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Enhancing Post-market Surveillance Through Laboratory Testing on Blue-green Algae Products in Malaysia

Bujang Nur Baizura*, Shamsuddin Noor'ain, Muhamad Muhammad Zhariff, Zahari Siti Hajar, Shahrir Mohamed Shahrizan, Gunasagaran Vinomalar, Mohamed Ali Norleen, Khoo Yvonne Siew Khoon, Ghafar Zakiah Abdul

National Pharmaceutical Regulatory Agency (NPRA), Ministry of Health, Petaling Jaya, Malaysia

Abstract

Algal blooms present naturally in marine and fresh water ecosystems are harvested as ingredients in health supplement products. Blue-green algal supplements (BGAS) such as *Aphanizomenon flos-aquae*, can be contaminated with highly potent toxins, most common being microcystin-LR (MC-LR). Recently, the Pharmacovigilance Section of NPRA has received adverse drug reaction (ADR) reports including liver and kidney toxicities suspected to be related to products containing *Aphanizomenon flos-aquae* in the local market. The objectives of this study were: (1) to develop and validate a MC-LR quantification test method and (2) to use quality control test results as a complement to managing product safety issues. During investigation on ADR reports concerning *Aphanizomenon flos-aquae* products, samples obtained through ADR reporting and market surveillance were used to develop and validate a test based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. Testing results have been applied to supplement other investigational activities which together support follow-up regulatory actions on the ADR reports received by NPRA. This research report illustrates an example in which a test method developed in-house can serve to supplement other regulatory components in handling product safety issues.

Keywords

Microcystin-LR, *Aphanizomenon flos-aquae*, Test Method Development, Adverse Drug Reaction, Complementary and Alternative Medicinal Products

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1. Introduction

Algal blooms consisting of microalgae (layterm: seaweed) and cyanobacteria (aquatic and photosynthetic bacteria) present naturally in marine and fresh water ecosystems. While providing nutrients, their presence occasionally raises a concern due to the production of highly potent toxins in the ecosystem. Microcystins (MCs) are a group of toxins most commonly associated with blue-green algae like *Aphanizomenon flos-aquae*. MCs are structurally monocyclic heptapeptides, containing two variable L-amino acids (as basis for congener naming) [1]. To date, there are over 100 congeners of MC identified, with microcystin-leucine arginine (MC-LR) most advancely characterised [2].

Toxins associated with microalgae and cyanobacteria can harm human through contact and accidental ingestion of contaminated water and food. MCs have been suggested to cause cases of toxic ingestion leading to hepatotoxicity, nephrotoxicity, neurotoxicity and tumour growth promotion in animals [1, 3]. Therefore, some health authorities have set limits based on leading routes of ingestion, as follows: (1)

* Corresponding author
E-mail address: baizura@npra.gov.my (B. N. Baizura)

drinking water (1μg/L drinking water or 0.04 μg MC-LR/kg BW/day), and (2) blue-green algal supplements (BGAS) (1μg/g dry weight or 0.04 μg MC-LR/kg BW/day) [4]. Additionally, ingestion can come from: (3) contaminated food (vegetables, fish, shellfish), and (4) accidental contact and ingestion during recreational use of water. Among possible routes of MC-LR ingestion, BGAS carries the highest risk of toxicity due to ingestion of concentrated microalgae and cyanobacteria products.

In affluent societies associated with emerging and advanced economies, the increasing intake of health supplements is encouraged by a thriving industry. Based on an analysis by Technavio [5], the health supplement market in Malaysia was predicted to grow at a compound annual growth rate (CAGR) of 7% from 2015 to 2019. This market growth can be linked to the rise in aging population in this region. Products which are high on demand among this age group are those consumed to prevent age-related disorders, such as those which can improve the digestive system, those which can help maintain blood sugar levels and also the ones that can maintain youth and good physique [5]. In another article outlining the Malaysian-Japanese collaboration on research and development of commercial microalgae products, the author noted that a rising cost of healthcare in this region also contributed to an increased demand of nutraceuticals [6]. Some Malaysian consumers have claimed to take *Aphanizomenon flos-aquae*-containing products for diabetes mellitus, leg pain, and to boost immunity (personal communications with product regulators).

