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Quantitative determination of isoflavones and coumestrol in soybean by column liquid chromatography

L.S. Hutabarat[a], H. Greenfield[a,*], M. Mulholland[b]

[a]*Department of Food Science and Technology, University of New South Wales, Australia P.O. Box 1, Sydney, NSW 2052, Australia*
[b]*Department of Chemistry, University of Technology, Sydney (UTS), Australia, P.O. Box 123, Sydney, NSW 2007, Australia*

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Abstract

Four different stationary phases and a variety of solvents in varying proportions were examined in this study. Daidzein, genistein, formononetin, biochanin A and coumestrol were separated within 24 min on a phenyl column with acetonitrile–water (33:67, v/v) as eluent. The proposed method showed an acceptable repeatability with a RSD of quantitation <6%. The mean recoveries of daidzein, genistein, formononetin, biochanin A and coumestrol from soybean ranged from 89 to 104%. The identity of the individual analytes was confirmed by LC–MS–MS. The four isoflavones and coumestrol were isolated from soybean by hydrolysis with acid and heat. Neutralization of the soybean samples prior to identification did not alter the concentration of daidzein and genistein in soybean.

Keywords: Soybean; Isoflavones; Coumestrol; Daidzein; Genistein; Formononetin; Biochanin A

1. Introduction

The potential impact of phytoestrogens on human and animals has led an ever-increasing interest in the study of these compounds in foods, particularly legumes. The common phytoestrogens found in soybean are isoflavones and coumestrol. Daidzein, genistein, formononetin and biochanin A which possess estrogenic activity are simple isoflavonoids. Coumestrol has also been reported to have 30- to 100-times the estrogenic activity of the isoflavones [1]. Both isoflavones and coumestrol are classified into a subclass of flavones. Isoflavones have a basic skeleton of 3-phenylchroman biogenetically derived by an aryl migration mechanism from the 2-phenylchroman skeleton of flavones [2]. Coumestrol is a coumarin-like compound with a close structural relationship to stilbestrol as well as to the natural estrogen, estradiol [2,3].

A successful method for the quantitative determination of daidzein, genistein, and biochanin A in soybean previously developed by these authors [4] could not separate daidzein, genistein, formononetin, biochanin A and coumestrol satisfactorily. In the present study, further work was performed to separate these analytes by means of a rapid liquid chromatographic (LC) procedure. The extraction and hydrolysis methods for the isolation of the isoflavones and coumestrol from dried, cooked and

*Corresponding author.

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canned soybeans, tofu and soymilk were also studied.

2. Experimental

2.1. Reagents and materials

Genistein, biochanin A and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, USA), daidzein from ICN (Aurora, OH, USA), and formononetin and coumestrol from Indofine (Belle Mead, NJ, USA). Hydrochloric acid, sodium hydroxide, acetic acid and sodium acetate were purchased from Ajax (Auburn, Australia), and dimethylsulfoxide (DMSO) from Merck (Kilsyth, Australia). Acetonitrile (HPLC-grade), methanol and isopropanol were obtained from EM Science (Gibbstown, NJ, USA), and ethanol from Chem-Supply (Gillman, Australia). Millipore water was used for all mobile phases. Dried soybeans (*Glycine max*) from McKenzie's (Altona, Australia), canned soybeans from Master Foods (Wyong, Australia), and Vitalife soymilk from Australian Natural Foods (Taren Point, Australia) were purchased from a local supermarket. Cooked soybean was prepared in the laboratory by boiling dried soybean from McKenzie's in water for 2 h and draining.

2.2. LC

Four columns examined were Alltima Cyano 100A (150×4.6 mm I.D., 5 μm) from Alltech (Baulkham Hills, Australia); and Symmetry (C_8) (150×3.9 mm); C_{18} Nova Pak (150×3.9× mm I.D., 4 μm) reversed-phase; and Phenyl Nova-Pak (150× 3.9 mm I.D., 4 μm), all three from Waters (Milford, MA, USA). All the LC analyses were performed with a Waters liquid chromatograph from Waters Australia (Rydalmere, Australia). The samples were injected with a Model 717 plus automatic sample injector and the samples were pumped by a Model 600 pump. The analytes were monitored and determined by UV-photodiode array detection (Model 996) from 200 to 400 nm.

