FAST LC-MS DETECTION OF CYANOBACTERIAL PEPTIDE HEPATOTOXINS – METHOD DEVELOPMENT FOR DETERMINATION OF TOTAL CONTAMINATION LEVELS IN BIOLOGICAL MATERIALS

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Helille.
Koska olen kiitollinen niin paljosta.

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A. List of original publications:

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Vesa O. Sipiä, Milla-Riina Neffling, James S. Metcalf, Sonja M.K. Nybom, Jussi A.O. Meriluoto, Geoffrey A. Codd
Nodularin in feathers and liver of eiders (*Somateria mollissima*) caught from the western Gulf of Finland in June–September 2005
Harmful Algae, Volume 7, Issue 1, 2008, pp. 99-105

II.
Milla-Riina Neffling, Emilie Lance, Jussi Meriluoto
Detection of free and covalently bound microcystins in animal tissues by liquid chromatography–tandem mass spectrometry
Environmental Pollution, Volume 158, Issue 3, 2010 pp. 948-952

III.
Emilie Lance, Milla-Riina Neffling, Claudia Gérard, Jussi Meriluoto, Myriam Bormans
Accumulation of free and covalently bound microcystins in tissues of *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacteria or dissolved microcystin-LR exposure
Environmental Pollution, Volume 158, Issue 3, 2010 pp. 674-680

IV.
Milla-Riina Neffling, Lisa Spoof, Jussi Meriluoto
Rapid LC–MS detection of cyanobacterial hepatotoxins microcystins and nodularins—Comparison of columns
Analytica Chimica Acta, Volume 653, Issue 2, 2009, pp. 234-241

V.
Milla-Riina Neffling, Lisa Spoof, Michael Quilliam, Jussi Meriluoto
LC-ESI-Q-TOF-MS for faster and accurate determination of microcystins and nodularins in serum
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B. Contribution of the author

The work was designed, conducted and manuscripts prepared by Milla-Riina Neffling, Biochemistry, Department of Biosciences, Åbo Akademi University, in Turku, under the supervision of Dr. Jussi Meriluoto and with help from Dr. Lisa Spoof, with the following exceptions:

I. The work was initiated and the manuscript prepared by Dr. Vesa Sipiä with the help from M-R Neffling and Dr. J. Meriluoto. ELISA assays for the toxins in feathers were performed at the University of Dundee, at the laboratory of Prof. Geoffrey A. Codd. Contamination levels in the mussel tissues were determined by Sonja Nybom.

II. Experimental laboratory work was conducted together with Dr. Emilie Lance

III. Dr. Emilie Lance designed and conducted the experimental biological work (snail exposure), experimental analytical and data-processing work was conducted by M-R Neffling, together with Dr. Emilie Lance. The analytical methods and experimental work designed and performed in Turku. The interpretation of the biological results and manuscript writing was done by Dr. Emilie Lance with the help from M-R Neffling.

IV. Purified toxins were provided by Dr. Lisa Spoof.

V. The first part, choosing a suitable analytical HPLC column, was designed and conducted in Canada, at the Institute of Marine Biosciences, National Research Council Canada, under the supervision of Dr. Michael A. Quilliam. The second part was designed and conducted in Turku. Purified toxins were provided by Dr. Lisa Spoof.

C. Other publications

Publications which were not included into the PhD thesis, but in which a co-author position obtained during the PhD. studies

Lisa Spoof, Milla-Riina Neffling, Jussi Meriluoto
Separation of microcystins and nodularins by ultra-performance liquid chromatography
Journal of Chromatography B, Volume 877, Issue 30, 2009, pp. 3822-3830

Lisa Spoof, Milla-Riina Neffling, Jussi Meriluoto
Fast separation of microcystins and nodularins on narrow-bore reversed-phase columns coupled to a conventional HPLC system
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Milla-Riina Neffling

E. Abbreviations

4-PB	4-phenylbutyric acid
5-PP	5-phenylpentanoic acid
A (Ala)	Alanine
AcN	Acetonitrile
AcO	Acetyl
Adda	(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid
AdmAdda	AcetyldemethylAdda
BMAA	β-N-methylamino-L-alanine
BuOH	Buthanol
C (Cys)	Cysteine
CID	Collision induced dissociation
D (Asp)	Aspartic acid
Da	Dalton
DAD	Diode-array detector
DC	Direct current
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
dm	demethyl (e.g. in dmMC, demethyl-microcystin)
dmAdda	demethylAdda
DW	Dry weight
E (Glu)	Glutamic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
F (Phe)	Phenylalanine
FAIMS	Field-asymmetric waveform ion mobility mass spectrometry
FT	Fourier transform
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometer
FWHM	Full width at half maximum
G (Gly)	Glycine
GC	Gas chromatography
hI	Homoisoleucine
HOAc	Acetic acid
HPLC	High-performance liquid chromatography
hR	Homoarginine
IC_{50}	Compound concentration that gives 50% inhibition on enzyme activity
I.D.	Inner diameter (of an HPLC column)
IM-MS	Ion mobility-mass spectrometry
L (Leu)	Leucine
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LD_{50}	Dose that is lethal to 50% of the test animals
LOD	Limit of detection
LOQ	Limit of quantitation
M (Met)	Methionine
MALDI	Matrix-assisted laser desorption/ionisation
MC	Microcystin
MC-LR eq/l	Microcystin-LR equivalents / liter (used in UV-HPLC detection)
Mdha	N-methyldehydroalanine
MeOH	Methanol
MMPB	2-methyl-3-methoxy-4-phenylbutyric acid

MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MS^n	Sequential mass spectrometry fragmentations (n rounds)
MRM/ SRM	Multiple reactant monitoring/ Single reactant monitoring
m/z	Mass to charge ratio
NMeSer	N-methylserine
Nod	Nodularin
P (Phe)	Phenylalanine
PP	Protein phosphatase
PPIA	Protein phosphatase inhibiton assay
Q	Quadrupole mass analyser (filter)
QQQ	Triple quadrupole mass analyser
R (Arg)	Arginine
RF	Radio frequency
RP	Reversed-phase
Rs	Resolution
S (Ser)	Serine
SIM/ SIR	Single ion monitoring/ single ion recording
SPE	Solid-phase extraction
S/N	Signal to noise –ratio
TOF	Time-of-flight
UHPLC	Ultra high-performance liquid chromatography
UPLC	Waters Corporation's registered trademark for Ultra-Performance Liquid Chromatography
UV	Ultraviolet
V (Val)	Valine
W (Trp)	Tryptophan
Y (Tyr)	Tyrosine
Å	Ångström (10^{-10} m, 0.1 nm)

1. Introduction and review of the literature

Liquid chromatography – mass spectrometry

Liquid chromatography - mass spectrometry (LC-MS) is a powerful analytical tool. It is based on the separation of compounds of interest by high-performance liquid chromatography, ionisation of the sample analytes in an ionisation source, and analyte detection by mass spectrometry. Mass spectrometer is a very universal, or generic, detection method, but still a highly selective one.
The current applications for LC-MS vary from purely chemical analysis of small molecules, such as reaction and synthesis products and drug molecules to analysis of different types of biological macromolecules. The instrumentation is used in elemental analysis, petrol and pharmaceutical industry, food safety analysis, clinical diagnostics, environmental analysis and in analysis of biological systems with applications such as proteomics and metabolomics, etc.

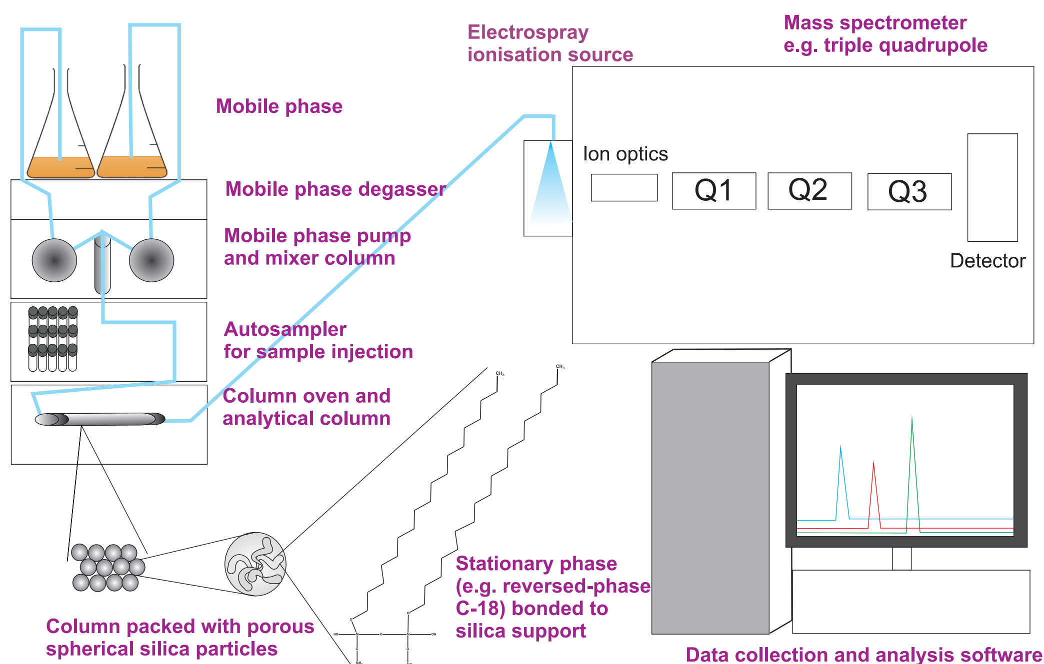

Figure 1.1
Schematic presentation of a LC-ESI-MS-MS instrumentation. The HPLC parts include the mobile phase reservoirs, degasser, pump with mixing chamber, automatic sampling system (autosampler) and analytical column in a thermostated column oven. The column is usually packed with spherical porous silica particles that have been covered, or bonded with the stationary phase material (e.g. reversed-phase C-18 material). The flow from analytical column is then directed to the ionisation source (electrospray) of the detector MS (e.g. a triple quadrupole) where the separated analytes give rise to signals that are recorded and later on processed with data-analysis software.

The general concept of liquid chromatography - mass spectrometry is separation of compounds based on different physico-chemical properties and detection based on ionic mass of the analytes. The wide applicability brings together experts from different fields, such as physics, chemistry, engineering, biology, medicine, pharmacy, etc. The field of mass spectrometry is highly dynamic, as it is continuously driven forward by different developments from different fields of science to improve various applications.
The current trend is instrumentation to meet the requirements of higher speed (higher throughput) without compromising on sensitivity, accuracy and stability. Also data-analysis software has been improved for more efficient data-mining.

1.1. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) separates compounds based on the interaction and physico-chemical preference of the analyte between stationary phase and mobile phase.
The analytes are driven further within the column packed with particles with the help of liquid mobile phase, which is pumped through the column with high pressure. The compounds interacting more strongly with the stationary phase than with the mobile phase will be retained in the column for longer. The interaction between the stationary phase and the analyte is determined by the material the porous particles are covered with, called bonded phase, as well as with the properties of the mobile phase. The stationary phase can be hydrophilic (e.g. silica surface in normal phase chromatography with increasing gradient of polar solvent in the mobile phase) or hydrophobic (reversed-phase chromatography with increasing gradient of organic solvent in the mobile phase). More specifically, the bonded phase material can be alkene chains of different length, chains of carbon and heteroatoms, specific interacting groups (phenyl rings, chiral residues etc.), ion exchange material etc. [1].

Recent developments in HPLC aim at faster separation and better sensitivity and include higher-pressure pumps (called ultra high-performance liquid chromatography, UHPLC or UPLC) [2-5] that allow the use of small particle size (2 µm or less in diameter) columns with high flow rates. Earlier the typical pressure limit for a HPLC system was around 400 bars, whereas now the limit in instruments provided by many different manufacturers (such as Waters Ultra-Performance Liquid Chromatography system) is around 1000 bars.
HPLC instrumentation consists of mobile phase reservoirs, pump (with gradient mixer), autosampler with injector, thermostated column compartment, an analytical column and detector (see figure 1.1). All of these different parts need to be connected for the mobile phase flow, and create the total system volume. The current developments aim for reduced total system volume and higher-pressure tolerance to obtain faster and more sensitive analysis methods.
Also the autosamplers for sample injection have become faster and the dead volumes inside the pumps have been decreased into µl range. All of these improvements aim for faster throughput and higher efficiency.
The recent developments have improved the throughput, the separation and therefore resulted in faster and more sensitive analysis enabling the analysis of complex sample within a single run [4].

The separated analytes can be detected by different means, for example by fluorescence, (UV-) light absorption, light scattering, electric conductivity or by mass spectrometry. With UV-detection, the absorbance can be specified to a certain wavelength, depending on the absorbance spectra of the analyte of interest. This detection method therefore gives two levels of information, retention time and absorbance at a specified wavelength. Further verification can be obtained by inspecting the absorbance spectra obtained by a diode array detector (DAD).

1.1.1. Different modes of separation and the effect of particle and pore size

HPLC can be operated on several different modes of separation: in addition to normal and reversed-phase chromatography, there are ion exchange, hydrophilic interaction, chiral, aromatic (π-π) interaction, other specific functional group interactions, but also size exclusion and immunoaffinity separation modes [1]. The method for separation is therefore very versatile. In general, one aims for well-resolved and narrow HPLC peaks with high signal response.

Van Deemter equation

$H = A + (B/u) + (Cu)$

H = HETP (Height equivalent of theoretical plates) (The lower value indicates higher efficiency)
u = Mobile phase linear velocity
A, B, and C are constants which account for contributions to band broadening
A = Eddy diffusion term (e.g. directly proportional to the particle size, due to the presence of stationary phase particles and therefore different flow channels/ paths for the molecules)
B = Longitudinal diffusion term (depending on viscosity etc. of the mobile phase)
C = Resistance to mass transfer coefficient (takes into account the diffusion in the mobile phase and in the stationary phase. Affected by parameters such as particle size, mobile phase viscosity and temperature)

The van Deemter equation states that with particle size below 2 μm the efficiency does not significantly diminish with higher flow rates [6]. The smaller particle size allows tighter packing of the columns, which produces higher backpressures. On the other hand, even higher flow rates can be used since the tighter packing decreases the interparticle (outside, between particles) diffusion as well as the diffusion of analytes between stationary and mobile phase (mass transfer).
Higher flow rates enable the gradients and compounds to be pushed through more efficiently, and result in narrower and higher peaks. Another parameter that is beneficial in reducing mass transfer is elevated temperature in the analysis [6] due to reduction in mobile phase viscosity. Nowadays temperatures up to 90°C are used, whereas earlier the highest recommended temperature for silica based column particles was 40°C. Conventional silica-based particles may in high temperatures undergo hydrolysis of the Si-O-Si bond that binds the bonded phase on the silica support [1]. Furthermore, the column material is made more tolerant against phase collapse towards high percentage of aqueous solvent by for example endcapping chemistries. Endcapping also reduces unwanted analyte interactions

with the silica support. In small particle size columns the interaction surface is increased since particle packing is more efficient. This way the chromatographic performance is better, the efficiency higher, the peaks sharper and higher, even with short, steep gradients and short run times.
Already there is a number of reports on utilising UPLC in multiple types of separation analysis [7].

1.1.2. Chromatographic performance

The chromatographic performance of a column can be assessed with several different parameters, including chromatographic peak width and height, resolution, selectivity and signal to noise ratios.

Peak width is a parameter describing the peak width in time it takes for the peak to elute. It is often described as peak width at half maximum (full width at half maximum, FWHM). The peak height is described as signal abundance (counts on detector, i.e. absorbance abundance or abundance of ions hitting the detector plate in MS). Peak symmetry is important in the aspect of column performance, and for example for integration. Most common problems in peak symmetry are peak tailing or fronting, which makes it more difficult to determine the end or the start of the signal peak. Asymmetry is indicative of poor analyte elution from the column and of inhomogeneous interaction with the stationary phase. It may also be caused by too high sample volume or mass that exceeds the loading capacity of the column.

Resolution (Rs) is a measure of how well two analytes are separated from each other.
Resolution is calculated with the equation

$$Rs = [1.18(t_2 - t_1)]/[W_{0.5,1} + W_{0.5,2}]$$

where t_1 and t_2 correspond to compounds' retention times and the $W_{0.5,1}$ and $W_{0.5,2}$ correspond to the peak widths at half maximum for the respective peaks. Resolution value 1 corresponds to 2% overlap of the analyte peaks (in case of Gaussian shaped peaks)[8], baseline resolution is achieved at resolution values of 1.5 or higher.

Selectivity (a) describes the spread of the analytes across the chromatogram. The value is calculated with the help of retention factors (k´), measure of retention of analytes in relation to unretained ($t_R = t_0$) peak. Selectivity is the ratio between the retention factor of a later eluting analyte of interest (k'_B) and the retention factor of an earlier eluting analyte of interest (k'_A).

Retention factor: $k'_A = (t_{R,A} - t_0)/t_0$

Selectivity: $a = k'_B / k'_A$

A suitable retention factor for an analyte is commonly considered to be between 2 to 10, to be certain that the analyte does not elute too early and that column and

the gradient in use are not retaining the analyte for an unnecessary long time. If the retention factor is too low, the analyte may elute too close to t_0, which means that the peak has not been retained in the column. This indicates that no separation from eventual disturbing sample components occurs.
Selectivity values can be compared for the assessment of gradient functionality, i.e. how the selectivity values change when changing the gradient steepness, mobile phase or column.

Signal to noise assessment in chromatography gives the ratio of the signal intensity of a peak divided by the signal intensity of the same signal outside the peak area, the background (at a random range of time). With signal to noise one correlates the peak height into the background noise level, and in this way is able to evaluate limit of detection (S/N 3) and limit of quantitation (S/N 10). This measure is often used for overall measure of the peak detection sensitivity and reliability.
The above mentioned parameters are used for method developed for better separation and detection limits [1].

1.1.3. Separation of peptides by HPLC

The principles of peptide separation have been extensively studied by many groups throughout the years (e.g. [9]). The retention of peptides is dependent on the properties of the analytes, the peptides, such as hydrophobities, charge states and sterical effects of the constituting amino acids [9].
Peptide separation has been given a lot attention during the years, [10], especially since the emerge of proteomics applications with MS detection, and therein the shotgun method, where proteins are enzymatically (e.g. with trypsin) digested into peptides and the peptides detected and related to the parent proteins [11]. The expected retention times are considered important as the identification of the detected tryptic peptides can be strengthened by the theoretical estimation of the peptide's retention time. The comparison of the retention times can be used to add certainty in the analysis.
The most common separation mode for peptides is the reversed-phase (RP) chromatography on hydrophobic C-18 material. Due to the charged and hydrophilic residues and the N- and C-terminus, the peptides elute with low concentrations of organic modifier. For the mobile phase, acetonitrile has been found to be superior over other organic solvents such as methanol [9] due to lower viscosity, and therefore more effective mass transfer. Trifluoroacetic acid as a pH modifier has benefits in separation and detection due to effective ion pairing and low light absorbance [12]. However, trifluoroacetic acid is not the best option for MS detection due to impaired ionisation caused by ion pairing.

1.2. Mass Spectrometry

In mass spectrometry (MS) a compound is detected based on its mass to charge ratio (*m/z*). Mass for a given compound is specific – it is the sum of the masses of the atoms that constitute the molecule, or in the case of MS, the ion. MS can be used for both qualitative and quantitative work. Qualitative, identification and structural elucidation of a compound, can be achieved by looking at the analyte mass, preferentially accurate mass, and fragmentation patterns. Quantitation can be achieved by relating the obtained signal abundance with an abundance created by known concentration of the analyte.
The major obstacle in mass spectrometry was for long the ionisation process. An MS instrument cannot process or detect nonionised molecules. The MS detector needs to receive ions in gas phase, that is, not in physical contact with solvent molecules. Previously only small organic compounds that easily evaporated could be separated with gas chromatography and analysed by mass spectrometer. Especially biological macromolecules are not easy to get into gas phase. The developments in the ionisation have brought mass spectrometry into field of biochemistry and biology.

In MS there are parameters that assess the analytical quality, such as mass resolution, mass accuracy, signal to noise ratio, linearity on a dynamic range, quantitational accuracy and precision, sensitivity and selectivity (assessing probability for false positives or negatives).

All of the above mentioned parameters are more or less dependent on the sample matrix, which is all the other compounds than the analyte itself in a sample. The matrix may interfere with analyte detection (be isobaric, i.e. compounds with identical *m/z* –ratios), by affecting analyte ionisation (ion suppression or enhancement) or by simply giving too strong of a signal that masks the detector from the lower abundance analyte responses (mainly a concern in the ion trap instruments).

LC-MS in general is a selective detection method, where levels of certainty, or, selectivity, can be added by defining specific fragments of a precursor ion to be monitored, by looking at a specific collisionally induced fragment of the analyte of interest (tandem mass spectrometry). The other way is to look at a narrow *m/z* range (10-20 mDa) for the analyte. Both of these means greatly improve the selectivity of the method by reducing the probability of false positives and therefore giving further benefits in quantitative studies.

In low-resolution instruments, in quadrupoles and triple quadrupoles, as well as in spherical and linear ion traps, the resolution is often defined as Δm, FWHM or as the m_2-m_1, where m_2 and m_1 are separated by 50% valley of the peak height. The resolution therefore refers to a small number, 1 or less. The resolution in these above-mentioned instruments is often 1 *m/z* unit across the detected *m/z* range. Therefore these instruments are often referred to as unit resolution instruments.
Mass resolving power is often defined by m/Δm, where Δm is defined as the peak width at half height (Full width at half maximum, FWHM), or where Δm is defined

as the m_2-m_1, where m_2 and m_1 are separated by 50% valley of the peak height. Therefore the value for resolving power is always a large number.
Good mass resolution is required for the elucidation and verification of molecular formula of an ion. It is also used for determining the ion's charge state. Detecting the exact masses of ions significantly reduces the false possibilities in the calculation of atomic compositions for the ions.

Instrument's mass accuracy defines how correctly the *m/z* value can be determined. MS instruments need to be calibrated with a known set of compounds (e.g. sodium formate clusters) in order to achieve mass accuracy. One can have a very high mass resolution but still lack mass accuracy if the calibration is off.
The accuracy is often given as error in parts per million, ppm: $[(m_{exp} - m_{exact})/m_{exact}]*10^6$,
where m_{exp} is the experimentally obtained *m/z* for the ion and m_{exact} is the theoretically calculated exact *m/z* of the ion. The exact *m/z* is calculated by the summation of the constituent atoms' exact masses divided by the charge. The exact mass is not the same as the usually given molecular weight of a molecule, which in turn corresponds to the average mass of the compounds' different isotopic forms and their masses.

