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

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Key Words
EQuan, Exactive, Orbitrap, algal toxins, cyanobacteria, water analysis, online concentration

Goal
To develop a column-switching technique based on online preconcentration and high-resolution, full-scan Thermo Scientific™ Orbitrap™ mass spectrometry to obtain fast and accurate results for the determination of algal toxins in drinking water.

Introduction
When the density of the colonies of *Microcystis* and *Nodularia* cyanobacteria surpass a certain level, they produce hepatotoxic substances called microcystins and nodularins, respectively,[2] while *Anabaena* and *Aphazinomenon* are known to produce a neurotoxin called anatoxin.[3] 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 (Figure 1).[4,5] Moreover, nodularins produced by *Nodulariais* are peptide-based hepatotoxins similar to microcystins.

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.[1] Prior research has shown that the microcystins -YR, -RR, and -LR (Figure 1) 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 in Korea as part of a candidate list for drinking water standards.

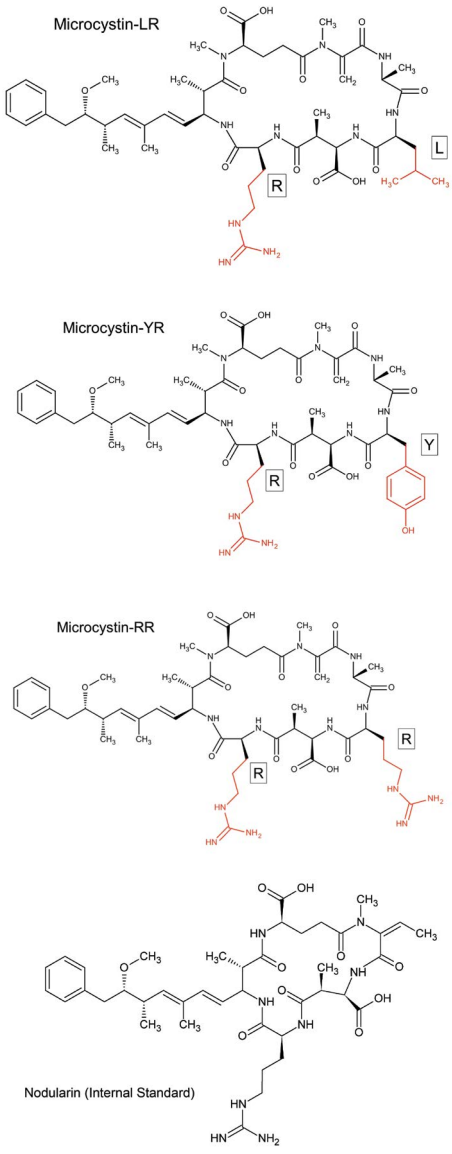

Figure 1. Structures of the cyclic peptide microcystins and nodularin

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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) or photodiode array detection. More recently, the analysis time has been reduced and the sensitivity improved through the use of liquid chromatography–mass spectrometry (LC-MS/MS) applying electrospray ionization (ESI).[7-13] The conventional SPE process required for all of these methods uses a great deal of time and solvent.

An online preconcentration and injection method can shorten the sample pretreatment process and help detect trace amounts of target substances, while an Orbitrap-type high-resolution mass spectrometry method takes into account the retrospective aspect of data, making possible both 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 by reducing the run time and the amount of solvent necessary. The method was applied to raw and treated water from water purification plants and river systems.

Experimental

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. Solvents were of residual pesticide grade. Water was double distilled by reverse osmosis.

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

Compound	Name (CAS)	Formula	Molecular Weight
Microcystin	Microcystin-LR (101043-37-2)	$C_{49}H_{67}N_{10}O_{12}$	995.1717
	Microcystin-RR (111775-37-4)	$C_{49}H_{75}N_{13}O_{12}$	1038.1997
	Microcystin-YR (101064-48-6)	$C_{52}H_{72}N_{10}O_{13}$	1045.1873
Nodularin	Nodularin (118399-22-7)	$C_{41}H_{60}N_8O_{10}$	824.9627

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 1000 pg/mL. An external standard method was used for calibration curve verification and sample identification. Then, the ratio of peak areas according to the concentration of standard solution were calculated.

