Talanta 84 (2011) 638–643

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Rapid surface plasmon resonance immunobiosensor assay for microcystin toxins in blue-green algae food supplements

Tatiana Vinogradova[a], Martin Danaher[b,*], Andrew Baxter[a], Mary Moloney[b], Danielle Victory[b], Simon A. Haughey[c]

[a] *Xenosense Ltd., Belfast, Northern Ireland*
[b] *Food Safety Department, Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland*
[c] *IAFLU, Queen's University Belfast, Northern Ireland*

ARTICLE INFO

Article history:
Received 1 September 2010
Received in revised form 2 January 2011
Accepted 16 January 2011
Available online 22 January 2011

Keywords:
SPR-biosensor
Microcystins
Blue-green algae
Food supplements

ABSTRACT

A surface plasmon resonance (SPR) immunobiosensor assay was developed and validated to detect microcystin toxins in *Spirulina* and *Aphanizomenon flos-aquae* blue-green algae (BGA) food supplements. A competitive inhibition SPR-biosensor was developed using a monoclonal antibody to detect microcystin (MC) toxins. Powdered BGA samples were extracted with an aqueous methanolic solution, centrifuged and diluted in HBS-EP buffer prior to analysis. The assay was validated in accordance with the performance criteria outlined in EU legislation 2002/657/EC. The limit of detection (LOD) of the assay was calculated from the analysis of 20 known negative BGA samples to be 0.561 mg kg^{-1}. The detection capability (CCβ) of the assay was determined to be ≤0.85 mg kg^{-1} for MC-LR. The biosensor assay was successfully applied to detect MC-LR toxins in BGA samples purchased on the Irish retail market. MC-LR was detected in samples at levels ranging from <0.5 to 2.21 mg kg^{-1}. The biosensor results were in good agreement with an established LC–MS/MS assay. The assay is advantageous because it employs a simple clean-up procedure compared to chemical assays and allows automated unattended analysis of samples unlike ELISA.

1. Introduction

Microcystins (MCs) are potent toxins produced by a number of cyanobacterial genera (often referred to as blue-green algae, BGA) most notably, widespread *Microcystis aeruginosa,* from which these toxins were first isolated and their name derived [1]. BGA is the former name for what we now call cyanobacteria, but for the purpose of this paper blue-green algae is used to be consistent with the term used for food supplements. MCs have now been shown to be produced by species of the planktonic genera *Anabaena*, *Aphanocapsa*, *Hapalosphon*, *Microcystis*, *Nostoc, Oscillatoria* and *Planktothrix*, though not all strains of these organisms produce toxins [2,3]. The cyanobacterial toxins are bound to the cell, but they are released in water when the cell dies, when it is lysed or compromised. MCs are a large group of 800–1000 Da cyclic heptapeptides with five non-protein and two protein amino acids (Fig. 1) [4]. It is these two protein amino acids that distinguish MCs from one another, while the other amino acids are more or less constant between variant MCs. Using amino acid single letter code nomenclature, each MC is designated a name depending on the variable amino acids which complete their structure. The most common and highly toxic MC-LR [5] contains the amino acids leucine (L) and arginine (R) in these variable positions. The other MCs which are naturally widespread and dangerous for humans and animals are MC-LW, -YR, -LF, and the structurally related nodularin [6].

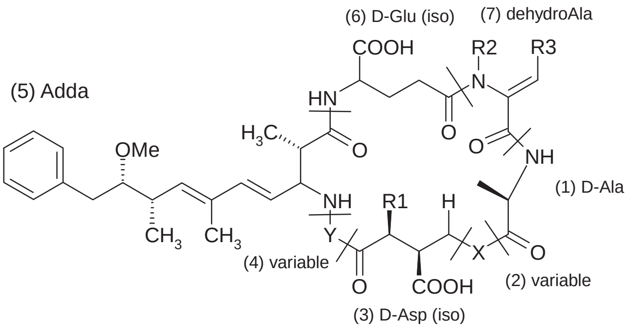
The biological effect of MCs is mainly based on the inhibition of the key enzymes in cellular regulation, namely protein phosphatases 1 and 2A, to which they bind covalently [7]. No antidotes are known. Symptoms of acute poisoning from MCs include skin irritation, vomiting, diarrhea, and fatal acute liver failure [8]. A long-term effect from exposure to small doses of MCs in humans also includes primary liver cancer, which has also been reported [9] and studied by experimental models [10].

