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## Original Article

# Rapid characterization of metabolites in soybean using ultra high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-Q-TOF-MS/MS) and screening for $\alpha$ -glucosidase inhibitory and antioxidant properties through different solvent systems



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## ABSTRACT

This work was the first to investigate on the simultaneous characterization of metabolite profiles in soybean using UPLC-ESI-Q-TOF-MS/MS. Twenty two compositions were observed within 14 min from the methanol extract and confirmed as twelve isoflavones of three types and ten soyasaponins (Ab, Af, I–III,  $\alpha$ g,  $\beta$ g,  $\beta$ a,  $\gamma$ g, and  $\gamma$ a). Moreover, the patterns of two chemicals showed considerable differences in seven solvent systems by HPLC analysis and their optimal extraction was achieved by 70% methanol (isoflavone: 4102.69  $\mu$ g/g; soyasaponin: ten peaks). The second abundant isoflavones were detected in 50% methanol (4054.39  $\mu$ g/g), followed by 30% methanol, 100% methanol, 10% methanol,  $\text{CH}_2\text{Cl}_2$ , and acetone extracts with 3134.03, 2979.49, 1681.33, 366.19, and 119.00  $\mu$ g/g, respectively. Soyasaponins exhibited similar tendencies as those of isoflavones. The highest total phenolic was found as  $2.10 \pm 0.05$  mg GAE/g in 70% methanol with remarkable differences by comparing other extracts. Specifically, this extract showed potent  $\alpha$ -glucosidase inhibitory (81%) and antioxidant capacities (DPPH: 93% and ABTS: 95%) at a

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concentration of 1.0 mg/mL. Our results may be contributed to enhancing the value to functional foods and evaluating the secondary metabolites concern to antioxidant properties using solvent system in soybean.

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## 1. Introduction

Phytochemicals are widely distributed in plants, fruits, flowers, crops, and vegetables [1–4], and they have been renowned to possess beneficial effects on human health such as anticancer, antioxidant, antidiabetic, and antimicrobial properties [3–5]. In this regard, phytochemicals are currently a great interest in the food and medicinal industries. Among various edible sources, legumes (soybean, lentil, chick pea, broad bean, cowpea, adzuki bean, etc.) are one of the most important crops due to the presence of many phytochemicals and their medicinal abilities [1,6,7]. Specifically, soybean [*Glycine max* (L.) Merr.] has been widely used as nutritious food material for many decades because of its valuable health beneficial properties, including anticarcinogenic, anti-oxidative, anticancer, antimetastatic, and antiestrogenic capacities [8–12]. This species is increasingly interested in natural source with high protein and oil contents as well as various metabolites (phenolics, isoflavones, triterpenoids, saponins, vitamins, tocopherols, and amino acids) [8–10]. Interestingly, isoflavone and saponin have been categorized as the most crucial groups for the pharmaceutical and functional aspects [7,12]. Soybean isoflavone has been considered as excellent secondary metabolite regarding numerous human health benefits (cancer, cardiovascular diseases, osteoporosis, and menopausal symptoms) [11,12]. This component is also known to be a group of active natural plant defense material as phytoalexin against pathogenic fungi and insect feeding [13]. Moreover, it is well established that saponins of pentacyclic triterpenoid oleanane skeleton, in which the predominant component in soybean and other legumes are associated with protective activities against cancers, diabetes, and cardiovascular diseases as well as antiviral, antimicrobial, and hepatoprotective effects [7,14,15]. For these above reasons, several researchers have focused on isoflavone and saponin analyzes for the development of functional foods and dietary supplements from soybean.

Until now, numerous analytical techniques (HPLC-UV, DAD-MS, FLD-MS and ESI-MS, TLC, GC, CE-ED, HPLC-ELSD, and ELISA) have been widely used for the quantitative and qualitative analyzes of isoflavones and saponins [1,8,9,16,17]. Despite the many procedures, these methods are a little uncomfortable as long analytical time, lack of reproducibility, relative complicate and expensive apparatus. Furthermore, there are few published studies on the fully separation and simultaneous determination of the isoflavone and saponin compositions with the accurate mass values. Their profiles have also never been examined at the short time with rapid and high resolution UPLC-ESI-TOF-MS system. Thus, the

accurate mass measurements provided by UPLC coupled with TOF-MS/MS can be contributed to the knowledge for the rapid isolation and identification of the complicated secondary metabolites in natural products.

Many literatures have demonstrated that phytochemicals play essential roles in preventing human diseases as well as developing of dietary supplements and pharmaceuticals [3–5]. In this regard, several scientists investigated the optimal extraction skills of phytochemicals according to solvent, temperature, and time from crops and plants [18,19]. To our knowledge, little intelligence has available on the comparison and determination of isoflavones and saponins through extraction solvent conditions in soybean. Several researchers are currently being conducted to identify natural antioxidants and  $\alpha$ -glucosidase inhibitory compounds owing to the safety factor. In particular, the antioxidant properties of crops are considered to have higher synergistic ability in crude metabolite extract compared to the activity of a single compound [20]. Based on the above observations, our work was designed to analyze the antioxidant capacities against radical scavenging effects and  $\alpha$ -glucosidase inhibitory effects by spectrophotometric assay as well as the development of sensitive and simple practical approach method using ESI-Q-TOF-MS technique from soybean extract.

The primary purpose of the current article was to demonstrate an effective approach through simultaneous identification of 12 isoflavones and 10 soyasaponins in soybean using UPLC-ESI-Q-TOF-MS analysis. In addition, our work was the first to examine changes in two phytochemical profiles according to the different extraction solvent systems. We also evaluated the antioxidant properties against DPPH and ABTS radicals,  $\alpha$ -glucosidase inhibitory effects, and total phenolic contents in various soybean extracts.

## 2. Materials and methods

### 2.1. Plant material and chemicals

The Korean soybean cultivar (cv. Taekwangkong, yellow soybean) was selected in the present research. This cultivar planted at the experimental fields of the National Institute of Crop Science, Milyang, Gyeongsangnam-do, Korea, during 2015. After harvesting, soybean seeds were freeze-dried in a deep freezer (HKF-51, Korea) and stored at  $-40$  °C prior to analysis. Analytical grade water, acetonitrile, and trifluoroacetic acid (TFA) were purchased for HPLC and UPLC-TOF-MS/MS analyses from J.T. Baker (Phillipsburg, NJ, USA). The three malonyl and three acetyl isoflavone glucosides were obtained from Sigma Chemical Co. (St. Louis, MO).  $\alpha$ -Glucosidase (EC

3.2.1.20), Folin-Cicalteu phenol reagent, gallic acid, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), potassium persulphate, *p*-nitrophenol- $\beta$ -D-glucopyranoside (*p*-NPG), acarbose, Sephadex LH-20, acetic acid, formic acid, and DMSO- $d_6$  were also purchased from Sigma Chemical Co. (St. Louis, MO, USA). To isolate isoflavone glucoside and aglycone materials, silica gel 60 Rp-18 (40–63  $\mu$ m) and TLC aluminum sheets RP-18 F<sub>254</sub> were obtained from Merck (Darmstadt, Germany). Sep-Pak cartridges were purchased from Waters Co. (Milford, MA, USA) and all analytical grade chemicals were used in the current study.

## 2.2. Instruments

To evaluate the radical scavenging activities, the absorbance was measured using a Beckman DU650 UV–Vis spectrophotometer (Beckman Coulter, Fullerton, USA). Total phenolic content and  $\alpha$ -glucosidase inhibitory effect were also evaluated by a Beckman spectrophotometer. The isolated isoflavones were identified using a Bruker AM 500 (<sup>1</sup>H NMR at 500 MHz, <sup>13</sup>C NMR at 125 MHz) spectrometer (Bruker, Karlsruhe, Germany) using DMSO- $d_6$  with tetramethylsilane (TMS) as the internal standard. For isoflavone analysis, HPLC was performed using an Agilent 1200 series (Boeblingen, Germany) quaternary pump, Agilent 1200 series diode array detector, and LichroCART 125-4 HPLC-Cartridge (Lichrophore 100 RP-18e; Merck KGaA, Darmstadt, Germany) column. The UPLC-ESI-TOF-MS skill was performed with an UPLC (Agilent 1200 series) equipped with a triple quadrupole time-of-flight tandem mass spectrometry (LC/MS-Triple TOF™ 5600+, AB SCIEX, Foster City, CA). The mass data were obtained using a Micro-mass QTOF Premier™ mass spectrometer (Waters Corporation, Milford, MA, USA).

## 2.3. Preparation of calibration curve and sample for isoflavone and soyasaponin analyses

For quantitative analysis, six isoflavones (three glucosides and three aglycones) were prepared from soybean seeds as the method described in our previous research [21]. Sample was pulverized for 5 min using a HR 2860 coffee grinder (Philips, Drachten, Netherlands). The powdered seeds (1.0 g, 60 mesh) were extracted using 20 mL of individual solvent such as CH<sub>2</sub>Cl<sub>2</sub>, acetone, 10% methanol, 30% methanol, 50% methanol, 70% methanol, and 100% methanol in a shaking incubator at 25 °C for 12 h. The supernatant was centrifuged at 3000 rpm for 5 min using VS-6000CFN (VISION, Seoul, Korea) and filtered through a syringe filter (0.45  $\mu$ m, Whatman Inc., Maidstone, UK) before injection into a HPLC and UPLC-ESI-TOF/MS. The stock solution of isoflavone standard was prepared by dimethylsulphoxide (DMSO) to give a 1000  $\mu$ g/mL concentration. The peak area of the individual standard was integrated with the HPLC chromatogram at 254 nm and plotted against the concentration to create a linear curve. The calibration curves were established on seven points (1, 5, 10, 20, 40, 80, and 100  $\mu$ g/mL) by DMSO dilution of each stock solution. Each isoflavone curve was obtained with high linearity of  $r^2 > 0.999$ .

