STUDY TITLE

Method Validation for Determination of Nitrapyrin and 6-CPA in Water

DATA REQUIREMENTS

SANTE/2020/12830, rev.1
OCSPP 850.6100
Dir98-02

EXPERIMENTAL

Sample Origin, Preparation and Storage

The analytical method was validated for drinking, surface and ground water.

Matrix Type	Origin, preparation and storage
Drinking water (tap water)	Obtained locally at the test facility. The water specimens was taking freshly before analysis. No further sample preparation was necessary.
Surface water	Obtained from River Schussen, near Bad Schussenried. Water specimens were placed in a refrigerated room, where they were stored at all times unless removed for analysis. Before taking aliquots for analysis, the sample material was allowed to reach room temperature and shaken thoroughly. After shaking, particulates were allowed to settle for a moment before beginning with the analysis.
Ground water	Obtained from Landeswasserversorgung (LW) Langenau. Water specimens were placed in a refrigerated room, where they were stored at all times unless removed for analysis. Before taking aliquots for analysis, the sample material was allowed to reach room temperature and shaken thoroughly. After shaking, particulates were allowed to settle for a moment before beginning with the analysis.

Instrumentation

Details of GC-MS/MS analysis of nitrapyrin are given in Appendix 1.

Details of LC-MS/MS analysis of 6-CPA are given in Appendix 2.

Calculation of Standard Calibration Curve

For nitrapyrin:

Calculation of a standard curve was performed by injection of calibration standards and acquisition of peak areas for the following transitions of analyte:

Nitrapyrin	*m/z* 196/160 (quantitative)
	m/z 194/158 (confirmatory)

The linearity of detector response was evaluated using calibration solutions in matrix. Calibration functions using linear regression with 1/x weighting (performed by the Chromeleon 7 Instrument control and data acquisition software) at ≥ 5 different concentrations ranging from 0.50 ng/mL to 50 ng/mL (0.010 to 1.0 µg/L sample equivalent concentration) were used to evaluate the final sample volumes. Examples for calibration plots and response factor/residuals vs. concentration diagrams are displayed in Figure *3* to Figure *14*.

For 6-CPA:

Calculation of a standard curve was performed by injection of calibration standards and acquisition of peak areas for the following transitions of analyte:

6-CPA	*m/z* 156/112 (quantitative)
	m/z 158/114 (confirmatory)

The linearity of detector response was evaluated using calibration solutions in neat solvent. Calibration functions using linear regression with 1/x weighting (performed by the Analyst 1.7.1 Instrument control and data acquisition software) at ≥ 5 different concentrations ranging from 0.40 ng/mL to 40 ng/mL (0.010 to 1.0 µg/L sample equivalent concentration) were used to evaluate the final sample volumes. Examples for calibration plots and response factor/residuals vs. concentration diagrams are displayed in Figure *15* to Figure *17*.

Confirmation of Residue Identity

Confirmation was performed to demonstrate the selectivity of the primary transition by monitoring one additional transition. Reagent blanks, unfortified matrix control samples and matrix control samples fortified at the lowest level of quantitation for each analyte/matrix combination were provided to demonstrate the selectivity of the method.

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" ("MITTELWERT" in German Excel) function of the Microsoft Excel (Office 2016) spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a fortification level of one matrix type was calculated using the "STDEV" ("STABW" in German Excel) function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the average, and then multiplying by 100. Statistical treatment of data included also the calculation of regression equations and the correlation coefficients (r) for describing the linearity of the calibration curves (Chromeleon 7 Instrument control and data acquisition software and curves for nitrapyrin and Analyst 1.7.1 Instrument control and data acquisition software and curves for 6-CPA).

The calculated LOD and LOQ was determined using the standard deviation of the residue levels found in the LOQ recovery sample extracts (in µg/L). The calculated LOD is three times the standard deviation and the calculated LOQ is ten times the standard deviation of the residue levels found in the LOQ recovery sample extracts.

The following average values (n = 5) were obtained:

Calc. [µg/L]	Nitrapyrin	
	m/z 196->160	*m/z 194->158*
Drinking water		
SD[a]	0.00156	0.00117
LOD[b]	0.00468	0.00351
LOQ[c]	0.0156	0.0117
Surface water		
SD[a]	0.00279	0.00388
LOD[b]	0.00837	0.0116
LOQ[c]	0.0279	0.0388
Ground water		
SD[a]	0.00160	0.00286
LOD[b]	0.00481	0.00857
LOQ[c]	0.0160	0.0286

[a] standard deviation of analysed concentrations from samples fortified at the level of LOQ.
[b] 3 x standard deviation.
[c] 10 x standard deviation.

Calc. [µg/L]	6-CPA	
	m/z 156->112	*m/z 158->114*
Drinking water		
SD[a]	0.00327	0.00399
LOD[b]	0.00980	0.0120
LOQ[c]	0.0327	0.0399
Surface water		
SD[a]	0.00283	0.00201
LOD[b]	0.00848	0.00602
LOQ[c]	0.0283	0.0201
Ground water		
SD[a]	0.00496	0.00459
LOD[b]	0.0149	0.0138
LOQ[c]	0.0496	0.0459

[a] standard deviation of analysed concentrations from samples fortified at the level of LOQ.
[b] 3 x standard deviation.
[c] 10 x standard deviation.

Stability of standard solutions and final volumes

Nitrapyrin was found to be stable for at least 90 days in isooctane solution(s) and for at least 110 days in acetonitrile solution(s) when stored under refrigerated condition in the dark. The analyte was found to be stable for at least 17 days in xylene solution(s) and stored under refrigerated condition in the dark. Results showed that the difference between freshly prepared and stored working solutions was lower than 10 %.

