Supplementary Data Sheet 1

A combined LC-MS metabolomics- and 16S rRNA sequencing platform to assess interactions between herbal medicinal products and human gut bacteria *in vitro*: A pilot study on willow bark extract

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Supplementary Data

1 Table S6: Details on identification of compounds significantly changing over incubation time

#	RT (min)	measured m/z [ion species]	indicative MS/MS fragments (relative intensity)	Monoisotopic mass	Molecular formula	Δ (ppm)	Identification (+InchI key for unambiguously identified compounds)	Literature/Database
1	1.33	377.0862 $[M+Cl]^-$	377 (60) 341 (100) 215 (20) 179 (25) 161 (5) 139 (5) 119 (10) 89 (20)	342.1166	$C_{12}H_{22}O_{11}$	4.14	saccharose[b]	http://www.hmdb.ca/
2	1.43	133.013 $[M-H]^-$	133 (40) 115 (100) 97 (10) 89 (10) 73 (10) 71 (45)	134.0203	$C_4H_6O_5$	-1.35	malic acid[b]	https://metlin.scripps.edu/ [b]
3	1.63	191.019 $[M-H]^-$	191 (30) 173 (5) 129 (10) 111 (100) 87 (35) 85	192.0263	$C_6H_8O_7$	1.94	citric acid[a] KRKNYBCHXYNGOX-UHFFFAOYSA-N	

			(25)					
4	1.96	117.018 [M-H]$^-$	117 (20) 99 (10) 73 (100)	118.0252	$C_4H_6O_4$	-2.42	succinic acid[a] KDYFGRWQOYBRFD-UHFFFAOYSA-N	
5	2.65	157.0492 [M-H]$^-$	157 (100) 139 (2) 113 (5) 95 (100)	158.0565	$C_7H_{10}O_4$	-2.00	isopropylmaleate[b]	http://www.hmdb.ca/
6	4.93	153.0179 [M-H]$^-$	153 (35) 109 (100)	154.0252	$C_7H_6O_4$	-2.32	protocatechuic acid[a,+] YQUVCSBJEUQKSH-UHFFFAOYSA-N	(1)$^+$
7	5.17	305.067 [M-H]$^-$	305 (50) 261 (10) 221 (10) 219 (15) 179 (20) 175 (100) 167 (15) 165 (20) 147 (35) 139 (15) 137 (15) 125 (60)	306.0742	$C_{15}H_{14}O_7$	5.12	(epi)gallocatechin[b,#]	https://metlin.scripps.edu/ [b]; (2)[#]

8	5.63	109.028 [M-H]$^-$	109 (100) 91 (3) 81 (3)	110.0352	$C_6H_6O_2$	-2.99	catechol[b]	https://metlin.scripps.edu/ [b]
9	6.08	329.0882 [M-H]-	175 (15) 169 (15) 167 (100) 152 (10) 123 (15) 108 (10)	330.0957	$C_{14}H_{18}O9$	5.26	vanillic acid hexoside[b,#]	(3)[b], (4)[#]
10	6.72	321.075 [M+^{35}Cl]$^-$ (35); 323.072 [M+^{37}Cl]$^-$ (12); 331.104 [M-H+HCOOH]$^-$ (100)	123 (100)	286.1055	$C_{13}H_{18}O_7$	3.78	salicin[a, #] NGFMICBWJRZIBI-UJPOAAIJSA-N	(5)[#], (6)[#]
11	6.98	226.0539 [M-H]$^-$	199 (7) 181 (100) 159(7) 153 (20) 139 (5) 131 (5)	227.0611	$C_{10}H_{13}O_3NS$	1.94	saligenin-cysteine adduct[b]	

