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Research Article

Standardization of Protodioscin Quantification in Tribulus Terrestris Linn to Resolve Analytical Variances

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Abstract

Tribulus terrestris is a highly potent medicinal plant belonging to the caltrop family. This study reviews its wide range of therapeutic applications and health benefits. The plant exhibits strong diuretic and anti-urolithiatic properties, making it effective for promoting urination and preventing kidney stones. Additionally, it serves as an antimicrobial and anthelminthic agent to combat infections and parasites. Cardiotonic and hepatoprotective actions ensure the protection of vital organs like the heart and liver. It effectively manages metabolic health through Hypolipidemic and antidiabetic activities that lower cholesterol and blood sugar levels. Furthermore, the plant offers pain relief, reduces inflammation, and prevents muscle spasms due to its analgesic, anti-inflammatory, and antispasmodic traits. As an aphrodisiac and immunomodulator, it enhances sexual health and strengthens the immune system. Finally, its antioxidant, anti-tumor, and anti-cariogenic properties help neutralize cellular damage, fight cancer cells, and protect dental health, highlighting its vast pharmaceutical potential The active compound, protodioscin, was identified and measured in the fruit extract of Tribulus terrestris using advanced testing methods. First, a UV spectrophotometer confirmed the compound by showing its peak light absorption at 205 nm. Then, TLC and HPLC testing methods were used to find the exact amount of protodioscin present in the fruit. Protodioscin is the substances of potential therapeutic interest are steroidal saponins. The following furostanol saponins-protodioscin, neoprotodioscin and their respective sulphates methylprotodioscin, prototribestrin, methylprorotribestrin and many more. Overall review of protodioscin medicinal applications like kidney and urinary tract health, sexual and reproductive wellness, athletic performance and muscle building and general health and wellness.

Keywords

Tribulus Terrestris Linn, Protodioscin, Liquid Chromatography-Mass Spectroscopy, High Performance Liquid Chromatography

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1. Introduction

Tribulus terrestris Linn, is a traditional me decimal herb, belongs to Zygophyllacaceae Caltrop family, commonly known as "Gokhru" in Indian traditional medicine and in traditional European medicine agency respectively (EMA/HMPC/886105/2022). *Tribulus terrestris* Linn, has diversified uses in agriculture, ayurvedic as well as allopathic system of medicines with thousands of years of clinical application [5]. T. terrestris contains steroids, saponins, flavonoids, alkaloids, unsaturated fatty acids, vitamins, tannins, resins, nitrate potassium, aspartic acid and glutamic acid [5]. Steroidal saponins and diosgenin is isolated from this plant [7]. It is very rich in proteins and calcium [3]. Dried fruits contain semi drying oil, peroxides, diastase, and traces of glycosides, resins, protein and large amount of inorganic matters [1].

Tribulus terrestris Linn, North and North West India's (Primary Hub) states are Rajasthan, Punjab, Haryana and Gujarat. Central India and indo-genetic Plains are Madhya Pradesh and Uttar Pradesh. And sub Himalayan foothills Uttarakhand, Jammu Kashmir and himachal pradesh. The extracts and active components of *Tribulus terrestris* Linn, demonstrated multiple biological activities, including acclaimed diuretic, Anti-urolithiatic, Antimicrobial, Anthelminthic, Cardio tonic, Anti-inflammatory, Hypolipidemic, Immuno-modulatory, Antispasmodic, Analgesic, Aphrodisiac, Anti diabetic, Anti-tumor, Hepato-protective, Anti-cariogenic, Anti-oxidant (*wjpmr, 2025, 11(2), 107-115*;) The principal bioactive compound of gokhru is protodioscin.

It is a naturally occurring Steroidal saponin (specifically a *furostanol saponin*) [7].

IUPAC name (3β,22R,25R)-26-(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside. Beside the Protodioscin is a compound found in several plants, including the *Trigonella* and *Dioscorea* families. In this study, scientists extracted protodioscin from *Tribulus terrestris* using methanol, then purified and verified the compound.

Tribulus terrestris is used to make natural, hormone-free health supplements. Because the strength of the plant changes depending on where it grows, scientists use a main compound called protodioscin to check the quality of different water and alcohol extracts [6]. They accurately measure this compound using high-tech tools: the Shimadzu LC-2050 (HPLC) and the Shimadzu 8045 (LC-MS/MS).

