EURL for Cereals and Feeding stuff
National Food Institute
Technical University of Denmark

Validation Report 32

Determination of pesticide residues in wheat, rye, oat and rice by LC-MS/MS and GC-MS/MS

(QuEChERS method)

Susan Strange Herrmann
Mette Erecius Poulsen
December 2019

CONTENT:

1. Introduction

This report describes the validation of the QuEChERS method combined with GC-MS/MS and LC-MS/MS. The method was tried validated for 31 pesticides and metabolites by both LC-MSMS and GC-MSMS in wheat, rye, oat and rice. The QuEChERS method is an extraction method which has been developed to be Quick, Easy, Cheap, Efficient, Rugged and Safe. The method is most commonly used on fruit, vegetables and cereals[1]. The pesticides included in the validation study and the reason for including them is presented in Appendix 3.

2. Principle of analysis

Sample preparation: The cereal samples are milled with a sieve at 1 mm.

The extraction procedure is outlines in Appendix 4 and described briefly in the following.

Extraction: Water and acetonitrile is added and the sample is shaken and a salt and buffer mixture is added and the sample is shaken again.

Clean-up: After centrifugation the supernatant is transferred to a clean tube and put in -80 degree freezer for minimum 15 minutes. The extracts are then allowed to thaw until almost liquid state and then centrifuged. At this point an aliquot is withdrawn and filtered, diluted 1:1 with acetonitrile and analysed by LC-MS/MS. The rest of the supernatant is transferred to a tube containing PSA and $MgSO_4$. After shaking and an additional centrifugation step the final extract is diluted 1:1 with acetonitrile to obtain the same matrix concentration as in the matrix matched calibration standards.

Quantification and qualification: The final extracts are analysed by GC-MS/MS. Crude extract withdrawn before PSA clean-up was analysed by LC-MS/MS.

GC-MS/MS: The pesticide residues were separated on a DB5-MS column and analysed by triple quadrupole operating in the multiple reaction monitoring mode (MRM) with electron energy at 70 eV, source temperature at 180°C and transfer line at 250°C. The injection volume was 1 µl. For each pesticide minimum two sets of precursor and product ions were determined. One for quantification and one for qualification. The MRM transitions for the pesticides and degradation products are given in Appendix 1a.

LC-MS/MS: The pesticide residues are separated on a reversed-phase column and detected by tandem mass spectrometry (MS/MS) by electrospray (ESI). The validation includes pesticides determined in positive and negative mode. All pesticides were detected in the MRM mode. For each pesticide or metabolite a precursor ion and 2 product ions were determined. One product ion for quantification and one for qualification. The MRM transitions for the pesticides and degradation products sought validated are given in Appendix 1b.

3. Validation design

The method was sought validated for 31 pesticides or metabolites in oat, rice, rye and wheat, see **Appendix 1**. The validation was performed on 5-6 replicates on oat, rice, rye and wheat at each of the three spiking levels; 0.002, 0.005, 0.01 and 0.05 mg/kg. A blank sample of each cereal commodity is included.

4. Calibration curves and linearity

The calibration curve is determined by the analysis of each of the analysts at least 4 calibration levels within the range of 0.3 to 33.3 ng/ml. The quantification was performed from the mean of two bracketing calibration curves. The calibration curves were fitted to a linear curve. The majority of the correlation coefficients (R) were higher or equal to 0.99 but none were lower than 0.97. Thus, good linearity was observed within the relevant concentration range.

5. Specificity

The ion ratios for sample extracts were within ±30% (relative) of average of relevant calibration standards from same sequence. The ion ratios may vary slightly depending on concentration level and in some cases the average of calibration standard are based on the lower calibration levels for the low spike samples.

6. Precision – repeatability and internal reproducibility

Repeatability was calculated for all pesticides and degradation products on all three spiking levels (0.002, 0.005, 0.01 and 0.05 mg/kg). Repeatability is given as the relative standard deviation on the result from two or more analysis at the same sample, done by the same technician, on the same instrument and within a short period of time.

Repeatability (RSD_r) in this validation was calculated from the 5-6 replicate determinations. Repeatability were calculated as given in ISO 5725-2[2].

