

Quantitation of Microcystins in Water by Direct Injection and Online SPE LC/MS/MS Systems

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Abstract

This Application Note compares direct injection and online SPE methods using liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) to analyze microcystins (MCs) in water. These include MC-RR, MC-HtyR, MC-LR, MC-HiLR, MC-LA, and MC-LF. The online SPE LC/MS/MS system eliminates time-consuming and laborious offline SPE extraction steps. Both the direct injection and online SPE method achieved limits of detection (LODs) of low or sub-ng/L levels, which are much lower than the provisional guidelines for drinking water expressed by the World Health Organization (WHO).

Introduction

MCs are cyclic peptides with a general structure of cyclo [-D-Ala-R²-D-MeAsp-R⁴-Adda-D-Glu-Mdha-], where R² and R⁴ are variable L-amino acids (Figure 1, Table 1). The two variable amino acids and methylation/demethylation of the other amino acids creates a large variety of MCs. More than 80 MCs have been identified, and in this experiment, six of the best-known MCs were chosen as target compounds.

MCs are naturally occurring toxins produced by certain genera of cyanobacteria. Reports suggest that MCs are hepatotoxic and potentially carcinogenic¹.

HPLC with UV detection has been used to analyze MCs. Analytical methods are shifting more towards MS detection. There is a potential risk of chronic exposure to MCs in drinking water supplies, which has prompted the WHO to issue a provisional guideline of 1 µg/L as the maximum concentration of total MC-LR (free plus cell-bound) in drinking water. Many national and regional governments have adopted this guideline value. Recently, strict regulation has been applied in some regions of China, especially for urban water supply systems. Method detection limits for direct injection lower than 0.05 µg/L are challenging for most MS/MS systems. An online SPE system coupled with MS is necessary for low concentration detection.

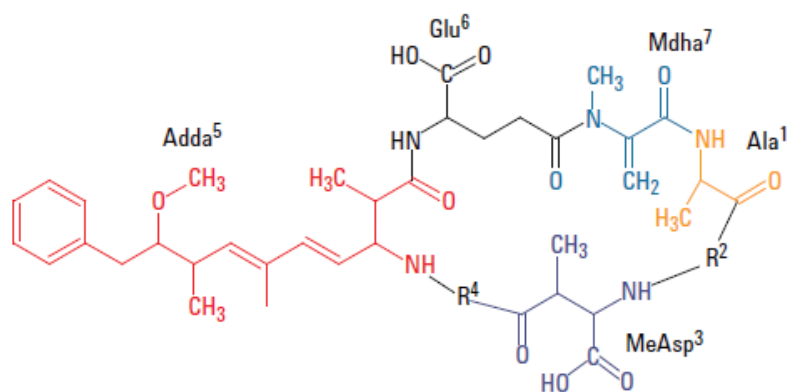


Figure 1. Structure of MCs. MC nomenclature is based on the L-amino acids present at R² and R⁴ positions.

Table 1. The composition of R²/R⁴ for different MCs.

MC	R ²	R ⁴	Molecular formula	Exact mass
LR	Leu	Arg	C ₄₉ H ₇₄ N ₁₀ O ₁₂	994.5488
RR	Arg	Arg	C ₄₉ H ₇₅ N ₁₃ O ₁₂	1037.5658
LA	Leu	Ala	C ₄₆ H ₆₇ N ₇ O ₁₂	909.4848
LF	Leu	Phe	C ₅₂ H ₇₁ N ₇ O ₁₂	985.5161
HtyR	Homo-L-Tyr	Arg	C ₅₃ H ₇₄ N ₁₀ O ₁₃	1058.5437
HiIR	Homo-L-Ile	Arg	C ₅₀ H ₇₆ N ₁₀ O ₁₂	1008.5644

Experimental

Standards

MC standards were purchased from Enzo Life Science International.

Instrumentation

Table 2. Instrument configuration.

