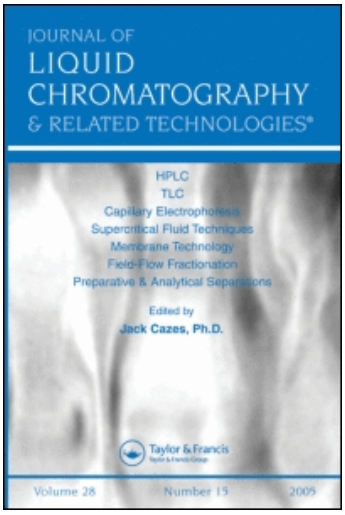

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of the Lignan Secoisolariciresinol Diglucoside from Flaxseed (*Linum Usitatissimum* L.) by HPLC

Juan Chen[a]; Xia Liu[a]; Yan-Ping Shi[a]; Chun-Yan Ma[b]
[a] State Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, P. R. China [b] College of Chemical and Environmental Science, Hebei University, Baoding, P. R. China

To cite this Article Chen, Juan , Liu, Xia , Shi, Yan-Ping and Ma, Chun-Yan(2007) 'Determination of the Lignan Secoisolariciresinol Diglucoside from Flaxseed (*Linum Usitatissimum* L.) by HPLC', Journal of Liquid Chromatography & Related Technologies, 30: 4, 533 — 544
To link to this Article: DOI: 10.1080/10826070601093853
URL: http://dx.doi.org/10.1080/10826070601093853

Journal of Liquid Chromatography & Related Technologies®, 30: 533–544, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601093853

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Juan Chen, Xia Liu, and Yan-Ping Shi
State Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, P. R. China

Chun-Yan Ma
College of Chemical and Environmental Science, Hebei University, Baoding, P. R. China

Abstract: A high performance liquid chromatographic (HPLC) technique coupled with photodiode array (PDA) detection was developed for determining secoisolariciresinol diglucoside (SDG) in extracts of flaxseed from different locations in Gansu province, China. The optimized method was achieved for the separation and detection of selected constituents using acetonitrile-1% aqueous acetic acid (15:85, v/v) as the mobile phase at a flow rate of 1.0 mL/min, and 280 nm as the detection wavelength. SDG showed good linearity in a relatively wide concentration range ($r = 0.9999$). Intra- and inter-assay accuracy and precision were all lower than 5.0%. Sample extracting and hydrolyzing procedures were intensively explored, treating of extraction methodology, solvent, time, and hydrolysis temperature, and alkali concentration. The recovery of the method was 95.3% with a relative standard deviation (RSD) of 0.67%. Different samples of flaxseed varied considerably in the content of SDG.

Keywords: High performance liquid chromatography, *Linum usitatissinum*, Secoisolariciresinol diglucoside, Lignan, Pharmaceutical analysis

Address correspondence to Yan-Ping Shi, State Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, P. R. China. E-mail: shiyp@lzb.ac.cn

INTRODUCTION

Plant lignans, natural products resulting from the phenylpropanoid metabolic pathway, are structurally characterized by the coupling of two phenylpropanoid units by a bond between the β-positions in the propane side chains and occur widely in a variety of foods, particularly whole grains and legumes. Recently, lignans have been paid considerable attention owing to their possible application in the fields of pharmacy and nutrition. Lignans have been found to possess a variety of demonstrated biological properties,[1] such as anti-tumorigenic,[2] anti-estrogenic,[3,4] anti-oxidant, and anti-aromatase properties.[5] Flaxseed is the richest known source of lignans, with lignan production at 75–800 times of other oil seeds, cereals, legumes, fruits, and vegetables.[6] Secoislariciresinol diglucoside (SDG) is the major lignan in flaxseed (*Linum usitatissinum* L.) and the principal precursor of the mammalian lignans enterodiol and enterolactone, which are present in body fluids of humans and animals and have been known for their demonstrated anticarcinogenic properties.[7,8] Owing to the great significance of SDG as a bioactive compound with potential application in pharmacy and nutrition, the appropriate analysis of SDG in flaxseed will play an essential part of any research involving the efficacy, the safety, and therapeutic reproducibility of the consumption of flaxseed.

