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Simultaneous Detection and Quantification of Aflatoxin M1, Eight Microcystin Congeners and Nodularin in Dairy Milk by LC-MS/MS

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Abstract: Dairy milk holds a prominent position as a widely consumed food, particularly among infants and children. However, it is crucial to address the presence of multiple natural toxic compounds that may co-occur in dairy milk to ensure its safety prior to consumption. Aflatoxin M1 (AFM1), an emerging mycotoxin of interest, is a potential contaminant in the milk of animals who ingest aflatoxin B1 (AFB1). The toxin is regulated in the European Union under Commission Regulation No 1881/2006. Unfortunately, there is a notable lack of data concerning the transfer of various emerging microbial contaminants into dairy milk and, therefore, their natural occurrences. In this study, a simple and sensitive LC-MS/MS method was developed and validated for the quantification of multiple cyanotoxins (microcystin congeners and nodularin) and AFM1 by the main analytical guidelines. Toxins are extracted with methanol 80%, followed by an SPE clean-up step before LC-MS/MS analysis. The LOQ was fixed at 1 µg/L for the nine cyanotoxins and 0.05 µg/L for AFM1. Recoveries were measured between 82.67% and 102%. To the best of our knowledge, there are no other LC-MS/MS methods available for the simultaneous quantification of cyanotoxins and mycotoxins in milk.

Keywords: cyanotoxins; aflatoxin M1; dairy milk; liquid chromatography–mass spectrometry; food safety; method development

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1. Introduction

Consumption of contaminated food leads to acute and chronic toxicity for animals and humans. This contamination could be associated with potential multiple adverse effects. This is an eminent hazard to human health from ingesting animal-derived food, such as milk containing toxins produced by various organisms [1,2].

Milk is the world's second-most consumed beverage after water, as it is a fundamental element in the diet of all age groups and the primary source of nourishment for infants and children [3]. According to the Food and Agriculture Organization (FAO), the European population consumes 2.6 times more dairy milk than the global population [1]. In 2022, the European Union consumed 23.8 million metric tons of milk, as reported by the Statista research department [4]. Therefore, it is crucial that milk is free of toxic compounds harmful to humans.

Mycotoxins are defined by their diverse organic structures containing heteroatom on the functional groups. These toxins can be ingested and, in high quantities, cause negative effects on human health [2]. Aflatoxins (AFs) represent a major class of mycotoxins. The primary producers of AFs are fungi from *Aspergillus* genus [5], and their production can be

impacted by different environmental conditions (e.g., drought, high temperature, substrate composition, etc.) [6]. Among AFs, aflatoxin B1 (AFB1) is the most toxic and is described as carcinogenic, teratogenic, and mutagenic [7]. In 2012, the IARC listed AFB1 as group I carcinogen [8]. Residues of AFB1 might be found in milk and animal products after consumption of contaminated feedstuff [9]. This is especially true in developing countries where regulations are limited.

AFB1 is metabolised in the liver to form the 4-hydroxy derivative aflatoxin M1 (AFM1). The structure of AFM1 is highlighted in Figure 1C. This hydroxylated metabolite is excreted in milk via mammary glands. It has been reported that between 0.3% and 6.2% of aflatoxin B1 is converted into aflatoxin M1, varying based on the type of diet, ingestion rate, digestion rate, and hepatic biotransformation capacity [10]. Poisoning from the consumption of AFs is known as aflatoxicosis. The symptoms of aflatoxicosis in mammals are lethargy, lack of appetite, rough and/or pale coat hair, ataxia, and fatty liver [11]. AFM1 is listed as a Group 2B carcinogen by the IARC, and because of this high toxicity, Europe has set a limit of 0.05 µg/kg and 0.025 µg/kg for raw milk, heat-treated milk, and milk for adults and infants, respectively [8,12].

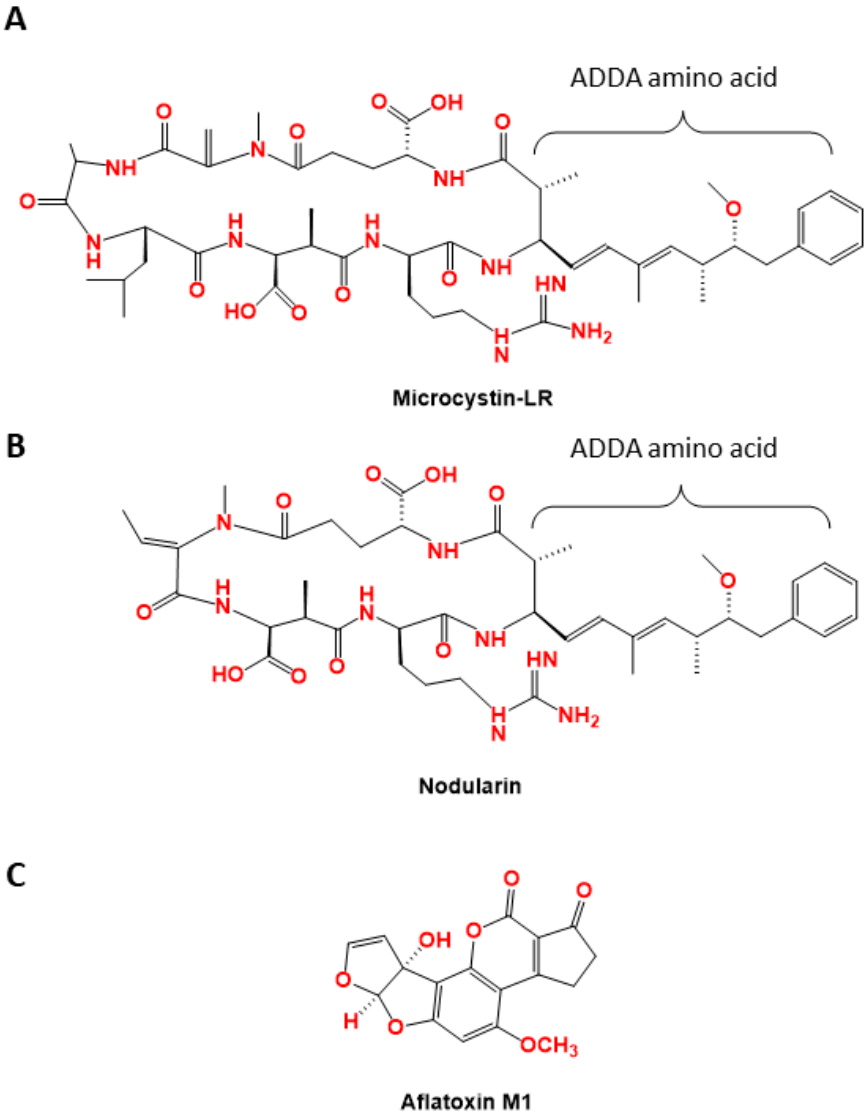


Figure 1. Chemical structures for the three toxins examined in this study. (**A**) Structure of cyanotoxin microcystin-LR; (**B**) structure of the cyanotoxin nodularin; (**C**) structure of mycotoxin aflatoxin M1.

