Appendix 4

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

Screening Validation Report 1

Screening of pesticide residues in cereals by UPLC-TOF

(QuEChERS method)

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CONTENT:

1. Introduction

Qualitative multi residue methods, especially those involving automated MS-based detection, offer laboratories a cost-effective means to extend their analytical scope to analytes which potentially have low probability to be present in the samples. The more commonly occurring analytes should continue to be sought and measured using quantitative MRMs.

This report describes the screening validation of the QuEChERS method combined with UPLC-TOF MS. The method was validated for 35 pesticides in cereals at the screening detection limit, 0.01 mg/kg.

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].

The method validated here is based on the procedure for dry matrixes (<30% water content) according to the document CEN/TC 275/WG 4 N 0204 (CEN document). Even though cereals have a fat content of about 2%[2] no attempt has been made to remove the fat from the extract, e.g. freezing out as proposed in the CEN document, since no problems caused by fat has been observed.

2. Principle of analysis

Cold water/ice water, acetonitrile and an internal standard are added to the milled sample. The sample is shaken and a salt and buffer mixture is added and the sample is shaken again. 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 obtained.

Screening:

Different cereal samples were spiked at 0.01 mg/kg with a mix of pesticide standards (Dr. Ehrenstorfer), extracted by and analysed by UPLC-QTOF (UPLC: Dionex RSLC; TOF: Bruker Daltronics, MaXis).

LC conditions:

Column: Acclaim RSLC C18 2.2m 2.1x100mm (Dionex)

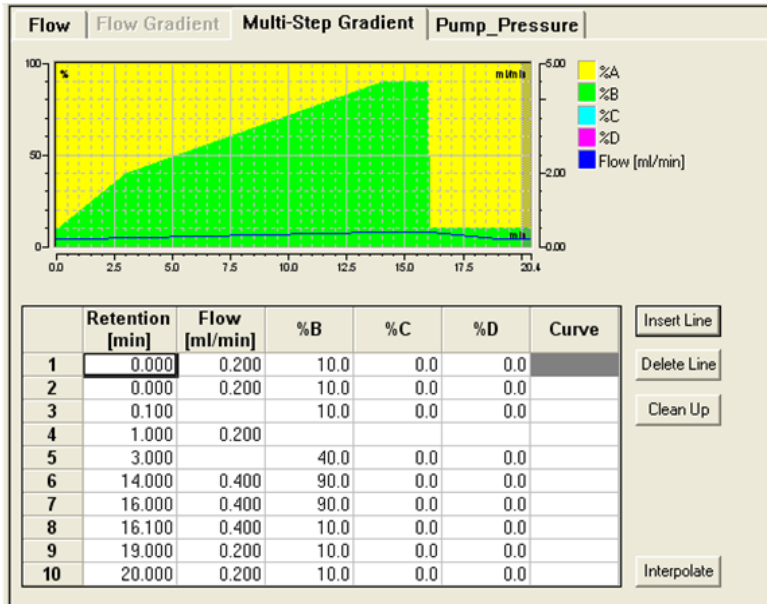
Eluents: ESI(+): A: H_2O/MeOH 90/10 (v/v), B: MeOH (both contain 5mM NH_4formate, 0.01% HCOOH)

Gradient and flowrate: see figure 1

Injection volume: 1µl

Runtime: 20 min

Temperature: 30°C



	Retention [min]	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	0.200	10.0	0.0	0.0	
2	0.000	0.200	10.0	0.0	0.0	
3	0.100		10.0	0.0	0.0	
4	1.000	0.200				
5	3.000		40.0	0.0	0.0	
6	14.000	0.400	90.0	0.0	0.0	
7	16.000	0.400	90.0	0.0	0.0	
8	16.100	0.400	10.0	0.0	0.0	
9	19.000	0.200	10.0	0.0	0.0	
10	20.000	0.200	10.0	0.0	0.0	

Figure 1: Figure and table of the multistep gradient used

TOF conditions:

TOF instrument: Bruker Maxis QTOF (only operated as TOF)

Ionisation mode: ESI positiv

Calibration was performed externally prior to a sample series with a sodium formate solution, and additionally internally for each chromatogram by injecting the calibrant at the beginning of each run.

Database:

Automated target detection for the pesticide standard mix spiked to cereal extracts can be achieved by automated peak detection on the EICs expected for the $[M+H]^+$, $[M+NH_4]^+$ or $[M+Na]^+$ ions of each compound in a database. A database containing names, sum formulas, exact masses and

retention times for 100 pesticides was set up. Based on accurate mass and known retention times the compounds present in the sample are identified. For each identification candidate additionally the theoretical isotope abundance pattern is compared to the experimental obtained isotopic pattern.

