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INTERNATIONAL STANDARD

ISO 5504

Second edition
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Oilseed residues — Determination of total isothiocyanate content and vinylthiooxazolidone content

Tourteaux de graines oléagineuses — Dosage des isothiocyanates et de la vinylthiooxazolidone

Reference number
ISO 5504:1992(E)

Foreword

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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.

International Standard ISO 5504 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 2, *Oleaginous seeds and fruits*.

This second edition cancels and replaces the first edition (ISO 5504:1983), the Scope and Expression of results of which have been technically revised.

Annexes A and B of this International Standard are for information only.

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Oilseed residues — Determination of total isothiocyanate content and vinylthiooxazolidone content

1 Scope

This International Standard specifies a method for the determination of the total isothiocyanate (ITC) content and 5-vinylthiooxazolidone (VTO) content produced by the enzymic hydrolysis of glucosinolates in oilseed residues of the *Brassica* family.

Under the operating conditions described, the method does not allow determination of free isothiocyanates and free vinylthiooxazolidone.

It has been concluded from the results of interlaboratory tests that, owing to the accuracy of the method, it is not suitable for use on products having contents of less than

2 µmol/g of ITC

6 µmol/g of VTO

Annex A gives, for information, a method of determining the isothiocyanate content by argentimetry. This method is not applicable, however, to oilseed residues of the *Brassica* family having low glucosinolates contents.

NOTE 1 For rapeseeds, two methods for the determination of glucosinolates are given in ISO 9167-1 and ISO 9167-2.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 734:1979, *Oilseeds residues — Determination of hexane extract (or light-petroleum extract), called "oil content"*.

ISO 5502:1992, *Oilseed residues — Preparation of test samples*.

3 Principle

After removal of the fats and oil (de-fatting), if necessary, and drying of the oilseed residue, enzymatic hydrolysis of the glucosinolates, extraction of isothiocyanates (ITC) with dichloromethane or chloroform and of vinylthiooxazolidone (VTO) with diethyl ether.

Determination of ITC by gas chromatography and of VTO by ultraviolet spectrometry.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of at least equivalent purity.

4.1 Reagents for determination of ITC

4.1.1 Dichloromethane, or, failing this, **chloroform**.

WARNING — Take special precautions when handling chloroform. Avoid prolonged exposure to chloroform by, for example, carrying out all operations, so far as possible, in a fume cupboard.

4.1.2 *n*-Hexane, or, failing this, **light petroleum** (boiling range 40 °C to 60 °C).

4.1.3 Buffer solution, of pH 7, commercially available, or, for example, a solution prepared as follows.

Measure 35,3 ml of 0,1 mol/l citric acid monohydrate ($C_6H_8O_7.H_2O$) solution (21,01 g/l solution) into a

200 ml one-mark volumetric flask and dilute to the mark with a 0,2 mol/l solution of disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (35,61 g/l solution). Check the pH and, if necessary, adjust.

4.1.4 Enzyme source, myrosidase (thioglucosidase), available commercially.

NOTE 2 It is also possible to use an enzyme source prepared from white mustard seed (*Sinapis alba* L.)[1], as follows.

Finely grind the white mustard seed so that at least 80 % will pass through a sieve of aperture size 280 µm.

Remove the oil and fat from the grindings using **cold** hexane or, failing this, **cold** light petroleum (4.1.2), carrying out the extraction using a method which will permit extraction until not more than 2 % of oil remains in the product and without the temperature exceeding 30 °C. For example, use a double-walled Soxhlet apparatus, or carry out extractions by grinding with hexane in a microgrinder cooled by running water. Remove traces of solvent at ambient temperature, preferably using a slight current of air.

Store the enzyme source thus obtained at 4 °C in a hermetically sealed glass bottle. Under these conditions, it can be kept for about 6 weeks, but it is preferable to use a freshly prepared enzyme source.

It is recommended that a blank test be performed to ensure that the enzyme source does not contain ITC in quantities which could significantly affect the results.

4.1.5 Butyl isothiocyanate, 2 mmol/l standard solution in dichloromethane or chloroform (4.1.1).

It is preferable to prepare a 20 mmol/l standard stock solution [230,4 mg of butyl isothiocyanate in 100 ml of dichloromethane or chloroform (4.1.1)] and to prepare a 10^{-1} dilution before use.

