Isoflavones and Their Conjugates in Soy Foods: Extraction Conditions and Analysis by HPLC–Mass Spectrometry

Stephen Barnes,*,†,‡,§ Marion Kirk,§ and Lori Coward†

Departments of Pharmacology and Biochemistry and UAB Comprehensive Cancer Center Mass Spectrometry Core Facility, University of Alabama at Birmingham, Birmingham, Alabama 35294

Identification of isoflavone glycosidic conjugates from several soy products was carried out by HPLC–mass spectrometry. Positive ion mass spectra obtained using the heated nebulizer–atmospheric pressure chemical ionization interface gave the most sensitive and structurally useful information about each isoflavone conjugate. Although extraction of isoflavones from soy products with 80% aqueous methanol at room temperature was just as efficient as at 60–80 °C, extraction at higher temperatures caused changes in isoflavone composition and should be avoided. Soybeans and defatted soy flour (which had been minimally heated during their preparation) contained mostly isoflavone 6″-*O*-malonylglucoside conjugates, with lesser quantities of the β-glucosides and only trace amounts of 6″-*O*-acetylglucoside conjugates. Soy milk, tofu, and soy molasses, each of which involves heating to 100 °C during their manufacture, contained mostly isoflavone β-glucosides. Toasted soy flour and an isolated soy protein had moderate amounts of each of the isoflavone conjugates.

Keywords: *Isoflavones; mass spectrometry; electrospray ionization; atmospheric pressure chemical ionization; high-pressure liquid chromatography; soy food materials*

INTRODUCTION

The isoflavone genistein (5,7,4′-trihydroxyisoflavone) has attracted a great deal of recent interest because of its property as an *in vitro* inhibitor of protein tyrosine kinases (Akiyama et al., 1987), many of which form part of growth factor-stimulated signal transduction cascades in normal and transformed cancer cells (Peterson and Barnes, 1993). Several groups have suggested that genistein is the agent in soy which accounts for the apparent association between increases in soy food consumption and reduction of cancer risk (Setchell et al., 1984; Barnes et al., 1990; Adlercreutz et al., 1991; Messina and Barnes, 1991).

In soybeans and foods derived from soy, isoflavones are found in concentrations ranging from 0.1 to 5 mg/g (Coward et al., 1993). Given their high molar absorbance values, relatively straightforward reversed-phase HPLC methods have been used for analysis of isoflavones (Eldridge, 1982; Farmakalidis and Murphy, 1985; Setchell et al., 1987; Kudou et al., 1991; Coward et al., 1993). Typically the isoflavones have been extracted from soy with a hot aqueous polar solvent such as methanol or acetonitrile, either by simple mixing or by Soxhlet extraction. These methods led to the identification of the aglucones and the β-glucoside conjugates as the principal components of the extracts. However, other isoflavone glycosidic conjugates are present in soy. Farmakalidis and Murphy (1985) identified isoflavone 6″-*O*-acetylglucosides (6-OAcGlc) in toasted soy flakes (Figure 1). These authors suggested that the much higher content of these conjugates in 80% aqueous acetonitrile extracts compared with 80% aqueous methanol extracts was a function of the greater solubilizing power of 80% aqueous acetonitrile. In addition, Kudou et al. (1991) reported that in the soybean hypocotyl and cotyledon the isoflavones were predominantly in the form of 6″-*O*-malonylglucoside (6-OMalGlc) conjugates (Figure 1). They suggested that hot aqueous alcohol extraction caused these conjugates to undergo heat-induced de-esterification to form β-glucosides.

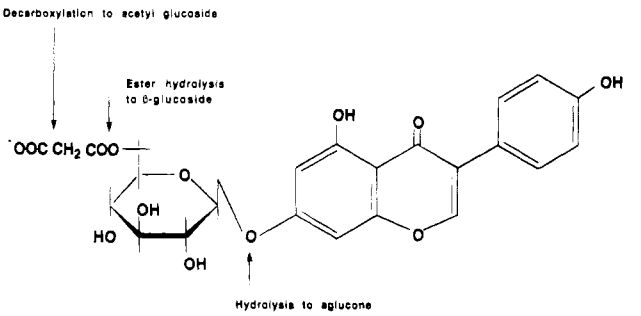

Figure 1. Chemical structure of genistein 6″-*O*-malonylglucoside. The sites of chemical modification to yield the 6″-*O*-acetylglucoside and the unesterified β-glucoside are marked with arrows.

Since many investigators are currently using soy food products in clinical and experimental studies to explore the relationship between soy consumption and reduction in risk of cardiovascular disease (Bakhit et al., 1994; Potter et al., 1994) and cancer, it is crucial to determine the qualitative and quantitative composition of isoflavones in selected soy foods.

In this study we have examined the extraction of isoflavones from several commercially available soy food materials with particular reference to the effect of heating during this process. To facilitate the identification of the isoflavone conjugates in the extracts, we have taken advantage of the ability of heated nebulizer–atmospheric pressure chemical ionization (HN–APCI)

* Address correspondence to this author the Department of Pharmacology, University of Alabama at Birmingham, Birmingham, AL 35294-0019 [telephone (205) 934-7117; fax (205) 934-8240].

† Department of Pharmacology.

‡ Department of Biochemistry.

§ UAB Comprehensive Cancer Center Mass Spectrometry Core Facility.

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Table 1. Effect of Mixing Time and Heat on the Extraction of Isoflavones[a] from Soy Milks

	room temperature			60 °C		
	1 h	2 h	24 h	1 h	2 h	4 h
soy milk 1						
daidzin	54.7 ± 2.3	53.1 ± 0.7	51.1 ± 3.6	52.6 ± 1.1	53.2 ± 0.2	54.1 ± 0.7
daidzein	3.7 ± 0.5	3.3 ± 0.45	2.6 ± 0.2	3.8 ± 1.3	3.1 ± 0.5	3.0 ± 0.4
genistin	89.6 ± 2.0	86.0 ± 1.5	84.1 ± 4.8	87.8 ± 1.5	86.3 ± 1.1	87.1 ± 0.3
genistein 6-OMalGlc	18.4 ± 1.0	18.2 ± 1.0	16.6 ± 1.1	16.3 ± 1.0	16.1 ± 1.0	15.1 ± 0.7
genistein	3.4 ± 0.9	3.6 ± 0.5	2.8 ± 0.4	5.4 ± 2.0	3.2 ± 0.5	3.1 ± 0.6
glycitin	3.6 ± 0.7	3.5 ± 0.3	3.2 ± 0.5	3.5 ± 0.3	3.8 ± 0.1	3.8 ± 0.4
total	173 ± 7	168 ± 3	160 ± 10	169 ± 5	166 ± 1	166 ± 2
soy milk 2						
daidzin	31.9 ± 2.2	32.5 ± 3.3	32.0 ± 0.6	30.7 ± 0.6	31.0 ± 1.6	30.7 ± 2.0
daidzein	2.1 ± 1.1	1.2 ± 1.1	0.9 ± 0.9	1.6 ± 0.2	0.7 ± 1.2	nd[b]
genistin	50.2 ± 2.0	50.6 ± 3.7	50.1 ± 0.4	49.0 ± 1.5	49.3 ± 1.3	51.8 ± 4.4
genistein 6-OMalGlc	6.4 ± 0.9	7.0 ± 2.2	6.5 ± 1.8	5.6 ± 0.2	7.3 ± 2.0	6.7 ± 0.6
genistein	1.5 ± 0.7	1.5 ± 0.4	1.3 ± 0.2	0.8 ± 0.7	1.9 ± 0.7	1.2 ± 0.1
glycitin	4.4 ± 0.8	4.6 ± 0.9	4.5 ± 0.7	4.0 ± 0.1	4.2 ± 0.6	4.2 ± 0.6
total	96.5 ± 7.6	97.4 ± 9.5	95.2 ± 2.2	91.8 ± 1.9	94.4 ± 5.2	94.6 ± 6.7

[a] Micrograms per gram. Mean ± SD of triplicate measurements. [b] nd, not detected.

