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Identification of flavone aglycones and glycosides in soybean pods by liquid chromatography–tandem mass spectrometry

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Abstract

High-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry was used to identify flavone aglycones and glycosides in soybean pods. Tandem mass spectrometry (MS–MS and MS–MS–MS) and photodiode array detection were also utilized in flavone characterization. A total of seven flavone aglycones and glycosides were identified. Among them three flavone aglycones—apigenin, 7,4′-dihydroxyflavone, and luteolin—and two flavone glycosides—apigenin-7-*O*-β-D-glucoside, and luteolin-7-*O*-β-D-glucoside—were unambiguously identified based on their abundant $(M+H)^+$ ions, UV spectra, retention time, and tandem mass spectrometric analysis compared with authentic standards. The tentative identification of two flavone glycosides as 7,4′-dihydroxyflavone-7-*O*-β-D-glucoside and apigenin-7-*O*-β-D-glucoside-6″-*O*-malonate was based on UV spectra, $(M+H)^+$ ions, and tandem mass spectrometry. This is the first report identifying flavone aglycones and glycosides in soybean pods.
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Keywords: Soybean; Flavonoids; Flavones; Isoflavones; Apigenin; Dihydroxyflavone; Luteolin; Aglycones; Glycosides

1. Introduction

Flavonoids are polyphenolic compounds commonly found in many plants, vegetables, and flowers. The flavonoid family comprises 15 classes of compounds, including the flavones, flavonols, flavanones, chalcones, and isoflavones (isoflavonoids) [1,2]. Although flavonoids are ubiquitous, isoflavones are found only in a few legumes, particularly soybean. Isoflavones have been shown to play a role in the prevention of hormone-dependent cancers [3–5] and the health benefits of a diet rich in isoflavones include a reduced risk of heart disease, osteoporosis, and menopausal symptoms [6–9]. Although isoflavones are the predominant flavonoid in soybean seeds, other flavonoids are present in different soybean tissues [10].

Research by Graham [10] has shown that soybean leaf tissue undergoes a programmed shift from isoflavone to flavonol metabolism 3 days after germination and mature soybean leaves are composed mainly of the glycosides of kaempferol, quercetin, and isorhamnetin. These flavonoids serve several functions within the plant and may have agricultural value. Flavonols and flavones have been shown to exhibit estrogenic and antiestrogenic ac-

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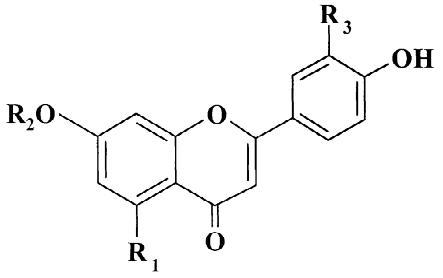
tivities in vitro and may be beneficial to human health [11]. Also, several flavonoids have been shown to inhibit the growth of fungi [12,13] and have been indicators of seed maturity [14,15], further enhancing their value for agricultural use. Although the flavonoid composition of soybean leaves and seeds has been well characterized, the flavonoid composition of soybean pods has not been determined.

The purpose of this study was to identify flavonoid (flavone) aglycones and major flavone glycosides in soybean pods using high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Fig. 1 lists the seven flavone aglycones and glycosides discussed in this report. Several HPLC and MS methods have been developed to analyze flavonoids in both soybean plant tissues and other soybean-derived foods [10,16–25]. To facilitate the identification of flavone aglycones and glycosidic conjugates, we have taken advantage of the ability of atmospheric pressure chemical ionization (APCI) to introduce flavones directly into the mass spectrometer. Positive ion APCI mass spectra detailed protonated molecules as base peaks with little fragmentation. Tandem mass spectrometry (MS–MS) was utilized for structural elucidation of flavone aglycones. Using a method called source-induced dissociation, increasing fragmentations in the source region, assisted in the identification of flavone glycosides. Triple mass spectrometry (MS^3) was utilized to confirm the structure of each flavone glycoside. UV–Vis spectra were obtained using photodiode-array detection and aided in flavone characterization.



