TECHNICAL SPECIFICATION

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Determination of hydroxytyrosol and tyrosol content in extra virgin olive oils — Reverse phase high performance liquid chromatography (RP-HPLC)

Détermination de la teneur en hydroxytyrosol et tyrosol dans les huiles d'olive vierges extra — Chromatographie liquide à haute performance en phase inverse (CLHP-RP)

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Foreword

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils.*

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Biophenolic compounds of a secoiridoid nature and typical of extra virgin olive oil (*Olea europaea* L.), are derived from oleuropein and ligstroside and are correlated to different beneficial health effects for human beings other than particular sensorial characteristics. The biophenolic compounds contain, in an esterified form, two aromatic alcohols, namely hydroxytyrosol and tyrosol. The method given in this document is based on an extraction of the biophenolic fraction with a methanol/water solution and a subsequent hydrolysis reaction to produce free tyrosol and hydroxytyrosol[1][2].

Determination of hydroxytyrosol and tyrosol content in extra virgin olive oils — Reverse phase high performance liquid chromatography (RP-HPLC)

1 Scope

This document specifies a method for the quantitative determination of hydroxytyrosol and tyrosol content in extra virgin olive oils using reverse phase high performance liquid chromatography (RP-HPLC) with spectrophotometric detection. The method is also applicable to all other olive oils of a different commercial category.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
hydroxytyrosol and tyrosol
aromatic alcohols present in extra virgin olive oil typical of *Olea europaea* L. species as free or bound form

4 Principle

Hydroxytyrosol and tyrosol, present in free and esterified forms, are extracted from the oil with a methanol/water solution and then submitted to hydrolysis reaction with a 10 % of sulphuric acid ethanolic solution. The components are identified by means of HPLC and a spectrophotometric detector at 280 nm. The amount of free aromatic alcohols is calculated with the use of an external standard.

5 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade.

5.1 Ortho-phosphoric acid, a volume fraction of 85 %.

5.2 Methanol chromatographic grade.

5.3 Acetonitrile chromatographic grade.

5.4 Water chromatographic grade.

5.5 Ethanol, a volume fraction of 96 %.

5.6 Sulphuric acid, a volume fraction of 96 %.

5.7 Methanol/water solution, 80/20 v/v.

5.8 Reference sample: hydroxytyrosol or 2-(3,4-Dihydroxyphenyl)ethanol, e.g. Extrasynthese, (Cedex, France)[1)].

5.9 Reference sample: tyrosol, e.g. Sigma Aldrich (Germany)[1)].

5.10 Standard calibration solution of hydroxytyrosol and tyrosol.

Prepare the external standard calibration solution of hydroxytyrosol and tyrosol as follows.

Weigh accurately, to the nearest 0,1 mg, about 25 mg of hydroxytyrosol (5.8) and tyrosol (5.9) in a graduated 50 ml flask (6.2) and make to volume with a solution of methanol/water 80/20 v/v (5.7). Transfer 1 ml of this solution in another 10 ml flask and fill to volume with the same solution of methanol/water 80/20 v/v (5.7). The final concentration will be 50 mg/l of each external standard. Inject 20 µl of this solution in the HPLC system. The solution is stable for at least six months at –20 °C.

5.11 Hydrolysis solution, consisting of ethanol/water/sulphuric acid 50/40/10 v/v/v.

6 Apparatus

Usual laboratory glassware and the following.

6.1 Analytical balance suitable for weighing to an accuracy of within ±0,1 mg.

6.2 10 ml and 50 ml calibrated flasks, class A.

6.3 1 000 µl and 5 000 µl electronic pipette or manual pipette.

6.4 10 ml test tube, with a screw cap.

6.5 Mixer, type vortex.

6.6 Ultrasonic extraction bath.

6.7 PVDF (polyvinyl difluoride) syringe filters, 0,45 µm, 13 mm.

6.8 Centrifuge, able to operate at 5 000 r/min.

6.9 5 ml plastic syringe.

6.10 Thermostatic bath.

6.11 Analytical system, comprising a HPLC ternary pump with a degassing system equipped with an HPLC column, RP 18 reverse phase. The following column has proven to be adapted for the

1) Extrasynthese (Cedex, France) and Sigma Aldrich (Germany) are examples of a companies that make suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

determination (internal diameter 4,6 mm, length 25 cm, size 5 µm, 100 Å, type Spherisorb ODS2[2)]) with a UV spectrophotometric detector at 280 nm and integration system. A photodiode array detector (PDA) for spectra recording could be used to facilitate peak identification, by matching the hydroxytyrosol and tyrosol spectra in the sample extract with the spectra of the external standard.

A system for analysis data and integration is needed.

7 Sampling

It is important that an intact oil sample is delivered to the laboratory, which has not been damaged or modified during transport or storage. A representative sample is considered for the purpose of the analysis. A recommended sampling method is given in ISO 5555.

