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Species-specific Standardisation of Licorice by Metabolomic Profiling of Flavanones and Chalcones

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Abstract

Introduction—Major phenolics from licorice roots (*Glycyrrhiza* sp.) are glycosides of the flavanone liquiritigenin (F) and its 2'-hydroxychalcone isomer, isoliquiritigenin (C). As the F and C contents fluctuate between batches of licorice, both quality control and standardisation of its preparations become complex tasks.

Objective—To characterise the F and C metabolome in extracts from *Glycyrrhiza glabra* L. and *Glycyrrhiza uralensis* Fisch. ex DC. by addressing their composition in major F–C pairs and defining the total F:C proportion.

Material and methods—Three types of extracts from DNA-authenticated samples were analysed by a validated UHPLC/UV method to quantify major F and C glycosides. Each extract was characterised by the identity of major F–C pairs and the proportion of Fs among all quantified Fs:Cs.

Results—The F and C compositions and proportions were found to be constant for all extracts from a *Glycyrrhiza* species. All *G. uralensis* extracts contained up to 2.5 more Fs than *G. glabra* extracts. Major F–C pairs were B-ring glycosidated in *G. uralensis*, and A-/B-ring apiosyl-glucosidated in the *G. glabra* extracts. The F:C proportion was found to be linked to the glycosidation site: the more B-ring F–C glycosides were present, the higher was the final F:C proportion in the extract. These results enable the chemical differentiation of extracts from *G. uralensis* and *G. glabra*, which are characterised by total F:C proportions of 8.37:1.63 and 7.18:2.82, respectively.

Conclusion—Extracts from *G. glabra* and *G. uralensis* can be differentiated by their respective F and C compositions and proportions, which are both useful for further standardisation of licorice botanicals.

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SUPPORTING INFORMATION

Supporting information providing 1D ¹H NMR and ¹³C NMR data of major Fs and Cs, purity of isolated metabolites, quantitative results linked to the validation of the UHPLC-UV method, extraction yields, and finally quantitative results obtained for all extracts, can be found in the online version of this article.

Keywords

Chalcones; flavanones; licorice; metabolomic profile; *Glycyrrhiza*

Introduction

Licorice roots consist of the dried unpeeled or peeled, whole or cut roots and stolons of *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch. ex DC. and/or *Glycyrrhiza inflata* Batalin. The genus *Glycyrrhiza* (Leguminosae) comprises around 20 known species, half of which are pharmacopoeial ingredients for herbal preparations. Among all the *Glycyrrhiza* species, the most widely used in commerce are *G. glabra* ('European licorice') and *G. uralensis* ('Chinese licorice'). According to various Pharmacopeia, the roots from these two principal species can be used alternatively or together in traditional medicine (Zhang and Ye, 2009) or as dietary supplements (EMA/ HMPC, 2011). The main chemical constituents of licorice roots are triterpene saponins, with glycyrrhizin predominating at 3–13% (w/w) of the dried roots, followed by polyphenols, which account for 1–5% of the dried roots. The latter are generally characterized by a high abundance of glycosides of isoliquiritigenin (2'-hydroxychalcone, LigC) and its isomer liquiritigenin (flavanone, LigF) (Nomura *et al.*, 2002; Zhang and Ye, 2009). Well-established major glycosides of LigF and LigC are liquiritin, liquiritin apioside and liquiritigenin 7-*O*-apiosyl-glucoside in the flavanone (F) series, as well as isoliquiritin, isoliquiritin apioside and licuraside in the chalcone (C) series (Fig. 1). As emphasized by Nomura and Fukai (1998) these trivial names are frequently confused throughout the literature (Shi *et al.*, 2012), mainly as a result of typographical errors or English translation. Additionally, these widely used names do not indicate the position of the glycosidation on the phenolic core. So as to avoid further confusion, the present study proposes a nomenclature that indicates for each major glycosides the type and position of the glycosidation on the equivalent aglycone LigF/LigC (Fig. 1).

Flavanones (F) and 2'-hydroxychalcones (C) are known to be biosynthetically related (Ralston *et al.*, 2005), and are also known to be chemically interchangeable. Recently it has been demonstrated that the interconversion between LigF and its chalcone isomer LigC occurs in aqueous solution as a function of time and temperature, reaching a final LigF:LigC equilibrium defined by the proportion of 9:1 (Simmler *et al.*, 2013). Furthermore, this unavoidable F–C isomerisation or interconversion is known to affect the glycosidated analogues, as previously demonstrated for F and C glycosides from citrus preparations (Gil-Izquierdo *et al.*, 2003; Caccamese and Chillemi, 2010), and from alcoholic extracts of the aerial parts of *Lippia salviaefolia* Cham. (Funari *et al.*, 2011). Moreover, it has been suggested that the isomerisation of LigC-4-*O*-Glc (isoliquiritin, C) to LigF-4'-*O*-Glc (liquiritin, F) was likely to occur during various treatment steps of licorice samples, from the sun drying of fresh roots, to storage, and finally preparation of a boiling alcoholic extract (Nomura and Fukai, 1998). Given that both Fs and Cs are prone to interconversion in aqueous solution or aqueous alcoholic extracts, it can be assumed that both Fs and their C isomers are likely to coexist within the same licorice preparation. The determination of the *F–C composition* (qualitative descriptor of the F and C metabolome), and the *F:C proportion* (quantitative descriptor of the relative abundance of metabolites in the F and C

metabolome) in various extracts are potentially representative of a flavanone:chalcone equilibrium in complex natural matrices (Simmler *et al.*, 2013). Testing this hypothesis requires the simultaneous quantitation of the metabolomic mixture of both isomers in licorice preparations. As previously described for the LigF–LigC pair, each major quantified F–C pair could then be defined by a value representing its F:C proportion in a given crude extract, thus, reflecting a potential *in situ* F:C equilibrium.

