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Development, Validation and Application of a Targeted LC-MS Method for Quantification of Microcystins and Nodularin: Towards a Better Characterization of Drinking Water

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Abstract: Cyanotoxins can be produced in surface waters by cyanobacterial blooms, mostly during summer and early autumn. Intoxications would result from consumption of water contaminated with the potent hepatotoxins, microcystins and nodularin. Therefore, the WHO has set a guideline value for drinking water quality concerning one congener of microcystin. Consequently, the design of a validated, public reference method to detect and quantify the hepatotoxins in drinking water is necessary. During this study, a method was developed to quantify cyanotoxins (eight microcystin congeners and nodularin) in water using liquid chromatography coupled with tandem mass spectrometry. Additionally, bottled and tap water samples were tested for the presence of cyanotoxins. No cyanotoxins were detected in any of the collected water samples. However, quality controls and the results of a proficiency test show the validity of the method.

Keywords: water; microcystin; liquid chromatography; mass spectrometry; method validation; matrix effect

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1. Introduction

Water availability and quality are two crucial factors contributing to a healthy and well-functioning society. However, due to climate change and pollution, access to safe freshwater sources diminishes. Increasing salinization, increasing sea levels and the presence of organic and non-organic pollutants are some of the causes of the problem. Moreover, due to the increasing human population, water demand is also increasing, while water reserves in aquifers, groundwater and fossil water are decreasing. These resources can only slowly be replenished. An increase in water storage deficits in Europe was observed after the dry summers of 2018 and 2019 compared to the water storage deficits after the droughts during the summers of 2003 and 2015, as shown by the GRACE and GRACE-FO data record [1]. A high water storage deficit was also observed in Belgium in 2018 and 2019 [1]. Freshwater is not only used for consumption but also for industrial processes, agriculture and other activities. In Flanders, 10% of the total consumed water is used for agriculture [2]. Currently, to produce tap water, ground and surface waters are used equally in Flanders, while in Wallonia, up to 80% of tap water originates from groundwater [3–5]. The remaining 20% is captured from the river Meuse, old mining sites and six dams [6,7]. Increasing the use of surface water could be necessary to meet the current and future water demand. However, the switch to surface water is accompanied by certain pitfalls, as mentioned by the Flanders Environment Agency [8].

One of these pitfalls is the development of cyanobacterial blooms in these water bodies. These proliferations of certain cyanobacterial taxa are favored by environmental and meteorological factors and their prediction is still under study [9–12]. The presence of these blooms can have a detrimental effect on the water quality by producing compounds that lead to foul tastes and odors, or possibly worse, toxic compounds also known as cyanotoxins.

A major group of cyanotoxins are the hepatotoxins, categorized as such due to their main toxicological effect. Two other structurally related hepatoxins are the microcystin congeners (MCs) and nodularin (NOD) (Figure 1). Both contain in their structure an (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA) group connected to a peptide ring. However, NOD's ring contains five peptides, whereas the MCs have a heptacyclic peptide ring [13,14]. Both toxins inhibit protein phosphatase 1 (PPI) and 2A (PPIIA), disrupting cell growth and metabolism [15,16]. When ingested, these toxins are transported by the bile salts to the liver, potentially causing liver damage [17–19].

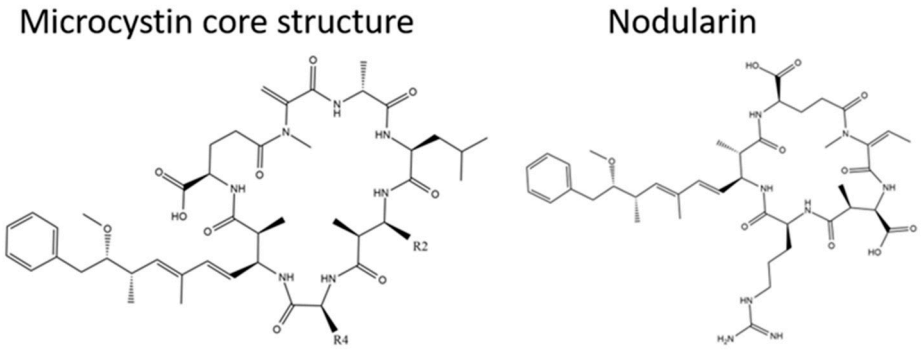

Figure 1. Microcystin core and nodularin structures. For microcystin, the two variable amino acids are annotated as R2 and R4.

