EURL-MP-method_010 (version 1)
Determination of cyanogenic glycosides in food and feed by LC-MS/MS

Analyte group:	**Plant toxins** – cyanogenic glycosides
Analyte(s):	Amygdalin
	Linamarin
	Linustatin
	Lotaustralin
	Neolinustatin
	Prunasin

Commodity group:	Food and feed
Commodities validated:	Linseed (flax seed), linseed cake, almond and apricot kernels, cassava flour, and products containing these ingredients

Technique:	Liquid chromatography / Tandem Mass Spectrometry (LC-MS/MS)

Modifications compared to previous version:
Not applicable

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1 Introduction

Cyanogenic glycosides (CNGs) are secondary metabolites produced by a variety of plants, including several species consumed as food. Well known sources of CNGs are bamboo shoots (*Bambusa vulgaris*), cassava (*Manihot esculenta*), lima beans (*Phaseolus lunatus*), linseed or flax (*Linum usitatissimum*), sorghum (*Sorghum bicolor*) and stone fruits of the genus *Prunus*, such as apricot and almond. When the plant cells are damaged, for example by grinding or chewing, CNGs come in contact with plant-specific β-glycosidases, such as linamarase in cassava and amygdalase in stone fruits. Consequently, CNGs are converted to unstable cyanohydrins and this ultimately results in the formation of toxic hydrocyanic acid (HCN).

The European Commission has established a maximum limit for the presence of hydrocyanic acid, in the form of bound CNGs, in a number of food products: 20 mg HCN equivalents/kg (mg HCN eq./kg) in apricot kernels, 35 mg HCN eq./kg in almonds, 50 mg HCN eq./kg in fresh cassava roots, 10 mg HCN eq./kg in cassava and tapioca flour, 150 mg HCN eq./kg in linseed for the final consumer and 250 mg HCN eq./kg for linseed not placed on the market for the final consumer [1]. Maximum limits are also in place for marzipan and similar products (50 mg HCN eq./kg), canned stone fruits (5 mg HCN eq./kg) and alcoholic beverages (35 mg HCN eq./l) [2]. Maximum limits for products intended for feed applications, such as linseed, are currently under discussion within the European Commission.

2 Scope

The method described here has been validated for the determination of six intact cyanogenic glycosides, amygdalin, linamarin, linustatin, lotaustralin, neolinustatin and prunasin in products of low water content such as linseed (flax seed), linseed cake, almond and apricot kernels, cassava milling products, and products containing one of these ingredients, in the range 1-100 mg HCN eq./kg, and by subsequent dilution to 1-10,000 mg HCN eq./kg by liquid chromatography tandem mass spectrometry (LC-MS/MS). The LOQ was determined at 1 mg HCN eq./kg. The method is not suited for products with high water content such as fresh cassava root.

3 Principle

The cyanogenic glycosides are extracted from the sample by mixing 1 g homogenised material with 40 ml methanol/water/formic acid (25/74/1) (v/v/v) on a rotary tumbler for 30 min. The samples are centrifuged, and a portion of the supernatant is diluted with 1% formic acid, filtered and analysed by LC-MS/MS. CNGs are quantified by matrix-matched calibration (MMC) in an extract of a (surrogate) blank material. The CNG concentration added to the MMC calibrants is presented as mg HCN eq./kg. Dhurrin is used as internal standard.

4 Reagents

All reagents and solvents shall be of quality for LC analysis, unless otherwise specified.

4.1 Analytical standards

4.1.1 Amygdalin

4.1.2 Linamarin

4.1.3 Lotaustralin

4.1.4 Linustatin

4.1.5 Neolinustatin

4.1.6 Prunasin

4.1.7 Dhurrin (IS)

4.2 Chemicals

4.2.1 Methanol, LC-MS grade

4.2.2 Formic acid, 98-100%

4.2.3 Water, LC-MS grade
Water purified by a Milli-Q purification system with a minimal resistance of 18.2 MΩ/cm can be used as well.

4.2.4 Acetonitrile, LC-MS grade

4.3 Solutions and reagent

4.3.1 Extraction solvent: 1% formic acid in methanol/water (25/75) (v/v)
Mix 250 ml of methanol (4.2.1) with 750 ml of water (4.2.3). Add 10 ml of formic acid (4.2.2) and mix. This solution is stored at room temperature and can be used for 3 months.

4.3.2 Dilution solvent: 1% formic acid in water
Mix 1 ml formic acid (4.2.2) with 100 ml of water (4.2.3). This solution is stored at room temperature and can be used for 3 months.

4.3.3 Mobile phase A: 0.1% formic acid in water
Mix 1 ml formic acid (4.2.2) with 1000 ml of water (4.2.3). This solution is stored at room temperature and can be used for 3 months.

4.3.4 Mobile phase B: methanol (4.2.1)

Note: acetonitrile (4.2.4) can be used as well.

4.4 Standard solutions

4.4.1 Stock solutions (250 µg HCN eq./ml)
Accurately weigh (± 0.02 mg) into separate glass bottles (5.1.3) between 5 and 10 mg of the CNG analytical standards (4.1.1 to 4.1.7). When the analytical standard is only available in a quantity of 5 mg or less, the entire content of the container is used. In that case the weight reported by the supplier is used. Flush the contents of the bottle three times with methanol (4.2.1) to dissolve and collect all material.

