View metadata, citation and similar papers at core.ac.uk
brought to you by CORE

Acta Pharm. **68** (2018) 97–107
https://doi.org/10.2478/acph-2018-0002

Short communication

HPTLC determination of diosgenin in fenugreek seeds

BARBARA KRÓL-KOGUS[1]
KHENIFI MOHAMMED LAMINE[2]
PIOTR MIGAS[1]
MESSAOUD BOUDJENIBA[2]
MIROSŁAWA KRAUZE-BARANOWSKA[1*]

[1] *Department of Pharmacognosy with Medicinal Plants Garden Medical University of Gdańsk 80-416 Gdańsk, Poland*

[2] *Laboratoire d'Ethnobotanique et Substances Naturelles Ecole Normale Supérieure de Kouba B.P 92 Kouba, 16050 Algerie*

Accepted October 15, 2017
Published online November 3, 2017

A new HPTLC-densitometric method for diosgenin determination in fenugreek seeds was established after optimization of the conditions for efficient saponin extraction and acid hydrolysis. Several procedures were tested, the best of which was a three-step Soxhlet extraction, followed by hydrolysis of the obtained methanolic extract with 2 mol L^{-1} H_2SO_4. Best diosgenin separation from other hydrolysis products was obtained on HPTLC $Si60_{F254}$ plates using a mixture of *n*-heptane/ethyl acetate (7:3, *V*/*V*) and modified anisaldehyde as a spraying reagent. The method was preliminarily validated and the determined amounts of diosgenin in fenugreek seeds of Polish and African origin were found to be similar and ranged from 0.12–0.18 %.

Keywords: fenugreek (*Trigonella foenum-graecum)*, diosgenin, HPTLC, quantitative analysis

Fenugreek seeds are a well-known source of different steroidal saponins of both spirostane and furostane type. These compounds are derivatives of several aglycones, the most abundant of which is diosgenin (5,25-α-spirostan-3β-ol) (Fig. 1) (1). Diosgenin possesses a number of biological activities, including anticancer, hypolipidemic, anti-inflammatory and antidiabetic ones (2). It also plays a beneficial role in the cardiovascular system and reduces bone loss in osteoporosis (2). On the other hand, the influence of

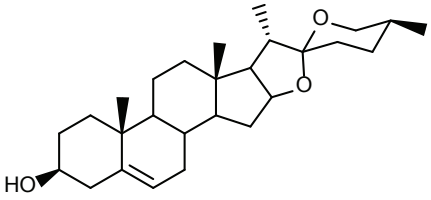

Fig. 1. Chemical structure of diosgenin (5,25-α-spirostan-3β-ol).

* Correspondence; e-mail: krauze@gumed.edu.pl

diosgenin and other steroidal saponins on human sex hormones remains vague and research results are ambiguous or even inconsistent (3). Nowadays, apart from being applied internally in the form of gruel or externally in the form of cataplasms, fenugreek seeds are also a constituent of many dietary supplements with antidiabetic, nutritious, strengthening and anti-acne properties. As these preparations are getting increasingly popular, the industry faces new challenges connected not only with effectiveness, but also with safety of the initial raw herbal material. As for multidirectional biological activity of diosgenin, reliable analytical methods for its determination in fenugreek seeds are needed.

It has been shown that the level of diosgenin found in plant material depends not only on cultivation or storage conditions (4) but is also associated with the analytical procedure used (5). Sapogenin analysis in plant material is confronted with several difficulties such as the presence of ballast compounds, insufficient extraction and acid hydrolysis or formation of artifacts (1, 5, 6). Therefore, the conditions of extraction and acid hydrolysis of steroidal saponins from fenugreek seeds have to be optimized.

For diosgenin quantification in plant extracts, several chromatographic methods were established (6–10). As HPLC and GC require specialized and expensive equipment, TLC remains a good alternative and hyphenated with densitometry provides a useful tool for its quantification in plant material.

The aim of this work was to establish a TLC-densitometric method for the estimation of diosgenin content in fenugreek seeds of Polish and African origin. Different analytical procedures were tested and compared. The best methods for extraction, acid hydrolysis and TLC determination of diosgenin in the analyzed plant material were established.

