TECHNICAL SPECIFICATION

**ISO/TS 12788**

First edition
2022-05

# 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*



Reference number
ISO/TS 12788:2022(E)



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Published in Switzerland

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# Foreword

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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.