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

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Supporting information

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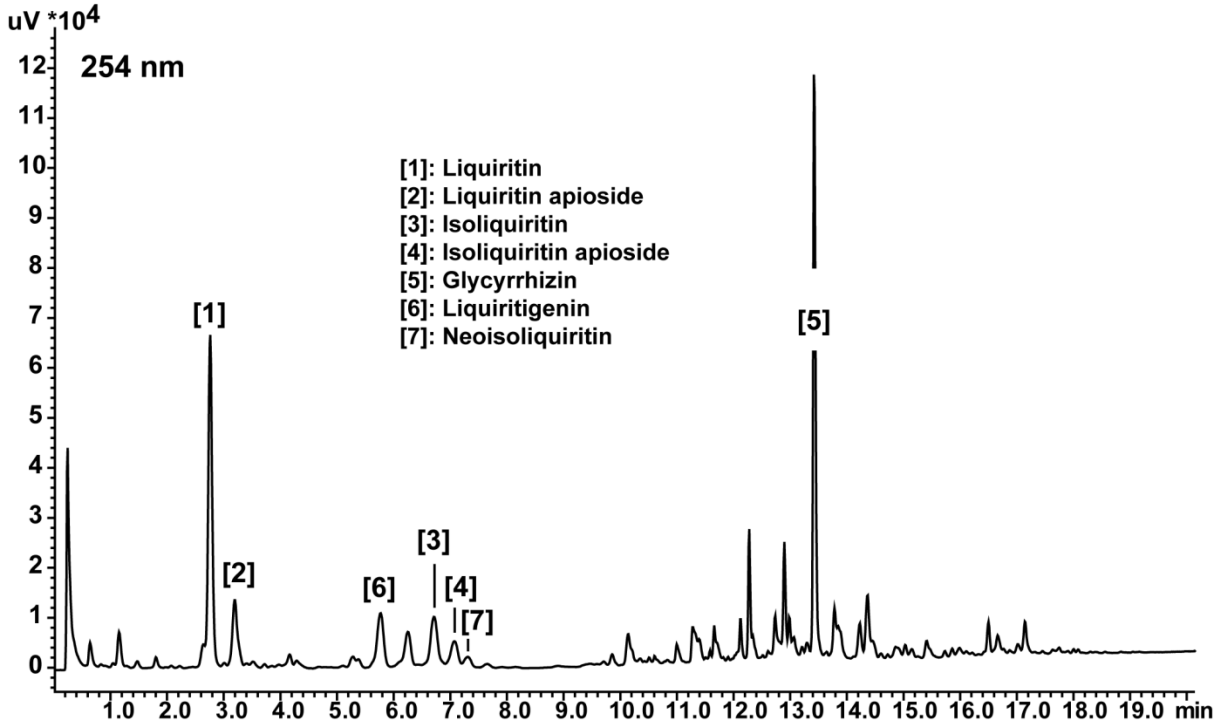
S1. UHPLC-UV quantitation of major secondary metabolites in *G. uralensis* crude extract

Name	Rt (min)	Conc. 1	Area	Conc.2	Area	Conc.3	Area	% w/w	%w/w	%w/w	mean	stdv
Liquiritin (1)	2.76	0.392	888857	0.380	861004	0.372	843799	3.922	3.798	3.722	**3.81**	**0.10**
Liquiritin api (2)	3.19	0.152	218726	0.149	213134	0.144	206158	1.485	1.485	1.436	**1.47**	**0.03**
Isoliquiritin (3)	6.71	0.072	380003	0.074	388347	0.071	377103	0.736	0.736	0.715	**0.73**	**0.01**
Isoliquiritin api (4)	7.08	0.032	157683	0.034	168687	0.033	164726	0.337	0.337	0.329	**0.33**	**0.00**
Glycyrrhizin (5)	13.43	0.315	335209	0.316	336149	0.323	343904	3.160	3.160	3.234	**3.18**	**0.04**
Liquiritigenin (6)	5.77	0.052	221636	0.054	230079	0.052	222169	0.543	0.543	0.523	**0.54**	**0.01**
Neoisoliquiritin (7)	7.31	0.011	64223	0.012	67212	0.012	66103	0.117	0.117	0.115	**0.12**	**0.00**
Licuraside (8)	7.65	0.004	17823	0.006	24698	0.008	31165	0.062	0.062	0.079	**0.07**	**0.01**
Total quantified Major secondary Metabolites by UHPLC-UV								10.46	10.33	10.26	**10.35**	**0.10**

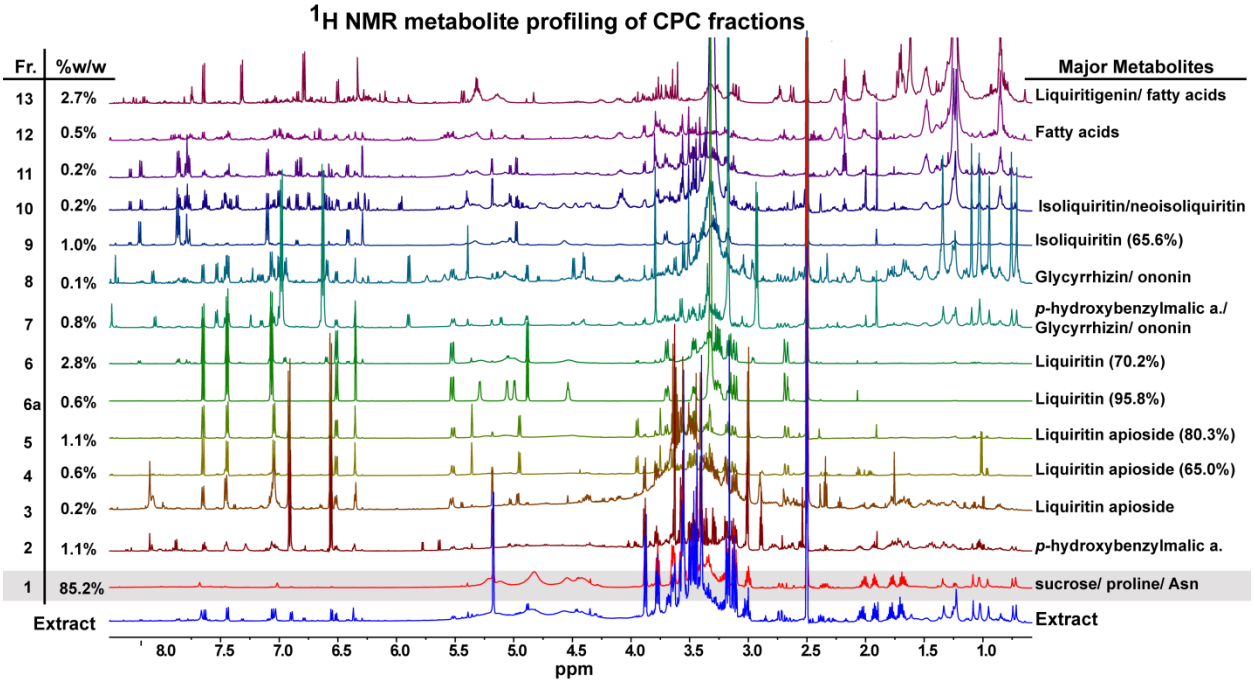


All flavanones (compounds **1**, **2** and **6**) were quantified at 275 nm. All chalcones (compounds **3**, **4**, **7** and **8**) were detected and quantified at 360 nm. Finally Glycyrrhizin **5** was quantified at 254 nm.

The UHPLC-UV chromatogram of *G. uralensis* crude extract (10 mg/mL) at 254 nm (2 µL injected) is represented on the left.