2.3. LC–tandem mass spectrometry (MS–MS)

Analysis was performed on an API 300 triple-quadrupole mass spectrometer from Perkin-Elmer Sciex (Thornhill, Canada) equipped with an Apple Macintosh System 8.0 computer for data analysis. The isoflavones and coumestrol from a standard solution and from soybean were separated by reversed-phase LC on a 150×3.9 mm I.D., 4 μm, Phenyl Nova-Pak column at a flow-rate of 1 ml/min using an isocratic system of acetonitrile–water (33:67, v/v). Positive ions from solutes were introduced into the mass spectrometer following their generation by atmospheric pressure chemical ionization (APCI) caused by a corona discharge needle in the heated nebulizer interface of the instrument.

2.4. Preparation of standard solutions for calibration

The preparation of stock standard solutions for checking the purity of the authentic compounds and for calibration purposes used the procedure described by Hutabarat et al. [4]. A 5-μl volume of the standard solution were injected into the LC system for 30 min at a flow-rate of 0.80 ml/min.

2.5. Extraction of foods

A series of experiments was designed to determine the extraction efficiency of the analytes from dried soybean, cooked soybean, canned soybean, tofu and soymilk. Cooked soybean, canned soybean and tofu were freeze–dried with a Lyovac GT2 freeze dryer (Leybold-Heraeus, Koln, Germany) before milling through a Fritsch Mill/Pulverisette (Fritsch, Oberstein, Germany). Dried soybeans were also milled through a Fritsch Mill/Pulverisette. Each of the dried, cooked and canned soybean and tofu powders from the three purchases were pooled and mixed before analysis. All foods were analyzed in duplicate. A 1-g amount of food was extracted with 10 ml of 2 *M* HCl and 40 ml of 96% ethanol and refluxed with heating in a water bath at 100°C in 2 *M* HCl for 1, 2, 4 or 6 h. The extract solutions were adjusted to 50 ml with 96% ethanol. A 1.2-ml volume of the mixture was placed in a 1.5-ml Eppendorf tube (Bonnet, Taren Point, Australia) and was then cen-

trifuged at 800 *g* for 20 min in a centrifuge, Model Hettich EBA 12, (Tullingen, Germany). The clear supernatant was passed through a PTFE micro filter (0.20 μm pore size I.D.; 25 mm) (Dublin, CA, USA) before analysis by LC. The other experiments were carried out by extraction of 1 g of sample with 10 ml of 2 *M* HCl and 40 ml of 96% ethanol without refluxing, and extraction of 1 g of sample with 50 ml of 96% ethanol only, followed by refluxing with heating in a water bath at 100°C for 1, 2, 4 or 6 h.

The same experiment was carried out for soymilk. The extraction and hydrolysis procedures were similar to those for dried soybean. However, 25 ml of soymilk and HCl at pH 0.4, 0.6, 0.8, 1.0, 1.4 or 7.2 were used.

2.6. Neutralization

pH was adjusted to 2, 3, 4, 5, 6 or 7 by adding sodium hydroxide into the extracted samples. The mixtures were then centrifuged at 800 *g* for 20 min. The clear supernatants were filtered and stored in the freezer at −20°C for up to 28 days. The recoveries of the five analytes were determined by injecting 5 μl of solution into the LC system. The stability of the four isoflavones and coumestrol during storage in alkali and acid was also studied by adding NaOH or HCl to a mixture of the standards. The mixtures were then centrifuged at 800 *g* for 20 min and were stored in the freezer at −20°C for up to 28 days. The recovery was determined by LC analysis.