Microcystins (MCs) and Nodularin (NOD) are common cyanotoxins characterised as hepatotoxins. Produced by multiple bacterium taxa, such as *Microcystis*, *Planktothrix*, *Nostoc*, *Anabaena*, *Dolichospermum* and *Oscillatoria* [13], MCs are cyclic heptapeptides containing a specific ADDA moiety (i.e., (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid) as observed in Figure 1A. The amino acids at positions 2 and 4 are variables, and other parts can be methylated, demethylated, and/or acetylated, leading to a high number of congeners. Actually, more than 300 congeners are described, and the two variable amino acids are also used for the nomenclature of MCs [14]. For example, MC-LR, the most prevalent congener in Western Europe, is a MC containing leucine (L) and arginine (R) as variable amino acids [13]. The associated toxicity is also variable because of the structural difference between congeners [14]. For example, the median lethal dose (LD_{50}) for MC-RR is lower than MC-LR, but the uptake of MC-LF and MC-WR is more efficient than for MC-LR, which could indicate a higher in vivo toxicity [15,16]. Concerning NOD, it is structurally similar to MCs, containing the ADDA moiety but it includes an acyclic pentapeptide (Figure 1B). This toxin is synthetised by organisms of *Nodularia* genus [17,18].

Cyanotoxins are generally produced and contained within actively growing cyanobacterial cells. In an algal bloom, the release of these toxins into the surrounding environment as dissolved toxins usually occurs during cell death or lysis. Cyanotoxins are considered emerging toxins and have been frequently detected in fresh and brackish waters. Therefore, drinking water is a source of potential exposure. The occurrence of cyanotoxins in drinking water depends on various steps during its production, such as their original levels in raw source water and the efficiency of treatment methods at removing cyanobacteria and cyanotoxins [19,20]. Consequently, a European directive from 2020 fixed a guideline of 1 µg/L in drinking water for MC-LR [21]. However, this configuration ignores other MC congeners that are also found in nature. Therefore, a study in 2022 established a protocol for the detection of 8 MC congeners and NOD in drinking water and highlighted the good quality of Belgian bottled and tap water [22]. Other potential routes of exposure have been described, such as recreational exposure, cyanobacteria-based food supplements or contaminated crops [18,23]. Concerning the potential transfer of MCs and NOD in milk, sparse results are available. Effectively, only a few studies based on livestock are published but rely on a limited number of animals and with analytical methods not considered reliable. In this context, the EFSA published an external report explaining that further research is needed regarding the possible transfer of MCs to milk or meat [23].

Additionally, experimental studies have highlighted that MC exposure may play a role in the origin but also in the progression of diverse cancers [24,25]. In 2010, MC-LR was classified as a Group 2B carcinogen by the IARC (International Agency for Research on Cancer) [26].

Reliable, accurate, and sufficiently sensitive methods are required to detect and quantify cyano- and myco-toxins. A wide range of extraction and analytical methods have been developed [19,27–31]. The first part of the sample treatment is the extraction. An adequate extraction procedure is required depending on the objective that has been set. A rigorous choice of the solvent mixture during the extraction process is essential to make sure it is compatible with the detection technique. For MCs and NOD, methanol is frequently used at a variable concentration ranging from 75% to 100%, according to the matrices [32–34]. In opposition, ACN is the most used solvent for AFM1 extraction [35,36]. The use of clean-up steps in the analysis has been largely reported and concerns different procedures such as liquid-liquid partitioning, solid-phase extraction (i.e., SPE), or immunoaffinity column (i.e., IAC) [23,37]. Regarding the detection techniques used for the analysis of food toxins, they can be classified as chemical (capillary electrophoresis, gas or liquid chromatography coupled with UV, FLD, or MS) or biological (bioassays, immunoassays, cytotoxicity assays, or receptor binding assays). Characterisation and/or validation studies are used to assess the performance of these techniques. In this context, different guidelines are available and can be followed during the validation process [23].

Liquid Chromatography-Mass Spectrometry (LC-MS) has gained significant interest and popularity in the analysis of cyanotoxins and mycotoxins due to its numerous advantages that address the complexities associated with these harmful compounds. The combination of separation capabilities (liquid chromatography) with detection and identification power (mass spectrometry) provides a comprehensive approach to cyanotoxin and mycotoxin analysis. The high sensitivity of the occurring trace levels detection in complex samples but also selective detection enables the differentiation between multiple toxins and their variants. Moreover, this method offers flexibility and adaptability with modifications to different toxins and matrices by adjusting parameters, such as column type, mobile phase composition, and ionisation techniques [38,39].

A literature search revealed multiple studies aimed at the detection of MCs or AFM1 in various matrices [1,32,40–42], but none of these combine the detection of both types of toxins simultaneously. Even if some methods were developed for multi-toxin detection, they usually gathered toxins from the same origin. Effectively, numerous methods are designed for the detection of multiple mycotoxins in diverse matrices, such as the method proposed by Flores-Flores and Gonzalez-Penas, consisting of the analysis of 15 mycotoxins in cow milk [36]. Similarly, Haddad et al. developed a method to detect nine cyanotoxins, including a few MC congeners and NOD, in fish tissues [43]. The co-occurrence of different natural toxins together or in combination with other chemical contaminants has driven an increase in the interest in analytical methods addressing the simultaneous detection of various classes of analyte, commonly based on LC-MS techniques [44]. However, if a combined analysis of cyano- and myco-toxins is required, two separate analyses need to be performed, resulting in extra efforts and an increased time for analysis. Consequently, it is essential to develop multi-analyte methods that simultaneously detect different classes of toxins. Additionally, there was a lack of information concerning the potential synergistic and negative effects caused by contamination with diverse classes of toxins concurrently. For MCs and AFM1, both being hepatotoxic, this potential synergy could be even more harmful to consumers.

