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National Food Institute
Technical University of Denmark

Validation Report 25

Quantitative determination of pesticide residues in cereals

by LC-QTOF (HRMS)

(QuEChERS method)

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December 2017

CONTENT:

1. Introduction

This report describes the validation of the QuEChERS method combined with LC-QTOF accurate mass determination. Accurate mass determination using the LC-QTOF was initially intended only for screening purposes, i.e. qualitative analysis. However with increasing resolution, increasing sensitivity as well as wider dynamic range of the high resolution MS systems it has become more realistic to also perform quantitative analysis on these instrument. 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]. Our inhouse QuEChERS procedure is outlined in Appendix 2.

The aim of the present validation study was therefore to evaluate whether the HRMS systems available (Agilent's 6550 iFunnel QTOF LC/MS) can provide data which allow for quantitative analysis.

2. Principle of analysis

Sample preparation:
The samples were 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 clean tube and put in -80 degree freezer. When the extract is almost thawed it is centrifuged and the supernatant is transferred to a tube containing PSA and $MgSO_4$. An aliquot was withdrawn prior to this clean-up step and analysed by LC-MS/MS. 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 LC-ESI-QTOF.

LC-ESI-QTOF analysis:
The pesticide residues are separated on a reversed-phase column (Acclaim RSLC C18 2.2m 2.1x100mm from Dionex, Thermo Scientific (Sunnyvale, California)) and detected by tandem mass spectrometry (MS/MS) by electrospray (ESI). The validation includes pesticides determined in positive mode. Gradient LC elution was performed using 0.1% formic and 5 mM ammoniac in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The gradient started with 10% B and was kept for 1 min, followed by a linear gradient to 40% B up to 3 min, followed by a linear gradient to 90% B up to 14 min. This level of B was kept for 2 minutes and

then returned to the start level of 10% B, which were kept for 4 min before next injection. The flow rate started and 0.2 ml/min and was increased to 0.4 ml/min using a linear gradient from 3 to 14 min. The flow was kept at 0.4 ml/min for 2 min and then changed back to 0.2 ml/min at 16 min. The LC system was the same used in positive and negative mode. The injection volume was 5 µl.

The instrument was operated in the "All Ion" approach in which four different experiments are performed switching between collision energies of 0, 10, 20 and 40 V with no isolation of single ions in the quadrupole. All ions with m/z-values of 50-1600 were led through the quadrupole into the collision cell, where the different collision energies were applied and then finally into the TOF instrument obtaining accurate masses. The instrument operated in 2 GHz Extended mode, which gives a higher dynamic range, and the slicer mode was high resolution: around 15.000 at m/z-values of 300. The mass axis was calibrated in the m/z-range 50-1600 using the tuning mix from Agilent containing 10 compounds with masses from 118-2721. A reference solution was sprayed into a separate nebulizer during analysis to give a continuous calibration during analysis. Reference masses of m/z 112.985587 and 966.000725 were used in negative mode and m/z 121.050873 and 922.009798 in positive mode.

The quantitative data-analysis was performed using MassHunter TOF Quantitative Analysis (version B.05.20). The ions used as quantifier ions and qualifier ions were ions available in the PCDL made available for pesticide analysis by Agilent.

3. Validation design

The method was sought validated for 84 pesticides or metabolites in wheat, barley, oat and rice for the quantitative analysis and determination of LOQ and for wheat, barley, oat and rice for the qualitative analysis and determination of SDL.

Validation design quantitative analysis:
The validation was performed on 5 replicates of wheat, barley, oat and rice at two spiking levels; 0.01 and 0.1 mg/kg (total of 25 samples). The calibration standard were matrix matched with rye matrix.

4. Linarity

The calibration curve is determined by the analysis of each of the analysts at least 4 calibration levels, i.e. 5, 10, 33.3 and 100 ng/ml. The quantification was performed from the mean of two bracketing calibration curves.

On the LC-ESI-QTOF the calibration curves were in general best fitted to a quadratic curve. The three lowest calibration points generally fitted well with a linear curve. Regardless of the choice of curve fit the quantification of the relevant spike levels were very similar. The majority (75%) of the correlation coefficients (R) were higher or equal to 0.99. The poorest fit (R2=0.913) to the quadratic line was obtained for bromadiolone.