## 2.4. UPLC-ESI-Q-TOF-MS/MS conditions

The isoflavone and soyasaponin profiles in various extracts of soybean seeds were analyzed on a triple TOF™ 5600+ system coupled to UPLC system. Separation was performed in a reverse phase column (Agilent ZORBAX EclipsePlus C18 RRHD, 1.8  $\mu$ m, 50 mm  $\times$  2.1 mm, Agilent Technologies, Santa Clara, CA) with a flow of 0.2 mL/min. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with Duo-Spray™ source and the mass scan range was set at *m/z* 100–2000 for both TOF-MS and TOF-MS/MS scan using a resolution of 37700. The other mass spectrometric conditions were programmed as follows: ion source voltage, 5500 V; ion source heater, 400 °C; curtain gas, 25 psi; nebulizer gas, 50 °C (GS1 50 and GS2 50); collision energy, 10 eV (MS) and 35 eV (MS/MS); Declustering potential 100. The analyst TF software (version 1.7) program combined with the information-dependent acquisition (IDA) packing was used to acquire MS/MS data. The mobile phase was composed of methanol (elution A) and 0.1% formic acid in water (elution B) using a gradient elution of 25% elution B (0–5 min), from 25% to 50% of elution B (5–13 min), from 50% to 80% elution B (13–20 min).

## 2.5. HPLC conditions for isoflavone analysis

The isoflavone content was carried out using an HPLC analysis. The total running time was 45 min at a flow rate of 1.0 mL/min and the column temperature was set at 25 °C. The mobile phase was performed of 0.1% acetic acid (v/v) in water (eluent A) and acetonitrile (eluent B). The gradient was programmed as follows: 0–20 min, 20% B; 30 min, 25% B; 45 min, 35% B, and then held for 5 min before returning to the initial conditions. A 20  $\mu$ L sample of the crude extract was analyzed for an analytical C<sub>18</sub> column (Lichrophore 100 RP-18e, 5  $\mu$ m, 125 mm  $\times$  4 mm, Merck KGaA, Darmstadt, Germany), and the detection was recorded at 254 nm.

## 2.6. Determination of total phenolic content

Total phenolic content in soybean extract was determined using the Folin-Ciocalteu spectrophotometric procedure according to the previous research [11]. The diluted extract with each solvent was shaken for 1 min, and then 0.2 M Folin-Ciocalteu (1.0 mL) was added. After 3 min, 15% sodium carbonate solution (800  $\mu$ L) was added and entire solution was incubated for 30 min at 25 °C. The absorbance of the solution was measured at 750 nm and all terminations were carried out in triplicate. The data were obtained by reporting the absorbance from the gallic acid standard curve (50, 100, 250, 500, 750, and 1000 mg/L) and the result was expressed as mg of gallic acid equivalents ( $\mu$ g GAE/g).

## 2.7. Determination of antioxidant activities

To determine antioxidant properties, the DPPH and ABTS radical scavenging capacities in various extracts of soybean seeds were evaluated as described by Lee et al. [2,9]. The powdered sample (1.0 g, 60 mesh) was extracted with each solvent (20 mL) for 12 h at 25 °C. The crude extract was filtered through a Whatman No. 42 filter paper, and then the

supernatants were immediately measured by UV–Vis absorption spectrophotometer for scavenging effects against radicals. For determining the DPPH radical scavenging ability, a solution of 1 mM DPPH in methanol was stirred for 30 min and the solution absorbance was adjusted to 0.70 at 517 nm using methanol. The solvent extract (each 0.1 mL) or positive control (BHT) (0.1 mL) of seven concentrations (200, 100, 50, 25, 10, 5, and 1  $\mu\text{g}/\text{mL}$ ) was mixed with methanol (0.49 mL) and 1 mM DPPH methanolic solution (0.1 mL). The mixture was vortexed for 1 min, and incubated for 20 min at 25 °C in darkness. The decrease of absorbance was evaluated at 517 nm and its scavenging rate was calculated as a percentage using the following equation:

$$[(1 - A_t/A_o) \times 100\%]$$

× ( $A_t$ : absorbance of test sample,  $A_o$ : absorbance control)

The scavenging activity of ABTS radical was confirmed as the capacity of different substances to scavenge the ABTS<sup>•+</sup> radical cation by comparison of a Trolox (positive control) [2,9]. This radical cation was generated by reacting the 7 mM ABTS<sup>•+</sup> solution with 2.45 mM potassium persulfate, and the mixture was maintained in darkness at 25 °C for 10–14 h. The ABTS<sup>•+</sup> solution was melted with ethanol to an absorbance of  $0.70 \pm 0.02$  at 734 nm. For 5 min at room temperature, the ABTS<sup>•+</sup> solution (0.9 mL) was mixed with 0.1 mL sample and then measured using a spectrophotometer. The concentrations of sample and positive control were evaluated as those of DPPH radical. This effect was determined as a percentage by the following formula:

$$[(1 - A_t/A_o) \times 100\%]$$

× ( $A_t$ : absorbance of test sample,  $A_o$ : absorbance control)

### 2.8. Determination of $\alpha$ -glucosidase inhibitory activity

The  $\alpha$ -glucosidase inhibitory effect was evaluated by the previous method with slight modification [22]. The sample for this capacity was prepared by 1 g of the grounded seeds in distilled water (10 mL), and then centrifuged at 3,500g for 5 min. The reaction mixture of this enzyme contained the sample extract (100  $\mu\text{L}$ ) and phosphate buffer (0.1 M, pH 7.0, 100  $\mu\text{L}$ ) containing  $\alpha$ -glucosidase solution (1.0 U/mL) and was incubated at 25 °C for 10 min. After incubation, 50 mM *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (PNP-G, 50  $\mu\text{L}$ ) was added. The resultant solution was mixed, and the enzyme activity was determined by monitoring the *p*-nitrophenol released from PNP-G at a wavelength of 405 nm. The  $\alpha$ -glucosidase (1.0 U) is defined as the amount of enzyme liberating 1.0  $\mu\text{M}$  of PNP/min. The  $\alpha$ -glucosidase inhibitory effect was expressed as percentage (%) inhibition and calculated by the following equation:

$$\alpha - \text{Glucosidase inhibitory effect (\%)} = [(A - B/A)] \times 100$$

where A = absorbance of 0.1 M phosphate buffer +  $\alpha$ -glucosidase solution + 50 mM PNP-G solution, B = absorbance of soybean extract +  $\alpha$ -glucosidase solution + 50 mM PNP-G solution.

### 2.9. Statistical analysis

All the measurements (radical scavenging activities, total phenolic content,  $\alpha$ -glucosidase inhibitory effect and

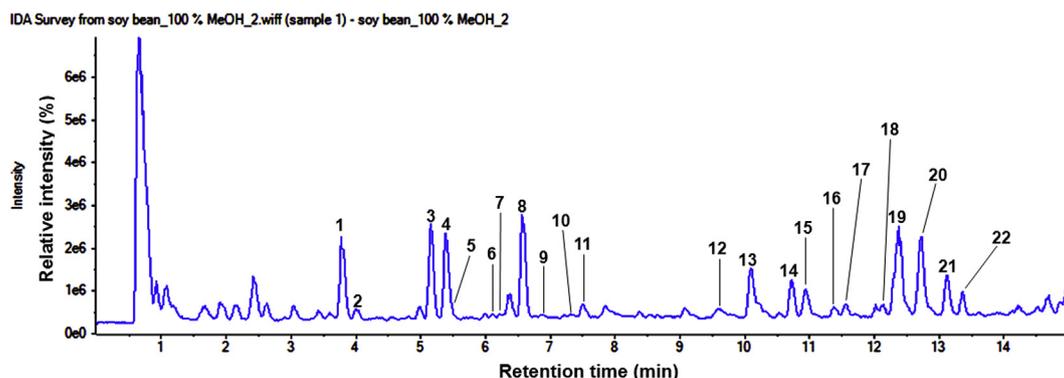
isoflavone content) were made in triplicate. The results were expressed as mean values using Sigma Plot 2001 (Systat Software Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Characterization of isoflavone and soyasaponin profiles using UPLC-ESI-Q-TOF-MS/MS analysis

It is well established that the UPLC system coupled with mass spectrometry is widely used as one of the most important analytical techniques for investigated of molecular weights of secondary metabolite profiles in foods and natural plants [1,2,23,24]. This method offers significant information for the characterization through assignment of individual peak, because the product ions were produced from the fragmentation of a selected precursor ion [2,23,24]. For these reasons, we characterized the isoflavones and soyasaponins in soybean seeds using the mass values of UPLC-ESI-Q-TOF-MS/MS technique. As illustrated in Fig. 1, a complete chromatographic separation of various phytochemicals (12 isoflavones and 10 soyasaponins), including major and minor peaks reached within 14 min. Our method showed high resolution and sensitivity as well as rapid separation. The identification of 22 compositions was based on the comparison of mass spectral data (MS, MS/MS and fragment ion values) and retention time ( $t_R$ ) with those of the earlier reported data (Table 1). Moreover, the chemical structures of identified phytochemicals are shown in Fig. 2.

