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Determination of microcystins in blue-green algae, fish and water using liquid chromatography with ultraviolet detection after sample clean-up employing immunoaffinity chromatography

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Abstract

Anti-microcystin LR immunoaffinity cartridges were evaluated for their ability to selectively remove microcystins from extracts of blue-green algae, fish and water samples for subsequent analysis by liquid chromatography with UV absorbance detection at 238 nm. Blue-green algae and fish samples were extracted with 75% methanol in water. A portion of the extract was diluted and passed through an immunoaffinity cartridge. Water samples were applied directly to the cartridge. The cartridge was rinsed with water and 25% methanol in water. The microcystins were eluted with 80% methanol in water containing 4% acetic acid. It was found that the cartridges were effective in isolating the microcystins from blue-green algae, fish and water samples, resulting in extracts that were clean enough to enable direct LC–UV detection down to ~0.03 μg/g in the blue-green algae and fish samples, and as low as 0.02 ng/ml for water samples. The cartridges were found to have a capacity of ~200 ng each for a mixture of microcystins RR, YR, LR and LA, or as much as 525–800 ng for individual compounds. Recoveries through the complete analytical procedure ranged from 64 to 115% (all values) with an overall average of ~80% at spiking levels of 0.5–4.0 μg/g for the microcystins in blue-green algae. The average recoveries ($n=8$) from spiked (0.1–0.5 μg/g) fish samples were 73% for RR, 79% for YR, 81% for LR and 77% for LA, while from the spiked (2.0–0.04 ng/g) tap and river water samples ($n=6$), recoveries were 78% for RR, 86% for YR, 94% for LR and 89% for LA.

Keywords: Clean-up methods; Immunoaffinity cartridges; Microcystins; Toxins

1. Introduction

Microcystins are a group of natural toxins known to be produced by certain types of freshwater cyanobacteria (blue-green algae) and which have been shown to be liver toxins and tumour promoters [1–4]. They have been of concern in many areas of the world because of their possible contamination of drinking water supplies [5]. As a result, there has been much research carried out on the development of analytical methods for them in potable waters. Some of the more popular methods include phosphatase assay [6–8], ELISA immunoassay [9–13] and liquid chromatography (LC) with UV [14,15] or mass spectrometric (MS) detection [16–20]. Each of these methods measures different characteristics of

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the microcystins and thus can provide different information on the microcystin content of unknown samples. For example, the phosphatase assay measures inhibition of an enzyme while the ELISA test is based on molecular recognition by certain antibodies. The LC–MS approach measures discreet chemical fragments of the microcystins after a chromatographic separation step. The first two approaches measure total microcystins relative to a single microcystin standard, usually microcystin LR, and do not provide any information on the actual microcystin composition in the sample. However, these techniques are well suited to rapid screening since little sample preparation, other than dilution, is required. The LC–MS method can be much more definitive since it provides structural information for the identification of the microcystins. However, it is not particularly suited to the detection of microcystins for which there are no standards available. All methods may suffer from a certain percentage of false positives and negatives due to matrix effects. In careful, well controlled analyses these are usually at a minimum. However, for regulatory purposes it is prudent to confirm positive findings by an independent technique.

In 1999, Health Canada carried out a national survey of blue-green algal health food products for microcystins. In developing the analytical method, LC–MS was chosen as the procedure of choice. However, results were confirmed both by ELISA and colorimetric phosphatase assay [21]. During this work attempts were made to use LC with UV detection as a screen prior to LC–MS analysis. Unfortunately, we found that the methods available in the literature employing SPE-C_{18} and SPE-silica, usually applied to water samples [13,22,23], were not at all suitable for the quantitative determination of microcystins in blue-green algal products at concentrations of 1.0 μg/g or less. The main reason was that the extracts contained too much coextracted material that interfered in the LC–UV detection. In the present work, we report on the results of evaluating immunoaffinity chromatography (IAC) for the sample purification. We have previously shown that IAC can provide excellent sample clean-up for a variety of toxic chemicals in biological samples at trace concentrations while at the same time using little or no organic solvents (other than alcohols) in the extraction and clean-up procedure [24–28].

