Facultad de Ciencias Químicas

Departamento de Química Analítica, Nutrición y Bromatología

VNiVERSiDAD D SALAMANCA

CAMPUS DE EXCELENCIA INTERNACIONAL

Desarrollo de metodologías analíticas para el análisis de isoflavonas en legumbres.

Development of analytical methodologies for the analysis of isoflavones in legumes.

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DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE ANALYSIS OF ISOFLAVONES IN LEGUMES.

Memoria que para optar al Grado de Doctor por la Universidad de Salamanca presenta la licenciada Lara Pérez Martín.

Salamanca, 26 de Mayo de 2015

Fdo: Lara Pérez Martín

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Salamanca, 26 de Mayo de 2015

Fdo: M.M.Delgado Zamarreño

Fdo: M. Bustamante Rangel

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1. AIM

The nutritional properties of foods are under constant surveillance with a view to controlling their quality. In recent years there has been increasing interest in knowing, besides nutritional properties, the bioactive properties of foodstuffs. Bioactive compounds are naturally present in food and cause some healthy effects. Among these compounds are species such as phytoestrogens, and among these, most notably the isoflavones.

The overall objective of the present research is to develop analytical methodologies that facilitate the analysis of isoflavones in widely consumed foods.

In this regard, pulses are foods with high contents of proteins, carbohydrates, fibre and other micronutrients such as vitamins. Furthermore, pulses contain bioactive compounds, among which are isoflavones. Among the legumes, soya has high levels of these substances, and this pulse has been studied in depth as regards its bioactive properties. The consumption of soya is not very high in Spain, unlike other legumes such as chickpeas, lentils or faba beans, such that it would be of great interest to have data concerning both its nutritional and bioactive properties.

Analytical methodologies include a sample treatment step and another step in which the separation, identification and quantification of analytes take place. In food analysis, one of the fundamental problems arising from the complexity of the matrices stems from analyte extraction prior to determination. In the determination of bioactive compounds in foods, sample treatment is a critical step and sometimes limits the development of analytical methodologies.

Traditional methods proposed tedious time-consuming sample treatments which needed large amounts of organic solvent and possibly introduced errors in the determination step. Accordingly, there is a need to develop new methods with reduced organic solvent consumption and sample manipulation in order to avoid clean-up steps and, if possible, allow the pre-concentration of the analytes in order to increase extraction efficiency. *Automation of the sample treatment or of some of the steps involved is also important.*

Thus, one of the aims of this work is to develop sample treatments that meet these requirements and are also compatible with liquid chromatography or capillary electrophoresis as separation and measurement methods. Moreover, isoflavones are found in plants in free or glycosylated derivatives with different bioavailabilities. Another factor to be considered is the use of treatments to isolate analytes in the form in which they are naturally present in the samples.

The extraction methods proposed to achieve this aim are *Pressurized Liquid Extraction (PLE)* and the *QuEChERS extraction* method *(Quick, Easy, Cheap, Effective, Rugged and Safe).*

Most applications using PLE have focused on the determination of contaminants in environmental samples, few of them being used in foods. The QuEChERS methodology has

primarily focused on the extraction of pesticide residues from fruits and vegetables, and there are almost no references concerning its use in extracting substances that are present in samples naturally.

In this research, sample treatments were based on extraction with pressurized liquids, and a modified QuEChERS method adapted to samples of legumes in order to extract the compounds naturally present in these matrices in the form of glycosides and aglycones. In the case of the QuEChERS methodology, a study addressing the issue of placing the sample and solvent in contact for extraction was also carried out, since this point has been considered important for improving the efficiency in this kind extraction.

Liquid chromatography is the technique most widely used in the determination of bioactive compounds for all types of sample. Capillary electrophoresis has been little used in the analysis of phytoestrogens. In this work, the aim was to use both liquid chromatography and capillary electrophoresis for analyte separation after applying the sample treatments developed.

Detection was carried out using mass spectrometry coupling both LC and CE to ESI-MS with a view to improving the selectivity and sensitivity provided by spectrophotometric detection. In the case of liquid chromatography, the mass spectrometry detector used was MS/MS in tandem, with the triple quadrupole version.

Ultimately, the methodologies developed were applied to samples of chickpeas, lentils and beans in order to study the possibility of classifying the different types of pulses according to their variety, taking into account the observed isoflavone profiles.

2. RESULTS AND DISCUSSION

2.1. Pressurized Liquid Extraction

Research article: Analytical and Bioanalytical Chemistry 404 (2) (2012) 361

"Pressurized Liquid Extraction as a simple preparation method for the analysis of isoflavones in pulses".

2.2. Extraction by the QuEChERS method

Research article: Talanta 100 (2012) 320

"A modified QuEChERS method as sample treatment before the determination of isoflavones in foods by ultra-performance liquid chromatography-triple quadrupole mass spectrometry".

2.3. Study the placing on contact between sample and solvent in the QuEChERS methodology

Research article: Phytochemical Analysis 25 (2013) 170

"Comparative study of the methodology used in the extraction of isoflavones from legumes applying a modified QuEChERS approach".

2.4. Determination of isoflavone contents in foodstuff by Capillary Electrophoresis

Research article: Current Analytical Chemistry 11 (2015) 117

"Determination of isoflavones in legumes by QuEChERS-capillary electrophoresis-electrospray ionization-mass spectrometry".

2.5. Classification of pulses based on their isoflavone content

Research article: Not yet published

"Classification of different varieties of chickpeas, lentils and beans based on their isoflavone content using multivariate analysis"

Anal Bioanal Chem
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TECHNICAL NOTE

Pressurized liquid extraction as a sample preparation method for the analysis of isoflavones in pulses

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Abstract In this work, we describe a rapid and simple analytical method that exploits pressurized liquid extraction (PLE) and liquid chromatography with diode array detection for the determination of isoflavones in samples of Spanish pulses. Confirmation of the analytes present was performed using ion-trap mass spectrometry. To optimize the PLE extraction, variables such as the dispersing agent, type of solvent and sample amount, and the experimental parameters, such as temperature and the number of extraction cycles, were studied. Separation was carried out using a reverse-phase C18 with polar endcapping as the stationary phase and acetonitrile/water with 0.2 % of formic acid, under a gradient regime, as the mobile phase. Optimal extraction of formononetin and biochanin-A from chickpeas with PLE was achieved using Hydromatrix as a dispersant agent, methanol/water (50:50), a temperature of 90 °C, and three cycles. The same optimal conditions—except methanol/water (75:25)—for solvent extraction were obtained for the extraction of daidzin, genistin, and formononetin from lentils. Recoveries ranged from 97 to 110 %, and standard deviations lower than 20 % were obtained. The contents obtained for daidzin in lentils using the proposed method were not significantly different from those obtained using another official method of analysis.

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Keywords Isoflavones · Pressurized liquid extraction · Chickpeas · Lentils · Liquid chromatography

Introduction

The nutritional properties of foods are under constant surveillance with a view to controlling their quality. In this regard, pulses are foods with high contents of proteins, carbohydrates, fibre, and other micronutrients such as vitamins. Recently, the importance of bioactive compounds in human health has been confirmed and attracted considerable attention; hence, there is a need to develop new methods for their analysis. Pulses contain this type of compound, among which are isoflavones [1]. Among the legumes, soya [2] has high levels of these substances, and it has been studied in depth as regards its bioactive properties. The consumption of soya is not very high in Spain, unlike other legumes such as chickpeas and lentils, such that it would be of great interest to have data concerning both its nutritional and bioactive properties.

Isoflavones belong to a large group of natural substances present in plants. Their structure is based on a chromane skeleton with phenyl substituents at position C3. The structures vary as a function of the degree of methylation, hydroxylation, and glycosidation [3]. In light of this, the most common forms are daidzein, genistein, and glycitein, and the conjugated forms daidzin, genistin, and glycitin. There are also minor free forms such as formononetin and biochanin-A [4]. Both the presence and amount of isoflavones in plants, fruits, and vegetables may vary, depending on different factors such as variety, the year of harvest, storage, climatic conditions, etc. [5]. Interest in the consumption of isoflavones has increased in recent years owing to their possible influence in the prevention of certain

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diseases or problems associated with menopause, cardiovascular disease, osteoporosis, and breast, prostate, and colon cancer [6–9].

From the point of view of the analysis of these substances in foods, one of the main problems is the complexity of the matrices and the need to isolate the analytes before their determination. Different authors have studied the sample treatments used for the determination of isoflavones, above all in soya products and derivatives [10, 11]. The isolation of these compounds is carried out using acid hydrolysis and/or alkaline saponification prior to analyte separation and quantification [12–14]. Although the treatment most commonly used is solvent extraction using traditional methodology, in discontinuous mode or with a Soxhlet device [15], currently, the use of stirring or sonication, such as ultrasonic-assisted extraction, is recommended [16–18]. Other authors have used methodologies in which extraction is carried out at high temperature and high pressure, above its critical point, such as supercritical fluid extraction (SFE), or not reaching this supercritical state, as is the case of pressurized liquid extraction (PLE). Different investigators have performed the extraction of isoflavones using SFE extraction from soybean cake [19], soybean flour [20], and soybean meal [21], but PLE is more widely used than SFE for the extraction of isoflavones. PLE has been used to extract isoflavones from soy foods [22] using only one type of extractant or performing two successive extractions with two different-polarity solvents [23]. Some authors have compared PLE and other extraction methods [24]. PLE also has been used for other plant matrices such as *Trifolium* L. species [25]. However, we have not found any references concerning the extraction of isoflavones in pulses, in which the contents of these compounds are lower.

Thus, one of the aims of the present work was to study extraction by pressurized liquids for the isolation of isoflavones in pulses widely consumed in Spain prior to their chromatographic determination via high-performance liquid chromatography LC-DAD. Confirmation of the analytes present was performed using LC-ion-trap MS.

Experimental

Instruments

The LC system used was a Hewlett-Packard Agilent 1100 Series (Waldbronn, Germany) that included a quaternary pump, a degasser, an autosampler, and a diode array detector. Chromatographic separation was achieved under gradient conditions using a Phenomenex (Torrance, CA, USA) Synergi 4 μ Hydro-RP 80A column (250×4.6 mm). Temperature was maintained at 25 °C. The injection volume was 20 μL. Quantification was performed at 254.1 nm. Agilent ChemStation software for LC was used for data acquisition and analysis.

The LC/MSD Trap XCT (Waldbronn, Germany) ion-trap mass spectrometer was equipped with an electrospray (ESI) source with a nebuliser spacer. The ESI parameters were 3,500 V as capillary voltage, 11 $L min^{-1}$ as drying gas flow at a temperature of 350 °C, and 60 psi as the nebuliser pressure. The trap parameters were a maximum accumulation time of 200 ms at an *m*/*z* ranging from 60 to 400 u. A narrow isolation width of 4 u was selected.

Samples were ground with a Foss (Barcelona, Spain) Knifetec™1095

Extractions were achieved using a Dionex (Germany) ASE™ 100 pressurized liquid extraction system equipped with 11-mL stainless steel extraction cells and 250-mL Dionex bottles for extract collection.

Chemicals and materials

Daidzin CAS RN [552-66-9], genistin CAS RN [529-56-9], daidzein CAS RN [486-66-8], and biochanin-A CAS RN [491-80-5] were obtained from Sigma-Aldrich (Steinheim, Germany), and genistein CAS RN [446-72-0] and formononetin CAS RN [485-72-3] were from Fluka (Steinheim, Germany). The organic solvents—HPLC-grade methanol (MeOH) and acetonitrile (ACN)—were obtained from Merck (Darmstadt, Germany), and formic acid was from Fluka (>98 %, Steinheim, Germany). Varian Hydromatrix® and washed sea sand (supplied by Scharlab S.L., Barcelona, Spain) and C18 from Sigma-Aldrich were used. Ultra-high-quality (UHQ) water was purified in an ElgaStat UHQ water purification system (High Wycombe, UK). Samples of chickpea from Fuentesaúco (Zamora, Spain) and lentils from la Armuña (Salamanca, Spain) were purchased from local markets.

Procedure

The ground samples (2–3 g of legumes) were mixed with the dispersant agent (Hydromatrix) in order to prevent the aggregation of sample particles and were then placed in 11-mL extraction cells. After closure, the cells were placed in the oven of the ASE system. The optimized conditions were as follows: oven temperature, 90 °C; pressure, 110 bars, and three cycles, with a static time of 5 min. The extraction solvent depended on the nature of the sample to be extracted: methanol/water 50:50 and 75:25 for chickpeas and lentils, respectively. After extraction, a volume of clean solvent was pumped into the sample cell, and the solvent was then purged from the cell with nitrogen. The extracts were diluted to 25.0 mL and filtered through 0.45-μm nylon

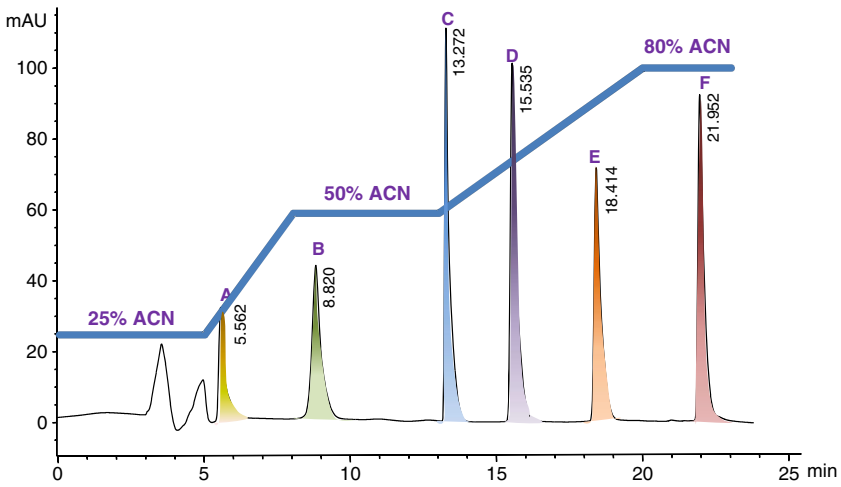

Fig. 1 Optimized mobile phase gradient and chromatogram of standard solutions using optimised LC conditions

membranes before their injection (20 μL) into the chromatographic system. Three replicate extractions and duplicate LC analyses of each extract were carried out for each sample.

Separations were carried out under gradient conditions. The mobile phase was a solution of ACN/H_2O with 0.2 % of formic in a gradient regime. The flow rate was 0.7 mL/min. Column temperature was maintained at 25 °C. Detection was carried out at 254.10 nm for all substances.

Results and discussion

Optimisation of LC method

Because β-glycosides and aglycons have similar structures, an isocratic regime cannot be used. The first study carried out here addressed the optimization of the gradient regime. Different mixtures of MeOH/H_2O and ACN/H_2O were tested as mobile phases, and in nearly all cases, mixtures of acetonitrile afforded better resolution than methanol. In all cases, the presence of formic acid produced a pH lower than the p*K*a of isoflavones, and consequently, the isoflavones were not dissociated. The best gradient regime to obtain good resolution was as follows: ACN/0.2 % formic acid in H_2O 75:25 for 5 min, passing to 50:50 in 8 min and holding for 13 min, passing to 80:20 in 20 min and holding for 23 min, and finally passing to 75:25 in 30 min. Under these conditions, all the isoflavones eluted before 25 min (Fig. 1).

The detection of isoflavones was carried out using a UV–vis diode array detector. All analyses were performed at 254.1 nm, the wavelength corresponding to the absorption maximum for most isoflavones. A good linear relationship was observed between peak areas and analyte concentrations (Table 1) in the range of 0.10 to 15 mg/L. The detection and quantification limits are also included.

Optimization of the pressurized liquid extraction of isoflavones

Optimization of the extraction conditions generally involves study of the extraction solvent, the sample amount, temperature, pressure, the extraction time, and the number of extraction cycles. In previous studies addressing PLE extraction for vitamins [26], it was observed that the most important parameters in the extraction yield were the type of extraction solvent and the amount of sample used; the remaining variables had a less marked effect on the extraction process.

Influence of the type of dispersing agent

The samples were mixed with dispersant to prevent the aggregation of particles and so that extraction would occur

Table 1 Calibration curves and detection limits: Y (peak area)=$(a\pm S_a)+(b\pm S_b)X$ (in milligrams per litre)

Isoflavone	Intercept	Slope	R^2	LOD[a] (μg/L)	LOQ[a] (μg/L)
Daidzin	$(-1.1\pm0.1)10^1$	$(2.46\pm0.02)10^1$	0.999	73	243
Genistin	$(-1.0\pm0.8)10^1$	$(6.33\pm0.05)10^1$	0.999	28	93
Daidzein	$(-0.38\pm0.4)10^1$	$(11.3\pm0.1)10^1$	0.998	16	53
Genistein	$(3.2\pm0.4)10^1$	$(11.90\pm0.09)10^1$	0.999	15	50
Formononetin	$(-0.1\pm0.9)10^1$	$(9.73\pm0.04)10^1$	0.9999	18	60
Biochanin-A	$(1.6\pm1.6)10^1$	$(9.59\pm0.08)10^1$	0.999	19	63

[a]LOD and LOQ for a signal-to-noise ratio of 3 and 10, respectively

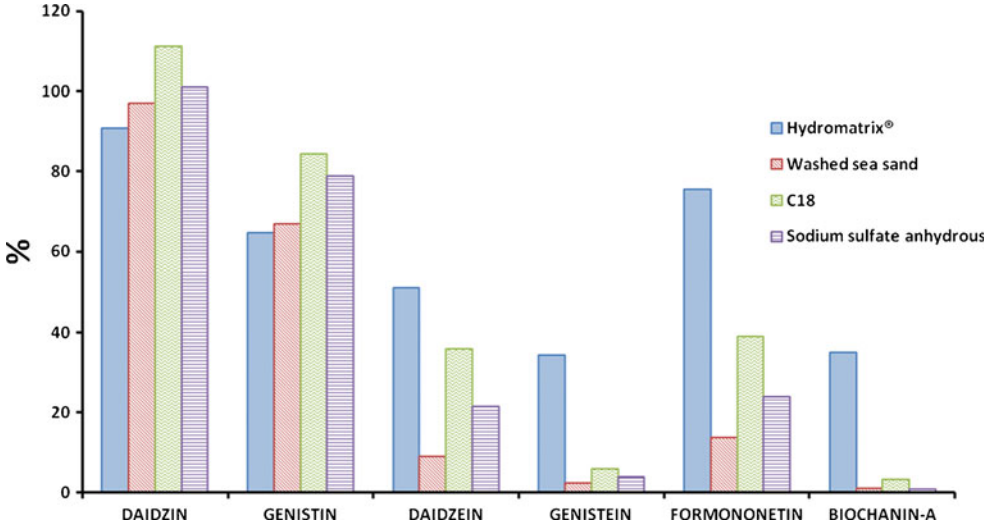

Fig. 2 Influence of the dispersant agent on the percentage of extraction of isoflavones. Dispersants spiked with 5 mg/L each isoflavone, 100 % methanol as extractant, 90 °C, and three cycles of extraction were used

homogeneously. Four dispersants were tested: Washed sea sand, Hydromatrix and anhydrous sodium sulphate, and even C18, in the event of it being required for a cleanup step. The whole method was as follows: at 90ºC, three cycles of extraction with 100 % methanol as extractant were applied to each dispersant spiked with analytes at two concentration levels, to finally achieve 1.00 and 5.00 mg/L in 25.0 mL of extract.

The extracts obtained were injected, and the analytical signals were compared with those obtained from the same standard solutions injected directly with no extraction process. As can be seen in Fig. 2, the dispersants tested had no influence on the extraction of compounds with high polarity, such as isoflavone glucosides, but they did influence the extraction of aglycones. Hydromatrix® afforded the best results, and hence, this was chosen as dispersant.

Influence of the extraction solvent

The extraction solvent must solubilise the analytes with minimum co-extraction of other matrix components, and it must be compatible with the chromatographic system. In addition, the polarity of the solvent should be close to that of the analytes. Here, polar solvents, such as acetonitrile, methanol, ethanol, and mixtures of these with water, were tested. The best results were obtained when methanol/water was used, and hence, a more exhaustive study was carried out in order to minimize the adsorption of the isoflavones. Hydromatrix was spiked with 1.00 mL of isoflavone standards (24 μg/mL of genistin and 75 μg/mL for the others). In all cases, the extracts were bought up to 25.0 mL. The extractions were carried out with different methanol/water mixtures (100; 75:25, 50:50, 10:90); a temperature of 90 °C and three cycles were used.

The results showed that when the extractant polarity decreased, the analytical signal increased (Fig. 3). The highest values were obtained for methanol/water percentages higher than 75:25. Under these conditions, upon studying reproducibility, relative standard deviations of 4.4, 6.0, 4.0, 9.1, 1.9, and 3.0 % were obtained for daidzin, genistin, daidzein, genistein, formononetin, and biochanin-A, respectively.

Optimisation of sample amount, number of cycles, and temperature

In order to apply PLE to pulses, optimization of the amount of sample was performed by weighing amounts of sample, chickpea or lentil, ranging from 1 to 5 g, completing the volume of the cell with Hydromatrix. The extractions were carried out with 75:25 methanol/water as the extraction solvent, using a single extraction cycle. The relationship between the amount of sample and the analytical signal was linear up to 4.0 g. In light of this, sample amounts of 2–3 g were weighed to carry out extraction.

