Electronic Supplementary Material (ESI) for Food & Function.

Hydroxytyrosol and tyrosol sulfate metabolites protect against oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells.

Angela Atzeri, Ricardo Lucas, Alessandra Incani, Pablo Peñalver, Alberto Zafra-Gómez, M. Paola Melis, Roberto Pizzala, Juan C. Morales and Monica Deiana

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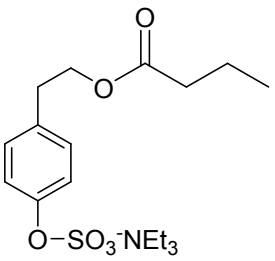
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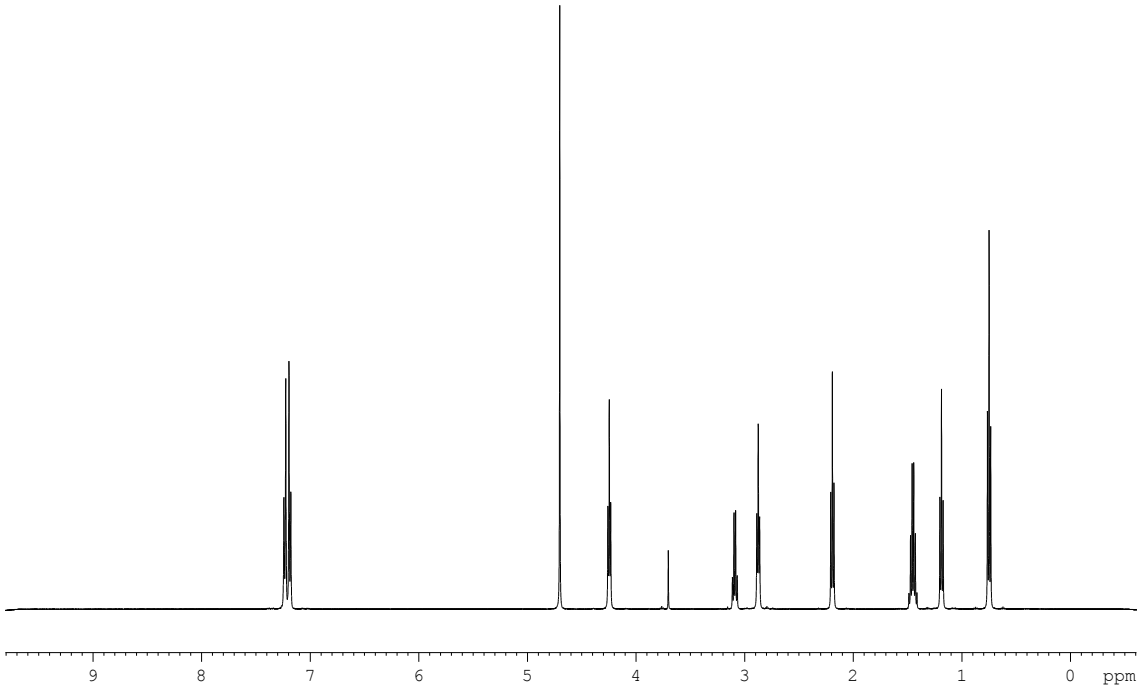
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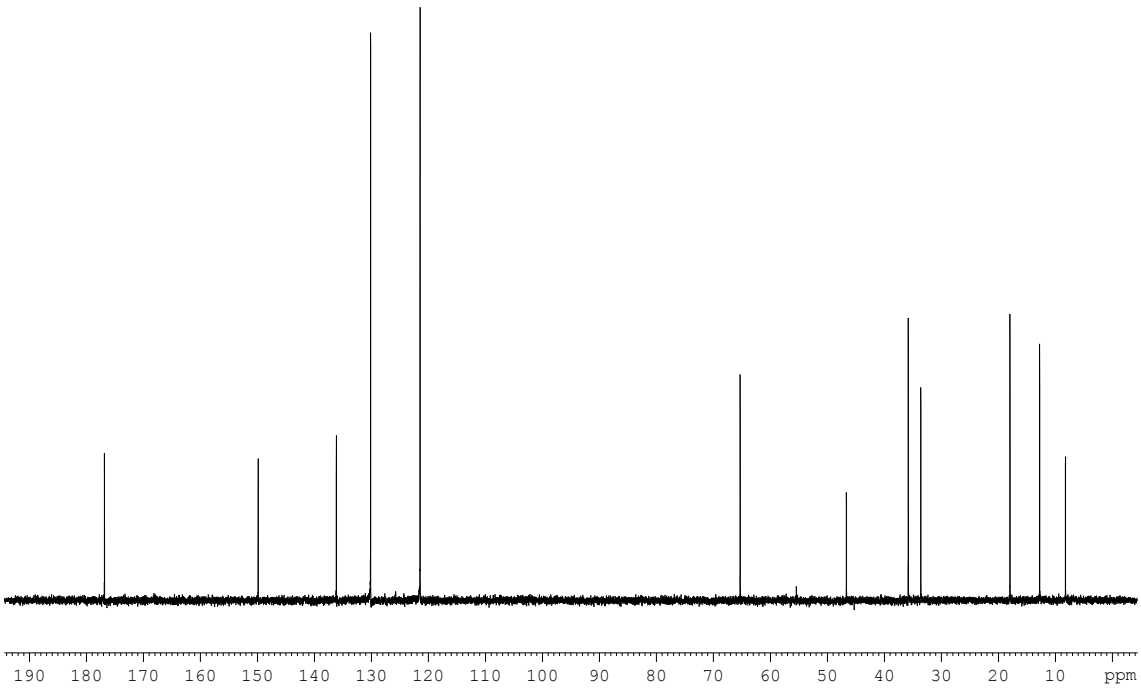
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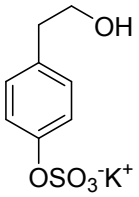
