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Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography–mass spectrometry

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Abstract

An improved method for extraction and analysis of isoflavones from soy protein, soy foods and nutritional supplements is presented. The method uses acetonitrile extraction without acidification, with apigenin as internal standard. Samples extracted in acetonitrile–water are diluted to 50% acetonitrile and directly injected for gradient HPLC separation on a C_{18} reversed-phase column. This method saves significant time during sample preparation and improves accuracy and precision. Conventional and rapid HPLC analysis methods compatible with the extraction scheme were developed. During development of the methods, unexpected minor forms of malonyl and acetyl isoflavones were discovered in extracts of soy proteins and in pure isoflavone standard preparations. By LC–triple MS, these peaks have identical composition to the respective 6″-*O*-malonyl- and 6″-*O*-acetyl-isoflavones from which they form. These minor forms are believed to be malonyl and acetyl isoflavones where the site of attachment is a hydroxyl other than the 6′-OH of the glucose. These compounds can represent significant minor isoflavone components of foods, which contain high concentrations of malonyl or acetyl isoflavones.

Keywords: Food analysis; Isoflavones; Proteins; Daidzin; Glycitin; Genistin; Daidzein; Glycitein; Genistein

1. Introduction

Isoflavones are phytochemicals found in a variety of plants including soybeans. They may contribute to many of the health benefits attributed to soy foods (see for example Ref. [1] for a review). In the United States, health claims for foods containing soy protein are now permitted and commercial preparations of isoflavone extracts from soy are sold as nutritional supplements.

The three isoflavones daidzein, glycitein and genistein each exist as four forms in soy food products (Fig. 1). In nature, the 6″-*O*-malonyl-β-glucosides of the isoflavones are the predominant forms. The 6″-*O*-acetyl-β-glucoside-, β-glucoside-, and aglucone forms develop from the 6″-*O*-malonyl-β-glucosides during processing of soybeans and soy foods or during sample preparation and analysis [2–5].

Extraction of isoflavones from soy foods is a difficult challenge. Extraction methods prior to 1991 usually utilized refluxing alcohol [6,7], after the method of Eldridge, resulting in complete conversion of 6″-*O*-malonyl- and 6″-*O*-acetyl-isoflavone-β-glucosides to β-glucoside and aglucone forms. Methods that are more recent have utilized room tempera-

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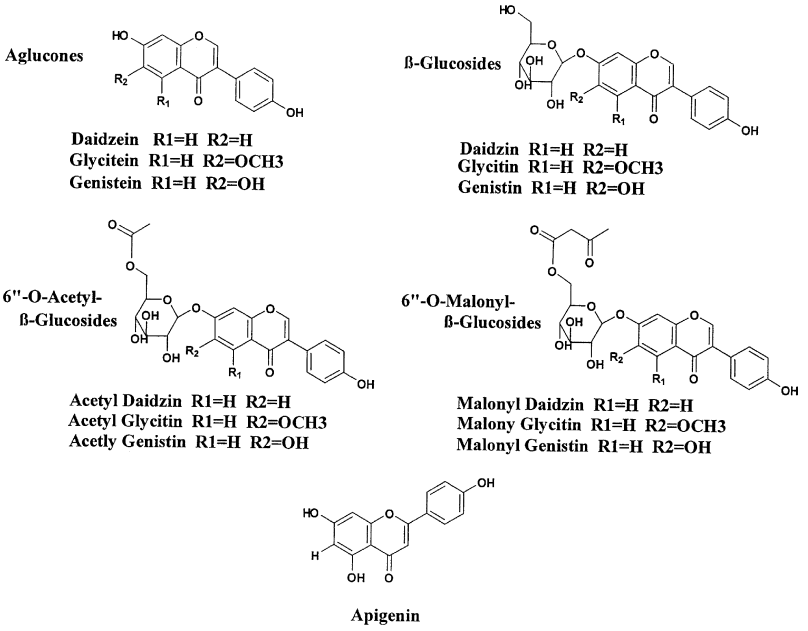

Fig. 1. Structures of soy isoflavones and apigenin internal standard.

ture or chilled extractions [5,8]. Some procedures still utilize 80% methanol [5]. Wang and Murphy [8] utilized acidified acetonitrile at room temperature for extraction of isoflavones from foods, but follow this step with rotary evaporation at less than 30°C to remove the acidified acetonitrile followed by dissolution in 80% (v/v) methanol. This laborious procedure makes extraction and analysis of large numbers of samples difficult. The US Department of Agriculture–Iowa State University Isoflavone Database [9] states that ''acid addition to extraction solvent'' is ''highly recommended''. The utility of acidifying samples will be discussed.

Reversed-phase high-performance liquid chromatography (HPLC) with UV detection [2–16] has been the method of choice for isoflavone analyses for many years. Liquid chromatography–mass spectrometry (LC–MS) [3,5,13,17–21] has also been widely used, especially for clinical studies of isoflavone metabolites in animals and humans. Our current studies center mostly on LC–UV methods, because most food laboratories are not equipped with LC–MS equipment for routine analyses. The extraction methods described in the current paper are applicable to either LC–UV or LC–MS methods. LC–MS for identification of isoflavone compounds will be discussed.

The need for an internal standard is crucial in evaluation of soy food and soy protein samples, which have varying moisture content and are not fully soluble in the extraction solvent. Song et al. have proposed synthesis of 2,4,4′-trihydroxydeoxybenzoin (THB) [22] for this purpose. This standard is not commercially available, and must be synthesized in the laboratory. Coward and co-workers at the University of Alabama [3,15] used fluorescein as internal standard. Nguyenle et al. [7] used acetophenone. Both fluorescein and acetophenone have a very different chemical structure from the isoflavones, and are likely to have different extraction efficiencies. Barnes et al. [21] proposed use of apigenin, the flavone analog of the isoflavone genistein (Fig. 1) as in internal standard for LC–MS, but did not resolve apigenin from genistein with the isocratic HPLC method proposed in that work, differentiating the two by differing MS fragment ions.

This paper presents an improved technique for extraction and HPLC analysis of isoflavones from food systems, including a better, commercially avail-

able internal standard. Quality control results and validation data for the method will be discussed. The problem of quantifying forms that do not have readily available standards will be reviewed.

Pure standards of the malonyl and acetyl isoflavones were acquired for this study. Their properties in solution will be reviewed. LC–MS for confirmation of peak identity and the discovery of unexpected minor forms of isoflavones in analysis of both soy proteins and of authentic pure standards will be presented.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile, acetic acid and Spectranalyzed-grade dimethyl sulfoxide (DMSO) were from Fisher Scientific (Pittsburgh, PA, USA). The six commonly available isoflavone standards (daidzin, glycitin, genistin, daidzein, glycitein, and genistein) were obtained from three different suppliers: LC Labs. (Woburn, MA, USA), Indofine (Sommerville, NJ, USA) and Plantech UK (Reading, UK). Standards from all three suppliers were found to be of comparable quality. Malonyl and acetyl isoflavones (malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin and acetyl genistin) were obtained from LC Labs. Apigenin was from Indofine.

2.2. Apparatus

HPLC separations for method development and validation were carried out on a system from Shimadzu Scientific Instruments (Columbia, MD, USA), consisting of two LC-10AT pumps, an SIL-10A autosampler, a SPD-10AV detector, and an SCL-10AVvp system controller. Gradient control, data acquisition and analysis were provided by a computer running Shimadzu Class VP software version 5.03. A 500-μl binary static mixer from ASI (Analytical Scientific Instruments, Richmond, CA, USA) was used to improve gradient reproducibility. Solvents were degassed with a Phenomenex (Torrance, CA, USA) vacuum degasser. Column temperature was controlled using an Eppendorf CH-30 column heater with a TC-50 controller (Supelco, Bellefonte, PA, USA).