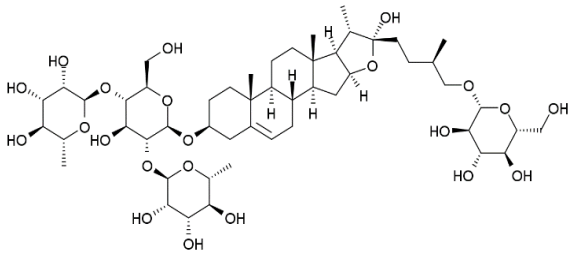

Figure 1. *Protodioscin.*

2. Materials and Methods

Gokhru Plant Fruit: The raw *Tribulus terrestris* fruits were purchased from a vendor in Haridwar and officially verified by the Patanjali Research Foundation Herbarium (PRHF). The fruits were dried in the shade, ground into a coarse powder, and stored in airtight zipper bags for later use.

Chemicals: All chemicals and solvents used were of high-grade quality (reagent and analytical grade) purchased from Merck Ltd (Mumbai, India). The reference standard for protodioscin (98.0% pure) was sourced from Sigma Aldrich (Phytolab, Germany.

Extraction of crude Protodioscin: Dried fruit powder (250 g) was extracted three times using a Soxhlet apparatus with a water-and-methanol mix (80:20) at 60 ℃ for three hours each time. A rotary evaporator removed the solvent under vacuum pressure, leaving 25.62 g of dry extract, which was weighed, calculated for percentage yield, and stored in a refrigerator for analysis [1].

3. Determination of Absorption Maxima

UV spectrophotometer: To measure the UV spectra, a Shimadzu UV-1800 double-beam spectrophotometer was used with quartz cuvettes (1 cm path length) across a 190-800 nm wavelength range [8]. Before testing, all glassware was cleaned with a chromic and sulphuric acid solution, rinsed with double-distilled water, and dried in a hot air oven. To prepare the sample, 10 mg of protodioscin was dissolved in 10% methanol in water inside a 100 mL volumetric flask to create a 100 µg/mL stock solution. This final solution was scanned to determine the exact absorption peak lambda max of protodioscin [2].

4. Identification and Quantification

TLC (Thin layer chromatography): To identify protodioscin quickly and affordably, a TLC method was chosen because it tests multiple samples at once while saving time and solvents. Merck silica gel plates (60 F254) were used, and glass capillaries spotted the samples and reference standard in duplicate [4]. The plate was placed in a chamber pre-saturated for two hours with a toluene: ethyl acetate (7:3) mobile phase, letting the solvent travel 6.0 cm. The completed plates were viewed under 254 nm and 366 nm UV light to determine and match the Rf values.

5. HPLC (High Performance Liquid Chromatography)

Protodioscin was measured in the *Tribulus terrestris* fruit extract using a Shimadzu i-series LC 2050 HPLC system equipped with a quaternary pump and a PDA detector. Separation was achieved at 25 ℃ on an RP-18 column (150 mm × 4.6

mm, 5 µm) with a 1.0 mL/min flow rate. The mobile phase consisted of a gradient mix of Milli-Q water (A) and 100% acetonitrile (B), using 10% methanol in water as the diluent. Psoralen was monitored at 247 nm, and the retention time for the protodioscin standard was confirmed at 12.897 minutes [6]. All data were collected and processed using LabSolutions software.

5.1. Standard Preparation

A 10 mg reference standard of protodioscin was dissolved in the diluent and mixed using a sonicated for 15 minutes.

5.2. Sample Preparation

5.2.1. Raw Material

Four grams of raw material was boiled with 10 mL of diluent on a water bath for 15 minutes, cooled, and filtered into a 50 mL volumetric flask. To maximize recovery, the plant residue was extracted two more times under the same conditions, blending all cooled filtrates together into the flask [8]. The combined mixture was then centrifuged at 5000 rpm for 5 minutes and cleared through a 0.45 µm filter right before HPLC injection.