2. Experimental

2.1. Reagents

All chemicals and reagents were analytical grade materials. Methanol was the only organic solvent employed in the sample extraction and clean-up. Doubly deionized water was used throughout. Microcystin LR and RR were obtained as analytical standards from Sigma, St. Louis, MO. Microcystin YR and LA were from Calbiochem-Novabiochem, La Jolla, CA. Nodularin was received as a gift from A. Sadiki, Environmental Health Directorate, Health Canada. Stock solutions were prepared in methanol and diluted as required with H_2O for use as working solutions. Phosphate buffered saline (PBS) was prepared by dissolving 2.68 g Na_2HPO_4 and 8.76 g sodium chloride in 1 l H_2O. The pH was adjusted to pH 7.4 with 0.1 *M* phosphoric acid. The blue-green algae samples were obtained from health food outlets from across Canada. The fish samples (salmon, rainbow trout and pickerel) were purchased at a local fish market.

2.2. Immunoaffinity cartridges

Anti-microcystin LR polyclonal antibodies were produced in the Food Research Division, Health Canada from New Zealand white rabbits. The resulting antibodies, isolated from the antisera using affinity chromatography (Avid-Chrom gel) were immobilized, using previously published techniques, on Sepharose CL-4B or activated silica gel supports [29,30]. Approximately 100–200 mg of the immunosorbents were packed into individual cartridges for evaluation. The cartridges were washed and stored in PBS at 4°C when not in use. For storage of the cartridges longer than 1 week, sodium azide was added to the PBS solution (0.02% w/v) to prevent mould and bacterial growth.

2.3. Liquid chromatography

The equipment consisted of a Hewlett-Packard Series 1100 Automated Quaternary LC3D System including a quaternary pump, a vacuum degasser, an autosampler, a diode-array detector set at 238 nm, an LC 3D ChemStation for data acquisition and processing, and a Symmetry LC-C_{18} column (5 μm

ODS, 150×3.9 mm I.D.) (Waters). Mobile phase A was acetonitrile–water (20+80) containing 0.05% (v/v) trifluoroacetic acid (TFA) and mobile phase B, acetonitrile–water (80+20) containing 0.04% (v/v) TFA. The chromatographic run consisted of a linear gradient from 10 to 29% B over 7 min then to 60% B over another 7 min and finally up to 80% B over 1 min. The flow-rate was 1 ml/min. Retention times were ~5.4 min for microcystin RR, 7.3 min for nodularin, 8.1 min for YR, 8.6 min for LR and 13.4 min for LA.

2.4. Algae and fish sample extraction

A 3-g portion of ground algae (using a coffee grinder) or fish (skinless, boneless and homogenized by a mincer-chopper) sample was placed into a 100-ml beaker. A 20-ml volume of methanol–water (75+25) was added and the contents mixed for 3 min using a Polytron homogenizer. The mixture was then transferred to a 40-ml polypropylene centrifuge tube and centrifuged at 4500 rpm (3600 *g*) for 10 min at room temperature. The supernatant was transferred to a clean graduated 40-ml glass tube. The residue was remixed with 10 ml of the methanol–water (75+25) and recentrifuged at 4500 rpm (3600 *g*) for 10 min at room temperature. The supernatant was transferred to the first portion and the volume made up to 30 ml with methanol–water (75+25).

2.5. Water sample analysis

River and tap water were used directly without extraction after filtration through a 0.45-μm disc (type HA, Millipore). Normally 5–15 ml of water sample were passed through the immunoaffinity cartridge. For improved sensitivity, volumes of water from ~20 to 500 ml were concentrated using a roto-evaporator at 35°C to a volume of ~5–15 ml prior to application. This concentration step had no influence on the recovery of the toxins through the procedure.

