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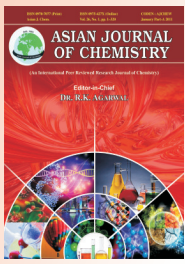

Large-Scale Extraction and Purification of Microcystin-LR by Macroporous Resins

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An effective method for large-scale extraction and purification of microcystin-LR (MC-LR) was established. 660 mL supernatant centrifugated from algae serum was absorbed by resin D101. The impurities on D101 resin were removed by 40 % acetic acid (3 L), deionized water (2.5 L), 20 % acetone (2 L) and 10 % ethanol (2 L) in turn. Thus microcystin-LR on D101 resin was purified. The adsorbed microcystin-LR was eluted from the resin with 70 % methanol (9 L). The eluent was concentrated by rotary evaporation at 80 °C. The quality of microcystin-LR in the crude extract was 2075.5 μg. The recovery rate was 79.71 %. In addition, the method of getting microcystin-LR by preparative chromatography is established. The purity of the product was 95 %.

Keywords: Microcystin-LR, Macroporous resin, Extraction, Purification.

INTRODUCTION

Microcystins (MCs) are cyanobacterial secondary metabolites[1], it is cyclic heptapeptide molecules and molecular weight is 800-1100. Most of them are water soluble and have good chemical durability[2]. Microcystins are highly toxic with liver as the target organ. Inside the hepatocytes, microcystins bind to protein phosphatases1 and 2A by covalent bonding[3], inhibit activity, it can cause hepatomegaly and make animal dead, it also has a high capacity to induce tumors in liver[4]. Microcystins cause the potential health hazard to aquatic animals and plants and humans[5]. At least 85 variants are known so far[6]. The World Health Organization (WHO) has suggested the concentration of microcystins in drinking water cannot be more than 1.0 μg/L[7]. Most studies have reported the high potential carcinogenic effect of long-term drinking water contaminated by microcystins[8]. Among all of the variants of microcystins , microcystin LR (MC-LR) is the most common, toxic and acute harmful[9]. Considerable researches on microcystins have been carried out, such as its characterizations[10], environmental behavior[11], toxicity assessment and toxic mechanism[12-13], but many problems still existing. In the study of cyanophycean toxin, the demands for standard microcystins increase. Microcystins variants are varied, it need standard microcystins to identify during the research[14]. However, highly purified microcystin-LR is so expensive that further research of microcystins in China is hindered. There have been many reports on miniature extraction and purification of microcystin-LR[15-18], but large-scale purification methods have not been reported. This paper is mainly about the method on extraction and purification of microcystin-LR by macroreticular resin. The product of microcystin-LR was later isolated by high performance liquid chromatography (HPLC) from crude product and identified by electrospray mass spectrometry. This study is significant to obtain large-scale highly purified microcystin-LR.

EXPERIMENTAL

Algae paste was collected from Chaobai river in Tianjin. The microcystin-LR standards were purchased from Alexis Company. Methanol, ethanol, acetic acid and acetone of analytical grade, trifluoro-acetic acid (TFA) of HPLC grade, were all form Beijing Chemical Works. Ultra-pure water was obtained from a Milli-Q Synthesis system (Millipore Ltd., USA). Resin D101 was obtained from Tianjin Guangfu Fine chemical industry research institute.

Detection conditions of HPLC: High performance liquid chromatography (HPLC, SHIMADZU, Japan), retrofitted with UV detection and LC Solution Lite workstation was used for identification of microcystin-LR. HPLC analysis was performed using LC column (Inertsil ODS-5 μm, 250 × 4.6 mm, DIKMA, USA). The mobile phase was methanol: ultra-pure water (0.1 % TFA) = 60: 40 (v/v), flow rate was 1 mL/min and injection volume was 20 μL. The column temperature was 25 °C and the detection wavelength was 239 nm.