Blue-green microalgae like spirulina and chlorella have long been known as superfoods owing to their high concentrations of vitamins, minerals, antioxidants and proteins. Other forms of microalgae are used to for weight loss, cancer, fibromyalgia, arthritis, stress, fatigue, depression, heart disease, high cholesterol, diabetes and improve memory and digestion. BGAS are commonly consumed "super food", as a result of active marketing of their putative beneficial effects, e.g. weight loss, increased energy and detoxification. Some BGAS products are even marketed as supplements for children with Attention Deficit Hyperactivity Disorder (ADHD) [1].

The biomass of microalgae and cyanobacteria as starting materials to produce health supplement products is obtained from either natural environment, controlled cultivation from open ponds, or specialised closed-system bioreactors [7]. Cultivation in open systems is subject to a risk of contamination with weed algal species and other organisms. *Aphanizomenon flos-aquae,* a blue-green alga harvested from Upper Klamath Lake in Oregon has been found to be contaminated with MC produced by a co-existing algal species, *Microcystis aeruginosa* [4]. Products containing *Aphanizomenon flos-aquae* are widely consumed in the United States, Canada and Europe. Health Canada, the national regulatory agency in Canada screened products containing cyanobacteria and detected microcystin in non-Spirulina products. Hence, Canadian consumers have been advised to exert caution when consuming these products [8].

In Malaysia, products containing algae such as *Aphanizomenon flos*-aquae, *Spirulina platensis* and *Chlorella pyrenoidosa* are controlled through activities at the National Pharmaceutical Regulatory Agency (NPRA), the regulatory authority of its Ministry of Health. Unlike Health Canada at which such products are controlled as health supplements, products containing only *Aphanizomenonflos-aquae* as a single active ingredient are registered as natural (traditional) products, while products containing a combination of *Aphanizomenon flos-aquae* with other active ingredients are regulated either as traditional products or health supplements depending on the other ingredients in the formulation. A traditional product consists of naturally occuring substances from plants, animals or minerals, or their parts thereof, in unextracted or crude extract forms [9]. A health supplement is defined as a product providing a concentrated source of nutrients or other substances (include animal/mineral/botanical sources) that is used to supplement a normal diet in order to maintain, enhance and improve the health function of human body [9]. Together, both product categories are controlled as complementary and alternative medicine (CAM) products. CAM products must undergo an evaluation process on its product content and labelling information, manufacturing process quality assurance, and laboratory testing at NPRA prior to authorisation to market to Malaysian consumers. Table 1 summarises CAM products containing *Aphanizomenon flos-aquae* registered from 2003 until July 2018.

Table 1. Health supplement products containing *Aphanizomenon flos-aquae* registered in Malaysia as of July 2018.

Year of registration	No. of registered products containing *Aphanizomenon flos-aquae*		% of local (Malaysian) manufactured products
	Registered as health supplement products (combination with other active ingredients)	Registered as traditional products (single active ingredient)	
2003-2008	0	1	100
2009-2014	4	6	60
2015- July 2018	3	0	0
Total	7	7	50
	14	13	50

Registered CAM products containing *Aphanizomenon flos-aquae* are either locally manufactured, or imported from Germany and New Zealand. In addition to single active ingredient traditional products, those registered as health supplements contain a combination of *Aphanizomenon flos-aquae* with several other active ingredients in a product, such as ascorbic acid (Vitamin C), borage oil, Coenzyme Q10, fish oil, and squalene. All registered products have been formulated as capsules containing powder. The approved product claims include: (1) traditionally used for health and strengthening of the body, and to invigorate vital energy, (2) traditionally used for general health / wellbeing, and (3) used as a health supplement.

