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Quantitative determination and structural characterization of isoflavones in nutrition supplements by liquid chromatography–mass spectrometry

L.J. Chen [a], X. Zhao [a,b,*], S. Plummer [c], J. Tang [c], D.E. Games [d]

[a] *Division of Cancer Biotherapy, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China*
[b] *Department of Gynecology and Obstetrics, Second University Hospital, West China Medical School, Sichuan University, Chengdu 610041, China*
[c] *Cultech Ltd., York Chambers, York Street, Swansea SA1 3NJ, UK*
[d] *Mass Spectrometry Research Unit, University of Wales, Swansea SA2 8PP, UK*

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Abstract

In this paper, an accurate and route method was developed to quantitative determine daidzein, genistein, glycitein, daidzin, glycitin, 6″-*O*-acetyldaidzin, 6″-*O*-acetylglycitin and 6″-*O*-acetylgenistin contents in selected high and low isoflavones in nutrition supplements by on line liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry (LC–APCI-MS). Improved extraction and hydrolysis methods of the isoflavones from three nutrition supplements were also studied and a rapid extraction method was developed. Comparison of different MS^2 and MS^3 spectra of isoflavones and some unknown compounds were also explored and proposed pathway fragments of nine isoflavones were first systematically suggested.

Keywords: Isoflavones; Nutrition supplement; APCI-MS; Proposed pathway fragments

1. Introduction

Many methods can be used to quantify isoflavones. Gas chromatography–mass spectrometry (GC–MS) is a common technique to identify and quantify isoflavones in soy and soy foods [1]. Although this technique is sensitive and accurate, it requires time-consuming purification, hydrolysis and the preparation of volatile derivatives, since the conjugated forms cannot be directly analysed by GC–MS. CE is a relatively new separation technique compared to other chromatographic methods. This method can offer advantages of a rapid, high-resolution separation (up to 10^6 theoretical plates) with sample volumes in the nanoliter range, resulting in excellent mass detection limits. Aramendia et al. [2] explored the use of on-line CE–MS for separation and characterization of selected isoflavones. This method can provide fast separation of genistein, daidzein, biochaninA and isoliquiritigenin, however, pseudobatigenin, formononetin and biochaninA co-migrated in this system. In addition, sensitivity of this system relied on many factors and optimum analytical signal for this system is not stable which resulted in the bad reproducibility and accuracy. High performance liquid chromatography [3–5] (HPLC) has a widespread application in phytoestrogens research, and has proved to be a highly effective analytical tool. HPLC method combined with ultraviolet absorbance (UV) has been extensively used for analyses of isoflavones of coumestrol, daidzein, genistein, formonetin, and biochaninA in legumes [6–7]. The weakness of these detection methods is their non-specificity leading to the possibility of sample matrix interference.

Mass spectrometry is currently the most sensitive and selective analytical method for the identification of unknown compounds from crude and partially purified samples of natural supplements [8] and has been applied to soy isoflavones analysis [9], Griffith and Collison [10] reported an improved

* Corresponding author. Tel.: +86 28 854 22 566; fax: +86 28 8540 4578.
E-mail address: xia-zhao@126.com (X. Zhao).

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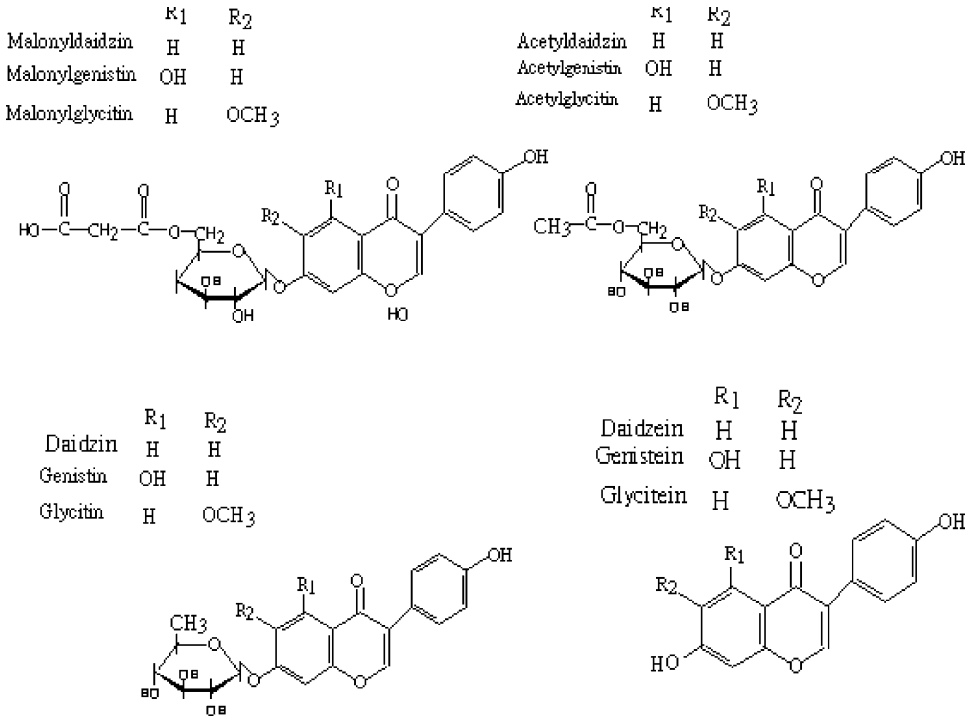


Fig. 1. Structures of isoflavones in soy food.

methods for the extraction and analysis of isoflavones by LC and LC–MS, but his work only focused on the analysis of aglycone isoflavones in soy food and acetyl isoflavones were not analysed.

In this paper, an integrated approach consisting of HPLC, LC–MS–MS has been used for the quantification and identification of isoflavones in nutrition supplements and the principal objective was to development a route quantitative and qualitative method to determine daidzein, genistein, glycitein, daidzin, glycitin, genistin, 6″-*O*-acetyldaidzin, 6″-*O*-acetylglycitin and 6″-*O*-acetylgenistin contents in nutrition supplements (see all structures of isoflavones in Fig. 1). A successful method for the quantitative determination of nine isoflavones was developed by on line LC–APCI-MS. The extraction and hydrolysis methods were also studied. Structural characterisations of unknown components in the nutrition supplements were explored. Comparisons of different MS^2 and MS^3 spectra of isoflavones and some unknown compounds were also explored and proposed pathway fragments of nine isoflavones were first systematically suggested.

2. Experimental

2.1. Apparatus

A Hewlett-Packard 1100 HPLC system (Hewlett-Packard, Wilmington, DE, USA) was interfaced to a LCQ Ion Trap mass spectrometer (Finnigan MAT, San Jose, CA, USA).

