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

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Chemical compounds studied in this article:
Formic acid (PubChem CID: 284)
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Cyanidin-3,5-di-glucoside chloride (2-(3,4-dihydroxyphenyl)-3-(|A-d-glucopyranosyloxy)-7-hydroxychromenium-5-yl A-d-glucopyranoside chloride) (PubChem CID: 164999)
Malvidin-3,5-di-glucoside chloride (2-(4-hydroxy-3,5-dimethoxyphenyl)chromenylium-3,5,7-triol;chloride) (PubChem CID: 69512)
Cyanidin-3-glucoside (kuromanin) ((2S,3R,4S,5S,6R)-2-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromenylium-3-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol) (PubChem CID: 441667)
Delphinidin-3-glucoside (myrtillin) ((2S,3R,4S,5S,6R)-2-[5,7-dihydroxy-2-(3,4,5trihydroxyphenyl)chromenylium-3-yl] oxy-6-(hydroxymethyl)oxane-3,4,5-triol;chloride) (PubChem CID: 165558)
Malvidin-3-glucoside (oenin) chloride (2-[5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)chromenylium-3-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol;chloride) (PubChem CID: 12313693)
Silica (dioxosilane) (PubChem CID: 24261)
Fluorescein sodium salt (disodium;2-(3-oxido-6-oxoxanthen-9-yl) benzoate) (PubChem CID: 10608)
AAPH (2,2'-azobis[2-methylpropionamidine] dihydrochloride) (2-[(1-amino-1-imino-2-methylpropan-2-yl)diazenyl]-2methylpropanimidamide] dihydrochloride) PubChem CID: 76344)

ABSTRACT

An efficient process for the purification of anthocyanin monomeric 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 μmol TE/μmol), and malvidin-3-glucoside the lowest (0.851 ± 0.032 μmol TE/μmol). A mechanistic pathway preview is suggested for the anthocyanins scavenging free radical activity by hydrogen transfer.

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Trolox (6-hydroxy-2,5,7,8-tetramethylchro mane-2-carboxylic acid) (PubChem CID: 40634)
Ascorbic acid ((2R)-2-[(1S)-1,2-dihydroxye thyl]-3,4-dihydroxy-2H-furan-5-one) (PubChem CID: 54670067)
Monobasic potassium phosphate (potassium;dihydrogen phosphate) (PubChem CID: 516951)
Dibasic potassium phosphate (dipotassium; hydrogen phosphate) (PubChem CID: 24450)
Potassium hydroxide (potassium; hydroxide) (PubChem CID: 14797)

Keywords:
Blueberries
Vaccinium angustifolium Aiton and *Vaccinium myrtilloides* Michaux
Anthocyanins
Preparative-HPLC
ORAC

1. Introduction

The wild blueberries from Lake Saint-Jean region (Quebec, Canada) belong to the genus *Vaccinium angustifolium* Aiton and *Vaccinium myrtilloides* 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 anthocyanidin moiety is derived from the flavylium or 2-phenylbenzopyrilium 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, & Belkacemi, 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 preparation 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 CO_2 and pressurized liquids (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.