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# Quantification of poliphenols by different validated methods with reference to the heath-claim

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# INTRODUCTION

## HEALTH CLAIM FOR "OLIVE OIL POLYPHENOLS"

### EU regulation 432/2012

"Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"

⇨ The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil.

## OBJECTIVE:

Comparison of three different analytical procedures, already published in the scientific literature or tested/validated in the IOC, ISO and Oleum project frameworks, for the determination of HYTY and TY

# MATERIALS AND METHODS

## Samples

Six different olive oil samples, characterized by a wide range in terms of phenolic compounds content.

## Methods by hydrolysis: extraction of HYTY and TY

### Method 1. Direct hydrolysis

**(Romero and Brenes, 2012; *J. Agric. Food Chem*, 60, 9017–9022; with some modifications)**

**Sample preparation:**

- 1.25 g of oil
- 25 mL HCl 2M
- Agitation 800 rpm, 25-30 °C, 5h
- 1 mL aqueous phase
- Filter: 0.45 µm, cellulose acetate
- Aproximative sample preparation time: 5.5 h

**HPLC conditions:**

- Elution by a gradient
- Phase A: $H_2O/H_3PO_4$ (95:0.5)
- Phase B: $MeOH/H_2O$ (1:1)
- Initial conditions: 90% A
- Flow: 1 mL/min
- Column: RP-HPLC C18 (4.6 mm i.d. X 250 mm; 5 µm particle size)
- Injection volume: 20 µL
- Analysis time: 35 min

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# MATERIALS AND METHODS

## Methods by hydrolysis: extraction of HYTY and TY

### Method 2. Hydrolysis after phenolic extraction
**(PD ISO/TS 23942:2020)**

**Sample preparation:**

- 2 g of oil
- 5 mL MeOH/$H_2O$ (80:20)
- Vortex 1 min
- Ultrasound 15 min
- Centrifuge 5000 rpm, 25 min
- Filter 1 mL aqueous phase
- Dry at 40 °C under $N_2$
- Add 1 mL EtOH/ $H_2O$/ $H_2SO_4$ (50:40:10)
- Bath 40 °C, 1 h
- Ambient T, by a night
- Filter: 0.45 µm, PVDF
- Aproximative sample preparation time: 3 h (overnight)

**HPLC conditions:**

- Elution by a gradient
- Phase A: $H_2O$/$H_3PO_4$ (0.2 %)
- Phase B: MeOH/ACN (1:1)
- Initial conditions: 96% A
- Flow: 1 mL/min
- Column: RP-HPLC C18
  (4.6 mm i.d. X 250 mm; 5 µm particle size)
- Injection volume : 20 µL
- Analysis time: 82 min

# MATERIALS AND METHODS

## Methods by hydrolysis: extraction of HYTY and TY

### Method 3. Hydrolysis after phenolic extraction

**(OLEUM Project, Tsimidou et al., 2019; *Molecules*, 24(6): 1044)**

**Sample preparation:**

- 2 g of oil
- 1 mL MeOH/$H_2O$ (80:20), vortex 30 s
- 5 mL MeOH/$H_2O$ (80:20), vortex 1 min
- Ultrasound 15 min
- Centrifuge 2745 rpm, 25 min
- Filter aqueous phase, 0.22 µm, PVDF
- 200 µL aqueous phase + 200 µL $H_2SO_4$ 1 M, vortex 2 s
- Bath 80 °C, 2 h
- Dilute with 200 µL MeOH/$H_2O$ (80:20)

X 3

- Mix 3 extracts
- Filter: 0.22 µm, PVDF
- Aproximative sample preparation time: 4 h

**UPLC conditions:**

- Elution by a gradient
- Phase A: $H_2O$/$H_3PO_4$ (0.2%)
- Phase B: MeOH/ACN (1:1)
- Initial conditions: 96% A
- Flow: 0.45 mL/min
- Column: Shim-pack XR-ODS III (2 mm i.d. X 75 mm; 1.6 µm particle size)
- Injection volume : 3 µL
- Analysis time: 20.5 min

# Methods by hydrolysis: extraction of HYTY and TY

## Method 3. Hydrolysis after phenolic extraction

### (OLEUM Project, Tsimidou et al., 2019; *Molecules*, 24(6): 1044)

- **Method 3a**

Calculation made by external calibration, without considering the compounds A and B and the correction factors proposed in the framework of OLEUM project)

