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Short communication

Determination of steroidal glycosides in *Yucca gloriosa* flowers by LC/MS/MS

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ABSTRACT

An high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) method, was developed for the quantitative analysis of the steroidal glycosides occurring in *Yucca gloriosa* flowers. The HPLC experiments were performed by means of an octadecyl-modified reversed-phase C-18 column and a binary mobile phase system under gradient elution conditions. The fragmentation patterns of steroidal saponins were analyzed by ESI–MS^n in positive ion mode and a specific multiple reaction monitoring MS/MS detection was developed for their quantitative determination. The described method provides high sensitivity and specificity for quantitative determination of the steroidal glycosides in *Y. gloriosa* flowers. Quantification was performed against an external calibration line obtained using each pure steroidal glycoside. Short- and long-term repeatabilities of the methods were better than 3 and 6%, respectively. The method was validated according to EMEA guidelines and applied to real samples.

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1. Introduction

Among the species of *Yucca* genus (Agavaceae), the best known is *Yucca schidigera*, a plant growing in California and Mexico, used in Indian traditional medicine. The extract of this plant finds wide commercial utilization for its high saponin content [1]; it is approved by Food and Drug Administration as a food additive, regarded with the "GRAS" label (Generally Recognised as Safe) [1]. Based on the applications of *Y. schidigera* products, we deemed it of interest to investigate another species of the same genus, *Y. gloriosa*. *Y. gloriosa* L. is a perennial, evergreen bush cultivated in eastern Georgia which easily adapts unfavorable climatic and soil conditions [2]. Previous investigations carried out on *Y. gloriosa* led to the isolation of steroidal saponins from different parts of the plant [2,8] (Fig. 1). Steroidal glycosides are superficially active substances and accordingly they have membranotrophic properties. When administered into the cultured plants, they act as phytohormones, thus a preparation based on the steroidal glycoside extract from *Y. gloriosa* flowers, named «Alexin», has been investigated as a plant growth stimulant [9]. Low concentration water solutions (0.0025–0.005%) of Alexin increased the productivity of agricultural and medicinal plants such as wheat, haricot bean, soy bean, potato, tomato, maize by 20–55% [9]. At the same time, Alexin is reported to improve the quality and to provide ecologically clean production. Alexin underwent state registration and was put into production. Previous phytochemical investigation on the steroidal saponins occurring in the flowers of *Y. gloriosa* led to the isolation of the spirostane saponins named yuccaloesides A, B, C, E (**1, 3, 5, 6**), degalactotigonin (**2**), and gitogenin 3-O-α-L-rhamnopyranosyl-β-lycotetraoside (**4**) [2–8]. These compounds show as aglycons smilagenin (**1**), tigogenin (**2**, **3**, **5**, **6**) and gitogenin (**4**) and possess sugar chains ranging from two to six sugar units. An interesting antifungal activity was shown by these compounds, particularly against yeast strains and several *Candida* isolates [10].

Steroidal saponins found in this species are not detectable by HPLC–UV analysis for the lack of an UV chromophore. Mass spectrometry could represent a candidate detection method, and in addition improvement in selectivity and specificity could be raised by using tandem mass spectrometry [11–14]. Thus, in the present study an LC/MS/MS method was developed for the quantitative analysis of steroidal glycosides in the MeOH extract of *Y. gloriosa* flowers.

2. Material and methods

2.1. Chemicals

Solvents used for the extraction were of high purity and purchased from Carlo Erba (Milano, Italy). HPLC grade methanol, acetonitrile and trifluoroacetic acid were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC grade water (18 mΩ) was prepared using a Millipore

