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SHORT COMMUNICATION

Development of a validated UPLC–qTOF-MS/MS method for determination of bioactive constituent from *Glycyrrhiza glabra*

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KEYWORDS

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Ultrasonication;
Ultra-performance liquid chromatography quadrupole time of flight mass spectrometer (UPLC–qTOF-MS/MS)

Abstract An ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC–qTOF-MS/MS) method was developed and validated for the simultaneous determination of glycyrrhizin and glycyrrhetic acid. These analytes were separated on a reverse phase C18 column using a mobile phase of acetonitrile:2% acetic acid in water (75:25, v/v) with a flow rate of 200 μL/min. The qTOF-MS was operated under multiple reaction monitoring (MRM) mode using the electrospray ionization (ESI) technique with positive ion polarity. A comparison of three different extraction techniques i.e. accelerated solvent extraction (ASE), extraction under ultrasonic waves (USW) and the classical extraction by percolation (CE) method was done and quantification of these extracts was also carried out by the proposed method.

1. Introduction

Glycyrrhizin is a water-soluble triterpenoid glycoside, which is responsible for the characteristic sweet taste of liquorice. Glycyrrhizin (glycyrrhizic acid) is chemically 3-O-(2-O-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl)-3β-hydroxy-11-oxo-18β, 20β-olean-12-en-29-oic acid. Roots of *Glycyrrhiza glabra* (liquorice) are extensively used in herbal medicines to treat diseases such as phthisis, contagious hepatitis, bronchitis and ague [1] and are also used in the treatment of various ailments such as spleen, sore

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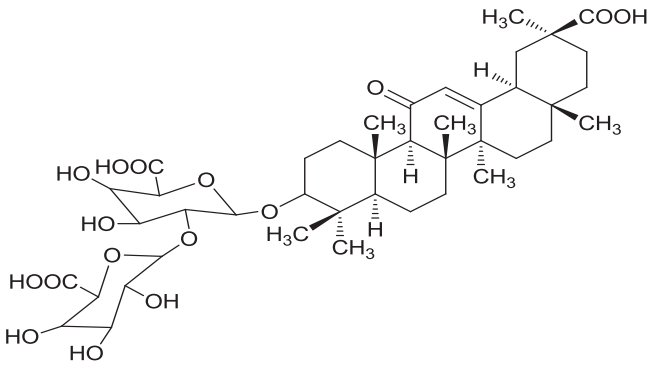

Fig. 1 Structure of glycyrrhizin.

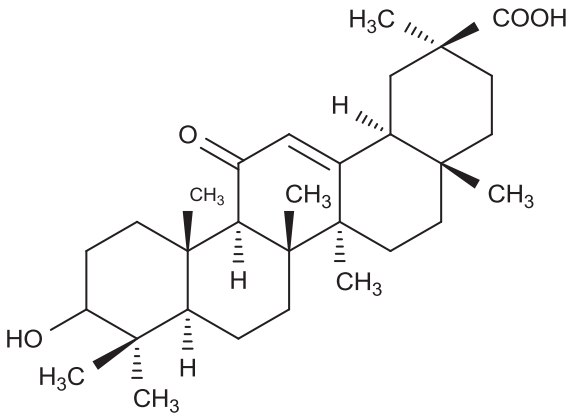

Fig. 2 Structure of glycyrrhetic acid.

throat, bronchitis, liver, kidney and ulcers [2–4]. Liquorice extract is largely used in confectionery, as a masking agent or taste corrective in pharmaceutical formulations and also to improve the taste of beer. It has anti-inflammatory, antiallergenic, antihepatotoxic, antiulcer and antiviral properties [5–7]. Glycyrrhizin presents in the roots as potassium or calcium salt and its content varies from 2% to 15% depending on plant species, as well as geographic and climatic conditions of its habitat.

Furthermore, it has been found to prevent the development of hepatic carcinoma from hepatitis C and has been demonstrated *in-vitro* to be an antiviral agent against SARS-associated corona virus [8,9]. Keeping in view the importance of plants in Ayurvedic and herbal formulations, it was decided to undertake a comparative study of various extraction procedures of this plant and find out an efficient method for the extraction of glycyrrhizin and glycyrrhetic acid. Moreover, method development and validation for the determination of glycyrrhizin (Fig. 1) and glycyrrhetic acid (Fig. 2) in extract obtained from different extraction procedures by ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC–qTOF-MS/MS) have not been reported, prior to this investigation.

