Analysis of Acidic Pesticides in Wheat Flour Samples by LC-MS(/MS) using the QuEChERS Method

(incl. optional alkaline hydrolysis to release covalently bound compounds)

The **Wheat Flour** sample is already homogeneous and can be employed as is. **NOTE: This protocol refers to the use of 5g sample for sample preparation.**

Apparatus and Consumables:

- Sample processing equipment, for example Stephan UM 5 universal or Robot-Coupe Blixer
- Automatic pipettes, suitable for handling volumes of 10 to 100 µl, 200 to 1000 µl and 1 to10 ml.
- 50 ml centrifuge tubes with screw caps, for example: a) 50 ml Teflon® centrifuge tubes with screw caps (e.g. Nalgene/Rochester, USA; Oak-ridge, article-no. 3114-0050) or b) disposable 50 ml centrifuge tubes (e.g. Sarstedt/Nümbrecht, Germany, 114x28 mm, PP, article-no. 62.548.004)
- 10 ml solvent-dispenser for acetonitrile
- Centrifuges, suitable for the centrifuge tubes employed in the procedure and capable of achieving at least 3000 rounds per minute (rpm)
- Powder funnel, to fit to the openings of the centrifuge tubes
- Injection vials, 1,5 ml, suitable for GC and LC auto-sampler

Optional:

- Plastic cups (stackable), for example flame photometer cups 25 ml article no. 10-00172 from GML-Alfaplast/Munich, Germany (>1000 pieces) or from b) JURO-LABS/Henfenfeld, Germany (> 100 pieces). These are used for the storage of the buffer-salt mixture portions which are used for each sample.
- Sample divider, to automatically portion salts and sorbents, for example from Retsch/Haan, PT 100 or Fritsch/Idar-Oberstein, Laborette 27 or Bürkle/Lörrach, Repro high-precision sample divider.

Chemicals:

- Acetonitrile, HPLC quality
- Sodium chloride
- Disodium hydrogencitrate sesquihydrate, for example Aldrich No. 359084 or Fluka No. 71635

- Trisodium citrate dehydrate, for example Sigma No. S4641 or Riedel-de Haën No. 32320
- Magnesium sulphate, anhydrous, grit, for example Fluka No. 63135, NOTE: Phthalates can be removed in a muffle furnace by heating to 550 ℃ (e.g. overnight)
- Sodium hydroxide, c = 5 mol/l (5N): 2 g sodium hydroxide are dissolved in some ml of water; and filled to 10 ml.
- Sulfuric acid, c = 2.5 mol/l (5 N), **Carefully** dilute 13.9 mL concentrated H_2SO_4 in 100 mL water
- Water (deionized)
- **ISTD-Solutions for Test samples:** Containing 10µg/mL one or more of the following compounds
  - (2,4,6-Trimethyl-Phenoxy)-acetic acid (e.g. Sigma Aldrich S236055)
  - (4-chloro-3,5-dimethyl-phenoxy)-acetic acid (e.g. Sigma Aldrich S236071)
  - (3-chloro-4-methyl-phenoxy)-acetic acid (e.g. Sigma Aldrich R539236)
  - N,N′-bis(4-Nitrophenyl)urea (Nicarbazin) (e.g. Dr. Ehrenstorfer C15508000), due to limited solubility prepare stock solution at 20 µg/mL in acetonitril, dimethylformamid addition increases solubility
- **ISTD-Solutions for Calibration Standards:** Containing 1µg/mL one or more of the following compounds Prepare a 1:10 dilution of the abovementioned solution.

Note: *In this protocol, the ISTD-mixture is added after the neutralization step. It could be also added before, for example in cases when alkaline hydrolysis step is assisted by a mixer to break up sample participles and allow a better interaction with the matrix. However, in this case Nicarbazin should not be used as ISTD since it may experience losses during alkaline hydrolysis.*

SAMPLE PREPARATION

1) WEIGHING:

Weigh 5 g of the wheat flour sample in the 50 mL centrifuge vial

Note: *The sample amount can be changed. In this case, however, all solvent and salt additions should be also scaled accordingly.*

2) PREPARATION OF SALT-MIXTURES FOR PARTITIONING

A sufficient number of small containers (e.g. stackable plastic vessels) are loaded with

4 g ± 0,2 g magnesium sulphate anhydrous,

1 g ± 0,05 g sodium chloride,

1 g ± 0,05 g trisodium citrate dihydrate and

0,5 g ± 0,03 g disodium citrate sesquihydrate.

