**Objectives:** As the first FDA-approved phosphodiesterase type 5 inhibitor, sildenafil (SDF) is widely used in the treatment of erectile dysfunction due to its strong pharmacodynamic activity. Since many food supplements are now involved in illegal adulteration, the presence of SDF in food supplements is very important because of their toxicological risks. In this study a simple fast, reliable high-performance liquid chromatography method with ultraviolet (UV) detector has been developed and validated for SDF analysis in herbal dietary supplements (HDSs).

**Materials and Methods:** 10 mM phosphate buffer containing 0.1% triethylamine (pH 3.5) and acetonitrile (65:35, v/v), as mobile phase was applied isocratically to a reverse phase C18 analytical (4.6×250 mm, 5 µm) column. Chromatographic separation was achieved by a C18 reverse-phase analytical column 4.6×250 mm, 5 µm particle size, using acetonitrile, with 10 mM phosphate buffer containing 0.1% triethylamine (65:35, v/v, pH 3.5) as a mobile phase. The mobile phase flow rate was 1 mL min⁻¹ and the column temperature was 35°C. The UV detector was set at 293 nm. The liquid-liquid extraction method used in the study provided a simple and practical method for the recovery of SDF in HDSs and their obtained values ranged from 87.6 to 111.7%.

**Results:** The method showed linearity with an excellent correlation coefficient (r²>0.999). Moreover, it was specific and sensitive with the limit of quantification, 6.5 ng mL⁻¹. Intraday and interday method precision was ±8.2 (relative standard deviation %). Intraday and interday method accuracy was between -4.0 and 7.1 (RE%). The method was strong according to the robustness test results obtained from UV detection, mobile phase buffer pH, column temperature, and flow rate changes. The described procedure was simple, fast, precise, and feasible for routine adulteration analysis of SDF, especially in food control or toxicology laboratories. This method was successfully applied to 50 individual solid and liquid form HDSs. The described procedure was found to be simple, rapid, precise and feasible for routine adulteration analysis of SDF, especially in food control or toxicology laboratories.

**Conclusion:** The results showed that 37 out of 50 samples of HDSs (represented 74.0%) examined contained SDF between 0.01 and 465.47 mg/g, 150.87±127.48 (mean ± standard deviation), which could lead to serious health problems and might even be fatal for consumers. The described procedure was found to be simple, rapid, precise and feasible for routine adulteration analysis of SDF, especially in food control or toxicology laboratories.

**Key words:** Sildenafil, adulteration, herbal dietary supplements, validation, high-performance liquid chromatographic-ultraviolet detection
The treatment of certain heart impairments. This side effect, which occurs in combination with nitrates and can cause risk of death, is very important toxicologically. Clinical studies have also reported blindness in one eye as an adverse effect of administration of SDF. SDF also has a high affinity for phosphodiesterase type 6 (PDE-6), which is a retinal enzyme involved in phototransduction. The inhibition of PDE-6 can result in a situation known as blue tinge, which prevents the ability to distinguish between blue and green colors. Although only 3% of patients report visual disturbances, this blue-green impairment can cause problems when fulfilling certain tasks. For example, this degradation can lead to problems for pilots during night flights or adverse meteorological conditions.

Because people consider that natural substances are safer and healthier than synthetic derivatives, herbal dietary supplements (HDSs) have widely increased as alternatives to chemically synthetic products recently. The adulteration with SDF in varying doses of herbal products sold for the treatment of erectile dysfunction is a serious potential health hazard. Although drugs containing SDF need to be prescribed under medical supervision, they are used without prescription in many countries, including Turkey. The sale of these medicines without medical advice causes patients to use them in high doses to gain more effect. It is known that the adulteration of HDSs with SDF is illegal and when a patient who is undergoing medical treatment with SDF also uses HDSs adulterated with SDF serious results may emerge. However, the presence of HDSs containing SDF marketed without controls in Turkey and around the world is frequently reported.

Therefore, consumption of HDSs that might be adulterated with SDF can produce serious toxic effects. For the purposes of quality control and product safety, analytical procedures need to be established that can selectively detect and quantify SDF in dosages in adulterated herbal products.

