Application Note

Food Testing & Agriculture

Agilent

Trusted Answers

Determination of Isoflavones in Soybean by LC/MS/MS

Authors

Maria Carolina Blassioli-Moraes and Mirian Fernandes Furtado Michereff
Embrapa Recursos Genéticos e Biotecnologia,
Brasília, Brazil

Claudimir Lucio do Lago
Department of Fundamental Chemistry,
Institute of Chemistry,
University of São Paulo, Brazil

Daniela Daniel
Agilent Technologies, Inc.

Abstract

This Application Note describes a sensitive and reliable method for the determination of seven isoflavones in soybean samples by liquid chromatography/tandem mass spectrometry (LC/MS/MS). The best separation of daidzein, genistein, glycitein, daidzin, genistin, genistein, and rutin was obtained on a reversed-phase C18 column (Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm, 1.8 µm, p/n 959758-902) under gradient elution. The proposed method is simple, fast, and presented a linear calibration with correlation coefficients greater than 0.998. The limits of detection (LODs) and limits of quantification (LOQs), based on the signal-to-noise ratio (S/N), were in the range of 0.7 to 6.7 and 2.3 to 22.5 ppb, respectively. The method was successfully used to determine isoflavones in soybean samples.

Introduction

Soybean (*Glycine max*) is a complex food matrix containing low starch, approximately 20 % oil, and 40 % high-quality protein, in addition to several important bioactive compounds. Soybean products are consumed worldwide as both foods and food additives. They can be cooked and eaten, or used to make other products such as tofu, soy milk, and soy sauce. Soybean is also used as an additive in processed foods to enhance texture, flavor, or nutritional content. It is commonly used as a vegetarian alternative to conventional products to produce soy infant formula, soy yogurt, veggie burgers, and so forth. In addition to high protein and nutrient content, soybean also contains isoflavones, compounds similar to the female hormone estrogen. Isoflavones may be present in soybean foods and supplements as aglycones (daidzein, genistein, and glycitein), glycosides (daidzin, genistin, and genistein), or malonyl- and acetyl-glycosides. The potential impact of phytoestrogens on humans and animals has fueled an ever-increasing interest in the study of these compounds in foods, especially in soybean. These compounds also have an influence on a plants insect resistance and can therefore in certain situations allow crop yields to be increased, ensuring greater food security.

Analytical methods for the determination of phytoestrogens in edible plants, plant products, and biological matrices include gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). These can be coupled with various techniques such as ultraviolet absorption (UV), electrochemical detection (ED), fluorescence detection, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy.

In addition, immunoassays are adopted to analyze isoflavones in food products and biological samples. Each method has its own advantages and limitations. This study develops and validates a method for the determination of seven isoflavones (daidzin, glycitin, rutin, genistin, daidzein, glycitein, and genistein) in soybean samples using LC/MS/MS. Figure 1 shows the molecular structures of isoflavones analyzed in this work.

