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The determination of glycyrrhizic acid in *Glycyrrhiza uralensis* Fisch. ex DC. (Zhi Gan Cao) root and the dried aqueous extract by LC–DAD

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Abstract

A rapid, sensitive and specific reversed phase high-performance liquid chromatographic (LC) method with photodiode array detection (DAD) has been developed for the determination of glycyrrhizic acid in both the raw herb and a commercially prepared dried aqueous extract of *Glycyrrhiza uralensis* Fisch. ex DC. root (Zhi Gan Cao, liquorice). It was determined that extracting the raw herb in aqueous methanol (50:50 v/v) by sonication for 2 × 30 min was the most efficient sample preparation. Baseline resolution of the glycyrrhizic acid peak was achieved on a Varian Polaris RP C18-A (250 mm × 4.6 mm, 5 μm packing) column using an isocratic mobile phase consisting of 0.5 v/v aqueous phosphoric acid and acetonitrile in the ratio 60:40 v/v. Chromatograms were monitored between 200 and 400 nm for peak purity assessments, with quantitation performed at 254 nm. Glycyrrhizic acid calibration curves in the concentration range of 14–558 μg/ml were prepared on the day of analysis. Curve fitting was by the least-squares method, with correlation coefficients of >0.9998 obtained each time. The average recovery at three spike levels (50, 100, 200%) was of 95.91 ± 1.05% and 98.36 ± 3.45% (±S.D., $n = 7$) for the spiked raw herb and dried aqueous extract respectively. The limit of detection and limit of quantitation was 0.52 and 1.72 mg/g respectively for the raw herb, and 0.75 and 2.51 mg/g respectively for the dried aqueous extract. Identity confirmation of the chromatographic peak was achieved by (−) electrospray ionisation tandem mass spectrometry. The concentration of glycyrrhizic acid in the root and dried aqueous extract was found to be 31.1 ± 0.2 and 40.4 ± 0.3 mg/g (±S.D., $n = 7$) respectively.

Keywords: Zhi Gan Cao; *Glycyrrhiza uralensis* Fisch. ex DC.; Glycyrrhizic acid; Glycyrrhizin; LC; DAD; Validation

1. Introduction

The root of *Glycyrrhiza uralensis* Fisch. ex DC. (Zhi Gan Cao, liquorice) has been used medicinally for over 2000 years in China and Japan where it is generally prescribed by herbalists as a component in formulations. The principle pharmacologically active ingredient in the root of the plant is glycyrrhizic acid (also referred to as glycyrrhizin), which is a glycosylated saponin with a pentacyclic triterpenic structure bound to two glucuronic acid units as seen in Fig. 1 [1].

The name 'Glycyrrhiza' is derived from ancient Greek, meaning 'sweet root'. Glycyrrhizic acid is 170 times sweeter than sucrose [2] and is widely used as a natural flavouring agent in tobacco, food and confectionery [3] products.

Glycyrrhizic acid is primarily localised in the root, with undetectable amounts in the rest of the plant [4]. The glycyrrhizic acid content (as the potassium and calcium salt) in the root has been reported as between 2 and 15 w/w, depending on the species, geographic location, climatic conditions and season of harvest [4,5]. Two aglycone forms of glycyrrhizic acid also exist, namely 18β-glycyrrhetinic acid and 18α-glycyrrhetinic acid, though they are present only in very small quantities (0.03–0.25 w/w) in the root [4].

Glycyrrhizic acid belongs to the class of natural substances that have been proven to be of considerable medicinal value by modern pharmacological testing. It has been demonstrated to have antiinflammatory, antiulcerous, antidotal, antiallergic, immunomodulating and antiviral properties [6–16]. Glycyrrhizic acid has been among the most important natural

