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UHPLC determination of total hydroxytyrosol (Htyr) and tyrosol (Tyr) content in olive oil

FIT FOR THE PURPOSE OF THE HEALTH CLAIM INTRODUCED BY THE EC REGULATION 432/2012 FOR "OLIVE OIL POLYPHENOLS"

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Principle

❖The UHPLC profile of the **extracted polar fraction** (PF) of the oil **before and after acid hydrolysis** is recorded by means of **diode array detection (280 nm)**. The acid hydrolysis of the bound forms of **Hydroxytyrosol (Htyr)** and **Tyrosol (Tyr)** gives rise to free **Htyr** and **Tyr**, the content of which can then be accurately quantified using commercially available standards. This content is expressed as total **Htyr** and **Tyr** (mg/20 g of oil) after correction for molecular weight differences between free and bound forms.

❖ The method is applicable to **all edible types of olive oil** (EC Regulation 1234/2007)

❖This **in house validated** (Tsimidou et al., 2019, Molecules, 24(6), 1044) ultra-high liquid chromatographic (UHPLC) procedure covers:

- the PF extraction step,
- the PF hydrolysis step,
- the chromatographic analysis in the UHPLC scale,
- quantification and result expression

The Procedure Step by Step

PF extraction

2.0 g of VOO are accurately weighted (± 0.001) in a 10 mL screw-cap tube

Then, 1 mL of methanol:water, 80:20 v/v is transferred to the previously weighed sample.

The tube is sealed and the sample is vortexed for exactly 30 s.

Then, 5 mL of methanol:water, 80:20 v/v are added

and the sample is further vortexed for exactly 1 min

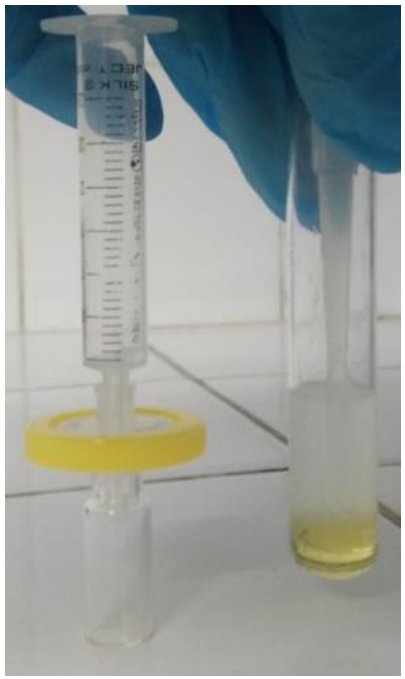
Extraction is carried out in an ultrasonic bath for 15 min at room temperature.

Ultrasonic frequency (kHz) 37
Ultrasonic power effective (W) 80

At the end of the extraction procedure

Centrifuge at 2745 g/min for 25 min

At the end of centrifugation

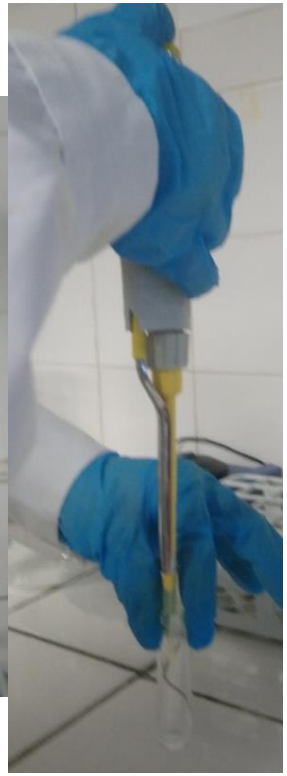


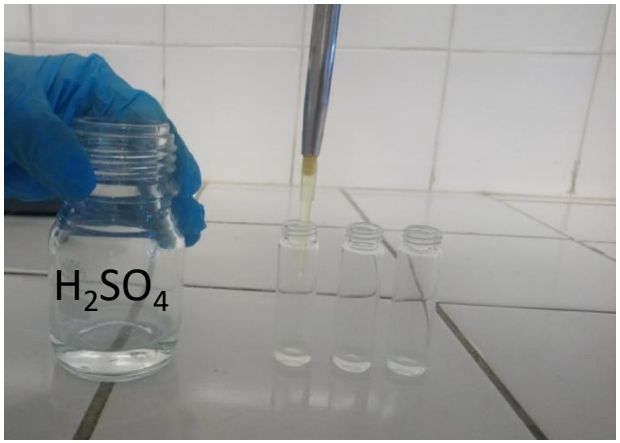
Part of the isolated polar fraction (PF) is filtered through 0.22 µm PVDF membrane

