.000	0.220 ± 0.001	0.091 ± 0.003
13	52.5	5	28.6	0.036 ± 0.001	0.163 ± 0.006	0.055 ± 0.006	0.041 ± 0.001	0.184 ± 0.013	0.081 ± 0.002
14	100	62.5	28.6	0.043 ± 0.001	0.169 ± 0.007	0.057 ± 0.002	0.048 ± 0.002	0.284 ± 0.020	0.070 ± 0.007
15	5	62.5	11.4	0.029 ± 0.002	0.177 ± 0.005	0.073 ± 0.002	0.062 ± 0.002	0.220 ± 0.006	0.102 ± 0.003

[a] Denotes center points

[b] Run 5 of sodium phosphate/ethanol ATPF was based on one independent experiment due to the column overflowing after several other attempts

flavonoids and TPC. The results were then validated experimentally, and Eq. 6 was used to compare the predicted and experimental values.

$$\text{Deviation}\ (\%) = \left|\frac{\text{Experimental value}-\text{Predicted value}}{\text{Predicted value}}\right| \times 100 \qquad (6)$$

High Performance Liquid Chromatography Analysis for Optimized Conditions

Prior to analysis, the top phase from the optimized extraction was dried using a centrifugal evaporator (Genevac EZ-2, SP Scientific, New York, USA) and then dissolved in distilled water. This aqueous solution was loaded onto a 500-mg/2.8-mL C18 solid phase extraction cartridge (Thermo Scientific, Rockwood, TN, USA) which had been preconditioned with methanol and equilibrated with water. The cartridge was then washed with acidified water, 0.1% (v/v) formic acid, to remove the salt. The desired compounds were subsequently eluted with a solution of 75% (v/v) aqueous ethanol with 0.1% (v/v) formic acid, followed by 100% ethanol. These organic fractions were then dried under nitrogen. Prior to HPLC analysis, the organic fractions were dissolved in 1 mL of 50% (v/v) methanol.

HPLC analysis was conducted using an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) equipped with a quaternary pump (Agilent G1311A), an autosampler (G1329A), a column compartment with temperature controller (G1316A), and a diode array detector (G1315B). The column (Zorbax SB-C18, 1.8 μm, 4.6 mm × 50 mm, Agilent, USA) was eluted with a mobile phase consisting of (A) 0.1% (v/v) formic acid in Milli-Q water and (B) 0.1% (v/v) formic acid in acetonitrile using a gradient of 1% to 99% B in 25 min at a flow rate of 0.3 mL/min. The injection volume was 1 μL, and the column temperature was maintained at 40 °C. Elution was monitored at 320 and 250 nm on the diode array detector. Chlorogenic acid, rutin, luteolin-7-*O*-glucoside, and diosmin were quantified using linear regression equations obtained from five-point calibration curves. These compounds were selected for HPLC analysis as they had been identified and quantified in haskap leaves (Dawson 2017).

Results and Discussion

Response Surface Regression

The coefficients for response surface equations are shown in Table 2. All the models were statistically significant at $P < 0.01$ for ATPF using ammonium sulfate/ethanol and $P < 0.05$ for ATPF using sodium phosphate/ethanol, except for the CGA yield from ATPF using sodium phosphate/ethanol. Upon detailed examination, only the interaction between sample loading and air flow rate (A*C) was significant with a P value of 0.002. Its R^2 value of 0.3887 shows that this model for CGA yield can only account for 38.87% of the variability of the response around its mean. Our previous study also showed insignificant models for CGA yields obtained from the glucose/1-propanol ATPS (Chong et al. 2020). In our analysis, blocking was included between the first and second independent experiment to minimize bias and reduce unexplained variability. It was found that the blocks were insignificant except for flavonoids yield using sodium phosphate/ethanol.