Table 2. Effect of Mixing Time and Heating to 60 °C on the Extraction of Isoflavones[a] from a Soy Protein Isolate

	room temperature			60 °C		
isoflavone	1 h	2 h	24 h	1 h	2 h	4 h
daidzin	168 ± 5	166 ± 6	181 ± 3	181 ± 1[b]	189 ± 6[c]	199 ± 1[d]
daidzein 6-OMalGlc	189 ± 5	183 ± 4	181 ± 3	174 ± 2[b]	166 ± 3[c]	161 ± 5[d]
daidzein 6-OAcGlc	52 ± 3	52 ± 2	51 ± 1	52 ± 2	51 ± 2	52 ± 1
daidzein	52 ± 5	58 ± 7	57 ± 3	56 ± 13	66 ± 5	66 ± 5
genistin	289 ± 7	288 ± 12	314 ± 8	320 ± 3[b]	335 ± 5[c]	357 ± 4[d]
genistein 6-OMalGlc	392 ± 10	397 ± 2	383 ± 11	369 ± 4[b]	357 ± 8[c]	341 ± 9[d]
genistein 6-OAcGlc	80 ± 6	81 ± 4	81 ± 3	80 ± 1	82 ± 5	84 ± 3
genistein	49 ± 7	45 ± 11	43 ± 3	56 ± 10[b]	59 ± 4[c]	55 ± 9[d]
glycitin	53 ± 4	57 ± 9	58 ± 7	55 ± 1	59 ± 3	59 ± 1
glycitein 6-OMalGlc	42 ± 2	43 ± 1	38 ± 3	41 ± 2	38 ± 3	34 ± 1
total	1365 ± 50	1371 ± 38	1387 ± 6	1385 ± 26	1402 ± 29	1408 ± 18

[a] Micrograms per gram. Mean ± SD of triplicate measurements. [b] Significantly different ($p < 0.05$) from 1 h room temperature extraction. [c] Significantly different ($p < 0.05$) from 2 h room temperature extraction. [d] Significantly different ($p < 0.05$) from 24 h room temperature extraction.

Table 3. Extraction of Isoflavone Conjugates[a] from Toasted Soy Flour with 80% Aqueous Methanol or 80% Aqueous Acetonitrile–HCl

isoflavone	80% methanol	80% acetonitrile–HCl
daidzin	406 ± 17	394 ± 11
daidzein 6-OMalGlc	260 ± 12	243 ± 11
daidzein 6-OAcGlc	223 ± 8	227 ± 12
genistin	489 ± 20	484 ± 3
genistein 6-OMalGlc	318 ± 12	314 ± 12
genistein 6-OAcGlc	181 ± 3	186 ± 12
glycitin	98 ± 3	101 ± 3
glycitein 6-OMalGlc	85 ± 3	82 ± 5
total daidzein conjugates	890 ± 36	863 ± 34
total genistein conjugates	988 ± 35	984 ± 25
total glycitein conjugates	186 ± 6	182 ± 8
total isoflavones	2061 ± 76	2030 ± 66

[a] Micrograms per gram. Mean ± SD of triplicate measurements.

and modified electrospray (ESI) interfaces to introduce the isoflavone conjugates directly into the mass spectrometer without the necessity of hydrolysis or derivatization.

MATERIALS AND METHODS

Materials. Soybeans and EdenSoy soy milk were obtained from a National food store chain specializing in vegetable products. Hypocotyls were separated from the cotyledons by hand. Defatted soy flour and an isolated soy protein (Supro) were kindly donated by Protein Technologies International, St. Louis, MO. Soy molasses and toasted soy flour were provided by the Archer Daniels Midland Co., Decatur, IL, and tofu was from Morinaga Nutritional Foods, Inc., Torrance, CA.

HPLC grade methanol and acetonitrile and the disodium salt of fluorescein were used without further purification. Double-distilled water used was filtered through a 0.45 μm pore size Superpore nylon membrane.

Daidzein and genistein and their β-glucoside conjugates were isolated and purified as described elsewhere (Walter, 1941; Coward et al., 1993).

Extraction of Isoflavones from Soy Foods. Portions of soy material (500 mg) were mixed with 4 mL of either 80% aqueous methanol or 80% aqueous acetonitrile–0.1% HCl (v/v), and the internal standard fluorescein (1 mg, as a concentrate in the same solvent) was added to each. The mixtures were then placed in a tumbling mixer for 1, 2, and 24 h at room temperature (22 °C). A second set of samples were extracted at 60 °C in a water bath for 1, 2, and 4 h. Aliquots of each mixture were clarified by centrifugation at 14000g in an Eppendorf centrifuge prior to reversed-phase HPLC analysis. In another set of experiments, extraction was carried out at 80 °C for periods of up to 4 h.

HPLC Analysis. Reversed-phase HPLC analysis of isoflavones was carried out on a 25 cm × 4.6 mm Aquapore C_8 column (Applied Biosystems, Foster City, CA). Elution was carried out at a flow rate of 1.5 mL/min using a solvent gradient consisting of a linear increase from 0 to 50% of acetonitrile (solvent B) in water in a background of either 0.1% trifluoroacetic acid or 2 or 10 mM ammonium acetate (solvent A) over periods ranging from 10 to 30 min, followed by 100% solvent B for 5 min. The column was equilibrated in solvent A prior to chromatography. Eluted isoflavones were detected

