

DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR PROTODIOSCIN IDENTIFICATION AND QUANTIFICATION IN HERBAL SUPPLEMENTS WITH *TRIBULUS TERRESTRIS* L.

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Abstract: *Tribulus terrestris* L. (TT) became a very popular plant due to its effects in sexual disorders. The pharmacological actions seem to be related with its content in sterolic saponins. Protodioscin has been reported to be the main compound from TT products with anabolic effects. Because of the popularity of the plant, numerous herbal supplements are consumed around the world. Quantification of steroidal saponins through HPLC methods is sometimes difficult. Several methods have been proposed but not all are reproducible. The aim of this study was to validate a LC MS/MS method for quantification of protodioscin in herbal supplements. Two herbal supplements found on the Romanian market have been chosen for this study. The compounds were separated on a C18 column with ammonium acetate buffer and acetonitrile as mobile phase. The ionization was performed in electrospray negative mode and detection of protodioscin was made by monitoring the sum of ions m/z 737.41, m/z 739.42 and m/z 755.42. In one of the herbal supplements, protodioscin was under the limit of quantification. In conclusion a simple and rapid method is proposed for the quantification of protodioscin in herbal supplements.

Keywords: *Tribulus terrestris*, protodioscin, LC-MS/MS.

1. Introduction

Tribulus terrestris L. (Zygophyllaceae family) is a popular plant that received great interest in the last decade. Because of its content in steroidal saponins the extracts from *Tribulus terrestris* (TT) are consumed as anabolic and sexual enhancers (Zhu et al., 2017). Protodioscin is thought to be responsible for the main pharmacological actions of TT products, although contradictory results have

been obtain in the recent years regarding the pharmacological effects, especially the anabolic effect (Neychev & Mitev, 2016). This suggest either that the herbal drugs collected from different regions contain significantly different compounds, or that the experiments were performed with extracts with different organs from TT.

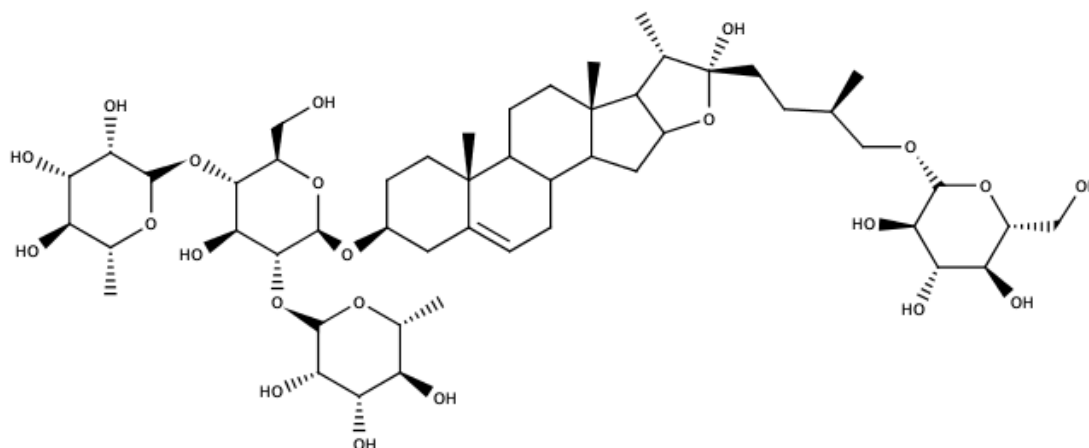


Fig. 1. Chemical structure of Protodioscin

However, due to the geographical influences, the chemical composition of TT products varies in large limits (Dinchev et al., 2008). As a response to the high demand of TT extracts, the availability and variability of herbal supplements increased with the risk of quality diminishment. Standardization and quality controls are necessary for the safety and phytoequivalence of the supplements. Today, LC–MS/MS is the most used technique in bioanalysis, and offers numerous possibilities for sensible detection of chemical compounds found in very low concentrations. Although there are some published papers with validated HPLC-DAD methods for protodioscin identification, these are hardly reproducible (Ganzera et al., 2001).

