Joint FAO/IAEA Centre
Nuclear Techniques in Food and Agriculture

Manual of Standard Operating Procedures for Selected Chemical Residue and Contaminant Analysis


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Manual of Standard Operating Procedures for Selected Chemical Residue and Contaminant Analysis

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Foreword

Laboratories are central to national food safety control systems and conduct routine testing and monitoring of chemical residues such as veterinary drugs and pesticides as well contaminants such as mycotoxins and toxic metals, among others. The IAEA and the Food and Agricultural Organization of the United Nations (FAO), through the Joint FAO/IAEA Center of Nuclear Techniques in Food and Agriculture, helps laboratory scientists to enhance the performance of these laboratories so that Member States safeguard the health of consumers and promote the market competitiveness of their foodstuff. One of the gaps to fill in these laboratories is the lack of analytical methods.

This publication presents several analytical methods in form of standard operating procedures (SOPs) that can be used for stepwise analysis of residues of chemicals including veterinary drugs, pesticides, and contaminants such as mycotoxins and toxic metals in food and related matrices. This arises from support provided to a number of IAEA/FAO Member States through capacity enhancement activities under regional and interregional arrangements. The publication also includes information on how the methods were validated. Some work on toxic metals in water is also presented since unsafe water can contribute to unsafe food.

The primary beneficiaries of the publication will be food and environmental safety laboratories involved in testing and monitoring of veterinary drug and pesticide residues, mycotoxins and toxic metals in food and water. The publication is also expected to enhance laboratory performance and competence through use of reliable analytical techniques. It is suitable for applied research on these chemical contaminants/hazards as well.

Twenty–one scientists from seven countries were involved in drafting the SOPs that constitute this publication.

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Abbreviations and acronyms

µg	Microgram
µg/kg	Microgram per kilogram
µl	Microliter
µm	Micrometer
AAS	Atomic absorption spectrometry
ACN	Acetonitrile
AF	Aflatoxins
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AHD	1–Amino Hydantoin
AMOZ	3–amino–5–morpholino–methyl–1,3–oxazolidinone
amu	Atomic mass unit
AOAC	Association of Official Agricultural Chemists
AOZ	3–amino–2–oxazolidinone
AR	Analytical reagent
AR grade	Analytical Reagent grade
Avg	Average
BG	Brilliant green
C18	Carbon 18
CAC	Codex Alimetarius Commission
CAP	Chloramphenicol
CCα	Decision limit
CCβ	Detection capability
Conc	Concentration
CRL	Community Reference Laboratories
CS	Calibration Standard
CV	Coefficient of variation
Cv	Crystal violet
DMSO	Dimethyl sulfoxide
DMSO	Dimethyl Sulfoxide
dSPE	Dispersive solid-phase extraction
EC	European Commission
EDTA	Ethylene diamine tetra acetic acid
EI	Electron Ionization
ELISA	Enzyme–Linked Immunosorbent Assay
EPI	Enhanced product ion
ESI	Electrospray ionization
EU	European Union

ε	Absorptivity
FAO	Food and Agricultural Organization of the United Nation
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B2
FLD	Fluorescence Detection/Detector
GC	Gas chromatography
GCB	Graphitized carbon black
GC–MS/MS	Gas chromatography–tandem mass spectrometry
H_2O	Water
HCOOH	Formic acid
HPLC	High–Performance Liquid Chromatography
HPLC–FLD	High Performance Liquid Chromatography–Fluorescence Detection
IAC	Immunoaffinity Column
IAEA	International Atomic Energy Agency
ICP–OES	Inductively Coupled Plasma–Optical Emission Spectrometry
IP	Identification point
K_2HPO_4	Di–potassium hydrogen phosphate
KOH	Potassium Hydroxide
kV	Kilovolt
LC–MS/MS	Liquid chromatography-tandem mass spectrometry
LCV	Leucocrystal violet
LMG	Leucomalachite green
LOD	Limit of detection
LOQ	Limit of quantification
LWC	Laboratory working concentration
M	Molar
m/z	Mass–to–charge ratio
MG	Malachite green
ml/min	Milliliters per minute
mM	millimolar
mmol/l	Millimole per liter
mol/l	Mole per liter
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MRPL	Minimum required performance limit
MU	Measurement uncertainty
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBA	2–nitrobenzaldehyde
NFs	Nitrofurans
nm	Nanometers
OTA	Ochratoxin A

PBS	Phosphate Buffered Saline
ppb	parts per billion
PPE	personal protective equipment
ppm	Parts per million
PSA	Primary Secondary Amine
psi	Pounds per square inch
PTFE	Polytetra–fluoroethylene
QQQ	Triple quadrupole
QuEChERS	Quick easy cheap effective rugged and safe
R^2	Coefficient of determination
Rcf (g)	Relative centrifugal force
rpm	Revolutions per minute
RSD	Relative standard deviation
S/N	Signal to noise ratio
SEM	Semi carbazide
SOP	Standard Operating Procedure
SPE	Solid–Phase Extraction
SRM	Selective Reaction Monitoring
STDEV	Standard Deviation
TMB	Tetramethylbenzidine
Tris	Tris (hydroxymethyl) aminomethane
UHPLC	Ultra–High–Performance Liquid Chromatography
UPLC	Ultra–performance liquid chromatography
UV	Ultraviolet
v/v	Volume by volume
w/w	Weight by weight
WS	Working Standard
α	Alpha
β	Beta
λ	Wavelength
μg/ml	Microgram per milliliter
σ	Sigma

1 Introduction

1.1 Background

The publication is a product of regional and interregional capacity enhancement activities supported by the Joint FAO/IAEA and conducted between 2016 and 2019 to facilitate the testing and monitoring of selected veterinary antimicrobial and pesticide residues, mycotoxins and toxic metals in order to safeguard consumer safety.

Multi-hazard methods were developed/validated and applied to national residue programs in some countries involved in the projects. Other Member State laboratories that did not take part can therefore benefit through dissemination of the analytical methods compiled as SOPs. While the publication covers a limited scope of residues, contaminants and tools, it addresses common laboratory needs including lack of analytical methods in many Member States and it is therefore worth disseminating.

1.2 Objective

The purpose of this publication is to facilitate the operations of food safety testing laboratories especially those involved in the routine testing and monitoring/control of residues of certain veterinary drug and pesticide residues, mycotoxins and toxic metals in food and related samples. The document can also support relevant research work.

1.3 Scope

This publication consists of analytical methods, in form of SOPs, for testing certain veterinary drug and pesticide residues, mycotoxins and toxic metals in food and related samples. It covers several stable-isotope based chromatographic-spectrometric and rapid and cost-effective screening techniques. These methods and thus SOPs were produced based on needs identified by more than 28 countries that participated in regional/interregional capacity enhancing projects. The information in the publication is presented in a step-by-step manner to better guide laboratory analysts on procedures.

1.4 Structure

The publication starts with SOPs addressing the analysis of mycotoxins including aflatoxins B1, B2, G1, G2; fumonisins and ochratoxins in a range of food and related matrices such as milk, edible vegetable oil, cereals/grains and feed. This is followed by SOPs on analysis of a range of multiple veterinary drug residues. These include amphenicols (thiamphenicol and chloramphenicol), anthelmintics, stilbenes, resorcylic acid derivatives, growth promoters; beta agonists, sulfonamides, macrolides, tetracyclines, quinolones, nitrofurans and a range of anticoccidials. The matrices include shrimps, meat, liver and eggs among others. The publication then concludes with SOPs on pesticide residues in matrices such as cereals, edible oils, fruits and vegetable; triphenylmethane and related dyes in aquaculture products, as well as toxic metals in food and water.

2

Detection of aflatoxins B1, B2, G1, G2 and M1 in milk, edible vegetable oil and animal feed/ ingredient by HPLC–FLD

2.1 Introduction

Aflatoxins (AFs) are a group of toxic secondary metabolites produced by several species of fungi such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) are the major naturally occurring aflatoxins and are known to contaminate a variety of agricultural products during their growth, harvest and storage. Aflatoxin M1 (AFM1), which is the 4-hydroxylated metabolite of AFB1, is excreted in the milk of mammals that have ingested AF B1–contaminated feed. AFs are carcinogenic, teratogenic and mutagenic to humans and animals, and hence, other contamination of food/feed remains a global food safety issue that has forced regulatory authorities in many countries to limit the aflatoxin levels in food/feed. These aflatoxins in milk, feed/ingredients and vegetable oils can be determined quantitatively using the HPLC–FLD method.

2.2 Scope and objective

This procedure describes the screening of AFM1, AFB1, AFB2, AFG1 and AFG2 in milk, and AFB1, AFB2, AFG1 and AFG2 in edible vegetable oil and animal feed/ ingredients as applicable, using the High–Performance Liquid Chromatography–Fluorescence Detection method (HPLC–FLD) at limits of detection (LOD) ranging from 0.01 µg/kg (or µg/l) to 0.5 µg/kg (or µg/l).

2.3 Principle of the method

Samples are prepared by mixing with an extraction solution [salt and methanol/ water (for feed and vegetable oil) or salt (for milk)] and blending at high speed (feed and vegetable oil) or by centrifuging (milk). Coarse/particulate material and precipitates are removed from the sample extract through separate filtration steps. The sample extract is then cleaned up using monoclonal antibody–based affinity

chromatography. In this step, when the extract is applied to the immunoaffinity column, which is bound with antibodies specific to aflatoxins, aflatoxins in the extract bind to antibodies on the column. Impurities in the column are then removed by several washing steps. Aflatoxins bound to antibodies are eluted by passing methanol (for feed and vegetable oil) or methanol/water (for milk) through the column and are detected by the HPLC–FLD system after postcolumn photochemical derivatization.

2.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may present a serious health risk for the laboratory workers. Aflatoxins are proven carcinogens and require appropriate facilities and gear. Suitable gloves should be worn when chemicals/ aflatoxin standards are handled, and the work should be performed in a fume hood.

2.5 Materials

The following reagents and chemical(s) are applicable: Ultrapure water or water of equivalent quality; methanol (HPLC grade); acetonitrile (HPLC Grade) and salt (analytical grade).

2.5.1 Equipment/instruments and consumables

The following apparatus and material are applicable:

Analytical balance (OHAUS); Filtering device for solvents and water; Phenex™ Nylon Filter Membranes (0.20 µm) (Phenomemex®); Laboratory blender (Waring); Grinder (Moulinex®); Laboratory centrifuge (Centurion scientific Ltd.); Vortex mixer (Velp); Fluted filter papers (24 cm) (Vicam); Glass microfiber filter papers (1.5 µm) (Vicam); Measuring cylinder (25 ml, 50 ml 100 ml, 250 ml); Volumetric flask (50 ml); Stopwatch; Glass beakers; Conical flasks; Glass funnels; Polypropylene tubes with screw cap (15 ml and 50 ml); Disposable syringes (10 ml, 20 ml and 50 ml); 18G syringe needles; Micropipettes (10 µl–100 µl, 100 µl–1 000 µl, 500 µl–5000 µl (Eppendorf); Immunoaffinity column (AflaTest®, Vicam); Vacuum Pump (Cole Parmer); Extraction manifold with a vacuum chamber, rack and stopcocks (Agilent); Amber colour HPLC vials; Glass HPLC vial inserts with bottom polymer feet (250 µl); HPLC–FLD system (Agilent 1100 series); Postcolumn photochemical reactor (PHRED).

2.5.2 Solutions

The solutions (and how they are prepared) include:

- Methanol/water (10/90, v/v): Mix 10 ml of methanol with 90 ml of water;
- Methanol/water (60/40, v/v): Mix 60 ml of methanol with 40 ml of water;
- Methanol/water (80/20, v/v): Mix 80 ml of methanol with 20 ml of water;
- Mobile phase A consisting of ultrapure water filtered through a 0.20 µm nylon membrane filter;
- Mobile phase B, which is acetonitrile filtered through a 0.20 µm nylon membrane filter;
- Mobile phase C is made of methanol filtered through a 0.20 µm nylon membrane filter.

2.5.3 Standards

2.5.3.1 Standard solutions of aflatoxins

The analytical standards include: AFB1 at 2 mg/l (Fermentek Ltd., Jerusalem, Israel); AFB2 at 0.5 mg/l (Fermentek Ltd., Jerusalem, Israel); AFG1 at 2 mg/l (Fermentek Ltd., Jerusalem, Israel); AFG2 at 0.5 mg/l (Fermentek Ltd., Jerusalem, Israel); AFM1 at 0.5 mg/l (Fermentek Ltd., Jerusalem, Israel).

2.5.3.2 Working standard solutions

a) Working standard 1 (WS 1, 100µg/l)

WS 1 contains all five AFs, each at 100 µg/l and is used to spike the blank milk samples. To prepare WS1, pipette 200 µl each from AFM1, AFB2 and AFG2 standard solutions, and 50 µl each from AFB1 and AFG1 standard solutions and mix with 300 µl of methanol.

b) Working standard 2 (WS 2, 200 µg/l)

WS 2 contains AFB1, AFB2, AFG1 and AFG2, each at 200 µg/l, and is used to spike blank vegetable oil samples and blank feed samples. To prepare WS 2, mix 200 µl each from AFB2 and AFG2 standard solutions and 50 µl each from AFB1 and AFG1 standard solutions.

c) Working standard 3 (WS 3, 50 µg/l)

WS 3 contains all five AFs, each at 50 µg/l and is used to prepare the calibration standards. To prepare WS 3, pipette 200 µl each from AFM1, AFB2 and AFG2 standard solutions, and 50 µl each from AFB1 and AFG1 standard solutions and mix with 1 300 µl of mobile phase [water/methanol/acetonitrile (50/30/20, v/v/v)].

2.5.3.3 Calibration standards

External calibration standards are used in the procedure. Prepare the calibration standards by mixing WS 3 (50 µg/l) with the mobile phase according to Table 1.

When stored in the dark at -20°C, calibration standards are stable for at least four months.

TABLE 1. Preparation of calibration standards

Calibration Standard	Concentration (µg/l)	Preparation	
		Volume of WS 3 (µl)	Volume of mobile phase[1] (µl)
CS 1	0.25	10	1 990
CS 2	0.5	20	1 980
CS 3	1	30	1 470
CS 4	3	60	940
CS 5	5	100	900
CS 6	10	300	1 200
CS 7	20	600	900

[1]Mobile phase: Water/methanol/acetonitrile (50/30/20, v/v/v)

2.6 Procedure

2.6.1 Sample preparation

a) **Milk:** Measure 50 ml of liquid milk into a 50 ml polypropylene tube.

b) **Vegetable oil:** Weigh 25 g (±0.05 g) of vegetable oil into a 50 ml polypropylene tube.

c) **Feed/ingredient:** Grind the feed/ingredient sample using a grinder and weigh 25 g (±0.05 g) of the ground sample into a 50 ml polypropylene tube or a glass beaker.

2.6.2 Quality control samples

Blank samples and spiked samples should be run as quality control samples with each batch of test samples.

a) **Blank samples:**

- A milk sample (50 ml) that does not contain AFM1, AFB1, AFB2, AFG1 and AFG2 is used as a blank milk sample.
- A vegetable oil sample (25 g) and a feed or corn sample (25 g) that do not contain AFB1, AFB2, AFG1 and AFG2 are used as blank samples.

b) **Spiked samples:**

- Milk samples are spiked with all five AFs, each at 0.5 µg/l. Measure a 50 ml blank milk sample using a 50 ml volumetric flask and discard 250 µl of the sample. Then add 250 µl of WS 1 (100 µg/l) to the milk sample to spike at 0.5 µg/l level.
- Vegetable oil samples and feed samples are spiked with AFB1, AFB2, AFG1 and AFG2, each at 2 µg/kg. To spike the vegetable oil samples, add 250 µl of WS 2 (200 µg/l) to 25 g of the blank oil sample.
- To spike the feed samples, add 250 µl of WS 2 (200 µg/l) to 25 g of blank feed sample.

NOTE: *Let spiked samples stand for 30 minutes (min) before continuing with the rest of the procedure.*

2.6.3 Sample extraction

The following steps are followed during extraction:

Milk:

a) Add 1 g (±0.05 g) of NaCl to the test portion (unknown, blank and spiked samples) in a 50 ml polypropylene tube.

b) Close the caps tightly and mix well by vortexing or shaking vigorously by hand. Centrifuge the samples at 2 000 rcf for 10 min.

c) After the centrifugation, poke a hole into the bottom of the polypropylene tube, using an 18G syringe needle, and carefully remove the bottom layer of the milk (i.e. skim portion, ~30 ml) into a 50 ml syringe for analysis without disturbing the top fat layer.

d) Filter the skim sample through a fluted filter paper and collect the filtrate into a clean container.

Vegetable oil:

a) Place the test portion (unknown, blank and spiked) in a clean blender/jar.

b) Add 5 g (±0.05 g) of NaCl and 125 ml of methanol/water (60/40, v/v) into the blender jar.

c) Close the blender jar tightly and blend at high speed (~ 22 000 rpm) for 1 min.

d) Remove the cover from the jar and filter the extract through a fluted filter paper into a glass container.

e) Pipette 20 ml of the filtered extract into a 50 ml polypropylene tube.

f) Add 20 ml of purified water to the tube to dilute the extract and mix well by shaking by hand.

Feed/ingredients:

a) Place the test portion (unknown, blank and spiked samples) in a clean blender jar.

b) Add 2.5 g (±0.05 g) of NaCl and 100 ml of methanol/water (80/20, v/v) to the blender jar.

c) Close the blender jar tightly and blend at high speed (~22 000 rpm) for 1 min.

d) Remove the cover from the jar and filter the extract through a fluted filter paper.

e) Collect filtrate into a clean glass container and discard the supernatant.

f) Pipette 10 ml of the filtered extract into a 50 ml polypropylene tube.

g) Add 40 ml of purified water to the tube to dilute the extract and mix well by shaking with a hand.

h) Filter the diluted extract through 1.5 µm glass microfiber filter into a clean container or a 50 ml polypropylene tube.

2.6.4 Immunoaffinity column chromatography

NOTE: *Remove the top cap from the AflaTest® IAC and cut the bottom ⅛ inch off the end of the cap with a sharp blade or scissors. Using this top cap as a coupling, attach the syringe barrel to the IAC. Pipette out or pour the desired volume of extract or diluted extract into the syringe barrel, and then remove the bottom cap from the IAC. Achieve the required flow rate (given for each matrix below) through the column, using gravity, a vacuum pump or by pressing the plunger as necessary.*

Vegetable oil:

a) Pass 5 ml of filtered diluted extract of oil through the AflaTest®IAC at a rate of about 1 drop until air comes through column.

b) Pass 10 ml of purified water through the column at a rate of about 1–2 drop/s.

c) Pass another 10 ml of purified water (1–2 drop/s) until air comes through the column.

d) Elute the column with 1 ml of methanol at a rate of 1 drop or slower and collect the eluate.

e) Double dilute the eluate with purified water and transfer to an HPLC vial.

Feed/ingredients:

a) Pass 20 ml of filtered diluted feed extract through the AflaTest® IAC at a rate of about 1 drop until air comes through the column.

b) Pass 10 ml of purified water through the column at a rate of about 1–2 drop/s.

c) Pass another 10 ml of purified water (1–2 drop/s) until the air comes through the column.

d) Elute the column with 1 ml of methanol at a rate of about 1 drop or slower and collect the eluate.

e) Double dilute the eluate with purified water and transfer to an HPLC vial.

2.6.5 Instrumental analysis (HPLC–FLD)

HPLC–FLD analysis is carried out using an Agilent 1100 HPLC system consisting of a micro–vacuum degasser, (G1379A), quaternary pump (G1311A), auto–sampler (G1313A), thermostatted column compartment (G1316A) and a fluorescence detector (FLD), G1321A, Agilent 1 200 series). A photochemical reactor (PHRED) is connected between the HPLC column and the fluorescence detector for on-line postcolumn derivatization of aflatoxins. Conditions of HPLC–FLD analysis are as follows:

a) Separation mode: reversed phase chromatography

b) Column: Phenomenex Luna C18 (2) 100A (4.6×250 mm, 5 µm particle size)

c) Column temperature: 40°C

d) Injection volume: 10 µl

e) Run time: 12 min

f) Flow rate: 1 ml/min

g) Mobile phase: water/methanol/acetonitrile (50/30/20, v/v/v)

h) Elution mode: isocratic

i) FLD settings: 360 nm (excitation), 450 nm (emission)

2.6.6 Interpretation of results

The concentrations of aflatoxin in milk, vegetable oil and feed/ingredient samples are calculated using the respective calibration curves.

HPLC run is considered valid if the following results are achieved.

- The calibration curves of each AF should have a coefficient of determination (R^2) of ≥ 0.95.
- Recovery of AFs in spiked samples are be in the ranges shown in Table 2.

TABLE 2. Recovery of AFs from spiked samples

Matrix	Analyte	Spike level (µg/l or µg/kg)	Recovery range (%)
Milk	AFM1	< 1	50–120
	AFB1		
	AFB2		
	AFG1		
	AFG2		
Vegetable oil, animal feed /ingredients	AFB1	1–10	70–120
	AFB2		
	AFG1		
	AFG2*		

* For AFG2 in vegetable oil, the acceptable range of recovery is 50%–120%

If the above two conditions are true, the test samples can be interpreted as follows:

Positive samples:

- Milk samples with corresponding HPLC–FLD peak area for AFM1, B1, B2, G1 and G2 above 0.5 µg/l level

- Vegetable oil samples with corresponding HPLC–FLD peak area for AFB1 above 1 µg/kg and/or peak area for total aflatoxin level (AFB1, AFB2, AFG1, and AFG2) above 4 µg/kg
- Feed samples with corresponding HPLC–FLC peak area for AFB1 above 1 µg/kg
- Any other result should be reported as negative.

2.6.7 Method validation

This is done following guidelines for standard method performance requirements (AOAC, 2012). One of the parameters to validate is method precision/variability. Precision of the HPLC–FLD method was considered at two levels: repeatability (intra–assay) and intermediate precision (within laboratory reproducibility). They were expressed as %RSD values.

Chromatographic selectivity of the AFM1, AFB1, AFB2, AFG1 and AFG2 is determined by calculating the peak resolution between adjacent peaks, using Eq. (1).

$$Rs = \frac{RT2 - RT1}{0.85(W2 + W1)} \quad \ldots\ldots(1)$$

Where: Rs = peak resolution; RT2 = retention time of peak 2; RT1 = retention time of peak 1; W1 = width of peak 1 at half peak height; W2 = width of peak 2 at half peak height.

Most validation guidelines recommend peak resolution above 1.5 since measuring peak areas accurately becomes difficult when resolution drops below 1.2.

a) Validation for milk

The Tables 3–5 below summarize results of method precision, selectivity and measurement uncertainty for mycotoxin testing in milk:

TABLE 3. Precision of the method for milk

Analyte	Day	Repeatability (%RSD)	Intermediate precision (%RSD)
AFM1	1	9.8	10.8
	2	12.9	
AFB1	1	9.6	8.5
	2	5.6	
AFB2	1	9.8	9.5
	2	8.6	
AFG1	1	8.3	8.6
	2	8.8	
AFG2	1	10.0	10.4
	2	11.0	

TABLE 4. Selectivity of the method for milk

Peaks	Peak resolution
AFM1–AFG2	1.74
AFG2–AFG1	2.52
AFG1–AFB2	2.51
AFB2–AFB1	3.00

TABLE 5. Validation summary and uncertainty calculation of the method for milk

	AFM1	AFG2	AFG1	AFB2	AFB1
Mean Conc. (Negative Control)	0.005	0.001	0.004	0.001	0.004
STDEV (Negative Control)	0.003	0.001	0.003	0.001	0.004
LOD (Mean × 3)	0.014	0.003	0.012	0.002	0.011
Avg. Conc. of spiked samples at 0.5 ppb	0.500	0.500	0.500	0.500	0.500
STDEV (spiked samples at 0.5 ppb)	0.051	0.048	0.039	0.044	0.039
Avg. % Recovery	84.71	84.19	93.67	85.13	79.57

	AFM1	AFG2	AFG1	AFB2	AFB1
Avg. Conc. of spiked samples at 0.5 ppb	0.500	0.500	0.500	0.500	0.500
STDEV (spiked samples at 0.5 ppb)	0.051	0.048	0.039	0.044	0.039
Sample Size	8.0	8.0	8.0	8.0	8.0
Standard Uncertainty	0.02	0.02	0.01	0.02	0.01
Degrees of Freedom	7.0	7.0	7.0	7.0	7.0
95% t–Value	2.365	2.365	2.365	2.365	2.365
95% Confidence Deviation	0.04	0.04	0.03	0.04	0.03
95% Confidence Interval (Min)	0.46	0.46	0.47	0.46	0.47
95% Confidence Interval (Max)	0.54	0.54	0.53	0.54	0.53
Percentage Uncertainty	8.53	8.03	6.59	7.39	6.59

b) Validation for vegetable oil

The Tables 6–8 summarize results of method precision, selectivity and measurement uncertainty for mycotoxin testing in vegetable oil.

TABLE 6. Precision for vegetable oil method

Analyte	Day	Repeatability (%RSD)	Intermediate precision (%RSD)
AFB1	1	8.15	14.8
	2	18.8	
	3	14.8	
AFB2	1	15.1	14.3
	2	10.2	
	3	17.8	
AFG1	1	15.6	14.9
	2	13.7	
	3	15.7	
AFG2	1	18.7	15.4
	2	6.38	
	3	16.0	

TABLE 7. Selectivity of the method for vegetable oil

Peaks	Peak resolution
AFG2–AFG1	2.45
AFG1–AFB2	2.43
AFB2–AFB1	2.95

TABLE 8. Validation summary and uncertainty calculation of the method for vegetable oil

	AFG2	AFG1	AFB2	AFB1
Mean Conc. (Negative Control)	0.005	0.019	0.005	0.010
STDEV (Negative Control)	0.004	0.019	0.005	0.010
LOD (Mean × 3)	0.02	0.06	0.02	0.03
Avg. Conc. of spiked samples at 2 ppb	2.00	2.00	2.00	2.00
STDEV (spiked samples at 2 ppb)	0.25	0.26	0.26	0.25
Avg. % Recovery	42.64	93.37	92.17	87.61

	AFG2	AFG1	AFB2	AFB1
Avg. Conc. of spiked samples at 2 ppb	2.00	2.00	2.00	2.00
STDEV (spiked samples at 2 ppb)	0.25	0.26	0.26	0.25
Sample Size	9.0	9.0	9.0	9.0
Standard Uncertainty	0.08	0.09	0.09	0.08
Degrees of Freedom	8.0	8.0	8.0	8.0
95% t–Value	2.306	2.306	2.306	2.306
95% Confidence Deviation	0.20	0.20	0.20	0.19
95% Confidence Interval (Min)	1.80	1.80	1.80	1.81
95% Confidence Interval (Max)	2.20	2.20	2.20	2.19
Percentage Uncertainty	9.80	10.01	9.81	9.73

c) Validation for feed

The precision of the HPLC–FLD method was considered in terms of repeatability. For AFB1, AFB2, AFG1 and AFG2 %RSD values were 3.5%, 3.7%, 5.4% and 2.2%, respectively.

The Tables 9 and 10 below summarize results of method selectivity and measurement uncertainty for mycotoxin testing in feed.

TABLE 9. Selectivity of the method for feed

Peaks	Peak resolution
AFG2–AFG1	2.45
AFG1–AFB2	2.43
AFB2–AFB1	2.95

TABLE 10. Validation summary and uncertainty calculation of the method for feed

	AFG2	AFG1	AFB2	AFB1
Mean Conc. (Negative Control)	0.003	0.013	0.004	0.009
STDEV (Negative Control)	0.001	0.009	0.005	0.002
LOD (Mean × 3)	0.01	0.04	0.01	0.03
Avg. Conc. of spiked samples at 2 ppb	2.0	2.0	2.0	2.0
STDEV (spiked samples at 2 ppb)	0.04	0.11	0.07	0.07
Avg. % Recovery	85.85	96.57	75.19	62.25

	AFG2	AFG1	AFB2	AFB1
Avg. Conc. of spiked samples at 2 ppb	2.00	2.00	2.00	2.00
STDEV (spiked samples at 2 ppb)	0.04	0.11	0.07	0.07
Sample Size	3.00	3.00	3.00	3.00
Standard Uncertainty	0.03	0.06	0.04	0.04
Degrees of Freedom	2.00	2.00	2.00	2.00
95% t–Value	4.30	4.30	4.30	4.30
95% Confidence Deviation	0.11	0.27	0.19	0.18
95% Confidence Interval (Min)	1.89	1.73	1.81	1.82
95% Confidence Interval (Max)	2.11	2.27	2.19	2.18
Percentage Uncertainty	5.49	13.53	9.30	8.77

2.6.8 Schematic diagram of extraction procedure

The schematics below summary the processes for extracting mycotoxins from milk, vegetable oil and feed.

a) Milk

TEST SAMPLE	Measure 50 ml of a milk sample into a 50 ml polypropylene tube
SAMPLE EXTRACTION	1. Add 1 g (±0.05 g) of NaCl to sample and mix well. 2. Centrifuge sample at 2 000 g for 10 min. 3. Remove the bottom skim layer, using an 18G needle. 4. Filter the skim sample through fluted filter paper and collect filtrate. 5. Filter the filtered skim sample through 1.5 µm glass microfiber filter and collect filtrate.
COLUMN CHROMATOGRAPHY	1. Pass 10 ml of filtered skim milk through IAC (1 drop) until air comes through the column. 2. Remove the loading syringe barrel from the column. 3. Fill the column headspace with methanol/water (10/90, v/v). 4. Attach the new syringe barrel to the column and pass 10 ml of methanol/water (10:90, v/v) through IAC (1–2 drop/s). 5. Pass another 10 ml of methanol/water (10/90, v/v) until the air comes through. 6. Elute the column by passing 1 ml of methanol/water (80:20, v/v) (1 drop or slower) through the IAC. 7. Double dilute the eluate with purified water and transfer to the HPLC vial.

b) Vegetable oil

TEST SAMPLE	1. Weigh 25 g (±0.05 g) of vegetable oil into a 50 ml polypropylene tube.
SAMPLE EXTRACTION	1. Place test sample in the blender jar. 2. Add 5 g (±0.05 g) of NaCl. 3. Add 125 ml of methanol/water (60/40, v/v). 4. Blend at high speed (~22 000 rpm) for 1 min. 5. Pour extract into a fluted filter paper and collect filtrate. 6. Discard the supernatant.
EXTRACT DILUTION	1. Pipette 20 ml of filtered extract into a clean vessel. 2. Add 20 ml of purified water and mix well. 3. Filter through 1.5 µm glass microfiber filter into a clean vessel.

COLUMN CHROMATOGRAPHY	1. Pass 5 ml of filtered diluted extract through IAC (1 drop) until air comes through. 2. Pass 10 ml of purified water through IAC (1–2 drop/s). 3. Pass another 10 ml of purified water until air comes through the IAC. 4. Elute column by passing 1 ml of methanol though the IAC (1 drop/s or slower). 5. Double dilute the eluate with purified water and transfer to HPLC vial.

c) Feed

TEST SAMPLE	1. Grind the feed/ingredient sample and weigh 25 g (±0.05 g) of ground sample.
SAMPLE EXTRACTION	1. Place test sample in the blender jar. 2. Add 2.5 g (±0.05 g) of NaCl. 3. Add 100 ml of methanol/water (80/20, v/v). 4. Blend at high speed (~22 000 rpm) for 1 min. 5. Pour extract into a fluted filter paper and collect extract. 6. Discard the supernatant.
EXTRACT DILUTION	1. Pipette 10 ml of filtered extract into a clean vessel. 2. Add 40 ml of purified water and mix well. 3. Filter through a 1.5 µm glass microfiber filter into a clean vessel.
COLUMN CHROMATOGRAPHY	1. Pass 20 ml of filtered diluted extract through IAC (1 drop) until air comes through. 2. Pass 10 ml of purified water (1–2 drop/s) through the IAC. 3. Repeat the previous step until air comes through the IAC. 4. Elute aflatoxins by passing 1 ml of methanol through the IAC (1 drop or slower). 5. Double dilute the eluate with purified water and transfer to the HPLC vial.

3

Determination of Aflatoxins (B1, B2, G1 and G2) in milled cereals and grains by HPLC–FLD

3.1 Introduction

Contamination of foodstuffs with mycotoxins is one of the most concerning problems in food and feed safety globally. Mycotoxins are secondary metabolites produced by filamentous fungi, having toxic and destructive biological effects on animal and human tissues. A group of toxins produced by the common mould *Aspergillus flavus* is known as aflatoxins. Based on their fluorescent property they are named as B1, B2 (exhibit blue fluorescence) and G1, G2 (those exhibiting green fluorescence). The hydroxy toxins M1 and M2 produced by cattle after feeding on contaminated feeds are also known as milk toxins. *Aspergillus flavus* thrives mainly on nuts, oil seeds, cereals, pulses and dried fruits. Wet and humid preharvest conditions as well as improper postharvest storage of foods are conducive for the growth and proliferation of the fungi. Moisture content of above 9%, high humidity and temperatures above 30°C, are conducive for production of aflatoxins. The fluorescent property of aflatoxins is widely used for their detection and determination in foods. Immuno–affinity cleanup methods have been found to be very effective in the selective cleanup of toxins from other interfering materials. HPLC separation followed by postcolumn derivatization of the toxins improves the fluorescence of B1 and G1 without affecting the fluorescence of B2 and G2 which makes the technique very sensitive and suitable for the determination of aflatoxins.

3.2 Scope and objective

This method is applicable for the determination of aflatoxins (B1, B2, G1 and G2) at ppb levels in maize grains, peanut and milled cereal samples.

3.3 Principle of the method

Aflatoxins are extracted, purified, separated by reversed phase liquid chromatography and then derivatized using a UVE™ – Photochemical Postcolumn Derivatization unit before detection by fluorescence.

3.4 Safety considerations and precautions

The laboratory is a high–risk area and therefore proper care should be taken while handling the chemicals and reagents. Use proper protective equipment and wear laboratory coats; safety glasses for eye protection, and gloves to protect the skin from toxic and corrosive materials. Use respirators is ventilation if inadequate.

3.5 Materials

3.5.1 Reagents and solvents

The following reagents and chemical are applicable: water (HPLC grade); sodium chloride (AR grade) and methanol (HPLC grade).

3.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Aflatoxin immunoaffinity columns; Volumetric flasks; Analytical balance; Ultra-turax homogenizer; Vortex mixer; Reversed phase C–18 column (particle size 5 µm, 250×4.6 mm or equivalent); Membrane filter (0.45 µm); Ultrasonic bath; Reagent bottle; Falcon tubes; Filter manifold, Millipore/SPE manifold, IST; Filter paper, qualitative No. 4 or equivalent; Glass microfiber filter papers; PTFE filter; Autosampler vials; Glass tubes; Photochemical Postcolumn Derivatization; Micropipette 5 µl–100 µl, adjustable; Vacuum manifold; HPLC with a Fluorescence detector; UV Spectrophotometer.

3.5.3 Solutions

Preparation of mobile phases.

- Measure 700 ml of methanol in a measuring cylinder.
- Measure 300 ml of distilled water in a measuring cylinder.
- Mix 1 and 2 thoroughly and degas.
- Mobile phase A: Measure 1 000 ml of methanol (HPLC Grade) into the mobile phase bottle and place in ultrasonic bath and shake for 15 min.
- Mobile phase B: Measure 1 000 ml of Water (HPLC Grade) into the mobile phase bottle and place in ultrasonic bath for 15 min.

3.5.4 Standards

The analytical standards include: Aflatoxin B1, B2, G1 and G2 standards (Sigma or equivalent) with stated concentrations of each component such as 20 µg/ml of each component and purity of ≥ 98% of the component.

3.5.4.1 Preparation of individual standard stock solution (1000 mg/l):

This is prepared in µg/ml as shown in Eqs. (2)–(4)

$$Weight\ of\ standards\ (corrected,\ a) = \frac{[weight\ of\ standards \times Purity]}{100} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$Volume\ of\ solvent\ (ml,\ b) = \left[Weight\ of\ solvent\text{-}specific\ gravity\right] \quad \ldots\ldots\ldots\ldots\ldots (3)$$

$$Calculated\ Conc.\ \left(\frac{\mu g}{ml}\right) = \left[Corrected\ weight\ of\ standard\ \frac{(a)}{}\ volume\ of\ solvent\ (b)\right] x\ 1000 \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

- Label the container with laboratory code, name of chemical, concentration in µg/ml, name of solvent, date of presentation, date of expiry.
- Enter the data in the respective logbook and registers.

Dissolve 10 mg of each aflatoxin B1, B2, G1 and G2 standard in methanol and make up to 10 ml in a volumetric flask. If required, determine their purity by a standard molar extinction coefficient method.

3.5.4.2 Preparation of intermediate standard stock solutions:

- Before use, the stock solution is sonicated and vortexed thoroughly.
- From the above standard stock using Eq. (5), intermediate standard solutions with concentration of 4 µg/ml for individual aflatoxins is prepared in required volume.

$$C_1V_1 = C_2V_2 \quad \ldots\ldots (5)$$

Where C_1 and V_1 are the concentration and volume of the original standard; C_2 and V_2 are the intended concentration and volume, respectively.

- Take 100 µl of each aflatoxin stock solution (1 000 mg/l) into 100 ml volumetric flask and dilute with methanol.
- Note: Already prepared aflatoxin mixes of 1 mg/l from reputable manufacturers can be used if they are certified reference material with certificate of analysis containing measurement uncertainty and coverage factor and traceable

3.5.4.3 Analysis by UV–Spectrophotometer:

- Since aflatoxins are photosensitive and get degraded upon exposure to light during storage, their accurate concentration is determined by UV spectroscopy following Eqs. (6) and (7):

$$A = \varepsilon lc \quad \ldots\ldots (6)$$

where A is absorbance; ε is absorptivity; l is pathlength

Absorbance = molar absorptivity x path length of the cuvette (1 cm) x concentration

$$Concentration\left(\frac{mg}{ml}\right) = \frac{A\,x\,Mol.wt \times 1\,000}{molar\,absorptivity\,x\,path\,length} \quad \ldots\ldots (7)$$

- This is considered as the correct concentration which is applied for preparation of working standard solution mixture and calibration standards.

3.5.4.4 Preparation of working standard solution mixture:

- A working standard solution mixture (100 ppb) is prepared from individual intermediate standard at 4 mg/l.
- Application of correction factor to the concentration of individual intermediate standard stock solution assures correct preparation of further mixtures and dilutions.

3.5.4.5 Working standard (4 µg/l)

Prepare working standard at 4 µg/l of the mycotoxins by taking 40 µl of 1 mg/l solution into 10 ml volumetric flask and making to the mark with methanol/water (50/50, v/v).

3.5.4.6 Calibration standards

The calibrants are prepared as follows (Table 11).

TABLE 11. Preparation of calibration standards

Stock concentration (µg/l)	Volume of stock (ml)	Volume dilution	Actual concentration (µg/l)	Method factor	Method Conc. (µg/kg)
4.0	2	n.a.	4.0	8	32
4.0	2	2	2.0	8	16
2.0	2	2	1.0	8	8
1.0	2	2	0.5	8	4
0.5	2	2	0.25	8	2
0.25	2	2	0.125	8	1
0.125	2	2	0.0625	8	0.5
0.0625	2	2	0.03125	8	0.25

n.a: No dilution

NOTE *From the table, standards equivalent to 0.25 µg/kg, 4 µg/kg, 8 µg/kg, 16 µg/kg and 32 µg/kg are used for calibration.*

3.6 Procedure

This follows modifications as reported elsewhere (AOAC, 2000; AOAC, 2002).

3.6.1 Sample preparation

Nuts and grains

a) Grind the entire quantity of the sample (2 kg–5 kg) using a romer laboratory mill or equivalent.

b) Mix thoroughly to get a well homogenized sample. Take 200 g of the homogenized samples and grind further to fine particle size with a bench mill.

Peanut butter

c) Transfer the entire sample from bottles into a dry vessel.

d) Mix well to get a homogenized sample.

Milled cereals

e) Mix thoroughly to get a well homogenized sample.

Feeds and samples

f) Grind the sample if necessary, using a romer laboratory mill or equivalent, and mix thoroughly to get a well homogenized sample.

3.6.2 Quality control samples

a) At least one quality check should be included with each batch of analysis, and results should be entered into controls charts to monitor the trend.

b) The analyst should prepare an additional control sample by spiking a blank sample at 5 µg/kg using a different batch of the stock standard not used for creating a calibration curve.

c) Leave this control sample in a fridge overnight after spiking for use the following day.

d) The rest of the portion may also be used for proficiency testing at known concentration and by analyzing it alongside each batch of samples.

3.6.3 Reagent blank (RB)

a) Contain the same volumes of all reagents used in the processing of the samples.

b) Be carried through complete procedure and contain the same acid concentration in the final solution as the sample solution used for analysis.

c) Be prepared at the same time and under the same conditions.

3.6.4 Sample extraction

a) Weigh 20 g (±0.0010 g) of the sample in a reagent bottle and add 4 g of sodium chloride.

b) Add 100 ml of methanol/water (70/30, v/v) and blend with ultra turax at 25 000 rpm for 3 min.

c) Filter the extract using a qualitative filter paper into a clean 50 ml falcon tube.

d) Pipette 7.5 ml of the extract into a clean 50 ml falcon tube.

e) Dilute the extract with 15 ml of distilled water and vortex for 1 min.

f) Filter the dilute extract through a glass microfibre filter into a clean 50 ml falcon tube (if filtrate is still turbid, vortex for 5 min at maximum speed).

NOTE *If a microfibre filter is not available, vortex for 5 min at maximum speed and filter through normal qualitative filter paper.*

3.6.5 Sample cleanup

a) Remove two end caps of an immunoaffinity column (Aflatest or equivalent).

b) Attach the column to a reservoir syringe and place into a vacuum manifold.

c) Pass 7.5 ml of filtered diluted extract (equivalent to 0.5 g of sample) through an Aflatest/equivalent column at a rate of about 1–2 drop/s until the air comes through the column.

d) Pass 10 ml of distilled water through the column at a rate of about 2 drops.

e) Repeat the previous step with another 10 ml of distilled water.

f) Remove the excess water in the system by suction.

g) Place the glass tubes into the manifold.

h) Add 1 ml of methanol into the immunoaffinity column.

i) Allow 3–4 drops of methanol into the glass tubes, and then close the taps of the manifold.

j) Allow methanol to soak for 5 min.

k) Then elute the column at a rate of 1–2 drop/s into the glass tubes.

l) Add another 1 ml of methanol and elute the column in the same way after allowing methanol to soak for 5 min.

m) Remove the excess methanol by suction.

n) Remove the glass tubes from the manifold and add 2 ml of HPLC water.

o) Cap and vortex for 1 min and filter with 0.45 µm PTFE filter into an auto sampler vial.

3.6.6 Instrumental analysis (HPLC–FLD)

Analysis of aflatoxins is carried out using an HPLC instrument with FLD. The HPLC analytical conditions are provided below:

- Separation mode: reversed phase chromatography
- Analytical column: L 5 RP C–18 (4.6×250 mm, particle size 5 µm) or equivalent
- Column temperature: 40°C
- Post column reactor coil temperature: 40°C
- Injection volume: 40 µl
- Flow rate: 0.65 ml/min
- Run time: 20 min
- Mobile phase: A is Methanol, B is Water
- A is 50%, B is 50%, Mobile phase: Methanol/water (50/50, v/v)
- Elution mode: Isocratic
- FLD settings: 362 nm (excitation), 455 nm (emission)

3.6.7 Calculation and interpretation of results

$$Aflatoxin,\ ppb = \frac{AxCxV}{A1xW} \quad \ldots\ldots (8)$$

WHERE: V is volume of sample made up, if any
W is weight of sample in grams
A is average area of sample peak
A1 is average area of standard peak
C = Concentration of standard (µg/l)

3.6.8 Method validation

The precision (repeatability and reproducibility) and accuracy of the method is determined using a portion of proficiency testing sample. Information on the validation tests is provided in Table 12.

TABLE 12. Summary of validation experiments

	% Accuracy	Linearity	LOD	LOQ	Working range (ppm or mg/l)	%RSD repeatability	%RSD reproducibility
G2	88.5	r2=0.99999	0.06	0.25	0.25–32.0	5.2	5.2
G1	85.9	r2=0.99999	0.125	0.25	0.25–32.0	10.1	9.9
B2	79.8	r2=0.99999	0.060	0.25	0.25–32.0	4.2	4.2
B1	81.8	r2=0.99998	0.125	0.25	0.25–32.0	3.2	3.2

3.6.9 Schematic diagram of extraction procedure

SAMPLE EXTRACTION

1. Weigh 20 g (±0.0010 g) of sample in a reagent bottle.
2. Add 4 g of NaCl.
3. Add 100 ml of methanol/water (70/30, v/v).
4. Blend with ultra turrax at 25000 rpm for 3 min.
5. Filter extract using a qualitative filter paper into a 50 ml falcon tube.
6. Pipette 7.5 ml of extract into a 50 ml falcon tube.
7. Dilute the extract with 15 ml of water.
8. Vortex for 1 min.
9. Filter the diluted extract through a glass microfiber filter into a 50 ml falcon tube.

SAMPLE CLEANUP

1. Pass 7.5 ml of filtered diluted extract through Aflatest column (1–2 drop/s) until air comes through the column.
2. Pass 10 ml of distilled water (2 drops).
3. Remove excess water by suction.
4. Pass another 10 ml of distilled water (2 drops) and remove excess water by suction.
5. Place glass tubes into the manifold.
6. Add 1 ml methanol to the immunoaffinity column.
7. Allow 3–4 drops of methanol into the glass tubes and close the taps of the manifold.
8. Allow methanol to soak for 5 min.
9. Elute the column into the glass tube (1–2 drop/s).
10. Add another 1 ml of methanol and repeat steps 7, 8 and 9.
11. Remove excess methanol by suction.
12. Add 2 ml of water to eluate.
13. Vortex for 1 min.
14. Filter with 0.45 µm PTFE filter into autosampler vial.

4

Detection of Fumonisins B1, B2 and B3 in animal feed/feed ingredients by HPLC–FLD

4.1 Introduction

Fumonisins are a naturally occurring group of mycotoxins produced mainly by species of *Fusarium* fungi. They are found frequently in corn and corn–based products. Among the different Fumonisin analogues known, Fumonisins B1 (FB1), B2 (FB2) and B3 (FB3) are the most important regarding occurrence and toxicity. FB1 is the most prevalent and most toxic of all FBs and can be found not only in corn but also in other crops such as rice, sorghum, beans and soybeans etc.

Fumonisins can have significant health effects on livestock and other animals. While the evidence for adverse health effects in humans are currently inconclusive, there are concerns that exposure to fumonisins may contribute to various serious adverse health outcomes such as birth defects. Fumonisins (B1 and B2) are also considered possible human carcinogens. Because of the health concerns, several countries and regulatory authorities have established maximum tolerable levels of fumonisins in animal feed/ingredients. Fumonisins in animal feed/feed ingredients can be determined quantitatively using the HPLC–FLD method. In the European Union (EU), limits have been established at 60 mg/kg and 20 mg/kg for the sum of FB1 and FB2, respectively.

4.2 Scope and objective

This SOP describes the procedure for the screening of Fumonisins B1, B2 and B3 in animal feed/ingredients, using the HPLC–FLD. It is also aimed at ensuring the quality and reproducibility of results.

4.3 Principle of the method

Samples are prepared by mixing with an extraction solution (salt and methanol/water) and blending at high speed. Coarse particulate material and precipitates are removed from the sample extract by two filtration steps. The sample extract is then cleanedup using monoclonal antibody–based affinity chromatography. When

the filtered extract is applied to the immunoaffinity column (IAC), which is bound with antibodies specific to FB1, FB2 and FB3, fumonisins in the extract bind to the antibodies. Impurities in the IAC are then removed by washing with Phosphate Buffer Saline (PBS). Fumonisins bound to antibodies are eluted by passing methanol and water through the IAC. These are then derivatized (pre-column) by adding a fumonisin developer mix and are detected by a reversed phase HPLC–FLD system set at 333 nm (excitation) and 477 nm (emission).

4.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may be a serious health risk for the laboratory workers. Further, fumonisins are possible human carcinogens. Suitable gloves should be worn when these chemicals are handled, and the work should be performed in a fume hood.

4.5 Materials

4.5.1 Reagents and solvents

The following reagents and chemical are applicable: Ultrapure water or water of equivalent purity; Methanol (HPLC grade); Acetonitrile (HPLC Grade); Salt (NaCl) (Analytical grade); Acetic acid (Analytical grade); 10×PBS; Fumonisin developer A (Vicam, USA); Fumonisin developer B (Vicam, USA).

4.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Analytical balance (Range 0.0001g–150000 g, Last digit 0.0001g, OHAUS); Filtering device for solvents and water; Phenex™ Nylon Filter Membranes, 0.20 µm (Phenomemex®); Laboratory blender (Waring); Grinder (Moulinex®); Vortex mixer (Velp); Fluted filter papers (24 cm) (Vicam, USA); Glass microfiber filter papers (1.5 µm) (Vicam, USA); Measuring cylinders (25 ml, 50 ml 100 ml, 250 ml); Volumetric flask (5 ml, 10 ml, 50 ml); Stopwatch; Glass beakers; Conical flasks; Glass funnels; Polypropylene tubes with screw cap (50 ml); Disposable syringes (10 ml, 20 ml); Micropipettes (10 µl –100 µl, 100 µl –1000 µl, 500 µl –5000 µl) (Eppendorf/Nichipet); Immunoaffinity column (FumoniTest®, Vicam, USA); Vacuum Pump (Cole Parmer); Extraction manifold with a vacuum chamber, rack and stopcocks (Agilent); Microcentrifuge tubes (2 ml); Amber colour HPLC vials; Glass HPLC vial inserts with bottom polymer feet (250 µl); HPLC–FLD system (Agilent 1100 series).

4.5.3 Solutions

The solutions (and how they are prepared) include:

- Methanol/water (80/20, v/v): Mix 800 ml of methanol with 200 ml of water.
- 1×PBS: Mix 100 ml of 10×PBS with 900 ml of water.
- Mobile phase A: Acetonitrile filtered with 0.20 µm filter.
- Mobile phase B: Water adjusted to pH 2.6 with acetic acid and filtered with 0.20 µm filter.
- Fumonisin developer solution: Add 2.5 µl of developer B to 1 ml of developer A and mix well. This mixed developer solution is stable for 5 days at room temperature.
- Acetonitrile/water (50/50, v/v): Mix 5 ml of acetonitrile with 5 ml of water.
- Methanol/water (50/50, v/v): Mix 5 ml of methanol with 5 ml of water.

4.5.4 Standards

4.5.4.1 *Fumonisin* standard solutions

The analytical standards include: FB1: 50 mg/l (Fermentek Ltd., Jerusalem, Israel); FB2: 50 mg/l (Fermentek Ltd., Jerusalem, Israel); FB3: 50 mg/l (Fermentek Ltd., Jerusalem, Israel).

4.5.4.2 Mixed standard solution (10 mg/l)

- Mix 200 µl each from FB1, FB2 and FB3 standard solutions with 400 µl of methanol/water (50/50, v/v) to prepare a mixed standard solution at 10 mg/l of each fumonisin.
- This mixed standard solution should be stored in the dark at -20°C.

4.5.4.3 Calibration standards

Prepare the calibration standards on the day of the analysis by diluting the mixed solution 1 (MS1) with methanol/water (50/50, v/v) as shown in Table 13.

TABLE 13. Preparation of calibration standards

Calibration standard	Concentration (mg/l)	Volume of 10 mg/l standard (µl)	Volume of methanol/ water (50/50) (µl)
CS 1	5	25	25
CS 2	3.5	17.5	32.5
CS 3	2	10	40
CS 4	1	5	45
CS 5	0.5	2.5	47.5
CS 6	0.1	2.5	247.5

Calibration standards should be prepared on the day of the analysis and one by one because derivatization standards should be injected afterwards – within 3 min – like the sample. Calibration standards should be derivatized by the addition of a fumonisin developer solution before the injection into HPLC.

4.6 Procedure

4.6.1 Sample preparation

a) Grind the feed/ingredient sample using an appropriate grinder.

b) Weigh 25 g (±0.05 g) of the ground sample into a 50 ml polypropylene tube for the analysis.

4.6.2 Quality control samples

A blank sample and a spiked sample should be run as quality control samples with each batch of test samples.

a) **Blank sample**

- A blank feed sample that does not contain fumonisins is used.
- Weigh 25 g (±0.05 g) of the blank sample.

b) **Spiked sample**

- A blank sample is fortified with a known amount of FB1, FB2 and FB3. Weigh 25.50 g (±0.05 g) of ground blank feed sample.

- Fortify the blank sample to yield 0.5 mg/kg concentration of each of FB1, FB2 and FB3 by adding 250 µl each of FB1, FB2 and FB3 standard solutions (50 µg/ml).

4.6.3 Sample extraction

a) Place the sample (unknown sample, blank sample and spiked sample) in a clean blender jar.

b) Add 2.5 g (±0.05 g) of NaCl and 100 ml of methanol/water (80/20, v/v) to the blender jar, and blend at high speed (~22 000 rpm) for 5 min.

c) Remove the cover from the jar and filter the extract through a fluted filter paper.

d) Collect filtrate into a clean glass container and discard the supernatant.

e) Pipette 10 ml of the filtered extract into a 50 ml polypropylene tube.

f) Add 40 ml of 1×PBS to the tube and mix well by shaking.

g) Filter the diluted extract through a 1.5 µm glass microfiber filter into a clean glass container or a 50 ml polypropylene tube.

4.6.4 Immunoaffinity column chromatography

- Remove the top cap from the FumoniTest® immunoaffinity column (IAC), and cut the bottom 1/8 inch off the end of the cap with a sharp blade or scissors.
- Using this top cap as a coupling, attach the syringe barrel to the IAC.
- Pipette or pour the desired volume of diluted extract into the syringe barrel, and then remove the bottom cap from the IAC.
- Achieve the required flow rate through the column, using gravity, a vacuum pump or by pressing the plunger as necessary.

Sample cleanup

a) Pass 20 ml of filtered diluted extract of feed through the FumoniTest® IAC at a rate of about 1 drop until air comes through the column.

b) Wash the column by passing 10 ml of 1×PBS through the IAC at a rate of about 1–2 drop/s until air comes though the column.

c) Elute the column with 1 ml of methanol at a rate of 0.5 drops followed by 1 ml of water at a rate of 0.5 drops.

d) Collect the eluate in a 2 ml micro centrifuge tube.

e) Mix 50 µl of the eluate with 50 µl of fumonisin developer solution thoroughly in a HPLC vial and inject into the HPLC.

4.6.5 Instrumental analysis (HPLC–FLD)

Analysis of the Fumonisin is conducted using HPLC system (Agilent 1 100) consisting of a degasser (G1379A), quaternary pump (G1311A), autosampler (G1313A), column oven with a thermostat (G1316A), and fluorescence Detector (Agilent 1200 series, G1321A). Conditions for chromatographic separation are given below:

a) Separation mode: reversed phase chromatography

b) Analytical column: Zorbax Eclipse XDB C18 (4.6 × 250 mm, particle size 5 µm)

c) Guard column: Zorbax SB–C18 (4.6 × 12.5 mm, particle size 5 µm)

d) Column temperature: 40°C

e) Flow rate: 1 ml/min

f) Injection volume: 50 µl

g) Run time: 22 min

h) Mobile phase: acetonitrile (A), acetic acid in water pH 2.6 (B)

i) Elution mode: isocratic

j) Fluorescence wavelengths: 335 nm (excitation), 440 nm (emission)

k) Software: ChemStation for LC 3D (Rev. A. 10.02) software.

4.6.6 Interpretation of results

The concentration of fumonisins in samples is calculated using the respective calibration curve.

The HPLC run is considered as valid if the following results are achieved:

1) The calibration curves of FB1, FB2 and FB3 should have a coefficient of determination ≥0.95.

2) Recovery of FB1, FB2 and FB3 in samples fortified at 0.5 mg/kg are between 60% and 120%.

If the above two conditions are true, the test samples can be interpreted as follows:

- Negative samples: Samples with HPLC–FLD peak areas below the corresponding peak areas for sum of FB1 and FB2 at 1 mg/kg.
- Positive sample: Samples with HPLC–FLD peak areas above the corresponding peak areas for sum of FB1 and FB2 at 1 mg/kg.

4.6.7 Method validation

Trueness is determined following recommended guidelines (AOAC, 2012) by calculating recovery of FB1, FB2 and FB3 in blank samples spiked with each fumonisin at 0.5 mg/kg. Precision is determined in terms of repeatability (intra–assay precision) and intermediate precision (within–laboratory reproducibility) and is expressed as relative standard deviations (RSD%) of measured analyte concentration in samples spiked at 0.5 mg/kg as shown in Table 14 while uncertainty of measurement is presented in Table 15.

TABLE 14. Summary of method validation

Analyte	Spike level (mg/kg)	Mean recovery (%)	Repeatability (RSD%)	Intermediate precision (RSD%)
FB1	0.5	101.9	12.2	12.2
FB2	0.5	93.8	4.5	4.9
FB3	0.5	64.7	9.3	14.0

Chromatographic separation between the fumonisins is evaluated by calculating the peak resolution, using Eq. (9):

$$Rs = \frac{RT2 - RT1}{0.85(W2 + W1)} \quad \text{.....(9)}$$

where, Rs is peak resolution; RT2 is retention time of peak 2; RT1 is retention time of peak 1; W2 is width of peak 2 at half peak height.

W1 is width of peak 1 at half peak height. The peak resolutions for FB1–FB2; and FB2–FB3 in this SOP are 14.6 and 1.4, respectively.

TABLE 15. Validation summary (measurement uncertainty)

	FB1	FB2	FB3
Limit of detection (mg/kg)	0.080	0.0006	0.005
Limit of quantification (mg/kg)	0.267	0.0017	0.017
Avg. Conc. of spiked samples at 0.5 mg/kg	0.525	0.5	0.611
STDEV (spikes samples at 0.5 mg/kg)	0.049	0.018	0.024
Sample Size	6	6	5
Standard Uncertainty	0.020	0.007	0.011
Degrees of Freedom	5	5	4
95% t–Value	2.571	2.571	2.776
95% Confidence Deviation	0.052	0.018	0.030
Percentage uncertainty	9.889	3.689	4.881

4.6.8 Schematic diagram of extraction procedure

TEST SAMPLE

1. Grind the feed/ingredient sample and weigh 25 g (±0.05 g) of ground sample.

↓

SAMPLE EXTRACTION AND DILUTION

1. Place test sample in the blender jar.
2. Add 2.5 g (±0.05 g) of NaCl.
3. Add 100 ml of methanol/water (80/20, v/v).
4. Blend at high speed (~22 000 rpm) for 5 min.
5. Pour the extract into a fluted filter paper and collect extract.
6. Pipette 10 ml of filtered extract into a clean vessel.
7. Add 40 ml of 1×PBS and mix well.
8. Filter through 1.5 µm glass microfiber filter into a clean vessel

↓

SAMPLE EXTRACTION

1. Pass 20 ml of filtered diluted extract through IAC (1 drops) until air comes through.
2. Pass 10 ml of 1×PBS (1–2 drop/s) through the IAC.
3. Elute fumonisins by passing 1 ml of methanol through IAC (0.5 drop/s or slower).
4. Also pass 1 ml of water through IAC (0.5 drop/s or slower).
5. Collect the eluate (2 ml).
6. Mix 50 µl of eluate with 50 µl of fumonisin developer solution.
7. Inject to HPLC system after 3.5 min.

5

Confirmatory method for the determination of residues of nitrofurans in animal tissues by LC–MS/MS

5.1 Introduction

Nitrofurans (NFs) are a group of broad–spectrum antimicrobials. Due to health concerns, nitrofurans are now prohibited for use in food–producing animals in most jurisdictions. The use of the nitrofurans furazolidone, furaltadone, nitrofurantoin and nitrofurazone as veterinary drugs for food–producing animals is banned by the EU for instance. The minimum required performance limit (MRPL) for nitrofurans in poultry muscle and shrimps is set at 1 µg/kg by Commission Decision 2003/181/EC (EC, 2003) and amending Decision 2002/657/EC (EC, 2002). Because nitrofurans are absorbed, metabolised and distributed very rapidly, only their metabolites are detectable in muscle, liver and kidney as tissue–bound residues. The marker residues identified are 3-amino–2-oxazolidinone (AOZ) for furazolidone, 3-amino–5-methyl-morpholino–2-oxazolidinone (AMOZ) for furaltadone, 1-amino–hydantoin (AHD) for nitrofurantoin and semicarbazide (SEM) for nitrofurazone.

5.2 Scope and objective

This method is applied for confirmatory analysis of furazolidone, furaltadone, nitrofurantoin and nitrofurazone as their tissue bound residues AOZ, AMOZ, AHD and SEM in animal tissue, below the EU MRPL of 1 µg/kg.

5.3 Principle of the method

Meat samples are extracted with a mixture of water, HCl and 2-nitrobenzaldehyde (2-NBA) solution. The pH is adjusted with di–potassium hydrogen phosphate and NaOH; samples vortexed and centrifuged with ethyl acetate and the supernatant evaporated to dryness under Nitrogen. This is later dissolved in acetic acid (and mobile phase) filtered through 0.45 µm filters prior to analysis by LC–MS/MS system. Since nitrofurans bind to proteins, the metabolites should first be liberated through mild acid hydrolysis. After derivatization with 2-NBA and extraction from the matrix, the nitrofuran metabolites are determined by LC–MS/MS.

5.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may present a serious health risk for the laboratory workers. Suitable gloves should be worn when handling these chemicals are handled, and the work should be performed in a fume hood.

Nitrofurans are susceptible to photodegradation. During sample preparation and analysis and therefore, samples should be protected from light to avoid photo-degradation.

5.5 Materials

5.5.1 Reagents and solvents

The following reagents and chemicals are applicable:

Deionized water; Methanol; Ethyl acetate; HCl; 2-NBA; Nitrogen gas; Acetic acid; Ammonium acetate; Dipotassium hydrogen phosphate (K_2HPO_4); NaOH.

5.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: 0.45 µm filters; Centrifuge; Vortex mixer; Freezer (-20°C); Food processor; Analytical balance; Polypropylene tubes; Micropipettes; Rotary shaker; Centrifuge; Nitrogen gas; Water bath; Ultrasonic bath; Autosampler vials; FLEXAR binary HPLC pump or equivalent; FLEXAR automatic injector or equivalent; Thermo Hypersil Gold column: 50 × 2.1 mm, 3 µm grain size; Precolumn C18, 3.5 µm, 2.1×10 mm.

5.5.3 Solutions

The solutions (and how they are prepared) include:

- 50 mM 2-NBA solution: Weigh 189 mg ± 1 mg of nitrobenzaldehyde in an amber colored flask and make up to 25 ml with methanol.
- 0.1 M dipotassium hydrogen phosphate: Weigh 8.71 g of dipotassium hydrogen phosphate (anhydrous) in a 500 ml flask. Add water and make up to 500 ml.
- 0.5 mM ammonium acetate: Weigh 3.8 g (±0.1 g) of ammonium acetate into a 100 ml flask. Dissolve with water and make up to 100 ml. Transfer 1 ml of this solution (0.5 M ammonium acetate) to a 1 litre flask and adjust the volume with water.
- Acetic acid 0.01%: Pipette 1 ml of acetic acid into a 100 ml flask and adjust the volume with water. Transfer 500 µl of this solution (1% acetic acid) to a 50 ml vial and adjust the volume with water.
- 1 M NaOH: Weigh 4 g of sodium hydroxide in a beaker and dissolve with 50 ml of water. Transfer to a 100 ml flask and make up the volume with water.

5.5.4 Standards

Relevant reference/analytical standards in Table 16 are used.

TABLE 16. Analytical standards used

Compound Family	Molecule	Internal Code	Batch Number	Purity	Origin/ manufacturer	Storage Temperature °C	Stock solution concentration
Nitrofurans	AMOZ	171–4	SZBD049XV	99.90%	Sigma	-20	1g/l
Nitrofurans	AMOZ–D5	173–2	SZBD042XV	99.10%	Sigma	-20	1g/l
Nitrofurans	AOZ	170–2	SZBD196XV	99.60%	Sigma	-20	1g/l
Nitrofurans	AOZ–D4	172–2	SZBD015XV	99.30%	Sigma	-20	1g/l
Nitrofurans	SEM–HCl	183–3	MKBL5740V	99.7%	Sigma	-20	1g/l
Nitrofurans	AHD	174–2	G0813	98.20%	Santa cruz	-20	1g/l

5.5.4.1 Stock solutions (1 000 mg/l)

- Stock solutions of each standard (AOZ, AMOZ, AHD, and SEM) at 1 000 mg/l are prepared independently in methanol by dissolving well and transferring the contents of each vial into polypropylene bottles. Stock solutions are stable for at least two years if stored in a -20°C freezer.
- To prepare stock solution of each internal standard (AOZ–D4 and AMOZ–D5) at 1 000 mg/l, weigh the amount of powder corresponding to 1 mg of active substance, and dissolve and adjust with 1 ml of methanol.
- Prepare from the 1 mg/l solution containing each standard, a solution of 100 µg/l in methanol. This solution can be stored for 1 week in the refrigerator (≤ + 4°C).

5.5.4.2 Intermediate solutions (10 mg/l)

- From the stock solutions, intermediate solutions are prepared at 10 mg/l containing each standard in methanol. This solution can be kept for 6 months in the refrigerator (≤ + 4 °C).
- From the 10 mg/l solution, a solution containing each standard at 100 µg/l is prepared in methanol. This solution can be kept for 3 months in a refrigerator (≤ + 4°C).
- In addition, from the 100 µg/l solution containing each standard, a solution of 10 µg/l is prepared in methanol. This solution can be stored for 1 week in the refrigerator (≤ + 4°C).

5.6 Procedure

5.6.1 Sample preparation

- Meat samples are thawed to room temperature and lean meat portions (fat removed) individually minced in a domestic food processor.
- Weigh 1.0 g (±0.1 g) into 50 ml polypropylene tubes and add 6 ml of methanol/water (50/50, v/v).
- Shake for 15 min on a rotary shaker at 100 rpm/min followed by centrifugation for 10 min at 3 500 rcf at 4°C.
- Remove the supernatant and add 6 ml of a methanol/water mixture (75/25, v/v).
- Shake again for 10 min at 100 rpm/min, and centrifuge for 10 min at 3 500 rcf at 4°C.
- Remove the supernatant and add 6 ml of methanol to the remaining material; shake for 10 min and centrifuge for 10 min at 3 500 rcf and remove the supernatant again.

- Finally, add 2 ml of water to the extract.
- Agitate on the vortex for 20 s and centrifuge for 10 min at 3 500 rcf at 4°C and remove the supernatant.
- Add 50 µl of the internal standard solution to the mixture.

5.6.2 Negative control sample and calibration range of spiked samples

- Prepare 5 tubes each containing 1 g (± 0.1 g) of sample of matrix free of nitrofurans.
- Add 50 µl of the working solution and a mixture of internal standards at 0.1 µg/ml in each tube.
- The first tube is considered as a negative control sample, the other tubes are considered as samples spiked at 0.55 µg/kg, 15 µg/kg, 1.5 µg/kg after adding 50 µl, 100 µl, 150 µl and 200 µl of the working solution (a mixture of standards at 0.01 µg/ml).

5.6.3 Extraction procedure (derivatization)

- Add 4 ml of water, 0.5 ml of 1 mol/l HCl, and 150 µl of 2-NBA solution.
- Mix gently and agitate for 10 s in vortex.
- Protect from the light and incubate in a water bath at 37°C for 16 hrs.

5.6.4 Extraction of NPAOZ, NPAMOZ, NPAHD and NPSEM derivatives

- Add 5 ml of 0.1 mol/l dipotassium hydrogen phosphate followed by 0.12 ml of 1 mol/l NaOH.
- Shake for about 15 s by vortexing and check the pH with a strip.
- Adjust the pH to 7 (±0.5) by adding NaOH if necessary.
- Add 5 ml of ethyl acetate and shake for 20 min at 100 rpm.
- Centrifuge for 5 min at 3 000 g and 4°C.
- Transfer the supernatant into a 10 ml plastic tube.
- Re–extract with 3 ml of ethyl acetate and shake for 20 min at 100 rpm, followed by centrifugation for 10 min at 3 000 rcf and 4°C.
- Transfer the supernatant to the same plastic tube.
- Evaporate mixture to dryness under N_2 at 45°C.
- Dissolve with 400 ml of acetic acid 0.01% and place into the ultrasonic tank for about 1 min.
- Transfer content to microcentrifuge tubes and centrifuge at 13 200 rpm for 20 min at 4°C.
- Remove the supernatant using a 1 ml syringe and filter through a 0.45 µm filter (diameter 13).

5.6.5 Instrumental analysis (LC–MS/MS)

The LC–MS/MS system consists of a binary UHPLC pump (FLEXAR or equivalent), and auto sampler (FLEXAR or equivalent) from PerkinElmer, API4000 triple quadrupole mass spectrometer with Ion Source Turbo Spray from AB Sciex. The conditions include:

a) Analytical column: Thermo Hypersil Gold column (50 × 2.1 mm, particle size 3 µm).

b) Column temperature: 30°C

c) Injection volume: 40 µl

d) Run time: 6 min

e) Flow rate: 500 µl/min

f) Mobile Phase: Ammonium acetate 0.5 mmol/l (A), methanol (B)

g) Elution mode: Linear gradient (Table 17).

TABLE 17. Mobile phase gradient

Time (min)	Solvent A (%)	Solvent B (%)
0.1	65	35
1.5	30	70
2.5	30	70
3.5	65	35
6.0	65	35

5.6.6 Mass spectrometry

Analysis is carried out using an electrospray ionization source in positive mode. The operational conditions for this mode include:

a) Ionspray voltage: 5.5 kV

b) Source temperature: 450°C

c) Curtain gas: 10 psi

d) Ion source gases 13 psi and 9 psi.

The optimal multiple reaction monitoring (MRM) parameters are summarized in Table 18.

TABLE 18. MRM parameters

	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	DP	CE	CXP
NP–AOZ–D4	240.1	134.0	100	66	19	8
NP–SEM	209.0	166.0 191.9	100 100	41 46	15 17	8 4
NP–AOZ	236.2	134.0 104.1	100 100	36 36	17 29	6 8
NP–AHD	249.1	134.0 104.1	100 100	46 46	21 31	6 8
NP–AMOZ–D5	340.2	296.2	100	41	23	8
NP–AMOZ	335.1	262.1 291.1	100 100	79 76	23 25	12 8

5.6.7 Calculation and interpretation of results

The presence of nitrofurans in extracts is confirmed when defined criteria (EC, 2002) are met as follows:

i) The relative retention time of the detected analyte should be identical to that of the spiked samples within ± 2.5%.

ii) At least two specific transitions of each nitrofurans should be detected in the samples to be analyzed with S/N > 3.

iii) The relative intensities of the transitions in the extracts should be compared with the relative intensities from spiked samples (within the same concentration range) and fall within the following tolerable values (Table 19).

TABLE 19. LC–MS/MS acceptance criteria for signal intensity

Ion m/z Relative intensity (% base peak) base peak) base)	LC–MS/MS
> 50%	± 20%
> 20%–50%	± 25%
> 10%–20%	± 30%
≤ 10%	± 50%

Quantification is done based on the major transition and considering the internal standard. The calibration line is established from the control sample and spiked samples.

5.6.8 Method validation

The method provides reliable identification of unauthorized substances (requiring a minium of 4 identification points) and substances with an established MRL and quantification of analytes for MRL compounds, as indicted elsewhere (EC, 2002). LC–MS/MS methods fulfill these requirements with one precursor ion and at least two transition product ions (1.5 IP each one).

Specificity

- Twenty blank samples of animal tissue from different sources are analyzed to verify the absence of interfering peaks.
- Each molecule is specifically identified by two minimum transitions (parent ion > product ions) and retention time. Specificity is evaluated from the examination of chromatograms.

Linearity

- The linearity of the analytical method is validated using matrix calibration curves for each compound at different concentration levels to prevent matrix effects.
- For each series, the equation of the calibration ranges is calculated from blank and spiked samples at 0.1 µg/kg, 0.2 µg/kg, 0.35 µg/kg and 0.5 µg/kg.
- The equation of the calibration curve is established. The R^2 coefficient of the calculated regression curves should always be above 0.97 (Table 20).

TABLE 20. Coefficient of determination (R^2)

Analytes	Slope a		Ordinate b		Coef det. R^2	
	Signal 1 (+intense)	Signal 2 -intense)	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 -intense)
NP–AOZ	1.20	0.56	-0.002	0.03	0.9997	0.998
NP–AMOZ	0.40	0.098	-0.003	-0.0004	0.998	0.999
NP–AHD	1.07	0.52	0.001	0.01	0.9995	0.9989
NP–SEM	0.94	0.68	0.15	0.14	0.998	0.998

Recovery

The % recovery is determined using Eq. (10) following the procedure summarized in Table 21.

$$Recovery\ (\%) = \frac{Experimental\ Concentration\ x\ 100}{Theoretical\ Concentration} \quad \text{.....(10)}$$

TABLE 21. Analytical method recovery

Analyte	Theoretical concentration (µg/kg)	Experimental concentration (µg/kg)	Recovery (%)
NP–AOZ	1	0.97	97
NP–AMOZ	1	1.05	105
NP–AHD	1	0.93	93
NP–SEM	1	1.02	102

Repeatability

- Repeatability (intraday precision) is evaluated by performing replicates
- Within–laboratory reproducibility (interday precision) is evaluated by analyzing samples over the course of three consecutive days.
- For all the measured compounds, the analyzed recovery should comply with certain established ranges (EC, 2002) with acceptable coefficients of variation (CVs) below 20% as shown in Table 22.

TABLE 22. Coefficient of variation on reproducibility

Analyte	CV% (Signal)	
	Signal 1 (+intense)	Signal 2 (-intense)
NP–AOZ	8.7	11.2
NP–AMOZ	12.4	11.9
NP–AHD	7.2	10.8
NP–SEM	8.9	8.0

Repeatability of Relative Retention Time and Ratio

The method is repeatable (EC, 2002) and the CVs obtained should be less than 2.5%. Tables 23 and 24 summarize the relative retention times and ratios.

TABLE 23. Repeatability of relative (retention) time

Analyte	%CV (RT)
NP–AOZ	0.1
NP–AMOZ	0.6
NP–AHD	0.1
NP–SEM	0.1

TABLE 24. Relative ratios

Analyte	%CV (Ratio)
NP–AOZ	6.9
NP–AMOZ	5.2
NP–AHD	6.9
NP–SEM	6.9

Accuracy

This is determined using Eq. (11)

$$Accuracy = \frac{Estimated\ concentration - Theoretical\ concentration\ x\ 100}{Theoretical\ concentration} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (11)$$

Ruggedness

The method is rugged as elaborated in Table 25.

TABLE 25. Method ruggedness

Analytes	Theoretical concentration µg/kg		Estimated concentration µg/kg		Accuracy %	
	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1(+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)
NP–AOZ	1	1	0.97	0.87	-3.2	-12.7
NP–AMOZ	1	1	1.05	1.06	4.8	6.0
NP–AHD	1	1	0.93	0.84	6.5	16.2
NP–SEM	1	1	1.02	0.90	2.3	-10.1

The decision limit (CCα) and detection capability (CCβ)

The CCα and CCβ are determined as guided elsewhere (EC, 2003) with α and β of ≤ 1% and 5%, respectively. An overview of validation results for CCα and CCβ is shown in Table 26.

TABLE 26. Decision limit and detection capability

Analyte	Decision limit CCα (µg/kg)		Detection capability CC β (µg/kg)	
	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)
NP–AOZ	0.013	0.07	0.02	0.09
NP–AMOZ	0.01	0.02	0.01	0.03
NP–AHD	0.03	0.06	0.03	0.08
NP–SEM	0.16	0.64	0.2	0.78

The decision limits obtained should all be well below the recommended concentration (CRL, 2007) with reasonable uncertainty values (Table 27).

TABLE 27. Method uncertainty

Molecule	Standard deviation (signal 1)	Uncertainty (2*U)
NP–AOZ	0.1	0.2
NP–AMOZ	0.07	0.14
NP–AHD	0.05	0.10
NP–SEM	0.08	0.16

Evaluation of identification criteria

The CCβ value (the second transition in MS/MS) calculated for the different analyte is less than MRPL and it could be identified in at least 19 out of 20 different representative samples (Table 28).

TABLE 28. Summary of CCβ values

Analyte	Spiking level (µg/kg)	Identification criteria (/20)	CCβ (µg/kg) Most intense signal
NP–AOZ	1	20	0.09
NP–AMOZ	1	20	0.03
NP–AHD	1	20	0.08
NP–SEM	1	20	0.78

5.6.9 Schematic diagram of extraction procedure

SAMPLE PREPARATION

1. Weigh 1.0 g (± 0.1 g) was weighed into a 50 ml polypropylene tube.
2. Add 6 ml of methanol/water (50/50, v/v).
3. Shake for 15 min on a rotary shaker at 100 rpm/min.
4. Centrifuge at 3 500 rcf at 4°C for 10 min.
5. Eliminate the supernatant and add 6 ml of methanol/water (75/25, v/v).
6. Shake for 10 min at 100 rpm/min.
7. Centrifuge 3 500 rcf at 4 °C for 10 min.
8. Eliminate the supernatant and add 6 ml of methanol.
9. Shake for 10 min.
10. Centrifuge at 3 500 rcf at 4 °C for 10 min.
11. Eliminate the supernatant.
12. Add 2 ml of water and vortex for 20 s.
13. Centrifuge at 3 500 rcf at 4 °C for 10 min.
14. Add 50 µl of the internal standards solution.

↓

DERIVATIZATION

1. Add 4 ml of water, 0.5 ml of 1 mol/l HCl, and 150 µl of 2-NBA solution.
2. Mix gently and vortex for 10 s.
3. Incubate in a water bath at 37°C for 16 h (protect from light).

↓

EXTRACTION OF DERIVATIVES

1. Add 5 ml of 0.1 mol/l dipotassium hydrogen phosphate and 0.12 ml of 1 mol/l NaOH.
2. Vortex for 15 s.
3. Adjust pH to 7 (±0.5) with NaOH.
4. Add 5 ml of ethyl acetate and shake at 100 rpm for 20 min.
5. Centrifuge at 3 000 g at 4°C for 5 min.
6. Transfer the supernatant into a plastic tube.
7. Re–extract with 3 ml of ethyl acetate, shake 20 min at 100 rpm/min.
8. Centrifuge at 3 000 g at 4°C for 5 min.
9. Combine supernatant with previous supernatant in the tube.
10. Evaporate to dryness under nitrogen at 45°C.
11. Dissolve the dry residue in 400 µl of acetic acid 0.01%.
12. Place in an ultrasonic bath for 1 min.
13. Transfer solution to microcentrifuge tube.
14. Centrifuge at 13 200 rpm at 4°C for 20 min.
15. Filter the supernatant though 0.45 µm filter into an autosampler vial.

6

Detection of Ochratoxin–A in animal feed/feed ingredients by HPLC–FLD

6.1 Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species including *Aspergillus ochraceus, A. carbonarius, A. niger* and *Penicillium verrucosum*. It is found in a wide variety of agricultural commodities worldwide, ranging from cereal grains to dried fruits to wine and coffee. OTA contamination in animal feed can have major economic impacts on the livestock and food industry. OTA is known to cause nephrotoxicity and renal tumours in a variety of animal species. As a result of OTA contamination in animal feed, animal products intended for human consumption can also become contaminated. Although human health effects of OTA are less well characterized, various studies have linked OTA exposure with the human diseases Balkan endemic nephropathy and chronic interstitial nephropathy, as well as other renal diseases. OTA has also been classified as a possible carcinogen to humans (Group 2B). Because of health concerns, several countries and regulatory authorities have established maximum tolerable levels of OTA in animal feed. OTA in animal feed/ feed ingredients can be determined quantitatively using HPLC–FLD method.

