Supplementary information

Flow bioprocessing of citrus glycosides for high-value aglycone preparation

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Contents

1. General information

NMR spectra were recorded on Brucker Avance 600 MHz spectrometer employing the residual signal of the deuterated solvent as internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz (Hz). Merck Silica gel 60 F254 (aluminium foils) plates were used for TLC analysis (Merck Life Science S.r.l., Milan, Italy); flash column chromatography was performed on Merck Silica gel (230–400 mesh) (Merck Life Science S.r.l., Milan, Italy). Detection of TLC analyses has been performed under UV light at 254 and 365 nm or revealed by a solution of vanillin (2%) and H_2SO_4 (1%) in EtOH. Organic solutions were concentrated using a Buchi rotary evaporator below 40 °C at 25 torr. Morphological investigation was carried out with a LEO 1430 (Carl Zeiss, Oberkochen, Germany) Scanning Electron Microscope operating at at 20 kV and 3×10^{-3} Torr, SEI detection mode. Before analysis, samples were sputter-coated with gold to a thickness of approximately 10 nm using a Semprep 2 sputter coater (Nanotech Ltd, Prestwick, UK) at 10 mA. Enzyme localization was observed using a Nikon A1 laser scanning confocal microscope with an excitation laser (λ: 488 nm) and the emission filter 500-550 nm for fluorescein signals, while excitation laser (λ: 561 nm) and the emission filter 570-620 nm for rhodamine signals. HPLC analyses were performed using a Merck-Hitachi LaChrom Liquid Cromatograph with L-7200 autosampler, L-7100 pump and L-7400 UV-detector. Analyses were carried out using the following gradient: 90/10 (v/v) H_2O milliQ/ACN until 60% of ACN for 30 min (t0min → t30min); reaching 100% ACN at 35 min (t30min → t35min), maintaining 100% ACN for 5 min (t35min → t40min); λ = 280 nm; flow rate: 0.7 mL/min; column: LiChroCART (250 x 4.6 mm x 5 µm). Continuous flow biotransformations were performed using a R2$^+$/R4 Vapourtec flow reactor equipped with Omnifit® glass columns (6.6 mm i.d. × 100 mm length).

2. Chemicals

All reagents and solvents were obtained from commercial suppliers and were used without further purification.

3. Cloning, overexpression and purification of HOR

Protein expression and purification were performed following previously reported protocols by Delgado *et al.*[1] Figure S1 shows pellet, crude extract, flow through fractions and pure protein analyzed by SDS-PAGE. The monomer of HOR is 52.1 kDa. Typically, starting from 1 L culture it is possible to obtain 55 mg of pure protein.

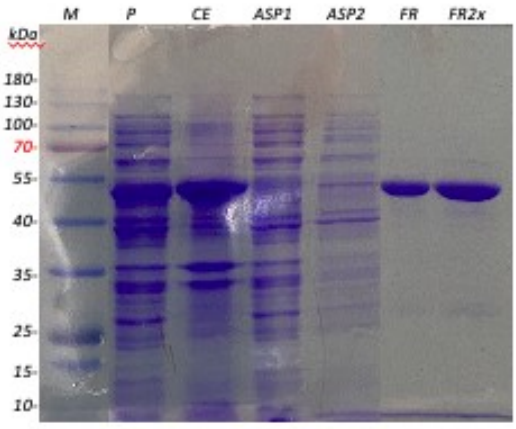

Figure S1: M: marker, P: pellet, CE: crude extract, ASP1, ASP2: flow through fractions, FR and FR2x: pure protein at different sample concentrations.

4. Activity assay of HOR and RN free enzyme

HOR free enzyme activity measurements were performed following previously reported protocols by Delgado *et al.* [1] Specific activity was 3 U/mg.

RN free enzyme activity measurements were performed spectrophotometrically at 420 nm by determining the formation of *p*-nitrophenol (PNP) at 25 °C in half microcuvette (total volume 1 mL) for 2 min. One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 µmol of *p*-nitrophenyl α-L-rhamnopyranoside (NPRP) per minute under reference conditions, namely 10 µL of the suitable enzyme dilution and 990 µL of NPRP 0.01 M in HEPES buffer 0.05 M, pH 7.5. Specific activity was 6 U/mg.

5. Immobilization onto agarose matrix

Aldehyde agarose immobilization was performed as previously described by Dall'Oglio *et al.*[2] The immobilization results in terms of retained activity and stability (after a week and after a month at 4 °C) at different concentration of enzyme (1, 5, 10 mg/g_{matrix}) are reported in Table S1. Data were collected in duplicates and the results expressed as average of the obtained data.