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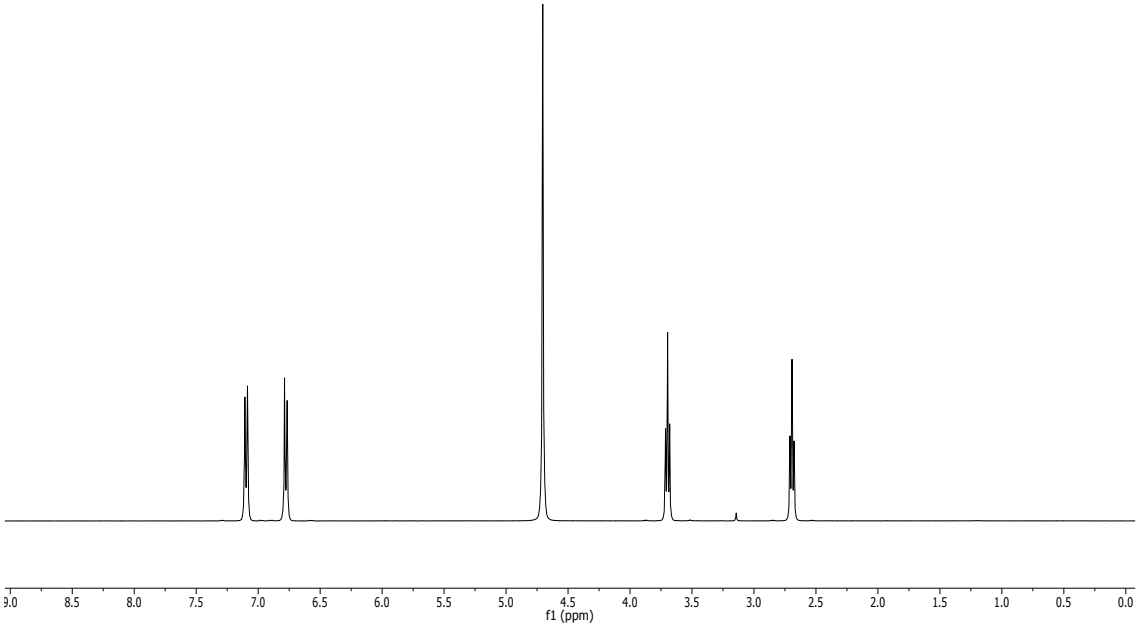
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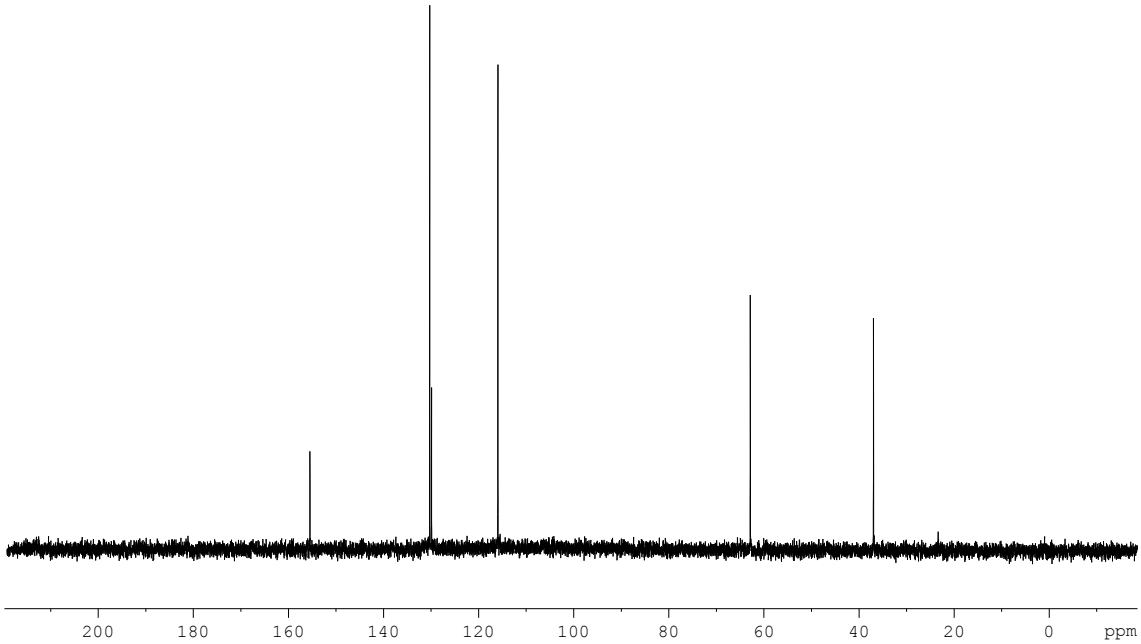
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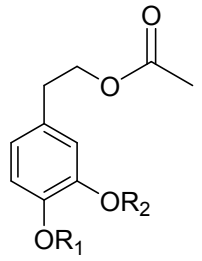
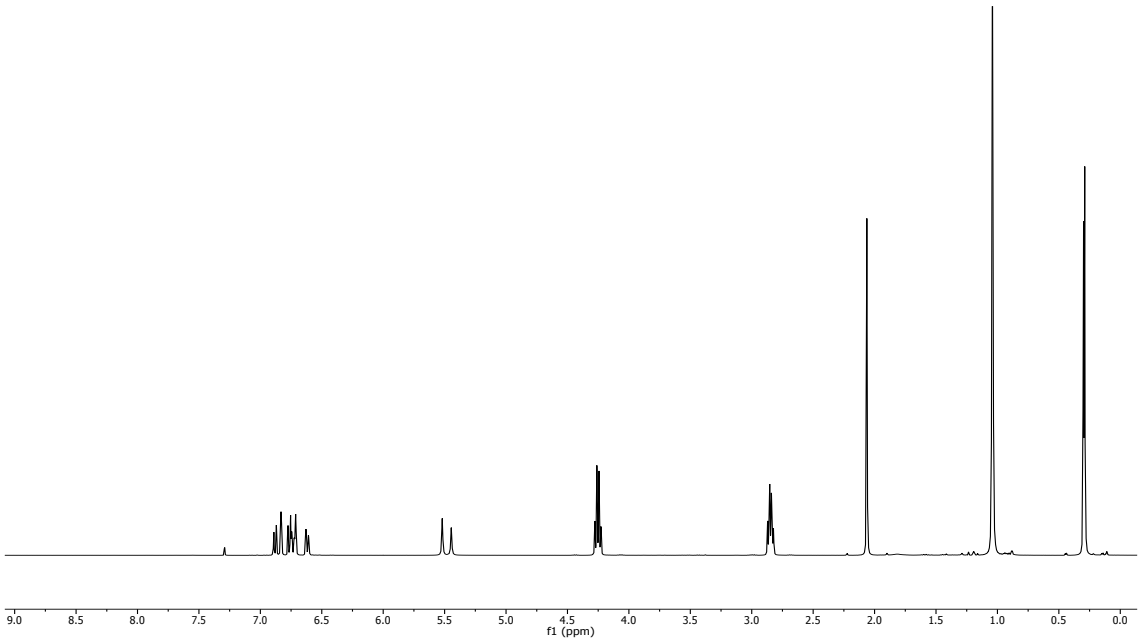
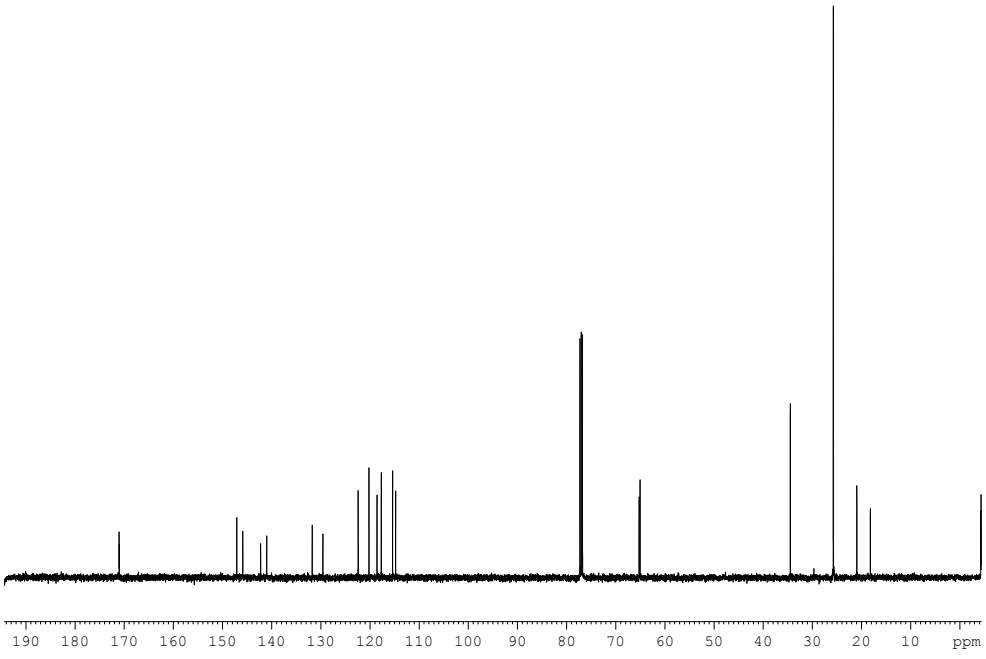
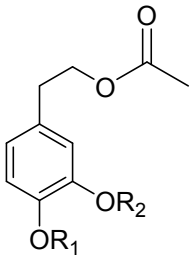