Agilent 1290 Infinity II Online SPE system	Model no.
Agilent 1290 Infinity II Flexible Pump	G7104A
Agilent 1290 Infinity II High Speed Pump	G7120A
Agilent 1290 Infinity II Multisampler	G7167B
Analytical head, 900 µL	G4267-60046
Sample loop flex, 900 µL right	G7167-68900
Multidraw kit for Multisampler	G7167-68711
Triple-height drawer kit (3H)	G7167-60022
Agilent 1290 Infinity II Multicolumn Thermostat	G7116B
Agilent online SPE starter set (includes one 2-position/10-port valve and two Agilent Bond Elute online SPE cartridges, PLRP-S, 2.1 × 12.5 mm)	G4742A
Agilent 1290 Infinity Valve Drive	G1170A
Agilent 6470 Triple Quadrupole LC/MS with Agilent Jet Stream technology	G6470A

Chromatographic conditions and MS parameters

Table 3. LC parameters for direct injection.

Parameter	Value								
Analytical column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 μm (p/n 695975-302)								
Column temperature	45 °C								
Injection volume	10 μL								
Draw and eject speed	100 μL/min; 400 μL/min								
Needle wash	Methanol (MeOH), 5 seconds								
Mobile phases	A) 0.1 % formic acid (FA) + 1 mM ammonium fluoride (NH ₄ F) in water (H ₂ O) B) 0.1 % FA in acetonitrile (ACN)								
Stop time	10 minutes								
Post time	3 minutes								
Flow rate	0.4 mL/min								
Gradient	<table border="1"> <thead> <tr> <th>Time (min)</th> <th>%B</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>30</td> </tr> <tr> <td>8</td> <td>95</td> </tr> <tr> <td>10</td> <td>95</td> </tr> </tbody> </table>	Time (min)	%B	0	30	8	95	10	95
Time (min)	%B								
0	30								
8	95								
10	95								

Table 4. LC parameters for online SPE injection.

Parameter	Value										
Analytical column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 μm (p/n 695975-302)										
Column temperature	45 °C										
Trapping columns	2 × PLRP-S cartridges, 2.1 × 12.5 mm (p/n 5982-1271); 6 mL screw top vials (p/n 9301-1377), screw caps (p/n 9301-1379), preslit septa (p/n 5188-2758)										
Injection volume	1,800 μL										
Draw and eject speed	1,000 μL/min; 4,000 μL/min										
Needle wash	MeOH, 5 seconds										
Mobile phases	A) 0.1 % FA + 1 mM NH ₄ F in H ₂ O B) 0.1 % FA in ACN										
Stop time	14 minutes										
Post time	3 minutes										
Flow rate	0.4 mL/min										
Gradient	<table border="1"> <thead> <tr> <th>Time (min)</th> <th>%B</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>30</td> </tr> <tr> <td>4</td> <td>30</td> </tr> <tr> <td>12</td> <td>95</td> </tr> <tr> <td>14</td> <td>95</td> </tr> </tbody> </table>	Time (min)	%B	0	30	4	30	12	95	14	95
Time (min)	%B										
0	30										
4	30										
12	95										
14	95										

Table 5. Flexible pump parameters for SPE loading and washing.

Time (min)	Function	Parameter
0	Pump for loading	Pump 4 minutes; flow: 1.5 mL/min; H ₂ O
4	Valve change position	Increase valve position (switch valve)
4.1	Pump for washing	Pump 1 minute; flow: 1.5 mL/min; H ₂ O
5	Pump for washing	Pump 5 minutes; flow: 1.5 mL/min; ACN
10	Pump for reloading	Pump 7 minutes; flow: 1.5 mL/min; H ₂ O

Table 6. MS parameters for both online SPE and direct injection methods.

Parameter	Value
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Drying gas temperature	350 °C
Drying gas flow	10 L/min
Nebulizer	20 psi
Capillary voltage	4,500 V
Nozzle voltage	500 V
Delta EMV	300 V

Table 7. MRM parameters for MCs.

