INTERNATIONAL STANDARD

ISO 9167-1

First edition
1992-07-01

Rapeseed — Determination of glucosinolates content —

Part 1: Method using high-performance liquid chromatography

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Graines de colza — Dosage des glucosinolates —
Partie 1: Méthode par chromatographie liquide à haute performance

INTERNATIONAL ISO

Reference number
ISO 9167-1:1992(E)

Foreword

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International Standard ISO 9167-1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 2, *Oleaginous seeds and fruits*.

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ISO 9167 consists of the following parts, under the general title *Rapeseed — Determination of glucosinolates content*:

— *Part 1: Method using high-performance liquid chromatography*

— *Part 2: Method using X-ray fluorescence spectrometry*

Annex A of this part of ISO 9167 is for information only.

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International Organization for Standardization
Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

Rapeseed — Determination of glucosinolates content —

Part 1:
Method using high-performance liquid chromatography

1 Scope

This part of ISO 9167 specifies a method for the determination of the content of the different glucosinolates in rapeseeds (colza) using high-performance liquid chromatography.

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NOTES

1 This method does not determine glucosinolates which are substituted on the glucose molecule, but these compounds are of little importance in commercial rapeseed.

2 A rapid method for the determination of glucosinolates content using X-ray fluorescence spectrometry is the subject of ISO 9167-2.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 9167. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 9167 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 664:1990, *Oilseeds — Reduction of laboratory sample to test sample.*

ISO 665:1977, *Oilseeds — Determination of moisture and volatile matter content.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

3 Principle

Extraction of glucosinolates by methanol, then purification and enzymatic desulfatation on ion-exchange resins. Determination using reversed-phase high-performance liquid chromatography (HPLC) with elution gradient and ultraviolet detection.

4 Reagents

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

4.1 Methanol, HPLC grade, 70 % (V/V) solution.

4.2 Sodium acetate, 0,02 mol/l at pH 4,0.

4.3 Sodium acetate, 0,2 mol/l solution.

4.4 Imidazole formate, 6 mol/l solution.

Dissolve 204 g of imidazole in 113 ml of formic acid in a 500 ml one-mark volumetric flask. Make up to the mark with water.

4.5 Internal standard, use either **sinigrin monohydrate** (potassium allylglucosinolate monohydrate, $M_r = 415{,}49$) (see 4.5.1) or, for rapeseed (cultivated or self-propagated) in which sinigrin is present naturally, **glucotropaeolin** (benzylglucosinolate, potassium salt, $M_r = 447{,}52$) (see 4.5.2).

For rapeseed with a low glucosinolate content (< 20 µm/g), reduce the internal standard concentration (1 mmol/l to 3 mmol/l) in 4.5.1 and 4.5.2.1 .

4.5.1 Sinigrin monohydrate

4.5.1.1 Sinigrin monohydrate, 5 mol/l solution.

Dissolve 207,7 mg of potassium allylglucosinolate monohydrate in water in a 100 ml one-mark volumetric flask. Make up to the mark with water.

The solution thus prepared may be stored in a refrigerator at approximately 4 °C for up to a week or in a freezer at − 18 °C for a longer period.

4.5.1.2 Sinigrin monohydrate, 20 mmol/l solution.

Dissolve 831,0 mg of potassium allylglucosinolate monohydrate in water in a 100 ml one-mark volumetric flask. Make up to the mark with water.

The solution thus prepared may be stored in a refrigerator at approximately 4 °C for up to a week or in a freezer at − 18 °C for a longer period.

4.5.1.3 Purity check

Use one or more of the following three tests:

— HPLC analysis using the method specified in this part of ISO 9167;

— analysis of the intact sinigrin by HPLC (ion-pair technique);

— analysis of the desulfated and silylated sinigrin by gas chromatography.

For each test, the chromatogram shall show only one major peak representing at least 98 % of the total peak area.

Confirm the purity by determining the quantity of glucose released after hydrolysis with myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). Measure the glucose by enzymatic means. The use of a commercially available test kit facilitates the determination. Take into account any free glucose present; this is determined in the same way but without addition of myrosinase. The molar concentration of glucose measured should be at least 98 % of the molar concentration of the sinigrin solution tested.

4.5.2 Glucotropaeolin

NOTE 3 Glucotropaeolin is sometimes difficult to separate from other natural minor glucosinolates.

4.5.2.1 Glucotropaeolin, 5 mmol/l solution.

Dissolve 233,8 mg of glucotropaeolin in water in a 100 ml volumetric flask. Make up to the mark with water.

