
EURLMP-method_012 (version 1)
Determination of quinolizidine alkaloids in lupin seeds, food products and feed
by LC-MS/MS

Analyte group: Plant toxins – quinolizidine alkaloids

Analyte(s):

Albine
Anagryne
Angustifoline
trans-13 α -Cinnamoyloxylupanine
Cytisine
Epilupinine
Gramine
13 α -Hydroxylupanine
Isolupanine
Lupanine
Lupinine
Methylcytisine
Multiflorine
Sparteine
Thermopsine

Commodity group: Food and feed

Commodities validated: Lupine seeds, cereal-based food products, meat and milk imitates, milk, compound feeds

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

Modifications compared to previous version:

Not applicable

Method drafted by:

EU Reference Laboratory for mycotoxins & plant toxins in food and feed (EURL-MP)
Wageningen Food Safety research (WFSR), part of Wageningen University & Research
Akkermaalsbos 2, 6708 WB, Wageningen, the Netherlands
eurl.mycotoxins-planttoxins@wur.nl

Notices:

This method has been drafted as guidance for EU National Reference Laboratories on mycotoxins and plant toxins in food and feed. It has been produced with the utmost care. However, WFSR does not accept liability for any claims based on the contents of this document.

Any reference to specific manufacturers' products is mentioned only for the convenience of users. They do not constitute an endorsement by the EURL and do not imply exclusion of similar alternatives.

The use of this document can involve hazardous materials, operations and equipment. This document does not address safety issues associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

© 2022 Wageningen Food Safety Research, Wageningen University & Research, institute within the legal entity Wageningen Research Foundation. Reproduction is authorised provided the source is acknowledged.

Suggested Citation: EURLMP-method_012 v1, 2022, Determination of quinolizidine alkaloids in lupin seeds, food products and feed by LC-MS/MS, WFSR Wageningen University & Research.

Table of contents

1	Introduction	4
2	Scope	4
3	Principle	4
4	Reagents	4
	4.1 Analytical standards	5
	4.2 Chemicals	5
	4.3 Solutions and reagents	5
	4.4 Standard solutions	6
5	Materials & equipment	6
	5.1 Materials	7
	5.2 Equipment	7
6	Procedures	8
	6.1 Preparation of the test samples	8
	6.2 Test portion	8
	6.3 Lupin seeds, sweet and bitter (L)	8
	6.4 Cereal-based food products, meat and milk imitates and compound feeds (P)	10
	6.5 Milk (M)	11
7	LC-MS/MS analysis	13
	7.1 Injection sequence	13
8	Evaluation and calculations	14
	8.1 Verification of linearity of LC-MS/MS measurement	14
	8.2 Identification of QAs in the samples	14
	8.3 Quantification of QAs in the samples	15
	8.4 Final result	16
9	References	17
	Annex A.1 Checklist for sample preparation lupin seeds (L)	18
	Annex A.2 Checklist for sample preparation cereal-based food products, meat and milk imitates and compound feeds (P)	20
	Annex A.3 Checklist for sample preparation milk (M)	22
	Annex B Example of LC-MS/MS conditions	24
	B.1 LC conditions	24
	B.2 MS conditions	24
	B.3 LC-MS/MS example chromatograms of quinolizidine alkaloids in meat imitate fortified at 1 mg/kg	26

1 Introduction

Quinolizidine alkaloids (QAs) are substances, which mainly occur in *Lupinus* (lupin) species and other plants of the *Genisteae* tribe. More than 500 lupin species, comprising wild and domesticated species, have been described worldwide. QAs are secondary metabolites for defense against pathogens and herbivores. The level of QAs depends on genotype, presence of pathogens, environmental effects and soil characteristics. More than 170 QAs have been identified in different lupin species and the alkaloid pattern is highly variable among different species [1]. Lupin seeds are used as alternative protein sources in foods such as bakery products, imitation milk and meat products. Lupins and lupin-derived products are also used as feed materials. When lupin is fed to ruminants, quinolizidine alkaloids can transfer to the milk. Consumption of lupin with high levels of alkaloids can cause severe toxic effects in humans and for animals they act as “anti-nutritional factors”. The alkaloids can be removed by soaking the lupin beans in (salty) water, or sweet lupin varieties can be used.

Currently, no MLs are set for QAs in food and feed in the EU, but Australia has set a maximum level of 200 mg QAs/kg lupin flour, lupin kernel flour, lupin kernel meal and lupin hulls and for sparteine a maximum level of 5 mg/kg in beverages [2].

2 Scope

This method describes the quantitative determination of 15 QAs: albine, anagyrene, angustifoline, *trans*-13-cinnamoyloxylupine, cytosine, epilupinine, gramine, 13-hydroxylupanine, isolupanine, lupanine, lupinine, methylcytosine, multiflorine, sparteine and thermopsine, in lupine seeds, cereal-based food products, meat and milk imitates, milk and in compound feeds. The method is suited for the range 1 to 200 mg/kg in lupin seeds, 0.2 to 40 mg/kg in cereal-based food products, meat and milk imitates and compound feeds and 0.02 to 4 mg/l in milk. Samples with a higher QA content can be analysed after dilution of the extract.

3 Principle

The alkaloids are extracted from 2 g or ml of homogenised sample with 40 ml methanol/water 50/50, containing 1% formic acid. The mixture is shaken for 30 min on a rotary tumbler, centrifuged and a portion of the supernatant is further diluted with water and transferred to a 500 µl filter vial. The samples are analysed by injection of 2-5 µl on a reverse-phase column to separate the analytes, followed by MS/MS detection. Quantification is performed by external matrix calibration in blank extract.

4 Reagents

All chemicals should at least be of pro-analysis quality or higher. With “water” is meant water purified by a Milli-Q purification system with a minimal resistance of 18.2 MΩ/cm. References to products or producers are just for general information and do not imply that other products or producers with the same or similar characteristics are ruled out.

4.1 Analytical standards

- 4.1.1 **Albine**, as hydrochloride
- 4.1.2 **Anagyrine**, as hydrochloride
- 4.1.3 **Angustifoline**
- 4.1.4 ***trans*-13 α -Cinnamoyloxylupanine**
- 4.1.5 **Cytisine**
- 4.1.6 **Epilupinine**, as hydrochloride
- 4.1.7 **Gramine**
- 4.1.8 **13 α -Hydroxylupanine**
- 4.1.9 **D- α -Isolupanine**, as perchlorate
- 4.1.10 **D-Lupanine**, as hydrochloride
- 4.1.11 **(-)-Lupinine**
- 4.1.12 **Methylcytisine**
- 4.1.13 **Multiflorine**
- 4.1.14 **(-)-Sparteine**
- 4.1.15 **Thermopsine**

4.2 Chemicals

- 4.2.1 **Formic acid**, 98-100%
- 4.2.2 **Ammonium carbonate ((NH₄)₂CO₃)**, HPLC quality
- 4.2.3 **Methanol**, LC-MS grade
- 4.2.4 **Ammonia**, 25%

4.3 Solutions and reagents

- 4.3.1 **Extraction solvent**, methanol/water/formic acid (50:50:0.1, v/v/v)
Mix 500 ml methanol (4.2.3), 500 ml water and 1 ml formic acid (4.2.1) in a bottle of 1000 ml.
This solution is stored at room temperature and can be used for 3 months.

4.3.2 Dilution solvent, methanol/water (10:90, v/v)

Mix 100 ml methanol (4.2.3) and 900 ml water in a bottle of 1000 ml. This solution is stored at room temperature and can be used for 3 months.

4.3.3 Mobile phase A, 10 mM ammonium carbonate in water, pH 9

Dissolve 0.96 g ammonium carbonate (4.2.2) in 1000 ml water. Check the pH with a pH meter (5.1.12) and adjust, when necessary, the pH to 9.0 ± 0.1 by adding formic acid (4.2.1) or 25% ammonia (4.2.4). This solution is stored at room temperature and can be used for 1 month.