As dairy milk is regularly consumed daily all over the world, it is essential to develop a powerful method to detect multiple classes of toxins using one analysis, which is less described in the literature. The presence of aflatoxin in milk is due to dairy cows ingesting aflatoxin-contaminated feed. However, the presence of emerging cyanotoxins in dairy milk is also important to consider since dairy cows could drink water contaminated with these cyanotoxins. The current work aimed at developing and validating the first LC-MS/MS method for the simultaneous detection and quantification of nine cyanotoxins and one mycotoxin in milk.

2. Materials and Methods

2.1. Reagents and Chemicals

All the solvents used in the current study were of LC-MS grade. Acetonitrile (ACN) and formic acid (HCOOH 99%) were purchased from Biosolve (Biosolve, Valkenswaard, The Netherlands). Ultra-purified water (H_2O) was obtained from a Milli-Q system (Millipore, Overijse, Belgium). The resistivity and total organic carbon in H_2O were 18.2 ohms and 2–3 ppm, respectively. Analytical standards of eight MCs and NOD were obtained from Enzo Life Sciences® (Enzo Life Science, Antwerp, Belgium) as a solid powder and stock solutions were prepared in pure methanol (MeOH), and intermediate dilutions were prepared in a mixture MeOH:Milli-Q 50:50 (v/v) supplemented with 1% acetic acid. The eight MCs used in the study are MC-WR, MC-YR, MC-LW, MC-LY, MC-LR, MC-LA, MC-LF, and MC-RR (W = tryptophan, R = arginine, Y = tyrosine, L = leucine, A = alanine, F = phenylalanine). AFM1 and $^{13}C_{17}$ AFM1 were purchased by Sanbio as a solid powder (Sanbio, Uden, The Netherlands). AFM1 toxin stock and intermediate solutions were prepared in a mixture ACN:MeOH 50:50 (v/v), while the internal standard $^{13}C_{17}$ AFM1 stock and intermediate solutions were diluted in ACN. All stock and intermediate solutions were stored at −20 °C until further use.

2.2. Sample Preparation and SPE Clean-Up Phase

Target analytes were extracted from each milk sample (1 mL) by the addition of 3 mL of a mixture of 80% methanol (MeOH80%, v/v) and sonicated for 15 min (2510 Ultrasonic Cleaner, Branson, Danbury, CT, USA). Samples were incubated for 15 min in an overhead shaker (Reax 2, Heidolph, Schwabach, Germany). Thereafter, the samples were centrifuged for 15 min at 12,000$\times$ g (Sorvall Legend X1, Thermo Scientific, Waltham, MA, USA). The supernatants were recovered, and 26 mL of ultra-purified milli-Q water was added. Samples were then purified and concentrated by solid-phase extraction (i.e., SPE).

Hydrophobic bond Elut C18, containing silica sorbent, SPE cartridges (500 mg, 6 mL) from Agilent (Agilent, Machelen, Belgium), were conditioned with 6 mL of pure MeOH followed by 6 mL ultra-purified milli-Q water adjusted at pH 11. The samples were then loaded and passed through the SPE cartridges at a rate of 1 drop per sec. Afterwards, cartridges were vacuum dried for 5 min before the samples were eluted with 3 mL of MeOH80% into plastic tubes. Finally, samples were filtered through 0.2 µm RC-syringe filters (Phenomenex Inc., Utrecht, The Netherlands) into amber glass vials and subjected to LC-MS/MS analysis.

2.3. Matrix-Matched Calibration Curve

External calibration curves were prepared by serial dilution in MeOH80% to prepare solutions at concentrations ranging from 0.1 µg/L to 25 µg/L for MCs and NOD and from 0.005 µg/L to 25 µg/L for AFM1. An internal standard, $^{13}C_{17}$ AFM1, was added to our AFM1 solutions to reach a final concentration of 1 µg/L. For the preparation of the matrix-matched calibration curve, toxin standards were diluted in series in a blank matrix. The blank matrix represented a milk sample containing no detectable toxins and was treated similarly to other samples. The final mixture obtained after the extraction, clean up, and filtration steps were used for the dilution.

2.4. Operation of LC-MS/MS Instrument

Target analytes were detected and quantified using a XEVO TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionisation source operated in positive mode (ESI+). The initial separation of the toxins was performed with a Waters Acquity UPLC H-class (Waters) on a 1.7 µm, 2.1 mm $\times$ 100 mm Waters Acquity BEH C18 column preceded by a Waters Acquity BEH C18 1.7 µM VANGUARD PRE-Col (Waters).

The mobile phase comprised a milli-Q water phase (phase A) and an ACN phase (phase B). In both phases, A and B, 0.025% formic acid was added. The flow rate used was fixed at 0.5 mL/min, 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 fixed at 60 °C, the sample manager was at 10 °C, and the sample injection volume was 10 µL.

The MS parameters were set as follows: the source temperature was 150 °C, and the desolvation temperature was 450 °C. The capillary voltage was 1.0 kV. Cone and desolvation gas flows were set at 150 L/h and 1000 L/h, respectively. The collision gas flow was fixed at 0.15 mL/min, and the source offset was 50 V.

The MassLynx V4.2 SCN1040 (Waters©) software was used to operate and control the UHPLC-MS/MS instrument during the analysis, and toxin concentrations were calculated by the TargetLynx extension included in the MassLynx V4.2 (Waters©, Eten-Leur, The Netherlands) software based on dilution factors and the calibration curve, which was prepared in the blank matrix (i.e., sample extract containing no toxins) to correct the matrix effect. Additionally, quality control samples were added during each run to calculate the recovery of the analytes. The blank matrix sample was spiked with a mixture of all the toxin standards at a final concentration of 5 µg/L for each of the MC congeners, NOD and AFM1. For the internal standard, the concentration was 1 µg/L.

2.5. Method Validation Procedure

The validation study was performed using blank milk samples. Briefly, aliquots of blank milk were fortified with a toxin mixture of eight MC congeners as well as NOD and with AFM1 and $^{13}C_{17}$ AFM1. All the targeted cyanotoxins were spiked at 1, 5, and 10 µg/L, and AFM1 was spiked at 0.05, 1, and 10 µg/L. The internal standard, $^{13}C_{17}$ AFM1, was added to reach a final concentration of 1 µg/L. The procedure was conducted in triplicate and repeated on three different days. The following multiple parameters were evaluated to assess the method's validity: specificity, LOD, LOQ, recovery, reproducibility, repeatability, measurement uncertainty, linearity, and matrix effect.