The dilution factor may be modified depending on the assumed ITC content of the product.

4.1.6 Gases for gas chromatography

Carrier gas: carefully dried nitrogen containing less than 10 mg of oxygen per kilogram.

Auxiliary gases: hydrogen and air.

4.2 Reagents for the determination of VTO

4.2.1 Diethyl ether, spectrometric grade.

4.2.2 Anti-foaming agent, for example octan-2-ol.

4.2.3 Buffer solution, of pH 7 (see 4.1.3).

4.2.4 Enzyme source (see 4.1.4).

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Apparatus for preparing the test sample and drying the test portion

5.1.1 Sieve, of aperture size 280 µm.

5.1.2 Apparatus for the extraction of fat and oil, by the method specified in ISO 734, if necessary.

5.1.3 Grinder

5.1.4 Desiccator

5.1.5 Analytical balance

5.1.6 Electric oven, capable of being maintained at 103 °C ± 2 °C.

5.1.7 Conical flask, of 25 ml capacity.

5.2 Apparatus for the determination of ITC

5.2.1 Chromatograph, with a flame ionization detector and a recorder, comprising, for example, a column of length 2 m, external diameter 3,2 mm and internal diameter 2 mm, filled with 10 % (*m*/*m*) diethylene glycol succinate (DEGS) on GAS/CHROM P 150 µm to 180 µm (80 to 100 mesh) (treated with hexamethyldisilazane).

5.2.2 Stirrer/shaker system, for conical flasks, or a **magnetic stirrer**.

5.2.3 Microsyringe, of 1 µl capacity.

5.2.4 Pipettes, of 5 ml and 10 ml capacity.

5.3 Apparatus for the determination of VTO

5.3.1 Spectrometer, preferably with a recorder, suitable for measurements in the ultraviolet, with silica cells of 10 mm optical path length.

5.3.2 Stirrer/shaker system, for conical flasks, or a **magnetic stirrer**.

5.3.3 Conical flasks, of 100 ml and 200 ml capacity.

5.3.4 One-mark volumetric flasks, of 25 ml and 100 ml capacity.

5.3.5 Beaker, of 50 ml capacity.

1) Use seed in which more than 85 % of the grains germinate in less than 72 h and which is less than 2 years old.

5.3.6 Separating funnel, of 50 ml capacity.

5.3.7 Pipette, of 2 ml capacity.

6 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport and storage.

Sampling is not part of the method specified in this International Standard. A recommended method is given in ISO 5500.

7 Preparation of test sample

See ISO 5502.

7.1 Oilseed residues having oil contents less than 5 % (*m*/*m*)

As de-fatting is not necessary for oil contents below 5 % (*m*/*m*), use the oilseed residues as received after grinding, if necessary, so that at least 80 % of the grindings obtained pass through the sieve (5.1.1).

As results are expressed relative to the de-fatted sample, perform a parallel determination of the oil content of the oilseed residue, using the method described in ISO 734 or any other suitable method.

7.2 Oilseed residues having oil contents greater than or equal to 5 % (*m*/*m*)

If the oil content is greater than or equal to 5 % (*m*/*m*) (pressed oilseed residues), carry out de-fatting of 10 g of oilseed residue using the procedure specified in ISO 734. After elimination of the solvent, grind, if necessary.

8 Procedure

8.1 Determination of ITC

8.1.1 Test portion

Transfer approximately 2,2 g of the test sample (clause 7) to a 25 ml conical flask (5.1.7), which has been previously dried and weighed to the nearest 1 mg. Place in the oven (5.1.6), set at 103 °C, for at least 8 h, and allow to cool in the desiccator (5.1.4) to room temperature.

NOTE 3 If a determination of VTO has also been requested (see 8.2.1), simultaneously dry the test portion to be used for the determination of VTO in the beaker.

Weigh the conical flask to the nearest 1 mg, and determine the mass of the de-fatted, if necessary, and dried test portion (about 2 g).

