

# Identification and Determination of Phenolic Compounds in Rapeseed Meals (*Brassica napus* L.)

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## Abstract

This study aimed in the identification and quantification of phenolic compounds in different four varieties of rapeseeds (*Brassica napus* L.) using LC-MS and HPLC. LC-MS analysis guided to identify 12 phenolic compounds including sinapine, sinapine(4-*O*-8')guaiaacyl, feruloyl choline(4-*O*-8')guaiaacyl, kaempferol-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside, kaempferol-3-*O*- $\beta$ -sophoroside, cyclic spermidine-alkaloid, feruloyl choline(5-8')guaiaacyl, kaempferol-sinapoyl-trihexoside, 1,2-di-*O*-sinapoyl-gentiobiose, 1,2-di-*O*-sinapoyl-glucose, feruloyl choline(4-*O*-8')guaiaacyl-di-sinapoyl, and sinapine(4-*O*-8')guaiaacyl-di-sinapoyl, respectively. Total phenolic compounds in rapeseed meals were ranged from 38.50 (2012 "Sunmang") to 63.95 mg/g dry weight (DW) (2014 Jeju local cultivar). Main phenolic compositions were cyclic spermidine-alkaloid, kaempferol, feruloyl choline, and sinapine. Sinapine was predominant compound among all the samples ranged from 29.74 to 52.24 mg/g DW (mean 79% of total phenolic contents). This study provided the information for the variation of phenolic compounds between the varieties of rapeseeds and the cultivation periods. Furthermore, this information can be developed for bio-energy industry as a reducer of the cost of the bio-energy products.

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## Keywords

LC-MS Analysis, HPLC Analysis, Rapeseed Meal, Phenolic Compounds

## 1. Introduction

Rapeseed (*B. napus* L.) is one of *Brassica* vegetables, produced in many countries such as Australia, Canada, China, Europe, India, the Russian Federation, and the United States [1]. Rapeseed production amount in the world market is highest in China (11,760 ton, 35.7%) and EU (11,384 ton, 34.6%) [2]. Rapeseed has been used for landscape plant because of its distinct yellow flowers. Recently, however, it is mainly cultivated for its oil which is used for bio diesel or bio ethanol. Rapeseed is one of the main materials of bio-diesel (83%) and utilizations of residues of rapeseed such as rapeseed meal, stem and flower also have been interested by oilseed industries in order to reduce the production expenses [3]. There is a lot of utilization of rapeseed residues due to their high nutritive value and functional compounds such as amino acids, carotenoids, flavonoids, and glucosinolates [4]. Thus, rapeseed meals have been used for organic fertilizer, feed stuff for livestock, adhesive hydrolyzed by enzymes [5].

Functional compounds among other compounds of rapeseed residues are phenolics in rapeseed meal, which this study focused on. Flavonoids belonged to phenolic compositions are polyphenols which have  $C_6C_3C_6$  skeletons. They are a large family of secondary plant metabolites contained anti-oxidation, anti-inflammation, anti-allergic effects, etc. [6]. Cartea *et al.* (2011) identified that *p*-coumaric, sinapic and ferulic acids, and main flavonoids are kaempferol, quercetin, and cyanidin from the leaves and roots of rapeseed meal but not on the seeds or meals [7]. On the other hand, Oskoueian *et al.* (2011) reported other phenolic acids such as (gallic and syringic acids) and flavonoids (apigenin, kaempferol, naringenin) in rapeseed meal [8]. These identified phenolic compounds have organic acids and sugar moiety in their chemical structure. These sugar moieties have significant influence on the identification and quantification of the phenolic compounds by analytical methods. However, the phenolic compounds with aglycone moiety were acid hydrolyzed to eliminate the sugar in order to compare and quantify the exact phenolic compounds [8] [9]. In this study, analytical methods were established to identify and quantify the phenolic compounds present in rapeseed meal.

## 2. Materials and Methods

### 2.1. Chemical Reagents

The HPLC-grade methanol was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA). Tert-Butyl hydroquinone (TBHQ) was obtained from Alfa Aesar (Heysham, Lancashire, UK) and hydrochloric acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sinapine was purchased from Chem. Faces (Wuhan, China) and kaempferol was purchased from Wako, Japan. Ultra-pure water used in this study was made by PURE LAB Option-Q system (ELGA Lab Water, VWS Ltd., UK).

### 2.2. Plant Materials

Two types of rapeseeds cultivated in Jeju Island (Jeju local cultivar) and Muan (“Sunmang”) were annually provided from 2011 to 2013 by the Bio-Energy Crop Center, National Institute of Food Science (Muan, Korea). The Jeju local cultivar was cultivated from October to May, whereas “Sunmang” was grown from October to June. After rapeseeds were harvested and de-oiled, the residue, rapeseed meal, were provided to chemical analyzes for phenolic compositions. In 2014, imported rapeseed meal from India was obtained from the Department of Animal Resource and Science, Dankook University.

