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Simultaneous quantitation of free and conjugated phytoestrogens in Leguminosae by liquid chromatography–tandem mass spectrometry

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Abstract

Phytoestrogens are diphenolic compounds that are present in several edible plants and are particularly abundant in soybeans. Because of their estrogenical, antriestrogenical, anticarcinogenic and antioxidant activities in animal and humans, they became of great interest. Dietary factors are considered important in determination of risks, in fact, studies have revealed beneficial or protective effects of the consumption of vegetables, in particular soy and soybean products. So that in the present paper the simultaneous determination of eight isoflavones and coumestrol in vegetables is reported.

The quantitative analysis has been made by means of LC separation combined with tandem mass spectrometry. In particular, a new simple and fast extraction methodology and a clean-up, based on cold aided de-fatting, is proposed. Method performance was evaluated by comparison with a reference procedure.

The developed procedure was then used for a survey of phytoestrogens concentration in some selected vegetables.
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Keywords: Food analysis; LC–MS/MS; Coumestrol; Isoflavones; Phytoestrogens

1. Introduction

The term phytoestrogen is used in a general way to define a wide variety of non-steroidal compounds found in plant cells or arising from the in vivo metabolism of some precursors present in many edible plants [1]. They are important for the normal growth and development of plants. Their main function is to protect them from phytopathogenous organisms and UV radiations and are present in more than 300 plants [2,3].

Isoflavones represent one of the most characteristic classes of phytoestrogenic compounds in higher plants. They play an important role not only for vegetables but also for animals, including humans. They are very abundant in particular in the Leguminosae family (Fabacae) and recently many other vegetables have been reported to contain these compounds as well [4]. Many studies and reviews are reported [5–9] concerning the health properties of phytoestrogens as hormone substitutes with respect to estradiol (in particular the genistein) [4,10–16].

More recently, however, it has been reported that the hormone replacement therapy is not so safe and effective as previously thought [17].

These are the reasons why large attention has been devoted to develop analytical methodologies capable of phytoestrogen determination in vegetable matrices, like soy, soy-based products and other vegetables. In the recent past, analysis of isoflavones had been performed by using gas chromatography technique with mass spectrometry detection (GC–MS), later the reversed phase-high performance liquid chromatography (RP-HPLC) was gradually introduced [10,18–21]. Now, liquid chromatography coupled with mass spectrometry is the most used technique.

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Isoflavones can be present in plants as aglycones and also as glycosides, malonylglycosides and acetylglycosides [22]. Previously, the analysis of the total isoflavones content, including not only the aglyconic but also the various conjugated forms, has been performed by a hydrolysis step for converting the conjugated forms into the corresponding free substances [23].

In the present work, the simultaneous determination of free and conjugated representative isoflavones and coumestrol in vegetable samples is reported. In particular, a methodology for extraction and clean-up procedures, useful to determine free isoflavone and two representative conjugated without any acid hydrolysis is proposed. Moreover, to quantify total content of each isoflavone (free and ester conjugated forms), an alkaline hydrolysis of the glucoside ester linkage was used.

The extracted analytes were determined by means of LC–MS/MS and this procedure has been applied to the analysis of isoflavones and coumestrol in different kinds of vegetables purchased in Italian stores and commonly consumed in the Italian diet.

2. Experimental

2.1. Chemicals and reagents

Standards of isoflavones: genistin (GEN-GLY) (purity 95%), daidzin (DAID-GLY) (purity 98%), trihydroxyisoflavone (TRI) (purity ≥ 97%), daidzein (DAID) (purity 98%), genistein (GEN) (purity ≥ 97%), formononetin (FORM) (purity ≥ 99%), biochanin-A (BIO-A) (purity ≥ 97%), coumestrol (COUM) (purity ≥ 98%), glycitein (GLY) (purity 97%) and isoflavone (I.S. purity ≥ 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemical structures and trivial names of all the compounds are shown in Fig. 1.

Acetonitrile and methanol, both HPLC grade, as well as, acetic acid were supplied by Carlo Erba (Milan, Italy). Trifluoroacetic acid (TFA) and sodium hydroxide were obtained from Sigma-Aldrich (Milan, Italy). Deionized water was further purified using a Milli-Q (Millipore, Bedford, MA, USA) apparatus. Standard stock solutions of each compound were prepared by dissolving the standards in a suit-