From a biological perspective, LigC and LigF have been reported to display closely related biological activities. For instance, both isomers have been demonstrated to exhibit not only estrogenic, but also anti-inflammatory, as well as anti-cancer, hepato- and neuroprotective properties (Simmler *et al.*, 2013). In view of their potential metabolism and/or deglycosidation by microorganisms from the gut flora (Kamei *et al.*, 2005), glycosides of LigF and LigC are also believed to contribute to the *in vivo* biological activities reported for their aglycone counterparts (Asl and Hosseinzadeh, 2008). Therefore, glycosides of LigF and LigC could be regarded as either pro-drugs or bio-equivalent agents of the corresponding LigF–LigC aglycones. Furthermore, licorice extracts enriched in LigF–LigC glycosides demonstrate *in vitro* estrogenic activity (Hajirahimkhan *et al.*, 2013). Finally, the pharmacological properties of an extract cannot be attributed solely to a few constituents (e.g. LigF, LigF-4'-O-Glc, glycyrrhizin), but rather are due to the combined action of multiple chemical entities (Folashade *et al.*, 2012). Accordingly, another widely used biological marker of licorice roots is glycyrrhizin (Asl and Hosseinzadeh, 2008). This saponin glycoside undergoes deglycosidation prior to its intestinal adsorption, which eventually leads to the generation of glycyrrhetic acid. The latter is known for its corticoid-like anti-inflammatory properties, which are also associated with unwanted side effects (Isbrucker and Burdock, 2006).

To date, several analytical methods have been reported for the quantitative determination of major phenolics and/or glycyrrhizin from licorice extracts, including HPLC (Wang and Yang, 2007) with UV or MS detection (Montoro *et al.*, 2011), capillary electrophoresis (Rauchensteiner *et al.*, 2005) or electrochromatography (Chen *et al.*, 2009), and recently UPLC/UV combined with electrospray and quadrupole time-of-flight (QTOF) MS detection (Zhou *et al.*, 2013). All these methods were applied to analyse extracts prepared from one, two, or three of the major pharmacopoeial *Glycyrrhiza* species, or even from mixtures of them (Wu *et al.*, 2013). All these studies focused on the validation of the analytical method associated with the quantitation of the principal Fs, and/or Cs, and/or glycyrrhizin in the extracts, comparing absolute concentrations as a quality control measure. Given the biological properties of LigF, LigC and glycyrrhetic acid, the quantitation of the corresponding glycosides is proposed as a means of characterisation and standardisation of licorice preparations. Relevant to this approach is a recent study demonstrating that the content of both LigF-4'-O-Glc (liquiritin, F) and glycyrrhizin can greatly fluctuate between batches of *G. uralensis*, despite all being cultivated under the same conditions and harvested after 5 years of growth (Kojoma *et al.*, 2011). Hence, considering the natural and substantial variation of bioactive constituents, between batches of the same *Glycyrrhiza* species, quality control and standardisation of licorice preparations become a complex task.

Interestingly, both the determination of major coexisting F–C pairs and the evaluation of the global F:C proportion in licorice extracts have not yet been undertaken. It is assumed herein that the F–C composition and the defined proportionality between Fs and Cs, reflecting an F:C equilibrium, could be used for further standardisation of licorice preparations. In order to provide a solid botanical basis for the present study, three different samples of DNA-authenticated *G. glabra* and *G. uralensis* were investigated. The first aim of the present study was to identify the major coexisting F/C pairs, and to define the F/C composition of licorice extracts obtained by hydro-alcoholic maceration with 95% ethanol (EtOH) during 24 h at room temperature (RT). All extracts were analysed by UHPLC/ UV with a validated method used for the quantitation of major Fs and Cs, LigF-LigC glycosides and glycyrrhizin. The second aim was to evaluate the impact of different extraction processes on the F–C compositions. Therefore, each licorice sample was also extracted by maceration (for 24 h, at RT) and reflux (for 30 min, at 100 °C) processes with 70% aqueous EtOH. These preparations, the extraction under reflux in particular, were chosen to probe their ability to modify the chemical composition of the final licorice extract by initiating an isomerisation, which in turn would alter the resulting F–C composition. Finally, combining the results obtained from both species, the third aim was to determine the principal differences between the *G. glabra* and *G. uralensis* metabolomes with regard to their F–C composition.

Experimental

Plant material and authentication

Three different batches of dried roots from *G. uralensis* Fisch. ex DC. were purchased from a local supplier (Chicago, IL), and from Starwest Botanicals (Sacramento, CA). Each batch was attributed a code: BC 624 and BC 689/BC 716, respectively. Batches from *G. glabra* L. were obtained from Nature Med S.r.l (Cosenza, Italy), Herb Pharm LLC (Williams, OR), or were collected at the Indiana botanical garden, and were attributed the following codes: BC 693/BC 694, BC 695 and BC 044, respectively. All plant materials were identified through DNA authentication (Kondo *et al.*, 2007a, b) and a series of macroscopic and microscopic analyses, comparing the plants with voucher specimens from the Field Museum (Chicago, IL, for *G. glabra* L. FM 7172717 and for *G. uralensis* × *glandulifera* FM 2174544).

Isolation and purification of reference standards

For the isolation of secondary metabolites, DNA-authenticated *G. uralensis* (BC 624, 998 g) and *G. glabra* (BC 044, 500 g) were extracted by percolation with MeOH at RT (weight root powder (g)/volume solvent (mL): 1:20). Freeze-drying yielded 269 g of *G. uralensis* crude extract representing 27% w/w (weight extract/weight roots), and 100 g of *G. glabra* crude extract representing 20% w/w. LigF [5] and LigC [6] were isolated from the CHCl₃ partition (3.5% w/w of the extract) of *G. uralensis*, as previously described (Simmler *et al.*, 2013). LigF-4'-O-Glc [3] and LigC-4-O-Glc [4] were isolated from the EtOAc fraction of *G. uralensis* (5.8% w/w of the extract), and LigF-4'-O-ApiGlc [1], LigC-4-O-ApiGlc [2], LigF-7-O-ApiGlc [9] and LigC-4'-O-ApiGlc [10] were isolated from the EtOAc fraction of *G. glabra* (14.0% w/w of the extract). For that purpose, medium-pressure liquid chromatography (MPLC, column i.d. × length = 2.5 × 30 cm) using MCI-CHP-20P (Sigma-Aldrich, St Louis, MO, part no. 13630-U SUPELCO) as adsorbent was performed on each

