Using Solid Phase Extraction Disk Technology to Research Optimal Concentration Methodology for Analysis of Microcystins Found in Natural Tsukui Lake Waters and Drinking Water by Liquid Chromatography

Global monitoring of microcystin congeners is necessary to maintain a safe environment that is low in toxicity for our global food and water supplies. Multiple methods exist that demonstrate a wide range of SPE methodologies for concentrating large volume water samples prior to HPLC analysis, but for the typical laboratory, a series of experiments provide data to support that using both disk SPE and automated disk SPE achieve the greatest efficiency with high recoveries and effective reproducibility for spiked laboratory purified water samples and Tsukui Lake water samples from Tsukui County in Kanagawa, Japan.

> *"Renewed global attention is focused on monitoring cyanobacteria, specifically microcystins, to maintain a safe environment that is low in toxicity for our global food and water supplies."*

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Introduction:

Blue-green algae, also known as cyanobacteria, is commonly found in many global sources of freshwater and can be visibly detected as natural algae blooms upon accumulation.[1-4] Cyanobacteria has been widely studied to determine toxin levels and assess the impact on aquaculture and their lifecycles. Not all blue-green algae blooms are toxic, and through scientific research, it has been determined that from 25 – 75% of the cyanobacteria blooms tested are considered to be categorised as toxic.[5]

All microcystins are cyclic hepatotoxins produced by cyanobacteria that are most commonly located in surface water.[1] There are multiple congeners of microcystins, each with slight variations from one another, including a pair of L-amino acids. All congeners contain a total of seven common amino acids.[6,7] Microcystins are toxic and common, often affecting the liver and digestive system within the human population when ingested through contaminated drinking water and/or contaminated food sources, such as fish.[8,9]

To reduce the health impacts of this serious hepatotoxin impacting global animal and human health, the WHO has published a recommended guideline to limit microcystins in drinking water to 1 µg/L microcystin-leucine (L) arginine (R) for short term exposure and 0.1 µg/L for long-term exposure. In addition, the USEPA is considering adding microcystins to the UCMR 4 list of candidates for contaminant monitoring of public water systems under EPA Method 544, which is currently under development. In this EPA method, six microcystin congeners (LR, YR, RR, LA, LF, and LY) are proposed for screening.[10,11]

Routine testing of microcystins in drinking water commonly involves the use of SPE to concentrate a large volume of water sample for low level detection of the microcystin congeners. Significant research provides recovery and reproducibility of microcystin congeners using a variety of SPE techniques, such as on-line SPE, cartridge SPE, and disk SPE. For the typical laboratory that performs multiple large water contaminant methods, a series of experiments outlined in this research provides data to support that higher recoveries and more efficient reproducible concentration of both spiked laboratory purified water samples and Tsukui Lake water samples from Tsukui County in Kanagawa, Japan are obtained from using disk SPE and automated SPE-DEX 4790 disk SPE prior to HPLC analysis.

Materials & Methods:

Reagents

All reagents used were laboratory grade.

- Reagent water
- Methanol
- Acetone
- Potassium dihydrogen phosphate solutions (KH_2PO_4)
- Phosphoric acid
- Trifluoroacetic acid (0.1%)
- Sodium hydroxide
- Hydrochloric acid
- Purified nitrogen

Water Samples & Spiking Levels

A range of volumes and spiking levels were used in a variety of purified water samples (0.5 to 2.5 µg/L spiking level) and Tsukui Lake water samples from Tsukui County in Kanagawa, Japan (2.5 µg/L spiking level).

Microcystin Congener	CAS Registry Number
Microcystin-LR	101043-37-2
Microcystin-RR	111755-37-4
Microcystin-YR	101064-48-6

Table 1: Microcystin Congener Compounds Used for Spiking.

Solid Phase Extraction Methodologies & Experiments

Several experiments were performed using a matrix of SPE disk sorbents and parameters to determine the optimal set of conditions for microcystin recovery, efficiency, and reproducibility using both automation and manual techniques. Figure 1 provides the full step-by-step details of the automated and manual SPE methods performed prior to the evaporation and analysis steps. A range of disks from the Atlantic™ C18 to the 3M™ Empore™ were used for the different experiments (Figure 2).