The stability of nitrapyrin in final volume of samples was assessed by reinjection of selected samples fortified at the level of 10xLOQ after at least 11 days of refrigerated storage against freshly prepared matrix- matched standards solutions. The mean recoveries determinated in the stored samples were within the range 70 – 120% with a relative standard deviation of ≤ 20 %.

Results showed that 6-CPA is stable in stock and fortification (methanol) and calibration solutions (methanol:water, 25:75 (v/v)) for at least 48 days when stored under refrigerated conditions in the dark and the difference between freshly prepared and stored working solutions was lower than 10 % .

The stability of 6-CPA in final volume of samples was assessed by reinjection of the samples fortified at the level of 10xLOQ after at least 11 days of refrigerated storage against freshly prepared calibration solutions.

The 6-CPA is shown to be stable in the final volume of samples for 11 days of refrigerated storage as the mean recoveries determined in the stored samples were within the range of 70 - 120 % with a relative standard deviation of ≤ 20 %.

Detailed results are given in Table *22* to Table *25*.

Matrix Effects

Matrix effects were evaluated by comparing the response of the analyte fortified in a processed control extract to the response of the analyte fortified in neat solvent. The calculation for the matrix effect is as follows:

$$\text{Matrix Effect} = 100 \times \frac{\text{Peak Area in MMS - Peak Area in Cal. In Solvent}}{\text{Peak Area in Cal. In Solvent}}$$

A negative value for the matrix effect indicates matrix suppression, and a positive value for matrix effect indicates matrix enhancement.

The experimental details regarding determination of the matrix effects were recorded in the raw data file. For nitrapyrin, the effect of matrix on the GC-MS/MS detector was assessed by comparing the mean peak areas of three injections of a matrix-matched standard solutions against the mean response of three injections of standard solutions prepared in solvent at an equivalent concentration. The matrix effct was checked at two levels (5.0 and 50 ng/mL). For 6-CPA, the effect of matrix on the LC-MS/MS detector was assessed by comparing the mean peak areas of three preapared matrix-matched standard solutions against the mean response of three standard solutions prepared in solvent at an equivalent concentration.

Matrix effects for the quantitative and confirmatory transitions were calculated, and the results are summarized in the Table *20* and Table *21*. Significant matrix effects (>20%) were observed for nitrapyrin in water matrices. No significant matrix effects (≤20%) were observed for 6-CPA in water matrices.

Carry Over

Carry-over was assessed during the validation by injecting a reagent blank sample after the highest concentration calibration standard.

For each analyte, the analyte response in the reagent blank samples were less than 30% of the peak response of the calibration standard at the LOQ concentration for the analyte.

CONCLUSIONS

The analytical method for the determination of nitrapyrin and 6-CPA in drinking water, surface water and ground water has been demonstrated to be satisfactory in terms of accuracy, precision, linearity and specificity. The method was validated over the concentration range of 0.05 µg/L – 0.5 µg/L for both analytes with a limit of quantitation of 0.05 µg/L for each analyte.

Table 1 Identities and Structures of Analyte(s)

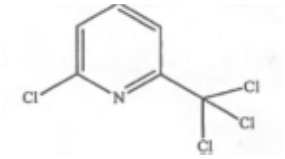
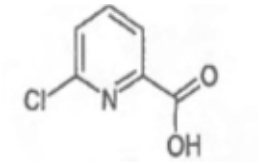
Common Name	Structural Formula and Chemical Name
Nitrapyrin (TSN003679-0002) Formula: $C_6H_3Cl_4N$ Molecular Mass: 230.90 g/mol Lot-No: V43-037266-95	 2-Chloro-6-(trichloromethyl)pyridine
6-CPA (X163214) (AGR029021) Formula: $C_6H_4ClNO_2$ Molecular Mass: 157.55 g/mol Lot-No: 188	 6-chloropicolinic acid

Figure 18. Example Calculation for Quantitative Determination of Nitrapyrin in Drinking Water

Residues are quantified using external standards in matrix.

Sample volume (V_{sample}) = 100 mL

Final volume (V_{End}) = 2.0 mL

Residue R (µg/L) were calculated for the fortified specimens as follows:

Residue R (µg/L) = C_{End} x V_{End} / V_{sample} = C_{End} (ng/mL) x M (mL/ mL)

Multiplier M = [(V_{End}) / (V_{sample}) = [(2.0 mL / (100 mL)] = 0.020 mL/mL

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = (R / $R_{fortified}$) x 100 %

Example:
The calculation of the residue and % recovery of the analyte are exemplified below for the drinking water sample P21-09121-1266 fortified at 0.050 µg/L (LOQ) with Nitrapyrin. The final volume was examined by GC-MS/MS in run file S21-01921GCMS5#534.wiff (see *Figure 26*). The final concentration (C_{End}) was determined to be 1.89 ng/mL (0.0379 µg/L) for the primary transition (*m/z* 196 → 160).

Residue R (µg/L) = 1.89 (ng/mL) x 0.02 (mL/mL) = 0.0379 µg/L

Rec. = (R/ $R_{fortified}$) x 100%
= (0.0379 µg/L / 0.050 µg/L) x 100%
= 76%

The values reported in the tables are calculated with full precision, but displayed with rounding. Minor / insignificant discrepancies may be observed when recalculated.

Figure 19. Example Calculation for Quantitative Determination of 6-CPA in Drinking Water

Residues are quantified using external standards in solvent.