4.3.4 Mobile phase B, methanol (4.2.3)

4.4 Standard solutions

4.4.1 Stock solutions (200 µg/ml)

Accurately weigh into separate amber coloured glass bottles (5.1.2) between 5 and 6 mg \pm 0.02 mg (5.1.8) of the QAs (4.1.1 to 4.1.15). However, when the standard is only available in a quantity of 3 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 (4.2.3) to dissolve and collect all material. Add a volume of methanol (4.2.3) in such a way that the concentration of the solution is 200 µg/ml. 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.

4.4.2 Mixed standard solution MSS1 (5 µg/ml)

Pipette 500 µl of each of the stock solutions of 200 µg/ml (4.4.1) of albine, anagryrine, angustifoline, *trans*-13-cinnamoyloxylupine, cytisine, epilupinine, gramine, 13-hydroxylupanine, isolupanine, lupanine, lupinine, methylcytisine, multiflorine, sparteine and thermopsine in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at -20°C.

4.4.3 Mixed standard solution MSS2 (500 ng/ml)

Pipette 2 ml of mixed standard solution MSS1 of 5 µg/ml (4.4.2) in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at -20°C.

4.4.4 Mixed standard solution MSS3 (50 ng/ml)

Pipette 2 ml of mixed standard solution MSS2 of 500 ng/ml (4.4.3) in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at -20°C.

4.4.5 Working standard solution (10 ng/ml)

Pipette 20 µl of the mixed standard solution 500 ng/ml (4.4.3) in a vial and add 980 µl dilution solvent (4.3.2) and mix. Prepare a fresh solution every new day of analysis.

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 **Centrifuge tubes**, 12 and 50 ml, polypropylene, with screw cap
- 5.1.2 **Amber coloured glass bottle**, 20, 30, 40 or 60 ml, with screw cap
- 5.1.3 **PTFE Filter vial**, mini-UniPrep, 0.45 µm
- 5.1.4 **Compressor for filter vials**
- 5.1.5 **Various pipettes** – use positive displacement pipettes for solutions prepared in methanol
- 5.1.6 **Volumetric flasks**, calibrated
- 5.1.7 **Analytical balance**, accuracy: 0.02 mg
- 5.1.8 **Laboratory balance**, accuracy: 0.01 g
- 5.1.9 **Mechanical vertical or horizontal shaker or rotary tumbling machine**, adjustable
- 5.1.10 **Laboratory shaker** (Vortex mixer)
- 5.1.11 **Centrifuge**, suitable for 12 and 50 ml centrifuge tubes
- 5.1.12 **pH meter**

5.2 Equipment

- 5.2.1 **LC-MS/MS system** with the following components:
- 5.2.2 **LC pump**, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.
- 5.2.3 **Injection system**, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0.1%.
- 5.2.4 **Analytical column**, capable of retaining the target QAs and capable of baseline separation of compounds with the same molecular mass.
- 5.2.5 **Column oven**, capable of maintaining a constant temperature of 50 °C.
- 5.2.6 **Tandem mass spectrometer (MS/MS)**, capable of ionisation of the compounds in positive mode, performing Multiple Reaction Monitoring (MRM), with a sufficiently wide dynamic range and capable of unit mass separation, equipped with a computer-based data processing system. Any ionisation source giving sufficient yield may be employed.

6 Procedures

This document describes the determination of quinolizidine alkaloids in food (lupine seeds, cereal-based food products, meat and milk imitates, milk) and feed (lupine seeds, compound feeds) on the basis of external matrix matched calibration in blank extract.

In Annex A.1-3, the steps described in section 6.3.1 to 6.3.4 are shown in the format of checklists.

6.1 Preparation of the test samples

Samples are ground through a sieve of 1 mm or smaller. Samples with a water content of more than 15% are stored at -20 °C. Frozen samples are left to thaw overnight at 4-6 °C or on the day of analysis in a water bath of 40 °C.

6.2 Test portion

For lupin seeds, food products and compound feeds the amount of homogenised test sample examined is 2.0 ± 0.05 g. For liquid food products the amount of homogenised test sample examined is 2.0 ± 0.05 ml.

6.3 Lupin seeds, sweet and bitter (L)

6.3.1 Matrix matched calibration standards MMC(L)1-9

The calibration standards are prepared by addition of mixed standard solutions to the blank sample extract according to Table 1. Use as a blank material soy or maize flour, which are devoid of the QAs analysed in this method.

Prepare a diluted blank extract by weighing a test portion of 2 g ground blank material in a PP tube of 50 ml (5.1.1). Add 40 ml of extraction solvent (4.3.1), shake vigorously and extract for 30 minutes on a rotary tumbling machine (5.1.9). Centrifuge the sample for 15 minutes at 3500 rpm (5.1.11). Transfer 1 ml of the supernatant to a PP tube of 50 ml (5.1.1). Add 49 ml dilution solvent (4.3.2) and mix. Prepare calibration standards in 500 µl filter vials (5.1.3) using diluted blank extract and mixed standard solutions MSS1 (4.4.2), MSS2 (4.4.3) and MSS3 (4.4.4) according to Table 1. Mix and close the vials with the help of a compressor (5.1.4).

Table 1: Preparation of matrix-matched calibration standards for lupin seeds, sweet and bitter (MMC(L))

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Mixed standard solution MSS3 50 ng/ml (4.4.4) (µl)	Mixed standard solution MSS2 500 ng/ml (4.4.3) (µl)	Mixed standard solution MSS1 5 µg/ml (4.4.2) (µl)	Diluted blank extract (6.3.1) (µl)
MMC(L)1	0	0	-	-	-	500
MMC(L)2	1	1	10	-	-	490
MMC(L)3	2.5	2.5	25	-	-	475
MMC(L)4	5	5	50	-	-	450
MMC(L)5	10	10	-	10	-	490
MMC(L)6	25	25	-	25	-	475
MMC(L)7	50	50	-	50	-	450
MMC(L)8	100	100	-	-	10	490
MMC(L)9	200	200	-	-	20	480

6.3.2 Quality control samples for lupin seeds QC(L)

6.3.2.1 Quality control sample recovery, QC_{Rec(L)} (QC 10 mg/kg)

Weigh 0.5 ± 0.05 g of the blank material of 6.3.1 into a 12 ml PP tube (5.1.1). Add 1,000 μ l of mixed standard solution MSS 1 of 5 μ g/ml (4.4.2) and mix. Wait 30 minutes before starting the extraction procedure. Add 9 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 min at 3500 rpm (5.1.11). Transfer 100 μ l of the supernatant to a 12 ml PP tube (5.1.1). Add 4.9 ml dilution solvent (4.3.2) and mix. Transfer 500 μ l of the diluted sample extract to a filter vial (5.1.3). Mix and close the vial with the help of a compressor (5.1.4).

6.3.2.2 Quality control sample limit of quantification, QC_{LoQ(L)} (QC 1 mg/kg)

Weigh 0.5 ± 0.05 g of the blank material of 6.3.1 into a 12 ml PP tube (5.1.1). Add 100 μ l of mixed standard solution MSS1 of 5 μ g/ml (4.4.2) and mix. Wait 30 minutes before starting the extraction procedure. Add 10 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (2.1.9) and centrifuge for 15 min at 3500 rpm (5.1.11). Transfer 100 μ l of the supernatant to 12 ml PP tube (5.1.1). Add 4.9 ml dilution solvent (4.3.2) and mix. Transfer 500 μ l of the diluted sample extract to a filter vial (5.1.3). Mix and close the vial with the help of a compressor (5.1.4).