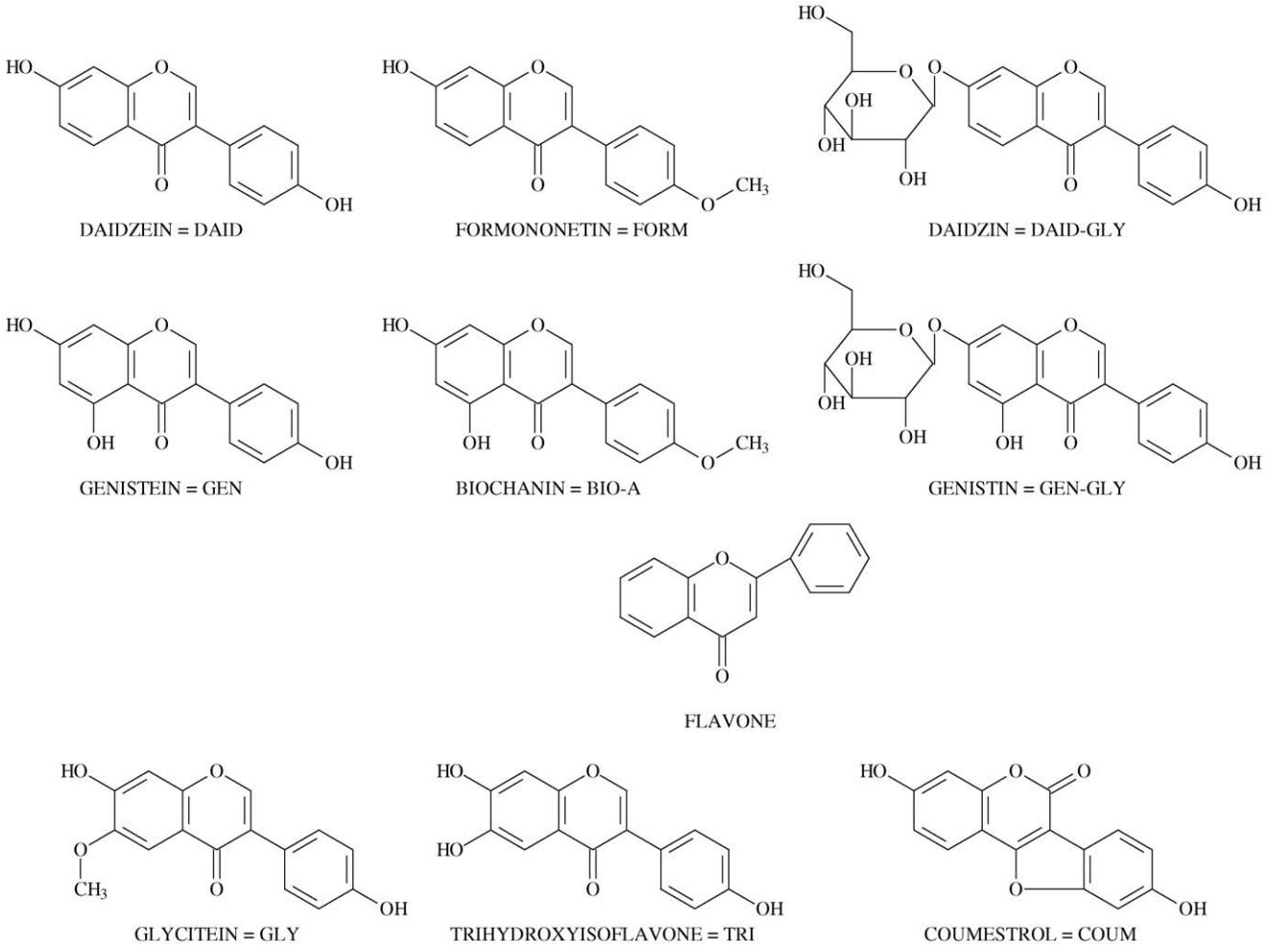

Fig. 1. Chemical structures and common names of analyzed phytoestrogens.

able amount of methanol or acetonitrile to achieve a concentration of 1 $\mu g\,\mu L^{-1}$. From these solutions a new stock solution, containing each of the nine compounds at a concentration of 100 ng μL^{-1} was prepared. Working solutions were prepared by diluting with a suitable amount of solvent each solution. All solutions were kept at −20 °C and allowed to equilibrate at room temperature before use. The cartridge employed for SPE (solid phase extraction) were 6 mL, 200 mg Oasis HLB, purchased from Waters (Milford, MA, USA). These cartridges were stored without any particular precaution. 0.45 μm PTFE filters were obtained from Millipore (Billerica, MA, USA).

2.2. Instrumentation

For the extraction of analytes Polytron homogenizer (Cinematica, Kriens CH) was used.

