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 to 10 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 °C (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\(_2\)SO\(_4\) 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, dimethylformamide 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 particules 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 H₂SO₄ 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 CALIBRATION 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 advisable 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, \( \rho = 0,1 \text{ ml glacial acetic acid /l} \)

- **Mobile phase B2**
  Acetic acid solution in acetonitrile, \( \rho = 0,1 \text{ ml glacial acetic acid /l} \)

- **Column temperature**
  40 °C

- **Injection volume**
  5 µl

**Table 1 — Flow rate and elution gradient:**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (µl/min)</th>
<th>Mobile phase A₂ (%)</th>
<th>Mobile phase B₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>300</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>22</td>
<td>300</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>22,1</td>
<td>300</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>30</td>
<td>300</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>
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)

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Pesticide</th>
<th>1st Transition</th>
<th>2nd Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Q1</td>
<td>Q3</td>
</tr>
<tr>
<td>1</td>
<td>2,4,5-T</td>
<td>253</td>
<td>195</td>
</tr>
<tr>
<td>2</td>
<td>2,4-D</td>
<td>219</td>
<td>161</td>
</tr>
<tr>
<td>3</td>
<td>2,4-DB</td>
<td>247</td>
<td>161</td>
</tr>
<tr>
<td>4</td>
<td>4-CPA</td>
<td>185</td>
<td>127</td>
</tr>
<tr>
<td>5</td>
<td>Bentazon</td>
<td>239</td>
<td>132</td>
</tr>
<tr>
<td>6</td>
<td>Bromoxynil</td>
<td>274</td>
<td>79</td>
</tr>
<tr>
<td>7</td>
<td>Dicamba</td>
<td>219</td>
<td>175</td>
</tr>
<tr>
<td>8</td>
<td>Dichlorprop</td>
<td>233</td>
<td>161</td>
</tr>
<tr>
<td>9</td>
<td>Fenoprop</td>
<td>267</td>
<td>195</td>
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<tr>
<td>10</td>
<td>Fenoxaprop-P</td>
<td>332</td>
<td>260</td>
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<tr>
<td>11</td>
<td>Fluroxypyr</td>
<td>253</td>
<td>195</td>
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<tr>
<td>12</td>
<td>Imazethapyr</td>
<td>288</td>
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<tr>
<td>13</td>
<td>Ioxynil</td>
<td>370</td>
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<td>14</td>
<td>MCPA</td>
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<td>141</td>
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<td>15</td>
<td>MCPB</td>
<td>227</td>
<td>141</td>
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<tr>
<td>16</td>
<td>Mecoprop</td>
<td>213</td>
<td>141</td>
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<tr>
<td>17</td>
<td>Naphthyloxyacetic acid</td>
<td>201</td>
<td>143</td>
</tr>
<tr>
<td>18</td>
<td>Picloram</td>
<td>239</td>
<td>195</td>
</tr>
<tr>
<td>19</td>
<td>Triclopyr</td>
<td>254</td>
<td>196</td>
</tr>
</tbody>
</table>

|    | 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 $PR_{\text{cal mix }} = A_{\text{cal mix }}^{\text{pest}} / A_{\text{STD}}^{\text{cal mix }}$ of each calibration level against the mass of active substance in the standard solution $m_{\text{cal mix }}^{\text{pest}} (C_{\text{pest}} x V_{\text{cal mix }}^{\text{pest}})$. The corresponding calibration graph is

$$PR_{\text{cal mix }} = a_{\text{cal}} x m_{\text{pest}}^{\text{cal mix}} + b_{\text{cal}}$$

(1)

The mass fraction $w_R$ of the pesticide in the sample is calculated using the peak ratio of pesticide and internal standard $PR_{\text{sample }} = A_{\text{pest}}^{\text{sample}} / A_{\text{STD}}^{\text{sample }}$ obtained from final extract as

$$w_R = \frac{PR_{\text{sample }} - b_{\text{cal}}}{a_{\text{cal}}} \times \frac{1}{m_a} \times \frac{m_{\text{STD}}^{\text{sample}}}{m_{\text{cal mix }}^{\text{pest}}} (\frac{mg}{kg})$$

(2)

**Variables used:**

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

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