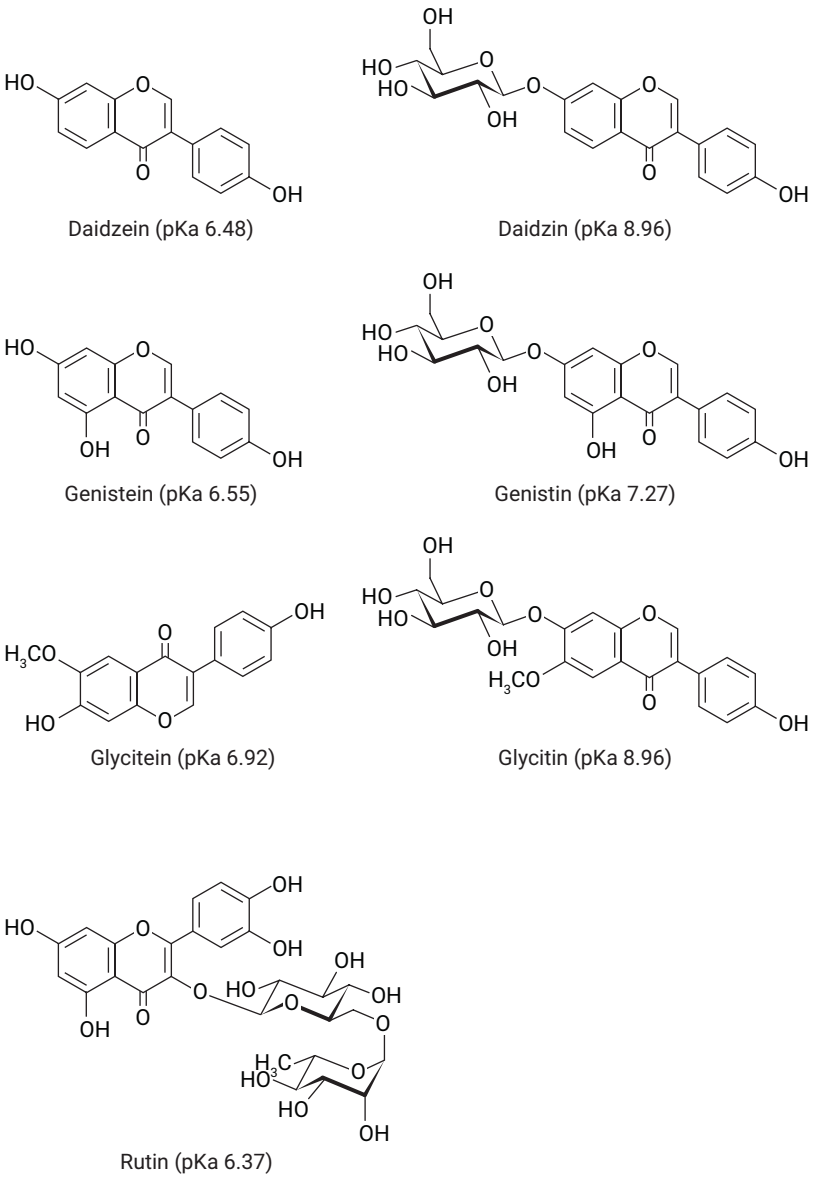

Figure 1. Molecular structure of isoflavones analyzed with predicted pKa values (calculated at www.chemicalize.org (accessed July, 2018).

Experimental

The mass spectrometer was operated in positive multiple reaction monitoring (MRM) mode using two specific transitions for each isoflavone. The most intense transition was used for quantification, and the other was used as a qualifying ion. Table 1 lists the retention time (RT), monitored ions, and other MS/MS acquisition parameters used for the identification and quantification of isoflavones in soybean.

Sample preparation

Samples of soybean seeds from two different cultivars, Dowling (resistant to sucking insects) and Silvania (susceptible to sucking insects), were obtained from the Embrapa Cerrados Research Center (Brasília, DF, Brazil). The samples were homogenized using liquid nitrogen in a porcelain mortar and pestle to obtain a fine flour. An aliquot of 2.0 g was weighed and added to a glass vial (15-mL) containing 10 mL of methanol and 2 mL of aqueous 0.1 M HCl.
The samples were sonicated for 20 minutes at room temperature. Then, the supernatant was separated and filtered through a filter paper, followed by a second filtration step using a syringe filter with a 0.45 µm PTFE membrane. The solvent was removed under low pressure using a rotary evaporator, and the final volume was adjusted to 2 mL of methanol.