EXPERIMENTAL

Chemicals

Ethyl acetate, acetone, methanol, ethanol, chloroform, *n*-heptane, cyclohexane, phosphomolybdic acid, acetic acid, isopropanol and sulphuric acid were obtained from POCH (Polskie Odczynniki Chemiczne, Poland). Anisaldehyde, vanillin, phosphoric acid and antimony chloride were from Sigma-Aldrich (USA) and *n*-hexane was from J. T. Baker (India). Reference substance, diosgenin (5,25-α-spirostan-3β-ol, 100 % purity) was obtained from PhytoLab (Germany).

Plant material

The seeds of *Trigonella foenum-graecum* L. (Fabaceae) were obtained from three Polish suppliers (Lewandowski, Kawon, Flos). African fenugreek seeds were harvested in spring (April, 2012) from the Bin El Ouiden region (north-eastern Algeria).

Sample preparation

Dried fenugreek seeds (2.0 g) were pulverized, extracted and hydrolyzed according to different procedures from the literature (4, 6, 8, 10, 11) and are presented below. Two types

of seeds were analyzed: non-defatted and defatted by exhaustive extraction with petroleum ether and/or chloroform.

Defatted/non-defatted seeds were extracted with methanol under reflux (50 mL, 2 h, 80 °C) or exhaustively in a Soxhlet apparatus. Non-defatted seeds were also extracted with methanol by ultrasound extraction (10, 20 or 30 min, 10 mL, 25 °C). In addition, to test effective purification methods, the extract from non-defatted seeds was diluted to 100 mL with distilled water and defatted with *n*-hexane (3 × 50 mL) according to a modified method of Kim *et al*. (11).

Acidic hydrolysis

Dried defatted/non-defatted fenugreek seeds and the obtained methanol extracts were hydrolyzed with 2 mol L^{-1} sulphuric acid in solutions: aqueous, 50 % methanol or 70 % isopropanol ($H_2SO_{4(aq)}$, $H_2SO_{4(MeOH)}$ and $H_2SO_{4(iso)}$, resp.) (2 h, 80 °C) or 2.5 mol L^{-1} sulphuric acid in ethanol or water (4 h, 80 °C). For 2.0 g of fenugreek seeds (or an equivalent amount of extract), 50 mL of a hydrolytic agent was used.

After acid hydrolysis, the hydrolyzates were cooled and aglycones were extracted with chloroform (3 × 50 mL). Combined chloroform extracts were concentrated under reduced pressure and diluted with chloroform to 10 mL in a volumetric flask.

Preparation of standard solutions

Diosgenin was dissolved in chloroform (1 mg mL^{-1}). Calibration curves were plotted using diosgenin standard solutions in the concentration range from 25 to 900 ng μL^{-1}.

TLC/HPTLC instrumentation and experimental conditions

Separations were performed in an Automatic Development Chamber ADC2 (Camag, Germany). Bands of the extracts (1 μL) and the standard (1 μL) were applied with the aid of an automatic sample applicator AS-30 (Desaga, Germany). After visualization, plates were heated on a Thermoplate S (Desaga).

Qualitative and quantitative analyses of diosgenin were performed on glass TLC $Si60_{F254}$ plates and HPTLC $Si60_{F254}$ plates (both 20 × 5 cm) (Merck, Germany), resp. Standards (1 μL, $n = 3$ for each concentration) and samples (1 μL, $n = 6$) were applied onto plates in a single cycle of application (application speed 15 s μL^{-1}) as 5-mm bands, placed 7 mm from the bottom edge and 10 mm from the lateral edge of the plate. The distance between bands was 10 mm. Chromatograms were developed using the mobile phase *n*-heptane/ethyl acetate (7:3, *V*/*V*) to a distance of 45 mm in a saturated ADC2 chamber (saturation time 10 min), at 22 ± 2 °C temperature and humidity of 47 ± 2 %. After drying (15 min), the separated bands were visualized by spraying with a modified anisaldehyde reagent and heating at 105 °C for 3 min. Densitometric analysis was performed 5 min after derivatization. Densitograms were obtained through linear scans at two wavelengths, namely, 426 and 590 nm. Densitometer parameters were: slit width 4 mm, slit height 0.02 mm, number of measurements per point 4, resolution of measurement 0.025.