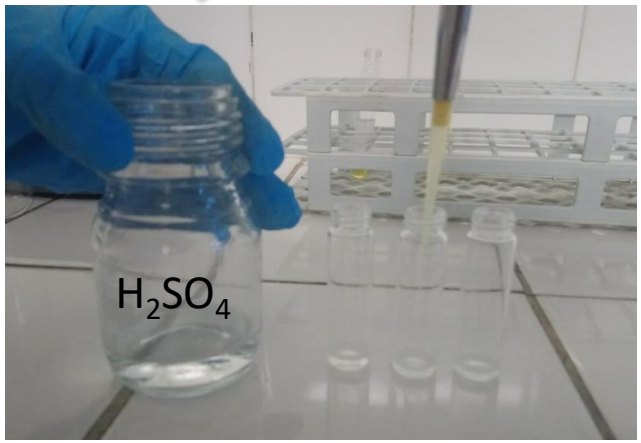
UHPLC-DAD

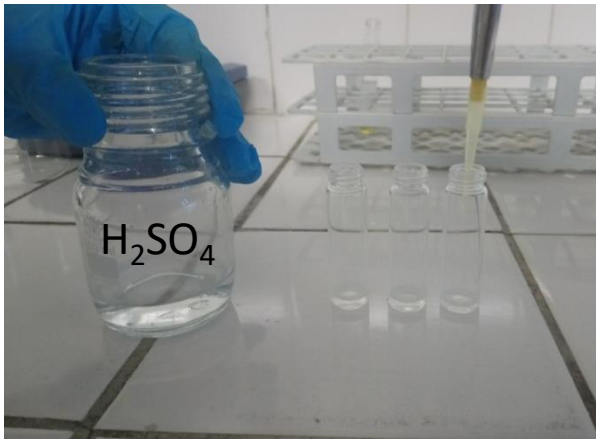
Hydrolysis of PF

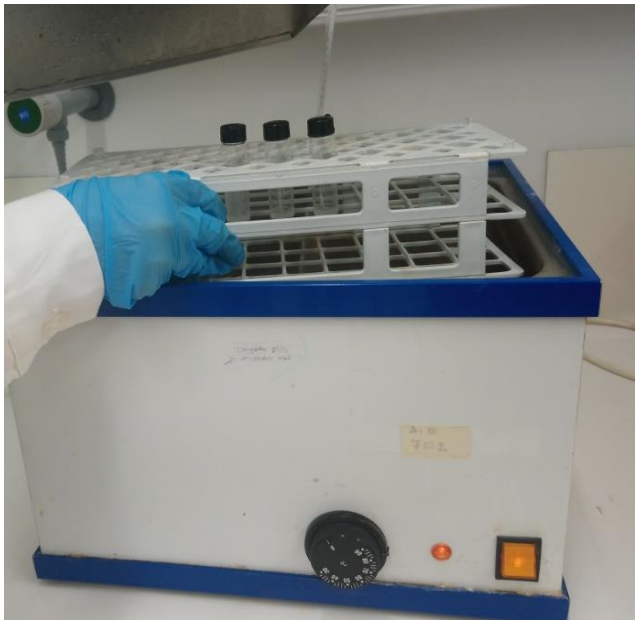
An aliquot (200 µL) of polar fraction is mixed with 200 µL of H_2SO_4 solution (1M). The procedure is carried out in triplicate