2.2. Chemicals and materials

A Lurna RP C_{18} column, 150 mm × 4.6 mm (USA) with 5 μm packing was chosen for the sample analysis. HPLC grade solvents of methanol, water, formic acid, acetic acid and acetonitrile were purchased from Fisher Chemicals (Loughborough, UK). Genistein, daidzein, glycitein and biochaninA were obtained from Aldrich. Genistin, glycitin and daidzin were obtained from Indofine (Somerville, NJ, USA). Acetyldaidzin, acetylglycitin and acetylgenistin were purchased from Plantech (Reading, UK). All solvents were filtered using a vacuum filtration system (Millipore) through a 0.45 μm nylon membrane filter (Altech Associates, Lancashire, UK), and degassed prior to use. Gases used included oxygen free nitrogen (OFN), helium and air, which were purchased from BOC (Surry, UK).

Stock standard solutions were prepared by accurately weighing, on a five-place analytical balance to the nearest 0.01 mg. 0.01 g daidzin, 0.01 g glycitin. 0.01 g genistin, 0.01 mg daidzein, 0.01 mg genistein and 0.01 mg glycitein were dissolved into 50 ml methanol solution and the final concentrations were 200 μg/ml, separately. These solutions were stable for at least 2 months at room temperature and were used in recovery experiments with soy sample and preparation of working standards. Acetylglucoside isoflavones were available in limited quantities and no stable in room temperature, so acetyl isoflavones forms were always dissolved freshly into dimethyl sulfoxide (DMSO) and diluted in a single step to final concentration prior

to use. All standard were kept in the ultrasonic bath for 10 min to confirm the complete dissolving and stored at 0 °C.

2.3. LC–MS separation conditions and quantitative parameters

2.3.1. Extraction procedures

Approximately, 0.2000 g of soy nutrition supplement was accurately weighed into a screw cap 20 ml test tube. Four milliliters of water was added to the sample, and then 16 ml volume of acetonitrile was added and shaken briefly to mix. Sample tube was extracted for 40 min on a sonic bath under 25 °C. After 40 min, 1 ml of samples were centrifuged for 10 min at 2000 × *g* to pellet insoluble matter and eliminate foam. A portion of the supernatant was removed with a syringe, filtered through a 0.45 μm PVDF filter into a sample vial and diluted with 80% acetonitrile to 1 ml and analysed by HPLC. This extraction procedure minimized handling of the samples during preparation. The sample was extracted directly by 80% acetonitrile and transferred to the HPLC system without evaporation procedure, and acetonitrile was also the mobile phase solvent in on line HPLC–MS analysis.

2.3.2. LC–APCI-MS method

LC–ESI-MS and LC–APCI-MS were explored in our study and LC–APCI-MS with negative mode was found to effect on the separation and quantitation.

2.3.3. Recovery experiment

Three samples of the soy nutrition supplements, each spiked with known quantities of reference standards in low (0.5 μg/ml), middle (5 μg/ml) and high concentrations (20 μg/ml), were together extracted according to the same extraction method. The isoflavones in these six samples were determined by LC–MS. The ratio of the found to the expected isoflavone contents in the spiked samples was the recovery efficiency of the extraction.

3. Results and discussion

3.1. Chromatographic conditions and quantitation method by LC–APCI-MS

An optimised separation method was investigated by LC–APCI-MS. Fig. 2 shows the separation gradient elution programmes.

Fig. 2 indicates that negative LC–APCI-MS can provide good resolutions for all isoflavone. On the other hand, the entire column flow was directly into the mass spectrometer without the split of flow rate and a stable flow rate was guarantied in all analytical processes.

3.2. Comparison of different extraction methods

Extraction of isoflavones from soy foods is a difficult challenge. Extraction of isoflavone from soy supplements has commonly been achieved with hot methanol and water mixture with different ratios of volume. Eldridge [11] reported complete extraction by stirring soy samples in aqueous methanol for 4 h at 60 °C. After removal of the solid residue by filtration, the filtrate was directly assayed for isoflavone by HPLC without further sample treatment. But this method results in complete conversion of 6″-*O*-malonyl – and 6″-*O*-acetyl-isoflavones to β-glucoside and aglycone forms. Wang and Murphy [12] utilized acidified acetonitrile at room temperature for extraction of isoflavones from foods, but follow this step was to remove the acidified acetontrile at less than 30 °C and then to dissolve the residue in 80% (v/v) methanol. This laborious procedure makes extraction and analysis of large numbers of sample difficult. In our study, acetonitrile, ethanol and methanol were evaluated for their efficiencies in extraction isoflavones from soy nutrition foods. Extractions were carried out with and without the addition of hydrochloric acid, and acetonitrile as an extract solvent can obtain high concentration, so acetonitrile was chosen to be an extracting solvent. In the other hand, the sample can be directly subjected to LC–MS without evaporation process because the acetonitrile was chosen to be mobile phase solvent in our analysis.

3.3. The effect of supersonic time on the extraction efficiencies

Supersonic bath extraction of sample can greatly shorten the time of extract but the temperature was controlled under 25 °C in order to avoid the desertification to β-glucoside from acetyl glucoside form. Our studies indicate that the concentrations of isoflavones maintain constant in 20–50 min of supersonic time. Too long supersonic time will result in the deesterification of acetyl isoflavones. In order to avoid the hydrolysis of acetyl isoflavones in high temperature, sample should maintain at a room temperature in supersonic period.

3.4. The effect of refluxing time on the extract efficiencies

Most papers reported the determination of isoflavones by refluxing sample in acidified methanol by converting all acetyl and glycoside forms into aglycone isoflavones. In our study, hydrolysis of isoflavones was explored. After refluxing 60 min, all acetyl isoflavones converted into their glycosides and aglycones. Increasing the refluxing time resulted in the conversion of glycosides into aglycones. About refluxing 4 h, all glycoside isoflavones were converted to aglycones. One important phenomenon was that the aglycone became unstable when the refluxing time was increased to 4 h, and the concentration of genistein was found to decrease and a new unknown peak was detected. Hence, the hydrolysis

