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Metabolic profiling of roots of liquorice (*Glycyrrhiza glabra*) from different geographical areas by ESI/MS/MS and determination of major metabolites by LC-ESI/MS and LC-ESI/MS/MS

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ABSTRACT

Liquid chromatography electrospray mass spectrometry (LC-ESI/MS) has been applied to the full characterization of saponins and phenolics in hydroalcoholic extracts of roots of liquorice (*Glycyrrhiza glabra*). Relative quantitative analyses of the samples with respect to the phenolic constituents and to a group of saponins related to glycyrrhizic acid were performed using LC-ESI/MS. For the saponin constituents, full scan LC–MS/MS fragmentation of the protonated (positive ion mode) or deprotonated (negative ion mode) molecular species generated diagnostic fragment ions that provided information concerning the triterpene skeleton and the number and nature of the substituents. On the basis of the specific fragmentation of glycyrrhizic acid, an LC–MS/MS method was developed in order to quantify the analyte in the liquorice root samples. Chinese *G. glabra* roots contained the highest levels of glycyrrhizic acid, followed by those from Italy (Calabria).

1. Introduction

Liquorice root (*Glycyrrhiza glabra*) is used worldwide as a natural sweetener and, in certain cases, as a flavour additive in the preparation of candies and speciality foods. In Italy, for example, a traditional liquor is prepared from *G. glabra* collected in the Calabria region. Additionally, powdered liquorice root is commonly employed as a herbal drug in the formulation of Ayurvedic and Chinese medicines, and reportedly possesses anti-spasmodic, anti-diabetic, anti-depressive, hepatoprotective, expectorant and memory-enhancing activities [1–4].

The main constituent of liquorice root is glycyrrhizic acid [**1**; glycyrrhizin; (3β,18α)-30-hydroxy-11,30-dioxoolean-12-en-3-yl-2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid], which exhibits anti-inflammatory, anti-viral, anti-allergenic, anti-ulcer and anti-oxidative properties, and is also believed to have chemo-preventive activity against cancer and AIDS [5–14]. On the other hand, the intake of high levels of liquorice extract is known to increase blood pressure, an effect caused principally by the presence of **1** [15].

Along with **1**, some 400 or so other secondary compounds have been detected in and/or isolated from *G. glabra* and related species. The majority of these components are flavonoids or triterpene saponins, and they are considered to be responsible for most of the therapeutic activities of the plant. Some liquorice saponins, however, give rise to negative side effects including salt retention and hypokalaemic hypertension [16]. As is the case for most secondary metabolites, the flavonoid and saponin profiles of liquorice are subject to considerable variability according to geographic area, state of plant maturity, environmental conditions, harvesting and processing. Since changes in the composition of the plant material could affect its therapeutic activity, strict quality control is critical to ensure the efficacy and safety of liquorice root in medicinal use. For this purpose, it is necessary to obtain a complete chemical characterization of the species and to develop sensitive, accurate and high-resolution methods for the simultaneous qualitative and quantitative analyses of liquorice flavonoids and saponins.

A number of techniques, including liquid chromatography (LC), capillary zone electrophoresis and micellar kinetic capillary electrophoresis, have been applied to the determination and quantification of glycyrrhizic acid in fresh or dried liquorice roots, in root extracts, in formulations of *Glycyrrhiza* species, and in biological samples [17,18]. However, these methods all depend on the analysis of isolated **1** or of the corresponding aglycone after hydrol-

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ysis. The occurrence in liquorice roots of various other saponins with structures related to that of glycyrrhizic acid, together with a large number of phenolic compounds, has been reported [19]. A review of the progress, since 2000, in the development of chromatographic methods widely employed in the quality control of liquorice is available [20].

In the present study, a qualitative and quantitative evaluation of the secondary metabolites present in liquorice root has been carried out using LC coupled with electrospray mass spectrometry (ESI/MS) and tandem MS (ESI/MS/MS) in order to obtain a full metabolite profile of the drug.

2. Materials and methods

2.1. Chemicals

Solvents used for 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 (Bedford, MA, USA) Milli-Q purification system. Glycirrizic acid standard was purchased from Extrasynthese (Geney, France).

2.2. NMR analysis

NMR experiments were performed on a Bruker BioSpin (Rheinstetten, Germany) model DRX-600 spectrometer at 300 K with samples dissolved in CD_3OD. Standard pulse sequences and phase cycling were used to acquire DQF-COSY, HSQC and HMBC spectra, and the data were processed using UXNMR software. Isolated compounds were characterized by NMR and MS by comparison of the spectral data with those available in the literature.

2.3. HPLC–UV

Reversed-phase HPLC was carried out on an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector and a G-1365B multiple wave detector. Separations were performed on an RP C18 column μ-bondapak 300 mm × 7.6 mm (Waters, Milford, MA). The air-dried root extract was dissolved in methanol (10 mg/1 ml), and chromatographed by HPLC–UV using a μ-bondapak column at flow rate of 2 ml min^{-1}. Linear gradient elution with a mobile phase comprising water acidified with 0.05% trifluoroacetic acid (solvent **A**) and acetonitrile acidified with 0.05% trifluoroacetic acid (solvent **B**) commenced at 100:0 (**A:B**) and attained 20:80 in 55 min.

2.4. ESI/MS, LC-ESI/MS and LC-ESI/MS/MS analyses

2.4.1. ESI/MS

The electrospray ionisation (ESI) source of a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was tuned with a standard solution of **1** in methanol (1 μg ml^{-1}) infused at a flow rate of 10 μl min^{-1} with a syringe pump. The mass spectrometer was operated in the positive ion mode with a capillary voltage of 5 V, spray voltage of 5 kV, tube lens offset of −10 V, capillary temperature of 280 °C, and sheath gas (nitrogen) flow rate of 80 (arbitrary units). In the negative ion mode the spray voltage was −39 kV and the tube lens offset was 55 V, whilst the remaining parameters were unaltered.

ESI/MS data for **1** were acquired in a similar manner using an Applied Biosystems (Foster City, CA, USA) API2000 triple quadrupole instrument. In this case the mass spectrometer was operated in the positive ion mode with optimised conditions: declustering potential 100 eV, focusing potential 200 eV, entrance potential 5 eV, collision energy 10 eV, and collision cell exit potential 5 eV. In the negative ion mode declustering potential −100 eV, focusing potential −200 eV, entrance potential 5 eV, collision energy 10 eV, and collision cell exit potential 5 eV. In all cases, the MS spectra were acquired and elaborated using the software provided by the manufacturer.

2.4.2. LC-ESI/MS

Qualitative LC-ESI/MS was performed using a Thermo Finnigan SpectraSystem HPLC equipped with a Waters (Milford, MA, USA) Symmetry Shield RP C18 column (150 mm × 2.1 mm i.d.) and coupled to a LCQ Deca ion trap. Linear gradient elution with a mobile phase comprising water acidified with 0.05% trifluoroacetic acid (solvent **A**) and acetonitrile acidified with 0.05% trifluoroacetic acid (solvent **B**) commenced at 100:0 (**A**:**B**) and attained 20:80 in 55 min. The mobile phase was supplied at a flow rate of 250 μl min^{-1}, and the column effluent was injected directly into the ESI source. The mass spectrometer was operated in the negative and positive ion modes with a capillary voltage of 5 V, spray voltage of 5 kV, tube lens offset of 35 V, capillary temperature of 280 °C, and sheath gas (nitrogen) flow rate of 80 (arbitrary units).