Apigenin (**7**): R_1=OH, R_2=H, R_3=H, M_r=270
Apigenin-7-*O*-β-D-glucoside (**3**): R_1=OH, R_2=glucosyl, R_3=II, M_r=432
Apigenin-7-*O*-β-D-glucoside-6"-*O*-malonate (**5**): R_1=OH, R_2=6"-*O*-malonylglucosyl, R_3=H, Mr=518
7,4'-Dihydroxyflavonc (**4**): R_1=H, R_2=H, R_3=II, M_r=254
7,4'-Dihydroxyflavone-7-*O*-β-D-glucoside (**1**): R_1=H, R_2=glucosyl, R_3=H, M_r=416
Lutcolin (**6**): R_1=OII, R_2=II, R_3=OII, M_r=286
Lutcolin-7-*O*-β-D-glucoside (**2**): R_1=OII, R_2=glucosyl, R_3=OII, M_r=448

Fig. 1. Structures of the flavone aglycones and glycosides in soybean pod extract.

2. Materials and methods

2.1. Chemicals

Apigenin, 7,4′-dihydroxflavone, luteolin, apigenin-7-*O*-β-D-glucoside, and luteolin-7-*O*-β-D-glucoside were obtained from Indofine (Somerville, NJ, USA). The solvents acetonitrile (HPLC grade) and ethanol were purchased from Aldrich (Milwaukee, WI, USA). Water treated with a Millipore system was used during sample preparation procedures and HPLC analyses.

2.2. Soybean pod sample preparation

Soybean pods (*Glycine max* ''Tomahomare'') were harvested at the Southern Regional Research Center in New Orleans in 2000. Flavones were extracted from soybean pods (0.3-0.6 g) homogenized (Tekmar Tissuizer) in 80% aqueous ethanol (1.5 ml). Homogenate was heated at 50 °C for 1 h, cooled, and centrifuged at 14 000 *g* for 10 min. An aliquot (100 μl) of supernatant was analyzed by HPLC.

2.3. Chromatography system for HPLC–UV–Vis and HPLC–APCI-MS

For both UV-photodiode array detection and MS, HPLC analyses were performed on a Waters 600E System Controller combined with a Waters UV–Vis 996 detector (200–400 nm) following established methods [23]. Separations were carried out using a Vydac Multiring C_{18} (250×4.6 mm; 5 μm) reversed-phase column. A guard column containing the same packing material was used to protect the analytical column. Elution was carried out at a flow-rate of 1.0 ml/min with the following solvent system: A=acetic acid–water (pH 3.0), B=acetonitrile; 5% B to 65% B in 90 min, then 65% B to 100% B in 10 min followed by holding at 100% B for 5 min.

2.4. HPLC–APCI-MS analysis

The mass spectrometer utilized was a Finnigan MAT LCQ ion trap (San Jose, CA, USA) equipped with a heated nebulizer atmospheric pressure chemical ionization interface. HPLC effluent at 1 ml/min was introduced directly into the interface without splitting using a source temperature of 500 °C. Positive ion mode was used with a sprayer needle voltage of 4 kV. The capillary temperature was 210 °C. The full scan mass spectra of the flavones from m/z 100–1000 were measured using 500 ms for collection time and three micro scans were summed. Tandem mass spectrometry was performed at a collision energy of 23% for MS–MS analyses. For MS^3 of flavone glycosides, the collision energy was set to 19% during first stage of MS–MS, and set to 23% for second stage of MS–MS analysis. During the initial stages of analyses, the instrument was set to measure total ion chromatograms (TICs) in full scan MS mode to measure protonated $(M+H)^+$ ions. Second, full scan source-induced dissociation was used to screen the pod extract for 7,4′-dihydroxflavone (m/z 255), apigenin (m/z 271), and luteolin (m/z 287). Third, MS–MS was used to characterize both flavone aglycones and glycosides. Lastly, MS^3 was utilized to fragment flavone glycosides to their corresponding aglycone, and further product ion mass spectra from the aglycone were matched to standard flavones.

