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National Food Institute
Technical University of Denmark

Validation Report 8

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

(QuEChERS method)

Appendix 2

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CONTENT:

1. Introduction

This report describes the validation of the QuEChERS method combined with GC-MS/MS and LC-MS/MS. The method was sought validated for 32 pesticides, isomers and degradation products in rice and wheat. The method has previously been validated for 45 pesticides by LC-MS/MS detection, 62 pesticides by GC-MS/MS detection and 62 pesticides by GC-MS.

The QuEChERS method has an extraction and clean-up step, which has been developed to be Quick, Easy, Cheap, Efficient, Rugged and Safe. The method is most commonly used on fruit and vegetables[1].

2. Principle of analysis

Sample preparation:

Cold water/ice water and acetonitrile is added to the milled sample.

Extraction:

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 tube with 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 calibration standards.

For the LC-MS/MS analysis the extraction is followed by adding internal standard and the extract is filtered into HPLC vials.

In Appendix 4 is the procedure presented in a flow diagram.

Quantification and qualification:

GC-MS/MS: The final extract is analysed GC/MS/MS (electron energy 70 eV, source temp. 180°C, transfer line GC interface 250°C). The injection volume is 4 µl.

LC-MS/MS: The pesticide residues are separated on a reversed-phase column and detected by tandem mass spectrometry (MS/MS) by electrospray (ESI).

Selectivity and specificity:

GC-MS/MS: GC-MS/MS is a highly selective method, and thereby highly specific. Two MRM transitions were used (two parent and two daughter ion) one for quantification and another transition for qualification. Parent and daughter ions are presented in appendix 1.

LC-MS/MS: The validation includes pesticides determined with both positive and negative ESI. $^{13}C_6$-carbaryl was used as internal standard for quantification. All pesticides were detected in the multiple reaction monitoring mode (MRM). For each pesticide precursor ion and 2 product ions (where possible) 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 1.

3. Validation design

The method was south validated for 32 pesticides, isomers or degradation products (see appendix 1) in rice and wheat. The validation was performed on 5-6 replicates at each of the three spiking levels; 0.01, 0.02 and 0.1 mg/kg. A blank sample was included.

4. Chromatograms and calibration curves

The calibration curve is determined by the analysis of each of the analysts at, at least 4 calibration levels, i.e. 0.003, 0.01, 0.033 and 0.1 µg/ml. The calibration curves were best fitted to a linear curve. The quantification was performed from the mean of two calibration curves surrounding the samples. The majority of the correlation coefficients (R) were higher or equal to 0.98. Examples of chromatograms obtained when analysing the extracts by GC-MS/MS are presented in figure 1. Examples of calibration curves are presented in figure 2.