Signal to noise ratio in mass spectrometry is defined from the mass spectra, where the signal for analyte *m/z* peak is compared to the background, i.e. unspecific signals. The unspecific background may arise from chemical noise from sample components, which is sample specific noise, or electrical, instrumental background noise. With the help of signal to noise –values the limit of detection (LOD; S/N 3; signal detectable from the background) and quantitation (LOQ; S/N 10) can be determined.

Sensitivity in MS is commonly described by the values of limit of detection or limit of quantitation for a given compound in a matrix, the term relating to signal to noise values. This definition, however, is stated to be incorrect by Price et al. [13]. According to their definition, sensitivity is the lowest detectable signal in a system where all the parameters are strictly determined. Sensitivity parameter therefore should be reported with units coulombs (C) per microgram of the injected analyte, or as pressure [13] of the analyte in gas form. Therefore in general description of instrument performance one should use the definition of "S/N –values" instead of "sensitivity".

Quantitative accuracy in MS is generally considered to be acceptable in the range of ±20% [14]. The precisions between MS measurements done on separate days, and between MS measurements between different sample lots (of the same type of tissue) are often described to vary [15]. However, the accuracy, obtained with the help of internal or external standards, is often good [16]. The lack of inter-day precision in MS is due to inter-day signal response fluctuations, which is caused for example by the differences in ion source cleanliness (affecting ionisation) etc.

Linearity on a dynamic range is important in quantitation. The dynamic range corresponds to the signal responses in a given range of concentrations of analytes,

and in mass spectrometry this range between LOQ to signal saturation is expected to be 3 to 4 orders of magnitude depending on the instrumentation.

In mass spectrometry all of the isotopic forms of the compound are recorded according to their abundance, and the isotopic pattern produced is additional information. The isotopes differ by the number of neutrons in the atoms, e.g. the presence of ^{13}C is statistically 1%. Therefore, in a molecule containing 50 carbon atoms, the probability of at least one of them being ^{13}C is 0.01*50= 0.5, and therefore the isotopic pattern has as a second *m/z* peak (in addition to the monoisotopic ion species) in the abundance of ½ of the first isotope *m/z* peak in the mass spectra. As the number of carbon atoms increases, the probability of having at least one (or further on, two, three, or four etc.) ^{13}C increases, and therefore the relation between the two *m/z* peaks' abundance is changed accordingly. Parts of the elementary composition can sometimes be deduced from a characteristic isotopic pattern. As an example, in the presence of chlorine (^{35}Cl 76% ^{37}Cl 24%) or bromine (^{79}Br 50.5%, ^{81}Br 49.5%) give characteristic isotopic patterns. The isotopic patterns are especially useful in high-resolution instruments where experimentally obtained spectra can be matched to theoretically calculated spectra for compound identification [17].
Most importantly, the isotopic pattern can be used to reveal the charge state of the ion. With organic compounds, singly charged ions have the one mass unit isotopic peaks separated by 1 *m/z* (Δm =1 from 1 neutron difference, z= 1), doubly charged ions by 0.5 *m/z* (Δm =1, z= 2), and triply charged ions by 0.333 *m/z* (Δm =1, z= 3) etc.

1.2.1. Ionisation techniques that enable the use of MS with biological macromolecules

The discoveries in the 1990's, electrospray ionisation (ESI) [18] and matrix-assisted lased desorption/ionisation (MALDI) [19], enabled the mass spectrometry instrumentation to be utilised for biological samples for analysis of macromolecules. Biological macromolecules, such as proteins, peptides, carbohydrates and nucleic acids, are water soluble, and neither evaporates easily, nor are thermally stable and therefore not suitable for the traditional ionisation methods. For quantitative purposes one often uses the ESI source. Before these ionisation sources, sources such as chemical ionisation (CI), electron ionisation (EI) and fast atom bombardment (FAB) and secondary ion mass spectrometry (SIMS) were used for the ionisation of smaller, and usually thermally stable compounds. Nowadays there are even further possibilities for ionisation sources, one can choose atmospheric pressure chemical ionisation (APCI) or atmospheric pressure photoionisation (APPI) or any of the traditional methods. However, the main ionisation source nowadays for the biological analytes is the ESI. See table 1.1 for the summary of different ionisation sources currently used in LC-MS and in analysis of spotted biological materials or tissue sections in mass spectrometry.

Electrospray ionisation coupled to liquid chromatography
In ESI the mobile phase from LC is directed through a metal capillary that carries high voltage. The solvent is pushed through the needle, and the voltage induces charges, i.e. ionises molecules within the solvent passing through. pH modifiers,

such as acids in the mobile phase, are used to fortify the ionisation. The charged solvent then forms a fine mist of droplets, repelling each other because of the same polarity of charge. The solvent is further vaporised with the help of heated gas flow, and therefore the droplets become even smaller. The charge remains on the surface of the droplet and at a certain point, when the droplet is highly concentrated with charged ions, the repulsion breaks the droplet and gas phase ions are released into the source by coulombic explosion. The formed ions are thereafter pulled into the mass spectrometer by another voltage, and further inside in the instrument by increasing vacuum and with the help of voltages of the ion optics.

The ESI process ionises most analytes, especially in the positive ion mode. When the solution is acidified, many different types of compounds in the sample matrix will easily obtain positive charge in the ESI source. This creates high background noise from the sample and also increases the possibility for matrix effects.

ESI is a flow dependent ionisation process, where the ionisation efficiency is dependent on the flow-rate, the liquid composition (solvent properties such as surface tension and boiling point), pH, and the salt concentration of the solvent introduced [20]. In general one uses acidic volatile pH modifiers with the positive mode detection (ions are created by protonisation) and pH close to neutral or basic in the negative mode detection (deprotonated ion species). However, for the LC separation acidic pH is preferred and Schiesel [21] noticed higher signal intensities in negative mode with mobile phase pH 5 than with pH 9.
The APCI source in connection to LC is often less sensitive to matrix effects than ESI source [22]. Furthermore, the negative mode in both ESI and APCI sources give lower ionisation efficiency (less compounds are ionised into the $[M\text{-}H]^-$ species than to the $[M+H]^+$ species. This means that if a compound of interest is readily ionised into the negative species it might be useful to analyse the compound in that mode in order to have reduced background noise and thereafter higher S/N values and better sensitivity [22].

Characteristic to ESI are the multiply charged species, and a series of different charge states of the same molecule. One compound may be seen in several different charge-states depending on the number of ionisable groups. Multiple charged ions that have more than one ionisable group within the structure, create richer fragmentation patterns in tandem MS, and therefore give more structural information of the ion.
The ESI ionises molecules also with ions other than protons, and therefore creates further ion species, e.g. with Na^+, K^+, NH_4^+ adducts in positive mode or Cl^- adducts in negative mode. In ESI the detection and quantification of a given compound can be determined on multiple spectral components, with singly/multiply ionised protonated or adducted molecules, and/or with fragment ions from the original molecule.

Nonvolatile solutes may induce ion suppression matrix effect by competing of the ionisation with the analyte [23] in the solvent droplets in the ESI source [20]. High concentrations of analytes, matrix compounds or too high of a water content in the spray solution may produce disturbances in the conductivity of the ESI spray [24].

Too high a concentration of ions causes too high of a current and therefore high electrostatic pull in the spray that negatively effects the ionisation by initiating erratic and uneven behaviour of the spray [24].
The other prominent effect, ion enhancement, is less throughoutly described in the literature, but the principle might be the same: nonvolatile matrix compounds are less efficiently transferred into the gas phase. Therefore these matrix components remain in the droplets, and drive the other compounds, such as the analytes of interest, on the surface of the droplet and from thereon into the gas phase.

The sensitivity of the ESI process increases with decreasing flow-rate, and the efficiency of the ionisation increases with decreasing analyte concentration, as charge competition becomes negligible [23]. Highly surface-active compounds (such as reserpine) are not easily affected by the sample matrix [23], but ionise well despite other components present in the sample. On the other hand, the addition of such surface-active analyte will decrease the ionisation efficiency of other compounds in the sample [23]. The ESI ionisation process has been shown to be linear (with pure standards) until a certain "saturation" point, where the concentration of the analyte is too high to allow all the analytes of interest to be efficiently ionised [23].
Post-column infusion of the analyte can be helpful in the estimation of the matrix effect during a whole chromatographic run [16, 25]. In this procedure, the analyte is at a constant rate and concentration infused into the ion source via a T-piece while a blank (control) sample is run in the LC -system into the MS ionisation source via the other opening of the T-piece. In this way, ideally, the signal response in the MS should stay the same throughout the sample run. Bonfiglio and co-workers as well as King and co-workers have shown that in reality the response changes in the ESI source depending on the sample [20, 25].

Matrix-assisted laser desorption/ionisation
In MALDI the sample is spotted on a metal plate and covered with matrix. The matrix is of crucial role, since it is responsible of both extracting the sample analytes and giving the basis for the soft ionisation. The sample and the matrix should be evenly mixed, or the deposition of the matrix should be made homogeneously throughout on top of the sample spot in order to create good and reproducible measurements. Laser hits the matrix, which absorbs the energy and utilises it for the ionisation and disintegration of the plated matrix. The matrix ions may give the charge to the analyte molecules (co-ionise them) and in the MALDI source evaporate so that only the analyte molecules reach the mass spectrometer.
The advantage of MALDI is the ionisation of large molecules. Often, the produced ions are singly charged, and the analyser for larger molecules should therefore have the ability to detect high *m/z* ratios, such as the TOF detector (see section 1.2.2.1.).
MALDI can also be used on analysis of tissue sections to obtain the spatial information of molecules by imaging mass spectrometry (for review of imaging MS see [26]).
Table 1.1 presents different MS ionisation sources currently used for LC-MS and for analysis of spotted biological materials or tissue sections. Information presented in table 1.1 has been compiled from [27] and from instrument manufacturers.

Table 1.1 Different MS ionisation sources

Ionisation source	Ionisation method	Application	Molecules	Ion characteristics	Strengths	Weaknesses
Electrospray Ionisation (ESI)	Ionisation induced by applying voltage to analytes and solvent, evaporation of excess solvent with auxiliary gas.	LC-MS	Wide range of masses and compounds, polar, even non-volatile and thermally labile	Protonated and adduct ions, single or multiple charges	Multiple charged ions, good for rich fragmentation pattern, and for the detection of high mass compounds	Background noise, matrix effects
Atmospheric Pressure Chemical Ionisation (APCI)	Electrons from corona discharge induce ionisation on solvent carrier reagent (N_2, H_2O) that ionises the analytes, reaction in high temperature.	LC-MS	Molecules of MW up to 1200 Da (2000 Da). Even nonpolar, and thermally stable compounds	$[M+H]^+$, $[M-H]^-$ Mainly singly charged	Low matrix effects, good S/N spectrum. Also nonpolar analytes ionised. Tolerates high flow rates.	Limited mass range, limited to thermally stable compounds
Atmospheric Pressure PhotoIonisation (APPI)	Photon dopant or an analyte with benzene ring receives photon energy, and protonates. The dopant transfers the proton further to the analyte.	LC-MS	Small, rather hydrophobic molecules ionised as well	$[M+H]^+$, $[M-H]^-$ Mainly singly charged	Selectivity	Low efficiency
Matrix-Assisted Laser Desorption Ionisation (MALDI)	UV (e.g. 337 nm) or IR laser energy induces ionisation and evaporation of matrix, ion transfer in gas phase in close proximity to the solid phase.	Spotted solutions, off-line from HPLC separation, tissue sections for imaging	Wide range of MWs up to 100 000 Da, different types compounds	Mainly singly charged molecules, ionisation and selectivity matrix dependent	Soft ionisation, wide mass range, sample preparation easy	Mainly only singly charged ions. Off-line from LC separation
Secondary Ion Mass Spectrometry (SIMS)	Primary ion beam of Ar, Xe, Ga or Au ions bombarded onto sample surface, analytes ionised by energy and charge of the primary beam.	Surfaces, spotted solutions, off-line from HPLC separation, tissue sections for imaging	Small molecules, of MWs up to 1000 Da. With matrix-enhanced SIMS higher, up to 5000-10 000Da.	Protonated and adduct ions, ion clusters, mainly singly charged ions.	High spatial resolution, small, high resolution ion beam	Low mass range, low efficiency

Information presented in this table has been compiled from [27] and from instrument manufacturers.

1.2.2. Different types of mass analysers

Mass analysers can be divided into four different subgroups: 1) magnetic sector 2) time-of-flight (TOF), 3) quadrupole (Q) and 4) Fourier transform (FT) analysers. Of these four groups mainly the three last mentioned are used for biological molecules. The magnetic sector instruments are mainly used in the study of smaller molecules with a flexible, but narrow mass range. The TOF and the quadrupole type instruments can be used for quantitative analysis, the TOF having a far wider mass range and higher resolution than the Qs, whereas the QQQs have the shortest duty cycles, and are most sensitive and reliable for the quantitative purposes. FT instruments are mainly used for high accuracy mass studies for especially structural determination.

1.2.2.1. Time-of-flight (TOF) instruments

TOF has the easiest mass analysis principle to understand: the ions are separated by their flight time. Ions are given a certain kinetic energy by an accelerator, the amount of energy depending on the charge of the ions, but not depending on the mass of the ions. Therefore the lower mass to charge ratio ions gain higher speed. The flight time is dependent on speed and therefore the smaller *m/z* ions arrive to the detector plate first. To have even higher resolution in the detection the ions can be reflected back to the field free flight tube in order to double the flight path and therefore double the difference created by the different *m/z* in the ions. The TOF instrumentation has gained its current usability due to the inventions made in the 1980s, such as the use of reflectrons for longer flight path [28] and delayed extraction, or time-lag focusing, for sharper mass peaks. Other important developments have been the orthogonal accelerator and the speed of the analog-to digital converters for the data acquisition. Before these developments the instrumentation was lacking the current main advantage of having high mass resolving power with fast scan rates.

1.2.2.2. Quadrupole (Q) instruments

Transmission quadrupole mass spectrometers, or, quadrupoles (Q) are mass filters that only let through a specified mass range (i.e. one mass unit) at a time. The electric field created by the radio frequency (RF) amplitude and constant direct current (DC) potential stabilises only one mass range at a time, and all the other ions are discarded. Although capable of only unit resolution, for a quantitative study the most popular instrument still is a triple quadrupole (QQQ). Usually, in the first quadrupole the ion of interest is selected, in the second fragmented and in the third scanned, or a fragment of interest selected (see figure 1.2). With a QQQ instrument there are many scanning modes available (see figure 1.2), and some useful options for function that are not possible in other types of instruments.

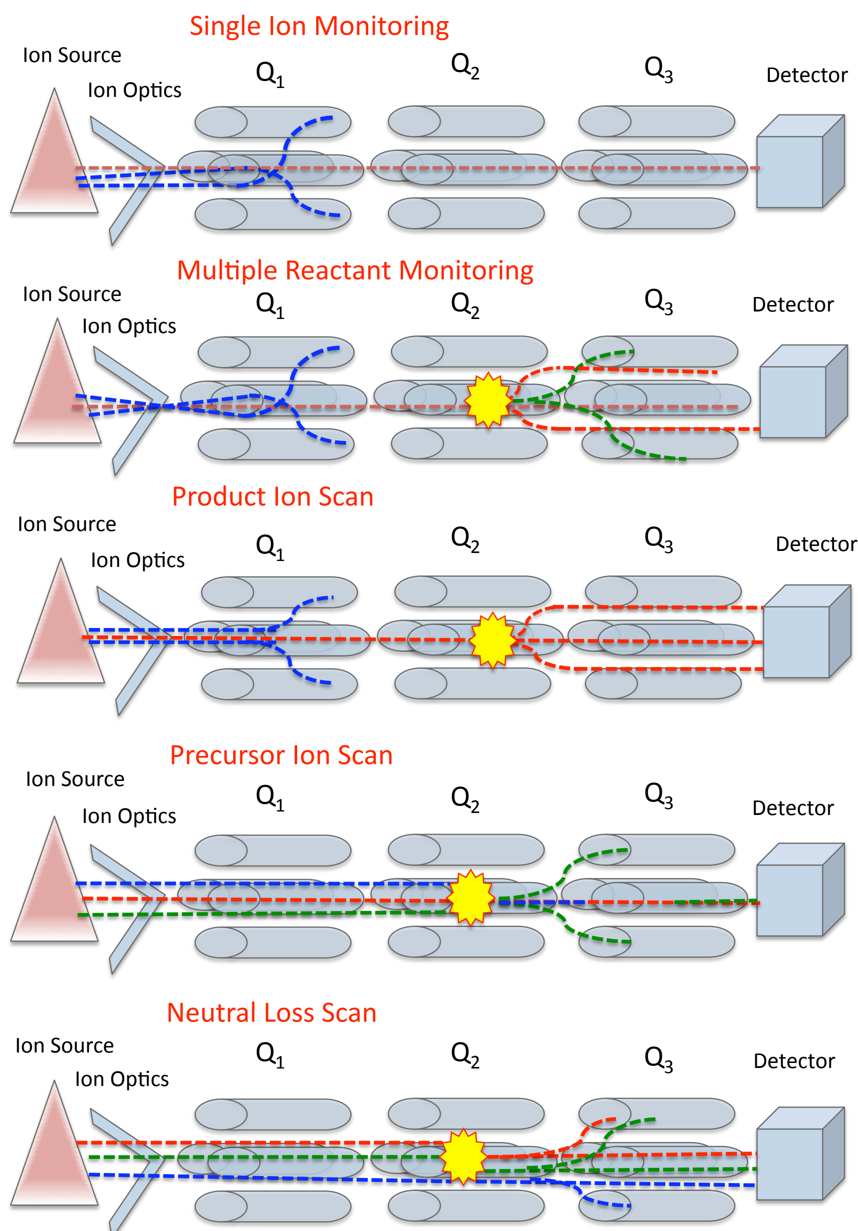

Figure 1.2. Triple quadrupole functions

In single ion monitoring (SIM) the first Q is selecting, only letting through, one *m/z* at a time and Q2 and Q3 are on RF mode only, letting through everything (and not fragmenting anything).

Multiple reactant monitoring, (MRM) functions so that the Q1 only lets through one *m/z* at a time, the selected ion is fragmented in Q2, and from these, only the selected fragments are let through Q3, one at a time.

Product ion scan functions in a similar way as MRM, but the product ions (fragments) are scanned in Q3 (all *m/z*, one at a time on a specific scan range).

Precursor ion scan utilises the Q1 filter to only let through one *m/z* to Q2 at a time, these ions are then fragmented, and only if a specific fragment (at which *m/z* the Q3 is sitting on) is present, will the detector receive signal.

Neutral loss scan functions so that the one *m/z* is let through in Q1 at a time, the selected ion fragmented in Q2 and the Q3 is set to let through only ions that have a determined mass difference to the ion let through in Q1.

The characteristic functions include neutral loss scan and precursor ion scan. In neutral loss scan the loss of certain mass, [X] (functional group, e.g. phosphate, or water molecule), gives rise to a fragment that has a mass of M-X which is the filtering mass to charge ratio for the third quadrupole before the ions hit the detector. In precursor ion scan, the first quadrupole is scanning, whereas the third quadrupole sits on one specific mass to charge ratio. The second quadrupole is fragmenting the ions from the first quadrupole. The precursor ion scan function is good for scanning for compounds with a similar functional group such as metabolites of a common drug / other compound, or other similar fragmentation product (see figure 1.2).

1.2.2.3. Spherical and linear ion trap instruments

Quadrupole type ion trap mass spectrometers, linear or spherical, three-dimensional (a Paul trap) ion traps have the same physical principals for ion detection and selection as quadrupoles, but the functions are rather different. The ion traps can be used for sequential stabilisation- fragmentation and ion ejection events and in this way further structural information, specific fragments, are obtained from molecular species. In other words, whereas the different functions for ion selection and fragmentation in QQQs are separated by space, in ion traps those functions occur in one place, the ion trap, but the different functions are separated by time. Whereas in a QQQ an ion can only be fragmented in the second quadrupole (plus, less specifically, in the ion source by applying higher voltage) the fragmentation may in a quadrupole ion trap be repeated over and over again, to create MS^n fragmentation. Furthermore the quick scanning, trapping and sequential ejection of ions of a wide range of *m/z* gives advantage in the detection of previously unknown, or unexpected analytes in a sample.

Some of the same type of functions as for the triple quadrupole can, in principle, be performed in a spherical ion trap, however with a major difference: whereas in a true QQQ MRM -experiment only one fragment ion species at a time is let through, in an ion trap the whole fragmentation pattern is scanned, and then a specific fragment is afterwards, during data-analysis chosen for analysis. The main drawback in using the ion trap for MRM is the time cycle: The trap needs to take in, stabilise in the trap, and successively eject all of the trapped ions to the detector. This creates a far longer duty cycle time than just swiftly changing the DC current for ion filtering in a QQQ instrument.

1.2.2.4. Fourier transform (FT) instruments

FT (Fourier transform) instruments nowadays include two types of mass analysers, the ion cyclotron resonance (ICR) [29] and Orbitrap [30] type instruments. The ICR instruments require a high magnetic field, and therefore a large magnet for the ion stabilisation into a circular ion path, orbital, whereas in the Orbitraps the ions are stabilised into an axial oscillation ion path around an electrode, or orbital. The fact that the great magnet is not needed in the Orbitrap offers great benefits in instrument maintenance and operating costs. The FT instruments utilise the mathematical equation Fourier transform to convert the

detected signal frequency of the oscillating ions passing through space between detector plates into *m/z* spectra of the ions in the cell. The frequency of the oscillation is *m/z* dependent, the signal intensity is dependent on the number of ions included in the oscillating package of ions. The advantage of the FT instruments is the in theory unlimited mass resolution, and therefore very high specificity of the ions detected. The down-side is the long duty cycle time it takes for the analysis to be made. Rummel and co-workers reported mass resolving power of over 300000 with the duty cycle of 30 s [31] in a 9.4 T instrument.

In table 1.2 different types of mass analysers are listed. Since the mass accuracy is dependent on calibration and signal intensity etc. and sensitivity is dependent on several factors (calibration, ionisation source, analyte, sample matrix, instrument tuning etc.) those parameters are not included.
Information presented in table 1.2 has been compiled from[27], from instrument manufacturers and from[31].