3. Results and discussion

3.1. Identification of the flavone aglycones

The UV–Vis spectra of flavones exhibit two major absorption peaks: band I (usually 300–380 nm) and band II (usually 240–280 nm) [26]. HPLC and total ion chromatograms of the soybean pod extract are shown in Fig. 2 using the band II absorption at 260 nm. Several components are clearly shown which appear to be flavonoids. UV–Vis spectra confirm the identity of these components as flavones due to the intense band I absorption at 330–350 nm. To further characterize individual components of the extract, MS and MS–MS spectra were obtained. In Fig. 3A, the extracted ion chromatogram for the ion m/z 271 is shown detailing a single intense peak at 59.7 min. A mass spectrum of the peak at 59.7 min is shown in Fig. 3B detailing a base peak at m/z 271. The MS–MS spectrum of m/z 271 (Fig. 3C) matched the fragmentation spectrum of apigenin. Using these techniques it was possible to identify three flavone aglycones in soybean pod extract. The retention time, UV λ_{max}, and $[M+H]^+$ values and the identifications for individual peaks are listed in Table 1. Using standards for each identified flavone aglycone, retention times, UV–Vis spectra, and MS–MS spectra matched each flavone aglycone from soybean pod extract.

3.2. Identification of flavone glycosides from soybean pod

Source-induced dissociation was used to screen the soybean pod extract for flavone glycosides. By inducing fragmentation within the source region of the mass spectrometer, all characteristic aglycone ions of each flavone glycoside are clearly detailed showing a characteristic loss of 162 u indicative of a hexose sugar. In Fig. 4A, the extracted ion chromatogram of m/z 255 shows the 7,4′-dihydroxflavone aglycone at 49.9 min (peak 4) and one prominent glycoside of 7,4′-dihydroxflavone (m/z 417 from MS spectrum) at a retention time of 36.8 min (peak 1). In Fig. 4B, the apigenin aglycone appears at 59 min (peak 7) and two glycosides of apigenin (peak 3—m/z 433 at 43.5 min and peak 5—m/z 519 at 49.9 min) are shown in the extracted ion chromatogram of m/z 271. Fig. 4C shows the luteolin aglycone at 53.3 min (peak 6) and one glycoside of luteolin (peak 2—m/z 449 at 40.1 min) using the extracted ion chromatogram of m/z 287.

MS and MS–MS spectra were utilized to further characterize each flavone glycoside in the soybean pod extract. In Fig. 5A, the MS spectrum of the glycoside of apigenin at 43.5 min (peak 3) shows an intense molecular ion $[M+H]^+$ at m/z 433. The MS–MS spectrum in Fig. 5B confirms the loss of 162 u that is indicative of a hexose sugar. In order to confirm the structure of the ion at m/z 271 as that of apigenin an MS^3 experiment was performed. Fig. 5C details the MS^3 spectrum of m/z 433>271>. The progeny ions found at m/z 247, 229, 225, 171, 153, 145, and 119 are identical to that of apigenin. The