6.2 Scope and objective

This SOP describes the procedure for screening of ochratoxin A in animal feed and feed ingredients, using HPLC–FLD. The SOP also aims at ensuring the accuracy, quality and reproducibility.

6.3 Principle of the method

Samples are prepared by mixing with an extraction solution (salt and methanol/ water) and blending at high speed. Coarse particulate material and precipitates are removed from the sample extract by two filtration steps. A sample extract is then cleanedup using monoclonal antibody–based affinity chromatography. When the extract is applied to the immunoaffinity column (IAC), which is bound with antibodies specific to OTA, OTA in the extract bind to antibodies. Then impurities in the IAC are removed by several washing steps. OTA bound to antibodies are eluted with methanol and are detected by the HPLC–FLD system set at 335 nm (excitation) and 440 nm (emission).

6.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may present a serious health risk for the laboratory workers. Further, OTA is considered a possible carcinogen to humans. Suitable gloves should be worn when chemicals/OTA standards are handled, and the work should be performed in a fume hood.

6.5 Materials

6.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Ultrapure water or water of equivalent purity; Methanol (HPLC grade); Acetonitrile (HPLC Grade); Salt (NaCl) (Analytical grade); Acetic acid (Analytical grade); 10×PBS.

6.5.2 Equipment/instruments and consumables

The following apparatus and materials are applicable: Analytical balance (Range 0.0001g–150000 g, Last digit 0.0001g, OHAUS); Filtering device for solvents and water; Phenex™ Nylon Filter Membranes, 0.20 µm (Phenomemex®); Laboratory blender (Waring), Grinder (Moulinex®); Laboratory centrifuge (Centurion scientific Ltd.); Vortex mixer (Velp), Fluted filter papers, 24 cm, (Vicam); Glass microfiber filter papers, 1.5 µm (Vicam); Measuring cylinders (25 ml, 50 ml, 100 ml, 250 ml); Volumetric flask (5 ml, 10 ml, 50 ml); Stopwatch; Glass beakers; Conical flasks; Glass funnels; Polypropylene tubes with screw cap (15 ml and 50 ml); Sterile disposable syringes (10 ml, 20 ml); Micropipettes, 0.5 µl –10 µl, 2 µl –20 µl, 10 µl –100µl, 100 µl –1 000 µl, 500 µl–5 000 µl (Eppendorf/ Nichipet); Immunoaffinity column (OchraTest®, Vicam); Vacuum Pump (Cole Parmer); Extraction manifold with a vacuum chamber, rack and stopcocks (Agilent); Amber colour HPLC vials; Glass HPLC vial inserts with bottom polymer feet, 250 µl; HPLC–FLD system (Agilent 1 100 series).

6.5.3 Solutions

The solutions (and how they are prepared) include:

- Methanol/water (80/20, v/v): Mix 400 ml of methanol with 100 ml of water.
- 1×PBS: Mix 100 ml of 10×PBS with 900 ml of water.
- Mobile phase A (methanol): Methanol filtered through a 0.20 µm nylon membrane filter.
- Mobile phase B (acetonitrile): Acetonitrile filtered through a 0.20 µm nylon membrane filter.
- Mobile phase C (0.4% acetic acid): Dilute 2 ml of acetic acid up to 500 ml with water and filter through 0.20 µm nylon membrane filter.

6.5.4 Standards

6.5.4.1 Standard solution

The analytical standard is Ochratoxin A at 10 mg/l (Fermentek Ltd., Jerusalem, Israel).

6.5.4.2 Working standard solution (WS1, 500 µg/l)

a) Working standard solution 1 (WS1, 500 µg/l)

WS 1 (500 µg/l) is used to spike blank feed/ingredient samples and to prepare calibration standards. To prepare WS1, dilute 250 µl of OTA standard solution (10 mg/l) with the mobile phase up to 5 ml in a 5 ml volumetric flask. Store this solution at -20°C.

b) Working standard solution 2 (WS2, 50 µg/l)

WS 2 (50 µg/l) is used to prepare calibration standards. To prepare WS 2, dilute 500 µl of WS1 (500 µg/l) up to 5 ml with the mobile phase in a 5 ml volumetric flask. Store this solution at -20°C.

6.5.4.3 Calibration standards

External calibration standards are used in the procedure. Prepare seven calibration standards by diluting WS 1 (500 µg/l) or WS 2 prepared above with a mobile phase according to Table 29. Once prepared, these calibration standards are stable for up to 4 months at -20°C.

TABLE 29. Preparation of calibration standards

Calibration Standard	Concentration (µg/l)	Preparation		
		Volume of WS 1 (µl)	Volume of WS 2 (µl)	Volume of mobile phase (µl)
CS 1	0.5	n.a.	100	9900
CS 2	5	n.a.	100	500
CS 3	25	n.a.	500	
CS 4	50	100	n.a.	900
CS 5	100	200	n.a.	800
CS 6	150	300	n.a.	700
CS 7	250	500	n.a.	500

n.a: No WS volume added

6.6 Procedure

6.6.1 Sample preparation

Grind the feed/ingredient sample using the grinder and weigh 25 g (±0.05 g) of the ground sample into a 50 ml polypropylene tube.

6.6.2 Quality control samples

Blank samples and spiked samples should be run as quality control samples with each batch of test samples.

a) Blank sample:

- A blank feed sample containing no OTA is used as the negative control.
- Weigh 25 g (±0.05 g) of ground blank feed sample into a 50 ml polypropylene tube.

b) Spiked sample:

- A blank feed/ingredient sample fortified with a known amount of OTA is used as the positive control.
- Weigh 25.50 g (±0.05 g) of ground blank feed sample.
- Fortify the blank sample to yield 10 µg/kg concentration of OTA by adding 500 µl of the above described working solution (WS1).
- Let the fortified sample stand for 15 min before continuing with the procedure.

6.6.3 Sample extraction

- Place the test portion (unknown, blank and spike samples) in a clean blender jar.
- Add 2.5 g (±0.05 g) of NaCl and 100 ml of methanol/water (80/20, v/v) to the blender jar.
- Close the blender jar tightly and blend at high speed (~22 000 rpm) for 1 min.
- Filter the extract through a fluted filter paper and collect the filtrate into a clean glass container and discard the supernatant.
- Pipette out 10 ml of the filtered extract into a 50 ml polypropylene tube.
- Add 40 ml of 1×PBS to the tube and mix well by shaking.
- Filter the diluted extract through 1.5 µm glass microfiber filter into a clean container or a 50 ml polypropylene tube.

6.6.4 Immunoaffinity column chromatography

NOTE: *Remove the top cap from the OchraTest® Immunoaffinity column (IAC), and cut the bottom 1/8 inch off the end of the cap with a sharp blade/ scissors. Using this top cap as a coupling, attach the syringe barrel to the IAC.*

a) Pipette or pour the desired volume of diluted extract into the syringe barrel and then remove the bottom cap from the IAC.

b) Achieve the required flow rate through the IAC, using gravity, a vacuum pump or by pressing the plunger as necessary.

c) Pass 20 ml of filtered diluted extract of feed through the OchraTest® IAC at a rate of about 1 drop until air comes through the column.

d) Wash the IAC by passing 10 ml of 1×PBS through the column at a rate of about 1–2 drops.

e) Pass 10 ml of purified water (1–2 drops) until air comes through the column.

f) Elute the column with 1.5 ml of methanol at a rate of about 1 drop or slower and collect the eluate in an HPLC vial.

6.6.5 Instrumental analysis (HPLC–FLD)

Analysis of OTA is carried out using an Agilent 1 100 HPLC system consisting of a micro–vacuum degasser, (G1379A), quaternary pump (G1311A), auto–sampler (G1313A), thermostatted column compartment (G1316A), and a fluorescence detector (G1321A, Agilent 1 200 series). The analytical conditions are as follows:

a) Separation mode: reversed phase chromatography

b) Analytical column: Zorbax Eclipse XDB C18 (4.6×250 mm, particle size 5 µm)

c) Guard column: Zorbax SB–C18 (4.6×12.5 mm, particle size 5 µm)

d) Column temperature: 30°C

e) Injection volume: 25 µl

f) Flow rate: 1 ml/min

g) Run time: 15 min

h) Mobile phase: methanol (A), acetonitrile (B), 0.4% acetic acid in water (C) (35/35/30, v/v/v)

i) Elution mode: isocratic

j) FLD settings: 333 nm (excitation), 477 nm (emission).

6.6.6 Interpretation of results

The concentration of OTA in a sample will be calculated using the calibration curve.

The HPLC run is considered valid if the following results are achieved:

- The calibration curve of OTA should give a coefficient of determination / correlation coefficient (R^2) ≥ 0.95.
- The recovery controls or quality control samples fortified at 10 µg/kg should yield recoveries between 70% and 110%.

If the above two conditions are true, the test samples can be interpreted as follows.

Positive samples:

- Samples with corresponding HPLC–FLD peak area for OTA above 50 µg/kg.
- Any other result should be reported as negative.

6.6.7 Method validation

Trueness is determined by calculating recovery of OTA from blank samples spiked with OTA at 10 µg/kg. Precision is determined in terms of repeatability (intra–assay precision) and intermediate precision (within–laboratory reproducibility) and is expressed as relative standard deviations (RSD%) of measured analyte concentration in samples spiked 10 µg/kg. Table 30 and 31 summarize recovery, precision and measurement uncertainty findings.

TABLE 30. Method recovery and precision

Analyte	Spike level (µg/kg)	Mean recovery ± SD (%)	Repeatability (RSD%)	Intermediate precision (RSD%)
OTA	10	86.3 ± 2.1	3.4	4.1

TABLE 31. Measurement uncertainty

Parameter	OTA
Limit of Detection (µg/kg)	0.08
Limit of Quantification (µg/kg)	0.25
Avg. Conc. of spiked samples at 10 µg/kg	10.00
STDEV of spiked samples at 10 µg/kg	0.57
Sample Size	9
Standard Uncertainty	0.19
Degrees of Freedom	8
95% t–Value	2.31
95% Confidence Deviation	0.44
Percentage Uncertainty	%

6.6.8 Schematic diagram of the extraction procedure

TEST SAMPLE	1. Grind the feed/ingredient sample and weigh 25 g (±0.05 g) of ground sample.
↓	
SAMPLE EXTRACTION	1. Place test sample in the blender jar. 2. Add 2.5 g (±0.05 g) of NaCl. 3. Add 100 ml of methanol/water (80/20, v/v). 4. Blend at high speed (~22 000 rpm) for 1 min. 5. Pour extract into a fluted filter paper and collect extract.
↓	
EXTRACT DILUTION	1. Pipette 10 ml of filtered extract into a clean vessel. 2. Add 40 ml of 1×PBS and mix well. 3. Filter through 1.5 µm glass microfiber filter into a clean vessel.
↓	
COLUMN CHROMATOGRAPHY	1. Pass 20 ml of filtered diluted extract through IAC (1 drop/s) until air comes through. 2. Pass 10 ml of 1×PBS (1–2 drop/s) through the IAC. 3. Pass 10 ml of purified water until air comes through the IAC. 4. Elute OTA by passing 1.5 ml of methanol through IAC (1 drop or slower). 5. Collect eluate to HPLC vial and inject.

7

Multiclass and multimatrix method for analysis of veterinary drug residues in foods

7.1 Introduction

Veterinary drugs are widely used in food producing animals to cure or prevent diseases and to increase productivity. However, this may result in residues of the veterinary drugs in the food. Therefore, to ensure food safety it is very important to analyze residues of the veterinary drugs in animal products. When testing individual analytes, laboratories may use an analytical method for every family of veterinary drugs. This is costly and cumbersome and thus currently, the use of multiclass methods has become more and more frequent. This method enables simultaneous analysis of seven classes of veterinary drug residues in six different animal products.

7.2 Scope and objective

This liquid chromatography tandem mass spectrometry (LC–MS/MS) method can be used for the simultaneous determination of 34 veterinary drug residues, belonging to seven classes (Stilbenes, Amphenicols, Beta–Agonist, Growth promoters, Sulphonamides, Resorcylic Acid Derivative and Benzimidazoles) in a number of animal products including: beef, pork, poultry, fish, shrimp, milk, honey and eggs. The method was successfully validated, and it has proven to be suitable for regulatory purposes. The scope is further summarized in Table 32.

TABLE 32. Scope of the analytical method

Analyte	Working range (µg/kg)
Amphenicols: Thiamphenicol	10–150
Chloramphenicol	0.10–1.5

(con't)

TABLE 32. Scope of the analytical method (con't)

Analyte	Working range (µg/kg)
Benzimidazoles: Albendazole Albendazole sulfone Albendazole amine sulfone Albendazole Sulfoxide Febantel Fenbendazole Fenbendazole sulfone Flubendazole Levamisole Mebendazole Mebendazole amine Oxfendazole Oxibendazole Oxibendazole amine Thiabendazole	5.0–75
Stilbenes: Diethylstilbestrol Hexestrol Dienestrol	1.0–15
Resorcylic Acid Derivative: Zeranol	1.0–15
Growth Promoter: Ractopamine	1.0–15
Beta-Agonist: Clenbuterol	0.10–1.5
Sulfonamides: Sulfadiazine Sulfamethazine Sulfaguanidine Sulfanilamide Sulfathiazole Sulfadoxine Sulfaclozine Sulfachloropyridazine Sulfadimethoxine Sulfamerazine Sulfamethizole Sulfamethoxazole Sulfamonomethoxin Sulfapyridine Sulfaquinoxaline Sulfisoxazole Trimethoprim	10–150

7.3 Principle of the method

The method is based on detection, quantification and confirmation of the different compounds using Triple Quadrupole Mass Spectrometer, with Electrospray ionization. Samples are extracted with acetonitrile and then cleanedup by freezing out before the LC–MS/MS analysis.

7.4 Safety considerations and precautions

Always wear gloves, safety googles, laboratory coat. Remember that acetonitrile vapors are dangerous and work in an appropriate fume hood.

7.5 Materials

7.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Deionized water, Acetonitrile, HCl, Acetic acid and Methanol.

7.5.2 Equipment/instruments and consumables

The following apparatus and materials are applicable: Sample grinder; Analytical Balance; Electronic balance; Volumetric flasks; Micropipettes; Water bath; Liquid dispensers from 1 to 10 ml; Multivortex; Vortex; Turbovap evaporator; Centrifuge; Freezer; 0.22 µm syringe filter; Chromatographic column Zorbax Eclipse Plus C18, 4.6 mm×50 mm, 1.8 µm; LC Agilent 1 200 series Binary pump with QQQ Mass spectrometer, Agilent 6,410.

7.5.3 Solutions

The solutions (and how they are prepared) include:

Mobile phase:

- Solvent A–500 ml of deionized water is mixed with 60 µl of glacial acetic acid to achieve a 2 mmol/l concentration of acetic acid. This is then passed through a 0.45 µm filter with the help of a vacuum system.
- Solvent B–Mass grade acetonitrile (unfiltered); HCl 0.5 mol/l: Add 25 ml of concentrated HCl in a 500 ml tube with 250 ml of deionized water and fill up to 500 ml with deionized water.

7.5.4 Standards

7.5.4.1 Reference standard

High purity analytical standards of the specific substances summarized in Table 33 are used and stored in freezing.

TABLE 33. Reference/analytical standards

Analyte	Brand	Part number
Amphenicols		
Thiamphenicol	Sigma Aldrich	16715
Chloramphenicol	Sigma Aldrich	31667
beta-Agonists		
Ractopamine	Sigma Aldrich	34198
Zilpaterol	Sigma Aldrich	32379
Carazolol	Dr. Ehrenstorfer	10968000
Levamisole	Sigma Aldrich	31742
Salbutamol	Sigma Aldrich	46725
Clenbuterol	Sigma Aldrich	C5423
Sulfonamides		
Sulfaguanidine	Sigma Aldrich	32402
Sulfanilamide	Sigma Aldrich	46874

(con't)

TABLE 33. Reference/analytical standards (con't)

Analyte	Brand	Part number
Sulfathiazole	Sigma Aldrich	46902
Sulfadiazine	Sigma Aldrich	35033
Sulfapyridine	Dr. Ehrenstorfer	c 17000100
Sulfamethizole	Sigma Aldrich	46802
Sulfamethoxypyridazine	Sigma Aldrich	S7257
Sulfamerazine	Sigma Aldrich	46826
Sulfamethazine	Sigma Aldrich	46802
Sulfamonomethoxine	Sigma Aldrich	32091
Sulfachloropyridazine	Sigma Aldrich	46778
Sulfadoxine	Sigma Aldrich	31736
Sulfamethoxazole	Sigma Aldrich	31737
Sulfisoxazole	Sigma Aldrich	31739
Sulfaquinoxaline	Sigma Aldrich	45662
Sulfadimethoxine	Sigma Aldrich	46794
Sulfaclozine	Sigma Aldrich	32421
Trimethoprim	Sigma Aldrich	46984
Resorcylic Acid derivative		
Zeranol	Sigma Aldrich	Z0292
Stilbenes	Sigma Aldrich	
Diethylstilbestrol	Sigma Aldrich	46207
Dienestrol	Sigma Aldrich	46190
Hexestrol	Sigma Aldrich	46320
Benzimidazoles		
Thiabendazole	Sigma Aldrich	45684
Oxibendazole	Sigma Aldrich	32924
Albendazole	Sigma Aldrich	A 4673
Fenbendazole	Sigma Aldrich	F5396
Mebendazole	Sigma Aldrich	M 2523
Albendazole Sulfoxide	Sigma Aldrich	54029-12-8
Oxfendazole	Sigma Aldrich	34176
Albendazole sulfone	Sigma Aldrich	32178
Fenbendazole sulfone	Witega	BI024
Levamisole	Sigma Aldrich	31742
Flubendazole	LGC Standards	LGCFOR0463
Febantel	Sigma Aldrich	33981
Albendazole amine sulfone	Sigma Aldrich	5156
Oxibendazole amine	Sigma Aldrich	32166
Mebendazole amine	Sigma Aldrich	35403

7.5.4.2 Stock solution (1 000 mg/l)

About 10.0 mg of analyte or internal standard is measured and brought to 10 ml with acetonitrile in a volumetric flask. For the expression of the exact concentration, the purity of the analytical standard is considered. These solutions are stored in freezing for up to two years.

7.5.4.3 Intermediate solutions (50 mg/l)

These solutions are stored in a freezer and have a shelf life of one year.

7.5.4.4 Sulfonamides Mix A

Take an aliquot of the necessary volume of the stock solutions of the sulfamethazine, sulfathiazole, sulfaguanidine, sulfanilamide, sulfaclozine, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadoxine and sulfamethoxypyridazine to obtain a final concentration of 50 mg/l in 15 ml volumetric flask and complete to volume with acetonitrile.

7.5.4.5 Sulfonamides Mix B + Amphenicols

Take an aliquot of the necessary volume of the stock solutions of sulfamerazine, sulfamethizol, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, trimethoprim, and thiamphenicol to obtain a final exact concentration of 50 mg/l in a 15 ml volumetric flask and complete to volume with acetonitrile.

7.5.4.6 Benzimidazoles Mix A

Take an aliquot of the necessary volume of the stock solutions of albendazole, fenbendazole, mebendazole, thiabendazole, albendazole sulfoxide and oxibendazole to obtain a final concentration of 50 mg/l in a 15 ml volumetric flask and complete to volume with acetonitrile.

7.5.4.7 Benzimidazoles Mix B

Take an aliquot of the necessary volume of the stock solutions of oxfendazole, febantel, levamisole, albendazole sulfone, albendazole 2-aminosulfone, fenbendazole sulfone, flubendazole, oxibendazole amine, mebendazole amine to obtain a final concentration of 50 mg/l in a 15 ml volumetric flask and complete to volume with methanol.

7.5.4.8 Stilbenes + Ractopamine

Take an aliquot of the necessary volume of the stock solutions of diethylstilbestrol, dienestrol, hexestrol, ractopamine, α-zearalanol to obtain a final concentration of 50 mg/l in a 15 ml volumetric flask and complete to volume with acetonitrile.

7.5.4.9 Clenbuterol + Chloramphenicol

Take an aliquot of the necessary volume of the stock solutions of clenbuterol and Chloramphenicol to obtain a final concentration of 50 mg/l in a 15 ml volumetric flask and complete to volume with acetonitrile.

7.5.4.10 Working standard solutions

These solutions are stored by freezing and have a shelf life of six months.

Take an aliquot of the volume indicated in Table 34 into the same volumetric flask of 25 ml and complete volume with acetonitrile.

TABLE 34. Working standard solution

Class or analyte	Volume (µl)	Starting dissolution	Final concentration (µg/l)
Sulfonamides	2 500	Sulfonamides Mix A Sulfonamides Mix B+Amphenicols	5 000
Thiamphenicol			
Benzimidazoles	1 250	Benzimidazoles Mix A and B	2 500
Stilbenes	250	Stilbenes + Ractopamine	500
Ractopamine			
Zeranol			
Clenbuterol	25	Clenbuterol + Chloramphenicol	50
Chloramphenicol			

7.6 Procedure

7.6.1 Sample preparation

Defrost the sample at room temperature in a water bath, preventing the exchange of water between the samples and the bath.

a) **Tissue:** Prepare a homogenous sample by cutting pieces of different part of the sample. Grind approximately 200 g of sample.

b) **Milk:** Shake the bag or carton of milk to obtain a homogeneous sample.

c) **Eggs**

- Mix at least 5 eggs
- Weigh 5.0 g of samples and add 50 µl of the internal standard working solution.

7.6.2 Quality control samples

- Weigh 6 blank samples for the calibration curve and 1 blank sample for each additional matrix of the set of samples, for spike control.
- Add to all the samples 50 µl of the internal standard working solution.
- Spike calibration curve and control samples according to Table 35.

TABLE 35. Calibration curve and controls

Calibration point	Volume of working solution (µl)
STD 0	0
STD 1	10
STD 2	20
STD 3	50
STD 4	100
STD 5	150
Control	10

7.6.3 Sample extraction

a) Let the sample stand for 10 min.

b) Only for honey, add 5 ml of 0.5 mol/l HCl and heat in a water bath at 40°C for approximately 1 hr.

c) Add 10 ml of acetonitrile and shake by hand to disperse.

d) Shake in a multivortex for 5 min. Centrifuge at 4 000 rpm for 5 min.

7.6.4 Sample cleanup

a) Place the samples in a freezer for at least 4 hrs (Critical point: Maximum temperature -20°C).

b) Centrifuge at 4 000 rpm at -20°C for 5 min, immediately after removing them from the freezer.

c) Take a 5 ml aliquot of the acetonitrile layer and place it in a 15 ml tube (Critical point: take the aliquot quickly to avoid the temperature raise).

d) Evaporate to dryness at 40°C in a TurvoVap or appropriate evaporator.

e) Reconstitute with 500 µl of acetonitrile and 500 µl of deionized water.

f) Filter with a 0.22 µm syringe filter, and transfer to an autosampler vial.

7.6.5 Instrumental analysis (LC–MS/MS analysis)

The following conditions apply:

a) analytical column: Zorbax Eclipse Plus C18 (4.6×50 mm, particle size 1.8 µm)

b) Column temperature: 35°C

c) Flow rate: 0.3 ml/min

d) Injection volume: 10 µl

e) Run time: 14.6 min

f) Mobile phase: Deionized water 2 mmol/l acetic acid (A), acetonitrile (B)

g) Elution mode: gradient elution.

The mobile phase conditions are summarized in Table 36.

TABLE 36. Mobile phase gradient

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow (ml/min)
0.25	95.0	5.0	0.300
6.00	5.0	95.0	0.300
11.50	5.0	95.0	0.300
11.60	95.0	5.0	0.600
14.60	95.0	5.0	0.600

The mass spectrometric condition and dynamic MRM are summarized as follows (Table 37):

TABLE 37. MS parameters in positive ionization mode

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Fragmentor	CE	CV
Albendazole sulfone	298	266	8.11	165	18	4
Albendazole sulfone	298	159	8.11	165	34	4
Albendazole sulfone–D3	301.1	159	8.09	160	46	4
Albendazole sulfoxide	282.1	240.1	7.57	122	9	7
Albendazole sulfoxide	282.1	159.1	7.57	122	41	7
Albendazole sulfoxide–D3	285.2	243.1	7.56	122	9	7
Albendazole	266	234	9.32	155	20	4
Albendazole	266	191	9.32	155	35	6
Albendazole–D3	269	234	9.3	155	20	4
Clenbuterol	277.21	203	7	103	13	7
Clenbuterol	277.21	168.1	7	103	29	7
Clenbuterol	277.21	132.1	7	103	32	7
Febantel	447.1	415.1	10.5	115	10	4
Febantel	447.1	383	10.5	115	18	4
Fenbendazole sulfone	332.1	300	8.6	155	22	4
Fenbendazole sulfone	332.1	159	8.6	155	42	4
Fenbendazole sulfone–D3	335.1	300	8.57	155	22	4
Fenbendazole	300	268	9.66	165	20	3
Fenbendazole	300	159	9.66	165	40	3
Fenbendazole–D3	303	268	9.66	155	20	3
Flubendazole	314.1	282	8.98	135	18	4
Flubendazole	314.1	122.9	8.98	135	34	4
Levamisole	205.1	178	6.57	120	22	4
Levamisole	205.1	123	6.57	120	32	4
Mebendazole amine	238.1	133	7.1	150	42	4
Mebendazole amine	238.1	105	7.1	150	26	4
Mebendazole	296	264	8.83	130	20	3
Mebendazole	296	105	8.83	130	36	6
Mebendazole–D3	299	264	8.81	130	26	3
Oxfendazole	316	191	8.04	135	18	4
Oxfendazole	316	158.8	8.04	135	34	4
Oxfendazole–D3	319	159	8.03	135	34	4
Oxibendazole	250	176	8.58	160	30	6
Oxibendazole amine	192.2	149.9	7.15	100	18	4
Oxibendazole amine	192.2	108	7.15	100	34	4
Oxibendazole–D7	257	177	8.54	160	30	1
Ractopamine	302.3	164.1	6.8	98	13	7
Ractopamine	302.3	107.1	6.8	98	37	7
Ractopamine–D6	308.3	107.1	6.8	98	37	7

(con't)

TABLE 37. MS parameters in positive ionization mode (con't)

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Fragmentor	CE	CV
Salbutamol–D6	246.6	148.9	4	85	15	4
Sulfaclozine	285	108	8.5	120	29	4
Sulfaclozine	285	92	8.5	120	29	4
Sulfachloropyridazine	285.1	156	8.08	95	13	4
Sulfachloropyridazine	285.1	92.1	8.08	95	33	4
Sulfachloropyridazine–$^{13}C_6$	291.1	98.2	8.1	95	28	4
Sulfadiazine	251.1	108.1	7.13	96	20	4
Sulfadiazine	251.1	92.1	7.13	96	28	4
Sulfadiazine–$^{13}C_6$	257.1	98.1	7.14	96	28	4
Sulfadimethoxine	311.1	156.1	8.54	110	21	4
Sulfadimethoxine	311.1	92	8.54	110	33	4
Sulfadimethoxine–D6	317.1	92	8.51	110	33	4
Sulfadoxine	311	156	8.18	110	17	4
Clenbuterol	277.2	168.1	6.5	103	29	7
Clenbuterol	277.2	132.1	6.5	103	29	7
Clenbuterol	277.2	203	6.5	103	13	7
Ractopamine	302.3	164.1	6.5	98	13	7
Ractopamine	302.3	107.1	6.5	98	37	7
Ractopamine–D6	308.3	107.1	6.5	98	37	7
Trimethoprim–D9	300.2	123	6.5	105	29	4

TABLE 38. MS parameters in negative mode

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Fragment	CE	CV
Chloramphenicol	321	257	8.3	120	3	4
Chloramphenicol	321	194	8.3	120	4	6
Chloramphenicol	321	151.9	8.3	120	9	3
Chloramphenicol–D5	326	157	8.3	125	9	1
Dienestrol	265.1	249	9.8	140	20	4
Dienestrol	265.1	171	9.8	140	15	6
Diethylstilbestrol	267.1	250.9	9.7	140	20	4
Diethylstilbestrol–D8	275.1	258.9	9.7	140	20	3
Hexestrol	269.2	134	9.8	120	5	3
Hexestrol	269.2	119	9.8	120	45	4
Thiamphenicol	354	290	7.5	130	5	3
Thiamphenicol	354	240	7.5	130	9	4
Zeranol	321.2	303.2	9.3	140	15	6
Zeranol	321.2	277	9.3	140	20	3

7.6.6 Calculation and interpretation of results

Calculate results using a response factor as in Eq. (12):

$$RF = \frac{A_{Std} \times C_{IS}}{A_{IS} x C_{Std}} \quad \text{.....(12)}$$

RF is Response factor

AStd is Integrated abundance of the quantitation ion for analyte standard

AIS is Integrated abundance of the quantitation ion for the internal standard

Cstd is Concentration of analyte standard in µg/kg

CIS is Concentration of the internal standard in µg/kg

For results to be acceptable, for duplicate samples, each replicate should be within ± 10% of the mean value.

7.6.7 Method validation

Selectivity

The method for multiresidue analysis LC–MS/MS is considered a confirmatory technique due to its high selectivity. For each compound there is a precursor ion and at least two product ions which ensures the selectivity. This is thus considered a confirmatory method with 4 identification points (EC, 2002).

In addition, matrix matched calibration curves are compared with solvent curves to determine matrix effects. For those analytes in which a difference greater than 20% is obtained, this is attributed to matrix effect (Table 39).

TABLE 39. Matrix effect on analytes

Analyte	Matrix Effect
Amphenicols	
Thiamphenicol	Yes
Chloramphenicol	No
Beta-Agonist	
Ractopamine	Yes
Clenbuterol	Yes
Sulfonamides	
Sulfaguanidine	Yes
Sulfanilamide	Yes
Sulfathiazole	No
Sulfadiazine	Yes
Sulfapyridine	No
Sulfamethizole	No
Sulfamethoxypyridazine	Yes
Sulfamerazine	No

(con't)

TABLE 39. Matrix effect on analytes (con't)

Analyte	Matrix Effect
Sulfamethazine	No
Sulfamonomethoxine	Yes
Sulfachloropyridazine	Yes
Sulfadoxine	No
Sulfamethoxazole	No
Sulfisoxazole	No
Sulfaquinoxaline	No
Sulfadimethoxine	No
Sulfaclozine	No
Trimethoprim	No
Resorcylic acid derivative	
Zeranol	Yes
Stilbenes	
Diethylstilbestrol	No
Dienestrol	No
Hexestrol	No
Benzimidazoles	
Thiabendazole	No
Oxibendazole	No
Albendazole	No
Fenbendazole	No
Mebendazole	No
Albendazole Sulfoxide	No

With these results we can conclude that for the correct quantification of the analytes that presented a matrix effect, the analysis should be performed using a calibration curve of the corresponding matrix.

Linearity, Range and Sensitivity

Proper linearity is demonstrated for each compound with matrix calibration curves. Linearity and analytical sensitivity are documented by means of the average of 5 matrix curves used during validation (Table 40).

TABLE 40. Linearity and sensitivity

Analyte	Linearity (R^2)	Sensitivity (RR* /µg/kg)
Amphenicols		
Thiamphenicol	0.991	0.3814
Chloramphenicol	0.986	0.8727
Beta–Agonist		
Ractopamine	0.992	0.0304
Clenbuterol	0.952	0.0385
Sulfonamides		
Sulfaguanidine	0.987	0.0098
Sulfanilamide	0.997	0.0318
Sulfathiazole	0.967	0.0375
Sulfadiazine	0.999	0.0141
Sulfapyridine	0.999	0.0214
Sulfamethizole	0.999	0.0169
Sulfamethoxypyridazine	0.9979	0.0279
Sulfamerazine	0.999	0.0234
Sulfamethazine	0.999	0.0302
Sulfamonomethoxine	0.999	0.0333
Sulfachloropyridazine	0.999	0.0175
Sulfadoxine	1.000	0.0145
Sulfamethoxazole	0.999	0.0162
Sulfisoxazole	0.999	0.017
Sulfaquinoxaline	0.999	0.0175
Sulfadimethoxine	0.999	0.0202
Sulfaclozine	0.999	0.0263
Trimethoprim	0.991	0.0991
Resorcylic acid derivative		
Zeranol	0.993	2.6365
Stilbenes		
Diethylstilbestrol	0.994	0.3265
Dienestrol	0.993	0.1467
Hexestrol	0.991	0.3520
Benzimidazoles		
Thiabendazole	0.999	0.0241
Oxibendazole	0.997	0.0200
Albendazole	0.999	0.0391
Fenbendazole	0.999	0.0375
Mebendazole	0.999	0.0419
Albendazole Sulfoxide	0.999	0.0354

All analytes show an adequate linearity (>0.95).

Limits of Detection (LOD) and Quantification (LOQ)

For the estimation of the limits of detection and quantification (Table 41), calibration curves from a matrix using Eqs. (13) and (14):

$$LOD = Blank_{\text{average}} + 2.33\, s_b \quad \ldots\ldots (13)$$

$$LOQ = LOD + 1.64\, s_b \quad \ldots\ldots (14)$$

S_b is the standard deviation of the signal in the blank samples.

TABLE 41. Limits of detection and quantification

Analyte	LOD (µg/kg)	LOQ (µg/kg)
Amphenicols		
Thiamphenicol	5.9	10
Chloramphenicol	0.11	0.19
Beta-Agonist		
Ractopamine	0.52	0.77
Clenbuterol	0.084	0.12
Sulfonamides		
Sulfaguanidine	4.8	7.1
Sulfanilamide	6.3	10.8
Sulfathiazole	2.4	3.5
Sulfadiazine	4.1	7.0
Sulfapyridine	3.1	5.3
Sulfamethizole	3.6	6.1
Sulfamethoxypyridazine	5.1	8.7
Sulfamerazine	3.1	5.3
Sulfamethazine	2.2	3.8
Sulfamonomethoxine	4.0	6.9
Sulfachloropyridazine	3.0	5.1
Sulfadoxine	2.5	4.2
Sulfamethoxazole	2.7	4.7
Sulfisoxazole	3.5	6.0
Sulfaquinoxaline	3.5	5.9
Sulfadimethoxine	3.2	5.5
Sulfaclozine	2.2	3.7
Trimethoprim	1.2	2.0
Resorcylic acid derivative		
Zeranol	0.59	1.0

(con't)

TABLE 41. Limits of detection and quantification (con't)

Analyte	LOD (µg/kg)	LOQ (µg/kg)
Stilbenes		
Diethylstilbestrol	0.78	1.3
Dienestrol	1.0	1.7
Hexestrol	0.95	1.6
Benzimidazoles		
Thiabendazole	2.6	4.5
Oxibendazole	1.2	1.8
Albendazole	1.1	1.9
Fenbendazole	1.4	2.3
Mebendazole	1.5	2.5
Albendazole sulfoxide	2.8	4.8

The results obtained are below the MRLs and MRPL, established for these compounds.

Recovery

Recovery is calculated in fortified samples at three concentration levels (L1, L3 and L5) in each matrix and the outcome is detailed in Table 42.

TABLE 42. Results of recovery studies

Analyte	Average Recovery (%)
Amphenicols	
Thiamphenicol	105.2
Chloramphenicol	98.2
Beta-Agonist	
Ractopamine	108.0
Clenbuterol	110.8
Sulfonamides	
Sulfaguanidine	98.5
Sulfanilamide	104.7
Sulfathiazole	99.8
Sulfadiazine	101.6
Sulfapyridine	100.8
Sulfamethizole	101.0
Sulfamethoxypyridazine	101.6
Sulfamerazine	102.1
Sulfamethazine	101.6
Sulfamonomethoxine	103.5
Sulfachloropyridazine	101.0

(con't)

TABLE 42. Results of recovery studies (con't)

Analyte	Average Recovery (%)
Sulfonamides (con't)	
Sulfadoxine	102.0
Sulfamethoxazole	100.6
Sulfisoxazole	102.1
Sulfaquinoxaline	101.2
Sulfadimethoxine	98.1
Sulfaclozine	100.7
Trimethoprim	100.5
Resorcylic acid derivative	
Zeranol	100.9
Stilbenes	
Diethylstilbestrol	98.8
Dienestrol	92.6
Hexestrol	91.3
Benzimidazoles	
Thiabendazole	101.1
Oxibendazole	98.8
Albendazole	101.4
Fenbendazole	101.9
Mebendazole	102.5
Albendazole Sulfoxide	105.9

The results obtained for the recovery are within recognized provisions (EC, 2002; CAC, 2014).

7.6.10 Precision

Repeatability is assessed from recovery studies and is expressed as the highest coefficient of variation obtained for each compound.

The within–laboratory reproducibility is reported as the highest variation coefficient obtained from all the tests performed for validation. In all cases, the coefficient of global variation, calculated by grouping the variances of the different validation tests, carried out on different days and by different analysts, should present a lower result than the repeatability. The highest variability is reported as intermediate precision.

The uncertainty is calculated with the maximum variation obtained (intermediate precision) multiplied by a coverage factor of 2 (k = 2) which gives a confidence level of 95 %. To obtain the expanded uncertainty of a result in µg/kg, the uncertainty is multiplied by the concentration obtained. Table 43 summarizes the CV and measurement uncertainty.

TABLE 43. Precision and measurement uncertainty

Analyte	Coefficient of Variation (%)	Uncertainty
Amphenicols		
Thiamphenicol	12.9	0.26
Chloramphenicol	13.4	0.27
Beta–Agonist		
Ractopamine	13.4	0.27
Clenbuterol	21.7	0.43
Sulfonamides		
Sulfaguanidine	21.3	0.43
Sulfanilamide	21.9	0.44
Sulfathiazole	14.1	0.28
Sulfadiazine	14.4	0.29
Sulfapyridine	10.1	0.20
Sulfamethizole	12.3	0.25
Sulfamethoxypyridazine	13.4	0.27
Sulfamerazine	11.1	0.22
Sulfamethazine	10.8	0.22
Sulfamonomethoxine	13.2	0.26
Sulfachloropyridazine	12.5	0.25
Sulfadoxine	15.6	0.31
Sulfamethoxazole	9.4	0.19
Sulfisoxazole	12.6	0.25
Sulfaquinoxaline	16.9	0.34
Sulfadimethoxine	13.7	0.27
Sulfaclozine	14.8	0.30
Trimethoprim	13.1	0.26
Resorcylic acid derivative		
Zeranol	10.9	0.22
Diethylstilbestrol	8.7	0.17
Dienestrol	14.5	0.29
Hexestrol	21.9	0.44
Benzimidazoles		
Thiabendazole	16.5	0.33
Oxibendazole	13.7	0.27
Albendazole	10.0	0.20
Fenbendazole	10.1	0.20
Mebendazole	10.2	0.20
Albendazole Sulfoxide	16.2	0.32

Ruggedness

In order to test the ruggedness of the method, sample are injected in three different instruments, Thermo TSQ Endura, Agilent 6410 and Agilent 6420 and analyzed. The differences obtained are below the maximum variation of the method (intermediate precision), and therefore, the method is considered robust and any of the three instruments are suitable for analysis of the targeted samples.

7.6.11 Schematic diagram of extraction procedure

SAMPLE EXTRACTION	1. Weigh 5.0 g of sample. 2. Add 50 µl of the internal standard working solution. 3. Let the sample stand for 10 min. 4. For honey: add 5 ml of 0.5 mol/l HCl and heat in water bath at 40°C for approximately 1 hr. 5. Add 10 ml of acetonitrile and shake by hand to disperse. 6. Shake in a multivortex for 5 min. 7. Centrifuge at 4 000 rpm for 5 min.
↓	
SAMPLE CLEANUP	1. Place the samples in a freezer for at least 4 hrs (Critical point: Maximum temperature -20°C). 2. Remove the samples from the freezer. 3. Centrifuge immediately at 4 000 rpm at -20°C for 5 min. 4. Take a 5 ml aliquot of the acetonitrile layer into a 15 ml tube (avoid temperature rise). 5. Evaporate to dryness at 40°C in a TurvoVap or alternative. 6. Reconstitute with 500 µl of acetonitrile and 500 µl of deionized water. 7. Filter with a 0.22 µm syringe filter, and transfer to autosampler vial.

8

Detection of chloramphenicol residues in shrimp/fish muscle and meat by ELISA

8.1 Introduction

Chloramphenicol (CAP) is a broadspectrum antimicrobial substance effective against both gram positive and gram–negative bacteria. Although it is sometimes used in therapeutic doses to treat serious infections in humans, a safe level of human exposure to CAP has not been identified because of its toxic effects. Adverse effects include allergies, antibacterial resistance, carcinogenicity, genotoxicity, fetotoxicity, and nondose–dependent fatal aplastic anaemia in humans. In veterinary practice, CAP is often used as a cheap and effective drug to treat diseases associated with pathogens that have become resistant to other commonly used antibiotics. Use of even low doses of CAP in food producing animals can result in its residues in edible animal tissues. Due to the potential public health risks and unpredictable effects, many countries [e.g. European Union (EU), USA, China and Canada] have prohibited the use of CAP in food producing animals including aquaculture, and presence of CAP residues in food is illegal in the EU. Despite the ban, illegal use of CAP in food producing animals remains a possibility due to its broadspectrum activity, availability and low cost. Being a banned substance, CAP does not have an MRL; instead it has an MRPL of 0.3 ppb in the EU.

8.2 Scope and objective

This SOP describes the procedures of an Enzyme Immuno Sorbent Assay (ELISA) to screen CAP in shrimp/fish muscle and meat. Moreover, the EU's analytical methods used for detection of CAP residues in meat and aquaculture products should meet the MRPL of 0.3 µg/kg.

8.3 Principle of the method

This is a competitive enzyme immunoassay where CAP in a standard or sample compete with CAP conjugated to an enzyme for with sheep antibodies to rabbit IgG on a 96 well ELISA plate. The plate is incubated for at least 30 min and unbound drug washed off and tetramethylbenzidine (TMB) is used as a chromogen to visualize the drug conjugate. The substrate reaction is stopped using an acid (sulfuric) and the colour intensity read at 450 nm. The absorbance read is inversely proportional to the concentration of the CAP residue in the sample.

8.4 Safety considerations and precautions

Wear disposable latex gloves before handling samples and while pipetting the reagents and CAP standards. Once handling is over, wash hands thoroughly with soap and water. Where possible operate in a fume hood. All the kits should be stored at 2°C–8°C in a dark place.

8.5 Materials

8.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Distilled deionized/ultrapure water; Ethyl acetate; Nitrogen gas; Iso–octane; Trichloromethane and *n*–hexane.

8.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: ELISA reader (Thermo scientific); Tissue homogenizer (Ultra Turrex T 25 Basic); Micropipettes (Gilson/ Eppendorf/Nichipet); Laboratory Centrifuge (Centurion Scientific Ltd./HERMLE Z 160 M); Vortex mixer (Velp); Multidispenser; Analytical balance (Range 0.0002 g – 0.1000 g, Last digit 0.0001 g, Sartorius); Analytical balance (Range 0.0002 g–150 000 g, Last digit 0.0001 g, OHAUS); Refrigerator/freezer; Test tube rotator (GFL); Dry block incubator with sample concentrator (Techne, DB.3A); Glass test tubes; Polypropylene centrifuge tubes (15 ml, 50 ml); Microcentrifuge tubes (2 ml); Parafilm; CAP FAST ELISA 5091 CAPF kit (EuroProxima), including sample dilution buffer, rinsing buffer (20×), substrate solution, stop solution, conjugate solution, antibody solution, and CAP standards (0.04 µg/l, 0.1 µg/l, 0.2 µg/l, 0.4 µg/l, 1.0 µg/l, 4.0 µg/l and 100 µg/l).

8.5.3 Solutions

The solutions (and how they are prepared) include:

Rinsing buffer (1×)

- Dilute 2 ml of concentrated rinsing buffer (20×) in the ELISA kit with 38 ml of water.
- Prepare the rinsing buffer freshly before use.

Sample dilution buffer

- Dilute 3 ml of sample dilution buffer in the ELISA kit with 9 ml of water.
- This is adequate for about 10 samples.
- Prepare the sample dilution buffer freshly before use.

Conjugate solution

- Reconstitute the vial of lyophilized conjugate (CAP–HRPO) in the ELISA kit with 4 ml of reconstitution/zero standard buffer provided with the kit.
- Mix thoroughly and keep in the dark until use.
- The solution is stable for 2 months at 2°C–8°C in the dark.

Antibody solution

- Reconstitute the vial of lyophilized antibodies with 4 ml of reconstitution/zero standard buffer.
- Mix thoroughly and keep in the dark until use.
- The solution is stable for 6 months at 2°C–8°C in the dark.

8.5.4 Standards

- The ELISA kit contains CAP standards at 0.04 µg/l, 0.1 µg/l, 0.2 µg/l, 0.4 µg/l, 1.0 µg/l, 4.0 µg/l and 100 µg/l levels. This procedure, however, does not require the use of these standards as no calibration curve is constructed.
- A blank matrix is spiked with 10 µg/l CAP, and to prepare this solution, mix 30 µl of 100 µg/l CAP standard solution with 270 µl of water.

8.6 Procedure

8.6.1 Sample preparation

Remove the samples (shrimp, fish or meat) from the freezer (-20°C) and thaw the samples by keeping at room temperature or overnight in the refrigerator.

a) Shrimp

- Randomly pick three shrimps and remove the carapace and slice into small pieces using a knife and a cutting board.
- Homogenize and weigh 3 g (±0.05 g) of tissue for the analysis and record the sample weight.

b) Fish and meat

- A chunk of muscle tissue about 25 g–30 g is cut and sliced into small pieces.
- Homogenize the sample and weigh 3 g (±0.05 g) of tissue for the analysis and record the sample weight.

8.6.2 Quality control samples

Blank samples and spiked samples should be run as quality control samples with each batch of test samples.

a) Blank samples

A known blank sample [3 g (±0.05 g)] from the same matrix as the test sample can be used as the negative control (blank sample).

b) Spiked samples

Take 3 g (±0.05 g) of the known blank sample of the same matrix as the test sample, and fortify it using the 10 µg/l CAP standard solution (Table 44).

TABLE 44. Fortification of samples with CAP

Concentration of CAP in the sample (µg/kg)	Volume to add from 10 µg/l CAP (µl)
0.1	30
0.3	90
0.5	150

Let the fortified sample stand for 15 min at room temperature before continuing with the next step.

8.6.3 Sample extraction

a) Add 6 ml of ethyl acetate to 3 g (±0.05 g) of the test portion (unknown, blank and spiked samples), and mix for 10 min using the test tube rotator.

b) Centrifuge the sample at 2 000 rcf for 10 min.

c) After centrifugation, pipette 4 ml of ethyl acetate into a glass tube.

d) Evaporate the ethyl acetate to dryness at 50°C under a mild stream of nitrogen, using a dry block incubator with a sample concentrator.

e) Dissolve the fatty residue in 1 ml of either iso–octane/trichloromethane (2:3, v/v) or *n*–hexane.

f) Add 1 ml of the sample dilution buffer and vortex for 1 min.

g) Centrifuge at 2 000 rcf for 10 min.

h) Pipette a 50 µl portion of the upper layer (if iso–octane/trichloromethane is added in the previous step) or a 50 µl portion of the bottom layer (if *n*–hexane is added in the previous step) for the test.

8.6.4 ELISA protocol

a) Pipette 100 µl of reconstitution/zero standard buffer in duplicate (well A1, A2).

b) Pipette 50 µl of reconstitution/zero standard buffer in duplicate (well B1, B2).

c) Pipette 50 µl of each fortified sample (i.e. 0.1 µg/kg, 0.3 µg/kg and 0.5 µg/kg) in duplicate (well C1, C2 to E1, E2) and 50 µl of the blank sample in duplicate (well F1, F2).

d) Alternatively, a single well may be used instead of duplicating the above steps.

e) Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

f) Add 25 µl each of the conjugate (CAP–HRPO) and antibody solution into all wells but A1 and A2 and then rock the covered plate for at least 1 min followed by incubation in the dark at room temperature for 30 min.

g) Discard the solution from the microtiter plate and wash 3 times with rinsing buffer. Each cycle of rinsing includes pipetting 300 µl of the rinsing buffer into the wells and emptying the wells. After the 3rd washing cycle, tap the plate onto a paper towel to make sure the wells do not contain any rinsing buffer.

h) Transfer 100 µl of substrate to each well; and incubate for 15 min at room temperature.

i) Add 100 µl of a stop solution to each well and then read at 450 nm.

8.6.5 Interpretation of results

Assay is considered valid if absorbance of the sample spiked at 0.1 µg/kg is greater than that of the sample spiked at 0.3 µg/kg, and absorbance of the sample spiked at 0.5 µg/kg is lower than that of the sample spiked at 0.3 µg/kg.

If the above conditions are true, the test sample can be interpreted as follows:

- Positive samples

Samples with absorbance values below the absorbance of the sample fortified at 0.3 µg/kg.

- Negative samples

Samples with absorbance values above the absorbance of the sample fortified at 0.3 µg/kg.

8.6.6 Method validation

Repeatability

Precision of the ELISA method for each matrix is assessed using repeatability data (intra–assay), which are expressed as %RSD values (Table 45).

TABLE 45. ELISA method repeatability

Matrix	Repeatability (%RSD)
Shrimp	20.3
Chicken	16.4
Fish	11.6

Selectivity

This ELISA kit uses a specific antibody raised in rabbits against protein conjugated CAP. The reactivity pattern of this antibody is:

Cross reactions: Chloramphenicol: 100%

Chloramphenicol glucuronide: 100%

Thiamphenicol:< 1%

Florfenicol:< 1%

The measurement uncertainty values are summarized in Table 46.

TABLE 46. Calculation of uncertainty at 0.3 ppb

Average recovery at 0.3 ppb (µg/kg)	75.31	%
LOD	0.02	ppb
Uncertainty calculation at 0.3 ppb		
Mean	0.300	ppb
Sample Standard Deviation	0.051	
Sample Size	9	
Standard Uncertainty	0.017	
Degrees of Freedom	8	
95% t–Value	2.306	
95% Confidence Deviation	0.040	ppb
95% Confidence Interval (Min)	0.260	ppb
95% Confidence Interval (Max)	0.340	ppb
Percentage uncertainty	7.58	%

8.6.7 Schematic representation of the ELISA procedure

TEST SAMPLE	1. Cut thawed 25 g–30 g of shrimp, fish or meat into smaller pieces. 2. Homogenize the tissue and weigh 3 g (±0.05 g).
↓	
FORTIFICATION	1. Fortify the blank sample by adding 30 µl, 90 ul and 150 µl of 10 µg/l CAP soulution.
↓	
EXTRACTION	1. Add 6 ml of ethyl acetate to the test portion. 2. Mix 10 min in the test tube rotator. 3. Centrifuge at 2 000 rcf for 10 min. 4. Pipette out 4 ml of ethyl acetate layer into a glass tube. 5. Evaporate the ethyl acetate in the glass tube at 50°C under mild N2 gas flow. 6. Dissolve the fatty residue in 1 ml of either iso–octane/trichloromethane or *n*–hexane. 7. Add 1 ml of sample dilution buffer and vortex for 1 min. 8. Centrifuge at 2 000 rcf for 10 min. 9. Pipette out 50 µl portion from bottom or top layer depending on the extraction solution.
↓	
ELISA PROCEDURE	1. Pipette 100 µl of reconstitution/zero standard buffer in duplicate (well A1, A2). 2. Pipette 50 µl of reconstitution/zero standard buffer in duplicate (well B1, B2). 3. Pipette 50 µl of each fortified sample (i.e. 0.1 µg/kg, 0.3 µg/kg and 0.5 µg/kg) in duplicate (well C1, C2 to E1, E2). 4. Pipette 50 µl of each sample solution in duplicate into the remaining wells. 5. Add 25 µl each of conjugate (CAP–HRPO) and antibody solution into all wells but A1 and A2 and then roak the covered plate for at least 1 min followed by inclubation in the dark at room temperature for 30 min. 6. Discard the solution from the microtiter plate and wash 3 times by adding 300 µl of rinsing buffer to each well. After the final rinse, tap the plate onto a paper towel. 7. Add 100 µl of substrate solution to each well 8. Incubate the plate for 15 min at room temperature and add the 100 µl of stop solution before reading at 450 nm.

TABLE 47. Schematic representation of ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2										
B	B1	B2										
C	C1	C2										
D	D1	D2										
E	E1	E2										
F	F1	F2										
G												
H												

A1, A2: Blank wells
B1, B2: Zero Standard
C1, C2:0.1 µg/kg sample
D1, D2: 0.3 µg/kg sample
E1, E2: 0.5 µg/kg sample
F1, F2: Negative Control

9

Determination of macrolide residues in animal tissues by LC–MS/MS

9.1 Introduction

Macrolides, a group of antimicrobials produced by a range of Streptomyces strains, are commonly used in veterinary medicine. Examples of these drugs include erythromycin, gamithromycin, spiramycin, tilmicosin, tylosin, tylvalosin (all macrolides), azithromycin (an azalide) and tulathromycin (a triamilide). They are absorbed well after oral administration (except for erythromycin base) and distribute extensively to tissues, especially the lungs, liver and kidneys. In the USA, EU, and many other countries, MRLs for macrolides in edible animal tissues have been set up. Therefore, an LC–MS/MS method is established for the determination of macrolide antimicrobials in animal derived foods.

9.2 Scope and objective

This publication describes the procedure for monitoring residues and the metabolites of macrolides in animal and fish tissues, as well as in processed foods using LC–MS/MS. The macrolides of concern are tiamulin, lincomycin, spiramycin, tylosin, tilmicosin and josamycin.

9.3 Principle of the method

Tissue samples are blended with EDTA–McIlvaine buffer solution. After centrifuging, the extracts containing the macrolide are cleanedup by passing through MCX SPE cartridges. Macrolides are eluted from the cartridge with 5% ammonia in methanol. The eluate is evaporated to dryness and reconstituted with 0.1% formic acid. The extracted residues are examined using LC–MS/MS under electrospray ionization (ESI).

9.4 Safety considerations and precautions

Wear disposable gloves when handling all types of tissue sample. Wash hands thoroughly with soap and water after handling tissue samples. All operations using solvents should be conducted in a fume hood. Rinse all glassware used before putting it in the washing up tray. Avoid inhalation and skin contact with the standard powders and the stock solutions.

Other hazards are listed in Table 48.

TABLE 48. Hazards and precautions

Procedure Step	Hazard	Recommended Safe Procedures
Macrolide standards	Can cause kidney damage	Wear PPE when handling standards.
Acetonitrile	Flammable, toxic, may be fatal if inhaled or absorbed.	Use only in a fume hood. Avoid breathing fumes. Keep away from flame or heat.
Methanol	Flammable, harmful if swallowed.	Use only in a fume hood. Avoid breathing fumes. Keep away from flame or heat.
Formic acid, concentrated	Harmful if inhaled. Causes skin and eye burns.	Use only in fume hood. Wear PPE, avoid skin contact.

9.5 Materials

9.5.1 Reagents and solvents

The following reagents and chemicals are applicable except where stated: Distilled/de–ionized water; Citric acid monohydrate (Ajax, AR grade) or equivalent; Disodium hydrogen phosphate dihydrate (BDH, AnalaR); Disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA) (Ajax, AR grade) or equivalent; Methanol; Acetonitrile and Ammonia solution, 32%.

9.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Beakers of various sizes; Duran bottles, 1 000 ml with solvent bottle dispenser (10 ml); Volumetric flasks, various sizes; Falcon (centrifuge) tubes with lids (50 ml); Test tubes (borosilicate glass) – 10 ml capacity; HPLC insert, vials and caps; Homogenizer (Silverson L5M–A); Mincer (Kenwood 8B07); Centrifuge (Jouan CR3i and microcentrifuge type Jouan A14); Top pan balance (Mettler PB1502); Analytical balance (Mettler AX26); Nitrogen evaporator Turbo–Vap LV, (Zymark) Caliper Life Science; Water purification system, Milli–Q Integral 5 A10 Pure (Millipore); Millipore filter apparatus; Water bath (Memmert); Vortex mixer (Thermolyne); pH meter (Crison 2001); Pipettor, microadjustable, 50 µl, 100 µl, 500 µl and 1 000 µl and tips; OASIS MCX SPE column, 60 mg/3 ml, Waters or equivalent; SPE vacuum manifold and tank; Reservoir adaptor caps; Syringes, plastic disposable (10 ml); Vacuum flask; Vacuum pump; Micromass Quattro Ultima Pt LC–MS/MS, fitted with an ESP probe; HPLC system, Waters 2695; HPLC Column: Columbus (150 × 2 mm, 5 µm) (Phenomenex) or equivalent.

9.5.3 Solutions

a) Extraction solution: McIlvaine buffer

Phosphate buffer (0.2 mol/l): Dissolve 35.6 g of disodium hydrogen phosphate in water and make up to 1 litre.

Citric acid (0.1 mol/l):

- Dissolve 21.01 g of citric acid monohydrate in about 300 ml of water and make up to 1 litre.
- Mix 385.5 ml citric acid (0.1 mol/l) with 614.5 ml phosphate buffer (0.2 mol/l).

- Check that the pH is at 4.0 (±0.05). Adjust if necessary.
- To the combined solution, add 37.22 g of EDTA and dissolve.
- Store at 2°C–8°C in a refrigerator for a month.

b) Ammonia solution pH 12.0

- Pipette 5 ml ammonium solution 32% in a flask.
- Add 90 ml of water and adjust to pH 12.0 with ammonium solution 32%.

c) Methanol/Ammonium solution pH 12.0 (10/90, v/v)

- Add 10 ml of methanol and fill up to 100 ml with ammonia solution pH 12.0.

d) Ammonia solution pH 12.0/methanol (5/95, v/v)

- Pipette 5 ml ammonia solution pH 12.0 into a volumetric cylinder.
- Make up to 100 ml with methanol.

e) HPLC mobile phase A (0.1% formic acid)

- Place 1 ml of formic acid in a 1 litre cylinder containing 999 ml of water.
- Mix and filter through a 0.45 µm filter under vacuum.
- Store in a reagent bottle at room temperature. Stable for 3 months.

f) HPLC mobile phase B (0.1% formic acid in acetonitrile)

- Place 1 ml of formic acid in a 1 litre cylinder containing 999 ml of acetonitrile.
- Filter through a 0.45 mm filter under vacuum and store in a reagent bottle at room temperature. Stable for 3 months.

9.5.4 Standards

9.5.4.1 Reference standards

The analytical standards include: Tiamulin fumarate (Sigma Aldrich); Lincomycin (Sigma Aldrich); Spiramycin (Dr Ehrenstorfer, Augsburg, Germany); Tylosin (Dr Ehrenstorfer, Augsburg, Germany); Tilmicosin (Dr Ehrenstorfer, Augsburg, Germany); Roxithromycin (Dr Ehrenstorfer, Augsburg, Germany); Josamycin (Dr Ehrenstorfer, Augsburg, Germany).

9.5.4.2 Stock standards

NOTE: *Supplies of both standard material and deuterated internal standards are extremely limited. Fresh stock solutions should only be prepared when necessary.*

a) Macrolide standard solutions (1 000 mg/l)

- Weigh out the appropriate amount of macrolide standard and internal standard, considering the purity of the standard material in a 10 ml flask.
- Dissolve and make up to the mark with methanol.
- Transfer to a brown glass vial and store in a freezer at -20°C.

b) Internal standard solutions (100 mg/l)

- Weigh 2.5 mg (±0.025 mg) of roxithromycin into a 25 ml glass volumetric flask.
- Dissolve and make up to the mark with methanol.
- Transfer to a brown glass vial and store in a freezer at -20°C.

9.5.4.3 Intermediate standards

Macrolide Intermediate Standard Solutions (100 mg/l)

- Pipette 1 ml of each stock solution at 1 000 mg/l into individual 10 ml volumetric flasks.
- Add methanol, mix and make up to the mark.
- Transfer to a brown glass vial and store in a refrigerator at -20°C.

9.5.4.4 Working standards and internal standard solutions for matrix calibration

a) Mixed Macrolide Working Standard for Poultry Muscle

- Pipette an appropriate volume of individual intermediate standard at 100 mg/l into a 10 ml volumetric flask as indicated in Table 49.
- Add methanol, mix and make up to the mark.
- Transfer to a brown glass vial and store in a refrigerator at 4°C.

TABLE 49. Preparation of macrolide working standards when testing muscle

Compound	Initial conc. of individual intermediate std (mg/l)	Vol. of individual intermediate std. (µl)	Volume of methanol (ml)	Final conc. of mixed working std. (mg/l)
Tilmicosin	100	200	10	2.0
Tiamulin		200		2.0
Tylosin		200		2.0
Erythromycin		200		2.0
Lincomycin		400		4.0
Spiramycin		400		4.0

b) Mixed Macrolide Working Standard for Porcine Kidney

- Pipette an appropriate volume of individual intermediate standard (100 mg/l) or stock solution (1 000 mg/l) into a 10 ml volumetric flask as shown in Table 50.
- Add methanol, mix and make up to the mark.
- Transfer to a brown glass vial and store in a refrigerator at 4°C.

TABLE 50. Preparation of macrolide working standards when testing kidney

Compound	Initial conc. of std. solution (mg/l)	Vol. of standard solution (µl)	Volume of methanol (ml)	Final conc. of mixed working std. (mg/l)
Tiamulin	100	200	10	2.0
Tylosin	100	200		2.0
Erythromycin	100	400		4.0
Spiramycin	100	600		6.0
Tilmicosin	1 000	200		20
Lincomycin	1 000	300		30

c) **Internal Standard Solution (5 mg/l)**

- Pipette 500 µl of roxithromycin stock solution (100 mg/l) into a 10 ml volumetric flask.
- Add methanol, mix and make up to the mark.
- Transfer to a brown glass vial and store in a refrigerator at -20°C.

9.6 Procedure

9.6.1 Sample preparation

- For analysis, subsamples are taken from thin slices through the frozen sample to gain as representative a sample as possible.
- Homogenization of the whole sample is best avoided as loss of analyte through increased enzymatic activity may result.
- Weigh 2.0 g (±0.05 g) of minced sample into a plastic falcon tube.
- Add 100 µl and 200 µl of 5 mg/l internal standard into a blank tissue pellet poultry muscle and porcine/bovine kidney respectively.
- Vortex mix for homogenisation.
- Allow to stand for at least 10 min.

9.6.2 Quality control samples

Weigh 5 portions [2.00 g (±0.05 g)] of negative minced tissue into 50 ml centrifuge tubes.

a) **Blank Samples (2 portions)**

- Add 100 µl and 200 µl of 5 mg/l internal standard into a blank tissue pellet poultry muscle and porcine/bovine kidney, respectively.
- Vortex mix for homogenization.
- Allow to stand for ~10 min.

b) **Spike Samples (3 portions)**

- Add 100 µl and 200 µl of 5 mg/l internal standard into a blank tissue pellet poultry muscle and porcine/bovine kidney, respectively.
- Add 100 µl of mixed working standard with appropriate concentrations into a blank tissue pellet. Vortex mix for homogenisation.
- Allow to stand for ~10 min.

c) **Matrix calibration curve**

- Weigh 6 portions [2.00 g (±0.05 g)] of negative minced tissue into 50 ml centrifuge tubes.
- Add an appropriate volume of mixed working standard and internal standard with the appropriate concentration as for poultry muscle and kidney porcine/bovine as shown in Tables 51 and 52.

TABLE 51. Analytical standards for chicken muscle analysis

Label	Spike concentration (µg/kg)		Spike concentration (mg/l)		Mixed WS, volume (µl)	Mixed working I.S, 5 mg/l volume (µl)
	Set 1	Set 2	Set 1	Set 2		
Std 0	0	0	0	0	0	100
Std 1	25	50	250	500	25	100
Std 2	50	100	500	1 000	50	100
Std 3	100	200	1 000	2 000	100	100
Std 4	150	300	1 500	3 000	150	100
Std 5	300	600	2 250	6 000	300	100

Set 1 (Tilmicosin, tiamulin, tylosin, erythromycin), Set 2 (Lincomycin, Spiramycin)

TABLE 52. Analytical standards for porcine/bovine kidney muscle analysis

Label	Spike concentration (µg/kg)					Mixed WS, volume (µl)	Mixed working I.S, 5 mg/l volume (µl)
	Tiamulin, Tylosin	Erythro	Spira	Linco	Spira		
Std 0	0	0	0	0	0	0	200
Std 1	25	50	75	250	375	25	200
Std 2	50	100	150	500	750	50	200
Std 3	100	200	300	1000	1500	100	200
Std 4	150	300	450	1500	2250	150	200
Std 5	300	600	900	3000	4500	300	200

Vortex mix for homogenization. Allow to stand for ~ 10 min.

9.6.3 Sample extraction

a) Add 20 ml of EDTA–Mcllvain buffer.

b) Homogenize at full speed for at least 20 s.

c) Mix with a rotative stirrer at 230 rpm for 10 min.

d) Centrifuge the sample at 4 000 rpm for 15 min.

e) Apply supernatant to SPE for further cleanup.

9.6.4 Solid Phase Extraction (SPE) cleanup

a) Condition the OASIS MCX SPE cartridges (60 mg/3 ml) with 3 ml methanol followed by 3 ml water, then 3 ml EDTA–Mcllvain buffer (if the tubes are to be left for more than 5 min after conditioning, close the tap and leave at least 200 µl of liquid covering the sorbent bed).

b) Fit a plastic syringe barrel and syringe adaptor to the top of the SPE tube.

c) Load the sample supernatant into the SPE tube.

d) Allow the solution to drip through the sorbent bed under gravity.

e) Wash the SPE tube with 3 ml methanol/ammonia solution pH 12 (10/90, v/v), allowing it to flow through under gravity.

f) Apply full vacuum for ~ 10 min or 1–2 min by nitrogen to dry the SPE tubes.

g) Place labeled 10 ml test tubes into the SPE tank rack under the SPE tubes.

h) Elute the samples with 2×6 ml ammonia solution pH 12/methanol (5/95, v/v) at a flow rate of less than 1 ml/min.

i) Place the test tube containing the sample extract into a pre–heated evaporation bath at ~ 45°C.

j) Adjust the nitrogen flow such that the surface of the liquid ripples gently. (Avoid an excessive nitrogen flow rate as this could cause analyte loss).

k) Allow the sample solutions to evaporate to dryness.

l) Add 200 µl of 0.1% formic acid to the residue and mix the contents on a vortex mixer for 10 s–15 s and sonicate for ~ 5 min.

m) If the sample is turbid or contains particulates, centrifuge at 13 000 rpm.

n) Sample extracts should be stored at +4°C if not run immediately.

o) Transfer the sample extract to a labeled autosampler vial.

9.6.5 Instrumental analysis (LC–MS/MS)

The HPLC and MS conditions are indicated in Tables 53–63.

TABLE 53. HPLC parameters–inlet method

Pump A %	95 (Formic acid in H_2O, 100 %)
Pump B%	5 (Formic acid in acetonitrile, 100 %)
Pump C%	0
Pump D%	0
Run Time (min)	25
Flow (ml/min)	0.25
Degasser	Normal
Flow Ramps (min)	2

TABLE 54. HPLC parameters–column setup

Temperature (°C)	30
Temperature Limit (°C)	20
Low Pressure (bar)	0
High Pressure (bar)	300
Precolumn volume (µl)	0

TABLE 55. HPLC parameters–pump gradient

Time	A (%)	B (%)	C (%)	D (%)	Flow	Curve
0.00	95	5	0	0	0.25	1
10.00	60	40	0	0	0.25	6
15.00	20	80	0	0	0.25	6
18.00	20	80	0	0	0.25	6
20.00	95	5	0	0	0.25	6
25.00	95	5	0	0	0.25	6

TABLE 56. HPLC parameters–autosampler conditions

Sample Heater	Injection Parameters
Temperature (°C): 20	Injection Volume: 25 µl
Temperature Limit (°C): 20	Needle Depth: 1 mm
	Draw Speed (µl/s): Slow

TABLE 57. Electrospray source (tuning parameters)

Capillary:	3.35kV
Cone:	35 Vs
RF Lens 1:	35
Aperture:	0.0
RF Lens 2:	0.0
Source Temp (°C):	120
Desolvation Temp (°C):	350
Cone gas flow:	60 l/hr
Desolvation gas flow:	600 l/hr
Collision cell pressure:	3.00^{e-3} mbar

TABLE 58. Analyzer parameters (tuning parameters) – MS1

LM Resolution 1:	13.0
HM Resolution 1	13.0
Ion Energy 1	1.0 Volt
Entrance	1 Volt
Collision	43 Volts
Exit	1 Volt

TABLE 59. Analyzer parameters (tuning parameters) – MS2

LM Resolution 2	13.0
HM Resolution 2	13.0
Ion Energy	1.8 Volts
Multiplier	650 Volts

TABLE 60. MS method parameters (experiment setup)–function 1

Type	MRM
Ionization Mode	ES^+
Inter–Channel Delay	0.1 s
Inter–Scan Time (sec)	0.02 s
Span (Da)	0.0 Da
Start Time (min)	4 min
End Time (min)	12 min
Cone Voltage	35 V
Dwell Time	0.2 s

TABLE 61. Precursor, product ions and collision conditions

Name	Precursor ion (m/z)	Product ion (m/z)*	Collision energy (eV)
Lincomycin	407.47	359.09	18
		126.18	26
Tilmicosin	435.43	174.32	21
		694.93	15
Spiramycin	843.56		
		174.15	41
		101.17	42

*Most abundant production is in bold

TABLE 62. MS method parameters (experiment setup) –function 2

Type	MRM
Ionization Mode	ES^+
Interchannel Delay	0.1 s
Interscan Time	0.02 s
Span (Da)	0.0 Da
Start Time	10 min
End Time	25 min
Cone Voltage	35 V
Dwell Time	0.2 s

TABLE 63. Precursor, product ions and collision conditions

Name	Precursor ion (m/z)	Product ion (m/z)*	Collision energy (eV)
Tiamulin	494.34	192.03	21
		119.03	36
Tylosin	916.77	772.46	28
		174.14	40
Erythromycin	734.50	558.10	17
		576.18	20
Roxithromycin	837.64	679.43	22

*Most abundant product ion is in bold

9.6.6 Calculation and interpretation of results

- Calculation using response factor

Calibrate the instrument by analyzing a minimum of five calibration standards.

A response factor for macrolides metabolite is calculated as in Eq. (15):

$$RF = \frac{A_{Std} \times C_{IS}}{A_{IS} x C_{Std}} \quad \ldots\ldots(15)$$

RF is the response factor

A_{Std} is the integrated abundance of the quantitation ion for macrolide standard

A_{IS} is the integrated abundance of the quantitation ion for the internal standard

C_{std} is the concentration of macrolide standard in µg/kg

C_{IS} is the concentration of the internal standard in µg/kg

Calculate the mean response factor (RF_{mean}) of the five concentration levels.

Calculate the macrolide sample concentration Eq. (16):

$$Cmacrolide = \frac{A_{macrolide} x C_{IS}}{A_{IS} x (RF_{mean})} \quad \ldots\ldots(16)$$

$C_{macrolide}$ is the concentration of macrolide in µg/kg in the tissue sample

$A_{macrolide}$ is the integrated abundance of the ion for macrolide sample

A_{IS} is the integrated abundance of the quantitation ion for the internal standard

RF_{mean} is the mean response factor of analyte.

9.6.7 Calculation using linear regression equation

As an alternative to calculating the mean response factor, a linear regression curve can be generated from the initial calibration data by plotting the ratio of macrolide/IS–macrolide (response) versus macrolide concentration.

Using the response and associated ppb values, calculate slope, intercept, and correlation coefficient of the regression line and Eq. (17).

$$y = \frac{Macrolide_{peakarea}}{IS - macrolide_{peakarea}} = \text{response} \quad \text{.....(17)}$$

x is the macrolide concentration in µg/kg (ppb); IS the internal standard.

The linear equation shown on the chart represents the relationship between concentration (x–axis) and response (y–axis) for the compound. Results are calculated using the linear regression equation by Quanlynx software.

9.6.8 Criteria for acceptability of results

Residues of macrolide metabolites in samples are considered confirmed once all the following method performance criteria are met:

i) The relative retention time of macrolide metabolites in a sample obtained correspond to the ratio obtained in a standard matrix, with a maximum variation of 2.5%.

ii) The presence of the two product ions, from each macrolide metabolite molecular peak and presence of ion originating from their internal standards.

iii) The S/N for all diagnostic ions should be ≥ 3.

iv) The stability of the ion ratio between the two transitions for each macrolide metabolites in accordance with the tolerances recommended as shown in Table 64.

TABLE 64. Maximum permitted tolerances (%) for LC–MS/MS relative ion intensities (EC, 2002)

Relative intensity	Tolerances in LC–MS/MS (%)
≥50%	±20
≥20% <50%	±25
≥10% <20%	±30
<10%	±50

The Head of the laboratory Unit (HU) will ensure that blanks do not give rise to any signals that meet the appropriate identification point.

These analytes should not be present in food of animal origin. If the identified analyte concentration in a sample is equal to (or greater than) the MRL, the sample should be judged non-compliant. Any samples detected below this concentration will be reported as negative.

If duplicate determinations are carried out and one replicate reveals the presence of macrolide and the other does not, the HU will require that the analysis be repeated. The HU will ensure that percentage recovery values fall within the ranges.

It is normal practice to run several recoveries (minimum 2) in each analytical batch. Ideally all individual recovery values should fall inside the acceptable range, defined above. If the recovery of analyte from fortified known negative tissue falls outside the acceptable range, the HU should exercise professional judgement in deciding whether to accept some/all the results in the batch as follows or not:

- If percentage recoveries fall outside the acceptable range and the sample is negative the HS may accept the results in the batch.
- If 2 recoveries fall within the acceptable range, 1 recovery falls outside the acceptable range, and the sample is positive, the average mean recovery should be taken. If the mean recovery falls within the acceptable range, results for that batch should be accepted.

- If only one recovery falls within the acceptable range, 2 recoveries fall outside the acceptable range and the sample is positive, the HU should reject the entire batch and the analysis be repeated.

9.6.9 Method validation

This method is validated for the analysis of trace levels of macrolides in animal–derived food typically following known guidelines (EC, 2002).

Selectivity/ Specificity

Twenty blank samples of animal tissue from different sources are analyzed to verify the absence of interfering peaks. Each molecule is specifically identified by two minimum transitions (parent ion > daughter ion) and retention time. Specificity is evaluated from the examination of chromatograms.

Recovery

As indicated elsewhere (EC, 2002) when no certified reference material is available, trueness of measurements is assessed through recovery of additions of known amount of the analytes to a blank matrix. Therefore, trueness was obtained with blank sample spiked at 100 µg/kg–200 µg/kg in chicken muscle and 100 µg/kg–1 500 µg/kg in porcine/bovine kidney.

Linearity

The linearity of the analytical method is validated using the tissue calibration curves for each compound at different concentration levels to prevent matrix effects. For each series, the equation of the calibration ranges is calculated from blank and spiked samples accordingly, with regards to type of matrix and analytes. The equation of the calibration curve is established. The R^2 coefficient of the calculated regression curves is ≥0.97.

Reproducibility/ Repeatability

Repeatability (intraday precision) was evaluated performing replicates in a day and within-laboratory. Reproducibility (interday precision) was evaluated by analyzing samples over the course of three consecutive days. For all the measured compounds, the analyzed recovery was compliant within established ranges (EC, 2002). The CVs obtained are all less than 20%.

The decision limit and detection capability

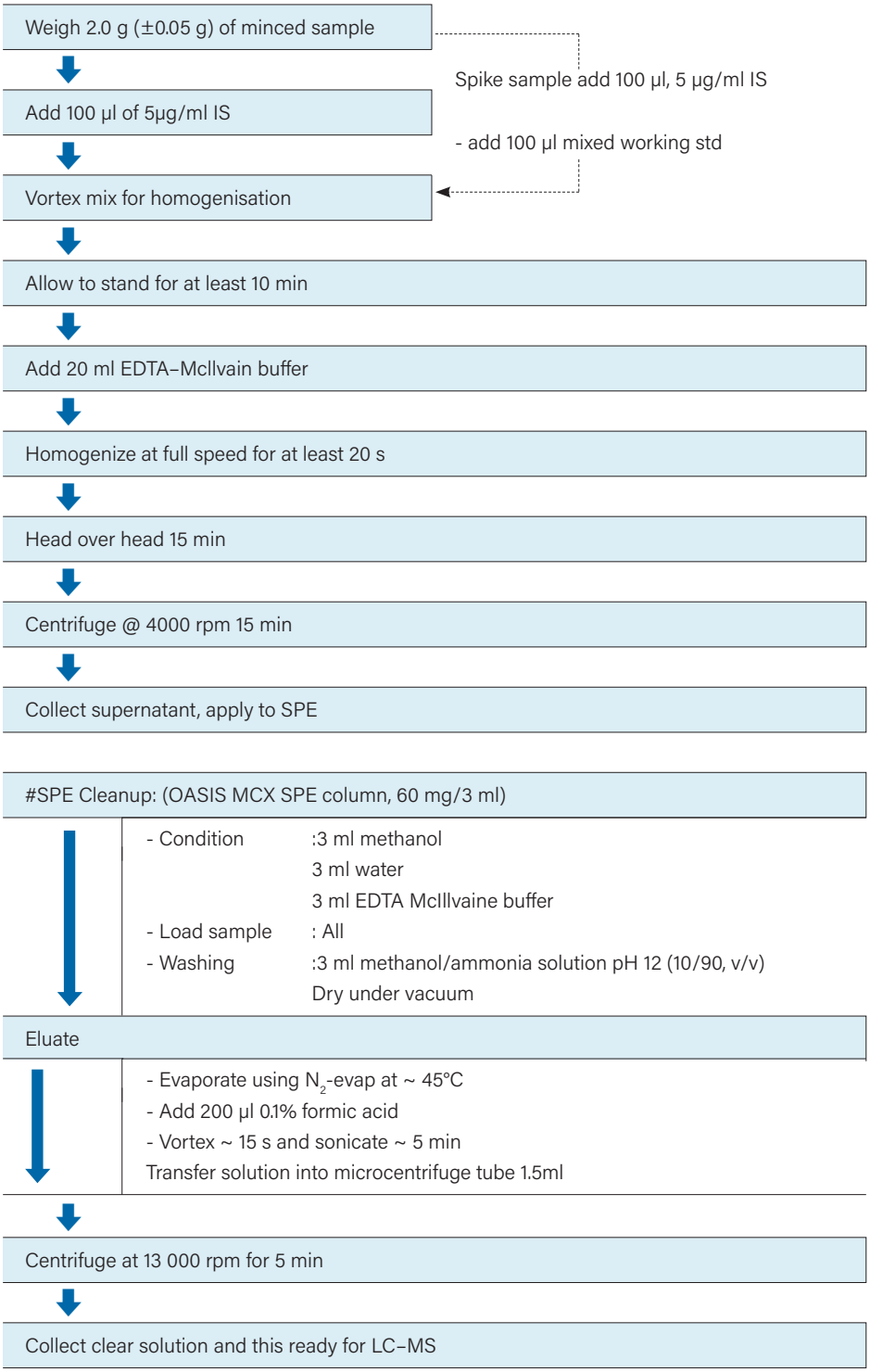
An overview of validation results for CCα and CCβ is shown in Table 65.

TABLE 65. Decision limit and detection capability

Analyte	Decision limit CCα (µg/kg)		Detection capability CCβ (µg/kg)	
	Signal 1 (+intense)	Signal 2 (–intense)	Signal 1 (+intense)	Signal 2 (–intense)
NP–AOZ	0,013	0.07	0.02	0.09
NP–AMOZ	0.01	0.02	0.01	0.03
NP–AHD	0.03	0.06	0.03	0.08
NP–SEM	0.16	0.64	0.2	0.78

The decision limits obtained are all well below the recommended concentrations (CRL, 2007)

9.6.10 Schematic diagram of the extraction procedure



10

Screening of veterinary drug residues in egg using by UPLC–MS/MS

10.1 Introduction

Veterinary drugs are used in chicken farms to control the disease of laying hens. However, these compounds can be transferred to and accumulate in the eggs. The presence of veterinary drug residues in eggs is a potential health risk for the consumer because the residual drugs can trigger allergic reactions or induce pathogen resistance to antibiotics used in human medicine.

