INTERNATIONAL STANDARD

ISO 5565-2

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Vanilla [*Vanilla fragrans* (Salisbury) Ames] —

Part 2: **Test methods**

*Vanille [*Vanilla fragrans *(Salisbury) Ames] —*

Partie 2: Méthodes d'essai

Reference number
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Foreword

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

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 part of ISO 5565 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 5565-2 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-committee SC 7, *Spices and condiments*.

This first edition of ISO 5565-2, together with ISO 5565-1, cancels and replaces ISO 5565:1982, which has been technically revised.

ISO 5565 consists of the following parts, under the general title *Vanilla [*Vanilla fragran*s (Salisbury) Ames]*:

— *Part 1: Specification*

— *Part 2: Test methods*

Vanilla [*Vanilla fragrans* (Salisbury) Ames] —

Part 2: Test methods

1 Scope

This part of ISO 5565 specifies test methods for the analysis of vanilla belonging to the species *Vanilla fragrans* (Salisbury) Ames, syn. *Vanilla planifolia* Andrews.

This part of ISO 5565 is applicable to vanilla in pods, cut in bulk, and in the form of powder. It is not applicable to vanilla extracts.

Three test methods for the analysis of vanilla are described in this part of ISO 5565:

a) the determination of moisture content in vanilla pods and powder (4.1);

b) the determination of vanillin, vanillic acid, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid by high-performance liquid chromatography (4.2);

c) the determination of vanillin content by an ultraviolet spectrometric method (4.3).

NOTE Specifications for vanilla are given in ISO 5565-1.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this part of ISO 5565. For dated references, subsequent amendments to, or revisions of, this publication do not apply. However, parties to agreements based on this part of ISO 5565 are encouraged to investigate the possibility of applying the most recent edition of the normative document 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 1042, *Laboratory glassware — One-mark volumetric flasks.*

3 Terms and definitions

For the purposes of this part of ISO 5565, the following term and definition apply.

3.1
moisture content
quantity of water entrained and collected in accordance with the method specified in this part of ISO 5565

NOTE It is expressed as a mass fraction in percent [formerly given as % (m/m)].

4 Test methods

4.1 Determination of moisture content in vanilla pods and powder

NOTE The general method described in ISO 939 is not applicable to vanilla.

4.1.1 Principle

The amount of water entrained by azeotropic distillation is determined using a water-immiscible organic liquid. The water is collected in a graduated tube.

4.1.2 Reagent

Use only reagents of recognized analytical grade, and distilled or demineralized water or water of equivalent purity.

4.1.2.1 Toluene

Saturate the toluene by shaking it with a small quantity of water and distil it. Use the distillate for the determination of the moisture.

4.1.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.1.3.1 Distillation apparatus, consisting of a glass flask heated by a suitable means and provided with a reflux condenser discharging into a receiver connected to the flask.

The connections between the receiver, the condenser and the flask are interchangeable ground glass joints. The receiver serves to collect and measure the condensed water, and to return the solvent to the flask. The assembly of the apparatus is shown in Figure 1 and the various components are described below.

4.1.3.1.1 Flask, of capacity 500 ml, of the shape shown in Figure 1 and made of heat-resistant glass, well annealed and as free as possible from striae and similar defects.

4.1.3.1.2 Reflux condenser, water cooled, made of glass, having a jacket approximately 400 mm long and an inner tube of diameter 9,5 mm to 12,5 mm.

The tip of the condenser to be inserted in the receiver may be ground off at an angle of 30° from the vertical axis of the condenser. When inserted into the receiver, the tip of the condenser shall be 6 mm to 7 mm above the surface of the liquid in the receiver after distillation conditions have been established.

4.1.3.1.3 Receiver, of capacity 5 ml, made of heat-resistant glass, well annealed and as free as possible from striae and similar defects, provided with ground glass joints, with the shape, dimensions and tolerances given in Figure 1.

