
**Oils of bergamot, lemon, citron and lime,
fully or partially reduced in bergapten —
Determination of bergapten content by
high-pressure liquid chromatography
(HPLC)**

iTeh STANDARD PREVIEW
*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 pression
(CLHP)*

[ISO 7358:2002](https://standards.iso.org/standards/sist/5a9dbcbb-05d9-401b-8dc0-3d03b44f2e38/iso-7358-2002)

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 7358 was prepared by Technical Committee ISO/TC 54, *Essential oils*.

Annex A of this International Standard is for information only.

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Oils of bergamot, lemon, citron and lime, fully or partially reduced in bergapten — Determination of bergapten content by high-pressure liquid chromatography (HPLC)

1 Scope

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

For essential oils having a bergapten content greater than or equal to a mass fraction of 0,001 %, the internal standard method is applicable.

For essential oils having a bergapten content between a mass fraction of 0,000 1 % and 0,001 %, the external standard method is applicable.

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2 Normative references

[ISO 7358:2002](#)

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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

ISO 8432:1987, *Essential oils — Analysis by high performance liquid chromatography — General method*

3 Principle

Liquid chromatography is based on the physico-chemical phenomena of adsorption, separation, ion exchange or exclusion. It enables a small quantity of essential oil to be analysed using a chromatographic column with an appropriate packing, under appropriate conditions, with identification of the different constituents and the quantitative determination of specific compounds.

4 Reagents

Use only reagents of recognized analytical grade.

DANGER — Attention is drawn to the hazard deriving from the specified use of chloroform, a toxic solvent.

4.1 Reference substance: bergapten, of known purity ≥ 95 %.

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4.2 Internal standard: coumarin, of known purity $\geq 98\%$.

4.3 Solvents.

4.3.1 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.

4.3.2 Hexane, HPLC grade, for use in the mobile phase (4.3.4).

4.3.3 Ethyl acetate, HPLC grade, for use in the mobile phase (4.3.4).

4.3.4 Mobile phase.

For the 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:

4.3.4.1 Hexane (4.3.2) and **ethyl acetate** (4.3.3), mixed in proportions of 80:20 by volume.

4.3.4.2 Hexane (4.3.2) and **chloroform** (4.3.1), mixed in proportions of 85:15 by volume.

5 Apparatus

5.1 HPLC system, consisting of the following.

5.1.1 Liquid chromatograph.

5.1.2 Column, 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 .

5.1.2 Solvent supply system, capable of supplying the mobile phase at a constant or programmed flow rate.

5.1.3 Solvent degassing system (optional).

5.1.4 UV detector, adjustable to wavelengths of 254 nm or 313 nm.

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

5.2 Volumetric flasks, of 15 ml to 150 ml capacity, depending on the quantity of bergapten assumed to be contained in the sample.

6 Sample preparation

Prepare the test sample as specified in ISO 356.

Dissolve any solid deposit by moderate heating.

7 Procedure

7.1 HPLC operating conditions

Adjust the flow rate of the mobile phase (4.3.4) so as to obtain good separation of the peaks corresponding to bergapten and coumarin from other essential oil components detectable by the UV detector (5.1.4). The flow rate is typically between 0,5 ml/min and 1 ml/min.

Follow the procedure specified in ISO 8432.

7.2 Determination

7.2.1 Internal standard method for essential oils with bergapten content $\geq 0,001$ % by mass

7.2.1.1 Optimization of HPLC chromatographic conditions

7.2.1.1.1 Separation

Verify that the bergapten is well separated from the other constituents of the essential oil in the chromatograms obtained. Secondly verify that the internal standard, coumarin (4.2), does not mask or coincide with any constituent of the essential oil. Determine the retention times of the bergapten and coumarin.

