Simple extraction and rapid quantitative analysis of isoflavones in soybean seeds

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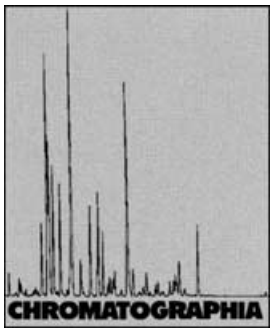

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Abstract

A simple extraction and rapid HPLC method, suitable to separate and quantify the isoflavonoids in soybean seeds, are proposed and discussed. The method has been applied to the separation and quantification of seed isoflavonoids in five naturally grown soy cultivars; the total amount of isoflavones ranged from 1.83 g kg^{-1} to 11.88 g kg^{-1} of fresh weight. The detection limits (200 $\mu g/kg^{-1}$), evaluated for genistein and daidzein, are presented together with a list of the soy polyphenols analysed by HPLC-DAD and HPLC-MS.

Keywords

Column liquid chromatography
Diode array and MS detection
Isoflavones
Soya bean seeds
Flavonoids

Introduction

Isoflavonoids are a category of polyphenols, mainly found in legumes such as soybeans [1–3]. The health benefits of soybean have been extensively investigated. Isoflavonoids may prevent certain cancers [4, 5], reduce the risk of osteoporosis [6], have a beneficial role in chronic renal disease [7], lower plasma cholesterol [8], exhibit an anti-atherosclerotic activity [9] and decrease the risk of coronary heart disease [10]; they have sterile or estrogenic activities [1]. Isoflavonoids may act as antioxidants and especially genistein shows one of the highest antioxidant activities among dietary flavonoids [11].

The chemical composition of soybean seeds has been thoroughly studied: genistein, daidzein and their glycosides have been the major isoflavones identified [12].

The identification and separation methods of isoflavones in food and biological fluid have been recently reviewed [13]. Among the different separation methods, HPLC plays an important role: the main improvements concern the kind of material from which isoflavones must be extracted and the detection limits. Also the time needed to perform the complete analysis plays an important role; in fact the presence of polyphenols in food increases both quality and commercial value since the food may subsequently be regarded as "health food".

The aim of this study was to optimise a rapid chromatographic method to characterise and quantify the isoflavones content of soybeans seeds, one of soy's edible parts. The method was then applied to five different soy cultivars grown under natural conditions.

Experimental

Sample Preparation and Extraction of Polyphenols

Plant Material

Seeds of five soybean cultivars (Ciesse "Emiliana", Pioner "Elvir", Asgrow "Kure", "Sapporo" and "Cresir"), grown in Italy, under natural conditions, were analyzed.

Standards

Authentic standards of daidzein genistein and formononetin were purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France).

Solvents

All the solvents used were of HPLC grade purity (BDH Laboratory supplies, England).

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Table 1. The linear solvent gradient system used in HPLC-DAD and HPLC-MS analysis of polyphenols in soy seeds. (Analysis was carried out during a 27-min period at flow rate of 0.8 mL min^{-1} using a 150 × 3.9 mm (4 μm) Nova Pak C18 column operating at 26 °C.)

Time	H_2O/H^+	CH_3CN
0.1	95%	5%
3	95%	5%
13	80%	20%
15	80%	20%
23	55%	45%
25	0	100%
27	0	100%

Table 2. The linear solvent gradient system used in HPLC-DAD and HPLC-MS analysis of polyphenols in soy seeds. (Analysis was carried out during a 14-min period at flow rate of 0.8 mL min^{-1} using a 150 × 3.9 mm (4 μm) Nova Pak C18 column operating at 26 °C.)

Time	H_2O/H^+	CH_3CN
0.1	91%	9%
12	70%	30%
13	0	100%
14	0	100%

Sample Preparation

The seeds were ground with a mill. A quantity of 100 mg of seed flour was extracted over one night with 30 mL of ethanol/water (70/30, *v*/*v*) adjusted to pH 2.0 by HCOOH. The raw ethanolic extracts were defatted with 15 mL of *n*-hexan. The ethanolic extracts were then evaporated to dryness under vacuum (Rotavapor 144 R, Büchi, Switzerland) at room temperature and finally rinsed with ethanol/water (70/30, pH 2.0) to a final volume of 1.5 mL.

Samples of 4 μL were analysed by HPLC-DAD (Diode Array Detector) and HPLC-MS for the qualitative and quantitative evaluation.