LC conditions

Instrument	Agilent 1290 Infinity II LC
Column	ZORBAX Eclipse Plus C18 2.1 mm × 100 mm, 1.8 µm (p/n 959758-902)
Column temperature	40 °C
Injection volume	1 µL
Mobile phase	A) Water with 0.1 % formic acid B) Acetonitrile
Gradient	Time (min) %A %B 0.0 90 10 0.5 90 10 6.0 50 50 7.0 10 90 8.0 10 90 8.01 90 10
Stop time	9 minutes
Flow rate	0.300 mL/min

MS conditions

Instrument	Agilent 6470 triple quadrupole LC/MS
Ion mode	AJS-ESI, positive ionization
Capillary Voltage	4,000 V
Sheath gas heater	300 °C
Sheath gas flow	10 L/min
Drying gas flow (N_2)	10 L/min
Drying gas temperature	300 °C
Nebulizer pressure	20 psi
VCharging	500 V

Table 1. RT and MS/MS acquisition parameters used for the identification and quantification of isoflavones in soybean.

Compound	RT (min)	Q1[a] (*m/z*)	Q3[b] (*m/z*)	CE[c] (V)	FE[d] (V)
Daidzin	3.54	417.1	255.0* 199.0	20 52	101
Glycitin	3.64	447.2	285.0* 270.0	12 52	101
Rutin	3.88	611.2	303.0* 85.1	24 56	101
Genistin	4.23	433.1	271.0* 153.0	20 60	96
Daidzein	5.47	255.1	199.0* 91.1	28 44	125
Glycitein	5.62	285.1	270.0* 118.0	28 52	125
Genistein	6.43	271.1	153.0* 91.1	32 44	135

a = precursor ion (Q1); b = fragment ion (Q3); c = collision energy; d = fragmentor energy.

Results and discussion

Isoflavones have acidic-basic characteristics, with pKa values ranging from 6.37 to 8.96 (see Figure 1). Accordingly, the mobile phase was acidified with 0.1 % aqueous formic acid to prevent the deprotonation of analytes, and to improve the shape of the chromatographic peaks. The mobile phase composition, gradient composition, and injected volume were optimized for separation efficiency and sensitivity. Figure 2 shows a representative dMRM chromatogram obtained for isoflavone standards under optimized conditions using the Agilent MassHunter Qualitative software (B.08.00).

The developed method has the potential to quantify isoflavones in soybean by standard addition, since it is not possible to find a blank for soybean. To check the linearity of the isoflavones standards, calibration curves were constructed with at least nine distinct levels of concentration with each measured in triplicate. Calibration curves were constructed with standard solutions with at least nine distinct levels of concentration in triplicate. The correlation coefficients of calibration curves were greater than 0.998, with relative standard deviations (RSDs) ranging from 0.2 to 3.6 % for run-to-run precision. The LODs and LOQs were determined with reference to the corresponding concentration to three and ten times, respectively, the baseline noise, in a time close to the RT measured around each isoflavone in matrix.