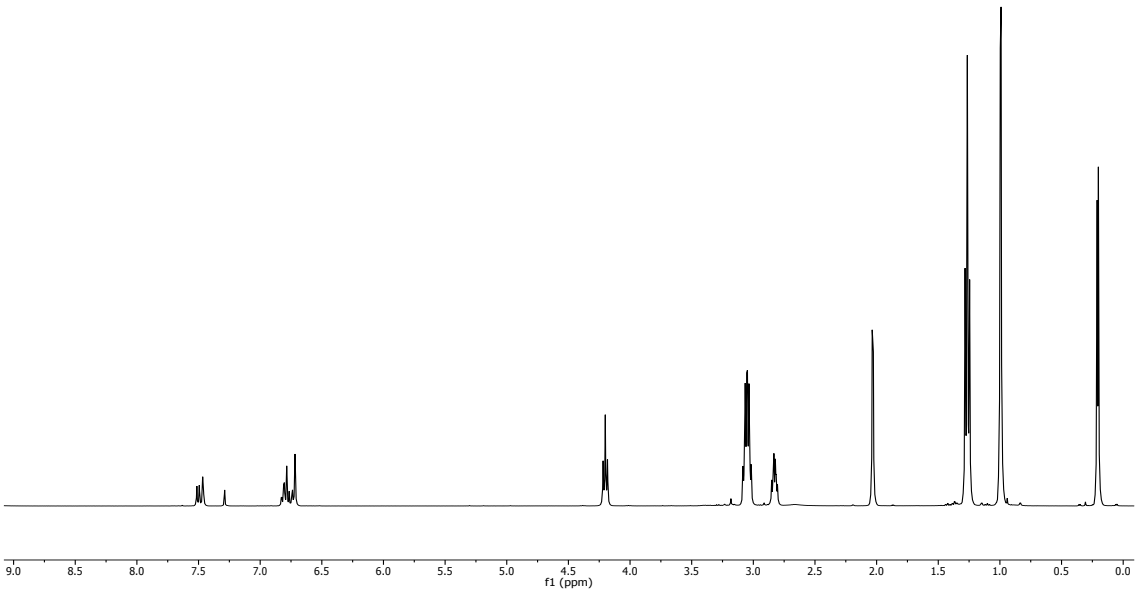
9 R_1=H, R_2=TBDMS
10 R_1=TBDMS, R_2=H

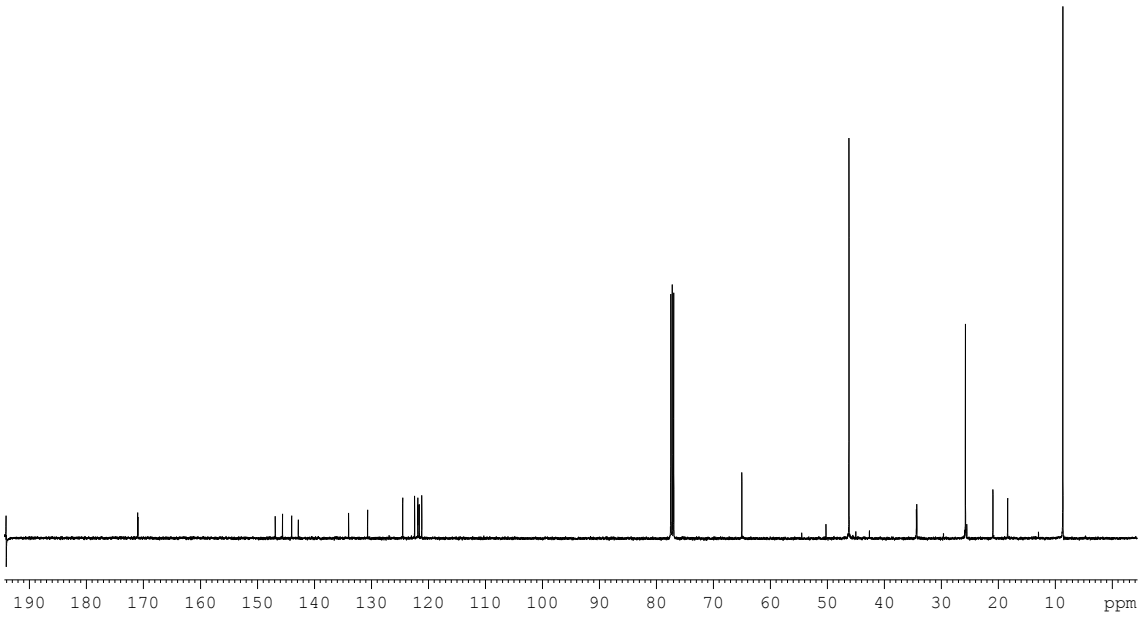


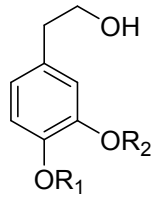




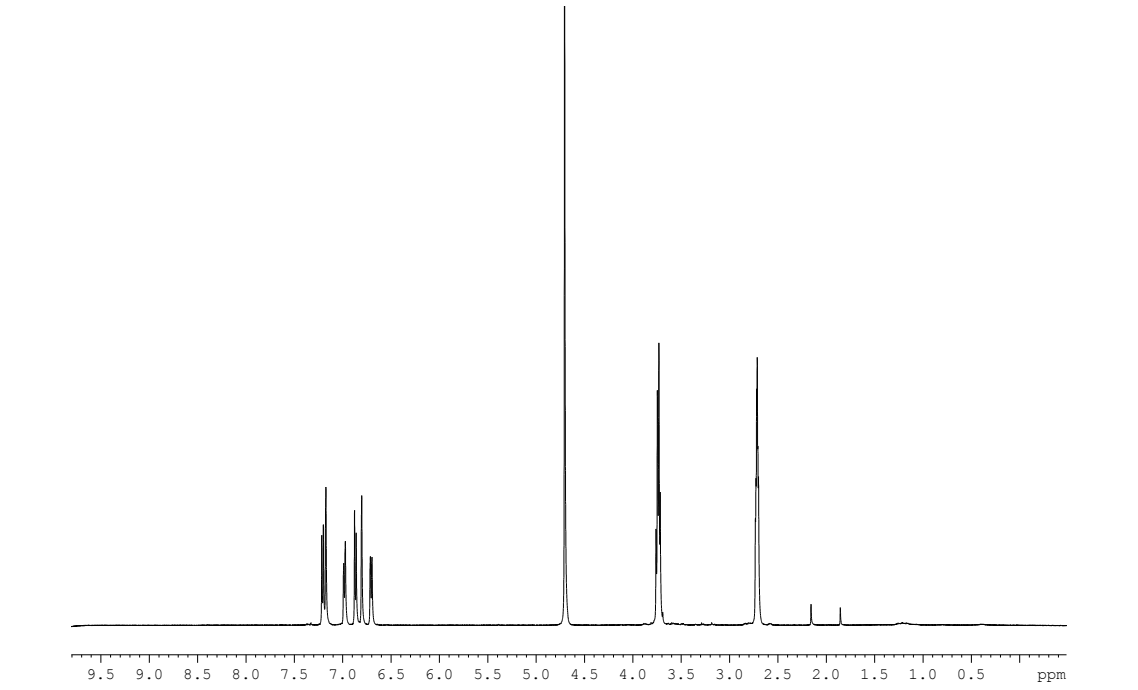
11 R_1=$SO_3^-NEt_3$, R_2=TBDMS
12 R_1=TBDMS, R_2=$SO_3^-NEt_3$