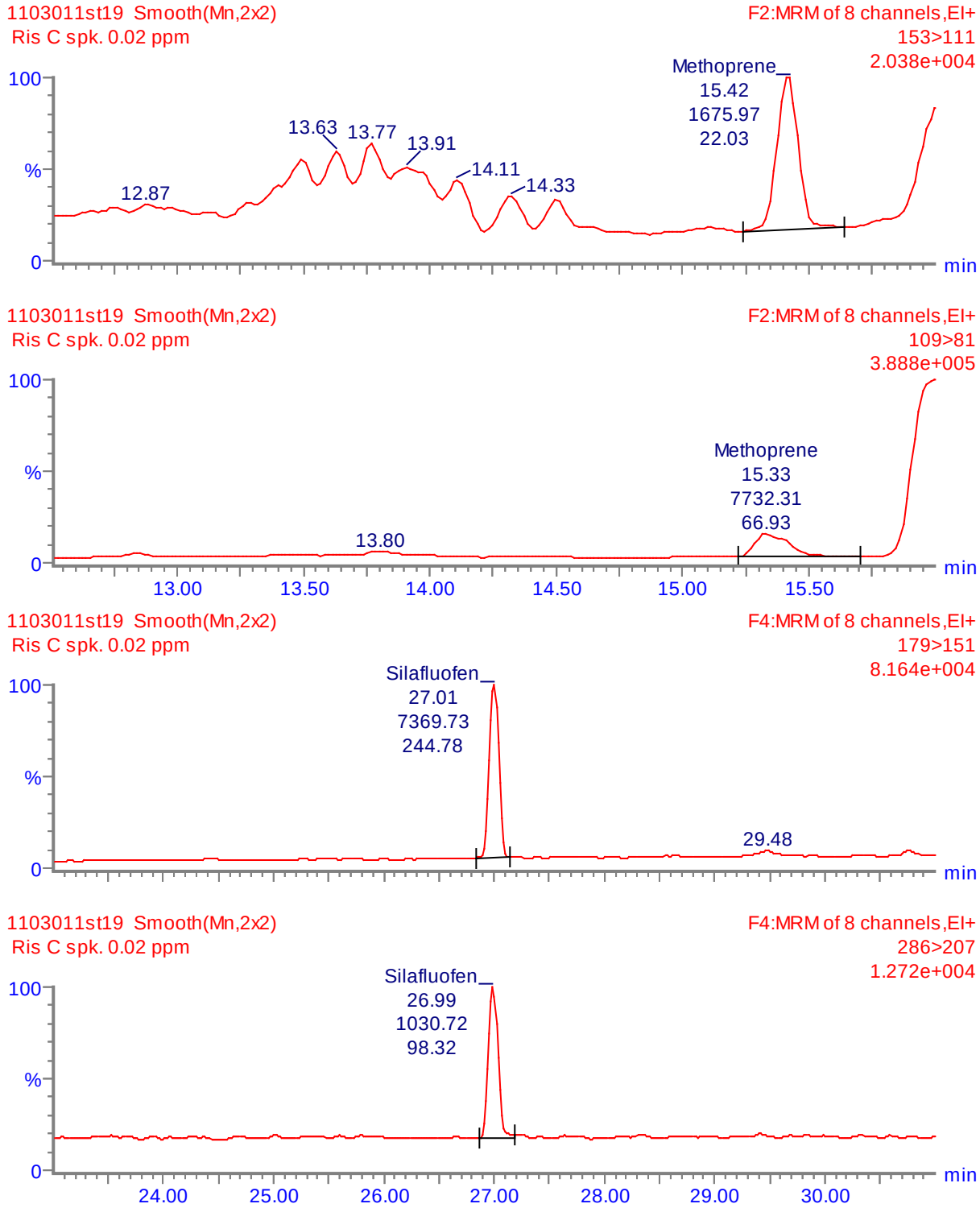

Figure 1: Examples of chromatograms for methoprene and silafluofen obtained when analysing extract of wheat spiked with 0.02 mg/kg (two MRM transitions are shown for each pesticide).

