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Rapeseed — Determination of glucosinolate content — Spectrometric method for total glucosinolates by glucose release

Colza — Détermination de la teneur en glucosinolates — Méthode spectrométrique pour les glucosinolates totaux par libération de glucose

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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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 34, *Food products*, Subcommittee SC 2, *Oleaginous seeds and fruits and oilseed meals*.

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.

Introduction

Glucosinolates are important antinutritional and flavour components of brassica oilseeds. They are particularly important in brassica seeds that have been modified to reduce the level of glucosinolates such as low glucosinolate types of rapeseed (canola). Determination of the level of glucosinolates in these seeds often reflects the commercial value and, certainly, discrimination between seeds with high levels of glucosinolates and those with low levels of glucosinolates is important both for commercial testing and scientific studies. This document provides a method for the estimation of total glucosinolates without requiring chromatographic apparatus. It complements ISO 9167, which is the reference method. This document is a Technical Specification as the precision data issued from the collaborative trial is not sufficient.

Rapeseed — Determination of glucosinolate content — Spectrometric method for total glucosinolates by glucose release

1 Scope

This document specifies a method for the determination of the content of the total glucosinolates in rapeseeds (colza) using visible spectrometry to determine the glucose released from glucosinolates by hydrolysis.

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 664, *Oilseeds — Reduction of laboratory sample to test sample*

ISO 665, *Oilseeds — Determination of moisture and volatile matter content*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
total glucosinolates
quantity of glucose released on hydrolysis by myrosinase and determined enzymatically

Note 1 to entry: The total glucosinolates is expressed as micromoles per gram of the seeds.

4 Principle

Total glucosinolates in a ground, full-fat brassica seed sample can be determined directly by estimation of glucose released on hydrolysis as the glucose unit is common to all glucosinolates. Hydrolysis of glucosinolates by myrosinase quantitatively releases glucose from the glucosinolates. The glucose is analysed using either glucose oxidase/peroxidase enzyme assay or glucose hexokinase/glucose-6-phosphate dehydrogenase enzyme assays.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and water conforming to grade 2 of ISO 3696.

5.1 Hydrochloric acid (2 M).

Dissolve 43 ml 36 % hydrochloric acid or 49 ml 32 % hydrochloric acid in 250 ml water.

5.2 Phenol tungstate reagent.

Dissolve 5,0 g sodium tungstate, 5,0 g sodium phosphate, dibasic, anhydrous, and 9,0 g sodium chloride in approximately 350 ml distilled water in a 500 ml volumetric flask. Adjust pH to 3,0 with 2 M hydrochloric acid (approximately 125 ml). Add 2,0 g phenol and make up to the mark with water.

5.3 Glucose determination reagents.

5.4 Glucose oxidase/peroxidase assay.

NOTE The reagents in this assay can be available in kit form. In this assay, β-D-glucose reacts with oxygen in the presence of glucose oxidase to form D-glucono-1,4-lactone and hydrogen peroxide. The hydrogen peroxide reacts with a colourless dye in the presence of peroxidase to give a coloured compound that can be measured spectrophotometrically.

5.5 Sodium phosphate buffer.

Dissolve 10 g sodium phosphate dibasic anhydrous in approximately 750 ml distilled water in a 1 l volumetric flask.

5.6 Glucose oxidase reagent.

Add 105,3 mg of glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* having activity of 25 000 units/100 mg or 131,7 mg of glucose oxidase having activity of 20 000 units/100 mg, to the sodium phosphate buffer mixture (5.5) and shake to mix.

5.7 Peroxidase colour reagent.

Dissolve 16,7 mg peroxidase (EC 1.11.1.7) type II from horseradish having an activity of 200 pupugallen units per mg solid and 333 mg 4-aminoantipyrine (4-amino-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one, 98 %) in a beaker with approximately 15 ml water. Add the contents of beaker to the 1 l volumetric flask containing the glucose oxidase (5.6) plus the sodium phosphate mixture (5.5) and fill with distilled water to obtain the glucose oxidase/peroxidase mixture. Store this solution in a brown bottle and refrigerate at between 2 °C and 4 °C.