Accuracy – Recovery

The accuracy was determined from recovery studies in which samples were spiked at three concentration levels (0.002, 0.005, 0.01 and 0.05 mg/kg) with the relevant pesticides, isomers and degradation products.

Robustness

The QuEChERS method has, in connection with the development of the method, been shown to be robust by Anastassiades et al. 2003[1].

Limit of quantification, LOQ

The quantification limits (LOQ) was determined as the lowest spike level for which the acceptance criteria (se Section 6) were meet.

7. Criteria for the acceptance of validation results

For the pesticides to be accepted as validated the following criteria for precision and trueness must to be fulfilled:

1. The relative standard deviation of the repeatability should be ≤20%[3].
2. The average relative recovery must be between 70 and 120%[3].

If the above mentioned criteria have been meet, the quantification limits, LOQs is stated.

The expanded uncertainty is calculated to demonstrate that it is less than 50%. The expanded uncertainty is given by:

$$U = \sqrt{RSD^2 + Bias^2 + (RSD^2/n)} * 2$$

Where RSD is the intra-laboratory uncertainty (RSD_R),
Bias is 100 minus the recovery,
RSD^2/n is the uncertainty of the bias,
n is the number of recoveries included in the bias and
2 is the coverage factor corresponding to 95% confidence level.

If the expanded uncertainty is higher than 50%, the analytical results must be corrected for recovery and the combined uncertainty is then given by:

$$U_c = \sqrt{RSD^2 + (RSD^2/n)}$$

Where RSD in this validation is the repeatability uncertainty (RSD_r),
RSD^2/n is the uncertainty of the bias,
n is the number of recoveries included in the bias and
2 is the coverage factor corresponding to 95% confidence level.

The bias/recovery used for correction will be the bias/recoveries determined for the individual analytes during the initial validation and/or ongoing method validation. However, if it is evaluated

that the type of sample being analysed is significantly different from the matrices employed for the method validation it is possible to correct for bias/recoveries based on recovery from spiked samples included in the analytical batch in question. However, minimum of 5 recovery samples must be included then.

The obtained results including recovery, RSD_r, RSD_R, expanded uncertainty (U, Uc and limit of quantification (LOQ) are presented in appendix 2.

8. Results and conclusion

The validation results obtained for the 31 pesticides or metabolites using LC-MSMS and GC-MSMS are presented in appendix 2. The lowest LOQ achieved were 0.002 mg/kg for 21 compounds, 0.005 mg/kg for four compounds and 0.01 for four compounds. The majority of the combined uncertainties were lower than 50%, indicating that recovery for correction is not needed. However it has been decided at our laboratory that all results shall be corrected for recovery when possible, regardless of the expanded uncertainty and the combined uncertainty will therefore apply. Two compounds, fenpicoxamid-X12326349 and spirotetramat-cis-enol, were not successfully validated in the present study. For fenpicoxamid-X12326349 identification of precursor and product ions was not successfully and for spirotetramat-cis-enol the quantification was compromised.

9. References

1 EN 15662:2008. Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method

2 ISO 5725-2:1994. Accuracy (trueness and precision) of measurement methods and results – Part2. Basic method for the determination of repeatability and reproducibility of standard measurement method. First edition. December 1994.

3 Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed, Document SANTE/11813/2017, 21–22 November 2017 rev.0,

4 Working document on pesticides to be considered for inclusion in the national control programmes to ensure compliance with maximum residue levels of pesticides residues in and on food of plant and animal origin, European Commission, Brussels, 2017. SANCO 12745 2013 rev 11.

