An efficient method for high-purity anthocyanin isomers isolation from wild blueberries and their radical scavenging activity

Nasima Chorfa, Sylvain Savard, Khaled Belkacemi

Abstract

An efficient process for the purification of anthocyanin monomer isomers from wild blueberries of Lake Saint-Jean region (Quebec, Canada) was developed and easy scalable at industrial purpose. The blueberries were soaked in acidified ethanol, filtered, and the filtrate was cleaned by solid phase extraction using silica gel C-18 and DSC-SCX cation-exchange resin. Anthocyanin-enriched elutes (87 wt.%) were successfully fractionated by preparative liquid chromatography. The major anthocyanins mono-galactoside, -glucoside and -arabinoside isomers of delphinidin, cyanidin, petunidin, peonidin and malvidin were isolated with a purity up to 100% according to their LC-MS and $^1$H NMR spectra. The oxygen radical absorbance capacity (ORAC) of the obtained pure anthocyanins was evaluated. Delphinidin-3-galactoside has the highest capacity (13.062 ± 2.729 l/mol TE/l mol), and malvidin-3-glucoside the lowest (0.851 ± 0.032 l/mol TE/l mol). A mechanistic pathway preview is suggested for the anthocyanins scavenging free radical activity by hydrogen transfer.
1. Introduction

The wild blueberries from Lake Saint-Jean region (Quebec, Canada) belong to the genus Vaccinium angustifolium Aiton and Vaccinium myrtillus Michaux are especially rich in flavonoids (anthocyanins, flavonols and proanthocyanidins) and other phenolic compounds (Moisan-Deserres, Girard, Chagnon, & Fournier, 2014). The beneficial health effects of blueberries have been widely reported, including their antioxidant capacity correlated with their anthocyanins content. Blueberry anthocyanins were reported as potent molecules used in the treatment of diabetic retinopathy (Nabavi et al., 2015) or cardiovascular risk factors (Kruger, Davies, Myburgh, & Lecour, 2014). Unfortunately, low extraction yields, instability and difficulties in obtaining pure anthocyanin with reasonable costs greatly hinder research on their bioactivity.

Anthocyanins are heterosides in which the aglycone or anthocyanin moiety is derived from the flavylium or 2-phenylbenzopyrylium cation. Among the 21 anthocyanidins described in the literature, six are widespread in fruits and vegetables: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Among these structures, five of them have been identified in blueberries, only pelargonidin was not detected (Nicoue, Savard, & Bellacemi, 2007).

Anthocyanin stability is favored in acidic environment with the glycosylation of hydroxyl groups and acylation of sugars (Gould, Davies, & Winefield, 2009). In aqueous media, anthocyanins are in equilibrium with four structures: the flavylium cations, neutral and anionic quinonic bases, carbinol pseudo-bases and chalcones. The content of these chemical structures depends mainly on the pH-value with the predominance of flavylium cations in highly acidic medium (pH < 2). As the pH-value increases, the red flavylium cations disappear by de-protonation of the hydroxyl groups in positions 5, 7 and 4' to produce quinonic bases with a blue coloration. In neutral or slightly acidic medium, flavylium cations hydration occurs in positions 2 and 4 to yield carbinol pseudo-bases which are then converted into open chalcones with a yellow coloration (Andersen & Markham, 2006). The degree and position of hydroxylation and methoxylation in the B-ring, the pattern of glycosylation and the completely conjugated structure of anthocyanins inducing electron delocalization are structural factors modulating the stability and polarity as well as the ability of anthocyanins to act as free radical scavengers (Jing et al., 2014).

There are numerous methods developed to evaluate radical scavenging activity of dietary antioxidant, and these may be classified into two mechanisms based on hydrogen atom transfer or electron transfer. The methods measuring hydrogen atom donating ability are most of the time a competitive reaction between antioxidant and substrate for generated peroxyl radicals through azo-compound decomposition, and they include low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter and crocin bleaching assays. The electron donating capability is estimated by the capacity of an antioxidant in the reduction of an oxidant which changes color when reduced, and it include Trolox equivalent antioxidant capacity, ferric reducing antioxidant power and 2,2-diphenyl-1-picrylhydrazyl assays (Shahidi & Zhong, 2015). Presently, ORAC is the most used assay because it combines both inhibition time and inhibition degree of anthocyanins ability to quench peroxyl radicals by hydrogen donation (Huang, Ou, & Prior, 2005; Prior, 2014).