In the present research, the identified phytochemicals were shown by the full scan positive ion mode. Firstly, we have demonstrated 12 isoflavone peaks from the methanol extract of soybean. Although many literatures have revealed the distribution of isoflavones in soybean [10,24,25], little information has been reported on the exactly molecular weights by various UPLC-ESI-Q-TOF-MS spectrums. The UPLC-ESI-Q-TOF-MS profile of peak 1 ( $t_R = 3.3$  min) exhibited the molecular ion  $[M+H]^+$  at  $m/z$  417.1185 and the fragmentation pattern of MS/MS spectrum was detected the signal at  $m/z$  255.0654  $\{[M+H]^+ - 162 \text{ amu}\}$  (Fig. 3A). This fragment ion ( $m/z$  255.0654) may be resulted from the loss of a glucose moiety (162 amu), as established by comparison with previous research [24]. In brief, this peak was elucidated by the loss of  $m/z$  162 from the molecular ion  $m/z$  417.1185 resulting in a fragment ion of daidzein aglycone [26]. Based on these observations, peak 1 was tentatively assigned as daidzin (1) of the elemental formula ( $C_{21}H_{20}O_9$ ) [24,26]. The ESI-TOF-MS spectrums of peaks 4 ( $t_R = 5.5$  min) and 6 ( $t_R = 6.1$  min) presented the molecular ions  $[M+H]^+$  at  $m/z$  503.1185 and  $m/z$  459.1288 and their characteristic fragment ions possessed  $m/z$  255.0655 and  $m/z$  255.0652 (Fig. 3D, F, and M). These above fragmentation ions were characterized as daidzein aglycone, with the losses of the  $m/z$  248 and  $m/z$  204 fragments. In other words, peaks 4 and 6 were coincident with daidzein+248 and daidzein+204, similar to the pattern of peak 1. Consequently, the fragmentation ions of two peaks were formed by the losses of a 6''-O-malonyl glucoside and 6''-O-acetyl glucoside moieties [24,27]. Therefore, peaks 4 and 6 were confirmed with malonyldaidzin (4) ( $C_{24}H_{22}O_{12}$ ) and acetyldaidzin (6) ( $C_{23}H_{22}O_{10}$ ) by comparison



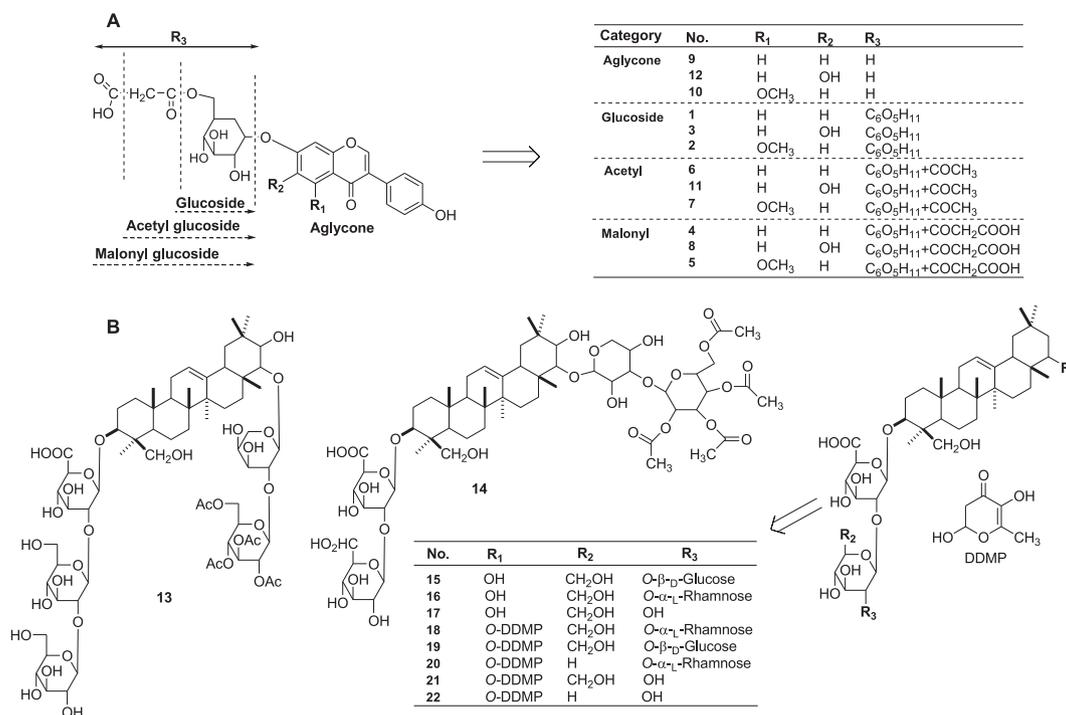
**Fig. 1** – Representative total ion chromatogram regarding the methanol extract of soybean seeds obtained by UPLC-ESI-Q-TOF/MS in positive ion mode.

with the previously reported data [28]. Furthermore, the full mass scan analysis of peak 9 ( $t_R = 6.9$  min) displayed an identical molecular ion  $[M+H]^+$  at  $m/z$  255.0651 (Fig. 3I) with the same patterns of peaks 1, 4, and 6 ( $m/z$  255.0654,  $m/z$  255.0655, and  $m/z$  255.0652). According to previous literatures, this peak was determined to be characteristic of daidzein (9) ( $C_{15}H_{10}O_4$ ) [26,28]. The UPLC ESI-Q-TOF-MS in the positive ion mode of peak 2 ( $t_R = 4.1$  min) exhibited the molecular ion  $[M+H]^+$  at  $m/z$  447.1286 and the one fragment ion at  $m/z$  285.0758 (Fig. 3B). This fragmentation at  $m/z$  285.0758 ( $[M+H]^+$ ) was attributed to the loss of  $m/z$  162 (glucose group, 6''-O-glucose moiety) by comparison with the earlier reported data [24,28]. Based on molecular weight, it was tentatively assigned as glycitin (2) ( $C_{22}H_{22}O_{10}$ ) in soybean isoflavones. Full TOF mass scan analysis of peak 5 ( $t_R = 5.6$  min) showed the molecular ion  $[M+H]^+$  at  $m/z$  533.1287 and a fragment ion at  $m/z$  503.1181, which was formed by the loss of 30 fragment (Fig. 3E) [23]. The fragment ion ( $m/z$  503.1181) resulted from the loss of a methoxy moiety ( $[M+H]^+ - 30$  amu ( $OCH_3 - H$ )) as the previously reported data regarding hydroxycinnamoyl residue in the acylated derivatives [23]. In addition, one major fragment ion at  $m/z$  285.0770 and one minor peak at  $m/z$  270.0526 were observed in TOF-MS/MS spectrum. The fragmentation at  $m/z$  285.0770 ( $[M+H]^+$ ) was characterized as glycitein aglycone and the fragment ion at  $m/z$  270.0526, which was formed by the loss of a methyl moiety ( $[M+H]^+ - 15$  amu). Based on the mentioned evidences, peak 5 was assumed to be malonylglycitin (5) with the formula ( $C_{25}H_{24}O_{13}$ ). The full mass scan analysis of peak 7 ( $t_R = 6.2$  min) was observed with  $[M+H]^+$  ion at  $m/z$  489.1389 with two fragment ions at  $m/z$  415.0869 and  $m/z$  411.0927 (Fig. 3G). Especially, the major fragmentation at  $m/z$  415.0869 was attributed to the loss of  $m/z$  74 (acetyl group:  $C_2H_3OH$ ,  $m/z$  44; methoxy group:  $OCH_3$ ,  $m/z$  30) by comparison with the previously reported data [29]. Moreover, the fragment ion at  $m/z$  285.1221 was produced from the parent ion at  $m/z$  489.1389 in MS/MS data. The MS<sup>2</sup> fragmentation pattern of the ion at  $m/z$  285.1221 is similar to those of peaks 2 and 5 (2:  $m/z$  285.0758 and 5:  $m/z$  285.0770). Therefore, the structure of peak 7 may be considered to contain a glycitein skeleton (Fig. 3M). According to the above described patterns, peak 7 was identified as acetylglycitin (7) ( $C_{24}H_{24}O_{11}$ ) by agreement with a previous research [24,29]. As presented in Fig. 3J, the TOF-MS