The specificity of the method is stated as sufficient in case the quantifier ion and the qualifier ion were present during detection. In addition, the ion ratio adhered to the regulation (EU) 2021/808 [45]. Furthermore, no residual signal should be detected in the blank samples above 1% of the signal intensity at the lowest spike concentration.

The LOQ is defined as the lowest validated concentration and for which the signal-to-noise (S/N) ratio is $\geq$10, while the LOD is defined as the lowest point of the calibration curve, considering the dilution factor, with an S/N ratio $\geq$3.

The boundaries set for the apparent recovery lay between 70% and 120%. In addition, the limits for reproducibility and repeatability were based on the Horwitz ratio. Reproducibility, representing the variability of the method over three different days of analysis, was evaluated as the average variance of the validation results. Repeatability was calculated as the coefficient of variation (CV) of the validation results and represents the variability during one analysis day. The measurement uncertainty was calculated as double the CV, and the upper threshold value was 50%.

A Mandel's fitting test was used to determine the linearity of the standard curve at the concentrations mentioned above for target cyanotoxins and AFM1. Nevertheless, in case the R^2 value of the linear fit was equal to or higher than 0.98, a linear fit was used for quantification.

Moreover, for each target analyte, the matrix effect in the milk was determined. An external calibration curve was performed in solvent solution (MeOH80%) and compared with a matched matrix calibration curve (in the blank matrix). The slopes of the resulting curves were compared using Student's *t*-test, and a matrix effect was deduced when slopes were significantly different (t(b) > t(95%)).

3. Results

3.1. Optimisation of the LC-MS/MS Parameters

Concerning the eight MC congeners and NOD, their MS/MS parameters were previously optimised by our group, which are described in Van Hassel et al. [22]. A summary of the MS/MS parameters is also outlined in Table 1. Parameter optimisation for AFM1 and the internal standard $^{13}C_{17}$ AFM1 was performed at 1 µg/L using a syringe infusion pump and a positive ionisation mode. Ideal fragmentation conditions were determined by changing the cone voltage and collision energies. Four product ions were selected for both AFM1 and $^{13}C_{17}$ AFM1. After the optimisation of sample preparation and toxin detection, the two most intense transitions were further selected for quantitative and qualitative purposes, as depicted in Table 1.

Similarly, the LC column, gradient, and mobile phases were also previously optimised based on the publication by Van Hassel et al. [22]. These parameters were applied for AFM1 and $^{13}C_{17}$ AFM1 and selected for further analyses. The chromatograms obtained for the investigated toxin standards spiked into the milk blank matrix are shown in Figure 2.

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

Toxin	Precursor Ion (m/z)	Quantifier Ion (m/z)	Collision Energy (eV)	Cone Voltage (V)	Qualifier Ion (m/z)	Collision Energy (eV)	Cone Voltage (V)
MC-WR	1068.4	135.3	70	100	213.1	60	100
MC-YR	1045.5	135.3	80	60	212.9	60	60
MC-LW	1025.4	134.9	60	60	213.1	50	60
MC-LY	1002.4	135.3	60	50	213.0	50	50
MC-LR	995.4	135.0	70	80	213.1	60	80
MC-LF	986.3	135.0	60	70	213.1	60	70
MC-LA	910.3	135.1	60	50	107.1	80	50
MC-RR	519.8	134.8	30	50	107.2	60	50
NOD	825.25	134.9	50	80	102.7	90	80
AFM1	329.0	273.0	22	22	259.0	22	22
$^{13}C_{17}$ AFM1	346.0	288.1	22	46	242.1	38	46

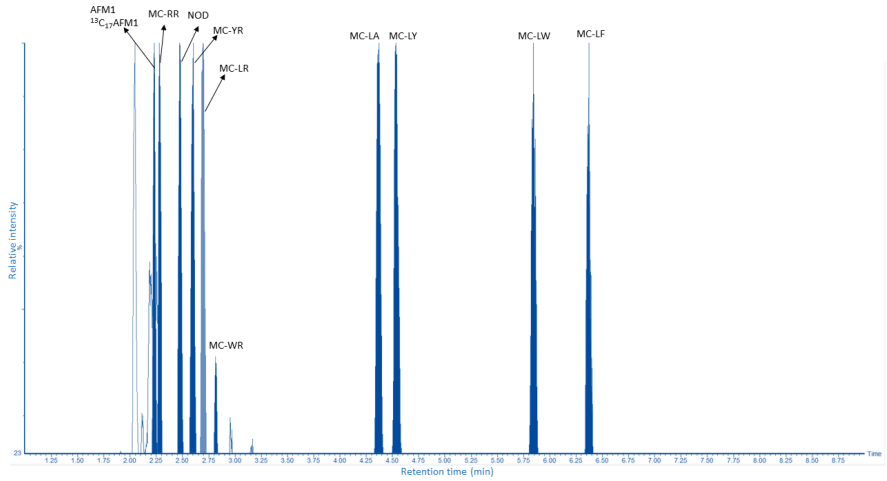


Figure 2. LC-MS/MS overlayed chromatograms for ten toxin standards spiked at 1 µg/L into a blank milk matrix.

3.2. Optimisation of Sample Preparation

Appropriate sample extraction is a prerequisite for the reliability of the analytical method. Therefore, the extraction conditions were optimised in a number of preliminary trials in order to select the best extraction protocol with a reduced sample treatment.

During the optimisation of the extraction process, its performance was evaluated by extraction yield experiments. Therefore, blank samples were spiked in triplicate, and recoveries were analysed. The protocol was based on one extraction step followed by a clean-up step by SPE. Milk is a complex matrix, and the components of milk (e.g., proteins, fats, sugars, and others) could cause problems during the extraction of the target analytes, explaining the need for a clean-up step. Finally, samples were filtrated before injection.

Firstly, based on the literature and physicochemical properties of the targeted analytes, two extraction solvents were tested. ACN is frequently used for mycotoxin extraction from milk [38–40], and MeOH80% was already used for cyanotoxin detection in drinking water and food supplements [22,32]. As highlighted in Table 2, the use of ACN as an extraction solvent resulted in lower recoveries compared with those obtained with MeOH80% as an extraction solvent. Effectively, recoveries for the different targets ranged between 49.5% and

89.3%, with ACN as the extraction solvent. In comparison, the use of MeOH80% resulted in recoveries between 87.7% and 113.3%.

