Determination of Steroidal Saponins in *Tribulus terrestris* by Reversed-Phase High-Performance Liquid Chromatography and Evaporative Light Scattering Detection

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Received 19 June 2000; revised 22 January 2001; accepted 1 February 2001

ABSTRACT: This paper describes the first analytical method suitable for the determination of steroidal saponins in *Tribulus terrestris*. A separation by high performance liquid chromatography (HPLC) was achieved by using a reversed-phase (RP-18) column, evaporative light scattering (ELS) detection, and a water/acetonitrile gradient as the mobile phase. The marker compound, protodioscin, was detected at a concentration as low as 10.0 µg/mL. Several different samples of plant material were successfully analyzed, and depending on origin and plant part used for extraction, significant differences in the composition of the saponins were observed. The analysis of market products showed considerable variations of 0.17 to 6.49% in the protodioscin content. © 2001 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 90:1752–1758, 2001

Keywords: Tribulus terrestris; HPLC; ELSD; saponins

INTRODUCTION

Tribulus terrestris (L.), puncture vine or small caltrops, belongs to the Zygophyllaceae family. It is a 10–60 cm high, annual herb, with pinnate leaves and yellow flowers.[1] Its carpel fruits are of a characteristic, stellate shape and are known as "Chih-hsing" in China or goathead in the USA. The plant can be found in arid climate regions around the world; for examples, in southern USA, Mexico, Spain, Bulgaria, India, and China.[2] The fruits and seeds are of great importance in oriental medicine because they are used as an aphrodisiac, diuretic and anthelmintic, as well as to treat coughs and kidney failure.[3,4] *T. terrestris* has the reputation of having anabolic effects as well and is available in the USA as a dietary supplement.

Previous publications on *T. terrestris* have reported the isolation of several steroidal saponins, with protodioscin (1) the most dominant, as well as alkaloids and flavonoids.[5–9] Pharmacological studies have shown that saponins are responsible for some of the pharmacological activities of the plant. It has been reported that they stimulate spermatogenesis, increase the activity of Sertoli cells, decrease urinary oxalate excretion, and reduce the activity of liver enzymes such as GAO (glycolate oxidase) and GAD (glycolate dehydrogenase).[10,11] Except for a fortuitous determination of the saponins in *T. terrestris* by photometry and the quantification of the aglycon diosgenin by HPLC, no analytical method has hither to been reported.[12,13] The explanation for this limited number of reports is most likely based on the fact that the saponins show very weak absorption, even in the short wavelength range, which renders a sensitive detection by ultraviolet

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Journal of Pharmaceutical Sciences, Vol. 90, 1752–1758 (2001)

(UV) spectroscopy impossible. This paper thus describes an HPLC method for the separation of the main saponin glycosides in puncture vine. From our previous experiences with a similar problem, an evaporative light scattering (ELS) detector was utilized.[14] The most dominant saponin 1 was used as a marker compound and quantified in samples of different origin as well as in several market products to demonstrate the utility of the new method.

EXPERIMENTAL SECTION

Materials

Plant material was provided by NutraSource Inc. (1300 Industrial Road, San Carlos, CA 94070). For the isolation of standard compounds, *T. terrestris* feedstock from Bulgaria (for 1), and Indian *T. terrestris* stems (for 3 and 4) were used. Two samples of Tribulus fruits (samples F and G) were bought as "Chih-hsing" in China by Mr. Zhao [National Center for Natural Products Research (NCNPR), The University of Mississippi] on local markets in China. The market products were purchased from supermarkets in Mississippi. Voucher specimens of all samples are deposited at the NCNPR.

All standard compounds and 22-*O*-methylprotodioscin were isolated in our laboratories, and identity and purity were confirmed by chromatographic [thin-layer chromatography (TLC), HPLC] and spectral [infrared (IR), one-dimensional (1D-) and two-dimensional (2D-) nuclear magnetic resonance (NMR), and high-resolution electrospray ionization mass spectrometry (HR-ESIMS)] methods.

Solvents (water, acetonitrile) were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation

One gram of the finely powdered plant material or market product was extracted three times with 3.0 mL of 50% aqueous acetonitrile by sonication for 10 min. After centrifugation, the extracts were combined in a 10.00-mL volumetric flask and the volume was adjusted to 10 mL with the solvent used for extraction. Market products containing *T. terrestris* extract were treated by the same procedure, only 100.00 mg of material were used for extraction. Prior to injection, all samples were filtered through a 0.45-µm Nylaflo filter (Gelman, Ann Arbor, MI). Every sample solution was injected in triplicate; standard deviations were less than 2.66% for all experiments.