Several methods, based mainly on thin-layer chromatography (TLC),[9] gas chromatography (GC),[10] high performance liquid chromatography (HPLC),[11–14] HPLC mass spectrometry (HPLC-MS),[15] HPLC nuclear magnetic resonance spectroscopy (HPLC-NMR),[16] have been developed to monitor and quantitate SDG in flaxseed. Results from these studies have been inconsistent, probably owing to the differences in methodology, most of them using complex sample pretreatment. It is well known, that the uncertainty of an analytical method is dominated by the repeatability of the sample preparation including extraction from the matrix and cleanup step, each involving risks for contamination, loss of sample, and unexpected reaction of the lignans. SDG in flaxseed may require special pretreatment before the analysis, since SDG is present in the plant as ester-linked oligomers or even polymers, consisting of SDG and hydroxymethyl glutaric acid (HMGA).[17,18] This oligomeric or polymeric lignan derivative appears to be readily soluble in aqueous methanol or ethanol, but alkaline hydrolysis is subsequently needed to release free SDG. Although several sample preparation procedures, including various steps and combinations of enzymatic,[19] acidic,[20,21] and alkaline hydrolysis,[13] before or after extraction, have been developed for analyzing SDG in food and feed, the methodologies reported are variable and factors influencing the extraction efficiency have not been sufficiently investigated.

Despite limited information to elucidate molecular structure, the HPLC-UV method is still the most popular technique with its rapidity, simplicity, and convenience.[22–24] In this work, a HPLC-UV method was developed for the analysis of secoisolariciresinol diglucoside (SDG) in

flaxseed, and several factors influencing the extraction efficiency including extraction solvent, extraction time, extraction method, and alkali concentration and the reaction temperature and time of alkaline hydrolysis, were thoroughly investigated. The optimized method was used to study the variation in the content of SDG in flaxseed samples from 100 different locations in Gansu province, China.

EXPERIMENTAL

Chemicals

The reference standard, secisolariciresinol diglucoside (SDG) (structure shown in Figure 1), was the kind gift from Gansu Institute of Agriculture Science, China. A total of 100 sets of flaxseed were collected from different locations in Gansu province. Acetonitrile was of chromatographic grade and purchased from Institute for the Chemical Engineering of Huaiyin Plastic Product Factory (Jiangsu, China). Distilled and deionized water was used for the preparation of all samples and solutions. Other chemicals were of analytical grade and purchased from Tianjing Chemical Reagent Corportion (Tianjing, China).

Apparatus and Chromatography

The HPLC system (Milford, MA, USA) consisted of a Waters quaternary pump (Model Delta 600E), a photodiode array detector (Model 2996), and a

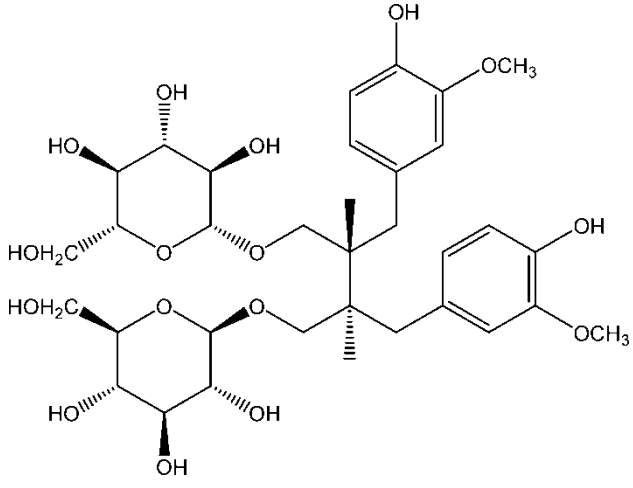

Figure 1. Structure of secoisolariciresinol diglucoside (SDG; 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butane-diglucoside).

manual injector. The chromatographic data were recorded and processed with Waters Millennium[32] software. The chromatographic separation of analytes was performed on a Kromasil C_{18} column (5 μm, 4.6 mm × 250 mm i.d.) (Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian, China). Compounds were eluted by a degassed mobile phase consisting of acetonitrile-1% aqueous acetic acid (15:85, v/v) at a flow rate of 1.0 mL/min. The analysis was monitored at 280 nm, and absorption spectra of the compounds were recorded between 200 and 400 nm. Helium (He) was used for degassing the mobile phase. The temperature of the column during analysis was maintained at 25°C. The injection volume was 10 μL each time.