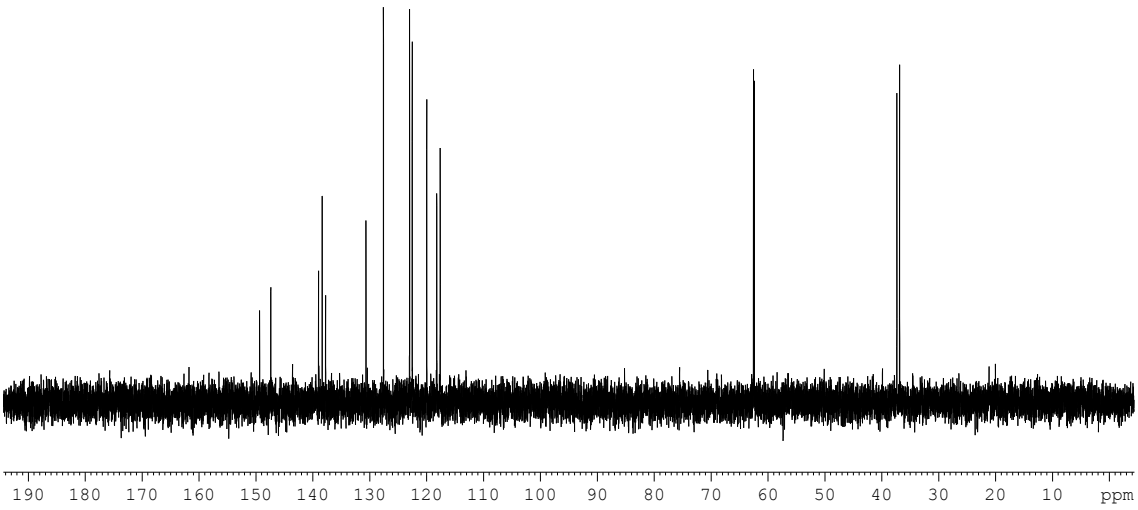






3 $R_1=SO_3^-K^+$, $R_2=H$
4 $R_1=H$, $R_2=SO_3^-K^+$





Tyr, HT and their sulfated metabolites stability/uptake in Caco-2 culture

a) Method description

a.1.- Instrument and software

A Waters Acquity UPLC^{TM} H-class system (Waters, Manchester, UK) was used for chromatographic separation. The column was an Acquity UPLC^{R} BEH C18 (2.1 x 100 mm, 1.7 μM). A QDA single quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray^{TM} electrospray ionization (ESI) source was used for metabolites detection. Empower 3 software was used for instrument control, peak detection and integration. Data analysis was done in Microsoft Excel.

a.2.- Samples preparation

Caco-2 cells were seeded at a density of about 10.000 cells/ml on Petri dishes and used 15 days post-seeding. Medium was then discarded and the cells were treated with the phenolic compounds (250 μM, in order to obtain detectable metabolites) in 10 ml of medium (2.5% FBS) for several incubation times (30 minutes, 1 hour, 18 hours and 24 hours). After incubation 1 ml of medium was collected and reserved for further UPLC analysis and the rest was thrown away. The Petri dishes were then washed with ice-cold PBS and 500 ml of lysis buffer (H_2O: MeOH 50: 50 v/ v + 0.1 % HCl) was added to the cells. The lysed cells were then scrapped, collected in eppendorf tubes and stored on ice for 45 minutes. Later on centrifugation of the samples (10 minutes at 4ºC and 12.000 g) was performed and the supernatants collected for UPLC studies.

All the samples were homogenised by vortexing and filtered through a 0.2 μm nylon filter prior to be injected in the instrument.

Control samples were also prepared incubating the tested phenolic compounds for 24h in absence of cells at 0°C or at 37°C, in medium or lysis buffer, to evaluate their stability.

a.3.- UPLC-MS conditions

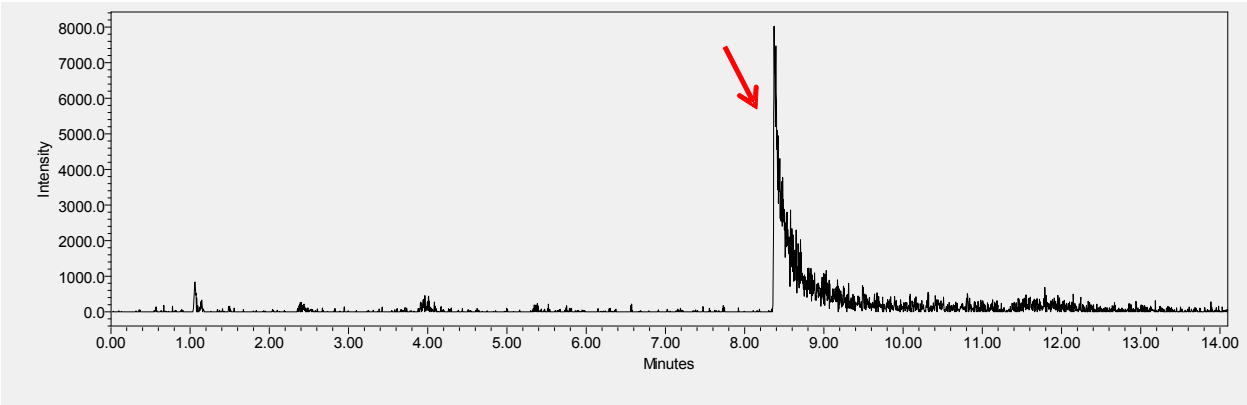
Chromatographic separation was performed using a binary gradient mobile phase consisting of H_2O+ NH_4OAC 1% at pH 4.6 (solvent A) and MeOH (solvent B). The flow rate was 0.4 ml/min, the column was maintained at 30ºC and the injected volume was 10 μl. gradient conditions were as follows: 97% of A for 2 min, then 93% of A for 5 more minutes and finally 0 % of A (100% B) for 3 minutes to clean the column and 4 more minutes at the initial conditions (97% of A) to equilibrate the column.