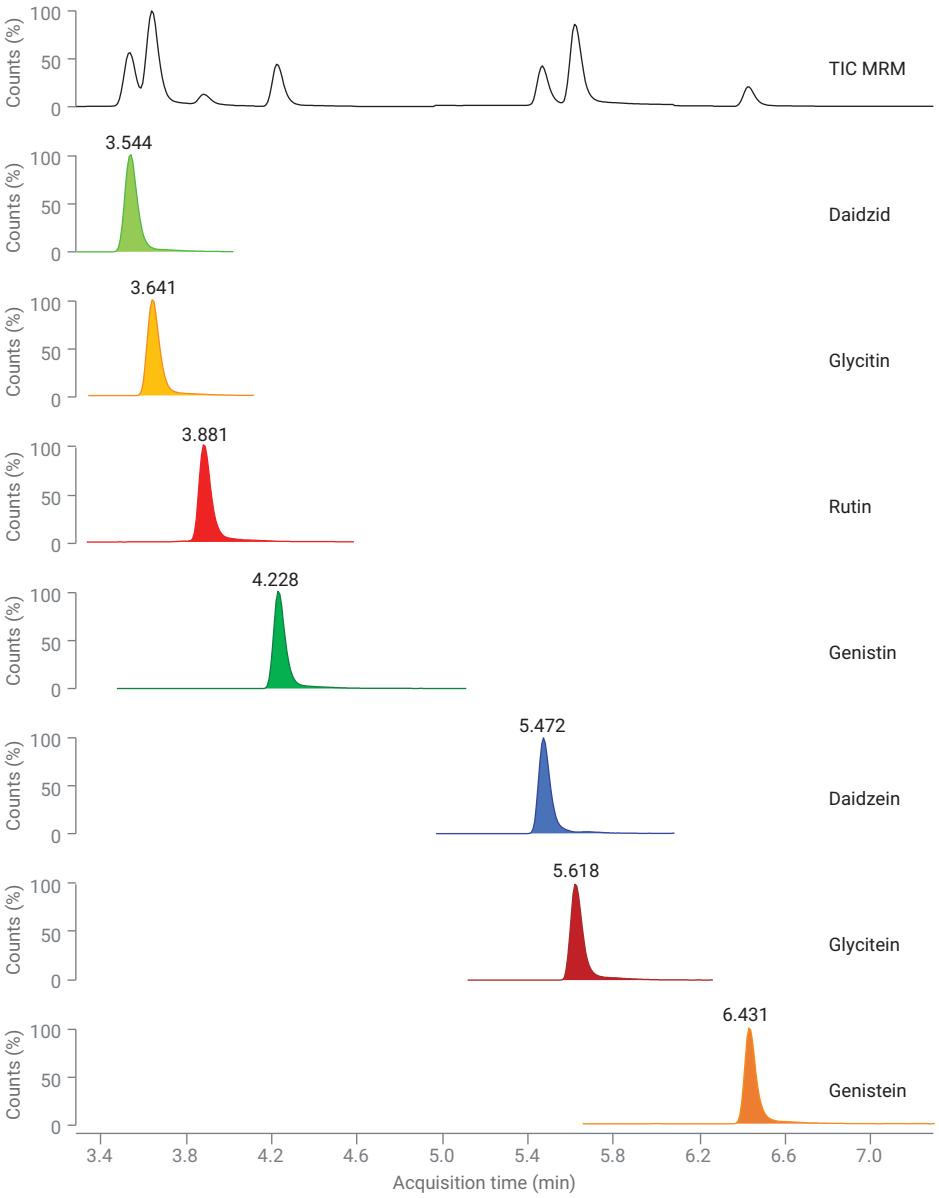

Figure 2. Normalized dMRM chromatogram at optimum conditions for the isoflavone standards at 40 ppb each, using the MassHunter qualitative software (B.08.00).

Table 2 shows the regression equations and other characteristic parameters for the developed method, while Figure 3 shows the calibration curve for genistin, using the MassHunter quantitative software (B.08.00).

Table 2. Figures of interest from the method developed for the determination of isoflavones in soybean by LC/MS/MS.

Compound	Linear range (ppm)	y = ax + b	R^2	LOD (ppb)	LOQ (ppb)
Daidzin	0.01 – 2.5	y = 664.2x – 5,044.9	0.998	0.7	2.4
Glycitin	0.01 – 2.5	y = 265.6x – 4,880.2	0.998	0.7	2.3
Rutin	0.04 – 5.0	y = 230.9x – 2,3876.8	0.999	6.7	22.5
Genistin	0.01 – 4.0	y = 155.5x – 173.3	0.998	0.7	2.3
Daidzein	0.01 – 1.3	y = 380.9x + 8215.7	0.998	1.3	4.4
Glycitein	0.01 – 2.5	y = 51.6x + 524.9	0.998	1.5	5.0
Genistein	0.01 – 1.3	y = 314.7x + 1,600.8	0.998	1.6	5.4

