Food Sci. Biotechnol. 23(6): 1753-1760 (2014)
DOI 10.1007/s10068-014-0240-z

RESEARCH ARTICLE

Antioxidant Activity of Phenolic Compounds from Canola (*Brassica napus*) Seed

Hyun-Il Jun, Dennis P. Wiesenborn, and Young-Soo Kim

Received January 24, 2014; revised June 14, 2014; accepted June 26, 2014; published online December 31, 2014
© KoSFoST and Springer 2014

Abstract The antioxidant activities of various extracts from canola (*Brassica napus*) seed were investigated using the DPPH assay, ABTS radical assay, and reducing power. An 80% methanol extract from canola seed was sequentially fractionated and separated according to the solvent polarity and Sephadex LH-20 chromatography, respectively. The antioxidant activities of various extracts and their total phenolic content (TPC) and total flavonoid content (TFC) were closely correlated, resulting in correlation coefficient values higher than 0.87. Of all extracts, the sub-fraction 3 had the highest TPC (462.3 μg/mg) and TFC (75.4 μg/mg) while it showed the lowest EC_{50} value (183.1 μg/mL). The EC_{50} value of sub-fraction 3 measured using the DPPH radical assay was 1.3 times lower than that of butylated hydroxytoluene (BHT). The major free phenolic compound was *trans*-sinapic acid (193.4 μg/mg) and sinapic acid derivatives identified as 1-*O*-β-D-glucopyranosyl sinapate and 1,2-di-*O*-sinapoyl-β-D-glucose in sub-fraction 3.

Keywords: canola seed, antioxidant activity, phenolic compound, sinapic acid, sinapic acid derivatives

Introduction

Seeds, fruits, and vegetables have recently been highlighted for the importance of some of their functional properties. One particularly important property is antioxidant activity, which is routinely characterized using various *in vitro* and *in vivo* systems. Phenolic compounds in plants play an important role as primary antioxidants or free radical terminators, suggesting their use to prevent the oxidative breakdown of lipids and to prevent chronic diseases from reactive oxygen species (ROS) (1-3). The potential antioxidant activity of food and medicinal plants is well-known to be associated with phenolic content, type, and structure (4,5).

Canola (*Brassica napus*) is in high demand for processing into animal feed and vegetable oils due to its favorable nutritional characteristics, and it has much higher content of phenolic compounds compared to other oilseeds (6,7). Phenolic acids, such as sinapic acid, gentisic acid, protocatechuic acid, vanillic acid, syringic acid, ρ-hydroxybenzoic acid, ρ-courmaric acid, caffeic acid, and ferulic acid, have been reported in canola seed (7). Of various phenolic acids in canola seed, the hydroxycinnamic acids (sinapic acid, caffeic acid, cinnamic acid, chlorogenic acid, coumaric acid, and ferulic acid, etc.) have attracted considerable attention due to their various biological activities, including antioxidant activity (8,9). In particular, sinapic acid and its derivatives are dominant phenolic antioxidants in canola seed and are well-known to be as effective as synthetic antioxidants (10). Therefore, canola seed might be an important source of natural antioxidants in the food industry as well as in the livestock industry for animal feeds. Nonetheless, few studies have been reported on isolation, characterization, and identification of phenolic antioxidants in canola seed.

The amount of phenolic compounds in canola extract is affected by the extraction method used, indicating that the extraction solvent and isolation steps should be considered to maximize the phenolic compounds in canola seed extracts (11-13). Therefore, the aims of this study were to evaluate the antioxidant activity of canola seed for use as a natural food preservative and to identify the sinapic acid and its

Hyun-Il Jun, Young-Soo Kim (✉)
Department of Food Science & Technology, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea
Tel: +82-63-270-2569; Fax: +82-63-270-2572
E-mail: ykim@jbnu.ac.kr

Dennis P. Wiesenborn
Department of Agricultural and Biosystems Engineering, North Dakota State University, Fargo ND 58105-6050, USA

derivatives with strong antioxidant activity. We obtained various extracts (80% methanol extract, fractions, and sub-fractions) from canola seed through multiple steps, and then determined their antioxidant activities and total phenolic content. Finally, the sinapic acid and its derivatives with strong antioxidant activity were identified using HPLC and ion-trap time-of-flight (IT-TOF) MS.

Materials and Methods

Materials and reagents Four varieties of canola (*B. napus*) seed [varieties IS7145RR, Invigor 5630(1), Invigor 5550, and Hyola] produced in North Dakota, USA in 2007 were ground using a Braun coffee grinder for 0.5 min, which was sufficient to pass them through a 150 μm sieve. Standards of Folin-Ciocalteau reagent, DPPH, ABTS, butylated hydroxytoluene (BHT), gallic acid, protocatechuic acid, chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid, *trans*-sinapic acid, salicylic acid, and *trans*-cinnamic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other reagents used in the study were of analytical grade.

