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Rapeseed and rapeseed meals — Determination of glucosinolates content — Method using high-performance liquid chromatography

Graines et tourteaux de colza — Dosage des glucosinolates — Méthode par chromatographie liquide à haute performance

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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*.

This first edition cancels and replaces ISO 9167-1:1992, which has been technically revised. It also incorporates the amendment ISO 9167-1:1992/Amd.1:2013. The main changes are as follows:

— rapeseed meals have been added to the scope with the addition of a new collaborative trial;

— in 9.2, methanol 70 % has been replaced by ethanol 50 % for lower toxicity[6];

— in 9.2, only one extraction is carried out instead of two;

— in 10.2 and E.5.1, the term “relative proportionality factor” has been used instead of “response factor”;

— the isocratic mode has been added in Annex E.

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

The glucosinolates in rapeseed can be analysed by chromatographic, enzymatic or spectroscopic methods. This document describes a chromatographic method with two conditions (gradient and isocratic) of elution for qualitative and quantitative analysis of individual glucosinolates in rapeseed and rapeseed meals. The method with gradient elution is considered as the reference method whereas the method with isocratic elution is considered as a simplified method and is presented in Annex E as information.

This document specifies a method using high-performance liquid chromatography (HPLC) with gradient elution as reference method. For the isocratic mode, the choice of the internal standard, the chromatographic conditions and the separation results are different from the reference method. These aspects are discussed in Annex E.

Rapeseed and rapeseed meals — Determination of glucosinolates content — Method using high-performance liquid chromatography

1 Scope

This document specifies a method for the determination of the individual glucosinolates content in rapeseeds and rapeseed meals using high-performance liquid chromatography with gradient elution.

This method was tested on rapeseeds and rapeseed meals (*Brassica rapa*, *Brassica napus* and *Brassica juncea*) but is applicable to other plant materials, on the condition that the occurring glucosinolates previously identified are described in this document. On the contrary, the quantitative analysis of the concerned glucosinolate(s) is not carried out.

NOTE This method does not determine glucosinolates that are substituted on the glucose molecule, but these compounds are of little importance in commercial rapeseed and rapeseed meal.

Annex A presents the results of the interlaboratory trials for the gradient elution HPLC method. Annex B presents how to check the titre of the prepared internal standard solution. Annex C presents how to prepare and test the purified sulfatase solution and how to check the desulphation step on the ion exchange column. Annex D presents the HPLC and column performance criteria qualification.

The analysis of glucosinolates content in rapeseed can also be done using an isocratic elution mode. This requires some modifications of the method (internal, standard, HPLC column and HPLC buffers), as described in Annex E.

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 771, *Oilseed residues — Determination of moisture and volatile matter content*

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

ISO 5502, *Oilseed residues — Preparation of test samples*

3 Terms and definitions

No terms and definitions are listed in this document.

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

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

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

4 Principle

Extraction of glucosinolates by a water-ethanol mixture, then purification and enzymatic desulfatation on ion-exchange columns. Determination using reverse phase liquid chromatography with gradient elution (reference method) or isocratic elution (rapid method) and detection by ultraviolet absorptiometry.

5 Reagents

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

5.1 Ethanol, volume fraction = 50 %.

5.2 Sodium acetate, c = 0,02 mol/l at pH 4,0, prepared by mixing sodium acetate, c = 0,02 mol/l and acetic acid, c = 0,02 mol/l as to obtain a solution having a pH = 4,0.

5.3 Sulfatase, *Helix pomatia*, type H1 purified and diluted as described in Annex C.

5.4 Imidazole formiate, c = 6 mol/l.

Dissolve 204 g of imidazole in 113 ml of formic acid in a 500 ml beaker. Transfer the mixture in a 500 ml cylinder and make up to 500 ml with water.

5.5 Internal standard.

Use either sinigrin (potassium allyglucosinolate monohydrate, M = 415,5 g/mol) (5.6) or glucotropaeolin (potassium benzylglucosinolate, M = 447,5 g/mol or tetramethylammonium benzylglucosinolate, M = 482,6 g/mol) (5.7). The glucotropaeolin may be used in a hydrated form, then the molar mass and the purity shall be known and taken into consideration for the preparation of the solution.

The choice of the internal standard will be conditioned by its perfect chromatographic separation from the other glucosinolates of the sample. The natural absence in the sample of the internal standard or of glucosinolates unseparated from the latter may be checked with a blank test (see 9.3).

With gradient elution on octyl or octadecyl stationary phases, sinigrin or glucotropaeolin can be used. However, glucotropaeolin is sometimes difficult to separate from other natural minor glucosinolates.

With isocratic elution on cyano propyl stationary phase (see Annex E), sinigrin cannot be used for rapeseed analysis because of the non-separation from the other glucosinolates. Glucotropaeolin shall be used instead.

In the most frequent cases (i.e. when rapeseeds have an assumed glucosinolates content between 10 µmol/g and 50 µmol/g inclusive) the internal standard is used in solution form at 20 mmol/l. For rapeseeds where the assumed glucosinolates content is less than 10 µmol/g or greater than 50 µmol/g, the concentrations of the internal standard solutions used per sample are given in Table 1.

Check the titre of the prepared internal standard solution as described in Annex B.

Table 1 — Concentration of the internal standard solution to use according to the assumed glucosinolates content of the sample

Glucosinolates content of the sample µmol/g	Concentrations of the internal standard solutions mmol/l
< 10	5
> 10 and < 50	20
> 50	40

5.6 Sinigrin solution.

The sinigrin solution with the required concentration (see Table 1) is prepared according to Table 2. Weigh the sinigrin to the nearest 0,5 mg and dissolve it in water in a 100 ml volumetric flask. Make up to the mark with water. The solution thus prepared may be stored in a refrigerator at approximately 4 °C up to a week or in a freezer at –18 °C for a longer period.