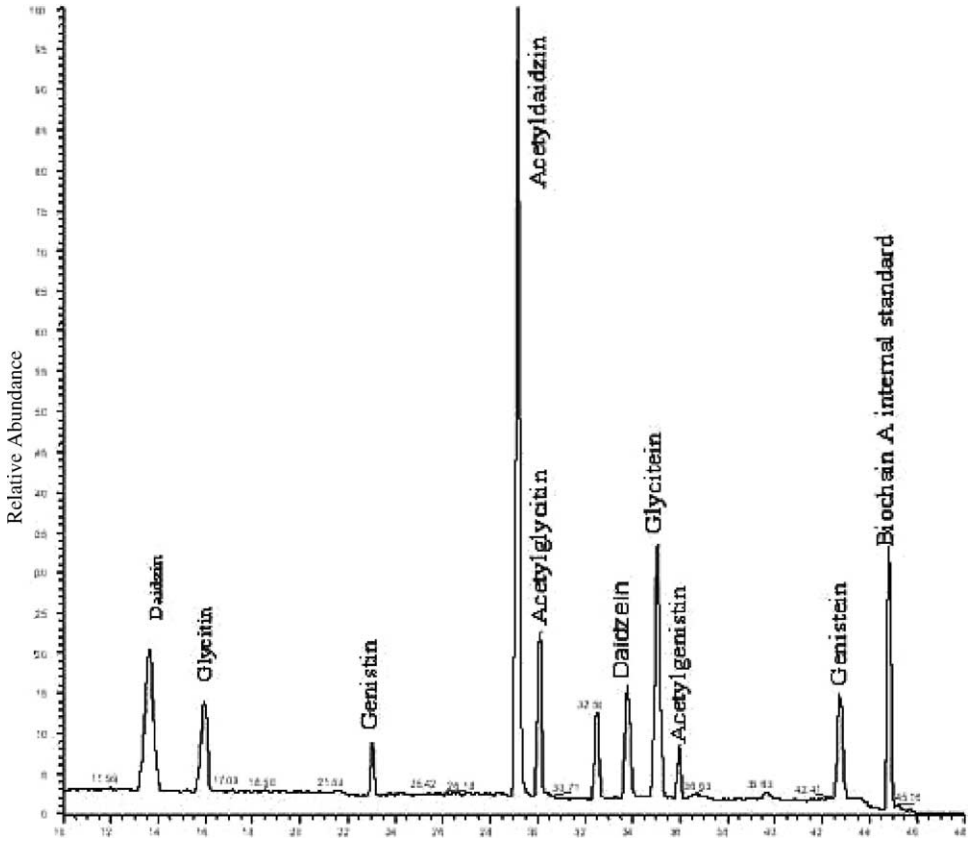


Fig. 2. LC–APCI-MS chromatogram of nine standard isoflavones. HPLC experimental conditions: reversed-phase column: Luna C_{18} (5 μ), 150 mm × 4.6 mm. The column was held constant at 12% B for 10 min of the run, followed by a two-step linear gradient, to 30% B over 30 min, then to 90% B over 2 min. The column was washed at 90% B for 6 min and equilibrated 10 min. MS conditions: vaporizer temperature: 500 °C; sheath gas flow rate: 90 arbitrary; discharge current: 5.0 V; capillary temperature: 170 °C, capillary voltage: −30 kV; electrospray voltage: 4.5 kV.

time should be considered carefully. In our study, hydrolysis method was avoided due to this reason.

3.5. Validation of analytical method

The validation of an analytical method was evaluated by accuracy and precision. The accuracy of a measurement is defined as the closeness of the measured value to the true value. Typically, accuracy is represented and determined by recovery studies and evaluated by standard deviation (SD) or relative standard deviation (RSD). The recoveries of daidzin, glycitin, genistin, daidzein, glycitein and genistein were measured by adding six standard isoflavones into the soy based nutrition supplements covering a range of concentrations of 0.5–6 μg/ml. The spiked samples were then processed through the whole extraction procedure. Their concentrations were determined by their individual standard curve. Table 1 shows the results of recoveries.

The method showed good recoveries that were close to 100%. The mean recoveries of the individual standards ranged from 102.7 to 111.0 for daidzein, 95.27 to 108.1 for glycitein. 97.27 to 104.0 for genistin, 93.50 to 100.4 for daidzin, 92.36 to 101.7 for glycitin and 94.67 to 101.9 for genistein. The higher recoveries (>100%) were found for lower amounts of spiked standards. This was probably due to some impurities co-eluting in the nutrition food that were significant at the lower spike levels. Precision can be defined as the degree of agreement among individual test results and can be evaluated by repeatability and reproducibility. The repeatabilities and reproducibilities of the method were evaluated by carrying out six replicate determinations on the same day and six on three different days. In the present study, inter-assay RSDs and intra-assay RSDs were also evaluated and shown in Table 2.

Table 2 shows that good repeatability and reproducibility are obtained by our method and can be used for quantitative determination.

3.6. LC–APCI-MS^2 and LC–APCI-MS^3 studies of the isoflavones structures of nutrition supplements

In our study, both LC–APCI-MS and LC–APCI-MS–MS with positive and negative ion modes were studied using a Finnigan MAT LCQ ion trap, and the negative ion ionisation methods were deemed superior. Negative LC–ESI-MS gave a lower level of sensitivities for acetyl isoflavones compared to negative LC–APCI-MS but the split has to be used to lower the flow rate into mass spectrometer. LC–APCI-MS

Table 1
Recoveries of isoflavones in different concentrations

Recoveries (%)	Daidzein	Glycitein	Genistin	Daidzin	Glycitin	Genistein
2	109.0	99.98	97.27	100	101.7	98.65
2	111.0	101.4	99.95	100.4	92.36	94.67
2	101.4	104.2	101.6	102.5	94.63	102.4
2	99.85	102.5	98.45	97.67	96.84	99.87
2	104.5	107.8	97.43	98.45	102.4	100.4
Mean ($n = 5$)	105.15	103.176	98.94	99.804	97.586	99.20
SD	4.79	3.01	1.83	1.87	4.38	2.87
RSD (%)	4.55	2.92	1.05	1.88	4.49	2.39
1	102.7	95.27	102.7	93.64	93.27	101.0
1	105.0	105.2	104	93.5	98.08	99.69
1	106.4	105.3	100.9	99.36	98.51	99.25
1	106.7	108.1	100.4	99.84	97.51	101.9
1	110.0	106.3	103.5	103.5	102.7	103.5
Mean ($n = 5$)	106.2	104.0	102.3	97.97	98.01	101.1
SD	2.67	5.04	1.59	4.32	3.35	1.72
RSD (%)	2.51	4.84	1.55	4.41	3.42	1.70
3	102.3	90.45	97.86	96.33	94.85	96.42
3	105.3	105.8	100.6	98.74	96.65	97.32
3	106.8	103.1	100.2	98.99	100.8	100.9
3	102.8	100.4	98.42	97.46	98.33	95.43
3	104.3	102.8	96.33	101.3	95.33	99.45
Mean ($n = 5$)	104.4	102.1	98.68	98.56	97.19	97.90
SD	1.92	2.80	1.75	1.86	2.43	2.24
RSD (%)	1.84	2.74	1.77	1.89	2.50	2.29

1, low concentration (0.5 μg/ml); 2, middle concentration (5 μg/ml); 3, high concentration (20 μg/ml).

in negative ion mode was found better than LC–ESI-MS in negative ion mode. So, LC–APCI-MS was used for qualitative and quantitative determination of isoflavones. Prior to LC–APCI-MS analysis of real sample, parameters such as the source voltage, the sheath and auxiliary gas pressures, the heated capillary temperature, the vaporizer temperature, discharge current, electrospray voltage, and the tube lens offset voltage, were optimised by loop injection of a standard solution (supplied by Finnegan) at 0.8 ml/min into the APCI sources, respectively.