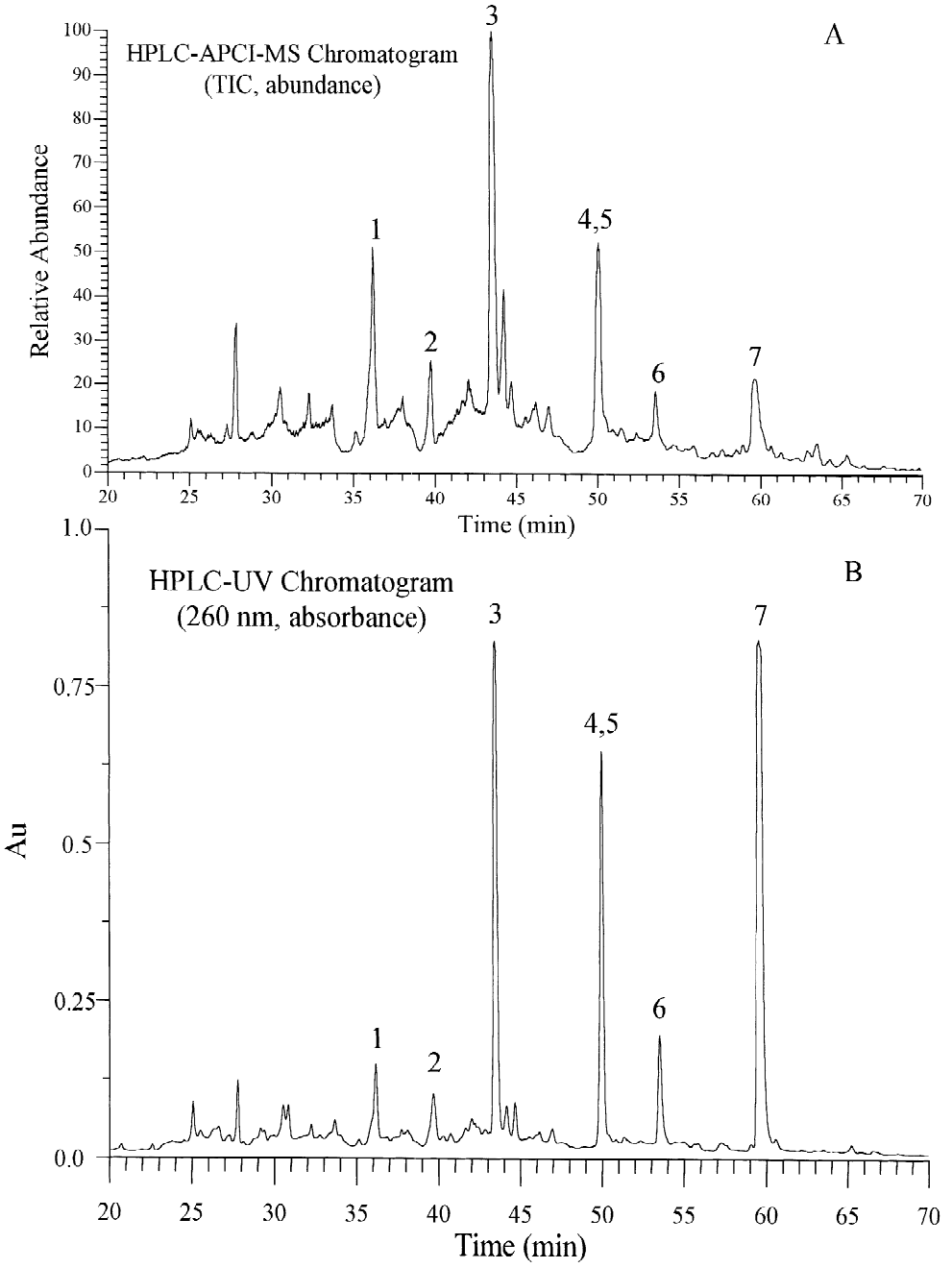


Fig. 2. Simultaneous HPLC–APCI-MS (A) and HPLC–UV (B) chromatograms of the soybean pod extract. Chromatographic conditions described in Section 2. The t_R value, MS, and UV λ_{max} of each compound are listed in Table 1.

structure for the flavone glycoside was identified as apigenin-7-*O*-β-D-glucoside. Using these techniques it was possible to unambiguously identify two flavone glycosides as apigenin-7-*O*-β-D-glucoside and luteolin-7-*O*-β-D-glucoside in the soybean pod extract using authentic standards for comparison.