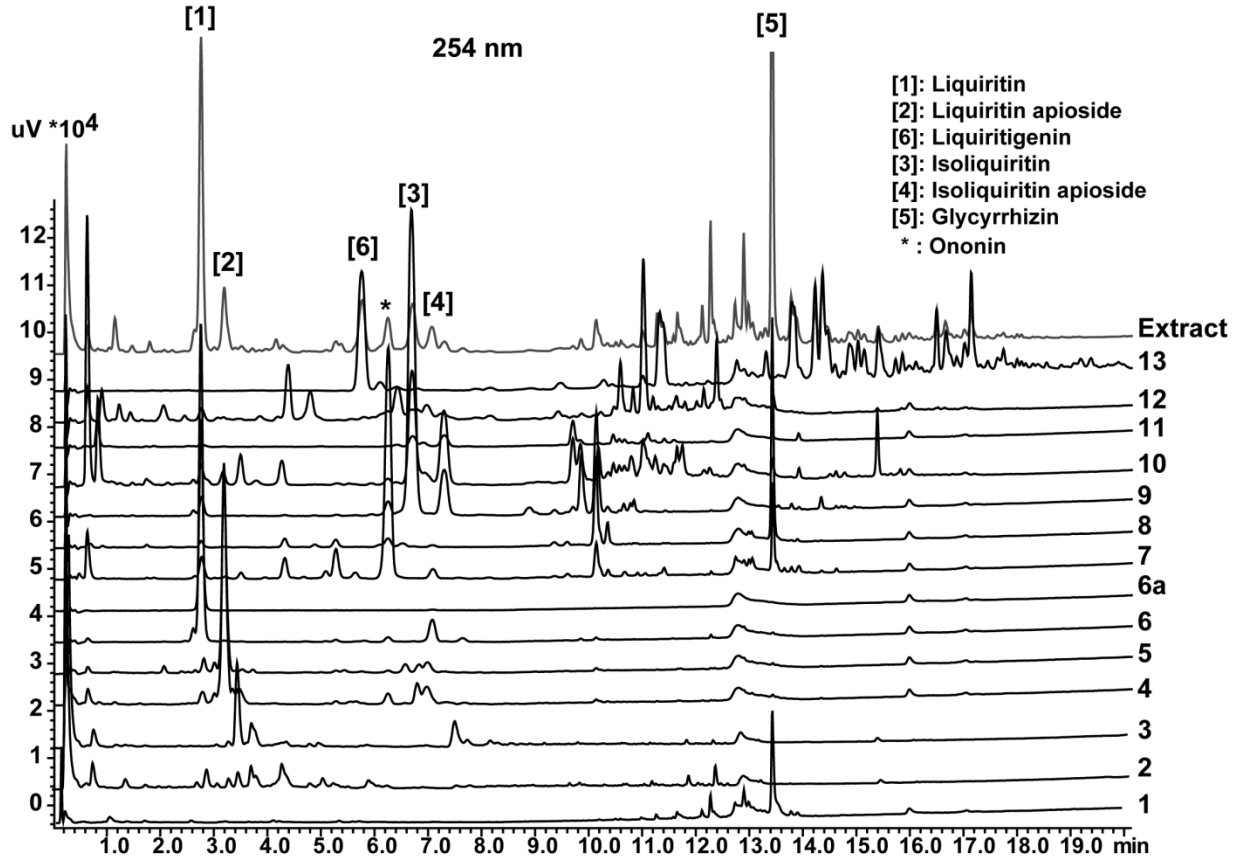
Kinetex C18-XB (50 x 2.1 mm, 1.7µm) eluted with a gradient composed of (A) H_2O + 0.1% formic acid (FA) and (B) ACN + 0.1% (FA) as follows: 8% to 11% B in 2 min, and during 30 sec, to 13% B at 4 min, to 15.5% B at 8 min, to 36% at 13 min, and during 30 sec, to 80% B at 21 min, and during 1 min, back to 8% B at 23 min (flow rate: 0.8 mL/min)

S2. Stacked ^{1}H NMR profiles of CPC fractions and *G. uralensis* crude extract



NMR sample of *G. uralensis* crude extract was prepared by precisely weighing 8 mg followed by the addition of 300 µL of DMSO-d_6. From this solution, 200 µL measured with a calibrated pipets (cat no:2-000-200, Drummond Scientific, Broomall, PA, UAS) were added into 3 mm, 7 in. standard NMR tubes. NMR samples of CPC fractions, and purified metabolites were prepared following the same procedure, by precisely weighing 3-5 mg (except for fraction 2: 8mg, fraction 7: 27mg, fractions 10 and 13: 10 mg). The 1D ^{1}H NMR spectra were acquired at 298 K under quantitative conditions using a 90° single-pulse experiment (DE = 39.71 μsec, D1 = 60.00 sec, P1 = 8.75 μsec, ds = 4, ns = 32, rg = 128) on a Bruker AVANCE 600.13 MHz. Fraction 7 was defined by 21.63 % w/w of glycyrrhizin (**5**) and contained also *p*-hydroxybenzylmalonic acid (**9**) as well as ononin. Fractions 9 to 11 contained isoliquiritin (**3**) and neoisoliquiritin (**7**). Fraction 13 obtained through the extrusion of the stationary phase contained all the non-polar metabolites including liquiritigenin (**6**) as identified by ^{1}H NMR analysis.

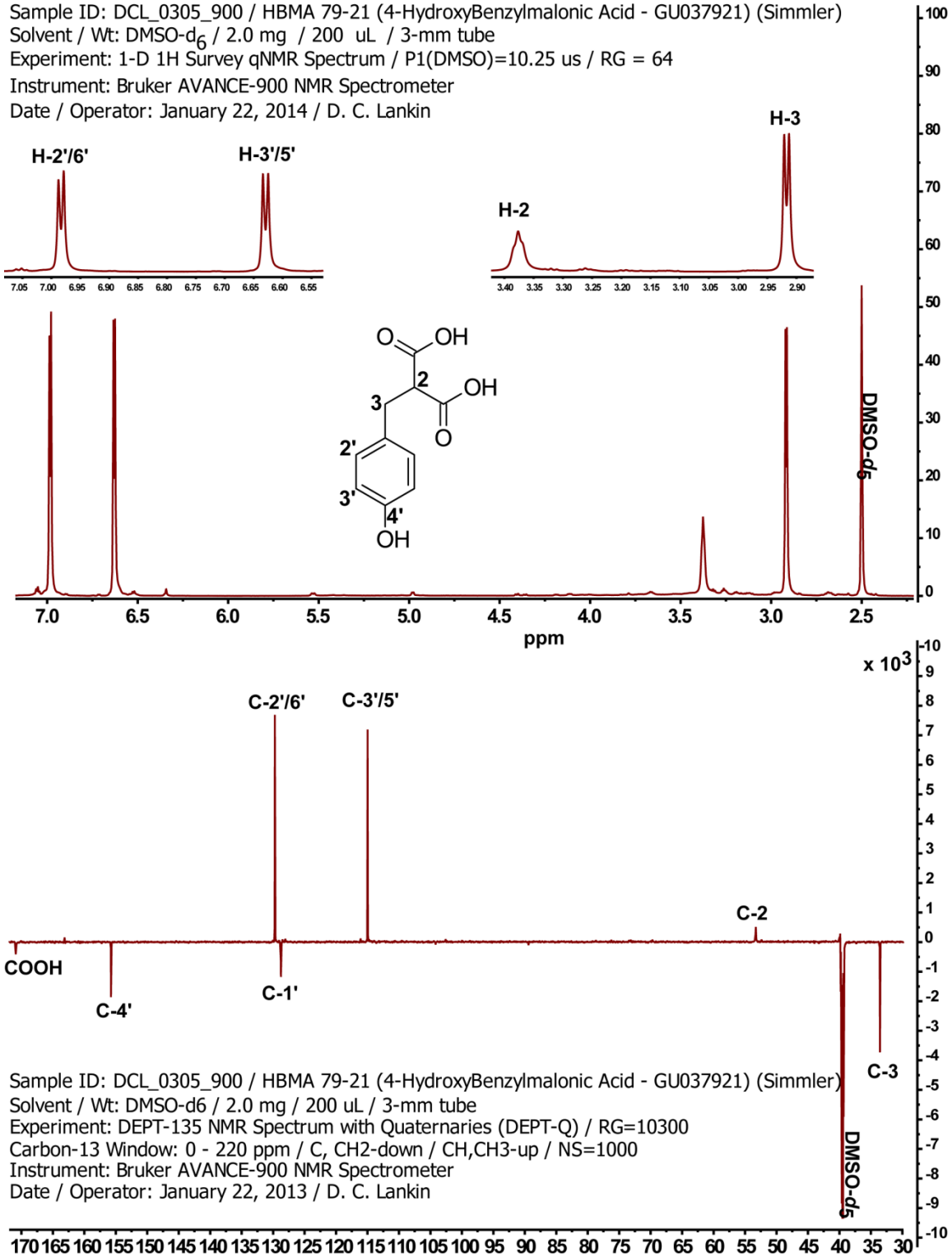
S3. Stacked UHPLC-UV profiles of CPC fractions and *G. uralensis* crude extract