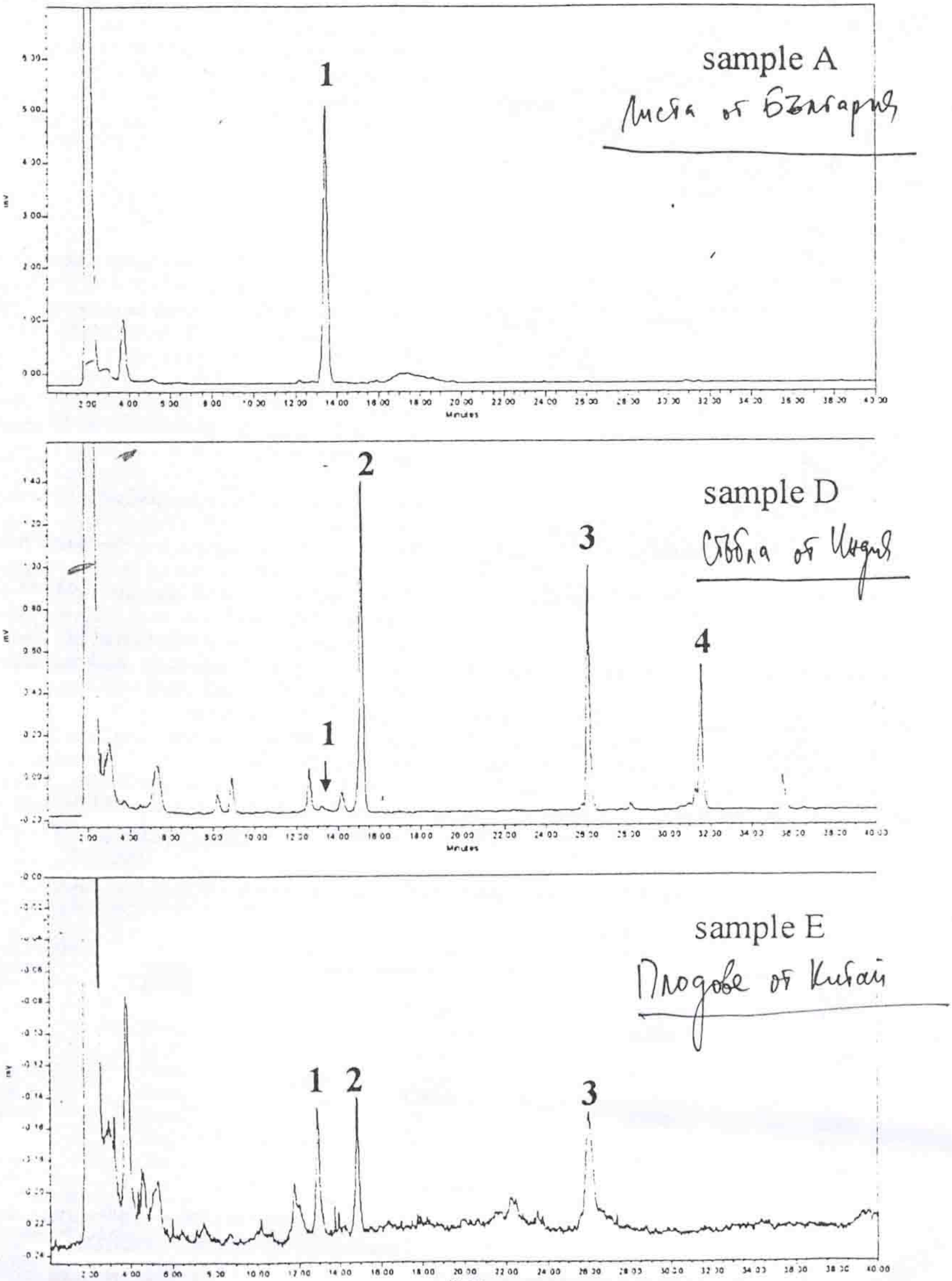
Calibration

Five milligrams of protodioscin standard was dissolved in 5.00 mL of 50% aqueous acetonitrile (stock solution); further calibration levels were prepared by diluting the stock solution with the same water/acetonitrile mixture. The resulting range for the concentration of 1 was from 31.2 to 1000.0 µg/mL. All calibration levels were injected in triplicate, and the calibration data obtained ($R^2 = 0.9977$ and $y = 1.49X - 0.221$) indicated linearity of the detector response in the range already mentioned. The response of an ELS detector is a function of the mass and follows an exponential relationship; therefore y in the regression equation stands for log response (area) and X for log concentration (in µg/mL).[15]