5. Validation parameters and criteria for quantitative analysis

Precision – repeatability

Repeatability was calculated for all pesticides and degradation products on both spiking levels (0.01 mg/kg and 0.1 mg/kg). Repeatability (RSD_r) in this validation was for LC-ESI-QTOF calculated from the 15 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 (0.01 mg/kg and 0.1 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) was meet.

The obtained results including recovery, RSD_r, Combined Uncertainty (U_c) and limit of quantification (LOQ) are presented in appendix 1.

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 $\leq 20\%$[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 analytical result is by default corrected for bias/recovery and the combined uncertainty is then given by:

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

Where RSD is the intra-laboratory uncertainty (RSD_R).

6. Validation parameters and criteria for qualitative analysis

Selectivity: An Un-spiked sample of each of the five cereal matrices were analysed and screened for false detects.

7. Results and conclusion

Of the 82 pesticides and metabolites sought validated on wheat, barley, oat and rice 68 were successfully validated. The results obtained for these 68 compounds are presented in Appendix 1. For 57 compounds an LOQ of 0.01 mg/kg was achieved and for the remaining 13 compounds an LOQ of 0.1 mg/kg was obtained. For the 14 compounds that were not possible to validate the reason was primarily high variation on the reproducibility and/or repeatability and/or low recovery (40-60%).

The present validation study show that even though the linear range of the Q-TOF is limited and a quadratic fit is the best fit in many cases quantification is possible with satisfactory precision. A number of analytes could not be validated and besides the reason mentioned above further investigations are needed in order to determine why these analytes perform poorly. The poor results may not be related to the use of Q-TOF but to poor extraction efficiencies and/or ionization efficiencies. If poor extraction efficiency and/or poor ionization efficiency is the reason then these compounds will also perform poorly if analysed by the traditional quantitative methods using tandem mass spectrometry.

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 Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed, Document No SANTE/11813/2017, 01/01/2018.

Appendix 1. Recoveries, repeatability (RSD_r) and Limit of Quantification (LOQ) for pesticides validated on three cereal commodities, wheat, barley, oat and rice using QuEChERS method and LC-ESI-QTOF.

			Spike level 0.01 mg/kg				Spike level 0.1 mg/kg				
	no.	Compound	Recovery %	RSD_r, %	RSD_R, %	Comb. Uncer-tainty (%)	Recovery %	RSD_r, %	RSD_R, %	Comb. Uncer-tainty (%)	LOQ
LC	1	Acibenzolar-S-methyl	83	11	10	11	91	6	7	8	0.01
LC	2	Ancymidol	71	5	10	10	77	14	*22*	*22*	0.01
LC	3	Anilofos	83	6	9	9	96	7	7	7	0.01
LC	4	Atraton	72	12	13	13	95	12	18	19	0.01
LC	5	Atrazine-Desethyl	*59*	12	*23*	*24*	96	6	5	5	0.1
LC	6	Azaconazole	86	9	13	13	93	9	18	19	0.01
LC	7	Aziprotryne	76	4	14	14	90	5	9	9	0.01
LC	8	Beflubutamid	*41*	20	*37*	*38*	89	5	13	13	0.1
LC	9	Benodanil	*66*	12	*23*	*24*	96	5	9	9	0.1
LC	10	Bensulide	101	9	18	19	108	10	12	13	0.01
LC	11	Benzoximate	71	7	11	11	92	6	8	8	0.01
LC	12	Bromadiolone	*9*	*69*	*131*	*134*	74	11	16	16	0.1
LC	13	Butachlor	120	9	10	11	92	6	8	8	0.01
LC	14	Butafenacil	107	6	7	7	115	11	13	14	0.01
LC	15	Butamifos	*65*	12	*21*	*22*	92	6	8	9	0.1
LC	16	Butylate	77	5	*24*	*25*	85	5	18	19	0.01
LC	17	Chloridazon	73	7	11	12	95	5	6	6	0.01
LC	18	Chloroxuron	90	6	15	16	90	8	20	20	0.01
LC	19	Coumachlor	92	12	*22*	*23*	94	12	15	15	0.01
LC	20	Crimidine	101	4	7	7	89	13	15	15	0.01
LC	21	Cycloate	72	5	13	13	94	5	7	8	0.01
LC	22	Daimuron	88	6	8	9	91	11	14	14	0.01
LC	23	Desmedipham	77	10	17	18	99	15	14	15	0.01
LC	24	Diallate (cis)	91	11	11	12	90	5	6	6	0.01