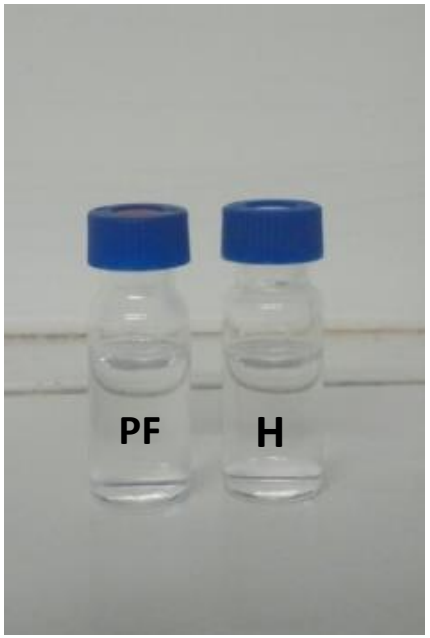
The mixture is maintained in a water bath at 80 °C for 2 h.

Each hydrolysate is diluted with 200 µL of the extracting solvent (methanol:water, 80:20 v/v) immediately after the 2 h of heating

The three replicates are combined to obtain a representative hydrolysate (H)

which is filtered through 0.22 µm PVDF membrane before injected into the chromatograph.

UHPLC-DAD



Load to autosampler

Injection in to the chromatograph

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Using **ultrapure water** and **filtering** all solvents at least via 0.45 µm membrane

Analytical Conditions for UHPLC-DAD				