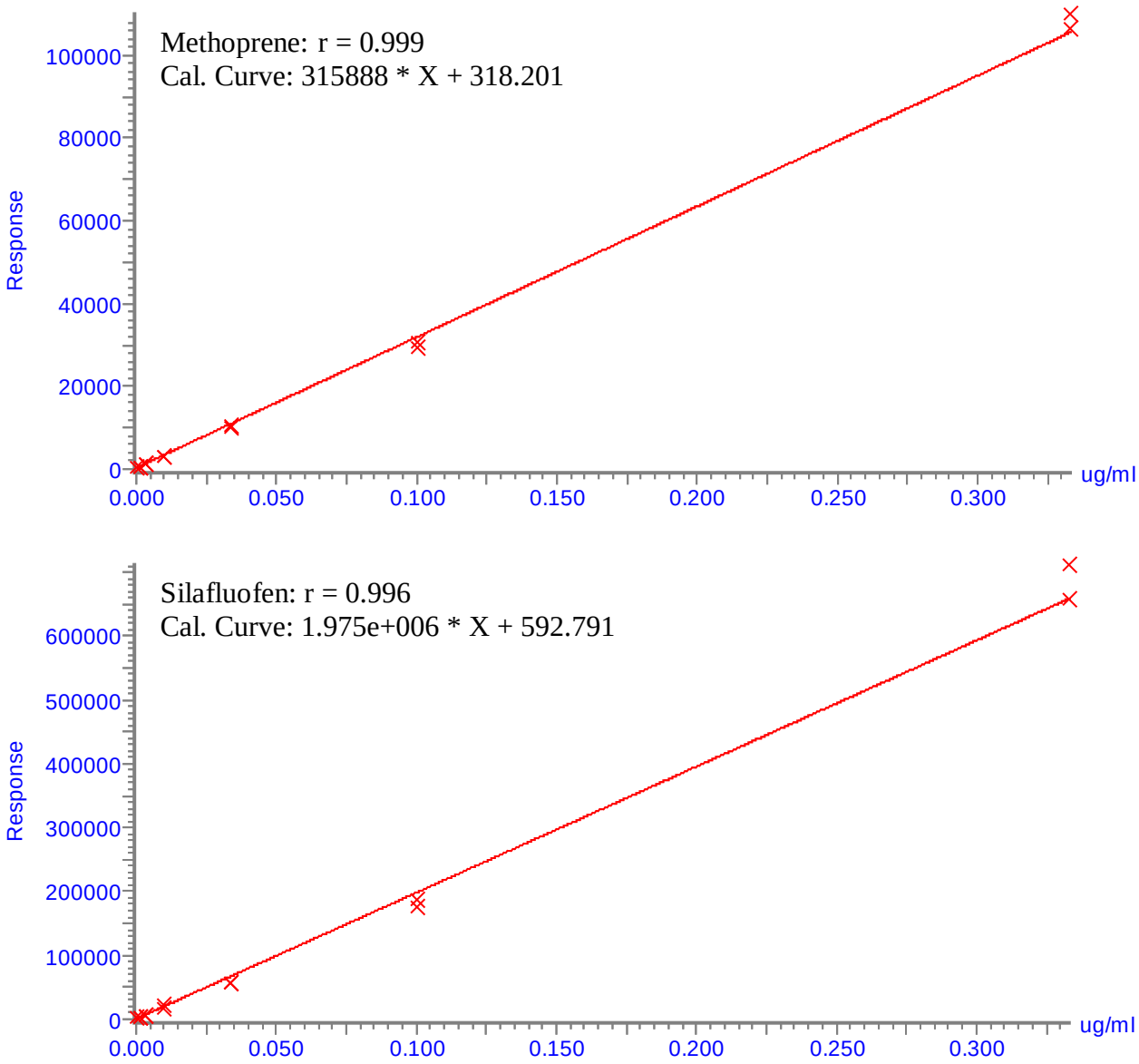

Figure 2. Examples of calibration curves for methoprene and silafluofen (concentrations from 0.003-0.1 µg/ml and 0.003-0.333 µg/ml, respectively)

5. Validation parameters

Precision – Repeatability

Repeatability was calculated for all pesticides and degradation products on all three spiking levels. 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 in this validation was calculated from the 5-6 replicate determinations. Repeatability were calculated as given in ISO 5725-2[2].

Appendix 2 and 3 shows the relative repeatability for the validated pesticides, isomers and degradation products.

Accuracy – Recovery

The accuracy was determined by recovery, samples were spiked at three concentration levels. In appendix 2 and 3 recovery, repeatability and limit of quantification (LOQ) are given for the validated pesticides, isomers and degradation products for all three spiking levels (0.01 mg/kg, 0.02 mg/kg and 0.1 mg/kg). Recoveries may be seen in appendix 2 and 3.

Robustness

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

Limit of quantification, LOQ

Quantification limits (LOQ) are calculated from the results at the lowest accepted spike level, as 6 times the standard deviation (absolute recovery). The quantification limits are given in appendix 2 and 3.

6. 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 must be less than or equal to the standard deviation proposed by Horwitz[3].
2. The average relative recovery must be between 70 and 120%[4].

If the above mentioned criteria have been meet, the detection limits have been calculated.

7. Results and discussion

LC-MS-MS compounds

The multi-residue method has been tested for 30 pesticides, isomers and degradation products in rice and wheat using LC-MS/MS. The relative repeatability (RSD_r) varied between 2-20 %, however most of the values were below 15%. For the majority of the pesticides the recovery was in the range of 75-100% at all three concentration levels.

For rice the criteria for acceptance were met for 24 out of 30 pesticides, isomers and degradation products (listed in Table 2-3). Trinexapac-ethyl could only be accepted for rice at the highest spike level and not at all for wheat. The recovery from rice was 70% and RSD_r of 19% at the highest spiking level. Thus trinexapac-ethyl would probably be more amenable to another type of method or may require a single-residue method.

For wheat the criteria for acceptance were met for 25 out of 30 pesticides, isomers and degradation products (listed in Table 1).