The effects of sample loading, flotation time, and air flow rate are illustrated in Fig. 2, where selected three-dimensional surface plots are shown. As surface plots illustrate interactions between two factors, the third factor was held at the zero level in coded units. Figure 2 a and c show that as sample loading increased from 5 to 100 mg, the yields for CGA and TPC decreased, which may have been due to the larger amount of leaves forming a barrier blocking the bubble flow to the top phase, resulting in lower yields. Similarly, other studies have attributed decreasing yields to an increased viscosity in the bottom phase preventing the bubbles from rising effectively (Koyande et al. 2019). Other studies have reported a volume exclusion effect, which can occur when there is reduced space in the top phase for the biomolecules to partition (Ng et al. 2020) therefore decreasing the yields.

In Fig. 2, it is evident that as the air flow rate increased from 11.4 to 28.6 mL/min, the yields for CGA and TPC increased. This behavior has been attributed to the higher air flow rates generating more air bubbles and increasing the surface area for the adsorption of biomolecules (de Araújo Padilha et al. 2018; Koyande et al. 2019); however, there is a limit to the maximum allowable air flow rate to avoid foam accumulation (Bi et al. 2013). Figure 2b shows that the flavonoids yield increased with increasing flotation time from 5 to 120 min. This can be attributed to an increase in the biomolecules adsorbing onto the bubble surface with the greater flotation time. Other studies reported similar trends with increased recoveries of isoflavone (Bi et al. 2010b) and proteins (Tham et al. 2019) obtained with increased flotation time.

Figure 2a also shows the interaction effects of sample loading and air flow rate. Here, the maximum CGA yield of about 0.052-mg/mg leaves from ATPF with sodium phosphate/ethanol is shown at a sample loading of 5 mg and air flow rate of 11.4 mL/min. However, high CGA yields of around 0.050-mg/mg leaves are also observed at a sample loading of 100 mg and air flow rate of 28.6 mL/min. The presence of these two scenarios with high CGA yields in the sodium phosphate/ethanol system may be due to a poor regression fit, as shown in Table 2. The highest flavonoids yield of about 0.220-mg RE/mg leaves was achieved at 120-min flotation time and

Table 2 Regression coefficients for yields obtained from ATPF

	Ammonium sulfate/ethanol ATPF[b]			Sodium phosphate/ethanol ATPF[b]		
Coefficient[a]	CGA Yield	Flavonoids yield	TPC yield	CGA yield	Flavonoids yield	TPC yield
β_0	0.00988	0.1737	0.059	0.0791	0.20857	0.08818
β_A	0.00018	− 0.02188	− 0.01125	− 0.000506	0.02192	− 0.0079
β_B	0.000285	0.01181	0.00537	N/A	0.02319	0.00181
β_C	0.000531	0.01181	0.00337	− 0.001954	N/A	− 0.00704
β_{AA}	N/A	N/A	0.00562	N/A	0.03626	N/A
β_{BB}	N/A	N/A	N/A	N/A	N/A	N/A
β_{CC}	N/A	N/A	N/A	N/A	N/A	N/A
β_{AB}	− 0.000003	N/A	N/A	N/A	0.01688	N/A
β_{AC}	N/A	N/A	N/A	0.000028	N/A	N/A
β_{BC}	N/A	N/A	N/A	N/A	N/A	N/A
R^2	0.7375	0.7084	0.8378	0.3887	0.8308	0.5286
R^2 (adj)	0.6829	0.6617	0.8040	0.2869	0.7940	0.4500
R^2 (pred)	0.5246	0.5603	0.7409	0.00	0.7139	0.3414
P (model)[c]	0.000	0.000	0.000	0.015	0.000	0.001
P (lack of fit)[c]	0.024	0.026	0.089	0.233	0.037	0.228
P (blocks)[c]	0.208	0.031	0.087	0.443	0.002	0.112

[a] β_0 is the estimated constant coefficient

β_A, β_B, and β_C are the estimated coefficients for linear effect terms

β_{AA}, β_{BB}, and β_{CC} are the estimated coefficient for quadratic terms

β_{AB}, β_{AC}, and β_{BC} are the estimated coefficient for interaction terms

A, B, and C are the uncoded independent variables for sample loading, flotation time, and air flow rate