5.8 Glucose hexokinase/Glucose-6-phosphate dehydrogenase glucose assay.

This assay is most conveniently carried out using commercially available kits. Glucose is first phosphorylated to glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase and adenosine-5-triphosphate. G-6-P, in the presence of glucose-6-phosphate dehydrogenase, is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The increase in absorption of NADPH at 334 nm, 340 nm or 365 nm is measured. The following are constituents of a typical kit.

a) Bottle containing dry ingredients including triethanolamine buffer – pH 7,6, NADP – 110 mg, ATP – 260 mg, magnesium sulfate, stabilizers to a total mass of 7,2 g. The contents of this bottle are diluted to 45 ml with water to make Solution 1.

b) Solution 2 (suspension): Bottle containing 1,1 ml enzyme suspension consisting of hexokinase (EC 2.7.1.1 about 320 U) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49 about 160 units).

NOTE Solution 1 is stable for four weeks at +4 °C and for two months at –20 °C and solution 2 is stable for one year at 4 °C.

5.9 Myrosinase solution.

Dissolve 12,5 mg myrosinase (beta-thioglucoside glucohydrolase EC 3.2.1.147, lyophilized, 200 U/g, from white mustard seed) per 1,0 ml distilled water, allowing for 0,5 ml per sample. A sufficient quantity of this solution to allow for the analyses planned should be made up fresh on the day of analysis. A method for preparing myrosinase from white mustard seed (*Sinapis alba* L.) is given in Annex A.

5.10 Sodium hydroxide (0,5 M).

Dissolve 20 g sodium hydroxide in approximately 800 ml distilled water in a 1 l beaker. Transfer solution to a 1 l volumetric flask and top up to 1 l with water.

5.11 Sodium acetate buffer (0,2 M).

Add 16,5 g sodium acetate anhydrous and 11,5 ml glacial acetic acid to 950 ml water in a 1 l volumetric flask. Adjust pH to 4,9 with 2 M hydrochloric acid and top up to 1 l with water.

5.12 Tris-HCl buffer.

Add 1,6 g tris(hydroxymethyl)aminomethane to 900 ml water in a 1 l volumetric flask. Adjust to pH 7,0 using 2 M hydrochloric acid and fill to 1 l with water.

5.13 Glucose solution, which is used to prepare a calibration curve to relate the absorbance of samples with the absorbance of standard glucose solutions. The calibration samples (5.13.2) should be prepared immediately prior to measurement on the spectrometer.

5.13.1 Stock solution.

Dry D-glucose (99 % purity) under vacuum at 40 °C for 4 h. In a 1 l volumetric flask, weigh approximately 1 g dry D-glucose to an accuracy of 0,1 mg. Add 1 g benzoic acid and make up to 1 l with water.

5.13.2 Calibration samples.

Accurately measure aliquots of the glucose stock solution (5.13.1) into individual 10 ml volumetric flasks. Add 2 ml phenol tungstate reagent (5.2) to each 10 ml volumetric flask. Carefully fill the volumetric flasks to the 10 ml mark with water. The preparation of the calibration samples is described in Table 1.

Table 1 — Calibration sample preparation description

Parameter	Calibration sample number						
	1	2	3	4	5	6	7
Volume of glucose stock standard solution (5.13.1)	0	0,10	0,20	0,50	1,0	1,5	2,0
Volume of phenol tungstate reagent (5.2)	2	2	2	2	2	2	2
Glucose concentration (µg/l)	0	0,010	0,020	0,050	0,100	0,15	0,200
Glucose content in 10 ml flask (µmol)	0	0,56	1,11	2,78	5,55	8,32	11,10

6 Apparatus

Usual laboratory apparatus and, in particular, the following shall be used.

6.1 Spectrometer, capable of operating in the visible region, in particular at 505 nm and equipped with cells of path length 1 cm, or, if the glucose hexokinase/glucose-6-phosphate dehydrogenase assay is used, a spectrometer capable of operating in the ultraviolet region between 334 nm and 365 nm.

6.2 Analytical balance, capable of displaying 0,000 1 g.

6.3 Centrifuge, capable of producing a radial acceleration of 5 000*g*, suitable for use with tubes (6.10).

6.4 Micro grinder, e.g. a coffee mill.

6.5 Tubes, (13 × 100) mm screw cap test tubes.

6.6 Water-bath, capable of being maintained at a temperature of 37 °C and equipped with a rack to hold chromatography columns (6.9).

