Analytical method **Valid from 01.07.2011**	**E_FP417.1**

ANALYSIS OF PESTICIDES IN CEREALS USING THE QUECHERS METHOD AND DETECTION BY GCMS, GC-MS/MS AND/OR LC-MS/MS

Principle: Cold water, acetonitrile and if necessary an internal standard is added to the milled sample. The sample is shaken and a salt and buffer mixture is added, the sample is shaken again. After centrifugation the supernatant is transferred to a tube with PSA and $MgSO_4$. After shaking and another centrifugation the extract is ready for analysis by GC-MS(/MS) and/or LC-MS/MS.

Scope of application: The method is used for the determination and quantification of pesticides, metabolites and isomers in cereals and cereal products (e.g. grain, bran etc.).

Official reference: Determination of pesticide residues using GC-MS(/MS) and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE – QuEChERS-method. CEN/TC 275 prEN 1556662:2007.

Validation:

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1. List of content

2. Scope of application

The method is based on the procedure for dry matrices (<30% water content) according to the document CEN/TC 275/WG 4 N 0204[1].

The method is used by the department for Food Chemistry at DTU, The National Food Institute, for the analysis of pesticides in cereals and cereal based products.

3. References

[1] Foods of plant origin – Determination of pesticide residues using GC-MS and/or LC-MS(/MS) following acetonitrile extraction/partitioning and cleanup by dispersive SPE – QuEChERS-method. CEN/TC 275 prEN 1556662:2007.

[2] Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed[1]. Document N° SANCO/10684/2009 or latest version.

4. Principles of the method

Cold water (ice water), acetonitrile and if necessary an internal standard is added to the milled sample. Note that it is very important that the entire sample is wetted thoroughly. The sample is shaken and a salt and buffer mixture is added, then the sample is shaken again. After centrifugation the supernatant is transferred to a tube

with PSA and $MgSO_4$. After shaking and another centrifugation the extract is ready detection by GC-MS (/MS) and/or LC-MS/MS.

5. Chemicals

All reagents and organic solvents are of Analytical Grade unless anything else is stated.

Solvents must not show any peaks at the same retention time as the pesticides analysed for. This is controlled by evaporating 10 ml solvent added 0.10 ml 10% propane diol on a vacuum evaporator, dissolving the residue in 1.00 ml acetonitrile and performs a chromatographic determination.

5.1 Acetonitrile (HPLC grade)

5.2 Sodium sulphate (Na_2SO_4), anhydrous, grit or powder. Heated to 500°C for 5 hours (to remove phthalates) and conditioned to room temperature covered with aluminium foil before use.

5.3 Sodium chloride

5.4 Trisodium citrate dehydrate (Sigma-Aldrich W302600 or similar)

5.5 Disodium hyrogencitrate sesquihydrate (Sigma-Aldrich 359084 or similar)

5.6 Formic acid (5% v/v, eg. 5 ml formic acid /100 ml acetonitrile)

5.7 Bondesil-Primary/Secondary Amine (PSA) 40 µm (Varian)

5.8 Prepacked test tubes 1: (5.2+5.3+5.4+5.5), Supelco dSPE citrate extraction tube (#55227-U).

5.9 Prepacked test tubes 2: (5.2+5.7), Supelco dSPE PSA clean-up tube 1 (#55228-U).

5.10 Sodium hydroxide (5N)

5.11 Sulfuric acid H_2SO_4

5.12 <u>Stock solutions</u>:

Dissolve at least 5 mg of the pesticide in toluene, or another suitable solvent, resulting in a concentration of 1 mg/ml (pesticides and stock solutions is stored at -18°C).

5.13 Standards for spiking and for external calibration. Prepare a standard solution of 20.0 µg/ml and 2.00 µg/ml by volumetrically dilution of the stock solutions (5.12) with acetonitrile.

When spiking, add standard to the sample resulting in a spike concentration of 0.01, 0.02 and 0.1 mg/kg cereal or cereal product.

Prepare at least three calibration standards at different concentration levels diluted with matrix. The matrix should match the sample or alternatively use standard addition at the same level as the sample with a positive find.
These standards are stable for 6 months when stored at -18°C in glass containers with air tight lids.

5.14 Internal standard. Triphenyl phosphate can be used as internal standard for correction of the results obtained by GC-MS(/MS) detection. Dilute a stock solution (5.10) with acetonitrile resulting in a concentration of 20 µg/ml.