The aim of this present study was to develop a new, simple and rapid LC MS/MS method for protodioscin quantification in herbal supplements.

2. Materials and Methods

Plant material

Tribulus terrestris products were bought from a local pharmacy. The first product had its origin in Poland, and the second one was from China. The voucher specimens were deposited at the herbarium of Pharmacognosy and Phytotherapy, George Emil Palade University of Medicine, Pharmacy, Science and

Tehnology of Târgu Mureș, Romania (TT-1.2-17 and TT-2.2-17).

Chemicals and reagents

Protodioscin was obtained from Cayman Chemical. HPLC grade Acetonitril was obtained from Scharlau. Water was obtained from a Millipore Direct-Q water purification system (Millipore, Bedford, MA, USA). Other reagents used were of analytical grade.

LC MS/MS analysis

LC MS/MS analysis was performed using a QTOF 4600 (AB Sciex), UHPLC Flexar FX-10 (Perkin Elmer). Chromatographic separation of protodioscin was performed on a Phenomenex Luna C18, 4.6 x 100mm, 3 μm column. The mobile phase consisted of a mixture of 70% 1mM ammonium acetate buffer (phase A) and 30% acetonitrile (phase B) and the flow rate was set at 0.600 mL/min. Sample injection volume was 5 μl. The analysis time was 10 minutes for the samples and 3 minutes for the standard solutions. Ionization of analytes was performed in electrospray negative mode and the source parameters were set as follows: Spray voltage: -4200V, vaporizer temperature: 600 °C, Ion Gas Source 1: 40, Ion Gas Source 2: 30, Curtain Gas: 25, Declustering Potential: -300,

Ion Release Delay: 100, Ion Release Width: 10. Protodioscin detection was performed by monitoring the sum of ions m/z 737.41, m/z 739.42 and m/z 755.42 resulted from m/z 1047.7 at a collision energy of 65V.

Preparation of standard solutions

Two stock solutions of 200 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ protodioscin in HPLC grade acetonitrile were prepared. These stock solutions were used to prepare the calibration standard solutions with concentrations of 0.5, 1, 3, 5, 6, 8, 10 and 15 $\mu\text{g/mL}$ protodioscin by diluting the appropriate amount of stock solution with a solvent made up of acetonitrile and ultrapure water, at a ratio of 3:7.

Preparation of sample solutions

From the herbal drug (origin China) extracts were prepared using ultrasound assisted extraction. The herbal drug was accurately weighed and was extracted twice with water (in a ratio of 1:5) at 60 °C for 60 minutes. The extract was filtered through a 0.45 μm microporous cellulose syringe filter, and an aliquot of this solution was used for the analysis.

The capsules (origin Poland) of finished product were analyzed using sample solutions prepared by accurately weighting to 10 mL volumetric flasks the powder equivalent to one capsule (550mg) from a larger pooled sample for assay testing, and the entire content of one capsule for uniformity of content testing. Samples were further diluted with 3 mL HPLC grade acetonitrile and 5 mL ultrapure water. The solution was placed in an ultrasonic water bath, sonicated at room temperature for 60 minutes, and was further diluted up to the mark with ultrapure water. Before analysis the

samples were filtered through a 0.45 μm microporous cellulose syringe filter.

Accuracy evaluation was performed by the standard addition method and the recovery was calculated.

Preparation of placebo solutions

In order to check the existence of any interference from the excipients contained in the capsules, placebo solutions were prepared. Approximately 5mg microcrystalline cellulose and 0.15 mg magnesium stearate were accurately weighed in a 10 mL flask and 3mL acetonitrile and 5mL ultrapure water were added. The samples were sonicated for 60 minutes and then were diluted up to the mark with water. The solution was passed through a 0.45 μm microporous cellulose syringe filter and transferred to a HPLC vial.