Table 1.2. Different types of MS instruments

Instrument type	Instrument	Strenght	Weakness	Mass resolving power	Speed	Commercial hybrid instruments	Application
Quadrupole analysers		MS/MS. Low cost.	Low resolution	~ 1500 * (Resolution 0.7 Da)		QQQ, Q-Trap, Q-TOF, Q-FT-ICR	Quantitative, small molecules up to 1500-3000 m/z, biological molecules
	Q	Ease of use	Ranges missed	~ 1500 * (Resolution 0.7 Da)	Continuous mode. Fast, in ms range.		
	QQQ	Speed. Specificity. Calibration required seldom.	Ranges missed	~ 1500 * (Resolution 0.7 Da)	Continuous mode. Fast, in ms range.		Small to medium size molecules
	Spherical/ linear ion trap	MS^n. Wide scan range/ scan.	Trap overload. Sensitivity. Low-mass cut-off in MSMS	~ 1500 * (Resolution 0.7 Da). Slower scans higher resolution (~0.3 Da)	Pulsed mode. Dependent on scan and duty cycle according to mode		Small to medium size molecules, (m/z up to 6000). Proteomics
Time of flight (TOF)		High upper mass limit. Scan over wide range. Speed. Good resolving power and mass accuracy.	Detector saturation. Requires calibration.	20000 – 40000 – 60000	Pulsed mode. Fast, 10-100 ms range.	Q-TOF, TOF-TOF	Wide range of molecule sizes. High resolution. Quantitation with narrow mass window. Proteomics. Default for MALDI and SIMS applications.
Fourier transform		Very high mass resolving power, good mass accuracy.	Speed. Detector saturation. Requires calibration.	Dependent on scan time. Very high, up to or over 100000	Pulsed mode. High resolution scans are slow.		High resolution, structural studies, identification of new compounds
	FT-ICR	Highest mass resolving power.	Speed in high resolution mode. Initial and operating costs high due to magnet.	Very high, up to or over 100000 (over 300000).		Linear trap-FT-ICR, Q-FT-ICR	Broad range masses.
	Orbitrap	Relatively cheap as compared to FT-ICR.	Speed in high resolution mode	Very high, up to or over 100000		Linear Trap-Orbi	Broad range of masses. Proteomics
Double focusing Magnetic sector		Good resolving power when well tuned. High collision energy MS-MS available.	Use requires high expertise. Instrument very big. Resolution vs. sensitivity.	As high as 20000. Sensitivity decreases with increase of resolving power	Continuous mode		Most applications on small molecules, nowadays instrument replaced by other instruments in most cases.

- In unit resolution instruments the mass resolution is the same throughout the span of the mass range, resolving power in the range of 140 – 4000

Information presented in this table has been compiled from [27, 31], as well as from instrument manufacturers.

1.2.3. Quantitative mass spectrometry analysis

LC-MS detection is well suited for quantitative analysis, provided that suitable standards are available. However, the sample needs to be prepared for the analysis. The importance of sample preparation was recognised by van Leeuwen [32], where differences in results were noticed in the same way treated samples in different laboratories. Hyötyläinen mentioned sample preparation as the main source of error in the analytical work-flow [33].

The purpose of sample preparation is to selectively extract, to recover, the analytes of interest from the matrix and leave the interfering compounds out of the processed sample. The purpose is also to concentrate the sample so that instrument sensitivity is enough for detection. As a general rule, all the samples need to be processed in some way prior to mass spectrometric analysis. The sample preparation, sometimes laborious, often is the bottleneck for sample throughput. In quantitative analysis precision in sample preparation is crucial in maintaining the samples as comparative. Sample preparation should consider aspects like analysis goal (multiple or one target), sample stability, available standards, and assessment of signal responses (spiking studies).

1.2.3.1. Analysis goal

In mass spectrometry many different analytes can be looked at simultaneously, but on the other hand, for quantitative purposes, all of the analytes of interest need a standard to relate the signal response to the concentration of the analyte. Multi-compound analysis also changes the sample preparation goal in the sense that highest selectivity cannot be maintained, but the purpose is to include all of the different analytes of interest in the processed sample [34].

1.2.3.2. The use of standards – internal standards vs. external standards

In quantitative analysis two types of standards can be used: internal standards (for example isotopically labelled analytes) or external standard. The internal standard is spiked into all samples in an equal amounts, whereas the eternal standard signal response will be related to the sample signal response from a separate (adjunct) run. In the case of an external standard, the standard can, and should be, the analyte itself.

The use of internal standards is not only recommended for the determination of sample preparation efficiency, but also required for the estimation of the instrument behaviour and signal responses. Internal standards were found beneficial in a systematic study of different types of biomolecules by Schiesel et al. [21, 35]. The usefulness was proven in both in analysis accuracy and interday precision.

1.2.3.3. Assessment of signal responses – spiking studies

Spiking studies are crucial in order to define the effect of the matrix to the analyte ionisation. Matrix effects are sometimes difficult to understand, especially matrix enhancement. Great matrix enhancement effects have been reported eg. in [36-37].

Schiesel et al [21, 35] found variable matrix effects for different compounds in the same matrix, and found matrix effects to be a major source of inaccuracies in case not corrected for by spiking studies [35]. Furthermore the instrument response fluctuation (ion source contamination due to extensive sample series with complex sample matrix) was found to decrease accuracy. These inaccuracies can be corrected by the utilisation of internal standards or by external standards [35].

1.2.4. Current trends in mass spectrometry

Mass spectrometry instrumentation can be used multiple ways. Nowadays the fast duty cycles and software development enable functions previously unavailable. Modes that enable the acquirement of different types of data in one run may include
- isotopic pattern triggered product ion scan
initiates fragmentation and scanning of a compound consisting of characteristic isotopic pattern (e.g. containing Cl or Br)
- enhanced product ion scan, for the use of an linear ion trap for a high quality MS spectra,
- polarity switching in single runs, where compounds can be screened with two different modes in a single run
- polarity switching used in negative precursor ion triggered positive product ion scan, can be used when the analyte shows well and with less noise on negative mode, but fragments poorly, for example gives only a water loss, on the negative mode.
- constant neutral loss scan for certain functional group or biological modification (e.g. glutathione adduct),
- MRM (of 100 transitions) triggered product ion scan, where multiple spectra can be obtained during one run,
- mass defect triggered MS-MS, where for example exogenous compounds rich in double bonds or sulphur, chlorine or phosphor can be surveyed by initiating fragmentation when negative mass defect is detected.

All of these modes have been utilised in drug metabolite research (reviewed in [38]). The usual product ion scan function benefits in selectivity from the utilisation of accurate mass (TOF or FT instrument) detection for the triggering precursor ion. The combination of a quadrupole to linear ion trap has brought benefits in MS-MS scans and MS^n measurements. Data mining and data processing after LC-MS runs are essential in pointing out the relevant observations.

The Orbitrap, released in 2005, has revolutionised the accurate mass field by providing FT-ICR comparable resolution without the large magnets and therefore space and electricity requirements. Whereas the FT-ICR utilises the circular path

oscillation frequency in the cell for the determination of spectra, in Orbitrap the recorded oscillation is in the axial movement around the spindle-shaped central electrode. The addition of double stage linear ion trap in the front gives benefits in speed in the first stage analysis, while only selected ions are selected for accurate mass measurement in the Orbitrap [30].
Another interesting instrument development is the coupling of ion mobility flight tube in front of a time-of-flight instrument [39]. This set-up has been shown to give benefits in the detection and identification of (highly) branched carbohydrates where the analysis might be very demanding due to many structural isomers [40] and in the detection of peptides and proteins [41].

1.3. Cyanobacteria and their toxic metabolites

Cyanobacteria are among the oldest organisms in the world: they were responsible for creating the oxygen atmosphere on earth (reviewed in [42]) and there is evidence of cyanobacteria already from about 3.5 billion years ago [43]. There are about 150 genera and 2000 species of cyanobacteria, they belong to the gram-negative bacteria ([44]). Cyanobacteria have vital roles in cycling biochemical nutrients, they function as nitrogen fixers, and therefore in the maintenance of biodiversity of microbial and higher organisms [45]. Most commonly cyanobacteria inhabit water bodies, lakes and seas, but they are encountered in most variable environments, desert sand and volcanic ash, in temperatures ranging from those of hot springs to the Arctic and Antarctica (reviewed in [42]). Also, cyanobacteria have roles as symbiots in different types of plants. The problems with cyanobacteria occur mostly in regions where water bodies are warm throughout the year and contain nutrients in excess.

Cyanobacterial blooms occur frequently all over the world. They cause hypoxia in the water bodies during bloom disintegration, leading into problems for fish and other organisms in the water bodies.
Besides the blooms themselves, the toxic genera of cyanobacteria (about 40 out of the 150 genera of cyanobacteria (reviewed in [46]) cause problems to the organisms depending on water resources. The oldest scientific report on animal toxicosis was written by Francis in 1878 [47]. Reported human exposure cases are from both short-term exposure incidents as well from long-term exposure. Short-term exposure cases in Brazil have been reported by Teixeira and co-workers from Paulo Afonso [48], and e.g. by Jochimsen and co-workers [49] from Caruaru. Short-term exposure cases from Australia have been reported by Byth [50] and Bourke and co-workers [51]. Long-term exposure has occurred in China, as described by Ueno and co-workers [52]. Less severe incidents have occurred for example in Sweden, reported by Annadotter [53] and in the UK by Turner and co-workers [54].

There are many different classes of biologically active compounds produced by the different species of cyanobacteria. The classes comprise of hepatotoxic cyclic peptides microcystins (MCs) and nodularins (Nod), cytotoxic cylindrospermopsin, neurotoxic anatoxin-a and –a(S), saxitoxin and saxitoxin analogues, neurotoxic amino acid β-N-methylamino-L-alanine (BMAA) and irritating agents (non-toxic) lipopolysaccharides. See table 1.3 for an overview of the produced toxins and their toxicities. The information in this table is mainly based on [55-57] and completed with information from [58-62].

Table 1.3 Toxins produced by cyanobacteria

Toxin	Structure	Primary producer (not exclusive)	Toxin class, target	Toxicity	Concern to Human health	Typical screening method
Microcystins	Cyclic heptapeptide with unusual amino acids (Adda), >100 variants	*Microcystis, Planktothrix, Anabaena, Nostoc*	Hepatotoxins, hepatocytes, liver, hepatopancreas. Specific molecular targets protein phosphatase 1 and 2A	LD_{50} for most MCs ca 50-300 µg kg^{-1} (mouse, i.p.), oral toxicity weaker	Longterm exposure via drinking water, recreation exposure, common in eutrophic freshwaters, traditional drinking water treatment is inadequate, chemically stable	ELISA, HPLC-DAD, LC-MS(-MS), protein phosphatase inhibition assay Standard HPLC-based method: ISO 20179:2005
Nodularins	Cyclic pentapeptide with unusual amino acids (Adda), ca 10 variants	*Nodularia*	Hepatotoxins, hepatocytes, liver, hepatopancreas	LD_{50} for most Nods 50-300 µg kg^{-1} (mouse, i.p.), oral toxicity weaker	Common in the Baltic Sea, recreational exposure, chemically stable	ELISA, HPLC-DAD, LC-MS(-MS), protein phosphatase inhibition assay
Cylindro-spermopsin	Cyclic guanidine alkaloid with an attached uracil ring	*Cylindro-spermopsis, Aphanizomenon, Anabaena*	Cytotoxins, affects many types of organs, liver, kidney etc. Inhibit protein synthesis, DNA damage	LD_{50} for cylindro-spermopsin 2.1 mg kg^{-1} (mouse, i.p., 24 h), 200 µg kg^{-1} (mouse, i.p., 5-6 days)	Long term exposure via drinking water, recreational exposure, present in extracellular form, chemically stable	ELISA, HPLC-DAD, LC-MS-MS
Anatoxin-a, Homo-anatoxin-a	Secondary amine, low molecular weight alkaloid	*Anabaena, Aphanizomenon, Planktothrix, Oscillatoria, Cylindrospermum*	Neurotoxic, mimic the effect of acetylcholine in nerve synapses but is not hydrolysed	LD_{50} 200-250 µg kg^{-1} (mouse, i.p.)	Recreational exposure (but degrades rapidly to non-toxic in natural waters)	HPLC-DAD, LC-MS-MS, GC methods
Anatoxin-a(S)	Organo-phosphate	*Anabaena*	Neurotoxic anticholin esterase	LD_{50} 20 µg kg^{-1} (mouse, i.p.)	Recreational exposure	LC-MS-MS

Table 1.3 Continued

Saxitoxins, also known as paralytic shellfish poisons	Carbamate alkaloids with different sulphation degrees. Some decarbamoyl variants. >20 variants, but not all are present in cyanobacteria	Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya Marine dinoflagellates	Neurotoxic, block sodium channels in nerve axons	LD_{50} 10 µg kg^{-1} (mouse, i.p.) for saxitoxin	Recreational exposure, accumulation in marine shellfish can cause lethal poisonings	HPLC with derivatisation and fluorescense detection, LC-MS-MS, ELISA
Beta-N-methyl-amino-L-alanine (BMAA)	Amino Acid	All cyanobacteria (??)	Neurotoxic, associated with chronic degenerative nerve diseases (ALS, Alzheimer)	Chronic toxicity (debated)	Long-term exposure via food	HPLC with fluorescence detector after derivatisation, LC-MS-MS
Lipopoly-saccharides	Lipid-sugar structures in cell walls of Gram negative bacteria	All cyanobacteria	Irritants, fever, gastrointestinal problems, allergenic responses	Regarded as non-lethal	Exposure to LPS may potentiate the effects of other toxins	*Limulus* amoebocyte lysate assay (LAL)

The information in this table has been reviewed in [55-57] and completed with information from [58-62].

1.3.1. Cyanobacterial peptide hepatotoxins

The structure of the cyanobacterial peptide hepatotoxins is unordinary to biological peptides, they comprise seven (MC) or five (Nods) amino acid residues in a cyclic structure, the amino acid residues being both common or uncommon (D-amino acids as well as other characteristics in the sidechains) amino acids (Figure 1.3). Characteristic Adda (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid is by definition found in all MC- and Nod -structures. There are over 100 variants of MCs described in the literature, the structure in general being cyclo(-D-Ala$^{(1)}$–X$^{(2)}$–D-MeAsp$^{(3)}$–Z$^{(4)}$–Adda$^{(5)}$–D-Glu$^{(6)}$–Mdha$^{(7)}$). Mdha stands for *N*-methyldehydroalanine. Table 1.4 presents the latest MC variants to have been published, and completes the lists compiled by Sivonen and Jones [55] and presented in Dr. Lisa Spoof's doctoral thesis [63]. There are about 10 variants of Nods.

Table 1.4. Recently reported novel MCs. Table compiled by Dr. Lisa Spoof, manuscript under preparation.

Microcystin	MW	Organism	Reference
[D-Asp$^{(3)}$,dmAdda$^{(5)}$]MC-LA	881	*Hapalosiphon hibernicus* BZ-3-1	[64]
[D-Asp$^{(3)}$]MC-VA	881	*Hapalosiphon hibernicus* BZ-3-1	[64]
[Asp$^{(3)}$]MC-LA	895	*M. aeruginosa* B2666	[65]
[D-Asp$^{(3)}$]MC-LA	895	*Hapalosiphon hibernicus* BZ-3-1	[64]
[D-Asp$^{(7)}$]MC-LA	895	*Hapalosiphon hibernicus* BZ-3-1	[64]
MC-VA	895	*Hapalosiphon hibernicus* BZ-3-1	[64]
[D-Asp$^{(3)}$]MC-LV	923	*Hapalosiphon hibernicus* BZ-3-1	[64]
[Asp$^{(3)}$,Dha$^{(7)}$]MC-YA	931	*M. aeruginosa*	[66]
[Asp$^{(3)}$]MC-LL	937	*M. aeruginosa* B2666	[65]
MC-LV	937	*Hapalosiphon hibernicus* BZ-3-1	[64]
[D-Asp$^{(3)}$]MC-RA	938	*Hapalosiphon hibernicus* BZ-3-1	[64]
[Gly$^{(1)}$, Asp$^{(3)}$]MC-LR	966	*Nostoc*, Miers valley, Antarctica	[67]
MC-AhR	967	*Microcystis* sp. Adityanagar pond, India	[68]
[Gly$^{(1)}$, Asp$^{(3)}$]MC-LhR	980	*Nostoc* Miers valley, Antarctica	[67]
MC-VR	980	*Microcystis* MK10.10, Vistula Lagoon, southern Baltic	[69]
[D-Asp$^{(3)}$, (E)-Dhb$^{(7)}$]MC-hIR	994	*P. rubescens*	[70]
[Gly$^{(1)}$, Asp$^{(3)}$, AdmAdda$^{(5)}$]MC-LR	994	*Nostoc*, Miers valley, Antarctica	[67],
[Gly$^{(1)}$, Asp$^{(3)}$, AdmAdda$^{(5)}$]MC-LhR	1008	*Nostoc*, Miers valley, Antarctica	[67]
[methylated Ala$^{(1)}$]MC-LR or [methylated Leu$^{(2)}$]MC-LR	1008	* Lake Suwa bloom	[71]
[Gly$^{(1)}$, Asp$^{(3)}$]MC-RR	1009	*Nostoc*, Miers valley, Antarctica	[67]
MC-ER	1010	*	[65]
MC-MR	1012	*	[72]
[Gly$^{(1)}$, Asp$^{(3)}$]MC-RhR	1023	*Nostoc*, Miers valley, Antarctica	[67]
[Asp$^{(3)}$]MC-RY	1031	* Uganda	[73]
[Gly$^{(1)}$, Asp$^{(3)}$, AdmAdda$^{(5)}$]MC-RR	1037	*Nostoc*, Miers valley, Antarctica	[67]
[MeAsp$^{(3)}$]MC-RY	1045	* Uganda	[73]

[Ser(7)]MC-YR	1048	* Lake Suwa bloom	[71]
[Gly(1), Asp(3), AdmAdda(5)]MC-RhR	1051	*Nostoc*, Miers valley, Antarctica	[67]
[NmeSer(7)]MC-YR	1063	* Uganda	[74]
MC-hRhR	1065	*	[75]
[9-AcO-Adda(5)]MC-RR	1066	*Planktothrix rubescens*, Lake Averno, Italy	[76]

* Producer not determined
Some abbreviations: hR homoarginine, AcO acetyl, AdmADDA acetyldemethylADDA, dhb dehydrobutyrine, hI homoisoleucine, NMeSer methylserine, Dha dehydroalanine

The characteristic amino acid Adda and the acidic group in the Glu residue are required for the MC and Nod toxicity, as well as the cyclic structure of the toxin. The MC variants differ in toxicity: in the literature reported values range from the very potent toxin MC-LR, LD_{50} (mouse i.p.) of 50 μg/kg, to MC-RR, LD_{50} (i.p.) of 600 μg/kg, and to the Adda -modified MC:s LD_{50} (i.p.) of over 1000 μg/kg (reviewed in [55]). Modification in the Adda structure or opening of the ring structure renders the molecule to a less toxic form (reviewed in [42]). The Adda amino acid on its own is not toxic [77].

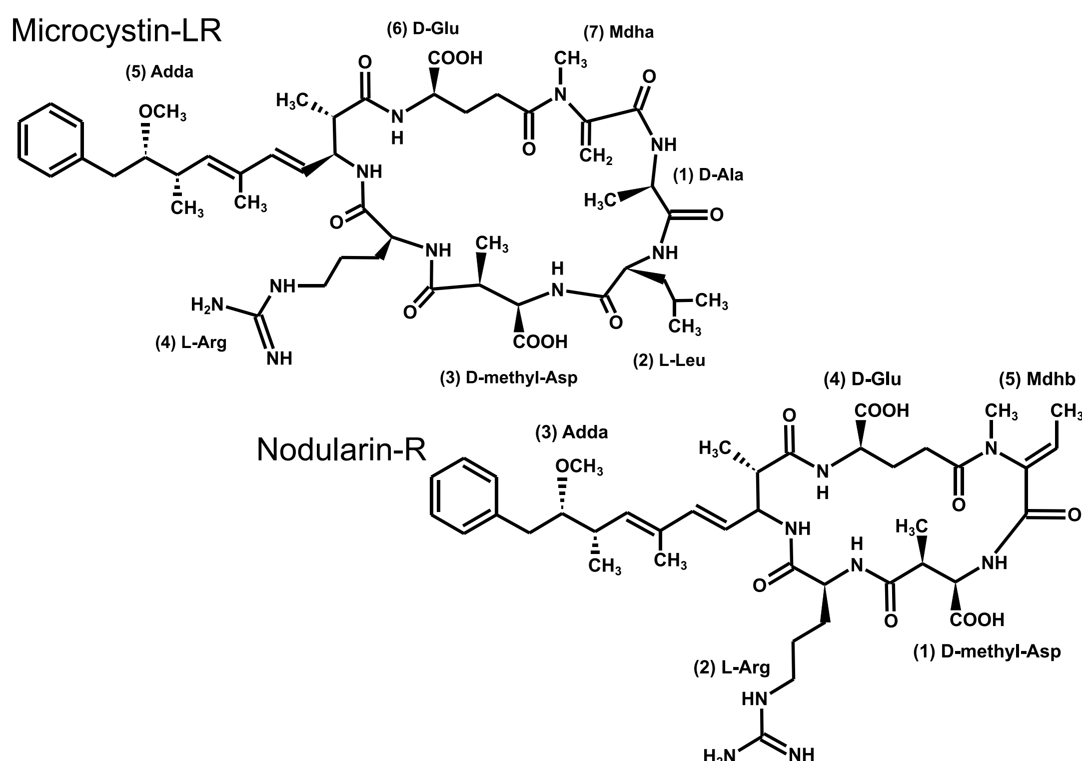

Figure 1.3. The structure of cyanobacterial hepatotoxic cyclic peptides, microcystins and nodularins. The structures have either seven or five amino acids in a cyclic structure, and contain some uncommon amino acids such as the Adda-amino acid. Adda has a conjugated diene in the side chain, which is a key element for the typical absorption spectra in UV-detection.

MCs and Nods are taken up into the cells, in animals mainly hepatocytes, through the organic anion transporting polypeptides (OATP) [78], formerly called bile acid

transport system, as they are unable to penetrate the cell membrane [79]. The more lipophilic congeners such as MC-LW and MC-LF were shown to interact more efficiently with a model membrane than MC-LR, suggesting more interactions with cell membranes [80].

