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Appendix 2

Validation Report 10

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

(QuEChERS method)

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CONTENT:

1. Introduction *3*

2. Principle of analysis *3*

3. Validation design *3*

4. Chromatograms and calibration curves *3*

5. Validation parameters *6*

6. Criteria for the acceptance of validation results *7*

7. Results and discussion *7*

8. Conclusions *8*

9. References *8*

Appendix 1. MRM transitions for the validated pesticides. *9*

Appendix 2. Recoveries, repeatability (RSD_r), internal reproducibility (RSDR) and Limit of Quantification (LOQ) for pesticides validated on 5 cereal commodities, wheat, oat, rye, rice and barley *10*

Appendix 3. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on wheat. *11*

Appendix 4. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on oat. *12*

Appendix 5. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on rye. *13*

Appendix 6. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on rice. *14*

Appendix 7. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQs) for pesticides validated on barley *15*

Appendix 8: Principles of the QuEChERS method for cereal extraction *16*

1. Introduction

This report describes the validation of the QuEChERS method combined with LC-MS/MS. The method was validated for 11 pesticides 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.

Quantification and qualification: The final extract is analysed GC/MS/MS. The pesticide residues are separated on a DB5-MS column and detected by tandem mass spectrometry (MS/MS) operating with electron energy at 70 eV, source temperature at 180°C and transfer line at 250°C. The injection volume was 4 µl. All pesticides were detected in the multiple reaction monitoring mode (MRM). For each pesticide two transistion were determined. One for quantification and one for qualification. The MRM transitions for the pesticides and degradation products 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 GC/MS/MS results (the LC/MS/MS results can be found in 'validation report no 8'. 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**.

