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Fast and Accurate Determination of Algal Toxins in Water Using Online Pre-concentration and UHPLC-Orbitrap Mass Spectrometry

Jae-Won Choi[1,†], Won-Seok Choi[1], YunS Kim[1], Sun-Hong Lee[1], Jonathan Beck[2], and Jennifer Massi[2]

[1]Water Quality & Analysis Center, K-water Convergence Institute, Daejeon, 34350, Korea
[2]Thermo Fisher Scientific, San Jose, CA, USA

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Concerns over algal toxins have been growing recently due their impact on rivers and drinking water treatment plants. The current accredited method allows conventional SPE and online SPE. These sample preparation techniques have no difficulty in achieving the LOQ of the guideline. SPE materials suitable for algal toxins are most commonly used with HLB-based sorbents with C18. On the other hand, MS/MS, which is a tandem MS, is the most widely used mass spectrometry method, but a full scan MS which has a high resolution (HR) and high accuracy performance also has a great advantage. The combination of online SPE and Orbitrap MS applied in this study provided a rapid and automated pretreatment method and precise and sensitive quantification of the detected compounds. The recovery rate was 83.9-113.7% and the PQL was 0.03-0.11 ng/mL. Determination of the family compounds of algal toxins is also very easy with an HR full scan MS and retrospective data review with a single measurement also has a big advantage.

Key words: EQuan, Orbitrap, Algal toxins, Cyanobacteria, Water analysis, Online pre-concentration

1. Introduction

The numerous dams built in Korea in the 1990s have contributed to the frequent occurrences of algal blooms in summer. This phenomenon is caused by excess nutrients, such as nitrogen (N) and phosphorous (P), that remain in the stagnant waters of the lake or river system after a heavy rainfall and lead to eutrophication. The combination of these nutrients with warmer water temperatures in summer (25-30°C) creates optimum growth conditions for algae. The algal bloom causes a rise in the pH level of the water, reduction in dissolved oxygen, asphyxiation of fish, and an unpleasant odor of the water. In addition, the fatty substances in the algal cells prevent condensation and sedimentation during the water purification process and cause obstructions in the filtration process.

When the density of the colonies of *Microcystis* and *Nodularia* cyanobacteria surpass a certain level, they produce hepatotoxic substances called microcystins and nodularins, respectively,[1)] while *Anabaena* and *Apha-zinomenon* are known to produce a neurotoxin called anatoxin.[2)] These toxins can cause deaths of wild animals and domestic livestock. Human poisoning can lead to gastrointestinal and allergy-like reactions and, in rare occasions, death. Of the cyanobacteria species, *Microcystis* has been observed to be dominant in the majority of eutrophication events. Microcystins, the toxins it produces, are cyclic peptides comprised of seven amino acids, each with a relatively large molecular mass ranging from 900 to 1,100 Da. There are approximately 60 to 85 variants of microcystins reported to date (Fig. 1).[3,4)] Moreover, nodularins produced by *Nodulariais* are peptide-based hepatotoxins sim-

[†]To whom correspondence should be addressed.
Tel: 82-42-629-4760, Fax: 82-42-629-4739, E-mail: choijw@kwater.or.kr