The presence of phenolic compounds not belonging to anthocyanins and other impurities inevitably interfere with the evaluation of the biological antioxidant activity of crude anthocyanin extracts (Diaconeasa, Florica, Rugina, Lucian, & Socaciu, 2014). Value-added high-purity anthocyanins are not yet commercially available. Although technologically difficult to realize, the preparation of the pure wild blueberry anthocyanins is a very promising task. In light of these issues, pure anthocyanin isolation and purification from plant sources is mandatory for the accurate quantification and bioactivity application needs. Therefore separation of anthocyanin molecules from vegetables has been carefully studied using techniques such as supercritical CO2 and pressurized liquid (Paes, Dotta, Barbero, & Martinez, 2014), pressurization and cold storage (Bodelon, Avizcuri, Fernandez-Zurbano, Dizy, & Prestamo, 2013), high performance counter-current chromatography (Choi et al., 2015), or solid phase extraction coupled with preparative high performance liquid chromatography (Wang, Yin, Xu, & Liu, 2014).

The complete and detailed structural studies on the anthocyanins extracted and purified from wild blueberries of Lake Saint-Jean (Quebec, Canada), together with their antioxidative activity are hardly reported or attempted. The objectives of this study relate to (1) determine the anthocyanin profile of wild blueberries from Saint-Jean Lake; (2) separate, purify and characterize the major anthocyanins for their structure elucidation; (3) evaluate the radical scavenging activity of the samples by the ORAC method and (4) suggest a mechanistic oxidation pathway for the reaction.
2. Materials and methods

2.1. Plant material

The plant material consisted on a mixture of wild blueberries harvested in the Lake Saint-Jean area (Quebec, Canada). They were 95% from *V. angustifolium* Aiton (common lowbush blueberry) and 5% from *V. myrtillus* Michaux (velvet-leaf blueberry). These blueberry species are wild plants naturally found in the mentioned region. They were harvested in August 2012 and provided by Les Bleuets Sauvages du Quebec Inc. (Saint-Bruno, Quebec, Canada). The fruits were stored at –20 °C for 24 h until use.

2.2. Chemicals

All chemicals were reagent grade unless otherwise stated. Formic acid and methanol were obtained from Fisher Scientific (Ottawa, Canada). Ethanol (95% v/v) was purchased from Les Alcools de Commerce, Inc. (Boucherville, Canada). Cyanidin-3,5-di-glucoside (cyanidin), malvidin-3,5-di-glucoside (malvin) chloride standards were purchased from Sigma–Aldrich (Milwaukee, US). Cyanidin-3-glucoside (kuromanin), delphinidin-3-glucoside (myrtillin) and malvidin-3-glucoside (oenin) chloride standards were purchased from ExtraSynthese (Genay, France). Silica gel packed C-18 reverse phase and cation-exchange resin DSC-SCX were purchased from ExtraSynthese (Genay, France). Deionized water was used for all solution preparation. Monobasic potassium phosphate was obtained from EMD-Millipore (Etobicoke, Canada). Phosphoric acid and methanol were obtained from Fisher Scientific (Ottawa, Canada). Cyanidin-3-glucoside (cyanidin), malvidin-3-glucoside (malvin) chloride standards were purchased from Sigma–Aldrich (Milwaukee, US). Cyanidin-3-glucoside (kuromanin), delphinidin-3-glucoside (myrtillin) and malvidin-3-glucoside (oenin) chloride standards were purchased from ExtraSynthese (Genay, France). Silica gel packed C-18 reverse phase and cation-exchange resin DSC-SCX were purchased from ExtraSynthese (Genay, France). Deionized water was used for all solution preparation.

2.3. Anthocyanins extraction and isolation

Anthocyanins were extracted from frozen berries of Saint-Jean Lake using acidified ethanol with small amount of phosphoric acid (0.02% v/v) at room temperature (21 °C). Typically, 10 kg of frozen berries were ground in acidified ethanol in a jacketed reactor with mechanical stirring (Savard, Tremblay, & Arsenault, 2013). Macerated blueberries were then filtered in a filter press with a porosity of 100 μm. The filtered solid consisted mainly of skins and seeds rich in protein, complex sugars, carbohydrates, lipids, fibers, minerals. The filtrate was filtered a second time with a semi-microporous filter cartridge to remove insoluble fine particles in ethanol. The second filtrate (1039.5 g) was evaporated to dryness under reduced pressure to remove ethanol dissolved in water. The solid residue was then subjected to a solid phase extraction (SPE) as described by Durst & Wrolstad, 2005 but modified for the needs of this work.