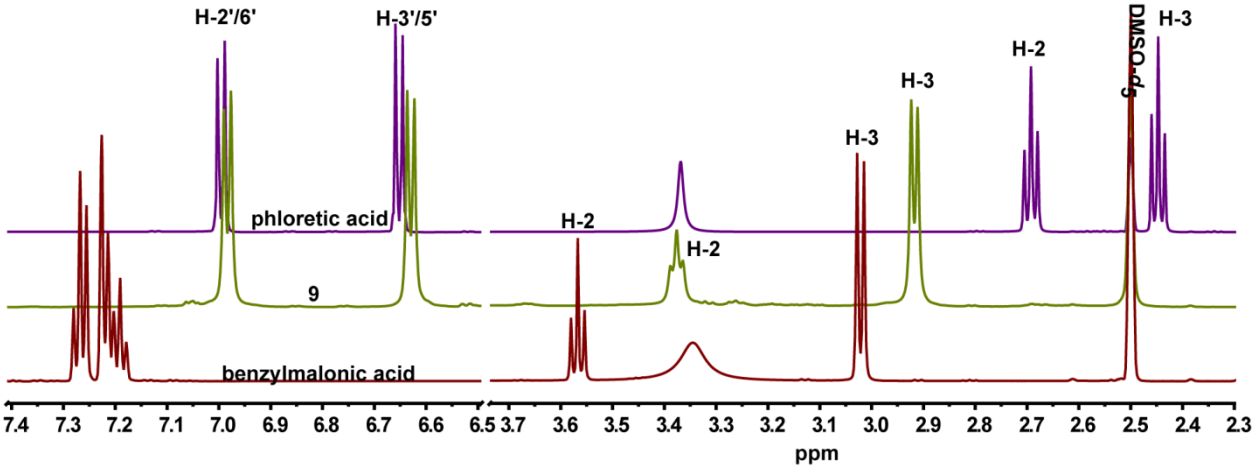
The crude extract was prepared at 10 mg/ mL, whereas fraction 1 was analyzed at 2.5 mg/mL. Both solutions were analyzed on a Kinetex C18-XB (50 x 2.1 mm, 1.7µm) and eluted with a gradient composed of (A) H_2O + 0.1% formic acid (FA) and (B) ACN + 0.1% (FA) as follows: 8% to 11% B in 2 min, and during 30 sec, to 13% B at 4 min, to 15.5% B at 8 min, to 36% at 13 min, and during 30 sec, to 80% B at 21 min, and during 1 min, back to 8% B at 23 min (flow rate: 0.8 mL/min). Fractions 1 to 3 contained almost no-UV-detectable compounds at 254 nm (except glycyrrhizin in fraction 1).

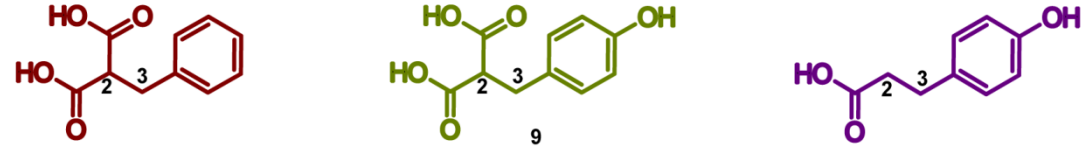
S4. ^{1}H and ^{13}C NMR spectra of *p*-hydroxybenzylmalonic acid

(900 and 225 MHz, DMSO-d_6)



S5. Comparative ^{1}H NMR data of benzylmalonic acid, *p*-hydroxybenzylmalonic acid, and phloretic acid (DMSO-d_6, 600 MHz)

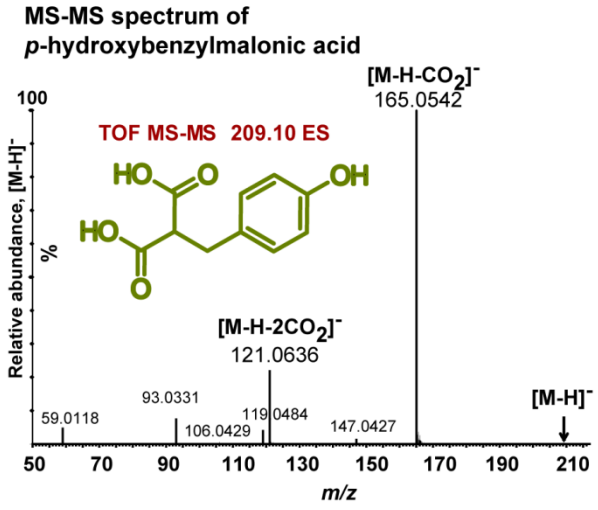


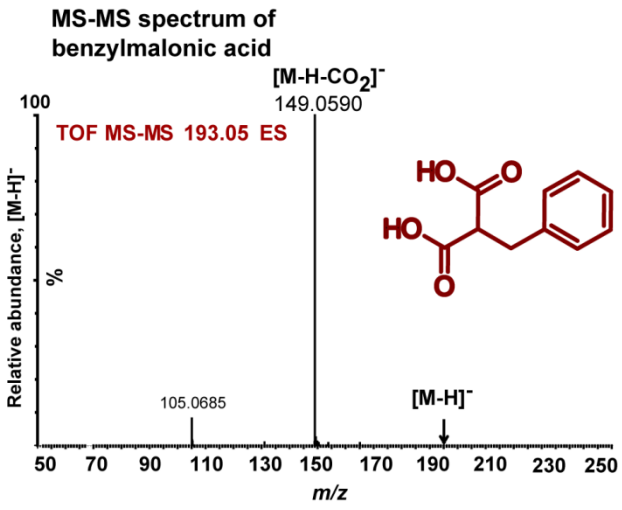


benzylmalonic acid | *p*-hydroxybenzylmalonic acid | phloretic acid

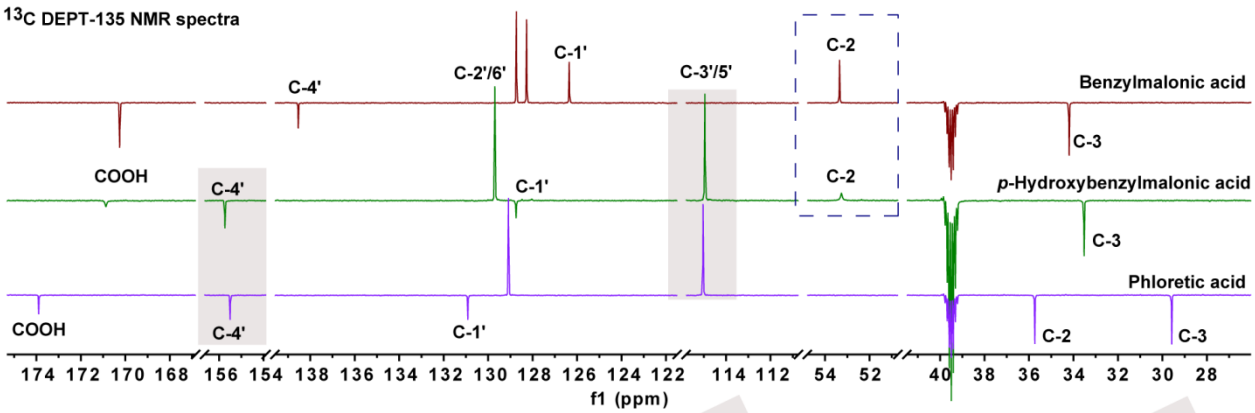
	benzylmalonic acid	*p*-hydroxybenzylmalonic acid	phloretic acid
Position	δ_H in ppm (*J* in Hz), pH 4-4.5 for all solutions		
H-2	3.567, *t*, (7.83)	3.376, *t*, (7.41)	2.692, *t*, (7.67)
H-3	3.021, *d*, (7.83)	2.917, *d*, (7.41)	2.447, *t*, (7.67)
H-2′/6′	7.249	6.983, *AA'XX'* (8.33/2.50/0.32)	6.997, *AA'XX'* (8.41/2.67/0.22)
H-3′/5′		6.630 *AA'XX'* (8.33/2.65/0.32)	6.653, *AA'XX'* (8.41/2.67/0.22)

