

# Application of Protein Phosphatase 1 Inhibition Assay and Reversed Phase Liquid Chromatography for the Detection of Microcystins in Algal Dietary Supplements

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## ABSTRACT

Protein phosphatase 1 inhibition assay and reversed phase high performance liquid chromatography were adjusted for the detection of microcystins in *Chlorella* and *Spirulina* dietary supplements. Various sample preparation procedures for the enzyme and chemical assays were tested by the toxin-spiked samples to improve the sensitivity and accuracy of these methods. A silica gel solid phase extraction coupled with a deactivated elution had remarkably reduced the matrix interference from sample extracts and improved the detection of microcystins in both assays. High performance liquid chromatography could give the composition of different microcystins in the sample if the resolution of separation and toxin standards were available. On the contrary, protein phosphatase 1 inhibition assay gave an accumulated inhibition activity of the sample that could be converted to MCYST-LR equivalent according to the IC<sub>50</sub> of this standard. A good sensitivity of 50 ppb of microcystin in dried algal samples, was reached using the methods established in this research. Both enzyme and chemical assays were applied for the detection of possible microcystin contamination in the commercial products of algal dietary supplement. It was found that all nine samples of algal product examined in this study were free from microcystins.

Key words: solid-phase extraction (SPE), microcystins, protein phosphatase 1 inhibition assay, high performance liquid chromatography (HPLC)

## INTRODUCTION

Microcystins (MCYSTs) are a group of cyclic heptapeptide toxins produced by freshwater blue-green algae (cyanobacteria), including species of *Microcystis*<sup>(1)</sup>, *Anabaena*<sup>(2,3)</sup>, *Nostoc*<sup>(4)</sup>, *Oscillatoria agardhii*<sup>(5,6)</sup>, and *Planktothrix rubescens*<sup>(7)</sup>. They are potent hepatotoxins against mammals, birds and fishes and cause immediate death from liver dysfunction and hemorrhage or a chronic effect of tumor promoting<sup>(8-10)</sup>. Human illness resulting from exposure to microcystins, though less common than poisoning of wild and domestic animals, has been reported<sup>(11,12)</sup>. Toxicity of microcystins is generally regarded as a function of inhibitions on the activities of protein phosphatases 1 (PP-1) and 2A (PP-2A), the two major serine/threonine protein phosphatase in eukaryotes<sup>(13,14)</sup>.

Microcystin contamination from toxic blue-green algal blooms in surface waters has attracted worldwide attentions for its potential health concern. The World Health Organization (WHO) thus developed a regulatory standard of 1.0 ppb MCYST-LR equivalent of microcystins in drinking water, and a tolerant daily intake (TDI) of 0.04  $\mu\text{g day}^{-1} \text{kg}^{-1}$ <sup>(15,16)</sup>. There are also concerns on the possible contamination of toxic blue-greens in the microalgal

cultures for dietary supplements or in natural waters where the commercial microalgae are harvested. Due to *Microcystis* contamination in Upper Klamath Lake (south Oregon) where the commercial blue-green dietary supplement products, *Aphanizomenon flos-aquae*, were harvested, the Oregon Health Division and the Oregon Department of Agriculture established a regulatory limit of 1.0  $\mu\text{g/g}$  for microcystins in blue-green algae-containing products. Risk assessment data showed that a rather high proportion (85/87) of such products from Upper Klamath Lake were contaminated by MCYST-LR, with 63 samples (72%) containing concentration higher than the official limit<sup>(17)</sup>. The fear of the possible microcystin contamination in blue-green algal dietary supplements has extended to other microalgal sources, such as *Chlorella* and *Spirulina* products. In many areas of the world, manufacturers of microalgal products are often required to label the level of microcystin contaminants by the consumers (i.e. personal communications with microalgae factories).