The major route of human exposure to cyanobacterial toxins is through the consumption of contaminated drinking water and this issue has received increasing attention around the world as a public health concern [11]. A less common route is the recreational use of lakes and rivers but absorption through skin contact is unlikely as the toxin does not readily cross cell membranes [12]. Potential risks from exposure to toxins in contaminated BGA health food products have received less attention [13]. BGA are harvested to produce blue-green algae food supplements (BGAS) advertised as healthy nutritionally balanced natural source of vitamins, minerals, and amino acids. However, the occurrence of toxic species during harvesting can result in the contamination of BGA products. The

---

* Corresponding author. Tel.: +353 1 8059500; fax: +353 1 8059550.
E-mail address: martin.danaher@teagasc.ie (M. Danaher).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.01.036



Microcystin	R1(3)	*X* (2)	*Y* (4)	R2 (7)	R3 (7)
MC-LR	CH_3	Leu	Arg	CH_3	H
MC-RR	CH_3	Arg	Arg	CH_3	H
MC-YR	CH_3	Tyr	Arg	CH_3	H
MC-LA	CH_3	Leu	Ala	CH_3	H
MC-LW	CH_3	Leu	Trp	CH_3	H
MC-LF	CH_3	Leu	Phe	CH_3	H

Fig. 1. Structure of different microcystin congeners. Adapted from Zeck et al. [28], Copyright (2001), with permission from Elsevier. Nodularin has the structure cyclo(D-*erythro*-β-methylisoAsp-L-Arg-Adda-D-*iso*-Glu-*N*-methyldehydroAla).

accumulated information on the MC toxicity, results of the BGAS surveys showing contamination of the BGAS with MCs [13–15] and increased interest in health foods and supplements including BGAS, lead to the discussion of the guideline for MC contents in BGAS and their Tolerable Daily Intake (TDA) [16–19]. The Oregon Health Division and the Oregon Department of Agriculture established a regulatory limit of 1 mg kg^{-1} for MCs in BGA-containing products [16]. This limit is enforceable for food supplements on sale in the State of Oregon. No federal guidelines set for microcystins in food supplements in the United States or in the European Union.

Several analytical methods, including ELISA immunoassay [14,20], protein phosphatase immunoassay [14], liquid chromatography (LC) with UV [15] or mass spectrometric (MS) detection [14,20], ultra performance liquid chromatography coupled to orthogonal acceleration time of flight mass spectrometry [21], and multiplex PCR [22], have been described for detection of MCs in BGAS. Affinity-based immunosensor techniques have been used recently to exploit the potential for analyte detection through the coupling of a biospecific binding protein i.e. antigen interaction via optical, piezoelectric or electrochemical signal transducers. A commercially available Biacore Q biosensor system based on the surface plasmon resonance (SPR) optical phenomenon exploits the non-labeled interaction of a specific binding protein with the covalently immobilised analyte of interest on a carboxymethyldextran modified gold sensor surface. The SPR detector monitors changes in resonance angle due to molecular interaction at the interface which is directly proportional to the change of mass at the surface. SPR biosensor assays employ label-free detection and have proven to be versatile, robust and capable of producing rapid and reliable results with minimum sample preparation [23]. In the past, assays have been developed using SPR instrumentation to analyse a wide range of analytes including veterinary drug residues [24,25], mycotoxins [26] and paralytic shellfish poisoning toxins [27].

In this work, a SPR-based immunosensor assay was used to detect MC-LR levels (<1 mg kg^{-1}) in BGAS using a simple sample preparation procedure that negates the need for lengthy clean-up procedures compared to chemical assays resulting in a reduction in solvent usage in the laboratory [14,15]. In comparison, to other screening assays the method is advantageous because of the automated real-time analysis of extracts, which eliminates the plate washing and incubation steps required for ELISA [14]. This should make the transfer of assays between laboratories easy through the reduction of operator effects normally associated with ELISA. The developed method was evaluated and cross reactivity of other MC congeners was determined. Following method validation, it was then used to detect MC-LR in naturally contaminated BGAS samples and the data were compared to those generated using an established LC–MS/MS method.

2. Materials and methods

2.1. Chemicals and reagents

Dimethylsulphoxide, DMSO, acetonitrile and methanol (all HiPerSolv grade) were supplied by BDH/VWR international Ltd. (Poole, England, UK). Dimethylsulphoxide, DMSO (Analar grade) was purchased from Sigma Aldrich. Ultra-pure water (18.2 MΩ cm) was generated using an in-house Milli Q Synthesis system. Sodium hydroxide (Analar grade) and sodium hypochlorite (GPR grade) were purchased from BDH/VWR international Ltd. CM5 sensor chips (research grade), an amine coupling kit containing, NHS (100 mM *N*-hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM HEPES pH 7.4 with 0.05 M NaCl, 3.4 mM EDTA and 0.005% P20 (v/v) were all obtained from GE Healthcare (Uppsala, Sweden). Cetyltrimethylammonium bromide (CTAB) was from Sigma Aldrich (Steinheim, Germany). MC-LR, -RR, -YR, -LF, -LW and nodularin were from Alexis Biochemicals (San Diego, U.S.A.). MC-LA and MC-LR Reference Material (RM), 1 μg mL^{-1} solution in ethanol (no. 33893) were from Sigma–Aldrich. Monoclonal antibody (MC10E7) raised against MC-LR coupled to a cationised ovalbumin carrier protein was purchased in its lyophilized form Squarix Biotechnology GmbH (Marl, Germany). The production and characterization of this antibody has been reported elsewhere [28]. Whatman ReZist syringe Filter units (polytetrafluoroethylene (PTFE), 13 mm/0.45 μm) were purchased from Fisher Scientific (Dublin, Ireland).