In the analyses of isoflavones of nutrition supplements by full scan negative LC–APCI-MS, Their molecular ion plus acetic acid of $[M - H + 60]^-$ ions of acetyl and glycoside isoflavones gave intense negative ion spectra at $m/z = 532.9$ for acetylgenistin, $m/z = 546.9$ for acetylglycitin, $m/z = 517$ for acetyldaidzin, $m/z = 491$ for genistin, $m/z = 475$ for daidzin and $m/z = 505$ for glycitin (see Fig. 3). All isoflavones at free aglycone ion constituted the base peaks at $m/z = 269$ for acetylgenistin, genistin and genistein, at $m/z = 253$ for acetyldaidzin, daidzin and daidzein and at $m/z = 283$ for acetylglycitin, glycitin and glycitein. Nine isoflavones were detected in the sample and malonyl isoflavones were not detected in three nutrition supplement samples. Three unknown components marked P1, P2 and P3, which have the same mass spectrum as acetylglycitin, were detected. In order to have enough information and identifications of the components

Table 2
Reproducibility of HPLC analysis of the soy nutrition supplements

Isoflavone	Concentration (mg/g)					
	Within-day			Between-days		
	Average ($n = 5$)	SD	RSD (%)	Average ($n = 5$)	SD	RSD (%)
Daidzin	20.79	0.458	1.87	21.33	0.70	4.19
Glycitin	14.78	0.565	3.82	14.92	0.32	9.09
Genistin	6.73	0.086	1.27	7.10	0.26	7.35
Acetyldaidzin	58.46	0.394	0.67	59.16	1.70	2.87
Acetylglycitin	5.37	0.064	1.19	5.37	0.14	2.67
Daidzein	0.827	0.086	10.39	0.62	0.24	7.94
Glycitein	0.369	0.014	3.79	2.35	0.07	2.83
Acetylgenistin	6.98	0.090	1.29	6.95	0.19	2.74
Genistein	0.154	0.008	5.19	2.37	0.05	1.93

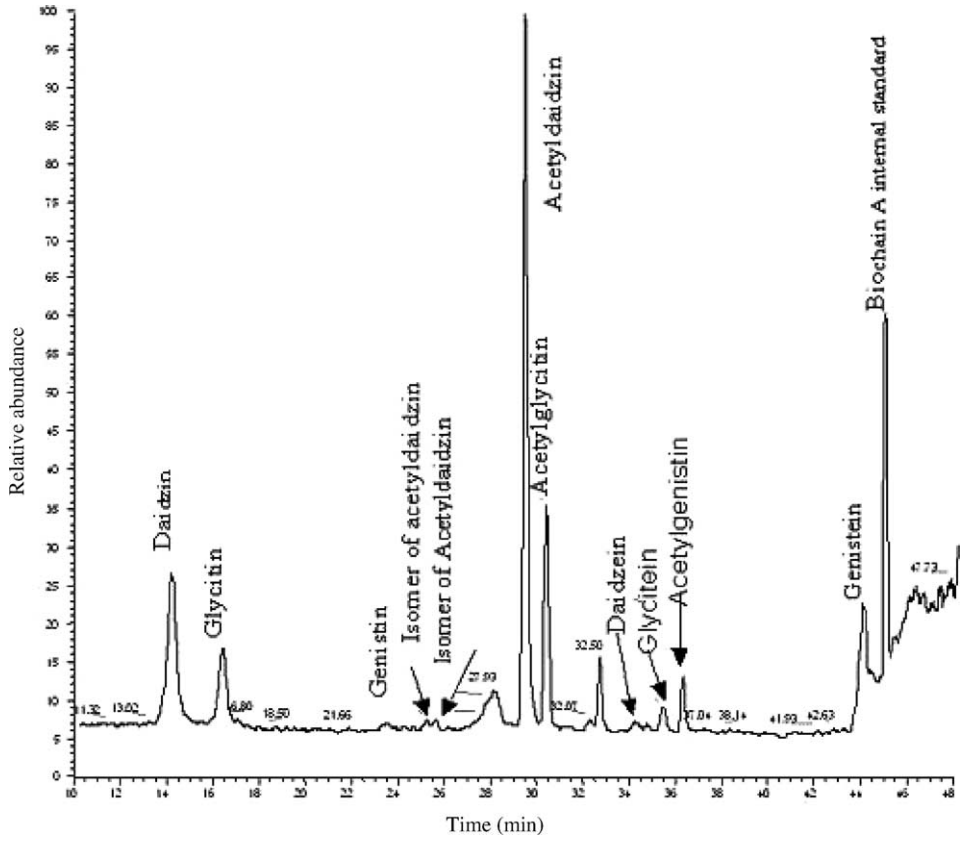


Fig. 3. TIC chromatogram of one soy supplement by negative LC–APCI-MS (experimental conditions, see Fig. 2).

under analyses, LC–APCI-MS^n was explored in our study.

3.6.1. LC–APCI-MS^2 analysis of the acetyl isoflavones

MS^n has been used to determine flavonoids existing as their glycosylated conjugates and their aglycone flavonoids structures [13]. Aramendia et al. [8] determined the structure of isoflavones by using HPLC–APCI-MS^n. The LCQ can perform MS^n operations in a step-wise manner, and precursor ions are isolated prior to a subsequent MS^n experiment. Hence, the isolation step ensures that the fragmentation spectra from next are less complex than traditional MS–MS data. Cunniff et al. [14] results have shown the powerful ability of MS^n in exposing core flavonoids. In our study, LC–APCI-MS–MS was operated by a Finnigan ion trap and the operating conditions applied were shown in Table 3.

The nomenclature used to define the various fragment ions has been proposed by Claeys and co-workers [15]. For free aglycones, the symbols $^{i,j}A^+$ and $^{i,j}B^+$ are used to designate primary fragment ions containing A- and B-ring, respectively. The superscripts i and j refer to the bonds of the C-ring that have been broken. These ions can lose small neutral fragments, such as H_2O and CO. These supplement ions are represented combining $^{i,j}A^+$ or $^{i,j}B^+$ with the lost fragments. In order to obtain accurate product ion fragments from the precursor ion, molecular ion of $[M-H]^-$ was chosen as precursor ion even though the $[M-H–CH_3COOH]^-$ were detected instead of ions of $[M-H]^-$ due to the use of acetic acid in the composition of the mobile phase. For acetyl isoflavones, the APCI-MS^2 spectra exhibited their fragments $[M-H-120]^-$ and $[M-H-90]^-$ characteristic of the cleavage of the *O*-glycoside moieties when the ions of $[M-H]^-$ are parent ions. Fig. 4 shows the proposed pathway of their fragmentations according to the proposal pathway of Claey.

