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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.

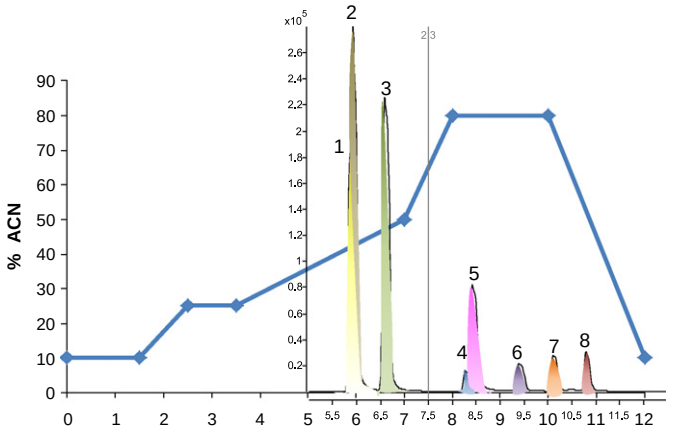


Fig. 1. Optimized elution gradient and TIC of standard solutions of daidzin (1), glycitin (2), genistin (3), daidzein (4), glycitein (5), genistein (6), formononetin (7) and biochanin A (8).

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).

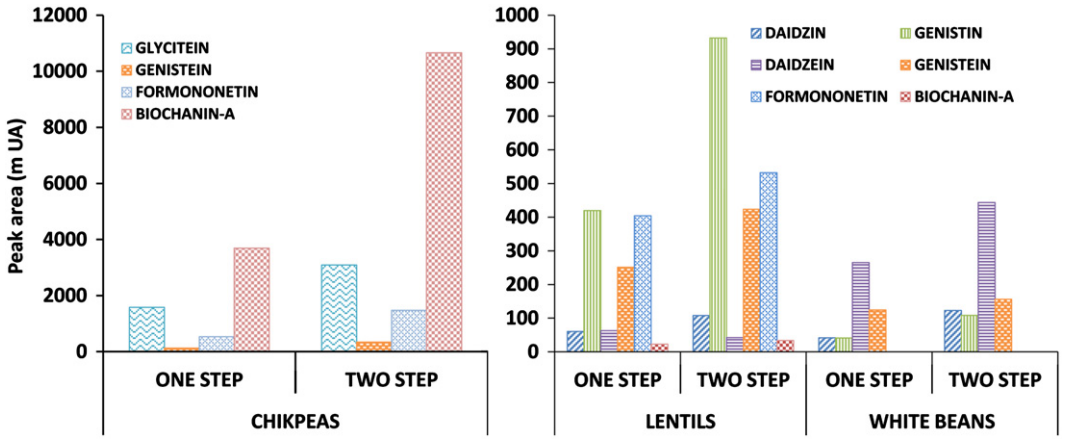


Fig. 2. Comparison of the analytical signals using one or two steps in the extraction process.