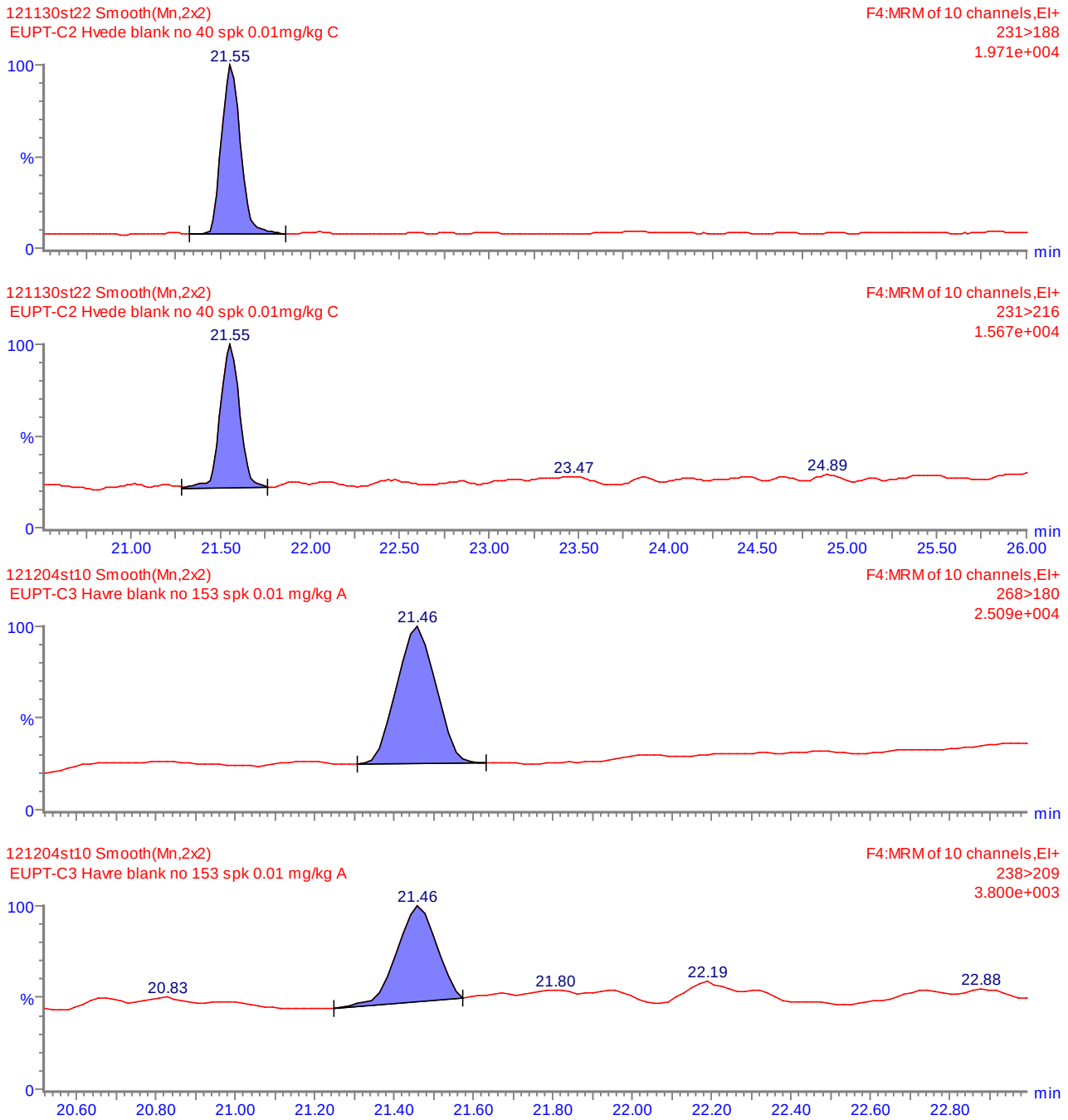

Figure 1: Examples of chromatograms for rimsulfuron/wheat and fenamidone/oat obtained when analysing extract spiked with 0.01 mg/kg (two MRM transitions are shown for each pesticide).