			120 (85)					
12	7.06	123.0439 $[M-H]^-$	123 (30) 121 (100) 93 (30)	124.0512	$C_7H_8O_2$	-1.51	saligenin[a,#] CQRYARSYNCAZFO-UHFFFAOYSA-N	(5)[#]
13	7.68	223.0604 [M-H]-, 259.0375 $[M+^{35}Cl]$-	223 (100) 179 (50) 138 (10)	224.0677	$C_{11}H_{12}O_5$	1.26	5-(3´,4´,5´-trihydroxyphenyl)-y-valerolactone[b,+]	(7)[+]
14	7.70	153.0179 $[M-H]^-$	153 (60) 109 (100) 108 (25)	154.0252	$C_7H_6O_4$	-2.25	gentisic acid[a] WXTMDXOMEHJXQO-UHFFFAOYSA-N	
15	7.72	137.023 $[M-H]^-$	137 (20) 93 (100)	138.0301	$C_7H_6O_3$	-3.51	4-hydroxybenzoic acid[a,+] FJKROLUGYXJWQN-UHFFFAOYSA-N	
16	8.88	225.076 $[M-H]^-$	225 (30) 207 (15) 181 (10) 163 (55) 123 (100) 101 (80)	226.0834	$C_{11}H_{14}O_5$	1.6	4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid[b,+]	(8)[b,+]

17	9.13	181.049 [M-H]$^-$	181 (80) 153 (85) 137 (100) 121 (20) 109 (20) 93 (5) 61 (7) 59 (45)	182.0570	$C_9H_{10}O_4$	2.07	dihydrocaffeic acid[a,+] DZAUWHJDUNRCTF-UHFFFAOYSA-N	(8)$^+$, (9)$^+$
18	9.23	577.1357 [M-H]$^-$	577 (7) 451 (15) 425 (25) 407 (85) 289 (100) 245 (17) 179 (5) 161 (25) 151 (5) 137 (10) 125 (85)	578.143	$C_{30}H_{26}O_{12}$	2.82	epi)catechin-(epi)catechin[b,#]	https://metlin.scripps.edu/[b], (2)[#]
19	9.62	289.0720[M-H]$^-$	289 (100) 245 (35) 221 (10) 205 (20) 203 (25) 179 (17) 175 (15) 151 (15) 137 (15) 125 (27)	290.0793	$C_{15}H_{14}O_6$	4.59	catechin[a,#] PFTAWBLQPZVEMU-DZGCQCFKSA-N	(2)[#]

20	9.79	223.0604 $[M-H]^-$	223 (70) 205 (5) 195 (10) 179 (30) 161 (10) 123 (100)	224.0677	$C_{11}H_{12}O_5$	1.44	4-oxo-5-(dihydroxyphenylvaleric)acid[b,+]	(8)[b,+]
21	9.87	153.0179 $[M-H]^-$	153 (30) 109 (100)	154.0251	$C_7H_6O_4$	-2.37	dihydroxybenzoic acid[b]	https://metlin.scripps.edu/[b]
22	10.52	131.0698 $[M-H]^-$	131 (90) 119 (35) 85 (100) 62 (5)	132.0771	$C_6H_{12}O_3$	-3.74	hydroxy(iso)caproic acid [b, +]	http://www.hmdb.ca[b]; (10)[+]
23	10.54	363.085 $[M+^{35}Cl]^-$, 365.083 $[M+^{37}Cl]^-$, 373.1143 $[M-H+HCOOH]^-$	175 (30) 123 (100) 59 (25)	328.1161	$C_{15}H_{20}O_8$	3.82	acetylsalicin[b,#]	(5)[b,#], (11)[#]
24	10.88	131.0698 $[M-H]^-$	131 (90) 119 (20) 85 (100)	132.0771	$C_6H_{12}O_3$	-3.68	hydroxy(iso)caproic acid[b, +]	http://www.hmdb.ca[b]; (10)[+]
25	10.92	353.0882	191 (100)	354.0955	$C_{16}H_{18}O_9$	4.48	chlorogenic acid[a,#]	(12)[#]