Quantitative analyses of both saponins and flavonoids were performed by HPLC–MS using a Thermo Finnigan SpectraSystem HPLC equipped with a Waters (Milford, MA, USA) Symmetry Shield RP C18 column (150 mm × 2.1 mm i.d.) and coupled to a LCQ Deca ion trap, by using positive ion mode detection.

2.4.3. LC–MS/MS

Quantitative analyses of glychyrrizic acid were performed on an Agilent (Palo Alto, CA, USA) 1100 HPLC system equipped with a Waters Symmetry Shield RP C18 column (150 mm × 2.1 mm i.d.) and coupled to an Applied Biosystems API2000 triple quadrupole instrument. The mobile phase and gradient elution program described in Section 2.3 were employed, and the column effluent was injected directly into the ESI source. The API2000 mass spectrometer was used in the tandem MS mode with multiple reaction monitoring (MRM). The instrument was operated in the positive ion mode with a declustering potential of 100 eV, focusing potential of 200 eV, entrance potential of 5 eV, collision energy of 80 eV, collision cell exit potential of 22.5 eV, ion spray voltage of 4500, and capillary temperature of 280 °C

2.5. Sources of plant material and preparation of extracts

Air-dried commercial samples of China, Iran, Turkey and Italy were obtained and authenticated by Prof. Mariateresa Russo. *G. glabra* roots from China (voucher n. 121 deposited in Università degli Studi Mediterranea di Reggio Calabria, Reggio Calabria, Italy) were collected in region Rurade, Nord Est of Keerksin's steppe; *G. glabra* roots from Iran (voucher n. 122 deposited in Università degli Studi Mediterranea di Reggio Calabria, Reggio Calabria, Italy) were collected in region Khuzestan, near Ahwaz city; *G. glabra* roots from Turkey (voucher n. 123 deposited in Università degli Studi Mediterranea di Reggio Calabria, Reggio Calabria, Italy) were collected in region Lago Manyas, near Karabey city, *G. glabra* roots from Italy (voucher n. 124 deposited in Università degli Studi Mediterranea di Reggio Calabria, Reggio Calabria, Italy) were collected in region Calabria.

For metabolic profiling, appropriate aliquots of root material (Chinese sample 3 g; Iranian sample 6.06 g; Turkish sample 3.5 g; Italian sample 3.04 g) were mixed with ethanol:water (1:1, v/v; sample to solvent ratio 1:5, w/v), subjected to ultrasonic agitation for 1 h, and stored overnight at room temperature. The resulting extracts were filtered and diluted 1:10 (v/v) with ethanol:water (1:1, v/v) prior to qualitative or quantitative analysis. In order to isolate liquiritin apioside, employed as external standard in flavonoid

assays, a 55.26 g sample of Chinese liquorice root was extracted in an exactly similar manner and the solvent removed from the filtered extract under vacuum at 30 °C in a rotary evaporator.

2.6. Preparation of standards

2.6.1. Synthesis of glycyrrhizic acid dimethyl ester

Glycyrrhizic acid (21.1 mg) was dissolved in anhydrous methanol (80 ml), acidified with 0.1 M hydrochloric acid (4 ml), and stirred in a sealed vessel at room temperature for 12 h. The esterification was selective and involved only the carboxyl group of the glucuronic acid moieties, the product was then purified by dissolving in ethyl acetate and crystallizing by hexane, and the final yield of the dimethyl ester was 20.8 mg (98%).

Glycyrrhizic acid dimethyl ester: brown powder; UV (MeOH) λ_{max} 254 nm; ESI/MS *m/z* 851.1 $[M+H]^+$; ^{1}H NMR (CD_3OD, 600 MHz), δ: 3.17 (dd, H-3, J = 5.0, 11.4 Hz), 2.47 (s, H-9), 5.61 (s, H-12), 1.05 (s, H-23), 0.82 (s, H-24), 1.15 (s, H-25), 1.16 (s, H-26), 1.45 (s, H-27), 0.86 (s, H-28), 1.19 (s, H-29), 4.53 (d, H-1′$_{glcrAc}$, J = 7.5 Hz), 3.52 (dd, H-2′$_{glcrAc}$, J = 7.5, 9.0 Hz), 3.61 (dd, H-3′$_{glcrAc}$, J = 9.0, 9.0 Hz), 3.57 (dd, H-4′$_{glcrAc}$, J = 9.0, 9.0 Hz), 3.87 (d, H-5′$_{glcrAc}$, J = 9.0 Hz), 4.67 (d, H-1″$_{glcrAc}$, J = 7.5 Hz), 3.32 (dd, H-2″$_{glcrAc}$, J = 7.5, 9.0 Hz), 3.42 (dd, H-3″$_{glcrAc}$, J = 9.0, 9.0 Hz), 3.57 (dd, H-4″$_{glcrAc}$, J = 9.0, 9.0 Hz), 3.83 (d, H-5″$_{glcrAc}$, J = 9.0 Hz), 3.80 (s, 6H, H-7′$_{glcrAc}$ and H-7″$_{glcrAc}$);