High performance liquid chromatography (HPLC), mono- or poly-cloned immunoassay, protein phosphatase inhibition assay, and animal toxicity assay for the microcystin contents or toxicities have been developed. There are advantage and disadvantage of these methods for the microcystin analysis in surface waters for drinking or recreation, and in microalgal extracts. Generally a pre-cleaning process is needed to reduce the matrix interference in

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microalgal extracts, while a pre-concentrating process is applied for the low microcystin content in water samples. In the past decade, we were able to isolate various microcystins from the local isolates of *Microcystis* species (Figure 1), and to develop methods for toxin analysis using these microcystins and cultures in lab<sup>(20-23)</sup>. Although the immunological method of ELISA has been reported to have the lowest detection limit of 20 ppb or less<sup>(24)</sup>, it could not differentiate the various immuno-cross reactivities of different toxins or distinguish these toxins in a mix<sup>(25)</sup>. Our previous studies indicated that protein phosphatase inhibition by microcystins might be corresponded to the mouse toxicity<sup>(23)</sup> and an HPLC with a proper sample preparation was sensitive enough and highly reproducible in the microcystin analysis<sup>(24-25)</sup>. It is also noted that a newly proposed ISO20179 method, using HPLC and UV detection for the microcystin analysis in drinking and surface waters is currently employed in an inter-calibration practice (Luckas, personal communication).

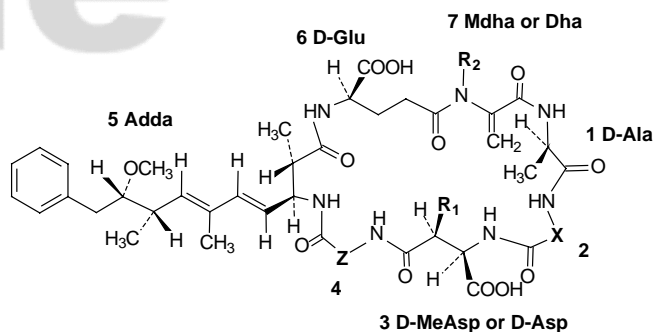
In this study we developed a solid-phase-extraction (SPE) sample preparation in conjunction with a modified protein phosphatase inhibition assay and an HPLC system for the quantitative analysis of microcystins in *Chlorella* and *Spirulina* food supplementary products. Sensitivity and accuracy of various SPE procedures were compared to optimize the sample preparation in the developed system.

## MATERIALS AND METHODS

### I. Protein Phosphatase 1 Inhibition Assay

Protein phosphatase 1 (PP-1) inhibition assay using *Escherichia coli* recombinant protein phosphatase 1  $\alpha$ -isoform of rabbit muscle (Cat. No. 539493, Calbiochem, San Diego, CA, USA) in a buffer solution containing 50 mM Tris-HCl at pH 7.0, 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 mM MnCl<sub>2</sub> and BSA of 0.2 mg/mL, was adopted following the protocol of Dr. En-Yuh Chang (Technical Service, Calbiochem). The assay used *p*-nitrophenyl phosphate (*p*-NPP) as substrate and the reaction product *p*-NP was detected at 405 nm using Spectra Fluor Plus microplate reader (Tecan AG, Hombrechtikon, Switzerland). The activity unit was defined as the phosphatase activity that hydrolyzed *p*-NPP to form 1 nmol *p*-NP min<sup>-1</sup>.

Standardization of enzyme reaction used the experimental solutions prepared as followed. Concentrations of MCYST-LR corresponding to 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, and 0.2 ng in 30  $\mu$ L methanol were prepared for calibration measurement. Enzyme solution was prepared by mixing 1.0  $\mu$ L of PP-1 (2500 unit/mL) in 2.5 mL buffer, while the substrate solution was prepared by dissolving 250 mM *p*-NPP in the same buffer. Different concentration of authentic MCYST-LR, control (zero concentration of microcystin) and various algal extracts in 30  $\mu$ L of methanol were mixed with 970  $\mu$ L of buffer to make the sample solutions.



