Asian Journal of Chemistry; Vol. 24, No. 3 (2012), 1322-1324

ASIAN JOURNAL OF CHEMISTRY

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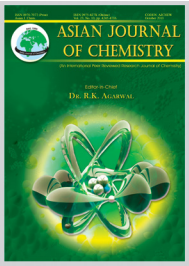

Thin Layer Chromatography Densitometric Determination of Soybean Isoflavones in Wild Soybean (*Glycine soja*) Seeds

MENG HUANG and DAICHENG LIU*

Department of Life Science, Shandong Normal University, 88 East Wenhua Road, Jinan-250014, P.R. China

*Corresponding author: Tel/Fax: +86 531 86185360; Email: liudch@sdnu.edu.cn, hmfriendly@163.com

(*Received*: 21 April 2011; *Accepted*: 12 November 2011) AJC-10651

A simple and selective method for qualitative and quantitative analysis of four soybean isoflavones in wild soybean seeds was validated. The isoflavones tested include daidzin, genistin, daidzein and genistein, which were identified by comparing with the standards and quantified simultaneously by thin layer chromatography densitometric method. Thin layer chromatography separation was performed on silica gel 60F254 [10 × 20 cm^2] using dichloromethane:methanol:acetic acid (10:2:0.1,v/v) as the mobile phase for two β-glucosides (daidzin and genistin); chloroform:methanol:acetic acid (9:1:0.15,v/v) for two aglycones (daidzein and genistein). Densitometric evaluation of the spots was performed with a thin layer chromatography scanner in reflection/absorption mode at 260 nm. Average recoveries of daidzin, genistin, daidzein and genistein were 97.45, 98.32, 97.87 and 99.74 %, respectively; the total content of the four isoflavones was 42.471 (mg/g), which is the highest level in glycine species. The method elucidated above is appropriate for detection of isoflavones in wild soybean due to its specificity, accuracy and reproducibility.

Key Words: *Glycine soja*, Thin layer chromatography densitometric method, Soybean isoflavones.

INTRODUCTION

Wild soybean (*Glycine soja*) is an origin of soybean (*Glycine max*)[1-3]. It mainly distributes in China and possesses many excellent characteristics for suboptimal natural environment adaptation, such as cold resistance, disease resistance and high saline-alkaline tolerance[4-6], which can be utilized for genetic modification of soybean[6], in addition, it's a rare and endangered plant in national secondary[3,7].

Wild soybean has a large isoflavone accumulation, because isoflavone, the secondary metabolite of wild soybean, is related to wild soybean's adaptation to the severe wild area. Moreover, wild soybean is a traditional chinese herbal medicine of great medicinal value and its pharmacological efficacities mostly profit from isoflavones[7,8].

Among several soybean components, isoflavone has various pharmacological activities attributing to healthy maintenance and recuperation, including phytoestrogen performance, anticarcinogenic function, antiosteoporosis action and atherosclerosis prevention, *etc*.[8-10].

Isoflavones are phenolic compounds, mainly existing in leguminous plants. There are twelve soybean isoflavones, among which, daidzein and genistein are the most notable forms owing to their pharmacological activity exhibition in human body[10-12] and partially existing in soybean as their β-glucosides (daidzin and genistin)[9]. Furthermore, no document about densitometric determination of isoflavones in wild soybean has been covered. The ultimate objectives of this research were to establish a simple and reliable method for detection of isoflavones and to demonstrate the high level of isoflavone content in wild soybean.

EXPERIMENTAL

The wild soybean was collected from the saline-alkaline soil beside Yellow river in Dong Ying City, China. All chemicals including solvents were of analytical grade. Four isoflavone standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Precoated thin layer chromatography silica gel 60F254 glass plates (10 cm × 20 cm) were purchased from Haiyang Chemical Co. (Qingdao, China).

Standard solution preparation: The solution of four isoflavone standards were prepared with accurately weighed β-glucosides and aglycones dissolved in methanol. The concentration of daidzin, genistin, daidzein and genistein was 0.2775, 0.2350, 0.2550, 0.3013 mg/mL, respectively.