5.2.2. Herbal Extract (Hydro-alcoholic and Aqueous)

Two grams of raw material was boiled with 10 mL of diluent on a water bath for 15 minutes, cooled, and filtered into a 50 mL flask. The plant residue was extracted two more times under the same conditions, pooling all cooled filtrates into the flask [8]. The combined liquid was centrifuged at 5000 rpm for 5 minutes and cleared through a 0.45 µm filter for HPLC analysis.

6. The Testing Followed the Specific Guidelines Recommended by the FDA, USP, and ICH

6.1. System Suitability

In system suitability we injected the six injection of reference standard protodioscin to check the parameters are RSD% of the area, RT and height, Theoretical plates and tailing factor. Result comes RSD of area is 0.114%, RT is 0.252%, height 0.465% and theoretical plate (USP2) 161617 and tailing factor 0.872. Then all parameters of system suitability is pass as per the ICH guideline [11].

6.2. Specificity

In this parameter we injected the replicate 20 injection of placebo. Their no peak interfere between the RT times of protodioscin, this parameters pass as per the ICH guideline [9].

6.3. Linearity

Protodioscin solutions at 9 levels were prepared from 1 ppm to 100 ppm of protodioscin reference standard concentration. At each level, analysis was carried out in triplicate. The peak areas versus concentrations data was evaluated by linear regression analysis.

Three data set corresponding to triplicate analysis were constructed and calculate the value of R^2 =0.9999324 and plot the linearity curve y = mx + b. this parameters pass as per the ICH guideline [9].

6.4. Limit of Quantification and Detection (LOD & LOQ)

The limit of detection (LOD) is pass at 2.5 ppm of reference standard protodioscin signal to noise is come 3.87 which equal to greater than 3.0. On other hand the limit of quantification (LOD) is pass at 10 ppm of reference standard protodioscin signal to noise is come 13.79 [9] which equal to greater than 10.0. Both result fulfilled the guideline of ICH.

6.5. Accuracy

we perform the recovery (Accuracy) of sample at two points 500 ppm and 1000 ppm in both raw material and herbal dry extract. Result of raw material at 500 ppm is 96.18% and at 1000ppm 97.90ppm on other side herbal dry extract recovery at 500ppm id 91.19% and 1000ppm is 101.06% [9]. both point of recovery lies between 80 to 120% as per guideline of ICH.

6.6. Repeatability (Precision)

In repeatability we prepared the sample of raw material and herbal extract in replicate an analyzed the RSD of the result which lie between in 2.0% or not. the result is come in raw material is 0.162% and herbal dry extract is 0.065% [10]. Which less than 2%.

6.7. Robustness

we perform the robustness on two parameter flow and oven temperature in flow and oven temperature. We check the RT, Area RSD which RSD not greater than 2%. In oven temperature RSD is 0.047% and in flow 0.610% of RT [10].

7. Results

7.1. Identification and Characterization

Compounds were identified and characterized using optimized UV, HPLC, and LC-MS spectral techniques. Under TLC conditions, the reference standard, raw material, and dry

extract all shared a retention factor R_f of 0.57 ±0.02, confirming the presence of protodioscin [13].

7.2. HPLC Chromatographic Analysis

The characteristic retention time (RT) chromatograms are illustrated in Figures 2, 3, 6, and 7. The protodioscin reference standard eluted at 12.992 minutes, while the protodioscin in both the raw material and dry extract eluted at 12.993 minutes. Quantitative peak area analysis showed that protodioscin is a major compound, with a purity concentration of 0.01%w/w in the raw material and 0.1%w/w in the herbal extract [16].

7.3. Method Validation

The analytical method was validated according to regulatory parameters:

1) *Linearity:* The calibration curve showed excellent linearity with an R^2 value of 0.9999324.
2) *Precision (Repeatability):* The method demonstrated high reproducibility, yielding a Relative Standard Deviation (RSD) of 0.162% for the raw material and 0.065% for the herbal extract.
3) *Sensitivity:* Limits of Detection (LOD) and Quantification (LOQ) were successfully established.
4) *Robustness:* The method remained reliable under minor variations in operating conditions [15]

7.4. Recovery (Spike Testing)

Accuracy was evaluated by spiking samples at two different concentrations:

Table 1. *Spike Testing.*

S.no	Sample Type	Spiked Concentration	Recovery Rate
7.4.1	Raw Material	100 ppm	96.18%
7.4.2	Raw Material	500 ppm	97.90%
7.4.3	Dry Extract	100 ppm	91.19%
7.4.4	Dry Extract	500 ppm	101.96%

7.5. Solution Stability

Stability testing of the reference standard solution revealed that it degraded after 15 days at room temperature. However, when stored in a refrigerator at 2 to 4 °C the solution remained stable for 45 days [12].