Furthermore, MCs are the cyanotoxins most commonly observed worldwide, with MC-LR being the most prevalent in Western Europe [13,20]. The World Health Organization (WHO) has set a guideline value of 1 μg L^{-1} for MC-LR in drinking water in 1994. Following the most recent assessment, the 1 μg L^{-1} MC-LR value is to be considered as a provisional guideline value for the lifetime exposure via drinking water (WHO, 2020) [21]. Additionally, a provisional guideline value for short exposure of 12 μg L^{-1} MC-LR was set based on the No Observed Adverse Effect Level (NOAEL) observed by Fawell et al. [22], the bodyweight of an adult (60 kg), assuming 100% exposure via drinking water and excluding the uncertainty factor of limited databases [23]. The short exposure is considered for a duration of maximum two weeks in one season until water treatment can be improved and the toxin removed. Due to the absence of the oral toxicity data for the other congeners, it is assumed that their values would be similar to MC-LR as the other congeners have a comparable activity. The sum of the congeners is currently calculated and presented as μg L^{-1} MC-LR equivalent to assess the intoxication risk without any equivalency factors taken into account [23]. Nevertheless, to describe the risk accurately, toxicity equivalency factors for the other MCs and NOD need to be determined as in the case of other toxins potentially present in drinking water.

The European drinking water directive recently selected the 1 μg L^{-1} MC-LR guideline value as a quality parameter for drinking water [24]. However, this directive does not include the other MCs, though a mixture of congeners is commonly found in nature.

Multiple methods have been developed to detect and quantify the MCs. HPLC-DAD was the first method used [25]. Later on, protein phosphates inhibition assays (PPIA) and ELISA tests were developed to quickly quantify the MCs and NOD. PPIAs were simple tests based on a colorimetric detection of the dephosphorylation of the p-nitrophenyl phosphate by PPI [19,26]. The presence of MCs prevents this process by inhibiting the PPI. A pitfall of this test is the lack of specificity for one particular MC or NOD. Therefore,

identification is not possible. Interactions with unintended compounds can also result in false-positive results or concentrations in abnormal ranges. ELISA assays have similar shortcomings. However, there is now a tendency to use liquid chromatography–tandem mass spectrometry (LC-MS/MS) and liquid chromatography high-resolution mass spectrometry (LC-HRMS) techniques for the detection of MCs and NOD in water, as well as other matrices [27–29]. The main advantage of these methods is that the identification of each congener is based on its physicochemical properties and molecular mass, while the concentration can be determined simultaneously. However, LC methods require pure standards for each specific congener, with the exclusion of high resolution mass spectrometry.

Only a limited number of fully described, validated LC-MS/MS methods are available to evaluate drinking water contamination by hepatotoxins, where the matrix effect has been investigated or taken into account during quantification [29–31]. Considering that these toxins could jeopardize public health, the development and validation of a quantification method for MCs and NOD and the evaluation of the drinking water quality in Belgium appeared worthwhile. A method was developed to quantify eight MCs (MC-LR, MC-RR, MC-LA, MC-LY, MC-YR, MC-WR, MC-LF and MC-LW) and NOD using a matrix-matched calibration curve. It was fully validated to analyze bottled water from several countries in Europe and tap water from different sources in Belgium.

2. Materials and Methods

2.1. Reagents

UHPLC-MS grade solvents (Biosolve B.V., Valkenswaard, The Netherlands) were chosen to prepare the mobile phases and dilution solvents. Milli-Q water and acetonitrile (ACN) were used as mobile phase A and phase B, respectively. All the toxin standards, eight MCs (MC-RR, MC-YR, MC-WR, MC-LR, MC-LA, MC-LF, MC-LW, MC-LY) and NOD were obtained from Enzo Life Sciences® (Enzo Life Sciences, Antwerp, Belgium) as a solid powder. The toxin stock solutions were diluted in MeOH. Intermediate dilutions for the toxin standards were made with a MeOH: Milli-Q water mixture (50:50), supplemented with 1% acetic acid. The stock and the intermediate solutions were stored at −20 °C.

2.2. Water Samples

Bottled water samples were obtained from major retail stores. A total of 51 water samples (various bottles and brands) were tested, with 23 samples of sparkling water and 28 samples of still water. The bottled water samples originate from all over Europe (Belgium, Luxembourg, Germany, France, Italy, The Netherlands). Furthermore, 24 tap water samples were included. These samples were collected in sterile amber glass bottles directly after opening the faucet. In the Flemish provinces, 18 samples were collected. Additionally, two samples were taken from faucets in the Brussels region, and four samples were collected in Wallonia. More details on the samples are provided in Tables S1 and S2. The geographic distribution of the tap water samples is presented in Figure S1.

Additionally, we participated to the Eurofins Abraxis microcystins proficiency testing program 2021-01.