Several techniques are reported for the determination of SDF in many kinds of food supplements and pharmaceutical formulations, namely flow injection analysis, thin layer chromatography (TLC), spectrophotometry, spectrofluorometry, high-performance liquid chromatography (HPLC)-ultraviolet (UV), HPLC-diode array detection, HPLC-electrospray ionization-mass spectrometry (ESI-MS), micellar electrokinetic chromatography, electrospray tandem (ESI)-MS, gas chromatography (GC)-MS, liquid chromatography (LC)-MS, and LC-MS. In addition, low analytical sensitivity, inadequate intraday and interday reproducibility values, and inappropriate recovery amounts cause problems with the use of these techniques.

**Figure 1.** Chemical structure of SDF (A) and CZP (B) used as an internal standard

SDF: Sildenafil, CZP: Clozapine
Spectrophotometer analyses can lead to false positive results due to chemical interactions with molecules with similar chemical properties through the absence of pre-separation through a column. TLC assays do not show sufficient analytical sensitivity in routine assays with the long analysis times and difficulties of quantification. Analysis of SDF by GC-MS does not show sufficient analytical sensitivity due to its chemical properties, that is its low transition to the gas phase. Although the LC-MS is a device with high analytical sensitivity, it is less common in toxicological analysis laboratories due to its high cost. Moreover, its per analysis cost is higher than that of other techniques. HPLC-UV-based analytical techniques provide sufficient analytical sensitivity and reproducibility to determine the amount of SDF in food supplements, biological fluids, and other many matrices.

Our study of the analysis of SDF content in HDSs with HPLC-UV provides a significant advantage with high analytical accuracy and precision, low cost per analysis, and fast retrieval of results. This method was based on separation with an analytical column 4.6×250 mm, 5 µm particle size, using acetonitrile, and 10 mM phosphate buffer (pH 3.5) containing 0.1% triethylamine (65:35, v/v) as the mobile solvent. The UV detector was set at 293 nm and the total analysis time was 7.5 min. Clozapine (C2P) was used as an internal standard (ISTD). The developed method was also validated with linearity, accuracy, precision, sensitivity, recovery, and robustness according to the ICH-2005 guideline. The validity of the developed method has been proved by analyzing 50 HDSs that were liquid (liquid and paste) and solid (tablet, capsule, and powder) form. The results showed that products sold as HDSs for the treatment of erectile dysfunction seriously threatened public health, since SDF content was found in terms of SDF contents (150.87±127.48, x± Standard deviation).

MATERIALS AND METHODS

Chemicals and reagents
Pure reference samples of SDF (Figure 1a) and CZP as the ISTD (Figure 1b) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol and acetonitrile were obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade triethyl amine, orthophosphoric acid, potassium dihydrogen phosphate, sodium carbonate, and sodium sulfate were purchased from Merck (Darmstadt, Germany). Ultrapure water was made by Elga Purelab (High Wycombe, United Kingdom) system. A polytetrafluoroethylene disc filter (0.45 µm) was used to purify the HDSs. Membrane filters with a pore size of 0.45 µm from Millipore (Burlington, MA, USA) were used for filtration of the mobile phase.

Instrumentation
The separation and quantification were performed by HP Agilent 1100 series (Palo Alto, CA, USA) HPLC system, equipped with a UV detector. A HPLC system was employed in the present study; it consisted of a gradient pump (G1311A), a degasser (G1312), a column oven (G1316A), a UV detector (G1314A), and a Rheodyne 7725i manual injector with a 20-µL sample loop. ChemStation® version 08.03 software was employed for data collection and handling.

Chromatographic conditions
Separations were carried out on an ACE-5 (Aberdeen, Scotland) reverse phase C8 analytical column (4.6×250 mm, 5 µm particle size). The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL min⁻¹ at 35°C. Chromatographic quantitation was conducted at 293 nm. The mobile phase consisted 10 mM phosphate buffer (containing 0.1% triethyl amine) and acetonitrile (65:35, v/v) before delivery into the HPLC system. The mobile phase pH was adjusted to 3.5 with 1 M phosphoric acid. It was degassed before every use over 30 min using an ultrasonic bath.

