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

Validation Report 9

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

(QuEChERS method)

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CONTENT:

1. Introduction

This report describes the validation of the QuEChERS method combined with LC-MS/MS. The method was validated for 19 pesticides and degradation products in wheat, oat , rye, rice and barley. 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, vegetables and cereals[1].

2. Principle of analysis

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

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 and put in -80 degree freezer. When the extract is almost thawed it is centrifuged and 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.

Quantification and qualification:

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 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 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 or degradation products in wheat, oat , rye, rice and barley. However, this reports only includes the LC/MS/MS results. The validation was performed on 5-6 replicates on each cereals commodity at each of the three spiking levels; 0.01, 0.02 and 0.1 mg/kg. A blank sample of each cereal commodity was included.

4. Chromatograms and calibration curves

The calibration curve is determined by the analysis of each of the analysts 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 bracketing calibration curves. The majority of the correlation coefficients (R) were higher or equal to 0.99. 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**.

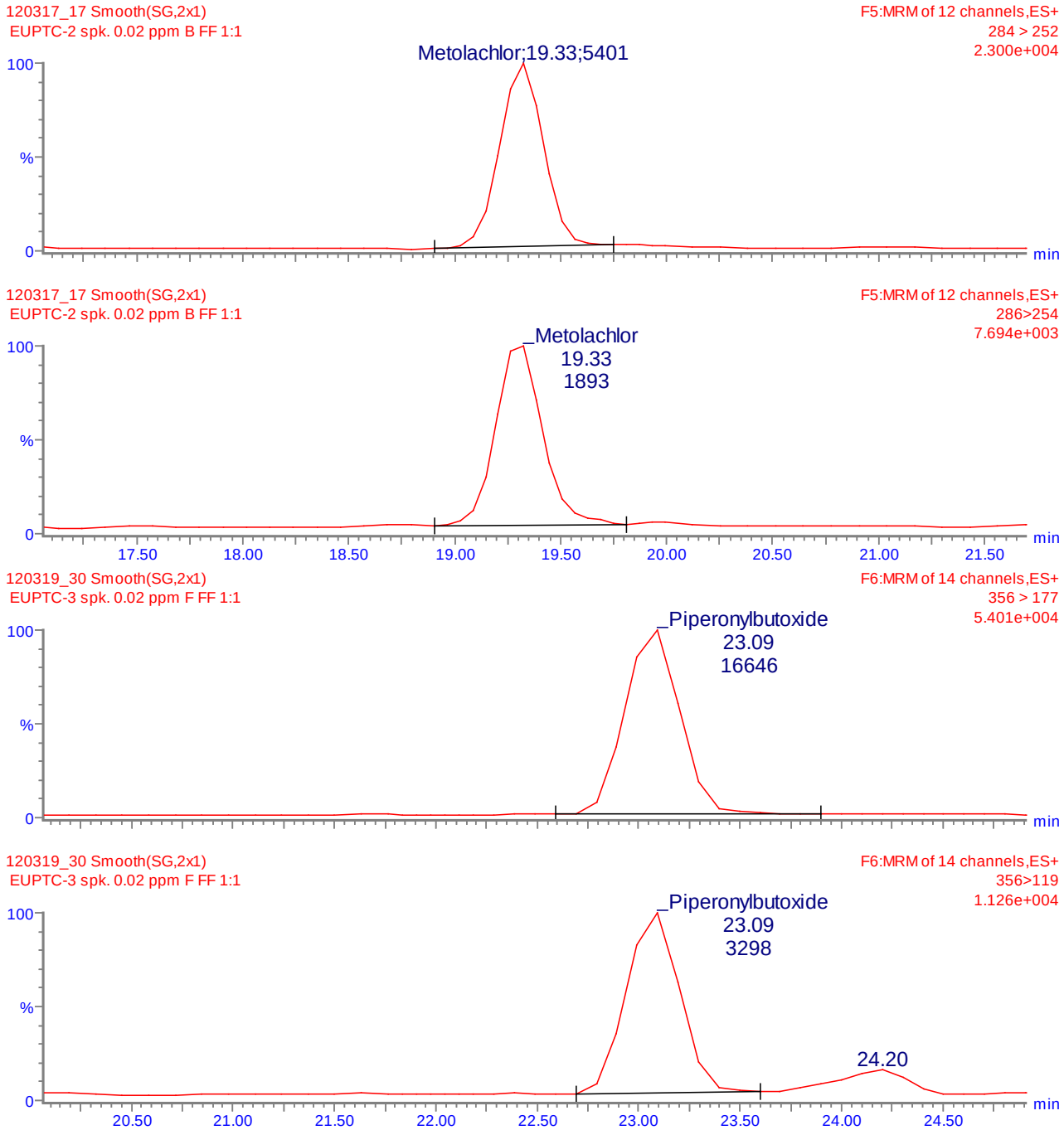

Figure 1: Examples of chromatograms for metolachlor/wheat and piperonylbutoxide/oat obtained when analysing extract spiked with 0.02 mg/kg (two MRM transitions are shown for each pesticide).