Table 2. Comparison of the two extraction solvents for the treatment of milk samples. Three volumes of solvent were added to milk samples initially spiked with 10 µg/L of toxins. The recovery represents the mean of three biological replicates and is expressed as a percentage.

Extraction Solvent	Recovery (%)									
	MC-WR	MC-YR	MC-LW	MC-LY	MC-LR	MC-LF	MC-LA	MC-RR	NOD	AFM1
Acetonitrile	49.5	69.3	88.0	84.0	68.0	89.3	85.0	56.7	50.7	43.7
MeOH80%	107.0	111.3	103.0	100.0	109.0	113.3	90.3	113.3	99.0	87.7

Regarding the potential diversity in milk composition depending on the type of samples (e.g., raw, whole, skim, etc.), it was decided to add a purification step, even if recovery was acceptable with MeOH80%. Indeed, the milk composition may vary depending on the animal species, the genetic factors, the breed, and feed, and adding a clean-up step could lead to avoiding any matrix effect coming from different milk compositions and reducing matrix interference at lower concentrations.

For the clean-up step, three cartridges were tested and compared: Oasis HLB® (3 cc, 60 mg) from Waters, HybridSPE®-Phospholipid (6 cc, 500 mg) from Merck (Merck, Hoeilaart, Belgium), and Bond Elut C18 (6 cc, 500 mg) from Agilent (Agilent, Machelen, Belgium). The first observation highlighted the absence of cyanotoxin detection when HybridSPE® cartridges were used (Table 3).

Table 3. Recovery comparison for clean-up step optimisation by testing three different types of cartridges. Milk samples were spiked at 10 µg/L. The recovery represents the mean of three biological replicates and is expressed as a percentage.

Cartridge	Recovery (%)									
	MC-WR	MC-YR	MC-LW	MC-LY	MC-LR	MC-LF	MC-LA	MC-RR	NOD	AFM1
HLB	40.7	65.3	81.0	75.0	65.3	79.0	73.3	50.7	57.3	74.7
HybridSPE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	88.3
Bond Elut	49.3	69.3	88.0	84.0	68.0	89.3	85.0	50.7	56.7	43.7

An additional experiment based on spike samples before and after the clean-up step proved that these cartridges were inefficient for cyanotoxin purification in milk (Table 4). Effectively, when milk samples were spiked before the SPE step, no cyanotoxins were detected, but when the samples were spiked just after the clean-up step (i.e., before the filtration step on the syringe filter), toxins were detected, and recoveries were globally correct.

Table 4. Recovery was obtained by using hybridSPE-phospholipid cartridges from Merck for the clean-up step. Milk samples were spiked at 10 µg/L before or after the clean-up step, and recoveries were compared. The recovery represents the mean of three biological replicates and is expressed as a percentage.

Spike	Recovery (%)									
	MC-WR	MC-YR	MC-LW	MC-LY	MC-LR	MC-LF	MC-LA	MC-RR	NOD	AFM1
Before SPE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	102.0
After SPE	67.0	114.0	103.0	104.0	115.0	103.0	110.0	109.0	113.0	93.0

Concerning the two other cartridge types tested, the recoveries obtained exposed that the Bond Elut C18 cartridges were the best compromise between the target analytes for the clean-up step.

In parallel, the elution step during the SPE was also optimised by testing various methanol percentages. Firstly, a two-step elution was tested with 60% methanol (MeOH60%, *v*/*v*) followed by MeOH80%. In comparison, a two-step elution was tested with MeOH80% and pure MeOH. The results showed (Figure 3A) that the use of 3 mL of MeOH60% was not sufficient to elute all toxins from the cartridges as a few percentages of some MC congeners (e.g., MC-WR, MC-LW, MC-LY, MC-LR, MC-LF, and MC-LA) and NOD were detected in the second 3 mL elution fraction (MeOH80%) in different proportions.

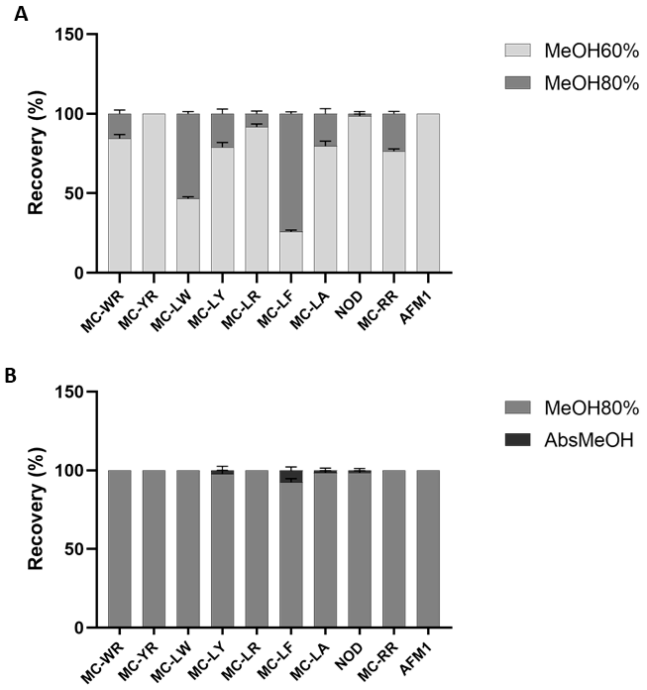


Figure 3. Bar charts comparing two optimisation protocols for the elution step during SPE. (**A**) Elution step with MeOH60% followed by MeOH80%. (**B**) Elution step with MeOH80% followed by pure MeOH. The clean-up step was performed on Bond Elut C18 cartridges (Agilent, Machelen, Belgium), and elution was achieved with 3 mL of solvent. Milk samples are spiked at 5 µg/L. The recovery represents the mean of three biological replicates ± SD and is expressed as a percentage.

Comparatively, the elution of the ten toxins with MeOH80% followed by a second elution with pure MeOH, led to an almost total recovery of the toxins in the first elution fraction (Figure 3B). Effectively, for six out of the ten toxins tested in this study, no toxin was detected in pure MeOH. Concerning the four other toxins, less than 10% were detected in the second elution fraction. Therefore, the combination of those results indicates that one elution step with 3 mL of MeOH80% was sufficient for a complete recovery.

3.3. Method Validation

The developed method was validated for the detection of 8 MCs, NOD, and AFM1 in milk according to the regulation (EU) 2021/808 [45].