Standard Solution Preparation

The reference standard of 6.4 mg SDG was accurately weighed and transferred into a 5 mL volumetric flask and dissolved in methanol to make a stock solution. The stock solution was stored at 4°C and brought to room temperature before use. Calibration standard working solutions were freshly prepared by appropriate dilution of the stock solution with the concentration of 1.28 mg/mL, 0.32 mg/mL, 0.08 mg/mL, 0.02 mg/mL, 0.01 mg/mL, 0.005 mg/mL, and 0.00125 mg/mL, respectively.

Sample Solution Preparation

Alkaline Hydrolysis of SDG

Experimental designs were created to find conditions of alkaline hydrolysis giving the highest yields of SDG. The effect of hydrolysis temperature, time, and aqueous sodium hydroxide (NaOH) concentration on the yield of SDG was studied, using a full factorial experimental design involving four temperatures (20, 40, 60, 80°C), five time periods (0.5, 1, 2, 4, and 12 h), and four concentrations of alkali (0.3, 0.5, 1, and 2 M) with two replicates.

The procedure was as follows: Flaxseed (10 g) was ground to pass through a 20 mash screen and defatted with 500 mL of *n*-hexane at room temperature in an ultrasonicator for 30 min. The *n*-hexane was removed by filtration and the defatted powder allowed to air dry. The defatted flaxseed was extracted with 200 mL of methanol in an ultrasonicator at room temperature. The methanol extract was decanted from the flaxseed and the wet flaxseed was re-extracted with another 200 mL of methanol. The methanol extract was separated from the flaxseed by filtration. The extracts were combined and concentrated to give a final volume of 200 mL with a rotary evaporator in a partial vacuum. An aliquot of the resulting solution (10 mL) was evaporated to dryness and was subjected to a base hydrolysis according

to the above experimental designs, and then acidified with 2 mol/L sulfuric acid to pH 3. The resulting hydrolysis solution was evaporated to dryness and the residue was dissolved in 10 mL of methanol, then filtered through a 0.45 μm filter membrane, and prepared for HPLC analysis.

Extraction Procedure of SDG from the Matrix

The influence of the extraction method on the extraction efficiency of SDG in flaxseed was investigated. Flaxseed (0.5 g) was ground and defatted with 50 mL of *n*-hexane at room temperature, in an ultrasonicator for 30 min. The defatted flaxseed was extracted twice using two different methods, i.e., reflux and ultrasonic extraction, respectively. The extract was filtered and evaporated to dryness and was subjected to a base hydrolysis with 10 mL of 1 mol/L aqueous sodium hydroxide for 1 h at 40°C, and then acidified with 2 mol/L sulfuric acid to pH 3. The following hydrolysis solution was evaporated to dryness and the residue was dissolved in 10 mL of methanol, then filtered through a 0.45 μm filter membrane and subjected to HPLC analysis.

The influence of extraction solvent on the extraction efficiency of SDG in flaxseed was investigated. The fladseed (0.5 g) was ground and defatted according to the above procedure. The defatted flaxseed was extracted twice with 50 mL of various solvents, involving pure and aqueous methanol (80%, 70%, 60%, 50%), pure and aqueous ethanol (80%, 70%, 60%, 50%), and 1, 4-dioxane–95% ethanol (1:1, v/v), respectively, and then followed by the same procedure described above.

The influence of extraction time on the extraction efficiency of SDG in flaxseed was also investigated. Four sets of 0.5 g of defatted flaxseed was refluxed 1, 2, 3, and 4 times, respectively, (every 60 minutes) with 50 mL 60% methanol, and then followed by the same procedure described above.

