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Orthogonal Analysis Underscores the Relevance of Primary and Secondary Metabolites in Licorice

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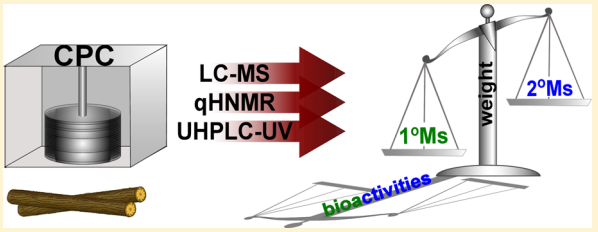
Supporting Information

ABSTRACT: Licorice botanicals are produced from the roots of *Glycyrrhiza* species (Fabaceae), encompassing metabolites of both plant and rhizobial origin. The composition in both primary and secondary metabolites (1°/2°Ms) reflects the physiologic state of the plant at harvest. Interestingly, the relative abundance of 1°Ms vs 2°Ms in licorice extracts remains undetermined. A centrifugal partition chromatography (CPC) method was developed to purify liquiritin derivatives that represent major bioactive 2°Ms and to concentrate the polar 1°Ms from the crude extract of *Glycyrrhiza uralensis*. One objective was to determine the purity of the generated reference materials by orthogonal UHPLC-UV/LC-MS and qHNMR analyses. The other objectives were to evaluate the presence of 1°Ms in purified 2°Ms and define their mass balance in a crude botanical extract. Whereas most impurities could be assigned to well-known 1°Ms, *p*-hydroxybenzylmalonic acid, a new natural tyrosine analogue, was also identified. Additionally, in the most polar fraction, sucrose and proline represented 93% (w/w) of all qHNMR-quantified 1°Ms. Compared to the 2°Ms, accounting for 11.9% by UHPLC-UV, 1°Ms quantified by qHNMR defined an additional 74.8% of *G. uralensis* extract. The combined orthogonal methods enable the mass balance characterization of licorice extracts and highlight the relevance of 1°Ms, and accompanying metabolites, for botanical quality control.



Licorice (*Glycyrrhiza* sp., Fabaceae) is one of the oldest and most popular herbal medicines included in the United States, Asian, and European Pharmacopoeias. The Fabaceae (Leguminosae) family comprises a variety of important agricultural and food plants such as *Glycine max* (soybean), *Phaseolus* sp. (beans), *Medicago sativa* (alfalfa), and *Arachis hypogea* (peanut), from which the fruits and sometimes the leaves are consumed as food and/or forage, respectively. Like many other genera from the Fabaceae family, *Glycyrrhiza* sp., in symbiosis with rhizobia, are able to fix nitrogen (N_2) from the atmosphere. The rhizobia comprise all the bacteria capable of inducing symbiotic N_2-fixing nodules in the roots or stems of legumes.[1] Interestingly, roots and stolons are the organs traditionally used for producing licorice botanicals, which are defined under the term *Glycyrrhizae radix* in various Pharmacopoeias.[2]

Major secondary metabolites (2°Ms) of licorice roots are triterpenoid saponins, at 3−5% (w/w) of the dried roots/rhizomes (referred to as roots in the following), followed by polyphenols, which account for 1−1.5% (w/w). Glycyrrhizin (**5**) is the predominant member of the former group, whereas the latter are generally characterized by a high abundance of glycosides of liquiritigenin (a flavanone; compounds **1**, **2**, and **6**) and its isomer, isoliquiritigenin (a 2′-hydroxychalcone; compounds **3**, **4**, **7**, and **8**).[3,4] In particular, the polyphenolic content of *Glycyrrhiza uralensis* (Chinese licorice), a perennial herb that grows primarily in the semiarid zones of Asia, is characterized by a high proportion of B-ring glycosides of liquiritigenin, namely, liquiritin (**1**) and liquiritin apioside (**2**) (Scheme 1).[5−7]

Among the major bioactive constituents of licorice roots, compounds **1** and **2** exhibit a range of pharmacological properties including antiallergic,[8] antitussive,[9] estrogenic,[10] chemopreventive,[11] and potentially anticancer[12] activities.[13] From an ecological perspective, the less polar liquiritigenin (**6**) and isoliquiritigenin are known to promote the formation of nodules by rhizobia (*Nod* gene inducers), which in turn are chemotactic to various primary metabolites (1°Ms) produced by the plant, such as sugars, dicarboxylic acids, and also phenolic acids.[14] Therefore, **1** and **2** are likely to be fundamental for plant development.[14,15] As a result, the composition of both 1°Ms and 2°Ms is expected to reflect the dynamic exchange between plant and rhizobia metabolites

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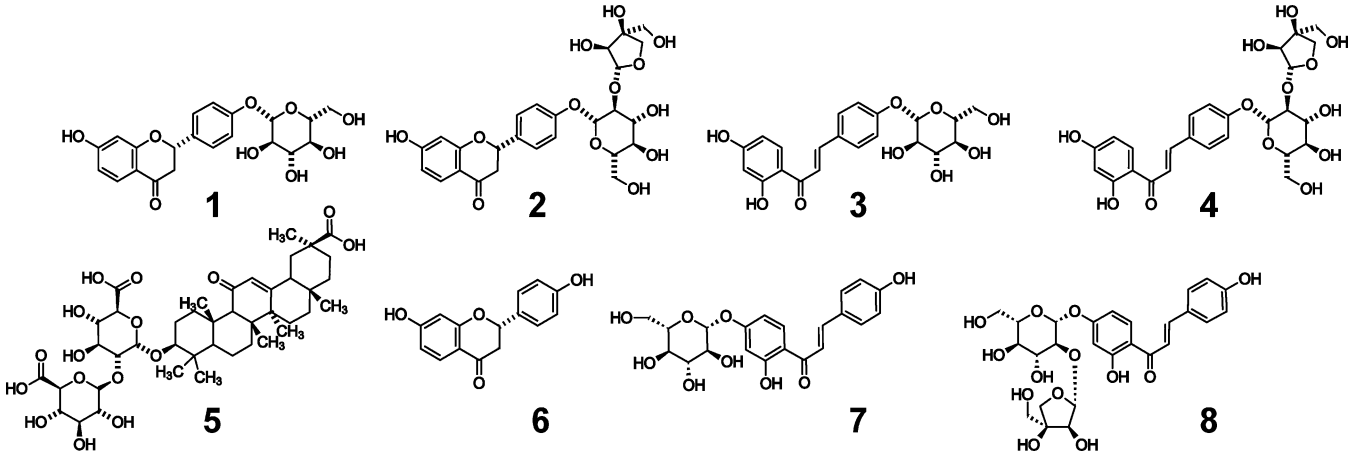
Scheme 1. Structures of Major 2°Ms Found in *G. uralensis* MeOH Extract[a].



[a]Major 2°Ms were quantified by UHPLC-UV[7] in *G. uralensis* MeOH extract prior to further CPC fractionation (Table S1, Supporting Information). Major flavanones were liquiritin (**1**) (3.81 ± 0.10% w/w crude extract) and liquiritin apioside (**2**) (1.47 ± 0.03% w/w crude extract). Glycyrrhizin (**5**) was the second major 2°Ms (3.18 ± 0.04% w/w crude extract). Other detected and quantified 2°Ms were isoliquiritin (**3**), isoliquiritin apioside (**4**), liquiritigenin (**6**), neoisoliquiritin (**7**), and licuraside (**8**).

in licorice roots and, therefore, the physiologic state of the plant at harvest time.

