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High-Performance Liquid Chromatography (HPLC) Method Validation for Simultaneous Quantitation of Five Phytoestrogenic Flavonoids

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ABSTRACT

A Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) method for simultaneous quantitation of five phytoestrogens namely daidzein, genistein, formononetin, and biochanin A, and quercetin was developed and validated through the evaluation of linearity, accuracy, precision, specificity, limit of detection and limit of quantitation in accordance with the ICH guidelines. The analysis was performed in a C18 column (150 x 4.6 mm, 5 µm) with optimized gradient elution using acetonitrile-water (0.1% trifluoroacetic acid) as mobile phase at a flow rate of 1.0 mL min^{-1} and sample injection volume of 10 mL. The retention times of the standards daidzein, quercetin, genistein, formononetin, and biochanin A were 4.42, 5.24, 7.85, 10.06, and 13.55 min, respectively with tailing factors ranging from 1.09 to 1.12 and a minimum resolution value of 3.74. Detection limits ranged from 0.339 to 0.964 ug/mL and quantitation limits ranged from 1.027 to 2.922 µg/mL with good linearity ($R^2 \geq 0.9967$) within 1.25 to 20 µg/mL concentrations of the standards. The method was also found to be accurate and precise based on percentage recovery ranging from 96.96% to 106.87% (intraday, n=3) and relative standard deviation of %RSD≤1.45% (intra-day, n=3) and %RSD≤2.35% (inter-day, n=5). The specificity of the method was evaluated based on the positivity of the minimum peak purity index during the quantitation of the target compounds from the spiked hydrolyzed and unhydrolyzed extract of *Cajanus cajan* ICPL 7035.

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KEYWORDS

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INTRODUCTION

Phytoestrogens are polyphenolic compounds from plants which have estrogen-like properties. They can bind to human estrogen receptor ERα and ERβ hence have been used as substitutes for estrogen in hormone-related therapy for a variety of hormone-related diseases such as endometriosis and menopausal disorders (Poluzzi et al, 2014; Desmawati D & Sulastri D, 2019). There are various classes of phytoestrogens that include isoflavones and flavonoids (Dixon 2004). Isoflavones have a distinct structure of 3-phenylchroman skeleton hydroxylated in 4'- and 7'- positions and may vary depending on the functional group substitution in carbons 5 and 6 (Mitani et al. 2005). Flavonoids, on the other hand, have a 2-phenylchroman structure. Figure 1 shows the structures of flavones, isoflavones, and 17-β-estradiol, while Figure 2 shows the structure of daidzein, genistein, formononetin, biochanin A, and quercetin.

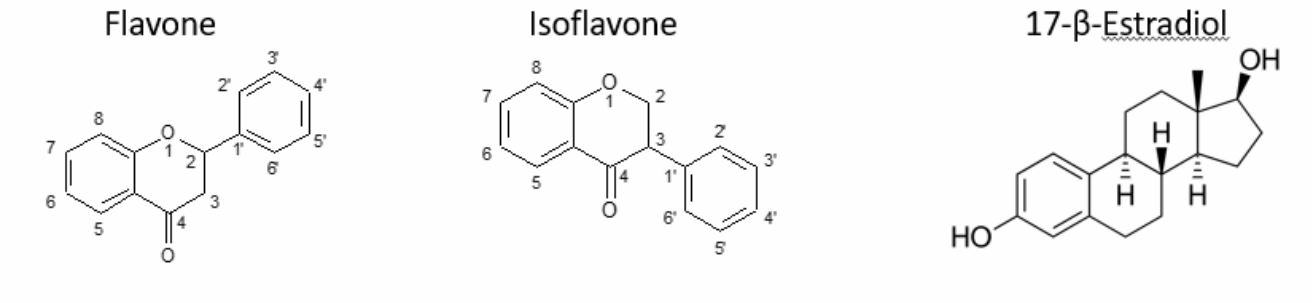

Figure 1: General structures of flavones, isoflavones, and 17-β-estradiol

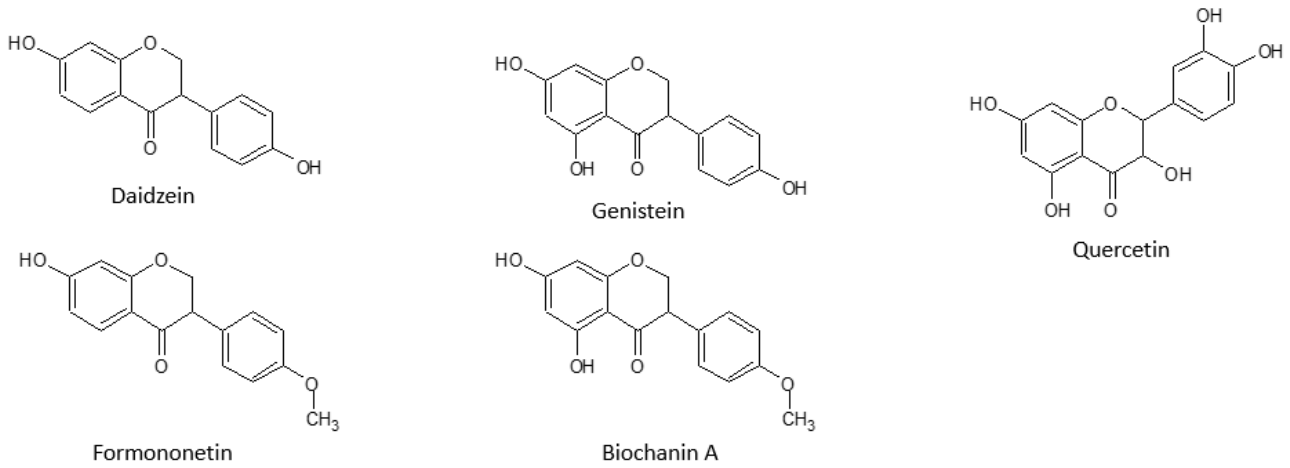

Figure 2: Structures of standards daidzein, formononetin, genistein, biochanin A, and quercetin.

Flavonoids were detected vegetables, tea, wine, and fruits (Mitani et al. 2005; Aidoo-Gyamfi et al. 2009; Panche et al. 2016). Isoflavonoids often present in the form of their respective O-glycosides are detected in flowering species while being almost exclusively produced by the members of the *Fabaceae* (legumes) plant family. The *Fabaceae* family is considered the second most important among crops for food sources, livestock feeds, and raw materials for industrial and agricultural products.