Preparation of canola seed extracts Defatted canola seed flour (100 g) was added to 0.5 L of 80% aqueous methanol and was stirred using a magnetic stirrer for 24 h at 25°C to extract phenolic antioxidants. After centrifuging at 5,000×*g* for 15 min, the supernatant was filtered through a filter paper (Whatman No. 1; Whatman, Little Chalfont, UK). This procedure was repeated with the pellet two times, and the filtrates were then pooled, concentrated by evaporation at 40°C and freeze-dried. The yield of this freeze-dried extract was 17.3%. Five batches of extract were prepared from each canola seed variety, and extracts from the same seed variety were pooled.

The methanol extract was fractionated sequentially in triplicate at room temperature as follows: first, distilled water (400 mL) was added to the freeze-dried methanol extract (76 g) and the solution was stirred for 6 h. Then it was washed sequentially with *n*-hexane (400 mL, 12 h), chloroform (400 mL, 12 h), ethyl acetate (400 mL, 12 h), and *n*-butanol (400 mL, 12 h) 3 times. Each of these 4 fractions were centrifuged, filtered, evaporated, and freeze dried as described above. Yields of *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water fractions from the 80% methanol extract were 12.4, 0.8, 1.3, 14.4, and 50.2%, respectively.

The ethyl acetate fraction was further fractionated by gel filtration chromatography as follows: first, freeze-dried extract (300 mg) was dissolved in 2 mL of 85% aqueous methanol, and it was then loaded onto a column (2.5×76 cm) packed with Sephadex LH-20 resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The sample was eluted with 85% methanol at a flow rate of 0.5 mL/min, and the filtrate was collected as 10 mL of aliquots using a fraction collector. Absorbance at 282 and 326 nm (UV-1650PC Spectrophotometer; Shimadzu Co., Kyoto, Japan) was used to isolate three principle peaks. Aliquots for each sub-fraction were pooled, evaporated, and freeze-dried using the same methods as described above. The yields of sub-fractions 1, 2, and 3 from the ethyl acetate fraction were 9.6, 55.2, and 19.2%, respectively. Each extract was then dissolved in 80% aqueous methanol at a concentration of 0-8 mg/mL to analyze for phenolic compound content and antioxidant activity.

Determination of total phenolic content (TPC) and total flavonoid content (TFC) TPC was measured using the method described by Dewanto *et al*. (14). Folin-Ciocalteu's phenol reagent (0.2 mL) and 5% Na_2CO_3 (3 mL) were added to each sample (0.1 mL). The mixture was shaken and allowed to stand for 1 h in the dark at room temperature. The absorbance was then measured at 725 nm (UV-1650PC Spectrophotometer; Shimadzu Co.). The standard was *trans*-sinapic acid, and total phenolic contents were expressed as *trans*-sinapic acid equivalents.

TFC was measured using the method of Jia *et al*. (15). The sample (500 μL) was mixed with 5% $NaNO_2$ (75 μL), and then the mixture was shaken and allowed to stand for 5 min at room temperature. The mixture was then sequentially mixed with 150 μL 10% $AlCl_3$, 500 μL 1 M NaOH, and 275 μL distilled water. The absorbance was measured at 510 nm using a spectrophotometer. Catechin was used as a standard, and total flavonoid content was expressed as catechin equivalent.

Antioxidant activity analysis DPPH radical scavenging ability (RSA) was measured using the method described by Brand-Williams *et al*. (16). The sample (0.2 mL) was mixed with 2.8 mL 60 μM DPPH, and the mixture was allowed to stand for 30 min in the dark at room temperature. The absorbance (A) was measured at 515 nm, and BHT was used for comparison. The DPPH RSA was calculated by using the following formula:

$$\text{DPPH RSA (\%)} = [(A_{515\,\text{nm of control}} - A_{515\,\text{nm of sample}})/A_{515\,\text{nm of control}}] \times 100$$

The half maximal effective concentration (EC_{50}) value (μg/mL) is the effective concentration at which the DPPH radical is scavenged by 50%, and it was calculated by interpolation of the data.

The ABTS radical cation scavenging ability (RCSA) was measured using the method described by Arts *et al*. (17). Each sample (30 μL) was mixed with 3 mL ABTS radical cation solution, and the mixture was allowed to

stand for 7 min in the dark at room temperature. The absorbance was then measured at 734 nm, and BHT was used for comparison. ABTS radical cation solution contained 2.45 mM potassium persulfate and 7 mM ABTS stock solution, and was allowed to stand for more than 12 h in the dark at room temperature prior to use. For the control, 5 mM phosphate-buffered saline (PBS, pH 7.4) was added to 2.45 mM ABTS radical cation solution to attain an absorbance of 0.70±0.02 at 734 nm. The ABTS RCSA was calculated according to the following formula:

$$\text{ABTS RCSA (\%)} = [(A_{734\text{ nm of control}} - A_{734\text{ nm of sample}})/A_{734\text{ nm of control}}] \times 100$$

The EC_{50} value (μg/mL) is the effective concentration at which the ABTS radical cation was scavenged by 50%.

The reducing power was measured using the method described by Oyaizu (18). The sample (1 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% $K_2Fe(CN)_6$. After incubating the mixture at 50°C for 20 min, 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 2,200×*g* for 5 min. Supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance was measured at 700 nm, and BHT was used for comparison. The EC_{50} value (μg/mL) is the effective concentration at which the absorbance was of 0.500 for reducing power.