Collection of samples and its preparation to analysis
Fifty-one different HDSs advertised to enhance sexual performance in men from individual brands were purchased online and from an herbal market in Sivas. All the solid drug and tablet samples were pulverized by a mortar. An exactly weighed 200 mg (liquid, solid, and powdered) sample was dissolved in a 10 mL mixture of methanol and 1.5% Na₂CO₃ (7:3, v/v). After that, the mixture was dried with 250 mg of Na₂SO₄. The extract was mixed over 10 min at 1200 rpm in a rotator shaker and dissolved in an ultrasonic bath over 30 min. The sample was centrifuged at 3000 rpm for 5 min and approximately 10 mL of the upper phase was transferred to a clean test tube and filtered by a 0.45-µm disc filter. Then 10 µL of sample filtrate and 10 µL of ISTD (100 µg mL⁻¹) were completed to 10 mL with the mobile phase and vortexed at 1200 rpm for 1 min. Finally, 20 µL of this mixture was applied to the liquid chromatograph under specified chromatographic conditions.

Preparation of standard solutions
A SDF stock solution (1 mg mL⁻¹) was prepared in methanol and stored at -20°C. It has been quantitatively determined that it is chemically stable for at least 3 months. Working solutions were prepared by main stock solution weekly in methanol at 10, 20, 30, 40, 60, 80, and 100 µg mL⁻¹ concentrations. The ISTD main stock solution (10 mg mL⁻¹) was prepared with methanol to yield a 100 µg mL⁻¹ working solution.

RESULTS AND DISCUSSION

Method validation
The method developed was validated in terms of specificity, linearity, accuracy, precision, sensitivity, recovery, and robustness. In order to obtain accurate and precise measurements in accordance with the International Harmonization Conference, the intraday and interday validity protocols were implemented taking into account the reproducibility of the method and instrument.24

Specificity
Specificity is the ability of the method to measure the analyte response in the presence of all the impurities that may arise from the analyte and other conditions. The UV detector was
set to a wavelength of 293 nm displaying optimum sensitivity. Figures 2a, 2b, and 2c show chromatograms of blank, spiked, and real samples illustrating the high resolution with no interference and too short separation time (7.5 min). The method demonstrated excellent chromatographic specificity with no endogenous interference at the retention times of SDF and CZP (6.2 and 6.7 min, respectively) as an ISTD.

**Linearity and selectivity**

After establishing the chromatographic conditions, the linearity of SDF was studied by preparing standard solutions at 7 different levels ranging from 10 to 1000 ng mL\(^{-1}\). Calibration was performed by linear regression of peak-area ratios of SDF to the ISTD versus the respective standard concentration. For each concentration 3 individual replicates were injected and linearity was obtained for SDF with high correlation coefficients (\(r^2\)) over 0.999 (Table 1). System suitability parameters are tabulated in Table 2.

**Precision and accuracy**

The precision and accuracy of the method were examined on 5 consecutive days. Precision, defined as relative standard deviation (RSD), was determined by five individual replicates at three different concentrations, which were 20, 100, and 500 ng mL\(^{-1}\) (n=5). Table 3 shows the RSD values of low, medium, and high concentrations (20, 100, and 500 ng mL\(^{-1}\), respectively) to present inter- and intraday precision. Accuracy, defined as relative error (RE%), was also determined for the same concentrations of analytes (Table 3).

**Recovery**

The recovery of the method was calculated by comparing the results obtained from the application of the standard sample prepared in methanol to the samples prepared in the same concentration in the herbal sample. The recovery results conducted at 20, 100, and 500 ng mL\(^{-1}\) are tabulated in Table 3.

**Sensitivity**

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve, according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines:24 (LOD=3.3\(\sigma\)/S, LOQ=10\(\sigma\)/S, where \(\sigma\) is the standard deviation of the response and S is the slope of the calibration curve). The LOD and LOQ values of the method were 1.94 ng mL\(^{-1}\) and 6.46 ng mL\(^{-1}\), respectively.

