
**Essential oils of bergamot, lemon,
bitter orange and lime, fully or
partially reduced in bergapten —
Determination of bergapten
content by high-performance liquid
chromatography (HPLC)**

*Huiles essentielles de bergamote, de citron, de bigarade et de
limette complètement ou partiellement privées de bergaptène —
Détermination de la teneur en bergaptène par chromatographie
liquide à haute performance (CLHP)*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 54, *Essential oils*.

This second edition cancels and replaces the first edition (ISO 7358:2002), which has been technically revised.

The main change to the previous edition is as follows:

- the addition of an alternative method using a reversed phase column.

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.

Essential oils of bergamot, lemon, bitter orange and lime, fully or partially reduced in bergapten — Determination of bergapten content by high-performance liquid chromatography (HPLC)

1 Scope

This document specifies a high-performance liquid chromatographic (HPLC) method using either an internal standard or external standard, for the determination of the bergapten content in essential oil of bergamot [*Citrus aurantium* ssp. bergamia (Risso et Poit.) Wight et Arn. ex Engl.], in essential oil of lemon [*Citrus limon* (L.) Burm. f.], in essential oil of bitter orange (*Citrus bigaradia* Risso) and in essential oil of lime [*Citrus aurantiifolia* (Christm.) Swingle and *Citrus latifolia* Tanaka], all of them fully or partially reduced in bergapten.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 356, *Essential oils — Preparation of test samples*

ISO 8432, *Essential oils — Analysis by high performance liquid chromatography — General method*

3 Terms and definitions

No terms and definitions are listed in this document.

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/>

4 Principle

The bergapten contents of test samples to be measured are determined after dilution by reversed phase HPLC or by normal phase HPLC with gradient elution, using an internal standardization method or an external standardization method and diode-array UV spectrometric detection.

5 Reagents

Use only the following reagents of recognized analytical grade.

5.1 Reference substance: bergapten (5-Methoxypsoralen), $C_{12}H_8O_4$, MW = 216,19 g/mol of known purity ≥ 95 %.

5.2 Internal standard: coumarin (1-Benzopyran-2-one), $C_9H_6O_2$, MW = 146,14 g/mol of known purity ≥ 98 %.

5.3 Solvents.

5.3.1 Solvents for normal phase HPLC.

5.3.1.1 Chloroform, of analytical purity, containing less than 2 % (volume fraction) of ethanol, for use in preparing the sample of essential oil containing the bergapten and internal standard as well as the mobile phase.

5.3.1.2 Hexane, HPLC grade, for use in the mobile phase ([5.3.1.4](#)).

5.3.1.3 Ethyl acetate, HPLC grade, for use in the mobile phase ([5.3.1.4](#)).

5.3.1.4 Mobile phase. Use solvents of a quality compatible with the detection system and prepare sufficient quantities for the complete analysis. Mix, for example, one of the following:

5.3.1.4.1 Hexane ([5.3.1.2](#)) and **ethyl acetate** ([5.3.1.3](#)), mixed in proportions of 80:20 by volume.

5.3.1.4.2 Hexane ([5.3.1.2](#)) and **chloroform** ([5.3.1.1](#)), mixed in proportions of 85:15 by volume.

5.3.2 Solvents for reversed phase HPLC. The solvent used for the mobile phase is a ternary mobile phase prepared with these three solvents as described in [Table 1](#) (see [8.2.2.3](#)).

5.3.2.1 HPLC grade **distilled water**, to be used in the mobile phase ([5.3.1.4](#)).

5.3.2.2 HPLC grade **acetonitrile**, to be used in the mobile phase ([5.3.1.4](#)).

5.3.2.3 HPLC grade **methanol**, to be used in the mobile phase ([5.3.1.4](#)).

5.3.2.4 Dilution solvent, mixture of **methanol** ([5.3.2.3](#)) and **acetonitrile** ([5.3.2.2](#)) in proportions of 80:20 by volume.