4.5.2.2 Glucotropaeolin, 20 mmol/l solution.

Dissolve 895,0 mg of glucotropaeolin in water in a 100 ml volumetric flask. Make up to the mark with water.

4.5.2.3 Purity check

Check the purity in accordance with the procedure described in 4.5.1.3.

4.5.2.4 Response factor

Verify that the response factors of glucotropaeolin, in comparison with sinigrin, correspond to those indicated in 9.2.

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4.6 Mobile phases

4.6.1 Eluant A: water, purified by passing it through an activated charcoal cartridge (e.g. Norganic Millipore[1] system) or water of equivalent purity.

4.6.2 Eluant B: acetonitrile, HPLC grade, 20 % (V/V) solution in purified water. The concentration may be modified in relation to the column used.

4.7 Ion-exchange resin, use either 4.7.1 or 4.7.2.

4.7.1 DEAE Sepharose CL-6B[2] suspension, available commercially ready for use, or an equivalent product.

4.7.2 DEAE Sephadex A25[2] suspension, prepared as follows.

Mix 10 g of DEAE Sephadex A25 resin (or an equivalent resin) in excess 2 mol/l acetic acid solution. Leave to settle. Add 2 mol/l acetic acid until the volume of the liquid is equal to twice the volume of the sediment.

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1) Norganic Millipore system is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of this product.

2) DEA Sepharose and Sephadex are examples of suitable products available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of these products.

4.8 Sulfatase, *Helix pomatia* type H1 (EC 3.1.6.1), having an activity of greater than 0,5 units of activity per millilitre of purified sulfatase solution.

Purify, test and dilute the sulfatase in accordance with the method described in 4.8.1 to 4.8.4.

4.8.1 Preparation of ion-exchange columns

Cut five Pasteur pipettes (5.9) 7 cm above the neck and place a glass wool plug (5.8) in the neck. Place the pipettes vertically on a stand and add to each a sufficient quantity of ion-exchange resin (4.7) such that, once the water has drained off, a volume of 500 µl of resin is obtained.

Pour 1 ml of the imidazole formate solution (4.4) into each pipette and rinse twice with 1 ml portions of water.

4.8.2 Purification

Weigh, to the nearest 0,1 mg, 25 mg of *Helix pomatia* type H1 (4.8), dissolve it in 2,5 ml of water and transfer 500 µl of this solution to each of the columns prepared in 4.8.1. Wash each column with 1,5 ml of water and discard the effluent. Then add 1,5 ml of the sodium acetate solution (4.3) and collect the eluates from the five columns in a test tube.

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Concentrate the eluates by filtration using a Millipore PTGC 11K25[3)] immersion filter until approximately 100 µl of liquid remains (sulfatase with a molar mass in excess of 5 000 is not removed). Add 2,5 ml of water and concentrate once more by filtration until approximately 100 µl of liquid remains. Dilute to 2,5 ml with water and store the purified sulfatase in a freezer at − 18 °C in small amounts in order to allow defrosting of the amount necessary for immediate use.

4.8.3 Test of the sulfatase activity

4.8.3.1 Preparation of a 0,15 mmol/l sinigrin solution, buffered to pH 5,8.

Prepare three solutions in succession as follows:

a) transfer 1 ml of acetic acid to a 500 ml one-mark volumetric flask and make up to the mark with water;

b) transfer 1 ml of ethylene diamine to a 500 ml one-mark volumetric flask and make up to the mark with water;

c) mix 73 ml of solution a) with 40 ml of solution b) and adjust the mixture to pH 5,8 using solution a) or solution b) as appropriate.

Pour 3 ml of the 5 mmol/l sinigrin solution (4.5.1.1) into a 100 ml one-mark volumetric flask and make up to the mark with solution c).

4.8.3.2 Test of activity

Using a pipette, transfer 2 ml of the buffered sinigrin solution (4.8.3.1) into the reference and measuring cells of the spectrometer (5.3) adjusted to a wavelength of 229 nm with a cell temperature of 30 °C. At time $t = 0$, add 50 µl of purified sulfatase (4.8.2) to the measuring cell and immediately switch on the recorder. Stop the recorder when the absorbance no longer varies (A_e), plot the tangent to the point $t = 0$ and measure its gradient $\Delta A / \Delta t$.