8.1.2 Determination

Add to the test portion, 5 ml of the buffer solution (4.1.3), 0,25 g of the enzyme source (4.1.4) and 10 ml, using the pipette (5.2.4), of the standard butyl isothiocyanate solution (4.1.5) (using a more dilute solution, if necessary), such that the height of the peak of the standard solution is of the same order as that of the highest peak of the test solution).

Shake for 2 h at room temperature using the stirrer/shaker system or the magnetic stirrer (5.2.2). Allow to separate, or centrifuge if necessary.

Using the microsyringe (5.2.3), take 1 µl of the dichloromethane or chloroform phase, avoiding taking any particles in suspension, and inject this into the chromatograph (5.2.1) controlled, for example, as follows:

temperature of the injector: 135 °C

temperature of the column: 90 °C

temperature of the detector: 130 °C

pressure or flow-rate of the carrier gas: 80 kPa[2] or 20 ml/min to 30 ml/min.

The order of elution is as follows:

allyl isothiocyanate,

butyl isothiocyanate,

butenyl isothiocyanate,

pentenyl isothiocyanate.

NOTES

4 For information, if using diethylene glycol succinate (DEGS) as the stationary phase, the retention times relative to butyl isothiocyanate are as follows:

allyl isothiocyanate: 0,70

butenyl isothiocyanate: 1,45

pentenyl isothiocyanate: 2,45

5 As the solution injected is very corrosive, clean the microsyringe immediately after use with a solvent.

2) 80 kPa = 0,8 bar

8.2 Determination of VTO

8.2.1 Test portion

Transfer approximately 2,2 g of the test sample (clause 7) to a 50 ml beaker (5.3.5), which has been previously dried and weighed to the nearest 1 mg. Place in the oven (5.1.6), set at 103 °C, for at least 8 h, and allow to cool in the desiccator (5.1.4) to room temperature.

Weigh the beaker to the nearest 1 mg, and determine the mass of the de-fatted, if necessary, and dried test portion (about 2 g).

8.2.2 Determination

Quantitatively transfer the test portion into a 200 ml conical flask (5.3.3) and add 70 ml of boiling buffer solution (4.2.3), using some to rinse the beaker. Allow to cool to about 30 °C, then add 0,50 g of the enzyme source (4.2.4) and a few drops of the anti-foaming agent (4.2.2). Shake for 2 h at room temperature using the stirrer/shaker system or the magnetic stirrer (5.3.2).

Immediately transfer quantitatively to a 100 ml one-mark volumetric flask (5.3.4), rinsing with water, and make up to the mark with water. Filter and collect the filtrate in a 100 ml conical flask (5.3.3). Shake gently for 30 s and, by means of the pipette (5.3.7), take 2 ml and place it in the 50 ml separating funnel (5.3.6). Carry out two extractions of the VTO using 10 ml of the diethyl ether (4.2.1) each time. Collect the ether layers in a 25 ml one-mark volumetric flask (5.3.4), and make up to the mark with diethyl ether.

Determine the absorption curve from 220 nm to 280 nm and subtract the absorbance read at 280 nm from that read at the maximum absorbance (which occurs at about 250 nm) in order to obtain the absorbance of the test portion.

If the absorbance values obtained are beyond the limits of the instrument, dilute the extracted test solution as necessary with diethyl ether.

8.2.3 Blank test

Carry out a blank test under the same conditions, omitting the test portion in order to determine the absorbance due to the enzyme source (4.2.4).

9 Expression of results

9.1 ITC content

The ITC content, expressed in micromoles per gram of dry matter content of the de-fatted[3] sample, is equal to

$$\frac{Q(\mathrm{ITC})}{S_e \times m_1} \times [4/3S_a + 4/4S_b + 4/5S_p]$$

where

m_1 is the mass, in grams, of the de-fatted and dried test portion (8.1.1);

Q(ITC) is the amount of butyl isothiocyanate, in micromoles, contained in 10 ml of solution (usually 20 µmol);

S_e is the area of the peak corresponding to butyl isothiocyanate;

S_a is the area of the peak corresponding to allyl isothiocyanate;

S_b is the area of the peak corresponding to butenyl isothiocyanate;

S_p is the area of the peak corresponding to pentenyl isothiocyanate.

NOTE 6 The values 4/3, 4/4 and 4/5 are the theoretical response coefficients of isothiocyanates.