### 2.3. Extraction of Phenolic Compounds

Extraction of phenolic compositions was performed following the standard method with slight modifications [10]. Rapeseed meal (dried powder 100 mg) was mixed thoroughly with 1 mL of 10% methanol contained 0.1% phosphoric acid and vortexed for 10 min. After that, the extract solution was incubated for 3 h at 37°C with vor-

texting every hour. Subsequently, it was centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was filtered through PTFE hydrophilic syringe filter (0.45 µm pore size, 13 mm diameter) prior to the quantification in HPLC.

## 2.4. LC-ESI-MS Analysis

Each peak of rapeseed meal separated using HPLC (Perkin Elmer Flexar, USA) for accurate LC-MS analysis was analyzed by 4000 Qtrap LC-MS/MS system. Mass analysis was carried out with 1.0 mL/min flow rate and positive ion mode. The UV spectrum was performed at 280 and 350 nm. The mobile phase consisted of (A) MeOH: water: acetic acid [5:92.5:2.5, (v/v/v)] + 0.01% TFA and (B) MeOH: water: acetic acid [95:2.5:2.5, (v/v/v)] + 0.01% TFA. The binary gradient used was as follows: 5% - 100% (0 - 13 min). The binary gradients were used as follows: 0 - 10 min, 0% - 20% B; 10 - 15 min, 20% - 40% B; 15 - 20 min, 40% B; 20 - 25 min, 40% - 50% B; 25 - 28 min, 50% B; 28 - 30 min, 50% - 0% B; 30 - 40 min, 0% B.

## 2.5. HPLC Analysis

Phenolic compounds in rapeseed meal were analyzed by Perkin Elmer Flexar (USA). Samples were separated on a Capcell PAK C18 column (4.6 × 250 mm, 5 µm particle size; Shiseido, Tokyo, Japan) maintained at 40°C. The flow rate was 1.0 mL/min, and the injection volume was 10 µL. The mobile phase consisted of (A) MeOH: water: acetic acid (5:92.5:2.5, v/v/v) and (B): water: acetic acid (95:2.5:2.5, v/v/v). Additionally, tri-floro acetic acid (TFA) was added in order to improve the peak separations. These gradients were used as follows: 0-10 min, 0% - 20% B; 10 - 15 min, 20% - 40% B; 15 - 20 min, 40% B; 20 - 25 min, 40% - 50% B; 25 - 28 min, 50% B; 28 - 30 min, 50% - 0% B; 30 - 40 min, 0% B. The absorbance was measured at 280 and 350 nm. Identification in the peaks of HPLC chromatogram of the phenolic compounds was based on spectra, standards, and literature data. External standards were used for identification and quantification, and results are presented as milligram per gram of dry weight [10].

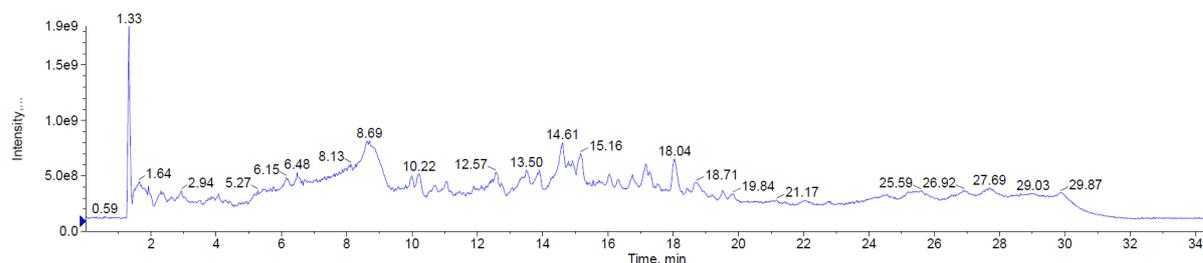
## 2.6. Acid Hydrolysis

Acid hydrolysis procedures were done to identify the unknown phenolic compounds. Phenolic compounds usually conjugated with sugars and organic acids were extracted by acid hydrolysis through two extraction methods (Extraction I and II) in order to eliminate the glycoside. The difference between extraction I and II was the time of adding acid into the extractions. In the Extraction I, 10% methanol contained 0.1% phosphoric acid, TBHQ (2 g/L), and 0.25 ml of 6 M hydrochloric acid was orderly added into 2.0 ml Eppendorf-tube containing rapeseed meal (100 mg), and then the mixture was extracted with vortexing for 10 min [11]. On the other hand, in the Extraction II, rapeseed meal was extracted with adding of 10% methanol contained 0.1% phosphoric acid together with TBHQ. And hydrochloric acid was then added with different ratios (1:1, 1:2, 1:3, v/v; labeled as EII-A, EII-B, EII-C, respectively) [12]. After each extraction process, the mixtures were centrifuged at 12,000 rpm at 4°C for 15 min. Finally, the extracts were passed through a 0.45 µm PTFE ACRODISC CR13 hydrophilic syringe filter before HPLC analysis.