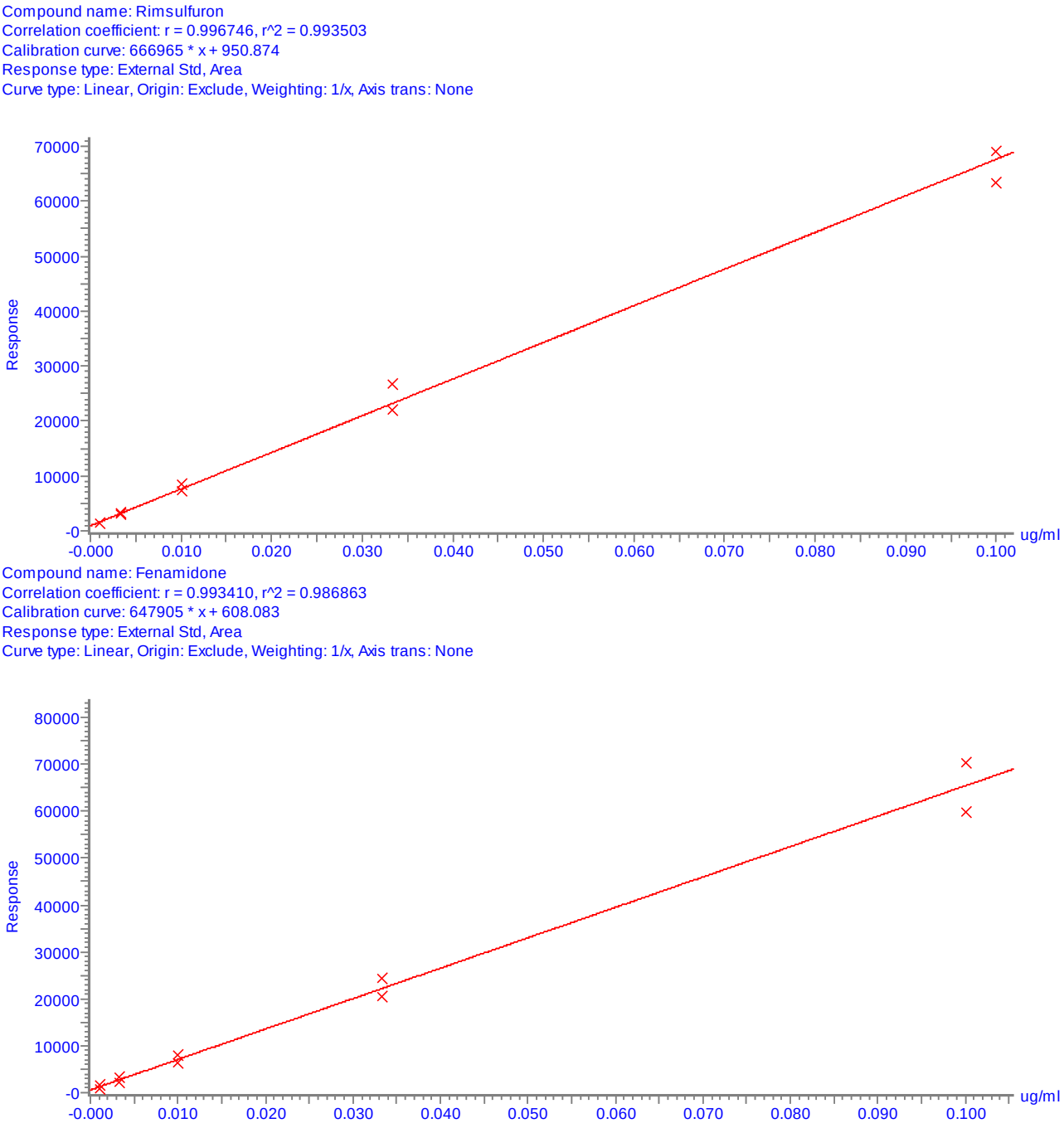

Figure 2. Examples of calibration curves for rimsulfuron and fenamidone (concentrations from 0.003-0.1 µg/ml)

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, because the individual cereal type is analysed on one occasion only. 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.

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 quantification limits, LOQs have been calculated.

7. Results and discussion

Overall validation on all 5 cereal types.

Bifenox, buprofezine, EPN, fenamidone, isoprothiolane, metolachlor, piperonyl-Butoxide, rimsulfuron and zoxamide were validated for all spike levels. The relative repeatability (RSD_r) varied between 6-32 %, however most of the values were below 15%. Recovery for this nine pesticides was in the range of 70-102% at all three concentration levels. 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 0.07 mg/kg). Validation for bifenazate was only accepted for the highest spike level. This was due to problems on several of the cereal types (see below). Likewise, validation for phenmedipham was not accepted at the lowest spike level (see below).

Validation on individual cereal type.

Wheat: Validation for bifenazate could not be accepted at the two lowest spike level (0.01 and 0.02 mg/kg).

Oat: The recoveries calculated on middle spike level (0.02 mg/kg) were significantly lower compared to the lowest and highest spike level (0.01 and 0.1 mg/kg) in oat and most spike levels for the four other cereal type. It is assumed the an error during the spike process hid influence the results. E.g. the automatic pipette may have been incorrectly adjusted. Consequently, the validation is accepted for 11 pesticides.

Rye: Validation for EPN could not be accepted at the lowest spike level (0.01) mg/kg and phenmedipham was not accepted for the two lowest spike level (0.01 and 0.02 mg/kg).

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

Barley: Validation for bifenazate was not accepted any of the spike levels due to low recoveries. Furthermore, rimsulfuron and EPN was not accepted at the lowest spike level (0.01 mg/kg).

The results for the accepted pesticides analysed on GC-MS/MS are listed in Appendix 2-7.

8. Conclusions

In conclusion 11 pesticides were validated on wheat, oat , rye, rice and barley for the QuEChERS method using GC-MS/MS for the detection.

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/12495/2011, 01/01/2012, European Commission, Brussels, 2012.

Appendix 1. MRM transitions for the validated pesticides.

GC-MS/MS		Retention time	Precursor ion-1	Product ion-1	CE	Precursor ion-2	Product ion-2	CE
1	Bifenazate	21.28	258	196	15	300	258	10
2	Bifenox	21.68	341	310	12	311	279	15
3	Buprofezin	17.13	249	193	10	305	249	10
4	EPN	21.11	169	141	10	169	77	13
5	Fenamidone	21.54	268	180	20	238	209	20
6	Isoprothiolane	16.59	290	118	15	290	204	15
7	Metolachlor	13.82	238	162	15	162	133	15
8	Phenmedipham	9.77	167	135	10	167	167	0
9	Piperonyl Butoxide	20.16	338	176	5	176	145	15
10	Rimsulfuron	21.64	231	188	15	231	216	10
11	Zoxamide	15.42	187	159	15	258	187	15

