SLOVENSKI STANDARD
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Krma - Metode vzorčenja in analize - Določanje pirolizidinskih alkaloidov v krmi z LCMS/MS

Animal feeding stuffs- Methods of sampling and analysis - Determination of pyrrolizidine alkaloids in animal feeding stuff by LCMS/MS

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung von Pyrrolizidinalkaloide in Futtermitteln mittels LC-MS/MS

Aliments pour animaux : Méthodes d'échantillonnage et d'analyse — Dosage des alcaloïdes pyrrolizidiniques dans les aliments pour animaux par CL-SM/SM

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European foreword

This document (prEN 17683:2021) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

Introduction

Pyrrolizidine alkaloids (PA) are secondary metabolites of flowering plants. Ingestion of high doses results in acute liver damage. In animal studies some PA have proven to be genotoxic carcinogens. Therefore, PA are undesired substances in food and feed [1], [2]. Poisoning in animals has been reported with known outbreaks attributed to *Heliotropium*, *Trichodesma*, *Senecio*, and *Crotalaria species*. In general, grazing animals will avoid PA-bearing plants. However, if weedy crops are used for the production of hay, silage or other plant derived feed materials the animals can no longer exercise discrimination when feeding because the toxins survive storage processes and are completely intermixed with the feed. Therefore, analytical methods for the control of PA levels in animal feed are needed [1], [2].

WARNING — The use of this protocol involves hazardous materials, operations and equipment. 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 health and safety practices and determine the compatibility with regulatory limitations prior to use.

1 Scope

This document describes a method for the quantitative determination of pyrrolizidine alkaloids (PA) in complete and supplementary feed and in forages by liquid chromatography tandem mass spectrometry (LC-MS/MS) after solid phase extraction (SPE) clean-up.

The method has been successfully validated in a collaborative trial for the matrices complete feed for horses, supplementary feed for horses, supplementary feed for rodents, hay, alfalfa and grass silage. Validation was carried out for the PA and concentrations ranges listed in Table 1. It was demonstrated that the PA isomeric pairs senecivernine and senecionine as well as senecivernine-N-oxide and senecionine-N-oxide cannot be determined individually due to insufficient chromatographic separation. However, the sums of the individual PA of the isomeric pairs were quantified with sufficient reproducibility. Co-elution of other PA-isomers not included in the scope of the method shall be taken into account. A list of potentially co-eluting isomers is presented in Annex E.

Although the calibration range of the method protocol is specified from 10 µg/kg to 300 µg/kg, the results of the collaborative study showed, that the dilution of sample extracts with blank sample extracts enables for the quantitation of concentrations exceeding the calibration range. Satisfactory reproducibility was achieved when quantifying up to 1428 µg/kg for individual PA and up to 887 µg/kg for the sum of isomeric pairs.

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

Tested pyrrolizidine alkaloid (PA)	Abbreviation	Tested concentration range[a] (µg/kg)	
		From	To
Echimidine	Em	20	435
Echimidine-N-oxide	EmN	5	30
Erucifoline	Er	20	245
Erucifoline-N-oxide	ErN	20	370
Europine	Eu	15	330
Europine-N-oxide	EuN	25	285
Heliotrine	Hn	25	280
Heliotrine-N-oxide	HnN	25	245
Jacobine	Jb	20	230
Jacobine-N-oxide	JbN	20	215
Lasiocarpine	Lc	20	350
Lasiocarpine-N-oxide	LcN	5	250
Intermedine	Im	25	560
Intermedine-N-oxide	ImN	5	395
Lycopsamine	La	25	500
Lycopsamine-N-oxide	LaN	20	280
Monocrotaline	Mc	20	360
Monocrotaline-N-oxide	McN	20	365
Retrorsine	Re	250	375
Retrorsine-N-oxide	ReN	5	285
Senecionine[b]	Sc	25	205
Senecionine-N-oxide[b]	ScN	5	300
Senecivernine[b]	Sv	20	205
Senecivernine-N-oxide[b]	SvN	5	165
Senkirkine	Sk	20	275
Seneciphylline	Sp	25	225
Seneciphylline-N-oxide	SpN	5	225
Trichodesmine	Td	5	250
Intermedine + Lycopsamine	Im+La	50	890
Intermedine-N-oxide + Lycopsamine-N-	ImN+LaN	5	645
Senecivernine + Senecionine	Sv+Sc	30	280
Senecivernine-N-oxide + Senecionine-N-	SvN+ScN	10	380

[a] Rounded figures

[b] Individual PA of the isomeric pairs Sv+Sc and SvN+ScN were not evaluated statistically due to insufficient chromatographic separation

NOTE 1 A second method was part of the method validation collaborative main trial. For this method PA-N-Oxides are reduced by adding zinc powder to the extract of the feed material. The following steps correspond to the first and main method. Quantitative results for each PA except the otonecine type PA senkirkine represent the sum of the free PA base and its corresponding N-oxide.