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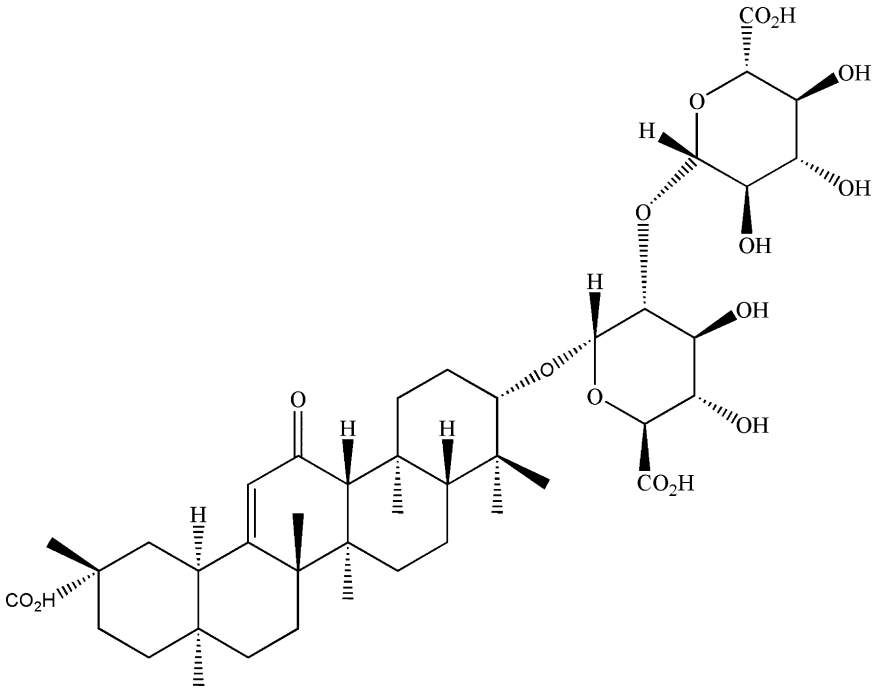

Fig. 1. The chemical structure of glycyrrhizic acid [1].

compounds capable of inhibiting the human immunodeficiency virus (HIV) and acceptable for the treatment of HIV patients [15–17]. Commercially, glycyrrhizic acid is present in the drug 'Glycyrram' used for inhibiting the activity of the Marburg virus [18] as well as being present in the drug 'Stronger Neo-Minophagen C' (SNMC) that has been successfully used for more than two decades in the treatment of viral hepatitis B and C [19–21]. Recently, it has been reported that prolonged administration of SNMC is capable of preventing the development of hepatocirrhosis [22].

There have been twenty six studies [23–49] which have analysed the glycyrrhizic acid content in liquorice roots by LC. Of these studies, the overwhelming majority used a UV detector, with chromatograms monitored at 254 nm [23–49]. Separation was exclusively performed on reversed phase C18 type columns with isocratic elution, mainly using acetonitrile as the organic component of the mobile phase [23,24,27,28,31–39,46] though methanol [26,29,30,39,40,42–45] or acetonitrile–methanol mixes [1,25] have also been employed. The use of acid modifiers in the aqueous component is common, with either acetic acid [1,24,25,28,33–39,42–44] or phosphoric acid [23,26,27,38,40] being the most popular. Numerous extraction methods have been used, with reflux [23,29,30,32–35,37,39,43] or leeching-type methods [1,24–26,39] being the most widely used, though sonication [38,42] shaking [46], pressurised hot water extraction [27] and microwave assisted extraction [28] have also been employed. The extraction solvent was generally methanol [24,35], ethanol [32,37], water [23,27,30,31,34,36,39,43,44] or mixes thereof [1,26,28,29,38,42], with acetone [25] and acetonitrile [33] also reported. The majority of analytical methods were for the purpose of quantitation [23,26–30,32–45], with only four methods used for qualitative screening [24,25,31,46]. It was uncommon for these methods to have been extensively validated, with the work by Sabbioni et al. [1] reporting the most thoroughly validated procedure. The calibration graph linearity was >0.999 for all quantitative methods [1]. The reported content of glycyrrhizic acid in the liquorice root ranged from 0.77 to 83.70 mg/g [30,29] though the result obtained would be strongly dependant on the quality of the herb used as well as the extraction method used.