Analytical Techniques and Equipment

HPLC-DAD Analysis

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, California, USA). Polyphenolic compounds were separated using a 150 × 3.9 mm (4 μm) Nova Pak C18 column (Waters Corporation, Massachusetts, USA) operating at 26 °C. The mobile phase was a four-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 by H_3PO_4) up to 100% CH_3CN during a 27-min period [Table 1] [14], and a three-step linear solvent system, starting from 91% H_2O (adjusted to pH 3.2 by H_3PO_4) up to 100% CH_3CN during a 14-min period [Table 2]. In both cases the flow rate was 0.8 mL min^{-1}.

HPLC-MS Analysis

HPLC-MS analyses were performed using a HP 1100 MSD API, ESI interface, coupled with a HP 1100L liquid chromatography equipped with a DAD detector (Agilent Technologies). The HPLC-MS analysis was performed using the same HPLC-DAD condition with water adjusted to pH 3.2 by HCOOH. Mass spectrometer operating conditions were: nitrogen gas temperature 350 °C at a flow rate of 12 L min^{-1}, nebulizer pressure 30 psi, quadrupole temperature 30 °C, and capillary voltage 3500 V. The mass spectrometer operated in positive and negative mode at 80–180 eV fragmentor values.

Results and Discussion

Recent papers have dealt with the separation of phytoestrogens in soybeans, soy foods and human biological fluids [13, 15]. In some of these works the proposed analytical methods involve the sample hydrolisation and quantitation of the obtained aglycons. Owing to the different biological activities of isoflavones [16], we used a non-hydrolytic method; therefore the HPLC method should allow separation either of the isoflavone aglycons and the glycosides. As regards the extraction, the choice of ethanol instead of acetonitrile [17] as extraction solvent was made on the basis of its lower boiling point, lower toxicity and lesser environmental problems. On the other way with our samples the difference in the extraction yield between the two solvents was lesser than 5% and all the compounds were extracted. Furthermore, notwithstanding the possibility of conversion from malonylglucoside derivative to glucoside ones [18] we did not found any difference in the ratio malonylglucoside/glucoside comparing two different extraction times (3 and 24 h). The extraction yield (95%) was controlled adding formononetine (7-hydroxy-4'-methoxy-isoflavone) as internal standard. The choice of this molecule was based on its absence in our samples, and on its retention time which falls in an empty zone of the chromatogram (R_t = 25.08 min).

The chromatographic profile of a seed flour extract recorded at 260 nm is reported in Fig. 1. The chromatogram was obtained with the Nova Pack C18 column, using the "long" method which has already successfully been used in the separation of isoflavones [14].

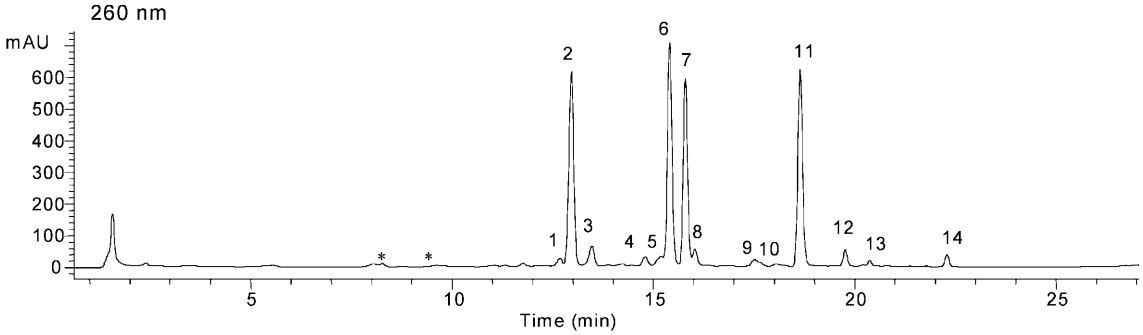
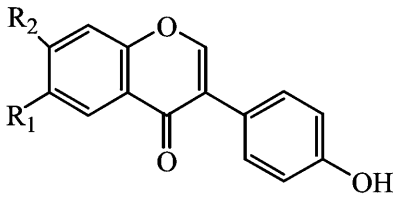
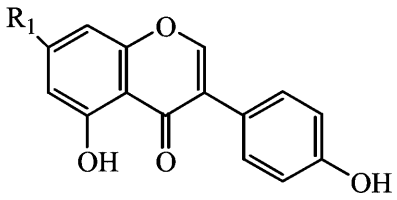