The importance of licorice as a dietary supplement and in traditional medicine emphasizes the need for thorough metabolite profiling of licorice preparations, together with the identification and quantitation of principal constituents. To achieve this goal, fractionation techniques that resolve a large variety of metabolites and facilitate the isolation of bioactive constituents are fundamental. Reproducibility, ease of scale-up, and high-throughput capabilities have always been a distinct advantage of countercurrent separation (CS) methods, especially when compared to solid-support liquid chromatography (LC).[16] Moreover, the use of (volatile) solvents, linked to the liquid nature of the stationary phase in CS, avoids irreversible adsorption, hence, favoring an optimal recovery. CS combined with orthogonal analytical techniques, such as quantitative ^{1}H NMR (qHNMR), UHPLC-UV, and LC-MS, can be a powerful tool for metabolite profiling of complex mixtures.[17]

High-speed countercurrent chromatography (HSCCC) has already been applied to the isolation of liquiritin derivatives. Isolation of compounds **1**, **2**, and **5** directly from the crude extract has been described recently by Xu and co-workers[18] (pH-zone refining HSCCC method) and Wang and co-workers (centrifugal partition chromatography, CPC).[19] Interestingly, both techniques were targeted toward the isolation of major 2°Ms in *G. uralensis*, regardless of the overall metabolite profiles of neighboring fractions. Moreover, in these studies, the final purity of the target metabolites was defined by LC-UV analyses, a method well known to be insensitive to residual solvents and UV-transparent metabolites, in particular 1°Ms such as sugars and amino acids. Coeluting impurities occur commonly as "hidden" or "underlying" peaks in the chromatographic profiles. Importantly, their presence constitutes the residual complexity (RC) of a given isolate.[20] The identity, purity, and determination of the RC portion of the material containing the target metabolites have to be performed accurately not only for quality control of the reference material but also to ensure meaningful interpretation of downstream biological results.[21,22]

A survey of the literature revealed that prior research on licorice emphasized the isolation and biological investigation of the most prominent 2°Ms, flavonoids and/or triterpene saponins. No specific attention is paid to 1°Ms, as they are widely regarded as being irrelevant for bioactivity. Two studies have addressed the 1°M composition in various *Glycyrrhiza* species, mostly for comparative purposes. In particular, Yang and co-workers used ^{1}H NMR metabolite profiling to demonstrate that the general composition in polar 1°Ms varied between *Glycyrrhiza* species.[23] Farag and co-workers combined GC-MS, LC-MS, and 1D NMR to compare the 1°Ms and 2°Ms profiles of four *Glycyrrhiza* species.[24] However, the relative abundance of 1°Ms vs 2°Ms in licorice extracts remains undetermined.

Therefore, the scope of the present study was (i) to develop a preparative CPC method to isolate the key 2°Ms, **1** and **2**, and to produce simultaneously a fraction enriched in polar 1°Ms; (ii) to demonstrate the potential of orthogonal analytical approaches combining LC-UV, MS, and qHNMR[25,26] to identify previously unrecognized compounds that contribute to the RC of the isolates; (iii) to evaluate the presence of 1°Ms in purified 2°Ms fractions; and (iv) to determine, in a crude *G. uralensis* extract, the relevance of both 1°Ms and 2°Ms by defining their mass balance.

RESULTS AND DISCUSSION

Large-Scale CPC Fractionation and Sample Recovery.

In general, the use of pH-zone refining in HSCCC and CPC is attractive in terms of high loading capacity and the ability to isolate large quantities (~100 mg) of target metabolites.[18] A large-scale CPC method that allowed targeted isolation of substantial amounts of the 2°Ms, **1** and **2**, while simultaneously concentrating the polar 1°Ms into a separate fraction was developed. Ideally, such a one-step CPC method would accommodate crude licorice extract and have a relatively high loading capacity (>10 g), greater than that previously achieved by pH-zone refining CPC (2 g per injection, V_{tot} = 300 mL).[18] Isolation of **1** and **2** has been achieved with EtOAc and H_2O,[18,19] both of which were used as starting points for the optimization of the two-phase solvent system (SS) conditions. However, considering the known pH-dependent interconversion of chalcones and flavanones,[27] pH-zone refining was ruled

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Table 1. Selection of Solvent Systems for the CPC Fractionation

	EtOAc/MeOH/H_2O			EtOAc/MeCN/H_2O		
volume ratio	5:1:5	5:1.9:5	5:2:5	5:2:5	5:4:7	5:6:9
			Partition Coefficient K (UP/LP)[a]			
liquiritin (**1**)	0.59	0.73	0.79	1.06	0.80	0.72
liquiritin apioside (**2**)	0.16	0.25	0.37	0.34	0.34	0.29
isoliquiritin (**3**)	2.39	2.24	2.25	3.64	1.93	2.09
isoliquiritin apioside (**4**)	0.76	0.45	0.95	1.20	0.92	0.82
glycyrrhizin (**5**)	0.04	0.05	0.14	0.13	0.27	0.16
liquiritigenin (**6**)	3.68	4.71	5.11	5.02	2.64	5.13
neoisoliquiritin (**7**)	2.11	2.00	1.80	2.57	1.59	1.77
licuraside (**8**)	0.67	0.75	0.59	0.84	0.67	0.63
			Settling Time			
without extract	24 s	20 s	17 s	13 s	14 s	7 s
with extract	>60 s	>60 s	>60 s	30 s	42 s	24 s

[a] K was calculated as metabolite concentration in the UP divided by its concentration in the LP (UP/LP).

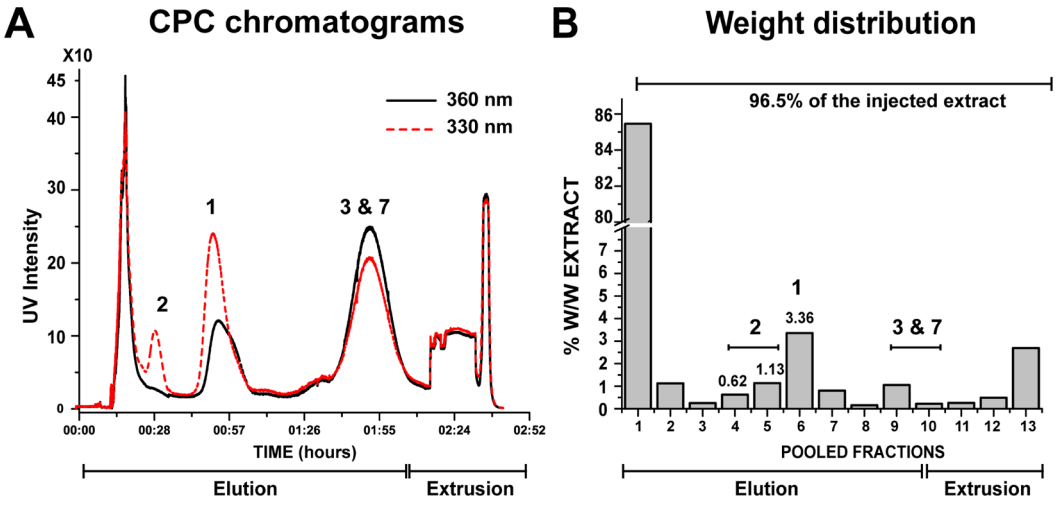

Figure 1. CPC chromatograms and weight distribution of collected fractions. (A) Large-scale CPC chromatograms recorded at 330 and 360 nm as a function of elution time in hours. The fractionation of *G. uralensis* crude extract (15 g) was performed in less than 3 h using the elution-extrusion (EECCC) mode, leading to a recovery of 96.5% of the injected material. The elution of the major flavanones, liquiritin (**1**) and liquiritin apioside (**2**), is visualized at 330 nm. (B) The weight distribution of each pooled CPC fraction highglights that fraction 1 accounts for 85.2% w/w of the crude extract.

out, as it involves a pH gradient, which would alter the characteristic chalcone–flavanone pattern in *G. uralensis*. Therefore, the SS used in the present study was free of any acid or base to minimize pH-induced chemical alteration.

The stationary phase retention volume ratio, S_f, can be predicted based on the settling time (separation of the two phases)[28] and is important when optimizing conditions for larger loading. Two intermediate solvents, MeOH and MeCN, were examined (Table 1). The settling times of the EtOAc/MeOH/H_2O SS were extended with the addition of *G. uralensis* crude extract (from 20 to more than 60 s). When MeCN was used as an intermediate solvent, the settling times were also increased in the presence of the crude extract, but to a lesser extent. On the basis of an optimized settling time, and taking into account the K value distribution of the screened metabolites (Table 1), the SS composed of EtOAc/MeCN/H_2O (5:6:9, v/v) was chosen for the fractionation of *G. uralensis* crude extract. The calculated selectivity factor for **1** and **2**, $\alpha = K_2/K_1$ (where $K_2 > K_1$), was >2, indicating an achievable baseline separation. Even though the separation efficiency of the target analytes was predicted, impurity profiling was still necessary to assess potential overlap and/or coelution with other constituents exhibiting similar K values.

