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Methods validation and analysis of Spirulina (*Arthrospira platensis*) microcystin contaminants by Enzyme-Linked Immunosorbent Assay (ELISA)

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Abstract

Spirulina (*Arthrospira platensis*) production dominates the global microalgae market due to its high nutritional value. Spirulina can be cultivated in ponds with quality control due to the possible growth of cyanotoxins like *Microcystis* sp. that produce microcystins. The Enzyme-Linked Immunosorbent Assay (ELISA) method was used and validated with linearity, precision, reproducibility, and accuracy parameters that met the criteria for microcystin analysis. The regression curve gave the linear regression, the value of the coefficient of correlation (r) 0.99 showed a very strong correlation, and coefficient determination (R^2) was 0.988 (R^2 > 0.90). The precision analysis in repeatability (RSDanalysis < 2/3RSD Horwitz) and reproducibility (RSDanalysis < RSD Horwitz) showed an acceptable value with a significance value of 0.191 (*p*-value <0.05) that showed no significance difference. The accuracy from spiking analysis with a recovery percentage of 79.90±17.97% was still within the range of 40% to 120%, with a LOD value of 0.1 µg/kg. Analysis completed on spirulina powder showed that the spirulina product has a good quality with microcystin levels of 1.39 ± 0.16 µg/kg (dry basis), 719 times below the predetermined limit of 1000 µg/kg based on the Oregon Health Division Regulation for blue-green algae (BGA).

Keywords: Cyanotoxin; ELISA validation; Microalgae cultivation; Microcystin analysis; Spirulina powder; Quality control.

Highlights

- Spirulina (*Arthrospira platensis*) dominates the global microalgae production due to its high nutritional value
- Spirulina product processing ensures quality control, concerns about cyanotoxin presence such as *Microcystis* sp. producing Microcystin
- Validated method parameters meeting the criteria for microcystin analysis

1 Introduction

Spirulina (*Arthrospira platensis*) is one of the most widely utilized food ingredients. Spirulina is a microalgae categorized as cyanobacteria or algae with green-blue pigments. Spirulina can photosynthesize

and naturally grows in water with high salt content and alkali in tropical and subtropical regions. Global microalgae production data in 2019 showed a total production of 56,455 tons and was dominated by spirulina at 56,208 tons cultivated in ten countries (Cai et al., 2021; Food and Agricultural Organization of the United Nations, 2021). The public's attraction to a healthy lifestyle has increased the demand for foods with high nutritional content. Spirulina protein content is a parameter that is favored because it has a high level of approximately 59% to 76% (dry basis) with a complete composition of essential amino acids. Other beneficial components of spirulina include phycocyanin, carotenoid including -carotene, zeaxanthin, and chlorophyll (Ghaeni et al., 2014). These components have a variety of health effects, including antioxidant, anticancer, antimicrobial, and provitamin A sources (Park et al., 2018; Safari et al., 2020; Soror et al., 2022).

Spirulina is grown in freshwater ponds at PT Algaepark Indonesia Mandiri in Klaten, Central Java, and processed into spirulina powder for commercial purposes. To prevent contamination in the form of other microalgae that can create toxins, including *Microcystis* sp., the condition of spirulina during the cultivation process needs to be considered to maintain its quality. This is critical since this species can create microcystin, a harmful toxin contaminant. It is necessary to examine the amounts of microcystin in spirulina since it is a crucial factor in the international trade of spirulina products.

Microcystin (MCs) is a cyclic heptapeptide with over 100 different forms of two amino acid variables. This component is hepatotoxic, meaning it harms the liver (Merel et al., 2013). Microcystin has a significant toxin potential because it may inhibit eukaryotic protein serine/threonine phosphates 1 and 2A (PP1 and PP2A), causing hyperphosphorylation of the cytoskeleton and apoptosis regulatory proteins. Due to the frequent occurrence of microcystin contamination in 1996 - 1997, this toxin was one of the primary factors behind the regulation of the maximum limit of 1µg/g based on Oregon Health Division regulations on blue-green algae (BGA) products (Gilroy et al., 2000).