It consists essentially of an upper chamber, together with a tube and ground joint leading to a flask and graduated tube. The graduated portion shall have a capacity of 5 ml when filled to the highest graduation mark.

The scale shall cover the range of 0 ml to 5 ml and shall be graduated at intervals of 0,10 ml. The graduation marks corresponding to each millilitre shall be numbered and carried completely round the tube. The graduation marks midway between the numbered marks shall be carried three-quarters of the way round, and the remaining marks shall be carried half-way round the tube. The error at any indicated capacity shall not exceed 0,05 ml.

4.1.3.1.4 Heat source, either an **oil bath** or an **electric heater,** provided with a sliding rheostat or other means of heat control.

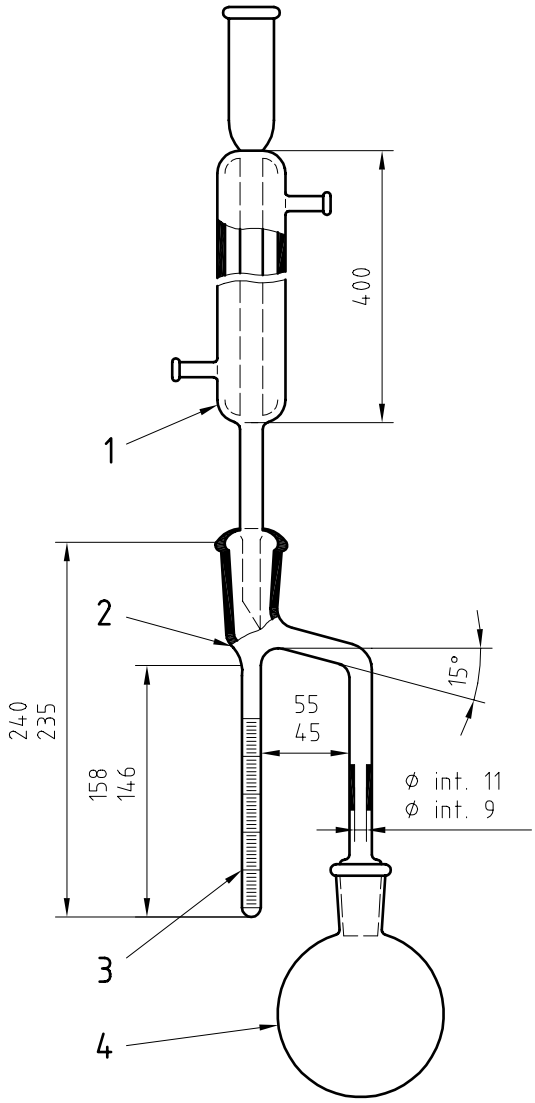
The temperature of the oil in the bath should not be very much higher than the boiling point of toluene.

4.1.3.1.5 **Copper wire**, long enough to extend through the condenser and with one end twisted into a spiral.

The diameter of the spiral shall be such that it fits snugly within the graduated portion of the receiver and yet can be moved up and down.

4.1.3.2 **Analytical balance**, accurate to 0,01 g.

Dimensions in millimetres



Key

1 Reflux condensor (4.1.3.1.2)
2 Receiver (4.1.3.1.3)
3 Graduated tube, of capacity 5 ml, graduated in 0,10 ml
4 Flask (4.1.3.1.1)

Figure 1 — Distillation apparatus

4.1.4 Preparation of test sample

4.1.4.1 Vanilla in pods, cut or in bulk

Prepare the test sample by cutting the vanilla pods into pieces of 5 mm maximum, taking care not to modify the moisture content.

4.1.4.2 Vanilla in powder

Thoroughly mix the laboratory sample.

4.1.5 Procedure

4.1.5.1 Preparation of apparatus

Clean the entire apparatus with a potassium dichromate/sulfuric acid cleaning solution to minimize the adherence of water droplets to the sides of the condenser and the receiver. Rinse thoroughly with water and dry completely before use.