Add a volume of methanol (4.2.1) in such a way that the concentration of the CNG in the solution is equal to 250 µg HCN eq./ml, as indicated in Table 1. Take into account the weight, the purity and the appearance form of the standard. The solutions can be used for 24 months when stored in the dark at ≤-20°C.

Table 1. Preparation of CNG stock solutions of 250 µg HCN eq./ml

Stock solution	Analyte	Molecular weight (amu)	MW ratio to HCN	Concentration of CNG representing 250 µg HCN eq./ml methanol
4.4.1.1	Amygdalin (4.1.1)	457.43	16.92	4229 µg/ml
4.4.1.2	Linamarin (4.1.2)	247.25	9.14	2286 µg/ml
4.4.1.3	Lotaustralin (4.1.3)	261.27	9.66	2416 µg/ml
4.4.1.4	Linustatin (4.1.4)	409.39	15.14	3785 µg/ml
4.4.1.5	Neolinustatin (4.1.5)	423.41	15.66	3915 µg/ml
4.4.1.6	Prunasin (4.1.6)	295.29	10.92	2730 µg/ml
4.4.1.7	Dhurrin (4.1.7)	311.29	11.51	2878 µg/ml

4.4.2 Mixed standard solution MSS1 (25 µg HCN eq./ml in methanol)
Pipette 1 ml of CNG stock solutions 4.4.1.1 to 4.4.1.6 in a 10 ml volumetric flask. Fill up to the mark with methanol (4.2.1). The solution can be used for 24 months when stored in the dark at ≤-20°C.

4.4.3 Mixed standard solution MSS2 (1000 ng HCN eq./ml in methanol)
Pipette 800 µl of mixed standard solution MMS1 (25 µg HCN eq./ml) (4.4.2) in a 20 ml volumetric flask. Fill up to the mark with methanol (4.2.1). The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.4.4 Mixed standard solution MSS3 (100 ng HCN eq./ml in methanol)
Pipette 1 ml of mixed standard solution MMS2 (1000 ng HCN eq./ml) (4.4.3) in a 10 mL volumetric flask. Fill up to the mark with methanol (4.2.1). The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.4.5 Internal standard (dhurrin) (IS) solution (5 µg HCN eq./ml in methanol)
Pipette 400 µl of dhurrin stock solution (4.4.1.7) in a 20 ml volumetric flask. Fill up to the mark with methanol (4.2.1). The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.4.6 Working standard solution CNGs and IS (25 ng HCN eq./ml in dilution solvent)
Pipette 25 µl of mixed standard solution MSS2 (1000 ng HCN eq./ml) (4.4.3) and 5 µl of internal standard solution (5 µg HCN eq./ml) (4.4.5) in a HPLC vial of 1 ml. Add 970 µl dilution solvent (4.3.2) and mix. The solution can be used for 12 months when stored in the dark at ≤-20°C.

5 Materials & equipment

Any reference to type and/or product is only to inform the user and to identify the equipment and does not imply exclusion of similar equipment.

Usual laboratory glassware and equipment, in particular the following, can be used:

5.1 Materials

5.1.1 Analytical balance, precision: 0.02 mg

5.1.2 Balance, precision: 0.02 g

5.1.3 Glass bottle, 4, 20, 30 or 60 ml, amber coloured, with screw cap

5.1.4 Overhead (rotary tumbler) or horizontal shaker

5.1.5 Vortex mixer

5.1.6 Various pipettes: use positive displacement pipettes for solutions prepared in methanol

5.1.7 Centrifuge tube, 50 ml, polypropylene, with screw cap

5.1.8 Centrifuge, suitable for 50 ml centrifuge tubes

5.1.9 PTFE filter vial, 500 µL (e.g. Mini-Uniprep™)

5.1.10 Compressor for filtervials

5.2 Equipment

5.2.1 LC-MS/MS system with the following components:

5.2.1.1 LC pump, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy

5.2.1.2 Injection system, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0.1%

5.2.1.3 Analytical column, capable of baseline separation of CNGs

5.2.1.4 Column oven, capable of maintaining a constant temperature of 50°C

5.2.1.5 Tandem mass spectrometer (MS/MS), capable of ionisation of the compounds in positive mode, performing Multiple Reaction Monitoring (MRM), and with a sufficiently wide dynamic range and capable of unit mass separation and equipped with a computer-based data processing system. Any ionisation source giving sufficient yield may be employed.

6 Procedures

This document describes the quantification of cyanogenic glycosides in products of low water content, such as linseed, almond, apricot kernel, cassava and tapioca flour, and in processed materials thereof, such as linseed cake, marzipan, nougat.

In Table 2 typical HCN concentrations that can be found in the various matrices are shown. The HCN concentration can vary considerably between products and between sweet and bitter varieties. Table 2 also shows the sample intake and the advised sample to solvent dilutions to be prepared for LC-MS/MS analysis.