Table 2 — Weight of sinigrin in 100 ml water for preparation of 5 mmol/l, 20 mmol/l and 40 mmol/l solutions

Sinigrin form	Molecular weight	Sinigrin weight		
	g/mol	g		
		5 mmol/l	20 mmol/l	40 mmol/l
Potassium monohydrate	415,5	0,207 7	0,831 0	1,662 0

5.7 Glucotropaeolin solution.

The glucotropaeolin solution with the required concentration (see Table 1) is prepared according to Table 3. Weigh the glucotropaeolin to the nearest 0,5 mg and dissolve it in water in a 100 ml volumetric flask. Make up to the mark with water. The solution thus prepared may be stored in a refrigerator at approximately 4 °C up to a week or in a freezer at –18 °C for a longer period.

Table 3 — Weight of glucotropaeolin in 100 ml water for preparation of 5 mmol/l, 20 mmol/l and 40 mmol/l solutions

Glucotropaeolin form	Molecular weight g/mol	Glucotropaeolin weight g		
		5 mmol/l	20 mmol/l	40 mmol/l
Potassium	447,5	0,223 7	0,895 0	1,790 1
Tetramethylammonium	482,6	0,241 3	0,965 2	1,930 3

5.8 Eluent A: water, purified by passing it through an activated charcoal cartridge or water of equivalent purity.

NOTE The use of insufficiently purified water can lead to ghost peaks during the analysis due to impurities eluted when the proportion of acetonitrile in the eluent increases.

5.9 Eluent B: acetonitrile, HPLC gradient grade, solution in purified water, volumic fraction = 20 %. The concentration may be modified in relation to the column used.

5.10 Rinsing solvent: acetonitrile, HPLC grade, solution in water, volumic fraction = 70 %.

5.11 Ion-exchange resin: DEAE Sephadex A25[1)] 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 total volume is equal to twice the volume of the sediment.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 HPLC apparatus with gradient or isocratic elution, column temperature adjustment at 30 °C and detection by ultraviolet absorptiometry at wavelength of 229 nm and, if possible, at wavelength of 275 nm.

An efficient column temperature regulation at 30 °C can be impossible when the ambient temperature is above 25 °C. An oven with a cooling-heating device is recommended in this case.

6.2 HPLC columns for gradient elution.

HPLC column containing an octyl (C8) or octadecyl (C18) stationary phase fixed to silica column packing, of particle size less than or equal to 5 µm.

The performance of the column should be checked regularly, preferably using a reference sample of rapeseed. In particular, the column shall not degrade desulfo-4-hydroxyglucobrassicin, an important but relatively unstable desulfoglucosinolate. Figure 1 shows an example of glucosinolates separations using the HPLC gradient mode. New columns shall be subjected to preliminary conditioning in accordance with the manufacturer's instructions so that reproducible results can be obtained.

6.3 pH-meter.

6.4 Microgrinder, for example, a coffee mill.

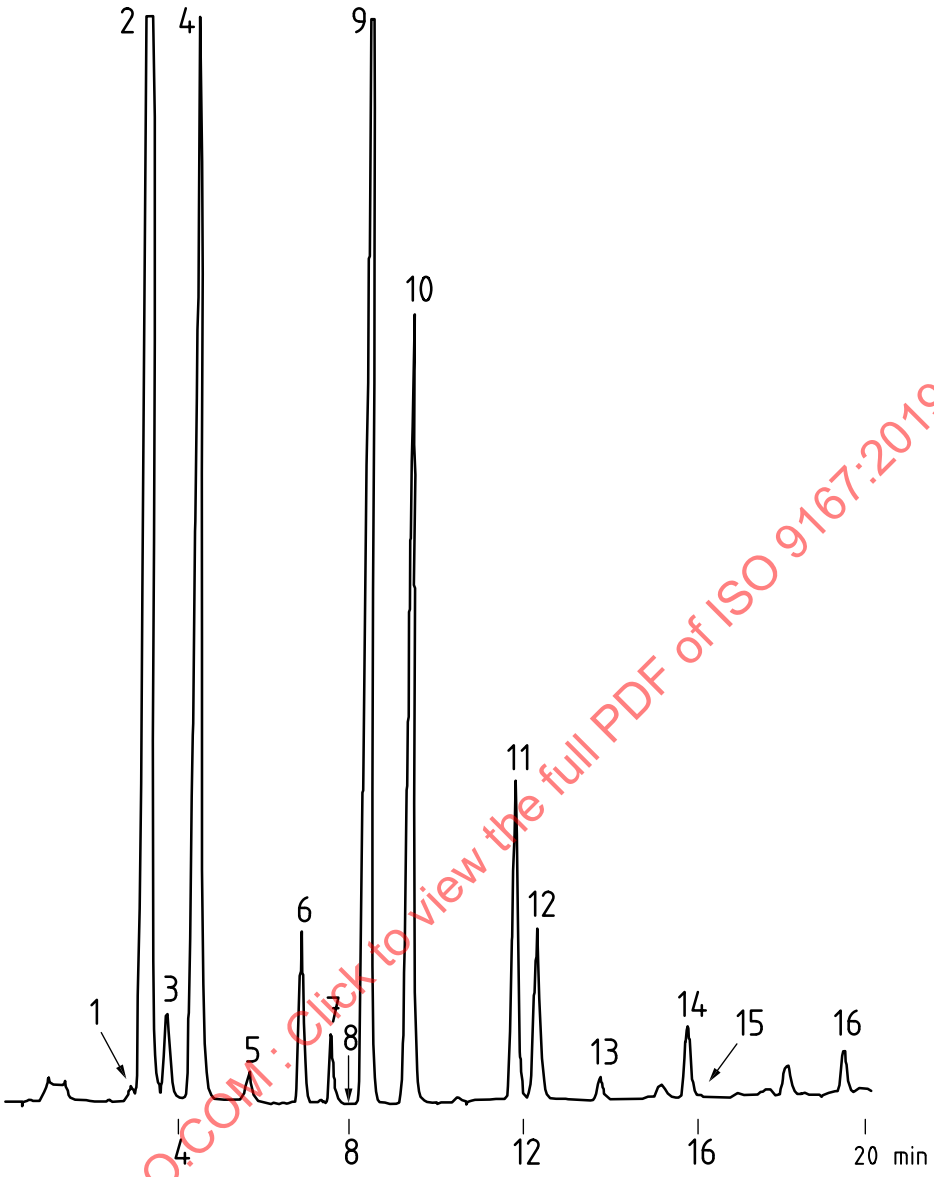
6.5 Centrifuge, suitable for use with the tubes (6.6), capable of obtaining a centrifugal acceleration of 5 000*g*.

