# Analysis of Phenoxyalkanoic Acids in Milk using QuEChERS method and LC-MS/MS

**Version 2 (last update: 05.05.14)**

## Short description

A method is presented for the analysis of acidic pesticides in milk. The pesticides are extracted using the QuEChERS method and analysed by LC-MS/MS following cleanup by freeze-out. The method does not involve a hydrolysis step and thus only covers the free acids. A variant which includes the conjugates will follow in a future version.

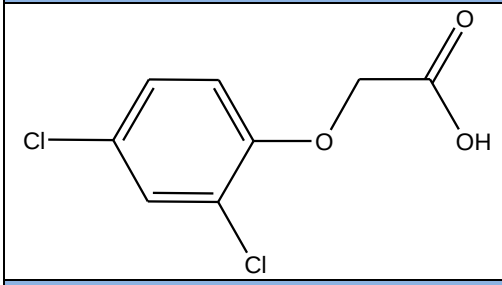
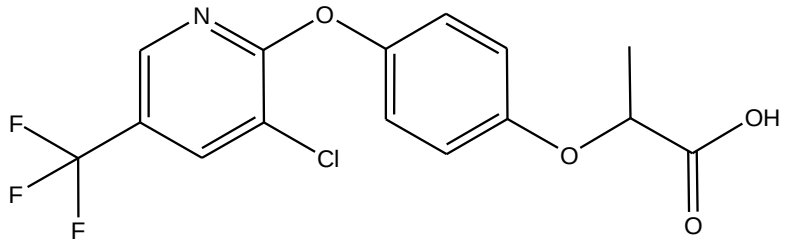
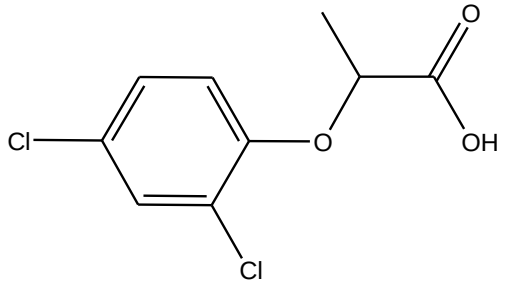
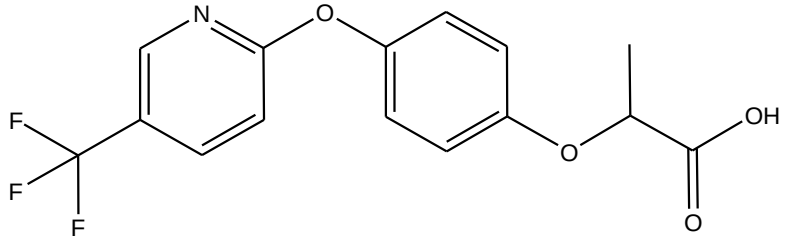
## Compound details

The group of phenoxy herbicides is subdivided into:

- Phenoxyacetic acid herbicides (e.g. 4-CPA, 2,4-D, MCPA, 2,4,5-T)
- Phenoxybutyric acid herbicides (e.g. 2,4-DB, MCPB)
- Phenoxypropionic acid herbicides (e.g. dichlorprop, fenoprop, mecoprop)
- Aryloxyphenoxypropionic acid herbicides (e.g. diclofop, fluazifop, haloxyfop, quizalofop)

altogether these are 49 different compounds [4].

As representatives of the group the following four herbicides were validated for milk.

| 2,4-D | Haloxyfop |
|---|---|
|  |  |
| **Dichlorprop** | **Fluazifop** |
|  |  |

**Residue definitions (commodity group AO, as of March 2014):**

- 2,4-D (sum of 2,4-D and its esters expressed as 2,4-D)
- Dichlorprop: sum of dichlorprop (including dichlorprop-P) and its conjugates, expressed as dichlorprop
- Fluazifop-P-butyl (fluazifop acid (free and conjugate))
- Haloxyfop including Haloxyfop-R: Haloxyfop-R and conjugates of haloxyfop-R expressed as haloxyfop-R

**Sources of Supply (exemplary):**
Analytical standards of phenoxy herbicides can be purchased from most producers of analytical standards.

**Apparatus and Consumables:**
Use materials described in the QuEChERS standard procedure (EN15662). As a mechanical shaker you can use a horizontally or vertically reciprocating shaker or a rotatory shaker (e.g. HS260 by IKA or GenoGrinder by Spex or SSL1 Labscale Orbital Shaker by Stuart). To filter the extract use e.g. polyester disposable syringe filters of 0.45 µm pore size.

