Analysis of Chlormequat and Mepiquat Residues in Foods of Plant Origin
(version 2; Jan 2009)

1. Aim and Scope

This manuscript describes a method for the analysis of the growth regulators residues chlormequat (CCC) and mepiquat in dry and water-containing foods of plant origin.

2. Short Description

Residues of CCC and mepiquat are extracted from the homogeneous and representative sample with methanol/water after addition of isotope labeled standards. The mixture is then centrifuged, filtered and analyzed by LC-MS/MS. The quantity of CCC and mepiquat in the sample is calculated as the amount of chlormequat cations and mepiquat cations, respectively. The amount is expressed in mg/kg foodstuff.

3. Apparatus and Consumables

Sample processing equipment, e.g. Mincer Stephan UM 5 universal
High performance dispenser, e.g. Ultra-Turrax
Beakers, e.g. 100 mL, tall shape
Test tubes, e.g. 10 mL, preferably thick-walled for centrifuging
Centrifuge, e.g. Labofuge A Heraeus
Syringes, e.g. 2 or 5 mL disposable syringes
Syringe filters, 0.45 µm pore size, e.g. MN-Chromafil
Glass vials, 1.8 mL volume
Automatic pipettes
LC-MS/MS: e.g. Agilent 1100 series with API 3000 detector from Applied Biosystems
HPLC column: MonoChrom MS 100x2mm; 5µ (Part No. 2080-100x020)
4. Chemicals

Unless otherwise stated,
- chemicals of recognized analytical grade must be used,
- water must be of appropriate purity,
- a “solution” is understood to be aqueous.

4.1. Chlormequat chloride stock solution

in methanol, \( c (C_5H_{13}NCl_2) = 1289 \, \mu g/mL \) (e.g. 64.5 mg/50 mL)
Note: the stock solution is equivalent to 1000 \( \mu g/mL \) chlormequat cation.

4.1.1. Chlormequat chloride dilution I

in methanol, stock solution diluted by a factor of 100
Note: this dilution contains 10 \( \mu g/mL \) chlormequat cation.

4.1.2. Chlormequat chloride dilution II

in methanol, stock solution diluted by a factor of 1000
Note: this dilution contains 1 \( \mu g/mL \) chlormequat cation.

4.1.3. Chlormequat chloride dilution III

in methanol, stock solution diluted by a factor of 10000
Note: this dilution contains 0.1 \( \mu g/mL \) chlormequat cation.

4.2. Mepiquat chloride stock solution

in methanol, \( c (C_7H_{16}NCl) = 1310 \, \mu g/mL \) (e.g. 65.5 mg/50 mL)
Note: the stock solution contains 1000 \( \mu g/mL \) mepiquat cation.

4.2.1. Mepiquat chloride dilution I

in methanol, stock solution diluted by a factor of 100
Note: this dilution contains 10 \( \mu g/mL \) mepiquat cation.
4.2.2. Mepiquat chloride dilution II
in methanol, stock solution diluted by a factor of 1000
Note: this dilution contains 1 µg/mL mepiquat cation.

4.2.3. Mepiquat chloride dilution III
in methanol, stock solution diluted by a factor of 10000
Note: this dilution contains 0.1 µg/mL mepiquat cation.

4.3. d4-Chlormequat chloride internal standard (ISTD) stock solution
in methanol, c (C5H9D4NCl2) = 100 µg/mL

4.3.1. To be added to the samples prior to extraction:
d4-Chlormequat chloride ISTD dilution I
in methanol, c (C5H9D4NCl2) = 10 µg/mL
Note: this dilution contains 7.81 µg/mL d4-chlormequat cation.

4.3.2. For the preparation of calibration standards:
d4-Chlormequat chloride ISTD dilution II
in methanol, c (C5H9D4NCl2) = 1 µg/mL
Note: this dilution contains 0.781 µg/mL d4-chlormequat cation.

4.4. d3-Mepiquat iodide internal standard (ISTD) stock solution
in methanol, c (C7H13D3NI) = 100 µg/mL

4.4.1. To be added to the samples prior to extraction:
d3-Mepiquat iodide ISTD dilution I
in methanol, c (C7H13D3NI) = 10 µg/mL
Note: this dilution contains 4.80 µg/mL d3-mepiquat cation.

4.4.2. For the preparation of calibration standards:
d3-Mepiquat iodide ISTD dilution II
in methanol, c (C7H13D3NI) = 1 µg/mL
Note: this dilution contains 0.480 µg/mL d3-mepiquat-cation.
4.5. Methanol
(CH₃OH) for HPLC

4.6. Acetonitrile
(CH₃CN) for HPLC

4.7. Ammonium acetate
(CH₃COONH₄), p.a.

4.8. Acetic acid (glacial acetic acid)
(CH₃COOH) minimum 96 g/100g
4.9. Calibration standards

**Dissolved in methanol/ water 1+1 (V/V).**

Prepare 6 calibration standards into a 10 mL volumetric flasks as specified in Table 1. and fill it to the calibration mark with methanol/water (1/1, v/v). Additionally a blank solution should be prepared containing the internal standard at the same concentration as in the calibration standards.