Mobile phase	Water/Phosphoric acid (0.2% v/v) (A), Methanol (B), Acetonitrile (C)			
Ternary gradient	time (min)	A %	B %	C %
	0	96	2	2
	11	50	25	25
	13	40	30	30
	17	0	50	50
	20	0	50	50
	20.5	96	2	2
Stop time	20.5 min			
Post time	9.5 min			
Column temperature	35 °C			
Flow rate	0.45 mL/min			
Injection volume	3 µL			
Run time	30 min			
Thermostat autosampler	6 °C			
Diode array detector	280 nm/band width ± 4 nm			
Peak width	5 s			

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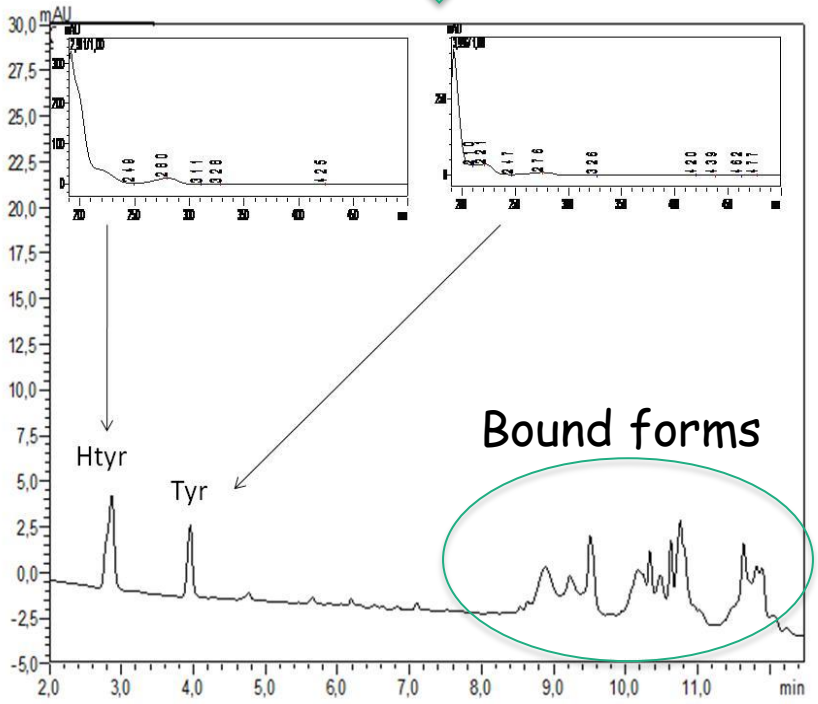
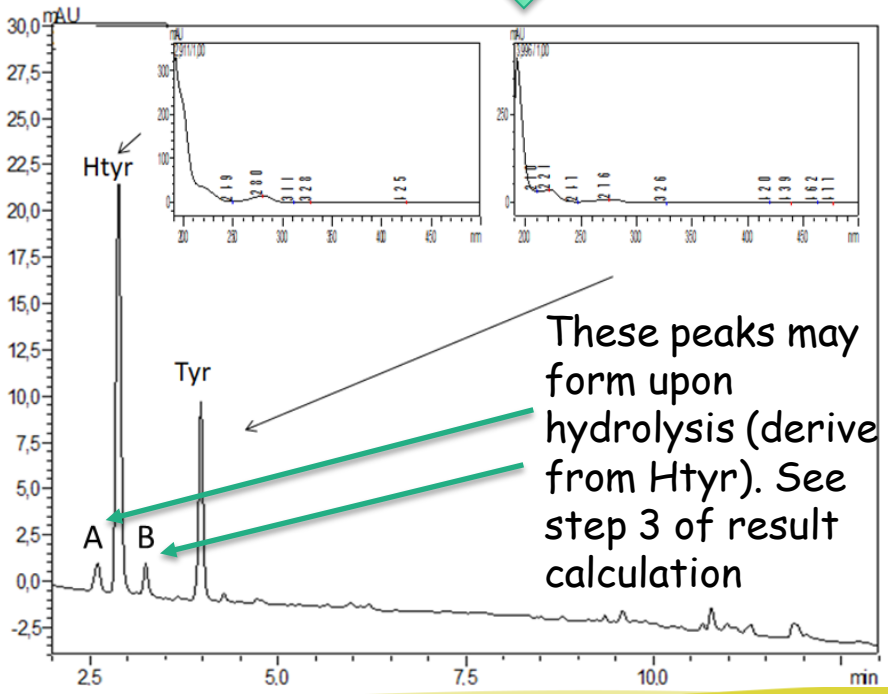
Column: 1.6 µm, 75 × 2.0 mm
(pressure tolerance= 100 Mpa)