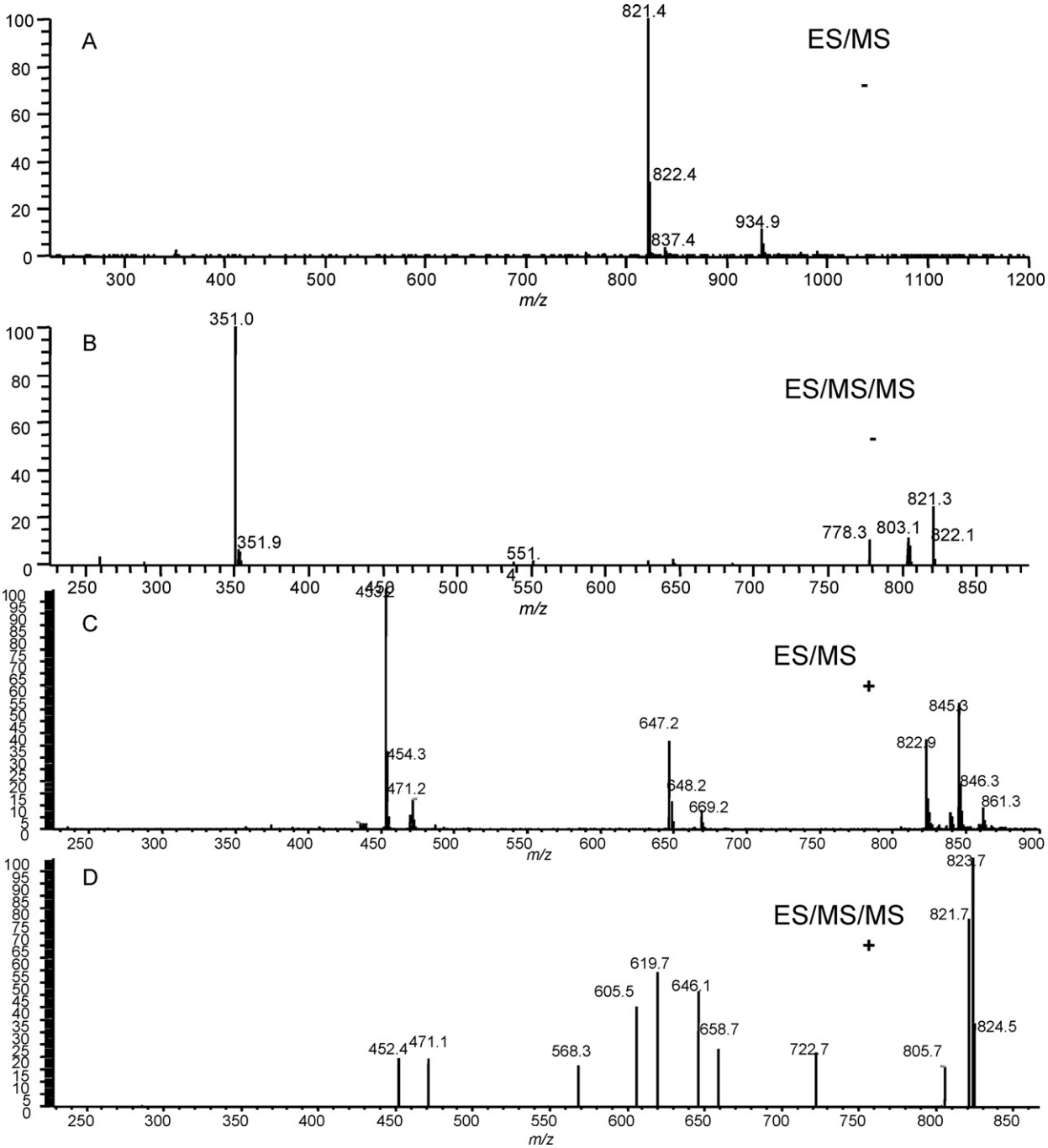

Fig. 1. ESI/MS (A and C) and ESI/MS/MS (B and D) spectra of glycyrrhizic acid obtained using an ion trap analyser operated in the negative ion mode (A and B) and positive ion mode (C and D).

^{13}C NMR (CD_3OD, 600 MHz), δ: 40.4 (C-1), 27.6 (C-2), 90.5 (C-3), 40.0 (C-4), 55.9 (C-5), 18.1 (C-6), 33.8 (C-7), 46.8 (C-8), 62.5 (C-9), 37.0 (C-10), 202.2 (C-11), 128.8 (C-12), 172.8 (C-13), 44.0 (C-14), 27.0 (C-15), 27.4 (C-16), 33.0 (C-17), 49.1 (C-18), 41.7 (C-19), 45.2 (C-20), 32.0 (C-21), 38.7 (C-22), 28.1 (C-23), 16.3 (C-24), 16.6 (C-25), 18.9 (C-26), 23.8 (C-27), 29.2 (C-28), 28.8 (C-29), 180.4 (C-30), 105.0 (C-1′$_{glcrAc}$), 83.7 (C-2′$_{glcrAc}$), 76.9 (C-3′$_{glcrAc}$), 72.6 (C-4′$_{glcrAc}$), 76.1 (C-5′$_{glcrAc}$), 171.2 (C-6′$_{glcrAc}$), 105.8 (C-1″$_{glcrAc}$), 75.8 (C-2″$_{glcrAc}$), 76.6 (C-3″$_{glcrAc}$), 72.6 (C-4″$_{glcrAc}$), 77.0 (C-5″$_{glcrAc}$), 170.7 (C-6″$_{glcrAc}$), 52.5 (C-7′$_{glcrAc}$ and C-7″$_{glcrAc}$).

2.6.2. Isolation of liquiritin apioside

A 2.5 g portion of the concentrated Chinese liquorice root extract (Section 2.3) was submitted to column chromatography over Sephadex LH-20 (100 cm × 5 cm i.d.; Pharmacia, Uppsala, Sweden) with methanol as mobile phase, and 50 ml × 8 ml fractions were obtained. Fractions 15–19 were bulked (224 mg) and further fractionated by preparative HPLC–UV using a Waters μ-Bondapak RP C18 column (300 mm × 7.6 mm i.d.). The mobile phase and gradient elution program described in Section 2.3 were employed, except that the flow rate of the mobile phase was 2 ml min^{-1}. The peak corresponding to liquiritin apioside (**24**) was detected at 254 and 350 nm at a retention time of 22.2 min, and 2.6 mg of the flavonoid were obtained.

Liquiritin apioside **(24)**: yellow powder; UV (MeOH) λ_{max} 350 nm; ESI/MS *m/z* 551.3 $[M+H]^+$; ^{1}H NMR (CD_3OD, 600 MHz), δ: 5.46 (dd, H-2, *J* = 3.2, 12.9 Hz), 3.05 (dd, H-3a, *J* = 12.9, 16.9 Hz), 2.75 (dd, H-3b, *J* = 3.2, 16.9 Hz), 7.74 (d, H-5, *J* = 8.9 Hz), 6.52 (dd, H-6, *J* = 2.0, 8.9 Hz), 6.38 (d, H-8, *J* = 2.0 Hz), 7.46 (d, H-2′ and H-5′, *J* = 8.9 Hz), 7.16 (d, H-3′ and H-6′, *J* = 8.9 Hz), glucose unit: 5.01 (d, H-1″$_{glc}$, *J* = 7.7 Hz), 3.65 (dd, H-2″$_{glc}$, *J* = 7.7, 9.0 Hz), 3.47 (dd, H-3″$_{glc}$, *J* = 9.0, 9.0 Hz), 3.40 (dd, H-4″$_{glc}$, *J* = 9.0, 9.0 Hz), 3.44 (m, H-4″), 3.70 (dd, H-6″a$_{glc}$, *J* = 4.5, 12.0 Hz), 3.91 (dd, H-6″b$_{glc}$, *J* = 2.5, 12 Hz), apiose unit: 5.48 (d, H-1‴$_{api}$, *J* = 1.6 Hz), 3.96 (d, H-2‴$_{api}$, *J* = 1.6 Hz), 4.08 (d, H-4‴a$_{api}$, *J* = 9.7 Hz), 3.81 (d, H-4‴b$_{api}$, *J* = 9.7 Hz), 3.56 (br s, H-5‴a-b$_{api}$); ^{13}C NMR (CD_3OD, 600 MHz), δ: 80.3 (C-2), 44.4 (C-3), 193.2 (C-4), 129.5 (C-5), 111.1 (C-6), 166.3 (C-7), 103.2 (C-8), 114.4 (C-4a), 165.2 (C-8a), 134.1 (C-1′), 128.4 (C-2′ and C-6′), 117.4 (C-3′ and C-5′), 158.8 (C-4′), glucose unit: 100.1 (C-1″$_{glc}$), 78.2 (C-2″$_{glc}$), 77.7 (C-3″$_{glc}$), 70.9 (C-4″$_{glc}$), 77.6 (C-5″$_{glc}$), 62.0 (C-6″$_{glc}$), apiose unit: 110.3 (C-1‴$_{api}$), 77.6 (C-2‴$_{api}$), 80.5 (C-3‴$_{api}$), 75.0 (C-4‴$_{api}$), 65.6 (C-5‴$_{api}$).

