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# Sensitive determination of microcystins in drinking and environmental waters


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**Keywords**

HPLC, SPE, microcystins, water, environmental


## Introduction

Waterblooms of cyanobacteria (blue-green algae) can produce potent toxins that have become a severe problem for eutrophic aquatic environments. Hepatotoxins are among the primary toxins produced by these species growing in lakes, ponds and rivers used as drinking water sources. Microcystins (structures shown in Figure 1) are hepatotoxins that exhibit tumor-promoting activity and are among the most commonly found cyanobacteria toxins. Microcystin contamination of drinking water at low nanomolar concentrations is considered a risk factor for cancer and microcystin-LR has been associated with most of the incidents of toxicity involving microcystins. Therefore, the World Health Organization (WHO) has proposed a provisional guideline concentration of 1.0 µg/L for microcystin-LR in drinking water.[1]

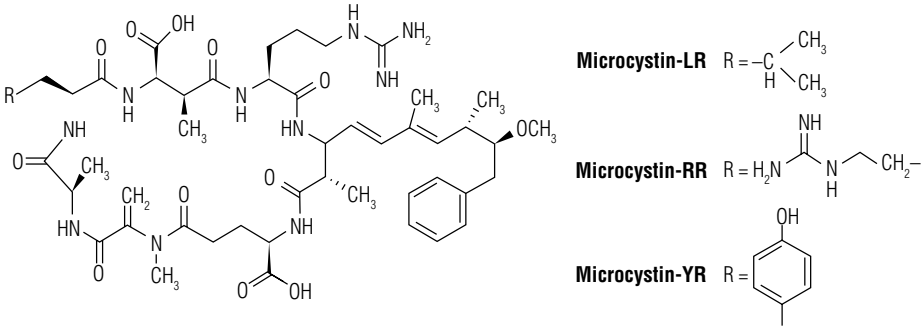


**Figure 1. Structures of microcystins.**

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The analytical approaches commonly used for microcystins include bioassay, chemical and biochemical methods. Bioassays have been used in screening but were found to be non-specific and/or more time consuming. Biochemical methods, such as enzyme-linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay (PPIA), are advantageous as screening methods due to their high sensitivity and ability to quickly treat a large number of samples; the disadvantage of these methods, however, is that they provide poor identification and have the potential for false positives. Reversed-phase high-performance liquid chromatography (HPLC) with UV detection, liquid chromatography mass spectrometry (LC-MS), and capillary electrophoresis are chemical methods that have been used for the identification and quantification of microcystins.[2]

The control of microcystins at 1.0 µg/L levels requires sensitive analytical methods and HPLC methods have been widely used for this purpose. Solid-phase extraction (SPE) is one of the main methods for sample extraction and preconcentration; however, the authors of Reference 3 suggest that the typically used SPE stationary phase (C18) does not supply good selectivity for trace analysis.[3] Immunoaffinity columns (IAC) modified with anti-microcystin-LR monoclonal antibodies on polypropylene stationary phases have been used for extraction with good selectivity for the HPLC analysis of microcystins,[3-5] but extensive use of this method is limited because an IAC is not commercially available for this application.

The authors have reported a simple, fast and effective target-cut on-line SPE method followed by HPLC with UV detection on an Thermo Scientific™ UltiMate™ 3000 Dual Gradient Standard LC system consisting of a dual gradient pump, autosampler and column oven equipped with one 2p–6p valve for the determination of trace amounts of vitamin $B_{12}$ added to beverages.[6] This on-line SPE method is different from a typical SPE method. The bound analyte on the SPE column is selectively eluted from the SPE column using a mobile phase gradient, just like the first dimension of a two-dimensional chromatography system. This reduces the number of interferences for sample analysis. While the SPE process is running, the analytical column is equilibrating. Just before the front portion of the analyte peak elutes from the SPE column, the SPE column is switched into the analytical flow path. As soon as the analyte is completely eluted from the SPE column, the SPE column is switched out of the analytical flow path and back to the SPE flow path. Therefore, only those interferences co-eluting with the analytes will enter the analytical column; thus, more interferences are removed. The volume of analyte cut from the SPE column is separated on the analytical column and detected by the UV detector. This target-cut on-line SPE method with dual function (analyte capture and partial separation) operates under automatic control of Thermo Scientific™ Chromeleon™ Chromatography Data System software and offers full automation, absence of operator influence and strict process control, compared to a typical off-line SPE method.

Here, the target-cut on-line SPE method followed by HPLC with UV detection was applied to the determination of three microcystins (-LR, -RR, and -YR) in drinking, tap and lake water. The three target analytes were co-eluted from the first column using chromatographic conditions that eliminated as many interferences as possible; then the analytes were sent to the analytical flow path and separated on the second column using the same type of stationary phase under different chromatographic conditions. This design takes advantage of the separation power of both columns and may eliminate interferences more efficiently than typical on- and off-line SPE methods. An additional dual-valve design is easy-to-use and convenient for method development.

The UltiMate 3000 Dual Gradient Standard LC system provides an efficient platform to fulfill the requirements of these designs. Sub-µg/L concentrations of microcystins-LR, -RR, and -YR spiked in water samples were determined, which exceeds the WHO requirement.