2.4. SPE method

The SPE column consists of a cylindrical glass tube with an internal volume of 4.7 L. Circa 750 g of C-18 type adsorbent (1.5 L) having the following characteristics: modified silica gel; distribution of particles 40–63 μm; organic load 0.38 mmol g⁻¹; carbon load 9.16%; was poured into the glass column after its dispersion in 2 L of 95% ethanol. At the completion of gel sedimentation at the bottom of the column, the residual solvent was eluted from the bottom of the column by gravity or by applying a small vacuum. The gel was subsequently conditioned in the column by eluting 2 L of 95% ethanol then 2-L of deionized water at a rate of 50 mL/min.

Typically, a fraction of the collected extracts (323 g dry extract) was dissolved in 1.6 L of water (ratio 1/5). The resulting solution was filtered through a 5 μm-porosity filter and was discharged at the head of the SPE column for the anthocyanin separation. About 8.24 g of insoluble materials were retained on the filter (2.5% extract). Blueberry extract dissolved in 1.6 L of water was then poured at the top of column. Water was eluted slowly and the extract was left adsorbed on the gel for 1 h. A first wash of the gel with water was achieved by circulating 4 L of water in the column at a flow rate of 50 mL/min. This step allows to elute sugars and inorganic substances (250 g dry) present in the extract. Other substances, including anthocyanins, remained adsorbed on the gel. Two successive gel washes were subsequently made with 95% ethanol (with respectively 3 and 1 L EtOH). This step allows desorbing the anthocyanins of the gel (the color quickly migrates to the bottom of the column). The elution rate was ~50 mL min⁻¹. To retrieve the anthocyanin concentrate dissolved ethanol solution in form solid, both fractions of ethanol collected were combined and evaporated under reduced pressure at a slightly lower temperature (65 °C). The collected solid was then stripped of its residual solvent (5% and 10% by weight of residual solvent (especially water after the first evaporation of the solvent) by evaporation under high vacuum (<1 mbar). About 22 g of anthocyanin concentrate (6.8% of initial blueberry extract) containing 50% of polyphenolic substances (0.54% yield compared to the mass of frozen blueberries used containing 88.2% moisture) were obtained in the form of a very fine blue powder soluble in water (Nicoue et al., 2007; Savard et al., 2013).

The fine blue powder (10 g) was then dissolved in acidified water (5% v/v HCOOH-H2O, pH 1.5) and put-down at the head of another 250 mL-SPE column for the anthocyanins purification. The adsorbent was a strong cationic exchange resin, DSC-SCX (40 g), functionalized with benzene sulfonic acid group (pKa < 1.0). The resin was conditioned in the column by eluting 1 L of 95% ethanol then 2 L of deionized water at a rate of 50 mL/min. The resin was equilibrated with 2 L of acidified water (5% v/v HCOOH-H2O, pH 1.5). It is worth to note that formic acid is a weaker acid than hydrochloric acid, and it is less expected to cause pigments degradation. A first wash of the resin was achieved by circulating 2 L of acidified water (pH 1.5) in the column at a flow rate of 50 mL min⁻¹. This step allows eluting uncharged components as chlorogenic acid, whereas anthocyanins as flavylium cations are adsorbed by the resin. It is necessary for the anthocyanins to be in an anionic (quinonic bases) or a neutral form (quinonic bases, carbinol bases and chalcones) at pH 2–6 to desorb from the DSC-SCX resin. Therefore, the anthocyanins were eluted from the resin with an ethanol gradient (30% and 60% v/v) in acidified water (5% v/v HCOOH-H2O, pH 2–3, 20 L). Eluates were acidified with formic acid to reproduce the chemically more stable flavylium cations at pH <2. The acidified elutes were combined and evaporated under reduced pressure and low temperature (40 °C) to remove ethanol. The obtained anthocyanins were concentrated in acidified water (pH 1.5) and stored at 4 °C.

The wash-water and elutes were analyzed by HPLC-DAD (λ = 520 nm) coupled to mass spectrometry (ESI Q-TOP MS⁺ – Coll Energy 5 V and 20 V) to evaluate the anthocyanins content.

The major anthocyanins monomeric isomers were isolated from eluates by preparative-HPLC. The apparatus was equipped with a Waters model 600 controller, a photodiode array detector 2996 adjusted at λ = 520 nm, and 2767 automated sample collector (Waters, Milford, Massachusetts, US). The column was a Xterra Prep MS C18 OD-BTM (5 mm, 5 μm, 19 × 100 mm i.d.) combining the properties of silica functionalized with organic groups. Elution
was carried out by using a gradient procedure with a mobile phase contained of acidified water (5% v/v formic acid, solvent A) and methanol (solvent B) (Wang et al., 2014). The elution conditions were as follows: solvent A: 0 min, 95%; 4 min, 80%; 12 min, 75%; 20 min, 67%; 26 min, 64%; 28 min, 55%; 32 min 45%; 34 min, 30%; 35 min, 95%. The flow rate was 20 mL min\(^{-1}\) and the column temperature 25 °C. The chromatogram was collected continuously every 15 s in test tubes during 30 min.