a = slope; b = intercept; R^2 = determination coefficient

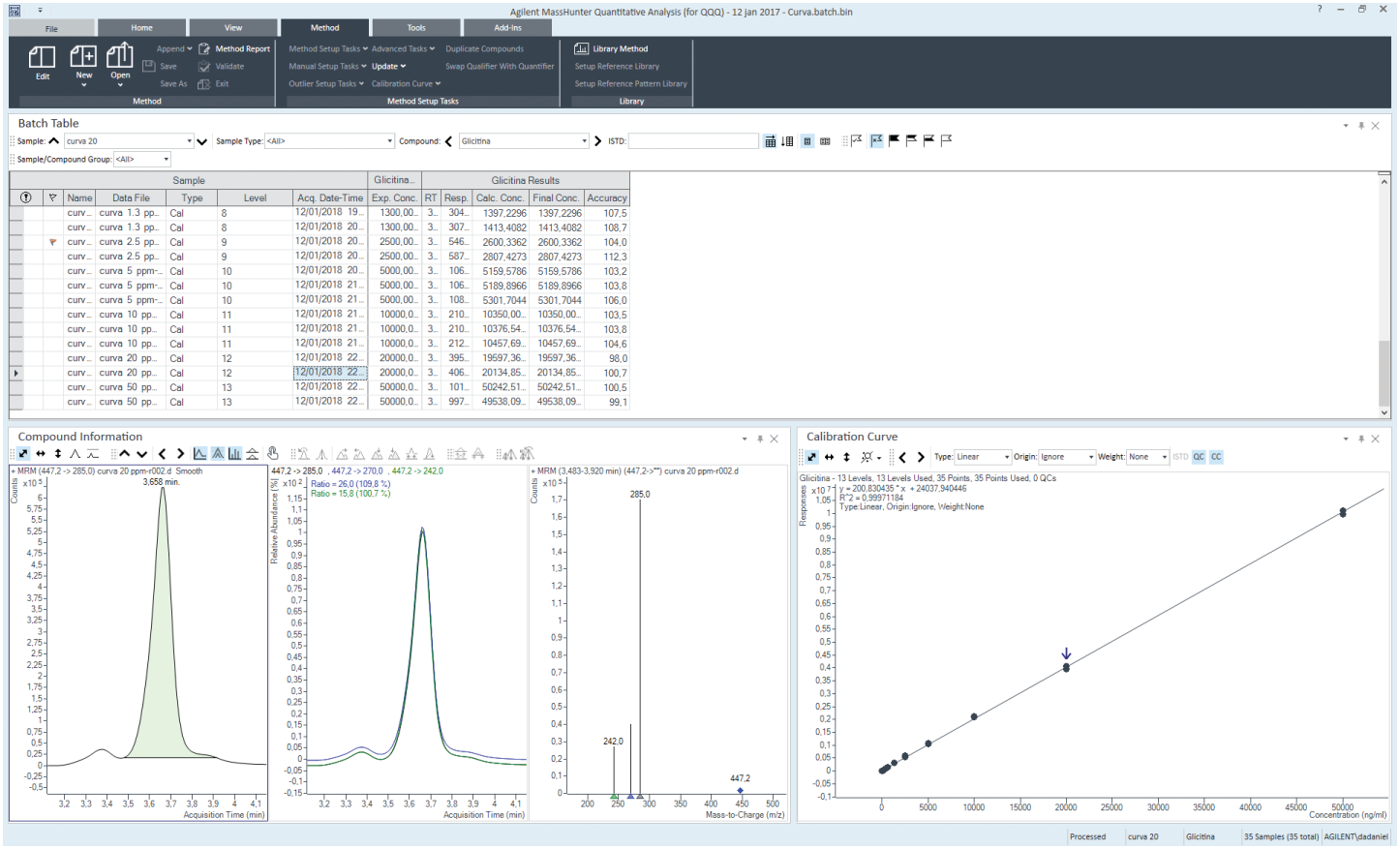

Figure 3. Calibration curve for genistin using MassHunter quantitative software (B.08.00).