Sample volume (V_{sample}) = 20 mL

Final volume (V_{End}) = 0.50 mL

Residue R (µg/L) were calculated for the fortified specimens as follows:

Residue R (µg/L) = C_{End} x V_{End} / V_{sample} = C_{End} (ng/mL) x M (mL/ mL)

Multiplier M = [(V_{End}) / (V_{sample}) = [(0.50 mL / (20 mL)] = 0.025 mL/mL

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = (R / $R_{fortified}$) x 100 %

Example:
The calculation of the residue and % recovery of the analyte are exemplified below for the drinking water sample P21-09121-76 fortified at 0.050 µg/L (LOQ) with 6-CPA. The final volume was examined by LC-MS/MS in run file S21-01921LCNo5#133.wiff (see Figure *47*). The final concentration (C_{End}) was determined to be 1.65 ng/mL (0.0413 µg/L) for the primary transition (*m/z* 156 → 112).

Residue R (µg/L) = 1.65 (ng/mL) x 0.025 (mL/mL) = 0.0413 µg/L

Rec. = (R/ $R_{fortified}$) x 100%
= (0.0413 µg/L / 0.050 µg/L) x 100 %
= 83%

APPENDIX 1 Analytical Method for Nitrapyrin

I. Reagents and Materials

Information pertaining to the identity and source of reagents used is summarised in Table I. Alternatively, equivalent reagents and materials may be used, unless specifically stated otherwise.

Table I. Identification of Reagents and Materials

- Acetonitrile, HPLC grade (VWR)
- Isohexane, ≥ 95% (Promochem)
- tert-butyl methyl ether, MTBE, ≥ 99.5% (Roth)
- Sodium chloride, 99.5% (Honeywell/ Sigma-Aldrich)
- Sodium sulfate anhydrous, p.a. grade 99% (Merck)
- p-Xylene, 99% (Sigma Aldrich)
- Isooctane, for pesticide residue analysis (VWR)
- Millipore Water (obtained at the test facility)

II. Instrumentation and Apparatus

Information pertaining to the identity of instruments and apparatus used is summarised in Table II. Alternatively, equivalent instrumentation and apparatus may be used, unless specifically stated otherwise.

Table II. Identification of Instrumentation and Apparatus

- Analytical Balance XS205DU (Mettler Toledo)
- Laboratory Balance ENTRIS2202I-1S (Sartorius)
- Ultrasonic bath Transsonic 460 (Elma Hans Schmidbauer)
- Evaporator Büchi Rotavapor R 210 V850 (Büchi)
- Evaporator Reacti-Vap (Thermo/Pierce)
- Various pipettes with disposable tips
- Nitrogen evaporation station (Thermo Scientific)
- Vortex mixer Reax top (Heidolph)
- Volumetric flasks
- Autosampler vials with caps
- Silanized glass wool, Supelco

III. Preparation of Standard Solutions

A stock solution of nitrapyrin was prepared by dissolving a weight of the test / reference item with the aid of an ultrasonic bath.

The stock solution was further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solutions for subsequent use as solvent calibration solutions and preparation of matrix-matched calibration solutions.

Matrix-matched calibration solutions were prepared using final sample extracts of control (untreated) samples of a respective matrix, which were then fortified with solvent standard solutions.

During the study, 2 sets of stock and calibration solutions were prepared in a similar way as described in the tables below. (The second set was used to determine the stability in solutions and final sample volumes)

All solutions were stored 1 °C to 10 °C in brown glass vials in the dark.

Examples of the typical dilutions that were carried out is presented in the following tables.

Table III. Preparation of a Stock Solution of Nitrapyrin in Acetonitrile

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (mg/mL)	Reference of standard solution produced
99.6%	10.04	10.00	10	1.0	ST21-09121-1000

* taken from the Certificate of Analysis

Table IV. Preparation of a Stock Solution of Nitrapyrin in Isooctane

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (mg/mL)	Reference of standard solution produced
99.6%	10.03	9.99	10	1.0	ST21-09121-1002

* taken from the Certificate of Analysis

Table V. Preparation of Fortification Solutions of Nitrapyrin in Acetonitrile

Reference of standard solution used	Concentration (µg/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
ST-24-09121-1000	1000	0.10	10	10	SP21-09121-1013
SP21-09121-1013	10	0.50	50	0.10	SP21-09121-1014
SP21-09121-1014	0.10	1.0	10	0.010	SP21-09121-1015A
SP21-09121-1015A	0.010	3.0	10	0.003	SP21-09121-1016

Table VI. Preparation of Intermediate Solvent Calibration Solutions of Nitrapyrin in Xylene

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
ST21-09121-1002	1000000	0.10	10	10000	K21-09121-1227
K21-09121-1227	10000	0.10	1.0	1000	K21-09121-1228
K21-09121-1227	10000	0.050	1.0	500	K21-09121-1229
K21-09121-1227	10000	0.010	1.0	100	K21-09121-1230
K21-09121-1229	500	0.10	1.0	50	K21-09121-1231
K21-09121-1229	500	0.040	1.0	20	K21-09121-1232
K21-09121-1229	500	0.020	1.0	10	K21-09121-1233
K21-09121-1231	50	0.10	1.0	5.0	K21-09121-1234
K21-09121-1231	50	0.040	1.0	2.0	K21-09121-1235
K21-09121-1231	50	0.020	1.0	1.0	K21-09121-1236
K21-09121-1231	50	0.010	1.0	0.50	K21-09121-1237

Table VII. Representative Preparation of Matrix-Matched Calibration Solutions

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
K21-09121-1228	1000	0.010	0.20	50	K21-09121-1238
K21-09121-1229	500	0.010	0.20	25	K21-09121-1239
K21-09121-1230	100	0.010	0.20	5.0	K21-09121-1240
K21-09121-1231	50	0.010	0.20	2.5	K21-09121-1241
K21-09121-1232	20	0.010	0.20	1.0	K21-09121-1242
K21-09121-1233	10	0.010	0.20	0.50	K21-09121-1243

IV. Laboratory Specimen Preparation

Specimen preparation as done in this method validation study is described in section "Sample Origin, Preparation and Storage" of this report.