3. Validation design

The method was sought validated for 38 pesticides in cereals. The validation was performed on 20 replicates, and blank material was spiked at the expected screening reporting limit (SDL), which in this case was 0.01 mg/kg. The validation was performed on wheat, rye, oat, barley (5 replicates each).

4. Chromatograms

Examples of a chromatogram obtained when analysing the extracts by UPLC-TOF are presented in figure 2. The chromatogram show the base peak chromatogram (BPC) overlaid with extracted ion chromatograms (EIC) of the compounds detected from the database. Below the chromatogram all detected traces with accurate mass measurements are shown (the list was cut off for fitting purposes).

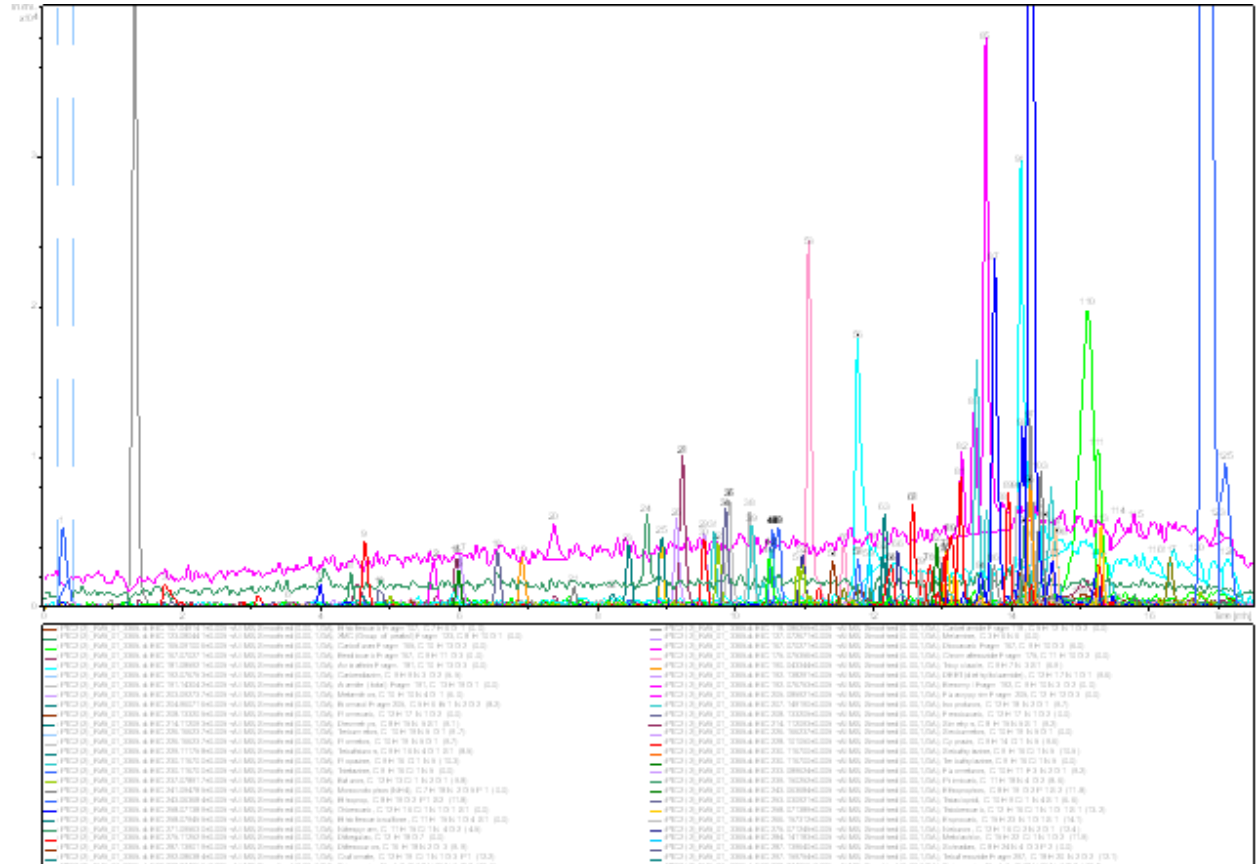


Figure 2: Chromatogram showing both base peak chromatogram overlaid with extracted ion chromatograms of the compounds detected from the database. The accurate mass of all the peaks detected.