Eggs are among the highest food sources of lecithin (phospholipids) and have significant amounts of fats. These co–extracted substances can lead to interference and ion suppression in the LC–MS analysis. Eight classes of veterinary drugs are included in this procedure.

10.2 Scope and objectives

The scope of this method includes: Chloramphenicol, Thiamphenicol, Florfenicol, Ciprofloxacin, Enrofloxacin, Norfloxacin, Tylosin, Erythromycin, Tilmicosin, Oxytetracycline, Chlortetracycline, Doxycycline, Ampicillin, Penicillin G, Sulfadiazine, Sulfathiazole, Sulfamethazine, Sulfaquinoxaline and Sulfadimethoxine. Others are CAP–D5, DMZ–D3, IPZ–D3, RNZ–D3, Enrofloxacin–D5, Norfloxacin–D5 and SMZ–$^{13}C_6$.

10.3 Principle of the method

Samples are treated with an acidified acetonitrile/water solvent to precipitate proteins, release bound residues and to extract the veterinary drugs from the egg. It is followed by simple pass-through Oasis Prime HLB cartridge cleanup to remove fats and phospholipids. The extracted residues are examined using UPLC–MS/MS under electrospray ionization (ESI) conditions.

10.4 Safety considerations and precautions

All operations using solvents should be conducted in a fume hood. Rinse all glassware used before putting it in the wash tray. Avoid inhalation of and skin contact with the standard powders and the stock solutions. Additional guidance is provided in Table 66.

TABLE 66. Hazards and precautions

Procedure Step	Hazard	Recommended Safe Procedures
Antimicrobial standards	Some individuals may have allergic reactions to certain β–lactams, sulfa, or other drugs.	Wear appropriate PPE to avoid dermal contact.
Formic acid, concentrated	Harmful if inhaled. Causes skin and eye burns.	Use only in fume hood. Wear personal protective equipment, avoid skin contact.
Acetonitrile	Flammable, toxic, may be fatal if inhaled or absorbed.	Use only in a fume hood. Avoid breathing fumes. Keep away from flame or heat.
Methanol	Flammable, harmful if swallowed.	Use only in a fume hood. Avoid breathing fumes. Keep away from flame or heat.

10.5 Materials

10.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Water OPTIMA™ LC/MS Grade or deionized water passed through Milli–Q Integral 5 A10 Pure (Millipore) Ultra Filtration System; Acetonitrile OPTIMA™ LC/MS Grade; Methanol OPTIMA™ LC/MS Grade; Formic acid; Ammonium acetate.

10.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Rotary stirrer – Heidolph Reax 2; Centrifuge – Jouan CR3i and microcentrifuge type Jouan A14; Balance – Mettler Top; Loading Model PB1502 capable of weighing 2 g (±0.01 g); Balance Analytical – Mettler Model AX2; 15 ml and 50 ml Polypropylene Centrifuge tubes (PP), 15 ml; Magnetic stirrer and stirbars; volumetric flasks; graduated cylinders; Pasteur pipettes; Repeating pipettes and tips; Beakers; Bottles; Weighing boats; Spatulas; Funnels; Bottle top volumetric dispensers; LC vials with screw cap lids (2 ml); Plastic screw cap vials – Polypropylene, 4 ml; Solid Phase Extraction – Oasis PRIME HLB, 3 ml, 60 mg; Waters Xevo TQ–XS or TQS MS/MS; Waters Acquity I–Class with MassLynx operating software; UPLC column – Acquity UPLC® BEH C18, 2.1×100 mm, 1.7 µm.

10.5.3 Solutions

- Acetonitrile/water (80%): Add 80 ml acetonitrile and make up to 100 ml with water in a 100 ml volumetric flask and mix.
- 0.2% formic acid in 80% acetonitrile/water: Add 0.2 ml formic acid to a 100 ml volumetric flask. Dilute to volume with 80% acetonitrile/water and mix.
- UPLC mobile phase A: 2 mmol/l ammonium acetate in water
    - Place 1 ml of formic acid in a 1 litre volumetric flask and make up to the mark with water.
    - Mix and filter through a 0.45 µm filter under vacuum.
    - Store in a reagent bottle at room temperature. This is stable for 3 months.

- Ammonium acetate (5 mol/l)
  - Weigh 96.35 g of ammonium acetate into a beaker.
  - Dissolve the salt with about 200 ml water.
  - Pour into a 250 volumetric flask and make up to 250 ml with water.
- Ammonium acetate (2 mmol/l)
  - Add 40 µl of the 5 mol/l ammonium acetate stock solution to a 100 ml volumetric flask.
  - Bring to volume with water.
- UPLC mobile phase B:2 mmol/l ammonium acetate in methanol
  - Add 40 µl of the above 5 mol/l stock solution into a 100 ml volumetric flask.
  - Dilute to volume with methanol.

10.5.4 Standards

10.5.4.1 Reference standard

The standards are listed in Table 67.

NOTE: *Equivalent standards/solutions may be substituted. Purity is considered when calculating standard concentrations*

TABLE 67. List of standards and source

Standard Analyte	Manufacturer	Standard Analyte	Manufacturer
Chloramphenicol	Dr. Ehrenstorfer	Dimetridazole	Fluka
Thiamphenicol	Dr. Ehrenstorfer	Ipronidazole	Witega
Florfenicol	Dr. Ehrenstorfer	Ronidazole	Fluka
Ciprofloxacin	Fluka	CAP–D5	Cambridge Isotope
Enrofloxacin	Witega	DMZ–D3	Witega
Norfloxacin	Fluka	IPZ–D3	Witega
Tylosin	Dr. Ehrenstorfer	RNZ– D3	WItega
Erythromycin	Sigma-Aldrich	Enrofloxacin–D5	Witega
Tilmicosin	Dr. Ehrenstorfer	Norfloxacin–D5	Dr. Ehrenstorfer
Oxytetracycline	Sigma–Aldrich	SMZ–$^{13}C_6$	Dr. Ehrenstorfer
Chlortetracycline	Sigma–Aldrich	Penicillin V	Dr. Ehrenstorfer
Doxycycline	Dr. Ehrenstorfer	Demeclocycline	Dr. Ehrenstorfer
Ampicillin	Dr. Ehrenstorfer		
Penicillin G	Dr. Ehrenstorfer		
Sulfadiazine	Dr. Ehrenstorfer		
Sulfathiazole	Dr. Ehrenstorfer		
Sulfamethazine	Dr. Ehrenstorfer		
Sulfaquinoxaline	Sigma–Aldrich		
Sulfadimethoxine	Fluka		

10.5.4.2 Stock standard solutions and internal standard stock solutions

Prepare each stock solution and internal standard stock solutions at approximately 1000 mg/l when adequate material is available in appropriate solvent. For each stock solution, calculate the amount of base material needed (eg. accounting for purity and/or water and sulfate content) to prepare at the required concentration.

10.5.4.3 Intermediate standard solutions

Prepare each group of intermediate standard and internal standards solutions for the analytes below (Tables 68 and 69) in 10 ml volumetric flasks. Make up the volume to 10 ml for each standard group in acetonitrile except for the β–lactam group, which is dissolved in water.

TABLE 68. Mixed intermediate standards in acetonitrile

No	Standard Group	Analyte	Stock Standard Conc. (mg/l)	Volume Stock Std. (ml)	Mixed Intermediate Std Conc. (mg/l)
1	Amphenicol	Chloramphenicol	1 000	0.36	36
		Thiamphenicol	1 000	5	500
		Florfenicol	1 000	2.5	250
2	Quinolone	Ciprofloxacin	1 000	0.25	25
		Enrofloxacin	1 000	0.25	25
		Norfloxacin	1 000	0.125	12.5
3	Macrolide	Erythromycin	1 000	0.5	50
		Tylosin	1 000	0.25	25
		Tilmicosin	1 000	0.125	12.5
4	Sulfonamide	Sulfamethazine	1 000	0.25	25
		Sulfadiazine	1 000	0.25	25
		Sulfathiazole	1 000	0.25	25
		Sulfaquinoxaline	1 000	0.25	25
		Sulfadimethoxine	1 000	0.25	25
5	Dapsone	Dapsone	1 000	1.25	125
6	Tetracycline	Oxytetracycline	1 000	1	100
		Chlortetracycline	1 000	1	100
		Doxycycline	1 000	0.125	12.5
7	Nitroimidazole	Dimetridazole	1 000	0.5	50
		Ipronidazole	1 000	0.5	50
		Ronidazole	1 000	0.5	50
8	β–lactam*	Ampicillin	1 000	0.5	50
		Penicilin G	1 000	0.125	12.5
		Penicillin V	1 000	500	50

* volume is made up to 10 ml using water

TABLE 69. Mixed intermediate internal standards in acetonitrile

No	Standard Group	Analyte	Stock Standard Conc. (mg/l)	Volume Stock Std. (µl)	Mixed Intermediate Std Conc. (mg/l)
1	Amphenicol	CAP–D5	1 000	50	5
2	Quinolone	Enrofloxacin–D5	1 000	500	50
		Norfloxacin–D5	1 000	1 000	10
3	Sulfonamide	SMZ–$^{13}C_6$	1 000	500	50
4	Tetracycline	DMC	1 000	500	50
5	Dapsone	Dapsone–D8	1 000	50	5
6	β–lactam	Penicillin V	1 000	500	50
7	Macrolide	Roxythromycin	1 000	500	50
8	Nitroimidazole	IPZ–D3	1 000	50	5
		IPZ–OH–D3	1 000	50	5
		DMZ–D3	1 000	50	5
		RNZ–D3	1 000	50	5

10.5.4.4 Working standard and internal standard for spiking and matrix calibration

Prepare the composite "Acetonitrile Mix" working solution for the veterinary drugs using the intermediate standard solutions above and the volumes listed in Table 70.

a) Pipet the calculated volume and as shown in table below into a 10 ml volumetric flask.

b) Dilute to 10 ml volume with acetonitrile.

c) Cap flask and mix.

d) Transfer solution into glass vials with screw cap lids.

TABLE 70. Mixed working standards in acetonitrile

No	Standard Group	Analyte	Mixed Intermediate Std Conc. (mg/l)	Solution Volume, (ml)	Mixed Working Std Conc. (mg/l)
1	Amphenicol	Chloramphenicol	36	0.1	0.36
		Thiamphenicol	500		5
		Florfenicol	250		2.5
2	Quinolone	Ciprofloxacin	25	1	2.5
		Enrofloxacin	25		2.5
		Norfloxacin	12.5		1.25
3	Macrolide	Erythromycin	50	1	5
		Tylosin	25		2.5
		Tilmicosin	12.5		1.25
		Tiamulin	25		2.5
		Lincomycin	25		2.5

(con't)

TABLE 70. Mixed working standards in acetonitrile (con"t)

No	Standard Group	Analyte	Mixed Intermediate Std Conc. (mg/l)	Solution Volume, (ml)	Mixed Working Std Conc. (mg/l)
4	Sulfonamide	Sulfamethazine	25	1	2.5
		Sulfadiazine			
		Sulfathiazole			
		Sulfaquinoxaline			
		Sulfadimethoxine			
5	Dapsone	Dapsone	125	0.1	1.25
6	Tetracycline	Oxytetracycline	100	1	10
		Chlortetracycline	100		10
		Doxycycline	12.5		1.25
7	Nitroimidazole	Dimetridazole	50	0.1	0.5
		Ipronidazole			
		Ronidazole			
		Metronidazole			

Prepare the composite working solution for the veterinary drugs contained in the "Beta–Lactam mix" using the intermediate standard solutions above and the volumes listed in Table 71.

a) Pipet the calculated volume of intermediate standards and internal standard into a 10 ml volumetric flask.

b) Dilute to 10 ml volume with water.

c) Cap flask and mix.

d) Transfer solution into plastic screw cap vials.

TABLE 71. Mixed working beta–lactam standards and internal standard in water

No	Standard Group	Analyte	Stock Standard Conc. (mg/l)	Volume Stock Std.(ml)	Mixed Intermediate Std Conc. (mg/l)
1	β–lactam Std	Ampicillin	50	1	5
		Penicilin G	12.5		1.25

Prepare the composite working solution for the isotopically labeled veterinary drugs used for internal standards concentrations shown in Table 71.

a) Pipet the calculated volume of intermediate IS into a 10 ml volumetric flask.

b) Make up the volume to 10 ml with acetonitrile.

c) Cap flask and mix.

d) Transfer into 4 glass vials.

TABLE 72. Mixed working internal standards in acetonitrile

No	Standard Group	Analyte	Mixed Intermediate Std Conc. (mg/l)	Solution Volume, (ml)	Mixed Working Std Conc. (mg/l)
1	Amphenicol	CAP–D5	5	1	0.5
2	Quinolone	Enrofloxacin–D5	50		5
		Norfloxacin–D5	10		1
3	Sulfonamide	SMZ–$^{13}C_6$	50		5
4	Tetracycline	DMC	50		5
5	Dapsone	Dapsone–D8	5		0.5
6	β–lactam	Penicillin V	50		5
7	Macrolide	Roxythromycin	50		5
8	Nitroimidazole	IPZ–D3	5		0.5
		IPZ–OH–D3	5		0.5
		DMZ–D3	5		0.5
		RNZ–D3	5		0.5

10.6 Procedure

10.6.1 Sample preparation

Weigh 2 g (±0.1 g) of homogenized whole chicken egg samples into labeled 50 ml polypropylene centrifuge tubes.

10.6.2 Quality control samples

Blank samples (1 sample) and spiked samples (2 portions for high and low concentrations) are used as quality control samples. Prepare the blank, spiked sample and unknown samples as in Table 73.

TABLE 73. Preparation of controls and samples

Label	Std Mix Series 1 in acetonitrile (µl)	Std Mix Series 2 in acetonitrile (µl)	Mixed Working internal Std (µl)
Blank	-	-	40
Spike 1	20	20	40
Spike 2	80	80	40
Unknown	-	-	40

Vortex all uncapped tubes for 10 s after addition of standards. Allow to stand at least 10 min before continuing with the extraction procedure.

10.6.3 Sample extraction

- Add 8 ml of 0.2% formic in acetonitrile/water (80/20, v/v) to all tubes. Cap tubes well. Vortex tube for 30 s and shake all tubes using an appropriate shaker for 30 min. Centrifuge the tubes at 4 000 rpm for 10 min.

- Load 0.5 ml supernatant through Oasis Prime HLB, 3 ml, 60 mg without vacuum. Discard extract. Load another 0.5 ml through the same SPE. Collect 0.2 ml filtrate. Dilute filtrate with 0.6 ml water. Vortex 30 s, centrifuge high speed ~13 000 rpm for 5 min.
- Inject into the LC/MS system.

10.6.4 Instrumental analysis (LC–MS/MS)

The instrument parameters may be optimized to ensure system suitability.

10.6.5 UPLC operating parameters

a) Mobile phase for residue analysis:

- Mobile Phase A – 2 mmol/l ammonium acetate in water
- Mobile Phase B – 2 mmol/l ammonium acetate in methanol
- Flush the column with mobile phases A and B (50/10, v/v) at a flow rate of 0.5 ml/min for 3 min. Change the mobile phase initial conditions to 100% A. Allow the column to equilibrate until the "delta" value on the pressure reading is < 20.

b) UPLC gradient programme (Table 74)

- Flow rate: 0.35 ml/min
  Pressure Limits: 200 psi minimum; 18 000 psi maximum
  Run time: 11 min.

TABLE 74. UPLC gradient programme

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Gradient
0	90	10	none
0.25	90	10	linear
6	10	90	linear
7.5	10	90	linear
7.6	90	10	linear
11	90	10	linear

c) Autosampler, column programme

Run time: 11 min

Injection volume: 5 µl

Weak wash solvent: acetonitrile/water (20/80, v/v)

Strong wash solvent: acetonitrile/water (50/50, v/v)

Sample temperature: 15°C

Column temperature: 45°C

10.6.6 Mass spectrometer operating parameters

Mass spectrometer calibration and resolution are to be done according to the manufacturer's specification using the manufacturer's supplied calibration solution.

10.6.6.1 Electrospray Source Parameters

a) Capillary: 0.5 kV

b) Cone (V): Variable – analyte dependent

c) Source Temperature: 500°C

d) Desolvation Temperature: 800°C

e) Cone Gas Flow: 150 l/hr

f) Desolvation Gas Flow: 1 000 l/hr

g) Collision Gas Flow: 0.15 ml/min

10.6.6.2 Analyzer Parameters

a) LM 1 Resolution: 2.73

b) HM 1 Resolution: 14.74

c) Ion Energy 1: 0.1

d) LM 2 Resolution: 2.73

e) HM 2 Resolution: 15

f) Ion Energy 2: 0.5

10.6.6.3 MS Method Parameters

a) Type: MRM

b) Ion Mode: ESI+ and ESI-

c) Dwell (s):

d) Start time (min):

e) End time (min):

10.6.6.4 Instrumental Settings – MRM Parameters.

These are summarized in Table 75.

TABLE 75. Instrument settings

Analyte	Ionization Mode	RT (min)	Window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z	Cone (V)	Collision Energy (V))
Chloramphenicol	Negative	3.60	3.3–4.0	0.2	321	152, 257	70	18, 12
Thiamphenicol	Negative	2.38	2.0–3.0	0.2	354	185, 290	52	22, 12
Florfenicol	Negative	2.92	2.6–3.2	0.1	356	185, 336	56	22, 10
CAP–D5	Negative	3.58	3.3–4.0	0.2	326	157	54	18
Ciprofloxacin	Positive	2.85	2.5–3.2	0.001	332	288, 314	32	18, 22
Enrofloxacin	Positive	4.97	4.6–5.5	0.01	360	245, 316	32	20, 22
Norfloxacin	Positive	2.85	2.5–4.5	0.001	320	233, 276	32	25, 20
Enrofloxacin–D5	Positive	4.89	4.6–5.4	0.01	365	245	16	26
Norfloxacin–D5	Positive	2.85	2.5–4.5	0.001	325	281	30	18
Erythromycin	Positive	5.28	4.6–5.8	0.001	734	158, 576	30	30, 20

(con't)

TABLE 75. Instrument settings (con't)

Analyte	Ionization Mode	RT (min)	Window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z	Cone (V)	Collision Energy (V))
Tylosin	Positive	5.71	5.1–6.2	0.001	916.5	101, 174	60	45, 40
Tilmicosin	Positive	5.77	5.4–6.4	0.001	869	174, 696	25	45, 40
Sulfamethazine	Positive	2.61	2.3–3.0	0.001	279	156, 186	46	18, 16
Sulfadiazine	Positive	1.59	1.2–2.2	0.001	251	108, 156	32	24, 14
Sulfathiazole	Positive	1.93	1.6–2.3	0.001	256	108, 156	12	22, 14
Sulfaquinoxaline	Positive	2.98	2.6–3.4	0.001	301	108, 156	54	26, 16
Sulfadimethoxine	Positive	3.01	2.6–3.4	0.001	311	108, 156	38	26, 20
SMZ-^{13}C6	Positive	2.61	2.4–2.9	0.001	285	186	60	16
Dapsone	Positive	2.04	1.9–2.28	0.003	249	108, 156	48	20, 14
Dapsone-D8	Positive	2.04	1.9–2.28	0.003	257	160	40	14
Oxytetracycline	Positive	2.98	2.7–3.3	0.01	461.2	426.2, 443.1	30	19, 13
Chlortetracycline	Positive	3.85	3.6–4.3	0.001	479.3	444.2, 462.2	30	20, 18
Doxycycline	Positive	3.13	2.7–3.6	0.01	445.2	154, 428.2	30	28, 20
DMC	Positive	3.36	3.0–4.4	0.001	465.2	448.2	30	18
Ampicillin	Positive	2.77	1.0–5.0	0.001	350	160, 174	35	12, 15
Penicillin G	Positive	4.00	3.4–4.5	0.01	335	160, 176	64	14, 10
Penicillin V	Positive	4.08	3.9–4.6	0.01	351	160	23	10
Dimetridazole	Positive	2.24	1.8–2.6	0.05	142	81, 96	54	14, 14
Ipronidazole	Positive	.351	3.1–3.8	0.001	170	109, 124	2	24, 18
Ronidazole	Positive	1.81	1.6–2.3	0.05	201	55, 140	32	20, 10
IPZ–D3	Positive	3.50	3.1–3.8	0.001	173	127	54	20
DMZ–D3	Positive	2.23	1.8–2.6	0.001	145	99	54	14
RNZ–D3	Positive	1.79	1.6–2.3	0.001	204	143	35	10

10.6.7 Sample set

These are run as follows:

i) External standard

ii) Blank sample (negative control)

iii) Spike sample at low concentration (positive control)

iv) Spike sample at high concentration (positive control)

v) Up to 27 samples

vi) External standard, spike sample

NOTE: *Placing solvent blanks in the sample injection sequence is prudent in case of carry-over. Additionally, you may include an additional external standard or spiked sample within the sample injection sequence to verify retention time and instrument response stability.*

10.6.8 Calculation and interpretation of results

Monitored ions for each analyte will be assessed as follows:

i) The ion for each analyte should be present.

ii) All product ions specified for ratio matching are present with a S/N of ≥ 3.

iii) Retention time for ions in the samples should match the retention time of the positive control within 2.5%.

iv) Ion ratios are calculated by dividing the area count of each diagnostic ion by the area count of the base ion. Ion ratios should be less than 1. If the ratio is not less than 1 for a sample set, the inverse of this ratio may be used.

v) The level of the ion in the blank (negative control) should be less than 10% of the spiked 1 (positive control).

A sample is screened positive for an analyte as follows: If the sample meets the screen positive criteria below, further confirmation tests should be conducted:

i) Sample ion ratio match the ratio of the positive control sample.

ii) The stability of the ion ratio between the two transitions for the sample in accordance with the tolerances recommended as shown in Tables 76.

TABLE 76. Maximum permitted tolerances (%) for LC–MS/MS relative ion intensities (EC, 2002)

Relative intensity	Tolerances in LC–MS/MS (%)
≥ 50%	±20
≥20% <50%	±25
≥10% <20%	±30
<10%	±50

10.6.9 Schematic diagram of extraction procedure

DETERMINATION OF MULTICLASS VETERINARY DRUGS IN EGG USING LC-MS/MS

11

Confirmatory method for the determination of beta–agonists in the liver by LC–MS/MS

11.1 Introduction

β–agonists or β–adrenergic agonists are synthetic drugs sometimes used for therapeutic purposes in veterinary medicine. They were introduced as growth-promoting agents and are a physiological analog of adrenalin. However, these compounds also belong to a class of illegal growth promoters that decrease the fat content in farm animals. The use of β–agonists as growth promoters has been banned by some authorities such as the EU. Nevertheless, these drugs are still used illegally, and could result in concern.

11.2 Scope and objective

The multiresidue method described in this SOP applies to detection and identification β–agonists in tissues of different species, by LC–MS/MS and ESI mode. The method applies to the following β–agonists: Clenbuterol, Brombuterol, Zilpaterol, Isoxsuprine Ractopamine, and Salbutamol.

11.3 Principle of the method

Thawed and chopped liver/meat samples are homogenized in a meat mincer using phosphate buffer and further spinned in a centrifuge. The supernatant is combined with methanol and cleaned using SPE material. The sample is then mixed with β–glucuronidase (for enzymatic hydrolysis), further extracted by centrifugation and an aliquot evaporated to dryness. This is then reconstituted in water/methanol (25/75, v/v/) and analyzed on an LC–MS/MS after separation with a C–18 column.

11.4 Safety considerations and precautions

This method requires proper knowledge by the operator of basic rules for handling chemicals and solvents. As far as possible, it should be implemented under a fume hood. All necessary precautions should be taken to avoid contamination when handling standards and throughout extraction and purification operations. Samples are stored in the freezer at ~ -18°C upon arrival at the laboratory until analysis. Use protective gear such as gloves, laboratory coats and gloves.

11.5 Materials

11.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Deionized water; Phosphate buffer; KOH; HCl; Methanol; Acetic acid; Ethyl acetate; Ammonia; Nitrogen gas (N_2); Acetonitrile and Ammonium acetate.

11.5.2 Equipment/instruments and consumables

The following apparatus and material and applicable: pH meter; Vortex mixer; Meat mincer; Micropipettes; Water bath; Centrifuge; Freezer (-20°C); Precision balance; Ultrasonicator; Autosampler vials and inserts; Nitrogen evaporator; UHPLC system with triple quadrupole mass spectrometer: API 4000 QTRAP ABSIEX with turbo ionspray interface and Analyst software; Analytical column: Thermo Hypersil Gold column (50×2.1 mm, particle size 3 µm); Pre column: C18 (2.1×10 mm, 3.5 µm).

11.5.3 Solutions

The solutions (and the how they are prepared) include:

- Potassium phosphate buffer, pH 5: Weigh 13.6 g of potassium dihydrogen phosphate in a 1-liter flask, add 950 ml of water and adjust to pH 5 with 1 M HCl. This solution is then filled up to 1 liter with water. This buffer can be used for a month when stored in a refrigerator.
- Ammonium acetate 25 mM, pH 9.2: Dissolve 1.93 g (± 0.1 g) of ammonium acetate in 900 ml of ultra–pure water; adjust the pH to 9.2 with ammonium hydroxide, check the pH with a pH meter.
- 1 mol/l KOH: Weigh 56.1056 g of KOH and add 1 000 ml of ultra–pure water.
- 25% HCl solution: (HCl density of 1.49 kg/l), weigh 372.5g (25% of 1 490 g/l) of HCl, and add it to 1 litre of ultra–pure water.

11.5.4 Standards

11.5.4.1 Reference standards

All standards (Table 77): Salbutamol–d9, Salbutamol, cimaterol–d7, Zilpaterol, Ractopamine–d5, Ractopamine, Clenbuterol–d9, Clenbuterol, Brombuterol, Isoxsuprine should be above 95 % purity.

TABLE 77. Analytical standards and related conditions

Family	Standard	Internal code	Batch Number	Purity	Origin/ Manufacturer	Amount (mg)	Storage temperature (°C)	Stock solution concentration
β–agonists	Salbutamol	186–2	279/10	99,90%	Berlin	10.56	-20	1 g/l
β–agonists	Isoxsuprine HCl	40–1	061M1159	99,00%	Sigma	5000,00	-20	1 g/l
β–agonists	Zilpaterol	62–4 a	I 1213	*	BC labo city	5	-20	1 g/l
β–agonists	Cimaterol–D7	46–1	212137	99,00%	Afer	25	-20	1 g/l
β–agonists	Ractopamine HCl	223–1	303/11	95,90%	BVL	13.72	-20	1 g/l
β–agonists	Ractopamine–D5	76–a	2–AVS–95–4	95%	TRC Canada	10	-20	1 g/l
β–agonists	Clenbuterol–D9	88–3	BCBL757V	99,90%	SIGMA	10	-20	1 g/l
β–agonists	Clenbuterol HCl	92–4	278/08	99,10%	BVL	13.06	-20	1 g/l

11.5.4.2 Stock solutions (1 000 mg/l)

Individual stock solutions at 1 000 mg/l are prepared by dissolving 1 mg of each compound in 1 ml of methanol. These are stored at -20°C in brown glass to prevent photo–degradation. Internal standards are also prepared in the same manner at a concentration of 1 000 mg/l. Stock solutions are stable for at least two years if stored in a -20°C freezer.

11.5.4.3 Intermediate solutions (1 mg/l and 0.1 mg/l)

Intermediate solutions containing 1 mg/l and 0.1 mg/l of each standard and each internal standard are prepared in methanol. These solutions (1 mg/l) are stable for one year and half a year for working solutions (0.1 mg/l and lower concentrations) if stored in a +4°C refrigerator.

11.5.4.4 Working solution (10 µg/l)

A mixture of all the standards at 10 µg/l (Clenbuterol, Brombuterol, Zilpaterol, Isoxsuprine Ractopamine, and Salbutamol) is prepared in methanol.

A working solution containing 0.01 µg/l of mixture of all internal standards (D9-clenbuterol, D7-cimaterol, D5-ractopamine, and D9-salbutamol) is prepared in methanol.

11.6 Procedure

11.6.1 Sample preparation

After cutting partially thawed samples into smaller pieces in a conventional meat mincer, 10 g of homogenized bovine liver is transferred to a 50 ml tube with 20 ml of phosphate buffer (pH 5).

11.6.2 Quality control samples

Calibration range.

Preparation details of spiked samples, blanks, and spiked samples for calibration are as follows:

- For blank samples: 250 µl of internal standard are added to each tube;
- Spiked samples: 250 µl of internal standard and 200 µl of working solution standard at 0.2 ppb
- Spiked samples for calibration: 5 tubes (Tube 1: blank, Tube 2: ½ MRL, Tube 3: 1MRL, tube 4: 1.5 MRL) containing each one 250 µl of internal standard and spiked at 100 ppb, 200 ppb, and 300 ppb, respectively.
- For the confirmatory purpose, 5 tubes containing 250 µl of the working solution are prepared: mixture of internal standards at 10 µg/l in each of the tubes and 100 µl, 200 µl, 350 µl and 500 µl of the working solution: mixture of standards at 0.2 ppb. The blanks, spiked samples, and matrix matched samples are extracted/processed same as study samples.

11.6.3 Extraction procedure

- After 250 µl of the working solution mixture of internal standards at 10 µg/l is added, vortex sample for 15 s at least and leave in contact for 10 min. The pH value should be checked and if necessary, the pH is adjusted to 5 (±0.3) using 1 mol/l KOH or 25% HCl.
- The homogenized samples are shaken for 20 min in a headover at 40°C, and then allowed to cool to room temperature.

- The pH is checked again and, if necessary, adjusted to 5 (± 0.3) using 1 mol/l KOH or 25% HCl.
- The samples are then centrifuged at 4°C for 30 min at 3 000 rpm and the supernatant transferred to another centrifuge tube. Extract the sample again with 10 ml of pH 5 phosphate buffer, centrifuge and combine the supernatant. Adjust the pH to 5 (±0.3) if necessary.
- Add 50 ± 5 µl of β–glucuronidase to the extract and vortex. Incubate for 60 min at 40 (±2) °C in a water bath. Let the samples cool to room temperature.
- The pH is adjusted to 6 using 5 mol/l KOH.
- Centrifuge at 3 000 rpm for at least 5 min, then add 200 µl of methanol to the supernatant.

11.6.4 Sample cleanup

- Extracted samples are cleanedup using CleanScreenDau (C8/benzosulfonic acid).
- SPE cartridges are preactivated with 2 ml of methanol 2 ml of ultrapure water and 2 ml of phosphate buffer (pH 6).
- After loading sample supernatants, the cartridges are washed with 1 ml of acetic acid 1 M.
- A strong vacuum is applied for at least 20 min, and then 2 ml of methanol added.
- The analytes are eluted with 6 ml of a mixture of ethyl acetate and ammonia in a ratio of 97:3, v/v with a maximum drop rate of 1 ml/min.
- The extract is evaporated under a stream of nitrogen at 35°C.
- After evaporation, take up with 200 µl of the water/methanol mixture (75/25, v/v) brought directly to the bottom of the tube and not on the walls.
- Agitate for at least 30 s and transfer to a screw vial containing a 150 µl insert.

11.6.5 Instrumental analysis (LC–MS/MS)

LC–MS/MS analysis is performed on a UHPLC system (PerkinElmer) consisting of a binary UHPLC pump (FLEXAR or equivalent), and auto sampler (FLEXAR or equivalent) coupled to an AB SCIEX QTRAP 4 000/5 500 triple quadrupole mass spectrometer (Framingham, MA, USA) with Ion Source Turbo Spray.

The chromatographic conditions are as follows:

a) Separation mode: reversed phase chromatography

b) Analytical column: Thermo Hypersil Gold column (50×2.1 mm, particle size 3 µm)

c) Precolumn: C18 (2.1×10 mm, 3.5 µm)

d) Column temperature: 25°C

e) Injection volume: 10 µl

f) Flow rate: 350 µl/min

g) Run time: 9 min

h) Mobile phase: ammonium acetate (pH 9.2) 25 mmol/l (A), acetonitrile (B)

i) Elution mode: linear gradient (Table 78).

TABLE 78. Mobile phase gradient

Time (min)	Solvent A (%)	Solvent B (%)
0.1	95	5
0.1	95	5
5.5	0	100
6.5	0	100
7.0	95	5
9.0	95	5

Mass spectrometric conditions: Mass analysis is carried out using an electrospray ionization source in positive mode. The operation conditions for this mode are as follows:

- Ionspray voltage: 5.5 kV
- Source temperature: 500°C
- Curtain gas: 40 psi
- Ion source gases 1 = 40 psi and 2 = 9 psi.

The optimal MRM parameters are summarized in Table 79.

TABLE 79. MRM parameters

Compound	Ion precursor (m/z)	Ions products (m/z)	Dwell time (ms)	DP	CE	CXP
Salbutamol–D9	249.3	149	25	56	27	8
Salbutamol	240.3	222.1 148.1	25 25	46 46	15 25	4 8
Cimaterol–D7	227.3	161	25	41	25	8
Zilpaterol	262,2	202.1 185.1	25 25	46 46	15 25	4 8
Ractopamine–D5	307.29	167.1	25	61	23	8
Ractopamine	302,3	164.2 121.1	25 25	51 51	23 31	8 8
Clenbuterol–D9	286.2	204	25	51	23	10
Clenbuterol	277.2	258.9 203	25 25	56 56	17 23	6 4
Brombuterol	367.1	348.1 292.1	25 25	51 51	17 27	8 14
Isoxsuprine	302.3	284.0 150.2 107.0	25 25 25	46 46 46	19 31 39	6 8 18

DP: Declustering potential
CE: Collision energy
CXP: Collision Cell exit potential

11.6.6 Calculation and interpretation of results

The presence of β–agonists in extracts is confirmed when defined criteria (EC, 2002) are met:

i) The relative retention time of the detected analyte should be identical to that of the spiked samples within ± 2.5%.

ii) At least two specific transitions of each β–agonist should be detected in the samples to be analyzed with a S/N > 3.

iii) The relative intensities of the transitions in the extracts should be compared with the relative intensities spiked samples (within the same concentration range) and respect the limits of the following tolerance values in Table 80.

TABLE 80. Relative intensity

Ion m/z Relative Intensity (% Base peak)	LC–MS/MS
> 50 %	± 20 %
> 20 to 50 %	± 25 %
> 10 to 20 %	± 30 %
≤ 10 %	± 50 %

Quantification is done based on the major transition considering the internal standard. The calibration line is established from the control sample and spiked samples.

11.6.7 Method validation

These methods provide reliable identification (at least 4 identification points) of unauthorized substances and substances with an established MRL and quantification of analytes (MRL compounds) following established guidelines (EC, 2002). LC–MS/MS methods fulfil these requirements with one precursor ion and at least two transition product ions (1.5 IP each one).

Specificity

Twenty blank liver samples from different sources are analyzed to verify the absence of interfering peaks. Each molecule is specifically identified by two minimum transitions (parent ion > product ion) and retention time.

Linearity

The linearity of the analytical method is validated using the matrix matched curves for each compound at different concentration levels to prevent matrix effects. For each series, the equation of the calibration ranges is calculated from blank and spiked samples at 0.1 µg/kg, 0.2 µg/kg, 0.35 µg/kg and 0.5 µg/kg. The equation of the calibration curve is established. The R^2 coefficient of the calculated regression curves is always above 0.97 (Table 81):

TABLE 81. The coefficient of regression

Analytes	Slope a		Ordinate b		R^2		
	signal 1 (+intense)	signal 2 (–intense)	signal 1 (+intense)	signal 2 (–intense)	signal 1 (+intense)	signal 2 (–intense)	
Salbutamol		8.55	7.75	0.02	0.03	0.99	0.99
Zilpaterol		0.87	0.59	0.002	0.005	0.98	0.98
Ractopamine		5.19	3.13	0.005	0.01	0.98	0.98
Clenbuterol		4.94	2.97	0.02	0.02	0.99	0.99
Brombuterol		2.26	2.24	0.002	0.001	0.98	0.99
Isoxsuprine		7.06	1.02	0.02	0.003	0.99	0.99

Repeatability

Repeatability (intraday precision) is evaluated performing replicates and within–laboratory reproducibility (interday precision) is evaluated by analyzing samples over the course of three consecutive days. For all the measured compounds, the analyzed recovery complies with established ranges (EC, 2002). The Coefficients of variation, relative time/ratio and ruggedness are summarized in Tables 82–84:

TABLE 82. Coefficient of variation for method repeatability

Analyte	CV% (Signal)	
	Signal 1 (+intense)	Signal 2 (–intense)
Salbutamol	32.3	28.3
Zilpaterol	27.9	22.5
Ractopamine	32.8	32.3
Clenbuterol	34.5	35.4
Brombuterol	18.5	23.0
Isoxsuprine	34.5	28.8

TABLE 83. Repeatability results

Analyte	CV% (TR)	CV% (Ratio)
Salbutamol	0.3	8.2
Zilpaterol	1.2	8.4
Ractopamine	0.4	6.3
Clenbuterol	0.6	6.2
Brombuterol	0.2	9.1
Isoxsuprine	1.3	14.6

TABLE 84. Method ruggedness

Analyte	Theoretical concentration (µg/kg)		Estimated concentration (µg/kg)		Ruggedness %	
	Signal 1 (+intense)	Signal 2 (–intense)	Signal 1 (+intense)	Signal 2 (–intense)	Signal 1 (+intense)	Signal 2 (–intense)
Salbutamol	0.2	0.2	0 .21	0.21	5.5	-2.6
Zilpaterol	0.2	0.2	0.22	0.22	11.9	18.9
Ractopamine	0.2	0.2	0.22	0.22	12.3	8.9
Clenbuterol	0.2	0.2	0.17	0.17	-14.2	-16.7
Brombuterol	0.2	0.2	0.18	0.18	-7.8	-16.5
Isoxsuprine	0.2	0.2	0.15	0.19	-24.2	-3.7

The decision limit and detection capability

An overview of validation results including CCα and CCβ is shown in Table 85.

TABLE 85. Decision limit and detection capability

Analyte	Decision limit CCα (µg/kg)		Detection capability CCß (µg/kg)	
	Signal 1 (+intense)	Signal 2 (–intense)	Signal 1 (+intense)	Signal 2 (–intense)
Salbutamol	0.004	0.01	0.01	0.02
Zilpaterol	0.005	0.01	0.01	0.02
Ractopamine	0.002	0.01	0.004	0.03
Clenbuterol	0.01	0.01	0.02	0.04
Brombuterol	0.003	0.001	0.01	0.001
Isoxsuprine	0.01	0.01	0.01	0.02

The decision limits obtained are all well below recommended concentrations (CRL, 2007).

Method uncertainty

Uncertainty of the method is equal to 2* the repeatability standard deviation (Table 86).

TABLE 86. Method uncertainty

Molecule	Standard deviation (Signal 1)	Uncertainty
Salbutamol	1.18	2.36
Zilpaterol	0.12	0.24
Ractopamine	0.76	1.52
Clenbuterol	0.60	1.2
Brombuterol	0.16	0.32
Isoxsuprine	0.74	1.48

12

Confirmatory method for determination of anthelmintics in poultry, bovine and ovine muscle by LC–MS/MS

12.1 Introduction

Anthelmintics are one of the most widely used groups of veterinary medicines in the world. They are used in prophylaxis and therapeutic treatment of parasitic infections in livestock animals including cattle, sheep and poultry. These drugs include various benzimidazole compounds, imidazothiazoles, macrocyclic lactones and flukicides. Organophosphorus compounds have also previously been used as anthelmintics. Concerns over the toxicity of some of these drugs led the EU to establish MRLs for their residues in tissues of food–producing animals.

12.2 Scope and objective

This method is applied for the identification (screening) and/or confirmation (identification and quantification) of anthelmintics in poultry, cattle and sheep muscle, by LC–MS/MS and ESI. The method is applied in particular to the following anthelmintics: Thiabendazole, Levamisole, 5 hydroxythiabendazole, Amino Mebendazole, Albendazole sulfonamide, Oxibendazole, Albendazole sulfoxide, Mebendazole, Albendazole sulfone, Fenbendazole, Flubendazole, Oxfendazole, Oxfendazole sulfone, Clorsulon, Ketotriclabendazole, Triclabendazole sulfone, Triclabendazole sulfoxide, Triclabendazole, Nitroxinil, Closantel, and Rafoxanide. The following deuterated compounds are used as internal standards: Thiabendazole $^{13}C_6$, Albendazole–2-aminosulfone–D3, Oxibendazole–D7, Albendazole–D3, Albendazole sulfoxide–D3, Mebendazole–D3, Albendazole sulfone D3, Oxfendazole–D3, Oxfendazole sulfone–D3, Fenbendazole–D3, Nitroxinil $^{13}C_6$, Triclabendazole–D3 and Rafoxanide $^{13}C_6$.

12.3 Principle of the method

The method involves in extracting a sample with acetonitrile, followed by phase separation from water. After centrifugation, the supernatant is evaporated to dryness at 50°C. The residue is reconstituted with 250 µl of 0.1% formic acid in water. Detection is then carried out on an ion-trap mass spectrometer by a multiple reaction monitoring (MRM) mode and an enhanced product ion (EPI) mode and ESI.

12.4 Safety considerations and precautions

This method requires knowledge by the operator on basic guidelines for handling chemicals and solvents. As far as possible, work should be done under a fume hood.

All necessary precautions should be taken to avoid contamination when handling standards and throughout extraction and purification operations. Samples are stored in the freezer at approximately -18°C upon arrival at the laboratory until analysis. Use a laboratory coat, eye protection and gloves.

12.5 Materials

12.5.1 Reagents and solvents

The following reagent and chemicals are applicable: Ultrapure water; Acetonitrile; DMSO; Formic acid and NaCl.

12.5.2 Equipment/instruments and consumables

The following apparatus and materials are applicable: UHPLC system with triple quadrupole mass spectrometer: API 4 000 QTRAP ABSIEX with turbo ionspray interface and Analyst software; Polypropylene bottles; Freezer (-20°C); Micropipettes; Domestic food processor; Analytical balance; Tissue homogenizer; Vortex mixer; Rotary shaker; Centrifuge; Nitrogen gas; Microcentrifuge tubes; Precision balance; Ultrasonic tank; Chromatographic column (Acquity UPLC): 100×2.1 mm, particle size 1.8 µm (C18) and Corresponding precolumn; FLEXAR binary HPLC pump or equivalent and FLEXAR automatic injector or equivalent.

12.5.3 Solutions

The solutions (and how they are prepared) include:

- DMSO/Acetonitrile (30/70, v/v) solution: For a volume of 100 ml, 30 ml of DMSO is added to 70 ml of acetonitrile.
- Mobile phase: for 100 ml of mobile phase A, 100 µl of formic acid is added to 100 ml of ultrapure water and 100 µl of formic acid in 100 ml of acetonitrile for the phase B.

12.5.4 Standards

All standards (Table 87) should be above 98% purity.

TABLE 87. Analytical standards used

Family	Molecule	Internal Code	Batch number	Origin/ Manufacturer	Storage temperature °C	Stock solution concentration
Anthelmintics	Levamisole HCl	67–17	111/02	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole–D3	72–3	913/04	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole sulfoxide	78–5	122/14	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole sulfone	80–8	126/09	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole–2-aminosulfone HCl	84–3	1242/03	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole sulfone–D3	86–3	912/04	BVL Berlin	-20	0.1 mg/ml

(con't)

TABLE 87. Analytical standards used (con't)

Family	Molecule	Internal Code	Batch number	Origin/ Manufacturer	Storage temperature °C	Stock solution concentration
Anthelmintics	Fenbendazole sulfone	87–6	052/12	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Oxfendazole	106–4	114/11	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Flubendazole–D3	108–4	1059/03	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Oxibendazole	144–3	121/10	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Oxibendazole–D7	157–3	1091/03	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Flubendazole	161–3	092/06	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Mebendazole– D3	165–3	1060/02	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Mebendazole	166–3	095/06	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Fenbendazole Sulfone–D3	169–4	1056/03	BVL Berlin	-20	0.1 mg/ml
Anthelmintics	Fenbendazole D3	97–3	979/03	Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole–2–aminosulfone D3 hydrochloride	66–2	090512	Witega	-20	0.1 mg/ml
Anthelmintics	5–Hydroxy thiabendazole	114–3	232133	Witega	-20	0.1 mg/ml
Anthelmintics	Albendazole	25–24	120/09	BERLIN	-20	0.1 mg/ml
Anthelmintics	Thiabendazole	39–4	112/08	Berlin	-20	0.1 mg/ml
Anthelmintics	Fenbendazole	13–7	113/10	Berlin	-20	0.1 mg/ml
Anthelmintics	Thiabendazole $^{13}C_6$	237–3	775/07	Berlin	-20	0.1 mg/ml
Anthelmintics	Oxfendazole–D3	117–3	1061/03	Berlin	-20	0.1 mg/ml
Anthelmintics	Albendazole sulfoxide–D3	60–3	914/06	Berlin	-20	0.1 mg/ml
Anthelmintics	Mebendazole–amine	41–3	132/08	Berlin	-20	0.1 mg/ml
Anthelmintics	Clorsulon	34–10	BCBV4517	99.30 %	100 mg	0.1 mg/ml
Anthelmintics	Triclabendazole sulfoxide	122–2	251718	—	100 mg	0.1 mg/ml
Anthelmintics	Triclabendazole sulfone	123–2 b	232328	—	50 mg	0.1 mg/ml
Anthelmintics	Triclabendazole	151–4	783194	98.9 %	100 mg	0.1 mg/ml
Anthelmintics	Nitroxynil	159–4	779306	99.2 %	100 mg	0.1 mg/ml
Anthelmintics	Nitroxynil–phenyl $^{13}C_6$	167–4–c	789037	99.70 %	10mg	0.1 mg/ml
Anthelmintics	Ketotriclabendazole	210–2	SZBD185XV	99.10%	10mg	0.1 mg/ml
Anthelmintics	Triclabendazole–D3	233–2	SZBE049XV	99.3%	10mg	0.1 mg/ml
Anthelmintics	Rafoxanide–$^{13}C_6$	239–2	SZBD028XV	99.4%	10 mg	0.1 mg/ml

(—): Purity information not provided by manufacturer

12.5.4.1 Stock solutions (100 mg/l)

- Stock solutions at 100 mg/l of active substances are prepared independently for each of the molecule in DMSO/acetonitrile (30/70, v/v).
- Dissolve well and transfer the contents of each vial into polypropylene bottles.
- Store stock solutions in a freezer at -20 °C for up 1 yr.

12.5.4.2 Working solutions

- A working solution containing a mixture of all the standards was prepared in a DMSO/acetonitrile (30/70, v/v).
- Working solutions are stable for at least 2 months in the freezer at -20°C protected from light.
- A working solution containing a mixture of all internal standards is prepared in the same manner. The preparation of all working solutions is performed according to the recommendations indicated in Table 88.

TABLE 88. Preparation of working solutions

Compound	volume to take from the stock solution at 100 mg/l and into vials of 20 ml DMSO/ACN (30/70, v/v) (µl)	Concentration in the spiking solution (mg/l)
Albendazole	200	1
Albendazole–2-aminosulfone	200	1
Albendazole sulfone	200	1
Albendazole sulfoxide	200	1
Thiabendazole	200	1
5-Hydroxy Thiabendazole	200	1
Rafoxanide	200	1
Ketotriclabendazole	450	2.25
Triclabendazole	450	2.25
Triclabendazole sulfone	450	2.25
Triclabendazole sulfoxide	450	2.25
Oxibendazole	200	1
Fenbendazole	100	0.5
Fenbendazole sulfone	100	0.5
Oxfendazole	100	0.5
Flubendazole	100	0.5
Mebendazole	120	0.6
Amino Mebendazole	120	0.6
Levamisole	20	0.1
Nitroxynil	800	4
Closantel	2 000	10
Clorsulon	70	0.35
Albendazole–D3	200	1

(con't)

TABLE 88. Preparation of working solutions (con't)

Compound	volume to take from the stock solution at 100 mg/l and into vials of 20 ml DMSO/ACN (30/70, v/v) (µl)	Concentration in the spiking solution (mg/l)
Albendazole–2-aminosulfone–D3	200	1
Albendazole sulfone–D3	200	1
Albendazole sulfoxide–D3	200	1
Thiabendazole $^{13}C_6$	200	1
Rafoxanide $^{13}C_6$	200	1
Triclabendazole–D3	450	2.25
Oxibendazole–D7	400	2
Fenbendazole–D3	100	0.5
Fenbendazole sulfone–D3	100	0.5
Oxfendazole–D3	100	0.5
Flubendazole–D3	100	0.5
Mebendazole–D3	120	0.6
Nitroxynil–$^{13}C_6$	1 600	8
Tetramisole–D5	40	0.2

12.6 Procedure

12.6.1 Sample preparation

- Meat samples are thawed to room temperature and lean meat portions (fat removed) individually minced in a domestic food processor.
- Samples of 2.0 g are weighed into 50 ml polypropylene tubes. 0.5 g of NaCl added and extracted by homogenization for 1 min in acetonitrile (15 ml).
- 200 µl of the mixture of standards are added to obtain positive controls at MRL, then 100 µl of internal standards mixture added to the extract.
- Levels of matrix matched standards (in µg/kg) for muscle are defined in Table 89:

TABLE 89. Anthelmintics MRL and spiking levels

	Metabolites	Level 1	Level 2	Level 3	Level 4	MRL
Albendazole	Albendazole–2-amino sulfone; Albendazole sulfone; Albendazole sulfoxide	50	100	150	200	100
Thiabendazole	5-Hydroxy Thiabendazole	50	100	150	200	100
Rafoxanide		50	100	150	200	30 cattle 100 sheep
Triclabendazole	Ketotriclabendazole; Triclabendazole sulfone; Triclabendazole sulfoxide	112.5	225	337.5	450	225

(con't)

TABLE 89. Anthelmintics MRL and spiking levels (con't)

	Metabolites	Level 1	Level 2	Level 3	Level 4	MRL
Oxibendazole		50	100	150	200	100
Fenbendazole	Fenbendazole sulfone; Oxfendazole	50	100	150	200	100
Flubendazole		25	50	75	100	50
Mebendazole	Amino Mebendazole	30	60	90	120	60
Levamisole		5	10	15	20	10
Nitroxynil		200	400	600	800	400
Closantel		500	1 000	1 500	2 000	1 000 cattle 1 500 sheep
Clorsulon		17.5	35	52.5	70	35

12.6.2 Samples extraction

- Acetonitrile (15 ml) is added to the mixture and shaken on vortex for 1 min and further for 10 min on the rotary shaker. Centrifuge at 4 000 rcf for 10 min.
- Take 9 ml of the supernatant into tubes and add 250 µl of DMSO to the extract and evaporate to dryness under a stream of nitrogen at about 50°C for 1 hr.
- Reconstitute the residue with 250 µl of the ultra–pure water solution at 0.1% formic acid and vortex, and
- Transfer the contents of the tubes into the eppendorf tubes and centrifuge for 10 min at 16 000 rpm and 4°C.

12.6.3 Calibration range

For confirmatory purposes, prepare 5 tubes containing 250 µl of the working solution: a mixture of internal standards at 10 µg/l is added to each of the tubes with 100 µl, 200 µl, 350 µl and 500 µl of the working standard solution (10 µg/l).

12.6.4 Instrumental analysis (LC–MS/MS)

The chromatography conditions performed using a UHPLC system (PerkinElmer) coupled to an AB SCIEX QTRAP 4000/5500 mass spectrometer (Framingham, MA, USA) include:

- Analytical column: Acquity UHPLC C18 (100×2.1 mm, particle size 1.8 µm) and corresponding precolumn
- Column temperature: 50°C;
- Injection volume: 20 µl;
- Flow rate: 500 µl/min;
- Run time: 15 min;
- Mobile phase: Ultrapure water with 0.1% formic acid, acetonitrile with 0.1% formic acid;
- Elution mode: Linear gradient (Table 90).

TABLE 90. Mobile phase gradient

Temps (min)	Gradient profile	Ultra pure water + 0.1 % HCOOH	Acetonitrile + 0.1 % HCOOH
0.1	0	95	5
1	1	95	5
2	6	85	15
6	6	50	50
11	6	2	98
12	6	2	98
13	6	95	5
15	1	95	5

Mass spectrometric analysis is carried out using an electrospray ionization source in positive and negative mode.

The operation conditions for the negative mode are as follows:

- Ionspray voltage: 5.5 kV
- Source temperature: 450°C
- Curtain gas: 25 psi
- Ion source gases 30 psi and 70 psi

The optimal MRM parameters are summarized in Table 91.

TABLE 91. MRM parameters in negative ionization mode

Compounds	Ion precursor (m/z)	Ions products (m/z)	Retention time (min)	Dwell time (ms)	DP	CE	CXP	Internal standard
Clorsulon	379.8 379.8	343.9 341.8	7.7	100 100	-51 -51	-28 -12	-4 -4	Nitroxynil $^{13}C_6$
Ketotriclabendazole	326.9 326.9	189.8 146.1	10.29	30 30	-71 -71	-32 -42	-2 -2	Triclabendazole D3
Triclabendazole sulfoxide	372.9 372.9	357.9 180.9	10.74	30 30	-151 -151	-30 -52	-4 -2	Triclabendazole d3
Triclabendazole sulfone	388.9 388.9	309.9 311.9	11.53	30 30	-91 -61	-24 -32	-4 -1	Triclabendazole D3
Triclabendazole	356.9 356.9	196.9 212.0	11.73	30 30	-96 -96	-44 -30	-2 -2	Triclabendazole D3
Triclabendazole–D3	359.9	196.9	11.71	30	-91	-44	-2	n.a
Nitroxynil	289.0 289.0	126.8 161.7	8.81	30 30	-46 -46	-34 -26	-1 -1	Nitroxynil $^{13}C_6$
Nitroxynil $^{13}C_6$	294.9	126.9	8.82	30	-61	-38	0	n.a
Closantel	662.9 662.9	344.7 317.0	13	80 80	-126 -126	-38 -36	-4 -4	Rafoxanide $^{13}C_6$
Rafoxanide	623.8 623.8	344.7 217.0	13.63	80 80	-101 -101	-34 -56	-4 -4	Rafoxanide $^{13}C_6$
Rafoxanide $^{13}C_6$	631.8	126.7	13.63	80	-96	-62	-4	n.a

DP: Declustering potentia CE: Collision energy CXP: Collision Cell exit potential n.a: No internal standards used

For the positive mode

- Source temperature: 450°C
- Curtain gas: 25 psi
- Ion source gases 30 psi and 70 psi

The optimal MRM parameters are summarized in Table 92:

TABLE 92. MRM parameters in positive ionization mode

Compounds	Ion precursor (m/z)	Ions products (m/z)	Retention time (min)	Dwell time (ms)	DP	CE	CXP	Internal standard
Levamisole	205.2 205.2	178.2 132.1	5.96	40 40	51 51	27 35	4 4	Tetramisole–D5
Albendazole–2-aminosulfone–D3	243	133.4	8.07	40	66	39	4	n.a.
Albendazole sulfone	240.1 240.1	133.1 198.1	8.07	40 40	66 66	35 21	4 4	Albendazol–2-aminosulfone–D3
Thiabendazole	202.1 202.1	175.1 131.3	8.10 8.10	40 40	61 61	31 39	4 4	Thiabendazol–$^{13}C_6$
Thiabendazole $^{13}C_6$	208.1	181.2	8.08	40	66	33	4	n.a.
Albendazole sulfoxide–D3	285.1	243.1	8.26	24	33	15	4	n.a.
Amino Mebendazole	238.1 238.1	105.1 133.1	8.39 8.39	24 24	76 76	33 47	4 4	Mebendazole–D3
Albendazole sulfoxide	282.1 282.1	240.1 208.2	8.27 8.27	24 24	36 36	17 27	4 4	Albendazole sulfoxide–D3
Oxibendazole	250.1 250.1	218 176	8.59 8.59	24 24	41 41	21 33	4 4	Oxibendazole–D7
Oxibendazole–D7	257.1	225.3	8.58	24	28	23	4	n.a.
Oxfendazole–D3	319.1	159.2	8.56	24	66	39	4	n.a.
Albendazole sulfone–D3	301.1	224.1	8.62	24	56	27	4	n.a.
Albendazole sulfone	298.1 298.1	266.1 159.1	8.62 8.62	24 24	51 51	21 45	4 4	Albendazole sulfone–D3
Oxfendazole	316.1 316.1	159.1 191.1	8.57 8.57	24 24	41 41	41 27	4 4	Oxfendazole–D3
Albendazole–D3	269.1	234.1	9.04	40	46	21	4	n.a.
Albendazole	266.2 266.2	234 191	9.05 9.05	40 40	46 46	21 37	4 4	Albendazole–D3
Mebendazole–D3	299.1	264	9.07	40	56	23	4	n.a.
Oxfenbendazole sulfone–D3	335	300.1	8.56	40	81	25	4	n.a.
Oxfenbendazole sulfone	332.1 332.1	300.2 159	9.06 9.06	40 40	51 51	27 49	4 4	Oxfenbendazole sulfone–D3
Mebendazole	296.3 296.3	264.1 105	9.08 9.08	40 40	56 56	23 43	4 4	Mebendazole–D3
Flubendazole	314.1 314.1	282 123.1	9.27 9.27	40 40	46 46	23 47	4 4	Mebendazole–D3

(con't)

TABLE 92. MRM parameters in positive ionization mode (con't)

Compounds	Ion precursor (m/z)	Ions products (m/z)	Retention time (min)	Dwell time (ms)	DP	CE	CXP	Internal standard
Fenbendazole	300.1 300.1	268 159	9.69 9.69	100 100	71 71	23 43	4 4	Fenbendazole–D3
Fenbendazole–D3	303.2	268.2	9.67	100	78	25	4	n.a.
5-Hydroxy Thiabendazole	218.1 218	191.1 147.1	7.96 7.96	40 40	66 66	31 41	4 4	Thiabendazole–$^{13}C_6$
Tetramisole–D5	210.3	183.1	8.07	40	46	27	4	n.a.

n.a.: No internal standards used

12.6.5 Interpretation of results

Confirmation: The method has been validated and presence of anthelmintics residues confirmed following specific criteria/guidelines (EC, 2002):

i) The relative retention time of the detected analyte should be identical to that of the spiked samples within ±2.5%.

ii) At least two specific transitions of each anthelmintic should be detected in the samples to be analyzed with a S/N ratio of > 3.

iii) The relative intensities of the transitions in the extracts should be compared with the relative intensities of spiked samples (within the same concentration range) and meet the following tolerance requirements in Table 93:

TABLE 93. Relative intensity

Ion m/z relative intensity (% of base peak)	LC–MS/MS
> 50%	± 20%
> 20 to 50%	± 25%
> 10 to 20%	± 30%
≤ 10%	± 50%

Quantification: This is done using the major transition and the internal standard. The calibration curve is established from the control sample and spiked samples.

12.6.6 Method validation

These methods provide reliable identification (at least 4 identification points) of unauthorized substances and substances with an established MRL and quantification of analytes (MRL compounds) following established guidelines (EC, 2002) LC–MS/MS methods fulfil these requirements with one precursor ion and at least two transition product ions (1.5 IP each one).

Specificity

Twenty blank liver samples from different sources are analyzed to verify the absence of interfering peaks. Each molecule is specifically identified by two minimum transitions (parent ion > product ion) and retention time.

Linearity

This is determined using matrix matched standard curves for each compound. For each series, the equations of the calibration ranges are calculated from blank

and spiked samples at 0.1 µg/kg, 0 µg/kg, 2 µg/kg, 0.35 µg/kg and 0.5 µg/kg. The R^2 coefficient of the calculated regression curves are above 0.97.

Repeatability

Repeatability (intraday precision) is evaluated by performing replicates and within-laboratory

Reproducibility (interday precision) is evaluated by analyzing samples over the course of three consecutive days.

The decision limit and detection capability

The decision limit (CCα) is "the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant", and the detection capability (CCβ) as "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β". The α and β errors should be less than or equal to 1% and 5%, respectively (EC, 2002).

Method uncertainty

Uncertainty of the method is equal to 2 * the repeatability standard deviation (see Table 94).

TABLE 94. Method uncertainty

Molecule	Standard deviation (signal 1)	Uncertainty (µg/kg)
Levamisole	0.224	0.448
Albendazole sulfone	0.116	0.232
Thiabendazole	0.172	0.344
Amino Mebendazole	0.332	0.664
Albendazole sulfoxide	0.134	0.268
Oxibendazole	0.180	0.360
Albendazole sulfone	0.746	1.492
Oxfendazole	0.026	0.052
Albendazole	0.260	0.52
Oxfendazole sulfone	0.452	0.904
Mebendazole	0.148	0.296
Flubendazole	0.246	0.492
5-Hydroxy Thiabendazole	0.131	0.262
Clorsulon	0.012	0.024
Ketotriclabendazole	0.440	0.88
Triclabendazole sulfoxide	0.088	0.176
Triclabendazole sulfone	0.028	0.056
Triclabendazole	0.076	0.152
Nitroxynil	0.280	0.56
Closantel	3.316	6.632
Rafoxanide	0.034	0.068

The confirmation method for the determination of anthelmintics in ovine, bovine and poultry muscle by LC–MS/MS is validated accordingly (EC, 2002) with ranges between 0 μg/kg and 0.5 μg/kg. The decision limits of the method are higher than the MRL or the recommended value for each molecule (CRL, 2007).

12.6.7 Schematic diagram of extraction procedure

SAMPLE	1. Thaw meat samples and mince 2. Weigh 2 g of sample in to a 50 ml polypropylene tube 3. Add 0.5 g of Naal 4. Add 15 ml of acetonitrile and homogenize for 1 min 5. Add 100 μl of internal standard to the extract
↓	
EXTRACTION	1. Add 15 ml of acetonitrile and vortex for 1 min 2. Add 100 μl of internal standard to the extract 3. Shake for 10 min on rotary shaker 4. Centrifuge at 4 000 rcf for 10 min 5. Take 9 ml of supernatant and add 250 μl of DMSO 6. Evaporate to dryness using nitrogen at 50°C for 1 hr 7. Reconstitute the residue with 250 μl of ultrapure water solution at 0.1% formic acid and vortex 8. Transfer the solution to microcentrifuge tube 9. Centrifuge at 16 000 rpm at 4°C for 10 min

13

Confirmatory method for determination of anticoccidials in poultry muscle by LC–MS/MS

13.1 Introduction

Coccidiosis is a parasitic disease caused by protozoan residents in the intestinal epithelium. It occurs whenever animals are housed in small areas that are contaminated with coccidian oocysts. Historically, poultry has shown the greatest susceptibility to coccidiosis because of the intensive nature of most of the poultry industry. A wide range of drugs are available for the prevention and treatment of coccidiosis. The most used compounds are the carboxylic ionophores: monensin, lasalocid, narasin, salinomycin, maduramicin and semduramicin. These compounds are polyether antimicrobials. Another group of drugs for the prevention and treatment of coccidiosis are the coccidiostats: nicarbazine, toltrazuril, diclazuril, robenidine, clopidol, amprolium, halofuginone and ethopabate.

13.2 Scope and objective

The method is suitable for the identification (screening) and/or confirmation (identification and quantification) of coccidiostats in chicken meat by LC–MS/MS. The coccidiostats are: monensin, decoquinate, robenidine, lasalocid, narasin, salinomycin, maduramicin, diclazuril, nicarbazin, halofuginone and toltrazuril, toltrazuril sulfone, toltrazuril sulfoxide. Toltrazuril–D3, decoquinate–D5, robenidin–D8, halofuginone–$^{13}C_6$, methyl-diclazuril and dichloroisoeverninic acid–D6 are used as internal standards. This quantitative method should allow, after validation, the determination of the coccidiostat content in a concentration range up to twice the authorized limits. These limits are either MRL or Laboratory Working Concentration (LWC) defined by the laboratory (Table 95).

TABLE 95. MRL and laboratory working concentrations

Active pharmacological substance	Marker residue	MRL (µg/kg)	LWC (µg/kg)
Lasalocid	Lasalocid A	60	60
Toltrazuril	Toltrazuril sulfone	100	100
Halofuginone	Halofuginone	10	10
Diclazuril	Diclazuril	500	500
Avilamycin	Dichloroisoeverninic Acid	50	50
Robenidin	Robenidin	n.a	40
Monensin	Monensin	2	2
Narasin	Narasin	n.a	40
Salinomycin Sodium	Salinomycin	n.a	40
Maduramicin	Maduramicin	n.a	40
Decoquinate	Decoquinate	n.a	40

n.a: No assigned MRL

13.3 Principle of the method

The analytes are extracted from chicken samples mixed with acetonitrile, using a homogenizer and the content further spinned using a centrifuge and the supernatant evaporated to dryness. The residue is then reconstituted in 1 ml of acetonitrile/water (50/50, v/v) before analysis by LC–MS/MS.

13.4 Safety considerations and precautions

Operate in a fume hood wearing a laboratory coat and gloves. Use eye protection as necessary. All necessary precautions should be taken to avoid contamination when handling standards and throughout extraction and purification operations. Samples are stored in the freezer at approximately -18°C upon arrival at the laboratory until analysis.

13.5 Materials

13.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Deionized water; Acetonitrile; Ethanol; DMSO; Chloroform and Formic acid

13.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Microcentrifuge tubes; Volumetric flasks; Micropipettes; Centrifuge tubes; Glass tubes; Freezer (-20°C); Refrigerator; Micropipettes; Centrifuge; Vortex mixer; Nitrogen gas; Autosampler vials; Rotary agitator; UHPLC system with triple quadrupole mass spectrometer: API 4 000 QTRAP ABSIEX with turbo ion-spray interface and Analyst software; FLEXAR binary HPLC pump or equivalent; FLEXAR automatic injector or equivalent.

13.5.3 Solutions

The solutions (and how they are prepared) include:

- Ammonium formate solution + 0.1% HCOOH: Weight 315.5 mg of formate ammonium in a glass container and add 1 litre of ultrapure water to obtain a concentration of 5 mM of ammonium acetate.
- Add 1 000 µl of formic acid to the solution.
- Methanol + 0.1% HCOOH: 1 ml of formic acid is added to 1 litre of methanol.
- Mix Acetonitrile/Water: To get 1 litre of this solution, 500 ml of acetonitrile is added to 500 ml of ultrapure water.

13.5.4 Standards

The standards used are summarized in Table 96.

TABLE 96. Analytical standards used

Family compound	Molecule	Internal Code	Batch number	Purity	Origin/ manufacturer	Storage °C	Stock solution Concentration
Anticoccidials	Salinomycin	137–1	SZBB055XV	93.80%	Sigma Aldrich	-20	1mg/ml
Anticoccidials	Narasin	141–1	971117	98.00%	Berlin	-20	1mg/ml
Anticoccidials	Monensin	145–1	SZBB07OXV	86.30%	Berlin	-20	1mg/ml
Anticoccidials	Maduramicin	164–1	SZB9140XV	97.80%	Sigma	-20	1mg/ml
Anticoccidials	Diclazuril	203–2	134/04	—	Berlin	-20	1mg/ml
Anticoccidials	Methyl–diclazuril	216–3	662/08	—	Berlin	-20	1mg/ml
Anticoccidials	Toltrazuril–D3	234–2	729/08	—	Berlin	-20	1mg/ml
Anticoccidials	Toltrazuril	235–1	105/04	99.00%	Witega	-20	1mg/ml
Anticoccidials	Toltrazuril sulfone	238–3	103-07	—	Berlin	-20	1mg/ml
Anticoccidials	Dichloroisoeverninic acid	258–1	1320/01	99.00%	Witega	-20	1mg/ml
Anticoccidials	Dichloroisoeverninic D6	259–1	1348/01	99.00%	Witega	-20	1mg/ml
Anticoccidials	Halofuginone– $^{13}C_6$	262–2	1505-07	—	Berlin	-20	1mg/ml
Anticoccidials	Robenidine–D8	263–1	915/04	99.30%	Witega	-20	1mg/ml
Anticoccidials	Lasalocid	352–1	213/43	—	Berlin	-20	1mg/ml
Anticoccidials	Decoquinate–D5	358–2	BCBR9956V	99.50%	Sigma	-20	1mg/ml

(—) Information on purity not provided by manufacturer

13.5.4.1 Stock solutions (500 mg/l)

Stock solutions at 500 mg/l of active substances are prepared independently for each of the molecules in methanol. Halofuginone, Halofuginone–$^{13}C_6$, Dichloroisoeverninic acid, Dichloroisoeverninic acid–D6, Diclazuril and Methyl diclazuril are diluted in DMSO. Decoquinate and Decoquinate–D5 in chloroform.

If stock solutions are prepared at different concentrations, dilutions for the preparation of supplementation solutions should be adjusted. If stored in the freezer at -20°C, these can last for at least 2 yrs.

13.5.4.2 Working solutions

All the analytes are mixed in a 100 ml volumetric flask as recommended in following table, and then adjusted until the mark with acetonitrile. This solution is stable for half a year if stored in a refrigerator at + 4°C. The fortification standard solutions are prepared according to Table 97.

TABLE 97. Preparation of standard fortification solution

Analyte	Target concentration (µg/kg)	Concentration of the fortification solution (mg/l)	Volume (µl) of the stock solution to mix in a 100 ml volumetric flask*
Lasalocid	60	0.6	120
Monensin (1)	2	0.02	80
Salinomycin	40	0.4	80
Narasin	40	0.4	80
Maduramicin	40	0.4	80
Toltrazuril	100	1	200
Toltrazuril sulfone	100	1	200
Toltrazuril sulfoxide	100	1	200
Robenidine	40	0.4	80
Halofuginone (2)	10	0.1	80
Decoquinate (2)	40	0.4	80
Diclazuril	500	5	1000
Avilamycin	50	0,5	100

* (1) For Monensin, prepare a dilution of 1/20 in the acetonitrile before taking the volume needed to prepare the spiking solution.

* (2) For Halofuginone and decoquinate prepare a dilution of 1/4 in the acetonitrile before taking the volume needed to prepare the spiking solution.
Before adding the fortification solution of internal standards, prepare a dilution of 1/20 in acetonitrile. This can last for at least 6 months if stored at + 4°C.

13.5.4.3 Working solution of internal standards

All the solutions of the internal standard (500 mg/l) are mixed in a 100 ml volumetric flask as recommended in the table below, and then adjusted to the mark with acetonitrile. This solution if stored in a refrigerator at +4°C can last for 6 months. Preparation of the internal standards is summarized in Table 98.

TABLE 98. Preparation of internal standard solution

Analyte	Concentration of the spiking solution (mg/l)	Volume (in µl) of the stock solution to mix in a 100 ml volumetric flask
Toltrazuril–D3	1	200
Robenidine–D8	0.4	80
Halofuginone–$^{13}C_6$	0.1	20
Methyl–diclazuril	5	1 000
Avilamycin–D6	0.5	100
Decoquinate–D5	0.4	80

13.6 Procedure

13.6.1 Sample preparation

- Chicken samples are thawed to room temperature and lean meat portions (fat removed) are individually minced in a domestic food processor.
- Weigh 3.0 g (±0.1 g) into 50 ml polypropylene tubes.
- Add 6 ml of acetonitrile/water and shake for 30 min on a rotary shaker at 100 revolutions/min followed by centrifugation for 5 min at 4 460 rcf at 4°C.
- The supernatant is evaporated to dryness at 50°C.

13.6.2 Quality control samples

a) Positive control sample

- Weigh 3 g of a negative sample in a centrifuge tube.
- Add 100 µl of internal standard mixture.
- Add 300 µl of the standard working solution to obtain the positive controls at MRL/LWC.

b) Negative control sample

- Weigh 3 g of the ground/minced sample in a centrifuge tube.
- Add 100 µl of the internal standard mixture.

c) Spiked sample range

Weigh 3g of negative sample in 5 centrifuge tubes and add 100 µl of internal standards mixture for each tube.

- Tube 1 (1/4 MRL): add 75 µl of the standard working solution
- Tube 2 (1/2 MRL): add 150 µl of the standard working solution
- Tube 3 (1 MRL): add 300 µl of the standard working solution
- Tube 4 (1.5 MRL): add 450 µl of the standard working solution
- Tube 5 (2 MRL): add 600 µl of the standard working solution

13.6.3 Extraction procedure

- Add 6 ml of acetonitrile to the samples and vortex.
- Close the tubes and shake for 30 min with rotary agitator.
- Centrifuge the tubes for 5 min at 4,460 rcf, and then transfer the supernatant into glass tubes.

13.6.4 Sample cleanup

The supernatant is evaporated to dryness under nitrogen flow at about 50°C. After recovering the residues with 1 ml acetonitrile/water (50/50, v/v) and vortex; transfer the content into microcentrifuge tubes and then centrifuge for 5 min at 13 000 rcf. Put the extract in vials for injection and analysis.

13.6.5 Instrumental analysis (LC–MS/MS)

The HPLC system consists of a binary UHPLC pump (FLEXAR or equivalent), and auto sampler (FLEXAR or equivalent); Triple quadrupole mass spectrometer: API 5 500 QTRAP Applied Biosystems (or comparable) with turbo ionspray interface and Analyst software.

The LC conditions include:

- Analytical column: HSS C18 (100×2.1 mm, particle size 1.8 µm) and corresponding precolumn
- Column temperature: 50°C
- Injection volume: 10 µl
- Flow rate: 300 µl/min
- Run time: 20 min
- Mobile phase A: ammonium formate buffer (5mM) + 0.1% HCCOH
- Mobile phase B: methanol + 0.1% HCOOH
- Elution mode: linear gradient (Table 99)

TABLE 99. Gradient mobile phase conditions

Time (minute)	Ammonium formate buffer (5mM) + 0.1% HCCOH	Methanol + 0.1% HCOOH
0.1	98	2
2	98	2
3	2	98
14	2	98
15	98	2
20	98	2

- Mass analysis is carried out using an electrospray ionization source in positive and negative mode by a QTRAP API 5 500 mass spectrometer.
- The detection conditions for the negative mode are as follows: 449°C in the source temperature, curtain gas is about 10 (provide unit), entrance potential is 10, GS1 = 30, GS2 = 60, IS = -4 500 V and Collision activated dissociation (CAD) is medium.
- Regarding the positive mode: the source temperature is 500°C, curtain gas is 10 psi, entrance potential is 10, GS1 = 30, GS2 = 60, IS = 5 500 V and CAD is medium.
- The optimal MRM parameters are summarized in Tables 100 and 101.

TABLE 100. MRM parameters in negative ionization mode

	precursor ions (m/z)	Product ions (m/z)	Dwell time (ms)	DP	CE	CXP	Internal standard
Diclazuril	405.9 406.9	333.9 335.9	65 65	-65 -65	-28 -28	-19 -19	Methyl diclazuril
Dichloroisoeverninic acid	248.8 248.8	189.8 161.8	65 65	-70 -70	-28 -20	-16 -16	Dichloroisoeverninic acid–D6
Methyl diclazuril	421,2	280.2	65	-60	-33	-10	n.a
Dichloroisoeverninic Acid–D6	254.9	211.2	65	-70	-17	-16	n.a
Toltrazuril	424.2 424.2	424.2 42	65 65	-50 -50	-5 -60	-16 -16	Toltrazuril–D3
Toltrazuril sulfone	456.2 456.2	456.2 42.1	65 65	-50 -50	-5 -60	-16 -16	Toltrazuril–D3
Toltrazuril sulfoxide	440.2 440.2	42.1 371.1	65 65	-50 -50	-70 -24	-16 -16	Toltrazuril– D3
Toltrazuril–D3	427.2	42.1	65	-50	-60	-16	n.a

n.a: No internal standard used

TABLE 101. MRM parameters in positive ionization mode

	Precursor ions (m/z)	Product ions (m/z)	Dwell time (ms)	DP	CE	CXP	Internal standard
Decoquinate	418.56 418.56	372.2 204	40	66 66	55 33	12 22	Decoquinate–D5
Robenidine	334 334	155 138	40	81 81	29 35	8 8	Robenidine–D8
Halofuginone	416 416	120 100.2	40	76 76	31 41	8 6	Halofuginone–$^{13}C_6$
Lasalocid	608.4 608.4	591.2 337.2	40	51 51	15 25	18 8	Decoquinate–D5
Monensin	688.5 688.5	635.2 653.2	40	61 61	17 25	20 18	Decoquinate–D5
Salinomycin	768.5 768.5	733.1 715.1	40	66 66	27 35	20 18	Decoquinate–D5
Narasin NH_4	782.5 782.5	747.1 729.1	40	76 76	27 33	22 20	Decoquinate–D5
Narasin Sodium	787.5 787.5	431.1 531	40	101 101	65 67	10 8	Decoquinate–D5
Maduramicin	934.2 934.2	629.2 647.2	40	71 71	37 29	18 16	Decoquinate–D5
Decoquinate–D5	423.5	377,2	40	66	30	10	n.a
Robenidine–D8	342.19	159.12	40	81	24	8	n.a
Halofuginone– $^{13}C_6$	421.99	120.2	40	76	14	8	n.a

DP: Declustering potential CE: Collision energy CXP: Collision Cell exit potential n.a: No internal standards used

13.6.6 Calculation and interpretation of results

Confirmation

The method is validated and presence of anticoccidial residues confirmed following known guidelines (EC, 2002):

i) The relative retention time of the detected analyte should be identical to that of the spiked samples within ±2.5%.

ii) At least two specific transitions of each anticoccidials should be detected in the samples to be analyzed with a S/N> 3.

iii) The relative intensities of the transitions in the extracts should be compared with the relative intensities of spiked samples (within the same concentration range) and meet the limits of the following tolerance requirements:

TABLE 102. Maximum permitted tolerances for relative ion intensities

Ion m/z Relative intensity (% of base peak)	LC–MS/MS
> 50%	± 20%
> 20% to 50%	± 25%
> 10% to 20%	± 30%
≤ 10%	± 50%

Quantification:

This is done from the major transition and the internal standard. The calibration curve is established from the control sample and spiked samples.

13.6.7 Method validation

These methods provide reliable identification (at least 4 identification points) of unauthorized substances and substances with an established MRL and quantification of analytes (MRL compounds) following established guidelines (EC, 2002). LC–MS/MS methods fulfil these requirements with one precursor ion and at least two transition product ions (1.5 IP each one).

Specificity

- Twenty blank samples of muscle are analyzed to verify the absence of interfering peaks.
- Each molecule is specifically identified by two minimum transitions (parent ion > product ion) and retention time.

The two transitions of each molecule are observed at a retention time according to the dates of analysis:

- Negative control: verification of a chromatogram without interference with the blank at the retention time of the analytes. The absence of interfering peak at the retention time is verified for the two transitions of each analyte. Only the simultaneous presence of a peak for each of the transitions with a S/N > 3 makes it possible to identify the presence of each analyte.
- Positive control: the retention time of each analyte in the spiked sample corresponds to the standard retention times of each analyte injected on the same day.
- The method is proved selective if no interfering peaks are observed in the chromatogram.

Linearity

The linearity of the analytical method is validated using the matrix matched curves at different concentration levels. For each series, the equation of the calibration ranges is calculated from blank and spiked samples at 0 µg/kg, 0.5 µg/kg, 1 µg/kg, 1.5 µg/kg, 2 µg/kg MRL/LWC. The equation of the calibration curve is established with good regression coefficient (Table 103):

TABLE 103. The coefficient of regression

Analytes	Slope a	y-intercept b	Coef reg. r^2
Lasalocid	0.0138	0.0023	0.99
Toltrazuril	0.0476	0.0276	0.99
Toltrazuril sulfone	0.0348	0.0459	0.99
Toltrazuril sulfoxide	0.0221	0.0008	0.99
Halofuginone	0.4444	0.0472	0.99
Diclazuril	0.0046	0.0186	0.999
Avilamycin	0.0099	0.0111	0.99
Robenidine	0.1081	0.0206	0.99
Monensin	0.0064	0.0014	0.99
Narasin Sodium	0.0113	0.0003	0.99
Narasin NH_4	0.0391	0.002	0.99
Salinomycin Sodium	0.0208	0.0002	0.99
Maduramicine	0.0083	0.0003	0.999
Decoquinate	0.0116	0.0025	0.98

Repeatability

Repeatability is evaluated by analyzing samples over the course of three consecutive days and variations in signal, retention and ratios determined (Tables 104 and 105).

