SLOVENSKI STANDARD
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Krma: metode vzorčenja in analize - Ugotavljanje nepoškodovanih glukozinolatov v oljni ogrščici z LC-MS/MS

Animal feeding stuffs: Methods of sampling and analysis - Determination of intact glucosinolates in rapeseed by LC-MS/MS

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung von intakten Glucosinolaten in Rapssaat mittels LC-MS/MS

Aliments pour animaux - Méthodes d'échantillonnage et d'analyse - Dosage des glucosinolates intacts dans les matières premières pour aliments des animaux et les aliments composés par CL-SM/SM

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European foreword

This document (prEN 17853:2022) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs: Methods of sampling and analysis”, the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

Introduction

Glucosinolates are a group of plant produced secondary metabolites predominantly found in the family Brassicaceae (mustards and cabbages) ([1], [2], [3]). Many common vegetables such as broccoli, Brussels sprouts, cabbage and cauliflower, belong to this plant family. At the same time species from the genera *Brassica*, *Camelina*, *Crambe*, *Rhaphanus* and *Sinapis* are agricultural crops used for the production of plant oils, such as rapeseed oils. The press cake is used as animal feed material. Glucosinolates are considered undesirable substances in feed [4]. Glucosinolates in rapeseed and rapeseed products can also be measured after enzymatic desulfation by high-performance liquid chromatography (HPLC) coupled with UV detection. This method is described in standard EN ISO 9167 [5].

WARNING — This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health protection measures and to ensure that regulatory and legal requirements are complied with.

1 Scope

This document specifies a method for the determination of individual intact glucosinolates in oilseeds, oilseed products and complete feeds by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS).

The method described in this document has been successfully validated by collaborative trial in the following matrices: rape seed, camelina seed, *Brassica oleracea* seeds, mixed oilseeds, rape seed flakes, compound feed for bovine, porcine and poultry.

The method is applicable for the quantitative determination of epiprogroitrin, glucoalyssin, glucoarabin, glucobrassicanapin, glucobrassicin, glucocamelinin, glucoerucin, glucoiberin, gluconapin, gluconapoleiferin, gluconasturtiin, glucoraphanin, glucoraphenin, glucotropaeolin, homoglucocamelinin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, progoitrin, sinalbin and sinigrin.

The concentration ranges tested in the collaborative trial for each individual glucosinolate and for the total glucosinolate content are summarized in Table 1.

Table 1 — Summary of glucosinolate concentration ranges tested in the collaborative trial

	Number of samples with acceptable results	**Tested concentration range** mmol/kg		
		Min		**Max**
Epiprogoitrin	7	0,01		0,93
Glucoalyssin	6	0,02		2,10
Glucoarabin	3	0,31		6,15
Glucobrassicanapin	5	0,01		0,38
Glucobrassicin	5	0,02		0,31
Glucocamelinin	3	0,82		16,1
Glucoerucin	3	1,07		15,6
Glucoiberin	3	1,51		18,5
Gluconapin	6	0,23		1,68
Gluconapoleiferin	5	0,01		0,33
Gluconasturtiin	7	0,01		11,0
Glucoraphanin	5	0,01		3,11
Glucoraphenin	1		15,6	
Glucotropaeolin	2	0,03		18,3
Homoglucocamelinin	3	0,17		3,23
4-Hydroxyglucobrassicin	6	0,23		7,33
4-Methoxyglucobrassicin	1		0,16	
Neoglucobrassicin	5	0,01		0,13
Progoitrin	6	0,62		14,8
Sinalbin	4	0,01		41,1
Sinigrin	3	0,25		23,7
Total content	8	1,48		117,3

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.

<std>EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*</std>

<std>EN ISO 6498, *Animal feeding stuffs - Guidelines for sample preparation (ISO 6498:2012)*</std>

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:

- IEC Electropedia: available at https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp

4 Principle

Glucosinolates are extracted from the homogenized sample with methanol:water (70:30). After centrifugation, the extracts are diluted, filtered and measured by liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). In oilseeds and oilseed products individual glucosinolates are quantified by multi-level calibration using standards in aqueous solution. In compound feeds individual glucosinolates are quantified by multi-level calibration using standards in blank feed matrix extract.

5 Reagents

5.1 Analytical standards

Analytical standards should have a demonstrated purity of at least 90 %, preferably of 95 % or higher.

NOTE In this section glucosinolate standards are listed that are currently available from at least one commercial supplier. Depending on the intended application a selection of the listed glucosinolate standards can be used.