## Equipment and software

The UltiMate 3000 Dual Gradient Standard LC system was used, which includes:

- Thermo Scientific™ UltiMate™ 3000 SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
- Thermo Scientific™ UltiMate™ 3000 DGP-3600A or equal Thermo Scientific™ UltiMate™ 3000 DGP-3600SD Dual Gradient Standard Pump (P/N 5040.0061)
- Thermo Scientific™ UltiMate™ 3000 WPS-3000TSL Thermostatted Semi-Preparative Split-Loop Autosampler (P/N 5822.0028), equipped with a 2500 µL sample loop

- Thermo Scientific™ UltiMate™ 3000 TCC-3200 or equal Thermo Scientific™ UltiMate™ 3000 TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.000) with two 2p-6p switching valve
- Thermo Scientific™ UltiMate™ 3000 VWD-3400RS Variable Wavelength Detector (P/N 5074.0010), equipped with semi-micro flow cell, 2.5 µL, SST (P/N 6074.0360)
- Chromeleon Chromatography Data System software
- Thermo Scientific™ Orion™ 420A+ pH meter

## Reagents

- Deionized water, Milli-Q® Gradient A10 Water Purification System, Millipore Corporation
- Fisher BioReagents™ Acetonitrile ($CH_3CN$) and methanol ($CH_3OH$), HPLC grade
- Potassium dihydrogen phosphate ($KH_2PO_4$), dipotassium hydrogen phosphate ($K_2HPO_4$), and phosphoric acid ($H_3PO_4$), 85% (analytical grade), Sinopharm Chemical Reagent Co., Ltd, China

## Standards

100 µg of microcystins-LR (CAS 101043-37-2), -RR (CAS 111755-37-4), and -YR (CAS 101064-48-6), respectively, ≥95% (HPLC), Alexis Corporation.

Prepare stock standard solutions with 50 µg/mL concentrations by dissolving the standards with 2000 µL of methanol. Prepare the standard solutions used for the calibration curve by making appropriate dilutions of the stock standard solutions with water.

## Samples

Tap water samples were collected from local water sources in Shanghai, China. The lake water sample was collected at Zhangjiang High-Science and Technology Park located in the Pudong District of Shanghai, China. Bottled spring water samples were purchased from a supermarket in Shanghai. These samples were filtered through a 0.45 µm membrane (Millex-HN) prior to injection.

| Chromatographic conditions | |
|---|---|
| Column | On-Line SPE:<br>Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2),<br>3 µm, 3.0 × 33 mm (P/N 066276) |
| | Analytical:<br>Acclaim PA2, 3 µm, 3.0 × 150 mm (P/N 063705) |
| Column Temp. | 40 °C |
| Mobile Phases | For SPE:<br>A) 22.5 mM $KH_2PO_4$–2.5 mM $K_2HPO_4$ buffer (dissolve ~ 3.1 g of KH2PO4 and 0.44 g of $K_2HPO_4$ in 1 L of water)<br>B) $CH_3CN$<br>In gradient (Table 1) |
| | For separaton:<br>A: 0.05% (v/v) $H_3PO_4$ (dilute 0.6 mL of 85% $H_3PO_4$ to 1 L with water)<br>B: $CH_3CN$<br>In gradient (Table 1) |
| Valve Switching | Table 1 |
| Flow Rate | 0.7 mL/min for both SPE and separation |
| Inj. Volume | 2500 µL on the SPE column |
| UV Detection | 240 nm |

Table 1. Gradients and valve switching for target-cut on-line SPE and separation.

| Time (min) | Right Pump (for Separation) | | | Left Pump (for On-Line SPE) | | | Valve Switching | |
|---|---|---|---|---|---|---|---|---|
| | Flow Rate (mL/min) | Solvent A Buffer (%) | Solvent B $CH_3CN$ (%) | Flow Rate (mL/min) | Solvent A Buffer (%) | Solvent B $CH_3CN$ (%) | Left | Right |
| **0.00** | 0.7 | 85 | 15 | 0.7 | 80 | 20 | 6-1 | 1-2 |
| **5.00** | 0.7 | — | — | 0.7 | 80 | 20 | 6-1 | — |
| **6.95** | 0.7 | — | — | 0.7 | — | — | 6-1 | 6-1 |
| **7.00** | 0.7 | 85 | 15 | 0.7 | 65 | 35 | 6-1 | — |
| **7.35** | 0.7 | — | — | 0.7 | — | — | 6-1 | 1-2 |
| **7.50** | 0.7 | — | — | 0.7 | 20 | 80 | 6-1 | — |
| **8.50** | 0.7 | — | — | 0.7 | 20 | 80 | 6-1 | — |
| **8.60** | 0.7 | — | — | 0.7 | 80 | 20 | 6-1 | — |
| **12.0** | 0.7 | 41 | 59 | 0.7 | — | — | 6-1 | — |
| **12.1** | 0.7 | 85 | 15 | 0.7 | — | — | 6-1 | — |
| **15.0** | 0.7 | 85 | 15 | 0.7 | 80 | 20 | | — |

Table 2. Gradients and valve switching for traditional on-line SPE and separation.

| Time (min) | Right Pump (for Separation) | | | Left Pump (for On-Line SPE) | | | Valve Switching |
|---|---|---|---|---|---|---|---|
| | Flow Rate (mL/min) | Solvent A Buffer (%) | Solvent B $CH_3CN$ (%) | Flow Rate (mL/min) | Solvent A Buffer (%) | Solvent B $CH_3CN$ (%) | |
| **0.00** | 0.7 | 80 | 20 | 0.7 | 80 | 20 | 1-2 |
| **5.00** | 0.7 | 80 | 20 | 0.7 | 80 | 20 | 6-1 |
| **6.00** | 0.7 | — | — | 0.7 | 80 | 20 | 1-2 |
| **9.00** | 0.7 | 50 | 50 | 0.7 | 80 | 20 | — |
| **9.10** | 0.7 | 25 | 75 | 0.7 | 80 | 20 | — |
| **11.0** | 0.7 | 25 | 75 | 0.7 | 80 | 20 | — |
| **11.1** | 0.7 | 80 | 20 | 0.7 | 80 | 20 | — |
| **12.0** | 0.7 | 80 | 20 | 0.7 | 80 | 20 | — |

## Results and discussion

### Retention behavior of bicrocystins-RR, -YR, and -LR on the Acclaim PA2 column

The Acclaim Polar Advantage II (PA2) column is a polar-embedded column designed for enhanced hydrolytic stability within a wide range of pH values (pH 1.5 to 10) and compatibility with 100% aqueous mobile phases, overcoming the limitations of conventional C8 and C18 reversed-phase columns.