3.6.2. LC–APCI-MS^2 and LC–APCI-MS^3 analyses of glycoside and aglycone isoflavones

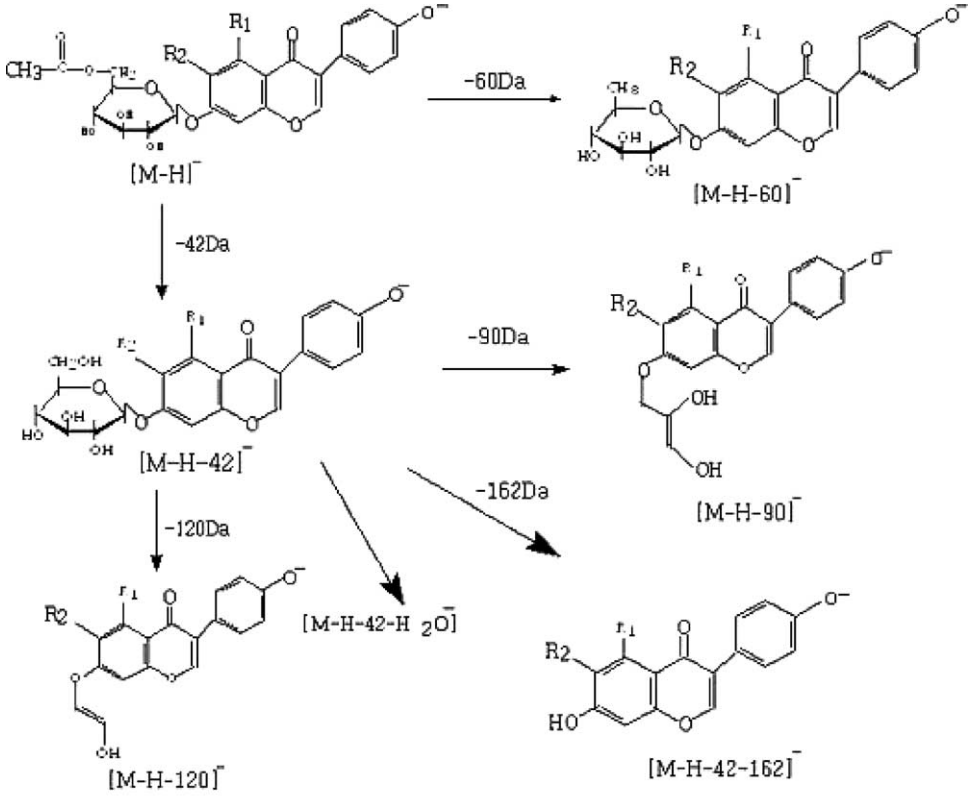
Glycoside isoflavones gave very similar fragments to the acetyl isoflavones when $[M-H]^-$ were selected as their precursor ions and product ions were recorded by LC–APCI-MS^2. LC–APCI-MS^2 studies of daidzein, genistein and glycitein can provide more information for their structural elucidation and identification of unknown compounds. The results of extensive studies give scope as a molecular fragmentation fingerprint with which to index and identify each target component within mixtures of unknown compounds. For daidzein, glycitein and genistein, different collision energies were required to provide a satisfactory fragmentation pattern, whilst retaining the presence of the $[M-H]^-$ precursor ions. For daidzein, $m/z = 224$ and 225 appear as major fragments in the negative APCI-MS^2 spectrum, implying losses of CHO, and CO, respectively. This type of fragmentation is indicative of phenol-containing compounds such as

Table 3
Major ions of isoflavones acquired by negative mode LC–APCI-MS–MS

Isoflavones	Precursor	Supplement ions (relative intensities)	Collision energy
Daidzin	415	142.3 (18), 143.5 (9), 236.6 (21) 252.3 (100), 253.4 (97), 295 (10). 319.2 (28), 415.2 (19)	35
Glycitin	445.4	124.8 (2), 268.3 (2), 282.2 (19), 283.2 (100), 325.2 (9), 355.2 (3), 430.2 (5), 445.4 (12)	30
Genistin	431.2	170 (1), 268.3 (100), 269.3 (67), 293.3 (1), 311.1 (16), 323.4 (3), 341.1 (5), 381.5 (1), 431.2 (11)	33
Acetyldaidzin	457.1	252.3 (100), 253.4 (73), 267.3 (4), 295.2 (27), 325.2 (4), 397.2 (5), 446 (4), 457.1 (5)	35
Acetylglycitin	487.3	268.2 (7), 282.3 (100), 283.2 (61), 325.2 (25), 355.2 (5), 427.3 (4), 472.2 (8), 487.3 (9)	
Daidzein	253.2	152.3 (14), 183.3 (21), 197.3 (64), 209.4 (25), 223.4 (29), 224.3 (100), 225.3 (69), 235.4 (14)	45
Glycitein	283.2	240.3 (0.1), 266.2 (0.1), 267.5 (0.6), 268.2 (100), 283.5 (0.3)	32
Acetylgenistin	473.2	267.3 (1), 268.3 (100), 269.3 (20), 311.2 (9), 323.3 (1), 341.3 (6), 413.2 (3), 473.3 (6)	35
Genistein	269.2	169.2 (26), 181.2 (27), 197.2 (39), 201.2 (50), 213.2 (28), 224.3 (43), 225.3 (100), 241.2 (47)	45
P1	283.2	82.8 (0.6), 140.1 (0.5), 158.6 (0.5), 167 (0.5), 255.4 (1.3), 267.5 (0.6), 268.2 (100)	32
P2	283.2	126.5 (0.4), 137.9 (0.8), 159.7 (0.4), 162.3 (0.5), 214.8 (0.4), 267.5 (1), 268.2 (100), 283.2 (0.3)	32
P3	283.4	124.6 (0.4), 207.2 (0.2), 219.3 (0.3), 267.3 (2.1), 268.2 (100), 272.9 (0.4), 283.4 (0.2)	32

baicalein, daidzein, genistein and chrysin. The appearance of $m/z = 196$ may suggest a further loss of CO or CHO from the other phenolic group. A notable ion at $m/z = 197$ may imply a loss of CO from $m/z = 225$ and rearrangement.

