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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/

# DOCUMENT TO DECLARE THE USE OF IOC METHODS FOR PHENOLIC COMPOUNDS DETERMINATION

## METHOD 1: COI/T.20/Doc. No 29/Rev.1 2017. DETERMINATION OF BIOPHENOLS IN OLIVE OILS BY HPLC

### PURPOSE

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

### PRINCIPLE

The method is based on the direct extraction of the BMP 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 ligustroside derivatives, lignans, flavonoids and phenolic acids is expressed in mg/kg of tyrosol.

## METHOD 2: DETERMINATION OF PHENOLIC COMPOUNDS IN OLIVE OILS BY SPE-HPLC-DAD

### PURPOSE

The standard provides guidance for the determination of phenolic compounds, free and bound, in olive oils following their extraction by solid phase extraction (SPE).

The phenolic compounds in olive oil may comprehend natural and oxidised derivatives of oleuropein and ligustroside, lignans, flavonoids and phenolic acids.

The fraction is analysed by HPLC-DAD to obtain the concentration of individual phenolic compounds and the total amount in mg or mmol per kg of oil.

**PRINCIPLE**

The method is based on the direct extraction of the phenolic compounds from olive oil by SPE on diol-phase cartridges and subsequent quantification directly by HPLC-DAD.

**Differences**

Both methods isolate the phenolic fraction from olive oils by different means: liquid-liquid extraction vs SPE extraction. In collaborative tests performed within the IOC, differences were observed when comparing the results obtained by the different methods due to the saturation of the liquid phase as the concentration of phenol increases (method COI/T.20/Doc. No 29 includes a range of determination, whereas the new method does not). The use of only an internal standard may underestimate flavonoids and ligustrosides. The new method also uses response factors for the different phenolic compounds whereas method No 29 quantifies using tyrosol and therefore may underestimate the true value.

**USES**

Method COI/T.20/Doc. No 29 may be used to perform a quick determination of phenols using a simpler method, whereas the new method can be used to determine the real concentration of phenols to fulfil an EFSA claim as well as the content of individual phenols (e.g., oleocanthal and oleoscein).

# METHOD OF ANALYSIS No 1

# DETERMINATION OF BIOPHENOLS IN OLIVE OILS BY HPLC

## 1. PURPOSE

This method describes a procedure for the extraction and HPLC quantification of 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 the direct extraction of BMP 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**.

[1] Vortex type.

**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/mi | 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%.**

**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 seconds.

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

Shake (3.5) for exactly 1 minute.

Extract in the ultrasonic bath (3.6) for 15 minutes 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 hour before analysis.

The chromatography column should be conditioned for at least 15 minutes 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 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 considered 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 minutes and store the column in methanol/acetonitrile 1/1 (V/V).

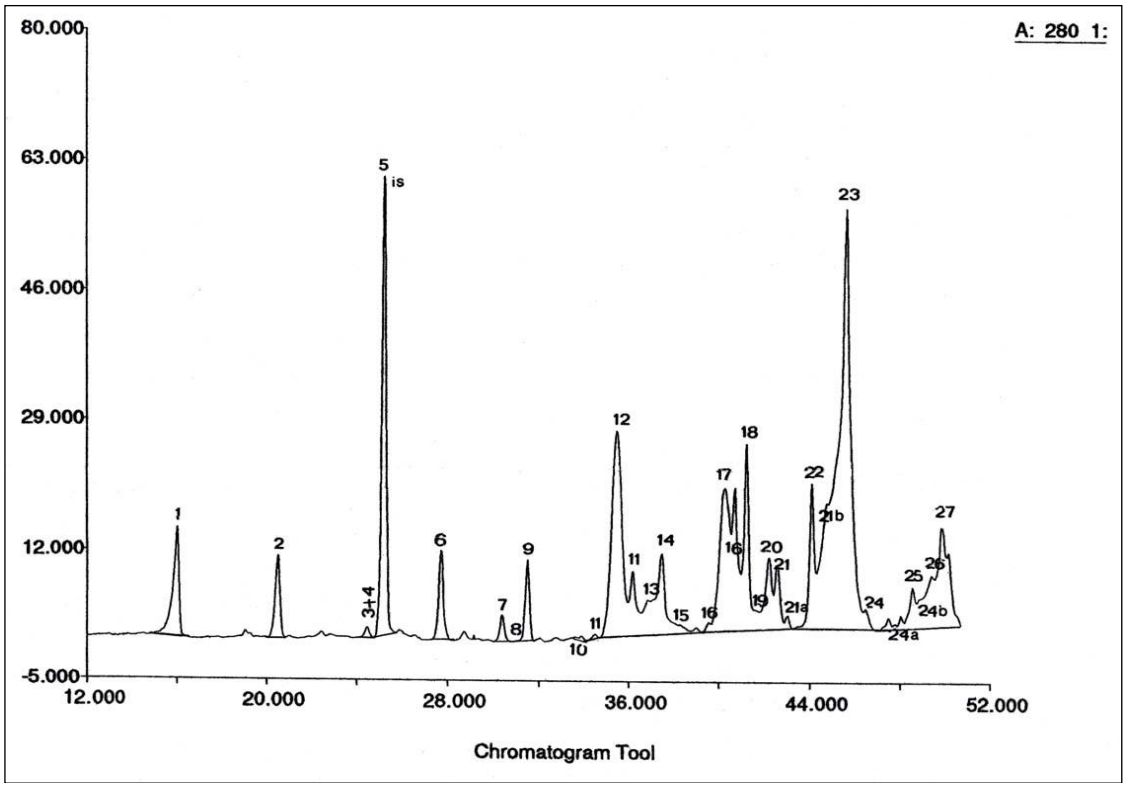



**Figure 1.** HPLC chromatogram recorded at 280 nm for biophenols profile present in an extra virgin olive oil

## 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 biophenols content of virgin olive oil

Biophenols 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 to no 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.

