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RESEARCH NOTE

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Rapid Identification of Radical Scavenging Compounds in Blueberry Extract by HPLC Coupled to an On-line ABTS Based Assay and HPLC-ESI/MS

Chul Young Kim, Hee Ju Lee, Eun Ha Lee, Sang Hoon Jung, Dong-Un Lee, Suk Woo Kang, Sae Jin Hong[1], and Byung-Hun Um*

Natural Product Research Center, KIST Gangneung Institute, Techno Valley, Gangneung, Gangwon 210-340, Korea
[1]Department of Applied Plant Science, Kangnung National University, Gangneung, Gangwon 210-702, Korea

Abstract This study employed high performance liquid chromatography (HPLC) coupled to an on-line $ABTS^+$ radical scavenging detection (RSD) system along with HPLC-electro spin impact/mass spectrometry (ESI/MS), to rapidly determine and identify antioxidant compounds occurring in blueberry extract. The extract was separated by HPLC, and then the radical scavenging activities of the separated compounds were evaluated by the on-line coupled $ABTS^+$-RSD system. The negative peaks of the $ABTS^+$-RSD system, which indicates the presence of antioxidant activity, were monitored by measuring the decrease in absorbance at 734 nm. The active components in the blueberry extract were identified by HPLC-ESI/MS using their MS spectra and retention times. According to the data acquired from the on-line HPLC-$ABTS^+$-based assay and HPLC-ESI/MS systems, the antioxidant compounds detected in the blueberry extract were identified as chlorogenic acid and 11 anthocyanins.

Keywords: blueberry, *Vaccinium corymbosum* L., antioxidant, free radical scavenging, anthocyanin

Introduction

Free radicals and reactive oxygen species, such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (·OH), and peroxyl radicals (ROO·), are deemed harmful to human health and trigger many diseases, such as cancer, coronary heart diseases, inflammatory disorders, arteriosclerosis, and aging (1-6). Antioxidants are considered possible protective agents against oxidative damage in the human body, and currently, the intake of antioxidants from food components is of great interest.

Plants are rich sources of natural antioxidants such as ascorbic acid, carotenoids, tocopherols, and polyphenols (7). The polyphenols, in particular, have attracted considerable interest due to their beneficial effects as antioxidants and their abundance in certain fruits, vegetables, and beverages (8-13).

Recently, high performance liquid chromatography (HPLC) hyphenated techniques such as liquid chromatography-mass spectrometry (LC-MS) and LC-nuclear magnetic resonance (NMR) were developed; facilitating the identification of various chemical structures of hundreds of compounds found in crude extracts (14). As a complement to these approaches, on-line performed bioassays allow one to efficiently determine the bioactivity that is associated with peaks in chromatograms. Furthermore, on-line antioxidant screening systems have been developed using post column reactions with 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagents (15-18).

*Corresponding author: Tel: +82-33-650-7206; Fax: +82-33-650-7299
E-mail: albertum@kist.re.kr
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A considerable number of polyphenolic compounds, including anthocyanins, flavonols, chlorogenic acid, and procyanindins are contained in blueberry, which may relate to its potential health benefits. Anthocyanins are the violet pigments of blueberries and are especially potent antioxidants. Several studies have indicated the potential antioxidant properties of anthocyanins (19-21). In addition, anthocyanins were reported as having physiological functions such as vision improvement effects (22) and anticancer (23,24) and anti-inflammatory (25) activities.

Accordingly, the aim of this study was the rapid identification of antioxidants in blueberry extract using the on-line coupled HPLC-$ABTS^+$ based assay along with HPLC-ESI/MS.

Materials and Methods

Chemicals and reagents The following reagents were used in the radical scavenging assays: ABTS, DPPH, 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), and potassium persulfate, which were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The ethanol for the assays was HPLC grade, and all HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other solvents were purchased from Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea).

Plant material A freeze-dried blueberry sample (ca. 1 g) was extracted with a 50 mL mixture of methanol, acetic acid, and distilled water at a ratio of 25:1:24 by ultrasonic extraction at room temperature for 1 hr; it was then filtered through filter paper into a 50-mL volumetric flask. The extract was brought up to volume with methanol and

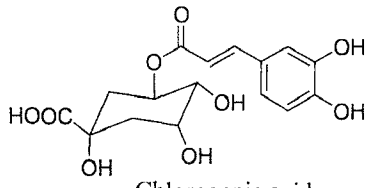
Table 1. Solvent gradient conditions for HPLC coupled to an on-line-ABTS$^+$ based assay

Time	Acetonitrile	0.1% TFA in H_2O
0	10	90
5	10	90
15	15	85
20	15	85
25	18	82
30	18	82
50	35	65

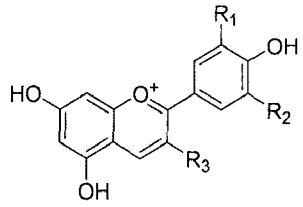
filtered prior to injection into the HPLC system.