It has been reported that both isoflavonoids and flavonoids exhibit a number of bioactivities including antioxidant, anti-inflammatory, anti-viral, anti-estrogenic, and anti-cancer properties, among others (Desmawati and Sulastri, 2019; Ullah et al. 2020). Daidzein and genistein are already used as substitutes for estrogen in hormone replacement therapy (HRT) to alleviate post-menopausal symptoms and estrogen-dependent diseases such as endometriosis, as estrogen has been reported to increase the risk of breast and endometrial cancer (Aidoo-Gyamfi et al. 2009). Daidzein and Genistein's precursors, formononetin and biochanin A, respectively possess lipid metabolism modulatory and neuroprotective effects (Blicharski and Oniszczuk 2017; Geng et al. 2022; Guo et al. 2019). Both isoflavones and flavonoids are receiving attention in research as health-promoting "nutraceuticals" (Dixon and Steele 1999).

For years, soybean has been the main dietary source of isoflavones daidzein, genistein, and glycitein, with genistein being the most abundant and glycitein being the least (Cho et al. 2020). Quantitative analyses of these isoflavones were focused on soy and its commercially available products using gas chromatography in tandem with mass spectrometry (GC/MS). In correlation with the diet, phytoestrogen levels in biological fluids were also measured by GC/MS. However, the availability of such expensive equipment renders simple screening assays for phytoestrogen content costly and requires profound analytical experience from the researcher. GC/MS methods are also time-consuming and require multiple sample preparation steps such as derivatization to increase the volatility of non-volatile compounds, particularly isoflavones (Franke et al. 1995; Hsu et al. 2010).

Reversed-phase high-performance Liquid Chromatography (RP-HPLC) coupled with photodiode array detectors (PDA) offers more cost-effective analysis for screening plant extracts for phytoestrogen content. Most of the existing HPLC methods for the quantification of phytoestrogen and their corresponding glycosides were validated and optimized for soy samples . Varying mobile phase compositions were reported in either isocratic or gradient flow (Franke et al. 1994; Rostagno et al. 2005; Cho et al. 2020). Among all developed HPLC assays, acidified water using acetic acid, trifluoroacetic acid (TFA), and formic acid were used as polar components, while organic solvents acetonitrile and methanol were the most common choice as nonpolar components.

Aside from soy, other species in the *Fabaceae* family such as red clover (*Trifolium pratense),* were reported to contain low amounts of daidzein and genistein while having higher amounts of formononetin and biochanin A (Lee et al. 2004). Plant parts aside from seeds, pods, and fruits were also reported to have considerable amounts of isoflavones in which some parts cannot be consumed and are discarded. Pigeon pea (*Cajanus cajan*), a local bean crop in Southeast Asia, was reported to have biochanin A from leaves and roots (Nix et al. 2015). Another study determined the coumestrol, daidzein, genistein, formononetin, and biochanin A content from legumes (Franke et al. 1994).

The method of extraction may vary depending on the target compound or analyte of the study which may impact the form of isoflavones. Some studies may limit the quantitation to aglycone forms, while others have tried to quantify both isoflavone aglycones and glycosides. The form of isoflavones largely depend on the raw materials used for extraction. Glycosides are mainly extracted from raw plant materials, while aglycone forms are extracted from processed food such as tofu and miso, with varying levels due to the enzymatic or acidic hydrolysis during food processing. (Koh and Mitchell 2007) . It is possible, however, to extract aglycone forms from raw plant materials by employing a hydrolysis step after the extraction. The optimum pH for the extraction of isoflavones from soybeans must be observed to maximize the extraction of targeted forms of isoflavones (Cho et al. 2020). Isoflavone aglycones were also reported to be less stable at alkaline pH than at acidic or neutral pH, such that the highest total soy isoflavones were measured at acidic pH using HCl concentration (HC) of 3.42 N within a total hydrolysis time (HT) of 205.5 min and reaction temperature (RT) of 44.6 °C (Chiang et al. 2001), which served as the basis for the hydrolysis reaction of this study.

Direct injection of plant extracts to the HPLC is possible, although the matrix of the sample may contain co-extractants which may affect the signal of the target isoflavones and may diminish the effectiveness of the column. Studies have incorporated pre-HPLC on-line SPE sample clean-up procedures (Mitani et al. 2005; Doerge et al. 2000), neither of which was applied to plant extracts. On-line SPE clean-up procedures also require the use of a trap column, which is less commonly available than conventional SPE cartridges. Rostagno et al. 2005 tested for the recovery of isoflavones through several solid phase extraction (SPE) cartridges prior to the HPLC analysis, reporting that the divinyl-benzene-co-N-vinylpyrrolidone SPE cartridge offers the highest recovery. Divinylbenzene-based polymer sorbents can significantly reduce the number of proteins and salts which are commonly found as co-extractants from biological samples (Guermouche and Gharbi 2004). Although isoflavones exist in plants as free aglycones, most of them still exist as glycosides. Since acid hydrolysis is applied to free the isoflavones from their glycosides, an additional acid neutralization step is normally employed before the HPLC analysis as silica-based sorbents are particularly unstable in extreme pH. At low pH (pH < 2), silyl ether linkages can be broken while silica can be dissolved in aqueous solutions at higher pH (pH > 7.5). Polymer-based sorbents SPE cartridges are reliably stable in a wide range of pH (pH 1 to 14), bypassing the acid neutralization step from the sample through a simple washing step in the solid phase extraction clean-up (Yawney et al. 2002).

In this study, an RP-HPLC method was developed and optimized for determining the amounts of four isoflavones namely, daidzein, genistein, formononetin, and biochanin A, and the flavonol quercetin. The method was validated using the parameters of limit of detection (LOD) and limit of quantitation (LOQ), linearity, precision, accuracy, and specificity. The development of a fast, efficient, and sensitive method for the quantitation of phytoestrogens would aid in the determination of plant products that can be used as natural dietary supplements and as a basis for the assessment of the relationship between the occurrence of estrogen-dependent diseases and the inclusion of phytoestrogen-rich plant products as part of the regular diet. This method may then be used to determine the phytoestrogen content of functional foods included in the Filipino diet in future experiments.