9.2 VTO content

The VTO content, expressed in micromoles per gram of dry matter content of the de-fatted[3] sample, is equal to

$$\frac{(A_E - A_B) \times 25 \times 100 \times 10^6}{15\,756 \times 1\,000 \times 2 \times m_2}$$

where

A_E is the absorbance of the test solution (8.2.2);

A_B is the absorbance of the blank test solution (8.2.3);

15 756 is the molar extinction coefficient, in litre reciprocal moles reciprocal centimetres, of the VTO;

m_2 is the mass, in grams, of the test portion (8.2.1).

A simplified formula may thus be derived where the VTO content, expressed in micromoles per gram of dry matter content of the de-fatted[3] sample, is equal to

$$\frac{(A_E - A_B) \times 79{,}33}{m_2}$$

3) In the case of oilseed residues which have not been de-fatted, it is necessary to take into account the oil content determined in clause 7.

Take into account any dilution of the test solution.

9.3 Special case: animal feeding stuffs

For animal feeding stuffs, the results are generally expressed relative to the product as received. Thus, if such a manner of expression of results is required, make the necessary corrections to take into account the water and oil contents.

10 Test report

The test report shall specify

— the method in accordance with which sampling was carried out (if known),

— the method used,

— the test result(s) obtained, and

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

It shall also mentioned all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report shall include all information necessary for the complete identification of the sample.

Annex A
(informative)

Determination of isothiocyanate content by argentimetry

A.1 Introduction

For laboratories which do not have gas chromatographic apparatus for determining ITC, it is recommended to use the following argentimetric method. This method, which was compared with the gas chromatographic method by inter-laboratory tests carried out at the international level, was shown to give comparable results, provided that the glucosinolate content was sufficiently high.

It is important to note, however, that the expression of results is different in the two methods; for gas chromatography, the results are expressed in micromoles of isothiocyanates detected, whilst, for argentimetry, the results are expressed conventionally in milligrams of butenyl isothiocyanate.

A.2 Principle

After de-fatting (if necessary) and drying of the oilseed residue, enzymatic hydrolysis of the glucosinolates, then azeotropic distillation of ITC in the presence of ethanol and collection in dilute ammonia solution. Action of silver nitrate on the thiourea derivatives thus formed.

Back titration of the excess silver nitrate using an ammonium thiocyanate solution.

A.3 Reagents and materials

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

A.3.1 Ethanol, 95 % (V/V).

A.3.2 Anti-foaming agent, for example octan-2-ol.

A.3.3 Nitric acid, solution, $c(HNO_3) = 6$ mol/l.

A.3.4 Ammonia, 10% (m/m) solution.

A.3.5 Ammonium iron(III) sulfate, 80 g/l solution.

A.3.6 Silver nitrate, standard volumetric solution, $c(AgNO_3) = 0{,}1$ mol/l.

A.3.7 Ammonium thiocyanate, standard volumetric solution, $c(NH_4SCN) = 0{,}01$ mol/l.

A.3.8 Buffer solution, of pH 4, commercially available, or, for example, a solution prepared as follows.

Dissolve 42 g of citric acid monohydrate in 1 l of water. Adjust to pH 4 using concentrated sodium hydroxide solution.

A.3.9 Enzyme source, myrosidase (thioglucosidase), or an enzyme source prepared using white mustard seed (*Sinapis alba* L.) (see 4.1.4).

A.3.10 Anti-bumping granules

A.4 Apparatus

Usual laboratory apparatus and, in particular, the following.

A.4.1 Apparatus for preparing the test sample and drying the test portion

See 5.1.

A.4.2 Apparatus for the determination of ITC

A.4.2.1 Electric oven, capable of being maintained at 25 °C or 40 °C.

A.4.2.2 Distillation apparatus, of the type shown in figure A.1, comprising in particular:

— a conical flask ⓐ, of 500 ml capacity;

— a round-bottomed flask ⓑ, of 250 ml capacity, with a graduation line corresponding to a volume of 70 ml.

A.4.2.3 Water-bath, containing ice.

A.4.2.4 Water-bath, containing boiling water.

A.4.2.5 Conical flask, of 100 ml capacity.

A.4.2.6 One-mark volumetric flask, of 100 ml capacity.