NOTE 2 Due to insufficient numbers of data for some analyte-matrix combinations statistical evaluation was not valid for standardization. Received data indicated the methods applicability in experienced laboratories with appropriate quality assurance measures. Therefore, the method description is included as an informative annex (Annex D).

2 Normative references

There are no normative references in this document.

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

4.1 General

A test portion of 2 g feed material is sonicated twice in aqueous sulphuric acid solution for PA extraction. After centrifugation, an aliquot of the supernatant is purified by solid phase extraction (SPE) using reversed phase (C18) material. PA are released from the cartridge using methanol. Subsequently, the eluate is evaporated to dryness and reconstituted in methanol/water (initial HPLC conditions).

For chromatographic separation, an RP-HPLC column is used with a binary gradient. Analytes are detected by triple stage quadrupole mass spectrometry. Quantification of PA is accomplished by means of matrix matched calibration.

4.2 Reagents

All chemicals should at least be of pro-analysis quality or higher. References to products or vendors are just for general information and do not imply that other products or producers with the same or similar characteristics are ruled out.

If not specified elsewise, use only reagents of analytical grade and solvents suitable for HPLC-MS/MS. Water shall be distilled in glass vessels or demineralized before use, or shall be of equivalent purity.

4.3 Analytical standards

4.3.1 Senecionine (Sc)

4.3.2 Senecionine-N-oxide (ScN)

4.3.3 Seneciphylline (Sp)

4.3.4 Seneciphylline-N-oxide (SpN)

4.3.5 Monocrotaline (Mc)

4.3.6 Monocrotatline-N-oxide (McN)

4.3.7 Retrorsine (Re)

4.3.8 Heliotrine (Hn)

4.3.9 Heliotrine-N-oxide (HnN)

4.3.10 Trichodesmine (Td)

4.3.11 Retrorsine-N-oxide (ReN)

4.3.12 Echimidine (Em)

4.3.13 Echimidine-N-oxide (EmN)

4.3.14 Intermedine (Im)

4.3.15 Intermedin-N-oxide (ImN)

4.3.16 Lycopsamine (La)

4.3.17 Lycopsamine-N-oxide (LaN)

4.3.18 Erucifoline (Er)

4.3.19 Erucifoline-N-oxide (ErN)

4.3.20 Senecivernine (Sv)

4.3.21 Senecivernine-N-oxide (SvN)

4.3.22 Jacobine (Jb)

4.3.23 Jacobine-N-oxide (JbN)

4.3.24 Lasiocarpine (Lc)

4.3.25 Lasiocarpine-N-oxide (LcN)

4.3.26 Europine (Eu)

4.3.27 Europine-N-oxide (EuN)

4.3.28 Senkirkine (Sk)

4.4 Chemicals

4.4.1 Formic acid 98 – 100 %

4.4.2 Methanol (MeOH) in LC-MS quality

4.4.3 Sulphuric acid 98 %

4.4.4 Ammonia 32 %

4.4.5 Ammonium formate in LC-MS quality

4.4.6 Acetonitrile

4.5 Solutions

4.5.1 Extraction solution

Prepare a 0,05 °M solution of aqueous sulphuric acid. Make up 2,665 ml of sulphuric acid (H2SO4) (see 4.4.3) to 1 l with water. The extraction solution can be used for 1 month when stored at room temperature.

4.5.2 Aqueous ammoniacal solution (for neutralization of extracts before SPE)

Dilute ammonia 32 % (see 4.4.4) with water in a 1 to 5 ratio by volume (V:V), e.g. mix 5 ml of ammonia 32 % with 20 ml of water.

The solution shall be prepared every working day.

4.5.3 Examples of HPLC mobile phase

- Eluent A:

  Weigh 315 mg ammonium formate (see 4.4.5) and dissolve in 5 ml of water in a 1000 ml volumetric flask, add 1 ml of formic acid (see 4.4.1) and make up to 1 l with water. The solution can be used for 1 month when stored at room temperature.

- Eluent B:

  Weigh 315 mg ammonium formate (see 4.4.5) and dissolve in 5 ml of water in a 1000 ml volumetric flask, add 1 ml of formic acid (see 4.4.1) and make up to 1 l with methanol (see 4.4.2). The solution can be used for 3 months when stored at room temperature.

4.5.4 PA stock solutions

To create a stock solution, weigh 1 mg to 10 mg (depending on the amount available per unit purchased) of a pyrrolizidine alkaloid standard in a 10 ml volumetric flask using an analytical balance (see 5.22) and make up to 10 ml with a suitable organic solvent like methanol or acetonitrile. The resulting concentration of the stock solution is 0,1 mg/ml to 1°mg/ml. Stock solutions are stable for at least 2 years when stored < −18 °C.