Analysis of free phenolic acids and sinapic acid derivatives by HPLC and LCMS-IT-TOF Free phenolic acids were measured using an HPLC analysis system (Sycam; Gilching, Germany) according to the method described by Jin *et al*. (19). A Sunfire C18 column (25 cm×4.6 mm, 5 μm; Waters Co., Milford, MA, USA) with a C18 guard column (2 cm×4.6 mm) was used at 25°C. The separated phenolic acids were detected at 280 and 325 nm by using a photodiode array detector (PDA, S3210; Sycam). Hydroxybenzoic acids and hydroxycinnamic acids were detected at 280 and 325 nm, respectively. The mobile phase consisted of 0.1% formic acid in 10% acetonitrile (solvent A) and 0.1% formic acid in 90% acetonitrile (solvent B). The flow rate was kept at 1 mL/min for a total run time of 45 min, and the gradient program was as follows: 100% A for 2 min, 100% A to 90% A in 4 min, 90% A to 37% A in 25 min, 37% A to 50% A in 10 min, 50% A to 100% A in 4 min, 100% A for 5 min.

The HR mass spectrum of a component in sub-fraction 3 was recorded on a Hybrid IT-TOF-MS (LCMS-IT-TOF; Shimazu Co.) with an electrospray ionization source (ESI-IT-TOF) in negative ion mode according to the method described by Li *et al*. (20). The sample was dissolved in methanol, and the injection volume was 10 μL. The column used was a LiChrospher RP-18 (12.5 cm×4.0 mm, 5 μm; Merck KGaA, Darmstadt, Germany) and was eluted at 30°C. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) and was eluted into a photodiode array detector and MS detector in order to detect and identify peaks. The flow rate was kept at 0.2 mL/min for a total run time of 30 min. The gradient program was as follows: 90% A to 20% A in 20 min, 20% A to 0% A in 5 min, only B for 5 min. UV-absorption was monitored at 280 nm. The LCMS-IT-TOF was operated under the following conditions: the ionization mode was a negative ion to increase sensitivity and maximize ionized fragment. Nitrogen was used as the nebulizer gas, and the flow was held at a rate of 1.5 L/min. The curve dissolution line (CDL) and block heater (BH) temperatures were kept at 200°C. Prove voltage and drying gas pressure were 4.5 kV and 200 kPa, respectively. Mass accuracy was calibrated with an external standard solution. Mass data were collected in the range from *m/z* 0 to *m/z* 700 for $MS^{1\text{-}3}$ and were then recorded by LC/MS solution version 3.4 software (Shimazu Co.), including a formula predictor to predict chemical formulas.

Statistical analysis All data were expressed as mean± standard deviation (SD) for at least triplicate analyses on the same sample. Data were analyzed by ANOVA and regression analysis using the SAS statistical analysis system (SAS Institute, Inc., Cary, NC, USA). Differences among samples were analyzed using Duncan's multiple range test ($p<0.05$). Correlations between phenolic compounds (i.e., TPC and TFC) and each of the EC_{50} values of antioxidant activity were calculated by using Pearson correlation.

Results and Discussion

Antioxidant activities of various extracts from canola seed The EC_{50} values of 80% methanol extract from various canola seed varieties on DPPH radicals are shown in Fig. 1. The extract from canola variety IS7145RR exhibited the lowest EC_{50} value (693 μg/mL), thereby demonstrating the best ability to stabilize reactive DPPH radicals via the electron donor mechanism (13). This indicated that the extract from variety IS7145RR had a higher content of antioxidant compounds and/or compounds with greater potency, and thus it was subsequently used for further antioxidant study.

Aqueous 80% methanol extracts from variety IS7145RR were fractionated sequentially depending on the polarity of the solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water). The ethyl acetate fraction, which showed the highest antioxidant activity, was further sub-fractionated by

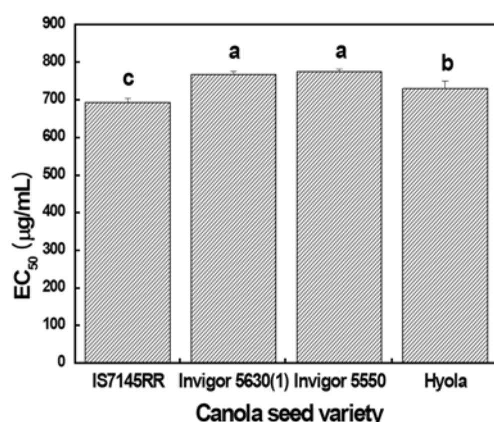

Fig. 1. EC50 values of 80% methanol extract from varieties of canola seed by DPPH radical assay. The vertical bars represent mean±standard deviation (*n*=3). Different lower case letters in the same bar are significantly different by Duncan's multiple test ($p<0.05$). EC50 values are expressed as effective concentration at which antioxidant activity using DPPH radicals were scavenged by 50%.

Sephadex LH-20 chromatography into three sub-fractions (sub-fractions 1-3). The antioxidant activities of these various extract fractions were then determined by using the DPPH radical and ABTS radical assays, and reducing power. Their antioxidant activities were dependent upon their concentration within the range of 0 to 8 mg/mL (data not shown). The results indicated that each extract from canola seed had antioxidant activities, playing a role as terminators of DPPH radical and ABTS radical cations, as well as reductants against ferrous ions in the sample matrix. The EC_{50} values of all extracts from canola seeds variety IS7145RR are shown in Table 1.