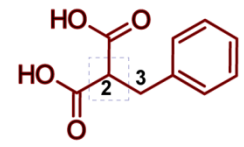
S6. Comparative MS-MS spectra of *p*-hydroxybenzylmalonic acid and benzylmalonic acid



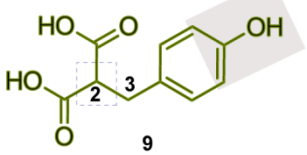


S7. Comparative 13C NMR spectra of benzylmalonic acid, *p*-hydroxybenzylmalonic acid, and phloretic acid (DMSO-d_6, 225 MHz)



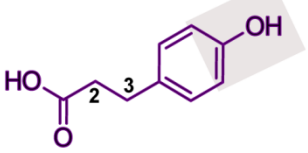


Benzylmalonic acid



9

p-hydroxybenzylmalonic acid

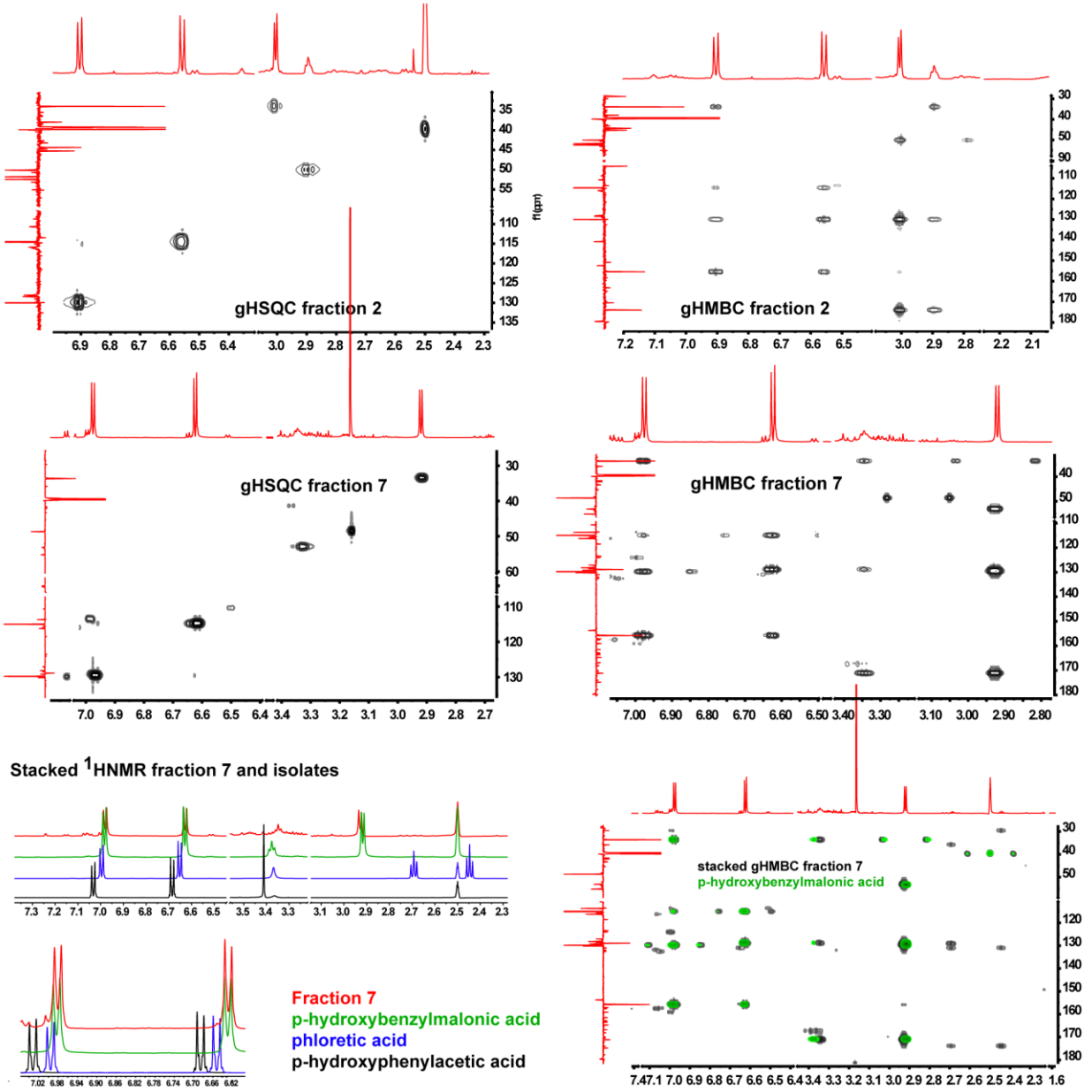


phloretic acid

	Benzylmalonic acid	*p*-Hydroxybenzylmalonic acid	Phloretic acid
Position	δ_C in ppm		
COOH	**170.18**	**170.93**	174.02
C-2	**53.30**	**53.20**	35.76
C-3	34.27	33.50	29.39
C-2′/6′	128.80	129.65	128.99
C-3′/5′	128.34	**114.96**	**115.20**
C-4'	138.67	**155.82**	**155.43**
C-1'	126.33	128.74	130.81