EtOAc fraction (1 g for *G. uralensis* and 2.5 g for *G. glabra*), with a gradient of MeOH/H₂O at 2.5 mL/min starting from 50% MeOH to 100% MeOH. Some excess of impure LigF-4'-O-Glc [3] precipitated (171.4 mg) from the EtOAc fraction of *G. uralensis* when dissolved in 50% MeOH prior to MPLC fractionation. After performing the MPLC, pure LigF-4'-O-Glc [3] (24.5 mg, 95.72% w/w) crystallized from a mixture of 60% MeOH in H₂O, while the fraction containing LigF-4'-O-ApiGlc [1] and LigF-7-O-ApiGlc [9] (618.2 mg) was obtained from 65% MeOH. LigC-4-O-Glc [4] (20.8 mg), LigC-4-O-ApiGlc [2], and LigC-4'-O-ApiGlc [10] (32.8 mg) crystallized in different vials from 65% MeOH. A third step using preparative HPLC was necessary to purify both LigF-4'-O-ApiGlc [1] and LigF-7-O-ApiGlc [9] from the enriched MPLC fraction. The purification was performed on a YMC-Pack-ODS-AQ (250 × 10 mm, 5 µm, part no. 102500531) column, eluting with a H₂O/ACN gradient from 90% to 80% H₂O in 30 min at 1.5 mL/min and with UV detection at 275 nm. All compounds were identified by means of one and two-dimensional NMR analyses (online Supporting information S1–S4), and confirmed by MS. Results were in accordance with spectral data reported previously (Nakanishi *et al.*, 1985; Fu *et al.*, 2005). The purity of each compound and the relative ratio of isomers (e.g. LigC-4-O-ApiGlc/LigC-4'-O-ApiGlc) in each sample were defined by quantitative ¹H-NMR (qHNMR) using the 100% method (online Supporting information S5). The glycyrrhizin standard (part no. 50531) used for the UHPLC/UV quantitation was purchased from Sigma-Aldrich.

Sample preparation

Each of the three samples per investigated species was extracted using 2 g of plant material for 40 mL of solvent (ratio 1:20), and washed with 20 mL of solvent after extraction. All samples were extracted by maceration, at RT during 24 h with 95% EtOH (USP 190 Proof), and with EtOH (USP 200 Proof)/H₂O (70:30). Samples were also extracted with EtOH/H₂O (70:30) under reflux at 100 °C for 30 min. Each extract was concentrated using a Thermo-Fischer Savant SC250 EXP speed vacuum overnight at 35 °C, and then freeze-dried with a Labconco Freezone 4.5 (Kansas City, MO, USA) so as to remove residual water. Sample BC 695 (2 × 2 g) was additionally extracted at 100 °C under reflux for 30 min, and at RT by maceration for 24 h with 50% and 30% aqueous EtOH.

Conditions of UHPLC analyses

Solutions of the crude extracts were prepared at 10 mg/mL in 100% MeOH or MeOH/H₂O (70:30) HPLC grade (Fisher Co. Ltd) and filtered (filter acrodisc CR 13 mm, 0.45 µm PTFE membrane) prior to injection (2 µL). The UHPLC analyses were performed on a Shimadzu UFLC equipped with a Kinetex XB-C18 (2.1 × 5.0 mm, 1.7 µm, 00B-4498-AN, Phenomenex) column and using a diode array detector (DAD, Shimadzu SPD-M20-A). The temperatures of the autosampler and the column oven were set at 4 and 40 °C, respectively. Post-run data analyses were processed with the Shimadzu Labsolution software package. The column was eluted with a gradient composed of (A) H₂O + 0.1% formic acid (FA) and (B) ACN + 0.1% (FA) as follows: from 8% to 11% B in 2 min, isocratic at 11% B during 30 s, to 13% B at 4 min, to 15.5% B at 8 min, to 36% B at 13 min, and isocratic at 36% during 30 s, to 80% B at 21 min and during 1 min, back to 8% B at 23 min (flow rate: 0.8 mL/min). Under those conditions, the retention times (*t_R*) of isolated metabolites were the following,

in the order of elution, for all Fs: 2.42 min (7), 2.53 min (3), 2.78 min (9), 2.94 min (1) and 5.36 min (5); and for all Cs: 6.33 min (3), 6.70 min (2), 6.90 min (8), 7.13 min (10), and 11.05 min (6). Glycyrrhizin eluted at 13.35 min. A standard curve containing the 10 reference standards was used for their quantitation in each extract. The area under the curve (AUC) was taken at 360 nm for all Cs, at 275 nm for all Fs, and at 254 nm for glycyrrhizin. Each *Glycyrrhiza* extract was examined in duplicate within the same sequence of analysis. Under the present chromatographic conditions, the limits of detection (LOD) and quantitation (LOQ) were determined at a signal-to-noise (S:N) ratio superior or equal to 3 and 10, respectively.

Validation of the UHPLC method

The UHPLC method, developed for the comparison of the different *Glycyrrhiza* extracts and the quantitation of the major F/C pairs, was evaluated for precision, accuracy, stability and repeatability. The relative standard deviation (%RSDs) obtained for each analysis was taken as a measure of precision and accuracy. The precision of the method was determined with the sample BC 716 (extracted by maceration with 70% EtOH) through the intraday (six replicates of the same extract analysed within 1 day) and interday variations (one extract analysed twice a day during 3 days). The accuracy was evaluated through the recovery measurement, using the sample BC 689 (extracted by maceration with 70% EtOH). Three different concentrations of mixed standard solutions were spiked into the known 70% EtOH maceration extract. The recovery was calculated as follows:

$$\text{recovery}(\%) = (\text{concentration measured} / \text{calculated maximum possible concentration}) \times 100$$

The reproducibility was measured through the quantitation of Cs, Fs and glycyrrhizin in the same BC 695 sample that was extracted by maceration in triplicate with 70% EtOH. The stability of the analysed extracts (BC 689 and BC 695, maceration with 70% EtOH) was measured using the same UHPLC method, and defining an analysis at 0, 4, 8, 16 and 24 h. During these analyses, all extracts were kept at 4 °C in the autosampler.