Appendix 1a. MRM transitions for GC-MS/MS for compounds validated

Name	RT	Parent Mass	Product Mass	Collision Energy
Aldimorph	13.34, 13.68, 14.03, 14.18, 16.14	128.1	70.1	15
Aldimorph	13.34, 13.68, 14.03, 14.18, 16.14	128.1	110.1	10
Bifenox	21.97	172.9	137.9	16
Bifenox	21.97	311	279	8
Bifenox	21.97	341.1	281	12
Clofentezine	22.52	102	50.9	12
Clofentezine	22.52	102	74.9	12
Clofentezine	22.52	137.6	102	12
Cyhalothrin-gamma	22.97	181.1	152.1	20
Cyhalothrin-gamma	22.97	197	141.1	10
Dentaotnium-benzoate	22.05	176.1	103	25
Dentaotnium-benzoate	22.05	176.1	105.1	15
Dentaotnium-benzoate	22.05	176.1	147.1	15
Diafenthiuron	18.2	311.1	254.1	15
Diafenthiuron	18.2	311.1	278.2	10
Diafenthiuron	18.2	311.1	296.1	10
Diphenylamine	10.45	168.1	139	38
Diphenylamine	10.45	168.1	167.1	14
Diphenylamine	10.45	169.2	167.1	22
Endrin-ketone	21.15	209	139.1	25
Endrin-ketone	21.15	245	173	25
Endrin-ketone	21.15	280.9	245	10
Endrin-ketone	21.15	316.9	281	10
Fenpicoxamid	10.45	128.1	102.1	25
Fenpicoxamid	10.45	128.1	127.1	20
Fenpicoxamid	10.45	143.1	128.1	15
Florpyrauxifen-benzyl	18.36	289	261	10
Florpyrauxifen-benzyl	18.36	304	261	15
Florpyrauxifen-benzyl	18.36	304	289	10
Flupyrsulfuron-methyl	17.43	299.1	216.1	20
Flupyrsulfuron-methyl	17.43	299.1	239	20
Flupyrsulfuron-methyl	17.43	299.1	256.1	15
Flutianil	27.24	200.1	199.1	10
Flutianil	27.24	231.1	216.1	10
Flutianil	27.24	426	231.1	10
Mefentrifluconazole	22.86	295	185.1	25
Mefentrifluconazole	22.86	295	232.1	15
Mefentrifluconazole	22.86	340	320	10
Methoxychlor	21.6	227.1	141.1	32
Methoxychlor	21.6	227.1	169.1	22
Methoxychlor	21.6	227.1	212.1	12

Appendix 1b. MRM transitions for LC-MS/MS for compounds validated.

Compound Name	RT	ESI mode	Precursor ion	Product ion	Collision Energy
Aldimorph	5.61	Positive	284.5	98.2	22
Aldimorph	5.61	Positive	284.5	130.2	21
Bifenox	6.62	Positive	359.2	310	7
Bifenox	6.62	Positive	359.2	341.8	5
Clethodim-sulfone(1)	3.62	Positive	392.2	164.1	21
Clethodim-sulfone(1)	3.62	Positive	392.2	300.1	9
Clethodim-sulfone(2)	4.88	Positive	392.2	300.1	9
Clethodim-sulfone(2)	4.88	Positive	392.2	208.1	15
Clethodim-sulfoxide(1)	3.67	Positive	376.2	206.1	12
Clethodim-sulfoxide(1)	3.67	Positive	376.2	164.1	16
Clethodim-sulfoxide(1)	3.67	Positive	376.2	298.1	11
Cletodim-sulfoxide(2)	4.92	Positive	376.2	206.1	12
Cletodim-sulfoxide(2)	4.92	Positive	376.2	298.1	11
Clofentezine	6.52	Positive	303	138	11.5
Clofentezine	6.52	Positive	303	102	30
Diafenthiuron	7.56	Positive	385.3	278.1	25
Diafenthiuron	7.56	Positive	385.3	236.1	37
DMST	4.01	Positive	215	106.1	13
DMST	4.01	Positive	215	77	43
DMST	4.01	Positive	215	151	5
Ethiprole	5.325	Positive	397	350.8	16
Ethiprole	5.325	Positive	397	254.9	33
Fenamiphos-sulfoxide	3.89	Positive	337.2	320.1	5
Fenamiphos-sulfoxide	3.89	Positive	337.2	171.1	20
Fenpicoxamid	6.83	Positive	615	239	22
Fenpicoxamid	6.83	Positive	615	515.2	12
Fenpicoxamid	6.83	Positive	615	124.1	74
Fenpicoxamid-sulfone	4.80	Positive	325.3	268.9	11
Fenpicoxamid-sulfone	4.80	Positive	325.3	296.9	7
Fenthion-oxon	4.97	Positive	263	216	20
Fenthion-oxon	4.97	Positive	263	231	30
Fenthion-sulfoxide	4.02	Positive	295	280	17
Fenthion-sulfoxide	4.02	Positive	295	109.2	26
Fenthion-sulfoxide	4.02	Positive	295	125	30
Florpyrauxifen-benzyl	6.60	Positive	439	91.2	16
Florpyrauxifen-benzyl	6.60	Positive	439	65.3	36
Flutianil	6.22	Positive	427	192	23
Flutianil	6.22	Positive	427	411	19
Flutianil	6.22	Positive	427	132	45
Formetanate	1.72	Positive	222	46.2	28
Formetanate	1.72	Positive	222	165.1	10
Hymexazol	1.88	Positive	100	54	12
Hymexazol	1.88	Positive	100	43	27
Lufenuron	7.18	Negative	511	176.7	30
Lufenuron	7.18	Negative	511	328.4	20