The CPC was performed in reversed phase mode (15 g of crude extract, aqueous [LP] mobile, S_f =71.8%) to achieve rapid elution of the polar 1°Ms. Phase I of the elution-extrusion (EECCC)[17] mode was completed after 129 min, immediately followed by sweep elution and extrusion (phases II and III, respectively), which completed the stationary phase recovery after 2 h and 40 min (Figure 1A). A total of 171 fractions were pooled according to their UV and TLC profiles, yielding 13 final fractions. The weight recovery was 96.5%, confirming that CS can essentially recover the entire injected sample, even when working with relatively large loadings (Figure 1B).

The dried fractions were analyzed using orthogonal methods (UHPLC-UV and qHNMR) to comprehensively evaluate their composition (Figures S2 and S3, Supporting Information). The weight distribution varied throughout the CPC run, with the fractions containing the two target compounds, **1** and **2**, representing only 0.62% and 3.40% w/w of the extract load, respectively. In contrast, the early eluting polar fraction containing the majority of the polar 1°Ms accounted for 85.2% w/w of the extract.

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Table 2. Purity Determination of 1 and 2 by qHNMR/LC-MS and UHPLC-UV.

			% purity (w/w)		identified major impurities	
Fr.	qty (mg)	principal metabolite	UHPLC-UV	qHNMR	qHNMR, LC-MS-MS	% (w/w)[a]
4	90	**2**	89.4	65.0	*p*-hydroxybenzylmalonic acid (**9**)	15.9
					sucrose (**10**)	12.1
					lactic acid (**La**)	7.0
5	165	**2**	91.6	80.3	*p*-hydroxybenzylmalonic acid (**9**)	9.2
					sucrose (**10**)	7.6
					isoliquiritin apioside (**4**)	1.5
6	408	**1**	89.9	70.2	isoliquiritin apioside (**4**)	
					licuraside (**8**)	12.8
					sucrose (**10**)	6.1
					p-hydroxybenzylmalonic acid (**9**)	5.0
					liquiritigenin-7-*O*-glucoside	2.9
6a	90	**1**	97.7	95.8	isoliquiritin apioside (**4**)	2.3
					licuraside (**8**)	

[a]The relative concentration (% w/w fraction) of all identified impurities was determined by qHNMR using the 100% method.[31,32]

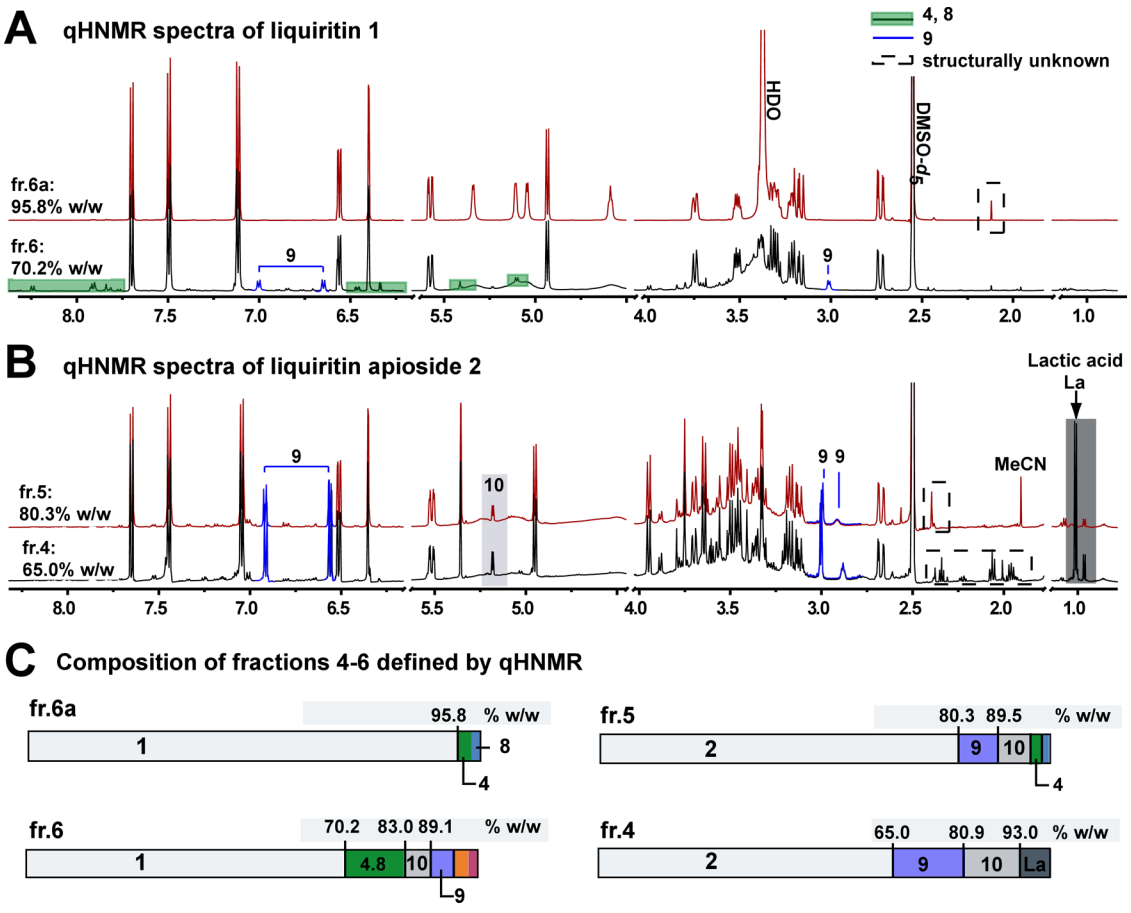

Figure 2. Purity determination of liquiritin (**1**) and liquiritin apioside (**2**) by qHNMR. (A, B) The analysis of the qHNMR spectra of liquiritin (**1**) and liquiritin apioside (**2**) enabled the structural identification of major impurities: *p*-hydroxybenzylmalonic acid (**9**, blue), sucrose (**10**, gray), lactic acid (**La**), and isoliquiritin apioside (**4**, green). Together, these impurities constitute the RC of the isolated flavanone materials. (C) Using the 100% qHNMR method, the relative concentration (% w/w) of **1** and **2** in each fraction was determined. Such results indicate the purity level and associated RC of each isolate. In fraction 6, liquiritigenin-7-*O*-glucoside (orange box) represented 2.9%, whereas isoliquiritin apioside (**4**) and licuraside (**8**, green) were the most predominant impurities.

Purity Determination of 1 and 2. In order to evaluate the efficiency of the large-scale CPC method, the purity and RC of the isolated compounds were determined by orthogonal analysis using UHPLC-UV/LC-MS-MS and qHNMR. Both target flavanones were shown to be present in fractions 4 to 6 as follows: **2** was identified in fractions 4 (vials 13 and 15) and 5 (vials 16 to 28); **1** was predominant in fraction 6 (vials 29–53), from which (vials 35–42) compound **1** crystallized spontaneously to give fraction 6a. The MS and ^{1}H NMR profiles of both isolates were in agreement with published data.[7,29,30] Together with NMR, LC-MS-MS contributed to the structural identification of both the target metabolites and