3. Results and discussion

3.1. Optimization of separation

Four stationary phases (C_8, C_{18}, phenyl, and cyano bonded silica) and seven solvents [acetonitrile, 1% or 10% aqueous acetic acid, isopropanol, methanol, ethanol, acetate buffer (pH 2.6) and water] were evaluated to optimize the separation of four isoflavones and coumestrol. No complete separation of the five analytes could be achieved on C_8, C_{18} or cyano columns with acetonitrile–1% aqueous acetic acid (33:67, v/v) [4], or a combination of acetonitrile with varying proportions of 1% or 10% aqueous acetic acid, water, or acetate buffer (pH 2.6) as eluent.

On the phenyl column, the five compounds could not be resolved using acetonitrile with varying proportions of methanol, ethanol, acetate buffer (pH 2.6), 10% aqueous acetic acid or isopropanol, or a combination of methanol with water or 1% aqueous acetic acid as eluent. Only the phenyl column with a mixture of acetonitrile and water or 1% aqueous acetic acid (33:67, v/v) could create the desired separation. The use of an eluent with acid was avoided in order to preserve the column; therefore, acetonitrile–water (33:67, v/v) was selected. This system produced sharp separation of coumestrol from genistein with all compounds eluted within 24 min (Fig. 1). The range of k' values was close to ideal (1.1–11.1) (Fig. 2).

The elution order of daidzein, coumestrol, genistein, formononetin, and biochanin A was different from that of Setchell and Welsh [5] and Franke et al. [6] who reported the elution order as daidzein, genistein, coumestrol, formononetin and biochanin A. The stationary phases and eluent used by these authors were C_{18} with methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v) and, C_{18} with a gradient system of acetonitrile and 1% acetic acid, respectively. The maximum absorption of daidzein was achieved at a wavelength of 249 nm with a shoulder at 302 nm, coumestrol at 342 nm with a shoulder at 244 nm, genistein at 259 nm, formononetin at 249 nm with a shoulder at 302 nm and biochanin A at 260 nm.

To confirm the correct identity of the peaks obtained by LC–UV, a mixture of five standards was analyzed by LC–MS–MS. The chromatogram obtained is shown in Fig. 3. No significant difference in the chromatographic resolution was observed between LC–UV detection and LC–MS–MS. The retention times obtained by LC–MS–MS were very close to those obtained by LC–UV. Selected peaks from Fig. 3 were then extracted using positive acquisition mode over a m/z range from 150 to 600. Peaks 1–5 showed a major ion product at m/z 254.6, 269.0, 270.8, 269.2 and 284.8, respectively (Fig. 4). Peaks 1–5 were identified as daidzein (M_w=254) (A), coumestrol (M_w=268) (B), genistein (M_w=270) (C), formononetin (M_w=268) (D) and biochanin A (M_w=284) (E). In conclusion, the elution order of