2.3. Sample Preparation

Water samples (5 mL), independent of their source, were adjusted to pH 11. Using solid-phase extraction (SPE), toxins were extracted from the water as follows: conditioning 6 mL MeOH 100%, equilibration with 6 mL Milli-Q water (pH 11) and elution with 5 mL MeOH (80%). The elute was purified using a Phenomenex 0.2 µm RC-syringe filter (Phenomenex Inc., Utrecht, The Netherlands) and was transferred in amber glass vials with an insert. Each analysis of drinking water samples was accompanied by a quality control (QC) containing all nine toxins at a concentration of 1 µg L^{-1}. A calibration curve was made in a blank water matrix ranging from 0.1 ng mL^{-1} to 20 ng mL^{-1}.

2.4. UHPLC-MS/MS Conditions

Toxins were analyzed on a Xevo-TQ-S from Waters© (Waters, Eten-Leur, The Netherlands). The initial separation of the toxins was performed with a Waters Acquity UPLC H-class (Waters, Eten-Leur, The Netherlands) on a 1.7 µm, 2.1 mm × 100 mm Waters Acquity BEH C18 column (Waters, Eten-Leur, The Netherlands) proceeded with a Waters Acquity BEH C18 1.7 µM VANGUARD PRE-Col (Waters, Eten-Leur, The Netherlands).

The mobile phase was composed of phase A (Milli-Q water) and phase B (acetonitrile (ACN)). Formic acid was added to both mobile phases at a ratio of 0.025%. The flow rate used was 0.5 mL min-1 and the applied gradient elution program was as follows for mobile phase B: 0 min, 2%; 1.00 min, 40%; 7.00 min, 55%; 7.20 min, 98%; 8.00 min, 98%; 9.00 min; 2%; 12 min, 2%. The column temperature was 60 °C, and the sample injection volume was 10 µL.

The mass spectrometer was operated in the ESI(+) mode. The MS parameters were set as follows: source and desolvation temperatures were 150 and 450 °C, respectively. The capillary voltage was 1.0 kV. Cone and desolvation gas flows were set at 150 and 1000 L h^{-1}, respectively. Collision gas flow was 0.15 mL min^{-1}. Source offset was 50 V.

2.5. Optimization of the MS/MS Conditions

Initially, detection parameters for the toxins were optimized individually at concentrations of 1 µg L^{-1}. The precursor mass was determined and used as the selection parameter during the collision. After the precursor ion fragmentation, two product ions with the highest intensity were selected as a qualifier or quantifier ion. Collision energy and cone voltage were then further optimized to maximize the signal intensity. An overview can be found in Table 1.

Table 1. MS/MS parameters used for ion fragmentation.

Toxins	Precursor Ion	Quantifier Ion (m/z)	Collison Energy (eV)	Cone Voltage (V)	Qualifier Ion (m/z)	Collison Energy (eV)	Cone Voltage (V)
MC-LR	995.4	135.0	70.0	80.0	213.1	60.0	80.0
MC-RR	519.8	134.8	30.0	50.0	107.2	60.0	50.0
MC-YR	1045.5	135.3	80.0	60.0	212.9	60.0	60.0
MC-WR	1068.4	135.3	70.0	100.0	213.1	60.0	100.0
MC-LY	1002.4	135.4	60.0	50.0	213.0	50.0	50.0
MC-LA	910.3	135.1	60.0	50.0	107.1	80.0	50.0
MC-LF	986.3	135.0	60.0	70.0	213.1	60.0	70.0
MC-LW	1025.4	134.9	60.0	60.0	213.1	50.0	60.0
NOD	825.2	134.9	50.0	80.0	102.7	90.0	80.0

The selectivity of the LC method was also optimized to minimize overlap between different toxin peaks. Different mixtures of mobile phases, such as methanol instead of ACN and neutral, acidified or alkalized versions of the mobile phases, were tested. Moreover, various total elution times and elution gradients were tested to provide the best elution pattern and peak shape, resulting in the use of the earlier described LC method. Peak selectivity is shown in Figure S2.

2.6. Method Validation Procedure

The validation study was performed using spiked bottled water. The following method parameters were evaluated: limit of detection (LOD), limit of quantification (LOQ), specificity, linearity, recovery, repeatability, reproducibility, matrix effects, and measurement uncertainty (MU).

During the validation experiments, three toxin mixtures with different concentrations (0.5 µg L^{-1}, 2.5 µg L^{-1} and 5 µg L^{-1}) for each toxin were selected. These concentrations were chosen around the 1 µg L^{-1} WHO guideline value for chronic exposure for accurate quantification [23]. In food applications, concentrations for validation would be chosen as

0.5, 1.0 and 1.5 maximum residue limit (MRL), and in the case of MCs in water, 0.5 μg L^{-1}, 1.0 μg L^{-1}, 1.5 μg L^{-1} [26]. Using this validation approach would result in a very narrow concentration range, which will not be able to accurately quantify higher concentrations of MCs present under validation in drinking or environmental water samples.