Statistical analysis

Quantitative data represent the mean $\pm$ standard deviation of independent experiments. Statistical comparison of results was made using analysis by Student's *t* test. Differences were considered significant (*) for $p < 0.05$.

Results and discussion

Sample preparation

The authenticity of botanical material is crucial in the process of quality and safety controls of herbal products and dietary supplements. Authenticity is also fundamental for the production of consistent and reproducible scientific results (Smillie and Khan, 2010). Botanical authentication of commercial licorice roots is even more complex: first, the acquired raw material is often powdered, making it unsuitable for macroscopic authentication; second, *Glycyrrhiza* species share very similar or near identical microscopic characteristics (WHO, 1999). In order to overcome this challenge, the present study

authenticated all root samples acquired from each *Glycyrrhiza* species by means of DNA-barcoding, following the work of Kondo *et al.* (2007b). Three different batches of DNA-authenticated *G. glabra* ('European licorice') and *G. uralensis* ('Chinese licorice') were first extracted with 95% EtOH (ratio plant:solvent of 1 g:20 mL) by a 24 h maceration at RT. This first extract was considered as a control and used to assess its composition in principal Fs and Cs as well as to characterise each F-C pair in the different samples. Secondly, in order to evaluate the impact of sample preparation on the final F-C composition and proportion, all licorice roots were extracted with 70% EtOH in H₂O by maceration (24 h, RT) and reflux (30 min, 100 °C) using the same ratio of plant:solvent. These traditional extraction methods were chosen for two reasons: they use environmentally safe and non-toxic solvents, and they are easy to implement at a larger scale for industrial production. Additionally, they represent methods that are traditionally used for herbal remedies, which are prepared for possible consumption by the patients.

Validation of the UHPLC/UV method

The separation of major Fs and Cs was performed on a Kinetex XB C₁₈-column, eluting with a slow H₂O-ACN gradient during the first 10 min (Fig. 2). The method enabled the critical separation of B- versus A-ring glycosides of both the Fs and the Cs. All principal Fs [1, 3, 5, 7, 9] were detected at 275 nm, eluting between 2 and 5 min, and all major Cs isomers [2, 4, 6, 8, 10] were detected at 360 nm in the 6 to 11 min elution window. The triterpene glycoside, glycyrrhizin, eluted much later at 13.34 min. The calibration curves for the 11 target analytes as well as the LODs and LOQs are summarised in Table 1. All calibration curves were linear within the test range. The validation of the UHPLC/UV method was performed using extracts from both licorice species prepared by maceration with 70% EtOH (Table 2, and online Supporting information S6). Precision, accuracy, reproducibility and stability were compared by their RSDs. The calculated RSDs for the intraday and interday variations in precision, calculated with BC 716, were between 0.60% and 9.39%. The overall RSDs were found to be higher when the quantified compound was present only in trace amounts in the extract. The accuracy was determined through the recovery of the quantified analytes in extracts from sample BC 689, which contained very low amounts of A-ring apiosyl-glucosides of LigF-LigC [9]-[10]. Therefore, the RSDs calculated for the F-C pair [9]-[10] were above 10%. The tested extracts were found to be stable when kept at 4 °C in the autosampler during 24 h (RSDs 5.32%). Furthermore, the RSDs calculated for the reproducibility with sample BC 695, which was extracted independently three times, were between 1.69% and 8.67%. As a whole, these results indicate that the method for the simultaneous quantitation of 11 analytes was sufficiently precise, accurate and reproducible for a metabolomic comparison of the F-C compositions and proportions in the investigated licorice extracts. Consequently, the validated UHPLC/UV method was used for the analysis of all extracts obtained from each of the *Glycyrrhiza* species (nine extracts per species).

Initial characterisation of major F/C pairs in licorice samples

Extracts obtained by maceration at RT with 95% EtOH were used as a control for the comparative analyses. This method, defined by a low percentage of water in the final solvent and a mild extraction temperature, was chosen to avoid initiating any F-C isomerisation.

Therefore, this extraction was chosen to determine the basic ('genuine') F–C composition that characterises the different licorice root samples. The quantitative results of major Fs, Cs and of glycyrrhizin for the six *Glycyrrhiza* samples are represented in Table 3. Results are expressed as mass percentages of a given analyte in the crude extract (% w/w crude extract). Subsequently, the sum of all quantified Fs and Cs allowed the determination of the total F:C proportions through the calculation of the percentage of total quantified Fs (% F) among all quantified Fs and Cs ($\%F = (\text{total Fs}) \times 100 / [\text{all (Fs + Cs)}]$).

The concentration of all quantified Fs and Cs differed largely between batches of roots within the same species investigated. Interestingly, *G. uralensis* extracts contained 1.5 to 2.5 times more Fs, and approximately identical amounts of Cs to that found in *G. glabra* extracts (Table 3). These results were in accordance with previous observations (Kondo *et al.*, 2007a, b; Zhou *et al.*, 2013). Therefore, the percentage of total quantified Fs and, thus, the F:C proportion in all *G. uralensis* extracts (F:C = 8.35:1.66) were found to be significantly higher than in *G. glabra* extracts (F:C = 7.20:2.80). The results also revealed that in *G. uralensis* samples the two major F–C pairs were [3]–[4] and [1]–[2], which all represent B-ring glycosides of the LigF–LigC pair, namely LigF-4'- and LigC-4-O-Glc (syn. liquiritin and isoliquiritin) and LigF-4'- and LigC-4-O-ApiGlc (syn. liquiritin apioside and isoliquiritin apioside). These two F–C pairs represented $62.1 \pm 11.8\%$ and $26.0 \pm 9.6\%$, respectively, of the total quantified Fs and Cs calculated for all *G. uralensis* extracts obtained with 95% EtOH (Fig. 2 and Table 4). In contrast, the major F–C pairs in *G. glabra* 95% EtOH extracts were [3]–[4] and [9]–[10], which are B- and A-ring apiosyl-glucosides of LigF and LigC, respectively. In all *G. glabra* 95% EtOH extracts, [3]–[4] represented $77.5 \pm 2.5\%$ and [9]–[10] accounted for $14.3 \pm 4.2\%$ of all quantified Fs and Cs (Fig. 2 and Table 4).