2.5. Anthocyanins quantification

Total anthocyanins were quantified by HPLC as described by (Durst & Wrolstad, 2005), using an Acquity Ultra Performance LC (Waters, Montreal, Canada) equipped with an Acquity UPLC™ BEH C18 (1.7 μm-2.1 × 50 mm i.d.) reverse-phase column, and an ACQUITY UPLC™ Tunable UV detector with dual wavelength ultraviolet/visible. Samples of 25 μL were injected by an autosampler at ambient temperature. The mobile phase was an elution gradient of 5% v/v formic acid in water and methanol. Absorbance was recorded at 520 nm. Cyanidin-3,5-di-glucoside chloride was used as external standard to evaluate anthocyanins concentration.

2.6. Anthocyanins identification

The structure was individually identified for each anthocyanin by coupling UPLC to a mass spectrometer Micromass/Waters Q-TOF micro (EIT Ltd, Illinois, US) equipped with an electrospray ion source (ESI) operated in positive mode, and a hybrid detector with a quadrupole filter (Q) for the ion separation. This was carried out according to their mass/charge ratio under an electric field, and a time of flight analyzer (TOF) based on the speed difference to reach the ion detector cell. The ions are positively charged in an electric field of 5 or 20 V. The LC-MS chromatogram associated a retention time to a mass spectrum for each ion detected. It is then possible to associate a chromatogram peak to an aglycone and anthocyanin molecular mass and therefore identifying a partial chemical structure. Another approach was used consisting to restrict the LC-MS chromatogram to an accurate mass of an anthocyan aglycone. It is thus possible to associate a retention time to the mass spectrum of an anthocyanin free from impurities (Barnes, Nguyen, Shen, & Schug, 2009).

Nuclear magnetic resonance (NMR) spectroscopy elucidated the anthocyanin’s chemical structure by determining for example the aglycone and sugars substitution. The proton NMR spectra were recorded with a spectrometer Agilent DRR 500 MHz, in the solvent \(d_2\)-HCOOH/d\(_2\)-H\(_2\)O 5:95 v/v, where 3000 scans were necessary to obtain a satisfactory resolution because of the low anthocyanin concentration.

Anthocyanin identification was based on a comparison of spectroscopic and chromatographic results of commercialized standards and literature data.

2.7. ORAC assay

The oxygen radical absorbance capacity (ORAC) procedure was carried out based on previous reported works (Cao, Verdon, Wu, Wang, & Prior, 1995; Ou, Hampsch-Woodill, & Prior, 2001; Wang, Cao, & Prior, 1997). The ORAC values of anthocyanins were evaluated by using fluorescein as the fluorescent probe with a fluorescence quantum yield >0.9 for a sensitive measurement (Magde, Wong, & Seybold, 2002), AAPH as a peroxyl radical generator found in body, and Trolox, a water-soluble vitamin E analogue as a control standard. The cell contained 6.1 × 10\(^{-8}\) mol L\(^{-1}\) fluorescein and 19.1 × 10\(^{-3}\) mol L\(^{-1}\) AAPH in 7.5 × 10\(^{-2}\) mol L\(^{-1}\) phosphate buffer (pH 7.4). Phosphate buffer was used as a blank. The reagents were mixed and incubated at 37 °C. Once AAPH was added, the initial fluorescence was measured until zero fluorescence occurred. Fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used with a Perkin-Elmer LS-5 (Norwalk, CT) spectrophotometer. The ORAC value refers to the net protection area under the quenching curve of fluorescein in the presence of an antioxidant, and it was calculated on the basis of a standard curve with 0.1, 0.2, 0.4, 0.8, 1.6, 3, and 6 μmol L\(^{-1}\) Trolox. ORAC values were expressed as μmol Trolox equivalent (TE) per gram or per μmol of anthocyanin molecule. Linear regression analyses of ORAC activity (Y) versus anthocyanin concentrations (X) with four data points of each compound were computed using Sigmaplot software.