A.4.2.7 Pipettes, of 10 ml and 25 ml capacity.

A.4.2.8 Filter paper

A.4.2.9 Reflux condenser, to fit the flask ⓑ of the distillation apparatus (A.4.2.2).

A.5 Preparation of test sample

Prepare the test sample as described in clause 7.

A.6 Procedure

A.6.1 Test portion

Transfer approximately 2,2 g of the test sample (A.5) to a conical flask (A.4.2.5), which has been previously dried and weighed to the nearest 1 mg. Place in the oven (5.1.6), set at 103 °C, for at least 8 h, and allow to cool in the desiccator (5.1.4) to room temperature.

Weigh the conical flask to the nearest 1 mg, and determine the mass of the de-fatted, if necessary, and dried test portion (about 2 g).

A.6.2 Hydrolysis

Quantitatively transfer the test portion to the 500 ml conical flask ⓐ of the distillation apparatus (A.4.2.2), add 100 ml of the buffer solution (A.3.8), using some to rinse the flask containing the test portion, and add 0,50 g of the enzyme source (A.3.9). Stopper the flask and shake gently, then place in the oven (A.4.2.1) for 16 h at 25 °C, or 3 h at 40 °C. During this period, shake the flask at regular intervals.

A.6.3 Preparation of the receiver flask

Transfer to the round-bottomed flask ⓑ of the distillation apparatus (A.4.2.2), 10 ml of the silver nitrate solution (A.3.6), measured from a pipette (A.4.2.7), and 2,5 ml of the ammonia solution (A.3.4).

Connect the flask to the distillation apparatus and place it in the water-bath containing ice (A.4.2.3). The end of the condenser tube shall be immersed in the silver nitrate/ammonia solution.

A.6.4 Distillation

Allow the conical flask (see A.6.2) to cool to room temperature, add a few anti-bumping granules (A.3.10) and a few drops of the anti-foaming agent (A.3.2), then connect it to the distillation apparatus. Using a funnel placed above the condenser, add to the contents of the flask 10 ml of ethanol (A.3.1) and place 3 ml of ethanol in the safety tube of the receiver flask.

Distil slowly until a total of 70 ml of liquid is obtained in the receiver flask.

A.6.5 Determination

Disconnect the receiver flask, and pour into it the ethanol contained in the safety tube. Attach a reflux condenser (A.4.2.9) and heat the flask for 30 min on a boiling-water-bath (A.4.2.4), then cool in cold water.

Transfer quantitatively the contents of the receiver flask to a 100 ml one-mark volumetric flask (A.4.2.6), rinsing the flask with water, and make up to the mark. Shake, and filter using a filter paper. Using a pipette (A.4.2.7), take 25 ml of the filtrate and place it in a 100 ml conical flask (A.4.2.5). Add 1 ml of the nitric acid solution (A.3.3), and 0,5 ml of the ammonium iron(III) sulfate solution (A.3.5) as indicator.

Titrate the excess silver nitrate with the ammonium thiocyanate solution (A.3.7) until a stable pink colour is obtained.

A.6.6 Blank test

Carry out a blank test using the same procedure, but omitting the test portion.

A.7 Expression of results

The ITC content, expressed as milligrams of butenyl isothiocyanate per gram of dry matter of the de-fatted[3] sample, is equal to

$$\frac{4(V_1 - V_2) \times c \times 56{,}59}{m_3}$$

where

m_3 is the mass, in grams, of the test portion (A.6.1);

V_1 is the volume, in millilitres, of ammonium thiocyanate solution used for the blank test (A.6.6);

V_2 is the volume, in millilitres, of ammonium thiocyanate solution used for the determination (A.6.5);

c is the exact concentration of the ammonium thiocyanate solution used.

A.8 Test report

The test report shall specify

— the method in accordance with which sampling was carried out (if known),

— the method used,

— the test result(s) obtained, and

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

It shall also mention all operating details not specified in this annex, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report shall include all information necessary for the complete identification of the sample.