$^{13}C_6$-carbaryl can be used as internal standard in the LC-MS/MS detection. A stock solution of 1 µg/ml is prepared (5.12), and a workingsolution of 0.1 µg/ml is prepared. The internal standard is added prior to injection on the LC-MS/MS and is used for correction of the detection.

These standards are stable for 6 months when stored at -18°C in glass containers with air tight lids.

6. Apparatus (Equipment)

6.1 Centrifugal mill (Retsch Ultra centrifugal mill ZM1000, sieve size 0.5 mm)

6.2 50 ml disposable centrifuge tubes with screw caps (Sarstedt 114x28mm, polypropylene, or similar)

6.3 15 ml disposable centrifuge tubes with screw caps (Sartsted 17 mm x 120mm, polypropylene or similar)

6.4 Mini uniprep polypropylen filter vials 500µl, Whatman, Cat. No. UN203NPUPP poresize 0.45µm.

6.5 Centrifuge. Heraeus Sepatech Megafuge 3.0 R, explosion proofed.

6.6 Gas chromatographic equipment:
GC-MS detection could be performed by a Finnigan Polaris (iontrap) equipped with temperature-programmable split less injector or similar equipment.

GC-MS/MS detection could be performed by a Waters Quattro Micro GC-MS/MS system.

The LC-MS/MS detection could be performed by a Agilent HPLC coupled with a Micromass Quattro Ultima Triple Quadrupole Instrument or similar instruments.

7. Sampling and sample preparation

Analysis is done in accordance with the latest version of the SANCO document "Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed"[2].

8. Procedure

8.1 Homogenisation and grinding: The laboratory sample is mixed and 50 g. is homogenised to flour by milling

For basic hydrolysis step 8.5-8.7 is followed. Hydrolysis is used to break covalent bonds between acidic pesticides and the matrix.

8.2 **Quality control analysis:** For every analytical series a spiked sample is always included for the determination of recoveries of the pesticides relevant for the sample/samples to be analysed. If the sample is without content a single determination is performed. Double determinations are typically performed for samples with content. The results obtained for the two double determinations are acceptable if the difference is ≤25%.
For recovery tests a maximum of 1.00 ml spiking standard is added.

8.2 **Extraction**:

- 5.0 g of sample is weighed into a 50 ml centrifuge tube. Add internal standard if required.

- Add 10 ml of cold deionised (demineralised) water, shake vigorously, it is very important that the sample is soaked thoroughly.
- Add 10.0 ml acetonitrile. Close the tube and shake vigorously by hand for 1 min (1st extraction), or by other shaking devices.
- Add a buffer-salt mixture consisting of: 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hyrogencitrate sesquihydrate. Shake for a few seconds after each addition to prevent lumps. Prepacked centrifugation tubes are available on the marked and can be used (5.8).
- Shake the centrifugation tubes vigorously for 1 min. (2nd Extraction with phase separation), or use shaking device.
- Centrifuge (3.4) the sample for 10 min. at 4500 rpm or until the supernatant is clear.
- Analysis of acidic pesticides (without hydrolysis) by LC-MS/MS is to be performed on this crude extract.

8.3 **Clean up**

Removal of co-extracted low soluble substances (fat, sugars etc.) (**optional**):

- Transfer an aliquot of 8 ml of the raw acetonitrile extract to a 15 ml. centrifuge tube and store for a minimum of 1 hours (or overnight) in the freezer (-80°C). Before centrifugation for 5. Min. at 5°C 4500 rpm, allow the extracts to become almost liquid.
- Alternatively draw the raw acetonitrile extract into a syringe with a ball of surgical cotton and freeze the whole syringe for 1 hour at -80°C and when the extracts has just thawed press the extract through the cotton.

Good results may be obtained for most matrixes without the freezing-out step (e.g. wheat) however less contaminants are injected in the GC/LC system.