- Automated Disk SPE System & Disk Details
  - o SPE-DEX® 4790 Automated Extractor System
  - o Envision® Platform
  - o Atlantic C18 SPE Disks (47mm)
  - o DryVap® Concentrator System
  - o DryDisk® Separation Membranes
- Manual Disk SPE Disk Details
  - o Manual SPE Vacuum Manifold
  - o 3M Empore C18-SD, C18-FF SDB-XC, and SDB-XD Disks

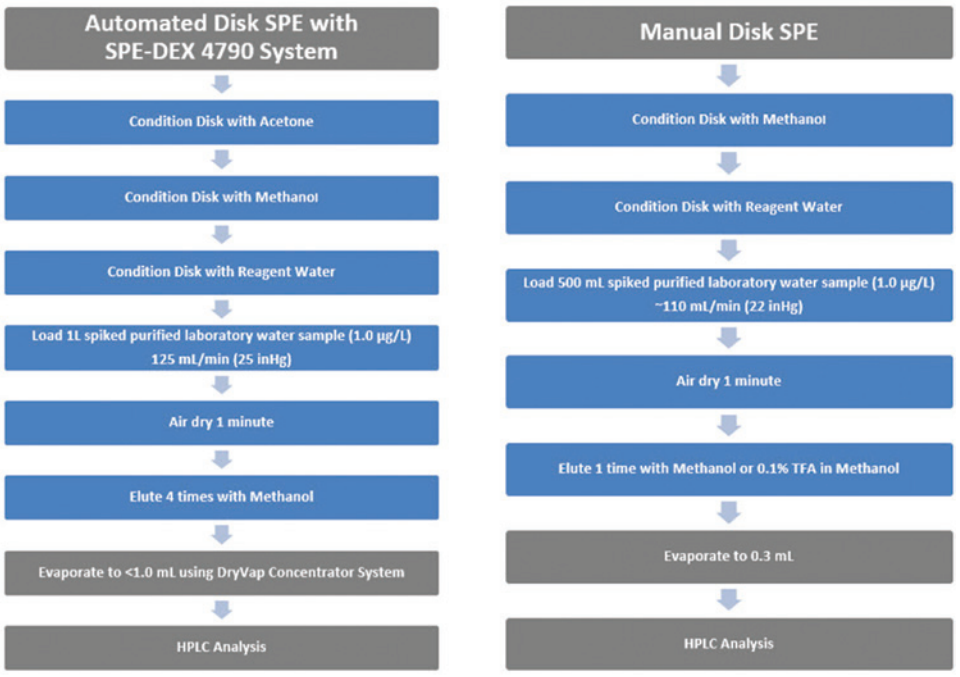

Figure 1: Automated Disk SPE with SPE-DEX 4790 System Methodology vs. Manual Disk SPE Methodology.

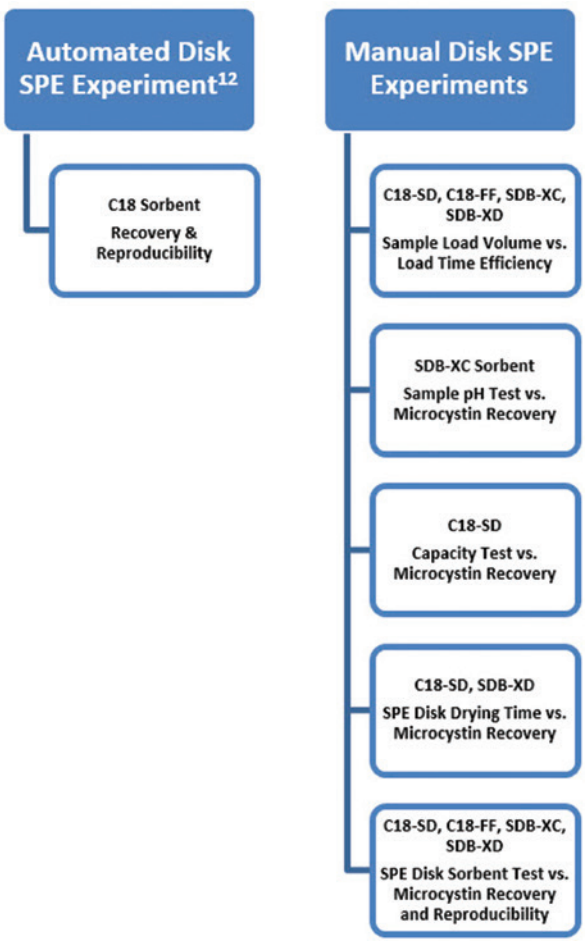

Figure 2: List of Automated and Manual Disk SPE Experiments.