Of the 5 fractions, the EC_{50} values of chloroform, ethyl acetate, and *n*-butanol fractions were lower for DPPH radical, ABTS radical, and reducing power assays, but *n*-hexane and water fractions were higher than those of 80% methanol extract. The ethyl acetate fraction exhibited the lowest EC_{50} values for DPPH radical assay, ABTS radical assay, and reducing power compared to those of other fractions, thereby showing that this fraction has the highest antioxidant activity. In addition, the EC_{50} values of the ethyl acetate fraction were 4.2, 5.3, and 4.1 times lower than those of the 80% methanol extract for DPPH radical assay, ABTS radical assay, and reducing power, respectively. The results indicated that some compounds involved in antioxidant activity might be selectively concentrated in the ethyl acetate fraction during fractionation of the 80% methanol extract from canola seed. Therefore, the choice of extraction solvent is a very important factor, because the solubility of antioxidant compounds found in canola seed is influenced by the polarity of the extraction solvents (13,21).

The UV-absorbance values of sub-fraction 1 at 282 and 326 nm were similar, while sub-fractions 2 and 3 showed significantly higher UV-absorbances at 326 nm than those seen at of 282 nm (Fig. 2). The sensitive UV-absorbance at 328 nm is attributed to sinapic acid and its derivatives in sub-fractions, having maximum absorbance in the range from 322 to 334 nm (12,21,22). This result might be explained by the fact that the contents of hydroxycinnamic acids were higher than those of hydroxybenzoic acids. Of the three sub-fractions, the EC_{50} of sub-fraction 3 had the lowest values for all three antioxidant activity assays, whereas the EC_{50} of sub-fraction 1 had the highest values. In particular, the EC_{50} value of sub-fraction 3 was lower for DPPH radical assay than that of the comparison (BHT). Another

Table 1. EC_{50} value of various extracts from canola seed

Extracts		EC_{50} value[1)] (μg/mL) of antioxidant activity		
		DPPH radical assay	ABTS radical assay	Reducing power
80% methanol extract		699.2±9.9$^{cC2)}$	2,501.4±8.0^{aC}	1,160.0±30.8^{bC}
Fractions	*n*-Hexane	1,607.7±60.0^{bB}	3,843.3±119.2^{aB}	1,585.9±3.8^{cB}
	Chloroform	289.8±5.7^{cE}	659.0±19.3^{aE}	401.7±3.4^{bE}
	Ethyl acetate	165.5±1.9^{cF}	470.4±0.3^{aF}	282.8±1.3^{bF}
	n-Butanol	304.0±1.4^{cD}	848.6±1.3^{aD}	486.3±25.8^{bD}
	Water	1,757.0±40.3^{cA}	5,397.0±7.5^{aA}	2,538.5±3.0^{bA}
Sub-fractions	Comparison[3)]	165.6±0.4^{aC}	163.7±0.3^{bD}	135.2±2.6^{cD}
	Sub-fraction 1	438.1±3.1^{bA}	607.0±5.1^{aA}	368.2±3.7^{cA}
	Sub-fraction 2	183.1±2.2^{cB}	407.6±16.2^{aB}	258.8±9.3^{bB}
	Sub-fraction 3	142.6±2.2^{cD}	367.6±10.6^{aC}	217.7±1.1^{bC}

[1)]EC_{50} values are expressed as effective concentration at which antioxidant activity using DPPH or ABTS cation radical were scavenged by 50%; and absorbance was 0.5 for reducing power, respectively.
[2)]Values are presented as mean±SD (*n*=3). Different small letters (a, b, and c, etc.) in the same row are significantly different by Duncan's multiple test ($p<0.05$). Different capital letters (A, B, and C, etc.) in the same column indicate a significant difference according to Duncan's multiple test ($p<0.05$).
[3)]Comparison is butylated hydroxytoluene (BHT).

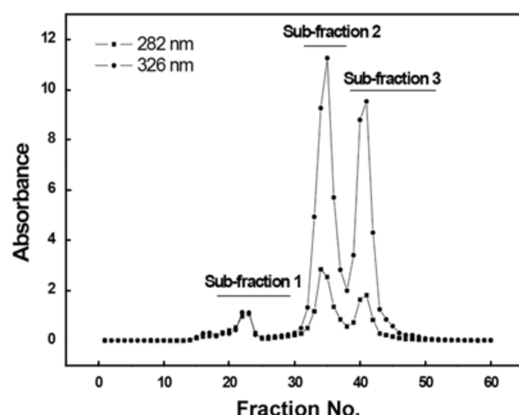

Fig. 2. Sephadex LH-20 chromatogram of sub-fractions from canola seed. Sephadex LH-20 column (2.5×76 cm) eluted with 85% methanol at a flow rate of 1 mL/min. Absorbance values are expressed as the filtrate equivalents (10 mL).

study also reported that their best fraction had a stronger antioxidant activity than BHT, indicating that the most effective phenolic compounds in the fractions can be isolated by Sephadex LH-20 chromatography (10).