Microcystin is analyzed using the Enzyme-Linked Immunosorbent Assay (ELISA) method, which detects and quantifies a specific substance using the antigen-antibody principle. This method enables more accurate and rapid time. ELISA uses a combination of antibodies and complicated enzyme substrates to identify sample analytes (Shah & Maghsoudlou, 2016). Microcystin can be conducted in three ways: biological by a mouse bioassay, biochemical using a protein phosphatase inhibition assay (PPIA) and ELISA, and chemical with high-performance liquid chromatography (HPLC) (Massey et al., 2020). In this research, the target antigen of microcystin was detected using an indirect competitive ELISA technique. This technique will create lighter colors when detecting more antigens. This analysis must be carried out appropriately to produce reliable results using a validated method.

Method validation was performed to assure consistency and analysis capability. The method validated for analyzing spirulina powder produced by PT Algaepark Indonesia Mandiri will increase global marketing. The analytical performance parameters are based on the Eurachem Guide, The Fitness Purpose of Analytical Methods in 2014, and AOAC (Association of Official Analytical Chemists, 2016). During the method validation procedure, methods are examined and will be utilized to analyze microcystin in spirulina powder. The purpose of this study is to determine the most effective extraction process for microcystin in spirulina products, validate the method for analyzing microcystin in spirulina products using the ELISA, and determine the level of microcystin in spirulina powder using the validated method.

2 Materials and methods

2.1 Reagents and materials

The products used in this study were commercial spirulina powder obtained from PT Algaepark Indonesia Mandiri, Klaten, Central Java. The powder was from cultured spirulina heated in an oven at 60 °C for 6 hours and produced spirulina flakes which ground to produce powder with a size of ≥ 80 mesh. Microcystin analysis

was performed with a microcystin ELISA kit with microcystin-LR (MC-LR) standard produced by PT Attogene, USA (catalog: EL2024-02), methanol, and demineralized water.

2.2 Microcystin extraction

Extraction was carried out with a 50% concentration of methanol solvent, with or without the sonication. Powdered spirulina samples were weighed 0.2 g using an analytical scale and homogenized with 0.8 mL methanol solvent for 5 minutes using a vortex, then treated with or without sonication for 5 minutes. The samples were then centrifuged (Eppendorf®Centrifuge 5810/5810R) at 3000xg for 10 minutes at 25 °C. The centrifuged supernatant was collected and analyzed with a microcystin analysis specific ELISA kit and analyzed on a 450 nm ELISA reader (Bio Rad iMark™ Microplate Absorbance Reader). The best microcystin extraction results can be observed in the best treatment precision and stable concentration results. This method was performed based on the instructions on the Attogene ELISA kit used.

2.3 Method validation

2.3.1 Linearity

The test used microcystin-LR (MC-LR) standards at concentrations of 0.00, 0.05, 0.10, 0.20, 0.40, and 2.00 µg/L in three repetitions. The percentage of absorbance for each standard is calculated after analysis. The method's linearity is assessed by constructing a linear regression equation ($y = a + bx$) and calculating the correlation coefficient (r) and coefficient of determination (R^2). A good correlation will result in a straight line on the curve with a value of r closer to +1 (Schober & Schwarte, 2018) and a coefficient of determination R^2 greater than 0.90, which is preferable if it is close to 1 (Frej, 2023). Analysis by immunoassay can show non-linear analytical results, so the analytical response obtained must be described and transformed to the actual conditions of the concentration or amount of analyte in the sample (ICH, 2005). Data transformation is performed on standard concentration data by entering the concentration value in the natural logarithm function (Ln (x)).

2.3.2 Accuracy

The spiking method was analyzed by adding a standard solution of 1 µg/kg microcystin to the spirulina powder sample to be examined. The standard added in six replicates of samples before extraction. The accuracy study will generate a percent recovery number as a reference in the method's acceptance. The acceptability levels in the analysis with a 1 µg/kg spike vary from 40% to 120% and the result will be in percent (Association of Official Analytical Chemists, 2016).

2.3.3 Precision and reproducibility

The precision approach was evaluated using six replicates (n = 6) on the same day and location. The analytical data is then processed to generate the RSD and RSDHorwitz values. The precision value is acceptable if the RSDa value is smaller than 2/3 RSDH (RSDa < 2/3 RSDH). This sentence may indicate that this procedure was meticulous. The reproducibility analysis was done using five repetitions (n = 5) with the same sample and analysis method on different days. The results obtained were processed to generate the RSD and RSDHorwitz values. Reproducibility values are accepted if RSDa < RSDH and will show the meticulousness of this method (Association of Official Analytical Chemists, 2016).