LC–MS experiments were performed using an HPLC system equipped with an LCQ ion trap mass spectrometer (Thermoquest, San Jose, CA, USA). The system consisted of a P4000 quaternary gradient pump, an AS3000 autosampler with column heater, an SN4000 system controller, a UV6000 diode array detector and an SCM1000 solvent degassing module. A 500-μl in line static mixer (Analytical Scientific Instruments) was used to improve gradient reproducibility. The HPLC system was controlled and LC–UV data were analyzed on a computer equipped with ChromQuest software. LCQ MS data were acquired simultaneously using Xcalibur software on the same computer.

Samples were mixed for extraction on a LabQuake rotator Model 415-110 (Barnstead-Thermoline, Dubuque, IA, USA). A clinical centrifuge (IEC Model HN-S; IEC, Needham Heights, MA, USA) with an eight-position angle rotor was used for centrifuging samples after extraction.

The HPLC column for the standard method was a YMC ODS-AM, 250×3 mm with 5 μm packing (Waters, Milford, MA, USA). The HPLC column for LC–MS work was a YMC ODS-AM 150 mm×3 mm with 3 μm packing. Rapid methods were also evaluated on an Alltima C_{18} Rocket column from Alltech (Deerfield, IL, USA), 53×7 mm, 3 μm packing.

Poly(vinylidene difluoride) (PVDF) filters, 13 mm, 0.45 μm pore size were from Scientific Resources (Eatontown, NJ, USA). Five milliliter plastic syringes were from Fisher.

2.3. Extraction procedures

Extraction conditions for soy protein, soy foods, and nutritional supplements were as follows. For soy protein and other foods, approximately 1 g of sample was accurately weighed into a screw cap 125 mm× 20 mm test tube. For nutritional supplements, commercial isoflavone preparations and other products containing high concentrations of isoflavones, smaller sample amounts were used. The amount of sample was chosen to contain approximately 10 mg total isoflavones, but no more than 1 g total. A 10-ml volume of acetonitrile was added to the sample and

swirled to suspend. A 6-ml volume of high-purity deionized water and exactly 0.500 ml internal standard solution (2000 μg/ml apigenin in DMSO) were added and shaken briefly to mix. Sample tubes were extracted for 2 h on a rotary mixer. After 2 h, 3.5 ml deionized water was added with brief shaking to mix, bringing the final acetonitrile concentration to 50% (v/v). 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 analyzed by HPLC.

2.4. HPLC methods

The standard HPLC method, used for most studies, was based on the work of Wang and Murphy [8]. A YMC ODS-AM column, 250×3 mm with 5 μm packing, was used. Injection volume was 5 μl. Solvent A was 0.1% (v/v) acetic acid in water; solvent B was 0.1% (v/v) acetic acid in acetonitrile. The flow-rate was 0.65 ml/min. Column temperature was maintained at 40°C. The gradient was started immediately upon injection. Gradient elution was from 10% to 30% B in a linear gradient over 60 min. The column was washed at 90% B for 3 min and equilibrated 10 min between runs at 10% B. Total sample to sample time was 73 min for this method. Detection was by UV absorbance at 260 nm. Peak areas were integrated for quantitation.

The modified method of Murphy et al. [23] was used in some experiments. All conditions were identical to the standard method except for the gradient. The column was held constant at 15% B for the first 5 min of the run, followed by a two-step linear gradient, to 29% B over 31 min, then to 35% B over 8 min. The column was washed at 90% B for 3 min and equilibrated 10 min at 10% B between runs. Total sample to sample time was 57 min for this method. Detection was by UV absorbance at 260 nm.

The rapid HPLC method utilized an Alltima C_{18} Rocket column, 53×7 mm with 3 μm packing (Alltech). Injection volume was 5 μl. Solvent A was 0.1% (v/v) acetic acid adjusted to pH 3.50 with ammonium hydroxide, solvent B was acetonitrile. The flow-rate was 3 ml/min. Column temperature was held constant at 40°C. The gradient was started immediately upon injection of the sample. Elution was by a two-step linear gradient to 27% B in 9 min, then to 29% B in the next 4 min. The column was washed at 90% B for 2 min and equilibrated 2 min at 10% B between runs. Total sample to sample time was 17 min for this method. Detection was by UV absorbance at 260 nm.

LC–MS analysis of newly discovered isoflavone forms was done on an LCQ ion trap mass spectrometer. The LCQ was used in the positive ion mode with electrospray ionization (ESI). Electrospray voltage was 4.5 kV. The heated capillary temperature was 200°C. The system was calibrated according to manufacturer instructions and was tuned to a molecular mass of 524.3 using the calibration solution. The LC–MS column was a YMC ODS-AM column, 150×3 mm with 3 μm packing. Injection volume was 5 μl. Solvent A was 0.1% (v/v) acetic acid in water; solvent B was 0.1% (v/v) acetic acid in acetonitrile. The flow-rate was 0.5 ml/min. Column temperature was maintained at 40°C. The gradient was started immediately upon injection and was linear from 10% to 30% B over 60 min. The column was washed at 90% B for 10 min and equilibrated 10 min at 10% B between runs. Detection was by data dependent triple MS (MS^3) on the LCQ mass spectrometer. The entire column flow was directed into the MS system.

Standards of the six readily available isoflavones were used for routine calibration and recovery experiments. Stock standard solution was prepared by accurately weighing, on a five-place analytical balance to the nearest 0.01 mg, the following into a 100-ml flask: 0.1 g daidzin, 0.025 g glycitin, 0.1 g genistin, 0.01 g daidzein, 0.01 g glycitein and 0.01 g genistein. Sufficient DMSO was added to bring the final mass to 50.00 g and exact concentrations were calculated. The solution was thoroughly mixed and stored at room temperature in tightly closed amber bottles until use. The solution was stable for at least 2 months at room temperature. This solution was used in recovery experiments with soy protein samples and to prepare working standards.

Working standards were prepared by accurately weighing 1, 2, 3, 4 or 5 g of the stock solution into a 50-ml class A volumetric flask, adding exactly 1.25 ml internal standard solution, and diluting to volume with 50% (v/v) acetonitrile. Diluted standards were

transferred immediately to tightly capped amber vials and stored refrigerated. Diluted standards were stable for at least 2 months.

Malonyl and acetyl isoflavones were available in very limited quantities and were not used for routine calibration. Calibration standards of the malonyl and acetyl forms were always prepared just prior to use, and were diluted in a single step to final concentration. Samples were weighed into an appropriate container, internal standard solution was added at a ratio of 0.5 ml per 20 ml final volume, and standards were diluted to final volume with 50% acetonitrile. Diluted standards of malonyl and acetyl isoflavones were not stable, and were used immediately after preparation or as described.

2.5. Quality control

Weekly five-point calibrations with the six readily available isoflavones were done on all HPLC systems. The precision of the internal standard method is such that negligible drift in calibration was seen. The six malonyl and acetyl isoflavones were routinely calibrated by ratio to the β-glucoside standards. Check standards of the six commercially available β-glucoside and aglucone isoflavones were analyzed prior to the first sample each day and at least once per 10 samples. Check samples of either a reference lot of a commercial 40% isoflavone preparation or a reference lot of soy protein were also run daily. Recoveries of the major isoflavone peaks and the total isoflavone recovery of quality control (QC) check standards and check samples were expected to be within 2% of the mean value for every QC analysis. Recovery of the apigenin internal standard was checked in every sample, and was expected to be greater than 98.5%. The six readily available standards spiked into soy proteins or soy food samples were recovered at between 99% and 101%. Quality data for the standard method was compiled similar to that of Murphy et al. [16] for comparison, as this is considered reference method as suggested in Ref. [9].