Liquid chromatography was carried out using a Perkin-Elmer binary LC pump 250 (Perkin–Elmer, Norwalk, CT, USA) equipped with a Rheodyne 7125 injector with a 50 μL loop. The column used was a LC-18 (25 cm × 4.6 mm i.d.) Alltima (Alltech, Deerfield, IL, USA) filled with particles of 5 μm average size, and a precolumn Supelguard (2 cm × 4.6 mm i.d.) supplied by Supelco.

The mass detection has been performed by means of a Perkin–Elmer/Sciex API 365 (Concorde, ON, Canada) equipped with a triple quadrupole analyzer and a TISP (turbo ion spray) interface. Data acquisition has been made with a Mass Chrom 1.1 software.

2.3. Sample preparation

Samples of different kinds of vegetables, were taken from in local supermarkets (species and varieties are described in Table 1) were ground with a blender to reduce them in a fine powder, during no more than 10 s to avoid heat degradation. The powder obtained was then sieved to obtain a sample homogeneous in granulometry (70–120 mesh). Portion (1.00 g) of this sample was transferred to a 50 mL disposable plastic centrifuge tube and suspended in 10 mL of CH_3OH/H_20 (80:20, v/v) solution; 3.00 μg of the volumetric internal standard flavone was then added. Analytes were then extracted from the matrix using a homogenizer. The sample was homogenized for 10 s, this amount of time was found to be optimal, because it allowed the complete disruption of the sample without overheating the solution. Excess heat, in fact, could lead to isoflavones degradation.

Following the homogenization, analytes were centrifuged at 10,000 rpm for 15 min. The supernatant was collected in a 25 mL volumetric flask. The solid residue was washed twice with a 7.5 mL aliquot of extracting solution and centrifuged again. Finally, all fractions were collected in the volumetric flask and the volume was carefully adjusted to 25 mL with the extracting solution.

2.4. Clean-up

The solution obtained with the described procedure was freezed overnight at −20 °C; thus part of the co-extracted compounds were precipitated. They are mainly proteins, waxes, lipids, oils that are able to interfere with the target analytes determination during the LC–MS/MS run, and to clog the chromatographic column. 1.0 mL aliquot of the supernatant extract, previously defreezed, was then filtered with a 0.45 μm PTFE filter, and 50 μL of solution were injected into the LC–MS/MS system.

When the concentration of target compounds was below the direct injection method detection limit, an SPE concen-

Table 1
Flavonoids concentrations ($\mu g\,g^{-1}$) detected in several kinds of Italian Leguminosae

Sample treatment	Scientific name, common name	Compound concentration ($\mu g\,g^{-1}$)								
		DAID-GLY	GEN-GLY	TRI	DAID	GLY	GEN	COUM	FORM	BIO-A
Freeze clean-up	***Glycine soja*, yellow soybean**	**738**	**1617**	**575**	**63**	**n.d.**	**88**	**n.d.**	**n.d.**	**n.d.**
	***Vicia faba*, broad bean**	**29**	**2**	**19**	**15**	**11**	**18**	**n.d.**	**n.d.**	**n.d.**
	***Pisum sativus*, pea**	**299**	**360**	**24**	**83**	**67**	**94**	**n.d.**	**n.d.**	**124**
Freeze clean-up and SPE	*Glycine soja*, green soybean	0.1	0.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Phaseulus vulgaris, yellow bean	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Phaseulus vulgaris, black bean	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	n.d.	n.d.
	Phaseulus vulgaris, "Spain" bean	0.7	2.2	0.3	0.2	n.d.	n.d.	0.3	n.d.	n.d.
	Phaseulus vulgaris, borlotti bean	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.
	Phaseulus vulgaris, bean with eye	0.2	0.6	n.d.	0.4	n.d.	n.d.	n.d.	n.d.	n.d.
	Phaseulus vulgaris, "lamon bean"	0.5	1.7	0.3	0.1	0.1	n.d.	0.1	n.d.	n.d.
	Phaseulus vulgaris, white bean	n.d.	n.d.	0.4	0.0	0.1	0.1	n.d.	n.d.	0.1
	Lupinus albus, lupin	n.d.	1.2	n.d.	1.0	n.d.	0.7	n.d.	n.d.	n.d.
	Cicer arietinum, chickpea	0.2	2.2	n.d.	0.2	8.9	n.d.	n.d.	n.d.	n.d.
	Eruum lens, "eston" lentil	1.0	5.7	0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Eruum lens, "pantelleria" lentil	n.d.	0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Vegetables in bold are those analyzed without the SPE concentration step. The reported values are a mean of three determinations, R.S.D. values range between 5 and 8%, for samples analyzed without the SPE concentration step and between 10 and 15%, for the others.

tration step was employed. For this purpose, a 200 mg Oasis HLB cartridge was employed. Before extraction, the cartridge was sequentially washed with 5 mL of Milli-Q water, 5 mL of CH_3OH/H_2O (50:50, v/v), containing CH_3COOH 0.5 mol L^{-1}, 5 mL of CH_3OH/H_2O and 5 mL of Milli-Q water.