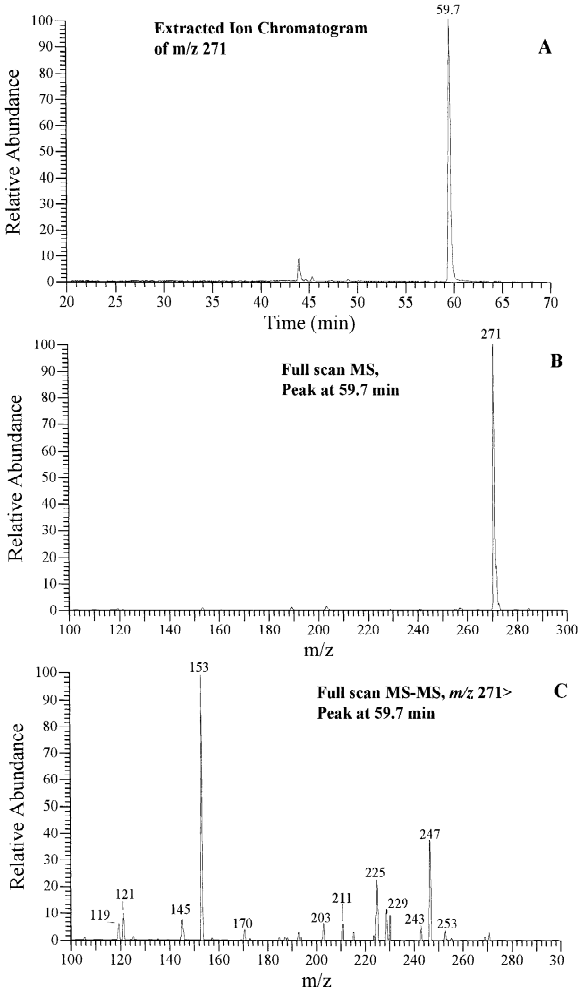


Fig. 3. Identification of apigenin aglycone from soybean pod extract. (A) HPLC–APCI-MS extracted ion chromatogram for the apigenin $[M+H]^+$ ion at m/z 271, (B) full scan MS spectrum of the peak at 59.7 min, (C) fragmentation spectrum (MS–MS) of the $[M+H]^+$ ion at m/z 271.

The tentative identification of two flavone glycosides as 7,4′-dihydroxyflavone-7-*O*-β-D-glucoside and apigenin-7-*O*-β-D-glucoside-6″-*O*-malonate is based on UV spectra, $(M+H)^+$ ions, and tandem mass spectrometry. The retention time, $[M+H]^+$ ions, major fragment ions (MS–MS), match of the MS^3 with aglycone standard MS–MS, and identifications are listed in Table 2.

As shown in Table 2, the predominant flavone glycosides identified in the soybean pod extract were the 7-*O*-β-D-glucosides of luteolin, 7,4′-dihydroxflavone, and apigenin. Also tentatively identified in soybean pod extract was apigenin-7-*O*-β-D-glucoside-6″-*O*-malonate with the $[M+H]^+$ ion at m/z 519 (peak 5). As shown in Table 2 (for the glycosides) the $[M+H]^+$ ion for apigenin-7-*O*-β-D-glucoside-6″-*O*-malonate is 86 u higher than its glycoside at m/z 433, which is characteristic for malonyl conjugates. Also shown are the similar UV spectra of the malonate and the glycoside of apigenin. This same process is currently being used to screen soybean pod extracts for malonates of luteolin and 7,4′-dihydroxflavone.

4. conclusions

HPLC–APCI-MS and MS–MS are valuable tools in the structural characterization of soybean pod flavone aglycones and glycosides. APCI-MS is considered a ‘‘soft’’ ionization technique and provides useful information on molecular ions present. APCI-MS–MS provides additional fragmentation spectra which assists in the unambiguous determination of structure, particularly in the characterization of flavone aglycones. Source induced dissociation pro-

Table 1
Flavone aglycones identified in soybean pod extracts based on UV λ_{max}, m/z values of $[M+H]^+$ ions, MS–MS match with aglycone standard MS–MS

Peak	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$ (m/z)	MS–MS match with the aglycone standard MS–MS	Identification
4	49.9	253sh, 312sh, 328	255	+	7,4′-Dihydroxyflavone
6	53.3	253, 266sh, 347	287	+	Luteolin
7	59.7	267, 336	271	+	Apigenin

sh, Shoulder.