2.6. IAC Sepharose and silica cartridges

2.6.1. Algae and fish samples

An aliquot of extract equivalent to 5–200 mg of sample was diluted with PBS to obtain a methanol concentration of less than 15% and then added to the cartridge, previously conditioned with 3 ml water and 3 ml of PBS. The flow-rate was maintained at ~1 ml/min. The cartridge was rinsed with 3 ml of PBS, followed by 3 ml of water and then 3 ml of methanol–water (25+75). The microcystins were then eluted with 6 ml of methanol–water (8+2) containing 4% (v/v) acetic acid. The effluent was collected in a 50-ml round bottom flask and evaporated at 35°C to dryness using a roto-evaporator, then redissolved in 0.5–1.0 ml of acetonitrile–water (2+8). A 100-μl volume of this solution was injected into the LC system for analysis. The cartridge was rinsed with 5 ml water and 5 ml PBS and stored for later use.

2.6.2. Water samples

A 5–15-ml aliquot of river water (up to 500 ml before the concentration) was added to a cartridge previously conditioned with 3 ml PBS. The flow-rate was maintained at ~1–2 ml/min. The cartridge was rinsed with 6 ml of methanol–water (25+75). For the samples which were concentrated by rotary evaporation, the 6 ml of methanol–water (25+75) was first added to the round bottom flask (as a rinse) before being passed through the cartridge. The microcystins were then eluted with 6 ml of methanol–water (8+2) containing 4% (v/v) acetic acid. The effluent was collected in a 50-ml round bottom flask and evaporated at 35°C to dryness using a roto-evaporator, then redissolved in 0.25–1.0 ml of acetonitrile–water (2+8). A 100-ml volume of this solution was injected into the LC system for analysis. The cartridge was rinsed with 5 ml water and 5 ml PBS and stored for later use.

Note that during the course of this work we found that certain types of 0.45-μm syringe filters contained impurities that caused significant interferences in the chromatograms. This is partly due to the non-selectivity of the UV absorption wavelength, 238 nm, used for detection. Thus, filters should be tested before use and washed if necessary before using.

3. Results and discussion

3.1. Immunoaffinity cartridge characterization

The polyclonal antibodies tested were produced

according to the procedure used for fumonisins [28]. Both the Sepharose and silica IAC cartridges retained the microcystins. The Sepharose cartridge was found to have a capacity of ~200 ng each for a mixture of microcystins RR, YR, LR and LA, or as much as 525–800 ng for individual compounds (760 ng RR, 800 ng YR, 570 ng LR and 525 ng for LA). The silica cartridge had a capacity of ~90–135 ng for each microcystin in a mixture. Both types of cartridge showed a gradual loss of antibody activity with repeated use. The capacity of the Sepharose based cartridge decreased by ~50% after 28 uses using standards and different algal extracts while the capacity of the silica based cartridge decreased by ~44% after three to four uses using standards only. Thus, the silica cartridges were not evaluated further. It is possible the immobilization of the antibodies was not particularly successful, since we have had good success with other antibodies immobilized on this material [24,25,27,28]. However, this was not investigated further.

To ensure that the anti-microcystin antibodies were responsible for the retention of the analytes by the Sepharose cartridges, a ''blank'' IAC cartridge was prepared using the same immobilization procedure but with antibodies that did not recognise the microcystins. All microcystins passed through the blank IAC unretained, indicating that the immobilized anti-microcystin antibodies were functioning and were responsible for microcystin retention.

The IAC clean-up procedure described in the Experimental for the Sepharose cartridges was obtained after many studies and found to be optimum for microcystins RR, LR, YR and nodularin. These microcystins were essentially quantitatively recovered from the IAC cartridges with only 5 ml of eluting solvent containing only 0.2% (v/v) acetic acid. Unfortunately, LA was not effectively recovered with this volume. It required as much as 14 ml of the elution solution for best recovery. Even then some LA occasionally remained on the cartridges even after reconditioning. As a result, it was necessary to carry out a blank clean-up cycle to ensure that no LA remained on the cartridges that could lead to false positives in subsequent samples. However, we found that by increasing the acetic acid concentration to 4% (v/v), LA was consistently recovered in only 6 ml of the elution solution and that no LA remained on the cartridges. The increased acid concentration did not affect the recoveries of the other microcystins, nor did it have a detrimental effect on the lifetime of the cartridges. The average recoveries ($n=7$) for 50–100 ng each of pure standards of these microcystins passed through three different IAC cartridges were 94% (range, 84–110%) for RR, 92% (range, 71–111%) for YR, 95% (range, 85–115%) for LR and 85% (range, 79–97%) for LA. Recoveries of nodularin were found to be >90% for $n=4$.