Extraction of microcystin-LR from algae paste: Algal cells were freeze-thawed for extraction. Next, the solution with

algae cells were centrifugated at 4 °C and 8000 rpm for 20 min. Microcystin-LR in supernatant was analyzed by HPLC. Microcystin-LR concentration was 4 μg/mL. Supernatant were stored at -10 °C and light-free.

Purification of microcystin-LR from supernatant: Supernatant (660 mL) was absorbed by resin D101, drip washed continuously with 40 % acetic acid (3 L, 80 mL/min), deionized water (2.5 L, 100 mL/min), 20 % acetone (2 L, 120 mL/min), 10 % ethanol (2 L, 120 mL/min) in order to purify microcystin-LR. The adsorbed microcystin-LR was eluted from the resin with 70 % methanol (9 L, 120 mL/min). The eluent was collected continuously in the brown flask and every 500 mL was in one flask. The eluent between 1.5-7.5 L was concentrated by rotary evaporation at 80 °C. The crude extract constant volume was 50 mL by adding 70 % methanol, then filtered through 0.45 μm organic filter paper and analyzed by HPLC to determine concentration and recovery of microcystin-LR. A large number of crude extract obtained was concentrated at 80 °C. New crude extract with high concentration of microcystin-LR was obtained (Fig. 1). The microcystin-LR recovery was calculated using the following equation:

$$R = \frac{m_{MC-LR[crude-extract]}}{m_{Mc-LR[supernatant]}} \times 100\ \% \quad (1)$$

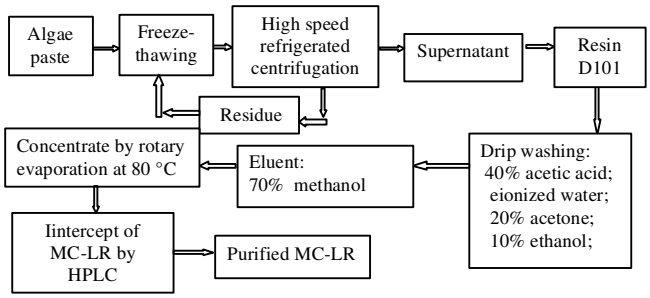

Fig.1. Process chart of microcystin-LR (MC-LR) purification

Preparation of microcystin-LR by HPLC from crude extract: 0.45 μm organic membranes were used to filter crude extract, 20 μl was injected into HPLC. A brown reagent bottle was used to collect the affluent, which was detected between 0.66-1.06 min after microcystin-LR's characteristic peak appeared. The volume of collection was nearly 0.4 mL per time, after repetitive operation, it could reach 30 mL. The concentration and purity of microcystin-LR in the collection was determined by HPLC and microcystin-LR was identified by mass spectrometry. The electrospray mass spectrometry analysis[19,20] was performed by Bruker Apex IV FTMS of Peking University.

RESULTS AND DISCUSSION

The small-scale extraction and purification of microcystin-LR by resin D101 had been established by our lab. In order to deal with the large amount of supernatant and obtain microcystin-LR with high concentration, small-scale experiment was scaled up.

Determination of optimal sample loading amount: The resin D101 column chromatography was performed on a low-pressure glass chromatographic column (24# F80 L = 610 mm 150 cm, Tianjin Xuehui Glass Inc, China) filled with 3 Kg resin D101. The length of the resin column was 60 cm. Supernatant (concentration of microcystin-LR is 4 μg/mL) was absorbed by column with flow rate of 30 mL/min. The eluent was collected sequently in the brown volumetric flask and every 50 mL was in one flask numbered in sequence. These eluent were concentrated to 10 mL, respectively. Finally, these crude extract was submitted to HPLC analysis[21]. The result was shown in Fig. 2: microcystin-LR appeared in the eluent after volume of 650 mL and reached to saturation until the volume of 850 mL. To avoid of wasting microcystin-LR, volume of 660 mL was chosen to be the optimal sample loading amount when microcystin-LR concentration was 4 μg/mL.