Mefentrifluconazole	6.42	Positive	398.1	70.3	17
Mefentrifluconazole	6.42	Positive	398.1	182	25
Methomyl	2.16	Positive	163	106	9.5
Methomyl	2.16	Positive	163	88	8
Monocrotophos	2.24	Positive	224	127	12.5
Monocrotophos	2.24	Positive	224	193	7.5
Monocrotophos	2.24	Positive	224	98	10
Oxathiapiprolin	5.44	Positive	540.3	500.1	21
Oxathiapiprolin	5.44	Positive	540.3	163	39
Oxathiapiprolin	5.44	Positive	540.3	167	24

Appendix 2. Recoveries, repeatability (RSD_r), internal reproducibility (RSDR), expanded uncertainty (U) and Limit of Quantification (LOQ) for pesticides validated on four cereal commodities, oat, rice, rye and wheat using QuEChERS.

Red numbers indicate that the recovery is not 70-120% recovery or that RSD is above 20% RSD.

		Spike level 0.002 mg/kg					Spike level 0.005 mg/kg					Spike level 0.01 mg/kg					Spike level 0.05 mg/kg					
	Compound	Recovery %	RSD_r %	RSD_R %	U %	Cu%	Recovery %	RSD_r %	RSD_R, %	U %	Cu%	Recovery %	RSD_r, %	RSD_R, %	U %	Cu%	Recovery %	RSDr %	RSD_R %	U %	Cu%	LOQ
LC	Aldicarb	104	8	12	25	12	98	20	22	45	22	105	19	20	42	20	104	13	20	41	20	0.002
GC	Aldimorph	96	3	16	33	16	86	3	9	34	9	87	3	10	33	10	91	3	6	23	7	0.002
LC	Aldimorph	102	13	16	33	17	101	8	21	44	22	93	15	24	50	24	101	14	16	33	17	0.002
GC	Bifenox	82	12	11	44	12	75	13	19	63	20	76	7	20	63	21	87	5	19	47	20	0.002
LC	Clethodim-sulfone	107	12	20	42	20	109	11	14	33	14	106	18	17	36	17	110	16	15	37	16	0.002
LC	Cletodim-sulfoxide	105	14	19	40	19	107	14	17	38	18	109	19	19	43	19	114	14	19	48	19	0.002
GC	Clofentezine						73	14	19	66	19	73	8	19	66	20	81	6	20	57	21	0.005
LC	Clofentezine	115	10	18	48	18	108	8	15	34	15	104	12	18	37	18	108	8	19	41	19	0.002
GC	Cyhalothrin-gamma						107	7	15	34	15	104	10	20	42	21	102	5	17	36	18	0.005
GC	Denatonium-benzoate	127	15	20	68	21	120	13	18	54	18	101	8	16	33	16	97	3	14	30	15	0.005
GC	Diafenthiuron											49	6	15	106	15	62	3	16	83	16	0.002
LC	Diafenthiuron	52	12	53	145	55	57	14	58	149	61	70	18	31	88	32	96	9	9	20	9	0.05
GC	Diphenylamine	69	5	11	67	12	75	4	8	54	9	78	6	19	59	19	92	3	6	20	7	0.01
LC	DMST	115	7	19	48	19	121	9	19	58	20	121	17	22	62	22	106	10	19	40	19	0.002
GC	Endrin-ketone	103	7	7	17	8	96	4	20	42	21	96	3	13	28	14	97	3	7	15	7	0.002
LC	Ethiprole	*120*	*5*	*29*	*72*	*30*	97	9	18	37	18	94	12	14	31	14	107	15	17	36	17	0.005
LC	Fenamiphos-sulfoxide	119	13	19	55	19	105	14	20	42	21	106	12	20	43	20	99	16	23	48	24	0.002
GC	Fenpicoxamid																91	6	17	39	17	0.05
LC	Fenpicoxamid	102	11	17	36	18	102	10	13	27	13	99	15	18	36	18	106	12	13	30	14	0.002
LC	Fensulfothion-sulfone	102	10	18	36	18	102	14	20	41	20	103	17	20	42	21	105	15	21	44	21	0.002
LC	Fenthion-oxon	98	9	13	26	13	99	7	14	29	15	100	14	15	32	16	101	11	14	28	14	0.002
LC	Fenthion-sulfoxide	*133*	*7*	*23*	*82*	*24*	*125*	*19*	*26*	*74*	*27*	132	10	19	75	19	106	11	17	37	18	0.01