Compound	Precursor ion (<i>m/z</i>)	MS1 res.	Product ion (<i>m/z</i>)	MS2 res.	Dwell (ms)	Fragmentor (V)	Collision energy (eV)	Polarity
LR	995.6	Unit	213	Unit	40	200	58	Positive
	995.6	Unit	135	Unit	40	200	110	Positive
RR	520.1	Unit	213	Unit	40	140	35	Positive
	520.1	Unit	135	Unit	40	140	30	Positive
LA	910.5	Unit	213	Unit	40	170	50	Positive
	910.5	Unit	135	Unit	40	170	75	Positive
LF	986.5	Unit	213	Unit	40	170	68	Positive
	986.5	Unit	135	Unit	40	170	78	Positive
HtyR	1,059.6	Unit	106.7	Unit	40	220	147	Positive
	1,059.6	Unit	103	Unit	40	220	155	Positive
HilR	1,009.6	Unit	106.7	Unit	40	210	128	Positive
	1,009.6	Unit	103	Unit	40	210	160	Positive

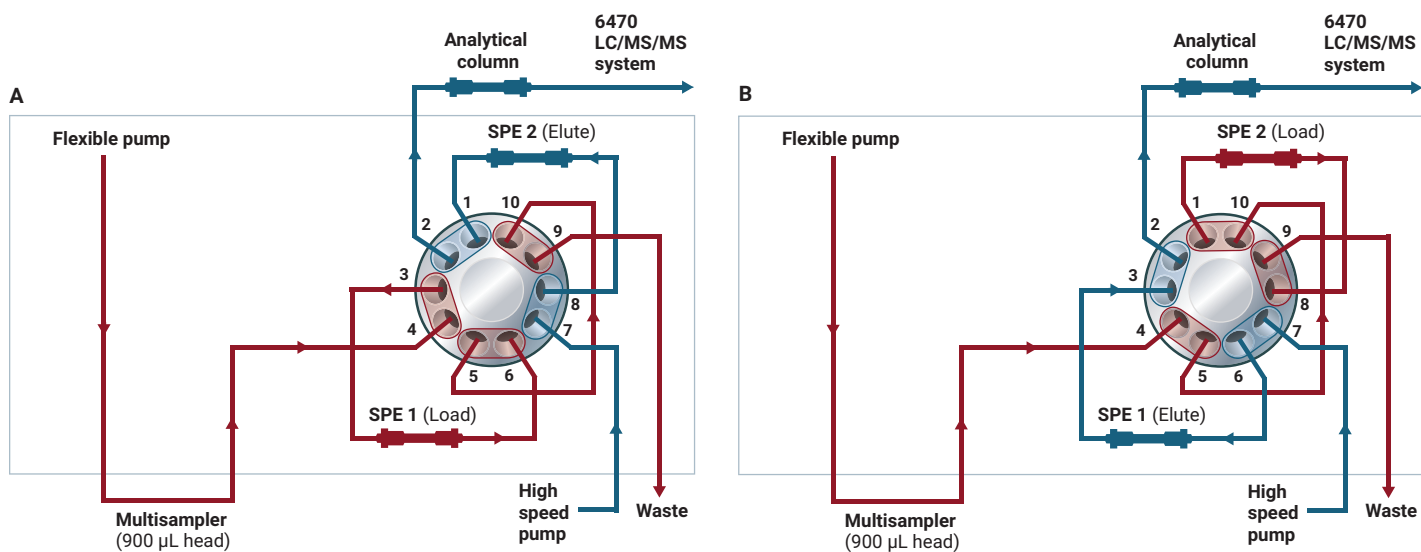


Figure 2. Valve positions for loading and eluting.

Results and discussion

Figure 3 shows MRM chromatograms of the MCs analyzed by direct injection (A) and online SPE (B). $[M+2H]^{2+}$ was chosen as the precursor ion for MC-RR, while $[M+H]^+$ was used as the precursor ion for the other MCs to provide the best sensitivity. The MassHunter Optimizer

Software automatically optimized each MS parameter, including fragmentor, CE voltage, and ion source temperature, flow and voltage.