Sample Collection and Storage

A total of 173 raw and treated water samples were collected from 59 facilities at the Han (18 sites), Nakdong (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 three days of delivery.

Pretreatment and Instrumental Analysis

Online preconcentration 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™ preconcentration column (20 x 2.1 mm, 12 µm particle size) and a Thermo Scientific™ Hypersil GOLD™ analytical column (50 x 2.1 mm, 1.9 µm particle size) was used. The allowable liquid sample injection range was 1 to 20 mL, and in 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). 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 2. EQuan MAX chromatography conditions used

Pump 1				Pump 2			
Hypersil GOLD aQ (preconcentration column)				Hypersil GOLD (analytical column)			
Time	%A	%B	µL/min	Time	%A	%B	µL/min
0.00	98	2	1000	0.00	98	2	400
1.01	98	2	1000	1.00	98	2	400
1.20	98	2	100	2.00	2	98	400
3.50	98	2	100	3.50	2	98	400
4.00	98	2	1000	3.51	98	2	400
				4.00	98	2	400
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							

Table 3. Exactive Orbitrap MS operating conditions

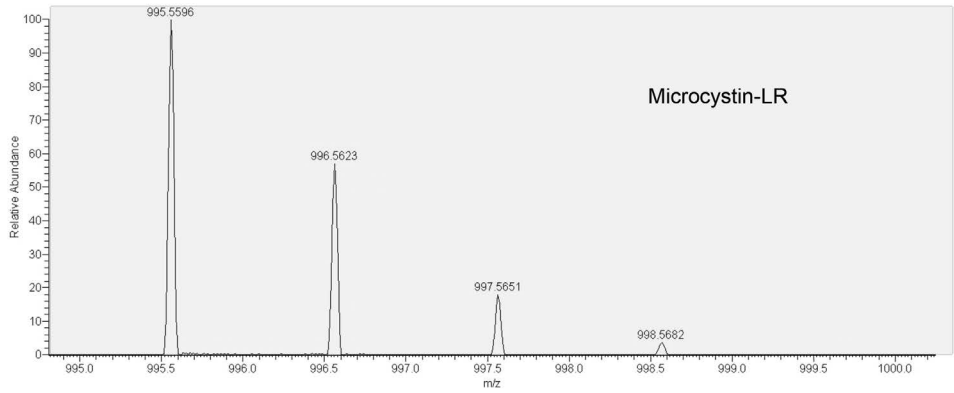
Parameter	Setting
Scan range	*m/z* 150–1100
Resolving power	50,000 (FWHM at *m/z* 200)
Polarity	Positive
Measured *m/z*	995.5543 MC-LR 519.7898 MC-RR 1045.5344 MC-YR 825.4501 Nodularin
Ionization source	Electrospray
Spray voltage	4000 V
Capillary temperature	340 °C
Capillary voltage	37 V
Tube lens voltage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

