

Analysis of phytoestrogens from eleven soybean cultivars using LC-MS approach

Hung-Yu Lin¹, Dinar S. Saputri¹, Sahri Yanti¹, Dinesh Chandra Agrawal¹, Wen-Goang Yang², Wei-Jyun Chien^{1*}

¹ Department of Applied Chemistry, Chaoyang University of Technology, Taichung City 413310, Taiwan

² Department of Leisure Services Management, Chaoyang University of Technology, Taichung City 413310, Taiwan

ABSTRACT

The beneficial effects of soybean isoflavones for the human body have been confirmed, and such as antioxidant activity and suppresses cancer occurrence. Therefore, soybean is widely used as food in the world. In the past few decades, several soybean varieties have been developed. A high-performance liquid chromatography electro-spray ionization with tandem mass spectrometry (HPLC-ESI-MS/MS) approach was developed and validated to determine isoflavone in 11 soybean varieties of Taiwan. The separation of 12 isoflavones was realized with a Phenomenex F5 column (2.1 × 50 mm, 1.7 μm) and using acetonitrile as mobile phase over a total analytical run time of 15 min. An Agilent 6470 triple quadrupole mass spectrometer (QQQ) with multiple reaction monitoring (MRM) was employed in ESI positive mode was achieved for isoflavone analysis. In this method validation includes the linear relationship in the range of 3.0 to 800 ng/mL ($R^2 > 0.99$); extraction recovery (71.2% to 93.6%); matrix effect (71.3% to 84.9%); intra- and inter-day accuracy were within 81 to 118% and intra- and inter-day precision below 15%. The LC-MS approach was successfully achieved to assess isoflavone of 11 soybean varieties, and the average total isoflavone content was 7150 ng/mL. In addition, this approach can contribute to robust applications as a workflow for the stable and sensitive detection of soybean seeds.

Keywords: Isoflavone, Soybean, Phytoestrogens, High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

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Corresponding Author:

Wei-Jyun Chien

wjchien@gm.cyut.edu.tw

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1. INTRODUCTION

Soybean (*Glycine max* L.) is one of Taiwan's essential agricultural products. According to the color and utilization of seed coats, several varieties, including soybeans, black beans, yellow beans, and edamame (immature green soybean). Soybean has a wide range of uses as oil, tofu, bean sprouts, soy milk, and soy sauce that can also be used as an essential raw material for the producing soap, glycerin, and hardened oil in the industry. In the Taiwanese's daily diet, soybeans are used as daily food to supplement plant-based protein. According to modern nutritional analysis, soybeans have high isoflavones of nutritional value that have become a worldwide health food (de Oliveira Silva et al., 2018). Isoflavones are structurally similar to the female hormone 17-β-estradiol and have biological activities (Nemitz et al., 2015). Therefore, isoflavones called phytoestrogens can have estrogenic or anti-estrogenic effects (Kurahashi et al., 2009). Isoflavones have been found to beneficial effect on enhanced bone formation due to exhibit estrogenic activity and promotion of insulin-like growth factor (IGF-I) production (Harada et al., 2007). Isoflavones have been proved in breast cancer prevention, which allows them to bind to both estrogen receptors (ER) for activate the signaling pathway of apoptotic genes like Bcl-2, P53, Caspase-3, and BRCA-2

(Takagi et al., 2015). Isoflavones have antioxidative effects and anti-inflammatory actions to inhibit endothelial cell dysfunction, thus reducing atherosclerosis and cardiovascular diseases (Lee et al., 2004).

Soy isoflavones are commonly found in legumes, and their content has been affected by the variety, growth stage, and planting conditions (Teekachunhatean et al., 2013). Isoflavones have been classified into four chemical forms in seed soybean, which are (a) Aglycones (Daidzein; genistein; glycitein), (b) Glycosides (Daidzin; genistin; glycitin), (c) Acetylglycosides (Acetyldaidzin; acetylgenistin; acetylglycitin), and (d) Malonylglycosides (Malonyldaidzin; malonylgenistin; malonylglycitin) (Mayo et al., 2019). Several studies have reported that malonylglycosides are the largest and aglycones least abundant of isoflavones in soybean (Azam et al., 2020). However, aglycones have been confirmed to be more biologically active and have human health benefits than other isoflavones (Hsiao et al., 2020).