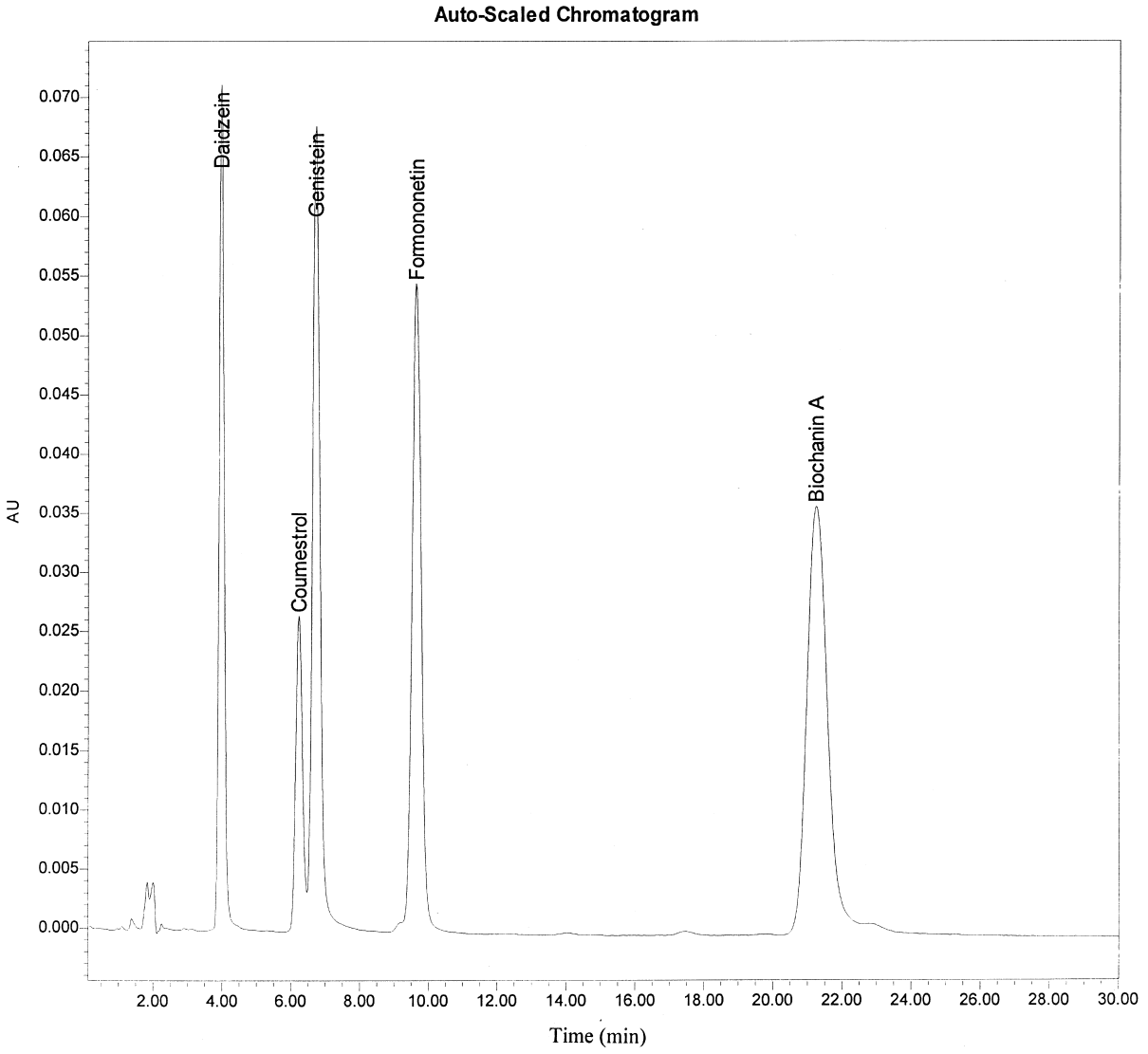

Fig. 1. LC–UV chromatograms of standards daidzein, coumestrol, genistein, formononetin and biochanin A using a phenyl column and acetonitrile–water (33:67, v/v) as eluent.

daidzein, coumestrol, genistein, formononetin, and biochanin A was confirmed.

3.2. Analytical performance

The analytical performance is summarized in Table 1. The detector responses were linear with the coefficient of determination $r^2>0.999$ over the concentration range from 0.02 to 11 mg/l expected for soybean foods. The detection limits of each compound were monitored at the analytes' absorption maximum and were calculated based on a signal-to-noise ratio of 3:1. The detection limits for daidzein, coumestrol, genistein, formononetin and biochanin A were found to be 47, 82, 76, 75,and 224 n*M*, respectively. This study used UV detection for coumestrol, while Wolfbeis and Schafner used fluoresence detection which determines coumestrol