3. Results and discussions

3.1. Anthocyanin identification and quantification

The anthocyanin profiles of blueberries extract obtained from the solid phase extraction (SPE) with C-18 silica gel is depicted in Table SI-1, with the detailed ESI-MS and HPLC-DAD data including retention times, molecular ion peaks, MS\(^2\) fragments, and the anthocyanins concentration in mg of Cyanin equivalent/g extract enriched in anthocyanins after C-18 solid phase extraction. The total anthocyanin content is circa 500 mg of Cyanin equivalent/g extract or ~50% wt%, where up to 22 different anthocyanins have been appropriately identified. This total anthocyanin concentration was 290 mg/100 g fresh blueberries, comparable to those reported previously for the same berry fruits (Mi, Howard, Prior, & Clark, 2004; Paes et al., 2014). Malvidin-3-glucoside and Peonidin-3-glucoside have the highest contributions with 65 and 57 mg Cyanin equivalent/g extract, respectively. These results are consistent with those reported previously for similar blueberry fruits (Barnes et al., 2009; Wang et al., 2014). Fig. SI-1 shows HPLC-DAD chromatogram (λ = 520 nm) of the blueberry extract where 22 peaks are clearly shown. In fact, the MS analysis allowed the identification of 21 different anthocyanins as depicted in Table SI-1. This difference is attributed to the overlap problems or difficulty to differentiate all glucoside, galactoside and arabinoside derivatives.

Durst and Wrolstad (2005) reported the anthocyanins chemical structure effects on the HPLC retention time (RT) using C-18 reverse phase column where the hydroxylation and glycosylation products appeared in shorter retention time, while the methylation and acylation products were observed at longer retention time. Under standard analysis conditions, the elution order began first with delphinidin derivatives, followed by cyanidin-, petunidin-, pelargonidin-, peonidin- and malvidin-based molecules. The chromatogram peaks of blueberry extract (Fig. SI-1 and Table SI-1) followed this elution order, and among the six anthocyanidins commonly isolated in berries, only pelargonidin was not present (Nicoue et al., 2007). It can be noticed that the anthocyanidins are condensed with the same sugars (galactose, glucose and arabino-rose) for which some of them are esterified with acetic acid. The acylated anthocyanins represents circa 20% of total anthocyanins detected where malvidin-3-(6"-acetyl)glucoside is the major one (28 mg of Cyanin equivalent/g extract, see Table SI-1). The presence of this acylated anthocyanin molecule was verified according to its MS spectra (Fig. SI-2). Some authors (Gao & Mazzo, 1994; Giovaneli & Buratti, 2008) reported that the main difference between the cultivated and wild blueberries resides in the absence of the acylated anthocyanins. However, the extraction process was carried out with a very small acid amount (0.02% v/v phosphoric acid) to avoid the acylated anthocyanins hydrolysis (Gao & Mazzo, 1994).

Also, it is worth to mention the presence of anthocyanidin cores linked to pentose moiety such as reported delphinidin-3-arabinoside,
cyanidin-3-arabinoside and malvidin-3-arabinoside with interesting concentrations of 19, 14 and 29 mg of Cyanin equivalent/g extract, respectively. These anthocyanins represent 13 wt.% of total anthocyanins detected.

3.2. Anthocyanins purification and their isolation

To isolate the anthocyanins and purify them, some washing and elution steps were necessary to carry out as described before. The mono-glycoside derivatives of delphinidin and cyanidin have the more important amount in 30% ethanol elute (pH 2). Meanwhile, mono-glycoside derivatives of delphinidin and cyanidin have the elution steps were necessary to carry out as described before. The recovery yield of total anthocyanins was 61% of the total content (19, 14 and 29 mg of Cyanin equivalent/g extract, respectively). These anthocyanins represent 13 wt.% of total anthocyanins detected. After that, it was possible to isolate major derivatives of anthocyanidins in elute fractions according to the elution pH.

The DSC-SCX capacity, resulting in the binding site, was achieved considering that a slight anthocyanins amount (3%) was flushed at the washing step with acidified water (pH 1.5) (Table 1). The recovery yield of total anthocyanins was 61% of the total concentration detected in the C-18 extract, suggesting that the anthocyanins dissociation at pH 2–3 was not completed where the chemical transformation of the flavylium cations into neutral compounds was not also completely accomplished. This could be attributed to strong ionic interaction and a low cationic exchange ability due to a hydrolysis of labile acyl group in acidic media. This is confirmed by malvidin-3-glucoside concentration in the DSC-SCX elute fractions (66 mg of Cyanin equivalent/g extract) higher than in the C-18 extract (65 mg of Cyanin equivalent/g extract) (see Table 1).

The DSC-SCX adsorbent was also tested instead of DSC-SCX, as the hydroxyl groups of the silica polymer were functionalized with carboxyl propyl phase and K⁺ counter ion at pKa of 4.8. DSC-WCX is a weak cation-exchange and less than 1 wt.% anthocyanins were able to adsorb on it at pH 1.5 (5%v/v HCOOH-H₂O).

