Analysis of Microtoxins in the Nakdong River Watershed

Jung Min Park[1], Jae Jung Lee[1], Dong Jin Hwang[1], Sang Yong Yang[1], Sang Hak Lee[2] and In Kyu Jung[2]

[1]*Nakdong River Water Environment Laboratory, NIER, Ministry of Environment, Taegu, 702-170, Korea*
[2]*Department of Chemistry, Kyungpook National University, Taegu, 702-701, Korea*

The different methods such as HPLC, indirect- and direct-ELISA were employed for the analysis of microtoxins and the results of each method were compared in terms of the detection limit and accurary. Three toxins, microcystin-RR, -LR and -YR were clearly separated by HPLC using 0.05 M methanol and phosphate buffer used as a solvent system. The calibration curves for the toxins were linear in the range of 5 ng to 50 ng. The standard curves for the immunoassay of microcystin obtained by direct and indirect ELISA are compared. The linear responses of inhibitions of binding by microcystin in the direct and indirect competitive ELISA were in the range of 10 ng to 1000 ng and 50 pg to 160 pg, respectively. Distribution of microtoxins at 11 sites in the Nakdong river and several lakes in Korea was also studied. The most dominant microcystin variant in the test sites was found to be microcystin-RR.

Key words : HPLC, ELISA, Microtoxins, SPME

1. Introduction

Since the first studies on algae bloom was reported in 1870 in Australia due to the death of the animals caused by Nodularia, many other research have been done in America, Canada, England and Japan. The material that is the cause was found to be Microcystin[1)]. In Korea since July 1992 in the Nakdong River, every year in the summer months there has been bloom caused by algae and in 1993 in the Daecheong Lake Microcystis has been detected up to 4.6×108 cell/㎖ [2)]. Until now there were no reports of animals or people harmed by the abnormal increase of the algae but in 1996 a minute amount of peptide toxins was detected in the tap water purified from the water of the Nakdong river and cause a lot of arguments on the issue of the safety of tap water.

The abnormal increase of the algae has become a serious problem. The cause of biological odor in the tap water was growth of algae and bacteria and the decomposition of animal remains and the discharge of waste. And the growth of algae in the water and the strange taste and odor in relation with these causes has long since been reported[3-4)]. These materials are caused by various types of bacteria and takes on the odor of dirt or mold according to the concentration. In this study samples of algae and water in the downstream areas of the Nakdong river were taken and various extraction methods were used to detect microcystin and the distribution characteristics of microcystin in the Nakdong river watershed were examined.

Corresponding Author ; Jungmin Park, Nakdong River Water Environment Laboratory, NIER, Ministry of Environment, Taegu, 702-170, Korea
Phone : +82-53-944-0592
E-mail : shalom@me.go.kr

2. Experimental Methods

2.1 Solid Phase Extraction

Solid Phase Extraction was done using the Tekmar AutoTrace SPE(Solid Phase Extraction) Workstation. Preprocessing was done using 6 ㎖, 1000 ㎎, C18 cartridge and 6 ㎖, 1000 ㎎, C8 cartridge, 6 ㎖, 1000 ㎎, CN cartridge, 6 ㎖, 1000 ㎎, Si cartridge, and in the case of the C18 cartridge the sample loading were done at rates of 2, 5, 10, 15 ㎖/min. When using the CN cartridge 0.5M acetic acid was used instead of methanol for the conditioning and 30% acetonitrile was used.

2.2 TLC method

Ethylacetade-isopropane-water was used for the solvent and the detection was done in a capped

glass bottle of about 500 ㎖ and then adding iodine. The TLC plate was placed in the glass bottle with the iodine and the distance when changing to brown were measured.

2.3 HPLC method

The HPLC method was based on the Harada method[5]. The conditions of solvent and column are shown in Table 1. The microcystin in the sample was identified based on a comparison with the retention time of the standard material. The concentration of microcystin in the sample was calculated from the peak area using a standard curves.

Table 1. HPLC operation condition

Model	Waters 600 Controller, Waters pump, Waters 996 PDA detector
Column	μBondapakTM C18 10 ㎛(3.9×150mm)
Mobile phase	Methanol : 50μM Phosphate buffer = 52 : 48(at pH 3.0)
Flow rate	1.0 ㎖/min
Injection volume	10 ㎕
Detection wavelength	238 nm
Detection limit	Microcystin-RR : 2.00 ng Microcystin-YR : 3.03 ng Microcystin-LR : 2.40 ng(S/N = 3)

2.4 ELISA

This study used the ELISA plate commercialized kit developed by Mitsubishi.