In the last 10 years, many analytical extractions, separation, and detection methods for isoflavones in soybean have been reported (López-Fernández et al., 2020). In particular, high-performance liquid chromatography (HPLC) coupled with electro-spray ionization (ESI) and triple quadrupole mass spectrometry (QqQ) is a robust analytical approach for qualitative and quantitative (Lin et al., 2020). The normal-phase and reverse-phase are significant types of HPLC columns that depend on the respective stationary phase materials. Although the reverse-phase column separates isoflavones, that is the most convenient method for quantification and quantitation in soybean. The Kinetex F5 column belongs to the reversed phase; that stationary phase is bonded with pentafluorophenyl (PFP) (Lin et al., 2021). The column provides hydrogen bonding interactions and dipole-dipole interactions mechanism for polar compounds; hydrophobic, aromatic and π - π interactions for aromatic rings or carbon-containing organic compounds. As explained earlier, isomeric, aromatic, and conjugated compounds show unique separation selectivity on the F5 column compared to traditional reversed-phase columns (Chien et al., 2020).

Since 1981, mass spectrometry has become a necessary technique for different scientific disciplines (analytical chemistry, physics, life sciences, and medical sciences) (Qasim 2017). Indeed, the tandem mass spectrometry (MS/MS) provides significantly more reliable qualitative fragment ion on QqQ; analyte via the first quadrupole Q1 (precursor ion) for selection, and second quadrupole Q2 (CID, collision-induced dissociation) for ion fragmentation, and the third quadrupole Q3 (product ions) scans for quantification in multiple reaction monitoring (MRM) modes (Famiglini et al., 2021). Several LC-MS/MS methods have been developed to characterize of isoflavones in soybean (Gómez et al., 2018; Prabakaran et al., 2018). However, few studies have been reported 12 isoflavones in soybean varieties of Taiwan (Iwashina et al., 2018). The purpose of this study was to determine isoflavones in common soybean varieties by using LC-MS/MS. Moreover,

provide more information for soybean breeders to improve on new varieties in the future.

2. MATERIALS AND METHODS

2.1 Seeds of the Soybean Cultivars

Total 11 varieties of soybean seeds including Hualien 1 (HL1), Hengchun (HC), Kaohsiung 7 (KH7), Kaohsiung 10 (KH10), Tenstone (TS), Tainan 3 (TN3), Tainan 5 (TN5), Tainan 8 (TN8), Tainan 9 (TN9), Tainan 10 (TN10) and Tainan 11 (TN11) were harvested in the summer of 2021 and provided by Wu, C. H. from Tainan District Agricultural Research and Extension Station (Muchang Hsinhua District, 71246 Tainan, Taiwan, ROC). The intact black soybeans were washed and air-dried, after which they were homogenized process, and stored at -4°C before analysis.

2.2 Materials and Reagents

HPLC-grade formic acid (FA), acetonitrile (ACN) and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q Gradient water system (Milford, USA). Twelve standard isoflavone compounds (daidzein, daidzin, genistein, glycitein, glycitein, genistin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin and malonylglycitin) were purchased from Sigma-Aldrich (St. Louis, USA). The chemical structure of isoflavones were shown Fig. 1.

2.3 Isoflavones Extraction from Soybean

The soybean powder sample (1 g) with 10 mL of water/acetonitrile/formic acid (70:30:0.5, v/v/v) was transferred into a 50 mL centrifuge tube in an ultrasonic-assisted extraction bath at 40°C for 10 min. Then supernatant (3 mL) was divided into a 15 mL centrifuge tube and were centrifuged at 2,550 rpm for 180 seconds. Then the supernatant (1 mL) was filtered with syringe filters (PVDF, $0.45\ \mu\text{m}$, 13 mm, Phenomenex) and stored at -4°C until LC-MS analysis.

2.4 LC-MS/MS with MRM Conditions

The isoflavone compounds were analyzed in soybean using a liquid chromatography–mass spectrometry system (LC system: Agilent 1260 infinity binary system; MS system: Agilent 6470 Triple Quadrupole, Agilent, CA, USA) and with the scan mode of multiple reaction monitoring (MRM). 12 isoflavone compounds were separated by Agilent 1260 LC using UHPLC Kinetex F5 Column ($2.1 \times 50\ \text{mm}$, $1.7\ \mu\text{m}$, Phenomenex). The mobile phases consisted of 0.1% formic acid in water (mobile phases A) and 0.1% formic acid in acetonitrile (mobile phases B) with an injection volume of $1.0\ \mu\text{L}$ at 40°C for column chamber. The LC gradient elution was performed at $0.35\ \text{mL/min}$ as follows, starting with 5.0% B for 0.5 min, followed by a linear ramp to 40% B over 7 min, then increased to 100% B

at 0.1 min, remaining for 2.9 min. Finally, recovered to 5.0% B at 0.1 min, then equilibrated for 2.9 min. All analyte retention times were described in Table 1.