V. Sample Volume(s) and Fortifications

Control (untreated) specimens of each water type were fortified prior to extraction with the fortification solutions as described below.

Table VIII. Summary of Sample Volumes and Fortifications

Fortified analyte	Matrix	Sample volume (mL)	Reference of fortification solution used	Concentration of fortification solution (µg/mL)	Volume of fortification solution added (mL)	Fortification level (µg/L)
Nitrapyrin	Water	100	SP21-09121-1014	0.10	0.50	0.50
		100	SP21-09121-1015A	0.010	0.50	0.050
		100	SP21-09121-1016	0.003	0.50	0.015

VI. Sample Work-Up Procedure Used

1. Transfer 100 mL of water into a 250 mL separatory funnel.
2. Fortify if necessary with the appropriate spiking solution, as described above.
3. Add 20 mL of isohexane, 20 mL of MTBE and 5 g of NaCl. Manually shake for 1 min. Allow the phases to separate.
4. Transfer the lower (aqueous) phase into a 150 mL beaker.
5. Filter the upper (organic) layer through a glass funnel filled with silanized glass wool and 10 g Na_2SO_4. Collect the filtered extract into a pre-weighed 100 mL pear shaped flask.
6. Put the aqueous phase back to the 250 mL separatory funnel. Rinse the 150 mL beaker with 20 mL of isohexane and add it into the 250 mL separatory funnel containg the aqueous phase. Manually shake for 1 min. Allow the phases to separate.
7. The aqueous phase can be discarded now.
8. Filter the upper (organic) layer into the same glass funnel filled with silanized glass wool and 10 g Na_2SO_4 and collect the filtered extract into the same pre-weighed 100 mL pear shaped flask from point 5.
9. Wash the funnel containing silanized glass wool and 10 g Na_2SO_4 with 10 mL isohexane.Collect the isohexane into the same pre-weighed 100 mL pear shaped flask.
10. Add 2.0 mL of xylene (as a keeper) to the combined organic phases.
11. Evaporate with Evaporator Rotavapor R 210 V850 (Büchi) at 35°C to < 2.0 mL. Adjust the volume with xylene to 2.0 mL gravimetrically.

VII. Chromatographic and Mass Spectrometric Conditions

A summary of the chromatographic and mass spectrometric conditions used for quantification is included in the following table:

Table IX. Summary of chromatographic and mass spectrometric conditions

GC operating conditions		
GC-MS/MS system	Thermo TSQ 8000 Evo triple quadrupole GC-MS/MS system, consisting of Trace 1310 gas chromatograph equipped with TriPlus RSH autosampler, split/splitless injector, triple quad mass spectrometer with chemical and electron ionization mode, and Chromeleon Software	
Column	Agilent DB-1701 (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness)	
Carrier Gas	Helium (constant flow: 1.2 mL/min; gas saver flow: 20 mL/min)	
Injection port	Split / Splitless (split flow 20 mL/min)	
Injection mode	Splitless	
Purge time/Splitless time	2.0 min	
Purge Flow	5.0 mL/min	
Injection volume	1 µL	
Injector temperature	220 °C	
Column oven temperature program	50 °C (hold for 1 min), ramp with 15 °C/min to 280 °C (hold for 2.0 min)	
Transfer line temperature	280°C	
Retention time	Nitrapyrin: approx. 10.4 min	
Mass spectrometer operating conditions		
Ionisation mode	Electron Impact Ionisation (Positive)	
Ion source temperature	250°C	
Analyte(s) monitored	Mass transition monitored *(m/z)*	Collision energy (CE) [V]
Nitrapyrin	196 → 160[#] (pos)	15
Nitrapyrin	194 → 158 (pos)	15

[#] proposed and used for quantification but both mass transitions listed can be used for quantification.

VIII. Special Precautions

As described at the corresponding section.

Additional precautions:

- The following liner is recomended to be used: Restek Topaz liner, Splitless, Single Taper, 4mmx6.5x78.5 for Thermo GCs (Cat#23446), (without glasswool).
- Because the evaluation was done using matrix-matched standard, during one injection series, it is not recomended to alternate calibration solutions in neat solvent with matrix-matched standard or final samples extracts injections. (GC system have to be equilibrated always with matrix).

IX. Calculation of Results

Quantification was performed using calibration plots with a minimum of five (5) different concentration levels covering the required calibration range.

The injection of matrix-matched standard solutions was spread over the whole analytical sequence.

SOFTWARE CALCULATION

The linearity of the detection system is demonstrated by use of standard solutions covering a working range which is equivalent to no more than 20% of the LOQ and at least +20% of the highest analyte concentration level in a sample extract.

A linear regression is performed with 1/x weighting. The correlation coefficient (R) must be greater or equal to 0.995 meaning that the coefficient of determination (R^2) must be greater or equal to 0.99.

A linear calibration function ($y = a + b \cdot x$) as determined by Chromeleon Software was used to calculate the analyte concentration in final extracts as follows:

$C_{End} =$	$\frac{A_A - a}{b}$
C_{End}	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with Chomeleon software.
a	y -axis Intercept of the calibration curve (counts)
b	Slope of calibration curve (counts x mL/ng)

RESULT CALCULATION

X. Method Flow Chart

Let the surface and ground water sample allow to reach room temperature.

⇩

Shake the sample. If necessary, let the particulates allowed to settle down.

⇩

Transfer 100 mL of water sample into a separatory funnel.

⇩

Fortify the specimen if necessary.

⇩

Add 20 mL of isohexane, 20 mL of MTBE and 5 g of NaCl.