		$[M-H]^-$					CWVRJTMFETXNAD-JUHZACGLSA-N	
26	10.95	865.1994 $[M-H]^-$	739 (3) 713 (5) 577 (15) 543 (7) 525 (5) 451 (15) 449 (15) 425 (20) 407 (55) 289 (50) 287 (45) 261 (7) 245 (15) 243 (25) 161 (30) 125 (100)	866.2067	$C_{45}H_{38}O_{18}$	2.47	(epi)catechin-(epi)catechin-(epi)catechin[b,#]	(13), (14)[b]; (2), (12)[#]
27	11.38	407.1120 $[M-H+^{35}Cl]^-$; 417.1408 $[M-H+HCOOH]^-$	209 (100) 194 (10) 175 (50) 145 (10)	372.1421	$C_{17}H_{24}O_9$	4.44	syringin[b,#]	(15)[b], (6)[#]
28	12.34	163.0751 $[M-H-CO_2]^-$, 207.0654 $[M-H]^-$,	207 (100) 163 (75) 122 (20)	208.0726	$C_{11}H_{12}O_4$	0.07	5-(dihydroxyphenyl)-y-valerolactone[b,+]	(8), (1)[b,+]

		243.0425 $[M+^{35}Cl]^-$, 245.04396 $[M+^{37}Cl]^-$						
29	12.40	447.1514 [M-H+HCOOH]$^-$	401 (100) 293 (3) 269 (45) 233 (5) 161 (30) 143 (3) 113 (10) 101 (20) 89 (5) 71 (15)	402.1539	$C_{18}H_{26}O_{10}$	4.50	benzyl-hexoside-pentoside[b]	(16)[b]
30	12.42	319.0462 [M-H]$^-$	319 (5) 301 (15) 257 (15) 215(15) 193 (100) 179 (20) 175 (25) 167 (10) 151 (10) 125 (40)	320.0534	$C_{15}H_{12}O_8$	4.16	ampelopsin[b,#]	(17)[b], (6)[#]
31	12.86	383.0083 [M-H]$^-$	383 (20) 303 (100) 285 (80)	384.01626	$C_{15}H_{12}O_{10}S$	1.56	diyhdroquercetin sulfate[b]	(18)[b]

			275 (10) 259 (5) 241 (3) 217 (3) 177 (25) 153 (3) 125 (40)					
32	12.89	447.1513 [M-H+HCOOH]$^-$	401 (50) 269 (100) 175 (5) 161 (35) 113 (15) 101 (20) 89 (3) 85 (7) 71 (17)	402.1541	$C_{18}H_{26}O_{10}$	4.95	benzyl-hexoside-pentoside[b]	(16)[b]
33	14.39	363.0854 [M-H+^{35}Cl]$^-$; 365.0825 [M-H+^{37}Cl]$^-$; 373.1141 [M-H+HCOOH]$^-$	175 (3) 123 (100) 59 (33)	328.1159	$C_{15}H_{20}O_8$	3.42	acetylsalicin[b,#]	(5)[b,#]
34	14.65	165.0543 [M-H]$^-$, 121.0643 [M-H-CO_2]$^-$, 119.0486	136 (5) 121 (100) 119 (45)	166.0616	$C_9H_{10}O_3$	-1.88	3-(3-hydroxyphenyl)propionic acid[a, +] QVWAEZJXDYOKEH-UHFFFAOYSA-N	(8)[a,+]