**Robustness**

Significant changes were not observed in the analytical signals upon changing the UV wavelength value (±2 nm) (p>0.05), mobile phase buffer pH (±0.3) (p>0.05), column temperature (±4°C) (p>0.05), or mobile phase flow rate (±0.1 mL min\(^{-1}\)) (p>0.05). The statistics were evaluated by SPSS 15 - Kruskal-Wallis test. In addition, changes in analyst, analytical column, the source of chemical, and/or solvent did not lead to significant changes in chromatographic signals. Robustness experiments demonstrated that the method created data of acceptable precision and accuracy. After the data from the validation tests were found to be appropriate for safe analysis, the survey of the SDF in real HDSs was initiated.

**Analysis of samples**

A quantitative investigation of SDF contents in 50 HDSs used for the treatment of erectile dysfunction sold in an herbal market and on the Internet was conducted. Solid and liquid supplement samples were prepared according to the sample preparation procedure.

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**Table 1. The analytical parameters of the proposed HPLC method**

| Parameter                     | SDF            |
|-------------------------------|----------------|
| Calibration curve (ng mL\(^{-1}\)) | 10.0-1000.0    |
| Slope                         | 0.001          |
| Standard error of slope       | 0.00013        |
| Intercept                     | -0.0028        |
| Standard error of intercept   | 0.0573         |
| Coefficient of determination (\(r^2\)) | 0.9992       |
| LOD (ng mL\(^{-1}\))          | 1.9            |
| LOQ (ng/mL)                   | 6.5            |
| Retention time for SDF (min)  | 6.2            |
| Retention time of IS (min)    | 6.7            |

*Number of repeated measurement n=6, LOD: Limit of detection, LOQ: Limit of quantification SDF: Sildenafil, HPLC: Hight performance liquid chromatography

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**Table 2. System suitability parameters**

| Parameters | \(T_n\) | K | \(\alpha\) | (Rs) | Symmetry factor |
|------------|---------|---|---------|------|----------------|
| SDF        | 6.2     | 5.6 | 1.1     | 3.3  | 1.0            |
| IS         | 6.7     | 6.3 | -       | -    | 1.0            |

\(T_n\)=Retention time, K= Capacity factor for SDF and IS, where k=the capacity factor for drug and IS,

\(\alpha\)=Separation factor, calculated as \(K_2/K_1\), Rs= Resolution 2 (t2-t1) / (w1+w2), where t2 and t1 are the retention times of the SDF and IS and w2 and w1 are the half-peak width of the drug and IS, respectively.
procedure explained above. The measurement results are given in Table 4. Sample chromatograms are shown in Figure 3.

CONCLUSION

In our study, the presence of SDF was quantitatively investigated in 50 products described as ginseng, panax, epimedium, herbal mixtures, and herbal supplements on product packages by the developed and also validated method in accordance with ICH (2005) Guidelines. The SDF positive samples known as HDSs were in the form of pills, capsules, powders, syrups, liquids, and pastes. These food supplements that were positive for SDF analysis were all different shades of green and brown. Some of the products analyzed gave the impression that they were malodorous herbal medicines. In addition, some analyzed herbal products were enriched with chocolate, lemon juice, and other sweeteners. In addition, there was a malodorous product that was difficult for a person to swallow in one go because of its size, 3 cm in diameter. Powdered products were usually brown and gray in color and had a sharp spice smell. Liquid products were light green. It is thought that these processes are intended to convince users that the products are natural.

It has been reported that SDF is a safe drug for therapeutic use. However, the use of SDF-containing medical tablets as well as a user who considers taking a natural product will cause an overdose. The tendency to use more of the recommended product for the expected increase in activity, as well as the product thought to be natural, is often common in individuals who use food supplements. It is also known that the use of SDF, in combination with organic nitrates significantly increases systemic blood pressure lowering effects. An overdose can create a serious risk for the cardiovascular system. It is known that individuals who frequently use these products are men over 50 years old and cardiovascular system diseases increase in this group during this period.