6.7 Heating block, to hold tubes (6.5) maintaining a temperature of 95 °C.

6.8 Ion exchange resin, weak anion exchanger with high binding capacity, pore size 30 000 Da exclusion limit, working pH 2 to 9 and 3 meq/g to 4 meq/g ion exchange capacity (DEAE-Sephadex® A-25[1)] can be used).

6.9 Chromatography columns, (0,8 × 4) cm polypropylene columns (9 cm total column height) which hold up to 2,0 ml of chromatography media and 10 ml of eluant or sample in an integral reservoir, equipped with stopcock and stopper and a suitable stand. Pasteur pipettes, 150 mm long packed with glass wool may be suitable.

6.9.1 Preparation of ion exchange columns:

a) Place 100 mg of ion exchange resin (6.8) into columns (6.9). This amount has a theoretical capacity of at least 300 microequivalents.

b) Attach stopcocks to columns.

c) Fill each column with approximately 10 ml distilled water, letting approximately 2,0 ml run through the open stopcock.

d) Close the stopcock and cap the column loosely.

e) Let column-packing swell for a minimum of 16 h.

f) Run the following through the columns, without allowing the column to run dry: 5,0 ml 0,5 M sodium hydroxide (5.10), followed by 10 ml distilled water, then 5,0 ml 0,2 M sodium acetate buffer (5.11), then 10 ml water.

g) Lose the stopcock and cap the column loosely until sample is applied.

6.10 Concentrating tubes, 12 ml capacity. Suitable for centrifugation at 5 000*g*.

6.11 Glass cuvettes, 1 cm path length (disposable cuvettes may be used).

7 Sampling

Sampling should have been carried out in accordance with ISO 21294. If large non-oleaginous foreign bodies have been separated before the reduction of the laboratory sample, allowance shall be made for this in the calculation.

1) DEAE-Sephadex® A-25 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. Equivalent products may be used if they can be shown to lead to the same results.

8 Preparation of the test sample

8.1 Reduction of test sample to laboratory sample

Prepare the laboratory sample in accordance with ISO 664.

8.2 Moisture and volatile matter content

Determine the moisture and volatile matter content of the test sample in accordance with ISO 665, prior to sampling for grinding. If the seeds have a moisture and volatile matter content in excess of 10 % (mass fraction), dry them to less than 10 % (mass fraction) using a current of air at approximately 45 °C. If the seeds have been treated, wash them with dichloromethane and dry them in a current of air at ambient temperature.

8.3 Grinding

Grind the seeds in the microgrinder (6.4) for 20 s. Mix then grind for a further 5 s.

9 Procedure

9.1 Test portion

Weigh approximately 200 mg measured to the nearest 0,1 mg of ground seed (see 8.3) into a (13 × 100) mm screw cap test tube (6.5). Record this mass as *m*.

9.2 Extraction of glucosinolates

9.2.1 General

A procedure for testing the efficiency of extraction and subsequent analytical procedures is given in Annex B.

9.2.2 Principle

Heat is applied to ground canola seed samples to denature the natural myrosinase, keeping the glucosinolates intact during extraction. Glucosinolates, extracted with boiling water, are applied to a column packed with a weak anionic exchange resin that binds the glucosinolates. Myrosinase is applied to the column, releasing glucose from all glucosinolates present. The resulting glucose is eluted from the columns with water.

9.2.3 Operating mode

Place the test tube, uncovered, in a 95 °C heat block (6.7) for 15 min.

NOTE It is useful to measure the temperature with a thermometer inserted into a control sample of 200 mg ground seed similar to the samples being tested placed in a tube (6.5). Once the thermometer has reached a stable temperature of 95 °C, the control and thermometer can be removed.

Add approximately 2,0 ml boiling water using a heated Pasteur pipette to the test tube. Heating can be achieved by flushing the pipette several times in the boiling water prior to use. Cap, shake and return the tube to the heating block for another 5 min. Remove the test tube from the heat block and allow to cool. Add 2 ml room temperature water to the test tube and shake. Centrifuge at 5 000*g* for 10 min. Pour the supernatant into a 12 ml graduated concentrating tube (6.10). Add 2,0 ml room temperature water and repeat two more times. Adjust the volume in the 12 ml graduated concentrating tube to 10,0 ml with distilled water.

9.3 Hydrolysis and collection of glucose from glucosinolates by myrosinase and column chromatography

Using a class A pipette, accurately apply an aliquot of the supernatant (see 9.2.3) according to Table 2, avoiding any lipid layer which can have formed, to a prepared column (6.9), and add sufficient distilled water to make the total aliquot and water equal 5 ml, and allow the column to drain.