		[M-H-HCOOH]$^-$						
35	16.61	209.081 [M-H]$^-$	209 (100) 191 (35) 165 (25) 151 (15) 147 (10) 123 (20)	210.0882	$C_{11}H_{14}O_4$	0.45	4-hydroxy-5-(hydroxyphenyl))valeric acid [b,+]	(8)[b,+]
36	17.78	303.0514 [M-H]$^-$	303 (15) 285 (85) 275 (15) 259 (15) 217 (15) 179 (10) 177 (25) 175 (30) 125 (100)	304.0585	$C_{15}H_{12}O_7$	4.62	dihydroquercetin[a, +] CXQWRCVTCMQVQX-LSDHHAIUSA-N	(6)[#]
37	17.89	433.1143 [M-H]$^-$	433 (15) 271 (100) 313 (5) 151 (25) 119 (5)	434.1215	$C_{21}H_{22}O_{10}$	3.64	naringenin hexoside[b,#]	(5)[#]
38	17.99	137.0229 [M-H]$^-$	93 (100)	138.0302	$C_7H_6O_3$	-3.29	salicylic acid[a] YGSDEFSMJLZEOE-UHFFFAOYSA-N	(5)[#]

39	18.34	473.1665 [M-H+HCOOH]$^-$	427 (15) 293 (40) 233 (7) 161 (85) 149 (30) 143 (10) 131 (30) 125 (40) 113 (35) 101 (45) 89 (100) 85 (15) 73 (10) 71 (90) 59 (45)	428.1683	$C_{20}H_{28}O_{10}$	3.08	rosarin[b]	(19)[b]
40	18.49	459.107 [M+^{35}Cl]$^-$; 461.1031 [M+^{37}Cl]$^-$; **469.1353** [M-H+HCOOH]$^-$; 486.1256	423 (30) 317 (5) 161 (40) 155 (100) 137 (35) 123 (80) 111 (75) 101 (10) 83 (30) 71 (15)	424.1371	$C_{20}H_{24}O_{10}$	1.79	salicortin [b,#]	(5)[b,#]
41	18.84	433.1143	271 (100) 313 (5)	434.1215	$C_{21}H_{22}O_{10}$	3.09	naringenin-hexoside ((-)-	(5)[b,#]

		$[M-H]^-$	151 (25)				naringenin-5-glucoside?) [b,#]	
42	19.65	209.0809 $[M-H]^-$, 245.058 [M+Cl]-, 293.0341	209 (100) 191 (10) 165 (3) 147 (3) 135 (7) 121 (3) 109 (3)	210.0882	$C_{11}H_{14}O_4$	0.31	5-(dihydroxyphenyl)valeric acid[b,+]	(8)[b,+]
43	19.66	473.1664 [M-H+HCOOH]$^-$	427 (20) 293 (70) 233 (15) 191 (20) 161 (65) 149 (40) 143 (10) 131 (25) 125 (40) 113 (30) 101 (40) 89 (100) 71 (85) 59 (45)	428.1690	$C_{20}H_{28}O_{10}$	2.94	cinnamyl-(6`-O-xylopyranosyl)-O-glucopyranoside[b]	(19)[b]
44	20.70	463.0884 $[M-H]^-$	463 (55) 301 (70) 300 (100) 271 (5)	464.0957	$C_{21}H_{20}O_{12}$	4.27	hyperoside[a,#] OVSQVDMCBVZWGM-DTGCRPNFSA-N	(20)[#]

			255 (3) 179 (3) 151 (3)					
45	20.95	487.1827 [M-H+HCOOH]$^-$	441 (15) 340 (3) 307 (50) 247 (30) 205 (5) 175 (20) 163 (55) 145 (15) 125 (75) 119 (45) 113 (7) 103 (70) 101 (90) 97 (15) 89 (60) 85 (20) 81 (10) 73 (40) 71 (57) 59 (100)	442.1840	$C_{21}H_{30}O_{10}$	2.80	cinnamylhexoside-deoxyhexoside [b]	https://metlin.scripps.edu/[b]
46	21.52	489.1983 [M-H+HCOOH]$^-$	443 (95) 297 (100) 161 (25) 143 (3) 113 (10) 101 (30) 89 (5) 83	444.2001	$C_{21}H_{32}O_{10}$	3.42	dihydrocinnamylhexoside-deoxyhexoside[b]	(21)[b]