The MCs and Nods are potent and specific inhibitors of protein phosphatases (PPs) 1 and 2A [81-83]. MCs, but not the related Nod toxins [84], form a covalent bond with target proteins, PPs, after hours of exposure [85]. Both covalent and noncovalent interactions with the PPs give rise to effective inhibition. The IC_{50} of the most common variant, MC-LR for the mammalian PP1 has been reported as 1.7 nM and for mammalian PP2A 0.04 nM [82]. For Nod-R the corresponding IC_{50} values were 1.8nM and 0.026 nM respectively [86].
The noncovalent interaction is directed by the Adda side chain burying into a hydrophobic pocket in the protein phosphatase [87]. The covalent bond is formed between *N*-methyldehydroalanine (Mdha) residue double bond and the cysteine 273 residue in PP1 [87-88], or the corresponding residue cysteine 269 in PP2A [89] (Figure 1.4).

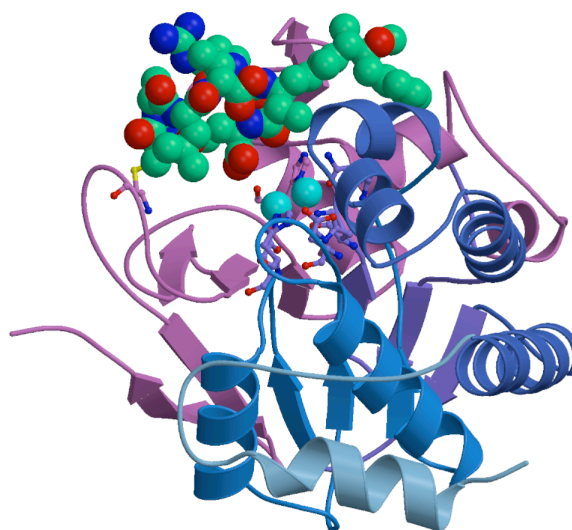

Figure 1.4.
Three-dimensional structure of MC-LR covalently bound to the target protein, protein phosphatase 1, obtained by x-ray crystallography. The MC-LR, and the covalent bond counterpart the Cys 273 residue are in ball and stick model, whereas the protein secondary structures are shown in ribbons (alpha helices) and sheets (beta sheets). The manganese ions required for the activity in the active site are rendered as cyano-coloured balls. PDB ID 1fjm; Goldberg and co-workers [87]

In a wide selection of eukaryotic organisms, including mammals, bivalves, zooplankton, and plant species, the IC_{50} for protein phosphatase 1 and 2A inhibition by MC-LR falls into the narrow range of below 0.1-0.25 nM [83, 90-91]. The protein phosphatase variant PP2A from maize (*Zea mays*) has the lowest reported IC_{50}: 0.03 nM [92]. Since more than 97% of protein phosphates specifically dephosphorylate the serine or threonine residue-linked phospho-groups, the inhibitory effect of MCs to the Ser/Thr-phosphatases is likely to

interfere with many pathways in the cell (see [93]). The result of these toxins is the overall increase in phosphorylation of the regulatory proteins. The main target for the toxins in mammals, fish and birds is the liver.

A phosphoproteomics study by Tachi and co-workers [94] suggests that the morphological changes such as swollenness and darker colour of liver tissue, and apoptosis would be a consequence of PP2A inhibition, which is a common target for both MCs and okadaic acid [95]. The acute toxicity of MCs (not occurring with OA in liver cells) would then be related to PP1 inhibitory effects [94].

Mikhailow and co-workers have shown that also ATP synthase is a target for MC inhibition [96]. This might explain the apoptotic effect of high concentrations of MCs in cells [96]. The possible other candidates for MC toxicity in mouse liver were studied by Imanishi and Harada [95], where they found that in addition to the PPase family also some calmodulin binding proteins, striatins specifically bind to MC-LR.

1.3.2. Bioaccumulation and concentrations of peptide hepatotoxins in animal and plant tissues

The levels of cyanobacterial toxins in natural waters have been reported to vary between 0 to up to 25000 μg/l (reviewed in [42]). The usually reported extracellular concentrations of MCs (not including the occasion of a heavy bloom breaking down) are, however, much lower, in the range of 0.1-10 μg/l (reviewed in [55]).

There are extensive reviews on the subject of animal and plant exposure to cyanobacterial toxins [97-98]. Accumulation occurs in bivalves, molluscs, fish and aquatic plants through filtering the contaminated water and by consumption of toxic cyanobacteria. Accumulation has also been shown up in the trophic level in the food web by animals feeding on contaminated food, such as mussels and aquatic snails by predatory fish, birds etc. [99] (see fig. 1.5). Baltic Sea ecosystem has been found to contain Nod on many organism levels (reviewed in [100]): Baltic sea *Nodularia spumigena* has been reported to produce up to 18100 μg of Nod/g dry weight (DW) phytoplankton [101], releasing up to 18100 μg of Nod/l [102] into the water bodies. Despite these very high toxin concentration values of only up to 2 μg of Nod/g DW tissue has been reported from higher organisms (mussels) in the Baltic Sea [103] (see figure 1.5. for information compiled by Harri Kankaanpää). The main accumulation site is the hepatopancreas in bivalves and the liver in fish and birds.

MC -accumulation in fish, mussels and aquatic snails has been thoroughly inspected (see review [98]). Majority of the accumulation studies have been conducted with the ELISA method that is not designed for tissue analysis, but for water samples (see section 1.5.2.2). Therefore cross-reactivity and matrix effects and extraction efficiency may have affected the accurate determination of the concentrations. Furthermore, the total amount of toxin (free, plus covalently bound to the target proteins) remains a question when the toxins are extracted from the tissue. The true values of the total toxin concentrations might have been

underestimated. It is also unclear if the covalently bound toxins are bioavailable for the next trophic level [104]. Furthermore, the possible biotransformation products of the toxins might not be detected with ELISA.

There are not many studies published of MC transfer from one trophic level to another in the food chain. In an extensive study of three years lake monitoring, food chain starting from filter feeder zooplankton (mainly *Daphnia galeata*) was found to be contaminated with MCs, whereas food chain starting from another filter feeder zebra mussel *Dreissena polymorpha* was found to be rather clean from MCs [105]. The concentrations found in the first consumer organism were for the zooplankton, *Daphnia galeata*, up to 1000 µg of MC/g DW, on average in the range of 63-211 µg of MC/g DW, and for the zebra mussel *Dreissena polymorpha* up to 30 µg of MC/g DW, on average in the range of 2-12 µg of MC/g DW [105].

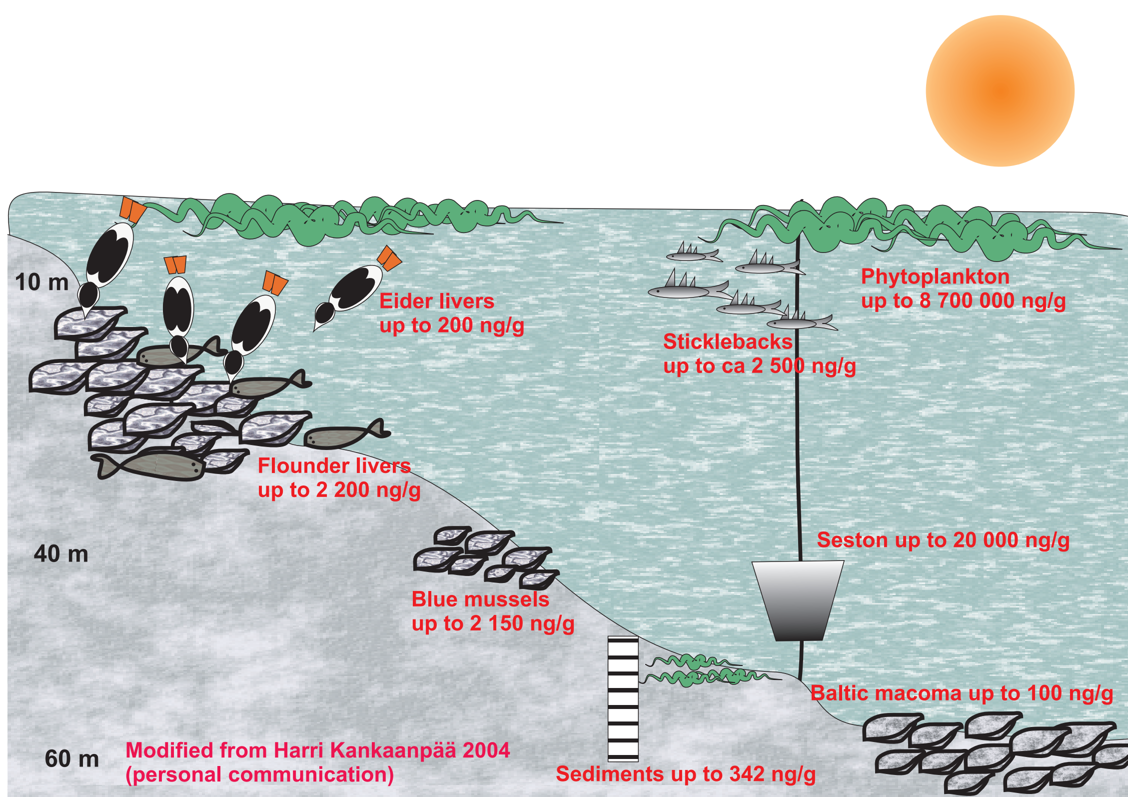

Figure 1.5.
In the Baltic sea the aquatic organisms such as mussels, flounders and stickelbacks have been shown accumulate high amounts of Nod-R in their tissues. The eider ducks that consume mussels as a part of their diet accumulate the toxin as well. The figure is modified from Harri Kankaanpää's (Finnish Environment Institute, Marine Research Centre) presentation in 2004.

1.3.2.1. Fish, bivalves and aquatic snails

Reported toxin levels in different aquatic organism tissue range between 436 µg/g DW (in freshwater snails exposed in laboratory conditions) [106], and to 420 µg/g DW (bivalves in a hypereutrophicated lake in Japan) [107] to maximum of 49.7 µg/g (in laboratory experiment exposed fish) and in general in the range of few

hundred ng of MCs/g DW in crab and fish tissue (reviewed in [98]). As the main target organ in fish is the liver, only minute amounts of the toxins have been detected in the fish muscle tissues [99]. In the case of a heavy blooms in a Japanese lake the difference between accumulation into different species of bivalves was noticeable, from a max value 12.6 μg of MCs/g for *Anodonta woodiana* to 297 μg/g in *Cristaria plicata* and 420 μg/g in *Unio douglasiae* [107].
Interestingly, the bivalves are able to survive despite the considerable accumulation of MCs in the tissues, although their IC_{50} protein phosphatase 2A was determined to 0.25 nM [83, 91]. Mussel insensitivity towards okadaic acid and the marine dinoflagellate diarrhetic shellfish poison (DSP) toxin has been reported [108], but the mechanism explaining insensitivity towards MC has not yet been identified [97].

1.3.2.2. Birds

In a study by Chen et al. MCs in the small intestinal and stomach contents as well in the livers of the ducks and water birds were in the range of 12 - 42.0 ng/g DW [109]. In the same study the egg yolk also showed some accumulation with concentrations in the range of 7 - 15 ng/g DW tissue.
Analyses of Baltic Sea eiders by ELISA and LC-MS showed that eider liver samples contained 3 to 180 ng of Nod/g DW [110]. In a similar study conducted in a different year eider liver samples contained Nod up to 199 ng/g DW and muscle samples up to 21 ng/g DW as measured by LC-MS [111].
Birds are known to accumulate some toxins into their feathers [112-113]. The first report of cyanobacterial hepatotoxins, MCs, in bird feathers was done by Metcalf *et al.* [114]. These birds, lesser flamingos, had earlier been reported to have been exposed to cyanobacterial toxins. The reported concentrations from different tissues were in stomach, intestine and fecal pellets of 196 ng/g fresh weight of MCs and 4.34 μg/g fresh weight of anatoxin-a [115].

1.3.2.3. Humans

WHO has provided a provisional guideline of 1 μg/l of MC-LR in drinking water [116]. This guideline is based on a 44-day long exposure study on pigs [117]. However, Ueno et al. [52] have criticised this value and suggested a stricter guideline value of 13 ng/l MCs, especially for drinking water consumed in areas of high occurrence of primary liver cancer. Their study is based on water samples collected in China to estimate the effects of long-term exposure of humans to cyanobacterial toxins [52] (see more in human risk assessment, section 1.4.). In a second, more recent study from China Chen and co-workers have detected MCs on average 0.39 ng/ml in human serum samples taken from people exposed to the MCs below the range of WHO guideline values [118]. The authors had also looked for indicators of MC-induced liver damage from the blood samples [118] and found positive correlation between MC concentrations and concentrations of enzymes alanine- and aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase. They conclude that either the exposure levels had in reality been higher than what their data shows, or that the WHO guideline values would need to be reassessed since liver damage had already occurred [118].

In the extensively studied Caruaru dialysis clinic case in 1996 the human poisonings could be traced back to MCs and cylindrospermopsin with high confidence. The patients had been exposed to the toxins by short-term exposure via dialysis. In this outbreak of acute liver failure 116 of 131 patients experienced adverse effects of exposure, 100 developed acute liver failure and 76 of them died. The evidence for MC exposure has been shown with number of samples that had been taken from the patients receiving the contaminated water through renal dialysis and the concentrations of MCs in liver and serum tissues have been reported [49, 119-121]. The serum concentrations varied between patiences, in the range of 7-31 ng/ml serum [122] and 50-472 ng/g of fresh weight in livers [119].

1.3.2.4. Plants

Contamination of plants by MCs have been studied e.g. in [37, 123-124]. Contamination occurs usually by irrigating the plants with contaminated water. The accumulation of these toxins into plants constitute a hazard of toxins in food in the same way as by contaminated fish, mussels or crabs. Furthermore MCs may affect the crop size. Plant protein phosphatases are susceptible to damage by MC exposure [92, 125]. In plants these proteins regulate essential functions such as ion channel activity, carbon and nitrogen metabolism and gene expression as well as growth and developmental processes (reviewed in [126]). The negative alleopathic effect of MC-LR on aquatic plants (macrophytes) was shown in [127], starting from concentrations as low as 1µg/l. In general the exposure studies have been performed with very high concentrations of MCs, higher than naturally relevant. However, the species –specific sensitivity to MCs would suggest that the overall alleopathich effect of MCs to higher plants would not be important, or that plants have developed means to overcome and protect themselves from MC exposure (reviewed in [126]). Quantitative studies on MC accumulation into plant material show low (1-2.6 ng/g fresh weight; [37]) to up to 650 ng/g fresh weight [128] amounts of MCs to accumulate into terrestrial plant materials via normal exposure routes (irrigating) and higher amounts (up to 110 µg/g fresh weight) in aquatic plants ([129]; reviewed in [126]).

1.3.2.5. Covalently bound toxins

There are not many studies concerning the covalently bound MCs in tissues. However, it has been shown that the MCs covalently bind to the target proteins [84]. Accumulation has been shown to occur in vivo by a MMPB method [130-132] as well as by radiolabelled MCs [133]. Recently, the covalent accumulation was proven by histopathology analysis of aquatic snail tissues [134].

MCs are oxidised with 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) method [135] to carboxylic acid MMPB to assess the total amount of MCs in tissue. The MMPB is formed from the characteristic Adda amino acid (Figure 1.6, formation of MMPB from MCs). The otherwise inaccessible covalently bound MCs can be determined in this way. MMPB has been utilised for the detection total MC concentrations in cyanobacterial blooms [135], sediments [136], and tissues (e.g. [130-131, 137]).

Lake soils and sediments have been considered a difficult matrix for MC and Nod extraction [136], but in a study by Chen *et al.* [138] acidic EDTA-sodium pyrophosphate solution was found to give good yield, about 90% efficiency in toxin extraction from lake soils and sediment samples. Before this, due to the difficulties in extracting MCs and Nods from the sample, the MMPB method was utilised for the analysis of MCs in soils and sediments [136].

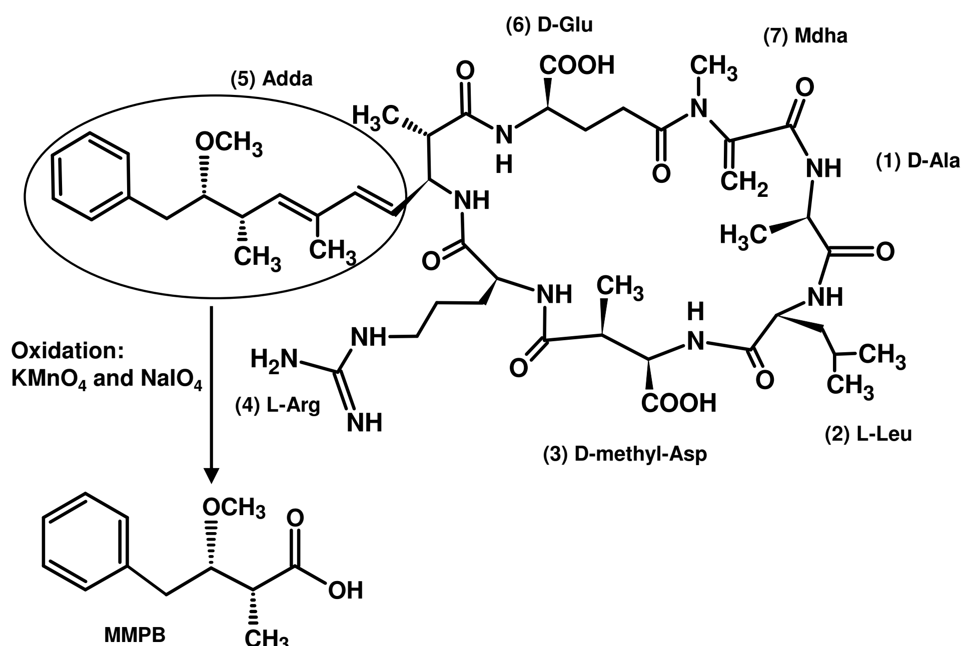

Figure 1.6. The Adda amino acid in the MCs and Nod is oxidised into MMPB by the use of oxidating agent (KMnO4, NaIO4, or ozone). The covalent bond between the MC and target protein, protein phosphatase, is formed with the Mdha (7) residue in the structure.

The proportion of the covalently bound toxins from the total contamination have been reported to vary in different organisms and exposure conditions: about 24% in salmon liver [131], from 1% to 38% in mussels [137], from 6% to 75 % in human serum samples in [130] determined with the MMPB –method. From 87% to 95% of covalently bound MCs were found in salmons utilising isotopically labelled MC by Williams and co-workers [133]. However, Williams and co-workers [132] reported a ratio of more than 99.9% of covalently bound MCs in mussel tissues with analysis conducted with the MMPB -method.

1.4. Risk assessment for human health

Cyanobacteria are not regarded as waterborne pathogens in the safety assessment concerning production of drinking water, since they are unable to colonise, invade and grow in human and animal hosts [45]. However, cyanobacteria do produce variety of bioactive compounds, which may develop hazard to human health when present in water bodies. Many of the bioactive compounds are peptides with enzyme inhibition activities in e.g. mammalian cells. Some of the compounds have potential pharmaceutical use but many of the bioactive compounds are potent toxins in mammals and/or aquatic organisms.

There is much qualitative evidence of cause and effect for MCs and Nods, but the data has been inadequate to establish a clear dose-response relationship for human risk assessment. However, for the estimation of health hazard, following aspects should be taken under consideration:

The total risk of a certain toxin is evaluated on the basis of following aspects:
- toxic properties of the substance,
- dose responses,
- possible routes for human exposure and
- prevalence and concentrations of the toxin.

1.4.1. Toxic properties

(see also section 1.3., table 1.3)
As reported in literature, (see review [45]) problems caused by cyanobacteria are encountered around the world and problems related to safe drinking water production are common. Human health effects from cyanobacterial toxicosis are diverse, including gastroenteritis, nausea, vomiting, fewer, flu-like symptoms, sore throat, blistered mouth, ear- and eye irritation, rashes, abdominal pain, visual disturbance, kidney and liver damage etc [45]. As an example of the effects of long-term exposure, the cancer incidence rate in general in China is half of that in Australia, but the incidence rate for primary liver cancer is about ten fold higher [117]. Furthermore, the liver cancer incidence rate in people Qidong province of China, is more than ten fold in people drinking pond water (where cyanobacteria may occur) as compared to people drinking deep well water [139]. Many cyanotoxins are persistent and they are capable of bioaccumulation [99].

The cyclic peptide toxins microcystins are hepatotoxins and tumour promoters produced by freshwater cyanobacteria, including genera *Microcystis, Anabaena* and *Planktothrix*, which commonly form mass occurrences in eutrophic surface waters. The presence of MCs in drinking and bathing waters has been recognized as a human health hazard by the World Health Organization and a provisional guideline level for MC-LR equivalents in drinking water has been introduced at 1 μg/l [116]. It is already known that acute exposure to high MC concentrations causes liver damage [49]. Chronic exposure is believed to promote liver tumour formation [52], and MCs are risk factors in the development of liver cancer. Nodularin is not only hepatotoxic and tumour promoter, but also direct carcinogen

[140]. The Baltic Sea cyanobacterium *Nodularia spumigena* contains practically always Nod-R which bioaccumulates into Baltic Sea biota.

In the total risk assessment concerning toxic cyanobacteria and human health one needs to consider the possibility of multiple toxin producer genera and toxins. The assessment of safe concentrations of a toxin in drinking water is not straightforward: the toxins within a natural bloom extract better simulate the natural exposure route for these toxins than purified toxins, but the synenergism from other compounds, or therefore the effect of the toxins themselves cannot be assessed [99]. In the assessment of long-term exposure effect of cylindrospermopsin Humpage and Falconer [141] found some differences when administrating an extract or purified toxin to Swiss albino mice. A natural bloom sample may be constituted of various bioactive compounds, depending on the genera and taxa of the cyanobacteria in the sample, growth environment, etc. Therefore the bloom sample might not be comparable to clean toxins, or to other type of bloom materials.

1.4.2. Dose responses

Detailed information on the exposure to cyanobacteria and their toxins (concentrations) are almost always lacking in the reported human illness and death cases. Therefore health hazard assessment relies on studies done on animals, and dose-effect relationship requires some extrapolation. The World Health Organization recommendation is max. 1 μg of MC-LR/l in drinking water [116].