2. Experimental

2.1. Materials and chemicals

The plant material was collected from the local market and the voucher specimen of the material was deposited in herbarium division of the institute with accession no. 0453. The 30% ammonium hydroxide solution used in extraction was from Qualigens (Mumbai, India) whereas glacial acetic acid was purchased from Sisco Labs (Mumbai, India). Commercial grade 95% ethyl alcohol was distilled and used for extraction of the plant material. All solvents/chemicals used were of HPLC grade and obtained from E-Merck (Mumbai, India).

Glycyrrhizin and glycyrrhetic acid used as standards were isolated from the roots of *G. glabra*. The isolated Glycyrrhizin and glycyrrhetic acid were identified on the basis of NMR and mass spectral data. The purity of standards was >97%.

2.2. Instruments

ASE-300 used for accelerated solvent extraction was from Dionex and the Ultrasonication bath used was from Toshon (Ajmer, India). The percolator used was indigenous and was fabricated by the engineering division of the institute.

A UPLC–qTOF-MS system (Synapt, Waters, USA, equipped with MassLynx acquisition software, version 4.1) was used and experimental conditions were column, C18 (50 mm × 2.1 mm); particle size, 1.7 μm (Acquity, BEH); flow rate, 200 μL/min; mobile phase, acetonitrile:2% acetic acid in water (75:25, v/v); injection volume, 1 μL. The analyte infusion experiments were performed using an in-built syringe pump. A UPLC–qTOF-MS with ESI interface was used for MS/MS analysis. ESI parameters were capillary voltage, 2.7 kV for positive mode; source temperature, 100 °C; desolvation temperature, 200 °C; cone gas flow, 50 L/h and desolvation gas flow, 550 L/h. The multiple reaction monitoring (MRM) mode was used to monitor the transitions (845→823→453 and 493→471→177) of glycyrrhizin and glycyrrhetic acid, respectively. The major ions observed in the positive ion ESI spectra were *m/z* 845.1873 [M+Na], 867.1644 [M+K], 823.2040 [M+H] (Fig. 3) and *m/z* 493.2053 [M+Na], 471.2287 [M+H] (Fig. 4) for glycyrrhizin and glycyrrhetic acid, respectively.

2.3. Isolation of marker compounds

Roots of *G. glabra* (500 g) were pulverized and mixed with 50% aqueous methanol at ambient temperature. The mixture was filtered after 18 h to obtain the extract and plant residue. The extraction was repeated under similar conditions three times. Pooled extract was treated with NaCl and filtered again. The filtrate was treated with H_2SO_4 to precipitate glycyrrhizin which was dissolved in hot distilled water and filtered again. The filtrate was evaporated to dryness under reduced pressure.

The residue was crystallized and re-crystallized to obtain glycyrrhizin (4 g).

Glycyrrhizin (1 g) was mixed with 12 mL hot distilled water and 25% H_2SO_4 (10 mL) was added dropwise while shaking. The contents were refluxed for 8 h and cooled. The precipitate was filtered under suction and washed till filtrate was neutral to pH paper. The precipitate was dried and crystallized and re-crystallized to obtain glycyrrhetic acid (400 mg).

2.4. *Extraction methods*

Extractions were carried out by using three different techniques [accelerated solvent extraction (ASE), extraction under ultrasonic waves (USW) and the classical extraction by percolation (CE)].

The plant material was extracted with different solvents (liquid ammonia at different concentrations, 50% ethyl alcohol, acetic acid at different concentrations and water) at different pH which were used in case of ASE and USW. The concentration of liquid ammonia used was 30% NH_4OH and it was considered as 100% in the study.