1 mg/g_{matrix}	Average activity (%)	Weekly average stability (%)	Monthly average stability (%)	**5 mg/g_{matrix}**	Average activity (%)	Weekly average stability (%)	Monthly average stability (%)	**10 mg/g_{matrix}**	Average activity (%)	Weekly average stability (%)	Monthly average stability (%)
HOR	53	52	48	HOR	22	23	21	HOR	12	12	10
RN	35	33	30	RN	15	15	14	RN	7	6	6

Table S1.HOR/RN immobilization onto glyoxyl-agarose

6. Quantification and activity assay of HOR and RN immobilized enzyme

Protein quantification during the immobilization procedure has been performed *via* Bradford assay as previously reported by Dall'Oglio *et al.*[2] The activity of imm-RN/imm-HOR/imm-RN-HOR was determined by weighing an appropriate amount of immobilized enzyme (5-10 mg) into a 15 mL reaction tube with cap, followed by the addition of 10 mL reaction mixture (0.05 M HEPES buffer pH 7.5, containing 0.01 M *p*-nitrophenyl β-D-glucopyranoside or *p*-nitrophenyl α-L-rhamnopyranoside). The reaction mixture was shaken at 25 °C, 150 rpm and the absorbance at 420 nm was recorded every minute as single reading using half-microcuvettes (total volume 1 mL). The imm-RN/imm-HOR specific activity (U/mg) is defined as µmol of *p*-nitrophenol formed for minute for mg of immobilized enzyme.

7. Labeling of HOR and RN

Fluorescent label was done according with reported methodologies by Velasco-Lozano *et al.*[3] An enzyme (HOR/RN) solution was mixed (1:1 molar ratio) with fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) (from a stock solution 10 mg/mL in DMSO) in 0.05 M HEPES buffer pH 7.5 and incubated for 1 h with gentle agitation at 25 °C in darkness. The excess of FITC/RITC was removed *via* dialysis against HEPES buffer 0.05 M pH 7.5 until the flow through becomes colorless.

8. TMO preparation

TMO has been synthesized following previously reported protocols by Pellis *et al.*[4]

9. Small scale batch reactions

Batch reactions using free, agarose-immobilized enzymes or the co-immobilized system were performed in 10 mL screw cap tubes; 2 mL reaction mixture composed of 0.05 M HEPES buffer pH 7.5/TMO 50:50, containing 5 mg/mL of substrates (*i.e.,* HES/RT or Glu-HP/IQ), 1 mg/mL of free enzyme or 50 mg of imm-enzyme (enzymatic loading RN: 5 mg/$g_{agarose}$, HOR: 1 mg/$g_{agarose}$, imm-RN-HOR: 5 $mg_{RN}/g_{agarose}$, 1 $mg_{HOR}/g_{agarose}$,), were left under gentle shaking at 40 °C. 100 µL aliquots were collected at different reaction times (30 min, 1 h, 2 h, 5 h, 24 h) for TLC analysis (acetone/EtOAc/CH_3COOH/H_2O 7:2:0.5:0.5). After evaporation, the samples were re-suspended in the mobile phase for HPLC analysis. The retention times were: hesperidin (HES): 16.3 min, hesperetin-7-O-glucoside (Glu-HP): 19.9 min, hesperetin (HP): 26.7 min; rutin (RT): 11.6 min, isoquercetin (IQ): 16.2 min, quercetin (Q): 24.8 min, confirmed by comparison with commercially available standards.

Table S2. Comparison between batch reaction with free and immobilized enzymes

Entry	Substrate	Enzyme[b]	m.c. (%)[a]	Time (h)[b]
1	HES	Free-RN	15	14
2	Glu-HP	Free-HOR	18	2
3	RT	Free-RN	30	3
4	IQ	Free-HOR	32	0.5
5	HES	Imm-RN	30	6
6	Glu-HP	Imm-HOR	41	0.5

7	RT	Imm-RN	50	1
8	IQ	Imm-HOR	80	1.5
9	HES	Imm-RN-HOR	60	1
10	RT	Imm-RN-HOR	70	0.5

[a]m.c. = molar conversion. [b]Time corresponding to maximum conversion.

10. Flow preparation of glucoside-intermediates and aglycones

A glass column (6.6 mm i.d.) was filled with 1 g of imm-RN (5 mg/g_{resins}), imm-HOR (1 mg/g_{resins}) or imm-RN-HOR (5 $mg_{RN}/g_{agarose}$, 1 $mg_{HOR}/g_{agarose}$) (packed bed reactor volume: 1.2 mL). A 10 mg/mL solution of starting material in TMO and HEPES buffer 0.05 M pH 7.5 were prepared. The two solutions were mixed in a T-piece and the resulting segmented flow stream (1:1) was directed into the column packed with the biocatalyst. For small scale flow biotransformations, 2 mL loops have been used. The flow rate was varied and optimized dependently on the selected residence time. After extraction of the exiting flow stream, both the organic and aqueous phases were analyzed by HPLC using the above reported conditions (paragraph 8). The organic phase containing the desired products were evaporated and when necessary column chromatography (*n*-hexane:EtOAc 1:1 for glucoside intermediates; *n*-hexane:EtOAc 6:4 for aglycones) was performed to yield the final compounds.