Note: *The use of a sample divider, as shown above under Apparatus and Consumables, can considerably facilitate this task. The complete mixture is also commercially available (e.g. from Supelco Cat No.: 55227-U)*

3) WATER-ADDITION:

Add **10 mL of water**

4) ALKALINE HYDROLYSIS STEP (OPTIONAL)

Note: *This step is performed to break-up any covalent bonds between acidic pesticides and matrix-components.*

- Add 300µl of a 5N NaOH solution (this brings pH to a value of ca. 12).

- Tightly close the tube and shake vigorously for 1 min. (by hand or with a powerful mechanic shaker)**.**

- Let the mixture stand for 30 min occasionally shaking it (e.g. every 10 min.)

5) NEUTRALIZATION STEP (OPTIONAL)

Add 300µl of a 5N H_2SO_4 solution

6) Acetonitrile- and ISTD-Addition:

Add **10 ml of acetonitrile** (e.g. using a solvent dispenser) followed by 100µL of the ISTD-solution to the sample

7) FIRST EXTRACTION:

Tightly close the centrifuge vial and **shake vigorously for 1 min. (by hand or with a powerful mechanic shaker)**

8) SALT-ADDITION:

Add the prepared salt-mixture (see 2)).

Note: *When running series of samples, a short shaking of each sample immediately after salt-addition helps to avoid the formation of big salt-conglomerates. Should these still be formed, continue normally with the procedure.*

9) SECOND EXTRACTION (AND PARTITIONING):

Tightly close the centrifuge vial and **shake vigorously for 1 min. (by hand or with a powerful mechanic shaker).**

10) CENTRIFUGATION:

Centrifuge **for 5 min.** (at 3000 g).

11) CLEANUP BY FREEZING (Optional):

7 mL of the Extract are transferred into a PP-Centrifuge vial and placed in a freezer for at least 2 hours (e.g. over night), this procedure removes most of the co-extracted fat as well as other components with limited solubility in acetonitrile.

12) EXTRACT-TRANSFER:

1 mL of each extract is transferred into an HPLC-autosampler-vial to be used for LC-MS/MS

Note: *An equalization of the volume of the test sample extract with that of the calibration solutions may be necessary to equalize matrix induced effects*

PREPARATION OF MATRIX-MATCHED CALIBARTION STANDARDS

13) PREPARATION OF CALIBRATION STANDARDS AND VOLUME ADJUSTMENT FOR THE EXTRACTS OF THE RECOVERY AND SAMPLES

Take a 5 g **blank matrix** portion and **proceed sample preparation (1-11) exactly the same way as described for the test sample**. However, **DO NOT ADD ISTD**. Instead of ISTD add acetonitrile of the same volume (here 100µL).

- Transfer sufficient 1 mL aliquots of the blank extract to HPLC-autosampler vials.
- Add exactly 1/10th of the ISTD portion added to the test samples in each of them (It is advisceable to add the same volume of the 10-fold diluted ISTD solution added to the test samples).
- Add pesticide standard solutions as required to prepare a calibration curve covering the appropriate concentration range (Example: 1µg would correspond to 1 mg/kg in the sample).
- Equalize the total volumes of the test sample extracts and calibration solutions.

Measurement

LC-MS/MS MEASUREMENT CONDITIONS

Any suitable LC and MS/MS conditions may be used. Below you will find some MS/MS parameters that you may use.

Proposed LC-MS/MS conditions:

Column	Zorbax XDB C18, length 150 mm, inner diameter 2,1 mm, particle size 3,5 µm
Mobile phase A2	Acetic acid solution in water, ρ = 0,1 ml glacial acetic acid /l
Mobile phase B2	Acetic acid solution in acetonitrile, ρ = 0,1 ml glacial acetic acid /l
Column temperature	40 ℃
Injection volume	5 µl

Table 1 — Flow rate and elution gradient:

Time min	**Flow rate** µl/min	**Mobile phase A_2** %	**Mobile phase B_2** %
0	300	80	20
20	300	0	100
22	300	0	100
22,1	300	80	20
30	300	80	20

Table 1: List of some acidic compounds and MRM parameters in ESI neg. mode (DP= Declustering Potential [V], and CE=Collision Energy [V], valid for Applied Biosystems API-3000 instrument)