Then a 5 mL aliquot taken from the freeze-purified extract was filtered with a 0.45 μm PTFE filter and diluted to 100 mL with Milli-Q water. This solution was passed through the cartridge by vacuum aspiration. The vacuum was regulated in order to obtain a flow rate of 10–15 mL min^{-1}. After the whole sample was passed, the vacuum was reduced and the cartridge was washed with 10 mL of Milli-Q water to remove any salt or water-soluble compound present on the sorbing material surface. After that the cartridge was washed with 2 mL of CH_3OH/H_2O (15:85, v/v), containing CH_3COOH 0.5 mol L^{-1}; this step allowed to eliminate basic substances eventually co-extracted. The cartridge was then kept to dry under vacuum aspiration for 30 min to eliminate the water still contained in the column. As a additional precaution, any residual water that might be present in the column and interfere with the evaporation step was eliminated by slowly washing the tube with 200 μL of CH_3OH under vacuum. This volume resulted suitable to wash the column without eluting the analytes under consideration. All the above-mentioned washing solutions were discarded. The final elution step was performed with 10 mL of CH_3OH. The extract coming off the column was collected in a 10 mL round bottom glass vial and completely evaporated in a 30 °C thermostatic water bath, under a gentle nitrogen stream. The residue was re-solubilized with 200 μL of a CH_3CN:H_2O (25/75, v/v). A 50 μL aliquot of solution was injected into the LC–MS/MS system.

2.5. Glycoside esters saponification

For total isoflavone determination a hydrolysis step was introduced [24]. A 5.0 mL aliquot of filtered freeze clean-up methanolic extract was put in a 10 mL round bottomed tube and equilibrated at room temperature. Then, 150 μL of a 5.0 mol L^{-1} NaOH solution were added and the sample was slowly vortex mixed at room temperature for 10 min. Finally, the saponification reaction was stopped with 25 μL of glacial acetic acid.

2.6. Instrumental conditions

The chromatographic analysis was performed using a gradient solvent program. The initial composition of the mobile phase was acetonitrile–water (20:80, v/v), containing 10 mmol L^{-1} TFA.

The gradient was programmed to linearly increase the amount of organic phase until 85% in 30 min. To clean the column the amount of acetonitrile was increased to 100% and kept constant for 5 min. The eluent flow rate in the LC system was 1 mL min^{-1}, but only one fifth of the column effluent was splitted and transferred into the mass spectrometer. The mass spectrometric working conditions were: the turbo ion spray (TISP) operated in positive ion (PI) mode, with nitrogen as turbo gas heated at 350 °C. Mass calibration and resolution adjustments on the resolving quadrupoles were performed automatically by using a polypropylene glycol (PPG) 10^{-5} mol L^{-1} solution introduced via a Model 11 Harvard infusion pump. The peak-width was set on both resolving quadrupoles at 0.7 Th (measured at half-height) for all MS and MS/MS experiments. The final data acquisition was performed by working in MRM mode (multiple reaction monitoring) after a preliminary tuning process. For each analyte under examination, the optimization of tuning parameters was obtained by connecting directly to the source the infusion pump in continuous flow mode (10 μL min^{-1}). The standard solutions contained 10 ng μL^{-1} of each compound in acetonitrile–water solvent (50:50, v/v) with 10 mmol L^{-1} TFA.

MS and MS/MS spectra were collected in full-scan mode over the range *m/z* 50–450, using a *m/z* 0.5 step size and a 10 ms dwell-time. The LC–MS/MS analyses were performed in a MRM mode. The first quadrupole (Q1) was set to a defined *m/z* value (precursor ion), in the second quadrupole (Q2-collision cell) this ion is fragmented by CAD (collisionally activated dissociation) with nitrogen at 10 mTorr pressure. At the end three characteristic product ions will be detected in the third quadrupole (Q3).

To enhance the sensitivity, the acquisition time was divided in seven periods; in Table 2 are reported, the acquisition intervals, the selected ionic transition and the collision energies for each compound.

