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Short communication

Development and validation of an isocratic high-performance liquid chromatographic method for quantitative determination of phytoestrogens in soya bean

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Abstract

High-performance liquid chromatography (HPLC) is the technique most often used for the separation and quantification of phytoestrogens in various plants and food products. A number of HPLC methods using gradient elution have been developed for determination of phytoestrogens. These HPLC methods have been applied to the separation and quantification of phytoestrogens in various sample matrices. However, gradient elution methods are time consuming as they necessitate lengthy equilibration times and they require specialised pumps. This paper describes the development and validation of a reversed-phase HPLC method with isocratic conditions for the analysis of daidzein, genistein and biochanin A in soya beans using flavone as an internal standard. The proposed method has an overall elution time of about 24 min which compares well with elution times of 20–60 min for the gradient methods. Results for the linearity, sensitivity, precision and accuracy of the method are presented. © 1998 Elsevier Science B.V.

Keywords: Food analysis; Soybean; Phytoestrogens; Estrogens; Daidzein; Genistein; Biochanin A; Flavone

1. Introduction

Plant foods contain a large number of compounds such as phytoestrogens which, although possessing no nutritional properties, do appear to have bioactive roles, e.g. protection against certain cancers. Studies have suggested that phytoestrogens possess a similar activity to the natural hormone, estrogen. It is the further investigation of these properties that prompts the interest in developing a rapid, accurate and precise method for their analysis in foods.

Various techniques have been applied to the analysis of phytoestrogens in plants, human fluids and food products. A gas–liquid chromatographic (GLC) method was developed by Naim et al. [1] to isolate and quantify isoflavonoid phytoestrogens in 1974. To assay phytoestrogens by GLC a derivatization step is required making the sample preparation cumbersome. HPLC does not require derivatization and thus many workers in the 1980s and 1990s have attempted to use this technique for phytoestrogens. Several HPLC methods have been tested using various mobile phases and columns. The most commonly used HPLC method applies a linear gradient for the separation and quantitative analysis of isoflavonoids compounds from soya bean and many soya bean based products [2–8]. Murphy [9] and Franke et al. [10,11] have also reported on the use of

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HPLC with non-linear gradient elution for the determination of isoflavonoid and coumestan phytoestrogens from many legumes and legume-based products.

West et al. [12] used HPLC analysis to separate the isomeric trihydroxyisoflavones and genistein from soya bean with isocratic conditions. However, this method was not applicable to phytoestrogens other than genistein.

More recently isotope dilution gas chromatography with mass spectrometry has been applied for the isolation and quantitative analysis of phytoestrogens in various plant foods and food products [13]. This method allows both quantitative and qualitative information to be sought, however, derivatization is again required. The instrumentation is very expensive and so is the production of the relevant isotopic standards. Thus this method is unsuitable for routine analysis.

Phytoestrogens exist in plant and food products as complex mixtures, therefore most investigators prefer gradient conditions for HPLC to maximise the separation of these mixtures. As previously mentioned, gradients require equilibration times and special HPLC pumps hence it was considered worthwhile to investigate the potential for an isocratic separation. The work described in this paper succeeded in obtaining an isocratic HPLC method to separate daidzein, genistein and biochanin A in soya beans. The starting point of the method optimization was based on the conditions described by Franke et al. [10,11].

2. Experimental

2.1. Apparatus

The HPLC analysis was carried out on a Waters liquid chromatograph with an auto sampler model 717 plus and a model 600 controller pump connected to a photodiode-array detector model 996 (Waters Australia, Rydalmere, Australia). The column used from Waters (Waters, Milford, MA, USA) was a reversed-phase C_{18} Nova-Pak (150×3.9 mm I.D.; 4 μm). The optimized mobile phase was 33% acetonitrile (A) and 67% water–acetic acid (99:1, v/v) (B) at a flow-rate of 0.80 ml/min. The wavelength ranged from 200 to 400 nm throughout the chromatogram and each peak is plotted using the wavelength that provides a maximum response. This facility is available on the HPLC system employed for this work, however, a single wavelength of 280 nm was used for most of the work as this is more easily used on a wide range of detectors. The maximum absorption of daidzein was 249.8 nm with a shoulder at 301.9 nm; the maxima for genistein and biochanin A were 259.2 nm, and for flavone 254.5 nm with a shoulder at 297.1 nm.