The activity of the sulfatase (i.e. the production of 1 micromole of desulfated sinigrin per minute at 30 °C and pH 5,8), expressed in units of activity per millilitre of sulfatase solution, is equal to

$$\frac{\Delta A}{\Delta t} \times \frac{V}{\Delta \varepsilon} \times \frac{1000}{50} \times 10^6$$

where

$\Delta A / \Delta t$ is the gradient of the tangent to the point $t = 0$, in absorbance units per minute;

V is the volume, in litres, of the reacting medium (i.e. $2,05 \times 10^{-3}$ l);

$\Delta \varepsilon$ (approximately 1 500 $l \cdot mol^{-1} \cdot cm^{-1}$) is the difference between the molar extinction coefficients of sinigrin and of desulfosinigrin at 228 nm, i.e.

$$\Delta \varepsilon = \frac{A_e}{lc}$$

where

A_e is the difference between the absorbance at equilibrium of the desulfated sinigrin and the absorbance at time $t = 0$;

l is the path length of the cell, in centimetres (i.e. 1 cm);

c is the concentration of desulfated sinigrin at equilibrium, in moles per litre, i.e.

$$c = \frac{0,15 \times 10^{-3} \times 0,95 \times 2}{2,05}$$

$$= 1,39 \times 10 \text{ mol/l}$$

0,95 is the yield at equilibrium of the desulfatation of the sinigrin.

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3) Millipore PTGC 11K25 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of this product.

Alternatively, the activity of the sulfatase may be calculated using the following simplified formula, where the activity is given by the expression

$$\frac{\Delta A \times 5{,}7}{\Delta t A_e}$$

4.8.4 Dilution

Using a pipette, transfer 1 ml of purified sulfatase (4.8.2) to a 10 ml one-mark volumetric flask. Make up to the mark with water and mix.

Divide the solution into small quantities and store in a freezer at − 18 °C.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 High-performance liquid chromatograph, suitable for obtaining an elution gradient and control of the temperature of the column at 30 °C, connected to an ultraviolet detector permitting measurements to be made at a wavelength of 229 nm.

5.2 Chromatography column for HPLC, type C_{18} or C_8, of particle size less than or equal to 5 µm, for example[4)]

Lichrosorb RP18 column, ⩽ 5 µm (150 mm × 4,6 mm)

Spherisorb ODS2 column, ⩽ 5 µm (250 mm × 4 mm; 250 mm × 5 mm)

Novapak C18 column, 4 µm (150 mm × 4 mm)

Lichrospher RP8 column, ⩽ 5 µm (125 mm × 4 mm)

Nucleosil C18 column, ⩽ 5 µm (200 mm × 4 mm)

The performance of the column should be checked regularly, preferably using a reference sample of colza desulfoglucosinolate[5)]. In particular, the column shall not degrade 4-hydroxyglucobrassicin, an important but relatively unstable glucosinolate.

New columns shall be subjected to preliminary conditioning in accordance with the manufacturer's instructions so that reproducible results can be obtained.

5.3 Double-beam spectrometer, capable of operating in the ultraviolet region of the spectrum, and at a controlled temperature of 30 °C, equipped with quartz cells of path length 1 cm and a recording system.

5.4 Microgrinder, for example a coffee mill.

5.5 Centrifuge, suitable for use with the tubes (5.6), capable of obtaining a centrifugal acceleration of 5 000*g*.

5.6 Polypropylene tubes, of 6 ml capacity.

5.7 Water-bath or other **heating apparatus**, capable of maintaining a temperature of 75 °C ± 1 °C.

5.8 Glass wool

5.9 Pasteur pipettes, 150 mm long, and **a suitable stand,** or any other appropriate apparatus.

6 Sampling

Sampling should have been carried out in accordance with ISO 542.

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.

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7 Preparation of the test sample

Reduce the laboratory sample in accordance with ISO 664.

If the seeds have a moisture and volatile matter content in excess of 10 % (*m*/*m*), dry them using a current of air at approximately 45 °C.

The impurities level is generally 2 % (*m*/*m*). If sinigrin is found in the sample, carry out the test on pure seed and analyse the impurities separately.

Determine the moisture and volatile matter content of the test sample in accordance with ISO 665.

If the seeds have been treated, wash them with dichloromethane.

Grind the seeds in the microgrinder (5.4) for 20 s. Mix the meal and then grind for a further 5 s.

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4) The examples given are suitable products available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of these products.

5) Reference samples of colza desulfoglucosinolate may be obtained from the Community Reference Bureau.