In the optimization process, different stationary phases and mobile phases were tested (Table I).

Table I. The chromatographic systems (adsorbent type and mobile phase) tested

Mobile phase	Stationary phase	R_f
Chloroform (100 %) (multidevelopment)	TLC $Si60_{F254}$	0.1
Ethyl acetate (100 %)	TLC $Si60_{F254}$	0.88
Isopropanol (100 %)	TLC $Si60_{F254}$	0.87
Diethyl ether (100 %)	TLC $Si60_{F254}$	0.87
Butanol (100 %)	TLC $Si60_{F254}$	0.88
Methanol/water (4:1, *V*/*V*)	TLC $Si60_{F254}$ HPTLC RP-18	0.85 0.15
Chloroform/acetone (7:3, *V*/*V*)	TLC $Si60_{F254}$	NU[a]
Chloroform/acetone (8:2, *V*/*V*)	TLC $Si60_{F254}$	0.77
Chloroform/acetone (9:1, *V*/*V*)	TLC $Si60_{F254}$	0.7
Dichloromethane/acetone (9:1, *V*/*V*)	TLC $Si60_{F254}$	0.76
Cyclohexane/acetone (1:1, *V*/*V*)	TLC $Si60_{F254}$	0.87
Hexane/acetone (4:1, *V*/*V*)	TLC $Si60_{F254}$	0.43
Isopropanol/*n*-heptane (1:12, *V*/*V*)	TLC $Si60_{F254}$	0.1
Isopropanol/petroleum ether (1:12, *V*/*V*)	TLC $Si60_{F254}$	0.05
Hexane/ethyl acetate (4:1, *V*/*V*)	TLC $Si60_{F254}$	0.31
Hexane/ethyl acetate (6:1, *V*/*V*)	TLC $Si60_{F254}$ HPTLC RP-8 HPTLC Si60 DIOL	0.25 0.63 0.24
n-Heptane/ethyl acetate (1:1, *V*/*V*)	TLC $Si60_{F254}$	0.63
n-Heptane/ethyl acetate (1:2, *V*/*V*)	TLC $Si60_{F254}$	0.79
n-Heptane/ethyl acetate (2:1, *V*/*V*)	TLC $Si60_{F254}$	0.52
n-Heptane/ethyl acetate (3:1, *V*/*V*)	TLC $Si60_{F254}$	0.34
n-Heptane/ethyl acetate (4:1, *V*/*V*)	TLC $Si60_{F254}$	0.27
n-Heptane/ethyl acetate (7:3, *V*/*V*)	TLC $Si60_{F254}$ HPTLC$Si60_{F254}$ Lichrospher HPTLC $Si60_{F254}$ TLC $Si60_{F254}$ Lux Plate	0.47 0.47 0.37 0.41
n-Heptane/ethyl acetate (10:3, *V*/*V*)	TLC $Si60_{F254}$	0.30
Petroleum ether/ethyl acetate (7:3, *V*/*V*)	TLC $Si60_{F254}$	0.54
Chloroform/methanol/water (90:5:5, *V*/*V*/*V*)	TLC $Si60_{F254}$	0.61
Chloroform/methanol/water (13:6:2, *V*/*V*/*V*)	TLC $Si60_{F254}$ HPTLC RP-8	0.89 0.84
Butanol/water/acetic acid (4:1:1, *V*/*V*/*V*)	TLC $Si60_{F254}$	0.94

[a] NU – not useful, band heavily blurred

TLC $Si60_{F254}$ (particle size of 10–12 μm); HPTLC $Si60_{F254}$ (particle size of 5–6 μm); Lichrospher HPTLC $Si60_{F254}$ (spherical silica 60, particle size 7 μm); TLC $Si60_{F254}$ Lux Plate (silica gel 60 with a unique polymeric binder added, very adherent, hard surface resistant to damage, very smooth); HPTLC RP-8 (silica gel 60 with octyl groups); HPTLC RP-18 (silica gel 60 with octadecyl groups); HPTLC Si60 DIOL (silica gel 60 with cyano propyl groups).