			(7) 81 (10) 73 (15) 71 (20)					
47	21.90	433.1149 $[M-H]^-$	271 (100) 175 (5) 151 (15)119 (5)	434.1215	$C_{21}H_{22}O_{10}$	1.58	naringenin-7-glucoside[a,#] DLIKSSGEMUFQOK-SFTVRKLSSA-N	(5)[#]
48	22.34	441.0963 $[M+^{35}Cl]^-$; 451.125 [M-H+HCOOH]$^-$	283 (60); 163 (7); 121 (100)	406.1268	$C_{20}H_{22}O_9$	3.28	salireposide [b,#]	(5)[b,#]
49	24.15	149.0593 $[M-H]^-$	149 (100) 121 (50) 105 (5) 93 (10)	150.067	$C_9H_{10}O_2$	-0.35	phenylpropionic acid[b,+]	http://www.hmdb.ca[b]; (1),(22), (23)[+]
50	24.79	501.1169 $[M+^{35}Cl]^-$; 502.1203; 503.1141 $[M+^{37}Cl]^-$; 511.1457 [M-H+HCOOH]$^-$; 528.1355	465 (50) 423 (5) 405 (20) 299 (2) 271 (2) 155 (100) 137 (70) 123 (85) 111 (75) 109 (25)	466.1475	$C_{22}H_{26}O_{11}$	1.86	acetylsalicortin [b,#]	(5)[b,#]

			83 (60) 59 (40					
51	24.97	423.1659 $[M-H]^-$	163 (20) 145 (100)	424.173	$C_{21}H_{28}O_9$	4.23	grandidentatin/isograndidentatin [b,#]	(24)[b], (11)[#]
52	25.40	433.1138 [M-H]-	433 (5) 313 (5) 271 (100) 151 (30) 119 (5)	434.1211	$C_{21}H_{22}O_{10}$	3.99	isosalipurposide[b,#]	(5)[b,#]
53	25.46	423.1666 $[M-H]^-$	163 (20); 145 (100)	424.174	$C_{21}H_{28}O_9$	4.02	grandidentatin/isograndidentatin [b,#]	(24)[b], (11)[#]
54	25.87	193.0859 $[M-H]^-$	193 (100) 175 (25) 149 (10)	194.093	$C_{11}H_{14}O_3$	0.00	hydroxyphenylvaleric acid[b,+]	(8)[b,+]
55	27.51	435.1299 $[M-H+HCOOH]^-$	389 (100) 283 (15) 123 (5)	390.132	$C_{20}H_{24}O_9$	3.07	benzoylsalicin [b,#]	(5)[b,#]

56	30.77	271.061 [M-H]$^{-}$	271 (100) 177 (10) 151 (60) 119 (20) 107 (7) 93 (7)	272.0683	$C_{15}H_{12}O_5$	4.58	naringenin[a,#] FTVWIRXFELQLPI-UHFFFAOYSA-N	(25)[#]
57	32.46	649.1785 [M-H+HCOOH]$^{-}$	603 (10) 543 (5) 465 (20) 447 (10) 405 (10) 341 (3) 155 (40) 137 (45) 123 (47) 111 (100) 109 (45) 93 (10) 83 (35) 59 (20)	604.1893	$C_{29}H_{32}O_{14}$	2.45	HCH-acetylsalicortin[b,#]	(26)[b], (27)[#]
58	35.54	563.1329 [M+^{35}Cl]-; 565.1292 M+^{37}Cl]- 573.1616 [M-H+HCOOH	527 (7) 405 (15) 155 (30) 137 (25) 123 (10) 121 (100) 11 (20) 109 (10)	528.163	$C_{27}H_{28}O_{11}$	1.73	tremulacin [b,#]	(5)[b,#]

		]$^-$; 590.1515	83 (17)					

a: identified by comparison with authentic reference compound; b: tentative identification based on monoisotopic mass and comparison with fragmentation patterns in literature or databases (HMDB, Metlin, Massbank, mzCloud); structural isomers cannot be ruled out

described in the literature in Salix sp.