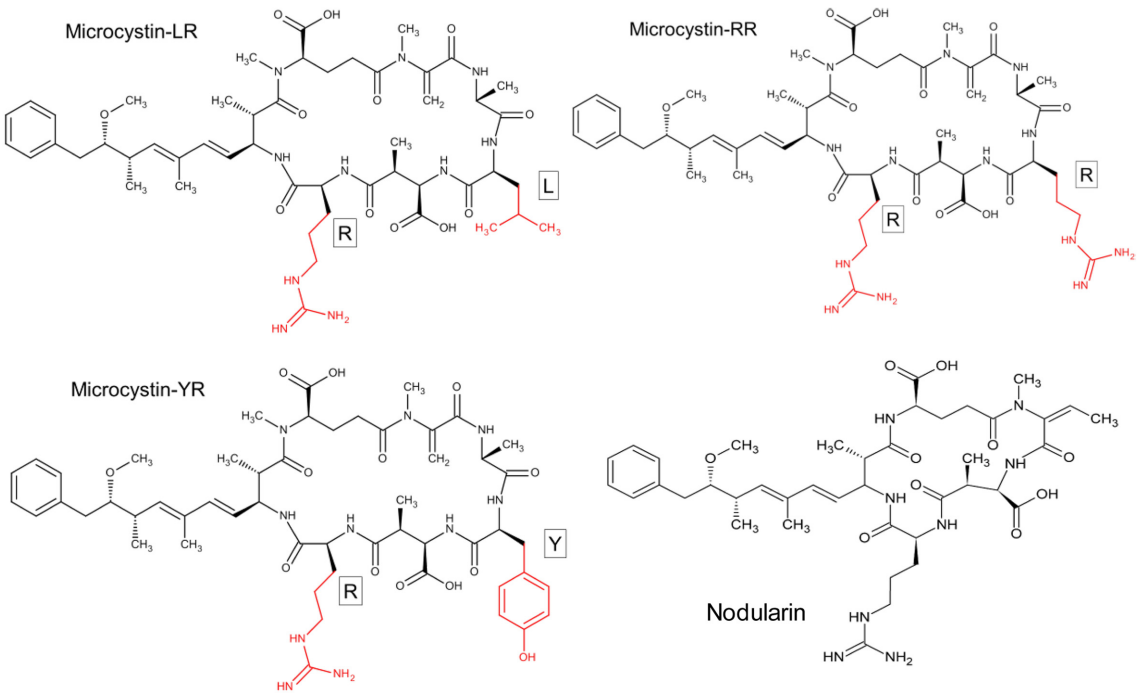

Fig. 1. Structures of the cyclic peptide microcystins and nodularin.

ilar to microcystin.

According to the World Health Organization (WHO), microcystins are chemically stable and can have an adverse impact on human health if present in a water supply source.[5)]

Prior research has shown that the microcystins -YR, -RR, and –LR are the most common isomers detected, and that microcystin-LR is the most toxic. Based on these results, the WHO has set forth a water quality guideline specifying that the microcystin-LR concentration be maintained below 1 ng/mL. This guideline is currently being used as a in candidate lists of Korean drinking water standard.

In Korea, when an algal bloom is forecasted, samples from the water supply source are collected and the chlorophyll-a concentration and the cyanobacteria cell number are measured. Based on the results, the situation is categorized into one of the following situations: ‘algal bloom watch,’ ‘algal bloom alert,’ or ‘algal bloom.’ In the latter two situations, the cyanotoxins, mainly microcystin-LR, are analyzed.[6)] Accurate analysis of multiple samples within a short time is required in order to monitor the multiple points of the water supply source and each of the processes taking place at water purification plants.

Traditionally, cyanotoxins have been measured by performing extraction and concentration through solid-phase extraction (SPE) followed by high-performance liquid chromatography with ultraviolet detection (HPLC/UVD).[7-9)] More recently, the analysis time has been reduced and the sensitivity improved through the use of ultra-high-performance liquid chromatography with photodiode array detection (UHPLC/PDA) or liquid chromatography-mass spectrometry (LC-MS/MS) applying electrospray ionization (ESI).[10-13)] However, these methods require pre-treatment of the samples, and the conventional SPE method requires a great deal of time and solvent. In an attempt to omit these pre-treatment processes, an HPLC method using

Table 1. Chemical formula and molecular weight of target algal toxins

Compound	Name (CAS)	Formula	Molecular Weight
Microcystin	Microcystin-LR (101043-37-2)	$C_{49}H_{67}N_{10}O_{12}$	995.2
	Microcystin-RR (111755-37-4)	$C_{49}H_{75}N_{13}O_{12}$	1038.2
	Microcystin-YR (101064-48-6)	$C_{52}H_{72}N_{10}O_{13}$	1045.2
Nodularin	Nodularin (118399-22-7)	$C_{41}H_{60}N_8O_{10}$	824.9

column switching has been used,[14] and more recently, a direct injection of the filtered sample into an LC-MS/MS has been reported.[15]

An online pre-concentration and injection method can shorten the sample pre-treatment process and help detect trace amounts of target substances, while an Orbitrap-type high-resolution mass scanning method takes into account the retrospective aspect of data, making it possible for accurate identification of the analyzed toxins and post-process quantitation of microcystin isomers. Therefore, we combined these two techniques for the identification and quantitation of microcystin-RR, -YR and -LR as well as nodularin. Then, an optimized method was developed to enhance the reliability and economic efficiency and was applied to raw and treated water from water purification plants and river systems.

2. Material and Methods

2.1. Reagents

Microcystin-LR, RR, and YR were procured from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) in a dried crystal form. Nodularin was procured from Cayman Chemical (CA, USA) in a dissolved form (500 μg in 500 μL of ethanol). Information on each of the standard materials is summarized in Table 1. Structural information of target toxins is illustrated in Fig. 1. Solvents were of residual pesticide grade. Water was double distilled by reverse osmosis.