MATERIALS AND METHODS

Chemicals and Reagents

Isoflavone standards daidzein (≥98%, synthetic), genistein (≥98%, HPLC), formononetin (≥98%, HPLC), and biochanin A (≥97%, TLC) were purchased from Sigma-Aldrich, Inc (St. Louis, Missouri, USA). HPLC grade solvents acetonitrile and methanol, and AR grade methanol were purchased from RCI LabScan (Bangkok, Thailand). AR grade trifluoroacetic acid (TFA), hydrochloric acid (HCl), dimethylsulfoxide (DMSO), and flavonoid standard quercetin (pharmaceutical secondary standard) were purchased from commercial sources and used without further purification. Divinylbenzene SPE Cartridges were purchased from Waters™ (Ireland) (OASIS HLB PRiME, 6 cc, 200 mg). Type 1 Ultrapure water was purified using Waters Simplicity® Water Purification System. All standards are HPLC Grade and were pre-analyzed with spectral peak purity for the confirmation of spectral purity (Peak Purity Index ≥ 0.999).

Preparation of Standard Solutions

Isoflavone standards were each dissolved in DMSO to prepare 4,000 mg mL^{-1} stock solutions. Stock solutions were subjected to sonication for 10 min to ensure complete dissolution of the standards. The resulting solutions were stored in 1.5 mL polypropylene tubes at -40 °C. On the day of the analysis, the solutions were slowly thawed at room temperature, and calculated volumes were obtained from the stock solution to prepare solutions for the construction of a calibration curve (20 mg mL^{-1} – 1.25 mg mL^{-1}), accuracy and precision analysis (4, 8 and 12 mg mL^{-1}) and sample spike solution (~8 mg mL^{-1} per standard).

Solid Phase Extraction (SPE)

OASIS PRiME HLB SPE Cartridges were used for the SPE clean-up step. Initially, the applied procedure was described in a previous study (Rostagno et al. 2005), scaled down for a lesser sample quantity. Briefly, the cartridge was conditioned with 6 mL methanol (1 mL min^{-1}) followed by equilibration using 6 mL of 1:1 methanol-water (1 mL min^{-1}). The dissolved extracts were loaded into the SPE (1 mL min^{-1}), the cartridge was washed with 6 mL water (1 mL min^{-1}) and finally eluting the analytes with 6 mL HPLC Grade methanol (1 mL min^{-1}). The eluate was collected and evaporated to dryness in a speed vacuum concentrator at 50°C . The resulting dry residue was dissolved in 1 mL of the mobile phase and filtered through 0.45 mm syringeless HPLC vials. Three concentrations (25, 75, and 150 mg mL^{-1}) were used to validate the percentage recovery of the standards through the SPE. These concentrations represent low, mid, and high concentrations that are within the calibration curves used in the SPE recovery study.

HPLC Analysis

The HPLC method used was based on the optimized parameters from the method validation of this study. The order of elution of the isoflavone standards was determined by analysing separate solutions containing 20 mg mL^{-1} of analytes, separated through a 150 x 4.6 mm ID RP-18 Column with a linear gradient elution profile starting with 5% solvent B (acetonitrile) in solvent A (0.5% TFA in water (% v/v)) at 0 min, gradually increasing to

95% solvent B over 60 min. The resulting chromatogram was compared with a standard solution containing all five analytes. Succeeding analyses were modified to design the optimum linear gradient elution profile at a constant flow rate of 1 mL min^{-1} and injection volume of 10 µL. Ultimately, the optimized elution time program starts with 35% Solvent B at 0 min, increasing gradually to 80% solvent for 13 min, a steady increase in solvent B by 5% per minute over 2 min, followed by an isocratic flow using 95% solvent B for the next 2.5 min, and lastly a column equilibration step for 10 min at 35% solvent B concentration for a total runtime of 27.5 min per sample. The photodiode array detector was set to read the absorbance at 254 nm. All peaks were analysed and integrated using the Shimadzu LabSolutions Software version 5.81. Solvent blanks of 35% acetonitrile-water (% v/v) and HPLC grade methanol were used to subtract the interference of the corresponding sample solvent.

Method Validation

Linearity, Limit of Detection, and Limit of Quantitation

Using the optimized HPLC method C, calibration curves were constructed by plotting the concentration (abscissa) versus the peak area (ordinate) of the standards using five calibration points serially diluted from 20 mg mL^{-1} to 1.25 mg mL^{-1}. The linearity was evaluated by the linear equation, coefficient of variation, and the Y-intercept. The LOD and LOQ were calculated with signal-to-noise ratios of 3.3 and 10, respectively.

Accuracy and Precision

Accuracy and precision were determined by quantitative analysis of 3 concentrations for each of the standards. The ICH Guidelines have recommended that the accuracy and precision of a method must be evaluated at 50%, 100%, and 150% assay concentrations (4, 8, and 12 ug/mL in the case of this study). Precision was expressed as the standard deviation or the degree of reproducibility of the method under normal operating conditions.

Application of the Method

A variant of *C. cajan* (pigeon pea, ICPL 7035) was used as the test sample for the validated method. Initially, a total of 2 kilograms of fresh *C. cajan* seeds were individually cut open and freeze-dried at -50°C under 0.3 mBarr pressure for at least 4 days or until complete evaporation using Labconco Freezone 2.5 lyophilizer. The seeds were ground to fine powder and sieved using a 2 mm metal sieve. Approximately 1.5 grams of the dried powder were extracted with 60% acetonitrile-water (% v/v) through 35 kHz ultrasonication at room temperature for 60 min in three replicates. The extracts were centrifuged for 10 min at 5,000 rpm 25°C, followed by separation from the plant material through pipetting. For the acid hydrolysis reaction, the extract was mixed with 200 mL of 3.42 N HCl solution and incubated in a water bath with a controlled temperature of 80°C for 205.5 min. Both the hydrolyzed and unhydrolyzed extracts were evaporated to dryness using a speed vacuum concentrator at 50°C and the resulting residue was dissolved in 1:1 methanol-water (%v/v). The resulting solution was passed through the SPE and the methanol eluate was evaporated to dryness. The residue was dissolved in 0.5 mL of the mobile phase (35% Acetonitrile-water) and filtered through a 0.2 µm syringe filter. The resulting solution was spiked with 5 mL of spike solution and analysed using the validated method (method C).

Data and Statistical Analysis

Data analysis and peak integration for all standards and samples were performed directly using the LabSolutions software, with the peak integration baseline set at 0 mAU after subtracting the chromatogram of the blank solvent. All concentrations were obtained as mean ± standard deviation among technical replicates. Linear regression analysis and the limits of detection and quantitation were determined using GraphPad Prism 6. The analysis of variance (ANOVA) and descriptive statistics for retention time was performed using Microsoft Excel 2016.

