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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 g/mol	Sinigrin weight 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.

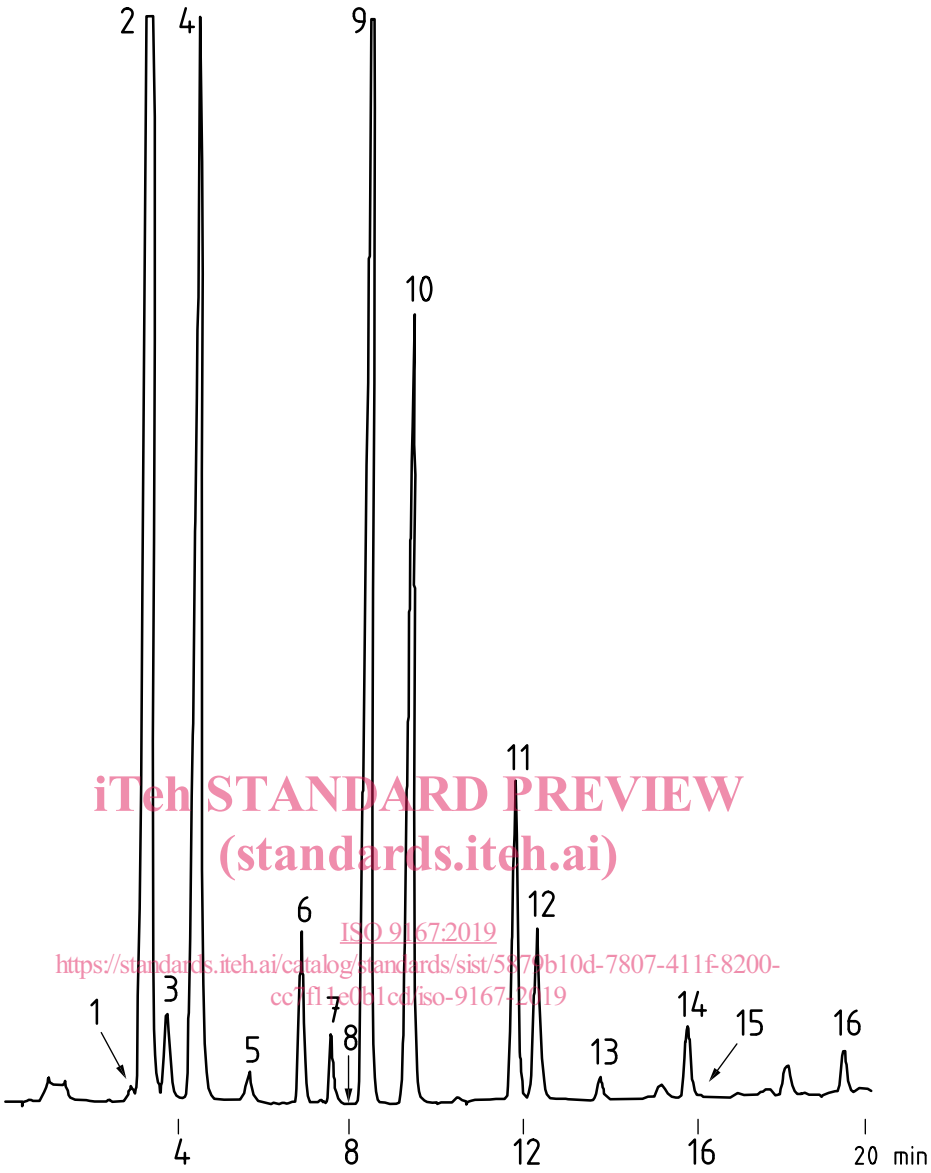
6.5 Centrifuge, suitable for use with the tubes (6.6), capable of obtaining a centrifugal acceleration of 5 000*g*.

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 micrograinder (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].