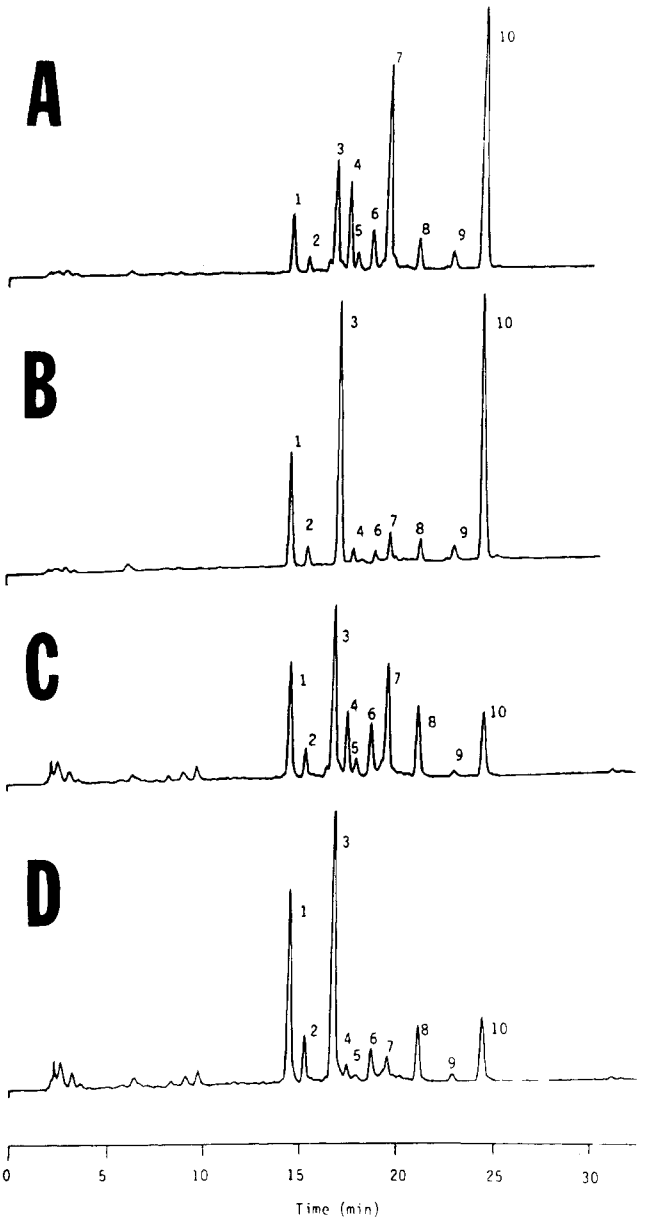

Figure 2. Reversed-phase HPLC analysis of isoflavones in soy protein isolate (A, B) or toasted soy flour (C, D) extracted with 80% aqueous methanol at room temperature (A, C) or at 80 °C for 4 h (B, D). Peak identification: 1, daidzin; 2, glycitin; 3, genistin; 4, daidzein 6-OMalGlc; 5, glycitein 6-OMalGlc; 6, daidzein 6-OAcGlc; 7, genistein 6-OMalGlc; 8, genistein 6-OAcGlc; 9, genistein; 10, fluorescein (internal standard). Analyses were performed on a 25 cm × 4.6 mm C_8 Aquapore column using a linear elution gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid over 30 min at a flow rate of 1.5 mL/min. Isoflavones were detected by their absorbance at 262 nm.

by their absorbance at 262 nm. Quantitative data for daidzein, daidzin, genistein, and genistin were obtained by comparison to known standards. Since Kudou et al. (1991) have shown that the molar extinction coefficients of the daidzein and genistein 6-OMalGlc conjugates approximated those of daidzin and genistin, respectively, the concentrations of the 6-OMalGlc and 6-OAcGlc conjugates were calculated from standard curves for the corresponding β-glucoside. Since pure standards were not available, concentrations of glycitein were calculated from the daidzein standard curve and the concentrations of the glycitein 6-OMalGlc and 6-OAcGlc conjugates from the daidzin standard curve. In all cases, the concentrations reported in the Tables 1–3 are for the equivalent unconjugated aglucones, thereby permitting comparison of the isoflavone content independently of the chemical form(s) present.

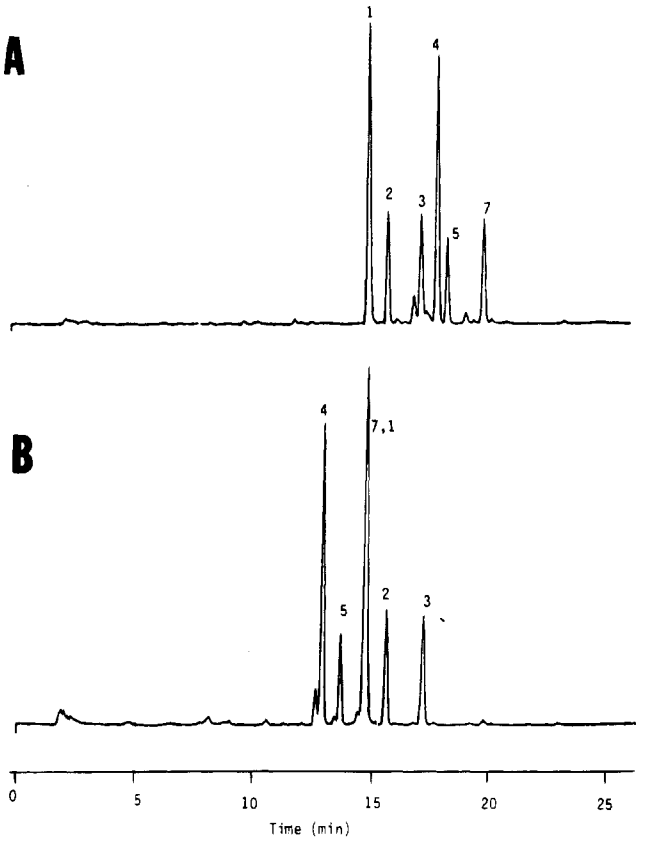

Figure 3. Effect of replacing 0.1% trifluoroacetic acid (A) with 10 mM ammonium acetate (B) on chromatographic separation of isoflavone conjugates from soy hypocotyl by reversed-phase HPLC. Other conditions and peak identification are as in Figure 2.

Mass Spectrometry Analysis. Analyses were performed on an API III triple-quadrupole mass spectrometer (PE-Sciex, Thornhill, ON) equipped with two MacIntosh Quadra 950 computers for data analysis. The isoflavones in the soy extracts were separated by reversed-phase HPLC on a 10 cm × 4.6 mm Aquapore C_8 column at a flow rate of 1.0 mL/min using a linear 0–50% gradient of acetonitrile (5%/min) in 0.1% acetic acid or 10 mM ammonium acetate. Positive and negative ions from eluted solutes were introduced into the mass spectrometer following their generation by atmospheric pressure chemical ionization caused by a corona discharge needle in the heated nebulizer interface of this instrument.

Isoflavone conjugates were also separated by reversed-phase HPLC on a 10 cm × 2.1 mm Aquapore C_8 column at a flow rate of 0.2 mL/min, using a 0–50% acetonitrile gradient (5%/min) in aqueous 2 mM ammonium acetate. The column eluate was split 1:1, with 100 μL/min going to the IonSpray interface. Positive and negative ion mass spectra were recorded in this mode, with orifice potentials of 70 and −60 V, respectively.

RESULTS

Extraction Conditions. Maximum recovery of the isoflavones from soy milk and the isolated soy protein with 80% aqueous methanol sufficient for reproducible quantitative measurements was obtained by tumbling for 2 h (Tables 1 and 2). Furthermore, for both soy products, there were no significant differences in overall recovery of isoflavones when extraction was performed at room temperature as opposed to 60 °C (Tables 1 and 2).