3. Results and Discussion

3.1 Solid phase extraction

Solid phase extraction was used to extract 3 different types of microcystin. 1 ㎖ of 2ppm standard microcystin mixture was added to 1000 ㎖ of distilled water. Methanol was used for the extraction solvent(in the case of the CN cartridge 30% acetonitrile) and comparison was done between the four phase types, C18(Octadecyl bonded silica), C8(Octyl bonded silica), Si and CN. The final amount was decided to be 2 ㎖. The recovery rate was different according to the polarity of the solvent and material that was examined. 100 % recovery rate was set to be the peak area achieved by not preprocessing using SPE and adding 1ppm standard mixture directly into HPLC. When using the C18 cartridge the sample was loaded into the SPE at rates of 2, 5, 10, 15 ㎖/min. Other experiments were done at a fixed rate of 5 ㎖/min and was preprocessed. The average recovery rate and %RSD for each cartridge is shown in Table 2.

When using the C18 cartridge and differing the loading rate, micocystin-RR showed an average recovery rates of 99.18, 93.62, 88.06, 88.63 % showing that the rate fell when the speed was increased. microcystin-YR showed rates of 91.34, 90.65, 90.78, 92.14 % showing that it was relatively not affected by the loading speed while microcystin-LR showed rates of 96.55, 96.04, 91.89, and 91.71%. The %RSD was good at a fixed loading rate of 15 ㎖/min for the three different types of microcystin than other loading rates. The C8 cartridge showed that the average recovery rate and %RSD was lower than the C18 cartridge. The Si cartridge showed an average recovery rate for microcystin-RR, microcystin-YR, microcystin-LR at 12.81, 22.09, 11.74 %, respectively and the %RSD at 19.48, 13.66, 19.24 %, respectively.

The efficiency fell significantly compared with the C18, C8 cartridge even though the Si cartridge was a polar cartridge type. All of the experiment conditions were the same but the difference was thought to be because the cohesion between microcystin and Si is relatively weak. The CN cartridge had a recovery rate of over 100 % for all three types and the %RSD was also good at 2.12 ~ 3.43. This is because the arginine type guanidine of microcystin-RR and -LR has a resonant structure in acidic conditions. This resonant structure has a vary stable form. This guanidine in acidic conditions has positive ions and has a positive polarity which is attracted to the negative polarity of the cyano of the CN cartridge and thus is more attracted to the microcystin than the ODS cartridge.

3.2 TLC Method

The thin layer chromatography TLC was done using a Merck experiment TLC, a glass sheet coated with silica gel, cut 9cm by 3cm. The solvent used was ethylacetate : isopropanol :

Table 2. The average recovery rate and %RSD.

			Microcystin-RR	Microcystin-YR	Microcystin-LR
C18	15 mℓ/min	average recovery rate	99.78	91.34	96.55
		%RSD	2.11	1.99	2.15
	10 mℓ/min	average recovery rate	93.62	90.65	96.04
		%RSD	8.67	3.42	2.66
	5 mℓ/min	average recovery rate	88.06	90.78	91.89
		%RSD	5.24	3.51	3.44
	2 mℓ/min	average recovery rate	88.63	92.14	91.71
		%RSD	2.61	2.59	3.17
C8	5 mℓ/min	average recovery rate	84.56	88.67	92.87
		%RSD	7.91	5.72	5.28
Si	5 mℓ/min	average recovery rate	12.81	22.09	11.74
		%RSD	19.48	13.66	19.24
CN	5 mℓ/min	average recovery rate	100.7	101.33	100.16
		%RSD	2.96	3.43	2.12

water(3.5 : 2.5 : 6) and the result was microcystin -RR at 0.35, microcystin-YR at 0.48, and microcystin-LR at 0.54. The advantage of TLC is that if a proper solvent is selected the material may be detected very quickly with just a small amount.