6.3.3 Extraction and sample preparation procedure for lupin seeds, sweet and bitter (L)

Weigh a test portion of 2.0 ± 0.05 g in a tube of 50 ml (5.1.1). Add 40 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 minutes at 3500 rpm (5.1.11). Transfer 100 μ l of the supernatant to a 12 ml PP tube (5.1.1). Add 4.9 ml dilution solvent (4.3.2) and mix. Transfer 500 μ l of the diluted sample extract to a filter vial (5.1.3). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:1,000 and the dilution factor is 1.

6.3.4 Analysis of lupin seeds, sweet and bitter (L) with high QA concentrations

Diluted sample extracts of 6.3.3 that contain one or more QAs exceeding the range of the calibration curve are diluted with blank soy extract and reanalysed.

- When the estimated concentration in the sample is in the range of 200 to 1,000 mg/kg, the extract is diluted 5 times. Transfer 100 μ l of the diluted sample extract of 6.3.3 to a filter vial (5.1.3) and add 400 μ l diluted blank extract (6.3.1). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:5,000 (dilution factor = 5).
- When the estimated concentration in the sample is in the range of 1,000 to 5,000 mg/kg, the extract is diluted 25 times. Transfer 20 μ l of the diluted sample extract of 6.3.3 to a filter vial (5.1.3) and add 480 μ l diluted blank extract (6.3.1). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:25,000 (dilution factor = 25).
- When the estimated concentration in the sample is in the range of 5,000 to 20,000 mg/kg, the extract is diluted 100 times. Transfer 5 μ l of the diluted sample extract of 6.3.3 to a filter vial (5.1.3) and add 495 μ l diluted blank extract (6.3.1). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:100,000 (dilution factor = 100).

6.4 Cereal-based food products, meat and milk imitates and compound feeds (P)

6.4.1 Matrix matched calibration standards MMC(P)1-9

The calibration standards are prepared by addition of standard solution to the blank sample extract according to Table 2. Use as a blank material a bakery product, pasta or meat imitate product, soy milk or a compound feed, in which according to a previous analysis, no QAs were detected. The blank sample should be representative for the samples to be analysed.

Prepare a diluted blank extract by weighing a test portion of 2 g ground blank material in a PP tube of 50 ml (5.1.1). Add 40 ml of extraction solvent (4.3.1), shake vigorously and extract for 30 minutes on a rotary tumbling machine (5.1.9). Centrifuge the sample for 15 min at 3500 rpm (5.1.11). Transfer 1 ml of the supernatant to a 12 ml PP tube (5.1.1), add 9 ml dilution solvent (4.3.2) and mix. Prepare calibration standards in 500 µl filter vials (5.1.3) using diluted blank extract and mixed standard solutions MSS1 (4.4.2), MSS2 (4.4.3) and MSS3 (4.4.4) according to Table 2. Mix and close the vials with the help of a compressor (5.1.4).

Table 2: Preparation of matrix-matched calibration standards for quantification for cereal-based food products, meat and milk imitates and compound feeds (MMC(P))

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Mixed standard solution MSS3 (4.4.4) (µl)	Mixed standard solution MSS2 (4.4.3) (µl)	Mixed standard solution MSS1 (4.4.2) (µl)	Diluted blank sample extract (6.4.1) (µl)
MMC(P)1	0	0	-	-	-	500
MMC(P)2	1	0.2	10	-	-	490
MMC(P)3	2.5	0.5	25	-	-	475
MMC(P)4	5	1	50	-	-	450
MMC(P)5	10	2	-	10	-	490
MMC(P)6	25	5	-	25	-	475
MMC(P)7	50	10	-	50	-	450
MMC(P)8	100	20	-	-	10	490
MMC(P)9	200	40	-	-	20	480

6.4.2 Quality control samples for cereal-based food products, meat and milk imitates and compound feeds QC(P)

6.4.2.2 Quality control sample recovery, QC_{Rec(P)} (QC 2 mg/kg)

Weigh 1.0 ± 0.05 g of blank sample of 6.4.1 into a 50 ml PP tube (5.1.1). Add 400 µl of mixed standard solution MSS1 of 5 µg/ml (4.4.2) and mix. Wait 30 minutes before starting the extraction procedure. Add 20 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 min at 3500 rpm (5.1.11). Transfer 500 µl of the supernatant to a 12 ml PP tube (5.1.1), add 4.5 ml dilution solvent (4.3.2) and mix. Transfer 500 µl of the diluted sample extract to a filter vial (5.1.3). Mix and close the vial with the help of a compressor (5.1.4).

6.4.2.2 Quality control sample limit of quantification, $QC_{LOQ(P)}$ (QC 0.2 mg/kg)

Weigh 1.0 ± 0.05 g of blank sample of 6.4.1 into a 50 ml PP tube (5.1.1). Add 40 μ l of mixed standard solution MSS1 of 5 μ g/ml (4.4.2) and mix. Wait 30 minutes before starting the extraction procedure. Add 20 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 min at 3500 rpm (5.1.11). Transfer 500 μ l of the supernatant to a 12 ml PP tube (5.1.1), add 4.5 ml dilution solvent (4.3.2) and mix. Transfer 500 μ l of the of the diluted sample extract to a filter vial (5.1.3). Mix and close the vial with the help of a compressor (5.1.4).

6.4.3 Extraction and sample preparation procedure for cereal-based food products, meat and milk imitates and compound feeds (P)

Weigh a test portion of 2.0 ± 0.05 g in a tube of 50 ml (5.1.1). Add 40 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 minutes at 3500 rpm (5.1.11). Transfer 500 μ l of the supernatant to a 12 ml PP tube (5.1.1), add 4.5 ml dilution solvent (4.3.2) and mix. Transfer 500 μ l of the diluted sample extract to a filter vial (5.1.3). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:200 and the dilution factor is 1.

6.4.4 Analysis of samples cereal-based food products, meat and milk imitates and compound feeds (P) with high QA concentrations

Diluted sample extracts that contain one or more QAs exceeding the range of the calibration curve are diluted with diluted blank extract and reanalysed.

- When the estimated concentration in the sample is in the range of 40 to 200 mg/kg, the extract is diluted 5 times. Transfer 100 μ l of the diluted sample extract of 6.4.3 to a filter vial (5.1.3) and add 400 μ l diluted blank extract (6.4.1). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:1,000 (dilution factor = 5).
- When the estimated concentration in the sample is in the range of 200 to 1,000 mg/kg, the extract is diluted 25 times. Transfer 20 μ l of the diluted sample extract of 6.4.3 to a filter vial (5.1.3) and add 480 μ l diluted blank extract (6.4.1). Mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:5,000 (dilution factor = 25).

6.5 Milk (M)

6.5.1 Matrix matched calibration standards MMC(M)1-9

The calibration standards are prepared by addition of standard solution to the blank sample extract according to Table 3. Use as a blank material a cow's milk sample, in which according to a previous analysis no QAs were detected.

Prepare a diluted blank extract by weighing a test portion of 2 ml blank milk in a PP tube of 50 ml (5.1.1). Add 18 ml of extraction solvent (4.3.1), shake vigorously and extract for 30 mins on a rotary tumbling machine (5.1.9). Centrifuge the sample for 15 min at 3500 rpm (5.1.11) (see note 1). Transfer 5 ml of the supernatant to a 12 ml PP tube (5.1.1), add 5 ml water and mix. Prepare calibration standards in 500 μ l filter vials (5.1.3) using diluted blank extract and mixed standard solutions MSS1 (4.4.2), MSS2 (4.4.3) and MSS3 (4.4.4) according to Table 3. Mix and close the vials with the help of a compressor (5.1.4).

Note 1: supernatants that remain turbid after centrifugation can be prefiltered through a 5 μ m membrane filter.