To determine isoflavones in four soybean samples obtained from Embrapa Cerrados Research Center (Brasília, DF, Brazil), the consistency of the proposed method was evaluated by applying it to real samples, using standard addition methodology to avoid matrix effects. The RSD was lower than 10.2 %. Table 3 summarizes these results. The values obtained also confirmed previously published results, which showed that the Dowling cultivar produces a lower amount of the isoflavones identified here compared to Silvania soybean cultivar.

Table 3. Concentration (µg/g of soybean) of isoflavone in soybean samples (n = 3) as well the RSD (%) values.

Compound	Silvania 1	Silvania 2	Dowling 1	Dowling 2
Daidzin	3.8 ± 0.3 (7.9 %)	2.8 ± 0.2 (7.1 %)	1.6 ± 0.1 (6.2 %)	1.6 ± 0.1 (6.2 %)
Glycitin	3.6 ± 0.2 (5.5 %)	2.2 ± 0.2 (9.1 %)	1.3 ± 0.1 (7.6 %)	1.4 ± 0.1 (7.1 %)
Rutin	0.025 ± 0.002 (8.0 %)	0.036 ± 0.002 (5.5 %)	0.036 ± 0.002 (5.5 %)	0.029 ± 0.002 (6.9 %)
Genistin	20.1 ± 1.5 (7.4 %)	16.9 ± 1.3 (7.7 %)	6.9 ± 0.5 (7.2 %)	11.8 ± 1.2 (10.2 %)
Daidzein	0.080 ± 0.003 (3.7 %)	0.081 ± 0.004 (4.9 %)	0.031 ± 0.003 (9.7 %)	0.072 ± 0.004 (5.5 %)
Glycitein	0.108 ± 0.005 (4.6 %)	0.260 + 0.004 (1.5 %)	0.055 ± 0.002 (3.6 %)	0.079 ± 0.003 (3.8 %)
Genistein	0.036 ± 0.002 (5.5 %)	0.035 ± 0.001 (2.8 %)	0.025 ± 0.002 (8.0 %)	0.046 ± 0.003 (6.5 %)

Conclusion

We have shown that LC/MS/MS is well suited to determine isoflavones in soybean samples. The proposed method presented a linear response with excellent precision data for replicate injections and LODs lower than 7 ppb. In addition, the method is simple, fast, and lasts less than nine minutes per sample. It presents excellent potential for application in food analysis laboratories, not only for the analysis of soybean isoflavones, but also for other types of matrices.

References

1. Mujić, I.; *et al.* Isoflavone content and antioxidant properties of soybean seeds, *Croat. J. Food Sci. Technol.* **2011**, *3(1)*, 16-20.
2. Anderson, J. J. B.; *et al.* Health potential of soy isoflavones for menopausal women, *Public Health Nutr.* **1999**, *2(4)*, 489-504.
3. Wu, Q.; Wang, M.; Simon, J. E. Analytical methods to determine phytoestrogenic compounds, *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci*. **2004**, *812(1-2)*, 325-355.
4. Delgado-Zamarreño, *et al.* A modified QuEChERS method as sample treatment before the determination of isoflavones in foods by ultra-performance liquid chromatography-triple quadrupole mass spectrometry, *Talanta* **2012**, *100*, 320-328.
5. Konar, N.; *et al.* Determination of conjugated and free isoflavones in some legumes by LC–MS/MS, *J. Food Compost. Anal.* **2012**, *25(2)*, 173-178.
6. Michereff, M. F. F.; *et al.* Volatiles mediating plant-herbivory-natural enemy interaction in resistant and susceptible soybean cultivars. *J. Chem. Ecol*. **2011**, *37(3)*, 273-285.
7. Michereff, M. F. F.; *et al.* Effect of resistant and susceptible soybean cultivars on the nymphal development, fecundity and mortality of Euschistus heros (Hemiptera:pentatomidae). *1st American Latin Chemical Ecology Meeting*. Abstract Books. Colonia del Sacramento, October 17th-20th, **2010**, *138*, Uruguay.

www.agilent.com/chem

Printed in the USA, August 8, 2018
5994-0162EN

Agilent
Trusted Answers