TPC and TFC of various extracts from canola seed The TPC and TFC in various extracts from canola seed are expressed as *trans*-sinapic acid and catechin equivalents in Table 2. Also, regression analysis for correlations between phenolic compounds and their EC_{50} values of antioxidant activities was performed, and their correlation coefficients (R) and slopes were obtained. The TPC in various extracts varied from 20.0 μg/mg for the water fraction to 462.3 μg/mg for sub-fraction 3. The results were consistent with those seen in a previous report which stated that various fractions from canola hull were separated by Sephadex LH-20 chromatography (23). On the other hand, the TPCs in chloroform, ethyl acetate, and *n*-butanol fractions were higher than those present in the 80% methanol extract, those present in *n*-hexane and water fractions were lower. Our data showed a similar pattern with the previous report in which ethanol extracts from canola seed contained TPC in the range of 52.2 to 70.9 μg/mg (13). Moreover, sub-fraction 3 showed the highest TPC (462.3 μg/mg) as well as the highest antioxidant activity, followed by the ethyl acetate fraction (389.7 μg/mg), indicating that antioxidant activity might be directly correlated with phenolic compounds due to the presence of their hydroxyl groups (4,24).

The TFC of 80% methanol extract showed 34.9 μg/mg. Among the fractions and sub-fractions, the ethyl acetate fraction and subfraction 3 showed the highest values (66.7 and 75.4 μg/mg), resulting in lower content than their TPC. However, it is a similar pattern compared to those of each TPC in various extracts from canola seed. The TFC of 80% methanol extract resulted in higher content than that of our previous study in which the TFC of four extracts (i.e., 100% methanol, 100% ethanol, 100% acetone, and water extract) of canola meal ranged from 8.6 to 21.3 μg/mg (25). This discrepancy might be due to the isolation method of canola seed and meal, suggesting that 80% methanol aqueous mixture solvent when used as a crude extract solvent was more effective than 100% methanol, 100% ethanol, 100% acetone, and water extract solvent.

The *R* values between phenolic compounds and each EC_{50} value of three antioxidant activities were higher than 0.87, indicating a strong relationship between the two parameters. Previous research has also shown significant correlation between antioxidant activity and TPC in seven rapeseed varieties, resulting in *R* values ranged between 0.87 and 0.95 (26). In particular, the higher slope value of

Table 2. Total phenolic content (TPC) and total flavonoid content (TFC) of various extracts from canola seed

Extracts		TPC[1)] (μg/mg)	TFC[2)] (μg/mg)	Regression analysis[3)] for EC_{50} values of antioxidant activity		
				DPPH radical assay	ABTS radical assay	Reducing power
80% methanol extract		71.9±0.4^{aD}	34.9±1.3^{bB}[4)]			
Fractions	*n*-Hexane	43.0±1.1^{aE}	23.1±3.1^{bC}	$Y=-3.40X_1+1{,}425.37$ (R=0.87)	$Y=-10.05X_1+4{,}055.51$ (R=0.88)	$Y=-4.34X_1+1{,}838.25$ (R=0.87)
	Chloroform	278.2±3.3^{aB}	66.8±0.7^{bA}			
	Ethyl acetate	389.7±4.8^{aA}	66.7±0.5^{bA}			
	n-Butanol	228.4±4.4^{aC}	36.2±1.7^{bB}			
	Water	20.0±0.7^{F}	9.7±0.3^{bD}			
Sub-fractions	Sub-fraction 1	263.7±7.1^{aC}	53.0±0.5^{bC}	$Y=-24.63X_2+1{,}791.99$ (R=0.88)	$Y=-73.25X_2+5{,}161.62$ (R=0.90)	$Y=-31.94X_2+2{,}329.97$ (R=0.90)
	Sub-fraction 2	371.3±8.1^{aB}	62.1±1.2^{bB}			
	Sub-fraction 3	462.3±11.2^{aA}	75.4±0.5^{bA}			

[1)]Total phenolic content expressed as *trans*-sinapic acid equivalent per mg of extract, fraction, and sub-fraction.
[2)]Total flavonoid content expressed as μg catechin equivalent per mg of extract, fraction, and sub-fraction.
[3)]X_1, X_2, Y, and R represent total phenolic content, total flavonoid content, EC_{50} values of antioxidant activity, and correlation coefficient, respectively.
[4)]Values are presented as mean±SD (n=3). Different small letters (a, b, and c, etc.) in the same row are significantly different by Duncan's multiple test ($p<0.05$). Different capital letters (A, B, and C, etc.) in the same column indicate a significant difference according to Duncan's multiple test ($p<0.05$).