5.1.1 Epiprogoitrin (2-(S)-hydroxy-3-butenyl glucosinolate) or its potassium salt (CAS 19237-18-4)

5.1.2 Glucoalyssin (5-(methylsulfinyl)pentyl glucosinolate) or its potassium salt (CAS 499-37-6)

5.1.3 Glucoarabin (9-(methylsulfinyl)nonyl glucosinolate) or its potassium salt (CAS 67920-64-3)

5.1.4 Glucobrassicanapin (4-pentenyl glucosinolate) or its potassium salt (19041-10-2)

5.1.5 Glucobrassicin (3-indoylmethyl glucosinolate) or its potassium salt (CAS 4356-52-9)

5.1.6 Glucocamelinin (10-(methylsulfinyl)decyl glucosinolate) or its potassium salt (CAS 67884-10-0)

5.1.7 Glucoerucin (4-methoxythiobutyl glucosinolate) or its potassium salt (CAS 21973-56-8)

5.1.8 Glucoiberin (3-methylsulfinylpropyl glucosinolate) or its potassium salt (CAS 554-88-1)

5.1.9 Gluconapin (3-butenyl glucosinolate) or its potassium salt (CAS 19041-09-9)

5.1.10 Gluconapoleiferin (2-(S)-hydroxy-4-pentenyl glucosinolate) or its potassium salt (CAS 19764-03-5)

Gluconapoleiferin is only available as a 1:1 mixture of 2-S and 2-R isomers. A purity of 50 % is taken for 2S-gluconapoleiferin.

5.1.11 Gluconasturtiin (2-phenylethyl glucosinolate) or its potassium salt (CAS 499-30-9)

5.1.12 Glucoraphanin (4-methylsulfinylbutyl glucosinolate) or its potassium salt (CAS 21414-41-5)

5.1.13 Glucoraphenin (4-methylsulfinylbutenyl glucosinolate) or its potassium salt (CAS 28463-24-3)

5.1.14 Glucotropaeolin (benzyl glucosinolate) or its potassium salt (CAS 499-26-3)

5.1.15 Homoglucocamelinin (11-(methylsulfinyl)undecyl glucosinolate) or its potassium salt (CAS 186037-18-3)

5.1.16 4-Hydroxyglucobrassicin (4-hydroxy-3-indoylmethyl glucosinolate) or its potassium salt (CAS 83327-20-2)

5.1.17 4-Methoxyglucobrassicin (4-methoxy-3-indoylmethyl glucosinolate) or its potassium salt (CAS 83327-21-3)

5.1.18 Neoglucobrassicin (1-methoxy-3-indoylmethyl glucosinolate) or its potassium salt (CAS 5187-84-8)

5.1.19Progoitrin (2-(R)-hydroxy-3-butenyl glucosinolate) or its potassium salt (CAS 585-95-5)

5.1.20 Sinalbin (4-hydroxybenzyl glucosinolate) or its potassium salt (CAS 16411-05-5)

5.1.21 Sinigrin (2-propenyl glucosinolate), monohydrate or its potassium salt (CAS 3952-98-5)

5.2 Chemicals

5.2.1 Methanol (HPLC grade)

5.2.2 Acetic acid (98-100 %)

5.2.3 Water

Water of LC-MS grade, double-distilled or water of grade1 as defined in EN ISO 3696.

5.3 Standard solutions

5.3.1 General

Accurately weigh (6.1) between 5 and 10 mg of each standard (5.1.1-5.1.21) into a separate amber-coloured glass bottle of 4 ml (6.8). Add a volume of extraction solvent (5.4.1) to produce a solution with a concentration of 10 µmol/ml. Take into account the weight, the purity and the appearance form of the standard (see NOTES 1-4). In Table 2 example calculations are given for the preparation of 1 ml stock solution.

NOTE 1 Glucosinolate standards are typically available as potassium salts. Some glucosinolate standards additionally contain one molecule of water.

NOTE 2 Most analytical standards of individual glucosinolates are typically obtained in 5-10 mg quantities. Since glucosinolates are highly hygroscopic compounds it is preferable to use the solid standard only once.

NOTE 3 Depending on the intended application, a selection of the standard solutions listed below can be used.

NOTE 4 The stock standard solutions are stable for 24 months when stored below < −18 °C. Methanol:water (70:30) is the preferred solvent because it provides a better stability of glucosinolates than water.