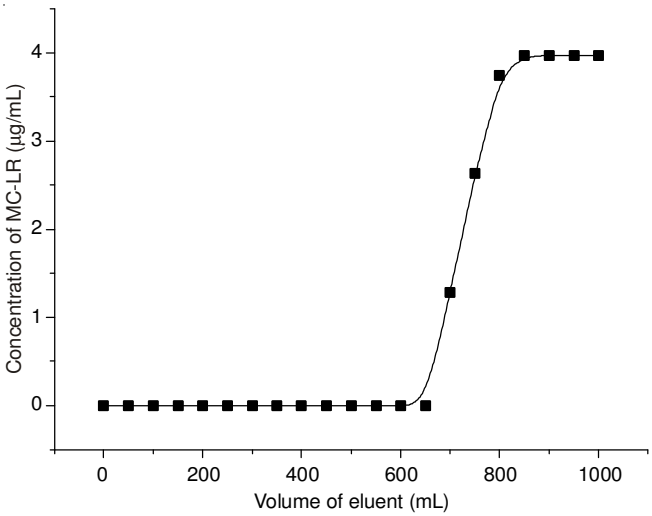

Fig. 2. Determination of optimal sample loading amount

Determination of drip washing volume: According to the two factors proportion of small-scale experiment and large-scale one-volume of resin and sample loading, the drip washing volume was scaled up too. The details of experimental condition and recovery of microcystin-LR were shown in Table-1. Experiment I was small-scale; II was large-scale. The microcystin-LR recovery of II was higher than that of I. It may because the amount of microcystin-LR used in II was quite larger than I, during the drip-washing, the proportion of wastage decreased and the recovery was higher than I. So the optimal condition of drip washing was 40 % acetic acid (3 L), deionized water (2.5 L), 20 % acetone (2 L), 10 % ethanol (2 L).

Determination of eluent volume: Supernatant (660 mL) was absorbed by a column; the condition of drip washing was the same as experiment II. Eluent was 70 % ethanol. The fraction was collected continuously in the brown flask and every 500 mL was in one flask numbered in sequence. These eluent were concentrated to 10 mL, respectively[22] and submitted to HPLC analysis. The elution curve of microcystin-LR was shown in Fig. 3. microcystin-LR appeared in the eluent after 1.5 L and the concentration reached to maximum at 3 L; then it decreased and disappeard at 9 L. In order to wash all microcystin-LR down, 9 L was chosen to be the optimal volume of 70 % ethanol. Because the volume of eluent was large, it needed a lot time to be concentrated, eluent between 1.5-7.5 L were chosen to be concentrated by rotary evaporation.

TABLE-1
EFFECT OF DRIP WASHING VOLUME ON MICROCYSTIN-LR (MC-LR) RECOVERY

Experiment	Volume of sample loading (mL)	Volume of 40 % acetic acid (L)	Volume of deionized water (L)	Volume of 20 % acetone (L)	Volume of 10 % ethanol (L)	Recovery of MC-LR (%)
I	8	0.10	0.05	0.15	0.15	61.36
II	660	3	2.5	2	2	76.37

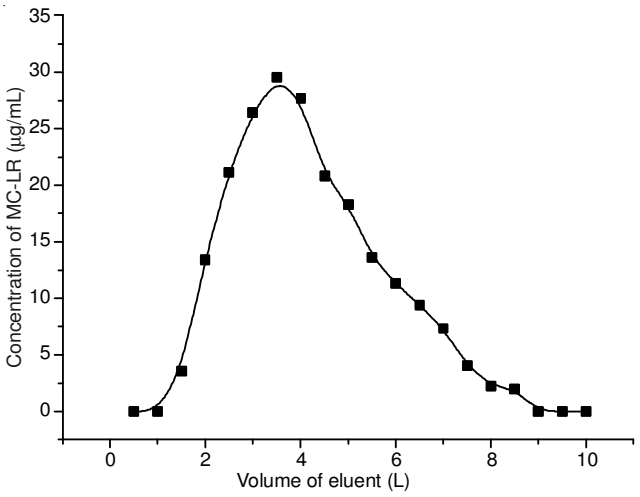

Fig. 3. Elution curve of microcystin-LR (MC-LR) in 660 mL supernatant

After repeated experiments, it was confirmed that the average recovery of microcystin-LR was 79.71 %.