[b] N/A not applicable; factor was not significant. The equations were averaged over blocks

[c] A 99% confidence interval was used for ammonium sulfate/ethanol ATPF while a 95% confidence interval was used for sodium phosphate/ethanol ATPF

28.6-mL/min air flow rate while the highest TPC yield of 0.077-mg/mg leaves was reached at a low sample loading of 5 mg and high air flow rate of 28.6 mL/min (Fig. 2 b and c, respectively).

Optimization of ATPF

The operating conditions were optimized for maximum CGA yield, flavonoids yield, and TPC yield from haskap leaves. For all responses, the weight factor was set to 1 to ensure equal importance on the desirability. Table 3 shows two optimized solution for each ATPF system, individual desirability values, and validation results. The overall desirability, D is a measure of how well the goals for the three responses were optimized, and ranges from 0 to 1, where 1 represents the ideal situation and 0 indicates that one or more responses are out of their acceptable limits.

The optimized parameters for ATPF using ammonium sulfate/ethanol were 5-mg leaves, 120-min flotation time, and 28.6 mL/min for the air flow rate, while the optimized

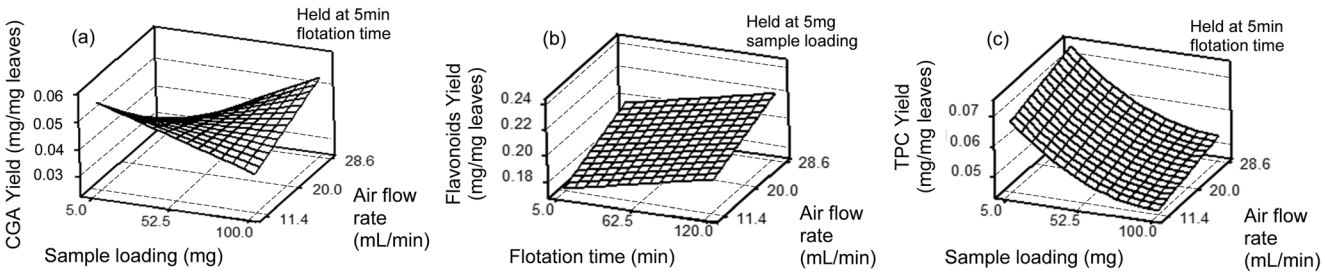

Fig. 2 Interaction effects on **a** CGA yield from sodium phosphate/ethanol ATPF, **b** flavonoids yield, and **c** TPC yield from ammonium sulfate/ethanol ATPF

Table 3 Optimized parameters of ATPF, desirability, and validation results for CGA yield (mg/mg leaves), flavonoids yield (mg RE/mg leaves), and TPC yield (mg GAE/mg leaves)

Responses	Individual desirability, *d*	Predicted value	Experimental value	Deviation (%)
$(NH_4)_2SO_4$/ethanol: 5-mg leaves, 120-min flotation time, 28.6-mL/min air flow rate. $D = 0.81$[a]				
CGA yield	0.88	0.058	0.035	39.7
Flavonoid yield	0.69	0.219	0.307	40.0*
TPC yield	0.86	0.085	0.097	14.6
NaH_2PO_4/ethanol: 5-mg leaves, 120-min flotation time, 11.4-mL/min air flow rate. $D = 0.61$[a]				
CGA yield	0.61	0.056	0.034	39.3
Flavonoid yield	0.45	0.229	0.193	15.6
TPC yield	0.82	0.105	0.093	11.7

[a] *D*: overall desirability

*Value is outside of 99% prediction interval

parameters for ATPF using sodium phosphate/ethanol were 5-mg leaves, 120-min flotation time, 11.4 mL/min for the air flow rate. Their composite desirabilities of 0.81 and 0.61 are considered "very good" and "satisfactory" (Lazic 2006).