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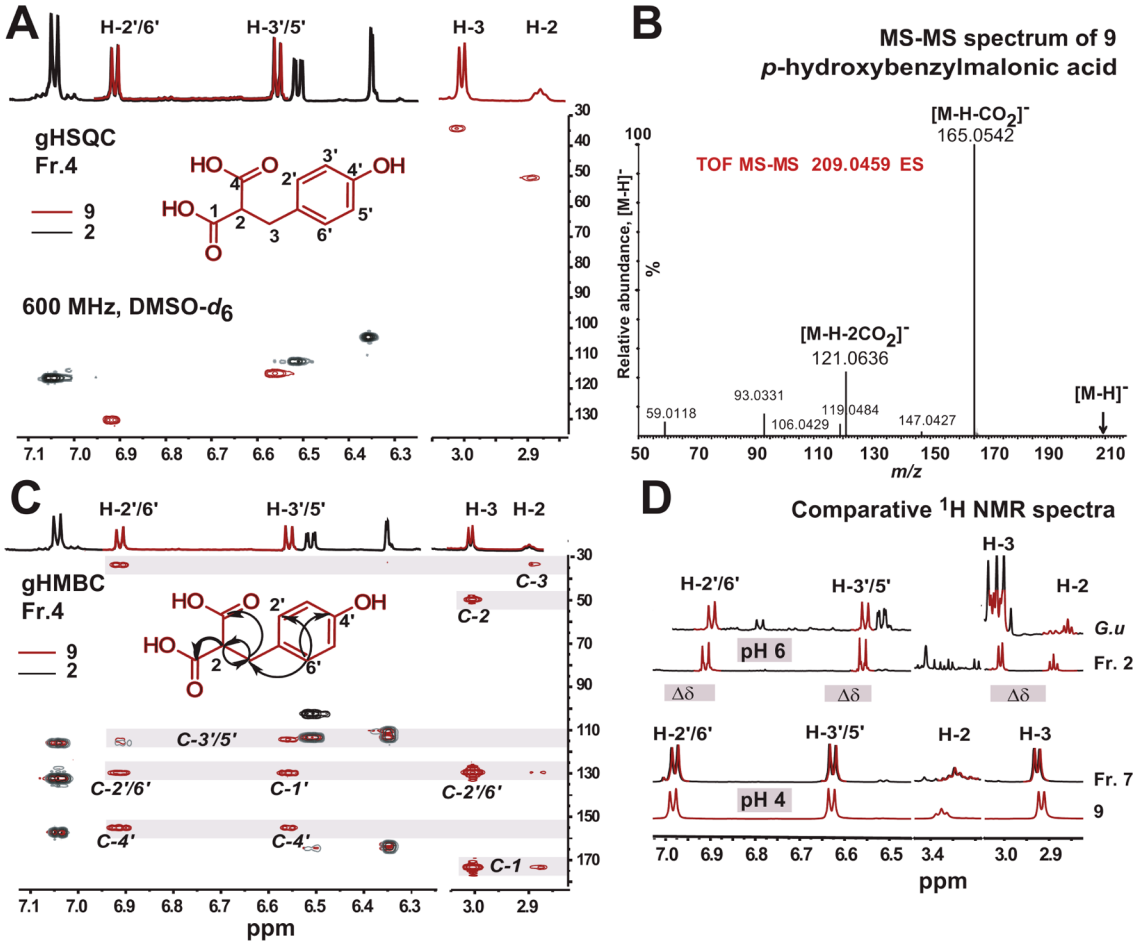

Figure 3. Structural identification of *p*-hydroxybenzylmalonic acid in fraction 4 and comparative ^{1}H NMR profiles of fractions 2 and 7. (A, C) By evaluating the difference between the HSQC (A) and HMBC (C) spectra of pure **2** (black, and 2D correlations in gray) and fraction 4, all the 2D NMR signals of *p*-hydroxybenzylmalonic acid (**9**, in red) could be assigned in fraction 4. (B) The MS-MS spectrum of **9** indicated the presence of two carboxylic acid functions and a molecular formula of $C_{10}H_{10}O_5$. (D) NMR analyses of neighboring fractions revealed the presence of **9** in fractions 2 and 7 (Supporting Information S8 and S9).

Table 3. Comparative NMR Spectroscopic Data of *p*-Hydroxybenzylmalonic Acid in Mixtures and as Purified Metabolite (^{1}H, 600 MHz; ^{13}C 225 MHz, in DMSO-d_6).

	p-hydroxybenzylmalonic acid (**9**)				
	in fractions 2–6		in fraction 7	purified	
pos.	δ_H, mult. (J)[a,b]	δ_C, type	δ_H, mult. (J)[a,b]	δ_H, mult. (J)[a,b]	δ_C, type
1 and 4		174.02, C_2			170.96, C_2
2	2.895, t (5.10)	53.31, CH	3.348, t (7.28)	3.376, t (7.41)	53.31, CH
3	3.009, d (5.10)	33.03, CH_2	2.930, d (7.28)	2.917, d (7.41)	33.03, CH_2
1′		128.88, C			128.88, C
2′/6′*	6.910, **AA**′XX′ (8.29/2.63/0.40)	129.95, CH	6.980, **AA**′XX′ (8.33/2.65/0.32)	6.983, **AA**′XX′ (8.33/2.65/0.32)	129.41, CH
4′-OH		155.78, C			155.78, C
3′/5′*	6.559, AA′**XX**′ (8.29/2.63/0.40)	114.79, CH	6.628 AA′**XX**′ (8.33/2.65/0.32)	6.629 AA′**XX**′ (8.33/2.65/0.32)	114.79, CH
pH[c]	5–6			4	

[a]All chemical shifts are given in ppm, and all coupling constants in Hz. [b]Full ^{1}H and ^{13}C NMR profiles (DMSO-d_6, 900 and 225 MHz) of isolated *p*-hydroxybenzylmalonic acid are available in the Supporting Information (Figure S4). The full spin parameters of the AA′XX′ pattern were determined by HiFSA using the Perch NMR software tool (v.2011.1, PERCH Solutions Ltd., Kuopio, Finland), in order to define the exact chemical shifts and coupling constants (Figure S10, Supporting Information). [c]As the pH of the solution decreases, a downfield shift of both aromatic protons as well as of H-2 can be observed, whereas the signal of proton H-3 is shifted upfield.

coeluted impurities. Purity determination was performed with UHPLC-UV and qHNMR, using the 100% method in both instances (Table 2, Figure 2).[31,32] It should be noted that, in general, UHPLC-UV overestimates purity whenever UV-transparent metabolites such as sugars are present. In contrast, ^{1}H NMR offers nearly universal detection of protons and bears the specific advantage of simultaneous access to both qualitative and quantitative information. The 100% method reflects the

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relative concentration of a targeted compound compared to its coeluted impurities.

One impurity (**9**) was detected by comparing the qHNMR spectra of fractions 4−6 (Figure 2A and B). The spectrum of fraction 4 provided evidence of the presence of a phenolic ring exhibiting a characteristic AA′/XX′ pattern centered at 6.665 and 6.890 ppm (2H each, J = 8.29 Hz) and two up-field signals, a doublet at 3.010 ppm (2H, J = 5.10 Hz) and a triplet at 2.895 ppm (1H, J = 5.10 Hz). LC-MS analysis using negative ion electrospray revealed a deprotonated molecule $[M - H]^-$ of m/z 209.0459, suggesting a molecular formula of $C_{10}H_{10}O_5$ (Figure 3B). The fragmentation pattern of **9** was similar to that of benzylmalonic acid (reference no. 100773, Sigma-Aldrich). In addition, both compounds produced extensive loss of CO_2 during in-source fragmentation, further suggesting a structural similarity. Collectively, these results indicated that the common impurity, **9**, should bear a *p*-hydroxyphenolic ring and two carboxyl functions. These observations were compatible with **9** being *p*-hydroxybenzylmalonic acid (CAS no. 90844-16-9). It is noteworthy that due to extensive in-source decarboxylation, **9** could have been easily misidentified as phloretic acid. This possibility could only be ruled out by meticulous comparison of the ^{1}H NMR profiles of fractions 4−6, as well as careful consideration of the MS spectra of benzylmalonic acid (Figures S5−S7, Supporting Information). Malonic acid derivatives are known to spontaneously decarboxylate at temperatures near 170 °C.[33] As such, the phenomenon of in-source decarboxylation of dicarboxylic acids has to be carefully considered during MS analysis.

The second major impurities detected by qHNMR profiling were the 1°Ms, sucrose (**10**) (anomeric proton δ_H 5.174, d, J = 3.74 Hz) in fractions 4 to 6 and lactic acid (methyl protons δ_H 1.019, d, J = 6.08 Hz) in fraction 4. Other detected impurities in fractions 5 and 6 were mostly chalcone and flavanone isomers (e.g., isoliquiritin apioside (**4**) and licuraside (**8**), Table 2), which had partition coefficients similar to those of compounds **1** and **2**.