6.6 Polypropylene tubes, of 6 ml capacity.

6.7 Water-bath or other **heating apparatus**, capable of maintaining a temperature of 75 °C ± 3 °C.

6.8 Pasteur pipettes fitted with glass wood, 150 mm long, and a suitable stand, or any other appropriate apparatus.

1) DEAE Sephadex A25 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.



Key

1	desulfoglucoiberin	9	desulfogluconapin
2	desulfoprogoitrin	10	desulfo-4-hydroxyglucobrassicin
3	desulfoepiprogoitrin	11	desulfoglucobrassicanapin
4	desulfosinigrin	12	desulfoglucotropaeolin
5	desulfoglucoraphanin	13	desulfoglucobrassicin
6	desulfogluconapoleiferin	14	desulfogluconasturtiin
7	desulfoglucoalyssin	15	desulfo-4-methoxyglucobrassicin
8	desulfosinalbin	16	desulfoneoglucobrassicin

Figure 1 — Example of a typical chromatogram of rape seeds with gradient elution

7 Sampling

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

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 21294[4] for oilseeds and ISO 5500[1] for oilseed meals.

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.

8 Preparation of the test sample

Reduce the laboratory sample in accordance with ISO 664 for oilseeds and ISO 5502 for oilseed meals.

If the seeds have a moisture and volatile matter content in excess of w = 10 %, dry them beforehand using a current of air at 45 °C ± 5 °C.

The impurities level is generally 2 % (mass fraction). If sinigrin is found in the sample (with the blank), analyse the impurities separately, as the sinigrin may stem from seeds of adventitious cruciferae, which are impurities in rapeseed.

If the seeds have been treated, wash them with dichloromethane and dry them in a current of air at ambient temperature.

Reduce the sample to two sub-samples of 20 g each.

Determine the moisture and volatile matter content of a sub-sample in accordance with ISO 665 for oilseeds and ISO 771 for oilseed meals or an adequate procedure.

Grind the seeds of other sub-sample in the microgrinder (6.4) for 20 s. Mix, then grind for a further 5 s. Weigh the prepared sample (see 9.1) immediately to avoid modification of the moisture and volatile matter content.

9 Procedure

9.1 Test portion

Label two tubes (6.6) as A and B and transfer 200 mg for oilseeds and 100 mg for oilseed meals, weighed to the nearest 0,5 mg, of the prepared test sample (see Clause 8) to each tube. Use tube A for the test sample and use the tube B as blank sample, if necessary.

9.2 Extraction of glucosinolates

Place the tubes in the water-bath or other heating apparatus (6.7), set at 75 °C and leave for 1 min. Add 3 ml of boiling ethanol solution (5.1) and then immediately add, to tube A, 200 µl to the nearest 3 µl of internal standard solution prepared according to the HPLC elution mode and the assumed glucosinolate content of the sample (5.5).

The temperature of the ethanol solution shall be as close as possible to the boiling point to ensure a rapid denaturation of the enzyme myrosinase which generally occurs in the test portion.

NOTE Non-denatured myrosinase can break down the glucosinolates in a few minutes.

Continue heating at 75 °C for a further 10 min, shaking the tubes at regular intervals. Adjust the volume in each tube A and B to approximately 4 ml with water, mix and then centrifuge at an acceleration of 5 000g for 3 min.

Transfer the supernatant liquid from each tube to two other tubes (6.6) labelled A′ and B′. Adjust the volume in each tube A′ and B′ to approximately 5 ml with water and mix.

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

NOTE With ethanol 50 % as solvent, a one-step extraction was found efficient enough[6].

9.3 Blank test

If required (5.5), carry out a blank test using the same procedure (see 9.2) on a test portion taken from the same test sample, but omitting the internal standard solution (sinigrin or glucotropaeolin) and replacing it by 200 µl of water, in order to detect and quantify any sinigrin or glucotropaeolin (or coeluting compound) present in the test portion.

9.4 Preparation of ion-exchange columns

Cut the required number of Pasteur pipettes fitted with glass wood (6.8), i.e. one pipette per combined extract, so as to leave a volume of 1,2 ml above the neck. Place the pipettes vertically on a stand.

Transfer 0,5 ml of a well-mixed suspension of ion-exchange resin (5.11) to each pipette and allow to settle.