## 3. Results and Discussion

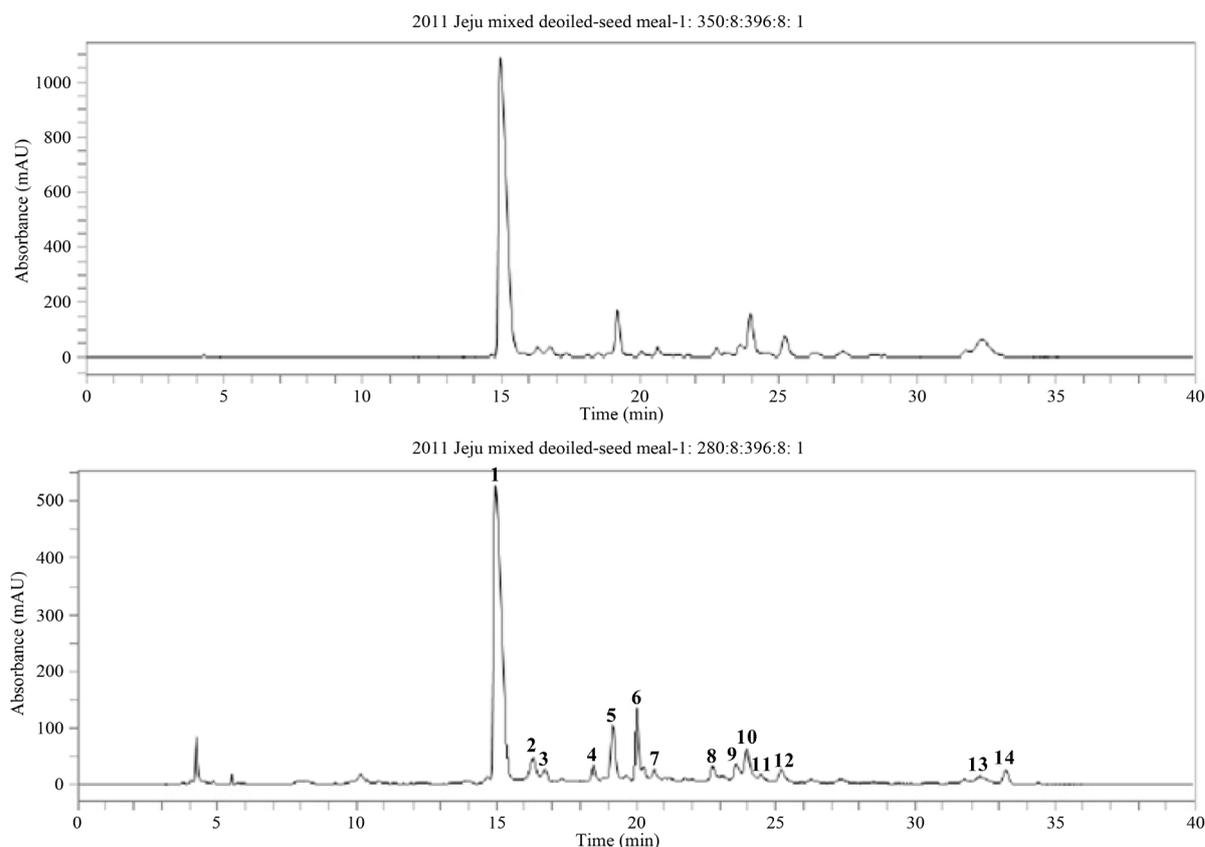
### 3.1. Identification of Phenolic Compounds by LC-ESI-MS Analysis

Phenolic compounds were identified with range of 8.69 - 19.84 retention times (RT) in total ion chromatogram, TIC (Figure 1). The RT is not matched exactly with Figure 2 and Figure 3 because HPLC analysis was performed with different company of analysis instrument. In addition, LC-MS was the first step in this research. The analytical method, especially entire time of the analysis, was modified reasonably after LC-MS process. The identified phenolic compounds including systematic and common names and the principal ions are listed in Table 1. Twelve phenolic and two unknown compounds were detected in all rapeseed meal extracts. Each compound was identified from their protonated molecular ions  $[M+H]^+$  and one main productions, corresponding to the adducts  $[M+Na]^+$ , compared to the literature information. The phenolic compounds namely [sinapine, sinapine (4-*O*-8')guaiacyl, feruloyl choline(4-*O*-8')guaiacyl, kaempferol-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside, kaempferol-3-*O*- $\beta$ -sophoroside, unknown-1, cyclic spermidine-alkaloid, feruloyl choline(5-8')guaiacyl, kaemp-



Note: because LC-MS analysis was first step in this study, the retention time (RT.) of this TIC is not matched exactly with fig.1 and 2. RT. 8.69 is the sinapine in this TIC.