Aramendia have suggested a fragmentation pathway for genistein and daidzein relating to ions at m/z 135 and 91 by negative APCI-MS–MS, but in our case, the ion of $m/z = 133$ was detected instead of $m/z = 135$. The ion of $m/z = 91$ is a very important fragment of the structure elucidation of flavonoids and isoflavones since this fragment results from the ion of the B-ring, which indicated an OH group in the B-ring of daidzein, but the signal of ion at $m/z = 91$ is very weak for daidzein in our case The ion of $m/z = 181$ is possibly formed by the loss of O from $m/z = 197$ and the ion of $m/z = 153$ is



	R_1	R_2	m/z
Acetyldaidzin	H	H	457
Acetylgenistin	OH	H	473
Acetylglycitin	H	OCH_3	487

Fig. 4. Proposed pathway fragmentations of acetyl isoflavones.

further loss of CO from $m/z = 197$. According to our elucidation, the ion of $m/z = 197$ may imply one OH group in the A-ring from daidzein and the ion of $m/z = 213$ should be observed from the mass spectrum of genistein in its APCI-MS^2 due to two OH groups in the ring B.

The MS^2 fragmentation mass spectrum of genistein shows similarities to that observed for daidzein and the ions of $m/z = 225$ and 197 are observed as expected since the two compounds are very similar. However, the ion of $m/z = 106.8$ is observed with a very weak signals instead of m/z 91, indicating two OH groups in the B-ring of genistein. From the mass spectrum of genistein, the fragments at $m/z = 225$ and 224 indicate respective losses of $m/z = 44$ and 45, which could be the losses of CO_2 or CH_2CHOH, and CO_2H or CH_3CHOH. The fragment of m/z 213 is observed as we expected and may result from the loss of CO from $m/z = 241$ and imply two OH groups in the ring A. So, it is possible to differentiate the daidzein and genistein from the $m/z = 213$ ion. Further inspection of the daidzein and genistein standard solutions were acquired by performing MS^3 and MS^4 analysis on the base peak product from the MS^2 study. Indeed, we can obtain significant value in ascertaining differences or similarities between identical m/z results of the two different compounds by using MS^3 to MS^4. The proposed pathways of aglycone isoflavones were suggested and shown in Fig. 5.

The negative LC–APCI-MS^2 mass spectrum of glycitein only gave a strong ion at $m/z = 268$ $[M - H–CH_3]^-$, even increasing the collision induced energy (CID) to 45%. This result was also observed in the unknown peaks 1–3 when the same conditions of APCI-MS^2 were used. As a rule, isoflavones with a methoxy group (biochaninA, glycitein and glycitin) exhibit one fragment ion at $m/z = [M - H–CH_3]^-$ when a high extraction cone voltage is used. In order to further investigate the structure information especially for the unknown compounds, further MS^3 studies of the glycitein and unknown peaks 1–3 were performed.

3.7. LC–APCI-MS^3 Analyses of glycitein, peaks 1–3

For the glycitein, as we expected, the negative APCI-MS^2 mass spectrum only gave the base peak of $m/z = 268$ and peaks 1–3 exhibit the same mass spectra as that of the glycitein in negative APCI-MS^2. These fragments are not enough to differentiate them. Further inspection of glycitein peaks 1–3 were acquired by performing MS^3 on the base product ions resulting from the MS^2. Peaks 1–3 gave the same precursor $[M - H]^-$ at $m/z = 517$ ion and product ion at $m/z = 283$. We can conclude that peaks 1–3 all are isomers of acetylglycitin. However, the differences of their structure can still be obtained by their MS^3 spectra. For peak 1, the mass spectrum of negative APCI-MS^3 gives a very different pattern from that of glycitein, indicating their different structures. We cannot elucidate the structure of peak 1 due to low concentration. We can still conclude that peak 1 belongs to the 6″-*O*-acetyl glycoside due to the precursor presence of fragment of $[M - H–CH_3CO - 162]^-$ and peak 1 is the isomer of acetylglycitin. These fragments are not enough to differentiate

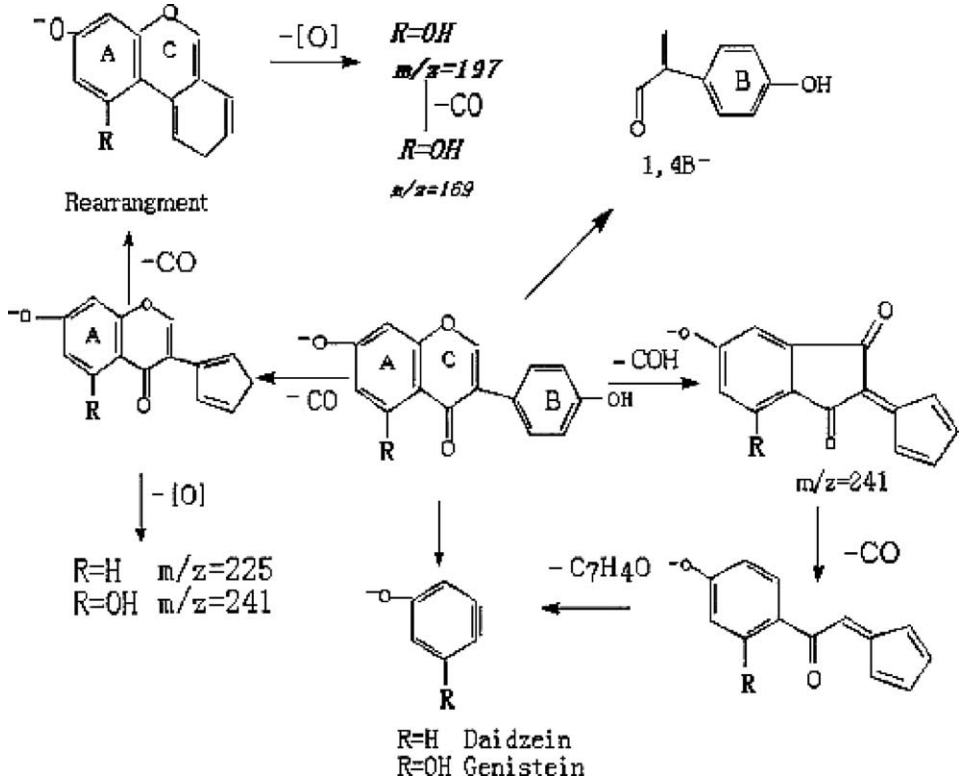


Fig. 5. Proposed pathway fragmentations of aglycone isoflavones.