The lowest calibration level (LCL) was 0.0033 µg/ml and most of the LOQs are above 0.006 mg/kg. Flucythrinate could only be accepted for wheat at the highest spiking level and not at all for rice. The recovery at the highest spiking level for what was 102% and RSD_r 9%. However for rice and the lower spiking levels for wheat the recoveries where to low and RSD_r was too high.

The pesticides, isomers and degradation products which have been validated are presented in table 1 and 2. The tables divided the compounds into two groups, one for the compounds for which the acceptance criteria could be meet (accepted) and those which could not meet the acceptance criteria (not accepted).

The results for the pesticides which were accepted for LC-MS/MS are listed in Appendix 2.

Naled was not accepted because of to low recoveries. The RSD_r was acceptable. The low recoveries is probably due to the fact that naled can be reduced to dichlorvos. Thus the residues of naled should be determined as the sum of naled and dichlorvos. Because the possible degradation of naled to dichlorvos it is not possible to distinguish whether or the residue originates from naled, dichlorvos or a combination of the two.

Table 1: Compounds validated and not accepted as validated for **rice** with analysis by LC-MS/MS.

Rice		
Accepted (24 compounds)		
Amidosulfuron	Flonicamid	Pirimiphos-Methyl
Bendiocarb	Florasulam	Pyraclofos
Chlorantraniliprole	Flutolanil	Thiobencarb
Chromafenozide	Isoprocarb	Tralkoxydim
Cinosulfuron	Isoxathion	Trichlorfon
Dicrotophos	Methoprene	Tricyclazole
Diflubenzuron	Nitenpyram	Triflumizole
Dinotefuran	Oxadiazon	Trinexapac-ethyl
Not accepted (6 compounds)		
Bensultap	Naled	Triforine
Flucythrinate	Thiocyclam-hydrogenoxalate	
Imazapyr		

Table 2: Compounds validated and not accepted as validated for **wheat** with analysis by LC-MS/MS.

Wheat		
Accepted (25 compounds)		
Amidosulfuron	Florasulam	Pyraclofos
Bendiocarb	Flucythrinate	Thiobencarb
Chlorantraniliprole	Flutolanil	Tralkoxydim
Chromafenozide	Isoprocarb	Trichlorfon
Cinosulfuron	Isoxathion	Tricyclazole
Dicrotophos	Methoprene	Triflumizole
Diflubenzuron	Nitenpyram	Triforine
Dinotefuran	Oxadiazon	
Flonicamid	Pirimiphos-Methyl	
Not accepted (5 compounds)		
Bensultap	Naled	Trinexapac-ethyl
Imazapyr	Thiocyclam-hydrogenoxalate	

Thiocyclam-hydrogenoxalate and **imazapyr** gave low recoveries and for imazapyr also to high RSD_r. The analysis of these too acidic pesticides may be more sussecfull if the raw extract (without freezing out and PSA clean-up) where analysed. **Trinexapac-ethyl** could only be validated at the higest spiking level for rice, and from to the literature it was expected to be more GC amenable. For **bensultap** the recoveries where too low. At the moment we do not have a suggestion for a solution for this problem. In the literature only results obtained by GC with FPD are available.

GC-MS-MS compounds

The multi-residue method has been tested for 15 pesticides, isomers and degradation products in rice and wheat using GC-MS/MS. The relative repeatability (RSD_r) varied between 3 to 20 %, however most of the values were below 15 %. For the majority of the pesticides the recovery was in the range of 75-100% at all three concentration levels. Though the recovery of silafluofen where only 63-66% from rice and 69-75% from wheat. However RSD_r was low (3-14%) if looking at all spike levels and where 8-12 at the lowest spike level. The validation results where therefore found acceptable though notice should be taken of the low recovery. Tralomethrin is a pyrethroid wich are typically GC amenable. Acceptable validation data were also obtained, however by GC-MS and GC-MS/MS it is not possible to distinguish between deltamethrin and tralomethrin. Tralomehtrin can debrominate to deltamethrin in the GC systems and perhaps also in the homogenate and the method is therefore not specific. Tralomethrin is not on the positive list in the EU list and this situation can therefore only occur for samples originating from third countries or because of illegal uses.