- **Method 3b**

Calculation made considering the compounds A and B, and the correction factors established in the OLEUM method

### 1.8. Calculations and Data Analysis

**1.8.1. Expression of Results**

The total amount of **Htyr** and **Tyr** is calculated as the sum of their free and bound forms after correction for molecular mass differences between free and bound forms (see scheme of the method). Correction factors are introduced in the quantification of individual total **Htyr** (2.2) and **Tyr** (2.5), which are obtained by dividing the mean molecular mass of the most known bound forms of **Htyr** and **Tyr** (343 amu) by the molecular mass of **Htyr** (154 amu) and **Tyr** (138 amu), respectively*. The results for each of them are expressed as mg/20 g oil (with two decimal points). Their sum is then rounded to the nearest integer. Taking into calculation for the total **Htyr** content the peak areas of peaks A and B, when present in the chromatograms, no correction for recovery is necessary.

**NOTE: Bound forms used for the derived correction factors were: tyrosol glucoside (300.30 amu), aldehydic form of ligstroside aglycone (362.12 amu), dialdehyde form of ligstroside aglycone (304.34 amu), aldehyde form of oleuropein aglycone (378.37 amu), oleuropein aglycone (378.37 amu), (carboxymethylated) dialdehyde form of oleuropein aglycone (378.37 amu), (decarboxymethylated) dialdehyde form of oleuropein aglycone (320.34 amu), (decarboxymethylated) aldehyde form of oleuropein aglycone (320.34 amu).*

**Equation**: **Total Htyr and Tyr** (mg/20 g oil) = $[Htyr_{free*}] + [Tyr_{free*}] + 2.2 \times [Htyr_{hydrolysate} - Htyr_{free}] + 2.5 \times [Tyr_{hydrolysate} - Tyr_{free}]$.

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

**NOTE: free Htyr or Tyr refers to the analysis of the PF prior to hydrolysis*

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# MATERIALS AND METHODS
## Methods without hydrolysis: extraction of the total phenolic fraction
### Method A. ISOLATION OF PHENOLIC COMPOUNDS BY SPE-DIOL

INTERNATIONAL OLIVE COUNCIL

COI/T.20/Doc. No 29/Rev.2
June 2022

ENGLISH
Original: ITALIAN

**Sample preparation:**

- 2.5 g of oil
- 500 µL IS
- Shake and evaporate (6-7 min) under $N_2$
- Activate the cartridge SPE: 6 mL MeOH, 6 mL *n*-hexane
- Dissolve the oil sample (6 mL *n*-hexane)
- Placed sample onto the activated SPE cartridge
- Leave it enter into the cartridge
- Wash the sample container (6 mL *n*-hexane), add it to the cartridge and discard
- Add 4 mL hexane:ethyl acetate (85:15) and discard
- Elute the analytes with 10 mL MeOH and collect
- Evaporate in a rotary evaporator
- Dissolve the residue: 500 µL, MeOH/$H_2O$ (1:1)
- Filter: 0.45 µm, cellulose acetate
- Aproximative sample preparation time: 3 h

**HPLC conditions:**

- Elution by a gradient
- Phase A: $H_2O$/$H_3PO_4$ (95.5:0.5)
- Phase B: MeOH/AcN (1:1)
- Initial conditions: 95% A
- Flow: 1 mL/min
- Column: RP-HPLC C18 column (4.0 mm i.d. x 250 mm; 5 µm particle size)
- Injection volume : 20 µL
- Analysis time: 50 min

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# MATERIALS AND METHODS

## Methods without hydrolysis: extraction of the total phenolic fraction

### Method B. IOC method (COI/T. 20/Doc No 29)

**Sample preparation:**

- 2 g of oil
- 1 mL IS (syringic acid), shake 30s
- 5 mL MeOH/$H_2O$ (80:20)
- Vortex 1 min
- Ultrasound 15 min
- Centrifuge 5000 rpm, 25 min
- Take supernatant
- Filter: 0.45 µm, PVDF
- Aproximative sample preparation time: 1 h

**HPLC conditions:**

- Elution by a gradient
- Phase A: $H_2O$/$H_3PO_4$ (0.2 %)
- Phase B: MeOH/ACN (1:1)
- Initial conditions: 96% A
- Flow: 1mL/min
- Column: RP-HPLC C18
  (4.6) mm i.d. X 250 mm; 5 µm particle size)
- Injection volume : 20 µL
- Analysis time: 82 min