Microcystin (MCYST)	X	Z	R1	R2	Code
MCYST-LR	Leu	Arg	CH <sub>3</sub>	CH <sub>3</sub>	LR
MCYST-RR	Arg	Arg	CH <sub>3</sub>	CH <sub>3</sub>	RR
MCYST-FR	Phe	Arg	CH <sub>3</sub>	CH <sub>3</sub>	FR
MCYST-WR	Trp	Arg	CH <sub>3</sub>	CH <sub>3</sub>	WR
MCYST-RA	Arg	Ala	CH <sub>3</sub>	CH <sub>3</sub>	RA
[Dha <sup>7</sup> ]MCYST-LR	Leu	Arg	CH <sub>3</sub>	H	DhaLR
[Dha <sup>7</sup> ]MCYST-RR	Arg	Arg	CH <sub>3</sub>	H	DhaRR
[D-Asp <sup>3</sup> ]MCYST-RR	Arg	Arg	H	CH <sub>3</sub>	DARR
[D-Asp <sup>3</sup> ]MCYST-FR	Phe	Arg	H	CH <sub>3</sub>	DAFR
[D-Asp <sup>3</sup> ]MCYST-WR	Trp	Arg	H	CH <sub>3</sub>	DAWR

**Figure 1.** Structure of ten microcystins used as standards in this research.

(Mdha, methyldehydroalanine; MeAsp, methylaspartic acid; Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-deca-4,6-dienoic acid; Leu, leucine; Arg, arginine; Phe, phenylalanine; Trp, tryptophane.)

Enzyme reactions were carried out in 96-well microplates for 1 hr at 30°C before the absorption measurement by a microplate reader. The assay included three groups, the sample, control and blank, and each treatment had three replicates. In each well, 100  $\mu$ L of buffer, 50  $\mu$ L of sample solution and 50  $\mu$ L of enzyme solution were pre-incubated for certain period before the addition of 50  $\mu$ L of substrate solution, while the blank group used another 50  $\mu$ L of buffer in replace of the enzyme solution. In the optimization study of PP-1 assay, pre-incubation time of 2 and 10 min were tested. The inhibitory activities of toxin standards and testing samples were determined by the activity ratio of sample to control, and expressed as percentage (%) activity of sample against control according to the following equations. The absorbance increase represented the difference of absorbance at 405 nm after 1 hr of PP-1 enzyme reaction at 30°C.

1. Activity of control = (average absorbance increase of control) - (average absorbance increase of blank)
2. Activity of sample = (absorbance increase of sample) - (average absorbance increase of blank)
3. Percentage activity = (2) / (1)  $\times$  100%

The correlation curves plotted on the percentage activity to the concentration of toxin of 2 and 10 min pre-incubation time were compared and the concentration

required to inhibit 50% enzyme activity of that in control ( $IC_{50}$ ) of these samples was also calculated.

## II. High Performance Liquid Chromatography

An isocratic HPLC equipped with UV absorption at 238 nm was used for the analysis of microcystins. A Luna phenyl-hexyl 5  $\mu$ m column (4.6  $\times$  250 mm, Phenomenex, Torrance, CA, USA) and a mobile phase of 0.01 M ammonium acetate/acetonitrile (73/27 or 75/25, v/v) at a flow rate of 1.0 mL  $min^{-1}$  were applied. Authentic microcystins including MCYST-LR, MCYST-RR, MCYST-FR, MCYST-WR, MCYST-RA, [D-Asp<sup>3</sup>]MCYST-FR, and [D-Asp<sup>3</sup>]MCYST-WR that were previously prepared in our earlier work<sup>(18)</sup> were mixed to serve as the standard. The content of microcystins in each examined sample was determined based on the calibration curve of MCYST-LR within the range of 1- 900 ng.

## III. Analysis of Microcystins in Microalgal Dietary Supplements

Both HPLC and PP-1 inhibition assay were applied for the determination of possible microcystin contamination in microalgal dietary supplements. Nine samples of *Chlorella* or *Spirulina* dietary supplement products (Table 1) purchased from local health food stores were studied in this experiment. Three to four algal tablets were randomly taken from each product package and then grinded to powder before extraction. To improve the detection, both normal and reversed phase SPE were applied in the PP-1 assay and compared for their removal of non-microcystin matrix interference, while normal phase (Si) SPE was applied for sample preparation in HPLC analyses. Microcystin recovery was also determined for the normal phase SPE in PP-1 inhibition assay using toxin-spiked microalgal products.