2.7. Comparison with other extraction procedures

The efficiency of the developed methodology was evaluated by comparing our results with those obtained by using as reference extraction procedure that one reported by Murphy et al. [22]. The LC–MS/MS conditions were identical to those utilized for the developed methodology. The extraction procedure proposed involves the processing of a 2.00 g of milled soy sample. The sample was transferred in a flask and added with 20 mL of extracting solution (CH_3CN:H_2O, 53:47, v/v). The analytes were extracted by stirring the sample for 2 h at room temperature. Afterwards, the whole extract was filtered; 10 mL were then taken to dryness under vacuum in a rotarvapor. The temperature was kept below 30 °C. The residue was solubilized with 5 mL of CH_3OH:H_2O (80:20, v/v), and filtered with a 0.45 μm PTFE filter. A 50 μL aliquot of the extract was finally injected into the LC–MS/MS apparatus.

2.8. Quantification

Quantitative analysis was made with the standard addition method. This was made in two steps procedure: first a rough estimate of analyte quantity was made by relating the ratio between analytes' peak and that of the internal standard to those obtained from a standard solution (2.0 ng μL^{-1} of each

Table 2
Instrumental parameters pointed out for the best LC–MS/MS analysis of the flavonoids examined

Compounds	Windows	Acquisition time (min)	Fragments (*m*/*z*)	Collision energy (eV)
DAID-GLY	1	5.0–9.0	255 → 91 255 → 137 255 → 181	42
GEN-GLY	2	9.0–11.5	271 → 91 271 → 153 271 → 215	42
TRI	3	11.5–14.0	271 → 141 271 → 169 271 → 197	41
DAID	4	14.0–17.5	255 → 91 255 → 137 255 → 181	42
GLY	4	14.0–17.5	285 → 197 285 → 242 285 → 270	41
GEN	5	17.5–19.5	271 → 91 271 → 153 271 → 215	42
COUM	5	17.5–19.5	269 → 197 269 → 213 269 → 241	36
FORM	6	19.5–23.0	269 → 118 269 → 197 269 → 253	42
BIO-A	7	23.0–28.0	285 → 152 285 → 213 285 → 242	43
I.S.	7	23.0–28.0	223 → 95 223 → 103 223 → 121	43

analyte); then the extract was spiked with an amount of each analyte equal to the quantity estimated in the first step and re-analyzed. The value obtained by subtracting each analyte to internal standard areas ratio of spiked extract from those obtained from unspiked extracts was then used to quantify each analyte. If the extract was concentrated, the spike was made before the evaporation step, otherwise volume correction was considered.

3. Results and discussion

3.1. Optimization of instrumental conditions

Preliminary experiments were performed in order to find the best instrumental conditions for unequivocal identification of target compounds. Isoflavones can be detected either as protonated or deprotonated molecules using characteristic fragments with both the ionization interfaces commonly supplied with a mass spectrometer, namely, ESI and APCI [25].

For the optimization of the chromatographic conditions, a gradient elution able to separate all nine compounds and the internal standard in a reasonably short time was employed. It is well known, dealing with isoflavones, that using a neutral mobile phase (CH_3CN/H_2O) is impossible to gain a satisfying separation of the analytes with symmetric peaks and reproducible retention times. Consequently, an acidic eluent phase must be employed. trifluoroacetic acid, acetic acid, formic acid, ammonium formate or acetate buffers (pH < 4.3), were used for this purpose in the past. Anyways, it was noticed that using acetate or formate buffers, DAID and GEN showed a diffuse residual peak tailing. According to us, acid addition is better, since coupling a chromatographic technique with mass spectrometry involves to find the best compromise between separation and detector response. Or in other words, the entire methodology should be evaluated in terms of peak symmetry, resolution, length of analysis and MS signal intensity. Then even if is true that as Cuyckens and Claeys [26] discovered, ammonium formate buffer and negative ionization (NI) give higher ionic signal intensities in absolute value (measuring areas), we used TFA and PI since the chromatographic resolution and the S/N ratio were