3. Results and discussion

3.1. Validation of the standard HPLC method

Most HPLC–UV methods for isoflavones are relatively long. The standard method we have used, based on the work of Wang and Murphy [8], is in excess of 1 h per analysis. The advantage of long methods is that they are very highly resolving. In

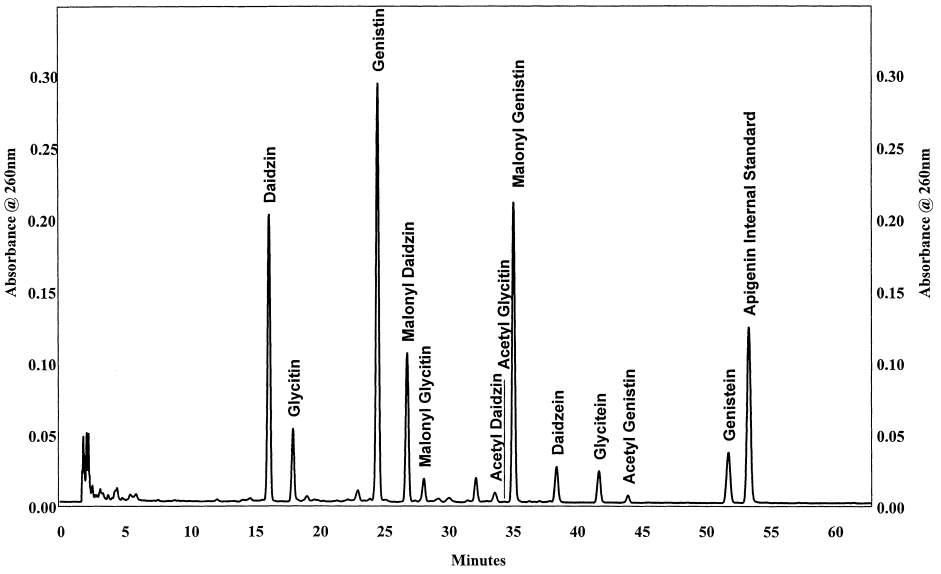

Fig. 2. Standard HPLC method, soy protein spiked with commercial standards.

complex matrices such as food systems, these methods give extremely high reproducibility and are nearly free from interference from the matrix. Fig. 2 shows a soy protein sample spiked with the six readily available standards, analyzed by the standard HPLC method. The 12 expected isoflavones are well resolved from one another, from background peaks, and from the apigenin internal standard. Linearity of the six standards was extremely good over the range specified, with r^2 values greater than 0.9998 for all six standards. Calibrations of our HPLC systems have varied by less than 1% over the past year. The limit of quantitation for the method is below 0.5 ppm for each isoflavone. The method is extremely reproducible. Relative standard deviations (RSDs) of check standards, compiled from 179 analyses of five different check standards over a four month period, were daidzin, 0.67%; glycitin, 0.66%; genistin, 0.58%; daidzein, 0.87%, glycitein, 0.65% and genistein, 0.50%. This resulted in an overall variability of approximately ±1.4%, which compares favorably with the ±5% reported by Murphy et al. [16] for standards. Samples which are fully soluble in the 50% acetonitrile sample solution, i.e., commercial isoflavone concentrates, showed similar variability of approximately 1%. Quality control results from our laboratory for a soy protein check sample are shown in Table 1. Average column lifetime using our standard method is about 2500 to 3000 samples.

3.2. Quantitation of malonyl and acetyl isoflavones

Malonyl and acetyl forms of isoflavones have not been readily available commercially, though they are beginning to be produced. Laborious syntheses or isolations from natural sources have been the normal method of acquiring these standards [8]. Quantitation of malonyl and acetyl forms has generally been accomplished by using the response factor of the corresponding β-glucoside and correcting for the molecular mass difference. This method is reported to give values that are close to correct [8,14,23]. During the course of this study, we obtained standards for all of the malonyl and acetyl isoflavones, in greater than 97% purity, through an arrangement with LC Labs. Only tiny amounts of malonyl glycitin and acetyl glycitin were available, but sufficient quantities of malonyl daidzin, malonyl genistin, acetyl daidzin, and acetyl genistin were obtained for several studies. Table 2 compares the expected response factors for malonyl daidzin, malonyl genistin, acetyl daidzin and acetyl genistin based on the respective β-glucoside response factors with those obtained gravimetrically. The response factors based

Table 1
Reproducibility of HPLC analysis of the soy protein check sample[a]

Isoflavone	Concentration (μg/g)					
	Within-day			Between-days		
	Average	SD	RSD (%)	Average	SD	RSD (%)
Daidzin	703.0	2.5	0.35	697.9	15.8	2.26
Glycitin	98.4	1.0	1.01	97.8	3.1	3.18
Genistin	864.2	3.5	0.40	857.9	15.9	1.86
Malonyl daidzin	69.0	0.8	1.10	69.5	2.7	3.94
Malonyl glycitin	13.2	0.8	6.10	12.1	2.8	22.87
Acetyl daidzin	73.5	1.8	2.45	69.3	9.1	13.10
Acetyl glycitin	8.0	1.4	17.10	10.0	6.9	69.69
Malonyl genistin	138.3	1.8	1.33	135.6	3.4	2.51
Daidzein	36.8	0.6	1.62	36.7	4.0	10.80
Glycitein	9.8	0.2	2.45	8.9	1.3	14.49
Acetyl genistin	64.4	0.3	0.50	65.6	1.3	1.96
Genistein	50.4	0.2	0.32	50.3	1.1	2.17
Totals	2129.0	8.1	0.38	2111.1	32.4	1.54

[a] A single lot of soy protein was analyzed as a check sample 42 times on 14 days over a 4-month period. Data are μg isoflavone/g protein.

Table 2
Comparison of gravimetric and molecular mass ratio response factors[a]

	Gravimetric	Molecular mass ratio
Malonyl daidzin	$4.70 \cdot 10^{-5}$	$5.11 \cdot 10^{-5}$
Acetyl daidzin	$4.12 \cdot 10^{-5}$	$4.67 \cdot 10^{-5}$
Malonyl genistin	$3.38 \cdot 10^{-5}$	$3.29 \cdot 10^{-5}$
Acetyl genistin	$2.71 \cdot 10^{-5}$	$3.01 \cdot 10^{-5}$

[a] Detection was by UV absorbance at 260 nm. Molecular mass ratio response factors were calculated by multiplying the response factor for the appropriate glucoside (daidzin or genistin) by the ratio of the molecular masses of the acetyl or malonyl form to the glucoside.

on gravimetric analysis are close to those predicted by molecular mass ratio method, but our results indicate the molecular mass method overestimates acetyl daidzin, malonyl daidzin and acetyl genistin by about 10%.

3.3. Apigenin as an internal standard

Apigenin is the flavone analog of genistein, 5,7,4′-trihydroxyflavone (Fig. 1). Barnes et al. [21] suggested apigenin as an internal standard for LC–MS analysis of isoflavones, but did not resolve apigenin from genistein by the isocratic HPLC method they used, discriminating between the two by MS fragmentation pattern. In the gradient HPLC methods reported here, genistein and apigenin are well resolved (Fig. 2). Several factors make apigenin an ideal internal standard for isoflavone determination. It is commercially available. It has a chemical structure very similar to the isoflavone aglucones and should extract with similar chemical interactions. It is slightly more hydrophobic than genistein, the most hydrophobic of the isoflavones. This is useful because it is possible to lose genistein and acetyl genistin, the two most hydrophobic isoflavone components, to hydrophobic surfaces during handling. Hydrophobic isoflavones can be lost to syringe filter housings, plastic transfer pipettes, plastic vials and plastic tubing within autosampler transfer lines. Liquid samples must be stored in glass containers. Storage in plastic containers can result in significant loss of isoflavones. Acetonitrile concentrations below 40% during sample preparation resulted in loss of the hydrophobic isoflavones, as shown in Table 3. Use of an internal standard slightly more hydrophobic than the most hydrophobic isoflavone assures that, if recovery of the internal standard is complete, no hydrophobic losses have occurred to the isoflavones. The THB standard of Song et al. [22] is also relatively hydrophobic. Lack of commercial availability of this compound makes it a less desirable choice.

3.4. Improved extraction procedure

The extraction procedure presented here minimizes handling of the samples during preparation. The key to the procedure is that, after extracting in acetonitrile–water–DMSO, the samples are introduced directly to the HPLC system, without drying

Table 3
Hydrophobic losses of isoflavone standards during handling[a]

Acetonitrile concentration (%)	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein	Internal standard
50	100	100	100	100	100	100	100
45	102	103	102	102	102	101	99
40	102	101	101	101	100	100	98
35	100	100	100	99	98	97	95
30	101	101	101	99	98	96	94
25	101	101	100	97	96	93	90
20	100	100	99	94	94	88	79
10	97	96	92	81	82	66	6

[a] Standards containing 40 μg/ml daidzin and genistin, 10 μg/ml glycitin and daidzein, 5 μg/ml glycitein and genistein and 100 μg/ml apigenin were fully dissolved and diluted at the indicated acetonitrile concentration from DMSO stock. Samples were filtered using plastic syringes through disposable 0.45-μm PVDF filters in plastic housings. Results are reported as a percent of the recovery in 50% acetonitrile.