As noted previously (Coward et al., 1993), coefficients of variation obtained using the method described declined as the total isoflavone content rose (5.8, 2.8, and 2.0% for soy milk 2, soy milk 1, and isolated soy protein,

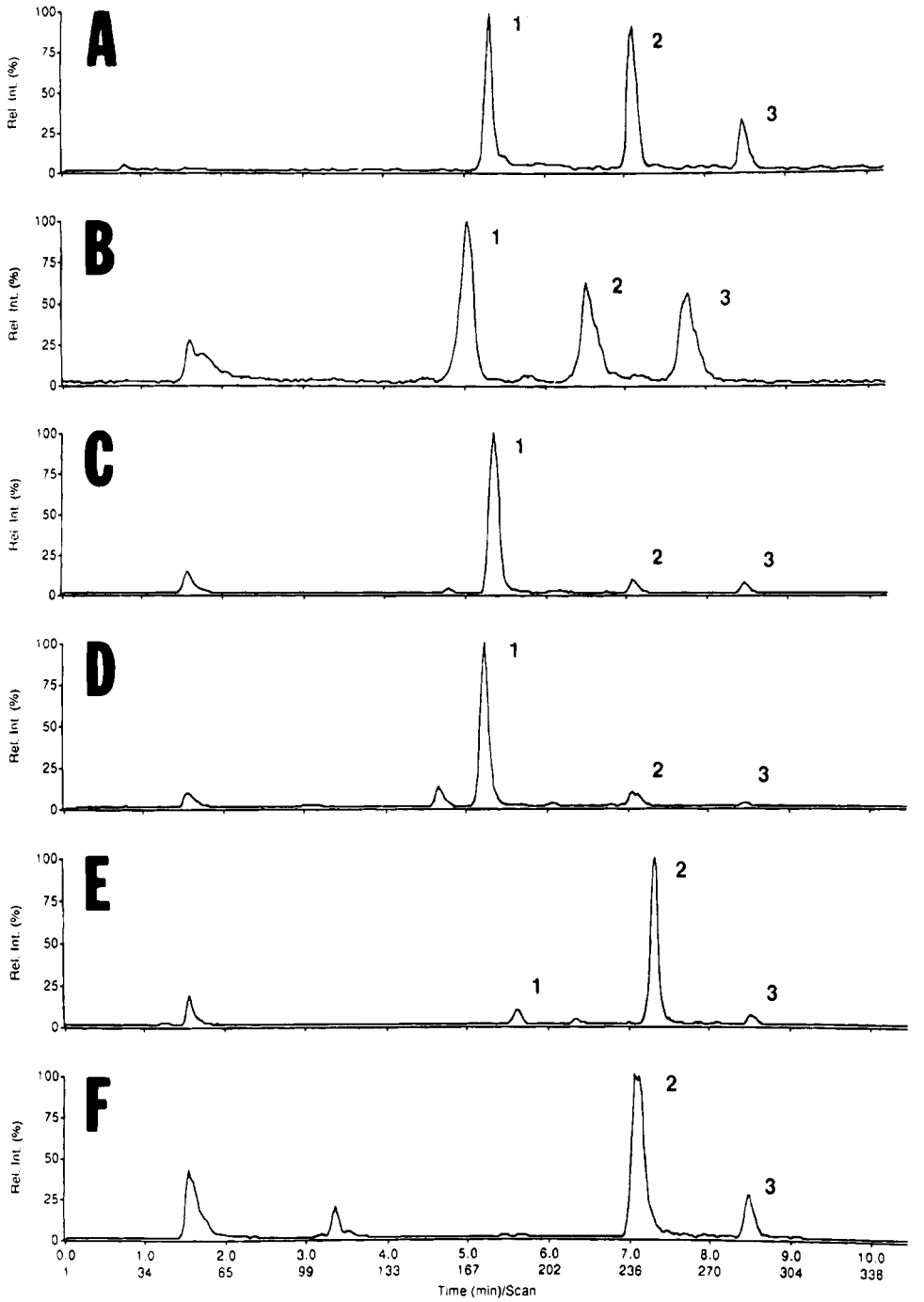

Figure 4. Selected positive ion chromatograms of genistein glycosidic conjugates in isolated soy protein (A), toasted soy flour (B), defatted soy flour (C), soybean hypocotyl (D), tofu (E), and soy molasses (F) following HPLC–APCI–HN–MS analysis on a 10 cm × 4.6 mm C_8 Aquapore reversed-phase column. The reconstructed ion chromatograms were obtained from the sum of the *m/z* 519, 475, and 433 ions. The mobile phase was a 0–50% acetonitrile gradient over 10 min in 2 mM ammonium acetate at a flow rate of 1 mL/min. Peak identification: 1, genistein 6-OMalGlc; 2, genistin; 3, genistein 6-OAcGlc. The individual analyses were performed over a 6 month period, accounting for the small differences in retention times, most noticeably in the case of toasted soy flour.

respectively). In addition, no differences were detected between the use of 80% aqueous methanol and 80% aqueous acetonitrile containing 0.1% HCl for the 2 h room temperature extraction of the total isoflavones in toasted soy flour (Table 3).

In the case of the soy milks, the isoflavones were present in the extracts almost entirely as their β-glucoside conjugates whether extracted at room temperature or at 60 °C. In contrast, for the isolated soy protein, although extraction at 60 °C did not change the total isoflavone concentration, it did cause significant changes in the composition of the isoflavone conjugates. At each point in the extraction, genistin and genistein concentrations were increased at the expense of genistein 6-OMalGlc (Table 2). Similar trends were also observed for daidzin, daidzein, and daidzein 6-OMalGlc. When the extraction temperature was increased to 80 °C, the conversion of the isoflavone 6-OMalGlc conjugates to the β-glucoside conjugates was much greater (Figure 2A,B) and was time-dependent. Heated extraction of toasted soy flour at 80 °C in 80% aqueous methanol also led to conversion of isoflavone 6-OAcGlc conjugates to their β-glucoside conjugates (Figure 2C,D). Even when kept at room temperature, isoflavones in 80% aqueous metha-