2.2. Standard solutions and calibration curves

The standard solutions containing the cyanotoxins were prepared by dissolving microcystin-LR, -RR, and -YR into methanol at 100 μg/mL and by dissolving nodularin in ethanol to a concentration of 10 μg/mL. Solutions were stored in a cold room at 4°C. Taking into consideration the sensitivity of the analysis method and the WHO guideline of a microcystin-LR concentration of 1 ng/mL, the solutions were diluted into six different concentrations within the range of 100 to 1,000-pg/mL. An external standard method was used for the calibration curve verification and sample identification. Then, the ratio of peak area according to the concentration of standard solution was calculated.

2.3. Sample collection and storage

In August 2011, a total of 173 raw and treated water samples were collected from 59 facilities at the Han River (18 sites), Nakdong River (18 sites), and Geum-Seomjin (19 sites) Rivers, and in the city of Geoje (4 sites), as well as 55 sites in the Han River basin measurement network area. All samples were refrigerated during transport, transferred directly to a cold room in the lab, and maintained at 4°C. Sample aliquots were analyzed within 3 days of delivery.

2.4. Pre-treatment and instrumental analysis

Online pre-concentration using column switching was applied as a means to minimize sample pre-treatment and shorten analysis time. A Thermo Scientific™ EQuan MAX™ online sample concentration UHPLC-MS system equipped with a Thermo Scientific™ Hypersil GOLD aQ™ pre-concentration column (20×2.1 mm, 12 μm) and a Thermo Scientific™ Hypersil GOLD™ C18 analysis column (50 ×2.1 mm, 1.9 μm) was used. The allowable liquid sample injection range was 1 to 20 mL, and in

Table 2. EQuan MAX chromatography conditions used

Pump 1				Pump 2			
Hypersil GOLD aQ (pre-concentration column)				Hypersil GOLD C18 (analytical column)			
Time	%A	%B	μL/min	Time	%A	%B	μL/min
0.00	98	2	1000	0.00	98	2	230
1.01	98	2	1000	1.00	98	2	230
1.20	98	2	100	3.00	98	2	230
8.00	98	2	100	7.00	98	2	230
8.00	98	2	1000	7.01	98	2	230
				8.00	98	2	230
Mobile phase A: 0.1% formic acid in water, B: acetonitrile				Mobile phase A: 0.1% formic acid in water, B: acetonitrile			
Column temperature: Ambient							
Injection volume: 1000 μL							

this study the sample injection amount was set at 1 mL after considering the WHO guideline, equipment sensitivity, peak shape, and concentration ratio of the online injection. The standard material for the calibration curve and all the samples used in the analysis were filtered through a 0.45 μm glass fiber (GF) membrane syringe filter. A Thermo Scientific™ Exactive™ Orbitrap mass spectrometer was operated in full-scan mode. Resolving power was set to 50,000 (FWHM at m/z 200) and mass accuracy was set to 5 ppm. The detailed conditions for the online sample concentration and injection and the operation of the Orbitrap mass spectrometer are summarized in Tables 2 and 3, respectively. For the post-analysis identification and quantitation, an external standard method was applied.

Table 3. Exactive Orbitrap MS operating conditions

Scan range	*m/z* 150-1100
Resolving power	50,000 (FWHM at *m/z* 200)
Polarity	Positive
Run time	10 min
Measured *m/z*	995.5543 MC-LR 519.7898 MC-RR 1045.5344 MC-YR 825.4501 Nodularin
Ionization source	Electrospray
Spray voltage	4000 V
Capillary temperature	340°C
Capillary voltage	37 V
Tube lens voltage	85 V
Skimmer voltage	22 V

3. Results and Discussion

3.1. High-resolution mass spectra of toxins

The standards were prepared at a concentration of 1 ng/mL each and injected using a syringe pump to observe the mass spectra. The molecular ion and carbon isotope spectra of microcystin-LR, -RR, -YR, and nodularin are shown in Fig. 2(a), 2(b). Four carbon isotopes can be observed for most compounds. Using this isotopic pattern, it is possible to match the experimentally recorded carbon isotopic distribution ratios to the theoretical isotopic ratio to provide confirmation of the toxin using the analysis software. Meanwhile, molecular ions were observed in nodularin at *m/z* 825 and the isotopic pattern was confirmed.

From the results of the syringe injection, the quantitation ions for microcystins -LR, -RR, and -YR and nodularin were set at 995.5543, 519.7898, 1045.5344, and 825.4501, respectively. The mass accuracy was 0.1-0.3 ppm, which satisfied the standard for compound identification set at 5 ppm. In addition, the scanning range for identification and quantitation of the target compounds was between *m/z* 400 and 1100 for simultaneous analysis. However, the minimum range was set at *m/z* 150 to allow confirmation and quantitation of various algal toxins, such as anatoxin generated by *Anabaena*, which occurs just as frequently during an algal bloom.