Table 3: Preparation of matrix-matched calibration standards for milk (MMC(M))

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/l)	Mixed standard solution MMS3 50 ng/ml (4.4.4) (μ l)	Mixed standard solution MMS2 500 ng/ml (4.4.3) (μ l)	Mixed standard solution MMS1 5 μ g/ml (4.4.2) (μ l)	Diluted sample extract (6.5.1) (μ l)
MMC(M)1	0	0	-	-	-	500
MMC(M)2	1	0.02	10	-	-	490
MMC(M)3	2.5	0.05	25	-	-	475
MMC(M)4	5	0.1	50	-	-	450
MMC(M)5	10	0.2	-	10	-	490
MMC(M)6	25	0.5	-	25	-	475
MMC(M)7	50	1	-	50	-	450
MMC(M)8	100	2	-	-	10	490
MMC(M)9	200	4	-	-	20	480

6.5.2 Quality control samples for milk QC(M)

6.5.2.1 Quality control sample recovery, $QC_{Rec(M)}$ (QC 1 mg/l)

Weigh 2 ml of blank sample of 6.5.1 into a 50 ml PP tube (5.1.1). Add 400 μ l of mixed standard solution MSS1 of 5 μ g/ml (4.4.2) and mix. Wait 30 minutes before starting the extraction procedure. Add 18 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 min at 3500 rpm (5.1.11) (see note 2). Transfer 250 μ l of the supernatant to a filter vial (5.1.3) and add 250 μ l water. Mix and close the vial with the help of a compressor (5.1.4).

Note 2: supernatants that remain turbid after centrifugation can be prefiltered (1 ml aliquot) through a 5 μ m membrane filter.

6.5.2.2 Quality control sample limit of quantification, $QC_{LOQ(M)}$ (QC 0.02 mg/l)

Weigh 2 ml of blank sample of 6.5.1 into a 50 ml PP tube (5.1.1). Add 80 μ l of mixed standard solution MSS2 of 500 ng/ml (4.4.3) and mix. Wait 30 minutes before starting the extraction procedure. Add 18 ml of extraction solvent (4.3.1) and shake vigorously. Extract for 30 minutes on a rotary tumbling machine (5.1.9) and centrifuge for 15 min at 3500 rpm (5.1.11) (see note 3). Transfer 250 μ l of the supernatant to a filter vial (5.1.3) and add 250 μ l water. Mix and close the vial with the help of a compressor (5.1.4).

Note 3: supernatants that remain turbid after centrifugation can be prefiltered (1 ml aliquot) through a 5 μ m membrane filter.

6.5.3 Extraction and sample preparation procedure for milk (M)

Weigh a test portion of 2 ml in a PP tube of 50 ml (5.1.1). Add 18 ml of extraction solvent (4.3.1) to the test portion, shake vigorously and extract for 30 mins on a rotary tumbling machine (5.1.9). Centrifuge the sample for 15 min at 3500 rpm (5.1.11) (see note 4). Transfer 250 μ l of the supernatant to a filter vial (5.1.3). Add 250 μ l water, mix and close the vial with the help of a compressor (5.1.4). The final sample to solvent ratio is 1:20 and the dilution factor is 1.

Note 4: supernatants that remain turbid after centrifugation can be prefiltered (1 ml aliquot) through a 5 µm membrane filter.

7 LC-MS/MS analysis

Chromatographic and mass spectrometric conditions may be chosen freely. The optimal measurement conditions strongly depend on the instrumentation used.

The chosen column dimensions and chromatographic conditions should be appropriate to obtain base line separation of isomers (i.e. lupinine and epilupinine; lupanine and isolupanine; anagyrine and thermopsine). For chromatographic separation mobile phases may be used over the range pH 2 to 12. Please note that an analytical column containing high pH-resistant cross-linked C18 reversed phase packing material is required for use with a mobile phase of pH higher than 7. It should also be noted that the retention times and separation of isomeric compounds is strongly influenced by the pH chosen. Optimal retention is obtained at pH ≥ 6 .

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. Preferably, product ions that are formed by the loss of water from the protonated molecular parent ion should not be selected. Select at least two precursor-to-product ion combinations to be included in the multiple reaction monitoring (MRM) method. Each chromatographic peak should be composed of at least 10 data points.

Check the system performance as well as the retention time for analysis of the samples using the working standard solution (WS) of 10 ng/ml (4.4.5). The analytical series should not be started before it is verified, by injection of the WS at least three times, that the system produces stable analyte retention times and that the sensitivity of the detector is sufficient and stable. The system should be able to detect the product ion with the lowest intensity with a S/N ratio of 25 or more for the analytes. the sensitivity is checked for the most critical component in each window. In case that matrix effects are expected, matrix matched calibration standard MMC5 of 10 ng/ml can be used to check the sensitivity of the instrument. Check for carry-over effects, by injecting the highest calibration standard, followed by a solvent injection. If the target compounds are present in the solvent injection, this may lead to a false-positive result in the samples. 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.

- Working standard solution (4.4.5) and/or MMC5, at least 3 times
- Extraction solvent (4.3.1)
- Calibration standards MMC(L)1-9 (6.3.1), MMC(P)1-9 (6.4.1) or MMC(M)1-9 (6.5.1)
- Extraction solvent (4.3.1)
- Quality control sample limit of quantification $QC_{LOQ(L)}$ (6.3.2.2), $QC_{LOQ(P)}$ (6.4.2.2) or $QC_{LOQ(M)}$ (6.5.2.2)
- Quality control sample recovery $QC_{Rec(L)}$ (6.3.2.1), $QC_{Rec(P)}$ (6.4.2.1) or $QC_{Rec(M)}$ (6.5.2.1)
- Extraction solvent (4.3.1)
- Sample extracts 6.3.3 (L), 6.4.3 (P) or 6.5.3 (M) (or diluted sample extracts 6.3.4 (L) or 6.4.4 (P))
- Extraction solvent (4.3.1)
- Calibration standards MMC(L)1-9 (6.3.1), MMC(P)1-9 (6.4.1) or MMC(M)1-9 (6.5.1)

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 standards (MMC(L)1-9, MMC(P)1-9 and MMC(M)1-9, see Table 1, Table 2 and Table 3) are used to determine the linearity of the LC-MS/MS system. For the MMC series the sum of the peak areas is plotted as function of the (theoretically) added concentration to the sample (mg/kg). Apply linear regression using the least squares method or apply weighted calibration (see note 5). The correlation coefficient of the line should be ≥ 0.990 . The deviation of the back calculated concentrations of the calibration standards from the true concentrations, using the calibration equation, should not exceed 20%.

Note 5: When linear regression is used it may be advised to construct for each MMC series two calibration curves, one using MMC 1 to 7 (low range calibration curve) and one using MMC 1 to 9 (full range calibration curve). Analytes for which the peak area falls within the low range MMC curve are calculated with the low range MMC curve. Analytes for which the peak area falls outside the short range MMC curve but inside the full range MMC curve are calculated with the full range MMC curve. Analytes for which the peak area falls outside the full range MMC curve are diluted and reanalysed.

8.2 Identification of QAs in the samples

Identify quinolizidine alkaloids in the samples by comparing retention time and ion ratio with that of the QAs in the matrix matched calibration standards (MMC) according to SANTE/11312/2021 [3].

QAs are considered present and identified when:

- the retention time (RT) of the peak observed for the analyte in the sample extract differs not more than 0.2 min from the average retention time as calculated (**Equation I**) from the matrix matched calibration standards MMC(L)2-9 (6.3.1), MMC(P)2-9 (6.4.1) or MMC(M)2-9 (6.5.1);
- the relative deviation of the ion ratio (D) is less than 30%. Compare the ion ratio (IR) of the analyte in the sample extract (IR_{sample}) with the average ion ratio (IR_{avg}) calculated from the matrix matched calibration standards MMC(L)2-9 (6.3.1), MMC(P)2-9 (6.4.1) or MMC(M)2-9 (6.5.1) by using **Equation II** and **Equation III**;
- in the matrix matched calibration standard MMC(L)1 (6.3.1), MMC(P)1 (6.4.1) or MMC(M)1 (6.5.1), no peak is present that exceeds 30% of the LOQ.