This work is the first to report a substantially validated LC–DAD method for the analysis of glycyrrhizic acid in both the raw liquorice root and its dried aqueous extract. It is also the first to use LC–ESI-MS/MS for identity confirmation of the analyte which meets the guidelines of the European Union Commission Directive 96/23/EC [47]. This investigation is part of a wider study conducted by the authors on the development of validated LC methods for the determination of bioactive ingredients in herbs and herbal extracts used in traditional Chinese medicines [48–50].

2. Experimental

2.1. Reagents and materials

The raw herb was obtained from Beijing Tong Ren Tang, Sydney Branch (Sydney, Australia) and the dried aqueous extract was obtained from a commercial source. The raw herb was authenticated against a certified reference sample of the dried root of *G. uralensis* Fisch. ex DC. (batch number HM25605AUTH_UWS) from Alchemists Pharmaceuticals (Santa Ana, USA) by LC–DAD.

The primary chemical reference standard of glycyrrhizic acid (ammonium salt, 94.53% purity) used for quantitation and

identity confirmation was purchased from Fluka (Seelze, Germany).

Methanol and ethanol (AR grade) used were purchased from Biolab (Mulgrave, Australia). Mallinckrodt Baker Inc. supplied the *n*-hexane used for the extraction solvent optimisation. Acetonitrile (LC grade) was from J.T. Baker (Phillipsburg, USA). Phosphoric acid (85%, AR grade) was from Biolab (Mulgrave, Australia). Ultra-pure water (>18.2 MΩ/cm) was obtained using an Elga (Buckinghamshire, UK) Purelab Prima 7 water purification unit.

2.2. Preparation of standard solutions and calibration

The glycyrrhizic acid stock solution (558 μg/ml) was prepared by dissolving the ammonium salt of glycyrrhizic acid (14.272 mg) in methanol (25 ml). Standard solutions in the concentration range 14–558 μg/ml were prepared and analysed. An 8-point calibration curve was drawn to determine system linearity, with a 6-point calibration curve constructed for quantitation. Calibration curves were prepared on the day of analysis, with each standard being injected in duplicate. Curve fitting was via the least-squares method.

2.3. Apparatus and chromatographic conditions

LC–DAD experiments were performed on a Varian (Walnut Creek, USA) chromatography system equipped with 2 × ProStar 210 solvent delivery modules, a ProStar 430 autosampler, a ProStar 500 column valve module and a ProStar 335 DAD detector. This system was controlled by the Varian Star MS workstation (Version 6.5). A tandem MS/MS detector with a negative electrospray ionisation ((−) ESI-MS/MS) interface was used for analyte identity confirmation. The LC system used was the same as for the LC–DAD method, except that a Varian 1200L triple quadrupole MS/MS detector replaced the DAD and only 20% of the total flow of 1 ml/min was diverted to the ESI source.

Separation was performed isocratically on a Varian Polaris RP C18-A (250 mm × 4.6 mm, 5 μm packing) column, with an isocratic mobile phase consisting of 0.5 v/v aqueous phosphoric acid–acetonitrile (60:40 v/v). The analysis time was 30 min, followed by a column wash of 100% acetonitrile for 10 min, then a period of re-equilibration to the previous mobile phase for a further 10 min. The mobile phase flow rate was 1.00 ml/min, and the column was maintained at 30 °C. The injection volume was 10 μl. The chromatograms were monitored between 200 and 400 nm for peak purity testing and identity confirmation, with quantitative analysis performed at 254 nm. Stronger identity confirmation was achieved by LC–ESI-MS/MS using the same chromatographic conditions as specified for the LC–DAD, except 0.1 v/v aqueous formic acid was used as the acid modifier. (-) ESI was operated using the following parameters: drying gas temperature, +400 °C; needle voltage, −5000 V; shield voltage, −500 V; capillary voltage, −99 V; detector voltage, +1850 V. The collision induced dissociation (CID) gas was argon. The MS conditions were determined by direct infusion into the MS of a 50 μg/ml glycyrrhizic acid standard in methanol and observing the area curves of the *m*/*z* ions generated by the collision cell breakdown of the operating software.