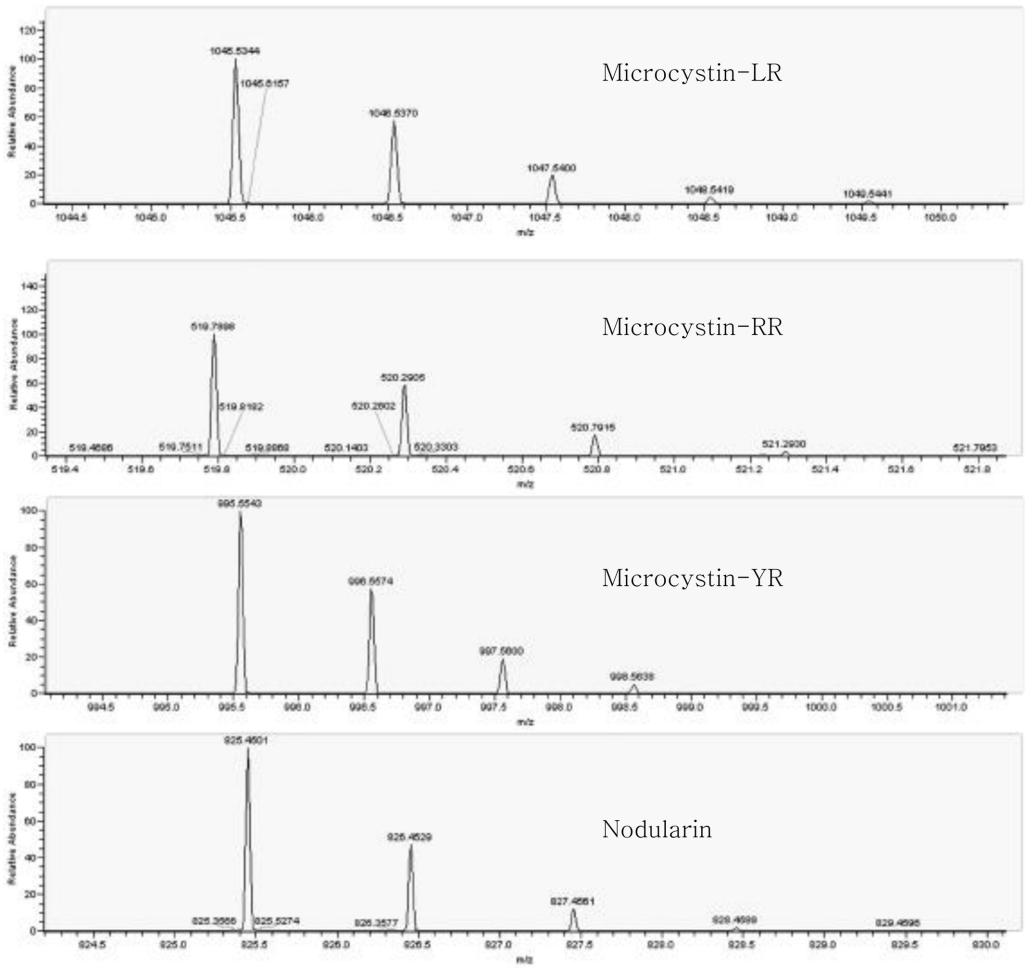

Fig. 2(a). Carbon isotope patterns by high-resolution, full-scan MS of microcystins and nodularin.

3.2. Optimization of the online pre-concentration method

In this study, 1 mL of each sample was used for the online pre-concentration method. During the five-minute analysis, adsorption and mobilization of the target toxin and column separation were carried out under the gradient conditions shown in Table 2. First, an injection of 1 mL of sample when the 0.1% formic acid and water / acetonitrile ratio was 98:2 led to the target toxin being adsorbed in the front part of the trap column and the remainder of the water sample being diverted to waste. Switching occurred inside the valve and then the proportion of the acetonitrile eluent was increased to 98% to transfer the adsorbed target toxin onto the analysis column and to the mass spectrometer. A summary of the analysis flow, including pre-treatment, is shown in Fig. 3.