Deviations between the experimental and predicted values were within the 99% prediction interval except for the flavonoids yield from ATPF using ammonium sulfate/ethanol. This is in keeping with the predicted R^2 value of 0.56 for the flavonoids yield in Table 2. It is possible that the 9-day period between the completion of the ammonium sulfate/ethanol ATPF experiments and the experimental validation could have contributed to the results outside of the 99% prediction interval. In a separate experiment to investigate the effect of frozen storage time on the bioactive content of the ground leaves, the flavonoids yield was found to increase by 0.027-mg/mg leaves after nine days (data not shown). This was also observed in other produce where transient increases were noted for some bioactive compounds with longer postharvest storage time (Kevers et al. 2007; Piljac-Žegarac and Šamec 2011; Sanchez-Ballesta et al. 2007).

Comparison of Optimized Parameters with Aqueous Two-Phase Extraction

Figure 3 shows the results from different extraction methods for ammonium sulfate/ethanol systems. Using the optimized parameters, ATPF with ammonium sulfate/ethanol yielded 0.035-mg CGA/mg leaves, 0.307-mg RE/mg, and 0.100-mg GAE/mg while ATPE resulted in 0.019-mg CGA/mg, 0.138-mg RE/mg, and 0.050-mg GAE/mg. This significant improvement in yield can be attributed to the role of the bubbles in ATPF, where the biomolecules are adsorbed onto the bubble surface and greater extraction results as the bubbles rise up to the top phase (de Araújo Padilha et al. 2018; Leong et al. 2018). Conventional extraction which was performed for 24 h yielded 0.057-mg CGA/mg leaves, 0.174-mg RE/mg, and 0.106-mg GAE/mg. Whereas extractions using water and conducted with and without flotation yielded 0.051- and 0.044-mg CGA/mg leaves, 0.133- and 0.140-mg RE/mg leaves, and 0.087- and 0.089-mg GAE/mg leaves, respectively. Although water extraction achieved yields that were comparable to those from conventional extraction, there are disadvantages associated with using water as the solvent, such as the high energy requirement for the removal of water (Chemat et al. 2019). Moreover, as water is a polar solvent, nonpolar compounds would have limited extractability in this solvent system. Other berry leaves such as blueberry leaves had 0.164-mg GAE/mg leaves (Routray et al. 2014) while Chilean berry leaves ranged from 0.11- to 0.13-mg GAE/mg leaves (López de Dicastillo et al. 2017).

Figure 4 shows the yield comparison between different extraction methods for sodium phosphate/ethanol systems. For ATPF using sodium phosphate/ethanol, yields of 0.034-mg CGA/mg, 0.19-mg RE/mg, and 0.09-mg GAE/mg were obtained while ATPE resulted in 0.037-mg CGA/mg, 0.19-mg RE/mg, and 0.09-mg GAE/mg. The results indicate that there was no significant difference between the yields obtained from ATPF and ATPE for sodium phosphate/ethanol systems.

Table 4 shows the partition coefficient and extraction efficiency of the optimized ATPF parameters with the values obtained by ATPE as a comparison. Here, the partition coefficient of 29.6 from ATPF with the ammonium sulfate/ethanol system indicates that the top phase is 29.6 times more concentrated than the bottom phase. All the partition coefficient values were more than 1, and this implies that partial purification had occurred as the top phase is more concentrated than the bottom phase. Table 4 also shows that ATPF using ammonium sulfate/ethanol had a significant increase in partition coefficient and extraction efficiency when compared to that obtained from ATPE for flavonoids and TPC. Moreover, the partition coefficient for CGA and flavonoids increased by 208% and 170% respectively with ATPF. A similar increase was reported by Bi et al. (2013), where an increase in concentration coefficient from 2.5 to 27 resulted when ATPF was used instead of ATPE to extract baicalin, a flavone glycoside.