#### *Effect of buffer pH value*

The pH value of the mobile phase buffer may affect the retention of microcystins-RR, -YR, and -LR. Changes in their retention behavior on the Acclaim PA2 stationary phase were investigated. Experiments showed that when the buffer pH value decreased from pH 6.5 to 2.7, the retention time of microcystins-YR and -LR increased and the resolution between them improved, whereas the retention time of microcystin-RR did not change. The three microcystins were separated at a pH value lower than 2.5. They co-eluted at approximately pH 6.0.

Thus, for the requirements addressed here, the PA2 column is a good choice as an SPE column for concentrating the three microcystins from large-volume water samples (tap water and beverages) and co-eluting them using mobile phase buffer with a high pH value (~6.0). The PA2 column is also a good choice as an analytical column for the separation using a mobile phase buffer with a low pH value.

#### *Effect of column temperature*

The effect of column temperature on the retention of microcystins-RR, -YR, and -LR on the Acclaim PA2 stationary phase was investigated. Increasing column temperature may shorten the retention time, and is a benefit to the separation of microcystins-YR and -LR, which have close retention times. For example, resolution ($R_s$) between the two compounds increased from 0.50 to 1.94 when the column temperature increased from 25 to 40 °C.

### Comparison of traditional and target-cut on-line SPE methods

The commonly used on-line SPE flow scheme (Figure 2A) couples the SPE column directly with the analytical HPLC column using one six-port (2p–6p) column valve. The filtered sample is injected directly onto the system and delivered to the SPE column for enrichment (1-2 position) using the left pump; the analytical column is equilibrated with the right pump at the same time. After the analytes are bound to the SPE column and impurities are washed out, the SPE column is switched into the analytical flow path to elute the bound analytes (6-1 position), then the analytes are separated on the analytical column and detected by the UV detector.

For the target-cut on-line SPE method, a small change in the flow scheme of the traditional on-line SPE mode reverses the flush direction on the SPE column (Figure 2B) and creates an on-line SPE system that can have a dual function to eliminate interferences more efficiently. The SPE process in this mode is different from that described in the traditional method. The bound analyte on the SPE column is selectively eluted from the SPE column using a mobile phase gradient, just like the first dimension of a two-dimensional chromatography system. As the SPE process (position 1-2) is running, the analytical column is equilibrating. Just before the front portion of the analyte peak elutes from the SPE column, the SPE column is switched into the analytical flow path (position 6-1). As soon as the analyte is completely eluted from the SPE column, the SPE column is switched out of the analytical flow path and back to SPE flow path (position 1-2). Therefore, only those interferences co-eluting with the analytes will enter the analytical column; thus, more interferences are removed.

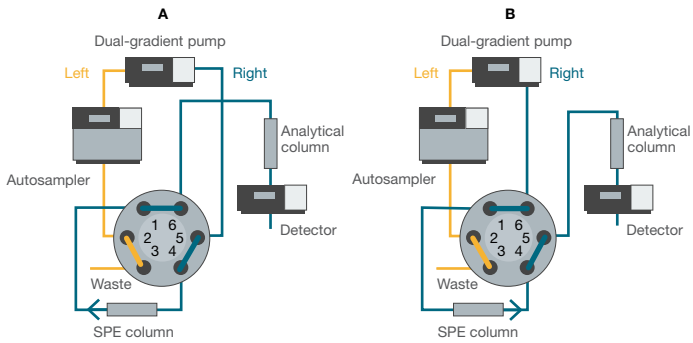


**Figure 2. Flow schematics for A) traditional and B) target-cut on-line SPE methods equipped with one 2p–6p valve for sample preparation and analysis.**

Figure 3 shows chromatograms of three types of water samples spiked with 1.0 µg/L each of microcystin-RR, -YR, and -LR standard using the traditional and target-cut on-line SPE methods, respectively. Tables 1 and 2 list the gradients and valve-switching times. Comparison of the two on-line SPE methods for analysis of different water samples demonstrates that the target-cut method may flush far fewer interferences to the analytical flow path, which is more efficient for analysis of the three microcystins in different water samples, whereas the traditional on-line SPE method is merely acceptable for the water samples.

| | |
|---|---|
| SPE Column: | Acclaim PA2, 3 µm, 3.0 × 33 mm |
| Anal. Column: | Acclaim PA2, 3 µm, 3.0 × 150 mm |
| Column Temp.: | 40 °C |
| Eluent for SPE: | $CH_3CN$–phosphate buffer ($22.5$ mM $KH_2PO_4$–$2.5$ mM $K_2HPO_4$)<br>A. Isocratic: 20% $CH_3CN$: 80% phosphate buffer (v/v)<br>B. In gradient: $CH_3CN$, 0–5 min, 20%; 7.0 min, 35%; 7.5–8.5 min, 80%; 8.6–15 min, 20% |
| Eluent for Separation: | $CH_3CN$–0.05% (v/v) $H_3PO_4$, in gradient<br>A. $CH_3CN$, 0–5.0 min, 20%; 9.0 min, 50%; 9.1–11 min, 75%; 11.1–12 min, 20%<br>B. $CH_3CN$, 0–7.0 min, 15%; 12 min, 59%; 12.1–15 min, 15% |
| Flow Rates: | 0.7 mL/min for SPE and separation |
| Injection Vol.: | 2500 µL on SPE column |
| Detection: | UV at 240 nm |