A comparison of the absolute amount introduced into the mass spectrometer using this method and that of the SPE shows that it has the same concentration-injection effect as pre-treating and concentrating a 200 mL sample into 2 mL and injecting 5 μL of the pre-concentrated sample. Thus, it is possible to perform a microanalysis without a separate pre-treatment. Also, this method uses UPLC-based chromatography and sharp peaks are obtained, as shown in Fig. 4. Therefore, the online pre-con-

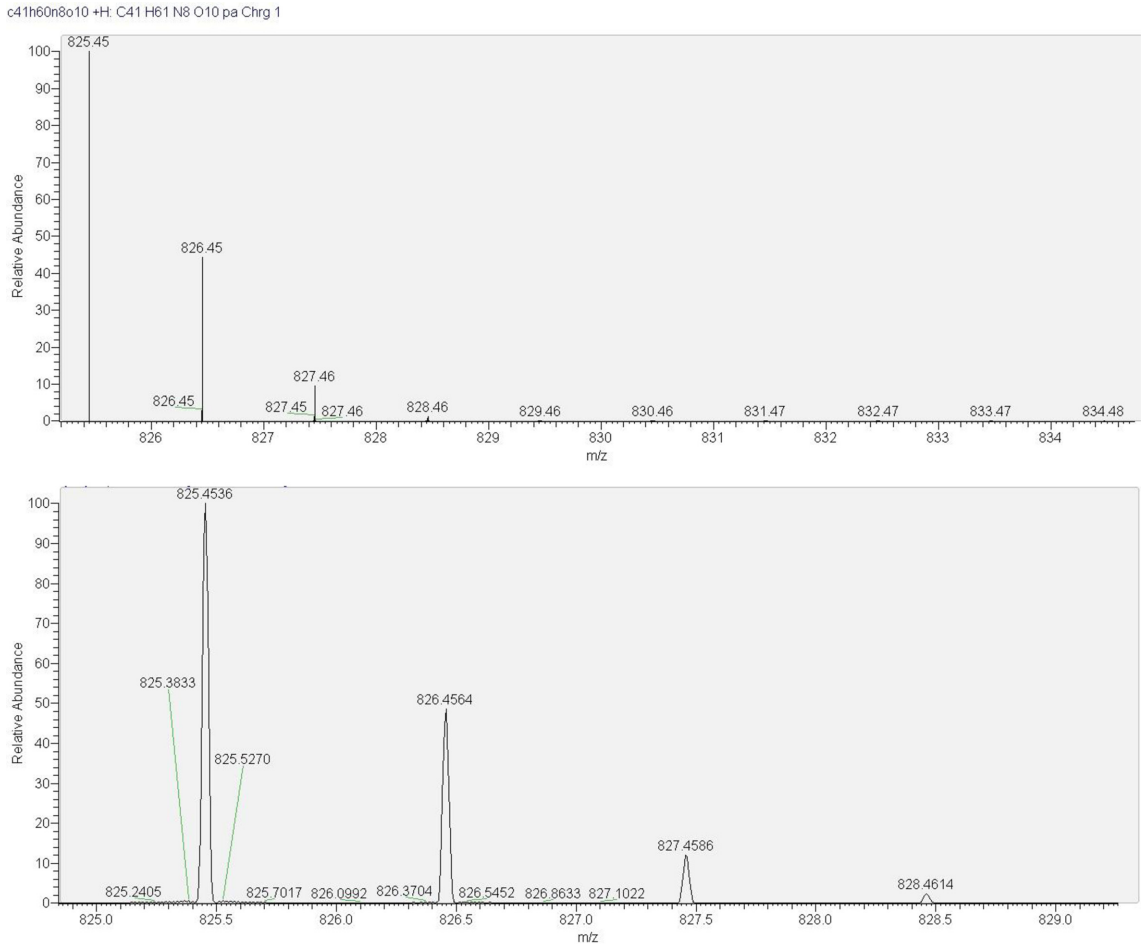

Fig. 2(b). Simulated spectrum of nodularin (top) compared to actual spectrum (bottom), confirming isotope pattern.