For each quantified F–C pair, the percentage of F was calculated so as to define the F:C proportion characterising the pair of isomers ($\%F = (F) \times 100 / (F + C)$ for a given F–C pair). Interestingly, when the F–C pair was glycosidated on the B-ring, the F–C proportion was relatively high, with a value of 8.35:1.65 for all 95% EtOH extracts, suggesting that more F than C isomers were extracted and/or produced by the plant (Table 4). However, when the F–C pair was glycosidated on the A-ring, the F:C proportion was found to be lower at 5.13:4.87 for all extracts, indicating that the quantities of both Fs and Cs remained quasi-equivalent in the extracts. For the LigF–LigC pair, the F:C proportion was found to differ between both species. As such, the calculated F:C proportion for each pair of isomers reflects the position of the glycosidation (A- vs. B-ring) of the aglycone pair. All *G. uralensis* extracts contained more B-ring glycosides of the LigF–LigC pair than the *G. glabra* extracts, leading to an overall higher F:C proportion: the more B-ring F and C glycosides were found in a given licorice extract, the higher was the global F:C proportion.

In summary, all *G. uralensis* extracts were found to contain more Fs and more B-ring glycosides of the LigF–LigC pairs than *G. glabra* extracts. The latter were relatively rich in A- and B-ring apiosyl-glucosides of the LigF–LigC pairs. Altogether, these characteristic F–C compositions lead to the expression of species characteristic F:C proportions in all investigated materials.

Impact of the extraction method (maceration vs. reflux) on the F/C metabolome

A second aspect of the study was to determine whether diverse extraction methods, differing in their potential to initiate F–C isomerisation reactions, could alter the global F–C composition and, thus, the proportion of each F–C pair. Possible isomerisation during extraction can lead to an increased production of Fs from their Cs precursors, which can be reflected by an increased value of the total F:C proportion in the final extract. In other words, the total F:C proportion would presumably increase for extractions performed with a higher proportion of water and/or at higher temperature. Hence, we initially expected to obtain a greater final F:C proportion in those extracts obtained by reflux with solvent containing 30% or more H₂O, compared with extracts obtained by RT maceration with the same solvent or with 95% EtOH.

To address these aspects, each batch of *Glycyrrhiza* species was extracted by maceration (24 h, RT) and reflux (30 min, 100 ° C) using the same alcoholic solvent composed of 30% H₂O in EtOH. Quantitative results obtained for the three different extracts were then compared in order to evaluate the extraction efficacy and the total F–C composition.

In general, the reflux processes gave better extraction yields (between 20 and 35% weight extract/weight roots powder) than the maceration (between 13 and 30% w/w) for any given *Glycyrrhiza* sample (online Supporting information S8). Interestingly, when quantitative results were expressed as mass percentages of the crude extract, although the amount of glycyrrhizin increased, the absolute concentrations of Fs and Cs were found to be lower in the extracts obtained with 70% EtOH than in the 95% EtOH extracts (Figs 3A and 4A). However, when the results were corrected by the extraction yields, expressed as mass percentages of the root powder, it was observed that all analytes were in fact extracted more efficiently by reflux or maceration with 70% EtOH (Figs 3B and 4B). One possible explanation could be that reflux and maceration produces higher extract yields for components of the primary metabolome such as free sugars (e.g. sucrose, fructose), but also glycyrrhizin. This results in a relative dilution of the total extracted Fs and Cs in the final extract. Consequently, when the aim is to obtain a licorice preparation with relatively high concentrations of Fs and Cs but relatively low abundance of glycyrrhizin, a maceration process using a solvent with a low proportion of H₂O (< 30%) in EtOH is the preferred choice.

The comparative quantitative results obtained for the three different preparations per *Glycyrrhiza* sample revealed that F and C isomers were extracted, while maintaining a constant F:C proportion (Figs 3C and 4C). All the total F:C proportions calculated for the three extracts of a given *Glycyrrhiza* sample were found to be equivalent (online Supporting information S9 for all extracts). These results suggest that the chosen preparation methods affected equally the extraction of all F and C isomers from licorice roots. Additionally, because the total F:C proportions remained constant in the three extracts from the same licorice species, it can be concluded that the presence of 30% of H₂O in EtOH does not lead to substantial amounts of isomerisation during extraction. In order to further study the impact of higher H₂O concentrations on potential isomerisation during extraction, sample BC 695 was further extracted by maceration and under reflux with 50% and 70% H₂O in

EtOH (Fig. 4C and online Supporting information S9). Interestingly, only a slight increase in the F:C proportion (7.81:2.19) was observed when the extraction was performed with 30% aqueous EtOH, compared with 95% EtOH (7.39:2.61). This might be the result of some F–C isomerisation during the extraction. Accordingly, in order to generate a slight increase in the total F:C proportion of a licorice extract, the hydro-alcoholic extraction solvent must contain at least 50% H₂O. The F:C proportions remain essentially unaltered with aqueous level below this value. Overall, the final F:C proportion in the aqueous extracts stayed in the same order of magnitude as those calculated from the 70% EtOH extracts (Fig. 4C).