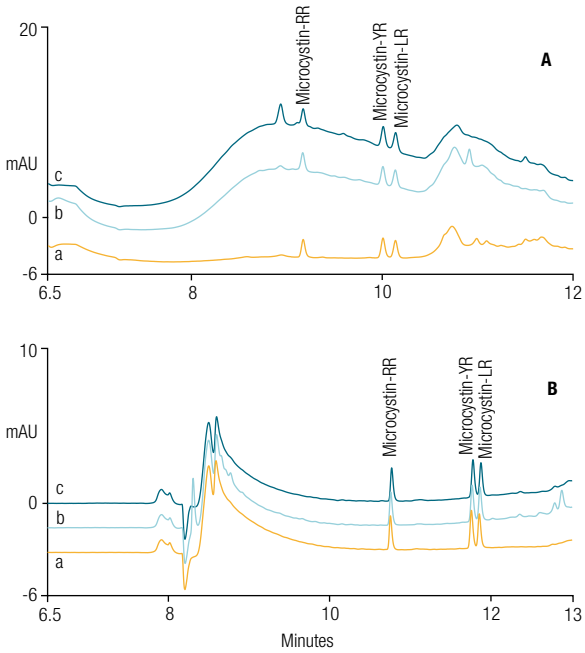


**Figure 3. Chromatograms of a) bottled spring water, b) tap water, and c) lake water spiked with 1 µg/L each of microcystin-RR, -YR, and -LR standard using A) traditional and B) target-cut on-line SPE methods.**

In practice, an additional 2p–6p valve may be used to construct a two-valve (2p–6p) system for convenient method development. The flow schematic of the two-valve configuration is shown in Figure 4. The left valve can be used to switch the SPE column or separation column into the flow path of the detector (Table 3).

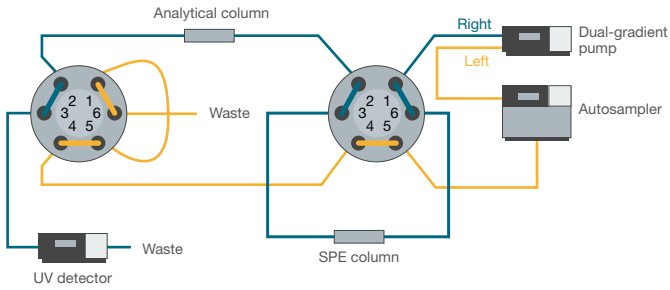


**Figure 4. Flow schematic for the target-cut on-line SPE method equipped with two 2p–6p valves.**

**Table 3. Valve position and related functions.**

| Position of Left Valve | Position of Right Valve | Description |
|---|---|---|
| 1-2 | 1-2 | Determine switching time of right valve during method development |
| 6-1 | 1-2 | Load sample and analysis |
| 6-1 | 6-1 | Transfer analytes from SPE column to analytical column |

## Evaluation of microcystins extraction using the target-cut on-line SPE method

### *Configuration of target-cut method*

This newly developed on-line SPE method with dual function (analyte capture and partial separation) automatically controlled by Chromeleon software was used for analysis of vitamin $B_{12}$.[6] In that application, it was easy to configure the instrument and set the method parameters for target-cut mode because there was only a single target analyte and the same mobile phases were used for SPE and separation.

For samples containing more than one target analyte, the choice of target-cut method parameters is important for the success of the on-line SPE method. In theory, the ideal approach would be to cut the analytes one by one from the first stationary phase (SPE column) to the second stationary phase (analytical column), thereby minimizing the interferences entering the analytical flow path. This approach is not recommended, however, because it may result in a complicated valve-switching process and affect the separation on the analytical column.

A simpler approach is to start the target-cut when the front shoulder of the first analyte peak is just eluting from the SPE column, then end when the tail of the last analyte peak elutes from the SPE column. This target-cut method is suitable for analytes with similar retention on the SPE column. For example, on the Acclaim PA2 SPE column, the retention times of microcystins-YR and -LR are similar but significantly different from that of microcystin-RR. For the determination of microcystins-RR, -YR, and -LR in a spiked tap water sample, the volume of cut analytes separated on the analytical column (Acclaim PA2 column) was large. As shown in Figure 5A, with the target-cut method, a large amount of interferences were still cut to the analytical flow path, which resulted in interference with the determination of microcystins at sub-µg/L concentrations.

| | |
|---|---|
| SPE Column: | Acclaim PA2, 3 µm, 3.0 × 33 mm |
| Anal. Column: | Acclaim PA2, 3 µm, 3.0 × 150 mm |
| Column Temp.: | 40 °C |
| Eluent for SPE: | $CH_3CN$–phosphate buffer ($22.5$ mM $KH_2PO_4$–$2.5$ mM $K_2HPO_4$) In gradient: $CH_3CN$, 0–5 min, 20%; 7.0 min, 35%; 7.5–8.5 min, 80%; 8.6–15 min, 20% |
| Eluent for Separation: | $CH_3CN$–0.05% (v/v) $H_3PO_4$ In gradient: $CH_3CN$, 0–7.0 min, 15%; 9.0 min, 50%; 12 min, 59%; 12.1–15 min, 15% |
| Flow Rates: | 0.7 mL/min for SPE and separation |
| Injection Vol.: | 2500 µL on SPE column |
| Detection: | UV at 240 nm |