Table 2. Typical HCN concentration range found in CNG-containing products and proposed sample dilutions for LC-MS/MS analysis

Matrix	Concentration range total CNGs (mg HCN eq./kg)	Sample intake (6.3.4) (g)	Regular sample to solvent ratio (6.3.4.1 or 6.3.4.2)	Additional sample to solvent ratio (6.3.5.1 or 6.3.5.2)
Linseed	100-250	1.0±0.02	1:1000	1:10,000
Linseed cake	100-500	1.0±0.02	1:1000	1:10,000
Almond, apricot (sweet)	1-50	1.0±0.02	1:1000	1:10,000
Almond, apricot (bitter)	500-5000	0.40±0.005	1:10,000	1:100,000
Marzipan, nougat	1-50	1.0±0.02	1:1000	1:10,000
Cassava/tapioca meal (sweet)	1-50	1.0±0.02	1:1000	1:10,000
Tapioca meal (bitter)	500-5000	0.40±0.005	1:10,000	1:100,000

In Annex A.1 and A.2, the steps described in section 6.3 are shown in the format of checklists.

6.1 Pre-treatment

Samples are ground under cryogenic conditions to avoid degradation of the CNGs. After homogenisation samples are stored at ≤-20°C.

6.2 Test portion

For products with a low total HCN content (<500 mg HCN eq./kg), the amount of homogenised test sample examined is 1.0 ± 0.02 g.

For products with a high total HCN content (>500 mg HCN eq./kg), the amount of homogenised test sample examined is 400 ± 5 mg.

6.3 Extraction, clean-up and preparation of test solutions

6.3.1 Preparation of matrix matched calibration standards (MMC1-8)

The calibration standards are prepared by addition of mixed standard solutions to a blank matrix extract.

In case no blank matrix sample is available, a surrogate blank sample extract is used as alternative. The following surrogate blank matrices can be used:
- for linseed use sesame seed or soybean,
- for linseed cake use soybean cake,
- for cassava or tapioca flour use maize flour,
- for almond and apricot kernel use hazelnut,
- for marzipan and nougat use white bean paste.

Work according to the procedure presented in section 6.5.1. Weigh a test portion of 1.0 g blank sample (or surrogate blank) in a PP tube of 50 ml (5.1.7). Add 40 ml of extraction solvent (4.3.1) to the test portion, shake vigorously and extract for 30 minutes on a rotary tumbler (5.1.4). Centrifuge the sample for 10 minutes (5.1.8) at 3,000 rpm and transfer of the supernatant 8 aliquots of 20 µl each to filter vials (5.1.9). Add standard solutions and solvents according to Table 3. Mix and close the vials with the help of a compressor (5.1.10).

Table 3. Preparation of matrix matched calibration standards (MMC) in blank extract

Code	HCN eq. in extract (ng/ml)	Conc. in sample (mg HCN eq./kg)*	MSS3 100 ng HCN eq./ml (4.4.4) (µl)	MSS2 1000 ng HCN eq./ml (4.4.3) (µl)	Dhurrin IS 5 µg HCN eq./ml (4.4.5) (µl)	Blank extract (6.3) (µl)	Methanol (4.2.1) (µl)	Dilution solvent (4.3.2) (µl)
MMC1	0	0	-	-	5	20	50	425
MMC2	1	1	5	-	5	20	45	425
MMC3	2.5	2.5	12.5	-	5	20	37.5	425
MMC4	5	5	25	-	5	20	25	425
MMC5	10	10	50	-	5	20	-	425
MMC6	25	25	-	12.5	5	20	37.5	425
MMC7	50	50	-	25	5	20	25	425
MMC8	100	100	-	50	5	20	-	425

*: The concentration in the sample that corresponds to a 1000-fold dilution. When a higher dilution is used, the corresponding concentration in the sample increases accordingly.

6.3.2 Quality control sample limit of quantification QC$_{LOQ}$ (1 mg HCN eq./kg)
Weigh a test portion of 1.0 g blank (surrogate) sample (see 6.3.1) in a PP tube of 50 ml (5.1.7). Add 40 µl of mixed standard solution MSS1 (25 µg HCN eq./ml) (4.4.2). Wait 30 min before starting the extraction procedure. Add 40 ml of extraction solvent (4.3.1) to the test portion, shake vigorously and extract for 30 minutes on a rotary tumbler (5.1.4). Centrifuge the sample for 10 minutes (5.1.8) at 3,000 rpm and transfer 20 µl of the supernatant to a filter vial (5.1.9). Add 5 µl of IS solution (5 µg HCN eq./ml) (4.4.5), 50 µl methanol (4.2.1) and 425 µl dilution solvent (4.3.2). Mix and close the vial with the help of a compressor (5.1.10).

6.3.3 Quality control sample recovery QC$_{Rec}$ (10 mg HCN eq./kg)
Weigh a test portion of 1.0 g of blank (surrogate) sample (see 6.3.1) in a 50 ml PP tube (5.1.7). Add 400 µl of mixed standard solution MSS1 (25 µg HCN eq./ml) (4.4.2) to the sample in the tube (QC$_{rec}$). Wait 30 min before starting the extraction procedure. Add 40 ml of extraction solvent (4.3.1) to the test portion, shake vigorously and extract for 30 minutes on a rotary tumbler (5.1.4). Centrifuge the sample for 10 minutes (5.1.8) at 3,000 rpm and transfer 20 µl of the supernatant to a filter vial (5.1.9). Add 5 µl of IS solution (5 µg HCN eq./ml) (4.4.5), 50 µl methanol (4.2.1) and 425 µl dilution solvent (4.3.2). Mix and close the vial with the help of a compressor (5.1.10).