TABLE 104. Coefficient of variation for signal intensity

Analyte	CV% (Signal)	
	Signal 1(+intense)	Signal 2(-intense)
Lasalocid	19.8%	18.4%
Toltrazuril	20.2%	6.0%
Toltrazuril sulfone	13.9%	5.7%
Toltrazuril sulfoxide	7.0%	15.3%
Halofuginone	21.8%	27.1%
Diclazuril	15.9%	15.0%
Avilamycin	18.2%	23.0%
Robenidine	11.5%	12.0%
Monensin	17.4%	20.3%
Narasin Sodium	27.0%	19.8%
Narasin NH_4	26.0%	28.1%
Salinomycine Sodium	27.5%	35.9%
Maduramicine	24.1%	23.2%
Decoquinate	6.5%	13.3%

TABLE 105. Coefficient of variation for the relative time and ratio

Analyte	CV% (RT)	CV% (Ratio)
Lasalocid	0.5 %	6.4 %
Toltrazuril	0.2 %	9 %
Toltrazuril sulfone	0.1 %	11.1 %
Toltrazuril sulfoxide	0.2 %	13.6 %
Halofuginone	0.1 %	8.4 %
Diclazuril	0.1 %	1.4 %
Avilamycin	0.1 %	12.6 %
Robenidine	0.2 %	3.2 %
Monensin	0.2 %	13.6 %
Narasin Sodium	0.3 %	14.9 %
Narasin NH_4	0.3 %	8.0 %
Salinomycine Sodium	0.1 %	11.4 %
Maduramicine	0.2 %	4.1 %
Decoquinate	0.1 %	11.5 %

Trueness

The bias values are all in accordance with the requirements of Decision 2002/657/EC (EC, 2002), which are calculated using Eq. (18) below and elaborated in Table 106.

$$Trueness\,\% = \frac{estimatedconcentration - theoreticalconcentration}{theoreticalconcentration} \quad \text{.....(18)}$$

TABLE 106. The percent trueness

Analytes	Theoretical concentration µg/kg	Estimated concentration µg/kg	Trueness %
Lasalocid	60	55.15	-8.1 %
Toltrazuril	100	114.28	6.7 %
Toltrazuril sulfone	100	105.91	5.9 %
Toltrazuril sulfoxide	100	103.69	3.7 %
Halofuginone	10	9.36	-6.4 %
Diclazuril	40	35.74	-10.6 %
Avilamycin	50	50.02	0 %
Robenidine	40	36.72	-8.2 %
Monensin	2	1.83	-8.5 %
Narasin Sodium	40	34.37	-14.1 %
Narasin NH_4	40	34.62	-13.4 %
Salinomycin Sodium	40	39.19	-2.0 %
Maduramicine	40	36.36	-9.1 %
Decoquinate	40	41.97	4.9 %

The decision limit and detection capability

An overview of validation results for CCα and CCβ is shown in Table 107.

TABLE 107. Decision limit and detection capability

Analyte	MRL/ LWC (µg/kg)	σHorwitz	CCα max	Decision limit CCα (µg/kg)	Detection capability CCβ (µg/kg)
Lasalocid	60	13. 2	86 4	73.13	108.39
Toltrazuril	100	22	144	142.31	213.25
Toltrazuril sulfone	100	22	144	130.38	169.32
Toltrazuril sulfoxide	100	22	144	115.58	130.53
Halofuginone	10	2.2	14 4	12,75	19.93
Diclazuril	500	110	720	564.49	765.44
Avilamycin	50	11	72	65.29	93.55
Robenidine	40	8.8	57 6	43.68	53.88
Monensin	2	0.44	2.88	2.42	3.46
Narasin Sodium	40	8.8	57.6	49.63	89.20
Narasin NH_4	40	8.8	57.6	49.43	86.31
Salinomycin Sodium	40	8.8	57.6	56.93	103.90
Maduramicine	40	8.8	57.6	50.71	83.77
Decoquinate	40	8.8	57.6	46.47	52.05

All calculated CCα values are well below CCα max for all analytes.

Method uncertainty

Uncertainty of the method is equal to 2× the repeatability standard deviation (Table 108).

TABLE 108. Method uncertainty

Molecule	Standard deviation (signal 1)	2U (µg/kg)
Lasalocid	0.151	0.302
Toltrazuril	1.033	2.07
Toltrazuril sulfone	0.520	1.05
Toltrazuril sulfoxide	0.160	0.32
Halofuginone	0.919	1.838
Diclazuril	0.331	0.662
Avilamycin	0.092	0.184
Robenidine	0.459	0.918
Monensin	0.002	0.004
Narasin Sodium	0.105	0.21
Narasin NH_4	0.353	0.706
Salinomycine Sodium	0.225	0.45
Maduramicine	0.073	0.146
Decoquinate	0.032	0.064

13.6.8 Schematic diagram of extraction procedure

SAMPLE	1. Weigh 3 g of chicken sample 2. Add 100 µl of internal standard mixture
↓	
SAMPLE EXTRACTION	1. Add 3 ml of acetonitrile and vortex 2. Close the tubes and shake for 30 min in rotary agitator 3. Centrifuge the tubes at 4 460 rcf for 5 min
↓	
SAMPLE CLEANUP	1. Transfer supernatant into a glass tube 2. Evaporate the supernatant to dryness under nitrogen at 50°C 3. Recover residue with acetonitrile/water, and vortex 4. Transfer the solution to a microcentrifuge tube 5. Centifuge at 13 000 rcf for 5 min 6. Transfer the extract into an autosampler vial for injection

14

Determination of residues of enrofloxacin and ciprofloxacin in chicken meat by HPLC–FLD

14.1 Introduction

Enrofloxacin is a synthetic, broadspectrum antimicrobial in the fluoroquinolone family, and it was the first fluroquinolone approved for use in animals. It is widely used in the poultry industry to treat respiratory and alimentary tract infections. Irrational use of enrofloxacin can leave its residues in animal products, and these residues can negatively affect human health and may promote the development of antimicrobial resistance. In addition, enrofloxacin is biotransformed in many species to ciprofloxacin, which is a pharmacologically active substance used in human therapeutics. To ensure consumer safety, various regulatory authorities have established MRL for enrofloxacin and ciprofloxacin in animal tissues. These residues in animal tissues can be determined quantitatively using the HPLC–FLD method.

14.2 Scope and objective

This SOP describes the procedure for determining residues of enrofloxacin and ciprofloxacin in chicken meat using HPLC–FLD method. The SOP also aims at ensuring the quality and reproducibility of laboratory procedures.

14.3 Principle of the method

Enrofloxacin and ciprofloxacin in chicken muscle are extracted using a mixture of acetonitrile/acetone and TRIS buffer (pH 9.1) following a homogenization step. The organic solvent is then evaporated under a mild flow of nitrogen to dryness, and residue is redissolved in tris buffer (pH 9.1). The sample is defatted by extraction with hexane. Finally, the resulting solution is filtered and injected into an HPLC instrument. Enrofloxacin and ciprofloxacin residues are analyzed using HPLC–FLD at 280 nm (excitation) and 450 nm (emission).

14.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Suitable gloves should be worn when these chemicals are handled, and the work should be performed in a fume hood at all possible times. Wear laboratory coats all the time, and wash hands thoroughly with water once the experiments are over.

14.5 Materials

14.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Ultrapure water or water of equivalent purity; Methanol (HPLC grade); Sodium hydroxide pellets (Analytical grade); Tris (hydroxymethyl) aminomethane (Biotechnology grade); Hydrochloric acid, 36% w/v (Analytical grade); *n*–Hexane (HPLC grade); Phosphoric acid, 8 5% w/w; Acetonitrile (HPLC grade); Acetone (HPLC grade).

14.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Analytical balance (Range 0.00001–0.1000 g, Last digit 0.0001 g (Sartorius); Analytical balance (Range 0.00001 g–150 000 g, Last digit 0.0001 g, OHAUS); Tissue homogenizer (Ultra Turrax T 25 Basic); Laboratory centrifuge (Centurion Scientific Ltd./ICE Centra 4B/Hermle Z160); Grinder (Moulinex); Glass tubes; Microcentrifuge tubes (2 ml); Dry block incubator with sample concentrator (Techne, DB.3A); Vortex mixer (Velp); Filtering device for solvents and water; Nylon membrane filters 0.20 µm (Phenex); PVDF syringe-driven filters (0.45 µm, 13 mm), PVDF (Whatman, 6872–1304); Disposable syringes (1 ml, 3 ml); Polypropylene tubes with screw cap (15 ml, 50 ml); pH meter (Mettler Toledo); Micro pipettes (10 µl–100 µl, 100 µl–1 000 µl, 500 µl–5000 µl) (Eppendorf /Nichiryo); Multidispenser (Eppendorf); Pasteur Pipettes; Vacuum Pump (Air cadet®, Cole-Parmer, Barrington, IL, USA); Amber colour HPLC vials; Glass inserts for HPLC vials and Nitrogen Gas.

14.5.3 Solutions

The solutions (and how they are prepared) include:

- Acetonitrile/acetone (70/30, v/v): Mix 140 ml of acetonitrile with 60 ml of acetone;
- Tris buffer (0.05 mol/l, pH 9.1): Dissolve 1.21 g of TRIS in 180 ml of water and adjust pH to 9.1 with HCl (~ 95 µl). Bring the volume up to 200 ml with water;
- Mobile phase A: Consists of acetonitrile filtered through a 0.20 µm filter;
- Mobile phase B (0.02 mol/l phosphoric acid): Dilute 1.368 ml of phosphoric acid up to 1 litre with water and filter with 0.20 µm filter;
- Sodium Hydroxide (NaOH), 1 mol/l: Dissolve 2.0 g of NaOH in 50 ml of water. NaOH pellets should be added slowly to water and the final volume is brought to 50 ml.

14.5.4 Standards

14.5.4.1 Standard substances

The analytical standards include: Enrofloxacin: purity > 98% (Sigma Aldrich, St Louis, MO, USA); Ciprofloxacin: Vetranal®; purity = 99%; (Sigma Aldrich, St Louis, MO, USA).

14.5.4.2 Stock standard solutions (200 mg/l)

- Prepare enrofloxacin stock standard solution at 200 mg/l (SSE) by dissolving 10 g ± 0.1 mg of enrofloxacin standard in 100 µl of 1 mol/l NaOH and then completing up to 50 ml with methanol.
- Prepare ciprofloxacine stock standard solution at 200 mg/l by dissolving (SSC) 10 mg ± 0.1 mg of ciprofloxacin standard in 100 µl of 1 mol/l NaOH and then completing up to 50 ml with methanol.

Stock standard solutions are stable for at least 10 months when stored in the dark at -20°C.

14.5.4.3 Mixed standard solution (MS1, 1 mg/l)

To prepare a mixed standard solution of enrofloxacin and ciprofloxacin, each at 1 mg/l, dilute 50 µl of enrofloxacin stock solution (SSE) and 50 µl of ciprofloxacin stock solution (SSC) up to 10 ml with TRIS buffer in a 10 ml volumetric flask.

This mixed standard solution is used to spike the blank samples and prepare the calibration standards and is prepared freshly before the analysis.

14.5.4.4 Calibration standards

External calibration standards are used in the method. Prepare 8 calibration standards freshly before each analysis by mixing the mixed standard solution (1 mg/l) with TRIS buffer as shown in Table 109.

TABLE 109. Preparation of calibration standards

Calibration standard name	Concentration (µg/l)	Preparation
CS 1	200	Mix 200 µl of MS1 with 800 µl of tris buffer
CS 2	150	Mix 150 µl of MS1 with 850 µl of tris buffer
CS 3	100	Mix 100 µl of MS1 with 900 µl of tris buffer
CS 4	50	Mix 50 µl of MS1 with 950 µl of tris buffer
CS 5	25	Mix 50 µl of MS1 with 1.950 µl of tris buffer
CS 6	10	Mix 20 µl of MS1 with 1.980 µl of tris buffer
CS 7	5	Mix 500 µl of CS6 with 500 µl of tris buffer
CS 8	1	Mix 100 µl of CS7 with 400 µl of tris buffer

14.6 Procedure

14.6.1 Sample preparation

- Thaw chicken samples at room temperature.
- Remove fat from the sample and grind 50 g of muscle thoroughly.
- Weigh 3 g (±0.05 g) of ground meat into a 50 ml polypropylene tube.

14.6.2 Quality control samples

A blank sample and a spiked sample should be run as quality control samples with each test batch.

a) **Blank sample:**
   - A chicken muscle sample containing no enrofloxacin and ciprofloxacin residues is used as the blank sample.
   - Weigh 3 g (±0.05 g) blank ground muscle into a 50 ml polypropylene tube.

b) **Spiked sample:**
   - A blank chicken muscle sample fortified with a known amount of enrofloxacin and ciprofloxacin is used as the spiked sample.

- Weigh 3 g (±0.05 g) of blank ground muscle into a 50 ml polypropylene tube and add 300 µl of mixed standard solution (MS1, 1 mg/l) to yield 100 µg/kg tissue concentration of each analyte.
- Let the sample stand for 15 min.

14.6.3 Sample Extraction

The following steps are followed during extraction:

a) Add 1 800 µl of TRIS buffer to unknown and blank samples. For spiked samples, add 1 500 µl of TRIS buffer.

b) Let the samples stand at room temperature for 10 min after addition of TRIS buffer.

c) Add 6 ml of acetonitrile/acetone (70/30, v/v) to the sample and homogenize for 2 min using a tissue homogenizer.

d) Centrifuge the homogenate at 3 000 rcf–4 000 rcf for 10 min at room temperature.

e) Transfer 2.6 ml of the supernatant to a glass tube and evaporate the solution to dryness at 50°C under mild nitrogen flow using a dry block incubator with sample concentrator, to dryness.

f) Dissolve the residue in 1.5 ml of TRIS buffer and vortex for 10 s.

g) Add 2 ml of *n*–hexane to the sample, vortex for 30 s and remove the hexane layer (upper layer) using either a pasteur pipette or a needle connected to a vacuum pump.

h) Repeat the above step with 2 ml of *n*–hexane.

i) Add 0.5 ml of *n*–hexane to the sample and transfer the solution to a 2 ml microcentrifuge tube.

j) Centrifuge the sample at 17 000 rcf for 5 min at room temperature.

k) Take ~750 µl of the bottom aqueous layer and filter it through a syringe–driven filter to an amber coloured HPLC vial.

14.6.4 Instrumental analysis (HPLC–FLD)

Analysis of the residues is carried out using an Agilent 1 100 HPLC system consisting of a microvacuum degasser, (G1379A), quaternary pump (G1311A), autosampler (G1313A), thermostatted column compartment (G1316A), and a fluorescence detector (G1321A, Agilent 1 200 series). Conditions of HPLC–FLD analysis are as follows:

a) Separation mode: reversed phase chromatography

b) Analytical column: Zorbax Eclipse XDB C18 column (4.6×150 mm, particle size 5 µm)

c) Guard column: Zorbax SB–C18 (4.6×12.5 mm, particle size 5 µm)

d) Column temperature: 50°C.

e) Flow rate: 1 ml/min

f) Injection volume: 20 µl

g) Total run time: 10 min

h) FLD settings: 280 nm (excitation), 450 nm (emission)

i) Mobile phase: 0.02 M H_3PO_4/acetonitrile (85/15, v/v)

j) Elution mode: isocratic elution

14.6.5 Interpretation of results

The concentrations of enrofloxacin and ciprofloxacin in samples are calculated using the respective eight-point calibration curves.

An HPLC run is considered as valid if the following results are achieved:

- The calibration curves of the two analytes should have a coefficient of determination (R^2) ≥ 0.95.
- Recovery of enrofloxacin and ciprofloxacin in samples fortified at 100 µg/kg should be above 55%.

If the above two conditions are true, the test samples can be interpreted as follows:

- Negative samples

  Samples with HPLC–FLD peak areas below the corresponding peak areas for the sum of ciprofloxacin and enrofloxacin at 100 µg/kg.
- Positive sample

  Samples with HPLC–FLD peak areas above the corresponding peak areas for the sum of ciprofloxacin and enrofloxacin at 100 µg/kg.

14.6.6 Method validation

Trueness is determined by calculating recovery of analytes in blank samples spiked with each analyte at three concentrations: 50 µg/kg, 100 µg/kg and 150 µg/kg. Precision is determined in terms of repeatability (intra–assay precision) and 'intermediate precision' (within–laboratory reproducibility) and expressed as relative standard deviations (RSD%) of measured analyte concentration in samples spiked at 50 µg/kg, 100 µg/kg and 150 µg/kg. Recovery and precision test results are summarized in Table 110.

TABLE 110. Method recovery and precision

Analyte	Spike level (µg/kg)	Mean recovery ± SD (%)	Repeatability (RSD%)	Intermediate precision (RSD%)
Enrofloxacin	50	61.6 ± 8.3	4.6	13.5
	100	64.5 ± 6.7	7.3	10.4
	150	57.9 ± 5.0	8.3	8.6
Ciprofloxacin	50	58.5 ± 5.1	2.8	8.7
	100	63.1 ± 5.7	5.4	9.1
	150	59.3 ± 2.3	3.8	3.9

Chromatographic separation between enrofloxacin and ciprofloxacin is evaluated by calculating the peak resolution using Eq. (19):

$$Rs = \frac{RT2 - RT1}{0.85(W2 + W1)} \quad \text{.....(19)}$$

where,

Rs is the peak resolution
RT2 is the retention time of peak 2
RT1 is the retention time of peak 1
W2 is the width of peak 2 at half peak height
W1 is the width of peak 1 at half peak height

Peak resolution between ciprofloxacin and enrofloxacin is 5.6.

Method measurement uncertainty. This is summarized in Table 111.

TABLE 111. Measurement uncertainty

	Enrofloxacin	Ciprofloxacin
Avg. Conc. of spiked samples at 100 µg/kg	0.9	1.9
STDEV (spikes samples at 100 µg/kg)	0.2	0.3
Sample Size	9	9
Standard Uncertainty	0.06	0.09
Degrees of Freedom	8	8
95% t–Value	2.306	2.306
95% Confidence Deviation	0.14	0.20
95% Confidence Interval (Min)	0.71	1.67
95% Confidence Interval (Max)	1.00	2.06

14.6.7 Schematic diagram of the HPLC procedure

TEST SAMPLE	1. Weigh 3 g ± 0.05 g of ground chicken meat into a 50 ml polypropylene tube.
↓	
FORTIFICATION SAMPLE	1. Add 300 µl of 1 mg/l mixed standard solution to blank test portion.
↓	
SAMPLE EXTRACTION	1. Add 1 800 µl of tris buffer to sample (pH 9.1). For positive control, add 1 500 µl of TRIS buffer. 2. Add 6 ml of acetonitrile/acetone (70:30, v/v). 3. Homogenize for 2 min. 4. Centrifuge the homogenate at 3 000 g–4 000 g at for 10 min. 5. Transfer 2.6 ml of supernatant to a glass tube. 6. Evaporate the sample to less than 1.5 ml at 50°C using nitrogen. 7. Bring the volume up to 1.5 ml with tris buffer (pH 9.1) and vortex for 10 s. 8. Add 2 ml of n–hexane to the sample, vortex for 30 s and remove upper hexane layer. 9. Repeat the previous step with another 2 ml of *n*–hexane. 10. Add 0.5 ml *n*–hexane to the sample. 11. Transfer the sample to a 2 ml microcentrifuge tube. 12. Centrifuge the sample at 17 000 g for 5 min. 13. Filter 750 µl of bottom aqueous layer using a syringe–driven filter. 14. Transfer to HPLC vial and inject.

15

Quantitative detection of tetracycline group (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) in muscle (red meat, poultry and fish) by LC–MS/MS

15.1 Introduction

Tetracycline antimicrobials (TCs), represented by oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DXC), are commonly used worldwide in veterinary therapy. However, the abundant and improper use of TCs may result in the presence of their residues in edible animal tissues, which can be toxic and dangerous to human health and potentially cause allergic reactions. In addition, low level doses of antimicrobials in foodstuffs consumed for long periods can lead to the spread of drug–resistant microorganisms (Masawat and Slater, 2007; Shalaby et al., 2001; Yu et al., 2011). To ensure human food safety, the EU established MRLs for OTC, TC, CTC and DXC, including 100 mg/kg in muscle.

15.2 Scope and objectives

This method is applied for detection of residues of tetracyclines (OTC, TC, CTC and DXC) in the following matrices of food of animal origin: red meat (bovine and sheep), poultry (chicken and turkey) and fish. Concentration of interest: 50 µg/kg – 400 µg/kg. MRL, zero tolerance, limit of interest: MRL: Oxytetracycline: 100 µg/kg, Tetracycline 100 µg/kg, Chlortetracycline: 100 µg/kg, Doxycycline: 100 µg/kg.

15.3 Principle of the method

Tetracycline residues in muscle tissue are extracted with 70% methanol and are detected by LC–MS/MS following separation with C18 reversed phase column.

15.4 Safety considerations and precautions

Laboratory safety procedures should be implemented. Gloves and goggles should be used. Chemical waste bottles should be used. All work should be carried out under a fume hood. Methanol (flammable and toxic), acetonitrile (flammable and harmful) and formic acid (toxic and corrosive) should be handled with care in the fume hood.

15.5 Materials

15.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Acetonitrile (analytical grade); Formic acid (analytical grade); Oxalic acid dihydrate (analytical grade); Methanol (analytical grade) and Titriplex, Na_2EDTA (analytical grade).

15.5.2 Equipment/instrument and consumables

The following apparatus and material are applicable: Centrifuge tubes (50 ml, and 15 ml); Volumetric flasks: (10 ml, 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, and 1 000 ml); Precision scales, CP 423 S (Sartorius, 1 mg hassasiyette); Micropipette (10 µl–100 µl, 20 µl–200 µl and 100 µl–1 000 µl; Eppendorf); Dispenser, 50 ml (Optifix Basic); Multiple vortex (Heidolph); Refrigerated Centrifuge, Rotina 35 R (Hettich); Vortex, Velp 2 x3 (Scientifica); Precision scales; SBP31 (Scaltec 0.1 mg sensitive); RC Filter, 0.45µm; Stainless Steel Knife; Blender (Waring); LC-MS/MS Instruments consisting of, Liquid Chromatography, (Agilent 1 200), Degasser, (Agilent 1 200), LC Pump, (Agilent 1 200), Autosampler, (Agilent 1 100), Mass Detector, (Agilent 6 460).

15.5.3 Solutions

The solutions (and how they are prepared) include:

- Mobile Phase A: Dissolve 0.126 g of oxalic acid in 200 ml of water and transfer this solution into a 1 litre volumetric flask. Add 2 ml of formic acid to the solution and complete it to 1 litre with water. This can be stored for 7 days at room temperature.
- Mobile Phase B: Add 1 ml of formic acid to 1 000 ml of acetonitrile. This can be stored for 30 days at room temperature.
- 70% Methanol: Pour 700 ml of methanol into a 1 litre volumetric flask and then complete to 1 litre with water.
- MNa_2EDTA: Dissolve 3.72 g of Na2EDTA in pure water and transfer the solution to a 100 ml volumetric flask. Complete to 100 ml with water.

15.5.4 Standards

15.5.4.1 Reference standards

The analytical standards include: Oxytetracycline Hydrochloride (Riedel De Haen, 46598, analytical grade); Tetracycline Hydrochloride (Fluka, 31741, analytical grade); Chlortetracycline Hydrochloride (Riedel De Haen, 46133, analytical grade); Doxycycline Hydrate (Fluka, 33429, analytical grade); Demeclocycline Hydrochloride (DMC, Sigma Aldrich, D6140, analytical grade).

15.5.4.2 Stock standard solutions

a) OTC stock standard (1 000 mg/l)

b) 10 mg (±0.01 mg) of pure OTC is weighed into a 10 ml volumetric flask and completed to volume with methanol. It can be stored for 6 months at -20°C in the dark.

c) TC stock standard (1 000 mg/l)

d) 10 mg ± 0.01 mg pure TC is weighed into a 10 ml volumetric flask and completed to volume with methanol. It can be stored for 6 months at -20°C in the dark.

e) CTC stock solution (1 000 mg/l)

f) Here 10 mg (±0.01 mg) pure CTC is weighed into a 10 ml volumetric flask and completed to volume with methanol. It can be stored for 6 months at -20°C in the dark.

g) DXC stock standard (1 000 mg/l)

h) Here 10 mg (±0.01 mg) pure DXC is weighed into a 10 ml volumetric flask and completed to volume with methanol. It can be stored for 6 months at -20°C in the dark.

i) DMC stock standard (1 000 mg/l)

Here 10 mg (±0.01 mg) pure DMC is weighed into a 10 ml volumetric flask and completed to volume with methanol. It can be stored for 6 months at -20°C in the dark.

15.5.4.3 Intermediate stock standard solutions

a) TC Intermediate mix standard (100 mg/l)
Here 600 µl of methanol is completed to 1 ml by adding 100 µl from the TC stock standard (1 000 mg/l). It can be stored for 6 months at -20°C in the dark.

b) TC Intermediate mix standard (10 mg/l)
Here 900 µl of methanol is completed to 1 ml by adding 100 µl of TC intermediate standard (100 mg/l).

c) TC Intermediate mix standard solution (2 mg/l)
Here 800 µl of methanol is completed to 1 ml by adding 200 µl of the TC intermediate stock standard (10 mg/l).

d) DMC intermediate standard solution (100 mg/l)
Here 900 µl of methanol is completed to 1 ml by adding 100 µl of the demeclocycline stock standard (1 000 mg/l). It can be stored for 3 months at -20°C.

e) DMC intermediate standard solution (10 mg/l)
Here 900 µl of methanol is completed to 1 ml by adding 100 µl of the demeclocycline intermediate standard (100 mg/l).

f) DMC intermediate standard solution (2 mg/l)
Here 800 µl of methanol is completed to 1 ml by adding 200 µl of the DMC intermediate standard (10 mg/l).

15.6 Procedure

15.6.1 Sample preparation

Adipose and nerve tissue is trimmed from red meat. Adipose tissue, nerve tissue and skin are trimmed from poultry, and fish samples are washed with water. Samples are chopped and at least 50 g of tissue used for homogenization.

15.6.2 Quality control samples

A reagent blank, a blank, four spiked samples and test samples should be included in each working set.

- Five blank samples known to be negative (without the residues tested) and 2 g ± 0.02 g fish samples to be analyzed are weighed into 50 ml polypropylene centrifuge tubes.

- Negative samples are marked as R (reactive blank), B (blank), S1 (0.5 MRL spike), S2 (1 MRL spike), S3 (2 MRL spike), and S4 (4 MRL spike). A Sample Accept Number is used for test samples.
- Pure water (2 ml) is added into the R tube. 50 µl, 100 µl 200 µl, 400 µl of the antimicrobial working standard solution are added into the S1, S2, S3 and S4 tubes, respectively.
- Add 100 µl of internal standard working solution to all tubes.

15.6.3 Sample extraction

a) Test tissue [2 g (±0.02 g)] is weighed into 50 ml polypropylene centrifuge tubes.

b) Then 100 µl internal standard (2 mg/l) is added and mixed for a few seconds using vortex mixer. A combination of 200 µl Na_2EDTA (0.1 mol/l) and 10 ml 70% methanol is added to the mix. Mix for 15 min using a vortex mixture.

c) Centrifuge the mixture at 4 000 rpm for 118 min at 3°C.

d) Add 200 µl of the supernatant to 1 800 µl of pure water and mix for a few seconds.

e) A 2 ml extract is filtered into the vial using a 0.45µm filter material and 20 µl of the sample is injected into the LC–MS/MS system.

15.6.4 Instrumental analysis (LC–MS/MS)

The LC conditions include:

a) Analytical Column: ZORBAX SB–C 18 (4.6 × 100 mm, particle size 3.5µm)

b) Column oven temperature: 35°C

c) Flow rate: 0.8 ml/min

d) Injection volume: 20 µl

e) Pressure limit: max 300 bar

f) Autosampler temperature: 10°C

g) Elution mode: gradient elution (Table 112)

TABLE 112. Mobile phase programme

Steps	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
1	00.00	90	10
2	01.00	90	10
3	07.50	49	51
4	08.00	90	10

The MS conditions include
Source

- Gas temperature: 350°C
- Gas flow: 9 l/min
- Sheath gas temperature: 400°C
- Sheath gas flow: 10 l/min
- Nebulizer: 40 psi
- Capillary: (positive) 4 000 V

Mass acquisitions parameters are also summarized in Tables 113 and 114.

TABLE 113. MS acquisition conditions

	Start Time	Source Type	DIV Valve	Delta EM +V	Delta EM -V	Stored
1	0.0	MRM	To Waste	0	0	-
2	4.5	MRM	To MS	100	0	+
3	8.0	MRM	To Waste	0	0	-

TABLE 114. MS acquisition results

Compound Name	Precursor ion	MS Res	Product ion	MS Res	Dwell	Fragment (V)	Collision energy	Polarity
Chlortetracycline	479	Wide	444	Wide	50	70	20	Positive
Chlortetracycline	479	Wide	154	Wide	50	70	25	Positive
*Demeclocycline	465	Wide	448	Wide	50	70	10	Positive
Oxytetracycline	461	Wide	444	Wide	50	70	10	Positive
Oxytetracycline	461	Wide	426	Wide	50	70	15	Positive
Doxycycline	445	Wide	428	Wide	50	70	15	Positive
Doxycyline	445	Wide	410	Wide	50	70	25	Positive
Tetracycline	445	Wide	410	Wide	50	80	15	Positive
Tetracycline	445	Wide	154	Wide	50	80	25	Positive

*Internal standard

15.6.5 Calculation and interpretation of results

a) Raw data – Final results:

Samples are arranged in the specified order on the device: First reactive blank, negative control sample, calibration samples, test samples, negative control sample and spike control sample (calibration sample at the MLR Level). This order shouldn't be changed.

Retention times of the compounds should be compatible with the standard. Maximum allowable shift time is 5%.

A single MRM detection is not enough. For each compound, the peak from the two MRM is required. The main transition ion in table enables quantitative analysis while the verification ion is used to define the compound of interest. The percent rate between smaller and greater MRM peaks which are obtained should be compatible with the standard (Table 115).

TABLE 115. Relative intensity

Relative intensity (% of base peak)	LC–MS, LC–MS^n (relative)
> 50%	± 20%
> 20% to 50%	± 25%
> 10% to 20%	± 30%
≤ 10%	± 50%

b) Calculations

- Determination is according to the internal standard method.
- A series of standards is prepared so that the amount of its internal standard is fixed while the analyte amount is variable (in different mx/ms value) and each chromatogram is identified. Draw a graph based on the value of Ax/As against the value of Mx/Ms; the Slope of the curve (linear) is the response factor.
- To determine the amount of analyte in the sample, the known amount of the internal standard is added to the sample.
- The value of Ax/Ais is calculated from the chromatogram of the sample positive internal standard mixture, and the concentration of analyte is calculated from the calibration curve.
- For example, for drawing the calibration curve as shown in the following table, the four standards are prepared:
  - Each standard has 5 mg/ml internal standard.
  - Values of Ax/Ais are taken from the graph against the value of Cx/Cis.
  - Calibration curve is the line whose slope is the response factor.

To analyze the sample, the known amount of internal standard is added to the sample and the mixture injected into the analytical instrument.

- Value of Ax/Ais is measured (details in Table 115).
- Cx/Cis value is determined from the calibration data and Cx is calculated using Eq. (20)

$$Cx = Cis\left(\frac{Cx}{Cis}\right) \quad \ldots\ldots (20)$$

Where, Ax is response for analyte; Ais is internal standard, As is standard; Mx is mass of internal standard; Mis is injected analyte; Ms is mass of standard; Cx is concentration of injected analyte; Cis is internal standard.

TABLE 116. Calculating test results

Standard	Analyte, mg/ml	Internal standard,mg/ml	Cx/Cis	Ax	Ais	Ax/Ais
1	2.50	5.00	0.500	120	600	0.200
2	5.00	5.00	1.000	241	601	0.401
3	10.00	5.00	2.000	480	600	0.800
4	25.00	5.00	5.000	1198	600	1.997

c) Acceptability Criteria:

- A blank injection should not have any positive value. i.e. no compound of interest detected in the blank test. No MRM pair be detected from the S1, S2, S3, S4 injections for each compound.
- The acceptable shift in retention time is a maximum of 5%, for the calibration curve the r2 ≥ 0.98. Values obtained from S2 should be compatible with the control chart for each compound.
- The validity of the test method should be supported by regular participation in proficiency testing schemes.

15.6.6 Method validation

- Validation is performed according to known guidelines (EC, 2002) concerning the performance of analytical methods and the interpretation of results.
- To verify the absence of interfering endogenous compounds around the retention time of the analytes, 10 blank samples are analyzed.
- Linearity and sensitivity are checked by injecting the analytes at different concentration levels to cover the whole working range.
- Calibration curves of the spiked blank samples for each tetracyclines are calculated by using a least squares linear regression analysis and then plotting the peak area of each analyte versus the analyte concentration.
- For the measurement of CCα, blank samples are spiked at the respective LOQ level and the MRL concentration of all the tetracyclines.
- CCα is calculated as the theoretical concentrations at the permitted limit plus 1.64 times the corresponding SD as in Eq. (21):

$$CC\alpha = CMRL + 1.64\,SD \quad (21)$$

- Where SD is the standard deviation.
- To obtain the CCβ values, several samples are spiked at the obtained CCα levels and injected into the chromatographic system.
- The CCβ calculation is done using the theoretical value of CCα previously obtained plus 1.64 times the corresponding SD as in Eq. (22):

$$CC\beta = CC\alpha + 1.64\,SD \quad (22)$$

- Accuracy and precision are also studied by analyzing three different concentration levels corresponding to 0.5×MRL, the MRL and 1.5×MRL.

15.6.7 Schematic diagram of extraction procedure

1. Weigh 2 g (±0.02 g) from test tissue into 50 ml polypropylene centrifuge tubes.
2. Add 100 µl internal standard (2 mg/l).
3. Mix for a few seconds using a vortex mixer.
4. Add 200 µl Na_2EDTA (0.1 mol/l) and 10 ml 70% methanol.
5. Mix 15 min on a vortex mixer.
6. Centrifuge the mixture at 4000 rpm for 118 min at 3°C.
7. Add 200 µl of supernatant into 1800 µl pure water.
8. Mix for a few seconds.
9. Filter 2 ml of extract into the vial.
10. Inject 20 µl of the sample into the LC–MS/MS system.

16

Detection and quantification of pesticide residues in cereals by GC–MS/MS

16.1 Introduction

Pesticides are still used widely to control animal and plants pests and diseases. However, residues of these substances may remain in the animal or plant products that are of public health and trade concerns and should be controlled with the help of testing laboratories using good analytical methods.

16.2 Scope and objectives

This procedure applies to cereal samples received by the Contaminants Monitoring Division (CMD) which includes commodity group 5: High starch and/or protein content and low water and fat content. It describes the procedure for food sample preparation, extraction and cleanup of pesticides from food matrices, GC–MS/MS analytical conditions, standards and calibration levels preparation and identification and result reporting.

16.3 Principle of the method

A modified QuEChERS method is used where a homogenised is extracted using acetonitrile and mixture of salts followed by reshaking and centrifugation. An aliquot of the organic phase is cleanedup using anhydrous $MgSO_4$ and PSA, shaken on a vortex mixer followed by dilution with acetonitrile/toluene.

16.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, some pesticides standards are considered a possible carcinogen to humans. Suitable gloves should be worn when chemicals/ pesticides standards are handled, and the work should be performed in a fume hood at all possible time.

16.5 Materials

16.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Distilled water; acetonitrile (LC–MS grade); toluene (HPLC grade) and formic acid.

16.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Freezer (-20°C; -80°C); Sample processing equipment (blender and grinder); Centrifuges for 50 ml, 15 ml and 2 ml centrifuge tubes; Vortex and/or shaker; Automatic pipettes (20 µl–200 µl, 100 µl –1000 µl and 1 ml –10 ml); Centrifuge tubes with screw caps (50 ml and 15 ml); Solvent–dispenser (for acetonitrile and toluene); Vials with caps for LC auto–sampler (2 ml); QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl, 1 g Na–citrate, 0.5 g disodium citrate sesquihydrate); dSPE tubes (150 mg $MgSO_4$, 25 mg PSA); Gas chromatograph–mass spectrometer (GC–MS/MS, Thermo Fisher Scientific).

16.5.3 Solutions

Measure 950 ml of acetonitrile in a litre–bottle and 50 ml of formic acid; mix well.

16.5.4 Standards

16.5.4.1 Reference/Analytical standards

The analytical/reference standards including those listed in Table 117 should be at purities of ≥ 98%.

TABLE 117. Analytical/reference standards

1	2,4'-DDE	55	delta–BHC	109	Isazophos	163	Pyraclophos
2	2,4'-DDT	56	Deltamethrin	110	Isodrin	164	Pyrazophos
3	2,4'-Methoxychlor	57	Diallate	111	Isopropalin	165	Pyridaben
4	2-Phenylphenol	58	Diazinon	112	Lenacil	166	Pyridaphenthion
5	4,4'-DDD	59	Dichlofluanid	113	Leptophos	167	Pyrimethanil
6	4,4'-DDE	60	Dichloroaniline, 3,4'–	114	Linuron	168	Pyriproxyfen
7	4,4'-DDT	61	Diclobenil	115	Malathion	169	Quinalphos
8	4,4'-Dichlorobenzo-phenone	62	Dicloran	116	Metalaxyl	170	Quintozene
9	4,4'-Methoxychlor olefin	63	Dieldrin	117	Metazachlor	171	Resmethrin
10	Acequinocyl	64	Dimethachlor	118	Methacriphos	172	Sulfotep
11	Acetochlor	65	Diphenamid	119	Methoxychlor	173	Sulprophos
12	Acrinathrin	66	Diphenylamine	120	Methyl parathion	174	tau-Fluvalinate
13	Alachlor	67	Disulfoton	121	Metolachlor	175	Tebuconazole
14	Aldrin	68	Edifenphos	122	Mevinphos	176	Tebufenpyrad
15	Allidochlor	69	Endosulfan ether	123	MGK 264	177	Tecnazene
16	Alpha–BHC	70	Endosulfan I	124	Mirex	178	Tefluthrin
17	Anthraquinone	71	Endosulfan II	125	Myclobutanil	179	Terbacil

(con't)

TABLE 117. Analytical/reference standards (con't)

18	Atrazine	72	Endosulfan sulfate	126	Nitralin	180	Terbuphos
19	Azinphos–ethyl	73	Endrin	127	Nitrofen	181	Terbutylazine
20	Azinphos–methyl	74	Endrin aldehyde	128	Nonachlor, cis–	182	Tetrachloroaniline, 2,3,5,6–
21	Benfluralin	75	Endrin ketone	129	Norflurazon	183	Tetrachlorvinphos
22	Beta–BHC	76	EPN	130	Oxadiazon	184	Tetradifon
23	Bifenthrin	77	Ethalfluralin	131	Oxyfluorfen	185	Tetrahydrophtha-limide
24	Biphenyl	78	Ethion	132	Paclobutrazol	186	Tetramethrin
25	Bromfenvinphos	79	Ethylan	133	Parathion	187	Tolclophos–methyl
26	Bromfenvinphos–methyl	80	Etofenprox	134	Pebulate	188	Tolylfluanid
27	Bromophos–methyl	81	Etridazole	135	Penconazole	189	Transfluthrin
28	Bromophos–ethyl	82	Fenamiphos	136	Pendimethalin	190	trans-Nonachlor
29	Bromopropylate	83	Fenarimol	137	Pentachloroaniline	191	Triadimefon
30	Bupirimate	84	Fenchlorphos	138	Pentachloroanisole	192	Triadimenol
31	Carbophenothion	85	Fenitrothion	139	Pentachlorobenzene	193	Triallate
32	Carfentrazone–ethyl	86	Fenpropathrin	140	Pentachlorobenzonitrile	194	Triazophos
33	Chlorbenside	87	Fenson	141	Pentachlorothioanisole	195	Tricyclazole
34	Chlordane, trans–	88	Fenthion	142	Permethrin, cis–	196	Triflumizole
35	Chlorfenapyr	89	Fenvalerate	143	Permethrin, trans-	197	Trifluralin
36	Chlorfenson	90	Fipronil	144	Phenothrin	198	Vinclozolin
37	Chlorfenvinphos	91	Fluazifop–p–butyl	145	Phorate		
38	Chlorobenzilate	92	Fluchloralin	146	Phosalone		
39	Chloroneb	93	Flucythrinate	147	Phosmet		
40	Chlorothalonil	94	Fludioxonil	148	Piperonyl butoxide		
41	Chlorpropham	95	Fluquinconazole	149	Pirimiphos–ethyl		
42	Chlorpyrifos	96	Fluridone	150	Pirimiphos–methyl		
43	Chlorpyrifos–methyl	97	Flusilazole	151	Pretilachlor		
44	Chlorthal–dimethyl	98	Flutolanil	152	Prochloraz		
45	Chlorthiophos	99	Flutriafol	153	Procymidone		
46	Chlozolinate	100	Folpet	154	Prodiamine		
47	cis–Chlordane	101	Fonofos	155	Profenofos		
48	Clomazone	102	gamma–BHC	156	Profluralin		
49	Coumaphos	103	Heptachlor	157	Propachlor		
50	Cycloate	104	Heptachlor epoxide	158	Propanil		
51	Cyfluthrin	105	Hexachlorobenzene	159	Propargite		
52	Cyhalothrin, lambda-	106	Hexazinone	160	Propisochlor		
53	Cypermethrin	107	Iodofenfos	161	Propyzamide		
54	Cyprodinil	108	Iprodione	162	Prothiofos		

16.5.4.2 Working solutions

a) Working solution 1 (W1, 10 µg/l)

- These are prepared from mixes of stock standard solution (100 µg/ml).
- Take 100 µl from each mix and complete with toluene so the final volume is 1 ml.
- Use it to prepare working solution 2 (WS2) at 1 µg/ml and to spike GC–MS/MS recovery samples.

b) Working solution 2 (W2, 1 µg/l)

- Prepare working solution 2 (W2) at 100 µg/l by taking 100 µl from W1 and completing with toluene up to 10 ml.
- Use it to prepare GC–MS/MS calibration levels.

16.5.4.3 Calibration standards

For quantitation, a matrix matched calibration curve is generated using known blank and related samples. The blank sample is subjected to the same extraction and cleanup procedure mentioned above. The final blank extract is used to prepare four calibration standards as below:

- Level 1 (0.1 µg/ml): Add 10 µl of WS1 + 990 µl final blank extract directly into an autosampler vial;
- Level 2 (0. 2 µg/ml): Add 20 µl of WS1 + 980 µl final blank extract directly into an autosampler vial;
- Level 3 (0.5 µg/ml): Add 50 µl of WS1 + 950 µl final blank extract directly into an autosampler vial;
- Level 4 (1 µg/ml): Add 100 µl of WS1 + 900 µl final blank extract directly into an autosampler vial.

The calibration curve should be valid until check standards deviation from the acceptable criteria (20% range of the corresponding calibration point value). If values deviate, then prepare a new calibration curve and check again.

16.6 Procedure

16.6.1 Sample preparation

Cereals:

a) Take ~200 g of the cereal sample and store at -20°C for at least 1 hr or overnight.

b) After freezing, weigh 100 g of the sample and transfer to a blender (preserve the other 100 g at -20°C);

c) Homogenize the sample into a fine free flowing powder.

Powder samples:

- Take 200 g of the sample and store at -20°C for at least 1 hr or overnight.

16.6.2 Sample extraction

a) Weigh 10 g (±0.1 g) of sample into a clean 50 ml tube;

b) Spike blank samples at a concentration of 0.05 mg/kg; let stand for 10 min;

c) Add 10 g of cold water and shake briefly;

d) Add 10 ml of acetonitrile;

e) Vortex for 1 min;

f) Add buffered QuEChERS extraction packet as indicated elsewhere (EC, 2015) and including: 4 g $MgSO_4$, 1 g NaCl, 1 g Na–citrate, 0.5 g disodium citrate sesquihydrate);

g) Vortex for 1 min;

h) Centrifuge at 5°C and 6 000 rpm for 10 min;

i) Transfer an aliquot (5 ml) of the acetonitrile phase (upper layer) into a 15 ml screw capped tube;

j) Place the tube containing the extract in the freezer at -80°C for at least for 15 min

k) Allow the extracts to become almost liquid, then cold centrifuge for 5 min at 5°C, 6 000 rpm.

16.6.3 Cleanup procedure

a) Transfer 1 ml of the supernatant to the ready–to–use dSPE tube (2 ml capacity, 150 mg $MgSO_4$, 25 mg PSA);

b) Vortex for 30 s;

c) Centrifuge for 5 min at 5°C and 6 000 rpm;

d) Filter the extract through 0.45 mm PTFE filter;

e) Take 0.25 ml of the filtrate;

f) Evaporate to almost dryness at 40°C using a gentle nitrogen flow (less than 5 bar);

g) Reconstitute in 0.5 ml of toluene and mix properly (dilution factor of 2);

h) Set sample amount at 1;

i) Set dilution factor at 2;

j) The final extract will be at a concentration of ~ 0.5 g/ml;

k) Sample is ready to be injected.

16.6.4 Instrumental analysis (GC–MS/MS)

The GC conditions are as indicated in Table 118:

TABLE 118. GC conditions

Instrument	Trace 1310 Gas Chromatograph (ThermoFisher Scientific – USA)
Analytical Column	TG–5SILMS (30 m, 0.25 mm ID, 0.25 µm)
Liner	SSL splitless liner, single taper,4 mm ID × 6.3 mm OD × 78.5 mm length
Injection Temp.	250°C
Injection Volume	1 µl
Injection Type	Splitless with surge (Hold 1 min)
Carrier Gas	Helium
Flow Type	Constant flow
Flow rate	1.4 ml/min
Oven Temp.	90 °C (hold 1 min) to 330 °C at 8.5 °C /min (hold 5 min)

TABLE 119. MS conditions

Instrument	TSQ™ Duo Triple Quadrupole Mass Spectrometer (ThermoFisher Scientific – USA)
Mode	Timed SRM
Scan rate	12 scans/peak
Scan range	50 amu–550 amu
Transfer Line Temp.	290°C
Analyzer Type	Quadrupole
Source Temp.	325°C
Electron Energy	70 eV
Solvent Delay Time	5 min
Ionization Mode	EI

16.6.5 Calculation and interpretation of results

a) Identification

- The retention time of any analyte in tested sample extract should correspond to that of the matrix matched calibration standard with a tolerance of ±0.1 min
- Extracted ion chromatograms of tested sample extracts should have peaks of similar shape and response ratio to those obtained from matrix matched calibration standards analyzed at comparable concentrations
- Chromatographic peaks from different selective ions for the analyte should fully overlap
- The ion ratio should not deviate more than 30% from the average ion ratios of all calibration levels.

b) Reporting results

- The results should be reported and expressed in mg/kg.
- Where the residue definition includes more than one analyte, the respective sum of analytes should be calculated as stated in the residue definition and should be used for checking compliance with the MRL.
- Residues for individual analytes below the reporting limits should be reported as <RL mg/kg.
- Where samples have been mixed well, the RSD of replicate results of the test portions should normally not exceed 30%.
- In general, residue data do not have to be adjusted for recovery when the mean recovery is within the range of 70%–120%.
- If residue data are adjusted for recovery, this should be stated in the report.
- The result should be reported together with the expanded measurement uncertainty (MU) as follows: Result = x ± U (units), with x is the measured value
- The sample is considered noncompliant if x-U > MRL.

16.6.6 Method validation

- Commodities used for fortification e.g. rice sample should be free of the pesticides analyzed. This product is chosen to represent group 5 (High starch low water content) commodities (EC, 2017).
- The validation method is performed at two spiking levels (0.01 mg/kg and 0.05 mg/kg).
- Five spiked samples and a blank are analyzed at each level (in total 11 samples are analyzed). Standard spiking solutions are prepared by mixing all the 198 pesticides used for the validation method at a concentration of 10 µg/ml in toluene.
- For the pesticides to be accepted as validated (EC, 2017) the following criteria for precision and trueness should be fulfilled:
- The repeatability RSD should be ≤20%
- The average relative recovery should be between 70% and 120%
- If the above-mentioned criteria have been met, the LOQ is stated.
- Linearity of the GC–MS/MS system is evaluated by assessing the signal responses of the target analytes from matrix matched calibration solutions prepared by spiking blank extracts at six concentration levels: 0.01 µg/ml, 0.02 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.15 µg/ml and 0.2 µg/ml. The six-point calibration curve should show excellent linearity with R2 ≥ 0.99 for all analytes in the scope of this study.

The limit of quantitation is defined (EC, 2017) as the lowest validated spike level meeting the method performance acceptability criteria. Generally good recovery values are obtained (Table 120).

TABLE 120. Recovery test results

		0.01 mg/kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
1	2,4'-DDE	5.5	86.1	5.5	89.9	0.0014	0.01	0.9995	17.45
2	2,4'-DDT	6.4	99.1	5.0	88.4	0.0019	0.01	0.9998	19.13
3	2,4'-Methoxychlor	5.4	109.0	3.2	93.8	0.0018	0.01	0.9997	20.10
4	2-Phenylphenol	9.0	**50.2**	2.3	**60.5**	0.0021	0.05	0.9956	9.96
5	4,4'-DDD	7.9	106.2	6.2	92.7	0.0025	0.01	0.9996	19.06
6	4,4'-DDE	7.5	88.4	6.6	87.0	0.0020	0.01	0.9995	18.14
7	4,4'-DDT	4.5	102.4	5.5	89.8	0.0014	0.01	0.9998	19.86
8	4,4'-Dichlorobenzo-phenone	9.3	107.4	3.4	97.4	0.0030	0.01	0.9998	16.10
9	4,4'-Methoxychlor olefin	7.4	105.3	4.7	92.3	0.0023	0.01	0.9998	19.66
10	Acequinocyl	**20.1**	**52.3**	8.7	86.4	0.0113	0.05	0.9992	24.32
11	Acetochlor	6.6	94.4	4.6	96.6	0.0019	0.01	0.9994	14.85
12	Acrinathrin	20.4	96.0	4.1	84.9	0.0059	0.01	0.9996	22.29
13	Alachlor	6.0	101.6	5.1	99.6	0.0018	0.01	0.9997	15.09
14	Aldrin	**27.9**	97.0	6.8	95.1	0.0097	0.05	0.9986	15.88
15	Allidochlor	**A**	**A**	7.0	98.1	0.0103	0.05	0.9997	6.55
16	Alpha–BHC	4.1	97.1	4.8	92.2	0.0012	0.01	0.9999	12.54

(con't)

TABLE 120. Recovery test results (con't)

		0.01 mg/kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
17	Anthraquinone	11.6	106.9	6.9	101.9	0.0037	0.01	0.9998	15.88
18	Atrazine	9.0	109.0	3.9	97.7	0.0029	0.01	0.9999	13.13
19	Azinphos–ethyl	13.0	95.7	2.1	83.2	0.0037	0.01	0.9996	22.45
20	Azinphos–methyl	6.8	115.4	3.5	83.1	0.0024	0.01	0.9998	21.73
21	Benfluralin	7.4	105.6	6.8	92.1	0.0023	0.01	0.9988	12.27
22	Beta–BHC	6.2	105.5	5.5	94.8	0.0020	0.01	0.9999	13.23
23	Bifenthrin	7.3	102.7	4.1	92.8	0.0022	0.01	0.9996	20.95
24	Biphenyl	**34.6**	75.6	2.9	86.7	0.0037	0.05	0.9997	8.01
25	Bromfenvinphos	8.9	106.6	6.0	92.7	0.0029	0.01	0.9998	17.87
26	Bromfenvinphos–methyl	7.4	101.2	2.9	93.8	0.0022	0.01	1	16.97
27	Bromophos methyl	7.6	100.5	2.1	92.2	0.0023	0.01	0.9998	16.44
28	Bromophos–ethyl	8.1	96.3	3.0	92.2	0.0023	0.01	0.9993	17.40
29	Bromopropylate	9.5	96.8	4.3	94.4	0.0027	0.01	0.9994	20.93
30	Bupirimate	6.9	106.2	6.0	100.5	0.0022	0.01	0.9993	18.43
31	Carbophenothion	4.2	110.4	5.6	95.3	0.0014	0.01	0.9995	19.61
32	Carfentrazone ethyl	9.8	109.4	7.1	97.6	0.0032	0.01	0.9996	19.65
33	Chlorbenside	10.2	83.9	1.9	87.9	0.0026	0.01	0.9993	17.20
34	Chlordane, trans–	7.0	98.4	6.2	91.4	0.0021	0.01	0.9994	17.33
35	Chlorfenapyr	14.5	**123.8**	8.0	104.5	0.0125	0.05	0.9983	18.70
36	Chlorfenson	7.8	103.6	6.5	93.5	0.0024	0.01	0.9995	17.82
37	Chlorfenvinphos 1	35.2	98.2	6.3	97.2	0.0104	0.01	0.9942	16.71
38	Chlorfenvinphos 2	2.6	115.7	10.1	103.3	0.0009	0.01	0.9994	16.97
39	Chlorobenzilate	10.6	92.3	6.2	96.6	0.0029	0.01	0.995	18.84
40	Chloroneb	7.3	96.8	2.8	92.5	0.0021	0.01	0.9999	9.93
41	Chlorothalonil	10.1	70.3	3.0	80.6	0.0027	0.01	0.9995	14.07
42	Chlorpropham	11.1	87.3	5.6	92.7	0.0029	0.01	0.9998	11.86
43	Chlorpyrifos	10.9	104.6	7.7	99.3	0.0034	0.01	0.9995	16.03
44	Chlorpyrifos–methyl	3.5	105.4	4.6	94.5	0.0011	0.01	0.9998	14.91
45	Chlorthal–dimethyl	4.3	89.5	5.2	98.3	0.0012	0.01	0.9994	16.16
46	Chlorthiophos 1	15.0	91.4	10.3	99.2	0.0041	0.01	0.9987	18.76
47	Chlorthiophos 2	13.6	101.8	4.8	87.3	0.0041	0.01	0.9998	18.95
48	Chlorthiophos 3	5.1	108.9	7.0	89.0	0.0017	0.01	0.9996	19.22
49	Chlozolinate	16.5	71.6	3.8	102.9	0.0036	0.01	0.9996	16.87
50	cis–Chlordane	16.0	120.1	11.0	94.6	0.0057	0.01	0.9996	17.69
51	Clomazone	8.6	101.7	4.0	95.8	0.0026	0.01	0.9998	13.20
52	Coumaphos	10.5	108.9	2.9	83.0	0.0034	0.01	0.9996	23.24
53	Cycloate	5.9	88.2	3.7	96.6	0.0016	0.01	0.9999	11.62
54	Cyfluthrin 1	15.5	82.4	15.9	100.9	0.0038	0.01	0.9993	23.64
55	Cyfluthrin 2	10.6	89.3	1.8	93.8	0.0028	0.01	0.9994	23.74

(con't)

TABLE 120. Recovery test results (con't)

		0.01 mg/kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
56	Cyfluthrin 3	16.6	77.3	7.4	90.0	0.0039	0.01	0.9986	23.84
57	Cyfluthrin 4	8.9	95.1	7.0	91.8	0.0025	0.01	0.9972	23.88
58	Cyhalothrin, lambda–	13.6	93.9	5.0	90.1	0.0038	0.01	0.9994	22.10
59	Cypermethrin 1	15.8	82.8	9.7	85.6	0.0039	0.01	0.9998	24.00
60	Cypermethrin 2	18.2	75.1	5.2	87.3	0.0041	0.01	0.9991	24.10
61	Cypermethrin 3	19.1	72.0	4.5	89.3	0.0041	0.01	0.9987	24.20
62	Cypermethrin 4	13.1	76.8	5.1	86.9	0.0030	0.01	0.9997	24.24
63	Cyprodinil	9.8	94.9	2.2	100.0	0.0028	0.01	0.9993	16.63
64	delta–BHC	8.0	99.7	4.9	96.0	0.0024	0.01	0.9988	13.96
65	Deltamethrin	9.9	91.1	6.4	97.5	0.0027	0.01	0.9993	25.90
66	Diallate 1	8.0	99.9	3.3	96.0	0.0024	0.01	0.9998	12.41
67	Diallate 2	16.3	74.7	5.1	93.5	0.0037	0.01	0.9999	12.62
68	Diazinon	6.5	100.1	5.4	96.7	0.0019	0.01	0.9998	13.78
69	Dichlofluanid	7.3	**26.3**	4.2	84.1	0.0052	0.05	0.9997	15.73
70	Dichloroaniline, 3,4'–	15.3	**49.9**	6.1	57.7	0.0053	0.05	0.9993	8.77
71	Diclobenil	12.5	83.7	3.7	92.8	0.0031	0.01	0.9998	7.47
72	Dicloran	**A**	**A**	6.5	83.8	0.0081	0.05	0.9992	12.84
73	Dieldrin	14.2	125.7	13.5	94.8	0.0054	0.01	0.9952	18.21
74	Dimethachlor	9.3	87.8	3.2	99.1	0.0024	0.01	0.9998	14.71
75	Diphenamid	20.1	87.5	5.0	103.5	0.0053	0.01	0.9997	16.46
76	Diphenylamine	11.3	77.5	5.7	75.3	0.0026	0.01	0.9997	11.47
77	Disulfoton	9.1	106.8	3.9	93.0	0.0029	0.01	0.9998	13.88
78	Edifenphos	8.0	106.0	3.9	93.8	0.0025	0.01	0.9995	19.71
79	Endosulfan ether	6.7	92.3	4.7	95.1	0.0018	0.01	0.9993	14.38
80	Endosulfan I	**34.7**	**185.4**	5.9	108.7	0.0096	0.05	0.9982	17.62
81	Endosulfan II	20.1	**134.8**	9.9	95.3	0.0141	0.05	0.9977	18.89
82	Endosulfan sulfate	9.8	**96.9**	6.1	93.1	0.0029	0.01	0.9998	19.8
83	Endrin	19.0	98.3	16.1	84.8	0.0056	0.01	0.9973	18.70
84	Endrin aldehyde	**23.8**	84.7	18.9	62.0	0.0176	0.05	0.9984	19.29
85	Endrin ketone	9.9	108.5	7.4	92.3	0.0032	0.01	0.9996	20.82
86	EPN	11.4	117.5	2.7	88.6	0.0040	0.01	0.9992	20.96
87	Ethalfluralin	8.2	101.7	10.3	95.2	0.0025	0.01	0.9997	11.99
88	Ethion	8.7	112.8	6.1	88.8	0.0030	0.01	0.9994	19.13
89	Ethylan	10.0	98.9	5.9	92.6	0.0030	0.01	0.9996	18.67
90	Etofenprox	8.0	88.5	5.3	92.9	0.0021	0.01	0.9996	24.33
91	Etridazole	6.9	80.2	4.6	89.2	0.0017	0.01	0.9998	9.12
92	Fenamiphos	17.6	101.4	7.3	88.8	0.0054	0.01	0.9998	17.88
93	Fenarimol	11.1	97.3	2.6	86.8	0.0032	0.01	0.9996	22.31
94	Fenchlorphos	3.3	103.3	4.4	92.6	0.0010	0.01	0.9999	15.25
95	Fenitrothion	4.8	103.9	6.5	105.4	0.0015	0.01	0.9992	15.55

(con't)

TABLE 120. Recovery test results (con't)

		0.01 mg/kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
96	Fenpropathrin	15.7	99.6	1.8	94.0	0.0047	0.01	0.9995	21.10
97	Fenson	6.8	101.2	4.4	100.1	0.0021	0.01	0.9997	16.30
98	Fenthion	6.2	102.2	4.4	99.5	0.0019	0.01	0.9995	15.99
99	Fenvalerate 1	9.2	87.6	5.0	94.7	0.0024	0.01	0.9997	25.05
100	Fenvalerate 2	8.4	81.3	6.2	86.3	0.0021	0.01	0.9999	25.28
101	Fipronil	13.4	117.0	7.9	99.8	0.0047	0.01	0.9999	16.99
102	Fluazifop–p–butyl	11.2	105.8	7.7	95.8	0.0035	0.01	0.999	18.64
103	Fluchloralin	8.5	117.4	3.3	99.5	0.0030	0.01	0.9974	13.88
104	Flucythrinate 1	10.6	86.4	5.2	91.8	0.0027	0.01	0.9999	24.23
105	Flucythrinate 2	9.8	88.8	5.7	93.3	0.0026	0.01	0.9999	24.45
106	Fludioxonil	8.5	112.6	5.9	95.5	0.0029	0.01	0.9989	18.11
107	Fluquinconazole	8.9	95.2	3.0	89.8	0.0025	0.01	0.9998	23.24
108	Fluridone	**A**	**A**	7.7	82.0	0.0095	0.05	0.9998	24.77
109	Flusilazole	10.1	98.7	4.2	94.3	0.0030	0.01	0.9995	18.37
110	Flutolanil	5.4	93.4	7.2	98.0	0.0015	0.01	0.9998	17.90
111	Flutriafol	6.6	99.9	7.0	97.5	0.0020	0.01	0.9993	17.74
112	Folpet	11.8	73.9	7.5	83.7	0.0026	0.01	0.9998	17.14
113	Fonofos	7.8	106.3	3.5	93.2	0.0025	0.01	0.9994	13.57
114	Gamma–BHC	7.2	96.1	4.8	93.7	0.0021	0.01	0.9996	13.37
115	Heptachlor	9.7	91.9	3.1	90.7	0.0027	0.01	0.9976	15.08
116	Heptachlor epoxide	4.0	104.1	5.2	98.9	0.0012	0.01	0.9989	16.81
117	Hexachlorobenzene	6.4	75.8	4.9	80.6	0.0015	0.01	1	12.72
118	Hexazinone	8.0	103.9	3.0	91.5	0.0025	0.01	0.9996	20.08
119	Iodofenfos	13.0	88.1	6.2	80.9	0.0034	0.01	0.9997	17.93
120	Iprodione	13.4	108.9	10.0	102.2	0.0044	0.01	0.9896	20.72
121	Isazophos	12.6	112.3	1.5	96.6	0.0042	0.01	0.9998	14.09
122	Isodrin	11.0	81.2	8.6	87.1	0.0027	0.01	0.999	16.55
123	Isopropalin	12.5	101.3	6.2	104.1	0.0038	0.01	0.9996	16.58
124	Lenacil	7.5	112.3	5.1	91.7	0.0025	0.01	0.9984	19.78
125	Leptophos	8.2	99.3	4.5	86.3	0.0025	0.01	0.9996	21.77
126	Linuron	**A**	**A**	6.3	94.7	0.0090	0.05	0.9988	15.62
127	Malathion	9.2	102.0	3.6	104.8	0.0028	0.01	0.9995	15.78
128	Metalaxyl	17.9	102.8	4.3	100.2	0.0055	0.01	0.9996	15.20
129	Metazachlor	8.1	86.8	3.4	102.9	0.0021	0.01	0.9997	16.75
130	Methacrifos	7.6	106.1	5.3	92.7	0.0024	0.01	0.9998	9.82
131	Methoxychlor	9.1	102.4	5.1	93.8	0.0028	0.01	0.9996	21.08
132	Methyl parathion	10.4	118.0	7.2	94.2	0.0037	0.01	0.9988	14.91
133	Metolachlor	8.0	88.9	3.2	105.1	0.0021	0.01	0.9996	15.92
134	Mevinphos	8.8	83.7	4.1	79.5	0.0022	0.01	0.9998	8.75
135	MGK 264 1	23.6	94.4	5.9	106.0	0.0067	0.01	0.9994	16.39
136	MGK 264 2	10.1	116.8	9.3	104.4	0.0035	0.01	0.9997	16.65

(con't)

TABLE 120. Recovery test results (con't)

		0.01 mg/kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
137	Mirex	9.3	81.5	2.0	83.5	0.0023	0.01	0.9999	21.99
138	Myclobutanil	11.6	99.7	7.1	95.3	0.0035	0.01	0.9997	18.30
139	Nitralin	11.0	107.8	3.3	96.1	0.0036	0.01	0.9998	20.52
140	Nitrofen	13.4	85.9	5.5	91.0	0.0034	0.01	0.9998	18.61
141	Nonachlor, cis–	9.8	94.9	9.0	95.5	0.0028	0.01	0.9995	19.16
142	Norflurazon	13.5	112.2	5.4	94.5	0.0045	0.01	0.9993	19.78
143	Oxadiazon	9.1	92.4	6.5	100.8	0.0025	0.01	0.9995	18.21
144	Oxyfluorfen	11.4	95.5	4.5	91.8	0.0033	0.01	0.9996	18.34
145	Paclobutrazol	5.8	105.7	4.8	101.0	0.0018	0.01	1	17.47
146	Parathion	6.5	103.7	4.4	104.4	0.0020	0.01	0.9994	16.05
147	Pebulate	8.7	101.7	3.5	99.1	0.0027	0.01	0.9998	9.17
148	Penconazole	7.9	99.7	3.0	99.6	0.0024	0.01	0.9994	16.80
149	Pendimethalin	11.4	110.0	7.3	101.5	0.0038	0.01	0.9985	16.77
150	Pentachloroaniline	5.0	90.3	1.3	93.9	0.0014	0.01	0.9998	14.49
151	Pentachloroanisole	8.6	89.3	3.9	85.6	0.0023	0.01	0.9997	12.86
152	Pentachlorobenzene	9.5	84.8	3.3	82.1	0.0024	0.01	0.9999	10.13
153	Pentachlorobenzonitrile	8.9	94.4	7.0	92.5	0.0025	0.01	0.999	13.54
154	Pentachlorothioanisole	8.4	84.8	5.1	90.9	0.0021	0.01	0.9999	15.67
155	Permethrin, cis–	13.0	82.1	4.6	89.2	0.0032	0.01	0.9998	22.96
156	Permethrin, trans–	17.0	80.6	4.0	85.3	0.0041	0.01	0.9996	23.10
157	Phenothrin 1	**A**	**A**	3.9	79.8	0.0047	0.05	0.9975	21.40
158	Phenothrin 2	21.0	83.1	5.7	91.9	0.0052	0.01	0.9996	21.52
159	Phorate	5.6	101.3	4.6	92.2	0.0017	0.01	0.9999	12.43
160	Phosalone	12.1	88.0	2.6	87.9	0.0032	0.01	0.9998	21.71
161	Phosmet	6.7	104.0	3.1	88.0	0.0021	0.01	0.9993	20.89
162	Piperonyl butoxide	8.3	110.7	4.2	90.7	0.0027	0.01	0.9997	20.31
163	Pirimiphos–ethyl	6.7	111.3	5.2	104.0	0.0022	0.01	0.9999	16.52
164	Pirimiphos–methyl	4.8	102.3	88.3	65.5	0.0015	0.01	0.9996	15.53
165	Pretilachlor	9.6	92.2	5.6	95.5	0.0027	0.01	0.9996	18.11
166	Prochloraz	12.6	81.7	8.5	79.9	0.0031	0.01	0.9989	23.31
167	Procymidone	14.6	113.8	6.1	102.6	0.0050	0.01	0.999	17.18
168	Prodiamine	10.2	81.3	6.4	102.8	0.0025	0.01	0.9983	15.60
169	Profenofos	4.4	105.6	3.6	89.1	0.0014	0.01	0.9996	18.05
170	Profluralin	5.1	**134.4**	3.6	93.8	0.0050	0.05	0.998	13.57
171	Propachlor	7.0	98.1	5.4	97.1	0.0021	0.01	0.9998	11.34
172	Propanil	**A**	**A**	15.8	92.8	0.0219	0.05	0.998	14.69
173	Propargite	8.8	115.5	4.8	94.6	0.0031	0.01	0.9995	20.22
174	Propisochlor	11.3	99.7	3.9	98.0	0.0034	0.01	0.9998	15.18
175	Propyzamide	8.9	101.3	2.6	90.5	0.0027	0.01	0.9986	13.57

(con't)

TABLE 120. Recovery test results (con't)

		0.01 mg/kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
176	Prothiofos	6.8	102.4	6.2	89.1	0.0021	0.01	0.9987	17.98
177	Pyraclofos	10.3	86.4	1.9	81.4	0.0027	0.01	0.9998	22.54
178	Pyrazophos	13.1	101.6	3.0	85.9	0.0040	0.01	0.9993	22.39
179	Pyridaben	12.5	90.3	3.0	82.9	0.0034	0.01	0.9996	23.11
180	Pyridaphenthion	9.1	117.3	4.5	89.2	0.0032	0.01	0.9994	20.81
181	Pyrimethanil	19.5	89.1	2.0	87.4	0.0052	0.01	0.9998	13.70
182	Pyriproxyfen	10.9	102.1	4.0	93.4	0.0033	0.01	0.9996	21.78
183	Quinalphos	7.8	112.5	3.0	98.9	0.0026	0.01	0.9997	17.03
184	Quintozene	18.2	87.6	3.1	88.5	0.0048	0.01	0.9996	13.49
185	Resmethrin 1	15.4	91.9	4.5	92.1	0.0043	0.01	0.9998	20.24
186	Resmethrin 2	**A**	**A**	15.0	85.4	0.0192	0.05	0.997	20.35
187	Sulfotep	5.3	111.7	5.5	93.1	0.0018	0.01	0.9996	12.32
188	Sulprofos	7.0	117.5	4.2	90.2	0.0025	0.01	0.9995	19.40
189	tau–Fluvalinate 1	6.8	81.8	11.2	90.5	0.0017	0.01	0.9999	25.26
190	tau–Fluvalinate 2	8.3	91.7	3.3	93.4	0.0023	0.01	0.9993	25.33
191	Tebuconazole	7.8	99.6	5.4	93.4	0.0023	0.01	0.999	20.14
192	Tebufenpyrad	9.3	103.9	4.3	96.8	0.0029	0.01	0.9995	21.17
193	Tecnazene	9.0	83.2	5.8	90.4	0.0023	0.01	0.9997	11.25
194	Tefluthrin	6.2	104.9	2.4	98.1	0.0019	0.01	0.9998	14.04
195	Terbacil	19.1	100.3	6.7	92.3	0.0057	0.01	0.9989	13.96
196	Terbufos	5.1	105.2	6.1	87.5	0.0016	0.01	0.9996	13.48
197	Terbutylazine	10.9	113.2	3.4	96.6	0.0037	0.01	0.9999	13.49
198	Tetrachloroaniline, 2,3,5,6–	11.0	78.8	4.1	90.0	0.0026	0.01	0.9997	11.54
199	Tetrachlorvinphos	6.3	110.7	6.6	94.1	0.0021	0.01	0.9997	17.58
200	Tetradifon	15.8	106.2	3.6	92.0	0.0050	0.01	0.9992	21.52
201	Tetrahydrophthalimide	10.0	**131.5**	6.4	96.5	0.0093	0.05	0.9997	9.47
202	Tetramethrin 1	18.5	70.8	5.9	89.3	0.0039	0.01	0.9998	20.82
203	Tetramethrin 2	12.4	106.7	3.3	92.0	0.0040	0.01	0.9993	20.96
204	Tolclofos–methyl	4.6	110.4	3.2	96.3	0.0015	0.01	0.9994	15.01
205	Tolylfluanid	7.7	**37.3**	3.0	87.6	0.0040	0.05	0.9996	16.90
206	Transfluthrin	6.8	105.4	2.2	98.8	0.0022	0.01	0.9998	15.00
207	trans–Nonachlor		13.6			113.4		3.8	
208	Triadimefon		7.2			88.7		4.1	
209	Triadimenol		**A**			**A**		8.2	
210	Triallate		5.2			93.3		3.3	
211	Triazophos		8.9			113.0		4.9	
212	Tricyclazole		**A**			**A**		10.9	
213	Triflumizole		17.4			93.4		6.0	
214	Trifluralin		7.0			94.5		7.1	
215	Vinclozolin		9.8			103.8		2.1	

Bold numbers: Recovery and/or RSD out of acceptable range
A: Peak shape error or no peak was detected.

16.6.7 Schematic diagram of extraction procedure

1. Weigh 10 g of homogenized/powdered sample into a clean 50 ml tube.
2. Add internal standard or spiking mix if required.
3. Add 10 g of cold water and shake briefly.
4. Add 10 ml acetonitrile.
5. Vortex for 1 min.
6. Add buffered QuEChERS extraction packet.
7. Vortex for 1 min.
8. Centrifuge for at 6 000 rpm for 10 min.
9. Transfer 6 ml of the acetonitrile phase (upper layer) into a 15 ml screw capped tube.
10. Place the tube containing the extract for 15 min in the freezer at (-80°C).
11. Centrifuge for at 6 000 rpm for 5 min.

1. Transfer 1 ml of the supernatant to the ready-to use dSPE tube
2. Vortex for 30 s
3. Centrifuge (5°C) for 5 min at 6 000 rpm
4. Filter the extract through 0.45 mm PTFE filter
5. Take 0.25 ml of the filtrate
6. Evaporate to almost dryness at 40°C using a slight nitrogen flow (less than 5 bar)
7. Reconstitute in 0.5 ml of toluene and mix properly
8. Inject into the GC–MS/MS

17

Detection and quantification of pesticide residues in cereals by LC–MS/MS

17.1 Introduction

Pesticides are still used widely to control animal and plants pests and diseases. However, residues of these substances may remain in the animal or plant products that are of public health and trade concerns and should be controlled with the help of testing laboratories using good analytical methods.

17.2 Scope and objectives

This procedure applies to cereal samples received by the Contaminants Monitoring Division (CMD). This SOP describes the procedure for food sample preparation, extraction and cleanup of pesticides from cereal samples, LC–MS/MS analytical conditions, standards and calibration level preparations, and identification and result reporting.

17.3 Principle of the method

Pesticides are extracted from cereals using the QuEChERS principle and analyzed by LC–MS/MS. The homogeneous sample is extracted with acetonitrile. After addition of salt, the mixture is shaken intensively and centrifuged for phase separation. An aliquot of the organic phase is frozen and then cleaned up with PSA and anhydrous $MgSO_4$ to remove the residual water. Small aliquot of extract is diluted with acetonitrile then acidified with 5% formic acid.

17.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, some pesticide standards are considered a possible carcinogen to humans. Suitable gloves should be worn when chemicals/pesticides standards are handled, and the work should be performed in a fume hood.

17.5 Materials

17.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Acetonitrile (MeCN) (LC–MS grade); 5% Formic acid solution in acetonitrile; Water (LC–MS grade); Methanol (LC–MS grade); Ammonium formate (LC–MS grade, ≥ 99.0%) and Formic acid LC–MS grade.

17.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Freezers (-20°C; -80°C); Sample processing equipment (blender and grinder); Refrigerated centrifuges for 50 ml, 15 ml and 2 ml centrifuge tubes; Vortex and/or shaker; Ultrasonic water bath; Automatic pipettes (e.g. for 20 µl–200 µl, 100 µl–1 000 µl and 1 ml–10 ml); Centrifuge tubes with screw caps (50 ml and 15 ml); Solvent–dispenser (for acetonitrile); Vials with caps for LC auto sampler (2 ml); Syringes (e.g. 2 ml disposable syringes); Syringes filters (0.45 µm pore size); QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate); dSPE tubes (150 mg $MgSO_4$, 25 mg PSA); LC–MS/MS (Shimadzu).

17.5.3 Solutions

The solutions (and how they are prepared) include:

- Mobile Phase (A): 2 mmol/l ammonium formate + 0.002% formic acid – Water
  - Add 0.126 g of ammonium formate into 1 000 ml volumetric flask
  - Add 500 ml of LC–MS grade water
  - Add 20 µl formic acid
  - Complete to 1 000 ml with LC–MS grade water
  - Sonicate for 10 min
- Mobile Phase (B): 2 mmol/l ammonium formate + 0.002% formic acid – Methanol
  - Add accurately 0.126 g of Ammonium formate into 1 000 ml volumetric flask
  - Add 500 ml of methanol
  - Add 20 µl formic acid
  - Complete to 1 000 ml with methanol
  - Sonicate for 10 min
- 5% formic acid in acetonitrile
  - Add 500 µl of formic acid in volumetric flask and complete to 10 ml with acetonitrile

17.5.4 Standards

17.5.4.1 Reference standards

The reference standards should be of the purity ≥ 98%.

17.5.4.2 Working solutions

a) Working solution 1 (W1, 10 µg/ml)

- This is prepared from the stock standard solution (100 µg/ml).
- Take 100 µl from each mix and complete with acetonitrile so the final volume is 1 ml.

- Use it to prepare working solution 2 (WS2) at 1 µg/ml and to spike LC–MS/MS recovery sample.

b) Working solution 2 (W2, 1 µg/l)

- Prepared from WS1 by taking 100 and complete with acetonitrile to 1 ml.
- Use it to prepare LC–MS/MS calibration levels.

17.5.4.3 Calibration standards

For quantitation, a matrix matched calibration curve is generated using known blank samples closely related to the tested samples. The blank sample is subjected to the same extraction and cleanup procedure. The final blank extract is used to prepare four calibration standards as follows:

- Level 1 (0.1 µg/ml): Add 10 µl of WS1 + 990 µl final blank extract directly in an auto sampler vial;
- Level 2 (0.2 µg/ml): Add 20 µl of WS1 + 980 µl final blank extract directly in an auto sampler vial;
- Level 3 (0.5 µg/ml): Add 50 µl of WS1 + 950 µl final blank extract directly in an auto sampler vial;
- Level 4 (1 µg/ml): Add 100 µl of WS1 + 900 µl final blank extract directly in an auto sampler vial.

The calibration curve should be valid until check standards deviated from the acceptable criteria (20% range of the corresponding calibration point value). If values deviate, prepare new calibration curve and check again.

17.6 Procedure

17.6.1 Sample preparation

Cereals:

a) Take 200 g of the cereal sample and store at (-20°C) for at least 1 hr or overnight.

b) After freezing, weigh 100 g of the sample and transfer to a blender (preserve the other 100 g at -20°C).

c) Homogenize the sample into a fine free flowing powder.

Powder samples:

- Take around 200 g of the sample and store at -20°C for at least 1 hr or overnight.

17.6.2 Sample extraction

a) Weigh 10 g (±0.1 g) of sample into a clean 50 ml tube

b) Spike blank samples at concentration 0.05 mg/kg. Let stand for 10 min

c) Add 10 g of cold water and shake briefly

d) Add 10 ml acetonitrile

e) Vortex for 1 min

f) Add buffered QuEChERS extraction packet as indicated elsewhere (EC, 2015) and including: (4 g $MgSO_4$, 1 g NaCl, 1 g Na–citrate, 0.5 g disodium citrate sesquihydrate)

g) Vortex for 1 min

h) Centrifuge (5°C) for 10 min at 6 000 rpm

i) Transfer an aliquot (5 ml) of the acetonitrile phase (upper layer) into a 15 ml screw capped tube

j) Place the tube containing the extract for at least 15 min in the freezer at -80°C

k) Allow the extracts to become almost liquid, then cold centrifuge (5°C) for 5 min at 6 000 rpm.

17.6.3 Cleanup procedure

a) Transfer 1 ml of the supernatant to the ready–to–use dSPE tube (2 ml capacity) contains 150 mg $MgSO_4$, 25 mg PSA;

b) Vortex for 30 s;

c) Centrifuge for 5 min at 5°C and 6 000 rpm

d) Filter the extract through 0.45 mm PTFE filter

e) Take 0.25 ml of the filtrate

f) Add 0.25 ml acetonitrile

g) Acidify at 1% with 5% formic acid in acetonitrile (10 µl to every 1 ml)

h) Set sample amount at 1

i) Set dilution factor at 2

j) The final extract has a concentration of ca. 0.5 g/ml

k) Sample is ready to be injected.

17.6.4 Instrumental analysis (LC–MS/MS)

Analyses involve the use of a High–Speed Analysis Method (for 646 Residual Pesticide Components) – LC–MS/MS Method Package - Residual Pesticides Version 2–SHIMADZU with UHPLC and mobile phase and MS conditions summarized in Tables 121–123.