RESULTS AND DISCUSSION

Chromatographic Separation

Preliminary analyses were conducted to investigate the retention times, tailing factors, and resolutions of the five phytoestrogens as shown in Table 1. The selection of the composition of the mobile phase was based on previous studies (Franke et al.1994; Grippo et al. 1999; Krenn et al. 2002; Wang et al. 2008) which were optimized using either an isocratic elution profile or a gradient elution profile using solvent A (0.1% aqueous TFA) and solvent B (100% ACN). The simultaneous identification of the peaks was based on both retention times and the similarity of the spectral scan from 190 nm – 800 nm. Individual solutions containing 20 mg mL^{-1} of each standard were analyzed using the optimized elution gradient (Figure 3) and spectral scans (Figure 4) for each standard were recorded.

Table 1: Retention times, tailing factors, and resolutions of the five phytoestrogen standards in three different chromatographic methods A, B and C.

Method	Phytoestrogen	Rt (min)	Tailing Factor	Resolution
A	Daidzein	11.56	1.47	
	Quercetin	12.6	1.45	4.13
	Genistein	14.61	1.42	7.8
	Formononetin	16.65	1.45	7.79
	Biochanin A	20.03	1.46	12.61
B	Daidzein	4.22	1.28	
	Quercetin	5.66	1.32	2.03
	Genistein	9.24	1.42	6.4
	Formononetin	11.77	1.48	7.22
	Biochanin A	15.48	1.5	12.37
C	Daidzein	4.44	1.15	
	Quercetin	5.25	1.15	3.56
	Genistein	7.82	1.09	10.7
	Formononetin	10.08	1.08	10.09
	Biochanin A	13.54	1.08	16.59

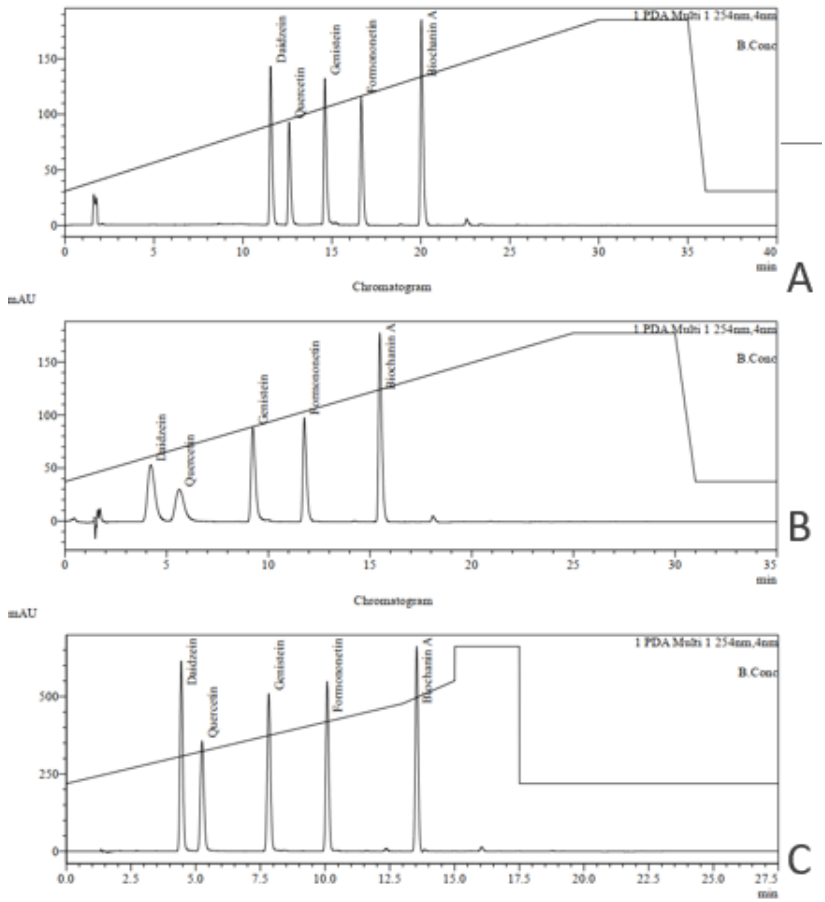

Figure 3: Chromatogram and elution profile for methods A, B, and C. Reversed-phase C18 Column 150 x 4.6mm ID; flow rate: 1mL/min; Solvent A: 0.1% aqueous TFA, Solvent B: acetonitrile; (A) Gradient: 0.00min - 20% B and allowed to increase linearly up to 95% solvent B for 30 min, followed by an isocratic run at 95% of solvent B for 5 min; (B) Gradient: started at 25% B and allowed to increased linearly to 95% B over 25 min; (C) Gradient: started at 35% B and allowed to increase linearly to 80% over 13 min, followed by 5% increase in B per minute for 2 min after which the % B was increased to 95% and allowed to isocratically run for 2.5 min.