2.4. Sample preparation

The liquorice root sample was ground to a fine powder using a spice grinder until it was fine enough to pass through a sieve (250 μm).

The extraction efficiency of glycyrrhizic acid from the powdered root sample was determined using 5 different solvents (with 5 replicates per solvent). The solvents tested were methanol, water, aqueous methanol (50:50 v/v), ethanol and hexane. The sample (approximately 0.5 g) was accurately weighed and transferred to a volumetric flask (100 ml). The extraction solvent (80 ml) was then added and the solution sonicated for 2 × 30 min, with the sample being allowed to cool between sonications before making up to volume with extraction solvent for analysis.

Extraction efficiency of glycyrrhizic acid from the sample was also tested using 5 different extraction methods (with 5 replicates per method) in order to determine which was most efficient in terms of analyte recovery and extraction time. The extraction methods tested were ultrasonication (referred to as sonication), reflux, Soxhlet and warm solvent extraction. Extraction efficiency was determined by quantifying the amount of analyte extracted initially and by further extracting the same root sample after the initial extraction, by the selected method for a further 4 h.

For the sonication experiments, the powdered sample (approximately 0.5 g) was accurately weighed into a volumetric flask (100 ml) and the extraction solvent (80 ml) added. Two assays of different sonication times were trialled. The first sample was sonicated for 2 × 30 min, with the sample being allowed to cool between sonications before making up to volume for analysis. The second sample followed the same procedure, but was sonicated for 2 × 60 min.

For the reflux experiment, the powdered sample (approximately 0.5 g) was accurately weighed into a round bottom flask (100 ml) and the extraction solvent (50 ml) added. The sample was refluxed for 30 min and allowed to cool before decanting into a volumetric flask (100 ml). The sample was then re-extracted with fresh solvent (30 ml) for 30 min and allowed to cool before decanting into the same volumetric flask. The extract was made up to volume with the extraction solvent before analysis.

For the Soxhlet extraction study, the powdered sample (approximately 0.5 g) was accurately weighed into a 100 ml cellulose extraction thimble and placed into a Soxhlet extraction apparatus. The extraction solvent (200 ml) was added to the round bottom flask (250 ml) and the sample extracted for 4 h. After cooling, the solvent was transferred to a volumetric flask (250 ml) and made up to volume for analysis.

For the warm solvent extraction method, the powdered sample (approximately 1.0 g) was accurately weighed into a conical flask (100 ml) and extraction solvent (100 ml) added. The mixture was allowed to stand in a warm (50 °C) water bath in for 2 h. The extraction solvent was then gravity filtered through Whatman (number 1, 185 mm) filter paper into a volumetric