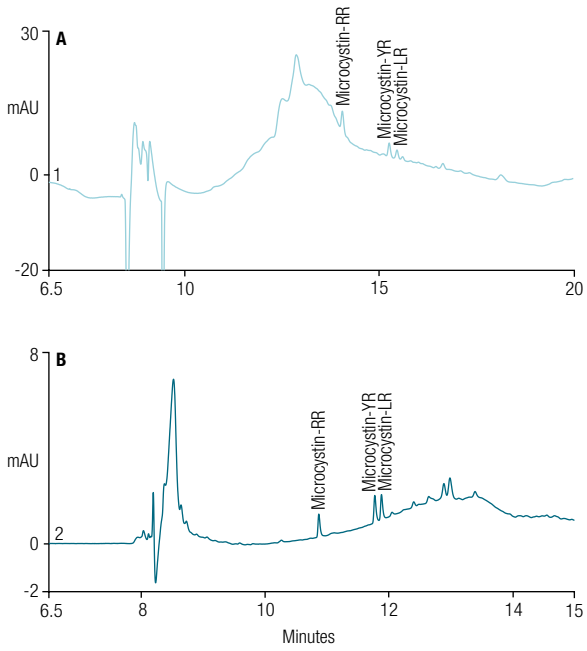


**Figure 5. Chromatograms of a tap water sample spiked with 0.5 µg/L each of microcystin-RR, -YR, and -LR standard using different target-cut modes. A) Valve-switching starts from microcystin-RR and ends at microcystin-LR when they are eluted from the SPE column. B) The three microcystins elute together from the SPE column.**

The appropriate target-cut method for a sample containing several target analytes is to use a mobile phase that will elute the analytes together (as one chromatographic peak) from the SPE column and then send them to the analytical flow path. Because the volume of cut target analytes is much smaller than that obtained by the alternate method, the co-eluted interferences may be much less; if so, the elimination of interferences will be more efficient.

Using the same determination of microcystins-RR, -YR, and -LR in a spiked tap water sample, Figure 5B shows the target-cut method with a $CH_3CN$–phosphate buffer (pH 6.0) mobile phase to elute analytes from the SPE column, and the analytical column using $CH_3CN$–0.05% $H_3PO_4$ (v/v, pH 2.2) mobile phase. Figure 5B shows that this approach does, in fact, have fewer interferences. Note that if the valve-switching times are inaccurate, the difference between the two mobile phases may affect separation of the three microcystins. Therefore, correctly setting valve-switching times is key to success of the target-cut on-line SPE method.

### *Determination of valve-switching times*

Based on the target-cut method in which all three analytes are eluted from the SPE column together, the valve-switching times for the extraction of microcystins-RR, -YR, and -LR can be estimated using the following equation:

$$t_{valve\text{-}switching\ 2} = t_{valve\text{-}switching\ 1} + (v_1/v_2) \times w_h$$

Where $t_{valve\text{-}switching\ 1}$ represents the first valve-switching time when the front shoulder of the analyte peak is just eluting from the SPE column at the flow rate for SPE; $t_{valve\text{-}switching\ 2}$ represents the second valve-switching time when the SPE column is switched out of the analytical flow path; $v_1$ and $v_2$ represent the flow rates for SPE and separation, respectively; and $w_h$ represents baseline peak width (min) of analytes on the SPE column.

Figure 6 shows the chromatogram of co-eluted microcystins-RR, -YR, and -LR on the SPE column. The front shoulder of the peak eluting from the SPE column at 0.7 mL/min ($v_1$) appears at 7.00 min ($t_{valve\text{-}switching\ 1}$). The peak is detected by the UV detector and the baseline peak width on the SPE column is 0.45 min ($w_h$). When the flow rate for the separation on the analytical column is also 0.7 mL/min ($v_2$), the second valve-switching time ($t_{valve\text{-}switching\ 2}$) calculated using the equation is 7.45 min.

The authors tried to have a slightly earlier $t_{valve\text{-}switching\ 1}$ and a delay in $t_{valve\text{-}switching\ 2}$ (0.10 min) to avoid losing microcystins when using different mobile phases for SPE and separation. Experiments showed that a 0.10 min delay in $t_{valve\text{-}switching\ 2}$ had no obvious effect, but 0.10 min earlier in $t_{valve\text{-}switching\ 1}$ resulted in the loss of microcystin-RR.

As shown in Figure 7, when $t_{valve\text{-}switching\ 1}$ = 7.00 min, all three microcystins were well retained; with 0.10 min earlier ($t_{valve\text{-}switching\ 1}$ = 6.90 min), a small part of microcystin-RR was lost; and with just 0.20 min earlier ($t_{valve\text{-}switching\ 1}$ = 6.80 min), microcystin-RR was lost completely, and more than half of microcystin-LR was lost as well. The authors hypothesize that this analyte loss was due to the cut volume obtained by using the slightly earlier time (0.2 min), which brought mobile phase of higher pH value (pH 6.0) and higher proportion of organic solvent ($CH_3CN$) to the analytical flow path before the analytes; this resulted in a change of the intrinsic equilibrium of the analytical column that significantly affected analyte retention. Therefore, the control of valve-switching time $t_{valve\text{-}switching\ 1}$ must be accurate.

