Quantitative Analysis of Microcystin-LR in Drinking Water Comparing On-Line Solid Phase Extraction and Direct Injection LC/MS/MS

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An efficient method for microcystin-LR in drinking water at the sub-ng/l level applying on-line solid phase extraction (SPE) with LC/MS/MS detection and comparing it to direct injection LC/MS/MS is presented. The performance of the method of direct injection did not significantly deviate from the SPE approach. However, the direct injection method used less sample volume (100 µl) in shorter analysis time (4 min) and allowed more sample throughput. Conversely, the uncertainty of measurement could be reduced using SPE.

Key words: On-line solid phase extraction; direct injection; microcystin; LC/MS/MS

Microcystin (MC) is one of the most widespread cyanobacteria toxins found worldwide in inland and coastal water environments. They are produced by cyanobacteria (blue-green algae) that are found naturally in blooms of freshwater, lakes, streams, ponds, and other surface waters at favourable eutrophic, warm and low turbulent conditions. There are many types of MC; microcystin-LR (MC-LR) is one of the more toxic and well-studied varieties. Health effects of MC-LR can be acute or chronic and have been known to cause a tumour, liver and kidney damage potentially. The most severe consequence of exposure to MC is death. The impacts of chronic or acute exposure to MC-LR in humans, especially at the lower levels are more common via drinking water. There is also a particular concern for livestock (Fitzgerald & Poppenga 1993; Kerr, McCoy & Eaves 1987;

Moore & Puschner 2012) and canine (Wood *et al.*, 2010; DeVries, Galey, Namikoshi & Woo 1993) exposure to MC via surface drinking water from contaminated lakes, ponds and rivers.

In response to continued harmful effects of MC-LR, the Ministry of Health Malaysia (MOH) added MC-LR to its Contaminants List of unregulated contaminant and uses the Drinking Water Quality Surveillance Programme (KMAM) to monitor this pollutant in public water systems. The monitoring will provide MOH with nationally representative data on the occurrences of MC-LR in drinking water, which can support future regulatory determinations and other actions to protect public health. Under the KMAM programme, the sampling activities are carried out at water

distribution system and three basic stations: water treatment intake; treatment plant outlet, and service reservoir outlet. The samples collected from these locations are monitored for MC-LR levels on a regular basis. The availability of rapid and low-cost assay is therefore essential to accommodate many routine MC analyses in water which uses no laborious technique.

Due to the increase of water sample testing demand, a high throughput analysis applying either on-line SPE or direct injection is essential to analyse a large number of samples quickly without using labourious manual SPE technique which may contribute to high variability in the analysis results.

In Malaysia, the maximum acceptable limit for MC-LR is set at 1 ng/ml by the National Standard Drinking water Quality which is also same with the World Health Organization recommended level of 1 ng/ml of MC-LR (free and cell bound) in drinking water for humans. Hence, an analytical method set-up which is robust and powerful for analysing drinking water contaminated with MC-LR at low levels is essential and needs to be in place. LC/MS/MS analysis with electrospray ionization (ESI) was the method of choice for this study. The LC technique with MS/MS detector was preferred for the analysis of polar and thermally labile compounds mainly due to its selectivity and sensitivity, enabling efficient and reliable detection, and quantitation of MC-LR in drinking water.

Trace level analysis often adds to the challenge of direct determination of the compound of interest by chromatographic

analysis, therefore demanding a sample preparation step that is often time consuming, tedious, and frequently overlooked. For a sensitivity analysis, an extraction and purification step are usually necessary. However, such analysis typically depends on the complexity of a sample. Here, the study compared the method performances of on-line solid phase extraction (SPE) and direct injection in determining MC-LR in drinking water. On-line SPE injection method has the advantage of reducing sample preparation steps and enabling effective pre-concentration and clean-up of samples. Alternatively, direct injection method allowed high sample throughput and shorter analysis time.

METHODOLOGY

Sample Preparation

Internal standard (ISTD) solution, solution was added into water sample at 0.1 ng/ml concentration level. Then, the sample was filtered using 0.2 μm nylon membrane filter, before extraction by on-line SPE and followed by UPLC-MS/MS analysis. The on-line SPE procedure consisted of three steps: loading; washing; and eluting the sample analyte through the SPE cartridge with the gradient flow of conditioning and rinsing. The extracts were then analysed by LC/MS/MS. The direct injection method only involved injecting of filtered water samples into LC/MS/MS for analysis with nil preparation. Data was acquired using electrospray ionization and multiple reaction monitoring (MRM) using one precursor ion/two product ion transitions per compound (*Table 1*).