Table 4
Extraction of isoflavones from soy protein[a]

	Daidzin	Glycitin	Genistin	Malonyl daidzin	Malonyl glycitin	Acetyl daidzin	Acetyl glycitin	Malonyl genistin	Daidzein	Glycitein	Acetyl genistin	Genistein
Current method[a]												
Mean (μg/g)	755.6	137.2	1079.4	46.3	14.1	59.5	1.3	80.6	28.1	10.7	58.5	36.1
SD	2.98	0.32	1.77	0.90	1.24	0.20	0.17	0.20	0.26	0.04	0.18	0.03
RSD (%)	0.39	0.23	0.16	1.93	8.77	0.34	13.11	0.24	0.94	0.38	0.30	0.09
80% (v/v) Methanol[b]												
Mean (μg/g)	723.6	129.3	1003.3	41.8	13.2	53.2	1.3	70.7	26.4	10.2	52.7	33.0
SD	0.23	0.47	1.88	0.41	0.59	0.43	0.29	0.49	0.07	0.15	0.21	0.15
RSD (%)	0.03	0.37	0.19	0.98	4.47	0.80	22.40	0.69	0.27	1.42	0.39	0.46
Acidified 60% (v/v) acetonitrile[c]												
Mean (μg/g)	738.1	136.3	1045.5	46.2	14.7	58.9	1.2	78.8	26.1	10.0	56.8	32.7
SD	6.16	0.78	6.60	0.17	0.06	0.22	0.07	0.32	0.15	0.10	0.23	0.42
RSD (%)	0.83	0.57	0.63	0.37	0.39	0.37	6.26	0.40	0.58	0.96	0.41	1.28
Comparison of the methods, normalized to the current method												
Current method	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
80% (v/v) Methanol	95.8	94.3	92.9	90.2	93.8	89.4	99.3	87.8	94.0	95.9	90.0	91.3
Acidified 60% (v/v) acetonitrile	97.7	99.3	96.9	99.7	104.2	99.0	89.1	97.8	93.1	94.0	97.1	90.5

[a] Three replicate 1-g samples of soy protein were extracted for 2 h at room temperature with (a) 10 ml acetonitrile+6 ml water+0.5 ml internal standard in DMSO; (b) 10 ml acetonitrile+2 ml 0.1 *M* HCl+5 ml water [22] or (c) 80% methanol [5]. Samples were analyzed by the standard HPLC method. Results are μg isoflavone per gram protein.

and transfer to 80% methanol as described in Refs. [8,9,22].

Table 4 shows the results of extraction of a soy protein sample, comparing the current procedure with use of 80% methanol [5] and acidified acetonitrile [22]. Use of 80% methanol was less efficient than acetonitrile in extracting malonyl isoflavones, acetyl isoflavones and aglucones than the current procedure. Acidified acetonitrile [22] was also less efficient than the current procedure, though the differences were smaller, with the primary difference in the extraction of the hydrophobic components, the three aglucones and acetyl genistin. The acidified acetonitrile method contained approximately the same concentration acetonitrile as the current method. The slight improvement in extraction efficiency we see in the current method could be due either to the lack of added acid or to the presence of the small quantity of DMSO contributed by the internal standard addition. This effect was not investigated in greater detail.

Table 5 shows the effect of acetonitrile concentration in the current method on extraction of isoflavones from two samples of soy proteins. The results indicate that 60% acetonitrile was the most effective acetonitrile concentration for extracting isoflavones from soy protein. Extraction time for a soy protein sample is shown in Table 6. Extraction time had only a small effect on recovery of isoflavones from soy protein. The vast majority of isoflavones were extracted in 5 min. Two-hour extractions were used as the standard method to insure complete extraction.

3.5. Effects of sample in high organic solvent on HPLC results

A relatively high concentration of organic solvent is required to extract all the forms of isoflavones and keep them in solution. Most previous isoflavone extraction methods either utilized 80% methanol as a solvent [3–7,15] or, after extracting in acidified water–acetonitrile mixtures, removed the solvent and dissolved the residue in 80% methanol [2,8,16,22,23]. In our laboratory, we found 60% acetonitrile was much better than 80% methanol for extracting isoflavones (Table 4). Therefore, acetonitrile extraction was used. We were not willing to

Table 5
Effect of acetonitrile concentration on efficiency of extraction of isoflavones from soy proteins[a]

Acetonitrile (%, v/v)	Daidzin	Glycitin	Genistin	Malonyl daidzin	Malonyl glycitin	Acetyl daidzin	Acetylglycitin genistin	Malonyl genistin	Daidzein	Acetyl genistin	Genistein	Totals
(A) Soy protein low in malonyl isoflavones												
10	258.8	40.9	152.8	26.3		13.6	3.4	22.8	3.7	7.6	2.6	539.0
20	562.1	78.5	575.4	67.0		54.0	12.7	102.9	6.7	34.9	7.1	1515.9
30	667.9	91.2	787.4	76.0		67.6	15.2	136.9	30.7	60.1	38.2	1989.8
40	676.8	91.8	812.9	72.4		67.3	18.6	135.0	34.6	63.8	45.3	2037.5
50	681.6	91.7	823.6	70.1		68.7	18.6	130.3	34.3	63.8	48.2	2047.9
60	684.0	92.7	828.1	70.3		68.8	19.2	132.1	34.3	64.6	48.0	2059.8
70	681.5	92.5	827.5	70.4		68.8	19.0	132.2	34.1	64.0	48.0	2055.5
80	679.2	92.2	826.6	69.1		65.0	17.2	130.2	32.8	63.8	47.9	2040.5
90	664.7	90.1	809.0	70.0		67.2	18.7	129.2	33.0	62.3	46.8	2008.6
100	663.8	90.2	807.8	69.5		66.6	18.1	128.9	31.8	62.3	45.7	2001.5
(B) Soy protein high in malonyl isoflavones												
20	209.0	59.8	299.2	848.9	126.7	81.1		1124.8		20.0		2795.8
30	243.0	69.8	338.9	881.9	139.7	88.0		1180.5		33.6		2995.1
40	256.1	74.3	354.2	878.0	142.4	89.4		1181.8		34.6		3028.2
50	259.3	74.5	356.6	857.8	152.8	94.3		1156.8		34.7		3002.2
60	245.4	64.2	335.7	914.5	168.0	96.7		1263.9		31.7		3133.1
70	213.5	54.0	320.6	586.4	105.0	78.4		894.9		38.8		2309.9
80	175.6	44.5	280.1	265.5	39.1	62.3		618.6		36.7		1538.7
90	222.9	57.2	306.4	604.4	105.4	74.6		961.0		32.9		2379.3
100	4.3	1.0	6.4	2.2	0.7	0.5		3.8		0.9		21.7

[a] Single analyses of two soy protein samples extracted at the indicated acetonitrile concentration. Results are μg isoflavone per gram protein. Isoflavone components at very low concentrations omitted from the table.

