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### 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)*

ICS: 71.100.60

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ISO copyright office  
CP 401 • Ch. de Blandonnet 8  
CH-1214 Vernier, Geneva  
Phone: +41 22 749 01 11  
Fax: +41 22 749 09 47  
Email: [copyright@iso.org](mailto:copyright@iso.org)  
Website: [www.iso.org](http://www.iso.org)

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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](http://www.iso.org/directives)).

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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 with 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](http://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 International Standard 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 aurantifolia* (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 8432:1987, *Essential oils — Analysis by high performance liquid chromatography — General method*

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## 3 Principle

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

## 4 Reagents

Use only reagents of recognized analytical grade.

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

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

### 4.3 Solvents

#### 4.3.1 Solvents for normal phase high performance liquid chromatography

**4.3.1.1 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.

4.3.1.2 **Hexane**, HPLC grade, for use in the mobile phase ([4.3.1.4](#)).

4.3.1.3 **Ethyl acetate**, HPLC grade, for use in the mobile phase ([4.3.1.4](#)).

#### 4.3.1.4 **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:

4.3.1.4.1 **Hexane** ([4.3.1.2](#)) and **ethyl acetate** ([4.3.1.3](#)), mixed in proportions of 80:20 by volume.

4.3.1.4.2 **Hexane** ([4.3.1.2](#)) and **chloroform** ([4.3.1.1](#)), mixed in proportions of 85:15 by volume.

#### 4.3.2 **Solvents for reversed phase high performance liquid chromatography**

The solvent used for the mobile phase is a ternary mobile phase prepared with these three solvents as described in the [table 1](#) (see [7.2.2.3.1](#)).

4.3.2.1 HPLC grade **distilled water**, to be used in mobile phase ([7.2.2.3.1](#)).

4.3.2.2 HPLC grade **acetonitrile**, to be used in the mobile phase ([7.2.2.3.1](#)).

4.3.2.3 HPLC grade **methanol**, to be used in the mobile phase ([7.2.2.3.1](#)).

4.3.2.4 Dilution solvent, mixture of **methanol** ([4.3.2.3](#)) and **acetonitrile** ([4.3.2.2](#)) in proportions of 80:20 by volume.

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## 5 **Equipment and apparatus**

### 5.1 **Common laboratory equipment**

5.1.1 **Volumetric flasks: 100 ml, 50 ml, 10 ml**

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

5.1.3 **Single-use syringes: 5 ml**

5.1.4 **PTFE filter: 0,45 µm**

5.1.5 **Vials for HPLC injection**

5.1.6 **Precision balance**

### 5.2 **HPLC system, consisting of the following**

#### 5.2.1 **Liquid chromatograph**

5.2.2 **Column for high performance liquid chromatography 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.

**5.2.3 Column for high performance liquid chromatography 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  $\mu\text{m}$  (or 5  $\mu\text{m}$ ) for example Zorbax Eclipse Plus C:18: 3,5  $\mu\text{m}$  (4,6  $\times$  150) mm agilent.

**5.2.4 A ternary pump system enabling programmed solvent gradients**

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

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

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

## 6 Sample preparation

Prepare the test sample as specified in ISO 356.

Dissolve any solid deposit by moderate heating.

## 7 Procedure

### 7.1 Operating conditions

Adjust the flow rate of the mobile phase ([4.3.1.4](#)) so as to obtain good separation of the peaks corresponding to bergapten and coumarin from other essential oil components detectable by the UV detector ([5.2.6](#)). The flow rate is typically between 1 ml/min and 1,5 ml/mn.

Follow the procedure specified in ISO [8432](#).

### 7.2 Determination

#### 7.2.1 HPLC in normal phase

##### 7.2.1.1 Internal standard method

##### 7.2.1.1.1 Optimization of HPLC chromatographic conditions in normal phase

##### 7.2.1.1.1.1 Separation

Verify that the bergapten is well separated from the other constituents of the essential oil in the chromatograms obtained. 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.

##### 7.2.1.1.1.2 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 (e.g. 10  $\mu\text{l}$ ) a solution containing a given amount (e.g. 10 mg) of coumarin ([4.2](#)) dissolved in chloroform ([4.3.1.1](#) e.g. 10 ml) into the HPLC. Then inject the same volume of a solution of the essential oil analyte diluted in chloroform ([4.3.1.1](#)). Adjust the mass concentrations of both of these solutions so as to obtain comparable peak areas.

### 7.2.1.1.2 Response factor $K$

Prepare a calibration solution as follows. In a volumetric flask (5.1.1) 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 (4.3.1.1).

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.

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

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).

### 7.2.1.1.3 Determination of bergapten

In a volumetric flask (5.1.1) 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 7.2.2.1, 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 (4.3.1.1, 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 [7.2.1.1.1](#), 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 ( $A_S$ ).

### 7.2.1.2 External standard method

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

## 7.2.2 HPLC in reversed phase

### 7.2.2.1 Preparation of the reference solutions for calibration:

For each calibration, the standard solutions are to be prepared using stock solutions of bergapten and coumarin, to be stored in a cool place at 4 °C.

#### 7.2.2.1.1 Stock solutions

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

#### 7.2.2.1.2 Working solutions

Using the stock solutions, prepare a standard range of 8 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 µl 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 µl 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 µl 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.