Method validation

The established HPTLC method was preliminarily validated in terms of specificity, linearity, precision, limits of detection (*LOD*) and quantification (*LOQ*), and robustness. Validation parameters for two wavelengths, 426 and 590 nm, were compared. Validation was performed with the aid of the obtained hydrolyzate of the methanol extract from exhaustive extraction in a Soxhlet apparatus as well as using diosgenin standard solutions.

Specificity was checked by performing two-dimensional separation of 1 μL of the hydrolyzed methanol extract from the Soxhlet apparatus on a HPTLC $Si60_{F254}$ plate in the presence of the reference substance (1 μL). In both dimensions, the chromatogram was developed with the help of a *n*-heptane/ethyl acetate mixture (7:3, *V*/*V*). Spots of analyzed compounds were visualized by a modified anisaldehyde reagent (1 min, 105 °C).

For linear dependence of the peak area on diosgenin concentration, it was plotted for standard solutions ranging from 25 to 900 ng μL^{-1} applied onto an HPTLC $Si60_{F254}$ plate. After developing and visualizing the chromatogram, densitometric analysis was performed.

Instrumental precision, expressed as RSD (%), was determined by linear scanning of bands of both the standard solution (n = 9) and sample (n = 9) after derivatization using a modified anisaldehyde reagent at 426 and 590 nm. Intra-day precision was determined by comparing the results of three sample analyses made on the same day. Inter-day precision was tested by comparing the results of sample analyses made on three different days. Intermediate precision of the method was tested by performing slight changes in the procedure; analyses were made by two different analysts using chemicals from different containers, whereas robustness testing was based on small deliberate changes in mobile phase composition and development distance.

Limit of detection (*LOD*) was determined as the concentration of diosgenin for which *S*/*N* ratio was 3:1 and limit of quantitation (*LOQ*) as the concentration of diosgenin for which *S*/*N* ratio was 10:1.

RESULTS AND DISCUSSION

Despite several analytical conditions already reported for the analysis of diosgenin in plant material, our experiments revealed some difficulties in their applications for fenugreek seeds. During the application of TLC chromatographic conditions formerly described by different authors for diosgenin analysis (6–10), the separation of diosgenin from other hydrolysis products in the analyzed extracts of fenugreek seeds was not achieved. This was probably associated with the differences in plant matrix composition and, as a result, the presence of additional compounds in the obtained hydrolyzates. Therefore, the new method was established.

Separation conditions

Stationary phase. – Optimization of the stationary phase was performed by the use of various adsorbents based on silica gel or chemically modified silica gel (10, 12), as presented in Table I. Among the adsorbents tested, the HPTLC $Si60_{F254}$ plates were optimal for

diosgenin separation since on the obtained HPTLC chromatogram, the band of diosgenin was narrower compared to the TLC chromatogram and was therefore better separated from other hydrolysis products.

Mobile phase. – Among the solvents tested, a mixture of *n*-heptane/ethyl acetate was the best. Firstly, analyses were performed with the mixture of *n*-hexane/ethyl acetate, but replacement of *n*-hexane by *n*-heptane resulted in better repeatability of separations due to lower volatility of *n*-heptane and consequently better stability of the mobile phase composition. Increase of the *n*-heptane/ethyl acetate ratio (2:1, 7:3, 3:1, 4:1, 10:3, *V*/*V*) resulted in a decrease of R_f, with an additional increase of R_s. The solvent consisting of *n*-heptane/ethyl acetate in the ratio 7:3, *V*/*V*, provided the best separation of diosgenin (R_f = 0.47, R_s = 14.8) from an unknown hydrolysis product in the analyzed fenugreek seed extract.

Derivatization reagent for diosgenin visualization

Several spraying agents were compared for diosgenin visualization on the TLC chromatogram. Among them, the most promising results were observed when using aromatic aldehydes. However, visualization with vanillin in phosphoric acid and a basic solution of anisaldehyde was not specific enough, since these agents colored intensively not only the diosgenin but also the accompanying compounds other than sapogenins. Composition of anisaldehyde reagent was, therefore, modified by diluting the basic solution with a mixture of methanol and acetic acid (85:15, *V*/*V*) in 1:1 ratio, which significantly improved the reaction specificity, giving a green tint with diosgenin but less intense coloration with ballast compounds.