2.2. Negative and positive control samples

Spirulina samples purchased in local shops and determined to be negative for MC residues by LC–MS/MS were used as negative controls for the biosensor detection system. Klamath Lake BGA found to contain <0.5 mg kg^{-1} were used as negative controls for LC–MS/MS. Klamath Lake BGA samples purchased in local shops and determined to contain MC-LR residues between <0.5 and 3 mg kg^{-1} by LC–MS/MS were used to measure the performance of the biosensor assay.

2.3. SPR-biosensor assay

2.3.1. Extraction procedure

The assay was suitable for detection of MC toxins from BGA food supplement samples in liquid, powder, capsule and tablet forms. Liquid (1 mL) or powder (1 g) samples were directly extracted. Representative samples of BGAS were prepared from the contents of 10 capsules or grinding 10 tablets into a fine powder with a mortar and pestle. Powder from a BGAS sample (1 g) was weighed out into a glass vial suitable for subsequent centrifugation, extracted by adding methanol:water (75:25, v/v,10 mL) and stirred for 1 h on a magnetic stirrer. The sample extracts were centrifuged at 4500 *g* for 10 min. A 100 μL aliquot of the supernatant was then carefully transferred into a glass vial and diluted 100 fold in HBS-EP buffer (9.9 mL).

2.3.2. *SPR-biosensor chip preparation*

Stable biosensor chip surfaces were produced through immobilisation of MC-LR using an amine coupling procedure in the presence of CTAB at pH 7.4 in an overnight incubation at room temperature. This chip surface was found to be stable for at least 1000 cycles. In brief, a CM5 chip was then equilibrated to room temperature and placed in a covered tray with wet paper to maintain high humidity environment. A solution of NHS/EDC (1:1, v/v, 50 μL) was then added to the chip surface to activate the surface. This solution was removed after 20 min and replaced with pre-mixed 25 μL of 5 mg mL^{-1} MC-LR and 25 μL of 0.6 mM CTAB in 10 mM HEPES pH 7.4 for overnight incubation. This MC-LR solution was replaced with 50 μL of 1 M ethanolamine–HCl pH 8.5 for 20 min to deactivate the surface. The surface was washed with water and dried under nitrogen. The surface response saturated with antibody was in the range of 120,00–16,000 RU. Each new flow cell was treated with the regeneration solution (40 mM NaOH, 20% acetonitrile) by five injections for 60 s at flow rate of 20 μL min^{-1} to condition the surface prior to using the cell for concentration analysis.

2.3.3. *SPR-biosensor analytical cycle*

The antibody concentration of 1 μg mL^{-1} was found to give the greatest sensitivity under the assay conditions. The stock antibodies diluted with HBS-EP to 1 μg mL^{-1} were mixed with a sample or with a MC-LR calibration solution prior to injection across the surface. The sample/calibration solution injection parameters were as follows 10% fraction of the antibody solution (1 μg mL^{-1}) at the flow rate of 13 μL min^{-1} for 960 s. The injection time is longer than typical SPR biosensor cycles but results in more sensitive detection of microcystin toxins. This improvement in sensitivity eliminates the need for lengthy sample concentration steps, which is generally the most time consuming step in residue analysis. This time saving can increase sample throughput and allow the potential reporting of test results during the day analysis. Regeneration of the surface was carried out by injecting 40 mM NaOH, 20% acetonitrile (v/v) across the chip surface at 20 μL min^{-1} for 30 s. The binding of antibody to the chip surface was measured in arbitrary resonance units (RU) as the change in surface plasmon resonance (SPR) signal between two report points, 10 and 30 s before and after each sample/calibration injection.

2.3.4. *Biosensor calibration*

MC-LR calibration curves at concentrations of 0.40, 0.59, 0.89, 1.33 and 2.00 ng mL^{-1} (1.5-times serial dilution) were prepared in HBS-EP buffer. This calibration range was selected because it covered the range typically found in BGAS. HBS-EP buffer was used as the zero concentration point. Biacore Q Control Software (3.0.4) was used to generate inhibition assay standard curves based on a four-parameter equation. The MC concentration in test samples was then read directly from the calibration curve.