The products listed in Table 1 have been granted marketing authorisation based on evaluation of Association of South East Asian Nations (ASEAN) Good Manufacturing Practice (GMP) for Traditional Medicines and Health Supplements implementation at the manufacturing plant, paper evaluation on fulfilment of registration requirements (e.g. labelling, raw material quality) and regulatory laboratory testing on heavy metal limits and microbial contaminations. The registration process at NPRA is guided by the Drug Registration Guidance Document (DRGD) [9] which contains a negative (banned) list of active ingredients in CAM products. The list is compiled based on evidence of poisoning (toxicity) when certain herbs are consumed by humans. MC-LR has been implicated in toxicity cases leading to death in animals, but has not been definitively linked to death due to toxicity in humans [3]. However, the International Agency for Research on Cancer (IARC) of the WHO has classified MC-LR as a possible human carcinogen (Group 2B), based on animal data [10]. As MC-LR toxicity in humans continues to be investigated by the scientific community, a limit of 1µg/g of MC-LR in the raw material of CAM products in Malaysia has been set, rather than placing *Aphanizomenon flos-aquae* on the banned ingredient list.

1.1. Post-approval Control on Quality and Safety of Registered Products in Malaysia

At NPRA, several regulatory activities are being carried out during post-marketing authorisation period in order to monitor the quality and safety of registered products. These activities involving surveillance, handling of product complaints and management of ADR reporting are executed with a pivotal aim of detecting previously unknown safety effects of registered medicinal products. During product registration, regulators' access to safety information is limited to findings in nonclinical toxicology and clinical trials. Based on NPRA's experience, discovery of new risks of drug use is fairly common after the registration phase. During 2017-2018, 49 directives were issued in relation to newly identified safety risks issued for registered products following market experience in Malaysia.

The Centre for Post-Registration of Product and Cosmetic Control at NPRA carries out risk-based sampling and laboratory testing on products in the Malaysian market in order to detect products of inferior quality including adulteration. Product samples that fail NPRA laboratory testing will be subject to regulatory actions such as product marketing suspension or cancellation of registration. In addition, press statements and alerts are disseminated to the public and healthcare professionals.

Occasionally, complaints such as product side effects are received from users. Following receipt of a complaint, NPRA will conduct an investigation, and discuss with the product registration holder if required. Samples of affected product batches are either obtained from the complainant or through surveillance in the market. Similar to surveillance, regulatory actions will be initiated accordingly.

Legislatively the Control of Drugs and Cosmetics Regulations (CDCR) 1984 mandates the reporting of ADRs by product registration holders. However, the ADR reporting system is heavily reliant on spontaneous, voluntary reporting at present. NPRA provides ADR reporting forms for healthcare professionals (widely known as the 'blue form'). Other than 'blue form', NPRA also had introduced the Consumer Side Effect Reporting Form (ConSERF) to facilitate consumer reporting. The management of both ADRs and complaints on product safety follow a common pathway of causality assessment, as shown in Figure 1.

1.2. Adverse Drug Reactions (ADRs) and Their Management at the National Regulatory Authority of Malaysia

On the pharmacovigilance front, NPRA has received adverse drug reaction (ADR) reports of suspected kidney toxicity with traditional products containing *Aphanizomenon flos-aquae* through its ADR reporting portal. The information received through the ADR report was provided to the NPRA surveillance team to be used in their sampling strategy as part of post-marketing surveillance activities. However, there is a lacking component of product testing in the current pharmacovigilance on *Aphanizomenon flos-aquae* containing products. Thus, the idea was mooted during a cross-functional meeting that a MC-LR quantitation test be developed in house to inform remedial actions such product de-registration or marketing withdrawal following an ADR report.

Since January 2012, a limit of 1µg/g of MC-LR in the raw material of CAM products in Malaysia has been set [9].

Applications to register *Aphanizomenon flos-aquae* - containing products must provide certificates of analysis on MC-LR content in finished products during evaluation process at NPRA. In the Natural Medicines Comprehensive Database, ADRs reported for *Aphanizomenon flos-aquae* include stomachache, diarrhoea, flatulence, and bloating. In Malaysia, adverse reaction reports from the consumers and healthcare practitioners are compiled by the National Centre for Drug Reaction Monitoring Centre at NPRA. Data from January 2016 until December 2017 (2-year period) show 20 adverse reaction reports with a total of 37 adverse events suspected to be associated with the *Aphanizomenon flos-aquae*-containing products. Of the reports received, majority were related to the System Organ Class (SOC) of Renal and urinary disorders (9 reports, 45.0%) followed by SOC of Skin and subcutaneous tissue disorders (7 reports, 35%) and SOC of Investigations (5 reports, 25%).