Dimensions in millimetres

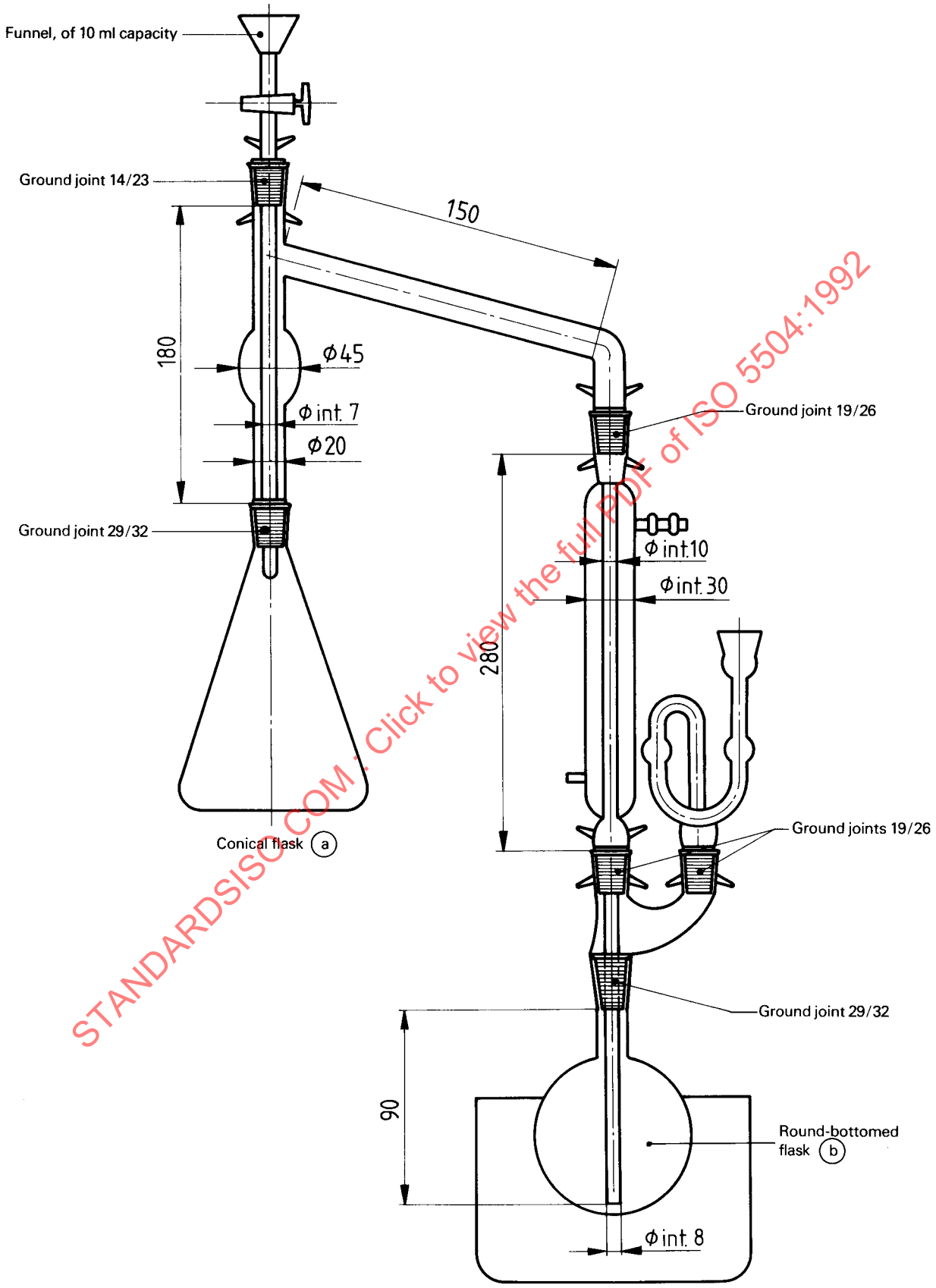

Figure A.1 — Example of apparatus for the distillation of isothiocyanates

Annex B
(informative)

Bibliography

[1] ISO 5500:1986, *Oilseed residues — Sampling.*

[2] ISO 9167-1:1992, *Rapeseed — Determination of glucosinolates content — Part 1: Method using high-performance liquid chromatography.*

[3] ISO 9167-2:—[4], *Rapeseed — Determination of glucosinolates content — Part 2: Method using X-ray fluorescence spectrometry.*

4) To be published.