Extraction by Direct Alkaline Hydrolysis[13]

Defatted flaxseed, 0.5 g, was continuously mixed with 4 mL distilled water and 5 mL 2 M aqueous sodium hydroxide for 1 h at 20°C by constant rotation. The hydroxide was acidified to pH 3 using 2 M sulfuric acid, and centrifuged for 10 min (2000 r/min). The supernatant was centrifuged in microcentrifuge tubes to a clear liquid phase. The liquid phase (1 mL) was mixed with 95% ethanol (9 mL) in 10 mL microcentrifuge tubes, left at room temperature for at least 10 min, and centrifuged for 10 min (2000 r/min), to precipitate and remove water soluble polysaccharides and proteins. The supernatant was filtrated through 0.45 μm filter membranes and prepared for analysis.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

SDG showed maximum absorbance at 220 nm and 280 nm, which is in accordance with the reports. To avoid the influence of acetic acid at 220 nm and obtain much higher sensitivity, 280 nm was selected as the detection wavelength in the subsequent studies.

According to the literature, owing to the phenolic feature of SDG, elution was made with an acidified eluent, which allowed for a satisfactory separation. To improve the peak shape (restrain the peak tailing), formic acid, phosphoric acid, and acetic acid were added as a mobile phase modifier to inhibit the dissociation of the phenolic hydroxyl group of SDG. The preliminary studies indicated that acetic acid provided more efficient separation.

Various proportions of acetonitrile, ranging from 60% to 15% (v/v) were tested for separating SDG. It is shown that the retention time of SDG increased and the resolution of SDG and the other unknown compounds were improved with decreasing acetonitrile concentration. When a proportions of acetonitrile concentration decreased to 15% (v/v), they could be completely separated. Therefore, acetonitrile-1% acetic acid (15:85, v/v) was used as the mobile phase to achieve the best separation efficiency and shorten the analytical time.

Linearity

The calibration was based on the five duplicate analyses of the working solutions of SDG at seven concentration levels in the range of 12.8–0.0125 μg. The regression equation and its correlation coefficient were calculated as follows: $y = 178630.0562\ x + 3036.9515$, $r = 0.9999$, where y is peak area and x is concentration of SDG. The limit of detection (LOD) of SDG was 6.25 ng at a signal-to-noise ratio of three. The limit of quantitation (LOQ) was 12.5 ng.

Method Validation

The reproducibility of the procedure was obtained from the relative standard deviation (RSD) of retention time and peak area calculated for five replicated injections of a standard solution. The values were 0.17% and 0.46%, respectively.

The stability of the assay was evaluated by intra-day variability. The standard solution was analyzed on five consecutive days, and the RSDs of retention time and peak area were all less than 5.0%.

The accuracy of the method was confirmed by analyzing the mixture prepared by adding suitable standard at five amounts levels, to the flaxseed

with a known content of SDG. The average recovery of SDG was 95.3% with an RSD of 0.67% (n = 5).

Evaluation of Alkaline Hydrolysis

The previous observation indicated that when aliquots of the unhydrolyzed extracts prepared from the flaxseed were analyzed by HPLC, no evidence of free SDG was detected, which suggested that SDG does not exist in a free form in flaxseed, but rather as an ester linked component of a polar complexity.[18] Therefore, a preliminary hydrolysis is necessary for the release of SDG from its complex forms. Although, several hydrolysis procedures have been used, the methodologies reported are variable, and hydrolysis factors influencing the yield of SDG have not been sufficiently investigated. The effect of alkaline hydrolysis after methanol extraction on the yield of SDG in flaxseed was determined by an experimental design. It is shown by Figure 2, that the yield of SDG reached the highest level at the temperature of 40° and 60°. The yield of SDG had already reached a high level after 1 h of hydrolysis and, then, with the hydrolysis time increasing continuously, the yield decreased. The aqueous sodium hydroxide concentration was also one of the influential factors on the yield of SDG in the procedure of hydrolysis. With the concentration of aqueous sodium hydroxide increasing from 0.1 mol/L to 1 mol/L, the yield of SDG increased and reached the highest level at 1 mol/L. So, SDG was subjected to an alkaline hydrolysis with 10 mL of 1 mol/L aqueous sodium hydroxide for 1 h at 40°C.

Evaluation of Extraction Efficiency

Contents of SDG from the different resulting solutions, which were prepared by different extraction procedures mentioned above, were calculated according to the equation for the calibration curve, and the extraction efficiency was compared.