6.3.4 Extraction and sample preparation

6.3.4.1 Extraction and sample preparation procedure for samples with low total HCN content (<500 mg HCN eq./kg)
Weigh 1.0 ± 0.02 g of sample in a 50 mL PP tube (5.1.7). Add 40 ml of extraction solvent (4.3.1) to the tube, shake vigorously and extract for 30 minutes on a rotary tumbler (5.1.4). Centrifuge the sample for 10 minutes (5.1.8) at 3,000 rpm. Make an extra dilution when needed according to 6.3.5. Transfer

20 μl of the supernatant to a filter vial (5.1.9). Add 5 μl of IS solution (5 μg HCN eq./ml) (4.4.5), 50 μl methanol (4.2.1) and 425 μl dilution solvent (4.3.2). Mix and close the vial with the help of a compressor (5.1.10). The resulting sample to solvent ratio (dilution factor) is 1,000.

6.3.4.2 Extraction and sample preparation procedure for samples with high total HCN content (>500 mg HCN eq./kg)

Weigh 400 ± 5 mg of sample in a 50 ml PP tube (5.1.7). Add 40 ml of extraction solvent (4.3.1) to the tube, shake vigorously and extract for 30 minutes on a rotary tumbler (5.1.4). Centrifuge the sample for 10 minutes (5.1.8) at 3,000 rpm. Make an extra dilution when needed according to 6.3.5. Transfer 5 μl of the supernatant to a filter vial (5.1.9). Add 15 μl blank extract, 5 μl of IS solution (5 μg HCN eq./ml) (4.4.5), 50 μl methanol (4.2.1) and 425 μl dilution solvent (4.3.2). Mix and close the vial with the help of a compressor (5.1.10). The resulting sample to solvent ratio (dilution factor) is 10,000.

6.3.5 Dilution of samples with CNG concentrations exceeding the calibration line

When the concentration of one or more of the analytes exceeds, or is expected to exceed, the working range of the calibration curve, the sample extract (6.3.4.1 or 6.3.4.2) is diluted ten-fold.

6.3.5.1 Dilution of samples with low total HCN content (<500 mg HCN eq./kg)

To prepare a ten-fold diluted sample extract, transfer 500 μl of the supernatant (6.3.4.1) to a test tube of 10 ml. Add 4.5 ml of dilution solvent (4.3.2) to the tube and mix. Transfer 20 μl of the supernatant to a filter vial (5.1.9). Add 20 μl blank supernatant (6.3.1), 5 μl of IS solution (5 μg HCN eq./ml) (4.4.5), 50 μl methanol (4.2.1) and 405 μl dilution solvent (4.3.2). Mix and close the vial with the help of a compressor (5.1.10). The resulting sample to solvent ratio (dilution factor) is 10,000.

6.3.5.2 Dilution of samples with high total HCN content (>500 mg HCN eq./kg)

To prepare a ten-fold diluted sample extract, transfer 500 μl of the supernatant (6.3.4.2) to a test tube of 10 ml. Add 4.5 ml of dilution solvent (4.3.2) to the tube and mix. Transfer 5 μl of the supernatant to a filter vial (5.1.9). Add 20 μl blank supernatant (6.3.1), 5 μl of IS solution (5 μg HCN eq./ml) (4.4.5), 50 μl methanol (4.2.1) and 420 μl dilution solvent (4.3.2). Mix and close the vial with the help of a compressor (5.1.10). The resulting sample to solvent ratio (dilution factor) is 10,000.

7   LC-MS/MS analysis

Chromatographic and mass spectrometric conditions may be chosen freely. The optimal measurement conditions strongly depend on the instrumentation used. However, important criteria and parameters with respect to the chromatographic separation and detection of the analytes are:

The chosen column dimensions and chromatographic conditions should be appropriate to obtain base line separation of the CNGs from compounds with the same mass-to-charge ratio. For chromatographic separation mobile phases may be used over the range pH 2 to 12. The retention times of the CNGs are not substantially influenced by the pH of the mobile phase. It should be noted that an analytical column containing a high pH-resistant cross-linked C18 reversed phase packing material generally is required for use with a mobile phase of pH higher than 7.

The injection volume should be optimised for the column dimension and the sensitivity of the mass spectrometric system. The use of large volume injections may result in distorted peak shapes, particularly for the analytes with shorter retention times.

The chosen mass spectrometric conditions should be appropriate to measure the analytes with sufficient sensitivity and specificity. Preferably, the protonated molecular parent ion should be selected as precursor ion and the product ions should be specific for the compound. Please note that in the example LC-MS/MS conditions provided (Annex B) the CNGs are analysed in their protonated hydrate form $(M+H_2O+H^+)$. These hydrate forms are much more sensitive than the corresponding protonated molecular ions. Select at least two precursor-to-product ion combinations to be included in the multiple reaction monitoring (MRM) method (see Table B.2.2 for example fragmentation conditions). Preferably, product ions that are formed by the loss of water from the protonated molecular parent ion should not be selected. Each chromatographic peak should be composed of at least 10 data points.