### (I) Sample preparation for HPLC

One hundred milligrams of each microalgal sample powder was extracted vigorously with 10 mL of methanol twice. Clear solution of both methanol extracts were collected by centrifugation and then dried with a rotary evaporator. Before HPLC analysis of microcystins in these

samples, they were treated by an SPE cleaning process. The SPE used a 0.6 cm i.d. column of 0.1 g silica gel (Baker silica gel 40  $\mu$ L flash chromatography packing, J. T. Baker, Phillipsburg, NJ, USA) which was pre-conditioned with 5 mL of ethyl acetate/isopropanol (4/3, v/v) before sample loading. Sample extracts were top-loaded on the SPE column in 0.5 mL of conditioning solution. They were first washed by 1.0 mL of the same solution and then eluted by 1.0 mL of methanol. The methanol effluent was dried and re-dissolved in 50  $\mu$ L of methanol, of which 10  $\mu$ L was analyzed by HPLC.

### (II) Sample preparation for PP-1 inhibition assay

A solution of 5 % acetic acid<sup>(26)</sup> was used for microalgal extraction and the extracts were subject for both normal and reversed phase SPE treatment before PP-1 inhibition assay. A reversed phase SPE using a 0.6 cm i.d. column filled with 0.1 g Cosmosil 40C18-PREP gel (40  $\mu$ m, Nacalai Tesque, Kyoto, Japan) was conditioned first with 1.0 mL of 90% methanol and then 1.0 mL of 5% acetic acid. Samples were top-loaded on the column and washed with 1.0 mL of 10% methanol before eluting with 1.0 mL of 90% methanol. The effluent were dried under N<sub>2</sub> stream and then re-dissolved in 1.25 mL of methanol. Thirty microliter of the methanol solution was mixed with 970  $\mu$ L of buffer as sample solution and analyzed by the PP-1 inhibition assay as described above. Results of PP-1 inhibition assay after C18 SPE sample preparation were compared with the results after Si SPE treatment. The Si normal phase SPE, the same as the sample preparation method for HPLC, was applied for the methanol extracts in PP-1 inhibition assay.

Two microalgal products, VD-C and VD-S, which were proven free from microcystin contamination in the previous analysis, were spiked with 1.0 ppm authentic MCYST-LR and subject for methanol extraction and Si SPE before PP-1 inhibition assay. Toxin contents obtained from PP-1 assay were compared between using and not using this Si SPE sample preparation.