For each concentration level, 5 mL of bottled water (from a source) was spiked from a standards mix solution containing each toxin at 100 μg L^{-1}, in triplicate. Additionally, a blank (bottled water) sample was included as a negative control. The validation experiment was repeated on three different days.

A calibration curve was established using a serial dilution of the toxins at 20 μg L^{-1}, 10 μg L^{-1}, 5 μg L^{-1}, 1 μg L^{-1}, 0.5 μg L^{-1}, 0.25 μg L^{-1}, and 0.1 μg L^{-1} for each toxin. The serial dilution was made in a blank water matrix to assess the matrix effects of the toxins in the water. To quantify the toxin content, the calibration curve was weighted at $1/X^2$.

The linearity of the curve was assessed based on the Mandle's fitting test [32]. This statistical test compares a linear model to a quadratic regression model based on the area under the peak for the different concentrations of a calibration curve.

The LOD and LOQ were accepted as the lowest point in the calibration curve (0.1 μg L^{-1}) and the lowest validated quantified concentration (0.5 μg L^{-1}), respectively, if the value of the signals was at least 3 times higher than the noise for LOD (S/N > 3) and 10 times higher for the LOQ (S/N > 10).

Furthermore, the specificity and selectivity of the signal were checked by monitoring the difference in elution time (<5%), the lack of signal in the negative control, the peak shape and the presence of both the quantifier and qualifier ion in spiked samples.

As part of the validation, the ion ratios were also taken into account, following the guidelines of the EU directive 2002/657/EC [33]. The tolerance of the ion ratio is determined based on the relative intensity of the qualifier compared to the quantifier. Inspired by the same directive, the Horwitz equation was used to calculate the reproducibility and repeatability, represented as the coefficient of variation (CV) and average variance, respectively. To determine the repeatability, spiking experiments were performed at three concentration levels in triplicate on the same day, while for within-laboratory reproducibility evaluation, the same experiments were carried out on three separate days.

During the validation, matrix effects were assessed by comparing the slopes of calibration curves prepared in the matrix extract and neat solvent (MeOH: Milli-Q water (50:50) + 1% acetic acid). The *t*-test was used for the statistical evaluation of the matrix effect data. The matrix effect can also be observed visually when the curves intersect.

Eventually, the concentrations of the spiked samples were measured. The data analysis was performed in TargetLynx extension of the MassLynx software (Waters©).

Furthermore, the recovery was calculated as the mean of means divided by the theoretical spiked concentration of each of the MCs, separately. The lowest and upper threshold values for the recovery from the spiked samples were 60.0% and 120.0%, respectively. The MU was calculated by multiplying the CV value for the reproducibility by 2 and adding the difference of the recovery from 100.0%. Both the recovery and MU were calculated for each concentration level. The upper threshold value was 80.0%.

Additionally, the sum of the measured concentrations for all the eight MCs and NOD in one spiked sample were taken, resulting in concentration levels of 4.5 μg L^{-1} total microcystin, 22.5 μg L^{-1} total microcystin and 45.0 μg L^{-1} total microcystin. Repeatability, reproducibility, recovery and MU were also calculated for these values and were evaluated using the same criteria as for the separate congeners.

3. Results

3.1. Method Validation

Initially, the analysis of blank bottled water samples demonstrated the absence of MCs toxins. This means that no peak with an S/N higher than three was detected at the expected retention time of the hepatotoxins from this study, pointing out the good specificity of the method.

Afterwards, the validity of a linear fit was determined for all the nine toxins using the Mandle's fitting test on three separate days [34]. For five out of nine toxins, the tests showed a preference for a linear model. The results were slightly more ambiguous for four MCs congeners (MC-RR, MC-LA, MC-LW and MC-YR) due to the preference of a quadratic model during at least one of the days. This ambiguity resulted from variations between different days for the residual standard deviation for both models. The residual standard deviations for both models on the same day were very similar. Moreover, the R^2 value of the linear model was higher than 0.99 and thus suitable for quantification (Table 2). In similar methods for quantification of the hepatotoxins, the R^2 was also used as selection criteria for the linear model [29,31,35,36]. The linear model was therefore selected for all nine toxins.

Table 2. Overview of validation results for eight microcystin congeners (MC), nodularin (NOD) and the sum of all toxins in water. Results for the recovery, repeatability, reproducibility, measurement uncertainty, R^2 and average signal to noise (S/N) for the limit of detection (LOD) and the limit of quantification (LOQ) are shown on average and at three concentration levels. * µg L^{-1} total microcystin.