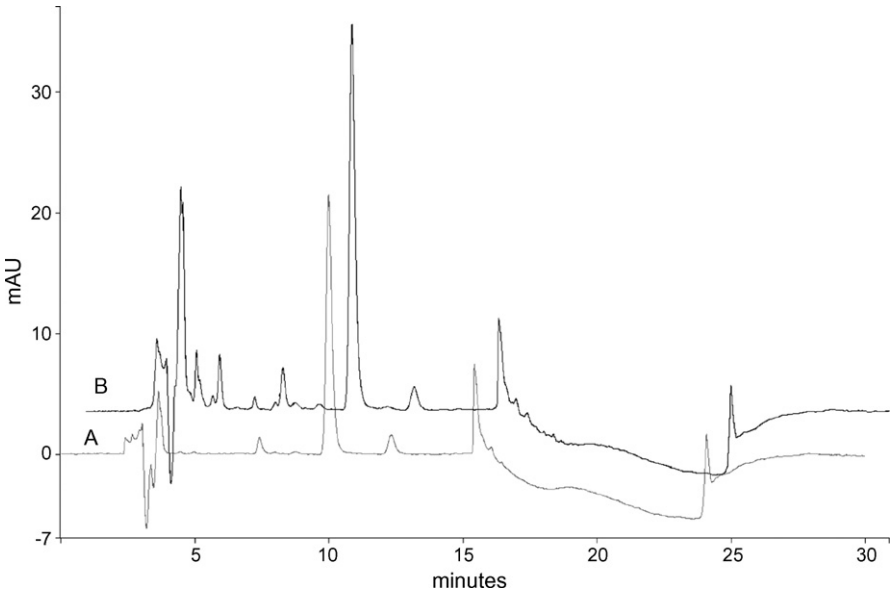

Fig. 2. Representative chromatograms of the superimposed and offset glycyrrhizic acid standard (A) and raw liquorice root (B), extracted using aqueous methanol (50:50 v/v) with sonication (2 × 30 min) and a mobile phase of acetonitrile and 0.5 v/v aqueous phosphoric acid (40:60 v/v), $\lambda_{detection}$ = 254 nm.

flask (250 ml). The sample was re-extracted with fresh solvent (100 ml) for a further 2 h. The solvent was then gravity filtered into the same volumetric flask and allowed to cool before making up to volume with the extraction solvent for analysis.

Three different membrane syringe filters were compared for their suitability for filtering the samples prior to LC analysis. The membranes tested were nylon, polytetrafluoroethylene (PTFE) and polyvinylidine difluoride (PVDF). Each study was carried out in duplicate.

3. Results and discussion

3.1. Authentication of the liquorice root sample

Authentication of the liquorice root used for method validation was by comparison with a certified reference. Macroscopic identification indicated no difference between the two samples. Comparison of the LC–DAD chromatograms of the extracts of both herb samples indicated that they were similar with some differences in peak intensities. Some differences were expected as the herbs came from different sources.

3.2. LC method

Ionisation suppression of glycyrrhizic acid was achieved by incorporating 0.5 v/v aqueous phosphoric acid in the aqueous component of the mobile phase. This acid was desirable because of its low UV cut-off point.

Though this work was originally designed to analyse both glycyrrhizic acid and 18β-glycyrrhetinic acid simultaneously, the amount of 18β-glycyrrhetinic acid in the liquorice sample used was too low to analyse, even after extract concentration. The 18β-glycyrrhetinic acid concentration in our extract appeared to be significantly lower than that reported in literature [4]. Another liquorice sample from a different supplier was analysed but it too showed undetectable amounts of 18β-glycyrrhetinic acid. Thus, subsequent assays focused on the determination of glycyrrhizic acid only, permitting the use of an isocratic mobile phase.

Baseline separation of glycyrrhizic acid was achieved, with a reproducible retention time of 9.23 ± 0.14 min (±S.D., n = 6). Representative chromatograms of the standard and sample extracts (Fig. 2) show that the analyte peak was symmetrical and well resolved from the co extractive peaks.

3.3. LC–DAD and LC–MS/MS identity confirmation

Analysis of the UV spectrum of both the sample and standard peaks indicated a high degree of agreement, with both exhibiting a maximum absorbance at 254 nm and a similar profile as shown in Fig. 3.

MS analysis of the analyte peak was also carried out for a higher degree identity confirmation. The presence of the molecular ion ([M−H], m/z 822) was taken as confirmation that the peak observed is that of glycyrrhizic acid in both the sample and standard solutions. MS/MS breakdown experiments of the glycyrrhizic acid peak, using argon as the CID gas, yielded two daughter ions from which identity confirmation was achieved as specified by European Commission Directorate for Agriculture [47]. The transition was m/z 822 → 351 and 822 → 193 m/z. The relative intensity for m/z 352 and 193 ions was 100% and 63% respectively for the glycyrrhizic acid standard and 100% and 64% respectively for the liquorice root sample as depicted in Fig. 4. The difference in intensity of the two daughter ions obtained for the standard and sample peaks were within the tolerance of ±20% as specified by the European Commission Directorate for Agriculture [47].

