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Journal of Chromatography A, 1045(2004) 217–222
JOURNAL OF CHROMATOGRAPHY A
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High-performance thin-layer chromatographic–densitometric determination of secoisolariciresinol diglucoside in flaxseed☆

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Received 4 May 2004; accepted 14 June 2004

Abstract

A HPTLC–densitometric method, based on an external standard approach, was developed in order to obtain a novel procedure for routine analysis of secoisolariciresinol diglucoside (SDG) in flaxseed with a minimum of sample pre-treatment. Optimization of TLC conditions for the densitometric scanning was reached by eluting HPTLC silica gel plates in a horizontal developing chamber. Quantitation of SDG was performed in single beam reflectance mode by using a computer-controlled densitometric scanner and applying a five-point calibration in the 1.00–10.00 μg/spot range. As no sample preparation was required, the proposed HPTLC–densitometric procedure demonstrated to be reliable, yet using an external standard approach. The proposed method is precise, reproducible and accurate and can be employed profitably in place of HPLC for the determination of SDG in complex matrices.

Keywords: Flaxseed; Secoisolariciresinol diglucoside; Glucosides

1. Introduction

Phytoestrogens, plant compounds that can interfere with oestrogen metabolism in humans and animals, have gained interest as dietary factors related to chronic diseases. Studies have indicated a relationship between phytoestrogens and hormone-dependent cancers [1]. Among these compounds there are the lignans enterodiol and enterolactone, produced in man from plant precursors of dietary origin by intestinal bacteria [2]; abundant literature exists on their discovery and their beneficial effects in humans [3–7]. Flaxseed has been found to be the richest source of the main precursor of these mammalian lignans reported as being secoisolariciresinol found in its glucosidic form, secoisolariciresinol diglucoside (SDG, Fig. 1), complexed in a polymeric ester structure not thoroughly known [8–13]. Several methods, based mainly on HPLC, have been developed to monitor and quantitate SDG content in flaxseed [14–22]. Results from these studies have been inconsistent probably due to differences in methodology, most of them using complex sample preparation. It is well known that the uncertainty of an analytical method is dominated by the repeatability of the sample preparation especially in the clean-up step. By a methodological point of view in these cases, procedures using internal standard or involving a minimum of sample manipulation are highly desirable. In a previous work [16] we proposed a simple GC internal standard method that could reliably quantitate SDG with accuracy and precision.

In this study an HPTLC method was considered, being an important feature of TLC the disposable stationary phase, therefore neither regeneration nor essential clean-up are required. Moreover, the progress in instrumentation has led HPTLC–densitometry to an improvement of the reliability making this technique competitive with respect to HPLC–UV detection so that a HPTLC–densitometric approach should be taken into consideration as an alternative to HPLC whenever it is hinted by the sample features. Accordingly, a simple

☆ Presented at the 15th International Symposium on Pharmaceutical and Biomedical Analysis, Firenze, Italy, May 2004.

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doi:10.1016/j.chroma.2004.06.042

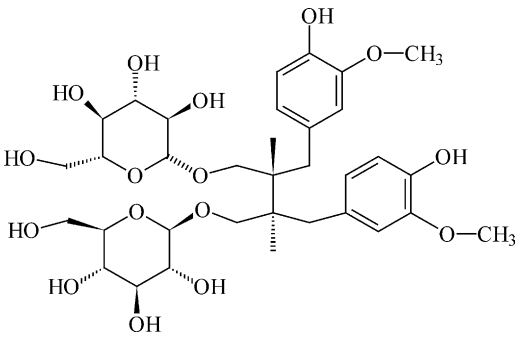

Fig. 1. Structure of secoisolariciresinol diglucoside (SDG; 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediyl bis-[R-(R*, R*)]-β-D-glucopyranoside).

HPTLC–densitometric method was developed in order to successfully quantitate SDG in defatted as well as in undefatted flaxseed.

2. Experimental

2.1. Apparatus

Densitometry was carried out with a Camag TLC Scanner 3 (Camag, Muttenz, Switzerland) fitted with a winCATS 1.2.3 software.

Samples were applied using the spray-on technique with a Camag Linomat 5 and developed in a Camag 10 cm × 10 cm horizontal chamber.