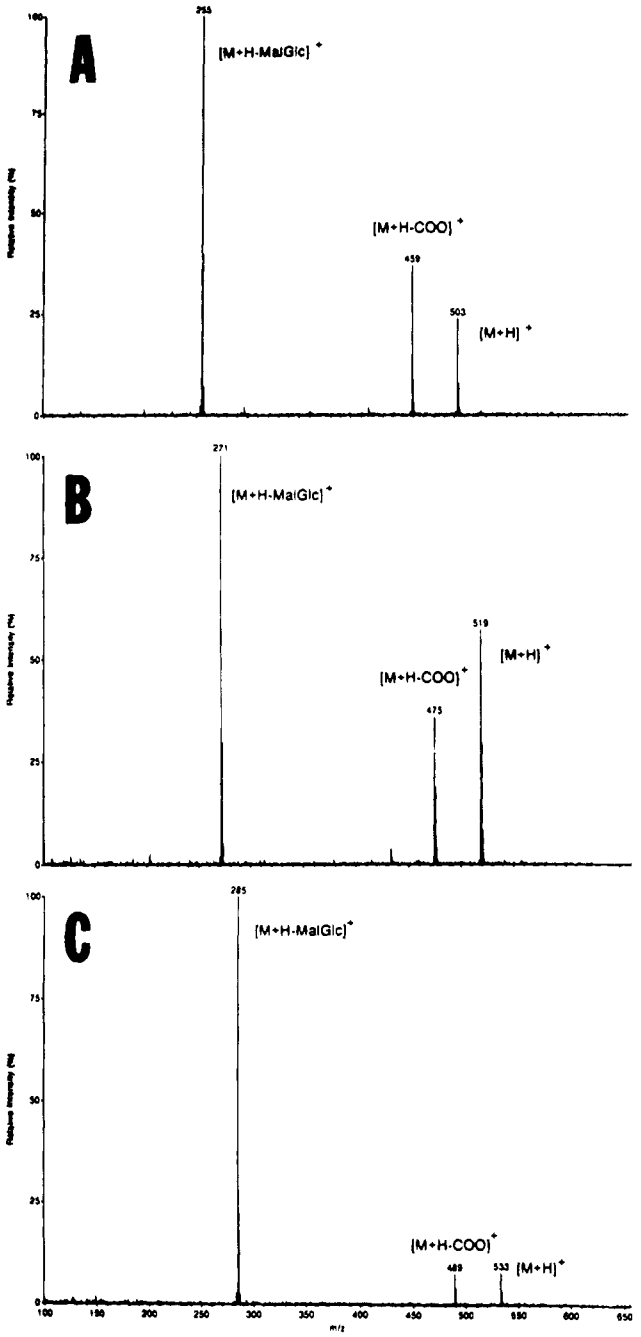

Figure 5. Positive ion mass spectra of daidzein (A), genistein (B), and glycitein (C) 6-OMalGlc conjugates in isolated soy protein separated by reversed-phase HPLC (see Figure 4).

nol extracts of the soy materials were converted gradually from the 6-OMalGlc forms to the β-glucosides (data not shown).

The use of acetic acid in the HPLC mobile phase (Kudou et al., 1991) did not lead to a significant improvement in resolution of the isoflavone conjugates compared to trifluoroacetic acid. When ammonium acetate was included in the aqueous buffer (as used for analysis by HPLC–MS), the elution volumes of the isoflavone 6-OMalGlc conjugates decreased sharply due to ionization of their carboxyl groups (Figure 3).

HPLC–Mass Spectrometry. When analyzed by HPLC–HN–APCI mass spectrometry in the positive ion mode, three different types of conjugates were identified for each isoflavone. In the case of defatted soy flour (Figure 4A) and soybean hypocotyls (Figure 4B), the principal form of genistein was the 6-OMalGlc conjugate. In contrast, in soy molasses (Figure 4C) and tofu (Figure 4D), the principal genistein conjugate was the β-glucoside. Toasted soy flour (Figure 4E) and isolated soy protein (Figure 4F) contained large amounts of the 6-OAcGlc conjugate. This pattern of distribution of conjugates was also the case for the other isoflavones in soy, daidzein (7,4′-dihydroxyisoflavone) and glycitein (7,4′-dihydroxy-6-methoxyisoflavone) (data not shown).

Table 4. Ions Observed for Isoflavone Conjugates by Atmospheric Pressure Chemical Ionization–Mass Spectrometry in the Negative Mode[a]

		relative abundance	
isoflavone	*m/z*	10 mM NH_4OAc	0.1% acetic acid
daidzein 6-OMalGlc	517 [M − COOH + CH_3COOH]⁻	36.5	66.7
	457 [M − COOH]⁻	14.2	32.6
	253 [M − H − MalGlc]⁻	100.0	100.0
daidzein 6-OAcGlc	517 [M − H + CH_3COOH]⁻	40.7	63.8
	457 [M − H]⁻	16.4	30.8
	253 [M − H − AcGlc]⁻	100.0	100.0
daidzin	475 [M − H + CH_3COOH]⁻	24.1	100.0
	415 [M − H]⁻	8.6	39.5
	253 [M − Glc]⁻	100.0	100.0
genistein 6-OMalGlc	533 [M − COOH + CH_3COOH]⁻	40.1	67.8
	473 [M − COOH]⁻	46.0	70.3
	269 [M − H − MalGlc]⁻	100.0	100.0
genistein 6-OAcGlc	533 [M − H + CH_3COOH]⁻	9.1	85.5
	473 [M − H]⁻	25.2	100.0
	269 [M − H − AcGlc]⁻	100.0	27.5
genistein	491 [M − H + CH_3COOH]⁻	25.5	69.2
	431 [M − H]⁻	28.1	100.0
	269 [M − H − Glc]⁻	100.0	94.2
glycitein 6-OMalGlc	547 [M − COOH + CH_3COOH]⁻	2.0	13.5
	487 [M − COOH]⁻	9.3	29.2
	283 [M − H − MalGlc]⁻	100.0	100.0
glycitein 6-OAcGlc	547 [M − H + CH_3COOH]⁻	17.5	100.0
	487 [M − H]⁻	7.7	49.3
	283 [M − H − AcGlc]⁻	100.0	34.8
glycitin	505 [M − H + CH_3COOH]⁻	5.1	82.6
	445 [M − H]⁻	1.8	29.7
	283 [M − H − Glc]⁻	100.0	100.0

[a] Mass spectra obtained during HPLC analysis (in a background of 10 mM ammonium acetate or 0.1% acetic acid) of an 80% aqueous methanol extract of toasted soy flour using the HN–APCI interface. Orifice potential was −60 V.

Isoflavone 6-OMalGlc Conjugates. The major ions in the HN–APCI positive ion mass spectrum of each isoflavone 6-OMalGlc conjugate when analyzed by HPLC in a background of 0.1% acetic acid (Figure 5) were the $[M + H]^+$ molecular ion, the $[M + H - COO]^+$ ion, and the $[M + H - MalGlc]^+$ aglucone ion. However, when analyzed by HPLC in a background of 10 mM ammonium acetate, the molecular ion was not observed.

In the negative ion mode, whether analyzed by HPLC in 0.1% acetic acid or in 10 mM ammonium acetate, the molecular $[M - H]^-$ ion for each isoflavone 6-OMalGlc was not observed. Instead, the $[M - MalGlc - H]^-$ aglucone ion was the principal ion. The other major ions were the $[M - COOH]^-$ ion and the [M − COOH + acetic acid]⁻ adduct ion (Table 4). At high orifice potential (−100 vs −60 V), the adduct ions were not detected.