11. HPLC analysis of flow biotransformations

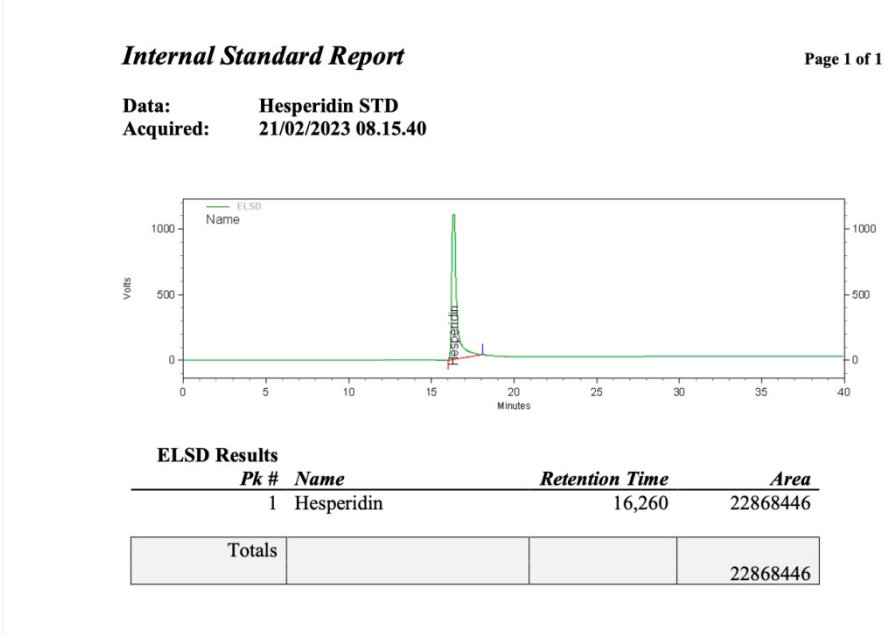

Figure S2: Hesperidin standard

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Data: **Hesp-7-O-glu STD**
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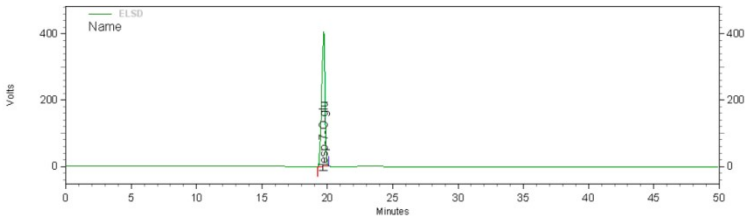

ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Hesp-7-O-glu	19,879	7821192
Totals			7821192

Figure S3: Hesperetin-7-O-glucoside standard

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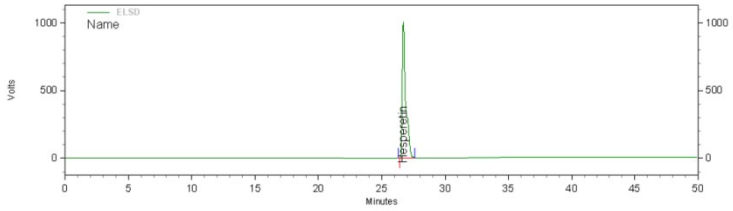
Data: **Hesperetin STD**
Acquired: **21/02/2023 10.06.27**



ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
2	Hesperetin	26,707	18892806
Totals			18892806

Figure S4: Hesperetin standard

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Data: **Flow RN Hesperidin Hesp-7-O-glu 30 min**
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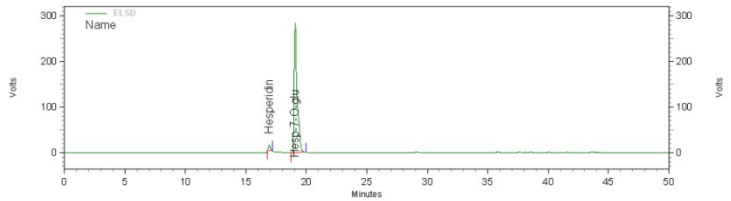

ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Hesperidin	16,919	120378
2	Hesp-7-O-glu	19,085	4852100
Totals			4972478

Figure S5: Hesperidin→hesperetin-7-O-glucoside flow biotransformation

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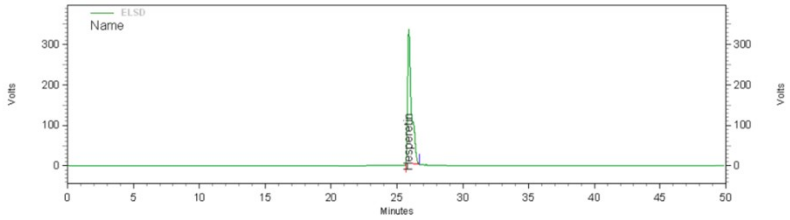
Data: **Flow HOR Hesp-7-O-glu Hesperetin 5 min**
Acquired: **21/02/2023 16.28.53**



ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Hesperetin	26,292	7276388
Totals			7276388

Figure S6: Hesperetin-7-O-glucoside→hesperetin flow biotransformation

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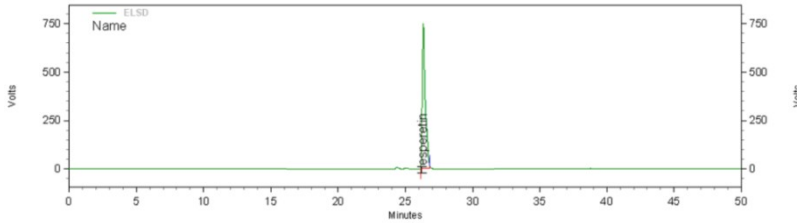
Data: **Flow coimm Hesperidin Hesperetin 5 min**
Acquired: **22/02/2023 10.11.47**