of peak 10 ( $t_R = 7.3$  min) presented the molecular ion ( $[M+H]^+$ ) at  $m/z$  285.0757 and its MS/MS spectrum showed the molecular ion at  $m/z$  285.0759 with a fragmentation at  $m/z$  270.0525. Especially, the fragment ion ( $m/z$  270.0525) was formed by the loss of the  $m/z$  15 fragment (Fig. 3M), based on the methyl moiety by comparison with published data [29]. Thus, peak 10 has already been evaluated as glycitein. The molecular ion ( $m/z$  285.0759) of this peak was also similar to those of glycitein derivatives 2 (glucoside), 5 (malonylglycoside), and 7 (acetylglycoside). For this reason, peak 10 was tentatively identified as glycitein (10) of  $C_{16}H_{12}O_5$  formula. The UPLC-Q-TOF-MS in the positive ion mode of peak 3 exhibited the molecular ion at  $m/z$  433.1129 (Fig. 3C). The one fragment ion at  $m/z$  271 (MS: 271.0602 and MS/MS: 271.0581) was attributed to the loss of  $m/z$  162 (glucose moiety) (Fig. 3M) [26,28,29]. On the basis of the described ions, peak 3 was assumed to be genistin 3 of the molecular formula of  $C_{21}H_{20}O_{10}$  in soybean isoflavones [24,26]. In the ESI-TOF-MS and MS/MS spectra, peak 8 ( $t_R = 6.7$  min) was detected as protonated ion  $[M+H]^+$  at  $m/z$  519.1131 and one fragment ion at  $m/z$  271.0607 ( $[M+H]^+ - 248$  amu) (Fig. 3H). This fragmentation ( $m/z$  271.0607) resulted from the loss of malonyl (86 amu) and glucose (162 amu) moieties. The fragment ion at  $m/z$  271.0607 was in agreement with the pattern of peak 3 ( $m/z$  271.0602). As a result, peak 8 was confirmed as malonylgenistin (8) with elemental formula of  $C_{24}H_{22}O_{13}$ . The TOF-MS data of peak 11 ( $t_R = 7.5$  min) showed the molecular ion ( $[M+H]^+$ ) at  $m/z$  475.1237 and the fragment signal at  $m/z$  271.0603 in MS/MS spectrum appeared to have similar fragmentation patterns by comparison of the MS/MS analyses of peaks 3 ( $m/z$  271.0602) and 8 ( $m/z$  271.0607) (Fig. 3K). In other words, the structure of peak 11 may be considered to contain a genistein skeleton through the losses of the protonated glucose and acetyl residues (C-7 site, 162 amu (glucose) + 42 amu (acetyl); 204 amu) (Fig. 3M). Based on the soybean isoflavones, peak 11 was assumed to be acetylgenistin (11) of  $C_{23}H_{22}O_{11}$  formula [24,28]. Peak 12 ( $t_R = 9.7$  min) was also elucidated by comparing their fragmentation ions concern to peaks 3, 8, and 11 as well as the described ion patterns (MS:  $m/z$  271.0601 and MS/MS  $m/z$  271.0603) (Fig. 3L). Therefore, this peak was confirmed with genistein (12) of  $C_{15}H_{10}O_5$  formula (Table 1). Although numerous researchers have established that

**Table 1 – Isoflavone and soyasaponin profiles in the methanol extract of soybean seeds by UPLC-ESI-Q-TOF-MS/MS analysis.**

Peak	t <sub>R</sub> (min)	Formula	Calculated mass [M] (m/z)	Calculated mass [M+H] <sup>+</sup> (m/z)	Observed molecular ion in MS [M+H] <sup>+</sup> (m/z)	Observed fragment ions in MS and MS <sup>2</sup> (m/z)	Identification	Reference
1	3.3	C <sub>21</sub> H <sub>20</sub> O <sub>9</sub>	416.1107	417.1180	417.1185	255.0654	Daidzin	Lee et al., 2008 [21]; Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
2	4.1	C <sub>22</sub> H <sub>22</sub> O <sub>10</sub>	446.1213	447.1286	447.1286	285.0758	Glycitin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
3	5.2	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	432.1056	433.1129	433.1129	271.0581	Genistin	Lee et al., 2008 [21]; Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
4	5.5	C <sub>24</sub> H <sub>22</sub> O <sub>12</sub>	502.1111	503.1184	503.1185	255.0655	Malonyldaidzin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
5	5.6	C <sub>25</sub> H <sub>24</sub> O <sub>13</sub>	532.1217	533.1260	533.1287	285.0770	Malonylglycitin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
6	6.1	C <sub>23</sub> H <sub>22</sub> O <sub>10</sub>	458.1213	459.1286	459.1288	255.0652	Acetyldaidzin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
7	6.2	C <sub>24</sub> H <sub>24</sub> O <sub>11</sub>	488.1319	489.1391	489.1389	285.1221	Acetylglycitin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
8	6.7	C <sub>24</sub> H <sub>22</sub> O <sub>13</sub>	518.1060	519.1133	519.1131	271.0607	Malonylgenistin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
9	6.9	C <sub>15</sub> H <sub>10</sub> O <sub>4</sub>	254.0579	255.0652	255.0651	255.0654	Daidzein	Lee et al., 2008 [21]; Lee et al., 2015 [9]; Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
10	7.3	C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>	284.0685	285.0758	285.0757	285.0759	Glycitein	Lee et al., 2008 [21]; Lee et al., 2015 [9]; Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
11	7.5	C <sub>23</sub> H <sub>22</sub> O <sub>11</sub>	474.1162	475.1235	475.1237	271.0603	Acetylgenistin	Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
12	9.7	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	270.0528	271.0601	271.0601	271.0603	Genistein	Lee et al., 2008 [21]; Lee et al., 2015 [9]; Dueñas et al., 2012 [27]; Shen et al., 2012 [28]
13	10.1	C <sub>67</sub> H <sub>104</sub> O <sub>33</sub>	1436.6460	1437.6533	1437.6532	975.5162, 727.8444, 331.1024	Soyasaponin Ab	Decroos et al., 2005 [14]; Ha et al., 2014 [1]
14	10.7	C <sub>61</sub> H <sub>94</sub> O <sub>28</sub>	1274.5932	1275.6010	1275.6006	945.5033, 813.4618, 331.1028	Soyasaponin Af	Guajardo-Flores et al., 2012 [34]
15	11.0	C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>	942.5188	943.5266	943.5245	491.2402	Soyasaponin I	Lee et al., 2014 [16]; Oh et al., 2016 [30]
16	11.3	C <sub>47</sub> H <sub>76</sub> O <sub>17</sub>	912.5083	913.5161	913.5152	617.4031, 441.3726, 423.3622	Soyasaponin II	Ha et al., 2014 [1]; Lee et al., 2014 [16]
17	11.5	C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	796.4609	797.4687	797.4682	274.2738, 149.0228	Soyasaponin III	Lee et al., 2014 [16]
18	12.1	C <sub>54</sub> H <sub>84</sub> O <sub>22</sub>	1084.5454	1085.5532	1085.5532	562.2529, 543.2804	Soyasaponin αg	Guajardo-Flores et al., 2012 [34]; Ha et al., 2014 [1]
19	12.4	C <sub>54</sub> H <sub>84</sub> O <sub>21</sub>	1068.5505	1069.5583	1069.5581	535.2826	Soyasaponin βg	Guajardo-Flores et al., 2012 [34]; Ha et al., 2014 [1]
20	12.8	C <sub>53</sub> H <sub>82</sub> O <sub>20</sub>	1038.5399	1039.5478	1039.5474	761.4460, 743.4331, 567.4041	Soyasaponin βa	Ha et al., 2014 [1]; Oh et al., 2016 [30]
21	13.1	C <sub>48</sub> H <sub>74</sub> O <sub>17</sub>	922.4926	923.5004	923.5004	462.2538, 424.3657	Soyasaponin γg	Guajardo-Flores et al., 2012 [34]; Lee et al., 2014 [16]; Oh et al., 2016 [30]
22	13.4	C <sub>47</sub> H <sub>72</sub> O <sub>16</sub>	892.4820	893.4899	893.4896	567.4032, 423.3629	Soyasaponin γa	Ha et al., 2014 [1]; Lee et al., 2014 [16]; Oh et al., 2016 [30]



**Fig. 2 – Chemical structures of isoflavones and soyasaponins found in the methanol extract of soybean seeds using UPLC-ESI-Q-TOF/MS analysis.**

soybean isoflavones exist twelve individual compositions, including four chemical groups (malonylglucoside, acetylglucoside, glucoside, and aglycone: daidzein, genistein, and glycitein) [10,17,24,28], to our knowledge, little information has been evaluated for the exactly MS and MS/MS values of isoflavone profile regarding the distribution four forms using UPLC coupled with TOF-mass technique. Herein, we devoted to the isoflavone profiling method through combining liquid chromatographic skill of rapid and adequate separation with mass spectrometry.