3.2. Application to algae samples

Fig. 1 shows chromatograms of a standard mixture directly analysed by LC and a spirulina blue-green algae extract, spiked to contain 1 μg/g of each microcystin, after the Sepharose-based IAC clean-up. It can be seen that the chromatograms are very similar demonstrating the very good selectivity of IAC for sample clean-up at the 1-μg/g concentration level. These chromatograms are typical of the results

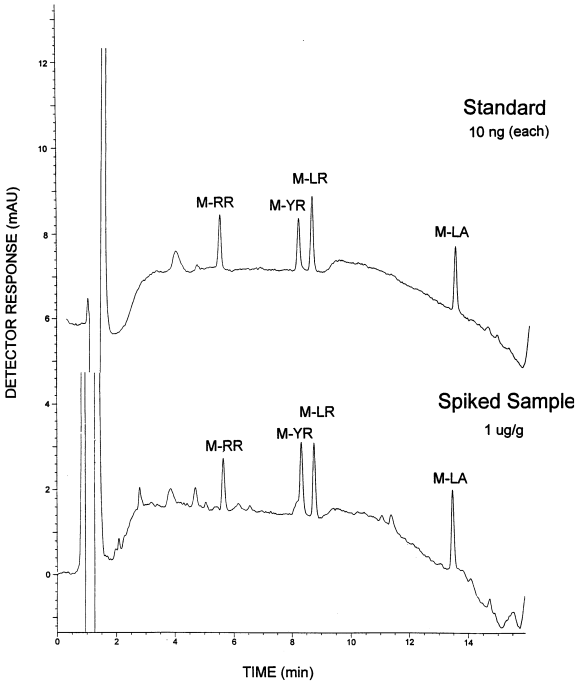

Fig. 1. Comparison of a direct standard mixture (10 ng each injected) and a spirulina sample spiked to contain 1 μg/g of each toxin, after IAC Sepharose cartridge clean-up.

obtained with many different blue-green algal samples. Average recoveries (n=6–8) from three different blank algae samples spiked at 1–4 μg/g for each microcystin were 79% (range 70–92%) for RR, 86% (73–104%) for YR, 82% (72–96%) for LR and 70% (50–84%) for LA. Estimated detection limits in algae samples were ~0.03 μg/g (3:1, signal-to-noise ratio).

Some algal samples exhibited matrix effects that decreased the recovery of the microcystins through the IAC clean-up procedure. One spirulina sample in particular always produced ~30%–60% recovery for the individual microcystins, regardless of the spiking level (1–4 μg/g) or amount of sample passed through the cartridges (25–100 mg). Because of this, it is important to verify quantitative results on unknown samples (particularly spirulina samples) by doing recovery studies on those specific samples by adding known amounts of standards and reanalysing them. However, for the majority of samples analysed recoveries were similar to those mentioned above.

