INTERNATIONAL OLIVE COUNCIL

COI/T.20/Doc. No 29/Rev.1
2017

ENGLISH
Original: ITALIAN

Príncipe de Vergara, 154 – 28002 Madrid – España Telef.: +34 915 903 638 Fax: +34 915 631 263 - e-mail: iooc@internationaloliveoil.org - http://www.internationaloliveoil.org/

METHOD OF ANALYSIS

DETERMINATION OF BIOPHENOLS IN OLIVE OILS BY HPLC

1. PURPOSE

This method describes a procedure for the extraction and HPLC quantification of the biophenolic minor polar (BMP) compounds in olive oils, such as the natural and oxidised derivatives of oleuropein and ligstroside, lignans, flavonoids and phenolic acids. The range of measurement is from 30 mg/kg to 800 mg/kg.

WARNING: This method may require the use of dangerous apparatus and chemicals or the performance of dangerous operations. It does not specify all the safety issues connected with its use. Users are therefore responsible for taking all appropriate safety measures beforehand and for observing any legal requirements.

2. PRINCIPLE

The method is based on direct extraction of the biophenolic minor polar compounds from olive oil by means of a methanol solution and subsequent quantification by HPLC with the aid of a UV detector at 280 nm. Syringic acid is used as the internal standard.

The content of the natural and oxidised oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids is expressed in mg/kg of tyrosol.

3. EQUIPMENT

3.1. **High-performance ternary gradient liquid chromatograph** (HPLC), equipped with C18 reverse-phase column (4.6 mm x 25 cm), type Spherisorb ODS-2 5µm, 100 A°, with spectrophotometric UV detector at 280 nm and integrator. Room temperature.

Spectral recording for identification purposes is facilitated by using a photodiode detector with a spectral range from 200 nm to 400 nm.

3.2. **Flasks,** 10 mL and 100 mL, Class A.

3.3. **Pipette,** 100 µL, 1000 µL and 5000 µL.

3.4. Test tubes, with screw cap, 10 mL.

3.5. Agitator for test tubes[1]

3.6. Ultrasonic extraction bath.

3.7. Syringe filters Ø13 mm, PVDF type 0.45 µm.

3.8. Centrifuge capable of working at a speed of 5000 min^{-1}.

3.9. Balance, accurate to ± 0.001 g.

3.10. Plastic syringes, 5 mL.

3.11. Usual laboratory glassware.

4. REAGENTS

Reagents should be pure HPLC chromatography grade.

4.1. Orthophosphoric acid, 85% (V/V).

4.2. Methanol, chromatography grade.

4.3. Acetonitrile, chromatography grade.

4.4. Water, chromatography grade.

4.5. Ternary linear elution gradient: water 0.2% H_3PO_4 (V/V) (A), methanol (B), acetonitrile (C). Elution solvents should be de-gassed.

Gradient elution should be performed as follows:

Gradient elution

Time min	Flow mL/min	A %	B %	C %
0	1.00	96	2	2
40	1.00	50	25	25
45	1.00	40	30	30
60	1.00	0	50	50
70	1.00	0	50	50
72	1.00	96	2	2
82	1.00	96	2	2

4.6. 2- (4 - hydroxyphenyl) ethanol (tyrosol) ≥ 98%.

4.7. 3,5 dimethoxy 4- hydroxy benzoic acid (syringic acid) ≥ 97%.

[1] Vortex type.

4.8. Extraction solution: methanol/water 80/20 (V/V).

4.9. Solution of external calibration standards (tyrosol and syringic acid). Accurately weigh 0.030 g of tyrosol (4.6) and 0.015 g of syringic acid (4.7) into a 10 mL volumetric flask (3.2). Make up to volume with the solution of methanol/water 80/20 (V/V) (4.8).

Transfer 100 µL (3.3) of the solution to a 10 ml volumetric flask. Make up to volume with the solution of methanol/water 80/20 (V/V) (4.8).

The concentrations of the external calibration solution are as follows: tyrosol 0.030 mg/mL, syringic acid 0.015 mg/mL.

The solution is stable if kept for three months in the refrigerator at +4 °C.