Determination of rotary evaporation temperature: Three samples of the crude extract (10 mL, 41.51 μg/L) were taken, adding 70 % methanol respectively to make the volume reach to 500 mL and evaporated to 10 mL at 70, 80 and 90 °C, respectively. The time was recorded. The concentration of microcystin-LR was detected. From Table-2, it could be seen that under the premise of the same volume after spin steaming, the recovery of the microcystin-LR decreased while the spin steaming temperature increased and the time was shortened. Considering the influence of recovery and time, 80 °C was chosen for experiment.

TABLE-2
EFFECT OF ROTARY EVAPORATION TEMPERATURE ON MICROCYSTIN-LR (MC-LR) RECOVERY

Temperature (°C)	Concentration of MC-LR (μg/mL)	Recovery of MC-LR (%)	Required time (min)
70	40.66	97.95	45
80	38.54	92.85	30
90	38.11	91.81	25

The stability of the experiment at 80 °C was tested. The time was obtained by record that it took 11 h to spin steam eluent from 6 L to 50 mL. In order to explore the influence of the long-time steam heating on the recovery, crude extract (50 mL, 60.65 μg/L) were taken, adding methanol (100 mL, 70 %) as the sample 1. The sample 1 was rotated for 11 h without vacuum by the rotary evaporator and then spins steamed for 0.5 h with vacuum until the volume reached to 50 mL; the reclaimed extract was numbered as sample 2. The results were shown in Fig. 4, after heated 11 h at 80 °C, the recovery of microcystin-LR was 91.87 % and it was acceptable. Hence, 80 °C was chosen as the rotary evaporation temperature.

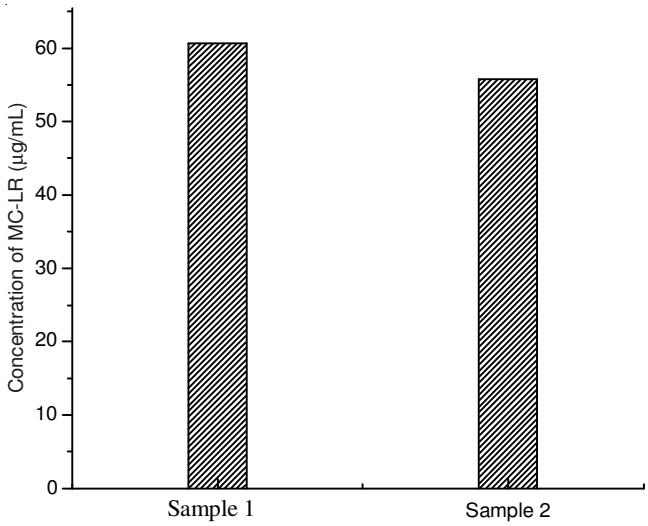

Fig. 4. Effect of rotary evaporation for 11h at 80 °C on microcystin-LR (MC-LR) recovery

Test experiment: According to the optimum experiment conditions, the test experiments were began to detect the recovery of microcystin-LR in supernatant. As Table-3 shown, the average recovery was 79.71 %. It could be certified that the method was stable and useful.