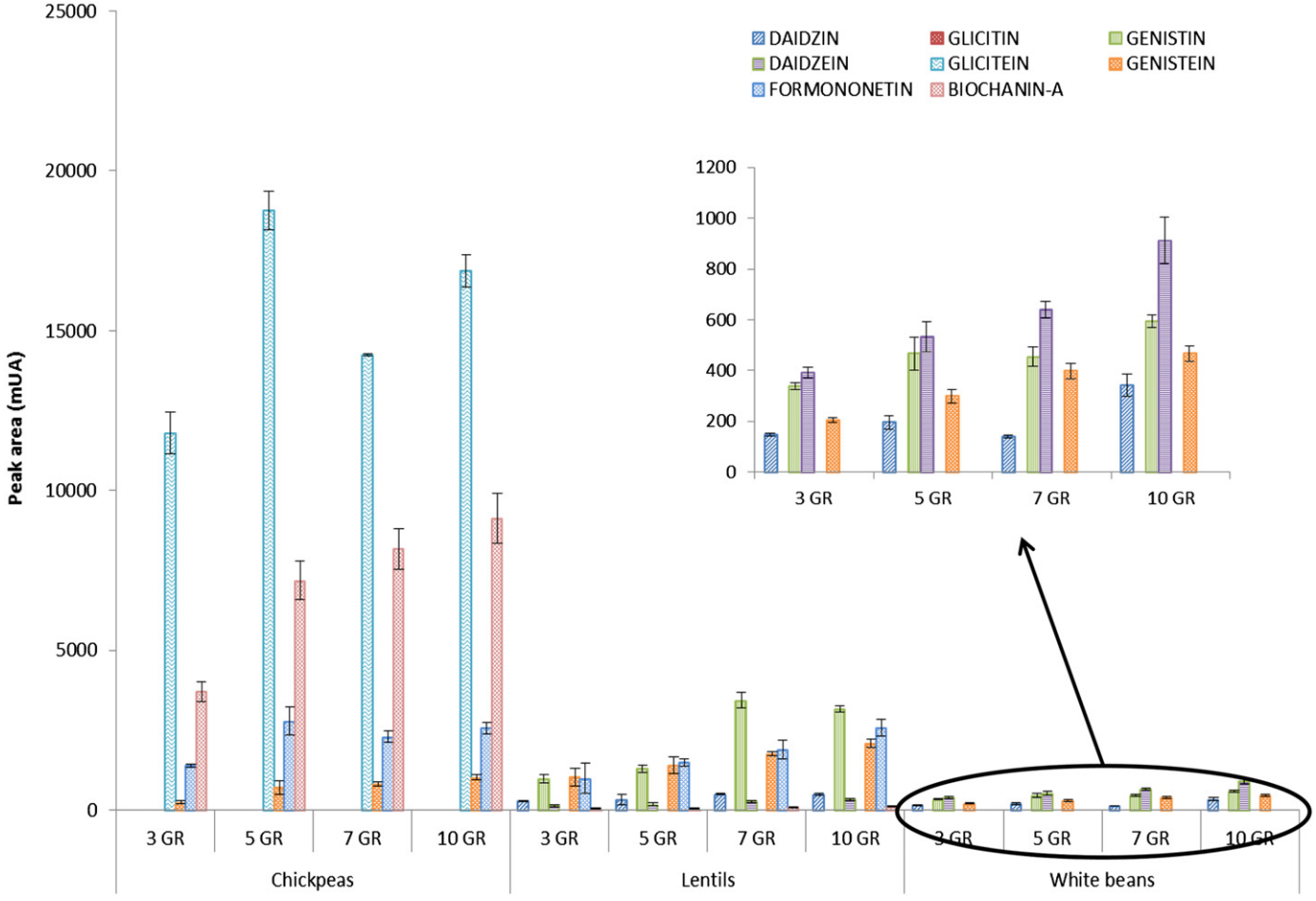


Fig. 3. Influence of sample amount on the analytical signal.