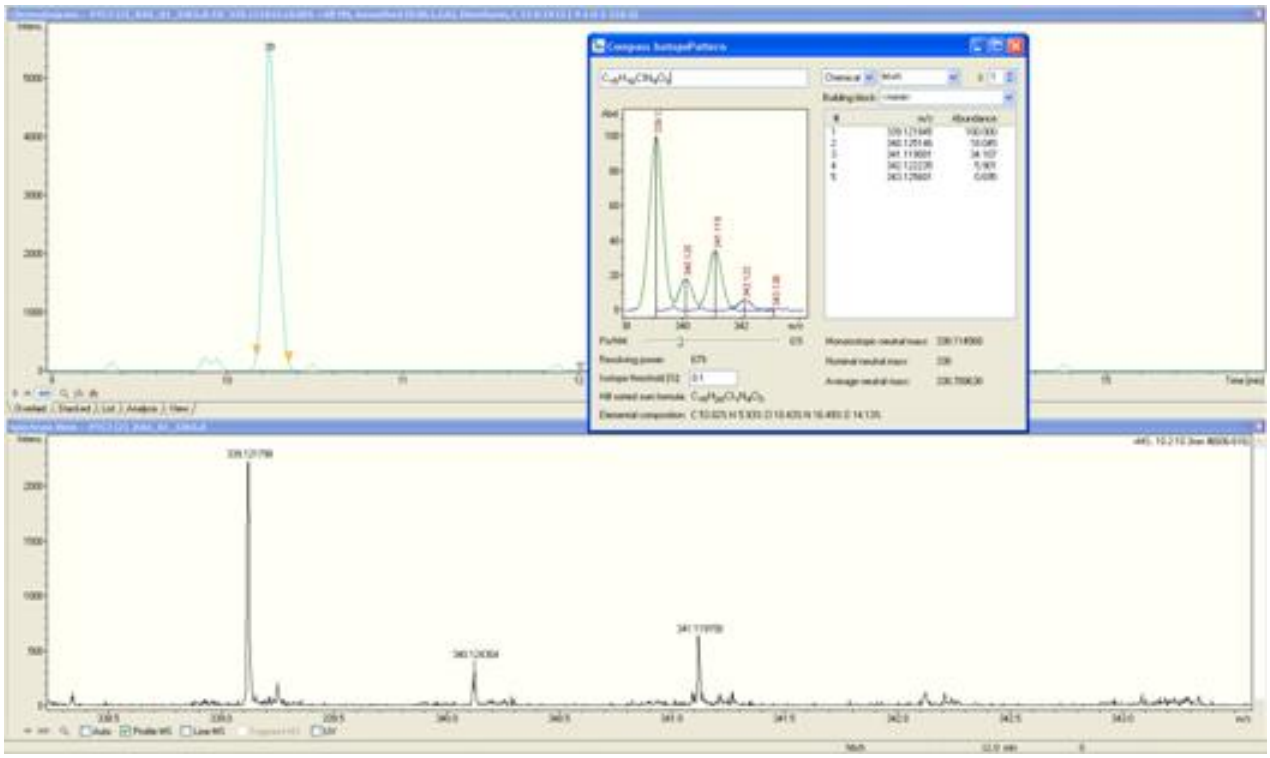


Figure 3: Top: Extracted ion chromatogram of the selected peak
Bottom: Measured isotope pattern from the selected peak
Small box right upper corner: Theoretical isotope pattern (from molecular formula)

For each extracted ion chromatogram a peak can be isolated and the measured isotopic pattern can be investigated, as shown in figure 3. The measured isotopic pattern may be compared/matched to the theoretical isotopic pattern for the given pesticide. The match is measured and if the given criteria are fulfilled, the probability for the correct formula is high.

5. Criteria for the acceptance of validation results

Screening Detection limit, LDL

The screening detection limit (SDL) of a qualitative screening method is the lowest concentration for which it has been demonstrated that a certain analyte can be detected in at least 95% of the samples (i.e. a false negative rate of 5% is accepted)[3].

Criteria for searching the database

For identification the database set up for pesticides was used. The probability of correct identification depend on which criteria's that are selected relating to the database search. Figure 4 is screen shot of the parameters selected for this validation. The mSigma value indicates a high probability of unequivocal identification. If the identification tolerance value is below 50 it indicates a high probability of correct formula. If the values from the screening report are above 50

and 5mDa respectively, the inputs are checked manually. Retention time is required for the attribution of the individual isomers.