Table 2 — Preparation of the sample mixture

Parameter	Expected glucosinolate level (µmol/g sample)			
	< 30	31 to 80	81 to 120	121 to 200
Volume of aliquot (ml) (V)	5,0	3,0	2,0	1,0
Volume of additional water (ml)	0	2,0	3,0	4,0
Glucosinolates in aliquot (µmol)	< 3,0	1,9 to 4,8	3,2 to 4,8	2,4 to 4,0

Rinse the column with 3 ml tris HCl buffer (5.12) rinsing the sides of the column and let drain. Carefully add 0,50 ml of myrosinase solution (5.9) to the column and allow the myrosinase solution to enter the column packing, leaving a slight meniscus over the packing. Close the column stopcock and cap the columns.

The 0,50 ml volume is approximately the dead volume of the columns described in 6.9. If other columns are used, this volume should be adjusted appropriately.

Incubate columns for 1 h at 37 °C in the water bath (6.6). Rinse the column four times with 1 ml water and catch the eluant in a clean 12 ml graduated concentrating tube (6.10).

After this point, do not wait more than overnight before completing the analysis.

9.4 Glucose determination

9.4.1 General

The glucose determination can be carried out by glucose oxidase/peroxidase or glucose hexokinase/glucose-6-phosphate dehydrogenase systems according to the availability of equipment or reagents.

9.4.2 Glucose oxidase/peroxidase glucose assay (5.4)

9.4.2.1 General

A deproteinizing phenol-tungstate reagent is added to the solution eluted from the columns to remove protein and other interfering substances, permitting enzyme activity in this assay. The glucose colour reagent contains glucose oxidase, peroxidase and 4-aminoantipyrine. Glucose oxidase specifically oxidizes glucose to gluconic acid and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide oxidizes 4-aminoantipyrine, an oxygen acceptor that turns a pink colour when oxidized. This coloured product absorbs light in the visible range (λ_{max} = 505 nm) and the colour is proportional to the amount of glucose present in the sample being tested. The following uses the reagents as given above. If a commercial kit is used, the calibration and testing should be carried out in accordance with the kit instructions.

9.4.2.2 Glucose calibration curve

This calibration curve will be used to compare the absorbance of samples with the absorbance of standard glucose solutions to determine the unknown glucose concentrations of the samples. The calibration should be repeated every time a new glucose colour reagent is prepared.

Place 1,0 ml aliquots of each standard dilution (5.13.2) into (13 × 100) mm screw cap test tubes (6.5).

Add 3,0 ml glucose colour reagent (5.7) to each test tube and mix. Incubate test tubes at 37 °C for 15 min in a water bath (6.6).

Measure absorbance of contents of test tube in a 1 cm cell on the spectrometer (6.1) at 505 nm. Correct the absorbance of the contents of each test tube for reagent absorbance, as shown by Formula (1):

$$A_{corr} = A_{measured} - A_{blank} \quad (1)$$

where

$A_{measured}$ is the absorbance of the reference tube contents;

A_{blank} is the absorbance of 0 ml of glucose solution.

Plot A_{corr} against the glucose concentration (µmol/ml) (absorbance as a function of glucose concentration; a linear relationship is expected). Calculate the slope a and y-intercept b of the best fit line relating glucose concentration (µmol/ml) with absorbance ($y = ax + b$).

9.4.2.3 Glucose measurement in samples

Add 2,0 ml phenol tungstate reagent (5.2) to the 12 ml graduated concentrating tube (9.3) and accurately dilute to 10,0 ml with water.

Prepare a reagent absorbance correction blank by adding 2,0 ml phenol tungstate reagent (5.2) to a clean 12 ml graduated concentrating tube and diluting to 10,0 ml with water.

Mix contents of the 12 ml graduated concentrating tubes well and centrifuge at 5 000*g* for 10 min.

Transfer 1,0 ml aliquot of the contents of each 12 ml graduated concentrating tube into clean (13 × 100) mm test tubes (6.5).

Add 3,0 ml glucose colour reagent (5.3) to each test tube, cap, mix and incubate test tubes for 15 min at 37 °C.

Measure the absorbance of the contents of test tube in a 1 cm cell on the spectrometer (6.1) at 505 nm.