⇩

Manually shake for 1 min. Allow the phases to separate.

⇩

Transfer the lower (aqueous) phase into a beaker. Filter the upper (organic) layer through a glass funnel filled with silanized glass wool and 10 g Na_2SO_4. Collect the filtered extract into a pointed flask.

⇩

Put the aqueous phase back to the separatory funnel. Rinse the beaker with 20 mL of isohexane and add it into the separatory funnel containg the aqueous phase. Manually shake for 1 min. Allow the phases to separate.

⇩

The aqueous phase can be discarded now.

⇩

Filter the upper (organic) layer into the same glass funnel filled with silanized glass wool and 10 g Na_2SO_4 and combine the filtered extracts.

⇩

Wash the funnel containing silanized glass wool and 10 g Na_2SO_4 with 10 mL isohexane and add 2.0 mL of xylene as a keeper.

⇩

Evaporate (35°C) to < 2.0 mL. Adjust the volume with xylene to 2.0 mL gravimetrically.

⇩

Transfer the sample extract into an AS Vial and analyse via GC-MS/MS.

XI. Safety Information

Reagents	H- and P-Codes	H- and P-Phrases
Acetonitrile	H225 H302, H312, H332 H319	• Highly flammable liquid and vapour. • Harmful if swallowed, in contact with skin, if inhaled. • Causes serious eye irritation.
	P210 P280 P305+P351+P338 P309+P311	• Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. • Wear protective gloves/protective clothing/eye protection/face protection. • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • IF exposed or if you feel unwell: call a POISON CENTER or doctor/physician.
Isohexane	H225 H304 H315 H336 H411	• Highly flammable liquid and vapour. • May by fatal if swallowed and eters airways. • Causes serious eye irritation. • May case drowsiness or dizziness. • Toxic to aquatic life with long lasting effects.
	P210 P301+P310 P302+P352 P331 P370+P378	• Kepp away from heat, hot sufaces, sparks, open flames and others ignition sources. No smoking • IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. • IF ON SKIN: Wash with plenty of soap and water. • Do NOT induce vormiting. • If case of fire: Use sand, carbon dioxide or powder extinguisher to extinguish.

Reagents	H- and P-Codes	H- and P-Phrases
Isooctane	H225 H304 H315 H336 H410	• Highly flammable liquid and vapour. • May be fatal if swallowed and enters airways. • Causes skin irritation. • May cause drowsiness or dizziness. • Very toxic to aquatic life with long lasting effects.
	P210	• Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
	P233 P240 P273 P301+P310 P302+P352 P304+P340 P331 P403+P235	• Keep container tightly closed. • Ground/bond container and receiving equipment. • Avoid release to the environment. • IF SWALLOWED: Immediately call a POISON CENTER/doctor/…. • IF ON SKIN: Wash with plenty of soap and water • IF INHALED: Remove person to fresh air and keep comfortable for breathing. • Do NOT induce vomiting. • Store in a well-ventilated place. Keep cool.
Xylene	H226 H304 H312+H332 H315 H319 H335 H373	• Flammable liquid and vopour. • May by fatal if swallowed and eters airways. • Harmful in contact with skin or if inhaled. • Causes skin irritation. • Causes serious eye irritation. • May cause respiratory irritation. • May cause damage to organs (central nervous system, liver, kidney) through prolonged or repeated exposure.
	P210	• Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
	P260	• Do not breathe mist/vapours.
	P280	• Wear protective gloves/protective clothing/eye protection/face protection.
	P301+P310	• IF SWALLOWED: Immediately call a POISON CENTER/ doctor.
	P303+P361+P353	• IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water (or shower).
	P305+P351+P338	• IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Reagents	H- and P-Codes	H- and P-Phrases
tert-butyl methyl ether (MTBE)	H225 H315 P210 P280 P302+P352	• Highly flammable liquid and vapour. • Causes skin irritation. • Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. • Wear protective gloves/protective clothing/eye protection/face protection. • IF ON SKIN: Wash with plenty of soap and water.
Sodium chloride	-	• No known significant effects or critical hazards. • Waste disposal - residue disposal of solid matter.
Sodium sulfate	-	• No known significant effects or critical hazards. • Waste disposal - residue disposal of solid matter.

APPENDIX 2 Analytical Method for 6-CPA

XII. Reagents and Materials

Information pertaining to the identity and source of reagents used is summarised in Table I. Alternatively, equivalent reagents and materials may be used, unless specifically stated otherwise.

Table X. Identification of Reagents and Materials

- Glacial acetic acid, 100 % (Merck).
- Hydrochloric acid, 32% (Chemsolute).
- Methanol, HPLC grade (VWR).
- Acetonitrile, HPLC grade (VWR).
- Methanol and water, LC-MS grade (VWR).
- Millipore water (supply at Test Facility).
- SPE cartridges, Bond Elut Mega BE-C18, 1 g, 6 mL (Agilent Technologies).

XIII. Instrumentation and Apparatus

Information pertaining to the identity of instruments and apparatus used is summarised in Table II. Alternatively, equivalent instrumentation and apparatus may be used, unless specifically stated otherwise.

Table XI. Identification of Instrumentation and Apparatus

- Balance XS205DU (Mettler Toledo)
- Ultrasonic bath Transsonic 460 (Elma Hans Schmidbauer)
- Ultrasonic bath Sonorex RK 100 (Bandelin Electronic)
- SPE extraction manifold (Baker).
- Evaporator Büchi Rotavapor R 210 V850 (Büchi)
- Evaporator Reacti-Vap (Thermo/Pierce)
- Various pipettes with disposable tips
- Nitrogen evaporation apparatus (Thermo Scientific)
- Vortex mixer Reax top (Heidolph)
- Volumetric flasks
- Plastic centrifuge tubes with caps, 50 mL.
- HPLC autosampler vials with caps

XIV. Reagent Solutions and Mobile Phases

Methanol:Water, 25:75, v/v

250 mL of methanol and 750 mL of Millipore water were mixed to ensure a complete homogeneous solution.