The Agilent 6470 Triple Quadrupole system was applied in positive mode under electro-spray ionization (ESI) for each isoflavone compound. The parameters of MRM data acquisition were: capillary voltage (4,130 V); sheath gas heater (290°C); sheath gas flow (9 L/min); drying gas flow (9 L/min); drying gas temperature (295°C); nebulizer pressure (19 psi); VCharging (510 V). Each isoflavone was used two transitions in MRM mode, the quantitative and qualitative ion. Table 1 represents each analyte and MS/MS acquisition parameter.

2.5 Preparation of Stock Solutions, Calibration and Quality Control (QC) Samples

Each isoflavone was mixed with 5% ACN at a concentration of 2,000 ng/mL. Then the stock of isoflavone

was prepared at calibration concentrations ranging (from 5, 20, 100, 200, 400, and 800 ng/mL) at six points. The quality control (QC) samples were prepared at two levels; the lower limit of quantification (5 ng/mL) and the upper limit of quantification (800 ng/mL). All isoflavone mix solutions and QC samples were stored at -4°C until analysis.

2.6 Method Validation

In accordance with the United States Food and Drug Administration (FDA) guidelines for bioanalytical assay validation to ensure this method was validated, including accuracy and precision, extraction efficiency and matrix effects, the limit of detection (LOD) and the lower limit of quantification (LLOQ), linearity and calibration curves. To calibration's linearity was evaluated using isoflavones standards at six concentrations range responding to peak area from 5.0 to 800 ng/mL. The correlation coefficients (R²) should be above 0.99 with a perfect positive correlation.

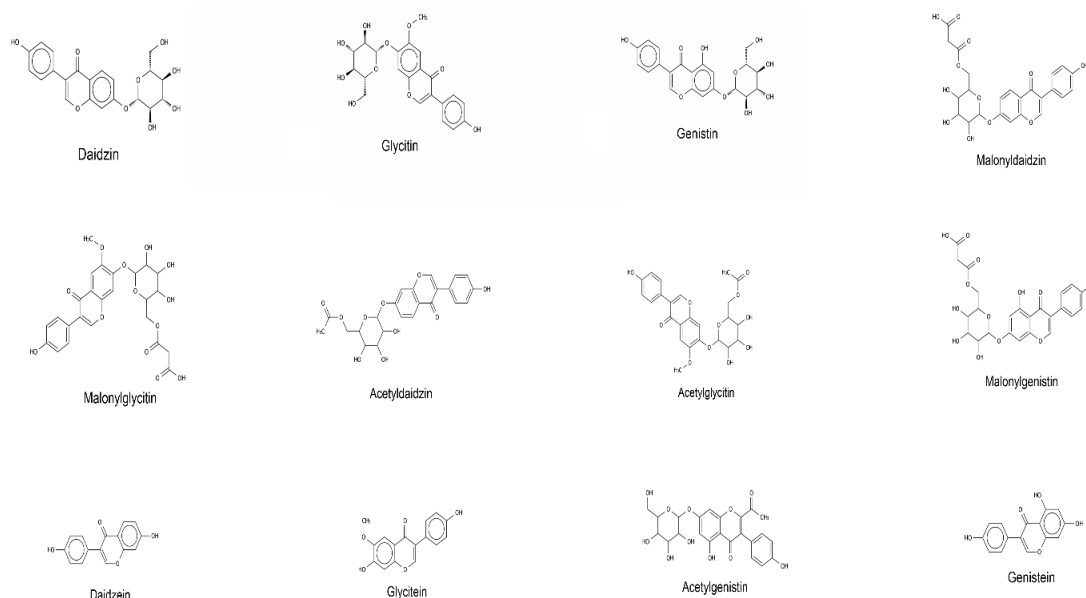


Fig. 1. The chemical structures of 12 isoflavones

Table 1. The acquisition parameters of multiple reaction monitoring for quantitative isoflavones analysis