The 17 laboratories that have IOC recognition at the time of writing took part in the collaborative test arranged by the Executive Secretariat of the IOC 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 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

# METHOD OF ANALYSIS No 2

# DETERMINATION OF PHENOLIC COMPOUNDS IN OLIVE OILS

## 1. PURPOSE

The standard provides guidance for the determination of phenolic compounds, free and bound, in olive oils following their extraction by solid phase extraction (SPE).

The phenolic compounds in olive oil may comprehend natural and oxidised derivatives of oleuropein and ligustroside, lignans, flavonoids and phenolic acids.

The fraction is analysed by HPLC-DAD to obtain the concentration of individual phenolic compounds and the total amount in mg or mmol per kg of oil.

## 2. PRINCIPLE

The method is based on the direct extraction of phenolic compounds from olive oil by SPE on diol-phase cartridges and subsequent quantification by HPLC-DAD.

# ANALYSIS OF THE PHENOLIC FRACTION OF OLIVE OILS

## 1. SCOPE AND FIELD OF APPLICATION

This method clarifies the isolation of the phenolic compounds, free and bound, from olive oils using SPE cartridges with diol-bonded phase.

## 2. METHODOLOGY

### 2.1. ISOLATION OF PHENOLIC COMPOUNDS BY SPE-DIOL

#### 2.1.1. APPLICABILITY

This method is applicable to the process of extracting the phenolic compounds, free and bound, by SPE cartridges with diol-bonded phase prior to analysis with HPLC-DAD.

#### 2.1.2. REAGENTS AND APPARATUS

2.1.2.1. Methanol, chromatographic quality.
2.1.2.2. n-Hexane, chromatographic quality.
2.1.2.3. SPE cartridge, diol-bonded phase, 3 mL (500 mg).
2.1.2.4. Ethyl acetate, chromatographic quality.
2.1.2.5. Eluting mixture, hexane:ethyl acetate (v/v 85:15).
2.1.2.6. Deionised or distilled Water.
2.1.2.7. Mixture, metanol:water (v/v 1:1).
2.1.2.8. Conic flask, 10 and 25 ml.
2.1.2.9. Vortex mixer.
2.1.2.10. Balance, accurate to ± 0.001 g.
2.1.2.11. Internal standard solution, *p*-hydroxyphenylacetic, 0,12 mg/mL and *o*-coumaric acid, 0,01mg/ml in methanol.
2.1.2.12. Rotary evaporator.
2.1.2.13. Solid phase extraction equipment (vacuum).

### 2.1.3. PROCEDURE

#### 2.1.3.1. Sample preparation

In a 10 mL conic flask (2.1.2.8), weigh approximately 2.5 g of oil, with a precision of 0.001g. Add 500µl of the internal standard solution (2.1.2.11). Shake it gently and evaporate for about 6-7 minutes ina very gentle current of nitrogen or in a rota-evaporator at room temperature.

#### 2.1.3.2. Isolation of the fraction

Place the SPE cartridge (2.1.2.3) in the SPE equipment (2.1.2.13). Activate the cartridge (2.1.2.3) by passing 6 ml of methanol (2.1.2.1) and 6 ml of n-hexane (2.1.2.2) without vacuum. Make sure the cartridge does not dry during elution.

The oil sample from 2.1.3.1 is diluted with 6 ml of n-hexane (2.1.2.2) and placed into the activated SPE cartridge. Let the sample enter the cartridge. Wash the flask (2.1.2.8) with 6 ml of n-hexane and place into the column. Leave it to run out of the cartridge and discard.

Elute with 4 mL of the eluting mixture (2.1.2.5) and discard. Elute now with 10 mL of methanol (2.1.2.1) and collect the elution in a 25 mL conic flask (2.1.2.8). Evaporate in a rotary evaporator at room temperature in a vacuum until dry.

Dissolve the residue in 500 µL of the methanol/water mixture (2.1.2.7). Then shake it vigorously with the aid of a vortex (2.1.2.9). Keep this final solution in dark and cool conditions for at least four hours before determination.

## 2.2. HPLC-DAD DETERMINATION OF THE ISOLATED PHENOLIC COMPOUNDS

### 2.2.1. SCOPE AND FIELD OF APPLICATION

Reference method quantifies the phenolic extract by HPLC at several UV wavelengths, phenols (except ferulic acid), cinnamic acid, and lignans are determined

at 280 nm using *p*-hydroxyphenylacetic acid as internal standard and flavones and ferulic acid are determined at 335 nm using *o*-coumaric acid as internal standard.