The mass spectrometer (MS) was operated with electrospray ionization (ESI) in negative ion mode $[M-H]^-$ and the data were collected by selected ion recording mode (SIR).

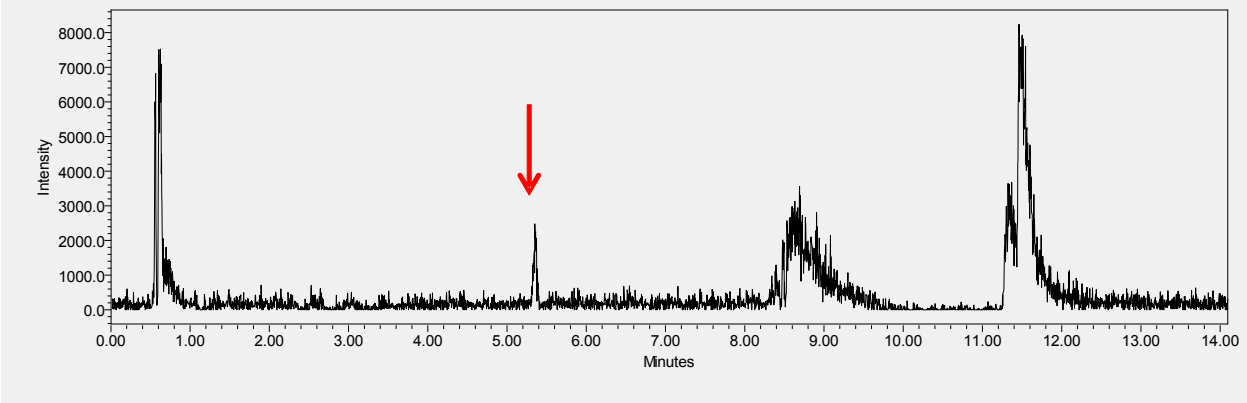
All compounds were quantified respect to its own calibration curve (1-250μM) and results were expressed as percentage of compound remaining.

b) Compounds identification

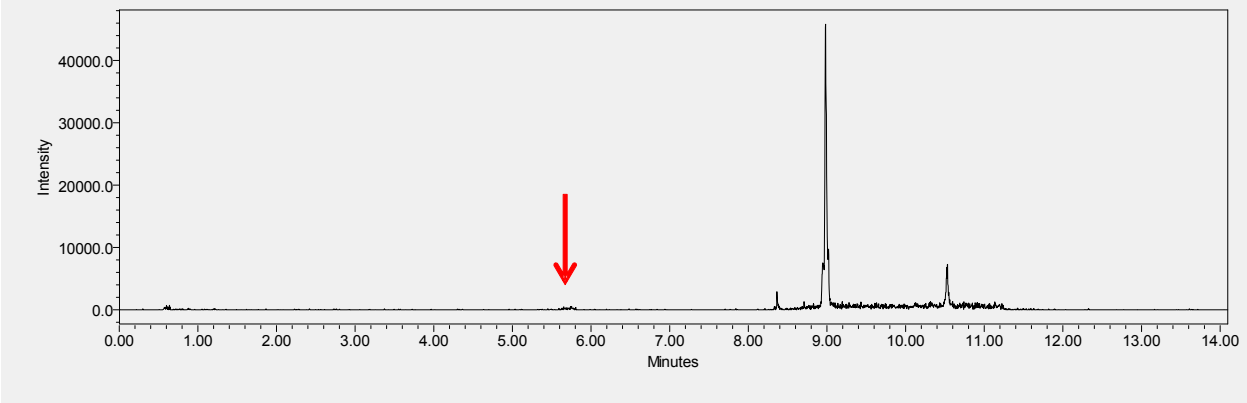
b.1.- PDA 280nm MW=137 (Tyr)



