

NOVEL METHODS FOR QUANTITATIVE ANALYSIS AND
EVALUATION OF EFFECTS OF CHRONIC EXPOSURE TO
MICROCYSTINS BY CAPILLARY ELECTROPHORESIS AND
METABOLOMIC STUDIES

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NATIONAL UNIVERSITY OF SINGAPORE

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Abstract/Summary

This thesis describes capillary electrophoresis (CE) methods for separation and quantification of cyanotoxins in water; and “metabolomic” and “metabonomic” approaches for investigation of the effect of exposure of a human cell line to low amounts of microcystins.

Incidences of toxic algal blooms in water bodies have increased. Toxic algae releases toxins into water bodies which puts water consumers at risk of exposure. Exposure to algal toxins is associated with harmful effects such as hepatotoxicity. Humans are at risk of non obvious exposure leading to chronic exposure to cyanobacterial toxins because contamination may not be visible to the naked eye. It is therefore important to develop analytical techniques which can adequately detect and quantify these toxins; moreover the effect of exposure to low amounts of microcystins in human has not been reported.

Capillary zone electrophoresis (CZE), micellar electrokinetic capillary electrophoresis (MEKC) and microemulsion electrokinetic chromatography (MEEC) were developed to determine microcystins LA, LF, LR, LW, RR, YR, nodularin (related hepatotoxin) and cylindrospermopsin, a hepatotoxic alkaloid. The CE methods were validated for use on a portable capillary electrophoresis instrument. Solid phase extraction (SPE) enabled cleanup and pre-concentration of a real sample and detection limits after SPE of the real sample spiked with microcystins were 0.90 µg/L (RR), 0.76 µg/L (YR), and 1.10 µg/L (LR), with relative standard deviation (% RSD) values of 9.9-11.7 % for peak area and 2.2-3.3 % for migration time respectively. SPE recoveries were 90.3 % (RR), 101.5 % (nodularin), 90.6 % (YR), and 88.2 % (LR). In MEEC, online pre-concentration with the aid of a solvent plug achieved a 2-10 fold increase in peak area and height and the detection limit was in the range of 0.15-3 µg/ mL. Freeze drying together with sample stacking was used to achieve detection limits of 0.2-1.1 µg/L. These methods can be used for routine water analysis to monitor

microcystins up to concentrations limits as set by the World Health Organisation (WHO) drinking water guidelines.

In the investigation of microcystin toxicity, HepG2 cells were incubated in media spiked with microcystins LR, RR, YR or a mixture of the three microcystins at different concentrations. Then aliquots of the media were sampled at specific time intervals, extracted and analysed using one dimensional proton nuclear magnetic resonance (^1H NMR) and direct injection mass spectrometry (DIMS). Data obtained was reduced by principal component analysis (PCA) using SIMCA P⁺ software. The use of PCA and “metabolic finger/foot printing” techniques, allowed a distinction between samples exposed to microcystins, those exposed to acetaminophen (positive control), and those that were not exposed (negative control samples).

Components responsible for the differences in patterns observed on the PCA plots were profiled and several metabolites were identified. Generally exposure to microcystins in the range of 1 ng/mL to 100 ng/mL interfered with the metabolisms of carbohydrates, amino acids, organic acids and lipids. The effects were more severe as concentration increased and more prominent for microcystin LR compared to microcystins RR and YR. The “metabolomic/metabonomic” approach demonstrated usefulness in studying toxicity due to microcystin exposure.

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List of Abbreviations

¹ HNMR	One dimensional proton nuclear magnetic resonance
Adda	2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid
BGE	Background electrolyte
C4D	Capacitively coupled contactless conductivity detector
CAPS	<i>N</i> -cyclohexyl-3-aminopropanesulfonic acid
CE	Capillary Electrophoresis
CSEI	Cation selective exhaustive injection
CZE	Capillary zone electrophoresis
DAG	Diacylglycerol
DIMS	Direct injection mass spectrometry
HPLC	High performance liquid chromatography
Id	Internal diameter
ITP	Isotachophoresis
LC	Liquid chromatography
MAPK	Microtubule-associated protein kinase
MEEKC/MEEC	Microemulsion electro chromatography
MEKC/MECC	Micellar electrochromatography
MS	Mass spectrometry
OATP	Organic anion transport peptide
P53	Protein 53 (tumor protein 53)
PI	Propidium iodide
PKC	Protein kinase C
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2 A
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction
TCA	Tricarboxylic acid
TO	Thiazole orange
UV	Ultra violet

CHAPTER 1- INTRODUCTION

1.0 Introduction

Presence of algae in our water bodies is a public health concern because algae not only affects the water aesthetically but also contains harmful toxins commonly referred to as cyanotoxins (microcystins and others). According to the world health organisation (WHO) drinking water guideline¹ the limit for microcystins in water is 1µg/L microcystin LR or its equivalents. The increased occurrence of algal blooms in water bodies has increased the presence of toxic algae which increases the likely hood of exposure of animals and humans to algal toxins. Since the toxins are harmful they need to be continuously monitored in our water sources using analytical techniques. In this study, capillary electrophoresis techniques were developed for the determination of cyanobacterial toxins (including microcystins, nodularin and cylindrospermopsin) in water and potential effects of exposure to microcystins were investigated. The effect of exposure of a human cell line to low amounts of microcystins was studied using HepG2 cell line by “metabolomic” and “metabonomic” approaches. Metabolites were profiled and pattern recognition tools were used to identify differences among the metabolite quantities due to exposure to microcystins. Algal toxins are explained in detail in the following section.

1.1. Algal Toxins

Algae are a diverse kind of life and play an important role in the ecology of rivers and estuaries. Algae range from single-celled microscopic plants to multi-cellular plants and have

different colours and shapes. Algae are widely distributed but the biggest percentage is found in the waters which cover 70 per cent of the earth's surface. Microscopic algae (microalgae) are a major component of plankton therefore they form the basis of aquatic food chains. They include diatoms, dinoflagellates, chlorophytes and cyanobacteria. Algae produce oxygen and are the primary producers of organic carbon in water bodies, are food for aquatic life such as fish and mussels as well as other animals like birds. The larger algae (macroalgae) are useful as a habitat for water dwelling organisms, provide shelter from predators and reduce soil erosion from the shore line ².

Increased intensity and frequency of algal blooms ^{3, 4, 5} is of concern, because algae interfere with the natural balance of plant and animal ecosystems in a water body ². Prokaryotic and eukaryotic microalgae produce a range of compounds with biological activities such as antibiotics, algaecides and toxins ⁶. The focus of this study was those algae that produce toxins. Several species of cyanobacteria produce potent toxins ^{2, 3, 6} and about 30 species of dinoflagellates (red tides) produce neurotoxins mainly the paralytic shellfish poisons (PSP) ².

Algal blooms incidents have recently increased especially with increased pollution of water bodies ⁶ and they are found in many eutrophic to hypertrophic water bodies throughout the world ³. The presence of blue green algae (also called cyanobacteria) in water bodies compromises the water quality. Their presence results in unpleasant colour, taste and odour of water and they are also capable of producing a wide range of potent toxins usually referred to as cyanotoxins ^{3, 7}.

There are about 40 genera of algae (cyanobacteria) that produce toxins but the main ones are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nostoc* and

Oscillatoria (Planktothrix). These organisms thrive in the presence of sunlight and warm temperature especially in polluted waters that are rich in nutrients and have a slow flow rate or are stagnant ³. They are also able to adapt to varying light conditions which results in their better survival compared to other algae ².

Cyanotoxins can be grouped into: cyclic peptides (hepatotoxic microcystins and nodularins), alkaloids (fresh water hepatotoxic cylindrospermopsin and neurotoxins such as anatoxin-a, anatoxin-a(s) and saxitoxins, the marine toxin aplysiatoxin, debromoaplysiatoxin and lyngbyatoxin-a), and lipopolysaccharides (LPS). All are biotoxins ^{3,6} and are known to cause acute and chronic poisoning to animals and humans but mostly to mammals rather than aquatic animals ⁶.

Microcystins are cyclic heptapeptides with molecular masses (RMM) in the range 500-4000 Da with the commonest in the range 800 -1100 ⁶. They are seven peptide-linked amino acids with two terminal amino acids of the linear peptide condensed to form a cyclic compound. Five of the amino acids are non-protein and mostly constant in microcystins. The other two, which distinguish microcystins from each other, are protein amino acids ³.

The general structure is: cyclo-(D-alanine - **X** - D-erythro- β -methyl aspartic acid - **Z** - 2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid-D-glutamate - N-methyldehydroalanine), short form: [cyclo-(D-alanine-X-D-MeAsp-Z-Adda-D-glutamate-Mdha)], in which **X** and **Z** represent the variable (protein) amino acids which can be leucine (L) and arginine (R) in microcystin LR, arginine (R) and arginine (R) in microcystin RR, Leucine (L) and tryosine (Y) in microcystin LY etc . Microcystins have

commonly been isolated from *Microcystis*, *Anabaena*, *Oscillatoria*, *Anabaenopsis* and *Aphanocapsa species*^{3,5,6}.

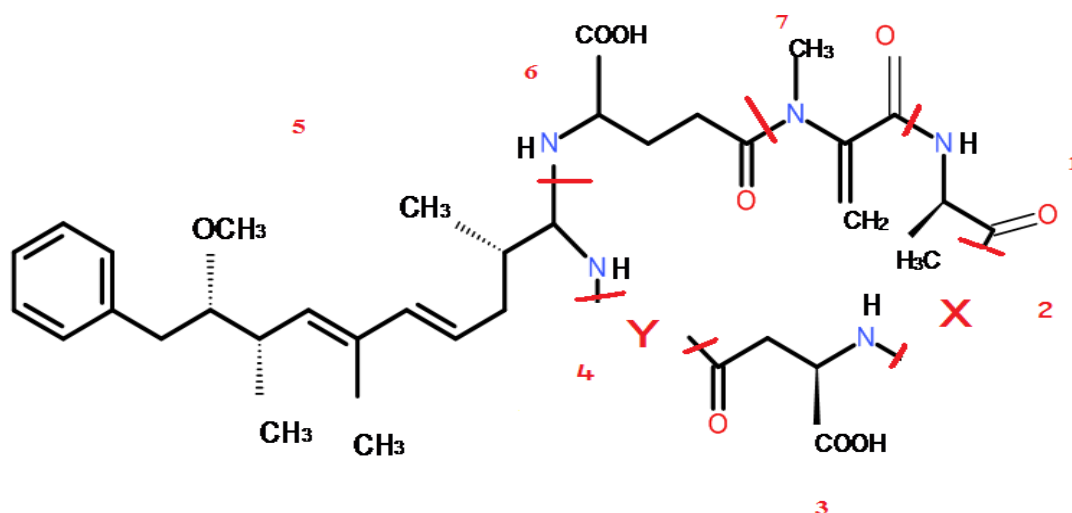


Figure 1. 1: General structure of a microcystin

Nodularins lack the two amino acids that distinguish microcystins and are therefore monocyclic pentapeptides. The general structure is: cyclo-(D-MeAsp-L-Arginine-Adda-D-glutamate-2-(methylamino)-2-dehydrobutyric acid [Mdhd]) and they can be isolated from *Nodularia spumigena*. Nodularin has a molecular mass of 824 Da^{3,6}. The Adda amino acid is a typical structure in all cyclic peptide toxins.