2.4. *LC–MS/MS assay*

2.4.1. *Sample extraction and clean-up*

An LC–MS/MS method was developed to detect MC toxins in blue-green algae based on the method developed by Lawrence et al. [14]. BGA samples (1 g) were weighed into tubes, methanol:water (75:25, v/v, 10 mL) was added and samples were homogenised (2.5 min) using a FASTH 21$^{\text{TM}}$ automated homogenisation unit. The system was capable of unattended homogenisation of 24 samples in a 15 min period. Sample extracts were centrifuged (2113 g) and filtered through Whatman No. 2 filter paper (Cat No. 1002 090). An aliquot of the sample extract (0.5 mL) was diluted with water (2 mL) and applied to a preconditioned Oasis® HLB SPE cartridge (60 mg, 3 mL). The cartridge was washed with methanol:water (30:70, v/v, 5 mL) and eluted with methanol:water (80:20, v/v, 4 mL). Sample extracts were evaporated to dryness at 50 °C under nitrogen. Extracts were reconstituted by sequential addition of methanol (400 μL) and water (600 μL) and votexing after each addition (2 min). Purified sample extracts were filtered through 0.45 μm PTFE filters and 10 μL was injected onto the LC–MS/MS system.

2.4.2. *Chromatographic conditions*

Chromatographic separation was carried out on a Waters Alliance 2795 HT separations module using a stainless steel Waters Atlantis C_{18} column (3 μm, 100 × 2.1 mm i.d.) maintained at 40 °C. The chromatographic separation was achieved using a binary gradient system comprised of Mobile phase A, 0.01% formic acid in water:ACN (90:10, v/v) and Mobile phase B, formic acid:ACN (0.1:99.9, v/v) pumped at a flow rate of 0.3 mL min^{-1}. Mobile phase was prepared daily and filtered using 0.45 μm filter membrane and degassed in an ultrasonic bath for 15 min. The gradient profile was as a follows (1) 0 min, 100%B, (2) 3.33–7.33 min, 33%B, (3) 7.5–12.4 min, 100%B, (4) 12.5 min, 100%A. Chromatographic separations were achieved in 14 min.

2.4.3. *MS/MS conditions*

MC-LR residues were detected using a Quattro Premier triple quadrupole mass spectrometer equipped with ESI interface (Waters, Milford, MA, USA). MS analyses were performed by atmospheric pressure electrospray ionisation in positive ion mode using selected reaction monitoring (SRM). The capillary voltage was set at 1 kV. The source and desolvation temperatures were set at 140 and 400 °C, respectively. The MS/MS conditions were optimised by tuning the cone voltage (75 V) and collision energy (CE) by infusing a 1000 ng mL^{-1} MC-LR standard solution. The two most abundant fragment ions produced from the molecular ions 995.5 > 213 (CE = 54) and 995.5 > 135 *m*/*z* (CE = 63). The nitrogen desolvation and cone gases were set at 1000 and 150 L h^{-1}, respectively. The system was controlled by Masslynx software and the results were processed by TargetLynx Software.

2.4.4. *LC–MS/MS calibration*

A 200 μg mL^{-1} working standard was prepared by dissolving 1 mg of MC-LR in 5 mL DMSO. Calibration curves were prepared by using solvent standards at concentrations of 0, 10, 20, 40, 100, 200 and 400 ng mL^{-1} in methanol: water (40:60, v/v). The preparation of matrix matched standards was not possible due to the low quantities and cost of the available MC-LR. Ion suppression and enhancement effects were monitored in each run through the analysis of BGA samples spiked in duplicate at levels of 1 and 5 mg kg^{-1}.

3. Results and discussion

3.1. *Antibody studies*

The specificity (CR, cross-reactivity) of the assay was evaluated using MC-LR, -RR, -LA, -LW, -LF, -YR and nodularin toxins (Fig. 2). The MC10E7 antibody recognises arginine amino acid residues in MC structural analogues. Thus, it was expected that MC10E7 could detect arginine containing MC-LR, -RR, -YR, and nodularin. Studies showed that the MC10E7 antibody was suitable for detecting several arginine containing analytes with cross-reactivities of 69%, 61%, 73% and 100% to MC-RR, MC-YR, nodularin and MC-LR respectively (Table 1). As expected, the MC10E7 antibody did not show cross-reactivity to MC-LA, -LW, and -LF. The LD_{50} values for MC-LR, MC-LA, MC-RR, MC-YR and nodularin were determined to be 50, 50, ≥500, ≥150 and 50 μg kg bw^{-1} in mice, respectively [29]. The analysis of BGAS demonstrates the risk of false positive results is low, with good agreement between biosensor and LC–MS/MS results, which indicates that MC-LR is the major arginine congener

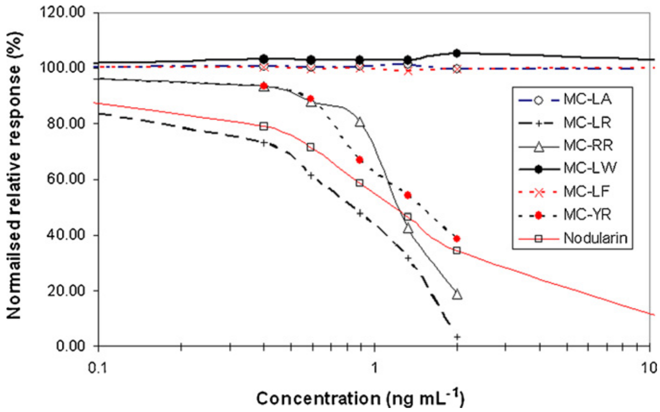


Fig. 2. Standard curves prepared for different MC standards prepared using MC10E7 antibody.