Table 6
Extraction time[a]

Time (min)	Concentration (μg/g)											
	Daidzin	Glycitin	Genistin	Malonyl daidzin	Malonyl glycitin	Acetyl daidzin	Malonyl genistin	Daidzein	Glycitein	Acetyl genistin	Genistein	Totals
5	253.1	76.6	340.3	890.0	147.6	95.1	1192.9	16.2	2.3	33.9	14.3	3062.3
10	248.8	79.9	345.2	888.5	151.7	86.0	1197.1	15.8	2.1	34.4	14.9	3064.5
20	253.6	82.9	351.0	900.7	151.5	85.9	1214.5	21.0	2.1	34.9	15.3	3113.3
30	250.6	84.3	348.2	895.9	151.0	84.9	1207.0	20.8	2.3	34.5	15.1	3094.6
60	253.4	93.4	352.9	896.9	146.5	85.1	1211.0	21.2	2.4	34.6	15.5	3112.6
120	252.4	93.1	352.4	895.6	147.4	84.9	1205.3	21.6	2.5	34.2	16.2	3105.5
180	254.1	92.4	356.3	897.8	145.7	85.0	1213.6	22.3	2.6	34.5	16.4	3120.7
240	251.8	90.5	354.3	892.1	144.2	71.4	1197.0	20.9	2.6	34.6	17.8	3077.2
Overnight	250.7	89.8	349.4	879.8	146.5	31.6	1188.8	21.8	3.2	34.8	17.1	3013.3
Average	252.0	87.0	350.0	893.1	148.0	78.9	1203.0	20.2	2.5	34.5	15.8	3084.9
SD	1.7	6.2	4.9	6.3	2.7	18.7	9.4	2.4	0.3	0.3	1.1	34.4
Correlation	0.267	0.714	0.757	0.099	−0.811	−0.773	0.053	0.579	0.901	0.100	0.958	0.235

[a] A 1-g amount of soy protein was extracted by the standard method for the indicated times at room temperature. Results are μg isoflavone per gram protein.

accept the transfer to 80% methanol as a handling step. Though isoflavones are soluble in 80% methanol, methanol would not seem to be the best choice of solvent for a variety of reasons. Our HPLC method, like most others for isoflavones, utilizes acetonitrile for elution [2–5,8,16,22,23]. This would seem to argue in favor of aqueous acetonitrile as a sample solvent. We have found isoflavone standards much more difficult to dissolve in 80% methanol than in 50–60% acetonitrile. Certainly, the manipulations to remove acetonitrile from samples in order to dissolve them in 80% methanol seem not to make sense. In addition to being very time consuming, such handling steps always increase variability in the results and can be a source of losses.

One reason methanol has been used as the solvent, aside from historical reasons (hot alcohol extraction was the first extraction procedure), is the peak distortion caused by injecting samples containing high concentrations of acetonitrile onto columns equilibrated at low acetonitrile concentrations. This problem is prevented by limiting the sample size. Fig. 3 shows the effect of injection volume on peak shape of the early eluting peaks using the standard method. With the samples dissolved in 50% (v/v) acetonitrile and the column equilibrated at 10% (v/v) acetonitrile, injection volumes greater than 5 μl on this 250×3.0 mm C_{18} column caused peak distortion of the three earliest peaks of interest, daidzin, glycitin and genistin, but volumes of 5 μl or less cause no distortion. The asymmetry of the daidzin peak when 5 μl samples were injected was approximately 0.94, with all other peaks of interest in the chromatogram having asymmetries of either 0.99 or 1.00. This made it possible to analyze samples extracted in the preferred solvent, acetonitrile, without the cumbersome drying step to transfer them to 80% (v/v) methanol. Using this method, samples extracted in acetonitrile were diluted slightly to bring the acetonitrile concentration to 50% (v/v), filtered and analyzed without additional handling. This greatly decreased the time required for sample preparation, compared with rotary evaporation of acetonitrile from samples.

3.6. Unexpected minor forms of malonyl and acetyl isoflavones

Regardless of whether the samples are prepared in 80% methanol or 50% acetonitrile, with or without added acid, neither the malonyl nor the acetyl standards are stable in solution. The expected degradation product of the malonyl and acetyl isoflavones

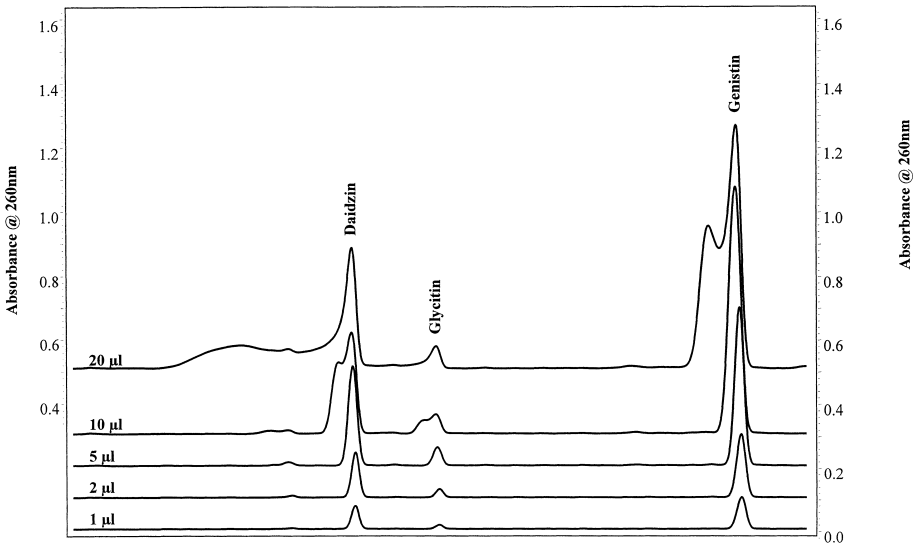

Fig. 3. Effect of injection volume on shape of early eluting peaks. HPLC analysis was by the standard method, with injection volumes changed as indicated.

in solution is their respective glucosides. As shown in Fig. 4, freshly prepared standards of 97–99% pure malonyl daidzin, malonyl genistin, acetyl daidzin and acetyl genistin develop several unexpected minor peaks not corresponding to the known isoflavones after a short time in solution. Peaks of the free β-glucoside also begin to appear in the standards after a few hours. Appearance of the new unexpected peaks corresponded to a similar decrease in the principle peak. In freshly prepared samples of pure standards, dissolved and immediately analyzed, peaks other than the primary peak are present at concentrations less than 1% of the concentration of primary component. After only a few hours, the new peaks plus the glucoside peaks will have peak areas equal to 10–15% of the principal component.