HPLC was performed with a Perkin-Elmer LC200 Series apparatus consisting of a quaternary pump with autosampler, a diode array UV–VIS detector and a Peltier column oven, under the control of Totalchrom 6.1 data handling software (Perkin-Elmer, Shelton, CT, USA).

2.2. Materials

Two types of flaxseed, Fink Linusit Gold (A), and Probios Flaxseed Gold (B) were purchased locally.

Pure SDG standard was obtained as previously described [23].

HPTLC precoated plates, silica gel Merck 60, F 254, 10 cm × 10 cm were used (Merck, Darmstadt, Germany).

All chemicals and solvents were of analytical grade and used as obtained.

2.3. Standard solution

For HPTLC purpose a SDG standard solution, containing 0.25 mg/mL, was prepared in a 10 mL volumetric flask by dissolving 2.5 mg of pure SDG in 1 mL of water and then diluting to volume with methanol.

2.4. Accuracy and assay procedure

Three solutions (containing 0.34, 0.45 and 0.60 μg/μL of pure SDG) were obtained individually spiking in 10 mL volumetric flasks, 1 mL of 0.1 M NaOH with 3.40, 4.55 and 6.05 mg of SDG, respectively, acidifying to pH 3 with 0.1 M HCl and diluting to volume with methanol. 10 μL of each solution were applied to the plates.

For the assay the following four samples were prepared.

100 mg of ethanol–1,4-dioxane extract of defatted flaxseed A (dry matter, obtained as previously described [9]) were subjected to alkaline hydrolysis with 3 mL 0.1 M NaOH at 40 °C for 2 h; then 0.5 mL of the hydrolysate were poured in a volumetric flask, acidified to pH 3 with 0.1 M HCl and diluted to 10 mL with methanol. For the assay 8 μL of this solution were applied.

500 mg of flaxseed A and 500 mg of flaxseed B were frozen in liquid N_2 and finely ground. Defatting was omitted and A and B were individually hydrolysed with 6 mL 0.1 M NaOH in the above described conditions. 1.0 mL of each hydrolysate was poured in a volumetric flask, acidified to pH 3 with 0.1 M HCl and diluted to 10 mL with methanol. For the assay 8 μL of each solution were applied.

500 mg of solvent-free defatted meal of flaxseed A were treated as above. For the assay 8 μL of this solution were applied.

2.5. Chromatography

Standard and sample solutions were applied bandwise (bandlength 6 mm, 70 nL/s delivery speed, track distance 8.4 mm, distance from left edge 12 mm) to the HPTLC plates. Plates were developed at room temperature in a Camag horizontal developing chamber in sandwich configuration with ethyl acetate–methanol–water–formic acid (77.0:13.0:l0.0:5.0, v/v) as mobile phase. The analyte was stable on the sorbent surface during development. The plates were scanned within 2 h; afterwards a progressive degradation was observed.

HPLC measurements were carried out using a 100 mm × 4.6 mm Chromolith Performance RP18 column (Merck) operated at 20 °C isocratically at 2.5 mL/min with a mobile phase of 0.01% aqueous formic acid–0.01% formic acid in acetonitrile (85:15, v/v); detection: UV absorbance at 282 nm; analysis time: 5 min.

2.6. Densitometry

The HPTLC plates were scanned in reflectance mode at 282 nm, D2 & W lamp, monochromator bandwidth 10 nm, slit dimensions 4.00 mm × 0.30 mm, scanning speed 5 mm/s, data resolution 25 μm/step.

3. Results and discussion

3.1. Specificity

Pure SDG and idrolysate specimens of the four samples considered were chromatographed simultaneously side by side. A good separation of SDG from other components was