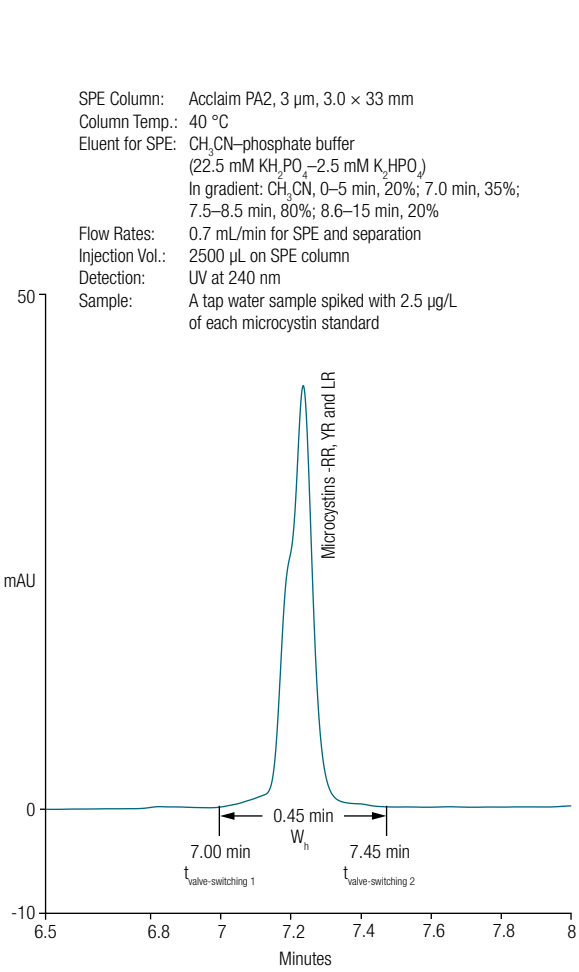


**Figure 6. Chromatography to determine valve-switching time for the target-cut on-line SPE method based on the configuration showed in Figure 4.**

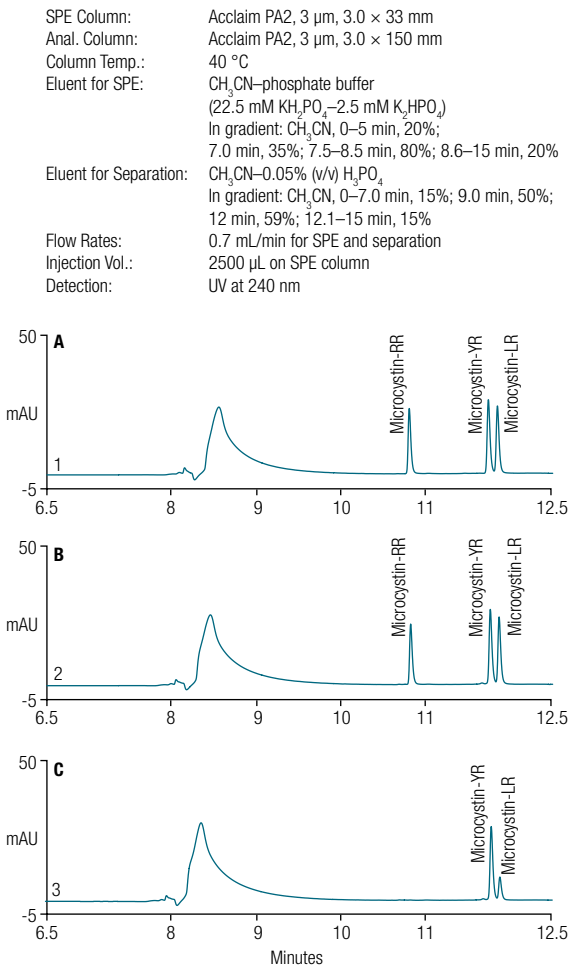


**Figure 7. Chromatograms of a mixture of microcystin-RR, -YR, and -LR standards with concentration 1.0 µg/L for each extracted at different valve-switching times: A) $t_{valve\text{-}switching\ 1}$ = 7.00 min, B) $t_{valve\text{-}switching\ 1}$ = 6.90 min, and C) $t_{valve\text{-}switching\ 1}$ = 6.80 min.**

### *Selection of SPE column format*

The effect of SPE column size on elimination of impurities using the target-cut on-line SPE method was investigated. Two Acclaim PA2 columns with different sizes, 4.6 × 50 mm and 3.0 × 33 mm, were used for SPE. As shown in Figure 8, interference elimination was slightly better on the larger column, which can be attributed to separation on the larger column being more efficient than that on the smaller one; therefore, fewer impurities enter the analytical flow path. However, the larger column did have a significant effect on separation on the analytical column due to more mobile phase being cut from SPE to the analytical flow path, which resulted in poor peak shape and less detection sensitivity for microcystins-YR and -LR. Therefore, the 3.0 × 33 mm Acclaim PA2 column was selected as the SPE column for this application.

| | |
|---|---|
| SPE Column: | Acclaim PA2, 3 µm, 3.0 × 33 mm<br>Acclaim PA2, 3 µm, 4.6 × 50 mm |
| Anal. Column: | Acclaim PA2, 3 µm, 3.0 × 150 mm |
| Column Temp.: | 40 °C |
| Eluent for SPE: | $CH_3CN$–phosphate buffer<br>(22.5 mM $KH_2PO_4$–2.5 mM $K_2HPO_4$)<br>In gradient: $CH_3CN$, 0–5 min, 20%;<br>7.0 min, 35%; 7.5–8.5 min, 80%; 8.6–15 min, 20% |
| Eluent for Separation: | $CH_3CN$–0.05% (v/v) $H_3PO_4$<br>In gradient: $CH_3CN$, 0–7.0 min, 15%;<br>12 min, 59%; 12.1–15 min, 15% |
| Flow Rates: | 0.7 mL/min for SPE and separation |
| Injection Vol.: | 2500 µL on SPE column |
| Detection: | UV at 240 nm |