The accuracy of the purity determination of target isolates, **1** and **2**, depends on the identity and the molecular weight of the impurities. In CS, these impurities result from possible stationary phase loss, coelution (similar K values) with the target analyte(s), and the comparatively wide Gaussian peak(s) of highly abundant constituents eluting earlier ("tailing effect"). The relative concentration (% w/w) of **9** vs **10** assessed by qHNMR was determined to be 15.9, 9.2, and 5.0 vs 12.1, 7.6, and 6.1% in fractions 4, 5, and 6, respectively (Table 2, Figure 2).

The single-step CPC produced 90 mg of purified **1** (95.8% by qHNMR) through spontaneous crystallization occurring in fraction 6 (K = 0.72), a phenomenon frequently observed in CS due to the physicochemical nature of multicomponent SSs. The mother liquor of fraction 6 yielded an additional 408 mg of **1** with 70.2% qHNMR purity (Table 2, Figure 2), compared to 89.9% by UHPLC-UV. The purity level of target compound **2** was determined by qHNMR to be only 65.0% and 80.3% in fractions 4 and 5, respectively, due to the coelution of 1°Ms. These observations led to the conclusion that polar 1°Ms were carried over with the aqueous mobile phase during the first 820 mL of elution (up to K = 0.72). In such cases, a second CS step, even with the same solvent system, would increase the purity of the isolates.

The presence of polar UV-transparent metabolites such as sucrose would have been missed if the purity control was performed only by UHPLC-UV and/or LC-MS analyses, as they generally elute at the solvent front on reverse phase and are usually diverted to waste prior to MS detection. Furthermore, they are not susceptible to ionization under general MS conditions.[24] Collectively, these results underscore the necessity of orthogonal analytical methods combining LC-UV/MS with qHNMR to accurately define the purity level of isolated metabolites and determine the structure of impurities.

Further Characterization of *p*-Hydroxybenzylmalonic Acid (9) in Mixtures. Interestingly, the natural occurrence of *p*-hydroxybenzylmalonic acid **9** has not been described previously. However, this phenolic acid has already been assigned a CAS number (90844-16-9) following chemical synthesis.[34] Moreover, **9** can be employed as a reagent in the synthesis of thyroxin derivatives.[35] A patent covering the use of any structural derivatives of **9** as stabilizers for plastic material (US patent 4,148,783) has been issued. Therefore, the question arises as to whether the presence of **9** in the CPC fractions represented a potential chemical contamination. In order to confirm the genuine nature of the presence of **9** in licorice fractions, additional 1D and 2D NMR experiments including COSY, HSQC, and HMBC were carried out on the mixtures, as well as on isolated **9**. Furthermore, the influence of the chemical environment on the structural elucidation was investigated. Finally, the identification and quantification of **9** were performed on crude extracts from different *Glycyrrhiza* species and extracts.

COSY experiments of fraction 4 clearly displayed short distance correlations between the methylene protons H-3 at 3.010 ppm from **9** and its methine proton H-2 at 2.895 ppm. On the other hand, short distance correlations were observed between the aromatic protons H-2′/6′ at 6.890 and H-3′/5′ at 6.665 ppm (Figure S9, Supporting Information). Moreover, HMBC experiments revealed long-range correlations between aromatic protons H-2′/6′ and the benzyl carbon C-3 (δ_C 53.31, δ_H 2.895), along with the C-4′ phenolic carbon (δ_C 155.78) (Figure 3C). Interpretation of the HMBC spectrum indicated that the H-3 benzylic proton correlated with the carboxylic acid functions (δ_C 174.02). Overall, 2D NMR analyses performed on the mixtures (fractions 4−6) also led to the structural identification of **9** as *p*-hydroxybenzylmalonic acid. Subsequent NMR analyses of the neighboring fractions indicated that **9** was concentrated in fraction 7, but was also present in fractions 2 and 3 (Figure 3D, Figures S8 and S9, Supporting Information). Considering that the CPC SS used deionized water (pH 6) and was not buffered, **9** may have eluted either as a partially negatively charged species or as a diacid. The potential occurrence of multiple forms of **9** explains its large volume of elution (>1.54 L) and, therefore, its presence in six consecutive fractions.

Compound **9** purified by semipreparative HPLC from fractions 2 and 4 was also subjected to extensive NMR analysis (Figure S11, Supporting Information). The ^{1}H chemical shifts of such a malonic acid derivative can be influenced by the chemical composition and, more importantly, the pH of the fraction from which it is characterized (Figure 3D and Figure S12, Supporting Information). Interestingly, the pH values of both fraction 7 and purified **9** were found to be ∼4, while the pH of fractions 2−6 was determined to be ∼5.5. Under acidic conditions (pH ∼4, fraction 7 and purified **9**), the aromatic protons, as well as that of H-2, were deshielded ($\Delta\delta$ = 0.07 ppm for H-2′/6′ and H-3′/5′, $\Delta\delta$ = 0.48 for H-2), while the H-3 resonance was shielded ($\Delta\delta$ = 0.09 ppm). Clearly, the

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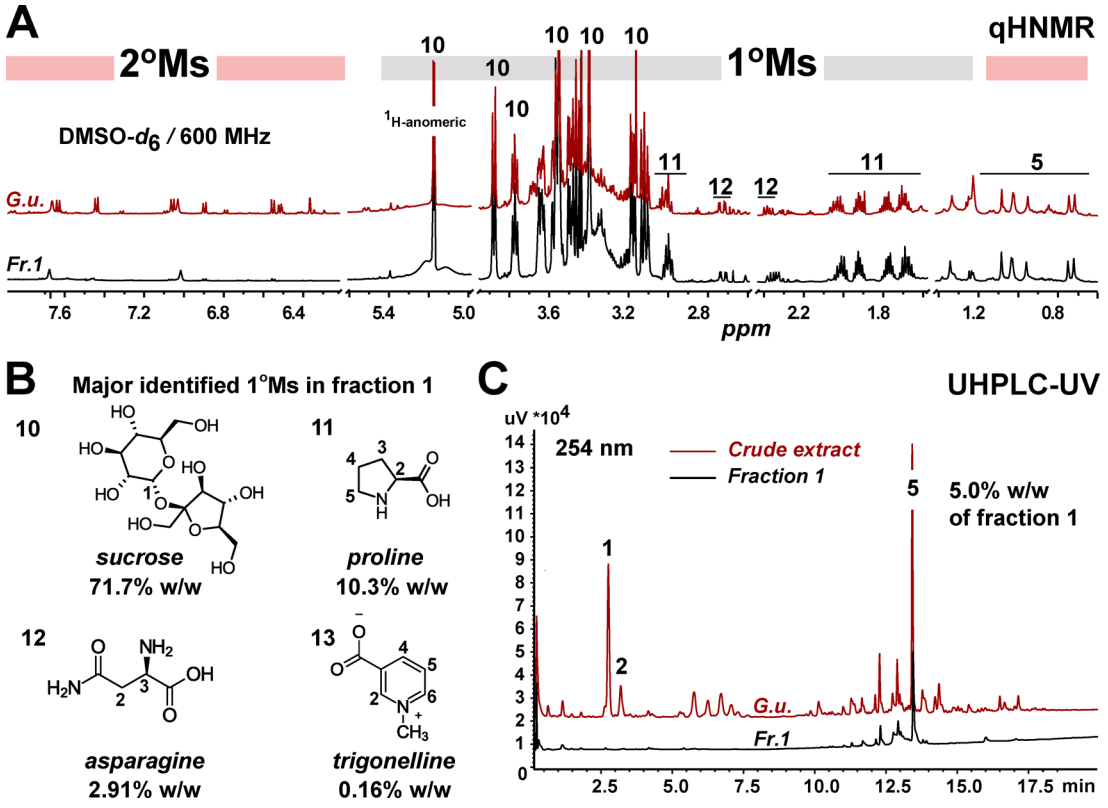

Figure 4. Comparative qHNMR spectra (A) and UHPLC-UV profiles (C) of fraction 1 and *G. uralensis* crude extract for identification of the major 1°Ms (B). (A) Comparative qHNMR spectra (600 MHz, DMSO-d_6) using an internal calibrant (3,5-dinitrobenzoic acid TraceCERT at 2.12 mM) enabled the identification and quantification of major 1°Ms in fraction 1 (Figures S14 and S15, Supporting Information). Sucrose (**10**), proline (**11**), and asparagine (**12**), but also glycyrrhizin (**5**), were defined as being the most abundant. (B) Structure and concentration of each identified 1°M determined by qHNMR in fraction 1. (C) The UHPLC-UV profile of fraction 1 displays only the presence of glycyrrhizin **5** (determined at 5% w/w) along with other minor saponins. The crude extract was prepared at 10 mg/mL, whereas fraction 1 was analyzed at 2.5 mg/mL (Kinetex C18-XB; 50 × 2.1 mm, 1.7 μm; gradient composed of H_2O and MeCN + 0.1% FA).

composition influences the final pH of the fractions and ultimately influences the form in which compound **9** exists (Figure 3D). This explains why the ^{1}H NMR signal pattern of pure **9** differed from those observed in fractions 2−5.