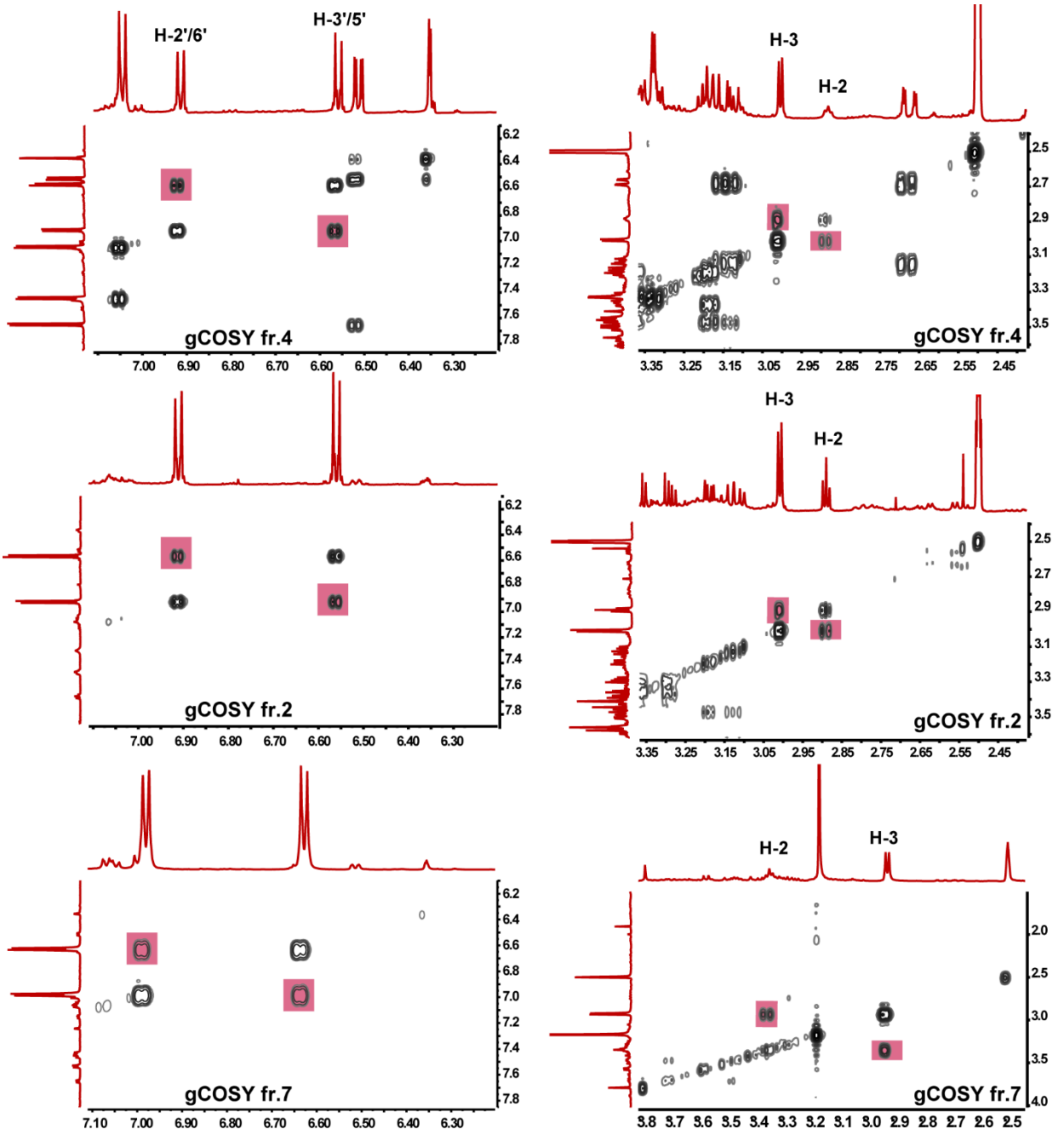
Dept-135 spectra were acquired on a 900 MHz spectrometer, using a RG value of 10300 for all phenolic acids: Phloretic acid (3.86 mg), ns 400; benzylmalonic acid (3.67 mg) ns 400; *p*-hydroxybenzylmalonic acid (~2 mg), ns 1000.

S8. HSQC/HMBC spectra of fractions 2 & 7 (600 MHz and 225 MHz, DMSO-d_6)



HSQC and HMBC spectra were acquired on a Bruker AVANCE 600.13 MHz, in DMSO-d_6 (Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All ^{13}C NMR spectra were acquired on a Bruker AVANCE 900 MHz spectrometer. Both HSQC and HMBC experiments confirmed the presence of *p*-hydroxybenzylmalonic acid (**9**) in fractions 2 to 7. Additionally, the HMBC spectrum of *p*-hydroxybenzylmalonic acid was compared with those from each fraction (example with the stacked gHMBC spectra of fraction 7 with HBMA). Even though the proton NMR spectra of **9** in fraction 2 and 7 were slightly different (see S7 and S8), the carbon spectra and HMBC correlations were identical between fractions 2 and 7.

S9. COSY spectra of fractions 2, 4 and 7 (600 MHz, DMSO-d_6)



Gradient COSY experiments were performed in DMSO-d_6, Bruker AVANCE 600.13 MHz spectrometer (ns = 4, ds =8, rg = 64, P1= 8.75 µsec). Fraction 4 contains liquiritin apioside (**2**) and *p*-hydroxybenzylmalonic acid (**9**), whereas fractions 2 and 7 concentrated mainly HBMA**.** The pH of fraction 7 was found to be 4, compared to the pH of fractions 2 and 4, which was around 6.