4.10. Preparation of the internal standard solution (syringic acid). Weigh accurately 0.015 g (4.7) of syringic acid into a 10 ml volumetric flask and make up to volume with the solution of methanol/water 80/20 (V/V) (4.8). Transfer 1 mL (3.3) of the solution to a 100 mL volumetric flask (3.2). Make up to volume with the solution of methanol/water 80/20 (V/V) (4.8). The final concentration is 0.015 mg/mL.

The solution is stable if kept for three months in the refrigerator at +4 °C.

5. PROCEDURE

5.1. Sample preparation

In a 10 mL screw-cap test tube (3.4) accurately weigh 2.0 g of olive oil.

Transfer 1 mL of the internal standard solution (4.10) to the previously weighed sample.

Seal with the screw cap and shake (3.5) for exactly 30 s.

Add 5 mL (3.3) of the methanol/water 80/20 (V/V) extraction solution (4.8).

Shake (3.5) for exactly 1 min.

Extract in the ultrasonic bath (3.6) for 15 min at room temperature.

Centrifuge at 5000 rev/min for 25 min (3.8).

Take an aliquot of the supernatant phase and filter through a 5 mL plastic syringe (3.10), with a 0.45 µm PVDF filter (3.7).

5.2. HPLC analysis

Switch on the UV spectrophotometer at least 1 h before analysis.

The chromatography column should be conditioned for at least 15 min with the elution solvent (initial composition) (water 0.2 % H_3PO_4 (V/V) /methanol/acetonitrile 96/2/2 (V/V/V)) (gradient elution).

A preliminary empty gradient chromatographic run should always be done (to make sure there are no interfering co-elution peaks) by injecting 20 µL of methanol/water 80/20 (V/V) (4.8) into the HPLC system.

Inject 20 µL of the external calibration standard solution (4.9) and record the chromatogram at 280 nm. Calculate the values of the response factors RF for 1 µg of tyrosol and 1 µg of syringic acid (6.1).

Calculate the ratio of the response factor of syringic acid to tyrosol, called $RRF_{syr/tyr}$. Note down the values (6.2).

Inject 20 µL of the final sample solution into the HPLC system and record the chromatogram at 280 nm.

Perform two independent determinations on the same sample and check that the results lie inside the precision values of the method.

Figure 1 shows a typical chromatogram of the biophenols in an extra virgin olive oil characterised by individual component.

The sum of the areas of the individual peaks should be taken into account to calculate the total content.

At the end of the day flush methanol/acetonitrile 1/1 (V/V) through the chromatographic column at a rate of 1.0 mL/min for at least 15 min and store the column in methanol/acetonitrile 1/1 (V/V).