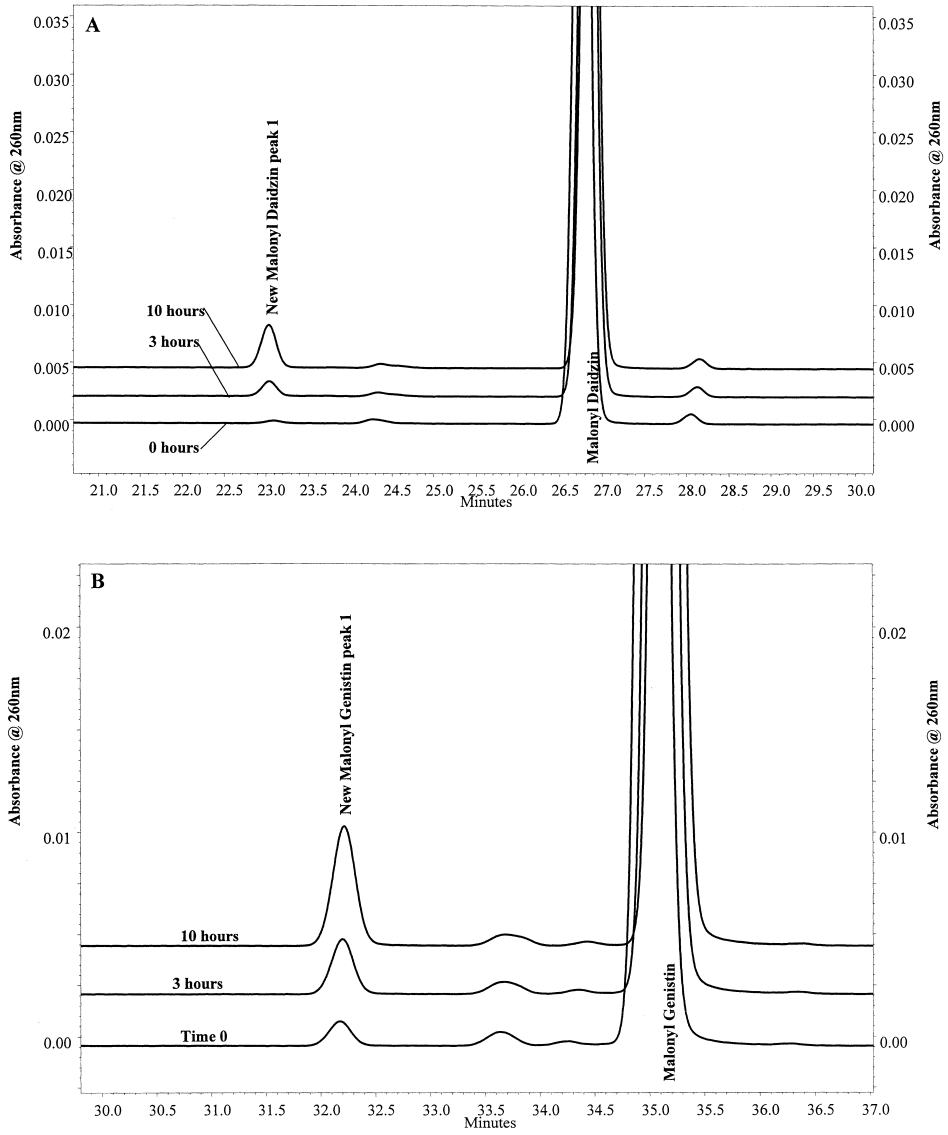

Fig. 4. New peaks appearing in pure standards of (A) malonyl daidzin, (B) malonyl genistin, (C) acetyl daidzin and (D) acetyl genistin. Samples were prepared as described in the text, and assayed by the standard HPLC procedure either immediately (time 0) or after the indicated number of hours.

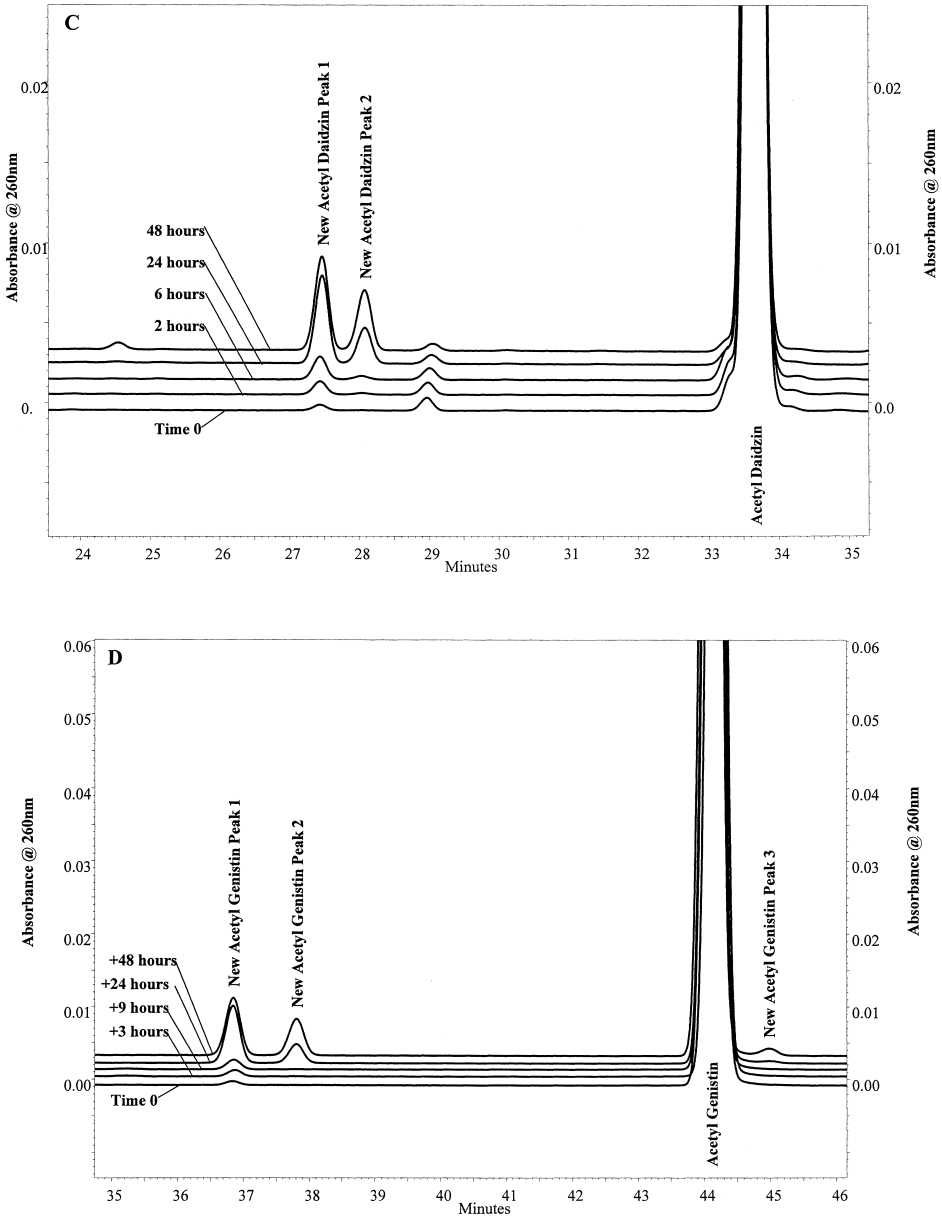

Fig. 4. (*continued*)

The time of appearance of the new, unknown peaks from malonyl daidzin and malonyl genistin is short enough that they should appear during the 2-h extraction process used for soy protein and food samples. Examination of chromatograms from soy protein samples containing high concentrations of malonyl isoflavones reveals that these peaks are present, as shown in Fig. 5. Close examination of chromatograms found in the literature reveals similar peaks present in the samples from a variety of investigators (Fig. 2 of Ref. [3], Fig. 4C and D of Ref. [5], Fig. 2 of Ref. [8], Fig. 1 of Ref. [23], Fig. 2 of Ref. [22]).

Identity of new peaks found in both standards and

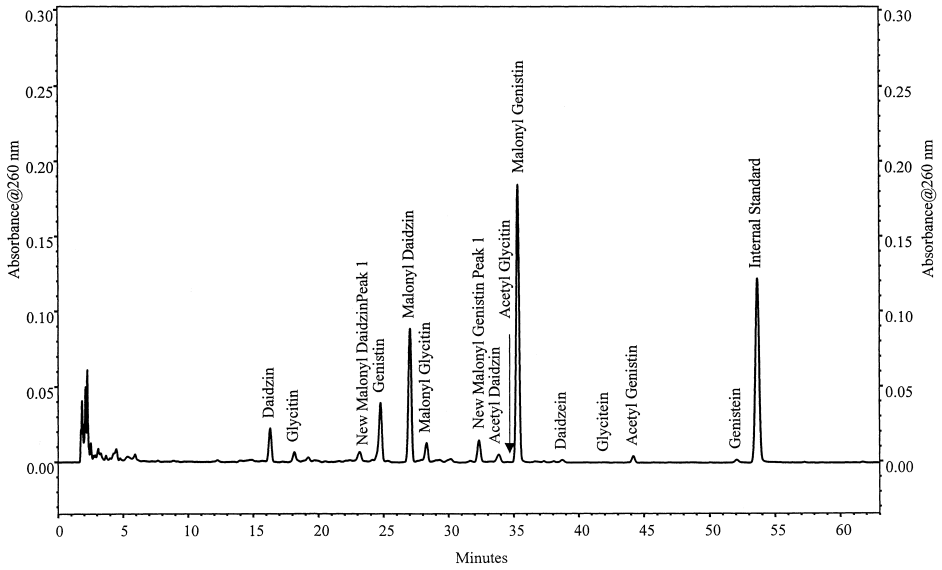

Fig. 5. Soy protein isolate HPLC analysis. ADM Nutrasoy 7B, a minimally processed soy protein, was extracted and analyzed by the standard method. New peaks malonyl genistin and malonyl glycitin peaks are labeled.

in soy protein was examined by ion trap LC–ESI-MS in the positive ion mode, using data dependent MS^3. This technique takes the most abundant ion found with full scan MS, and performs MS–MS on it, then performs MS^3 on the most abundant MS–MS ion. MS^3 results in nearly unequivocal identification of isoflavones. For malonyl, acetyl, and glucoside forms of isoflavones, the first stage MS gives an MH^+ ion for the intact molecule, MS–MS gives the aglucone core, and MS^3 fragments the core to a recognizable fingerprint.