Extraction and acid hydrolysis

Extraction of steroidal saponins from plant material requires carefully assorted conditions because during this process several disadvantageous reactions may occur (enzymatic hydrolysis in the aqueous medium, transacylation, esterification of acidic saponins or hydrolysis of ester groups) (5). Water and alcohols (methanol, ethanol, butanol or isopropanol and their mixtures) are the most commonly used extraction solvents (5). However, the use of water and aqueous alcohol for extraction of fenugreek seeds is strongly limited due to the presence of large amounts of polysaccharides in this plant matrix. Therefore, application of simultaneous extraction and acid hydrolysis of fenugreek seeds, either non-purified or previously defatted (5, 6), resulted in a low diosgenin concentration and the presence of several ballast compounds. For this reason, separate extraction of saponins before acid hydrolysis was considered indispensable. Several extraction procedures were examined, including direct extraction with methanol under reflux and ultrasound extraction, as well as the three-step Soxhlet extraction including exhaustive defatting (petroleum ether), purifying (chloroform) and saponin extraction (methanol). TLC analyses revealed that of all the methods tested, the hydrolyzate of methanol extract from the three-step Soxhlet extraction contained the highest amount of diosgenin with the lowest content of ballast compounds impeding densitometric analysis.

Acidic hydrolysis of steroidal saponins is usually performed with HCl (12) or H_2SO_4 (4, 6, 10, 11, 13) in methanol, ethanol, water or isopropanol (4, 6, 10, 11) . Hydrolytic conditions should provide an exhaustive process, avoiding adverse rearrangements and struc-

ture degradation leading to the formation of spirostadienes (1, 5, 6). As spirostadienes are formed from diosgenin during acid hydrolysis, their presence results in the understating of the real content of diosgenin in analyzed samples. Though HCl is commonly used, it was reported to increase the formation of dienes (1, 6). Therefore H_2SO_4 ($H_2SO_{4(aq)}$, $H_2SO_{4(MeOH)}$ or in $H_2SO_{4(iso)}$) was chosen for our experiments (6). In all the performed analyses, the $H_2SO_{4(MeOH)}$ and $H_2SO_{4(iso)}$ provided more contaminated extracts. The use of $H_2SO_{4(aq)}$ enabled us to solve this problem, since during the extraction of free aglycones from the post-hydrolytic mixture, two completely immiscible phases were used (water-chloroform). In addition, our experiments revealed that the application of more severe hydrolysis conditions (2.5 mol L^{-1} H_2SO_4, 4 h) did not increase the diosgenin level in the obtained hydrolyzate.

On the basis of the obtained results, the combination of the three-step Soxhlet extraction of fenugreek seeds and acid hydrolysis with aqueous 2 mol L^{-1} H_2SO_4 provided the clearest extract with the largest amount of diosgenin. Hence, this procedure was applied to the quantification of diosgenin in fenugreek seeds of Polish and African origin.

Qualitative analysis

Determination of diosgenin in the analyzed plant material was performed under optimized conditions on HPTLC $Si60_{F254}$ plates using the mobile phase *n*-heptane/ethyl acetate (7:3, *V*/*V*) (ADC 2, developing time 20 minutes) (Fig. 3). These parameters provided good separation of diosgenin with $R_f = 0.47$ and $R_s = 14.8$. By means of specific visualization with a modified anisaldehyde reagent, diosgenin was revealed only in hydrolyzate of the methanol extract from the Soxhlet apparatus, while it was absent in hydrolyzates of both ether and chloroform extracts (Fig. 2). This has proven that diosgenin derivatives were not extracted during the defatting and purifying of plant material. On the other hand, several sterol compounds (but not sapogenins) were detected in the hydrolyzates of ether and chloroform extracts as pink to violet spots, while no sterol compounds were observed in the methanolic extract. This confirmed the high degree of extraction effectiveness for providing an organic extract free from lipophilic ballast compounds.