TABLE-3
DETERMINATION RESULTS OF MICROCYSTIN-LR (MC-LR) RECOVERY

Times	Loading quantity of MC-LR (μg)	Recycling quantity of MC-LR (μg)	Recovery (%)
1	2640	2141	81.10
2	2640	2016	76.36
3	2640	2205	83.52
4	2640	2055	77.84
Average	2640	2104	79.71

Further purification of crude extract: The crude extract obtained by repetitive experiments was mixed and rotary evaporated to 15 mL. The liquid in the round bottom flask was sucked out by a straw and numbered as sample 1; then the attachments on the round bottom flask wall was washed down with 70 % methanol and numbered as sample 2. The two samples were centrifuged at 4000 rpm for 5 min and then supernatant was sucked out by a straw. Finally, the concentration of microcystin-LR was determined. As the Fig. 5 shown, there was no microcystin-LR in sample 1, but there were many other substances which retention time were between 2-6 min. Compared with sample 1, there were more microcystin-LR and less substances appeared between 2-6 min in sample 2. The characteristic peaks of microcystin-LR and other impurity could be clearly separated. So after further concentration and centrifugation, microcystin-LR in the crude extract could be

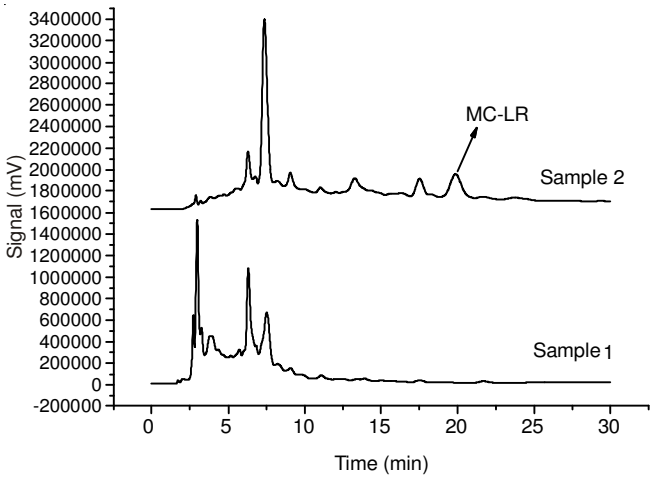

Fig. 5. Effect of concentrated by rotary evaporation and centrifugation on microcystin-LR (MC-LR) purification

further purified and the concentration could be highly improved, which was very beneficial for intercepting the highly purified microcystin-LR by HPLC.

Chromatography preparation of microcystin-LR: The effluent was collected when the microcystin-LR peak appeared in the detector, every 8 seconds for a sample, numbered in sequence, five more samples were intercepted after peak ended. 18 samples were obtained and determined the concentration separately. The result was shown in Fig. 6, microcystin-LR began to appear in the sample 3 and concentration reached maximum until the sample 6, 7, then it began to diminish gradually. Other substances were determined after microcystin-LR peak in sample 12. So, in order to receive highly concentrated and purified microcystin-LR, sample 4-8 were chosen to be collected; that was the fraction between 0.66-0.16 min after the microcystin-LR peak appeared. The result was shown in Fig. 7, microcystin-LR concentration was 7 μg/mL, purity was 95 %. microcystin-LR's molecular formula is $C_{49}H_{74}N_{10}O_{12}$. Molecular weight is 995[23]. According to Fig. 8-a, 8-b, electrospray mass spectrometry analysis of standard microcystin-LR and purified microcystin-LR could certify that the main product was microcystin-LR.