Fig. 6 shows MS^3 analysis of the 6″-*O*-malonyl parent, the new peaks formed from pure malonyl daidzin and malonyl genistin. In each case, the new unknown peaks formed have identical MH^+ mass ions to the parent isoflavone. Fragmentation by MS–MS and MS^3 shows structure identical to the malonyl isoflavone from which they form. MS^3 analysis of the glucoside peaks seen in these samples is also presented. Results were similar for the new peaks found in the pure acetyl isoflavone standards, with the new peaks showing structures very similar to the parent compound. Analysis of soy protein samples gave similar results, with new peaks for both malonyl daidzin and malonyl genistin matching the structure of the parent compound. These finding indicate these new peaks are different forms of the same isoflavones. Possibly, the malonyl or acetyl groups are exchanging to different hydroxyls on the glucose. Whatever the exact mechanism, these compounds are isoflavones which are present within a short time after preparation in all samples which contain large amounts of malonyl or acetyl isoflavones. The amount of isoflavone they contribute to the total sample can be significant, particularly if several hours pass between extraction and analysis of the samples. In Fig. 5, a soy protein isolate which was extracted for 2 h and analyzed a few hours after extraction, the area of new peak formed from malonyl daidzin was 11% of the area of the 6″-*O*-malonyl daidzin peak, and the area of the new peak formed from malonyl genistin was 9% of the 6″-*O*-malonyl genistin peak. These peaks can represent a significant portion of the total isoflavones analyzed in foods containing high concentrations of malonyl and acetyl isoflavones. The fact that this transition occurs is also important in handling and quantifying malonyl and acetyl isoflavone standards, which must not be allowed time in solution if accurate results are required.

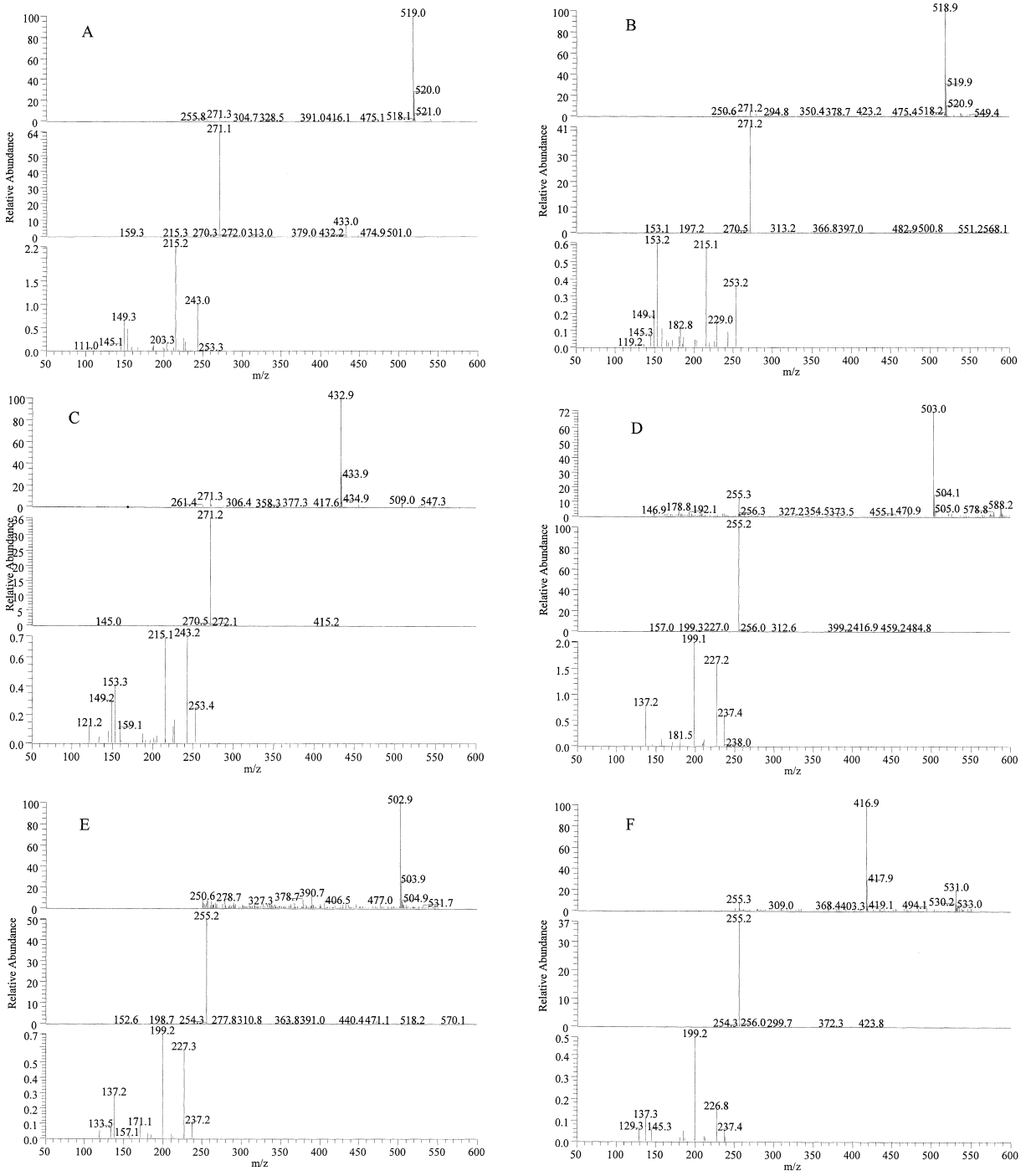

Fig. 6. LC–MS^3 analysis of new peaks in malonyl isoflavone standards. Full scan MS, MS–MS of the principle MS peak, and MS^3 of the principal MS–MS peak, for peaks appearing in pure samples of malonyl genistin (A–C) and malonyl daidzin (D–F). (A) 6″-*O*-malonyl genistin peak, (B) new peak 1 appearing in malonyl genistin, (C) genistin peak appearing in malonyl genistin, (D) 6″-*O*-malonyl daidzin peak, (E) new peak 1 appearing in malonyl daidzin, (F) daidzin peak appearing in malonyl daidzin.

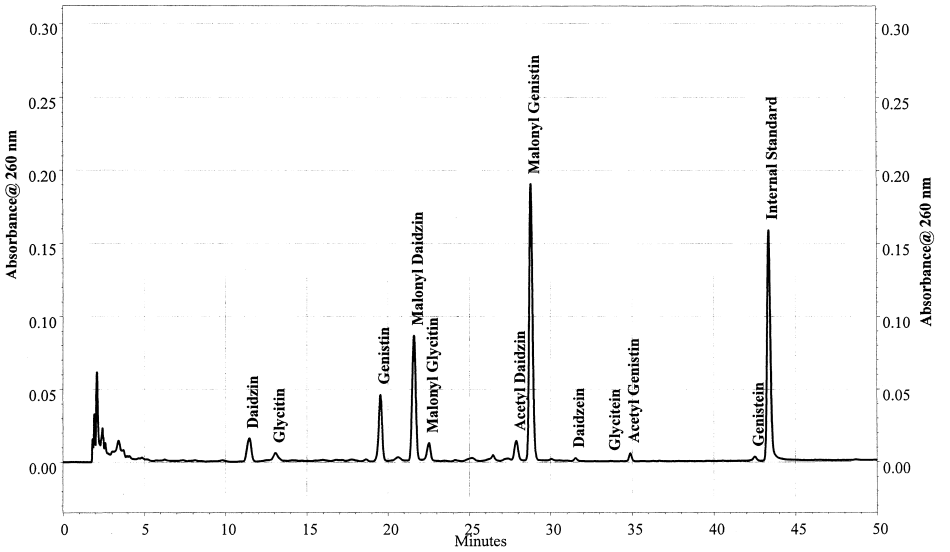

Fig. 7. HPLC analysis of isoflavones in soy protein by the HPLC method of Murphy et al. [23]. Samples were extracted by the current procedure and analyzed with gradient elution similar to that described in the reference.