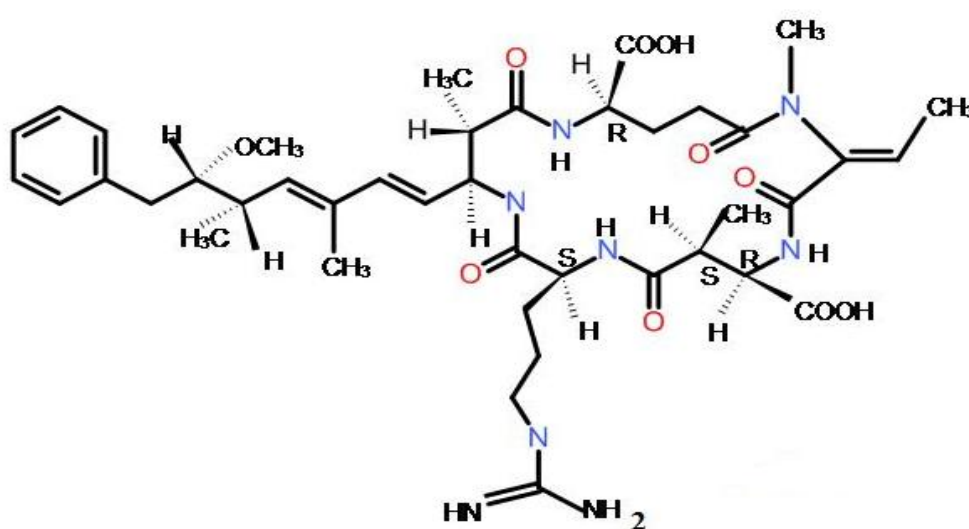


Figure 1. 2: General Structure of nodularin

Microcystins are relatively polar molecules (due to carboxyl, amino and amido groups) and the Adda residue gives them a partially hydrophobic character^{3, 8}. Therefore, they remain in the aqueous phase rather than being adsorbed on sediments or on suspended particulate matter⁸. Nodularins have similar chemical properties, so they can be determined with the same method as for microcystins. Determination of microcystins and nodularin in the same run by high performance liquid chromatography (HPLC) has been reported⁹ and capillary electrophoresis (CE) can also be used for their confirmatory/complementary determination.

Cylindrospermopsin is a tricyclic alkaloid cyanotoxin with a molecular mass of 415 Da that can be obtained from *Cylindrospermopsis raciborskii*. It is a cyclic guanidine alkaloid which is hepatotoxic and has been reported to cause severe liver damage but its symptoms are distinguishable from those of microcystins and nodularin⁶. Exposure to cylindrospermopsin is associated with severe hepatoenteritis and renal damage. It affects several organs including the liver, kidneys, lungs, heart, stomach, adrenal glands and the lymphatic system, with the liver exhibiting centrilobular necrosis¹⁰. Other variants of cylindrospermopsin are demethoxy-cylindrospermopsin and deoxy-cylindrospermopsin.

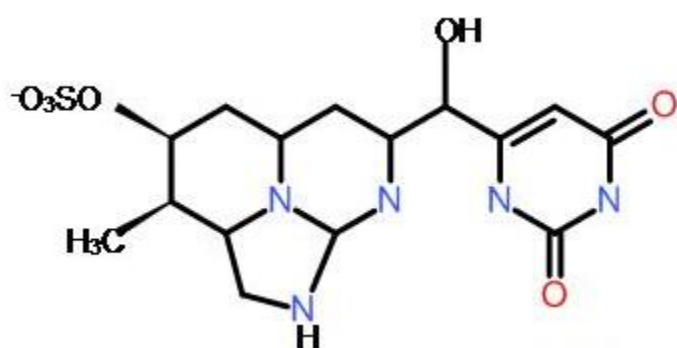


Figure 1. 3: Cylindrospermopsin

Cylindrospermopsin is hydrophilic, so it is often extracted and concentrated from water samples using graphitised carbon based sorbents³. The toxin concentration in water is considerable because cylindrospermopsin leaks into water under normal growth conditions¹⁰.

HPLC and CE methods for determination of cylindrospermopsin at the absorbance of 262 nm have been reported ¹¹ but there are very few reports about simultaneous determination of microcystins together with cylindrospermopsin yet the two types of toxins may coexist in the same water body.

Several methods have been developed for the determination of cyanotoxins in water, for example, the international organisation for standardisation method for determination of microcystins in water (ISO 20179:2005) is high performance chromatography (HPLC) with ultra violet (UV) detection at 238 nm after (solid phase extraction) SPE. Generally methods for analysis of cyanobacterial toxins are divided into screening methods and those which can be used for purposes of identification and quantification as summarised in table 1.1.

Among the physical methods for identification and quantitation, HPLC is the most commonly used. Total analysis times for conventional HPLC can take 45-60 minutes and the detection limits are 300-1000 µg/L without pre-concentration ^{12, 13}. A faster separation using a monolithic stationary phase was reported but the limit of detection was high (300 ng/mL) ¹³, even then, resolution between some analogues was poor. These detection limits are comparable to what can be achieved using capillary electrophoresis (CE) yet CE uses minute amounts of background electrolyte (e.g. 2 mL of BGE for a day) compared to HPLC (2-4 mL per minute of the run) ¹³. On the basis of solvent consumption CE is a more economical method.

Table 1. 1. Analytical Methods for determination of Cyanotoxins

Method	Comment
Mouse Bioassay (screen)	Mouse bioassay is a widely used method, but it lacks sensitivity and is not suitable for quantitation ^{14, 15} because a large number of mice would be involved and usually a license is required to use them. It is qualitative, determines the amount of sample needed to kill a mouse; therefore it is a screen for the ability of a sample to be toxic. However, it shows poor sensitivity and precision, may need a number of mice, and requires a license (animal test).
Protein phosphatase inhibition assay (screen)	Very sensitive. However, it lacks specificity as it will test positive in presence of other phosphatase inhibitors, moreover, it requires a purified enzyme and ³² P isotope has a short half life. It is expensive due to the radioactive ATP and cost of enzymes. they have poor specific identification ability ¹⁵ , usually used as a first screen ^{4, 14}
ELISA (screen)	Very sensitive but it may experience variable cross-reactivity, have poor specific identification ability ¹⁵ and therefore may underestimate concentration. So it is usually used as a first screen ^{4, 14}
HPLC	The most established and commonly used methods are based on HPLC ^{7, 15, 16} . However it can involve relatively lengthy analysis time and high solvent and chemical reagent consumption. More sensitive than mouse bioassay, and can distinguish the microcystins if standards are available. Using photodiode array (PDA) detection, characteristic UV spectra can be obtained to assist in identification of microcystins however, analysis time can be long and other analytes may absorb at the same wave length as microcystins. May not differentiate between the microcystin analogues.
LC-MS	Confirms analytes based on their mass spectra, sensitive and specific, equipment is expensive.
CE	Short analysis time, high separation efficiency, small sample volume, low solvent cost and little hazardous waste, however, It has low UV sensitivity due to small sample volumes used, analytes may need to be derivatised to improve sensitivity and detect with a fluorescence detector. Method requires further development to improve sensitivity.
CE-MS	Specific, equipment is expensive and interfaces still require development.

The use of CE to separate related analogues of microcystins was reported and it was demonstrated to result in better resolution compared to HPLC methods ¹². A simple CZE BGE can be used to separate microcystins in less than 20 minutes with detection limits (LOD) of 0.8 – 4.8 µg/mL ¹⁷. This LOD is comparable to LODs obtained using HPLC with UV detection yet the total run time in CE is shorter. Based on better resolution and shorter analysis time, there are significant advantages in using CE for determination of microcystins and CE can be an alternative technique for the determination of microcystins.

Vasas et al ^{11, 18} and Onyewuenyi et al ¹⁹ demonstrated that CE can be used to separate microcystins and the successful mode of CE employed was micellar electrokinetic chromatography (MEKC) using SDS. The use of capillary electrokinetic chromatography (CEC) has also been reported ²⁰. In most of these methods, about three microcystins were determined from algal masses and their determination in drinking water was not emphasized. Furthermore, there is a challenge of the high detection limits in CE methods compared to the requirement for the drinking water guideline of 1 µg/ L as proposed in WHO drinking water guidelines¹, but this can be solved using pre-concentration techniques.

In the first phase of our study, CZE and MEKC methods for the determination of four related cyanobacterial hepatotoxins (microcystin RR, microcystin YR microcystin LR and nodularin) in drinking water were investigated because they occur most frequently compared to other microcystins. Satisfactory separation was obtained and the methods were validated for the determination of hepatotoxins in drinking water. The methods demonstrated the potential to be applied to a wider range of microcystins because the resolutions between adjacent peaks were good. Microemulsion electrokinetic capillary electrophoresis (MEEC) was used for simultaneous separation of a bigger group of microcystins together with nodularin and cylindrospermopsin using sodium tetraborate as the back ground electrolyte (BGE) in the second phase. Good separation of the analytes was obtained. In this study, we developed capillary electrophoresis methods for a portable CE instrument to determine microcystins in water in order to harness the inherent advantages of CE (speed, resolution, and smaller solvent requirement) and to provide alternative methods to the conventional techniques available.