Subsequently, we identified ten peaks within a range of 10–13.5 min of UPLC-TOF chromatogram. As illustrated in Fig. 1, the UPLC-ESI-TOF chromatogram in the positive ion mode presented major and minor peaks with superior sensitivity and rapid separation. The most abundant peaks 19 and 20 ( $t_R = 12.4$  and  $12.8$  min) exhibited the molecular ions ( $[M+H]^+$ ) at  $m/z$  1069.5581 and  $m/z$  1039.5474 (Fig. 3T, U and Table 1), representing approximately 50% of the total peak area. Their chemical structures were confirmed with soyasaponin derivatives as soyasaponin  $\beta$ g (19) ( $C_{54}H_{84}O_{21}$ ) and soyasaponin  $\beta$ a (20) ( $C_{53}H_{82}O_{20}$ ), based on the earlier literatures [1,30]. Interestingly, the distribution of two compounds 19 and 20 was detected with high contents of about 90% in total saponin from soybean seeds of previously reported data [1]. These differences may be affected by various factors, including cultivars, environmental stresses, and agronomic conditions as earlier studies of soybean isoflavone and anthocyanin contents [9,25,26]. The remaining eight peaks were also observed as soyasaponin derivatives. Among various peaks, the major compositions 13, 14, 15 and 21

possessed identical molecular ions ( $[M+H]^+$ ) at  $m/z$  1437.6532 (13),  $m/z$  1275.6006 (14),  $m/z$  943.5245 (15), and  $m/z$  923.5004 (21) (Fig. 3N–P and V). Moreover, their retention times showed 10.1, 10.7, 11.0, and 13.1 min (Table 1). According to the above molecular ions and the earlier results, their structures were assigned as soyasaponin Ab (13) of  $C_{67}H_{104}O_{33}$ , soyasaponin Af (14) of  $C_{61}H_{94}O_{28}$ , soyasaponin I (15) of  $C_{48}H_{78}O_{18}$ , and soyasaponin  $\gamma$ g (21) of  $C_{48}H_{74}O_{17}$  [1,14,16]. The minor peaks 16, 17, 18, and 22 were observed with identical molecular ions ( $[M+H]^+$ ) at  $m/z$  913.5152,  $m/z$  797.4682,  $m/z$  1085.5532, and  $m/z$  893.4896 (Fig. 3Q–S and W) and their saponin compositions were tentatively assigned as soyasaponin II (16) ( $t_R = 11.3$  min,  $C_{47}H_{76}O_{17}$ ), soyasaponin III (17) ( $t_R = 11.5$  min,  $C_{42}H_{68}O_{14}$ ), soyasaponin  $\alpha$ g (18) ( $t_R = 12.1$  min,  $C_{54}H_{84}O_{22}$ ), and soyasaponin  $\gamma$ a (22) ( $t_R = 13.4$  min,  $C_{47}H_{72}O_{16}$ ) by comparison with published data (Table 1) [1,16,30]. Although the previous works showed that various saponin derivatives were present in soybean seeds [1,7,16,30], to the best of our knowledge, the current research was the first to characterize the 10 soyasaponins within 3.5 min according to the molecular weights of mass spectral data using rapid and reliable UPLC-ESI-TOF-MS/MS method. Soyasaponin compositions also exhibited considerable differences by comparison with those of previously published data [16,30]. Therefore, the contents and types of saponin in soybean may be affected by multiple factors such as cultivars, sources, and environmental conditions. Consequently, the results of the present work were undertaken to provide information on a rapid and sensitivity technique for the identification of twelve isoflavones and ten soyasaponins by UPLC-ESI-TOF-MS analysis from soybean seeds.

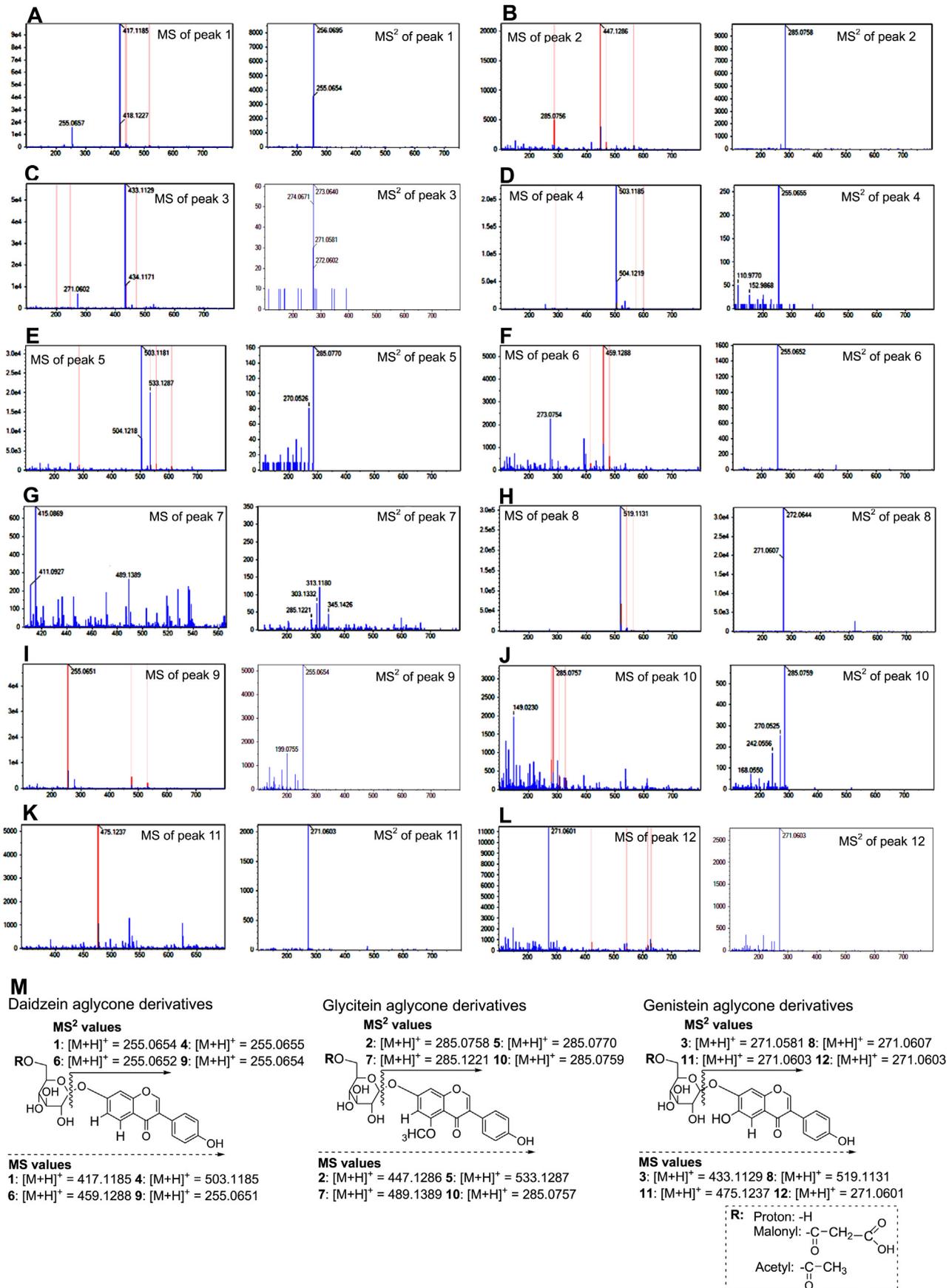


Fig. 3 – The positive ESI-TOF-MS and MS/MS spectra of isoflavones and soyasaponins. (A) daidzin (1), (B) glycitin (2), (C) genistin (3), (D) malonyldaidzin (4), (E) malonylglycitin (5), (F) acetylglycitin (6), (G) acetylglycitin (7), (H) malonylgenistin (8),

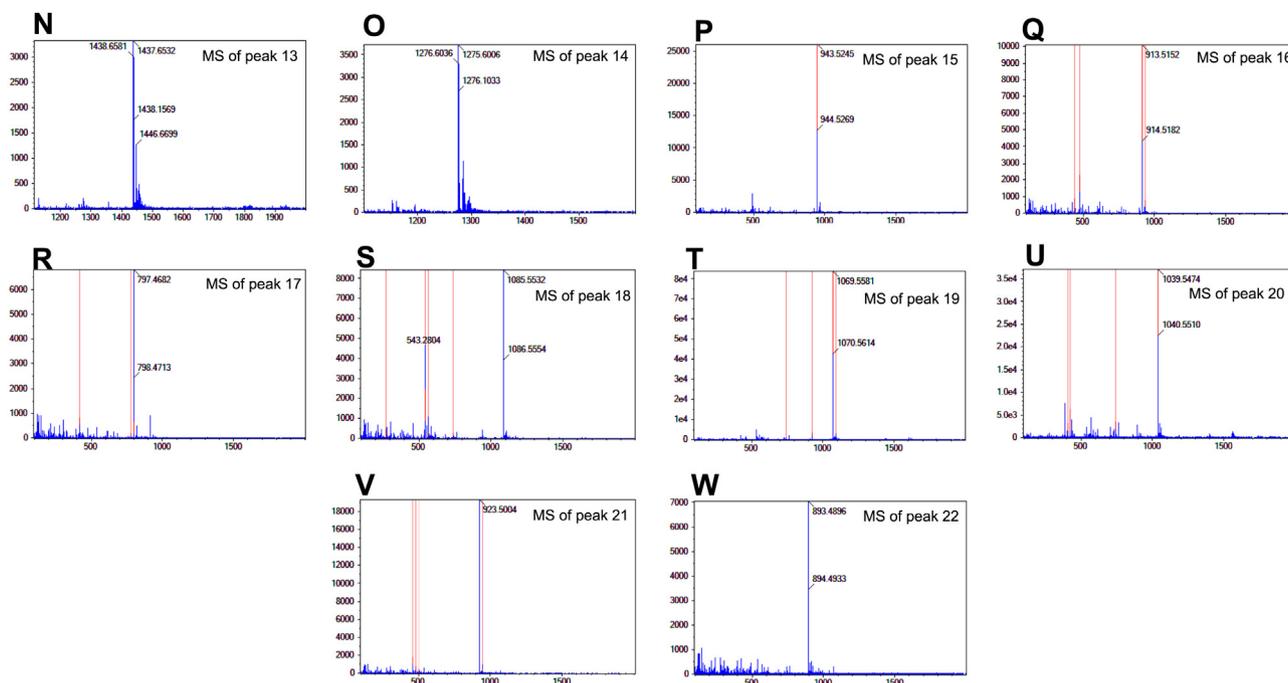


Fig. 3 – (continued).