2.4.1. *ASE*

The plant material was pulverized to a coarse powder and subjected to ASE. This method has been found to reduce the extraction time and increase the recovery of required constituents. ASE was performed at 1500 psi at 50 °C for 5 min and

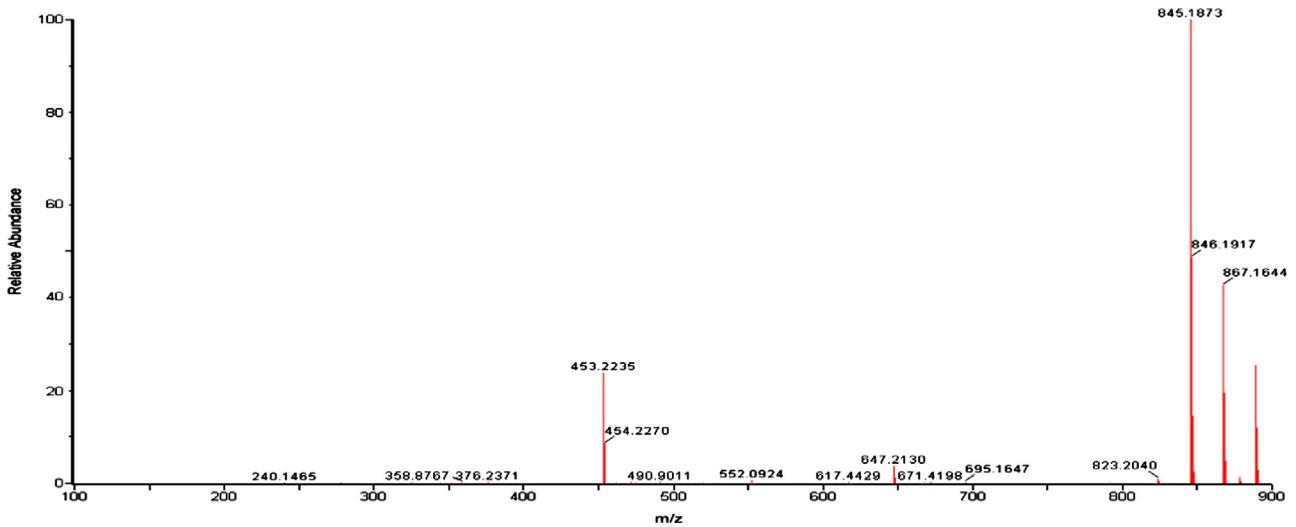

Fig. 3 UPLC–ESI-qTOF-MS spectra of glycyrrhizin showing its transition ion as well as molecular ions [M+H], [M+Na] and [M+K].