In some cases, a single extraction cycle is not able to extract analytes from the matrix quantitatively, and fresh solvent must

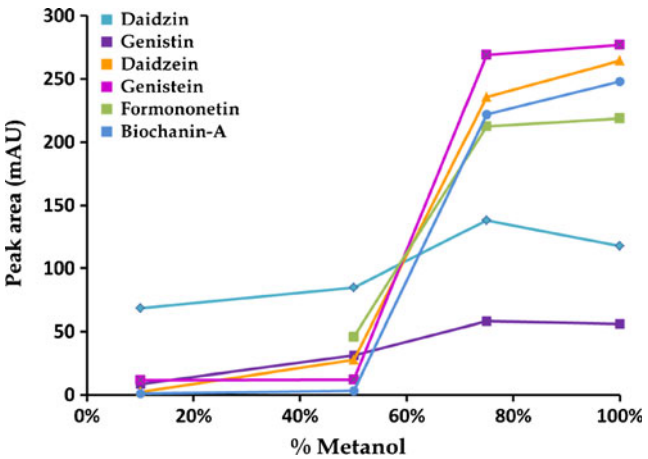

Fig. 3 Influence of extractant polarity on the extraction of isoflavones

Table 2 Amount obtained, in micrograms per gram, of a dry sample of each isoflavone obtained after application of the proposed method in dry and moistened chickpea and lentil samples

	Dry samples			Moistened 20 % samples
	MeOH 100 %[a]	MeOH 75 %[b]	MeOH 50 %[b]	MeOH 100 %[a]
Chickpeas				
Formononetin	0.87±0.3	4.7±0.9	4.3±0.6	4.0±0.6
Biochanin-A	1.2±0.1	1.2±0.7	4.2±0.7	3.2±0.3
Lentils				
Daidzin	15±1	13.1±0.8	15.8±1.4	10.0±0.7
Genistin	2.2±0.3	4.3±0.3	3.9±0.9	3.8±0.3
Formononetin	7.7±0.3	8.3±0.5	6.0±0.6	6.3±0.7

[a] Results from four replicate analyses

[b] Results from six replicate analyses

be introduced during the extraction process in order to maximize extraction efficiency. In the present work, no significant differences were observed when the number of cycles was increased, although better reproducibility was obtained when three or four cycles were used. As a result, it was decided to use three extraction cycles for all the PLE experiments.

It is well known that the use of solvents at elevated temperatures increases both the capacity of the solvents to solubilise analytes and the diffusion rate. In addition, high temperatures decrease the viscosity of solvents and debilitate strong solute–matrix interactions. A study of the oven temperature was carried out: temperatures of 55, 70, 80, 90, and 110 °C were evaluated at a pressure of 1,600 psi. Temperatures over 110 °C were not tested because cloudy extracts were obtained, probably due to the presence of proteins. The best values were obtained in the 70–90 °C range, and finally, a temperature of 90 °C was chosen.

Determination of isoflavones in pulses

When the PLE method was applied to samples of pulses, chickpeas, and lentils, the extraction of isoflavones using methanol as solvent was lower than when methanol/water mixtures were used. Some authors [10, 27] have reported that the extraction of isoflavones from soy-based foods with methanol/water or acetonitrile/water was higher than when methanol or acetonitrile was used as the extraction solvents, especially for the glycoside forms. A more exhaustive study addressing the presence of water was performed. Extractions of isoflavones from dry and wet (20 % moistened) pulse samples were carried out: for dry samples, methanol and methanol/water, at 75:25 and 50:50, were used as extraction solvents whereas for wet samples, only methanol was used. Table 2 shows the results obtained. For both chickpeas and lentils, the presence of water in the extraction solvent increased the extraction of isoflavones better than water in the samples. The best results were obtained when the solvents were methanol/water, 50:50, and methanol/water, 75:25, for chickpeas and lentils, respectively. Figure 4 shows the chromatogram obtained for each sample. Reproducibility was checked as the precision on different days (inter-day). The relative standard deviation values obtained, for six samples over five consecutive days, were 12.9 and 15.3 % for formononetin and biochanin-A, respectively, in chickpeas, and 7.1, 9.4, and 7.2 % for daidzin, genistin, and formononetin, respectively, in lentils.

Confirmation of the nature of the analytes (formononetin and biochanin-A in chickpeas and daidzin, genistin, and formononetin in lentils) was carried out with a LC/MS–MS ion-trap mass spectrometer using the conditions described in the "Experimental" section. The m/z $(M+H)^+$ of the precursor ion/product for each isoflavone were: 417/255 (daidzin), 433/271 (genistin), 255/137 (daidzein), 271/153 (genistein), 269/254 (formononetin), and 285/270 (biochanin-A). Moreover, the

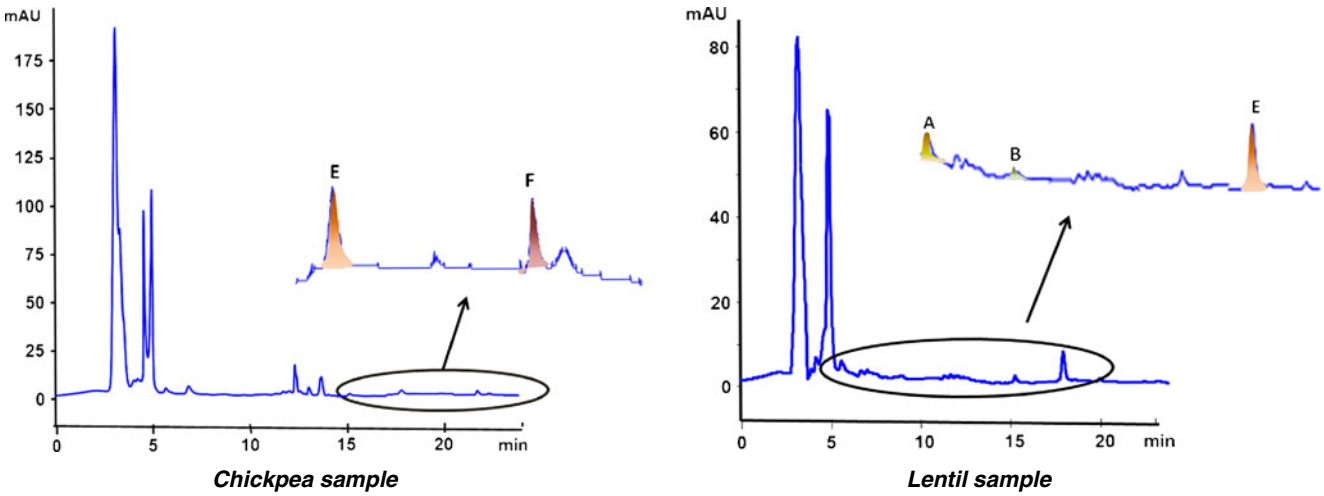

Fig. 4 Chromatograms obtained after application of the method to a sample of chickpeas or lentils. Solvent PLE extraction methanol/water 50:50 (*v*/*v*) for chickpeas and methanol/water 75:25 (*v*/*v*) for lentils. Analyte identification as in Fig. 1

specificity of the method was checked by the absence of any co-eluted peaks of the other components of the chickpea and lentil matrices at the retention times of isoflavones.

The recovery studies (three replicates) were carried out by spiking 2.7 g of sample with 1 mL of solution containing standards (0.96 mg/L of genistin and 3.00 mg/L for the others). PLE extraction and quantification were applied using the optimized parameters, described in the procedure reported in the "Experimental" section; the recoveries obtained (recovery (in percent)=(sample spiked−sample) 100/standard injected directly) were 109 and 107 % for formononetin and biochanin-A, respectively, in chickpeas, and 97, 103, and 99 % for daidzin, genistin, and formononetin, respectively, in lentils. These recoveries were considered satisfactory.

In order to validate the proposed method, lentil samples were analysed using this and the Association of Official Analytical Chemists (AOAC) official method 2008.03 [28] developed for soy-based foods. The AOAC method is recommended for samples with higher contents of isoflavones than those found in pulses; accordingly, only daidzin was quantified. Also, the method was modified slightly: 3.5 g of sample instead of 1.0 g and methanol/water/dimethylsulfoxide instead of acetonitrile/water/dimethylsulfoxide were used. The results obtained for daidzin contents applying both methods were 14.8±0.8 and 13.5±1 for the AOAC method and the proposed method, respectively. Comparison of the results using the *t* test (0.306) indicated that the methods were not significantly different.

Conclusions

An effective, simple, and reproducible method based on pressurized liquid extraction followed by liquid chromatography with diode array detection has been developed for the analysis of isoflavones in pulses. PLE has been applied for the first time for the isolation of isoflavones from this type of sample, whose contents in these substances are lower than those of soy-based foods. The extracts were filtered and injected directly into the LC system, with no additional cleaning step. With the proposed method, sample preparation is faster and easier than with the official AOAC method. Taking into account that the proposed method was validated and that the precision and recovery values obtained were acceptable, this methodology is suitable for control analyses of isoflavones in chickpeas and lentils.

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A modified QuEChERS method as sample treatment before the determination of isoflavones in foods by ultra-performance liquid chromatography–triple quadrupole mass spectrometry

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ABSTRACT

This paper reports the development of an analytical method for the determination of isoflavones in legumes using LC–MS/MS. A modified approach of the QuEChERS methodology was used to extract the analytes from the food samples. The proposed method includes a two-step extraction process and allows the determination of isoflavones in pulses without the need of a clean-up step. Use of this methodology for the extraction of natural occurring substances provides advantages such as simplicity and ease of use, especially taking into account the complexity of food matrices. The method was applied successfully for the determination of eight isoflavones, including aglycones and glucosides, in legumes of Spanish origin (chickpeas, lentils and beans from the region of Castilla y León). The target compounds were the glucosides daidzin, glycitin and genistin, and the aglycones daidzein, glycitein, genistein, formononetin, and biochanin A. The detection limits were in the 0.7 $\mu g\,L^{-1}$ to 1.5 $\mu g\,L^{-1}$ range for formononetin and glycitin respectively. Recoveries ranged from 72% to 119%, and standard deviations lower than 25% were obtained for the inter-day precision. The method described is precise, selective and not time-consuming.

1. Introduction

Isoflavones are a subgroup of phytoestrogens, which are natural plant substances with structures similar to 17-β-estradiol. The plant family most abundant in phytoestrogens is the *Leguminosae*. Soybeans are one of the richest sources of isoflavones in foods; they are also found in red clover, germs of alfalfa, and linseed, as well as in extracts of red wine [1,2].

Isoflavones have the ability to bind to estrogen receptors, depending on the degree of methylation or glycosylation of the hydroxyl groups [3]. Recent studies have shown the possibility of a duality in the estrogenic activity of these substances. Both agonist and antagonist activities that have been described are related with the amount of endogenous estrogens [4].

This group of substances has recently come into the limelight owing to increasing information about their positive effects in a variety of biological activities, such as the treatment of menopausal symptoms [5,6], as an alternative to hormone-replacement therapy (HRT), for cardiovascular disease [7,8], diabetes and obesity [9,10], for osteoporosis [11,12], and even for cancer (e.g., prostate [13] and endometrial cancer [14]). In this regard, the isoflavones with the highest clinical activity are daidzein, genistein and glycitein. These substances arise through both the hydrolysis of biologically inactive forms of glucoconjugates, and through the metabolism of biochanin A and formononetin. In the present work, these substances were analyzed, together with their glycosylated forms (daidzin, genistin and glycitin).

In food analysis, one of the fundamental problems arising from the complexity of the matrices is analyte extraction prior to chromatographic determination. In the determination of bioactive compounds in foods, sample treatment is a critical step and sometimes limits the development of analytical methodologies. In this case, sample treatment is a critical stage because isoflavones are relatively unstable compounds; glucoside esters tend to decrease with time, while the concentration of glycosides and aglycones increases [15].

The first study addressing the extraction of isoflavones was carried out by Eldridge in 1982 [16]. Usually, alkaline or acid hydrolysis with subsequent extraction of the analytes from the unsaponifiable fraction have been used. The most frequent techniques used for extraction include Soxhlet extraction, ultrasonic-assisted extraction (UAE), microwave assisted extraction (MAE), pressurized liquid extraction (PLE) or supercritical fluid extraction (SFE) [15,17,18].

In 2003, Anastassiades et al. [19] developed a new method for the extraction of a broad range of pesticide residues from fruits

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and vegetables (QuEChERS acronym of quick, easy, cheap, effective, rugged and safe) in contrast to the traditional methodology with multiple stages and the use of large amounts of sample. The method consisted of an initial extraction with acetonitrile followed by partitioning with magnesium sulphate, either alone or in combination with other salts, generally NaCl. After extraction, a clean-up step was performed using dispersive solid-phase extraction (d-SPE). This methodology has been modified, depending on the properties of the analyte, the matrix composition, and the techniques and equipment available in the laboratory [20]. The main changes include the addition of acetate [21,22] or citrate [23,24] buffers to avoid the degradation of certain pesticides, and the addition of water to dry samples to obtain the necessary moisture [25–27]. In the following clean-up step, d-SPE has been modified through the use of graphitized carbon black (GCB), C18 sorbent, or Florisil cartridges [28,29]. Other modifications proposed have been the use of dry ice to separate phases without the need for salting-out [30], and elimination of the clean-up step [31].

The QuEChERS method has mainly been applied for the determination of polar, middle polar and non-polar pesticide residues in food matrices [28]. Other compounds, such as antibiotics [32,33] and other veterinary drugs [34–36], steroids [37], and mycotoxins [38–42] have been also determined. To our knowledge, the use of this methodology for the extraction of naturally occurring substances is practically non-existent and the extraction of isoflavones in pulses has not been proposed.

Here we propose a procedure based on the QuEChERS methodology for the extraction of analytes, of different polarities, naturally present in samples, taking into account the advantage of the ease of application of this methodology to complex matrices such as food. One of the main advances provided by the proposed method is related to the elimination of the dispersive SPE step after extraction. Besides, in view of the different polarities of the analytes studied, a two-step extraction process was considered. The samples analyzed were legumes of Spanish origin (chickpeas, lentils and beans from the region of Castilla y León). In order to avoid the disadvantages of the QuEChERS methodology, in which sometimes preconcentration of compounds in the extracts is required, separation by liquid chromatography and detection by mass spectrometry with a triple quadrupole was used. This system has higher sensitivity as well as improved security of identification.

2. Experimental

2.1. Chemicals

The isoflavones studied, Daidzin (CAS RN 552-66-9), Glycitin (CAS RN 40246-10-4), Genistin (CAS RN 529-59-9), Daidzein (CAS RN 486-66-8), Glycitein (CAS RN 40957-83-3), Genistein (CAS RN 446-72-0), Formononetin (CAS RN 485-72-3), and Biochanin-A (CAS RN 491-80-5), were purchased from Sigma-Aldrich (Steinheim, Germany). The internal standard, 3′,4′-Dimethoxyflavone, was obtained from Extrasynthese (Genacy Cedex, France).

The organic solvents–acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH)–were of HPLC grade and were supplied by Merck (Darmstadt, Germany). Formic acid ($>98\%$) was from Fluka (Steinheim, Germany). Anhydrous magnesium sulfate, sodium chloride and trisodium citrate dihydrate ($Na_3Cit \cdot 2H_2O$) were from Scharlau (Barcelona, Spain). Disodium hydrogencitrate sesquihydrate ($Na_2HCit \cdot 1.5H_2O$) was from Sigma-Aldrich. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Spain) water purification system. All other chemicals used were of analytical reagent grade.

2.2. Samples

The analyzed samples were legumes coming from Castilla y León (Spain): chickpeas from Fuentesaúco (Zamora), and lentils and white beans from La Armuña (Salamanca). The samples were ground with a Knifetec^{TM} 1905 from Foss (Barcelona, Spain) before analysis. Sample preparation, as is indicated in Section 2.4, was carried out using a Vortex ZX Classic Velp Scientifica (Milan, Italy). The extracts were filtered through 0.22 μm PVDF Syringe filters (Scharlau).

2.3. Instrumentation

LC analyses were performed on a HP 1200 Series chromatograph from Agilent (Waldbronn, Germany) equipped with a binary pump, a membrane degasser, an autosampler, and a six-port valve. The analytical column was a $50 \times 4.6\ mm^2$ Zorbax Eclipse XDB-C18 with 1.8 μm particles (Agilent). The mobile phase consisted of an acetonitrile (solvent A) and 0.01% aqueous formic acid (solvent B) gradient, at a flow rate of 0.5 mL min^{-1}. The gradient elution was as follows: 0–1.5 min, 10% A; 1.5–2.5 min, 10–25% A; 2.5–3.5 min, 25% A; 3.5–7 min, 25–50% A; 7–8 min, 50–80% A; 8–10 min, 80% A, 10–12 min, 80–10% A. The analytical column was thermostated at 25 °C, and the injection volume was 10 μL.

Detection was carried out on a Triple Quad LC/MS 6410 (Agilent) equipped with an electrospray (ESI) source. ESI-MS spectra were acquired in positive-ion multiple reaction monitoring (MRM) mode. The conditions of the MS analysis were as follows: the electrospray capillary voltage was 3500 V and the nebulizer pressure was 35 psi. Nitrogen was used as a drying gas at the flow rate of 12 L min^{-1} at a temperature of 350 °C. The whole system was controlled by an Agilent Mass Hunter software, version B.04.01.

2.4. Sample preparation (QuEChERS methodology)

For sample treatment with the QuEChERS method, 5.0 to 7.0 g (depending on the kind of legume) of ground samples were taking in a 50 mL plastic centrifuge tube with screw cap. Extraction was carried out in two steps: firstly, 10 mL of ACN:H_2O (70:30, v/v) was added and the mixture was shaken for 5 min with a Vortex device; then, 5 mL of ACN was added and the mixture was shaken again for another 5 min. Following this, a mixture of 4 g of magnesium sulfate and 1 g of sodium chloride was added. The tube was immediately shaken vigorously for 1 min to prevent the formation of $MgSO_4$ conglomerates and centrifuged at 3000 rpm for 5 min. Finally, the extract was filtered through a 0.22 μm PVDF syringe filter before injection into the chromatographic system.

3. Results and discussion

3.1. Optimization of LC–MS

3.1.1. Optimization of chromatographic variables

Isoflavones have acidic-basic characteristics, with pK_a values ranging from 9.74 to 9.81 [43]. Accordingly, the mobile phase was acidified with 0.01% aqueous formic acid to prevent the deprotonation of analytes and to improve the shape of the chromatographic peaks. Mixtures of methanol–aqueous formic acid and acetonitrile–aqueous formic acid as mobile phases were compared in order to obtain the best chromatographic behavior. It was observed that the acetonitrile–aqueous formic acid gradient produced the best results.

Different types of gradient were tested to achieve the separation of the chromatographic peaks as well as their separation from the injection front, which is very useful in real samples. The optimized gradient was: 0–1.5 min, 10% A; 1.5–2.5 min, 10–25% A; 2.5–3.5 min, 25% A; 3.5–7 min, 25–50% A; 7–8 min, 50-80% A; 8–10 min, 80% A, 10–12 min, 80–10% A. The mobile phase consisted of an acetonitrile (solvent A) and 0.01% aqueous formic acid (solvent B). Fig. 1 shows the optimized gradient next to the TIC of a standard solution of the eight isoflavones studied.

3.1.2. Optimization of the mass spectrometry conditions

MS spectra were studied in both positive and negative modes. The positive mode was employed because it afforded the highest sensitivity. For the optimization of the MS conditions, the fragmentor voltage and collision energy were optimized by injection of the individual standard isoflavone solution directly into the mass spectrometer, using the optimum conditions for fragmentation.

The optimization of the precursor ion and product ions was carried out by direct injection of the individual standard isoflavone solution into the mass spectrometer. The Fragmentor voltage and collision energy were also optimized. These optimized parameters are shown in Table 1. The most abundant product ion was used as an identification point; the rest of the product ions were used for confirmatory analysis. The P.I. are also shown in Table 1.

3.2. Optimization of sample treatment (extraction conditions)

Optimization of the parameters involved in the extraction process was carried out using the three kinds of legume studied: chickpeas, lentils and white beans. The extraction method used for these experiments was as follows: the ground legume sample was mixed with the extraction solvent and the mixture was shaken for 5 min with a Vortex device. Then, a combination of magnesium sulfate and sodium chloride (4 g:1 g) was added and the mixture was shaken before centrifugation. The filtered extract was injected directly into the chromatographic system. The parameters studied were the type and volume of extraction solvent, the sample amount, the extraction time, salting-out, and clean-up.

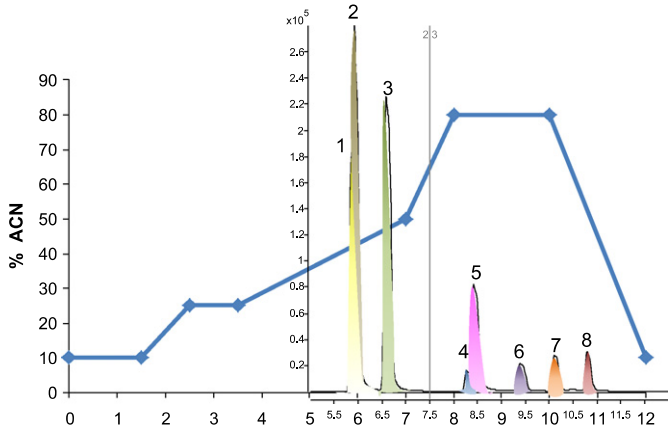

Fig. 1. Optimized elution gradient and TIC of standard solutions of daidzin (1), glycitin (2), genistin (3), daidzein (4), glycitein (5), genistein (6), formononetin (7) and biochanin A (8).

Table 1
LC–MS/MS conditions for the analysis of isoflavones

Compound	Fragmentor (*V*)	Precursor ion (*m*/*z*)	Collision energy (*V*)	Product ions (*m*/*z*)	I.P.[a]
Daidzin	110	417.1	12	255.1	2.5
Glycitin	90	447.1	8/40	285.1/270.1	4
Genistin	90	433.1	16	271.1	2.5
Daidzein	150	255.1	28/24/40	137/199.1 /91	5.5
Glycitein	150	285.1	24/32	270/242	4
Genistein	150	271.1	28/40	153/91.1	4
Formononetin	150	269.1	40/28	197.1/253	4
Biochanin A	150	285.1	24/40	152/213	4

[a] Identification points (IPs) according to Commission Decision 2002/657/EC.