If the available analytical balance does not provide sufficient accuracy, higher quantities shall be weighed.

NOTE Instructions of standard providers can indicate suitable solvents for the preparation of stock solutions but are not mandatory to be used.

4.5.5 Standard working solution (PA mixture), 1 µg/ml

Transfer respective volumes each PA stock solution (between 0,1 mg/ml to 10 mg/ml) into a volumetric flask and make up with acetonitrile (see 4.4.6), to achieve a concentration of 1 µg/ml for each PA contained in the mixture. Long term stability tests proved acetonitrile to be the most suitable solvent, but is not mandatory to be used. The PA mixture is stable for at least 2 years when stored < −18 °C.

If no baseline separation can be achieved for an isomeric pair (intermedine(-N-oxide) and lycopsamine (-N-oxide); senecivernine(-N-oxide) and senecionine(-N-oxide)), the sum concentration of both PA can be calculated via the calibration of one of them. The composition of the standard mix shall be prepared accordingly by including only one isomer of the pair.

5 Apparatus

Use laboratory equipment and, in particular, the following elements.

NOTE References to products, instruments or vendors are just for general information and do not imply that other products or producers with the same or similar characteristics are ruled out.

5.1 Various piston pipettes and multiple dispenser and respective tips

5.2 Analytical balance, capable of weighing to 0,1 mg

5.3 Compartment drier

5.4 Centrifugal mill with 0,5 mm sieve

5.5 Centrifuge for 50 ml centrifuge tubes, capable of at least 5 000 × g

5.6 Laboratory shaker

5.7 Overhead shaker

5.8 Evaporation station with or without vacuum support

5.9 Ultrasonic bath

5.10 Centrifuge tubes 50 ml

5.11 Test tubes 15 ml

5.12 Volumetric flasks, 10 ml and 20 ml

5.13 Folded filters

5.14 SPE cartridges: C18, 500 mg sorbent material

NOTE Supelco Discovery® DSC18 500 mg/6 ml is an example of a suitable product available commercially[1].

5.15 SPE vacuum chamber

5.16 Membrane filter 0,2 µm or 0,45 µm

Centrifugal filters should have > 0,5 ml capacity and contain modified nylon membrane. Other filtering tools can be used as well.

[1]Supelco Discovery® 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 CEN of this product.

5.17 HPLC vials 2 ml

5.18 Glass inserts, 250 µl conic for HPLC vials

5.19 Chromatographic column

C18 reversed phase packing material at acidic pH-conditions (pH 2-3), capable of baseline separation of analytes with identical molecular mass, separation of isomeric pairs is desirable.

5.20 LC-MS/MS system

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

6 Procedure

6.1 General

Animal feed is a complex matrix containing a wide range of ingredients in varying amounts leading to variable and sample dependent matrix effects (suppression/enhancement). For this reason, a quantification of the analytes shall be accomplished by matrix-matched calibration.

Comparison of matrix effects of different feed materials has shown that there might be a difference in signal suppression of around 50 % depending on the analyte-matrix combination. As usually a variety of feed materials shall be analysed in one sequence, a representative mix of blank feed materials can be used (e.g. 75 % different types of grass, like hay and silage, and legumes and 25 % cereals). If the sequence contains only samples of one specific feed material, a blank matrix as similar as possible to the sample matrix should be used for matrix-matched calibration.

Samples exceeding the calibration range can be diluted using blank sample extract. Recovery shall be checked with every series of samples proving the required range of recovery. Recovery correction is carried out if recovery is outside of 90 % - 110 % according to EURL-MP's Guidance document on performance criteria for the analysis of mycotoxins and plant toxins in food and feed [3] (under development).

6.2 Sample preparation

To determine the PA concentration that is representative for the entire sample, the sample material should meet the following characteristics: uniform particle size and a homogenous distribution. Therefore, an appropriate portion of the sample material is ground to a particle size of 0,5 mm (see 5.4) and homogenized for example using an overhead shaker (see 5.7).

Prior to grinding fresh feed samples (for example silage or grass) are dried at 40 °C for 18 h in a drying oven. Frozen samples are defrosted at room temperature before drying.

Dry samples are mixed with dry ice (ratio 2:1) and the mixture is allowed to stand for 10 minutes before grinding to a particle size of 0,5 mm using an ultra-centrifugal mill (see 5.4). The ground material is homogenized by shaking for example using an overhead shaker (see 5.7) for 1 h.

NOTE Grinding with ice has proven to obtain excellent grinding results due to shear forces and porosity of the frozen sample material.

If the test material cannot be ground to a particle size of 0,5 mm or smaller without generation of heat, increasing the weighed sample amount to at least 10 g is also possible to enhance the results representativeness for the sample. In order to keep a constant ratio of sample amount to extraction volume, the used volume of extraction solution (see 4.5.1) needs to be increased accordingly.