Comparison of *G. glabra* and *G. uralensis* extracts

For each *Glycyrrhiza* species, nine different extracts were produced (three extracts/batch). For each batch analysed, the identity of the major F–C pairs and the total F:C proportion remained constant in the three extracts. Therefore, results obtained from all nine *G. glabra* extracts were compared with the results obtained from all nine *G. uralensis* extracts, regarding their metabolomic F–C compositions and proportions (Fig. 5, online Supporting information S7 and S9). From a general point of view, a polarity shift for the major Fs and Cs was observed between *G. uralensis* and *G. glabra* extracts. In the *G. glabra* extracts, this shift was reflected by the presence of proportionally more apiosyl-glucosides of the LigF–LigC pair among all quantified Fs and Cs, with [1]–[2] being the major pair at 78.40% (Fig. 5A). Zhou *et al.* (2013), who quantified major B-ring glycosides of LigF in extracts from the three *Glycyrrhiza* species, made a similar observation. Additionally, *G. glabra* extracts contained proportionally more of the [9]–[10] Aring apiosyl-glucosides of LigF–LigC (15.42% of all Fs and Cs) than *G. uralensis* extracts. The latter contained more of the B-ring glycosides of LigF–LigC [1]–[2] (29.60% of all Fs and Cs) and [3]–[4] (58.38% of all Fs and Cs), and also more of the LigF–LigC aglycones [5]–[6] than *G. glabra* extracts.

In summary, extracts from each DNA-authenticated species could be characterised by two major LigF and LigC glycosides. The common major glycosides of the two investigated species were [1] and [2] (LigF-4' and LigC-4-*O*-ApiGlc). Additionally, the other major F–C pair in the *G. uralensis* extracts was [3]–[4] (LigF-4'– and LigC-4-*O*-Glc), while it was [9]–[10] (LigF-7– and LigC-4'-*O*-ApiGlc) in all *G. glabra* extracts. For all *Glycyrrhiza* extracts obtained from both species, the F:C proportion for a given pair of isomers correlated with the position of glycosidation (Fig. 5B). When Fs and Cs were glycosidated on the B-ring, the corresponding F represented 85% of the pair (F:C proportion = 8.46:1.54 (± 0.35 for both Fs and Cs)). In contrast, when Fs and Cs were A-ring glycosidated, the corresponding F represented only 47% of the pair (F:C proportion = 4.74:5.26 (± 0.19 for both Fs and Cs)).

As the major F–C pairs in *G. glabra* extracts were found to be structurally different from those in *G. uralensis*, and given that *G. glabra* extracts contained proportionally more A-ring glycosides, the total F:C proportion in all *G. glabra* extracts (7.18:2.82 (± 0.11)) was found to be significantly lower compared with *G. uralensis* extracts (8.37:1.63 (± 0.61)) (Fig. 5C). Accordingly, in the licorice extracts studied, higher proportions of A-ring glycosides correlated with lower total F:C proportions. Inversely, the more B-ring glycosides a given licorice extract contained, the higher was the total F:C proportion (Fig. 5A and C).

Finally, the composition in major Fs and Cs was obtained through the simultaneous UHPLC-based quantitation of 10 glycosides of LigF and LigC marker compounds. Interestingly, the calculated total F:C proportion was found to remain constant in all extracts obtained from a given *Glycyrrhiza* species. Neither the maceration nor the reflux with a hydro-alcoholic solvent containing less than 50% of H₂O had any significant effect on the total F:C proportion in the final extract. Moreover, *G. glabra* and *G. uralensis* extracts could be chemically differentiated according to their composition in major F:C pairs. This difference is reflected by the total F:C proportion in all extracts obtained from each species, regardless of the extraction method. In order to evaluate the natural variability and obtain representative values of species-characteristic F:C proportions, it will be necessary to extend the present study and analyse additional DNA-authenticated samples, following the same methodology described here. One potential limitation of the proposed method results from the limited commercial availability of some of the LigF–LigC glycosides, such as LigF-7–(4′)-*O*-ApiGlc, LigC-4–(4′)-*O*-ApiGlc, which are a prerequisite for calibration in LC-based quantitation. This limitation might be overcome by the use of qHNMR (Pauli *et al.*, 2012; Gödecke *et al.*, 2013), and efforts are ongoing to develop such a method for *Glycyrrhiza* extracts.

The quantitation of glycyrrhizin, major Fs and Cs, and the determination of the total F:C proportion not only contributes to the chemical characterisation of licorice extracts, but is also fundamental for a meaningful interpretation of biological results obtained with such extracts. To this end, the difference in the glycosidation patterns (mono- vs. doubly glycosylated, A- vs. B-ring) of the major F–C pairs in the *G. uralensis* and *G. glabra* extracts has to be considered as being potentially responsible for the expression of different *in vitro/in vivo* biological responses. This could also include differences in *in vitro* or *in vivo* deglycosidation, intestinal absorption, and/ or metabolism of these chemically distinct secondary plant metabolites. Therefore, the present metabolomic definition of the F–C composition, along with the determination of the total F:C proportions in *Glycyrrhiza* preparations, are suitable measures to be implemented into future quality control and standardisation workflow of licorice botanicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structures and nomenclature of the principal flavanones (F) and chalcones (C) in licorice roots. The major Fs and Cs in licorice roots are glycosides of liquiritigenin (LigF) and isoliquiritigenin (LigC). The site of glycosidation can be either on the A- (C-7 for Fs and C-4' for Cs) or the B-ring (C-4' for Fs and C-4 for Cs) of each LigF–LigC pair. The trivial names (italic font) found in the literature can be confusing (e.g. chalcone isomers [2] and [10]) and do not indicate the exact position of the sugar moiety. The use of non-uniform and non-informative trivial names for licorice flavonoids has been highlighted previously by Nomura and Fukai (1998). Therefore, the proposed nomenclature indicates the type and linkage of the sugar, considering the structure of the equivalent aglycone, either LigF for all Fs, or LigC for all Cs. The pairs [1]–[2], [3]–[4], [5]–[6], [7]–[8] and [9]–[10] represent F–C isomers. In grey font, the sugar moieties are abbreviated as ‘Glc’ for β -glucose and ‘ApiGlc’ for β -apiosyl- β -glucose.

Figure 2.