		Spike level 0.002 mg/kg					Spike level 0.005 mg/kg					Spike level 0.01 mg/kg					Spike level 0.05 mg/kg					
	Compound	**Recovery %**	**RSD_r %**	**RSD_R %**	**U %**	**Cu%**	**Recovery %**	**RSD_r %**	**RSD_R, %**	**U %**	**Cu%**	**Recovery %**	**RSD_r, %**	**RSD_R, %**	**U %**	**Cu%**	**Recovery %**	**RSDr %**	**RSD_R %**	**U %**	**Cu%**	**LOQ**
GC	Florpyrauxifen-benzyl						88	13	14	37	14	104	8	9	20	9	116	7	13	42	14	0.005
LC	Florpyrauxifen-benzyl	106	8	12	28	12	109	8	14	35	15	105	16	19	39	19	109	7	14	33	14	0.002
GC	Flupyrsulfuron-methyl	118	16	20	55	21	96	10	19	40	20	89	5	20	47	21	89	5	18	44	19	0.002
GC	Flutianil	97	7	8	18	9	95	4	10	23	10	104	3	12	26	12	103	2	5	13	6	0.002
LC	Flutianil	110	10	12	32	12	108	9	15	35	15	108	15	16	37	16	111	7	10	31	10	0.002
LC	Formetanate	72	16	20	70	21	72	12	17	66	17	85	7	15	43	16	88	10	16	40	16	0.002
LC	Hymexazol											90	15	15	37	15	94	10	14	30	14	0.01
LC	Lufenuron											118	12	40	90	41	72	29	44	106	45	0.01
GC	Mefentrifluconazole	104	6	7	18	8	107	4	14	31	14	107	4	9	23	9	104	3	9	19	9	0.002
LC	Mefentrifluconazole	*98*	*11*	*23*	*47*	*23*	100	7	19	39	20	99	13	20	41	20	106	9	15	33	15	0.005
LC	Methomyl	*122*	*9*	*12*	*51*	*12*	119	16	19	55	20	105	15	17	36	17	91	17	23	50	23	0.005
GC	Methoxychlor	72	9	15	64	15	77	6	6	47	6	83	4	8	38	8	93	4	7	20	7	0.002
LC	Monocrotophos	112	10	11	33	11	113	14	19	46	19	117	12	16	46	16	100	13	18	37	18	0.002
LC	Oxathiapiprolin	103	14	18	38	19	104	16	20	41	20	106	17	20	42	20	113	17	18	46	19	0.002

Appendix 3. List of compounds included in the validation study including reason for inclusion.

