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CH Priyadarshini
Department of Veterinary Biochemistry, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

P Eswara Prasad
Retd Professor and University Head, Department of Veterinary Biochemistry, College of Veterinary Science, Tirupati, Andhra Pradesh, India

K Aswani Kumar
Department of Veterinary Biochemistry, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

D Nagalakshmi
Associate Dean, College of Fisheries Science, Pebbair, Telangana, India

D Madhuri
Department of Veterinary Pathology, College of Veterinary Science, Korutla, Telangana, India

Corresponding Author:
CH Priyadarshini
Department of Veterinary Biochemistry, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

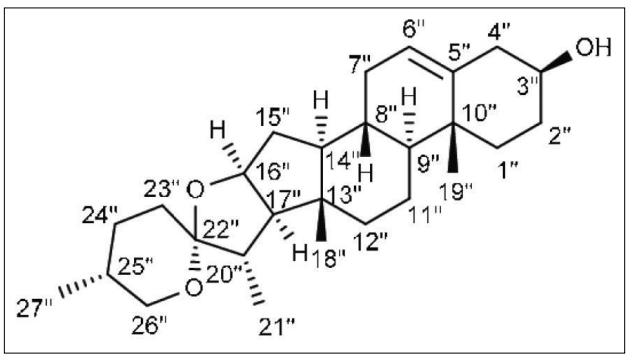
Quantitative determination of diosgenin in *Trigonella foenum graceum* seeds by HPLC method

CH Priyadarshini, P Eswara Prasad, K Aswani Kumar, D Nagalakshmi and D Madhuri

Abstract
Fenugreek (*Trigonella foenum-graecum*) seeds (FS) are rich in phytochemicals, one of them is steroidal saponins, diosgenin. Diosgenin has several properties in treatment of various disorders, inflammation, hypercholesterolemia, diabetes, several types of infections, antioxidant effect, different types of cancer. It also has shown estrogenic effect. Due to its pharmacological and industrial importance, several extraction and analytical procedures have been developed and applied over the years to isolate, detect, and quantify diosgenin. One of the best methods in determining the quantity of diosgenin is by HPLC technique. In HPLC method, an isocratic binary system of acetonitrile/water (90:10 v/v), a flow rate of 1 ml/min with UV at 203 nm and a temperature of 33 °C, the retention time (RT) of standards of 10, 50 and 100 ppm diosgenin showed 24.94, 24.933 and 24.927min respectively while aqueous and methanol extracts of FS showed 24.927 and 25.033 min respectively. The calibration curve constructed based on three dilutions of diosgenin standard at concentrations produced a linear regression (r = 0.995) and the concentration of diosgenin in methanol and aqueous extracts were 5.233 X 10^{-6} mg/ml and 151.649 X10^{-6} mg/ml respectively.

Keywords: *Trigonella foenum graecum* seeds, methanol and aqueous extracts of FS, diosgenin, HPLC

Introduction
Trigonella foenum graecum is a leguminous herb belonging to Fabaceae family, cultivated throughout the world especially in the Asia and North African countries [1]. The chemical composition of *Trigonella foenum graecum* seeds consists of 20-25% proteins, 45-50% dietary fiber, 20-25% mucilaginous soluble fibre, 6-8% fixed fatty acids and essential oils, and 2-5% steroidal saponins like diosgenin, gitogenin and yamogenin have also been identified and determined as the main component for its various biological effects [2]. The most abundant is diosgenin (5, 25-α-spirostan-3β-ol) (Fig. 1) [3]. Diosgenin possesses a number of biological activities, including anticancer, anti-inflammatory, effect of several infections, antioxidant effect and metabolic diseases like diabetes, obesity, dyslipidemia, hypercholesterolemia [4]. It also has effect on sex hormones, estrogen and testosterone [5]. Diosgenin is a white needle crystal or light amorphous powder with a proven thermal and chemical stability under various physical conditions The molecular formula of diosgenin is $C_{27}H_{42}O_3$, molecular weight is 414.62 gm, melting point is 204-207 °C, IUPAC name is 25R-spirost-5-en-3β-ol, UV absorption is 205 nm, soluble in non-polar solvents and stable at relatively temperature and light exposure [6].



[7]

Fig 1: Structure of diosgenin

Diosgenin occurs abundantly in plants such as *Dioscorea alata*, *Smilax china*, *Rhizoma polygonati* and *Trigonella foenum-graecum* [4, 8]. *Trigonella foenum-graecum* seeds are easily available and cheap in India. So diosgenin was determined in *Trigonella foenum-graecum* seeds. There are several standard methods developed in determining diosgenin [9] and one of the most reliable method is HPLC technique. So the present study was to quantitatively determine the concentration of diosgenin in *Trigonella foenum graecum* seeds in methanol and aqueous extracts by HPLC technique.