3. Results and discussions

Eight calibration standard solutions in a concentration range of 0.5 - 15.0 μg protodioscin/ mL were prepared and injected for linearity testing. The average calibration curve was plotted using a 1/ y^2 weighting and the accuracy of each calibration standard was calculated. Not more than two calibration standards (and not the lower or upper limit of quantification) of a calibration curve failed accuracy testing, the recalculated concentration being within $\pm 15\%$ compared to the nominal concentration. The calibration curve plotted had a correlation coefficient (R) of >0.99 and is thus considered linear throughout the interval of 0.5 - 15.0 $\mu\text{g/ mL}$.

The lower limit of quantitation was set as the smallest concentration in the standard calibration curve (0.5 $\mu\text{g/mL}$) (**Table 1**).

Table 1. Table appearance

Linearity	R = 0.9969
Concentration range	0.5-15.0 $\mu\text{g/mL}$
Lower limit of quantification (LLOQ)	0.5 $\mu\text{g/mL}$

The representative MS spectrum of protodioscin under negative ionization mode is illustrated in **Fig. 4** protodioscin was identified by comparing the mass spectrum, extracted ion chromatogram and retention time with reference standard. Protodioscin was detected at 2.24 min (**Fig. 3**).

HPLC-UV analysis usually has a number of drawbacks compared to LC-MS techniques, related mostly to the interference of other compounds when analyzing complex matrices, as sometimes it is not possible to chromatographically separate compounds or due to low concentrations not detectable with conventional UV-Vis detectors (Ganzera et al., 2001). In our previous research, we concluded that protodioscin was not detectable in using UV-Vis detection; therefore, an LC-MS method was developed. Although there are published papers were methods for quantitative and qualitative determinations of protodioscin in HPLC-UV were developed, HPLC-UV analysis of sterolic saponins is usually very difficult because these compounds do not have a chromophore and show a very weak UV absorption at very unspecific wavelengths (Lozano et al., 2017). Pavin et al. (2018) determined the protodioscin concentration using a HPLC method with the mobile phase consisted of 0.025% acetic acid in water (solvent A) and acetonitrile (solvent B) with a flow rate of 1.0 mL/min, DAD detection at 250 nm and protodioscin was detected at aprox 43 min. Shishovska et al. (2015) detected protodioscin at 200 nm, using a HPLC-DAD

method with acetonitrile and water as mobile phase and a flow rate of 1.0 mL/min. The characteristic fragmentation patterns of protodioscin were investigated using ESI-MS in negative mode. Due to ionization of protodioscin being performed in negative mode ammonium acetate, which helps deprotonation of compounds, was used as aqueous mobile phase.

The assay of the powder pooled from 10 capsules was 92.63 $\mu\text{g}/550\text{mg}$ powder (equivalent to content of one capsule). The uniformity of protodioscin content in capsules was tested on 5 separate capsules, the average concentration of protodioscin was 78.12 $\mu\text{g}/\text{capsule}$ and the relative standard deviation (RSD) was 11.51% (**Table 2**). Thus, it can be concluded that there are large variations among the capsules from the herbal supplement. Analysis of placebo samples revealed that the sample do not interfere with the detection of protodioscin.

It has been demonstrated that the content in steroidal saponins of TT preparations influences the pharmacological effects (Gauthaman et al., 2002). Different phytochemical profiles are linked with different outcomes in *in vitro* and *in vivo* experiments (Semerdjieva & Zheljzakov, 2019). Protodioscin was not identified in the herbal drug from China, and the results are in concordance with Dinchev et al. (2008), who reported great variations in the content of TT different organs collected from different countries.

Table 2. Uniformity control of protodioscin content in capsules

Sample	Protodioscin concentration $\mu\text{g}/\text{capsule}$	Min-Max (μg)	Average	RSD
1	67.45	67.45 – 91.70	78.12	11.51
2	91.70			
3	75.24			
4	81.00			
5	75.19			