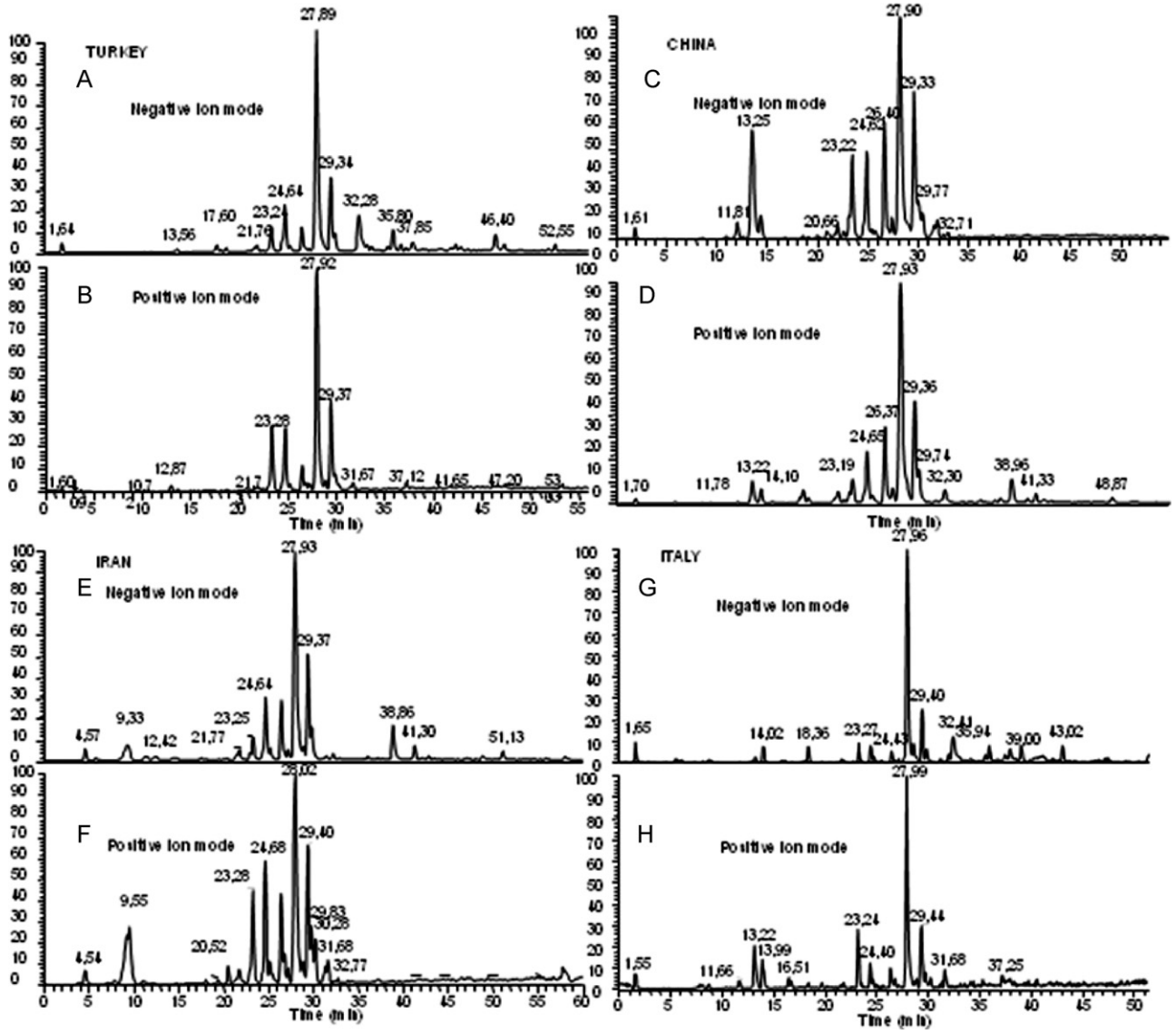

Fig. 2. LC-ESI/MS metabolite fingerprints of *Glycyrrhiza glabra* roots collected in Turkey (A and B), China (C and D), Iran (E and F) and Italy (G,and H) and obtained using an ion trap analyser operated in the negative ion mode (A, C, E, and G) and positive ion mode (B, D, F, and H).

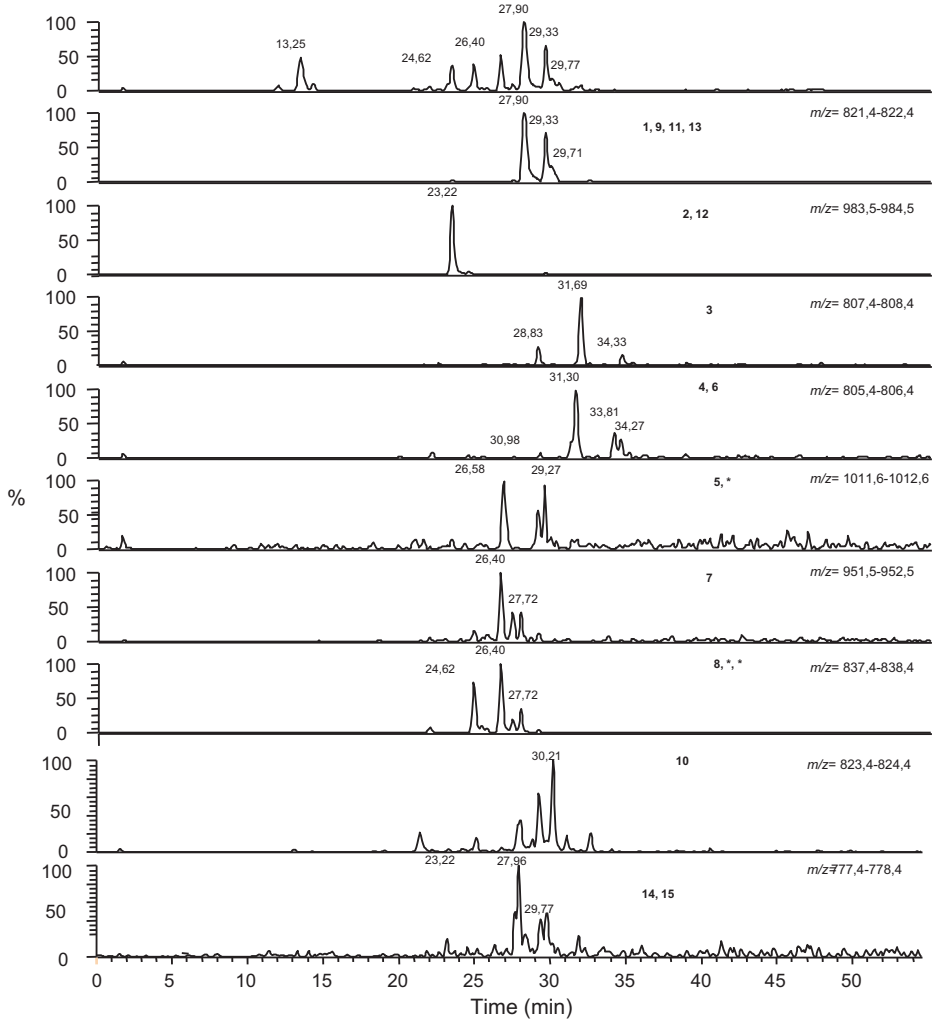

Fig. 3. LC-ESI/MS reconstructed ion chromatograms of *Glycyrrhiza glabra* roots collected in China.

2.7. Preparation of calibration curves

Stock solutions (1 mg ml^{-1}) of the external standards (ES; glycyrrhizic acid for saponins and liquiritin apioside for flavonoids) were prepared by dissolving each compound in methanol. Stock solutions were diluted with appropriate amounts of methanol to give solutions containing 10, 25, 50, 100 or 125 μg ml^{-1} of ES. To each standard solution was added an appropriate amount of internal standard (IS; glycyrrhizic acid dimethyl ester for saponins and rutin for flavonoids) to yield a final concentration of 50 μg ml^{-1}. Calibration curves were constructed by injecting each standard solution at each concentration level in triplicate. The ratios of the peak areas of the ES to those of the IS were calculated and plotted against the corresponding concentrations of the standard compounds using weighted linear regression to generate standard curves.