Retention time tolerance: 0.5 min

Identification tolerance: 10 mDa.

mSigma threshold < 100

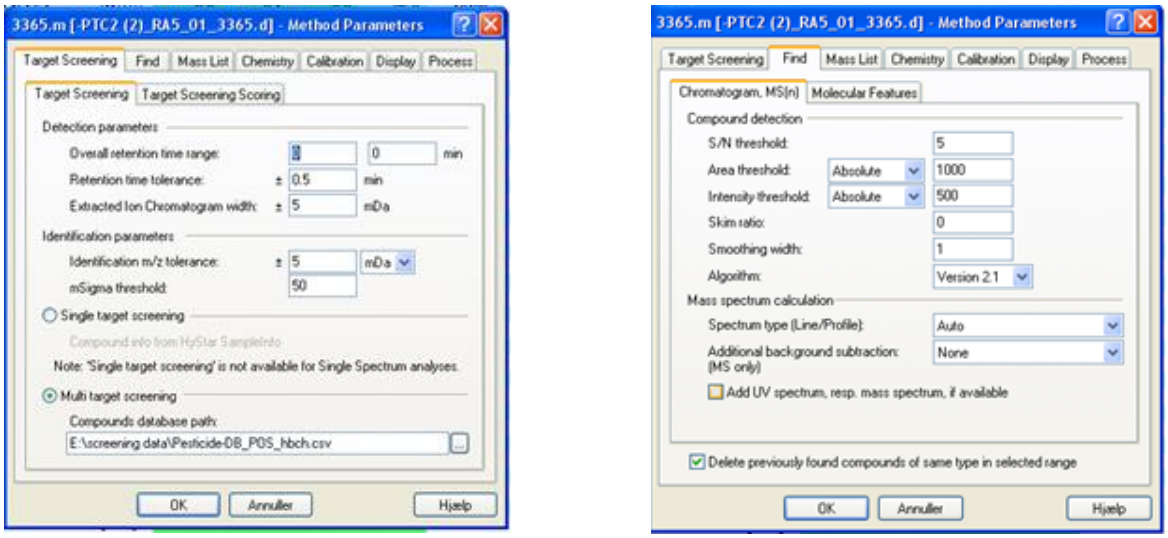


Figure 4: Parameters for the detection with database

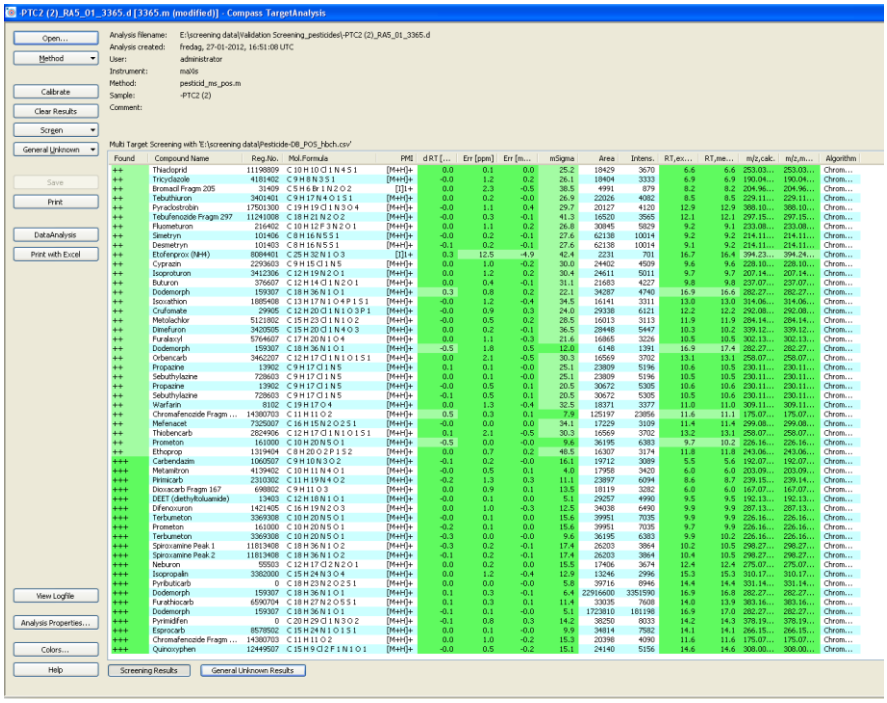


Figure 5: List of detected compunds form the database listed according to the degree of identification

When the software has finish the database searching, a table with identified compounds is shown, see figure 5. The table includes how well the compounds matches the measured accurate mass,

retention time and isotopic pattern compared to the theoretical, and rates the degree of unequivocal identification of the compound.