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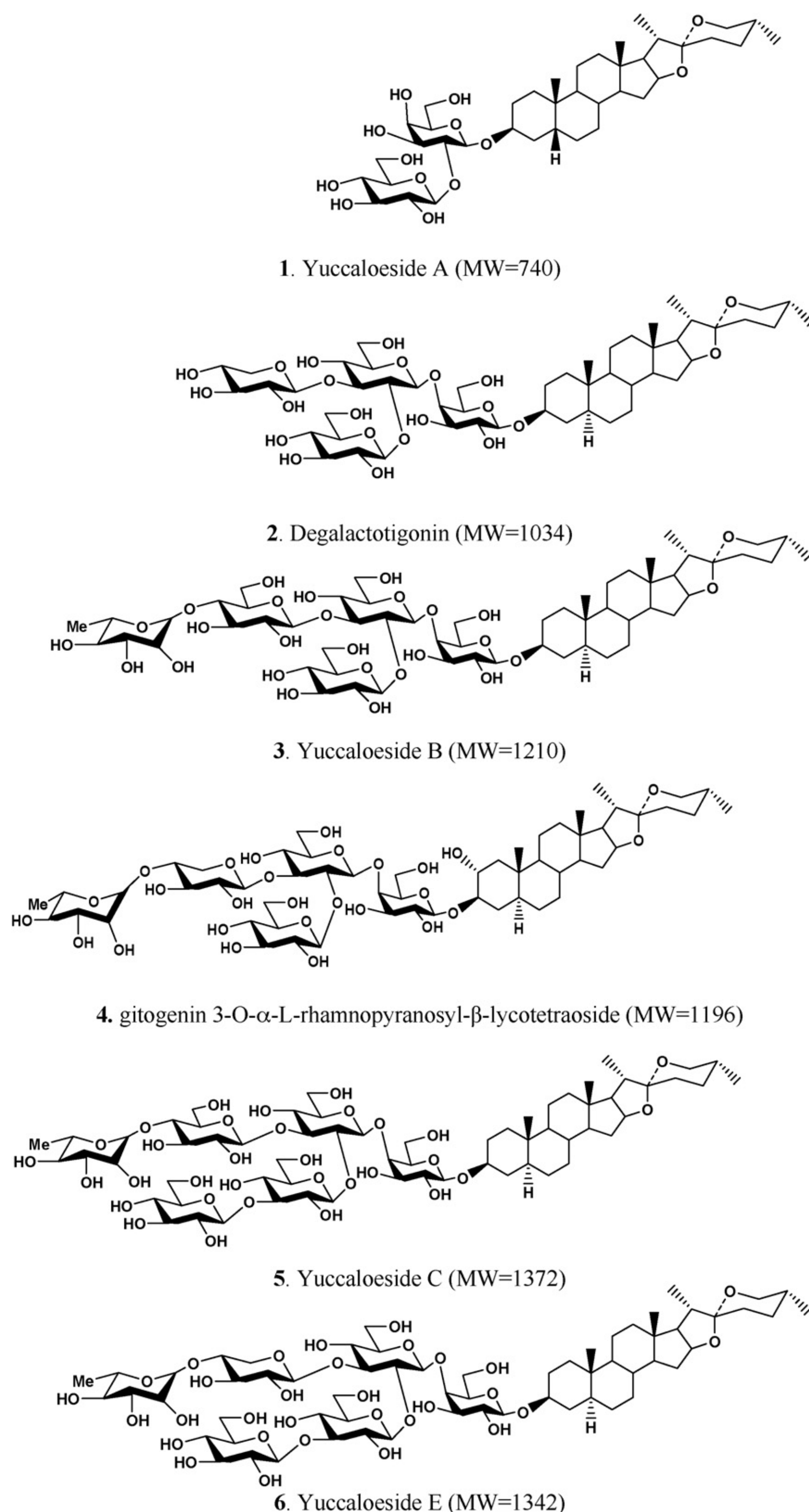

Fig. 1. Structures of compounds **1–6**.

(Bedford, MA, USA) Milli-Q purification system. Standards of yuccaloesides A, B, C and E, degalactotigonin and gitogenin 3-O-α-L-rhamnopyranosyl-β-lycotetraoside were isolated in our previous studies [10]. Each compound purity was over than 98% (measured by HPLC analysis), and they present stability in methanol solution.

2.2. Plant material and the preparation of extracts

The flowers of *Y. gloriosa* were collected in October 2008 in the experimental field of the Iovel Kutateladze Institute of Pharmacochemistry, Tbilisi, Georgia. A voucher specimen (no. 259) was deposited at the Institute of Pharmacochemistry.

In order to obtain the enriched saponin fraction, powdered flowers (100 g) were extracted with 70% MeOH (1 × 400 mL) at room temperature and then (2 × 300 mL) at 60 °C. The obtained extract was evaporated to dryness in vacuum at 40°, yielding a yellow-brown solid (27 g) that was suspended in water. After defatting with $CHCl_3$, the steroidal saponin fraction (10 g) was extracted with BuOH. 1 mg of the BuOH extract was dissolved in 1 mL of MeOH and centrifuged before analysis of 20 μL in chromatographic systems.