Table 1. Instrument condition and MS/MS setting of on-line SPE method *versus* direct injection.

Description/ Parameters	On-line SPE	Direct injection
LC column	Waters ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm × 100 mm	Waters ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm × 50 mm
SPE column	Waters Oasis HLB Direct Connect HP column, 20 µm, 2.1 mm × 30 mm	–
LC mobile phase	Deionised water and acetonitrile with 0.5% formic acid each.	
Run time	15 min	4 min
Injection volume	1500 µl	100 µl
MS condition	Electrospray ionization (ESI) at positive mode (ESI +ve) Capillary voltage: 0.5 kV Source temperature: 150°C Desolvation temperature: 350°C Desolvation gas flow: 650 L/hr Collision gas flow: 0.15 ml/min	
MRM setting (<i>m/z</i> : mass-to-charge; <i>CV</i> : cone voltage; <i>CE</i> : collision energy)	MC-LR: <i>m/z</i> (995.7 > 135.05), CV: 70V, CE: 70 eV <i>m/z</i> (995.7 > 213.1), CV: 70V, CE: 60 eV NOD (<i>as ISTD</i>): <i>m/z</i> (825.6 > 135.1), CV: 65V, CE: 60 eV	

Method Validation

A sequence of water samples, blanks and controls samples were analysed using the described method. The obtained data were evaluated with a calibration for MC-LR. MC-LR standards were prepared in deionised water over a range of 0.05 ng/ml to 10 ng/ml for on-line SPE and 0.1 ng/ml to 10 ng/ml for direct injection LC/MS/MS. Serial dilutions were obtained starting from 100 ng/ml concentration. The NOD ISTD was added to the standards and water samples at 0.1 ng/ml concentration.

For both methods, the LOD, signal-to-noise ratio ($3 \times \text{SD}$, $n = 10$, signal-to-noise >3:1) and LOQ, ($10 \times \text{LOD}$) were determined. Precision

and accuracy of the methods were evaluated with spiked samples of low concentrations.

RESULTS AND DISCUSSION

On-line SPE Method

The separation of the MC-LR was easier and distinct when using the on-line SPE method. *Figure 1* presents a total ion chromatogram (TIC) with a chromatographic separation retention time ratio ($\text{RT}_{\text{MC-LR}} / \text{RT}_{\text{NOD}}$) of 1.04. Selective detection was performed in MRM mode using two characteristic transitions for the compound. The ratio of both transitions (about 0.4) was used to confirm the presence of MC-LR in water. The calibration curve working

Figure 1. Total ion chromatogram for MC-LR spiked in tap water at concentration levels of 0.05 ng/ml and 0.02 ng/ml, respectively.

range of 0.05 ng/ml to 10.0 ng/ml is as presented in Figure 2. The calibration curve demonstrated a good linearity with correlation coefficient, $r^2 \geq 0.99$ and % deviation (% residual) within the acceptable range of $\pm 20\%$ from the actual value. The LOD and LOQ obtained were 0.005 and 0.05 ng/ml. Good results were obtained for MC-LR fortified in tap water samples at 0.5 ng/ml, 1 ng/ml and 1.5 ng/ml, with recoveries 97%,

102% and 102%, respectively (Table 2). The precision ($<5\%$ RSD) was rather satisfactory with results 3.3%, 3.9% and 2.5% for MC-LR fortified in tap water samples at 0.05 ng/ml, 0.1 ng/ml and 1.0 ng/mL, respectively (Table 2). The expanded combined relative uncertainty determined from the validation process was 10%.

Compound name: Microcystin-LR
Correlation coefficient: $r = 0.998274$, $r^2 = 0.996552$
Calibration curve: $0.613313 * x + -0.0200707$
Response type: Internal Std (Ref 2), Area * (IS conc. / IS area)
Curve type: Linear, Origin: Exclude, Weighting: $1/x$, Axis trans: None

Figure 2. Calibration curve for MC-LR from 0.05 ng/ml to 10 ng/ml.

Table 2. Comparison of results between on-line SPE method and direct injection.