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

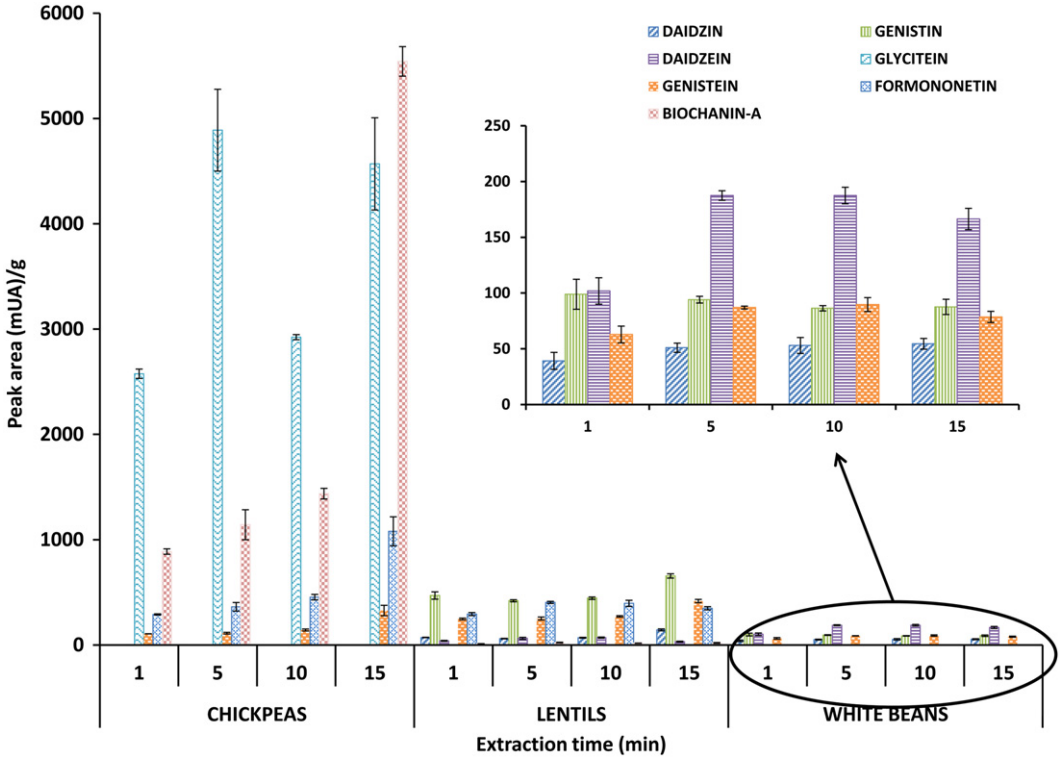


Fig. 4. Influence of extraction time on the extraction efficiency.