### 2.2.2. REAGENTS AND APPARATUS

**2.2.2.1.** Syringe filter of cellulose acetate (0.45 µm).

**2.2.2.2.** Eppendorf vial.

**2.2.2.3.** Methanol, chromatographic quality.

**2.2.2.4.** Acetonitrile, chromatographic quality.

**2.2.2.5.** Deionised or distilled water.

**2.2.2.6.** Phosphoric acid, analysis grade.

**2.2.2.7.** Eluting solvent A, water:phosphoric acid (v/v99.5:0.5).

**2.2.2.8.** Eluting solvent B, methanol:acetonitrile (v/v1:1).

**2.2.2.9.** HPLC equipped with a DAD detector and oven.

**2.2.2.10.** HPLC column RP-18 (4.0 mm i.d. x 250 mm; 5 µm particle size).

### 2.2.3. PROCEDURE

Filter the solution obtained in Part 1 with a syringe filter (2.1) and place the filtered solution in an Eppendorf vial (2.2).

Inject 20 µl of the filtered solution into the HPLC (2.9) equipped with a reverse phase column (2.10) maintained at 30 ºC. Perform the elution by a gradient (Table 1) using solvent A (2.7) and solvent B (2.8) at a flow rate of 1 ml/min. The detection wavelengths are 280 and 335 nm.

**Table 1. Gradient elution timetable**

| Time (min) | % A | % B |
|---|---|---|
| 0.0 | 95.0 | 5.0 |
| 15.0 | 70.0 | 30.0 |
| 30.0 | 62.0 | 38.0 |
| 35.0 | 62.0 | 38.0 |
| 40.0 | 55.0 | 45.0 |
| 45.0 | 47.5 | 52.5 |
| 50.0 | 0.0 | 100.0 |

## 2.2.4. EXPRESION OF RESULTS

### 2.2.4.1. Quantitative analysis

Calculate the mass fraction $w_i$ of the individual phenolic compounds using the response factors of each individual phenolic compound to the detector (see Annex 1), expressed as mg or mMol per kg of oil sample, as follows:

$$W_i = \frac{m_{IS} \times F_i \times A_i}{m \times F_{IS} \times A_{IS}}$$

where:

$A_i$ is the area the phenolic compound i.

AIS is the area of the internal standard.

Fi is the response factor of the phenolic compound i.

$F_{IS}$ is the response factor of the internal standard.

m is the mass of the test portion, in grams.

$m_{IS}$ is the mass of the internal standard, in milligrams.

The results are expressed to one decimal place in mg/kg.

$$W_i = \frac{m_{IS} \times F_i \times A_i}{m \times F_{IS} \times A_{IS} \times M_i}$$

where:

$A_i$ is the area the phenolic compound i.

AIS is the area of the internal standard.

Fi is the response factor of the phenolic compound i.

$F_{IS}$ is the response factor of the internal standard.

m is the mass of the test portion, in grams.

$m_{IS}$ is the mass of the internal standard, in milligrams.

$M_i$ is the molecular mass of the phenolic compound.

The results are expressed to three decimal places in mmol/kg.

## 3. TEST REPORT

The test report shall specify the method used to isolate the phenolic fraction and the conditions for the HPLC analysis. It shall also mention all operating details not specified in this Regulation, or regarded as optional, together with details of any incidents which may have influenced the results. The test report shall include all information necessary for the complete identification of the sample.

## 4. PRECISION

### Results of interlaboratory test

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

The trial consists of analysing five samples of extra virgin olive oil with one sample as duplicate.

- M1: EVOO
- M2: EVOO
- M3: EVOO
- M4: M2
- M5: EVOO

Some 20 laboratories participated in the trial, each of them with the corresponding code.

The results of the collaborative test organised by the IOC Executive Secretariat were statistically processed according to the rules laid down in the international standards. The data was initially reviewed using the recommendation of the INTERLABORATORY COLLABORATIVE STUDY. AOAC OFFICIAL METHODS OF ANALYSIS (2002). Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis.

Accuracy (trueness and precision) of measurement methods and results.
Outliers were examined by applying the initial revision and Cochran and Grubbs tests to the laboratory results for all the determinations (replicates a and b).

Laboratories eliminated in the initial revision: 9.

**The table lists:**

| | |
|---|---|
| n | number of participating laboratories after initial revision. |
| Outliers | number of laboratories with outlying values. |
| Mean | mean of the accepted results. |
| Median | |
| 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. |
| $RSD_r$ (%) | 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%.

SR reproducibility standard deviation.

$RSD_R$ (%) reproducibility coefficient of variation (SR x 100/mean).