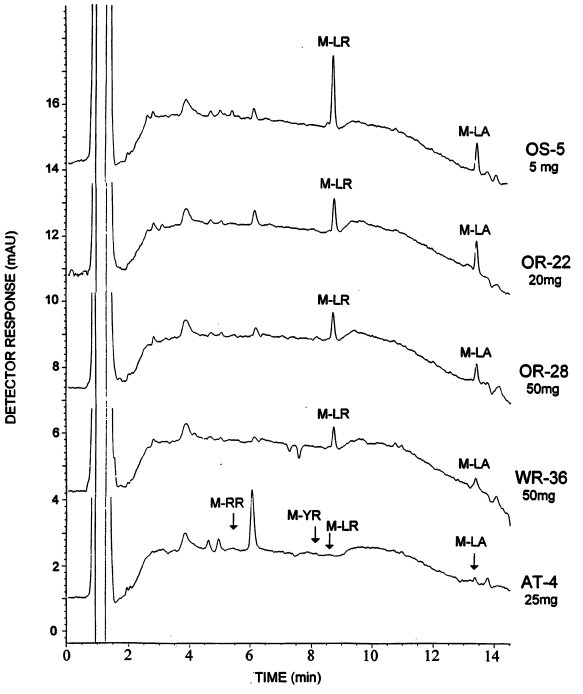

Fig. 2. Chromatograms of a blank and four naturally contaminated blue-green algae samples after passing different quantities of sample through the IAC cartridge: AT-4 blank (25 mg equivalent sample); WR-36 (50 mg equivalent sample) estimated to contain 0.8 μg/g of LR and 0.6 μg/g of LA; OR-28 (50 mg) estimated to contain 1.1 μg/g of LR and 0.7 μg/g of LA; OR-22 (20 mg) estimated to contain 3.2 μg/g of LR and 2.6 μg/g of LA; OS-5 (5 mg) estimated to contain 12.9 μg/g of LR and 5.9 μg/g of LA.

Since the IAC cartridges have a finite capacity to retain the microcystins, it is important that the sample extract being passed through the cartridges contains less total microcystins than the cartridge capacity, otherwise losses will occur. The experimental procedure developed here was designed to quantitate microcystins in blue-green algae samples at concentrations around 1 μg/g. Thus, the amount of sample taken for IAC clean-up was 50–100 mg to ensure that the capacity of the IAC cartridges would not be exceeded if they contained 1 μg/g of each microcystin. For highly contaminated samples, much less sample material must be passed through the cartridges so that capacity is not exceeded. For samples containing much less than 1 μg/g more sample extract can be passed through the cartridges to improve the detection limit. In the present work the maximum amount of sample passed through the cartridges was 200 mg, yielding a detection limit of ~0.03 μg/g.

Fig. 2 shows typical results of naturally contaminated blue-green algae samples containing a range of concentrations of microcystins LR and LA. These were the only two microcystins found in the samples. As can be seen, the chromatograms are very clean and the microcystins are readily identified and quantitated. A number of these samples were extracted in replicate and analysed to determine the repeatability of the method. A highly contaminated sample (n=3) gave a mean of 13.1 μg/g (range, 12.4–13.8 μg/g) for LR and 6.3 μg/g (range, 5.9–6.7 μg/g) for LA. Another sample (n=5) gave a mean of 1.1 μg/g (range, 0.8–1.3 μg/g) for LR and 0.9 μg/g (range, 0.7–1.0 μg/g) for LA. A low level sample (n=6) produced a mean of 0.17 μg/g (range, 0.14–0.20 μg/g) for LR and 0.47 μg/g (range, 0.38–0.68 μg/g) for LA.

A number of sample extracts were analysed by an independent liquid chromatography–mass spectrometric method (for details, see Ref. [21]) for comparison purposes. The results are shown in Table 1. It can be seen that there is a very good agreement between the two methods, indicating that the immunoaffinity-LC method provides comparable results for these toxins in blue-green algae samples. This is

Table 1
Comparison of LC–UV with LC–MS for the analysis of microcystins in blue-green algae samples (μg/g)

Sample	LC–UV		LC–MS	
	LR	LA	LR	LA
A	1.0	0.3	0.9	0.4
B	1.3	2.0	0.9	1.6
C	1.0	0.7	1.1	0.8
D	3.2	2.6	3.5	2.4
E	1.0	0.5	0.9	0.4
F	1.1	1.6	0.9	1.5

important since it demonstrates that the less expensive LC–UV method can be used to do routine monitoring of blue-green algae samples while confirmation of positive samples can be accomplished using LC–mass spectrometry with a good quantitative correlation expected between the two methods.