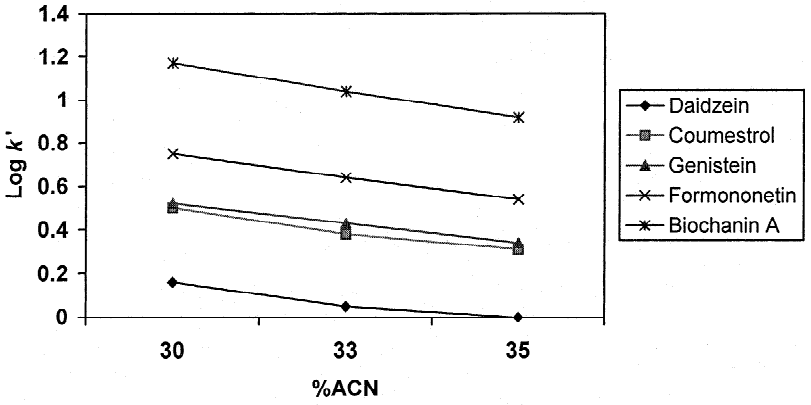

Fig. 2. Capacity factor (k') of daidzein, coumestrol, genistein, formononetin and biochanin A from 30 to 40% acetonitrile in water.

down to concentrations as low as 50 n*M* [7]. However, UV detection of coumestrol at a concentration of 82 n*M* in ethanol is acceptable.

The repeatability and reproducibility of the method were evaluated by carrying out six replicate determinations on the same day and six on three different days. Inter-assay relative standard deviations (RSDs) were 0.3–0.6% for elution times and

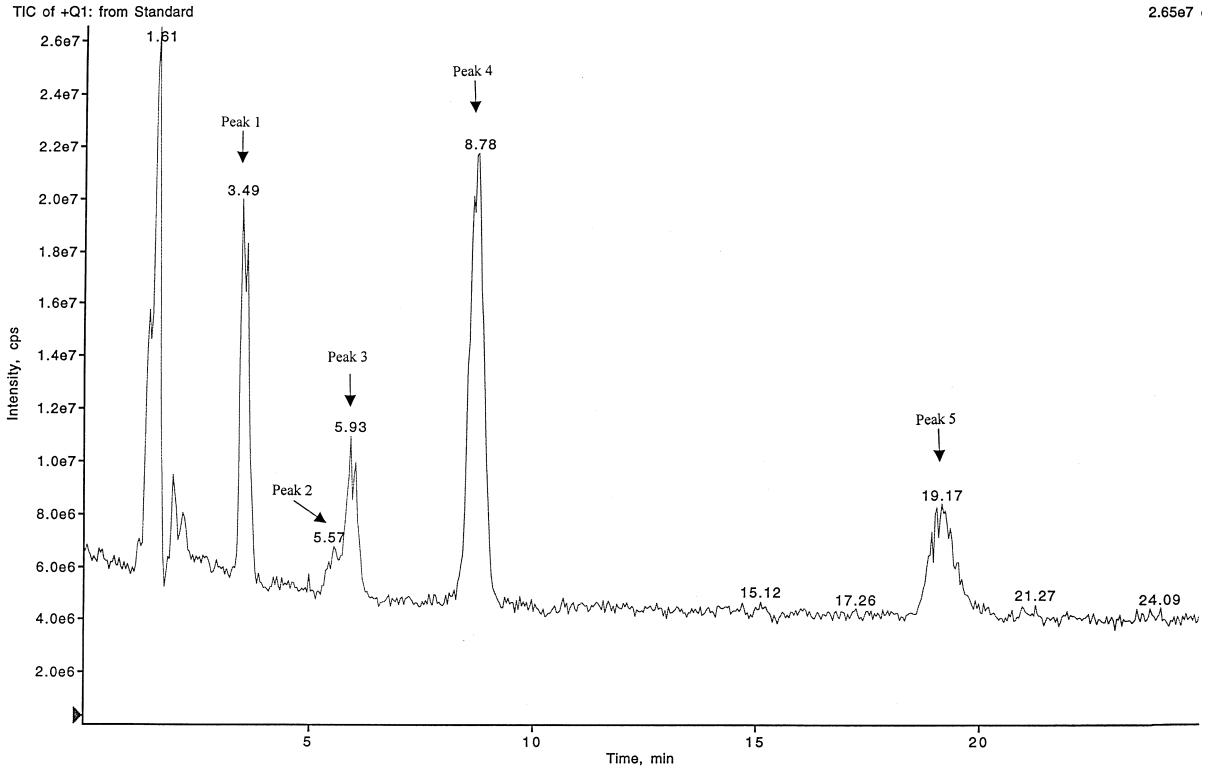

Fig. 3. LC–MS–MS chromatogram of standards daidzein, coumestrol, genistein, formononetin and biochanin A using a phenyl column and acetonitrile–water (33:67, v/v) as eluent.