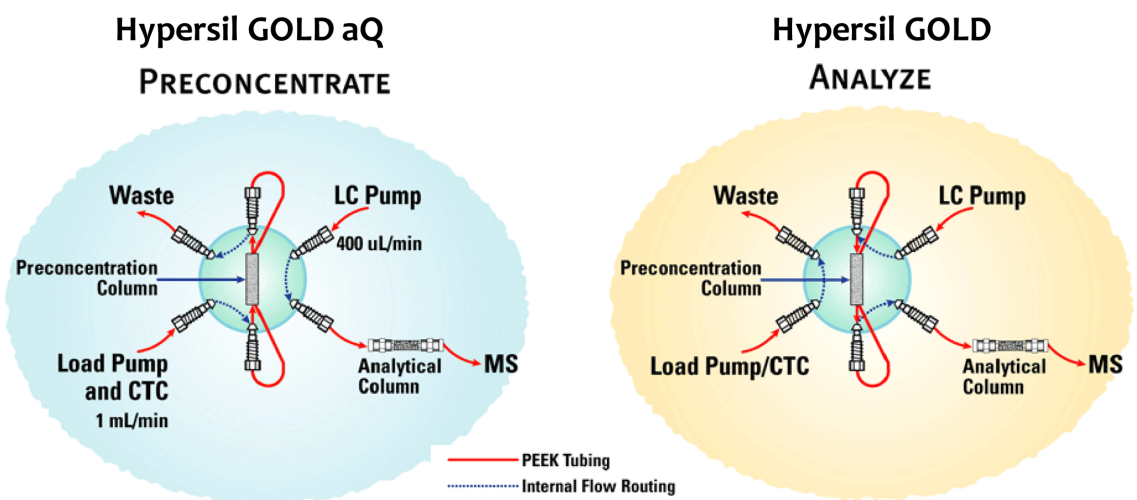

Fig. 3. Switching column method for on-line sample injection.

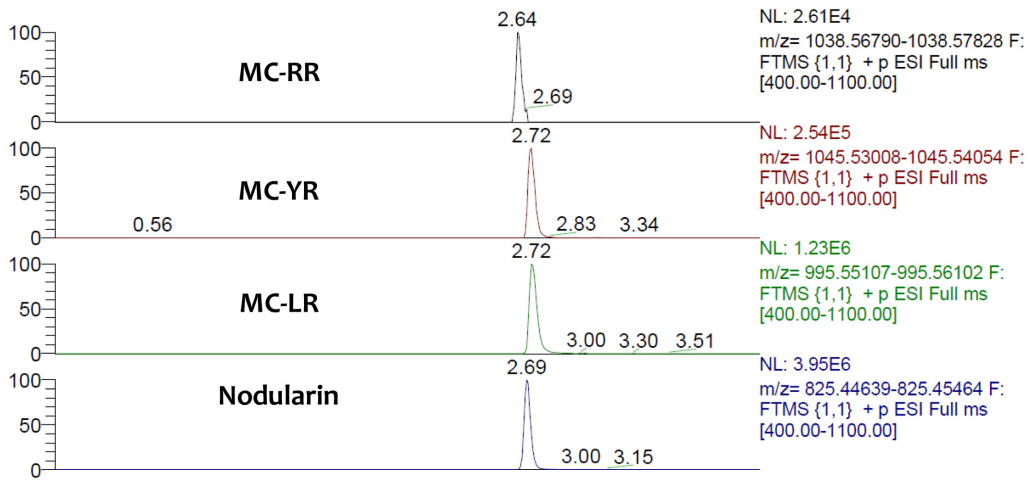

Fig. 4. Extracted chromatograms from full-scan data by UHPLC-Orbitrap mass spectrometer.

centration method does not result in peak broadening or reduced sensitivity.

The retention times for microcystin -LR, -RR, and -YR and nodularin using this method were between 2.6 and 2.8 min. Due to the application of a relatively short column and a simple solvent combination, mass separation occurs under high-resolution conditions at a resolving power of 50,000. Even if there is an overlap of retention times, identification and quantitation based on the difference of the precise mass unique to each of the toxins is possible as shown in Table 3. Thus, there was no actual interference between the toxins (Fig. 4).

The online sample pre-concentration method has been applied in the analysis of algal toxins,[14] but there have rarely been any cases in which 1 mL of sample was used for an analysis of trace amounts in pg/mL concentrations. The online injection method applies the principle of minimizing SPE and sending the concentrated amount to the analyzer. Compared to the conventional SPE method, which requires the use of 0.5 to 1 L sample, the online injection method effectively reduces the analysis time and amount of sample required. In a typical analysis with five samples, a conventional SPE method would require 8 hours for the filtration, solid-phase extraction, and concentration processes; 2.3 hours for instrumental analysis; and 1 hour for data analysis and quantitation, for a total of 12.3 hours. In contrast, the optimized method developed in this study requires 10 minutes for sample division and filtration, 0.8 hours for instrumental analysis with the application of UHPLC, and the same amount of time for data analysis and quantitation, for a total of 2 hours. This is an at least 80%-time savings. Other benefits of using this rapid pre-treatment method include enhanced productivity when there is a large amount of sample, reduced amounts of organic solvents, reduced labor for the pre-treatment process, and omission of a nitrogen concentration apparatus.