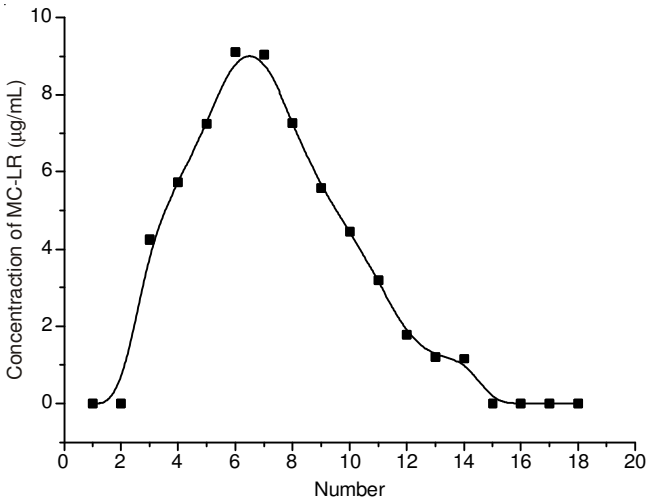

Fig. 6. Interception curves of microcystin-LR (MC-LR) by HPLC

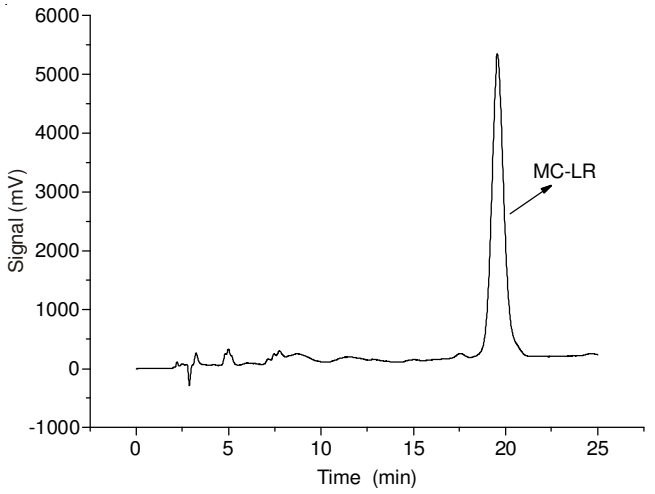

Fig. 7. HPLC chromatogram of purified microcystin-LR (MC-LR)

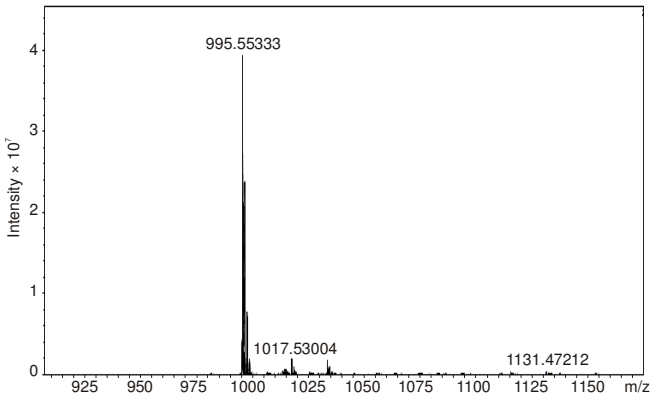

Fig. 8. (a) Electrospray mass spectrometry analysis of standard microcystin-LR

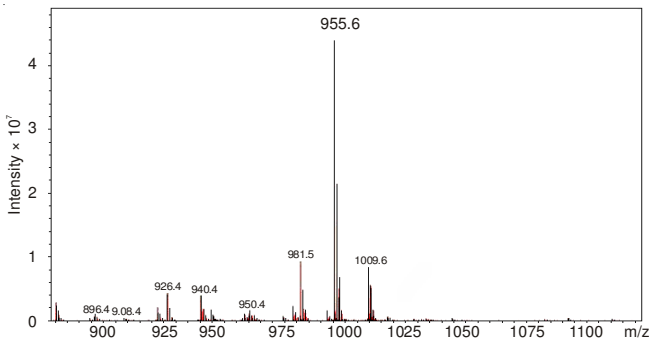

Fig. 8 (b) Electrospray mass spectrometry analysis of purified microcystin-LR

Conclusions

• An effective method for large-scale extraction and purification of microcystin-LR was established. 660 mL supernatant centrifugated from algae serum can be processed once by resin D101.40 % acetic acid (3 L), deionized water (2.5 L), 20 % acetone (2 L) and 10 % ethanol (2 L) were applied to get rid of the impurities from the resin. 70 % methanol (9 L) was used to elute the microcystin-LR from the resin. After concentrated by rotary evaporation at 80 °C. 2075.5 μg microcystin-LR was obtained, with the recovery rate of 79.71 %.

• The method of getting microcystin-LR by preparative chromatography was established, the purity of the product was 95 %.

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