Table 3. Free phenolic acid composition of 3 extracts from canola seed

Phenolic acids		80% Methanol extract (mg/g)	Ethyl acetate fraction (mg/g)	Sub-fraction 3 (mg/g)
Hydroxybenzoic acids	Gallic acid	10.4±0.2[aB1)]	5.8±0.7[bC]	2.7±0.1[cF]
	Protocatechuic acid	4.8±0.4[aC]	1.5±0.3[bE]	1.6±0.1[bG]
	Salicylic acid	ND[2)]	5.9±0.6[aC]	6.5±0.5[aD]
	p-Hydroxybenzoic acid	ND	2.7±0.2[bD]	9.0±1.4[aC]
Hydroxycinnamic acids	Caffeic acid	0.1±0.0[bE]	1.3±0.2[aE]	1.6±0.4[aG]
	Ferulic acid	ND	0.8±0.2[bF]	3.2±0.6a[EF]
	trans-Sinapic acid	41.5±0.4[cA]	141.3±5.7[bA]	193.4±6.7[aA]
	Chlorogenic acid	2.5±0.3[cD]	32.0±0.2[aB]	14.4±0.3[bB]
	trans-Cinnamic acid	ND	ND	3.7±0.7[aE]
Total		59.3±0.7[c]	191.2±6.6[b]	236.1±8.1[a]

[1)]Values are presented as mean±SD (n=3). Different small letters (a, b, and c, etc.) in the same row are significantly different by Duncan's multiple test (p<0.05). Different capital letters (A, B, and C, etc.) in the same column indicate a significant difference according to Duncan's multiple test (p<0.05).
[2)]ND, not detected

the DPPH radical assay might indicate that phenolic compounds in canola seed are a suitable antioxidant when compared to other antioxidant activity assays (16). In addition, previous reports on canola seed extract discussed implications in the reduction of lipid oxidation, which is the main cause of toxic and carcinogenic compounds in lipid-containing food products, such as meat and oil products (10,27). Phenolic compounds in canola seed extracts were highly correlated with antioxidant activity, suggesting that the phenolic compounds in canola seed could play a critical role in antioxidant activity. Based on our findings, consumption of canola seed and phenolic compounds concentrates from canola seed might protect against diseases which are associated with free radicals.

Composition of free phenolic acids in various extracts from canola seed The analysis of free phenolic acid composition in three extracts (80% methanol extract, ethyl acetate fraction, and sub-fraction 3) from canola seed is shown in Table 3. The total contents of free phenolic acid increased in order of 80% methanol extract, ethyl acetate fraction, and sub-fraction 3. In addition, hydroxycinnamic acids were major phenolic acids in all extracts. Hydroxycinnamic acids are a class which consists of more than one phenolic hydroxyl group with a C3-C6 skeleton. Their chemical structures of hydroxycinnamic acids are known to have a significantly higher antioxidant activity than those of hydroxybenzoic acids bearing no such functionalities (4,9). The results indicate that their chemical structures as well as the TPC also play key roles in antioxidant activity, depending on the number and position of free hydroxyl and methoxyl groups attached to the aromatic ring. Some phenolic acids, such as *p*-hydrobenzoic acid, ferulic acid, and *trans*-cinnamic acid were detected in sub-fraction 3 but were not detected in the 80% methanol extract. In addition, the most abundant phenolic acid in the three extracts was *trans*-sinapic acid, which represented 70 to 82% of the total phenolic acid content of those extracts. These results are consistent with those of previous reports in which sinapic acid (over 85.7% of the free phenolic acids) was determined to be a dominant phenolic antioxidant in canola seed (26,27).

Various sinapic acid derivatives as well as sinapic acid are present in *Brassica* vegetables including canola seed and might be also identified in canola seed (21,22,28). Identification of phenolic compounds in a given mixture is often very difficult to accomplish by HPLC due to its dependence on retention time. Therefore, the ESI-IT-TOF $MS^{1\text{-}3}$ analysis was used to further investigate the identification and structure of sinapic acid derivatives found in sub-fraction 3 from canola seed (Fig. 3). Three characteristic ion products for $[M\text{-}H]^-$ were observed at m/z 223.0613, m/z 385.1113, and m/z 591.1666 in the MS spectrum, respectively (Fig. 3A). The ion product for standard of *trans*-sinapic acid ($C_{11}H_{12}O_5$) was m/z 223.0620 (The calculated $[M\text{-}H]^-$ for $C_{11}H_{12}O_5$ was m/z 223.0612). One characteristic ion product ($C_{17}H_{22}O_{10}$) at m/z 385.1113 was identified as 1-*O*-β-D-glucopyranosyl sinapate, in which its structure was determined as a glycoside of sinapic acid, corresponding to sinapic acid (224 Da) and glucose moiety (162 Da), respectively (22,29). In particular, this compound is known to be associated most closely with antioxidant activity compared to various natural antioxidants presented in canola seed (30).

Two main ion products at m/z 223.0600 ($C_{11}H_{12}O_5$) and m/z 367.1015 ($C_{17}H_{20}O_9$) and two minor ion products at m/z 208.0380 and m/z 353.0851 were observed in the MS^2 spectrum for m/z 591.1666 (Fig. 3B). The results of MS^2 spectrum provided strong evidence that the methyl group (CH_3, 15 Da), presenting the characterized ion for the