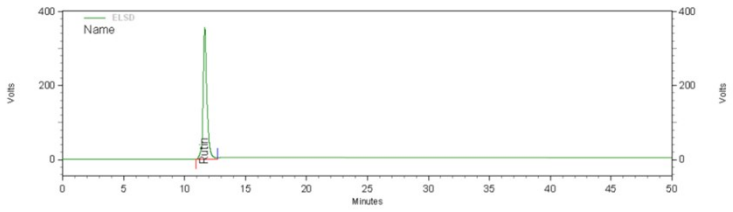
ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Hesperetin	26,405	11481384
Totals			11481384

Figure S7: Hesperedin→hesperetin flow biotransformation

Internal Standard Report

Data: **Rutin STD**
Acquired: **21/02/2023 11.09.45**



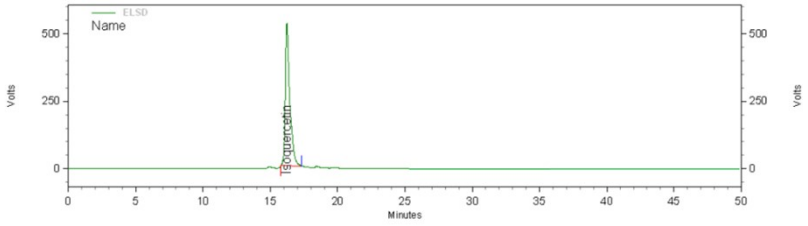
ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Rutin	11,651	7698639
Totals			7698639

Figure S8: Rutin standard

Internal Standard Report

Data: **Isoquercetin STD**
Acquired: **21/02/2023 12.17.44**



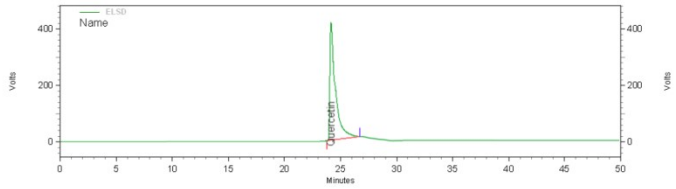
ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Isoquercetin	16,227	12060006
Totals			12060006

Figure S9: Isoquercetin standard

Internal Standard Report

Data: **Quercetin STD**
Acquired: **21/02/2023 13.23.11**



ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Quercetin	24,790	14595878
Totals			14595878

Figure S10: Quercetin standard

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Data: **Flow coimm Rutin Quercetin 5 min**
Acquired: **22/02/2023 11.58.36**

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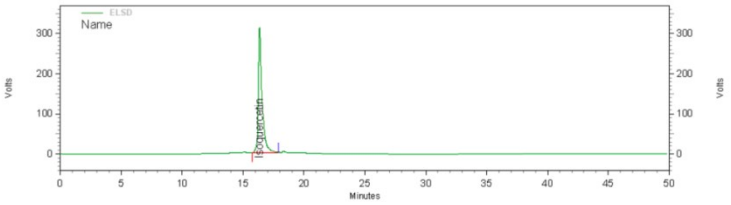
Data: **Flow RN Rutin Isoquercetin 5 min**
Acquired: **21/02/2023 15.33.28**



ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Isoquercetin	16,759	7240323
Totals			7240323

Figure S11: Rutin→isoquercetin flow biotransformation

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Data: **Flow HOR Isoquercetin Quercetin 1 min**
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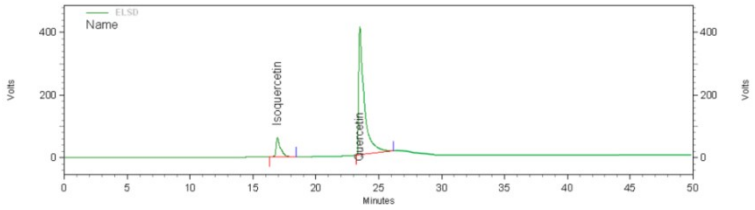

ELSD Results

Pk #	*Name*	*Retention Time*	*Area*
1	Isoquercetin	16,946	1550013
2	Quercetin	24,557	13560895
Totals			15110908

Figure S12: Isoquercetin→quercetin flow biotransformation

Figure S13: Isoquercetin→quercetin flow biotransformation

12. NMR spectra

Hesperetin-7-O-glucoside (Glu-HP): ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 12.04 (s, 1H), 9.11 (s, 1H), 6.94- 6.88 (m, 3H), 6.17-6.14 (m, 2H), 5.50 (dd, *J* = 12.2, 3.0 Hz, 1H), 5.00-4-96 (m, 1H), 3.78 (s, 3H), 3.66 (dd, *J* = 11.0, 4.7 Hz, 1H), 3.32-3.27 (m, 2H), 3.25- 3.14 (m, 5H). ^{13}C-NMR (150 MHz, DMSO-d_6) δ (ppm): 197.0, 165.3, 165.2, 162.6, 147.9, 146.4, 130.9, 117.8, 114.1, 112.0, 103.3, 99.6, 99.5, 96.4, 95.5, 78.5, 77.1, 76.2, 72.9, 69.4, 60.5, 55.7.