Comparative UHPLC/UV chromatograms of representative extracts from the roots of *G. glabra* (BC 695) and *G. uralensis* (BC 689). Chromatograms were observed at 275 nm for all Fs and 360 nm for all Cs. Extracts from *G. glabra*, represented here by BC 695, were mostly characterized by the F–C pairs [1]–[2] (LigF-4'- and LigC-4-O-ApiGlc) and [9]–[10] (LigF-7- and LigC-4'-O-ApiGlc), whereas the F–C pairs [1]–[2] and [3]–[4] (LigF-4'- and LigC-4-O-Glc) defined the extract obtained from the roots of *G. uralensis*, represented here by BC 689. An increase in the polarity of the major F–C pair was observed from *G. uralensis* containing monoglycosidated Fs and Cs to *G. glabra*, characterised by doubly glycosidated Fs and Cs. The LigF–LigC aglycones (F–C pair [5]–[6]) were found to be minor compounds in all analysed extracts from both species (see online Supporting information S7).

Figure 3.

Influence of the different extraction processes on the F–C composition in *G. uralensis* samples. (A) The concentration of each analyte represented in their order of UHPLC elution (flavanones **7**, **3**, **9**, **1** and **5**, chalcones **4**, **2**, **8**, **10** and **6**, and glycyrrhizin (Glycyrr.)) in the three different extracts obtained from *G. uralensis* BC 689. The major Fs were liquiritin (**3**, syn. LigF-4'-O-Glc) and liquiritin apioside (**1**, syn. LigF-4'-O-ApiGlc). The major Cs were isoliquiritin (**4**, syn. LigC-4-O-Glc) and isoliquiritin apioside (**2**, syn. LigC-4-O-ApiGlc). (B) Percentages of the initial powdered roots demonstrating that the extraction yields for all metabolites were better with 30% H₂O in EtOH. (C) The differences in extraction yields and total concentration of Fs and Cs in each extract, leading to a constant F:C proportion of around 9.02:0.98, represented by the % F = total (F) × 100/[total (F + C)]. See online Supporting Information S9 for all quantitative results. Ma, maceration; Re, reflux.

Figure 4.

Influence of the different extraction methods and solvent composition on the F:C proportion in *G. glabra* (BC 695). (A) The concentration of each analyte represented in their order of UHPLC elution (flavanones 7, 3, 9, 1 and 5, chalcones 4, 2, 8, 10 and 6, and glycyrrhizin [Glycyrr.]) in the three different extracts obtained from *G. glabra* BC 695. The major Fs were liquiritin apioside (1, syn. LigF-4'-O-ApiGlc) and LigF-7-O-ApiGlc (9). The major Cs were isoliquiritin apioside (2, syn. LigC-4-O-ApiGlc) and licuraside (10, syn. LigC-4'-O-ApiGlc). (B) Percentages of the initial powdered roots demonstrating that the extraction yields for all metabolites were better with 30% H₂O in EtOH. (C) Comparative F:C proportions in extracts obtained with increasing proportion of H₂O in EtOH (up to 70% H₂O). See online Supporting information S9 for all quantitative results. The average F:C proportion (total BC 695) determined by the %F = total (F) × 100/[total (F + C)], was around 7.59:2.41. Ma, maceration; Re, reflux.

Figure 5.

Evaluation of the F–C composition and total F:C proportion in all extracts from both *Glycyrrhiza* species. (A) The composition in major F–C pairs per *Glycyrrhiza* species for all analysed extracts (nine per species). Each F–C pair was classified according to its glycosidation pattern (glucoside vs. apiosyl-glucoside and A- vs. B-ring). n.d., not determined. (B) For each pair of isomers, the F:C proportion correlated with the position of the glycosidation on the equivalent aglycone. (C) Overall difference in F–C composition between *Glycyrrhiza* species and the total F:C proportion, which was found to be significantly lower in *G. glabra* than in *G. uralensis* extracts. Differences were considered significant (*) for $p < 0.05$.

Table 1

Calibration curves, LODs and LOQs of the target analytes

Quantified Compounds	t_R (min)	Standard curve x = concentration in mg/mL	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
LigF-4'- <i>O</i> -glucoside	2.53	$\text{AUC}_{275\text{ nm}} = 2253507x + 5135.95$ $R^2 = 0.9997$	3.75	15.00
LigF-7- <i>O</i> -glucoside	2.42			
LigF-4'- <i>O</i> -apiosyl-glucoside	2.94	$\text{AUC}_{275\text{ nm}} = 1421343x + 2045.64$ $R^2 = 0.9997$	5.00	85.00
LigF-7- <i>O</i> -apiosyl-glucoside	2.78			
LigF	5.36	$\text{AUC}_{275\text{ nm}} = 4051958x + 10098.43$ $R^2 = 0.9993$	3.90	15.60
LigC-4- <i>O</i> -glucoside	6.33	$\text{AUC}_{360\text{ nm}} = 5132334x + 9160.18$ $R^2 = 0.9993$	3.50	14.00
LigC-4'- <i>O</i> -glucoside	6.90			
LigC-4- <i>O</i> -apiosyl-glucoside	6.70	$\text{AUC}_{360\text{ nm}} = 4772846x + 2925.99$ $R^2 = 0.9996$	5.80	11.70
LigC-4'- <i>O</i> -apiosyl-glucoside	7.13			
LigC	11.05	$\text{AUC}_{360\text{ nm}} = 6361704x + 12740.11$ $R^2 = 0.9998$	1.95	7.81
Glycyrrhizin	13.34	$\text{AUC}_{254\text{ nm}} = 1056417x + 2275.29$ $R^2 = 0.9998$	1.85	14.84