For both rice and wheat the criteria for acceptance were met for out of 14 pesticides, isomers and degradation products (listed in Table 4 and Table 5, respectively). The lowest calibration level (LCL) was 0.0033 µg/ml and most of the LOQs are above or equal to 0.006 mg/kg.

The pesticides, isomers and degradation product which has been validated presented in table 4-6. The results for the pesticides which were accepted are listed in Appendix 3.

Table 4: Compounds validated and not accepted as validated for **rice** with analysis by GC-MS/MS.

Rice		
Accepted (14 compounds)		
Bendiocarb	Methoprene	Thiobenbarb
Flucythrinate	Oxadiazon	Tralomethrin
Flutolanil	Pirimiphos-methyl	Trichlorfon
Isoprocarb	Pyraclofos	Tricyclazole
Isoxathion	Silafluofen	
Not accepted (1 compounds)		
Trinexapac-ethyl		

Table 5: Compounds validated and not accepted as validated for **wheat** with analysis by GC-MS/MS.

Wheat		
Accepted (14 compounds)		
Bendiocarb	Methoprene	Thiobenbarb
Flucythrinate	Oxadiazon	Tralomethrin
Flutolanil	Pirimiphos-methyl	Trichlorfon
Isoprocarb	Pyraclofos	Tricyclazole
Isoxathion	Silafluofen	
Not accepted (1 compounds)		
Trinexapac-ethyl		

Acceptable validation results for **trinexapac-ethyl** could only be obtained for rice at the highest spiking level and only with LC-MS/MS determination. Trinexapac-ethyl has by others been reported to be GC amenable but in this study the recoveries were to low whereas the results showed good reproducibility. Before more work is put in to this compound, it should however be considered, whether or not it is relevant to monitor for. It has been shown that trinexapac-ethyl is rapidly degraded after it has been applied to the field.

For about half the pesticides included in the validation there has not been set a residue definition if referring to the MRL database[5]. For the rest of the pesticides validated here only the residue definition for flonicamid and triflumizole are different form only parent compound. The residue definition for flonicamid is according to the MRL database sum of flonicamid, TNFG and TNFA. The residue definition for triflumizole is Triflumizole and metabolite FM-6-1(N-(4-chloro-2-

trifluoromethylphenyl)-n-propoxyacetamidine), expressed as Triflumizole. These metabolites are not included in the validation performed here, thus further method validation would be required to meet the residue definition.

8. Conclusions

In conclusion 28 of 32 pesticides, isomers and degradation products were validated on rice and/or wheat for the QuEChERS method using GC-MS/MS and/or LC-MS/MS for the analysis. In total 12 compounds were validated on both GC-MS/MS and LC-MS/MS. Fourteen compounds were only validated on LC-MS/MS and 2 compounds were only validated on GC-MS/MS.

9. References

1 http://www.quechers.com/ or Anastassiades et al., J. AOAC Int., vol. 86, no. 2, p. 412, 2003

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

3 W. Horwitz, Anal. Chem., 1982; 54, 67A.

4 Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed, Document No SANCO/10684/2010, 01/01/2010, European Commission, Brussels, 2010.

5 EU Pesticides database available at http://ec.europa.eu/sanco_pesticides/public/index.cfm

Appendix 1. MRM transitions for the pesticides sought validated.

GC-MS/MS		Precursor ion-1	Product ion-1	Col. energi 1	Precursor ion-2	Product ion-2	Col. energi 2
1	Bendiocarb	166	151	10	223	166	6
2	Flucythrinate-1	199	157	5	451	199	10
3	Flucythrinate-2	199	157	5	451	199	10
4	Flutolanil	173	145	12	281	173	10
5	Isoprocarb	136	121	6	121	103	10
6	Isoxathion	177	130	6	313	177	6
7	Methoprene	153	111	5	109	81	5
8	Oxadiazon	258	175	5	302	175	10
9	Pirimiphos-Methyl	305	290	10	290	233	10
10	Pyraclofos	360	194	8	360	139	14
11	Silafluofen	179	151	8	286	207	8
12	Thiobencarb	100	72	4	125	89	12
13	Tralomethrin-1	253	93	14	253	172	18
14	Tralomethrin-2	253	93	14	253	172	18
15	Trichlorfon	185	93	15	145	109	10
16	Tricyclazole	189	162	8	189	161	16
17	Trinexapac-ethyl	224	151	5	151	95	5