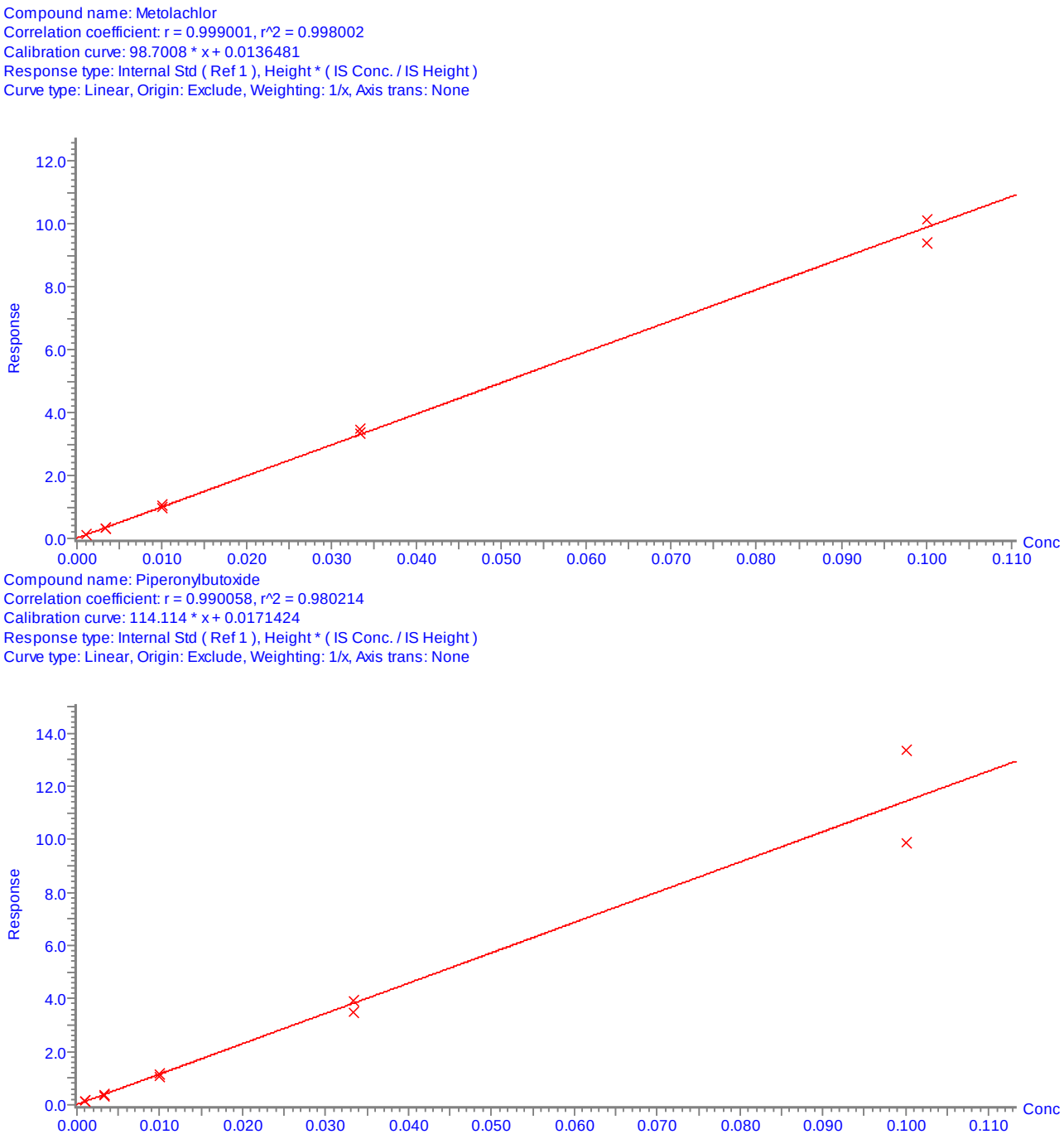

Figure 2. Examples of calibration curves for metolachlor and piperonylbutoxide (concentrations from 0.003-0.333 µg/ml)

5. Validation parameters

Precision – repeatability and internal reproducibility

Repeatability was calculated for all pesticides and degradation products on all three spiking levels, both for the individual cereal commodities and for the all commodities altogether. 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. The internal Reproducibility is calculated for the all the cereal commodities only as the individual commodities were only analyzed once. Internal reproducibility is relative standard deviation on results obtained under reproducibility conditions, with the same method on the same sample by different operators within a larger period of time. The In-house reproducibility is a combination of the repeatability variance and the in-house reproducibility

Repeatability and internal reproducibility in this validation was calculated from the 5-6 replicate determinations. Repeatability were calculated as given in ISO 5725-2[2].