Equation I: Deviation of the retention time (ΔRT)

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

where:

- ΔRT is the deviation of the retention time of the analyte in the sample extract, compared to the average retention time of the analyte in the MMC (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 present in the MMC (min)

Equation II: 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 MMC (%)
 IR_{sample} is the ion ratio of the analyte in the sample (%)
 IR_{avg} is the average ion ratio of the analyte in the MMC (%) (see note 6)

Note 6: 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 of the mass detector from calculation of the reference ion ratio.

8.3 Quantification of QAs in the samples

8.3.1 Recovery

Calculate the recovery of the QAs for lupin seeds with **Equation IV**, for cereal-based food products, meat and milk imitates and compound feeds with **Equation V** and for milk with **Equation VI**. The recovery should be between 70 and 120%.

Equation IV: Calculation of the recovery for lupin seeds (L)

$$R_{QC_{Rec(L)}} = \left(\frac{A_{QC_{Rec(L)}}}{A_{MMC(L)5}} \right) \times 100\%$$

where:

- $R_{QC_{Rec(L)}}$ is the recovery in sample $QC_{Rec(L)}$ of 10 mg/kg (%) (6.3.2.1)
 $A_{QC_{Rec(L)}}$ is the sum of the area of the product ions from the analyte in recovery sample $QC_{Rec(L)}$ QC 10 mg/kg (6.3.2.1)
 $A_{MMC(L)5}$ is the sum of the area of the product ions from the analyte in MMC(L)5 of 10 mg/kg (6.3.1)

Equation V: Calculation of the recovery for cereal-based food products, meat or milk imitates and compound feeds (P)

$$R_{\text{QCRec(P)}} = \left(\frac{A_{\text{QCRec(P)}}}{A_{\text{MMC(P)5}}} \right) \times 100\%$$

where:

$R_{\text{QCRec(P)}}$ is the recovery in recovery sample $\text{QC}_{\text{Rec(P)}}$ of 2 mg/kg (%) (6.4.2.1)

$A_{\text{QCRec(P)}}$ is the sum of the area of the product ions from the analyte in recovery sample $\text{QC}_{\text{Rec(P)}}$ of 2 mg/kg (6.4.2.1)

$A_{\text{MMC(P)5}}$ is the sum of the area of the product ions from the analyte in MMC(P)5 of 2 mg/kg (6.4.1)

Equation VI: Calculation of the recovery for milk (M)

$$R_{\text{QCRec(M)}} = \left(\frac{A_{\text{QCRec(M)}}}{A_{\text{MMC(M)7}}} \right) \times 100\%$$

where:

$R_{\text{QCRec(M)}}$ is the recovery in sample $\text{QC}_{\text{Rec(M)}}$ of 1 mg/kg (%) (6.5.2.1)

$A_{\text{QCRec(M)}}$ is the sum of the area of the product ions from the analyte in sample $\text{QC}_{\text{Rec(M)}}$ of 1 mg/kg (6.5.2.1)

$A_{\text{MMC(M)7}}$ is the sum of the area of the product ions from the analyte in MMC(M)7 of 1 mg/kg (6.5.1)

8.3.2 Quantification

Quantification of the analytes is based on comparison with the corresponding MMC curves. Calculate the concentration (C) of the analyte in the sample according to **Equation VII** (see note 7 and 8).

Equation VII: Concentration in the sample (C)

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

where:

C_{sample} is the concentration of the analyte in the sample (mg/kg)

A_{sample} is the sum area of product ions of the analyte in the sample

b is the intercept of the MMC

a is the slope of the MMC

DF is the dilution factor of the extract

R is the recovery of the QC sample obtained from Equation IV, V or VI (see note 8)

Note 7: optionally, the area of the quantifier ion only may be used in the calculations of analyte concentrations.

Note 8: when the recovery is between 90 and 110%, no correction for recovery is made.

8.4 Final result

The concentrations of QAs in food and feed samples are expressed as mg/kg.

9 References

- [1] European Food Safety Authority. Scientific opinion on the risks for animal and human health related to the presence of quinolizidine alkaloids in feed and food, in particular in lupins and lupin-derived products. EFSA Journal 2019; 17(11), 5860
- [2] Australia_New_Zealand_Food_Standards, Australia New Zealand Food Standards Code – Schedule 19 – Maximum levels of contaminants and natural toxicants - F2017C00333. COMLAW <https://www.legislation.gov.au/Details/F2017C00333>, 2017: p. 1-7.
- [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

Annex A.1 Checklist for sample preparation lupin seeds (L)

Technician:

Date:

Lab. Journal / page:

A.1 Checklist for sample preparation lupin seeds, sweet and bitter (L)

A.1.1 Extraction and sample preparation procedure (L)

Blank (6.3.1)	QC _{Rec(L)} 10 mg/kg (6.3.2.1)	QC _{Loq(L)} 1 mg/kg (6.3.2.2)	Samples (6.3.3)	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Weigh 2.0 ± 0.05 g of blank material in a tube of 50 ml Weigh 0.5 ± 0.05 g of blank material in a tube of 50 ml Weigh 2.0 ± 0.05 g of (test) sample in a tube of 50 ml
	<input type="checkbox"/>			Add 1 ml of mixed standard solution MSS1 of 5 µg/ml (4.4.2) and mix
		<input type="checkbox"/>		Add 100 µl of mixed standard solution MSS1 of 5 µg/ml (4.4.2) and mix
	<input type="checkbox"/>	<input type="checkbox"/>		Wait 30 min
	<input type="checkbox"/>	<input type="checkbox"/>		Add 10 ml of extraction solvent (4.3.1) and shake vigorously
	<input type="checkbox"/>			Add 9 ml of extraction solvent (4.3.1) and shake vigorously
<input type="checkbox"/>			<input type="checkbox"/>	Add 40 ml of extraction solvent (4.3.1) and shake vigorously
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Extract for 30 minutes on a rotary tumbling machine
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Centrifuge for 15 min at 3500 rpm
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 100 µl of the supernatant to a 12 ml PP tube, add 4.9 ml dilution solvent (4.3.2) and mix
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 500 µl to a filter vial, close and mix
			<input type="checkbox"/>	If content of one or more QAs > 200 mg/kg dilute according to section A.1.2
<input type="checkbox"/>				Transfer 1 ml of the supernatant to a 50 ml tube, add 49 ml dilution solvent (4.3.2) and mix
<input type="checkbox"/>				Prepare the matrix matched calibration standards MMC(L)1-9 (6.3.1) in filter vials according to Table A.1

A.1 Checklist for sample preparation lupin seeds, sweet and bitter (L)

Table A.1 Preparation of matrix-matched calibration standards (MMC(L)) for lupin seeds

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Mixed standard solution MSS3 50 ng/ml (4.4.4) (μ l)	Mixed standard solution MSS2 500 ng/ml (4.4.3) (μ l)	Mixed standard solution MSS1 5 μ g/ml (4.4.2) (μ l)	Diluted blank extract (6.3.1) (μ l)
MMC(L)1	0	0	-	-	-	500
MMC(L)2	1	1	10	-	-	490
MMC(L)3	2.5	2.5	25	-	-	475
MMC(L)4	5	5	50	-	-	450
MMC(L)5	10	10	-	10	-	490
MMC(L)6	25	25	-	25	-	475
MMC(L)7	50	50	-	50	-	450
MMC(L)8	100	100	-	-	10	490
MMC(L)9	200	200	-	-	20	480