2.8. Method validation

The LC–MS and LC–MS/MS methods were validated according to the European Medicines Agency (EMEA) guidelines relating to the validation of analytical methods [21]. Precision was evaluated at four concentration levels for each compound through triplicate intra-day assays and inter-day assays over 3 days. In all cases, the standard deviation was no higher than ±2.00% for either of the analytical methods. Specificity was defined as the non-interference by other analytes detected in the region of interest. In the case of the LC–MS method for flavonoids and saponins, no interfering peaks were encountered at the retention times of the investigated compounds. For the LC–MS/MS method, which was developed on the basis of the characteristic fragmentation of **1**, no other peaks interfered with the analyte in the MS/MS detection mode. Recoveries were estimated through the addition of pre-determined quantities of standard analytes to known amounts of liquorice root samples. Recovery was calculated from the difference between the amount of analyte measured in the spiked sample and the amount of analyte determined in the sample prior to spiking plus the amount of standard added. The mean recoveries for the LC–MS and LC–MS/MS methods were determined to be 100 ± 4% and 100 ± 2%, respectively. The calibration graphs, obtained by plotting the area ratios between ES and IS against the known concentration of each compound, were linear in the range of 25–125 μg ml^{-1} for all analytes. The limit of quantification (LOQ; equivalent to sensitivity), defined as the lowest concentration of analyte that could be quantified with acceptable accuracy and precision, was estimated by injecting a series of increasingly dilute standard solutions until the signal-to-noise ratio was reduced to 10. In the LC–MS method, LOQ values for the saponins and flavonoids were within the range of 10–70 ng/ml, whilst for the LC–MS/MS method, the LOQ value for **1** was 5 ± 0.4% ng/ml.

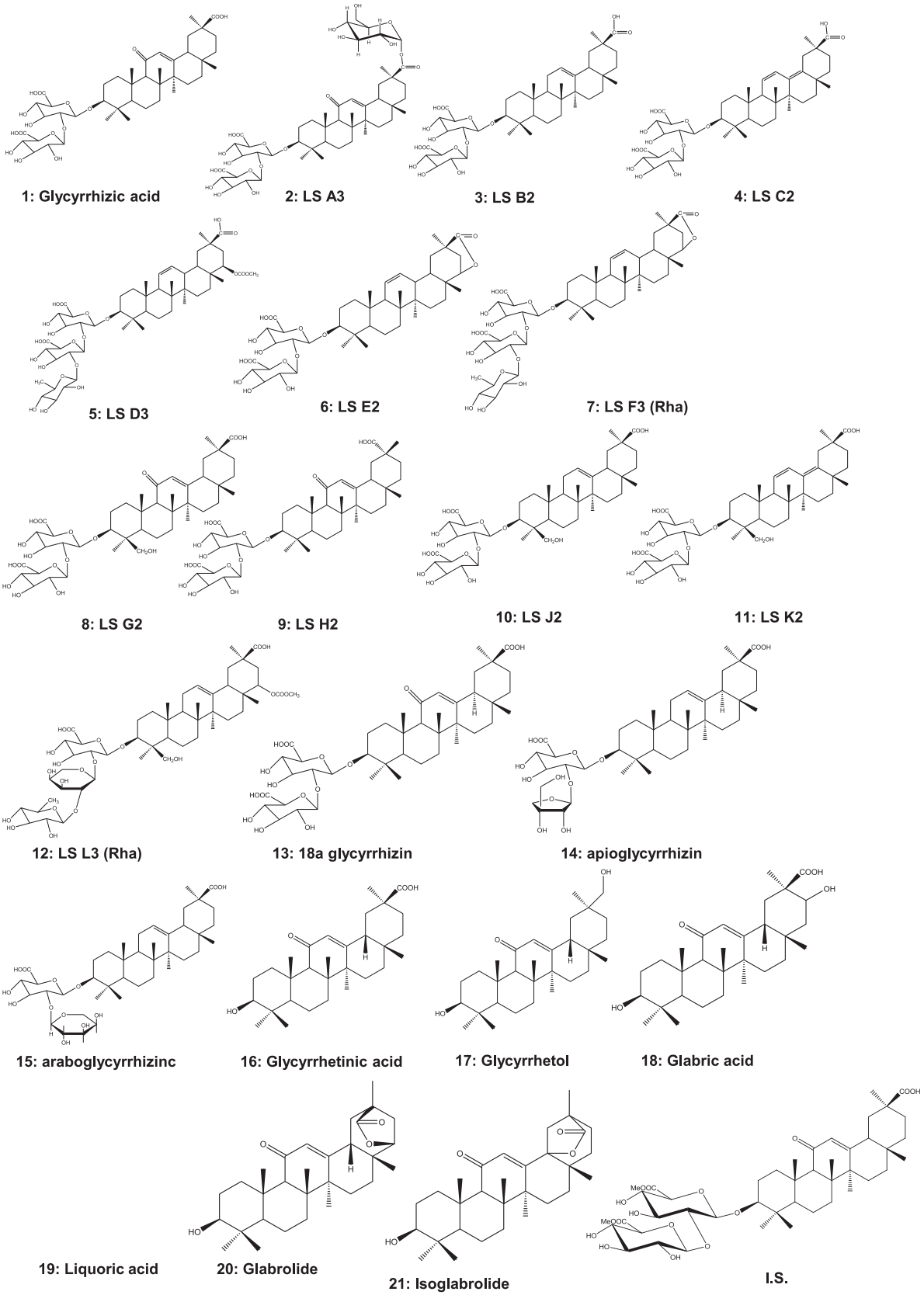

Fig. 4. Saponins identified in *Glycyrrhiza glabra* root extracts derived from different countries.