In order to purify and isolate the anthocyanins contained in 60% ethanol, the flavilium cations were converted into neutral quinonic bases. These elute fractions were immediately acidified with formic acid to prevent anthocyanins degradation because their cationic chemical structures are more stable than the neutral or anionic bases (Fossen, Cabrita, & Andersen, 1998). The use of an elution solvent at pH 4.5 (100% ethanol) resulted in the formation of unstable carbinol bases and chalcones which are hardly convertible into flavilium cations even at high formic acid concentration. Besides, it was not possible to collect the acetyl derivatives in the DSC-SCX elute fraction probably due to a hydrolysis of labile acyl group in acidic media. This is confirmed by malvidin-3-glucoside concentration in the DSC-SCX elute fractions (66 mg of Cyanin equivalent/g extract) higher than in the C-18 extract (65 mg of Cyanin equivalent/g extract) (see Table 1).

The ethanol gradient allowed elution of phenolic compounds. However, owing to ionic interactions between the flavilium cations and the anionic sulfonic groups, it was necessary to use a weak acidic eluent to release the major part of anthocyanins from the resin. Indeed, at pH 3 (60% ethanol), the flavilium cations were converted into neutral quinonic bases. These elute fractions were immediately acidified with formic acid to prevent anthocyanins degradation because their cationic chemical structures are more stable than the neutral or anionic bases (Fossen, Cabrita, & Andersen, 1998). The use of an elution solvent at pH 4.5 (100% ethanol) resulted in the formation of unstable carbinol bases and chalcones which are hardly convertible into flavilium cations even at high formic acid concentration. Besides, it was not possible to collect the acetyl derivatives in the DSC-SCX elute fraction probably due to a hydrolysis of labile acyl group in acidic media. This is confirmed by malvidin-3-glucoside concentration in the DSC-SCX elute fractions (66 mg of Cyanin equivalent/g extract) higher than in the C-18 extract (65 mg of Cyanin equivalent/g extract) (see Table 1).

The LC-MS spectra of collected fractions allowed the anthocyanins identification with the molecular ion [M]+ corresponding to the molecular mass of the solute, and the main fragmentation due to the loss of the sugar moiety. The fraction purity was calculated by dividing the anthocyanin peak area over the area of the whole HPLC chromatogram peaks. Therefore, the estimated purities for delphinidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside were 45%, 76%, 52%, 86% and 99%, respectively. The collected fractions with monomeric anthocyanin purity under 80% were re-chromatographed in a second preparative-HPLC processing to

Table 1

<table>
<thead>
<tr>
<th>Anthocyanins content (mg of Cyanin equivalent) determined by liquid chromatography in wash water and elutes issues of the solid phase extraction of blueberries extract on the cation exchange resin DSC-SCX.</th>
<th>mg of Cyanin equivalent (HPLC-DAD, λ = 520 nm)</th>
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<tr>
<td>ANTHOCYANINS Retention time (min)</td>
<td>C-18 Wash 5% v/v HCOOH-H₂O (pH 1.5)</td>
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<tr>
<td>Chlorogenic acid</td>
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<tr>
<td>Delphinidin-3-galactoside</td>
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<tr>
<td>Delphinidin-3-glucoside</td>
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<td>Total (mg of Cyanin equivalent)</td>
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<tr>
<td>Recovery (%)</td>
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*Note: Values are expressed as mg (C-18 extract, 5%v/v HCOOH-H₂O (pH 1.5) and DSC-SCX elute 30%v/v Ethanol /5% (HCOOH-H₂O) (pH 2.0) and 60%v/v Ethanol /5% (HCOOH-H₂O) (pH 3.0)).*
isolate the galactoside, glucoside and arabinoside isomeric anthocyanins with a fractionation time of 15 s (see Fig. 1). It was then possible to collect delphinidin-3-galactoside and glucoside, cyanidin-3-galactoside and glucoside, peonidin-3-galactoside and glucoside, and malvidin-3-galactoside, glucoside and arabinoside with a purity of 100% and a concentration ranged from 2 to 10 mg/mL according to their LC-MS analysis (Fig. SI-3).

To confirm the structure of the identified anthocyanins, the collected fractions were dried at 45 °C under vacuum and were subjected to $^1$H NMR analysis. Table SI-2 depicts the $^1$H NMR assignments of these anthocyanins, and their chemical shifts ($\delta_H$ in ppm) which are in agreement with those reported in the literature (Acevedo De la Cruz et al., 2012; Fossen & Andersen, 2006; McGhie, Rowan, & Edwards, 2006). However, the low anthocyanin concentration reduced the resolution of peaks and limited the measurement of the coupling constants.