Nr.	Pesticide	1st Transition				2nd Transition			
		Q1	Q3	DP	CE	Q1	Q3	DP	CE
1	2,4,5-T	253	195	-46	-18	253	159	-46	-38
2	2,4-D	219	161	-36	-16	219	125	-36	-36
3	2,4-DB	247	161	-36	-16	247	125	-36	-36
4	4-CPA	185	127	-56	-20	187	129	-51	-18
5	Bentazon	239	132	-56	-38	239	197	-56	-30
6	Bromoxynil	274	79	-41	-42	276	81	-41	-42
7	Dicamba	219	175	-31	-8	221	177	-31	-10
8	Dichlorprop	233	161	-41	-16	233	125	-41	-40
9	Fenoprop	267	195	-36	-16	267	159	-36	-40
10	Fenoxaprop-P	332	260	-41	-18	332	152	-41	-30
11	Fluroxypyr	253	195	-26	-18	253	233	-26	-10
12	Imazethapyr	288	244	-46	-20	288	201	-46	-32
13	Ioxynil	370	127	-46	-46	370	215	-46	-42
14	MCPA	199	141	-21	-20	201	143	-21	-20
15	MCPB	227	141	-26	-10	229	143	-26	-10
16	Mecoprop	213	141	-46	-22	213	105	-46	-44
17	Naphthyloxyacetic acid	201	143	-61	-20	201	115	-61	-54
18	Picloram	239	195	-31	-16	241	197	-31	-16
19	Triclopyr	254	196	-56	-14	256	198	-56	-16
ISTD	(4-chloro-3,5-dimethyl-phenoxy)-acetic acid	213	155	-45	-20	213	169	-45	-14
ISTD	(2,4,6-Trimethyl-Phenoxy)-acetic acid	193	135	-55	-22	193	149	-55	-14
ISTD	(3-chloro-4-methyl-phenoxy)-acetic acid	199	141	-45	-18	199	155	-45	-14
ISTD	N,N′-bis(4-Nitrophenyl)urea (Nicarbazin)	301	137	-31	-20	301	137	-31	-20

CALCULATION

Calibration: Determine the calibration functions for each active substance by **plotting the peak ratio** $PR^{cal\ mix}$ ($A_{pest}^{cal\ mix}$ / $A_{ISTD}^{cal\ mix}$) of each calibration level **against the mass of active substance in the standard solution** $m_{pest}^{cal\ mix}$ (C_{pest} x $V_{pest}^{cal\ mix}$) . The corresponding calibration graph is

$$PR^{cal\ mix} = a_{cal} \times m_{pest}^{cal\ mix} + b_{cal} \quad (1)$$

The mass fraction w_R of the pesticide in the sample is calculated using the peak ratio of pesticide and internal standard PR^{sample} (A_{pest}^{sample} / A_{ISTD}^{sample}) obtained from final extract as

$$w_R = \frac{(PR^{sample} - b_{cal})}{a_{cal}} \times \frac{1}{m_a} \times \frac{m_{ISTD}^{sample}}{m_{ISTD}^{cal\ mix}} \left(\frac{\text{mg}}{\text{kg}}\right) \quad (2)$$

Variables used:

Mass of pesticide in calibration mixture	$m_{pest}^{cal\ mix}$	µg
Mass of internal standard in calibration mixture	$m_{ISTD}^{cal\ mix}$	µg
Mass of internal standard added to test portion	m_{ISTD}^{sample}	µg
Concentration of pesticide in pesticide working solution	$C_{pes\ t}$	µg/ml
Volume of pesticide working solution used for preparation of calibration mixture	$V_{pest}^{cal\ mix}$	ml
Mass of test portion	m_a	g
Mass fraction of pesticide in the sample	w_R	mg/kg
Peak area of pesticide obtained from calibration mixture	$A_{pest}^{cal\ mix}$	(counts)
Peak area of ISTD obtained from calibration mixture	$A_{ISTD}^{cal\ mix}$	(counts)
Peak area of pesticide obtained from the final extract	A_{pest}^{sample}	(counts)
Peak area of ISTD obtained from the final extract	A_{ISTD}^{sample}	(counts)
Peak ratio obtained from calibration mixture	PR cal mix (dimensionless)	
Peak ratio obtained from final extract	PR sample(dimensionless)	
Slope of calibration graph using the simplified approach	a_{cal}	1/µg
Bias of calibration graph	b_{cal} (dimensionless)	

For any questions please contact: Michelangelo.Anastassiades@cvuas.bwl.de