LC-MS/MS ESI-		Precursor ion-1	Product ion-1	CV	CE	Precursor ion-2	Product ion-2	CE	CV
1	Amidosulfuron	368	259	40	15		109	40	21
2	Chlorantraniliprole	482	204	40	15	147	88	40	33
3	Diflubenzuron	309	156	46	15		93	46	40
4	Flonicamid	228	81	50	15		146	50	27
5	Florasulam	358	167	50	15		152	50	33
6	Tralkoxydim	328	254	16	21		212	16	35

LCMSMS ESI+		Precursor ion-1	Product ion-1	CV	CE	Precursor ion-2	Product ion-2	CV	CE
1	Bendiocarb	224	109	22	15		81	22	33
2	Bensultap	432	290	28	15		150	28	27
3	Chromafenozide	395	175	46	40				
4	Cinosulfuron	414	183	22	15		61	22	30
5	Dicrotophos	270	112	28	15	255	193	28	21
6	Dinotefuran	203	129	28	15		73	28	21
7	Flucythrinate	469	157	34	40		181	34	27
8	Flutolanil	324	262	33	35		242	33	25
9	Imazapyr	263	218	50	21		132	50	40
10	Isoprocarb	211	95	28	21		152	28	21
11	Isoxathion	314	105	16	15		97	16	40
12	Methoprene	279	219	10	10		237	10	10
13	Naled	398	127	50	15		109	50	39
14	Nitenpyram	271	189	28	15		126	28	33
15	Oxadiazon	362	220	22	27		239	22	21
16	Pirimiphos-Methyl	306	164	20	20		108	20	39
17	Pyraclofos	361	257	50	21		138	50	40
18	Thiobencarb	258	125	34	15		89	34	39
19	Thiocyclam-hydrogenoxalate	182	137	10	20		73	20	20
20	Trichlorfon	257	109	34	15		221	34	15
21	Tricyclazole	190	163	50	21		136	50	27
22	Triflumizole	346	278	16	15		73	15	21
23	Triforine	435	390	17	5		215	17	25
24	Trinexapac-ethyl	253	69	46	21		207	46	21

Appendix 2. Repeatability, recovery and limit of quantification for compounds validated on LC-MS-MS.

In the tables are presented repeatability and LOQ for compounds validated on LC-MS-MS. Values outside the acceptance criteria are marked by grey. The matrixes are rice and wheat.

Rice, QuEChERS with LCMSMS	Spike level mg/kg 0.01			Spike level mg/kg 0.02			Spike level mg/kg 0.10			
	Recovery, %	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**LOQ, mg/kg**
Amidosulfuron	73	4		93	17		103	19		0.010
Bendiocarb	89	18		94	19		97	8		0.011
Chlorantraniliprole	103	11		98	10		96	3		0.008
Chromafenozide	89	15		92	12		95	10		0.009
Cinosulfuron	67	19		65	10		67	10		0.008
Dicrotophos	*87*	*23*		96	19		100	20		0.022
Diflubenzuron	107	16		97	15		93	7		0.011
Dinotefuran				*113*	*121*		86	13		0.069
Flonicamid	81	15		92	13		96	7		0.014
Florasulam	89	10		81	15		77	7		0.006
Flutolanil	93	16		88	14		91	9		0.010
Isoprocarb	87	13		90	15		99	10		0.007
Isoxathion	93	12		92	14		101	7		0.007
Methoprene	*78*	*28*		71	20		76	13		0.017
Nitenpyram	*102*	*74*		*94*	*39*		90	9		0.049
Oxadiazon	92	18		82	15		86	6		0.011
Pirimiphos-Methyl	*86*	*27*		97	12		104	13		0.014
Pyraclofos	90	19		103	14		108	11		0.011
Thiobencarb	87	13		101	19		101	8		0.008
Tralkoxydim	79	13		75	10		77	7		0.007
Trichlorfon	*128*	*14*		*91*	*25*		92	12		0.067
Tricyclazole	*102*	*23*		*89*	*22*		91	8		0.044
Triflumizole	95	17		103	18		104	9		0.011
Trinexapac-ethyl	*86*	*34*		*88*	*53*		70	19		0.081