The effect of extraction method, solvent, and time on the extraction efficiency is summarized in Table 1 and Figure 3. Whether in methanol and ethanol extract, or in 1, 4-dioxane–95% ethanol (1:1, v/v) extract, the content of SDG with reflux is higher than that with ultrasonic extraction, which suggests that reflux is appropriate for the extraction of SDG from the matrix. However, in the literature, 80%, 70%, 50% aqueous methanol or even ethanol, 1, 4-dioxane–95% ethanol (1:1, v/v), were used as extraction solvents for SDG in flaxseed. The result obtained in Table 1 depicts that the content of SDG using 60% methanol as the extraction solvent is higher than those extracted by the other solvents under the reflux condition, which indicates that 60% methanol has a better extraction power than the other solvents. It was shown by Figure 3 that after refluxing twice, the content of

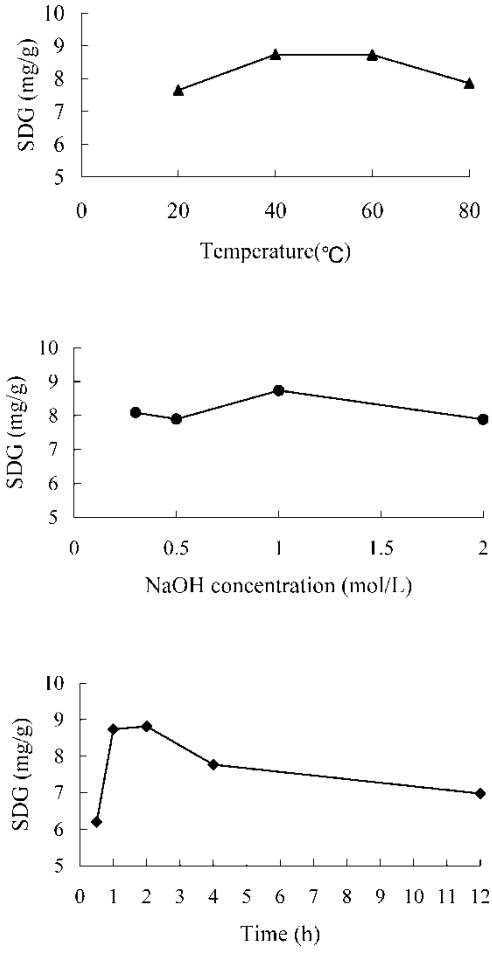

Figure 2. Effect of temperature (▲), time (◆), and NaOH concentration (●) on the yield of SDG in the procedure of alkali hydrolysis.

SDG almost did not increase with increasing extraction time, which indicates that SDG could be extracted completely after refluxing twice. The result of the recovery test also demonstrated that the extraction method was adequate and appropriate for the analysis.

Comparison of Alkaline Hydrolysis after Extraction with Direct Alkaline Hydrolysis

The result showed the yield of SDG obtained by direct alkaline hydrolysis (18.13 mg/g) was also equal to the yield obtained by alkaline hydrolysis

Table 1. Effect of extraction method and solvent on the yield of SDG (mg/g)

	Extraction method	
Extraction solvent	Ultrasonic	Reflux
Methanol	1.50	4.38
80% Methanol	4.45	7.84
70% Methanol	8.80	16.06
60% Methanol	8.38	18.02
50% Methanol	7.41	16.60
Ethanol	Undetected	2.50
80% Ethanol	6.36	8.99
70% Ethanol	6.94	15.27
60% Ethanol	6.71	14.76
50% Ethanol	6.37	14.01
1,4-Dioxane–95% ethanol (1:1)	6.19	14.16

after 60% methanol extraction (18.02 mg/g). However, relatively, the alkaline hydrolysis is simpler and faster.

Quantitation of SDG in Flaxseed

The optimum method was applied to the analysis of SDG in extracts of flaxseed from 100 different locations. The desired compound from flaxseed was identified by comparing both the retention times and UV spectra of SDG with that of the reference standard. The analyte was further confirmed by spiking the standard in the actual sample. To check the peak purity of the component, the eluates were monitored with the PDA detector at 200–400 nm. The three spectra corresponding to the up slope, apex, and down slope of each peak were normalized and superimposed. Peaks were considered pure when there was an exact coincidence among the three spectra. In addition, the peak

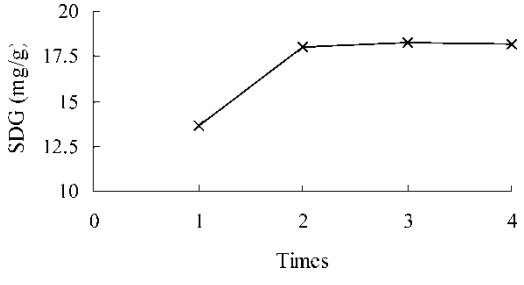

Figure 3. Effect of extraction times on the yield of SDG.