The amount of cyanotoxins in water bodies is of concern because that is the most likely mode through which animals and humans are exposed to the toxins. Exposure to cyanotoxins in high amounts has been reported to be hazardous, but the effect of exposure of humans to low amounts of these toxins had not been reported. Therefore, the toxic effects of microcystins LR, RR and YR were studied because they are the most frequently occurring microcystins (in Poland ^{4,5}, Thailand ⁵ and in Europe; in Africa, Australia and in China ³).

The effects of exposure of a human cell line to non cytotoxic concentrations of the most frequently occurring microcystins (LR, RR and YR) were investigated in this study using “metabolomics” and “metabonomics” approaches. HepG2 cell line was used and the cells were incubated in media containing different concentrations of the individual microcystins as well as media containing a mixture of the three microcystins because in a real situation, there is a likelihood of occurrence of more than one microcystin in a water body and thus a possibility of exposure to more than one microcystin at the same time. Aliquots of media were collected at different time intervals, extracted and analysed to obtain a finger/footprint of the metabolic profile after exposure.

The WHO drinking water guideline is 1µg microcystin LR per litre or its equivalent ¹, however, most of the literatures report investigations of microcystin toxicity at higher doses and studies of effects at a low dosage exposure over a long term (chronic toxicity) mainly focused on gene expression and DNA damage, for example it was reported that microcystin LR in low amounts (2- 20 µg/L) altered the profile of proteins in zebra fish. The proteins which were affected were involved in cytoskeleton assembly, macromolecule metabolism, oxidative stress and signal transduction ²¹. The metabolomics approach just like other global techniques offers a holistic analysis. By profiling all the metabolites, it is possible to obtain

an unbiased marker compared to conventional approaches of investigation of toxicity such as analysis of enzyme activity ²¹. Furthermore, there are reports describing the effects of microcystins on DNA strands, induction of oxidative damage and apoptosis, effect on glutathione levels and production of reactive oxygen species (ROS). The metabolic approach monitors the actual effect on the metabolic balance in effect giving a clear picture of what is going on in the organism and such an approach has not been reported before.

1.2 Statement of the Problem

The importance of water quality cannot be overstated. Fresh water shortages and water pollution are among the current environmental issues of concern ²². Lakes and reservoirs are vulnerable to overloading of nutrients and therefore eutrophication has become a serious problem ²³. Water eutrophication is of concern because it affects the normal balance of aquatic life. It increases turbidity as well as plant and animal biomass. As a result there is increased competition for nutrients and survival. Algal blooms are known to occur in high eutrophication conditions. These can include blue green algae (cyanobacteria) which produce a range of compounds that are of concern. For this research, the focus was on cyanobacterial toxins of the microcystin group. This is because microcystins are most commonly occurring cyanobacterial toxins worldwide and they are known to be responsible for poisoning of domestic and wild animals and in some instances humans^{2, 4, 5}. They are well known hepatotoxins; they inhibit the protein phosphatases inside hepatocytes thus causing liver damage. With increasing waste disposal into waters, eutrophication has increased and so have the occurrence of microcystins and the likelihood of poisoning from drinking contaminated

waters. There is therefore a need to develop an adequate analytical method for determination and quantification of cyanotoxins in water bodies to monitor contamination.

The ISO method for determination of microcystins is based on HPLC-UV but solid phase extraction has to be used to detect amounts in accordance with WHO water requirements; LC-MS methods have also been described with lower detection limits however they are characterised by long analysis time moreover most of these instruments are bench top sizes and are not easily applied in field. There is a need to develop an analytical method that can be applied on site. Capillary electrophoresis techniques are suitable in this application because they are known for shorter run times, they use small amounts of sample and solvent moreover a portable instrument was available. The methods developed for this portable system can be applied on site for quicker analysis and more representative information.

Microcystins are hepatotoxic and are said to induce necrosis at high doses (acute toxicity). They inhibit serine/threonine phosphatases 1 and 2A, cause cytoskeletal damage, liver necrosis and haemorrhage in the liver. Epidemiological studies have suggested that microcystins are one of the risk factors for the high incidence of primary liver cancer in certain areas of China ²⁴ and microcystin LR has been reported to induce oxidative DNA damage in human hepatoma cell line HepG2. Microcystins have been reported to be responsible for fatalities of animals and humans ^{25, 26}. Microcystin LR in low concentration (nM) caused a collapse of actin filaments in human primary hepatocytes ²⁷ and more recently microcystin were detected in sera of a chronically exposed human population in China ²⁸ which demonstrates a health risk of chronic exposure. Microcystin LR and nodularin were reported to induce time dependent intracellular glutathione alteration ^{29, 30} and production of reactive oxygen species (ROS) and to induce lipid peroxidation in rats ^{30, 31} which are

characteristic of oxidative stress. Oxidative lipid metabolism following acute hepatotoxicity^{32, 33}, induction of apoptosis and nephrotoxicity³⁴ have also been reported. Furthermore, Microcystin LR has been implicated in causing DNA strand breaks^{24, 29, 35, 36}. It is therefore evident that exposure to microcystins is hazardous; the issue at hand is to determine whether chronic exposure to low concentrations of microcystins may eventually lead to liver injury which would be a health risk and what mechanisms are involved. This is because in humans the likelihood of chronic exposure is higher than that of acute exposure in everyday life.

1.3 Scope of Research

1.3.1 Research Aims/Objectives

The general aim of this research was to develop analytical methods for determination of cyanobacterial toxins in water and to investigate the potential effect of exposure to microcystins using “metabolomic” and “metabonomic” approaches.

The specific objectives of this study were to:

- Develop capillary electrophoresis (CE) methods for determination of microcystins in drinking water
- Investigate the effect of exposure of a human cell line to non cytotoxic amounts of microcystins using proton nuclear magnetic resonance (¹H NMR) and mass spectrometry (MS).

In this study, CE technique was selected because of its advantages of good resolution and low solvent consumption. Methods for determination of cyanobacterial toxins in water were developed because contaminated water is the most common exposure route and the methods were developed for a portable capillary electrophoresis instrument to allow for determination of cyanobacterial toxins on site.

The current interest in cyanobacteria toxins research is mainly due to increased awareness of the harmful effects from exposure. Skin irritations, death of dogs and cattle have been linked to microcystin exposure ³⁷ and human fatalities were reported after exposure to cyanotoxins during dialysis in Brazil ^{26, 38}. Moreover exposure to low levels of hepatotoxins has been linked to development of tumours and cancers by an epidemiological study in China ³⁹. This increasing evidence shows that low levels of exposure may have chronic deleterious effects in humans ¹⁴. Therefore it is important to determine microcystins in drinking and other waters even at low concentrations because in addition to immediate effects attributed to exposure at high concentrations, long term exposure to low amounts of the toxins can also cause detrimental effects ²⁸.

Exposure to cyanobacterial toxins in human is not obvious and in most cases human are prone to chronic exposure to low concentrations of microcystin (e.g. in work related exposure such as in fishermen exposed to contaminated water, swimming in contaminated water). It is important to investigate whether continued exposure is hazardous. Different holistic approaches for investigation of biosystems can be used but "metabolomics" and "metabonomics" offer a direct representation of what is going on in the system before permanent features are observed on the phenotype.

The “metabolomics” approach was used to investigate the effects of microcystin toxicity because it generates a unique “finger/footprint” of the cellular processes going on. Compounds measured have a direct effect on the phenotype, the changes in metabolites can be monitored in a time and concentration dependent manner and the effect on the whole organism is determined. The results can be related to classical toxicological end points such as LC_{50} and compared to other techniques like “proteomics” and “genomics”, the components monitored can be directly related to exposure to xenobiotics for example if the xenobiotic binds or inhibits certain enzymes in effect controlling metabolism moreover metabolite monitoring is usually done for a whole organism/system.

Capillary electrophoresis the method of choice for this analysis is described in the following section.

1.4. Capillary Electrophoresis - Basic Principles

Electrophoresis can be defined as differential migration of electrically charged particles in a conductive medium under the influence of an electrical field ⁴⁰⁻⁴². The medium can be a gel (as in gel electrophoresis) or can be a liquid, in most cases a buffer solution. In capillary electrophoresis, the medium is contained in a small capillary ⁴⁰. Therefore capillary electrophoresis can be described as a separation of sample ions in a narrow bore (25 - 100 μm diameter) ⁴¹ or separation of a mixture in a capillary tube due to differing ionic mobilities induced by application of a high voltage along the capillary ⁴³. A schematic diagram of a capillary electrophoresis system is shown in figure 1.4.

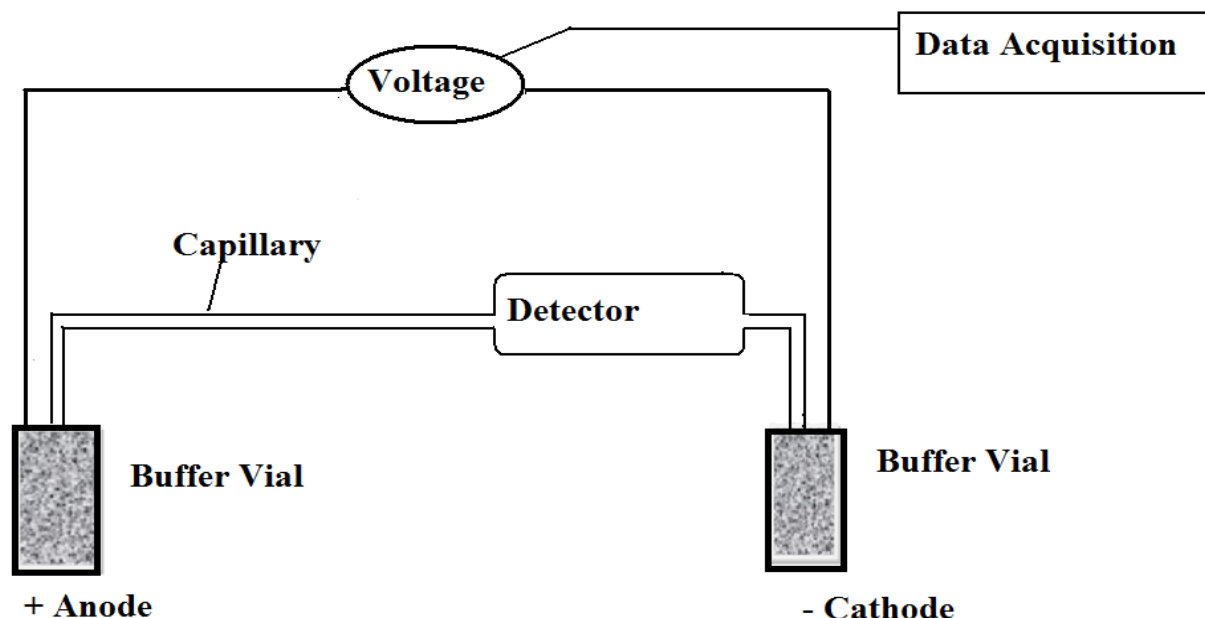


Figure 1. 4: Schematic diagram of capillary electrophoresis system

High performance capillary electrophoresis (HPCE) does not require the use of gels because the capillary walls provide mechanical support for the carrier electrolyte. Small diameter capillaries are used to obtain large ratios of surface area to volume, which enables sufficient heat dissipation and thus allows usage of high voltages necessary for quick and efficient separations⁴⁰. The emergence of HPCE solved some of the difficulties associated with gels such as the difficulty for automation. Furthermore, sample introduction can be performed in a repetitive manner, detection is on column, and the instrument output is easier to interpret since it resembles a chromatogram⁴⁰. Capillary electrophoresis is a commonly a serial technique compared to slab-gels which have a high throughput because multiple samples can be separated at once but capillary arrays have been developed to achieve high throughput⁴² and to overcome this limitation.