HPLC Analysis Conditions

Automated disk SPE analysis and manual disk SPE analysis were performed separately using slightly different HPLC conditions. See Table 2 for the HPLC details.

	Automated Disk SPE HPLC Conditions	Manual Disk SPE HPLC Conditions
HPLC System	LC 600	HP 1090
HPLC Column	Labtech C18 0.250 mm x 4.6 mm x 0.5 um	ODS 4.6 mm x 150 mm
Column Temperature	40° C	40° C
Mobile Phase	43/57 0.05 mol/L KH_2PO_4 (pH 3.0)/ MeOH	50/50 50mM KH_2PO_4 (pH3.3)/ACN
Flow Rate	1.0 mL/min	1.0 mL/min
Injection Volume	20 µL	20 µL
UV Detection Wavelength	238 nm	238 nm

Table 2: Automated Disk SPE and Manual Disk SPE HPLC Analysis Conditions

Results:

Chromatography

Microcystin standard solution (1 ppb) was injected to identify the three congeners (microcystin-RR, microcystin-YR, and microcystin-LR) and calculate recovery. The total run time to elute all three microcystins was 15 minutes (figure 3). The various experiments were established to test from one to all three of the congeners and provide a comparison of SPE disk sorbent material and efficiency with time and reproducibility between manual and automated SPE techniques.

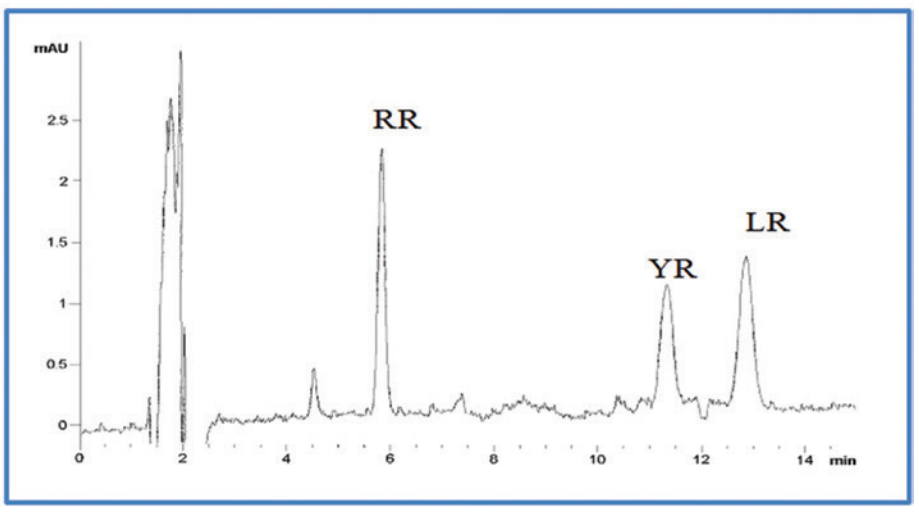

Figure 3: Example Microcystin Standard Solution Containing Three Congeners: Microcystin-RR, Microcystin-YR, and Microcystin-LR.

Automated Disk SPE Experiment – Using the SPE-DEX 4790 System for Recovery and Reproducibility[12]

Microcystin-LR Replicates	Recovery
1	99.57
2	97.26
3	98.05
4	98.78
5	99.73
Average	98.67
Standard Deviation	.0104
Relative Standard Deviation (%)	1.05

Table 3: Automated Disk SPE Recovery and Reproducibility Results of Microcystin-LR Replicates.

Microcystin-LR, being one of the most toxic congeners, was selected for disk SPE automation to determine recovery and reproducibility at the WHO levels of 1.0 µg/L. Five replicate 1L samples were processed. The Atlantic C18 disks (47mm) were used in combination with the SPE-DEX 4790 system for concentrating the 1L spiked laboratory water samples. Following SPE, the collected extract was evaporated using the DryDisk separation membrane in combination with the DryVap Concentrator System.