**Figure 1.** Total ion chromatogram (TIC) of phenolic compounds in rapeseed meal.



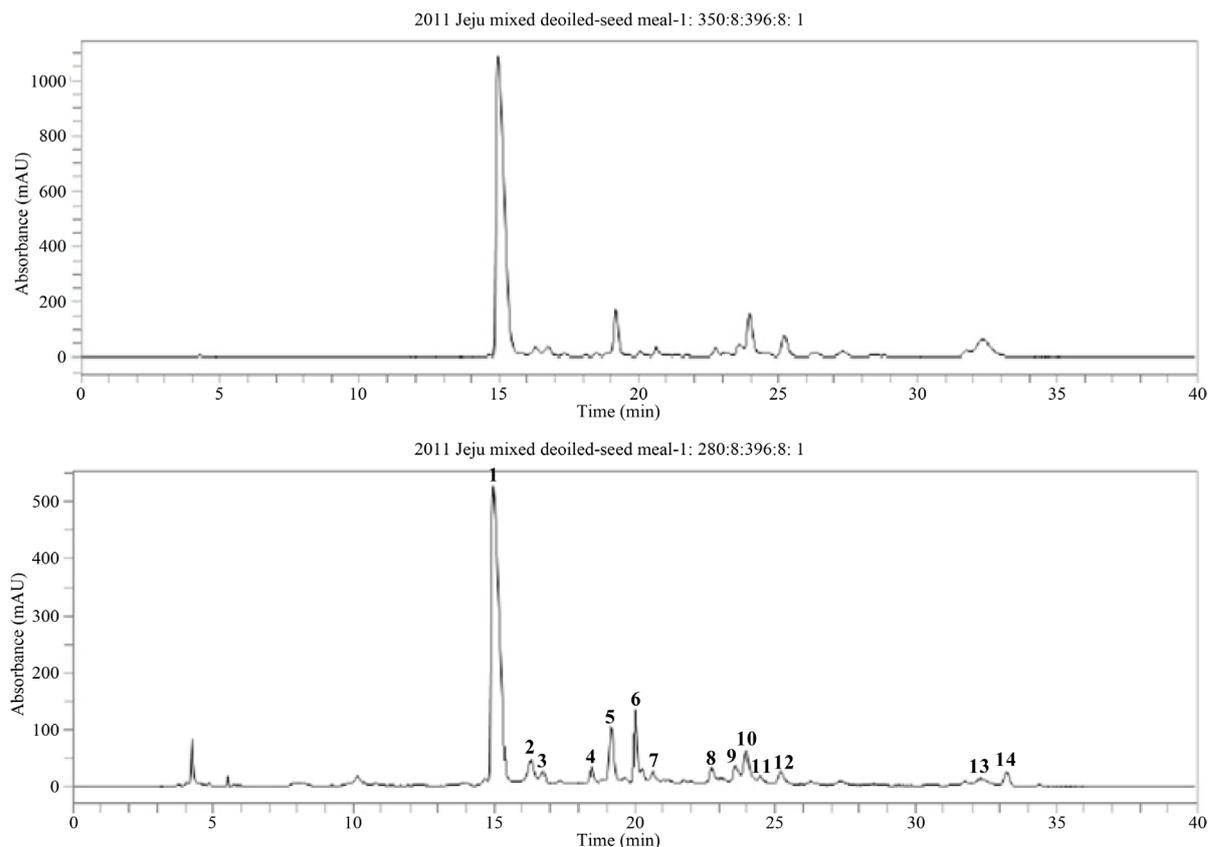
**Figure 2.** HPLC chromatogram of phenolic compounds in 2011 center meal (peak 13 was not detected in center meal).