3.3. Application to fish samples

Although the primary purpose of this work was to evaluate immunoaffinity chromatography for application to algae samples, we carried out some additional work to determine the usefulness of the approach to fish and water samples. Chromatograms of blank and spiked (0.25 μg/g) salmon samples after the Sepharose-based IAC clean-up are shown in Fig. 3. An unknown peak was observed at ~4.6 min (with variable intensity) although it never interfered with the quantitation of the microcystins. It appeared to be the result of an accumulation of a coextractive on the LC column, since the same peak was also obtained from a blank IAC run and even a direct standard injection. The average recoveries ($n=8$) from three different fish samples (salmon, rainbow trout and pickerel) spiked at 0.1–0.5 μg/g with each microcystin were 73% (range, 62–99%) for RR, 87% (range, 79–104%) for nodularin, 79% (range, 71–93%) for YR, 81% (range, 68–91%) for LR and 77% (range, 65–87%) for LA.

3.4. Application to water samples

Fig. 4 shows chromatograms after the Sepharose-

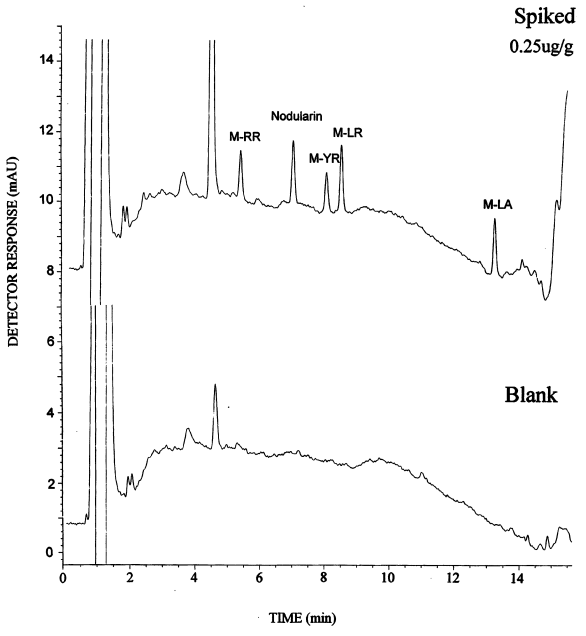

Fig. 3. Chromatograms of a salmon sample (200 mg equivalent) blank and spiked to contain 0.25 μg/g of each microcystin after IAC cartridge clean-up. Recoveries obtained were 65% RR, 84% nodularin, 77% YR, 78% LR and 75% LA.

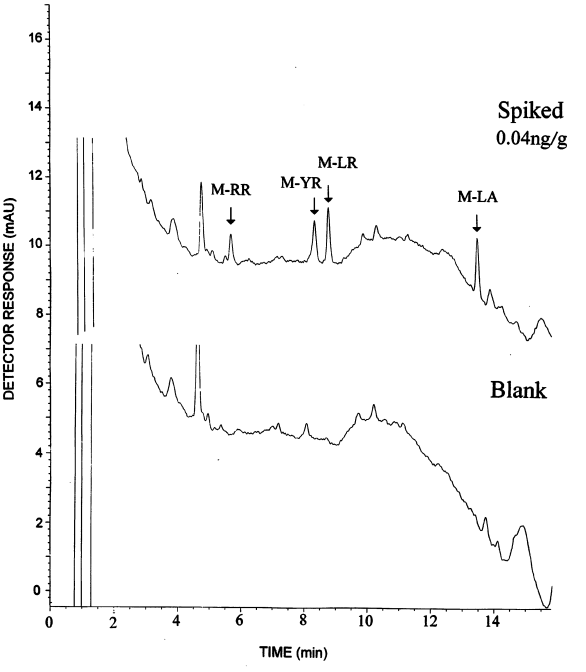

Fig. 4. Chromatograms of Ottawa River water samples, 500 ml, blank and spiked to contain 0.04 ng/g of each toxin, after IAC cartridge clean-up. Recoveries obtained were 91% RR, 84% YR, 92% LR and 93% LA.

based IAC clean-up of an Ottawa River water blank and one spiked to contain 0.04 ng/g of each microcystin. In these cases, 500 ml of each water sample was concentrated to ~10 ml before passing through the cartridge. As can be observed, the toxins are readily detected at this level. The average recoveries from spiked (2.0–0.04 ng/g) tap and river water samples ($n=6$) were 78% for RR, 86% for YR, 94% for LR and 89% for LA. The estimated detection limits were ~0.02 ng/ml. Local tap water was also analysed with similar results.

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