6 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

6.1 Common laboratory equipment

6.1.1 Volumetric flasks: 100 ml, 50 ml, 10 ml.

6.1.2 Pasteur pipettes; volumetric pipettes: 1 ml, 5 ml, 10 ml and 20 ml; **micropipette:** 500 µl.

6.1.3 Single-use syringes.

6.1.4 PTFE filter: 0,45 µm.

6.1.5 Vials for HPLC injection.

6.1.6 Precision balance.

6.2 HPLC system

6.2.1 Liquid chromatograph.

6.2.2 Column for HPLC in normal phase, made of stainless steel, of length between 150 mm and 250 mm, having an internal diameter between 4 mm and 5 mm and packed with a stationary phase consisting of granulated silica of HPLC quality, with a grain size of approximately 5 µm.

6.2.3 Column for HPLC in reversed phase, made of stainless steel, of length 150 mm (or 250 mm), having an internal diameter 4,6 mm and packed with a stationary phase C18 type with a grain size of approximately 3,5 µm (or 5 µm), for example Zorbax Eclipse Plus¹⁾ C:18: 3,5 µm (4,6 × 150) mm agilent.

6.2.4 A ternary pump system enabling programmed solvent gradients.

6.2.5 Solvent degassing system (optional), for example ultrasonic tank.

6.2.6 Detection system, adjustable to wavelengths of 254 nm or 312 nm, diode-array UV spectrometric detection system.

6.2.7 Recorder and (optional) **integrator**, suitable for this HPLC system.

7 Sample preparation

Prepare the test sample as specified in ISO 356.

Dissolve any solid deposit by moderate heating.

8 Procedure

8.1 Operating conditions

Adjust the flow rate of the mobile phase ([5.3.1.4](#)) so as to obtain good separation of the peaks corresponding to bergapten and coumarin from other essential oil components detectable by the UV detector ([6.2.6](#)). The flow rate is typically between 1 ml/min and 1,5 ml/min.

Follow the procedure specified in ISO 8432.

8.2 Determination

8.2.1 HPLC in normal phase

8.2.1.1 Internal standard method

8.2.1.1.1 Optimization of HPLC chromatographic conditions in normal phase

8.2.1.1.1.1 Separation

Verify that the bergapten is well separated from the other constituents of the essential oil in the chromatograms obtained. Next, verify that the internal standard, coumarin ([5.2](#)), does not mask or coincide with any constituent of the essential oil. Determine the retention times of the bergapten and coumarin.

1) Zorbax Eclipse Plus is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

8.2.1.1.1.2 Quantity of internal standard

The amount of coumarin (internal standard) added to the sample is considered suitable when the peak areas for the bergapten (in the essential oil) and the coumarin are approximately equal in the chromatograms. To determine this amount, inject a solution (e.g. 10 µl) containing a given amount (e.g. 10 mg) of coumarin (5.2) dissolved in chloroform (5.3.1.1, e.g. 10 ml) into the HPLC. Then inject the same volume of a solution of the essential oil analyte diluted in chloroform (5.3.1.1). Adjust the mass concentrations of both of these solutions so as to obtain comparable peak areas.

8.2.1.1.2 Response factor *K*

Prepare a calibration solution as follows. In a volumetric flask (6.1.1) of suitable volume, weigh, to the nearest 0,1 mg, about 20 mg of coumarin (5.2). In the same volumetric flask, weigh, to the nearest 0,1 mg, about 10 mg of bergapten (5.1) and dissolve both compounds in approximately 20 ml of chloroform (5.3.1.1).

Inject a suitable amount (see 8.2.1.1) of the calibration solution so as to remain within the detector sensitivity range.

Measure the peak areas of the chromatogram. A typical chromatogram of the reference substances is given in Annex A, Figure A.1, and the absorbance spectrum of bergapten is given in Annex B, Figure B.1.