TABLE 121. UHPLC conditions

Instrument	Nexera (2040c) LC system, Shimadzu
Analytical Column	Restek Raptor Biphenyl (100 × 2.1 mm, 2.7 µm) (Cat#: 9309A12)
Column Oven Temperature	35°C
Injection Volume	2 µl
Flow rate	0.4 ml / min
Mobile phase A	2 mmol/l ammonium formate + 0.002% formic acid – Water
Mobile phase B	2 mmol/l ammonium formate + 0.002% formic acid –Methanol

TABLE 122. Mobile phase gradient program

Step	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
1	0.01	97	3
2	1	90	10
3	3	45	55
4	10.5	0	100
5	12	0	100
6	12.01	97	3
7	15	97	3
Stop	15.01	97	3

TABLE 123. MS conditions

Nebulizing Gas Flow Rate	3 l/min
Drying Gas Flow Rate	10 l/min
Heating Gas Flow Rate	10 l/min
Interface Temperature	350°C
Desolvation Line Temperature	150°C
Block Heater Temperature	300°C
Ionization mode	ESI

17.6.5 Calculation and interpretation of results

a) Identification

- The retention time of any analyte in the tested sample extract should correspond to that of the matrix matched calibration standard with a tolerance of ± 0.1min.
- Extracted ion chromatograms of tested sample extracts should have peaks of a similar shape and response ratio to those obtained from matrix matched calibration standards analyzed at comparable concentrations.
- Chromatographic peaks from different selective ions for the analyte should fully overlap.
- The ion ratio should not deviate more than 30% (relative).

b) Reporting results

- The results should be reported and expressed in mg/kg.
- Where the residue definition includes more than one analyte, the respective sum of analytes should be calculated as stated in the residue definition and should be used for checking compliance with the MRL.
- Residues for individual analytes below the reporting limits should be reported as <MRL mg/kg.
- Where good mixing of samples has been undertaken, the RSD of replicate results of the test portions should normally not exceed 30%.

- In general, residue data do not have to be adjusted for recovery when the mean recovery is within the range of 70%–120%.
- If residue data are adjusted for recovery, then this should be stated in the report.
- The result should be reported together with the expanded measurement uncertainty (MU) as follows: Result = x ± U (units), with x representing the measured value
- The sample is considered non–compliant if x-U > MRL.

17.6.6 Method validation

- Commodities used for fortification may include rice samples free of the pesticides analyzed. This product may be chosen to represent group 5 (High starch low water content) defined commodities (EC, 2017.
- The validation method is performed at two spiking levels (0.01 mg/kg and 0.05 mg/kg).
- Five spiked samples and a blank are analyzed at each level. In total eleven samples are analyzed.
- Standard spiking solutions are prepared by mixing all the 184 pesticides used for the validation method at a concentration of 10 µg/ml in acetonitrile.
- For the pesticides to be accepted as validated the following criteria for precision and trueness should be fulfilled (EC, 2017:
- The repeatability RSD should be ≤ 20%
- The average relative recovery should be between 70% and 120%.
- If the above-mentioned criteria have been met, the quantification limits, LOQs is stated.
- Linearity of the LC–MS/MS system was evaluated by assessing the signal responses of the target analytes from matrix matched calibration solutions prepared by spiking blank extracts at six concentration levels corresponding to concentrations: 0.01 µg/ml, 0.02 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.15 µg/ml and 0.2 µg/ml. The six-point calibration curve should demonstrate excellent linearity with R2 ≥ 0.99 for all analytes in the scope of this study.
- The limit of quantitation as the lowest validated spike level meeting the method performance acceptability criteria (EC, 2017). The validation test results are presented in Table 124.

TABLE 124. Validation summary

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
1	(E)–Fenpyroximate	2.7	107.1	2.0	99.4	0.0009
2	Acephate	13.8	77.6	3.3	85.6	0.0032
3	Acetamiprid	7.7	82.6	1.8	100.0	0.0019
4	Acibenzolar–s–methyl	13.1	114.1	6.9	103.2	0.0045
5	Aldicarb	**20.0**	**65.5**	6.3	102.3	0.0097
6	Aldicarb–sulfone	4.5	88.9	1.8	101.7	0.0012

(con't)

TABLE 124. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
7	Aldicarb–sulfoxide	7.3	84.5	2.8	89.7	0.0018
8	Ametryn	8.8	119.4	3.3	100.2	0.0032
9	Aminocarb	12.9	82.0	3.8	92.2	0.0032
10	Avermectin B1a	**38.9**	**78.4**	15.7	88.8	0.0209
11	Azoxystrobin	6.5	97.9	2.9	97.7	0.0019
12	Benalaxyl	10.1	87.8	3.7	100.7	0.0027
13	Benzoximate	6.1	91.7	4.2	96.0	0.0017
14	Bitertanol	9.9	92.5	3.1	100.9	0.0028
15	Boscalid	7.8	84.4	6.0	97.6	0.0020
16	Bromuconazole	**14.0**	**67.5**	5.2	99.2	0.0077
17	Bupirimate	7.2	86.7	2.3	101.2	0.0019
18	Buprofezin	4.4	95.3	3.9	97.4	0.0013
19	Butafenacil	12.2	92.3	5.2	99.0	0.0034
20	Carbaryl (NAC)	7.4	81.7	1.6	98.7	0.0018
21	Carbendazim	9.8	79.6	3.2	90.8	0.0023
22	Carbetamide	9.9	99.5	1.7	100.8	0.0030
23	Carbofuran	9.9	110.1	10.5	120.9	0.0033
24	Carbofuran–3-hydroxy	19.4	73.4	3.3	104.0	0.0043
25	Carboxin	**36.0**	**39.9**	4.0	96.3	0.0058
26	Carfentrazone-ethyl	**31.2**	**106.3**	16.8	110.9	0.0280
27	Chlorantraniliprole	8.4	83.6	4.0	96.6	0.0021
28	Chlorfluazuron	**40.2**	**45.9**	3.7	103.9	0.0057
29	Chlorotoluron	4.7	89.4	2.7	100.0	0.0013
30	Chloroxuron	**24.4**	**70.4**	6.5	97.0	0.0095
31	Clethodim	14.0	81.5	6.2	89.0	0.0034
32	Clofentezine	12.6	82.8	4.8	96.7	0.0031
33	Clothianidin	8.2	80.7	1.9	98.3	0.0020
34	Cyazofamid	6.3	86.6	3.1	99.9	0.0016
35	Cycluron	11.0	106.0	5.9	104.9	0.0035
36	Cymoxanil	**32.8**	**102.5**	9.3	100.6	0.0140
37	Cyproconazole	7.7	85.3	12.4	91.8	0.0020
38	Cyprodinil	6.5	83.3	3.7	98.0	0.0016
39	Desmedipham	**18.7**	**57.8**	2.0	99.9	0.0029
40	Diclobutrazol	**17.2**	**58.5**	4.2	98.4	0.0062
41	Dicrotophos	**41.4**	**80.0**	11.4	94.6	0.0162
42	Diethofencarb	10.8	80.7	2.2	103.5	0.0026
43	Difenoconazole	7.3	93.0	2.4	99.9	0.0020

(con't)

TABLE 124. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
44	Diflubenzuron	11.1	85.2	2.5	102.4	0.0028
45	Dimethoate	**49.0**	**34.5**	4.0	99.6	0.0060
46	Dimethomorph	16.5	91.6	3.8	97.9	0.0045
47	Dimoxystrobin	4.6	91.2	2.0	98.3	0.0013
48	Diniconazole	12.0	90.7	2.0	97.7	0.0033
49	Dinotefuran	14.1	72.3	1.5	95.9	0.0031
50	Dioxacarb	**27.4**	**56.3**	3.7	102.5	0.0057
51	Diuron (DCMU)	26.7	96.3	9.3	103.7	0.0077
52	Doramectin	**83.1**	**42.6**	8.7	93.5	0.0122
53	Emamectin B1a	20.4	73.7	3.8	75.7	0.0045
54	Emamectin B1b	14.8	74.8	6.4	79.1	0.0033
55	Epoxiconazole	24.4	70.3	2.7	100.2	0.0052
56	Etaconazole	9.7	75.1	2.6	102.0	0.0022
57	Ethiofencarb	12.7	70.2	2.5	100.0	0.0027
58	Ethiprole	**24.4**	**67.1**	5.6	106.7	0.0089
59	Ethirimol	6.9	80.3	2.5	86.6	0.0017
60	Ethofumesate	**20.4**	**64.9**	4.2	103.0	0.0065
61	Etoxazole	11.3	76.8	2.3	98.0	0.0026
62	Famoxadone	13.0	101.2	7.4	96.7	0.0039
63	Fenamidone	8.4	94.7	5.1	101.1	0.0024
64	Fenarimol	11.3	85.0	3.7	97.3	0.0029
65	Fenazaquin	18.1	71.8	9.1	77.8	0.0039
66	Fenbuconazole	12.0	76.2	2.0	102.6	0.0027
67	Fenhexamid	17.3	75.4	6.3	97.0	0.0039
68	Fenobucarb	15.0	76.1	1.6	100.0	0.0034
69	Fenoxycarb	7.9	86.5	3.3	99.3	0.0021
70	Fenpropimorph	12.6	81.0	1.7	87.2	0.0030
71	Fenuron	14.4	105.0	6.1	109.8	0.0045
72	Fipronil	**23.1**	**68.4**	2.7	107.9	0.0043
73	Flonicamid	**28.6**	**64.7**	10.8	100.5	0.0162
74	Fluazinam	7.1	94.7	4.2	99.3	0.0020
75	Fludioxonil	9.9	86.4	2.5	103.7	0.0026
76	Flufenacet	16.7	72.6	2.8	103.7	0.0036
77	Flufenoxuron	18.1	70.5	2.6	104.7	0.0038
78	Fluometuron	12.0	79.3	3.3	103.1	0.0029
79	Fluoxastrobin	15.3	76.5	4.0	102.2	0.0035
80	Fluquinconazole	16.9	75.0	3.1	98.3	0.0038
81	Flusilazole	13.7	70.7	3.9	100.0	0.0029
82	Flutolanil	12.9	71.1	2.9	103.1	0.0028
83	Flutriafol	8.2	75.7	1.7	98.7	0.0019
84	Forchlorfenuron	**31.9**	**84.5**	3.4	95.7	0.0049

(con't)

TABLE 124. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
85	Formetanate	**3.4**	**122.3**	3.4	91.7	0.0046
86	Furalaxyl	5.1	85.6	2.2	101.9	0.0013
87	Furathiocarb	13.3	73.8	13.8	85.0	0.0029
88	Halofenozide	**45.9**	**34.6**	6.0	103.8	0.0094
89	Hexaconazole	9.6	87.0	3.9	97.7	0.0025
90	Hexaflumuron	**51.0**	**31.5**	1.7	112.9	0.0029
91	Hexythiazox	4.2	90.8	1.3	95.8	0.0012
92	Hydramethylnon	12.4	79.5	4.6	80.5	0.0030
93	Imazalil	7.5	87.5	3.0	96.7	0.0020
94	Imidacloprid	**94.0**	**23.3**	4.4	102.4	0.0068
95	Indoxacarb	13.4	77.3	4.0	113.2	0.0031
96	Ipconazole	11.2	76.2	2.0	99.5	0.0026
97	Iprovalicarb	14.7	82.3	2.6	98.3	0.0036
98	Isocarbophos	19.2	71.9	8.2	75.5	0.0041
99	Isoprocarb	7.7	89.7	2.9	102.1	0.0021
100	Isoproturon	7.7	93.7	2.3	100.2	0.0022
101	Ivermectin	**37.2**	**79.8**	12.9	86.9	0.0168
102	Kresoxim–methyl	9.6	76.2	2.5	102.3	0.0022
103	Linuron	7.5	94.2	3.2	101.6	0.0021
104	Mandipropamid	4.0	86.1	3.8	103.9	0.0010
105	Mefenacet	8.6	97.3	2.7	102.3	0.0025
106	Mepanipyrim	15.3	80.3	4.4	103.1	0.0037
107	Mepronil	9.3	88.0	4.9	104.8	0.0024
108	Metaflumizone	9.9	92.7	4.8	103.7	0.0027
109	Metalaxyl	12.3	105.3	5.0	102.8	0.0039
110	Metconazole	15.5	82.9	2.7	97.8	0.0039
111	Methabenzthiazuron	**18.4**	**56.6**	4.0	102.1	0.0061
112	Methamidophos	A	A	1.4	77.5	0.0017
113	Methiocarb	15.8	71.3	2.5	101.0	0.0034
114	Methomyl	**9.2**	**123.4**	1.5	117.9	0.0026
115	Methoprotryne	8.1	87.6	2.9	96.4	0.0021
116	Methoxyfenozide	11.8	88.2	5.4	101.2	0.0031
117	Metobromuron	**21.5**	**56.7**	4.7	97.3	0.0068
118	Metribuzin	9.5	81.9	3.5	97.2	0.0023
119	Mevinphos	2.9	108.2	1.6	103.5	0.0009
120	Mexacarbate	9.8	72.0	3.0	98.4	0.0021
121	Monocrotophos	4.0	95.8	1.1	96.2	0.0011
122	Monolinuron	11.5	74.5	6.2	101.2	0.0026
123	Myclobutanil	**46.1**	**68.3**	4.4	99.5	0.0066
124	Nitenpyram	**14.2**	**65.7**	3.4	96.0	0.0049

(con't)

TABLE 124. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
125	Novaluron	**27.3**	**87.7**	10.1	103.0	0.0156
126	Nuarimol	11.6	84.2	3.8	98.4	0.0029
127	Omethoate	19.2	87.3	2.9	90.7	0.0050
128	Oxadixyl	14.6	80.5	2.5	101.8	0.0035
129	Oxamyl	7.7	77.6	1.9	94.7	0.0018
130	Paclobutrazol	9.0	79.1	2.1	99.3	0.0021
131	Penconazole	12.0	79.5	3.8	102.0	0.0029
132	Pencycuron	13.1	76.2	3.4	102.6	0.0030
133	Phenmedipham	11.5	72.2	2.9	100.7	0.0025
134	Picoxystrobin	3.3	94.2	0.7	102.1	0.0009
135	Piperonyl–butoxide	9.5	83.3	4.1	95.2	0.0024
136	Pirimicarb	**6.3**	**130.2**	4.0	101.8	0.0061
137	Prochloraz	4.4	97.4	1.4	97.7	0.0013
138	Promecarb	13.4	70.8	2.6	99.3	0.0029
139	Prometon	4.8	101.5	0.6	97.3	0.0015
140	Prometryn	7.1	93.4	2.6	99.2	0.0020
141	Propargite	6.2	85.9	2.6	99.5	0.0016
142	Propham	19.6	117.6	5.2	102.7	0.0069
143	Propiconazole	15.6	72.0	3.4	99.8	0.0034
144	Propoxur	8.7	79.9	2.1	102.3	0.0021
145	Pymetrozine	8.1	86.8	2.9	66.3	0.0021
146	Pyracarbolid	16.1	70.4	2.0	100.9	0.0034
147	Pyraclostrobin	11.3	85.7	3.8	95.7	0.0029
148	Pyridaben	7.0	85.6	1.6	96.5	0.0018
149	Pyrimethanil	7.4	95.7	2.5	97.7	0.0021
150	Pyriproxyfen	12.0	70.8	3.0	101.1	0.0025
151	Quinoxyfen	8.5	79.8	2.7	92.6	0.0020
152	Rotenone	6.1	92.7	1.9	98.5	0.0017
153	Secbumeton	13.9	100.3	5.9	96.9	0.0042
154	Siduron	10.0	103.6	2.8	101.9	0.0031
155	Simetryn	**5.0**	**123.2**	2.0	100.0	0.0030
156	Spinetoram	11.5	70.0	3.1	72.7	0.0024
157	Spinosyn	**22.2**	**65.0**	7.6	76.7	0.0087
158	Spirodiclofen	8.2	83.6	2.8	98.1	0.0020
159	Spiromesifen	**64.1**	**30.7**	5.3	97.7	0.0078
160	Spirotetramat	18.8	94.9	14.8	92.6	0.0054
161	Tebuconazole	6.2	88.6	5.0	98.8	0.0016
162	Tebufenozide	13.8	82.1	4.5	100.0	0.0034
163	Tebufenpyrad	19.5	80.9	4.1	99.8	0.0047
164	Tebuthiuron	**18.7**	**57.1**	2.5	99.6	0.0037

(con't)

TABLE 124. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
165	Teflubenzuron	**250.6**	**9.4**	2.6	108.5	0.0043
166	Temephos	**28.0**	**89.9**	6.5	103.5	0.0100
167	Terbumeton	5.5	92.1	2.1	97.2	0.0015
168	Terbutryn	5.6	109.2	4.3	98.3	0.0018
169	Tetraconazole	**23.2**	**59.7**	3.3	101.9	0.0050
170	Thiacloprid	10.7	77.6	2.1	100.2	0.0025
171	Thiamethoxam	**81.6**	**35.8**	2.6	100.7	0.0039
172	Thidiazuron	**42.7**	**58.3**	3.5	88.5	0.0047
173	Thiobencarb	5.2	90.1	3.2	100.0	0.0014
174	Thiofanox	26.2	59.2	6.1	104.5	0.0096
175	Thiophanate–methyl	27.7	62.3	4.8	100.7	0.0072
176	Triadimefon	12.7	83.6	1.9	99.5	0.0032
177	Triadimenol	12.3	72.2	3.0	99.2	0.0027
178	Trichlorfon	**25.8**	**68.3**	7.1	97.7	0.0104
179	Tricyclazole	10.9	77.9	3.1	96.5	0.0025
180	Trifloxystrobin	17.7	73.5	2.8	102.6	0.0039
181	Triflumizole	2.2	95.0	1.8	98.5	0.0006
182	Triflumuron	8.9	89.0	3.4	101.2	0.0024
183	Vamidothion	2.3	103.5	1.7	98.7	0.0007
184	Zoxamide	10.2	70.3	2.0	102.6	0.0022

Bold numbers: Recovery and/or RSD out of acceptable range
A: Peak shape error or no peak was detected

Table 125. below details additional method performance parameters.

TABLE 125. LOQ, coefficients of determination (R^2) and concentration ranges for analysed pesticides

No.	Compound	LOQ	R^2	Instrumental concentration range
1	(E)–Fenpyroximate	0.01	0.9997	0.01–0.200
2	Acephate	0.01	0.9999	0.01–0.200
3	Acetamiprid	0.01	0.9997	0.01–0.200
4	Acibenzolar–s–methyl	0.01	0.9997	0.01–0.200
5	Aldicarb	0.05	0.9995	0.01–0.200
6	Aldicarb-sulfone	0.01	0.9999	0.01–0.200
7	Aldicarb-sulfoxide	0.01	0.9998	0.01–0.200
8	Ametryn	0.01	0.9999	0.01–0.200
9	Aminocarb	0.01	1.0000	0.01–0.200
10	Avermectin B1a	0.05	0.9937	0.01–0.200
11	Azoxystrobin	0.01	0.9996	0.01–0.200
12	Benalaxyl	0.01	0.9998	0.01–0.200

(con't)

TABLE 125. LOQ, coefficients of determination (R^2) and concentration ranges for analysed pesticides (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
13	Benzoximate	0.01	0.9989	0.01–0.200
14	Bitertanol	0.01	0.9997	0.01–0.200
15	Boscalid	0.01	0.9994	0.01–0.200
16	Bromuconazole	0.05	0.9998	0.01–0.200
17	Bupirimate	0.01	0.9998	0.01–0.200
18	Buprofezin	0.01	0.9996	0.01–0.200
19	Butafenacil	0.01	0.9993	0.01–0.200
20	Carbaryl (NAC)	0.01	0.9998	0.01–0.200
21	Carbendazim	0.01	0.9999	0.01–0.200
22	Carbetamide	0.01	0.9999	0.01–0.200
23	Carbofuran	0.01	0.9997	0.01–0.200
24	Carbofuran–3-hydroxy	0.01	0.9999	0.01–0.200
25	Carboxin	0.05	0.9954	0.01–0.200
26	Carfentrazone-ethyl	0.05	0.9972	0.01–0.200
27	Chlorantraniliprole	0.01	0.9999	0.01–0.200
28	Chlorfluazuron	0.05	0.9969	0.01–0.200
29	Chlorotoluron	0.01	0.9996	0.01–0.200
30	Chloroxuron	0.05	0.9989	0.01–0.200
31	Clethodim	0.01	0.9995	0.01–0.200
32	Clofentezine	0.01	0.9997	0.01–0.200
33	Clothianidin	0.01	0.9998	0.01–0.200
34	Cyazofamid	0.01	0.9999	0.01–0.200
35	Cycluron	0.01	0.9987	0.01–0.200
36	Cymoxanil	0.05	0.9989	0.01–0.200
37	Cyproconazole	0.01	0.9950	0.01–0.200
38	Cyprodinil	0.01	0.9999	0.01–0.200
39	Desmedipham	0.05	0.9992	0.01–0.200
40	Diclobutrazol	0.05	0.9988	0.01–0.200
41	Dicrotophos	0.05	0.9993	0.01–0.200
42	Diethofencarb	0.01	0.9998	0.01–0.200
43	Difenoconazole	0.01	0.9999	0.01–0.200
44	Diflubenzuron	0.01	0.9998	0.01–0.200
45	Dimethoate	0.05	0.9979	0.01–0.200
46	Dimethomorph	0.01	0.9998	0.01–0.200
47	Dimoxystrobin	0.01	0.9999	0.01–0.200
48	Diniconazole	0.01	1.0000	0.01–0.200
49	Dinotefuran	0.01	0.9998	0.01–0.200
50	Dioxacarb	0.05	0.9993	0.01–0.200
51	Diuron (DCMU)	0.01	0.9971	0.01–0.200

(con't)

TABLE 125. LOQ, coefficients of determination (R^2) and concentration ranges for analysed pesticides (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
52	Doramectin	0.05	0.9978	0.01–0.200
53	Emamectin B1a	0.01	0.9996	0.01–0.200
54	Emamectin B1b	0.01	0.9984	0.01–0.200
55	Epoxiconazole	0.05	0.9997	0.01–0.200
56	Etaconazole	0.01	0.9996	0.01–0.200
57	Ethiofencarb	0.01	0.9995	0.01–0.200
58	Ethiprole	0.05	0.9998	0.01–0.200
59	Ethirimol	0.01	0.9998	0.01–0.200
60	Ethofumesate	0.05	0.9951	0.01–0.200
61	Etoxazole	0.01	0.9998	0.01–0.200
62	Famoxadone	0.01	0.9993	0.01–0.200
63	Fenamidone	0.01	0.9995	0.01–0.200
64	Fenarimol	0.01	0.9993	0.01–0.200
65	Fenazaquin	0.01	0.9994	0.01–0.200
66	Fenbuconazole	0.01	0.9993	0.01–0.200
67	Fenhexamid	0.01	0.9999	0.01–0.200
68	Fenobucarb	0.01	0.9996	0.01–0.200
69	Fenoxycarb	0.01	0.9992	0.01–0.200
70	Fenpropimorph	0.01	0.9995	0.01–0.200
71	Fenuron	0.01	0.9998	0.01–0.200
72	Fipronil	0.05	0.9991	0.01–0.200
73	Flonicamid	0.05	0.9994	0.01–0.200
74	Fluazinam	0.01	0.9997	0.01–0.200
75	Fludioxonil	0.01	0.9998	0.01–0.200
76	Flufenacet	0.01	0.9993	0.01–0.200
77	Flufenoxuron	0.01	0.9996	0.01–0.200
78	Fluometuron	0.01	0.9999	0.01–0.200
79	Fluoxastrobin	0.01	0.9993	0.01–0.200
80	Fluquinconazole	0.01	0.9996	0.01–0.200
81	Flusilazole	0.01	0.9983	0.01–0.200
82	Flutolanil	0.01	0.9995	0.01–0.200
83	Flutriafol	0.01	0.9996	0.01–0.200
84	Forchlorfenuron	0.05	0.9991	0.01–0.200
85	Formetanate	0.05	0.9991	0.01–0.200
86	Furalaxyl	0.01	0.9998	0.01–0.200
87	Furathiocarb	0.01	0.9999	0.01–0.200
88	Halofenozide	0.05	0.9978	0.01–0.200
89	Hexaconazole	0.01	0.9995	0.01–0.200
90	Hexaflumuron	0.05	0.9915	0.01–0.200

(con't)

TABLE 125. LOQ, coefficients of determination (R^2) and concentration ranges for analysed pesticides (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
91	Hexythiazox	0.01	0.9997	0.01–0.200
92	Hydramethylnon	0.01	0.9997	0.01–0.200
93	Imazalil	0.01	0.9997	0.01–0.200
94	Imidacloprid	0.05	0.9978	0.01–0.200
95	Indoxacarb	0.01	0.9985	0.01–0.200
96	Ipconazole	0.01	0.9996	0.01–0.200
97	Iprovalicarb	0.01	0.9999	0.01–0.200
98	Isocarbophos	0.01	0.9996	0.01–0.200
99	Isoprocarb	0.01	0.9996	0.01–0.200
100	Isoproturon	0.01	0.9987	0.01–0.200
101	Ivermectine	0.05	0.9995	0.01–0.200
102	Kresoxim-methyl	0.01	0.9998	0.01–0.200
103	Linuron	0.01	0.9995	0.01–0.200
104	Mandipropamid	0.01	0.9995	0.01–0.200
105	Mefenacet	0.01	0.9997	0.01–0.200
106	Mepanipyrim	0.01	0.9993	0.01–0.200
107	Mepronil	0.01	0.9994	0.01–0.200
108	Metaflumizone	0.01	0.9999	0.01–0.200
109	Metalaxyl	0.01	0.9994	0.01–0.200
110	Metconazole	0.01	0.9995	0.01–0.200
111	Methabenzthiazuron	0.05	0.9984	0.01–0.200
112	Methamidophos	0.05	0.9997	0.01–0.200
113	Methiocarb	0.01	0.9992	0.01–0.200
114	Methomyl	0.05	0.9991	0.01–0.200
115	Methoprotryne	0.01	0.9998	0.01–0.200
116	Methoxyfenozide	0.01	0.9992	0.01–0.200
117	Metobromuron	0.05	0.9982	0.01–0.200
118	Metribuzin	0.01	0.9996	0.01–0.200
119	Mevinphos	0.01	0.9997	0.01–0.200
120	Mexacarbate	0.01	0.9997	0.01–0.200
121	Monocrotophos	0.01	0.9996	0.01–0.200
122	Monolinuron	0.01	0.9991	0.01–0.200
123	Myclobutanil	0.05	0.9992	0.01–0.200
123	Myclobutanil	0.05	0.9992	0.01–0.200
124	Nitenpyram	0.05	0.9995	0.01–0.200
125	Novaluron	0.05	0.9981	0.01–0.200
126	Nuarimol	0.01	0.9990	0.01–0.200
127	Omethoate	0.01	0.9982	0.01–0.200
128	Oxadixyl	0.01	0.9999	0.01–0.200
129	Oxamyl	0.01	0.9998	0.01–0.200

(con't)

TABLE 125. LOQ, coefficients of determination (R^2) and concentration ranges for analysed pesticides (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
130	Paclobutrazol	0.01	0.9998	0.01–0.200
131	Penconazole	0.01	0.9992	0.01–0.200
132	Pencycuron	0.01	0.9984	0.01–0.200
133	Phenmedipham	0.01	0.9952	0.01–0.200
134	Picoxystrobin	0.01	0.9995	0.01–0.200
135	Piperonyl–butoxide	0.01	0.9994	0.01–0.200
136	Pirimicarb	0.05	0.9985	0.01–0.200
137	Prochloraz	0.01	0.9996	0.01–0.200
138	Promecarb	0.01	0.9995	0.01–0.200
139	Prometon	0.01	1.0000	0.01–0.200
140	Prometryn	0.01	0.9997	0.01–0.200
141	Propargite	0.01	0.9999	0.01–0.200
142	Propham	0.01	0.9993	0.01–0.200
143	Propiconazole	0.01	0.9997	0.01–0.200
144	Propoxur	0.01	0.9998	0.01–0.200
145	Pymetrozine	0.01	0.9988	0.01–0.200
146	Pyracarbolid	0.01	0.9996	0.01–0.200
147	Pyraclostrobin	0.01	0.9995	0.01–0.200
148	Pyridaben	0.01	0.9999	0.01–0.200
149	Pyrimethanil	0.01	0.9998	0.01–0.200
150	Pyriproxyfen	0.01	0.9993	0.01–0.200
151	Quinoxyfen	0.01	0.9999	0.01–0.200
152	Rotenone	0.01	0.9998	0.01–0.200
153	Secbumeton	0.01	0.9998	0.01–0.200
154	Siduron	0.01	0.9998	0.01–0.200
155	Simetryn	0.05	0.9995	0.01–0.200
156	Spinetoram	0.01	0.9998	0.01–0.200
157	Spinosyn	0.05	0.9992	0.01–0.200
158	Spirodiclofen	0.01	0.9999	0.01–0.200
159	Spiromesifen	0.05	0.9962	0.01–0.200
160	Spirotetramat	0.01	0.9995	0.01–0.200
161	Tebuconazole	0.01	0.9997	0.01–0.200
162	Tebufenozide	0.01	0.9996	0.01–0.200
163	Tebufenpyrad	0.01	0.9999	0.01–0.200
164	Tebuthiuron	0.05	0.9985	0.01–0.200
165	Teflubenzuron	0.05	0.9926	0.01–0.200
166	Temephos	0.05	0.9977	0.01–0.200
167	Terbumeton	0.01	0.9998	0.01–0.200
168	Terbutryn	0.01	0.9996	0.01–0.200
169	Tetraconazole	0.05	0.9978	0.01–0.200

(con't)

TABLE 125. LOQ, coefficients of determination (R^2) and concentration ranges for analysed pesticides (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
170	Thiacloprid	0.01	0.9996	0.01–0.200
171	Thiamethoxam	0.05	0.9975	0.01–0.200
172	Thidiazuron	0.05	0.9999	0.01–0.200
173	Thiobencarb	0.01	0.9999	0.01–0.200
174	Thiofanox	0.05	0.9987	0.01–0.200
175	Thiophanate–methyl	0.05	0.9992	0.01–0.200
176	Triadimefon	0.01	0.9998	0.01–0.200
177	Triadimenol	0.01	0.9999	0.01–0.200
178	Trichlorfon	0.05	0.9991	0.01–0.200
179	Tricyclazole	0.01	0.9997	0.01–0.200
180	Trifloxystrobin	0.01	0.9993	0.01–0.200
181	Triflumizole	0.01	0.9996	0.01–0.200
182	Triflumuron	0.01	0.9997	0.01–0.200
183	Vamidothion	0.01	0.9996	0.01–0.200
184	Zoxamide	0.01	0.9995	0.01–0.200

17.6.7 Schematic diagram of extraction procedure and analysis

1. Weigh 10 g of homogenized/powdered sample into a clean 50 ml tube.
2. Add internal standard or spiking mix if required.
3. Add 10 g of cold water and shake briefly.
4. Add 10 ml acetonitrile.
5. Vortex or shake vigorously by hand for 1 min.
6. Add a buffered QuEChERS extraction packet.
7. Vortex or shake vigorously by hand for 1 min.
8. Centrifuge at 6 000 rpm for 10 min.
9. Transfer 6 ml of the acetonitrile phase (upper layer) into a 20 ml screw capped tube.
10. Place the tube containing the extract for at least 2 hrs in the freezer at (-80°C).
11. Transfer 1 ml of the supernatant to the required ready to use dSPE tube (2 ml capacity).
12. Vortex or shake vigorously by hand for 30 s.
13. Centrifuge at 6 000 rpm for 5 min.

Take 0.5 ml of the supernatant and add 0.5 ml of acetonitrile.

Acidify with formic acid 5% in acetonitrile (10 µl to every 1 ml).

Inject into the LC–MS/MS.

18

Detection and quantification of pesticide residues in edible oil by GC–MS/MS

18.1 Introduction

Pesticides are used widely to protect oil crops from pests/diseases and address challenges associated with weeds. Residues of these pesticides, mainly the lipophilics or persistent type can bioaccumulate in oilseed and can be a health risk to consumers. It is therefore important to test and regulate these chemicals.

18.2 Scope and objectives

This procedure applies to edible oil samples received by Contaminants Monitoring Division (CMD) which includes commodity group 4a: High oil content and very low water content. It describes the procedure for food sample preparation, extraction and cleanup of pesticides from food matrices, GC–MS/MS analytical conditions, standards and calibration levels preparation and identification and results reporting.

18.3 Principle of the method

A modified QuEChERS method where a homogenised sample is extracted using acetonitrile followed by reshaking and centrifugation is used. An aliquot of the organic phase is frozen and further centrifuged. An aliquot that is filtered and evaporated followed by reconstitution with toluene.

18.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, some pesticide standards are considered possible carcinogen to humans. Suitable gloves should be worn when chemicals/pesticides standards are handled, and the work should be performed in a fume hood.

18.5 Materials

18.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Acetonitrile (MeCN) (LC–MS grade); Water (LC–MS grade); Toluene (Pesticide grade).

18.5.1.1 Equipment/instruments and consumables

The following apparatus and material are applicable: Freezers (-20°C; -80°C); Nitrogen based evaporation system (TurboVap LV); Sample processing equipment (blender and grinder); Refrigerated centrifuges for 50 ml, 15 ml and 2 ml centrifuge tubes; Vortex and/or shaker; Automatic pipettes (e.g. for 20 µl–200 µl, 100 µl–1 000 µl and 1 ml–10 ml); Centrifuge tubes with screw caps (50 ml and 15 ml); Solvent-dispenser (for acetonitrile & toluene); Vials with caps for GC auto sampler (2 ml); Syringes (e.g. 2 ml disposable syringes); Syringes filters (0.45 µm pore size); QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate); Gas chromatograph-mass spectrometer (GC–MS/MS, Thermo Fisher Scientific).

18.5.2 Standards

18.5.2.1 Reference standards

All standards such as in Table 126 below should of high purity (≥98%).

TABLE 126. Pesticides used

GC-MSMS Pesticides							
1	2,4'-DDE	54	delta–BHC	107	Isopropalin	160	Pyrazophos
2	2,4'-DDT	55	Deltamethrin	108	Lenacil	161	Pyridaben
3	2,4'-Methoxychlor	56	Diallate	109	Leptophos	162	Pyridaphenthion
4	2-Phenylphenol	57	Diazinon	110	Linuron	163	Pyrimethanil
5	4,4'-DDD	58	Dichlofluanid	111	Malathion	164	Pyriproxyfen
6	4,4'-DDE	59	Diclobenil	112	Metalaxyl	165	Quinalphos
7	4,4'-DDT	60	Dicloran	113	Metazachlor	166	Quintozene
8	4,4'-Dichlorobenzo-phenone	61	Dieldrin	114	Methacrifos	167	Resmethrin
9	4,4'-Methoxychlor olefin	62	Dimethachlor	115	Methoxychlor	168	Sulfotep
10	Acequinocyl	63	Diphenamid	116	Methyl parathion	169	Sulprofos
11	Acetochlor	64	Diphenylamine	117	Metolachlor	170	tau–Fluvalinate
12	Acrinathrin	65	Disulfoton	118	Mevinphos	171	Tebuconazole
13	Alachlor	66	Edifenphos	119	MGK 264	172	Tebufenpyrad
14	Allidochlor	67	Endosulfan ether	120	Myclobutanil	173	Tecnazene
15	alpha–BHC	68	Endosulfan II	121	N–(2;4-Dimethyl-phenyl) formamide	174	Tefluthrin
16	Anthraquinone	69	Endosulfan sulfate	122	Nitralin	175	Terbacil
18	Azinphos–ethyl	71	Endrin aldehyde	124	Nonachlor, cis–	177	Terbutylazine
18	Azinphos–ethyl	71	Endrin aldehyde	124	Nonachlor, cis–	177	Terbutylazine

(con't)

TABLE 126. Pesticides used (con't)

GC-MSMS Pesticides							
19	Azinphos–methyl	72	Endrin ketone	125	Norflurazon	178	Tetrachloroaniline, 2,3,5,6-
20	Benfluralin	73	EPN	126	Oxadiazon	179	Tetrachlorvinphos
21	beta–BHC	74	Ethalfluralin	127	Oxyfluorfen	180	Tetradifon
22	Bifenthrin	75	Ethion	128	Paclobutrazol	181	Tetrahydrophthalimide
23	Biphenyl	76	Ethylan	129	Parathion	182	Tetramethrin
24	Bromfenvinphos	77	Etofenprox	130	Pebulate	183	Tolclofos–methyl
25	Bromfenvinphos–methyl	78	Etridazole	131	Penconazole	184	Transfluthrin
26	Bromophos–methyl	79	Fenamiphos	132	Pendimethalin	185	trans–Nonachlor
27	Bromophos–ethyl	80	Fenarimol	133	Pentachloroaniline	186	Triadimefon
28	Bromopropylate	81	Fenchlorphos	134	Pentachloroanisole	187	Triadimenol
29	Bupirimate	82	Fenitrothion	135	Pentachlorobenzene	188	Triallate
30	Carbophenothion	83	Fenpropathrin	136	Pentachlorobenzo-nitrile	189	Triazophos
31	Carfentrazone–ethyl	84	Fenson	137	Pentachloro-thioanisole	190	Tricyclazole
32	Chlorbenside	85	Fenthion	138	Permethrin, cis–	191	Triflumizole
33	Chlordane, trans–	86	Fenvalerate	139	Permethrin, trans–	192	Trifluralin
34	Chlorfenapyr	87	Fipronil	140	Phenothrin	193	Vinclozolin
35	Chlorfenson	88	Fluazifop–p–butyl	141	Phorate		
36	Chlorfenvinphos	89	Fluchloralin	142	Phosalone		
37	Chlorobenzilate	90	Flucythrinate	143	Phosmet		
38	Chloroneb	91	Fludioxonil	144	Piperonyl–butoxide		
39	Chlorothalonil	92	Fluquinconazole	145	Pirimiphos–ethyl		
40	Chlorpropham	93	Fluridone	146	Pirimiphos-methyl		
41	Chlorpyrifos	94	Flusilazole	147	Pretilachlor		
42	Chlorpyrifos–methyl	95	Flutolanil	148	Prochloraz		
43	Chlorthal–dimethyl	96	Flutriafol	149	Procymidone		
44	Chlorthiophos	97	Folpet	150	Prodiamine		
45	Chlozolinate	98	Fonofos	151	Profenofos		
46	cis–Chlordane	99	Gamma–BHC	152	Profluralin		
47	Clomazone	100	Heptachlor	153	Propachlor		
48	Coumaphos	101	Heptachlor epoxide	154	Propanil		
49	Cycloate	102	Hexazinone	155	Propargite		
50	Cyfluthrin	103	Iodofenfos	156	Propisochlor		
51	Cyhalothrin, lambda–	104	Iprodione	157	Propyzamide		
52	Cypermethrin	105	Isazophos	158	Prothiofos		
53	Cyprodinil	106	Isodrin	159	Pyraclofos		

18.5.2.2 Working solutions

a) Working solution 1 (W1, 10µg/ml)
   - This is prepared from stock standard solutions (100 µg/ml).
   - Take 100 µl from each mix and complete with Toluene so the final volume is 1 ml.
   - Use it to prepare working solution 2 (WS2) at 1 µg/ml and to spike GC–MS/MS recovery sample.

b) Working solution 2 (W2, 1 µg/l)
   - Prepare from WS1 by taking 100 and complete with Toluene to 1 ml.
   - Use it to prepare GC–MS/MS calibration levels.

18.5.2.3 Calibration standards

For quantitation, a matrix matched calibration curve is generated using known blank samples closely related to the tested samples. The blank sample is subjected to the same extraction and cleanup procedure mentioned above. This is to make sure that the matrix amount in each analyzed sample is constant. The final blank extract is used to prepare four calibration standards as below:

- Level 1 (0.1 µg /ml): Add 10 µl of WS1 + 990 µl final blank extract directly into an autosampler vial
- Level 2 (0.2 µg /ml): Add 20 µl of WS1 + 980 µl final blank extract directly into an autosampler vial
- Level 3 (0.5 µg /ml): Add 50 µl of WS1 + 950 µl final blank extract directly into an autosampler vial
- Level 4 (1 µg /ml): Add 100 µl of WS1 + 900 µl final blank extract directly into an autosampler vial.

The calibration curve should be valid until check standards deviate from the acceptable criteria (20% range of the corresponding calibration point value). If values deviate, then prepare new calibration curve and check again.

18.6 Procedure

18.6.1 Sample preparation

- Oil samples do not require preparation. Store the samples in a freezer (-20°C) until analysis.

18.6.2 Sample extraction

a) Weigh 5 g (±0.1 g) of oil sample into a clean 50 ml tube

b) Spike blank samples at a concentration of 0.05 mg/kg. Let stand for 10 min

c) Add 10 ml acetonitrile

d) Vortex for 1 min

e) Centrifuge for 5 min at 5°C and 6 000 rpm

f) Transfer an aliquot (5 ml) of the acetonitrile phase (upper layer) into a 15 ml screw capped tube

g) Place the tube containing the extract at -20°C or colder for at least 1 hr

h) Allow the extracts to become almost liquid, then cold centrifuge (5°C) for 5 min at 6 000 rpm.

18.6.3 Cleanup procedure

a) Take 2 ml of the extract and filter through 0.45 mm PTFE filter

b) Take 0.5 ml of the filtrate

c) Evaporate to almost dryness at 40°C using a gentle nitrogen flow (less than 5 bar)

d) Reconstitute in 0.5 ml of toluene and mix properly

e) Set sample amount at 0.5

f) Set dilution factor at 1

g) Sample is ready to be injected.

18.6.4 Instrumental analysis (GC–MS/MS)

The GC conditions are summarized in Table 127.

TABLE 127. GC conditions

Instrument	Trace 1310 Gas Chromatograph (ThermoFisher Scientific – USA)
Analytical Column	TG–5SILMS (30 m, 0.25 mm ID, 0.25 µm)
Liner	SSL Splitless Liner, Single Taper, 4 mm ID × 6.3 mm OD × 78.5 mm Length
Injection Temp.	250°C
Injection Volume	1 µl
Injection Type	Splitless with surge (Hold 1 min)
Carrier Gas	Helium
Flow Type	Constant flow
Flow rate	1.4 ml/min
Oven Temp.	90°C (hold 1 min) to 330°C at 8.5°C /min (hold 5 min)

The MS conditions are summarized in Table 128.

TABLE 128. MS conditions

Instrument	TSQ™ Duo Triple Quadrupole Mass Spectrometer (ThermoFisher Scientific - USA)
Mode	Timed SRM
Scan rate	12 scans/peak
Scan range	50 amu–550 amu
Transfer Line Temp.	290°C
Analyzer Type	Quadrupole
Source Temp.	325°C
Electron Energy	70 eV
Solvent Delay Time	5 min
Ionization Mode	EI

18.6.5 Calculation and interpretation of results

a) Identification

- The retention time of any analyte in the tested sample extract should correspond to that of the matrix matched calibration standard with a tolerance of ± 0.1 min.
- Extracted ion chromatograms of tested sample extracts should have peaks of a similar shape and response ratio to those obtained from matrix matched calibration standards analyzed at comparable concentrations.
- Chromatographic peaks from different selective ions for the analyte should fully overlap.
- The ion ratio should not deviate more than 30% (relative).

b) Reporting results

- The results should be reported and expressed in mg/kg.
- Where the residue definition includes more than one analyte, the respective sum of analytes should be calculated as stated in the residue definition and should be used for checking compliance with the MRL.
- Residues for individual analytes below the reporting limits should be reported as <RL mg/kg.
- Where good mixing of samples has been undertaken, the RSD of replicate results of the test portions should normally not exceed 30%.
- In general, residue data do not have to be adjusted for recovery when the mean recovery is within the range of 70%–120%.
- If residues data are adjusted for recovery, then this should be stated in the report.
- The result should be reported together with the expanded measurement uncertainty (MU) as follows: Result = x ± U (units), with x representing the measured value
- The sample is considered non–compliant if x-U > MRL.

18.6.6 Method validation

- Commodities used for fortification may include olive oil sample without pesticides analyzed. This product can be chosen to represent group 4a (High oil content and very low water content) commodities (EC, 2017).
- The validation method is performed at two spiking levels (0.01 mg/kg and 0.05 mg/kg).
- Five spiked samples and a blank are analyzed at each level. In total eleven samples are analyzed.
- Standard spiking solutions are prepared by mixing all the 193 pesticides used for the validation method at a concentration of 10 µg/ml in Toluene.
- The pesticides to be accepted as validated the following criteria for precision and trueness should be fulfilled (EC, 2017):
- The repeatability RSD should be ≤20%
- The average relative recovery should be between 70% and 120%
- If the above-mentioned criteria have been met the LOQ is stated.
- Linearity of the GC–MS/MS system is evaluated by assessing the signal responses of the target analytes from matrix matched calibration solutions prepared by

spiking blank extracts at six concentration levels corresponding to concentrations: 0.01 µg/ml, 0.02 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.15 µg/ml and 0.2 µg/ml.

- The calibration curves (six-point calibration points) should demonstrate excellent linearity ($R^2 \geq 0.99$) for all analytes in the scope of this study.
- The limit of quantitation is defined here as the lowest validated spike level meeting the method performance acceptability criteria (EC, 2017).

The validation test results are summarized in Table 129.

TABLE 129. Validation summary

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
1	2,4'-DDE	3.1	78.1	6.9	65.8	0.0007	0.01	0.9965	17.47
2	2,4'-DDT	4.3	79.1	4.9	61.1	0.0010	0.01	0.9974	19.16
3	2,4'-Methoxychlor	4.1	107.8	4.4	88.3	0.0013	0.01	0.9965	20.12
4	2-Phenylphenol	6.6	111.3	7.5	98.8	0.0022	0.01	0.9988	10.02
5	4,4'-DDD	4.4	98.5	4.3	80.6	0.0013	0.01	0.9976	19.08
6	**4,4'-DDE**	**2.7**	**67.7**	**6.1**	**55.6**	**0.0005**	**NA**	**0.9985**	**18.17**
7	4,4'-DDT	1.2	79.8	4.9	64.7	0.0003	0.01	0.9983	19.88
8	4,4'-Dichlorobenzophenone	4.5	115.0	4.6	79.4	0.0015	0.01	0.9988	16.12
9	4,4'-Methoxychlor olefin	4.7	112.4	4.9	87.0	0.0016	0.01	0.999	19.68
10	Acequinocyl	5.2	**157.9**	8.6	73.2	0.0094	0.05	0.9915	24.35
11	Acetochlor	7.8	99.5	8.5	97.7	0.0023	0.01	0.9976	15.20
12	Acrinathrin	6.1	**129.3**	5.9	108.3	0.0097	0.05	0.9959	22.31
13	Alachlor	7.4	119.6	8.6	98.9	0.0027	0.01	0.9985	15.11
14	Allidochlor	**A**	**A**	4.4	97.7	0.0064	0.05	0.9984	6.57
15	alpha–BHC	2.0	96.2	4.6	85.2	0.0006	0.01	0.9978	12.57
16	Anthraquinone	3.2	**128.4**	7.3	86.2	0.0095	0.05	0.9991	15.90
17	Atrazine	7.2	108.8	6.4	94.5	0.0023	0.01	0.9981	13.16
18	Azinphos–ethyl	4.2	**123.1**	4.6	102.8	0.0071	0.05	0.9946	22.47
19	Azinphos–methyl	7.5	115.9	5.0	105.3	0.0026	0.01	0.9955	21.75
20	Benfluralin	4.9	117.1	8.5	94.6	0.0017	0.01	0.9975	12.29
21	beta–BHC	5.8	95.7	5.9	86.6	0.0017	0.01	0.9983	13.39
22	Bifenthrin	4.0	96.3	6.3	83.8	0.0012	0.01	0.9984	20.97
23	Biphenyl	8.7	106.6	8.5	74.9	0.0028	0.01	0.998	8.03
24	Bromfenvinphos	5.2	117.2	3.2	101.3	0.0018	0.01	0.998	17.89
25	Bromfenvinphos–methyl	5.6	97.4	5.4	100.4	0.0016	0.01	0.9962	16.99
26	Bromophos methyl	1.9	111.9	3.2	86.7	0.0006	0.01	0.9966	16.47
27	Bromophos–ethyl	3.3	108.3	5.3	76.9	0.0011	0.01	0.9972	17.42
28	Bromopropylate	5.0	94.0	8.7	86.1	0.0014	0.01	0.9993	20.95
29	Bupirimate	5.6	113.9	6.2	100.0	0.0019	0.01	0.9981	18.45
30	Carbophenothion	5.3	111.8	3.6	87.7	0.0018	0.01	0.9976	19.63
31	Carfentrazone ethyl	7.4	105.9	7.6	100.7	0.0024	0.01	0.9986	19.67
32	Chlorbenside	4.1	101.1	4.9	72.8	0.0012	0.01	0.9986	17.23

(con't)

TABLE 129. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
33	Chlordane, trans–	19.0	85.5	7.2	71.8	0.0049	0.01	0.9984	17.37
34	Chlorfenapyr	13.0	113.1	16.8	105.1	0.0044	0.01	0.9906	18.72
35	Chlorfenson	4.2	106.2	4.7	92.8	0.0013	0.01	0.9979	17.84
36	Chlorfenvinphos 1	**A**	**A**	17.9	84.3	0.0226	0.05	0.9928	16.73
37	Chlorfenvinphos 2	5.2	98.2	3.6	102.2	0.0015	0.01	0.9965	16.99
38	Chlorobenzilate	7.2	81.1	8.5	89.7	0.0018	0.01	0.9968	18.86
39	Chloroneb	1.2	114.9	4.0	91.9	0.0004	0.01	0.9991	9.95
40	Chlorothalonil	8.4	**134.1**	11.4	98.8	0.0169	0.05	0.9991	14.10
41	Chlorpropham	8.1	112.9	9.2	93.0	0.0027	0.01	0.9982	11.88
42	Chlorpyrifos	5.3	110.8	3.8	87.9	0.0018	0.01	0.9966	16.06
43	Chlorpyrifos–methyl	5.5	120.1	7.2	94.3	0.0020	0.01	0.9986	14.94
44	Chlorthal–dimethyl	10.3	98.0	9.1	95.1	0.0030	0.01	0.9963	16.18
45	Chlorthiophos 1	**A**	**A**	14.1	101.1	0.0214	0.05	0.9963	18.79
46	Chlorthiophos 2	11.5	94.9	7.1	86.1	0.0033	0.01	0.9962	18.97
47	Chlorthiophos 3	3.1	96.1	4.4	86.2	0.0009	0.01	0.9973	19.24
48	Chlozolinate	12.2	96.9	7.0	99.2	0.0035	0.01	0.9976	16.89
49	cis–Chlordane	11.4	107.0	5.5	73.6	0.0037	0.01	0.996	17.71
50	Clomazone	6.7	111.6	7.3	101.2	0.0023	0.01	0.9974	13.22
51	Coumaphos	2.2	116.0	2.3	102.8	0.0008	0.01	0.9976	23.25
52	Cycloate	5.1	85.1	7.0	75.6	0.0013	0.01	0.9976	11.65
53	Cyfluthrin 1	7.2	109.0	5.7	99.7	0.0024	0.01	0.9956	23.66
54	Cyfluthrin 2	6.0	114.1	4.7	102.4	0.0021	0.01	0.9955	23.76
55	Cyfluthrin 3	5.9	107.1	5.5	101.7	0.0019	0.01	0.9964	23.86
56	Cyfluthrin 4	6.0	103.5	7.7	100.2	0.0019	0.01	0.9957	23.90
57	Cyhalothrin, lambda–	4.9	117.4	5.4	103.8	0.0017	0.01	0.9959	22.13
58	Cypermethrin 1	2.4	81.8	5.9	91.7	0.0006	0.01	0.9969	24.03
59	Cypermethrin 2	5.9	89.4	7.1	95.2	0.0016	0.01	0.9981	24.14
60	Cypermethrin 3	5.2	97.9	5.7	96.9	0.0015	0.01	0.999	24.23
61	Cypermethrin 4	9.6	110.7	7.3	94.9	0.0032	0.01	0.9998	24.27
62	Cyprodinil	7.6	90.5	8.4	78.7	0.0021	0.01	0.9971	16.65
63	Delta–BHC	7.9	94.3	7.5	87.9	0.0022	0.01	0.9982	13.99
64	Deltamethrin	6.4	98.1	7.0	98.5	0.0019	0.01	0.9968	25.93
65	Diallate 1	5.4	95.4	9.9	84.8	0.0016	0.01	0.9975	12.44
66	Diallate 2	8.2	105.0	8.4	87.6	0.0026	0.01	0.9949	12.65
67	Diazinon	5.2	109.7	5.5	95.8	0.0017	0.01	0.9974	13.81
68	Dichlofluanid	10.8	109.2	7.5	109.1	0.0035	0.01	0.9975	15.76
69	Diclobenil	5.8	100.3	6.3	87.6	0.0017	0.01	0.9994	7.50
70	Dicloran	5.3	**131.0**	10.7	97.2	0.0156	0.05	0.9986	12.87

(con't)

TABLE 129. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
71	Dieldrin	19.1	104.4	9.4	77.3	0.0060	0.01	0.9921	18.23
72	Dimethachlor	3.7	113.5	7.4	102.0	0.0013	0.01	0.9987	14.73
73	Diphenamid	6.8	107.6	9.5	100.2	0.0022	0.01	0.9981	16.48
74	Diphenylamine	4.9	104.9	7.7	91.3	0.0015	0.01	0.9987	11.49
75	Disulfoton	3.2	119.6	7.0	88.5	0.0012	0.01	0.9975	13.91
76	Edifenphos	2.9	**125.0**	3.6	100.2	0.0053	0.05	0.9962	19.74
77	Endosulfan ether	7.7	82.4	4.1	82.9	0.0019	0.01	0.9984	14.41
78	Endosulfan II	7.4	92.2	7.3	82.0	0.0020	0.01	0.9986	18.91
79	Endosulfan sulfate	6.1	118.4	4.5	99.7	0.0022	0.01	0.9983	19.83
80	Endrin	10.4	110.0	6.1	73.4	0.0034	0.01	0.999	18.71
81	Endrin aldehyde	12.9	115.1	8.8	86.3	0.0045	0.01	0.9974	19.32
82	Endrin ketone	9.6	**122.8**	6.6	92.8	0.0092	0.05	0.9995	20.84
83	EPN	8.0	**123.0**	6.6	97.2	0.0096	0.05	0.9966	20.98
84	Ethalfluralin	8.8	113.0	11.4	100.0	0.0030	0.01	0.9979	12.02
85	Ethion	4.0	117.5	4.6	99.4	0.0014	0.01	0.9974	19.16
86	Ethylan	5.5	94.4	4.6	85.0	0.0016	0.01	0.9948	18.69
87	Etofenprox	5.8	93.4	6.1	81.8	0.0016	0.01	0.9977	24.36
88	Etridazole	6.3	96.9	6.8	81.2	0.0018	0.01	0.9983	9.15
89	Fenamiphos	4.0	120.2	4.1	101.7	0.0014	0.01	0.9994	17.88
90	Fenarimol	6.3	102.2	4.8	90.2	0.0019	0.01	0.9975	22.33
91	Fenchlorphos	4.7	104.9	4.0	86.5	0.0015	0.01	0.9962	15.27
92	Fenitrothion	4.4	**124.9**	7.1	107.8	0.0115	0.05	0.9951	15.58
93	Fenpropathrin	10.3	106.1	7.3	95.0	0.0033	0.01	0.9959	21.12
94	Fenson	4.8	112.9	4.6	97.9	0.0016	0.01	0.9965	16.32
95	Fenthion	4.0	97.7	4.9	99.2	0.0012	0.01	0.9969	16.01
96	Fenvalerate 1	6.3	106.2	6.4	96.8	0.0020	0.01	0.9979	25.08
97	Fenvalerate 2	6.4	79.1	6.4	95.9	0.0015	0.01	0.9978	25.31
98	Fipronil	6.9	115.3	4.9	110.6	0.0024	0.01	0.999	17.01
99	Fluazifop–p–butyl	10.5	96.0	6.6	96.6	0.0030	0.01	0.998	18.66
100	Fluchloralin	7.8	**130.9**	7.0	98.7	0.0104	0.05	0.9976	13.91
101	Flucythrinate 1	6.5	115.6	5.9	106.5	0.0023	0.01	0.9971	24.26
102	Flucythrinate 2	6.1	115.9	6.3	108.7	0.0021	0.01	0.998	24.48
103	Fludioxonil	11.3	106.8	6.0	100.8	0.0036	0.01	0.9961	18.13
104	Fluquinconazole	6.6	107.4	5.2	98.9	0.0021	0.01	0.997	23.26
105	Fluridone	10.1	110.7	5.3	111.4	0.0033	0.01	0.9969	24.78
106	Flusilazole	4.1	103.6	3.9	102.8	0.0013	0.01	0.9987	18.39
107	Flutolanil	4.7	106.3	7.4	107.3	0.0015	0.01	0.9965	17.92

(con't)

TABLE 129. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
108	Flutriafol	5.5	114.4	7.0	103.3	0.0019	0.01	0.9968	17.76
109	Folpet	8.4	108.4	5.4	95.9	0.0027	0.01	0.9925	17.16
110	Fonofos	3.4	115.3	6.1	87.6	0.0012	0.01	0.9991	13.60
111	Gamma–BHC	4.7	100.1	4.4	88.1	0.0014	0.01	0.997	13.39
112	Heptachlor	4.4	77.9	5.1	66.6	0.0010	0.01	0.9977	15.11
113	Heptachlor epoxide	6.7	114.2	7.0	83.1	0.0023	0.01	0.9951	16.83
114	Hexazinone	5.6	**124.0**	6.6	107.1	0.0106	0.05	0.9974	20.11
115	Iodofenfos	5.4	109.8	3.8	81.8	0.0018	0.01	0.9958	17.96
116	Iprodione	14.5	87.0	9.6	101.5	0.0038	0.01	0.9931	20.74
117	Isazophos	7.9	98.5	12.7	112.7	0.0023	0.01	0.9969	14.11
118	Isodrin	12.8	**58.1**	10.6	52.1	0.0083	0.05	0.9987	16.58
119	Isopropalin	4.3	100.6	8.5	86.9	0.0013	0.01	0.9975	16.60
120	Lenacil	3.0	104.4	8.3	96.4	0.0009	0.01	0.9986	19.81
121	Leptophos	1.9	89.2	4.9	69.6	0.0005	0.01	0.998	21.80
122	Linuron	5.2	119.6	10.1	95.8	0.0019	0.01	0.9984	15.66
123	Malathion	4.0	118.2	3.4	106.5	0.0014	0.01	0.9974	15.81
124	Metalaxyl	7.3	113.2	6.8	103.3	0.0025	0.01	0.9981	15.23
125	Metazachlor	7.1	105.3	9.0	105.2	0.0022	0.01	0.9959	16.77
126	Methacrifos	2.1	109.9	4.5	102.0	0.0007	0.01	0.9987	9.86
127	Methoxychlor	5.6	103.4	6.2	93.3	0.0017	0.01	0.9968	21.10
128	Methyl parathion	3.6	**153.7**	7.2	106.1	0.0115	0.05	0.9988	14.93
129	Metolachlor	5.2	108.0	6.0	98.0	0.0017	0.01	0.9984	15.96
130	Mevinphos	4.7	**128.4**	5.4	109.0	0.0089	0.05	0.9996	8.78
131	MGK 264 1	15.6	106.0	8.8	94.8	0.0050	0.01	0.9971	16.42
132	MGK 264 2	11.9	**132.8**	9.5	101.0	0.0143	0.05	0.9973	16.68
133	Myclobutanil	6.3	111.7	7.4	103.8	0.0021	0.01	0.998	18.32
134	N– (2,4-Dimethylphenyl) formamide	**A**	**A**	10.3	106.3	0.0164	0.05	0.9994	9.51
135	Nitralin	9.7	**138.1**	9.6	102.6	0.0147	0.05	0.9966	20.54
136	Nitrofen	9.5	86.9	7.0	83.9	0.0025	0.01	0.9967	18.63
137	Nonachlor, cis–	9.3	82.5	8.2	67.1	0.0023	0.01	0.999	19.19
138	Norflurazon	7.0	119.5	7.4	105.8	0.0025	0.01	0.9982	19.80
139	Oxadiazon	7.3	90.1	7.7	97.5	0.0020	0.01	0.9974	18.23
140	Oxyfluorfen	10.9	102.8	8.7	95.7	0.0034	0.01	0.9965	18.35
141	Paclobutrazol	6.0	110.5	5.4	102.5	0.0020	0.01	0.9975	17.49
142	Parathion	3.6	118.7	6.3	101.2	0.0013	0.01	0.9981	16.08
143	Pebulate	3.1	87.3	7.1	79.1	0.0008	0.01	0.9991	9.19
144	Penconazole	4.8	105.3	5.5	94.7	0.0015	0.01	0.998	16.83

(con't)

TABLE 129. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
145	Pendimethalin	7.5	119.9	10.8	86.4	0.0027	0.01	0.9978	16.79
146	**Pentachloroaniline**	**7.9**	**66.1**	**9.4**	**59.7**	**0.0016**	**NA**	**0.9985**	**14.51**
147	**Pentachloroanisole**	**5.8**	**66.6**	**7.8**	**54.4**	**0.0012**	**NA**	**0.9978**	**12.88**
148	Pentachlorobenzene	3.5	74.8	5.5	47.3	0.0008	0.01	0.9986	10.15
149	Pentachlorobenzonitrile	9.7	84.0	11.4	77.4	0.0024	0.01	0.9956	13.56
150	**Pentachlorothioanisole**	**10.1**	**67.7**	**9.0**	**45.0**	**0.0020**	**NA**	**0.996**	**15.69**
151	Permethrin, cis–	4.6	85.8	6.8	76.7	0.0012	0.01	0.9963	22.98
152	Permethrin, trans–	5.5	89.0	5.9	81.2	0.0015	0.01	0.9977	23.12
153	Phenothrin 1	**A**	**A**	14.2	84.5	0.0180	0.05	0.99	21.43
154	Phenothrin 2	**22.9**	78.6	3.1	80.9	0.0038	0.05	0.9963	21.54
155	Phorate	5.6	119.4	5.4	92.5	0.0020	0.01	0.9976	12.45
156	Phosalone	5.8	120.8	4.8	102.2	0.0021	0.01	0.9977	21.73
157	Phosmet	4.9	**140.9**	4.2	106.6	0.0067	0.05	0.998	20.91
158	Piperonyl–butoxide	4.0	108.9	4.8	93.3	0.0013	0.01	0.9993	20.33
159	Pirimiphos–ethyl	6.5	116.2	4.6	93.0	0.0023	0.01	0.9972	16.55
160	Pirimiphos–methyl	3.7	101.2	5.2	94.0	0.0011	0.01	0.9977	15.61
161	Pretilachlor	9.3	115.9	6.3	98.9	0.0032	0.01	0.9959	18.13
162	Prochloraz	3.6	113.7	9.1	99.6	0.0012	0.01	0.9959	23.33
163	Procymidone	10.6	105.4	6.5	99.4	0.0033	0.01	0.9991	17.20
164	Prodiamine	14.4	114.2	12.2	94.4	0.0049	0.01	0.9982	15.62
165	Profenofos	9.1	116.5	5.0	91.3	0.0032	0.01	0.9978	18.07
166	Profluralin	6.7	**126.8**	10.9	92.0	0.0151	0.05	0.9996	13.59
167	Propachlor	7.9	117.7	6.9	102.3	0.0028	0.01	0.9977	11.37
168	Propanil	**A**	**A**	9.5	101.2	0.0145	0.05	0.9996	14.71
169	Propargite	5.5	114.2	8.4	99.2	0.0019	0.01	0.9971	20.24
170	Propisochlor	8.2	107.9	7.5	99.1	0.0026	0.01	0.9981	15.20
171	Propyzamide	6.6	**127.5**	8.3	98.7	0.0122	0.05	0.9974	13.59
172	Prothiofos	6.7	104.4	1.7	76.0	0.0021	0.01	0.9982	18.01
173	Pyraclofos	7.3	111.5	6.1	101.8	0.0024	0.01	0.9958	22.56
174	Pyrazophos	3.1	116.0	4.5	101.8	0.0011	0.01	0.998	22.41
175	Pyridaben	6.2	97.9	8.2	83.7	0.0018	0.01	0.9955	23.14
176	Pyridaphenthion	6.5	113.3	5.7	103.5	0.0022	0.01	0.9968	20.83
177	Pyrimethanil	5.6	109.8	8.1	82.9	0.0018	0.01	0.9979	13.72
178	Pyriproxyfen	6.5	94.4	7.1	85.5	0.0018	0.01	0.9975	21.81
179	Quinalphos	8.5	111.5	5.3	99.4	0.0028	0.01	0.9979	17.06
180	Quintozene	14.2	94.0	10.9	70.9	0.0040	0.01	0.9976	13.51
181	Resmethrin 1	16.2	92.8	9.9	86.2	0.0045	0.01	0.9966	20.26
182	Resmethrin 2	7.5	97.0	6.3	84.8	0.0022	0.01	0.998	20.38

(con't)

TABLE 129. Validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
183	Sulfotep	4.0	120.8	4.2	103.9	0.0014	0.01	0.9985	12.34
184	Sulprofos	3.3	105.0	4.1	88.2	0.0010	0.01	0.9958	19.42
185	tau–Fluvalinate 1	7.4	99.6	6.8	99.9	0.0022	0.01	0.9975	25.29
186	tau–Fluvalinate 2	6.8	102.4	5.6	99.7	0.0021	0.01	0.9972	25.35
187	Tebuconazole	5.8	107.0	6.5	98.8	0.0019	0.01	0.9983	20.16
188	Tebufenpyrad	4.9	92.2	9.1	84.2	0.0014	0.01	0.9981	21.20
189	Tecnazene	3.5	93.8	6.5	77.4	0.0010	0.01	0.9993	11.28
190	Tefluthrin	5.1	107.8	6.7	94.7	0.0017	0.01	0.9964	14.07
191	Terbacil	6.7	**135.6**	7.6	107.6	0.0123	0.05	0.9959	13.98
192	Terbufos	0.9	**124.0**	3.3	92.0	0.0045	0.05	0.997	13.50
193	Terbutylazine	1.9	109.6	3.5	92.5	0.0006	0.01	0.9972	13.51
194	Tetrachloroaniline, 2,3,5,6–	5.1	78.9	5.9	64.8	0.0012	0.01	0.9983	11.57
195	Tetrachlorvinphos	3.9	118.0	2.0	102.2	0.0014	0.01	0.9951	17.60
196	Tetradifon	5.2	95.2	3.7	81.4	0.0015	0.01	0.9977	21.55
197	Tetrahydrophthalimide	4.8	**134.5**	9.5	109.8	0.0156	0.05	0.9998	9.50
198	Tetramethrin 1	8.1	**132.9**	6.7	107.4	0.0109	0.05	0.9987	20.84
199	Tetramethrin 2	7.2	**126.1**	6.0	101.3	0.0091	0.05	0.9984	20.99
200	Tolclofos–methyl	2.8	109.6	2.7	91.5	0.0009	0.01	0.9986	15.03
201	Transfluthrin	5.6	118.3	6.8	93.1	0.0020	0.01	0.9977	15.02
202	trans–Nonachlor	22.9	71.4	8.3	64.0	0.0049	0.01	0.9967	17.81
203	Triadimefon	7.5	105.8	6.6	99.1	0.0024	0.01	0.999	16.13
204	Triadimenol	8.8	120.5	8.3	102.7	0.0032	0.01	0.9919	17.08
205	Triallate	6.5	83.1	5.8	73.0	0.0016	0.01	0.9984	14.12
206	Triazophos	7.1	102.0	7.2	100.2	0.0022	0.01	0.9968	19.45
207	Tricyclazole	**A**	**A**	10.9	91.9	0.0150	0.05	0.9945	18.14
208	Triflumizole	11.8	105.2	7.4	99.0	0.0037	0.01	0.997	17.26
209	Trifluralin	5.8	118.8	9.2	95.4	0.0021	0.01	0.9978	12.24
210	Vinclozolin	7.2	110.6	6.2	97.8	0.0024	0.01	0.9986	14.93

Bold numbers: Recovery and/or RSD out of acceptable range
A: Peak shape error or no peak was detected

18.6.7 Schematic diagram of extraction procedure

SAMPLE EXTRACTION	1. Weigh 5 g (±0.1 g) of oil sample into a clean 50 ml tube 2. Spike blank samples at concentration 0.05 mg/kg. Let stand for 10 min 3. Add 10 ml acetonitrile 4. Vortex for 1 min 5. Centrifuge for 5 min at 5°C and 6 000 rpm 6. Transfer an aliquot (5 ml) of the acetonitrile phase (upper layer) into a 15 ml screw capped tube 7. Place the tube containing the extract at -80°C at least for 1 hr 8. Allow the extracts to become almost liquid, then centrifuge for 5 min at 5°C and 6 000 rpm
↓	
SAMPLE CLEANUP	1. Take 2 ml of the extract and filter through 0.45 mm PTFE filter 2. Take 0.5 ml of the filtrate 3. Evaporate to almost dryness at 40°C using a slight nitrogen flow (less than 5 bar) 4. Reconstitute in 0.5 ml of toluene and mix properly 5. Sample is ready to be injected

19

Detection and quantification of pesticide residues in edible oil by LC–MS/MS

19.1 Introduction

Pesticides are used to protect oil crops from pests/diseases as well as weeds. These pesticides, especially the lipohilic or environmentally persistent ones can bioaccumulate in edible oil seeds and subsequently edible oil, thus presenting a risk to consumers. These should therefore be controlled through testing, monitoring and good practices.

19.2 Scope and objectives

This procedure applies to edible oil samples received by Contaminants Monitoring Division (CMD) which includes commodity group 4a: High oil content and very low water content. It describes the procedure for food sample preparation, extraction and cleanup of pesticides from food matrices, LC–MS/MS analytical conditions, standards and calibration levels preparation and identification and results reporting.

19.3 Principle of the method

Pesticides are extracted from oil seed using acetonitrile and concentrated through extraction using a centrifuge. Interfering material is removed, and a final extract dried and reconstituted in the mobile phase is analyzed by LC–MS/MS.

19.4 Safety considerations and precautions

The chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, some pesticide standards are considered a possible carcinogen to humans. Suitable gloves should be worn when chemicals/pesticides standards are handled, and the work should be performed in a fume hood.

19.5 Materials

19.5.1 Reagents and solvents

The following reagents and chemicals are applicable: acetonitrile (LC–MS grade); 5% formic acid solution in acetonitrile; Water (LC–MS grade); Methanol (LC–MS grade); Ammonium formate (LC–MS grade ≥99.0%) and formic acid LC–MS grade.

19.5.2 Equipment/instruments and consumables

The following apparatus and materials are applicable: Freezer (-20°C or below); Sample processing equipment (blender and grinder); Refrigerated centrifuges for 50 ml, 15 ml and 2 ml centrifuge tubes; Vortex and/or shaker; Ultrasonic water bath; Automatic pipettes (e.g. for 20 µl–200 µl, 100 µl–1 000 µl and 1 ml–10 ml); Centrifuge tubes with screw caps (50 ml and 15 ml); Solvent–dispenser (for acetonitrile and toluene); Vials with caps for LC auto sampler (2 ml); Syringes (e.g. 2 ml disposable syringes); Syringes filters (0.45 µm pore size); QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate); Liquid chromatograph–mass spectrometer (LC–MS/MS, Shimadzu).

19.5.3 Solutions

The solutions (and how they are prepared) include:

- Mobile Phase (A): 2 mmol/l ammonium formate + 0.002% formic acid – water
- Add 0.126 g of ammonium formate into 1 000 ml volumetric flask
- Add 500 ml of LC–MS grade water
- Add 20 µl formic acid
- Complete to 1 000 ml with LC–MS grade water
- Sonicate for 10 min
- Mobile Phase (B): 2 mmol/l ammonium formate + 0.002% formic acid – methanol
- Add 0.126 g of ammonium formate into 1 000 ml volumetric flask
- Add 500 ml of methanol
- Add 20 µl formic acid
- Complete to 1 000 ml with methanol
- Sonicate for 10 min
- 5% formic acid in acetonitrile
- Add 500 µl of formic acid in volumetric flask and complete to 10 ml with acetonitrile.

19.5.4 Standards

The reference standards and MRM conditions are summarized in Table 130.