# MATERIALS AND METHODS

## Methods without hydrolysis: extraction of the total phenolic fraction

### Method C. Folin-Ciocalteu method

**Sample preparation (method B without IS):**

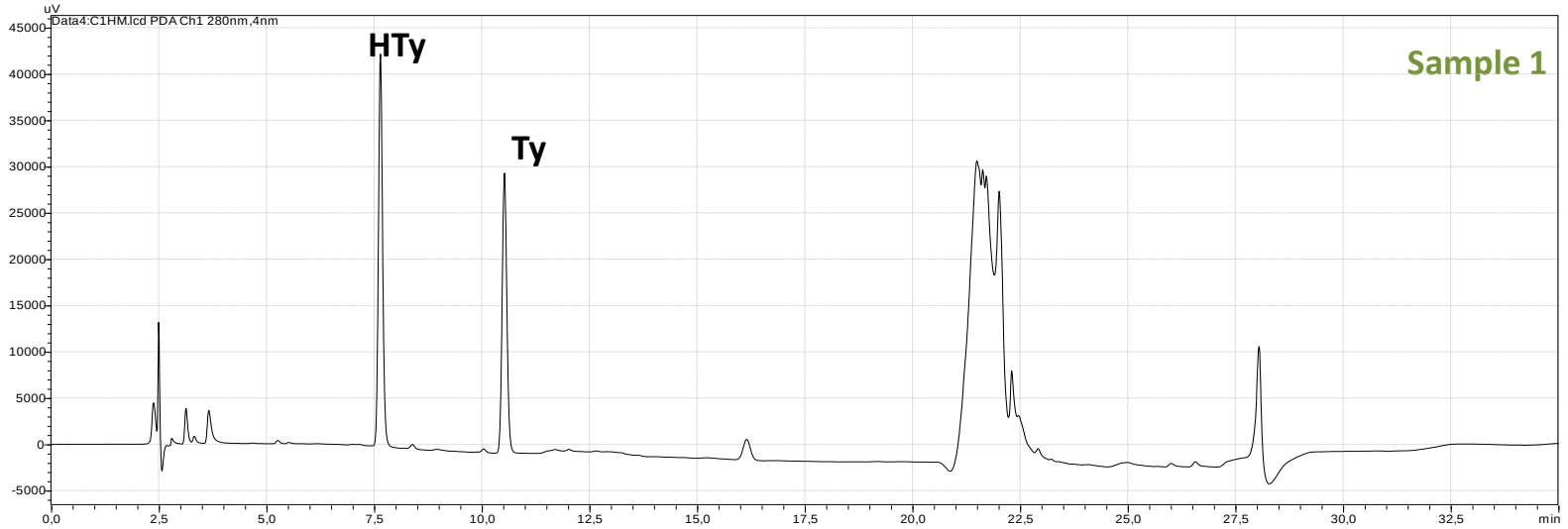
- 2 g of oil
- 6 mL MeOH/$H_2O$ (80:20)
- Vortex 1 min
- Ultrasound 15 min
- Centrifuge 5000 rpm, 25 min
- Take supernatant
- Filter: 0.45 µm, PVDF

**Determination of total phenolic:**

- 0.2 mL of the phenolic extract + 0.5 mL FC reagent + 2 mL of $Na_2CO_3$ (15%), in a 10 mL volumetric flask reaching the final volume with purified water
- Store for 2 h at room temperature
- Phenolic compounds were detected at 750 nm and quantified using gallic acid (PM 170) calibration curve

# RESULTS
# Methods by hydrolysis

## Method 1. Direct hydrolysis



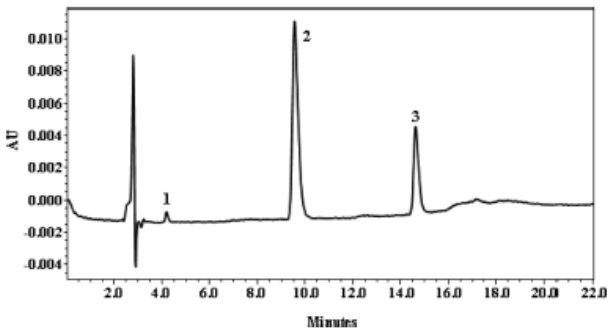




**Figure 2.** HPLC chromatogram of phenolic compounds obtained from extra virgin olive oil treated with 2 M HCl. They were monitored by UV detection at 280 nm. Peaks: (1) hydroxytyrosol glycol; (2) hydroxytyrosol; (3) tyrosol.