Table 5. Ions Observed in Positive and Negative Ion Spectra of Isoflavone Conjugates Generated by Electrospray Ionization–Mass Spectrometry[a]

isoflavone	ion	abundance (%)	ion	abundance (%)
daidzein 6-OMalGlc	255 $[M + H - MalGlc]^+$	2.5	253 $[M - H - MalGlc]^-$	18.1
	503 $[M + H]^+$	100.0	457 $[M - H - COO]^-$	5.8
	536 $[M + NH_4]^+$	9.8	501 $[M - H]^-$	100.0
	541 $[M + K]^+$	44.1	561 $[M - H + CH_3COOH]^-$	9.6
	579 $[M + 2K]^+$	8.2		
daidzein 6-OAcGlc	255 $[M + H - AcGlc]^+$	14.6	457 $[M - H]$	100.0
	459 $[M + H]^+$	100.0	517 $[M - H + CH_3COOH]^-$	8.4
	497 $[M + K]^+$	17.7		
daidzin	255 $[M - Glc]^+$	17.1	253 $[M - H - Glc]$	6.4
	417 $[M + H]^+$	100.0	415 $[M - H]^-$	11.6
	455 $[M + K]^+$	29.2	475 $[M - H + CH_3COOH]^-$	100.0
genistein 6-OMalGlc	271 $[M + H - MalGlc]^+$	0.0	269 $[M - H - MalGlc]^-$	39.7
	519 $[M + H]^+$	100.0	473 $[M - H - COO]^-$	16.7
	536 $[M + NH_4]^+$	13.1	517 $[M - H]$	100.0
	557 $[M + K]^+$	44.6	577 $[M - H + CH_3COOH]^-$	7.9
	595 $[M + 2K]^+$	7.5		
genistein 6-OAcGlc	271 $[M + H - AcGlc]^+$	14.9	473 $[M - H]^-$	30.4
	475 $[M + H]^+$	100.0	533 $[M - H + CH_3COOH]^-$	100.0
	513 $[M + K]^+$	12.0		
genistin	271 $[M + H - Glc]^+$	12.0	269 $[M - H - Glc]^-$	5.3
	433 $[M + H]^+$	100.0	431 $[M - H]^-$	26.3
	471 $[M + K]^+$	28.3	491 $[M - H + CH_3COOH]^-$	100.0
glycitein 6-OMalGlc	285 $[M + H - MalGlc]^+$	6.2	283 $[M - H - MalGlc]^-$	28.3
	533 $[M + H]^+$	100.0	531 $[M - H]^-$	100.0
	550 $[M + NH_4]^+$	10.7	591 $[M - H + CH_3COOH]^-$	12.1
	571 $[M + K]^+$	43.4		
	609 $[M + 2K]^+$	6.7		
glycitein 6-OAcGlc	285 $[M + H - AcGlc]^+$	26.9	nd[b]	
	489 $[M + H]^+$	100.0	nd	
	527 $[M + K]^+$	19.8	nd	
glycitin	285 $[M + H - Glc]^+$	54.0	283 $[M - H - Glc]^-$	6.3
	447 $[M + H]^+$	100.0	445 $[M - H]^-$	7.2
	485 $[M + K]^+$	41.2	505 $[M - H^+ CH_3COOH]^-$	100.0

[a] Spectra were generated by carrying out HPLC–MS on an 80% aqueous methanol extract of toasted soy flour. Isoflavone conjugates were separated by a 0–50% acetonitrile gradient in 10 mM ammonium acetate using a 2.1 mm × 10 cm C_8 reversed-phase column. [b] nd, not detected.

Positive ion mass spectra for each isoflavone 6-OMalGlc obtained with the IonSpray interface showed that under these conditions (no heating) the intact $[M + H]^+$ molecular ion was the most abundant (Table 5). Other major ions were ammonium and potassium adducts with the molecular ion ($[M + NH_4]^+$, $[M + K]^+$, and $[M + 2K]^+$ ions) and the aglucone ion $[M - MalGlc]^+$. In the negative ion mode, the molecular $[M - H]^-$ ion was the most abundant ion. Other major ions were the acetic acid adduct $[M - H +$ acetic acid$]^-$, $[M - COOH]^-$, and the aglucone ion $[M - H - MalGlc]^-$.

Isoflavone β-Glucoside Conjugates. Daidzin, genistin, and glycitin gave rise to two main ions using the HN–APCI interface, the molecular $[M + H]^+$ ion and the $[M - Glc + H]^+$ ion (Figure 6). Corresponding ions $[M - H]^-$ and $[M - Glc - H]^-$ were observed in negative ion mass spectra (Table 4). At an orifice potential of −70 V, the acetic acid adduct ions $[M - H +$ acetic acid$]$ were observed. Using the IonSpray interface, the molecular ions $[M + H]^+$ and $[M - H]^-$ were the most abundant ions in positive and negative ion spectra, respectively. The other ions in the positive ion spectra were the potassium adduct ion $[M + K]^+$ and the aglucone ion $[M + H - Glc]^+$. In the negative ion spectra, the acetate adduct ion $[M - H + CH_3COO]^-$ and the aglucone ion $[M - H - Glc]^-$ were observed (Table 5).

Isoflavone 6-OAcGlc Conjugates. These conjugates were the least abundant of the conjugates in all soy fractions tested, although significant amounts were detected in the toasted soy flour and the isolated soy protein. In the soybean hypocotyl and defatted soy flour they were hardly detectable when cold solvent extraction was used. They were also the least mobile of the glycosidic conjugates by HPLC in the presence of ammonium acetate. Using the heated nebulizer–APCI interface, they gave rise in the positive spectra to the molecular ion $[M + H]^+$ and the $[M - AcGlc + H]^+$ ion, the latter being the most abundant (Figure 7). Corresponding ions were observed in the negative ion spectra (Table 4); again, when using an orifice potential of −70 V, an acetic acid adduct with the molecular ion was observed. Using the IonSpray interface, the molecular ions $[M + H]^+$ and $[M - H]^-$ were the most abundant ions in positive and negative ion spectra, respectively (Table 5). Other major ions in positive ion spectra were the potassium adduct $[M + K]^+$ and the aglucone ion $[M + H - AcGlc]^+$, whereas in negative ion spectra only the acetate adduct $[M - H + CH_3COO]^-$ was observed. In the case of genistein 6-OAcGlc, the acetate adduct ion was the most abundant ion.

The relative sensitivity of HN–APCI and IonSpray for the detection of isoflavone glycosidic conjugates by HPLC–MS was assessed using an 80% aqueous methanol extract of toasted soy flour (Table 6). The highest sensitivity for each type of conjugate was observed for the positive isoflavone aglucone ions (*m/z* values 255, 271, and 285) generated in the HN–APCI interface. They were mostly 1.5–3-fold more intense than the