2.3. ESI–MS and ESI–MS/MS analyses

Full scan ESI–MS and collision induced dissociation (CID) ESI–MS/MS analyses of standard steroidal glycosides were performed on an Applied Biosystems (Foster City, CA, USA) API2000 spectrometer equipped with a triple quadrupole analyser. The analytical parameters were optimised by infusing a standard solution of compound **1** (1 μg/mL in methanol) into the source at a flow rate of 5 μL/min. The optimised parameters were: declustering potential 30 eV, focusing potential 100 eV, entrance potential 8 eV, collision energy 30 eV, and collision cell exit potential (CXP) 15 eV. Data were acquired in the positive ion MS and MS/MS modes.

2.4. HPLC–ESI–MS and HPLC–ESI–MS/MS analyses

Qualitative on-line HPLC–ESI–MS analyses of the saponin fraction were performed using a Thermo Finnigan (Thermo Electron) Spectra System HPLC coupled to a Thermo Electron LCQ Deca IT spectrometer. Analyses were carried out using a Waters (Milford, MA, USA) Atlantis C18 column (150 mm × 2.0 mm i.d.; 5 μm particle size) eluted with mixtures of water containing 0.05% trifluoroacetic acid, TFA (solvent A) and acetonitrile containing 0.05% TFA (solvent B) at a flow rate of 0.2 mL/min. Gradient elution started with 100% A and changed to 80:20 (A:B) in 35 min, then from 80:20 (A:B) to 100% B in 5 min. The flow from the chromatograph was injected directly into the ESI source, maintained at a temperature of 280 °C, and MS were measured under the optimised parameters indicated for the ESI–MS with nitrogen supplied at a flow rate of 80 (arbitrary units). MS data were acquired using the software provided by the manufacturer, and reconstructed ion chromatograms (RICs) were elaborated in order to identify steroid glycosides from their protonated molecular ions.

Quantitative on-line HPLC–ESI–MS/MS analyses of the extracts were performed using an Agilent 1100 HPLC system interfaced to an Applied Biosystems (Foster City, CA, USA) API2000 instrument. The chromatographic conditions were as described above for the qualitative analyses. The API 2000 ES source was tuned by infusing a standard solution of compound **1** (1 μg/mL in methanol) into the source at a flow rate of 10 μL/min. The optimised parameters were: declustering potential 30 eV, focusing potential 100 eV, entrance potential 8 eV, collision energy 30 eV, and collision cell exit potential (CXP) 15 eV The spectrometer was used in the MS/MS mode with MRM of fragmentation reactions selected for each glycoside as described below.

2.5. Calibration and quantification

In order to prepare the specific calibration plot for each compound under investigation, a sample (10 mg) of each standard was weighted accurately into a 10 mL volumetric flask, dissolved in methanol and the volume made up to the mark with methanol. The resulting stock solution was diluted with methanol in order to obtain reference solutions containing 2.5, 5, 10, 25 and 50 μg/mL of external standard. Peak areas of the external standard were plotted against the corresponding standard concentration using weighted linear regression to generate standard curves. All quantitative data were elaborated with the aid of Analyst software (Applied Biosystems).

3. Results and discussion

3.1. Qualitative LC–MS analysis of *Y. gloriosa* flower extracts

LC–MS is selective and sensitive enough to carry out the analysis of saponins [15–18]. For the present study a saponin fraction was obtained from the powdered flowers (100 g) and analyzed by HPLC–MS in positive ion mode.

Column and mobile phase selection in HPLC is determined, among the other aspects, by the combination of the compounds to be analyzed and the matrix where they occurr. In our work the use of a symmetry shield C18 column and a gradient elution allowed us to obtain a good separation of the steroidal glycosides occurring in the enriched saponin fraction of *Y. gloriosa* flowers. In LC–MS the glycosides displayed the pseudomolecular ion $[M+H]^+$. Positive ion electrospray LC/MS analysis obtained in the acquisition range of 190–1200 amu (atomic mass unity), total ion current (TIC) profile and reconstructed ion chromatograms (RICs), of the enriched saponin fraction from *Y. gloriosa* flowers are shown in Fig. 2. Compounds **1**–**6** were identified comparing their retention times and m/z values in the total ion current chromatogram to those of the selected standards. Reconstructed ion chromatograms were obtained for each m/z value of the standard compounds: m/z 741, compound **1**, m/z 1035, compound **2**, m/z 1211, compound **3**, m/z 1197, compound **4**, m/z 1373, compound **5**, m/z 1343, compound **6**, in order to improve the separation and the identification of the single compounds.