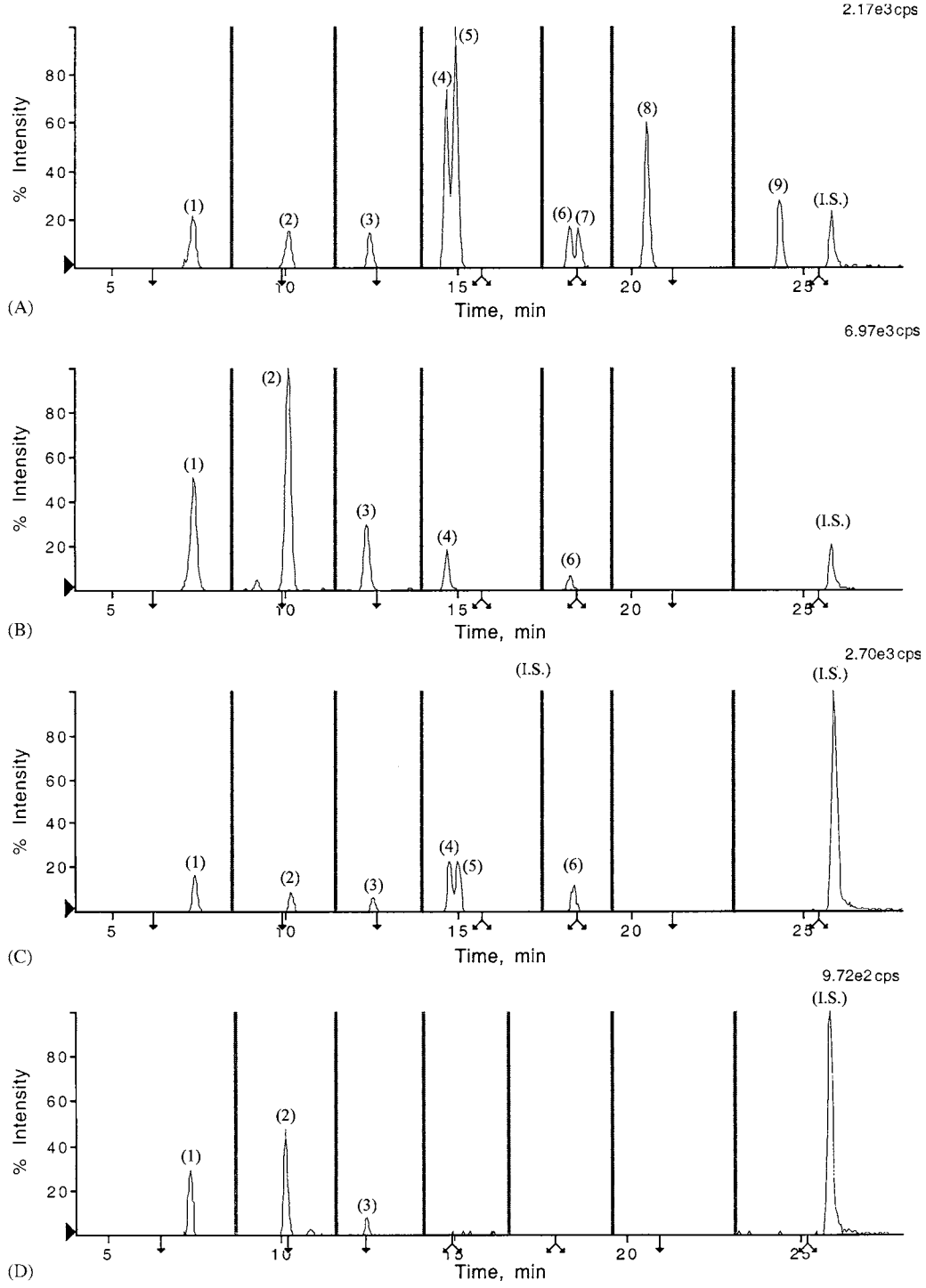

Fig. 2. MRM chromatogram obtained injecting (A) 200 ng of each analyte standard; (B) broad bean extract; (C) yellow soy extract; (D) "eston" lentil extract (1) DAID-GLY, (2) GEN-GLY, (3) TRI, (4) DAID, (5) GLY, (6) GEN, (7) COUM, (8) FORM, (9) BIO-A (I.S.) internal standard.

better in these conditions. Fig. 2 shows the chromatographic separation obtained in the experimental conditions used.

Employing acidic mobile phases implies to detect all the analytes in PI mode as proton adducts. Different TFA concentrations (5, 10, 15 and 25 mmol L^{-1}) were tested. The best choice resulted 10 mmol L^{-1}, since a clear increase of sensitivity was noticed when working with turbo ion spray (TISP). Lower concentration gave, as side effect, little peak broadening. Very little response was obtained using the APCI interface.

Optimization of MRM conditions were realized by injection of standard solutions of each analyte. As precursor ions for DAID and GEN, the ones corresponding to the proton adduct of the aglycones at *m*/*z* 255 and 271 were used. For

all the remaining analytes, the proton adduct $[M+H]^+$ was selected. The glucose molecule loss had been achieved by applying a higher voltage (61 V) to the skimmer cone that allowed the quantitative loss of the sugar moiety and very little further fragmentation. The conjugated compounds, indeed, are by nature very fragile, but on the contrary, the remaining fragment is resistant to further in-source fragmentation, even employing relatively high skimmer cone voltages. Further fragmentation of all the compounds was obtained in Q2 by applying a collision energy in the 36–43 V range. For every analyte, the three most intense characteristic fragment ions (Table 2) were selected. So that the confidence level of analyte identification was enhanced; it is to be noticed that in real samples, sometimes, interfering unassigned peaks having two daughter ions out of the three selected were present.