Analytical Method

HPLC analysis was performed on a Waters HPLC system (equipped with a 600F pump, a 712 WISP autosampler, a 600E system controller, and a 996 photodiode array detector, Waters, Milford, MA) and a Sedex 55 ELS detector (SEDERE, Alfortville, France). A Discovery C18 column (150 × 4.6 mm, 5 µm particle size) from Supelco (Bellefonte, PA) was used for all separations. The mobile phase, which consisted of water (A) and acetonitrile (B), was used for gradient elution from 80A/20B to 65A/35B in 15 min, then to 20A/80B in 20 min; this composition was then held for 5 min. The flow rate was adjusted to 1.0 mL/min, the detection wavelength was 200 nm, and 10 µL

Figure 1. Chromatogram of different *T. terrestris* samples (A: leaves from Bulgaria; D: stems from India; E: fruits from China) separated by RP-HPLC (column: Discovery C18, 5 µm particle size, 150 × 4.6 mm; mobile phase: water/acetonitrile, from 80/20 to 65/35 in 15 min, to 20/80 in 20 min, and held at that composition for 5 min; flow rate: 1.0 mL/min; detection: ELS; ELS parameters: 40 °C, 2.4 bar nitrogen, gain of 9; injected sample volume: 10 µL; temperature: ambient). Assignment of peaks according to Figure 2.



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of sample was injected. All separations were performed at ambient temperature. The ELS detector was set to a probe temperature of 40°C and a gain of 9, the nebulizer gas nitrogen adjusted to 2.4 bar. Peaks were assigned by spiking the samples with standard compounds and comparing the UV spectra (if applicable) and retention times.

RESULTS

Developing analytical methods for the quantification of saponins is challenging because most of these compounds have no chromophore and sensitive detection by UV is not possible. ELS detection, with detection based on scattering light and not absorption, proved to be a useful alternative. The separation of three *T. terrestris* samples by ELS detection is demonstrated in Figure 1 (sample A, leaves from Bulgaria; sample D, stems from India; and sample E, fruits from China; the corresponding structures of 1, 3, and 4 are given in Figure 2). Because the peaks are clearly separated and assignable, this detection technique seems to be the method of choice.

To develop and optimize a suitable HPLC system, several types of column material were assessed. The best results were obtained with RP-18 material with polar endcapping, like Aqua (Phenomenex, Torrance, CA) or Discovery from Supelco. The latter material was finally chosen because of a slightly better peak shape. During the isolation of 1, we noticed that the pure compound, once dissolved in methanol, immediately showed two spots on thin layer chromatographs. This second compound was isolated and identified as the acetal, 22-*O*-methylprotodioscin. To avoid the formation of this artifact, a water acetonitrile gradient was used as mobile phase and gave satisfactory results. The use of a buffer or the addition of modifiers did not further improve the separation.