3.2. Quantitative LC–ES(QqQ)/MS/MS analyses of *Y. gloriosa* extracts

A better accuracy in liquid chromatography tandem mass spectrometry for quantitative analyses is recognized for tandem in space mass spectrometry. Thus for quantitative purposes an accurate method on a mass spectrometer equipped with a triple quadrupole analyzer was developed for the analysis of steroidal glycosides in *Y. gloriosa* flowers. Fragmentation patterns were studied by analyzing a standard solution of 0.1 μg/mL for each investigated compound by ES–QqQ–MS. However, for compounds **1**–**6**, which were characterised by their glycosylated structures, the loss of the sugar chain was the predominant fragmentation, and these compounds were monitored by the transition from the specific protonated molecular ion $[M+H]^+$ to the corresponding aglycon ion $[A+H]^+$. The transitions employed to monitor these glycosides are summarised in Fig. 3.

Compounds could be detected with high sensitivity using the reported MS tandem conditions. The chromatographic profile obtained by MRM analysis exhibited all the peaks corresponding to the compounds under investigation, and with enough intensity for quantitative purposes. Fig. 3 shows LC/MS/MS profiles for the six compounds under investigation. Calibration curves were performed for all the six compounds used as external standards at different concentration levels (2.5, 5, 10, 25 and 50 μg/mL). The calibration curves obtained by plotting the external standard areas versus the known concentration of each compound were linear in the range 2.5–50 μg/mL for all the compounds. Five aliquots of each extract of *Y. gloriosa* flowers were analyzed in order to quantify their glycosidic content. Table 1 reports quantitative analysis results.

3.3. Validation

The HPLC–MS/MS assay was validated according to the European Medicines Agency (EMEA) guidelines relating to the validation

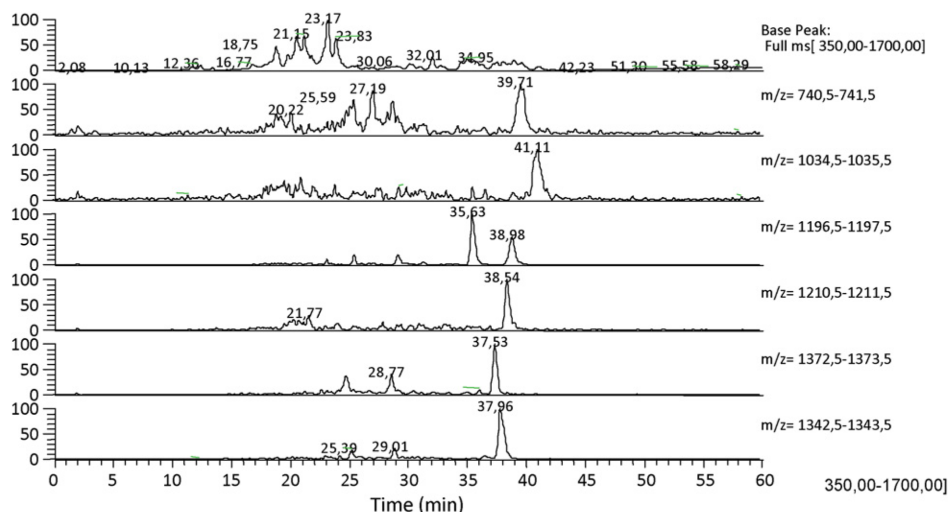

Fig. 2. LC/ES/MS analysis of steroidal glycosides in *Yucca gloriosa* flowers.