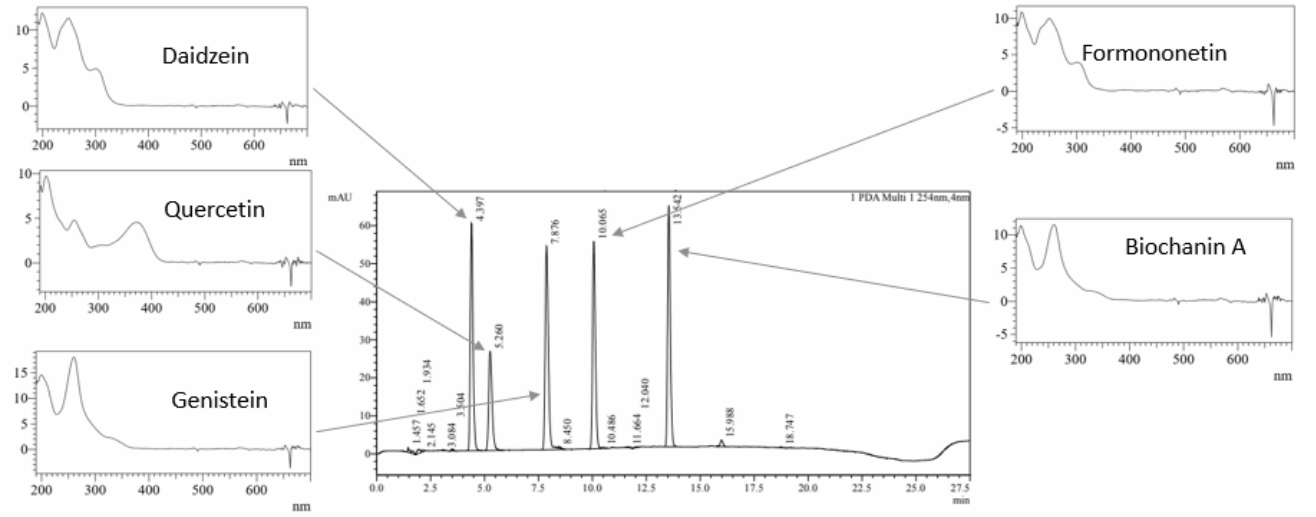

Figure 4: Chromatogram and absorption spectra of standards from 190nm - 700nm. Separation was done on a reversed-phase C18 Column 150 x 4.6mm ID; flow rate: 1mL/min; Solvent A: 0.1% aqueous TFA, Solvent B: acetonitrile; Gradient: 0.00min - 35% B, 13.00min - 80% B, 16.00min - 95% B, 18.00min - 95% B, 20.00min - 35% B, 27.50min – 35% B.

A 40-minute HPLC Method was conducted as a preliminary analysis to determine the elution order of the isoflavone standards (method A). Gradient elution using solvent A and solvent B was started with 20% solvent B and allowed to increase linearly up to 95% solvent B for 30 min, followed by an isocratic run at 95% of solvent B for 5 min. Using this elution gradient, daidzein was first to elute at 11.56 min, followed by quercetin (12.8 min), genistein (14.73 min), formononetin (16.78 min), and finally biochanin A (20.03 min) (Figure 3A and Table 1). The tailing factors obtained for all peaks were below the accepted values in the literature (less than 1.5) indicating good peak symmetry. Acceptable resolution values of greater than 4.13 were also observed among successive peaks as seen in Table 1. Method B was then conducted in an attempt to reduce the analysis time. In method B, the initial concentration of solvent B was increased to 25% and the mobile phase composition was allowed to increase linearly to 95% of solvent B over 25 min. This resulted in the faster elution time of all

peaks, with daidzein being detected first at 4.22 min and biochanin A detected last at 15.48 min (Figure 3B and Table 1). However, a noticeable increase in peak widths of daidzein and quercetin was observed as shown in Figure 3B, reducing the resolution between daidzein and quercetin to 2.03 and the resolution between quercetin and genistein to 6.40 as shown in Table 1. These resolutions were lower as compared to the resolutions obtained in method A. On the other hand, the resolutions between Genistein and formononetin and between formononetin and biochanin A did not significantly change when compared to their resolutions in method A (Table 1). To further reduce the analysis time to less than 30 min and improve the resolutions among the analytes, method C was performed. In method C, the initial concentration of solvent B was set to 35% and allowed to increase linearly to 80% over 13 min, followed by a 5% increase in B per minute for 2 min, after which solvent B was increased to 95%, and allowed to isocratically run for 2.5 min (Figure 3C). All methods end through an isocratic flow of 95% solvent B to ensure the elution of traces of the analytes followed by an equilibration step to 35% solvent B in preparation for succeeding analysis. For methods A and B, re-equilibration of the column was done for 4 min only. This resulted in slightly higher relative standard deviations on the retention times of the isoflavones during multiple batch analyses. A longer equilibration time of 10 min was used for method C to minimize the deviation in retention times between multiple analyses. The peak symmetry also improved in method C, represented by the tailing factors of 1.09 - 1.15 for all standards (Figure 3C and Table 2). A tailing factor closer to 1.0 indicates an almost symmetrical peak. High repeatability was also observed on the retention times using method C, with %RSD of not more than 1.95% for all standards. Therefore, method C was selected as the gradient elution program for this study. Table 2 shows that the analysis time of the validated method is generally shorter compared to most of the other published HPLC methods. Existing methods have analysis times ranging from 15 min up to as long as 60 min (Franke et al.1994; Grippo et al. 1999; Krenn et al. 2002; Wang et al. 2008).

Table 2: Retention times, tailing factors, and resolutions of the five phytoestrogen standards using the optimized HPLC method (Method C)

Phytoestrogen	Retention Time		Tailing Factor	Resolution	Number of Theoretical Plates (N)
	Mean, min	%RSD			
Daidzein	4.42 ± 0.04	0.9	1.12	1.00	7823
Quercetin	5.24 ± 0.07	1.25	1.11	3.74	8127
Genistein	7.85 ± 0.08	0.97	1.09	11.31	20461
Formononetin	10.06 ± 0.05	0.5	1.09	7.39	38346
Biochanin A	13.55 ± 0.06	0.41	1.09	17.09	73392

The choice of solvent for the optimum separation of the isoflavones was based on previous runs and from existing literature (Franke et al.199; Grippo et al. 1999; Krenn et al. 2002; Wang et al. 2008). Both acetonitrile and methanol have been used in the analysis of flavones and isoflavones combined with an acidified polar solvent such as water (Merken and Beecher 2000). Methanol is also a common choice due to its low cost and less toxicity than acetonitrile. However, acetonitrile has lower viscosity, shorter wavelength UV cut-off, and stronger dipole moment and elution strength in reversed-phase columns, allowing shorter elution and analysis times (Jung et al. 2020). Common acid modifiers for quantitation of isoflavones include acetic acid, perchloric acid, phosphoric acid, or formic acid (Merken and Beecher 2000), possibly acidifying both solvents in a binary mobile phase. The advantage of using trifluoroacetic acid (TFA) as a modifier for chromatography techniques is its volatility, eliminating the need for an acid neutralization step, particularly for mass spectrometry analysis. Acidifying both solvents with TFA in a binary mobile phase also eliminated the peak tailing. Although previous studies suggested that TFA does not improve the resolution of the peaks, especially for isoflavone conjugates (Gu and Gu 2001), the effect of either acetic acid or TFA as acid modifiers for the phytoestrogens was not thoroughly investigated in this study. Nevertheless, acceptable tailing factors are obtained for all peaks.