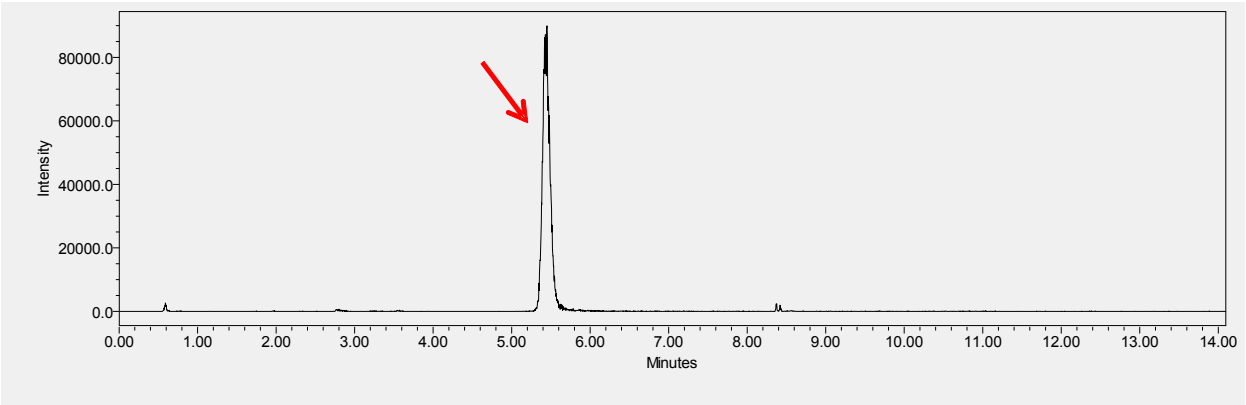
b.2.- PDA 280nm MW=217 (Tyr SO3)



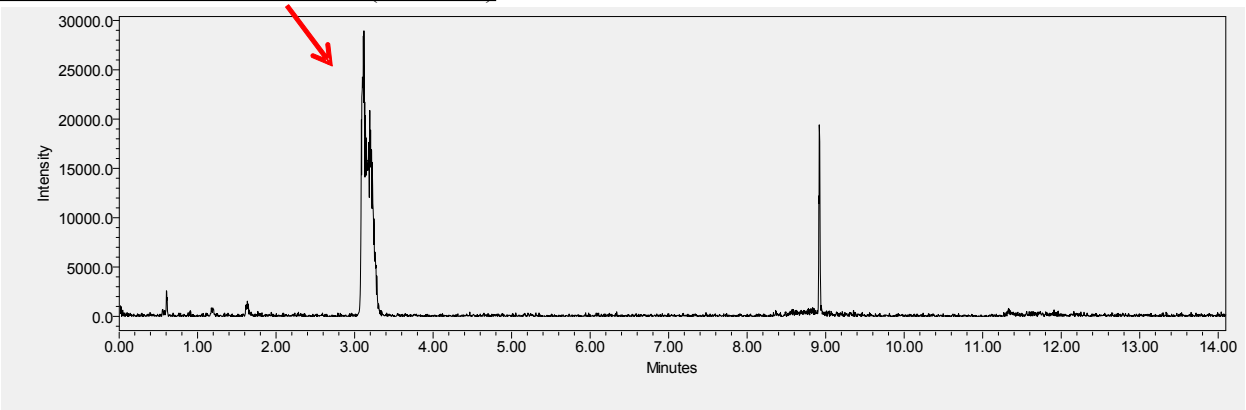
b.3.- PDA 280nm MW=311 (Tyr GlcA)



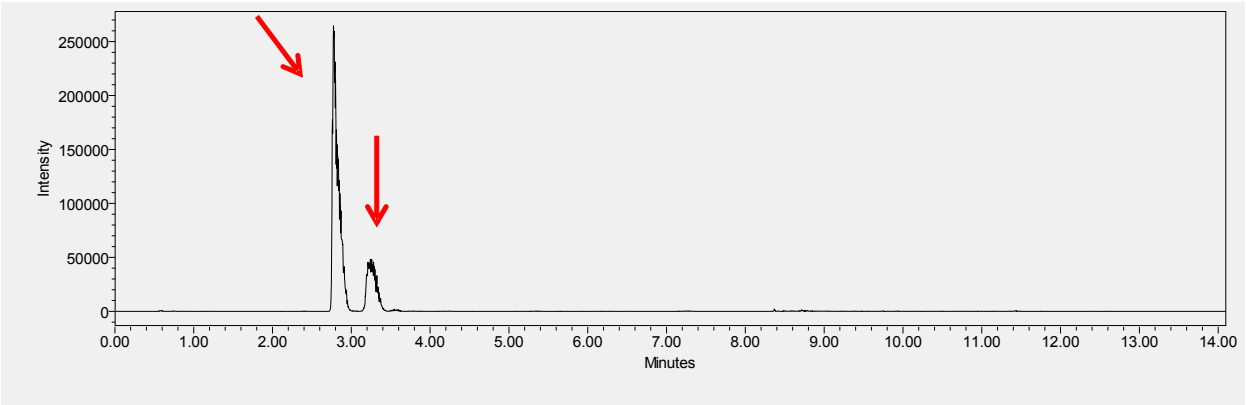
b.4.- PDA 280nm MW=153 (HT)



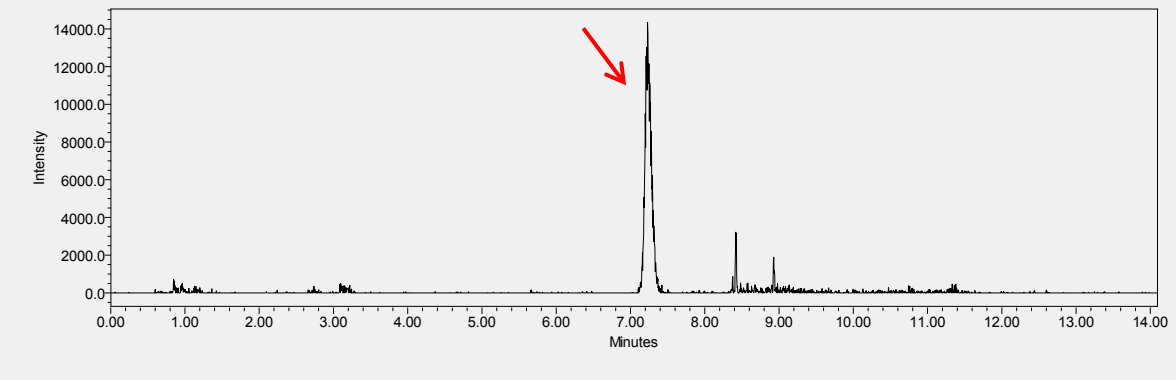
b.5.- PDA 280nm MW=233 (HT SO3)