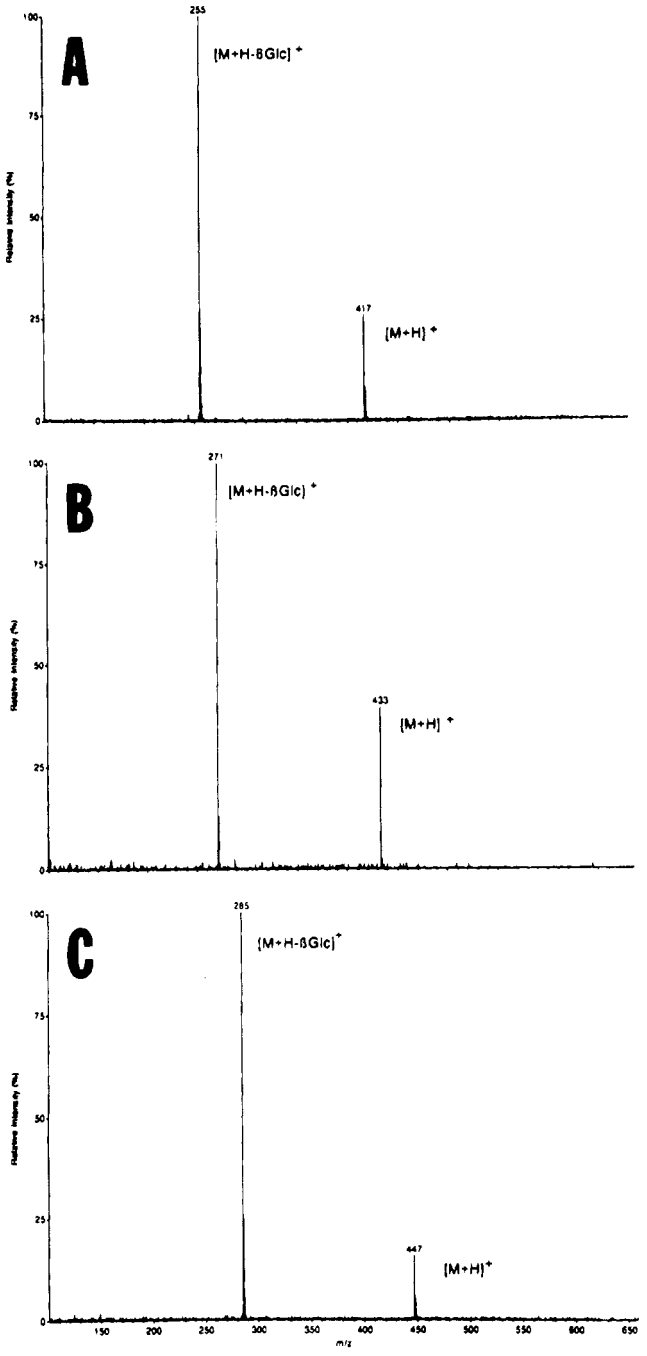

Figure 6. Positive ion mass spectra of daidzin (A), genistin (B), and glycitin (C) in isolated soy protein separated by reversed-phase HPLC (see Figure 4).

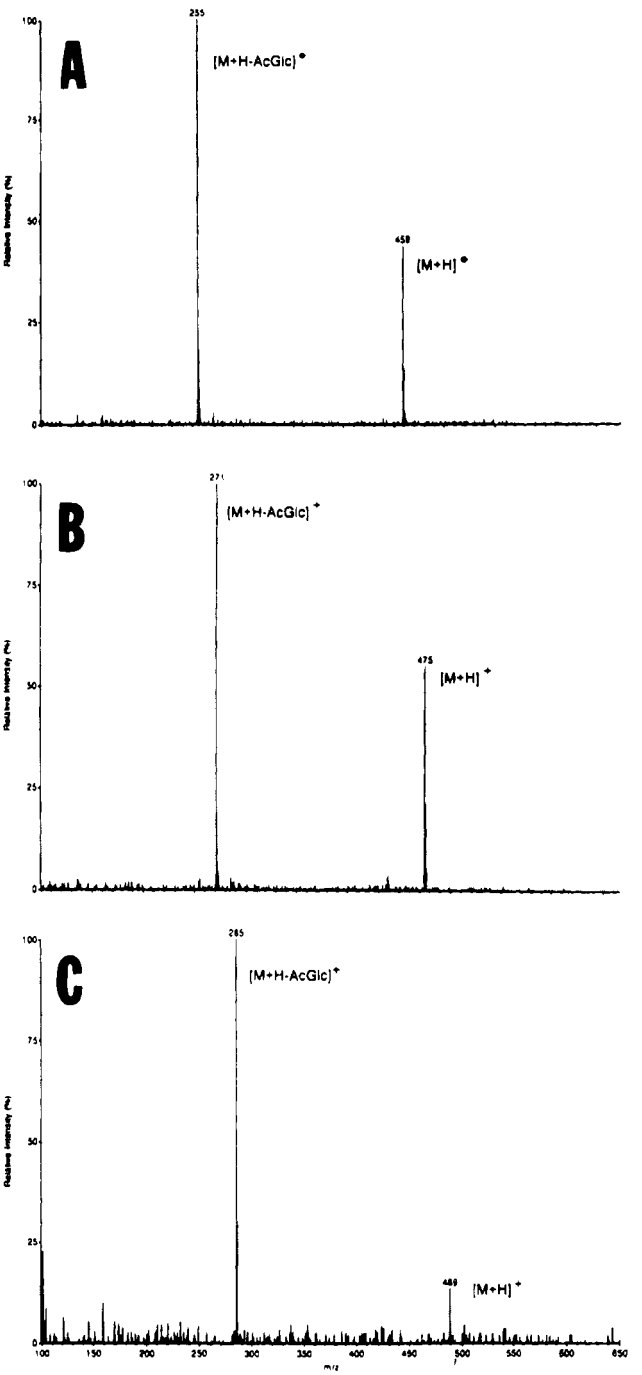

Figure 7. Positive ion mass spectra of 6-OAcGlc conjugates of daidzein (A), genistein (B), and glycitein (C) in isolated soy protein separated by reversed-phase HPLC (see Figure 4).

positive molecular ions $[M + H]^+$ generated in the IonSpray interface. The most abundant negative ions generated in the IonSpray interface tended to be less intense than their positive counterparts.

DISCUSSION

In the present study it has been shown that the extraction of isoflavone conjugates from the soy matrices tested occurs readily at room temperature in 80% aqueous methanol, being essentially complete within 1–2 h. Heating, as used by many previous investigators, is unnecessary and alters the isoflavone composition. At 60 °C, as used in a previous study from this laboratory (Coward et al., 1993), the magnitude of the heat-induced changes was considerably less than at 80 °C. In that study, recoveries of individual isoflavones and their β-glucosides added to alcohol-extracted (and isoflavone-depleted) soy flour ranged from 90 to 93% (Coward et al., 1993).

Farmakalidis and Murphy (1985) suggested that 80% aqueous methanol was not as good an extractant of 6-OAcGlc conjugates compared to 80% aqueous acetonitrile–0.1% HCl. However, data in this study show that these two solvents were equally good when used at room temperature. When extraction was carried out with 80% aqueous methanol at 80 °C for 1–4 h, the concentration of the isoflavone 6-OMalGlc conjugates and 6-OAcGlc conjugates (in toasted soy flour) declined as the β-glucoside conjugates rose. The de-esterification reaction was presumably a result of transesterification of the ester linkage between the malonate or acetate carboxyl group and the 6″-hydroxyl group of the glucose

Table 6. Maximum Ion Currents (Thousands of Counts) for the Principal Isoflavone Ions[a] Generated in the HN–APCI and IonSpray Interfaces