In order to ascertain its natural occurrence, compound **9** was quantified in the original *G. uralensis* crude MeOH extract by qHNMR (Figure 3D) to be 1.66%. Further investigations (Figure S13, Supporting Information) by qHNMR indicated that **9** could be detected in the crude extracts of all three *Glycyrrhiza* species at concentrations between 1.42% and 2.67%. The quantifications were performed utilizing the resonances of H-3′/5′ at 6.556 ppm.

Interestingly, Farag and co-workers had previously reported the ubiquitous presence of an "unknown compound" in *Glycyrrhiza* species.[24] This molecule also produced a molecular ion $[M - H]^-$ m/z = 209.0453, and we conclude that it is likely the same compound as **9**. In summary, our results confirm the identity and presence of *p*-hydroxybenzylmalonic acid (**9**) in licorice roots and indicated that **9** is neither a minor metabolite nor the result of a chemical contamination.

Identification and Quantification of Major 1°Ms. After the large-scale CPC fractionation, polar 1°Ms were assumed to be concentrated in the first fraction. Therefore, metabolite profiling of fraction 1 was performed by qHNMR in order to identify and quantify these 1°Ms. Interestingly, the UHPLC-PDA profile revealed the presence of only glycyrrhizin (**5**) at a concentration of 5.0%. However, further investigation by 1D/2D NMR (gCOSY, HSQC, HMBC) and qHNMR spectra led to the identification and quantification of major UV-transparent 1°Ms (Figure 4A and C).

Sucrose (**10**) was identified and quantified, utilizing its nonoverlapped anomeric proton resonance, a doublet (J = 3.74 Hz) at 5.174 ppm in DMSO-d_6. Proline (**11**) was also identified based on its diagnostic ^{1}H NMR resonances at 1.671 ppm (1H, J = −12.88/7.92/7.50/7.40/0.71 Hz, H-4a), 1.770 ppm (1H, J = −12.88/7.55/7.28/6.00/5.57 Hz, H-4b), 1.928 ppm (1H, J = −12.74/7.50/6.00/5.55 Hz, H-3a), and 2.003 ppm (1H, J = −12.74/8.87/7.92/7.28 Hz, H-3b). The H-2 resonance observed at 3.618 ppm (1H, ddd, J = 8.87/5.55/0.71 Hz) was partially obscured by resonances from **10**. The proton H-5 resonated at 3.201 ppm (1H, dddd, J = −11.21/7.40/5.57 Hz) and 2.994 ppm (1H, ddd, J = −11.20/7.55/7.40 Hz). Furthermore, the amino acid asparagine (**12**) was identified by its two double doublet resonances at 2.343 ppm (1H, dd, J = 16.49/8.89 Hz, H-2a) and at 2.724 ppm (1H, dd, J = 16.49/4.04 Hz, H-2b). The resonances of H-3 at 3.373 ppm (1H, dd, J = 8.89/4.04) were also partially obscured by hydroxy resonances from **10**. Lastly, trigonelline (**13**) was identified by its characteristic downfield proton at 9.923 ppm (1H, s, H-2), 8.891 ppm (1H, d, J = 5.90, H-6), 8.768 ppm (1H, d, J = 7.91, H-4), and 8.032 ppm (1H, dd, J = 7.91/5.90, H-5). The presence and identity of compounds **10** to **13** in fraction 1 were also confirmed by MS analyses, as described previously.[36] The ^{1}H NMR spectrum of fraction 1 was compared with the

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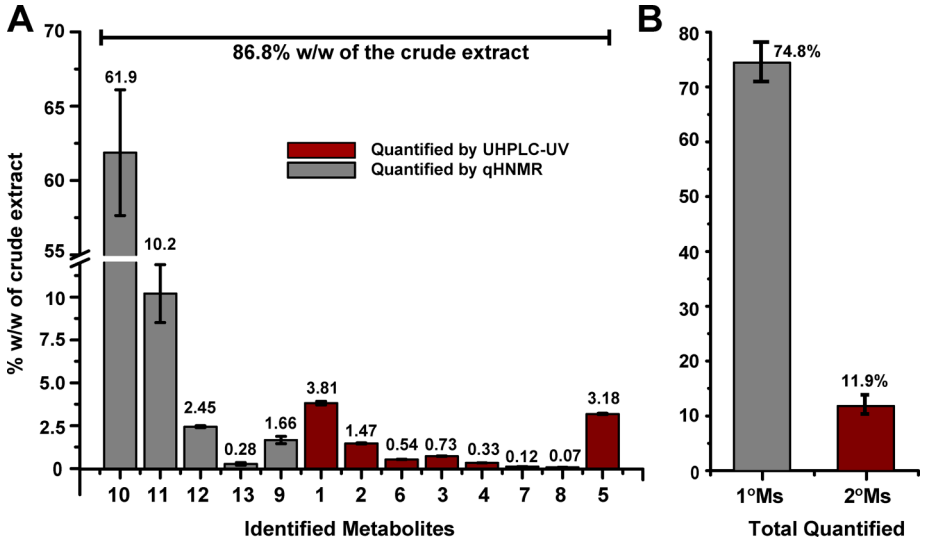

Figure 5. UHPLC-UV and qHNMR quantitative results of major identified 2°Ms and 1°Ms in *G. uralensis* crude extract. (A) Quantitative ^{1}H NMR was carried out in the crude MeOH extract from the roots of *G. uralensis*. All 1°Ms (gray) together with compound **9** were quantified by qHNMR. All 2°Ms (red) were quantified by a previously established UHPLC-UV method.[7] Results are expressed as mass percentages of the crude extract and highlight that sucrose (**10**) and proline (**11**) were the most concentrated of all quantified metabolites. Liquiritin (**1**) (3.81%) and glycyrrhizin (**5**) (3.18%) were the second most concentrated metabolites. (B) Sum of all quantified major 1°Ms (74.80%; gray) compared to the sum of all quantified 2°Ms (11.91%, red). Compounds: sucrose (**10**), proline (**11**), asparagine (**12**), trigonelline (**13**), *p*-hydroxybenzylmalonic acid (**9**), liquiritin (**1**), liquiritin apioside (**2**), liquiritigenin (**6**), isoliquiritin (**3**), isoliquiritin apioside (**4**), neoisoliquiritin (**7**), licuraside (**8**), glycyrrhizin (**5**).

spectrum from commercial reference compounds (Figures S14 and S15, Supporting Information) and were in agreement with previously published data.[23,37,38] All identified metabolites were quantified in fraction 1 using 3,5-dinitrobenzoic acid (Fluka, TraceCERT) as the internal calibrant (IC, 2.12 mM). By far, **10** was the most abundant metabolite (71.7% in fraction 1, qHNMR, anomeric proton), followed by **11** (10.3% of fraction 1, qHNMR, H-3 resonance), **12** (2.91% of fraction 1, qHNMR, H-3 resonance), and **13** (0.16% of fraction 1, qHNMR, H-4 and H-6). Hence, the sum of all identified and quantified 1°Ms represented 85.1% of fraction 1 and therefore around 72% of the crude extract (Figures 4 and 5). The present results are in agreement with the work by Farag and co-workers, who indicated that saccharides, especially sucrose, were the most abundant 1°Ms, followed by amino acids and fatty acids.[24]