3.3.1. Specificity

Blank samples did not present any toxin signals. All spiked samples showed signals for the quantifier and qualifier ions. In addition, the ion ratio between both was within the limits set by the European guidelines.

3.3.2. Linearity

The linearity was tested by evaluating the determination coefficient (R^2) on a seven-point calibration curve. It was estimated by a curve prepared in the blank matrix. A

determination coefficient was estimated for both quadratic and linear regression. For all toxins except MC-WR, R^2 values for the linear model were higher than 0.99.

An exception was observed with MC-WR, as its R^2 value was at 0.98 for both models. In similar methods, quantifying cyanotoxins and mycotoxins, the linear model was selected [28,31,32,46,47]. Therefore, this one was applied to all toxins in our study.

3.3.3. LOD and LOQ

The LOD was based on the lowest point of the calibration curve, considering the experiment's dilution factor. Moreover, the S/N value was ≥3, as described in the regulation (EU) 2021/808 [45]. Calibration curve concentrations were between 0.005 µg/L and 25 µg/L and 0.1 µg/L and 25 µg/L for AFM1 and the nine cyanotoxins, respectively. In this study, LOD was fixed at 0.3 µg/L and 0.015 µg/L for cyanotoxins (eight MCs and NOD) and AFM1, respectively. The LOQ for each toxin was determined as the lowest validated concentration. It was fixed at 1 µg/L and 0.05 µg/L for the nine cyanotoxins and AFM1, respectively. Moreover, S/N values were ≥10 for each analyte. All values are detailed in Table 5.

Table 5. Overview of results obtained during the validation. Various parameters are listed: recovery, repeatability, reproducibility, measurement uncertainty, S/N values, and the determination coefficient. All these parameters are evaluated at each concentration level for each toxin tested in this study.

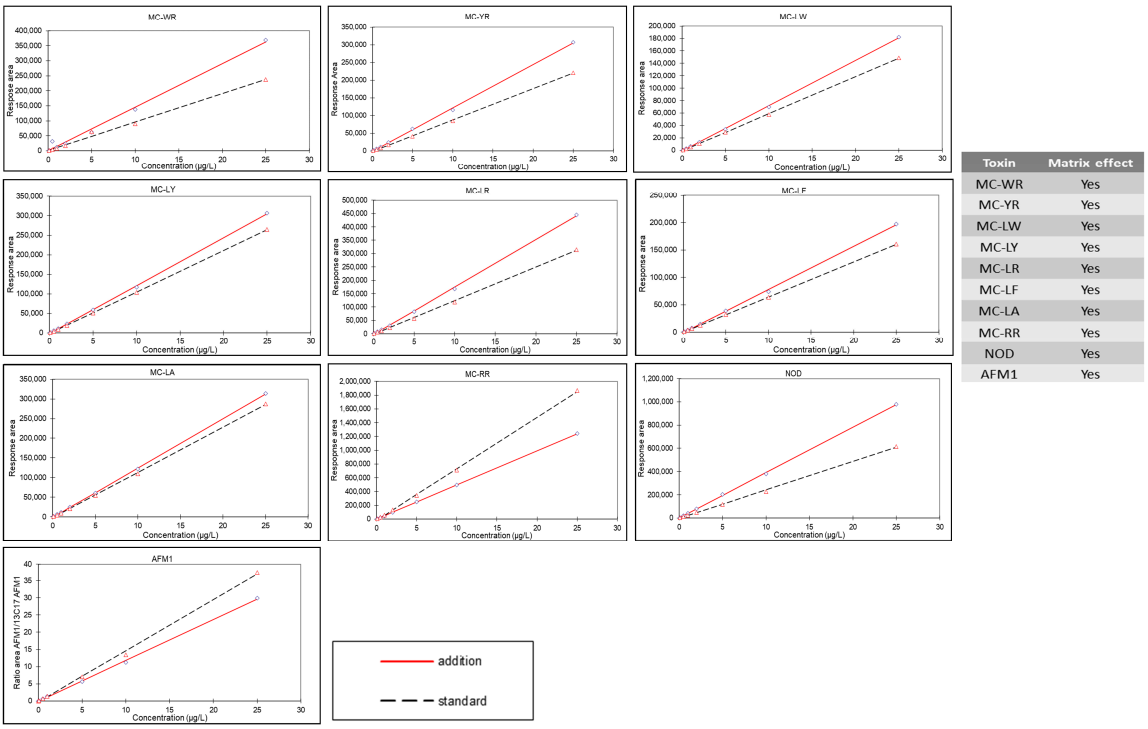
Toxin	Spiked Concentration (µg/L)	Recovery (%)	Repeatability (%)	Reproducibility (%)	Measurement Uncertainty (%)	Average S/N LOD	Average S/N LOQ	R^2
MC-WR	1	87.00	10.88	16.47	32.94	15.63	43.50	0.98
	5	71.00	6.52	9.47	18.94			
	10	107.00	12.56	13.24	26.48			
	Average	**88.33**	**9.99**	**13.06**	**26.12**			
MC-YR	1	104.00	9.03	14.86	29.71	13.38	36.35	0.99
	5	94.00	6.27	7.13	14.26			
	10	101.00	9.82	10.05	20.11			
	Average	**99.67**	**8.37**	**10.68**	**21.36**			
MC-LW	1	90.00	9.07	17.81	29.63	29.79	78.85	0.99
	5	75.00	3.06	6.07	12.13			
	10	91.00	13.54	14.54	29.08			
	Average	**85.33**	**8.56**	**12.81**	**27.90**			
MC-LY	1	91.00	10.35	18.54	37.07	19.34	56.56	0.99
	5	83.00	2.90	8.08	16.17			
	10	88.00	13.71	14.44	28.88			
	Average	**87.33**	**8.99**	**13.69**	**27.37**			
MC-LR	1	106.00	8.36	21.42	42.84	56.69	165.47	0.99
	5	91.00	5.93	5.93	11.86			
	10	104.00	8.89	9.10	18.20			
	Average	**100.33**	**7.73**	**12.15**	**24.30**			
MC-LF	1	88.00	9.30	20.91	41.83	23.58	73.48	0.99
	5	75.00	4.35	8.76	17.51			
	10	92.00	14.51	16.05	32.09			
	Average	**85.00**	**9.33**	**15.24**	**30.48**			
MC-LA	1	82.00	7.02	8.44	16.88	33.38	73.77	0.99
	5	82.00	3.72	8.44	16.88			
	10	84.00	14.50	16.54	33.08			
	Average	**82.67**	**8.41**	**11.14**	**22.28**			