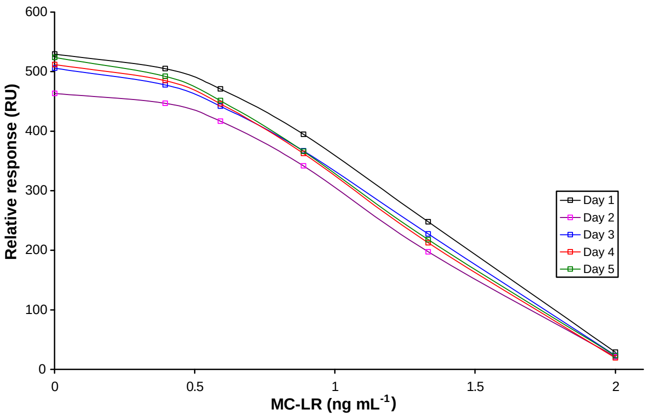


Fig. 3. Overlay of MC-LR calibration curves in HBS-EP buffer ($n = 5$ different days).

present in BGAS. There is potential for false negative results due to failure to detect non-arginine containing MCs. However, it has been shown by several groups that MC-LR is the major MC toxin detected in BGAS [14,15]. MC-LA has also been detected in samples in BGAS but generally at lower levels than MC-LR. Therefore, it can be concluded that the risk of false negative results is low and can be further reduced through the inclusion of MC-LA in the confirmatory LC–MS/MS assay.

However, all of the limits proposed for microcystins in water and food supplements use MC-LR as a marker residue. All of the internationally reported limits set for MC toxins in water use MC-LR (free plus cell bound) as the marker toxin [4,30,31]. These guidelines are believed to protect human health against exposure to other MCs (total MCs, free plus cell bound) that may also be present. Therefore, it can be concluded that the current assay, which uses an arginine specific monoclonal antibody is suitable for detecting microcystin toxins in BGAS.

3.2. Assay development

3.2.1. Biosensor assay

The development of stable and reusable sensor chip surfaces is critical for SPR immunobiosensor assays. Several approaches were investigated for immobilising immobilisation of MC-LR onto the dextran layer of the sensor chip surface. One of which was the MC-LR immobilisation using carboxyl groups (–COOH activation), another approach used the immobilisation of the amino group ($-NH_2$) of MC-LR (amine coupling). The effect of different factors on immobilisation were evaluated. These included MC-LR concentrations, acidic, neutral and alkaline conditions and the variation of immobilisation times (from 2 h to overnight). MC-LR immobilisation was carried out in the presence of a micelle forming agent (cetyltrimethylammonium bromide, CTAB) to enhance MC-LR solubility in water and improve immobilisation level. The best MC-LR surface immobilisation was achieved at concentration of 5 mg mL^{-1} using an amine coupling procedure in the presence of CTAB at pH 7.4 in an overnight incubation at room temperature. Optimised surfaces gave an Rmax with antibody MC10E7 >14,000 RU (concentration 200 μg mL^{-1}, injection volume 75 μL, flow rate 5 μL min^{-1}). Various surface regeneration solutions and assay conditions (contact time) were evaluated for the assay. A solution of 40 mM NaOH, 20% acetonitrile at an injection volume 10 μL and flow rate 20 μL min^{-1} gave satisfactory regeneration of the sensor surface. The variable parameters for the assay optimisation on MC-LR spiked samples included MC antibody concentration, MC-LR to antibody ratio, injection volume, and flow rate (or contact time).

Table 1
Cross-reactivity profile of MC toxins to MC10E7 antibody in HBS-EP buffer.

Analyte	[a]CR (%)
MC-LR	100
MC-RR	69
MC-YR	61
Nodularin	73
MC-LW	–
MC-LF	–
MC_LA	–

[a] Cross-reactivity of antibody to test MC at 50% inhibition ((IC_{50} MC-LR/IC_{50} test MC) × 100) in HBS-EP buffer.

The assay conditions were optimised to increase the assay dynamic range for the analyte concentration range from 0 to 2 ng mL^{-1} (equivalent of 2 mg kg^{-1} of BGA supplement) approximately from 450 RU to 250 RU on the chip surface. The assay sensitivity was increased by decreasing the antibody concentration to 1 μg mL^{-1} and antibody-to-analyte ratio down to 1:9. These conditions were suitable for screening BGAS contaminated with MC-LR under the regulatory limit 1 mg kg^{-1}. Chip surfaces were found to be very stable and showed good reproducibility over several days, which is demonstrated in Fig. 3.