Concentrations of 1°Ms vs 2°Ms in *G. uralensis* Crude Extract. qHNMR and UHPLC-UV were used to confirm the quantitative results previously obtained for fraction 1 and to assess the relative abundance of major 1°Ms vs 2°Ms in the crude extract. As such, all major UV−visible 2°Ms were quantified by UHPLC-UV using a previously validated method[7] (Supporting Information S1), whereas all major UV-transparent 1°Ms, plus the phenolic acid **9**, were quantified by qHNMR (Figure 5A). Combining these orthogonal analytical methods enabled the characterization and quantitation of 86.8% of *G. uralensis* crude extract. Compounds **10** (61.9%) and **11** (10.2%) were by far the most prevalent 1°Ms, thereby confirming the quantitative results obtained from fraction 1. Together, these compounds represented 93% of all quantified 1°Ms. In plant physiology, **10** and **11** are known to be natural osmoprotectants,[39] capable of maintaining the moisture level inside the roots in order to protect the plant cells against dehydration.[40] The third most concentrated 1°M was found to be **12** (2.45% of the crude extract), one of the 20 most common amino acids, which is also a nitrogen transport metabolite intensely synthesized in the legume roots.[41] The presence of excess **12** has been reported to be a marker of protein degradation under stress, such as drought, salinity, and nutrient deficiency.[42,43] The production of compounds **10**, **11**, and **12** has been reported to increase in the roots of various plant species, including those from the Fabaceae family, during a period of drought.[43,44] Therefore, the abundance of such metabolites (**10**−**12**) in licorice roots may be an indicator of drought stress at harvest time. Compound **13**, commonly found in seeds and root exudates of various Fabaceous plants and reported to be a *Nod* gene inducer at molar concentrations,[45,46] represented 0.28% of the crude extract. Interestingly, its occurrence in licorice roots is reported here for the first time.

From the perspective of botanical use, **10** contributes to the sweetness of the roots, together with the natural sweetener **5**. Moreover, the amino acid **11** is known to enter into the biosynthesis of proteins and collagen.[47] As a free amino acid, proline (**11**) has been described as a protein-solubilizing solute and as a protein folding agent both in vitro and in vivo.[48,49] The alkaloid **13** has also been attributed multiple health properties linked to the prevention of central nervous system diseases and diabetes.[50,51]

The flavanone, **1** (3.81%), and the saponin, **5** (3.18%), were found to be the second most prevalent metabolites. Interestingly, the dicarboxylic acid, **9** (1.66%), was more abundant than the flavanone apioside, **2** (1.47%), confirming the importance of this phenolic acid in licorice roots. From a structural perspective, compound **9** may be regarded as a chemical analogue of tyrosine, where the amino function is replaced by a carboxylic acid. The question remains whether **9**, like tyrosine, is a biogenetic precursor of various hydroxycinnamic acids and, thus, of licorice chalcones and flavanones.[52,53] Interestingly, in the root−rhizosphere environment, flavonoids can be degraded to phenolic derivatives by symbiotic rhizobia, mainly through the fission of the C-ring.[54−56] This group of compounds is considered as growth substrates for rhizobia. Therefore, **9** could be the result of either the plant or the

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rhizobia metabolism, highlighting its potential importance as a marker of plant−rhizobia metabolism.[55] From a pharmacological perspective, the only reported activity for **9** was in 1991, when Shechter and co-workers demonstrated that **9** inhibited the protein tyrosine kinase implicated in proliferative cell cycles as well as insulin-dependent lipogenesis (IC_{50} of 3.8 ± 0.1 mM).[57] Considering its natural occurrence in licorice roots, the pharmacological properties of **9** are deserving of further investigations.

Finally, the most abundant 2°Ms, including **9**, accounted for 11.9% of the crude extract, whereas the major 1°Ms alone represented 74.8% (Figure 5B). The combined quantitative results demonstrated that the 1°Ms were at least six times more concentrated than the 2°Ms in the crude MeOH extract.

In conclusion, the CPC method developed in this study enables the large-scale purification of the liquiritin derivatives in less than 3 h, producing reference materials with qHNMR purities of 70.2−95.8% for **1** and 65.0−80.3% for **2**. At the same time, the major 1°Ms, which represent 74.8% of the crude extract, were concentrated in a distinct fraction and subsequently identified and quantified by qHNMR. Additionally, orthogonal analyses by UHPLC-UV/LC-MS and qHNMR led to a comprehensive evaluation of the RC of the reference materials of **1** and **2**. One of the major coeluting impurities was identified as *p*-hydroxybenzylmalonic acid (**9**), isolated for the first time from a natural source, representing 1.66% of the *G. uralensis* extract. The identification of **9** along with the detection of UV-transparent 1°Ms as coeluting impurities illustrated the importance of orthogonal analyses (LC-UV/MS/qHNMR) for chemical profiling and purity determination. Finally, comparative quantitative results, obtained by UHPLC-UV for the major UV−visible 2°Ms and by qHNMR for the major 1°Ms, (Figure 5) enabled 86.8% of the original *G. uralensis* crude extract to be described and clearly underlined the abundance of these 1°Ms. This group of metabolites is implicated in the taste, sweetness, nutritional value, and potentially health effects of licorice preparations.[49−51,58] Consequently, qualitative and quantitative determination of both 1°Ms and 2°Ms, including *p*-hydroxybenzylmalonic acid (**9**), should be considered for the quality control of licorice botanicals. From a broader perspective, the existing but relatively sparse knowledge about the biological effects of (designated) 1°Ms also indicates that these small polar molecules warrant consideration as marker compounds, which may impact the overall biological profile of a plant extract.

■ EXPERIMENTAL SECTION

General Experimental Procedures. UHPLC analyses were performed on a Shimadzu UPLC 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 autosampler temperature was set at 4 °C, and the column oven temperature was set at 40 °C. Postrun data analyses were done with the Shimadzu LabSolution software package. Countercurrent separation was carried out on a hydrostatic centrifugal partition lab-scale instrument SCPC-1000 system, equipped with a 1000 mL column, and coupled with an Armen Spot Prep II system (Armen Instrument SAS, Saint Avé, France). The latter is equipped with a quaternary valve, a mixing chamber, a 50 mL/min pump, an automatic injection loop, and an automated back flush ascending/descending valve, coupled with a UV-DAD detector and a fraction collector all controlled by Armen CPC software. The injection capacity of the defined CPC instrument ranges from 100 mg to 30 g. All collected fractions were dried using a Thermo-Fischer Savant SC250 EXP speed vacuum equipped with an RVT4104 refrigerator vapor trap. Freeze-drying was performed on a Labconco Freezone 4.5 (Kansas City, MO, USA). NMR spectra were acquired on a Bruker AVANCE 600 MHz spectrometer equipped with a 5 mm TXI cryoprobe and on a Bruker AVANCE 900 MHz. Off-line data processing was performed using the Mnova NMR software package (v.6.0.2, MestreLab Research S.L., A Coruña, Spain). Standard NMR tubes of 3 mm, 7 in., were from Norell (part no. S-3-HT-7, Norell Inc., Landisville, NJ, USA). A precise Mettler Toledo XS105 dual range analytical balance was used to prepare all extracts and fractions for UPHLC and qHNMR analyses.

Reagents. All chemicals and reagents including HPLC-grade solvents were obtained from Fisher-Scientific (Hanover Park, IL, USA) or Sigma-Aldrich (St. Louis, MO, USA). For NMR acquisition, DMSO-d_6 (D 99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

Plant Material and Extraction. Dried roots of *G. uralensis* Fisch ex. DC were purchased from a local supplier (Chicago, IL, USA). The plant material (voucher codes BC 624 and BC 689) was identified through a series of microscopic analyses, comparing it to a voucher specimen from the Field Museum (Chicago, IL, USA, *G. uralensis* × *glandulifera* FM 2174544). DNA authentication was also performed according to Kondo and co-workers.[59] Other *Glycyrrhiza* species used for the quantification of **9** were identified following the same procedure. The powdered roots of BC 624 (1109.55 g) were exhaustively extracted by percolation with MeOH at room temperature (weight/volume ratio: 1:15). Freeze-drying yielded 282.76 g of crude extract, which represented 25.5% (w/w) of the powdered roots.