3.2. Extraction procedure

Several factors can influence the overall results obtained from an analysis but, dealing with phytochemicals, the most critical step is always the choice of the optimal extraction solution and procedure.

The identification of the appropriate solvent depends mostly on chemical structure of the analytes, solvent polarity, and the need to minimize co-extracted matrix interferences. Since target analytes are within a polarity range, a compromise must be found.

In the past, several solvents and solvent mixture were employed [27–30]. Murphy et al. in an early application have demonstrated that acetonitrile–water is better than methanol–water in isoflavones extraction. Moreover, they also demonstrated, more recently, that the extracting mixture efficacy is dependent from the matrix, and the optimal water percentage must be selected in each case depending on particular food analyzed. It has been also demonstrated that, in order to extract conjugate isoflavones without causing hydrolysis, the temperature must be kept below 30 °C in all steps of analysis, including evaporation.

We evaluated the behavior of two extracting mixtures: methanol–water and acetonitrile–water in different ratios. Methanol–water (80:20, v/v) turned out suitable in the case of freeze clean-up procedure because it yielded higher total isoflavone quantity, while acetonitrile water presents $\Delta H_{mix} > 0$ and it gives phase separation and icing phenomena. Regarding clean-up, low temperature fat precipitation was already used in a very early application [31] analyzing pesticides from plant extracts. More recently, this procedure has been applied to meat and fatty matrices gravimetric defatting [32]. We noticed that isoflavone freeze purification can be performed as well, after the gravimetric cold aided partial elimination of some co-extracted substances, mostly waxes and lipids. This could trap some analytes, however, the extracts, after freeze clean-up, were more clear and signal suppression phenomena were reduced. Moreover, the need of frequent cleaning of sampling cone and recalibration of the mass spectrometer were reduced [33].

Recovery determination in spiked solid samples is generally not considered representative of "natural" conditions. Analytes migration inside the solid phase and matrix–analyte interactions are processes requiring long times to achieve equilibration. On the other hand, long equilibration times may lead to low recovery due to analyte degradation. To evaluate the extraction efficiency, then, we compared our results with those obtained by means of the reference extraction method [22]. The same soy sample was analyzed by our and Murphy's method, results are comparable as shown in Table 3. As can be seen the differences do not exceed 5% error, but our procedure requires less sample manipulation and a simpler apparatus. Freezing procedure, in fact, even if time consuming, requires no further extract manipulation, organic solvents and allows to process many samples in the same batch.

After extraction the filtered sample was analyzed by LC–MS/MS. This methodology turned out to be suitable only for yellow soy, green peas, and broad beans. To detect the isoflavones in other vegetables, a concentration step based on solid-phase extraction was employed. To determine recoveries from the SPE cartridge, 5 mL of extracting solution was spiked with 200 ng of each analyte. This solution was then diluted to 100 mL with Milli-Q water. The overall recoveries were satisfactory, ranging between 75 and 88%, with the exception of trihydroxyisoflavone, which was 60%, probably due to its high sensitivity to oxidation by dissolved oxygen, to give the corresponding *o*-quinone.

To quantify also daidzein and genistein glucoside esters (acetyl and malonyl isoflavones esters) an alkaline hydrolysis step was introduced. Recently, reference standards of these compounds have been put on the market, but they are relatively expensive and unstable in solution. A mild saponification step instead was used to convert the isoflavone glucoside ester forms to the corresponding isoflavone glucoside forms. This approach allows direct comparison with stable, readily available, isoflavone parent and glucoside reference

Table 3
Comparison among results obtained by extracting the same yellow soy sample with described procedure and reference procedure [22]

Method	Compounds concentration ($\mu g\,g^{-1}$)								
	DAID-GLY	GEN-GLY	TRI	DAID	GLY	GEN	FORM	COUM	BIO-A
This work	738	1617	575	63	n.d.	88	n.d.	n.d.	n.d.
Reference	750	1678	564	60	n.d	86	n.d.	n.d.	n.d
Difference %	−1.6	−3.6	2.0	5.0	–	2.3	–	–	–

The reported values are a mean of three determinations, R.S.D. values range between 5 and 8%, for samples analyzed with this work extraction method, and between 6 and 8%, for samples analyzed with reference' method.

standards. Stopping the reaction by acidification after 10 min leaves the isoflavone glucoside intact, and therefore, it is possible to determine the relative proportion of parent isoflavone and glucoside present in the sample under investigation.