A.1.2 QA concentration in lupin seeds >200 mg/kg

Estimated content		
200 – 1,000 mg/kg	<input type="checkbox"/>	Transfer 100 μ l of diluted sample extract (6.3.3) to a filter vial and add 400 μ l diluted blank extract (6.3.1) (dilution factor = 5)
1,000 – 5,000 mg/kg	<input type="checkbox"/>	Transfer 20 μ l of diluted sample extract (6.3.3) to a filter vial and add 480 μ l diluted blank extract (6.3.1) (dilution factor = 25)
5,000 – 20,000 mg/kg	<input type="checkbox"/>	Transfer 5 μ l of diluted sample extract (6.3.3) to a filter vial and add 495 μ l diluted blank extract (6.3.1) (dilution factor = 100)

Annex A.2 Checklist for sample preparation cereal-based food products, meat and milk imitates and compound feeds (P)

Technician: _____

Date: _____

Lab. Journal / page: _____

A.2 Checklist for sample preparation cereal-based food products, meat and milk imitates and compound feeds (P)

A.2.1 Extraction and sample preparation procedure (P)

Blank (6.4.1)	QC _{Rec(P)} 2 mg/kg (6.4.2.1)	QC _{LoQ(P)} 0.2 mg/kg (6.4.2.2)	Samples (6.4.3)	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Weigh 2 ± 0.05 g of blank material in a tube of 50 ml
	<input type="checkbox"/>		<input type="checkbox"/>	Weigh 1 ± 0.05 g of blank material in a tube of 50 ml
			<input type="checkbox"/>	Weigh 2 ± 0.05 g of sample material in a tube of 50 ml
	<input type="checkbox"/>			Add 400 µl of mixed standard solution MSS1 of 5 µg/ml (4.4.2) and mix
		<input type="checkbox"/>		Add 40 µl of mixed standard solution MSS1 of 5 µg/ml (4.4.2) and mix
	<input type="checkbox"/>	<input type="checkbox"/>		Wait 30 min
	<input type="checkbox"/>	<input type="checkbox"/>		Add 20 ml of extraction solvent (4.3.1) and shake vigorously
<input type="checkbox"/>			<input type="checkbox"/>	Add 40 ml of extraction solvent (4.3.1) and shake vigorously
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Extract for 30 minutes on a rotary tumbling machine
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Centrifuge for 15 min at 3500 rpm
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 500 µl of the supernatant to a 12 ml PP tube, add 4.5 ml dilution solvent (4.3.2) and mix
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 500 µl to a filter vial, close and mix
			<input type="checkbox"/>	If content of one or more QAs > 40 mg/kg dilute according to section A.2.2
<input type="checkbox"/>				Transfer 1 ml of the supernatant to a 12 ml tube, add 9 ml dilution solvent (4.3.2) and mix
<input type="checkbox"/>				Prepare the matrix matched calibration standards MMC(P)1-9 in filter vials according to Table A.2

A.2 Checklist for sample preparation cereal-based food products, meat and milk imitates and compound feeds (P)

Table A.2 Preparation of matrix-matched calibration standards (MMC(P)) for quantification for cereal-based food products, meat and milk imitates, and compound feeds

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Mixed standard solution MSS3 50 ng/ml (4.4.4) (μ l)	Mixed standard solution MSS2 500 ng/ml (4.4.3) (μ l)	Mixed standard solution MSS1 5 μ g/ml (4.4.2) (μ l)	Diluted blank sample extract (6.4.1) (μ l)
MMC(P)1	0	0	-	-	-	500
MMC(P)2	1	0.2	10	-	-	490
MMC(P)3	2.5	0.5	25	-	-	475
MMC(P)4	5	1	50	-	-	450
MMC(P)5	10	2	-	10	-	490
MMC(P)6	25	5	-	25	-	475
MMC(P)7	50	10	-	50	-	450
MMC(P)8	100	20	-	-	10	490
MMC(P)9	200	40	-	-	20	480

A.2.2 QA concentration in samples >40 mg/kg

Estimated content		
40 – 200 mg/kg	<input type="checkbox"/>	Transfer 100 μ L of diluted sample extract (6.4.3) to a filter vial and add 400 μ l diluted blank extract (6.4.1) (dilution factor = 5)
200 – 1,000 mg/kg	<input type="checkbox"/>	Transfer 20 μ l of diluted sample extract (6.4.3) to a filter vial and add 480 μ l diluted blank extract (6.4.1) (dilution factor = 25)

Annex A.3 Checklist for sample preparation milk (M)

Technician: _____

Date: _____

Lab. journal / page: _____

Annex A.3 Checklist for sample preparation milk (M)

A.3 Extraction and sample preparation procedure (M)

Blank (6.5.1)	QC _{rec(M)} 1 mg/kg (6.5.2.1)	QC _{loq(M)} 0.02 mg/kg (6.5.2.2)	Samples (6.5.3)	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 2 ml of blank material in a tube of 50 ml
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 2 ml of sample material in a tube of 50 ml
		<input type="checkbox"/>		Add 400 µl of mixed standard solution MSS1 of 5 µg/ml (4.4.1) and mix
				Add 80 µl of mixed standard solution MSS2 of 500 ng/ml (4.4.2) and mix
	<input type="checkbox"/>	<input type="checkbox"/>		Wait 30 min
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Add 18 ml of extraction solvent (4.3.1) and shake vigorously
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Extract for 30 minutes on a rotary tumbling machine
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Centrifuge for 15 min at 3500 rpm ^{note 1}
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Transfer 250 µl to a filter vial, add 250 µl water, close and mix
<input type="checkbox"/>				Transfer 5 ml of the supernatant to a 12 ml tube, add 5 ml water and mix
<input type="checkbox"/>				Prepare the matrix matched calibration standards MMC(M)1-9 (6.5.1) in filter vials according to Table A.3

Note 1 Supernatants that remain turbid after centrifugation can be prefiltered (1 ml aliquot) through a 5 µm membrane filter.

Annex A.3 Checklist for sample preparation milk (M)

Table A.3 Preparation of matrix-matched calibration standards (MMC(M)) for milk

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/l)	Mixed standard solution MSS3 50 ng/ml (4.4.4) (μ l)	Mixed standard solution MSS2 500 ng/ml (4.4.3) (μ l)	Mixed standard solution MSS1 5 μ g/ml (4.4.2) (μ l)	Diluted sample extract (6.4.3.1) (μ l)
MMC(M)1	0	0	-	-	-	500
MMC(M)2	1	0.02	10	-	-	490
MMC(M)3	2.5	0.05	25	-	-	475
MMC(M)4	5	0.1	50	-	-	450
MMC(M)5	10	0.2	-	10	-	490
MMC(M)6	25	0.5	-	25	-	475
MMC(M)7	50	1	-	50	-	450
MMC(M)8	100	2	-	-	10	490
MMC(M)9	200	4	-	-	20	480

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.

Example conditions for the UPLC system

LC system:	Xevo TQ-S or TQ-XS
Analytical column:	Waters Acquity BEH C18 1.7 μm , 100 x 2.1 m
Column temperature:	50 °C
Mobile phase solvent A:	10 mM ammonium carbonate in water pH 9.0 (4.3.3)
Mobile phase solvent B:	Methanol (4.3.4)
Flow rate:	0.4 ml/min
Injection volume:	2-5 μl
Injection temperature	10 °C
Gradient program:	Table B.1

Table B.1 Gradient for the UPLC system

Time (min)	Mobile phase A (4.3.3) %	Mobile phase B (4.3.4) %
0	100	0
1	100	0
8	60	40
12	20	80
12.2	100	0
14.2	100	0

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

Parameter	Settings
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	1000 l/hr
CID gas, pressure	Argon; 4.3 10^{-3} mbar
Solvent discard	0-1.5 and 13-14.2 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 first two transitions are the preferred transitions used for quantification. The additional transitions can be used for additional confirmation or when there are problems with (one of) the preferred transitions. The retention times can differ from column to