On-line detection of radical scavenging activity The radical scavenging activity of the blueberry was determined using the on-line ABTS$^+$ assay according to the method of Stewart *et al.* (18) with some modification. A 2 mM ABTS$^+$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated overnight in the dark at room temperature, to allow for the stabilization of the radical. The ABTS$^+$ reagent was prepared by diluting the stock 8-fold in methanol. The blueberry extract (10 mL) was injected and separated using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with binary pumps, a diode array detection (DAD), a ultra violet (UV)/Vis detector, and an additional reagent pump. The analytical column was a reversed-phase Zorbax Eclipse XDB-C_{18} [150 mm length×4.6 mm i.d. and 5 μm particle size (Agilent Technologies)]. The mobile phase consisted of acetonitrile (solvent A) and water with trifluoroacetic acid (TFA) (0.1%, v/v) (solvent B). Table 1 presents the gradient programs that were used for the studied samples. DAD was performed in the 200-800 nm range, and the chromatographic profile was recorded at 520 nm. The sample injection volume was 10 μL and the flow rate was 0.4 mL/min. The analyses were performed at 40°C. The HPLC eluent from the DAD arrived at a 'T' piece, where the ABTS$^+$ was added. The ABTS$^+$ flow rate was 0.2 mL/min delivered by an additional Agilent 1200 pump. After mixing through a 1-mL loop maintained at 40°C, the absorbance was measured at 734 nm by a UV/Vis detector. Finally, the data were analyzed using ChemStation software (Agilent Technologies).

Identification of compounds by HPLC-ESI/MS The anthocyanins were identified by a Varian HPLC-MS system (Palo Alto, CA, USA). This system consisted of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200L triple quadrupole mass spectrometer equipped with an electrospray ionization source. The Varian MS workstation software (version 6.3) was used for data acquisition and processing. The HPLC conditions were the same as described above for the on-line detection of radical scavenging activity. The mass spectrometer conditions were as follows:positive ion mode; mass range=*m/z* 150-1,000; needle=5,000 V; shield=600 V; nebulizing gas pressure (N_2)=60 psi; drying gas (N_2) flow rate=20 psi; drying temperature=300°C.



Chlorogenic acid



Anthocyanin	R_1	R_2	R_3
Delphinidin-3-galactoside	OH	OH	Galactose
Delphinidin-3-glucoside	OH	OH	Glucose
Delphinidin-3-arabinoside	OH	OH	Arabinose
Petunidin-3-galactoside	OH	OCH_3	Galactose
Petunidin-3-glucoside	OH	OCH_3	Glucose
Petunidin-3-arabinoside	OH	OCH_3	Arabinose
Malvidin-3-galactoside	OCH_3	OCH_3	Galactose
Malvidin-3-glucoside	OCH_3	OCH_3	Glucose
Malvidin-3-arabinoside	OCH_3	OCH_3	Arabinose
Malvidin-3-acetyl-galactoside	OCH_3	OCH_3	Acetyl-galactose
Malvidin-3-acetyl-glucoside	OCH_3	OCH_3	Acetyl-glucose

Fig. 1. Structures of chlorogenic acid (1) and anthocyanins (2-12) in the blueberry extract.

Results and Discussion

On-line HPLC-ABTS$^+$ radical scavenging assay of blueberry extract The on-line coupling of separation and activity determination techniques has been achieved by mixing a solution combining a free radical with the eluate of an HPLC column (15-18). Such techniques allow for the rapid and selective detection of radical scavenging compounds in the presence of many other inactive constituents with minimal sample preparation. Here, we developed HPLC coupled to an on-line ABTS$^+$-based assay system and HPLC-ESI/MS, to rapidly identify radical scavenging compounds in blueberry extract. Following HPLC separation, the HPLC eluate was mixed with a stabilized solution of ABTS$^+$ radicals, and the solution was directed to a UV/Vis detector monitoring absorbance at 734 nm. The radical solution had a deep blue color, and any quenching of the ABTS$^+$ radical resulted in a loss of color, which was indicated by a negative peak on the absorbance profile being monitored at 734 nm.

Figure 1 shows the on-line HPLC-ABTS$^+$ analysis of the crude blueberry extract. Prior to the reaction with the ABTS$^+$ radical and the analysis of antioxidant potential at 734 nm (negative trace), 10 mL aliquots were analyzed by gradient reverse-phase HPLC with DAD at 520 and 280

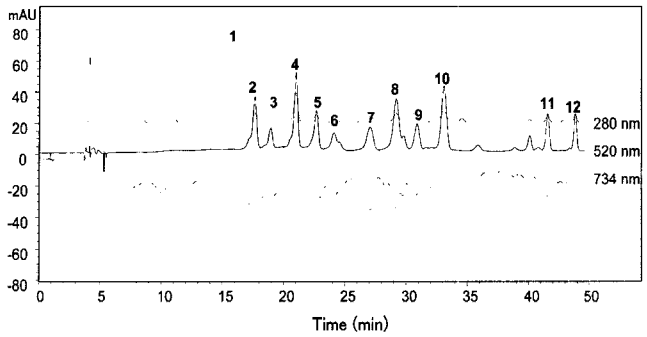


Fig. 2. On-line HPLC-ABTS$^+$ analysis of blueberry extract. Ten mL aliquots were analyzed by gradient reverse-phase HPLC with a DAD at 280 nm (positive trace, dotted line) and 520 nm (positive trace, solid line) prior to reaction with ABTS$^+$ radical and the analysis of antioxidant potential at 734 nm (negative trace).

nm (positive trace). The ABTS$^+$-based antioxidant activity profile showed that several compounds exhibited antioxidant activity. As shown in Fig.1, the on-line ABTS assay system was an efficient method for antioxidant purification, based on the activity-guided chromatographic separation and isolation of natural sources.