**Precision values for total phenol content, (mg/kg)**

| Variable | Samples | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| n | 11 | 11 | 11 | 11 | 11 |
| outliers | 1 | 1 | 2 | 2 | 2 |
| Mean (mg/kg) | 232.45 | 474.90 | 461.55 | 517.40 | 598.62 |
| Median (mg/kg) | 223.30 | 504.39 | 451.79 | 495.37 | 572.00 |
| r | 35.43 | 63.75 | 21.42 | 47.71 | 41.19 |
| $S_r$ | 12.527 | 24.602 | 7.573 | 16.870 | 15.019 |
| **$RSD_r$ (%)** | 5.61 | 4.51 | 1.68 | 3.41 | 2.46 |
| R | 96.27 | 210.65 | 147.80 | 175.38 | 145.48 |
| $S_R$ | 34.037 | 85.774 | 52.255 | 62.006 | 69.285 |
| **$RSD_R$ (%)** | 15.24 | 15.58 | 11.57 | 12.52 | 9.02 |

# ANNEX 1

## Figure 1. HPLC-DAD chromatogram of phenolic compounds isolated by SPE-diol

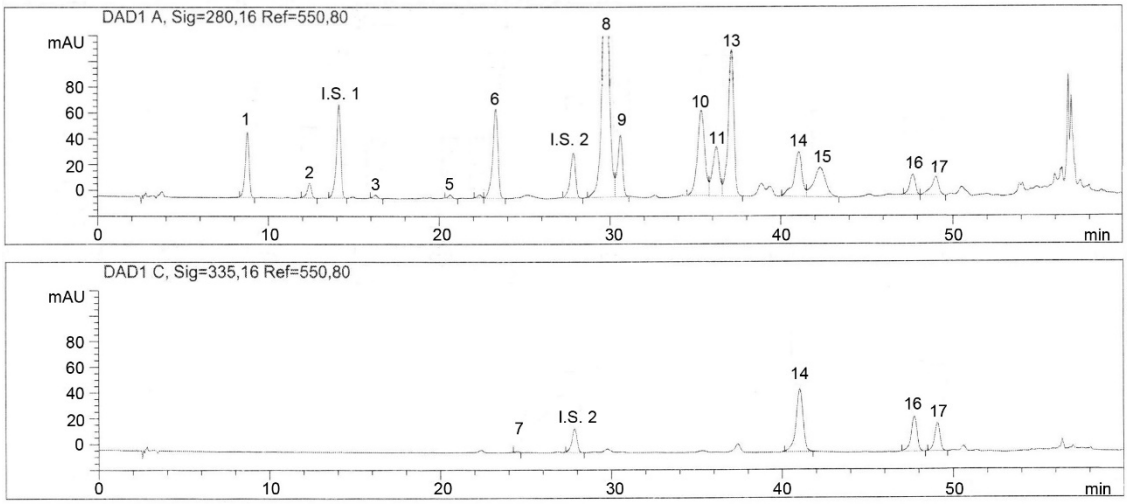



**Table 1.** Relative retention time and response factor of phenolic compounds isolated by SPE-diol and analysed by HPLC-DAD.

| HPLC peak | Compound | RRT (min) | Response factor |
|---|---|---|---|
| 1 | Hydroxytyrosol | 0.62[e] | 0.646[g] |
| 2 | Tyrosol | 0.88[e] | 0.829[g] |
| I.S. 1 | *p*-Hydroxyphenylacetic acid | 1.00 | 1.000 |
| 3 | Vanillic acid | 1.15[e] | 0.206[g] |
| 4 | Vanillin | 1.37[e] | 0.126[g] |
| 5 | *p*-Coumaric acid | 1.46[e] | 0.106[g] |
| 6 | Hydroxytyrosyl acetate | 1.65[e] | 0.788[g] |
| 7 | Ferulic acid | 0.88[f] | 0.542[h] |
| I.S. 2 | *o*-Coumaric acid | 1.00 | 1.000 |
| 8 | DDOA[a] | 2.11[e] | 1.303[g] |
| 9 | Isomer of AOA[c] | 2.17[e] | 1.093[g] |
| 10 | DDLA[b] | 2.50[e] | 1.843[g] |
| 11 | Pinoresinol | 2.56[e] | 0.197[g] |
| 12 | Cinnamic acid | ---- | 0.057[g] |
| 13 | 1-Acetoxypinoresinol | 2.63[e] | 0.584[g] |
| 14 | Luteolin | 1.47[f] | 0.836[h] |
| 15 | AOA[c] | 2.99[e] | 1.587[g] |
| 16 | Apigenin | 1.71[f] | 0.833[h] |
| 17 | AOL[d] | 3.47[e] | 2.121[g] |