6. Results and discussion

The 81 pesticides sought validated presented in table 1. Hereof 35 pesticides were accepted for validation at 0.01 mg/kg, here the compounds were detected in at least 95% of the samples, see table 1.

It was tested whether the criteria were set to strict, and if more pesticides would be accepted with less strict criteria. Changing the criteria as intensity and area threshold did not affect the identification, however setting the accurate mass and msigma value less strict allowed for a few additional pesticides to be validated.

Table 1: Compounds sought validated for screening in cereals. The pesticides accepted are marked with pale green.

Compounds	No. detected	% detected
3-hydroxy carbofuran	12	**60%**
Bensulfuron-methyl	17	**85%**
Bromacil	1	**5%**
Butralin	7	**35%**
Buturon	20	**100%**
Carbaryl	5	**25%**
Carbendazim	20	**100%**
Chlorbromuron	17	**85%**
Chlorotoluron	19	**95%**
Chlorsulfuron	7	**35%**
Chromafenozide	1	**5%**
Crufomate	20	**100%**
Cyanazine	1	**5%**
Cyprazin	19	**95%**
DEET (diethyltoluamide)	19	**95%**
Desmetryn	20	**100%**
Difenoxuron	20	**100%**
Diflubenzuron	9	**45%**
Dimefuron	20	**100%**
Dimethachlor	9	**45%**
Dinotefuran	7	**35%**
Dioxacarb	5	**25%**
Esprocarb	18	**90%**
Ethoprop (Ethoprophos)	20	**100%**
Etofenprox	13	**65%**
Fenhexamid	11	**55%**
Fenothiocarb	17	**85%**
Flonicamid	20	**100%**
Flumetsulam	19	**95%**
Fluometuron	20	**100%**
Forchlorfenuron	11	**55%**
Formetanate	1	**5%**
Furalaxyl	19	**95%**
Furathiocarb	20	**100%**
Imazalil	20	**100%**
Imazamox	13	**65%**
Imazapyr	9	**45%**
Imazaquin	17	**85%**
Imidacloprid	9	**45%**

Isopropalin	2	**10%**
Isoproturon	17	**85%**
Isoxathion	19	**95%**
Linuron	17	**85%**
Malaoxon	20	**100%**
Mefenacet	19	**95%**
Mepronil	18	**90%**
Metacriphos	1	**5%**
Metamitron	19	**95%**
Methoprene	11	**55%**
Metolachlor	18	**90%**
Metosulam	3	**15%**
Monuron TCA	19	**95%**
Neburon	19	**95%**
Nitenpyram	13	**65%**
Novaluron	3	**15%**
Orbencarb	12	**60%**
Oxadiargyl	8	**40%**
Oxadiazon	11	**55%**
Oxydemeton-methyl	20	**100%**
Paraoxon-methyl	20	**100%**
Profoxydim	14	**70%**
Prometon	19	**95%**
Propazine	20	**100%**
Pyraclostrobin	17	**85%**
Pyributicarb	20	**100%**
Pyrimidifen	14	**70%**
Quinoxyfen	20	**100%**
Sebuthylazine	20	**100%**
Simetryn	20	**100%**
Spiroxamine	20	**100%**
Tebuthiuron	20	**100%**
Terbumeton	20	**100%**
Thiacloprid	18	**90%**
Thidiazuron	9	**45%**
Thiobencarb	14	**70%**
Thiodicarb	2	**10%**
Thiophanate-methyl	13	**65%**
Tricyclazole	20	**100%**
Trifloxysulfuron	19	**95%**
Triflumizole	3	**15%**
Warfarin	10	**50%**

7. Conclusions

Only 35 compounds were validated at the expected screening detection limit at the criteria set for the database identification. It should be noted that the expected screening detection limit at 0.01 mg/kg may be too low for cereals, and should be 0.02 mg/kg instead. For future validation both levels should be tested. Similar validation was performed on fruit and vegetables, with spiking at SDL (0.01 mg/kg), here many more compounds were validated. This indicates that it should be taken into consideration if the SDL for cereals should be 0.02mg/kg.

For routine analysis non detects should be reported as <SDL mg/kg. If detected, a result can only be reported after confirmatory analysis using a quantitative method.

8. References

1 http://www.quechers.com/

2 The Composition of Foods – fourth edition by Erling Saxholt, Gyldendals, 1996.

3 Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed, Document N° SANCO/12495/2011, European Commission, Brussels, 2012.