b.6.- PDA 280nm MW=339 (HT GlcA)



b.7.- PDA 280nm MW=247 (Homov SO3)



c) Calibration curves

c.1.- Tyr
y = 268,85x - 1986,5
R^2 = 0,9921

c.2.- Tyr-SO3
y = 10,252x + 3437
R^2 = 0,9736

c.3.-Tyr-GlcA
y = 10,861x - 31,844
R^2 = 0,9741

c.4.- HT
y = 2320,6x + 15985
R^2 = 0,9959

c.5.- HT-SO3
y = 742,6x – 10960
R^2 = 0,9811

c.6.- HT-GlcA a
y = 5388,2x - 15128
R^2 = 0,9991

c.7.-HT-GlcA b
y = 1589,8x + 5286,2
R^2 = 0,9939

c.8.- Homov-SO3
y = 322,69x + 955,85
R^2 = 0,9983

Tyr-SO3, Tyrosol sulfate; Tyr-GlcA, Tyrosol glucuronide; HT-SO3, Hydroxytyrosol sulfate; HT-GlcA a,b, Hydroxytyrosol-3´-O-glucuronide and Hydroxytyrosol-4´-O-glucuronide; Homov-SO3, Homovanillic alcohol sulfate.

d) Analysis

Results expressed as percentage of compound remaining (100% value from 250µM data of the calibration curve in all the cases but Tyr-SO_3^-, where the Tyr-SO_3^- 250µM data at t=0 in culture medium was used instead)

d.1.- HT and HTSO3 derivatives:

Compound	Incubation time	Supernatants (from lysed cells)				Medium				Total
		HT	HT-SO_3	HT-GlcA	Homov-SO_3	HT	HT-SO_3	HT-GlcA	Homov-SO_3	
HT	30´	4,40	7,80	---	---	59,20	---	---	---	71,40
	60´	4,40	13,60	---	---	56,40	1,70	---	---	76,10
	18 h	3,90	22,40	---	12,80	48,80	32,50	0,20	0,60	121,2
	24 h	0,00	22,40	---	72,60	6,60	86,20	0,40	14,70	202,9
HT-SO_3	30´	---	26,20	---	2,10	21,70	26,20	---	---	76,20
	60´	---	2,20	---	2,20	26,30	69,20	---	---	99,9
	18 h	---	27,00	---	50,10	3,90	86,20	---	4,20	171,40
	24 h	---	8,00	---	51,30	0,00	36,10	0,20	9,90	105,50

Total values above a 100% (at longer incubation times) are due to errors in HT-$SO3^-$ determination due to the bad ionization of the compound which also renders in a calibration curve not as good as for the parent compound HT

HT controls (HT incubated in absence of cells):
- HT in medium at 0ºC for 24h: 58,30% HT remaining
- HT in medium at 37ºC for 24h: 10,50 % HT remaining
- HT in lysis buffer at 37ºC for 24h: 64,20 % HT remaining

HT-SO_3 controls (HT-SO_3 incubated in absence of cells):
- HT-SO_3 in medium at 0ºC for 24h: 19,90 % HT + 23,40 HT-SO_3 remaining
- HT-SO_3 in medium at 37ºC for 24h: 4,80 % HT + 8,40% HT-SO_3 remaining
- HT-SO_3 in lysis buffer at 37ºC for 24h: 27,75 % HT + 27,80% HT-SO_3 remaining

d.2.- Tyr and Tyr-SO_3 derivatives:

Compound	Incubation time	Supernatants (from lysed cells)		Medium		Total
		Tyr	Tyr-SO_3	Tyr	Tyr-SO_3	
Tyr	30´	2,90	---	50,70	---	53,60
	60´	3,00	---	45,10	3,35	51,45
	18 h	7,50	22,38	39,00	65,70	134,58
	24 h	3,70	16,87	69,20	81,50	171,27
Tyr-SO_3	30´	2,90	---	96,80	6,60	106,3
	60´	3,70	---	81,20	9,54	94,44
	18 h	8,00	32,51	70,70	89,97	201,18
	24 h	6,50	33,05	41,10	51,21	135,86

Total values above a 100% (at longer incubation times) are due to errors in Tyr-$SO3^-$ determination due to the bad ionization of the compound which also renders in a calibration curve not as good as for the parent compound Tyr.

Tyr controls (Tyr incubated in absence of cells):
- Tyr in medium at 0ºC for 24h: 38,70% Tyr remaining
- Tyr in medium at 37ºC for 24h: 39,70 % Tyr remaining
- Tyr in lysis buffer at 37ºC for 24h: 31,50 % Tyr remaining

Tyr-SO_3 controls (Tyr-SO_3 incubated in absence of cells):
- Tyr-SO_3 in medium at 0ºC for 24h: 64,20 % Tyr (0,00 Tyr-SO_3)remaining
- Tyr-SO_3 in medium at 37ºC for 24h: 73,60 % Tyr (0,00 Tyr-SO_3)remaining
- Tyr-SO_3 in lysis buffer at 37ºC for 24h: 35,40 % Tyr (0,00 Tyr-SO_3)remaining

Untreated cells:
- Cells incubated at 37ºC for 24h without any of the tested compounds, supernatant from lysed cells or medium: 0% of everything