**Extraction and Cleanup Procedure:**
*Extraction*: Weigh 10 g of milk, add 10 mL acetonitrile and internal standard (e.g. 100µL of an appropriately concentrated solution) and shake 15 min using a mechanical shaker. Add QuEChERS citrate-buffer mix, shake 1 min and centrifuge --> Raw Extract.
*Cleanup*: Transfer e.g. 6-8 mL raw extract into a vial and place it in a freezer for at least 2 h. Take the vial out of the freezer and either a) quickly pass 1mL through a syringe filter into an LC-MS/MS vial, paying attention to avoid heating up the mixture as this would progressively re-dissolve lipids; OR b) quickly decant 1 mL into an LC-MS/MS vial paying attention to leave precipitates behind and to avoid heating up; OR c) quickly pass some milliliters through a cotton-wool-filled funnel into a vessel and transfer 1mL filtrate into an LC-MS/MS vial.

For a more intensive cleanup (mainly removal of additional fat), optionally, transfer 3 mL of the extract that was cleaned-up via freeze-out into a 15 mL centrifuge and dilute with 3 mL water (1:1 dilution). Shake shortly to mix and filter 1 mL through as syringe filter into an LC-MS/MS vial.

***Alternative cleanup step**: take 3 mL of the raw extract, add 3 mL of water and mix with 150 mg C18-sorbent (no PSA, no MgSO4), shake, centrifuge and filter. The absolute amount of IS to be added to 1 mL calibration solution should optimally be 20 times lower than the absolute amount added to the samples at the beginning of the procedure.*

**Preparation of calibration standards:**
Matrix matched calibration standards are prepared using an extract of blank milk (not containing any of the pesticides of interest). The blank extract is produced as described above however without addition of an IS. 1mL final extract will represent approximately 1 g matrix.
*If a 1:1 dilution step is conducted (see above) 1mL final extract will represent approximately 0,5 g matrix. The absolute amount of IS to be added to 1 mL calibration solution should optimally be 20 times lower than the absolute amount added to the samples at the beginning of the procedure.*

**Measurement:**
Inject the extracts into LC-MS/MS instrument.

## Exemplary Instrumentation details for phenoxy alkanoic acids:

**Tab. 1: MRM Details negative mode:**

| Instrument | Waters Acquity, ABSciex API 4000 QTrap |
|---|---|
| **Ionisation mode** | ESI neg |
| **Column** | Acquity UPLC BEH Shield RP 18, 1.7 µm; 2.1 x 100 mm |
| **Pre-column** | Van Guard BEH Shield RP 18, 1.7 um |
| **Eluent A** | 0.01 % acetic acid in water (with 5% acetonitrile) |
| **Eluent B** | 0.01 % acetic acid in acetonitrile |
| **Gradient** | time / flow [µL min] / A% / B%<br>0 / 400 / 80 / 20<br>4 / 400 / 70 / 30<br>7 / 00 / 10 / 90<br>8.5 / 400 / 10 / 90<br>8.6 / 400 / 80 / 20<br>13.5 / 400 / 80 / 20 |
| **Internal Standard** | Nicarbazin |

**Tab. 2: MRM Details negative mode:**

| Transition Name | Rel. Sensitivity | Parent Mass | Daughter Mass | DP | CE | CXP | Mode |
|---|---|---|---|---|---|---|---|
| **2.4-D 219/161** | 1 | 219 | 161 | -50 | -18 | -9 | ESI neg. |
| **2.4-D 221/163** | 2 | 221 | 163 | -50 | -18 | -9 | ESI neg. |
| **2.4-D 219/125** | 3 | 219 | 125 | -50 | -38 | -7 | ESI neg. |
| **Dichlorprop 233/161** | 1 | 233 | 161 | -50 | -18 | -9 | ESI neg. |
| **Dichlorprop 235/163** | 2 | 235 | 163 | -50 | -18 | -7 | ESI neg. |
| **Dichlorprop 233/125** | 3 | 233 | 125 | -50 | -38 | -5 | ESI neg. |
| **Fluazifop 326/226** | 1 | 326 | 226 | -65 | -38 | -11 | ESI neg. |
| **Fluazifop 326/108** | 2 | 326 | 108 | -65 | -56 | -5 | ESI neg. |
| **Fluazifop 326/254** | 3 | 326 | 254 | -65 | -22 | -5 | ESI neg. |
| **Haloxyfop 360/288** | 1 | 360 | 288 | -70 | -20 | -15 | ESI neg. |
| **Haloxyfop 362/290** | 2 | 362 | 290 | -75 | -20 | -15 | ESI neg. |
| **Haloxyfop 360/196** | 3 | 360 | 196 | -70 | -52 | -9 | ESI neg. |