ferol-sinapoyl-trihexoside, 1,2-di-*O*-sinapoylgentiobiose, 1,2-di-*O*-sinapoylglucose, feruloyl choline(4-*O*-8') guaiacyl-di-sinapoyl, unknown-2, and sinapine(4-*O*-8')guaiacyl-di-sinapoyl] were identified based on fragmentation patterns of MS spectra and quantified based on the peak areas of HPLC chromatogram (**Figure 2** and **Figure 3**). MS data revealed that the main phenolic compounds (sinapine, kaempferol, feruloyl choline and cyclic spermidine-alkaloid) were conjugated with sugar such as glucose, sophorose, tri-hexoside, and guaiacyl. The HPLC peaks 1, 2, 4, 9, 10, 11, 12 and 14 have sinapoyl moiety derived from sinapine (sinapoyl choline;  $[M+H]^+$ ,  $m/z$  310), whereas, peaks 4, 5, and 9 have kaempferol ( $[M+H]^+$ ,  $m/z$  287). Peak 4 and 5 were identified as kaempferol-3-*O*-sinapoyl-sophorose-7-*O*-glucoside and kaempferol-3-*O*- $\beta$ -sophorose. Even though the two compounds were similar in structure, the mass spectra represented the difference in clear fragments. Their mass values ( $m/z$ ) were detected at 979 in peak 4 and at 611 in peak 5 [13], respectively. On the other hand, feruloyl choline and cyclic spermidine-alkaloid need to acid hydrolysis because of their molecular weights were not included in MS spectra library. Peaks 3, 8 and 12 were confirmed as feruloyl-choline and peak 7 has cyclic spermidine-alkaloid respectively. MS spectra of unknown-1 and 2 (peak 6 and 13) were similar to kaempferol compounds

**Table 1.** Identification of phenolics and their mass spectra in rapeseed meals.

No. <sup>a</sup>	Trivial names	Molecular weight	[M+H] <sup>+</sup> ( <i>m/z</i> )	MS/MS ( <i>m/z</i> )	References
1	Sinapine	310	311	251/207/175	[11]
2 <sup>b</sup>	Sinapine(4- <i>O</i> -8')guaiacyl	506	507	310/251/221/207/175	[13]
3	Feruloyl choline(4- <i>O</i> -8')guaiacyl	476	477	476/417/221/145	[13]
4 <sup>b</sup>	Kaempferol-3- <i>O</i> -sinapoyl-sophoroside-7- <i>O</i> -glucoside*	978	979	979/611/449/287	[14]
5	Kaempferol-3- <i>O</i> - $\beta$ -sophoroside*	610	611	611/449//287	[15]
6 <sup>b</sup>	Unknown-1	978	979	979/817/496/458/207	
7	Cyclic spermidin-alkaloid	496	497	496/478/408/351/325/251/207/175	[13]
8	Feruloyl choline(5-8')guaiacyl	458	459	458/399/381/369/207	[13]
9	Kaempferol-sinapoyl-trihexoside*	978	979	979/817/655/369	[13]
10	1,2-Di- <i>O</i> -sinapoylgentiobiose	754	-	777/531/369/207/175	[16]
11	1,2-Di- <i>O</i> -sinapoylglucose	592	-	615//391/369/207/	[13]
12	Feruloyl choline(4- <i>O</i> -8')guaiacyl-di-sinapoyl	682	683	682/623/403/221/207/145	[13]
13	Unknown-2	838	839	839/817/682/655/538/369	
14	Sinapine(4- <i>O</i> -8')guaiacyl-di-sinapoyl	712	713	712/653/207/179/175/147	[13]

<sup>a</sup>No., the elution order of flavonoid HPLC analysis in Figure 2; <sup>b</sup>only detected at 280 nm, but the others detected both at 280 and 350 nm.

**Figure 3.** HPLC chromatogram of phenolic compounds in 2011 Jeju mixed rapeseed meal.

(especially peak 9) but kaempferol molecular weight was not detected in them. Thus the analysis using acid hydrolysis treatment was needed in order to confirm the unknown compounds.

### 3.2. Quantification of Phenolic Compounds by HPLC Analysis

The kaempferol and sinapine was used for quantification of phenolic compounds as external standard in rapeseed meal. The contents of phenolic compounds in rapeseed meal are shown in **Table 2**. Total phenolic compound content of 2014 Jeju local cultivar was the highest (63.95) and 2012 “Sunmang” was the lowest (45.78 mg/g dry weight, DW) but there is little difference of contents in each sample.