Complete separation could be accomplished in 7 and 10 minutes for direct injection and online SPE methods, respectively. MC-RR, with the highest-abundance doubly charged

precursor ion, gave the most sensitive response. Peak shapes were consistent and reproducible.

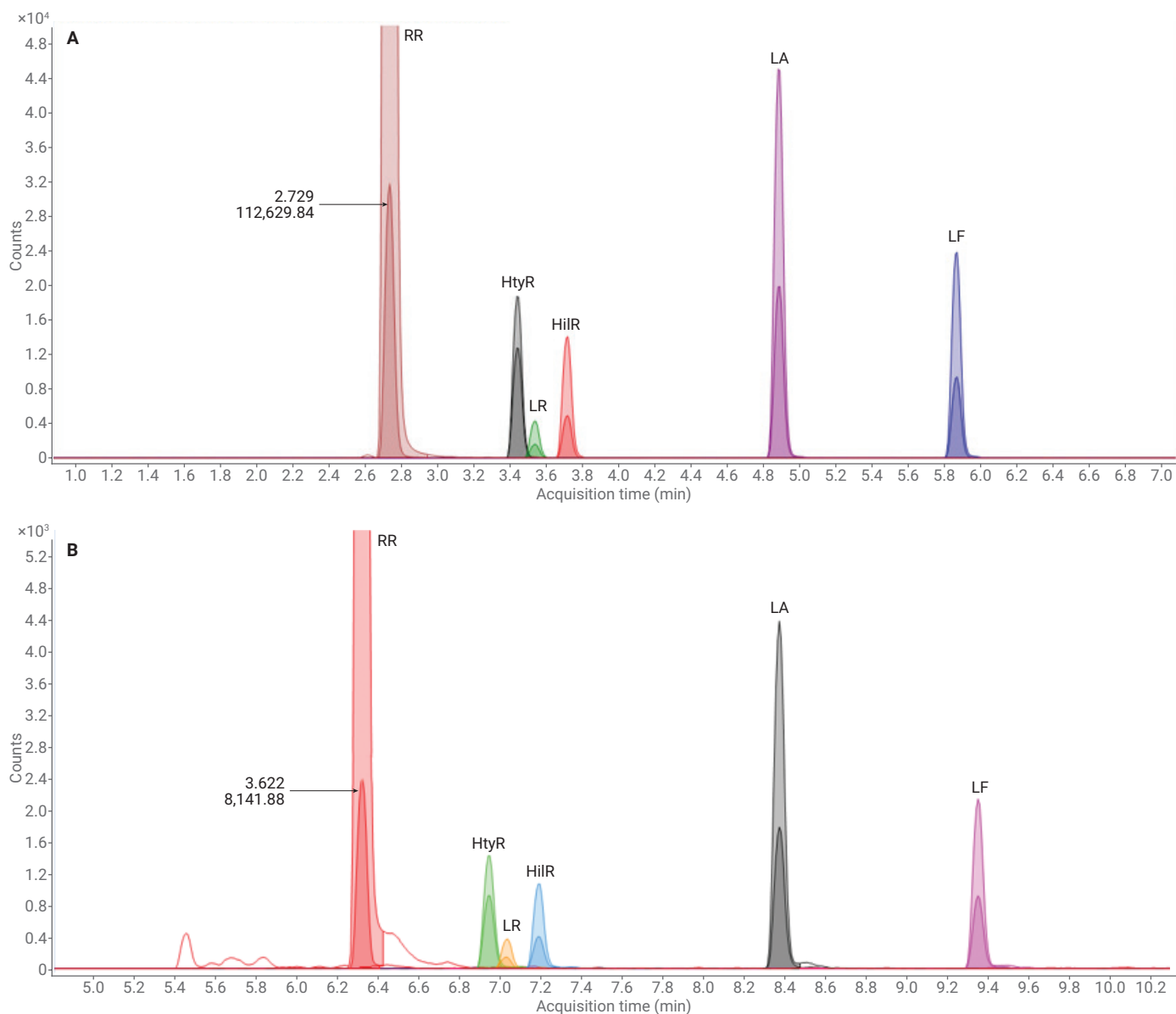


Figure 3. MRM chromatogram for direct injection (100 $\mu\text{g/L}$) and online SPE (2 $\mu\text{g/L}$).