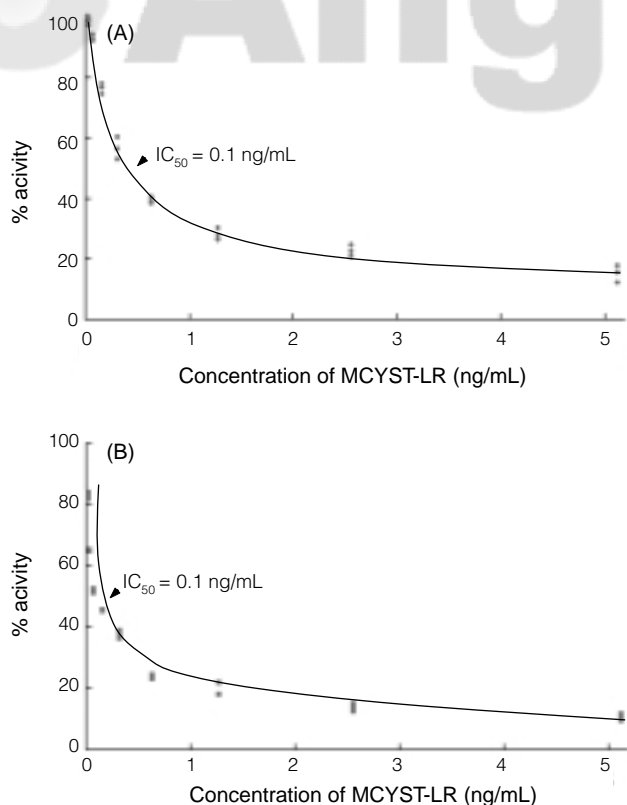
## RESULTS

### I. Optimization of PP-1 Inhibition Assay

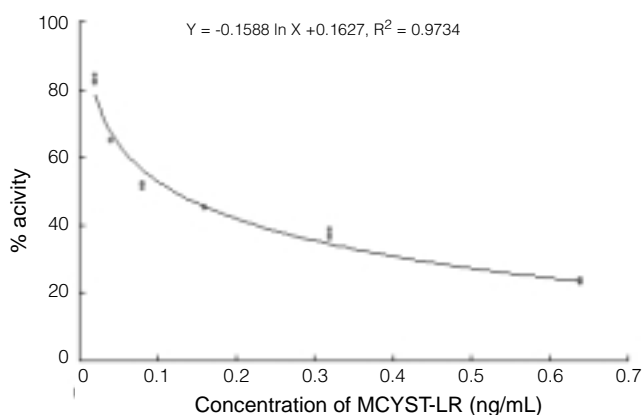
Activities of PP-1 decreased remarkably with the increase of MCYST-LR concentration (Figure 2) in the standardization of PP-1 inhibition assay using authentic MCYST-LR over the range of 5.12-0.04 ng/mL. It was also found that inhibition of phosphatase activity was enhanced when the pre-incubation of MCYST-LR and enzyme increased from 2 min to 10 min, especially in the lower concentration of MCYST-LR (Figure 2B). Concentration of 50% inhibition ( $IC_{50}$ ) of MCYST-LR on PP-1 activity was calculated as 0.1 and 0.5 ng/mL for the 2 and 10 min pre-incubation treatment, respectively (Figure 2 A and B).

**Table 1.** Microalgal dietary supplement products in study

Product	Code
Spichlo <i>Chlorella</i> Tablet	FE-C
Green Shine <i>Chlorella</i> Tablet	VD-C
Green Gem <i>Chlorella</i>	TC-C
Hou Lu Mei <i>Chlorella</i> Tablet	GB-C
Arkea <i>Chlorella</i> Tablet	AK-C
Green Shine <i>Spirulina</i>	VD-S
Green Gem <i>Spirulina</i>	TC-S
Arkea <i>Spirulina</i> Capsule	AK-S
Spailen <i>Spirulina</i>	NP-S



**Figure 2.** Inhibition of PP-1 activities by various amount of MCYST-LR which were pre-incubated with PP-1 for (A) 2 min, and (B) for 10 min.  $IC_{50}$  representing the concentration of MCYST-LR which poses the 50% inhibition of PP-1 activity.



**Figure 3.** Regression of the inhibition of PP-1 activities by MCYST-LR at different concentrations from 0.02 to 0.64 ng/mL, with a 10 min pre-incubation of enzyme and toxin.

A nonlinear regression curve based on the correlation of PP-1 activities and microcystin concentrations was revealed in Figure 3. A formula,  $Y = -0.1588 \ln X + 0.1627$  (where  $Y = \% \text{ activity of control}$ ,  $X = \text{concentration of MCYST-LR, ng/mL}$ ), using the natural logarithm function that best fit the curve of the toxin standard over the range of 0.02-0.64 ng/mL ( $R^2 = 0.9734$ ) was then established accordingly. The equation was thus served as the

calibration curve for quantitative determination of microcystin contamination in microalgal dietary supplements using PP-1 phosphatase inhibition assay. The least concentration of MCYST-LR, 0.02 ng/mL showed a PP-1 activity near 78% was determined as detection limit in PP-1 inhibition assay.

## II. Analysis of Microcystins in Microalgal Dietary Supplements

### (I) by HPLC

Standards of seven different microcystins were not well resolved by the reversed phase HPLC using a Luna phenyl-hexyl 5  $\mu\text{m}$  column and 0.01N ammonium acetate/acetonitrile (73/23) elution. However the resolution was much improved if the ratio of effluent composition was changed to 75/25. It was also found the sample preparation procedure of Si SPE removed the unidentified interference peaks that might confuse the qualitative identification of known microcystins. No microcystin was detected in the nine microalgal dietary supplement products of *Chlorella* and *Spirulina* collected from local health stores. This observation suggested that the concentration of microcystins in microalgal products was lower than 50 ppb, as estimated by the HPLC detection limit ( $S/N \geq 2$ ) of 1.0 ng for microcystin-LR and -RR at the sample size of 0.02 g dry algae.