Appendix 2-7 shows the relative repeatability and internal reproducibility for the validated pesticides 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 is listed in **Appendix 2-7**.

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 2-7**.

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

The 19 pesticides were validated for all spike levels. The relative repeatability (RSD_r) varied between 6-32 %, however most of the values were below 15%. For the majority of the pesticides the recovery was in the range of 70-110% at all three concentration levels. But in general the recoveries for cycloxydim was low for all commodities. The combined LOQs were in the range of 0.01-0.02 mg/kg, although some of the LOQs for specific commodities was seen to be higher (up to 0.08 mg/kg).

However, some of the pesticides were not validated on the the two lowest spike levels for some of the cereal commodities.

Wheat: Validation for tepraloxydim and tribenuron-methyl could not be accepted at the lowest spike level (0. 1) mg/kg.

Oat: All pesticides validated at all spike levels.

Rye: Validation for azimsulfuron, bensulfuron methyl, diuron, imidacloprid and triasulfuron could not be accepted at the lowest spike level (0. 1) mg/kg and cycloxydim was not accepted for the two lowest spike level (0.01 and 0.02 mg/kg).

Rice: Validation for cycloxydim and dicrotophos could not be accepted at the lowest spike level (0. 1) mg/kg and cycloxydim was not accepted for the two lowest spike level (0.01 and 0.02 mg/kg).

Barley: All pesticides validated at all spike levels.

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

8. Conclusions

In conclusion 19 pesticides and degradation products were validated on wheat, oat , rye, rice and barley for the QuEChERS method using LC-MS/MS for the analysis.

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 – Part2. 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 validated pesticides.

LC-MS/MS ESI-		Precursor ion-1	Product ion-1	CV	CE	Precursor ion-2	Product ion-2	CE	CV
1	Azimsulfuron	425	182	52	11	425	182	52	25
2	Bensulfuron methyl	411	149	21	30	411	182	21	20
3	Benzobicyclon	447	257	27	15	447	229	27	25
4	Cycloxydim	326	280	10	11	326	180	10	22
5	Dicrotophos	270	112	28	15	255	193	28	21
6	Diuron *)	231	186	16	15	231	150	16	15
7	Imidacloprid	256	209	21	15	256	175	20	20
8	Isoprothiolane	291	231	10	10	291	189	10	21
9	Mandipropamid	412	328	40	15	412	125	40	39
10	Mesotrione *)	338	291	16	15	338	212	16	27
11	Metamitron	203	104	34	33	203	77	34	33
12	Metolachlor	284	252	28	15	286	254	28	15
13	Nitenpyram	271	126	28	15	271	190	28	15
14	Piperonylbutoxide	356	177	10	15	356	119	10	33
15	Prothioconazole desthio	312	70	15	21	314	127	15	20
16	Tepraloxydim	342	166	25	25	342	250	20	20
17	Thifensulfuron-methyl *)	482	204	40	15	147	88	40	33
18	Triasulfuron	402	167	52	17	402	141	52	22
19	Tribenuron methyl	396	155	52	17	396	181	50	20

*) Pesticides analysed by negative ESI

Appendix 2. Recoveries, repeatability (RSDr), internal reproducibility (RSDR) and Limit of Quantification (LOQ) for pesticides validated on 5 cereal commodities, wheat, oat, rye, rice and barley.