**Tab. 3: MRM Details of Fluazifop and Haloxyfop in the ESI positive mode:**

| Transition Name | Rel. Sensitivity | Parent Mass | Daughter Mass | DP | CE | CXP | Mode |
|---|---|---|---|---|---|---|---|
| **Fluazifop 328/282** | 1 | 328 | 282 | 76 | 27 | 14 | ESI pos. |
| **Fluazifop 328/254** | 2 | 328 | 254 | 76 | 37 | 14 | ESI pos. |
| **Fluazifop 328/91** | 3 | 328 | 91 | 76 | 43 | 6 | ESI pos. |
| **Haloxyfop 362/316** | 1 | 362 | 316 | 81 | 25 | 18 | ESI pos. |
| **Haloxyfop 362/91** | 2 | 362 | 91 | 81 | 47 | 6 | ESI pos. |
| **Haloxyfop 364/318** | 3 | 364 | 318 | 71 | 27 | 6 | ESI pos. |

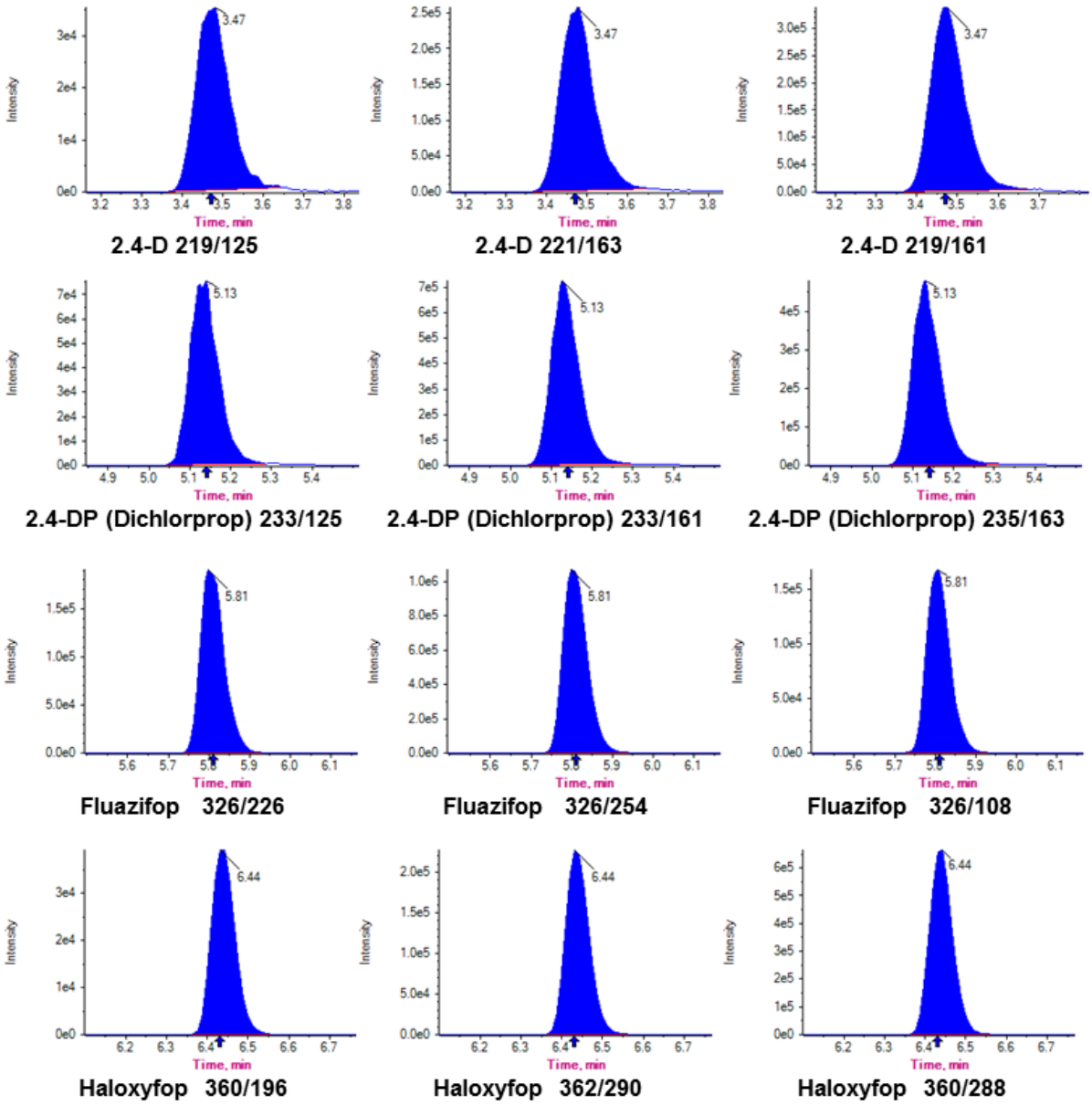



Fig 1: Chromatograms of 2,4-D, Dichlorprop, Fluazifop, Haloxyfop 0.1 µg/mL in Acetonitrile.

## Validation data:

**Tab. 4: Recovery experiments for acidic pesticides from Whole Milk, cleanup with ODS (n=5)**

| Spiking Level | 2.4-D | Dichlorprop | Fluazifop | Haloxyfop |
|---|---|---|---|---|
| | Recovery rates (RSDs) | | | |
| 0.1 mg/kg | 100.3% (2.5%) | 101.5% (2.0%) | 104.4 (3.4%) | 100.5% (4.9%) |
| 0.02 mg/kg | 98.0% (5.2%) | 99.4% (5.7%) | 102.2% (4.6%) | 105.8% (10.7%) |

## Validation data with modified methods:

**Tab. 5: Recovery experiments for acidic pesticides from Whole Milk (n=3), Cleanup: freeze-out -> filtration ->dilution with water (1:1)**

| Spiking Level | 2.4-D | Fluazifop | Haloxyfop |
|---|---|---|---|
| | Recovery rates (RSDs) | | |
| 0.05 mg/kg | 108% (2.1%) | 108 (1.4%) | 111% (3.8%) |

**Tab. 6: Recovery experiments for acidic pesticides from Whole Milk (n=5), Cleanup: dilution with water (1:1) + ODS 25mg/mL raw extract**

| Spiking Level | 2.4-D | Fluazifop | Haloxyfop |
|---|---|---|---|
| | Recovery rates (RSDs) | | |