| Isoflavone | CAS number | Formula (Da) | [M+H] ⁺ (m/z) | MRM transitions (m/z) | Fragmentor energy (V) | Collision energy (V) | Ret. Time (min) |
|------------------|-------------|---|--------------------------|-------------------------------------|-----------------------|----------------------|-----------------|
| Daidzin | 552-66-9 | C ₂₁ H ₂₀ O ₉ | 417 | 225 ^a / 199 ^b | 110 | 26 | 4.5 |
| Genistin | 529-59-9 | C ₂₁ H ₂₀ O ₁₀ | 433 | 271 ^a / 153 ^b | 110 | 22 | 5.2 |
| Glycitin | 40246-10-4 | C ₂₂ H ₂₂ O ₁₀ | 447 | 285 ^a / 270 ^b | 110 | 23 | 4.7 |
| Daidzein | 486-66-8 | C ₁₅ H ₁₀ O ₄ | 255 | 137 ^a / 91 ^b | 100 | 36 | 6.0 |
| Genistein | 446-72-0 | C ₁₅ H ₁₀ O ₅ | 271 | 153 ^a / 91 ^b | 100 | 37 | 7.2 |
| Glycitein | 40957-83-3 | C ₁₆ H ₁₂ O ₅ | 285 | 242 ^a / 118 ^b | 100 | 36 | 6.1 |
| Malonyl daidzin | 124590-31-4 | C ₂₄ H ₂₂ O ₁₂ | 503 | 255 ^a / 199 ^b | 155 | 30 | 5.3 |
| Malonyl genistin | 51011-05-3 | C ₂₄ H ₂₂ O ₁₃ | 519 | 271 ^a / 153 ^b | 155 | 30 | 5.9 |
| Malonyl glycitin | 137705-39-6 | C ₂₅ H ₂₄ O ₁₃ | 533 | 285 ^a / 270 ^b | 155 | 31 | 5.5 |
| Acetyl daidzin | 71385-83-6 | C ₂₃ H ₂₂ O ₁₀ | 459 | 255 ^a / 137 ^b | 125 | 22 | 5.8 |
| Acetyl glycitin | 134859-96-4 | C ₂₄ H ₂₄ O ₁₁ | 489 | 285 ^a / 270 ^b | 125 | 20 | 5.9 |
| Acetyl genistin | 73566-30-0 | C ₂₃ H ₂₂ O ₁₁ | 475 | 271 ^a / 268 ^a | 125 | 18 | 6.4 |

^a: quantitative ion ; ^b: qualitative ion; Ret. time: retention time

The LOD was defined the lowest standard concentration in this approach, and the signal-to-noise ratio over at least 3. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were defined at which the lowest and the highest calibration point on the calibration curve, and that can be provided with percentage relative error (%RE) within 80 and 120% for accuracy and relative standard deviation (%RSD) should be less than 15% for precision, with complying to intra-day and inter-day batch analysis. The extraction efficiency and matrix effect were assessed for five repeats of QC samples by comparing the peak area response. All matrix effect and extraction recovery were evaluated by $ME\% = [(A-B) / C] \times 100$ and $RE\% = (D/A) \times 100$, respectively. Where A is the peak area obtained from the post-extracted spiked sample; B is the peak area obtained from the post-extracted non-spiked sample; C is the peak area obtained from the standard in the blank solvent; D is the peak area obtained from the pre-extracted spiked sample.

3. RESULTS AND DISCUSSION

3.1 Solvent Extraction for Soybean Samples

Many analytical methods for extraction of isoflavones from soybean has been reported in the past ten years (Bustamante-Rangel et al., 2018). Some extraction techniques like as ultrasound-assisted extraction (Magiera et al., 2017), supercritical fluid extraction (Klejduš et al., 2010), QuEChERS (Bustamante-Rangel et al., 2013), solidphase extraction and liquid-liquid extraction. Compare other methods of extracting soy isoflavones from soybeans, the direct solvent extraction for isoflavone in soybean has become more than 65% of conventional methods (Torofunes et al., 2012). In previously studies reported several extraction solvents for various isoflavone, that were known as acetonitrile, acetone, ethanol, methanol, with added acid and without (Nile et al., 2021). Compared with acetonitrile, the main advantage of ethanol has low cost and less toxin for environment, and one significant disadvantage is low extraction efficiency for isoflavones. Although, the extraction of isoflavones was executed in an ultrasonic-assisted bath, and using 80% methanol with non-acidified has provided the best extraction method for isoflavone in point of these results. In this case, soybean samples were investigated with solvent direct extraction coupling ultrasonic-assisted extraction process in different ratios of acetonitrile and formic acid. As a result of this study, the solvent extraction selection as water/acetonitrile/formic acid (70:30:0.5, v/v/v) proved to be better because this obvious extraction is higher abundant than others (data not shown).

3.2 The HPLC-MS and MRM Method Development

At present, a variety of the separation and detection techniques of isoflavones in soybean sample have been reported. Including thin-layer chromatography, capillary