2.4 Analysis of microcystin content in spirulina

Microcystin levels in spirulina powder samples were measured using a validated method. Powder samples were weighed and extracted, then analyzed the microcystin level by kit ELISA Microcystin. The data was then analyzed using statistical analysis to obtain the value of microcystin levels. Acceptable microcystin level is regulated by the Oregon Department of Health's established standard for BGA products, which is 1 µg/g spirulina products. Microcystin consumption levels exceeding this limit could potentially be harmful if consumed (Gilroy et al., 2000).

2.5 Statistical analysis

Data of Microcystin extraction, method validation, and analysis of microcystin levels were analyzed with Microsoft Excel and Minitab statistical software with one-way Analysis of Variance (ANOVA), followed by Tukey's test if the probability value showed significant results (*p-value* < 0.05).

3 Results and discussion

3.1 Microcystin extraction

The results of microcystin extraction by solvent concentration treatment and sonicator selection are in Table 1. This study showed the microcystin content with different sonication treatments in extraction. Determination of the selected microcystin extraction was based on the RSDa and RSDH values obtained. A smaller RSDa value indicates a more precise or thorough analysis result and better repeatability.

Table 1. Microcystin content with different extraction treatments.

Sonication Treatment	Microcystin[1)] (µg/kg)	RSDa[2)] (%)	2/3 RSDH[3)] (%)
Yes	1.19 ± 0.26^{a}	22.10	29.39
No	1.34 ± 0.11^{a}	8.13	28.88

Same superscript letters following values on the same row are not significant $p < 0.05$. [1)]Values are the mean and standard deviation of four data (n=4). [2)]RSD analysis. [3)]RSD Horwitz.

Extraction with 50% methanol concentration and without sonication is a method that has a smaller standard deviation and RSDa values. The RSDa value obtained was also smaller than 2/3 RSDH (RSDa ≤ 2/3 RSDH), indicating that the extraction method used has good precision (Sengoku et al., 1999). Sonication is a method used for the pre-treatment process in extraction. This method can cause cell lysis then the intracellular part of the cell will be extracted so that *Microcystis* sp. can release microcystin (Both et al., 2015; Manubolu et al., 2018; Panhwar et al., 2018). The results showed that the microcystin content in the extraction method with sonication had a higher RSD analysis value and the repeatability of the analysis was not better than the extraction without sonication.

The t-test analysis used to do a comparison test between the two extraction techniques. The results showed that the *p*-value obtained was 0.378 (*p-value* > 0.05), indicating no significant difference between the two treatments (Xu et al., 2017). A comparison of the use of sonication with different times in microcystin extraction has also been conducted in the research of Jiang-qi et al., (2013) with time variations of 5, 10, 15, 20, 30, 40, and 50 minutes and found that there was no significant improvement in microcystin concentration as sonication time increased. As a result, the extraction approach without sonication is recommended since it is simpler and allows for a more thorough analysis of the microcystin concentration of spirulina powder.

3.2 Method validation

3.2.1 Linearity

A calibration curve (Figure 1) and linear regression equation were obtained to show the linearity of the data. The concentration values of the standard solution were transformed to obtain a linear curve and linear regression equation. The results showed that the linear regression equation obtained was y = 23.9 - 21.6x with a correlation coefficient value of 0.99 and a coefficient of determination of 0.988.

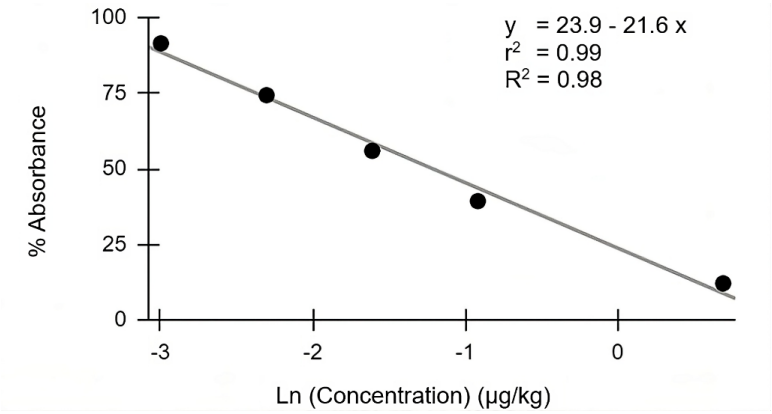

Figure 1. Standard curve of microcystin in linear form.