3.2.1. Optimization of the type and volume of extraction solvent

In order to optimize the extraction conditions, different solvents–acetonitrile, methanol, ethanol and water–and mixtures thereof at different proportions were investigated at two levels of sample concentration (3 and 5 g of chickpeas and lentils). It was found that for most of the isoflavones the best results were obtained when the extraction solvent was 80% acetonitrile. In this study, different extraction efficiencies were also observed, depending on the polarity of the analytes, and those of the extraction solvent. In view of the different polarities of the analytes studied, a two-step extraction process was considered. The aim of this approach was to extract the more polar analytes first and then, by decreasing the polarity of the extraction solvent, to extract of the less polar analytes. The most efficient approach to achieve the extraction of the analytes was to begin with ACN:H_2O (70:30, v/v), then adding 100% acetonitrile, achieving a final proportion of ACN:H_2O (80:20, v/v), considered optimum in the previous study. Fig. 2 shows the analytical signals obtained for the samples when the extraction was carried out using ACN:H_2O (80:20, v/v) (one-step) and a two-step extraction, decreasing the polarity of solvent.

Study of the extraction solvent volume revealed that extraction with a volume of solvent higher than that proposed in the original QuEChERS methodology improved the extraction yields. Thus, a total volume of 15 mL was chosen: first, 10 mL of ACN:H_2O (70:30, v/v) were added to the sample and the mixture was shaken for 5 min with a vortex device. Subsequently, 5 mL of 100% acetonitrile was added and the mixture was shaken again for another 5 min.

3.2.2. Optimization of sample amount and extraction time

In order to determine the most suitable sample amount to achieve extraction, extracts of the three types of samples studied–chickpeas, lentils and beans–were analyzed at four concentration levels: 3, 5, 7 and 10 g of dry solid sample. Fig. 3 shows the behavior of the different types of sample versus the extraction process. Whereas chickpeas and white beans reached saturation in the extraction, in lentils this behavior was not observed. It is important to point that the profiles of chromatographic peaks are kept for all cases. In light of this, a sample amount of 5 g was chosen to carry out the extraction of chickpeas and white beans and 7 g was chosen for lentils.

The QuEChERS methodology allowed analyte extraction in about 1 min. Taking into account that the isoflavones were present in their natural form in the samples analyzed and that their concentration was very low, we tested whether the increase in the extraction time might increase the efficiency of extraction. An experiment to determine the optimal conditions for the extraction of the isoflavones was conducted using a vortex device. The extractions were carried out using times ranging from 1 to 15 min. When the extraction time increased, a rise in the analytical signal was observed, but reproducibility decreased. Accordingly, an extraction time of 5 min was chosen as a compromise between the extraction yields and reproducibility (Fig. 4).

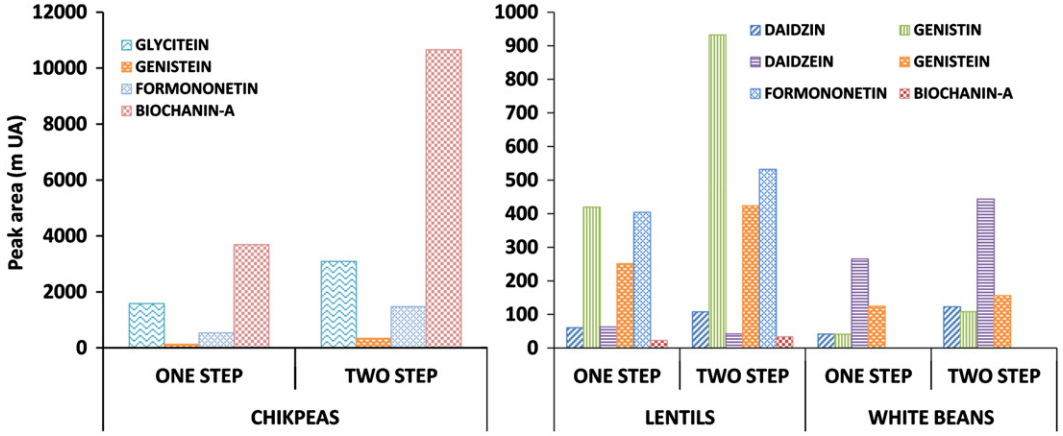

Fig. 2. Comparison of the analytical signals using one or two steps in the extraction process.

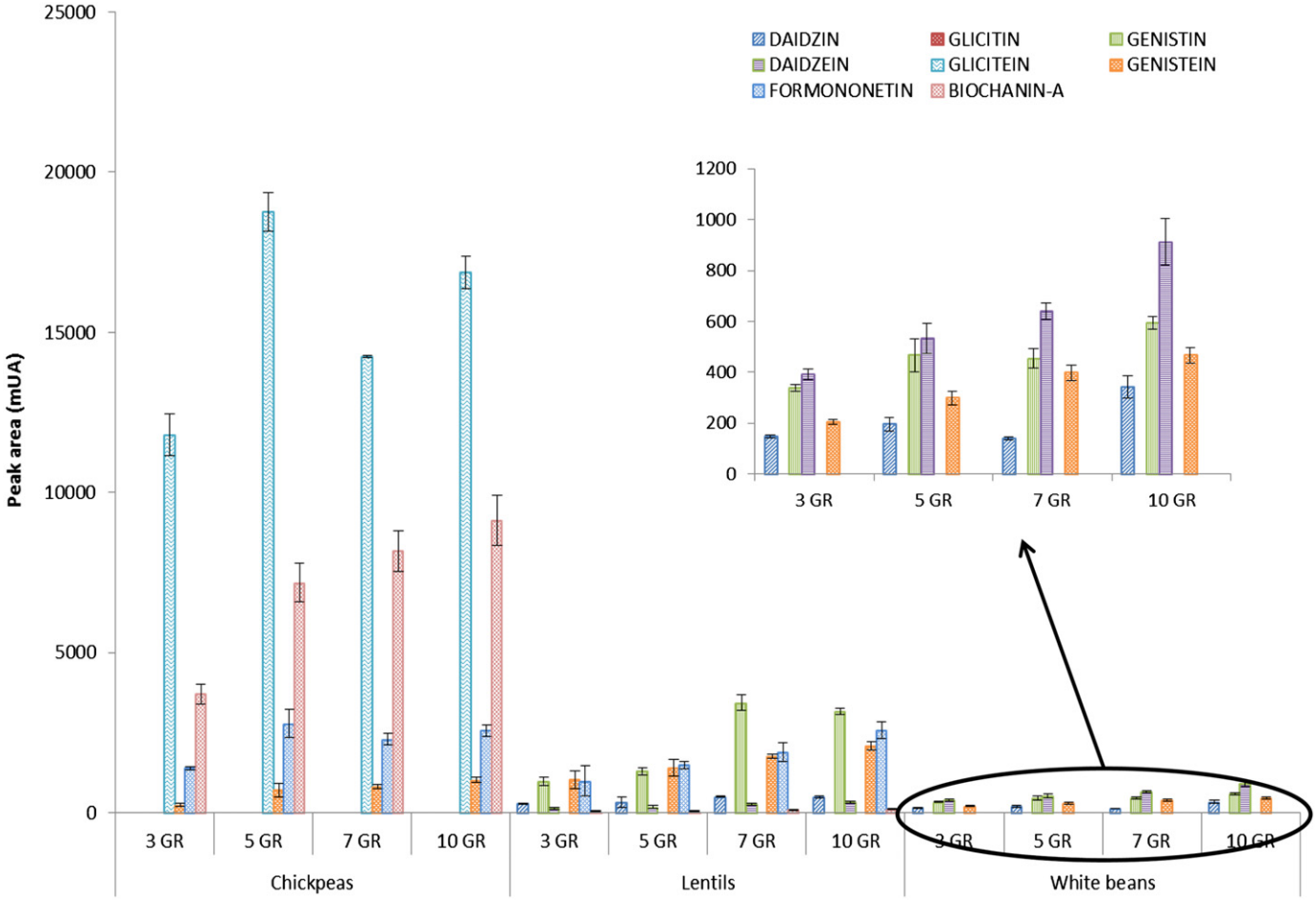

Fig. 3. Influence of sample amount on the analytical signal.

3.2.3. Optimization of salt addition and clean-up

In the QuEChERS methodology, phase separation was induced by the addition of various salts–avoiding the use of potentially toxic and expensive co-solvents. The salt most used is $MgSO_4$, which reduces the volume of the aqueous phase and facilitates the partitioning of polar analytes into the organic phase [28]. By varying the amount of NaCl added to the sample during partitioning with $MgSO_4$, it is possible to control the polarity range of the method and thus the amount of interferents in the extract. To avoid the presence of interferents in the extracts, the best option was to use a mixture of 4 g $MgSO_4$ and 1 g NaCl.

The original QuEChERS method has previously been modified by the addition of acetate or citrate buffers to prevent the degradation of certain pesticides. By buffering with citrate salts (1 g of $Na_3Cit \cdot 2H_2O$ and 0.5 g of $Na_2HCit \cdot 1.5H_2O$) the pH value was maintained at 5–5.5. In our case, taking into account the pK_a of the isoflavones studied, the addition of citrate buffer provided a pH-value that could be adequate for the quantitative extraction of

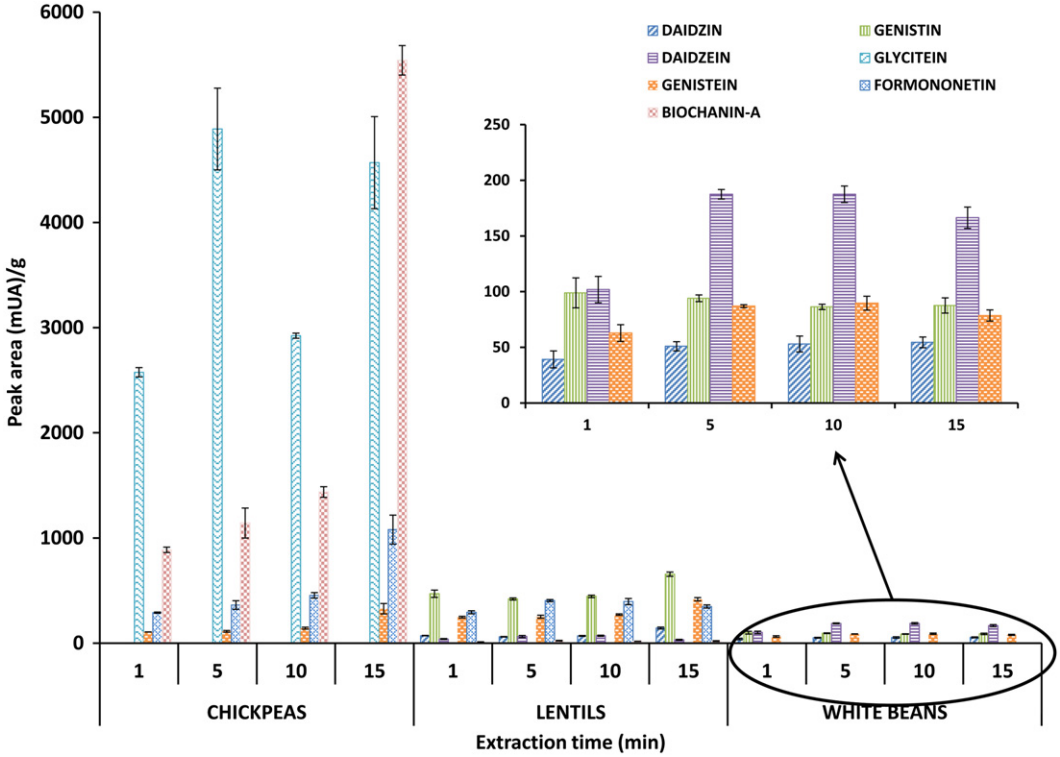

Fig. 4. Influence of extraction time on the extraction efficiency.

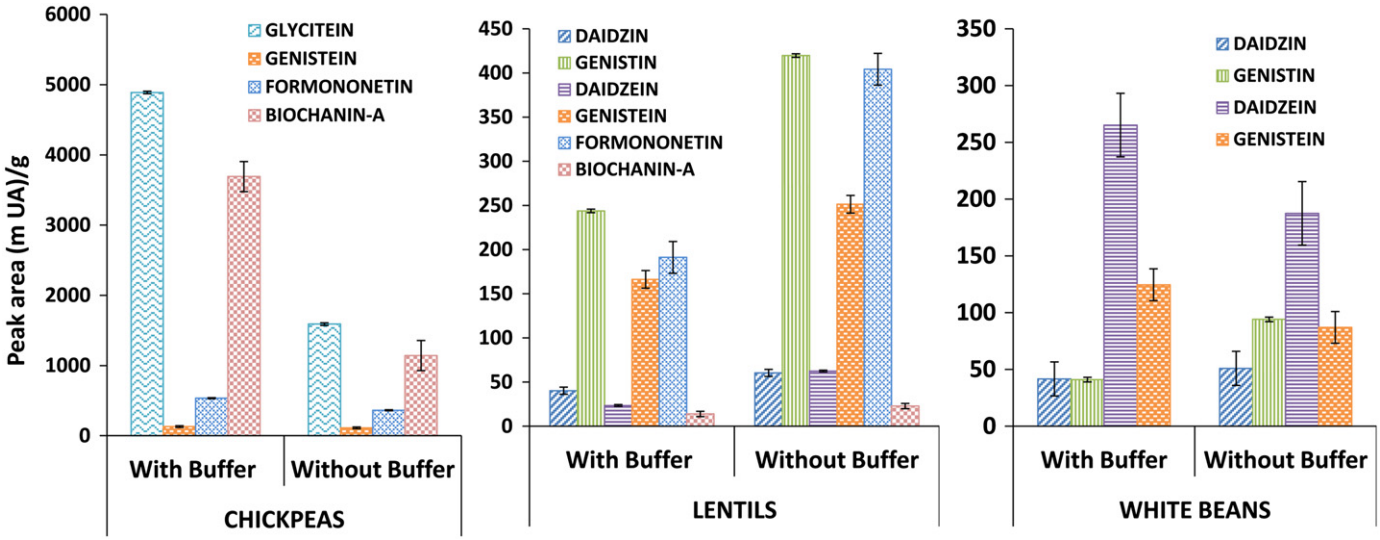

Fig. 5. Influence of the addition of citrate buffer.

protonated analytes, as well as the protection of alkaline groups. In order to find the best extraction conditions, 1 g of $Na_3Cit \cdot 2H_2O$ and 0.5 g of $Na_2HCit \cdot 1.5H_2O$ was added and the samples were shaken vigorously. Analysis of the results pointed to two types of behavior; in the case of the samples of chickpeas and white beans, analyte extraction was improved in the presence of citrate buffer, especially in the case of aglycones. This could be due to a higher presence of protonable amino acids in these matrices. However, in the case of the lentils, the addition of citrate buffer produced a decrease in the signal (Fig. 5). Therefore, for the chickpeas and white beans salting-out was achieved using the buffered method, whereas in the case of the lentils citrate no buffer was added.

Finally, a study was made of the clean-up step, using d-SPE with PSA or C18. In both cases it was found that neither the resolution of the chromatograms nor recovery was improved. Fig. 5 shows the behavior observed for the chickpea samples, without d-SPE (Fig. 6A) and when C18 (Fig. 6B), and PSA (Fig. 6C) were added for extract clean-up. The differences between A and B were insignificant, as expected, because this kind of sample contains relatively low amounts of lipids. However, when d-SPE was carried out with PSA, less interference was observed in the chromatogram, but the signal corresponding to the more polar analytes (such as genistin) or intermediate-polarity analytes (genistein) disappeared. Similar results were observed for white

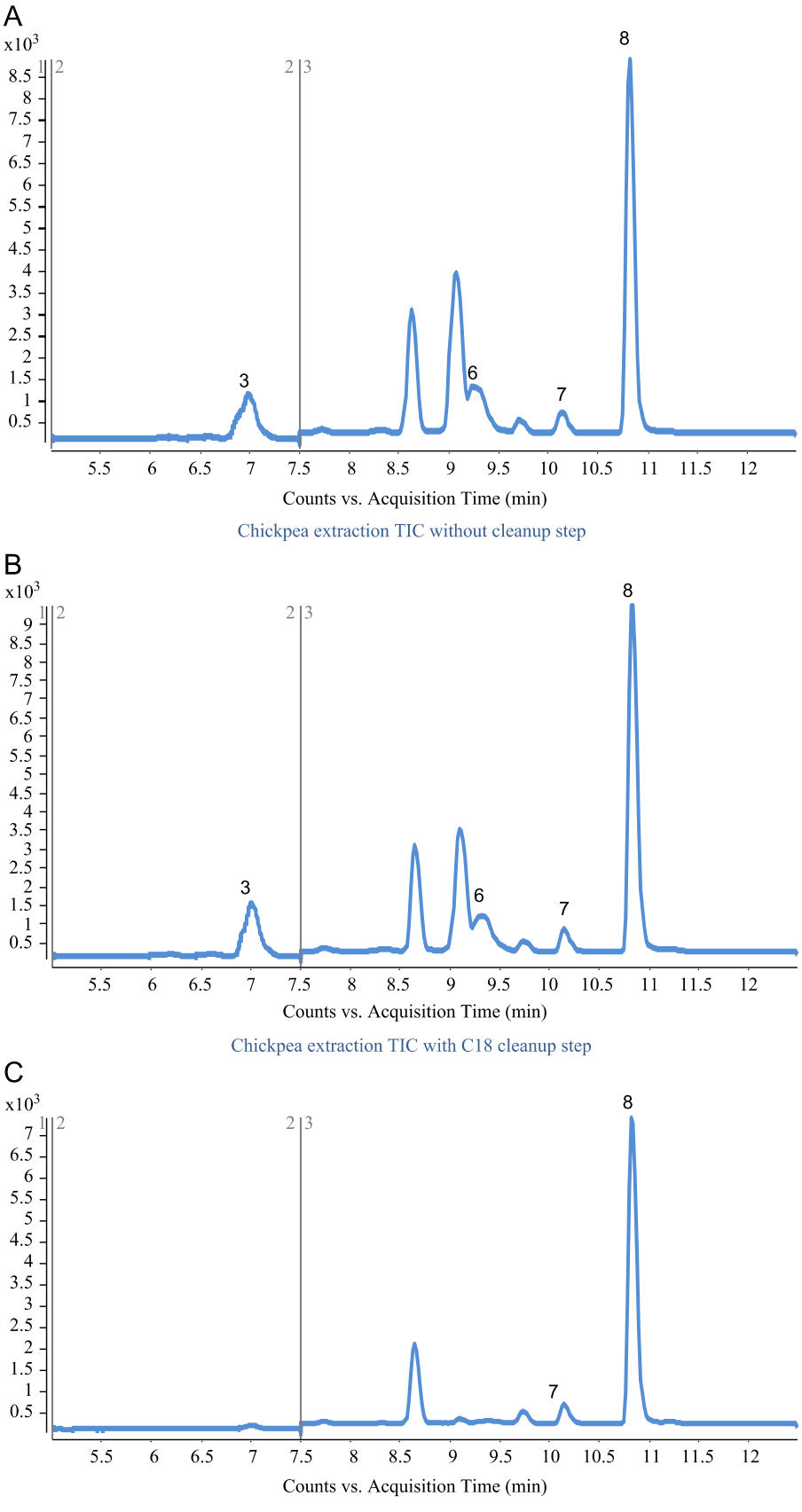

Fig. 6. Study of the clean-up step in the extraction of chickpeas samples: (A) without d-SPE, (B) d-SPE with C18, and (C) d-SPE with PSA.

Table 2
Analytical Characteristics of the QuEChERS–LC–MS/MS method applied to the analysis of isoflavones in legumes samples

Validation		Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein	Formononetin	Biochanin A
Calibration parameters	Intercept (ua)	203 ± 54	1197 ± 92	55 ± 31	49 ± 62	604 ± 399	28 ± 13	581 ± 60	25 ± 64
	Slope (ua μg L^{-1})	145 ± 2	180 ± 4	188 ± 1	28 ± 1	129 ± 8	33.3 ± 0.6	74 ± 3	13 ± 0.3
	R^2	0.9992	0.9985	0.9999	0.9957	0.9892	0.9992	0.9957	0.9988
	LOD[a] (μg L^{-1})	0.97	1.5	1.2	1.03	0.91	0.97	0.71	1.1
	MLOD[a,b] (μg/100 g)	0.40	1.8	0.30	1.6	1.2	1.1	1.5	0.8
Reproducibility as RSD %	Chickpeas	–	–	–	–	9.8	19.2	15.8	17.3
	Lentils	21.1	–	22.9	10.1	–	20.3	16.7	25.7
	White beans	25.8	–	6.9	21.6	–	12.5	–	–
Recovery	Chickpeas	107 ± 8[c]	[c]96 ± 11	85 ± 15[c]	84 ± 13[c]	75 ± 13	104 ± 20	75 ± 12	97 ± 13
	Lentils	113 ± 13	95 ± 11[c]	103 ± 17	107 ± 13	119 ± 15[c]	77 ± 12	72 ± 12	75 ± 14
	White beans	100 ± 11	112 ± 7[c]	104 ± 7	110 ± 7	109 ± 4[c]	96 ± 14	101 ± 14[c]	97 ± 13[c]

[a] $S/N=3$
[b] MLOD: Limit of detection of method
[c] Recovery of isoflavones not quantified in the samples.

beans and lentils. In light of this, the clean-up step was not necessary to increase the recovery of isoflavones from legumes. It should be noted that the time taken for the treatment of all samples was less than 20 min.