Table 5. *Cont.*

Toxin	Spiked Concentration (µg/L)	Recovery (%)	Repeatability (%)	Reproducibility (%)	Measurement Uncertainty (%)	Average S/N LOD	Average S/N LOQ	R^2
MC-RR	1	102.00	6.52	16.30	32.61	100.93	351.29	0.99
	5	88.00	3.99	11.80	23.60			
	10	91.00	7.19	7.19	14.38			
	Average	**93.67**	**5.90**	**11.76**	**23.53**			
NOD	1	110.00	7.42	19.87	39.74	145.25	493.49	0.99
	5	101.00	7.04	10.48	20.96			
	10	95.00	7.48	7.48	14.97			
	Average	**102.00**	**7.31**	**12.61**	**25.22**			
AFM1	0.05	118.00	14.65	15.03	30.06	4.38	12.53	0.99
	1	84.00	5.58	13.48	26.97			
	10	93.00	10.55	14.72	29.44			
	Average	**98.33**	**10.26**	**14.41**	**28.82**			

3.3.4. Matrix Effect

A matrix effect was observed by comparing a mix of toxins in the milk matrix and in the solvent. The results are depicted by the difference in slopes between the two calibration curves (Figure 4). Moreover, these slopes were statistically analysed by Student's *t*-test (Table 6). The average values measured over three different days of validation indicate that a matrix effect was detected. Therefore, the calibration curves of our milk blank matrix reduced the matrix effect and potential bias for the quantification results.



Toxin	Matrix effect
MC-WR	Yes
MC-YR	Yes
MC-LW	Yes
MC-LY	Yes
MC-LR	Yes
MC-LF	Yes
MC-LA	Yes
MC-RR	Yes
NOD	Yes
AFM1	Yes

Figure 4. Matrix effect analyses in milk matrix. Comparison of the response area of the calibration curve in solvent (i.e., standard) to a calibration curve in the blank matrix (i.e., addition) and the statistical analysis by Student's *t*-test. Concentrations for the calibration curves range from 0.1 µg/L to 25 µg/L for MCs and NOD and from 0.005 µg/L to 25 µg/L for AFM1. An internal standard, $^{13}C_{17}$ AFM1, was added to our AFM1 solutions to reach a final concentration of 1 µg/L. The table sums up the potential presence of a matrix effect for each of the ten toxins.

Table 6. Evaluation of a potential matrix effect. T(b) values were calculated and compared with the tabulated t at the 95% confidence level (2.23). If t(b) is higher than t(95%), a matrix effect is present. The t(b) value compares the slope of the standard (i.e., in MeOH80%) and the addition curve (i.e., in the milk blank matrix).

Toxin	MC-WR	MC-YR	MC-LW	MC-LY	MC-LR	MC-LF	MC-LA	MC-RR	NOD	AFM1
t(b) DAY 1	7.26	23.19	2.39	2.07	22.48	15.68	2.01	37.11	34.03	2.23
t(b) DAY 2	16.34	30.76	18.32	15.36	20.86	14.87	8.47	24.62	54.8	1.2
t(b) DAY 3	4.21	25.92	5.30	18.33	28.73	8.86	47.35	12.27	110.6	9.58
Average	**9.27**	**26.62**	**8.67**	**11.92**	**24.02**	**13.14**	**19.28**	**24.67**	**66.48**	**4.34**

3.3.5. Apparent Recoveries

The apparent recoveries were calculated individually at three different concentrations for the nine cyanotoxins and AFM1, as MCs and NOD were analysed at 1, 5, and 10 µg/L and AFM1 was analysed at 0.05, 1, and 10 µg/L. The internal standard $^{13}C_{17}$ AFM1 was spiked at 1 µg/L. Values are described in Table 5. The average recoveries were measured between 82.67% and 102.00% and were in good agreement with the range selected (i.e., 70–120%).

3.3.6. Reproducibility, Repeatability, and Uncertainty Measurement

The Horwitz ratio determined a maximum value of 14.70% for the average variance (i.e., repeatability) and 22.00% for the coefficient of variation (CV) (i.e., reproducibility). These parameters were calculated for each toxin at each concentration level, and the values were below their respective threshold, as detailed in Table 5. Therefore, the method was repeatable and accurate.

Finally, the average measurement's uncertainty values for all the toxins at all spiked levels were between 21.36% and 30.48% (Table 5). Judging from the results of this detailed validation, the method is suitable for the simultaneous detection of eight MCs, NOD, and AFM1.

4. Discussion

Toxin-free milk is fundamental to human health due to its daily consumption all over the world. In food matrices, multiple toxins can contaminate. Therefore, a European directive determined a guideline value of 1 µg/L for MC-LR in drinking water [21]. For AFM1, the European Commission fixed the maximal value at 0.05 µg/kg and 0.025 µg/kg for adults and infants, respectively [12].

Our method was based on one extraction step with MeOH80%, followed by a purification step through C18 cartridges. The LOQ was fixed at 1 µg/L and 0.05 µg/L for the nine cyanotoxins and AFM1, respectively. The LOQ at 1 µg/L was acceptable for MCs and NOD as this concentration represented the limit determined for drinking water by the WHO. Moreover, for children over three years old and adults, milk consumption decreases and becomes lower compared to water. In this case, the potential guideline value would be higher than the value for drinking water. Effectively, for babies over six months, with dietary diversification, the need for milk gradually decreases to reach around 500 mL until three years old. Then, the need will continue to be reduced compared with water [48].

This study describes the first method to extract and detect cyanotoxins in a milk matrix. Therefore, the comparison with other studies and methods is not easily feasible. Effectively, few results are available on MC detection in livestock or related products, and no data remains for European countries [23,49]. One study analysed milk from Australian cattle that drank water contaminated with *Microcystis aeruginosa*. HPLC and ELISA analyses revealed the absence of MCs in the milk [49]. In 2003, a second study highlighted the absence of MC-LR in cow milk, but MCs were administered to cows by gavage as freeze-dried cells containing a known amount of MCs [50]. However, numerous parameters can impact this type of large-scale experiment, such as the metabolism or the age of cows, the

toxin concentration, and the duration of the exposure. Additionally, these analyses did not take into account the MC congeners in the analytical method and methods were not clearly validated.