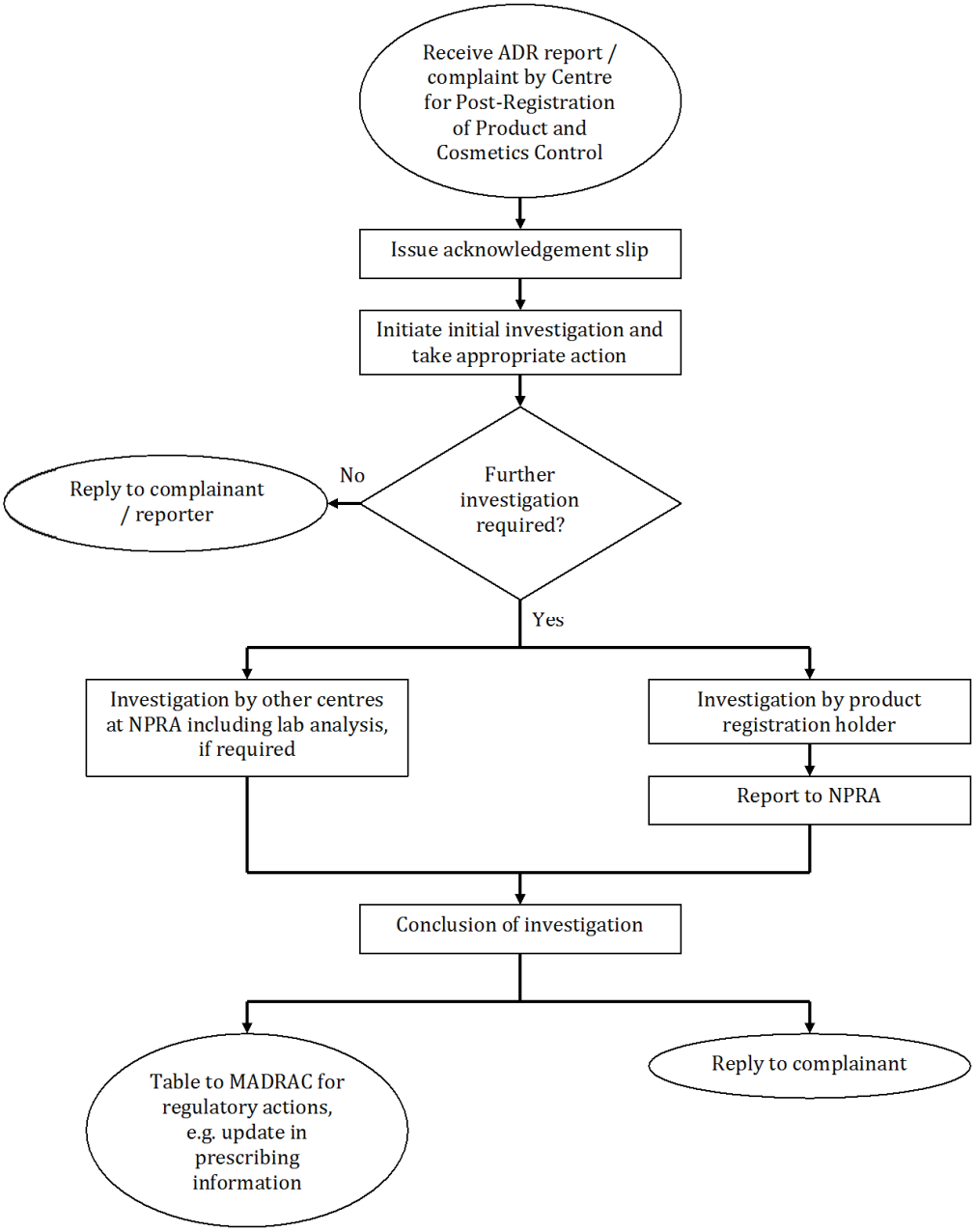

Figure 1. Procedure for management of ADRs and complaints on product safety at NPRA (MADRAC: Malaysian Adverse Drug Reactions Advisory Committee).