3.3. Method validation

3.3.1. Calibration curves and detection limits

Linearity was tested by the injection of standard mixtures of the eight isoflavones studied, in triplicate, at concentration levels ranging from 0.1 to 500 μg L^{-1} for biochanin A; from 0.1 to 100 μg L^{-1} daidzein and glycitein, and from 0.1 to 40 μg L^{-1} for the rest. These ranges were chosen taking into account the expected levels in the matrices studied. Calibration curves based on the peak area versus the standard concentration were obtained and good correlation coefficients ($R^2 > 0.998$) were obtained for all compounds (Table 2).

Detection limits, calculated on the basis of a signal-to-noise ratio (S/N) of 3, were between 0.71 μg L^{-1} for formononetin and 1.5 μg L^{-1} for glycitin. The method detection limits (MLOD) were also evaluated, considering a sample blank without isoflavone, in some cases, or low-level real matrix sample in others (Table 2).

Quantitative determinations, using ESI as an ion source, may be affected by ion suppression, which mainly occurs due to the co-elution of matrix compounds with the analytes. The matrix effect was evaluated by comparison of the response of the target compounds in spiked legumes samples (standard addition calibration) and in aqueous standards calibration. The slopes of the standard addition and calibration with aqueous standards were compared for each analyte in the samples of legumes using Student's t-test. In all cases p values > 0.05 were found, suggesting that there were no significant differences between the two methods. It is therefore possible to conclude that there was no matrix effect.

In order to increase the precision of method, calibration curves based on the internal standard (IS) method were also obtained. Several analytes were tested–apigenin, 4,4′-dimethoxychalcone, 7,8-dimethoxyflavone and 3′,4′-dimethoxyflavone. The latter was chosen as an IS because under the working conditions it was separated from the rest of analytes and it was ionized in the MS system. The concentration of the IS was also studied taking into account the analyte concentration obtained using the external standard quantification method. 10 mg L^{-1} of 3′,4′-dimethoxyflavone was added to standard mixtures of the isoflavones at the same concentration levels described above. The calibration curves based on the ratio between the peak areas of each standard and the internal standard versus the standard concentration showed good correlation coefficients ($R^2 > 0.995$) for all compounds.

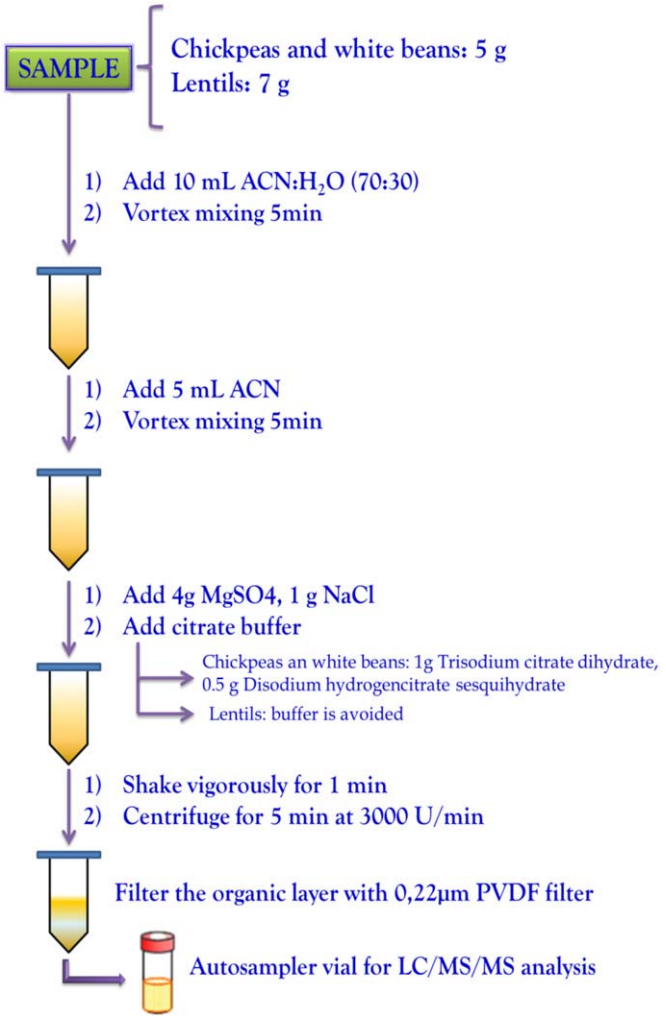

Fig. 7. Scheme of the proposed method.

In the absence of certified or standard materials, the method was validated by measuring the percentage of recovery after the addition of known amounts of standard to the samples: chickpeas, lentils and white beans. Recovery studies performed in

Table 3
Isoflavone contents (μg/100 g dry sample ± RSD) of legume samples using the external standard method (ES method) and the internal standard method (IS method)

	Chickpeas			Lentils			White beans		
Isoflavone	ES	IS	p^a	ES	IS	p^a	ES	IS	p^a
Daidzin	–	–	–	1.77 ± 0.33	1.3 ± 0.3	0.186	0.52 ± 0.23	–	–
Glycitin	–	–	–	–	–	–	–	–	–
Genistin	–	–	–	0.33 ± 0.15	0.3 ± 0.15	0.822	0.32 ± 0.15	0.27 ± 0.15	0.71
Daidzein	–	–	–	2.50 ± 0.30	2.1 ± 0.4	0.57	2.63 ± 0.3	2.13 ± 0.45	0.208
Glycitein	18 ± 3	18 ± 3	0.658	–		–			
Genistein	3.1 ± 0.4	3 ± 0.5	0.707	10.3 ± 0.5	9.3 ± 0.5	0.092	1.35 ± 0.35	1.02 ± 0.3	0.303
Formononetin	4 ± 1	3 ± 1	0.584	2.3 ± 1	1.5 ± 1	0.339	–	–	–
Biochanin A	474 ± 16	369 ± 79	0.109	1.1 ± 0.4	0.8 ± 0.2	0.329	–	–	–

[a] *p*-value obtained in Student's *t*-test

triplicate were carried out by spiking samples of legumes, before sample treatment, with the eight isoflavones studied at concentration levels close to those present in the original samples. When isoflavone was not naturally present in the samples the fortification level was 5 μg L^{-1}. Recovery %= (spiked sample–sample)/ amount added directly injected. Values obtained are shown in the Table 2. In all cases the recovery values were satisfactory, ranging from 72% to 119%.

Reproducibility was checked as the precision on different days (inter-day). The relative standard deviation (RSD) values obtained for eight samples over consecutive six days ranged between 25.8% for daidzin in white beans and 9.8% for glycitein in chickpeas. These are highly very acceptable values for these types of kind of complex sample.

3.4. Applicability of the optimized method

In order to verify the applicability of the proposed method (Fig. 7), commercial samples of chickpeas, lentils and white beans from Castilla y León were analyzed in triplicate. Quantification of the isoflavones was performed using the external standard and the internal standard methods. The results obtained from the analysis of the three kinds of legume sample in μg/100 g of dry sample are shown in Table 3. As may be seen the highest contents of isoflavones were found in chickpeas. Biochanin A and glycitein were major isoflavones in chickpeas, genistein in lentils, and daidzein in white beans.

Comparison of the results obtained with the external standard and the internal standard methods was achieved using Student's *t*-test. There were no significant differences between the results obtained with either quantification method (at a level of significance of 0.05) and it was possible to perform the quantification using the internal standard or external standard methods.

On comparing the proposed QuEChERS–LC–MS/MS method with others reported in the literature, which analyze free and conjugated phytoestrogens in legumes and also use LC–MS/MS, it may be concluded that the limits of detection obtained with the proposed method are similar to those reported by Konar et al. [44] and even better than those obtained with the other method proposed by Antonelli and colleagues [45]. From point of view of sample treatment, in this case QuEChERS extraction is simpler and less time-consuming than the extractions used by these authors.

4. Conclusions

In this work a modified QuEChERS approach was applied for the extraction of analytes naturally present in food samples. The proposed method includes a two-step extraction process and allows the determination of isoflavones in pulses without the need of a clean-up step. The extraction method is simple and easy to use, making it very suitable for complex matrices such as foods. The method developed was applied to determination of isoflavones in legumes of Spanish origin (chickpeas, lentils and beans, from the region of Castilla y León). The proposed method included extraction of the analytes using the QuEChERS methodology, followed by LC–MS/MS. This methodology permits determination of free and conjugated isoflavones in their natural form in pulses. The proposed method is precise, selective and not time- consuming.

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Comparative Study of the Methodology Used in the Extraction of Isoflavones from Legumes Applying a Modified QuEChERS Approach

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ABSTRACT:

Introduction – Isoflavones are phytochemicals of great interest because of their association with a large variety of positive effects on human health. The major sources of isoflavones in the diet are plants of the Leguminosae family, especially soybeans, although many other legumes more widely consumed in the Mediterranean diet have also been reported to contain these compounds. In previous work we extracted isoflavones from samples using a modified QuEChERS (Quick Easy Cheap Effective Rugged Safe) methodology.

Objective – To compare different methods for placing the sample and the solvent in contact to optimise the extraction of isoflavones from legumes (chickpeas, lentils and white beans) using a modified QuEChERS methodology.

Method – Five different approaches to sample agitation were tested: vortex agitation, thermostatted stirring agitation and thermostatted tray shaking, and a thermostatted ultrasound bath and an ultrasound probe. To evaluate the different methodologies a modified QuEChERS approach was used as the extraction method. The separation and quantification of isoflavones was carried out using liquid chromatography–triple quadrupole/mass spectrometry (LC–MS/MS).

Results – The best methods were found upon using a thermostatted shaking tray for the extraction of chickpeas and white beans and the ultrasound probe for lentil samples. These methods were chosen based on the highest amount of analytes obtained as well as the best recovery values.

Conclusion – Determination of isoflavones in foods may be affected by the different methods used to place the sample and the solvent in contact in the extraction step. The main advantages of the proposed extraction procedures are their simplicity, speed, reliability and low cost. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Agitation system; liquid chromatography–mass spectrometry; QuEChERS; isoflavone; legume

Introduction

Isoflavones are phytochemicals of great interest because of their association with a large variety of positive effects on human health, in particular the prevention of hormone-dependent cancers, cardiovascular disease, osteoporosis, adverse menopausal manifestations and age-related cognitive decline (Pilšáková *et al*., 2010).

The major sources of isoflavones in the diet are plants of the Leguminosae family, especially soybeans, alfalfa and red clover, although many other legumes have also been reported to contain these compounds. In plants, isoflavones mainly occur as glucoconjugates, which are biologically inactive and are hydrolysed in the human gut to their aglycones (with biological activity) (Mazur *et al*., 1998).

The analytical determination of isoflavones is usually carried out using liquid chromatography (Peñalvo *et al*., 2004; Zafra-Gómez *et al*., 2010; Konar *et al*., 2012) or capillary electrophoresis (Xiao *et al*., 2011; Bustamante-Rangel *et al*., 2012), although other techniques such as gas chromatography (Magiera *et al*., 2011) and inmunoanalysis (Bennetau-Pelissero *et al*., 2003) have also been used.

The preparation of samples of food matrices generally involves multiple steps such as drying, homogenisation, sieving, extraction, pre-concentration and derivatisation (Rostagno *et al*., 2009). Furthermore, this step may alter the composition of isoflavone mixtures (free or derivatives). When the aim is to determine the total isoflavone content, regardless of the chemical structure, the sample treatment usually involves hydrolysis. Three main strategies for performing the hydrolysis of isoflavones have been reported in the literature: acid (Konar *et al*., 2012; Toro-Funes *et al*., 2012), basic (AOAC, 2005; Delmonte *et al*., 2006) and enzymatic (Shao *et al*., 2011; Fiechter *et al*., 2013) hydrolysis. Usually, the extraction of isoflavones from foods is achieved by extraction with organic solvents such as pure or aqueous methanol (MeOH) (Eisen *et al*., 2003; Setchell and Cole, 2003; Tsai *et al*., 2007), ethanol (EtOH) (Cho *et al*., 2009) or acetonitrile (ACN) (Griffith and Collison, 2001; Lin and Giusti, 2005) at room (RT) or elevated temperature, using different modes of agitation and with or without the presence of acid (Lee *et al*., 2003; Lin and Giusti, 2005) (Table 1).

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Table 1. Conventional methods for the solvent extraction of isoflavones from foods

Sample	Agitation mode	Extraction solvent	Extraction time and temperature	Reference
Soy milk	Shaking	Methanol	2 h at RT	Eisen *et al.*, 2003
Soy foods (soy protein isolates and milks)	Refluxing	80% Methanol	1 h	Setchell *et al.*, 2003
Soybean	Inversion by hand	80% Methanol	30 min at 70 °C	Tsai *et al.*, 2007
Soybean sprout cotyledon	Reflux in a vertical mixer	80–90% Ethanol	100 min at 90 °C	Cho *et al.*, 2009
Soy drink	Inversion mixer	Ethanol	30 min at RT	Bustamante-Rangel *et al.*, 2012
Supplemented milk and juice	Vortex	20% Acetonitrile	1 min + 15 min precipitation at RT	Zafra-Gómez *et al.*, 2010
Soy foods and nutritional supplements	Rotary mixer	60% Acetonitrile	2 h at RT	Griffith *et al.*, 2001
Soy foods	Stirring	58% Acetonitrile	2 h at RT	Murphy *et al.*, 2002
Soybean	Stirring	58% Acetonitrile	2 h at RT	Lin *et al.*, 2005
Soybean	Stirring	Acetonitrile 0.1 M HCl	2 h at RT	Lee *et al.*, 2003

Currently, new methods are being developed to extract isoflavones from food matrices, including ultrasound-assisted extraction (Rostagno *et al.*, 2007b; Sun *et al.*, 2011), microwave-assisted extraction (Rostagno *et al.*, 2007a; Terigar *et al.*, 2010), pressurised liquid extraction (Zgórka, 2009; Delgado-Zamarreño *et al.*, 2012a) and supercritical fluid extraction (Zuo *et al.*, 2008; Klejdus *et al.*, 2010). These methodologies reduce sample manipulation, the extraction time (about 10 to 60 min) and solvent consumption, but require more expensive and complex equipment than conventional solvent extraction techniques.

In previous work we extracted isoflavones from both liquid (Bustamante-Rangel *et al.*, 2012) and solid (Delgado-Zamarreño *et al.*, 2012b) samples using solvent extraction, either alone or with the addition of salts, using a modified QuEChERS (Quick Easy Cheap Effective Rugged Safe) methodology. This latter approach was applied to the extraction of eight isoflavones, including aglycones and glucosides, from legume samples. The use of this methodology for the extraction of naturally occurring substances provides advantages such as speed (extraction time was about 10 min), simplicity and ease of use.

The aim of this study was to compare different methods for placing the sample and the solvent in contact in the extraction of isoflavones from legumes. To this end, three different kinds of legume samples (chickpeas, lentils and white beans) were analysed using conventional solvent extraction methods and ultrasound-assisted extraction. For this purpose, five different approaches for sample agitation were tested: vortex agitation, thermostatted stirring agitation and thermostatted tray shaking, and a thermostatted ultrasound bath and an ultrasound probe. To evaluate the different methods of placing the sample and solvent in contact, a modified QuEChERS approach was used in all cases because it provided greater efficacy in the extraction of such analytes in this kind of matrix. Considering the best extraction procedure for each type of legume, the methodologies developed were validated for the analysis of isoflavones in legumes using LC–MS/MS.

The importance of assessing these analytes in this kind of sample lies not only in health benefits but also in the novelty of the matrix, because the legumes tested here are more widely consumed in Europe than are soybeans.

Experimental

Chemicals

Isoflavone standards: daidzin (Di, CAS RN 552-66-9, ≥ 95%), glycitin (Gli, CAS RN 40246-10-4, analytical standard), genistin (Gi, CAS RN 529-59-9, ≥ 95%), daidzein (De, CAS RN 486-66-8, ≥ 98%), glycitein (Gle, CAS RN 40957-83-3, ≥ 97%), genistein (Ge, CAS RN 446-72-0, ≥ 98%), formononetin (For, CAS RN 485-72-3, ≥ 98%) and biochanin-A (Bio, CAS RN 491-80-5, analytical standard) were purchased from Sigma-Aldrich (Alcobendas, Madrid, Spain). This company also supplied formic acid (> 98%). The HPLC grade acetonitrile was supplied by Merck (Darmstadt, Hesse, Germany). Anhydrous magnesium sulphate, sodium chloride and trisodium citrate dihydrate ($Na_3Cit.2H_2O$) were from Scharlau (Barcelona, Cataluña, Spain). Disodium hydrogencitrate sesquihydrate ($Na_2HCit.1.5H_2O$) was from Sigma-Aldrich. Ultrahigh-quality (UHQ) water was obtained with a Wasserlab (Noain, Navarra, Spain) water purification system. All other chemicals used were of analytical reagent grade.

Samples

The samples analysed were legumes from the Region of Castilla y León (Spain): chickpeas were from the town of Fuentesaúco (Zamora), and lentils and white beans were from the La Armuña district (Salamanca). The samples were ground with a Knifetec™ 1905 from Foss (Barcelona, Spain) and were stored in a cool (4 °C), dry place in closed containers.

Instrumentation

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisted of an Agilent Technologies (Waldbronn, Baden-Württenmberg, Germany) 1200 Series chromatograph equipped with a binary pump, a membrane degasser, an autosampler, a six-port valve, a diode-array detector (DAD) and a 6410 LC–MS triple quadrupole (QqQ) mass spectrometer. The analytical column was a 50 × 4.6 mm Zorbax

Eclipse XDB-C18 with 1.8 mm particles (Agilent). The whole system was controlled by Agilent Mass Hunter software, Version B.04.01.

Extraction of isoflavones was carried out with the QuEChERS methodology using different systems for placing the sample in contact with the extraction solvent. The systems used were a Vortex ZX Classic Velp Scientifica (Milan, Lombardia, Italy) for vortex agitation. A Jenway 100 magnetic stirrer connected to an SBS immersion thermostat and a VWR thermostatted shaking tray (Radnor, PA, USA) were used for stirring methodologies, a Bandelin Electronic ultrasound bath (Berlin, Germany) coupled to the immersion thermostat and a Misonix Microson Ultrasonic Cell Disruptor probe (Farmingdale, NY, USA) were employed for the ultrasound-assisted approach.

Sample preparation

Extraction based on the QuEChERS methodology was optimised in a previous work (Delgado-Zamarreño *et al*., 2012b). The optimised QuEChERS method was applied to ground samples of the three types of legumes studied. Solvent extraction was carried out in two steps: first, 10 mL of acetonitrile:water (70:30, v/v) was added and the mixture was shaken for 5 min. Then, 5 mL of acetonitrile was added and the mixture was shaken for an additional 5 min, in both cases using each of the proposed agitation systems. Following this, 4 g of magnesium sulphate and 1 g of sodium chloride were added, with or without further addition of citrate buffer, depending on the type of legume and the agitation method, and the mixture was shaken vigorously for 1 min and centrifuged at 3000 rpm (1.000 g) for 5 min. The upper layer was filtered through a 0.22 μm PVDF syringe filter before injection into the chromatographic system.

The LC–MS/MS conditions

In a previous work chromatographic separation and MS/MS detection were optimised (Delgado-Zamarreño *et al*., 2012b). The mobile phase consisted of an acetonitrile (solvent A) and 0.01% aqueous formic acid (solvent B) gradient at a flow rate of 0.5 mL/min. The elution gradient was as follows: 0–1.5 min, 10% A; 1.5–2.5 min, 10–25% A; 2.5–3.5 min, 25% A; 3.5–7 min, 25–50% A; 7–8 min, 50–80% A; 8–10 min, 80% A, 10–12 min, 80–10% A. The analytical column was thermostatted at 25 °C and the injection volume was 2 mL. The QqQ mass spectrometer was equipped with an electrospray ionisation (ESI) source. The electrospray capillary voltage was set at 3500 V; the nebuliser pressure was set at 35 psi and nitrogen was used as the drying gas at 12 L/min and 350 °C. ESI/MS spectra were acquired in positive ion multiple reaction monitoring (MRM) mode.

Results and discussion

Optimisation of extraction procedures

Optimisation of the parameters involved in the extraction of isoflavones from legumes was conducted using three types of vegetables: chickpeas, lentils and white beans. The different methods tested of placing the sample and solvent in contact were vortex agitation, thermostatted stirring agitation and thermostatted tray shaking, and ultrasound-assisted extraction using an ultrasound bath or an ultrasound probe.

In all cases, extraction was carried out following the procedure described in above and schematised in Fig. 1. Extraction was performed in two steps owing to the difference in the polarity of the isoflavones studied. The decrease in the polarity of the extraction solvent permits the quantitative extraction of both the polar and less-polar analytes. It should be noted that no subsequent cleaning step was required because it was not necessary to remove interferences and the peak shapes were not improved.

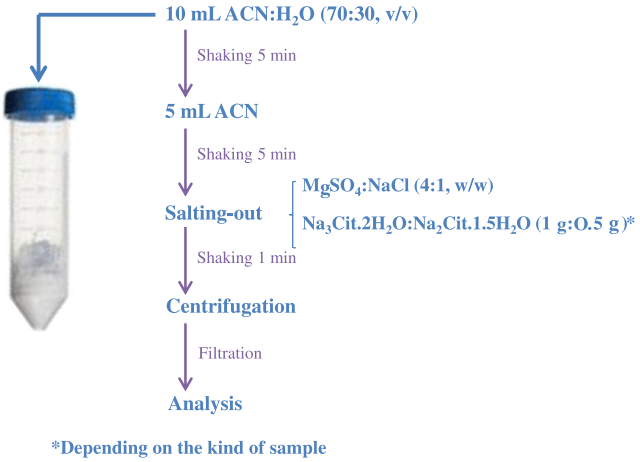

Figure 1. Schematic diagram of the extraction procedure.