Method Validation

Limits of Detection and Quantitation, and Linearity

Calibration curves were constructed using serially diluted solutions containing 20 µg mL^{-1} to 1.25 µg mL^{-1} of standards in five replicates over five consecutive days. Validation parameters were presented in Table 3 as equations of the line, linearity, LOD, and LOQ. Linear regression analysis confirmed that there is a linear relationship between the peak area and concentration for each standard, with r^2 values ≥ 0.997. Calculated LOD and LOQ values proved the sensitivity of the method and were able to detect the target compounds at the lowest concentration of 0.339 ug mL^{-1}.

Table 3: Linearity, limit of detection, and limit of quantitation of the optimized HPLC method for the quantitation of the five phytoestrogens within the linear range of 1.25 to 20 µg mL^{-1}.

Phytoestrogen	Equation	Linearity	LOD, µg mL^{-1}	LOQ, µg mL^{-1}
Daidzein	y = 0.0015x + 0.168	0.9996	0.339	1.027
Quercetin	y = 0.0022x + 0.2398	0.9977	0.693	2.101
Genistein	y = 0.0016x + 0.2011	0.9991	0.790	2.395
Formononetin	y = 0.001x + 0.1017	0.9996	0.964	2.922
Biochanin A	y = 0.001x + 0.1885	0.9993	0.394	1.194

Accuracy and Precision

The accuracy and precision of the method are presented in Table 4. Intra-day precision was determined by calculating the relative standard deviation (% RSD) of three replicates (n=3) and inter-day precision was determined by the analysis of three replicates over three consecutive days (n=5). The accuracy was calculated by dividing the mean concentration measured by the calculated concentration multiplied by 100%. The % recovery as well as the % RSD for all standards, were found to be within the acceptable range based on the ICH guidelines for intra- and inter-day variations.

Table 4: Intra-day and Inter-day accuracy and precision of the optimized HPLC method for the quantitation of the five phytoestrogens.

	Intraday, n=3				Interday, n=5		
Phytoestrogen	Concentration (µg mL^{-1}.)	Mean Accuracy	STDev	%RSD	Mean Accuracy	STDev	%RSD
Daidzein	4.2	106.50%	0.82	0.77%	107.30%	1.61	0.77%
	8.4	101.63%	0.2	0.19%	101.78%	0.58	0.57%
	12.6	100.29%	0.58	0.58%	100.4	0.62	0.62%
Quercetin	4.2	101.10%	0.49	0.49%	102.00%	2.40	2.35%
	8.4	99.44%	0.39	0.40%	99.53%	0.36	0.36%
	12.6	98.44%	0.61	0.62%	97.55%	0.52	0.53%
Genistein	4.3	104.40%	1.51	1.45%	104.30%	1.19	1.14%
	8.6	98.63%	0.69	0.70%	98.81%	0.67	0.68%
	12.9	98.36%	0.87	0.90%	96.14%	0.92	0.96%
Formononetin	4.2	102.40%	0.51	0.50%	103.00%	0.88	0.85%
	8.4	98.85%	0.11	0.11%	99.16%	0.61	0.62%
	12.6	98.96%	0.38	0.38%	97.30%	0.54	0.55%
Biochanin A	4.5	107.80%	0.07	0.65%	108.30%	0.99	0.91%
	9	103.26%	0.22	0.22%	103.44%	0.41	0.40%
	13.5	101.68%	0.41	0.40%	101.76%	0.31	0.31%

The retention times (RT) of all standards across intra-day and inter-day runs were recorded. Typically, column and ambient temperature are optimized to minimize the RT shift of standards in different temperatures. During intra-day analysis, nine successive runs were performed continuously in a span of 247.5 min or approximately 4 hours. In inter-day analysis, the ambient room temperature was maintained at 25°C. Table 5 shows the retention time of all standards in both intra-day and inter-day analysis. Pair-wise comparison (t-test) between successive intra-day and inter-day runs for three concentrations of 25, 75, and 150 mg mL-1 shows no significant difference in retention time ($p > 0.05$) thus, the column temperature does not significantly change or affect the retention times of the standards over the entire analytical period.

Table 5: Retention times (RT) of all standards during intra-day (n=3) and inter-day (n=3) analysis.

		Intra-day (n=3)				
	Standards	RT (min)			Mean (min)	Stdev
25 ppm	Daidzein	4.462	4.467	4.467	4.465	0.003
	Quercetin	5.306	5.317	5.315	5.313	0.006
	Genistein	7.925	7.939	7.932	7.932	0.007
	Formononetin	10.111	10.118	10.112	10.114	0.004
	Biochanin A	13.603	13.608	13.599	13.603	0.005
75 ppm	Daidzein	4.473	4.468	4.465	4.469	0.004
	Quercetin	5.324	5.315	5.310	5.316	0.007
	Genistein	7.948	7.936	7.932	7.939	0.008
	Formononetin	10.128	10.115	10.114	10.119	0.008
	Biochanin A	13.622	13.600	13.600	13.607	0.013
150 ppm	Daidzein	4.472	4.459	4.464	4.465	0.007
	Quercetin	5.321	5.300	5.307	5.309	0.011
	Genistein	7.945	7.923	7.928	7.932	0.012
	Formononetin	10.124	10.115	10.114	10.118	0.006
	Biochanin A	13.614	13.607	13.606	13.609	0.004

		Inter-day (n=3)				
	Standards	RT (min)			Mean	Stdev
25 ppm	Daidzein	4.462	4.444	4.414	4.440	0.024
	Quercetin	5.255	5.278	5.230	5.254	0.024
	Genistein	7.865	7.896	7.841	7.867	0.028
	Formononetin	10.066	10.092	10.053	10.070	0.020
	Biochanin A	13.555	13.583	13.539	13.559	0.022
75 ppm	Daidzein	4.440	4.450	4.400	4.430	0.026
	Quercetin	5.270	5.286	5.205	5.254	0.043
	Genistein	7.885	7.903	7.810	7.866	0.049
	Formononetin	10.081	10.094	10.034	10.070	0.032
	Biochanin A	13.569	13.584	13.518	13.557	0.035
150 ppm	Daidzein	4.445	4.427	4.398	4.423	0.024

	Quercetin	5.278	5.249	5.202	5.243	0.038
	Genistein	7.894	7.863	7.810	7.856	0.042
	Formononetin	10.088	10.069	10.033	10.063	0.028
	Biochanin A	13.574	13.555	13.516	13.548	0.030

Specificity

The specificity of the method was evaluated by comparing the UV spectral scan of a spiked sample against the spectral scan of the standards detected at 254 nm (Figure 5). As can be seen in Figure 5, the absorption spectra of the target compounds from the pigeon pea ICPL 7035 extract were very similar to the absorption spectra of the standards. This indicates that the peaks found in the pigeon pea ICEAP 7035 with the same retention times as those of the standards are the target compounds.