3.3. Oxygen radical absorbance capacity (ORAC)

The major anthocyanins isolated by preparative-HPLC were evaluated for their oxygen radical absorbance capacity against peroxyl radical (ROO$^\cdot$). The ORAC value ($Y$, expressed in μmol L$^{-1}$) Trolox equivalent versus the anthocyanin concentration ($X$) expressed in μmol L$^{-1}$ were plotted giving a linear correlation.

$$Y = a_0 + a_1 \times X$$  

The slope $a_1$, directly reflects the antioxidant potency (ORAC) against peroxyl radicals. A slope of 1.0 would have the same potency as Trolox, a water-soluble $\alpha$-tocopherol analogue (Wang et al., 1997).

The best fit as assessed by the correlation coefficient $R^2$, ORAC value, and the regression coefficients $a_0$ and $a_1$ are summarized in Table 2 for major identified anthocyanins as well as ascorbic acid used as a positive control test for the comparison purpose. As shown, all regression correlation coefficients $R^2$ are greater than 0.98, which reflects the linearity between the anthocyanin concentration and the ORAC value. Except malvidin-3-glucoside which displays the lowest slope (0.851 ± 0.032 μmol TE/μmol) similar to the Trolox potency, all other identified anthocyanins exhibit slopes greater than 1.0 signifying that they have a stronger antioxidant activity against peroxyl radicals than Trolox. Delphinidin-3-galactoside has the largest slope (13.062 ± 2.729 μmol TE/μmol) among the compounds tested and thirteen times higher than Trolox. The ortho-substitution to the functional 4‘-OH group in the B-ring (catechol nucleus) with electron-donating groups and/or hydrophobic groups may enhance the radical scavenging activity, because it could form intermolecular hydrogen bonds stabilizing the phenol radicals (Dangles, Fargeix, & Dufour, 2000). A general tendency makes that anthocyanidins lacking the O-dihydroxy group in the B-ring, such as peonidin, malvidin, and pelargonidin have lower ORAC values compared to cyanidin, delphinidin and petunidin ones (Wang et al., 1997). In the case of this study, it is clear that delphinidin-based anthocyanins outperform other types of anthocyanins in terms of ORAC antioxidant activity.

It is worth to mention that all identified major anthocyanins exhibit higher $a_1$ value than the ascorbic acid potency (0.687 ± 0.037 μmol TE/μmol) (Cao & Prior, 1998; Watanabe et al., 2012). These results confirm the superior antioxidant activity of the collected anthocyanins against peroxyl radicals.

In Table 2, the galactoside isomers of delphinidin, peonidin and malvidin have an ORAC value higher than the glucoside isomers, but this tendency is inverted for the cyanidin isomers. Moreover, arabinoside substituent increased remarkably the ORAC value of malvidin-based anthocyanin. Therefore, the aglycone pattern and the sugar moieties have a synergetic influence on the peroxyl radical scavenging activity of the anthocyanins (Bors, Heller, Michel, & Saran, 1990). This is clearly shown for malvidin-based anthocyanins where CS-sugar moieties could enhance significantly this activity.

In order, to get more insight into the oxygen radical absorbance capacity of anthocyanins, we attempted to elucidate the peroxyl radical scavenging mechanistic pathway based on the chemical structure of the oxidation products.

To establish the schematic reaction pathway of the peroxyl radical absorption by anthocyanins, AAPH was constrained to oxidation under specific conditions in the presence of malvidin-3-glucoside, the major anthocyanin in blueberry extract. For this purpose, malvidin-3-glucoside collected during the first preparative-HPLC (38 mmol L$^{-1}$) react with AAPH (153 mmol L$^{-1}$).
in phosphate buffer (75 mmol L\(^{-1}\), pH 7.4). The reaction media was incubated at 37 °C during 24, 48 and 72 h until anthocyanin depletion. The LC-MS chromatograms of malvidin-3-glucoside, AAPH and the oxidation media at different time are depicted in Fig. 2a-e. Two new peaks appear at the LC-MS retention time 11.6 and 15.9 min. These peaks were assigned to oxidation products.

As depicted in Fig. 2b, initially, AAPH presents two small peaks at LC-MS retention times of 3.3 and 4.7 min. These peaks are attributed to the presence of free peroxyl radicals and associated hydroperoxides, respectively according to their MS\(^{+}\) spectra (Figs. SI-4a and SI-4b). Moreover malvidin-3-glucoside exhibits a peak around 13 min (Fig. 2a). Owing to the reaction of this anthocyanin with radicals generated by AAPH, this peak disappears as the reaction evolves with time. Consequently, two new products are formed. Their peaks are shown at retention times of 11.6 and 15.9 min. The intensity of these peaks is amplified as the reaction time increases. These peaks are attributed to malvidin-3-glucoside degradation products via an absorption process of free radicals. It is worth to note that peroxy radical and hydroperoxide peaks increase over the reaction time because AAPH is in excess. This excess in AAPH was voluntarily introduced to accelerate the rate of the reaction and quickly distinguish the malvidin-3-glucoside degradation products.