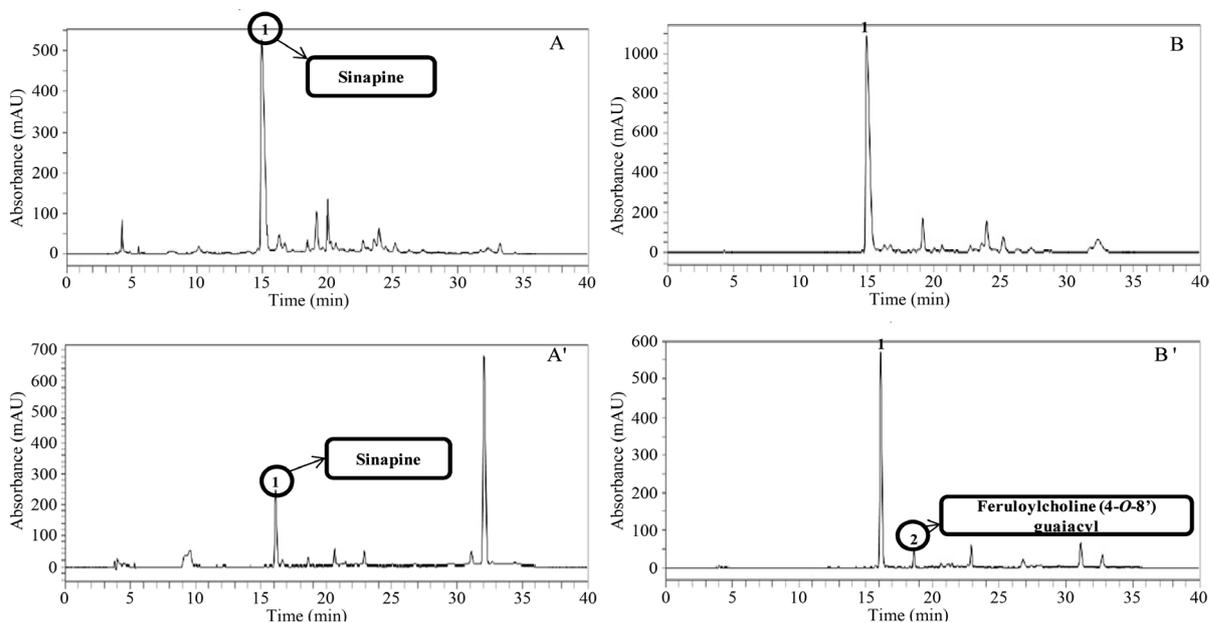
Sinapine and 1,2-di-*O*-sinapoyl-gentiobiose was predominant from the quantified compounds. Sinapine, one of main phenolic compounds was contained wide-ranging amounts in rapeseed meal, with levels in the following order: 2014 Jeju local cultivar (52.24) > 2014 Import (46.65) > 2011 Jeju local cultivar (41.43) > 2011 “Sunmang” (36.48) > 2012 Jeju local cultivar (34.59) > 2013 “Sunmang” (32.79) > 2012 “Sunmang” (29.74 mg/g DW). Except 2014 samples revealed highest contents, Jeju local cultivar has more amount of sinapine than “Sunmang” in each year. However total kaempferol compounds, sum of kaempferol-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside, kaempferol-3-*O*- $\beta$ -sophoroside, and kaempferol-sinapoyl-tri-hexoside was not detected with the same tendency. Total kaempferol content was higher in “Sunmang”. On the other hand, the contents of feruloyl choline and cyclic spermidine-alkaloid were so ambiguous that there were did not reveal any tendency, whereas Oskoueian *et al.* (2011) reported gallic and syringic acids as phenolic acids and apigenin, kaempferol and naringenin as flavonoids in the rapeseed meal [8].

[17] [18] reported that sinapine has anti-nutrition effects such as bitter, sour, astringency for non-ruminant animals. However [19] reported that, sinapine has beneficial effects because of the chemo-preventive, anti-tumor, anti-oxidant, and anti-mutagenic effects. Concretely, sinapine can be used as an active compound to enhance the