The x variable or regression coefficient is negative, indicating a negative influence between the variables. Based on the curve, if the concentration of microcystin increases, the percent absorbance will decrease. The value of the correlation coefficient (r) showed a correlation in a very strong category, which illustrates the existence of a linear relationship between variables x and y that affect each other as a whole. The coefficient of determination (R^2) value of 0.988 showed 98.8% of variable y, namely the percent absorbance, which can be explained based on the value of microcystin concentration. This result showed that the two variables had a linear relationship with appropriate parameters (Schober & Schwarte, 2018). Standard curve construction in analysis can explain the relationship between concentration as the independent variable and absorbance response as the dependent variable. A linear curve can indicate that the method used gives proportional results (Moosavi & Ghassabian, 2018).

3.2.2 Accuracy

The results of method accuracy in microcystin analysis in the addition of 1 µg/kg microcystin standard showed a percent recovery of 79.90 ± 17.97% of the six analyzed samples (n = 6). This value has met the criteria for accuracy acceptance requirements in the 40% to 120% (Association of Official Analytical Chemists, 2016). The recovery percentages were obtained by comparing the microcystin concentration spiked by the microcystin standard with the actual concentration and then multiplied by 100% (ICH, 2005). Method accuracy can determine the closeness of the analysis results. Research by Fan et al. (2022) has also analyzed the accuracy of surface water lakes and reservoirs in China. Samples were analyzed using the ELISA method and spiked with microcystin-LR standard concentrations of 0.15 to 3.00 µg/L. The analytical results obtained percent recoveries of 70-130% that met the acceptance criteria. This value also showed the close results of this study and indicated good method performance.

Research by Van Hassel et al. (2022) also analyzed the accuracy of microcystin-LR in spirulina samples spiked with microcystin using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) instruments and obtained an average recovery value of 83.75%. This value is still within the criteria of the accuracy parameter requirements used in the study, which is 69% to 104%. The difference in this value can be caused by differences in the analysis method and the type of sample.

3.2.3 Precision and reproducibility

Precision analysis was carried out with two parameters, namely precision and reproducibility, as shown in Table 2. Repeated analysis will obtain results that express the degree of closeness using the relative standard deviation or RSD analysis (RSDa). The precision parameter uses analysis with six repetitions (n = 6) on the same day. Reproducibility with five replicates (n = 5) on different days.

Table 2. Precision and reproducibility results in microcystin analysis method.

Precision			Reproducibility		
Microcystin[1)]	**RSDa[2)]**	**2/3 RSDH[3)]**	**Microcystin[1)]**	**RSDa[2)]**	**RSDH[3)]**
(µg/kg)	**(%)**	**(%)**	**(µg/kg)**	**(%)**	**(%)**
1.46 ± 0.18	12.22	28.49	1.30 ± 0.07	5.31	43.50

[1)]Precision and reproducibility values are the mean and standard deviation of 6 data (n=6) and 5 data (n=5), respectively. [2)]RSD analysis.
[3)]RSD Horwitz

Precision describes the degree of closeness utilizing relative standard deviation (RSDa) to determine the accuracy of the procedure by measuring data variance in an analysis result. Precision will determine precise and acceptable if the RSD value of the analysis is less than 2/3 RSD Horwitz (RSDa < 2/3 RSDH) and RSDa is less than RSDH for the reproducibility parameter (RSDa < RSDH) (Association of Official Analytical Chemists, 2016). The results concerning this research showed that precision and reproducibility met the acceptance criteria. The RSDa value obtained in the reproducibility parameter was smaller than the precision parameter. It might be due to the influence of laboratory conditions. Precision analysis was carried out on the same day with close analysis times, but it was suspected that in the time interval, there were reactions between samples and reagents that caused the variation of test results to be wider (Peris-Vicente et al., 2015).