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3.3 HPLC Method

The sample which was preprocessed using a solid phase extraction method was analyzed using HPLC. The HPLC method showed a retention time of 4.193min for microcystin-RR, 6.010min for microcystin-YR, and 7.710min for microcystin-LR. The retention time and the spectrum pattern at 238nm, was analyzed and measured. The chomatogram of the three types of microcystin using the analysis methods of the study are shown in Fig. 1. The calibration curve was obtained by making the microcystin mixture of 1, 3, and 5 ppm and then inserting 10 μℓ. Microcystin-RR showed that the r^2 was 0.9978, microcystin-YR was 0.9992, and microcystin-LR was 0.9999. This result showed a very good linearity. The detection limit when the S/N ratio was 3 was 0.13 mg/ℓ for microcystin-RR, and 0.18, 0.15 mg/ℓ for microcystin-YR and microcystin-LR respectively.

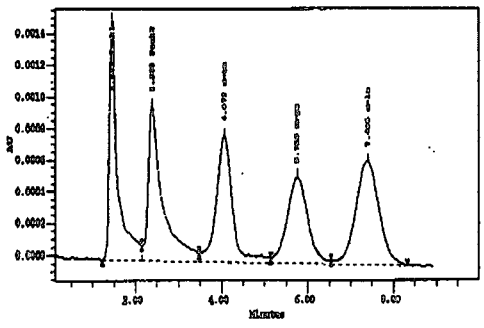

Fig. 1. HPLC chromatogram of Microcystin RR, -YR, -LR.

3.4 Enzyme Linked Immunosorbent Assay(ELISA) Method

The kit used in the ELISA method was a Mitsubishi product with the standard concentration for the calibration curve at 50, 100, 200, 400, 1600 ng/ℓ. The regression in the

Logistic line shows a satisfactory measurement line.

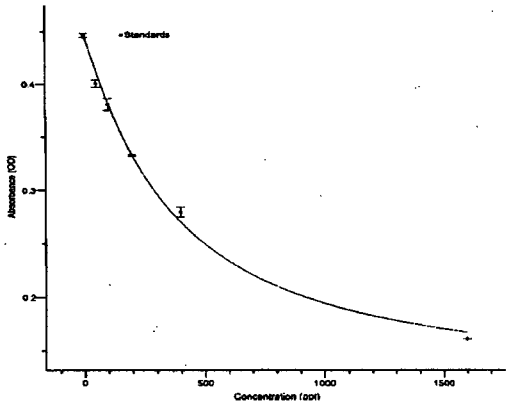

Fig. 2. Calibration curve for Microcystins.

3.5 Analysis of the Microcystin in research locations

When the lower limit of the measurement of the Algae toxic matter analysis using HPLC was set to 0.5 $\mu g/\ell$, none was detected in the lakes and streams during the inspection period. And in the case of the Algae materials, during the increase of algae in August the algae was gathered using plankton nets in the area of Nok San and Gangdong and freeze dried for analysis. The sample algae gathered at the GangDong area showed microcystin-RR at 167.3 $\mu g/g$ dry wt and microcystin-YR and microcystin-LR at 6.9, 29.6 $\mu g/g$ dry wt, respectively. The NokSan area showed microcystin-RR at 95.3 $\mu g/g$ dry wt and microcystin-YR and microcystin-LR at 2.1, 11.5 $\mu g/g$ dry wt respectively. Almost sites, microcystin variants were detected in the water sample via ELISA method.

4. Conclusions

In this study, three toxins, microcystin-RR, -LR and – YR were clearly separated with methanol ; 0.05M Phosphate buffer as a solvent system by HPLC. Linear relationships were obtained for the peak area vs concentrations of the toxins between 5 ng and 50 ng. The contents of microcystin in algae and water sample were monitored with HPLC. Microcystin RR, -LR, and –YR were identified in the algal material in August. The most dominant microcystin variant was microcystin –RR. The concentration of microcystin-RR in the bloom ranged from none-detectable to as high as 167.3 mg/g dry wt. No microcystin variants were detected in the water sample via HPLC Method. The standard curve for the immunoassay of microcystin by both direct and indirect ELISA are compared. The linear responses of inhibitions of binding by microcystin in the direct and indirect competitive ELISA were in the range of 10 to 1000 ng, 50 to 160 pg, respectively. Almost sites, microcystin variants were detected in the water sample via ELISA method.

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