**Table 1:** Pipetting scheme for calibration standards (exemplarily)

<table>
<thead>
<tr>
<th>No.</th>
<th>Concentration of Chlormequat /Mepiquat cation in calibration solution</th>
<th>Chlormequat cation</th>
<th>Mepiquat cation</th>
<th>d₄-chlormequat cation-</th>
<th>d₃-mepiquat cation</th>
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</thead>
<tbody>
<tr>
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<td>4.1.3/</td>
<td>4.1.2/</td>
<td>4.2.3/</td>
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<tr>
<td>0.1</td>
<td>µg/mL</td>
<td>1 µg/mL</td>
<td>1 µg/mL</td>
<td>1 µg/mL</td>
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</tbody>
</table>

Volumes to be added to each 10 mL vial (7 vials in total)

<table>
<thead>
<tr>
<th></th>
<th>Volumes to be added to each 10 mL vial (7 vials in total)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µL 100 µL 167 µL 167 µL</td>
</tr>
<tr>
<td>2</td>
<td>500 µL 500 µL 167 µL 167 µL</td>
</tr>
<tr>
<td>3</td>
<td>100 µL 100 µL 167 µL 167 µL</td>
</tr>
<tr>
<td>4</td>
<td>250 µL 250 µL 167 µL 167 µL</td>
</tr>
<tr>
<td>5</td>
<td>500 µL 500 µL 167 µL 167 µL</td>
</tr>
<tr>
<td>6</td>
<td>1000 µL 1000 µL 167 µL 167 µL</td>
</tr>
<tr>
<td>7</td>
<td>blank 167 µL 167 µL</td>
</tr>
</tbody>
</table>

* see corresponding sections in the text

**Note:** Following the instructions of this method, the content of d₄-chlormequat cation is ca. 13 ng/mL in all standard working solutions and samples and that of d₃-mepiquat cations ca. 8 ng/mL.
5. Procedure

5.1. Sample preparation

To obtain representative analytical portions from the laboratory sample, proceed as required by the respective regulations or guidelines. (For Germany follow the 5th recommendation of the GDCh working group for pesticides [Lebensmittelchemie 49 (1995) 40-42] or the monitoring handbook). Cryogenic milling is generally recommended for fruits and vegetables. In case of big units, cut them into small pieces with a knife (approx. 3 x 3 cm) and freeze them. Add some dry ice and comminute in frozen state with an industrial strength grinder, so that a powdery consistency is achieved.

5.2. Extraction

Weigh 20 g of the comminuted sample into an appropriate beaker (e.g. 150 mL).

**Note:** When analyzing dry samples such as grains, dry milk or flour, the sample amount should be reduced to 10 g followed by the addition of ISTD and water as described below.

Add 100 µL ε4-chlormequat chloride internal standard solution (4.3.1; ω = 10 µg/mL) and 100 µL ε3-mepiquat iodide internal standard solution (4.4.1; ω = 10 µg/mL), and enough water so that the sum of added and inherent water is ca. 20 mL. Add 40 mL methanol and homogenize with a disperser for 2 min.

Afterwards, centrifuge an aliquot of the dispersion (e.g. with 3500 g). Transfer about 2 mL of the supernatant into a syringe and filter the extract into a 1.8 mL-vial.

**Note:** When analyzing dry samples wait 10 min for the water to soak before adding methanol.

**Note:** The resulting volume of the supernatant (including the natural water content of the sample) should amount to ca. 60 mL.

5.3. HPLC measurement

Inject, in succession, the same volumes of the prepared extracts and the calibration standards (see 4.10) into the LC-MS/MS system. Run the system in the MRM-mode using the following mass transitions:
Chlormequat: m/z = 122/58 and 124/58
ISTD d₄-Chlormequat: m/z = 126/58

Mepiquat: m/z = 114/58 and 114/98
ISTD d₃-Mepiquat: m/z = 117/101

5.4. LC-MS-MS-System (exemplary conditions)
Degasser: Agilent 1100-series, G 1322 A
HPLC pump: Agilent 1100-series, BinPump G 1312 A
Autosampler: Agilent 1100-series, ALS G 1313 A
Column oven: Agilent 1100-series, ColComp G 1316 A
Column: MonoChrom MS 100x2mm; 5µ (Varian) + corresponding guard column
Column temperature: 40°C
Eluent A: 5 mmol/L NH₄ acetate + 0,1 % acetic acid
Eluent B: acetonitrile
Gradient: 5 % A in 2 min to 95 % A
Flow: 0,4 mL/min
Injection volume: 5 µL
Detector: API 3000 (Applied Biosystems)
Ion source: TurbolonSpray
Ionization mode: ESI positive
Ionspray voltage: 1500 V
Source temperature: 450 °C

6. Evaluation of results

6.1. Identification and quantification
The cationic form of chlormequat and mepiquat is to be used to express the residue content in the sample.
For identification, the relative intensities of the measured transitions of the relevant peaks, detected within the relevant retention time ranges, are compared with those
of chlormequat and mepiquat obtained from the calibration standard as well as with those of the respective ISTD obtained in the sample. In addition the peak shapes of the analytes and the respective ISTDs are compared.