electrophoresis (Dinelli et al., 2007), gas chromatography and liquid chromatography for separation assess; UV-visible, fluorescence, nuclear magnetic resonance and mass spectrometry for detection techniques (Yerramsetty et al., 2011). In addition, the HPLC-MS approach provides efficiency, sensitivity, and has become the most common methods for analysis and quantification. The Phenomenex kinetex core-shell F5 column provides five interaction mechanisms, such as hydrophobic retention, aromatic π - π electrons, dipole moment, steric interaction and hydrogen bonding for retention of 12 isoflavone compounds. The mobile solvent consisted of ACN with FA under gradient elution were described in section 2.4. When using ACN as an organic elution solvent, the peak resolution of isoflavone compounds was better than MeOH (data not shown). Meanwhile, shorten sample run time within 7.2 min to improve analysis efficiency and reduce column back pressure to maintain instrument stability. To compare the sensitivity and selectivity of 12 isoflavones under ESI mode, an Agilent Mass hunter Optimizer software that automatically optimizes the MS and MS/MS data acquisition was used. Table 1. shows each isoflavone MRM parameter including ESI polarity, analyte transitions (precursor ion and product ion pair), collision energy and fragmentor energy. In positive ion mode (ESI+) with correct voltage value exhibited the higher amount of peak area, and selection of transitions pair promoted specificity for all isoflavone analyte. This approach provides a complete MRM parameter for the analysis of isoflavone and demonstrates an efficient LC method to separate 12 analytes even though not in the ultra performance liquid chromatography (UPLC) or ultra high performance liquid chromatography (UHPLC) system (Yang et al., 2020). The MRM chromatogram of 12 isoflavone is shown in Fig. 2. Despite this, some progress has been made in this HPLC method, such as shorter column length for reducing analysis times; select sub-2 μ m particles size for sensitivity; choosing correct stationary phase and mobile phase for separation efficiency.

3.3 LOD, LOQ, Linearity, Precision and Accuracy

The HPLC-MS/MS quantification method has been validated a linear range from 3.0 to 800 ng/mL with a correlation coefficient R^2 over 0.99. That limit of detection (LOD) and limit of quantification (LOQ) was 1.5 mg/mL and 3.0 ng/mL for each isoflavone, respectively (Table 2). The results of precision and accuracy (inter-day and intra-day) were evaluated by QC samples which results are listed in Table 2. The data reveal that %RSD for precision ranged from 1.6% to 14.2% and %RE for accuracy ranged from 81.9 to 118.8%. These results demonstrated an acceptable analysis method for isoflavone analyte by LC-MS/MS within limits recommended by most U.S. FDA guidelines (Prathipati et al., 2019).

3.4 Extraction Efficiency and Matrix Effect

The extraction recovery and matrix effect were estimated at two QC points (concentration: 3.0 and 800 ng/mL). The estimation of extraction efficiency was added pre-spike or post-spiked QC with that in three repeats (Table 3). The mean extraction efficiency for isoflavones was 71.2% to 93.6%, revealing that the extraction process was recommended that soybean sample. The access of matrix effect on endogenous isoflavone compounds was compared by ESI ion suppression/enhancement. The mean matrix effect for isoflavones was in the range of 71.3% to 84.9%, that exhibiting there was no significant ion suppression in the analyses of the soybeans.

3.5 Application of LC-MS Approach for Soybean Varieties

The concentration of isoflavone compounds was quantified in a total of 11 soybean varieties from Taiwan using the LC-MS/MS method with the method was successfully validation established. The profile of individual isoflavones in 11 analyzed soybean varieties is presented in Fig. 3. The daidzin, genistin and malonylgenistin were the insignificant main isoflavones in the soybean samples, while acetyldaidzin, acetylglycitin and acetylgenistin were lower than the composition ratio of total isoflavone abundance (Kurzer et al., 1997). During the isoflavones group, TS cultivar contains the highest

daidzein, genistin and malonylgenistin; HC cultivar contains the highest glycitin, glycitein, malonuglycitin, acetyldaidzin, acetylglycitin and acetylgenistin; KH7 cultivar contains the highest daidzin and genistein; TN7 cultivar contains the highest malonyldaidzin and malonylgenistin, respectively. *In vitro* studies, isoflavones exhibit a considerable antioxidant activity, which especially daidzein is more potent antioxidant than the rest of isoflavones. *In vitro* study report, genistein, glycitein and daidzein have the phytoestrogen potency, which block the endogenous estrogen effects (Liu et al., 2017). In this context, the average total isoflavone content of 11 soybean varieties was 7150 ng/mL. Table 4. shows the mean total isoflavone contenttotal of soybean in 11 soybean varieties under five repeatability investigation with mean a ranging from 10,798.4 to 4,864.7 ng/mL. A higher intake of soybean isoflavone (30-60 mg/day) during maturing woman may reduce the risk of the breast cancer (Wada et al., 2013). The highest and lowest mean amount of total isoflavone was TS and HL1, respectively. Nowday, soybean are the main dietary intakes source of isoflavones in humans, which can be used as an alternative therapy for hormonal disorders (Muthyala et al., 2004). However, isoflavones do not play as hormones in plant, while as fungistatic, antibacterial, antiviral, and antioxidant properties during stress and microbe attacks (Bellou et al., 2012). The significant variations in concentration of isoflavone between soybean cultivars should be considered in soybean breeding.