1.4.1 Sample Introduction

In CE, a sample can be introduced into the capillary by electrokinetic injection or hydrodynamic injection. Electrokinetic sample injection is achieved by applying a high voltage at the inlet end of the capillary for a short time. Figure 1.5 shows sample introduction by electrokinetic injection. Analyte ions migrate into the capillary by a combination of electrophoretic migration of the ions and electroosmotic flow of the sample solution.

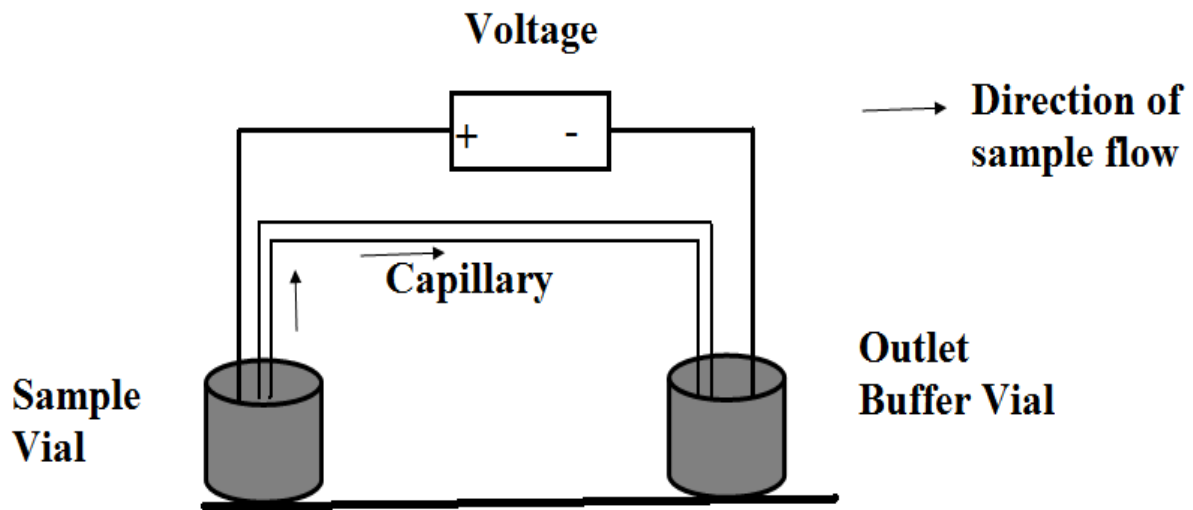


Figure 1. 5: Electrokinetic injection

The length of the sample zone introduced into the capillary (l_{inj} in cm) is described by:

$$l_{inj} = \int_0^{t_{inj}} (v_{eo} + v_{ei}) dt \quad (i)$$

Where: v_{eo} (in cm s^{-1}) is the electroosmotic velocity of the bulk solution

v_{ei} (in cm s^{-1}) is the electrophoretic velocity of ion i , and t_{inj} (in s) is injection time

For a short injection time,

$$l_{inj} = (v_{eo} + v_{ei})t \quad (ii)$$

$$v_{eo} = \mu_{eo}E \quad \text{and} \quad v_{ei} = \mu_{ei}E$$

Where,

E (in $V \text{ cm}^{-1}$) is the electric field strength = V/L , μ_{eo} (in cm^2/Vs) is the electroosmotic mobility, μ_{ei} (cm^2/Vs) is the electrophoretic mobility of ion.

The amount of sample injected in nL/s (m_i) will be proportional to the injection length i.e.

$$m_i = AC_i l_{inj} = AC_i(\mu_{eo} + \mu_{ei})Et \quad (iii)$$

where:

A is the cross sectional area of the capillary, C_i is the concentration of the ionic species and t is the injection time in seconds.

Hydrodynamic injection takes advantage of pressure differences between the inlet and outlet of the capillary which can be achieved by gravity, vacuum at the outlet or increasing the pressure at the inlet ⁴⁴. Hydrodynamic injection by gravity is sometimes referred to as siphoning. The sample vial is raised to a specific height (dH) for a short time interval and the sample is injected into the capillary due to the hydrostatic pressure difference created at the outlet end of the capillary as shown in figure 1.6.

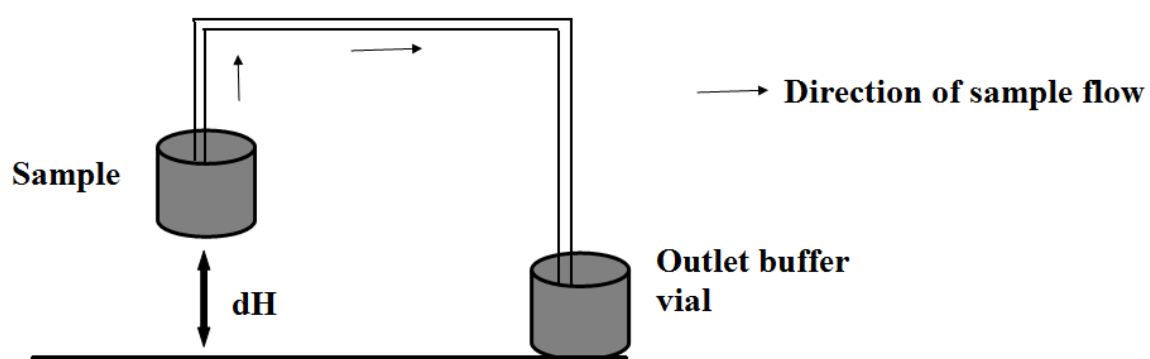


Figure 1. 6: Injection by gravity (siphoning)

Hydrodynamic injection by pressure/ vacuum is achieved by applying a low pressure (often 0.3 – 0.5 psi) at the inlet end or vacuum at the outlet end of the capillary as shown in figure 1.7.

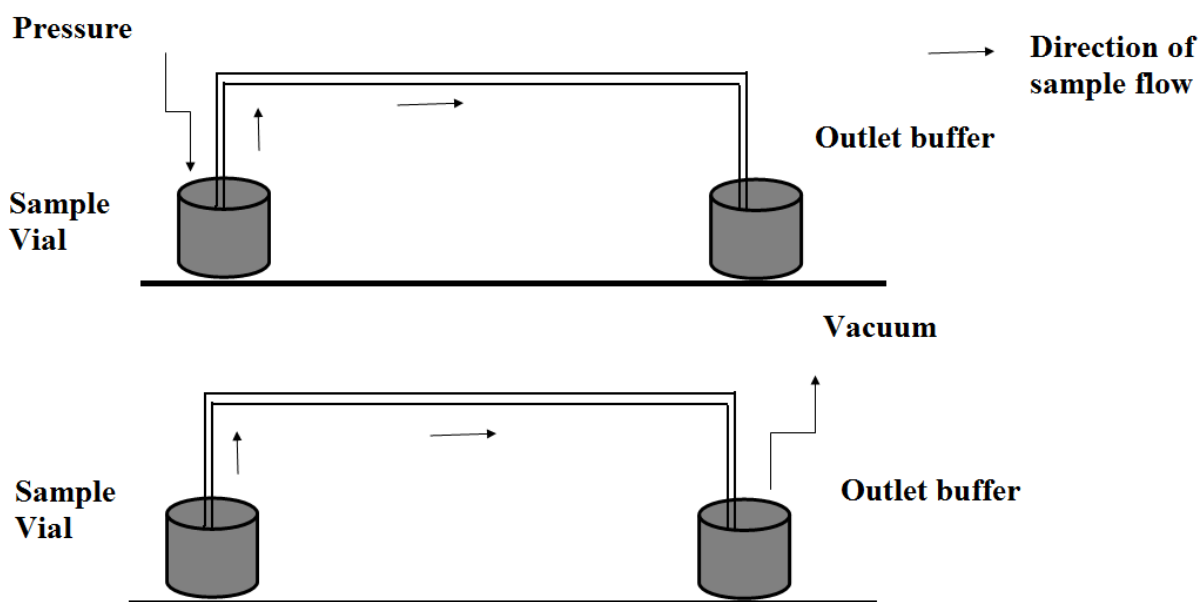


Figure 1. 7: Injection by pressure or vacuum

The length of the sample zone injected can be obtained from ⁴⁴:

$$l_{inj} = \int_0^{t_{inj}} v_{hf} dt \quad (iv)$$

Where: t_{inj} is the total injection time, v_{hf} is hydrodynamic flow velocity and

When the flow velocity is constant the length of the sample injected can be obtained from,

$$l_{inj} = v_{hf} t_{inj} \quad (v)$$

The flow velocity can be described by Poisseuille equation ⁴⁴:

$$v_{hf} = \frac{\Delta P d^2}{32 \eta L_t} \quad (vi)$$

Where,

ΔP is the pressure difference between the inlet and outlet of the capillary, d is the diameter of the capillary, η is the viscosity of the liquid and L_t is the capillary length.

For injection by gravity,

$$\Delta P = \rho g \Delta h \quad (vii)$$

Where: ρ is the density of the sample solution, g is acceleration due to gravity (9.8 m/s²)

⁴⁴ and Δh is the height to which the sample vial is raised.