However, SDF has been found in quantities of 0.01 to 465.47 mg/g (150.87±127.48, mean ± standard deviation) in 37 products with positive results, which could harm human health even after a single use (Table 4). It has been reported that these products have been sold at a higher rate than others with low SDF content. It has also appeared on product packages that the same products are more effective in the treatment of erectile dysfunction. It has been determined that some of the herbal products studied contain SDF in almost half of their weight.

This study was carried out due to the fact that there is no method in the literature that is analyzed with a simple, uncomplicated analytical device with low cost of analysis. In our study, a new HPLC-based analysis method was developed for the determination of illegal addition of SDF to herbal products and the method was validated and the applicability of the method was shown with 50 samples. The method developed has significant advantages, such as rapid analysis with a total analysis time of 7.5 min, a low sample requirement (0.2 g), and high analytical accuracy (LOD: 1.88 ng mL⁻¹). The method developed is noteworthy due to its intraday and interday reproducibility values, which were between 2.34 and 8.13 (RSD%) for precision and between -4.02 and 7.14 (RE%) for accuracy, and recovery values were between 87.57% and 111.65%. The method was also proven to be robust against changes in some analytical conditions such as mobile phase content, column temperature, and flow rate, as well as robustness tests. The method was also easily applied to 50 herbal products and the analyses were successfully completed in all products. In addition, during sample preparation and analysis only a small amount of solvent was used. Our study methodology was compared with the other HPLC study results in Table 5.

A total of 50 products marketed as herbal products for the treatment of erectile dysfunction were exposed to serious abuse. Measures to prevent international marketing of these products, which seriously threaten public health, are needed. Although the products examined are visually similar to a medicinal product, they are marketed as “herbal products” online and in markets selling herbal products. These synthetic

| Expected conc. (ng mL⁻¹) | Intraday (n=5) | Interday (n=5) | Observed concentration (n=5) |
|--------------------------|----------------|----------------|-----------------------------|
|                          | Estimated conc. ±SD | Precision (RSD%) | Accuracy (RE%) | Estimated conc. ±SD | Precision (RSD%) | Accuracy (RE%) | Values of the recovery (%) | Mean recovery %±SD |
| 20                       | 22.43±1.54 | 8.13 | 7.14 | 21.05±0.97 | 4.21 | 5.25 | 87.57±108.73 | 98.15±9.52 |
| 100                      | 106.47±2.43 | 2.34 | 6.47 | 104.98±6.05 | 5.90 | 4.98 | 92.08-103.61 | 97.85±5.83 |
| 500                      | 479.89±15.80 | 3.31 | -4.02 | 486.07±22.46 | 4.58 | -2.79 | 98.59-111.65 | 105.12±7.00 |

"Concentrations" are abbreviated as “conc.”, SDF: Sildenafil, SD: Standard deviation, RSD: Relative standard deviation, RE: Relative error.
### Table 4. Results of SDF values determined in HDSs