Currently there are no formal regulated automated disk SPE methods for processing large volume water samples for microcystin analysis; however, results are within typical recovery and reproducibility ranges for regulatory methods. Recoveries from automated disk SPE ranged from 97.26% to 99.73%, with a %RSD value of 1.05% (table 3). Based on the recovery data for five replicate samples processed using the automated SPE-DEX 4790 with the Atlantic C-18 disks, the values show strong correlation between samples processed, removing technician variability typically present with manual SPE methods. Low %RSD values indicate the Atlantic C-18 disks have optimal capacity and retention of microcystin-LR using automation for the SPE process.

Manual Disk SPE Experiments

Manual Sample Load Volume vs. Load Time Efficiency

A series of SPE disks were tested to determine load time efficiency at 22 in-Hg for different volumes of environmental water samples. Volumes from 50 – 500 mL were loaded to a SPE disk, and the time to achieve full sample loading was tracked. The greatest load efficiency was achieved using small sample volumes for all cartridges. When large volume sample loading is required, the SDB-XD disk was able to process 500 mL in less than 5 minutes, while the C18 disk required more than 20 minutes to process the same sample volume (figure 4).

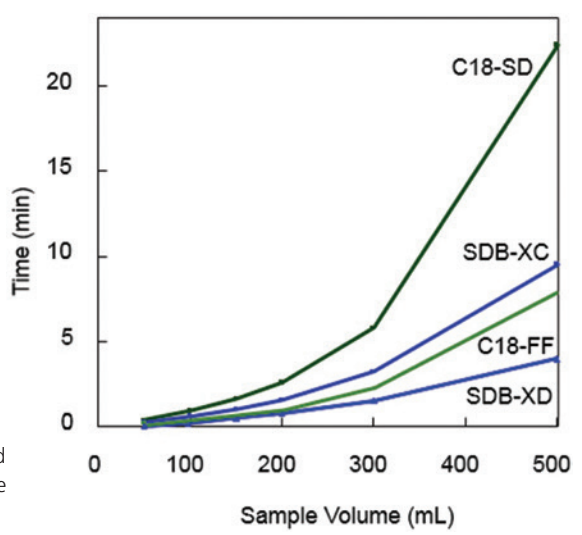

Figure 4: Manual Sample Load Volume vs. Load Time Efficiency.

Manual Sample pH Test vs. Microcystin Recovery

The SDB-XC disk was chosen for testing recovery of both microcystin-RR and microcystin-LR in the presence of acid or base conditions. The 500 mL spiked water samples were first adjusted for pH of 2, 3, 5, 7, 9, and 10 using hydrochloric acid and sodium hydroxide. Spiked water samples that were adjusted to pH 9 showed a slight decrease in recovery for microcystin-RR concentrated through the SCB-XC disk (figure 5). A recovery increase above 100% at pH 2 was obtained, and the use of a different disk sorbent material for low pH water samples may show more consistent recoveries near 100%.

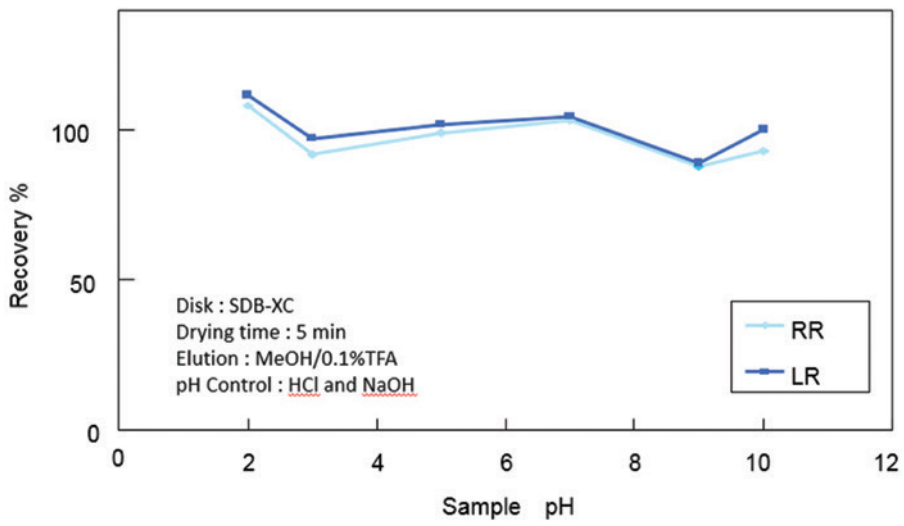

Figure 5: Manual Sample pH Test vs. Microcystin Recovery.