1 N Hydrochloric Acid in Water

900 mL of Millipore Water were filled into a 1 L Schott bottle. 100 mL of hydrochloric acid (32%) were added carefully to the same Schott bottle. The solution was mixed well.

0.1 N Hydrochloric Acid in Water

990 mL of Millipore Water were filled into a 1 L Schott bottle. 10 mL of hydrochloric acid (32%) were added carefully to the same Schott bottle. The solution was mixed well.

LC-MS Mobile Phase A: 0.01 % Acetic Acid in Water

To 1000 mL of LC-MS grade water 0.10 mL of concentrated acetic acid were added and mixed well to ensure complete homogeneous solution.

LC-MS Mobile Phase B: 0.01 % Acetic Acid in Methanol

To 1000 mL of LC-MS grade methanol 0.10 mL of concentrated acetic acid were added and mixed well to ensure complete homogeneous solution.

XV. Preparation of Standard Solutions

A stock solution of 6-CPA was prepared by dissolving a weight of the test / reference item in methanol with the aid of an ultrasonic bath.

The stock solution was further diluted to fortification solutions which were used for preparation of recovery samples and as (intermediate) standard solution for subsequent use as solvent calibration solutions and/or preparation of matrix-matched calibration solutions.

During the study, 2 sets of stock and calibration solutions were prepared in a similar way as described in the tables below. (The second set was used to determine the stability in solutions and final sample volumes)

All solutions were stored 1 °C to 10 °C in brown glass vials in the dark.

Examples of the typical dilutions that were carried out is presented in the following tables.

Table XII. Preparation of a Stock Solution of 6-CPA in Methanol

Purity of reference item* (%)	Weighed amount of reference item (mg)	Amount of analyte corrected for purity (mg)	Final volume (mL)	Equivalent concentration (mg/mL)	Reference of standard solution produced
99%	10.10	10.00	10	1.0	ST21-09121-1

* taken from the Certificate of Analysis

Table XIII. Preparation of Fortification Solutions of 6-CPA in Methanol

Reference of standard solution used	Concentration (µg/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (µg/mL)	Reference of standard solution produced
ST21-09121-1	1000	0.10	10	10	SP21-09121-4
ST21-09121-1	1000	0.050	50	1.0	SP21-09121-5
SP21-09121-4	10	0.50	50	0.10	SP21-09121-6
SP21-09121-4	10	0.150	50	0.030	SP21-09121-7

Table XIV. Preparation of Intermediate Solvent Calibration Solutions of 6-CPA in Methanol:Water (25:75, v/v)

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
ST21-09121-1	1000000	0.10	10	10000	K21-09121-8
K21-09121-8	10000	0.10	10	100	K21-09121-9
K21-09121-8	10000	0.050	10	50	K21-09121-10*

*Prepared but not used for further dilutions

Table XV. Preparation of Solvent Calibration Solutions of of 6-CPA in Methanol:Water (25:75, v/v)

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
K21-09121-8	10000	0.040	10	40	K21-09121-24
K21-09121-9	100	2.0	10	20	K21-09121-11
K21-09121-9	100	1.0	10	10	K21-09121-12
K21-09121-9	100	0.50	10	5.0	K21-09121-13
K21-09121-9	100	0.20	10	2.0	K21-09121-14
K21-09121-9	100	0.10	10	1.0	K21-09121-15
K21-09121-9	100	0.050	10	0.50	K21-09121-16
K21-09121-9	100	0.040	10	0.40	K21-09121-25

Table XVI. Preparation of Solvent Calibration Solution(s) of of 6-CPA in Methanol:Water (25:75, v/v) used for Preparation of Matrix-Matched and Calibration Solution Used to Check the Matrix-Effect

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
K21-09121-8	10000	0.40	10	400	K21-09121-42

Table XVII. Preparation of Calibration Solutions of 6-CPA in Neat Solvent (Methanol:Water (25:75, v/v))

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced
K21-09121-42	400	0.010	0.20	20	K21-09121-123
K21-09121-42	400	0.010	0.20	20	K21-09121-124
K21-09121-42	400	0.010	0.20	20	K21-09121-125

Table XVIII. Preparation of Matrix-Matched Calibration Solutions of 6-CPA in Final Blank Extract

Reference of standard solution used	Concentration (ng/mL)	Volume taken (mL)	Final volume (mL)	Equivalent concentration (ng/mL)	Reference of standard solution produced*
K21-09121-42	400	0.010	0.20	20	K21-09121-93
K21-09121-42	400	0.010	0.20	20	K21-09121-121
K21-09121-42	400	0.010	0.20	20	K21-09121-122

Exemplified for drinking water; the other matrix-matched standard solutions were prepared in a similar way.

XVI. Laboratory Specimen Preparation

Specimen preparation as done in this method validation study is described in section "Sample Origin, Preparation and Storage" of this report.

XVII. Sample Volume(s) and Fortifications

Control (untreated) specimens of each water type were fortified prior to extraction with the fortification solutions as described below.