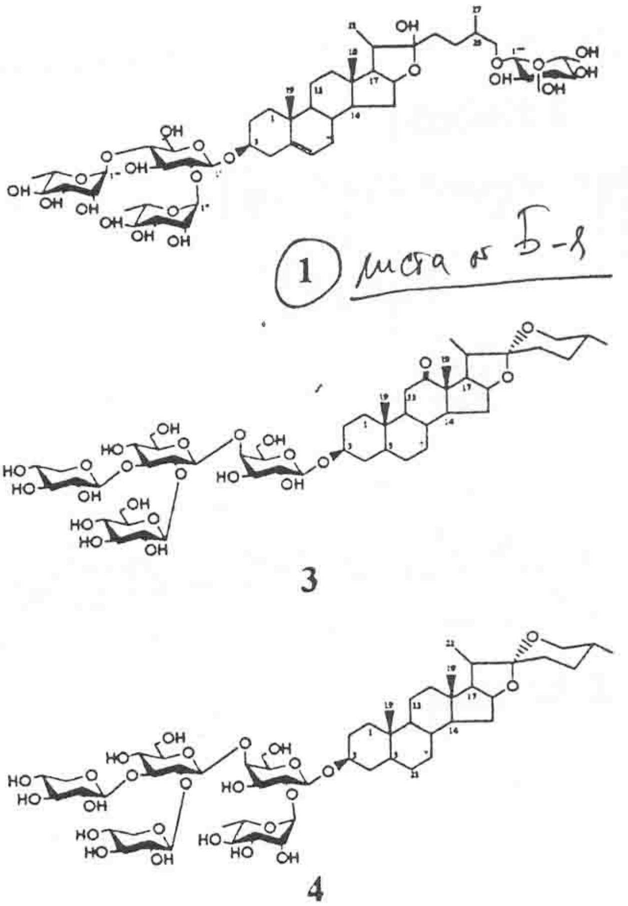
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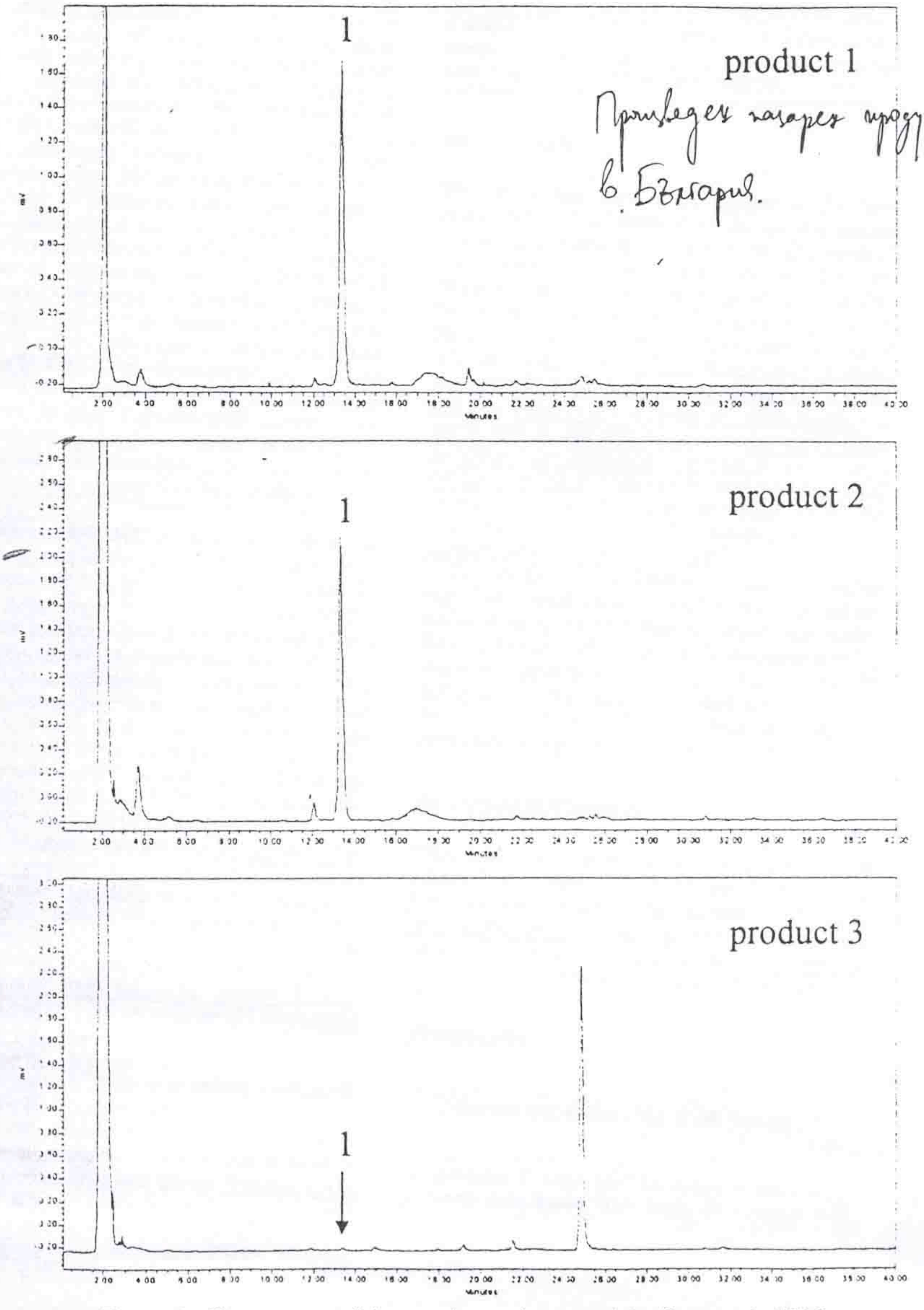
Figure 2. Structures of the saponins 1, 3, and 4.