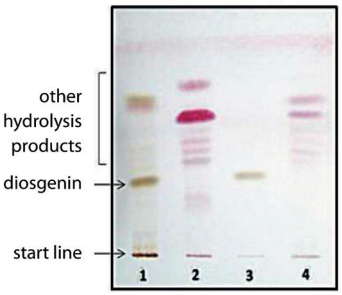

Fig. 2. TLC chromatogram of acid hydrolyzates (2 mol L^{-1} H_2SO_{4aq}, 80 °C, 2 h) of extracts from fenugreek seeds obtained by three-step extraction in the Soxhlet apparatus: 1 – hydrolyzate of methanolic extract; 2 – hydrolyzate of petroleum ether extract; 3 – diosgenin standard (1 mg mL^{-1}), 4 – hydrolyzate of chloroform extract. Stationary phase: TLC $Si60_{F254}$, mobile phase: *n*-heptane/ethyl acetate (7:3, *V*/*V*), derivatization: modified anisaldehyde reagent.

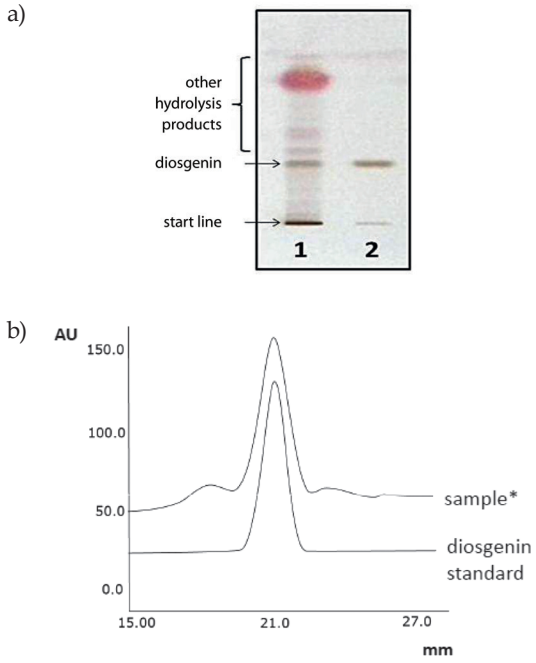

Fig. 3. a) HPTLC chromatogram and b) its densitogram (λ = 590 nm) of: 1 – hydrolyzate of the methanolic extract from fenugreek seeds, obtained by a combination of three-step Soxhlet extraction and acid hydrolysis with aqueous 2 mol L^{-1} $H_2SO_{4(aq)}$ (sample), 2 – diosgenin standard (250 ng mL^{-1}). Experimental conditions as in Fig. 2.

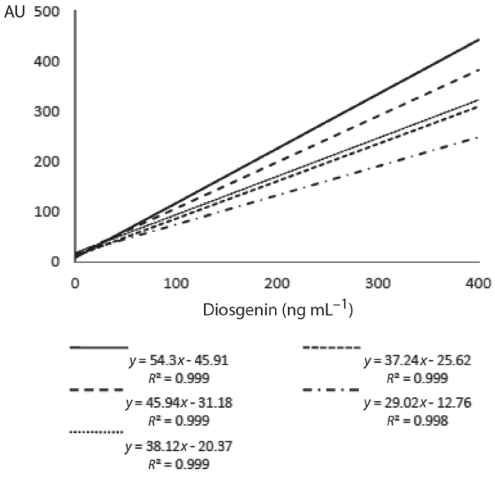

Fig. 4. Typical calibration curves for diosgenin on different TLC plates and the respective least square regression data.