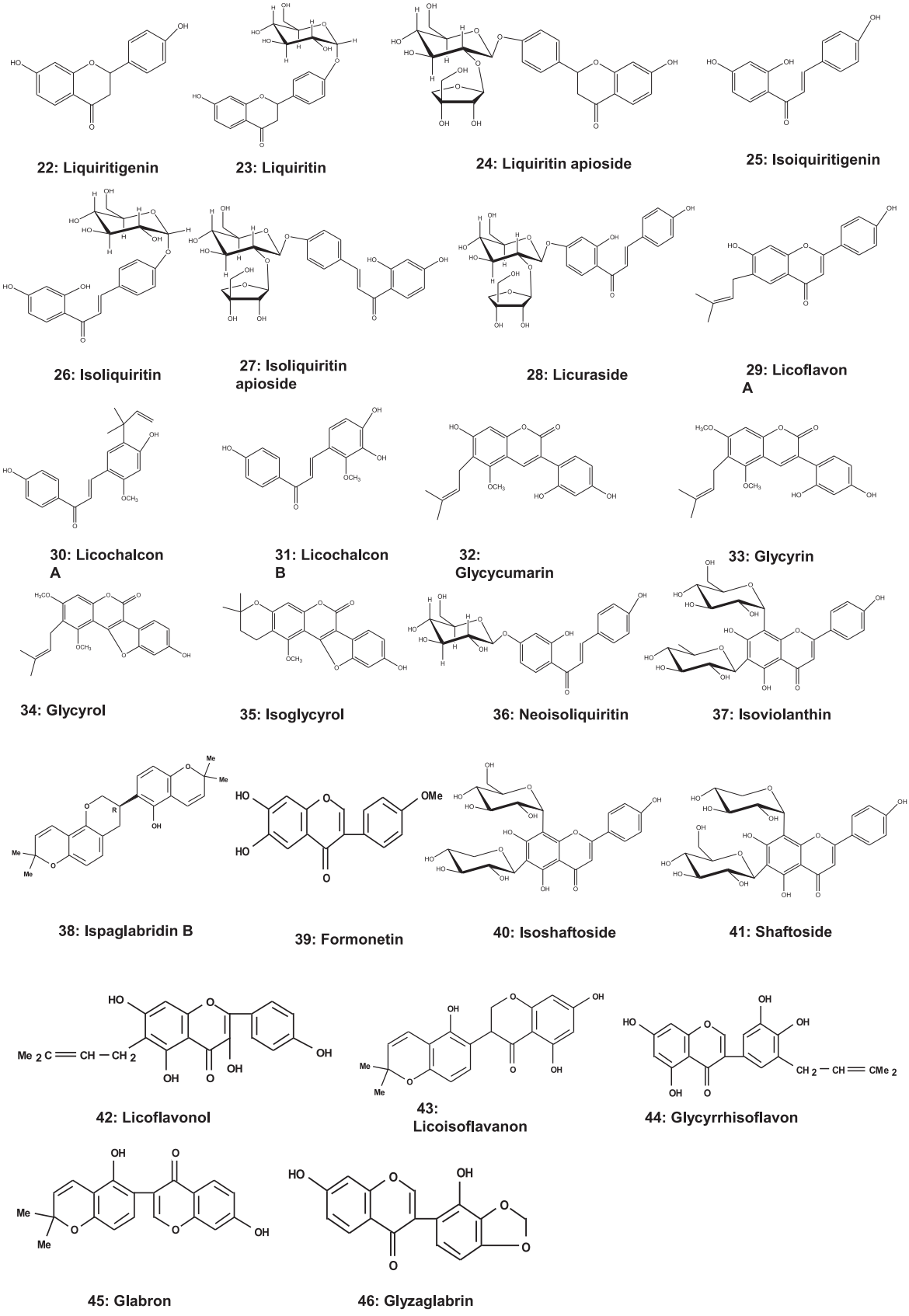

Fig. 5. Phenolic compounds identified in *Glycyrrhiza glabra* root extracts derived from different countries.

Table 1
Qualitative analyses by LC-ESI/MS and LC-ESI/MS/MS of saponins in the extracts of *G. glabra* derived from different geographic areas.

$[M-H]^-$	Retention time (min)	MS/MS fragments	Identity	Occurrence[a]
1011	26.58	–	**5**	C, Ir, T
983	23.22	821 803	**2**	C, Ir, T, It
983	23.22	821 803	**12**	C, Ir, T, It
951	26.4	935 891 777	**7**	C, Ir, T
837	24.62	819 775 661 643 350	**8**	C, Ir, T, It
823	30.21	–	**10**	C, Ir, T, It
821	27.90	805 761 647 350	**1**	C, Ir, T, It
821	29.33	803 759 645 350	**9**	C, Ir, T, It
821	–	805 761 647 350	**11**	-
821	–	805 761 647 350	**13**	–
807	31.69	–	**3**	C, Ir, T, It
805	31.30	787 761 710 629	**4**	C, Ir, T, It
777	27.96	–	**14**	C, Ir, T
777	29.77	–	**15**	C, Ir, T

[a] Sources of samples: C, China; T, Turkey; Ir, Iran; It, Italy.

2.9. Qualitative and quantitative analysis of liquorice root samples

For qualitative analysis, an aliquot (20 μl) of the diluted liquorice root extract was injected into the LC–MS system. For the quantitative determination of saponins and flavonoids by LC–MS, or of **1** by LC–MS/MS, an appropriate volume of IS (glycyrrhizic acid dimethyl ester for saponins and rutin for flavonoids) was added to the diluted liquorice root extract to give a final concentration of 50 μg ml^{-1}, and an aliquot (20 μl) of the mixture injected into the analytical system. Quantitative analyses were replicated five times for each liquorice root extract.

3. Results and discussion

3.1. ES/MS analysis of glycyrrhizic acid

Direct flow injection ESI/MS analyses of standard glycyrrhizic acid (**1**) were performed in the negative and positive ion modes using two mass spectrometers, one equipped with an ion trap analyser and the other with a triple quadrupole analyser. The ESI/MS and ESI/MS/MS spectra of **1** obtained in the negative and positive ion modes using the ion trap analyser are displayed in Fig. 1. The ESI/MS spectrum of **1** in the negative ion mode (Fig. 1A) showed predominantly the pseudomolecular ion peak $[M-H]^-$ at *m*/*z* 821, the MS/MS spectrum of which (Fig. 1B) exhibited a major fragment ion at *m*/*z* 351 arising from the loss of the triterpenic aglycone. More information was available from the ESI/MS spectrum in the positive ion mode (Fig. 1C), which presented fragmentation peaks at *m*/*z* 647 and 453 caused by the consecutive neutral loss of two glucuronic acid residues from the pseudomolecular ion $[M+H]^+$ at *m*/*z* 823. This fragmentation pattern was confirmed in the positive ion mode MS/MS spectrum (Fig. 1D).

The reason for the difference in fragmentation patterns between the two modes may be that the glucuronic acid residue was able to lose electrons in the negative mode and not capable of capturing a proton in the positive mode, where the electrons were kept from the aglycone. On this basis, it was possible to define the nature of the sugar moiety from the fragmentation pattern in the negative ion mode ESI/MS/MS, whilst in the positive ion mode the fragments originated mainly from the triterpene skeleton thus allowing the aglycone to be characterized. The fragmentation patterns described above were similar to those obtained with the triple quadrupole analyser (data not shown) and were also in accordance with a previous report [22].

3.2. Qualitative analysis of liquorice root extracts by LC-ESI/MS

In a preliminary study, hydroalcoholic extracts of liquorice roots were analysed by HPLC–UV with detection at 254 and 350 nm in order to optimise the chromatographic conditions. Based on the results obtained, a method involving a Symmetry Shield C18 column, eluted with a linear gradient of mobile phase comprising water and acetonitrile (each acidified with 0.05% trifluoroacetic acid), was developed and this allowed the separation of several saponins structurally related to **1** together with a number of phenolic.

LC-ESI/MS analysis was then applied for the full characterization of the different metabolites in the extracts of the roots of *G. glabra* obtained from China, Iran, Turkey and Italy (Calabria). Polarity switching from the positive to the negative ion mode was applied throughout the chromatographic runs in order to obtain ion current chromatograms in both modes and hence to detect as many triterpene saponins (in the negative ion mode) and phenolic compounds (in the positive ion mode) as possible. The chromatograms so obtained are displayed in Fig. 2, and they demonstrate that liquorice roots derived from different regions present dissimilar metabolic profiles. For each sample, a full MS scan, in the form of a total ion current chromatogram (TIC), was initially acquired, following which reconstructed ion chromatograms (RICs) were generated for each of the expected *m*/*z* values based on the molecular weights of the possible constituents. This step was very important in order to attain a high selectivity for the quantitative analysis.