Table 1. Amount (g/100g) of Protodioscin (1) in Different Samples and Market Products[a]

Sample Product	Plant Part Analyzed	Origin	Percentage of 1 in Product	δ^b ($n = 3$)
Sample A	Leave	Bulgaria	1.337	1.35
Sample B	Stem	Bulgaria	0.276	1.49
Sample C	Fruit	Bulgaria	0.245	2.02
Sample D	Stem	India	0.024	2.66
Sample E	Fruit	China	0.063	2.41
Sample F	Fruit	China	—	—
Sample G	Fruit	China	0.089	1.80
Product 1	Not specified (extract)	Bulgaria	6.492	0.55
Product 2	Aerial parts	Not specified	0.847	1.08
Product 3	Fruit, root (extract)	Not specified	0.176	1.89

[a] Data represent the means of three replicates.

[b] Standard deviations.



Произведен пазарен продукт
в България.

Figure 3. Chromatogram of three market products containing *T. terrestris*. HPLC conditions same as in Figure 1, with assignment of peaks according to Figure 2.

Test procedures for the assessment of the quality level of pharmaceutical products require the determination of certain analytical parameters like accuracy, precision, peak purity, linearity, and limit of detection. The accuracy of a method can be described as recovery. We spiked one sample (sample B) with a known amount of 1 and calculated the recovery rate; 98.7% of the theoretical amount of the compound was found. An indicator for precision is the standard deviation (δ). All samples were injected in triplicate and the standard deviation for 1 was less then 2.66% for all samples and far below the tolerated 5.0%. Peak purity was confirmed by injecting a concentrated solution of sample A (10 mg dissolved in 1 mL of 50% aqueous acetonitrile) and detecting by PDA (photodiode array) and ELS; no indications of impurities could be found. As mentioned in the Experimental Section, the calibration curve for 1 was linear from 31.2 to 1000.0 μg mL, with a correlation coefficient (R^2) of 0.9977. The limit of detection for protodioscin was 10.0 μg mL.

Because no analytical method for the determination of protodioscin was available so far, little is known about the distribution of this compound in different plant parts or variations in the content due to geographical location. Before investigating these points, we had to ensure that our method of extraction was sufficiently exhaustive. For this reason we extracted one sample (product 2) four times with 3.0 mL of the extraction solvent and analyzed each extract separately. The first extraction step yielded 65.0% of the total 1, the second 29.5%, and the third 5.5%. In the fourth extraction, no more protodioscin was detectable.

Several different samples from Bulgaria, India, and China were analyzed; if possible the samples were separated according to specific plant parts (leaves, fruits, stems). The results of this study are shown in Table 1 and reflect the substantial diversity among the samples (see also Figure 1). All the samples from Bulgaria (sample A to C) contained a rather high percentage of 1, with most of it in the leaves and less in stems and fruits. Sample D showed a totally different profile in the saponin content; 1 is only a minor compound (0.023%), but 2–4 are most dominant. Compounds 3 (Terrestrosin K) and 4 have been reported from *T. terrestris* previously.[5,7] Compound 2 is currently being isolated by our group. Samples E–G (fruits from China) contained either no or only small amounts of 1. Finally, three *T. terrestris* market products were analyzed (Figure 3). Product 1, manufactured in Bulgaria and containing a standardized plant extract, showed the highest content of protodioscin (6.49%), whereas the other two (0.85 and 0.17%) were significantly lower.

DISCUSSION

The assay described in this paper is the first report of an HPLC method for the determination of steroidal saponins in *T. terrestris*. The method is accurate and precise and was successfully used to analyze different samples of puncture vine. The results of this study showed large variations not only in the content but also in the composition of the saponins itself. *T. terrestris* leaves from Bulgaria had the highest percentage of 1, with >1.3%, whereas fruits generally seem to contain much less 1. All the samples originating from Bulgaria (also including a market product) were rather rich in 1; this result and the fact that samples like D show a totally different profile could be an indication for the presence of different chemotypes of *T. terrestris*.

The analysis of marketed products revealed significant product-to-product variations, which make a comparable usage of them impossible. Even if these products are only considered to be dietary supplements, some of their compounds are pharmacologically active and quality assurance is necessary. This method provides an important analytical tool towards that end.

ACKNOWLEDGMENTS

This work was supported in part by the United States Department of Agriculture, ARS Specific Cooperative Agreement No. 58-6408-7-012. The authors thanks NutraSource (San Carlos, CA) for providing the plant material and ChromaDex (Irvine, CA) for financial support of this project.

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