	HN–APCI positive		IonSpray positive		IonSpray negative	
isoflavone	*m/z*	ion current	*m/z*	ion current	*m/z*	ion current
daidzein 6-OMalGlc	255	850				
	459	230				
	503	226	503	271	501	102
daidzin	255	1631				
	417	408	417	485	475	379
daidzein 6-OAcGlc	255	950				
	459	379	459	485	517	335
genistein 6-OMalGlc	271	500				
	475	317				
	519	195	519	323	517	335
genistin	271	522				
	433	322	433	298	491	418
genistein 6-OAcGlc	271	450				
	475	300	475	261	533	216
glycitein 6-OMalGlc	285	210				
	489	33				
	533	26	533	101	531	33
glycitin	285	270				
	447	123	447	162	505	101
glycitein 6-OAcGlc	285	694				
	489	55	489	91	547	53

[a] Data using the HN–APCI were obtained by analysis of an 80% aqueous methanol extract of toasted soy flour by reversed-phase HPLC on a 10 cm × 4.6 mm i.d. Aquapore C_8 column using a 0–50% gradient of acetonitrile in 10 mM ammonium acetate over 0–10 min at a flow rate of 1 mL/min. In the case of the data obtained with the IonSpray interface, the same sample was analyzed on a 10 cm × 2.1 mm i.d. Aquapore C_8 column using a 0–50% gradient of acetonitrile in 10 mM ammonium acetate over 0–10 min at a flow rate of 0.2 mL/min.

moiety, yielding methyl malonate or methyl acetate and the isoflavone β-glucoside. This effect may explain the apparently lower concentrations of isoflavone 6-OAcGlc conjugates in 80% aqueous methanol extracts of toasted soy flour, as reported previously (Farmakalidis and Murphy, 1985). It should be noted that storage of extracts for extended periods even at room temperature would be expected to lead to gradual changes in the composition of isoflavone conjugates.

This study has also shown that an important source of the observed variation in isoflavone conjugate composition of different soy foods is the degree of heating the soy material is exposed to during its preparation. Isoflavone 6-OMalGlc conjugates are prone to both heat-induced decarboxylation (to form 6-OAcGlc conjugates) and de-esterification (to form β-glucoside conjugates) (Farmakalidis and Murphy, 1985; Kudou et al., 1991). Thus, soy foods prepared with an aqueous heating step, i.e., pressurized boiling water extraction to prepare full-fat soy milk, have a marked reduction of 6-OMalGlc conjugates compared to whole soybeans and to products in which heating was minimized. In the case of soy milk, soy molasses, a concentrate of a hot 65% aqueous ethanol extract of soy flour, and tofu, essentially complete de-esterification to the β-glucosides was the principal chemical change. Indeed, the predominance of the β-glucosides over other isoflavone conjugates in soy molasses has enabled investigators to isolate genistin (the β-glucoside of genistein) on a large scale from this matrix (Walter, 1941; Coward et al., 1993; Barnes et al., 1994a).

Since the isoflavone 6-OAcGlc conjugates are virtually absent from extracts of the soybean cotyledon and hypocotyl (Kudou et al., 1991; present study) but are present in large quantities in toasted soy flour and to a lesser extent in the isolated soy protein, it is apparent that they are formed during the drying process used to manufacture the latter products.

The present study also describes for the first time the application of HPLC–mass spectrometry with HN–APCI and IonSpray interfaces to the analysis of isoflavones. Isoflavones in soy foods are more readily detected using the aglucone ions generated in the HN–APCI interface than molecular ions or their adducts generated in the IonSpray interface.

The data confirm previous findings that, in addition to the β-glucoside conjugates, isoflavones in soy hypocotyl and cotyledon are also present as 6-OMalGlc conjugates (Kudou et al., 1991) and in toasted soy flour as 6-OAcGlc conjugates (Farmakalidis and Murphy, 1985). Even though the 6-OMalGlc conjugates contain a carboxylic acid group, positive ion mass spectra obtained with the HN–APCI interface were easier to interpret than negative ion mass spectra. The molecular $[M + H]^+$ ion was a prominent ion in the positive ion mass spectra when HPLC analysis was conducted in a background of acetic acid but not in a background of ammonium acetate. In contrast, the $[M - H]^-$ ion was absent in the negative ion mass spectra when HPLC analysis was conducted in a background of acetic acid or ammonium acetate. This suggests that the carboxylate ion more readily decarboxylates in the heated nebulizer than the protonated carboxylic acid.

The isoflavone β-glucosides and 6-OAcGlc conjugates yielded molecular ions in both positive and negative ion mass spectra, but the most abundant ion in each case was the aglucone ion. Therefore, to identify the conjugates of an isoflavone in serial mass spectra obtained following reversed-phase HPLC separation of food extracts, selected ion chromatograms were prepared with a combination of the aglucone ion and the molecular ions of the individual conjugates. This was important for

conjugates of glycitein since their mass spectra contained a much lower relative abundance of the molecular ion compared with daidzein and genistein conjugates.

Introduction of isoflavone β-glucoside and 6-OAcGlc ions into the mass spectrometer via the IonSpray interface resulted in a lower sensitivity than that using the HN–APCI interface. However, since sensitivity in the IonSpray interface is dependent on the concentration of the solute in the nebulized droplets rather than the amount of solute (as observed in the HN–APCI interface), the use of a capillary HPLC column, as used in the analysis of peptide digests, would markedly increase sensitivity in this mode. For example, in the case of a 0.3 mm i.d. capillary column, the theoretical improvement in sensitivity would be 50-fold over a 2.1 mm i.d. column. We have recently applied this technology to quantitatively and specifically detect 2.5 pmol of genistein in 1 mL samples of serum (Barnes et al., 1994b). This technical development would also permit the measurement of isoflavones in either very small samples of a food matrix or in food matrices in which the isoflavone content was less than 1–5 μg/g, the practical limit of detection using HPLC–UV analysis.

In contrast to the HN–APCI interface, the molecular ions of the 6-OMalGlc conjugates were the most abundant in both positive and negative ion mass spectra, probably since the sample was not heated (and minimally decarboxylated) before it entered the mass spectrometer. The β-glucosides and the 6-OAcGlc conjugates, not having a carboxylic acid group, readily formed adducts with the acetate ion, these ions being the most abundant in negative ion mass spectra. The extent to which this happened was reduced by increasing the orifice potential. The relative molar ion yields for the three types of conjugates and the three isoflavones varied only over a 2-fold range and were similar in positive and negative ion mass spectra (data not shown).

In summary, we have shown that the composition of isoflavone conjugates in soybeans and soy products is more complex than previously thought. In the present study we have developed a reproducible and accurate method for the extraction and analysis of the individual isoflavone conjugates using reversed-phase HPLC and HPLC–mass spectrometry. The variation in the relative amounts of each isoflavone conjugate between the different soy materials tested has important implications for previous, ongoing, and future animal and clinical studies of the effects of soy isoflavones. It is not known what effects 6″-*O*-substitution has on the susceptibility of the isoflavone conjugates to intestinal hydrolysis (and hence absorption). However, it should be anticipated that there will be differences in bioavailability and metabolism of the isoflavones dependent on the nature of their chemical form. Studies are urgently needed to address this issue.

ABBREVIATIONS USED

MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; HPLC, high-pressure liquid chromatography; 6-OAcGlc, 6″-*O*-acetylglucoside; 6-OMalGlc, 6″-*O*-malonylglucoside.

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