The WHO provisional guideline value of 1 μg/l of MC-LR is derived from information on two separate studies with 1) mice [142] and 2) with pigs [117]. The study on mice was performed for 90 days with pure MC-LR by gavage [143]. In the study on pigs, the toxicological assessment of the study was well planned. The total concentration of MCs in the extract the pigs were exposed to, was determined with mouse bioassay, ELISA assessment and protein phosphatase inhibition assay, all giving different values. In the HPLC analysis, it was said that none of the peaks in the chromatogram correspond to the standards of MC-LR or MC-RR, and that the major peak was tentatively identified as MC-YR. All of the different methods supported the conclusion that the toxicity to pigs was due to hepatotoxins in the sample, but the existence of other possible toxins was not ruled out. Uncertainty factors were used to determine a guideline value for the most potent and best-studied variant of the hepatotoxins, MC-LR [117].

The WHO guideline value has been derived based on a no-observed-adverse-effect-level (NOAEL) for mice and pigs, 40 μg/kg body weight of MCs, utilising uncertainty factor of 10 for 1) inter-species extrapolation, 2) intra-species variation and 3) less-than-lifetime-exposure, together adding up to a uncertainty factor of 1000. The calculations are made with following assumptions: adult body weight of 60 kg, drinking water consumption of 2 l daily, and an allocation factor for the exposure to be mainly from drinking water 0.8 [144]. The relevance of WHO´s guideline value to a long-term exposure in humans has been criticised, as it has been based on studies on pigs for only 44 days. In China, where the problem of

liver cancer and other related diseases is public concern, researchers Ueno and co-workers [52] suggest the recommendation for safe drinking water (taking into account long term exposure) to be lowered to a value under 0.013 µg/l of MCs. Ibelings and Chorus suggest that the allocation factor of 0.8 for drinking water is overestimated considering the possible high amount of toxin exposure resulting from contaminated food (mussels, prawns, fish etc. caught from the contaminated water bodies) [99].

A similar guideline value as for the MC-LR, of 1 µg/l for cylindrospermopsin has been proposed by Humpage and Falconer, based on a study on Swiss albino mice for 10-11 weeks [141].

1.4.3. Possible routes of human exposure

Possible routes of human exposure to cyanobacterial toxins include drinking or recreational water, contaminated food (e.g. shellfish, fish, and plants) or dietary supplements [104]; inhalation of contaminated water during showering and sauna or recreational use of water bodies, as well as skin contact during the same activities [145]. It is of importance to notice that not all drinking water treatment processes (e.g. boiling) are efficient in removing cyanobacterial toxins such as MCs from water. Activated carbon, ozone or reverse osmosis treatment is required [146].

The MCs and Nods are intracellular toxins, and the greatest toxin concentrations in water occur during cyanobacterial cell lysis, in example during bloom clearance by e.g. chemical agents, such as copper sulphate. Some of the reported (short term) human exposure incidences can directly be linked to bloom clearance actions in drinking water reservoirs (reviewed in [147]).

The accumulation concentrations in animal tissues are reviewed in section 1.3.2. Ibelings and Chorus [99] have reviewed the possibility of MC exposure through contaminated aquatic animals used as food, such as mussels, fish and prawns, and found that the tolerable daily intake value provided by the WHO is exceeded in many scenarios, both for the long-term as well as for the short-term exposure. They have compiled a table of reported toxin concentrations on several aquatic animals used as food [99]. They also calculated the relation of the possible exposure to the toxins by this contaminated food and the tolerable daily intake value for lifetime daily exposure. The amount of toxins reported in the literature for different tissues resulted in a relation of

- in fish from 0.03 up to 42.1,
- in mussels from 0.3 up to 23.5, and
- in crayfish and shrimp from 0.05 up to 14.3

times the tolerable daily intake value for an adult weighing 60-70 kg [99]. The accumulation of toxins from water sources into mussels and snails and from thereon to edible parts should therefore be taken into account when calculating total exposure to MCs.

As the cyanobacteria cause adverse effects also during recreational use of water bodies, visibly contaminated water bodies should be avoided, whether toxicity has been proven or not [45].

1.4.4. Prevalence and concentrations of peptide hepatotoxins

Hepatotoxin producing cyanobacteria have been reported from all the continents of the world (including Antarctica [148]). The blooms are often seasonal, usually having their peak in the warm summer months [55]. However, long-term blooms that may cause continuous long-term exposure can be considered even more hazardous to human health. Throughout the year lasting blooms have been reported from warm countries such as Brazil [149] and India [68]. Eleven different cyclic MCs and additional linear peptides were reported from a single bloom sample by Namikoshi and co-workers [150]. The versatility of MC variants in blooms makes the toxicity assessment more intricate.

The most harmful group of toxins as possible health hazard to humans is considered to be the cyclic peptide hepatotoxins MCs and Nods. They are especially of concern because they are not easily degraded: the toxins remain intact even after boiling. The other, nonpeptidic, toxins, such as the neurotoxic anatoxin-a, anatoxins-a(S) and saxitoxins do not remain in the water for as long, but are degraded e.g. by sunlight [55]. Cylindrospermopsins are described to be relatively stable toxins [55]. Furthermore the cyclic peptide toxins are found in animal and plant tissues as well as drinking and recreational water, and therefore multiple possible exposure routes exist.

1.4.5. Examples of reported incidents of cyanotoxins exposure

Caruaru dialysis center incident that claimed the lives of 76 people due to exposure to cyanotoxins through renal infusion is the best-studied case of human exposure to cyanotoxins [119, 121, 151]. New information on the long-term exposure to MCs has been provided by the study by Chen and co-workers [118]. In the incidents described in section 1.3.2.3, the tissue samples were verified to contain MCs, and the concentrations had been determined by methods available. However, several incidents, where MC exposure has been shown or presumed, have been reported, although no tissues were analysed to verify MC contamination.

In a Brasilian incident in 1988 affecting about 2000 persons, of which 88 died, the cause for toxicosis was with great propability determined to be toxicosis from MCs via drinking water [48]. Blood and fecal samples as well as samples from drinking water were subjected to virologic, bacteriologic, toxicologic and heavy metal analysis, and the cause was concluded to be compounds produced by cyanobacteria. However, the analytical possibilities for verification of MCs in the samples did not exist [48].

In Palm Island in Australia in 1979 150 people required medical attention after a heavy bloom in a drinking water reservoir [50, 51]. The bloom had been terminated with copper sulphate and the hospitalisations started to occur the following week. The causative agent was not identified with certainty, but the effects differ from the ones reported for MC exposure, and cylindrospermopsins have been suggested to have been responsible [152].

In Armidale, Australia, in 1981 water reservoir that was used for drinking water supply was treated with copper sulphate to terminate a heavy cyanobacterial bloom causing bad taste and odour. The population receiving the water from this reservoir and a population in the same area receiving their drinking water from another source, were screened for liver function indicators in plasma samples. The sampling occurred in time periods of six weeks, before, during, and after the bloom termination. The samples of individuals receiving their drinking water from the contaminated water supply showed significant increase in gamma glutamyl transferase during the bloom and its termination, indicating substantial liver damage [153] (reviewed in [147]).

In a very interesting case gastroenteritis that occurred in southern Sweden, drinking water line had been by accident connected to an untreated lake water line. Of the people using the water, the people drinking only coffee made of the water did not become ill (the water filtrated through the ground coffee beans) whereas people drinking tea (with the boiled water only flavoured with dipping the tea bag inside) did become ill [53]. This shows how the MCs (produced by *Plankttothrix agardhii*) are not degraded even by boiling the contaminated water [53].
In [147] cases of exposure to cyanobacterial toxins by recreational use of water bodies have been reviewed by Kuiper-Goodman and co-workers. The exposures were described to have happened by swimming and by canoeing (with eskimo-rolls). The effects of MC exposure have been described in general as nausea, vomiting, diarrhoea, irritation of skin and eyes, and in two cases pneumonia that required intensive care in a hospital [54].

Human risk assessment is still hampered by the lack of knowledge, and by the lack of specific detection methods that would be suitable for multiple types of matrices. A clear dose-response relationship has not been determined for humans.

1.5. Methods and sample preparation for quantitative analysis of cyanobacterial peptide hepatotoxins

Cyanobacteria appear in many different cell forms and structures. It is impossible to determine the toxicity of the strain by just looking at its appearance. Therefore methods for toxicity determination are required. The success of any analysis is dependent on the sample treatment performed. In an inter-laboratory study participated by 31 laboratories from 13 countries it was found that the various laboratory methods for detection and sample preparation of MCs and Nods gave satisfactory precision on cyanobacterial bloom field sample analysis. However, standardisation of methodologies would be required for comparable results [154].

1.5.1. Sample preparation for microcystin and nodularin analysis

The success of any analysis is highly dependent on the ability of the sample preparation to extract the toxins to be analysed. For different purposes and sample types some differences in sample preparation exist. However, the main point is to concentrate the toxins in the sample, at the same time as removing some of the disturbing sample matrix (sample clean-up) by solid phase extraction.
In bloom sample analysis the sample can be filtered, and the cyanobacterial material on the filter analysed, since the cyanobacterial peptide toxins exist in intact cells. The toxins become available for extraction by cell lysis. Therefore the cells need to be disrupted before extraction for example by freeze-drying and ultra sonication. Probe sonication was found to be best alternative for this purpose by Rapala and co-workers [155]. MCs and Nods have commonly been purified from water and cyanobacterial bloom samples by extraction with methanol:n-buthanol:water (20:5:75) [156], but also water (with or without acidification) and different concentrations of aqueous methanol have been employed [155, 157-159]. Fastner and co-workers found that for extracting cyanobacterial bloom samples 75% aqueous methanol is the most efficient solvent [160]. Sample preparation methods for MC and Nods have been reviewed by Smith and Boyer, and the information nicely collected into a table in [161].

Biological tissues, with usually low levels of toxins present, are difficult and variable matrices, for which the extraction efficiencies are highly dependent on tissue type. Tissues must be homogenised, the sample structure needs to be broken down (intricate in the case of rigid structures such as plant and feather material) and some additional sample preparation steps might be required, for example protein precipitation for serum samples. Tissue sample preparation requires more efficient concentration of the sample. Also, sample preparation steps for several samples should be performed at a time in the interest of saving time. For these purposes and especially for better overall cleanup one nowadays uses solid phase extraction (SPE), eg. reversed-phase C-18 material [157], CN - material [162] hydrophilic-lipophilic-balanced (Waters Oasis HLB) cartridge material [155] and immunoaffinity SPE [163].
A rather new and still costly SPE method utilises Molecular Imprinted Polymer (MIP) material [164].

1.5.2. Detection methods

1.5.2.1. Biological tests

A large amount of the knowledge collected in the past about MC and Nod prevalence and toxicity has been obtained by mouse bioassay. Pathological findings have been supported by organotropism studies made with radiolabelled toxins [165]. These studies have shown that the liver is the main target of MC and Nod [79]. However, the use of mouse bioassay is no longer encouraged due to the apparent drawbacks of it being unethical, unspecific and insensitive. The other biological assay often encountered in the detection of MCs is the brine shrimp (*Artemia salina*) biological assay, but this method as well suffers from being nonspecific and insensitive [98, 166].

1.5.2.2. Biochemical methods

Nucleic acid based methods

Polymerase chain reaction (PCR) has been used for cyanobacterial detection purposes for microcystin producing strains. The detection is done either with general primers for the peptide and microcystin synthetases, or with more specific primers targeting specific (one or several) of the peptide synthetase *mcy* genes. The *mcy* genes are from a gene cluster responsible for toxin production enzymes [167]. Quantitative PCR has the advantage of revealing the main producer of the toxins in a more complicated mixture of cyanobacterial strains in a sample. In quantitative PCR as well, the primers used have been targeting specific genes in the peptide synthatase gene cluster *mcy*A, B, E, and or D [168]. DNA microarrays have recently been utilised for the analysis of environmental water samples for MC and Nod production [169]. The DNA microarray chips offer an advantage over the more traditional genetic methods by quickly producing large amounts of data in an automated fashion.

The nucleic acid based tests are only applicable to determine the existence of toxic/ nontoxic cyanobacterial strains in water samples, not the toxins concentration, nor the possibility of toxins in other tissues than those containing cyanobacterial cells and genetic material.

ELISA

The most commonly used method for MC and Nod detection is most probably the ELISA (enzyme-linked immunosorbent assay) that is based on the detection of the specific interaction between antibodies and analytes, in this case the MCs and Nods. The antibodies are often developed against conjugates of the Adda-amino acid [170] or the most common MC variant MC-LR [171-172] or MC-RR [173]. ELISA is very sensitive detection method for water samples, but is not intended for other sample materials such as cyanobacterial bloom or animal tissues.

ELISA detection has some limitations such as the unequal cross reactivity between different variants of MCs and Nods that affects quantitation, as well as cross reactivity with sample matrix that causes problems in both qualitative and quantitative analysis. The ELISA is designed to be used with water samples, and the low detection limits enable water samples directly (without concentration) to be analysed in order to determine whether the WHO guideline value of 1 µg/l of

MC-LR has been exceeded or not. However, as the detection is based on molecular recognition and not toxicity, the different variants give different responses, but the response difference cannot be connected to toxicity. On the other hand, the possible matrix effects from different types of tissues can be severe, especially at lower levels of toxins where the sample cannot be diluted well. Therefore, all the different matrices should be assessed with controls and spiking studies before drawing conclusions of levels of MC or Nod.
ELISA can be done by two different methods, by direct competitive or by indirect noncompetitive way [174].

Protein phosphatase inhibition assay
The protein phosphatase inhibition assay (PPIA) is based on the inhibitory potency of MCs and Nods towards the known targets protein phosphatases. The assay is not recommended for analysis of complex tissue samples, because of the potential matrix interference towards protein phosphatase activity (by tissue components or other toxins such as okadaic acid), as well as masking of the inhibitory effects from the MCs by the sample's endogenous phosphatase activity [155].
The PPIA can have colorimetric detection (utilising dephosphorylation of p-nitrophenyl phosphate) or radioactively labelled ^{32}P, or by competitively binding to (^{125}I)MC-YR [174].

Both of the above-mentioned assays, ELISA and PPIA, are group-selective methods, and therefore the different variants cannot be separately identified and quantified. Furthermore the response for these analyses by the different variants may vary significantly, and also cross-reactivity with the matrix may potentially cause false positive results or mask the toxins from being detected [175].

1.5.2.3. Physico-chemical detection methods

HPLC
In HPLC the separation is most commonly achieved with reversed-phase (RP) C-18 stationary phase columns [176]. Modified RP-columns, such as the RP-amide column with amide functional group, has been shown to have its advantages in the separation of MCs [177]. UV- (e.g. DAD) detection of MCs and Nods is based on the characteristic absorbance spectra of the Adda amino acid in the structure. Trans-conjugated diene structure in Adda is responsible for the absorbance maximum at 238 nm. The residue Mdha contributes to the spectra as well.

Traditionally, the column performance in the separation of MCs and Nods have been tested by looking at some rather intricate pairs of MCs to separate, the pairs dmMC-LR/MC-LR, MC-LR/MC-YR and MC-LW/MC-LF. The task of separating a certain pair of MCs is not a trivial question, as the MCs have a rather wide range of hydrophobicities [178] and require a wider gradient in order for the whole range of toxins to be resolved.
The mobile phase solvents have traditionally been water-acetonitrile, or, in some cases water-MeOH [179], but this has been shown to produce wider, less intense peaks with peptidic analytes in general [9]. The pH in HPLC separation is commonly acidic in order to protonate the carboxylic groups present in the analyte. The acid is commonly either trifluoro acetic acid (low light absorbance and therefore suitable for UV-detection, but not suitable for MS because of ion

pairing) or formic acid (suitable for MS detection, not suitable for UV detection because of strong absorbance). Isocratic separation is still preferred in some laboratories, but gradient runs have benefits in separation of variants with variable hydrophilicity.
The addition of ammonium acetate may give advantages in resolving the demethylated variants from the methylated corresponding ones, such as dmMC-LR from MC-LR [155].

The great number of closely related variants make the separation and detection of the different variants more complex. Eleven different cyclic MCs and additional related linear peptides were reported from a single bloom sample by Namikoshi and co-workers [150, 180]. The lack of suitable standards causes problems with any type of analysis. The standards are required for the assessment of the compound behaviour, especially in complex matrices. There is a requirement of standards for the HPLC analysis, since the different variants are detected based on their retention time and UV-absorbance spectra. There are no major differences in the absorbance spectra for many of the variants, and therefore the different MCs cannot be distinguished based on the spectra [181]. However, MCs containing tyrosine (Y) or tryptophan (W) have additional functional groups contributing to the absorbance spectra and can this information can be utilised in toxin identification [176].
Early work on HPLC MC separation was conducted by [182] and [183] and in Prof. Harada's laboratory [157, 184]. From that time instrumentation and methods have developed towards faster analysis and higher throughput [176-177] and ultra high-performance separation with small particle size columns has recently been utilised by several research groups, by Wang and co-workers [185], Xu and co-workers [186], Ortelli and co-workers [187], and Oerhle and co-workers [188] as well as in our laboratory [189].

LC-MS
Complete separation of the different toxin variants is not a requirement in MS, since the analytes can be separately detected by their *m/z* ratio. No toxin-class specific probes are required. This also means that different toxin species even from different toxin classes can be quantitatively analysed in one analysis. Highly reliable analysis of natural products with LC-MS is often hindered by the lack of suitable standards. The lack of standards is especially apparent in areas of analysis where the analyte is as complex as the MCs and Nods: most toxins, and especially the isotopically labelled ones are not currently available. The lack of suitable well-defined standards of even the unmodified MCs have been acknowledged by many researches [175, 190-191]. However, the requirement of an internal standard is not plausible to be fulfilled for each toxin in a cyanobacterial extract. Therefore external standards (as standards in adjunct runs) of the compounds themselves (of the ones available) should be used.
LC-MS is an established tool in MC and Nod detection in water samples [176, 192] and in tissues [158], and some structural studies have also been conducted with MS instrumentation [65, 72, 193-194].

In general the hepatotoxin detection has developed in recent years especially due to the advances in HPLC-MS, but also in the qualitative analysis in bloom samples in the area of nucleic acid based detection methods.

MMPB –method
As described in section 1.3.2.5., the 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) method by [135] is used for the assessment of the total amount MCs in tissues, bloom samples and sediments (Figure 1.6 in section 1.3.2.5., formation of MMPB from MCs). With the MMPB method also the otherwise inaccessible covalently bound MCs can be determined from different tissues.

All MC variants are detected with equal intensity, since all MC variants containing Adda give rise to one MMPB molecule, i.e. not at all related to the toxicity levels of the different variants. With MMPB -method both the covalently and noncovalently bound toxins are detected.

The MMPB method can be performed with the Lemieux oxidation [135] using oxidative chemicals such as potassium permanganate and sodium perjodate at basic pH, or by ozonylation [195]. After the chemical reaction, oxidation, has been quenched with acid and some reducing agent, such as bisulfite and acid, the MMPB containing sample is cleaned up by liquid-liquid [131-132, 196] or solid phase extraction (SPE) [121, 197], and in case of GC-MS analysis derivatised to MMPB methylester with the help of trifluoroborate before detection [136].
The MMPB is then detected with LC-MS (or GC-MS) system. One can use MMPB-d_3, 5-phenyl valeric acid (5-PP) or 4-phenylbutyric acid (4-PB) as internal standards in the analysis.

2. Aims of this study

The overall aim of this project was to develop quantitative liquid chromatography - mass spectrometry (LC-MS) detection method for cyanobacterial peptide hepatotoxins present in animal tissues and biofluids. Thereon, these methods were used to answer relevant biological questions concerning exposed samples. The exposed samples were provided by collaborators (Drs. Harri Kankaanpää, Vesa Sipiä and Emilie Lance). The samples were used for both method development and validation and further on for analysis of the contaminated tissues. The sample preparation and LC-MS analysis were performed at instrument facilities at the Åbo Akademi University and at the University of Turku Instrument Centre. The first part of publication V was planned and performed at the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Canada.
The methodology used was developed further by

1) improving sample preparation
2) improving chromatographic separation and speed with higher efficiency fast chromatography
3) improving MS detection methods (including SRM transition and instrument parameter optimisation)

The methods used for the purpose of tissue analysis included extraction of the toxins, and chemical oxidation of the tissue to release the Adda-derived fragment MMPB. The resulting extracts were analysed with LC-MS equipment.
The sample preparation both for the free and covalently bound toxins was optimised from the existing methods in our laboratory. In LC-MS detection, low instrument performance was noticed with the most difficult samples. After the introduction of new instrument technologies the existing detection methods were optimised for faster and more accurate analysis. The test samples were first extracted and SPE-processed cyanobacterial bloom samples (publication IV) and then more intricate spiked serum samples (publication V).

The MS instrumentation used included a QQQ, a quadrupole ion trap, a spherical ion trap and a quadrupole-TOF instrument. MS instruments from three different instrument manufacturers (Waters (Micromass), Bruker and Applied Biosystems) were therefore used.

Specific aims

Publication I
Assessment of nodularin concentrations in eider duck feathers, livers and in their food supply, Baltic Sea blue mussels. The methods employed were ELISA and LC-MS. This was the first report of the cyanobacterial hepatotoxins (this case toxin Nod-R) detected in feathers with LC-MS instrumentation. Previously MCs had been reported in feathers in one publication, the detection therein was done with HPLC and ELISA. The detection method in publication I was LC-ESI-MS-MS (QQQ).

Publication II
Optimisation of MMPB method (sample preparation and LC-MS methodology) to be used in aquatic snail tissues and calf serum. The method assesses the total amount of microcystins, free form together with the covalently bound MCs in tissue. The extraction procedure for free MCs for the same tissues was assessed as well. The detection method in publication II was LC-ESI-MS-MS (QQQ).

Publication III
Utilisation of the optimised method described in publication II for the analysis of total, covalently bound and extractable MCs in aquatic snails exposed in naturally relevant concentrations in laboratory conditions. The detection method here was LC-ESI-MS-MS (QQQ). Confirmatory analysis was done with ELISA.

Publication IV
Analytical LC columns of different stationary phase materials were compared for the fast separation of 13 variants of MCs and Nods. The purpose was to test the suitability of fast resolution columns to the separation of MCs and Nods. The detection method here was LC-ESI-MS (spherical ion trap).