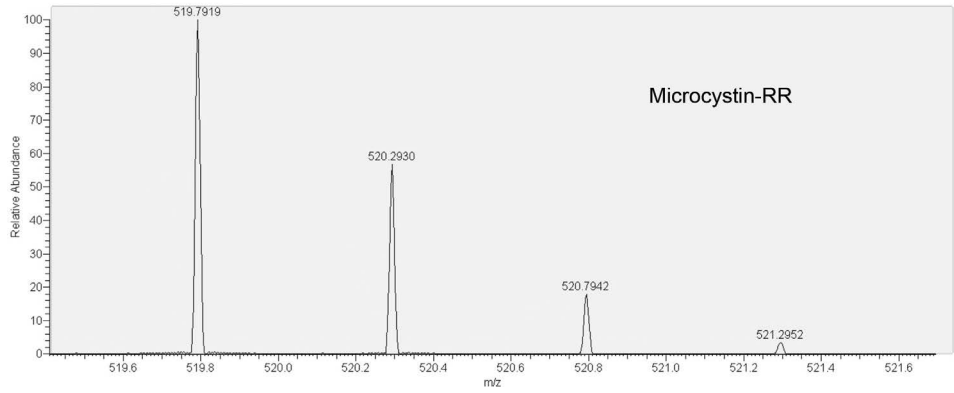
Results and Discussion

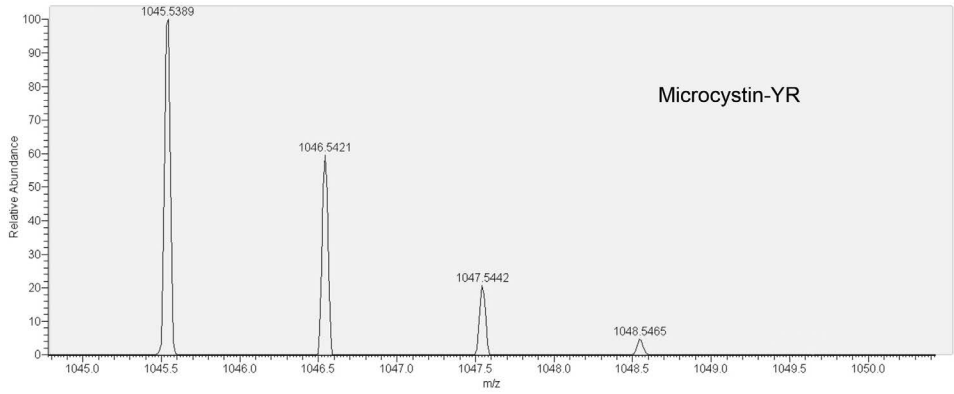
High-Resolution Mass Spectra of Toxins

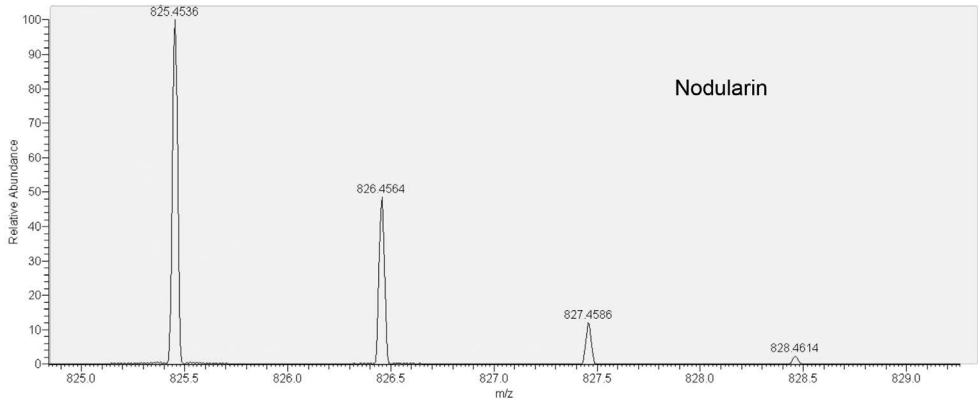
The standards were prepared at a concentration of 1 ng/mL each and injected using a syringe pump to observe the mass spectra. The molecular ion and carbon isotope spectra of microcystin-LR, -RR, -YR, and nodularin are shown in Figure 2a. Four carbon isotopes were observed for most compounds. Using this isotopic pattern, it was 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 (Figure 2b).