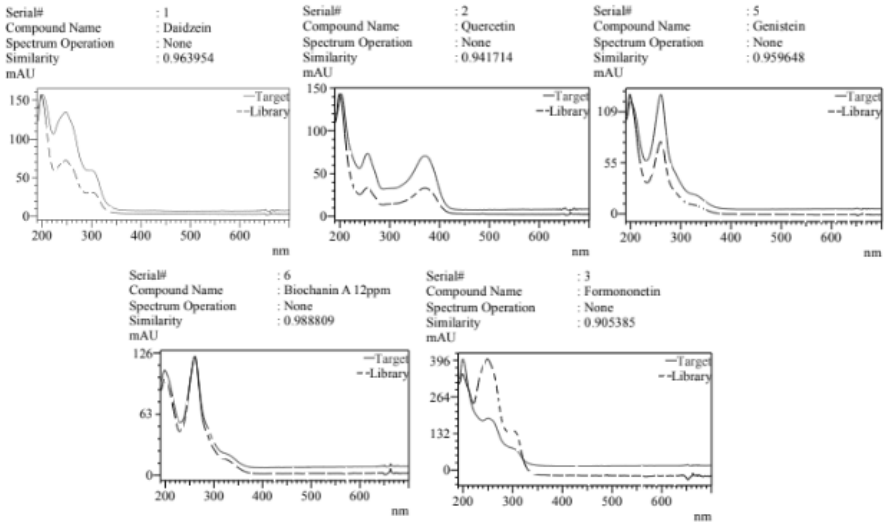

Figure 5: Comparison of absorption spectra of target compounds from analysis of *C. cajan* ICEAP 7035 extract (solid) against phytoestrogen standards (broken lines).

Solid Phase Extraction Recovery

The recovery of five standards from the SPE step was determined to assure the complete elution of the analytes during sample clean-up. Standard solutions of known concentrations were passed through the SPE and quantitated using the optimized HPLC method. Recoveries were performed in triplicates for three concentrations (25, 75, 150 mg mL^{-1}) which represent low, mid, and high concentrations within the calibration curve. The results of the recovery studies are shown in Table 6. The developed method showed acceptable accuracy with a minimum intra-day accuracy of 98%, up to a maximum accuracy of 107%. Intra-day precision also suggests that this study is repeatable for 4, 8, and 12 mg mL-1 concentrations at %RSD < 2.35%. The % recovery as well as the % RSD for all standards were found to be within the acceptable range based on the ICH guidelines for recovery studies.

Table 6: Percentage recovery of the five phytoestrogen standards using SPE pre-concentration prior to HPLC analysis

Phytoestrogen	Mean, µg mL^{-1}.	%Recovery	%RSD
Daidzein	24.39 ± 0.02	97.52	0.06
	73.32 ± 0.05	97.77	0.07
	147.52 ± 0.08	98.35	0.05
Quercetin	23.79 ± 0.41	95.18	1.71
	76.08 ± 0.90	101.44	1.18
	143.87 ± 2.46	95.92	1.71
Genistein	24.68 ± 0.02	98.73	0.06
	74.91 ± 0.14	99.88	0.19
	149.69 ± 0.72	99.79	0.48
Formononetin	25.69 ± 0.02	102.77	0.06
	76.15 ± 0.13	101.53	0.17
	153.13 ± 0.56	102.09	0.36
Biochanin A	25.00 ± 0.01	100.01	0.03
	73.36 ± 0.08	97.81	0.11
	149.65 ± 0.09	99.76	0.06

Note: The loading concentrations of all standards are 25, 75, and 150 mg mL^{-1}.

The SPE recovery was performed at higher concentrations in an attempt to determine the upper limit of the available SPE cartridge. Calibration curves were constructed for all standards using a concentration range from 6.25 to 200 µg mL^{-1}. These calibration curves showed good linearity with at least 0.9993 regression coefficient values for all standards (Supplementary Table 1). The recovery with the SPE method used for 25, 50 and 150 mg mL^{-1} concentrations of the standards was surprisingly high at the range of 95% - 102%, with the highest %RSD of 1.71% for quercetin suggesting high reproducibility even at high concentrations. It has been previously reported that divinylbenzene-based polymers, particularly HLB Prime SPE

cartridges, were able to retain soy isoflavones daidzein, genistein, and glycitein, together with their acetylated and malonylated forms (Rostagno et al. 2005) and were also used for the extraction of steroidal endocrine disrupting compounds (EDCs) from wastewater samples (Qi et al. 2019) and honey (Rodrigues-Gonzalo et al. 2010). It must be noted that no literature has reported the utilization of divinylbenzene-co-n-pyrrolidone SPE adsorbent for the extraction of quercetin, formononetin, and biochanin A which was done in this study. Another divinylbenzene-based adsorbent Isolute ENV+ cartridge was used for the recovery studies of two flavonoids quercetin and kaempferol, which proved better selectivity than C18 cartridges (Rostagno et al. 2005). However, the recovery of quercetin was only around 74% and the complete elimination of interfering compounds was still unachievable. The present study has shown that all five standards were retained and recovered from the SPE cartridge with recoveries not less than 95%.

Determination of Phytoestrogen Content of C. cajan ICPL 7035 seeds extract

The validated method was then applied to measure the amounts of the target compounds in ICPL 7035 extracts. Unhydrolyzed and acid-hydrolyzed extracts were analyzed for their phytoestrogen content. Table 7 shows the phytoestrogen content of ICPL 7035 reported as mg of target analyte per gram of dry weight of the sample. The chromograms of the unhydrolyzed and hydrolyzed extracts are shown in Figure 6 and Figure 7, respectively.