Publication V
Separation and accurate mass detection of MCs and Nods from spiked serum samples by utilising a small particle size column for separation and an accurate mass spectrometer for analyte detection. The choice of column was partly based on the study conducted in paper IV and the study done in the first part of publication V. The detection method in publication V was LC-ESI-MS (Q-trap; column selection) and LC-ESI-MS-MS (Q-TOF; spiked serum study).

3. Experimental procedures

3.1. Materials

Chemicals used in the laboratory work, e.g. for the oxidation in the MMPB method, are listed in the publications. The MMPB standard is available from Wako Pure Chemical Industries, Ltd (Osaka, Japan).
Solvents and solvent pH modifiers were of HPLC/LC-MS grade from known suppliers. Chromatographic mobile phase was typically water (purified to 18.2 MΩ cm (Milli-Q) in a Millipore, Bedford, MA, USA system) and acetonitrile acidified with 0.1 % formic acid.
The first part of publication V was conducted with mobile phase composed of water and acetonitrile, both modified with 50 mM formic acid and 2 mM ammonium formate (pH 2.3). In second part of publication V different mobile phases were tested: mobile phase consisting of aqueous part modified with formic acid and with/ without ammonium formate and organic part, acetonitrile, modified with formic acid. These mobile phases were tested for differences in separation and detection in the LC-MS system.

Cyanobacterial toxins were purified (by Lisa Spoof and Jussi Meriluoto) from cultured or field-collected cyanobacterial material. Toxins produced by strain PCC7820 have been reported to be MC-LR, -LY, -LW and –LF [198] and from strain NIES-107 mainly 3-demethyl-MC-RR, MC-RR, MC-YR, 3-demethyl-MC-LR and MC-LR (partially reported in [199]). MC-LA was purchased from Alexis Biochemicals (Lausen, Switzerland). The Nods were purified from Baltic Sea *Nodularia* [158] and identified in accordance with the fragmentation patterns published by Mazur-Marzec and co-workers [194].
Cyanobacterial bloom material used in publications II-IV was collected from Sulejow reservoir in Poland in 2002 by Dr. Tomasz Jurczak, and identified to contain *Microcystis aeruginosa* and *M. wesenbergii* [200]. The main toxins in the Sulejow material were MC-RR, MC-YR, MC-LR, MC-LA and MC-YA.
The used standard and sample mixes are described more in detail in publications.

3.2. Instrumentation

The work described in publications I-III were carried out on an Agilent 1100 binary pump HPLC system (Waldbronn, Germany) coupled to a Quattro Micro (from Waters, Manchester, UK) QQQ MS with an ESI source. The separation was achieved on a Purospher STAR RP-18e (Merck, Darmstadt, Germany) 30 mm × 4 mm i.d. column with 3 μm particles, thermostated to 40 °C.

In publication IV the instrumentation consisted of an Agilent Technologies (Waldbronn, Germany) 1200 Rapid Resolution (RR) LC coupled to a Bruker Daltonics (Bremen, Germany) HCT Ultra Ion Trap MS with an ESI ion source. The 1200 RR LC system included a model SL binary pump, vacuum degasser, model SL autosampler (set at 10 °C), and model SL column oven thermostated to 60 °C.
A similar LC system was used in publication V, coupled to an 1) Applied Biosystems 4000 QTRAP (Sciex, Canada, at the National Research Council, Halifax, Canada) Q-Trap MS, and 2) micrO-TOF (Bruker Daltonics, Bremen, Germany) Q-TOF MS.

In both studies (IV and V) multiple columns were used. The columns tested in publications IV and V are listed below:

- Ascentis Express from Supelco,
- Fortis C-18 from Fortis Technologies,
- Zorbax RR Eclipse plus and Zorbax RR Stable Bond from Agilent Technologies,
- Chromolith FastGradient from Merck,
- Synergi Fusion-RP, Hydro-RP, MAX-RP and Polar-RP from Phenomenex
- Waters Acquity BEH C-18 from Waters.

Column specifications are described in detail in publications IV and V.

3.3. Methods

3.3.1. Preparation of tissue samples for extraction of toxins from feather, liver, mussel, aquatic snail and serum samples (publications I-III, V)

Sample homogenisation

Sample preparation started with a careful dissection and homogenisation of the sample tissue. This part was mostly performed by our collaborators according to our instructions. The freeze-dried material is the easiest to work with for quantitative analysis, since in the dry material the different water contents in the sample does not compromise comparison between different samples.

Extraction

In general the sample preparation started with extraction of the toxin with or without the help of 1) probe sonication 2) bath sonication followed by centrifugation. The supernatant taken to analysis was evaporated, and/ or some water added in order to reduce the solvent strength before the sample is introduced to solid phase extraction (SPE) cartridges. In case of (fresh, i.e. not freeze-dried) serum, sample preparation differed from the other tissues in that the protein precipitation and extraction was combined and no sonication was used. The protein precipitation step was performed with acetonitrile acidified with TFA. Extraction was performed as described in detail in publications I-III, V.

3.3.2. Preparation of aquatic snail and serum tissue samples for the assessment of covalently bound microcystins with the MMPB method (publications II and III)

The tissue samples were treated with the following steps in the MMPB method:

1) trypsination
2) sample oxidation
3) quenching of oxidation
4) sample clean up with centrifugation and SPE
5) sample reconstitution into LC-MS applicable solvent

The samples were homogenised and weighed before protein cleavage by trypsination, which was performed at 37°C in phosphate buffer pH 7.5, for two hours under shaking.

The sample was oxidised for three hours, pH 9.0, in room temperature, with 0.1 M potassium permanganate and 0.1 M sodium perjodate. The reaction was quenched with 40% sodium bisulphite and 10% sulphuric acid and the sample mix then centrifuged. The supernatant was cleaned-up and concentrated by solid phase extraction (Waters Oasis HLB 30 mg cartridges). The final eluant of the SPE was evaporated to dryness on a heat-plate under argon flow, and the residue dissolved into 35% MeOH for LC-MS analysis. A flow chart of this process is given in publication II.

3.3.3. Chromatographic separation and MS detection of the hepatatoxins and MMPB

HPLC

The utilised LC-MS instrumentation was as described above (QQQ, Trap, Q-Trap and Q-TOF) coupled to HPLC instrumentation. MCs were separated with gradients typically with following conditions: mobile phase consisted of water and acetonitrile modified with formic acid with or without ammonium formate. The gradients started from 25% to 35% acetonitrile and were risen to 65% to 75 % acetonitrile before a washing step from 80% to 90% acetonitrile. Flow rate was typically 0.5 to 1.1 ml/min, the washing and equilibration was done with a higher flow rate (flow directed to the waste) when possible. The gradient specifications are described in more detail in publications (I-V).

MMPB was separated with a mobile phase system of 0.1% FA in water (A), and AcN (B), with the following gradient: from 40% to 70% B over 3 min, increase of B to 90% B over 0.1 min, which was held for 1 min, flow rate 0.5 ml/min, followed by equilibration at 40% B for 4 min, flow rate 1 ml/min.

Mass spectrometry detection

The MS parameters are described in detail in the publications. The most important parameters, the ion selection modes were:

Either single ion recording (SIR) or multiple reactant monitoring (MRM) mode with the QQQ and Q-trap. In SIR the monitored ions were commonly $[M+H]^{1+}$ or $[M+2H]^{2+}$ (MC-RR and dmMC-RR). In the MRM mode the monitored transitions were from $[M+H]^{1+}$ or $[M+2H]^{2+}$ to the characteristic fragment of *m/z* 135 from Adda amino acid. In both cases the monitoring occurred with a mass window of 1 Da. MMPB detection was carried out in the MRM mode with the strongest transitions from *m/z* 209 to *m/z* 91, 131 and 191;

Scanning function with scanning range from *m/z* 300 to 1200 was used in the ion trap instrument in order to include both the doubly and singly charged ion species;

The detection mode with the Q-TOF MS instrument was scanning function with automatic MS-MS mode with the scan range from *m/z* 50 to 1500. The range from lower *m/z* values was included in order to record the whole fragmentation pattern with the MS-MS function.

4. Results and discussion

The aim of this project was to develop and optimise LC-MS methods for the detection of cyanobacterial peptidic hepatotoxins from complex biological tissues. This goal is important for the determination of toxin exposure and contamination levels in different organisms. The biological tissues studied during the project, feathers and snail tissues, were analysed to answer relevant biological questions concerning toxin accumulation. These tissues as well as spiked serum samples were used to develop the methodology further. In the end, this knowledge may provide tools for the correct and accurate assessment of ecological effects and risks to human health by these toxins.

The results described here highlight the main results presented in detail in publications I-V.

4.1. Spiking studies

During the project it became obvious that the spiking studies play an important role in the valid detection and correct interpretation of the results. The instruments are getting better in separation of disturbing compounds, by better ionisation efficiencies and mass accuracies. Therefore, the statistical calculations on matrix effects, of signal suppression or enhancement will most probably become easier, but still should not be forgotten.
The spiking studies are essential in the estimation of the true signal-to concentration relationship in the studied complex matrices.

In case of the natural samples, it was impossible to perform the relative matrix effect assessment (the difference of matrix effects between specimens of the same species [15-16]) by spiking studies. The assessments of absolute matrix effects, i.e. matrix effect of a sample (control material) to a standard solution, were made by spiking on selected samples, controls, either on a blank sample or on sample where the toxin concentrations was determined to be below LOD.
Spiking was performed in the beginning of the sample treatment procedure, into untreated samples, and after sample clean-up in order to estimate recovery from the extraction, as well as to assess the matrix effects. The samples spiked with a known concentration of MCs and Nods in the beginning were compared with the samples spiked with the same concentration of the toxins after clean-up (in the end) for the assessment of extraction recovery. The samples spiked in the end of the sample preparation were compared to standards without any matrix in order to assess the matrix effects. [16]. Results for the different spiking studies are described below (see sections 4.3. and 4.4).

4.2. Extraction of nodularins from eider duck feathers (publication I)

Feather matrix was a laborious tissue type to handle, due to the difficulties in homogenising the sample. The long (2×1h) extraction periods in an ultrasonication bath were considered necessary for adequate toxin recovery, but could not be

tested due to the nature of the samples. Spiking studies could not be considered as reliable tool for the determination of extraction efficiency from the rigid structures, but were used in matrix effect determination. The values for Nod contamination were in correlation with the values determined for the liver tissue (see table 4.1). However, some differences were seen in ELISA and LC-MS results for the feather samples. The differences were most probably due to the fact that the analysed feather samples were not the exact same extracts, or even the exact same feathers between the two analysis methods, although from the same individual bird. Furthermore, utilisation of two different types of analytical methods, biochemical and physicochemical, is a source for differences in analysis performance, for example by differences in matrix disturbance.

Table 4.1.
Nod in eider duck tissues, livers and feathers, as determined by LC-MS. Confirmatory analyses were made with ELISA.

Species (sex)	Sample	Sampling time	LC-MS (μg/kg DW)	ELISA (μg/kg DW)
Eider (male)	Liver	3th June 2005	2.7	-
Eider (female)	Liver, Feather	28th Aug. 2005	7.7*, 12**	48.9**
Eider (female)	Liver, Feather	28th Aug. 2005	31.7*, 18**	-
Eider (female)	Liver, Feather	29th Aug. 2005	11.7*, 8**	-
Eider (female)	Liver, Feather	29th Aug. 2005	5.6*, 15**	-
Eider (female)	Liver, Feather	29th Aug. 2005	4.7*, 11**	-
Eider (female)	Liver, Feather	3rd Sept. 2005	47.6*, 43**	52.1**
Eider (female)	Liver, Feather	9th Sept. 2005	3.4*, 16**	6.5**

* Concentration in liver
** Concentration in feather

The bird feather tissue analysis may be regarded comparable to human hair analysis, and is of interest to a biologist assessing the toxins in the ecosystem. The feather analysis would be a non-invasive method for the survey or screening of exposure levels in eider ducks.
Futhermore, the eider ducks flesh is consumed by humans and the assessment of toxin, Nod, accumulation into eider ducks is therefore of relevance to human health. Nods have been shown to exist also in the eider duck muscle tissue in minor amounts 21 ng/g [111]. The exposure levels should therefore be assessed to assure that these carcinogens are not consumed by humans.

4.3. Extraction of free microcystins (publications II, III and V)

Sample preparation plays a crucial role in the analysis. One of the key elements is sample homogenisation before further sample treatment. After the analysis of aquatic snails tissues it was noticed that homogenisation of the tissue in liquid with Ultra-Turrax works well, either prior (to enable real homogenisation) or after freeze-drying (the problem of weighing heterogeneous sample material remains). The weighing of the freeze-dried material is somewhat difficult at times. The material may adhere to the tube walls and the particle density (weight related to size) differed quite substantially between tissue pieces. As a general rule even the samples from the same tissues of the same species may differ considerably

depending on exposure media or alternative feeding, as was noticed with the freeze-dried aquatic snail tissues.

The optimised extraction procedure for different tissues consisted of the following: Extraction of freeze-dried soft tissues of snails, fish livers, etc. with H_2O: MeOH: BuOH; 75:20:5, bath and probe sonication of the sample, solid phase extraction with a Waters Oasis HLB 30 mg cartridge, a washing step with 20% aqueous MeOH and elution with TFA-acidified 90% MeOH. The optimised procedure for the extraction of free MCs from aquatic snail tissues is presented in a workflow description in publication II.

The processing of the biological fluid, calf serum, consisted of an initial protein precipitation step with 80% acetonitrile containing 0.1% TFA, followed by the evaporation of the sample to dryness in glass vials under argon flow at 40°C, and then reconstitution in water for SPE. The SPE was performed with a Waters Oasis HLB cartridge with a wash step with 15% aqueous methanol containing 0.1 M acetic acid (HOAc) and elution with 80% methanol containing 0.1 M HOAc.

Table 4.2
Extraction efficiency from spiked serum and aquatic snail samples

Extraction efficiency / %					
Sample	Spiked with 100 µL of	MC-RR	MC-LR	MC-YR	Average
Serum 1 mL	Natural bloom extract	100.2	86.1	87.2	91.2
	Natural bloom extract 1:10	86.7	78.9	80.9	82.2
	Natural bloom extract 1:100	83.9	73.2	84.0	80.4
Serum 0.1 mL	Natural bloom extract 1:2	90.8	86.1	82.7	86.5
	Natural bloom extract 1:20	80.8	74.0	86.3	80.3
Snail 10 mg	Natural bloom extract	78.1	76.8	77.3	77.4
	Natural bloom extract 1:10	51.0	56.8	58.5	55.4
	Natural bloom extract 1:100	66.7	57.2	73.8	65.9
Average from different matrices and concentrations		79.8	73.6	78.8	77.4

The cyanobacterial natural bloom extract used for spiking was from Sulejow reservoir containing 0.6 µg MC-RR, 0.6 µg MC-LR, and 0.4 µg MC-YR. Dilutions of this mixture (1:2, 1:10, 1:20, 1:100) were used for spiking the samples. The values are averages from SIR and MRM measurements.

The methods described above gave good extraction efficiencies on spiked tissue samples (77% on average) for the free, extractable toxins (Table 4.2). The recoveries are in this selection best for the variant MC-RR (up to 100%) but in

general very similar (on average between 74% and 80%) for the different arginine-containing MC variants. Lower concentrations have lower recoveries. The recovery should always be checked for each tissue type separately, since the recoveries are dependent on many parameters in tissue, such as fat and protein content.
The concentrations of free, extractable MCs from aquatic snail tissues (determined by two methods, ELISA and LC-MS in publication III) are given in table 4.4.

The concentrations of the extracted MCs from the exposed aquatic snail tissues in publication III were determined with two different methods, LC-MS and ELISA. The results correspond to each other well, except for one outlier for the LC-MS measurements (week 3) and one for ELISA (week 4) (see fig. 4.1). The concentrations of extractable MCs show a clear trend of increase during exposure and decline during depuration periods (fig. 4.1).

The utilisation of second, confirmatory, detection method is strongly recommended especially in the case of low-level analysis where sample matrix may disturb the detection. This reduces the changes of obtaining false negative or false positive results.
ELISA and LC-MS are based on completely different analysis principles and therefore complete each other in the analysis. Whereas MS is a molecular weight based tool, ELISA is a group specific tool recognising different variants of the same group of compounds. However, individual analogues cannot be distinguished. In LC-MS one can miss a closely related compound due to the fact that the mass of that compound is not known or considered important, whereas with ELISA all the compounds having the molecular properties the antibodies are raised against will be detected. The possibility of cross-reactivity with matrix compounds, or poor cross-reactivity with some variants make the interpretation of ELISA analysis more difficult. This creates differences not only in the levels of possible matrix disturbances (different between methods) but also in the detection levels. As LC-MS methods have improved, they have become the methods of choice in many laboratories due to the ability to selectively identify and quantify the different variants of the analytes. In publication I, the ELISA and LC-MS did not correspond to each other as well as in publication II. This may be due to several factors. The individual feathers were different for the two separate analysis, the sample preparation was done in a different laboratory and the extraction procedure therefore was slightly different. In publication I the ELISA was mainly used as additional confirmatory assay, and not to support the concentration values obtained by LC-MS.

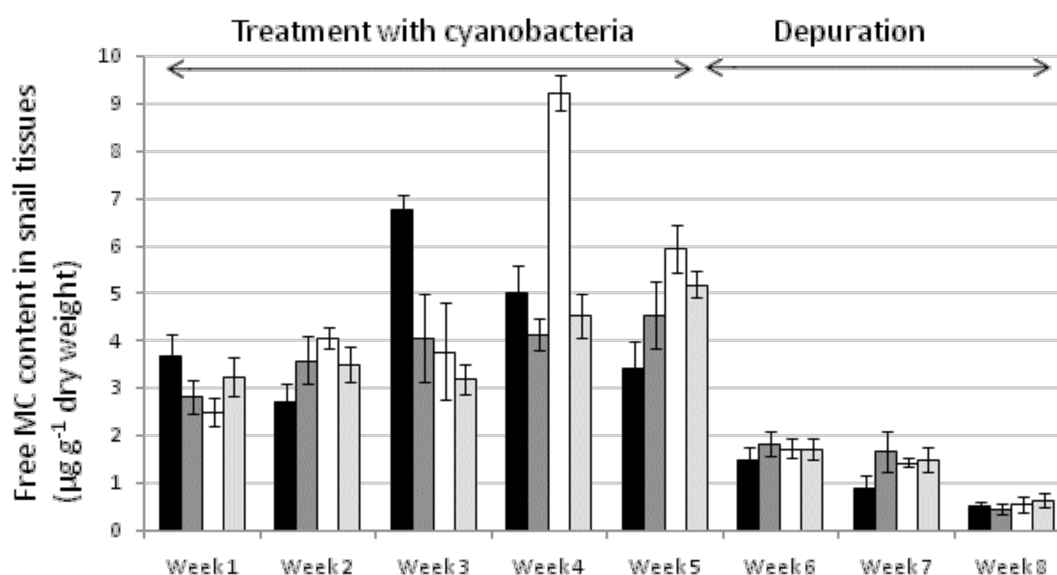

Figure 4.1
Values for free, extractable MCs in exposed aquatic snail tissues (±standard errors). The white and light grey bars correspond to concentrations of MCs from aquatic snails exposed to cyanobacterial suspension P2 (containing 33 µg/l of MCs; described in table 4.4) with lettuce and the black and deep grey bars to the corresponding experiments without lettuce. The values for black and white bars have been obtained with LC-MS measurements and the values for deep and light grey bars with ELISA.

4.4. MMPB methodology (publications II and III)

The MMPB method is of relevance to determine the total, both free and bound forms of microcystins.
It was considered important to trypsinate the sample prior to oxidation because of two different aspects: the toxin in the (target) protein binding pocket should be readily available for the oxidation process. As the oxidation is dependent on the tissue surrounding the toxins it was considered important to make the toxins more accessible in the oxidating solution. On the other hand the sample preparation and clean up after the oxidation and quenching the oxidation processes were difficult. The sample clogged the solid phase extraction cartridges. This was simplified by processing the samples by trypsination and by centrifuging the samples prior to SPE.

The optimised sample preparation procedure for the determination of total concentrations of MCs from aquatic snail tissues is given in a workflow description in publication II. The method includes seven steps of which one is trypsination for 2 h, one is oxidation for 3 hours, and the drying process takes additional 2-3 hours. Therefore the whole sample preparation process is time consuming, and even the other steps (reaction quenching and checking the pH, solid phase extraction and elution) in the procedure are laborious and time consuming when handling dozens of samples at a time.

As found in the spiking studies during the development of the method, the oxidation recovery remains to be the weak point in the MMPB method, together with the matrix effects in the LC-MS detection (see Table 4.3). The oxidation process is a balance between oxidising the MCs into MMPB molecule, oxidising the matrix surrounding the toxins and further oxidising the MMPB into smaller products. The sample oxidation recovery is the measure of the proportion of MCs in a tissue recovered as the oxidation product MMPB. Some of the MCs in the tissue may escape oxidation and some of the formed MMPB may be further oxidised and lost.
The oxidation recovery, extraction efficiency and the matrix effects were controlled by spiking of control samples as described above, but an additional step of spiking is required after the oxidation, to assess the oxidation recovery (comparison between samples spiked in the beginning and after oxidation). The number of required controls makes the method even more laborious, but the different recoveries must be assessed for each tissue type and oxidation treatment. The MMPB method is laborious and has not been utilised in many publications. Even the current method reported in publication II suffers from low sensitivity.

The poor oxidation recovery accounts for up to (or over) 70% loss of MC to MMPB material, and the matrix effect ion suppression up to 84% loss in signal in the used LC-MS instrumentation. The matrix effect were determined by control sample material with a known amount of MMPB and comparing the produced signal responses to the signal responses obtained from clean (without matrix) standard solutions.

Table 4.3 MMPB methodology recovery values
Oxidation recovery, extraction efficiency and matrix signal suppression effect on signal recovery by LC-MS detection, are given for serum and aquatic snail tissues.