TABLE 130. Pesticides, retention time and MRM transitions

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
1	(E) –Fenpyroximate	10.881	422.10>366.00	422.10>138.10	ESI +
2	Acephate	2.877	184.00>143.00	184.00>49.15	ESI +
3	Acetamiprid	5.509	223.10>126.10	223.10>55.95	ESI +
4	Acibenzolar–s–methyl	8.275	210.90>136.05	210.90>140.00	ESI +
5	Aldicarb	5.202	208.20>115.85	208.20>89.00	ESI +
6	Aldicarb–sulfone (Aldoxycarb)	4.005	240.10>86.20	240.10>148.15	ESI +
7	Aldicarb–sulfoxide	3.88	207.10>89.10	207.10>132.15	ESI +
8	Ametryn	6.763	228.10>68.00	228.10>96.00	ESI +
9	Aminocarb	5.364	209.00>122.10	209.00>152.05	ESI +
10	Azoxystrobin	8.966	404.00>371.95	404.00>328.95	ESI +
11	Benalaxyl	9.151	326.20>208.10	326.20>91.05	ESI +
12	Benzoximate	9.875	364.10>198.95	364.10>105.05	ESI +
13	Bifenazate	8.149	301.10>170.00	301.10>198.10	ESI +
14	Bitertanol	8.613	338.00>99.15	338.00>70.05	ESI +
15	Boscalid	7.73	343.00>271.95	343.00>270.95	ESI +
16	Bromuconazole	9.017	377.90>159.05	377.90>160.90	ESI +
17	Bupirimate	8.433	317.20>166.00	317.20>108.00	ESI +
18	Buprofezin	9.574	306.20>57.00	306.20>201.05	ESI +
19	Butafenacil	8.733	492.10>179.90	492.10>330.85	ESI +
20	Carbaryl (NAC)	6.006	202.10>145.10	202.10>127.00	ESI +
21	Carbendazim	4.85	192.10>160.15	192.10>132.15	ESI +
22	Carbetamide	5.168	237.10>192.10	237.10>118.15	ESI +
23	Carbofuran	5.948	222.10>165.00	222.10>123.15	ESI +
24	Carbofuran–3-hydroxy	4.744	255.00>163.15	255.00>220.05	ESI +
25	Carboxin	6.381	236.10>87.00	236.10>143.10	ESI +
26	Carfentrazone–ethyl	8.717	412.00>345.90	412.00>366.00	ESI +
27	Chlorantraniliprole	7.407	483.90>452.90	483.90>285.90	ESI +
28	Chlorfluazuron	10.162	539.90>382.85	539.90>158.00	ESI +
29	Chlorotoluron	5.854	213.10>72.20	213.10>46.10	ESI +
30	Chloroxuron	7.567	291.10>46.15	291.10>218.05	ESI +
31	Clethodim	9.199	360.10>164.15	360.10>166.05	ESI +
32	Clofentezine	9.483	303.00>138.15	303.00>102.10	ESI +
33	Clothianidin	4.551	250.00>132.05	250.00>169.10	ESI +
34	Cyazofamid	8.682	325.00>108.10	325.00>43.95	ESI +
35	Cycluron	6.356	199.20>46.20	199.20>88.95	ESI +
36	Cymoxanil	5.004	199.10>128.15	199.10>111.15	ESI +

(con't)

TABLE 130. Pesticides, retention time and MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
37	Cyproconazole	7.096	292.10>70.05	292.10>125.05	ESI +
38	Cyprodinil	8.379	226.10>93.00	226.10>108.00	ESI +
39	Cyromazine	2.918	167.20>68.00	167.20>59.90	ESI +
40	Desmedipham	6.727	318.00>136.10	318.00>301.00	ESI +
41	Diclobutrazol	7.569	328.00>70.10	330.00>70.10	ESI +
42	Dicrotophos	4.679	237.90>127.00	237.90>193.00	ESI +
43	Diethofencarb	6.908	268.20>124.15	268.20>152.00	ESI +
44	Difenoconazole	9.739	406.10>250.90	406.10>111.00	ESI +
45	Diflubenzuron	7.947	311.00>158.10	311.00>113.05	ESI +
46	Dimethoate	4.838	230.00>171.00	230.00>198.90	ESI +
47	Dimethomorph	8.662	388.10>165.10	388.10>301.00	ESI +
48	Dimoxystrobin	8.613	327.10>205.00	327.10>116.10	ESI +
49	Diniconazole	8.048	326.10>70.05	326.10>159.00	ESI +
50	Dinotefuran	3.776	203.15>114.15	203.15>87.00	ESI +
51	Dioxacarb	4.936	224.10>123.00	224.10>77.10	ESI +
52	Diuron (DCMU)	5.453	233.00>46.15	233.00>72.10	ESI +
53	Emamectin B1a	10.809	886.40>158.20	886.40>82.05	ESI +
54	Emamectin B1b	10.685	872.20>158.20	872.20>82.05	ESI +
55	Epoxiconazole	8.405	330.00>101.10	330.00>121.10	ESI +
56	Etaconazole	8.369	328.10>159.00	328.10>54.95	ESI +
57	Ethiofencarb	6.172	226.10>107.00	226.10>77.00	ESI +
58	Ethiprole	6.575	397.00>350.90	397.00>254.85	ESI +
59	Ethirimol	5.656	210.20>140.20	210.20>98.15	ESI +
60	Ethofumesate	7.576	304.10>121.10	304.10>240.95	ESI +
61	Etoxazole	10.193	360.10>113.05	360.10>141.10	ESI +
62	Famoxadone	9.345	392.20>331.05	392.20>237.95	ESI +
63	Fenamidone	7.629	312.10>65.00	312.10>236.00	ESI +
64	Fenarimol	7.891	331.00>268.00	331.00>139.10	ESI +
65	Fenazaquin	10.674	307.20>161.10	307.20>57.00	ESI +
66	Fenbuconazole	8.569	337.10>125.05	337.10>70.10	ESI +
67	Fenhexamid	7.405	302.10>97.10	302.10>55.05	ESI +
68	Fenobucarb	6.519	208.10>95.00	208.10>152.10	ESI +
69	Fenoxycarb	8.476	302.10>116.15	302.10>88.00	ESI +
70	Fenpropimorph	8.537	304.20>117.00	304.20>132.05	ESI +
71	Fenuron	4.655	165.00>46.10	165.00>72.15	ESI +
72	Fipronil	6.958	435.00>330.00	435.00>250.05	ESI -
73	Flonicamid	4.032	230.10>203.00	230.10>148.05	ESI +
74	Fluazinam	9.104	463.00>415.95	463.00>397.95	ESI -
75	Fludioxonil	6.623	247.00>180.15	247.00>126.15	ESI -
76	Flufenacet	8.063	364.10>124.05	364.10>194.00	ESI +
77	Flufenoxuron	9.758	489.00>158.10	489.00>140.90	ESI +

(con't)

TABLE 130. Pesticides, retention time and MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
78	Fluometuron	5.453	233.10>46.20	233.10>72.05	ESI +
79	Fluoxastrobin	9.55	458.80>188.10	458.80>111.00	ESI +
80	Fluquinconazole	8.608	376.00>349.00	376.00>307.00	ESI +
81	Flusilazole	8.354	316.10>165.10	316.10>247.00	ESI +
82	Flutolanil	7.33	324.10>261.90	324.10>242.00	ESI +
83	Flutriafol	6.433	302.10>70.05	302.10>123.00	ESI +
84	Forchlorfenuron	5.916	248.10>129.15	248.10>93.15	ESI +
85	Formetanate	4.144	222.20>165.20	222.20>93.10	ESI +
86	Furalaxyl	7.979	302.10>95.00	302.10>270.05	ESI +
87	Furathiocarb	10.147	383.20>195.00	383.20>167.05	ESI +
88	Halofenozide	6.796	331.10>105.20	331.10>275.00	ESI +
89	Hexaconazole	7.867	314.10>70.00	314.10>159.00	ESI +
90	Hexaflumuron	8.993	458.80>439.00	458.80>276.00	ESI -
91	Hexythiazox	10.38	353.10>228.00	353.10>168.00	ESI +
92	Hydramethylnon	9.11	495.10>151.00	495.10>323.00	ESI +
93	Imazalil	8.335	297.10>159.05	297.10>41.10	ESI +
94	Imidacloprid	5.171	256.10>209.00	256.10>174.95	ESI +
95	Indoxacarb	9.781	528.10>203.00	528.10>218.00	ESI +
96	Ipconazole	8.582	334.10>70.10	336.10>70.00	ESI +
97	Iprovalicarb	7.32	321.20>203.00	321.20>91.00	ESI +
98	Isoprocarb	5.989	194.10>95.00	194.10>77.00	ESI +
99	Isoproturon	6.131	207.20>46.10	207.20>165.00	ESI +
100	Kresoxim–methyl	9.044	314.10>267.00	314.10>235.00	ESI +
101	Linuron	6.503	248.80>160.00	248.80>182.05	ESI +
102	Mandipropamid	8.296	412.10>327.90	412.10>125.10	ESI +
103	Mefenacet	8.811	299.00>192.00	299.00>148.15	ESI +
104	Mepanipyrim	8.017	224.10>106.05	224.10>77.00	ESI +
105	Mepronil	7.582	270.20>228.00	270.20>91.05	ESI +
106	Mesotrione	4.588	357.00>227.90	357.00>104.00	ESI +
107	Metaflumizone	9.207	507.10>178.05	507.10>287.00	ESI +
108	Metalaxyl	7.093	280.10>192.05	280.10>220.00	ESI +
109	Metconazole	8.192	320.10>70.15	322.10>70.10	ESI +
110	Methabenzthiazuron	6.686	222.10>150.10	222.10>96.00	ESI +
111	Methamidophos	0.934	142.20>93.95	142.20>124.90	ESI +
112	Methiocarb	7.015	226.10>121.10	226.10>169.05	ESI +
113	Methomyl	4.42	163.00>87.90	163.00>58.00	ESI +
114	Methoprotryne	6.963	272.20>197.95	272.20>169.95	ESI +
115	Methoxyfenozide	7.806	369.20>312.95	369.20>149.15	ESI +
116	Metobromuron	6.032	259.00>170.00	259.00>148.10	ESI +
117	Metribuzin	5.765	215.10>187.10	215.10>49.10	ESI +
118	Mevinphos	4.902	225.10>127.00	225.10>109.00	ESI +

(con't)

TABLE 130. Pesticides, retention time and MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
119	Mexacarbate	6.992	223.10>151.15	223.10>136.15	ESI +
120	Monocrotophos	4.277	240.90>127.10	240.90>193.00	ESI +
121	Monolinuron	5.777	215.10>148.00	215.10>99.10	ESI +
122	Myclobutanil	7.659	289.10>70.05	289.10>125.00	ESI +
123	Nitenpyram	4.496	271.10>237.00	271.10>189.20	ESI +
124	Novaluron	8.575	493.00>158.00	493.00>141.05	ESI +
125	Nuarimol	7.233	315.10>251.95	315.10>207.00	ESI +
126	Omethoate	3.604	214.10>125.00	214.10>183.00	ESI +
127	Oxadixyl	6.573	296.20>219.05	296.20>279.05	ESI +
128	Oxamyl	4.367	237.10>72.10	237.10>90.00	ESI +
129	Paclobutrazol	6.803	294.10>70.10	294.10>125.05	ESI +
130	Penconazole	8.106	284.10>158.95	284.10>70.05	ESI +
131	Pencycuron	9.098	329.10>125.00	329.10>218.00	ESI +
132	Phenmedipham	6.748	318.10>168.10	318.10>300.90	ESI +
133	Picoxystrobin	8.823	368.00>145.10	368.00>205.00	ESI +
134	Piperonyl–butoxide	9.807	356.20>119.00	356.20>91.05	ESI +
135	Pirimicarb	6.715	239.20>72.00	239.20>182.05	ESI +
136	Prochloraz	9.477	376.00>307.95	376.00>70.00	ESI +
137	Promecarb	6.886	208.10>109.10	208.10>151.15	ESI +
138	Prometon	6.395	226.20>142.00	226.20>85.95	ESI +
139	Prometryn	7.337	242.10>158.00	242.10>200.15	ESI +
140	Propamocarb	4.019	189.20>102.15	189.20>144.20	ESI +
141	Propargite	10.244	368.20>231.10	368.20>175.10	ESI +
142	Propham	5.86	180.10>138.00	180.10>77.10	ESI +
143	Propiconazole	8.901	342.00>158.90	342.00>69.10	ESI +
144	Propoxur	5.684	209.90>93.10	209.90>168.15	ESI +
145	Pymetrozine	4.778	218.10>105.00	218.10>78.10	ESI +
146	Pyracarbolid	5.922	218.10>125.10	218.10>97.10	ESI +
147	Pyraclostrobin	9.801	388.00>194.10	388.00>164.05	ESI +
148	Pyridaben	10.982	365.20>147.10	365.20>117.00	ESI +
149	Pyrimethanil	7.047	200.10>107.00	200.10>82.00	ESI +
150	Pyriproxyfen	10.185	322.10>184.95	322.10>78.05	ESI +
151	Quinoxyfen	10.014	308.00>197.00	308.00>162.00	ESI +
152	Rotenone	10.602	395.10>213.00	395.10>192.00	ESI +
153	Secbumeton	6.4	226.20>57.00	226.20>100.15	ESI +
154	Siduron	6.584	233.20>137.10	233.20>77.00	ESI +
155	Simetryn	6.21	214.10>96.00	214.10>68.00	ESI +
156	Spinetoram	11.536	748.50>142.15	748.50>98.05	ESI +
157	Spinosyn	11.037	732.60>142.20	732.60>98.10	ESI +

(con't)

TABLE 130. Pesticides, retention time and MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
158	Spirodiclofen	10.458	411.10>313.05	411.10>71.10	ESI +
159	Spiromesifen	10.182	371.20>273.10	371.20>255.20	ESI +
160	Spirotetramat	8.528	374.10>330.05	374.10>302.00	ESI +
161	Spiroxamine Isomer	7.758	298.20>144.20	298.20>100.15	ESI +
162	Tebuconazole	7.888	308.20>70.05	308.20>125.05	ESI +
163	Tebufenozide	8.12	353.20>297.00	353.20>133.10	ESI +
164	Tebufenpyrad	9.195	334.20>145.15	334.20>147.20	ESI +
165	Tebuthiuron	5.661	229.10>116.00	229.10>172.00	ESI +
166	Temephos	10.93	467.00>418.80	467.00>125.00	ESI +
167	Terbumeton	6.672	225.90>170.00	225.90>114.10	ESI +
168	Terbutryn	7.337	242.10>68.00	242.10>157.95	ESI +
169	Tetraconazole	7.564	372.00>159.00	372.00>70.10	ESI +
170	Thiacloprid	6.06	253.00>126.05	253.00>90.10	ESI +
171	Thiamethoxam	4.72	292.00>211.10	292.00>181.10	ESI +
172	Thidiazuron	5.302	221.00>102.00	221.00>128.00	ESI +
173	Thiobencarb	8.895	257.80>125.10	257.80>89.00	ESI +
174	Thiofanox	6.149	241.20>184.00	241.20>57.20	ESI +
175	Thiophanate–methyl	6.232	343.00>311.00	343.00>93.00	ESI +
176	Triadimefon	7.483	294.10>69.00	294.10>196.95	ESI +
177	Triadimenol	6.874	296.10>70.05	298.10>70.05	ESI +
178	Trichlorfon	4.446	257.00>109.10	257.00>220.95	ESI +
179	Tricyclazole	6.904	190.10>136.00	190.10>109.00	ESI +
180	Trifloxystrobin	9.542	409.10>145.10	409.10>205.90	ESI +
181	Triflumizole	8.885	346.00>278.00	346.00>43.15	ESI +
182	Triflumuron	8.236	359.00>156.05	359.00>139.05	ESI +
183	Vamidothion	4.944	288.10>146.05	288.10>58.05	ESI +
184	Zoxamide	8.057	336.00>186.95	336.00>159.00	ESI +

19.5.5 Working solutions

a) **Working solution 1** (W1, 10 µg/ml)

- Prepared from all mixes of stock standard solution (100 µg/ml).
- Take 100 µl from each mix and complete with acetonitrile so the final volume is 1 ml.
- Use it to prepare working solution 2 (WS2) at 1 µg/ml and to spike LC–MS/MS recovery sample.

b) **Working solution 2** (W2, 1 µg/l)

- Prepared from WS1 by taking 100 µl and complete with acetonitrile to 1 ml.
- Use it to prepare LC–MS/MS calibration levels.

19.5.6 Calibration standards

For quantitation, a matrix matched calibration curve is generated using known appropriate blank samples. The blank sample is subjected to the same extraction and cleanup procedure mentioned above. This is to make sure that the matrix amount in each analyzed sample is constant. The final blank extract is used to prepare four calibration standards as below:

- Level 1 (0.1 µg /ml): Add 10 µl of WS1 + 990 µl final blank extract directly in an autosampler vial
- Level 2 (0.2 µg /ml): Add 20 µl of WS1 + 980 µl final blank extract directly in an autosampler vial
- Level 3 (0.5 µg /ml): Add 50 µl of WS1 + 950 µl final blank extract directly in an autosampler vial
- Level 4 (1 µg /ml): Add 100 µl of WS1 + 900 µl final blank extract directly in an autosampler vial

The calibration curve should be valid until check standards deviate from the acceptable criteria (20% range of the corresponding calibration point value). If values deviate, prepare new calibration curve and check again.

19.6 Procedure

19.6.1 Sample preparation

- Oil samples do not require preparation. Store the samples in a freezer (-20°C) until analysis.

19.6.2 Sample extraction

a) Weigh 5 g (±0.1 g) of oil sample into a clean 50 ml tube

b) Spike blank samples at concentration 0.05 mg/kg. Let stand for 10 min

c) Add 10 ml acetonitrile

d) Vortex for 1 min

e) Centrifuge for 5 min at 5°C and 6 000 rpm

f) Transfer an aliquot (5 ml) of the acetonitrile phase (upper layer) into a 15 ml screw capped tube

g) Place the tube containing the extract at -20°C or colder at least for 1 hr

h) Allow the extracts to become almost liquid, then centrifuge for 5 min at 5°C and 6 000 rpm

19.6.3 Cleanup procedure

a) Take 2 ml of the extract and filter through 0.45 mm PTFE filter material

b) Take 0.5 ml of the filtrate

c) Acidify with formic acid 5% in acetonitrile (10 µl to every 1 ml)

d) In lab solutions software: Set sample amount at 0.5

e) In lab solutions software: Set dilution factor at 1

f) The final extract has a concentration of ca. 0.5 g/ml

19.6.4 Instrumental analysis (LC–MS/MS)

The tool used is High–Speed Analysis Method (for 646 Residual Pesticide Components) – LC–MS/MS Method Package – Residual Pesticides Version 2–SHIMADZU. UHPLC, mobile phase and MS conditions are summarized in Tables 131–133.

TABLE 131. Summary of UHPLC conditions

Instrument	Nexera (2040c) LC system, Shimadzu
Analytical Column	Restek Raptor Biphenyl (100 × 2.1 mm, 2.7 µm) (Cat#: 9309A12)
Column Oven Temperature	35 °C
Injection Volume	2 µl
Flow rate	0.4 ml / min
Mobile phase A	2 mmol/l ammonium formate + 0.002 % formic acid - Water
Mobile phase B	2 mmol/l ammonium formate + 0.002 % formic acid - Methanol

TABLE 132. Mobile phase programme

Step	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
1	0.01	97	3
2	1	90	10
3	3	45	55
4	10.5	0	100
5	12	0	100
6	12.01	97	3
7	15	97	3
Stop	15.01	97	3

TABLE 133. MS conditions

Nebulizing Gas Flow Rate	3 l/min
Drying Gas Flow Rate	10 l/min
Heating Gas Flow Rate	10 l/min
Interface Temperature	350°C
Desolvation Line Temperature	150°C
Block Heater Temperature	300°C
Ionization mode	ESI

19.6.5 Calculation and interpretation of results

a) Identification

- The retention time of any analyte in the tested sample extract should correspond to that of the matrix matched calibration standard with a tolerance of ± 0.1 min.

- Extracted ion chromatograms of tested sample extracts should have peaks of similar shape and response ratio to those obtained from matrix matched calibration standards analyzed at comparable concentrations.
- Chromatographic peaks from different selective ions for the analyte should fully overlap.
- The ion ratio should not deviate more than 30% (relative).

b) Reporting results

- The results should always be reported and expressed in mg/kg.
- Where the residue definition includes more than one analyte, the respective sum of analytes should be calculated as stated in the residue definition and should be used for checking compliance with the MRL.
- Residues for individual analytes below the reporting limits should be reported as <MRL mg/kg.
- Where good mixing of samples has been undertaken, the RSD of replicate results of the test portions should normally not exceed 30%.
- Residues data do not have to be adjusted for recovery when the mean recovery is within the range of 70%–120%.
- If residues data are adjusted for recovery, then this should be stated in the report.
- The result should be reported together with the expanded measurement uncertainty (MU) as follows: Result = x ± U (units), with x representing the measured value
- The sample is considered non-compliant if x-U > MRL.

19.6.6 Method validation

- Commodities used for fortification may include olive oil sample without pesticides analyzed. This product can be chosen to represent group 4a (High oil content and very low water content) commodities (EC, 2017).
- The validation method is performed at two spiking levels (0.01 mg/kg and 0.05 mg/kg).
- Five spiked samples and a blank are analyzed at each level. In total eleven samples are analyzed.
- Standards spiking solutions are prepared by mixing all the 184 pesticides used for the validation method at a concentration of 10 μg/ml in Toluene.
- The pesticides to be accepted as validated the following criteria for precision and trueness should be fulfilled (EC, 2017):
  - The repeatability RSD should be ≤ 20%
  - The average relative recovery should be between 70% and 120%
- If the above-mentioned criteria have been met, the quantification limits, LOQs is stated.
- Linearity of the GC–MS/MS system is evaluated by assessing the signal responses of the target analytes from matrix matched calibration solutions prepared by spiking blank extracts at six concentration levels corresponding to concentrations: 0.01 μg/ml, 0.02 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.15 μg/ml and 0.2 μg/ml.
- The calibration curves (six–point calibration points) should demonstrate excellent linearity ($R^2 \geq 0.99$) for all analytes in the scope of this study.

- The limit of quantitation is defined (EC, 2017) as the lowest validated spike level meeting the method performance acceptability criteria.

The validation test results are summarized in Tables 134 and 135.

TABLE 134. Validation test results

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD%	Rec%	RSD%	Rec%	LOD
1	(E) –Fenpyroximate	5.2	76.0	3.4	82.8	0.0012
2	Acephate	12.9	98.2	4.1	101.2	0.0038
3	Acetamiprid	6.6	84.6	4.0	103.3	0.0017
4	Acibenzolar–s–methyl	13.9	**64.2**	5.9	77.8	0.0069
5	Aldicarb	18.8	74.8	9.9	102.1	0.0042
6	Aldicarb–sulfone	6.5	89.6	2.8	101.5	0.0018
7	Aldicarb–sulfoxide	18.3	95.5	4.0	101.7	0.0052
8	Ametryn	7.8	90.1	6.0	85.3	0.0021
9	Aminocarb	7.9	80.9	4.4	97.8	0.0019
10	Azoxystrobin	5.4	101.1	2.4	100.0	0.0016
11	Benalaxyl	10.2	84.7	3.7	96.9	0.0026
12	Benzoximate	17.5	67.2	4.1	90.4	0.0035
13	Bifenazate	5.2	102.5	4.3	92.6	0.0016
14	Bitertanol	8.2	88.0	4.3	94.5	0.0022
15	Boscalid	12.5	94.0	4.8	97.7	0.0035
16	Bromuconazole	18.6	**64.2**	4.4	92.2	0.0061
17	Bupirimate	5.3	73.9	4.5	94.7	0.0012
18	Buprofezin	14.2	**62.3**	3.8	84.4	0.0048
19	Butafenacil	9.2	89.4	5.6	95.9	0.0025
20	Carbaryl (NAC)	**23.0**	84.9	2.8	99.7	0.0043
21	Carbendazim	7.9	78.8	2.3	94.2	0.0019
22	Carbetamide	11.5	82.0	2.6	96.9	0.0028
23	Carbofuran	9.5	81.3	5.3	98.8	0.0023
24	Carbofuran–3-hydroxy	10.3	70.4	5.5	99.8	0.0022
25	Carboxin	18.8	**50.4**	5.7	98.3	0.0083
26	Carfentrazone–ethyl	**21.0**	**131.7**	12.2	103.5	0.0189
27	Chlorantraniliprole	16.8	90.8	2.8	98.3	0.0046
28	Chlorfluazuron	**72.4**	**23.4**	7.0	95.2	0.0100
29	Chlorotoluron	8.8	81.5	5.8	94.7	0.0021
30	Chloroxuron	14.0	81.7	8.5	93.2	0.0034
31	Clethodim	6.7	77.6	3.7	86.1	0.0016
32	Clofentezine	16.9	**63.1**	6.0	86.3	0.0077
33	Clothianidin	5.6	82.9	2.6	100.8	0.0014
34	Cyazofamid	7.2	83.2	3.1	98.9	0.0018
35	Cycluron	13.9	74.4	6.2	86.9	0.0031

(con't)

TABLE 134. Validation test results (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	**Compound**	**RSD%**	**Rec%**	**RSD%**	**Rec%**	**LOD**
36	Cymoxanil	**38.5**	77.8	7.8	100.1	0.0118
37	Cyproconazole	5.0	88.4	10.3	96.2	0.0013
38	Cyprodinil	13.3	**52.1**	4.3	77.0	0.0050
39	Cyromazine	14.7	103.1	7.6	91.3	0.0045
40	Desmedipham	17.8	81.6	2.4	102.5	0.0044
41	Diclobutrazol	18.4	**48.0**	5.0	90.6	0.0068
42	Dicrotophos	**31.6**	83.4	10.8	99.6	0.0161
43	Diethofencarb	13.5	70.2	6.1	94.8	0.0028
44	Difenoconazole	14.1	74.0	4.7	90.3	0.0031
45	Diflubenzuron	16.2	72.9	3.0	95.4	0.0035
46	Dimethoate	17.7	58.6	3.7	105.3	0.0059
47	Dimethomorph	6.7	91.7	5.7	95.3	0.0018
48	Dimoxystrobin	9.2	95.1	4.0	97.7	0.0026
49	Diniconazole	**23.3**	**55.1**	6.8	83.6	0.0085
50	Dinotefuran	12.7	70.0	4.6	100.2	0.0027
51	Dioxacarb	10.7	**58.2**	4.5	102.5	0.0070
52	Diuron (DCMU)	7.7	70.8	5.1	96.2	0.0016
53	Emamectin B1a	7.3	101.4	14.5	90.8	0.0022
54	Emamectin B1b	6.5	100.3	9.4	96.5	0.0019
55	Epoxiconazole	17.6	**59.5**	5.1	97.3	0.0074
56	Etaconazole	**23.0**	**57.0**	7.1	91.5	0.0097
57	Ethiofencarb	4.4	71.3	3.3	100.1	0.0009
58	Ethiprole	10.1	70.4	8.5	102.5	0.0021
59	Ethirimol	**31.9**	**126.3**	17.5	106.9	0.0280
60	Ethofumesate	**84.7**	86.6	7.8	103.8	0.0122
61	Etoxazole	8.6	**65.3**	3.1	83.3	0.0039
62	Famoxadone	12.1	91.4	4.5	93.6	0.0033
63	Fenamidone	16.6	89.1	3.5	97.5	0.0044
64	Fenarimol	17.7	78.8	6.3	84.0	0.0042
65	Fenazaquin	8.7	**48.1**	3.7	54.9	0.0030
66	Fenbuconazole	5.1	74.8	4.2	99.5	0.0011
67	Fenhexamid	8.8	85.0	7.3	84.3	0.0022
68	Fenobucarb	4.0	97.7	4.4	99.1	0.0012
69	Fenoxycarb	14.4	83.3	4.6	92.3	0.0036
70	Fenpropimorph	2.0	97.6	2.7	99.2	0.0006
71	Fenuron	18.5	86.0	7.8	89.2	0.0048
72	Fipronil	**23.9**	**66.5**	5.3	101.0	0.0080
73	Flonicamid	**27.8**	**48.6**	12.9	102.5	0.0198
74	Fluazinam	15.5	88.0	3.9	92.5	0.0041
75	Fludioxonil	12.3	76.1	4.8	96.2	0.0028

(con't)

TABLE 134. Validation test results (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD%	Rec%	RSD%	Rec%	LOD
76	Flufenacet	**23.3**	**54.5**	4.0	97.3	0.0058
77	Flufenoxuron	**38.3**	**48.5**	8.4	91.8	0.0116
78	Fluometuron	9.1	79.4	3.8	102.7	0.0022
79	Fluoxastrobin	17.6	82.6	5.3	102.2	0.0044
80	Fluquinconazole	7.6	71.9	5.1	92.0	0.0016
81	Flusilazole	8.9	74.6	4.4	100.7	0.0020
82	Flutolanil	13.6	84.4	3.3	107.4	0.0034
83	Flutriafol	15.3	71.1	7.4	98.8	0.0033
84	Forchlorfenuron	19.4	81.0	8.1	94.3	0.0047
85	Formetanate	9.4	108.7	4.5	91.9	0.0031
86	Furalaxyl	3.8	82.3	1.9	97.6	0.0009
87	Furathiocarb	6.1	80.5	1.7	93.0	0.0015
88	Halofenozide	17.4	**52.9**	7.3	100.3	0.0109
89	Hexaconazole	5.0	72.3	6.6	84.8	0.0011
90	Hexaflumuron	**101.5**	**29.9**	5.8	106.4	0.0092
91	Hexythiazox	9.7	73.2	3.4	76.0	0.0021
92	Hydramethylnon	11.0	97.8	2.3	100.5	0.0032
93	Imazalil	11.6	88.2	5.7	96.3	0.0031
94	Imidacloprid	**24.0**	**32.2**	6.1	107.3	0.0097
95	Indoxacarb	13.3	**64.8**	5.3	101.6	0.0081
96	Ipconazole	11.2	**60.1**	3.2	87.7	0.0042
97	Iprovalicarb	14.8	93.7	5.0	100.5	0.0042
98	Isoprocarb	12.3	87.5	3.8	97.8	0.0032
99	Isoproturon	11.7	89.9	4.8	95.8	0.0032
100	Kresoxim–methyl	10.2	75.9	5.9	98.7	0.0023
101	Linuron	8.8	78.5	4.3	92.1	0.0021
102	Mandipropamid	7.6	82.0	5.1	99.9	0.0019
103	Mefenacet	10.2	87.0	2.9	92.7	0.0027
104	Mepanipyrim	14.4	70.6	6.6	84.1	0.0031
105	Mepronil	13.9	70.9	5.4	96.1	0.0030
106	Mesotrione	14.1	**60.1**	5.6	100.7	0.0085
107	Metaflumizone	21.6	76.4	7.4	98.5	0.0050
108	Metalaxyl	9.3	90.6	3.3	95.9	0.0025
109	Metconazole	19.9	72.5	3.3	89.2	0.0043
110	Methabenzthiazuron	**23.0**	**49.4**	6.9	91.2	0.0094
111	Methamidophos	**A**	**A**	3.8	100.0	0.0057
112	Methiocarb	6.7	80.3	3.0	96.3	0.0016
113	Methomyl	5.6	84.3	3.8	93.3	0.0014
114	Methoprotryne	6.3	77.4	3.5	90.1	0.0015
115	Methoxyfenozide	8.5	88.8	6.2	98.1	0.0023

(con't)

TABLE 134. Validation test results (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD%	Rec%	RSD%	Rec%	LOD
116	Metobromuron	10.4	**66.3**	3.4	101.1	0.0052
117	Metribuzin	12.1	83.8	7.5	99.7	0.0030
118	Mevinphos	11.4	101.5	4.3	95.9	0.0035
119	Mexacarbate	3.0	73.3	3.5	96.1	0.0006
120	Monocrotophos	6.3	100.3	3.0	99.2	0.0019
121	Monolinuron	**28.3**	72.1	10.3	97.0	0.0150
122	Myclobutanil	**65.2**	**50.7**	5.0	99.9	0.0075
123	Nitenpyram	8.3	86.0	6.5	101.8	0.0022
124	Novaluron	**22.6**	**61.8**	7.9	96.9	0.0115
125	Nuarimol	9.5	83.8	4.7	88.8	0.0024
126	Omethoate	4.9	91.1	2.5	99.1	0.0014
127	Oxadixyl	14.7	90.6	6.5	98.7	0.0040
128	Oxamyl	14.0	98.4	3.1	101.9	0.0041
129	Paclobutrazol	10.0	87.1	2.5	96.4	0.0026
130	Penconazole	17.9	**54.4**	6.4	85.3	0.0082
131	Pencycuron	13.1	**55.6**	6.7	89.4	0.0090
132	Phenmedipham	6.0	81.9	3.6	98.2	0.0015
133	Picoxystrobin	4.0	92.6	1.6	97.8	0.0011
134	Piperonyl–butoxide	4.8	82.6	3.2	86.2	0.0012
135	Pirimicarb	6.7	94.5	3.0	92.6	0.0019
136	Prochloraz	4.3	80.9	2.3	91.7	0.0010
137	Promecarb	9.8	76.5	2.4	96.2	0.0023
138	Prometon	9.2	96.7	3.8	95.4	0.0027
139	Prometryn	8.8	79.7	4.1	88.9	0.0021
140	Propamocarb	4.0	**131.6**	2.6	96.3	0.0038
141	Propargite	5.2	71.7	3.8	89.9	0.0011
142	Propham	**23.2**	97.1	6.5	89.4	0.0087
143	Propiconazole	14.5	**67.0**	2.9	91.5	0.0040
144	Propoxur	6.2	84.6	8.8	104.7	0.0016
145	Pymetrozine	**24.4**	**55.7**	6.0	100.1	0.0090
146	Pyracarbolid	9.6	70.2	4.0	96.4	0.0020
147	Pyraclostrobin	11.0	81.6	4.1	93.3	0.0027
148	Pyridaben	7.0	82.1	1.8	74.2	0.0017
149	Pyrimethanil	8.3	76.2	2.4	80.2	0.0019
150	Pyriproxyfen	11.0	**52.1**	4.6	81.7	0.0057
151	Quinoxyfen	**20.6**	**46.4**	6.1	58.8	0.0054
152	Rotenone	6.9	88.4	4.4	96.4	0.0018
153	Secbumeton	9.7	87.9	4.3	93.4	0.0025

(con't)

TABLE 134. Validation test results (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD%	Rec%	RSD%	Rec%	LOD
154	Siduron	12.7	82.3	4.8	88.5	0.0031
155	Simetryn	9.3	102.4	2.1	88.2	0.0029
156	Spinetoram	17.1	93.1	17.3	92.1	0.0048
157	Spinosyn	6.9	105.2	16.4	113.1	0.0022
158	Spirodiclofen	6.6	74.1	5.6	85.6	0.0015
159	Spiromesifen	**51.7**	**18.9**	7.9	95.8	0.0114
160	Spirotetramat	9.8	96.2	4.0	93.8	0.0028
161	Spiroxamine Isomer	**57.3**	**41.5**	3.5	99.0	0.0052
162	Tebuconazole	12.9	76.7	3.1	89.7	0.0030
163	Tebufenozide	19.0	89.0	4.3	98.9	0.0051
164	Tebufenpyrad	15.8	**58.7**	7.4	77.0	0.0086
165	Tebuthiuron	7.6	92.4	10.5	105.2	0.0021
166	Temephos	20.1	77.4	6.5	94.5	0.0047
167	Terbumeton	6.4	84.8	2.3	96.4	0.0016
168	Terbutryn	**41.5**	100.1	4.4	85.0	0.0056
169	Tetraconazole	10.1	70.6	7.0	102.3	0.0021
170	Thiacloprid	6.6	71.0	3.6	101.9	0.0014
171	Thiamethoxam	**24.9**	**56.8**	6.1	108.1	0.0098
172	Thidiazuron	8.5	70.6	6.8	100.0	0.0018
173	Thiobencarb	7.3	**67.1**	3.3	80.2	0.0040
174	Thiofanox	**27.1**	**58.8**	9.7	104.1	0.0152
175	Thiophanate–methyl	**44.7**	**184.9**	13.1	107.4	0.0211
176	Triadimefon	4.6	86.8	4.1	97.9	0.0012
177	Triadimenol	9.2	80.3	2.8	96.3	0.0022
178	Trichlorfon	19.7	**214.2**	11.5	96.2	0.0166
179	Tricyclazole	16.4	71.1	5.6	94.8	0.0035
180	Trifloxystrobin	11.5	73.8	4.0	100.0	0.0026
181	Triflumizole	4.9	**67.3**	2.0	90.3	0.0027
182	Triflumuron	11.9	78.7	5.5	93.9	0.0028
183	Vamidothion	3.4	95.3	2.3	97.1	0.0010
184	Zoxamide	4.4	**56.8**	3.1	94.9	0.0044

Bold numbers: Recovery and/or RSD out of acceptable range
A: Peak shape error or no peak was detected

Additional validation test results are shown in Table 135.

TABLE 135. Additional validation test results

No.	Compound	LOQ	R^2	Instrumental concentration range
1	(E)–Fenpyroximate	0.01	0.9997	0.01–0.200
2	Acephate	0.01	0.9997	0.01–0.200
3	Acetamiprid	0.01	0.9997	0.01–0.200
4	Acibenzolar–s–methyl	0.05	0.9991	0.01–0.200
5	Aldicarb	0.01	0.9975	0.01–0.200
6	Aldicarb–sulfone	0.01	0.9996	0.01–0.200
7	Aldicarb–sulfoxide	0.01	0.9996	0.01–0.200
8	Ametryn	0.01	0.9992	0.01–0.200
9	Aminocarb	0.01	0.9991	0.01–0.200
10	Azoxystrobin	0.01	0.9997	0.01–0.200
11	Benalaxyl	0.01	0.9983	0.01–0.200
12	Benzoximate	0.01	0.9989	0.01–0.200
13	Bifenazate	0.01	0.9993	0.01–0.200
14	Bitertanol	0.01	0.9994	0.01–0.200
15	Boscalid	0.01	0.9989	0.01–0.200
16	Bromuconazole	0.05	0.9993	0.01–0.200
17	Bupirimate	0.01	0.9997	0.01–0.200
18	Buprofezin	0.05	0.9994	0.01–0.200
19	Butafenacil	0.01	0.9994	0.01–0.200
20	Carbaryl (NAC)	0.05	0.9997	0.01–0.200
21	Carbendazim	0.01	0.9997	0.01–0.200
22	Carbetamide	0.01	0.9997	0.01–0.200
23	Carbofuran	0.01	0.9996	0.01–0.200
24	Carbofuran–3-hydroxy	0.01	0.9990	0.01–0.200
25	Carboxin	0.05	0.9956	0.01–0.200
26	Carfentrazone–ethyl	0.05	0.9909	0.01–0.200
27	Chlorantraniliprole	0.01	0.9995	0.01–0.200
28	Chlorfluazuron	0.05	0.9927	0.01–0.200
29	Chlorotoluron	0.01	0.9995	0.01–0.200
30	Chloroxuron	0.01	0.9990	0.01–0.200
31	Clethodim	0.01	0.9994	0.01–0.200
32	Clofentezine	0.05	0.9985	0.01–0.200
33	Clothianidin	0.01	0.9997	0.01–0.200
34	Cyazofamid	0.01	0.9997	0.01–0.200
35	Cycluron	0.01	0.9995	0.01–0.200
36	Cymoxanil	0.05	0.9999	0.01–0.200
37	Cyproconazole	0.01	0.9942	0.01–0.200
38	Cyprodinil	0.05	0.9993	0.01–0.200
39	Cyromazine	0.01	0.9996	0.01–0.200

(con't)

TABLE 135. Additional validation test results (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
40	Desmedipham	0.01	0.9995	0.01–0.200
41	Diclobutrazol	0.05	0.9981	0.01–0.200
42	Dicrotophos	0.05	0.9984	0.01–0.200
43	Diethofencarb	0.01	0.9986	0.01–0.200
44	Difenoconazole	0.01	0.9992	0.01–0.200
45	Diflubenzuron	0.01	0.9997	0.01–0.200
46	Dimethoate	0.05	0.9986	0.01–0.200
47	Dimethomorph	0.01	0.9991	0.01–0.200
48	Dimoxystrobin	0.01	0.9997	0.01–0.200
49	Diniconazole	0.05	0.9997	0.01–0.200
50	Dinotefuran	0.01	0.9987	0.01–0.200
51	Dioxacarb	0.05	0.9996	0.01–0.200
52	Diuron (DCMU)	0.01	0.9931	0.01–0.200
53	Emamectin B1a	0.01	0.9995	0.01–0.200
54	Emamectin B1b	0.01	0.9992	0.01–0.200
55	Epoxiconazole	0.05	0.9990	0.01–0.200
56	Etaconazole	0.05	0.9984	0.01–0.200
57	Ethiofencarb	0.01	0.9989	0.01–0.200
58	Ethiprole	0.01	0.9986	0.01–0.200
59	Ethirimol	0.05	0.9996	0.01–0.200
60	Ethofumesate	0.05	0.9977	0.01–0.200
61	Etoxazole	0.05	0.9993	0.01–0.200
62	Famoxadone	0.01	0.9984	0.01–0.200
63	Fenamidone	0.01	0.9988	0.01–0.200
64	Fenarimol	0.01	0.9997	0.01–0.200
65	Fenazaquin	0.05	0.9995	0.01–0.200
66	Fenbuconazole	0.01	0.9986	0.01–0.200
67	Fenhexamid	0.01	0.9993	0.01–0.200
68	Fenobucarb	0.01	0.9996	0.01–0.200
69	Fenoxycarb	0.01	0.9992	0.01–0.200
70	Fenpropimorph	0.01	0.9998	0.01–0.200
71	Fenuron	0.01	0.9986	0.01–0.200
72	Fipronil	0.05	0.9993	0.01–0.200
73	Flonicamid	0.05	0.9970	0.01–0.200
74	Fluazinam	0.01	0.9997	0.01–0.200
75	Fludioxonil	0.01	0.9988	0.01–0.200
76	Flufenacet	0.05	0.9978	0.01–0.200
77	Flufenoxuron	0.05	0.9967	0.01–0.200
78	Fluometuron	0.01	0.9992	0.01–0.200

(con't)

TABLE 135. Additional validation test results (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
79	Fluoxastrobin	0.01	0.9986	0.01–0.200
80	Fluquinconazole	0.01	0.9992	0.01–0.200
81	Flusilazole	0.01	0.9990	0.01–0.200
82	Flutolanil	0.01	0.9989	0.01–0.200
83	Flutriafol	0.01	0.9993	0.01–0.200
84	Forchlorfenuron	0.01	0.9998	0.01–0.200
85	Formetanate	0.01	0.9993	0.01–0.200
86	Furalaxyl	0.01	0.9991	0.01–0.200
87	Furathiocarb	0.01	0.9997	0.01–0.200
88	Halofenozide	0.05	0.9952	0.01–0.200
89	Hexaconazole	0.01	0.9990	0.01–0.200
90	Hexaflumuron	0.05	0.9954	0.01–0.200
91	Hexythiazox	0.01	0.9998	0.01–0.200
92	Hydramethylnon	0.01	0.9993	0.01–0.200
93	Imazalil	0.01	0.9998	0.01–0.200
94	Imidacloprid	0.05	0.9956	0.01–0.200
95	Indoxacarb	0.05	0.9984	0.01–0.200
96	Ipconazole	0.05	0.9987	0.01–0.200
97	Iprovalicarb	0.01	0.9998	0.01–0.200
98	Isoprocarb	0.01	0.9998	0.01–0.200
99	Isoproturon	0.01	0.9990	0.01–0.200
100	Kresoxim–methyl	0.01	0.9991	0.01–0.200
101	Linuron	0.01	0.9997	0.01–0.200
102	Mandipropamid	0.01	0.9995	0.01–0.200
103	Mefenacet	0.01	0.9992	0.01–0.200
104	Mepanipyrim	0.01	0.9992	0.01–0.200
105	Mepronil	0.01	0.9990	0.01–0.200
106	Mesotrione	0.05	0.9993	0.01–0.200
107	Metaflumizone	0.01	0.9991	0.01–0.200
108	Metalaxyl	0.01	0.9993	0.01–0.200
109	Metconazole	0.01	0.9996	0.01–0.200
110	Methabenzthiazuron	0.05	0.9985	0.01–0.200
111	Methamidophos	0.05	0.9996	0.01–0.200
112	Methiocarb	0.01	0.9991	0.01–0.200
113	Methomyl	0.01	0.9998	0.01–0.200
114	Methoprotryne	0.01	0.9997	0.01–0.200
115	Methoxyfenozide	0.01	0.9995	0.01–0.200
116	Metobromuron	0.05	0.9993	0.01–0.200
117	Metribuzin	0.01	0.9998	0.01–0.200
118	Mevinphos	0.01	0.9996	0.01–0.200
119	Mexacarbate	0.01	0.9996	0.01–0.200

(con't)

TABLE 135. Additional validation test results (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
120	Monocrotophos	0.01	0.9998	0.01–0.200
121	Monolinuron	0.05	0.9989	0.01–0.200
122	Myclobutanil	0.05	0.9989	0.01–0.200
123	Nitenpyram	0.01	0.9992	0.01–0.200
124	Novaluron	0.01	0.9927	0.01–0.200
125	Nuarimol	0.01	0.9992	0.01–0.200
126	Omethoate	0.01	0.9998	0.01–0.200
127	Oxadixyl	0.01	0.9997	0.01–0.200
128	Oxamyl	0.01	0.9999	0.01–0.200
129	Paclobutrazol	0.01	0.9997	0.01–0.200
130	Penconazole	0.05	0.9984	0.01–0.200
131	Pencycuron	0.05	0.9982	0.01–0.200
132	Phenmedipham	0.01	0.9984	0.01–0.200
133	Picoxystrobin	0.01	0.9997	0.01–0.200
134	Piperonyl–butoxide	0.01	0.9996	0.01–0.200
135	Pirimicarb	0.01	0.9997	0.01–0.200
136	Prochloraz	0.01	0.9996	0.01–0.200
137	Promecarb	0.01	0.9996	0.01–0.200
138	Prometon	0.01	0.9992	0.01–0.200
139	Prometryn	0.01	0.9998	0.01–0.200
140	Propamocarb	0.05	0.9994	0.01–0.200
141	Propargite	0.01	0.9991	0.01–0.200
142	Propham	0.05	0.9994	0.01–0.200
143	Propiconazole	0.05	0.9989	0.01–0.200
144	Propoxur	0.01	0.9995	0.01–0.200
145	Pymetrozine	0.05	0.9984	0.01–0.200
146	Pyracarbolid	0.01	0.9996	0.01–0.200
147	Pyraclostrobin	0.01	0.9994	0.01–0.200
148	Pyridaben	0.01	0.9996	0.01–0.200
149	Pyrimethanil	0.01	0.9995	0.01–0.200
150	Pyriproxyfen	0.05	0.9976	0.01–0.200
151	Quinoxyfen	0.05	0.9995	0.01–0.200
152	Rotenone	0.01	0.9997	0.01–0.200
153	Secbumeton	0.01	0.9934	0.01–0.200
154	Siduron	0.01	0.9997	0.01–0.200
155	Simetryn	0.01	0.9992	0.01–0.200
156	Spinetoram	0.01	0.9998	0.01–0.200
157	Spinosyn	0.01	0.9993	0.01–0.200
158	Spirodiclofen	0.01	0.9986	0.01–0.200
159	Spiromesifen	0.05	0.9930	0.01–0.200
160	Spirotetramat	0.01	0.9992	0.01–0.200

(con't)

TABLE 135. Additional validation test results (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
161	Spiroxamine Isomer	0.05	0.9992	0.01–0.200
162	Tebuconazole	0.01	0.9999	0.01–0.200
163	Tebufenozide	0.01	0.9993	0.01–0.200
164	Tebufenpyrad	0.05	0.9973	0.01–0.200
165	Tebuthiuron	0.01	0.9996	0.01–0.200
166	Temephos	0.01	0.9991	0.01–0.200
167	Terbumeton	0.01	0.9996	0.01–0.200
168	Terbutryn	0.05	0.9520	0.01–0.200
169	Tetraconazole	0.01	0.9983	0.01–0.200
170	Thiacloprid	0.01	0.9993	0.01–0.200
171	Thiamethoxam	0.05	0.9992	0.01–0.200
172	Thidiazuron	0.01	0.9995	0.01–0.200
173	Thiobencarb	0.05	0.9995	0.01–0.200
174	Thiofanox	0.05	0.9966	0.01–0.200
175	Thiophanate–methyl	0.05	0.9986	0.01–0.200
176	Triadimefon	0.01	0.9996	0.01–0.200
177	Triadimenol	0.01	0.9994	0.01–0.200
178	Trichlorfon	0.05	0.9974	0.01–0.200
179	Tricyclazole	0.01	0.9995	0.01–0.200
180	Trifloxystrobin	0.01	0.9988	0.01–0.200
181	Triflumizole	0.05	0.9998	0.01–0.200
182	Triflumuron	0.01	0.9998	0.01–0.200
183	Vamidothion	0.01	0.9997	0.01–0.200
184	Zoxamide	0.05	0.9984	0.01–0.200

19.6.7 Schematic diagram of extraction procedure

1. Weigh 5 g (±0.1 g) of oil sample into a clean 50 ml tube
2. Spike blank samples at concentration of 0.05 mg/kg. Let this stand for 10 min
3. Add 10 ml acetonitrile
4. Vortex for 1 min
5. Centrifuge for 5 min at 5°C and 6 000 rpm
6. Transfer an aliquot (5 ml) of the acetonitrile phase (upper layer) into a 15 ml screw capped tube
7. Place the tube containing the extract at -80°C at least for 1 hr
8. Allow the extracts to become almost liquid, then centrifuge for 5 min at 5°C and 6 000 rpm

1. Take 2 ml of the extract and filter through a 0.45 mm PTFE filter.
2. Take 0.5 ml of the filtrate and add 0.5 ml of acetonitrile.
3. Acidify with formic acid 5% in acetonitrile (10 µl to every 1 ml).
4. Inject the sample into the LC–MS/MS

20

Detection and quantification of pesticide residues in fruits and vegetables by GC–MS/MS

20.1 Introduction

Pesticides in both crop and animal production to crop diseases and pests but their residues may end up in food for human consumption. This has public health and trade implication and should be controlled. Cost effective sample preparation techniques followed by confirmatory analysis are critical to control laboratories and programmes.

20.2 Scope and objectives

This procedure applies to food samples received by Contaminants Monitoring Division (CMD) that includes commodity group 1(High water content) and commodity group 2 (High acid content and high-water content) (CEN, 2008). It describes the procedure for food samples preparation, extraction and cleanup of pesticides from food matrices, GC–MS/MS analytical conditions, standards and calibration levels preparation and identification and results reporting.

20.3 Principle of the method

A modified QuEChERS method where a homogenised sample is extracted using acetonitrile and mixture of salts followed by shaking and centrifugation is used. An aliquot of the organic phase is cleanedup using anhydrous $MgSO_4$ and PSA, shaken on a vortex mixer followed by dilution with acetonitrile.

20.4 Safety considerations and precautions

The chemicals use in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, some pesticides standards are considered a possible carcinogen to humans. Suitable gloves should be worn when chemicals/pesticides standards are handled, and the work should be performed in a fume hood.

20.5 Materials

20.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Distilled water; Acetonitrile (LC–MS grade); Toluene (HPLC grade) and Formic acid.

20.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Freezers (-20°C and -80°C); Nitrogen based evaporation system (TurboVap LV); Sample processing equipment (Blender and grinder); Refrigerated centrifuges for 50 ml, 15 ml and 2 ml centrifuge tubes; Vortex and/or shaker; Automatic pipettes (e.g. for 20 µl–200 µl, 100 µl –1 000 µl and 1 ml – 10 ml); Centrifuge tubes with screw caps (50 ml and 15 ml); Solvent-dispenser (for acetonitrile & toluene); Vials with caps for GC auto sampler (2 ml); Syringes (e.g. 2 ml disposable syringes); Syringes filters (0.45 µm pore size); QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate); dSPE tubes (150 mg $MgSO_4$, 25 mg PSA) (150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB); GC–MS/MS (Thermo Fisher Scientific).

20.5.3 Standards

The reference standards (>98% purity) to use are listed in Table 136.

TABLE 136. Standards for GC–MS/MS analyses

1	2,4'-DDE	55	Delta–BHC	109	Isazophos	163	Prothiofos
2	2,4'-DDT	56	Deltamethrin	110	Isodrin	164	Pyraclofos
3	2,4'-Methoxychlor	57	Diallate	111	Isopropalin	165	Pyrazophos
4	2-Phenylphenol	58	Diazinon	112	Lenacil	166	Pyridaben
5	4,4'-DDD	59	Dichlofluanid	113	Leptophos	167	Pyridaphenthion
6	4,4-DDE	60	Dichloroaniline, 3,4'-	114	Linuron	168	Pyrimethanil
7	4,4'-DDT	61	Diclobenil	115	Malathion	169	Pyriproxyfen
8	4,4'-Dichlorobenzo-phenone	62	Dicloran	116	Metalaxyl	170	Quinalphos
9	4,4'-Methoxychlor olefin	63	Dieldrin	117	Metazachlor	171	Quintozene
10	Acequinocyl	64	Dimethachlor	118	Methacrifos	172	Resmethrin
11	Acetochlor	65	Diphenamid	119	Methoxychlor	173	Sulfotep
12	Acrinathrin	66	Diphenylamine	120	Methyl parathion	174	Sulprofos
13	Alachlor	67	Disulfoton	121	Metolachlor	175	tau-Fluvalinate
14	Aldrin	68	Edifenphos	122	Mevinphos	176	Tebuconazole
15	Allidochlor	69	Endosulfan ether	123	MGK 264	177	Tebufenpyrad
16	Alpha–BHC	70	Endosulfan I	124	Mirex	178	Tecnazene
17	Anthraquinone	71	Endosulfan II	125	Myclobutanil	179	Tefluthrin
18	Atrazine	72	Endosulfan sulfate	126	N-(2,4-Dimethyl-phenyl) formamide	180	Terbacil
19	Azinphos–ethyl	73	Endrin	127	Nitralin	181	Terbufos
20	Azinphos–methyl	74	Endrin aldehyde	128	Nitrofen	182	Terbutylazine

(con't)

TABLE 136. Standards for GC–MS/MS analyses (con't)

21	Benfluralin	75	Endrin ketone	129	Nonachlor, cis–	183	Tetrachloroaniline, 2,3,5,6-
22	Beta–BHC	76	EPN	130	Norflurazon	184	Tetrachlorvinphos
23	Bifenthrin	77	Ethalfluralin	131	Oxadiazon	185	Tetradifon
24	Biphenyl	78	Ethion	132	Oxyfluorfen	186	Tetrahydrophthalimide
25	Bromfenvinphos	79	Ethylan	133	Paclobutrazol	187	Tetramethrin
26	Bromfenvinphos–methyl	80	Etofenprox	134	Parathion	188	Tolclofos-methyl
27	Bromophos–methyl	81	Etridazole	135	Pebulate	189	Tolylfluanid
28	Bromophos–ethyl	82	Fenamiphos	136	Penconazole	190	Transfluthrin
29	Bromopropylate	83	Fenarimol	137	Pendimethalin	191	trans–Nonachlor
30	Bupirimate	84	Fenchlorphos	138	Pentachloroaniline	192	Triadimefon
31	Carbophenothion	85	Fenitrothion	139	Pentachloroanisole	193	Triadimenol
32	Carfentrazone ethyl	86	Fenpropathrin	140	Pentachlorobenzene	194	Triallate
33	Chlorbenside	87	Fenson	141	Pentachlorobenzonitrile	195	Triazophos
34	Chlordane, trans–	88	Fenthion	142	Pentachlorothioanisole	196	Triflumizole
35	Chlorfenapyr	89	Fenvalerate	143	Permethrin, cis–	197	Trifluralin
36	Chlorfenson	90	Fipronil	144	Permethrin, trans-	198	Vinclozolin
37	Chlorfenvinphos	91	Fluazifop–p–butyl	145	Phenothrin		
38	Chlorobenzilate	92	Fluchloralin	146	Phorate		
39	Chloroneb	93	Flucythrinate	147	Phosalone		
40	Chlorothalonil	94	Fludioxonil	148	Phosmet		
41	Chlorpropham	95	Fluquinconazole	149	Piperonyl butoxide		
42	Chlorpyrifos	96	Fluridone	150	Pirimiphos–ethyl		
43	Chlorpyrifos–methyl	97	Flusilazole	151	Pirimiphos–methyl		
44	Chlorthal–dimethyl	98	Flutolanil	152	Pretilachlor		
45	Chlorthiophos	99	Flutriafol	153	Prochloraz		
46	Chlozolinate	100	Folpet	154	Procymidone		
47	cis–Chlordane	101	Fonofos	155	Prodiamine		
48	Clomazone	102	gamma-BHC	156	Profenofos		
49	Coumaphos	103	Heptachlor	157	Profluralin		
50	Cycloate	104	Heptachlor epoxide	158	Propachlor		
51	Cyfluthrin	105	Hexachlorobenzene	159	Propanil		
52	Cyhalothrin–lambda	106	Hexazinone	160	Propargite		
53	Cypermethrin	107	Iodofenfos	161	Propisochlor		
54	Cyprodinil	108	Iprodione	162	Propyzamide		

20.5.4 Calibration standards

For quantitation, a matrix matched calibration curve is generated using known blank samples closely related to the tested samples. The blank sample is subjected to the same extraction and cleanup procedure mentioned above. This is to make sure that the matrix amount in each analyzed sample is constant. The final blank extract is used to prepare four calibration standards as below:

- Level 1 (0.01 µg /ml): Add 10 µl of WS1 + 990 µl final blank extract directly into an autosampler vial.
- Level 2 (0.02 µg /ml): Add 20 µl of WS1 + 980 µl final blank extract directly into an autosampler vial.
- Level 3 (0.05 µg /ml): Add 50 µl of WS1 + 950 µl final blank extract directly into an autosampler vial.
- Level 4 (0.1 µg /ml): Add 100 µl of WS1 + 900 µl final blank extract directly into an autosampler vial.
- The calibration curve should be valid until check standards deviate from the acceptable criteria (20% range of the corresponding calibration point value).
- If values deviate, prepare new calibration curve and check again

20.5.5 Working solutions

a) **Working solution 1** (W1, 10 µg/ml)
   - Prepare from all mixes of stock standard solution (100 µg/ml).
   - Take 100 µl from each mix and complete with Toluene so the final volume is 1 ml.
   - Use it to prepare working solution 2 (WS2) at 1 µg/ml and to spike GC–MS/MS recovery sample.

b) **Working solution 2** (W2, 1µg/ml)
   - This is prepared from WS1 by taking 100 µl and complete with Toluene to 1 ml.
   - Use it to prepare GC–MS/MS calibration levels.

20.6 Procedure

20.6.1 Sample preparation

a) Homogenize all the received sample, if possible, by a blender

b) Homogenize with dry ice, if needed, into a fine free flowing powder

c) Store the received samples in freezer at -20°C or colder until analysis.

20.6.2 Sample extraction

a) For samples with water content > 80%, weigh 10 g of homogenized sample into a clean 50 ml tube.

b) For other samples, add cold distilled water to rehydrate samples according to Table 137.

TABLE 137. Sample weight and treatment before extraction

Sample type	Sample weight (g)	Water added (g)	Note
Fruits and vegetables >80 % water content	10	n.a	
Fruits and vegetables 25 %–80 % water content	10	x	x = 10 g - water amount in 10 g sample
Dried fruits	5	7.5	

n.a: No water added

c) Spike blank samples at concentration 0.05 mg/kg. Let stand for 10 min

d) Add 10 ml of acetonitrile

e) Vortex for 1 min

f) Add buffered QuEChERS extraction packet as indicated elsewhere (EC, 2016) (4 g $MgSO_4$, 1 g NaCl, 1 g Na–citrate, 0.5 g disodium citrate sesquihydrate)

g) Adjust acidic pH (below 3) in matrices such as lemons, limes, currants and raspberry using 5 mol/l NaOH

h) Vortex for 1 min

i) Centrifuge for 5 min at 5°C and 6 000 rpm.

20.6.3 Cleanup procedure [dispersive solid-phase extraction (dSPE)]

Transfer 1 ml of the supernatant to the required ready–to–use dSPE tube (2 ml capacity) as shown in Table 138.

TABLE 138. Summary for sample cleanup

Sample type	dSPE type
General fruits and vegetables (e.g. zucchini, cabbage, cauliflower, head lettuce, celery, melon etc.)	150 mg $MgSO_4$, 25 mg PSA
Fruits and vegetables with fats and waxes	150 mg $MgSO_4$, 25 mg PSA, 25 mg C18
Pigmented fruits & vegetables with carotenoids and chlorophyll (Spanish, Red & Green sweet pepper, Carrots etc.)	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB (Higher concentration of GCB can be used with high concentration of pigment)
Pigmented fruits and vegetables with fats and waxes (eggplant etc.)	150 mg $MgSO_4$, 25 mg PSA, 7 mg GCB, 25 mg C18

a) Vortex for 1 min (2 min in case of GCB)

b) Centrifuge for 5 min at 5°C and 6 000 rpm to separate the solid material

c) Filter the extract through 0.45 mm PTFE filter

d) Take 0.25 ml of the filtrate

e) Evaporate to almost dryness at 40°C using a gentle nitrogen flow (less than 5 bar)

f) Reconstitute in 0.5 ml of toluene and mix properly (dilution factor of 2)

g) Set sample amount at 1

h) Set dilution factor at 2

i) The final extract has a concentration of ca. 0.5 g/ml

j) Sample is ready to be injected

20.6.4 Instrumental analysis (GC–MS/MS)

The GC parameters are summarized in the Table 139:

TABLE 139. GC conditions

Instrument	Trace 1310 Gas Chromatograph (ThermoFisher Scientific – USA)
Analytical Column	TG–5SILMS (30 m, 0.25 mm ID, 0.25 µm)
Liner	SSL Splitless Liner, single taper, 4 mm ID × 6.3 mm OD × 78.5 mm length
Injection Temp.	250°C
Injection Volume	1 µl
Injection Type	Splitless with surge (Hold 1 min)
Carrier Gas	Helium
Flow Type	Constant flow
Flow rate	1.4 ml/min
Oven Temp.	90°C (hold 1 min) to 330°C at 8.5°C /min (hold 5 min)

The MS parameters are summarized in Table 140:

TABLE 140. MS conditions

Instrument	TSQ™ Duo Triple Quadrupole Mass Spectrometer (ThermoFisher Scientific – USA)
Mode	Timed SRM
Scan rate	12 scans/peak
Scan range	50 amu–550 amu
Transfer Line Temp.	290°C
Analyzer Type	Quadrupole
Source Temp.	325°C
Electron Energy	70 eV
Solvent Delay Time	5 min
Ionization Mode	EI

20.6.5 Calculation and interpretation of results

a) Identification

- The retention time of any analyte in tested sample extract should correspond to that of the matrix matched calibration standard with a tolerance of ±0.1 min.
- Extracted ion chromatograms of tested sample extracts should have peaks of similar shape and response ratio to those obtained from matrix matched calibration standards analyzed at comparable concentrations.
- Chromatographic peaks from different selective ions for the analyte should fully overlap.
- The ion ratio should not deviate more than 30% from the average ion ratios of all calibration levels.

b) Reporting results

- The results should be reported and expressed in mg/kg.
- Where the residue definition includes more than one analyte, the respective sum of analytes should be calculated as stated in the residue definition and should be used for checking compliance with the MRL.
- Residues for individual analytes below the reporting limits should be reported as <MRL mg/kg.
- Where good mixing of samples has been undertaken, the RSD of replicate results of the test portions should normally not exceed 30%.
- Data on the residues do not have to be adjusted for recovery when the mean recovery is within the range of 70%–120%.
- If residue data are adjusted for recovery, then this should be stated in the report.
- The result should be reported together with the expanded measurement uncertainty (MU) as follows: Result = x ± U (units), with x representing the measured value.
- The sample is considered non-compliant if x-U > MRL.

20.6.6 Method validation

- Commodities used for fortification include cucumber, strawberry and jam samples and should be free of the pesticides analyzed.
- These products are chosen to represent group 1 (High water content), group 2 (High acid content) and group 3 (High sugar content) commodities (EC, 2017).
- The validation method is performed at two spiking levels (0.01 mg/kg and 0.05 mg/kg).
- Five spiked samples and a blank are analyzed at each level. In total eleven samples are analyzed.
- Standard spiking solutions are prepared by mixing all the 198 pesticides used for the validation method at a concentration of 10 μg/ml in toluene.
- For the pesticides to be accepted as validated the following criteria for precision and trueness (CEN, 2008) should be fulfilled:
- The repeatability RSD should be ≤20%
- The average relative recovery should be between 70% and 120%
- If the above-mentioned criteria have been met, the LOQs is stated.
- Linearity of the GC–MS/MS system is evaluated by assessing the signal responses of the target analytes from matrix matched calibration solutions prepared by spiking blank extracts at six concentration levels corresponding to concentrations: 0.01 μg/ml, 0.02 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.15 μg/ml and 0.2 μg/ml. The six-point calibration curve should demonstrate excellent linearity with $R^2 \geq 0.99$ for all analytes in the scope of this study.

The limit of quantitation is defined here as the lowest validated spike level meeting the method performance acceptability criteria (EC, 2017).

The method validation parameters are results are summarized in Table 141.

TABLE 141. Method validation summary

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
1	2,4'-DDE	5.5	86.1	5.5	89.9	0.0014	0.01	0.9995	17.45
2	2,4'-DDT	6.4	99.1	5.0	88.4	0.0019	0.01	0.9998	19.13
3	2,4'-Methoxychlor	5.4	109.0	3.2	93.8	0.0018	0.01	0.9997	20.10
4	2-Phenylphenol	9.0	**50.2**	2.3	**60.5**	0.0021	0.05	0.9956	9.96
5	4,4'-DDD	7.9	106.2	6.2	92.7	0.0025	0.01	0.9996	19.06
6	4,4'-DDE	7.5	88.4	6.6	87.0	0.0020	0.01	0.9995	18.14
7	4,4'-DDT	4.5	102.4	5.5	89.8	0.0014	0.01	0.9998	19.86
8	4,4'-Dichlorobenzophenone	9.3	107.4	3.4	97.4	0.0030	0.01	0.9998	16.10
9	4,4'-Methoxychlor olefin	7.4	105.3	4.7	92.3	0.0023	0.01	0.9998	19.66
10	Acequinocyl	**20.1**	**52.3**	8.7	86.4	0.0113	0.05	0.9992	24.32
11	Acetochlor	6.6	94.4	4.6	96.6	0.0019	0.01	0.9994	14.85
12	Acrinathrin	20.4	96.0	4.1	84.9	0.0059	0.01	0.9996	22.29
13	Alachlor	6.0	101.6	5.1	99.6	0.0018	0.01	0.9997	15.09
14	Aldrin	**27.9**	97.0	6.8	95.1	0.0097	0.05	0.9986	15.88
15	Allidochlor	**A**	**A**	7.0	98.1	0.0103	0.05	0.9997	6.55
16	Alpha–BHC	4.1	97.1	4.8	92.2	0.0012	0.01	0.9999	12.54
17	Anthraquinone	11.6	106.9	6.9	101.9	0.0037	0.01	0.9998	15.88
18	Atrazine	9.0	109.0	3.9	97.7	0.0029	0.01	0.9999	13.13
19	Azinphos–ethyl	13.0	95.7	2.1	83.2	0.0037	0.01	0.9996	22.45
20	Azinphos–methyl	6.8	115.4	3.5	83.1	0.0024	0.01	0.9998	21.73
21	Benfluralin	7.4	105.6	6.8	92.1	0.0023	0.01	0.9988	12.27
22	Beta–BHC	6.2	105.5	5.5	94.8	0.0020	0.01	0.9999	13.23
23	Bifenthrin	7.3	102.7	4.1	92.8	0.0022	0.01	0.9996	20.95
24	Biphenyl	**34.6**	75.6	2.9	86.7	0.0037	0.05	0.9997	8.01
25	Bromfenvinphos	8.9	106.6	6.0	92.7	0.0029	0.01	0.9998	17.87
26	Bromfenvinphos–methyl	7.4	101.2	2.9	93.8	0.0022	0.01	1	16.97
27	Bromophos methyl	7.6	100.5	2.1	92.2	0.0023	0.01	0.9998	16.44
28	Bromophos–ethyl	8.1	96.3	3.0	92.2	0.0023	0.01	0.9993	17.40
29	Bromopropylate	9.5	96.8	4.3	94.4	0.0027	0.01	0.9994	20.93
30	Bupirimate	6.9	106.2	6.0	100.5	0.0022	0.01	0.9993	18.43
31	Carbophenothion	4.2	110.4	5.6	95.3	0.0014	0.01	0.9995	19.61
32	Carfentrazone ethyl	9.8	109.4	7.1	97.6	0.0032	0.01	0.9996	19.65
33	Chlorbenside	10.2	83.9	1.9	87.9	0.0026	0.01	0.9993	17.20
34	Chlordane, trans–	7.0	98.4	6.2	91.4	0.0021	0.01	0.9994	17.33
35	Chlorfenapyr	14.5	**123.8**	8.0	104.5	0.0125	0.05	0.9983	18.70
36	Chlorfenson	7.8	103.6	6.5	93.5	0.0024	0.01	0.9995	17.82
37	Chlorfenvinphos 1	35.2	98.2	6.3	97.2	0.0104	0.01	0.9942	16.71
38	Chlorfenvinphos 2	2.6	115.7	10.1	103.3	0.0009	0.01	0.9994	16.97

(con't)

TABLE 141. Method validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
39	Chlorobenzilate	10.6	92.3	6.2	96.6	0.0029	0.01	0.995	18.84
40	Chloroneb	7.3	96.8	2.8	92.5	0.0021	0.01	0.9999	9.93
41	Chlorothalonil	10.1	70.3	3.0	80.6	0.0027	0.01	0.9995	14.07
42	Chlorpropham	11.1	87.3	5.6	92.7	0.0029	0.01	0.9998	11.86
43	Chlorpyrifos	10.9	104.6	7.7	99.3	0.0034	0.01	0.9995	16.03
44	Chlorpyrifos–methyl	3.5	105.4	4.6	94.5	0.0011	0.01	0.9998	14.91
45	Chlorthal–dimethyl	4.3	89.5	5.2	98.3	0.0012	0.01	0.9994	16.16
46	Chlorthiophos 1	15.0	91.4	10.3	99.2	0.0041	0.01	0.9987	18.76
47	Chlorthiophos 2	13.6	101.8	4.8	87.3	0.0041	0.01	0.9998	18.95
48	Chlorthiophos 3	5.1	108.9	7.0	89.0	0.0017	0.01	0.9996	19.22
49	Chlozolinate	16.5	71.6	3.8	102.9	0.0036	0.01	0.9996	16.87
50	cis–Chlordane	16.0	120.1	11.0	94.6	0.0057	0.01	0.9996	17.69
51	Clomazone	8.6	101.7	4.0	95.8	0.0026	0.01	0.9998	13.20
52	Coumaphos	10.5	108.9	2.9	83.0	0.0034	0.01	0.9996	23.24
53	Cycloate	5.9	88.2	3.7	96.6	0.0016	0.01	0.9999	11.62
54	Cyfluthrin 1	15.5	82.4	15.9	100.9	0.0038	0.01	0.9993	23.64
55	Cyfluthrin 2	10.6	89.3	1.8	93.8	0.0028	0.01	0.9994	23.74
56	Cyfluthrin 3	16.6	77.3	7.4	90.0	0.0039	0.01	0.9986	23.84
57	Cyfluthrin 4	8.9	95.1	7.0	91.8	0.0025	0.01	0.9972	23.88
58	Cyhalothrin, lambda–	13.6	93.9	5.0	90.1	0.0038	0.01	0.9994	22.10
59	Cypermethrin 1	15.8	82.8	9.7	85.6	0.0039	0.01	0.9998	24.00
60	Cypermethrin 2	18.2	75.1	5.2	87.3	0.0041	0.01	0.9991	24.10
61	Cypermethrin 3	19.1	72.0	4.5	89.3	0.0041	0.01	0.9987	24.20
62	Cypermethrin 4	13.1	76.8	5.1	86.9	0.0030	0.01	0.9997	24.24
63	Cyprodinil	9.8	94.9	2.2	100.0	0.0028	0.01	0.9993	16.63
64	delta–BHC	8.0	99.7	4.9	96.0	0.0024	0.01	0.9988	13.96
65	Deltamethrin	9.9	91.1	6.4	97.5	0.0027	0.01	0.9993	25.90
66	Diallate 1	8.0	99.9	3.3	96.0	0.0024	0.01	0.9998	12.41
67	Diallate 2	16.3	74.7	5.1	93.5	0.0037	0.01	0.9999	12.62
68	Diazinon	6.5	100.1	5.4	96.7	0.0019	0.01	0.9998	13.78
69	Dichlofluanid	7.3	**26.3**	4.2	84.1	0.0052	0.05	0.9997	15.73
70	Dichloroaniline, 3,4'-	15.3	**49.9**	6.1	57.7	0.0053	0.05	0.9993	**8.77**
71	Diclobenil	12.5	83.7	3.7	92.8	0.0031	0.01	0.9998	7.47
72	Dicloran	**A**	**A**	6.5	83.8	0.0081	0.05	0.9992	12.84
73	Dieldrin	14.2	125.7	13.5	94.8	0.0054	0.01	0.9952	18.21
74	Dimethachlor	9.3	87.8	3.2	99.1	0.0024	0.01	0.9998	14.71
75	Diphenamid	20.1	87.5	5.0	103.5	0.0053	0.01	0.9997	16.46
76	Diphenylamine	11.3	77.5	5.7	75.3	0.0026	0.01	0.9997	11.47

(con't)

TABLE 141. Method validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
77	Disulfoton	9.1	106.8	3.9	93.0	0.0029	0.01	0.9998	13.88
78	Edifenphos	8.0	106.0	3.9	93.8	0.0025	0.01	0.9995	19.71
79	Endosulfan ether	6.7	92.3	4.7	95.1	0.0018	0.01	0.9993	14.38
80	Endosulfan I	**34.7**	**185.4**	5.9	108.7	0.0096	0.05	0.9982	17.62
81	Endosulfan II	20.1	**134.8**	9.9	95.3	0.0141	0.05	0.9977	18.89
82	Endosulfan sulfate	9.8	96.9	6.1	93.1	0.0029	0.01	0.9998	19.80
83	Endrin	19.0	98.3	16.1	84.8	0.0056	0.01	0.9973	18.70
84	Endrin aldehyde	**23.8**	84.7	18.9	62.0	0.0176	0.05	0.9984	19.29
85	Endrin ketone	9.9	108.5	7.4	92.3	0.0032	0.01	0.9996	20.82
86	EPN	11.4	117.5	2.7	88.6	0.0040	0.01	0.9992	20.96
87	Ethalfluralin	8.2	101.7	10.3	95.2	0.0025	0.01	0.9997	11.99
88	Ethion	8.7	112.8	6.1	88.8	0.0030	0.01	0.9994	19.13
89	Ethylan	10.0	98.9	5.9	92.6	0.0030	0.01	0.9996	18.67
90	Etofenprox	8.0	88.5	5.3	92.9	0.0021	0.01	0.9996	24.33
91	Etridazole	6.9	80.2	4.6	89.2	0.0017	0.01	0.9998	9.12
92	Fenamiphos	17.6	101.4	7.3	88.8	0.0054	0.01	0.9998	17.88
93	Fenarimol	11.1	97.3	2.6	86.8	0.0032	0.01	0.9996	22.31
94	Fenchlorphos	3.3	103.3	4.4	92.6	0.0010	0.01	0.9999	15.25
95	Fenitrothion	4.8	103.9	6.5	105.4	0.0015	0.01	0.9992	15.55
96	Fenpropathrin	15.7	99.6	1.8	94.0	0.0047	0.01	0.9995	21.10
97	Fenson	6.8	101.2	4.4	100.1	0.0021	0.01	0.9997	16.30
98	Fenthion	6.2	102.2	4.4	99.5	0.0019	0.01	0.9995	15.99
99	Fenvalerate 1	9.2	87.6	5.0	94.7	0.0024	0.01	0.9997	25.05
100	Fenvalerate 2	8.4	81.3	6.2	86.3	0.0021	0.01	0.9999	25.28
101	Fipronil	13.4	117.0	7.9	99.8	0.0047	0.01	0.9999	16.99
102	Fluazifop–p–butyl	11.2	105.8	7.7	95.8	0.0035	0.01	0.999	18.64
103	Fluchloralin	8.5	117.4	3.3	99.5	0.0030	0.01	0.9974	13.88
104	Flucythrinate 1	10.6	86.4	5.2	91.8	0.0027	0.01	0.9999	24.23
105	Flucythrinate 2	9.8	88.8	5.7	93.3	0.0026	0.01	0.9999	24.45
106	Fludioxonil	8.5	112.6	5.9	95.5	0.0029	0.01	0.9989	18.11
107	Fluquinconazole	8.9	95.2	3.0	89.8	0.0025	0.01	0.9998	23.24
108	Fluridone	**A**	**A**	7.7	82.0	0.0095	0.05	0.9998	24.77
109	Flusilazole	10.1	98.7	4.2	94.3	0.0030	0.01	0.9995	18.37
110	Flutolanil	5.4	93.4	7.2	98.0	0.0015	0.01	0.9998	17.90
111	Flutriafol	6.6	99.9	7.0	97.5	0.0020	0.01	0.9993	17.74
112	Folpet	11.8	73.9	7.5	83.7	0.0026	0.01	0.9998	17.14
113	Fonofos	7.8	106.3	3.5	93.2	0.0025	0.01	0.9994	13.57

(con't)

TABLE 141. Method validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
114	Gamma–BHC	7.2	96.1	4.8	93.7	0.0021	0.01	0.9996	13.37
115	Heptachlor	9.7	91.9	3.1	90.7	0.0027	0.01	0.9976	15.08
116	Heptachlor epoxide	4.0	104.1	5.2	98.9	0.0012	0.01	0.9989	16.81
117	Hexachlorobenzene	6.4	75.8	4.9	80.6	0.0015	0.01	1	12.72
118	Hexazinone	8.0	103.9	3.0	91.5	0.0025	0.01	0.9996	20.08
119	Iodofenfos	13.0	88.1	6.2	80.9	0.0034	0.01	0.9997	17.93
120	Iprodione	13.4	108.9	10.0	102.2	0.0044	0.01	0.9896	20.72
121	Isazophos	12.6	112.3	1.5	96.6	0.0042	0.01	0.9998	14.09
122	Isodrin	11.0	81.2	8.6	87.1	0.0027	0.01	0.999	16.55
123	Isopropalin	12.5	101.3	6.2	104.1	0.0038	0.01	0.9996	16.58
124	Lenacil	7.5	112.3	5.1	91.7	0.0025	0.01	0.9984	19.78
125	Leptophos	8.2	99.3	4.5	86.3	0.0025	0.01	0.9996	21.77
126	Linuron	**A**	**A**	6.3	94.7	0.0090	0.05	0.9988	15.62
127	Malathion	9.2	102.0	3.6	104.8	0.0028	0.01	0.9995	15.78
128	Metalaxyl	17.9	102.8	4.3	100.2	0.0055	0.01	0.9996	15.20
129	Metazachlor	8.1	86.8	3.4	102.9	0.0021	0.01	0.9997	16.75
130	Methacrifos	7.6	106.1	5.3	92.7	0.0024	0.01	0.9998	9.82
131	Methoxychlor	9.1	102.4	5.1	93.8	0.0028	0.01	0.9996	21.08
132	Methyl parathion	10.4	118.0	7.2	94.2	0.0037	0.01	0.9988	14.91
133	Metolachlor	8.0	88.9	3.2	105.1	0.0021	0.01	0.9996	15.92
134	Mevinphos	8.8	83.7	4.1	79.5	0.0022	0.01	0.9998	8.75
135	MGK 264 1	23.6	94.4	5.9	106.0	0.0067	0.01	0.9994	16.39
136	MGK 264 2	10.1	116.8	9.3	104.4	0.0035	0.01	0.9997	16.65
137	Mirex	9.3	81.5	2.0	83.5	0.0023	0.01	0.9999	21.99
138	Myclobutanil	11.6	99.7	7.1	95.3	0.0035	0.01	0.9997	18.30
139	Nitralin	11.0	107.8	3.3	96.1	0.0036	0.01	0.9998	20.52
140	Nitrofen	13.4	85.9	5.5	91.0	0.0034	0.01	0.9998	18.61
141	Nonachlor, cis–	9.8	94.9	9.0	95.5	0.0028	0.01	0.9995	19.16
142	Norflurazon	13.5	112.2	5.4	94.5	0.0045	0.01	0.9993	19.78
143	Oxadiazon	9.1	92.4	6.5	100.8	0.0025	0.01	0.9995	18.21
144	Oxyfluorfen	11.4	95.5	4.5	91.8	0.0033	0.01	0.9996	18.34
145	Paclobutrazol	5.8	105.7	4.8	101.0	0.0018	0.01	1	17.47
146	Parathion	6.5	103.7	4.4	104.4	0.0020	0.01	0.9994	16.05
147	Pebulate	8.7	101.7	3.5	99.1	0.0027	0.01	0.9998	9.17
148	Penconazole	7.9	99.7	3.0	99.6	0.0024	0.01	0.9994	16.80
149	Pendimethalin	11.4	110.0	7.3	101.5	0.0038	0.01	0.9985	16.77
150	Pentachloroaniline	5.0	90.3	1.3	93.9	0.0014	0.01	0.9998	14.49

(con't)

TABLE 141. Method validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
151	Pentachloroanisole	8.6	89.3	3.9	85.6	0.0023	0.01	0.9997	12.86
152	Pentachlorobenzene	9.5	84.8	3.3	82.1	0.0024	0.01	0.9999	10.13
153	Pentachlorobenzonitrile	8.9	94.4	7.0	92.5	0.0025	0.01	0.999	13.54
154	Pentachlorothioanisole	8.4	84.8	5.1	90.9	0.0021	0.01	0.9999	15.67
155	Permethrin, cis–	13.0	82.1	4.6	89.2	0.0032	0.01	0.9998	22.96
156	Permethrin, trans–	17.0	80.6	4.0	85.3	0.0041	0.01	0.9996	23.10
157	Phenothrin 1	**A**	**A**	3.9	79.8	0.0047	0.05	0.9975	21.40
158	Phenothrin 2	21.0	83.1	5.7	91.9	0.0052	0.01	0.9996	21.52
159	Phorate	5.6	101.3	4.6	92.2	0.0017	0.01	0.9999	12.43
160	Phosalone	12.1	88.0	2.6	87.9	0.0032	0.01	0.9998	21.71
161	Phosmet	6.7	104.0	3.1	88.0	0.0021	0.01	0.9993	20.89
162	Piperonyl butoxide	8.3	110.7	4.2	90.7	0.0027	0.01	0.9997	20.31
163	Pirimiphos–ethyl	6.7	111.3	5.2	104.0	0.0022	0.01	0.9999	16.52
156	Permethrin, trans–	17.0	80.6	4.0	85.3	0.0041	0.01	0.9996	23.10
164	Pirimiphos–methyl	4.8	102.3	88.3	65.5	0.0015	0.01	0.9996	15.53
165	Pretilachlor	9.6	92.2	5.6	95.5	0.0027	0.01	0.9996	18.11
166	Prochloraz	12.6	81.7	8.5	79.9	0.0031	0.01	0.9989	23.31
167	Procymidone	14.6	113.8	6.1	102.6	0.0050	0.01	0.999	17.18
168	Prodiamine	10.2	81.3	6.4	102.8	0.0025	0.01	0.9983	15.60
169	Profenofos	4.4	105.6	3.6	89.1	0.0014	0.01	0.9996	18.05
170	Profluralin	5.1	**134.4**	3.6	93.8	0.0050	0.05	0.998	13.57
171	Propachlor	7.0	98.1	5.4	97.1	0.0021	0.01	0.9998	11.34
172	Propanil	**A**	**A**	15.8	92.8	0.0219	0.05	0.998	14.69
173	Propargite	8.8	115.5	4.8	94.6	0.0031	0.01	0.9995	20.22
174	Propisochlor	11.3	99.7	3.9	98.0	0.0034	0.01	0.9998	15.18
175	Propyzamide	8.9	101.3	2.6	90.5	0.0027	0.01	0.9986	13.57
176	Prothiofos	6.8	102.4	6.2	89.1	0.0021	0.01	0.9987	17.98
177	Pyraclofos	10.3	86.4	1.9	81.4	0.0027	0.01	0.9998	22.54
178	Pyrazophos	13.1	101.6	3.0	85.9	0.0040	0.01	0.9993	22.39
179	Pyridaben	12.5	90.3	3.0	82.9	0.0034	0.01	0.9996	23.11
180	Pyridaphenthion	9.1	117.3	4.5	89.2	0.0032	0.01	0.9994	20.81
181	Pyrimethanil	19.5	89.1	2.0	87.4	0.0052	0.01	0.9998	13.70
182	Pyriproxyfen	10.9	102.1	4.0	93.4	0.0033	0.01	0.9996	21.78
183	Quinalphos	7.8	112.5	3.0	98.9	0.0026	0.01	0.9997	17.03
184	Quintozene	18.2	87.6	3.1	88.5	0.0048	0.01	0.9996	13.49
185	Resmethrin 1	15.4	91.9	4.5	92.1	0.0043	0.01	0.9998	20.24
186	Resmethrin 2	**A**	**A**	15.0	85.4	0.0192	0.05	0.997	20.35

(con't)

TABLE 141. Method validation summary (con't)

		0.01 mg /kg		0.05 mg /kg					
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD	LOQ	R^2	R.T. (min)
187	Sulfotep	5.3	111.7	5.5	93.1	0.0018	0.01	0.9996	12.32
188	Sulprofos	7.0	117.5	4.2	90.2	0.0025	0.01	0.9995	19.40
189	tau–Fluvalinate 1	6.8	81.8	11.2	90.5	0.0017	0.01	0.9999	25.26
190	tau–Fluvalinate 2	8.3	91.7	3.3	93.4	0.0023	0.01	0.9993	25.33
191	Tebuconazole	7.8	99.6	5.4	93.4	0.0023	0.01	0.999	20.14
192	Tebufenpyrad	9.3	103.9	4.3	96.8	0.0029	0.01	0.9995	21.17
193	Tecnazene	9.0	83.2	5.8	90.4	0.0023	0.01	0.9997	11.25
194	Tefluthrin	6.2	104.9	2.4	98.1	0.0019	0.01	0.9998	14.04
195	Terbacil	19.1	100.3	6.7	92.3	0.0057	0.01	0.9989	13.96
196	Terbufos	5.1	105.2	6.1	87.5	0.0016	0.01	0.9996	13.48
197	Terbutylazine	10.9	113.2	3.4	96.6	0.0037	0.01	0.9999	13.49
198	Tetrachloroaniline, 2,3,5,6-	11.0	78.8	4.1	90.0	0.0026	0.01	0.9997	11.54
199	Tetrachlorvinphos	6.3	110.7	6.6	94.1	0.0021	0.01	0.9997	17.58
200	Tetradifon	15.8	106.2	3.6	92.0	0.0050	0.01	0.9992	21.52
201	Tetrahydrophthalimide	10.0	**131.5**	6.4	96.5	0.0093	0.05	0.9997	9.47
202	Tetramethrin 1	18.5	70.8	5.9	89.3	0.0039	0.01	0.9998	20.82
203	Tetramethrin 2	12.4	106.7	3.3	92.0	0.0040	0.01	0.9993	20.96
204	Tolclofos–methyl	4.6	110.4	3.2	96.3	0.0015	0.01	0.9994	15.01
205	Tolylfluanid	7.7	**37.3**	3.0	87.6	0.0040	0.05	0.9996	16.90
206	Transfluthrin	6.8	105.4	2.2	98.8	0.0022	0.01	0.9998	15.00
207	trans–Nonachlor	13.6	113.4	3.8	91.4	0.0046	0.01	0.9995	17.79
208	Triadimefon	7.2	88.7	4.1	105.0	0.0019	0.01	0.9999	16.11
209	Triadimenol	**A**	**A**	8.2	95.4	0.0118	0.05	0.9984	17.07
210	Triallate	5.2	93.3	3.3	93.4	0.0015	0.01	0.9996	14.10
211	Triazophos	8.9	113.0	4.9	97.4	0.0030	0.01	0.9999	19.42
212	Tricyclazole	**A**	**A**	10.9	76.2	0.0124	0.05	0.9975	18.13
213	Triflumizole	17.4	93.4	6.0	95.5	0.0049	0.01	0.999	17.23
214	Trifluralin	7.0	94.5	7.1	95.8	0.0020	0.01	0.9995	12.22
215	Vinclozolin	9.8	103.8	2.1	98.5	0.0031	0.01	0.9996	14.90

Bold numbers: Recovery and/or RSD out of acceptable range
A: Peak shape error or no peak was detected

20.6.7 Schematic diagram of extraction procedure

SAMPLE EXTRACTION	1. If sample water content is below 80%, rehydrate by adding cold distilled water (refer to relevant table). 2. Add internal standard or spiking mix if required. 3. Add 10 ml of acetonitrile 4. Vortex for 1 min. 5. Use ready to use buffered QuEChERS extraction as indicated elsewhere (EC, 2015). Tear the top of the packet and empty into the extraction tube. 6. Vortex for 1 min. 7. Centrifuge at 6 000 rpm for 5 min
	
SAMPLE CLEANUP	1. Transfer 1 ml of the supernatant to the ready to use dSPE tube 2. Vortex for 30 s 3. Centrifuge for 5 min at 5ºC and 6 000 rpm 4. Filter the extract through 0.45 mm PTFE filter 5. Take 0.25 ml of the filtrate 6. Evaporate to almost dryness at 40°C using a slight nitrogen flow s(less than 5 bar) 7. Reconstitute in 0.5 ml of toluene and mix properly 8. Inject into the GC–MS/MS

21

Detection and quantification of pesticide residues in food samples by LC–MS/MS

21.1 Introduction

Pesticides are still used in both crop and animal production to treat diseases and pests, but their residues may end up in food for human consumption. This has public health and trade implication and should be controlled. Cost effective sample preparation techniques followed by confirmatory analysis are critical to control laboratories and programmes.