Compound	**Reason for including in validation study 2019**
Aldicarb	On EU MACP. Lower LOQs to be tested.
Aldimorph	was detected in several screening analysis of FVST 2017 samples in sweet pepper and grape sample and was formerly used as a fungicide for the control of powdery mildew in cereal crops in EU but is no longer authorized.
Bifenox	Authorized in EU and used for cereals
Clethodim-sulfone	proposed included in the residue for clethodim by Reasoned opinion of 2018
Clethodim-sulfoxide	proposed included in the residue for clethodim by Reasoned opinion of 2018
Clofentezine	On EU MACP. Lower LOQs to be tested.
Cyhalothrin-gamma	Relevant for baby food and mentioned in the working document (sanco 12745 2013 rev 11).
Denatonium-benzoate	Support on appropriate LOQ/LOD. REGULATION (EU) 2019/973. Not authorized for food commodities therefore MRL should be set in Annex II to Regulation (EC) No 396/2005 at the specific LOD.
Diafenthiuron	In working document (sanco 12745 2013 rev 11)
Diphenylamine	On EU MACP. Lower LOQs to be tested.
DMST	Part of residue definition for tolylfluanid which is not authorized in EU, but is relevant for use on cereals outside EU and possible illegal use. Lower LOQs to be tested.
Endrin-ketone	Endrin-keton (delta-ketoendrin) relevant for products of animals origin (sanco 12745 2013 rev 11) and feed as part of the residue definition for Endrin
Ethiprole	Never notified and authorized in the EU though an application for setting MRL for import tolerance in rice was active in 2019
Fenamiphos-sulfoxide	Fenamiphos is in the EU MACP and fenamiphos-sulfoxide is part of the residue definition. Lower LOQs to be tested.

Fenpicoxamid	In working document (sanco 12745 2013 rev 11)
Fensulfothion-sulfone	Relevant for baby food
Fenthion-oxon	Fenthion is on EU MACP and fenthion-oxon is part of the residue definition. Lower LOQs to be tested.
Fenthion-sulfoxide	Fenthion is on EU MACP and fenthion-sulfoxide is part of the residue definition. Lower LOQs to be tested.
Florpyrauxifen-benzyl	New active substance
Flupyrsulfuron-methyl	Not authorized in EU. It is a post-emergent cereal herbicide designed for the control of problem grass weeds. The EU authorizations was withdrawn because it was suspected to be carcinogenic. Relevant to include in cases of unauthorized uses.
Flutianil	New active ingredient under EFSA evaluation.
Formetanate	On the EU MACP. An LOQ of 0.05 mg/kg was achieved at previous validation and it will be tested whether a lower LOQ can be achieved.
Hymexazol	Information on LOQ requested in connection with art. 12. An LOQ of 0.05 mg/kg was achieved previously and it will be tested whether a lower LOQ will be tested.
Lufenuron	On EU MACP
Mefentrifluconazole	Mefentrifluconazole is a relatively new conazole fungicide used to control disease on cereals. MRL application on 15/10-18.
Methomyl	On EU MACP
Methoxychlor	On EU MACP for products of animal origin. Is not authorized in EU but may illegally be used For use as insecticide on beef cattle, dairy cattle, goats, sheep, and swine and for spray treatment of barns, grain bins, mushroom houses, and other agricultural premises
Monocrotophos	On EU MACP. Lower LOQs to be tested.
Oxathiapiprolin	In working document (sanco 12745 2013 rev 11)

Appendix 4: Principles of the QuEChERS method for cereal extraction

QuEChERS for cereals (FP417)

Weigh 5 g (±0.05 g) of flour into a 50 ml single use centrifuge tube (red cap). Add internal standard and/or spike standard (maximum 25 µl)

Add a ceramic homogenizer and 10 g of cold water and shake briefly

Add 10 ml acetonitrile and shake vigorously by hand for 1 min. (1. extraction)

Add the prepared mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g Na_3 citrate dihydrate and 0.5 g Na_2H cirate sesquihydrate. Shake for a few seconds after each addition to prevent lumps.

Shake vigorously for 1 min. (2. Extraction with phase separation)

Centrifuge for 10 min at 4500 rpm

Transfer at least 8 ml of the extract to a 15 ml single use centrifuge tube and store in the freezer (-80˚C for 1 hour or over night). When the extract are almost thawed (i.e. About -40 ˚C) centrifugate (should be cold 5 C) for 5 min. at 4500 rpm.

Transfer 6 ml of the cold extract to a 15 ml single use centrifuge tube containing 150 mg PSA and 900 mg $MgSO_4$. Close the tube and shake vigorously for 30 seconds.

Centrifuge for 5 min. at 4500 rpm

Transfer 4 ml of the extract to a 15 ml single use centrifuge tube. Add 40 µl of 5% formic acid solution in acetonitrile (10 µl/ml extract). Dilute the extract 1:1 with acetonitrile

Transfer the final extract into auto sampler vials and analyse by GC and LC.