8 Procedure

8.1 Sample preparation

Weigh, with an analytical balance (6.1), 2 g of oil well-homogenized in a 10 ml conical test tube (6.4). Add, using a pipette (6.3), 5 ml of the methanol/water solution 80/20 v/v (5.7). Mix the solution with the help of a mixer for test tube type vortex (6.5) for 1 min and continue the extraction for 15 min in an ultrasonic bath (6.6) at room temperature. Centrifuge (6.8) at 5 000 r/min for 25 min. Filter an aliquot through a PVDF membrane syringe filter (6.7). Transfer 1 ml, using a pipette (6.3), of the filtered solution into another 10 ml test tube (6.4) and completely dry on a thermostatic bath (6.10) at a maximum temperature of 40 °C under nitrogen stream. Add 1 ml of hydrolysis solution (5.11) and mix, followed by reaction at 40° C for 1 h. Leave the solution at room temperature overnight. Then filter the solution using a PVDF membrane syringe filter (6.7).

8.2 HPLC analysis

8.2.1 General

Inject 20 µl of the sample into the HPLC system (6.11). The first sample injected as part of a series of analysis shall be a blank of a methanol/water solution 80/20 v/v (5.7). There shall be no interfering signals present during the chromatographic run at the same retention time of hydroxytyrosol and tyrosol.

8.2.2 HPLC conditions

The operating conditions given in Table 1 have proven to be adapted for the determination.

2) The Spherisorb ODS2 column is an example of suitable chromatographic column that is commercially available. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Table 1 — Operating conditions

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
Key A = water 0,2 % H_3PO_4 B = methanol C = acetonitrile				

The ternary gradient is programmed to enable an observation in the full chromatogram that hydrolysis is complete with no bound forms remaining in the extract. The chromatogram of the hydrolysed oil sample is compared with the chromatogram of the extract oil sample that has not been hydrolysed, which has been injected into the system under the same conditions, to determine whether hydrolysis is complete. Acetonitrile and methanol are always used in the same concentration ratio and therefore a binary system could be also used. Once the operators are experienced with the analysis, the elution time could be reduced by stopping the gradient after the elution of tyrosol, followed by washing the column for 10 min with B/C solvents in ratio of 50/50 v/v and then reconditioning for 10 min with A/B/C solvents 96/2/2 v/v/v.

The spectrophotometric detector (280 nm) shall be turned on an hour before the first analysis. The HPLC column shall be conditioned for at least 15 min before the gradient development with the initial solvent T = 0. Initially, inject 20 µl of external standard solution (5.10) and then, once the run is completed, inject 20 µl of the extracted sample solution (see 8.1).

Two independent determinations on the same sample shall be performed. The results have to respect repeatability values for a certain level of concentration. The result will be the average of the two independent determinations.

At the end of the day, the system shall be conditioned with methanol/acetonitrile 50/50 v/v at a flow of 1 ml/min for at least 15 min. Store the chromatographic column with the end fitting closed.

An example of a chromatogram is shown in Figure A.1.

8.2.3 Peak identification

Peak identification is carried out from the retention times by comparison with the external standard solution of known composition of hydroxytyrosol and tyrosol, which are eluted in the following order: hydroxytyrosol, tyrosol (see Annex A).

9 Expression of results

Calculate the areas of the two identified peaks through an electronic integrator, in mg/kg, using Formulae (1) and (2):

$$H = \frac{\left(A_{\text{H\_sample}}\right)\times 250\times\left(C_{\text{H}}\right)}{\left(A_{\text{H}}\right)\times m} \quad (1)$$

where

H is hydroxytyrosol content;

$A_{\text{H\_sample}}$ is the area corresponding to the peak of hydroxytyrosol of the sample recorded at 280 nm;

A_{H} is the area corresponding to the peak of hydroxytyrosol of the external standard recorded at 280 nm;

250 is the multiplication factor used to express the result, in mg/kg, taking into consideration a final extraction volume of 5 ml and an injection volume of 20 µl;

C_{H} is the quantity, in µg, of hydroxytyrosol injected as external standard;

m is the original sample mass, in grams.

$$T = \frac{\left(A_{\text{T\_sample}}\right)\times 250\times\left(C_{\text{T}}\right)}{\left(A_{\text{T}}\right)\times m} \quad (2)$$

where

T is tyrosol content;

$A_{\text{T\_sample}}$ is the area corresponding to the peak of tyrosol of the sample recorded at 280 nm;

A_{T} is the area corresponding to the peak of tyrosol of the external standard recorded at 280 nm;

250 is the multiplication factor used to express the result, in mg/kg, taking into consideration a final extraction volume of 5 ml and an injection volume of 20 µl;

C_{T} is the quantity, in µg, of tyrosol injected as external standard;

m is the original sample mass, in grams.

The total content is given by the sum of the content of the two components, as shown by Formula (3):

$$S = \Sigma\,(H + T) \quad (3)$$

where S is the total biophenolic compound contents.