S10. PMS file (PERCH NMR software) of *p*-hydroxybenzylmalonic acid pH4

```
CHEMICAL SHIFTS(PPM):
PROTON    2*SPIN= 1 SPECIES=1H    POPULATION(Y)=   1.00000
 H4'-OH    / 1   100.000005  1*1*1  STAT=Y  PRED= 9.311 RANGE= 1.000 WIDTH(Y)= 2.792 RESP(Y)= 0.5587
 H3        / 1     2.917166  1*1*2  STAT=Y  PRED= 2.855 RANGE= 0.222 WIDTH(Y)= 3.976 RESP(Y)= 0.9994
 H2        / 1     3.375814  1*1*1  STAT=Y  PRED= 3.597 RANGE= 0.342 WIDTH(Y)= 5.684 RESP(Y)= 0.9946
 H1-0H     / 1     8.137648  2*1*1  STAT=Y  PRED= 12.732 RANGE= 1.693 WIDTH(Y)= 2.608 RESP(Y)= 0.0506
 H2'-6'    / 1     6.983103  1*2*1  STAT=Y  PRED= 7.041 RANGE= 0.172 WIDTH(Y)= 3.158 RESP(Y)= 1.0000
 H5'-3'    / 1     6.629428  1*2*1  STAT=Y  PRED= 6.792 RANGE= 0.142 WIDTH(Y)= 3.005 RESP(Y)= 0.9812

COUPLING CONSTANTS(HZ):
 J17_19      7.4096   J H3     H2       STAT=Y  PRED= 9.380 RANGE= 4.600
 J25_22      2.5030   J H2'-6' H2'-6'   STAT=Y  PRED= 2.330 RANGE= 0.700
 J22_23      8.3318   J H2'-6' H3'-5'   STAT=Y  PRED= 8.410 RANGE= 0.410
 J22_24      0.3247   J H3'-5' H2'-6'   STAT=Y  PRED= 0.430 RANGE= 0.320
 J24_23      2.6487   J H3'-5' H3'-5'   STAT=Y  PRED= 2.830 RANGE= 0.700

CONTROL PARAMETERS:
       Solvent =  DMSO-D6(def. 99% enriched)
         1.000 =  Concentration (vol%, def=1.0%)
    0.00100000 =  Minimum line-intensity
    0.00100000 =  Diagonalization criterium (not in use)
  600.12700000 =  FIELD(1H,MHz), used to transform shifts to ppms
   21.02058921 =  Left frequency (ppm)
   -1.02059033 =  Right frequency (ppm)
         0.000 =  Acquisition time (s, for QMTLS)
         2.439 =  Line-width (for modes D, P & T, 0=use defaults)
   0.050459149 =  Data-point resolution (Hz)
        11.728 =  GAUSSIAN (%, 0=use default from INF)
        -1.107 =  Dispersion contribution (%, 0=use default from INF)
    0.00000000 =  Decoupling frequency (for DORES)

CONSTRAINTS (in equations X0 = 1.0)...use no empty lines
 IGNORE(HZ):  1549.454 to  1456.297

CARBON/DEC  2*SPIN=1  SPECIES=13C  POPULATION= 1.0000
 C2'-6'  / 1      129.887     2*1  STAT=DS PRED= 129.890 RANGE= 3.421 WIDTH= 6.739 RESP= 1.1266
 C3'-5'  / 1      115.039     2*1  STAT=DS PRED= 115.040 RANGE= 2.272 WIDTH= 6.739 RESP= 1.1044
 C4'     / 1      155.776     1*1  STAT=DS PRED= 155.780 RANGE= 1.561 WIDTH= 6.739 RESP= 0.3248
 C1'     / 1      129.457     1*1  STAT=DS PRED= 129.460 RANGE= 5.357 WIDTH= 6.739 RESP= 0.3346