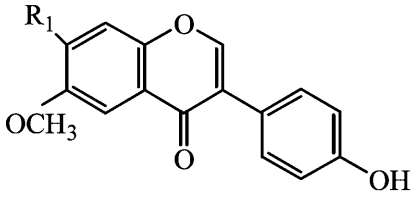
Fig. 1. Chromatographic profile, acquired at 260 nm by HPLC-DAD, of a hydroalcoholic extract from soy seeds at relative maximum of absorbance of isoflavonoids, obtained with a 150 × 3.9 mm (4 μm) Nova Pak C18 column. The elution method is reported in Table I. Polyphenolic compounds: **1** OH-daidzin, **2** Daidzin, **3** Glycitin, **4** Daidzein malonylglucoside, **5** Genistein glucoside, **6** Genistein glucoside, **7** Unknown daidzein malonylglucoside isomer, **8** Glycitein malonylglucoside, **9** Geinistein malonylglucoside, 1**0** Daidzein acetylglucoside, **11** Unknown genistein malonylglucoside isomer, **12** Daidzein, **13** Genistein acetylglucoside, **14** Genistein, * Caffeic acid derivatives



COMPOUNDS	R_2	R_1	MW
6-OH-Daidzein	OH	OH	270
Daidzein	OH	H	254
Daidzein glucoside	O-Gluc.	H	416
Daidzein malonyl glucoside	O-malonyl gluc.	H	502
Daidzein Acetyl glucoside	O-acetyl gluc.	H	458



COMPOUNDS	R_1	MW
Genistein	OH	270
Genistein glucoside	O-Gluc.	432
Genistein malonyl glucoside	O-malonyl gluc.	518
Genistein Acetyl glucoside	O-acetyl gluc.	474



COMPOUNDS	R_1	MW
Glycitein	OH	284
Glycitein glucoside	O-Gluc.	446
Glycitein malonyl glucoside	O-malonyl gluc.	532
Glycitein Acetyl glucoside	O-acetyl gluc.	488

Fig. 2. Structural formula of the main isoflavones detected in soy seeds

The isoflavones were identified by means of their retention times and UV-Vis spectra compared with commercial standards of genistein and daidzein. In the case of glycosides, whose standards are lacking, UV-Vis and mass spectra was used. The fragmentation pattern of genistein and daidzein malonylglucoside shows signals at m/z 519 and 271, and at m/z 502 and 255 respectively similar to those described by Griffith and Collison [20].

Individual polyphenols were quantified with a four-point regression curve ($r^2 \geq 0.9998$) operating in the range 0–10 μg on the basis of authentic standards, and the calibration was directly performed by HPLC-DAD.

The isoflavones detected in the soy seed extracts and their chemical structures are reported in Fig. 2.

In particular, genistein and glycitein derivatives were calibrated at 260 nm using genistein as reference compound; daidzein derivatives were calibrated at 305 nm using daidzein as reference compound. In all cases actual concentrations of derivatives were calculated after applying corrections for changes in molecular weight: knowing the molecular weight of each compound (PM_x), their actual concentration was obtained applying a multiplication factor of PM_x/PM_y, where PM_y is the molecular weight of the aglycons [19].

Since the overall time of analysis is quite large, we compared this method with a shorter one. Fig. 3 shows the chromatogram obtained with the second method described in the experimental section (Table 2). Even in this case we found all the compounds previously identified with the longer analysis procedure. The shorter method offers improvements with respect to a recent paper [20], in which two methods, the first conventional and the second rapid, applied to soy proteins and commercial food supplements (40% isoflavones), were developed and compared.

Among the advantages, there is the analysis time which let us obtain the same resolution of the longer method allowing achievement of a correct quantitative analysis; high values of polyphenols recovery (> 95%) with respect to a triplicate extraction procedure [14] with easy pre-treatments of the seed sample were also achieved. The other advantage concerns the use of a very