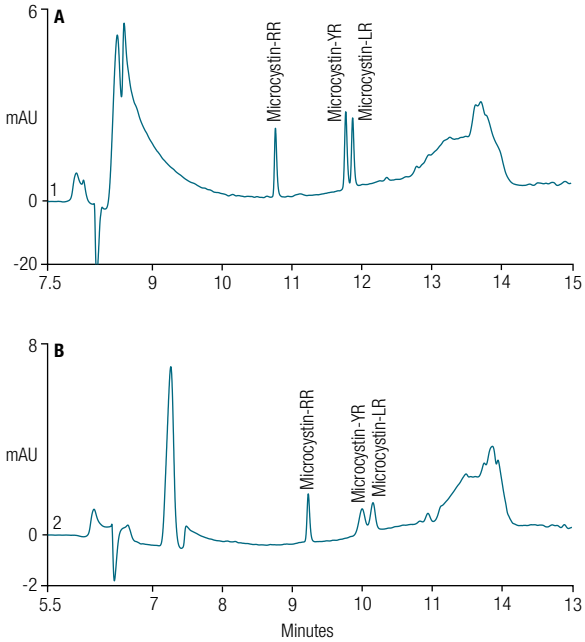


**Figure 8. Chromatograms of a lake water sample spiked with 1.0 µg/L each of microcystin-RR, -YR, and -LR standard using different size SPE columns: A) Acclaim PA2, 3 µm, 3.0 × 33 mm column, and B) Acclaim PA2, 3 µm, 4.6 × 50 mm column with the target-cut on-line SPE method in Table 1.**

## Method reproducibility, linearity, and detection limits

Method reproducibility was estimated by making six consecutive 2500 µL injections of a drinking water sample spiked with 0.5 µg/L of each microcystin standard. Retention time and peak area reproducibilities are summarized in Table 4 and show good precision. Figure 9 shows an overlay of chromatograms for the six consecutive injections.

**Table 4. Reproducibility for peak retention time and area.**

| Microcystins | Retention Time RSD | Peak Area RSD | Concentration of Standard (µg/L) |
|---|---|---|---|
| RR | 0.037 | 1.53 | 0.5 |
| YR | 0.028 | 1.59 | 0.5 |
| LR | 0.029 | 1.13 | 0.5 |

| | |
|---|---|
| SPE Column: | Acclaim PA2, 3 µm, 3.0 × 33 mm |
| Anal. Column: | Acclaim PA2, 3 µm, 3.0 × 150 mm |
| Column Temp.: | 40 °C |
| Eluent for SPE: | $CH_3CN$–phosphate buffer<br>(22.5 mM $KH_2PO_4$–2.5 mM $K_2HPO_4$) |
| Eluent for Separation: | $CH_3CN$–0.05% (v/v) $H_3PO_4$, in gradient<br>$CH_3CN$, 0–7.0 min, 15%; 12 min, 59%;<br>12.1–15 min, 15% |
| Flow Rates: | 0.7 mL/min for SPE and separation |
| Injection Vol.: | 2500 µL on SPE column |
| Detection: | UV at 240 nm |

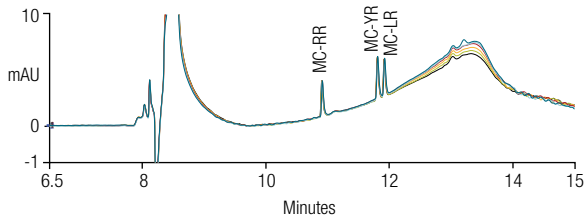


**Figure 9. Overlay of chromatograms of six consecutive injections of a drinking water sample spiked with 0.5 µg/L each of microcystin-RR, -YR, and -LR standard using the target-cut on-line SPE method in Table 1.**

Calibration linearity for microcystins-RR, -YR, and -LR was investigated by making three consecutive injections of a mixed standard prepared at five different concentrations. The external standard method was used to establish the calibration curve and to quantify these microcystins in samples. Excellent linearity was observed from 0.1 to 10 µg/L when plotting concentration versus peak area. Figure 10 shows a chromatogram of the three microcystins with concentrations of 0.1 µg/L each. Table 4 reports the data from the calibration as calculated by Chromeleon software.

Detection limits were calculated using the equation:

$$\text{Detection limit} = S_{t(n-1,\ 1-\alpha=0.99)}$$

Where S represents Standard Deviation (SD) of replicate analyses, n represents number of replicates, $t_{(n-1,\ 1-\alpha=0.99)}$ represents Student *t* value for the 99% confidence level with n – 1 degrees of freedom.

Method detection limits (MDL) were estimated using six consecutive injections of drinking water sample spiked with 0.5 µg/L of each microcystin standard to determine S (Table 5).

| | |
|---|---|
| SPE Column: | Acclaim PA2, 3 µm, 3.0 × 33 mm |
| Anal. Column: | Acclaim PA2, 3 µm, 3.0 × 150 mm |
| Column Temp.: | 40 °C |
| Eluent for SPE: | $CH_3CN$–phosphate buffer ($22.5$ mM $KH_2PO_4$–$2.5$ mM $K_2HPO_4$) In gradient: CH3CN, 0–5 min, 20%; 7.0 min, 35%; 7.5–8.5 min, 80%; 8.6–15 min, 20% |
| Eluent for Separation: | $CH_3CN$–0.05% (v/v) $H_3PO_4$, in gradient $CH_3CN$, 0–7.0 min, 15%; 12 min, 59%; 12.1–15 min, 15% |
| Flow Rates: | 0.7 mL/min for SPE and separation |
| Injection Vol.: | 2500 µL on SPE column |
| Detection: | UV at 240 nm |