 C3      / 1       33.080     1*1  STAT=DS PRED= 33.080 RANGE= 5.788 WIDTH= 6.739 RESP= 1.0614
 C2      / 1       53.977     1*1  STAT=DS PRED= 53.980 RANGE= 5.052 WIDTH= 6.739 RESP= 1.0719
 C1      / 1      169.033     2*1  STAT=DS PRED= 169.030 RANGE= 8.052 WIDTH= 7.239 RESP= 0.8138

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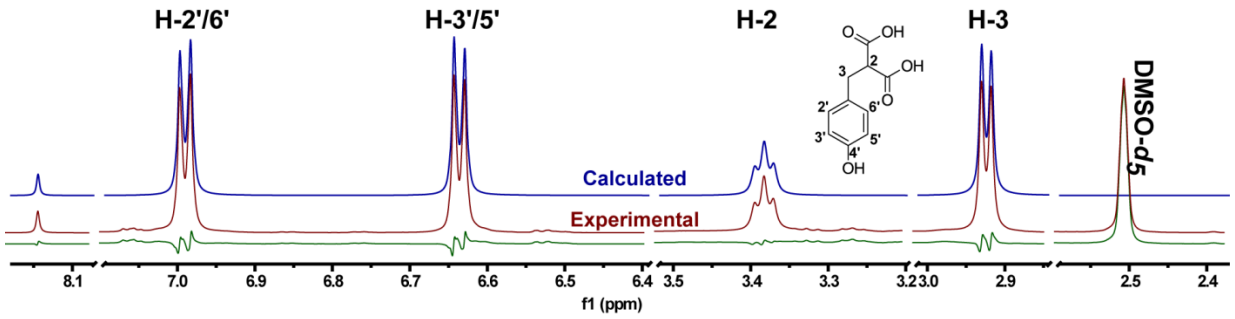
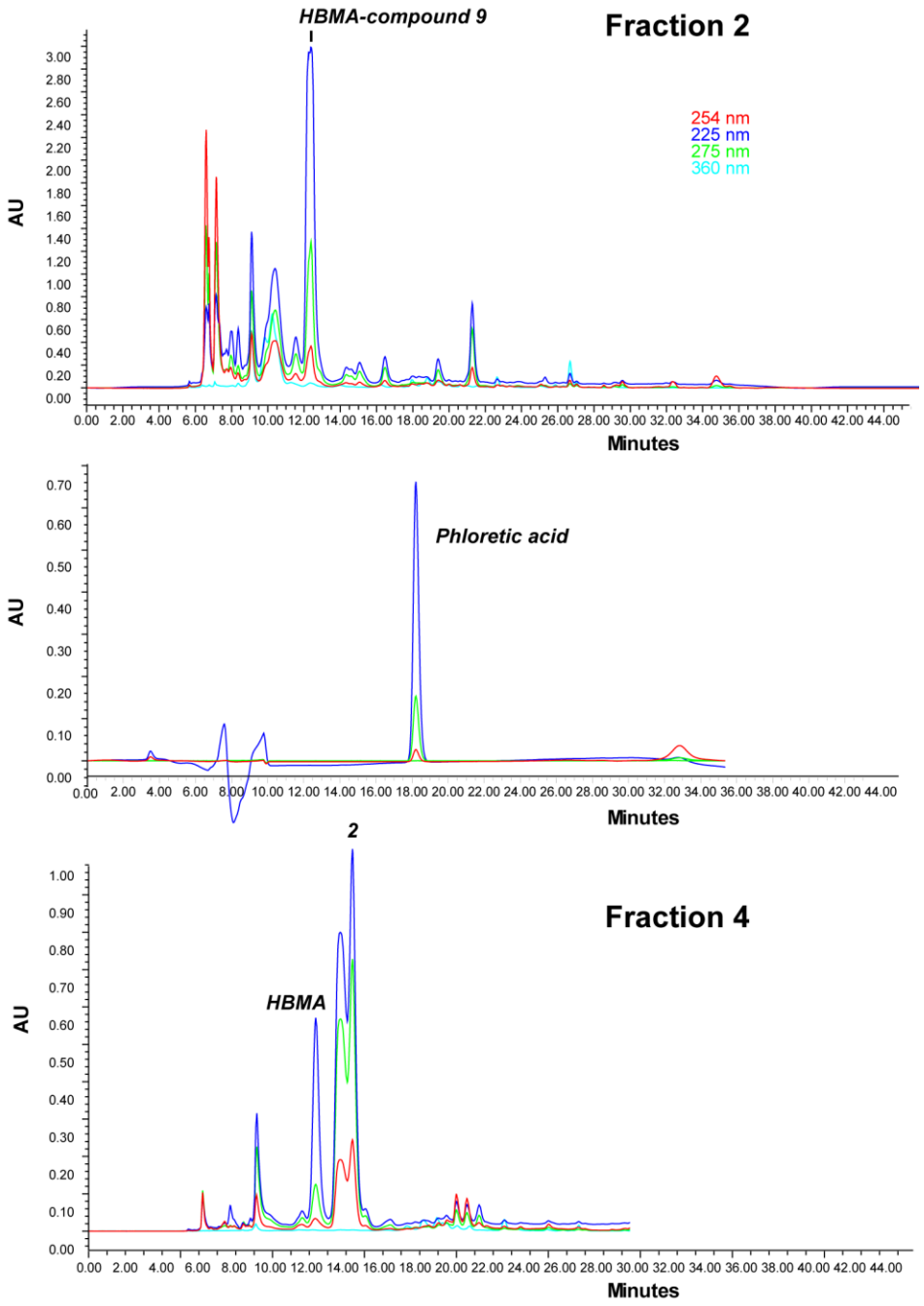
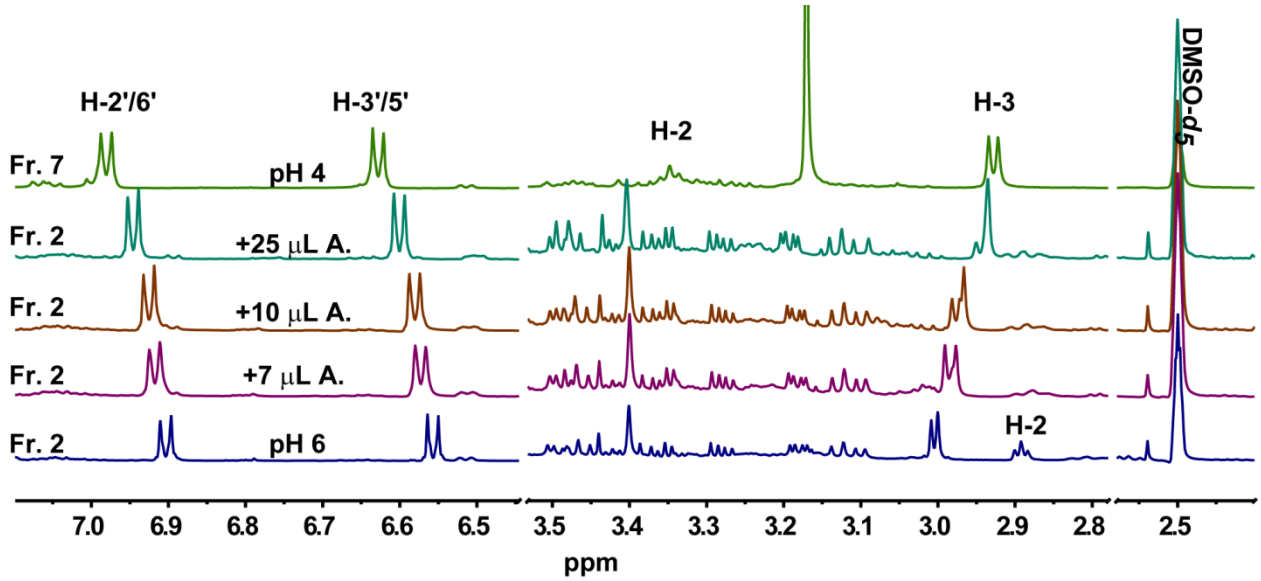


S11. Semi-preparative chromatograms for the isolation of *p*-hydroxybenzylmalonic acid.



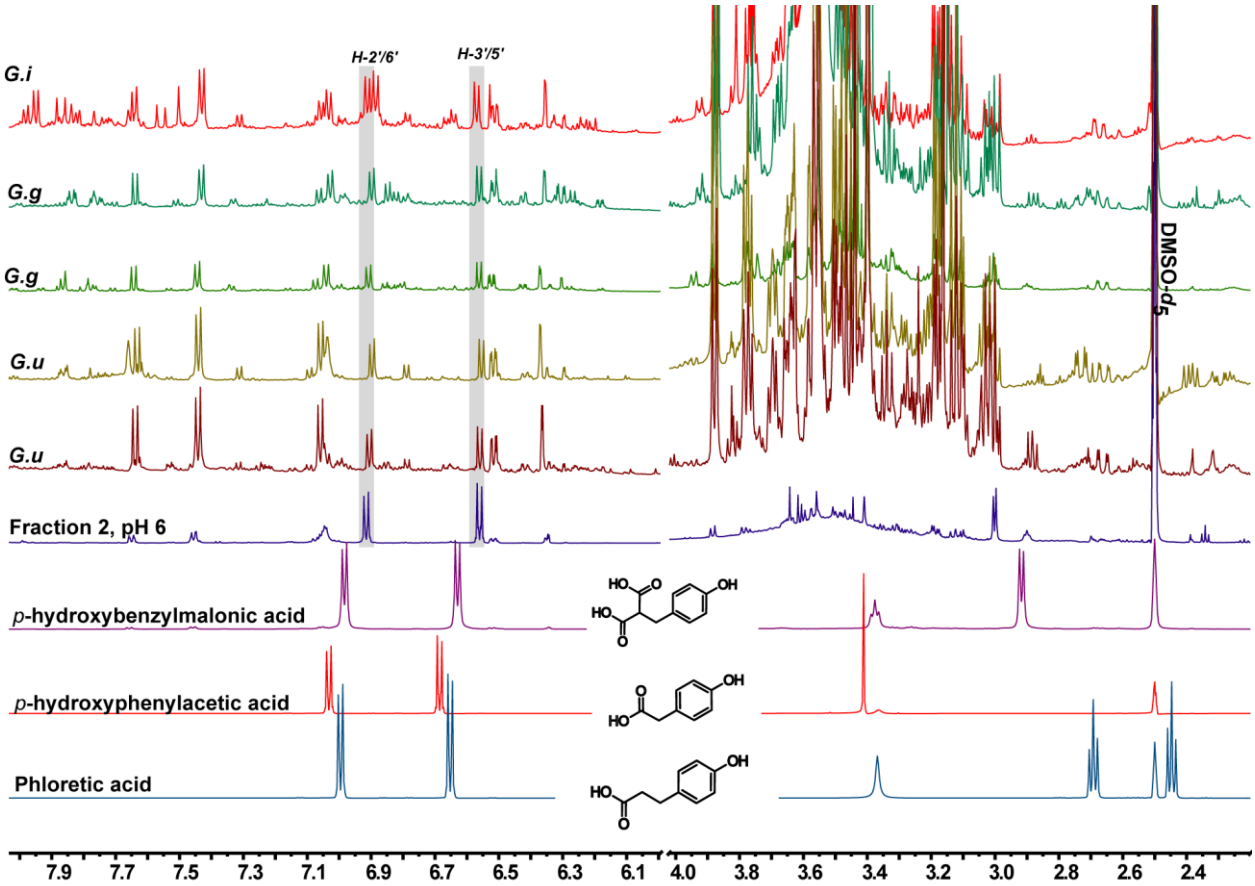
Both fractions 2 and 4 were prepared at 100 mg/mL in 50% MeOH HPLC-grade, and 50 µL were injected. The column YMC-Pack-ODS-AQ column (250 ×10 mm, 5 µM) was eluted with a gradient composed of (A) H_2O + 0.1% formic acid (FA) and (B) ACN + 0.1% (FA) as follows: from 25% B during 2 min to 40% B in 20 min and during 5 min back to 25% B in 5 min (flow rate: 2 mL/min). The retention times (t_R) were 13.15 min for *p*-hydroxybenzylmalonic acid (**9**), and 15.00 min for liquiritin apioside (**2**). Phloretic acid (0.5 mg/mL) was injected under the same LC conditions and used as a control.

S12. Exemplifying the pH influence on the chemical shifts of *p*-hydroxybenzylmalonic acid in fraction 2: comparative ^{1}H NMR profiles after addition of deuterated acetic acid.



For comparative purposes, the pH of fraction 2 and 7 was roughly estimated with pH strips (Whatman®, panpeha™), after diluting 100 µL of the NMR solution into 300 µL of deionized water. In order to exemplify the influence of the pH on the chemical shifts of protons from p-hydroxybenzylmalonic acid, different ^{1}H NMR spectra were recorded after addition of deuterated acetic acid (acetic acid d_4, CAS # 1186-52-3) into fraction 2. The results obtained clearly displayed a gradual down-field shift of the signals from the aromatic protons ($\Delta\delta = 0.07$ ppm), and from proton H-2 (closest to the carboxylic acids $\Delta\delta = 0.50$ ppm), whereas the signals from the benzyl protons (H-3) were shifted up-field ($\Delta\delta = 0.09$ ppm).

S13. Detection and quantification of *p*-hydroxybenzylmalonic acid in different *Glycyrrhiza* extracts (EtOH 95% extracts)



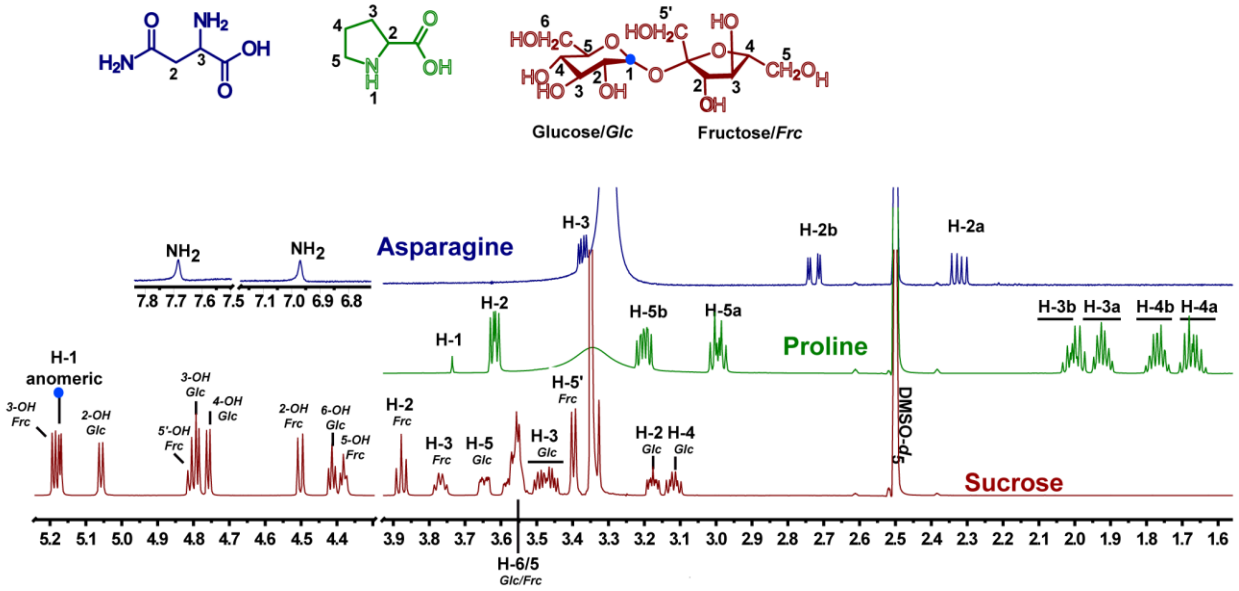