Toxins	Spiked Concentration (µg L^{-1})	Recovery (%)	Repeatability (%)	Reproducibility (%)	Measurement Uncertainty (%)	Average S/N for LOD (0.1 µg L^{-1})	Average S/N for LOQ (0.5 µg L^{-1})	R^2
MC-RR	0.5	96.00	5.03	9.12	18.25	361.85	1691.95	1.00
	2.5	97.00	2.04	4.99	9.98			
	5.0	103.00	6.39	7.98	15.96			
	Average	98.70	4.48	7.37	14.73			
NOD	0.5	95.00	5.23	7.14	14.29	193.38	7605.39	1.00
	2.5	98.00	2.80	5.08	10.15			
	5.0	103.00	7.26	8.10	16.20			
	Average	98.70	5.10	6.77	13.55			
MC-LA	0.5	90.00	4.66	6.43	12.85	60.53	222.93	1.00
	2.5	92.00	3.30	6.35	12.70			
	5.0	97.00	7.21	7.84	15.67			
	Average	93.00	5.06	6.87	13.74			
MC-LF	0.5	68.00	3.56	5.75	11.51	37.42	106.18	1.00
	2.5	66.00	4.87	14.05	28.09			
	5.0	72.00	7.04	7.04	14.08			
	Average	68.70	5.15	8.95	17.89			
MC-LR	0.5	88.00	2.27	7.19	14.37	95.72	432.98	1.00
	2.5	89.00	1.63	7.67	15.34			
	5.0	93.00	7.35	10.07	20.14			
	Average	90.00	3.75	8.31	16.62			
MC-LY	0.5	88.00	5.29	10.39	20.78	49.53	174.58	1.00
	2.5	88.00	2.70	9.41	18.82			
	5.0	94.00	6.11	10.94	21.88			
	Average	90.00	4.70	10.25	20.49			
MC-LW	0.5	53.00	8.68	16.23	32.45	42.34	98.27	1.00
	2.5	53.00	8.10	19.64	39.28			
	5.0	59.00	6.01	11.16	22.32			
	Average	55.00	7.60	15.67	31.35			
MC-YR	0.5	83.00	6.32	10.69	21.38	54.48	192.34	1.00
	2.5	87.00	4.17	15.80	31.61			
	5.0	91.00	5.30	15.34	30.67			
	Average	87.00	5.26	13.94	27.89			
MC-WR	0.5	62.00	11.31	19.64	39.29	55.80	254.99	1.00
	2.5	67.00	6.43	21.05	42.10			
	5.0	72.00	3.59	16.14	32.28			
	Average	67.00	7.11	18.94	37.89			
SUM	4.5 *	80.00	4.32	5.27	10.54	/	/	/
	22.5 *	82.00	2.99	8.59	17.18			
	45.0 *	87.00	5.92	7.08	14.15			
	Average	83.00	4.41	6.98	13.96			

After spiking the water matrix at the same concentrations as the calibration curve, a matrix effect was observed for all toxins based on the difference between the slopes using a *t*-test (Figure 2 and Table 3). The presence of a matrix effect substantiates the need for a calibration curve in the matrix.