Check the system performance as well as the retention time and time windows for analysis of the samples by injection of the working standard solution of 25 ng HCN eq./ml (4.4.6). Alternatively, matrix matched calibration standard MMC6 (25 ng/ml) (6.3) can be used to condition the mass spectrometer and analytical column. Inject the working standard solution or MMC6 at least five times or until stable RTs (less than 0.05 min difference) and sensitivity (less than 10% difference) are obtained.

Carry-over: The presence of the target compounds in a solvent injection is assessed. If the target compounds are present in the solvent injection, in a concentration which might lead to a false-positive result, the system should be cleaned before starting the analysis series.

Example LC-MS/MS conditions and example LC-MS/MS chromatograms are given in Annex B.

7.1 Injection sequence
Analyse the sample extracts in the order as given below.

- o   Standard working solution 25 ng HCN eq./ml (4.4.6) or calibration standard MMC6 25 ng HCN eq./ml (6.3.1), injected at least 6 times
- o   Dilution solvent (4.3.2)
- o   Matrix matched calibration standards (MMC1-8) (6.3.1)
- o   Dilution solvent (4.3.2)
- o   Quality control sample limit of quantification QC$_{LOQ}$ (1 mg HCN eq./ml) (6.3.2)
- o   Quality control sample recovery QC$_{Rec}$ (10 mg HCN eq./ml) (6.3.3)
- o   Dilution solvent (4.3.2)
- o   Sample extracts (6.3.4 and/or 6.3.5)
- o   Dilution solvent (4.3.2)
- o   Matrix matched calibration standards (MMC1-8) (6.3.1)

8   Evaluation and calculations
Peak areas are used for all subsequent calculations. For each injection, check peak assignment and integration for all measured transitions and adjust if needed.

8.1   Verification of linearity of LC-MS/MS measurement
The matrix matched calibration solutions MMC1-8 (6.3.1) are used to determine the linearity of the LC-MS/MS system. Plot the sum of the area of the product ions against the corresponding concentrations in ng HCN eq./ml. Construct a calibration curve using the least-square regression method (see note).

Linearity is demonstrated and the calibration curve is fit-for-purpose when the deviation of the back-calculated concentrations of the calibration standards from the true concentrations, using the calibration equation, do not exceed 20%. The correlation coefficient of the lines should be ≥0.990.

Note: two calibration curves are constructed, one using calibration standards MMC1 to 6 (low range calibration curve) and one using calibration standards MMC1 to 8 (full range calibration curve). Analytes for which the peak area falls within the low range calibration curve are calculated with the low range calibration curve. Analytes for which the peak area falls outside the low range calibration curve but within the full range calibration curve are calculated with the full range calibration curve. Analytes for which the peak area falls outside the full range calibration curve are diluted and reanalysed.

8.2   Identification of CNG in the samples

Identify the CNGs in the samples by comparing retention time and ion ratio with that of the matrix matched calibration solutions MMC1-8 (6.3.1) according to SANTE/11312/2021 [3].

CNGs are considered present and identified when:
a) in the blank QC sample MMC1 (6.3.1), the peak for the quantifier ion at the retention time of the plant toxin is below 30% of the LOQ;
b) the retention time (RT) of the peak observed for the CNGs in the individual calibration standards, QC samples and sample extracts differs not more than 0.1 min from the average retention time as calculated (**Equation I**) from the matrix matched calibration standards MMC2-8 (6.3.1);
c) the ion ratio (IR) in individual calibration standards, QC samples and sample extracts differs not more than 30% from the average ion ratio of the matrix matched calibration standards MMC2-8 (6.3.1) (=reference ion ratio) using **Equation II** and **Equation III**.

Note: for calculation of the reference ion ratio use only responses with an S/N > 10. For the higher concentrations, exclude peak areas exceeding the linear range from calculation of the reference ion ratio.

Equation I: Deviation of the retention time (ΔRT)

$$\Delta RT = RT_{sample} - RT_{avg}$$

where:
ΔRT	is the deviation of the retention time of the analyte in the sample extract (min), compared to the average retention time in the matrix matched calibration standards MMC2-8 (6.3.1) (min)
RT_{sample}	is the retention time of the analyte in the sample extract (min)
RT_{avg}	is the average retention time of the analyte in the matrix matched calibration standards MMC2-8 (6.3.1) (min)

Equation II: Calculation of the ion ratio (IR)

$$IR = \left(\frac{A_{low}}{A_{high}}\right) \times 100\%$$

where:
IR	is the ion ratio (%)
A_{low}	is the area of the product ion with the lowest intensity

A_{high} is the area of the product ion with the highest intensity

Equation III: Relative deviation of the ion ratio (D)

$$D = \left(\frac{IR_{sample} - IR_{avg}}{IR_{avg}}\right) \times 100\%$$

where:

D	is the relative deviation of the ion ratio of the analyte in the sample, compared to the average ion ratio of the analyte in the matrix matched calibration standards MMC2-8 (6.3.1) (%)
IR_{sample}	is the ion ratio of the analyte in the sample (%) (**Equation II**)
IR_{avg}	is the average ion ratio of the analyte in the matrix matched calibration standards MMC2-8 (6.3.1) (%) (**Equation II**)

Note: for calculation of the reference ion ratio use only responses with an S/N > 10. For the higher concentrations, exclude peak areas exceeding the linear range from calculation of the reference ion ratio.