Materials and Method

Fenugreek seeds were washed with distilled water to remove foreign material, and were then air dried. The seeds were ground into fine powder by using an electrical blender. Defatting of the fenugreek seeds powder was done by petroleum ether. Aqueous and methanolic extracts were prepared by taking 1 g of fenugreek seed powder in 20 ml of distilled water, 1 gm of fenugreek seed powder in 20 ml of methanol respectively in two conical flasks. The samples were agitated with continuous shaking on a shaker at room temperature for 24 hours. The extracts were transferred into 50 ml falcon tubes and centrifuged at 9000 rpm for 10 minutes by using a refrigerated centrifuge machine. The supernatant was collected [10].

Hydrolysis of steroidal saponin in FSA extract

Hydrolysis of steroidal saponins into diosgenin was performed as described by Król-Kogus *et al.* (2018) [5]. Both aqueous and methanolic extracts were mixed with 25 ml of 2 M H_2SO_4 separately. The mixture was then refluxed at 70 ºC for one hour and was centrifuged at 9000 rpm for five minutes. The supernatant was discarded and precipitate was freeze dried using a lyophilizer. The lyophilized samples were reconstituted with 1 ml methanol and filtered through 0.22 µ filter.

HPLC quantification of diosgenin in FSA extract

Quantification of diosgenin was performed using a HPLC instrument, (Agilent Technologies 1290 affinity, applying the standard method described by Warke *et al.* (2011) [11].

Preparation of Diosgenin Standard Solution

2 mg of diosgenin standard was weighted and dissolved in 1 ml methanol for 15 minutes. The solution later was diluted up to 2 ml with methanol (1 mg/ml). 1 ml solution from stock solution was pipetted out and diluted up to 10 ml with methanol (100 ug/ml or 100 ppm).

HPLC Chromatographic Conditions

Chromatographic estimation was performed in HPLC Agilent 1290 affinity using an equilibrated reverse phase C18 column (particle size 5 µm, 4.6 mm x 250 mm). The experimental conditions were an isocratic binary system of acetonitrile/water (90:10 v/v), a flow rate of 1 ml/min with 203 nm UV wavelength and a temperature of 33 °C. 5 µl standard solutions of diosgenin (ranging from 10, 50 and 100 pm) and a volume of 5 µl of sample extract were injected into HPLC and HPLC was run. The corresponding peak areas were plotted against the concentration of diosgenin injected. Peak identification was achieved by comparison of both the retention time (RT) and UV absorption spectrum for standard.

Results and Discussion

The retention time, areas and height of the diosgenin was as eluted and recorded as follows in the given Table 1.

Table 1: Retention time, average area, height of different diosgenin standards and extracts of *Trigonella foenum graecum* seeds

Diosgenin Standard (ppm)	RT (min)	Area	Area%	Height	Height %
10	24.940	17117527	0.9	408353	0.17
50	24.933	51599717	2.52	2129581	0.88
100	24.927	106756538	5.31	3975651	1.63
Trigonella foenum graecum seeds extract					
Aqueous	24.927	156.786231	1.91	5403160	0.9
Methanol	25.033	10.370445	0.15	321660	0.04

Calculation

Compound	Linearity	r^2 correlation co-efficient
Diosgenin	y = 1.000x +5.137	r^2 =0.995

Estimation of diosgenin in aqueous extract of Fenugreek seeds (FS)

156.786 = 1.000x + 5.137

1.000x = 156.786 - 5.137

$$x = \frac{156.786 - 5.137}{1.000}$$

x = 151.649 X 10^{-6} mg/ml

Concentration of diosgenin in aqueous extract of Fenugreek seeds is 151.649 X 10^{-6} mg/ml.

Estimation of diosgenin in methanol extract of Fenugreek seeds

10.370 = 1.000x + 5.137

1.000x = 10.370 - 5.137

$$x = \frac{10.370-5.137}{1.000}$$

x = 5.233 X 10^{-6}mg/ml

Concentration of diosgenin in methanol extract of Fenugreek seeds is 5.233 X 10^{-6}mg/ml.