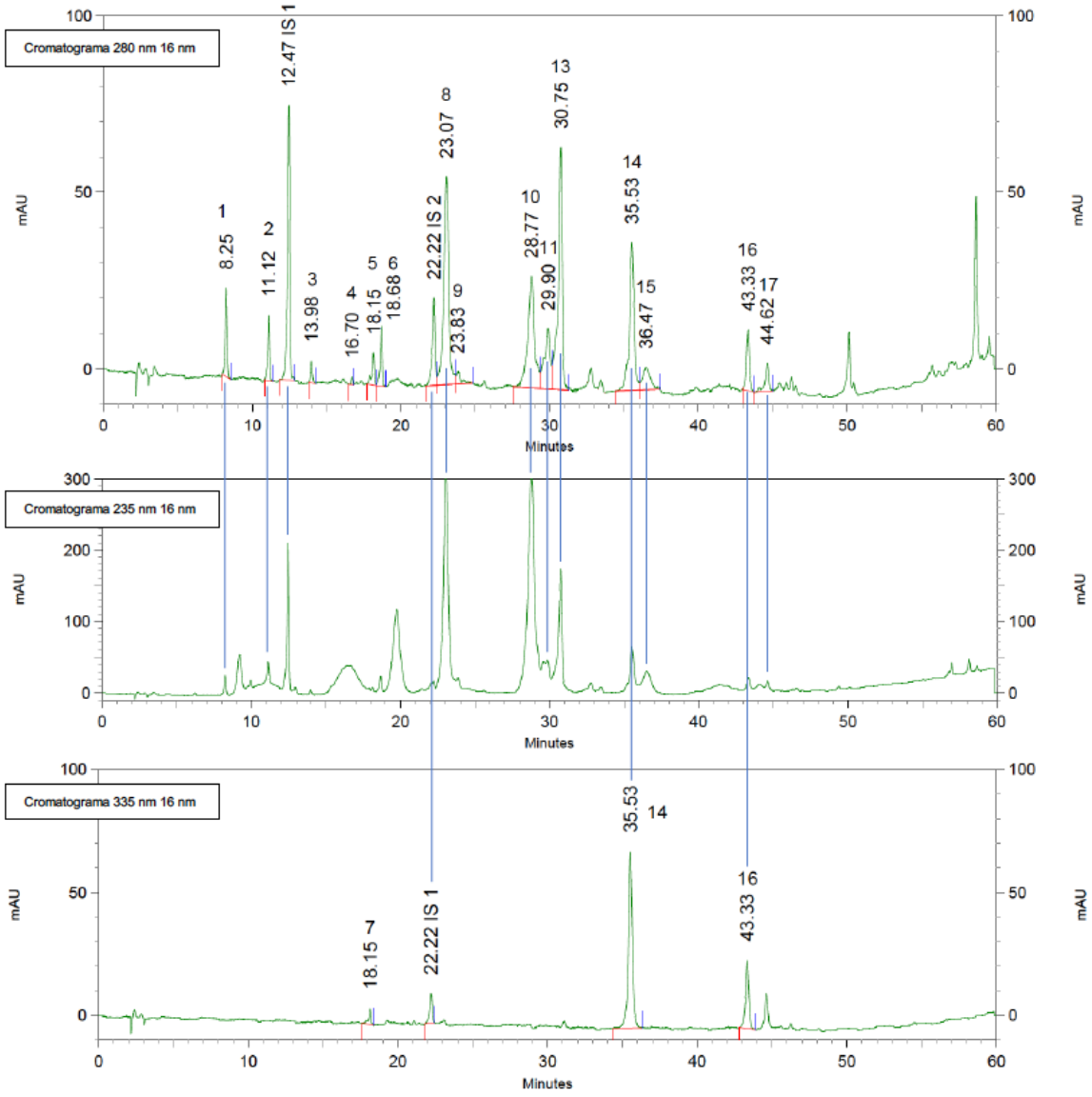
4

[a] dialdehydic form decarboxymethyl oleuropein aglycon. [b] dialdehydic form decarboxymethyl ligstroside aglycon. [c] aldehydic form oleuropein aglycon. [d]aldehydic form ligstroside aglycon. [e]relative retention time to I.S. 1. [f]relative retention time to I.S. 2. [g]relative response factor to I.S. 1. [h]relative response factor to I.S. 2.