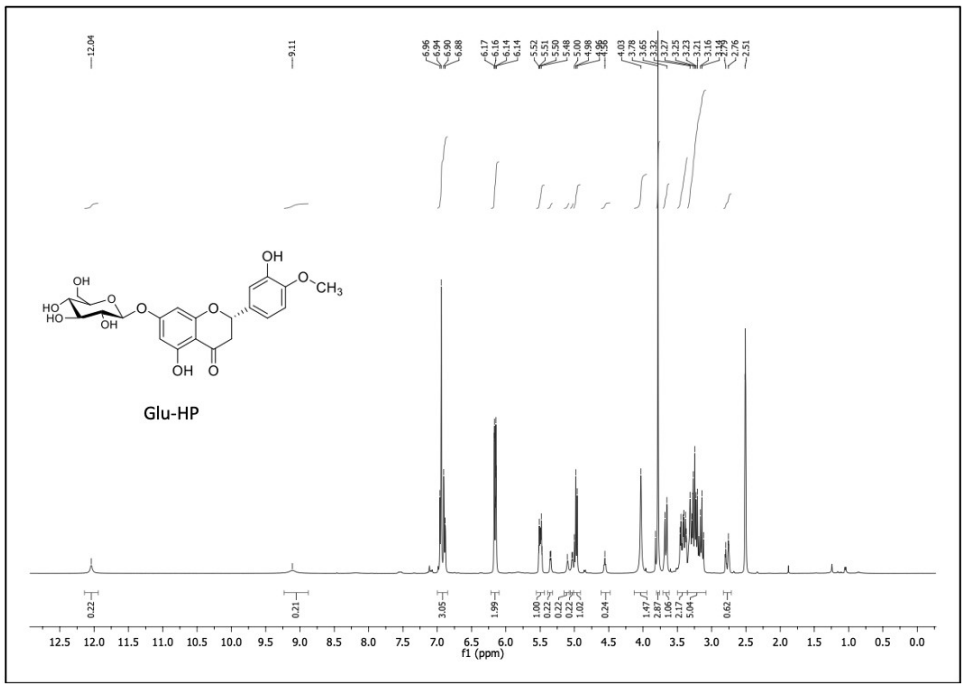

Figure S14: ^{1}H-NMR hesperetin-7-O-glucoside

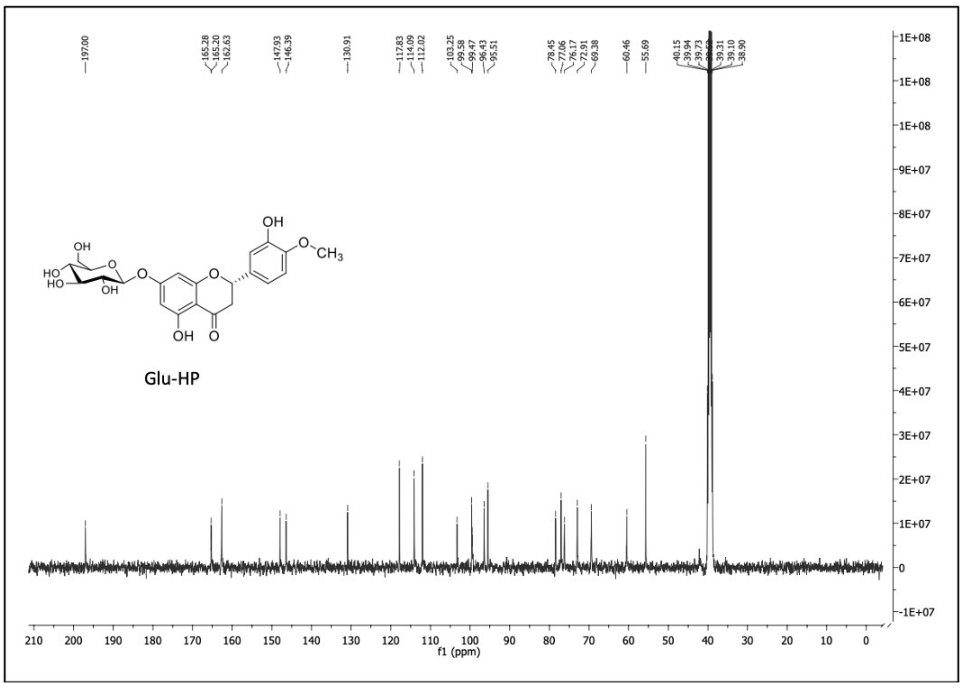

Figure S15: ^{13}C-NMR hesperetin-7-O-glucoside

Hesperetin (HP): ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 6.94-6.87 (m, 3H), 5.90 (d, *J* = 1.9 Hz, 1H), 5.88 (d, *J* = 1.9 Hz, 1H), 5.28 (dd, *J* = 12.3, 3.0 Hz, 1H), 3.81 (s, 3H), 3.05 (dd, *J* = 17.2, 12.3 Hz, 1H), 2.70 (dd, *J* = 17.2, 3 Hz, 1H). ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 197.6, 168.3, 165.4, 164.7, 149.3, 147.7, 133.1, 118.9, 114.5, 112.5, 103.4, 97.1, 96.2, 80.2, 56.4, 44.0.