Sample solution preparation: The wild soybean seeds (150 g) were grinded into granules, dryed at 50 ºC for 4 h. Then the seeds were treated with *n*-hexane for degreasing (50 ºC, Soxhlet extraction). Eighty per cent of ethanol was selected for exhaustively extracting isoflavones from wild soybean (70 ºC, Soxhlet extraction). The ethanol was removed by a rotary vacuum desiccator at 60 ºC, subsequently adding

acetic ether (ethyl acetate, m.f. $C_4H_8O_2$) (100 mL) thrice for extracting isoflavones from the the rest aqueous solution. Acetic ether was concentrated *in vacuo* to give a residue (3.116 g), dissolving accurately in 50 mL methanol and storing at -15 ºC for the next procedures.

A Camag (Muttenz, Switzerland) thin layer chromatography scanner 3 with Wincats 1.4.1 software was used for the scanning densitometry. Analysis was performed on 10 cm × 20 cm thin layer chromatography plates coated with silica gel 60F254. The cleaned plates were activated in an airoven at 110 ºC for 0.5 min. In an empty desiccator the active plates were cooled down to room temperature and equilibrated with the relative humidity of the laboratory atmosphere. Two mobile phases were adopted, dichloromethane: methanol:acetic acid (10:2:0.1,v/v) for the β-glucosides (daidzin and genistin), with the development distance of 12.5 cm; chloroform:methanol: acetic acid (9:1:0.15, v/v) for the aglycones (daidzein and genistein) with the development distance of 10 cm. The optimized chamber saturation time for mobile phases were 35 min, at room temperature (25 ºC ± 2). Standards and sample were applied to the plates as dots with diameters less than 3 mm, 1 cm apart by using a 10 µL microsyringe. Application volume of glucosides : sample solution 0.5 mL, standard solution 1, 2, 3, 4, 5 µL on the same plate; application volume of aglycones: sample solution 5 µL, standard solution 0.5, 1, 1.5, 2, 2.5 µL on the same plate. Each scanning repeats quintic. The densitometric evaluation was performed in the reflection/absorption mode at 260 nm by a deuterium lamp, slit width 6.00 mm × 0.90 mm, scanning speed 80 mm/s and data resolution 200 µm/step. Evaluation *via* peak areas with linear regression.

RESULTS AND DISCUSSION

The spectrodensitometric analysis (Fig. 1.) showed the maximum absorption of isoflavones was $\lambda_{max} = 260$ nm. Therefore, the ultraviolet wavelengh 260 nm was selected for densitometric determination.

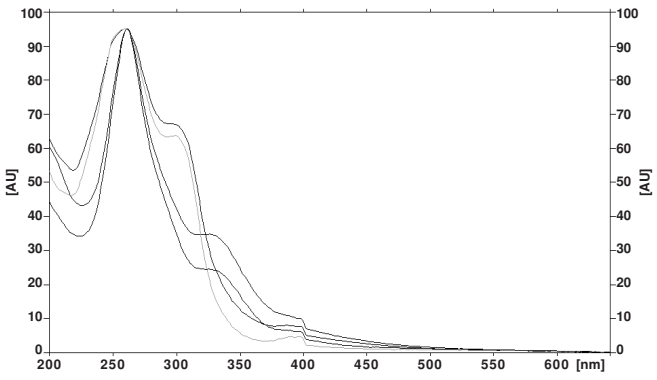

Fig. 1. Spectrodensitometric analysis graph of the four isoflavones, both the glucosides and the aglycones have the maximum absorption of λ = 260 nm.

The polarity of β-glucoside is much higher than aglycone by reason of the glycosyl. For acquiring ideal separation, two mobile phases were selected. The densitograms of β-glucosides and aglycones were in Fig. 2 and Fig. 3. Before the method in this paper validated,several thin layer chromatography methods for isoflavones in soybean were tried with results of bad separations[13-15] and many work had been done for confirming the appropriate mobile phases and other thin layer chromatography procedures, which could come to a conclusion that this method exactly adapt to wild soybean with high specificity.