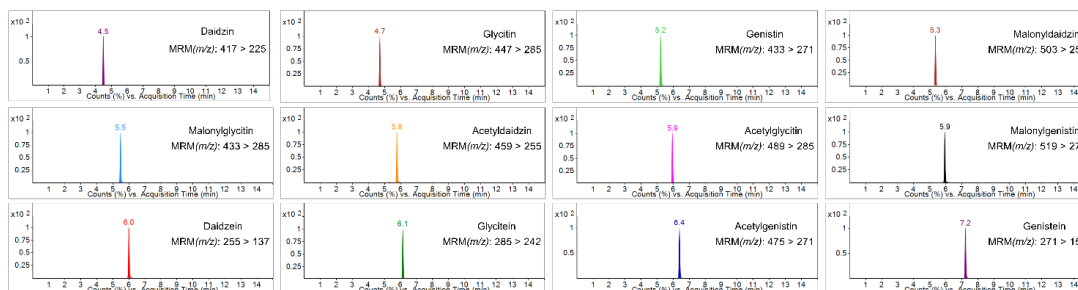


Fig. 2. The performance MRM chromatograms of 12 isoflavones standard in solvent

Table 2. Validation characteristics of method developed by LC-MS/MS

| Compound | Linear range (ng/mL) | Regression equation | R ² | Intra-day (n = 3) | | Intra-day (n = 3) | |
|------------------|----------------------|---------------------------|----------------|--------------------------------------|---|---------------------------------------|---|
| | | | | %Precision | %Accuracy | %Precision | %Accuracy |
| Daidzin | 3-800 | y = 1524.674x - 10488.106 | 0.996 | 4.8 ^a / 7.6 ^b | 93.7 ^a / 99.2 ^b | 11.5 ^a / 7.2 ^b | 116.7 ^a / 95.2 ^b |
| Genistin | 3-800 | y = 1175.958x - 14940.893 | 0.994 | 2.8 ^a / 13.8 ^b | 81.9 ^a / 116.7 ^b | 5.2 ^a / 7.4 ^b | 91.8 ^a / 101.1 ^b |
| Glycitin | 3-800 | y = 1635.342x - 15877.575 | 0.996 | 8.9 ^a / 7.3 ^b | 92.4 ^a / 94.3 ^b | 2.2 ^a / 11.3 ^b | 82.1 ^a / 107.3 ^b |
| Daidzein | 3-800 | y = 522.909x + 2444.278 | 0.999 | 9.3 ^a / 4.4 ^b | 95.5 ^a / 101.9 ^b | 8.0 / 2.9 ^b | 99.5 ^a / 94.8 ^b |
| Genistein | 3-800 | y = 279.413x - 4130.323 | 0.993 | 5.6 ^a / 3.7 ^b | 115.4 ^a / 103.5 ^t | 10.1 ^a / 13.5 ^b | 105.0 ^a / 93.2 ^b |
| Glycitein | 3-800 | y = 317.848x - 4934.912 | 0.997 | 4.9 ^a / 8.9 ^b | 94.1 ^a / 111.1 ^b | 9.1 ^a / 10.1 ^b | 104.9 ^a / 110.9 ^b |
| Malonyldaidzin | 3-800 | y = 1502.956x - 13631.386 | 0.997 | 7.7 ^a / 9.6 ^b | 104.7 ^a / 95.4 ^b | 14.7 ^a / 12.4 ^b | 97.7 ^a / 99.6 ^b |
| Malonyl genistin | 3-800 | y = 547.652x - 5875.301 | 0.995 | 6.8 ^a / 7.6 ^b | 96.4 ^a / 97.7 ^b | 11.4 ^a / 7.9 ^b | 86.4 ^a / 94.6 ^b |
| Malonyl glycitin | 3-800 | y = 480.243x - 4405.638 | 0.997 | 6.6 ^a / 6.1 ^b | 118.8 ^a / 116.9 ^b | 7.8 ^a / 6.7 ^b | 104.6 ^a / 106.1 ^b |
| acetyl daidzin | 3-800 | y = 3168.790x - 32413.268 | 0.998 | 7.8 ^a / 1.6 ^b | 99.9 ^a / 91.7 ^b | 5.1 ^a / 7.6 ^b | 90.2 ^a / 91.1 ^b |
| acetyl glycitin | 3-800 | y = 1520.396x - 27944.461 | 0.998 | 13.2 ^a / 2.6 ^b | 100.3 ^a / 93.0 ^b | 13.3 ^a / 10.1 ^b | 113.9 ^a / 112.9 ^b |
| acetyl genistin | 3-800 | y = 1814.083x - 27240.497 | 0.996 | 14.2 ^a / 9.7 ^b | 94.9 ^a / 108.1 ^b | 9.9 ^a / 8.7 ^b | 104.2 ^a / 109.7 ^b |

R²: coefficient of determination; ^a: QC in blank sample spiked with 3 ng/mL; ^b: QC in blank sample spiked with 800 ng/mL