The quantification of chlormequat and mepiquat is performed using the respective ISTDs. As the isotopically labeled ISTDs are added at the beginning of the procedure they do not only correct for matrix-induced signal shifts (matrix effects) but also for losses and volume differences at any stage of the procedure.

Note: the isotopically labeled ISTDs may contain small amounts of the non-labelled analytes. These amounts become more and more significant the lower the detected concentrations of pesticides are and the higher the amount of added ISTD is. This fact has to be considered when dealing with very low findings.

Example using 1-point calibration and assuming linear response:
For 20 g sample portion and approx. 60 ml total extraction volume 1 mL of the extract contains the equivalent of approx. 1/60th of the ISTD-portion added to the sample in (5.2). Aiming to have approximately the same concentration of ISTD in sample extract and calibration solution(s) the calibration standard is prepared (see 4.9) to contain exactly 1/60th of the ISTD portion added to the sample in (5.2) per mL.

Assumptions:
1) Peak area of ISTD obtained from the final extract: 100,000
Peak area of pesticide obtained from the final extract: 10,000
Peak ratio pesticide/ISTD for the final extract (PR_{sample}) = 0.1

2) Peak area of ISTD obtained from the calibration standard: 100,000
Peak area of pesticide obtained from the calibration standard: 20,000
Peak ratio pesticide/ISTD for the calibration solution (PR_{calib.}) = 0.2
Amount of pesticide contained in calibration solution: exactly 1 mg/mL

Knowing that the amount of ISTD added to the sample in 5.2 is 60 times higher than the amount of ISTD added to the calibration solution, PR_{sample} can be corrected by a factor of 60, i.e. PR_{sample-corr.} = 6.0.
**Calibration standard:** 1 µg of pesticide and 1 ISTD-portion result in a peak ratio of 0.2

**Sample extract:** x µg of pesticide and 1 ISTD-portion result in a peak ratio of 6.0.

Result: 20g sample contain $1\mu g \times 6.0 / 0.2 = 30 \mu g$ pesticide. This corresponds to 1.5 mg/kg sample.

Reliability of the Method:
Spiking experiments for chlormequat on tomato, strawberry, grapes and pears at levels between 0.1 and 1 mg/kg resulted in recoveries between 63.9 and 93.4 %, having a mean value of 87.7±8.6% (mean value±standard deviation).
Spiking experiments for mepiquat on tomato, strawberry and grapes at levels between 0.1 and 1 mg/kg resulted in recoveries between 80.4 and 102.0 %, having a mean value of 90.4±5.8% (mean value±standard deviation).

These recoveries were determined using matrix-matched calibrations, assuming exactly 60 mL extract volume and without correction of recovery by the ISTD.

### 7. References

Martin Vahl, Anette Graven, René K.Juhler
Analysis of Chlormequat residues un grain using liquid chromatography-mass spectrometry (LC-MS/MS); Fresenius J Anal Chem (1998) 361:817-820

German Official Procedure according § 35 LMBG L 00.00 76
Bestimmung von Chlormequat und Mepiquat in Lebensmitteln mittels HPLC-MS/MS December 2002

CEN EN 15054 Non fatty foods - Determination of chlormequat and mepiquat - LC-MS method

Lutz Alder, James R. Startin
Determination of Chlormequat and Mepiquat in Foods by Liquid Chromatography/Mass Spectrometry or Liquid Chromatography/Tandem Mass Spectrometry: Interlaboratory Study; Journal of AOAC International vol. 88, no. 6, 2005, 1762-1776

J Hau, S Riediker, N Varga, R H Stadler Determination of the plant growth regulator chlormequat in food by liquid chromatography-electrospray ionisation tandem mass spectrometry; J Chromatogr A. 2000 May 5;878 (1):77-86
**Table 10: Document History:**

<table>
<thead>
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<th>Action</th>
<th>When?</th>
<th>Changes introduced</th>
<th>Version</th>
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<td>V 1</td>
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<td>Document revision</td>
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<td>Section 4.9: <em>Calibration Standards</em>: major text revisions</td>
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<td>Section 7: <em>Evaluation of Results</em>: major text revision</td>
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<td>Section 8: <em>Reference List</em>: extended</td>
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<td>All other sections: correction of syntax, typos, minor text revisions</td>
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