Table II. Robustness testing and intermediate precision of the TLC method

Factor	Level of changes	R_f variability (RSD, %)
Mobile phase composition (*n*-heptane:ethyl acetate, *V*/*V*)[a]	7:3	0.6
	6.8:3.2	1.3
	7.2:2.8	1.7
Development distance (cm)[a]	4.0	1.3
	4.5	0.6
	5.0	0.6
Analysts[a]	2 different analysts	0.8
Solvents (*n*-heptane, ethyl acetate)[a]	2 different containers	0.2

[a] $n = 3$

Table III. Validation parameters of the established HPTLC-densitometric method for quantification of diosgenin in fenugreek seeds

Parameter	Detection wavelength (nm)	
	590	426
Linearity (ng mL^{-1})	50–900	200V800
Limit of detection, *LOD* (ng mL^{-1})	5.1	12.5
Limit of quantitation, *LOQ* (ng mL^{-1})	17	40
Instrumental precision (RSD, %)[a]	1.9 (diosgenin standard, 50 ng mL^{-1}, 1 μL) 1.6 (sample, 1 μL)	
Inter-day precision (RSD, %)[b]	1.0	22.1
Intra-day precision (RSD, %)[b]	1.3	9.0

[a] $n = 9$, [b] $n = 3$

Validation

Specificity of the method was confirmed by performing two-dimensional chromatography. On the obtained chromatogram of the methanol extract hydrolyzate, the diosgenin spot was clear and free from other co-eluting compounds. It is known that all color derivatives formed during the reaction of steroidal sapogenins with anisaldehyde possess absorption maxima at 510–620 nm while spirostane derivatives may additionally demonstrate a second maximum at 455–460 nm (5).

Some authors examined anisaldehyde-diosgenin derivatives at 428 (10), 430 (8) or 540 nm (14), while Kharat *et al*. (7) scanned the plates at 366 nm. In the current experiments, two absorption maxima, at 428 and 590 nm, were observed in diosgenin spectrum. After validation for both wavelengths, superior parameters were found for 590 nm (Table III). This wavelength was, therefore, chosen for quantitative analysis of diosgenin.

Quantitative analysis

Literature data show that diosgenin content in fenugreek seeds varies from 0.11 % (15) to 0.92 % in certain cultivars (6). The determined amount of diosgenin in fenugreek seeds of Polish origin was $0.13 \pm 0.00_2$ % (Lewandowski, Kawon) and $0.12 \pm 0.00_2$ % (Flos) and is considerably lower than in the herbal material from other parts of the world (4, 6, 10). However, the level of diosgenin was similarly low ($0.18 \pm 0.00_3$%) in the analyzed seeds originating from Algeria. Diosgenin concentration estimated by Trivedi *et al*. (10) in fenugreek seeds from Indian markets was more than five times higher (0.529–0.658 %). Moreover, diosgenin concentrations in fenugreek seeds of different origins (from African to Canadian) were also higher and varied between 0.42 and 0.98 % (4, 6). On the other hand, Indian cultivars with low diosgenin contents (0.113–0.135 %, *m*/*m*), similar to those determined in our plant material, were also reported (15).

CONCLUSIONS

A new quantitative HPTLC-densitometric method for diosgenin estimation was established together with the procedures of efficient extraction and acid hydrolysis of steroidal saponins in fenugreek seeds. Exhaustive three-step extraction in a Soxhlet apparatus (petroleum ether, chloroform, methanol) was the most effective, followed by acid hydrolysis of the methanol extract with 2 mol L^{-1} aqueous sulphuric acid. As a result of optimization, chromatographic separation was performed on the HPTLC Si60 $_{F254}$ plates with the use of the mobile phase of *n*-heptane/ethyl acetate (7:3, *V*/*V*) and a modified anisaldehyde reagent as spraying agent. Due to its specificity and sensitivity, fair precision and robustness, the established method may be used for diosgenin determination in fenugreek seeds as well as in other plant matrices. No significant differences were noticed in diosgenin contents between fenugreek seeds originating from various climate regions (African and European).