Table 2 — Preparation of stock standard solutions

	Molecular weight potassium form g/mol	**Weight standard for 1 ml standard solution of 10 µmol/ml** mg
Epiprogoitrin	427,48	4,2748
Glucoalyssin	489,63	4,8963
Glucoarabin	545,73	5,4573
Glucobrassicanapin	425,51	4,2551
Glucobrassicin	486,26	4,8626
Glucocamelinin	559,76	5,5976
Glucoerucin	459,61	4,5961
Glucoiberin	461,56	4,6156
Gluconapin	411,49	4,1149
Gluconapoleiferin	441,51	4,4151
Gluconasturtiin	461,16	4,6116
Glucoraphanin	475,66	4,7566
Glucoraphenin	473,58	4,7358
Glucotropaeolin	447,52	4,4752
Homoglucocamelinin	573,79	5,7379
4-Hydroxyglucobrassicin	502,56	5,0256
4-Methoxyglucobrassicin	516,59	5,1659
Neoglucobrassicin	516,59	5,1659
Progoitrin	427,49	4,2749
Sinalbin	463,52	4,6352
Sinigrin	397,47	3,9747

5.3.2 Epiprogoitrin (10 µmol/ml)

5.3.3 Glucoalyssin (10 µmol/ml)

5.3.4 Glucoarabin (10 µmol/ml)

5.3.5 Glucobrassicanapin (10 µmol/ml)

5.3.6 Glucobrassicin (10 µmol/ml)

5.3.7 Glucocamelinin (10 µmol/ml)

5.3.8 Glucoerucin (10 µmol/ml)

5.3.9 Glucoiberin (10 µmol/ml)

5.3.10 Gluconapin (10 µmol/ml)

5.3.11 Gluconapoleiferin (10 µmol/ml)

5.3.12 Gluconasturtiin (10 µmol/ml)

5.3.13 Glucoraphanin (10 µmol/ml)

5.3.14 Glucoraphenin (10 µmol/ml)

5.3.15 Glucotropaeolin (10 µmol/ml)

5.3.16 Homoglucocamelinin (10 µmol/ml)

5.3.17 4-Hydroxyglucobrassicin (10 µmol/ml)

5.3.18 4-Methoxyglucobrassicin (10 µmol/ml)

5.3.19 Neoglucobrassicin (10 µmol/ml)

5.3.20 Progoitrin (10 µmol/ml)

5.3.21 Sinalbin (10 µmol/ml)

5.3.22 Sinigrin (10 µmol/ml)

5.3.23 Mixed standard solution (100 nmol/ml)

Pipette in a 10 ml volumetric flask 100 µl of each stock solution 5.3.1-5.3.22 (10 µmol/ml) and fill to the mark with extraction solvent (5.4.1).

NOTE 1 Depending on the intended application, a selection of the standard solutions can be used.

NOTE 2 When stored below < −18 °C the solution is stable for 1 year. Extraction solvent (methanol:water (70:30)) is the preferred solvent because it provides a better stability of glucosinolates than water.

5.3.24 Mixed standard solution (10 nmol/ml)

Pipette 2 ml of mixed standard solution 100 nmol/ml (5.3.23) in a 20-ml volumetric flask and fill to the mark with extraction solvent (5.4.1).

NOTE When stored below < −18 °C the solution is stable for 1 year Extraction solvent (methanol:water (70:30)) is the preferred solvent because it provides a better stability of glucosinolates than water.

5.3.25 Mixed standard solution (1 nmol/ml)

Pipette 500 µl of mixed standard solution 100 nmol/ml (5.3.23) in a 50 ml volumetric flask and fill to the mark with extraction solvent (5.4.1).

NOTE When stored below < −18 °C the solution is stable for 1 year. Extraction solvent (methanol:water (70:30)) is the preferred solvent because it provides a better stability of glucosinolates than water.

5.3.26 Calibration solutions in aqueous solution

Prepare calibration solutions according to Table 3. Pipette directly in HPLC vials (see NOTE).

Table 3 — Preparation of calibration standards in aqueous solution

	Concentration (nmol/ml)	**Mixed standard solution 1 nmol/ml (5.3.25)** µl	**Mixed standard solution 10 nmol/ml (5.3.24)** µl	**Mixed standard solution 100 nmol/ml (5.3.23)** µl	**Water (5.2.3)** µl
Cal 1	0	0	0	0	1 000
Cal 2	0,01	10	0	0	990
Cal 3	0,02	20	0	0	980

	Concentration (nmol/ml)	Mixed standard solution 1 nmol/ml (5.3.25) µl	Mixed standard solution 10 nmol/ml (5.3.24) µl	Mixed standard solution 100 nmol/ml (5.3.23) µl	Water (5.2.3) µl
Cal 4	0,05	50	0	0	950
Cal 5	0,10	0	10	0	990
Cal 6	0,25	0	25	0	975
Cal 7	0,50	0	50	0	950
Cal 8	1,0	0	0	10	990
Cal 9	2,5	0	0	25	975
Cal 10	5,0	0	0	50	950

Calibration standards should be prepared each new day of analysis.