4.1.5.2 Test portion

Weigh, to the nearest 0,1 g, about 10 g of the test sample (4.1.4) such that the quantity of water collected will not exceed 4,5 ml.

4.1.5.3 Determination

Transfer quantitatively the test portion (4.1.5.2) to the distillation flask (4.1.3.1.1) using toluene (4.1.2.1). Add sufficient toluene (about 75 ml in all) to cover the sample completely and swirl to mix. Assemble the apparatus and fill the receiver (4.1.3.1.3) with the toluene by pouring it through the condenser (4.1.3.1.2) until it begins to overflow into the distillation flask. If necessary, insert a loose cotton plug in the top of the condenser or attach to it a small calcium chloride tube to prevent condensation of atmospheric moisture within the condenser tube.

In order to control refluxing, wrap the flask and tube leading to the receiver with a cloth for insulation. Heat the flask so that the distillation rate is about 100 drops per minute. When the greater part of the water has distilled over, increase the distillation rate to about 200 drops per minute and continue until no more water is collected. Purge the reflux condenser occasionally during the distillation with 5 ml portions of the toluene to wash down any moisture adhering to the walls of the condenser.

The water in the receiver may be made to separate from the toluene by occasionally moving a spiral copper wire up and down in the condenser and receiver, thus causing the water to settle at the bottom of the receiver. Reflux until the water level in the receiver remains unchanged for 30 min and then shut off the source of heat. Flush the condenser with toluene as required, making use of the spiral copper wire to discharge any moisture droplets.

Immerse the receiver in water at room temperature for at least 15 min or until the toluene layer is clear; then read the volume of water.

4.1.6 Expression of results

The moisture content, w, expressed as a percentage by mass, is equal to:

$$w = \frac{100\,V}{m}$$

where

V is the volume, in millilitres, of water collected;

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

It is assumed that the density of water is 1 g/ml exactly.

4.2 Determination of vanillin, vanillic acid, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid in vanilla in pods, bulk, cut or powder form, by high-performance liquid chromatography (Reference method)

4.2.1 Principle

A test portion is extracted and/or diluted (as necessary) then separated by high-performance liquid chromatography (HPLC), using an internal standard, then determined by ultraviolet spectrometry.

4.2.2 Reagents

Use only reagents of recognized analytical grade, and distilled or demineralized water or water of equivalent purity.

4.2.2.1 **Ethanol**, 96 % (volume fraction).

4.2.2.2 **Methanol**

4.2.2.3 **Dilute phosphoric acid**, $c(H_3PO_4)$ = 0,01 mol/l.

4.2.2.4 **Mobile phase** (for guidance).

Mix 75 parts of the dilute phosphoric acid (4.2.2.3) with 25 parts of the methanol (4.2.2.2). Filter through a membrane (4.2.3.3). Degas.

4.2.2.5 **Reference standards**, with a minimum purity 99 %.

4.2.2.5.1 **Vanillin** (4-hydroxy-3-methoxybenzaldehyde).

4.2.2.5.2 **Vanillic acid** (4-hydroxy-3-methoxybenzoic acid).

4.2.2.5.3 **4-Hydroxybenzaldehyde**

4.2.2.5.4 **4-Hydroxybenzoic acid**

4.2.2.6 **Internal standard**: acetylsalicylic acid, with a minimum purity of 99 %.

4.2.2.7 **Stock standard solutions**, preferably dissolved in the mobile phase.

4.2.2.7.1 **Vanillin stock solution**, of concentration 1 g/l, weighed to the nearest 0,001 g.

4.2.2.7.2 **Vanillic acid stock solution**, of concentration 0,1 g/l, weighed to the nearest 0,001 g.

4.2.2.7.3 **4-Hydroxybenzaldehyde stock solution**, of concentration 0,1 g/l, weighed to the nearest 0,001 g.

4.2.2.8 Working solution

The working solution is obtained by dilution of the stock solutions (4.2.2.7) preferably in the mobile phase (4.2.2.4) in such a manner that the final concentration of the working solution should be as given in 4.2.2.8.1 to 4.2.2.8.4.