| Sample number | The presentation of the product | The detected SDF amount (mg/g) | Sample number | The presentation of the product | The detected SDF amount (mg/g) | Sample number | The presentation of the product | The detected SDF amount (mg/g) |
|---------------|--------------------------------|-------------------------------|---------------|--------------------------------|-------------------------------|---------------|--------------------------------|-------------------------------|
| 1             | Dust                           | 0.01                          | 18            | Tablet                         | 48.51                         | 35            | Dust                           | ND                           |
| 2             | Capsule                        | ND                            | 19            | Dual tablet                    | 246.41                        | 36            | Dust                           | ND                           |
| 3             | Tablet                         | 151.78                        | 20            | Dust                           | ND                            | 37            | Tablet                         | 91.83                        |
| 4             | Capsule                        | 34.15                         | 21            | Capsule                        | 153.64                        | 38            | Dual tablet                    | 30.77                        |
| 5             | Tablet                         | 290.86                        | 22            | Tablet                         | 188.53                        | 39            | Dust                           | ND                           |
| 6             | Capsule                        | 1.95                          | 23            | Dual tablet                    | 261.89                        | 40            | Dust                           | ND                           |
| 7             | Capsule                        | ND                            | 24            | Tablet                         | 75.95                         | 41            | Tablet                         | 55.35                        |
| 8             | Liquid                         | 15.52                         | 25            | Tablet                         | 148.68                        | 42            | Tablet                         | 241.79                       |
| 9             | Capsule                        | 287.56                        | 26            | Tablet                         | 0.90                          | 43            | Capsule                        | 392.85                       |
| 10            | Tablet                         | 262.70                        | 27            | Tablet                         | 116.18                        | 44            | Dual tablet                    | 465.47                       |
| 11            | Dust                           | ND                            | 28            | Tablet                         | 344.70                        | 45            | Dust                           | ND                           |
| 12            | Tablet                         | 127.34                        | 29            | Dust                           | ND                            | 46            | Dust                           | ND                           |
| 13            | Tablet                         | 201.85                        | 30            | Dust                           | ND                            | 47            | Tablet                         | 91.72                        |
| 14            | Tablet                         | 0.02                          | 31            | Tablet                         | 288.74                        | 48            | Tablet                         | 104.76                       |
| 15            | Dust                           | ND                            | 32            | Capsule                        | 346.48                        | 49            | Capsule                        | 25.60                        |
| 16            | Dust                           | 0.40                          | 33            | Capsule                        | 168.53                        | 50            | Tablet                         | 247.32                       |
| 17            | Paste                          | 13.36                         | 34            | Dual tablet                    | 58.09                         |               |                                |                               |

Minimum detected concentration: 0.01

Maximum detected concentration: 465.47

Average: 150.87

Standard deviation: 127.48

SDF: Sildenafil, HDSs: Herbal dietary supplements, ND: Not detected

### Table 5. The validation parameters and chromatographic properties of SDF determination methods in the literature

| Study                  | LOQ       | Retention times (min) | Linearity | Linear range | Mobile phase                                                                 | Flow (mL min⁻¹) | Detector and detection value | Total analysis time (min) |
|------------------------|-----------|-----------------------|-----------|--------------|--------------------------------------------------------------------------------|-----------------|-----------------------------|---------------------------|
| Reddy and Reddy 2008⁷  | 8.2 ng mL⁻¹ | 4.1                   | 0.9990    | 0.1-30 µg mL⁻¹ | Phosphate buffer (pH 7.0) and acetonitrile/ (3:7, v/v)                      | 0.8             | UV 228                      | 15                        |
| Daraghmeh et al. 2001  | 12.2 µg mL⁻¹ | 9.5                   | 0.9999    | 2-12 µg mL⁻¹  | 64-257 µg mL⁻¹                                                                 | 1               | UV 240                      | 10                        |
| Dinesh et al. 2002     | 0.05 µg mL⁻¹ | 10.0                  | 0.9995    | 0.05-7.5 µg mL⁻¹ | Water and acetonitrile (48:52, v/v)                                         | 1               | UV 245                      | 10                        |
| Nagaraju et al. 2003   | -         | 5.2                   | 0.9950    | 90.5-99.9 µg mL⁻¹ | 0.05 M potassium dihydrogen orthophosphate and acetonitrile (3:7, v/v)     | 1               | DAD 230                     | 15                        |
| Yang et al. 2010       | 2 ng mL⁻¹  | 2.0                   | 1.0000    | 0.2- 200 µg mL⁻¹ | 30 mM ammonium formate (pH 3.0) and acetonitrile (7.3, v/v)                  | 1.3             | UV 230                      | 7                         |

SDF: Sildenafil, LOQ: Limit of quantification, UV: Ultraviolet, DAD: Diode array detection
products have been found to contain SDF in amounts that could threaten human health even after a single use.

In the present study, the established HPLC-UV method is fast, cheap, and accurate for reference laboratories concerned with food control or toxicology. The applicability of the developed method to herbal products was proved by analysis of 50 HDSs. At the same time, it was shown that herbal products marketed for the treatment of erectile dysfunction in the market contain high amounts of SDF, which can pose a risk to human health.

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