The assessment on all adverse reaction reports received at NPRA is conducted based on the World Health Organization – Uppsala Monitoring Centre (WHO-UMC) causality assessment system. In addition, all causality assessments were verified by the Malaysian Adverse Drug Reactions Advisory Committee (MADRAC), which consists of panel of experts from various medical fields. Reporting figures show the highest number of adverse events related to kidney injury

or kidney failure. The time to onset for the various adverse event varied from days up to 1 year after taking an *Aphanizomenon flos-aquae*-containing product. A diabetic patient was recorded as having defaulted his antidiabetic medications and developed acute kidney injury two months after consuming an unregistered product containing *Aphanizomenon flos-aquae*. However, considering that there are many other factors such as concomitant medications or supplements taken by the patients, drug or food interaction occurring at the time of event, time to onset of the adverse event, and underlying diseases, all of the Malaysian adverse reaction reports were only classified as "Possible", according to the WHO-UMC system. Furthermore, most *Aphanizomenon flos-aquae*-containing products were reported to contain multiple ingredients or herbs which add to the complexity in the causality assessment.

1.3. Development and Validation of a MC-LR Quantitation Method for Products Containing *Aphanizomenon flos-aquae*

Several methods have been developed to quantify MC-LR across environmental samples (e.g. water, biomass) and BGAS healthcare products. They include high-performance liquid chromatography with pulsed amperometric detector (HPLC-PAD) for lake water samples [11], HPLC-ELISA (enzyme-linked immunosorbent assay) for BGAS [12], Adda-ELISA (Adda moiety is present in all MC congeners)[13], DNA extraction and PCR analyses of mcyE gene in BGAS [13], colorimetric protein phosphatase inhibition assay (cPPIA) for water samples or BGAS [14], and liquid chromatography tandem mass spectrometry (LC-MS/MS) for BGAS [13, 15].

For our MC-LR quantitation method development, the LC-MS/MS method was selected due to the following reasons: (1) the availability of test equipment, i.e. an LC-MS/MS and experience in chemical analysis using this system, (2) LC-MS/MS is an established system for determination and quantitation of chemical compounds, and (3) high sensitivity and reliability compared to cPPIA and Adda-ELISA, based on findings by Heussner*et al.* [13].

1.4. Aim and Objectives

This project aims to develop a LC-MS/MS method to determine the MC-LR content in CAM products controlled at NPRA. Sample preparation from products containing *Aphanizomenon flos-aquae* was optimised and method performance parameters such as system suitability, specificity, accuracy, precision, limits of detection/quantitation, and linearity were assessed.

2. Materials and Methods

2.1. Materials

All chemicals used were of analytical grade. Reagents used to prepared LC-MS water solutions were as follows: formic acid purchased from Fluka, LC-MS grade methanol purchased from Merck and HPLC grade acetonitrile purchased from J. T. Baker. Ultra purified water generated in-house with the Milipore Simplicity 185 was used to prepare the sample solutions.

2.2. Standard Preparation

Microcystin-LR (MC-LR) standard 10 µg/ml readily diluted in methanol (analytical grade) was purchased from Sigma-Aldrich. Stock solutions of 1 µg/ml were prepared in methanol and stored at -50°C. Diluted stock solutions (5 ng/ml – 150 ng/ml) were prepared prior to analysis.

2.3. Sample Preparation

Samples were freshly prepared prior to analysis. The sample preparation procedure was constructed in reference to Roy-Lachapelle *et al.* [16]. As all registered products exist in the form of capsules containing powder, a health supplement sample in the form of capsules containing *Aphanizomenon flos-aquae* powder was purchased from a commercial supplier and was used to validate the developed method. The capsules were emptied and 300mg of the powder were weighed into a 5ml volumetric flask. Three ml acidified methanol solution (pH2, adjusted with formic acid) was used to dilute the powder, followed by sonication for 10 min, then made up to 5ml volume. The resulting solution was then centrifuged at 3500 rpm for 10 min. The supernatant was filtered through a 0.45 µm pore polytetrafluoroethylene (PTFE) membrane through a syringe filter. Acidified methanol (3ml) was then used to dilute the remaining residue, followed by sonication for 5 min and centrifugation at 3500rpm for 5 min. The resulting supernatant was combined with the earlier supernatant, evaporated to dryness under a stream of nitrogen, and diluted with 1ml of methanol/water (50:50, v/v, pH2). Finally, the solution was filtered through a 0.45 µm pore PTFE membrane and transferred into a 1.5ml vial for analysis.