3.3. Calibration curve assessment

To review the linearity, the calibration curve of the standard toxin mixture of microcystin -LR, -RR, and -YR and nodularin was measured repeatedly within the range 100 to 1,000 pg/mL. As shown in Fig. 5, the correlation coefficient for each of the toxins was between 0.9971 and 0.9996. Reproducibility was ±15%. This is an improvement compared to the quantitation range for algal toxins in the water quality test samples reported.[15] Also, it was deemed possible to perform a linearity assessment

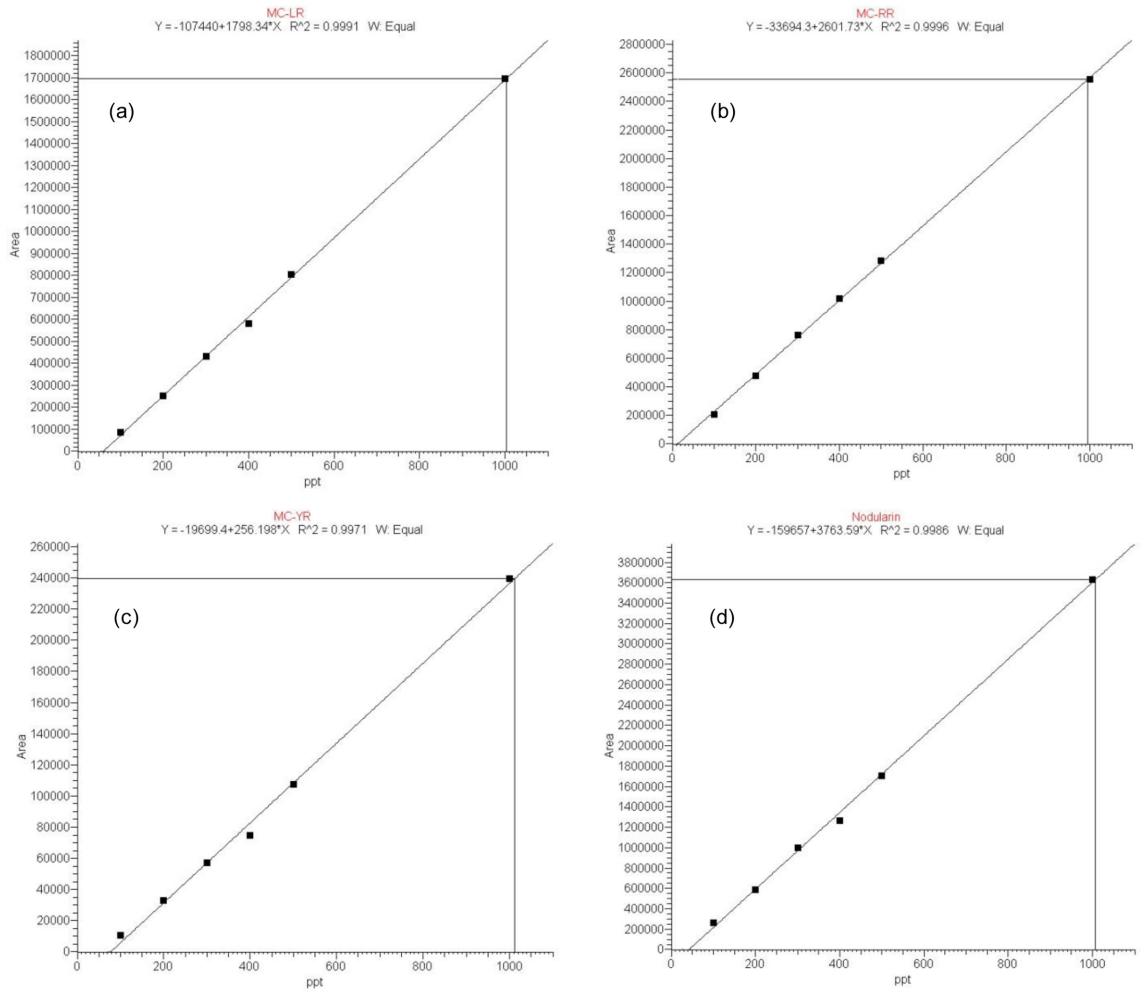

Fig. 5. Calibration curve of (a) microcystin-LR, (b) RR, (c) YR and (d) nodularin.

at lower concentrations if necessary in the future since the signal-to-noise ratio (S/N) was sufficient at the minimum concentration of 0.1 ng/mL. Thus, based on these results, we determined that the online pre-concentration high-resolution fall-scanning method has the equivalent trace quantitation capacity as the conventional solid-phase extraction and LC-MS/MS method.