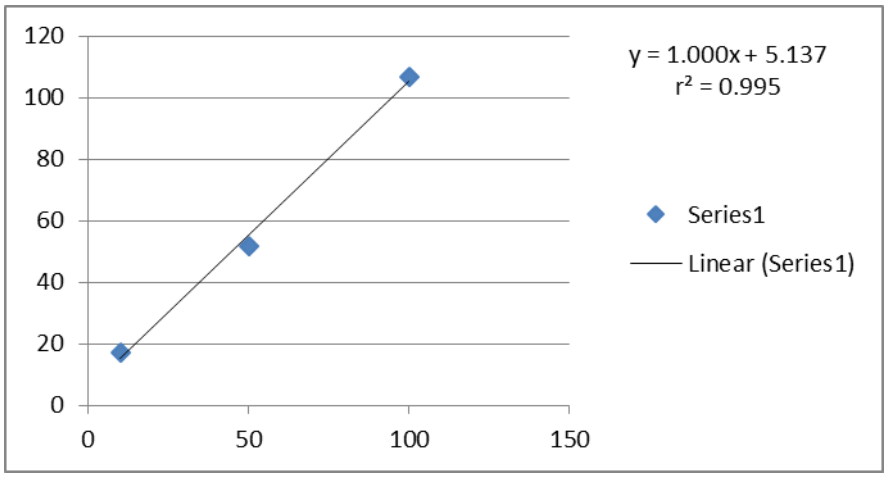


Fig 2: Linear regression curve for diosgenin in Fenugreek seeds

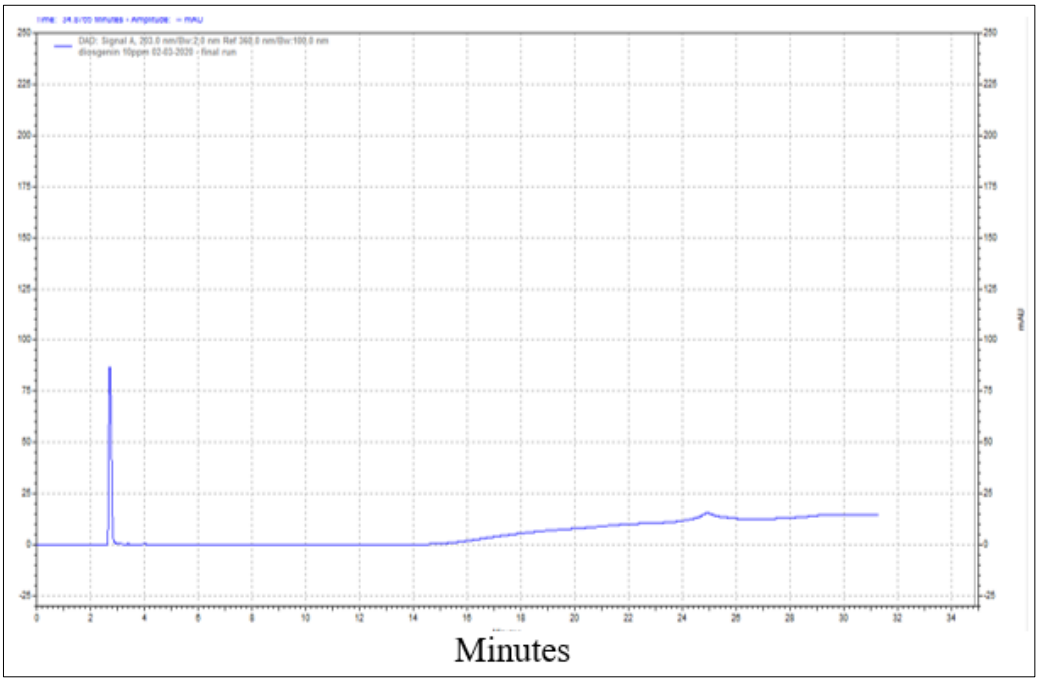


Fig 3a: Total ion chromatogram of diosgenin with RT for 10ppm concentration

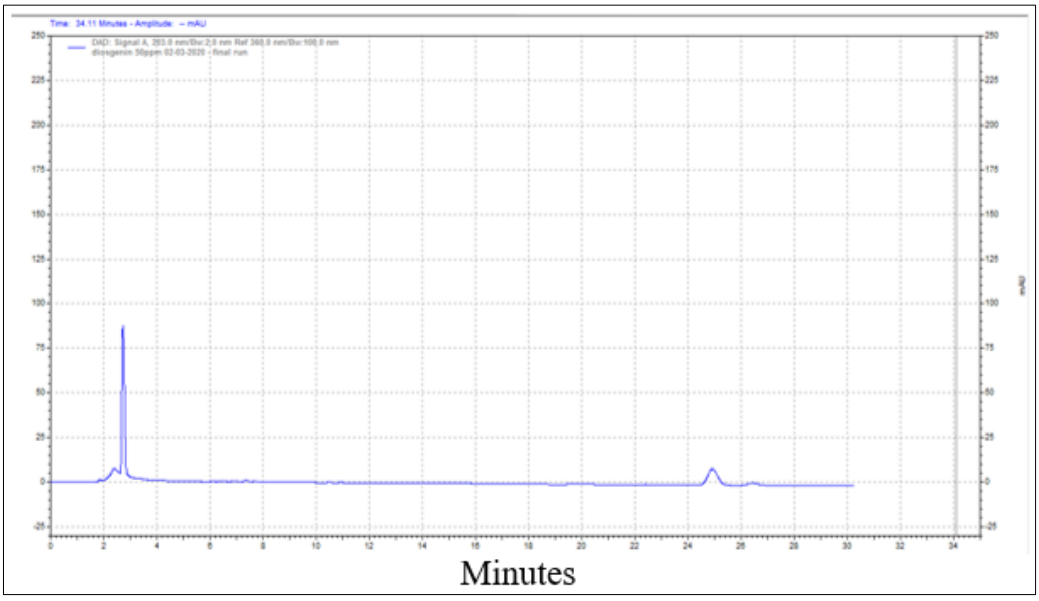


Fig 3b: Total ion chromatogram of diosgenin with RT for 50ppm concentration

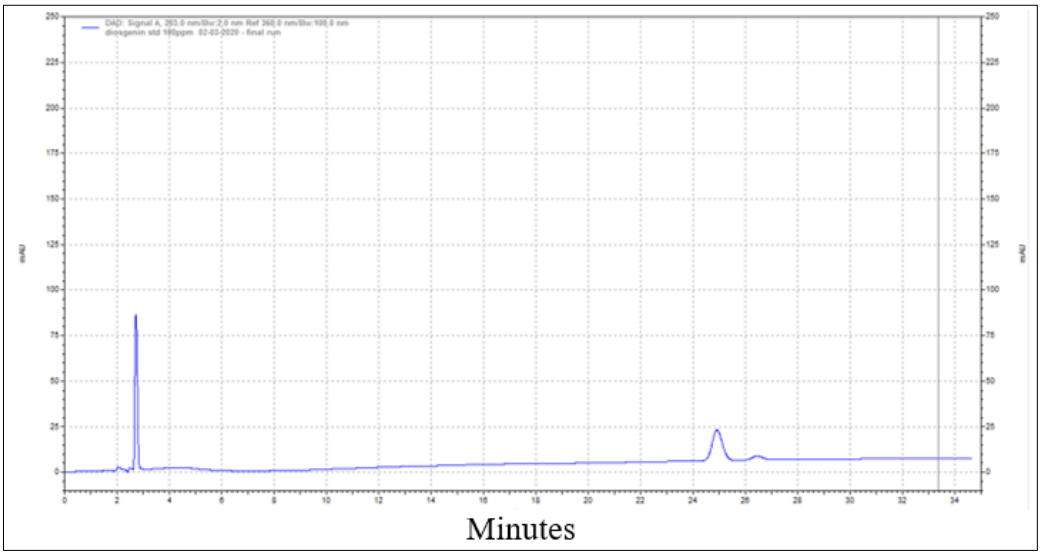


Fig 3c: Total ion chromatogram of diosgenin with RT for 100ppm concentration

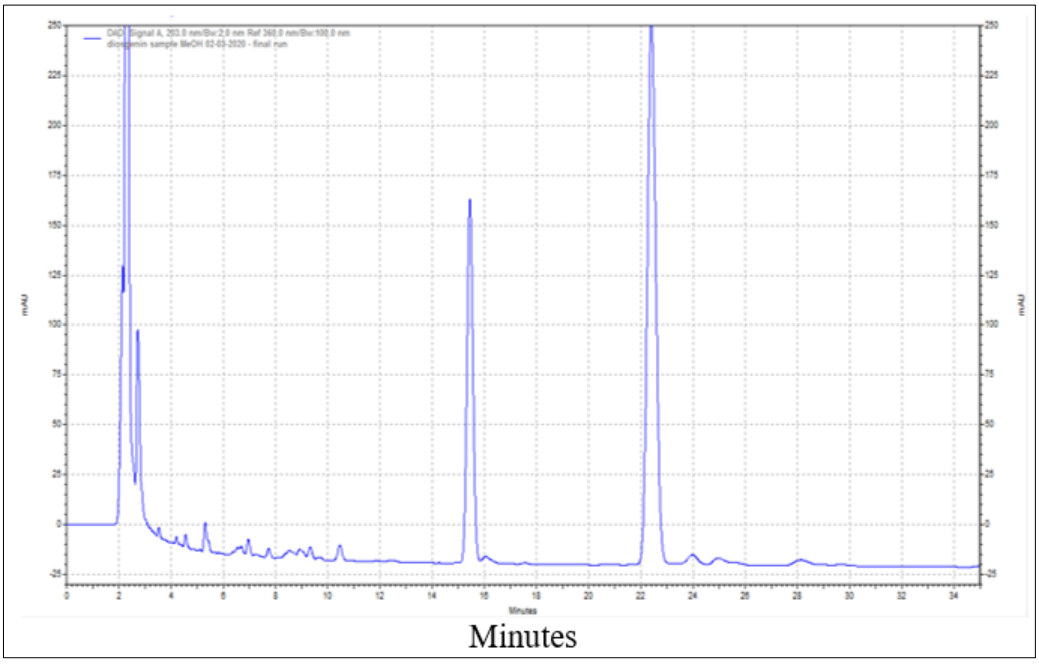


Fig 3d: Total ion chromatogram of diosgenin with RT in methanol extract of Fenugreek seeds

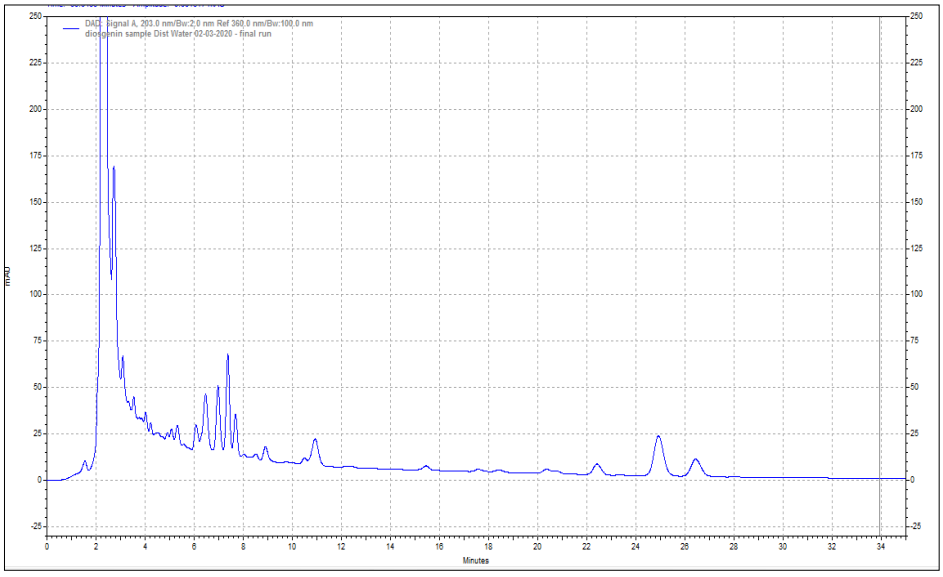


Fig 3e: Total ion chromatogram of diosgenin with RT in aqueous extract of Fenugreek seeds