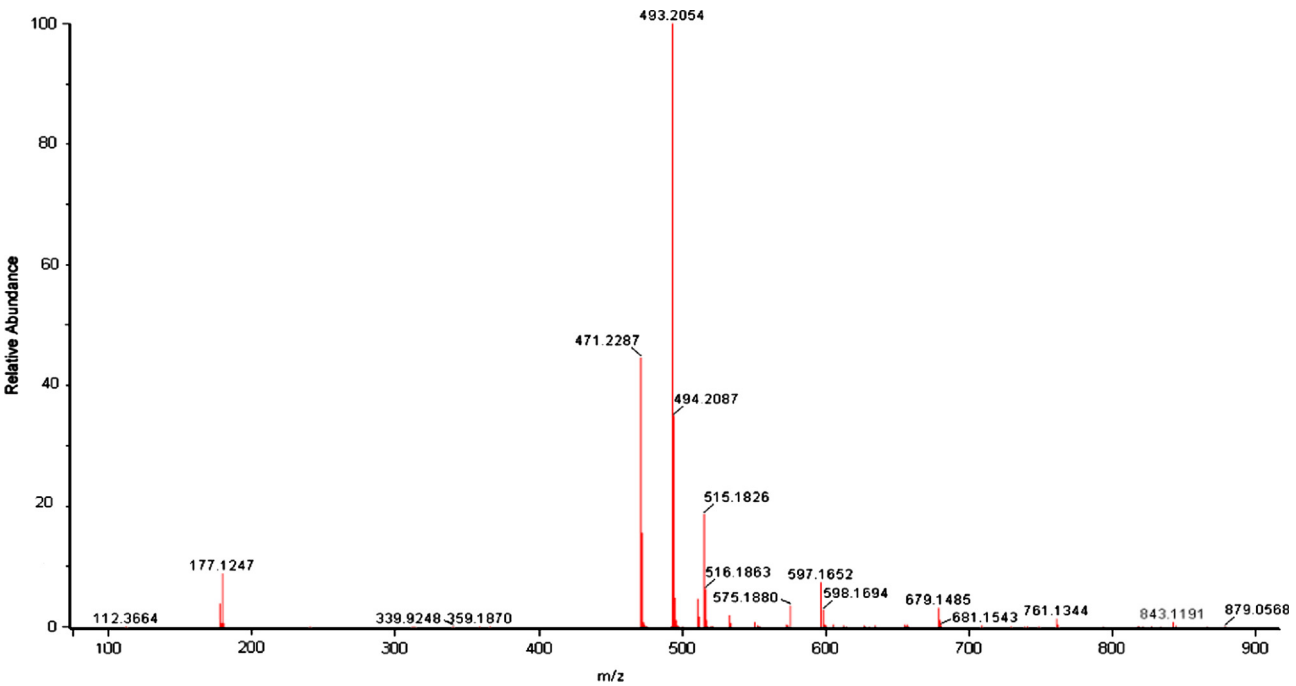

Fig. 4 UPLC–ESI-qTOF-MS spectra of glycyrrhetic acid showing its transition ion as well as molecular ions [M+H] and [M+Na].

10 min was static time. 7 g of coarse powder was taken in a 100 mL capacity cell and 130 mL of the solvent was used which included 30 mL of washing solvent. The marc left was extracted twice with the same solvent and the pooled extracts were dried under reduced pressure on a rotavapor. The different extracts obtained were analyzed for glycyrrhizin and glycyrrhetic acid by UPLC–qTOF-MS/MS.

2.4.2. USW

For extraction under ultrasonic waves 10 g of plant material and 40 mL of solvent at 50 °C were used. The extraction time was 20 min to achieve good extraction values. The marc left after filtration was extracted twice. All the three extracts were combined and dried under reduced pressure on the rotavapor. The extracts obtained were analyzed.

2.4.3. CE

It was carried out at ambient temperature by percolation with 50% ethyl alcohol. The solvent was changed after 18 h and three repeated extractions were performed. These extractions were combined and processed for analysis.

2.5. Preparation of standard solutions

Reference solutions of glycyrrhizin and glycyrrhetic acid were prepared by weighing 5 mg of each compound and transfering to 5 mL volumetric flasks, dissolving and diluting suitably with HPLC grade methanol. All the reference solutions (1 mg/mL) were covered with aluminum foil.

Working solutions of lower concentration were prepared by appropriate dilution of the stock solutions in methanol. 5 μL ($n=10$) each of the above solutions was injected into the UPLC–qTOF-MS system for preparation of standard curves. Quantification of glycyrrhizin and glycyrrhetic acid was done by the external standard curve method.

2.6. Sample preparation

Accurately weighed 25 mg of each extract was dissolved in fixed volumes (25 mL) of methanol and filtered through a 0.20 μm filter paper. 1 mL of this solution was diluted to 10 mL with HPLC grade methanol. 1 μL of each sample was injected into the UPLC–qTOF-MS system. The concentration of glycyrrhizin and glycyrrhetic acid in samples was determined by the external standard method of quantification.

2.7. Validation of method

The proposed method was validated by carrying out linearity, limits of detection (LOD) and quantification (LOQ), precision and accuracy studies.

Linearity was determined on the basis of calibration curves. Regression analysis was used to assess the linearity of the developed UPLC–qTOF-MS method. In the present study, linearity was studied for standard solutions of glycyrrhizin and glycyrrhetic acid respectively.

LOD and LOQ were measured based on the method described by the International Conference on Harmonization (ICH, 2005). The LOD and LOQ of glycyrrhizin and glycyrrhetic acid were determined on the basis of signal-to-noise ratio of 3 and 10, respectively.

The precision parameters were ascertained in three different concentrations (5, 25 and 200 ng/mL), 6 replicates each in 2 sets on the same day and on 3 consecutive days.

Accuracy was determined in terms of % recovery in two different concentrations (30 and 180 ng/mL), 6 replicates each in 2 sets on the same day and on 3 consecutive days.

Recovery study was performed by analyzing analytes at three different levels (10, 50 and 250 ng/mL) by comparing the mean peak areas ($n=6$ for each concentration) obtained from blank samples with those spiked on 3 consecutive days.