2.2. Reagents and materials

Genistein, biochanin A, and flavone were purchased from Sigma (St. Louis, MO, USA). Daidzein was obtained from ICN (Aurora, OH, USA), ethanol from CSR (Pyrmont, Australia), hydrochloric acid from Ajax (Auburn, Australia), dimethyl sulfoxide (DMSO) from Pierce (Rockford, IL, USA), and butylated hydroxytoluene (BHT) from Sigma. Acetonitrile (HPLC grade) and acetic acid were purchased from Rhone Poulenc (Clayton, Australia) and Bowyer cultivar of soya bean seeds (*Glycine max*) from Allgold Foods (Leeton, Australia). Millipore water was used for all mobile phases.

2.3. Purity of standard

In order to test the purity of the reference materials, genistein, biochanin A, daidzein and flavone were dissolved separately first in 20 μl DMSO follow by the addition of 96% ethanol. The purity of the standards was checked by injecting the stock solution into the HPLC. The solutions were then monitored at each individual compounds absorption maximum. The purity (percent) of the standards was calculated by dividing the peak area of the compounds by all peak areas in the chromatogram and multiplying by 100. All compounds were >95% pure and none were discarded [10].

2.4. Preparation of stock standard solution

The stock standard solution was prepared in duplicate according to the method of Franke et al. [10,11]. Approximately 10 mg each of daidzein, genistein, biochanin A and flavone were accurately

weighed and dissolved first in 20 ml DMSO followed by the addition of 96% of ethanol in water. The stock solutions were stored at −20°C prior to use.

2.5. Calibration curve

Each standard solution was diluted with mobile phase to seven different concentrations. The final concentrations of the solutions were in the range of 11 to 800 m*M*. Analysis of each concentration was replicated by injecting 30 ml three times.

2.6. Food sample extraction and sample spike

Whole soya bean seeds (dried, raw) were ground to a fine powder and extracted by the method described by Franke et al. [10,11] with slight modification. Samples were prepared in triplicate. The soya bean powder (1 g) was placed into a 50 ml round-bottomed flask. This was then spiked with various volumes of stock solution containing 20% of standard daidzein, genistein, and biochanin A. Then 2 *M* HCl (10 ml), 40 ml 96% ethanol containing 0.05% BHT and 20 ppm flavone were added. The mixture was then stirred and ultra-sonicated for 20 min followed by refluxing at 80°C for 1 h. The extract was cooled to room temperature and transferred into a 50 ml volumetric flask. The ethanol lost during refluxing was replaced. At that stage 1.2 ml of the mixture was placed in an 1.5 ml eppendorf tube (Bacto, Australia) and was centrifuged at 800 *g* for 10 min in a centrifuge model EBA 12 (Hettich Scientific, Australia). The clear supernatant was filtered through a PTFE micro filter (0.20 μm pore size I.D.; 25 mm) (Dublin, CA, USA) and was kept in an HPLC vial. The sample was stored at −20°C until HPLC analysis was carried out.

3. Results and discussion

3.1. Separation

The separation of phytoestrogens in soya beans was optimized in two stages, optimization of the solvent strength and optimization of the pH control.

Retention times were measured over a range of acetonitrile–water compositions. From the previous gradient method [10,11] it could be assumed that an isocratic separation would be found between 30% and 40% (v/v) acetonitrile in water–acetic acid (99:1, v/v). The mobile phase for previous gradient methods was acetonitrile (A) and water–acetic acid (90:10, v/v) (B). The elution system was gradient with 23% (v/v) A in B linearly to 70% A in B in 8 min followed by holding at 23% A in B for 12 min. This change to 23% from 70% acetonitrile occurred before all the peaks had eluted. This is not a sensible way to apply gradients, and the column is adversely affected by such a dramatic change in concentration of the mobile phase, and the associated pressure changes. It seems likely that this may have been a typographical error in the original paper and the intention was to hold at 70% rather than 23% for 20 min. However, all subsequent papers also used these unsuitable conditions.

The capacity factors (k') of the phytoestrogens were equal to the ratio of the difference of the relative retention time of analytes with the dead time. The dead time was estimated using the clear solvent front peak. A plot of log k' against solvent strength is displayed in Fig. 1. The chromatograms for both the standard solution and the spiked soya bean sample with different solvent strength are shown in Fig. 2. There was no separation which had an ideal k' range of 1–10, however, a compromise solution with a k' range of 0.92–14.16 was selected. The optimum composition was 33% acetonitrile and 67% water–acetic acid (99:1, v/v).

Efforts were made to reduce the amount of acetic acid in water (10%) used by the Franke et al. method [10,11] to 1% because of possible adverse effects of acid on the column. The reduction of acetic acid concentration did not cause noticeable deterioration in method performance. The capacity factors and R.S.D. values of daidzein, genistein, biochanin A and flavone using 10% acetic acid were 0.60±4.69, 1.24±1.52, 6.68±1.28 and 9.22±1.34, respectively.