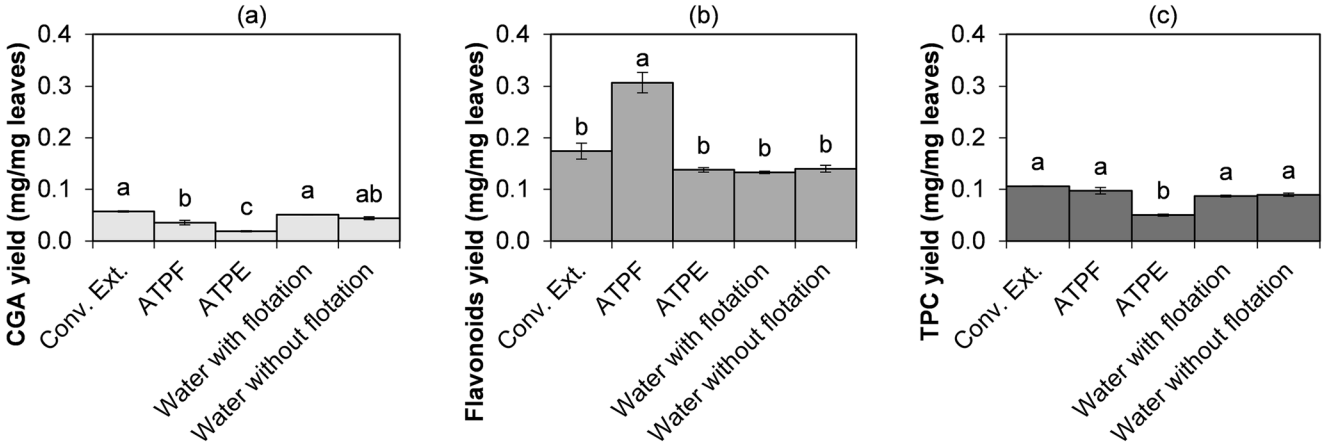

Fig. 3 Yield comparison between conventional extraction, ATPF and ATPE using ammonium sulfate/ethanol, water extraction with flotation, and water extraction without flotation. Different letters in each plot indicate significant difference between extraction methods. **a** Chlorogenic acid, **b** flavonoids, and **c** total phenolic content

ATPF also showed a 19.5% improvement of the partition coefficient for the recovery of polyphenols in comparison to that obtained from ATPE (de Araújo Padilha et al. 2018). In our work, there were no significant differences between the partition coefficients and extraction efficiencies from ATPF and ATPE using the sodium phosphate/ethanol ATPS. However, almost all the TPC obtained by ATPF using sodium phosphate/ethanol partitioned to the top phase, resulting in infinity partitioning.

Based on studies by other researchers (Bi et al. 2013; de Araújo Padilha et al. 2018), it was expected that ATPF would improve yields, however this was not observed in our work for the sodium phosphate/ethanol systems. To investigate this further, a separate experiment was done to investigate the effect of volumetric ratio (i.e., ratio of top phase to the bottom phase volume) on the extraction performance. A volumetric ratio of 1.6 was tested using 19.9 wt% sodium phosphate, 19.5 wt% ethanol, and 60.6 wt% water, which was based on the ATPS phase diagram from previous work (Chong et al. 2020), as a comparison to the volumetric ratio of 9 otherwise used in this study with sodium phosphate/ethanol. The results also showed that the partition coefficient and extraction efficiency of ATPF and ATPE for CGA and flavonoids did not have a significant difference while infinity partitioning was observed for TPC (data not shown). This strong affinity towards the top phase for TPC was also exhibited by laccase in a polyethylene glycol/phosphate ATPS (Blatkiewicz et al. 2018) and resulted in infinity partitioning. However, the large standard error of mean observed for the TPC partition coefficient in both ammonium sulfate/ethanol and sodium phosphate/ethanol systems of this study suggests that TPC may not be a suitable performance indicator.