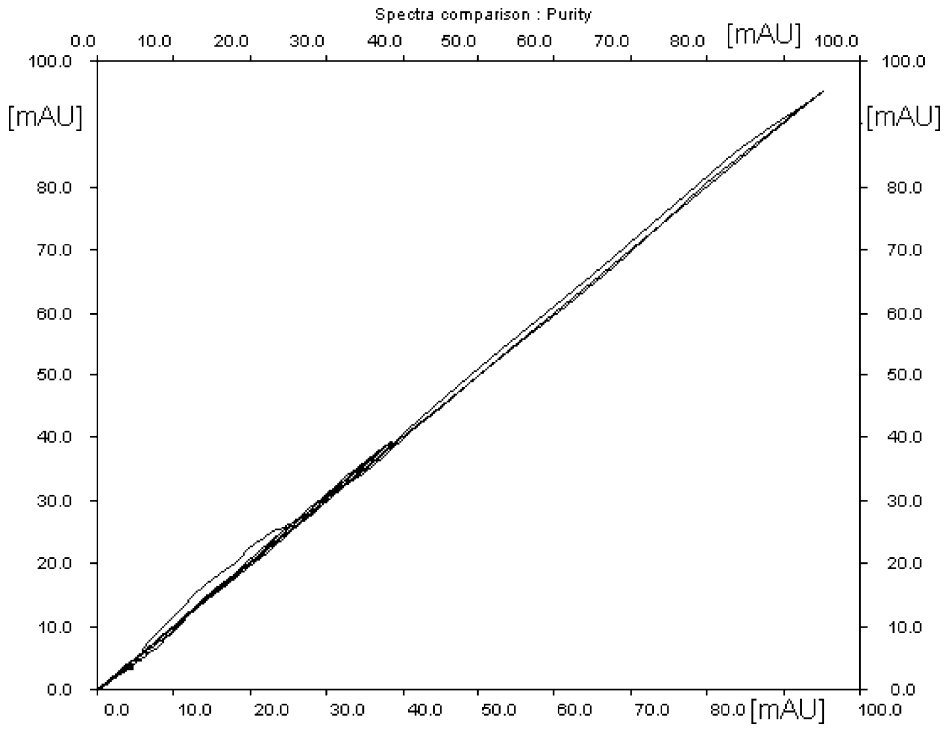

Fig. 2. Peak-purity correlation display of SDG present in the densitogram of undefatted flaxseed A (see Fig. 3d).

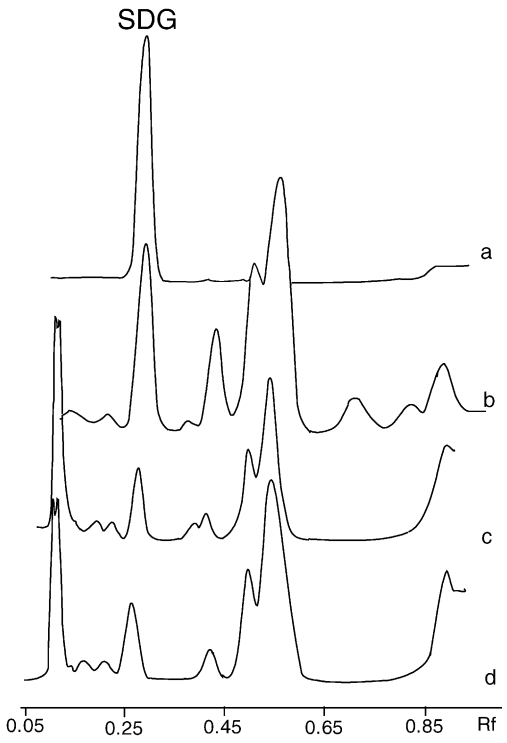

Fig. 3. HPTLC densitograms of SDG standard (a) and hydrolysis product of ethanol–1,4-dioxane extract of flaxseed A (b), defatted flaxseed A (c), undefatted flaxseed A (d).

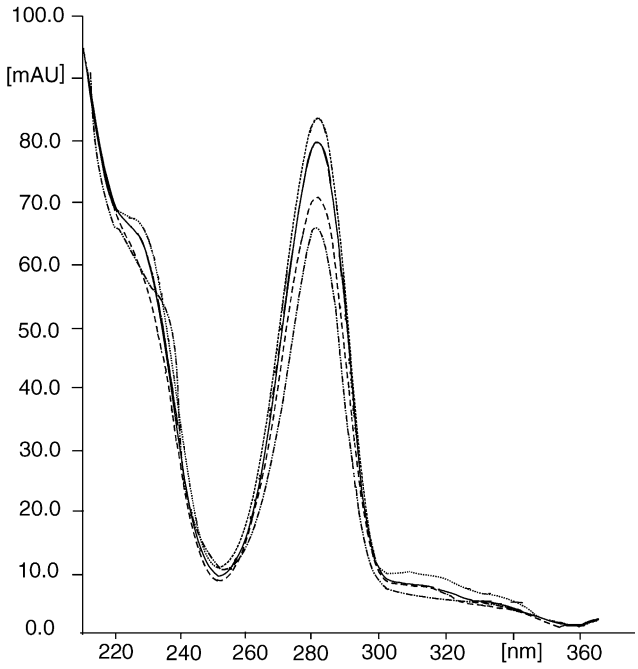

Fig. 4. UV spectra of standard SDG and SDG present in the hydrolysed samples obtained by HPTLC spot scanning from 200 to 380 nm.