| Sample | HyTy (mg/20 g) | S | Ty (mg/20 g) | S | HyTy+Ty (mg/20 g) |
|---|---|---|---|---|---|
| **Sample 1** | 6.27 | 0.64 | 6.64 | 0.72 | 12.91 |
| **Sample 2** | 6.06 | 0.33 | 4.35 | 0.09 | 10.40 |
| **Sample 3** | nd | nd | 1.16 | 0.04 | 1.16 |
| **Sample 4** | 0.76 | 0.05 | 2.26 | 0.04 | 3.02 |
| **Sample 5** | 2.22 | 0.15 | 3.59 | 0.21 | 5.81 |
| **Sample 6** | 1.88 | 0.08 | 3.07 | 0.03 | 4.95 |

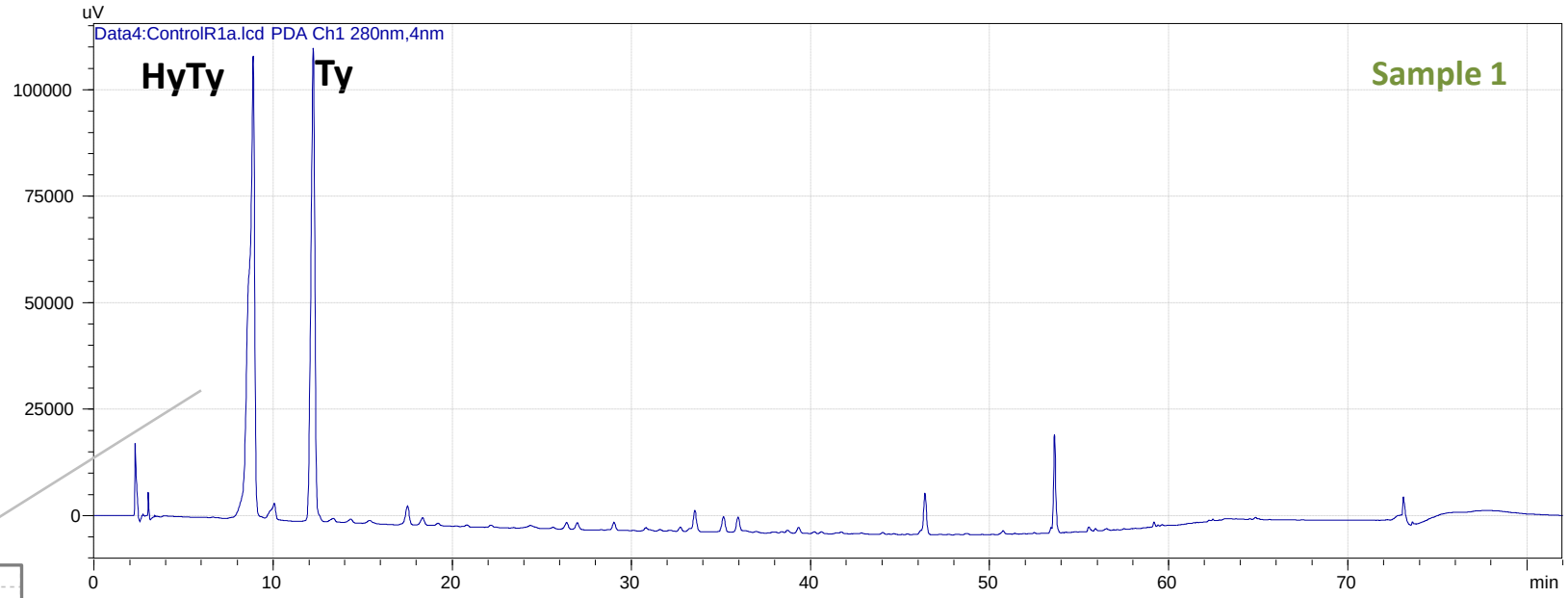
**Table 1. Gradient elution timetable**

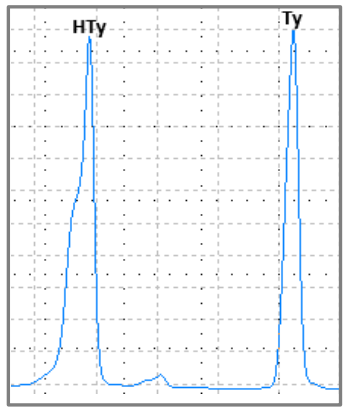
| Time (min) | %A | %B |
|---|---|---|
| 0 | 90 | 10 |
| 10 | 70 | 30 |
| 18 | 70 | 30 |
| 19 | 0 | 100 |
| 25 | 0 | 100 |
| 26 | 90 | 10 |
| 35 | 90 | 10 |