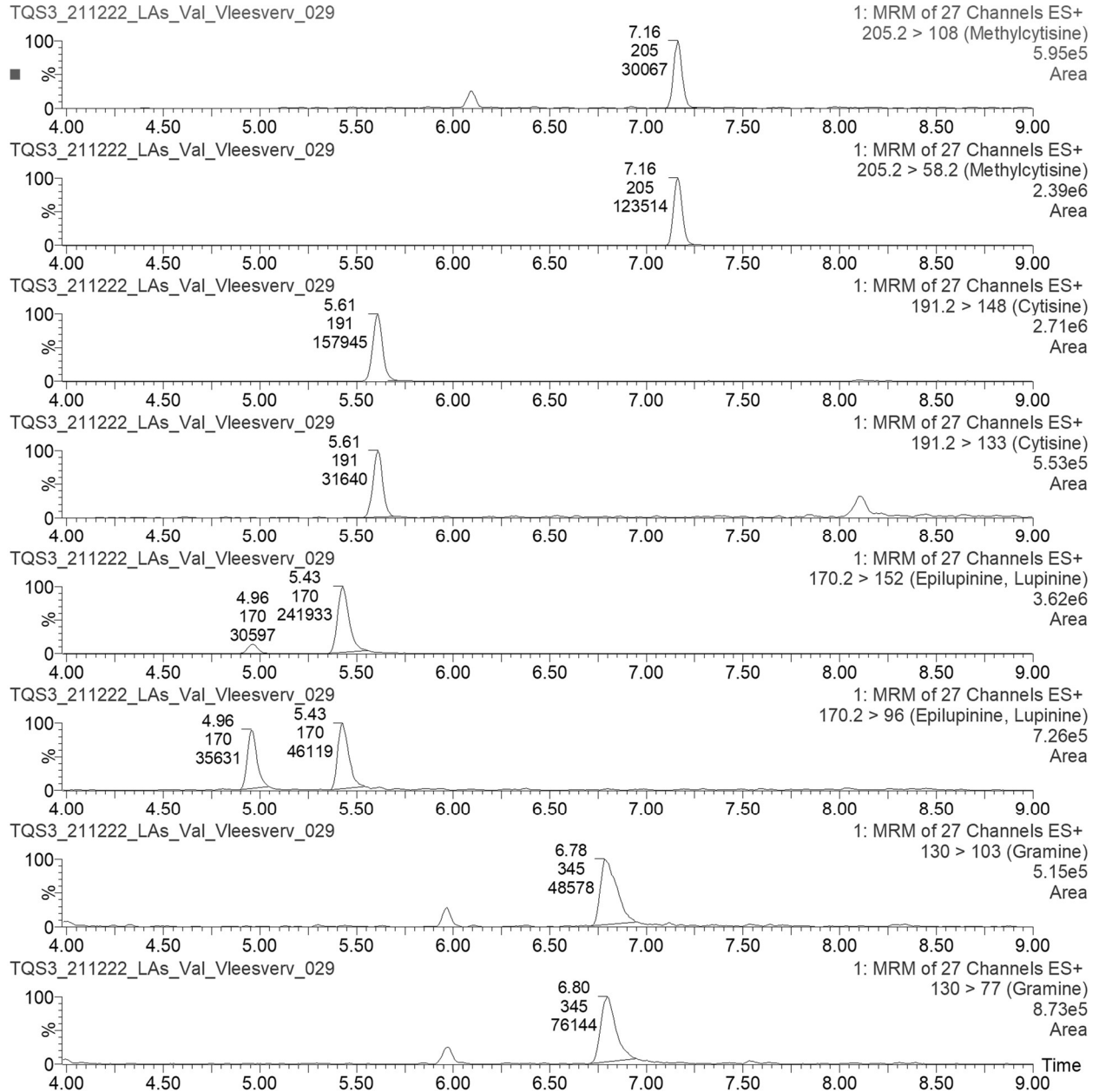
column and between HPLC systems. The retention times shown in Table B.2.2 are therefore indicative. The optimal dwell time is calculated automatically by the software of the instrument.

Table B.2.2 MS/MS fragmentation conditions for quinolizidine alkaloids

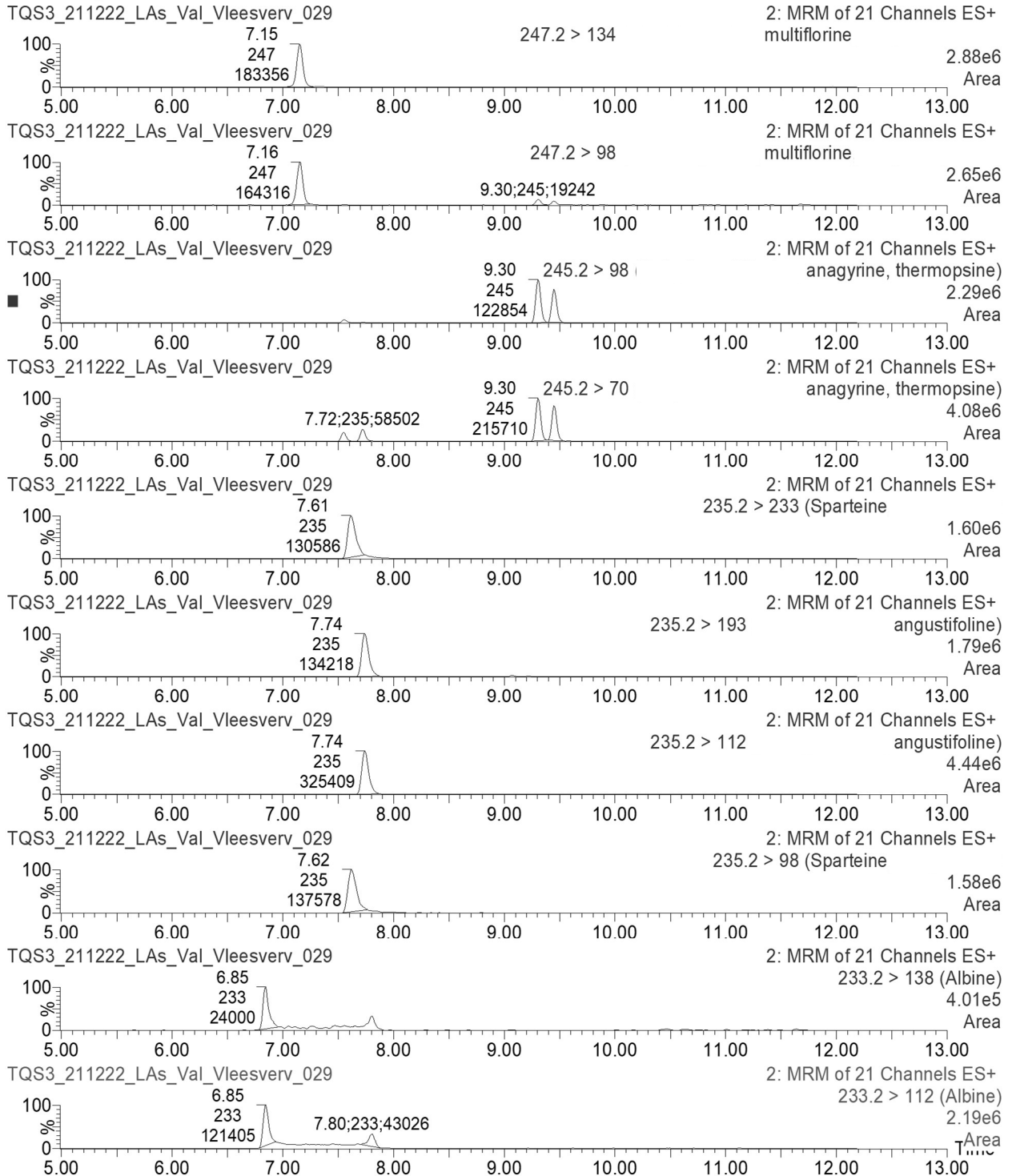
Compound	Precursor ion (m/z)	Cone voltage (V)	Product ion 1 (m/z)	Col. energy 1 (eV)	Product ion 2 (m/z)	Col. energy 2 (eV)	Product ion 3 (m/z)	Col. energy 3 (eV)	Product ion 4 (m/z)	Col. energy 4 (eV)	Indicative RT (min)
Gramine	130.0	30	77.0	20	103.0	25					5.85
Epilupinine	170.2	30	96.0	30	152.0	20	124.0	25	98.0	25	3.75
Lupinine	170.2	30	124.0	25	152.0	20	96.0	30	98.0	25	4.25
Cytisine	191.2	30	133.0	30	148.0	20					4.30
Methylcytisine	205.2	30	58.2	20	108.0	20					6.20
Albine	233.2	30	112.0	20	138.0	20	150.0	30			5.95
Angustifoline	235.2	30	112.0	20	193.0	30	114.0	30	70.0	30	7.10
Sparteine	235.2	30	98.0	30	233.0	30	70.0	30			6.70
Anagryne	245.2	30	70.0	45	98.0	35					9.05
Thermopsine	245.2	30	70.0	45	98.0	35					9.20
Multiflorine	247.2	30	70.0	40	112.0	25	134.0	20	84.0	40	6.40
Lupanine	249.2	30	114.0	30	136.0	30	98.0	30	84.0	30	6.90
Isolupanine	249.2	30	84.0	30	98.0	30	136.0	30	114.0	30	10.10
13-Hydroxy-lupanine	263.2	30	114.0	30	152.0	30	84.0	40	112.0	35	5.75
<i>trans</i> -13-Cinnamoyl-oxy-lupanine	395.2	30	98.0	40	112.0	30	247.0	30			11.55