Express this result without decimal digits.

Other peaks displayed in the chromatogram do not have to be considered.

10 Precision

10.1 Validation study

The details of the validation study and the method precision are given in Annex C. The obtained values cannot be applied to matrixes different from those indicated by the present method.

The repeatability values were obtained in accordance with ISO 5725-1, ISO 5725-2, ISO 5725-5 and ISO 5725-6 through a national validation study.

10.2 Repeatability, *r*

The absolute difference between two test results obtained by the same operator, with the same instruments and in the same operative conditions, on identical test material, over a short timescale shall not be over the *r* value for each concentration level in 95 % of cases.

10.3 Reproducibility, *R*

The difference between two single and independent results, obtained by different operators, in different laboratories, by using different equipment, on identical test material, shall not be over the *r* value for each concentration level in 95 % of cases.

11 Test report

The test report shall specify:

a) all information necessary for a correct sample identification;

b) the sampling method used if known;

c) the analysis method used in reference to this document, i.e. ISO/TS 23942;

d) all the operative details not specified in this document or regarded as optional, together with details of any incidents which may have influenced the test results;

e) the results obtained in mg/kg of oil, of the single aromatic alcohols and of their sum, when over the LOQ value (in accordance with Annex B), as indicated by formulae reported in the method;

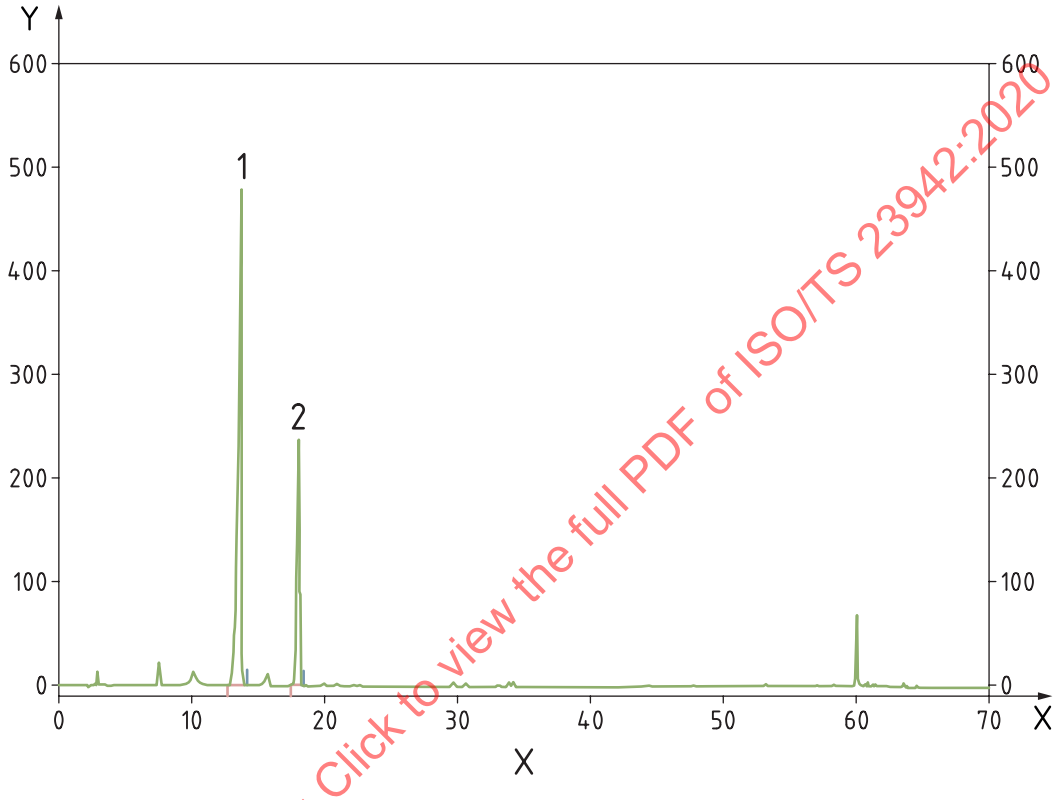
f) any deviations from the procedure;

g) any unusual features observed;

h) the date of the test.

Annex A
(informative)

Chromatogram 280 nm



Key

1 hydroxytyrosol
2 tyrosol
X time (in min)
Y mV

Figure A.1 — Example of a chromatogram

Annex B
(normative)

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated on the basis of the ratio signal/noise (S/N) based on the peak determination area compared with the area of the noise. LOD and LOQ were calculated as the concentration of hydroxytyrosol and tyrosol spiked to a blank sample, without the presence of these aromatic alcohols that produce a recognizable peak from the noise with a ratio S/N of 3 (LOD) and 10 (LOQ).

The LOQ for hydroxytyrosol and tyrosol using the analytical condition of the present method is 1 mg/kg of oil (S/N 10), while the LOD is 0,5 mg/kg (S/N 3).