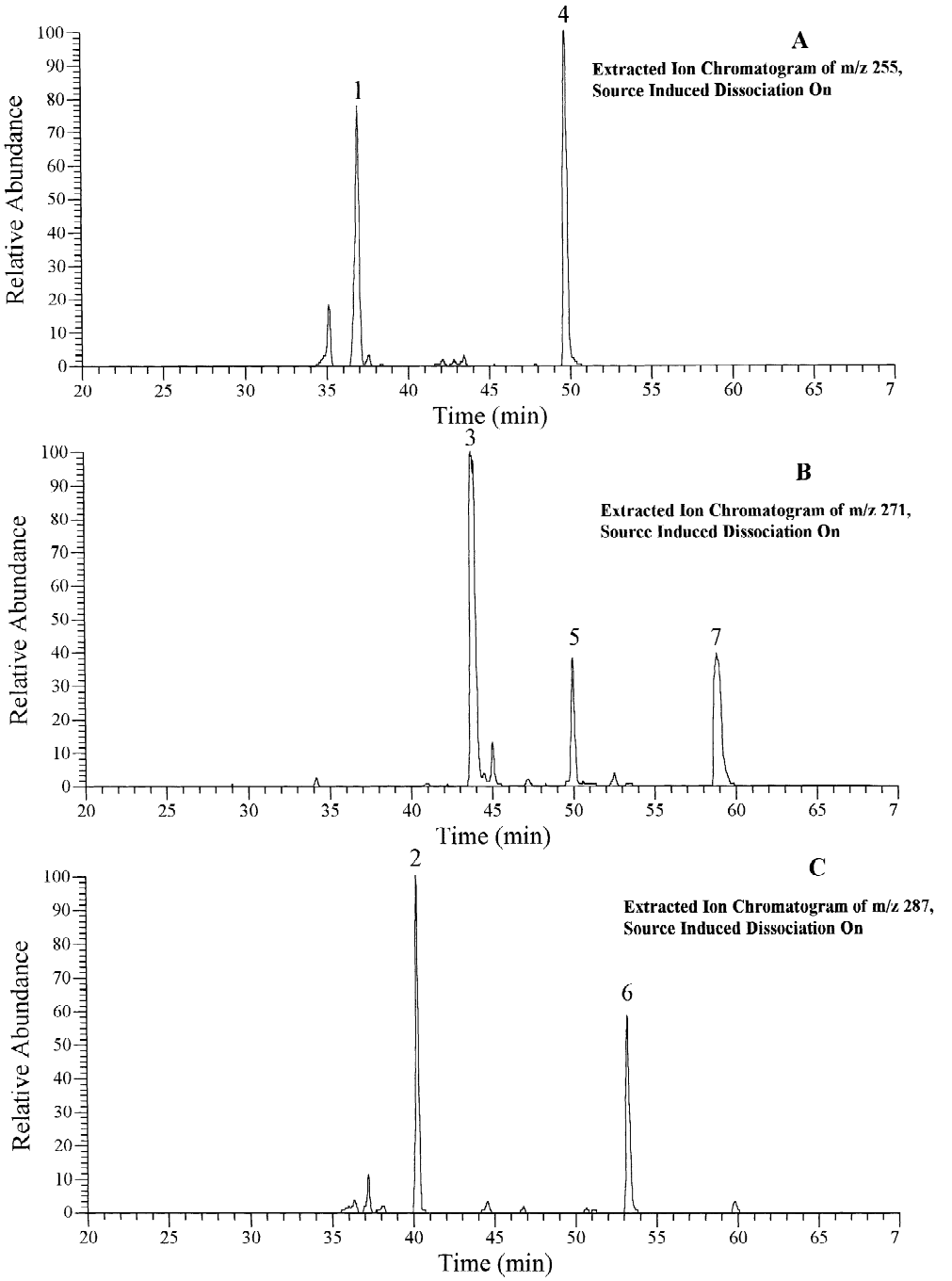


Fig. 4. Screening the soybean pod extract for flavone glycosides using source-induced dissociation. (A) Extracted ion chromatogram using source-induced dissociation for the $[M+H]^+$ ion at m/z 255 (7,4′-dihydroxyflavone), (B) extracted ion chromatogram using source-induced dissociation for the $[M+H]^+$ ion at m/z 271 (apigenin), (C) extracted ion chromatogram using source-induced dissociation for the $[M+H]^+$ ion at m/z 287 (luteolin).