Manual Capacity Test vs. Microcystin Recovery

The capacity test, or ability for a disk to hold both large volume and high concentration of compound while maintaining an acceptable recovery, is an important test for determining a method's range of operation. The C18-SD disk was chosen for this test, despite the inefficiency of the disk for volume loading above 300 mL (figure 4). The benefit of using C18-SD is its ability to hold an even capacity for microcystin-RR across a wide volume loading range of 200 mL to 2000 mL, as well as a wide microcystin congener concentration range from 0.5 ppb to 2.5 ppb (figure 6).

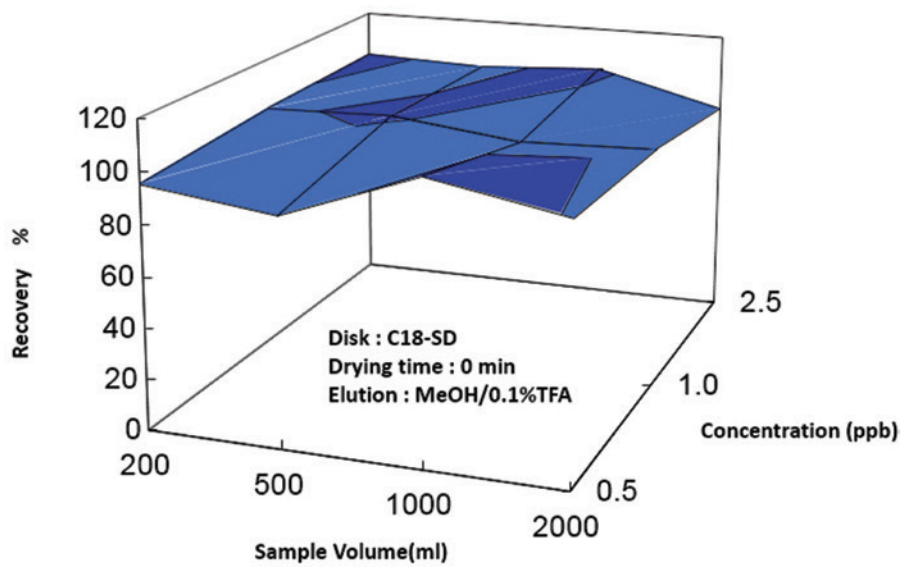

Figure 6: Manual Capacity Test vs. Recovery.

Manual SPE Disk Drying Time vs. Microcystin Recovery

The importance of drying time in the SPE method is critical for optimal recovery of microcystin congeners. General SPE disk drying is important for removal of water prior to elution to aid in efficient evaporation prior to final analysis; however, too much drying time prior to elution can reduce recovery values. A drying time test was performed on the two most effective SPE disks – C18-SD and SDB-XD. All three congeners were tested over a range of drying times from 0 to 80 minutes. Results show high recoveries from no drying to a 5 minute minimal drying time. Drying times greater than 5 minutes show slight reduction in recoveries, with the SDB-XD providing more consistent recoveries for all three microcystin congeners over the C18-SD SPE disk (figure 7).

Manual SPE Disk Sorbent Test vs. Microcystin Recovery and Reproducibility

SPE Disk 3M™ Empore™	Microcystins	Drying Time(min)					
		0	5	10	20	40	80
C18 SD	RR	100	103	85	86	78	39
	YR	101	95	83	82	64	22
	LR	99	103	89	89	70	30
SDB-XD	RR	95		84			71
	YR	100		95			70
	LR	102		99			83

Figure 7: Manual SPE Disk Drying Time vs. Microcystin.