Calculate the response factor *K* using Formula (1):

$$K = \frac{m_R \cdot A_{IS}}{m_{IS} \cdot A_R} \quad (1)$$

where

m_R is the mass, expressed in milligrams, of bergapten (reference substance) (5.1) added to the solution;

m_{IS} is the mass, expressed in milligrams, of coumarin (internal standard) (5.2) added to the solution;

A_R is the peak area, expressed in integrator units, corresponding to bergapten (reference substance) (5.1);

A_{IS} is the peak area, expressed in integrator units, corresponding to coumarin (internal standard) (5.2).

NOTE In ISO 8432 this formula is equivalent to the following:

$$K = \frac{m_R A_E}{m_E A_R}$$

where

m_R is the mass, expressed in milligrams, of bergapten (reference substance) (5.1) added to the solution;

m_E is the mass, expressed in milligrams, of coumarin (internal standard) (5.2) added to the solution;

A_R is the peak area, expressed in integrator units, corresponding to bergapten (reference substance) (5.1);

A_E is the peak area, expressed in integrator units, corresponding to coumarin (internal standard) (5.2).

8.2.1.1.3 Determination of bergapten

In a volumetric flask (6.1.1) of suitable volume (e.g. 15 ml) prepare the test solution. Weigh, to the nearest 0,1 mg, a suitable amount of coumarin (m_{IS}) (approximately 10 mg), as determined in 8.2.2.1, and a

portion of the essential oil (m_s) so as to obtain a chromatogram with equal peak areas for bergapten and coumarin.

Add chloroform (5.3.1.1, approximately 8 ml) and shake carefully to dissolve the coumarin.

It can be necessary to prepare several dilutions of the test solution to obtain a chromatogram with comparable peak areas for bergapten and coumarin because the bergapten content in the essential oils is unknown (fully or partially reduced in bergapten). Choose the volume of the volumetric flask, the quantities of coumarin and the volume of chloroform so as to meet this requirement.

Using the same HPLC operating conditions established in 8.2.1.1.1, inject a suitable amount of the test solution so as to remain within the detector sensitivity range.

Measure the peak areas of the chromatogram.

Measure and record the peak areas of the chromatogram corresponding to bergapten (A_x) and coumarin (A_{IS}).

8.2.1.2 External standard method

Follow the procedure for the external standard method as specified in ISO 8432.

8.2.2 HPLC in reversed phase

8.2.2.1 Preparation of the reference solutions for calibration

8.2.2.1.1 General

For each calibration, use the stock solutions of bergapten and coumarin to prepare the standard solution. Store the standard solution in a cool place at 4 °C.

8.2.2.1.2 Stock solutions

As accurately as possible weigh 50 mg of bergapten into a 100 ml flask and make up to volume with the dilution solvent. Homogenize the solution then leave it for 3 min in the ultrasonic bath to get stock solution. This stock solution contains almost exactly 500 mg/l of bergapten.

As accurately as possible weigh 50 mg of coumarin into a 50 ml flask and make up to volume with the elution solvent. Homogenize the solution then leave it for 3 min in the ultrasonic bath to get stock solution. This solution contains almost exactly 1 000 mg/l of coumarin.

8.2.2.1.3 Working solutions

Using the stock solutions, prepare a standard range of eight calibration points for bergapten concentration: 1 mg/l, 2,5 mg/l, 5 mg/l, 10 mg/l, 25 mg/l, 50 mg/l, 100 mg/l and 200 mg/l.

- 1 mg/l standard solution: into a 50 ml flask, transfer 100 μ l of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.
- 2,5 mg/l standard solution: into a 50 ml flask, transfer 250 μ l of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.
- 5 mg/l standard solution: into a 50 ml flask, transfer 500 μ l of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.
- 10 mg/l standard solution: into a 50 ml flask, transfer 1 ml of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.
- 25 mg/l standard solution: into a 50 ml flask, transfer 2,5 ml of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.

- 50 mg/l standard solution: into a 50 ml flask, transfer 5 ml of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.
- 100 mg/l standard solution: into a 50 ml flask, transfer 10 ml of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.
- 200 mg/l standard solution: into a 50 ml flask, transfer 20 ml of bergapten stock solution and 5 ml of coumarin stock solution then make up to volume with the dilution solvent.