8.3 Quantification of CNGs in the samples

8.3.1 Recovery
Calculate the recovery (Rec) for the QC_{rec} sample (6.3.3) using **equation IV**.

Equation IV: Calculation of the recovery (Rec)

$$Rec = \left(\frac{A_{QCrec}}{A_{MMC5}}\right) \times 100\%$$

where:

Rec	is the recovery (%)
A_{QCrec}	is the area of the product ions from the analyte in sample QC_{rec} of 10 mg HCN eq./kg (6.3.3)
A_{MMC5}	is the sum of the area of the product ions from the analyte in matrix matched calibration standard MMC5 of 10 ng HCN eq./ml (6.3.1).

The recovery must be between 70-120%.

8.3.2 Quantification
The concentration of the CNGs in the sample is calculated using **Equation V**. The content of an analyte in the sample is expressed in mg HCN eq./kg. Quantification takes place based on the sum of the areas of the two product ions and with correction for recovery (see note).

Equation V: Calculation of the concentration in the sample (C_{sample})

$$C_{sample} = \left(\frac{A_{sample} - b}{a}\right) \times DF \times \frac{1}{Rec}$$

where:

C_{sample}	is the concentration of the analyte in the sample (mg HCN eq./kg)
A_{sample}	is the sum of the area of the product ions obtained for the analyte in the sample
b	is the intercept of the matrix matched calibration standards curve
a	is the slope of the matrix matched calibration standards curve
DF	is the dilution factor of the extract (6.3.4, 6.3.5)
R	is the recovery of the QC_{rec} sample (**Equation IV**)

Note: When the recovery is between 90 and 110%, correction for recovery is not mandatory.

8.4 Final result

The concentration of the individual CNGs and the summed concentration of CNGs in the sample is expressed in mg HCN eq./kg.

9 References

[1] EU (2023) Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006.

[2] EU (2008) Regulation (EU) No 1334/2008 and its amendments.

[3] DG_SANTE, Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed SANTE/11312/2021. https://ec.europa.eu/food/system/files/2022-02/pesticides_mrl_guidelines_wrkdoc_2021-11312.pdf

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Annex A.1. Checklist sample preparation of products with low total HCN content (<500 mg HCN eq./kg)

Technician:	
Date:	
Lab. journal / page:	

Sample preparation and extraction (6.3)

MMC (6.3.1)	QC 1 (6.3.2)	QC 10 (6.3.3)	Samples (6.3.4.1)	
☐	☐	☐		Weigh 1 g of blank (surrogate) sample in a 50 ml PP tube
	☐			Add 40 µl of MSS 1 (4.4.2) and wait 30 min
		☐		Add 400 µl of MSS 1 (4.4.2) and wait 30 min
			☐	Weigh 1 ± 0.02 g of sample in a 50 ml PP tube
☐	☐	☐	☐	Add 40 ml of extraction solvent and shake vigorously
☐	☐	☐	☐	Extract for 30 minutes on a shaking machine
☐	☐	☐	☐	Centrifuge for 10 minutes at 3000 rpm
	☐	☐	☐	Transfer 20 µl supernatant, 5 µl IS solution (4.4.5), 50 µl MeOH and 425 µl dilution solvent to a filter vial
			☐	(Optional): 10-fold dilution: transfer 500 µl supernatant to a 10 ml tube and add 4.5 ml dilution solvent and mix
			☐	(Optional): 10-fold dilution: transfer 20 µl 10x diluted supernatant, 20 µl blank extract, 5 µl IS solution (4.4.5), 50 µl MeOH and 405 µl dilution solvent to a filter vial
☐				Prepare a MMS series in filter vials according to Table 1
☐	☐	☐	☐	Mix and close the vials with the help of a compressor

Table 1. Preparation of matrix matched calibration standards (MMC) in blank extract

	Name	HCN eq. extract (ng/ml)	HCN eq. sample (mg/kg)	MSS3 100 ng HCN eq./ml (4.4.4) (µl)	MSS2 1000 ng HCN eq./ml (4.4.3) (µl)	IS 5 µg HCN eq./ml (4.4.5) (µl)	Blank extract (6.3.1) (µl)	MeOH (µl)	Dilution solvent (4.3.2) (µl)
☐	MMC1	0	0	-	-	5	20	50	425
☐	MMC2	1	1	5	-	5	20	45	425
☐	MMC3	2.5	2.5	12.5	-	5	20	37.5	425
☐	MMC4	5	5	25	-	5	20	25	425
☐	MMC5	10	10	50	-	5	20	-	425
☐	MMC6	25	25	-	12.5	5	20	37.5	425
☐	MMC7	50	50	-	25	5	20	25	425
☐	MMC8	100	100	-	50	5	20	-	425

Annex A.2 Checklist sample preparation of products with high total HCN content (>500 mg HCN eq./kg)

Technician:
Date:
Lab. journal / page:

Sample preparation and extraction (6.3)

Columns: MMC (6.3.1), QC 1 (6.3.2), QC 10 (6.3.3), Samples (6.3.4.2)