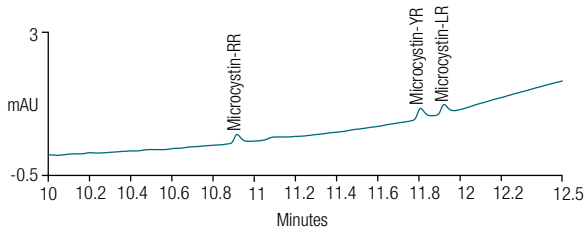


**Figure 10. Chromatogram of a mixed solution with concentrations of 0.1 µg/L each of microcystin-RR, -YR, and -LR standard using the target-cut on-line SPE method in Table 1.**

**Table 5. Reproducibility for peak retention time and area.**

| Microcystin | Regression Equations | r (%) | Range of Standards µg/L | RSD for Calibration Curve | MDL* (µg/L) |
|---|---|---|---|---|---|
| RR | A = 0.0844 c - 0.0027 | 99.997 | 0.1–10 | 0.91 | 0.028 |
| YR | A = 0.1054 c - 0.0022 | 99.994 | 0.1–10 | 1.25 | 0.028 |
| LR | A = 0.0942 c + 0.0030 | 99.994 | 0.1–10 | 1.21 | 0.028 |

*The single-sided Student *t* test method (at the 99% confidence limit) was used for determining MDL, where the standard deviation (SD) of the peak area of six injections is multiplied by 4.03 to yield the MDL.

| | |
|---|---|
| SPE Column: | Acclaim PA2, 3 µm, 3.0 × 33 mm |
| Anal. Column: | Acclaim PA2, 3 µm, 3.0 × 150 mm |
| Column Temp.: | 40 °C |
| Eluent for SPE: | $CH_3CN$–phosphate buffer<br>(22.5 mM $KH_2PO_4$–2.5 mM $K_2HPO_4$)<br>In gradient: $CH_3CN$, 0–5 min, 20%;<br>7.0 min, 35%; 12min, 59%; 12.1–15 min, 15% |
| Eluent for Separation: | $CH_3CN$–0.05% (v/v) $H_3PO_4$<br>In gradient: $CH_3CN$, 0–7.0 min, 15%;<br>7.5–8.5 min, 80%; 8.6–15 min, 20% |
| Flow Rates: | 0.7 mL/min for SPE and separation |
| Injection Vol.: | 2500 µL on SPE column |
| Detection: | UV at 240 nm |
| Samples: | A. Tap water<br>B. Lake water<br>C. Bottled spring water |

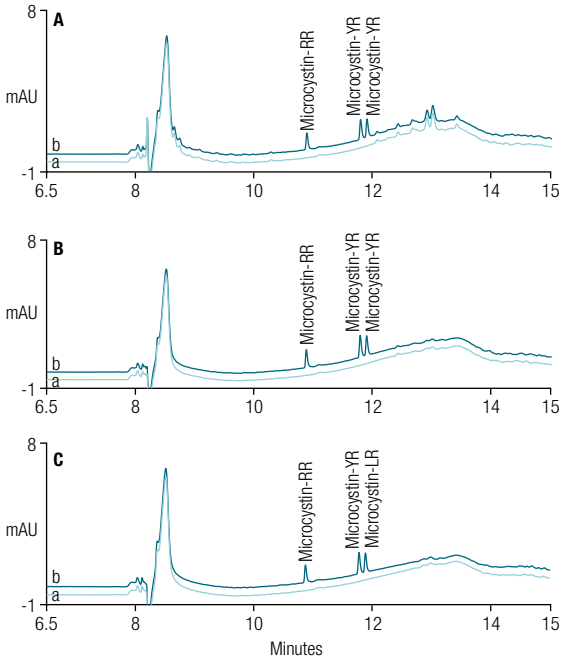


**Figure 11. Overlay of chromatograms for A) water sample and B) the same sample spiked with 0.5 µg/L each of microcystin-RR, -YR, and -LR standard using the target-cut on-line SPE method in Table 1.**

## Sample analysis

Figure 11 shows chromatograms of tap water, lake water and bottled spring water samples, as well as the same samples spiked with 0.5 µg/L of each microcystin standard. None of the three samples had detectable microcystins. Recoveries for each standard in all three samples ranged from 92% to 100%, thus indicating that the analysis method is accurate (Table 6).

## Conclusion

Figure 11 shows chromatograms of tap water, lake water, and bottled spring water samples, as well as the same samples spiked with 0.5 µg/L of each microcystin standard. None of the three samples had detectable microcystins. Recoveries for each standard in all three samples ranged from 92% to 100%, thus indicating that the analysis method is accurate (Table 6).

**Table 6. Analysis results of Microcystins-RR, -YR, and -LR in the samples.**

| Sample | Tap Water | | | | Lake Water | | | | Bottled Spring Water | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Micro-cystin | Detected (µg/L) | Added (µg/L) | Found (µg/L) | Recovery (%) | Detected (µg/L) | Added (µg/L) | Found (µg/L) | Recovery (%) | Detected (µg/L) | Added (µg/L) | Found (µg/L) | Recovery (%) |
| RR | ND | 0.50 | 0.48 | 96 | ND | 0.50 | 0.55 | 110 | ND | 0.50 | 0.49 | 98 |
| YR | ND | 0.50 | 0.46 | 92 | ND | 0.50 | 0.51 | 102 | ND | 0.50 | 0.48 | 96 |
| LR | ND | 0.50 | 0.48 | 96 | ND | 0.50 | 0.51 | 102 | ND | 0.50 | 0.49 | 98 |

ND - Not detected

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