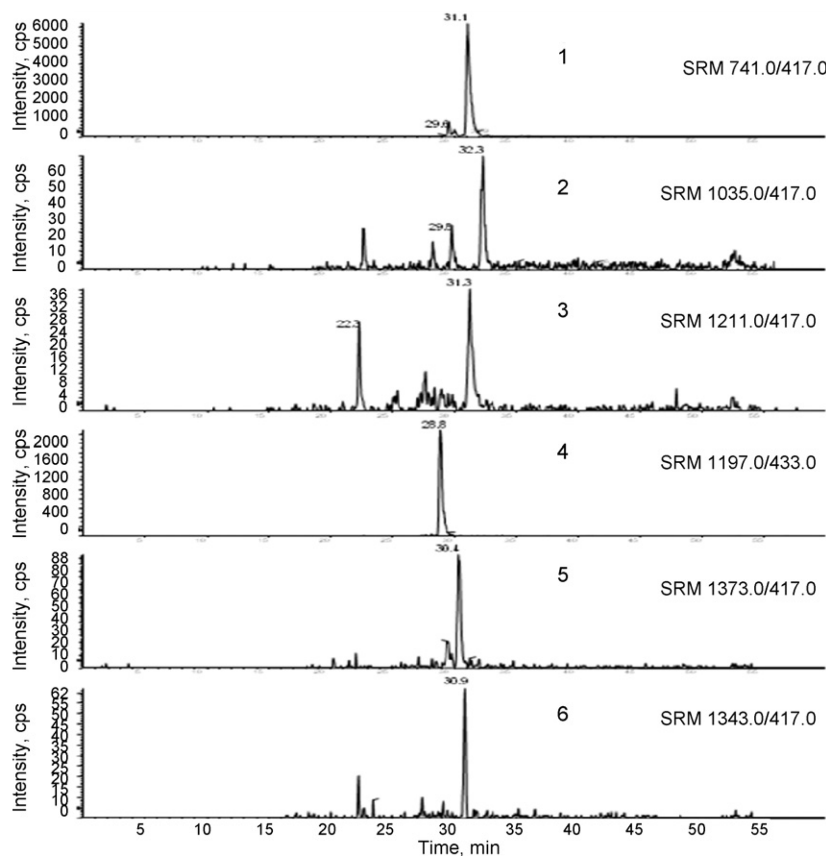

Fig. 3. LC/ES/MS/MS MRM (multiple reaction monitoring) analysis of steroidal glycosides in *Yucca gloriosa* flowers.

Table 1
Calibration curve data for the analysis of compounds **1–6**.

Compound	Precursor ion [M+H]+	Product ion [A+H]+	R^2	Calibration curve
1	741	417	0.983	$y = 28.88x + 44.86$
2	1035	873	0.976	$y = 17.57x + 6.79$
3	1211	1049	0.989	$y = 7.494x + 108.36$
4	1197	595	0.985	$y = 10.14x + 66.20$
5	1373	1211	0.998	$y = 49.17x + 62.82$
6	1343	1181	0.992	$y = 25.85x - 18.52$

of analytical methods [19]. The method based on the characteristic fragmentation reactions of steroidal glycosides was highly specific with no any other peak interfering at the retention times of the marker compounds (compounds **1**–**6**) in the MRM chromatograms. The intra-day accuracy and precision were calculated by analysing three samples of compound **1** at three different concentration levels, namely, 1, 5 and 10 μg/mL, in the same day. Inter-day estimates were performed over three consecutive days. The standard deviation was <5%. The calibration graphs, obtained by plotting the area obtained from external standard against the known concentration of external standard (for each compound) was linear in the range of

Table 2
Quantitative results for compounds **1**–**6** by MRM LC/ESI/MS/MS.

Compound	LOQ	LOD	Dried flowers concentration ($\mu g\,mg^{-1}$) (% S.D.)
1	18 $ng\,mL^{-1}$	4.1 $ng\,mL^{-1}$	11.35 (1.48)
2	21 $ng\,mL^{-1}$	0.9 $ng\,mL^{-1}$	5.83 (0.13)
3	11 $ng\,mL^{-1}$	2.1 $ng\,mL^{-1}$	48.35 (2.47)
4	11 $ng\,mL^{-1}$	1.8 $ng\,mL^{-1}$	54.03 (3.44)
5	15 $ng\,mL^{-1}$	4.3 $ng\,mL^{-1}$	8.90 (1.32)
6	14.8 $ng\,mL^{-1}$	3.7 $ng\,mL^{-1}$	22.64 (0.81)

1–50 μg/mL. The limit of quantification (LOQ), defined as the lowest concentration of compound quantifiable with acceptable accuracy and precision, was determined by injection of a series of diluted standard solutions until a signal-to-noise ratio of 10 was attained. The LOQ values calculated for the six compounds under investigation were less than 15 ng/mL. Table 2 reports validation data of the method developed for quantitative analysis of compounds **1–6**.

In conclusion *Y. gloriosa* flowers can be considered a rich source of steroidal glycosides. An LC/MS/MS multiple reaction monitoring quantitative method was developed, validated and applied to real samples of *Y. gloriosa* flowers.

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