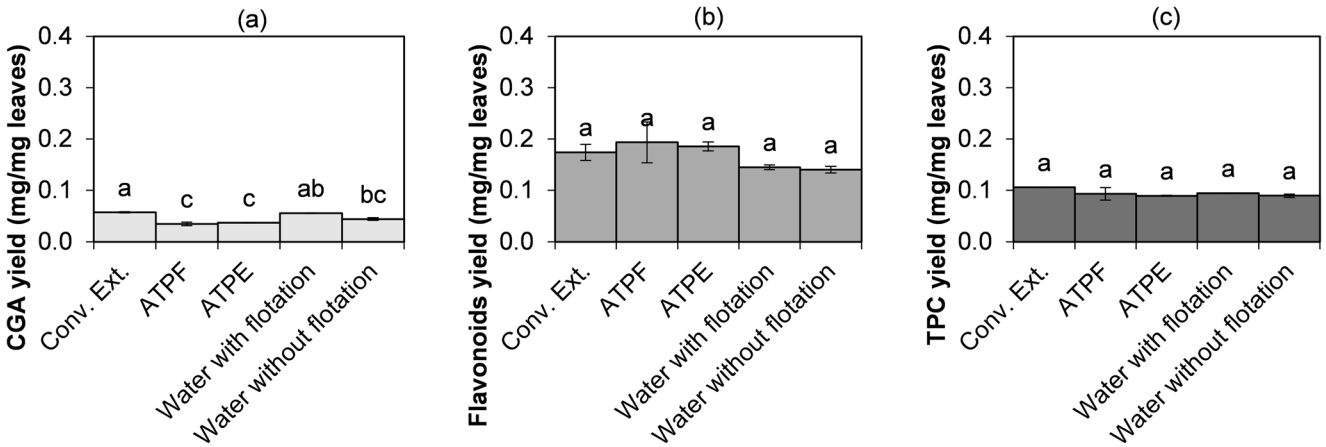

Fig. 4 Yield comparison between conventional extraction, ATPF and ATPE using sodium phosphate/ethanol, water extraction with flotation, and water extraction without flotation. Different letters in each plot indicate significant difference between extraction methods. **a** Chlorogenic acid, **b** flavonoids, and **c** total phenolic content

Table 4 Comparison of partition coefficient, *k*, and extraction efficiency, *EE* (%), of ATPF and ATPE for ammonium sulfate/ethanol and sodium phosphate/ethanol systems. Different superscript letters indicate significant difference between ATPF and ATPE of the same system

Performance indicators (Average ± SEM)	Ammonium sulfate/ethanol		Sodium phosphate/ethanol	
	ATPF	ATPE	ATPF	ATPE
k CGA	29.6 ± 8.6[a]	9.6 ± 0.5[a]	11.1 ± 5.3[a]	10.2 ± 1.2[a]
EE CGA (%)	97.1 ± 0.8[a]	92.2 ± 0.3[b]	98.7 ± 0.6[a]	98.9 ± 0.1[a]
k flavonoids	62.4 ± 4.3[a]	23.1 ± 1.9[b]	6.7 ± 1.7[a]	7.9 ± 0.5[a]
EE flavonoids (%)	98.7 ± 0.1[a]	96.6 ± 0.3[b]	98.3 ± 0.4[a]	98.6 ± 0.1[a]
k TPC	231.4 ± 16.5[a]	161.6 ± 123.6[a]	∞	104.8 ± 97.2
EE TPC (%)	99.6 ± 0.0[a]	98.8 ± 0.9[a]	99.6 ± 0.4[a]	99.2 ± 0.7[a]