Description/ Parameters	Direct injection	Online SPE
Separation (RT_{MC-LR} / RT_{NOD})	2.26/1.61 = 1.40	8.92/8.59 = 1.04
Linearity (internal standard calibration)	0.1 – 10 ng/ml ($r^2 \geq 0.99$)	0.05 – 10 ng/ml ($r^2 \geq 0.99$)
LOD = $3 \times SD$ (In tap water)	0.0192 ng/ml	0.0050 ng/ml
LOQ = $10 \times LOD$ (In tap water)	0.0959 ng/ml	0.0501 ng/ml
Precision (% RSD) (In tap water)	0.5 ng/ml: 5% (n = 10) 0.1 ng/ml: 4% (n = 10) 1 ng/ml: 17% (n = 30)	0.05 ng/ml: 3.3% (n = 10) 0.1 ng/ml: 3.9% (n = 10) 1 ng/ml: 2.5% (n = 20)
Relative recovery (%) (In tap water)	0.1 ng/ml: 119% (n = 20) 1 ng/ml: 106% (n = 10)	0.5 ng/ml: 97% (n = 10) 1 ng/ml: 102% (n = 10) 1.5 ng/ml: 102% (n = 10)
Expanded combined relative uncertainty (U_{cr})	19%	10%

Direct Injection Method

The analysis time of direct injection method was only 4 min with a chromatographic separation retention time ratio of 1.40 (Figure 3). This allows high throughput sample analysis. Calibration curve for the range, 0.1 to 10.0 ng/ml displayed a good linearity with correlation coefficient, $r^2 \geq 0.99$ and $\pm 20\%$ deviation (Figure 4). The LOD and LOQ determined were 0.019 ng/ml and 0.096 ng/ml, respectively. The recovery results of MC-LR fortified in tap water samples (Table 2) at 0.1 ng/ml and 1 ng/ml were 119% and 106%. Precision results were acceptable with $<20\%$ RSD determined as 5%, 4% and 17% for MC-LR fortified in tap water samples at 0.5 ng/ml, 0.1 ng/ml and 1.0 ng/ml respectively (Table 2). The expanded combined relative uncertainty determined from the validation process was 19%.

Comparison between Two Methods

The validation data showed that the direct injection method did not significantly deviate from the on-line SPE method. The LOD and LOQ obtained for both methods were well below than the NSDWQ standard requirements of 1 ng/ml. The slightly improved sensitivity for on-line SPE method could be explained by a lower LOD and LOQ than direct injection method due to injection of larger sample volume (1500 μ l) compared with 100 μ l for direct injection. Large sample volume injection was able using on-line SPE due to concentration enrichment of analyte in the SPE cartridge, thus enhance its sensitivity. However, the analysis time of on-line SPE method was much longer (15 min) compared with direct injection (4 min) as longer gradient flow time was required for multi-steps of conditioning, loading, washing,

and elution. The recovery results obtained for both methods were considered acceptable as they fall within the range of 70% to 120% [6].

Both LC-MS/MS methods were also compared using statistical significance tests. The F-test was applied to determine the precision variability between the two methods, while the *t*-test was applied to determine if there was a significant difference between these methods. The calculated F-value, 0.03 and *t*-value, 0.22, were below the critical values of 2.42 and 2.01, respectively at 95% confidence level. Although measurement uncertainty for on-line SPE method was found to be almost two times lower than direct injection, the significance tests outcomes indicated that there were no significant differences between these methods.

CONCLUSION

On the whole, all validation results of both methods fall within the acceptable limits that complied with EU Commission Decision 2002/657/EC (Anon. 2002) and EURACHEM (Anon. 2014) guidelines requirements. Also, the statistical data proved that the two methods were equally precise and there was no significant difference between these methods. Both methods were rather simple, rapid, precise, accurate and sensitive and could be deployed for routine analysis. However, the advantage of direct injection over on-line SPE method was the former which used smaller sample volume (100 μ l) and shorter analysis time (4 min) thus enabled high sample throughput.

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Compound name: Microcystin-LR
 Correlation coefficient: $r = 0.998658$, $r^2 = 0.997319$
 Calibration curve: $0.405756 * x + -0.00651749$
 Response type: Internal Std (Ref 2), Area * (IS conc. / IS area)
 Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Figure 4. Calibration curve for Microcystin-LR from 0.1 ng/ml to 10 ng/ml.

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