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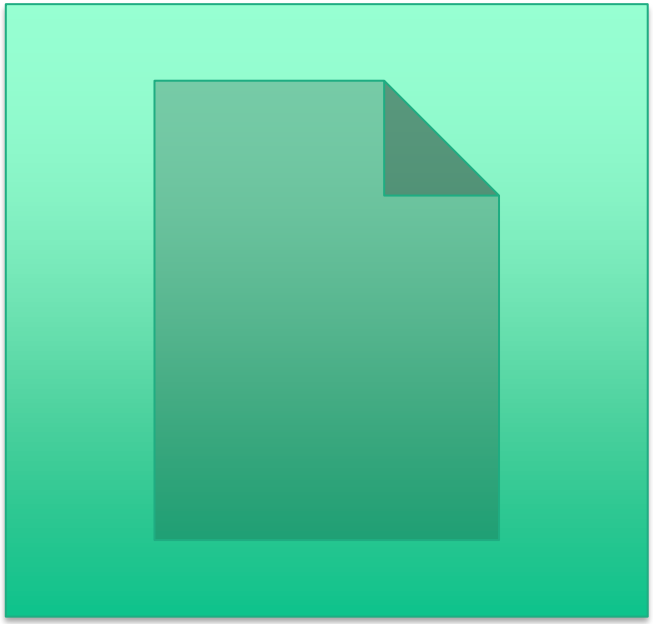
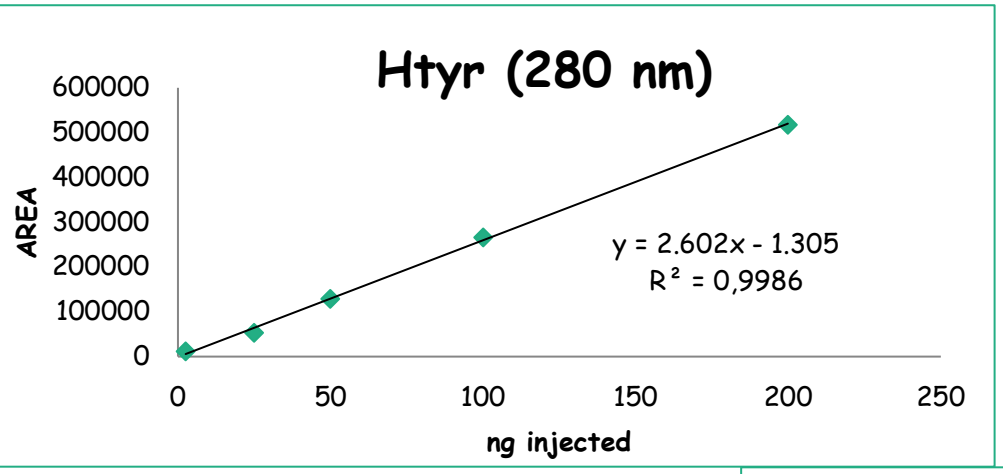
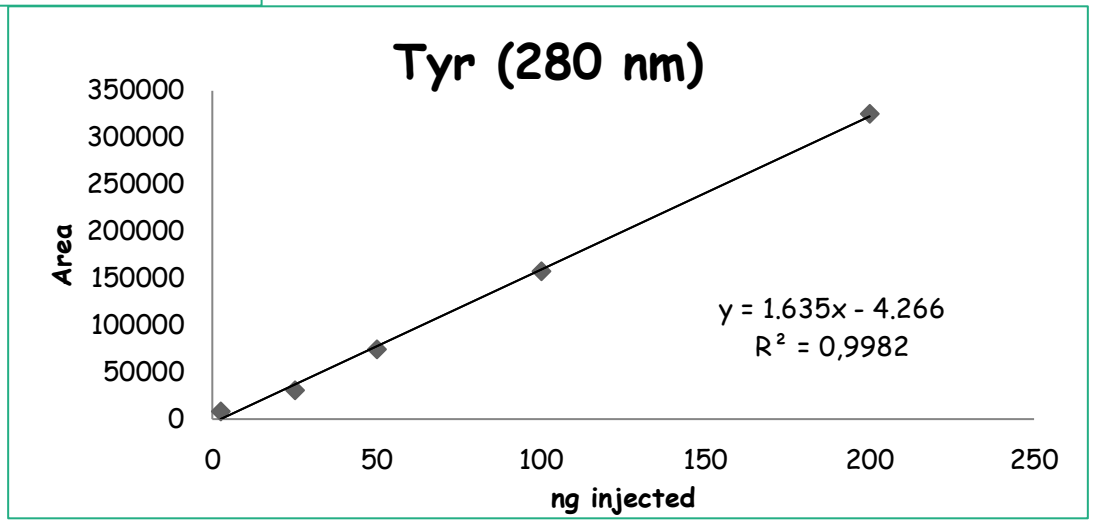
Calculation example