Table 3. Investigation of extraction recovery and matrix effect for the 12 isoflavones in Tainan No. 11 soybean cultivar

| Compound | Spiked concentration (ng/mL) | Extraction Recovery (n = 3) | | Matrix Effect (n = 3) | |
|------------------|------------------------------|-----------------------------|------|-----------------------|------|
| | | Mean | %RSD | Mean | %RSD |
| Daidzin | 3 | 92.2 | 8.2 | 81.7 | 6.5 |
| | 800 | 71.8 | 10.7 | 75.2 | 4.7 |
| Genistin | 3 | 91.2 | 8.2 | 78.3 | 8.3 |
| | 800 | 82.6 | 8.8 | 78.7 | 9.8 |
| Glycitin | 3 | 85.3 | 12.2 | 81.9 | 9.3 |
| | 800 | 86.6 | 3.5 | 74.4 | 4.6 |
| Daidzein | 3 | 71.2 | 7.4 | 74.6 | 11.6 |
| | 800 | 86.5 | 2.1 | 77.1 | 9.6 |
| Genistein | 3 | 71.5 | 12.3 | 72.4 | 7.9 |
| | 800 | 73.8 | 7.6 | 78.2 | 6.5 |
| Glycitein | 3 | 74.6 | 13.3 | 75.5 | 13.1 |
| | 800 | 88.2 | 6.8 | 71.5 | 12.3 |
| Malonyl daidzin | 3 | 85.8 | 4.4 | 78.2 | 3.6 |
| | 800 | 72.7 | 4.7 | 81.2 | 7.6 |
| Malonyl genistin | 3 | 87.3 | 2.4 | 71.3 | 11.7 |
| | 800 | 86.9 | 13.8 | 84.9 | 12.3 |
| Malonyl glycitin | 3 | 81.8 | 5.7 | 81.4 | 4.2 |
| | 800 | 78.2 | 2.3 | 83.4 | 11.1 |
| Acetyl daidzin | 3 | 80.4 | 2.5 | 73.6 | 2.4 |
| | 800 | 74.3 | 9.3 | 79.9 | 3.4 |
| Acetyl glycitin | 3 | 93.6 | 1.8 | 73.7 | 2.5 |
| | 800 | 91.5 | 12.6 | 79.8 | 1.9 |
| Acetyl genistin | 3 | 82.4 | 7.5 | 72.1 | 10.5 |
| | 800 | 71.4 | 11.3 | 80.6 | 5.9 |

Table 4. Mean total isoflavone amount in 11 soybean cultivars (unit: ng/mL)

| | List of soybean cultivars | | | | | | | | | | |
|------------------|---------------------------|---------|--------|--------|---------|--------|--------|--------|--------|--------|--------|
| | HL1 | HC | KH7 | KH10 | TS | TN3 | TN5 | TN8 | TN9 | TN10 | TN11 |
| Repeatability #1 | 4978.4 | 10459.0 | 6865.1 | 5908.2 | 9270.7 | 8518.3 | 6772.0 | 8358.0 | 5412.0 | 5512.2 | 5068.0 |
| Repeatability #2 | 4829.0 | 10359.5 | 7210.3 | 6062.5 | 10331.8 | 8459.0 | 6719.0 | 8248.6 | 5280.8 | 5485.2 | 5171.8 |
| Repeatability #3 | 4769.1 | 10437.2 | 7355.1 | 6231.0 | 11606.5 | 8348.8 | 6289.1 | 8569.2 | 5256.3 | 5495.5 | 5091.4 |
| Repeatability #4 | 4870.1 | 10232.6 | 7137.4 | 6147.5 | 11387.3 | 8365.6 | 6740.0 | 8580.3 | 5318.8 | 5495.3 | 4998.1 |
| Repeatability #5 | 4876.8 | 10368.6 | 7120.6 | 6214.0 | 11395.7 | 8247.6 | 6765.9 | 8560.2 | 5252.9 | 5457.5 | 4987.9 |
| Mean | 4864.7 | 10371.4 | 7137.7 | 6112.6 | 10798.4 | 8387.9 | 6657.2 | 8463.3 | 5304.2 | 5489.1 | 5063.5 |
| SD | 76.7 | 88.6 | 178.3 | 132.1 | 988.5 | 104.6 | 206.9 | 151.2 | 65.8 | 20.2 | 75.0 |
| %RSD | 1.6 | 0.9 | 2.5 | 2.2 | 9.2 | 1.2 | 3.1 | 1.8 | 1.2 | 0.4 | 1.5 |