3. Results and discussions

3.1. Validation of HPLC method

3.1.1. Linearity

In the proposed method, linearity was studied in the concentration range of 5–250 ng/mL of standard solutions of glycyrrhizin and glycyrrhetic acid respectively. Calibration curves were linear over a large concentration range and established good linear regression. The correlation coefficients (0.99104, 0.99782) and the linear regression equations ($y=4680.05x-1787.21$, $y=48.10x-198.75$) indicate high degrees of correlation and good linearity of the method for glycyrrhizin and glycyrrhetic acid, respectively.

3.1.2. LOD and LOQ

The LODs of glycyrrhizin and glycyrrhetic acid were 1.7 and 1.0 ng/mL, respectively. The LOQs for glycyrrhizin and glycyrrhetic acid were 5 and 3 ng/mL, respectively.

3.1.3. Precision and accuracy

The results of intra-day and inter-day precision and accuracy for determination of glycyrrhizin and glycyrrhetic acid are

Table 1 Precision (% RSD) of glycyrrhizin and glycyrrhetic acid ($n=6$).

Analytes	Concentration (ng/mL)	RT (RSD, %)		Peak area (RSD, %)	
		Intra-day	Inter-day	Intra-day	Inter-day
Glycyrrhizin	5	0.93	1.28	1.90	1.75
	25	0.87	1.11	0.98	0.79
	200	1.86	2.86	3.64	1.39
Glycyrrhetic acid	5	0.78	1.28	0.67	0.98
	25	1.20	0.97	0.63	0.91
	200	1.19	1.03	1.32	1.70

Table 2 Accuracy (% recovery) of glycyrrhizin and glycyrrhetic acid.

Analytes	Amount added (ng/mL)	Amount obtained (ng/mL)	Recovery (%)	RSD (%)
Glycyrrhizin	30	29.96	99.86	1.02
	180	182.76	101.53	0.46
Glycyrrhetic acid	30	30.00	100.00	1.29
	180	179.98	99.98	0.87

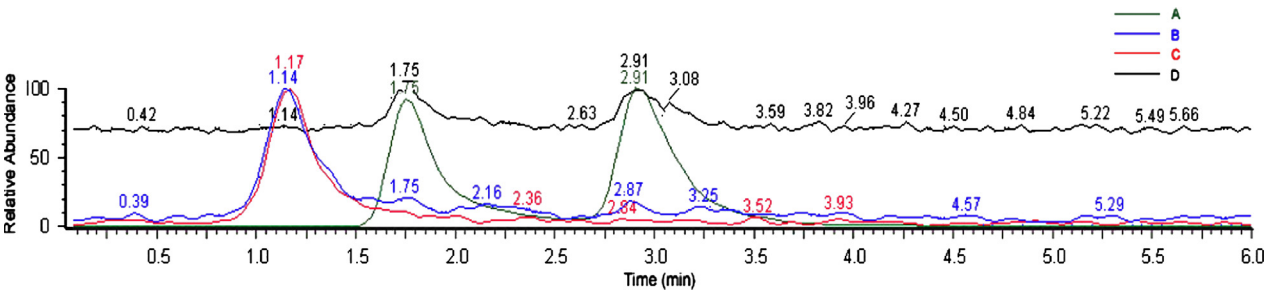

Fig. 5 Typical chromatograms. (A) Extracted ion chromatograms of glycyrrhetic acid showing α and β isomers; (B) extracted ion chromatograms of transition ion of glycyrrhizin showing m/z 453.2235; (C) extracted ion chromatograms of glycyrrhizin showing *m/z* 845.1873 and (D) total ion chromatogram of both glycyrrhizin and glycyrrhetic acid.

Table 3 Amounts of glycyrrhizin obtained from *Glycyrrhiza glabra* by accelerated solvent extraction and ultrasonication extraction methods.

Solvent used for extraction	Amount of glycyrrhizin obtained (%)			
	Accelerated solvent extraction	Ultrasonication	Percolation	pH
Ammonia	12.70	10.12	8.23	11.85
50% ammonia	8.09	6.57	5.00	11.45
25% ammonia	6.37	5.29	4.12	11.25
10% ammonia	4.72	4.34	3.90	10.80
5% ammonia	4.06	4.30	3.60	10.64
50% ethyl alcohol	4.12	4.00	2.82	—
Acetic acid	3.17	3.00	2.17	0.22
50% acetic acid	2.86	2.93	1.80	1.85
25% acetic acid	2.03	2.65	1.69	2.41
10% acetic acid	0.96	1.06	0.84	2.85
5% acetic acid	0.72	0.56	0.42	2.98
Water	3.50	3.48	3.40	7.20

summarized in Tables 1 and 2, respectively. The overall values of precision for intra-day variations were between 0.87% to 3.64%, and 0.63% to 1.32%, and inter-day variations were between 0.79% to 2.86% and 0.91% to 1.70% for the analytes, correspondingly.