HPLC Analysis

While the spectrophotometer provides an economical and rapid method to analyze a broad spectrum of compounds, HPLC analysis offers higher precision when quantifying individual compounds. Figure 5 illustrates the yield of four compounds quantified using HPLC for conventional extraction, in comparison to ATPF and ATPE for ammonium sulfate/ethanol and sodium phosphate/ethanol systems. The retention times are included in Table 5. For the conventional extracts, the yields obtained by HPLC were 0.021-mg CGA/mg, 0.014-mg rutin/mg, 0.001-mg luteolin-7-*O*-glucoside/mg, and 0.003-mg diosmin/mg leaves. The optimized ATPF extracts using ammonium sulfate/ethanol recorded the largest yield increase of 49.9%, 96.4%, 652.1%, and 160.6% for chlorogenic acid, rutin, luteolin-7-*O*-glucoside, and diosmin respectively when compared to conventional extracts. These findings support the significant increase observed in the partition coefficients for CGA and flavonoids from ATPF using ammonium sulfate/ethanol compared with sodium phosphate/ethanol in Table 4. The other remaining extraction systems resulted in varying yields. For instance, the optimized ATPF extracts using sodium phosphate/ethanol had 0.0190-mg CGA/mg, 0.016-mg rutin/mg, 0.001-mg luteolin-7-*O*-glucoside/mg, and 0.003-mg diosmin/mg leaves while the optimized ATPE extracts using sodium phosphate/ethanol had 0.019-mg CGA/mg, 0.017-mg rutin/mg, 0.001-mg luteolin-7-*O*-glucoside/mg, and 0.003-mg diosmin/mg leaves. The yields were not statistically significant different except for diosmin extracted by ammonium sulfate/ethanol ATPF. In comparison, our previous ATPE work on a different batch of Aurora haskap leaves (Chong et al. 2020) had a maximum yield of 0.005-mg/mg leaves for the bioactive compounds. The chlorogenic acid and rutin yields are much higher in the present study. This difference could be due to varying environmental conditions such as soil quality, precipitation, and light intensity (Boyarskikh et al. 2015; Senica et al. 2018).

The yields obtained using HPLC and UV-Vis spectrophotometry methods for chlorogenic acid and rutin were compared. The CGA yield obtained from ATPF with ammonium sulfate/ethanol was similar (0.03- to 0.04-mg/mg leaves) when using UV-Vis spectrophotometry (Fig. 3) and HPLC (Fig. 5). For conventional extracts, the CGA yield from UV-Vis spectrophotometry (Fig. 3) was about 2.8 times higher than the CGA yield from HPLC (Fig. 5). This difference may be caused by the interference of other components such as CGA isomers, as well as solvent-related effects with the ATPS that could have affected the absorption when using the UV-Vis method, while the HPLC measurement was very specific. The remaining chlorogenic acid and rutin yields

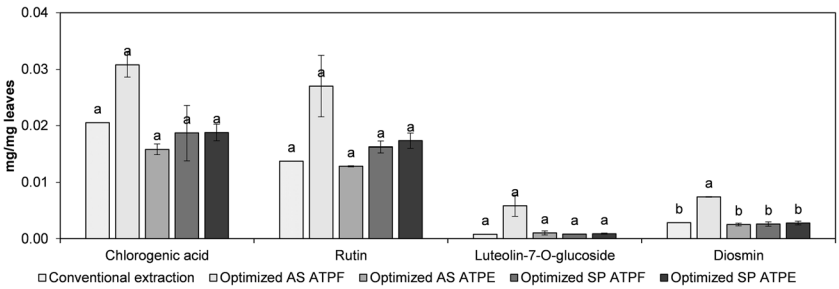

Fig. 5 Yield comparison of chlorogenic acid, rutin, luteolin-7-*O*-glucoside, and diosmin as detected by HPLC. AS: Ammonium sulfate/ethanol system, SP: Sodium phosphate/ethanol system. Different letters for each compound indicate significant differences between extraction methods

Table 5 Wavelength, retention time, and content of selected bioactive compounds from HPLC. Retention times and yields reported for ATPF and APTE extracts are an average of two independent experiments