RSD – Relative standard deviation

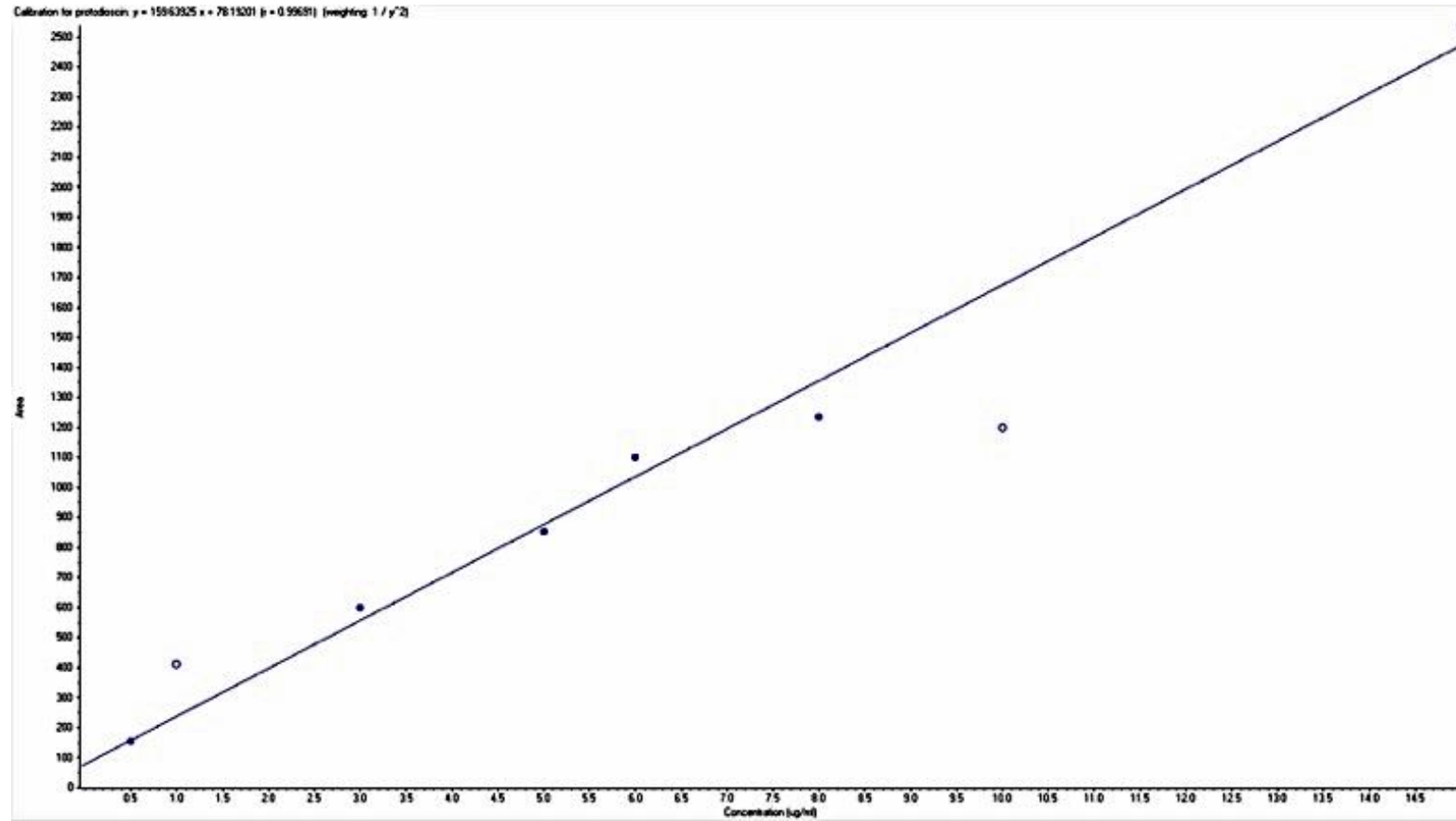


Fig. 2. Calibration curve

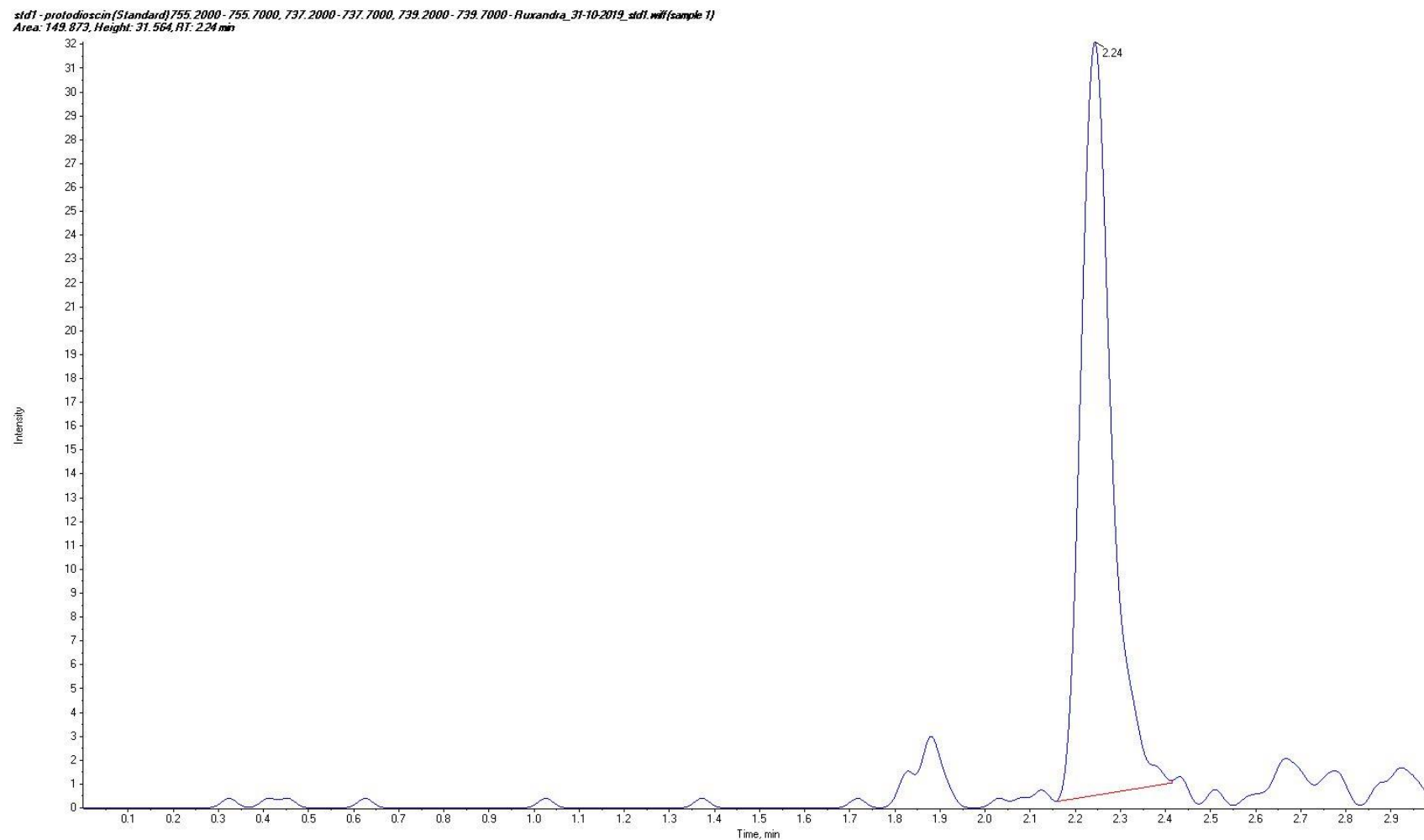


Fig. 3. Chromatogram of LLOQ protodioscin solution

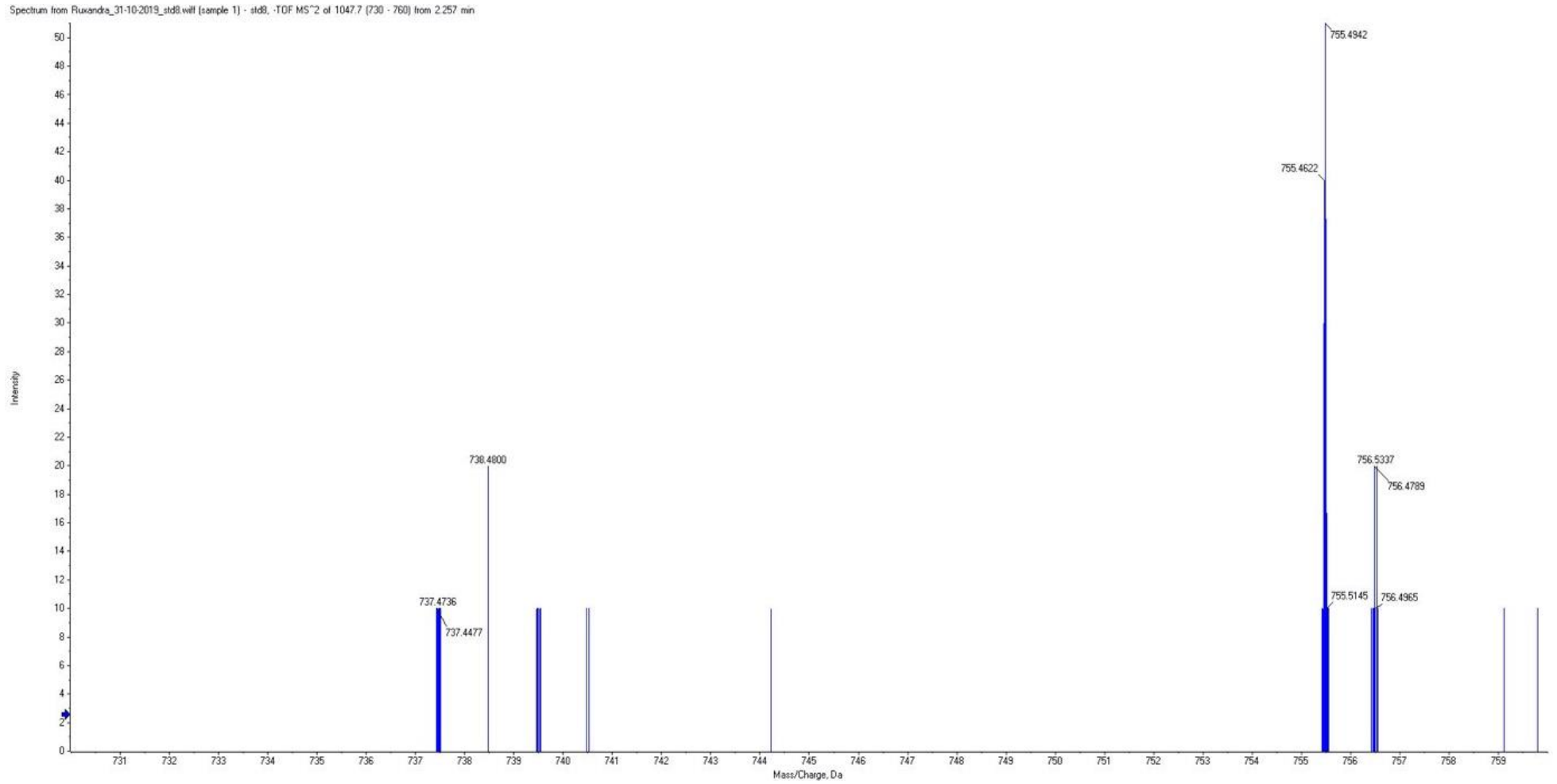


Fig. 4. MS/MS mass spectra of protodioscin

The content of protodioscin, for example, was lower in the Asian countries and the highest content was determined in the samples from Europe.

The industry of food supplements and therefore their consumption has increased constantly in the recent years. Because the quality control of supplements is usually minimal, and the standardization processes are scarce, the effects are in the best case

Although it was not the primary aim of this study, we have demonstrated that two different supplements with TT found on the Romanian market, have significantly different concentrations.

These findings demonstrate that the same outcome is impossible to achieve after the consumption of these supplements.

Conclusions

A rapid and reproducible LC-MS method was developed, which can be applied to other food supplements based on *Tribulus terrestris* extracts. As previously reported great differences are in the marketed supplements with *T. terrestris*, therefore therapeutic efficacy it is rightly questioned.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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