21.2 Scope and objectives

This procedure applies to food samples received by Contaminants Monitoring Division (CMD) that includes commodity group 1(High water content) and commodity group 2 (High acid content and high–water content) (CEN, 2008). It describes the procedure for food samples preparation, extraction and cleanup of pesticides from food matrices, LC–MS/MS analytical conditions, standards and calibration levels preparation and identification and results reporting.

21.3 Principle of the method

A modified QuEChERS method where a homogenised sample is extracted using acetonitrile and mixture of salts followed by shaking and centrifugation is used. An aliquot of the organic phase is cleanedup using anhydrous $MgSO_4$ and PSA, shaken on a vortex mixer followed by dilution with acetonitrile.

21.4 Safety considerations and precautions

The chemicals use in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, some pesticides standards are considered a possible carcinogen to humans. Suitable gloves should be worn when chemicals/ pesticides standards are handled, and the work should be performed in a fume hood at all possible time.

21.5 Materials

21.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Acetonitrile (LC–MS grade); 5% Formic acid solution in acetonitrile; Water (LC–MS grade); Methanol (LC–MS grade); Ammonium formate (LC–MS grade ≥ 99.0%); Formic acid LC–MS grade.

21.5.2 Equipment/instruments and consumables

The following apparatus and materials are applicable: Freezer (-20°C or below); Sample processing equipment (blender and grinder); Refrigerated centrifuges for 50 ml, 15 ml and 2 ml centrifuge tubes; Vortex and/or shaker; Ultrasonic water bath; Automatic pipettes (e.g. for 20 µl – 200 µl, 100 µl – 1 000 µl and 1 ml – 10 ml); Centrifuge tubes with screw caps (50 ml and 15 ml); Solvent–dispenser (for acetonitrile & toluene); Vials with caps for LC & GC auto sampler (2 ml); Syringes (e.g. 2 ml disposable syringes); Syringes filters (0.45 µm pore size); QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl, 1 g Na–citrate, 0.5 g disodium citrate sesquihydrate); dSPE tubes (150 mg $MgSO_4$, 25 mg PSA) (150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB); LC–MS/MS (Shimadzu).

21.5.3 Solutions

The solutions (and how they are prepared) include:

- Mobile Phase (A): 2 mmol/l ammonium formate + 0.002% formic acid – Water
- Add 0.126 g of ammonium formate into 1 000 ml volumetric flask
- Add 500 ml of LC–MS grade water
- Add 20 µl of formic acid
- Complete to the 1 000 ml mark with LC–MS grade water
- Sonicate for 10 min
- Mobile Phase (B): 2 mmol/l ammonium formate + 0.002% formic acid – Methanol
- Add 0.126 g of ammonium formate into 1 000 ml volumetric flask
- Add 500 ml of methanol
- Add 20 µl formic acid
- Complete to 1 000 ml with methanol
- Sonicate for 10 min
- 5 % formic acid in acetonitrile
- Add 500 µl of formic acid in volumetric flask and complete to 10 ml with acetonitrile.

21.5.4 Standards

The reference standards used and MS parameters (MRM transitions) are summarized in Table 142.

TABLE 142. MRM transitions

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
1	(E)–Fenpyroximate	10.991	422.10>366.00	422.10>138.10	ESI +
2	Acephate	2.924	184.00>143.00	184.00>49.15	ESI +
3	Acetamiprid	5.59	223.10>126.10	223.10>55.95	ESI +
4	Acibenzolar–s–methyl	8.382	210.90>136.05	210.90>140.00	ESI +
5	Aldicarb	5.266	208.20>115.85	208.20>89.00	ESI +
6	Aldicarb–sulfone (Aldoxycarb)	4.049	240.10>86.20	240.10>148.15	ESI +
7	Aldicarb–sulfoxide	3.917	207.10>89.10	207.10>132.15	ESI +
8	Ametryn	6.878	228.10>68.00	228.10>96.00	ESI +
9	Aminocarb	5.442	209.00>122.10	209.00>152.05	ESI +
10	Azoxystrobin	9.089	404.00>371.95	404.00>328.95	ESI +
11	Benalaxyl	9.264	326.20>208.10	326.20>91.05	ESI +
12	Benzoximate	9.986	364.10>198.95	364.10>105.05	ESI +
13	Bitertanol	8.723	338.00>99.15	338.00>70.05	ESI +
14	Boscalid	7.841	343.00>271.95	343.00>270.95	ESI +
15	Bromuconazole	9.136	377.90>159.05	377.90>160.90	ESI +
16	Bupirimate	8.545	317.20>166.00	317.20>108.00	ESI +
17	Buprofezin	9.682	306.20>57.00	306.20>201.05	ESI +
18	Butafenacil	8.852	492.10>179.90	492.10>330.85	ESI +
19	Carbaryl (NAC)	6.098	202.10>145.10	202.10>127.00	ESI +
20	Carbendazim	4.899	192.10>160.15	192.10>132.15	ESI +
21	Carbetamide	5.229	237.10>192.10	237.10>118.15	ESI +
22	Carbofuran	6.04	222.10>165.00	222.10>123.15	ESI +
23	Carbofuran–3-hydroxy	4.79	255.00>163.15	255.00>220.05	ESI +
24	Carboxin	6.496	236.10>87.00	236.10>143.10	ESI +
25	Carfentrazone–ethyl	8.831	412.00>345.90	412.00>366.00	ESI +
26	Chlorantraniliprole	7.516	483.90>452.90	483.90>285.90	ESI +
27	Chlorfluazuron	10.268	539.90>382.85	539.90>158.00	ESI +
28	Chlorotoluron	5.94	213.10>72.20	213.10>46.10	ESI +
29	Chloroxuron	7.675	291.10>46.15	291.10>218.05	ESI +
30	Clethodim	9.321	360.10>164.15	360.10>166.05	ESI +
31	Clofentezine	9.601	303.00>138.15	303.00>102.10	ESI +
32	Clothianidin	4.593	250.00>132.05	250.00>169.10	ESI +
33	Cyazofamid	8.799	325.00>108.10	325.00>43.95	ESI +
34	Cycluron	6.456	199.20>46.20	199.20>88.95	ESI +
35	Cymoxanil	5.063	199.10>128.15	199.10>111.15	ESI +
36	Cyproconazole	7.199	292.10>70.05	292.10>125.05	ESI +

(con't)

TABLE 142. MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
37	Cyprodinil	8.488	226.10>93.00	226.10>108.00	ESI +
38	Desmedipham	6.855	318.00>136.10	318.00>301.00	ESI +
39	Diclobutrazol	7.675	328.00>70.10	330.00>70.10	ESI +
40	Dicrotophos	4.727	237.90>127.00	237.90>193.00	ESI +
41	Diethofencarb	7.043	268.20>124.15	268.20>152.00	ESI +
42	Difenoconazole	9.851	406.10>250.90	406.10>111.00	ESI +
43	Diflubenzuron	8.055	311.00>158.10	311.00>113.05	ESI +
44	Dimethoate	4.891	230.00>171.00	230.00>198.90	ESI +
45	Dimethomorph	8.784	388.10>165.10	388.10>301.00	ESI +
46	Dimoxystrobin	8.724	327.10>205.00	327.10>116.10	ESI +
47	Diniconazole	8.151	326.10>70.05	326.10>159.00	ESI +
48	Dinotefuran	3.816	203.15>114.15	203.15>87.00	ESI +
49	Dioxacarb	4.991	224.10>123.00	224.10>77.10	ESI +
50	Diuron (DCMU)	5.521	233.00>46.15	233.00>72.10	ESI +
51	Emamectin B1a	10.909	886.40>158.20	886.40>82.05	ESI +
52	Emamectin B1b	10.787	872.20>158.20	872.20>82.05	ESI +
53	Epoxiconazole	8.516	330.00>101.10	330.00>121.10	ESI +
54	Eprinomectin	10.913	914.60>186.20	914.60>112.00	ESI +
55	Etaconazole	8.48	328.10>159.00	328.10>54.95	ESI +
56	Ethiofencarb	6.273	226.10>107.00	226.10>77.00	ESI +
57	Ethiprole	6.681	397.00>350.90	397.00>254.85	ESI +
58	Ethirimol	5.728	210.20>140.20	210.20>98.15	ESI +
59	Ethofumesate	7.69	304.10>121.10	304.10>240.95	ESI +
60	Etoxazole	10.293	360.10>113.05	360.10>141.10	ESI +
61	Famoxadone	9.464	392.20>331.05	392.20>237.95	ESI +
62	Fenamidone	7.741	312.10>65.00	312.10>236.00	ESI +
63	Fenarimol	7.998	331.00>268.00	331.00>139.10	ESI +
64	Fenazaquin	10.785	307.20>161.10	307.20>57.00	ESI +
65	Fenbuconazole	8.684	337.10>125.05	337.10>70.10	ESI +
66	Fenhexamid	7.509	302.10>97.10	302.10>55.05	ESI +
67	Fenobucarb	6.623	208.10>95.00	208.10>152.10	ESI +
68	Fenoxycarb	8.592	302.10>116.15	302.10>88.00	ESI +
69	Fenpropimorph	8.643	304.20>117.00	304.20>132.05	ESI +
70	Fenuron	4.704	165.00>46.10	165.00>72.15	ESI +
71	Fipronil	7.059	435.00>330.00	435.00>250.05	ESI -
72	Flonicamid	4.073	230.10>203.00	230.10>148.05	ESI +
73	Fluazinam	9.211	463.00>415.95	463.00>397.95	ESI -
74	Fludioxonil	6.727	247.00>180.15	247.00>126.15	ESI -
75	Flufenacet	8.174	364.10>124.05	364.10>194.00	ESI +
76	Flufenoxuron	9.862	489.00>158.10	489.00>140.90	ESI +

(con't)

TABLE 142. MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
77	Fluometuron	5.522	233.10>46.20	233.10>72.05	ESI +
78	Fluoxastrobin	9.677	458.80>188.10	458.80>111.00	ESI +
79	Fluquinconazole	8.724	376.00>349.00	376.00>307.00	ESI +
80	Flusilazole	8.466	316.10>165.10	316.10>247.00	ESI +
81	Flutolanil	7.434	324.10>261.90	324.10>242.00	ESI +
82	Flutriafol	6.543	302.10>70.05	302.10>123.00	ESI +
83	Forchlorfenuron	6.003	248.10>129.15	248.10>93.15	ESI +
84	Formetanate	4.178	222.20>165.20	222.20>93.10	ESI +
85	Furalaxyl	8.087	302.10>95.00	302.10>270.05	ESI +
86	Furathiocarb	10.252	383.20>195.00	383.20>167.05	ESI +
87	Halofenozide	6.91	331.10>105.20	331.10>275.00	ESI +
88	Hexaconazole	7.971	314.10>70.00	314.10>159.00	ESI +
89	Hexaflumuron	9.105	458.80>439.00	458.80>276.00	ESI -
90	Hexythiazox	10.489	353.10>228.00	353.10>168.00	ESI +
91	Imazalil	8.438	297.10>159.05	297.10>41.10	ESI +
92	Imidacloprid	5.237	256.10>209.00	256.10>174.95	ESI +
93	Indoxacarb	9.888	528.10>203.00	528.10>218.00	ESI +
94	Ipconazole	8.69	334.10>70.10	336.10>70.00	ESI +
95	Iprovalicarb	7.422	321.20>203.00	321.20>91.00	ESI +
96	Isocarbophos	10.784	307.00>57.05	307.00>161.10	ESI +
97	Isoprocarb	6.078	194.10>95.00	194.10>77.00	ESI +
98	Isoproturon	6.228	207.20>46.10	207.20>165.00	ESI +
99	Kresoxim–methyl	9.16	314.10>267.00	314.10>235.00	ESI +
100	Linuron	6.609	248.80>160.00	248.80>182.05	ESI +
101	Mandipropamid	8.413	412.10>327.90	412.10>125.10	ESI +
102	Mefenacet	8.929	299.00>192.00	299.00>148.15	ESI +
103	Mepanipyrim	8.125	224.10>106.05	224.10>77.00	ESI +
104	Mepronil	7.694	270.20>228.00	270.20>91.05	ESI +
105	Metaflumizone	9.314	507.10>178.05	507.10>287.00	ESI +
106	Metalaxyl	7.221	280.10>192.05	280.10>220.00	ESI +
107	Metconazole	8.3	320.10>70.15	322.10>70.10	ESI +
108	Methabenzthiazuron	6.804	222.10>150.10	222.10>96.00	ESI +
109	Methamidophos	1.1	142.20>93.95	142.20>124.90	ESI +
110	Methiocarb	7.146	226.10>121.10	226.10>169.05	ESI +
111	Methomyl	4.467	163.00>87.90	163.00>58.00	ESI +
112	Methoprotryne	7.107	272.20>197.95	272.20>169.95	ESI +
113	Methoxyfenozide	7.915	369.20>312.95	369.20>149.15	ESI +
114	Metobromuron	6.13	259.00>170.00	259.00>148.10	ESI +
115	Metribuzin	5.855	215.10>187.10	215.10>49.10	ESI +
116	Mevinphos	4.954	225.10>127.00	225.10>109.00	ESI +

(con't)

TABLE 142. MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
117	Mexacarbate	7.139	223.10>151.15	223.10>136.15	ESI +
118	Monocrotophos	4.318	240.90>127.10	240.90>193.00	ESI +
119	Monolinuron	5.865	215.10>148.00	215.10>99.10	ESI +
120	Myclobutanil	7.665	289.10>70.05	289.10>125.00	ESI +
121	Neburon	7.532	274.80>57.00	274.80>87.95	ESI +
122	Nitenpyram	4.542	271.10>237.00	271.10>189.20	ESI +
123	Novaluron	8.682	493.00>158.00	493.00>141.05	ESI +
124	Nuarimol	7.336	315.10>251.95	315.10>207.00	ESI +
125	Omethoate	3.645	214.10>125.00	214.10>183.00	ESI +
126	Oxadixyl	6.692	296.20>219.05	296.20>279.05	ESI +
127	Oxamyl	4.412	237.10>72.10	237.10>90.00	ESI +
128	Paclobutrazol	6.918	294.10>70.10	294.10>125.05	ESI +
129	Penconazole	8.214	284.10>158.95	284.10>70.05	ESI +
130	Pencycuron	9.213	329.10>125.00	329.10>218.00	ESI +
131	Phenmedipham	6.87	318.10>168.10	318.10>300.90	ESI +
132	Picoxystrobin	8.935	368.00>145.10	368.00>205.00	ESI +
133	Piperonyl–butoxide	9.913	356.20>119.00	356.20>91.05	ESI +
134	Pirimicarb	6.832	239.20>72.00	239.20>182.05	ESI +
135	Prochloraz	9.593	376.00>307.95	376.00>70.00	ESI +
136	Promecarb	7.004	208.10>109.10	208.10>151.15	ESI +
137	Prometon	6.495	226.20>142.00	226.20>85.95	ESI +
138	Prometryn	7.438	242.10>158.00	242.10>200.15	ESI +
139	Propamocarb	4.052	189.20>102.15	189.20>144.20	ESI +
140	Propargite	10.349	368.20>231.10	368.20>175.10	ESI +
141	Propham	5.948	180.10>138.00	180.10>77.10	ESI +
142	Propiconazole	9.016	342.00>158.90	342.00>69.10	ESI +
143	Propoxur	5.764	209.90>93.10	209.90>168.15	ESI +
144	Pymetrozine	4.825	218.10>105.00	218.10>78.10	ESI +
145	Pyracarbolid	6.018	218.10>125.10	218.10>97.10	ESI +
146	Pyraclostrobin	9.915	388.00>194.10	388.00>164.05	ESI +
147	Pyridaben	11.088	365.20>147.10	365.20>117.00	ESI +
148	Pyrimethanil	7.189	200.10>107.00	200.10>82.00	ESI +
149	Pyriproxyfen	10.295	322.10>184.95	322.10>78.05	ESI +
150	Quinoxyfen	10.122	308.00>197.00	308.00>162.00	ESI +
151	Rotenone	10.734	395.10>213.00	395.10>192.00	ESI +
152	Secbumeton	6.502	226.20>57.00	226.20>100.15	ESI +
153	Siduron	6.693	233.20>137.10	233.20>77.00	ESI +
154	Simetryn	6.31	214.10>96.00	214.10>68.00	ESI +
155	Spinetoram	11.631	748.50>142.15	748.50>98.05	ESI +

(con't)

TABLE 142. MRM transitions (con't)

No	Pesticide	R.T. (min)	Transition 1	Transition 2	Ionization Mode
156	Spinosyn	11.137	732.60>142.20	732.60>98.10	ESI +
157	Spirodiclofen	10.56	411.10>313.05	411.10>71.10	ESI +
158	Spiromesifen	10.279	371.20>273.10	371.20>255.20	ESI +
159	Spirotetramat	8.639	374.10>330.05	374.10>302.00	ESI +
160	Spiroxamine Isomer	7.855	298.20>144.20	298.20>100.15	ESI +
161	Tebuconazole	7.996	308.20>70.05	308.20>125.05	ESI +
162	Tebufenozide	8.225	353.20>297.00	353.20>133.10	ESI +
163	Tebufenpyrad	9.308	334.20>145.15	334.20>147.20	ESI +
164	Tebuthiuron	5.742	229.10>116.00	229.10>172.00	ESI +
165	Teflubenzuron	9.411	378.80>339.00	378.80>358.90	ESI -
166	Temephos	11.042	467.00>418.80	467.00>125.00	ESI +
167	Terbumeton	6.781	225.90>170.00	225.90>114.10	ESI +
168	Terbutryn	7.438	242.10>68.00	242.10>157.95	ESI +
169	Tetraconazole	7.673	372.00>159.00	372.00>70.10	ESI +
170	Thiabendazole	5.457	202.00>131.15	202.00>175.00	ESI +
171	Thiacloprid	6.165	253.00>126.05	253.00>90.10	ESI +
172	Thiamethoxam	4.77	292.00>211.10	292.00>181.10	ESI +
173	Thidiazuron	5.364	221.00>102.00	221.00>128.00	ESI +
174	Thiobencarb	9.009	257.80>125.10	257.80>89.00	ESI +
175	Thiofanox	6.25	241.20>184.00	241.20>57.20	ESI +
176	Thiophanate–methyl	6.346	343.00>311.00	343.00>93.00	ESI +
177	Triadimefon	7.589	294.10>69.00	294.10>196.95	ESI +
178	Triadimenol	6.971	296.10>70.05	298.10>70.05	ESI +
179	Trichlorfon	4.488	257.00>109.10	257.00>220.95	ESI +
180	Tricyclazole	7.029	190.10>136.00	190.10>109.00	ESI +
181	Trifloxystrobin	9.651	409.10>145.10	409.10>205.90	ESI +
182	Triflumizole	8.994	346.00>278.00	346.00>43.15	ESI +
183	Triflumuron	8.344	359.00>156.05	359.00>139.05	ESI +
184	Vamidothion	4.997	288.10>146.05	288.10>58.05	ESI +
185	Zoxamide	8.164	336.00>186.95	336.00>159.00	ESI +

21.5.5 Working solutions

a) **Working solution 1** (W1, 10 µg/ml)

- Prepare from all mixes of stock standard solutions (100 µg/ml).
- Take 100 µl from each mix and complete with acetonitrile so the final volume is 1 ml.
- Use it to prepare working solution 2 (WS2) at 1 µg/ml and to spike LC–MS/MS recovery sample.

b) **Working solution 2** (W2, 1 µg/l)

- Prepared from WS1 by taking 100 and complete with acetonitrile to 1 ml.
- Use it to prepare LC–MS/MS calibration levels.

21.5.6 Calibration standards

For quantitation, a matrix matched calibration curve is generated using known blank samples closely related to the tested samples. The blank sample is subjected to the same extraction and cleanup procedure mentioned above. This is to make sure that the matrix amount in each analyzed sample is constant. The final blank extract is used to prepare four calibration standards as below:

- Level 1 (0.1 µg /ml): Add 10 µl of WS1 + 990 µl final blank extract directly into an autosampler vial;
- Level 2 (0.2 µg /ml): Add 20 µl of WS1 + 980 µl final blank extract directly into an autosampler vial;
- Level 3 (0.5 µg /ml): Add 50 µl of WS1 + 950 µl final blank extract directly into an autosampler vial;
- Level 4 (1 µg /ml): Add 100 µl of WS1 + 900 µl final blank extract directly into an autosampler vial.
- The calibration curve should be valid until check standards deviated from the acceptable criteria (20 % range of the corresponding calibration point value).
- If values deviate, then prepare new calibration curve and check again.

21.6 Procedure

21.6.1 Sample preparation

Vegetables and fruits

a) Chop (rough cuts) around 200 g of the sample and store at -20°C for at least 1 hr or overnight;

b) Homogenize all the received sample, if possible, using a blinder;

c) Homogenize with dry ice, if needed, into a fine free flowing powder;

d) Store the received samples in a freezer at -20°C or colder until analysis.

Powder and liquid samples

a) Store at -20°C for at least 1 hr or overnight;

b) After freezing, weigh 100 g of the sample and transfer to a blinder (if needed), and preserve the other 100 g at -20°C;

c) Homogenize with dry ice (if needed) into a fine free flowing powder;

d) Proceed with the extraction procedure.

21.6.2 Sample extraction

a) For samples with water content > 80%, weigh 10 g of homogenized sample into a clean 50 ml tube.

b) For other samples, add cold distilled water to rehydrate samples according to the Table 143.

TABLE 143. Sample handling and weight

Sample type	Sample weight (g)	Water added (g)	Note
Fruits and vegetables >80 % water content	10	n.a	
Fruits and vegetables 25%–80% water content	10	x	x = 10 g - water amount in 10 g sample
Dried fruits	5	7.5	
Honey	5	10	

n.a: No water added

c) Spike blank samples at concentration 0.05 mg/kg. Let stand for 10 min

d) Add 10 ml of acetonitrile

e) Vortex for 1 min

f) Add buffered QuEChERS extraction packet as indicated (EC, 2015) and include: 4 g $MgSO_4$, 1 g NaCl, 1 g Na–citrate, 0.5 g disodium citrate sesquihydrate

g) Adjust acidic pH (below 3) in matrices such as lemons, limes, currants and raspberry using 5 mol/l NaOH

h) Vortex for 1 min

i) Centrifuge for 5 min at 5°C and 6 000 rpm

21.6.3 Cleanup procedure [dispersive solid-phase extraction (dSPE)]

a) Transfer 1 ml of the supernatant to the required ready to use dSPE tube (2 ml capacity) as shown Table 144.

TABLE 144. Summary of sample cleanup

Sample type	dSPE type
General fruits and vegetables (e.g. zucchini, cabbage, cauliflower, head lettuce, celery, melon etc.)	150 mg $MgSO_4$, 25 mg PSA
Fruits and vegetables with fats and waxes	150 mg $MgSO_4$, 25 mg PSA, 25 mg C18
Pigmented fruits & vegetables with carotenoids and chlorophyll (Spanish, Red & Green sweet pepper, Carrots etc.)	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB (Higher concentration of GCB can be used with high concentration of pigment)
Pigmented fruits and vegetables with fats and waxes (eggplant etc.)	150 mg $MgSO_4$, 25 mg PSA, 7 mg GCB, 25 mg C18

b) Vortex for 1 min (2 min in case of GCB)

c) Centrifuge for 5 min at 5°C and 6 000 rpm to separate the solid material

d) Filter the extract through 0.45 mm PTFE filter

e) Take 0.25 ml of the filtrate

f) Add 0.25 ml acetonitrile

g) Acidify with 5% formic acid in acetonitrile (10 µl to every 1 ml)

h) In labsolutions software: Set sample amount at 1

i) In labsolutions software: Set dilution factor at 2

j) The final extract has a concentration of ca. 0.5 g/ml

k) Sample is ready to be injected for analysis

21.6.4 Instrumental analysis (LC–MS/MS)

The following tool is used: High–Speed Analysis Method (for 646 Residual Pesticide Components)—LC–MS/MS Method Package—Residual Pesticides Version2 —SHIMADZU.

The LC, mobile phase and MS conditions/parameters are summarized in Tables 145 and 146.

TABLE 145. Summary of LC conditions

Instrument	Nexera (2040c) UHPLC system, Shimadzu
Analytical Column	Restek Raptor Biphenyl (100×2.1 mm, 2.7 µm) (Cat#: 9309A12)
Column Oven Temperature	35°C
	2 µl
Flow rate	0.4 ml/min
Mobile phase A	2 mmol/l ammonium formate + 0.002% formic acid – Water
Mobile phase B	2 mmol/l ammonium formate + 0.002% formic acid – Methanol

The mobile phase as reported elsewhere (Alghareb and Alfararjeh, 2018) starts with 97% of (A) run for a 1 min and dropped to 45 in 3 min; B is the maintained at 100 % until the 12th min when it is dropped to 3% until the 15th min.

TABLE 146. Summary of MS conditions

Nebulizing Gas Flow Rate	3 l/min
Drying Gas Flow Rate	10 l/min
Heating Gas Flow Rate	10 l/min
Interface Temperature	350°C
Desolvation Line Temperature	150°C
Block Heater Temperature	300°C
Ionization mode	ESI

21.6.5 Calculation and interpretation of results

a) Identification

- The retention time of any analyte in the tested sample extract should correspond to that of the matrix matched calibration standard with a tolerance of ± 0.1 min.
- Extracted ion chromatograms of tested sample extracts should have peaks of a similar shape and response ratio to those obtained from matrix matched calibration standards analyzed at comparable concentrations.

- Chromatographic peaks from different selective ions for the analyte should fully overlap.
- The ion ratio should not deviate more than 30% (relative).

b) Reporting results

- The results should always be reported and expressed in mg/kg.
- Where the residue definition includes more than one analyte, the respective sum of analytes should be calculated as stated in the residue definition and should be used for checking compliance with the MRL.
- Residues for individual analytes below the reporting limits should be reported as <MRL mg/kg.
- Where good mixing of samples has been undertaken, the RSD of replicate results of the test portions should normally not exceed 30%.
- Residue data do not have to be adjusted for recovery when the mean recovery is within the range of 70%–120%.
- If residues data are adjusted for recovery, this should be stated in the report.
- The result should be reported together with the expanded measurement uncertainty (MU) as follows: Result = x ± U (units), with x representing the measured value
- The sample is considered non–compliant if x-U > MRL.

21.6.6 Method validation

- Commodities used for fortification are an equal mix of cucumber, strawberry and jam samples and are free of the pesticides analyzed.
- These produce are chosen to represent group 1 (High water content), group 2 (High acid content) and group 3 (High sugar content) commodities (EC, 2017).
- The validation is performed at two spiking levels (0.01 mg/kg and 0.05 mg/kg).
- Five spiked samples and a blank are analyzed at each level. In total eleven samples are analyzed.
- Standard spiking solutions are prepared by mixing all the 185 pesticides used for the validation method at a concentration of 10 μg/ml in acetonitrile.
- The pesticides to be accepted as validated the following criteria for precision and trueness (EC, 2017) should be fulfilled:
- The repeatability RSD should be ≤ 20%
- The average relative recovery should be between 70% and 120%
- If the above-mentioned criteria have been met, the LOQ is stated.
- Linearity of the LC–MS/MS system is evaluated by assessing the signal responses of the target analytes from matrix matched calibration solutions prepared by spiking blank extracts at six concentration levels corresponding to concentrations: 0.01 μg/ml, 0.02 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.15 μg/ml and 0.2 μg/ml.
- The six-point calibration curve should demonstrate excellent linearity with $R^2 \geq 0.99$ for all analytes in the scope of this study (AOAC, 2012)

The limit of quantitation is defined here (EC, 2017) as the lowest validated spike level meeting the method performance acceptability criteria.

The method validation study is summarized in Tables 147 and 148.

TABLE 147. Summary of method validation

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
1	(E)–Fenpyroximate	4.4	109.7	4.7	99.7	0.0015
2	Acephate	4.7	102.5	2.6	94.1	0.0014
3	Acetamiprid	5.4	101.7	2.6	102.8	0.0016
4	Acibenzolar–s–methyl	8.8	102.1	4.5	102.3	0.0027
5	Aldicarb	10.3	80.5	5.6	106.3	0.0025
6	Aldicarb–sulfone	3.4	97.1	1.5	101.4	0.0010
7	Aldicarb–sulfoxide	5.8	120.5	2.4	101.0	0.0021
8	Ametryn	4.8	116.4	5.0	98.6	0.0017
9	Aminocarb	5.7	92.6	1.6	98.2	0.0016
10	Azoxystrobin	3.5	114.5	2.6	102.8	0.0012
11	Benalaxyl	5.4	108.4	2.6	105.2	0.0018
12	Benzoximate	5.6	93.0	3.2	99.9	0.0016
13	Bitertanol	7.0	107.8	4.8	103.7	0.0023
14	Boscalid	12.4	117.7	6.5	100.6	0.0044
15	Bromuconazole	7.9	94.2	4.0	105.6	0.0022
16	Bupirimate	6.5	98.8	3.5	102.0	0.0019
17	Buprofezin	7.3	103.0	4.2	100.5	0.0022
18	Butafenacil	5.9	102.8	3.7	101.3	0.0018
19	Carbaryl (NAC)	6.0	109.4	1.9	107.9	0.0020
20	Carbendazim	5.2	99.2	2.8	99.3	0.0016
21	Carbetamide	5.0	97.9	3.2	100.9	0.0015
22	Carbofuran	3.4	114.1	2.2	112.7	0.0012
23	Carbofuran–3-hydroxy	5.1	89.9	4.0	100.6	0.0014
24	Carboxin	**31.2**	**56.7**	5.7	105.4	0.0091
25	Carfentrazone–ethyl	**34.4**	**135.8**	9.6	115.6	0.0166
26	Chlorantraniliprole	7.3	107.6	3.0	101.0	0.0023
27	Chlorfluazuron	**22.3**	**68.1**	6.4	106.8	0.0102
28	Chlorotoluron	8.1	96.5	4.3	101.8	0.0024
29	Chloroxuron	12.8	95.4	6.0	103.3	0.0037
30	Clethodim	5.2	88.2	2.9	88.2	0.0014
31	Clofentezine	6.3	96.2	5.0	104.0	0.0018
32	Clothianidin	3.6	96.6	2.8	104.1	0.0010
33	Cyazofamid	4.9	102.7	4.6	106.4	0.0015
34	Cycluron	18.9	82.8	4.9	100.1	0.0047
35	Cymoxanil	18.2	**129.8**	5.3	110.4	0.0087
36	Cyproconazole	15.3	81.9	7.3	103.5	0.0037
37	Cyprodinil	4.3	97.4	3.2	101.5	0.0012
38	Desmedipham	4.5	88.6	3.2	109.3	0.0012
39	Diclobutrazol	11.6	92.2	5.3	105.5	0.0032

(con't)

TABLE 147. Summary of method validation (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
40	Dicrotophos	14.2	**155.2**	7.8	108.7	0.0126
41	Diethofencarb	5.8	82.8	3.4	101.7	0.0014
42	Difenoconazole	4.5	102.7	1.6	101.4	0.0014
43	Diflubenzuron	8.4	95.2	3.9	104.9	0.0024
44	Dimethoate	14.4	83.2	4.8	109.8	0.0036
45	Dimethomorph	7.0	105.4	2.1	100.0	0.0022
46	Dimoxystrobin	3.0	106.7	3.0	102.5	0.0010
47	Diniconazole	10.5	94.8	5.4	101.5	0.0030
48	Dinotefuran	5.1	89.6	3.0	97.2	0.0014
49	Dioxacarb	6.9	85.7	4.8	99.6	0.0018
50	Diuron (DCMU)	6.8	93.8	5.4	104.5	0.0019
51	Emamectin B1a	11.9	94.4	3.2	95.7	0.0034
52	Emamectin B1b	9.8	89.6	5.1	94.7	0.0026
53	Epoxiconazole	12.5	87.2	4.2	103.8	0.0033
54	Eprinomectin	**40.4**	**103.2**	9.5	111.4	0.0797
55	Etaconazole	9.4	78.4	4.4	102.3	0.0022
56	Ethiofencarb	8.3	79.1	1.9	101.8	0.0020
57	Ethiprole	11.3	81.0	3.6	108.8	0.0027
58	Ethirimol	6.1	98.7	3.2	93.5	0.0018
59	Ethofumesate	20.3	70.1	7.2	106.7	0.0043
60	Etoxazole	7.4	101.5	3.4	103.7	0.0022
61	Famoxadone	8.4	**121.9**	6.4	102.2	0.0098
62	Fenamidone	7.4	107.6	4.4	102.4	0.0024
63	Fenarimol	10.3	96.5	4.3	101.5	0.0030
64	Fenazaquin	12.7	105.6	12.9	93.6	0.0040
65	Fenbuconazole	6.6	89.0	3.3	102.8	0.0018
66	Fenhexamid	13.5	85.8	2.8	94.5	0.0035
67	Fenobucarb	8.9	110.2	3.5	108.0	0.0030
68	Fenoxycarb	11.7	97.2	4.1	103.1	0.0034
69	Fenpropimorph	3.6	116.7	2.1	102.3	0.0013
70	Fenuron	20.1	99.8	15.3	89.0	0.0060
71	Fipronil	2.9	106.1	3.7	110.2	0.0009
72	Flonicamid	**42.8**	**61.9**	7.5	105.7	0.0119
73	Fluazinam	4.8	113.6	2.4	102.5	0.0017
74	Fludioxonil	8.9	106.4	5.9	99.0	0.0028
75	Flufenacet	13.7	84.8	4.0	106.0	0.0035
76	Flufenoxuron	8.8	73.4	3.3	100.4	0.0019
77	Fluometuron	7.6	94.6	3.5	106.3	0.0022
78	Fluoxastrobin	9.1	102.5	3.0	106.5	0.0028

(con't)

TABLE 147. Summary of method validation (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
79	Fluquinconazole	13.4	98.4	3.1	104.6	0.0039
80	Flusilazole	9.5	92.0	5.3	105.5	0.0026
81	Flutolanil	3.7	98.9	3.3	106.7	0.0011
82	Flutriafol	11.4	89.7	3.1	102.4	0.0031
83	Forchlorfenuron	16.0	90.7	4.1	97.2	0.0044
84	Formetanate	4.2	104.6	2.7	89.2	0.0013
85	Furalaxyl	2.9	102.1	2.3	102.4	0.0009
86	Furathiocarb	4.3	99.8	2.6	105.2	0.0013
87	Halofenozide	13.7	**52.7**	5.0	107.4	0.0080
88	Hexaconazole	7.9	96.4	5.6	100.5	0.0023
89	Hexaflumuron	9.8	**54.4**	3.8	116.0	0.0067
90	Hexythiazox	2.7	112.1	1.4	105.0	0.0009
91	Imazalil	7.9	99.6	3.1	99.7	0.0024
92	Imidacloprid	**41.3**	**38.6**	3.8	110.0	0.0062
93	Indoxacarb	10.1	81.4	7.0	111.7	0.0025
94	Ipconazole	7.6	95.7	3.4	107.2	0.0022
95	Iprovalicarb	8.2	119.0	3.7	104.2	0.0029
96	Isocarbophos	12.7	107.6	10.4	92.2	0.0041
97	Isoprocarb	A	A	3.6	106.7	0.0058
98	Isoproturon	12.2	94.9	3.8	101.2	0.0035
99	Kresoxim–methyl	10.2	90.8	2.7	106.5	0.0028
100	Linuron	2.8	96.3	2.8	103.5	0.0008
101	Mandipropamid	5.6	100.1	2.6	104.7	0.0017
102	Mefenacet	5.3	99.1	3.3	101.2	0.0016
103	Mepanipyrim	15.1	85.3	6.3	103.8	0.0039
104	Mepronil	10.4	96.4	4.0	105.8	0.0030
105	Metaflumizone	10.8	98.1	5.0	98.7	0.0032
106	Metalaxyl	6.8	107.1	2.4	102.5	0.0022
107	Metconazole	8.6	104.9	3.4	105.2	0.0027
108	Methabenzthiazuron	9.0	70.4	2.7	104.0	0.0019
109	Methamidophos	A	A	1.8	86.1	0.0023
110	Methiocarb	12.9	104.8	3.6	108.5	0.0041
111	Methomyl	5.8	112.6	3.0	104.7	0.0020
112	Methoprotryne	7.6	111.5	3.4	101.3	0.0026
113	Methoxyfenozide	9.1	89.8	5.5	103.7	0.0025
114	Metobromuron	9.6	91.7	4.1	106.2	0.0026
115	Metribuzin	6.3	102.0	3.4	103.8	0.0019
116	Mevinphos	4.6	105.4	2.4	98.6	0.0014
117	Mexacarbate	6.4	96.7	3.5	104.4	0.0019
118	Monocrotophos	3.5	108.0	1.2	99.0	0.0011

(con't)

TABLE 147. Summary of method validation (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
119	Monolinuron	10.1	88.0	3.2	105.7	0.0027
120	Myclobutanil	7.3	71.9	19.7	85.4	0.0016
121	Neburon	10.5	102.7	12.6	95.7	0.0032
122	Nitenpyram	8.6	94.2	2.3	98.1	0.0024
123	Novaluron	**32.8**	**60.1**	7.5	97.4	0.0109
124	Nuarimol	10.1	95.5	3.4	102.6	0.0029
125	Omethoate	15.5	117.3	7.4	101.4	0.0054
126	Oxadixyl	6.6	106.8	3.1	99.4	0.0021
127	Oxamyl	2.7	113.7	2.3	101.0	0.0009
128	Paclobutrazol	3.3	98.9	3.0	107.1	0.0010
129	Penconazole	7.5	84.7	4.4	99.8	0.0019
130	Pencycuron	14.9	81.7	2.2	106.4	0.0037
131	Phenmedipham	5.1	86.5	3.4	103.6	0.0013
132	Picoxystrobin	3.4	110.9	2.5	102.7	0.0011
133	Piperonyl–butoxide	5.6	107.0	2.3	104.1	0.0018
134	Pirimicarb	2.9	113.0	2.9	96.7	0.0010
135	Prochloraz	4.7	109.7	1.8	102.0	0.0015
136	Promecarb	6.7	105.8	3.9	108.4	0.0021
137	Prometon	3.9	113.7	2.9	101.7	0.0013
138	Prometryn	5.4	111.4	2.9	100.2	0.0018
139	Propamocarb	5.3	110.0	2.6	89.7	0.0017
140	Propargite	4.2	97.1	1.8	102.2	0.0012
141	Propham	**20.4**	100.4	6.5	100.8	0.0098
142	Propiconazole	3.8	95.9	2.7	106.3	0.0011
143	Propoxur	5.5	92.0	1.9	104.6	0.0015
144	Pymetrozine	15.1	72.5	13.1	52.3	0.0033
145	Pyracarbolid	16.1	82.8	2.0	103.9	0.0040
146	Pyraclostrobin	3.9	105.3	3.9	103.9	0.0012
147	Pyridaben	4.0	108.0	3.2	105.1	0.0013
148	Pyrimethanil	5.3	107.8	3.5	100.6	0.0017
149	Pyriproxyfen	14.3	80.0	3.6	104.5	0.0034
150	Quinoxyfen	6.6	96.5	5.8	101.3	0.0019
151	Rotenone	3.4	111.1	2.2	99.7	0.0011
152	Secbumeton	9.2	109.2	5.4	98.1	0.0030
153	Siduron	8.2	92.1	1.4	101.7	0.0023
154	Simetryn	2.1	116.0	3.3	98.7	0.0007
155	Spinetoram	7.3	92.1	12.1	91.3	0.0020
156	Spinosyn	5.4	**127.0**	7.0	107.5	0.0113
157	Spirodiclofen	A	A	5.5	108.9	0.0090
158	Spiromesifen	**34.5**	**56.8**	6.4	108.5	0.0105

(con't)

TABLE 147. Summary of method validation (con't)

		0.01 mg /kg		0.05 mg /kg		
No.	Compound	RSD %	Rec %	RSD %	Rec %	LOD
159	Spirotetramat	5.0	111.0	3.2	101.2	0.0016
160	Spiroxamine Isomer	2.9	117.4	1.4	100.5	0.0010
161	Tebuconazole	5.9	102.6	3.2	101.6	0.0018
162	Tebufenozide	13.8	100.2	4.8	105.5	0.0041
163	Tebufenpyrad	11.4	107.0	6.9	103.7	0.0036
164	Tebuthiuron	4.0	81.6	3.6	107.6	0.0010
165	Teflubenzuron	11.0	**66.1**	6.5	115.1	0.0113
166	Temephos	14.0	106.9	7.2	106.6	0.0045
167	Terbumeton	4.3	101.5	2.8	102.9	0.0013
168	Terbutryn	9.6	103.9	3.7	102.1	0.0030
169	Tetraconazole	8.9	84.2	4.5	107.6	0.0023
170	Thiabendazole	19.1	100.7	4.2	95.5	0.0058
171	Thiacloprid	4.2	87.4	2.0	102.9	0.0011
172	Thiamethoxam	19.9	80.9	3.4	106.1	0.0048
173	Thidiazuron	7.8	87.0	6.3	99.4	0.0020
174	Thiobencarb	3.9	98.7	1.3	102.5	0.0012
175	Thiofanox	**A**	**A**	5.4	112.8	0.0092
176	Thiophanate–methyl	**A**	**A**	7.5	98.7	0.0111
177	Triadimefon	4.8	96.3	2.6	101.0	0.0014
178	Triadimenol	6.0	94.1	2.5	105.7	0.0017
179	Trichlorfon	**23.6**	106.6	7.2	102.9	0.0111
180	Tricyclazole	5.9	89.6	2.9	99.0	0.0016
181	Trifloxystrobin	8.0	96.9	4.6	105.9	0.0023
182	Triflumizole	4.2	110.8	2.7	102.0	0.0014
183	Triflumuron	3.3	102.3	2.0	101.4	0.0010
184	Vamidothion	3.6	113.1	2.1	98.8	0.0012
185	Zoxamide	5.7	81.1		1.8	105.0

Bold numbers: Recovery and/or RSD out of acceptable range
A: Peak shape error or no peak was detected

TABLE 148. Summary of method validation, LOQ

No.	Compound	LOQ	R^2	Instrumental concentration range
1	(E)–Fenpyroximate	0.01	0.9999	0.01–0.200
2	Acephate	0.01	1.0000	0.01–0.200
3	Acetamiprid	0.01	0.9999	0.01–0.200
4	Acibenzolar–s–methyl	0.01	0.9994	0.01–0.200
5	Aldicarb	0.01	0.9990	0.01–0.200
6	Aldicarb–sulfone	0.01	0.9997	0.01–0.200
7	Aldicarb–sulfoxide	0.01	0.9997	0.01–0.200
8	Ametryn	0.01	0.9997	0.01–0.200
9	Aminocarb	0.01	0.9996	0.01–0.200

(con't)

TABLE 148. Summary of method validation, LOQ (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
10	Azoxystrobin	0.01	0.9996	0.01–0.200
11	Benalaxyl	0.01	0.9996	0.01–0.200
12	Benzoximate	0.01	0.9995	0.01–0.200
13	Bitertanol	0.01	0.9997	0.01–0.200
14	Boscalid	0.01	0.9996	0.01–0.200
15	Bromuconazole	0.01	0.9993	0.01–0.200
16	Bupirimate	0.01	0.9999	0.01–0.200
17	Buprofezin	0.01	0.9997	0.01–0.200
18	Butafenacil	0.01	0.9998	0.01–0.200
19	Carbaryl (NAC)	0.01	0.9994	0.01–0.200
20	Carbendazim	0.01	0.9999	0.01–0.200
21	Carbetamide	0.01	0.9998	0.01–0.200
22	Carbofuran	0.01	0.9997	0.01–0.200
23	Carbofuran–3-hydroxy	0.01	0.9994	0.01–0.200
24	Carboxin	0.05	0.9964	0.01–0.200
25	Carfentrazone–ethyl	0.05	0.9919	0.01–0.200
26	Chlorantraniliprole	0.01	0.9998	0.01–0.200
27	Chlorfluazuron	0.05	0.9934	0.01–0.200
28	Chlorotoluron	0.01	0.9999	0.01–0.200
29	Chloroxuron	0.01	0.9992	0.01–0.200
30	Clethodim	0.01	0.9998	0.01–0.200
31	Clofentezine	0.01	0.9993	0.01–0.200
32	Clothianidin	0.01	0.9999	0.01–0.200
33	Cyazofamid	0.01	0.9998	0.01–0.200
34	Cycluron	0.01	0.9966	0.01–0.200
35	Cymoxanil	0.05	0.9982	0.01–0.200
36	Cyproconazole	0.01	0.9966	0.01–0.200
37	Cyprodinil	0.01	0.9998	0.01–0.200
38	Desmedipham	0.01	0.9993	0.01–0.200
39	Diclobutrazol	0.01	0.9993	0.01–0.200
40	Dicrotophos	0.05	0.9990	0.01–0.200
41	Diethofencarb	0.01	0.9989	0.01–0.200
42	Difenoconazole	0.01	0.9997	0.01–0.200
43	Diflubenzuron	0.01	0.9998	0.01–0.200
44	Dimethoate	0.01	0.9980	0.01–0.200
45	Dimethomorph	0.01	0.9997	0.01–0.200
46	Dimoxystrobin	0.01	0.9998	0.01–0.200
47	Diniconazole	0.01	0.9993	0.01–0.200
48	Dinotefuran	0.01	0.9998	0.01–0.200
49	Dioxacarb	0.01	0.9994	0.01–0.200
50	Diuron (DCMU)	0.01	0.9996	0.01–0.200

(con't)

TABLE 148. Summary of method validation, LOQ (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
51	Emamectin B1a	0.01	0.9999	0.01–0.200
52	Emamectin B1b	0.01	0.9996	0.01–0.200
53	Epoxiconazole	0.01	0.9993	0.01–0.200
54	Eprinomectin	0.05	0.9989	0.01–0.200
55	Etaconazole	0.01	0.9993	0.01–0.200
56	Ethiofencarb	0.01	0.9990	0.01–0.200
57	Ethiprole	0.01	0.9972	0.01–0.200
58	Ethirimol	0.01	1.0000	0.01–0.200
59	Ethofumesate	0.01	0.9973	0.01–0.200
60	Etoxazole	0.01	0.9996	0.01–0.200
61	Famoxadone	0.05	0.9998	0.01–0.200
62	Fenamidone	0.01	0.9993	0.01–0.200
63	Fenarimol	0.01	0.9997	0.01–0.200
64	Fenazaquin	0.01	0.9992	0.01–0.200
65	Fenbuconazole	0.01	0.9996	0.01–0.200
66	Fenhexamid	0.01	0.9996	0.01–0.200
67	Fenobucarb	0.01	0.9999	0.01–0.200
68	Fenoxycarb	0.01	0.9999	0.01–0.200
69	Fenpropimorph	0.01	0.9995	0.01–0.200
70	Fenuron	0.01	0.9997	0.01–0.200
71	Fipronil	0.01	0.9999	0.01–0.200
72	Flonicamid	0.05	0.9987	0.01–0.200
73	Fluazinam	0.01	0.9995	0.01–0.200
74	Fludioxonil	0.01	0.9996	0.01–0.200
75	Flufenacet	0.01	0.9998	0.01–0.200
76	Flufenoxuron	0.01	0.9984	0.01–0.200
77	Fluometuron	0.01	0.9999	0.01–0.200
78	Fluoxastrobin	0.01	0.9992	0.01–0.200
79	Fluquinconazole	0.01	0.9999	0.01–0.200
80	Flusilazole	0.01	0.9980	0.01–0.200
81	Flutolanil	0.01	0.9987	0.01–0.200
82	Flutriafol	0.01	0.9996	0.01–0.200
83	Forchlorfenuron	0.01	0.9996	0.01–0.200
84	Formetanate	0.01	0.9995	0.01–0.200
85	Furalaxyl	0.01	0.9999	0.01–0.200
86	Furathiocarb	0.01	0.9999	0.01–0.200
87	Halofenozide	0.05	0.9938	0.01–0.200
88	Hexaconazole	0.01	0.9994	0.01–0.200
89	Hexaflumuron	0.05	0.9943	0.01–0.200
90	Hexythiazox	0.01	0.9999	0.01–0.200
91	Imazalil	0.01	1.0000	0.01–0.200

(con't)

TABLE 148. Summary of method validation, LOQ (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
92	Imidacloprid	0.05	0.9943	0.01–0.200
93	Indoxacarb	0.01	0.9968	0.01–0.200
94	Ipconazole	0.01	0.9996	0.01–0.200
95	Iprovalicarb	0.01	0.9986	0.01–0.200
96	Isocarbophos	0.01	0.9989	0.01–0.200
97	Isoprocarb	0.05	0.9997	0.01–0.200
98	Isoproturon	0.01	0.9999	0.01–0.200
99	Kresoxim–methyl	0.01	0.9998	0.01–0.200
100	Linuron	0.01	0.9998	0.01–0.200
101	Mandipropamid	0.01	0.9998	0.01–0.200
102	Mefenacet	0.01	0.9999	0.01–0.200
103	Mepanipyrim	0.01	0.9990	0.01–0.200
104	Mepronil	0.01	0.9997	0.01–0.200
105	Metaflumizone	0.01	1.0000	0.01–0.200
106	Metalaxyl	0.01	0.9999	0.01–0.200
107	Metconazole	0.01	0.9999	0.01–0.200
108	Methabenzthiazuron	0.01	0.9977	0.01–0.200
109	Methamidophos	0.05	0.9998	0.01–0.200
110	Methiocarb	0.01	0.9993	0.01–0.200
111	Methomyl	0.01	0.9998	0.01–0.200
112	Methoprotryne	0.01	0.9998	0.01–0.200
113	Methoxyfenozide	0.01	0.9998	0.01–0.200
114	Metobromuron	0.01	0.9992	0.01–0.200
115	Metribuzin	0.01	0.9999	0.01–0.200
116	Mevinphos	0.01	1.0000	0.01–0.200
117	Mexacarbate	0.01	0.9996	0.01–0.200
118	Monocrotophos	0.01	0.9998	0.01–0.200
119	Monolinuron	0.01	0.9985	0.01–0.200
120	Myclobutanil	0.01	0.9987	0.01–0.200
121	Neburon	0.01	0.9980	0.01–0.200
122	Nitenpyram	0.01	0.9983	0.01–0.200
123	Novaluron	0.05	0.9990	0.01–0.200
124	Nuarimol	0.01	0.9986	0.01–0.200
125	Omethoate	0.01	0.9855	0.01–0.200
126	Oxadixyl	0.01	1.0000	0.01–0.200
127	Oxamyl	0.01	0.9999	0.01–0.200
128	Paclobutrazol	0.01	0.9997	0.01–0.200
129	Penconazole	0.01	1.0000	0.01–0.200
130	Pencycuron	0.01	0.9986	0.01–0.200
131	Phenmedipham	0.01	0.9956	0.01–0.200
132	Picoxystrobin	0.01	0.9998	0.01–0.200

(con't)

TABLE 148. Summary of method validation, LOQ (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
133	Piperonyl–butoxide	0.01	0.9998	0.01–0.200
134	Pirimicarb	0.01	1.0000	0.01–0.200
135	Prochloraz	0.01	0.9998	0.01–0.200
136	Promecarb	0.01	0.9997	0.01–0.200
137	Prometon	0.01	0.9997	0.01–0.200
138	Prometryn	0.01	0.9999	0.01–0.200
139	Propamocarb	0.01	0.9998	0.01–0.200
140	Propargite	0.01	0.9996	0.01–0.200
141	Propham	0.05	0.9997	0.01–0.200
142	Propiconazole	0.01	0.9995	0.01–0.200
143	Propoxur	0.01	0.9992	0.01–0.200
144	Pymetrozine	0.01	0.9985	0.01–0.200
145	Pyracarbolid	0.01	0.9993	0.01–0.200
146	Pyraclostrobin	0.01	0.9997	0.01–0.200
147	Pyridaben	0.01	1.0000	0.01–0.200
148	Pyrimethanil	0.01	0.9998	0.01–0.200
149	Pyriproxyfen	0.01	0.9992	0.01–0.200
150	Quinoxyfen	0.01	0.9998	0.01–0.200
151	Rotenone	0.01	0.9999	0.01–0.200
152	Secbumeton	0.01	0.9997	0.01–0.200
153	Siduron	0.01	0.9996	0.01–0.200
154	Simetryn	0.01	0.9998	0.01–0.200
155	Spinetoram	0.01	0.9995	0.01–0.200
156	Spinosyn	0.05	0.9998	0.01–0.200
157	Spirodiclofen	0.05	0.9989	0.01–0.200
158	Spiromesifen	0.05	0.9915	0.01–0.200
159	Spirotetramat	0.01	0.9998	0.01–0.200
160	Spiroxamine Isomer	0.01	0.9997	0.01–0.200
161	Tebuconazole	0.01	0.9997	0.01–0.200
162	Tebufenozide	0.01	0.9997	0.01–0.200
163	Tebufenpyrad	0.01	0.9996	0.01–0.200
164	Tebuthiuron	0.01	0.9981	0.01–0.200
165	Teflubenzuron	0.05	0.9963	0.01–0.200
166	Temephos	0.01	0.9992	0.01–0.200
167	Terbumeton	0.01	0.9997	0.01–0.200
168	Terbutryn	0.01	0.9993	0.01–0.200
169	Tetraconazole	0.01	0.9994	0.01–0.200
170	Thiabendazole	0.01	0.9999	0.01–0.200
171	Thiacloprid	0.01	0.9992	0.01–0.200

(con't)

TABLE 148. Summary of method validation, LOQ (con't)

No.	Compound	LOQ	R^2	Instrumental concentration range
172	Thiamethoxam	0.01	0.9988	0.01–0.200
173	Thidiazuron	0.01	0.9999	0.01–0.200
174	Thiobencarb	0.01	1.0000	0.01–0.200
175	Thiofanox	0.05	0.9969	0.01–0.200
176	Thiophanate–methyl	0.05	0.9989	0.01–0.200
177	Triadimefon	0.01	0.9999	0.01–0.200
178	Triadimenol	0.01	0.9993	0.01–0.200
179	Trichlorfon	0.05	0.9973	0.01–0.200
180	Tricyclazole	0.01	0.9993	0.01–0.200
181	Trifloxystrobin	0.01	0.9997	0.01–0.200
182	Triflumizole	0.01	0.9997	0.01–0.200
183	Triflumuron	0.01	0.9998	0.01–0.200
184	Vamidothion	0.01	0.9996	0.01–0.200
185	Zoxamide	0.01	0.9986	0.01–0.200

21.6.7 Schematic diagram of extraction procedure

SAMPLE EXTRACTION

1. If water content of sample is below 80%, rehydrate by adding cold distilled water (refer to the table).
2. Add internal standard or spiking mix if required.
3. Add 10 ml of acetonitrile
4. Vortex for 1 min.
5. Use ready to use buffered QuEChERS extraction packet as indicated elsewhere (EC, 2015). Tear the top of the packet and empty into the extraction tube.
6. Vortex for 1 min.
7. Centrifuge at 6 000 rpm for 5 min.

↓

SAMPLE CLEANUP

1. Transfer 1 ml of the supernatant to the dSPE tube (choose dSPE type depending on the matrix)
2. Vortex or shake vigorously by hand for 30 s (2 min in case of GCB).
3. Centrifuge at 6 000 rpm 5 min.
4. Take 0.25 ml of the filtrate
5. Add 0.25 ml acetonitrile
6. Acidify with 5% formic acid in acetonitrile (10 µl to every 1 ml)
7. Sample is ready to be injected

22

Confirmatory method for determination of triphenylmethane dyes in aquaculture products by LC–MS/MS

22.1 Introduction

Malachite green (MG), a dye originally used in the textile industry, is used illegally in the aquaculture industry to control ecto–parasites and fungal infections on fish eggs, fingerlings and adult fish due to low cost, high efficacy and lack of alternatives. The veterinary use of dyes is forbidden by regulations such as in the EU (EEC, 1990). Proof of its usage can be found in the fish products. The criteria for unambiguous identification of these analytes are defined elsewhere (EC, 2002). The MRPL for residue confirmation methods is 2 µg/kg for the sum of MG and its metabolite leucomalachite green (LMG); the same limit will soon apply to triphenylmethane.

22.2 Scope and objective

This method allows the determination of residues of MG, LMG, Crystal violet (CV), and leucocrystal violet (LCV) in aquaculture products with a decision limit below the MRPL. The following method has been adapted for the screening, quantification and confirmation of the dyes in fish.

22.3 Principle of the method

The method involves extraction of dyes from the matrix with hydroxylamine hydrochloride (5 g/l) solution and acetonitrile. Quantification is by an HPLC with C18 type grafted silica column and positive mode detection with tandem mass spectrometry (MS/MS).

22.4 Safety considerations and precautions

All the solutions containing dyes should be protected from light. This method requires knowledge by the operator of the basic rules for handling chemicals and solvents. As much as possible, work should be implemented under a fume hood. All necessary precautions should be taken to avoid contamination when handling standards and throughout extraction and purification operations. Samples are to be stored in a freezer at approximately -18°C until analysis.

22.5 Materials

22.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Ultrapure water; Acetonitrile for HPLC; Methanol for HPLC; Formic acid; Ammonium acetate and Hydroxylamine hydrochloride.

22.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: FLEXAR binary HPLC pump or equivalent; FLEXAR automatic injector or equivalent; Phenomenex column: RP–C18, 4 µm, 50×2 mm; Precolumn C18, 3.5 µm, 2.1×10 mm; Liquid chromatograph-mass spectrometer (LC – MS/MS): API 4 000 Applied Biosystems (or comparable) with turbo ion-spray; interface and Analyst software; Analytical balance; Volumetric flasks; Micropipettes; Centrifuge tubes; Vortex mixer; Centrifuge; Rotatory shaker; Meat mincer; Rotatory agitator; 0.45 µm filters.

22.5.3 Solutions

The solutions (and how they are prepared) include:

- Hydroxylamine hydrochloride (5 g/l): Weigh 500 mg of hydroxylamine hydrochloride in 100 ml of demineralized water.
- Ammonium acetate solution (2 mmol/l): Prepare a solution of 0.2 mol/l ammonium acetate (1.45 g in 100 ml of demineralized water) then dilute to the 1/100th.
- Preparation mobile phase

  Mobile phase A:

  - Add 1.54 g of ammonium acetate to 90 ml of water and mix thoroughly until all salts are dissolved;
  - Filter solution through a 0.2 µm HPLC–certified Nylon filter
  - Add water to the 95 mark;
  - Add water to final volume of 100 ml, add 100 µl of formic acid, degas and transfer to mobile phase container;

  Mobile phase B:

  - Add 1 ml of ammonium acetate 2M to a flask, complete to 100 ml with Acetonitrile;
  - Acidify with 100 µl of formic acid.

22.5.4 Standards

22.5.4.1 Reference Standards

The standards include: Malachite Green (MG - min 95%) Sigma type; Leucomalachite green (LMG ≥95%) Sigma type; Bright green (BG ≥ 95%) Sigma type; Deuterated leucomalachite D5 (LMG – D5, ≥95%) Witega type.

22.5.4.2 Stock solutions (1 000 mg/l)

Individual stock (IS) solutions (1000 mg/l) are prepared by dissolving 1 mg of each compound in 1 ml of methanol. These can be stored for up to 6 months at -20°C.

22.5.4.3 Intermediate solutions (10 mg/l)

Using individual stock solutions, an intermediate solution is prepared with a concentration of 10 mg/l for each dye in methanol. These intermediate solutions can be stored up to 3 months in a fridge (+4°C).

22.5.4.4 Working solutions

Working solutions are prepared by diluting a mix of the intermediate solutions in water to get the following concentrations: 5 µg/l, 10 µg/l, 15 µg/l, 20 µg/l and 40 µg/l.

22.5.4.5 Internal standard

An intermediate solution of LMG–D5 (internal standard) is prepared by diluting the stock solution in methanol to get a 10 mg/l solution. This solution can be stored for up to 3 months in the fridge (+4°C). By diluting this solution in methanol, a working solution of the internal standard is obtained with a concentration of 10 mg/l.

22.6 Procedure

22.6.1 Sample preparation

a) After cutting partially thawed samples into smaller pieces in a conventional meat mincer, 1 g of homogenized meat is transferred to a centrifuge tube with 900 µl of ultrapure water.

b) After adding 100 µl of the internal standard solution (LMG–D5) at a 40 µg/l concentration, mix the solution using a vortex then let it rest for 10 min.

22.6.2 Quality control samples

a) Prepare blank and spiked samples by preparing 6 tubes each containing 1 g ± 0.1 g of the used matrix that does not contain any dye.

b) Add 100 µl of internal standard in each tube.

c) Add 900 µl of ultrapure water in the first tube to get a blank sample.

d) Add 100 µl of each working solution (at 5 µg/l, 10 µg/l, 15 µg/l, 20 µg/l and 40 µg/l) then 800 µl of ultrapure water in each tube to get spiked samples with 0.5 µg/kg, 1 µg/kg, 1.5 µg/kg, 2 µg/kg and 4 µg/kg concentration respectively.

e) Mix each tube using the vortex and let it rest for 10 min.

22.6.3 Sample extraction

a) Add 1 ml of hydroxylamine hydrochloride solution (5 g/l).

b) Close the tubes, and mix using the vortex and let it stand for 10 min.

c) Add 4 ml of acetonitrile.

d) Close the tubes and agitate using the rotatory agitator at 100 rpm.

e) Centrifuge at 3 000 rpm and 4°C for 10 min.

22.6.4 Sample cleanup/filtration

Filter the supernatant using 0.45 µm filters. Inject 20 µl into the LC–MS/MS.

22.6.5 Instrumental analysis (LC–MS/MS)

LC–MS/MS analysis is performed on a UHPLC system (PerkinElmer) coupled to an AB SCIEX QTRAP 4 000/5 500 mass spectrometer (Framingham, MA, USA).

22.6.6 Chromatography conditions

The conditions include:

- Mode of separation: reversed phase chromatography
- Analytical column: RP–C18 (50×2 mm, particle size 4 µm)
- Column temperature: 30°C Injection volume: 20 µl
- Flow rate: 350 µl/min
- Run time: 12 min
- Mobile phase: Solvent A: H2O + 2 mmol/l ammonium acetate + 0.1% formic acid; Solvent B: Acetonitrile + 2 mmol/l ammonium acetate + 0.1% formic acid

The mode of elution is summarized in Table 149.

TABLE 149. Mobile phase programme

Time (min)	Solvent A (%)	Solvent B (%)
0.1	95	5
4	5	95
6	5	95
6.5	95	5
12	95	5

22.6.7 Mass spectrometric conditions

Mass analysis is carried out using an electrospray ionization source in positive mode. The operation conditions are as follows.

- Ionspray voltage: 5 kV
- Source temperature: 700°C
- Curtain gas = 30 psi
- Ion source gases 1 = 40 psi and 2=60 psi.
- Collision activated dissociation CAD; High and Entrance potential EP = 10.

The optimal MRM parameters are summarized in Table 150.

TABLE 150. MRM parameters

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Dwell time (ms)	DP	CE	CXP
MG1	329.2	313.2	100	126	51	8
MG2	329.2	208.1	100	126	49	4
BG1	385.2	341.1	100	116	53	10
BG2	385.2	297.0	100	116	73	8
LMG1	331.2	239.2	100	101	41	6
LMG2	331.2	223.2	100	101	75	10
LMG- D5	336.2	239.2	100	101	75	10

DP: Declustering potential CE: Collision energy CXP: Collision Cell exit potential

22.6.8 Injection sequence

This method can be used in the implementation of residue monitoring and control plans in biological matrices as well as routine analyses.

In routine screening, the samples are injected preferably in the following order:

i) A standard solution of MG at 0.2 µg/l equivalent to 1µg/kg

ii) Injection of water/acetonitrile

iii) 1 blank sample

iv) 1 sample spiked at 1 µg/kg

v) Injection of water/acetonitrile

vi) Batch of unknown samples

vii) 1 sample spiked at 1 µg/kg

viii) A standard solution of malachite green at 0.2 µg/l equivalent to 1 µg/kg

If the number of unknown samples is more than 10, spiked samples can be injected in between.

For confirmation, samples are tested preferably in the following order:

i) A standard solution of MG at 0.2 µg/l equivalent to 1 µg/kg

ii) A blank sample and a range of spiked samples

iii) Injection of water/acetonitrile

iv) Samples to be confirmed and their duplicates

v) Injection of water

vi) A blank sample and a range of spiked samples

A standard solution of MG at 0.2 µg/l equivalent to 1 µg/kg.

22.6.9 Calculation and interpretation of results

The method is validated and the presence of tryphenylmethane in extracts confirmed following established criteria (EC, 2002).

i) The relative retention time of the detected analyte should be identical to that of the spiked samples within ± 2.5%.

ii) Two specific transitions of each dye should be detected in the samples to be analyzed with a S/N of > 3.

iii) The relative intensities of the transitions in the extracts should be compared with the relative intensities of spiked samples (within the same concentration range) and respect the limits of the following tolerance (Table 151).

TABLE 151. Ion intensities and MS/MS

Ion m/z relative intensity (% of base peak)	LC-MS/MS
> 50%	± 20%
> 20% to 50%	± 25%
> 10% to 20%	± 30%
≤ 10%	± 50%

For the quantification:

Quantification is achieved using the major transition considering the internal standard. The calibration curve is established from the control sample and spiked samples.

22.6.10 Method validation

These methods provide reliable identification (at least 4 identification points) of unauthorized substances and substances with an established MRL and quantification of analytes (MRL compounds) following established guidelines (EC, 2002). LC–MS/MS methods fulfil these requirements with one precursor ion and at least two transition product ions (1.5 IP each one). LC–MS/MS methods fulfil these requirements with one precursor ion and at least two transition daughter ions (1.5 IP each one).

a) Specificity

- Twenty blank fish samples from different sources are analyzed to verify the absence of interfering peaks.
- Each molecule is specifically identified by a minimum of two transitions (parent ion ->daughter ion) and retention time.
- Specificity is evaluated from the examination of chromatograms.

b) Linearity

- The linearity of the analytical method is validated using the tissue calibration curves for each compound at different concentration levels to prevent matrix effects.
- For each series, the equation of the calibration ranges is calculated from blank and spiked samples at 0.5 µg/kg; 1 µg/kg; 1.5 µg/kg; 2 µg/kg and 4 µg/kg.
- The R^2 coefficient of the calculated regression curves should be above 0.97 (Table 152).

TABLE 152. Coefficient of regression

Analytes	Slope a		y-intercept b		R^2	
	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)
MG	12.7	2.93	-2.42	-0.33	0.99	0.99
BG	14.81	5.16	-3.45	-1.54	0.989	0.98
LMG	5.63	1.51	-0.82	-0.23	0.989	0.98

MG: Transition 1: 329.2/313.2 BG: Transition1: 385.2/341.1 LMG: Transition1:331.2/239.2
Transition 2: 329.2/208.1 Transition 2: 385.2/297 Transition2: 331.2/223.2

c) Repeatability

Repeatability (intraday precision) is evaluated by performing replicates in a day and within–laboratory reproducibility (interday precision) is evaluated by analyzing samples over the course of three consecutive days. For all the measured compounds, the analyzed recovery should be compliant with established ranges (EC, 2002). The results are summarized in Table 153 and 154.

TABLE 153. Coefficient of variation

Analyte	CV% (Signal)	
	Signal 1 (+intense)	Signal 2 (-intense)
MG	21.5	26.9
BG	19.3	24.5
LMG	23.0	22.4

TABLE 154. Repeatability of relative time and ratio

Analyte	CV% (TR)	CV% (Ratio)
MG	0.5	11.9
BG	1	10.0
LMG	0.2	10.4

d) Trueness

This is calculated as in Eq. (23)

$$Trueness\ (\%) = \frac{(Estimated\ concentration - theoretical\ concentration)}{Theoretical\ concentration} \times 100 \ldots\ldots\ldots (23)$$

The results of method trueness are summarized in Table 155.

TABLE 155. Summary of method trueness

Analytes	Theoretical concentration (µg/kg)		Estimated concentration (µg/kg)		Trueness%	
	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)
MG	1	1	0.71	0.73	-29.5	-26.8
BG	1	1	0.83	0.75	-17.3	-24.8
LMG	1	1	0.62	0.54	-37.8	-46.4

The decision limit and detection capability

An overview of the validation results for CCα and CCβ is shown in Table 156.

TABLE 156. Decision limit and detection capability

Analyte	Decision limit CCα (µg/kg)		Detection capability CCβ (µg/kg)	
	Signal 1 (+intense)	Signal 2 (-intense)	Signal 1 (+intense)	Signal 2 (-intense)
MG	0.01	0.03	0.01	0.08
BG	0.02	0.01	0.03	0.03
LMG	0.03	0.08	0.06	0.15

The decision limits obtained are all well below recommended concentrations (CRL, 2007).

Method uncertainty

Uncertainty of the method is equal to 2* the repeatability standard deviation (Table 157).

TABLE 157. Summary of method uncertainty

Molecule	Standard deviation (signal 1)	2U (µg/kg)
MG	1.95	3.9
BG	2.42	4.84
LMG	0.84	1.68

22.6.11 Schematic diagram of extraction procedure

1. Weigh 1 g of homogenized meat.
2. Transfer sample to a centrifuge tube with 900 µl of water.
3. Add 100 µl of internal standard solution (LMG–D5).
4. Vortex and leave the sample for 10 min.
5. Add 1 ml of hydroxylamine hydrochloride solution (5 g/l).
6. Close the tubes and vortex.
7. Let the sample rest for 10 min.
8. Add 4 ml of acetonitrile.
9. Close the tubes and agitate using rotatory agitator at 100 rpm.
10. Centrifuge at 3 000 rpm at 4°C.
11. Filter the supernatant using 0.45 µm filter and transfer to an autosampler vial.

23

Determination of Co, Ni, Cu, Zn, Cd, Fe, Cr and Pb in water

23.1 Introduction

"Heavy metals" are natural elements characterized by their rather high atomic mass and their high density. They can be found all through the crust of our planet. Some heavy metals such as copper, selenium, or zinc are essential trace elements, with functions indispensable for various biological processes and human metabolism. Many heavy metals e.g. iron, zinc, tin, lead, copper, tungsten, etc. are of outstanding technological significance. On the other hand, many of them, e.g. mercury, cadmium, arsenic, chromium, thallium, lead, and others, exert toxic effects even at low concentration when continuously ingested through contaminated food and water.

Methods available for the determination of minerals in water and foods include atomic absorption spectrometry (AAS). The AAS involves converting a sample to gaseous atoms (atomization) that absorb radiation. These are excited using a specific absorption line emitted from a hollow cathode lamp made up of the element under determination. Only atoms of the same element, which have specific energy levels, are excited making the technique very specific to the element under analysis. The absorbance is linear over a concentration range. Different techniques are available within AAS for atomization which include: (i) flame atomization air/acetylene, N2O/ acetylene etc. (ii) vapor generation technique cold and hot vapor generation, (iii) graphite furnace technique makes it convenient for determination at ppm/ppb level.

23.2 Scope and objective

The method describes the procedure for the determination of cobalt, nickel, copper, zinc, chromium, cadmium and lead in water by flame AAS. The method is applicable to dissolved, acid extracted and total metals in water.

23.3 Principle of the method

The AAS measures chemical elements in food, water and environmental samples through measurement of radiation absorbed. It is selective because each element has different energy levels and produces specific narrow absorption lines separated using a monochromator. The amount of energy in form of photons and wavelength are then measured. The organic matter of a homogenized sample is removed by ashing or by wet digestion technique, mineral matter is dissolved in acid and different elements are determined by AAS.

23.4 Safety considerations and precautions

Operate in a fume hood as much as possible and wear protective gear such as laboratory coats, safety glasses, chemical resistant gloves and masks/respirators where possible and necessary. Avoid touching components of the AAS that should not be accessed during operation.

All glassware should carefully be soaked in nitric acid (1.5 mol/l) before being rinsed with distilled/deionized water prior to use. Take care in preparing and handling standards since they can be easily contaminated.

23.5 Materials

23.5.1 Reagents and solvents

These include high purity analytical grade deionized water and nitric acid (Q = 1.4 g/ml).

23.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: pH meter; Volumetric flasks; Conical flasks; Polypropylene containers; Borosilicate glass containers; Micropipettes; Hot plate; AAS.

23.5.3 Solutions

The solutions (and how they are prepared) include

- Nitric acid (1.5 mol/l): Add 100 ml of nitric acid to 600 ml of water and dilute to 1000 ml.
- Nitric acid (0.03 mol/l): Add 1 ml of nitric acid to 400 ml of water and dilute to 500 ml with water.

23.5.4 Standards

23.5.4.1 Standard solutions of metals

For each element to be determined, take the appropriate weight of the pure metal or metal salts of known composition and purity to obtain the required concentration. Industrial prepared standard metal solutions of known concentrations may also be used. Store each of the Standard solutions in either polyethylene or borosilicate glass containers.

23.5.4.2 Reference standards

Reference standards should be obtained from a reputable manufacturer and should be accompanied with a certificated of analysis with measurement uncertainty reported and coverage factor (Plasma HIQU or equivalent).

23.5.4.3 Working and Intermediate standard solutions

Prepare the appropriate intermediate and working standard solutions depending on the calibration range of the parameter to be determined.

23.5.4.4 Calibration solutions

At least three calibrants are prepared covering the concentration range to determine as summarized in Table 158.

TABLE 158. Preparation of calibration solutions

Analyte	Concentration (mg/l)	Vol. taken (ml)	Vol. made up (ml)	Final Concentration (mg/l)
Copper	1 000	0.100	50	2.0
	1 000	0.050	50	1.0
	1 000	0.025	50	0.5
	2	1.250	50	0.05
Lead	1 000	0.200	50	4.0
	1 000	0.100	50	2.0
	1 000	0.050	50	1.0
	4.0	1.250	50	0.1
	4.0	2.500	50	0.2*
Cadmium	1 000	0.050	50	1.0
	1 000	0.025	50	0.5
	1 000	0.0125	50	0.25
	1.0	0.500	50	0.01
	1.0	1.000	50	0.02*
	100	0.050	50	0.1*
Nickel	1 000	0.200	50	4.0
	1 000	0.100	50	2.0
	1 000	0.050	50	1.0
	4	1.250	50	0.1
Chromium	1 000	0.200	50	4.0
	1 000	0.100	50	2.0
	1 000	0.050	50	1.0
	4.0	1.250	50	0.1
Iron	1 000	0.200	50	4.0
	1 000	0.100	50	2.0
	1 000	0.050	50	1.0
	4.0	1.250	50	0.1
Cobalt	1 000	0.200	50	4.0
	1 000	0.100	50	2.0
	1 000	0.050	50	1.0
	4.0	1.250	50	0.1
	4.0	2.500	50	0.2*
Zinc	1 000	0.500	50	10
	10	0.600	50	0.12
	10	0.400	50	0.08
	10	0.200	50	0.04
	10	0.100	50	0.02

* used as check and control solutions

23.6 Procedure

23.6.1 Sample handling

For samples which may not require concentration and are to be analyzed for the above metals, treatment should be done using nitric acid (1.4 g/ml) and maintaining the pH between 1 and 2.

23.6.1.1 Preliminary digestion for metal analysis

The sample is digested and concentrated depending on the anticipated concentration in the sample using nitric acid. The suggested sample volumes are indicated in Table 159.

NOTE: *If the digested volume exceeds the digestion vessel capacity add the sample as evaporation proceeds. Minimize volumes of acids used since acids used in digestion will add metals to the samples and blanks.*

TABLE 159. Metal concentration and sample volume

Estimated metal concentration (mg/l)	Sample volume (ml)
< 0.1	1 000
0.1–10	100
10–100+	10

NOTE: *When samples are concentrated during digestion (e.g. > 100 ml sample used) determine metal Recovery for each matrix digested. 0.05 mg/l spike should be determined by spiking one of the samples with 5ml of 10 mg/l stock solution.*

23.6.1.2 Quality control samples

An analyst should perform the required quality checks, and at least one quality check should be included with each batch of analysis.

23.6.2 Preparation of sample blanks and spikes/positive controls.

Carry out a procedure with a spike test in parallel with the determination using the same procedure, and quantities of all the reagents. One of the test portions is measured twice; one portion is labeled as spiked. The volume of standard stock used for spiking can be calculated as in Eq. 24:

$$V = \frac{X\,x\,Y}{Z} \quad \ldots\ldots (24)$$

Where V is the volume of the standards; X is the volume of sample taken for digestion and Y is the required spike concentration; Z is the concentration of stock standard used for spiking.

Control sample of known concentration is used as instrument performance control.

23.6.3 Nitric acid digestion

The sample is digested as reported elsewhere (APHA-AWWA-WEF, 1999) with some modification

a) Add at least 5 ml of conc. HNO3 to the sample and cover appropriate material

b) Boil to evaporate the solvent and add more acid until the samples is fully digested (when you see a clear solution)

c) Transfer/rinse content into a volumetric flask using water (at least 5 ml, twice)

d) Let cool and analyze.