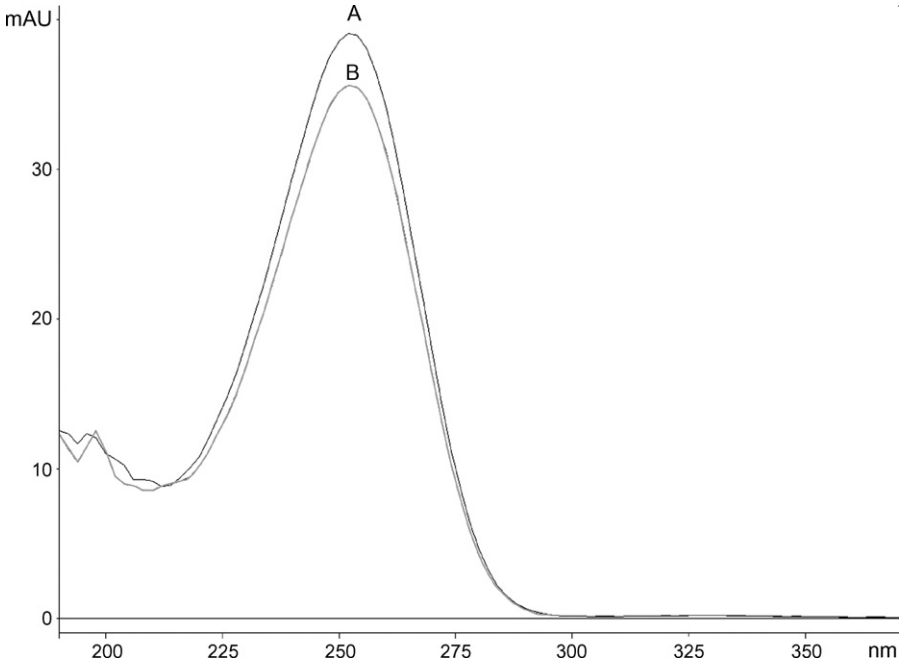

Fig. 3. A comparison of the UV spectra obtained at the apex of the glycyrrhizic acid peak of both the standard and sample chromatogram (A and B respectively).

3.4. Extraction efficiency determination

Due to the relatively polar nature of glycyrrhizic acid, volatility losses would be minimal during extraction. Previous studies indicate that glycyrrhizic acid was thermally stable up to 65 °C [26]. The presence of both hydrophobic and hydrophilic moieties in the molecule means that a variety of organic and aqueous solvents may have been suitable for its extraction. As can be observed in Table 1, an aqueous methanol solution (50:50 v/v) extracted the most analyte. This observation is in harmony with other studies which found aqueous ethanol to be the most effective solvent for the extraction of glycyrrhizic acid.

From Table 2, it can be seen that of the 5 extraction methods tested, sonication (2×30 min) proved to be the most effective. The slightly lower result obtained for the longer sonication time (2×60 min) could be due to some analyte decomposition caused by extended heating as the solution warms up during the sonication process.

Confirmation that the sonication technique was an efficient extraction method was achieved by re-extracting the root

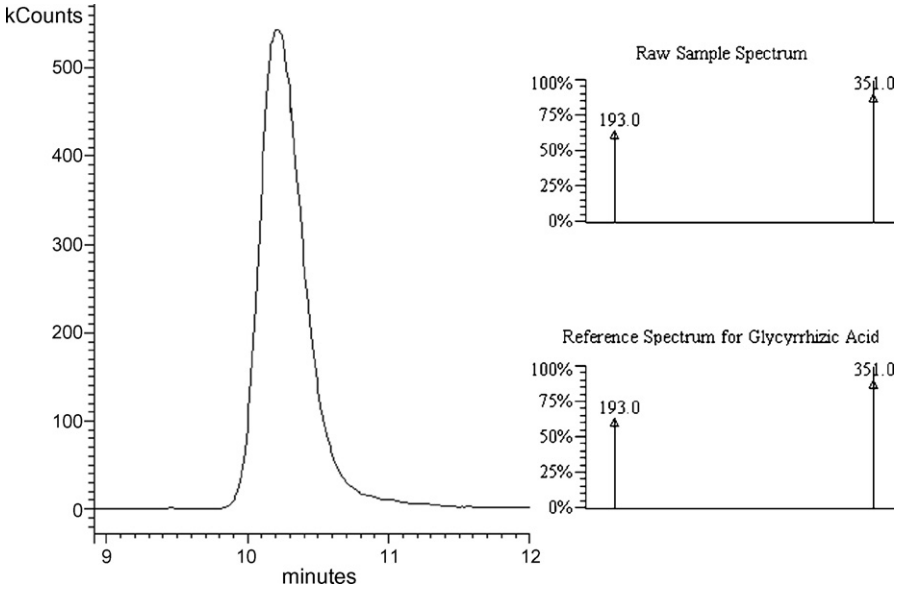

Fig. 4. A MS/MS chromatogram of the liquorice root sample extract and a comparison of its SIM spectrum to the standard SIM spectrum.