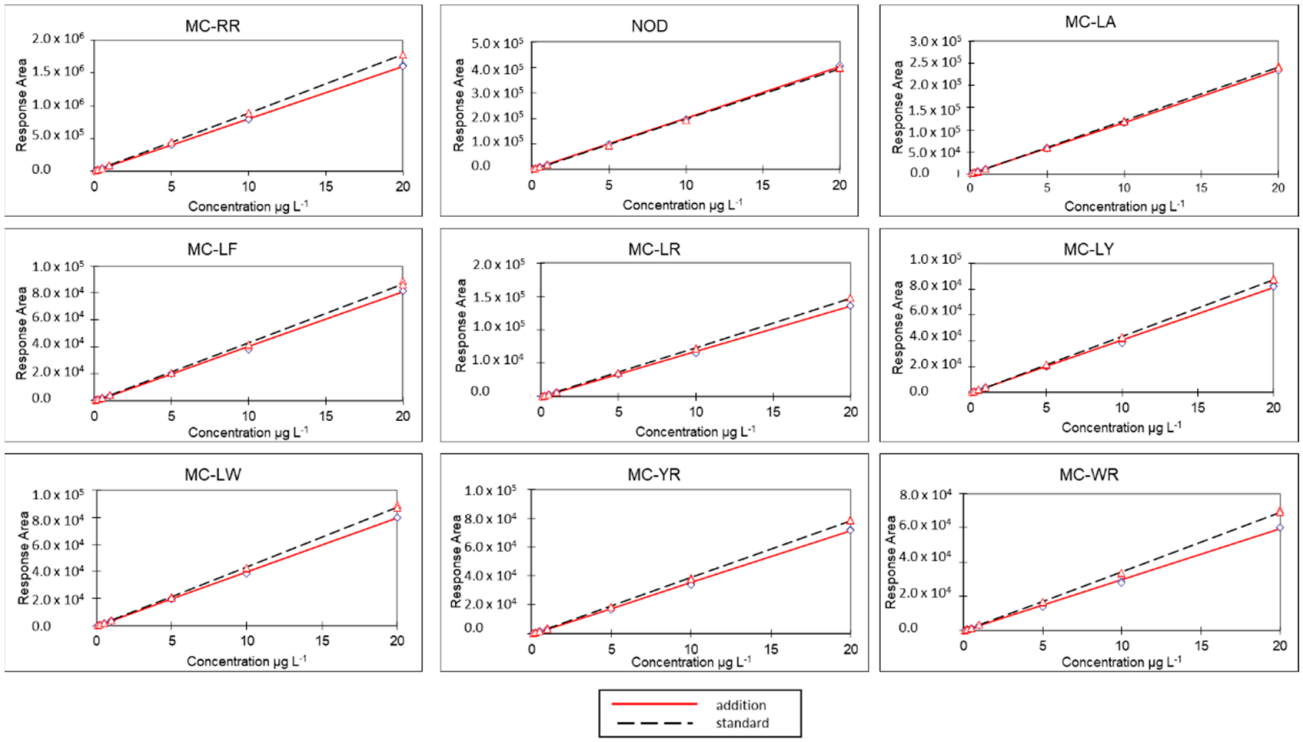

Figure 2. Matrix effect assessment for the different microcystin congeners and nodularin in drinking water. The presence of a matrix effect was established by comparing the difference in slope based on a student *t*-test. However, also a visual assessment can be made. If the curve runs in parallel, there is no matrix effect. If this is not the case, there is a matrix effect. All toxins displayed matrix effect in drinking water.

Table 3. Values for the calculated t (b) is compared with the tabulated t at the 95% confidence level. If t (b) is higher than t (95%), a matrix effect is present.

	MC-RR	NOD	MC-LA	MC-LF	MC-LR	MC-LY	MC-LW	MC-YR	MC-WR
t (b)	53.59	3.34	7.92	5.57	11.54	7.53	11.93	9.17	12.02
t (95%)	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06

Finally, our validation confirmed the accurate detection and quantification of the nine toxins. The retention time stability, peak shape, selectivity and ion ratios were all within the pre-set boundaries. Moreover, the LOD (0.1 µg L^{-1}) and LOQ (0.5 µg L^{-1}) for all nine toxins had a ratio of signal to noise higher than 3 and 10, respectively, as shown in Table 2. However, the recoveries for MC-LF, MC-LW and MC-WR were below the acceptable limit of 70.0% (Table 2 and Figure 3). These low recoveries are possibly due to retention of the more hydrophobic MCs on the plastic tubes used during pH adjustment. Rinsing these tubes with higher amounts of organic solvent would solve this [37] but cause early elution of MCs during SPE resulting in a decrease in recovery of the more hydrophilic MCs. Moreover, the lower recoveries for the hydrophobic MCs obtained with our method will suffice, as the results of samples' analyses will always be corrected with the value of the recovery of a quality control (QC) sample, and the results for reproducibility, repeatability and MU were acceptable. During sample analysis, acceptable recoveries for the QCs of MC-LF, MC-LW and MC-WR will need to be between 30.0% and 90.0% or they will be labelled as

non-conform. The recoveries for the conform toxins ranged from 87.0% to 98.7% on average (Table 2 and Figure 3), with acceptable recoveries for the QC during sample analysis being between 60.0–120.0%. MUs for all the MCs and NOD were calculated to be between 10.0% and 42.1% (Table 2). The average variance (repeatability) and CV (reproducibility) for all the MCs and NOD were within the bounds set by the Horwitz ratio, 14.7% and 22.0%, respectively (Table 2 and Figure 3).

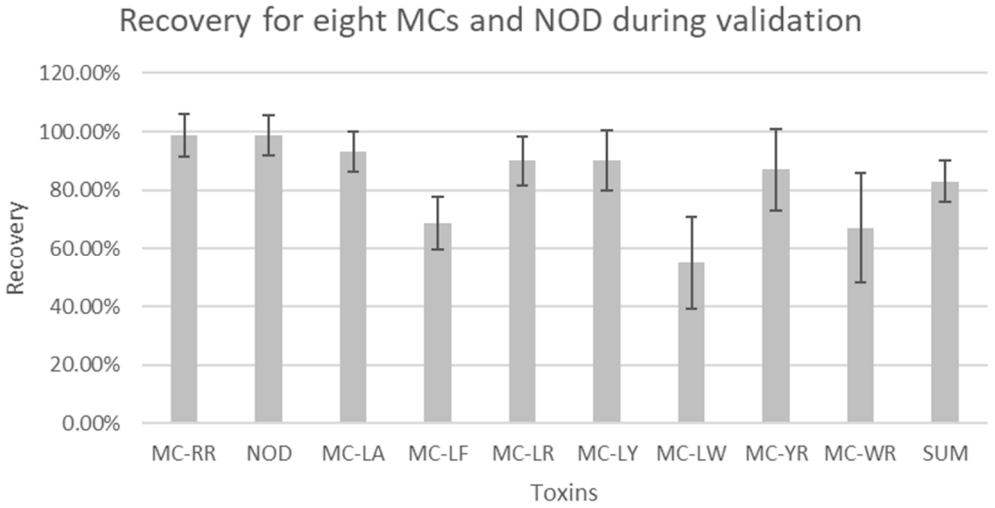

Figure 3. Recoveries for all eight microcystin congeners (MCs), nodularin (NOD) and their sum, are presented in the graph. The error bars represent the reproducibility.