Calculate the corrected absorbance A_{corr} for the sample by subtracting the absorbance of the blank tube from the absorbance of the sample tube, as shown by Formula (1).

9.4.3 Glucose hexokinase/glucose-6-phosphate dehydrogenase assay (5.8)

9.4.3.1 General

Kits for this assay are available from commercial suppliers. The methodology supplied with the kit should be followed. The following instructions make use of the reagents described in 5.8 a) and b).

9.4.3.2 Glucose calibration curve

This calibration curve is used to compare the absorbance of samples with the absorbance of standard glucose solutions to determine the unknown glucose concentrations of the samples. The calibration should be repeated every time a new glucose colour reagent is prepared.

Place 1,0 ml aliquots of each standard dilution (5.13.2) into cuvettes (6.11).

Add 1,0 ml of Solution 1 [5.8 a)] to the cuvettes and mix.

Wait 3 min and read the absorbance of Solution A1 at 365 nm.

Add 0,02 ml of Solution 2 (suspension) to the cuvettes and mix.

Wait 15 min and read the absorbance of Solution A2 at 365 nm.

Wait 5 min more and read the absorbance of Solution A2 at 365 nm. If the absorbance is stable, record A2, otherwise repeat the readings at 5 min intervals until the absorbance is stable.

Let $A_{measured}$ = A2 –A1 for the glucose containing aliquots and A_{blank} = A2 – A1 for the 0 glucose solution.

Correct the absorbance of the contents of each test tube for reagent absorbance, as shown by Formula (1).

Plot A_{corr} against the glucose concentration (µmol/ml) (absorbance as a function of glucose concentration; a linear relationship is expected). Calculate the slope a and y-intercept b of the best fit line relating glucose concentration (µmol/ml) with absorbance ($y = ax + b$).

Calibration samples 6 and 7 (5.13.2) can be too concentrated for this assay. In this case, do not use these results.

9.4.3.3 Glucose measurement in samples

Add 2,0 ml phenol tungstate reagent (5.2) to the 12 ml graduated concentrating tube (9.3) and accurately dilute to 10,0 ml with water.

Prepare a reagent absorbance correction blank by adding 2,0 ml phenol tungstate reagent to a clean 12 ml graduated concentrating tube and diluting to 10,0 ml with water.

Mix the contents of 12 ml graduated concentrating tubes well and centrifuge at 5 000g for 10 min.

Transfer 1,0 ml aliquot of the contents of each 12 ml graduated concentrating tube into a cuvette (6.11). Add 1,0 ml of Solution 1 [5.8 a)] to the cuvettes and mix.

Wait 3 min and read the absorbance of Solution A1 at 365 nm.

Add 0,02 ml of Solution 2 (suspension) to the cuvettes and mix.

Wait 15 min and read the absorbance of Solution A2 at 365 nm.

Wait 5 more min and read the absorbance of Solution A2 at 365 nm. If the absorbance is stable, record A2, otherwise repeat the readings at 5 min intervals until the absorbance is stable.

Let $A_{measured}$ = A2 – A1 for the glucose containing aliquots and A_{blank} = A2 – A1 for the 0 glucose solution.

Correct the absorbance of the contents of each test tube for reagent absorbance, as shown by Formula (1).

10 Calculation of glucosinolate content

The absorbance of a sample is compared to the absorbance of standard glucose solutions to determine the amount of glucose present, which is equal to the amount of total glucosinolates.

Calculate the amount of glucose C_G, equal to the total amount of glucosinolates in the sample, as shown by Formula (2):

$$C_G = \frac{A_{corr} \times a + b}{m \times V} \quad (2)$$

where

C_G is the glucosinolate content of the sample, in micromoles per gram;

A_{corr} is the corrected absorbance (see 9.4.2.3 or 9.4.3.3);

a and b are the slope and intercept from the calibration curve (see 9.4.2.2 or 9.4.3.2), respectively;

m is the mass of the sample (9.1), in milligrams;

V is the volume of the aliquot (9.3), in millilitres.

11 Expression of results

Express the results as micromoles of total glucosinolates per gram to one decimal place.

12 Precision

12.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex C. The values derived for this interlaboratory test are not necessarily applicable to concentration ranges and matrices other than those given.