**Table 2.** Phenolic compound contents (mg/g dry wt.) in rapeseed meals ( $n = 3$ ).

No. <sup>a</sup>	Trivial names	2011		2012		2013		2014	
		Jeju local cultivar	“Sunmang”	Jeju local cultivar	“Sunmang”	“Sunmang”	Jeju local cultivar	Imported	
1	Sinapine	41.43 ± 2.60	36.48 ± 1.82	34.59 ± 6.05	29.74 ± 4.81	32.79 ± 1.05	52.24 ± 3.64	46.65 ± 1.13	
2	Sinapine(4- <i>O</i> -8')guaiacyl	1.05 ± 0.20	0.85 ± 0.12	0.69 ± 0.32	0.62 ± 0.11	0.62 ± 0.15	0.59 ± 0.20	2.28 ± 0.14	
3	Feruloyl choline(4- <i>O</i> -8')guaiacyl	0.63 ± 0.04	0.61 ± 0.06	0.39 ± 0.18	0.69 ± 0.14	0.77 ± 0.07	0.07 ± 0.06	0.83 ± 0.24	
4	Kaempferol-3- <i>O</i> -sinapoyl-sophoroside-7- <i>O</i> -glucoside*	0.09 ± 0.02	0.06 ± 0.01	0.04 ± 0.03	0.07 ± 0.01	0.08 ± 0.00	0.84 ± 0.22	0.24 ± 0.24	
5	Kaempferol-3- <i>O</i> - $\beta$ -sophoroside*	0.70 ± 0.03	0.98 ± 0.11	0.58 ± 0.06	0.82 ± 0.13	1.07 ± 0.07	0.25 ± 0.15	0.59 ± 0.48	
6	Unknown-1	1.45 ± 1.26	ND <sup>b</sup>	0.11 ± 0.10	0.21 ± 0.03	0.27 ± 0.04	0.83 ± 0.48	0.14 ± 0.12	
7	Cyclic spermidine-alkaloid	0.40 ± 0.04	0.40 ± 0.10	0.41 ± 0.06	0.38 ± 0.06	0.44 ± 0.07	0.69 ± 0.35	ND	
8	Feruloyl choline(5-8')guaiacyl	0.60 ± 0.10	0.85 ± 0.06	0.47 ± 0.16	0.56 ± 0.20	1.24 ± 0.13	0.42 ± 0.03	0.12 ± 0.10	
9	Kaempferol-sinapoyl-tri hexoside*	0.41 ± 0.02	0.60 ± 0.04	0.22 ± 0.16	0.43 ± 0.07	1.00 ± 0.03	0.34 ± 0.04	0.16 ± 0.00	
10	1,2-Di- <i>O</i> -sinapoylgentiobiose	4.28 ± 0.34	5.47 ± 0.29	2.65 ± 1.55	3.80 ± 0.59	6.63 ± 3.03	5.13 ± 1.48	1.19 ± 0.06	
11	1,2-Di- <i>O</i> -sinapoylglucose	0.32 ± 0.08	0.12 ± 0.14	0.03 ± 0.04	0.07 ± 0.12	0.42 ± 0.03	ND	ND	
12	Feruloyl choline(4- <i>O</i> -8')guaiacyl-di-sinapoyl	0.65 ± 0.04	0.72 ± 0.03	0.60 ± 0.10	0.40 ± 0.05	0.44 ± 0.04	ND	ND	
13	Unknown-2	0.29 ± 0.50	0.00 ± 0.00	0.01 ± 0.02	0.04 ± 0.06	ND	1.69 ± 0.24	1.01 ± 0.15	
14	Sinapine(4- <i>O</i> -8')guaiacyl-di-sinapoyl	0.64 ± 0.03	0.96 ± 0.04	0.39 ± 0.10	0.67 ± 0.13	0.02 ± 0.03	0.87 ± 0.25	1.10 ± 0.34	
<b>Total</b>		<b>52.95 ± 4.77</b>	<b>48.10 ± 2.35</b>	<b>41.18 ± 7.94</b>	<b>38.50 ± 6.28</b>	<b>45.78 ± 4.33</b>	<b>63.95 ± 6.07</b>	<b>54.20 ± 2.41</b>	

<sup>a</sup>No., the elution order of phenolics by HPLC analysis; <sup>b</sup>ND, not detected; \* quantified with kaempferol standard, but the others with sinapine standard. Note: In 2013, Jeju local cultivar is not cultivated and imported sample was cultivated in India.



\*Peak 1 and 2 were identified by LC-MS analysis.

**Figure 4.** The comparison of HPLC chromatogram of EII-B (sample:acid = 1:2) in 2011 Jeju mixed meal; A: before acid treatment at 280 nm, B: at 350 nm, A': after acid treatment at 280 nm, B': at 350 nm.

effects of doxorubicin used for chemotherapy on cancer. Therefore, sinapine has been attracted researchers by its applications. Kaempferol is one of the flavonol belonged to flavonoids. Flavonoid compounds also have pharmacological properties such as anti-oxidative, anti-allergic, anti-inflammatory, anti-diabetic, hepato- and gastro-protective, anti-viral, and anti-neoplastic activities [11].

### 3.3. The Analysis of Rapeseed Meal under Acid Hydrolysis Treatment

Acid hydrolysis treatment was done to identify the unknown phenolic compounds present in the rapeseed meal. To confirm the unknown compounds (peak 6 and 13) acid hydrolysis treatment was conducted by various extraction methods. Extraction II was selected for acid hydrolysis because the peaks pattern of the extraction II was simpler and separated more clearly and the differences between extraction I and non-acid hydrolysis treatment samples were not significant. LC-MS analysis was performed to confirm what the peaks of HPLC chromatogram were exactly using EII-B that presented most clear peaks among Extraction II. Sinapine (310/251/175) and feruloyl choline(4-O-8)guaiacyl (mass spectra; 476/417/221/145) were detected by LC-MS analysis. However the mass spectra of other peaks were detected with different aspects from non-acid hydrolysis treatment samples, and it was hard to determine that they were certain compounds (Figure 4). That reasons were able to find from analysis data of sinapine. The content of sinapine was expected to increase after acid treatments because many compounds including sinapine have sinapoyl moiety in their structure but the content were rather decreased in fact. Sinapine seems to be decomposed by high concentration of acid. Thus proper ratio of the samples to the acid will be needed for the accurate results of phenolic compound aglycone in further research.