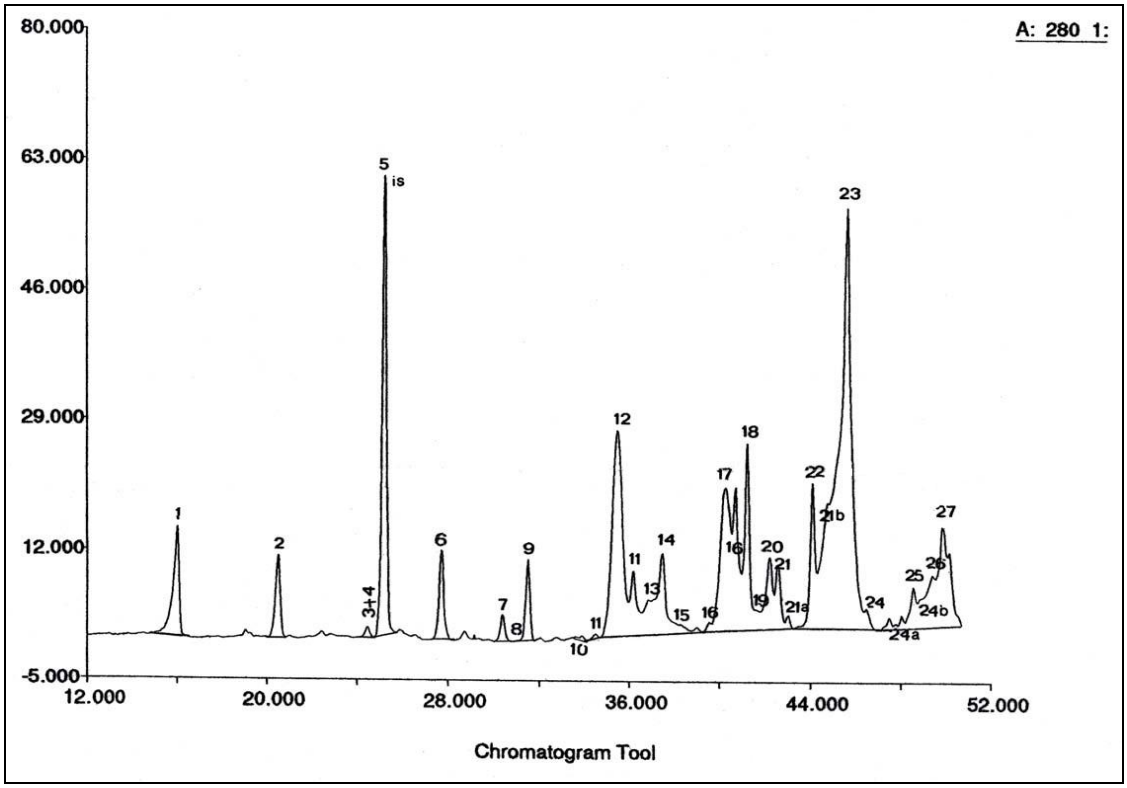

Figure 1. HPLC chromatogram recorded at 280 nm for biophenols profile present in an extra virgin olive oil

6. EXPRESSION OF RESULTS

6.1. Calculation of the response factors of the external calibration standards (RF)

$RF_{1\ \mu g}$ (syringic acid) = Area syringic acid/ µg syringic acid injected

$RF_{1\ \mu g}$ (tyrosol) = Area tyrosol/ µg tyrosol injected

6.2. Calculation of the ratio between the two response factors (RRF)

$RRF_{syr/tyr} = RF_{1\ \mu g}$ (syringic acid)/ $RF_{1\ \mu g}$ (tyrosol)

The value of $RRF_{syr/tyr}$ should be constant and should lie inside the range 5.1 ± 0.4. It enables the final result to be expressed as tyrosol, using syringic acid as the internal standard.

6.3. Calculation of the biophenol content of virgin olive oil

Biophenol content (hydroxytyrosol, tyrosol, natural and oxidised oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids), expressed in mg/kg, is calculated by measuring the sum of the areas of the related chromatographic peaks (identification in **Table 1**) according to the following formula, the result is expressed without decimal place.

$$(\text{mg/kg}) = \frac{(\Sigma A) \times 1000 \times RRF_{syr/tyr} \times (\text{W syr. acid})}{(\text{A syr. acid}) \times (\text{W})}$$

where:

(ΣA): is the sum of the peak areas of the biophenols (hydroxytyrosol, tyrosol, natural and oxidised oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids) recorded at 280 nm;

A syr. acid: is the area of the syringic acid internal standard recorded at 280 nm;

1000: is the factor used to express the result in mg/kg;

W: is the weight of the oil used, in g;

$RRF_{syr/tyr}$: is the multiplication coefficient for expressing the final results as tyrosol;

W syr. acid: is the weight, in mg, of the syringic acid used as internal standard in 1 mL of solution added to the sample.

Table 1
Identification of biophenols peaks.
Maximum absorbance (max UV abs) values and relative retention times (RRT)*