Vortex agitation

The modified QuEChERS method developed previously (Delgado-Zamarreño *et al*., 2012b) was followed for sample treatment using vortex agitation. Phase separation in the QuEChERS methodology is induced by the addition of different salts. Generally, magnesium sulphate, which reduces the volume of the aqueous phase and enhances the distribution of polar analytes in the organic phase, and sodium chloride, which helps to control the polarity range and interfering substances in the extract, are used. The amount of these salts was optimised at a ratio of 4 g magnesium sulphate to 1 g sodium chloride and this was used in all later studies. The addition of citrate buffer in order to achieve quantitative extraction of the isoflavones was also studied. In the case of chickpeas and white beans, the extraction of major analytes was significantly improved by adding citrate buffer at a ratio of $Na_3Cit.2H_2O$:$Na_2HCit.1.5H_2O$:water (1 g:0.5 g). In the case of lentils, the addition of citrate buffer produced a decrease in the signal.

Mix-stirring methodologies

A conventional method for the extraction of isoflavones was developed by Griffith and Collison (2001) and was modified by Murphy *et al*. (2002) using mix-stirring. Rostagno (Rostagno *et al*., 2009) reported that the efficiency of the extraction of isoflavones from foods was higher in the presence of a certain amount of water and a slight increase in temperature. It should be noted that the temperature chosen must be sufficient to increase the extraction efficiency without causing degradation of the target compounds. Two mix-stirring approaches were tested: thermostatted stirring agitation and thermostatted tray shaking.

Thermostatted stirring agitation. This methodology was carried out using a magnetic stirrer and an immersion thermostat. Bibliographic and experimental studies addressing the analysis of isoflavones and their temperature stability led us to choose a working temperature of 60 °C for the thermostat bath. Under these conditions, extraction was enhanced without causing degradation of the compounds studied.

The main parameters for optimising this system were the addition or not of citrate buffer, which could facilitate the extraction, and the amount of sample.

To explore the first possibility, several samples of each type of legume were analysed with and without the addition of buffer. It

was observed that in all cases the addition of citrate did not enhance the extraction of analytes using this methodology.

The amount of sample is an important parameter when performing extraction. In our case it was known that a sample amount of 5–7 g, depending on the type of matrix, was optimal for achieving an efficient extraction with the QuEChERS method. To achieve better contact between the sample and the solvent, smaller amounts of sample were tested. It was found that smaller amounts of sample favoured the movement of the stirrer inside the tube containing the sample, facilitating contact with the extraction solvent and thereby increasing extraction efficiency. As can be seen in Fig. 2A, an increase in the analytical signal was achieved for lower sample amounts; this behaviour was observed for all three types of matrices.

This method, although effective, is slow when performing several replicas because it can only carry out the extraction of one sample at a time. For this reason, a thermostatted shaking tray, which allows the simultaneous extraction of 12 samples at once and also ensures a constant temperature, was tested.

Thermostatted tray shaking. The device used to carry out this methodology was a VWR thermostatted shaking tray. The temperature was set at 60 °C, and the extractions were accomplished using the salting-out step. The influence of sample amount was examined for the three types of matrix studied. As shown in Fig. 2B, the influence of the amount of sample differed for the various types of matrix. Greater amounts of sample favoured extraction in the case of lentils and white beans, while the opposite was seen in the case of chickpeas.

The difference between the behaviour observed with both stirring methodologies was because in the first case a magnetic stirrer was introduced into the sample; on increasing the amount of sample, the movement of the stirrer was hindered. However, on using the shaking tray no device was introduced into the sample and agitation occurred throughout the sample simultaneously. In the case of chickpeas, extraction efficiency decreased with the increase in the amount of sample. This may be because the ground chickpea powder had a grainy appearance, thicker than that of the lentils or white beans. Thus, the movement of the powder is hindered when the amount of sample is increased, worsening the extraction yield.

Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) uses high frequency sounds in order to detach the target compound under study from the matrix. Thus, the solute passes rapidly from the solid to the solvent phase. The commercial ultrasound devices most commonly used are ultrasound baths and ultrasound probes.

Thermostatted ultrasound bath. An ultrasound bath is the most economical way to accomplish UAE. To carry out this methodology, the ultrasound bath was coupled to an immersion thermostat. As in the case of the stirring methodologies, the bath temperature was thermostatted at 60 °C.

Some authors (Rostagno *et al.*, 2009) have reported that the extraction efficiency of isoflavones in soy is increased by the action of ultrasound in comparison with the traditional stirring method using methanol, ethanol or acetonitrile at room temperature. In order to check this improvement in the extraction of samples with lower isoflavone contents, such as legumes, extractions were performed on the three types of matrix.

As stated previously, the parameters affecting the extraction process were the addition of citrate buffer and the amount of sample. Using this methodology, the addition of citrate buffer enhanced the extraction of analytes for chickpeas and white beans, as in vortex agitation.

To find the most appropriate amount of sample to perform ultrasound-assisted extraction, the three types of legume under study were extracted by weighing sample amounts lower than those used in the conventional QuEChERS methodology. As shown in Fig. 3A, extraction efficiency increased with the amount of sample. As in the case of tray shaking, extraction was enhanced by an increase in the sample amount because the agitation of the sample matrix occurred simultaneously throughout the tube.

In view of the good results provided by the ultrasound bath for the extraction of isoflavones from these matrices using the salting-out step, the use of an ultrasound probe was tested.

Ultrasound probe. Currently, ultrasound probes containing a microtip to facilitate the cavitation phenomenon have begun to be used for ultrasound-assisted extraction. This device provides an increase in extraction efficiency. Extractions of different sample amounts of the three types of legume were achieved using the salting-out step. The results obtained for lentil samples are

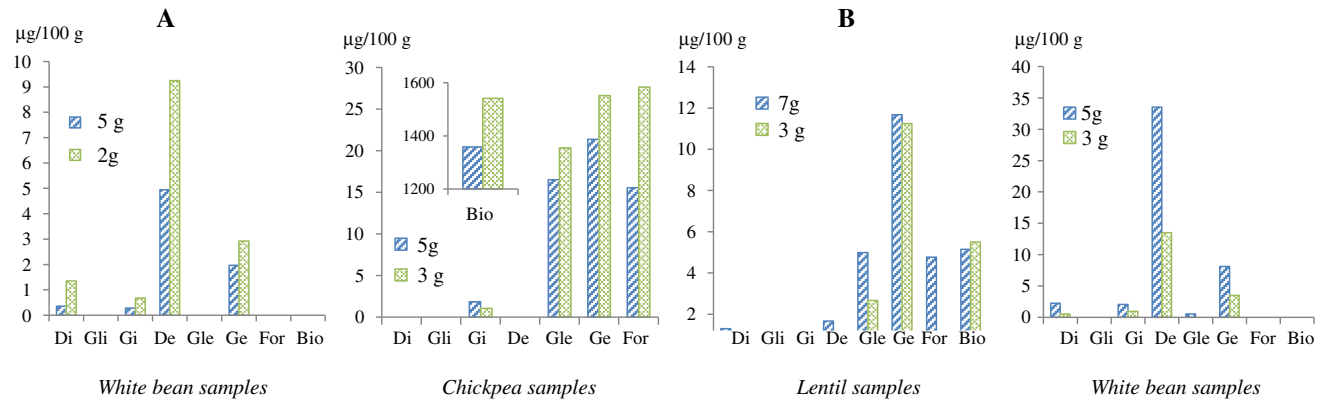

Figure 2. Influence of sample amount on the isoflavone content extracted with mix-stirring methodologies: (A) thermostatted stirring agitation; (B) thermostatted tray shaking; Di, daidzin; Gli, glycitin; Gi, genistin; De, daidzein; Gle, glycitein; Ge, genistein; For, formonetin; Bio, biochanin-A.

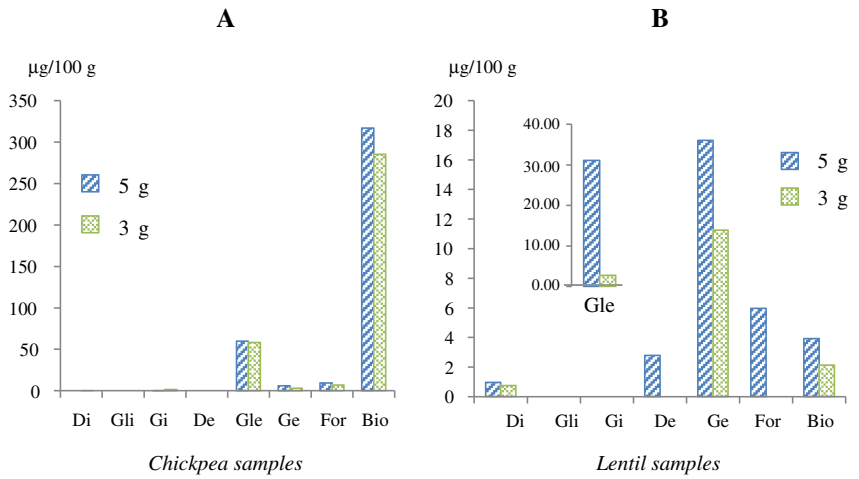

Figure 3. Influence of sample amount on the isoflavone content extracted with ultrasound-assisted extraction-based methodologies: (A) ultrasound bath; (B) ultrasound probe; Di, daidzin; Gli, glycitin; Gi, genistin; De, daidzein; Gle, glycitein; Ge, genistein; For, formonetin; Bio, biochanin-A.

shown in Fig. 3B. As can be seen, on increasing the sample amount the analytical signal was enhanced. This shows that the movement of the sample occurs simultaneously throughout the matrix, and accordingly an increase in the amount of sample will increase the extraction yield.

Method validation

Linearity, detection limit and quantification limit. Linearity was evaluated by injection of standard mixtures of the target isoflavones, in triplicate, at concentration levels ranging from 0.1 to 1000 mg/L for biochanin A, and from 0.1 to 200 mg/L for the other isoflavones. These ranges were chosen taking into account the expected levels in the samples analysed. The results for the regression equations, the correlation coefficients of the calibration curves, and the detection and quantification limits (LOD, S/N = 3; LOQ, S/N = 10) for the analytes under optimised conditions are summarised in Table 2. As can be seen, good linear correlation coefficients ($R^2 > 0.992$) were found between the peak area (y) and the concentration of isoflavones (x). The LODs and LOQs were in the range of 0.12–0.47 mg/L and 0.41–1.56 mg/L, respectively, showing the excellent sensitivity of the method.

Table 2. Analytical characteristics of the method

	Intercept $a \pm s_a$	Slope $b \pm s_b$	R^2	LOD[a]	LOQ[b]
Daidzin	726 ± 562	338 ± 6	0.999	0.36	1.19
Glycitin	3037 ± 826	531 ± 9	0.999	0.12	0.41
Genistin	35 ± 1106	462 ± 11	0.999	0.36	1.19
Daidzein	114 ± 134	51 ± 1	0.998	0.22	0.75
Glycitein	1828 ± 1006	373 ± 22	0.992	0.43	1.43
Genistein	24 ± 244	68 ± 3	0.997	0.21	0.7
Formononetin	1433 ± 432	123 ± 4	0.997	0.47	1.56
Biochanin-A	149 ± 227	17.6 ± 0.5	0.998	0.33	1.09

[a]mg/L; S/N = 3.
[b]mg/L; S/N = 10.

Application of the methodologies to real samples and comparison of the methods. In order to check the applicability of the proposed methodologies for the extraction of isoflavones, samples of chickpeas, lentils and white beans from the Region of Castilla y León (Spain) were analysed in quadruplicate.

The extractions were carried out considering the previously optimised parameters summarised in Table 3. Quantifications were performed using the external standards method. The results obtained for the analysis of each of the three types of pulse according to each method tested are shown in Table 4. As can be seen, considerable variability was observed.

For the three types of legume, the highest contents of most of the analytes were obtained using thermostatted tray shaking or the ultrasound probe. This may be because the agitation induced by these methodologies is achieved homogeneously and simultaneously at all points in the matrix. When vortex agitation or the ultrasound bath was used the samples became compacted; this hindered contact between the extraction

Table 3. Optimised conditions for each extraction methodology

Method	Pulse	Sample amount (g)	Addition of citrate buffer
Vortex	Chickpeas	5	Yes
	Lentils	7	No
	White beans	5	Yes
Thermostated stirring	Chickpeas	2	No
	Lentils	2	No
	White beans	2	No
Thermostated tray	Chickpeas	3	Yes
	Lentils	7	No
	White beans	5	Yes
Ultrasound bath	Chickpeas	5	Yes
	Lentils	7	No
	White beans	5	Yes
Ultrasound probe	Chickpeas	5	Yes
	Lentils	7	No
	White beans	5	Yes

Table 4. Isoflavone content (mg/100 g) in pulse samples depending on the type of extraction method

		Di	Gi	De	Gle	Ge	For	Bio
Chickpeas	Vortex				18 ± 3	3.1 ± 0.4	4 ± 1	474 ± 16
	Thermostatted stirring		1.23 ± 0.05		55.5 ± 5	5.5 ± 0.5	9.8 ± 1	581 ± 13
	Thermostatted tray		1.92 ± 0.1		9.77 ± 0.2	46.67 ± 2.5	98 ± 2	4794 ± 171
	Ultrasound bath		0.23 ± 0.03		59.8 ± 4.2	5.9 ± 0.3	9.3 ± 0.6	317 ± 8
	Ultrasound probe		1.56 ± 0.06		10.7 ± 0.6	8.1 ± 0.6	26 ± 0.9	1436 ± 81
Lentils	Vortex	1.77 ± 0.33	0.33 ± 0.15	2.5 ± 0.3		10.3 ± 0.5	2.3 ± 1	1.1 ± 0.4
	Thermostatted stirring	2.87 ± 0.1	0.45 ± 0.01	2 ± 0.4	2.82 ± 0.2	14 ± 1	8.8 ± 0.5	18 ± 3
	Thermostatted tray	1.47 ± 0.06	0.44 ± 0.04	2.9 ± 0.4	4.1 ± 0.1	28 ± 1	13 ± 0.6	18 ± 1
	Ultrasound bath	0.77 ± 0.24	0.38 ± 0.03	4.4 ± 0.3	17.85 ± 2.4	15.3 ± 0.3	5.7 ± 0.6	2.03 ± 0.3
	Ultrasound probe	1.32 ± 0.06	0.51 ± 0.04	3.2 ± 0.4	4.4 ± 0.8	27 ± 1	15.2 ± 0.6	18 ± 1
White beans	Vortex	0.52 ± 0.23	0.32 ± 0.15	2.63 ± 0.3		1.35 ± 0.35		
	Thermostatted stirring	2.3 ± 0.45	1.45 ± 0.05	8 ± 0.5		2.3 ± 0.45		
	Thermostatted tray	2.24 ± 0.27	2.05 ± 0.12	33.53 ± 2.1		8.1 ± 4.8		
	Ultrasound bath	0.74 ± 0.24	0.37 ± 0.03	8.7 ± 0.3		2.06 ± 0.27		
	Ultrasound probe	2.12 ± 0.27	1.79 ± 0.03	30.3 ± 1.8		7.8 ± 0.3		

Di, daidzin; Gi, genistin; De, daidzin; Gle, glycetein; Ge, genistein; For, formonetin; Bio, biochanin-A.

solvent and the matrix. Agitation by stirring also provided good results, especially for chickpeas. However, this methodology was discarded because it provided lower reproducibility, probably because the movement of the stirrer would be hampered in matrices that compact easily. Use of the thermostatted tray shaker was suitable because agitation does not occur only in a particular part of the sample – as in the case of stirring – and therefore extraction could not be affected by the movement of the stirrer. Use of the ultrasound probe provided good extraction yields due to its greater potency with respect to the bath.

Recovery, repeatability (intraday precision) and reproducibility (interday precision). In order to select the most appropriate procedure for each type of matrix, recovery and precision studies were performed using the methods considered most suitable.

Recovery was determined by the addition of a known amount of standard to samples of chickpeas, lentils and white beans. The amount of each isoflavone added was close to those present in the original samples. When isoflavones were not naturally present in the sample the fortification level was 5 mg/L. The samples were spiked before sample treatment using each of the abovecited methodologies and were analysed in triplicate. The recoveries obtained for each analyte, matrix and method are shown in Table 5. Recoveries were in the 70–120% range, which was satisfactory and in accordance with the values stipulated in Commission Decision 2002/657/EC.

The analytical precision of the data from the intraday (four times per day) and interday (eight samples over two months) determinations was measured in real samples of the three legume types. The results are shown in Table 5 for each analyte, matrix and method. The RSD values were lower than 12% for intraday precision, which can be considered acceptable for this type of methodology. The RSD values for interday precision ranged from 7 to 39%. Taking into account that RSD values should be lower than 22% to fulfil the criteria of CD 2002/657/EC, the method chosen for chickpeas and white beans was the thermostatted shaking tray. This methodology provided satisfactory results as regards both recovery and precision; moreover, it allowed the simultaneous extraction of up to 12 samples. In the case of lentil samples, the most appropriate method was the ultrasound probe because the reproducibility obtained with the thermostatted shaking tray was lower. Lentil powder was more finely ground than that of the other pulses and hence became more compacted when added to the extraction solvent. This may be because lentils have a lower fat content than chickpeas and white beans and the movement of the tray did not always provide optimum contact between the sample and the extraction solvent, while the ultrasound probe was more successful in this sense. Figure 4 shows the chromatograms corresponding to each kind of sample.

Summary

In the present work a comparative study of five different agitation techniques for the extraction of isoflavones from legume samples was made, including vortex agitation, ultrasound probe and stirring agitation, thermostatted tray shaking and the use of an ultrasound bath with temperature control. These methodologies were evaluated using a modified QuEChERS approach as the extraction method. The separation and quantification of isoflavones were carried out using LC–MS/MS. The main advantages of the proposed extraction procedures were their

Table 5. Recovery, repeatability and reproducibility of the most suitable methodologies for each kind of sample

Study	Pulse	Method	Di	Gli	Gi	De	Gle	Ge	For	Bio
Recovery (%)	Chickpeas	Thermostatted tray	82	96	82	88	113	78	70	80
		Ultrasound probe	70	82	80	75	97	81	94	89
	Lentils	Thermostatted tray	83	102	72	95	109	99	106	116
		Ultrasound probe	71	86	74	81	101	83	97	120
	White beans	Thermostatted tray	90	92	78	108	96	95	84	75
		Ultrasound probe	71	81	72	105	97	92	78	118
Intraday precision (RSD %)	Chickpeas	Thermostatted tray			4.4		4.5	4.6	3.3	2.7
		Ultrasound probe			1.9		5.2	11	4.7	4.7
	Lentils	Thermostatted tray	10		4.3	4.6	9.5	9.2	3.5	12
		Ultrasound probe	2.2		3.4	4.7	6.0	1.2	3.9	5.5
	White beans	Thermostatted tray	8.6		3.9	2.1		4.6		
		Ultrasound probe	9.2		4.7	6.6		3.8		
Interday precision (RSD %)	Chickpeas	Thermostatted tray			17		6.8	17	20	19
		Ultrasound probe			20		7.4	13	23	13
	Lentils	Thermostatted tray	19		18	30	39	32	17	27
		Ultrasound probe	14		14	20	12	10	19	13
	White beans	Thermostatted tray	15		11	15		13		
		Ultrasound probe	19		15	16		8.3		

Di, daidzin; Gi, genistin; De, daidzin; Gle, glycetein; Ge, genistein; For, formonetin; Bio, biochanin-A.

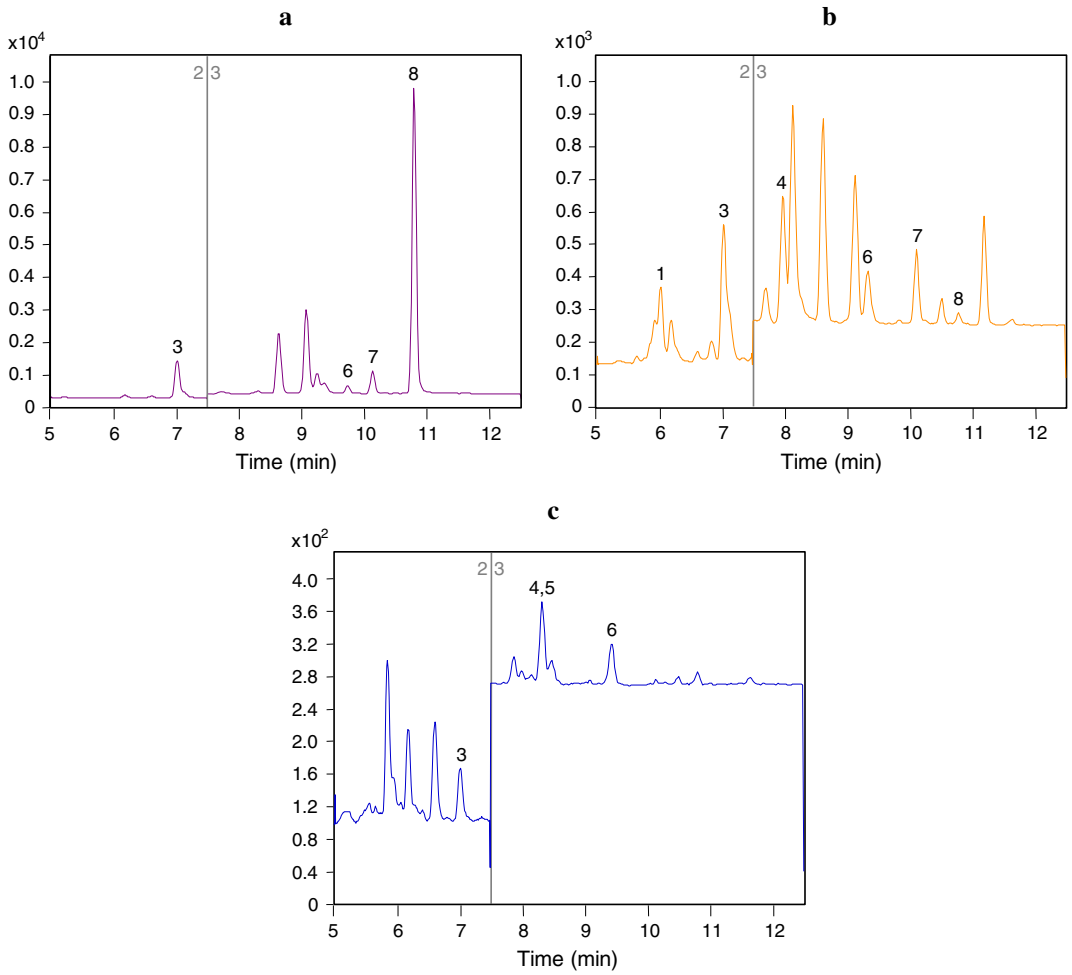

Figure 4. Total ion chromatograms (TIC) obtained using QuEChERS with LC–MS/MS analysis of legume samples: (a) chickpeas, (b) lentils and (c) beans. Numbered peaks are: (1) daidzin, (2) glycitin, (3) genistin, (4) daidzein, (5) glycitein, (6) genistein, (7) formonetin and (8) biochanin-A.

simplicity, speed, reliability and low cost. Optimal extraction conditions were attained using the thermostatted shaking tray or ultrasound probe as the contact method between the matrix and solvent. We propose the use of a thermostatted shaking tray for the extraction of chickpeas and white beans, whereas for lentil samples the best method would be the ultrasound probe. These methods were chosen based on the highest amount of analytes obtained as well as the best recovery values. The importance of the agitation mode in the extraction of analytes should be noted.