achieved (R_f = 0.29, $A_{0.05}$ = 0.98). WinCATS software afforded automatic calculation of SDG peak purity in each sample by comparing the overlaid spectra measured within the SDG peak in both the peak-flanks and at peak-maximum. As an example, the resulting peak purity correlation display of SDG from undefatted flaxseed A is reported in Fig. 2.

Table 1
Precision and acccuracy from the calibration curve validation experiments

Calculated (μg)	Michaelis–Menten calibration			Polynomial calibration			Linear calibration		
	Found (μg) (mean, n = 3)	RSD (%)	Bias (%)	Found (μg) (mean, n = 3)	RSD (%)	Bias (%)	Found (μg) (mean, n = 3)	RSD (%)	Bias (%)
3.25	3.20	1.62	−1.54	3.32	1.66	2.15	3.76	2.09	15.6
5.50	5.59	1.80	1.60	5.61	1.85	2.00	5.52	1.80	0.40
8.25	8.21	1.80	−0.50	8.05	1.80	−2.42	8.35	2.22	1.21

Table 2
Accuracy and repeatability of the method

Applied (μg)	Found (mean, n = 3) (μg)	RSD (%)	Bias (%)
3.4	3.42	0.29	0.6
4.5	4.56	2.09	1.3
6.0	6.25	2.26	3.6

Table 3
In situ precision at 1.00 and 5.50 μg levels

Determination (6 replicates)	Scanning runs	Area counts mean (1.00 μg)	RSD (%)	Area counts mean (5.50 μg)	RSD (%)
1	6	4882.67	0.31	20058.08	0.15
2	6	4935.85	0.17	19986.08	0.17
3	6	5072.81	0.52	19443.62	0.23
4	6	5161.39	0.17	19780.38	0.20
5	6	5186.04	0.38	20025.30	0.22
6	6	5093.05	0.25	19580.36	0.17
1–6	36	5055.30	2.41	19812.30	1.29

Table 4
Assay for SDG in real samples

Sample	Found (mg*) (n = 3)	RSD (%)
Undefatted flaxseed A	14.15	2.21
Defatted flaxseed A	13.95	2.00
Ethanol–1,4-dioxane extract of defatted flaxseed A	8.16	2.03
Undefatted flaxseed B	17.95	2.55

* Referred to 1 g of undefatted dry matter.

The selectivity of the separation and the specificity of the detection were shown by the densitograms and comparison of spectra, respectively (Figs. 3 and 4).

3.2. Linearity

Fig. 3a shows the densitogram of the standard SDG to be used for calibration. It was obtained after accurate optimization of the operative conditions chiefly affected by the sample delivery speed, slit dimensions and scanning speed. For proving linearity five level calibration points were obtained in triplicate over a range of 1.00–10.00 μg of the analyte, by applying 4, 13, 22, 33 and 40 μL of the standard solution. As often it occurs in TLC, in the case of determination by scanning in reflection mode, a quasi-linear correlation (R^2 = 0.97558; sdv = 8.03) was obtained. If the range was reduced (3.50–10.00 μg) the equation for the curve y = 1909x + 7852 (n = 15) could be calculated by linear regression analysis assuring method linearity with correlation coefficient R^2 = 0.9951. As the concentration of SDG in natural matrices was not predictable, it was preferred a calibration in the wider range (1.00–10.00 μg) based on a Michaelis–Menten regression optimization, $y = -890.97 + (50527.45 + x)/(9.07x)$, that showed to give very low residual standard deviation of the standard points (sdv = 1.15) and good accuracy.

No significant day-to-day variability was observed.