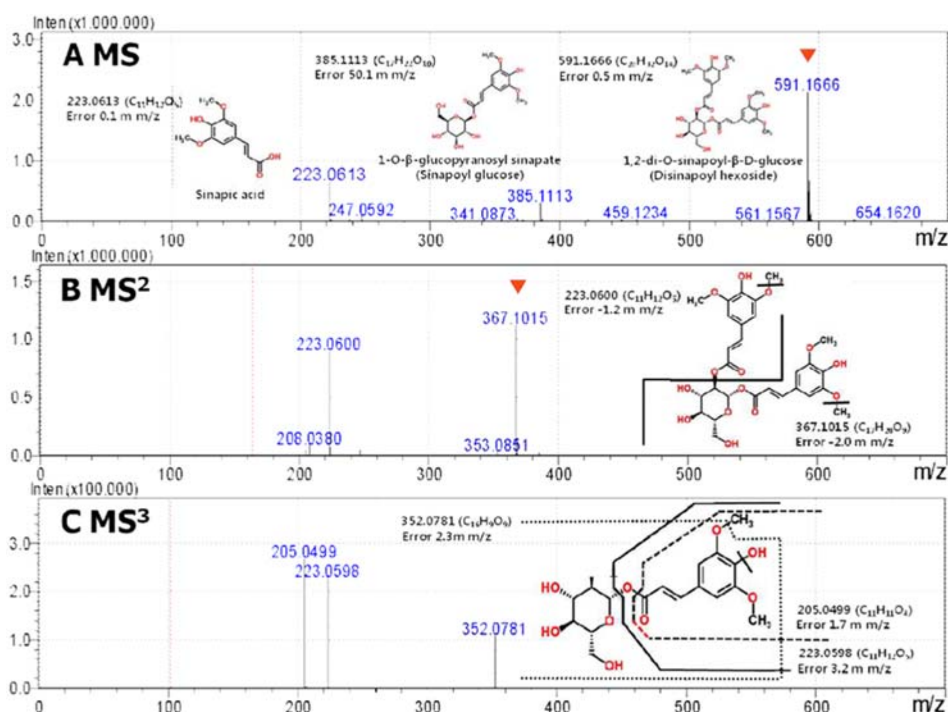

Fig. 3. Mass spectrum for sub-fraction 3 from canola seed by ESI-IT-TOF MS1-3 in negative ion mode. (A) MS, (B) MS^2, (C) MS^3.

hydroxycinnamic acids, was fragmented from the main molecule $C_{11}H_{12}O_5$ (*m/z* 223208) and $C_{17}H_{20}O_9$ (*m/z* 367353). In addition, according to the MS^3 spectrum for *m/z* 367.1015 (Fig. 3C), 3 characteristic ion products were observed at *m/z* 205.1499, *m/z* 223.0598, and *m/z* 352.0781. The first characteristic ion product at *m/z* 223.0598, presenting the characterized ion for sinapic acid (224 Da), indicated that the loss of sugar moiety (144 Da) from the mother molecule $C_{11}H_{12}O_5$ resulted in a fragment (*m/z* 367223). The second characteristic ion product at *m/z* 352.0781 indicated that the loss of the methyl group (15 Da) from the mother molecule results in a fragment (*m/z* 367352). These results similarly showed a fragmentation pattern to the MS^2 spectrum. The third characteristic ion product at *m/z* 205.1499 indicated that the loss of hydroxyl group (18 Da) from one main molecule (sinapic acid) results in a fragment (*m/z* 223205) and/or loss of sugar moiety (162 Da) from the mother molecule results in a fragment (*m/z* 367205). Therefore, its structure, based on the $MS^{1\text{-}3}$ analysis for *m/z* 591.1666 was identified as 1,2-di-*O*-sinapoyl-β-D-glucose (31).

In conclusion, the sub-fraction 3 from canola seed showed a higher antioxidant activity relative to a synthetic antioxidant (BHT) in the DPPH radical assay, and it had the highest TPC, TFC, and total phenolic acid in addition to *trans*-sinapic acid, which was the most abundant free phenolic acid, and sinapic acid derivatives, such as 1-*O*-β-D-glucopyranosyl sinapate and 1,2-di-*O*-sinapoyl-β-D-glucose in sub-fraction 3. As a result, sub-fraction 3 from canola seed may be useful as a natural antioxidant, having strong antioxidant activities. However, further studies might be needed to investigate antioxidant activity for reducing the lipid oxidation in a high-fat food system and the more detailed structure of sinapic acid derivatives in canola seed.

Acknowledgments The authors gratefully acknowledge assistance of the North Dakota Agricultural Experiment Station.

Disclosure The authors declare no conflict of interest.

References

1. Kalt W. Effect of production and processing factors on major fruit and vegetable antioxidants. J. Food Sci. 70: R11-R19 (2004)
2. Saita E, Kishimoto Y, Tani M, Lizuka M, Toyozaki M, Sugihara N, Kondo K. Antioxidant activites of *Perilla frutescens* against low-density lipoprotein oxidation in vitro and in human subjects. J. Oleo Sci. 61: 113-120 (2012)
3. Li C, Tang Z, Huang M, Tao N, Feng B, Huang S. Antioxidant efficacy of extracts produced from pickled and dried mustard in rapeseed and peanut oils. J. Food Sci. 70: C394-C400 (2012)
4. Rice-Evan CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20: 933-956 (1996)
5. Jacobo-Velázouez DA, Cisneros-Zevallos L. Correlations of antioxidant activity against phenolic content revisited: A new approach in data analysis for food and medicinal plants. J. Food Sci. 74: R107- R113 (2009)
6. Shahidi F, Naczk M. An overview of the phenolics of canola and rapeseed: Chemical, sensory and nutritional significance. J. Am. Oil

