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 µg/mL (e.g. 64.5 mg/50 mL)
Note: the stock solution is equivalent to 1000 µg/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 µg/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 µg/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 µg/mL chlormequat cation.

4.2. Mepiquat chloride stock solution

in methanol, c (C7H16NCl) = 1310 µg/mL (e.g. 65.5 mg/50 mL)
Note: the stock solution contains 1000 µg/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 µg/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 ($C_5H_9D_4NCl_2$) = **100 µg/mL**

4.3.1. To be added to the samples prior to extraction:

d_4-Chlormequat chloride ISTD dilution I
in methanol, c ($C_5H_9D_4NCl_2$) = **10 µg/mL**
Note: this dilution contains 7.81 µg/mL d_4-chlormequat cation.

4.3.2. For the preparation of calibration standards:

d_4-Chlormequat chloride ISTD dilution II
in methanol, c ($C_5H_9D_4NCl_2$) = **1 µg/mL**
Note: this dilution contains 0.781 µg/mL d_4-chlormequat cation.

4.4. d_3-Mepiquat iodide internal standard (ISTD) stock solution

in methanol, c ($C_7H_{13}D3NI$) = **100 µg/mL**

4.4.1. To be added to the samples prior to extraction:

d_3-Mepiquat iodide ISTD dilution I
in methanol, c ($C_7H_{13}D3NI$) = **10 µg/mL**
Note: this dilution contains 4.80 µg/mL d_3-mepiquat cation.

4.4.2. For the preparation of calibration standards:

d_3-Mepiquat iodide ISTD dilution II
in methanol, c ($C_7H_{13}D3NI$) = **1 µg/mL**
Note: this dilution contains 0.480 µg/mL d_3-mepiquat-cation.

4.5. Methanol

(CH_3OH) for HPLC

4.6. Acetonitrile

(CH_3CN) for HPLC

4.7. Ammonium acetate

(CH_3COONH_4), p.a.

4.8. Acetic acid (glacial acetic acid)

(CH_3COOH) 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)

No.	Concentration of Chlormequat /Mepiquat cation in calibration solution↓	Chlormequat cation		Mepiquat cation		d_4-chlormequat cation-	d_3-mepiquat cation
		4.1.3*/ **0.1 µg/mL**	4.1.2*/ **1 µg/mL**	4.2.3*/ **0.1 µg/mL**	4.2.2*/ **1 µg/mL**	4.3.2*/ **1 µg/mL**	4.4.2*/ **1 µg/mL**
		Volumes to be added to each 10 mL vial (7 vials in total)					
1	**0.001 µg/mL**	100 µL		100 µL		167 µL	167 µL
2	**0.005 µg/mL**	500 µL		500 µL		167 µL	167 µL
3	**0.01 µg/mL**		100 µL		100 µL	167 µL	167 µL
4	**0.025 µg/mL**		250 µL		250 µL	167 µL	167 µL
5	**0.05 µg/mL**		500 µL		500 µL	167 µL	167 µL
6	**0.10 µg/mL**		1000 µL		1000 µL	167 µL	167 µL
7	**blank**					167 µL	167 µL

* see corresponding sections in the text

Note: *Following the instructions of this method, the content of d_4-chlormequat cation is ca. 13 ng/mL in all standard working solutions and samples and that of d_3-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 d_4-chlormequat chloride internal standard solution (4.3.1; ω = 10 µg/mL) and 100 µL d_3-mepiquat iodide internal standard solution I (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 d_4-Chlormequat: m/z= 126/58

Mepiquat: m/z= 114/58 and 114/98
ISTD d_3-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℃
Eluent A: 5 mmol/L NH_4 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: TurboIonSpray
Ionization mode: ESI positive
Ionspray voltage: 1500 V
Source temperature: 450 ℃

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. $PR_{sample\text{-}corr.}$= 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 1µg x 6.0 / 0.2 = 30 µg 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

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Table 10: Document History:

Action	When?	Changes introduced	Version
Drafting of the present document	Aug.-Oct. 2008		**V 1**
Placing of the present document in CRL-Website	Nov. 2008		**V 1**
Document revision	Jan. 2009	**Section 4.9**; *Calibration Standards:* major text revisions **Section 7**; *Evaluation of Results*: major text revision **Section 8**; *Reference List*: extended **All other sections**: correction of syntax, typos, minor text revisions	**V 2**