### 3.2. Comparisons of isoflavones and soyasaponins in different soybean extracts through solvent systems

Until now, numerous researchers have reported on the variation and comparison of the isoflavone and saponin contents from soybeans and soybean foods [10,24,31]. Many studies have also demonstrated that the extraction effects of these above phytochemicals are associated with various factors, including time, solvent system, and temperature [19,32,33]. Among them, solvent contributed to the most important factor for the metabolite extraction [19]. In particular, methanol exhibited good extraction results due to the high polarity [34]. The mixture of methanol with distilled water also provides a wonderful effect for metabolite extraction [35]. We investigated isoflavone and saponin derivatives according to the methanol and water ratios (100, 70, 50, 30, and 10%) in a shaking incubator at 25 °C for 12 h. Moreover, we examined the recovery of two chemical constituents in comparison with other solvents such as CH<sub>2</sub>Cl<sub>2</sub> and acetone. Even though time and temperature may be considered as key factors for increasing metabolite extraction, their contents have a tendency to decrease owing to the degradation and oxidation of molecules by high temperature and longtime extraction [36]. As presented in Table 2, twelve isoflavones were observed in the various extracts by HPLC. The individual and total isoflavone contents showed remarkable differences in the solvent systems. Among different methanol-water solvents, the highest isoflavone contents was 4102.69 μg/g for the 70% methanol extract, while the lowest was 1681.33 μg/g in the

10% methanol extract (Table 2). In other words, total isoflavone content of the 70% methanol extract was 2 times higher than that of the 10% methanol extract (Fig. 4A and D). This phenomenon is similar to the results of previous researches on flavonoids in peanut pods [37]. Four isoflavone types and their contents may be affected by the chemical structure properties according to the extraction solvents. The remaining three methanol extracts exhibited high variations as the following order: 50% methanol (4054.39 μg/g) > 30% methanol (3134.03 μg/g) > 100% methanol (2979.49 μg/g) (Table 2 and Fig. 4B, C, and E). Our results are in agreement with the earlier literatures that phytochemical contents showed considerable differences into aqueous-organic solvent mixture [32,35].

To investigate the other organic solvents on the extraction efficiency of isoflavones, the powdered soybean seeds were extracted with CH<sub>2</sub>Cl<sub>2</sub> and acetone for 12 h at room temperature. Although extraction of phenolic compounds in crops, vegetables, and natural plants depends on organic solvents, their contents were not affected remarkably in low polarity solvents such as CH<sub>2</sub>Cl<sub>2</sub> and acetone (Fig. 4F and G) [19]. Concretely, the CH<sub>2</sub>Cl<sub>2</sub> extract was detected only three isoflavones of malonylglycitin (337.25 μg/g), malonylgenistin (26.60 μg/g), and daidzein (2.34 μg/g), whereas the acetone extract showed the lowest total isoflavone content with 119.00 μg/g (Table 2). On the basis of our findings, the mixtures of aqueous-organic polar solvent provide a powerful effect for isoflavone contents in comparison with those of the solvents of low polarity. Therefore, the methanol-water system may be the appropriate solvent condition for the best extraction of

(I) daidzein (9), (J) glycitein (10), (K) acetylgenistin (11), (L) genistein (12), (N) soyasaponin Ab (13), (O) soyasaponin Af (14), (P) soyasaponin I (15), (Q) soyasaponin II (16), (R) soyasaponin III (17), (S) soyasaponin αg (18), (T) soyasaponin βg (19), (U) soyasaponin βα (20), (V) soyasaponin γg (21), and (W) soyasaponin γa (22).

**Table 2 – Comparison of isoflavone contents in different soybean extracts through solvent systems.**

Solvent extraction	Isoflavone content ( $\mu\text{g/g}$ ) <sup>a</sup>											
	Glucoside			Malonylglucoside			Acetylglucoside			Aglycone		
	Di (1)	Gly (2)	Gi (3)	MDi (4)	MGLy (5)	MGi (8)	AcDi (6)	AcGly (7)	AcGi (11)	De (9)	Gle (10)	Ge (12)
10% MeOH	87.25	30.36	41.02	492.25	65.05	545.52	ND <sup>b</sup>	ND	ND	242.96	28.97	147.95
30% MeOH	273.50	71.47	196.74	949.93	89.41	1130.97	tr <sup>c</sup>	ND	ND	239.77	24.57	157.67
50% MeOH	626.91	149.46	630.86	1009.62	93.28	1250.41	11.14	2.43	1.21	135.81	13.29	129.97
70% MeOH	874.34	185.03	974.84	839.81	79.21	1056.84	5.24	2.34	1.17	43.47	ND	40.40
100% MeOH	870.72	182.33	989.62	346.71	43.92	446.03	67.37	0.84	0.42	18.50	ND	13.03
CH <sub>2</sub> Cl <sub>2</sub>	ND	ND	tr	337.25	ND	26.60	ND	ND	ND	2.34	ND	ND
acetone	44.56	ND	5.36	45.58	ND	21.16	ND	ND	ND	ND	ND	2.34

Di, Daidzin; Gly, Glycitin; Gi, Genistin; MDi, Malonyldaidzin; MGLy, Malonylglucoside; AcDi, Acetyldaidzin; MGli, Malonylgenistin; AcGly, Acetylglucoside; De, Daidzein; Gle: Glycetein; AcGi: Acetylglycetin; Ge: Genistein.

<sup>a</sup> All values are expressed as mean of triplicate experiments on dry weight basis.

<sup>b</sup> ND: not detected.

<sup>c</sup> tr: trace (<0.002  $\mu\text{g/g}$ ).

isoflavone contents in soybean. The individual isoflavone content was remarkably different, especially, malonylgenistin and malonyldaidzin which were the predominant compositions with a range of 46–66% of total isoflavone content in the methanol-water mixtures except 100% methanol extract (Fig. 4A–G and Table 2). Their contents also exhibited higher contents with 62 and 66% in the 10 and 30% methanol extracts than other extracts (Table 2). In more details, the malonylglucoside group exhibited the highest content with 2353.31 and 2170.31  $\mu\text{g/g}$ , representing 58.0 and 69.2% in the 50 and 30% methanol extracts, while the low contents were found in the CH<sub>2</sub>Cl<sub>2</sub> and acetone extracts with 363.85 and 66.74  $\mu\text{g/g}$ . The second major type showed isoflavone glucoside with 2042.67 and 2034.21  $\mu\text{g/g}$  of 69.0 and 49.6% in total content from the 100 and 70% methanol extracts. Especially, daidzin and genistin of glucoside group showed relatively high contents when compared to the other isoflavones (Table 2). As a result, the malonylglucoside type had the highest isoflavone content in all extraction solvent systems, followed by glucoside and aglycone, whereas the lowest was determined in acetylglucoside type. However, previous studies have confirmed that soybean isoflavones contributed in the order of malonylglucoside, glucoside, acetylglucoside, and aglycone forms [38,39]. These differences suggest that soybean isoflavones are positively correlated with environmental stresses (climate factor and soil state), agricultural practice, cultivation skill, extraction condition, and genotype as the results of earlier researches [9,10,16,25,26]. We documented that isoflavone contents of methanol solvent with high polarity does afford good extraction results by comparison with those of low polarity. We also evaluated the first time that the optimal solvent condition of isoflavone extraction was detected by employing 70% methanol.

For gather more information on extraction effects through solvent conditions, we investigated soyasaponin compositions. As shown in Fig. 4H–N, the chromatographic separation of ten soyasaponins was achieved in 10–13.5 min due to the high resolution and rapid separation technique. The individual and total soyasaponin peaks exhibited considerable differences among the extraction solvents (Fig. 4H–N). Especially, soyasaponin  $\beta\text{g}$  (19) and soyasaponin  $\beta\text{a}$  (20) were the most abundant compounds, representing approximately

47% of the total saponin peak area in the 100% methanol extract (Fig. 4L). The distribution of soyasaponin  $\beta\text{g}$  (19) was observed with the highest peak area (29%) and the second major peak was detected in soyasaponin  $\beta\text{a}$  (20) with 18% of total peak area. Interestingly, these two peaks showed significant differences by comparison with previously reported data (about 90% of the total peak area) [1]. This phenomenon suggests that soyasaponin compositions and their contents may vary depending on environmental factors, cultivars, and genetics as those of isoflavones in soybean [34]. The efficiency of the remaining compounds were in the following order: soyasaponin Ab (13) > soyasaponin  $\gamma\text{g}$  (21) > soyasaponin Af (14) > soyasaponin I (15) > soyasaponin  $\gamma\text{a}$  (22). In addition, soyasaponin II (16) and soyasaponin III (17) were present in the lowest areas by comparison with other compositions. Furthermore, the 100 and 70% methanol extracts exhibited all soyasaponin compositions, while the CH<sub>2</sub>Cl<sub>2</sub> and acetone extracts were not observed their peaks (Fig. 4M and N). In individual compositions, soyasaponins Ab (13), Af (14), I (15), II (16), and III (17) were observed in all methanol-water mixture extracts, and soyasaponin  $\alpha\text{g}$  (18) was only detected in the 50% methanol extract except 70 and 100% methanol extracts (Fig. 4J–L). These reasons suggest that the glucose moieties in soyasaponin structures may be positively affected to the high polarity of the methanol or methanol-water mixture solvents. Thus, solvents with high polarity had higher extraction effects when compared to the solvents of low polarity as supported by previous data [34,35]. Overall, the 70% methanol and 100% methanol are found to be the appropriate solvents for the maximum extracts of soyasaponins in soybean. We can consider that the appropriate extraction solvent of soyasaponins may be a key factor in determining the quality and functional characteristic of soybean.