The t-test analysis was used to conduct a comparison test on both validation parameters. The results showed a *p*-value greater than 0.05 and a significance value of 0.191. This conclusion explains why there was no significant difference between the microcystin data of the two parameters (Xu et al., 2017). The results indicated that the analyzed approach was consistent. The ELISA approach was used to analyze the precision and reproducibility of microcystin in surface water. The obtained values ranged from 3 to 11% and 6 to 15%, respectively (Fan et al., 2022). Analysis using LC-MS/MS was also performed on spirulina samples containing the microcystin-LR standard. The RSD values for precision and reproducibility were 4.72% and 11.64%, respectively (Van Hassel et al., 2022). Both of these outcomes are acceptable in each analytical approach.

3.2.4 Limit of detection

The analytical method's detection limit was determined by PT Attogene's microcystin ELISA kit (catalog: EL2024-02). The detection limit appears at 0.1 µg/kg. This value indicates that analyte concentrations less than 0.1 µg/kg cannot be identified using this method. The detection limit is a term used to describe the analytical limit of a method for detecting analytes (ICH, 2005).

3.3 Microcystin content in spirulina powder

The amounts of microcystin in spirulina powder can be determined using repeatability and reproducibility analysis. The average microcystin level was 1.39 ± 0.16 µg/kg (dry basis). This result was 719 times lower than the Oregon Department of Health's maximum intake restriction for BGA products which is 1000 µg/kg. According to World Health Organisation (WHO) guidelines, the tolerated daily intake (TDI) of microcystin is 0.04 µg/kg BW/day (World Health Organization, 2020). Assuming a body weight of 60 kg in an estimated

daily intake (EDI) of 2.4 µg/day, that is, the maximum amount of spirulina powder that can be ingested per day is comparable to 1.73 kg. Microcystin analysis of spirulina products from various countries has also been performed (Table 3). A comparison of results was carried out using microcystin data analyzed by different methods and sample types. This study also analyzed imported spirulina powder samples from Hawaii and China of the microcystin concentrations. The results in Table 3 show that the microcystin content of spirulina produced in Indonesia and analyzed in this study had the lowest value compared to others. Imported products analyzed by similar methods showed higher microcystin levels but still at consumable values. A comparison of microcystin concentrations found in different countries was also analyzed.

Table 3. Microcystin concentration in spirulina products from different countries.

Sample Source	Sample Type	Extraction Methods	Type of MCs Target	Analysis Method	Microcystin (µg/kg)	Reference
Indonesia	Powder	Methanol:Water(50:50)	MC-LR	ELISA	1.39 ± 0.16	This Research
China 1	Powder	Methanol:Water (50:50)	MC-LR	ELISA	1.64 ± 0.41	This Research
China 2	Powder	Methanol:Water (50:50)	MC-LR	ELISA	1.93 ± 0.07	This Research
Hawaii	Powder	Methanol:Water (50:50)	MC-LR	ELISA	4.67 ± 0.12	This Research
Prancis	*Flakes*	Methanol:Water (75:25)	MCs	ELISA	150 - 1310	(Pinchart et al., 2023)
Prancis	*Flakes*	Methanol:Water (80:20)	MC-LR	UHPLC-MS/MS	*ND - 15	(Pinchart et al., 2023)
			MCs		22 - 227	
Republic of Greece	Supplement	Methanol:water (75:25), Ultrasonication (15 W, 22.5 kHz)	MCs	ELISA	35.7 – 583.5	(Papadimitriou et al., 2021)
Pacific, Hawaii	Supplement (tablet, capsule, powder)	Lemieux oxidation	MCs (MMPB)	UHPLC	250 - 840	(Roy-Lachapelle et al., 2017)
Belgium	Supplement	Metanol:water (80:20)	MCs	HPLC	<LOD (22.5 µg/kg)	(Van Hassel et al., 2022)

*ND = Not Detected.