Wheat, QuEChERS with LCMSMS	Spike level mg/kg 0.01			Spike level mg/kg 0.02			Spike level mg/kg 0.10			
	Recovery, %	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**LOQ, mg/kg**
Amidosulfuron	98	16		86	16		70	5		0.010
Bendiocarb	86	11		93	13		97	8		0.006
Chlorantraniliprole	106	15		100	10		77	4		0.010
Chromafenozide	81	10		97	11		98	6		0.005
Cinosulfuron	*63*	*10*		80	16		75	5		0.016
Dicrotophos	90	5		98	12		103	4		0.003
Diflubenzuron	*98*	*21*		102	17		75	7		0.021
Dinotefuran	77	12		79	17		87	14		0.006
Flonicamid	105	19		92	11		68	3		0.013
Florasulam	89	10		93	12		68	2		0.006
Flucythrinate	*43*	*98*		*72*	*49*		102	9		0.060
Flutolanil	96	10		99	12		94	4		0.007
Isoprocarb	90	10		100	13		93	5		0.006
Isoxathion	84	9		99	13		100	5		0.005
Methoprene	*79*	*15*		*89*	*21*		88	9		0.050
Nitenpyram	83	18		89	14		89	4		0.010
Oxadiazon	89	8		91	15		88	5		0.005
Pirimiphos-Methyl	91	10		101	12		101	3		0.006
Pyraclofos	92	11		103	12		99	3		0.007
Thiobencarb	91	8		101	10		103	6		0.005
Tralkoxydim	75	13		72	15		*62*	*5*		0.006
Trichlorfon	*99*	*25*		96	17		101	7		0.019
Tricyclazole	89	9		83	11		80	6		0.005
Triflumizole	91	6		101	12		102	6		0.003
Triforine	*91*	*61*		*90*	*71*		89	19		0.107

Appendix 3. Repeatability, recovery and limit of quantification for compounds validated on GC-MS-MS.

In the tables are presented repeatability and LOQ for compounds validated on GC-MS-MS. Values outside the acceptance criteria are marked by grey. The matrixes are rice and wheat.

Rice, QuEChERS with GCMSMS	Spike level mg/kg 0.01		Spike level mg/kg 0.02		Spike level mg/kg 0.10		
	Recovery, %	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**LOQ, mg/kg**
Bendiocarb	90	13	84	9	78	7	0.008
Flucythrinate	73	13	78	11	82	5	0.006
Flutolanil	89	16	88	11	84	7	0.009
Isoprocarb	87	11	83	10	78	5	0.006
Isoxathion	88	19	80	7	73	6	0.011
Methoprene	90	20	83	9	82	7	0.012
Oxadiazon	84	15	87	12	88	4	0.008
Pirimiphos-methyl	91	18	92	10	88	4	0.011
Pyraclofos	84	15	84	10	79	7	0.008
Silafluofen	63	12	63	13	66	7	0.005
Thiobenbarb	83	15	88	12	90	4	0.008
Tralomethrin	87	12	76	12	85	9	0.007
Trichlorfon	*56*	*28*	88	20	102	4	0.022
Tricyclazole	73	15	73	5	78	9	0.007

Wheat, QuEChERS with GCMSMS	Spike level mg/kg 0.01		Spike level mg/kg 0.02		Spike level mg/kg 0.10		
	Recovery, %	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**LOQ, mg/kg**
Bendiocarb	*122*	*32*	113	13	75	20	0.018
Flucythrinate	108	7	102	12	91	8	0.009
Flutolanil	*46*	*12*	*62*	*18*	75	6	0.029
Isoprocarb	91	6	84	11	75	5	0.004
Isoxathion	*151*	*4*	108	5	72	12	0.006
Methoprene	96	13	93	13	80	4	0.008
Oxadiazon	*122*	*6*	106	12	88	4	0.015
Pirimiphos-methyl	79	12	82	14	79	5	0.006
Pyraclofos	*128*	*5*	105	10	70	17	0.013
Silafluofen	75	8	70	14	69	3	0.004
Thiobenbarb	*28*	*19*	*64*	*8*	82	3	0.017
Tralomethrin	*82*	*30*	100	15	109	10	0.018
Trichlorfon	110	4	111	9	106	8	0.003
Tricyclazole	*58*	*18*	70	11	73	7	0.009

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.