+ described in the literature as intestinal metabolite

2 Identification details for selected compounds:

Compound **11**: The compound was tentatively assigned as salicin-cysteine adduct, based on its molecular formula and MS/MS fragmentation pattern. Since cysteine adducts are no common microbial metabolites, we performed the following control experiment in order to find out how the observed adduct is formed: pure saligenin was mixed with cysteine (1 mg/ml each) in PBS buffer containing no human fecal suspension. In order to test whether the adduct is formed during analysis in the ESI source, one sample was measured directly after mixing saligenin and cysteine, however, no saligenin-cysteine adduct was detectable. Therefore, two further samples were prepared in the same way and incubated for 24 h, one at room temperature, and one at 37°C, mimicking the experimental conditions of HFS incubation. Indeed, after incubation, the adduct was detectable, with trace levels in the sample incubated at RT and high levels in the sample incubated at 37°C, indicating that the compound is an artefact which is spontaneously formed under the applied experimental conditions (Figure S1).

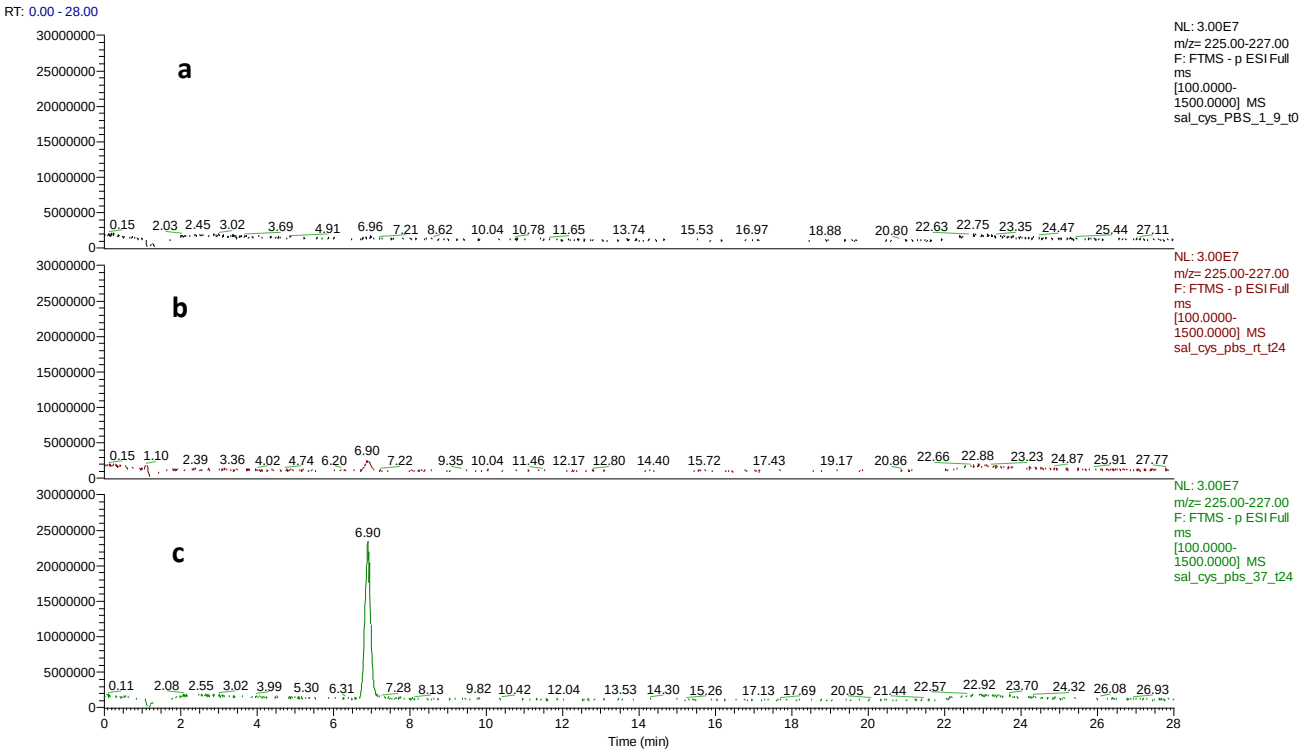

Figure S1: ESI negative mode extracted ion chromatograms (m/z 225-227) of saligenin-cysteine mixture in PBS buffer **(a)** directly after mixing saligenin with cysteine **(b)** after 24 h of incubation at room temperature **(c)** after 24 h of incubation at 37°C.

Compounds **23** and **33** were tentatively identified as acetylsalicins based on their molecular formula and on their MS/MS fragmentation patterns that were similar to the ones described for fragilin (6´-acetylsalicin) by Kammerer et al(5). The occurrence of fragilin has been described in pharmaceutical willow bark preparations; therefore, we assume that one of the two detected isomers is fragilin. The second isomer might bear the acetyl group in a different position. For example , 2´- and 3´-O-acetylsalicin have been isolated from *S. pseudolasiogyne* (11).

Compounds **27**, **31**, **35** and **40** all possess the same HR mass and molecular formula, namely $C_{21}H_{22}O_{10}$.There are several structural isomers possessing this molecular formula described in *Salix* sp.: the flavonone glycosides (+)- and (-)-naringenin-5-glucoside (salipurposide) and naringenin-7-