The solutions prepared in this manner can be preserved for some days in the dark, at a temperature of 4 °C.

4.2.2.8.1 **Vanillin working solution**, of concentration at 0,1 g/l.

4.2.2.8.2 Vanillic acid working solution, of concentration 0,008 g/l.

4.2.2.8.3 4-Hydroxybenzaldehyde working solution, of concentration 0,008 g/l.

4.2.2.8.4 4-Hydroxybenzoic acid working solution, of concentration 0,002 g/l.

4.2.2.9 Internal standard solution

Dissolve acetyl salicylic acid (4.2.2.6) in the mobile phase (4.2.2.4) to give a concentration of about 0,6 g/l, weighed to the nearest 0,001 g.

NOTE The concentrations should tally approximately with that found in the sample solution.

4.2.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.2.3.1 Airtight grinder.

4.2.3.2 One-mark volumetric flasks, of capacities 100 ml and 200 ml, complying with the requirements of ISO 1042.

4.2.3.3 Membranes, of porosity 0,45 µm.

4.2.3.4 Continuous-extraction apparatus, of the Soxhlet type, comprising the following.

4.2.3.4.1 Soxhlet extractor, of capacity 125 ml.

4.2.3.4.2 Condenser

4.2.3.4.3 Flask, of capacity 250 ml, with ground glass neck.

4.2.3.4.4 Cartridge

4.2.3.4.5 Heat source, either an **electric heating mantle** or an **oil bath**, provided with a sliding rheostat or other means of heat control, and with a temporized circuit breaker.

4.2.3.5 High-performance liquid chromatograph, fitted with the following.

4.2.3.5.1 Spectrometric detector, allowing measurement at a wavelength of 254 nm.

4.2.3.5.2 Injection device, for introducing a test sample of 10 µl to 100 µl.

4.2.3.5.3 Column, the packing of which consists of C_{18} bonded silica of 5 µm to 10 µm particle size.

4.2.3.6 Syringe, for high-performance liquid chromatography.

4.2.3.7 Analytical balance, with an accuracy of 0,001 g.

4.2.4 Preparation of test sample

4.2.4.1 Vanilla in pods, cut or in bulk

Grind the sample and homogenize it thoroughly.

4.2.4.2 Vanilla in powder

Thoroughly mix the sample.

4.2.5 Test portion

Weigh, to the nearest 0,001 g, 20 g of the prepared sample (4.2.4.1 or 4.2.4.2).

4.2.6 Extraction

Extract the test portion (4.2.5) in the extraction apparatus (4.2.3.4) with about 200 ml of ethanol (4.2.2.1) for 16 h; i.e. 25 to 30 rotations of solvent.

Use quantities of vanilla pods or vanilla powder and of the extraction solvent to lead to an extract being obtained which has a maximum concentration of 100 g of pods per litre.

Transfer the extract to a 200 ml volumetric flask (4.2.3.2). Wash the flask of the extraction apparatus several times with small quantities of ethanol and add the washings to the volumetric flask.

Dilute to the mark with ethanol and mix well. Thus, a solution with a known titre is obtained.

Make a one-in-ten dilution of this solution with the mobile phase (4.2.2.4).

4.2.7 Procedure

4.2.7.1 Chromatographic operating conditions

Conduct the analysis at atmospheric temperature under the conditions specified in 4.2.3.5 using the mobile phase (4.2.2.4) with a flow rate of about 1 ml per minute.

4.2.7.2 Calibration

4.2.7.2.1 Inject the reference standard and the internal standard separately, and then as a mixture. Adapt the operating conditions so that the resolution factors of the peaks of all the compounds are at least equal to 1.

4.2.7.2.2 Inject the sample as prepared in 4.2.6 after filtering it through a membrane (4.2.3.3). Check that the internal standard does not interfere with any of the constituents of the sample.