3.4. Recovery rate and detection limit

To assess the recovery rate of the optimized method, seven 20 mL samples were taken from the 1 L sample of the raw water collected from the Dae-Cheong Dam in which the target toxins were not detected. Then, microcystin -LR, -RR, and -YR and nodularin were added to prepare a solution with 0.50.1 ng/mL of each. The solution was then filtered through the 0.45 μm glass fiber filter and repeated analysis was conducted to measure the recovery rate for each toxin. As shown in Table 4, the recovery rates for microcystin -LR, -RR, and -YR and nodularin were 113.7%, 70.3%, 103.7% and 83.9% respectively. The recovery rates for the three types of microcystin toxins in the conventional SPE method were reported to be 70% to 110%.[15,16)]

Table 4. Validation results of the analytical method

Compound	Fortified Amount (μg/L)	MDL (μg/L)	PQL (μg/L)	Recovery (%)	RSD (%)
Microcystin-LR	0.1	0.009	0.03	113.7	2.5
Microcystin-RR	0.1	0.013	0.04	70.3	5.3
Microcystin-YR	0.1	0.035	0.11	103.7	10.9
Nodularin	0.1	0.009	0.03	83.9	3.7

MDL: $SD \times t = SD \times 3.14$, (n=7, 1-a=0.99), PQL: $SD \times 10$
(Ref: Standard Methods 20th Edition, 1030C Method Detection Level)

Also, as shown in Table 4, the degree of precision of this method was calculated to be 2.5-10.9%. The method detection limit (MDL) was 0.009-0.035 ng/mL and the practical quantitation limit (PQL) was 0.03-0.11 ng/mL. The MDL set forth in the WHO guidelines with respect to microcystin-LR is ten hundred times higher than what was achieved. Considering that the tentative guidelines observed overseas for microcystin-LR, such as 1 ng/mL in Australia, 0.3 ng/mL in Japan, 0.5 ng/mL in Canada, and 1 ng/mL by WHO, it was deemed that it was possible to apply the method suggested in this study for the purpose of monitoring of environmental samples at the detection limit proposed.

3.5. Application to environmental samples

The method was used on the samples collected from the water purification facilities in August. The treated water was not filtered additionally prior to the experiment, whereas the raw water and river water samples were treated in an ultrasonic extraction apparatus for 30 min before being filtered through a 0.45 μm glass fiber filter. Also, one sample of cyanobacteria from lake water that was separately stored was analyzed based on the sonicated extract as a comparison sample. The four target algal toxins detected in the raw and treated water from the water purification facilities and the river water were well below the quantitation limit and were considered to be not detected. On the other hand, molecular ions of microcystin-LR and -RR were detected in the sonicated extract of the cyanobacteria and were identified through a comparison of the mass spectrum ratio of the carbon isotope of the standard toxin (Fig. 2). However, as this sample was of cyanobacteria containing a large amount of water, and the accurate dilution ratio was not considered, quantitation was not performed based on the liquid concentration. It took approximately 16 hours to complete the calibration curve and analysis of the blank sample and all the samples. It was determined that the method could be used to rapidly analyze a large number of samples, to reduce the amount of labor and solvent necessary, and to contribute to making quick responses in the field.

4. Conclusion

It is difficult to forecast algal blooms; therefore, rapid diagnosis of cyanotoxins produced by cyanobacteria is an important element in making quick responses at water intake and purification facilities. In this study, a combination of the online pre-concentration and injection method and the high-resolution, full-scanning mass spectrometer method was used to assess algal toxins including microcystin-LR and applied to environmental samples. Based on the results, the following conclusions were reached:

Microanalysis can be performed without a complex pre-treatment procedure. The online pre-concentration method produces 200 times the concentration effect compared to the solid-phase extraction method, even with a small sample of 1 mL. When combined with the high-resolution, full-scanning mass spectrometer method, the method produced a linearity that was equivalent to that of the SPE and LC-MS/MS method. The recovery rate was over 70%

and the degree of precision was within 10%. At the same time, the method detection limit (MDL) and the practical quantitation limit (PQL) were determined to be 0.009-0.035 ng/mL and 0.03-0.11 ng/mL, respectively. Based on these results, it was deemed to have the same microanalysis performance as the conventional method.

The application of the online pre-concentration method decreased the analysis time at least by 80% compared to the conventional method and also reduced the amount of labor, solvent, and solid-phase cartridge cost required. Productivity was further enhanced with more samples and, thus, it is expected to substantially improve economic efficiency.

Combining the instrumental analysis with the use of a high-resolution, full-scanning mass spectrometer makes it possible to detect non-target compounds. Thus, this method could be utilized for retrospective search and simultaneous quantitation of algal toxins with similar physicochemical properties such as anatoxin (mol. wt.: 165) and aplysiatoxin (mol. wt.: 672).

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