MMC	QC 1	QC 10	Samples	Step
☐	☐	☐		Weigh 1 g of blank (surrogate) sample in a 50 ml PP tube
	☐			Add 40 µl of MSS 1 (4.4.2) and wait 30 min
		☐		Add 400 µl of MSS 1 (4.4.2) and wait 30 min
			☐	Weigh 400 ± 5 mg of sample in a 50 ml PP tube
☐	☐	☐	☐	Add 40 ml of extraction solvent and shake vigorously
☐	☐	☐	☐	Extract for 30 minutes on a shaking machine
☐	☐	☐	☐	Centrifuge for 10 minutes at 3000 rpm
	☐	☐	☐	Transfer 5 µl supernatant, 15 µl blank extract, 5 µl IS solution (4.4.5), 50 µl MeOH and 425 µl dilution solvent to a filter vial
			☐	(Optional): 10-fold dilution: transfer 500 µl supernatant to a 10 ml tube and add 4.5 ml dilution solvent and mix
			☐	(Optional): 10-fold dilution: transfer 5 µl 10x diluted supernatant, 20 µl blank extract, 5 µl IS solution (4.4.5), 50 µl MeOH and 420 µl dilution solvent to a filter vial
☐				Prepare a MMC series in filter vials according to Table 1
☐	☐	☐	☐	Mix and close the vials with the help of a compressor

Table 1. Preparation of matrix matched calibration standards (MMC) in blank extract

	Name	HCN eq. extract (ng/ml)	HCN eq. sample (mg/kg)	MSS3 100 ng HCN eq./ml (4.4.4) (µl)	MSS2 1000 ng HCN eq./ml (4.4.3) (µl)	IS 5 µg HCN eq./ml (4.4.5) (µl)	Blank extract (6.3.1) (µl)	MeOH (µl)	Dilution solvent (4.3.2) (µl)
☐	MMC1	0	0	-	-	5	20	50	425
☐	MMC2	1	10	5	-	5	20	45	425
☐	MMC3	2.5	25	12.5	-	5	20	37.5	425
☐	MMC4	5	50	25	-	5	20	25	425
☐	MMC5	10	100	50	-	5	20	-	425
☐	MMC6	25	250	-	12.5	5	20	37.5	425
☐	MMC7	50	500	-	25	5	20	25	425
☐	MMC8	100	1000	-	50	5	20	-	425

Annex B.   Example of LC-MS/MS conditions

B.1   LC conditions
The equipment and measuring conditions shown here are provided as an example. Other analytical equipment, columns, mobile phases and gradient conditions may work equally well.

Table B.1.1. Example conditions for the UPLC system

LC system	Waters Acquity UPLC
Analytical column	Waters Acquity BEH C18 1.7 µm, 100 x 2.1 mm
Column temperature	50°C
Vial tray temperature	10°C
Mobile phase solvent A	0.1% formic acid
Mobile phase solvent B	Methanol
Flow rate	0.4 ml/min
Injection volume	2-5 µl

Table B.1.2 Gradient for the UPLC system

Time (min)	Mobile phase A (4.3.3) (%)	Mobile phase B (4.3.4) (%)
0.0	100	0
2.0	100	0
7.0	85	15
9.0	20	80
9.2	20	80
9.4	100	0
11.5	100	0

Note: acetonitrile can also be used as mobile phase B. This will result in small retention time shifts.

See Annex B.3 for an example LC-MS/MS chromatogram.

B.2   MS conditions
The conditions given in Table B.2 are guidelines; in practice adjusted settings may be required to obtain an optimal performance of the LC-MS/MS system.

Table B.2.1 Example for MS conditions (Waters Xevo TQ-S or TQXS system)

Parameter	Settings
Ionisation mode	ESI positive
Capillary voltage	3.0 kV
Cone voltage	30 V
Source temperature	150°C
Desolvation temperature	600°C
Cone gas flow	150 L/hr
Desolvation gas flow	800 L/hr
CID gas, pressure	Argon; 4.3 10^{-3} mbar
Solvent discard	0-2 and 10-11.5 min

The precursor ions fragment to structurally related products ions. In Table B.2.2 the theoretical masses of the precursor ion and corresponding product ions are shown. Depending on the instrument, a deviation of ± 0.3 Da is allowed. All transitions shown in Table B.2.2 are included in the MS method installed on the LC-MS/MS. The retention times can differ from column to column and between LC systems. The retention times shown in Table B.2.2 are therefore indicative.