12.2 Repeatability

The absolute difference between the two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the repeatability limit, see Formula (3):

$$r = 0{,}23\,\bar{x} - 0{,}41 \tag{3}$$

12.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than values found for representative commodities shown in Annex C, see Formula (4):

$$R = 0{,}39\,\bar{x} - 1{,}56 \tag{4}$$

12.4 Critical difference

12.4.1 General

When the difference between two averaged values obtained from two test results under repeatability conditions is to be assessed, the repeatability limit cannot be used. Critical difference shall be used instead.

12.4.2 Comparison of two groups of measurements in one laboratory

The critical difference (D_r) between two averaged values ($\bar{x}$) obtained from two test results under repeatability conditions is equal to Formula (5):

$$D_r = 0{,}16\bar{x} - 0{,}29 = c3 \tag{5}$$

NOTE c3 is always lower than or equal to r.

12.4.3 Comparison of two groups of measurements in two laboratories

The critical difference (D_R) between two averaged values obtained in two different laboratories from two test results under repeatability conditions is equal to Formula (6):

$$D_R = 2{,}77\sqrt{s_R^2 - 0{,}5s_r^2} = c4 \quad (6)$$

where

s_r is the standard deviation of repeatability;

s_R is the standard deviation of reproducibility.

NOTE c4 is always lower than or equal to R.

13 Test report

The test report shall specify:

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

— the sampling method used, if known;

— the test method used, with reference to this document, i.e. ISO/TS 12788;

— all operating details not specified in this document, or regarded as optional, together with details of any incident which could have influenced the test result(s);

— the test result(s) obtained;

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

Annex A
(informative)

Extraction and purification of myrosinase from *Sinapis alba* L., also called white or yellow mustard

A.1 General

This annex describes a procedure to extract and purify myrosinase from defatted *Sinapis alba* (yellow mustard or white mustard) seeds.

A.2 Apparatus

A.2.1 Mill, capable of reducing 250 g of white mustard seed to a fine powder without excessive heat. A water cooled hammer mill, a blade mill or several coffee grinders may be suitable.

A.2.2 Refrigerated centrifuge, capable of 16,500*g* with 250 ml load in each compartment.

A.2.3 Centrifuge tubes, 250 ml.

A.2.4 Büchner funnel, top diameter 140 mm, fitted to a 2 l vacuum flask.

A.2.5 Filter paper. Whatman #4 or #52, 12,5 cm or Schleicher & Schuell Numbers 604 or 1574 or NucleoBond MN 617 or 1672 may be suitable[2)].

A.2.6 Plastic jug, 2 l.

A.2.7 Glass bottles, 1 l, for extracting oil from ground seed with petroleum ether.

A.2.8 Beaker, 3 l.

A.2.9 Measuring cylinders, 2 l.

A.2.10 Sintered glass funnel, with a receiving flask, 1 l.

A.2.11 Large bucket, 20 l, for dialysing enzyme solution.

A.2.12 Freeze dryer.

A.2.13 Vacuum pump or a vacuum aspirator, connected to the Büchner Funnel (A.2.4).

A.2.14 Dialysis tubing, made of cellulose membrane, molecular weight cut-off between 12 000 and 16 000.

2) Whatman, Schleicher & Schuell and NucleoBond are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

A.3 Materials

A.3.1 White or yellow mustard seeds (*Sinapis alba*, L.).

Seeds should be of good quality, reasonably fresh and well matured. The moisture content should be less than 9 %.

A.3.2 Petroleum ether.

CAUTION — Petroleum ether is flammable. The vapour and liquid are harmful by inhalation, ingestion and skin contact. Protective equipment (laboratory coats, gloves and safety glasses) shall be used. An operating fume cupboard shall be used when handling the solvent.

A.3.3 Cold distilled water, 2 l, at 4 °C.

A.3.4 Ethanol, 96 %.

CAUTION — Ethanol is flammable. The vapour and liquid are harmful by inhalation, ingestion and skin contact. Protective equipment (laboratory coats, gloves and safety glasses) shall be used. An operating fume cupboard shall be used when handling the solvent.

A.3.5 Ethanol, 90 %.

Dilute 940 ml of 96 % ethanol to 1 000 ml with distilled water, and store in the freezer at –20 °C.

Ethyl alcohol anhydrous (CAS[3)] 64-17-5) can also be used. Prepare a 1 l solution of 90 % aqueous ethanol by mixing 900 ml of ethyl alcohol anhydrous with 100 ml of deionized/distilled water. The solution shall be cold before use. Store it in the freezer at –20 °C.