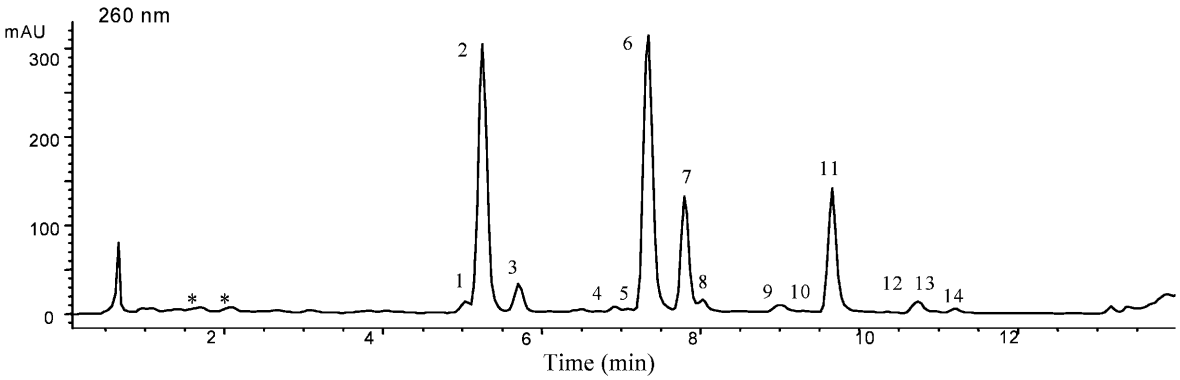


Fig. 3. Chromatographic profile, acquired at 260 nm by HPLC-DAD, of a hydroalcoholic extract from soy seeds at relative maximum of absorbance of isoflavonoids, obtained with a 150 × 3.9 mm (4 μm) Nova Pak C18 column. The elution method is reported in Table 2. Peak identification as in Fig. 1

Table 3. Quali-quantitative comparison of soy seeds: cv. Emiliana, Elvir, Kure, Sapporo and Cresir. Data are expressed in mg kg^{-1} of fresh weight of seed flour. Average value ± SD of three soy samples

Compounds	Emiliana	Elvir	Kure	Sapporo	Cresir
Genistein glucoside	1860 ± 26.9	780 ± 10.5	1260 ± 19.9	264 ± 3.5	192 ± 2.3
Genistein glucoside	75 ± 1.3	37 ± 0.8	75 ± 1.6	45 ± 0.9	30 ± 0.7
Genistein malonyl glucoside	37 ± 0.9	38 ± 0.9	38 ± 0.9	n.d.	n.d.
Genistein malonyl glucoside	2235 ± 22.6	865 ± 7.9	2235 ± 24.6	1240 ± 17.7	981 ± 10.8
Genistein acetyl glucoside	66 ± 1.1	66 ± 1.3	66 ± 1.5	n.d	n.d
Genistein	75 ± 1.6	37 ± 1.0	37 ± 0.8	15 ± 0.4	15 ± 0.3
Total	4348 ± 54.4	1823 ± 22.4	3711 ± 49.3	1564 ± 22.5	1218 ± 14.1
Daizein glucoside	3197 ± 41.7	1106 ± 17.1	1906 ± 21.7	123 ± 2.1	123 ± 2.0
Daizein malonyl glucoside	296 ± 3.5	148 ± 3.0	222 ± 3.7	30 ± 0.7	30 ± 0.6
Daizein malonyl glucoside	3409 ± 28.7	889 ± 9.8	2001 ± 27.1	445 ± 5.2	415 ± 5.0
Daizein acetyl glucoside	traces	traces	traces	traces	traces
Daizein	187 ± 3.4	75 ± 1.6	75 ± 1.9	n.d.	n.d.
Total	7089 ± 77.3	2218 ± 31.5	4204 ± 54.4	598 ± 8.0	568 ± 7.6
Glycitein glucoside	186 ± 3.7	62 ± 1.4	248 ± 4.9	49 ± 1.0	25 ± 0.4
Glycitein malonyl glucoside	221 ± 4.1	74 ± 1.5	221 ± 4.5	30 ± 0.6	15 ± 0.3
Total	407 ± 7.8	136 ± 2.9	469 ± 9.4	79 ± 1.6	40 ± 0.7
OH-daizein glucoside	38 ± 0.8	traces	traces	n.d.	n.d.
Total	38 ± 0.8	traces	traces	n.d.	n.d.
Caffeic acid derivatives	225 ± 4.8	187 ± 3.6	75 ± 1.1	30 ± 0.7	60 ± 1.4

small quantity of sample (100 mg) and a low elution rate (i.e. 0.8 mL min^{-1}), both of which are important factors, in particular when a large number of samples have to be analyzed, resulting in a minor consumption of expensive solvents and a consequential care for the environment.

The detection limits, evaluated for genistein and daidzein in all analyzed extracts, are 200 μg kg^{-1} for both methods. The detection limits were calculated based on a signal-to-noise ratio of 3:1 of the minor component. This is a good value which falls in the low region of HPLC results (100–3300 μg kg^{-1}), as reported in a recent review [19].

The results achieved for five different soy flours obtained from soy cultivars grown under natural conditions are listed in Table 3.

From a nutritional point of view, the most interesting results concern cv Emiliana, which exhibits a very high content of isoflavones (11.88 g kg^{-1} of fresh weight) when compared with the literature [21]. In the case of cv. Kure, the ratio between genistein and daidzein derivatives shows the highest value (0.88); this is important since genistein is the most biologically active isoflavone, which shows antimicrobial activity and is also an important nutraceutical molecule found in soybean seeds [22].

The method proposed seems very interesting in characterizing soy cultivars which are used in human nutrition since it is very sensitive in evaluating biologically active molecules, even if they are present in small amounts.

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