glucoside, as well as the chalcone glucoside isosalipurposide. Since these compounds share the same MS/MS fragmentation pattern, they cannot be discriminated on this basis. However, the chalcone isosalipurposide has a distinct UV spectrum (λ_{max} 370 nm vs. λ_{max} 280 nm for naringenin glycosides).

When we analyzed authentic naringenin-7-glucoside, the compound could be assigned to peak **47**. Low levels of **47** were detectable at t0 in WBE 10 mg/ml. In WBE 2 mg/ml, the levels of **47** were that low that it was not included in the processed LCMS data. However, inspection of the raw data showed that trace amounts of compound **47** were present in WBE 2 mg/ml at t0 as well. Concerning the two major naringenin hexoside peaks, we tentatively assigned compound **37** to (+)-naringenin-5-glucoside and compound **41** to its diastereomer (-)-naringenin-5-glucoside on the basis of their elution order described in earlier studies(5). Peak **52**, eluting at 25.40 min, was tentatively assigned to the chalcone isosalipurposide due to its typical UV spectrum and due to the elution order that was in accordance with the one in the study by Kammerer et al. (5).

Compound 28: The compound´s fragmentation pattern was in accordance with that of 5-(dihydroxyphenyl)valerolactone(8).

Compound 20: Based on its MS/MS fragment m/z 123, that was reported for 4-oxo-5-(dihydroxyphenyl)valeric acid, compound **20** was tentatively assigned to this metabolite(8).

Compounds **42** and **35** shared the same monoisotopic mass (m/z 209.0809), however, their MS/MS fragmentation patterns slightly differed: In its MS/MS spectrum, compound **42** displayed a fragment with m/z 135, which was not present in the MS/MS spectrum of compound **35**. According to Tagakaki et al.(8), this fragment occurs in the MS/MS spectrum 5-(3,4-dihydroxyphenyl)valeric acid, while it does not occur in the MS/MS spectrum of 4-hydroxy-5-(3-hydroxyphenyl)valeric acid. Therefore, the major peak with m/z 209.0809 eluting at 19.65 min (**42**) was tentatively assigned as 5-

(dihydroxyphenyl)valeric acid, while the minor peak (m/z 209.0809, RT 16.61 min) (**35)** was tentatively assigned as 4-hydroxy-5(-hydroxyphenyl)valeric acid.

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