3.2. Application of the Method in Drinking Water

The validated UHPLC-MS/MS method was subsequently used to investigate the contamination of drinking water available on the Belgian market and Belgian tap water. In total, 51 samples of bottled water and 24 samples of tap water were collected (Tables S1 and S2) and analyzed during six different analysis days.

During the analysis, QCs were added to each analysis series to ensure the quality of the procedure. The recoveries of the QCs were calculated and were acceptable for each of the six analysis days (Table 4). The precision of the analysis was taken into account by assessing the standard deviation of the retention time, which should be below 0.05%. Additionally, the R^2 values for the calibration curve were above 0.99, showing acceptability of the curve. The relative standard deviation (RSD) was calculated with the recovery and ranged between 1% to 10% for the different hepatotoxins. However, no MCs or NOD could be detected in the samples taken from stores or tap water.

Table 4. Average recoveries for quality controls (QC) of eight microcystin congeners and nodularin obtained from 6 different days of sample analysis.

MC-RR	NOD	MC-LA	MC-LF	MC-LR	MC-LY	MC-LW	MC-YR	MC-WR
97.00%	90.00%	81.00%	61.00%	72.00%	74.00%	43.00%	77.00%	60.00%

Additionally, results were satisfactory ($|z| < 2$) for the Eurofins Abraxis microcystins proficiency testing program 2021-01.

4. Discussion

The screening results from bottled and tap water revealed that no MCs or NOD could be found, indicating that there is currently no safety risk for the population regarding contamination of drinking water with these hepatotoxins, based on this study of 75 samples. As expected, bottled water is not contaminated because it is generally exploited from sources or springs, where cyanobacterial blooms do not occur. On the other hand, tap

water can be extracted from surface water and is thus at risk of contamination. However, in Belgium, groundwater sources are more frequently used than surface waters. In Flanders, only eight surface water bodies are used in four of the nine distribution districts. Moreover, most of the surface water originates from the river Meuse and the Albert Canal. For Flanders, tap water was sampled from distribution districts that exploit groundwater, which might explain the lack of toxins. In Wallonia, 80% of the water is extracted from groundwater. Most of the surface water also originates from the river Meuse [5,38].

Furthermore, Belgian tap water is diligently treated before it is distributed, reducing the likelihood of toxins being present in the water. One instance of a well-described treatment process for surface water uses grids and microsieves, flocculation, sand filtration, active carbon filters, consecutively, with additional chemical treatments if necessary to ensure the quality of the water [39]. Although the risk of MCs contaminated tap water seems small at present, our validated methods could be useful in case of any suspected contaminations. Due to extended dry periods, water reserves have dwindled every summer since a few years (e.g., in Flanders, four districts reported 'lower than normal' groundwater levels in 2020) [38]. This problem is expected to increase due to climate change, and alternate water sources, such as surface water, could be necessary to meet the water demand. Moreover, the flowing water bodies such as the river Meuse and Albert Canal, are also susceptible to droughts, meaning that other water bodies might need to be used in the future to supplement our water demand. Stagnant water bodies, such as lakes and reservoirs, are more likely to harbor potentially toxic cyanobacteria blooms. Multiple studies have already shown that bloom occurrence in different water bodies used as drinking water catchment is common [25,31,40–49]. Therefore, using the newly developed analytical methods to analyze contaminants, especially during summer and autumn, will ensure drinking water quality.

However, most studies found toxins in the raw water before but rarely after treatment. Although water treatment is still not yet universally available worldwide, certain techniques could remove cyanotoxins from raw water. The use of active carbon has been shown to remove cyanobacterial cells and, to a certain extent, free toxins from the raw water [44,50–57]. The use of chemical agents, such as chlorine, permanganate or ferrate, can inactivate cyanobacterial cells and, in some cases, degrade certain toxins [44,58–60]. Advance oxidation by ultrasound, ozone, UV, H2O2 or a combination of the latter two is also effective in degrading certain toxins and deactivating cyanobacterial cells [54,61–68]. Yet, the number of cyanobacterial cells and the concentration of the toxins influence the effectiveness of these methods. Therefore, accurate toxin quantification is vital for a successful treatment. Our validated UHPLC-MS/MS method could be applied for these control purposes.