Table XIX. Summary of Sample Volumes and Fortifications

Fortified analyte	Matrix	Sample volume (mL)	Reference of fortification solution used	Concentration of fortification solution (µg/mL)	Volume of fortification solution added (mL)	Fortification level (µg/L)
6-CPA	Water	20	SP21-09121-5	1.0	0.010	0.50
		20	SP21-09121-6	0.10	0.010	0.050
		20	SP21-09121-7	0.030	0.010	0.015

XVIII. Sample Work-Up Procedure Used

Sample Extraction

1. Dispense 20 mL of water sample into a 50 mL plastic centrifuge tube.
2. Fortify if necessary with the appropriate spiking solution, as described above. Shake shortly hy hand for homogenization.
3. Add 2.0 mL of 1 N hydrochloric acid. Cap and mix.

Solid-Phase Extraction

1. Place a C18 SPE column on an SPE vacuum manifold.
2. Rinse the column with 5.0 mL of acetonitrile and discard the rinse solvent.
3. Condition the SPE column with 5.0 mL of 0.1 N HCl. Do not allow the column bed to dry. Discard the conditioning solution.
4. Transfer the entire sample from the Step 3 (Sample Extraction) to the SPE column and draw through at approximately 1-2 mL/minute using a vacuum if gravity flow is inadequate to achieve the approximate flow rate of 1-2 mL/minute. Discard the column effluent.
5. Add 2.0 mL of 0.1 N HCl to the SPE column and draw through at approximately 1-2 mL/minute. Discard the column effluent.
6. Use vacuum to dry the SPE column for 40-45 minutes. Do not use a vacuum pressure less than -10 inches Hg and do not exceed the 45 minute drying time or analyte loss may occur.
7. Elute 6-CPA into a clean 15 mL glass culture tube with 5.0 mL of acetonitrile using a flow rate of approximately 1 mL/minute.
8. Once the column stops dripping, apply vacuum to collect the remaining eluent from the SPE column.
9. Evaporate the acetonitrile eluate just to dryness under a stream of nitrogen. A heating block temperature setting of 35ºC may be used. Do not exceed the 35ºC heating block temperature and do not over dry or analyte loss will occur. Evaporating the solvent to less than 100 µL then manually taking to dryness is recommended.
10. Redissolve the dried sample residue by pipetting 0.50 mL of methanol:water (25:75, v/v) into the culture tube.
11. Vortex mix for about 10 seconds at a speed adequate to thoroughly wash the sides of the culture tube.
12. Transfer the sample solution to a low volume recovery autosampler vial and cap. Store refrigerated.

XIX. Chromatographic and Mass Spectrometric Conditions

A summary of the chromatographic and mass spectrometric conditions used for quantification is included in the following table:

Table XX. Summary of chromatographic and mass spectrometric conditions

Chromatographic conditions						
HPLC system	Agilent 1290 HPLC System (Vacuum Solvent Degasser, Binary HPLC Pump), PAL System HTC-xt Autosampler and MayLab MistraSwitch column oven					
Pre-column	Phenomenex AJO 9000/AJO 8782					
Column	Acquity UPLC HSS T3, 100 mm x 2.1 mm, 1.8 µm, Waters					
Column oven temperature	40 °C					
Injection volume	10 µL					
Mobile phases	Eluent A: Water containing 0.01 % (v/v) acetic acid Eluent B: Methanol containing 0.01 % (v/v) acetic acid					
Gradient	Time [min]	% Eluent A	% Eluent B	Flow [µL/min]		
	0.00	95	5	300		
	0.10	95	5	300		
	4.00	30	70	300		
	4.50	30	70	300		
	4.51	5	95	300		
	5.50	5	95	300		
	5.51	95	5	300		
	7.50	95	5	300		
Retention time(s)	Approx. 3.8 min					
Mass spectrometric conditions						
MS system	Sciex 5500 Triple Quadrupole mass spectrometer with Turbo IonSpray ESI source					
Ionisation type	Electrospray (ESI, TurboIon Spray)					
Polarity	Negative ion mode					
Scan type	MS/MS, Multiple Reaction Monitoring (MRM)					
Capillary voltage (IS)	-4500		Ionspray turbo heater (TEM)		450 °C	
Curtain gas (CUR)	20 (arbitrary units)		Gas flow 1 (GS1)		40 (arbitrary units)	
Collision gas (CAD)	9 (arbitrary units)		Gas flow 2 (GS2)		60 (arbitrary units)	
Analyte monitored	Mass transition monitored *(m/z)*	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
6-CPA	156->112[#]	-35	-10	-16	-19	300
	158->114	-35	-10	-16	-19	500

[#] proposed and used for quantification but both mass transitions listed can be used for quantification.

XX. Special Precautions

Were described at the corresponding section.

XXI. Calculation of Results

Quantification was performed using calibration plots with a minimum of five (5) different concentration levels covering the required calibration range.

The injection of standard solutions was spread over the whole analytical sequence.

SOFTWARE CALCULATION

The linearity of the detection system is demonstrated by use of standard solutions covering a working range which is equivalent to no more than 20% of the LOQ and at least +20% of the highest analyte concentration level in a sample extract.