| 0.025 mg/kg | 101% (1.5%) | 101% (2.6%) | 97% (1.0%) |

## Impact of cleanup with PSA:

**Tab. 7: Recovery experiments for acidic pesticides from QuEChERS raw extracts of Whole Milk applying different cleanup steps**

| Compound | Mean Recovery of cleanup step in % (spiking on raw extract of whole milk at level corresp. to 0.1 mg/kg) | |
|---|---|---|
| | Cleanup: d-SPE with… | |
| | ODS 25 mg/mL (n=2) | ODS/PSA 25 mg/mL each (n=2) |
| 2.4-D | 100 | 47 |
| 2.4-DP (Dichlorprop) | 101 | 51 |
| Fluazifop | 104 | 50 |
| Haloxyfop | 100 | 55 |

**NOTE: Phenoxyalkanoic acids show reduced recoveries when PSA sorbent is used in dSPE cleanup step**!!

## Impact of cleanup on dry residue of extracts

| Cleanup approach | Dry residue % | Notes |
|---|---|---|
| None (Raw milk extract) | 100% | 3,3 mg dry residue/mL (=0,033%), compared to ca. 12% dry residue in whole milk |
| dSPE w. C18 (25 mg/mL) | 64% | w. additional PSA/$MgSO_4$ (25/150 mg/mL) 53% |
| Freeze-out and decanting though cotton | 44% | w. additional PSA/$MgSO_4$ (25/150 mg/mL) 35% |
| 1:1 dilution w. water -> filtration | 35% | Calculated on basis of non-diluted extract (x2) |
| 1:1 dilution w. water + simultaneous dSPE w. C18 (25 mg/mL) -> filtration | 23% | Calculated on basis of non-diluted extract (x2). **Substantial recovery losses of QACs observed!!** |
| Freeze-out -> filtration ->dilution with water (1:1) -> filtration | 10% | Calculated on basis of non-diluted extract (x2) |

Recoveries can also be found at **www.eurl-pesticides-datapool.eu**

## References

[3] The ePesticide Manual, ISBN 1-901396-31-2, The British Crop Protection Council
[4] http://www.alanwood.net/pesticides/class_herbicides.html