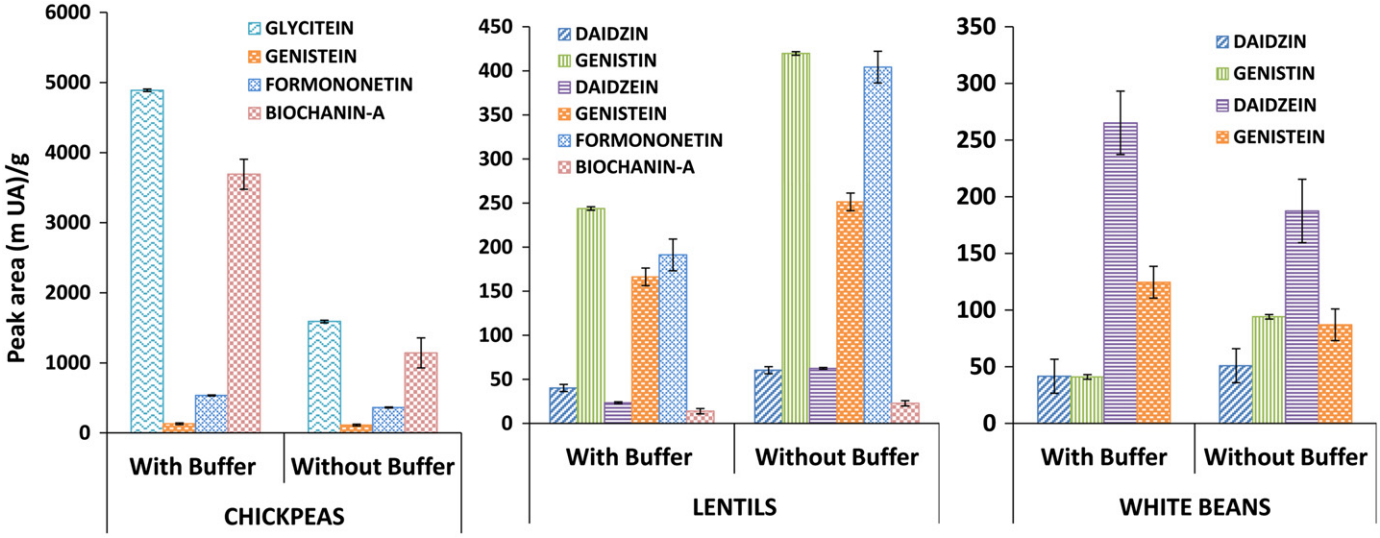
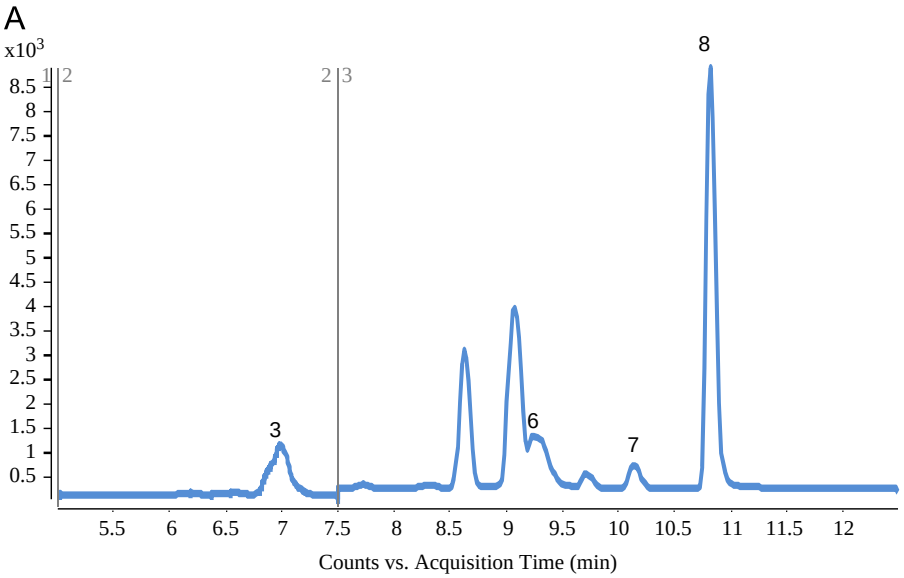
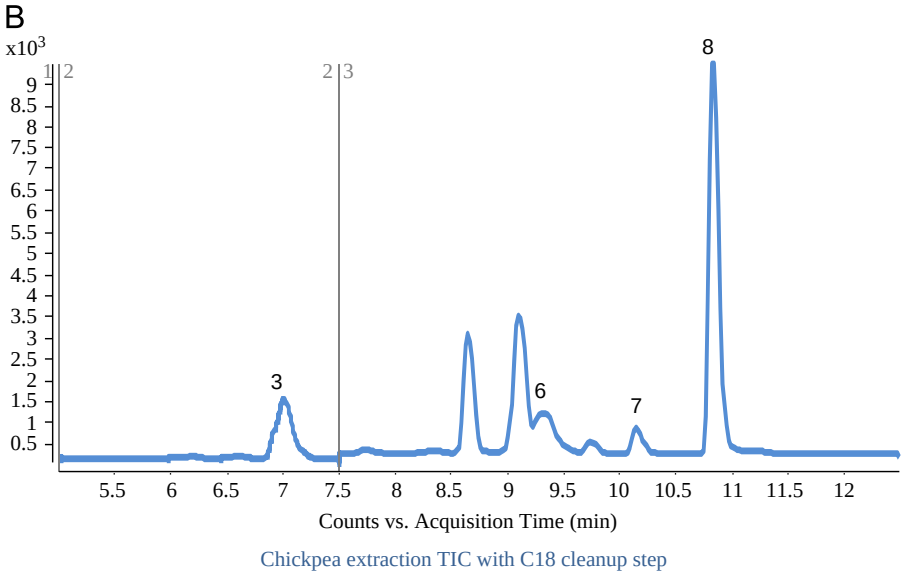


Fig. 5. Influence of the addition of citrate buffer.