Mobile phase pH showed an effect on the retention time of the analytes. A decrease in mobile phase pH decreased the retention time. The use of 1% acetic acid (pH 2.7) in the HPLC mobile phase led to slightly different resolution of the phytoestrogens compared to 10% acetic acid (pH 2.2). The

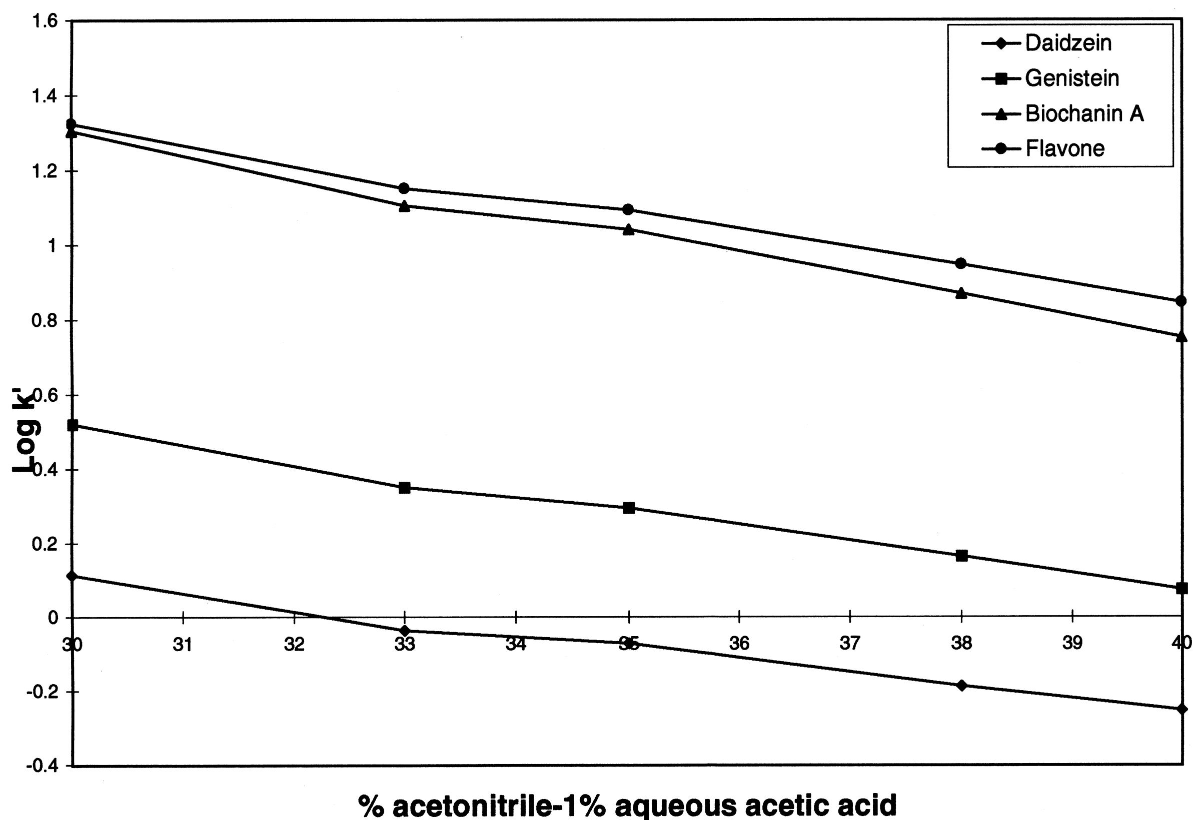

Fig. 1. Capacity factor (k') of standard daidzein, genistein, biochanin A and internal standard of flavone from 30 to 40% acetonitrile in 1% aqueous acetic acid.

rest of the conditions were not altered and are described in Section 2.

The separation of all analytes was achieved in less than 24 min with the mobile phase composition at 33% acetonitrile and 67% water–acetic acid (99:1, v/v). Daidzein eluted at 3 min ($k'=0.92$), genistein at 5 min ($k'=2.24$), biochanin A at 21 min ($k'=12.76$) and flavone (the internal standard) at 23 min ($k'=14.16$). This overall run time is more rapid than some of the gradient methods described by other investigators [4,6–12,14].

3.2. Validation of the method

3.2.1. Linearity

Linear curve fits were obtained from seven different concentrations using duplicate stock solutions and three replicate injections. Each analyte showed a linear fit with a R.S.D. of >0.999. This compares well with other methods ([10,11]).

3.2.2. Precision

The proposed HPLC method showed acceptable repeatabilities for each of daidzein, genistein and biochanin A. R.S.D. values were obtained from six injections of a mixture of phytoestrogens and the largest values were 1.26% and 1.51%.