Selection of the CPC Solvent Systems. The composition of a two-phase solvent system (SS) was selected according to the partition coefficient (K) of the target compounds in the crude extract, as well as the settling time of the selected SS. Approximately 5 mg of the crude extract was weighed in a 4 mL vial, to which 1.0 mL of the selected SS was added. The tube was shaken vigorously to equilibrate the sample thoroughly between the two phases. The upper phase (UP) and lower phase (LP) were separated and dried overnight in a Savant SC250 EXP speed vacuum. Each dried and separated UP and LP was diluted in 200 μL of HPLC-grade MeOH for further UHPLC-UV analysis. The K value was expressed as a peak area of the target compound in the UP divided by that in the LP.

Fractionation and Isolation by CPC. The fractionation was performed with EtOAc/MeCN/H_2O (5:6:9 v/v/v) in an isocratic and descending mode. The SCPC-1000 (column volume 1030 mL) was filled with the organic UP at a flow rate of 50 mL/min. The rotation speed, adjustable from 0 to 1500 rpm, was set at 1200 rpm. The system was equilibrated at a flow rate of 20 mL/min with an S_f of 71.8% (V_S = 739.54 mL and V_m = 290.46 mL). *G. uralensis* crude extract (15.003 g), diluted in 21 mL of UP and 21 mL of LP, was injected into the column. Fraction collection was set up for 20 mL/fraction and started after 300 mL of mobile phase elution. The extrusion after 2.6 column volumes and during 30 min was performed at a flow rate of 50 mL/min, while the fraction collection was set for 25 mL/fraction. CPC chromatograms were obtained by UV detection, using a UV-DAD detector at 254, 330, and 350 nm, as well as UV-scan from 200 to 600 nm. CPC fractions were pooled according to their UV and TLC profiles, leading to a total of 13 final fractions. TLC was performed on Alugram silica gel plates (SiO_2 F_{254}, Macherey-Nagel Gmbh & Co.), eluted with EtOAc/MeOH/AcOH/H_2O (8:1:1:2) and revealed with 5% H_2SO_4/vanillin reagent. The fractions were pooled as follows: Fraction 1: vials 1 to 6 (from 0 to 80 mL after the first 300 mL), fraction 2: vials 7 to 10, fraction 3: vials 11 to 12, fraction 4: vials 13 to 15, fraction 5: vials 16 to 28, fraction 6: vials 29 to 53, fraction 7: vials 54 to 76, fraction 8: vials 76 to 79, fraction 9: vials 80 to 105, fraction 10: vials 106 to 120, fraction 11: vials 121 to 130, fraction 12: vials 131 to 156, fraction 13: vials 157 to 171.

Preparative HPLC for the Purification of *p*-Hydroxybenzylmalonic Acid. Preparative HPLC was performed on a Waters 600 apparatus equipped with a YMC-Pack-ODS-AQ column (250 × 10 mm, 5 μM), a PDA detector (Waters 2996), and an autosampler (Waters 717plus). Each of the fractions 2 and 4 was prepared at 50 mg/mL in 50% HPLC-grade MeOH, and 50 μL was injected. The

column was eluted with a gradient composed of (A) H_2O + 0.1% formic acid (FA) and (B) ACN + 0.1% FA as follows: from 25% during 2 min, to 40% B in 20 min, and during 5 min back to 25% B in 5 min (flow rate 2 mL/min). The retention times (t_R) were 12.35 min for **9** and 15.00 min for compound **2**. After concentration, samples were freeze-dried for 12 h and placed under vacuum prior to NMR analyses. A total of 10 injections were performed, leading to the isolation of 5 mg of **9** (purity: 88.3% by qHNMR).

UHPLC-UV Analyses of Fractions and Crude Extract. *G. uralensis* crude extract and fractions obtained by CPC were prepared at 10 and 2.5 mg/mL, respectively, and isolated compounds were prepared at 0.5 mg/mL. Samples were diluted in 100% MeOH or MeOH/H_2O (70:30) HPLC grade (Fisher Co. Ltd.) and filtered (filter Acrodisc CR 13 mm, 0.45 μm PTFE membrane) prior to injection (2 μL). UHPLC analyses were performed following a published method.[7] *G. uralensis* crude extract was examined in triplicate.

LC-MS-MS of Fractions 4 to 7. LC-MS-MS analysis was carried out using a Waters 2695 solvent delivery system connected to a Water SYNAPT quadrupole/time-of-flight (Q/TOF) mass spectrometer operated in the negative ion electrospray mode. Separations were carried out using a Waters XBridge C_{18} column (2 × 50 mm, 2.5 μm particle size), eluted with a mobile phase consisting of 0.1% FA (solvent A) and 95% MeCN + 0.1% FA (solvent B) with a linear gradient from 10% to 60% B in 10 min. The flow rate was 0.25 mL/min, and the column was thermostated at 30 °C. High-resolution accurate mass measurements (HR-MS) were performed at the resolving power of 10 000 fwhm using Leu-enkephaline as the lock mass. Product ion spectra were recorded at 15 or 25 eV using argon as the collision gas. For identification of compounds, molecular compositions and tandem mass spectra were compared with the standard spectra from public (MassBank) and in-house generated databases as well as with spectra published in the primary literature.

qHNMR Analyses of Fractions and Purity Determination. The NMR samples of *G. uralensis* crude extract and the CPC fractions were prepared by precisely (0.01 mg) weighing 8 mg of the extract and 3−5 mg of the fractions, followed by the addition of 300 μL of DMSO-d_6 using a Pressure-Lok gas syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA) for accurate volume delivery. From this solution, 200 μL measured with calibrated pipets (cat. no. 2-000-200, Drummond Scientific, Broomall, PA, USA) was added into 3 mm standard NMR tubes. All 1D ^{1}H NMR spectra were acquired at 298 K under quantitative conditions using a 90° single-pulse experiment (DE = 39.71 μs, D1 = 60.00 s, P1 = 8.75 μs, ds = 4, ns = 32) at 600 MHz. The 1D ^{13}C NMR spectra of **9** and fractions 2 and 7 were acquired at 900 MHz (DEPT-Q experiment). ^{1}H and ^{13}C chemical shifts (δ) were expressed in ppm with reference to the residual solvent signal of DMSO-d_5 (^{1}H spectrum: 2.500 ppm, and ^{13}C spectrum: 39.525 ppm). The following processing scheme was used: a mild Lorentzian-to-Gaussian window function (line broadening = −0.3 Hz, Gaussian factor = 0.01) was applied, followed by zero filling to 256k acquired data points before Fourier transformation. After manual phasing, a fifth-order polynomial baseline correction was applied. The purity was calculated by qHNMR using the 100% method.[31,32] For absolute quantitation of 1°Ms in the crude extract and in fraction 1, qHNMR was performed using 3,5-dinitrobenzoic acid (Fluka, TraceCERT, purity P = 99.54% w/w, lot no. BCBH8381V) as internal calibrant (IC). A stock solution of the IC was prepared at 6.35 mM in a mixture of D_2O/DMSO-d_6 (1.5:7). NMR samples of fraction 1 (~5 mg) and of crude extract (~8 mg) were prepared by precision weighing as described above, except that their dilution was performed in 200 μL of DMSO-d_6 and 100 μL of IC stock solution (2.12 mM final concentration). The pH of each solution was determined using pH strips (Whatman, Panpeha) after diluting 100 μL of the remaining DMSO-d_6 solution into 300 μL of deionized H_2O. The 1D ^{1}H NMR spectra were acquired under quantitative conditions using 64 transients (DE = 39.71 μs, D1 = 60.00 s, P1 = 9.20 μs, rg = 64). The same data processing was performed as described above. The signal-to-noise ratio (S/N) was ≫200:1, leading to an uncertainty level of 1% for the integration procedure.[60,61]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Quantitative results of 2°Ms by UHPLC-UV; metabolite profiling of CPC fractions by qHNMR and UHPLC-UV; 1D and 2D NMR spectra of fractions 2 and 7; comparative ^{1}H NMR and MS spectra of benzylmalonic acid and *p*-hydroxybenzylmalonic acid (**9**); qHNMR results for the quantification of **9** in different *Glycyrrhiza* extracts; and the ^{1}H NMR profiles of the identified 1°Ms in DMSO-d_6 and D_2O are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): G.A. is CEO of and shareholder in Armen Instrument. C.L. is an employee of Armen Instrument. The other authors declare no competing financial interest.

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