2.4. Instrumental Analysis for Quantitation

The experiment was carried out on a LCMS-TQ 8045 (Shimadzu) equipped with a heated electrospray ionization source (ESI) coupled to a Nexera X2 HPLC system (Shimadzu), comprising of a Nexera X2 SIL-30AC autosampler and LC-30AD pump. The instrument control and data processing was conducted using LabSolutions software. Separations were carried out using a Phenomenex Kinetex C_{18}

(100 × 2.1 mm, 1.7 µm) column. The column was coupled to a Phenomenex UHPLC C_{18} guard column (2.1 mm) and operated at 40°C. The autosampler was maintained at 5°C to avoid sample degradation. The isocratic mobile phase consisted of acetonitrile / 0.1% v/v formic acid with the ratio of 35:65. The mobile phase was pumped at a flow rate of 0.3ml/min. Samples were injected at a volume of 5 µl.

The ESI was operated in a positive MRM mode. The MS parameters were optimised to the following: DL temperature: 250°C; heat block temperature: 400°C; interface temperature: 300°C; drying gas flow: 10 L/min; nebulising gas flow: 3 L/min and heating gas flow: 10 L/min. MRM transitions were optimised using auto optimisation feature of LabSolutions software.

3. Results

3.1. Optimisation of Sample Preparation

Different sample preparation techniques were employed to find a suitable method that could extract the highest amount of microcystin-LR from the sample. Among the techniques were: direct dissolve in pure methanol, solid phase extraction (SPE), and extraction with acidified methanol and varying proportions of methanol and water. From this research, extraction with acidified methanol together with 50% aqueous methanol yields the highest amount of approximately 87 ppb of Microcystin-LR from the sample. Direct dissolve in pure methanol and SPE, yielded approximately 37 ppb and 6 ppb, respectively. Sequential extraction, combining acid and methanol/water demonstrated better extraction [17].

3.2. Quantification of Microcystin-LR (MC-LR) Method Setup

The MRM transition optimisation shows that precursor ion doubly charged, at m/z 498.40 was more dominant for microcystin-LR, as compared to single charged positive and negative ions. Signals at 135.10 and 861.50 were the main product ions (see Figure 1). Method optimisation shows that microcystin-LR is eluted at 1.8 min, with allowances of 10% variation in retention time. The total runtime required was 3.5 min.

Concentration of microcystin-LR was calculated using the following equation:

$$\text{Concentration in Sample (µg/g)} = \frac{\text{Measured Concentration from LCMS–TQ (µg/ml)}}{\text{Sample weight (g)}} \times \text{Dilution Factor} \times \text{Standard purity}$$