A


995.5596
996.5623
997.5651
998.5682
Microcystin-LR
Relative Abundance
m/z


519.7919
520.2930
520.7942
521.2952
Microcystin-RR
Relative Abundance
m/z


1045.5389
1046.5421
1047.5442
1048.5465
Microcystin-YR
Relative Abundance
m/z


825.4536
826.4564
827.4586
828.4614
Nodularin
Relative Abundance
m/z

B

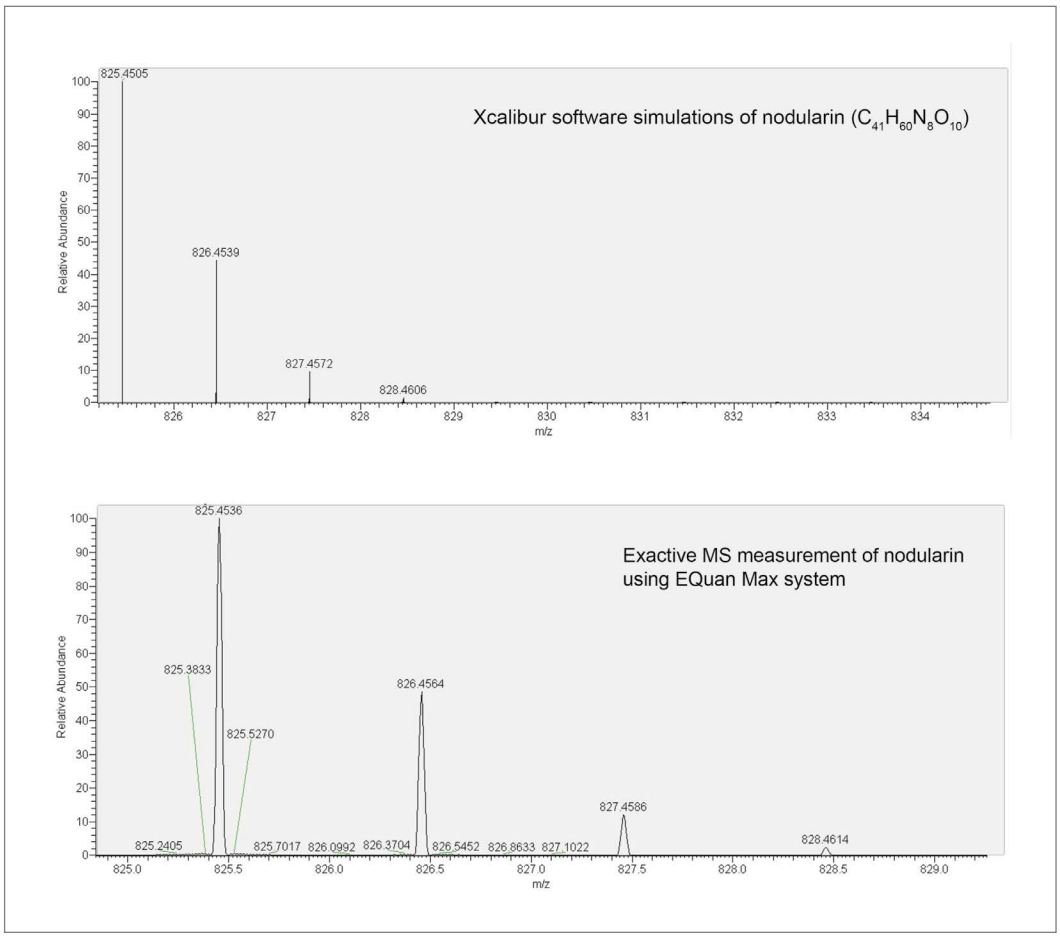


Figure 2. A) Carbon isotope patterns by high-resolution, full-scan MS of microcystins and nodularin, and B) simulated spectrum of nodularin (top) compared to actual spectrum (bottom), confirming isotope pattern.

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. 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.

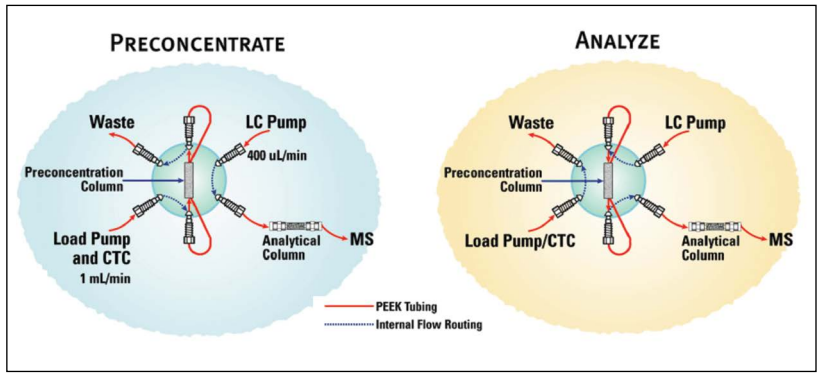


Figure 3. Switching column method for on-line sample injection

Optimization of the Online Preconcentration Method

In this study, 1 mL of each sample was used for the online preconcentration 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. The valve was then switched to postion 2 for elution from the SPE column onto the analytical column using 98% acetonitrile. A summary of the analysis flow, including online SPE, is shown in Figure 3.