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# Method 2. Hydrolysis after phenolic extraction
## (PD ISO/TS 23942:2020)



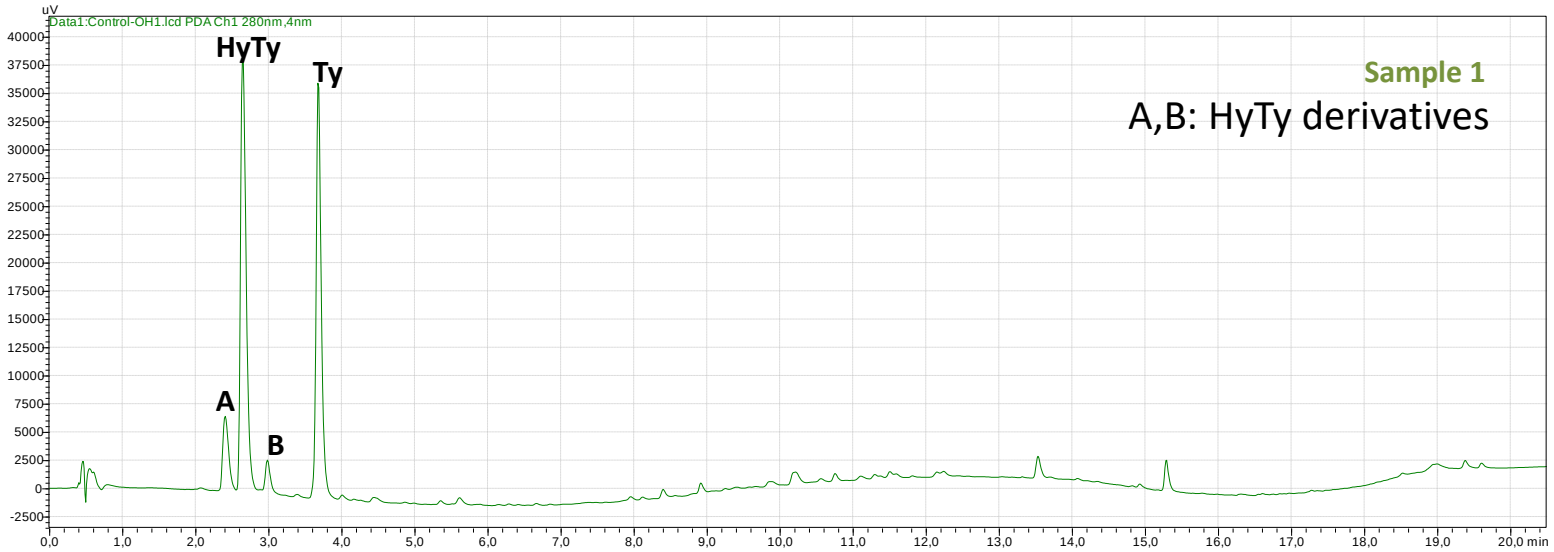



| Sample | HyTy (mg/20 g) | S | Ty (mg/20 g) | S | HyTy+Ty (mg/20 g) | INTERNATIONAL COLABORATIVE TRIAL 2021 HyTy+Ty (mg/20 g) |
|---|---|---|---|---|---|---|
| **Sample 1** | 6.24 | 0.59 | 5.87 | 0.60 | 12.11 | - |
| **Sample 2** | 5.89 | 0.08 | 3.71 | 0.05 | 9.61 | 8.65 |
| **Sample 3** | 0.15 | 0.00 | 0.86 | 0.01 | 1.02 | 0.99 |
| **Sample 4** | 1.37 | 0.03 | 2.17 | 0.08 | 3.54 | 2.90 |
| **Sample 5** | 2.32 | 0.20 | 2.77 | 0.23 | 5.09 | 4.90 |
| **Sample 6** | 1.97 | 0.04 | 2.29 | 0.04 | 4.26 | 3.94 |