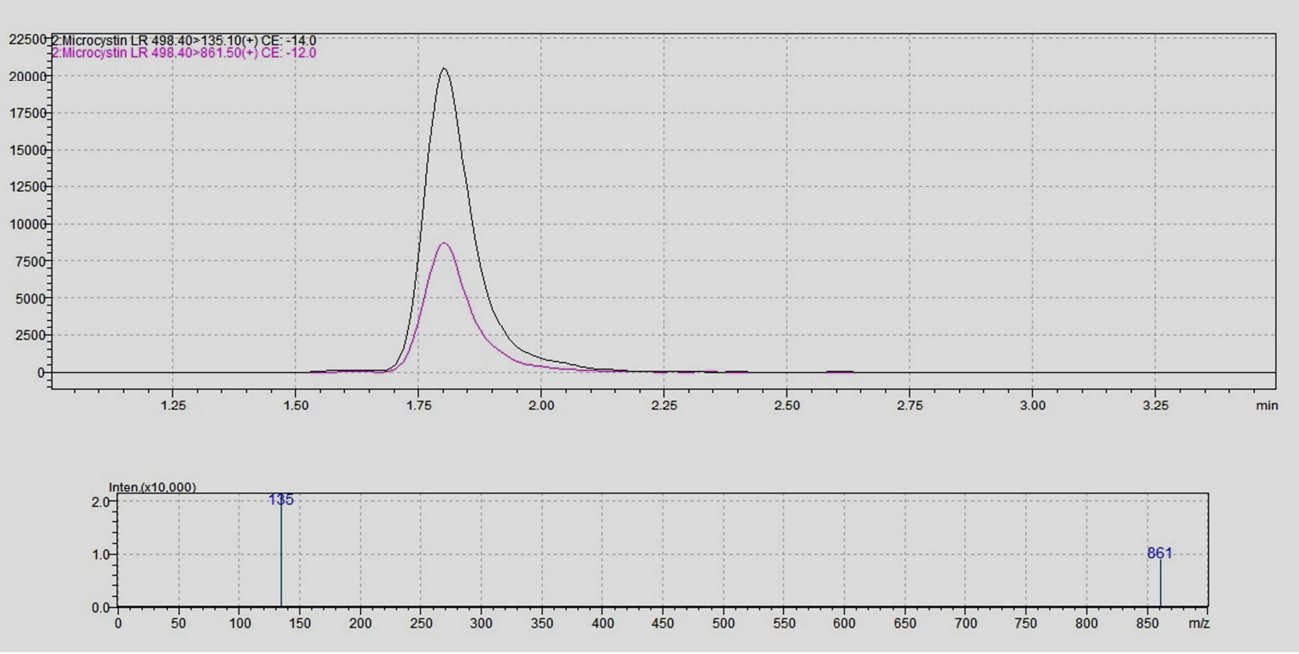

Figure 2. Mass chromatogram of Microcystin-LR (498.40>135.10, 498.40>861.50).

3.3. Method Validation Study

The following parameters were performed to validate the method: specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, intermediate precision and matrix effect as according to the International Conference on Harmonisation (ICH) guideline [18].

3.3.1. Specificity, LOD, LOQ and Linearity

Specificity was performed to ensure there are no interferences at the retention time of microcystin-LR. Comparisons were made between chromatograms of standard

at LOQ level, diluent, mobile phase (acetonitrile) and sample (results not shown). No interferences were observed, showing that the method is specific for its intended use.

LOD was set at 0.5 ng/ml (calculated at signal/noise ratio of ~3) and LOQ at 5 µg/ml (calculated at signal/noise ratio of ~10). Linearity was evaluated across a concentration range of 5 ng/ml (LOQ) to 150 ng/ml (n = 8). As shown in Figure 3, the graph plotted for peak area responses versus their respective concentrations demonstrated a linear range and utilises 1/C weighing. The calibration curve correlation coefficient (r2) was 0.996.

3.3.2. Accuracy, Precision and Intermediate Precision

Accuracy was evaluated at three different concentration: 30, 40 and 50 ng/ml. Concentration level at 30 and 50 ng/ml was prepared in three replicates, whereas concentration level at 40 ng/ml was prepared in six replicates, to perform precision simultaneously with accuracy. Validation samples were prepared by spiking the appropriate amount of standard in the sample matrix. Each sample was then injected for evaluation. The procedure for concentration level at 40 ng/ml was repeated by a different analyst and on a different day. These parameters were determined by comparing the calculated concentration with the true value spiked into the matrix. As seen in Table 2, the accuracy was within 70 – 125% and % RSD of all the determinations in precision were less than 7.3%.

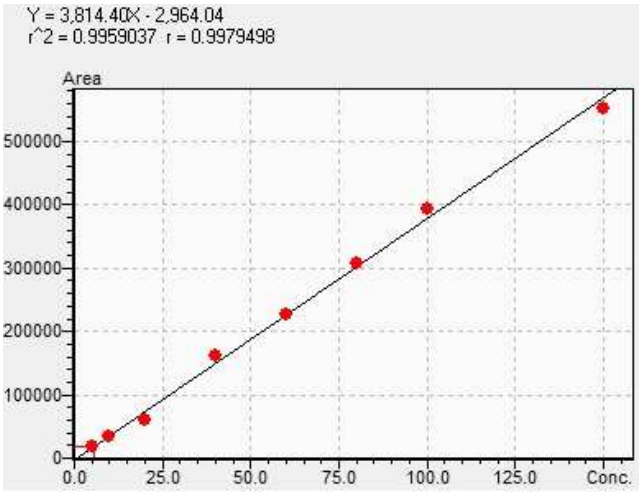

Figure 3. Linearity graph of Microcystin-LR standards (5 – 150 ng/ml).