The molecular structure of malvidin-3-glucoside degradation products are analyzed by MS-MS spectroscopy according to their molecular mass ([M\(^{+}\)] = 452.5 at \(t_g = 11.6\) min, and [M\(^{+}\)] = 466 at \(t_g = 15.9\) min (Figs. SI-5a and SI-5b). The chemical structure of the oxidation products allow suggesting an oxidation mechanistic pathway as follows: The reaction starts by the thermal decomposition of AAPH into alkyl, peroxy and oxy radicals as depicted in Scheme SI-1 and also previously reported (Karoui, Chalier, Finet, & Tordo, 2011; Kohri et al., 2009; Wahl, Zeng, Madison, DePinto, & Shay, 1998). The MS\(^{+}\) spectra of the peroxy radical ([M\(^{+}\)] = 154) localized at the LC-MS retention time of 3.3 min is depicted in Fig. SI-4a, and the corresponding hydroperoxide ([M\(^{+}\)] = 155) is localized at \(t_g = 4.7\) min.

Anthocyanins are efficient chain-breaking antioxidant because they can easily transfer a hydrogen atom to reactive peroxy radicals, as the phenoxyl radical generated can be stabilized by resonance allowing different mesomeric forms (Scheme SI-2). The phenoxyl radical is stable and do not extract hydrogen from other substance, but it can be hydrolyzed in the phosphate buffer (pH 7.4), and breakdown its structure into an acid and a phenol derivatives as previously reported (Castaneda-Ovando, Pacheco-Hernandez, Paez-Hernandez, Rodriguez, & Galan-Vidal, 2009; Fleschhut, Kratzer, Rechammer, & Kulling, 2006; Tsuda, Ohshima, Kawakishi, & Osawa, 1996). This is verified by the anthocyanins discoloration after reacting with AAPH, and the oxidation product does not absorb at 520 nm. Castaneda-Ovando et al. (2009) reported anthocyanin stability and the color variation with pH, and they concluded that the changes in the color of these compounds are more significant in the alkaline region due to their instability.

The acid and phenol groups generated by the anthocyanin breakdown structure may transfer hydrogen atoms to peroxy radicals and react with reactive free radicals to generate oxidation products (Tsuda et al., 1996). The mechanistic pathway of the malvidin-3-glucoside degradation products is depicted in Scheme 1, according to the MS-MS fragmentations.

The MS-MS spectra for the molecular mass [M\(^{+}\)] = 453 assigned to the oxidation product having a retention time of 11.6 min exhibits a major fragment with a mass of m/z = 301. It can be attributed to condensation of the phenolic derivatives with peroxy radicals. Another peroxy radical condensation occurring on this fragment results in the formation of products having a molecular mass m/z of 452.5 (see Scheme 1).
The degradation products of malvidin (phenolic derivative and syringic acid) can also co-condense through a hydrogen transfer process, and then react with alkyl radical. This allows forming a product with a molecular mass \( m/z \) of 466 (see Scheme 1).

Therefore, more than two radicals can be scavenged by an anthocyanin molecule contributing for maintaining its antioxidative activity. Further experiences demonstrated that identical reaction pathway is followed by other anthocyanins namely, delphinidin-, cyanidin-, and peonidin-mono-glycosides, with the same degradation product localized at the LC-MS retention time of 11.6 min having a molecular mass \([M^+]=452.5\). These observations confirmed the ORAC values portrayed in Table 2. The antioxidant potency of anthocyanins is enhanced with the accessibility of hydroxyl groups, but also by the liability of the sugar moieties.

4. Conclusions

The total anthocyanin content in blueberries of Lake Saint-Jean region (Quebec, Canada) was 290 mg/100 g, and twenty derivatives were identified. The major anthocyanins are the mono-glycoside isomers of malvidin, peonidin, cyanidin and delphinidin with a content of 163, 93, 73, and 91 mg of Cyanin equivalent/g extract respectively. Two successive solid phase extractions on hydrophobic silica gel (DSC-C18) and cationic exchange resin (DSC-SCX), and
a fractionation by preparative HPLC were required to isolate anthocyanin molecules with purity up to 100%. Delphinidin-3-galactoside has the highest oxygen radical absorbance capacity (13.062 ± 2.729 μmol TE/μmol), and malvidin-3-glucoside the lowest (0.851 ± 0.032 μmol TE/μmol). A suggested mechanism pathway describing the anthocyanins degradation into a phenol and acid derived can explain the high antioxidant activity of anthocyanins.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.076.

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