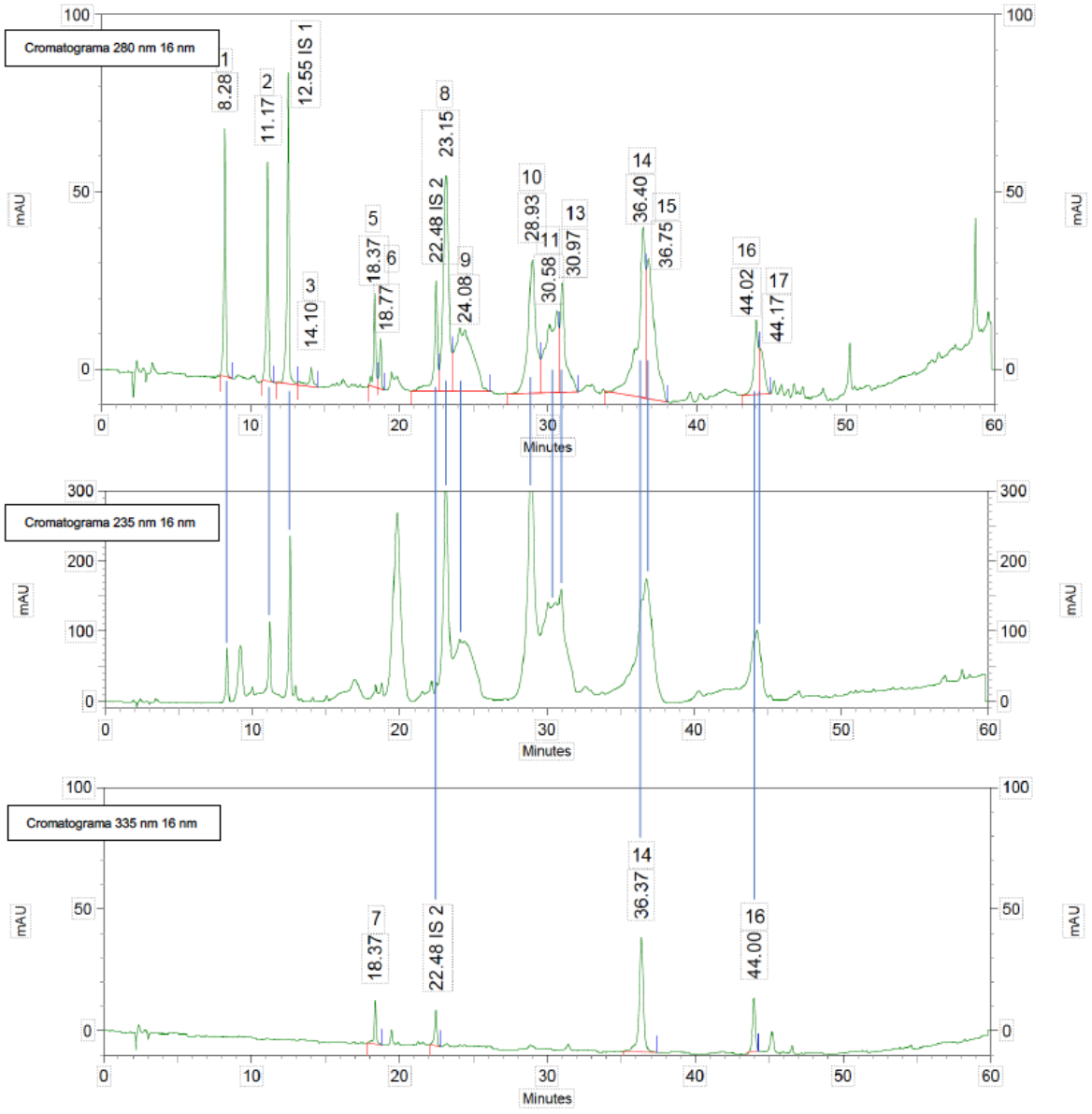
## ANNEX 2: EXAMPLES OF DIFFERENT PROFILES OF PHENOLIC COMPOUNDS

The example chromatograms may help identify the different phenols with the aid of the different wavelength. The lines are the connection between different phenols in the different chromatograms.