The TIC profile of an extract of Chinese liquorice root, obtained by LC-ESI/MS using the ion trap analyser in the negative ion mode, is shown in Fig. 3. As may be observed, the majority of components were efficiently separated, and it was possible to recognise peaks corresponding to the protonated molecular ions of various saponins. Individual compounds were tentatively identified by comparison of their *m*/*z* values in the TIC profile with those of selected compounds described in literature, and RICs were generated for specific compounds expected to be present in the sample (Fig. 3). Thus, the RIC related to *m*/*z* 821 showed one peak at a retention time of 27.9 min corresponding to glycyrrhizic acid (**1**) and two further peaks at 29.3 and 29.7 min, which were most probably associated with compounds **9**, **11** or **13** (Fig. 4 and Table 1). In a similar manner, RICs related to *m*/*z* values of 1011, 983, 951, 837, 823, 807, 805, 777 indicated the possible presence of compounds **5**, **2**, **12**, **7**, **8**, **10**, **3**, **4** and **6**, **14** and **15**, respectively. For *m*/*z* values 1011 and 837, the RICs showed two peaks each, which may indicate the presence of unidentified components with identical pseudomolecular ions.

Further LC-ESI/MS/MS experiments were carried out on-line using the dependent scanning mode in which the MS software selected ions of certain intensity for further fragmentation experiments on the basis of a set of parameters predetermined by the operator. The positive and negative ion fragmentation patterns so-obtained enabled comprehensive qualitative analyses of the saponins in the extracts of *G. glabra* from different geographic areas to be carried out (Table 1). Additionally, the LC-ESI/MS/MS data allowed the qualitative determination of phenolic compounds present in *G. glabra* extracts as shown in Fig. 5 (data not shown). It appears that Chinese liquorice root is the most complex with respect to both saponin and flavonoid content.

3.3. Quantitative analyses of glycyrrhizic acid in liquorice root extracts by LC-ESI/MS/MS

In order to obtain accurate data concerning the amounts of glycyrrhizic acid in roots of *G. glabra* derived from different geographic areas, a quantitative LC-ESI/MS/MS method was developed. ESI/MS/MS spectra were recorded following the direct introduction of standard **1** into the ESI source of an LC/MS instrument equipped with a triple quadrupole analyser. The transition from the specific pseudomolecular ion $[M+H]^+$ of **1** to the corresponding aglycone ion $[A+H]^+$ was selected to monitor this analyte, whilst glycyrrhizic acid dimethyl ester was chosen as internal standard on the basis of its structure, chromatographic and MS behaviour. The MRM method therefore involved the precursor/product transitions from *m*/*z* 823.0 to 453.0 for **1** and from *m*/*z* 851.0 to 453.0 for the IS. The calibration curves obtained by plotting the area ratios between ES (**1**) and IS against known concentration of both compounds were linear in the range of 10–125 $\mu g\,ml^{-1}$ with r^2 values of >0.98.

Table 2
Quantitative analyses by LC-ESI/MS/MS of glycyrrhizic acid in the extracts of *G. glabra* derived from different geographic areas.

Source of liquorice root sample	Concentration of glycyrrhizic acid (mg/g dried plant material)	
	Mean ($n = 5$)	Standard deviation
China	53.26	2.15
Iran	32.21	1.02
Turkey	33.47	1.85
Italy	51.91	1.21

Some variations were detected in the content of glycyrrhizic acid in liquorice root samples derived from different geographic locations (Table 2). Whilst Chinese *G. glabra* roots contained the highest levels of **1**, followed by those from Italy (Calabria), all samples attained a content of the analyte of near to 4%, as specified by the European Pharmacopoeia [23].

3.4. Quantitative analyses of saponins and flavonoids in liquorice roots by LC-ESI/MS

Compound **14** was isolated by semipreparative HPLC–UV (by using the same chromatographic conditions reported for liquiritine apioside in the Section 2) and characterized and identified by NMR data [24]. The compounds were obtained with a purity of 95%, not

Table 3
Quantitative analyses by LC-ESI/MS of saponins and flavonoids in the extracts of *G. glabra* derived from different geographic areas.

	Relative[b] content (mg/g dried plant material)			
	Mean ± standard deviation (n = 5)			
	China	Iran	Turkey	Italy
Saponins[a]				
1	53.90 ± 2.21	37.32 ± 1.11	32.52 ± 1.25	55.96 ± 1.25
2	5.87 ± 0.25	6.55 ± 0.12	6.19 ± 0.41	7.97 ± 0.22
12	4.88 ± 0.55	6.13 ± 0.24	5.92 ± 0.36	5.15 ± 0.14
3	5.03 ± 0.04	12.09 ± 0.19	5.14 ± 0.14	11.16 ± 0.21
4, 6	Trace	3.74 ± 0.12	Trace	4.67 ± 0.41
14	4.01 ± 0.09	3.87 ± 0.11	Trace	4.75 ± 0.12
15	4.01 ± 0.21	3.49 ± 0.85	Trace	4.00 ± 0.17
10	19.19 ± 2.14	8.22 ± 0.57	10.13 ± 0.85	8.97 ± 0.45
8	23.23 ± 2.31	22.78 ± 1.54	14.24 ± 0.57	18.70 ± 1.21
Flavonoids[c]				
23	2.33 ± 0.01	1.26 ± 0.05	2.38 ± 0.11	3.11 ± 0.15
26	2.07 ± 0.02	7.21 ± 0.21	3.10 ± 0.09	2.55 ± 0.04
36	2.67 ± 0.05	4.61 ± 0.07	trace	1.85 ± 0.01
28, 27	5.16 ± 0.14	8.81 ± 0.07	3.86 ± 0.07	3.26 ± 0.02
24	6.86 ± 0.03	23.21 ± 2.04	9.09 ± 1.01	6.06 ± 0.04
37	5.08 ± 0.05	27.30 ± 1.25	4.99 ± 0.10	1.61 ± 0.05
40, 41	5.52 ± 0.09	2.96 ± 0.08	Trace	1.74 ± 0.08
38	Trace	4.16 ± 0.07	Trace	Trace
46	Trace	Trace	Trace	3.17 ± 0.05
29	Trace	Trace	Trace	7.48 ± 0.14
30	Trace	Trace	Trace	2.84 ± 0.41

[a] Numbering corresponds to structures shown in Fig. 4.
[b] Content relative to glycyrrhizic acid dimethyl ester for saponins and rutin for flavonoids.
[c] Numbering corresponds to structures shown in Fig. 5.

permitting to use it as standards for quantitative analysis. The isolation permitted to distinguish the peak of compound **2** by the peak of compound **14**, and successively to quantify both the compounds.

Saponins and flavonoids in liquorice root samples were quantified from positive ion LC-ESI/MS spectra obtained using an LC–MS instrument equipped with an ESI source and an ion trap analyser. For each component, an RIC related to the appropriate m/z value was generated. The calibration curves obtained by plotting the area ratios between ES and IS against the known concentration of both compounds were linear in the range of 10–125 $\mu g\,ml^{-1}$ with r^2 values of >0.98.

Five aliquots of each sample of *G. glabra* were analysed using the above method and the results are reported in Table 3. The Turkish sample was characterized by a low content of **1** and related saponins, while the Calabrian sample presented a higher content of **1** and a larger number of saponins but in smaller quantities. The Chinese sample also showed high levels of saponins **8** and **10**. A larger number of flavonoids were present in the Iranian and Calabrian samples, although higher amounts of these compounds were found in Chinese and Iranian samples.