4. CONCLUSION

In soybean, the biosynthesis of isoflavone through the phenylpropanoid pathway (Jung et al., 2000), and isoflavones have been found to assist protect the happening of cardiovascular diseases and some types of cancer (Xiao et al., 2018). This approach has shown that LC-MS/MS is successfully suited to quantitative isoflavones in 11 soybean cultivars. Further, we improved the existing extraction process under 30% acetonitrile with 0.5% formic acid to enhance extraction efficiency and optimized the LC method for separating 12 isoflavones by F5 column within 8 min. The MRM method was successfully quantitated on isoflavone in soybean samples, then the highest and the

lowest total amount of flavonoids are TS and HL1, respectively. Soy isoflavones have been regarded as health benefits for human, whereas this study describe an LC-MS/MS approach and provide a workflow for isoflavone quantitative in soybean, which is worth considering in future soybean breeding and agricultural sciences studies.

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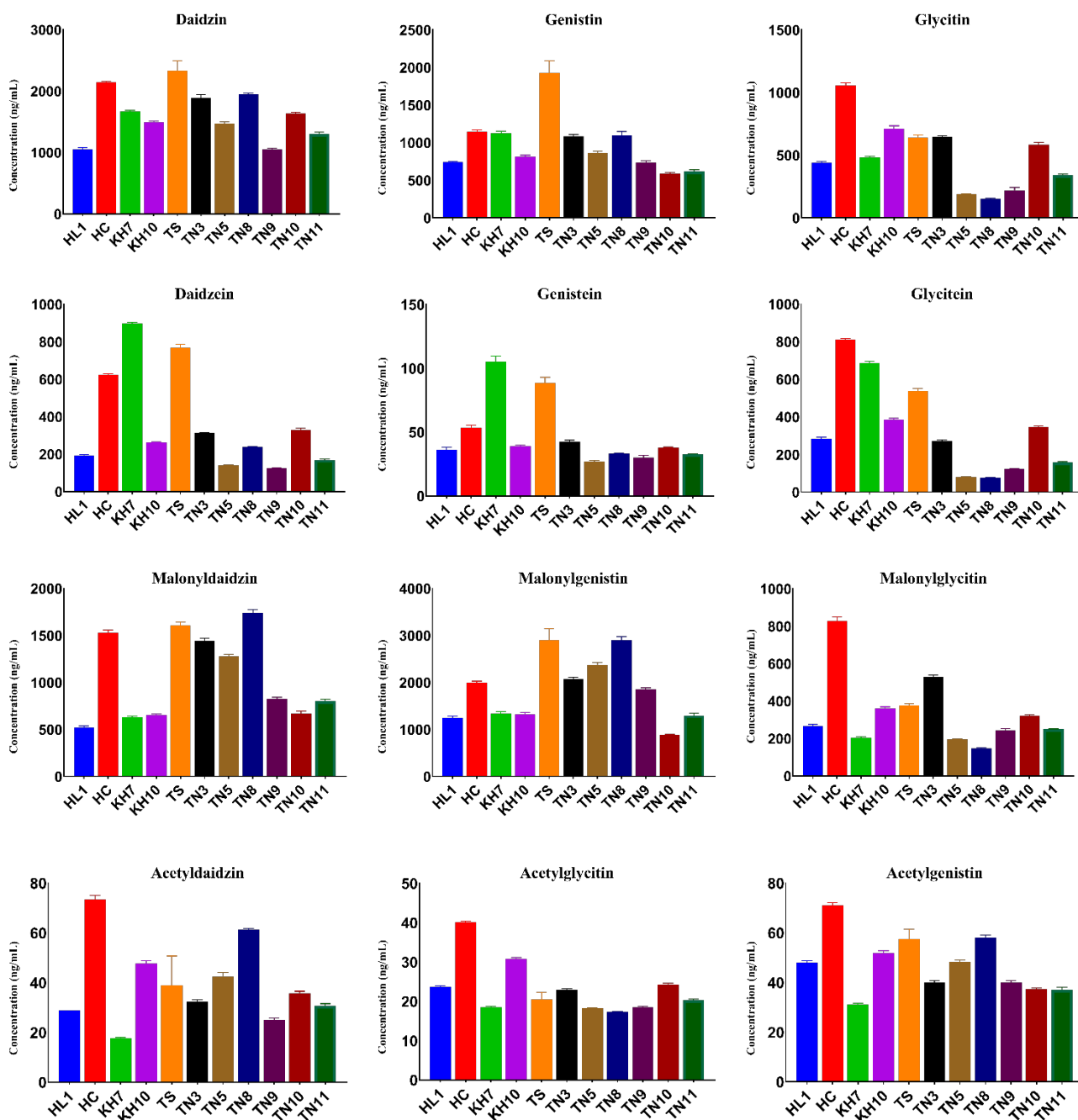


Fig. 3. Profile of individual isoflavone in 11 soybean varieties of Taiwan

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