### (II) By PP-1 Inhibition Assay

Protein phosphatase 1 inhibition assays of various microalgal products are presented in Table 2. Data of MCYST-LR equivalent containing in each sample were converted from the % activity and the calibration curve of standard. Two distinct sample preparations, including acetic acid (5%) and methanol extract, combined with their respective reversed phase SPE and normal phase SPE treatment, were applied in these enzyme assays. The inhibition of PP-1 activity by methanol extracts that were followed by Si SPE treatment were not pronounced (97.2-104.0% of the control), comparing with those of acetic acid extract and C18 SPE treatment. It could be asserted that the content of microcystins presented as MCYST-LR equivalent in microalgal products were lower than 40 ppb, based on the detection limit of 0.02 ng/mL microcystin-LR and the sample size of 480  $\mu\text{g}$  dried algae/mL in the enzyme assay. A lower enzyme activities (62.8-82.0% of the control) in the samples of acetate extract showed C18 SPE sample clean-up procedure were not able to remove the interference components that reduced the absorbance at 405 nm. Converted MCYST-LR contents in these samples from  $IC_{50}$  of PP-1 assay did not agree with the results obtained from HPLC.

The effectiveness of Si normal phase SPE in improving the PP-1 inhibition assay on microalgal products was deduced from the toxin contents analyzed in the

**Table 2.** Protein phosphatase 1 inhibition assays of microalgal products through two sample preparations of reversed and normal phase solid-phase-extraction respectively. (Values presented are mean  $\pm$  standard deviation of 3 replications.)

Products	Code	Reversed phase <sup>a</sup>		Normal phase <sup>b</sup>	
		% activity	MCYST-LR equivalent (ppb)	% activity	MCYST-LR equivalent (ppb)
<i>Chlorella</i>	FE-C	68.4 $\pm$ 0.8	78.2 $\pm$ 0.9	98.5 $\pm$ 1.0	< 40
	VD-C	67.7 $\pm$ 5.2	81.7 $\pm$ 6.3	97.2 $\pm$ 2.8	< 40
	TC-C	66.9 $\pm$ 1.8	85.9 $\pm$ 2.3	99.1 $\pm$ 3.3	< 40
	GB-C	71.3 $\pm$ 2.0	65.1 $\pm$ 1.8	98.1 $\pm$ 6.5	< 40
<i>Spirulina</i>	AK-C	63.8 $\pm$ 2.1	104.4 $\pm$ 3.4	103.6 $\pm$ 6.0	< 40
	VD-S	82.0 $\pm$ 7.2	< 40	102.6 $\pm$ 2.7	< 40
	TC-S	68.0 $\pm$ 5.1	80.2 $\pm$ 6.0	98.6 $\pm$ 5.7	< 40
	AK-S	66.0 $\pm$ 0.2	90.9 $\pm$ 2.7	102.0 $\pm$ 5.5	< 40
	NP-S	62.8 $\pm$ 3.6	111.2 $\pm$ 6.4	104.0 $\pm$ 11.4	< 40

<sup>a</sup>Acetic acid (5%) extract of samples, treated with C18 solid-phase-extraction.

<sup>b</sup>Methanol extract of samples, treated with Si solid-phase-extraction.

methanol extract before and after Si SPE sample preparation of MCYST-LR-spiked microalgal products. It was found the Si SPE treatment could recover almost 100% of the inhibition of PP-1 by MCYST-LR (Table 3).