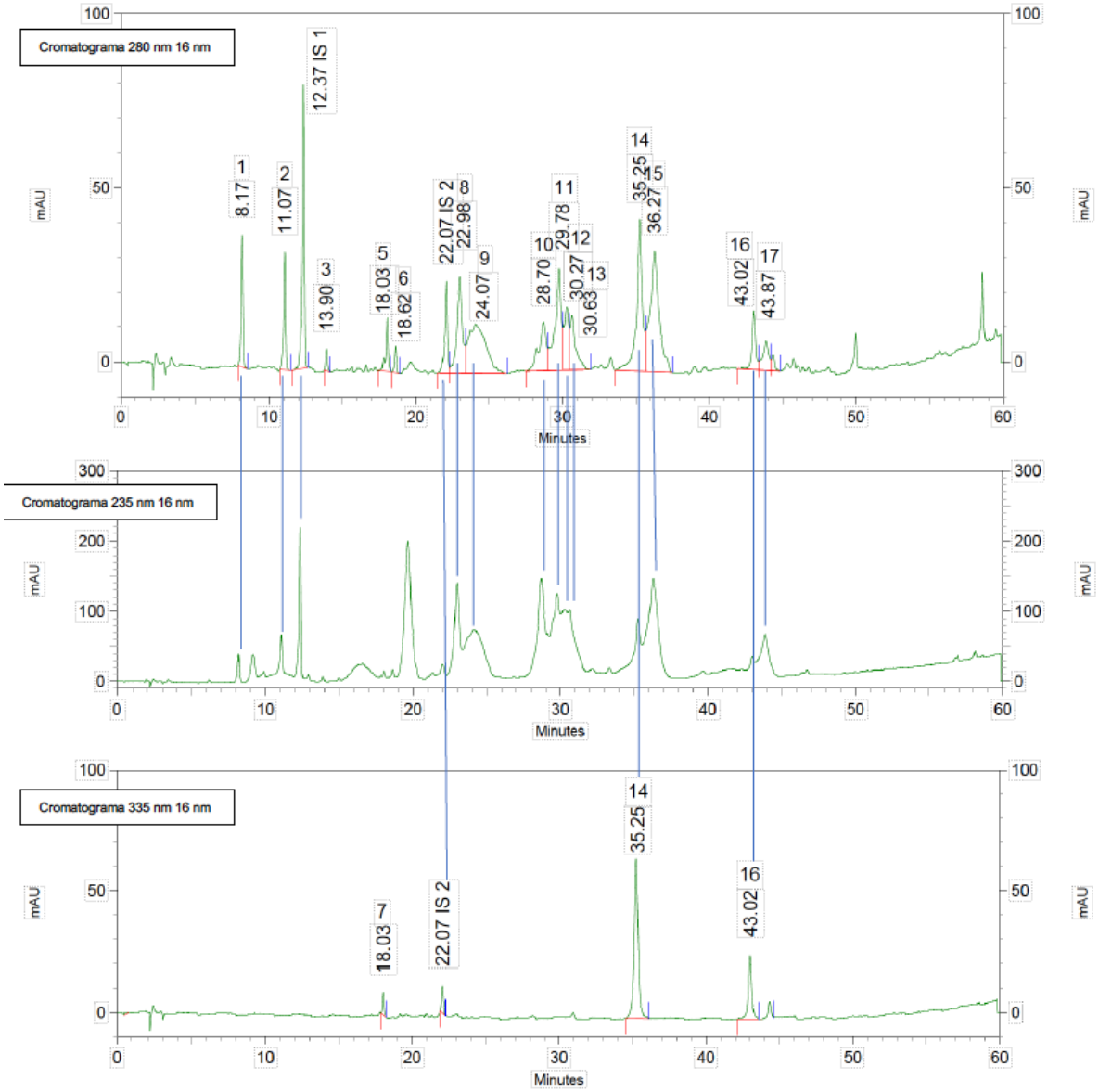
### OIL 1



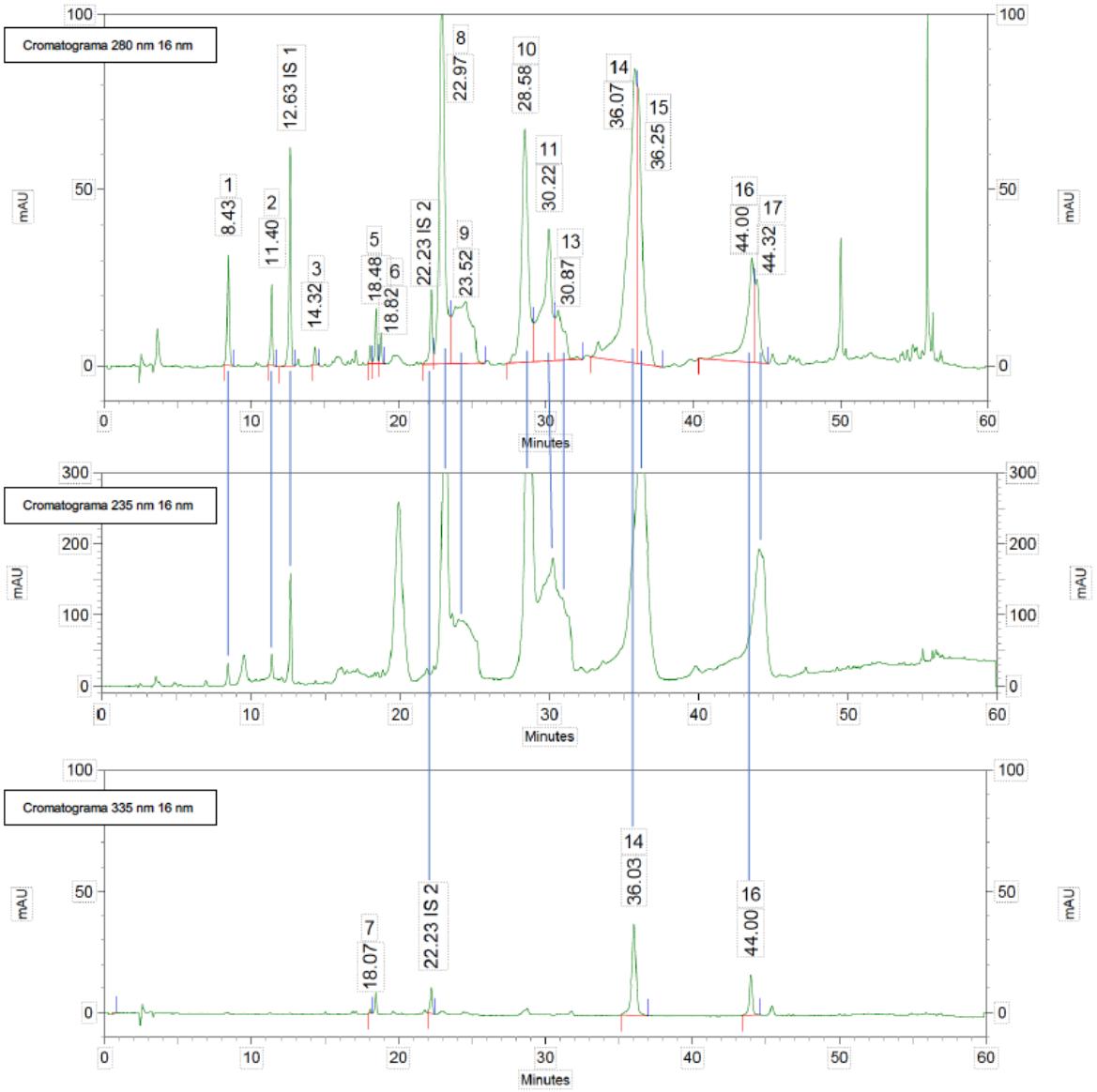
**OIL 2**


Cromatograma 280 nm 16 nm
1 8.28
2 11.17
12.55 IS 1
3 14.10
5 18.37
6 18.77
8 23.15
22.48 IS 2
9 24.08
10 28.93
11 30.58
13 30.97
14 36.40
15 36.75
16 44.02
17 44.17
mAU
Minutes
Cromatograma 235 nm 16 nm
Cromatograma 335 nm 16 nm
7 18.37
22.48 IS 2
14 36.37
16 44.00