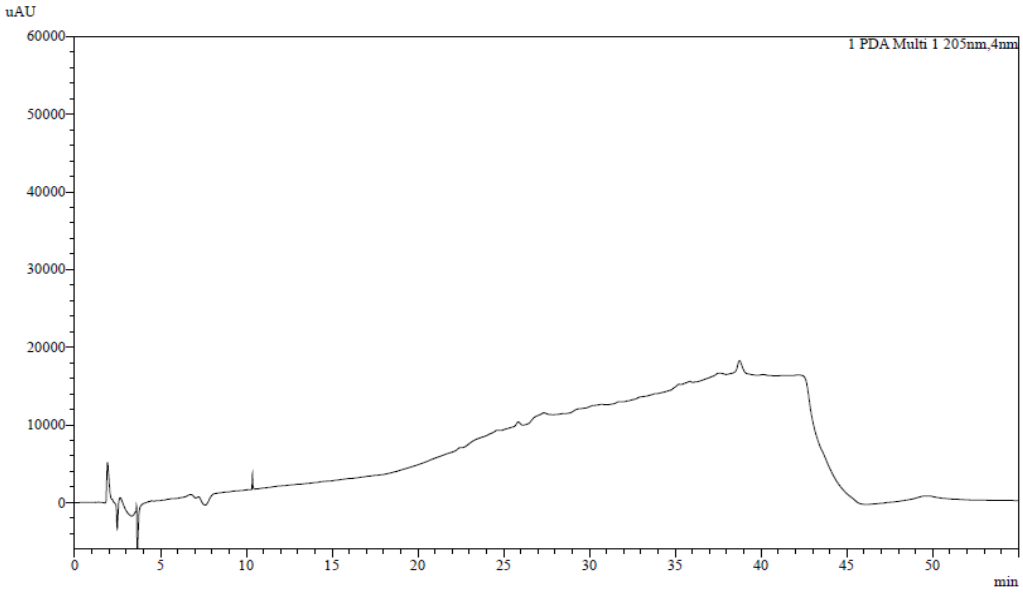

Figure 2. *HPLC chromatogram of the protodioscin blank.*

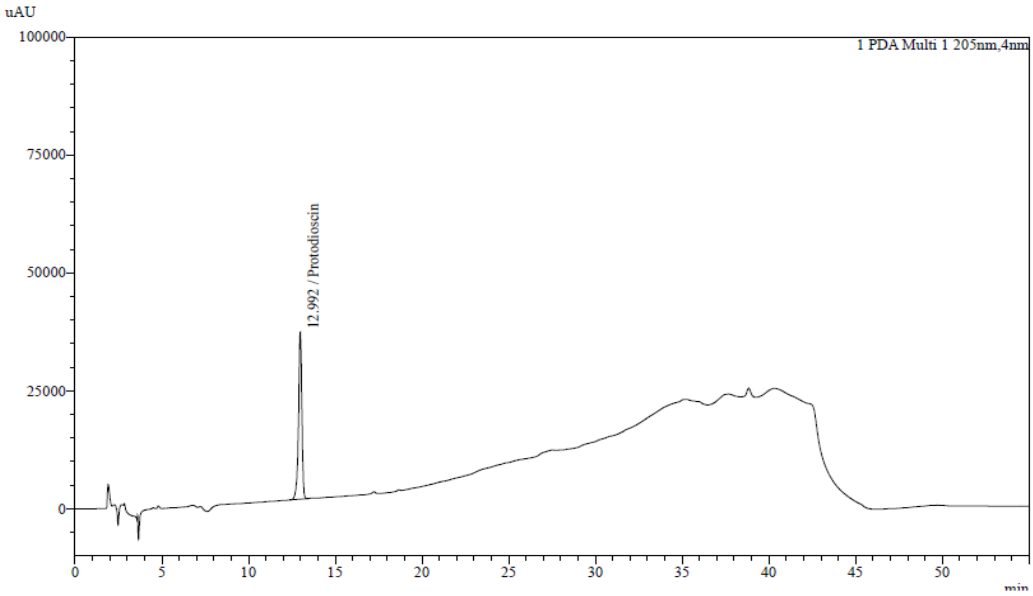

Figure 3. *Representative HPLC chromatogram of the protodioscin reference standard.*