Table 2. Precision, intermediate precision and accuracy for Microcystin-LR.

Analyte					
Microcystin-LR	**Precision (% RSD)**		**Accuracy (%recovery)**		
	40ng/ml (100%)		**30ng/ml (80%)**	**40ng/ml (100%)**	**50ng/ml (120%)**
	1.72		115.76	119.8	110.49
		Mean recovery	115.35		
	Intermediate Precision (% RSD) (n = 12)				
	3.54				

3.3.3. Matrix Effect

In order to determine whether there were any interferences present from the sample matrix, comparison were made between the slope of post spike sample and slope of non spike sample. The following equation was employed to determine the matrix effect:

$$\text{Matrix Effect, . ME (\%)} = \frac{\text{Slope}_{\text{matrix-matched}} - \text{Slope}_{\text{solvent}}}{\text{Slope}_{\text{solvent}}} \times 100\%$$

The calculated result of matrix effect shows that the differences between the two slopes are less than 10%, therefore no interferences from the sample matrix is expected.

4. Discussion

4.1. Current Limitations and Potential Enhancements on the Test Method

Due to the utilisation of only one brand of Aphanizomenon flos-aquae containing product in this test method validation project, NPRA will continue the validation using different brands of *Aphanizomenon flos-aquae* containing products, concurrently while testing the products sent in for routine analysis. The test method will be updated based on latest validation results in the future. There is also an option to expand current test method beyond detection and quantitation of MC-LR, i.e. by establishing a dose-related effect curve for serum level testing in users, in order to refine causality assessment of MC-LR in ADRs received. Nevertheless, the current test method sufficiently supports ADR management activities at NPRA.

4.2. Changes Made to Management of ADR Reports at NPRA After Development of a Validated Test Method

Currently, in addition to causality assessment, samples of registered products were obtained and tested at NPRA laboratory for MC-LR content using the validated test method. Further, product safety and quality tests such as

microbial contamination tests, heavy metal tests and adulterant screening tests were also conducted. In totality, quality control tests at NPRA contribute towards safety assurance on registered products. Test results assist in performing regulatory actions such as issuance of warning letters to the product registration holders, or amendments to product registration status, e.g. suspension. Furthermore, trending analysis performed on test results, e.g. MC-LR content in monitored product batches aids in post-marketing surveillance by ensuring consistent quality of products in the Malaysian market. Some of the reported adverse reactions are related to products determined to be unregistered with the local Drug Control Authority (DCA). In this instance, NPRA has alerted the Pharmacy Enforcement Division for further actions.

5. Conclusion

An MC-LR quantification test method has been developed and validated at NPRA. This method potentially becomes a routine quality control test method on products containing *Aphanizomenon flos-aquae* to ensure registered products comply with the MC-LR limit set by NPRA. In this paper, we have described how such quality control testing can complement not only product registration, but also post-marketing surveillance activities and ADR management based on MC-LR limit set by NPRA. We also discussed potential expansion of MC-LR testing in ADR reporting management at NPRA. Additionally, investigation outcome at NPRA also informs enforcement action to remove products of inferior quality found in the Malaysian market. Ultimately, regulatory activities at NPRA serve to protect the safety of Malaysians, as aptly demonstrated by this report on *Aphanizomenon flos-aquae*-containing traditional and health supplements.

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Author Contributions

N. B. B. designed experiments, generated and analyzed data. M. Z. M., S. H. Z. and M. S. S. prepared samples and performed experiments. Z. A. G. conceptualized, guided and planned the project. V. G. performed literature search for the introduction section of the paper. Y. K. S. K., N. S. and N. M. A. wrote the paper with input from the other authors. All authors approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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