Rinse the pipettes with 2 ml of the imidazole formiate (5.4) and then twice with 1 ml portions of water.

9.5 Purification and desulfatation

9.5.1 Carry out the operations given in 9.5.2 to 9.5.5 for each extract.

9.5.2 Transfer 1 ml or 0,5 ml of the extract (see 9.2) depending on the assumed glucosinolate content of the sample (see Table 4) to a prepared column (see 9.4) without disturbing the resin surface and allow to drain. Add two 1 ml portions of the 0,02 M, pH 4,0 sodium acetate buffer (5.2), allowing the buffer to drain after each addition.

Table 4 — Volume of extract transferred to the ion-exchange column according to the expected glucosinolate content of the sample

Glucosinolate content of the sample µmol/g	Volume of extract ml
< 50	1,0
> 50	0,5

9.5.3 Add to the column 75 µl of diluted purified sulfatase solution (see C.4.4). Leave to act approximately 15 h at ambient temperature.

9.5.4 Place a tube (6.6) under the column to collect the eluate. Elute the desulfoglucosinolates obtained with two 1 ml portions of water, allowing the water to drain after each addition.

NOTE The complete elution of the desulfoglucosinolates from the ion exchange column can be checked by using an additional portion of water (1 ml) for elution. The chromatography of this additional portion will not reveal any desulfoglucosinolate in significant amount.

9.5.5 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 one week.

9.6 Chromatography with gradient elution

9.6.1 General

The reference method uses a gradient elution with octyl or octadecyl stationary phase column (6.2) and eluents (5.8, 5.9 and 5.10).

9.6.2 Adjustment of the apparatus

9.6.2.1 General

Adjust the column temperature to 30 °C and the detector wavelength to 229 nm. Programme the HPLC apparatus (6.1) so as to rinse the column by passing of eluent B at a flow rate of approximately 1 ml/min. When the baseline is stable, assume the initial conditions of the elution gradient (eluent A) and allow the system to establish equilibrium during 5 min.

If the baseline remains unstable after passing the eluent B during 20 min, use the rinsing eluent (acetonitrile 70 %) to clean the column, then use again eluent B during 5 min and assume the initial conditions (eluent A).

When the system is balanced with eluant A, the sample shall be injected without delay. If not, traces of organic compounds occurring in water can be concentrated onto the column and be released during the gradient elution. Coelution with desulfoglucosinolates may then occur.

9.6.2.2 Analysis

Inject into the chromatograph not more than 50 µl of the desulfoglucosinolate solution obtained in 9.5.5 and vary the concentration in acetonitrile of the mobile phase according to a linear gradient (from 0 % to 25 % within approximately 20 min).

Use an elution gradient appropriate to the column employed.

The following elution gradients are given as examples and may be modified to give optimum separations according to the columns used (see Annex D).

EXAMPLE 1 RP18 column, 5 µm (150 mm × 4,6 mm): Pass 100 % of eluent A (5.8) for 1 min. Apply a linear elution gradient over 20 min until 0 % of eluent A and 100 % of eluent B are obtained. Apply a linear elution gradient over 5 min until 100 % of eluent A and 0 % of eluent B are obtained. Pass 100 % of eluent A for 5 min to establish equilibrium.

EXAMPLE 2 RP8 column, 5 µm (125 mm × 4 mm): Pass 100 % of eluent A for 2 min 30 s. Apply a linear elution gradient over 18 min until 0 % of eluent A and 100 % of eluent B are obtained. Pass 100 % of eluent B for 5 min. Apply a linear elution gradient over 2 min until 100 % of eluent A and 0 % of eluent B are obtained. Pass 100 % of eluent A for 5 min to establish equilibrium.

9.6.2.3 Examination of chromatograms

9.6.2.3.1 Identification of the peaks

Using this chromatographic method, the desulfated derivatives of the rape glucosinolates can be separated fairly easily and the order of elution is generally as shown in Figure 1. However, a few problems, which can be solved after slight modification of the elution gradient or after change of the column type, can exist for the separation of the following desulfoglucosinolates:

— desulfoepiprogoitrin and desulfoglucosinigrin;

— desulfogluconapoleiferin and desulfoglucoalyssin;

— desulfoglucobrassicanapin and desulfoglucotropaeolin;

— desulfogluconasturtiin and desulfo-4-methoxyglucobrassicin.

The identification of the peaks may be carried out by comparison with a chromatogram stemming from a standard sample (6.2) or by UV detection at a specific wavelength (275 nm for indolic glucosinolates).

9.6.2.3.2 Quantification

Only take into account the peaks which correspond to desulfoglucosinolates and where the area exceeds 1 % of the total surface of the areas of these peaks.

Make certain that the integration measurement device operates in a suitable manner for badly separated peaks and for high intensity peaks. If necessary, reinject the desulfoglucosinolate solution (see 9.5.5) after diluting with water.

NOTE In spite of the choice of two internal standards, the chromatogram of the blank analysis (tube B, see 9.2) can show a peak with the same retention time than the internal standard used in the tube A (see 9.2). Nevertheless, the calculation of the glucosinolates content can be possible by elimination of the influence of the coeluting peak on the internal standard peak area. If the coeluting peak area is less than the half of the internal standard area, the former can be subtracted from the latter with a correction made in comparison with the areas of another major peak present in both chromatograms. The test report specifies this calculation mode.