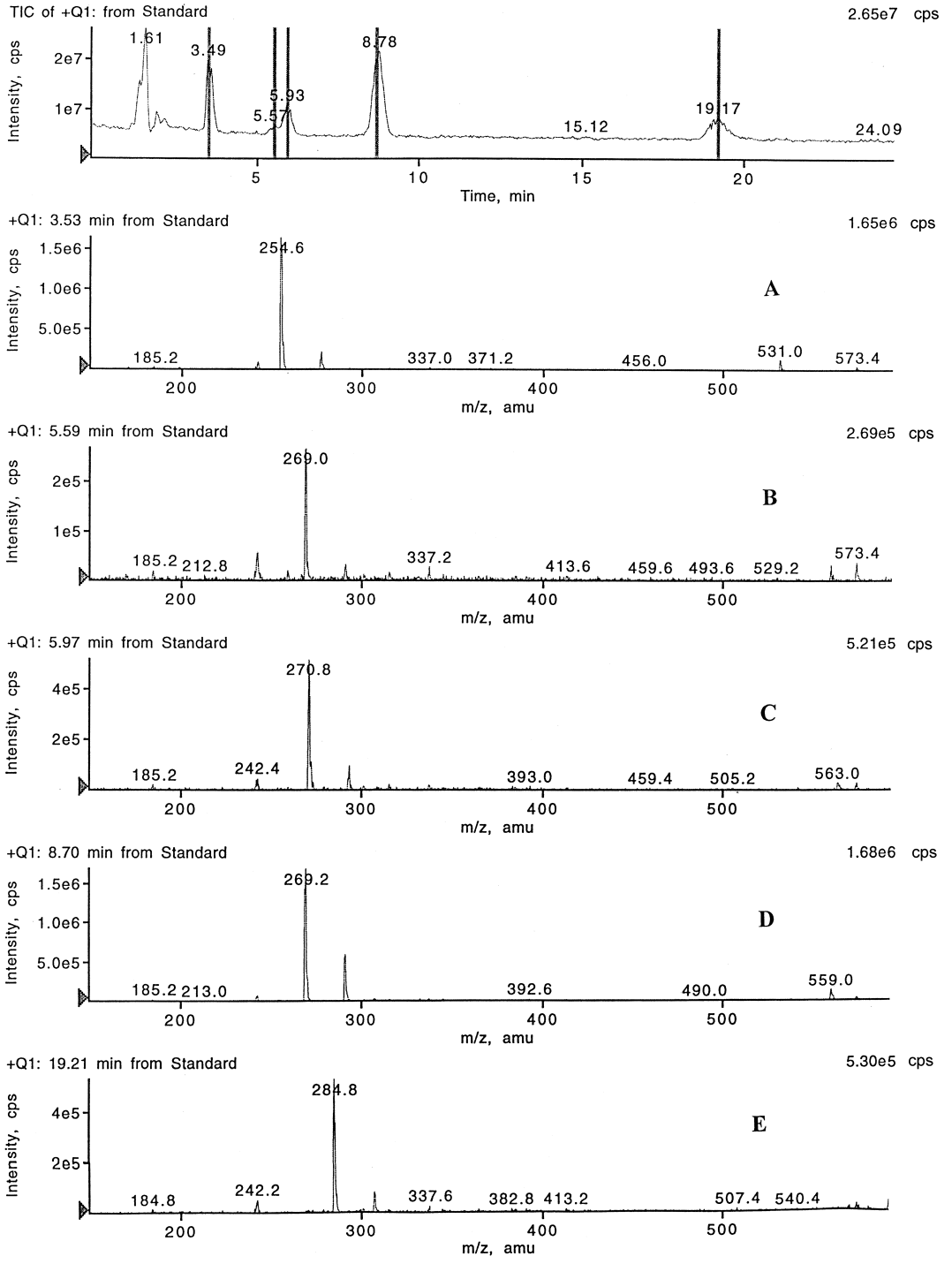

Fig. 4. Positive ion APCI mass spectra recorded during the LC–MS–MS analysis shown in Fig. 3 including (A) peak 1 is daidzein at the *m*/*z* of 254.6, (B) peak 2 is coumestrol at the *m*/*z* of 269.0, (C) peak 3 is genistein at the *m*/*z* of 270.8, (D) peak 4 is formononetin at the *m*/*z* of 260.2, and (E) peak 5 is biochanin A at the *m*/*z* of 284.8.

Table 1
Data on analytical performance

Compound	Linearity r^2	Precision (RSD, %)			
		Inter-assay		Intra-assay	
		Retention time	Quantitative analysis	Retention time	Quantitative analysis
Daidzein	0.999	0.3	3.8	1.0	4.0
Coumestrol	0.999	0.6	3.1	1.6	6.4
Genistein	0.999	0.4	5.7	1.5	4.0
Formononetin	0.999	0.5	0.7	1.6	2.4
Biochanin A	0.999	0.6	3.9	2.1	6.8

0.7–5.7% for quantitative analysis of the individual compounds. Intra-assay RSDs were 1.0–2.1% for elution times and 2.4–6.8% for quantitative analysis of individual compounds.

UV scans of daidzein and genistein isolated from both soybean and soybean spiked with authentic compounds were found to have the same absorbance patterns as the standards. Positive ion spectra of daidzein and genistein isolated from soybean were also similar to their standards. The method of extraction and hydrolysis in these experiments closely followed the procedure of Franke et al. [6]. The method has the advantage that it is relatively rapid. The recoveries of daidzein, coumestrol, genistein, formononetin and biochanin A were determined by adding the standards into the soybean foods over the concentration range 0.4–3 mg/l. The spiked samples were then processed through the entire procedure. All experiments were carried out in triplicate. The mean recoveries of each compound were 104% for daidzein, 94% for coumestrol, 93% for genistein, 99% for formononetin, and 89% for biochanin A.