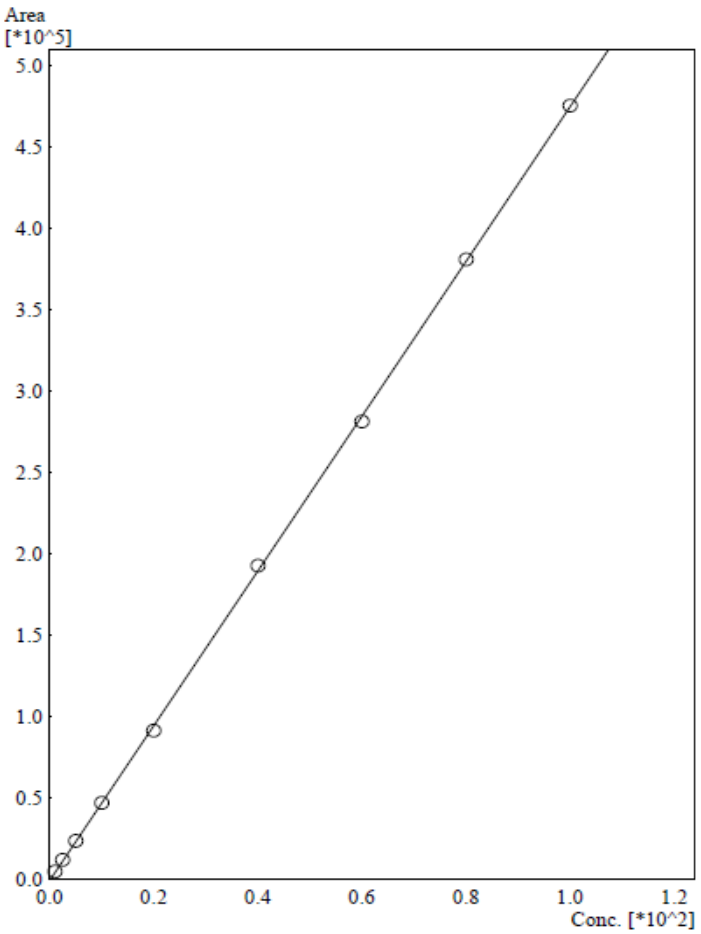

Figure 4. *Linearity Plot of Protodioscin Reference Standard by HPLC.*

#	Conc.(Ratio)	MeanArea	Area	RF %RSD
1	1	4883	5069	3.373028
			4755	
			4826	
2	2.5	12087	12069	1.053714
			12222	
			11969	
3	5	23760	23860	0.430113
			23764	
			23656	
4	10	47159	47100	0.277143
			47067	
			47308	
5	20	91526	91531	0.271044
			91275	
			91771	
6	40	192838	187941	4.353077
			188041	
			202531	
7	60	281449	281645	0.329321
			282262	
			280440	
8	80	380992	381162	0.081189
			381180	
			380635	
9	100	475593	475784	0.042613
			475613	
			475381	