## 4. Conclusion

Twelve phenolic compounds and two unknown compounds in rapeseed meal were identified by LC/MS/MS system at 280 and 350 nm. Cyclic spermidine-alkaloid, feruloyl choline, kaempferol, and sinapine were identified as the main phenolic compounds in LC-MS data. The feruloyl choline has very similar structure with sinapine like Figure 5 [20]. Sinapine was detected with largest amounts (mean 79%), and the other compounds have just slight differences on each. The quantification and acid hydrolysis treatment would be provided for utilization of rapeseed meal not only for feed stocks or fertilizer but also cosmetic industries or health functional food for human such as protein beverage and biscuit [9]. Furthermore, these utilizations can be developed for bio-energy industry as reducer of cost of the bio-energy products.

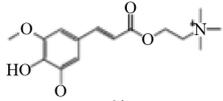
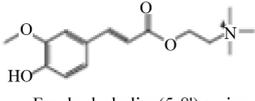
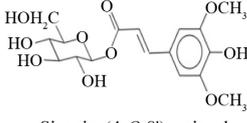
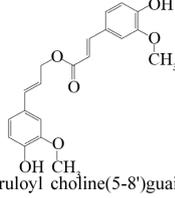
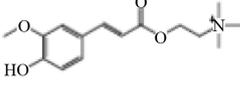
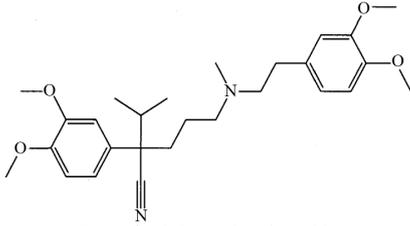
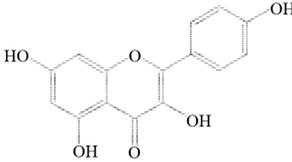
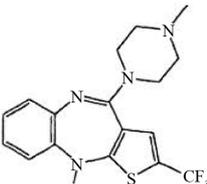
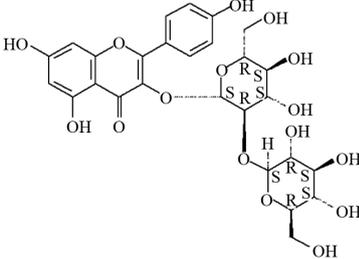
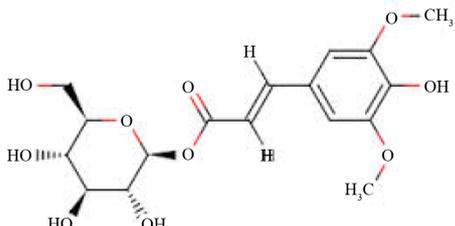
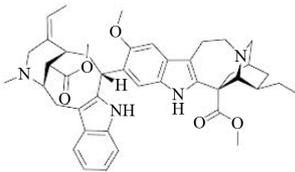
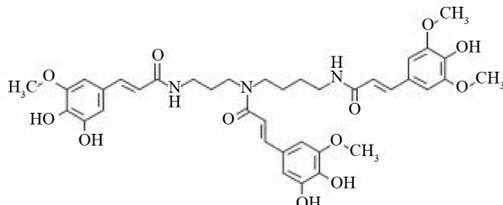
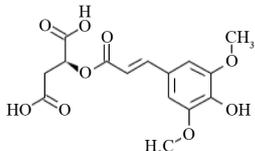
Phenolic compounds identified in rapeseed meals and their chemical structure	
 Sinapine	 Feruloyl choline(5-8')guaiaicyl
 Sinapine(4-O-8')guaiaicyl	 Feruloyl choline(5-8')guaiaicyl
 Feruloyl choline(4-O-8')guaiaicyl	 Kaempferol-sinapoyl-tri hexoside
 Kaempferol-3-O-sinapoyl-sophoroside-7-O-glucoside	 1,2-Di-O-sinapoylgentiobiose
 Kaempferol-3-O-β-sophoroside	 1,2-Di-O-sinapoylglucose
 Cyclic spermidine-alkaloid	 Feruloyl choline(4-O-8')guaiaicyl-di-sinapoyl
 Sinapine(4-O-8')guaiaicyl-di-sinapoyl	

Figure 5. Chemical structure of main phenolic compounds in rapeseed meal.

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