With the direct injection method, excellent linearity in detector response was observed over the range of 0.01 to 100 ng/mL for MC-RR, 0.05 to 100 ng/mL for MC-HtyR, MC-HiLR,

MC-LA, and MC-LF, and 0.1 to 100 ng/mL for MC-LR, respectively. With the online SPE method, the linear range was 0.0002 to 2 ng/mL for MC-RR, 0.001 to 2 ng/mL for MC-HtyR, MC-HiLR, MC-LA, and

MC-LF, and 0.008 to 2 ng/mL for MC-LR, respectively. Figures 4 and 5 show the calibration curves.

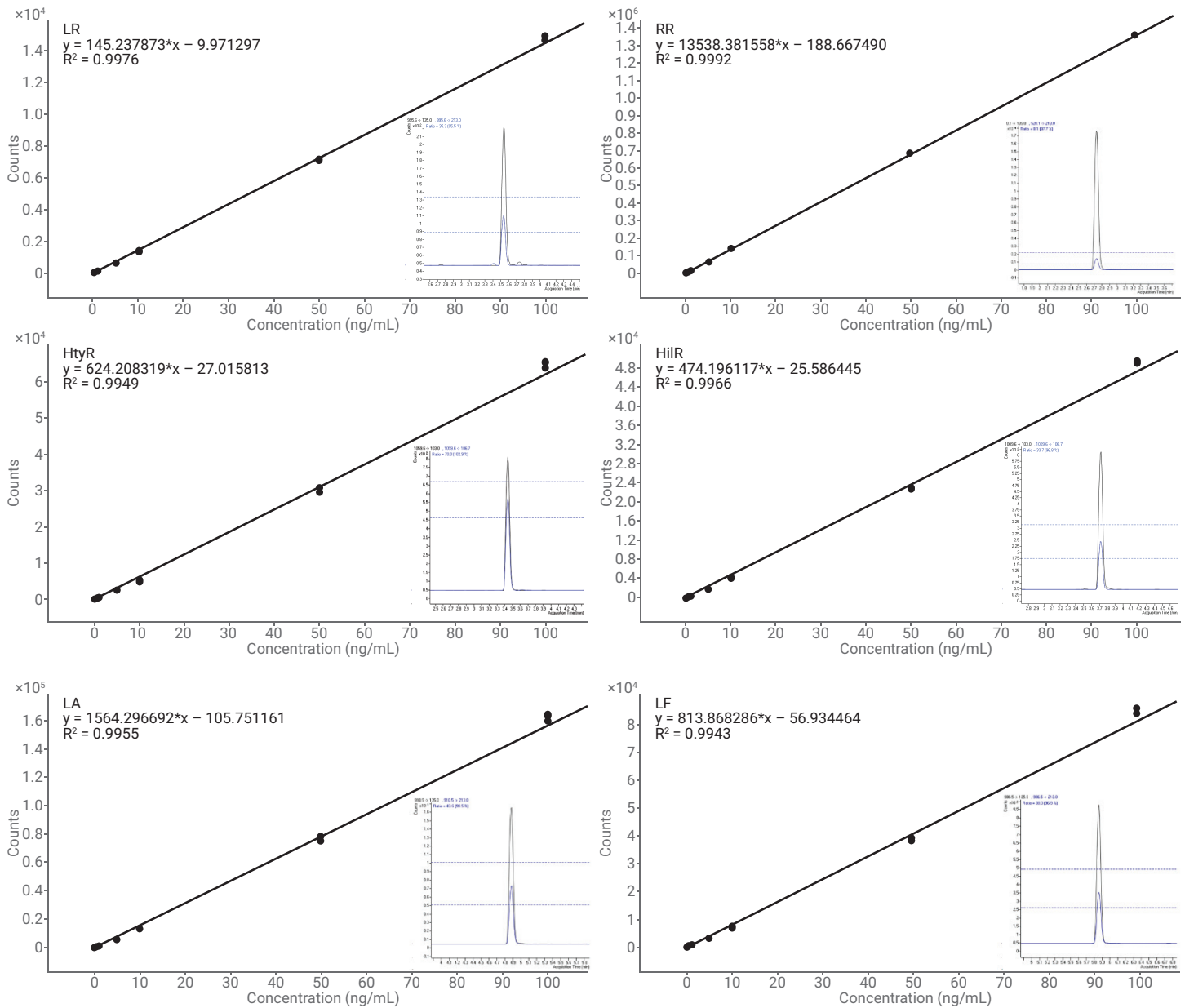


Figure 4. Calibration curves for quantitation of MCs with the direct injection method.