Table 2

Precision, accuracy and stability of the quantitative UHPLC/UV method

Extract	% RSDs					
	Precision		Accuracy ^a	Stability ^b 4 °C	Stability ^c 4 °C	Reproducibility ^d
	Intraday	Interday				
LigF-7- <i>O</i> -Glc [7]	3.10	2.63	n.d.	4.27	n.d.	n.d.
LigF-4'- <i>O</i> -Glc [3]	1.21	0.60	4.5	0.74	2.28	3.39
LigF-7- <i>O</i> -ApiGlc [9]	6.93	4.60	13.1	7.81	3.94	1.69
LigF-4'- <i>O</i> -ApiGlc [1]	2.66	2.82	1.2	2.33	0.76	3.00
LigF [5]	9.39	3.09	2.7	5.32	5.28	6.93
LigC-4- <i>O</i> -Glc [4]	2.71	0.17	3.5	0.65	8.55	5.97
LigC-4- <i>O</i> -ApiGlc [2]	2.84	1.10	1.3	2.04	1.18	3.74
LigC-4'- <i>O</i> -Glc [8]	3.83	1.24	5.4	1.16	8.54	8.67
LigC-4'- <i>O</i> -ApiGlc [10]	6.83	5.09	12.0	4.98	2.05	4.26
LigC [6]	7.71	4.95	8.8	2.69	n.d.	n.d.
Glycyrrhizin	1.34	0.63	2.0	0.36	1.00	1.98

The validation of the UHPLC method was performed using extracts of both *Glycyrrhiza* species. Percentage RSDs were found to be higher for all compounds found in trace amounts in a given extract.

^a Accuracy was calculated with extracts of *G. uralensis* (BC 689), where compounds [9] and [10] were found in trace amounts.

^b Results obtained for *G. uralensis* (BC 624).

^c Results obtained for *G. glabra* (BC 695), n.d. = not detected (below the LOD).

^d Reproducibility was calculated with BC 695 (see online Supporting information S6 for quantitative results).

Table 3

Quantitative results for different samples of *G. glabra* and *G. uralensis* extracted by maceration with 95% EtOH (24 h, RT)

Compounds	Extracts	<i>G. glabra</i>			<i>G. uralensis</i>		
		BC 694	BC 695	BC 693	BC 689	BC 624	BC 716
Flavanones (F)	LigF-7-O-Glc	n.d.	0.22 ± 0.00	n.d.	0.20 ± 0.09	0.15 ± 0.00	0.29 ± 0.01
	LigF-4'-O-Glc	0.14 ± 0.01	0.45 ± 0.02	0.19 ± 0.01	4.95 ± 0.24	6.69 ± 0.03	3.17 ± 0.01
	LigF-7-O-ApiGlc	0.53 ± 0.02	0.67 ± 0.10	0.42 ± 0.04	0.07 ± 0.01	0.07 ± 0.00	0.17 ± 0.02
	LigF-4'-O-ApiGlc	3.17 ± 0.02	6.28 ± 0.07	2.90 ± 0.03	1.50 ± 0.09	2.22 ± 0.02	2.35 ± 0.00
Chalcones (C)	LigF	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	0.27 ± 0.02	1.02 ± 0.03	0.07 ± 0.00
	LigC-4-O-Glc	0.03 ± 0.00	0.07 ± 0.00	0.03 ± 0.00	0.37 ± 0.03	1.29 ± 0.02	0.56 ± 0.01
	LigC-4-O-ApiGlc	0.91 ± 0.02	1.87 ± 0.03	0.87 ± 0.01	0.15 ± 0.01	0.45 ± 0.01	0.48 ± 0.03
	LigC-4'-O-Glc	0.06 ± 0.02	0.17 ± 0.01	0.03 ± 0.00	0.16 ± 0.01	0.22 ± 0.02	0.33 ± 0.01
Glycyrrhizin	LigC-4'-O-ApiGlc	0.51 ± 0.01	0.50 ± 0.01	0.51 ± 0.02	0.05 ± 0.00	0.05 ± 0.01	0.22 ± 0.01
	LigC	0.05 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.28 ± 0.00	0.03 ± 0.00
		0.73 ± 0.03	1.07 ± 0.03	0.88 ± 0.01	3.33 ± 0.14	1.06 ± 0.26	1.26 ± 0.26
		3.89	7.48	3.57	7.00	10.14	6.05
Total quantified F		1.55	2.64	1.48	0.79	2.30	1.61
Total quantified C		71.51%	73.93%	70.69%	89.85%	81.50%	78.98%
Percentage quantified F ^a		7.15:2.85	7.39:2.61	7.07:2.93	8.98:1.02	8.15:1.85	7.90/2.10
F:C proportion							
Total F:C proportion			7.20:2.80 (±0.17) ^b			8.35:1.65 (±0.57) ^b	

Results are expressed as % w/w of the crude extract. Data represent mean ± SD resulting from three analyses of the same extract (*n* = 3). Data in bold indicate the major F-C pairs in each extract; n.d. = not detected (below the LOD).

^a Total (F) × 100/total (F + C).

^b Standard deviation based on the percentage values for all Fs and Cs.

Table 4
Identification of the major F–C pairs and determination of the flavanone proportion (%) F) for each pair of isomer

Ring	F–C pairs	<i>G. glabra</i> (95% EtOH)			<i>G. uralensis</i> (95% EtOH)		
		BC batch #		693	BC batch #		689
		%		flavanone/pair ^b	%		flavanone/pair
B	LigF-4'- and LigC-4-O-ApiGlc ([1]–[2])	77.5 ± 2.5		78	77	78	91
	LigF-4'- and LigC-4-O-Glc ([3]–[4])	3.6 ± 1.2		82	86	86	93
	LigF–LigC ([5]–[6])	1.7 ± 0.6		54	78	60	82
A	LigF-7- and LigC-4'-O-Glc ([7]–[8])	n.d.		n.d.	56	n.d.	56
	LigF-7- and LigC-4'-O-ApiGlc ([9]–[10])	14.3 ± 4.2		51	57	45	58

Data represent mean ± SD resulting from three different extracts/species. Data in bold indicate the major F–C pairs for each *Glycyrrhiza* species. Each F–C pair was classified according to their site of glycosidation, either A-ring or B-ring of the equivalent LigF–LigC aglycone.

^a (F + C) × 100/[all (F + C)] = percentage of a given pair among quantified Fs and Cs.

^b F × 100/(F + C) for a given F–C pair.