Dispersive SPE with PSA (amino sorbent):

- Transfer 6 ml of cold acetonitrile extract to a PP-disposable centrifugation tube containing 150 mg PSA (25 mg per ml. Extract) and 900 mg $MgSO_4$ (150 mg per ml. Extract). Prepacked tubes may be used (5.9).
- Close the centrifugation tube and shake vigorously for 30 seconds.
- Centrifuge for 5 min. at 4500 rpm (hereafter immediately acidify the extract, pH is adjusted to pH=8.4, see below)

8.4 **Extract stabilisation by acidification**

- Transfer an aliquot of 4 ml. of the cleaned-up extract into a 15 ml centrifuge-tube; make sure that no sorbent particles are transferred as well. Slightly acidify the extract by adding 40 µl of 5% formic acid solution in acetonitrile (5.6).
- Dilute the pH-adjusted extract 1:1 with acetonitrile to obtain same matrix concentration as in the calibration standards.
- Transfer the pH-adjusted extract into autosampler vials and analyse by GC-MS, GC-MS/MS or LCMSMS.

8.5 **Basic hydrolysis (optional).**

- 5.0 g sample is weighed into a 50 ml. centrifuge tube (internal standard is added if needed).
- 10 ml deiononised/demineralised water and 300µl 5N NaOH solution is added (pH~12). Shake vigorously for 1 min immediately after addition.
- The sample is left to stand for 30 min, however the sample has to be shaken every 10. Min.
- 300µl 5N H_2SO_4 is added (neutralizes the sample).

8.6 **Extraction (basic hydrolysis)**

- Add 10 ml acetonitrile and 100µl intern standard to the sample, shake vigorously for 1 min.
- Add a salt and buffer mixture consisting of 4 g $MgSO_4$, 1 g NaCl, 1 g trinatrium citrate dihydrat and 0,5 g dinatrium hyrogencitrate sesquihydrate. Shake shortly after each addition of the salt to avoid lumps. Prepacked centrifugation tubes (5.8) may be used. If $MgSO_4$ grit is used, be aware that more $MgSO_4$ (about 1 gram extra) has to be added to remove the excess of water.
- Shake the centrifugation tubes vigorously for 1 min. (2. extraction with phase separation).
- Centrifuge the sample for 10 min. at 4500 rpm.

8.7 **Clean-up (basic hydrolysis)**

- Transfer 8 ml of the crude extract to a 15 ml centrifuge tube and store in -80°C freezer for at least 1 hour, or store in freezer overnight. Hereafter centrifuge at 5°C, the supernatant is transferred in a 15 ml centrifuge tube.

- The extract is diluted 1:1 with acetonitrile to obtain the same concentration of matrix in the sample as in the calibration standards.
- 400µl diluted extract is transferred to HPLC-plastic vials with build-in polypropylene filters (6.4). For each 400 µl sample or standards, 40 µl of internal standard (0.1 µg/ml) is added to the vials before filtration. Hereafter analysis of the extract on LC-MS/MS.

8.8 **Gas chromatographic determination**

The samples, including the quality control samples, are analysed by GC-MS, GC-MS/MS and/or LC-MS/MS. Quantification is based on two calibration curves (standards analysed before and after the samples). The curves are base on 3-5 matrix matched standard solutions, covering the relevant concentration range.

Matrix-matched standards are prepared by adding a representative matrix gained by the same extraction method and cleanup procedure as for the sample(s).

If the content of a sample is not within the range of the calibration curves the content of the sample can not be quantified. Samples with contents lower than the concentrations covered by the calibration curve are reanalysed with another set of calibration standards of lower concentration. Samples with contents higher than the concentrations higher than the concentrations covered by the calibration curve are diluted to an appropriate concentration and reanalysed.

Performance characteristics for GC-MS (iontrap),
Injection volume: 8 µl, PTV split injection
Temperatures:
Injector: Base temp. 80°C. Evaporation temp. 81°C, 4°C/sekund til 280°C i 1 min.
Column: DB5-MS (30mx0.25 mmID., film thickness 0.025µm), RESTEK (Rxi®-5ms, 30 m., 0.25 mmID, 0.25 µm df) or another type suited for the purpose.
Column temp: 2.5 min at 60°C, 30°C /min to 180°C, 5°C /min to 280° for 5 min, 40°C/min to 300 for 6 min.
Ion Source: 250°C
Transfer line: 280°C
MS scanning in EI-mode from 50-400 amu.
The compounds validated by GC-MS are presented in Appendix 1.

Example of performance characteristics for GC-MS/MS

Injection volume: 8 µl
Column: Restek Rxi-5ms 30 meter, 0.25 mm ID, 0.25µm coating (or similar).