Our validation shows that the method can quantify eight microcystin congeners (MC-LR, MC-RR, MC-YR, MC-LY, MC-LA, MC-WR, MC-LF and MC-LW) and nodularin. However, overall recoveries for MC-WR, MC-LF and MC-LW were lower than for the other toxins, and thus the acceptable limit for recovery had to be reduced for these hepatotoxins. Interestingly, this reduced recovery primarily affected the hydrophilic compounds, which was also observed in Zervou et al., where SPE was also used [30].

The LOQ is defined as the concentration level where a compound can be quantified with certain precision and accuracy during the method validation. This definition would only be applicable for our lowest validation point, 0.5 µg L^{-1}. Pekar et al. accurately determined concentrations at 0.1 µg L^{-1} for some MCs, while for NOD, MC-YR, MC-LW, MC-LY, MC-LF, this was only possible at a concentration of 0.5 µg L^{-1} [31]. During the analysis, 0.5 µg L^{-1} was chosen as the lowest level because it would be distinguishable from the 1 µg L^{-1} guideline value proposed by the WHO. Lower concentrations would be of little concern during monitoring campaigns, even though the feasibility of quantifying concentrations below 0.5 µg L^{-1} could be assessed. Instead, Turner et al. used an alternative term to approximate this definition of LOQ, namely limit of reporting (LOR). The LORs for the toxins ranged between 0.3 and 1.3 ng mL^{-1} [29]. Zervou et al. used an alternate

approach, first determining the LOD based on statistics and then defining the LOQ as 3 times the LOD [30]. They were able to obtain LODs in the ng L^{-1} range. However, the lower LOD values can be explained by the higher sample volume used during SPE and the increased concentration of the extract after a nitrogen stream evaporation. Moreover, higher sample volumes and up concentration would increase the reporting time, which is an important factor when analyzing samples for contaminations. Our LOD is sufficient to accurately assess contaminations close to the WHO guideline values, with a fast reporting time ($\pm$1 day).

The LOD of the toxins analyzed in our method was set at 0.1 µg L^{-1} as the lowest point of the calibration point and thus the lowest point for which we could assess the signal to noise ratio. Further investigation could result in lower values, but this would have little benefit to swiftly assess the public health threat.

The matrix effect in water seems to be variable depending on the source of the water used for validation. Pekar et al. reported a matrix effect for nearly all toxins in water, while Turner et al. found a matrix effect for only a few [31]. On the other hand, our study shows that a matrix effect could be measured for most MCs compared to the calibration solution MeOH:H2O 50:50 (v/v) solution with 1% acetic acid. However, the physicochemical properties (salts, metals and other micronutrients) of the water can probably influence the matrix effect. Therefore, we suggest that our approach could be a valuable asset to incorporate into a method design to analyze multiple water samples in one run by using a calibration curve in the water matrix. However, the water-based calibration curve could provide variability due to common adsorption by plastic lab equipment [37]. The SPE was used to collect the toxins in an organic solvent (MeOH 80%) before injection of the UHPLC-MS/MS, preventing this adsorption.

5. Conclusions

Our UHPLC-MS/MS method is the first publicly described and fully validated method for quantifying eight MCs and NOD for water in Belgium. This method shows reasonable specificity, linearity of the matrix-matched calibration curves, matrix effect, precision parameters, recovery, repeatability, reproducibility and measurement uncertainty for MC-RR, NOD, MC-LA, MC-LR, MC-LY, MC-YR, MC-WR, MC-LF and MC-LW in drinking water. However, the initial threshold recovery values for MC-WR, MC-LF and MC-LW were not reached. The obtained recoveries were sufficient for a valid method due to acceptable values for reproducibility and MU. All parameters for the other toxins were within the preset parameters.

Additionally, the implementation of our method on 51 bottled (from Europe) and 24 tap water (from Belgium) samples from different sources is exceptional. Dependent on the region in Belgium, hepatotoxins in drinking water are evaluated with undisclosed methods, which makes a comparison of the methods and results impossible. However, none of the nine hepatotoxins were detected during our study, showing that Belgian drinking water is most likely safe for the consumer. The QC measured during sample analysis does provide proof that our method is capable of quantifying the MCs and NOD, which was further supported by our participation in the Eurofins Abraxis proficiency test, resulting in satisfactory z-values.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14081195/s1, Figure S1: Map showing the distribution of the tap water sampling in Belgium, Figure S2: The elution peaks and the intensity shown for the eight microcystin congeners and nodularin, Table S1: Overview of bottled water based on their country of origin, total volume of water and count of individual samples, Table S2: Overview of tap water samples based on region and count of individual samples.

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