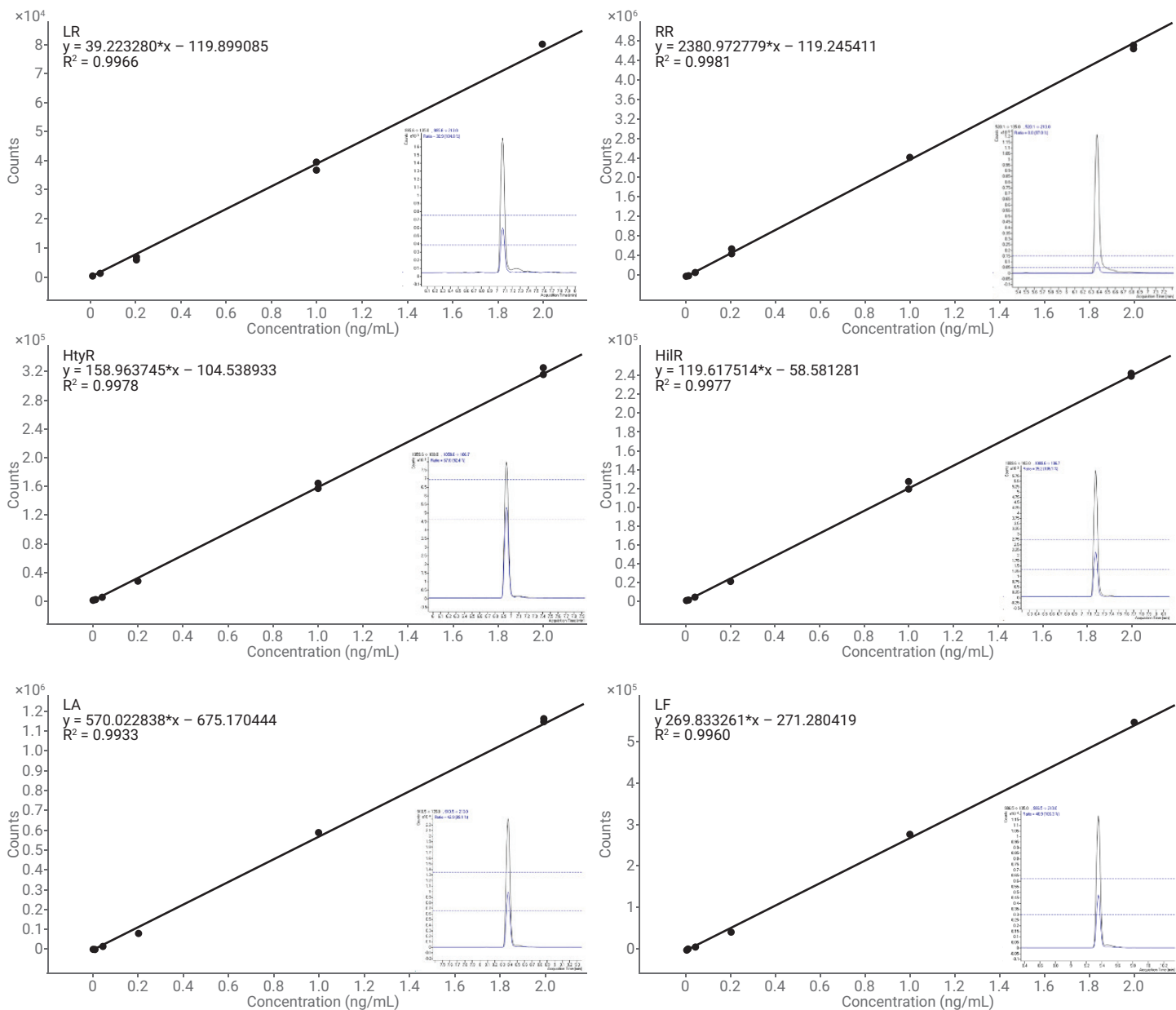


Figure 5. Calibration curves for quantitation of MCs with the online SPE method.

LODs and limits of quantitation (LOQs) were measured for each MC (Table 8). For direct injection, sensitivity of 2 to 20 ppt could be achieved, which is far below the WHO recommended guideline level of 1 ppb. However, some regional regulations concerning urban water supply systems stipulate method detection limits of 20 ng/L for MC-RR, and 100 ng/L for MC-LR3. Sensitivity was adequate for direct injection using the 6470 triple quadrupole LC/MS system, but using online SPE can enable less sensitive instruments to reach much lower LODs.

A recovery test was performed by injecting spiked surface water at 500 ppt and 2 ppt in the direct injection and online SPE method, respectively (n = 5). For both methods, recoveries between 75 and 110 % were achieved for all MCs. The online SPE method had a lower recovery than direct injection, possibly due to trapping and eluting of compounds on the SPE cartridge, but recoveries were still acceptable for all MCs (Table 9).

Analysis of real water samples

Three samples of surface water around the Chengdu district were collected, resulting in one positive and two negative samples when analyzed by the online SPE method. Additionally, due to its low concentration level, the positive sample could only be quantitated by the online SPE method, while the direct injection method, with a 10 µL injection volume, returned a nondetect (ND) for all MCs tested.