A comparison of the absolute amount introduced into the mass spectrometer comparing online and offline SPE shows that online SPE has the same concentration-injection effect as pretreating and concentrating a 200 mL sample into 2 mL and injecting 5 µL of the preconcentrated sample. Thus, it is possible to perform a direct injection, online SPE with small volumes microanalysis without a separate using a large offline, pretreatment step. Also, this method uses UPLC-based chromatography and sharp peaks are obtained, as shown in Figure 4.

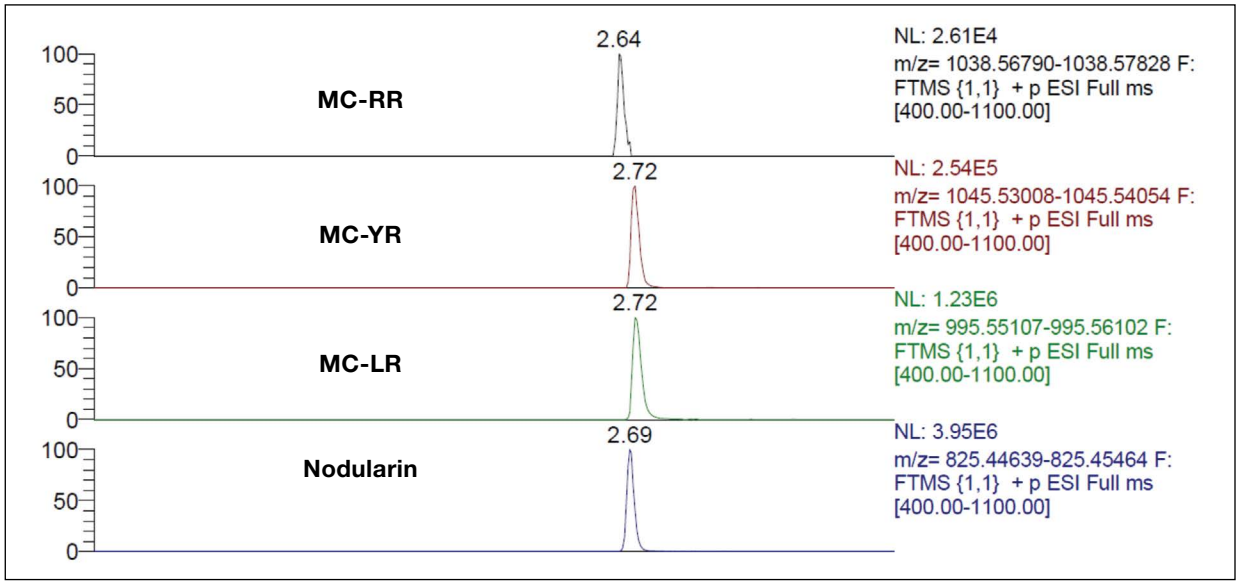


Figure 4. Extracted chromatograms from full-scan data by UHPLC-Orbitrap mass spectrometer

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

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. Therefore, 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 (Figure 4).

Compared to the conventional SPE method, which requires the use of 0.5 to 1 L sample, the online injection method effectively reduced 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 required 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 80% time savings. Other benefits of using this rapid pretreatment method include enhanced productivity when there is a large amount of sample, reduced use of organic solvents, reduced labor for the pretreatment process, and omission of a nitrogen concentration apparatus.

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 Figure 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.[13] Also, it was deemed possible to perform a linearity assessment 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 preconcentration high-resolution full-scan MS method has the equivalent trace quantitation capacity as the conventional method of solid-phase extraction and LC-MS/MS.