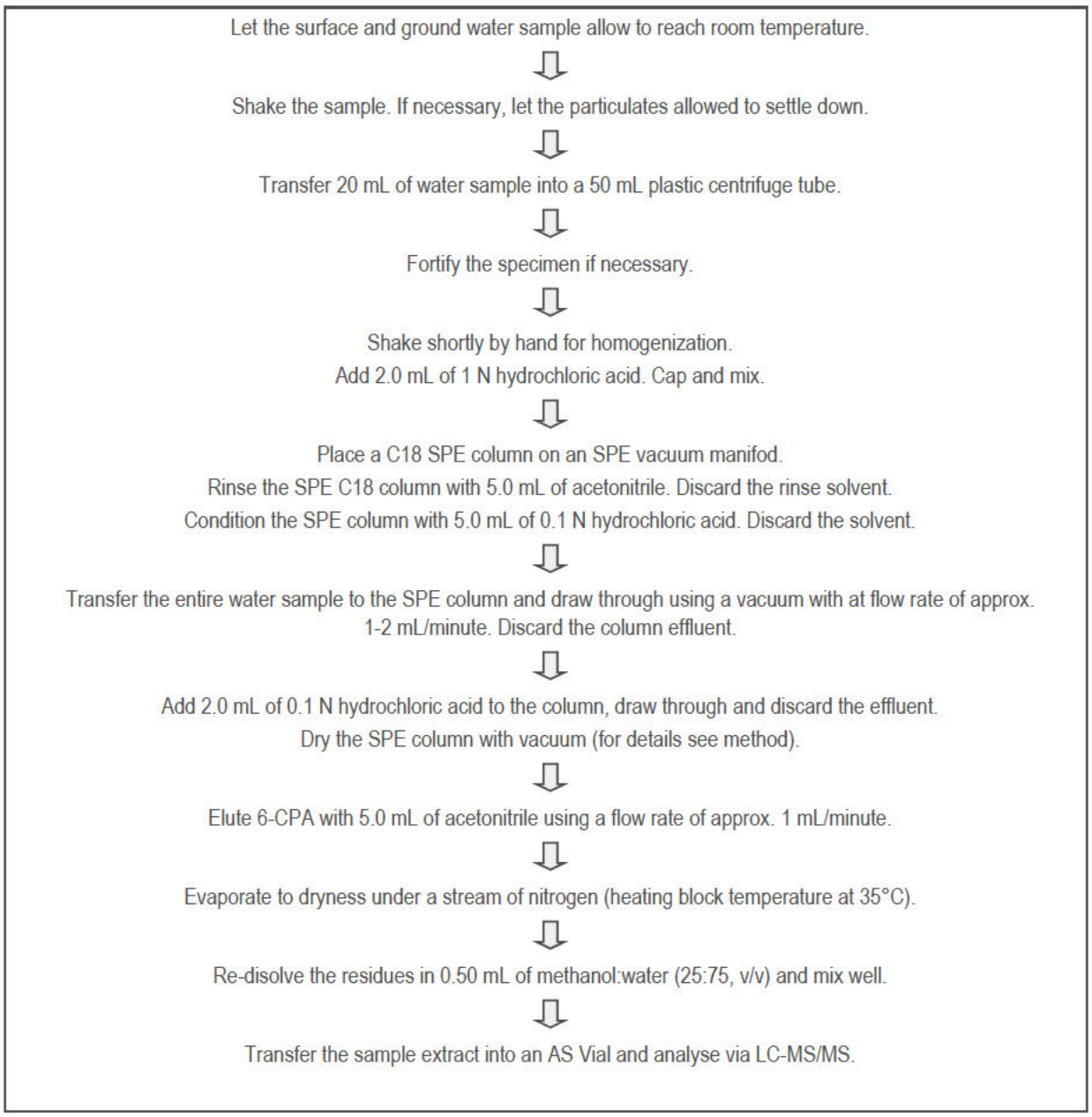
A linear regression is performed with 1/x weighting. The correlation coefficient (R) must be greater or equal to 0.995 meaning that the coefficient of determination (R^2) must be greater or equal to 0.99.

A linear calibration function ($y = a + b \cdot x$) as determined by Analyst Software was used to calculate the analyte concentration in final extracts as follows:

$C_{End} =$	$\frac{A_A - a}{b}$
C_{End}	Concentration of analyte in final extract (ng/mL) (x)
A_A	Peak area of analyte in the final solution (counts) (y) as obtained by integration with Analyst software.
a	y -axis Intercept of the calibration curve (counts)
b	Slope of calibration curve (counts x mL/ng)

RESULT CALCULATION

XXII. Method Flow Chart



XXIII. Safety Information

Reagents	H- and P-Codes	H- and P-Phrases
Methanol	H225 H301+H311+H331 H370	• Highly flammable liquid and vapour. • Toxic if swallowed, in contact with skin or if inhaled. • Causes damage to organs • Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
	P210 P280	• Wear protective gloves/protective clothing/eye protection/face protection.
	P302+P352+P312	• IF ON SKIN: Wash with plenty of soap and water, and call a POISON CENTER/doctor/… if you feel unwell.
	P304+P340+P311	• IF INHALED: Remove person to fresh air and keep comfortable for breathing and call a POISON CENTER/doctor/…
	P370+P378	• In case of fire: use extinguishing powder or dry sand.
	P403+P235	• Store in a well-ventilated place. Keep cool.
Acetonitrile	H225 H302, H312, H332 H319	• Highly flammable liquid and vapour. • Harmful if swallowed, in contact with skin, if inhaled. • Causes serious eye irritation.
	P210 P280	• Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. • Wear protective gloves/protective clothing/eye protection/face protection.
	P305+P351+P338	• IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
	P309+P311	• IF exposed or if you feel unwell: call a POISON CENTER or doctor/physician.

Reagents	H- and P-Codes	H- and P-Phrases
Acetic acid	H226 H314 P210 P280 P305+P351+P338 P303+P361+P353 P301+P330+P331 P310	• Flammable liquid and vapour. • Causes severe skin burns and eye damage. • Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. • Wear protective gloves/protective clothing/eye protection/face protection. • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • IF ON SKIN: Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower. • IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. • Immediately call a POISON CENTER/doctor/…
Hydrochloric acid	H290 H314 H335 P280 P305+P351+P338 P301+P330+P331 P308+P310	• May be corrosive to metals. • Causes severe skin burns and eye damage. • May cause respiratory irritation. • Wear protective gloves/protective clothing/eye protection/face protection. • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. • IF exposed or concerned: Immediately call a POISON CENTER/doctor/…