Wheat, oat, rye, rice and barley - QuEChERS	Spike level mg/kg	Horwitz, %		Spike level mg/kg	Horwitz, %		Spike level mg/kg	Horwitz, %		
	0.01	32		0.02	29		0.1	23		
	Recovery, %	**RSD_r, %**	**RSD_R, %**	**Recovery, %**	**RSD_r, %**	**RSD_R, %**	**Recovery, %**	**RSD_r, %**	**RSD_R, %**	**LOQ**
Azimsulfuron	96	24	27	90	12	17	92	9	12	0.02
Bensulfuron methyl	72	20	26	67	12	19	66	10	17	0.01
Benzobicyclon	96	22	29	*84*	20	*26*	*87*	11	22	0.01
Cycloxydim	69	21	27	60	17	35	61	14	22	0.01
Dicrotophos	109	16	18	110	10	10	105	14	14	0.01
Diuron *)	109	15	18	110	11	11	91	8	8	0.01
Imidacloprid	105	32	32	106	17	21	110	12	12	0.02
Isoprothiolane	110	12	13	103	6	7	99	9	9	0.01
Mandipropamid	105	14	14	104	11	13	99	11	11	0.01
Mesotrione *)	106	21	31	90	16	21	76	10	20	0.02
Metamitron	102	29	29	107	27	27	109	17	17	0.02
Metolachlor	107	17	17	104	6	6	98	10	10	0.01
Nitenpyram	86	30	30	95	21	22	100	13	14	0.02
Piperonylbutoxide	104	15	14	99	9	11	91	13	14	0.01
Prothioconazole desthio	95	28	30	87	16	18	95	13	15	0.02
Tepraloxydim	101	25	32	74	21	24	71	12	22	0.02
Thifensulfuron-methyl *)	99	18	23	82	12	21	63	11	22	0.01
Triasulfuron	70	20	27	72	13	17	73	11	17	0.01
Tribenuron methyl	108	28	31	87	24	27	85	15	20	0.02

*) Pesticides analysed by negative ESI

Appendix 3. Recoveries, repeatability (RSDr) and Limit of Quantification (LOQs) for pesticides validated on wheat.

Wheat - QuEChERS	Spike level mg/kg	Horwitz, %		Spike level mg/kg	Horwitz, %		Spike level mg/kg	Horwitz, %		
	0.01	32		0.02	29		0.1	23		
	Recovery, %	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**LOQ**
Azimsulfuron	110	30		95	9		98	14		0.02
Bensulfuron methyl	71	21		64	6		65	12		0.01
Benzobicyclon	114	29		103	29		111	16		0.02
Cycloxydim	78	32		62	14		66	12		0.01
Dicrotophos	110	29		103	7		105	19		0.02
Diuron *)	93	29		114	10		95	10		0.02
Imidacloprid	119	31		79	17		106	11		0.02
Isoprothiolane	96	27		106	6		106	14		0.02
Mandipropamid	82	27		116	8		100	14		0.01
Mesotrione *)	92	30		96	14		79	12		0.02
Metamitron	107	37		109	23		107	19		0.02
Metolachlor	112	31		105	4		101	13		0.02
Nitenpyram	77	19		81	10		96	15		0.01
Piperonylbutoxide	100	29		109	7		103	12		0.02
Prothioconazole desthio	85	16		103	14		110	17		0.01
Tepraloxydim				74	19		71	7		0.03
Thifensulfuron-methyl *)	89	23		80	11		62	13		0.01
Triasulfuron	63	17		65	7		72	13		0.01
Tribenuron methyl				92	20		74	15		0.03

*) Pesticides analysed by negative ESI

Appendix 4. Recoveries, repeatability (RSDr) and Limit of Quantification (LOQs) for pesticides validated on oat.

Oat - QuEChERS	Spike level mg/kg	Horwitz, %		Spike level mg/kg	Horwitz, %		Spike level mg/kg	Horwitz, %		
	0.01	32		0.02	29		0.1	23		
	Recovery, %	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**Recovery, %**	**RSD_r, %**		**LOQ**
Azimsulfuron	106	10		103	9		100	9		0.01
Bensulfuron methyl	81	6		84	6		81	6		0.01
Benzobicyclon	104	12		*87*	10		*88*	8		0.01
Cycloxydim	76	7		78	14		77	7		0.01
Dicrotophos	117	8		108	7		99	8		0.01
Diuron *)	120	6		109	10		90	9		0.01
Imidacloprid	105	12		113	17		113	8		0.01
Isoprothiolane	112	7		100	6		97	6		0.01
Mandipropamid	105	10		95	8		95	16		0.01
Mesotrione *)	114	13		104	14		89	11		0.01
Metamitron	107	28		116	23		107	15		0.02
Metolachlor	106	5		101	4		97	8		0.01
Nitenpyram	85	26		99	10		95	15		0.01
Piperonylbutoxide	108	7		91	7		91	15		0.01
Prothioconazole desthio	93	28		87	14		89	7		0.02
Tepraloxydim	114	23		91	19		89	11		0.02
Thifensulfuron-methyl *)	114	13		104	11		83	11		0.01
Triasulfuron	88	22		84	7		88	7		0.01
Tribenuron methyl	119	30		101	20		107	10		0.02

*) Pesticides analysed by negative ESI

Appendix 5. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on rye.