Electron energy:	70 eV
Source temp:	180°C
Transfer line:	250°C
Interface:	250°C

Example of performance characteristics for LC-MS/MS:
Injection volume: 10 µl
Column: Genesis C_{18} column or another suited column
HPLC: Injection volume 10 µl
Flow 0.30 ml/min
Gradient:

Time	Eluent A	Eluent B
0.0	100	0
2.0	50	50
20	0	100
24	0	100
25.1	100	0
30	100	0

MS/MS: Electro spray in positive mode (ESI+)

Capillary	1.00
Cone voltage	varies for different pesticides
Source temperature	120°C
Desolvation temperature	350°C
Cone gas flow	50 l/h
Desolvation gas flow	550 l/h
Collision energy (CE)	varies for different pesticides
Pirani pressure	1.7e-3 mbar
LM resolution 1; 2:	13.5
HM resolution 1; 2:	13.5
Ion Energy 1:	0.5
Ion Energy 2:	2.0
Multiplier	650
SPAN	0.1

Internal standard for quantification (13C6-carbaryl).

9. Screening for pesticides and calculation of contents

Screening for the content of pesticide residues is performed on the basis of retention times.

The concentration of a pesticide in a sample is calculated on the basis of the standard calibration curve. The formula for the regression line is defined by y= αx+β, where α is the slope of the line and β is the intercept with the y-axis. The calibration curve is drawn up by the relative responses obtained when analysing the standards in relation to the internal standard (RP/RIP) as a function of the standard concentration S (Table 1).

The content in the sample extract is calculated on the basis of the formula for the calibration curve:

$$K(\mu g / ml) = \frac{(RS / RIS) - b}{a}$$

The content of a sample (the cereal) can hereafter be calculated by:

$$Content(mg / kg)(C) = \frac{K \cdot V1}{G} = \frac{K\mu g / ml \cdot 10ml}{5g}$$

Table 1 The calculation parameters for the method, symbols and values

Concentration in cereals	C	µg/g (calculated)
Concentration in sample extract	K	µg/ml
Sample size	G	5.00 g
Volume of extraction solvent	V1	10.0 ml
PARAMETERS:	STANDARD:	SAMPLE:
Pesticide concentration	S	C
GC or LC-response pesticide	RS	RP
GC or LC-response, internal standard	RIS	RIP

Quality criteria

For positive identification of a pesticide by GC-MS it is required that the retention time and mass spectrum for sample and standard coincide. For positive identification by GC-MS/MS and LC-MS/MS it is required that the retention time of two ion transitions (quantifier and qualifier ions) coincide with that of the standard. The relative retention time of the analyte should for GC correspond to that of the calibration solution with a tolerance of ±0.5%. This has been found to be achievable for most analytes for a matrix like wheat, whereas for a matrix like rice larger

tolerances are needed. Thus for some matrixes and for some analytes a whither tolerance is needed (up to ±2%), when using a method with limited cleanup.

According to "Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed" (SANCO/10684/2009, or later versions) should the relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, correspond to those of the calibration standard of similar concentration when analysing by full scan and SIM. In the table below are presented the maximum tolerances for deviations between obtained intensities for sample and calibration standard.

Relative Intensity (% of base peak)	EI-GC-MS (relative)
> 50 %	± 10 %
> 20 % to 50 %	± 15 %
> 10 % to 20 %	± 20 %
≤ 10 %	± 50 %

Recoveries from spiked samples should be within the range of 70-110 %. For some pesticides and difficult matrixes or when using lower levels of spiking (<0.1 mg/kg) a larger range of recoveries can be found acceptable. These requirements are in accordance with "Method validation and Quality Control Procedure for Pesticide Residue Analysis in Food and Feed", ° SANCO/10684/2009, where it is stated that the recoveries for a single analysis should be within the range of 60-140%.

An uncertainty budget is prepared for the determination of the uncertainty of the method.

10. Safety precautions

The laboratory work is performed according to "The safety regulations at the "DTU, National Food Institute".

The pesticides are of different classifications, e.g. 'hazardous for your health' or 'carcinogenic'. The handling of the pesticides both in concentrated and diluted state should be performed in fume hood and gloves should be worn.

Instruments, where flammable organic solvents are used, are handled in accordance with the prescriptions in the safety regulations.

Toxic and flammable reagents and organic solvents shall be handled wearing protective gloves and in fume hood.