Quantification of diosgenin in aqueous and methanol extracts of Fenugreek seeds (FS)

A satisfactory result with sharp well defined and resolved peak with minimum tailing was achieved using a mobile phase consisting acetonitrile: water in the ratio of 90:10 v/v. Chromatogram from the HPLC analysis showed the peak area for diosgenin standard 10 ppm, 50 ppm and 100 ppm were recognized at retention time (RT) 24.940, 24.933 and 24.927 min respectively (Fig. 3a, b, c), whereas the peak area for aqueous and methanol extracts were recognized at RT 24.927 and 25.033 minutes (Figure 3d, e), both at 203 nm UV wavelength. A calibration curve that was constructed based on three dilutions of diosgenin standard at concentrations of 10, 50 and 100 ppm produced a linear regression (r = 0.995) as shown in Fig 2. The concentration of diosgenin in FS seeds in aqueous and methanol extract as calculated using regression analysis is found to be 151.649 $X10^{-6}$ mg/ml and 5.233 X 10^{-6} mg/ml respectively. In findings of Ahmed *et al*., (2016) [10], retention time of diosgenin was approximately 11.089 minute and concentration of diosgenin at 203nm wavelength and linear regression (r) was 0.999 using HPLC with mobile phase acetonitrile: water 90:10 ratio. Other findings showed that the retention time was 10 min at 205nm wavelength by HPLC method [13]. Hence it is concluded that the sample preparation is simple and the HPLC method provides nanogram sensitivity and adequate linearity and repeatability.

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