B.3 LC-MS/MS example chromatograms of quinolizidine alkaloids in meat imitate fortified at 1 mg/kg

V.v. +1 rep 1

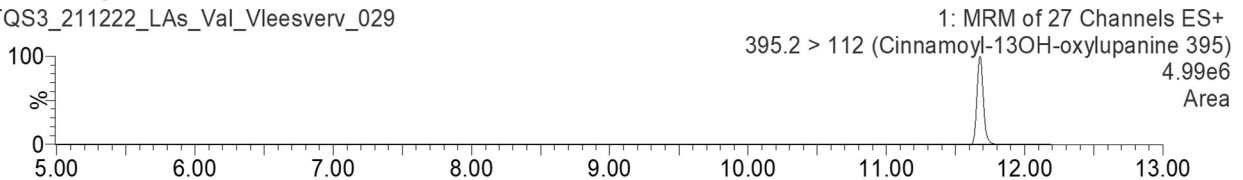


V.v. +1 rep 1

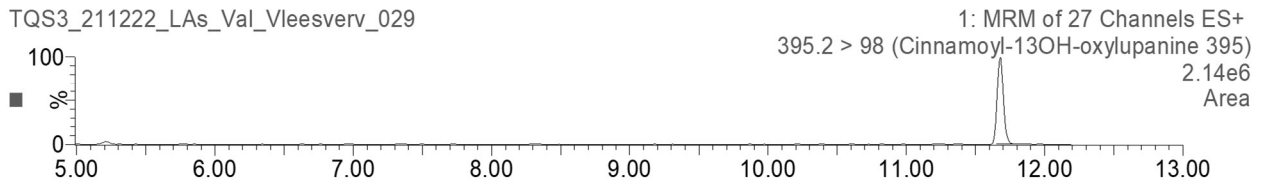


V.v. +1 rep 1

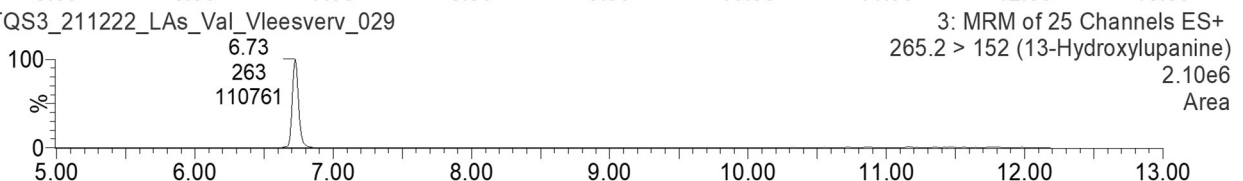
TQS3_211222_LAs_Val_Vleesverv_029



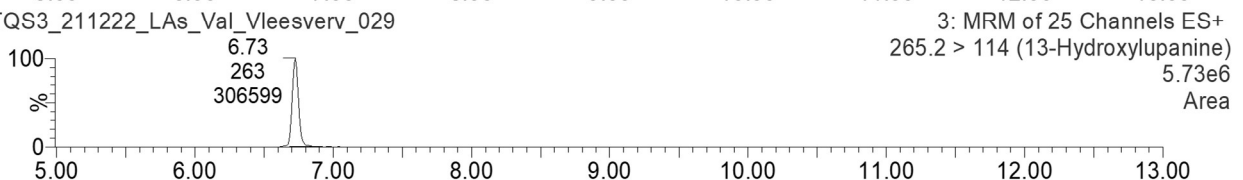
TQS3_211222_LAs_Val_Vleesverv_029



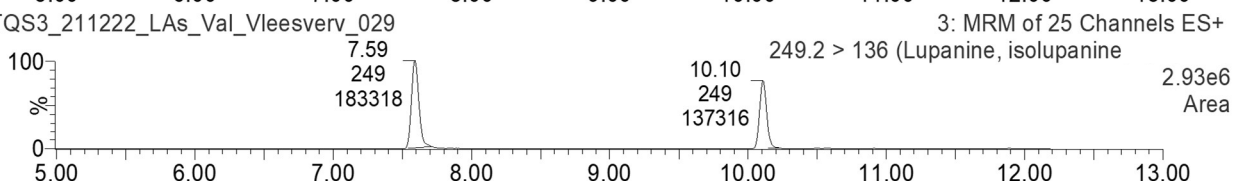
TQS3_211222_LAs_Val_Vleesverv_029



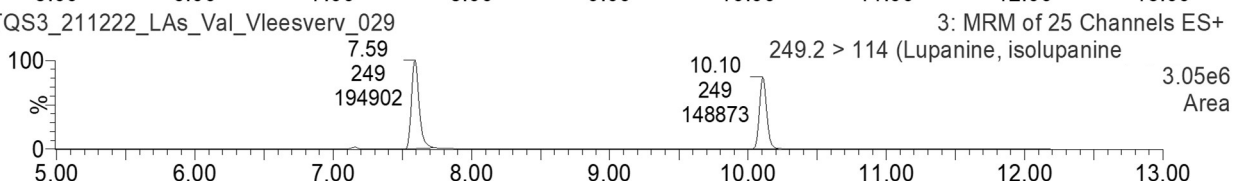
TQS3_211222_LAs_Val_Vleesverv_029



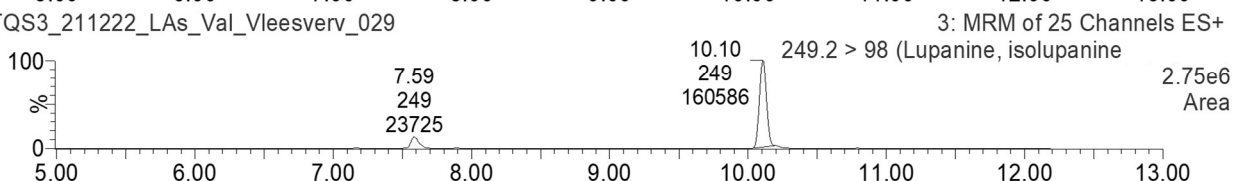
TQS3_211222_LAs_Val_Vleesverv_029



TQS3_211222_LAs_Val_Vleesverv_029



TQS3_211222_LAs_Val_Vleesverv_029



TQS3_211222_LAs_Val_Vleesverv_029