10 Expression of results

10.1 Calculation of the content of each glucosinolate

The content C_g of each glucosinolate, expressed in micromoles per gram of dry matter of the product is equal to Formula (1):

$$C_g = \frac{A_g}{A_s} \times \frac{n}{m} \times \frac{K_g}{K_s} \times \frac{100}{(100-w)} \quad (1)$$

where

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

A_s is the numeric value of the peak area, in integrator units, corresponding to the desulfoglucosinolate used as internal standard;

K_g is the numeric value of the relative proportionality factor of the desulfoglucosinolate;

K_s is the numeric value of the relative proportionality factor of the desulfoglucosinolate used as internal standard;

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

n is the numeric value of the quantity, in micromoles, of internal standard added to the tube in 9.2;

w is the numeric value of the moisture and volatile matter content, expressed in percentage by mass, of the test sample.

If it is desired to express the result relative to a specific moisture and volatile matter content, w_s (e.g. w_s = 9 %), multiply the result C_g obtained for dry matter (as above) by Formula (2):

$$\frac{100-w_s}{100} \quad (2)$$

10.2 Relative proportionality factors

The relative proportionality factors (Ki) given in Table 5 shall be adopted.

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

Table 5 — Relative proportionality factors (*K*i) to adopt

	Desulfo-glucosinolate	*K*i
1	Desulfoglucoiberin	1,07
2	Desulfoprogoitrin	1,09
3	Desulfoepiprogoitrin	1,09
4	Desulfosinigrin	1,00
5	Desulfoglucoraphanin	1,07
6	Desulfogluconapoleiferin	1,00
7	Desulfoglucoalyssin	1,07
8	Desulfosinalbin	0,50
9	Desulfogluconapin	1,11
10	Desulfo-4-hydroxyglucobrassicin	0,28
11	Desulfoglucobrassicanapin	1,15
12	Desulfoglucotropaeolin	0,95
13	Desulfoglucobrassicin	0,29
14	Desulfogluconasturtiin	0,95
15	Desulfo-4-methoxyglucobrassicin	0,25
16	Desulfoneoglucobrassicin	0,20
17	Other desulfoglucosinolates	1,00

10.3 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 content of each glucosinolate (the corresponding peak area of which is greater than 1 % of the sum total of the peak areas).

11 Precision

11.1 Interlaboratory test

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

11.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test materials 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 glucosinolates contents lower than 20 µmol/g and 4 µmol/g for glucosinolates contents ranging from 20 µmol/g to 35 µmol/g.

11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test materials in different equipment, should not be greater than 4 µmol/g for glucosinolate contents lower than 20 µmol/g and 8 µmol/g for glucosinolate contents ranging from 20 µmol/g to 35 µmol/g.

12 Test report

The test report shall specify the following:

— all 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 9167;

— all operating details not specified in this document, or regarded as optional, together with details of any incidents which may 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)

Results of interlaboratory trials — Gradient elution HPLC method

An interlaboratory test, carried out at the international level in 1988, in which 11 laboratories participated, each of which carried out two determinations on each sample A, B, C and D, gave the statistical results (evaluated in accordance with ISO 5725:1986[2]) shown in Table A.1. The gradient elution method was used.

Table A.1 — Statistical results of the 1988 interlaboratory test — Gradient elution

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

An interlaboratory test, carried out at the international level in 2014, in which 18 laboratories participated, each of which carried out two determinations on each sample A to F, gave the statistical results (evaluated in accordance with ISO 5725:1986[2]) shown in Table A.2. The gradient elution method was used.

Table A.2 — Statistical results of the 2014 interlaboratory test

Sample	A	B	C	D	E	F
Sample type	*Brassica napus* meal	*Brassica napus* seed	*Brassica juncea* meal	*Brassica juncea* seed	*Brassica napus* (canola) seed	*Brassica napus* (canola) meal
Number of participating laboratories	18	18	15	18	18	18
Number of laboratories retained after eliminating outliers	16	18	13	17	18	16
Mean glucosinolates content[a]	7,83	14,58	156,97	15,07	10,14	1,70
Standard deviation of repeatability, s_r[a]	0,15	0,18	1,99	0,36	0,52	0,10
Coefficient of variation of repeatability (%)	1,9	1,2	1,3	2,4	5,2	6,1
Repeatability limit, r (2,83 × s_r)[a]	0,43	0,51	5,57	1,02	1,47	0,29
[a] Expressed in µmol/g dry matter.						

Table A.2 *(continued)*

Sample	A	B	C	D	E	F
Standard deviation of reproducibility, s_R[a]	1,33	1,34	30,14	1,91	1,27	0,36
Coefficient of variation of reproducibility (%)	17,0	9,2	19,2	12,7	12,5	21,0
Reproducibility limit, R (2,83 × s_R)[a]	3,72	3,75	84,4	5,34	3,56	1,00
[a] Expressed in µmol/g dry matter.						

Annex B
(normative)

Checking of the titre of the prepared internal standard solution

B.1 Determination of purity

Make certain that the solution does not contain glucosinolates other than the internal standard by analysing the solution according to 9.5 to Clause 11.

B.2 Determination of the titre

B.2.1 Principle

The titre is determined either by an independent method for measuring the glucosinolates (glucose released) or by calibration with the present method and a reference material.

B.2.2 Glucose release method

Determine the titre of the solution by measuring the stoechiometric quantity of glucose released after complete hydrolysis with the myrosinase (thioglucoside glucohydrolase EC 3.2.3.1). The hydrolysis will be carried out on ion exchange columns[5] and the yield of the hydrolysis will be checked by using different durations as for checking activity of sulfatase (see C.4.5).