The obtained LOQ for AFM1, 0.05 µg/L, was equivalent to the maximal value for adults fixed by the European Commission. However, we were not able to reach a lower LOQ because of the matrix interferences at lower concentrations. AFM1 is mainly studied for its potential presence in milk and dairy products, and LOQ values obtained by different authors cannot often be compared due to the different procedures and criteria used [7,10,36,46,51]. Taking into account all described detection methods, the method with the lowest LOQ was obtained by ELISA and the value was fixed at 0.0028 µg/kg [52]. When focusing on procedures using LC-MS/MS analysis with the same type of equipment as used during this study (triple quadrupole), the LOQ values were between 0.01 and 0.05 µg/kg [36,53–56]. The variability of the extraction and purification steps in all these methods proved the importance of careful processing on the final result and the difficulties of comparing procedures to each other. Even if, for LC-MS/MS-based methods, LOQ values were higher than values obtained by ELISA, it is important to note that the first-mentioned approaches targeted multiple mycotoxins simultaneously. Similarly, a higher LOQ could be expected when developing a multimethod for AFM1 and cyanotoxins in comparison with ELISA or single-target instrumental methods.

The LOD was fixed at 0.3 µg/L and 0.015 µg/L for the examined cyanotoxins and for AFM1, respectively, representing the lowest point of the calibration curves, concentrations at which we assessed a correct S/N ratio. There was no need to optimise those values as the LOD obtained here was sufficient to swiftly evaluate public health.

Many factors can impact the efficiency of a new LC-MS/MS detection method, and the situation is still problematic when complex matrices are involved. Even if the detection technique used is fundamental, it is one of the last steps of the analytical chain; consequently, sample treatment is equally (if not more) important as it will affect the final result. Among the literature, there is a huge variability of methods used for the extraction and purification of the selected toxins in multiple matrices [1,40,57,58].

The first step, the extraction of toxins from the matrix, is usually based on the use of an organic polar solvent, such as ACN, acetone, or MeOH, as reviewed by Massey et al. [39]. Commonly, MCs and NOD are extracted from food matrices using MeOH at variable percentages [22,32,47,59]. Aflatoxins, however, are generally extracted using ACN [35,36,46,55]. In our case, a mix of MeOH:H_2O 80:20 (v/v) represented the best option for simultaneous extraction of both types of toxins.

Milk is a complex matrix rich in proteins and lipids, explaining the importance of a purification step before injecting samples for LC-MS/MS analysis. The inclusion of clean-up steps in the analysis of cyano- or myco-toxins has been widely reported, but mainly to reduce their interferences in matrix studies. However, this also creates an inaccuracy about the actual toxin(s) present in the matrix through losses during processing, whereby some are overlooked. Therefore, our study highlights the importance of an optimised clean-up procedure that overlooks nothing. Our study revealed that hybridSPE cartridges from Merck were not adapted for MCs and NOD in a milk matrix. We decided to test this column among others because of their specificities to remove endogenous protein and phospholipid interferences, which are highly represented in milk. However, only AFM1 was recovered. In the literature, few studies are using this type of cartridge for food matrix studies. Effectively, two studies highlighted the efficiency of hybridSPE for the analysis of the Human breast milk metabolome [60] and lipidome [61]. Little information is available concerning their use for the specific extraction of cyano- or myco-toxins. One study, by Altaner et al., tested these cartridges for the isolation of MCs in tissue samples, but their results were inconclusive [28]. In this study, they suggest using Waters Oasis HLB columns.

HLB Oasis (Waters) and C18 cartridges (Agilent, Machelen, Belgium) were selected for comparison because of their common use in numerous methods focusing on MCs or aflatoxins extracted from various food matrices. For example, Xie and Park [62] selected HLB cartridges to purify MC-LR and MC-RR from fish. However, the extraction was

performed with a mix of butanol:MeOH:H_2O, the detection of toxins was performed using HPLC (not LC-MS/MS), and their LOQ was determined to be 3.1 µg/g. Another study showed the efficiency of HLB cartridges for the detection of MC-LR and MC-RR in fish, vegetables, and soil sediments, in which matrix-specific extraction solvents were suggested, and the lowest spiked concentration was 0.25 µg/L [27]. HLB cartridges were found to be better for MC congener extraction from various vegetables, including eggplant and cucumber, indicating that the matrix effect on extraction protocol is meaningful [33]. However, the efficiency of C18 cartridges works well for the isolation of several MC congeners in mussels and fish samples, where methanol at a concentration from 70% to 80% was used as the extraction solvent [63,64]. AFM1 is investigated because of its potential to contaminate various milk and dairy products, and a broad range of purification techniques are applied. The most popular one is the use of an immunoaffinity column for the purification step, followed by HPLC-FD analysis [65–69]. Although procedures involving purification with C18 or HLB SPE cartridges have also been conducted [46,47,51]. All these procedures were based on the use of ACN as the extraction solvent, an additional evaporation step, and the resuspension of the extract in water prior to the purification step by SPE.

The available publications available for LC-MS/MS techniques for food/feed studies are extensive. For most, if not all, the need to reach low levels of LOQ and improve performance drives all procedural development. It is sometimes difficult to compare procedures because of the variability in parameters among methods. Nevertheless, there is a lack of studies on the concurrent detection of multiple toxins from different origins. This study developed and validated a method for simultaneous LC-MS/MS detection of nine cyanotoxins and AFM1 in the milk matrix with LOQ values close to thresholds defined by WHO and the European Commission.

5. Conclusions

Across the globe, milk has an overwhelmingly positive reputation among consumers because of its numerous health and nutritional benefits. Moreover, elevated temperature and climate changes indirectly influence milk production and quality as a consequence of shifts in the availability and quality of feed and water. Therefore, milk can be contaminated with a wide array of contaminants. While the carryover of some of these toxins, such as AFM1, into milk is an established fact, the presence of other emerging natural contaminants has not been confirmed or extensively studied yet. A 2016 report by the EFSA shared definitive conclusions regarding the potential transfer of MCs into milk, which cannot be accurately assessed, emphasizing the need for more precise analytical methods. With this method, LOQ values range from 0.05 to 1 µg/L and LOD values are comprised between 0.015 and 0.03 µg/L. This is the first work describing a validated LC-MS/MS-based method that can be widely used for accurately analysing the natural (co-)occurrence of multiple MCs as well as NOD and AFM1 in dairy milk samples.

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