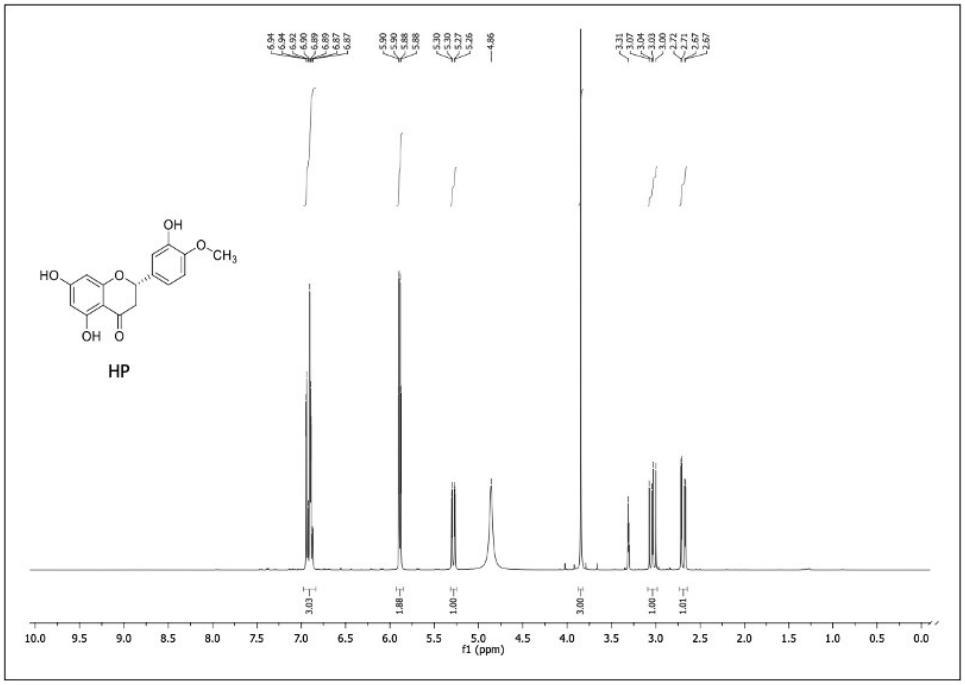

Figure S16: ^{1}H-NMR hesperetin

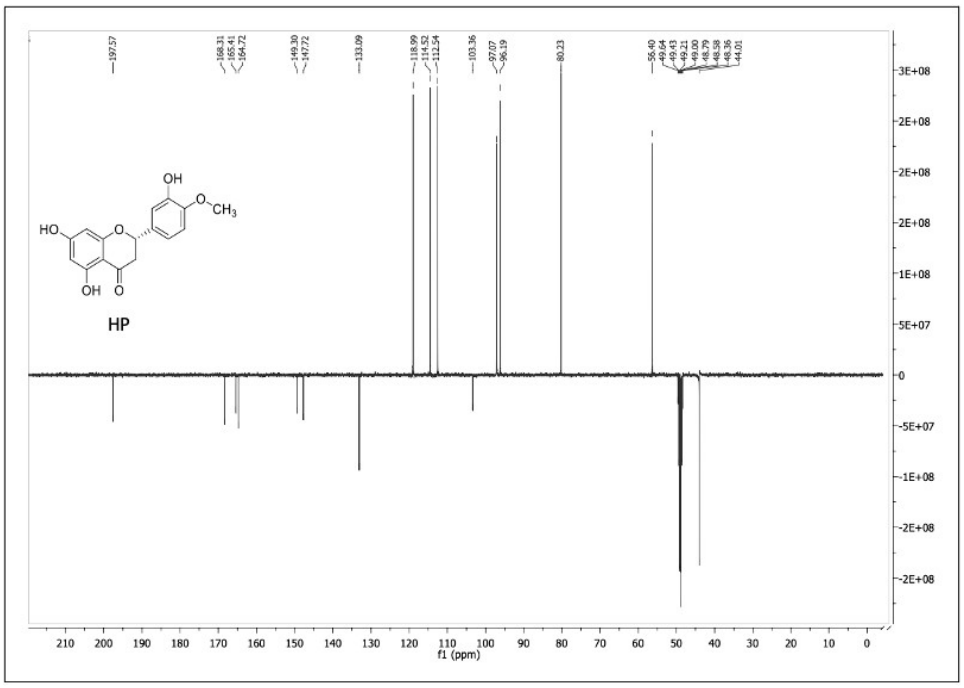

Figure S17: ^{13}C-NMR hesperetin

Isoquercetin (IQ): ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 7.73 (d, *J* = 2.2 Hz, 1H), 7.60 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.89 (d, *J* = 8.5 Hz, 1H), 6.41 (d, *J* = 1.7, Hz, 1H), 6.22 (d, *J* = 1.7, Hz, 1H), 5.27 (d, *J* = 7.6, Hz, 1H), 3.73 (dd, *J* = 12.0, 2.5 Hz, 1H), 3.58 (dd, *J* = 12.0, 5.5 Hz, 1H), 3.53-3.43 (m, 2H), 3.39-3.33 (m, 1H), 3.26-3.24 (m, 1H), 3.24-3.22 (m, 1H). ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 179.5, 166.0, 163.0, 159.0, 158.4, 149.8, 145.9, 135.6, 123.2, 123.1, 117.6, 115.9, 105.7, 104.4, 99.9, 94.7, 78.4, 78.1, 75.7, 71.2, 62.5.