B.2.3 Calibration by a reference material

Use with a theoretical titre, the internal standard solution where the real titre have to be determined, to analyse a sample of rape having a certified glucosinolate content. Use the same method of elution (gradient or isocratic) than for the expected use of the internal standard solution and carry out several replicates to evaluate the precision of the calibration. The real titre of the internal standard solution is calculated as shown by Formula (B.1):

$$IS_r = IS_{th} \times c_c / c_{th} \quad \text{(B.1)}$$

where

IS_r is the numeric value of the real titre of the internal standard;

IS_{th} is the numeric value of the theoretical titre of the internal standard;

c_c is the numeric value of the certified glucosinolates content of the reference material;

c_{th} is the numeric value of the glucosinolates content of the reference material determined with IS_{th}.

B.3 Relative proportionality factors

Verify that the relative proportionality factor of glucotropaeolin, in comparison with sinigrin, corresponds to those indicated in 10.2 with a tolerance of 10 %.

Annex C
(normative)

Preparation and test of purified sulfatase solution and checking of the desulphation step on ion-exchange columns

C.1 General

The sulfatase solution can be purified by two methods based on different principles. Method A is more efficient but less simple than method B. The purified sulfatase activity is measured in order to prepare "ready to use" solutions. The activity of the sulfatase solution is checked in analysis conditions (on the ion exchange columns).

C.2 Principle

C.2.1 Purification

Method A: The sulfatase is purified by ion exchange and ultrafiltration.

Method B: The sulfatase is purified by fractionated precipitation.

C.2.2 Activity measurement

The activity is calculated form the initial speed of the desulfatation of sinigrin. The decrease of the absorbance of the sinigrin solution at 229 nm is measured with a spectrometer.

C.2.3 Activity checking on ion exchange columns

The desulfatation activity is checked by carrying out a kinetic study of the reaction on glucosinolates linked on the ion exchange column.

C.3 Reagents and apparatus

C.3.1 Sulfatase, *Helix pomatia*, type H1 (EC 3.1.6.1).

C.3.2 DEAE Sepharose 6B-CL[2] **suspension,** available commercially, ready for use, or an equivalent product.

C.3.3 Ion exchange columns for method A purification.

Cut five Pasteur pipettes (6.8) 7 cm above the neck and place a glass wool plug in the neck. Install the pipettes vertically on a stand and add to each a sufficient quantity of ion exchange resin (C.3.2) such that, once the water has drained off, a 0,5 ml volume of resin is obtained.

Pour 1 ml of the imidazole formiate solution (5.4) into each pipette, then rinse them twice with 1 ml of water.

C.3.4 Sodium acetate, 0,2 mol/l solution.

2) DEAE Sepharose 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.

C.3.5 Sinigrin solution, 0,15 mmol/l buffered to pH 5,8 for measurement of activity.

Prepare the following three solutions in succession:

a) transfer 1 ml of glacial 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 to pH 5,8 using solution a) or b) as appropriate.

Pour 3 ml of the 5 mmol/l sinigrin solution (5.6) into a 100 ml one-mark volumetric flask and make up to the mark with solution c).

C.3.6 Ultra-filtration unit (immersion filter or membrane filter) of which the maximum nominal molar weight is 10 Kg/mol[3)].

C.3.7 Centrifuge tube of 10 ml capacity.

C.3.8 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 if possible with a recording system.

C.4 Procedures

C.4.1 Method A purification

Weigh, to the nearest 1 mg, 25 mg of sulfatase *Helix pomatia* type H1 (C.3.1), dissolve it in 2,5 ml of water and transfer 0,5 ml of this solution into each of the columns prepared in C.3.3. Wash each column with 1,5 ml of water and discard the effluent. Next, add 1,5 ml of the sodium acetate solution (C.3.4) then collect and combine the eluates from the five columns in a test tube.

Concentrate the eluates using an ultra-filtration unit (C.3.6) 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 0,1 ml of liquid remains. Dilute to 2,5 ml with water and store at −18 °C if the activity is not checked the same day. It is recommended to condition the sulfatase solution in several batches, to avoid successive freezing and thawing out as it is recommended to store the unused solution at −18 °C.

C.4.2 Method B purification

Weigh, to the nearest 1 mg, 75 mg of sulfatase *Helix pomatia* type H1 (C.3.1) in a 10 ml centrifuge tube (C.3.7). Dissolve it in 3 ml of water and 2 ml of ethanol by mixing with vortex during 3 min. Centrifugate at 5 000 r/min during 1 min and discard the precipitate. To the supernatant, add 8 ml of ethanol and mix by vortex during 3 min. After 45 min of decanting, centrifugate at 5 000 r/min during 10 min. Discard the supernatant and dissolve the precipitate in 5 ml of water and store at −18 °C if the activity is not checked the same day. It is recommended to condition the sulfatase solution in several batches, so as to avoid successive freezing and thawing out.

3) The Millipore PLGC 11K25 immersion filter or the Millipore Ultra free-CL filtration unit 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.

C.4.3 Activity measurement of the purified sulfatase

C.4.3.1 Using a pipette, transfer 2 ml of the buffered sinigrin solution (C.3.5) into the reference and measuring cells of the spectrometer (C.3.8) adjusted to a wavelength of 229 nm with a cell temperature of 30 °C. At time t = 0, add 50 µl of purified sulfatase (see C.4.1 or C.4.2) 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 $\Delta A/\Delta t$.

NOTE If no recorder is available for plotting the absorbance, the data can be read on the spectrometer display and manually reported on a graph. The beginning of the reaction will be noted accurately to plot the tangent to the point t = 0.