3.3. Extraction of foods

Dried, cooked, canned soybean, tofu and soymilk examined in this study were found to contain only daidzein and genistein (see Fig. 5 for dried soybean). The levels of daidzein and genistein in raw, cooked and canned soybean, tofu and soymilk were significantly higher with acid hydrolysis (pH 0.6) than without acid hydrolysis (pH 7). The level of daidzein and genistein in dried soybean, cooked soybean and tofu increased with the increase in refluxing times. A 4 h time of reflux was sufficient to give the highest yield of daidzein and genistein from canned soybean. The higher level of daidzein and genistein in soymilk was achieved after refluxing for 6 h at higher pH (0.8–0.4). The maximum levels of daidzein and genistein that could be isolated and identified were 96 mg and 61 mg/100 g for dried soybean, respectively, 92 mg and 69 mg/100 g for cooked dried soybean, respectively, 26 mg and 65 mg/100 g for canned soybean, respectively, 3.6 mg and 3.8 mg/100 g for soymilk, respectively. All the values are presented on a wet mass basis.

Neutralization was carried out on the samples after the extraction of the samples because the pH of the samples after extraction was found to be less than 1. The mean recoveries of daidzein and genistein in soybean did not change after adjusting with sodium hydroxide up to pH 6 or after storage up to 28 days at −20°C ($P>0.05$). This result was supported by a study on the stability of standard daidzein and genistein during storage in alkali and acid. The mean recoveries of daidzein (105±6.2%) and genistein (102±2.7%) did not change significantly ($P>0.05$) after the addition of sodium hydroxide (pH 10–12) or hydrochloric acid (pH 3–6) to a standard solution and storage up to 28 days at −20°C.