Table 4
Major ions with relative high intensities acquired by negative LC–APCI-MS^3

Component	First-stage precursor ion and CID (%)	Second-stage precursor ion and CID (%)	Third-stage precursor ion and intensity (%)
Daidzein	253 (44)	225 (40)	95.1 (71), 115.1 (16), 169.3 (100), 197.2 (54), 200.4 (13), 215.2 (8)
Genistein	269 (42)	225 (40)	180.3 (61), 181.7 (59), 195.3 (100), 197.1 (65), 211.4 (38)
Glycitein	283 (35)	268.3 (35)	224.4 (3), 239.5 (7), 240.3 (100), 267.3 (7), 268.2 (9)
Peak 1	283 (34)	268 (35)	187.9 (2), 203 (3), 213.6 (2), 224.3 (7), 239.1 (4), 240.2 (100), 267.3 (4), 268.0 (3)
Peak 2	283 (34)	268 (35)	157.3 (2), 212.3 (3), 239.4 (3), 240.3 (100), 253 (2), 267.4 (2) 268.1 (6)
Peak 3	283 (34)	268 (35)	224 (5), 239.3 (9), 240.2 (100), 267.2 (1), 268.1 (5)

them. Further inspection of their structural identification need be done.

The MS^3 experiments of the ion at $m/z = 283$ of peaks 1–3 display product ions due to the losses of 15 (CH_3), 29 (CHO), and 28 (CO), representing a methylated flavonoid. Attempts were made to elucidate the structure of the ion at $m/z = 283$ on the basis of its products. For this purpose, we compared the MS^3 data of the standard methylated isoflavones glycitein; $[M-H]^-$ 283 Da) with those of the ion at $m/z = 283$ of peaks 2 and 3. We found that the MS^3 mass spectrum of peak 3 is very similar to that of glycitein. On the basis of these pieces of evidence, peak 2 is proposed to be 5-methoxy-acetyldaidzin. We could not elucidate their structures merely based on MS^2 and MS^3 data, but we could conclude that peaks 2 and 3 are all isomer of acetylglycitin. Although the elucidation of unknown compounds is still difficult without the aid of reference materials, we can still get some useful structure information of unknown compounds. Table 4 shows their major ions with relative high intensities acquired by negative LC–APCI-MS^3 and Fig. 6 shows the proposed pathway fragments of glycitein.

3.8. Linear response curve for LC–APCI-MS analysis

Most quantitative determinations of isoflavones in foods or nutrition foods were measured in aglycones after the hydrolysis and only six standard materials were needed instead of 12 [16–17]. However, our studies of optimisation extraction of isoflavones indicate that some unknown compounds will hydrolyse to aglycone isoflavones and inaccurate results could be obtained. In present study,

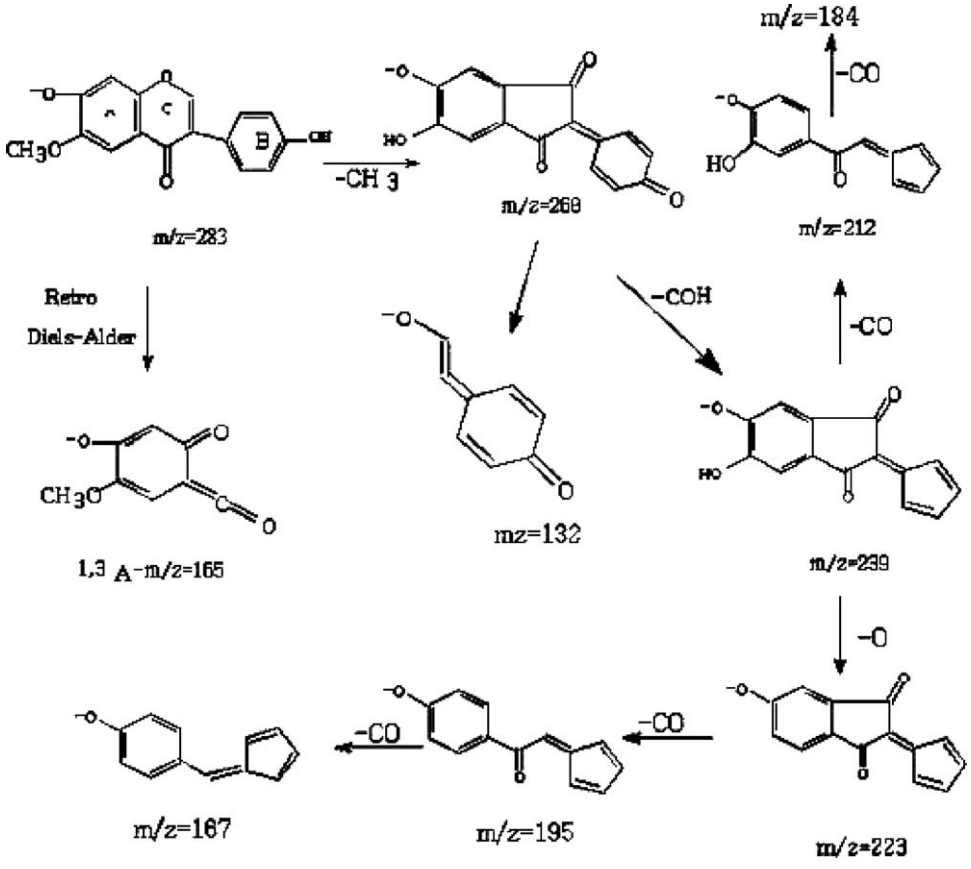


Fig. 6. Proposed pathway fragmentations of glycitein.