3.2.2. Sample preparation

The objective was to develop a simple procedure for isolating MC toxins from BGA food supplements. Initial experiments investigated the efficiency of different extraction solvent mixtures, namely 0%, 25%, 50%, and 75% methanol in water. Water was found to be an unsuitable extraction solvent because powdered BGA samples formed into a colloidous suspension. Extraction solvents consisting of 50% or 75% methanol gave best results. Although both 50% and 75% methanol extraction followed by centrifugation and 50- to 100-fold supernatant dilution are suitable for the optimised assay conditions, 75% methanol extraction and supernatant dilution from 75- to 100-fold displayed minimal matrix effect. Influence of methanol on the assay has been tested and no influence has been seen at up to 2% methanol remaining in the sample after dilution. Thus, sample extraction with 75% methanol followed by centrifugation and supernatant dilution has been chosen as the most suitable method of sample preparation for the assay. A number of different purification approaches were investigated to reduce non-specific binding effects including (1) purification on Strata-X® columns or Water Oasis® HLB SPE columns; (2) sample filtration through 0.22 and 0.45 μm nitrocellulose and PVDF membranes or their combination (3) sample centrifugation with subsequent supernatant dilution in HBS-EP buffer. Purification of sample extracts by SPE resulted in a low total recovery of MC toxins. Filtration through nitrocellulose and PVDF membranes resulted in a 20% loss in MC recovery. As sample filtration did not show any improvement in

Table 2
Summary of biosensor validation results.

Validation parameter	Fortification level (mg kg^{-1})	Mean concentration (mg kg^{-1})	Accuracy (%)	CV (%)
LOD[a] ($n = 20$)	0	0.561	–	3.1
CCβ[b] ($n = 20$)	0.85	0.906	107	6.6
WLr[c] ($n = 18$ each level)	0.85	0.886	101	5.2
	1.00	0.981	100	4.8
	1.5	1.403	97	6.7

[a] The LOD (limit of detection) = response for 20 negative minus 2 × S.D.
[b] CCβ was determined by spiking 20 samples at 0.85 mg kg^{-1}.
[c] The within laboratory repeatability was determined by assaying samples spiked at 0.85, 1.0 and 1.5 mg kg^{-1} ($n = 6$ each level) on three separate days.

elimination of the matrix effect, centrifugation and supernatant dilution were selected as the simplest sample clean up protocol to perform. The final sample preparation protocol was rapid and simple while maintaining high analyte recovery. The inclusion of a homogenisation step in the sample preparation protocol was also evaluated using naturally positive BGAS samples. This study showed that the similar MC concentrations were determined without homogenisation.

3.3. *Method validation*

The method was validated according to European method validation criteria as outlined in 2002/657/EC legislation [32]. A qualitative approach was used to validate the method to determine the performance factor, CCβ (detection capability). CCβ is the concentration at which a substance can be identified as positive (>LOD) with a statistical certainty of (1 − β), where β = 5%. The limit of detection (LOD) and selectivity of the assay were measured through the analysis of 20 different negative *Spirulina* samples, which represent a cross-section/population of BGA products and sample matrices. *Spirulina* is normally grown in artificial water reservoirs under controlled conditions and is a pure culture with no contaminants of other cyanobacteria. Therefore, *Spirulina* supplements can be considered free of MCs and used as blank samples. Negative samples used for the LOD validation were determined to be free of MC-LR toxin by LC–MS/MS. The LOD was 0.561 mg kg^{-1} and determined from the mean background (508.8 RU) minus two standard deviations (2 × 15.8 RU). Subsequently, CCβ was determined by spiking the same range of negative samples ($n = 20$) at a concentration above the LOD (Table 2). An arbitrary spiking level of 0.85 mg kg^{-1} was selected for the CCβ experiment. CCβ is the concentration at which a substance can be identified as positive (>LOD) with a statistically uncertainty of 1 − β. In this experiment, CCβ was determined to be less than 0.85 mg kg^{-1} because all spiked samples were found to contain MC residues as positive (>LOD). Recovery of MC-LR from spiked *Spirulina* samples was validated in three independent experiments carried out on different Biacore Q instruments on different days. Each experiment included three series of six replicates fortified with MC-LR at 0.85, 1.00, and 1.50 mg kg^{-1} of BGAS. Coefficient of variation (CV) of the negative samples fortified with 0.850, 1.000, and 1.500 μg of MC-LR per 1 g of the supplement fell in the range from 2.5% to 5.7% for repeatability of the assay. MC-LR recovery from the fortified samples was 101% for 0.850 mg kg^{-1}, 100% for 1 mg kg^{-1}, and 97% for 1.50 mg kg^{-1}. In addition, the ruggedness and reproducibility of the assay was evaluated through technology to the residue laboratories at Teagasc, where the assay was verified to be fit for purpose.