3.7. Development of rapid methods of isoflavone analysis

The extraction and sample injection procedures described here work well with a variety of HPLC analysis methods. The method of Murphy et al. [23] with our sample introduction method is shown in Fig. 7. This method is highly resolving, and is suitable for analysis of isoflavones in complex food matrices. While this method is somewhat shorter that the standard method, it is still a very long method.

Rapid methods are also possible for isoflavone analysis, but are not as rigorous, especially in complex protein or food systems. Fig. 8 shows a rapid method developed in our laboratory. This method allows analysis of samples with a 17 min sample to sample cycle time, including a high acetonitrile column wash. The method looks very good with standards (Fig. 8A), and works very well for commercial isoflavone preparations (Fig. 8B). With soy protein, the resolution is not as good as with longer methods, but would still be adequate for most analyses (Fig. 8C).

4. Conclusions

The method described for extraction and HPLC analysis of isoflavones provides a means to speed extraction and analysis of isoflavones using the preferred solvent, acetonitrile. The elimination of the need to evaporate the acetonitrile greatly reduces the time and effort in sample preparation, as well as improving reproducibility.

The technique of limiting sample injection volume should allow this extraction method to be used with most of the HPLC analysis procedures currently used for isoflavones.

The finding that both malonyl and acetyl isoflavones undergo changes as artifacts of preparation, leading to unexpected forms, is important in determining correct values for isoflavone content of foods. Failure to consider these forms will result in significant errors in calculating the isoflavone content if the malonyl and acetyl isoflavones are high in concentration.

The rapid HPLC method described here will allow very rapid analysis of isoflavones, and may be particularly useful for analysis of purified commercial preparations. It may be possible to adapt such a method to analysis of nutritional supplements, if matrix interference from other components do not interfere. Such rapid methods are less accurate for analysis of food systems, where the matrix complexity is a major concern, but still provide rapid analysis that would be acceptable for most needs.

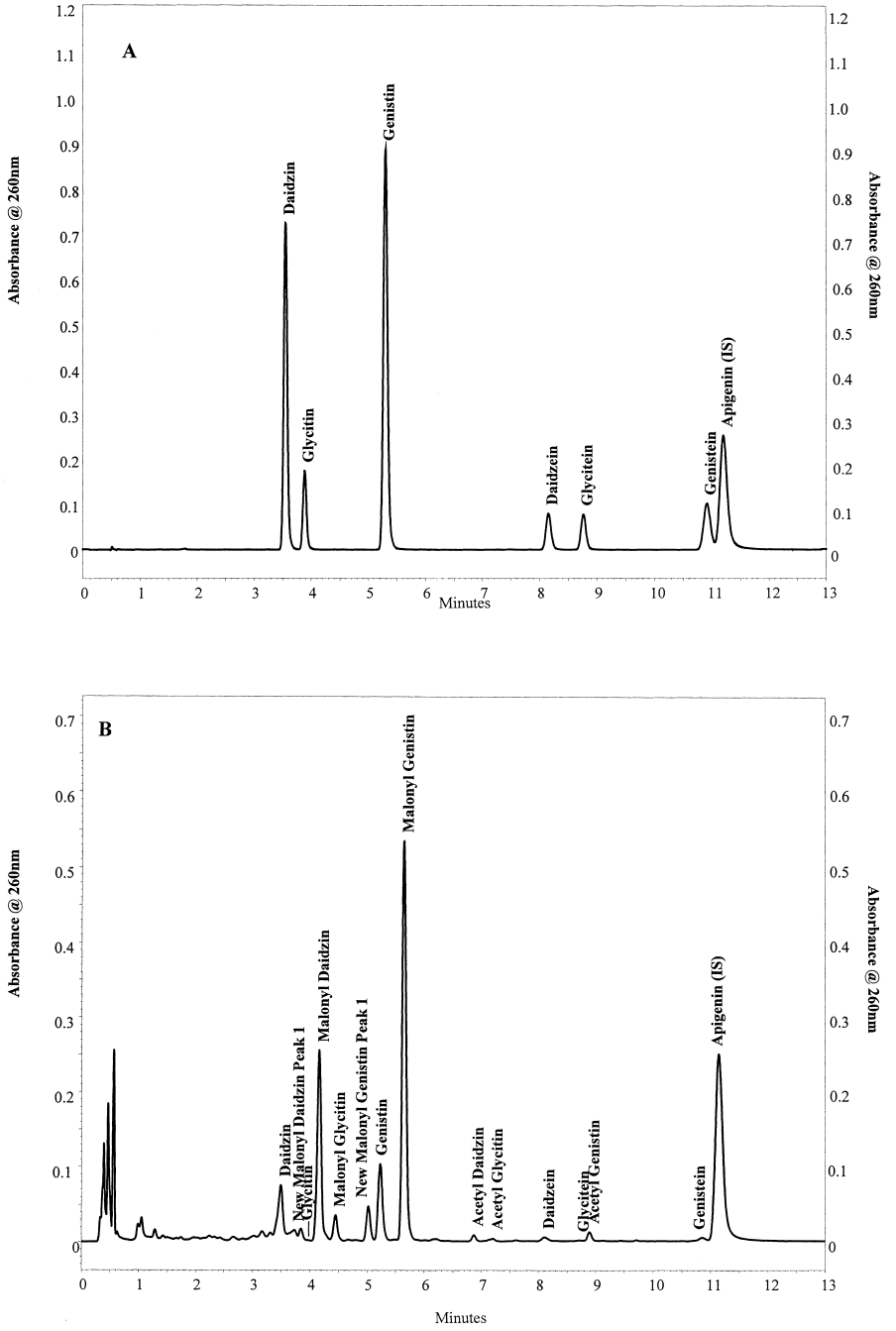

Fig. 8. Rapid method for analysis of isoflavones. (A) Standards; (B) commercial 40% isoflavone concentrate; (C) soy protein.

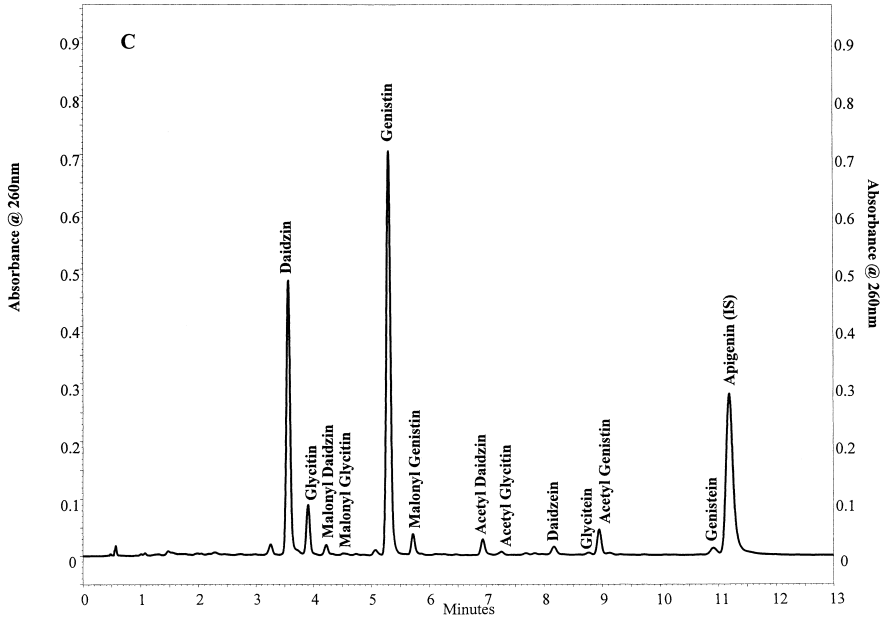

Fig. 8. (*continued*)

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