C.4.3.2 The activity of the sulfatase solution represents the number of micromoles of desulfated sinigrin per minute at 30 °C per millilitre of this solution. Activity is measured according to the simplified Formula (C.1):

$$a = \frac{\Delta A \times 5{,}7}{\Delta t \times A_e} \tag{C.1}$$

where

a is the numeric value of activity, in activity units per millilitre (ua/ml);

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

A_e is the numeric value of the difference between the absorbance at equilibrium of the desulfated sinigrin and the absorbance at time t = 0.

C.4.3.3 The calculation of the activity may be detailed as described in C.4.3.4 to C.4.3.5.

C.4.3.4 According to the definition of the activity, as shown by Formula (C.2):

$$a = \frac{\Delta N \times 10^6}{\Delta t \times v \times 10^3} = \frac{\Delta N \times 10^3}{\Delta t \times v} \tag{C.2}$$

where

ΔN is the number of moles of desulfated sinigrin during Δt;

v is the numeric value of the volume, in litres, of the sulfatase solution introduced into the reacting medium (i.e. 50 × 10^{-6} l);

10^6 is the numeric value of the factor for converting moles into micromoles;

10^3 is the numeric value of the factor for converting litres into millilitres.

$\Delta N = \Delta c \times V$

where

V is the numeric value of the volume, in litres, of the reacting medium (i.e. 2,05 × 10^{-3});

Δc is the numeric value of the variation in concentration of sinigrin, in moles per litre, during Δt.

C.4.3.5 According to Beer-Lambert's law, as shown by Formula (C.3):

$$\Delta c = \frac{\Delta A}{\Delta \varepsilon \times 1} \tag{C.3}$$

where

ΔA is the numeric value of the difference in absorbance between Δt at the beginning of the reaction;

$\Delta \varepsilon$ is the numeric value of the difference (in the region of 1 500 $l.mol^{-1}.cm^{-1}$) of the molar extinction coefficients of sinigrin and of desulfosinigrin at 229 nm;

1 is the numeric value, in cm, of the path length.

$\Delta \varepsilon$ may be expressed with the aid of A_e and c_e according to Formula (C.4):

$$\Delta \varepsilon = \frac{A_e}{1 \times c_e} \tag{C.4}$$

where

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

c_e is the numeric value of the concentration of the desulfosinigrin at equilibrium, in moles per litre, namely

$$c_e = c_d \times r \times \frac{V}{V+v} = 1{,}39 \times 10^{-4} \text{ mol/l}$$

c_d is the numeric value of the concentration of the original sinigrin solution ($0{,}15 \times 10^{-3}$ mol/l);

r is the numeric value, of the yield at equilibrium of the desulfatation of the sinigrin (0,95).

It is deduced from the expressions of $\Delta\varepsilon$, Δc, ΔN and a:

$$\Delta c = \frac{\Delta A \times c_e}{A_e} \quad \Delta N = \frac{\Delta A \times v \times c_e}{A_e} \quad \text{and} \quad a = \frac{\Delta A \times V \times c_e}{\Delta t \times A_e \times v} \times 10^3$$

C.4.4 Preparation of "ready to use" solutions

Dilute the purified sulfatase with water so as to obtain, as a function of the activity measured in C.4.3, a solution containing 0,25 ua/ml.

EXAMPLE If the measurement of the activity of a solution of purified sulfatase having a volume of 1,5 ml gives 0,75 ua/ml, this solution is diluted with 3 ml of water so as to obtain 4,5 ml of solution with an activity of 0,25 ua/ml.

Since the dilution factor can be subsequently modified on the basis of the results of the checking of the desulfatation (see C.4.5), it is recommended to store a stock of undiluted purified sulfatase (see C.4.1 or C.4.2).

C.4.5 Checking of the sulfatase activity on ion-exchange columns

Since glucosinolates are desulfated at different speeds, it is necessary, in order to have quantitatively accurate results, to make certain that the glucosinolates transferred to the ion exchanger are completely desulfated at the end of the time period chosen for desulfatation (15 h).

For this, examine the kinetics of the desulfatation under true operating conditions in the following manner.

— Prepare a volume of extract of approximately 8 ml by conducting, for example, four extractions according to the normal procedure (see 9.2), with addition of the internal standard, of a control sample and by collecting the 2 ml of extract produced by each extraction;

— Transfer 1 ml of extract to each of the six ion-exchange columns and conduct the desulfatation in accordance with the normal procedure described in 9.5 but with respective desulfatation times of 1 h, 2 h, 4 h, 8 h, 16 h and 32 h (transfer the extracts and the sulfatase at the same time and eluate each column after respectively 1 h, 2 h, 4 h, 8 h, 16 h and 32 h). Analyse the six eluates by HPLC in accordance with the normal procedure described in 9.6 and record the areas of the principal peaks for the six analyses.

— Next, plot the curves (desulfoglucosinolate peak area) = f (desulfatation duration) for the principal glucosinolates and the internal standard. The plane of each curve corresponds to the complete desulfatation of the glucosinolates.

The start of the plane of the most slowly desulfated glucosinolate shall be situated at a time well under 15 h (e.g. 8 h).

On the contrary, the dilution factor of the purified sulfatase should be reduced in order to increase the activity of the sulfatase solution transferred to the columns (increasing the volume of sulfatase transferred to the column does not improve the desulfatation yield).

The value of the peak area of the internal standard at the step can be used as a basis of verification for everyday analyses, since the conditions for introduction of the internal standard have remained unchanged.

The checking of the desulfatation is necessary when the operating conditions vary significantly (type of ion-exchange column, concentration of the sulfatase being used, ambient temperature).