Table 8. LODs and LOQs for both injection methods.

MCs	Direct injection		Online SPE	
	LOD (ppt)	LOQ (ppt)	LOD (ppt)	LOQ (ppt)
RR	10	50	0.05	0.2
HtyR	50	100	0.2	1
LR	100	500	0.5	2
HilR	50	100	0.2	1
LA	50	100	0.2	1
LF	50	100	0.2	1

Table 9. Recovery (%) from both injection methods.

MCs	Direct injection		Online SPE	
	Average	RSD (%)	Average	RSD (%)
RR	98.02	1.78	80.38	5.83
HtyR	99.50	4.49	86.01	7.55
LR	94.79	4.20	76.85	5.27
HilR	93.70	5.27	86.25	8.83
LA	97.03	5.47	84.15	6.39
LF	100.26	7.06	82.41	3.01

Table 10. MC detection results for samples (ND = not detected).

MCs	RR	HtyR	LR	HilR	LA	LF
Sample 1	ND	ND	ND	ND	ND	ND
Sample 2	ND	ND	ND	ND	ND	ND
Sample 3	7.40 ppt	ND	73.41 ppt	4.39 ppt	3.20 ppt	ND

Conclusion

A simple and sensitive method for the quantitative determination of MCs was developed. Direct injection and online SPE methods using the 6470 triple quadrupole LC/MS system were compared. The LOQs obtained both by the direct injection and online SPE method were lower than the provisional guideline value established by the WHO for MC-LR concentrations in drinking water. The online SPE method method also satisfied recently published strict, regional water supply system guidelines for detection levels required in China. MC-RR and other MCs were detected in one surface water sample, which could only be quantitated by the online SPE method due to its lower detection levels.

References

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