3.4. *Application the assay to natural samples*

The suitability of this assay was evaluated through its application to a range of BGA food supplements that were purchased on the Irish retail market. Samples were initially screened using the biosensor assay developed and samples were subsequently analysed using a confirmatory LC–MS/MS based on the Lawrence et al. method [14]. The presence of MC-LR residues in samples was confirmed using retention time and ion ratio criteria as outlined in 2002/657/EC criteria. The results from this comparison are shown in Table 3. The assay comparison included BGA samples determined to contain MC-LR between <0.5 and 2.21 mg kg^{-1}. This comparison showed good agreement between the two assays and demonstrated that the immunobiosensor assay was an effective screening tool for MC toxins in BGA food supplements. The results indicate a low false positive rate for the biosensor assay compared to LC–MS/MS. The R^2 value of a plot of biosensor results versus LC–MS/MS results was 0.9893 (slope = 0.9455 and intercept = 0.0568). The repeatability of the biosensor assay was also tested through application to eight different naturally contaminated BGA samples from four different manufacturers (Fig. 4). The repeatability of the assay for incurred samples was typically less than 5%.

Table 3
Comparison of biosensor assays and LC–MS/MS for the analysis of MC-LR residues in negative and positive blue-green algae samples.

Sample	Biosensor assay		LC–MS/MS
	Concentration (mg kg^{-1})	Interpretation (LOD = 0.561 mg kg^{-1})	Concentration (mg kg^{-1})
1	ND	Negative	ND
2	1.821	Positive	1.89
3	1.826	Positive	1.89
4	ND	Negative	ND
5	2.531	Positive	2.21
6	0.583	Positive	0.64
7	0.812	Positive	0.99
8	0.609	Positive	0.66
9	0.68	Positive	0.65
10	ND	Negative	ND
11	ND	Negative	ND
12	0.769	Positive	0.93
13	0.546	Negative	<0.5

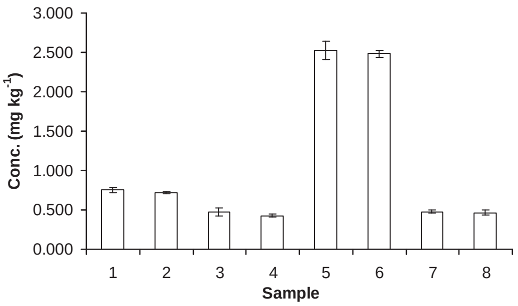


Fig. 4. Evaluation of reproducibility of assay for detecting MC-LR in eight different Klamath Lake blue-green algae samples ($n = 6$)

4. Conclusions

A new assay has been developed to detect MC algal toxins in BGA food supplements. The assay uses a simple and rapid sample preparation step prior to detection by SPR immunobiosensor. The assay is sufficiently sensitive to detect MC toxins to less than 1 mg kg^{-1}. The assay was evaluated through application to Klamath Lake BGA samples purchased on the Irish market. The SPR biosensor assay has been proven to be a robust analytical system for the detection MC toxins in BGA food supplements. The results of the biosensor assay show good correlation with an established LC–MS/MS assay when applied to naturally contaminated BGAS. LC–MS/MS has been used by several groups for the determination of MC toxins in BGAS analysis and is generally regarded as the gold standard because of its selectivity and sensitivity. This demonstrates that the biosensor assay is a viable alternative for the rapid screening of MC toxins. The biosensor assay reported in this paper has several advantages over LC–MS/MS including rapid sample preparation, limited number of consumables required and reduction in solvent waste. An additional advantage of the biosensor assay is that it can detect potential contamination from emerging MC toxins in food supplements. It is proposed that the technique can be used as a tool by both producers and regulatory laboratories to monitor the safety of BGAS. A mini-survey was carried out of *Spirulina* and Klamath Lake BGAS sold on the Irish market. In agreement with other studies, Klamath Lake BGAS were found to be contaminated with MC-LR in the range <0.5 to 2.21 $\mathrm{mg\,kg^{-1}}$. Levels of MC-LR in *Spirulina* samples were all <0.5 $\mathrm{mg\,kg^{-1}}$. It is estimated from the product label that the exposure to MC-LR from BGAS would be 8 $\mu\mathrm{g\,day^{-1}}$ based on the maximum daily intake (six capsules per day). The product came in a container of 120 capsules, which indicates the maximum exposure could be as much as 160 μg over a 20 day period. A more extensive survey and exposure assessment is required of to provide a more accurate exposure assessment, which should include other potential sources of MC-LR including drinking water, fish, fruit and vegetables.

Acknowledgement

This research was funded by the *safe*food, the Food Safety Promotion Board (Research contract 2004-04CR-06).

References

[1] R.W. Zurawell, H. Chen, J.M. Burke, E.E. Papras, J. Toxicol. Environ. Health B 8 (2005) 1–37.

[2] C.B. Lopez, E.B. Jewett, Q. Dortch, B.T. Walton, H.K. Hudnell. Scientific Assessment of Freshwater Harmful Algal Blooms. Interagency Working Group on Harmful Algal Blooms, Hypoxia, and Human Health of the Joint Subcommittee on Ocean Science and Technology. Washington, DC. (2008).