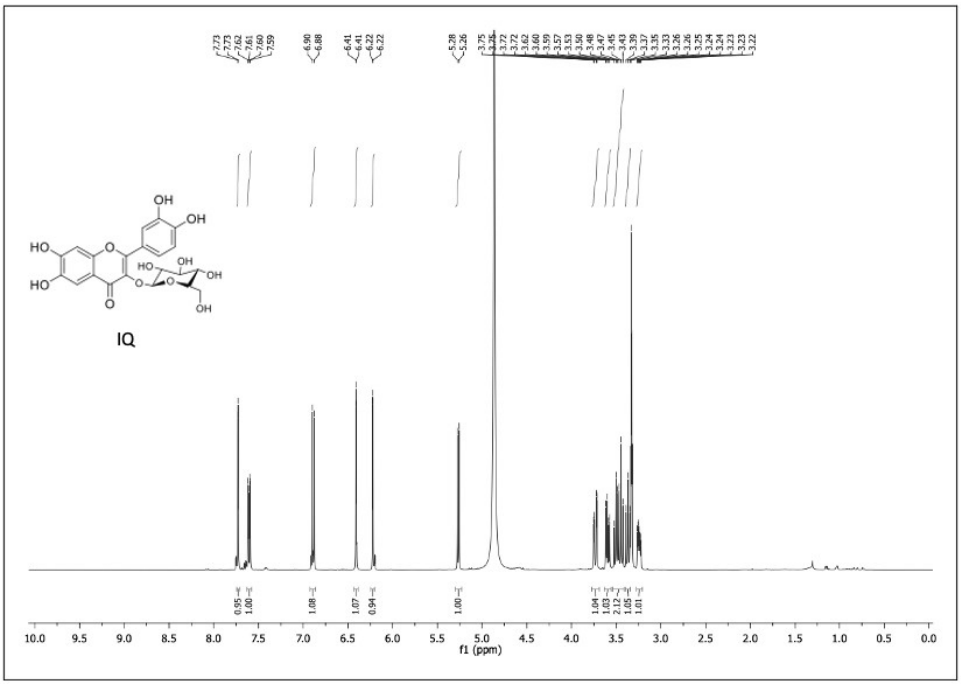

Figure S18: ^{1}H-NMR isoquercetin

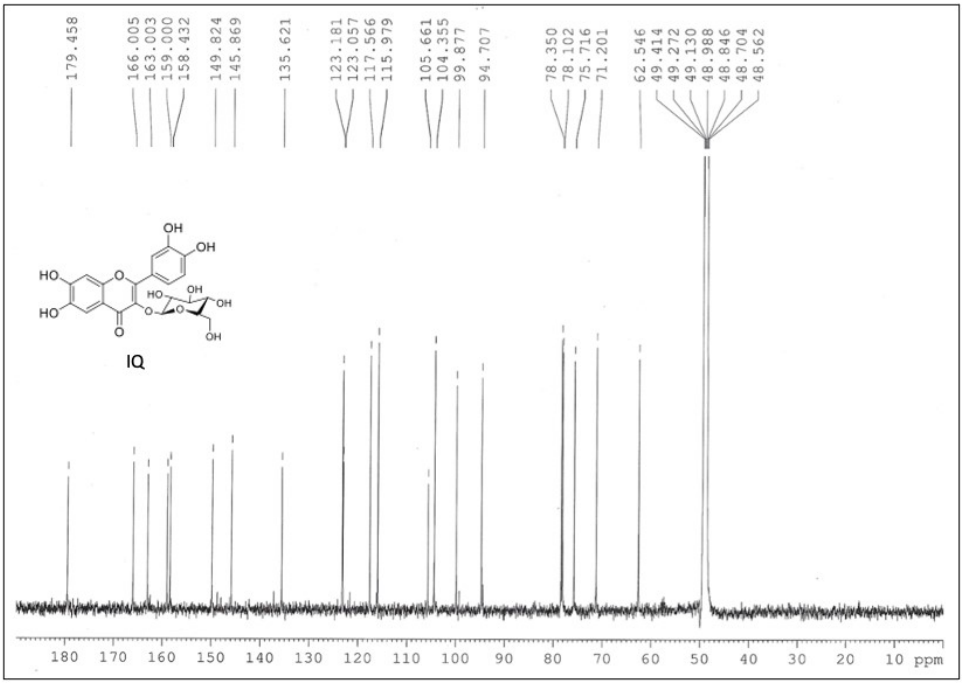

Figure S19: ^{13}C-NMR isoquercetin

Quercetin (Q): ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 7.73 (d, *J* = 2.1 Hz, 1H), 7.62 (dd, *J* = 8.7, 2.1 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.38 (d, *J* = 2.1, Hz, 1H), 6.18 (d, *J* = 1.8, Hz, 1H). ^{13}C-NMR (150 MHz, DMSO-d_6) δ (ppm): 175.8, 163.9, 160.7, 156.1, 147.7, 146.8, 145.0, 135.7, 121.9, 119.9, 115.6, 115.0, 102.9, 98.2, 93.3.