Compounds	Wavelength (nm)	Retention time (min)	Yield (mg/mg dry weight leaves)
Conventional extract			
CGA	320	9.641	0.021
Rutin	250	11.421	0.014
Luteolin-7-*O*-glucoside	250	11.834	0.001
Diosmin	250	12.444	0.003
Optimized extract for $(NH_4)_2SO_4$/ethanol ATPF			
CGA	320	9.651	0.031
Rutin	250	11.449	0.027
Luteolin-7-*O*-glucoside	250	11.859	0.006
Diosmin	250 nm	12.468	0.007
Optimized extract for $(NH_4)_2SO_4$/ethanol ATPE			
CGA	320	9.644	0.016
Rutin	250	11.440	0.013
Luteolin-7-*O*-glucoside	250	11.839	0.001
Diosmin	250	12.464	0.003
Optimized extract for NaH_2PO_4/ethanol ATPF			
CGA	320	9.644	0.019
Rutin	250	11.438	0.016
Luteolin-7-*O*-glucoside	250	11.852	0.001
Diosmin	250	12.469	0.003
Optimized extract for NaH_2PO_4/ethanol ATPE			
CGA	320	9.629	0.019
Rutin	250	11.438	0.017
Luteolin-7-*O*-glucoside	250	11.833	0.001
Diosmin	250	12.465	0.003

extracted by ATPE using ammonium sulfate/ethanol, and by ATPF and ATPE using sodium phosphate/ethanol were consistent between both analytical methods. The relationship between the two methods were also statistically examined with Pearson correlation. Chlorogenic acid had a low, positive, and insignificant linear correlation ($r = 0.370$, n = 9, $p > 0.05$) while flavonoids had a very strong, positive linear correlation between spectrophotometric method and HPLC method ($r = 0.928$, n = 9, $p < 0.05$). Due to the visibly non-monotonic relationship for chlorogenic acid, the Spearman rank correlation could not be applied. In a study comparing spectrophotometric and HPLC methods for the determination of CGA in coffee beans, the CGA percentage obtained by UV-Vis spectroscopy was slightly higher than those from the HPLC method (Belay and Gholap 2009). The authors attributed the difference to the minor interference of water-soluble compounds present in the sample. In our study, the different trends for the CGA yields may be due to the sample preparation step using SPE cartridges for HPLC but not for UV-Vis spectroscopy. It is worth noting that although there was a high Pearson correlation for flavonoids, the HPLC method used only a single compound rutin, as the standard, while the UV-Vis spectrophotometric method measured an array of flavonoids.

Conclusion

This study demonstrated that aqueous two-phase flotation improved the extraction performance of bioactive compounds from haskap leaves. The spectrophotometric assays for ATPF using ammonium sulfate/ethanol increased the partition coefficient by 208% for chlorogenic acid and 170% for flavonoids when compared to ATPE. It was further confirmed by HPLC quantification where ATPF using ammonium sulfate/ethanol increased yields by 49.9%, 96.4%, 652.1%, and 160.6% for chlorogenic acid, rutin, luteolin-7-*O*-glucoside, and diosmin respectively, in comparison to ATPE. However, there was no significant difference between ATPF and ATPE for sodium phosphate/ethanol systems in terms of yield, partition coefficient, nor extraction efficiency. With that, ammonium sulfate/ethanol ATPF is preferred for its enhanced ability to recover bioactive compounds. Potential applications of the ATPF extracts include food and bioproducts, and future work may integrate ATPF with mechanical processes such as ultrasound and pulsed electric field to improve its efficiency.

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Authors' Contributions Kar Yeen Chong: methodology, software, validation, formal analysis, investigation, writing—original draft, visualization. Roumiana Stefanova: methodology, formal analysis, investigation, resources, writing—review and editing, visualization. Junzeng Zhang: conceptualization, formal analysis, resources, writing—review and editing, visualization. Marianne Su-Ling Brooks: conceptualization, resources, supervision, project administration, funding acquisition, writing—review and editing.

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Data Availability Data will be made available upon request.

Code Availability Not applicable

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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