			Spike level 0.01 mg/kg				Spike level 0.1 mg/kg				
	no.	Compound	Recovery %	RSD_r, %	RSD_R, %	Comb. Uncer-tainty (%)	Recovery %	RSD_r, %	RSD_R, %	Comb. Uncer-tainty (%)	LOQ
LC	25	Dichlormid	*64*	*25*	*29*	*30*	77	9	22	22	0.1
LC	26	Dichlorobenzamide	*59*	15	*33*	*34*	85	11	17	18	0.1
LC	27	Diclobutrazol	92	7	13	13	92	8	10	10	0.01
LC	28	Dimethenamid	81	5	17	17	90	8	12	13	0.01
LC	29	Dimethylvinphos	79	7	14	14	95	5	8	9	0.01
LC	30	Dinex	89	8	8	8	90	12	11	11	0.01
LC	31	Diphenamid	83	7	10	11	88	10	12	12	0.01
LC	32	Dodemorph	83	6	11	11	83	9	17	18	0.01
LC	33	Etaconazole	81	19	19	19	98	9	16	17	0.01
LC	34	Ethiprole	*52*	*30*	*31*	*32*	101	8	9	9	0.1
LC	35	Fensulfothion	95	8	16	17	99	13	*24*	*25*	0.01
LC	36	Flucycloxuron	71	16	15	15	90	13	12	12	0.01
LC	37	Fluridone	87	6	7	7	98	5	6	6	0.01
LC	38	Flurprimidol	79	8	11	11	92	5	10	10	0.01
LC	39	Hexazinone	91	7	10	11	93	9	12	12	0.01
LC	40	Imazamethabenz-methyl	72	11	15	15	100	11	16	16	0.01
LC	41	Imibenconazole	*69*	8	10	11	84	11	12	12	0.01
LC	42	Inabenfide	*56*	9	*28*	*29*	87	12	19	19	0.1
LC	43	Iprobenfos	81	7	12	13	95	7	9	10	0.01
LC	44	Isazofos	*68*	6	*26*	*27*	85	5	16	17	0.1
LC	45	Isocarbamide	*64*	13	*21*	*21*	85	13	18	19	0.1
LC	46	Mefenpyr-diethyl	89	7	7	7	97	7	7	7	0.01
LC	47	Metazachlor	82	7	10	10	80	17	*23*	*24*	0.01
LC	48	Metobromuron	82	9	12	12	95	8	12	13	0.01
LC	49	Metrafenone	81	7	8	9	93	6	11	12	0.01
LC	50	Napropamide	86	7	10	10	94	7	9	9	0.01
LC	51	Norflurazon	79	14	16	17	81	11	*21*	*22*	0.01

			Spike level 0.01 mg/kg				Spike level 0.1 mg/kg				
	no.	Compound	Recovery %	RSD_r, %	RSD_R, %	Comb. Uncer-tainty (%)	Recovery %	RSD_r, %	RSD_R, %	Comb. Uncer-tainty (%)	LOQ
LC	52	Pentanochlor	70	7	16	16	84	3	10	10	0.01
LC	53	Pethoxamid	91	5	6	6	100	6	8	8	0.01
LC	54	Pretilachlor	81	4	10	11	92	5	7	7	0.01
LC	55	Prometryn	86	19	20	21	87	8	17	18	0.01
LC	56	Propachlor	72	10	16	16	79	17	*25*	*26*	0.01
LC	57	Propaphos	72	8	*29*	*30*	91	7	12	12	0.01
LC	58	Quizalofop-ethyl	72	6	10	10	84	7	8	8	0.01
LC	59	Rabenzazole	79	5	18	19	88	8	16	16	0.01
LC	60	Schradan	78	9	11	11	86	6	9	9	0.01
LC	61	Siduron	103	4	7	7	98	6	9	9	0.01
LC	62	Spirodiclofen	*60*	18	*29*	*30*	79	11	*21*	*21*	0.1
LC	63	Tebupirimfos	77	5	12	12	94	7	11	12	0.01
LC	64	Tebutam	85	5	11	12	91	6	13	13	0.01
LC	65	Thenylchlor	70	6	17	18	90	6	12	13	0.01
LC	66	Thiazopyr	84	5	*25*	*26*	97	7	18	19	0.01
LC	67	Tolfenpyrad	*66*	5	12	12	93	8	10	11	0.1
LC	68	Tribufos	73	7	10	10	91	8	9	9	0.01

Appendix 2: 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.