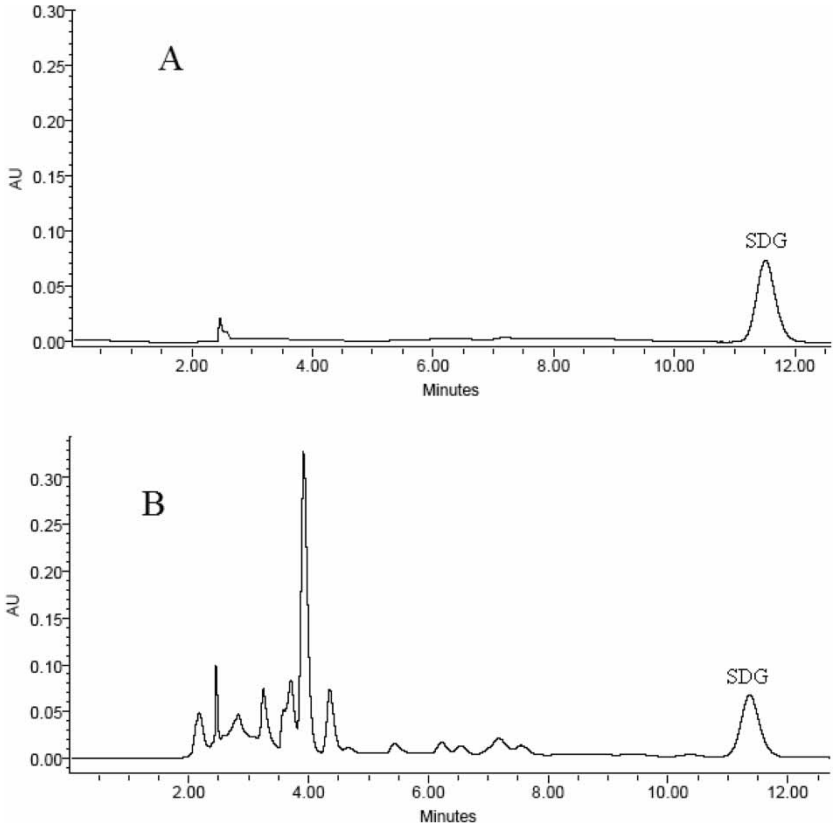

Figure 4. Chromatograms of standard (A) and the extract of flaxseed (B). Mobile phase: acetonitrile-1% acetic acid (15:85, v/v). Detection wavelength: 280 nm.

purity angle theory proposed by Waters Millennium[32] software was used to check the peak purity. Figure 4 illustrates the typical chromatograms of SDG along with the extract from flaxseed. The SDG and the other unknown compounds were well resolved within 11 min under the optimum condition described above. The obtained results showed that the amount of SDG present in these various samples is significantly different at the range of 5.67–12.96 mg/g. The large variation of the specific component concentrations is probably attributed to different locations, which demonstrate, once again, the importance and necessity of quality control of plant products.

CONCLUSION

A method was developed for the analysis of secoisolariciresinol diglucoside (SDG) in flaxseed. The analytical method involves extraction of defatted

flaxseed with 60% methanol, a base hydrolysis with 10 mL of 1 mol/L aqueous sodium hydroxide for 1 h at 40°C, and quantitative determination by high performance liquid chromatography (HPLC). The method was then applied to study the variation of SDG content in flaxseed from 100 cultivars in Gansu province, China. The SDG content varied between 5.67 and 12.96 mg/g.

ACKNOWLEDGMENTS

The work was supported by National Natural Science Foundation of China (No. 20475057) and Natural Science Foundation of Gansu Province (No. 3ZS051-A25-077).

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Received September 25, 2006
Accepted October 22, 2006
Manuscript 6949