Identification of active compounds by HPLC-ESI/MS

The chromatographic analyses of the blueberry extract, which were monitored at 280 and 520 nm, showed the existence of 1 phenolic acid and 11 major anthocyanins, respectively (Fig. 2). Table 2 shows the results of the HPLC-PDA-ESI/MS analysis of the blueberry extract.

The UV spectrum of peak **1** showed a maximum absorbance at 326 nm and a shoulder at 296 nm, which are typical of caffeic acid derivatives. The mass analysis of peak **1** showed a $[M+H]^+$ ion at *m/z* 355 and $[M+Na]^+$ at *m/z* 377 for ESI-MS in the positive ion mode. This compound was identified as chlorogenic acid based on the UV and MS spectra and previously reported literature data (26,27).

The individual anthocyanins (peak **2-12**) were identified mainly by their HPLC retention times, elution order, and MS spectra compared to previously reported data (28,29). The determination of molecular weights by ESI-MS showed that only 3 widespread anthocyanins, delphinidin (*m/z* 303), petunidin (*m/z* 317), and malvidin (*m/z* 331), occurred in the blueberry extract. The aglycon moieties of peak **2** and **3** were identified as delphinidin because their MS spectra showed the same pseudomolecular ion peak at *m/z* 465 and MS/MS fragments at *m/z* 303. The MS spectra indicated that these 2 compounds contained delphinidin linked to 1 hexose. Based on the retention times and elution order data of the literature, compound 2 was identified as delphinidin 3-galactoside and compound 3 as delphinidin 3-glucoside (28-30).

The MS spectrum of peak **4** showed that compound 4 contained delphinidin linked to one pentose (M+ *m/z*=435; MS/MS *m/z*=303). When compared to the literature data, peak **4** was determined as delphinidin 3-arabinoside.

Peak **5** and **6** showed an M^+ ion at *m/z* 479 and a MS/MS fragment at *m/z* 317, suggesting they contained petunidin linked to 1 hexose. Upon comparing their MS spectra and retention times to the literature, peak **5** and **6** were identified as petunidin-3-galactoside and petunidin-3-glucoside, respectively. For peak **7**, an M^+ ion at *m/z* 449 and a MS fragment at *m/z* 317 revealed it was petunidin linked to arabinose. Peaks **8-12** contained malvidin (MS/MS *m/z*=331) as their aglycon. Peaks **8-10** were identified as malvidin-3-galctoside, malvidin-3-glucoside, and malvidin-3-arabinoside, respectively, after comparing their retention times and MS spectral data with the literature. When anthocyanin sugar moieties are acetylated, a loss of polarity results, increasing the retention time (28). Peak **11** and **12** were thought to contain malvidin linked to acetylated hexose based on their increased retention times (40.4 and 43.1 min, respectively) and M^+ ions (*m/z*=535) and MS fragments (*m/z*=331). By comparing their data to the literature (28), peak **11** and **12** were identified as malvidin-3-acetyl-galactoside and malvidin 3-acetyl-glucoside, respectively.

Table 2. Peak assignment, retention time (RT), and MS spectral data of anthocyanins detected in blueberry extract

Peak	RT (min)	Identification	*m/z* [M$^+$]	Fragments
1	14.6	Chlorogenic acid	355[1)], 377[2)]	
2	15.8	Delphinidin-3-galactoside	465	303
3	17.4	Delphinidin-3-glucoside	465	303
4	19.8	Delphinidin-3-arabinoside	435	303
5	21.6	Petunidin-3-galactoside	479	317
6	22.8	Petunidin-3-glucoside	479	317
7	25.1	Petunidin-3-arabinoside	449	317
8	26.8	Malvidin-3-galactoside	493	331
9	28.9	Malvidin-3-glucoside	493	331
10	31.6	Malvidin-3-arabinose	463	331
11	40.4	Malvidin-3-acetyl-galactoside	535	331
12	43.1	Malvidin-3-acetyl-glucoside	535	331

[1)]$[M+H]^+$, [2)]$[M+Na]^+$.

In this study, blueberry anthocyanins were analyzed using HPLC coupled to an on-line-ABTS$^+$ based assay system. One phenolic acid (chlorogenic acid) and 11 major anthocyanins were identified using HPLC-ESI/MS and their antioxidant activities were detected simultaneously. Overall, HPLC coupled to an ABTS$^+$ based assay along with HPLC-ESI/MS analysis allows for the rapid identification of antioxidants in a natural product.

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