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Determination of Isoflavones in Legumes by QuEChERS-Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry

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Abstract: In this study, a capillary electrophoresis-mass spectrometry method was applied to the determination of isoflavones in samples with low content in these analytes, such as legumes. Sample stacking was used to enhance the sensitivity of the technique. Extraction of the analytes was carried out by a modified approach of QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology, in which the extracts were injected directly into electrophoretic system coupled with mass spectrometry detector. The developed method was validated and applied for the determination of isoflavone aglycones and glycosides in legume samples. The values of the validation parameters and the isoflavone contents found were compared with those obtained by LC-MS/MS. Both methods provided similar results and were comparable in terms of recovery (in the 80-120% range), precision (lower than 10 %) and limits of detection (ranging from 0.20 to 1.8 μg L^{-1}).

Keywords: CE-ESI-MS, LC-ESI-MS/MS, Isoflavone, legume, QuEChERS.

1. INTRODUCTION

Isoflavones are compounds of the family of flavonoids whose chemical structure is similar to that of estradiol and are also considered as phytoestrogen due to their oestrogenic activity. They have been associated with several beneficial health effects, such as the prevention of cancer [1, 2], cardiovascular disease [3, 4], osteoporosis [5, 6], as well as for the relief of menopausal symptoms [7, 8].

Isoflavones are found primarily in the Leguminosae family, being more abundant in soybeans, alfalfa and red clover; other legumes also contain these components, to a lesser proportion [9]. In vegetables, isoflavones are found mainly as biologically inactive glucoconjugates and are hydrolyzed in the intestine to their biologically active form, aglycon. [10].

Quantitative analysis of isoflavones is generally accomplished with liquid chromatography or capillary electrophoresis. Recently, several reviews have been published, including current trends in sample preparation [11], separation, identification and determination methods [11, 12] for the analysis of isoflavones from foods. The most widely used analytical technique for the separation of isoflavones is reversed phase liquid chromatography using C18 columns; mixtures acidified water: methanol and acidified water:acetonitrile are the most common mobile phases. Extraction of isoflavones from food samples is usually carried out by solvent extraction, Soxhlet extraction, solid-phase extraction, accelerated solvent extraction, ultrasound-assisted extraction, etc. Some of these methods are tedious, expensive and time-consuming. In previous works, we isolated isoflavones from soy-based foods and legume samples [14, 15] using a simple approach of the QuEChERS methodology developed by Anastassiades and co-workers [16]. This extraction method affords advantages in the analysis of naturally present components in the samples, since it is simple, rapid (extraction time ~ 10 min) and easy to use. This approach was successfully applied to the isolation of aglycones and glycoside isoflavones from chickpeas, lentils and beans. These samples show much lower isoflavone content than soy or soy-based products. In that work, the separation and determination of isoflavones were accomplished using ultra-performance liquid chromatography–triple quadrupole mass spectrometry.

The objective of this work was to evaluate the use of capillary electrophoresis in the determination of isoflavones in samples with low contents of these compounds, such as legumes. One of the major disadvantages of CE is its poor detection limit, which results from the small inner diameter of the separation column. An easy way to enhance CE sensitivity is to use the sample-stacking technique [17]. This method focuses on preconcentration analytes using different properties such as differences in conductivity, pH, ionic strength, viscosity, etc., between the background electrolyte (BGE) and the sample matrix [18]. The sample stacking phenomenon arises from the movement of ions through the separation limit between the zone containing the sample and the zone containing BGE [19]. The background zone has higher conductivity than the sample ions zone. When voltage is applied, the electric field is higher in the area of the sample, which makes the analytes migrate at a considerably higher speed in this segment. This causes the analytes to move rapidly towards the border between the two areas and preconcentration occurs.

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The objective of this study was to compare the L7C and CE techniques for the determination of isoflavones in pulse samples. Thus, both methods were applied for the analysis of three different kinds of legumes (chickpeas, lentils and white beans). One of them was a previously reported method using LC-MS/MS [15] and the other was the CE-MS method, which has been developed and validated in this work. Analyte extraction and sample clean-up were carried out using a modified QuEChERS approach which offers high extraction efficiency for the studied analytes in this type of matrix. Linearity, LOD, precision, accuracy and their applicability in real legume samples were compared.

The use of CE has the advantage of a lower consumption of organic solvents and high resolution capabilities. It is necessary to take into account that the sensitivity of capillary electrophoresis is less than that of LC-MS / MS. In a previous work [14] soy based foods were analyzed using CE- MS. However, soy based foods contain higher levels of isoflavones than the test samples: 10 to 20 times compared to chickpea, and up to 100 times in the case of lentils. Moreover, the matrices are different.

2. EXPERIMENTAL

2.1. Reagents and Chemicals

The isoflavone standards (glycitin, daidzin, genistin, formononetin, biochanin A, glycitein, daidzein and genistein) and the internal standard apigenin, as well as disodium hydrogencitrate sesquihydrate ($Na_2HCit.1.5H_2O$), were supplied by Aldrich (Alcobendas, Madrid, Spain). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Formic acid (>98%) was supplied by Fluka (Steinheim, Germany). Other salts employed ($MgSO_4$, NaCl and $Na_3Cit.2H_2O$ were from Scharlau (Barcelona, Spain). Ultra-high quality (UHQ) water was supplied by a Wasserlab (Spain) water purification system. Stock solutions of isoflavones were prepared in methanol and preserved at -18 °C.

The samples used were commercially available pulses from the Castilla y León Region (Spain).

2.2. Apparatus

CE-MS System

Electrophoretic separations were performed using a Hewlett-Packard HP^{3D} Capillary Electrophoresis system (Agilent Technologies, Waldbron, Germany) endowed of a diode array detector (DAD); wavelength was set at 254 nm. MS was achieved with an Agilent LC/MSD SL (Agilent technologies) single quadrupole mass spectrometer. CE-DAD and CE-MS data were analyzed using Agilent HP ChemStation software, version B.02.01 SR1. CE-MS coupling was performed using an Agilent coaxial sheath-liquid sprayer (Agilent technologies). The sheath liquid (isopropanol/water (1:1, v/v) was dispensed by an Agilent 1100 series pump, with a 1:100 flow-split ratio. The ESI conditions were as follows: capillary voltage, 3500 V; drying gas flow, 6 L min^{-1}; drying gas temperature, 350 °C. In a previous work [20] we have proved that the resolution and stability of the signals were improved when a programmed nebulizing gas pressure (PNP) was applied. Thus, no nebulizing gas pressure was applied during injection; separation was performed at a low pressure of 1 psi, and detection was carried out at a pressure of 4 psi, considered to be optimal for the ionization of analytes. Detection was performed in positive-ion mode. Analyte quantification was carried out using the extracted ion electropherograms for the protonated molecules; the ions monitored were: m/z 447 for Gli, 417 for Di, 433 for Gi, 269 for For, 285 for Bio and Gle, 255 for De, and 271 for Ge and IS, respectively. Although there was coincidence on m/z of extracted ions their migration times were different and it was possible the quantification.

Fused-silica capillaries (50 μm id, 87.5-cm total length) from Polymicro Technologies (Phoenix, AZ, USA), were supplied by Composite Metal Services Ltd (West Yorkshire, UK). and were used throughout the work. Conditioning of the new capillaries was accomplished by washing with 1.0 M sodium hydroxide, UHQ water, and the background electrolyte (BGE) for 10 min each. Between consecutive analyses, the capillary was washed for 2 min with the BGE.

Hydrodynamic injections were carried out at 50 mbar for 60 s. A potential of 25 kV was applied during analysis, and the temperature of the capillary was kept at 25 °C. The BGE consisted of 15 mM aqueous ammonium acetate at pH 11.0

LC-MS/MS System

LC analyses were carried out on an Agilent HP 1200 Series chromatograph consisting of a binary pump, a membrane degasser, an autosampler, and a six-port valve. Separation was performed on a 50 x 4.6 mm Zorbax Eclipse XDB-C18 column (Agilent) with 1.8-μm particles. Acetonitrile (solvent A) and 0.01% aqueous formic acid (solvent B) at a flow rate of 0.5 mL min-1 was used as the mobile phase, using the following gradient: 0 to 10%A in 1.5 min, 10 to 25% A in the next min, and kept for 1 min; 25 to 50 % A in the following 3.5 min; 50 to 80 % in 1 min, and kept for 2 min, and finally 80 to 10 % A in 2 min. Column temperature was maintained at 25 °C; the injection volume was 2 μL. Detection was performed using a Triple Quad LC/MS 6410 (Agilent) equipped with an electrospray (ESI) source. Quantification was achieved using positive-ion multiple reaction monitoring (MRM) mode. MS conditions were the same as used in a previous work [15]. The ions monitored m/z (Precursor/Products ions) were: 447.1/255.1 for Gli, 417.1/285.1/270.1 for Di, 433.1/271.1 for Gi, 269.1/197.1/253 for For, 285.1 /152/213 for Bio, 285.1/270/242 for Gle, 255.1/137/199.1/91 for De, and 271.1/ 153/91.1 for Ge.

2.3. Sample Preparation

Samples were ground with a Knifetec™ 1905 supplied by Foss (Barcelona, Spain), before extraction.

Extraction was carried out following the QuEChERS methodology optimized in a previous work.[15] An appropriate amount of sample – 3g for chickpea and bean samples and 7 g for lentils- was extracted in two steps: first, using 10 mL of 70 % aqueous ACN for 5 min, and then using 5 mL of pure ACN again for 5 min. In all cases, agitation was performed using two different systems to place the sample and the solvent in contact: a VWR thermostatted shaking tray (Radnor,

Pennsylvania, USA), and a Microson Misonix Ultrasonic Cell Disruptor probe (Farmingdale, NY, USA). After extraction, a mixture of $MgSO_4$: NaCl (4:1, w/w) together with a citrate buffer (in the extraction of chickpeas and beans) were added, and the mixture was shaken vigorously for 1 min and centrifuged at 3000 rpm during 5 min. No additional cleaning step was required. The upper layer was filtered through 0.22 μm PVDF syringe filters (Scharlau). The extract obtained was directly injected into the chromatographic system, or diluted with water at a 1:2 (v/v) ratio for injection into CE system.

3. RESULTS AND DISCUSSION

3.1 Method Validation

The CE proposed method for the analysis of isoflavones in legume samples, as well as, the LC method, were validated by means of linearity, detection and quantification limits, precision and accuracy, using the conditions described in the experimental section.

For the CE method, linearity was studied in the 10 to 1000 μg L^{-1} range (n=10). The results showed good linearity ($R^2 > 0.998$) between peak area ratios (isoflavone/internal standard) and concentration (Table **1**). For the LC method, the calibration curves were tested at concentration levels ranging from 0.1 to 500 μg L^{-1} for biochanin A; from 0.1 to 100 μg L^{-1} for daidzein and glycitein, and from 0.1 to 40 μg L^{-1} for the rest of the isoflavones. Calibration curves based on the external standard quantification also showed good correlation coefficients ($R^2 > 0.998$).

Detection limits were calculated on the basis of a signal-to-noise ratio (S/N) of 3. The LOD for the CE method ranged from 0.20 μg L^{-1} for glycitein to 1.8 μg L^{-1} for daidzin (Table **1**). The LOD for the LC method (ranging between 0.71 and 1.5 μg L^{-1} for formononetin and glycitin respectively) were similar to those obtained with the CE method.

Repeatability was evaluated by determination of the intra-day precision; a standard solution of the eight isoflavones at 100 μg L^{-1} was analized in quadruplicate on the same day. The relative standard (RSD) deviations ranged from 2.8 to 6.9 % for the CE method, and from 1.0 to 4.6 % for the LC method. Reproducibility was calculated over four different days, by applying both methods to a standard solution at 100 mg L^{-1}. The relative standard deviations (RSD) for the inter-day assays ranged from 4.7 to 9.6 % for the CE method, and from 3.0 to 7.5 % for the LC method.

Since no certified reference material was available commercially, a recovery study with spiked samples was necessary. Samples of the three kinds of legume in study were spiked with standard isoflavones at concentration levels close to those expected in the matrices. The samples were analysed in triplicate using the QuEChERS-CE-MS and the QuEChERS-LC-MS/MS methods, applying the two modes of placing the sample and the solvent in contact. In all cases, recoveries were in the 80-120% range.

3.2. Analysis of Isoflavones in Legume Samples

In a previous study we found that the isolation of isoflavones from pulse samples depends to a large extent on how the sample and the extractant are placed in contact. To corroborate this, samples of the three types of legume studied here were analyzed using the two methodologies that provided the best results in that study: with a thermostatted shaking tray and with an ultrasonic probe. In all cases, extractions were performed using the modified QuEChERS approach described in Section 2.2. This method provided high extraction efficiency for these analytes in this type of matrix. The extracts obtained were analyzed using capillary electrophoresis and liquid chromatography in order to compare both results.

3.2.1. Analysis of Isoflavones by QuEChERS-CE-MS

The extracts obtained after applying the QuEChERS methodology contained a high percentage of organic solvent (80 % ACN). These extracts cannot be injected directly into the CE system owing to the low stability of the capillary under these conditions. A study was therefore conducted to optimize the maximum amount of sample to be injected. Thus, the extracts were diluted at ratios ranging from 1:10 to 6:10 (ACN percentages from 8 to 48%). It was observed that

Table 1. Analytical characteristics of the CE-MS method.

Isoflavone	Intercept ± RSD	Slope ± RSD	R^2	LOD[a]	RSD[b]	RSD[c]
Gli	$(-1 \pm 1)\ 10^{-2}$	$(4.46 \pm 0.04)\ 10^{-3}$	0.9982	1.1	4.2 %	5.8 %
Di	$(-1 \pm 1)\ 10^{-2}$	$(4.99 \pm 0.04)\ 10^{-3}$	0.9985	1.8	3.2 %	6.8 %
Gi	$(-5 \pm 9)\ 10^{-3}$	$(3.55 \pm 0.03)\ 10^{-3}$	0.9983	1.7	3.8 %	4.7 %
For	$(-1 \pm 1)\ 10^{-2}$	$(5.49 \pm 0.04)\ 10^{-3}$	0.9983	0.7	6.0 %	9.0 %
Bio	$(1.2 \pm 0.8)\ 10^{-2}$	$(3.08 \pm 0.02)\ 10^{-3}$	0.9987	1.2	6.9 %	9.6 %
Gle	$(2 \pm 4)\ 10^{-2}$	$(3.00 \pm 0.02)\ 10^{-2}$	0.9985	0.20	4.5 %	8.8 %
De	$(-2.1 \pm 0.5)\ 10^{-2}$	$(4.34 \pm 0.02)\ 10^{-3}$	0.9996	1.0	3.7 %	5.5 %
Ge	$(-2.7 \pm 0.9)\ 10^{-2}$	$(3.78 \pm 0.03)10^{-3}$	0.9981	1.4	2.8 %	5.8 %

[a]Limit of Detection (μg L^{-1}): S/N=3.
[b]Intra-day precision, calculated by analysing a standard solution containing 100 μg L^{-1} of each isoflavone four times on the same day.
[c]Inter-day precision, calculated by analysing a standard solution containing 100 μg L^{-1} of each isoflavone on four different days.

for percentages above 40% peak width increased and reproducibility decreased. Accordingly, the extracts were diluted at a ratio of 1:2, obtaining a final ACN percentage of 40 %.

Taking into account the low content of isoflavones present in these pulses and the fact that the samples had to be diluted, we addressed the possibility of using on-line preconcentration techniques. The simplest way to perform the on-line concentration of diluted samples, enhancing separation efficiency and analytical sensitivity, is sample stacking. Sample stacking can be performed in the hydrodynamic and electrokinetic injection modes and here both modes were tested for the preconcentration of isoflavones under the working conditions described above. The method that provided the best results was the normal stacking mode (NSM) [21]. This is accomplished by dissolving the sample in a low-conductivity matrix (in our case water) and then injecting the resulting sample solution hydrodynamically. Preconcentration occurs due to the abrupt change in electrophoretic velocity between the low-conductivity matrix and the BGE. Polarity switching was also tested, obtaining poorer results. In NSM, injection times up to 60 seconds could be applied without causing any loss of resolution and efficiency. The (Fig. **1**) shows the influence of injection time on analytical signal which reaches constant values since 50 seconds, injection time of 60 s was chosen.

Applying the optimized conditions in these previous studies (sample dilution 1:2 with UHQ, the presence of 40% ACN, and an injection time of 60 s), samples of beans, chickpeas and lentils were analyzed using the two above modes for placing the sample and solvent in contact. The contents found (μg/100g ± RSD) are shown in Table **2**. Fig. (**2**) shows the extracted ion electropherograms obtained for a standard solution of the isoflavones studied at 50 μg L^{-1} and for samples of the three legumes analyzed.

It should be noted that the contents of glycitin found for all three sample types were below the limit of quantification. Genistin could not be determined using the CE method due to the co-migration of an unknown compound. In light of this, the data for glycitin and genistin are not included in Table **2**.

To evaluate the precision of the proposed method, samples of chickpeas, lentils and beans were subjected to the entire analytical procedure, using the two modes of placing the sample and the solvent in contact, in quadruplicate on the same day (intra-day precision) and on four different days (inter-day precision) respectively. The RSDs were lower than 10 % for the intra-day precision and lower than 16 % for the inter-day precision.

3.2.2. Analysis of Isoflavones by QuEChERS-LC-MS/MS

The extracts corresponding to the three types of samples, obtained after applying the QuEChERS methodology using the two different modes of agitation, were injected

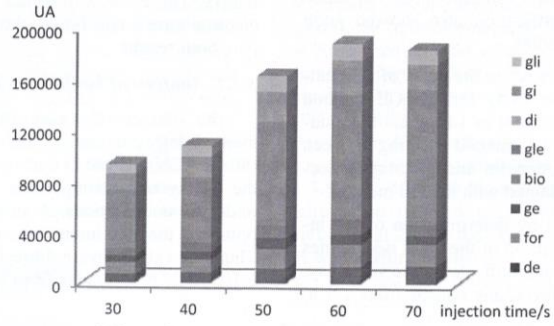

Fig. (1). Influence of injection time on area of electrophoretic peak. Analyte identification: gli: glycitin, gi: genistin, di: daidzin, for: formononetin, bio: biochanin A, gle: glycitein, de: daidzein, ge:genistein.

Table 2. Isoflavone contents (μg/100g ± RSD) of legumes (n=3) according to the CE method.

Isoflavone	Chickpeas		Lentils		Beans	
	Shaking tray	US probe	Shaking tray	US probe	Shaking tray	US probe
De	nd	nd	3.2 ± 0.7	2.8 ± 0.3	6.2 ± 0.1	6.6 ± 0.2
For	28 ± 2	10.5 ± 0.3	5.0 ± 0.6	6.5 ± 0.4	nd	nd
Ge	6.2 ± 0.3	nd	5.8 ± 0.6	10.0 ± 0.7	4.8 ± 0.7	4.5 ± 0.2
Bio	1360 ± 80	742 ± 24	4.9 ±0.6	5.4 ± 0.6	5 ± 1	5 ± 1
Gle	0.35 ± 0.04	nd	0.7 ± 0.1	1.4 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
Di	2.1 ± 0.1	1.3 ± 0.6	0.91 ± 0.5	0.98 ± 0.5	2.2 ± 0.6	3.2 ± 0.8

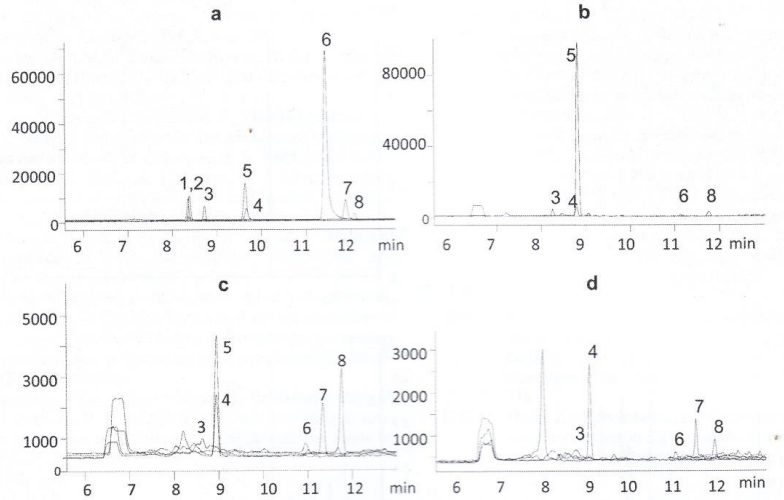

Fig. (2). Extracted ion electropherograms for CE-ESI-MS analysis of a standard solution of the isoflavones studied at 50 μg L^{-1} (**a**), and samples of the three legumes analyzed: chickpeas (**b**), lentils (**c**) and beans (**d**). Conditions as in text. Analyte identification: glycitin (1), genistin (2), daidzin (3), formononetin (4), biochanin A (5), glycitein (6), daidzein (7), genistein (8).

directly into the LC-MS/MS system. The contents found for the LC method are shown in Table **3**. Fig. (**3**) shows the total ion chromatograms obtained for a standard solution of isoflavones studied and for samples of the three legumes analyzed.