In general the pressure required for a normalized injection length (effective injection volume),

$$\Delta P = 8 \eta \frac{x_{inj}}{t} \left(\frac{L_t}{r} \right)^2 \quad (viii)$$

Where:

x_{inj} is the normalised injection length, t is the injection time and r is the capillary internal diameter.

By varying the pressure and injection time the sample zone length injected can be controlled⁴⁴. Hydrodynamic injection can be regarded as a universal injection mode in CE since is not biased by the sample or matrix but there is a need to control the pressure to optimise injection. This was the method employed to introduce analytes into the capillary in this research.

1.4.2 Sample Separation

Sample ion migration in capillary electrophoresis occurs as a result of the interplay between the electrophoretic mobility of the sample ions and the electroosmotic force. This determines the migration speed and the order of migration of the sample ions.

1.4.2.1 Electrophoretic Mobility

Upon application of a high voltage, ionic species in the sample migrate towards the electrode of opposite charge. Their speed or electrophoretic mobility and their direction are determined by their charge and mass^{42, 44}.

Electrophoretic velocity of an ion i , $v_{ei} = \mu_{ei}E$ (ix)

$$\mu_{ei} = \frac{q}{6\pi\eta r} \quad (x)$$

Where:

μ_{ei} is the electrophoretic mobility, q is the charge on the particle, η is the viscosity of the buffer and r is the radius of the particle.

The mobility of an ion is directly proportional to its charge and inversely proportional to the radius of the ion and the viscosity of the buffer solution. Therefore, highly charged ions with a smaller size will migrate towards the oppositely charged electrode faster in a solution of low viscosity.

1.4.2.2 Electroosmotic Mobility

Fused silica capillaries are the most commonly used capillaries in CE. When the pH is above 3, the inner surface is ionised⁴⁴. The ionised silanol (SiO^-) groups attract cationic species from the buffer forming a double layer. The ionic layer that is formed has a positive charge density which decreases as the distance from the wall increases. The double ion layer closest to the surface of the capillary (inner Helmholtz layer/stern layer) is static but the layer towards the inner part of the capillary (outer Helmholtz plane) is more diffuse.

Under an applied field, cations in the diffuse outer layer migrate towards the cathode at the same time carrying water of hydration molecules with them. This in turn causes the entire bulk of the solution to follow course due to hydrogen bonding between water of hydration and buffer water molecules as illustrated in figure 1.8.

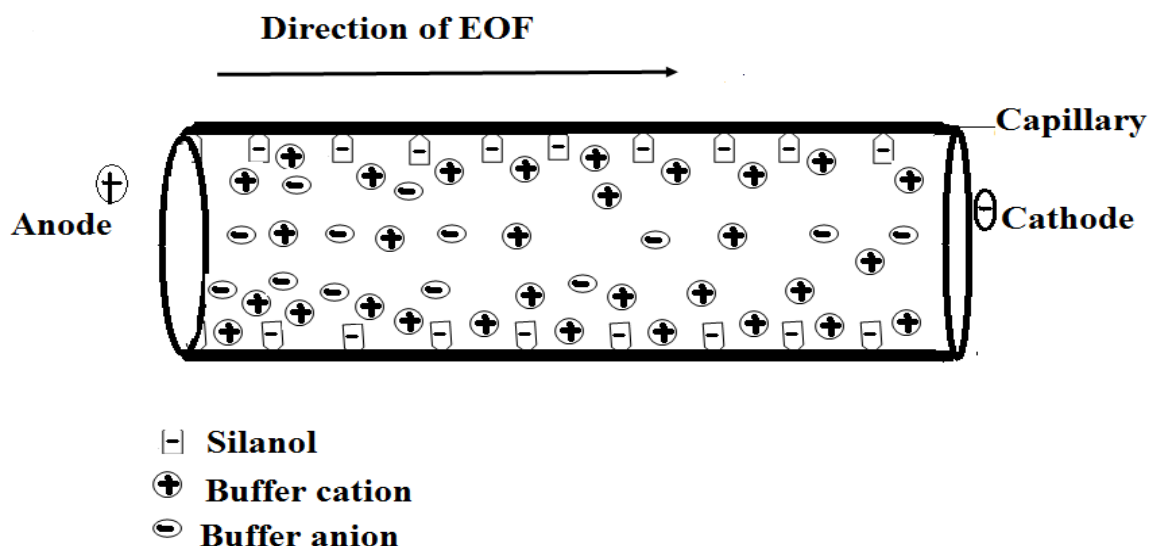


Figure 1. 8: Illustration of EOF inside a fused silica capillary

This force is called electroosmotic flow (EOF) and it leads to an overall movement of the bulk solution towards the cathode if the silica capillary wall surface is bare. In this way all species are carried towards the cathode in the order: cations, neutral ions and anions. Figure 1.9 illustrates the order of migration for a mixture of cations, anions and neutral species. EOF can be manipulated by changing the pH of the solution or coating the capillary wall resulting into an increase, a reduction, elimination or it can even be forced to change direction ⁴⁴.

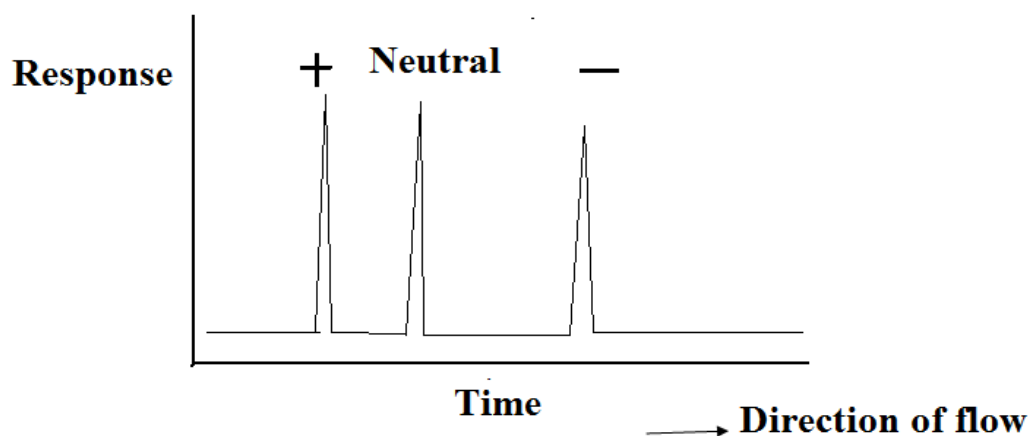


Figure 1. 9: Illustration of the order of migration of ions in CE

The migration of the ion is due to electrophoretic mobility and electroosmotic mobility.

$$v_i = \mu_{app} E = (\mu_{ei} + \mu_{eo}) E \quad (xi)$$

Where: μ_{ei} is the electrophoretic mobility μ_{eo} is the electroosmotic mobility

μ_{app} Apparent mobility E is the electric field strength

EOF has a flat profile rather than a parabolic profile (figure 1.10) which occurs in pressure driven flows. This profile is a significant feature of EOF driven separations because it reduces solute band broadening caused by differences in the bulk velocity and in effect it improves separation efficiency compared to HPLC^{40, 42, 44}.

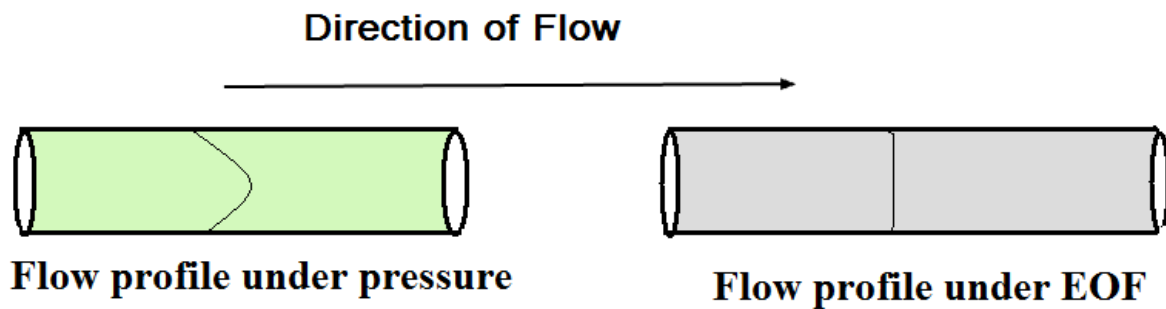


Figure 1. 10. Illustration of EOF profile

1.4.3 Detection in Capillary Electrophoresis

Conventional high performance capillary electrophoresis (HPCE) was developed based on high performance liquid chromatography (HPLC). The most common standard detection methods that are commercially available are based on measurement of optical characteristics such as absorbance and fluorescence^{42, 45, 46}. Detection by UV absorbance is versatile and well suited for UV-absorbing analytes. For non UV-absorbing analytes indirect measurement can be used although sensitivity is usually low. Indirect measurement is done by spiking excess amount of chromophoric species. Sensitivity in both cases is also limited by the short optical path lengths available⁴⁶.

Detection by laser induced fluorescence (LIF) is one of the most sensitive detection methods for many biochemical species, but it is limited by cost, the limited choice of excitation wavelength, time and labour intensive procedures involved in labelling non-fluorescent analytes, however not all compounds can easily be rendered fluorescent⁴².

Electrochemical techniques such as amperometry, potentiometry and conductivity are not as widely used because of poor sensitivity. Amperometry and potentiometry are applied to detect specific analytes (those that can undergo redox reactions), but conductivity is a universal principle which in theory can be applied to all charged analytes and to uncharged analytes separated by micellar electro kinetic chromatography (MEKC)^{42, 45, 46}.

1.4.4 Modes of Capillary Electrophoresis

Capillary electrophoresis (CE) has various modes which use narrow-bore capillaries e.g. fused-silica to separate different compounds with differing mechanisms of separation. Separation of analytes is based on differences in charge, size and hydrophobicity. The different modes include:

Capillary zone electrophoresis (CZE) is one example of free-solution CE (FSCE). This is the simplest form of CE, in which a narrow bore capillary is filled with a homogeneous background electrolyte (BGE) and a constant field is applied across the ends of the capillary. The separation mechanism is based on differences in the charge-to-size ratio of the analytes^{42, 44}. The important parameters to manipulate so as to obtain the best separation are: applied voltage, the choice of buffer and its ionic strength as well its pH. Organic modifiers can also be added to improve separation⁴³.