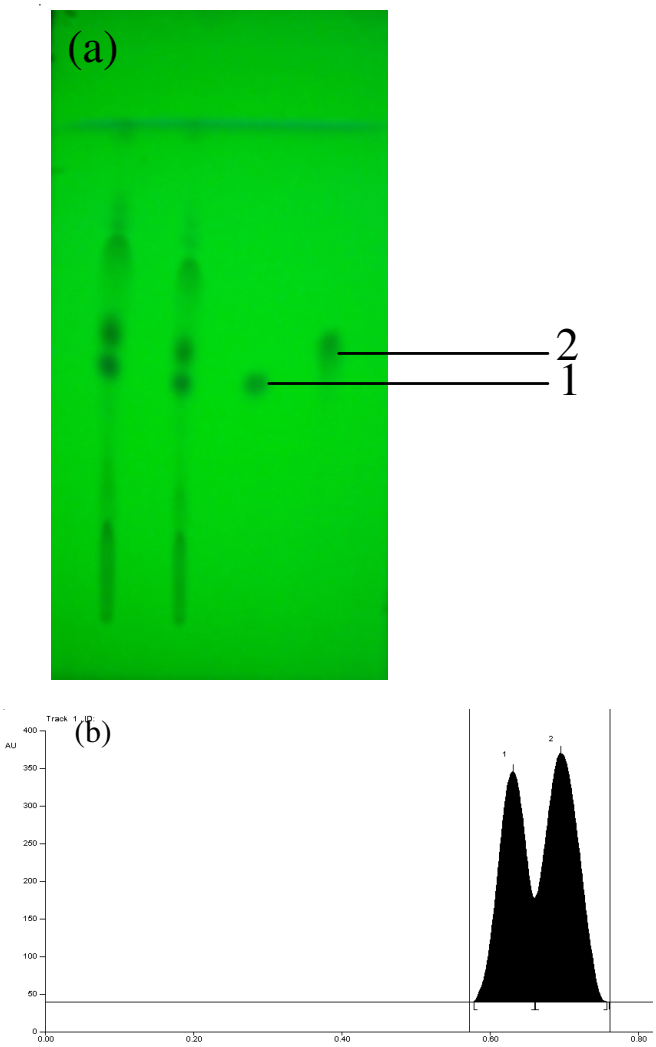

Fig. 2. (1) daidzin, (2) genistin; (a) The picture was taken by digital camera. (b) Densitogram of β-glucosides, though the resolution showed in (b) is not very good, the picture of the thin layer chromatography plate can verify a satisfied resolution of daidzin and genistin

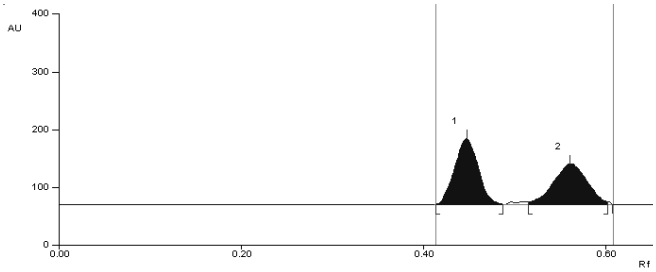

Fig. 3. Densitogram of daidzein (1) and genistein (2), which have a good resolution

The average recoveries of daidzin, genistin, daidzein and genistein were 97.45, 98.32, 97.87 and 99.74 %, respectively. The limit of detection and the limit of quantification, which could be monitored were 135 µg/mL and 1000 µg/mL. The

TABLE-1
RESULTS OF DENSITOMETRIC DETERMINATION

Isoflavone	Linear regression equation	Correlation coefficient	Linear range (mg)	Avg. content (mg/g)	RSD (%)
Daidzin	Y = 1258.8300 + 9.5320X	0.99719	0.2775-1.3875	24.646	1.79
Genistin	Y = 3360.4012 + 10.1354X	0.99937	0.2350-1.1750	56.322	1.41
Daidzein	Y = 2439.3234 + 7.3538X	0.99847	0.1275-0.6375	1.708	1.86
Genistein	Y = 2069.1854 + 9.8379X	0.99832	0.1507-0.7533	2.266	1.59

Annotation: Y is the response and X is the amount of isoflavone

densitometric data were presented in Table-1, which indicated that the precision and accuracy were acceptable. Total isoflavone content in soybeans can range from 0.4 mg/g to 9.5 mg/g in general[16-18]. Though only four isoflavones were detected in this paper, the total content of them was 42.471 mg/g (Table-1), which was distinctly higher than that in soybean.

Conclusion

Four isoflavones were determined qualitatively and quantitatively by thin layer chromatography densitometric method, which permitted a reliable determination and can be used for isoflavone analysis in wild soybean with advantages of speediness, flexibility and low cost. Moreover, wild soybean with high content of isoflavones is of enormouse development value and has vast prospect in scientific research and technical production.

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