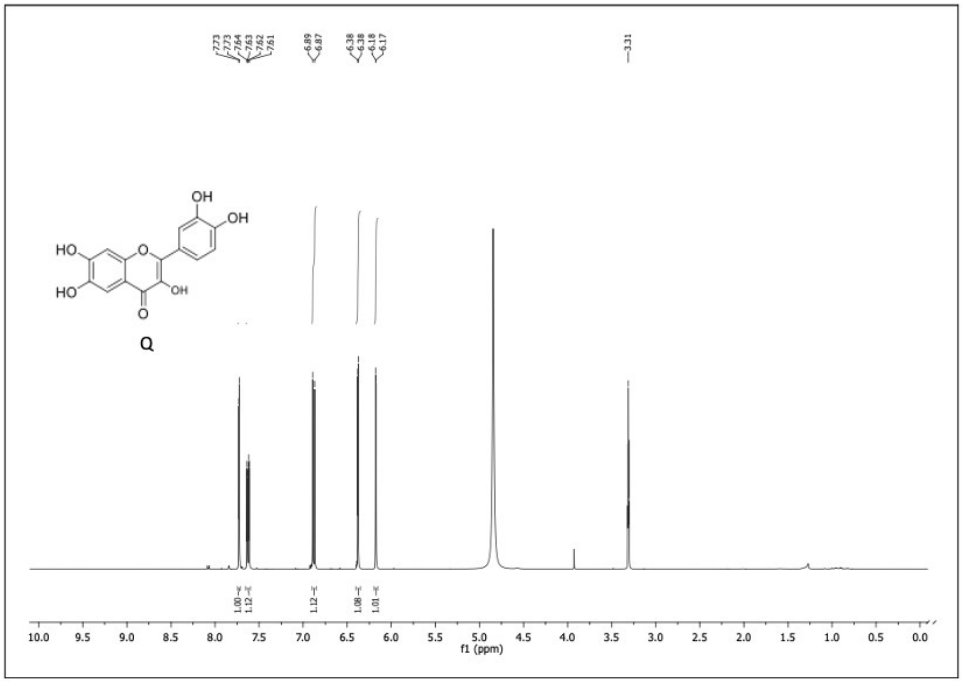

Figure S20: ^{1}H-NMR quercetin

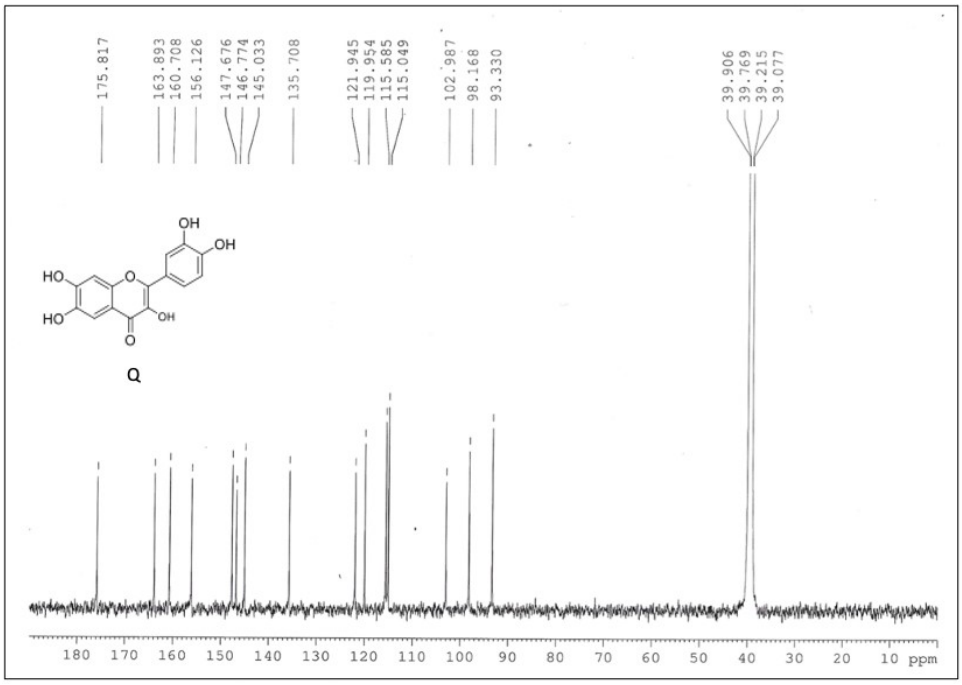

Figure S21: ^{13}C-NMR quercetin

13. References

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