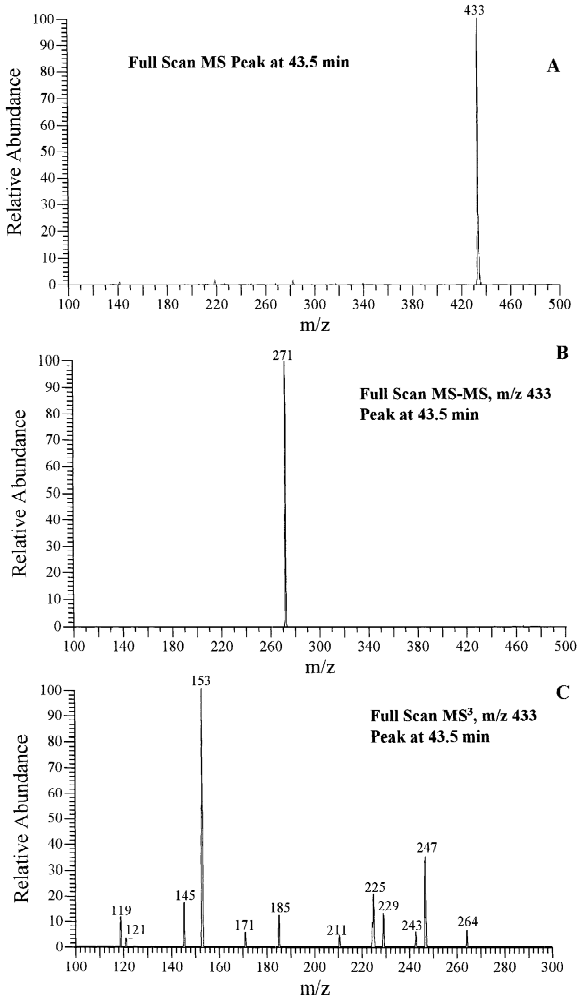


Fig. 5. Identification of apigenin-7-*O*-β-D-glucoside from soybean pod extract. (A) Full scan MS spectrum of the peak at 43.5 min (Fig. 4), (B) full scan MS–MS spectrum of the $[M+H]^+$ ion at m/z 433, (C) full scan MS^3 spectrum of the $[M+H]^+$ ion at m/z 271 (433>271>).

vides a screening technique whereby the glycosides of each flavone present are easily identified. To further characterize each flavone glycoside, the MS^3 spectrum of each glycoside provides an unambiguous method to elucidate structure. HPLC–APCI-MS can be easily combined with photodiode array detection under the same conditions and the UV–Vis spectra obtained provide additional confirmatory data particularly useful for soybean pod flavone identification.

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Table 2
Flavone glycosides identified in soybean pod extracts based on m/z values of $[M+H]^+$ ions (MS), major fragment ions (MS–MS), and the match of the MS^3 with the aglycone standard MS–MS

Peak	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$ (m/z)	Y_1^+ in MS–MS	MS^3 match with aglycone standard MS–MS	Identification
1	36.3	253sh, 312sh, 328	417	255	+	7,4′-Dihydroxyflavone-7-*O*-β-D-glucoside
2	39.8	253, 266sh, 347	449	287	+	Luteolin-7-*O*-β-D-glucoside
3	43.5	267, 335	433	271	+	Apigenin-7-*O*-β-D-glucoside
5	50.1	267, 336	519	271	+	Apigenin-7-*O*-β-D-glucoside-6″-*O*-malonate

sh, Shoulder.

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