Capillary gel electrophoresis (CGE) employs polymers in solution which are used to create a molecular sieve to allow analytes having similar charge-to-mass ratios to be resolved^{42, 44}. Separation of analytes is based on differences in size.

Capillary isotachopheresis (ITP) is a free solution focusing technique based on the migration of the sample components between leading and terminating electrolytes. Solutes with mobilities intermediate to those of the leading and terminating electrolytes stack into sharp, focused zones^{42, 44} and are usually separated by CZE.

Micellar electrokinetic capillary chromatography (MECC OR MEKC) is also a free solution technique where surfactants are added to the buffer solution at concentrations over their

critical micellar concentration (cmc) so that they can form micelles. Separation is based on the differential partition between the micelle and the buffer. MEKC is applicable to both charged and neutral analytes and is very useful in separation of hydrophobic compounds ^{42, 44}. Addition of organic modifiers to the mobile phase decreases the extent of partition of non polar components into the micelle phase in effect increasing their mobility. This further improves selectivity ⁴³.

Microemulsion electrokinetic chromatography (MEEKC or MEEC) is a CE technique in which solutes partition into moving oil droplets in a buffer. The micro-emulsion droplets are usually formed by sonicating immiscible heptane or octane with water to make an “oil in water” emulsion even though a “water in oil” emulsion can also be considered. A surfactant such as sodium dodecyl sulphate (SDS) is added at relatively high concentrations to stabilize the emulsion. This allows the separation of both aqueous and water-insoluble compounds. The surfactant helps to reduce the surface tension between the oil and aqueous phases and the surface tension is further reduced by incorporating a co-surfactant in most cases a short chain alcohol such as butanol which stabilises the microemulsion system ⁴⁷. Chromatographic separation occurs by partition of solutes between the oil phase and the aqueous phase with hydrophobic compounds staying in the oil phase and as a result they are separated from the hydrophilic compounds which elute faster. Hydrophobic analytes in the oil phase are covered in negative charge when an anionic surfactant is used and since it will migrate against the EOF, they stay longer in the capillary ⁴⁸. Separation in MEEC is based on hydrophobicity/hydrophilicity as well as electrophoretic migration; this technique can therefore be applied over a wide range of compounds.

Non-aqueous capillary electrophoresis (NACE) allows additional selectivity options in methods development and is also valuable for the separation of water-insoluble compounds. The medium is composed of organic solvents and solvents of a relatively high permittivity, low viscosity, and low vapor pressure are preferred. Parameters such as resolution, analysis time and selectivity can be easily adjusted by the composition of the medium or background electrolyte. Separation of compounds by NACE can be achieved when the analytes are charged or in presence of a charged additive and has mainly used in pharmaceutical and environmental analyses ⁴⁹.

Capillary electro-chromatography (CEC) can be described as a hybrid technique which combines the high separation efficiency of CZE with HPLC but uses an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. The capillary is packed with a stationary phase or the stationary phase may be synthesised in situ to make a monolithic capillary. The different stationary phases available offer an advantage of selectivity ⁴³ and CEC is also used during on-column analyte concentration to pre-concentrate a given sample prior to separation by CZE ^{42, 44}. CEC is greatly impacting on CE techniques but is still limited by development in instrumentation and stationary phases.

Capillary isoelectric focusing (CIEF) is a free solution CE technique in which solutes are separated by focusing them at their isoelectric points. The charge-to-mass ratio for each solute determines its migration, the solute will not migrate when it is neutral, at its isoelectric point, and this is the principle applied in isoelectric focusing. A pH gradient is generated between the cathode and anode using carrier ampholytes. Each solute will migrate to a point where its net charge is zero (isoelectric point or pI), at this point migration stops and the sample is focused into a tight zone. The zone is then mobilized past the detector by either

pressure or chemical means. This technique is applied to amphoteric compounds such as proteins^{42, 44}.

In the first phase of this study, the simplest modes of CE, CZE and MEKC were developed for determination of microcystin LR, microcystin RR, microcystin YR, and nodularin. In the next phase, an MEEC method was developed for determination of a wider range of cyanobacterial hepatotoxins microcystin LA, microcystin LF, microcystin LR, microcystin LW, microcystin LY, microcystin RR, microcystin YR, nodularin and a hepatotoxic alkaloid cylindrospermopsin in drinking water. Satisfactory separation was obtained for all the three modes of CE and the methods were validated for the determination of hepatotoxins in water.

1.4.5 Pre-concentration Techniques in Capillary Electrophoresis

As described earlier, capillary electrophoresis (CE) is a powerful separation technique. It has continued to develop with application in various fields such as environmental analysis, pharmaceutical and clinical analysis. This is largely due to its inherent advantages of high speed, high efficiency of separation and it is environmentally friendly due to the use of small amounts of sample and reagent compared to other separation methods like HPLC⁵⁰. The limiting factor however is poor sensitivity when on-column UV detection is employed. This limitation is due to small loaded sample volume and the narrow optical path length since small capillary internal diameter (id) is used^{50, 51}.

Different techniques have been used to improve sensitivity in CE. These are generally divided into instrument modifications mainly to increase the optical path length such as use

of a bubble cell, Z-shaped or multiple-reflection capillaries; and sample pre-concentration techniques to increase the amount of sample injected.

Sample pre-concentration can be achieved by off line methods such as solid phase extraction. In recent trends online concentration techniques have been used ⁵¹ because they are not as time consuming as off line methods.

1.4.6 Online Sample pre-concentration methods

These techniques rely on manipulation of the electrophoretic mobility of the sample ions for example in stacking (large volume sample stacking, field amplified stacking, pH-mediated stacking, dynamic pH junction stacking, use of solvent plugs); or partitioning into a stationary phase or a pseudo stationary phase which affects pre-concentration of the sample ions e.g. use of monoliths and sweeping ^{50, 51 52}. These techniques can be performed individually or in combination.

1.4.6.1. Stacking

Stacking is based on the differences in the electrolyte properties between the sample and the BGE. Sample stacking occurs when sample ions move across a boundary which separates the region of the sample ions from the rest of the capillary containing the BGE. Examples include sample stacking, pH mediated stacking. Alternatively stacking can be achieved by taking advantage of chromatographic effects such as in using monoliths or using

complexation effects such as the effect achieved by solvent gradients in certain buffers like borate buffer⁵⁰.

Large volume sample stacking (LVSS) pre-concentration technique involves injection of a sample dissolved in a low conductivity background electrolyte (BGE) or in water for a long injection time. It can also be performed with polarity switching to reverse the direction of the EOF⁵³. This method is sometimes referred to as reversed electrode polarity stacking mode and can be applied in microemulsion electrochromatography⁵⁴. In positive polarity, anionic components will move towards the detector and stack at the boundary between the sample zone and the BGE, while the cations and neutral species exit the capillary at the inlet end. When the current reaches its original value, the polarity is quickly switched to the original one leading to a reversal of EOF and separation of the sample ions occurs by CZE. Injection time must be optimized and current monitored. This method is useful for low mobility ions and is not useful for simultaneous separation of cations and anions⁵¹.

In field amplified sample stacking (FASS) the sample is dissolved in a low-conductivity BGE. A sample solution is injected into the capillary for an injection time usually longer than the normal (5-10 s) injection time. When a high voltage is applied, a greater electric field develops across the sample zone causing the ions to migrate more rapidly. At the boundaries between the sample zone and the BGE, the electric field strength suddenly decreases and migration becomes slower, causing the sample ions to be focused near the boundaries. The analytes will finally be carried towards the detector by the EOF whose mobility is greater than that of the sample ions^{50, 51}. This method works well with electrokinetic injection and besides injection voltage, sample injection volume has to be optimized to obtain adequate sample injection while leaving sufficient column length for separation.

Another pre-concentration technique that can be used for online sample pre-concentration is pH mediated stacking where the sample is prepared in a high-ionic strength medium and is electrokinetically injected into the capillary. Then, a plug of strong acid is injected and a positive separation voltage is applied. The sample solution is titrated by the acid to create a neutral high-resistance zone. Therefore a greater field will develop across the neutral zone causing the ions to migrate faster. As a result, the analytes are stacked at the boundary between the titrated zone and the BGE followed by CZE separation⁵¹. The use of pH can also be applied by injecting a sample in matrix whose pH is different from that of the BGE. This is referred to as dynamic pH-junction stacking and it is applicable to weakly ionic analytes that have mobilities which change as a function of buffer pH. This method is mainly applied to low-mobility analytes, and does not work well for neutral species⁵⁰.

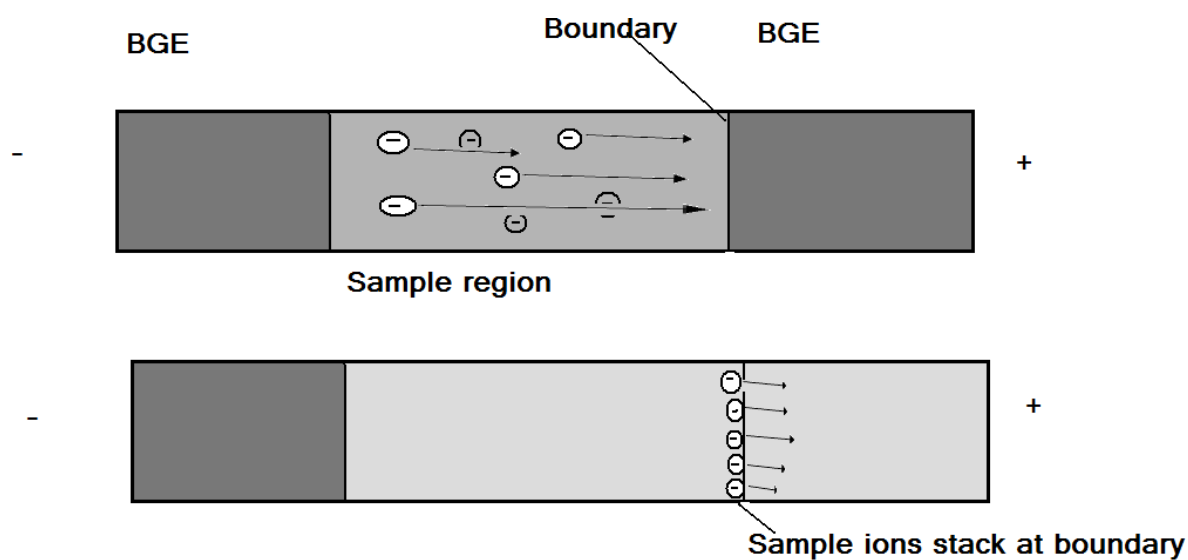


Figure 1. 11: Illustration of sample stacking for anions

1.4.6.2 Sweeping

Sweeping in electrokinetic chromatography can also be used for sample pre-concentration. The sweeping technique was originally developed for the online pre-concentration of neutral analytes in electrokinetic chromatography (EKC). Sweeping can be used whether the sample matrix has a similar or different conductivity from the BGE however it should be devoid of the pseudo stationary phase (PS) ⁵⁰. The principle involves accumulation of sample/analytes by the pseudo stationary phase which fills and penetrates the sample zone on application of voltage. Separation is by electrophoresis which causes the charged PS to move and penetrate the sample zone. Sweeping is applicable to analytes that have a strong affinity to the PS irrespective of the charge.