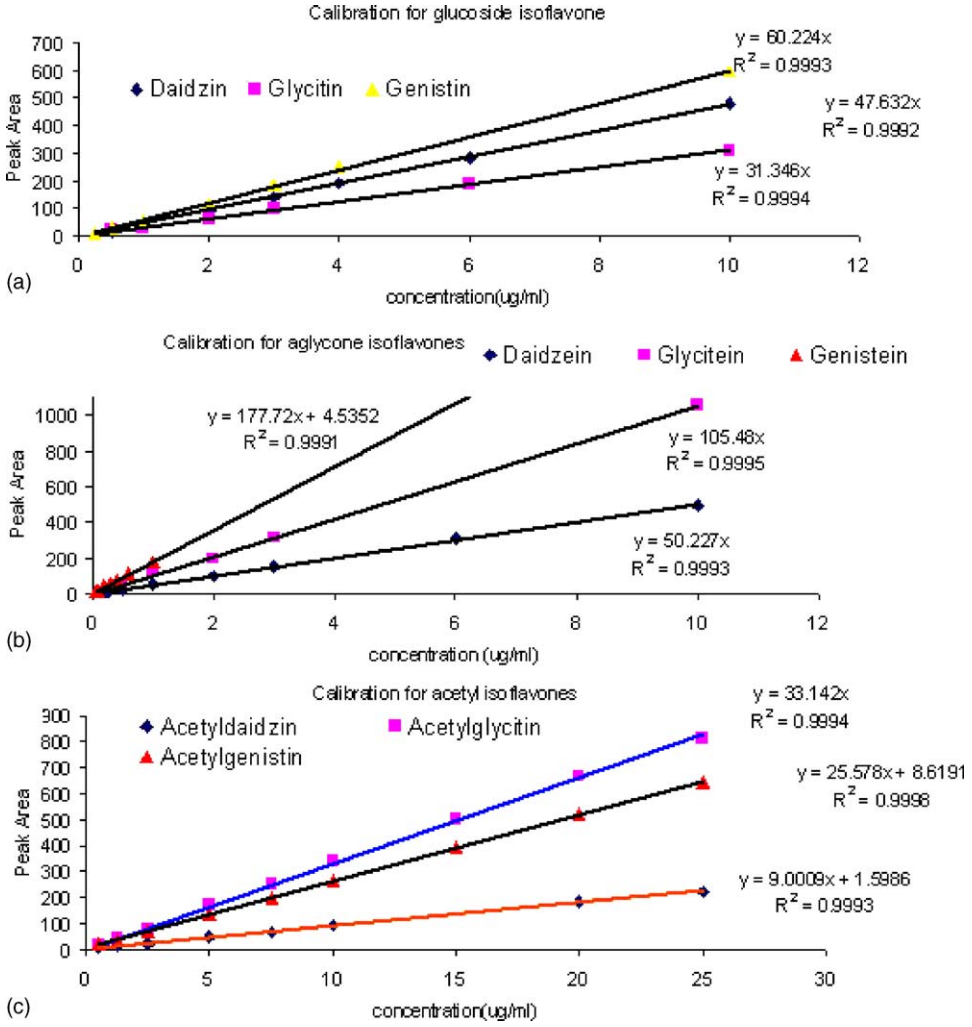


Fig. 7. Calibration curves for isoflavones.

Table 5
Isoflavone concentration of the soy supplements in ground samples

Supplement	Supplement (powder) (mg/g)[a]	Supplement 2[a] (capsule 0.7286[b]) (mg/capsule)[b]	Supplement[a] (powder) (mg/g)[a]
Daidzin	20.79	5.084	4.15
Glycitin	14.78	2.582	2.598
Genistin	6.73	1.542	0.858
Acetyldaidzin	58.46	0.605	12.98
Acetylglycitin	5.37	0.302	1.738
Daidzein	0.827	0.059	0.844
Glycitein	0.369	0.016	0.216
Acetylgenistin	6.98	0	1.03
Genistein	0.154	0.014	0.414
Total isoflavone	111.5	Total isoflavone: 10.20 mg Quercetin: 49.80 mg	24.83
The values provided by manufacture	100	Total isoflavone: 15 mg Quercetin: 50 mg	20

[a] Mean value of the triplicate.
[b] Weight of the capsule itself is not included.

malonyl isoflavones were not detected in our all nutrition supplement samples, So nine standard isoflavones were used and their calibration curves were measured. Fig. 7 gave all calibration curves of isoflavones and the calibration ranges of acetyl isoflavones are 0–25 μg/ml, glycoside isoflavones 0–10 μg/ml and aglycone isoflavones 0–10 μg/ml.

3.9. Isoflavone concentrations of nutrition supplements

Three nutrition supplements were quantified by our methods. In sample of 10% soya nutrition supplements, acetyl isoflavones were its prominent compounds and malonyl isoflavones were not detected. It indicates that the contents and isoflavones forms can vary due to different fermentation process. Glycoside and aglycone isoflavones only contained small portion of whole isoflavones. Their results were shown in Table 5.

4. Conclusion

In the present study negative HPLC–APCI-MS method to analyse the isoflavones was developed and three soy nutrition supplements were analysed and quantified in order to evaluate whether the isoflavone contents data given by the producers correspond with the measured value.

This method described for extraction and HPLC-MS analysis of isoflavones provides a means to speed extraction and analysis of isoflavones using the preferred solvent, acetonitrile. Acetonitrile is is also used as mobile phase for HPLC-MS analysis, hence, the elimination of the need to evaporate the acetonitrile process greatly reduce the time and effort in sample preparation. The good reproducibility and recoveries were also obtained by this method. The spectra of LC–MS^2 and LC–MS^3 of isoflavones were systematic discussed and proposed pathway fragments were suggested based on the experimental results.

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References

[1] J. Tekek, D.D. Keukeleire, H. Rong, E. Daeseleire, C. Van Peteghem, J. Agric. Food Chem. 47 (1999) 5059.
[2] M.A. Aramendia, V.I. Garcia, F. Lafont, J.M. Marinas, J. Chromatogr. A 707 (1995) 327.
[3] A. Edelmann, J. Diework, K.C. Schuster, B. Lendl, J. Agric. Food Chem. 49 (2001) 1139.
[4] J. Tekek, E. Daeseleire, A. Heeremans, C. Van Peteghem, J. Agric. Food Chem. 47 (3489) (1999) 111.
[5] W. Wang, Y. Tanaka, Z. Han, J. Cheng, J. Agric. Food Chem. 42 (1995) 1584.
[6] P.A. Ireland, S.Z. Dziedzic, J. Chromatogr. 325 (1985) 275.
[7] J. Hu, S.O. Lee, S. Hendrich, P.A. Murphy, J. Agric. Food Chem. 50 (2002) 2587.
[8] M.A. Aramendia, I. Garcia, F. Lafont, J.M. Marinas, Rapid Commun. Mass Spectrom. 9 (1995) 503.
[9] S. Barnes, M. Kirk, L. Coward, J. Agric. Food Chem. 42 (1994) 2466.
[10] A.P. Griffith, M.W. Collison, J. Chromatogr. A 913 (2001) 397.
[11] A.C. Eldridge, J. Agric. Food Chem. 30 (1982) 353.
[12] H.J. Wang, P.A. Murphy, J. Agric. Food Chem. 42 (1994) 1666.
[13] C. Borges, P. Martinho, A. Martins, Rapid Commun. Mass Spectrom. 15 (2001) 1760.
[14] J. Cunniff, P. Tiller, M. Harvey, A. Land, The Power of MSn, Finnigan, San Jose, 2000, p. l.
[15] Y. Ma, Q.M. Li, H. Van den Heuvel, M. Claeys, Rapid Commun. Mass Spectrom. 11 (1357) (1997) 1975.
[16] L. Coward, M. Kirk, N. Albin, S. Barnes, Clin. Chim. Acta 247 (1996) 121.
[17] S. Barnes, C.C. Wang, M. Smith-Johnson, M. Kirk, J. Med. Foods 2 (1999) 111.