Appendix 2. Recoveries, repeatability (RSD_r), 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**
Bifenazate									72	10	10		0.04
Bifenox	84	15	25		83	12	25		86	12	19		0.01
Buprofezine	99	13	13		96	11	20		90	7	17		0.01
EPN	85	32	32		77	21	28		86	13	20		0.02
Fenamidone	78	19	26		78	9	17		90	8	16		0.01
Isoprothiolane	102	14	18		96	8	18		92	8	19		0.01
Metolachlor	102	14	19		99	6	20		94	6	19		0.01
Phenmedipham					86	23	23		80	19	20		0.02
Piperonyl-Butoxide	98	11	18		90	7	13		84	6	12		0.01
Rimsulfuron	70	23	31		75	11	27		87	9	9		0.01
Zoxamide	86	15	24		81	9	9		81	7	7		0.01

Appendix 3. Recoveries, repeatability (RSD_r) 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**
Bifenazate							75	15		0.07
Bifenox	74	21		100	8		99	18		0.01
Buprofezine	107	21		111	9		100	9		0.01
EPN	80	24		98	21		103	16		0.01
Fenamidone	68	23		79	11		99	11		0.01
Isoprothiolane	105	24		108	6		101	12		0.02
Metolachlor	108	24		117	2		108	7		0.02
Phenmedipham	89	29		99	24		94	23		0.02
Piperonyl-Butoxide	111	16		95	6		79	9		0.01
Rimsulfuron	83	18		96	8		104	12		0.01
Zoxamide	94	23		94	2		89	9		0.01

Appendix 4. Recoveries, repeatability (RSD_r) 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**
Bifenazate	70	18		*43*	*14*		71	6		0.01
Bifenox	84	17		*57*	*22*		80	14		0.01
Buprofezine	104	7		79	7		82	3		0.00
EPN	84	12		*60*	*23*		70	8		0.01
Fenamidone	85	12		*59*	*8*		84	9		0.01
Isoprothiolane	96	3		79	7		92	4		0.002
Metolachlor	100	7		74	5		74	5		0.004
Phenmedipham	103	31		81	6		85	17		0.02
Piperonyl-Butoxide	101	6		81	6		88	4		0.004
Rimsulfuron	71	25		*43*	*11*		70	9		0.01
Zoxamide	77	10		*53*	*8*		79	8		0.005

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**
Bifenazate	87	17		82	14		71	10		0.04
Bifenox	112	5		90	11		74	8		0.00
Buprofezine	99	7		90	13		77	10		0.00
EPN				73	22		83	10		0.02
Fenamidone	100	11		89	10		75	9		0.01
Isoprothiolane	106	6		95	11		76	10		0.00
Metolachlor	100	6		95	9		82	8		0.00
Phenmedipham							75	10		0.05
Piperonyl-Butoxide	101	7		96	9		82	6		0.00
Rimsulfuron	82	15		79	12		75	9		0.01
Zoxamide	90	9		86	13		86	7		0.00

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**
Bifenazate					74	5	0.02
Bifenox	87	11	96	11	103	2	0.01
Buprofezine	105	15	116	13	109	5	0.01
EPN					95	13	0.07
Fenamidone	79	19	87	6	106	4	0.01
Isoprothiolane	119	10	116	3	113	4	0.01
Metolachlor	119	11	120	4	116	2	0.01
Phenmedipham			76	29	87	14	0.03
Piperonyl-Butoxide	105	8	100	3	97	5	0.00
Rimsulfuron	79	8	83	7	106	4	0.00
Zoxamide	107	14	95	6	102	4	0.01

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**
Bifenazate							
Bifenox	71	16	71	10	75	8	0.01
Buprofezine	81	10	*86*	10	*79*	4	0.00
EPN			76	13	80	13	0.01
Fenamidone	65	19	78	10	83	6	0.01
Isoprothiolane	83	17	84	11	77	6	0.01
Metolachlor	83	15	88	9	90	3	0.01
Phenmedipham	70	26	81	17	68	9	0.01
Piperonyl-Butoxide	73	16	76	10	75	6	0.01
Rimsulfuron			*62*	*15*	81	7	0.01
Zoxamide	63	12	75	13	74	3	0.00

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.