The precision of the LC method was also evaluated by injection of the three kinds of matrices studied (chickpeas, lentils and beans) subjected to the entire analytical procedure, using the two modes of placing the sample and the solvent in contact. The RSDs were lower than 10 % for the intra-day precision (n=4) and lower than 20 % for the inter-day precision (n=4).

3.2.3. Comparison of the CE and LC Methods

A t-test was used to verify whether there were statistically significant differences between the results obtained with the two methods. The level of significance was determined; when this value was greater than 0.05, which was chosen as the minimum level of significance, the null hypothesis was accepted: there were no significant differences among the results obtained by the two methods. These values were greater than 0.05, except for genistein in chickpeas and lentils, and glycitein in all cases.

A possible explanation for the observed differences between both methodologies for glycitein is that on using CE-ESI-MS method, the m/z ratios corresponding to glycitein and biochanin A coincided. Although it is possible to determine these compounds separately because they migrate at different speeds, it was observed that biochanin A may inhibit the ionization of glycitein. Owing to this, very low contents of this analyte were obtained.

Table 3. Isoflavone contents (μg/100g ± RSD) of legumes (n=3) according to the LC method.

	Chickpeas		Lentils		Beans	
	Shaking tray	**US probe**	**Shaking tray**	**US probe**	**Shaking tray**	**US probe**
De	nd	nd	2.9 ± 0.4	3.1 ± 0.1	6.5 ± 0.2	6.2 ± 0.7
For	29 ± 2	11.8 ± 0.7	5.4 ± 0.5	6.6 ± 0.3	nd	nd
Ge	13.8 ± 0.7	8.1 ± 0.6	14 ± 1	13 ± 1	6.0 ± 0.7	8.0 ± 0.3
Bio	1371 ± 32	679 ± 35	3.8 ±0.6	3.9 ± 0.8	5 ± 1	9 ± 2
Gle	6.3 ± 0.6	10.7 ± 0.6	4.1 ± 0.1	1.3 ± 0.1	0.13 ± 0.04	0.19 ± 0.02
Di	nd	nd	1.5 ± 0.6	0.70 ± 0.06	1.2 ± 0.1	1.3 ± 0.1
Gi	0.33± 0.02	0.49 ± 0.04	0.10 ± 0.01	0.12 ± 0.02	2.3 ± 0.2	2.4 ± 0.1

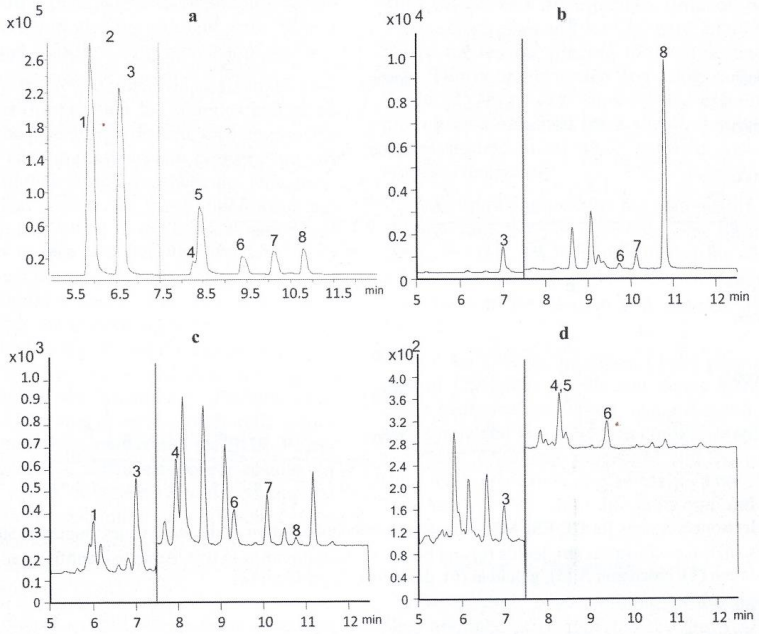

Fig. (3). Total ion chromatograms for LC-ESI-MS/MS analysis of a standard solution of the isoflavones studied at 50 μg L^{-1} (**a**), and samples of the three legumes analyzed: chickpeas (**b**), lentils (**c**) and beans (**d**). Conditions as in text. Analyte identification: 1, daidzin; 2, glycitin; 3, genistin; 4, daidzein; 5, glicitein ; 6, genistein ; 7, formononetin; 8, biochanin A .

4. CONCLUSION

A simple and sensitive capillary electrophoresis method with mass spectrometry detection for the determination of isoflavones in legume samples has been developed. The drawback of the low sensitivity of CE was resolved successfully by diluting the extracts in water and by injecting the resulting sample solution hydrodynamically, *i.e.* applying the normal stacking mode. The extraction of the analytes and sample clean-up were carried out with a previously developed modified QuEChERS approach. The proposed method was validated, and was then applied to the analysis of isoflavones in several legume samples. The values of the validation parameters and the isoflavone contents found were compared with those obtained by liquid chromatography. Both methods provided similar results and are comparable in terms of recovery, precision and limits of detection.

In view of these results it can be concluded that both methods provide similar results for the analysis of aglycones -except glycitein-; however, LC provided the best results for isoflavone glycoside analysis.

CONFLICT OF INTEREST

The authors confirm that this article content has not conflict of interes

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"***Classification of different varieties of chickpeas, lentils and beans based on their isoflavone content using multivariate analysis***"

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2.5.1.1. Abstract

Food legumes (*Fabaceae*) form an important part of the human diet, providing a high proportion of proteins, fats, carbohydrates, dietary fibres, B-group vitamins and minerals. Several *Fabaceae* species are acknowledged for their high levels of secondary metabolites. Isoflavones are among the most well-known examples of these compounds, being recognized for their varied types of biological activity. Isoflavone profiles are characterized in four species of *Fabaceae* genera (*Cicer arietinum*, *Lens culinaris* Medicus, *Phaseolus vulgaris* and *Phaseolus multiflorus*). Here, in particular, three ecotypes of the variety *macrocarpum*, three varieties of the species *Lens culinaris* Medicus and two subspecies with different varieties of the species *Phaseolus vulgaris* and *Phaseolus multiflorus* were analysed. The analysis of isoflavones was carried out using HPLC-MS/MS. To extract the analytes, a modified QuEChERS approach was used. The chromatographic peak areas obtained, after scaling in Pareto mode, were used to build statistical models. Both supervised and unsupervised techniques were applied for the different types of pulses used in the study. The results obtained revealed a similarity between the different ecotypes of the same variety of chickpeas and significant differences among varieties and subspecies belonging to the same species. Other factors besides the genotypic features, such as origin or agricultural practice, also contribute to the isoflavone content. Here, one of the statistical methods was validated by internal validation, obtaining satisfactory results.

2.5.1.2. *Introduction*

Legumes are the second most important crop as a source of food, feed for livestock and raw materials for industry. The importance of legumes is also associated with their secondary metabolites, in particular phytoestrogens. This family (*Fabaceae*) often comprises edible seeds. There are 13000 species of known legumes, of which about 20 are consumed by humans [1].

The three main classes of phytoestrogens are isoflavones, lignans and coumestans [2] and their biosynthesis depends heavily on the environment and plant species in question [3].

Isoflavones in particular are a subclass of flavonoids with a chemical structure similar to that of estradiol. They are mainly found as glycosides, conjugated with carbohydrates, which may be esterified with acetyl or malonyl groups. Flavonoids and isoflavonoids, these two groups of compounds have phenolic groups in both ends of the molecule, making them unique from the chemical, biochemical and biological point of view and giving the molecules exceptional stability [4].

Depending on the methylation or glycosylation of the hydroxyl groups, the ability of isoflavones to bind to estrogen receptors varies. Recently, the possibility of a duality in the estrogenic activity of isoflavones, which depends on endogenous estrogen concentrations, has been studied [5-7]. This underscores the beneficial effects gained from the consumption of this type of phytoestrogen for humans, such as the treatment of menopausal symptoms [8,9]; as an alternative to hormone-replacement therapy (HRT); in cardiovascular disease [10,11]; diabetes and obesity [12,13]; in osteoporosis [14,15], and even in cancer (e.g., prostate [16] and endometrial cancer [17]).

Isoflavones are typically associated with soybean, despite being synthesized by many other *Fabaceae* plants, such as alfalfa, clover, pea, peanut, beans, etc [2]. It is known that the amount of isoflavones present in species such as the legume family is influenced by genotype, agricultural practices, the time of ripening and harvesting, storage, weather conditions, processing conditions, etc [18]. Owing to this , there are many reports that show that the isoflavone content of legumes is strongly influenced by the genotype [18-20], agricultural practices [21], climatic factors [22], etc.

The *Fabaceae* species selected for this study are native to the Mediterranean areas of Europe, specifically Spain, and are characterized by their high protein content, good nutritional quality and good hay and silage quality. Also, they are grown easily.

A modified QuEChERS approach described previously [23] was implemented for the analysis of isoflavones in legume samples, followed by separation and quantification using HPLC-MS/MS. The data from HPLC-MS/MS are three-dimensional (retention time, mass and abundance) and therefore had to be pre-treated. The chromatographic peak areas obtained, after scaling in Pareto mode, were used to build statistical models. Both supervised and unsupervised techniques were applied.

2.5.1.3. *Material and methods*

- *Standards and reagents*

Isoflavone standards: Daidzin (Di, CASRN552-66-9), Glycitin (Gli, CAS RN40246-10-4), Genistin (Gi, CASRN529-59-9), Daidzein (De, CAS RN 486-66-8), Glycitein (Gle, CASRN40957-83-3), Genistein (Ge, CASRN 446-72-0), Formononetin (For, CASRN485-72-3), and Biochanin-A (Bio, CAS RN491-80-5) were purchased from Sigma-Aldrich (Alcobendas, Madrid, Spain). The organic solvents –acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH) – were of HPLC grade and were supplied by Merck (Darmstadt, Germany). Formic acid (>98%) was from Fluka (Alcobendas, Madrid, Spain). Anhydrous magnesium sulphate, sodium chloride and trisodium citrate dehydrate (Na_3Cit $.2H_2O$) were from Scharlau (Barcelona, Spain). Disodium hydrogen citrate sesquihydrate (Na_2HCit $.1.5H_2O$) was from Sigma-Aldrich. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Spain) water purification system. All other chemicals used were of analytical reagent grade.

- *Samples*

The samples analysed were purchased from local supermarkets.

Only the variety *macrocarpum* of the specie *Cicer arietinum* is commercialized in Spanish supermarkets; specifically, five ecotypes of this variety. In this study only three ecotypes were analysed, Blanco Lechoso (BL), Castellano (CS) and Pedrosillano (PD). Twenty chickpea samples from nine different producers from Andalucía and Castilla-León (Spain) were analysed. The samples analysed were labelled with the name of producer: "Fuentesaúco", "Pico Pardal", "Maragato", "Gordo" "Organically Grown", "Mexican" and "Castellano" "Pedrosillano" and "Blanco Lechoso".

The lentils most widely cultivated in Spain are of the species *Lens culinaris* Medicus. Three different varieties of this species exist: *Lens culinaris* Medicus var. *vulgaris (*including "Rubia de la Armuña" and "Rubia Castellana"*), Lens culinaris* Medicus var. *variabilis* and *Lens culinaris* Medicus var. *dupuyensis.* Twenty lentil samples from eight different producers of these three varieties in Castilla-León (Spain) were studied. The samples analysed were labelled with the name of producer.

Finally, twenty bean samples from twelve different producers in Castilla-León and Asturias (Spain) were analysed. In this case, two different species were analysed (*Phaseolus vulgaris* and *Phaseolus multiflorus*), including two different subspecies from *Phaseolus vulgaris*, spp. *nanus* and spp. *volubilis*. The samples studied were "Blanca Riñón" and "Blanca Redonda "from *Phaseolus vulgaris* spp. *nanus;* "Granja fabada", "Plancheta" and " Negrita" from *Phaseolus vulgaris* spp. *volubilis* and "Judión" from the species *Phaseolus multiflorus*.

- *Extraction procedure*

Before extraction, samples were ground with a Knifetec™ 1905 from Foss (Barcelona, Spain) and were stored in a cool, dry place in closed containers.

Extraction was carried out following the QuEChERS methodology optimized in a previous work [24]. Depending on the type of matrix, an appropriate amount of sample was extracted in two steps: first, 10 mL of ACN: H_2O (70:30, v/v) was added and the mixture was shaken for 5 min; then, 5 mL of ACN was added and the mixture was stirred again for 5 min. The chickpea and bean extractions were carried out using a VWR thermostatted shaking tray (Radnor, Pennsylvania, USA), while lentil extractions were accomplished using a Microson Misonix Ultrasonic Cell Disruptor probe (Farmingdale, NY, USA). After extraction, a mixture of $MgSO_4$: NaCl (4:1, w/w) together with citrate buffer (in the case of chickpeas and beans) was added, and the mixture was shaken vigorously for 1 min and centrifuged at 3000 rpm for 5 min. The upper layer was filtered through a 0.22 µm PVDF syringe filter before injection into the chromatographic system.

- *HPLC determination of isoflavones*

LC analyses were performed on a HP 1200 Series chromatograph from Agilent (Waldbronn, Germany) equipped with a binary pump, a membrane degasser, an autosampler, and a six-port valve. The analytical column was a 50x4.6 mm Zorbax Eclipse XDB-C18 with 1.8 µm particles (Agilent). The mobile phase consisted of an acetonitrile (solvent A) and 0.01% aqueous formic acid (solvent B) gradient at a flow rate of 0.5 mL min^{-1}. The gradient elution was as follows: 0–1.5min, 10% A; 1.5– 2.5 min, 10–25% A; 2.5–3.5min, 25% A; 3.5–7min, 25–50% A; 7–8 min, 50–80% A; 8–10min, 80% A, 10–12min, 80–10% A. The analytical column was thermostatted at 25°C, and the injection volume was 2 µL. Detection was carried out on a Triple Quad LC/MS6410 (Agilent) equipped with an electrospray (ESI) source. ESI-MS spectra were acquired in positive-ion multiple reaction monitoring (MRM) mode. The conditions of the MS analysis were as follows: the electrospray capillary voltage was 3500 V and the nebulizer pressure was 35 psi. Nitrogen was used as a drying gas at a flow rate of 12 L min^{-1} and a temperature of 350 °C. The whole system was controlled by Agilent MassHunter software, version B.04.01.

- *Statistical analysis*

Data analysis was performed with multivariate statistical methods, using SIMCA P+ version 13.0 software (Umetrics, Sweden). Pareto scaling was applied to the data prior to the development of the chemometric models. Principal component analysis (PCA), an unsupervised model, and partial least squares discriminant analysis (PLS-DA), a supervised model, were used for the discrimination and classification of legumes.

Principal component analysis (PCA) is one of the most widely used unsupervised techniques in multivariate data analysis because it is easy to interpret. PCA helps to reduce the

dimensionality of the data while retaining most of the information insofar that all the variable relationships are studied simultaneously [25].

This mathematical process aims to reduce the complexity of the data, identify the inherent trend of a set of experimental measurements, and classify the samples according to their similarities and differences, providing graphic visualization in the space defined by the principal components. The principal components were displayed as a set of scores (t), which highlights clustering or outliers, and a set of loadings (p), which highlights the influence of input variables on t [26].

Partial least squares discriminant analysis (PLS-DA) is a supervised model that uses information about the identity of each group of samples to generate a mathematical model that optimizes the separation between classes and allows the class to which new samples belong to be predicted.

PLS-DA is a classical PLS regression where the dependent variables (Y-matrix) are categorical and express the fact of belonging or not to a particular class. The matrix consisted of as many columns as there were classes and an observation took a value of one for the class it belonged to and zero for the rest [27]. PLS-DA provides rugged models, with predictive capability, that allow the differences between two sample groups to be found.

Validation of data models (PLS-DA) is essential to check whether the conclusions obtained are reliable according to the information provided by the model. For this, the parameters R^2 (total variance explained by the model) and Q^2 (predictive capacity of the model) were evaluated.

R^2 and Q^2 were calculated by cross-validation. In cross-validation, the prediction capacity of the model is determined by developing a model with part of the data set (training set) and using another part of the data (test set) for testing the model. Both the training and test sets contain samples representative of each class. This procedure, consisting of model development and model testing, is repeated several times so that the samples will have the same probability of being used as the training and as the test sets [28]. The results were compared with the original data and the quadratic sum or errors, divided by the initial sum of squares, and subtracted from 1, affording the value of Q^2 on a scale comparable to that of R^2[29].

2.5.1.4. Results and discussion

To study the isoflavone content of chickpeas, lentils and beans, all extractions were performed in triplicate and each replica was quantified in duplicate. Estimation of the content was carried out with the external calibration method.

Data were expressed as means ± standard deviations (µg / 100 g). The isoflavone profiles varied greatly among the species assayed, even among varieties from the same species. In

all cases, the aglycone content found was higher than the corresponding glucosides. As an example, the isoflavone compositions of the lentils analysed are shown in table 1.

- *Principal Component Analysis (PCA)*

Using the variables scaled with the Pareto mode, principal component analysis (PCA) models capable of classifying (among species, variety and ecotype) each class of legume based on its isoflavone content were constructed.

Figure 1 shows the PC2xPC1 score plot resulting from the application of PCA to the scaled results for chickpeas, lentils and beans respectively.

Figure 1.a-1 shows the PCA corresponding to chickpea samples, with a total explained variance >98% (R^2 (cum)) and a predictive capacity >85% (Q^2 (cum)). As can be seen, in figure 1.a-1 the chickpeas were denominated according to the label of the producer. Only the "Castellano", "Pedrosillano" and "Blanco Lechoso" ecotypes were considered as such. The samples designated "Maragato", "Gordo" or "Pico Pardal "can be considered to be from the "Pedrosillano" ecotype based on their isoflavone content and morphology. The sample denominated "Fuentesaúco" by the producer could be considered as being of the "Castellano" ecotype or the "Pedrosillano" ecotype, depending on the origin of sample and its isoflavone content. The same producer provided samples of different ecotypes and hence contamination among samples was possible. Figure 1.a-2 shows the PCA model generated taking into account only the three ecotypes studied. As can be seen, it was not possible to classify among the different ecotypes of the same variety of chickpeas, *macrocarpum.*

Figure 1.b-1 shows the PCA corresponding to the lentil samples, with a total explained variance >93% (R^2 (cum)) and a predictive capacity >85% (Q^2 (cum)). In this case, three different varieties of the same species (*Lens culinaris* Medicus) were analysed; *vulgaris, variabilis,* and *dupuyensis.* The samples were labelled with the label of the producer, "Rubia Castellana", Rubia de la Armuña", "Pardina" and "Verdina". Samples of unknown variety were identified considering the similarity (colour and morphology) to those known and, as can be seen, nearly all the samples of the same variety lie very close on the score plot. "Rubia Castellana" and "Rubia de la Armuña" belong to the same variety, *vulgaris*, but their isoflavone contents are very different. The formononetin content in "Rubia de la Armuña" sample is greater than in the "Rubia Castellana" sample. Figure 1.b-2 shows the PCA model generated for lentil samples as a function to the three different varieties: *vulgaris, variabilis* and *dupuyensis*

Finally, the classification of the bean samples is shown in figure 1c. Figure 1.c-1 shows the PCA model corresponding to the bean samples labelled by the producer. As may be seen, two perfectly differentiated groups were found. These groups corresponded to two different species studied, *Phaseolus vulgaris* and *Phaseolus multiflorus*. Only two samples belonged to *Phaseolus multiflorus*, namely samples Gh and Jv. The other samples, belonging to *P. vulgaris*, can be classified in two subspecies: *nanus* and *volubilis*, as can be seen in fig

1.c-2. In this figure some samples- Rls, Rl, Rvb and Rrg - belonging to the subspecies *nanus* are separated from the other samples of this subspecies; the isoflavone contents are also very different from those of the *volubilis* sample.

The later PLS-DA study carried out could improve the PCA classification.

- *Partial least squares discriminant analysis (PLS-DA)*

As mentioned above, a supervised technique -partial least squares discriminant analysis (PLS-DA) was conducted. This analysis allowed a more effective separation between classes, since the variables affecting the separation were known. Before PLS-DA, an HCA (*Hierarchical Cluster Analysis*) was carried out.

Analysis of the results confirmed the hypothesis made with PCA. For chickpea samples, a clear separation between "Blanco Lechoso" and the other two ecotypes, "Castellano" and "Pedrosillano", was observed in HCA and PLS-DA. Figure 2a shows the results obtained.

In the case of lentil samples (Figure 2b), on analysing the results provided by HCA it was observed that the samples were classified in four groups. The results obtained with this supervised technique corroborated the discrimination between "Rubia de la Armuña" lentils and the rest of the samples, as in the principal component analysis. As mentioned above, this variety of lentils had a higher content of aglycones; specifically with regard to the formononetin content. The explanation for this is similar to that invoked for the case of the chickpeas, i.e., the isoflavone content, which was dependent upon the origin and producer of the sample. However, separation among groups was better in lentil samples than in chickpea samples.