Homogenize each prepared standard solution by stirring then pass through a 0,45 µm PTFE filter into a vial before injection in the HPLC.

8.2.2.2 Preparation of citrus essential oils samples

To determine the content of citrus essential oils with the standard range created previously (8.2.2.1.3), it is necessary to dilute the samples before injection in the HPLC.

Into a 10 ml flask, accurately weigh 0,3 g of essential oil of bergamot and 1 ml of coumarin stock solution (8.2.2.1.2). Make up to volume with the dilution solvent (5.3.2.4).

For the essential oils with low bergapten content, adapt the test portion. For example, in a 10-ml flask, weigh accurately about 4 g of bergamot essential oil partially reduced in bergapten and weigh accurately about 1 ml of coumarin stock solution. Fill to volume with dilution solvent (5.3.2.4).

Homogenize each prepared sample by stirring then pass through a 0,45 µm PTFE filter into a vial before injection in the HPLC.

8.2.2.3 Determination by HPLC and internal standardization of bergapten content in essential oils of bergamot

The HPLC experimental conditions are:

- Injection volume: 10 µl.
- Oven temperature: 25 °C.
- Eluent flow rate: 1 ml/min.
- UV detection conditions: diode-array UV detection system, wavelength = 312 nm.

Elution solvent is described in Table 1.

Table 1 — Mobile phase: the elution solvent is a ternary mixture

Time min	Water %	Methanol %	Acetonitrile %
0	65	30	5
25	32	63	5
35	0	63	37
37	65	30	5
40	65	30	5

9 Calculation — Normal phase or reversed phase HPLC

9.1 Internal standard method

Using the response factor K determined in 8.2.1.1.2, calculate the mass fraction, expressed as a percentage, of bergapten, w_x , in the essential oil using Formula (2):

$$w_x = K \left(\frac{m_{IS} \cdot A_x}{m_s \cdot A_{IS}} \right) \times 100 \% \quad (2)$$

where

K is the response factor calculated as in Formula (1) (see 8.2.1.1.2);

m_{IS} is the mass, expressed in milligrams, of the coumarin added as the internal standard in the test sample (see 8.2.1.1.3);

m_s is the mass, expressed in milligrams, of the essential oil in the test sample (see 8.2.1.1.3);

A_x is the peak area, expressed in integrator units, corresponding to bergapten in the test sample (see 8.2.1.1.3);

A_{IS} is the peak area, expressed in integrator units, corresponding to the coumarin (see 8.2.1.1.3).

NOTE In ISO 8432:1987, 10.1, the mass fraction, expressed as a percentage, of bergapten, c_x , in the essential oil is equivalent to Formula (2) as follows:

$$c_x = \frac{A_x m_E K}{A_E m_x} \times 100 \%$$

where

A_x is the peak area, expressed in integrator units, corresponding to bergapten in the test sample (see 8.2.1.1.3);

A_E is the peak area, expressed in integrator units, corresponding to the coumarin (see 8.2.1.1.3);

m_x is the mass, expressed in milligrams, of the essential oil in the test sample (see 8.2.1.1.3);

m_E is the mass, expressed in milligrams, of the coumarin added as the internal standard in the test sample (see 8.2.1.1.3);

K is the response factor calculated as in Formula (1) (see 8.2.1.1.2).

9.2 External standard method

Calculate the bergapten content in accordance with ISO 8432.

10 Precision — Repeatability

For K and for the expression of results (%), take the mean value of several tests (at least three) carried out on the same sample. The different values (K or %) used to calculate this mean shall not differ by more than $\pm 5 \%$.

11 Test report

The test report shall include the following information:

- a) the details of the HPLC system;
- b) a reference to this document, i.e. ISO 7358:2021;
- c) the characteristics of the column (material, dimensions, packing, stationary phase);
- d) the characteristics of the detector (optional) and the operating conditions;
- e) the characteristics of the mobile phase (flow rate and nature);
- f) identification of the test sample (quantity injected and final dilution);
- g) the results obtained;
- h) the date of the test.

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