### 3.3. Comparisons of antioxidant effects against DPPH and ABTS radicals in different soybean extracts through solvent systems

DPPH and ABTS radical scavenging techniques are widely reported methods for screening antioxidants in crops, fruits, vegetables, and edible plants [2,9,10,19,25]. These radicals have been used extensively in the food industry due to their

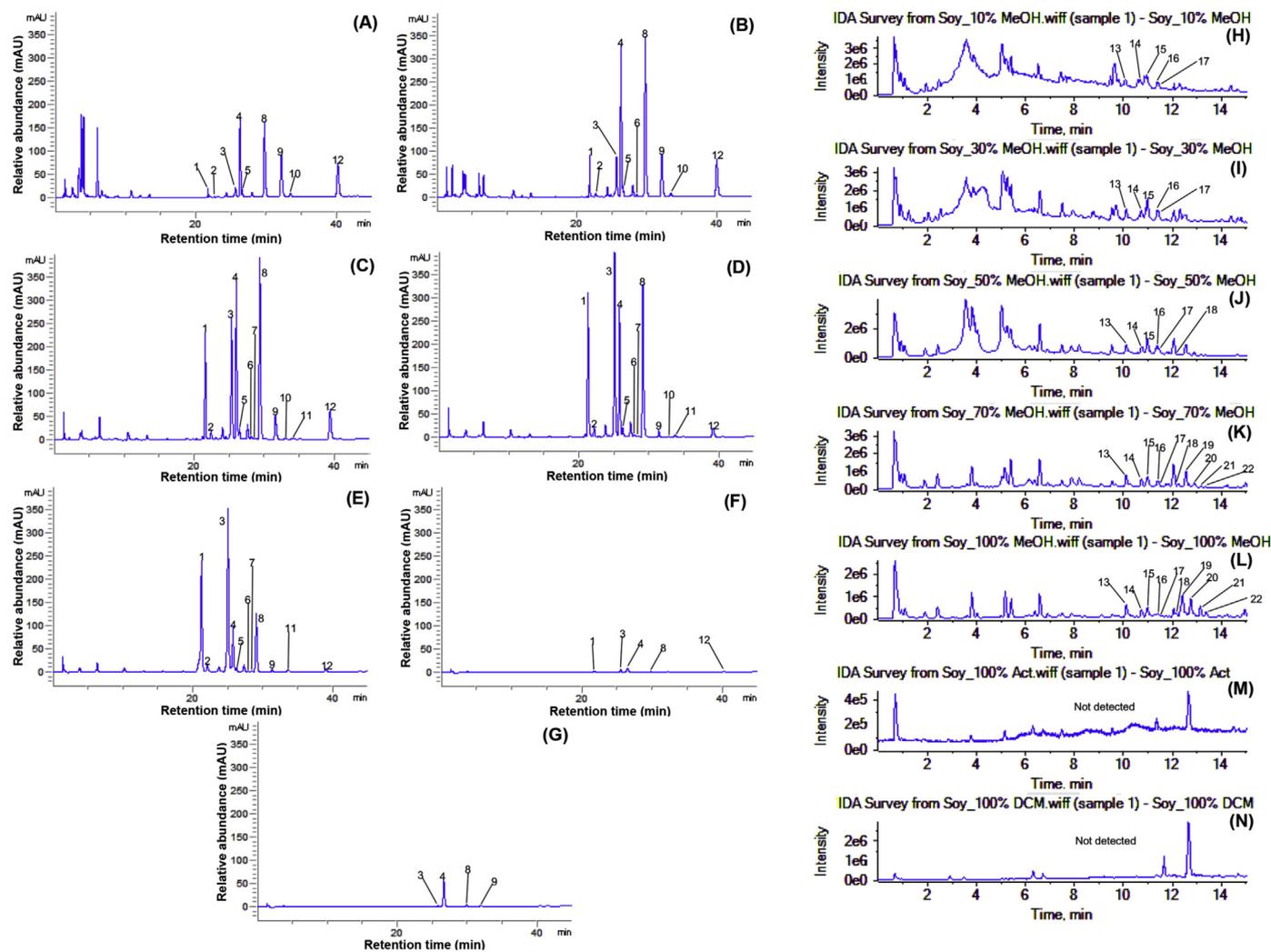


Fig. 4 – Comparisons of HPLC chromatograms concern to isoflavones and soyasaponins in various solvent systems. Isoflavone peak; (A) 10% methanol extract, (B) 30% methanol extract, (C) 50% methanol extract, (D) 70% methanol extract, (E) 100% methanol extract, (F) acetone extract, and (G)  $\text{CH}_2\text{Cl}_2$  extract; Soyasaponin peak; (H) 10% methanol extract, (I) 30% methanol extract, (J) 50% methanol extract, (K) 70% methanol extract, (L) 100% methanol extract, (M) acetone extract, and (N)  $\text{CH}_2\text{Cl}_2$  extract.

characteristics through simplicity, easy control, and cost effectiveness by the spectrophotometric skill [9,10]. Therefore, changes in antioxidant capacities of various soybean extracts at different solvent systems were performed with scavenging effects against DPPH and ABTS radicals. Although several researchers have reported the antioxidant properties of soybeans and soy products [25–27], the earlier works have never been evaluated the variation of the antioxidant capacities regarding radical scavenging activities by different solvent extracts. Also, black, brown, and green soybeans were well known as significantly high antioxidant capacities owing to the polymerized procyanidins, anthocyanins, and isoflavones [9,10,25,26,38], however there is little information available concern to antioxidant activities against the radical scavenging abilities of yellow soybean. In the present study, we investigated DPPH and ABTS radical scavenging effects in the various solvent extracts from yellow soybean (cv. Taekwangkong). The capacities of antioxidant were determined by comparing the percentage inhibition regard to the formation of radicals according to the different solvent extracts (methanol-water mixtures: 100, 70, 50, 30, and 10% methanol, CH<sub>2</sub>Cl<sub>2</sub>, and acetone) and positive controls (DPPH: BHT and ABTS: Trolox) as described in previous studies [9,10,25,26]. Firstly, the scavenging activities against DPPH radical of the methanol extract and BHT increased with increasing concentrations (0.1, 0.2, 0.5, 0.7, 1.0, 2.0, 3.0, and 4.0 mg/mL). Even though 100% radical scavenging effects were detected at 4.0, 3.0, and 2.0 mg/mL, the antioxidant effects on the radical scavengers were carried out at 1.0 mg/mL owing to the changes in the dose dependent contents and the inhibition rate in the sample. The DPPH radical effects in the methanol extract of soybean at different concentrations were as follows: 75% at 1.0 mg/mL, 68% at 0.7 mg/mL, 63% at 0.5 mg/mL, 52% at 0.2 mg/mL, and 31% at 0.1 mg/mL. This phenomenon suggests that the phytochemical contents (isoflavone, phenolic acid, and other phytochemicals) in soybean extract may be responsible for the major contribution to the antioxidant properties against DPPH radical [9,26]. Although the inhibition percentage of this radical in our previous work was 87% at a concentration of 1.0 mg/mL [9], the scavenging effect in the current research was detected low inhibition rate with 75% at the same concentration. These differences may be affected by the environmental conditions and genetic [25,38]. Moreover, the methanol extract showed lower activities than those of BHT in all concentrations. Based on the above preliminary experiments, we investigated antioxidant effects against DPPH radical of various concentrations from different soybean extracts. Significant differences of this radical scavenging effect were found in soybean extracts and their concentrations. The scavenging activities of positive control (BHT) and each extract increased with increasing concentrations. Even though all extracts exhibited lower effects than those of BHT at the same concentration, soybean extract may be considered as important natural antioxidants. The 1.0 mg/mL concentration of all extracts showed the highest scavenging abilities in comparison with those of other concentrations. In detail, the 70% methanol extract exhibited the highest DPPH radical scavenging effect with 93%, while the lowest capacity was observed in the CH<sub>2</sub>Cl<sub>2</sub> extract with 26% (Table 3). Especially, the 70% methanol extract showed higher