8 Procedure

8.1 Test portion

Label two tubes (5.6) A and B and transfer 200 mg, weighed to the nearest 0,1 mg, of the prepared test sample (clause 7) to each tube.

8.2 Extraction of glucosinolates

8.2.1 Place the tubes in the water-bath or other heating apparatus (5.7) set at 75 °C and leave for 1 min. Add 2 ml of boiling methanol solution (4.1) and then immediately add

— to tube A, 200 µl of 5 mmol/l internal standard solution (4.5.1.1), and

— to tube B, 200 µl of 20 mmol/l internal standard solution (4.5.1.2).

8.2.2 Continue heating at 75 °C for a further 10 min, shaking the tubes at regular intervals. Mix the contents of each tube and then centrifuge at an acceleration of 5 000*g* for 3 min. Transfer the supernatant liquid from each tube to two other tubes (5.6) labelled A′ and B′.

8.2.3 Add to the two tubes containing the solid residue 2 ml of boiling methanol solution (4.1) and reheat for 10 min in the water-bath or other heating apparatus (5.7) set at 75 °C, shaking the tubes at regular intervals.

Centrifuge for 3 min and then add the supernatant liquid from the two tubes to the respective supernatant liquids retained in 8.2.2.

8.2.4 Adjust the volume of the combined extracts to approximately 5 ml with water and mix.

These extracts may be kept for 2 weeks if stored in the dark in a freezer at − 18 °C.

8.3 Preparation of ion-exchange columns

Cut the required number of Pasteur pipettes (5.9), i.e. one pipette per combined extract, so as to leave a volume of 1,2 ml above the neck and place a glass wool plug (5.8) in the neck of each pipette. Place the pipettes vertically on a stand.

Transfer 0,5 ml of a well-mixed suspension of ion-exchange resin (4.7) to each pipette and allow to settle.

Rinse the pipettes with 2 ml of the imidazole formate (4.4) and then twice with 1 ml portions of water.

8.4 Purification and desulfatation

8.4.1 Carry out the operations given in 8.4.2 to 8.4.5 for each combined extract.

8.4.2 Transfer 1 ml of the extract (8.2.4) to a prepared column (8.3) without disturbing the resin surface and allow to drain. Add two 1 ml portions of the sodium acetate buffer (4.2), allowing the buffer to drain after each addition.

8.4.3 Add to the column 75 µl of diluted purified sulfatase solution (4.8.4). Leave to act overnight at ambient temperature.

8.4.4 Place a tube (5.7) under the column to collect the eluate.

Elute the desulfoglucosinolate obtained with two 1 ml portions of water, allowing the water to drain after each addition.

8.4.5 Mix the eluate well. If not used immediately for chromatography, the eluate may be stored in the dark in a freezer at − 18 °C for up to 1 week.

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8.5 Blank test

If required (see 9.3), carry out a blank test using the same procedure on a test portion taken from the same test sample, but omitting the sinigrin internal standard solution in order to detect and quantify any sinigrin present in the test portion.

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8.6 Chromatography

8.6.1 Adjustment of the apparatus

Adjust the chromatograph to give:

a flow-rate of the mobile phase (4.6), depending on the nature of the column (see 8.6.2), of generally of the order of 1 ml/min,

a column (5.2) temperature of 30 °C, and

a detection wavelength of 229 nm.

8.6.2 Analysis

Operating in accordance with the instructions for the apparatus, inject into the chromatograph not more than 50 µl of the desulfoglucosinolate solution obtained in 8.4.4.

Use an elution gradient appropriate to the column employed.

NOTES

4 The following elution gradients are given as examples.

a) Lichrosorb RP18 column, ⩽ 5 µm (150 mm × 4,6 mm)

- pass 100 % of eluant A (4.6.1) for 1 min
- apply a linear elution gradient over 20 min until 0 % of eluant A and 100 % of eluant B (4.6.2) are obtained
- apply a linear elution gradient over 5 min until 100 % of eluant A and 0 % of eluant B are obtained
- pass 100 % of eluant A for 5 min to establish equilibrium

b) Lichrospher RP8 column, ⩽ 5 µm (125 mm × 4 mm)

- pass 100 % of eluant A for 2 min 30 s
- apply a linear elution gradient over 18 min until 0 % of eluant A and 100 % of eluant B are obtained
- pass 100 % of eluant B for 5 min
- apply a linear elution gradient over 2 min until 100 % of eluant A and 0 % of eluant B are obtained
- pass 100 % of eluate A for 5 min to establish equilibrium

5 The gradient profiles may be modified to give optimum separations according to the columns used.

8.6.3 Examination of chromatograms

Take into account only those peaks having an area greater than 1 % of the sum total of the peak areas.