Sorbents were tested for optimal recovery and reproducibility of microcystin RR, microcystin YR, and microcystin LR by testing the elution solvent and comparing the recovery and reproducibility for each solvent used. Methanol and methanol + 0.1% TFA elution solvents were used to compare the three sorbents: C18, SDB XC, and SDB XD. Even a small acidic component to the elution can generate significant recovery differences.

Both C18 and SDB XC sorbents required methanol + 0.1% TFA to generate adequate recoveries (>90%). Recoveries were very comparable between the two sorbents. With acidified methanol, the C18 sorbent generated a mean microcystin congener recovery of 99%, while SDB XC generated a mean microcystin congener recovery of 96%. Reproducibility (%RSD) results from both C18 and SDB XC sorbents were <7% for each microcystin congener.

The SDB XD sorbent reacted differently, generating acceptable recoveries with both elution solvents. The highest mean microcystin congener recovery (96%) was generated using methanol without acidification. With acidified methanol, the mean recovery of microcystin congeners was 90%. Slightly higher reproducibility results were generated using the SDB XD sorbent when compared to the C18 and SDB XC sorbents, with the %RSD results <12% for each microcystin congener.

The diversity of the SDB XD was tested with a sample of spiked Tsukui Lake water to verify that methanol would generate acceptable and comparable recoveries and reproducibility for all microcystin congeners. Results confirm that SDB XD with methanol as the elution solvent generates a mean 95% recovery of all microcystin congeners in Tsukui Lake water compared to the 96% recovery from laboratory pure water. Reproducibility for all microcystin congeners was <4% using the SDB XD with the spiked Tsukui Lake water sample.

Disk Type	Sample	Elution	Microcystins Recovery (%RSD)		
			RR	YR	LR
C18-SD	Pure Water	MeOH	ND	14(65)	11(23)
	↓	MeOH/0.1% TFA	97(3.8)	99(3.9)	106(6.1)
SDB-XC	↓	MeOH	ND	ND	ND
	↓	MeOH/0.1% TFA	96(4.6)	93(5.8)	100(3.9)
SDB-XD	↓	MeOH	92(11.1)	96(5.1)	98(5.0)
	↓	MeOH/0.1% TFA	91(5.2)	90(11.8)	90(6.7)
SDB-XD	Tsukui Lake Water	MeOH	95(2.4)	99(1.7)	95(3.4)

Figure 8: Manual SPE Disk Sorbent Test vs. Microcystin Recovery and Reproducibility.

Conclusion:

Renewed global attention is focused on monitoring cyanobacteria, specifically microcystins, to maintain a safe environment that is low in toxicity for our global food and water supplies. The WHO has established safe microcystin levels of 1 µg/L in drinking water, with the USEPA considering adding microcystins to the UCMR 4 list of candidates for contaminant monitoring of public water systems. Using SPE disks, testing of 500 mL and 1 L samples of natural water has been shown to be efficient and effective to confirm presence and screen for levels of the most toxic form – microcystin-LR.

Automated disk SPE was efficiently performed without manual technician bias using the Atlantic C18 disks in combination with the SPE-DEX 4790 system for concentrating the 1L spiked laboratory water samples. Mean recovery results using methanol elution were 98.67%, with a %RSD value of 1.05%, indicating the Atlantic C-18 disks have optimal capacity and retention of microcystin-LR using automation for the SPE process.

Manual disk SPE was also used for concentrating microcystin water samples, using 3M Empore C18-SD, C18-FF SDB-XC, and SDB-XD disks with a manual vacuum manifold. It is important to note that sorbent materials vary by manufacturer, and this was evident in the manual SPE disk results. A wide range of tests for load volume, pH, capacity, drying time prior to elution, and sorbent comparison were performed to generate the optimal capacity and retention of three microcystin congeners (microcystin-RR, microcystin-YR, and microcystin-LR). Optimal conditions were determined at 2.5 µg/L with 500 mL of laboratory pure water and spiked Tsukui Lake water sample using the SDB-XC sorbent. Both methanol and acidified methanol generated acceptable recoveries; however, methanol generated the optimal mean microcystin congener recoveries of 95% in Tsukui Lake water compared to the 96% recovery from laboratory pure water.

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