Sample spiked with	Oxidation recovery / %	Extraction efficiency / %	Matrix effects, signal in spiked samples/ %
Serum			
1 mL 1.6 μg MC	31.6	85.6	18.4
1 mL 160 ng MC	39.5	97.2	16.3
0.1 mL 800 ng MC	28.9	70.7	34.8
0.1 mL 80 ng MC	33.9	73.0	37.3
Aquatic snail tissue			
10 mg 8.8 μg MC	38.4	62.4	25.8
10 mg 1.6 μg MC	36.2	76.3	18.0
10 mg 160 ng MC	22.4	44.9	27.4

In addition to being able to determine the total concentrations of MCs, the MMPB method also benefits from being dependent only on one standard in the analysis. All of the MC and Nod variants are detected as MMPB. The production and therefore availability of an MMPB internal standard, for example an isotopically labelled MMPB, is far more probable than production of multiple isotopically labelled MC standards.

The property of all MCs being detected as one molecule can be compared to ELISA assay, where the determination of the individual variants is not possible. However, in MMPB the signal response is directly proportional to the toxins available whereas in ELISA the different congeners are bound to the antibodies with different affinities and therefore give different quantitative responses.

The determined concentrations of covalently bound toxins (total amount of MCs determined by MMPB method – extractable amounts of MCs in aquatic snail tissues) are approximately half of the total contamination burden in exposed aquatic snail tissues (see table 4.4). Concentrations of toxins from the tissues for the initial exposure period and for the depuration period have been listed in table 4.4. Exposure media was on two separate experiments a bit different (described in table 4.4). In table 4.4 the extractable toxins have been analysed by two methods, with ELISA and LC-MS. The cysteine and glutathione conjugates of MCs were looked for during LC-MS analysis, but their concentrations were below limit of detection.

Table 4.4 Extractable and covalently bound MCs in aquatic snail tissues

Sample: aquatic snails fed with:	Exposure with *P. agardhii* suspension P1 Containing 7.8 ± 2.3% of dmMC-LR, 27.4 ± 2.6% of dmMC-RR, 64.8 ± 3.1% of MC-YR			Exposure with *P. agardhii* suspension P2 Containing 6.9 ± 0.8% of dmMC-LR, 90.5 ± 0.9% of dmMC-RR, 2.5 ± 0.4% of MC-YR		
	Free MCs (μg/g DW)	Bound MCs (μg/g DW)	% bound MCs	Free MCs (μg/g DW)	Bound MCs (μg/g DW)	% bound MCs
End of 5 week treatment period						
Cyan5	25 ± 5.5	9.2 ± 2.7	27 ± 5.2	3.3 ± 0.5	6.6 ± 2.0	67 ± 7.4
Cyan5Lt	12 ± 1.7	2.6 ± 0.9	18 ± 4.2	3.4 ± 1.0 (only ELISA)	-	-
Cyan33	32.4 ± 5.2	38 ± 7.9	54 ± 7.1	4.0 ± 1.1	5.1 ± 2.5	56 ± 8.0
Cyan33Lt	-	-	-	5.6 ± 1.0	3.9 ± 1.0	41 ± 5.6
End of 3 week depuration period						
Cyan5	1.9 ± 0.3 (only ELISA)	-	-	0.3 ± 0.1	< LOD	-
Cyan5Lt	0.9 ± 1.2	1.7 ± 0.2	65 ± 9.3	0.2 ± 0.04	< LOD	-
Cyan33	5.3 ± 2.0	15 ± 5.2	74 ± 14	0.5 ± 0.2	5.1± 0.7	91 ± 7.6
Cyan33Lt	-	-	-	0.6 ± 0.2	4.7 ± 0.6	89 ± 4.2

Snails were kept in two P. agardhii suspensions with different percentages of toxins dmMC-LR, dmMC-RR and MC-YR produced. Both suspensions were of a total concentration of 5 μg MC-LReq/l without additional feeding (Cyan5) and with lettuce ad libitum (Cyan5Lt); or of a total concentration of 33 μg MC-LR eq/l without additional feeding (Cyan33) and with lettuce ad libitum (Cyan33Lt).
Four snail bodies per treatment were mixed and 4 measures per mix were assessed using LC- MS and ELISA methods for free MCs assessment and using the MMPB method and LC-MS for total (free + bound) MC assessment.
Bound concentrations obtained by calculating [Bound MCs = total (MMPB) MCs - free (extracted) MCs].

The results from the study presented in publication III show that the percentage of covalently bound toxins is not the same all the time: the trend appears to be such that the covalently bound toxins are present in higher proportions in the longer exposed snails and after depuration, as compared to the proportions of the covalent bound toxins in the beginning of the exposure (see table 4.4 and fig. 4.2). Figure 4.2 describes the trend of proportion of the covalently bound MCs during exposure and depuration periods.

Due to the high amounts of covalently bound MCs in the tissues of *Lymnaea stagnalis* found in publication III it seems unlikely that the only target for covalent binding in the tissue would the protein phosphatases. Up to 37 μg/g of covalently bound toxins were found. The percentages of covalently bound toxins, as compared to extractable toxin amounts, are comparable to values found by others [130, 133, 137]. If all of the covalently bound toxins would be bound to protein phosphatases (for example the PP2A, either a dimer ~101kDa or a trimer approximately 155-173 kDa, see [201]; one binding site per phosphatase) the amount of covalently inhibited protein phosphatases in the tissue would be 3.7 mg – 6.4 mg/g, which is unrealistic. Imanishi and Harada presented a study to reveal proteins binding to MCs by a proteomics method [95] combined with affinity chromatography [202-203] to fish out proteins binding to MCs. This kind of experimental set-up could be useful in finding out other possible counterparts for MC binding in different tissues. However, in that study the MCs were bound to the resin by the double bond in the (Mdha) structure, which could be crucial for interactions with some of the target proteins.

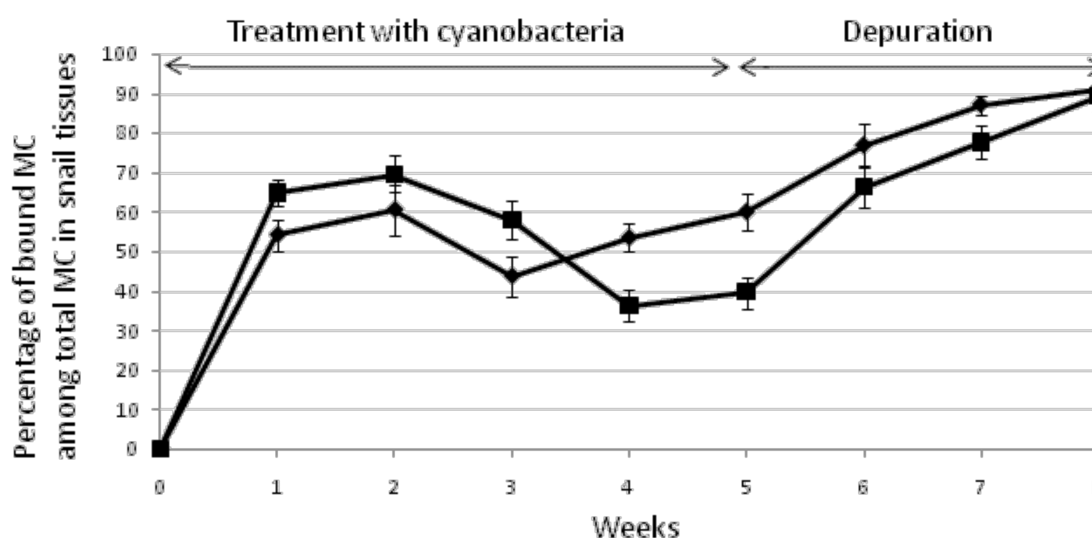

Figure 4.2
The proportion of covalently bound toxins in aquatic snails (in percentages of the total amount of MCs detected, ±standard errors). The data points marked with squares describe the values from exposure experiments conducted with cyanobacterial suspension P2 (containing 33 μg/l of MCs; described in table 4.4.) with lettuce and the data points marked with diamonds in a similar setup without lettuce.

Aquatic snail tissues might not be considered as relevant to human health by direct exposure, but they are interesting study organisms due to the fact that they directly consume cyanobacteria and are therefore possibly exposed to high amounts of toxins. Aquatic snails are consumed by higher organisms in the ecosystem. Therefore the detection of toxins from snails is important considering studies of toxin transfer to the following trophic level.

The toxicity or the availability of the covalently bound MCs (studied in publication III) to the next trophic level is unclear [104], and this aspect can only be studied if useful methods for the determination of the covalently bound toxins exist. In publication III it was found that approximately half of the MCs in tissues are covalently bound. During depuration (in nature corresponding to bloom ending) the proportion of covalently bound toxins increases, probably due to quicker elimination of the free MCs from the tissues as well as more MCs being covalently bound. The implications not only to the organisms exposed to MCs, but also to the next trophic level, warrant this method to be used in further studies.
The snail tissues are very complex sample matrix, and it is therefore anticipated that the methods developed for the snail tissues would be adequate for other types complex biological tissue analysis, such as analysis of liver tissues.

4.5. Chromatographic development of the LC-MS analysis

Recent instrument developments have driven LC-MS forward in the aspects of speed, detection limits, accuracy and certainty in identification. In my research two of the publications (IV and V) concern the utilisation of small particle size columns. I have taken advantage of the new abilities of the instrumentation available, and optimised the detection to be performed in fast and accurate manner.
The following aspects were considered important in the chromatographic method development:
- Higher performance pumps to be able to utilise smaller particle size columns. The benefits of the smaller particle size columns have been described before.
- Fast autosamplers that enable the run times to be shortened, as the difference to the older systems is 4-5 fold.
- Higher column temperatures have been found beneficial in RP separations due to more efficient partitioning between the mobile and stationary phases [204-205]. In our study in publication IV, with high flow rates, the columns were kept at 60°C, which is generally regarded as the highest recommended column temperature for silica-based stationary phase. The robustness of these columns at the elevated temperature was not systematically tested in this study, but the performance was stable for more than 1000 injections per column in [206].
In general, better performance is now easily achievable with modern instrumentation and columns.

The current technologies enabled the reduction in sample analysis time for water and cyanobacterial samples to 3 min 15 s in publication IV (high throughput, with a high flow rate up to 1 ml/min) and to 8.5 min for serum samples in publication V (ion source limited the flow rate to 0.5 ml/min).

4.5.1. Comparison of HPLC columns in the separation of microcystins and nodularins within extracted bloom samples (publication IV)

Chromatograms

Fig. 4.3 shows chromatograms of four columns, Ascentis Express, Zorbax Eclipse plus, Chromolith FastGradient and Synergi Fusion-RP, all representing different column technologies and manufacturers with 1 min gradient, total analysis time of 3 min and 15 s (including equilibration and injection) for the separation of 13 MC and Nod variants.

The mobile phase consisted of water and acetonitrile both acidified with formic acid, the gradient rise was from 25 % acetonitrile to 75% acetonitrile over 1 min. The flow was adjusted according to column inner diameter (I.D.), 1 ml/min for the 2 mm I.D. columns and 1.102 ml/min for the 2.1 mm I.D. columns. Columns were thermostated to 60°C.

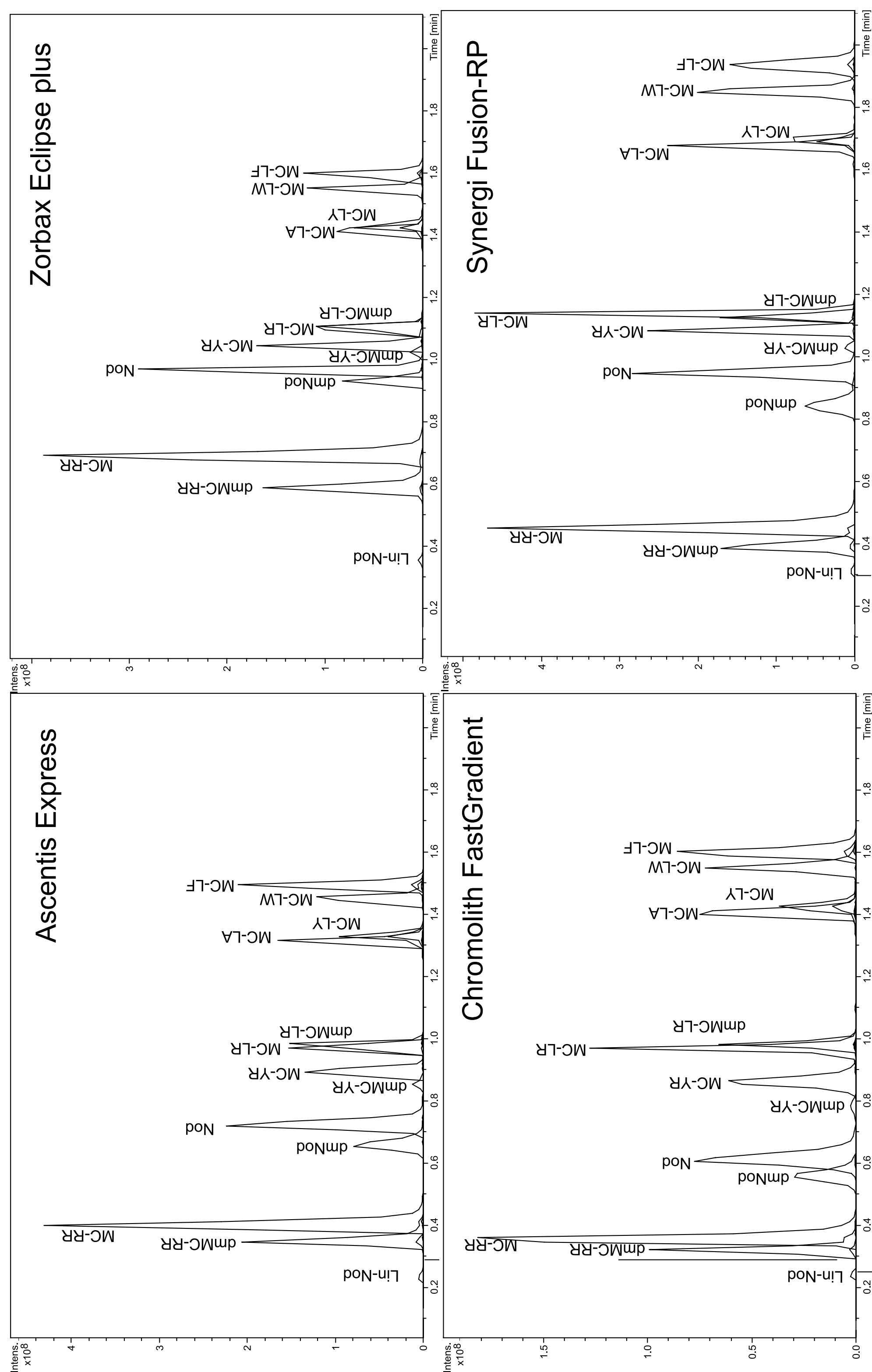

Figure 4.3
Extracted ion chromatograms produced by four different LC columns for the separation of 13 MCs and Nods in a standard mix. The whole run time per sample was 3 min 15 s from injection to injection. The mobile phase used here was water-acetonitrile based, pH modified with formic acid, from 25% to 75% acetonitrile over 1 min.

Chromatographic performance

Values of the different chromatographic parameters are shown in table 4.5.

The chromatographic resolution assessment was made by calculating Rs values for six pairs of compounds. All of the tested columns, except for the Fortis C-18, were able to resolve the MC-YR/MC-LR pair (Rs > 1.0, 2% or less overlap of the peaks) with all gradients. Using the Ascentis Express, Chromolith FastGradient, Zorbax Eclipse plus and Synergi Hydro-RP, dmMC-LR eluted after MC-LR. The mobile phase used in publication IV was FA-modified mobile phase. The elution order for the pair dmMC-LR / MC-LR with the C-18 material with acidic mobile phase is in the literature described to be dmMC-LR before MC-LR, but the order is reversed with ammonium acetate mobile phase [155, 177].

The Synergi Fusion-RP had four analyte pairs with resolution values higher than 1 (dmMC-RR/MC-RR, dmNod/Nod, MC-LR/MC-YR, MC-LW/MC-LF), thus performing best in this category. Also other columns gave good resolutions indicating that the new column technologies are working well with these compounds.

The most symmetrical peaks (although some tailing did appear with the first eluting compounds dmMC-RR and MC-RR) and narrowest peak widths were produced by Zorbax Eclipse plus column (average FWHM 0.028 min for the 8 main toxins in the standard mix, with the 1 min gradient). The Phenomenex Synergi columns' peaks were also very narrow; for the 1-min gradient the Hydro-RP and MAX-RP produced peaks 0.028 min and 0.029 min wide on average, respectively (see table 4.5).

S/N evaluation was conducted with a natural bloom extract further diluted. The concentrations of the toxins were 60 ng/ml MC-RR, 66 ng/ml MC-YR and 44 ng/ml MC-LR in the extract, and the S/N values for the Synergi and Zorbax Eclipse plus columns still stayed well above 40 (except for MC-YR for Synergi Fusion-RP, 33). From here we can approximate that the limit of quantitation (S/N 10) would in our methodological setup be close to 15 ng/ml or lower for MC-RR, MC-YR and MC-LR.

Table 4.5 presents resolution values, peak widths (FWHM), number of peaks and S/N values for 1 min gradient for the eight tested columns in the separation of MCs and Nods. The Rs values better than 1, as well FWHM peak widths lower than 0.03 min are boldfaced. The natural bloom extract refers to sample material obtained from Sulejow reservoir in Poland and the Dissect function to an automatic peak finding tool in Bruker Daltonics Data-analysis software. More details can be found in publication IV.

Table 4.5 Column comparison by peak resolution, width and number, and signal S/N -values

Column name	Resolution for pairs of MCs and Nods in 1 min gradient						Average Peak width in 1 min gradient (FWHM) in min for 8 MCs and Nods*		Number of peaks identified as peaks by the Dissect function; natural bloom extract; Average of three runs			Average signal to noise values for 1 min gradient, Sulejow extract**		
	dmMC-RR/ MC-RR	dmNod/ Nod	MC-YR/ MC-LR	dmMC-LR/ MC-LR	MC-LA/ MC-LY	MC-LW/ MC-LF	Average of 8 main	std for 8 main	2 min gradient	1.5 min gradient	1 min gradient	MC-RR	MC-LR	MC-YR
Ascentis Express	0.95	**1.02**	**1.69**	-	0.21	0.81	0.0302	0.005	45	44	33	25.8	28.2	30.2
Fortis C-18	0.77	0.43	0.56	-	0.14	0.83	0.0484	0.007	32	33	30	12.9	21.2	26.8
Zorbax Eclipse plus	**1.81**	0.71	**1.19**	-	0.19	0.82	**0.0282**	0.003	51	46	40	42.7	46.0	59.3
Chromolith FastGradient	0.59	0.68	**2.07**	-0.28	0.28	0.95	0.0346	0.008	42	42	42	60.4	42.8	32.2
Synergi Fusion_RP	**1.33**	**1.63**	**1.13**	0.19	0.35	**1.52**	0.0310	0.008	48	46	42	55.3	33.6	58.6
Synergi Hydro-RP	0.94	0.42	**1.09**	-0.15	-	0.63	**0.0276**	0.004	49	45	39	81.9	69.8	82.7
Synergi MAX-RP	**1.86**	0.85	**1.14**	-	-	**1.28**	**0.0288**	0.004	47	45	41	52.9	60.9	61.0
Synergi Polar-RP	0.78	0.89	**1.28**	-	0.35	0.40	**0.0295**	0.004	43	42	34	72.9	47.0	52.4

The natural bloom extract refers to sample material obtained from Sulejow reservoir in Poland and the Dissect function to an automatic peak finding tool in Bruker Daltonics Data-analysis software. More details can be found in publication IV.

* Eight main toxins here refers to MC-RR, dmNod-R, Nod-R, MC-YR, MC-LR, MC-LA, MC-LW and MC-LF

** The concentrations of the toxins were 60 ng/ml MC-RR, 66 ng/ml MC-YR and 44 ng/ml MC-LR

4.5.2. Chromatographic performance in publication V – column comparison and spiked serum sample study

The tested mobile phase in the first part of publication V, column comparison study, was ammonium formate-formic acid –based and tested with two different flow rates. Interestingly, a slower (less steep gradient), did not provide better resolution with these fast separation columns in the first part of the study with extracted bloom material. The calculated Rs -values were approximately the same between the 30 min (10%-80% B) and the 5 min (30%-75% B) gradients, or even slightly better with the 5 min gradient (see table 4.6). In table 4.6 the values that represent the best ones obtained in particular test are boldfaced. In publication V, the standard mix of 9 MC and Nod variants differed slightly (fewer toxins, did not include linear Nod, dmNod-R, dmMC-YR or MC-LA) from the one used in publication IV.

The Phenomenex Synergi Hydro-RP column was chosen from the first part of the study in publication V for the separation of MCs and Nod in serum matrix. This column worked well, i.e. there was no clogging, increased backpressures or shifts in retention times even with the more complex serum sample matrix. The column was tested with mobile phase containing either both, ammonium formate and formic acid, or only formic acid. The two mobile phases performed somewhat differently (see figure 4.4). The ammonium formate-formic acid based mobile phase separated the chromatographically very similar compounds dmMC-LR / MC-LR, whereas the formic acid based mobile phase was not able to do so when using the Hydro-RP column.

The spiked serum samples were analysed in the following chromatographic conditions: the mobile phase water (A) and acetonitrile (B), either modified only with formic acid or with formic acid plus ammonium formate (chromatograms presented in figure 4.4). The flow rate was set to 0.5 ml/min, which is the highest flow rate recommended for the MS ionsource in the Q-TOF instrument. The gradient was as follows: 28% B to 38 % B over 3.5 min, up to 75% B over 0.2 min hold 75% B for 0.9 min.

Table 4.6. Some chromatographic performance parameters for the columns tested in publication V, with long (30 min), 5 min, and short (1.1 min) gradient separation, based on ammonium formate + formic acid containing mobile phase.
The best values for every assessment (per gradient) are boldfaced.