The spirulina powder product in Pinchart et al. (2023) study was dried spirulina flakes with a diameter of 1.5 mm. Spirulina flakes were analyzed using the Adda-ELISA Kit, and the results revealed differences in the level of microcystin. Another investigation, also using the Adda-ELISA Kit, was conducted by Papadimitriou et al. (2021) with samples of spirulina supplements offered in the Republic of Greece. Supplement samples were from spirulina grown in open ponds in Europe, Asia, the United States, and Australia, according to the label on the package. The ELISA method used in this research was indirectly competitive using the epitope of b-amino acid 6E-Adda for the antibody recognition, and was constructed a standard curve using Microcystin-LR (MC-LR). The three investigations with the same method were compared, and the microcystin concentration found in this study was lower than in the others. The difference also could be due to the different targets used in the analysis. Because the Adda amino acid (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl deca-4,6-dienoic acid) is a specific structure present in microcystin, it is possible to detect all sorts of specific amino acids bound. This approach can even detect products that are degraded but still contain Adda chains (Birbeck et al., 2019).

Microcystin analysis using different methods was also conducted by Roy-Lachapelle et al. (2017) research, which analyzed 14 commercial spirulina supplement products with tablet, capsule, and powder variations obtained from supermarkets and online purchases in Canada. The four products harvested in Hawaii, one from the Pacific, and the other nine were not specified. Microcystins were detected in three brands of 14 spirulina products with accumulated values in Table 3. The methods used were Laser diode thermal desorption (LDTD) and Ultra-high performance chromatography (UHPLC) coupled with High-Resolution Mass Spectrometry (HRMS) can be analyzed with high detection and quantitation performance. This study uses the Lemieux oxidation method on the Microcystin Adda chain with potassium permanganate and sodium periodate which can produce 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) so that microcystins can generally be detected. The results obtained are the total microcystins contained in the product and are not specific to one type of microcystin, because MMPB is a unique marker of microcystins that can be found in all types of microcystins (Massey et al., 2020).

Analysis of microcystin concentration in supplement samples was also carried out in the study of (Van Hassel et al., 2022) using High-Performance Liquid Chromatography (HPLC) in Belgium. The detection limit of this method was 22.5µg/kg, so the concentrations below this value cannot be detected. The study showed results below the limit of detection (LOD) and the undetectable levels can be caused by the complexity of the pre-treatment process on supplement samples so that microcystins are not fully detected in the analysis process. Research by Pinchart et al. (2023) also conducted a further analysis using UHPLC-MS/MS with a separation process using mobile phases of water and acetonitrile. The analysis results of the total microcystin concentration obtained were much smaller than the analysis by ELISA on the same sample in the research. This is because the ELISA method in the study might be able to detect congeners that were undetected by the UHPLC-MS/MS. The Adda-ELISA method can react with microcystins that still have the Adda group structure even though they have degraded and can increase the food safety of the product. The HPLC method requires more complex sample pre-treatment to eliminate chemicals from the sample extract. As a result, it was speculated that microcystins were not discovered correctly during the analysis process.

The lower microcystin concentration in this study could be due to the specific target analysis used on microcystin-LR. The analysis of microcystin-LR is representative because it is the most common type of microcystin with the highest toxicity (Massey & Yang, 2020). Elisa method in general is known for its high sensitivity and ability to detect specific targets. Differences in the physical shape of the sample may also affect this difference because the extraction method used also differs depending on the type of sample used. The spirulina powder used was cultivated in controlled ponds that were regularly observed with a microscope, and the pond conditions were regularly monitored by testing optical density, temperature, pH, salinity, and light intensity. Environmental control of the culture pond is also suspected to affect microcystin concentrations as the growth of *Microcystis* sp. can be affected by the environmental conditions in which the species lives (Jiang & Zheng, 2018).

4 Conclusion

The best microcystin extraction was found with 50% methanol solvent without sonication. The microcystin analysis method in spirulina powder has also been validated. The linearity parameter has met the criteria and resulted in a linear relationship between the analysis variables. Precision and reproducibility resulted in RSDa of 12.22% and 5.31%, which have met the acceptance criteria and accuracy parameters and have qualified as a valid method with 79.90 ± 17.97% recovery. The detection limit value used in the analysis was 0.1 µg/kg. Analysis of spirulina powder microcystin levels showed lower values compared to the maximum consumption limit based on the Oregon Department of Health for blue-green algae products, making it safe for consumption.

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