For routine a 3-point calibration curve, covering the working range, was used as described in the monograph of the European Pharmacopoeia [24]

3.3. Accuracy and precision

Accuracy was assessed by comparing the results with the analysis of the standard reference material over the range of

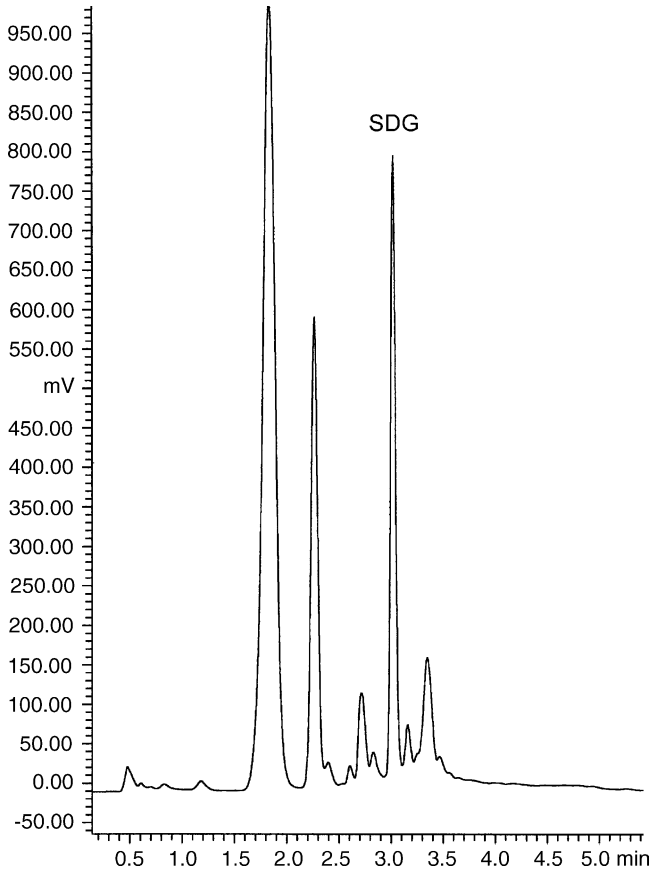

Fig. 5. Typical HPLC chromatogram (monolithic column) of the hydrolysis product from ethanol–1,4-dioxane extract of flaxseed A recorded at 282 nm by diode array detector.

80–120% of the amount corresponding to the mid point of the curve. The accuracy measurements were performed both with Michaelis–Menten, polynomial and linear regression, demonstrating the better fit and validity of Michaelis–Menten calibration (Table 1).

Accuracy assessed with the method of standard additions at three concentration levels (nine replicates) gave good results with bias ranging from 0.6 to 3.6%. The repeatability data were available from the replicate analyses of the samples in the accuracy study (Table 2).

The instrumental precision was also determined at all levels (6 replicates and 6 scanning runs) giving RSD values within 1.29% (third level) and 2.41% (lowest level). Data for the lowest and middle points are reported in Table 3.

3.4. Assay

The method was tested by analysing the content of SDG in the four samples considered. The statistical analysis of the results is reported in Table 4.

It is to be noted that the SDG determination is unaffected by the massive presence of the linseed oil (defatted versus undefatted flaxseed A).

Here again, the levels of SDG detected in the alkaline hydrolysis of whole seeds are higher than in the extract, as previously reported [16,22].

3.5. HPTLC–densitometry versus HPLC

The hydrolysis product of ethanol–1,4-dioxane extract of defatted flaxseed A was also analysed by HPLC. The chromatogram shows that a very satisfactory resolution of the components can be obtained in a short time using a modern monolithic HPLC column. SDG appears well resolved among the other glucosides (Fig. 5). However, these results are restricted to defatted samples. On the other hand, whole undefatted samples are freely analysed by the present procedure, which represent a considerable advantage in terms of simplicity and rapidity of execution over the widespread HPLC approach.

4. Conclusion

The developed HPTLC–densitometric procedure fits precision and accuracy usually requested by official methods and can be used for the direct quantitative determination of SDG both in simple and in complex matrices being reliable yet using an external standard approach. Chromatography of all samples takes only 5 min. All in all, including application and evaluation, the analysis of 14 samples requires about 42 min, i.e. 3 min per sample compared with 30–45 min using HPLC methods recently reported in the literature [17,21].

In conclusion, rapidness, high-throughput and cost-effectiveness of planar chromatography should be preferred in this case, especially for routine applications, to the comparatively more time-consuming and cost intensive HPLC not least because of exacting steps in sample preparation which can be omitted as solvent defatting of flaxseed meal.

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