Laboratories shall prove that the performance of their homogenization procedure used is sufficient.

6.3 Extraction

Weigh 2,0 g ± 0,1 g of the homogenized sample into a centrifuge tube (see 5.10).

1) **Extraction step 1:** Add 20 ml of the extraction solution (see 4.5.1) to the sample and mix to wet the sample material completely before extraction by ultra-sonication (see 5.9) for 15 min at ambient temperature.

   NOTE Extraction can be accomplished by other techniques than ultra-sonication (e.g. using an over-head shaker), if sufficient extraction efficiency was shown during in-house validation.

2) **Centrifugation**: Centrifuge the tube for 10 min ± 2 min at 3800 × g (see 5.5). Transfer the supernatant (extract 1) into a clean test tube and use the sediment for the second extraction step.

3) **Extraction step 2**: Add 20 ml of extraction solution (see 4.5.1) to the sediment. Shake the centrifuge tube vigorously to distribute the sample (the sample can also be stirred if necessary) and extract again by ultra-sonication* for 15 min at ambient temperature.

4) **Centrifugation:** Centrifuge the tube for 10 min ± 2 min at 3800 × g (see 5.5) and combine the supernatant (extract 2) with extract 1.

5) **Neutralization:** Adjust the pH of the combined extracts to pH 7 using the neutralization solution (see 4.5.2). Check the pH value using indicator strips. Usually, about 500 µl to 1000 µl of the solution are required.

6) **Filtration:** Pass the complete neutralized extract through a filter (e.g. folded filters see 5.1.3) or centrifuge alternatively. Use an aliquot of the filtered supernatant for SPE. Repeat the filtration step in case of larger quantities of remaining particles in the solution. Thereby, blockage of SPE cartridges can be avoided.

6.4 SPE procedure

1) **Conditioning step 1:** Load 5 ml of methanol (see 4.4.2) onto the SPE cartridge and let it pass through under normal pressure.

2) **Conditioning step 2:** Load 5 ml of water onto the SPE cartridge and let it pass through under normal pressure.

3) **Sample load:** Load 10 ml of the sample extract (combined supernatants from Clause 6.3) onto the SPE cartridge and let it pass through without letting the cartridge run dry.

4) **Washing step:** Wash the SPE cartridge 2 × 5 ml of water.

5) **Drying of cartridges:** Dry the cartridges by applying vacuum for 5 - 10 min (use the vacuum chamber (see 5.15).

6) **Elution of PA:** Elute the analytes from the cartridges using 2 × 5 ml methanol (see 4.4.2). The eluate is dried under a nitrogen stream at 50 °C ± 5 °C, or according to the recommendations for the evaporation equipment used.

NOTE If alternative SPE cartridges are used, solvent volumes for conditioning, sample loading, washing and eluting can be adapted. Depending on the solid phase material, it might be necessary to protect the stationary from running dry until the washing step.

6.5 Reconstitution of the sample

Dissolve the residue in 1 ml of methanol/water (5/95, v/v) by shaking (see 5.7).

Filter the reconstituted sample extracts through a 0,2 to 0,45 µm membrane filter (see 5.16). When using centrifugal filters, 500 µl of the sample are centrifuged at 20 000 × g for 10 min ± 3 min. Transfer 200 µl of the filtrate into an HPLC vial (5.17) with a glass insert (see 5.18).

6.6 Quality control samples

In every sample batch, recovery as a sum of extraction efficiency, clean-up and LC-MS/MS detection shall be checked. Preferably, a reference material or well characterized positive samples should be used. If no reference material is available, recovery can be determined by spiking a representative feed material (see 6.1) that is free from PA. Spiking procedure (example):

EXAMPLE Spike 2 g of the blank feed material with PA working solution (see 4.5.5), vortex or mix by hand vigorously and leave open for 1 hour to allow the solvent to evaporate. Analyse the spiked sample alongside with the unknown samples.

NOTE Addition of 50 µl of PA working solution (see 4.5.5) were used in the method validation study and worked well.

6.7 Calibration by means of matrix matched standards (MMS)

For the correction of matrix effects, a matrix matched calibration is used. In order to obtain the same matrix strength in the MMS and the samples, blank feed shall be processed as described in Clause 6 to receive blank matrix extract. Afterwards, MMS are prepared by mixing different volumes of the standard working solution with the blank matrix extract. The volume of matrix extract should account for at least 80 % in each MMS solution. Low concentration calibration levels can be prepared by diluting higher MMS levels with blank feed extract. Table 2 shows an exemplary scheme for the preparation of MMS.

Samples higher concentrated than the highest calibration level should be diluted using blank feed extract to obtain PA levels in the calibration range.