4.2.7.2.3 Mix 10 ml of the standard solution obtained in 4.2.2.7 with 10 ml of the internal standard solution (4.2.2.9). Filter on the membrane and inject.

The response coefficients (K_i) are given by the equation:

$$K_i = \frac{m_i \times h_S}{m_S \times h_i}$$

where

K_i is the response coefficient of the constituent i to be determined;

m_i is the mass of the constituent i in the injected solution;

m_S is the mass of the internal standard in the injected solution;

h_i is the height of the peak of the constituent i measured on the chromatogram;

h_S is the height of the peak of the internal standard measured on the chromatogram.

NOTE In the equation, the heights of the peaks may be replaced by the areas.

4.2.7.3 Determination

4.2.7.3.1 Mix 10 ml of the sample solution prepared in accordance with 4.2.6 with 10 ml of the internal standard solution (4.2.2.6). Filter through the membrane.

4.2.7.3.2 Inject the solution obtained in 4.2.7.3.1 under the conditions fixed in 4.2.7.2.3.

The percentages of the substances to be determined are calculated by the equation:

$$m_i = \frac{K_i \times h_i \times m_S}{h_S}$$

where the symbols are the same as in 4.2.7.2.3.

NOTE In the equation, the heights of the peaks may be replaced by the areas.

4.3 Determination of the vanillin content of vanilla pods, cut, in bulk and the form of powder, by an ultraviolet spectrometric method

4.3.1 Principle

The vanillin contained in a test portion is extracted with ethanol.

The vanillin in the ethanolic solution is determined by a spectrometric method.

4.3.2 Reagents

Use only reagents of recognized analytical grade, and distilled or demineralized water or water of equivalent purity.

4.3.2.1 **Ethanol,** 96 % (volume fraction), for UV spectrometry.

4.3.2.2 **Sodium hydroxide solution**, c(NaOH) ≈ 1 mol/l.

4.3.2.3 **Vanillin** (4-hydroxy-3-methoxybenzaldehyde).

4.3.3 Apparatus

Usual laboratory equipment and, in particular, the following.

4.3.3.1 **Airtight grinder**

4.3.3.2 **One-mark volumetric flasks**, of capacities 100 ml and 250 ml, complying with the requirements of ISO 1042.

4.3.3.3 **Pipettes**, to deliver 10 ml, 20 ml and 25 ml.

4.3.3.4 **Desiccator**, containing an efficient desiccant.

4.3.3.5 **Extraction apparatus**, as described in 4.2.3.4 to 4.2.3.4.5.

4.3.3.6 **Spectrometer**, suitable for making measurements in the ultraviolet region.

4.3.3.7 **Silica cells**, for spectrometry, having optical path lengths of 1 cm.

4.3.3.8 **Weighing bottle**, of capacity 25 ml, with an airtight stopper.

4.3.3.9 **Syringe**, for high-performance liquid chromatography.

4.3.3.10 **Analytical balance**, with an accuracy of 0,001 g.

4.3.4 Procedure

4.3.4.1 Determination of specific absorbance of vanillin

4.3.4.1.1 Preparation of standard solutions

In the weighing bottle (4.3.3.8) weigh, to the nearest 0,001 g, about 30 mg of the vanillin (4.3.2.3) which has been previously dried in the desiccator (4.3.3.4). Dissolve it in about 20 ml of the ethanol (4.3.2.1) and transfer it quantitatively to a 250 ml volumetric flask (4.3.3.2). Rinse the weighing bottle several times with the ethanol and pour the washings into the volumetric flask. Dilute to the mark with the ethanol and mix well (solution A1).

Pipette 25 ml of solution A1 into a 100 ml volumetric flask (4.3.3.2). Dilute to the mark with the ethanol and mix well (solution B1).

Pipette 10 ml of solution B1 into a 100 ml volumetric flask. Add about 60 ml of the ethanol and 2 ml of the sodium hydroxide solution (4.3.2.2). Mix well. Dilute to the mark with the ethanol and mix well (solution C1).