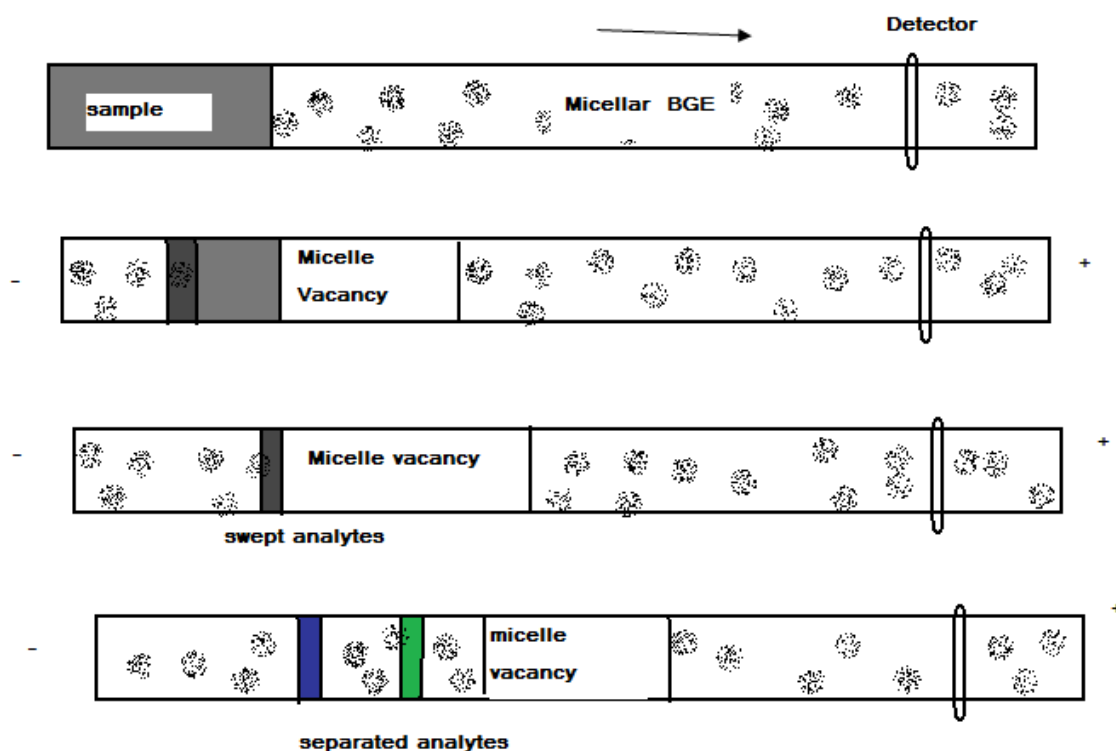


Figure 1. 12: Sweeping in a homogeneous electric field

1.4.6.3 Combination of Pre-concentration Techniques

Dynamic pH Junction–Sweeping: This technique combines dynamic pH junction and sweeping. The sample is prepared in a matrix of a different pH from that of the BGE without the PS. This combination of focusing techniques focuses both neutral and weakly ionic species, and in so doing improves the performance of stacking compared to conventional sweeping or the dynamic pH junction technique used alone⁵⁰. The focusing effects of buffer pH, borate complexation and SDS partitioning have been reported to result in enhanced analyte band narrowing improving the overall sensitivity.

Selective-exhaustive injection–sweeping (SEI-sweep): In SEI- sweep, sample stacking in combination with electrokinetic injection is performed over a much longer period of time than usual (e.g. 400 s). The electrokinetic injection is selective for either cationic or anionic analytes. Prolonged electrokinetic injection can deplete the sample solution of analytes in the inlet vial therefore a fresh solution of the sample is required for every new injection to achieve reproducibility. Samples are prepared in low conductivity matrix and the method works well under suppressed EOF conditions⁵⁰.

For cationic analytes the method used is cation selective exhaustive injection–sweeping (CSEI-sweep), where the capillary is first filled with nonmicellar BGE at low pH to suppress the EOF. This is followed by a high conductivity buffer (HCB) and a water plug. The water plug during the CSEI step helps to maintain a high electric field at the tip of the capillary, which allows the cationic analytes to enter the capillary at high velocity improving the stacking effect. The water plug and the HCB create a long sample zone to achieve a higher concentration than would normally be achieved in the original BGE solution. In the absence

of a water plug, analytes stack at the injection point and this affects field enhancement. The high-conductivity buffer (HCB) improves the total stacking effect of the analytes. It allows the injection of sample ions over a long period thus, increases the amount of sample injected and creates a narrower stacked zone after the CSEI step⁵⁰.

Anionic analytes are focused using anion-selective exhaustive injection–sweeping (ASEI-sweep). In this method, cationic micelles are used as carriers for the anions. EOF is also suppressed and polarities are reversed compared to CSEI. The anionic sample solution is injected electrokinetically with negative polarity and the separation voltage is applied at positive polarity⁵⁰.

Some modifications of the SEI-sweep have been employed including using a microemulsion solution instead of a micelle, or the use of an acid plug ASEI-sweep or a basic plug for CSEI-sweep. These have been reported to improve the total stacking time and in effect detection sensitivity. Use of a salt in high salt stacking electrochromatography has also been reported⁵⁵. In this research, large volume injections together with a solvent plug were used in attempts to pre-concentrate the sample online and improve the limits of detection for the analytes studied in water.

1.5. Metabolomics and Metabonomics - Introduction

Metabolomic techniques have been widely applied in several studies for example in investigation of disease pathologies for the identification of biomarkers of disease ⁵⁶, in toxicology investigations ^{57,58,59}, in toxicity assessment of drugs and other studies to investigate differences in individuals' metabolisms⁶⁰. In this project, the “metabolomics” and “metabonomics” approach was used to investigate the toxicity of microcystins which are potent hepatotoxins with the aim of identifying markers for hepatotoxicity as a result of exposure to low concentrations of microcystins LR, YR and RR.

“Metabonomics” is defined as "a quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification". “Metabolomics” on the other hand refers to “a comprehensive analysis in which all the metabolites of a biological system are identified and quantified” ^{61, 62}. Living organisms have a range of endogenous small molecules such as sugars, amino acids and organic acids in addition to macromolecules such as proteins and nucleic acids. Most of these are products and or intermediates of metabolic reactions although some may be externally acquired from their environment. A comprehensive analysis of the organism's metabolome (total small molecule complement of the cell ⁶¹) is therefore very important for understanding cellular function ⁶³.

There is a growing interest in systems biology, a field which uses holistic approaches to describe biological interactions. Components of a system and their interactions are studied to understand their total effect on an organism or a biosystem. This interest led to the development of the “-omic” studies including “genomics”, “proteomics” and

“metabolomics”. According to Dettmer, Aronov and Hammock⁶⁴ integration of the responses of an organism to external stimuli at the genomic, transcriptomic and metabolomic levels can lead to a better understanding of the biochemical and biological processes in a complex system. Figure 1.13 is a schematic diagram of the relationship between genomics, proteomics and metabolomics.

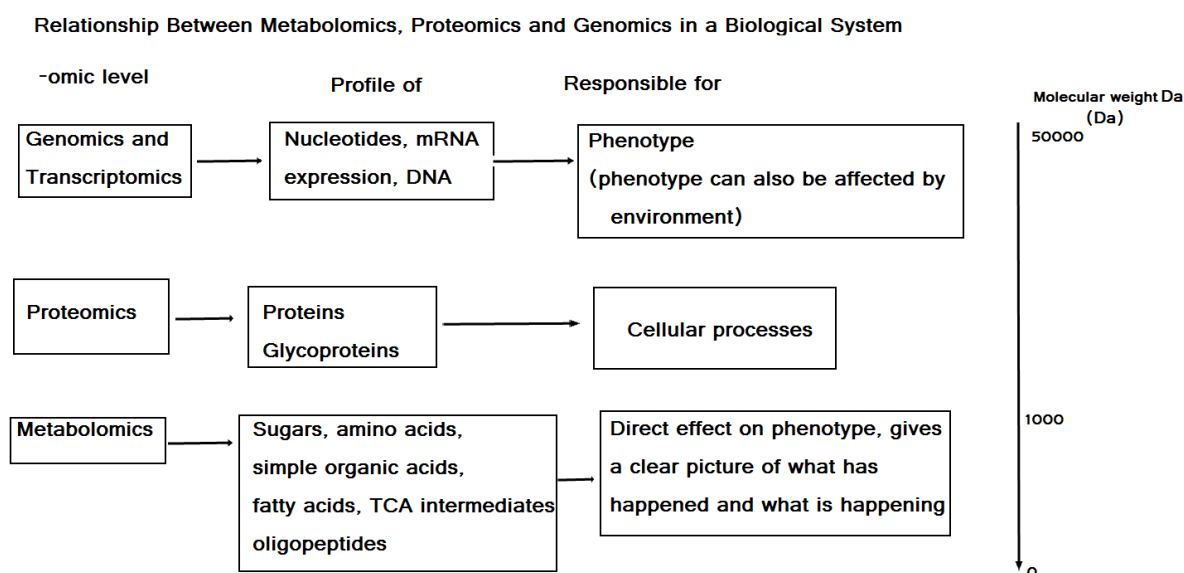


Figure 1. 13: Schematic of the relationship between genomics, proteomics and metabolomics

Genomics describes the genotype (genetic makeup) and is largely responsible for the phenotype (observable trait) therefore it is responsible for what can happen, however other factors such as the environment can also affect the phenotype. Genetic information in DNA is transcribed by messenger RNA which carries this information into the ribosome where protein synthesis occurs. In proteomics, proteins are profiled and studied to understand their structure and function so as to understand how they affect the biosystem. It leads to an understanding of different cellular processes in the cell. Metabolic profiling focuses on a group of metabolites related to a specific metabolic pathway or on a class of compounds. Mainly low molecular weight compounds which are substrates, intermediates and or end

products of biochemical reactions are analyzed. These compounds have a direct effect on the phenotype of the cell or organism or biosystem and as such provide a clear picture of what has happened and what is happening in the biosystem ⁶⁴.