A.3.6 Ethanol, 70 %.

Dilute 730 ml of 96 % ethanol to 1 000 ml with distilled water, and store in the freezer at –20 °C.

Ethyl alcohol anhydrous (CAS 64-17-5) can also be used Prepare a 1 l solution of 70 % aqueous ethanol by mixing 700 ml of ethyl alcohol anhydrous (CAS 64-17-5) with 300 ml of deionized/distilled water. The solution shall be cold before use. Store it in the freezer at –20 °C.

A.3.7 Sodium chloride.

Dissolve and dilute 90 g of NaCl to 18 l with distilled water to obtain a NaCl solution at 0,5 % NaCl.

Ensure all the NaCl crystals are dissolved.

A.3.8 Sinigrin monohydrate (formula weight 415,49 g/mol).

Prepare a solution of sinigrin monohydrate at 3 µmol/ml by dissolving 125 mg of sinigrin monohydrate in water and diluting to 100 ml in a volumetric flask.

A.4 Procedure

A.4.1 Sample preparation

Weigh two 250 g batches of white mustard seeds (A.3.1). Grind the two batches in the mill (A.2.1), taking care not to produce excessive heat. Store each batch in a separate 1 l bottle (A.2.7).

3) CAS Registry Number® is a trademark of CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

If the meal is not for immediate extraction, cap and store at –20 °C.

Add 750 ml of petroleum ether (A.3.2) into one of the bottles. Wait for at least 30 min.

Agitate the bottle regularly during the oil extraction period to maximize the effectiveness of the process.

Filter through a fast filter paper (A.2.5) using the Büchner funnel (A.2.4) under vacuum (A.2.13) and allow to dry for 1 h.

Discard the filtrate.

NOTE 1 Petroleum ether can be reclaimed from the filtrate using a rotary evaporator.

Wash the residue twice with 100 ml of petroleum ether.

NOTE 2 This process removes the seed coat, the seed oil and other unwanted materials.

Continue the vacuum for a further 30 min to dry the meal. Repeat with the second bottle.

Add the two extracted meals into a 2 l plastic jug (A.2.6).

A.4.2 Myrosinase extraction

Add 1 200 ml of cold distilled water (A.3.3) to the meal (see A.4.1) and mix thoroughly. Keep the mixture at 4 °C for 1 h with occasional stirring. Pour the mixture into 250 ml centrifuge tubes and centrifuge with refrigeration at 8 000*g* for 15 min.

Do not overfill the centrifuge tubes. Excess sample in the tube can cause leakages to occur.

Discard the pellet. Transfer the supernatant into a beaker (A.2.8). Add, with vigorous stirring, an equal volume of 90 % ice-cold ethanol (A.3.5).

NOTE 1 A white stringy precipitate is produced.

Transfer the precipitate to centrifuge tubes (A.2.3). Centrifuge the precipitate, with refrigeration, at 16,300*g* for 18 min. Discard the supernatant. Re-suspend the precipitate with a minimum amount (approximately 15 ml) of 70 % ice-cold ethanol (A.3.6).

Use a stirring rod or large spatula to break the pellet into small pieces when re-suspending the precipitate in 70 % ethanol.

Centrifuge with refrigeration at 16,300*g* for 18 min. Discard the supernatant. Briefly wash the precipitate with distilled water (approximately 2 ml) and discard the supernatant. Suspend the precipitate in 400 ml of cold distilled water (A.3.3) and centrifuge at 8 000*g* for 18 min.

NOTE 2 The precipitate can be difficult to break apart. Use a stirring rod or large spatula to break the precipitate into small pieces when re-suspending the precipitate in cold distilled water.

Discard the pellet.

A.4.3 Myrosinase purification

Pour the myrosinase solution (see A.4.2) into dialysis tubes which have been soaked in boiling distilled water just prior to use. Dialyse the myrosinase solution against 0,5 % NaCl (A.3.7) for 16 h. Secure the dialysis tubing at the top. Stir the salt solution occasionally.

Ensure that the myrosinase solution is completely immersed to ensure an effective dialysis process.

Remove the salt solution and replace with distilled water. Dialyse the solution against running water for at least 6 h. Combine the myrosinase solution and filter through a sintered glass Büchner filter funnel (A.2.10) under vacuum (A.2.13). This final filtration removes impurities in the liquid. Pour the filtered

myrosinase solution into the freeze-dryer containers, then cap and freeze overnight at –20 °C. Freeze-dry the myrosinase according to the procedure prescribed for the freeze dryer (A.2.12).

