Development of a Standardised Analytical Method for the Detection and Quantitation of Melamine and Related Substances in Aqueous Media − Short Method Description −

The method can be used for the determination of melamine (MEL), ammeline (AMN), ammelide (AMD), cyanuric acid (CYA), cyromazine (CYRO) and hexamethoxymethylmelamine (HMMM) in water. Quantification of the substances is achieved by calibration with analytical standards in drinking water. For all substances except for HMMM, isotope-labelled internal standards are applied to correct for intensity variations (Table 1).

Table 1: Manufacturers of applied calibration standards and isotope-labelled internal standards.

Standard	**Manufacturer**
MEL	Dr. Ehrenstorfer
AMN	Cambridge Isotope Laboratories
AMD	Cambridge Isotope Laboratories
CYA	Dr. Ehrenstorfer
CYRO	Dr. Ehrenstorfer
HMMM	TRC
IS-MEL-^{13}C3	Dr. Ehrenstorfer
IS-AMN-^{13}C3	TRC
IS-AMD-^{13}C3	TRC
IS-CYA-^{13}C3	TRC
IS-CYRO-d4	TRC

For most of the samples, the method can be used with direct injection, or, for wastewater, after dilution of samples. If lower LOQ values need to be achieved, pre-concentration by evaporation can be applied (Table 2). However, it should be noted that in wastewater, HMMM should only be measured with direct injection, due to significant matrix effects during evaporation.

Table 2: Sample preparation steps with direct injection and pre-concentration by evaporation.

<table>
<tr><th colspan="2">Direct injection</th></tr>
<tr><td>Sample volume</td><td>1 mL</td></tr>
<tr><td>Sample pH</td><td>Original</td></tr>
<tr><td>Internal standards</td><td>See Table 1</td></tr>
<tr><td>Sample dilution</td><td>Dilution of 100 µL with 900 µL acetonitrile</td></tr>
<tr><th colspan="2">Pre-concentration by evaporation</th></tr>
<tr><td>Pre-concentrator</td><td>Vacuum pre-concentrator (RVC 2-33 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany)</td></tr>
<tr><td>Sample volume</td><td>10 mL</td></tr>
<tr><td>Sample pH</td><td>Original</td></tr>
<tr><td>Internal standards</td><td>See Table 1</td></tr>
<tr><td>Pre-concentration programme</td><td>
<table>
<tr><td></td><td>Start</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td></tr>
<tr><td>Time (h:m)</td><td></td><td>00:03</td><td>04:00</td><td>00:05</td><td>04:00</td><td>00:05</td><td>04:00</td></tr>
<tr><td>Temperature (°C)</td><td>30</td><td>40</td><td>40</td><td>35</td><td>35</td><td>30</td><td>30</td></tr>
<tr><td>Vacuum (mbar)</td><td></td><td>10</td><td>10</td><td>10</td><td>10</td><td>10</td><td>10</td></tr>
<tr><td>Rotational speed</td><td></td><td>900</td><td>900</td><td>1200</td><td>1200</td><td>1200</td><td>1200</td></tr>
</table>
</td></tr>
<tr><td>Reconstitution of the dry residue</td><td>In 225 µL acetonitrile + 25 µL ultrapure water</td></tr>
</table>

Measurements are performed by high-performance liquid chromatography (HPLC) and MS/MS detection. The applied instrumentation and analytical conditions are listed in Table 3. Table 4 shows the resulting retention times and MS parameters for each target analyte and the available isotope-labelled internal standards.

Table 3: Summary of the analytical conditions.

HPLC system	HPLC Infinity 1290 (Agilent Technologies)
Injection volume	10 µL
Separation column	Luna HILIC column (150 x 2 mm, 3 µm, Phenomenex) with HILIC security guard cartridge (4 x 2 mm, Phenomenex)
Column temperature	30 °C
Eluents	A: acetonitrile B: ultrapure water + 10 mM ammonium acetate + 0.1% formic acid
Elution programme	Isocratic elution for 10 min: 90% A, 10% B
Flow rate	0.4 mL/min
MS/MS system	API5500 Triple Quad (Sciex)
Interface	Electrospray ionisation (ESI)
Ionisation mode	Positive (MEL, AMN, CYRO, HMMM) and negative (CYA, AMD)
Detection mode	Multiple reaction monitoring (MRM)
Mass transitions	See Table 4

Table 4: Retention times and MS parameters for detection of target analytes and internal standards.

Substance	Ionisation mode	Retention time (min)	Q1	Q3	DP (V)	EP (V)	CE (V)	CXP (V)
MEL	Positive	3.1	127	68* 85	106	10	37 25	10 12
AMN	Positive	3.5	128	86* 43.1	96	10	23 55	12 6
AMD	Negative	2.5	127	83.9* 42	−50	−10	−16 −28	−7 −5
CYA	Negative	1.5	127.9	42.1* 85	−45	−10	−26 −14	−5 −7
CYRO	Positive	2.0	167	68* 85	91	10	43 25	10 12
HMMM	Positive	1.2	391.1	177.1* 359.2	31	10	39 11	12 26
IS-MEL-^{13}C3	Positive	3.1	130	87.1*	120	10	25	6
IS-AMN-^{13}C3	Positive	3.5	131	88* 71	131	10	23 37	10 12
IS-AMD-^{13}C3	Negative	2.4	130	86* 43	−55	−10	−16 −36	−5 −5
IS-CYA-^{13}C3	Negative	1.5	130.9	43.1*	−45	−10	−38	−9
IS-CYRO-d4	Positive	2.0	171	86*	71	10	27	10

The method has been validated concerning recovery of evaporation, calibration linearity, matrix effects and reproducibility. Stability tests showed no analyte degradation in glass, PE, or PS containers over 28 days, regardless of the storage temperature. For drinking water, the method results in the limits of quantification (LOQ) given in Table 5. In other matrices, LOQ may vary depending on necessary sample dilution.

Table 5: LOQ values of target analytes in matrix drinking water.

Substance	LOQ with direct injection	LOQ with evaporation
MEL	0.10 µg/L	0.010 µg/L
AMN	0.20 µg/L	0.010 µg/L
AMD	0.10 µg/L	0.010 µg/L
CYA	1.0 µg/L	0.050 µg/L
CYRO	0.10 µg/L	0.010 µg/L
HMMM	0.10 µg/L	0.010 µg/L