Rye - QuEChERS	Spike level mg/kg	Horwitz, %	Spike level mg/kg	Horwitz, %	Spike level mg/kg	Horwitz, %	
	0.01	32	0.02	29	0.1	23	
	Recovery, %	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**LOQ**
Azimsulfuron			93	13	93	7	0.02
Bensulfuron methyl			71	7	66	14	0.02
Benzobicyclon	110	27	*90*	20	*87*	10	0.02
Cycloxydim					*56*	*23*	0.08
Dicrotophos	92	11	115	6	112	13	0.01
Diuron *)			106	9	86	8	0.02
Imidacloprid			109	21	115	2	0.03
Isoprothiolane	112	9	106	5	99	12	0.01
Mandipropamid	106	1	109	11	96	7	0.01
Mesotrione *)	116	6	81	14	70	5	0.01
Metamitron	80	3	105	9	112	19	0.01
Metolachlor	104	9	103	5	97	14	0.01
Nitenpyram	105	2	105	17	104	12	0.01
Piperonylbutoxide	104	15	99	9	91	13	0.01
Prothioconazole desthio	113	20	79	14	90	18	0.01
Tepraloxydim	91	12	69	13	67	12	0.01
Thifensulfuron-methyl *)	114	21	67	11	51	10	0.01
Triasulfuron			80	10	71	13	0.01
Tribenuron methyl	119	15	88	24	82	21	0.01

*) Pesticides analysed by negative ESI

Appendix 6. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on rice.

Rice - QuEChERS	Spike level mg/kg	Horwitz, %	Spike level mg/kg	Horwitz, %	Spike level mg/kg	Horwitz, %	
	0.01	32	0.02	29	0.1	23	
	Recovery, %	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**LOQ**
Azimsulfuron	76	17	82	8	91	6	0.01
Bensulfuron methyl	64	14	60	11	63	6	0.01
Benzobicyclon	93	16	*80*	10	*84*	9	0.01
Cycloxydim					56	16	0.05
Dicrotophos					112	3	0.05
Diuron *)	103	15	107	12	91	5	0.01
Imidacloprid	110	18	114	19	117	8	0.01
Isoprothiolane	115	9	103	6	101	4	0.01
Mandipropamid	106	8	104	8	107	8	0.01
Mesotrione *)	106	16	97	15	86	7	0.01
Metamitron	115	23	103	25	108	18	0.02
Metolachlor	104	9	108	4	102	4	0.01
Nitenpyram	62	16	103	18	108	9	0.01
Piperonylbutoxide	96	6	99	5	93	5	0.01
Prothioconazole desthio	98	20	78	10	100	6	0.01
Tepraloxydim	92	26	73	18	78	16	0.01
Thifensulfuron-methyl *)	102	17	89	12	64	4	0.01
Triasulfuron	58	12	67	16	73	10	0.01
Tribenuron methyl	112	18	92	12	88	17	0.01

*) Pesticides analysed by negative ESI

Appendix 7. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on barley.

Barley - QuEChERS	Spike level mg/kg	Horwitz, %	Spike level mg/kg	Horwitz, %	Spike level mg/kg	Horwitz, %	
	0.01	32	0.02	29	0.1	23	
	Recovery, %	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**Recovery, %**	**RSD_r, %**	**LOQ**
Azimsulfuron	84	5	76	10	80	6	0.01
Bensulfuron methyl	60	9	57	15	55	11	0.01
Benzobicyclon	67	19	*62*	16	*63*	5	0.01
Cycloxydim	53	11	41	3	49	3	0.01
Dicrotophos	116	8	114	4	104	4	0.01
Diuron *)	120	10	114	12	92	7	0.01
Imidacloprid	102	17	117	14	113	4	0.01
Isoprothiolane	110	8	101	8	95	3	0.01
Mandipropamid	118	15	99	12	96	5	0.01
Mesotrione *)	73	17	70	18	60	5	0.01
Metamitron	90	27	98	28	114	13	0.01
Metolachlor	107	2	103	4	96	5	0.01
Nitenpyram	76	5	79	24	94	16	0.01
Piperonylbutoxide	110	5	94	5	85	3	0.01
Prothioconazole desthio	91	15	88	10	87	9	0.01
Tepraloxydim	84	24	64	11	53	13	0.01
Thifensulfuron-methyl *)	78	7	70	16	57	11	0.01
Triasulfuron	64	18	64	18	60	10	0.01
Tribenuron methyl	97	18	94	16	76	8	0.01

*) Pesticides analysed by negative ESI

Appendix 8: 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.