NOTE The calibration points required depend on the concentrations expected in the samples (see Clause 7.3), the dilution factor used (see Clause 7.3) and the dynamic range of the mass spectrometer (see Clause 8). It is advised to use all calibration standards, but at least calibration standards Cal 1,3-8.

5.4 Reagents

5.4.1 Extraction solvent: methanol:water (70:30)

Mix 700 ml methanol (5.2.1) with 300 ml water (5.2.3). The solvent is stored at room temperature and can be used for one month.

5.4.2 Mobile phase A: 0,1 % acetic acid in water

Pipette 1 ml of acetic acid (5.2.2) in 1 000 ml water (5.2.3). The solvent is stored at room temperature and can be used for one month.

NOTE The acetic acid concentration can be adjusted in the range of 0,01 % to 0,1 % to optimize the retention of the analytes on the analytical column.

5.4.3 Mobile phase B: methanol (5.2.1)

5.5 Quality control material

An appropriate material: a reference material (5.6), or a material with known natural contamination, or a blank material fortified with known amounts of the glucosinolates, is included in each series and used for quality purposes.

5.6 Reference materials

Reference materials can be used for internal quality control purposes. Example reference materials are:

5.6.1 ERM®-BC366R Rapeseed

5.6.2 ERM®-BC190R Rapeseed

5.6.3 ERM®-BC367R Rapeseed

6 Apparatus

Usual laboratory equipment and, in particular, the following items

6.1 Analytical balance with a precision of 0,02 mg

6.2 Analytical balance with a precision of 1 mg

6.3 (Micro)grinder

6.4 Polypropylene centrifuge tubes 50 ml with screw cap

6.5 Water bath

Capable of maintaining a temperature of 75 °C ± 1 °C.

6.6 Vortex mixer or minishaker

6.7 Vertical or horizontal shaker adjustable

6.8 Amber coloured glass bottle 4 ml size with screw cap

6.9 HPLC autosampler vial glass or polypropylene 1,5 ml

6.10 Centrifuge suitable for use with the 50 ml centrifuge tubes (6.4)

6.11 Volumetric flasks calibrated 10, 20, 50 ml

6.12 Calibrated micrometric pipette(s)

6.13 HPLC system consisting of:

6.13.1 Autosampler thermostated

Capable of maintaining a temperature of 10 ± 1 °C.

6.13.2 Binary pump system

Capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

6.13.3 Column oven thermostated

Capable of maintaining a temperature of 50 ± 1 °C.

6.13.4 Analytical column

Containing C18 reversed phase packing material, capable of the base-line separation of analytes with identical molecular mass. A C18 stationary phase with charged surface hybrid chemistry has shown to work well.

6.13.5 Pre-column, optional

Containing the same stationary phase material as the analytical column and with appropriate dimensions.

6.14 Tandem mass spectrometer

Capable of performing multiple selected reaction monitoring in negative mode, with a sufficiently wide dynamic range, sufficient scan speed and capable of unit mass separation and equipped with a computer-based data processing system. Any ionization source giving sufficient yield may be employed.

7 Procedure

7.1 Sample pre-treatment

Laboratory samples should be taken and prepared in accordance with European legislation [6] where applicable or, in any other case with EN ISO 6498.

Homogenize samples in a grinder (6.3) to < 1 mm.

7.2 Test portion

7.2.1 Oilseeds and oilseed products

The amount of homogenized oilseed or oilseed product sample (7.1) examined is 1,0 ± 0,02 g.

The amount of homogenized oilseed or oilseed product sample may be reduced to 0,5 ± 0,01 g. The amount of extraction solvent (Clause 7.3.1) shall in that case be reduced accordingly.

7.2.2 Compound feed

The amount of homogenized compound feed sample (7.1) examined is 1,0 ± 0,02 g.

7.3 Extraction

7.3.1 Oilseeds and oilseed products

Weigh a test portion of 1,0 ± 0,02 g homogenized sample (7.2.1) into a centrifuge tube of 50 ml (6.4). Add 25 ml extraction solvent (5.4.1), vortex for 10 s and place the tube in a water bath at 75 °C (6.5) (see NOTE 1). Heat the sample for 15 min and then let cool down to room temperature. Place the tube for 60 min in a shaker rotating at moderate speed (6.7).