The overall values of the accuracy were between 0.46% to 1.02% and 0.87% to 1.29% for the analytes, correspondingly. The recovery range varied from 99.4% to 102.8%.

3.1.4. *Specificity*

The specificity of the method was examined by analyzing the blank sample (extract of *G. glabra*) and blank extract spiked with a known concentration of glycyrrhizin and glycyrrhetic acid; additional peaks were not observed except the peaks of interest.

3.2. *Determination of glycyrrhizin and glycyrrhetic acid in different extracts*

Glycyrrhetic acid used as a standard in the study showed separate peaks for α and β isomers in the extracted ion chromatogram (EIC) (Fig. 5A). Glycyrrhizin appeared in the EIC at 1.17 min (Fig. 5C). α and β isomers of glycyrrhetic acid exhibited peaks at retention times of 1.75 and 2.91 mins in the total ion chromatogram (TIC; Fig. 5D), respectively. As the β isomer was predominant in case of glycyrrhetic acid and it was quantified in the

Table 4 Amounts of glycyrrhizin obtained from *G. glabra* by accelerated solvent extraction, ultrasonication and percolation extraction methods in 50% ethyl alcohol.

Analytes	Accelerated solvent extraction	Ultrasonication	Percolation
Glycyrrhizin (%)	4.12	4.00	2.82
Glycyrrhetic acid (%)	0.19	0.17	0.11

different extracts. Retention times of glycyrrhizin (1.17 min) and glycyrrhetic acid (2.91 min) are shown in the TIC (Fig. 5D).

The quality of extraction procedure of a plant material is reflected in the percentage of a compound of interest extractable by a particular process. After extraction, the desired compound can be purified by normal reported methods of purification. Extraction values of glycyrrhizin from *G. glabra* with water and 50% aqueous ethanol were almost the same as with ASE and USW and were observed to be 3.50%, 3.48%, 3.40% and 4.12%, 4.00%, 2.82%, respectively (Table 3). The comparison of extraction values in 50% aqueous ethanol by CE at ambient temperature with extraction under USW and ASE in the same solvent exhibited no significant increase in the yield of glycyrrhizin. However, in acidic conditions (pH 0.22) a maximum of 3.00% of glycyrrhizin was extracted, whereas under basic conditions (pH 11.85) the extractive value was to the tune of 12.70% (Table 3). As *G. glabra* is one of the major plants used commercially, the ASE method with ammonium hydroxide is the most appropriate method of extraction. By this process the amount of extractable glycyrrhizin increases by more than 50% as compared to extraction by CE under similar conditions.

The amount of glycyrrhizin and glycyrrhetic acid in the extract obtained by different extraction methods (Table 4) with 50% ethyl alcohol was 4.12% and 0.19% by ASE, and 4.00% and 0.17% by USW whereas 1.71% and 0.11% was observed by CE, respectively. It is clear from the results that ASE with liquid ammonia is the most suitable method for extraction of glycyrrhizin from the roots of *G. glabra*.

However, several extraction techniques have already been reported to extract these analytes by the various authors [10–12] but there is no evidence available in the literature in which proposed extraction techniques were used and comparison of different extraction techniques was carried out. Several analytical techniques were used for the determination and quantification of glycyrrhizin and glycyrrhetic acid from plasma, extract and herbal preparations, which include TLC, HPLC [13] and LC–MS/MS [14,15].

The methods reported in the literature have several disadvantages, long separation times, poor resolution and complicated solvent mixtures with gradient elution for determination and quantification of glycyrrhizin and glycyrrhetic acid. These methods are not selective and time-consuming, and pretreatment of a sample or complicated mobile phase is required.

Separation of the analytes was achieved within 5 min in the proposed method with simple isocratic elution. In recent times, UPLC with qTOF-MS is widely considered as an analytical technique for better quality data in terms of increased detection limits and chromatographic resolution with greater sensitivity [16].

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