Column	Resolution			Capacity factor		Selectivity [a]
	dmMC-LR / MC-LR	MC-YR / MC-LR	MC-LW / MC-LF	dmMC-RR	MC-LF	MC-LF / dmMC-RR
Long gradient: from 10 to 80% of solvent B over 30 min						
Waters Acquity BEH C-18	**1.3**	2.7	2.8	19.6	33.1	1.7
Phenomenex Hydro-RP	0.1	2.0	**2.9**	19.7	39.8	2.0
Phenomenex Fusion-RP	0.1	1.9	1.8	18.5	38.5	**2.1**
Phenomenex Max-RP	0.1	1.9	2.7	19.3	38.0	2.0
Agilent Zorbax RR	0.6	**3.1**	2.8	20.6	35.6	1.7
5 min gradient: from 30 to 75% of solvent B over 5 min						
Waters Acquity BEH C-18	**1.3**	2.7	1.4	3.7	13.5	3.6
Phenomenex Hydro-RP	0.3	2.9	**2.0**	2.4	16.6	7.0
Phenomenex Fusion-RP	0.0	2.8	1.5	2.3	16.1	**7.1**
Phenomenex Max-RP	0.1	2.9	1.7	2.4	16.0	6.7
Agilent Zorbax RR	0.6	**3.1**	1.4	4.2	14.8	3.5
Short gradient: from 35 to 65% of solvent B over 1.1 min						
Waters Acquity BEH C-18	**0.7**	2.1	1.5	1.6	7.5	4.6
Phenomenex Hydro-RP	0.0	**2.3**	**1.7**	0.8	9.0	**11.7**

[a] Selectivity of the column has been calculated as the ratio of capacity factors for the last eluting compound, MC-LF and the first eluting compound, dmMC-RR.

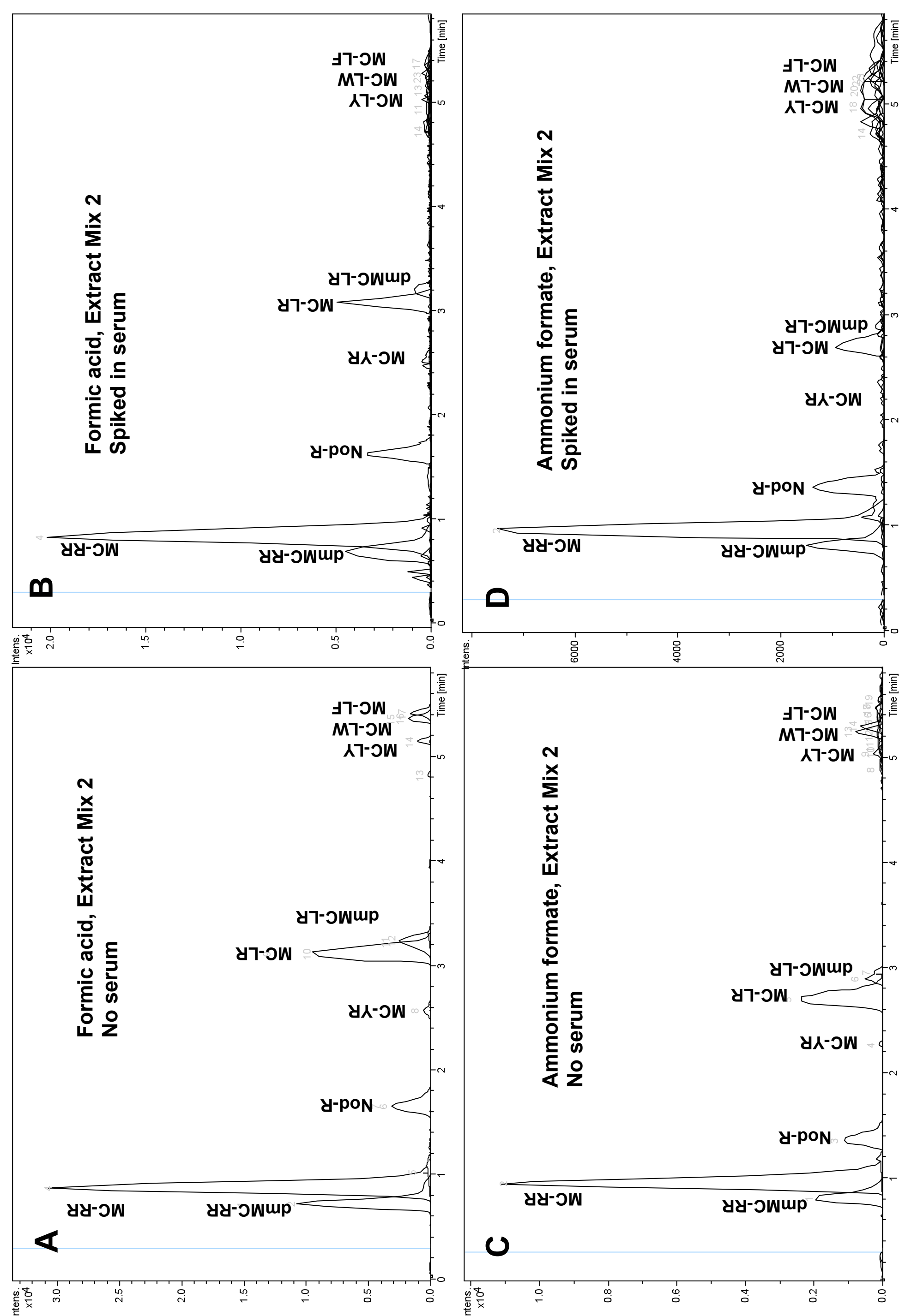

Figure 4.4
Chromatogram of nine MCs and Nod-R spiked in serum matrix, separated utilising two different mobile phase systems, only formic acid (panels A and B), or ammonium formate + formic acid modified (panels C and D) water- acetonitrile mobile phases. Chromatographic conditions described in the text as well as in publication V. The toxin mix was analysed without (panels A and C) or within a serum matrix (panels B and D).

4.6. Mass spectrometry detection used in this study

QQQ instrumentation

In publications I-III the instrument was a triple quadrupole MS instrument used in the SIM or MRM mode. The instrument performance suffered from matrix effects and from low sensitivity. With the detection of MCs the matrix effects and signal fluctuations during sample series were corrected by using external standards in adjunct runs. The external standards were added in the sample series after every third or fifth sample.

Feather sample analysis on SIR mode (see figure 4.5) showed adequate selectivity over disturbing background signals from matrix to clearly detect Nod. Out of the Nod variants looked for (dmNod-R, Nod-R and LinNod-R) only Nod-R was recorded above LOD. The spiking studies were performed on feather samples earlier determined to be negative for Nods.

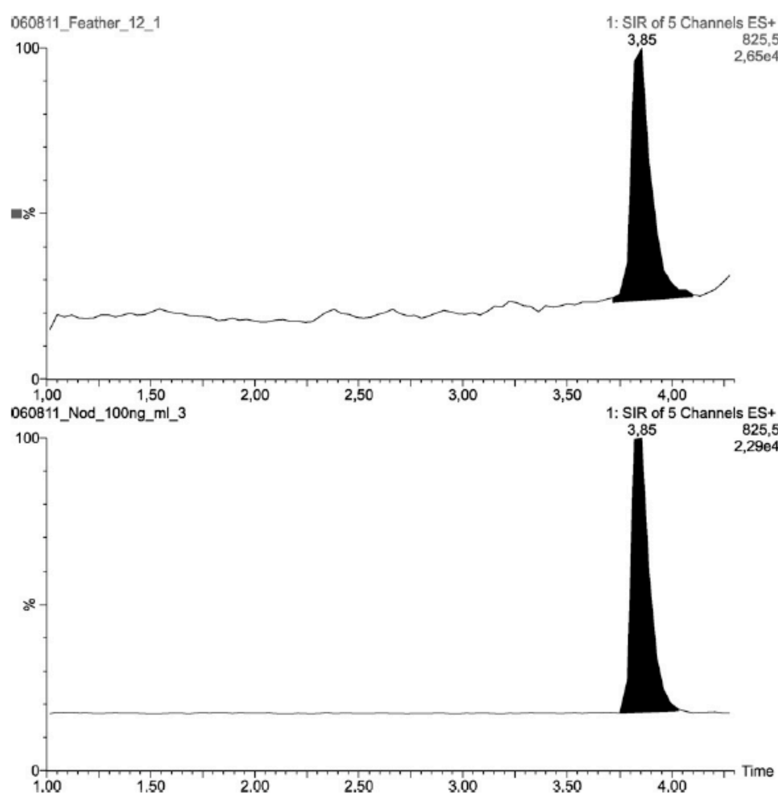

Figure 4.5
Chromatogram of an eider feather extract. The SIR mode analysis performs well and gives a distinct peak for Nod-R in the complex feather sample. Upper panel shows a SIR trace form a feather sample, the lower panel chromatogram of Nod-R standard (2 ng on column). The chromatographic conditions described in publication.

The results from QQQ MS analysis of aquatic snail samples (publications II-III), were calculated back to correspond the amounts in the beginning, and the amount of free MCs was expressed in µg of MCs/g DW tissue. The detection threshold was determined to be 0.1µg of MCs/g DW tissue for the variants MC-RR and dmMC-RR,

and 0.2 µg/g of MCs in DW tissue for the variants MC-LR, dmMC-LR and MC-YR. During the sample series the signal response fluctuated considerably, and the matrix effects (signal suppression or signal enhancement) and their effect on signal response were found to differ depending on the MC variant.
The limits of quantitation utilising the QQQ (in publication II and III) for spiked serum samples were estimated to be approximately 10 ng/ml MCs in vial (corresponding to 2 ng/ml MCs in serum samples), for the different arginine-containing variants.
The MRM mode of detection was utilised in the MMPB analysis for better selectivity, but suffered from signal suppression effects (table 4.3). The negative mode could have offered benefits in reducing the ion suppression with the detection of MMPB, but due to very low signal response in the MS, the negative mode could not be used. Furthermore, the compounds considered as good candidates for internal standards, 5-PP and 4-PB, failed to give signal response with the QQQ instrument in positive mode.
The signal fluctuation and suppression effects were corrected for by the use of external standards in both MC and Nod and MMPB analysis.

Spherical ion trap instrumentation
In publication IV the instrument used was a spherical ion trap that was well suited for the fast detection of multiple masses (a mass range) This detector was coupled to the mobile phase with an ESI source which is compatible with high mobile phase flow rate (1 ml/min) from the HPLC. The detection limits in ion trap instrumentation are somewhat higher than in triple quadrupole systems. However, the fast and efficient scan rates make ion traps well suitable for fast gradient analysis. The limit of quantitation for MC-RR was estimated to be in the range of 15 ng/ml or less in vial for MC-RR, MC-YR and MC-LR.

Scanning of wider range of ions enables doubly and singly charged ions, adduct ions, as well as some possible metabolites of the compounds to be detected within the same run. Data-analysis tools from Bruker offer a nice possibility for peak picking called Dissect. This function finds signals increasing and decreasing in the same time frame and groups these ion species into one peak (compound). This is a good tool for determining peaks, but also to detect the different ion species that the compound of interest produces in the ESI source. This information can be useful in the development of fragmentation modes. Furthermore, the Dissect tool may help in the determination if a new peak would be of a novel compound of interest. Ion source fragmentation patterns and multiple ion species for compounds may have similarities within a group of analytes, and therefore give indications if the novel compound would belong to that group.
For molecular identity confirmatory purposes one needs to select, stabilise, and fragment a precursor ion, which is more time consuming in an ion trap than for example in a Q-TOF system. However, the spherical ion trap offers the possibility for MS^n analysis, which can be useful especially in structure determination. The ion source in the ion trap used in publication IV was able to tolerate high flow rates of mobile phase, which is an important development considering the utilisation of the high throughput LC columns and methods.

Q-TOF instrumentation

Although found beneficial in the separation of certain MC pairs in publication V, the ammonium formate containing mobile phase was found to give signal intensities of only approximately half of the signal intensities produced with the formic acid mobile phase (figure 4.4).

The narrow mass window used in the publication V worked well in reduction of the background noise (as can be seen in fig. 4.6). LC-ESI-Q-TOF instrumentation gave a clear advantage in quantitative analysis when utilising a narrow mass window (±20 mDa) as compared to a wider detection mass window. A narrow window can only be used if the instrument is appropriately calibrated. In our case the calibration was done in each run separately (post-acquisition, during data-analysis) by having a segment in the beginning of each run for the acquisition of the calibrant spectra. In our case the calibrant used was sodium formate clusters.

The limit of detection in spiked serum samples (publication V) for the variant dmMC-LR was determined to be below 5ng/ml in vial, or below 25 pg on column (in treated serum matrix sample). This corresponds to below 1 ng/ml in the original spiked serum sample. The lower limit for linear quantitation in this system for the different arginine-containing variants varied from 5 ng/ml (dmMC-RR) to 85 ng/ml (MC-YR) calculated back into start concentrations in spiked serum.

Fragmentation can be used to give further confirmation in analyte identification, (see fig 4.7) as well as the MS spectrum with different ion species (charge states and adducts) of the same compound. MC-RR and dmMC-RR were detected mainly as doubly protonated ion species, whereas MC-LR produced a richer MS spectra with sodium adduct, singly and doubly charged and some fragmentation species detected (see fig. 4.7). In publication V you may find the *m/z* accuracies for the different toxins detected with the Q-TOF MS.

Obtaining the confirmatory fragments from the analytes with Q-TOF-MS instrumentation can be done in two ways: By using two different (MS only and MS–MS) runs for more reliable quantitation and separate confirmation; or, as in our case, within a single run utilising a preference list in the auto MS-MS function for the presumed analytes. The MS-MS spectra obtained from the serum samples were not always of good quality due to too low signal intensity. If full fragmentation patterns are considered essential for the analyte identification, one may tune the quadrupole fragmentation for each analyte to produce better spectra in a targeted analysis.

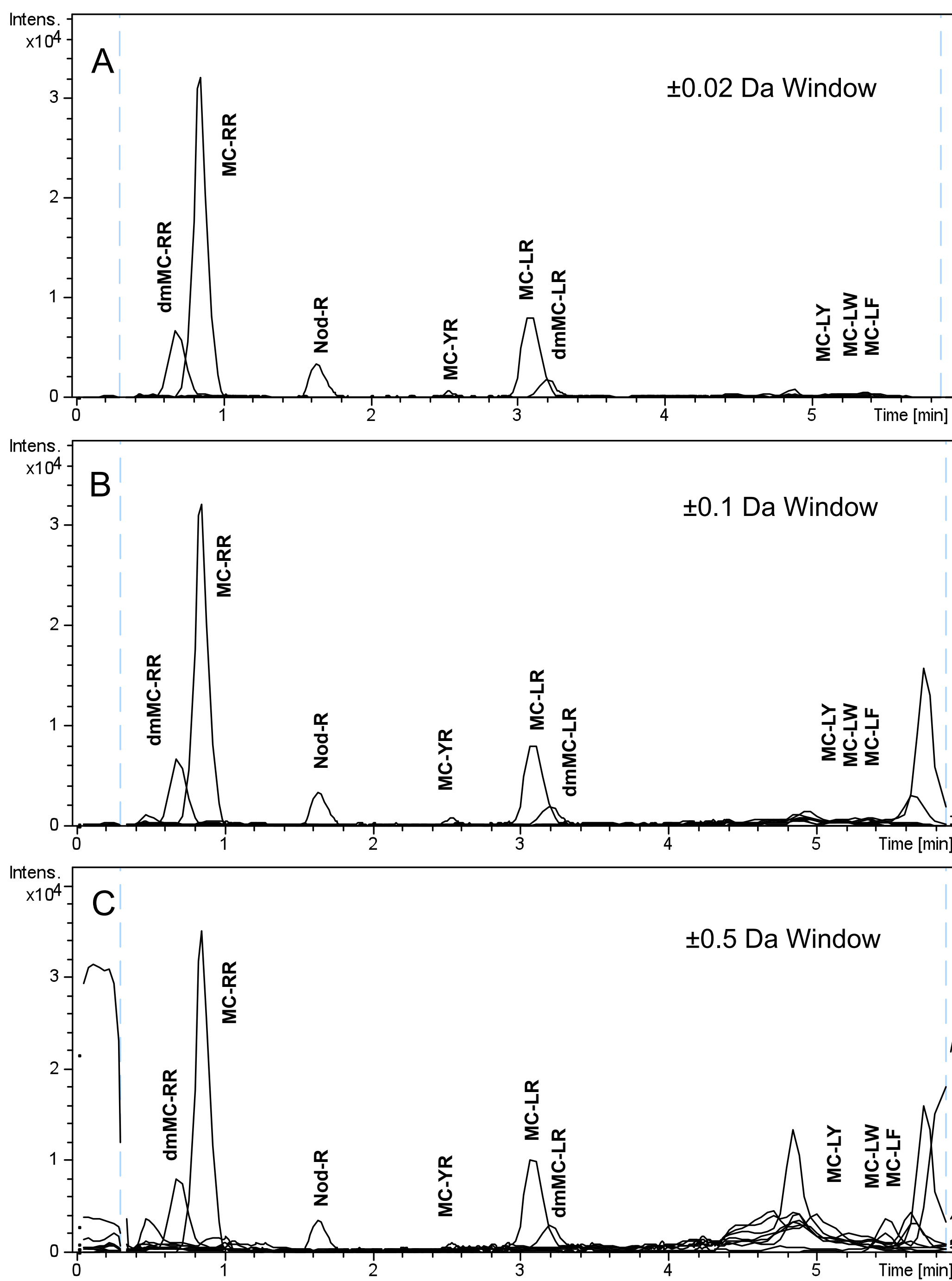

Figure 4.6 The effect on detection selectivity of three different detection mass windows. The sample here was serum matrix spiked with MCs and Nods, detected with an LC-ESI-Q-TOF instrument.

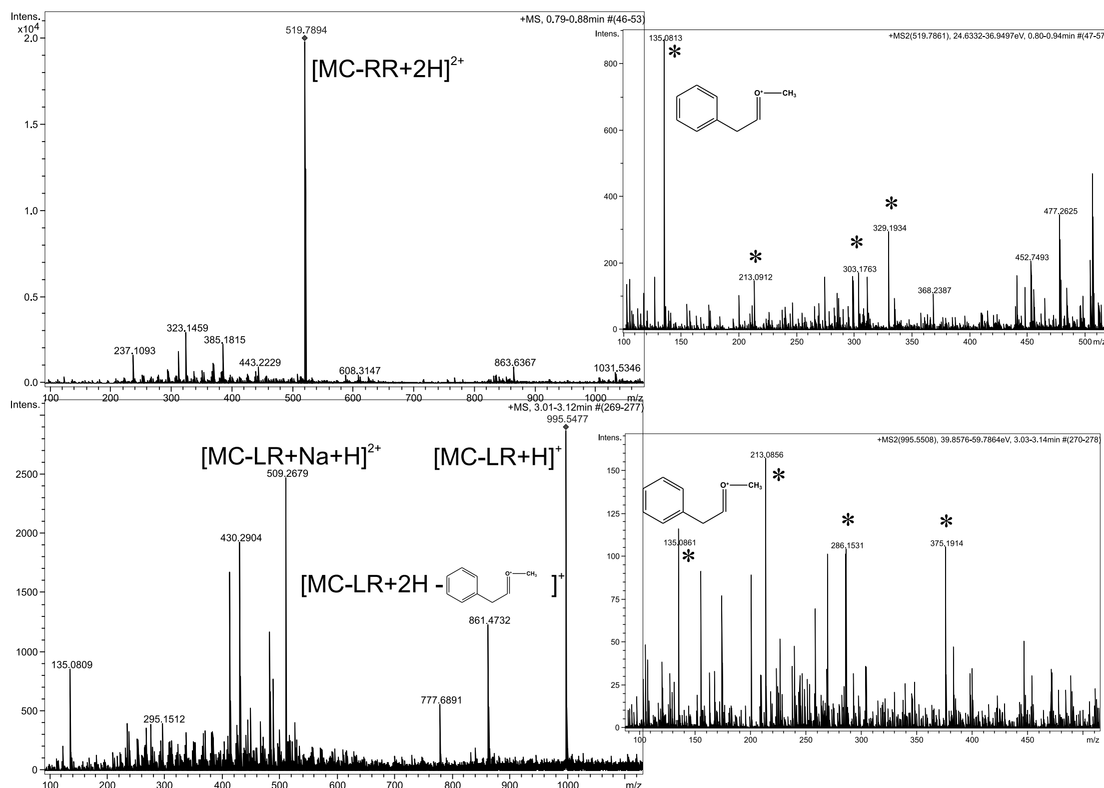

Figure 4.7
Spectral components and fragmentation spectra obtained by the MS and MS-MS mode, respectively from spiked serum samples. * -marked fragments have been identified from the literature to be indicative for MCs.

High-resolution MS detection is available with the current instrumentation at fast (MS only) to moderately fast (MS-MS) scan rates, which makes the Q-TOF instrumentation compatible with fast resolution LC systems.

During this study, fast and accurate LC-MS methods for the detection of MCs and Nods were developed. The methods are compatible with biological tissues.

5. Conclusions

Cyanobacterial peptide hepatotoxins have ecological effects and constitute a health hazard to human populations all over the world. The exposure routes include both drinking water and contaminated food, which therefore need to be analysed for assessment of contamination.

New developments in the field of LC-MS have driven the analytical possibilities forward. The utilisation of these new instruments and methods is of essence due to high demand in regulatory needs and requirements for higher throughput.

The analysis of water, bloom and food samples is of high importance due to possibility of contaminated drinking water, but scientists are also more and more interested in studying exposed (animal) tissues. The tissue analysis requires more from analytical instrumentation due to the complex matrix and low levels of toxins. The advances in the LC-MS field have answered to this need.

The biological questions that arise during a project can be answered with further development and optimisation of existing methods to give results with good reliability.
The analysis of biological tissues with LC-MS is possible but requires input on the level of sample preparation, LC separation and MS detection.

This PhD project contributed to the following aspects in the LC-MS analysis of cyanobacterial peptide hepatotoxins:

Excellent separation of 13 cyanobacterial cyclic heptapeptides could be achieved with total run times of 3min 15 s (including equilibration and injection) with new columns and rapid resolution HPLC and MS technologies in publication IV. Due to the overall excellent performance of the columns it is suggested that the small particle size columns, which enable short run times should be considered for routine MC and Nod analysis, for example in the context of the ISO method 20179 (Water quality – determination of microcystins – method using solid phase extraction and high-performance liquid chromatography with ultraviolet detection).

In publication V the MC and Nod analysis was optimised for accurate mass instrumentation with Q-TOF MS, which gave benefits in both qualitative and quantitative analysis of difficult sample matrices.

In addition to methodological advances, the PhD study produced quantitative information concerning toxin concentrations in biological specimens.

- the cyanobacterial toxin nodularin was with high certainty detected in a new matrix, eider duck feathers in publication I.

- the optimised MMPB method (publication II) enabled the determination of total MC -contamination levels in aquatic snail tissues (publication III).

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