3.3. LODs and precision estimation

The real LOD value for each analyte can not be determined with accuracy, since standard solutions do not take in account matrix effect, which varies from one matrix to another. However, by assuming a linear detector response and considering a signal to noise ratio > 3 as decision criterion, we estimated that all the analytes were detectable at values greater than 2 $\mu g\,g^{-1}$, using the direct injection method, while greater than 0.02 $\mu g\,g^{-1}$ using the SPE concentration step.

The precision of the proposed method is ranging between 5 and 8%, as R.S.D. of real samples analyzed without the SPE concentration step, and between 10 and 15%, for the others.

3.4. Legumes samples analysis

The extraction procedure, based on homogenization and freeze clean-up, has been applied to 15 different kinds of dried vegetables, purchased in different local stores (Table 1). In Fig. 2, are reported the chromatograms obtained analyzing three different vegetables.

Quantitative analysis was achieved by measuring the ratio between the areas of each analyte and the I.S. area and relating to those obtained from a sample fortified with a proper known amount of each standard. This amount depended on the analyzed vegetable, and was roughly estimated by comparing the obtained areas with those of a standard mixture. The amount to be spiked was then based on to the same quantity of the estimated one. Sample matrix effects, found out in real samples have been corrected by means of standard addition method.

The concentrations ($\mu g\,g^{-1}$) found in each vegetable are reported in Table 1. The data listed in bold refer to those analyzed with the direct injection method. All other data refer to samples where the SPE extraction was necessary. The most abundant concentrations were found in the yellow soy, 738 and 1617 $\mu g\,g^{-1}$ for daidzin and genistin, respectively. The aglycones are about 5–10% of this concentration.

Also, regarding trihydroxyisoflavone, great attention must be put to its identification; indeed several unidentified peaks can be found in real sample chromatograms. These peaks have all the characteristic transitions of trihydroxyisoflavone, but a retention time slightly different. By inspecting the various fragment signals intensity, it can be seen that their ratio is different respect to that of standard trihydroxyisoflavone; the observed peak might be assigned to an isomer of TRI, most likely a genistein conjugate. As a consideration to assess with sureness the presence of TRI, at least three fragments have to be considered and the ratio's variation of their relative intensities must be less than 20% with respect to that measured with standard solutions.

Table 4
Total daidzin and genistin concentrations ($\mu g\,g^{-1}$) determined after alkaline hydrolysis of glycosides esters of analyzed vegetable extracts

Scientific name, common name	Compound concentration ($\mu g\,g^{-1}$)	
	Total DAID -GLY	Total GEN -GLY
***Glycine soja*, yellow soybean**	1251	2589
***Vicia faba*, broad bean**	33	29
***Pisum sativus*, pea**	415	520
Glycine soja, green soybean	0.2	0.3
Phaseulus vulgaris, yellow bean	n.d.	0.2
Phaseulus vulgaris, black bean	0.2	n.d.
Phaseulus vulgaris, "Spain" bean	0.7	2.2
Phaseulus vulgaris, borlotti bean	n.d.	n.d.
Phaseulus vulgaris, bean with eye	0.5	0.2
Phaseulus vulgaris, "lamon bean"	0.4	0.2
Phaseulus vulgaris, white bean	n.d.	n.d.
Lupinus albus, lupin	0.5	1.6
Cicer arietinum, chickpea	0.4	2.6
Eruum lens, "eston" lentil	1.0	5.7
Eruum lens, "pantelleria" lentil	n.d.	0.5

Vegetables in bold are those analyzed without the SPE concentration step. The reported values are a mean of three determinations, R.S.D. values range between 5 and 8%, for samples analyzed without the SPE concentration step and between 10 and 15% for the others.

In order to estimate the total isoflavone concentration of each isoflavone (due to other conjugated forms), an alkaline hydrolysis was performed, using the methodology reported by Klump et al. [24]. The sample, after extraction, hydrolysis and clean-up, was analyzed again by the described LC–MS/MS procedure. The results are listed in Table 4, the contribution to the total quantity, due to other conjugates (mainly the acetyl and malonyl glycosides esters), is given by the difference between the values found with and without hydrolysis.

4. Conclusions

In the present paper, the applicability of LC–MS/MS technique for the simultaneous determination of isoflavones and coumestrol in complex food matrices like vegetables has been demonstrated. The extraction method and clean-up are simple to perform and do not require a complicated apparatus.

The proposed method is easy to perform, gives results reproducible and comparable with those reported by other authors. Therefore, it can be used for determining the isoflavones distribution among the edible plant species, and also for estimating the phytoestrogens average intake in the Italian diet.

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