4. Conclusions

Isocratic LC–UV on a phenyl column and acetonitrile–water (33:67, v/v) as eluent can be used to separate and quantitate isoflavones and coumestrol in soybean. This method is superior to published gradient LC methods because it is rapid, simple and less detrimental to column life. Separation could be achieved within 24 min and the isoflavones and coumestrol in standard solutions and in samples could be determined at concentration as low as 47 n*M*.

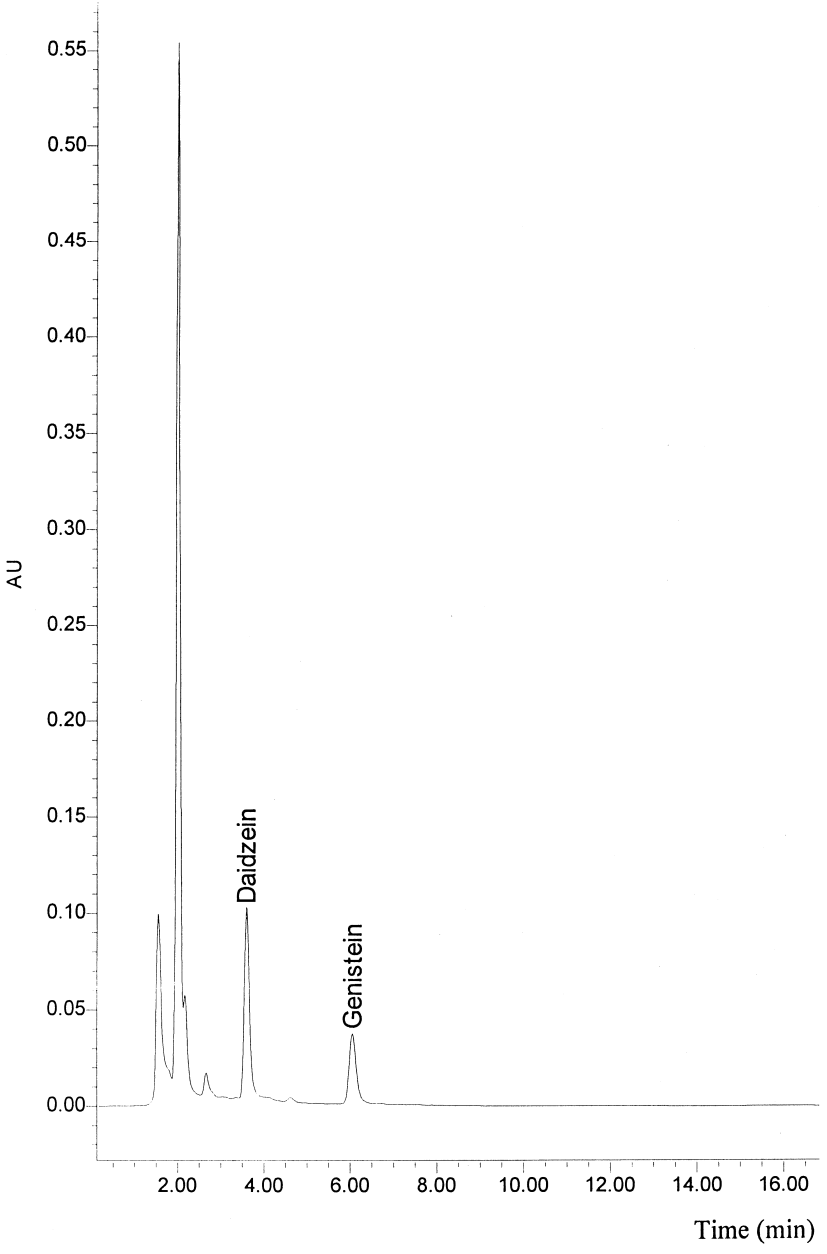

Fig. 5. LC–UV chromatograms of daidzein and genistein from dried soybean seeds using a phenyl column and acetonitrile–water (33:67, v/v) as eluent.

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