Table 7: Phytoestrogen content of *C. cajan* ICPL 7035 seed extracts

	Mean Concentration (µg/g dry weight)				
Sample	Daidzein	Quercetin	Genistein	Formononetin	Biochanin A
ICPL 7035 (unhydrolyzed)	0.70 ± 0.08	1.31 ± .037	2.44 ± 0.75	0.78 ± 0.16	0.91 ± 0.67
ICPL 7035 (hydrolyzed)	4.93 ± 0.26	11.00 ± 1.26	7.06 ± 3.81	2.20 ± 0.71	1.51 ± 0.09

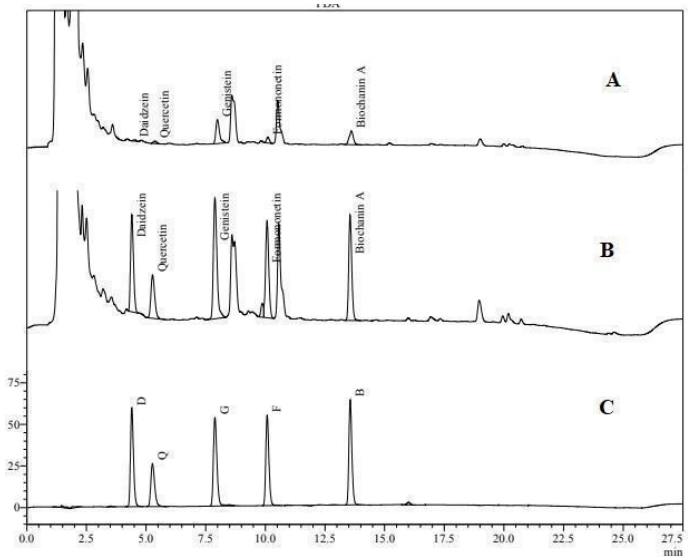

Figure 6: Chromatograms of (A) unhydrolyzed extract of *C. cajan* ICPL 7035; (B) unhydrolyzed extract of *C. cajan* ICPL 7035 spiked with the five phytoestrogens; (C) standard solutions of the five phytoestrogens. Separation was conducted on a reversed-phase C18 Column 150 x 4.6mm ID; flow rate: 1mL/min; Solvent A: 0.1% aqueous TFA, Solvent B: acetonitrile; Gradient: 0.00min - 35% B, 13.00min - 80% B, 16.00min - 95% B, 18.00min - 95% B, 20.00min - 35% B, 27.50min - 35% B

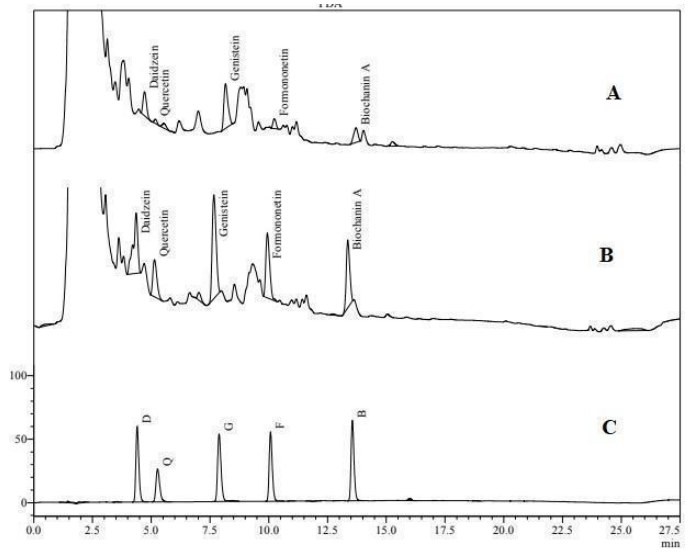

Figure 7: Chromatograms of (A) hydrolyzed extract of *C. cajan* ICPL 7035; (B) hydrolyzed extract of *C. cajan* ICPL 7035 spiked with the five phytoestrogens; (C) standard solutions of the five phytoestrogens. Separation was conducted on a reversed-phase C18 Column 150 x 4.6mm ID; flow rate: 1mL/min; Solvent A: 0.1% aqueous TFA, Solvent B: acetonitrile; Gradient: 0.00min - 35% B, 13.00min - 80% B, 16.00min - 95% B, 18.00min - 95% B, 20.00min - 35% B, 27.50min - 35% B

In this study, methanolic seed extracts of the variant of *C. cajan* (pigeon pea, ICPL 7035) were analysed with the optimized SPE-HPLC procedure. For the sample preparation step, the SPE method described above was unable to eliminate the peaks prior to the elution of daidzein. These peaks were presumed to be unhydrolyzed glycosides of isoflavones from *C. cajan.* The attached glycosidic moiety in the glycosides increases the hydrophilicity of the molecules and, therefore, is eluted earlier in reversed-phase chromatography (Kren 2008). Glycosylated forms were also retained by the SPE and therefore, not eliminated from the sample. Acid hydrolysis resulted in the increase in aglycone content of the extract, particularly that of quercetin which increased eight times as compared to the quercetin content of the unhydrolyzed extract. This was due to the conversion of glycosylated isoflavones to free aglycones which was also observed in increasing HCl concentrations and prolonged reaction time in previous studies (Yeo and Kim 2002; Delmonte and Rader 2006) The amount of quercetin was determined from the leaves of the *C. cajan* in a previous study (Zu et al. 2006) but no studies have reported the presence of such amounts of quercetin in the seeds. An increase in concentrations for daidzein, formononetin, and biochanin A was also observed from the acid hydrolysed sample, suggesting successful hydrolysis of the isoflavone aglycones.

CONCLUSIONS

An HPLC method for the simultaneous separation of phytoestrogenic isoflavones namely daidzein, quercetin, genistein, formononetin, and biochanin A was successfully developed and validated. The separation among the five phytoestrogens was supported by good resolution values and tailing factors. The solid-phase extraction procedure was also proven to be applicable as a clean-up step for plant extracts with a high percentage recovery for all five phytoestrogens. The validated method can be used to determine the presence and quantify of the analytes in *C. cajan* seed extracts. It is recommended that recovery study be conducted by spiking the samples with the analytes prior to extraction to further validate the SPE method.

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CONFLICT OF INTEREST

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

JCDC designed and conducted the experiments and wrote the manuscript. NSQ and MGN assisted in the study design, interpretation of the results, and revision of manuscript. MCV and CMM helped in obtaining the *C. cajan* samples and developing the extraction protocol and contributed to writing the manuscript.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1: Supplementary Table 1. Linear regression for the constructed calibration curve at higher concentrations (6.25 to 200 mg mL^{-1}).

Phytoestrogen	Equation	Linearity
Daidzein	y = 0.0018x + 0.6657	0.9999
Quercetin	y = 0.0041x - 3.3368	0.9999
Genistein	y = 0.0017x + 0.8198	0.9993
Formononetin	y = 0.0018x + 0.3146	0.9999
Biochanin A	y = 0.0017x + 0.4649	0.9999