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





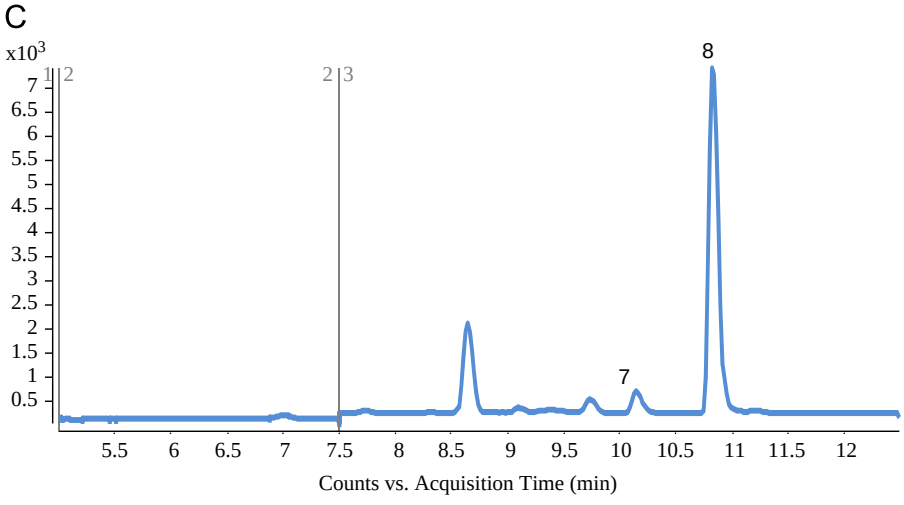


Fig. 6. Study of the clean-up step in the extraction of chickpeas samples: (A) without d-SPE, (B) d-SPE with C18, and (C) d-SPE with PSA.

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.

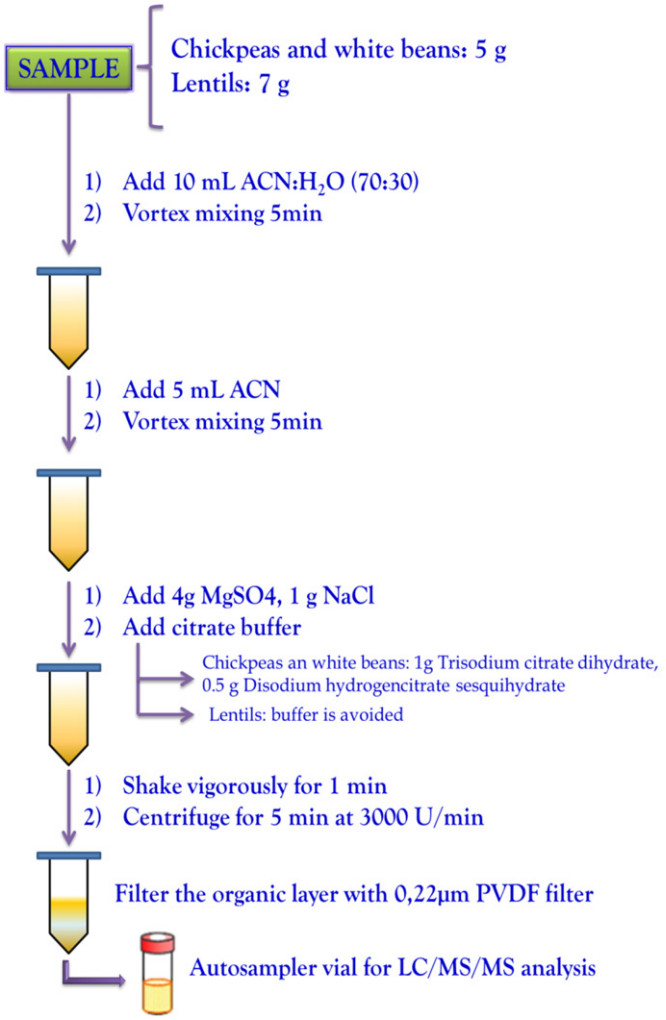


Fig. 7. Scheme of the proposed method.

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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