The order of elution of the peaks with a type C_{18} column and a suitable elution gradient (see the examples given in 8.6.2) is generally as shown in figure 1.

9 Expression of results

9.1 Calculation of the content of each glucosinolate

The content of each glucosinolate, expressed in micromoles per gram of dry matter of the product, is equal to

$$\frac{A_g}{A_s} \times \frac{n}{m} \times K_g \times \frac{100}{100 - w}$$

where

A_g is the peak area, in integrator units, corresponding to desulfoglucosinolate;

A_s is the peak area, in integrator units, corresponding to desulfosinigrin;

K_g is the response factor of desulfoglucosinolate (9.2);

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

n is the quantity, in micromoles, of internal standard added to the tube in 8.2;

w is the moisture and volatile matter content, expressed as a percentage by mass, of the test sample.

If it is desired to express the result relative to a specified moisture and volatile matter content w_s [e.g. $w_s = 9$ % (m/m)], multiply the result obtained for dry matter (as above) by

$$\frac{100 - w_s}{100}$$

9.2 Response factors

The following response factors shall be adopted.

NOTE 6 These response factors have been determined experimentally and have been fixed by concensus between the various laboratories who took part in the test; they may need to be revised in due course.

1	Desulfoglucoiberin	1,07
2	Desulfoprogoitrin	1,09
3	Desulfoepi-progoitrin	1,09
4	Desulfosinigrin	1,00
5	Desulfoglucoraphanin	1,07
6	Desulfogluconapoleiferin	1,00
7	Desulfoglucoalyssin	1,07
8	Desulfogluconapin	1,11
9	Desulfo-4-hydroxyglucobrassicin	0,28
10	Desulfoglucobrassicanapin	1,15
11	Desulfoglucotropaeolin	0,95
12	Desulfoglucobrassicin	0,29
13	Desulfogluconasturtin	0,95
14	Desulfo-4-methoxyglucobrassicin	0,25
15	Desulfoglucobrassicin	0,20
16	Other desulfoglucosinolates	1,00

9.3 Calculation of the total glucosinolate content

The total glucosinolate content, expressed in micromoles per gram of dry matter of the product, is equal to the sum of the contents of each glucosinolate (the corresponding peak area of which is greater than 1 % of the sum total of the peak areas).

If the difference between the total glucosinolate content results using both concentrations satisfy the requirements for repeatability (see 10.2), there is no

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contamination of the internal standard. In this case take as the result the arithmetic mean of the two determinations.

10 Precision

10.1 Results of inter-laboratory test

An inter-laboratory test, carried out at the international level in 1988, in which 11 laboratories participated, each of which carried out two determinations on each sample, gave the statistical results (evaluated in accordance with ISO 5725) shown in table 1.

Table 1 — Statistical results of inter-laboratory test

Sample	Rapeseed A	Rapeseed B	Rapeseed C	Rapeseed D
Number of laboratories retained after eliminating outliers	11	11	11	11
Mean glucosinolate content (µmol/g dry matter)	20,6	14,1	4,9	25,6
Standard deviation of repeatability, s_r	1,7	0,6	0,3	0,8
Coefficient of variation of repeatability	8,5 %	4,4 %	6,7 %	3,3 %
Repeatability, $2{,}83 s_r$	4,9	1,7	0,9	2,4
Standard deviation of reproducibility, s_R	3,4	2,5	1,5	2,4
Coefficient of variation of reproducibility	17 %	18 %	31 %	9,4 %
Reproducibility, $2{,}83 s_R$	9,6	7,1	1,4	6,8

10.2 Repeatability

The absolute difference between 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, should not be greater than 2 µmol/g for glucosinolate contents less than 20 µmol/g, and 4 µmol/g for glucosinolate contents within the range 20 µmol/g to 35 µmol/g.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than 4 µmol/g for glucosinolate contents less than 20 µmol/g, and 8 µmol/g for glucosinolate contents within the range 20 µmol/g to 35 µmol/g.

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11 Test report

The test report shall specify the method used and the result obtained. It shall also mention all operating details not specified in this part of ISO 9167, or regarded as optional, together with details of any incidents which may have influenced the result.

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