23.6.4 Instrumental analysis

- AAS, fitted with hollow cathode lamps for the appropriate metals or electrodeless discharge lamps, and with a suitable device for allowing the correction of the nonspecific absorbance and with a nebulizer burner with an acetylene–air flame.
- Before carrying out the spectrometric measurements, set up the spectrometer according to the manufacturer's instructions as summarized in the laboratory procedure.
- Aspirate the blank and zero the instrument. Then aspirate each standard in turn into the flame. Plot a graph with the metal contents, in milligrams per litre, of the calibration solutions as abscissae and the corresponding values of absorbance as ordinates. In case the instrument has software that plots the graph, there is no need for plotting.

23.6.5 Test portion

- Rinse the nebulizer by aspirating water containing nitric acid (0.03 mol/l). Atomize solvent blank and zero the instrument. Place a test portion of the acidified sample as prepared before. Atomize the sample and determine its absorbance.
- In case the absorbance of the sample is beyond the maximum absorbance of the highest standard, dilute the sample and include the dilution factor in the final calculation of results.

23.6.6 Blank test

Carry out a procedure blank test sample i.e. sample that does not contain the analyte, that is brought through the entire measurement procedure and analyzed in the same manner as a test sample in parallel with the determination, by the same procedure, using the same quantities of all the reagents as in the sampling and determination, but replacing the test portion with water.

23.6.7 AAS interferences

Effort should be made to address chemical, ionization and spectral interferences and correct for false test results.

All metals are not equally stable in the sample, especially if it contains only nitric acid, not nitric acid and hydrochloric acid. The sample should be analyzed as soon as possible.

Liquids (Water/mineral water, etc.): Mix individual units and store in an airtight container.

23.6.8 Calculation and interpretation of results

By reference to the calibration graph of each metal; for the unknown sample determine the concentrations corresponding to the absorbance of the test portion and of the blank.

Calculate the actual concentration from Eq. (25):

$$Conc.\ element,\ ppm = (Cs - Cb) \times D.F \quad \ldots\ldots (25)$$

Where:

Cs is the concentration (mg/l) of element in the sample solution.

Cb is the concentration (mg/l) of element in the blank solution.

D.F. is the dilution factor, if any, and it is the ratio of final volume of diluted or digested solution (in ml) to the sample size (in ml).

The validation results are further highlighted in Table 160.

TABLE 160. Summary of method validation

	Accuracy	Linearity	LOD	LOQ	Working range (ppm)	%RSD repeatability	%RDS reproducibility
Cd	98%-113%	r^2=0.996	0.05	0.1	0.05-2	2%	2%
Cu	96.7%-102%	r^2=0.997	0.005	0.009	0.05-1	5%	7%
Ni	103%-111%	r^2=0.998	0.05	0.1	0.1-4	5%	6%
Pb	99%-108%	r^2=0.996	0.05	0.1	0.2-4	5%	7%

23.6.9 Schematic diagram of extraction procedure

1. The sample is digested and concentrated depending on the anticipated concentration in the sample using nitric acid. The suggested sample volumes are indicated below;

Estimated metal concentration (mg/)	Sample volume (ml)
< 0.1	1 000
0.1–10	100
10–100+	10

2. Digest samples as indicated above

1. Transfer filtrate to 100 ml volumetric flask with two 5 ml portions of water, adding these risings to the volumetric flask. Cool dilute to the mark and mix thoroughly.
2. Weigh 0.1 g to 1 g of sample into a PTFE vessel
3. Add 3 ml of HCl + 9 ml HNO_3.
4. Digest the mixture in microwave for 30 min using the optimized program. (Program # 4)
5. Cool the PTFE vessel,
6. Transfer the contents and make up to 50 ml in a volumetric flask with deionized water.
7. Filter the solution through Whatman 42 filter paper, if required.
   The sample is now ready for analysis.

SAMPLE PREPARATION (OPEN DIGESTION) - DRY ASHING TECHNIQUE

1. Weigh 1 g–10 g of sample into a dry silica dish/crucible, or pyrex glass beaker
2. Add 1.5 ml of 15% magnesium acetate
3. Heat on a hot plate to completely char the sample
4. Place container with sample in a muffle furnace maintained at 450°C
5. Ash for 4 hrs–6 hrs.
6. Remove sample container with sample and place it in a desiccator and cool to room temperature.
7. If the ashing is incomplete (denoted by black specks) add few drops of distilled water, return to muffle and ash again for 2 hrs.
8. Place the crucible in a desiccator and allow to cool.
9. Add 1 ml of deionized water, 1 ml of conc. HCl, heat it slowly to dissolve all the minerals and bring it to near dryness
10. Add another 5 ml each of water and 1 ml HCl, warm to dissolve, transfer into a 10 ml or 50 ml volumetric flask using deionized water and make up to volume.
11. Filter the solution through Whatman 42 filter paper, if required.
    The sample is now ready for analysis.

WET DIGESTION TECHNIQUE

1. Weigh 1 g–10 g of sample into a dry silica dish/ crucible, or pyrex glass beaker
2. Add 10 ml nitric acid and allow for digestion for about 1 hr at room temperature
3. Add 10 ml of sulphuric and slowly digest on a hot plate untill all the organic matter is destroyed.
4. When sample tends to turn to black, add 1 ml– 2 ml of nitric acid to assist oxidation.

   Cool and make up to 10 ml or 50 ml in a volumetric flask.

   Filter the solution through Whatman 42 filter paper, if required.
   The sample is now ready for analysis.

24

Simultaneous determination of minerals/metals in foods by ICP–OES

24.1 Introduction

"Heavy metals" are natural elements characterized by their rather high atomic mass and their high density. They can be found all through the crust of our planet. Some heavy metals like copper, selenium, or zinc are essential trace elements, with functions indispensable for various biological processes also driving the entire human metabolism. Many heavy metals are of outstanding technological significance, e.g. iron, zinc, tin, lead, copper, tungsten, etc. However, on the other hand, many of them, e.g. mercury, cadmium, arsenic, chromium, thallium, lead, and others, exert toxic effects even at low concentration when continuously ingested through consumption of contaminated food and water.

Several methods available for the determination of minerals in water and foods include inductively coupled plasma atomic emission spectroscopy (ICP–AES) also referred to as inductively coupled plasma optical emission spectrometry (ICP–OES) elaborated in this SOP.

24.2 Scope and objective

This SOP describes the procedure for simultaneous determination of elements in food samples (fruits, vegetables, carbonated/non-carbonated juices, sodas, milled cereals, fish) and may be applicable to other matrices with appropriate sample preparation technique(s). The method is accredited for determinization of minerals and metals such as but not limited to, Al, Ba, Cr, Cu, Fe, Mn, Ni, P, Pb, V, and Zn. Other minerals and metals the method analyses include Na, K, Ca, Mg, Cd, Co, Mo, Sr and As. However, the results for latter group of elements should be reported as not accredited.

24.3 Principle of the method

A prepared solution containing analyte elements is aspirated into the plasma generated by ICP source, the atomized elements produce characteristic emission spectral lines, which are separated by a simultaneous optical spectrometer. The intensity of the spectral line of an element is proportional to its concentration. A homogeneous food or alternate sample is digested with an acid and analyzed.

24.4 Safety considerations and precautions

Operate in a fume hood; use protective gear including a mask or safety glasses, lab coat and gloves. Ensure glassware is cleaned well and rinsed with water, 1% nitric acid and deionized water prior to use. Dilute samples with high percentage of total dissolved solids.

24.5 Materials

24.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Acids used in the preparation of standards and samples should be of high purity grade or equivalent. Deionized water: deionized water from Milli–Q gradient/Milli–Q, Millipore or equivalent; Conc. hydrochloric acid (AR grade or equivalent); Conc. nitric acid (AR grade or equivalent).

24.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: Argon gas (99.99% purity or more); Filter paper (Whatman 42 or equivalent); 0.45 µm filter, Aqueous, Millipore or equivalent; Micro pipette, 1 000 µl and 5 000 µl, calibrated; Micro pipette (10 µl–1 000 µl, 100 µl–1 000 µl, and 500 µl–5 000 µl); Glass pipettes, bulb, 5 ml, 10 ml, 20 ml and 25 ml, calibrated; Volumetric flasks class B or better (25 ml, 50 ml, 100 ml and 500 ml); ICP–OES; Computer with monitor; Hot plate; Drying Oven; Furnace.

24.5.3 Solutions

The solutions (and how they are prepared) include:

1% Nitric acid:

- Measure 700 ml of distilled water into 1 000 ml volumetric flask and the add 10 ml of concentrated nitric acid through the wall of the flask.
- Swirl the flask with its contents and make up to the mark of the volumetric flask.

10% Nitric acid:

- Measure 700 ml of distilled water into 1 000 ml volumetric flask and the add 100 ml of concentrated nitric acid through the wall of the flask.
- Swirl the flask with its contents and make up to the mark.

10% Hydrogen peroxide

- Measure 700 ml of distilled water into 1 000 ml volumetric flask and the add 333.3 ml of 30% hydrogen peroxide solution through the wall of the flask.
- Swirl the flask with its contents and make up to the mark.

24.5.4 Standards

24.5.4.1 Reference standards

Reference standards should be obtained from a reputable manufacturer and should be accompanied with a certificate of analysis with measurement uncertainty and coverage factor reported.

24.5.4.2 Standard solutions (1 000 mg/l)

Al, Ba, Cd, Co, Cu, Cr, Mn, Fe, Ni, Pb, Mo, Sr, V, Zn, Na, K, Ca, Mg, P, As (Plasma HIQU or equivalent). Revalidate an expired standard. Standards with high concentrations e.g. 10 000 mg/l can be used but should be diluted to 1 000 mg/l.

24.5.4.3 Mixed standard solution (100 mg/l)

Mixed standard solution of 100 mg/l (calibration check standard): Plasma HIQU or equivalent.

24.5.5 Calibration standards

Minerals and metals (Na, K, Ca, Mg, P, As) other than those accredited by this method may be included in the calibration curve. However, the results for these elements should be reported as not accredited.

24.5.5.1 Mixed standards stock solution (50 mg/l)

- Pipette 25 ml of standard solution (1 000 mg/l) into a 500 ml volumetric flask and make up to volume with 1% nitric acid solution. This is stable for up to 6 months in capped plastic containers when stored in a refrigerator.

24.5.5.2 Mixed standard intermediate *Solution* 1 (10 mg/l)

- Pipette 20.0 ml of mixed standard stock solution (50 mg/l) into a 100 ml volumetric flask and make up to volume with 1% nitric acid. This is stable for up to 6 months in capped plastic containers when stored in a refrigerator.

24.5.5.3 Mixed standard intermediate Solution 2 (1.0 mg/l)

- Pipette 10 ml mixed standard intermediate solution-1 (10 mg/l) into a 100 ml volumetric flask and make up to volume with 1% nitric acid. This is stable for up to 1 month in capped plastic containers when stored in a refrigerator.

24.5.5.4 Mixed standard intermediate Solution 3 (0.1 mg/l)

- Pipette 10 ml of mixed standard intermediate solution–2 (1 mg/l) into a 100 ml volumetric flask and make up to volume with 1% nitric acid.

Prepare the calibration standards as summarize in Table 161.

TABLE 161. Preparation of calibration curve

Std. No.	Conc. of Std. (mg/l)	Volume taken (ml)	Volume made up (ml)	Conc. of calibration Std. (mg/l)	Shelf life of Standard (Months)
10	50	40	100	20	6
9	10	n.a	n.a	10	6
8	50	10	100	5	3
7	10	25	100	2.5	3
6	1.0	n.a	n.a	1.0	1
5	10	5	100	0.50	1
4	1.0	25	100	0.25	1
3	0.10	n.a	n.a	0.10	Prepare fresh
2	0.10	10	100	0.01	Prepare fresh
1	0.01	10	100	0.001	Prepare fresh

n.a: No additional solvent taken

24.5.6 Calibration check standards

- Calibration check standards should be prepared by an independent analyst.

24.5.6.1 Mixed calibration-check standard solution (10 mg/l)

- Pipette 10 ml of 100 mg/l calibration solution into a 100 ml volumetric flask and bring to the mark using 1% nitric acid. This is stable for up to 6 months in capped plastic containers when stored in a refrigerator.

24.5.6.2 Mixed calibration–check standard solution (5 µg/ml)

- Pipette 5 ml of calibration check standard solution 100 mg/l into a 100 ml volumetric flask and make up to volume with 1% nitric acid solution.
- Transfer the solution into a screw capped plastic bottle and store in a refrigerator. The solution is stable for 3 months.

24.5.6.3 Mixed standard Intermediate Solution 2 (1 mg/l)

- Pipette 10 ml of stock solution (10 mg/l) into a 100 ml volumetric flask and make up to volume with 1% nitric acid solution.
- Transfer the solution into a screw capped plastic bottle and store in a refrigerator. The solution is stable for 3 months.

24.5.7 The calibration blank sample

- This is prepared by diluting 1 ml of concentrated nitric acid in 100 ml deionized water.
- Prepare enough quantity to be used to flush the system between standards and samples.

24.6 Procedure

24.6.1 Sample handling

a) Collect samples in plastic bags, airdry, grind and then oven dry at 105°C for 3 hrs.

b) Grind the sample and store in a desiccator until the analysis time (moisture in the original sample should be determined, if results need to be expressed on wet weight basis).

24.6.2 Quality control samples

a) An analyst should perform the required quality checks. At least one quality check should be included with each batch of analysis. Refer to a laboratory Quality Assurance Procedure.

b) The analyst should prepare an additional control sample by spiking a blank sample at 2 mg/l using a different batch of the stock standard not used for creating a calibration curve which includes at least Pb, Cd, Ni, Cu and analyzing it alongside each batch of samples.

24.6.3 Reagent blank (RB)

This solution contains all the reagents used in sample preparation (at the same volume) and should be run through the analytical procedures; should mimic the final analytical solution.

24.6.4 Sample preparation (microwave digestion)

a) Weigh accurately about 0.1 g to 1 g of fine sample into a PTFE vessel.

b) Add 3 ml of HCl + 9 ml HNO3.

c) Digest the mixture in a microwave for 30 min using the optimized program. (Programme # 4).

The microwave conditions are as follows (Table 162).

TABLE 162. Microwave digestion program

Step	Time (Min)	Power (W)
1	2	250
2	2	0
3	6	250
4	10	400
5	10	650

Cool the PTFE vessel, quantitatively transfer the contents and make up to 50 ml in a volumetric flask with deionized water.

24.6.5 Sample preparation (open digestion)

Removal of organic matter

a) **Dry ashing (all foods)**

- Accurately weigh 1 g–10 g of sample into a clean dry silica dish/crucible, or pyrex glass beaker, add 1.5 ml of 15% magnesium acetate, heat on a hot plate to completely char the sample, place the crucible/silica dish or pyrex beaker in a muffle furnace maintained at 450°C and allow to ash for 4–6 hrs.
- Remove the crucible/silica dish or pyrex beaker from the muffle and place it in a desiccator and cool to room temperature.
- If the ashing is incomplete (denoted by black specks etc.), add a few drops of distilled water, carefully dry on a hot plate avoiding spurting of the sample, return the crucible to muffle and ash again for 2 hrs.
- Place the crucible in a desiccator and allow to cool.
- If the ashing is still not complete, add a few drops of hydrogen peroxide/ nitric acid (10%), dry the sample on a hot plate and return the crucible/silica dish or pyrex beaker to the muffle furnace and allow to ash.
- Cool the crucible in a desiccator, add 1 ml of deionized water, 1 ml of concentrated hydrochloric acid, heat it slowly to dissolve all the minerals and bring it to near dryness.
- Add another 5 ml each of water and 1 ml hydrochloric acid, warm to dissolve, transfer quantitatively into a 10 ml or 50 ml volumetric flask using deionized water and make up to volume.
- Dilute the sample if necessary, to get the mineral into the linear concentration range of the instrument using deionized water.

b) Wet digestion

- Accurately weigh about 1 g–10 g of sample into a 250 ml conical flask.
- Add 10 ml nitric acid and allow for digestion for about 1 hr at room temperature.
- Add 10 ml of Sulphur acid and slowly digest on a hot plate until all the organic matter is destroyed.
- When the sample tends to turn black, add 1 ml– 2 ml of nitric acid to assist oxidation.
- Cool and make up to 10 ml or 50 ml in a volumetric flask.
- Filter the solution through Whatman 42 filter paper, if required.
- The sample is now ready for analysis.

24.6.6 Instrumental analysis (ICP–OES)

This is a Spectro Blue, Simultaneous ICP–OES, with background correction, autofit multicalibration curve fitting, signal to background ratio (SBR) and signal to root background ratio.

Operating conditions

- Power = 1.45 kW
- Plasma Flow = 13 l/min
- Aux. Flow = 1.2 l/min
- Neb. Flow = 0.75 l/min
- Replicate read time =10 s
- Sample Uptake time = 30 s
- Rinse Time = 5 s
- Pump rate = 30 rpm
- Instrument stabilization delay= 15 s

24.6.7 Interferences

Note spectral interferences including, although not limited to : (1) overlap of spectral line from another element; (2) unresolved overlap of molecular band spectra; (3) background contribution from continuous or recombination phenomena; and (4) background contribution from stray light from the line emission of high concentration elements.

24.6.8 Construction of calibration curves

- Use the ICP software and prepared calibration solutions injection in alternation with deionized water to remove the memory effects.
- The calculated R^2 values should be ≥ 0.95.

24.6.9 Checking calibration curves with calibration mixed standards

- Aspirate at least one calibration–check standard solutions and record the value ensuring that there is no deviation affecting the R^2.

24.6.10 Sample Analysis

This is determined by aspirating the prepared food or related sample solutions with the concentration calculated from the calibration curve. Substances at lower concentrations can be calculated using a diluted calibration point.

24.6.11 Calculation and interpretation of results

By reference to the calibration graph, determine, for each metal, the concentrations corresponding to the absorbances of the test portion and of the blank using Eq. (26).

$$Conc.\ Element, \frac{mg}{kg} = \frac{Cs - Cb}{M} \times V \times D.F. \quad \ldots\ldots (26)$$

Where:

Cs is the concentration (mg/l) of element in the sample solution.

Cb is the concentration (mg/l) of element in the blank solution.

M is the weight of the sample taken in gram

D.F. is the dilution factor, if any.

V is the final volume made up in ml

24.6.12 Method validation

Analytical method development is summarized in Table 163.

TABLE 163. Method development summarized

	Accuracy	Linearity	LOD	LOQ	Working range (ppm)	%RSD repeatability	%RSD reproducibility
Cd	89.4%–113%	r^2=1	0.001	0.009	0.01-1	5.5%	4%
Cu	99.4%–117	r^2=0.999	0.05	0.1	0-2	8.6%	7%
Ni	99%–120%	r^2=1	0.05	0.07	0-1	1.7%	1.6%
Pb	95%–117%	r^2=0.999	0.05	0.1	0-1	1%	1%

24.6.13 Schematic diagram of extraction procedure

SAMPLE PREPARATION (MICROWAVE DIGESTION) TECHNIQUE	1. Weigh 0.1 to 1 g of sample into a PTFE vessel 2. Add 3 ml of HCl + 9 ml HNO_3. 3. Digest the mixture in microwave for 30 min using the optimized program. (Program # 4 4. Cool the PTFE vessel, 5. Transfer the contents and make up to 50 ml in a volumetric flask with deionized water. 6. Filter the solution through Whatman 42 filter paper, if required. The sample is now ready for analysis.

SAMPLE PREPARATION (OPEN DIGESTION)	**DRY ASHING TECHNIQUE** 1. Weigh 1 g–10 g of sample into a dry silica dish/crucible, or pyrex glass beaker 2. Add 1.5 ml of 15% magnesium acetate 3. Heat on a hot plate to completely char the sample 4. Place container with sample in a muffle furnace maintained at 450°C 5. Ash for 4 hrs–6 hrs. 6. Remove sample container with sample and place it in a desiccator and cool to room temperature. 7. If the ashing is incomplete (denoted by black specks) add few drops of distilled water, return to muffle and ash again for 2 hrs. 8. Place the crucible in a desiccator and allow to cool. 9. Add 1 mL of deionized water, 1 ml of conc. hydrochloric acid, heat it slowly to dissolve all the minerals and bring it to near dryness 10. Add another 5 ml each of water and 1 ml hydrochloric acid, warm to dissolve, transfer into a 10 ml or 50 ml volumetric flask using deionized water and make up to volume. 11. Filter the solution through Whatman 42 filter paper, if required. The sample is now ready for analysis.

	WET DIGESTION TECHNIQUE 1. Weigh 1 g–10 g of sample into a dry silica dish/crucible, or pyrex glass beaker 2. Add 10 ml nitric acid and allow for digestion for about 1 hr at room temperature 3. Add 10 ml of sulphuric and slowly digest on a hot plate till all the organic matter is destroyed. 4. When sample tends to turn to black, add 1 ml – 2 ml of nitric acid to assist oxidation. 5. Cool and make up to 10 or 50 ml in a volumetric flask. 6. Filter the solution through Whatman 42 filter paper, if required. The sample is now ready for analysis.

25

Determination of dye residues in aquaculture products by HPLC–MS/MS

25.1 Introduction

Malachite green (MG), a dye originally used in the textile industry, is used illegally in aquaculture industry to control ectoparasites and fungal infections in fish eggs, fingerlings and adult fish due to low cost, high efficacy and lack of alternatives. Therefore, the use of MG and Gentian Violet (GV), also known as Crystal Violet (CV) has been banned in many countries/regions including the United States of America and the European Union (EU). All dyes used as antifungal and antiparasitic drugs in fish production are banned in the EU. Likewise, according to Annex II of the Commission Decision 2002/657/CE (EC, 2002) there is a minimum required performance limit (MRPL) of 2 μg/kg to accurately determinate MG and its metabolites. The same MRPL should be considered for other veterinary dyes such as CV. Malachite green is metabolized by fish to leucomalachite green (LMG). Thus, when fish exposed to MG reach the consumer, the amount of LMG present in the fish is expected to be higher than that of MG. As LMG is the predominant residue found in fish tissues following exposure to MG, it is the residue of primary concern from a safety point of view. LMG is a major concern due to its link to induce hepatic cancer in mice studies. CV is similarly considered to be genotoxic and carcinogenic, its metabolite leucogentian violet or leucocrystal violet (LCV) is similar in structure to LMG therefore, it is possible that LCV could also be a more potent carcinogen than CV. Both GV and LGV (leucogentian violet) are readily interconvertible in the body, and so it is likely that exposure to CV will also result in exposure to LCV.

25.2 Scope and objective

This method enables the determination of residues of MG, LMG, CV, LCV in aquaculture products.

25.3 Principle of the method

This method is based on the extraction of MG, LMG, CV, LCV with a mixture (4:1, v/v) of acetonitrile and hydroxylamine 5 g/l from homogenized tissue. Extracts are then filtered and directly analyzed by LC–MS/MS. Chromatographic separation is performed by gradient elution.

25.4 Safety considerations and precautions

Always wear gloves, safety googles, laboratory coat. Remember that acetonitrile vapors are extremely dangerous, work in an adequate fume hood.

25.5 Materials

25.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Deionized water type I; Acetonitrile (LC/MS); Acetonitrile (ACS analysis); Acetic Acid (ACS analysis, 99%); Hydroxylamine hydrochloride (ACS analysis); Ammonium acetate (LC/MS); Ammonium hydroxide (ACS analysis, 25%).

25.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: UHPLC with Thermo Scientific brand binary pump Ultimate 3 000 model, with TSQEndura mass spectrometer detector; HPLC column: Waters Symmetry C18–150 mm × 4.6 mm, 5 µm and 100 A°, PN WAT045905. Precolumn: Waters Symmetry C18, 20 mm × 3,9 mm, 5 µm, PN WAT054225; Analytical balance (sensitivity: 0.0001 g; linearity ± 0.0002 g); Electronic balance (sensitivity: 0,01 g; linearity ± 0.02 g); Sample homogenizer; Centrifuge with cooling; Ultrasonic bath; Rotary shaker; Multivortex (vortex mixer); Polypropylene centrifuge tubes with screw cap, 15 ml; Volumetric flask (Class A), 10 ml, 25 ml and 50 ml; Vacuum filtration system for mobile phases; Nylon filters for solvents, 47 mm × 0.22 µm; Syringe Filters with Nylon Membrane for samples, 13 mm × 0.22 µm; Plastic disposable syringes, 3 ml; Vials for autosampler, 2 ml; Glass beakers, 10 ml; Micropipettes of variable volumes; Bottles for HPLC.

25.5.3 Solutions

The solutions (and how they are prepared) include:

- Hydroxylamine solution (5 g/l): Weigh 0.5 g of hydroxylamine hydrochloride, bring to 100 ml with deionized water and stir until dissolved. Prepare fresh daily.
- Buffer 0.1 mol/l ammonium acetate, pH 4.5 (Mobile phase A): Weigh 7.7 g of ammonium acetate, dissolve in approximately 100 ml of deionized water.
- Add approximately 8.5 ml of glacial acetic acid. Check the pH and adjust with ammonium hydroxide if necessary. Bring to 1 litre, stir and press through a 0.22 µm nylon (or alternative) filter.
- Acetonitrile (Mobile phase B): 100% (volume) acetonitrile is poured through 0.22 µm filter material before mixing.

25.5.4 Standards

25.5.4.1 Reference standards

Analytical standards include the following:

- Malachite Green Oxalate, analytical standard quality
- Malachite Green–D5 picrate, analytical standard quality (internal standard)
- Leucomalachite Green, analytical standard quality
- Leucomalachite Green–D5, analytical standard quality (internal standard)
- Crystal Violet, analytical standard quality
- Crystal Violet–D6, analytical standard quality (internal standard)
- Leucocrystal Violet, analytical standard quality
- Leucocrystal Violet–D6, analytical standard quality (internal standard)

25.5.5 Stock solutions

25.5.5.1 MG approximately 200 mg/l

- Weigh the approximate amount of malachite green oxalate to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile.
- Store in a freezer for a maximum period of 3 months.

25.5.5.2 *MG*–D5 approximately 200 mg/l

- Weigh the approximate amount of MG–D5 picrate to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask and fill with acetonitrile.
- Store in a freezer for a maximum period of 3 months.

25.5.5.3 LMG approximately 200 mg/l

- Weigh the approximate amount of LMG to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and bring to volume with acetonitrile.
- Store in a freezer for a maximum period of 3 months.

25.5.5.4 LMG–D5 200 mg/l

- Weigh the approximate amount of LMG–D5 to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile.
- Store in a freezer for a maximum period of 3 months.

25.5.5.5 CV approximately 200 mg/l

- Weigh the approximate amount of CV to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and bring to volume with acetonitrile.

25.5.5.6 CV–D6 approximately 200 mg/l

- Weigh the approximate amount of CV–D6 to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and bring to volume with acetonitrile.

25.5.5.7 LCV approximately 200 mg/l

- Weigh the approximate amount of LCV to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile.

25.5.5.8 *LCV*-D6 of approximately 200 mg/*l*

- Weigh the approximate amount of LCV–D6 to prepare 50 ml of a solution at a concentration of 200 mg/l.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile.

  NOTE: *Consider the purities and the gravimetric factor of the standards when preparing the stock solutions.*

25.5.6 Intermediate solutions

25.5.6.1 MG (2 mg/l)

- Pipette the corresponding volume of the MG stock solution.
- Transfer to a 25 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.2 *LMG (2 mg/*l)

- Pipette the corresponding volume of the LMG stock solution.
- Transfer to a 25 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.3 *MG*-D5 (2 mg/l)

- Pipette the corresponding volume of the MG–D5 stock solution.
- Transfer to a 25 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.4 LMG-D5 (2 mg/l)

- Pipette the corresponding volume of the stock solution of LMG–D5.
- Transfer to a 25 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.5 CV (2 mg/l)

- Pipette the corresponding volume of the CV stock solution.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.6 *CV*-D6 (2 mg/l)

- Pipette the corresponding volume of the CV–D6 stock solution.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.7 LCV (2 mg/l)

- Pipette the corresponding volume of the LCV stock solution.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.6.8 LCV-D6 (2 mg/l)

- Pipette the corresponding volume of the stock solution of LCV–D6.
- Transfer to a 50 ml amber volumetric flask, stir until dissolved and fill with acetonitrile. Prepare fresh daily.

NOTE: *Working solutions and calibration points should be prepared fresh daily. The working solutions correspond to a mixture of the intermediate solutions of MG, LMG, CV and LCV, therefore, the intermediate solutions of VM–D5, VLM–D5, CV–D6 and LCV–D6 should not be used for their preparation.*

25.5.7 Work solutions

25.5.7.1 Work solution No. 1 (2.5 µg/l)

- Pipette 12.5 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.2 Work solution No. 2 (5 µg/l)

- Pipette 25 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.3 Work solution No. 3 (10 *µg/l*)

- Pipette 50 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.4 Work solution No. 4 (20 *µg/l*)

- Pipette 100 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.5 Work solution No. 5 (30 µg/l)

- Pipette 150 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.6 Work solution No. 6 (40 µg/l)

- Pipette 200 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.7 Work solution No. 7 (50 µg/l)

- Pipette 250 µl of each of the intermediate solutions of MG and LMG, CV and LCV, into a 10 ml volumetric flask containing approximately 2 ml of acetonitrile.
- Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.7.8 Internal standard MG–D5 (10 µg/l)

- Pipette 250 µl of the MG–D5 intermediate solution.
- Transfer to a 50 ml volumetric flask containing approximately 10 ml of acetonitrile. Stir until dissolved and fill to the mark with acetonitrile. Prepare fresh daily.

25.5.8 Internal standards

25.5.8.1 Internal standard MG–D5 (10 µg/l)

- Pipette 250 µl of the MG–D5 intermediate solution.
- Transfer to a 50 ml volumetric flask containing approximately 10 ml of acetonitrile.
- Stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.8.2 Internal standard LMG–D5 (10 µg/l)

- Pipette 250 µl of the intermediate solution of LMG–D5.
- Transfer to a 50 ml volumetric flask containing approximately 10 ml of acetonitrile.
- Stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.8.3 *Internal standard CV*–D6 (10 µg/l)

- Pipette 250 µl of the intermediate CV–D6 solution.
- Transfer to a 50 ml volumetric flask containing approximately 10 ml of acetonitrile.
- Stir until dissolved and fill with acetonitrile. Prepare fresh daily.

25.5.8.4 Internal standard LCV–D6 (10 µg/l)

- Pipette 250 µl of the intermediate solution of LCV–D6.
- Transfer to a 50 ml volumetric flask containing approximately 10 ml of acetonitrile.
- Stir until dissolved and fill with acetonitrile. Prepare fresh daily

25.6 Procedure

25.6.1 Sample preparation

Depending on the nature of the sample/matrix, the following indications should be followed prior to homogenization:

- Fish fillet: quarter and cut into small pieces.
- Whole fish: remove the scales and viscera and divide the muscle into squares taking randomly from different parts of the total sample.
- Unprocessed fish pieces: homogenize the entire sample.
- Crustaceans: quartered, remove the head, shell and legs, and split the meat into small pieces.

Place the sample in the food processor and crush it until a ground muscle mass is obtained.

NOTE: *The size of the test sample should be at least 50 g.*

a) Weigh approximately [1.00 g (±0.01 g)] of each of the samples.

b) Add 100 µl of each of the internal working standard solutions (MG–D5, LMG–D5, CV–D6, LCV–D6 of 10 µg/l).

25.6.2 Quality control samples

Parallel to the analytical samples, spiked samples, reagent blanks, sample blanks, and certified reference materials (optional) are prepared in 15 ml polypropylene centrifuge tubes as described below.

25.6.2.1 Reagent blank

- Take an aliquot of 1 ml of deionized water.
- Add 100 µl of each of the internal working standard solutions.

25.6.2.2 Sample blank

- Weigh approximately 1.00 g (±0.01 g) of a blank sample (sample that does not contain the analyte).
- Add 100 µl of each of the internal working standard solutions.

25.6.2.3 Spiked samples for calibration

- Prepare 7 tubes with approximately 1.00 g (±0.01 g) of a blank sample.
- To each sample add a specified volume of work mix solutions.
- Add 100 µl of each of the internal standard solutions.

The following table details the volume and the standard solution to be added according to the levels stated in Table 164.

TABLE 164. Preparation of the calibration curve

Level	Concentration (mg/kg)	Standard solution	Volume (µl)
1	0.25	Work mix solution 1	100
2	0.50	Work mix solution 2	100
3	1.0	Work mix solution 3	100
4	2.0	Work mix solution 4	100
5	3.0	Work mix solution 5	100
6	4.0	Work mix solution 6	100
7	5.0	Work mix solution 7	100

25.6.2.4 Spiked samples

- Prepare 3 tubes with approximately 1.00 g (±0.01 g) of a blank sample (the same used as sample blank).
- Add 100 µl of the 20 µg/l working mixture to each sample.
- Add 100 µl of each of the internal working standard solutions.

25.6.2.5 Quality control material, QC (Optional)

- Weigh approximately 1.00 g (±0.01 g) of a quality control material (proficiency test remnant, QC in matrix prepared by a provider).
- Add 100 µl of each of the internal working standard solutions.

25.6.3 Sample extraction

a) Add 300 µl of water.

b) Add 1 ml of hydroxylamine solution.

c) Stir in a multivortex or single vortex mixer for 15 s and leave in the dark for approximately 10 min.

d) Add 4 ml of acetonitrile.

e) Shake for approximately 20 min on rotary shaker.

f) Put inside an ultrasonic bath for approximately 15 min.

g) Centrifuge for 16 min at 4°C and 3 000 s^{-1}.

25.6.4 Sample cleanup

a) Filter with 0.22 µm filter for samples.

b) Collect at least 250 µl of the filtered extract in the autosampler vial.

25.6.5 Instrumental analysis (LC–MS/MS)

LC–MS/MS analysis is conducted using UHPLC with Thermo Scientific brand binary pump Ultimate 3 000 model, with TSQ Endura triple quadrupole mass spectrometer.

25.6.5.1 LC conditions

- Analytical column: Waters Symmetry C18 (4.6×150 mm, particle size 5 µm and 100 A°), PN WAT045905.
- Pre–column: Waters Symmetry C18 (3.9×20 mm, particle size 5 µm), PN WAT054225.
- Column temperature: 35°C
- Flow rate: 0.4 ml/min
- Injection volume: 20 µl
- Run time: 21 min
- Mobile phase: Buffer 0.1 mol/l ammonium acetate, pH 4.5 (Mobile phase A)
- Acetonitrile (Mobile phase B)
- Elution mode: gradient elution (Table 165).

TABLE 165. Mobile phase gradient

Time (min)	Flow (ml/min)	% A	% B
-1.00	Equilibration		
0.00	0.400	40	60
8.50	0.400	40	60
8.60	0.400	0	100
18.00	0.400	0	100
18.10	0.400	40	60
21.00	Stop run		

25.6.5.2 Mass spectrometer conditions

Ion source conditions

- Ion Source Type: H–ESI
- Positive Ion (V): 3 700
- Sheath Gas (Arb): 25
- Aux gas (Arb): 5
- Sweep gas (Arb): 4
- Ion transfer tube temp (°C): 342
- Vaporizer temp (°C): 358
- SRM conditions
- Cycle time (sec): 0.5
- Q1 Resolution (FWHM): 0.7
- Q3 Resolution (FWHM): 0.7
- CID Gas (mTorr): 2.5.

Fragmentation patterns are summarized in Table 166.

TABLE 166. MS transitions

Analyte	Transition
MG	329.183 > 313.111
MG–D5	334.183 > 318.111
LMG	331.183 > 239.111
LMG–D5	336.213 > 239.040
CV	372.213 > 356.143
CV–D6	378.213 >362.151
LCV	374.213 > 358.143
LCV–D6	380.243 > 364.222

Inject 20 µl of each of the final extracts, in the following sequence:

1) Acetonitrile
2) Reagent Blank
3) Blank sample
4) Calibration curve
5) Acetonitrile
6) Spiked Sample 1
7) Spiked Sample 2
8) Spiked Sample 3
9) Acetonitrile
10) QC
11) Acetonitrile
12) Samples
13) Acetonitrile
14) Repeating two points of the curve (verification)
15) Acetonitrile

25.6.6 Calculation and interpretation of results

The calibration curve is prepared from matrix (matrix matched) and the results expressed directly in µg/kg. To obtain the result us Eq. (27):

$$Conc = \frac{\frac{S}{ISS} - b}{m} \quad \text{.....(27)}$$

Where:

Conc is the concentration in the sample extract (µg/kg)

m is the slope

S is the signal

ISS is the internal standard signal

b is the intercept on the y axis

The concentration of malachite green should be reported as the sum of MG and its green metabolite LMG (in µg/kg), for which no gravimetric factor is required. The concentration of CV should be reported as the sum of CV and its LCV metabolite (in µg/kg), for which no gravimetric factor is required.

Confirmation

In accordance with the criteria defined elsewhere (EC, 2002), the identity of a compound is confirmed when:

i) The presence of the analyte is observed in the two chromatograms corresponding to the characteristic transitions for that compound. Both peaks should have a S/N≥ 3.

ii) The resulting S/N ratio obtained by dividing the signal of the least abundant peak by the signal of the peak corresponding to the most abundant transition should coincide with the S/N of the nearest reference standard, taking into account the following tolerances which depend on the relative intensity with respect to the base peak (Table 167).

TABLE 167. Relative intensity

Relative intensity (% of base peak)	Tolerance
> 50 %	± 20 %
> 20–50 %	± 25 %
> 10–20 %	± 30 %
≤ 10 %	± 50 %

Acceptance criteria

1) The linearity of the calibration curve expressed as a coefficient of determination (R^2) should not be less than 0.99.

2) The average percentage recovery for each analyte in spiked samples should be between 70% and 110%.

3) In the case of analyzing QCs, the data obtained should meet the criteria assigned by the manufacturer.

4) The CV of each analyte in the internal controls should be less than 32% according to acceptable guidelines [7].

5) The difference in the response of the verification of calibration points read at the end of the sequence in comparison with the corresponding ones in the calibration curve should not exceed ± 20%.

6) The average recovery percentage of spiked samples and the sensitivity of the calibration curves (slope) should be plotted.

7) The detection limit for the determination of the sum of MG and LMG should not exceed 2 µg/kg.

25.6.7 Method validation

The performance characteristics obtained for MG and LMG are indicated in Table 168.

TABLE 168. Method performance parameters for MG and LMG

Parameter	Results	
	MG	LMG
Linearity (r^2)	0.9966	0.9994
Working range	(0.50 to 5.0) µg/kg	(0.50 to 5.0) µg/kg
Sensitivity ((S/SSTDI)/conc)	1.0296	1.0827
Recovery (%)	97.4	99.1
Intermediate precision of 0,5 µg/kg to 2 µg / kg (CV %)	4.6	2.6
Intermediate precision of 3 µg/kg to 4 µg/kg (CV%)	3.9	3.8
Limit of detection, LOD (µg/kg)	0.17	0.17
Limit of Quantification, LOQ (µg/kg)	0.25	0.25
Uncertainty	U exp= 2 x CV* x Conc	U exp= 2 x CV* x Conc

25.6.8 Schematic diagram of the extraction procedure

TEST SAMPLE	1. Weigh 1.00 g (±0.01 g) of ground muscle mass in a 15 ml centrifuge tube. 2. Add 100 µl of each of the internal working standard solutions (MG–D5, LMG–D5, CV–D6, LCV–D6 of 10 µg/l).
	↓
EXTRACTION	1. Add 300 µl of water. 2. Add 1 ml of hydroxylamine solution. 3. Stir in multivortex or single vortex for 15 s. 4. Leave the sample in the dark for 10 min. 5. Add 4 ml of acetonitrile. 6. Shake for 20 min in a rotary shaker. 7. Put inside an ultrasonic bath for 15 min. 8. Centrifuge at 3 000 rpm at 4°C for 16 min.
	↓
CLEANUP	1. Filter with 0.22 µm filter. 2. Transfer > 250 µl of the extract into Autosampler vial.

26

Determination residues of chloramphenicol, thiamphenicol and florfenicol in shrimp, fish and meat by LC–MS/MS

26.1 Introduction

Use of veterinary drugs for therapy, prophylaxis, metaphylaxis and growth promotion is a common practice in animal production to enhance the productivity. However, the presence of harmful levels of drug residues in food of animal origin resulting from use of these drugs has now become a global human health concern. Chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF), classified as amphenicols, are broad-spectrum antibiotics that have been used widely in veterinary medicine. Because of the deleterious effects on human health, their use in food-producing animals including aquaculture is regulated in many countries. For instance, CAP, which is used widely in human and veterinary medicine, can cause bone marrow depression, aplastic anaemia and acute leukaemia in humans. No safe limit has been established for CAP residues, and therefore, many countries including member states of the European Union (EU), United States and Canada, have banned use of CAP in food producing animals. The European Commission established a minimum required performance limit (MRPL) for CAP in food of animal origin at a level of 0.3 μg/kg, which defines the minimum content of CAP that need to be detected and confirmed (EC, 2003). TAP and FF, which were introduced to substitute CAP, are allowed in the EU, but with maximum residue limits (MRL) in edible animal tissues. Use of TAP and FF has also increased in food-producing animals after prohibition of CAP and establishment of MRLs for TAP and FF. Despite the legal ban and MRLs, these drugs can be misused because of their effectiveness, low cost and ready availability, posing a threat to human health. Determination of drug residues in animal products is therefore important to ensure consumer safety.

26.2 Scope and objective

The LC–MS/MS method can be used for simultaneous determination of residues of chloramphenicol, thiamphenicol and florfenicol in shrimp, fish and meat matrices. The method allows quantification of the CAP residues below the EU MRPL of 0.3 μg/kg and TAP and FF residues below MRL of 50 μg/kg and 100 μg/kg, respectively, in muscle of food producing species. To ensure the quality and reproducibility of the procedure.

26.3 Principle of the method

Drug residues in the tissues are extracted using ethyl acetate. Extraction is facilitated by vortexing and rotating. Ethyl acetate layer is evaporated to dryness using a mild flow of nitrogen. Dry residue is redissolved in water, after which defatting is performed by extraction with hexane followed by centrifugation. Aqueous layer is filtered and injected to LC–MS/MS system for analysis.

26.4 Safety considerations and precautions

Chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Suitable gloves must be worn when these chemicals are handled, and experiment is conducted. Work should be performed in a fume hood at all possible time.

26.5 Materials

26.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Type I water (ultrapure water); Methanol (LC–MS grade); Ethyl acetate (HPLC grade); Ammonium acetate (for HPLC, purity > 98.0%); *n*–hexane (HPLC grade).

26.5.2 Equipment/instruments and consumables

The following apparatus and material are applicable: The Analytical balance (Ohaus/ Sartorius); Polypropylene tubes (15 ml and 50 ml); Grinder (Moulinex); Tissue homogenizer (Ultra Turrax® T25 basic/ Ultra Turrax® T 25 digital); Micropipettes (0.5–10 µl, 100–100 µl, 100–1 000 µL, 500–5 000 µl) (Eppendorf/Nichiriyo); Volumetric flasks (5 ml and 10 ml); Spatulas; Pasteur pipettes; Plastic weigh boats; Test tube rotator (GFL); Glass tubes; Vortex mixer (Velp); Centrifuge (Eppendorf/Hermle; Vacuum pump (Cole pamer); Nitrogen evaporator (N–EVAP, Organomation); Nitrogen gas (for sample concentration, purity 99.95%); Nitrogen generator (Claind); Argon gas for mass spectrometry; Microcentrifuge tube (1.5 and 2 ml); Nylon syringe filters (0.45 µm, diameter 13 mm); Disposable syringes (1 ml or 3 ml); Autosampler vials; Polypropylene inserts for autosampler vials; Filtering device for solvents and water (consisting of filtering cup, filter head, conical flask and clamp); Nylon membrane filters (0.45 µm); Analytical column: Shimadzu Shim-pack XR–ODS; UHPLC system (Shimadzu Nexera X 2); Triple quadrupole mass spectrometer (Shimadzu LC–MS 8040) with Electrospray ionization (ESI) interface; Software: LabSolutions (version 5.93).

26.5.3 Solutions

The solutions (and how they are prepared) include:

- Mobile phase solvent A (0.5 mM ammonium acetate): Dissolve 0.0197 g of ammonium acetate in 500 ml of water and filter through 0.45 um filter.
- Mobile phase solvent B (methanol): Methanol is filtered through 0.45 um filter material.

26.5.4 Standards

The analytical standards include: Chloramphenicol, Thiamphenicol and Florfenicol (Sigma Aldrich, above 95%).

26.5.4.1 Stock standard solutions (1 000 mg/l)

Prepare individual stock standard solutions of CAP, TAP and FF at 1 000 mg/l by dissolving 10 mg of standard in 10 ml of methanol in a volumetric flask. Stock standard solutions are stored at -20°C in the dark.

NOTE: *Molecular formula, purity and water content should be carefully considered when calculating the amount of substance to be dissolved.*

26.5.4.2 CAP intermediate standard solution (3 mg/l)

Prepare CAP intermediate standard solution at 3 mg/l, by diluting 30 µl of 1 000 mg/l CAP stock standard solution up 10 ml with methanol in a volumetric flask. Prepare this standard daily.

26.5.4.3 Mixed standard solution 1 (MS 1) (30 µg/l CAP, 3 mg/l TAP and FF)

Dilute 30 µl of TAP stock standard solution (1 000 mg/l), 30 µL of FF stock standard solution (1 000 mg/l), and 100 µl of CAP intermediate standard solution (3 mg/l) in a volumetric flask up to 10 ml with water. MS 1 is used to spike the samples. Prepare this standard daily.

26.5.4.4 Mixed standard solution 2 (MS 2) (3 µg/l CAP, 300 µg /l TAP and FF)

Dilute 1 mL of MS 1 with water up to 10 mL in a volumetric flask. This mixed standard solution is further diluted to prepare the calibration standard solutions. Prepare this standard daily.

26.5.4.5 Calibration standards

Matrix matched calibration procedure is used in the analysis. Weigh five portions of 3 g (± 0.05 g) of blank sample (matrix blanks) as the same matrix to be analyzed into five 50 ml polypropylene tubes. Extract the samples following the sample extraction procedure. After evaporating the samples with nitrogen, redissolve the dry blank extracts in 0.5 ml of respective calibration standards (containing CAP, TAP and FF) prepared in water according to Table 169. Calibration standards should be prepared daily.

TABLE 169. Preparation of calibration standard

Calibration level	Volume from MS (µl)	Volume of water (µl)	CAP concentration (µg/l)	TAP and FF concentration (µg/l)
1	250	1250	0.5	50
2	375	1125	0.75	75
3	500	1 000	1	100
4	750	750	1.5	150
5	1 000	500	2	200

26.6 Procedure

Test portion

- Randomly pick three shrimps from the sample brought to the laboratory and then remove the carapace.
- Grind the muscle tissue and collect into a 50 ml conical tube.

- From the fish samples brought to the laboratory, grind the muscle tissue and collect into a 50 ml conical tube.
- Remove fat from the meat samples.
- Grind the meat and collect into a 50 ml conical tube.
- Weigh 3 g (± 0.05 g) of sample into a 50 ml conical tube for analysis.

Negative control

- Shrimp, fish or meat that does not contain residues of CAP, TAP and FF is used as a negative control.
- Weigh 3 g (± 0.05) g of sample into a 50 ml polypropylene tube.

Positive control

- A blank sample spiked with CAP at 0.3 µg/kg and with TAP and FF at 30 µg/kg level is used as the positive control.
- Add 30 µl of the spiking solution (MS 1) to 3 g (± 0.05) g of the blank sample.
- Leave the sample at room temperature for 15 min before proceeding with the sample extraction.

26.6.1 Sample extraction

a) Weigh 3 g (± 0.05 g) of ground sample (shrimp, fish or meat) into a 50 ml polypropylene tube.

b) Add 6 ml of ethyl acetate to the sample and vortex for 1 min.

c) After rotating the tubes for 10 min in a test tube rotator, centrifuge the samples at 3 200 g for 15 min at room temperature.

d) Transfer 5 ml of supernatant to a glass tube and evaporate it to dryness at 45°C using a mild flow N2 in a nitrogen evaporator.

e) Redissolve the dry residue in 500 µl of water and vortex for 1 min. The five blanks extracts used to prepare the calibration standards are redissolved in respective calibration standards (containing CAP, TAP and FF) prepared in water.

f) Add 1 ml of *n*–hexane to the sample and vortex for 1 min to defat the sample.

g) Transfer the contents into a 2 ml microcentrifuge tube.

h) Centrifuge the samples at 15 000 g for 10 min at room temperature.

i) Filter the aqueous layer (bottom layer) through 0.45 µm nylon syringe filter and transfer to autosampler vial with an insert.

j) Inject to LC–MS/MS after allowing sample to equilibrate to 15°C of autosampler temperature.

26.6.2 Instrumental Analysis (LC–MS/MS)

LC–MS/MS analysis is carried out using an UHPLC instrument interfaced to a triple quadrupole mass spectrometer. Chromatographic and mass spectrometric conditions used in the analysis are shown in Tables 170–172.

TABLE 170. UHPLC conditions

Instrument	Nexera X2 Ultra High Performance Liquid Chromatograph (Shimadzu, Japan)
Mode of separation	Reversed phased
Analytical column	Shimadzu Shim-pack XR-ODS (3 × 50 mm, particle size 2.2 µm)
Column temperature	30°C
Injection volume	20 µl
Total run time	5 min
Autosampler temperature	15°C
Flow rate	0.3 ml/min
Mobile phase solvent A	0.5 mM ammonium acetate
Mobile phase solvent B	methanol
Elution mode	Gradient elution (see Table 170 for gradient program)

TABLE 171. Mobile phase gradient

Time (min)	Solvent B (%)
0.01	50
0.80	50
1.50	80
1.51	50
5.00	50

TABLE 172. MS conditions

Instrument	LCMS 8040 Triple quadrupole mass spectrometer (Shimadzu, Japan)
Ion source	Electrospray ionization (ESI) in negative mode
Capillary voltage	-3.5 kV
Nebulising gas flow rate (N_2)	3 l/min
Drying gas flow rate (N_2)	10 l/min
Desolvation Line temperature	250°C
Heat block temperature	400°C
CID gas pressure (Ar)	230 kPa

Data acquisition is performed in scheduled multiple reaction monitoring mode (sMRM). The two most intense transitions are monitored for each analyte. Optimized MRM conditions are shown in Table 173.

TABLE 173. MRM conditions for CAP, TAP and FF

Analyte	Precursor m/z	Product m/z	Dwell time (ms)	Q1 pre-rod bias (V)	Collision energy (V)	Q3 pre-rod bias (V)
CAP	321.2	152.1[a]	96	15	18	12
		257.1	96	15	12	14
TAP	354.2	185.1[a]	36	16	21	16
		290.1	36	16	13	11
FF	356.2	336[a]	63	17	11	20
		219.05	63	17	13	12

[a] quantifier ion

26.6.3 Interpretation of results

Identity confirmation

Drug residues in sample are considered confirmed once all the following method performance criteria are met.

i) The retention time of the analyte in test sample should correspond to that in the matrix matched calibration standard with a tolerance of ± 0.1 min.

ii) Presence of two product ions from each molecular ion, and signal-to-noise ratio > 3 for all diagnostic ions.

iii) Ion ratio or relative intensities of the detected ions between the two transitions for each drug should correspond to those of the matrix matched calibration standard (at comparable concentrations) within the following tolerances as in Table 174.

TABLE 174. Maximum permitted tolerances for LC-MS/MS relative ion intensities

Relative intensity (% of base peak)	Tolerances (%) (relative)
> 50%	± 20
> 20% to 50%	± 25
> 10% to 20%	± 30
≤ 10%	± 50

Criteria for acceptability of results

i) Calibration curve for each analyte shall give a coefficient of determination (R^2) greater than or equal to 0.95.

ii) Recovery of analytes in spiked samples should be between 70% and 120%.

Results reporting

i) Results for each analyte should be reported in µg/kg, after correction for recovery.

ii) Results below the limit of detection should be reported as "not detected".

iii) Results between the LOD and LOQ should be reported as "detected below LOQ".

iv) Results above the LOQ should be reported with the numerical value.

26.6.4 Method Validation

Selectivity

Selectivity of the analytical method is evaluated by analyzing blank shrimp and fish samples. No co-eluting peaks from endogenous compounds are detected at the retention times of the analytes.

Recovery

Recovery of analytes is assessed by analyzing blank shrimp and fish samples spiked with CAP (0.3 µg/kg), TAP (30 µg/kg) and FF (30 µg/kg); See Table 174).

Precision

Precision was evaluated as the repeatability (intra–assay precision) and intermediate precision (within-laboratory reproducibility), by analyzing three replicates of blank samples spiked with CAP (0.3 µg/kg), TAP (30 µg/kg) and FF (30 µg/kg) on different days (See Table 174).

Limits of detection and quantification

The LOD and LOQ are calculated based on the signal–to–noise ratio method, in which signal–to–noise ratio of 3 and 10 are considered LOD and LOQ, respectively (see Table 175).

TABLE 175. Method performance characteristics

Matrix	Analyte	Recovery (%)	RSDr (%)	RSDi (%)	LOD (µg/kg)	LOQ (µg/kg)
Shrimp	CAP	80.5 ± 15.3	8.2	19.0	0.02	0.05
	TAP	72.0 ± 22.5	14.0	31.3	0.42	1.26
	FF	90.5 ± 17.7	14.6	19.6	0.86	2.59
Fish	CAP	81.5 ± 15.7	4.5	19.3	0.01	0.03
	TAP	55.5 ± 23.5	11.3	23.5	0.47	1.43
	FF	82.3 ± 20.5	9.80	20.5	0.75	2.28

RSD_r - repeatability; RSD_i - intermediate precision

26.6.5 Schematic diagram of the extraction procedure

TEST SAMPLE	1. Weigh 3 g (± 0.05 g) of ground sample into a 50 ml polypropylene tube.
	
SPIKING	1. Add 30 µl from mixed standard solution (MS 1) to blank sample. 2. Leave the sample at room temperature for 15 min.
	
SAMPLE EXTRACTION	1. Add 6 ml of ethyl acetate to the sample. 2. Vortex the sample for 1 min. 3. Rotate the samples for 10 min in a test tube rotator. 4. Centrifuge the samples at 3 200 g for 15 min at room temperature. 5. Transfer 5 ml of supernatant to a glass tube. 6. Evaporate the supernatant do dryness at 45°C with N2 7. Redissolve the dry residue in 500 µl of water (blank extracts used for matrix-matched calibration standards must be redissolved in standards prepared in water) 8. Vortex for 1 min. 9. Add 1 ml of n-hexane to the sample. 10. Vortex for 1 min. 11. Transfer the contents into a 2 ml microcentrifuge tube. 12. Centrifuge the samples at 15 000 g for 10 min at room temperature. 13. Filter the bottom aqueous layer through 0.45 µm nylon syringe filter. 14. Transfer the filtrate into an autosampler vial with an insert. 15. Inject to LC–MS/MS system.

27

Determination of veterinary drug residues in meat and egg by LC–MS/MS

27.1 Introduction

Veterinary drugs are used frequently in animal production for therapy, prophylaxis, metaphylaxis and growth promotion in food-producing animals. Although this practice is intended to improve productivity, irrational use of veterinary drugs, such as drug misuse or not adhering to withdrawal times, can lead to the presence of harmful levels of drug residues in food of animal origin, through which consumers can become exposed sub-therapeutic doses of drugs. Such exposures are known to be associated with human health hazards such as hypersensitivity reactions, carcinogenic, mutagenic, and teratogenic effects, tumour induction, and effects on fertility. Furthermore, continuous exposure to drug residues can negatively influence the intestinal microflora and may promote the development of antimicrobial-resistant strains of bacteria in humans, which has now become a global concern. Because societies rely heavily on animal products such as meat and egg for nutrition, the presence of veterinary drug residues in animal products has become a global food safety concern. To ensure consumer safety, many countries and regulatory authorities have either banned the use of certain veterinary drugs in food-producing animals or established MRLs for veterinary drugs in edible animal tissues (EC, 2003; EC, 2010). These drug residues present in animal tissues such as meat and eggs can be detected and quantified using LC–MSMS.

27.2 Scope

The LC–MS/MS method can be used for simultaneous determination of 30 veterinary drugs belonging to 4 classes in eggs and meat. The applicable veterinary drugs are tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline and demeclocycline), fluoroquinolones (enrofloxacin, ciprofloxacin, norfloxacin and flumequine), beta lactams (penicillin G, amoxicillin, ampicillin, cloxacillin, cefalexin, cefoperazone and penicillin V), sulfonamides (sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadimidine, sulfadoxin, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, sulfathiazole, dapsone). The method allows quantification of the residues below the MRLs or MRPL established by the European Union. This is to ensure the quality and reproducibility of the procedure.

27.3 Principle of the method

Veterinary drug residues in the sample are extracted using a mixture of acetonitrile/methanol/and EDTA. EDTA is added to prevent chelation of tetracyclines to metals. Extraction is facilitated by ultrasonication, rotating and vortexing. Sample extract is then cleaned up using solid-phase extraction using C18 material and is defatted using hexane. Sample is then dried using mild flow of nitrogen and then redissolved in 0.1% formic acid in water and then injected to LC–MS/MS.

27.4 Safety considerations and precautions

Chemicals used in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Suitable gloves must be worn when chemicals are handled, and the work shall be performed in a fume hood at all possible time.

27.5 Materials

27.5.1 Reagents and solvents

The following reagents and chemicals are applicable: Type 1 water (ultrapure water); methanol (LCMS grade); acetonitrile (LCMS grade); EDTA; formic acid (HCOOH); *n*–hexane.

27.5.2 Equipment/instrument and consumables

The following apparatus and material are applicable: Analytical balance (Ohaus/Sartorius); polypropylene tubes (15 ml and 50 ml); grinder (Moulinex); tissue homogenizer (Ultra Turrax® T25 basic/Ultra Turrax® T 25 digital); micropipettes (0.5–10 µl, 100–100 µl, 100–1 000 µl, 500–5 000 µl) (Eppendorf/Nichiriyo); volumetric flasks (5 ml and 10 ml); spatulas; plastic weigh boats; test tube rotator (GFL); vortex mixer (Velp); ultrasonic bath (Bransen); centrifuge (Eppendorf/Hermle); C18 SPE cartridges (Chromabond, 500 mg); 12 position extraction manifold (Agilent); vacuum pump (Cole parmer); Nitrogen evaporator (N-EVAP, Organomation); Nitrogen gas (for sample concentration, purity 99.95%); Nitrogen generator (Claind); Argon gas for mass spectrometry; Nylon syringe filters (0.45 µm, 13 mm); Disposable syringes (1 ml or 3 ml); Microcentrifuge tubes (1.5 ml and 2 ml); Pasteur pipettes; Autosampler vials; polypropylene inserts for autosampler vials; filtering device for solvents and water (consisting of filtering cup, filtering head, conical flask and clamp); Nylon membrane filters (0.45 µm); UHPLC system (Shimadzu Nexera 2); Triple quadrupole mass spectrometer (Shimadzu LCMS 8040) with Electrospray ionization (ESI) interface; Software: LabSolutions (version 5.93).

27.5.3 Solutions

The solutions (and how they are prepared) include:

0.1 M EDTA

- Mix 2 ml of 0.5 MEDTA 8 ml of water.

Extraction solvent

- Mix 80 ml of acetonitrile, 30 ml of methanol and 10 ml of 0.1 M EDTA.

Mobile phase solvent A (0.1% formic acid in water)

- Dilute 0.5 ml of formic acid up to 500 ml with water and filter through 0.45 um filter.

Mobile phase solvent B (0.1% formic acid in methanol)

- Dilute 0.25 ml of formic acid up to 250 ml with methanol and filter through 0.45 um filter.

27.5.4 Standards

27.5.4.1 Stock standard solutions (1 000 mg/l)

Weigh 10 mg (± 0.1 mg) of the standards and prepare the individual stock standard solutions at 1 000 mg/l by dissolving in 10 ml of the solvent in a volumetric flask. Solvent for each standard is given in the Table 176. Store the stock standard solution at -20°C in dark.

NOTE: *Molecular formula, purity and water content should be considered when calculating the amount of substance to be dissolved.*

TABLE 176. Analytical standards used

Analyte	Manufacturer	Catalogue Number	Solvent
Tetracycline hydrochloride	Sigma-Aldrich	31741	Methanol
Oxytetracycline hydrochloride	Sigma-Aldrich	46598	Methanol
Chlortetracycline hydrochloride	Sigma-Aldrich	46133	Methanol
Doxycycline hyclate	Sigma-Aldrich	33429	Methanol
Demeclocycline	Sigma-Aldrich	D6140	Methanol
Enrofloxacin	Sigma-Aldrich	33699	100 µl of 2 M NaOH and Methanol
Ciprofloxacin	Sigma-Aldrich	33434	100 µl of 2 M NaOH and Methanol
Norfloxacin	Sigma-Aldrich	33899	100 µl of 2 M NaOH and Methanol
Flumequine	Sigma-Aldrich	45735	100 µl of 2 M NaOH and Methanol
Penicillin G potassium salt	Sigma-Aldrich	46609	Water
Penicillin V potassium salt	Sigma-Aldrich	46616	Water
Amoxicillin trihydrate	Sigma-Aldrich	31586	Water
Ampicillin tryhydrate	Sigma-Aldrich	31591	Water
Cloxacillin sodium salt monohydrate	Sigma-Aldrich	46140	Water
Cefalexin	Sigma-Aldrich	33989	acetonitrile/ water (50/50)
Cefoperazone dihydrate	Sigma-Aldrich	32426	acetonitrile/ water (50/50)
Sulfachlorpyridazine	Sigma-Aldrich	46778	Methanol
Sulfadiazine	Sigma-Aldrich	S8626	Acetonitrile
Sulfadimethoxine	Sigma-Aldrich	46794	Methanol
Sulfadimidine	European Pharmacopoeia	S1900000	Methanol
Sulfadoxin	Sigma-Aldrich	31736	Methanol
Sulfamerazine	Sigma-Aldrich	S8876	Methanol

(con't)

TABLE 176. Analytical standards used (con't)

Analyte	Manufacturer	Catalogue Number	Solvent
Sulfamethazine	Sigma-Aldrich	46802	Methanol
Sulfamethizole	Sigma-Aldrich	46842	Methanol
Sulfamethoxazole	Sigma-Aldrich	31737	Methanol
Sulfamethoxypyridazine	Sigma-Aldrich	46858	Methanol
Sulfapyridine	Sigma-Aldrich	31738	Methanol
Sulfaquinoxaline	Sigma-Aldrich	45662	DMSO and Methanol
Sulfathiazole	Sigma-Aldrich	46902	Methanol
Dapsone	Sigma-Aldrich	46158	Acetonitrile

27.5.4.2 Mixed standard solution 1 (MS 1) (10 mg/l)

Mix 100 µl of each of the stock standard solution (1 000 mg/l) in a 10 ml volumetric flask and complete up to 10 ml with methanol. This mixed standard solution is used to spike the blank samples, and to prepare the calibration standards in 0.1% formic acid in water. Store the mixed standard solution at -20°C in dark.

27.5.4.3 Mixed standard solution 2 (MS 2) (200 µg/l)

Dilute 200 µl of MS 1 (10 mg/l) up to 10 ml in a volumetric flask with 0.1% formic acid in water to prepare mixed standard solution at 200 µg/l. MS 2 should be prepared daily.

27.5.4.4 Calibration standards

a) Matrix matched calibration procedure is used in the analysis.

b) Weigh five portions of 3 g (± 0.05 g) of blank sample as the same matrix to be analyzed into five 50 ml polypropylene tubes.

c) Extract the samples following the sample extraction procedure.

d) After the sample has been evaporated with nitrogen, redissolve the dry blank extracts in 0.5 ml of respective calibration standards (containing all 30 analytes) prepared in 0.1% formic acid in water.

e) Prepare the calibration standards by diluting MS 2 (200 µg/l) with 0.1% formic acid in water according to Table 177.

TABLE 177. Preparation of calibration standards

Concentration of calibration standard (µg/l)	Volume of MS 2 (200 µg/l) (µl)	Volume of 0.1% formic acid in water (µl)
10	100	1 900
25	250	1 750
50	500	1 500
75	750	1 250
100	500	500

Calibration standards in 0.1% formic acid in water must be prepared daily.

27.6 Procedure

Test portion

- Mix the contents of five eggs and blend at high speed.
- Remove fat from the meat samples (chicken, beef and pork) and grind around 100 g of meat in a grinder.
- Weigh 3 g (± 0.05 g) of ground meat/ blended eggs in to a 50 ml polypropylene tube for the analysis. Record the sample weight.

Negative control

- Meat or eggs samples without veterinary drug residues are used as negative control samples.
- Weigh 3 g (± 0.05 g) of blank sample in to a 50 ml polypropylene tube.

Positive control

- A blank sample (meat or eggs) spiked with the 30 veterinary drugs, each at 100 µg/kg, is used as a positive control.
- Weigh 3 g (± 0.05 g) of blank sample into a 50 ml polypropylene tube, and then add 30 µl of the 10 mg/l MS 1 to yield 100 µg/kg tissue concentration of all veterinary drugs.
- Let the spiked sample stand 15 min at room temperature before proceeding with the sample extraction.

27.6.1 Sample extraction

a) Weigh 3 g (± 0.05 g) of sample (egg or meat) into a 50 ml polypropylene tube and add 12 ml of extraction solvent.

b) Vortex the samples for 1min. Sonicate the samples for 15 min at room temperature in an ultrasonic bath. A

c) After sonication, rotate the tubes for 15 min in a test tube rotator.

d) Centrifuge the sample at 3 000 g for 15 min at room temperature.

e) Clean up 6 ml of the supernatant using solid-phase extraction cartridges as described below. Before the cleanup, condition the SPE columns as described below.

Solid phase extraction (SPE) cleanup

- Connect the extraction manifold to the vacuum pump.
- Fix the SPE cartridges to the stopcock valves attached to the extraction manifold.
- Condition the SPE columns, by passing pass 3 ml of methanol and then washing with 3 ml of water.
- Do not allow the sorbent material to dry completely.
- Load 6 ml of the supernatant of the centrifugation and pass through the column at a flow rate less than 1 drop per second and collect the eluate at the bottom of the SPE cartridge into a 15 ml polypropylene tube.
- Achieve the desired flow rate through the SPE column, using gravity or applying vacuum to the extraction manifold as necessary.
- After the SPE cleanup, add 6 ml of *n*–hexane to the sample and vortex for 1 min and then discard the hexane layer.
- Repeat the defatting with another 6 ml of hexane.

- Transfer 1 mL of the sample into a 15 ml polypropylene tube and evaporate it to dryness at 45°C using a mild flow of nitrogen in a nitrogen evaporator.
- Redissolve the dry residue in 500 µl of 0.1% formic acid in water. Blank extracts used to prepare matrix matched calibration standards are redissolved in respective calibration solutions (10 µg/l, 25 µg/l, 50 µg/l, 75 µg/l and 100 µg/l) prepared in 0.1% formic acid in water.
- Filter the sample through 0.45 µm syringe filter and transfer to an autosampler vial with a polypropylene insert.
- Inject the sample to LC–MS/MS.

27.6.2 Instrumental Analysis (LC-MS/MS)

LC-MS/MS analysis is carried out using an UHPLC instrument interfaced to a triple quadrupole mass spectrometer. Chromatographic and mass spectrometric conditions used in the analysis are given in Tables 178–180.

TABLE 178. LC conditions

Instrument	Nexera X2 Ultra High Performance Liquid Chromatograph (Shimadzu, Japan)
Mode of separation	Reversed phased
Analytical column	Shimadzu Shim-pack XR-ODS (3 × 50 mm, particle size 2.2 µm)
Column temperature	30°C
Injection volume	5 µL
Total run time	12 min
Autosampler temperature	15°C
Flow rate	0.4 mL/min
Mobile Phase solvent A	0.1% formic acid in water
Mobile phase solvent B	0.1% formic acid in methanol
Elution mode	gradient elution (see Table 4)

TABLE 179. Mobile phase gradient

Time (min)	Solvent B (%)
0.01	20
3.75	40
6	100
8	100
8.01	20
12	20

TABLE 180. MS conditions

Instrument	Triple quadrupole mass spectrometer (LCMS 8040, Shimadzu, Japan)
Ion source	Electrospray ionization (ESI) positive ion mode
Capillary voltage	+4.5 kV
Nebulizing gas flow rate (N_2)	3 L/min
Drying gas flow rate (N_2)	10 L/min
Desolvation Line temperature	250°C
Heat block temperature	400°C
CID gas pressure	230 kPa

Data acquisition is performed in scheduled multiple reaction monitoring mode (sMRM). Optimized MRM conditions are given in Table 181.

TABLE 181. MRM conditions

Analyte	Precursor ion *m/z*	Product ions *m/z*	Dwell time (ms)	Q1 pre-rod bias (V)	Collision energy (V)	Q3 pre-rod bias (V)
Amoxicillin	366.1	114.0	11	-24	-20	-25
		349.15	11	-24	-8	-27
Sulfadiazine	251.1	156	33	-12	-16	-17
		92.15	33	-12	-29	-17
Sulfathiazole	256	108.1	12	-18	-25	-21
		92.15	12	-24	-27	-17
Sulfapyridine	250	92.1	12	-22	-32	-18
		156	12	-23	-17	-16
Sulfamerazine	265.1	92.1	10	-13	-34	-17
		156	10	-13	-17	-16
Tetracycline	445.2	410.2	10	-15	-17	-23
		427.25	10	-15	-12	-18
Norfloxacin	320.1	302.1	5	-11	-18	-23
		276.25	5	-11	-16	-22
Sulfadimidine	279.2	186.05	3	-19	-16	-22
		124.05	3	-18	-21	-26
Ciprofloxacin	332.2	314.1	9	-23	-19	-24
		288.15	9	-23	-15	-23
Sulfamethazine	279.2	186	3	-19	-15	-15
		92.15	3	-19	-30	-20
Sulfamethizole	270.9	156.05	5	-17	-12	-19
		92.1	5	-17	-24	-20
Dapsone	249.2	156.1	5	-16	-12	-18
		92.05	5	-11	-23	-20

(con't)

TABLE 181. MRM conditions (con't)

Analyte	Precursor ion *m/z*	Product ions *m/z*	Dwell time (ms)	Q1 pre-rod bias (V)	Collision energy (V)	Q3 pre-rod bias (V)
Cefalexin	348.2	158	2	-12	-9	-19
		174.1	2	-12	-13	-21
Oxytetracycline	461.3	426.15	7	-16	-18	-24
		443.1	7	-16	-11	-25
Enrofloxacin	360.2	316.3	5	-24	-18	-25
		342.2	5	-16	-20	-26
Sulfamethoxypyridazine	281.1	108.15	9	-20	-28	-20
		156.05	9	-14	-17	-17
Ampicillin	350.1	106.15	2	-12	-20	-23
		192.1	2	-12	-14	-16
Demeclocycline	465.2	448.15	17	-16	-16	-25
		430.15	17	-16	-20	-24
Sulfachloropyridazine	285	156.05	2	-19	-13	-19
		108.05	2	-19	-22	-23
Sulfamethoxazole	254.2	156.15	3	-17	-14	-19
		92.05	3	-17	-27	-19
Sulfadoxin	311.1	156	22	-16	-20	-17
		92.1	22	-16	-34	-17
Chlortetracycline	479.2	444.2	3	-10	-21	-25
		462.1	3	-17	-16	-26
Cefoperazone	646.3	143.25	8	-22	-32	-29
		530.3	8	-22	-10	-30
Sulfadimethoxine	311.2	156.05	15	-21	-19	-18
		92.05	15	-21	-33	-20
Doxycycline	445.2	428.2	5	-30	-15	-24
		154.1	5	-30	-27	-18
Sulfaquinoxaline	301	156.05	24	-15	-17	-17
		92.05	24	-15	-37	-19
Flumequine	262.2	244.1	33	-17	-16	-29
		202	33	-17	-31	-23
Penicillin G	335.2	160	11	-23	-11	-19
		176.2	11	-11	-12	-14
Cloxacillin	436.2	160	11	-15	-14	-19
		277	11	-15	-13	-16
Penicillin V	351.1	160.15	21	-12	-11	-13
		114.15	21	-12	-29	-24

27.6.3 Interpretation of Results

Identity confirmation

Drug residues in sample are considered confirmed once all the following method performance criteria are met.

i) The retention time of the analyte in test sample should correspond to that of the matrix-matched calibration standard with a tolerance of ± 0.1 min.

ii) Presence of two product ions from each molecular ion, and signal-to-noise ratio > 3 for all diagnostic ions.

iii) Ion ratio or relative intensities of the detected ions between the two transitions for each drug should correspond to those of the matrix-matched calibration standard (at comparable concentrations) within the tolerances in Table 182.

TABLE 182. Maximum permitted tolerances for LC–MS/MS relative ion intensities

Relative intensity (% of base peak)	Tolerances (%) (relative)
> 50%	± 20
> 20% to 50%	± 25
> 10% to 20%	± 30
≤ 10%	± 50

Criteria for acceptability of results

i) Calibration curve for each analyte shall give a coefficient of determination (R^2) greater than or equal to 0.95.

ii) Recovery of analytes in spiked samples should be between 70% and 120%.

Results reporting

i) Results for each analyte must be reported in µg/kg.

ii) Results below the limit of detection should be reported as "not detected".

iii) Results between the limit of detection and limit of quantification should be reported as "detected below LOQ".

iv) Results above the LOQ should be reported with the numerical value.

27.6.4 Method Validation

Selectivity

Selectivity of the analytical method was evaluated by analyzing blank egg and meat samples. Co-eluting peaks from endogenous compounds were not detected at the retention times of the analytes.

Recovery

Recovery of analytes was assessed by analyzing blank egg and meat sample spiked with all the analytes (each at 100 µg/kg) (Table 182).

Precision

Precision was evaluated as the repeatability (intra–assay precision) and intermediate precision (within laboratory reproducibility), by analyzing three

replicates of blank samples spiked with all analytes (each at 100 µg/kg) on different days (See Table 182).

Limits of detection and quantification

The LOD and LOQ were calculated based on the signal-to-noise ratio method, in which signal-to-noise ratio of 3 and 10 are considered LOD and LOQ, respectively (Table 183).

TABLE 183. Method performance characteristics

Analyte	Recovery (%)	RSD_r (%)	RSD_i (%)	LOD (µg/kg)	LOQ (µg/kg)
Amoxicillin	53.6 ± 9.7	8.8	18.0	1.50	4.53
Sulfadiazine	59.0 ± 12.1	14.4	20.5	0.28	0.87
Sulfathiazole	60.8 ± 8.5	9.0	13.9	0.32	0.97
Sulfapyridine	61.6 ± 5.8	8.5	9.5	0.28	0.84
Sulfamerazine	62.7 ± 7.4	9.8	11.8	0.36	1.07
Tetracycline	53.4 ± 5.8	5.0	10.9	5.05	15.3
Norfloxacin	53.3 ± 5.9	5.8	11.1	1.16	3.52
Sulfadimidine	64.6 ± 10.9	11.5	16.9	0.60	1.83
Ciprofloxacin	51.8 ± 5.3	6.5	10.3	0.79	2.38
Sulfamethazine	62.9 ± 9.3	8.9	14.7	0.23	0.70
Sulfamethizole	65.7 ± 14.1	11.7	21.5	0.27	0.80
Dapsone	92.0 ± 12.3	9.2	13.3	1.57	4.77
Cefalexin	44.4 ± 15.3	16.7	34.4	2.48	7.51
Oxytetracycline	51.1 ± 10.8	13.5	21.1	0.48	1.44
Enrofloxacin	49.8 ± 8.0	6.7	16.1	1.01	3.06
Sulfamethoxypyridazine	58.6 ± 11.8	11.6	20.2	0.28	0.84
Ampicillin	54.0 ± 15.6	10.7	28.9	0.95	2.84
Demeclocycline	49.3 ± 4.7	4.9	9.6	1.18	3.59
Sulfachloropyridazine	75.8 ± 18.1	18.5	23.9	1.06	3.23
Sulfamethoxazole	69.4 ± 15.7	12.3	22.6	0.21	0.64
Sulfadoxine	65.4 ± 10.1	9.7	15.4	0.32	0.96
Chlortetracycline	42.7 ± 3.1	5.7	7.2	1.53	4.64
Cefoperazone	59.6 ± 16.1	17.1	27.0	1.28	3.87
Sulfadimethoxine	68.7 ± 18.0	27.2	26.2	0.55	1.66
Doxycycline	53.8 ± 3.6	6.6	6.7	1.36	4.12
Sulfaquinoxaline	33.1 ± 5.1	14.5	15.3	0.22	0.66
Flumequine	56.0 ± 3.8	7.2	6.9	0.75	2.26
Penicillin G	139.8 ± 39.4	16.2	28.2	7.14	21.64
Cloxacillin	71.4 ± 15.8	18.1	22.1	1.84	5.57
Penicillin V	56.8 ± 19.3	25.0	34.0	1.95	5.85

RSDr - repeatability; RSDi - intermediate precision

27.6.5 Schematic diagram of extraction procedure

TEST SAMPLE	1. Weigh 3 g (± 0.05 g) of ground meat or egg into a 50 ml polypropylene tube.
SPIKING	2. Add 30 µl from mixed standard solution (10 mg/l) to blank sample.
SAMPLE EXTRACTION	3. Add 12 ml of extraction solvent. 4. Vortex the sample for 1 min. 5. Sonicate the sample for 15 min at room temperature. 6. Rotate the tubes for 15 min in a test tube rotator. 7. Centrifuge the samples at 3 000 g for 15 min at room temperature.
SPE CLEAN UP OF SAMPLE	8. Pass 3 ml of methanol through the SPE column. 9. Pass 3 ml of water through the SPE column. 10. Load 6 ml of the supernatant onto the SPE column and pass at rate of 1 drop per second flow rate. 11. Collect the eluate at the bottom of the SPE column in a 15 ml polypropylene tube.
SAMPLE EXTRACTION	12. Add 6 ml of *n*–hexane, vortex for 1 min and discard hexane layer. 13. Repeat the previous step with another 6 ml of *n*–hexane. 14. Transfer 1 ml of sample into a 15 ml polypropylene tube. 15. Evaporate the sample to dryness at 45°C using nitrogen. 16. Redissolve the dry residue in 500 µl of 0.1% formic acid in water. 17. Redissove the blank extract for matrix-matched calibration standards in standards prepared in 0.1% formic acid in water. 18. Filter the sample through 0.45 µm syringe filter and transfer to an insert. 19. Inject to LC–MS/MS.

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Manual of Standard Operating Procedures for Selected Chemical Residue and Contaminant Analysis

Food safety is an important global public health and trade matter, with chemical hazards occupying centre stage due to associated acute and chronic health outcomes. There is also an increasing need to address antimicrobial resistance concerns. While food remains a major vehicle for exposure to these hazards, related matrices cannot be ignored. Animal feed for instance may contain drug or pesticide residues as well as mycotoxins that could carry-over to food either as parent compounds or their metabolites of toxicological relevance. Contaminated water is also another medium of potential exposure to food hazards.

A concerted effort is required to address the need for a safe food supply and one critical stakeholder is the testing laboratory. While this requires trained and capable analysts as well as reliable instrumentation, analytical methods are a major need. Development and validation – to ensure fitness of purpose – and availability of these methods is a necessity. This manual, consisting of several Standard Operating Procedures (SOPs), presents another opportunity for laboratories to address gaps in analytical methods and/or expand their options. The manual contains techniques for analyzing certain mycotoxins such as aflatoxins, fumonisin and ochratoxin in matrices that include milk, edible vegetable oil and animal feed etc. A range of veterinary drug residues including permitted and prohibited substances in animal matrices including fish, are also addressed. Several pesticide residues in cereals, fruits and vegetables are also covered. A couple of methods for analysis of selected metals are also presented. In general, the scope of SOPs here includes screening, quantitative and confirmatory methods. While these tests and analytes only cover a narrow gap for the needs of a routine testing or research laboratory, the manual is expected to fill some gaps. Future editions should expand the scope.

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