Table 1
Results of the extraction solvent optimisation for extraction of glycyrrhizic acid from liquorice root

Extraction solvent	Glycyrrhizic acid extracted (mg/g)	R.S.D. (%)
Water	26.87	3.77
Aqueous methanol (50:50 v/v)	28.91	1.96
Methanol	22.84	4.29
Ethanol	4.64	30.40
Hexane	n.d.[a]	–

[a] n.d.: not detected.

Table 2
Results of the extraction method optimisation for the extraction of glycyrrhizic acid from liquorice root

Extraction method	Glycyrrhizic acid extracted (mg/g)	R.S.D. (%)
Sonication (2 × 30 min)	28.74	0.49
Sonication (2 × 60 min)	27.45	3.45
Warm solvent	24.95	13.76
Soxhlet	25.27	3.61
Reflux	28.81	1.10

that remained after the sonication (2 × 30 min), for a further 8 × 30 min, using a fresh aliquot of solvent. The filtrate was then concentrated 20-fold by rotary evaporation and analysed. The results indicated that 99.67% of the glycyrrhizic acid was extracted by the initial procedure. Extraction efficiency analysis on the reflux sample (the next most efficient extraction technique) indicated that 99.44% of glycyrrhizic acid was extracted in the initial extraction. Since the sonication and reflux extraction results were comparable, the sonication method was chosen due its ease of use.

The results from the optimisation of the syringe membrane filter studies are presented in Table 3, where it can be observed that PVDF retained the least amount of glycyrrhizic acid compared to the other filter types. The PVDF membrane filter was used for all subsequent assays.

3.5. Method validation

All calibration curves showed good linearity (>0.9998). Inter-day precision of retention time was 1.47% R.S.D., and the peak area precision was 0.96% R.S.D. ($n = 6$ replicate injections of a standard solution). The limit of detection (LOD; determined from 3 times the S.D. of 7 replicate analyses) of the method was found to be 0.52 mg/g in the raw herb and 0.75 mg/g in the dried aqueous extract. The limit of quantitation (LOQ; determined from 10 times the S.D. of 7 replicate analyses) was found to be 1.72 mg/g in the raw herb and 2.51 mg/g in the dried aqueous extract. The average recovery ($n = 7$, 3 fortification levels) of glycyrrhizic acid was 95.91 ± 1.05% and 98.36 ± 3.45% from the root and dried aqueous extract respectively. The glycyrrhizic acid concentration, corrected for recovery, was found to be 31.1 ± 0.2 mg/g in the root and 40.4 ± 0.3 mg/g (±S.D., $n = 7$) in the dried aqueous extract. This is in agreement with another study [4] which quotes the amount of glycyrrhizic acid in the dried root as between 2.1 and 7.6 w/w.

Table 3
The percentage of glycyrrhizic acid retained in each of the membrane filters tested

Membrane	Glycyrrhizic acid retention (%)[a]
Nylon	3.07
PVDF	1.40
PTFE	2.14

[a] Based on the ratio between the filtered and unfiltered glycyrrhizic acid peak area.

4. Conclusions

The proposed LC method with DAD detection, coupled with a simple and rapid extraction procedure, was suitable for the analysis of glycyrrhizic acid in the liquorice root and its dried aqueous extract, in terms of analysis time, selectivity and quantitative recovery. Analyte identity confirmation was achieved by comparison of the UV spectrum of the sample and standard peaks as well as by ESI-MS/MS where two daughter ions were produced by CID.

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