Table B.2.2 Example MS/MS fragmentation conditions for cyanogenic glycosides

Component	Indicative RT (min)	Precursor ion (m/z)	Cone voltage (V)	Product ion 1 (m/z)	Collision energy 1 (eV)	Product ion 2 (m/z)	Collision energy 2 (eV)	Product ion 3 (m/z)	Collision energy 3 (eV)
Linamarin	3.55	265.2	20	85.0	20	163.0	10	180.0	10
Lotaustralin	5.90	279.2	20	85.0	20	163.0	10	180.0	10
Prunasin	8.10	313.2	20	85.0	20	163.0	10	180.0	10
Dhurrin (IS)	5.55	329.2	20	85.0	20	163.0	10	180.0	10
Linustatin	4.55	427.2	20	85.0	25	145.0	20	325.0	10
Neolinustatin	6.15	441.2	20	85.0	25	145.0	20	325.0	10
Amygdalin	7.90	475.2	20	85.0	25	145.0	20	325.0	10

B.3   LC-MS/MS example chromatogram of a maize flour sample extract spiked at 10 ng HCN eq./ml

STD M4 maismeel

STD M4 maismeel

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 329.2 > 180 (Dhurrin) 1.48e6 Area — 5.53 / 329 / 66650

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 329.2 > 163 (Dhurrin) 3.49e5 Area — 5.53 / 329 / 16482

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 329.2 > 85 (Dhurrin) 5.56e5 Area — 5.53 / 329 / 23954

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 313.2 > 180 (Prunasin) 5.02e5 Area — 8.09 / 313 / 17267

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 313.2 > 163 (Prunasin) 6.09e5 Area — 8.09 / 313 / 19297

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 313.2 > 85 (Prunasin) 4.56e5 Area — 8.09 / 313 / 15309

STD M4 maismeel

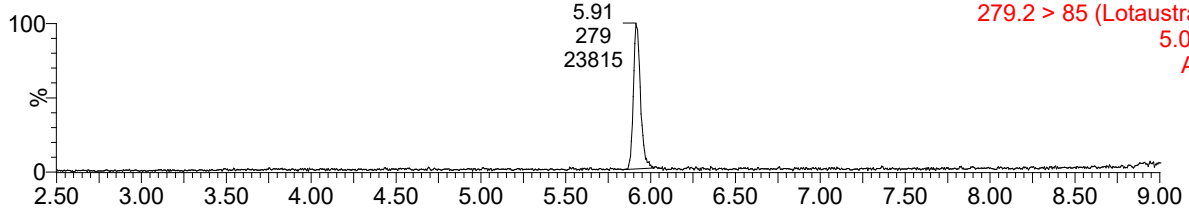

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 279.2 > 180 (Lotaustralin) 4.76e5 Area
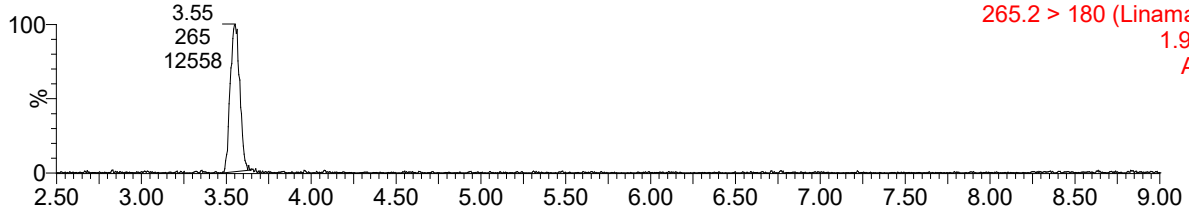5.92 / 279 / 22154

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 279.2 > 163 (Lotaustralin) 6.52e5 Area
5.92 / 279 / 30085
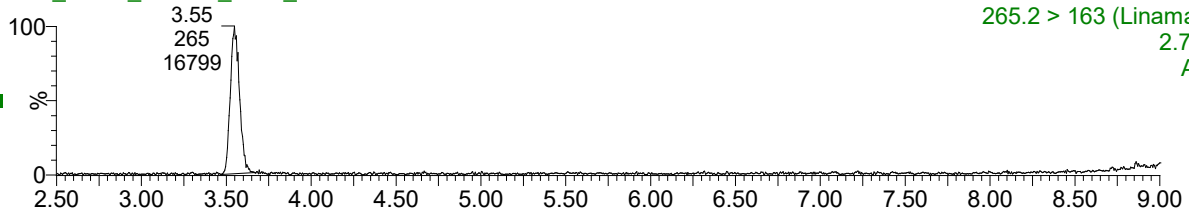
TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 279.2 > 85 (Lotaustralin) 5.06e5 Area
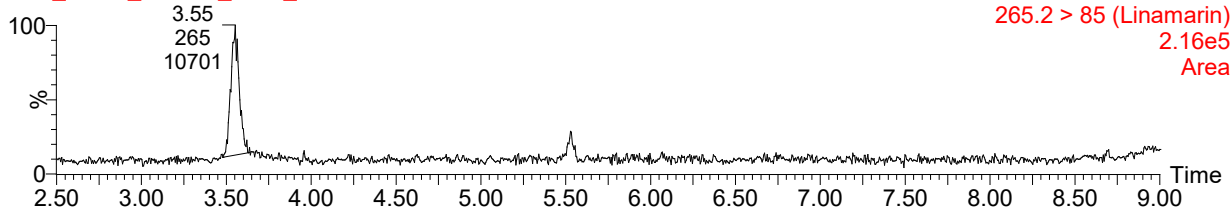5.91 / 279 / 23815

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 265.2 > 180 (Linamarin) 1.94e5 Area
3.55 / 265 / 12558

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 265.2 > 163 (Linamarin) 2.70e5 Area
3.55 / 265 / 16799

TQXS_EURL_210218_CNG_017 — MRM of 24 Channels ES+ 265.2 > 85 (Linamarin) 2.16e5 Area
3.55 / 265 / 10701