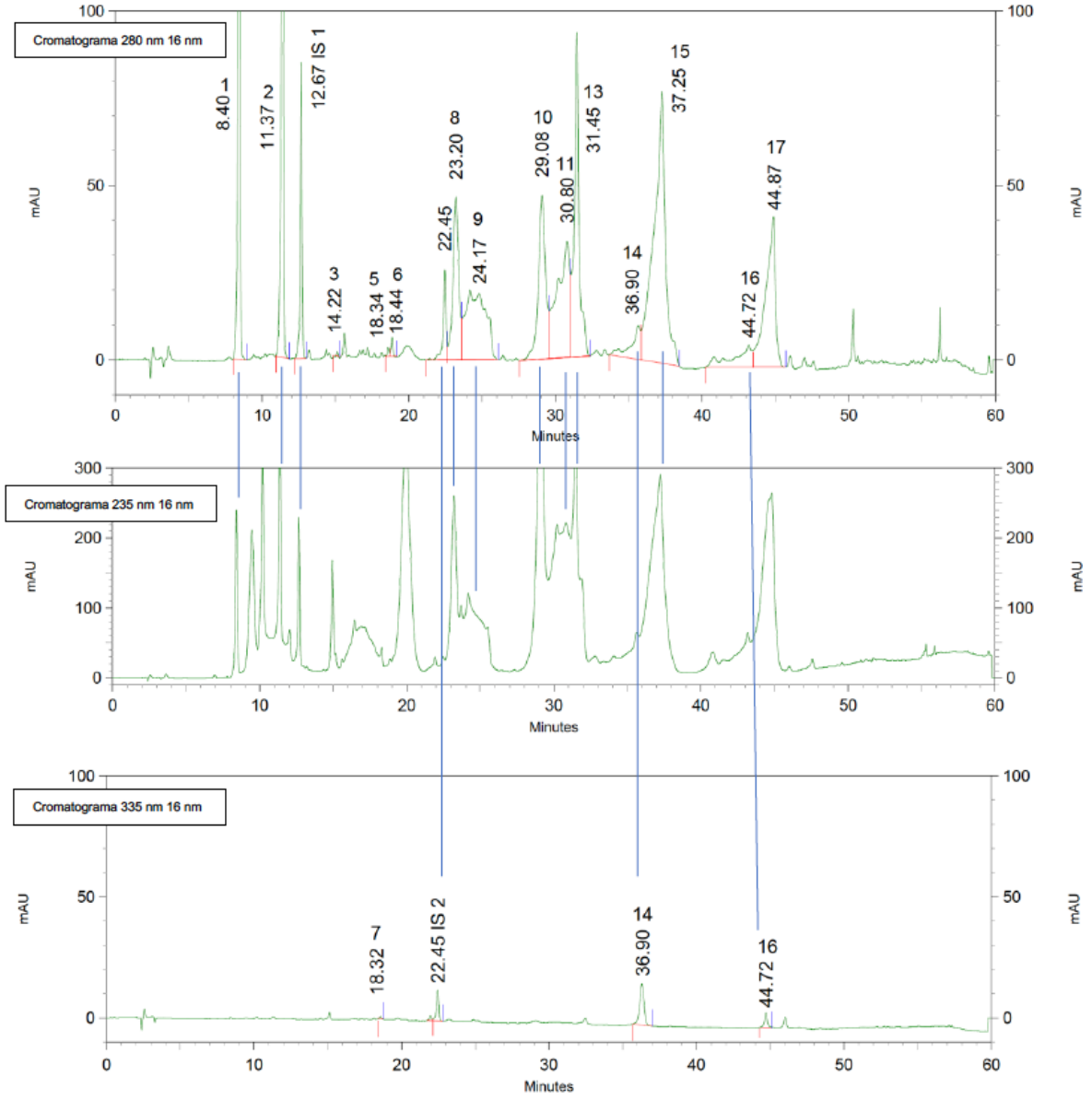
**OIL 3**


Cromatograma 280 nm 16 nm
Cromatograma 235 nm 16 nm
Cromatograma 335 nm 16 nm
mAU
Minutes
1 8.17
2 11.07
12.37 IS 1
3 13.90
5 18.03
6 18.62
22.07 IS 2
8 22.98
9 24.07
10 28.70
11 29.78
12
30.27
13 30.63
14 35.25
15 36.27
16 43.02
17 43.87
7 18.03

**OIL 4**


Cromatograma 280 nm 16 nm
mAU
Minutes
1 8.43
2 11.40
12.63 IS 1
3 14.32
5 18.48
6 18.82
22.23 IS 2
8 22.97
9 23.52
10 28.58
11 30.22
13 30.87
14 36.07
15 36.25
16 44.00
17 44.32
Cromatograma 235 nm 16 nm
mAU
Minutes
Cromatograma 335 nm 16 nm
mAU
Minutes
7 18.07
22.23 IS 2
14 36.03
16 44.00

**OIL 5**


Cromatograma 280 nm 16 nm
1 8.40
2 11.37
IS 1 12.67
3 14.22
5 18.34
6 18.44
8 22.45
23.20
9 24.17
10 29.08
11 30.80
13 31.45
14 36.90
15 37.25
16 44.72
17 44.87
mAU
Minutes
Cromatograma 235 nm 16 nm
Cromatograma 335 nm 16 nm
7 18.32
IS 2 22.45
14 36.90
16 44.72