Centrifuge the tube for 5 min at 2 000 *g* at room temperature (6.10). Transfer 5 µl of the supernatant to a HPLC vial (6.9) and add 995 µl of water and close the vial (see NOTE 2).

NOTE 1 An extraction solvent containing 70 % methanol gives the best compromise between analyte stability and extraction efficiency. An extraction solvent with a higher organic content (e.g. 80 % methanol) increases the stability of the analytes (slow degradation by myrosinase), but also results in a lower extraction efficiency. An extraction solvent with a lower organic content (e.g. 60 % methanol or 50 % ethanol) results in a comparable extraction efficiency, but also in a strongly increased myrosinase activity. This can result in partial degradation of the analytes and in a reduced reproducibility. ISO 9167:2019 describes an alternative extraction procedure for rapeseed and rapeseed meals using 50 % ethanol as extraction solvent. This alternative extraction procedure was not tested in this collaborative trial.

NOTE 2 In specific products concentrations can be present that exceed the working range of the calibration curve. For these products the extract is further diluted with water to obtain a concentration that falls within the working range of the calibration curve.

7.3.2 Compound feed

Weigh a test portion of 1,0 ± 0,02 g homogenized sample (7.2.2) into a centrifuge tube of 50 ml (6.4). Add 25 ml extraction solvent (5.4.1), vortex for 10 s and place the tube in a water bath at 75 °C (6.5). Heat the sample for 15 min and then let cool down to room temperature. Place the tube for 60 min in a shaker rotating at moderate speed (6.7)

Centrifuge the tube for 5 min at 2 000 *g* at room temperature (6.10). Transfer 25 µl of the supernatant to a HPLC vial (6.9) and add 975 µl of water and close the vial.

7.3.3 Recovery sample for compound feed

For preparation of the recovery sample take a representative blank feed sample (5.5) (see NOTE), preferably the same that is used for the preparation of the calibration range in blank extract (7.3.4).

Weigh a test portion of 1,0 ± 0,02 g homogenized sample (7.2.2) into a centrifuge tube of 50 ml (6.4). Add 1,00 ml of mixed standard solution 100 nmol/ml (5.3.22) to the sample. This is equivalent to 0,1 mmol/kg. Vortex for 10 s.

Add 25 ml extraction solvent (5.4.1), vortex for 10 s and place the tube in a water bath at 75 °C (6.5). Heat the sample for 15 min and then let cool down to room temperature. Place the tube for 60 min in a shaker rotating at moderate speed (6.7).

Centrifuge the tube for 5 min at 2 000 *g* at room temperature (6.10). Transfer 25 µl of the supernatant to a HPLC vial (6.9) and add 975 µl of water and close the vial.

NOTE A sample shown by a preceding analysis not to contain the target analytes in a concentration above the limit of detection.

7.3.4 Preparation of calibration standards in blank feed extract

For preparation of the calibration curve in matrix take a representative blank feed sample (5.5) (see NOTE).

Weigh a test portion of 1,0 ± 0,02 g homogenized sample (7.2.2) into a centrifuge tube of 50 ml (6.4). Add 25 ml extraction solvent (5.4.1), vortex for 10 s and place the tube in a water bath at 75 °C (6.5). Heat the sample for 15 min and then let cool down to room temperature. Place the tube for 60 min in a shaker rotating at moderate speed (6.7)

Centrifuge the tube for 5 min at 2 000 *g* at room temperature (6.10). Transfer an aliquot of 1 ml to a new centrifuge tube of 50 ml (6.4), add 39 ml of water and mix well. This diluted extract is used to prepare calibration solutions according to Table 4. Pipette directly in HPLC vials.

NOTE A sample shown by a preceding analysis not to contain the target analytes in a concentration above the limit of detection.

Table 4 — Preparation of calibration standards in blank feed extract

	Concentration nmol/ml	Mixed standard solution 1 nmol/ml (5.3.25) µl	Mixed standard solution 10 nmol/ml (5.3.24) µl	Mixed standard solution 100 nmol/ml (5.3.23) µl	Blank feed extract (7.3.4) µl
Cal 1	0	0	0	0	1 000
Cal 2	0,01	10	0	0	990
Cal 3	0,02	20	0	0	980
Cal 4	0,05	50	0	0	950
Cal 5	0,10	0	10	0	990
Cal 6	0,25	0	25	0	975
Cal 7	0,50	0	50	0	950