7.2.1.1.2 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 oil) and the coumarin are approximately equal in the chromatograms. To determine this amount, inject (e.g. 10 μ l) a solution containing a given amount (e.g. 10 mg) of coumarin (4.2) dissolved in chloroform (4.3.1, e.g. 10 ml) into the HPLC. Then inject the same volume of a solution of the essential oil analyte diluted in chloroform (4.3.1). Adjust the mass concentrations of both of these solutions so as to obtain comparable peak areas.

7.2.1.2 Response factor K

Prepare a calibration solution as follows. In a volumetric flask (5.2) of suitable volume, weigh, to the nearest 0,1 mg, about 20 mg of coumarin (4.2). In the same volumetric flask, weigh, to the nearest 0,1 mg, about 10 mg of bergapten (4.1) and dissolve both compounds in approximately 20 ml of chloroform (4.3.1).

Inject a suitable amount (see 7.2.1.1) 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.

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Calculate the response factor K using equation (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) (4.1) added to the solution;

m_{IS} is the mass, expressed in milligrams, of coumarin (internal standard) (4.2) added to the solution;

A_R is the peak area, expressed in integrator units, corresponding to bergapten (reference substance) (4.1);

A_{IS} is the peak area, expressed in integrator units, corresponding to coumarin (internal standard) (4.2).

NOTE In ISO 8432 this equation 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) (4.1) added to the solution;

m_E is the mass, expressed in milligrams, of coumarin (internal standard) (4.2) added to the solution;

A_R is the peak area, expressed in integrator units, corresponding to bergapten (reference substance) (4.1);

A_E is the peak area, expressed in integrator units, corresponding to coumarin (internal standard) (4.2).

7.2.1.3 Determination of bergapten

In a volumetric flask (5.2) 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 7.2.1.2, 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 (4.3.1, approximately 8 ml) and shake carefully to dissolve the coumarin.

It may 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 7.2.1.1, 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}).

A typical chromatogram is given in annex A.

7.2.2 External standard method for essential oils with bergapten content ranging from 0,000 1 % to 0,001 % by mass

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

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8 Calculation

8.1 Internal standard method for essential oils with bergapten content $\geq 0,001$ % by mass

Using the response factor K determined in 7.2.1.2, calculate the mass fraction, expressed as a percentage, of bergapten, w_x , in the essential oil using Equation (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 equation (1) (see 7.2.1.2);

m_{IS} is the mass, expressed in milligrams, of the coumarin added as the internal standard in the test sample (see 7.2.1.3);

m_s is the mass, expressed in milligrams, of the essential oil in the test sample (see 7.2.1.3);

A_x is the peak area, expressed in integrator units, corresponding to bergapten in the test sample (see 7.2.1.3);

A_{IS} is the peak area, expressed in integrator units, corresponding to the coumarin (see 7.2.1.3).

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

$$c_x = \frac{A_X m_E K}{A_E m_x} \times 100 \% \quad (2)$$

where

A_X is the peak area, expressed in integrator units, corresponding to bergapten in the test sample (see 7.2.1.3);

A_E is the peak area, expressed in integrator units, corresponding to the coumarin (see 7.2.1.3);

m_x is the mass, expressed in milligrams, of the essential oil in the test sample (see 7.2.1.3);

m_E is the mass, expressed in milligrams, of the coumarin added as the internal standard in the test sample (see 7.2.1.3);

K is the response factor calculated as in equation (1) (see 7.2.1.2).

8.2 External standard method for essential oils with bergapten content ranging from 0,000 1 % to 0,001 % by mass

Calculate the content of bergapten content in accordance with ISO 8432.

9 Precision

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9.1 Repeatability: essential oils with bergapten content $\geq 0,001$ % by mass

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 ± 5 %.

9.2 Repeatability: essential oils with bergapten content between 0,000 1 % and 0,001 % by mass

Take the mean value of several tests (at least three) carried out on the same sample. The values used to calculate this mean shall not differ by more than ± 20 %.

10 Test report

The test report shall include the following information:

- a) the details of the HPLC system;
- b) a reference to this International Standard, i.e. ISO 7358;
- 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.