REFERENCES

1. W. G. Taylor, M. S. Zaman, Z. Mir, P. S. Mir, S. N. Acharya, G. J. Mears and J. L. Elder, Analysis of steroidal sapogenins from amber fenugreek (*Trigonella foenum-graecum*) by capillary gas chromatography and combined gas chromatography/mass spectrometry, *J. Agric. Food Chem.* **45** (1997) 753–759; https://doi.org/10.1021/jf960200n
2. Y. Y. Chen, Y. M. Tang, S. L. Yu, Y. W. Han, J. P. Kou, B. L. Liu and B. Y. Yu, Advances in the pharmacological activities and mechanisms of diosgenin, *Chin. J. Nat. Med*. **13** (2015) 578–587; https://doi.org/10.1016/S1875-5364(15)30053-4
3. B. Król-Kogus and M. Krauze-Baranowska, Fenugreek – source of compounds with hormonal activity? (Kozieradka pospolita – źródło związków o aktywności hormonalnej?), *Farm. Pol.* **68** (2012) 39–41.
4. W. G. Taylor, H. J. Zulyniak, K. W. Richards, S. N. Acharya, S. Bittman and J. L. Elder, Variation in diosgenin levels among 10 accessions of fenugreek seeds produced in western Canada, *J. Agric. Food Chem.* **50** (2002) 5994–5997; https://doi.org/10.1021/jf020486y
5. K. Hostettmann and A. Marston, *Saponins*, Cambridge University Press, Cambridge 1995, pp. 123–124, 126–127, 144, 177–178.

6. W. G. Taylor, J. L. Elder, P. R. Chang and K.W. Richards, Microdetermination of diosgenin from fenugreek (*Trigonella foenum-graecum*) seeds, *J. Agric. Food Chem.* **48** (2000) 5206–5210; https://doi.org/10.1021/jf000467t
7. S. N. Kharat, R. Singh and V. D. Mendhulkar, Quantitative analysis for diosgenin content in *Elephantopus scaber* (Linn.) by HPTLC using successive solvent extraction method, *Pharm. Lett.* **7** (2015) 236–244.
8. V. K. Ghosh, S. G. Bhope, V. V. Kuber and A. D. Sagulale, An improved method for the extraction and quantitation of diosgenin in *Tribulus terrestris,* L., *J. Liq. Chrom. Rel. Tech.* **35** (2012) 1141–1155; https://doi.org/10.1080/10826076.2011.615101
9. M. Amir, A. Ahmad, N. Siddique, M. Mujeeb, S. Ahmad and W. Siddique, Development and validation of HPTLC method for the estimation of diosgenin in vitro culture and rhizome of *Dioscorea deltoidea*, *Acta Chrom.* **24** (2012) 111–121; https://doi.org/10.1556/AChrom.24.2012.1.10
10. P. D. Trivedi, K. Pundarikakshudu, S. Rathnam and K. S. Shah, A validated quantitative thin-layer chromatographic method for estimation of diosgenin in various plant samples, extract, and market formulation, *J. AOAC Int.* **90** (2007) 358–363; https://doi.org/10.1080/0363904080243059
11. C. M. Kim, K. H. Son, S. H. Kim and H. P. Kim, Steroidal sapogenin contents in some domestic plants, *Arch. Pharm. Res.* **14** (1991) 305–310; https://doi.org/10.1007/BF02876875
12. L. Wang, X. Wang, X. Yuan and B. Zhao, Simultaneous analysis of diosgenin and sarsasapogenin in A*sparagus officinalis* byproduct by thin-layer chromatography, *Phytochem. Anal.* **22** (2011) 14–17; https://doi.org/10.1002/pca.1244
13. Y. Peng, Z. Yang, Y. Wang, Z. Liu, J. Bao and Y. Hong, Pathways for the steroidal saponins conversion to diosgenin during acid hydrolysis of *Dioscorea zingiberensis* C. H. Wright, *Chem. Eng. Res. Des.* **89** (2011) 2620–2625; https://doi.org/10.1016/j.cherd.2011.06.008
14. D. Sobolewska, Z. Janeczko, I. Podolak and A. Szerłomska, Densitometric analysis of diosgenin in methanolic extracts of *Allium ursinum* collected at different times during plant development, *J. Planar Chrom. - Mod. TLC* **22** (2009) 305–307; https://doi.org/10.1556/JPC.22.2009.4.13
15. O. Laila, I. Murtaza, M. Z. Abdin, S. Ahmad, N. A. Ganai and M. Jehangir, Development and validation of HPTLC method for simultaneous estimation of diosgenin and quercetin in fenugreek seeds (*Trigonella foenum-graceum*), *ISRN Chromatogr.* **2014** (2014) Article ID 583047, 8 pages; https://doi.org/10.1155/2014/583047