Finally, linear discriminant analysis was applied to the bean samples. First, a clear separation between two species, *P. multiflorus* and *P. vulgaris*, can be observed in the dendrogram (Figure 2c). On analysing the results provided by the second branch of the dendrogram, classification in subspecies was possible (three in the case of P. *vulgaris*: *nanus, volubilis* and another unknown one). The samples denominated Rls, Rl, Rvb and Rrg were separated from the *nanus* and *volubilis*. Even though the producer had labelled these samples as var. "Riñon" (the *nanus* spp.), the isoflavone contents found were very different from those of this subspecie. The isoflavone content in biochanin-A was higher than in the rest of samples.
In light of the above it may be concluded that PLS-DA affords a better classification than the PCA model.

- *Method validation*

After generating the models, they were validated to check the reliability of the conclusions based on the information provided by them. For this, the parameters R2 (total variance explained by the model) and Q2 (predictive capability of the model) were evaluated with the permutation method using a "Scrambling" validation. The permutation

method revealed that the high predictability of the models was not due to over-fitting of the data. Furthermore, as can be seen in Figure 3, the models were statistically valid since the Q2 intercepts were less than 0.05. The results obtained were (0.0, -0.245), (0.0, -0.425) and (0.0, -0.287) for chickpea, lentil and bean samples respectively.

2.5.1.5. Conclusions

The objective of this work was to classify different varieties of legumes according to their isoflavone contents. For this, the data on chromatographic peak areas were scaled using the Pareto mode. Unsupervised and supervised techniques such as PCA, HCA and PLS-DA were applied respectively.

In the case of the samples of lentils and beans, according to the results generated using PCA models it seems that the different species and varieties can be distinguished. By contrast, for chickpeas there is no discrimination among them, because that the samples analyzed are ecotypes of the same variety. Despite this, it has been described that the isoflavone content is influenced not only by the genotype of the species, but also by the origin of the samples, the agricultural practices used, or even the time of harvest. Using a PCA model allows a classification among species and varieties, but not of ecotypes.

The results afforded by PLS-DA corroborated the above. A clear separation among the three varieties of lentil samples was observed, including the separation between "Rubia de la Armuña" and "Rubia Castellana", subvarieties from *Lens culinaris* Medicus var. *vulgaris*. The case of the bean samples was similar. A differentiation between two species of beans was observed and a separation between the spp. *nanus* and spp. *volubilis* was observed.

The case of the chickpea samples was different. The separation of chickpea samples by PLS-DA was barely possible because the samples analysed were different ecotypes of the same variety, *macrocarpum*.

The relevance of this study is clear since the differences between pulses of a given variety based on its isoflavone content are corroborated.

2.5.1.6. *Figures and tables*

Table 1: Isoflavone content of lentil samples (µg/100g)

	Daidzin	Glycitin	Genistin	Dadzein	Glycitein	Genistein	Formononetin	Biochanin-A
Al	1.32 ± 0.06	0.79 ± 0.01	0.51 ± 0.04	3.2 ± 0.4	4.4 ± 0.8	27 ± 1	15.2 ± 0.6	18 ± 1
Ald	*Nd*	*nd*	*nd*	0.12 ± 0.04	*nd*	5.1 ± 0.2	0.9 ± 0.2	*nd*
Ach	*Nd*	*nd*	*nd*	*nd*	*nd*	3 ± 0.2	*nd*	1.2 ± 0.4
Li	*Nd*	*nd*	*nd*	*nd*	*nd*	2.4 ± 0.2	*nd*	*nd*
Ps	*Nd*	*nd*	*nd*	*nd*	*nd*	1.6 ± 0.9	*nd*	*nd*
Pl	0.17 ± 0.02	*nd*	*nd*	0.3 ± 0.1	*nd*	3.9 ± 0.9	0.9 ± 0.2	3 ± 0.2
Pv	0.43 ± 0.02	0.47 ± 0.02	0.25 ± 0.06	*nd*	*nd*	7.0 ± 0.4	1.9 ± 0.1	2.1 ± 0.9
Pci	*nd*	*nd*	*nd*	*nd*	*nd*	1.9 ± 0.2	*nd*	0.8 ± 0.4
Pba	*nd*	*nd*	0,09 ± 0,01	*nd*	*nd*	2.9 ± 0.2	0.22 ± 0.02	1.0 ± 0.4
Pa	*nd*	*nd*	*nd*	*nd*	*nd*	2.4 ± 0.2	0.18 ± 0.02	1.1 ± 0.4
Ph	*nd*	*nd*	*nd*	*nd*	*nd*	1.6 ± 0.4	*nd*	1.8 ± 0.9
Ca	*nd*	0.11 ± 0.01	0.18 ± 0.01	*nd*	*nd*	4.0 ± 0.2	1.17 ± 0.06	1.9 ± 0.4
Cv	0.086 ± 0.006	0.1 ± 0.01	0.18 ± 0.01	2,52 ± 0,09	*nd*	10.4 ± 0.4	4.64 ± 0.09	2.3 ± 0.4
Cci	*nd*	*nd*	0.28 ± 0.01	*nd*	*nd*	2.6 ± 0.2	0.82 ± 0.06	3.1 ± 0.4
Vv	0.25 ± 0.02	0.06 ± 0.01	0.11 ± 0.06	*nd*	*nd*	32.3 ± 0.9	8.4 ± 0.2	4.6 ± 0.9
Ea	*nd*	*nd*	*nd*	*nd*	*nd*	4.4 ± 0.2	0.31 ± 0.06	*nd*
Rh	*nd*	*nd*	*nd*	*nd*	*nd*	4.5 ± 0.2	0.38 ± 0.06	0.9 ± 0.4
Ec	*nd*	0.15 ± 0.004	*nd*	*nd*	*nd*	7.4 ± 0.2	0.87 ± 0.06	1.1 ± 0.4
Lh	0.15 ± 0.02	0.08 ± 0.01	0.28 ± 0.06	*nd*	*nd*	3.1 ± 0.4	2.6 ± 0.1	1.9 ± 0.9
Lp	*nd*	*nd*	0.19 ± 0.02	0.28 ± 0.06	*nd*	3.4 ± 0.2	3.1 ± 0.2	*nd*

Figure 1 Principal component analysis of isoflavones contents of legume samples. A) Chickpeas; a-1) Samples denominated by label of manufacturer, a-2) Samples denominated by theirs ecotypes. B) Lentils, b-1) Samples denominated by label manufacturer, b-2) Samples denominated by theirs varieties. C) Beans, c-1) Samples denominated by label manufacturer. Blue circle and green circle were used to note Phaseolus vulgaris and Phaseolus Multiflorus species respectively. c-2) Samples denominated by theirs species and subspecies

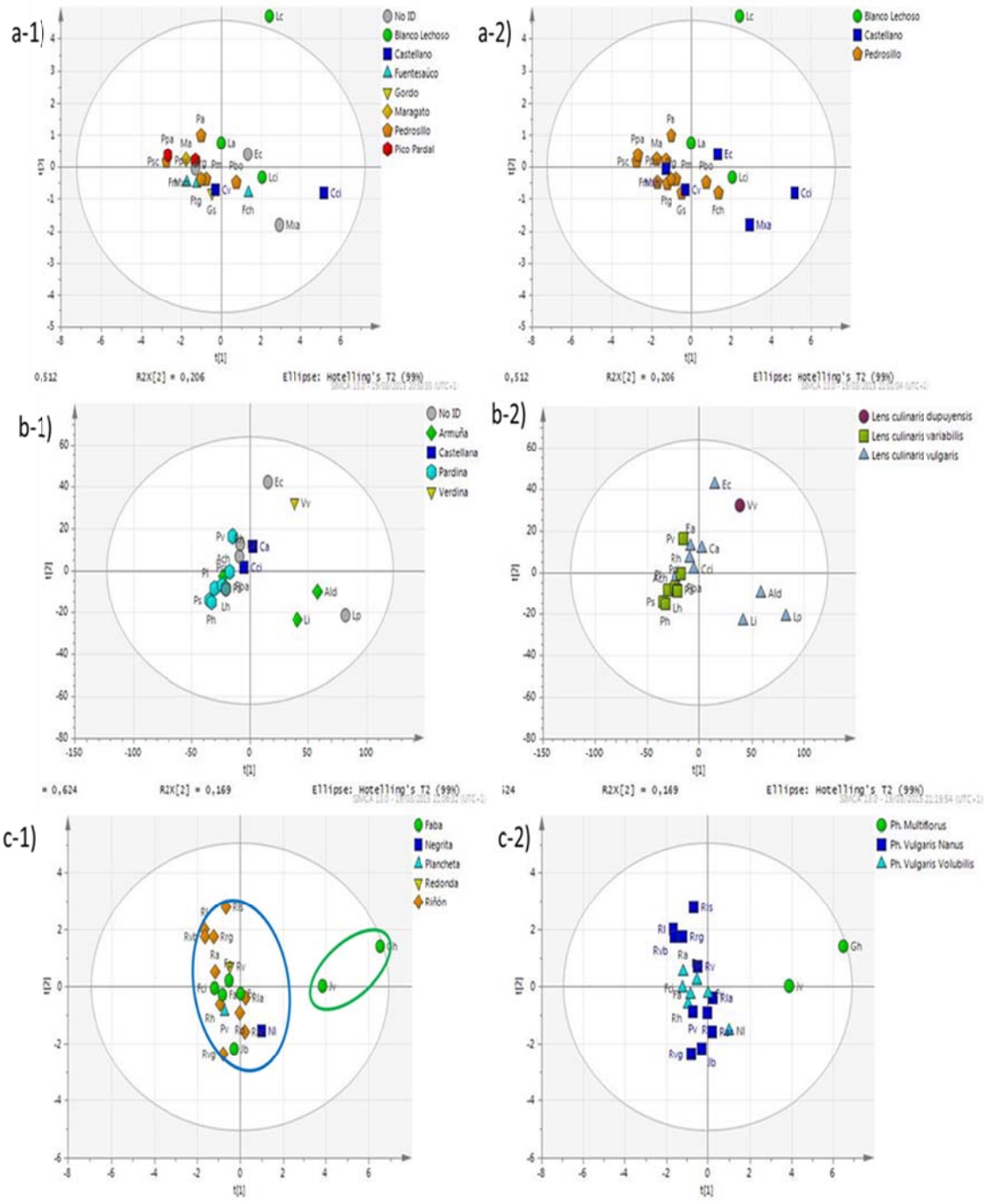


Figure 2 Hierarchical analysis cluster and Partial least squares discriminant analysis of isoflavones contents of legume samples. A) Chickpeas; Group 1:"Blanco Lechoso", Group 2:"Castellano", Group 3:"Pedrosillano" B) Lentils; Group 1: "Verdina", Group 2:"Rubia de la Armuña", Group 3: "Rubia Castellana", Group 4: "Pardina" C) Beans; Group 1: "Phaseolus Multigflorus", Group 2: "Unknown", Group 3." Phaseolus vulgaris nanus", Group 4: "Phaseolus vulgaris volubilis"

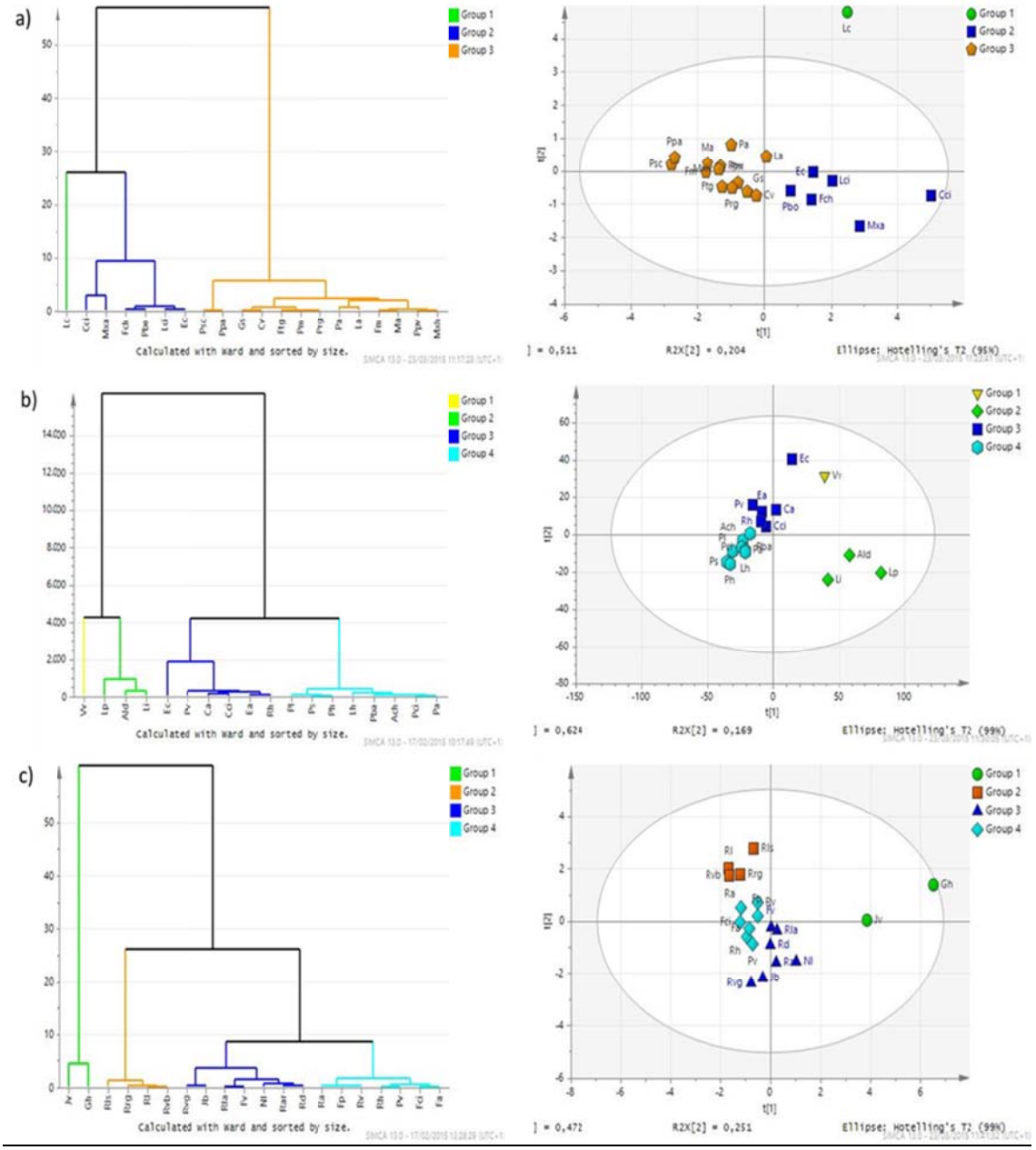
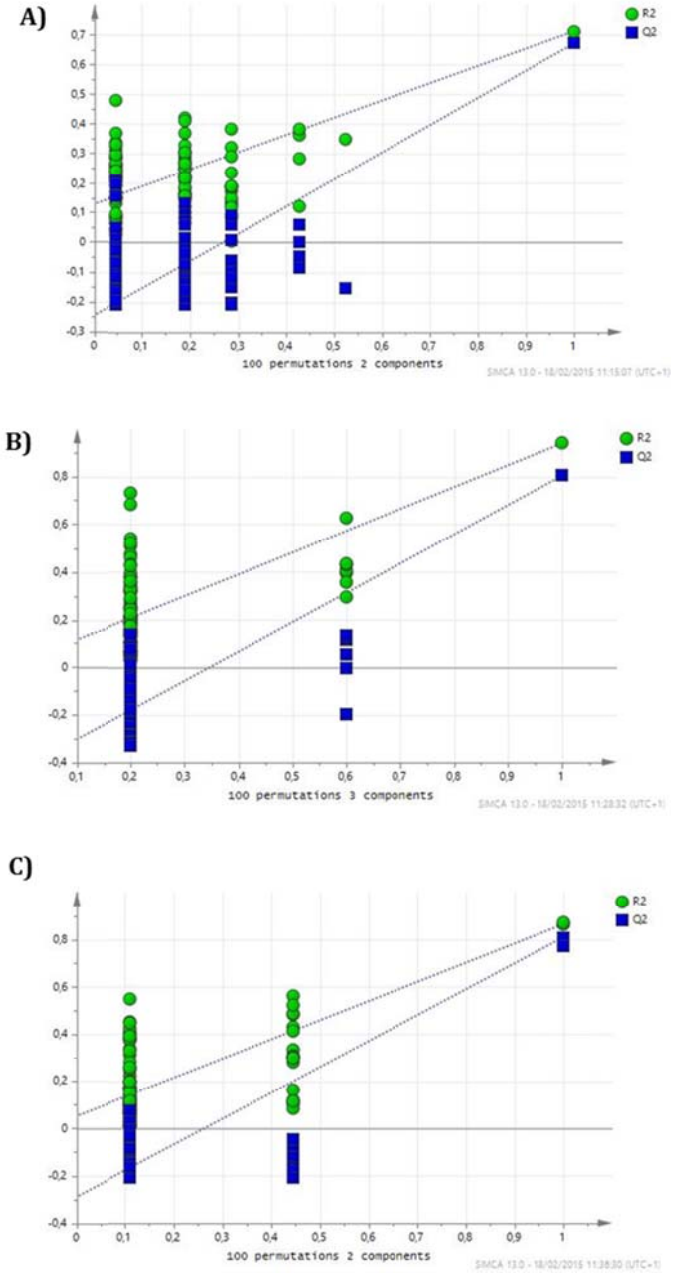


Figure 3: Scrambling validation of the model. A) Chickpeas, B) Lentils, C) Beans



2.5.1.7. References

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3. CONCLUSIONS

The aim of this research has been achieved; i.e. the development of analytical methodologies to analyze isoflavones in matrices as complex as food, and to determine these substances in the form in which they are present naturally in vegetables widely consumed in Spain, although found in low concentrations and even at trace levels in some cases.

The optimized sample treatments are simple and compatible with subsequent determinations, which were performed with liquid chromatography and capillary electrophoresis, with detection by mass spectrometry. The separation and detection methods used were also optimized for each case.

Pressurized liquid extraction (PLE), which has found few applications in food analysis, is a type of extraction that is valid as sample treatment for isoflavones in legumes. The optimized extraction method stands out for its simplicity, not requiring any extract cleaning step prior to determination, and has the advantage of sample treatment automation, which makes the method highly suitable for routine treatment.

As is known, extraction with QuEChERS methodology has usually been employed as sample treatment in the analysis of pesticides and other contaminants in food. This work has shown that it is possible to use it to isolate analytes naturally present in samples, obtaining good results in their determination. It is also concluded that the manner of placing the extractant and the sample in contact affects the extraction efficiency. Using QuEChERS methodology, the extraction of isoflavones was carried out quickly, easily and efficiently, minimizing the consumption of solvents and the cost of the analyses.

For the determination of analytes, the coupling of both liquid chromatography and capillary electrophoresis with mass spectrometry has allowed highly sensitive and selective methods to be developed. Study of the fragmentation of the analytes of interest allows the different transitions to be tracked, ensuring their identification with a high degree of reliability.

In general, it may be concluded that the methods developed are fast, sensitive and selective, and no tedious sample treatments are required. These methods have been validated using parameters such as recovery, repeatability and reproducibility, and can be applied in the routine analyses mandatory in food quality control.

More specifically it may be concluded that pressurized liquid extraction (PLE) allows extraction before the HPLC-DAD determination of isoflavones, with detection limits of 15-73 $\mu g\ L^{-1}$. The proposed methodology is easy to apply. There are no bibliographic references to this type of extraction for the determination of isoflavones in these legumes.

Regarding the extraction method by QuEChERS used here, this is able to simultaneously determine isoflavones in free and glucosylated form by applying two-step extraction with a decreased polarity of the extracting solvent. Elimination of the final cleaning step increases

the speed and simplicity of the method. The determination of isoflavones by UHPLC-MS / MS detection limits yielded 0.7 - 1.5 μg L^{-1}. A subsequent study of the contact between sample and solvent allowed the performance characteristics of the method and its applicability in facilitating routine analysis to be improved. The most suitable stirring systems are a thermostatted tray and an ultrasound probe. The former allows the analysis of multiple samples simultaneously, which is of great interest when analyzing large numbers of samples. In case of samples of chickpeas and beans, both methods offer adequate results; however, better results were obtained in lentil samples with an ultrasound probe.

Besides chromatographic methods, a capillary electrophoresis-mass spectrometry method was developed for the determination of isoflavones. Comparison of the results obtained by LC-ESI-MS / MS and CE-ESI-MS allows us to conclude that there are no significant differences between these methodologies, both being similar in terms of precision, recovery and detection limits. However, it should be noted that the CE-ESI-MS method is more suitable for the analysis of aglycones, while LC-ESI-MS / MS can be applied, and with better results, for the determination of both glycosides and aglycones.

The proposed methodologies were suitable for performing routine analysis of different types of legumes. Application of the QuEChERS-LC-MS/MS method to a large number of samples has allowed the classification of chickpeas, lentils and beans according to their isoflavone content, applying different multivariate analysis techniques (PCA, HCA, PLS-DA).

The statistical models proposed allowed the differentiation of lentil samples according to the three varieties under study, *Lens culinaris* Medicus var. *variabilis Lens culinaris* Medicus var. *dupuyensis* and *Lens culinaris* Medicus var. *vulgaris*, including different sub-varieties. In the case of the bean samples, the models allowed the differentiation of the species *Phaseolus multiflorus* and *Phaseolus vulgaris,* including the subspecies, *nanus* and *volubilis*. In the case of the chickpea samples, the tested samples were ecotypes of the same variety and hence the differentiation among classes with PLS-DA was barely possible. The proposed statistical models were validated, corroborating their robustness and quality.