4. Conclusions

The LC-ESI/MS and LC-ESI/MS/MS methods developed for the qualitative and quantitative determination of saponins and phenolic compounds in *G. glabra* extracts were specific and sensitive for the analytes studied. In particular, polarity switching in the MS gave very good results in terms of specificity for the two classes of compounds investigated. The qualitative analysis of *G. glabra* roots derived from different countries revealed that some phenolic compounds were indicative of the area of collection, thus allowing the origin of the plant material to be established readily. Samples of liquorice roots from China and Italy were the most rich in glycyrrhizic acid. Additionally, a number of saponins related to glycyrrhizic acid were found to be present in the studied extracts in large amounts, in some cases in quantities comparable with that of glycyrrhizic acid itself. For these compounds, it could be of interest to evaluate the biological activities in comparison with those of **1**.

References

[1] T. Mae, H. Kishida, T. Nishiyama, M. Tsukagawa, E. Konishi, M. Kuroda, Y. Mimaki, Y. Sashida, K. Takahashi, T. Kawada, K. Nakagawa, M. Kitahara, A licorice ethanolic extract with peroxisome proliferator-activated receptor-{gamma} ligand-binding activity affects diabetes in KK-Ay Mice, abdominal obesity in diet-induced obese C57BL mice and hypertension in spontaneously hypertensive rats, J. Nutr. 133 (2003) 3369–3377.
[2] D. Dhingra, A. Sharma, Antidepressant-like activity of *Glycyrrhiza glabra* L. in mouse models of immobility tests, Prog. Neuropsychopharmacol. Biol. Psychiatr. 30 (2006) 449–454.
[3] D. Dhingra, M. Parle, S.K. Kulkarni, Memory enhancing activity of *Glycyrrhiza glabra* in mice, J. Ethnopharmacol. 91 (2004) 361–365.
[4] B.S. Ko, J.S. Jang, S.M. Hong, S.R. Sung, J.E. Lee, M.Y. Lee, W.K. Jeon, S. Park, Changes in components glycyrrhizin and glycyrrhetinic acid, in raw *Glycyrrhiza uralensis* Fisch, modify insulin sensitizing and insulinotropic actions, Biosci. Biotechnol. Biochem. 71 (2007) 1452–1457.
[5] R.S.H. Finney, G.F. Somer, The antiinflammatory activity of glycyrrhetinic acid and derivatives, J. Pharm. Pharmacol. 10 (1958) 613–620.
[6] R. Pompei, O. Flore, M.A. Marcialis, A. Pani, B. Loddo, Glycyrrhizic acid inhibits virus growth and inactivates virus particles, Nature 281 (1979) 689–690.
[7] H. Nishino, K. Kitagawa, A. Iwashima, Antitumor-promoting activity of glycyrrhetic acid in mouse skin tumor formation induced by 7,12-dimethylbenz[a]anthracene plus teleocidin, Carcinogenesis 5 (1984) 1529–1530.
[8] J.K. Kim, S.M. Oh, H.S. Kwon, Y.S. Oh, S.S. Lim, H.K. Shin, Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages, Biochem. Biophys. Res. Commun. 345 (2006) 1215–1223.
[9] N.P. Visavadiya, A.V.R.L. Narasimhacharya, Hypocholesterolaemic and antioxidant effects of *Glycyrrhiza glabra* (Linn) in rats, Mol. Nutr. Food Res. 50 (2006) 1080–1086.
[10] S.J. Dong, A. Inoue, Y. Zhu, M. Tanji, R. Kiyama, Activation of rapid signaling pathways and the subsequent transcriptional regulation for the proliferation of breast cancer MCF-7 cells by the treatment with an extract of *Glycyrrhiza glabra* root, Food Chem. Toxicol. 45 (2007) 2470–2478.
[11] E.H. Jo, S.H. Kim, J.C. Ra, S.R. Kim, S.D. Cho, J.W. Jung, S.R. Yang, J.S. Park, J.W. Hwang, O.I. Aruoma, T.Y. Kim, Y.S. Lee, K.S. Kang, Chemopreventive properties of the ethanol extract of chinese licorice (*Glycyrrhiza uralensis*) root: induction of apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells, Cancer Lett. 230 (2005) 239–247.
[12] J. Ma, W.L. Peng, D. Liang, N.Y. Fu, D.B. Pang, A.L. Xu, The extract of *Glycyrrhiza uralensis* Fisch induces apoptosis in MGC-803 cells and its molecular mechanisms, Biomed. Res. 21 (2000) 129–137.
[13] F. Chen, K.H. Chan, Y. Jiang, R.Y.T. Kao, H.T. Lu, K.W. Fan, V.C.C. Cheng, W.H.W. Tsui, I.F.N. Hung, T.S.W. Lee, Y. Guan, J.S.M. Peiris, K.Y. Yuen, In vitro susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds, J. Clin. Virol. 31 (2004) 69–75.
[14] J. Cinatl, B. Morgenstern, G. Bauer, P. Chandra, H. Rabenau, H. Doerr, Glycyrrhizin an active component of liquorice roots, and replication of SARS-associated coronavirus, Lancet 361 (2003) 2045–2046.
[15] S. Ishida, Y. Sakiya, T. Ichikawa, S. Awazu, Pharmacokinetics of glycyrrhetic acid, a major metabolite of glycyrrhizin, in rats, Chem. Pharm. Bull. 37 (1989) 2509–2513.
[16] J. Heikens, E. Fliers, E. Endert, M. Ackermans, G.V. Montfrans, Liquorice-induced hypertension—a new understanding of an old disease: case report and brief review, Neth. J. Med. 47 (1995) 230–234.
[17] D.R. Lauren, D.J. Jensen, J.A. Douglas, J.M. Follet, Efficient method for determining the glycyrrhizin content of fresh and dried roots, and root extracts, of Glycyrrhiza species, Phytochem. Anal. 12 (2001) 332–335.
[18] P. Wang, S.F.Y. Li, H.K. Lee, Determination of glycyrrhizic acid and 18-beta-glycyrrhetinic acid in biological fluids by micellar electrokinetic chromatography, J. Chromatogr. A 811 (1998) 219–224.
[19] I. Kitagawa, Licorice root. A natural sweetener and an important ingredient in Chinese medicine, Pure Appl. Chem. 74 (2002) 1189–1198.
[20] Q. Zhang, M. Ye, Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice), J. Chromatogr. A 1216 (2009) 1954–1969.
[21] QualityGuidelines: Validation of Analytical Procedures – Text and Methodology (ICH Q2), London, European Medicines Agency Available at: http://www.emea.europa.eu/pdfs/human/ich/038195en.pdf, 1995.
[22] M. Ye, S.H. Liu, Z. Jiang, Y. Lee, R. Tilton, Y.C. Cheng, Liquid chromatography/mass spectrometry analysis of PHY906, a Chinese medicine formulation for cancer therapy, Rapid Commun. Mass Spectrom. 21 (2007) 3593–3607.
[23] European Pharmacopoeia, 2nd ed., Council of Europe, Strasbourg, 1995.
[24] I. Kitagawa, M. Sakagami, F. Hashiuchi, J.L. Zhu, M. Yoshikawa, R. Jiali, Apioglycyrrhyzin and araboglycyrryzin, two new sweet oleanane type triterpene oligoglycosides from the root of *Glycyrrhiza inflata*, Chem. Pharm. Bull. 37 (1989) 551–553.