4.3.4.1.2 Preparation of reference solution

Prepare a reference solution by pipetting 2 ml of the sodium hydroxide solution (4.3.2.2) into a 100 ml volumetric flask, and diluting to the mark with the ethanol. Mix well.

4.3.4.1.3 Determination

Record the spectrum of solution C1 relative to that of the reference solution (4.3.4.1.2) over the wavelength range 250 nm to 420 nm, using the spectrometer (4.3.3.6) and the cells (4.3.3.7).

4.3.4.1.4 Calculation

Maximum absorption occurs at (350 $\pm$ 3) nm and its absorbance should be between 0,2 and 0,8.

Draw a baseline from about 270 nm to 380 nm.

Note the maximum absorbance (A_{max}) and the absorbance at the baseline at the same wavelength (A_{base}).

Calculate the specific absorbance ($E\ ^{1cm}_{1\%}$) of vanillin from the following equation:

$$E\ ^{1cm}_{1\%} = \frac{100\,(A_{max} - A_{base})}{m}$$

where m is the mass, in milligrams, of vanillin used to prepare the solution.

4.3.4.2 Preparation of test sample

4.3.4.2.1 Vanilla as pods, cut or in bulk

Grind the sample and mix well.

4.3.4.2.2 Vanilla powder

Mix the sample well.

4.3.4.3 Test portion

Weigh, to the nearest 0,001 g, about 20 g of the prepared sample (4.3.4.2.1 or 4.3.4.2.2).

4.3.4.4 Extraction

Extract the test portion (4.3.4.3) in the extraction apparatus (4.3.3.5) with about 200 ml of ethanol (4.3.2.1) for 16 h; i.e. 25 to 30 rotations of solvent.

Transfer it to a 250 ml volumetric flask (4.3.3.2). Rinse the flask of the extraction apparatus several times with small quantities of ethanol, and pour the washings into the volumetric flask (4.3.3.2).

Make up to the mark with the ethanol and mix well (solution A2).

4.3.4.5 Preparation of reference solution

Prepare a reference solution by pipetting 2 ml of the sodium hydroxide solution (4.3.2.2) into a 100 ml volumetric flask (4.3.3.2). Dilute to the mark with the ethanol. Mix well.

4.3.4.6 Determination

Pipette 25 ml of solution A2 into a 100 ml volumetric flask. Dilute to the mark with the ethanol and mix well (solution B2).

Pipette 20 ml of solution B2 into a 100 ml volumetric flask. Dilute to the mark with the ethanol and mix well (solution C2).

Pipette 10 ml of solution C2 into a 100 ml volumetric flask. Add about 60 ml of the ethanol and 2 ml of the sodium hydroxide solution (4.3.2.2). Dilute to the mark with the ethanol and mix well (solution D2).

Record the spectrum of solution D2 relative to that of the reference solution 4.3.4.5 over the wavelength range 250 nm to 420 nm, using the spectrometer (4.3.3.6) and the cells (4.3.3.7).

4.3.4.7 Expression of results

The vanillin content, w_v, expressed as a percentage by mass of the sample, is equal to:

$$w_v = \frac{50\,000\,(A_{max} - A_{base})}{E\,^{1cm}_{1\%} \times m}$$

where

A_{max} is the maximum absorbance;

A_{base} is the absorbance at the baseline at the same wavelength;

$E\,^{1cm}_{1\%}$ is the specific absorbance of vanillin (4.3.4.1.4);

m is the mass, in grams, of the test portion used for extraction.

If it is desired to express the result relative to the dry matter content, take into account the moisture content of the product.

5 Test report

For the three methods described in 4.1, 4.2 and 4.3, the test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this part of ISO 5565;

— all operating details not specified in this part of ISO 5565, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test result(s) obtained, and if it is related to dry matter or not;

— if the repeatability has been checked, the final quoted result obtained.

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