1. Construction of calibration curves of **Htyr** and **Tyr**





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2. Calculate the concentration of the free **Htyr** [$Htyr_{free}$] and the free **Tyr** [Tyr_{free}] by analyzing the PF prior to hydrolysis

F11 | =(C11+1305)/2602

	A	B	C	D	E	F	G	H	I	J
8	Before hydrolysis									
9	Sample		AREA (280 nm)							*Htyr free*
10	injection 3 ul		Htyr			ng/3µL	ng/6mL	µg/6 mL	µg/g	mg/20g
11			17030			7,046503	14093,01	14,09301	7,046503	0,14
12	Sample A		17512			7,231745	14463,49	14,46349	7,231745	0,14
13										

C11 | =(B11+4266)/1635

	A	B	C	D	E	F	G
8	Before hydrolysis						
9	Sample	AREA (280 nm)					*TYR free*
10	injection 3 ul		ng/3µL	ng/6mL	µg/6mL	µg/g	mg/20g
11		11403	9,583486	19166,97	19,16697	9,573912	0,19
12	Sample A	12063	9,987156	19974,31	19,97431	9,977179	0,20

To be transferred to the columns B and D in the final result sheet

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3. Calculate the concentration of the **hydroxytyrosol hydrolysate** [**Htyr**$_{hydrolysate}$] after acid hydrolysis of PF, by **summing the peak areas of A, Htyr and B peaks when present in the chromatogram**

E5 | =SUM(B5:D5)

	A	B	C	D	E	F	G	H	I	J	K
1											
2					After hydrolysis						
3	Sample		AREA (280 nm)								*Htyr after hydrolysis*
4	injection 3 ul	A	Htyr	B	Sum of the areas A,Htyr,B	ng/3µL	ng/600µL	ng/6 mL	µg/6 mL	µg/g	mg/20g
5	Sample A	25074	83494	20413	128981	50,07148	10014,3	300428,9	300,4289	150,2144504	3,00
6		25811	83707	21010	130528	50,66603	10133,21	303996,2	303,9962	151,9980784	3,04
7											

4. Calculate the concentration of the **tyrosol hydrolysate** [**Tyr**$_{hydrolysate}$] after acid hydrolysis of PF

=(B5+4266)/1635

	A	B	C	D	E	F	G	H
			After hydrolysis					
Sample		AREA (280 nm)						*TYR after hydrolysis*
injection 3 ul			ng/3µL	ng/600µL	ng/6 mL	µg/6 mL	µg/g	mg/20g
Sample A		46768	31,21346	6242,691	187280,7	187,2807	93,64037	1,87
		46585	31,10153	6220,306	186609,2	186,6092	93,30459	1,87

To be transferred to the columns C and E in the final result sheet

5. Calculate the total Htyr and Tyr (mg/20g oil) as:

$$[Htyr_{free}] + [Tyr_{free}] + 2.2 \times [Htyr_{hydrolysate} - Htyr_{free}] + 2.5 \times [Tyr_{hydrolysate} - Tyr_{free}]$$

Final result sheet

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Sample	HTYR free mg/20g oil	HTYR after hydrolysis mg/20g oil	TYR free mg/20g oil	TYR after hydrolysis mg/20g oil	HTYR derived from bound forms	TYR derived from bound forms	correction factor for HTYR bound	correction factor for TYR bound	total HTYR bound mg/20 g oil	total TYR bound mg/20 g oil	total HTYR & TYR mg/20 g oil	total HTYR & TYR mg/20 g oil (average)	final result (total HTYR & TYR mg/20 g
2		0,14	3,00	0,19	1,87	2,86	1,68	2,2	2,5	6,30	4,20	10,84	10,86	11
3		0,14	3,04	0,20	1,87	2,90	1,67			6,37	4,17	10,88		
4	Sample A													

Column **F**: **Htyr derived from bound forms** = $[Htyr_{hydrolysate} - Htyr_{free}]$= column C-column B

Column **G**: **Tyr derived from bound forms** = $[Tyr_{hydrolysate} - Tyr_{free}]$= column E-column D

The result obtained with two decimals is then rounded to the nearest integer

❖Correction factors are introduced in the quantification of total bound **Htyr** (2.2) and total bound **Tyr** (2.5)*

Column **H**: = Total bound Htyr= 2.2 x $[Htyr_{hydrolysate} - Htyr_{free}]$= 2.2 x column F

Column **I**: = Total bound Tyr= 2.5 x $[Tyr_{hydrolysate} - Tyr_{free}]$= 2.5 x column G

*See Tsimidou et al., 2019, Molecules, 24(6), 1044 for explanation

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Pros and Cons of the proposed method

- **Pros**: Short elution time
  unequivocal peak assignment
  commercial availability of standards
  high number of samples analyzed per day
  environmentally friendly, ease of application by common laboratories.
  provides information about the freshness (as Htyr and Tyr is low or absent in fresh oil)

- **Cons**: Need to use correction factors for result expression to account for the difference in MW of the bound forms

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In House Validated UHPLC Protocol for the Determination of the Total Hydroxytyrosol and Tyrosol Content in Virgin Olive Oil Fit for the Purpose of the Health Claim Introduced by the EC Regulation 432/2012 for "Olive Oil Polyphenols"

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