3.2.3. Recovery

The recoveries of daidzein, genistein and biochanin A were measured by the addition of the standards into the soya bean samples over a concentration range from 2 to 20 μg/ml. The spiked samples were then processed through the whole procedure.

The method showed good recoveries that were

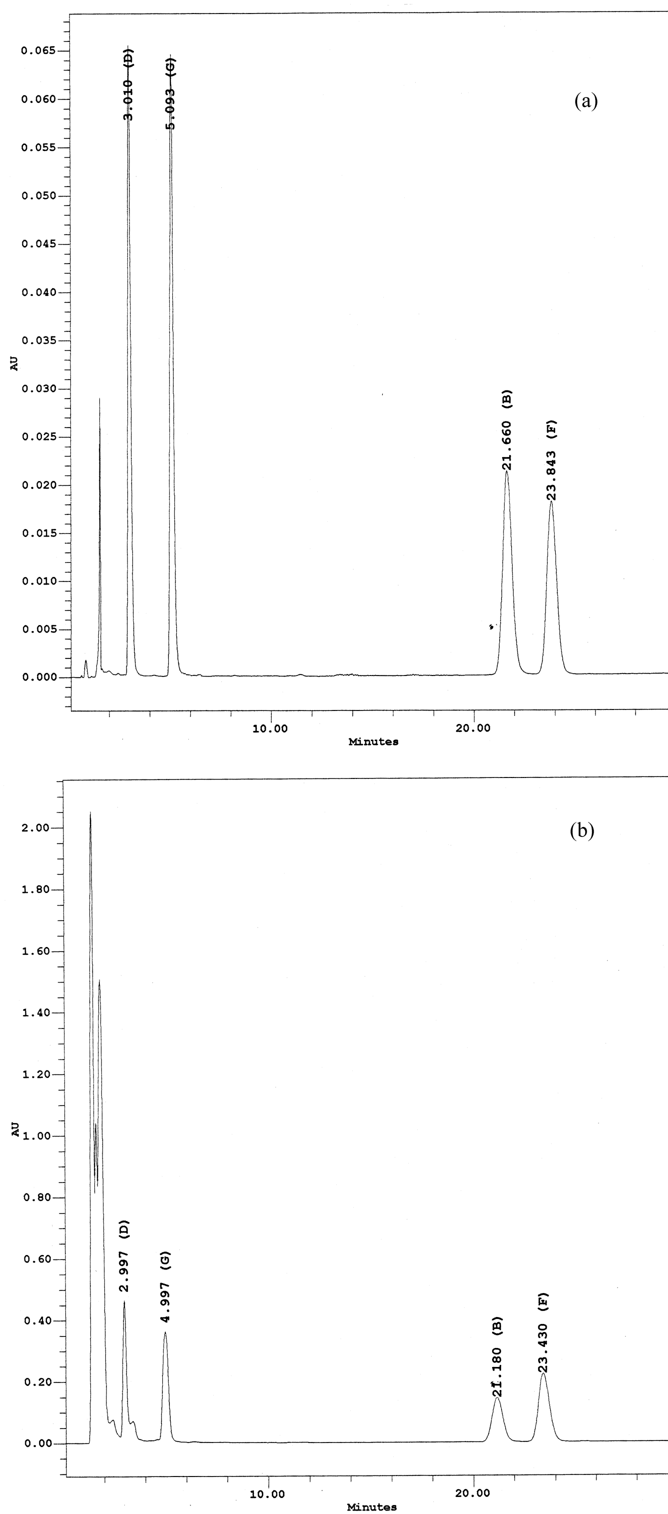

Fig. 2. Chromatogram of (a) standard and (b) soya bean spiked with standard (D, daidzein, G, genistein, B, biochanin A and internal standard F, flavone); mobile phase: 33% acetonitrile and 67% acetic acid–water (1:99, v/v).

close to 100%. The mean recoveries of the individual standards ranged from 100 to 127% for daidzein, 101 to 114% for genistein and 97 to 135% for biochanin A. The higher recoveries (>100%) were found for the lower amounts of spiked standard. This was probably due to small amounts of co-eluting impurities in soya bean which were more significant at the lower spike levels.

4. Conclusions

In conclusion, in the proposed procedure, the isocratic elution conditions resulted in shorter separation times for a mixture of daidzein, genistein and biochanin A compared to previous methods. Daidzein and genistein produced the sharpest peaks, while the biochanin A peak exhibited some broadening but was still within an acceptable range. This is supported by the high linearity of the response to concentration of standards. This method was chosen for further work on phytoestrogens in legumes as it was quicker, safer and less detrimental to column life than the other methods described in the literature. This method was also selected due to its suitability for automation and treatment of large numbers of legume samples.

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