The 1D ^{1}H NMR spectra were acquired at 298 K under quantitative conditions using a 90° single-pulse experiment (DE = 39.71 μsec, D1 = 60.00 sec, P1 = 8.75 μsec, ds = 4, ns = 18, rg = 256) on a Bruker AVANCE 600.13 MHz. All samples were prepared in 300 µL DMSO-d_6. *Glycyrrhiza glabra* extracts (*G.g*) *Glycyrrhiza inflata* (*G.i*) and *Glycyrrhiza uralensis* (*G.u.*) were exactly weighted at 8 mg. Fraction 2 concentrated *p*-hydroxybenzylmalonic acid (24.56% w/w, 31.8 mM), and had a pH of 6 equivalent to the one measured for the different crude extracts. The pH of isolated *p*-hydroxybenzylmalonic acid (84 mM) was estimated at ~ 4, the pH of phloretic acid (192 mM) and *p*-hydroxylphenylacetic acid (189 mM) was found to be 4.5.

The protons signals used for the quantification of *p*-hydroxybenzylmalonic acid in all extracts were those from the aromatic protons H-3'/5'. The quantification was performed using an internal calibrant IC (3, 5-dinitrobenzoic acid, TraceCERT, Purity 99.54% w/w) at 2.12 mM.

	G.u-1	*G.u-2*	*G.g-1*	*G.g-2*	*G.i*
Weight Extract (mg)	7.77	7.96	8.21	7.90	7.58
Abs. Int. IC-1H	824.2	864.4	886.4	882.9	857.8
Abs. Int H-3'/5'-1H	682.8	846.7	1024.0	15117	1209.9
Concentration (% w/w)	**1.42**	**1.64**	**1.87**	**2.67**	**2.47**

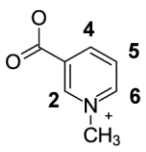
Abs. Int = Absolute Integral

S14. ^{1}H NMR profiles of identified 1°Ms (DMSO-d_6, 600 MHz)



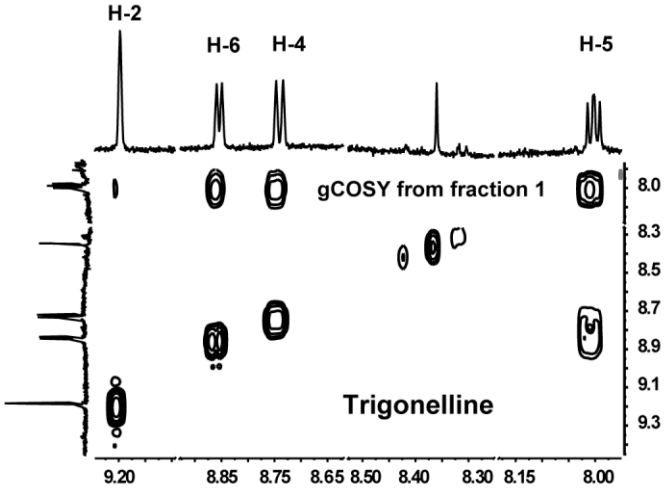
Proton signals used for quantitation in the qHNMR spectrum of *G. uralensis* crude extract

Position	Sucrose	Proline	Asparagine	Trigonelline
	δ_H in ppm (*J* in Hz)			
H-1 anomeric	5.174, *d* (3.7)			
H-3a H-3b		1.992,*dddd*(-12.74/7.50/6.00/5.55) 2.014, *ddddd*(-12.74/8.87/7.92/7.28/7)		
H-2b			2.724, *dd* (16.56, 4.04)	
H-2 H-4 H-6				9.923, *s* 8.768, *d* (7.91) 8.891, *d* (5.90)

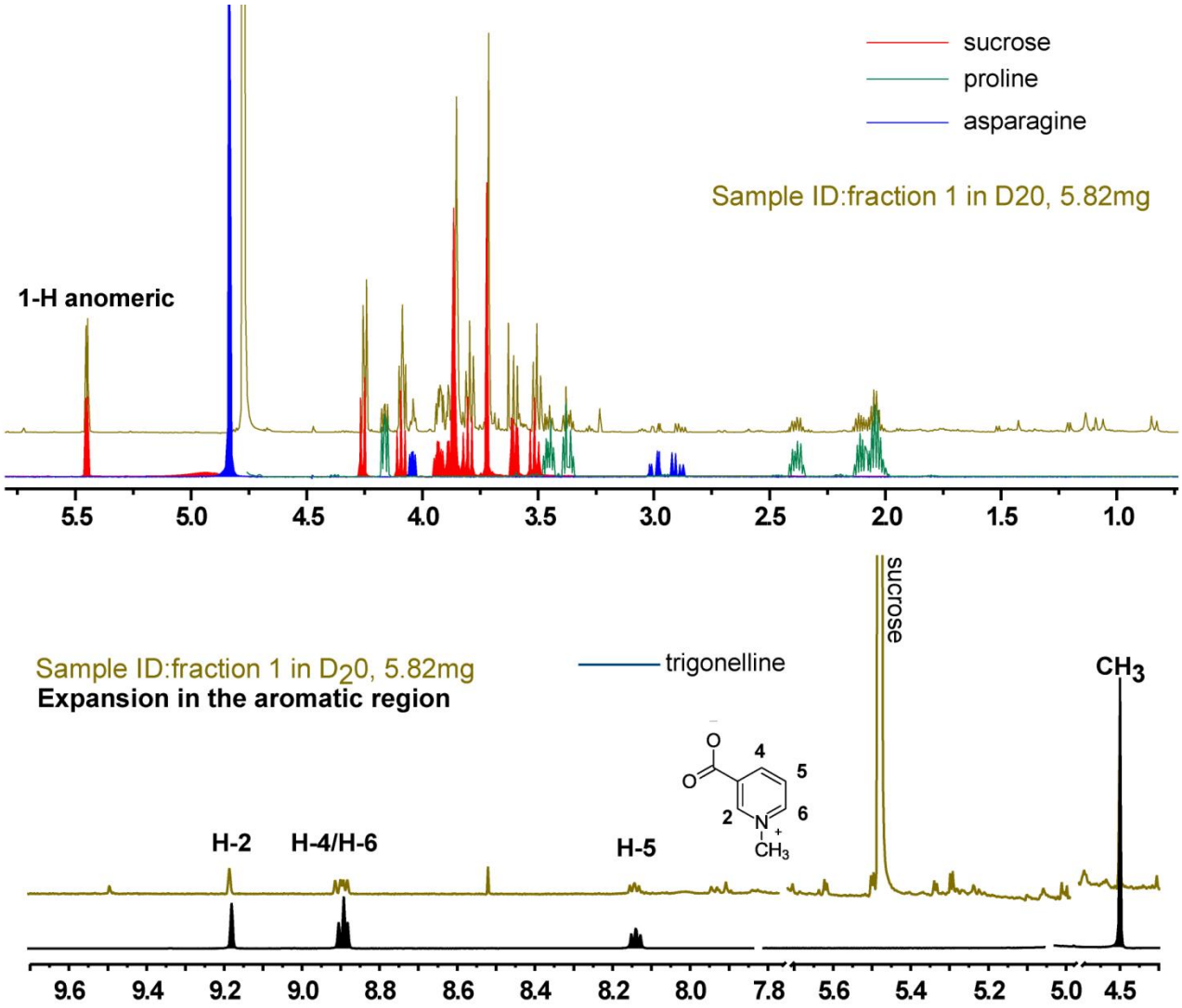


Trigonelline

Position	δ_H *in ppm (J in Hz)*	δ_C
H-2	9.923 s	146.18
CH3	4.346, s	48.15
H-4	8.768 *d* (7.91)	144.21
H-5	8.032,*dd*(7.91/5.90)	126.64
H-6	8.891 *d* (5.90)	145.06



S15. Stacked ^{1}H NMR spectra of fraction 1 with identified 1°Ms (D_2O, 600 MHz)



The ^{1}H NMR profile of fraction 1 was recorded at 600 MHz in D_2O (Bruker AVANCE 600.13 MHz), and compared with the ^{1}H NMR profiles of each metabolite obtained from the Human Metabolome Database (http://www.hmdb.ca). Comparative results obtained in DMSO-d_6 and D_2O clearly confirmed the identity of each major primary metabolite in fraction 1 (supporting information S13 and S14).