Figure 5. *Linearity study of protodioscin reference standard by HPLC.*

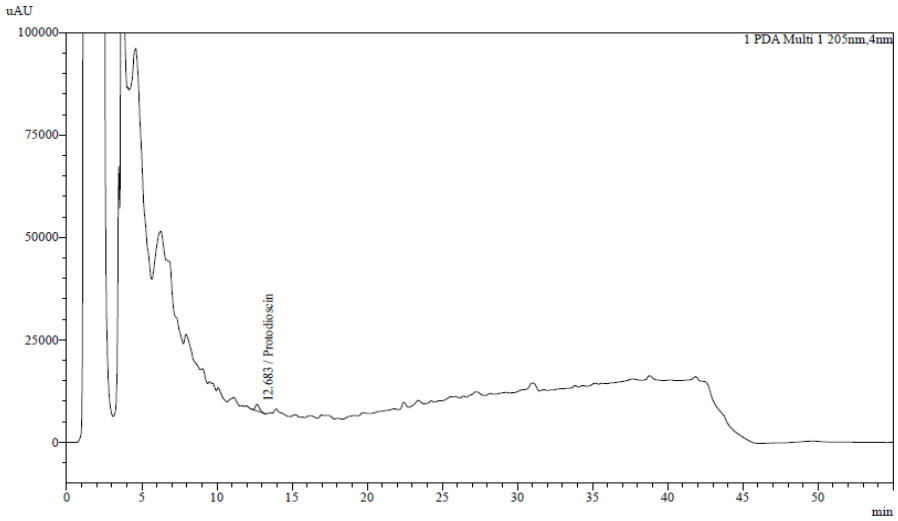

Figure 6. *HPLC Chromatographic Profile of* Tribulus terrestris *Linn. Raw Material.*

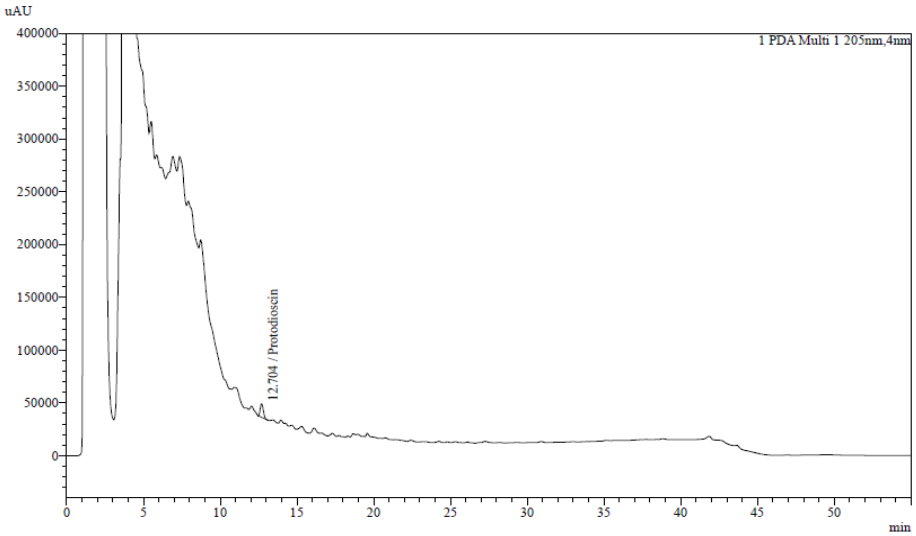

Figure 7. *HPLC chromatogram showing the presence of protodioscin in Tribulus terrestris Linn. herbal dry extract.*

8. Conclusions

A simple, sensitive HPLC-PAD and LCMS technique was demonstrated and justified for the characterization and quantitative estimation of the main protodioscin in the fruit raw material and herbal extract of *Tribulus terrestris* Linn. The HPLC results confirmed the existence of protodioscin in the raw material and herbal extract of *Tribulus terrestris* Linn fruit. It is concluded that the highest amount of protodioscin was found in the fruit and herbal extract of of *Tribulus terrestris* Linn [14].

Abbreviations

HPLC	High Performance Liquid Chromatography
LCMS	Liquid Chromatography-Mass Spectrometry
TLC	Thin Layer Chromatography
FDA	Food and Drug Administration
ICH	International Conference on Harmonization
USP	United States Pharmacopeia

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Author Contributions

Shri Ram Bharat: Conceptualization
Suman Kumar Jha: Project administration
Rajeev Saini: Project administration
Brijesh Kumar: Funding acquisition
Satyavrat Fore: Supervision
Prashant Yadav: Visualization
Rishabh Kumar Prajapati: Formal Analysis, Investigation, Methodology, Validation, Writing – original draft
Pragati Singh: Visualization

Data Availability Statement

All data available in hard and softcopy in our software.

Conflicts of Interest

The authors declare no conflicts of interest.

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