[3] J.K. Fawell, J. Hart, H.A. James, W. Parr, Water supply 11 (1993) 109–121.

[4] WHO, Geneva, Guidelines for Drinking Water Quality, Vol. 1, 3rd edition, 2004, pp. 407–408.

[5] N. Gupta, S.C. Pant, R. Vijayaraghavan, P.V. Rao, Toxicology 188 (2003) 285–296.

[6] W.W. Carmichael, Sci. Am. 270 (1994) 78–86.

[7] M. Craig, H.-A. Luu, T.L. McCready, D. Williams, R.J. Andersen, C.F.B. Holmes, Biochem. Cell. Biol. 74 (1996) 569–578.

[8] W.W. Carmichael, S.M. Azevedo, J.S. An, R.J. Molica, E.M. Jochimsen, S. Lau, K.L. Rinehart, G.R. Shaw, G.K. Eaglesham, Environ. Health Perspect. 109 (2001) 663–668.

[9] Y. Ueno, S. Nagata, T. Tsutsumi, A. Hasegawa, M.F. Watanabe, H.D. Park, G.C. Chen, G. Chen, S.Z. Yu, Carcinogenesis 17 (1996) 1317–1321.

[10] R. Nishiwaki-Matsushima, T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W.W. Carmichael, H. Fujiki, J. Cancer Res. Clin. Oncol. 118 (1992) 420–424.

[11] WHO, Geneva, Cyanobacterial Toxins: Microcystin-LR in Drinking-Water, 2003, p. 9.

[12] J.E. Eriksson, L. Grönberg, S. Nygård, J.P. Slotte, J.A.O. Meriluoto, Biochim. Biophys. Acta 1025 (1990) 60–66.

[13] D.J. Gilroy, K.W. Kauffman, R.A. Hall, X. Huang, F.S. Chu, Environ. Health Perspect. 108 (2000) 435–439.

[14] J.F. Lawrence, B. Niedzwiadek, C. Menard, B.P. Lau, D. Lewis, T. Kuper-Goodman, S. Carbone, C. Holmes, J. AOAC Int. 84 (2001) 1035–1044.

[15] J.F. Lawrence, C. Menard, J. Chromatogr. A 922 (2001) 111–117.

[16] D.J. Gilroy, K.W. Kauffman, R.A. Hall, X. Huang, F.S. Chu, Environ. Health Perspect. 108 (2000) 1–7.

[17] D.J. Shaeffer, P.B. Malpas, L.L. Barton, Ecotox. Environ. Saf. 44 (1999) 73–80.

[18] D. Dietrich, S. Hoeger, Toxicol. Appl. Pharm. 203 (2005) 273–289.

[19] Microcystins in algae products used as food supplements. DFG – Senate Commission on Food Safety, English text. (2007).

[20] M. Bruno, M. Fiori, D. Mattei, S. Melchiorre, V. Messineo, F. Volpi, S. Bogialli, M. Nazzari, Nat. Prod. Res. 20 (2006) 827–834.

[21] D. Ortelli, P. Edder, P. Jan, Anal. Chim. Acta 617 (2008) 230–237.

[22] M.L. Saker, M. Welker, V.M. Vasconcelos, Appl. Microbiol. Biotechnol. 73 (2007) 1136–1142.

[23] G.A. Baxter, J.P. Ferguson, M.C. O'Connor, C.T. Elliott, J. Agric. Food Chem. 49 (2001) 3204–3207.

[24] J. Keegan, M. Whelan, M. Danaher, S. Crooks, R. Sayers, A. Anastasio, C. Elliott, D. Brandon, A. Furey, R. O'Kennedy, Anal. Chim. Acta 654 (2009) 111–119.

[25] S.A. Haughey, G.A. Baxter, J. AOAC Int. 89 (2006) 862–867.

[26] J.P. Meneely, M. Sulyok, S. Baumgartner, R. Krska, C.T. Elliott, Talanta 81 (2010) 630–636.

[27] K. Campbell, S.A. Haughey, H. van den Top, H. van Egmond, N. Vilarino, L.M. Botana, C.T. Elliott, Anal. Chem. 82 (2010) 2977–2988.

[28] A. Zeck, A. Eikenberg, M.G. Weller, R. Niessner, Anal. Chim. Acta 441 (2001) 1–13.

[29] R.W. Zurawell, H. Chen, J.M. Burke, E.E. Prepas, J. Toxicol. Environ. Health Part B 8 (2005) 1–37.

[30] National Health and Medical Research Council and Natural Resource Management Ministerial Council (2004) National Water Quality Management Strategy Australian drinking water guidelines 6.

[31] Health Canada (2002) Guidelines for Canadian drinking water quality: Cyanobacterial toxins – MC-LR. Water Quality and Health Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario. (http://www.hc-sc.gc.ca/waterquality).

[32] Anon., European Commission Decision 2002/657/EC, Off. J. Eur. Commun. L221 (2002) 1–32.