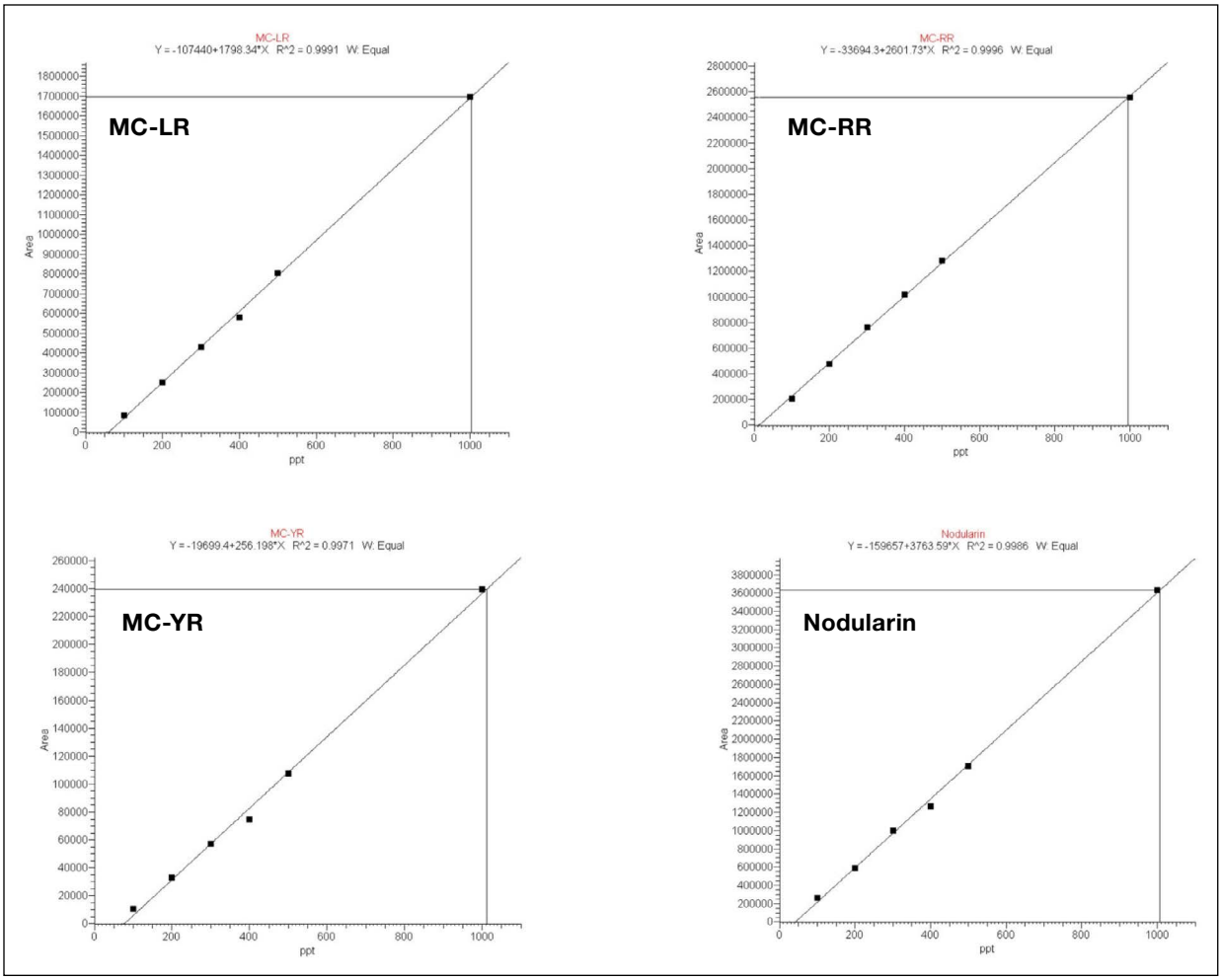


Figure 5. Calibration curve of microcystin-LR, RR, YR and nodularin

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 Daecheong Dam in which the target toxins were not detected. Then, microcystin-LR, -RR, and -YR and nodularin were added to prepare a solution with 0.1 ng/mL of each. The solution was then filtered through the 0.45 μm glass fiber filter and repeated analyses were 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%.[13,14] 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 0.15–0.51 ng/mL. The MDL set forth in the WHO guidelines with respect to microcystin-LR is a hundred times higher than what was achieved. These results are well below the guidelines set forth 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.

Table 4. Validation results of the analytical method

Compund	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 x t = SD x 3.14, (n=7, 1-a=0.99), PQL: SD x 10

(Ref: Standard Methods 20th Edition, 1030C Method Detection Level)

Application to Environmental Samples

The method was used on the samples collected from the water purification facilities. 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. 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 were detected in cyanobacteria lake water sample and were identified through a comparison of the mass spectrum ratio of the carbon isotope of the standard toxin (Figure 2). 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.

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-scan mass spectrometry 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 pretreatment procedure. The online preconcentration 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-scan mass spectrometry 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 performance as the conventional method.
- The application of the online preconcentration method decreased the analysis time 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 high-resolution, full-scan mass spectrometry 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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