NOTE Freeze drying can take about three days to complete.

Store the lyophilised enzyme in a jar at –20 °C until required.

A.4.4 Activity of myrosinase

The activity of the myrosinase may be determined by assaying a 1 ml aliquot of sinigrin solution (A.3.8) by the method given in this document. The assay should yield a result of 3 µmol of sinigrin. If further activity determination is needed, refer to Reference [5].

Annex B
(informative)

Determination of recovery of glucosinolates

B.1 General

This annex describes a procedure for estimating the recovery of glucosinolates from different seed matrices by the method.

B.2 Principle

Glucosinolates are estimated in both a representative sample of the matrix and from a similar size sample to which has been added more glucosinolates in the form of sinigrin (standard addition). The recovery can be calculated from comparing the amounts of glucosinolates found in both the representative sample and the sample with the known amount of added glucosinolates.

B.3 Reagents

Use the reagents in Clause 5 and the following.

B.3.1 Sinigrin monohydrate solution, 5 µmol/ml.

Dissolve 208 mg of sinigrin monohydrate in water and dilute to volume in a volumetric flask according to Table B.1.

Table B.1 — Sinigrin solution preparation description

Parameter	Expected glucosinolate level (µmol/g)			
	< 30	31 to 80	81 to 120	120 to 200
Volume of flask (ml)	100	50	25	10
Glucosinolates in 0,200 ml ($m_{singrin}$)	1,0	2,0	4,0	10
Added glucosinolate content µmol/g (based on 0,200 ml)	5	10	20	50

B.4 Apparatus

Use the apparatus in Clause 6.

B.5 Procedure

B.5.1 General

Follow Clauses 7 and 8.

B.5.2 Test portion

Weigh approximately 200 mg, measured to the nearest 0,1 mg, of ground seed (see 8.3) into a (13 × 100) mm screw cap test tube (6.5). Record this mass as *m*.

B.5.3 Test portion for additional sinigrin addition

Weigh approximately 200 mg, measured to the nearest 0,1 mg, of ground seed (see 8.3) into a (13 × 100) mm screw cap test tube (6.5). Record this mass as m_{sample}.

B.5.4 Extraction of glucosinolates

Place test tubes (see B.5.2 and B.5.3), uncovered, in a 95 °C heat block (6.7) for 15 min. It is useful to measure the temperature with a thermometer inserted into a control sample of 200 mg ground seed similar to the samples being tested placed in a tube (6.5). Once the thermometer has reached a stable temperature of 95 °C, the control and thermometer can be removed.

Add approximately 2 ml boiling water using a heated Pasteur pipette to the test tubes. Heating can be achieved by flushing the pipette several times in the boiling water prior to use.

Add 0,200 ml of sinigrin monohydrate solution (B.3.1) to the tube (see B.5.3) from the solution prepared appropriate to the sample being tested.

B.5.5 Purification, hydrolysis and analysis

Complete the analysis using the method given in 9.2 to 9.4. Report the corrected absorbance for the two samples as "A" for the sample without added sinigrin and "A_S" for the sample with added sinigrin.

B.6 Calculation of recovery

The absorbance of a sample is compared to the absorbance of standard glucose solutions to determine the amount of glucose present, which is equal to the amount of total glucosinolates.

Calculate the amount of glucose equal to the total amount of glucosinolates from the sample without added sinigrin as shown by Formula (B.1):

$$C_G = \frac{(A \times a + b) \times 10}{m + V} \quad \text{(B.1)}$$

where

C_G is the glucosinolate content of the sample, in micromoles per gram;

A is the corrected absorbance for the sample without added sinigrin;

a and b are the slope and intercept from the calibration curve;

m is the mass of the sample;

V is the volume of the aliquot.

Calculate the amount of glucosinolates added to the sample C_S as shown by Formula (B.2):

$$C_S = \frac{m_{singrin}}{m_{sample}} \quad \text{(B.2)}$$

where

C_S is the glucosinolate content of the sample with added sinigrin, in micromoles per gram;

$m_{sinigrin}$ is the amount of sinigrin added to the sample from Table B.1;

m_{sample} is the mass of the sample (in grams) to which the sinigrin was added.