Chem. Soc. 69: 917-924 (1992)
7. Naczk M, Amarowicz R, Sullivan A, Shahidi F. Current research developments on polynolics of rapeseed/canola: A review. Food Chem. 4: 489-502 (1998)
8. Yoon BH, Jung JW, Lee JJ, Cho YW, Jang CG, Jin CB, Oh TH, Ryu JH. Anxiolytic-like effects of sinapic acid in mice. Life Sci. 81: 234-240 (2007)
9. Cheng JC, Dai F, Zhou B, Yang L, Liu ZL. Antioxidant activity of hydroxycinnamic acid derivatives in human low density lipoprotein: Mechanism and structure-activity relationship. Food Chem. 104: 132-139 (2007)
10. Wanasundara U, Amarowiz R, Shahidi F. Isolation and identification of an antioxidant component in canola meal. J. Agr. Food Chem. 42: 1285-1290 (1994)
11. Xu L, Diosady LL. Rapid method for total phenolic acid determination in rapeseed/canola meals. Food Res. Int. 30: 571-574 (1997)
12. Wanasundara U, Amarowiz R, Shahidi F. Partial characterization of natural antioxidant canola meal. Food Res. Int. 28: 525-530 (1996)
13. Hassas-Roudsri M, Chang PR, Pegg RB, Tyler RT. Antioxidant capacity of bioactives extraction from canola meal by subcritical water, ethanolic and hot water extraction. Food Chem. 114: 717-726 (2009)
14. Dewanto V, Wu X, Adonm KK. Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agr. Food Chem. 50: 3010-3014 (2002)
15. Jia Z, Tang M, Wu J. The determination of flavonoid contents in mulberry and they scavenging effects on superoxide radicals. Food Chem. 64: 555-559 (1999)
16. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol. 28: 25-30 (1995)
17. Arts MJ, Haenen GR, Voss HP, Bast A. Antioxidant capacity of reaction products limits the applicability of the trolox equivalent antioxidant capacity (TEAC) assay. Food Chem. Toxicol. 42: 45-49 (2004)
18. Oyaizu M. Studies on products of browning reaction-antioxidant activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr. 44: 307-315 (1986)
19. Jin YC, Liu HL. Yuan K. Simultaneous determination of seven effective constituents in the leaves of bamboo by reversed phase high performance liquid chromatography (RP-HPLC). J. Med. Plants Res. 5: 5630-5635 (2011)
20. Li Q, Yan G, Ge T. A fragmentation study of two compounds related to 4'-demethylepipodophyllotoxin in negative ion electrospray ionization by MS^n ion-trap time-of-flight mass spectrometry. Rapid Commun. Mass Sp. 22: 373-378 (2008)
21. Khattab R, Eskin M, Aliani M, Thiyam U. Determination of sinapic acid derivatives in canola extracts using high-performance liquid chromatography. J. Am. Oil Chem. Soc. 87: 147-155 (2010)
22. Siger A, Czubinski J, Dwiecki K, Kachlicki P, Nogala-Kalucka M. Identification and antioxidant activity of sinapic acid derivatives in *Brassica napus* L. seed meal extracts. Eur. J. Lipid Sci. 115: 1130-1138 (2013)
23. Amarowicz R, Naczk M, Shahidi F. Antioxidant activity of various fraction of non-tanin phenolics of canola hulls. J. Agr. Food Chem. 48: 2755-2759 (2000)
24. Kuti JO. Antioxidant compounds from four *Opuntia* cactus pear fruit varieties. Food Chem. 85: 527-533 (2004)
25. Jun HI, Wiesenborn DP, Kim YS. Antioxidant of various solvent extracts from canola meal. Korean J. Food Preserv. 18: 59-64 (2011)
26. Szydlowska-Czerniak A, Trokowsk K, Karlovits G, Szlyk E. Determination of antioxidant capacity, phenolic acids, and fatty acid composition of rapeseed varieties. J. Agr. Food Chem. 58: 7052-7509 (2010)
27. Brettonnet A, Hewavitarana A, Dejong S, Lanari MC. Phenolic acids composition and antioxidant activity of canola extracts in cooked beef, chicken pork. Food Chem. 121: 927-933 (2010)
28. Cartea ME, Francisco M, Soengas P, Velasco P. Phenolic compounds in *Brassica* vegetables. Molecules 16: 251-280 (2011)
29. Amarowicz R, Shahidi. Chromatographic separation of glucopyranosyl sinapate from canola meal. J. Am. Oil Chem. Soc. 71: 551-552 (1994)
30. Wanasundara UN, Shahidi F. Canola extract as an alternative natural for canola oil. J. Am. Oil. Chem. Soc. 71: 817-822 (1994)
31. Engels C, Schieber A, Gänzle MG.. Sinapic acid derivatives in defatted oriental mustard (*Brassica juncea* L.) seed meal extracts using UHPLC-DAD-ESI-MS^n and identification of compounds with antibacterial activity. Eur. Food Res. Technol. 234: 535-542 (2012)