Annex D
(informative)

HPLC system and column performance criteria qualification

D.1 General

This procedure can be adopted to qualify any HPLC column or system not previously used for this document.

D.2 Procedure

D.2.1 Sinigrin and glucotraopaeolin peak shapes

Prepare standards as specified in 5.5. Using the working internal standard solution, inject no more than 50 µl onto the HPLC column. Carefully examine the shape of the peak(s). It should be symmetrical and should not show a leading shoulder or a chair-like appearance. A leading shoulder or a chair-like appearance indicates excessive injection volume. Make sure that the injection volume did not exceed 50 µl. If full loop injection was used, the problem could be caused by excessive dead volume where the loop attached to the injector. Replace the loop to correct this problem. A larger diameter column (4,6 versus 3 mm) may also correct the problem.

D.2.2 Resolution of internal standards

On the same chromatogram, examines the peaks for sinigrin and glucotraopaeolin. Determine the retention time for both internal standards. Both should be baseline resolved from one another. Figure 1 shows examples of good chromatograms.

D.2.3 Critical resolution

Prepare and analyse a rapeseed extract as describe in the standard. Ensure that the peaks for a) desulfoglucoiberin (peak 1) and desulfoprogoitrin (peak 2), b) desulfoglucobrassicanapin (peak 11) and desulfoglucotropaeolin (peak 12), and c) desulfogluconasturtiin (peak 14) and desulfo-4-methoxyglucobrassicin (peak 15) are fully resolved from one another (see the chromatograms in Figure 1). Some HPLC columns are not capable of resolving these peaks and are not suitable for this method. Some HPLC columns might change the elution order of the peaks. These columns can be used for this method provided that the peaks are correctly identified and fully resolved from one another. One cause of poor resolution might be inadequate mixing of the A and B solvents on the HPLC system, which will be evident as peaks that are too broad and which vary in retention time from run to run. This can be corrected by addition of a mixer.

D.3 System suitability

D.3.1 General

A system suitability step shall be performed to ensure that the HPLC system is working correctly. Before running any test solutions, demonstrate the repeatability and lack of carryover of the HPLC system as described in D.3.2 to D.3.3.

D.3.2 System artefacts

The first two injections of the day, a blank standard in succession, must produce no artefact. Artefacts present in both runs indicate impurities on the system and will be present in every run. Artefacts in the first chromatogram and absent in the second one indicate a build-up of impurities in the system. In this case, the first sample of every analytical test shall be a blank solution.

D.3.3 Carryover — System precision

Analyse five replicate analyses of the internal standard solution. Calculate the concentration of each analyte and calculate mean, standard deviation and relative standard deviation (RSD) of the results. The RSD for all peaks should be ideally < 0,2 %. If the RSD > 0,5 %, the system is not suitable for the analysis and the cause of the problem should be found and shall be corrected.

NOTE Reversed-phase HPLC column: any C8 and C18 reversed phase (RP) columns that pass the performance criteria requirements are acceptable. However, many C8 and C18 RP columns of identical particle size differ by their pore size, surface area, carbon load and purity of the silica. Some columns are also endcapped to minimize the effect of the non-reacted silanol groups. These characteristics will give the various C18 and C8 columns different polarity and hydrophobicity properties, therefore not all C8 and C18 columns are suitable for this analysis and column choice is critical in achieving desired resolution.

Annex E
(informative)

Elution in the isocratic mode

E.1 Internal standard

With isocratic elution on cyano propyl stationary phase, sinigrin cannot be used for rapeseed analysis because of the non-separation from the other glucosinolates. Glucotropaeolin (potassium benzylglucosinolate, M = 447,5 g/mol or tetramethylammonium benzylglucosinolate, M = 482,6 g/mol) (5.7) shall be used instead. The glucotropaeolin may be used in a hydrated form, then the molar mass and the purity shall be known and taken into consideration for the preparation of the solution.

See Table 1 to determine the concentration of the internal standard solution to use according to the assumed glucosinolate content of the sample and see Table 2 to determine the weight of glucotropaeolin to prepare a 100 ml water solution.

E.2 Mobiles phases for isocratic elution

E.2.1 Eluent.

Mixture of water and acetonitrile, filtered at 0,22 µm. The proportion of acetonitrile depends on the HPLC column. It can be, for example,

— for Lichrospher CN: from 0 % to 5 %, and

— for Nucleosil CN and Hypersil CN: around 10 %.

NOTE Acetonitrile can be replaced without drawback by methanol (with the proportion in water doubled). Using methanol can lightly modify the selectivity of the separation in comparison with acetonitrile.

E.2.2 Rinsing solvent, acetonitrile, HPLC grade, 25 % (volume fraction) solution in water.

E.3 Column for isocratic elution

HPLC column of size 250 mm × 4 mm or 250 mm × 4,6 mm, containing a cyanopropyle stationary phase, bound to silica column packing, of particle size less than or equal to 5 µm.

The characteristics of selectivity and retention of the selected column shall enable the desulfoglucosinolates separation as shown on Figure E.1.

New columns shall be subjected to preliminary conditioning in accordance with the manufacturer's instructions so that reproducible results can be obtained. In particular, before first using, the column shall be rinsed with pure ethanol to eliminate the storage solvent which is generally immiscible with water.

The performance of the column should be checked regularly, preferably using a reference sample of rapeseed desulfoglucosinolates. In particular, the column shall not degrade desulfo-4-hydroxyglucobrassicin, an important but relatively unstable desulfoglucosinolate. The peaks should be symmetrical and should not show a leading shoulder or a chair-like appearance. The internal standard should be well resolved from the other peaks.