effect than BHT (91%). The scavenging capacities of the remaining extracts decreased in the following order at the same concentration (1.0 mg/mL): 50% methanol (85%) > 30% methanol (79%) > 100% methanol (75%) > 10% methanol (61%) > acetone (39%) (Table 3). Although the increase of DPPH activities does not coincide with increasing isoflavone contents, our results suggest that the antioxidant abilities against DPPH radical may be correlated to the phenolic contents including isoflavones in soybean extract as previously reported literatures [25,26]. Other phytochemicals may also be responsible for the major portion of antioxidant activities on this radical [21,26,34]. Therefore, the antioxidant properties against DPPH radical exhibited considerable differences in soybean extracts of seven solvent systems. The scavenging effect of the individual extract on ABTS radical generated by potassium persulphate was compared to Trolox (positive control). All extracts had lower activities than positive control (96%), and their effects increased with increasing concentration, as was detected in the results obtained from the DPPH radical. The highest ABTS scavenging effects of all extracts were obtained at a concentration of 1.0 mg/mL, as those of DPPH radical. The 70% methanol extract had the highest scavenging activity, whereas the CH<sub>2</sub>Cl<sub>2</sub> extract had the lowest effect (Table 3). In more detail, the highest inhibition percentage of potent free radical scavenger was 95% in the 70% methanol extract (1.0 mg/mL) and the effects of the remaining extracts were in the following order: 90% (50% methanol extract) > 84% (30% methanol extract) > 80% (100% methanol extract) > 73% (10% methanol extract) > 57% (acetone extract) > 49% (CH<sub>2</sub>Cl<sub>2</sub> extract) (Table 3). Based on the above considerations, the antioxidant abilities against ABTS radical were observed significant differences in various extracts and their concentrations. All extracts also exhibited higher ABTS radical scavenging activities than those of the DPPH assay. These results suggest that the inhibition of ABTS radical may be attributed to the scavenging abilities of chain breaking and hydrogen donating antioxidants in soybean extract by comparison with the inhibition of hydrogen donating activities on DPPH radical [40,41]. Consequently, the antioxidant effects against DPPH and ABTS radicals were correlated with isoflavone contents [9,10]. Phenolic acids and other metabolites in soybean extract may be also affected in radical scavenging activities [42]. Furthermore, the appropriate extraction solvent can be considered as 70% methanol system due to the highest isoflavone contents and antioxidant activities. It is confirmed that the 70% methanol extract of soybean may be attributed as a potent radical scavenger for development of nutraceuticals and functional foods. The comparison of the isoflavone contents and antioxidant properties in various soybean extracts regarding solvent systems was demonstrated for the first time in this research.

#### 3.4. Comparison of $\alpha$ -glucosidase inhibitory activities in different soybean extracts through solvent systems

It is commonly established that the  $\alpha$ -glucosidase inhibition plays important roles in controlling postprandial hyperglycemia [43]. Also, soybean phytochemicals are associated with the inhibitory properties against  $\alpha$ -glucosidase as previously reported literatures [44,45]. However, the determination of

**Table 3 – Comparisons of antioxidant and  $\alpha$ -glucosidase inhibitory activities as well as total phenolic. Contents in different soybean extracts through solvent systems.**

Solvent extraction	Radical scavenging activity (%) <sup>a</sup>		$\alpha$ -Glucosidase inhibitory activity (%) <sup>a</sup>	Total phenolic content (mg GAE/g) <sup>b</sup>
	DPPH radical	ABTS radical		
10% MeOH	61 <sup>c</sup>	73	37	0.93
30% MeOH	79	84	55	1.47
50% MeOH	85	90	76	1.95
70% MeOH	93	95	81	2.10
100% MeOH	75	80	49	1.45
CH <sub>2</sub> Cl <sub>2</sub>	26	49	17	0.57
acetone	39	57	24	0.68
Positive control	91 (BHT)	96 (Trolox)	79 (acarbose)	–

<sup>a</sup> All values of samples and positive controls are expressed as the mean of triplicate determinations.

<sup>b</sup> All values of samples are expressed as the mean of triplicate determinations.

<sup>c</sup> Radical scavenging and  $\alpha$ -glucosidase inhibitory effects as well as total phenolic contents in the 70% methanol extract of samples and positive controls were carried out at 1.0 mg/mL.

this enzyme inhibition in soybean extracts on different solvent systems has not been extensively evaluated. We investigated the  $\alpha$ -glucosidase inhibitory effects by comparing the percentage of inhibition through the solvent systems at 1.0 mg/mL concentration according to the results of antioxidant properties. In our experiments, when the concentrations of solvent extract increased, the inhibitory activities of this enzyme increased and their variations showed significant differences in solvent systems. The highest effect was observed in the 70% methanol extract with inhibition of 81%, while the CH<sub>2</sub>Cl<sub>2</sub> extract was detected the lowest capacity (17%) (Table 3). The  $\alpha$ -glucosidase inhibitory abilities of the remaining extracts occurred in the following order: 50% methanol extract (76%) > 30% methanol extract (55%) > 100% methanol extract (49%) > 10% methanol extract (37%) > acetone extract (24%) (Table 3). The patterns of  $\alpha$ -glucosidase inhibition were similar to those of antioxidant activities and total phenolic contents. Although the inhibitory activities against  $\alpha$ -glucosidase of most extracts were lower than the acarbose effect (positive control, 79%), the value of the 70% methanol extract was slightly higher than positive control. It is confirmed that isoflavones and other phenolic compounds in soybean may be considered as potent  $\alpha$ -glucosidase inhibitory properties and an important natural sources for pharmaceuticals and functional foods. In addition, these above findings are in agreement with earlier research that the inhibitory effects against  $\alpha$ -glucosidase of soybean can be correlated with the phenolic compounds [46]. The variations of inhibitory effects against this enzyme were demonstrated for the first time in different solvent systems.

### 3.5. Comparison of total phenolic contents in different soybean extracts through solvent systems

It is established that total phenolic contents in foods, crops, and edible sources depend on many factors such as extraction solvent, extraction time, extraction temperature, and sample ratio [19,32,33,42]. Numerous researches have also reported that antioxidant abilities are positively correlated with the total phenolic contents [9,23,26,30,40] and solvents of high polarity including methanol have been used to afford good extraction of total phenolic contents [34,35]. We investigated

total phenolic contents in soybean extracts (1.0 mg/mL) of different solvent systems due to the highest DPPH and ABTS radical scavenging properties. As considering the antioxidant effects, total phenolic contents varied significantly, depending on the solvent systems. The highest total phenolic content was observed with  $2.10 \pm 0.05$  mg GAE/g in the 70% methanol extract, whereas the CH<sub>2</sub>Cl<sub>2</sub> extract exhibited the lowest content with  $0.57 \pm 0.02$  mg GAE/g (Table 3). The rank order of the remaining extracts was consistent with those of the antioxidant abilities. Their contents were as follows, in decreasing order: 50% methanol extract > 30% methanol extract > 100% methanol extract > 10% methanol extract > acetone extract with  $1.95 \pm 0.07$ ,  $1.47 \pm 0.02$ ,  $1.45 \pm 0.05$ ,  $0.93 \pm 0.01$ , and  $0.68 \pm 0.01$  mg GAE/g, respectively (Table 3). These above results are similar to those of the previous literatures [11,47]. Our study reveals that the antioxidant activities on DPPH and ABTS radicals in soybean may be significantly affected by the total phenolic contents. We evaluated for the first time the comparison and determination of total phenolic content in soybean extracts according to the solvent systems. We believe that the 70% methanol extract of soybean may contribute significantly as potent free radical scavengers and good natural source for healthy foods owing to the total phenolic content and radical scavenging effect.

## 4. Conclusion

In the present research, we characterized the isoflavone and saponin profiles in 14 min by UPLC-ESI-Q-TOF-MS skill of rapid and high resolution analytical method from soybean. Two phytochemical profiles were identified as twelve isoflavones and ten soyasaponins, particularly, malonylglucoside isoflavones (4, 5, and 8) and soyasaponin  $\beta$ g (19) and soyasaponin  $\beta$ a (20) were the abundant components. Moreover, our study was the first to demonstrate comparisons of isoflavone and saponin compositions from various extraction solvents. Two chemical groups showed significant differences in solvent systems, interestingly, the 70% methanol extract was obtained the appropriate solvent for the best extraction due to the highest isoflavone content ( $4102.69 \mu\text{g/g}$ ) and ten soyasaponin peaks. The remaining three methanol extracts

were observed with high variations as the following order: 50% methanol (4054.39  $\mu\text{g/g}$ ) > 30% methanol (3134.03  $\mu\text{g/g}$ ) > 100% methanol (2979.49  $\mu\text{g/g}$ ). The  $\text{CH}_2\text{Cl}_2$  and acetone extracts showed very low isoflavone contents with 366.19 and 119.00  $\mu\text{g/g}$ . All soyasaponins were detected in the 100 and 70% methanol extracts, while the  $\text{CH}_2\text{Cl}_2$  and acetone extracts were not observed with only one soyasaponin peak. The scavenging effects against DPPH and ABTS radicals as well as total phenolic contents also differed considerably in different solvent extracts. Specifically, the 70% methanol extract exhibited the highest antioxidant effect (the scavenging activities on DPPH radical: 93%; ABTS radical: 95% at 1.0 mg/mL) and total phenolic content (2.10  $\pm$  0.05 mg GAE/g). In addition, this extract was observed to have the highest  $\alpha$ -glucosidase inhibitory capacity with 81%. It is confirmed that strong  $\alpha$ -glucosidase inhibitory and radical scavenging capacities of soybean extract are positively correlated with high total phenolics including isoflavones. This work has helped to establish for quantitative and qualitative analyzes of phytochemicals from the extracts of soybeans and soy products. Future researches are still required to investigate the potential biological activities and soyasaponin contents to the development of human health functional foods.

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