## DISCUSSION

Chronic exposures to low level microcystins in contaminated drinking water or microalgal dietary supplements may pose potential health risks to the general public. Thus a fast, sensitive, accurate, and convenient methodology is essential for the routine monitoring of microcystins in drinking water, dietary supplements and other food materials. Previous literatures have demonstrated that protein phosphatase inhibition assays, as well as HPLC methods provided precise and reproducible analyses of microcystins<sup>(14,24,25)</sup>. In this study we indicated a reversed phase HPLC coupled with a sample preparation of silica gel SPE is effective in the analysis of microcystin in microalgal dietary supplements. Detection limit of the method has been determined to reach as low as 50 ppb, the same level of sensitivity an ELISA method may reach<sup>(22)</sup>. High performance liquid chromatography has its advantages of lower cost per sample and quantitative determination of each microcystin while comparing with the ELISA immunoassay.

Similar Si SPE applied in HPLC analysis was also applied in PP-1 inhibition for the methanol extract of microalgal products and was proven an efficient sample preparation operation in this study. It removed the constituents that interfered with the detection of PP-1 reaction product. Previous studies indicated that methanol extract of non-toxic *Microcystis*<sup>(19)</sup> and mussel<sup>(25)</sup> gave higher signal of PP-1 reaction product. Such matrix effect was speculated from indigenous phosphatases<sup>(25)</sup> or salts (M. Quilliam, personal communication) in the assayed samples. In the present study, interferences caused by salts and indigenous phosphatase were unlikely to happen, since methanol was used for sample extraction. On the contrary, acetic acid extract of algal supplement caused nonspecific inhibition of

**Table 3.** Toxin contents before and after Si SPE sample preparation using PP-1 inhibition assay on MCYST-LR-spiked microalgal products. (Values presented are mean  $\pm$  standard deviation of 3 replications.)

Samples	Percentage of the spiked MCYST-LR analyzed	
	Extract before SPE	Extract after SPE
VD-C	89.2 $\pm$ 8.2	101.9 $\pm$ 9.4
VD-S	92.4 $\pm$ 3.6	102.2 $\pm$ 5.6

PP-1 activity even after treatment with C18 SPE. In addition, Si SPE showed a good recovery of microcystin in the toxin-spiked experiment. Our data in the HPLC analysis of *Microcystis* extracts showed the same high recovery using Si SPE sample preparation for HPLC, not only to MCYST-LR but also to other microcystins<sup>(19)</sup>. It was also evident by the MALDI-TOF analysis of the toxin profiles before and after this SPE treatment (unpublished data). Thus, the use of silica gel SPE is recommended in sample preparation following methanol extraction for the PP-1 inhibition assay of microalgal dietary supplements. Although a reversed phase SPE has been generally applied as a sample preparation for water sample in HPLC analysis, Si SPE sample preparation is more convenient in operation for the methanol extract of microalgal samples, since the nonpolar components that may interfere the following reversed phase HPLC analysis can be clarified by this normal phase SPE sample preparation.

Previous report showed that PP-1 inhibition assay method was not sensitive enough in the detection of microcystins at ppb level<sup>(22)</sup>. However, PP-1 inhibition assay can be improved by increasing the pre-incubation time of enzyme and MCYST-LR or sample extracts before the addition of enzyme substrate. In an earlier trial we premixed the sample extracts and substrate solution before the adding of enzyme solution, as a routine procedure for the okadaic acid analysis by PP-2A inhibition assay<sup>(28)</sup>, in which no time was allowed for the microcystin to counteract PP-1. The results showed poor reproducibility and poor sensitivity. Various premix time in a range of 2 to 10 min were applied for the interaction of toxin and enzyme solution appeared in papers using protein phosphatase inhi-

bition assay for microcystin detection<sup>(29-33)</sup>. However, there was no comparison of these pre-mix-time differences. In this study we found that 10 min pre-incubation was sufficient to have a fast and pronounced inhibition response in the PP-1 inhibition assay and a detection limit of 0.02 ng/mL for MCYST-LR. It is equivalent to 40 ppb of MCYST-LR in microalgal products, almost reach the sensitivity of the polyclone ELISA method<sup>(22)</sup>.

In conclusion, both PP-1 inhibition assay and HPLC reach the required sensitivity and precision for microcystin analysis in microalgal dietary supplements, if coupled with a silica gel solid-phase-extraction pre-cleaning. All the microalgal dietary supplement products analyzed in this study are practically free from microcystin contamination.

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