Peak No	Biophenols	RRT*	Max. UV abs. nm
1	Hydroxytyrosol	0.62	230-280
2	Tyrosol	0.80	230-275
3	Vanillic acid	0.96	260
4	Caffeic acid	0.99	325
5	Syringic acid (internal standard)	1.00	280
6	Vanillin	1.10	310
7	Para-coumaric acid	1.12	310
8	Hydroxytyrosyl acetate	1.20	232-285
9	Ferulic acid	1.26	325
10	Ortho-coumaric acid	1.31	325
11;11a	Decarboxymethyl oleuropein aglycone, oxidised dialdehyde form	-	235-280
12	Decarboxymethyl oleuropein aglycone, dialdehyde form	1.45	235-280
13	Oleuropein	1.48	230-280
14	Oleuropein aglycone, dialdehyde form	1.52	235-280
15	Tyrosyl acetate	1.54	230-280
16;16a	Decarboxymethyl ligstroside aglycone, oxidised dialdehyde form	1.63	235-275
17	Decarboxymethyl ligstroside aglycone, dialdehyde form	1.65	235-275
18	Pinoresinol, 1 acetoxy-pinoresinol	1.69	232-280
19	Cinnamic acid	1.73	270
20	Ligstroside aglycone, dialdehyde form	1.74	235-275
21;21a;21b	Oleuropein aglycone, oxidised aldehyde and hydroxylic form	-	235-280
22	Luteolin	1.79	255-350
23	Oleuropein aglycone, aldehyde and hydroxylic form	1.87	235-280
24;24a;24b	Ligstroside aglycone, oxidised aldehyde and hydroxylic form	-	235-275
25	Apigenin	1.98	230-270-340
26	Methyl-luteolin	-	255-350
27	Ligstroside aglycone, aldehyde and hydroxylic form	2.03	235-275

(*) The relative retention time is calculated with respect to the retention time of syringic acid. Identification is performed by HPLC-MS.

7. TEST REPORT

The test report should specify the following information:

(a) The reference of this method.
(b) The test results, expressed in mg/kg of oil (no decimal places).
(c) The RRF value used for calculations.
(d) Any departure from this method, made by agreement between the parties concerned or for any other reason.
(e) The identification details of the laboratory, the date on which the test was performed and the signature of the test supervisor.

PRECISION VALUES

1. Analysis of the collaborative test results

The precision values of the method are given in the attached table.

Seventeen laboratories holding IOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 2008. The laboratories were from eight different countries.

Sample A – Extra virgin olive oil (Italy)
Sample B – Extra virgin olive oil (Spain)
Sample C – Extra virgin olive oil (Tunisia)
Sample D – Extra virgin olive oil (Slovenia)
Sample E – Extra virgin olive oil (Greece)
Sample R – Extra virgin olive oil (Italy)

The results of the collaborative test organised by the IOC Executive Seecretariat were statistically processed according to the rules laid down in the international standards ISO 5725.

Accuracy (trueness and precision) of measurement methods and results.

Outliers were examined by applying the Cochran and Grubbs tests to the laboratory results for all the determinations (replicates a and b).

The table lists:

n	number of participating laboratories.
Outliers	number of laboratories with outlying values.
Mean	mean of the accepted results.
r	value below which the absolute difference between two single independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time may be expected to lie with a probability of 95%.
Sr	repeatability standard deviation.
RSDr	(%) repeatability coefficient of variation (Sr x 100 / mean).
R	value below which the absolute difference between two single test results obtained with the same method on identical test material in different laboratories with different operators using different equipment may be expected to lie with a probability of 95%.
S_R	reproducibility standard deviation.
RSD_R	(%) reproducibility coefficient of variation (SR x 100/mean).

Precision values for total biophenol content, (mg/kg)

	A	B	C	D	E	R
n	17	17	17	17	17	17
outliers	3	3	1	2	2	2
mean	694	573	153	343	297	301
r	29	36	18	24	22	17
S_r	10.4	12.7	6.4	8.7	7.7	6.2
RSD_r(%)	1.5	2.2	4.2	2.5	2.6	2.1
R	100.8	83.7	59.6	62.7	77.0	32.2
S_R	36.0	29.9	21.3	22,4	27,5	11,5
RSD_R(%)	5.2	5.2	14.0	6.5	9.3	3.8

2. References

ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results
Part 1: General principles and definitions.

ISO 5725-2:1994 Accuracy (trueness and precision) of measurement methods and results
Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method.

ISO 5725:5:1998 Accuracy (trueness and precision) of measurement methods and results
Part 5: Alternative methods for the determination of the precision of a standard measurement method.

ISO 5725:6:1994 Accuracy (trueness and precision) of measurement methods and results
Part 6: Use in practice of accuracy values