# Method 3. Hydrolysis after phenolic extraction (OLEUM Project)

## RESULTS
## Methods by hydrolysis




| Sample | HyTy (mg/20 g) | S | Ty (mg/20 g) | S | HTy+Ty (mg/20 g) |
|---|---|---|---|---|---|
| **Sample 1** | 6.42 | 0.01 | 7.23 | 0.01 | 13.65 |
| **Sample 2** | 6.03 | 0.01 | 4.37 | 0.00 | 10.40 |
| **Sample 3** | 0.20 | 0.00 | 1.11 | 0.00 | 1.31 |
| **Sample 4** | 1.20 | 0.00 | 2.36 | 0.00 | 3.56 |
| **Sample 5** | 2.62 | 0.00 | 3.56 | 0.00 | 6.18 |
| **Sample 6** | 1.99 | 0.00 | 2.92 | 0.00 | 4.91 |

Method 3a: calculation made without considering the compounds A and B and the correction factors established in the oleum method

| Sample | A, B, HyTy e Ty (mg/20 g oil) | S |
|---|---|---|
| **Sample 1** | 35.24 | 0.05 |
| **Sample 2** | 25.56 | 0.01 |
| **Sample 3** | 3.08 | 0.01 |
| **Sample 4** | 8.52 | 0.01 |
| **Sample 5** | 14.97 | 0.02 |
| **Sample 6** | 12.20 | 0.01 |

Method 3b: calculation made considering the compounds A and B,and the correction factors established in the oleum method

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# Comparison of methods by hydrolysis

| Sample | Method 1 HyTy+Ty (mg/20 g) | Method 2 HyTy+Ty (mg/20 g) | Method 3a HyTy+Ty (mg/20 g) | Method 3b A, B, HyTy e Ty (mg/20 g oil) | Method 3b/Method 1 ratio |
|---|---|---|---|---|---|
| **Sample 1** | 12.91[a] | 12.11 [a] | 13.65 [a] | 35.24 | 2.73 |
| **Sample 2** | 10.40[a] | 9.61[b] | 10.40[a] | 25.56 | 2.46 |
| **Sample 3** | 1.16[b] | 1.02[c] | 1.31[a] | 3.08 | 2.65 |
| **Sample 4** | 3.02[b] | 3.54[a] | 3.56[a] | 8.52 | 2.82 |
| **Sample 5** | 5.81[a] | 5.09[b] | 6.18[a] | 14.97 | 2.57 |
| **Sample 6** | 4.95[a] | 4.26[b] | 4.91[a] | 12.20 | 2.46 |

*Different letters in the same row indicate statistically significant differences (ANOVA Tukey sHSD test,p≤ 0.05

# Total phenolic determination by Folin-Ciocalteu method

The quantification is done using the MW of gallic acid (170)

| Sample | Phenols concentration (mg/Kg) | S | Phenols concentration (mg/20 g) |
|---|---|---|---|
| **Sample 1** | 606.2 | 22.5 | 12.12 |
| **Sample 2** | 525.6 | 11.4 | 10.51 |
| **Sample 3** | 175.0 | 22.5 | 3.50 |
| **Sample 4** | 204.4 | 7.9 | 4.09 |
| **Sample 5** | 275.9 | 27.4 | 5.52 |
| **Sample 6** | 280.4 | 9.5 | 5.61 |

Correction of molecular weights of simple / complex phenols. E.g. MW HyTy = 154, MW Ty = 138 and MW Oleuropein = 540

# Conclusions: comparison of three different hydrolytic methods

The three hydrolytic methods give **comparable results**

From the samples analyzed there is **no evidence of an underestimation** by any of the methods

All methods quantify as tyrosol and hydroxytyrosol (or gallic acid for the Folin-Ciocalteu) and therefore **a correction factor should be applied**

Analyzes are ongoing to compare the results with IOC methods that quantify individual polyphenols

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# Comparison of three different methodologies for the determination of hydroxytyrosol and tyrosol in relation to the health claim for olive oils

**Patricia Garcia Salas, Ilaria Grigoletto, Enrico Valli, Alessandra Bendini, Tullia Gallina Toschi**

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