Bioanalytical approaches for measurements of effects of xenobiotic exposure on biosystems have mainly been based on investigations at genetic level (genomics) or at the level of expression of cellular proteins (proteomics). Techniques such as proteomics and transcriptomics are powerful and generate a wealth of information about the different responses of a system to external factors but they are expensive, labour intensive ⁶¹ and they do not put in consideration the dynamic metabolic status of the whole organism.

“Metabolomics” and or “metabonomics” investigates the organism’s metabolome and therefore complements the information obtained by genomics and proteomics ⁶¹ and integration of information at the genomic, transcriptomic, proteomic, and metabonomic levels provides a comprehensive understanding of a system’s biology ^{62, 64}. The important characteristic of “metabolomics” is that it provides information about the quantities of substances interest in an organism as well as the conditions in which they are present for example diseased versus normal state, and in effect the dynamic behaviour of an organism’s metabolism can be understood ⁶³. Furthermore, the metabolome is the most predictive of the phenotype ⁶⁴, so its profile is key to understanding what is going on in the biosystem.

The terms “metabonomics” and “metabolomics” are used to refer to measurement of low molecular weight metabolites (up to ~1000 Da) ⁶⁵ and their intermediates in a biological system in response to external stimuli ⁶⁵. Generally the aim is to generate an endogenous

metabolite profile, a unique fingerprint of the cellular processes that occur in a biological system, i.e. a comprehensive report of all the small molecules in the biosystem⁶¹. It provides many opportunities to investigate and understand changes caused by external stimuli⁶⁵. For the purpose of this report, the term “metabolite finger/footprinting” was adopted to describe “metabonomics” where patterns of metabolites are the main differentiation tool; and “metabolite profiling” to describe “metabolomics” where the metabolites are identified and quantified.

The study of metabolites in an organism provides a comprehensive signature of the physiological state of the organism and gives an insight into its biochemical process. The metabolome when monitored as a function of time allows for understanding the dynamics of homeostasis as well as the effects of external substances on the system⁶⁶ because metabolites play a significant role as building blocks and energy transporters in biological systems.

Metabolites and small molecules investigations started in the early 1970s. At the time, studies were mainly concerned with alterations in biological systems such as those caused by disease and inborn errors of metabolism. This was followed by studies on effects of external factors such as stress, drugs and infection with viruses on the organism’s metabolic profile in the 1980s and has been growing ever since facilitated by the development in analytical and data processing techniques⁶⁶.

1.5.1 Analytical Techniques in Metabolomics

Metabolomics involves quantification of a large number of compounds. Comprehensive analysis is challenging due to the complexity and dynamics of the metabolome, therefore complementary approaches of “metabolic profiling” (metabolite identification) and “metabolic finger/footprinting” (pattern recognition techniques) are often used in metabolome analysis. Due to the large number of compounds and the large dynamic range, high throughput techniques are used. An ideal analytical technique should be able to identify and quantify every component present, however, this is a challenge considering that metabolites have a range of chemical properties and span a wide concentration range (pg - µg per litre). Therefore it is important to strike a balance between minimizing sample preparation to avoid sample loss and removing larger and more abundant components so as to increase the dynamic range and detect components present in smaller amounts ⁶⁶.

Techniques used include: proton nuclear magnetic resonance spectroscopy (¹H NMR), direct injection mass spectrometry (DIMS), liquid chromatography - mass spectrometry (LC-MS), gas chromatography - mass spectrometry (GC-MS) and capillary electrophoresis often with a mass spectrometer (CE-MS). The data obtained is usually interpreted using multivariate statistics and chemometric techniques ^{62, 63}. The analytical techniques mentioned above, their advantages and drawbacks are described in the following section.

The conventional technique used for metabolite analysis is ¹H NMR spectroscopy. ¹H NMR is used to examine the proton spectrum of the sample and as such every component with a proton can be detected and thus has a wide applicability. The spectrum usually consists of many peaks with each peak representing a different chemical environment based on resonance of the ¹H nuclei. This is the basis of chemical classification. Generally, more stable

structures are positioned to the left and more flexible structures to the right so components are in the order: aromatics, sugars, organic acids and aliphatic compounds from left to right on the spectrum based on their chemical shift⁶⁵. ¹H NMR requires minimal sample preparation, generates a wealth of information about the compound classes and chemistries simultaneously with high analytical precision in a short time. It provides information on molecular structure for pure compounds or those in mixtures, has a quantitation ability because peak area/intensity is directly proportional to concentration and is non destructive of the sample. However, it has the limitation of poor sensitivity for compounds in very low concentration (ng per mL) so it is useful for profiling components in the higher concentration (µg per mL and above) range of the metabolome^{62, 65, 66}.

Mass spectroscopy (MS) is widely applied in metabolomics. It has the ability to provide information about the specific molecular masses and mass to charge ratios of the compounds. In direct injection mass spectrometry (DIMS) there is no chromatographic separation. DIMS is a high throughput technique and takes a short overall analysis time (~2 min) per sample. The short analysis time increases inter-sample reproducibility and improves the accuracy in subsequent cluster analysis. However, chemical isomers cannot be distinguished by this rapid screening technique and in the case of electrospray ionization there is concern about ion suppression due to matrix effects⁶⁴.

When MS is coupled with a separation technique such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis it becomes even more powerful because in addition to mass to charge values and molecular masses, compounds can be separated and isomers are identified. In LC, different columns are often used in order to separate a wide range of compounds (with different properties) and quantitation may be variable due to

variability in ionisation and ion suppression effects⁶². With a wide range of columns that are available, and developments in LC such as two dimensional LC, ultra high performance LC (UPLC), ultra fast LC (UFLC) the application of LC-MS in metabolomics is poised to increase.

Gas chromatography is readily available in many laboratories. When coupled to MS it is a highly sensitive technique (due to the high sensitivity achieved by the mass spectrometer) and gives good results for volatile compounds. Non-volatile compounds can be derivatised and GC-MS has the advantage of a bigger established compound library compared to LC-MS. It is a robust technique and can be applied in more than one dimension in the analysis of complex mixtures⁶⁷.

Capillary electrophoresis with mass spectroscopy detection has also been applied to metabolomics. Compounds are mainly separated based on the mass to charge ratio and detected in the mass spectrometer^{62, 63}.

1.5.2 Applications of Metabolomics in Toxicity Assessment

The genomics approach for toxicological assessment involves observation of altered gene expression after exposure to xenobiotics. The challenge here is that there is no direct relationship between gene regulation or expression and the control of cellular systems. This is because the majority of DNA is non-coding and protein coding sequences or genes cannot function as isolated units and therefore require neighbouring genes and/or non-coding DNA

⁶¹. Furthermore there may be some post translational modifications not related to the xenobiotic.

The proteomic approach measures production or changes in cellular proteins after exposure to xenobiotics or in response to other pathophysiological processes. Generally it involves separation and detection or characterisation steps and can therefore be slow and labour intensive. Proteomics can give an insight into understanding the mechanism of toxicity and provides information about biomarkers of exposure to chemicals or biomarkers of disease ⁶¹.

Genomics and proteomics are very useful techniques however it is difficult to relate their findings to classical indices of toxicity and toxicological end points because they do not normally involve measurements of a detailed time-course response to xenobiotics, and the measurements are generally not in a multi-organ system ⁶¹. The metabolomic approach investigates *in vivo* or *in vitro* systems and it provides real time information on responses due to exposure of xenobiotics or other conditions.

Furthermore, certain effects of exposure to xenobiotics may not be related to gene or protein alteration but exposure to foreign material may result in a disturbance of the normal fluxes of endogenous metabolites in the organism by direct chemical reactions, by binding to key enzymes that control metabolism. If the disturbance is significant, it can result into toxic effects affecting the whole organism. Abnormal cellular processes in a biosystem are directly related to biological fluid composition ⁶¹, different foreign substances produce characteristic changes in the concentrations and patterns of endogenous metabolites in biological fluids, so profiling these metabolites can help to understand the mechanism and sites of the processes.

Since the metabolome is close to the phenotype, metabolites profiling provides a better representation of the effects of a biochemical process in a biosystem. For this reason, “metabolomics” and or “metabonomics” techniques rather than “proteomic” or “transcriptomics” techniques were used for investigation of the toxicity of low amounts of microcystins in our study.

1.5.3 Toxicity of Microcystins

Microcystins are reportedly toxic to different multi-cellular organisms such as birds ⁶⁸, fish and mammals ³. In vertebrates, toxic effects of microcystins are linked to inhibition of serine-threonine phosphatases ^{69, 70} which leads to alteration of the cytoskeleton ^{70, 71} in the cells in effect destroying the cell structure.

Several studies investigating the effect of acute exposure and acute microcystin toxicity have been reported ^{72 32 21, 27} however in humans, the main concern is chronic exposure to low microcystin concentrations ⁷⁰ by direct ingestion through contaminated water or eating contaminated seafood or through contact with contaminated water in recreational activities such as swimming or work related exposure such as in fishing, leading to dermal exposure and inhalation. While several organs like kidney, brain, heart and reproductive organs were found to be affected by microcystins ^{69, 70}, the liver is the primary target of microcystin toxicity. This is because microcystins are large molecules with a partial polar character and therefore cannot easily diffuse through the cell membrane. Their transport is facilitated by organic anion transport peptides (OATP), mainly expressed in the liver.

Recent research suggests that chronic exposure to low amounts of microcystins (2 -20 µg/L) is deleterious as demonstrated through effects such as alteration of the liver structure and protein profiles in zebra fish ²¹ and indication of hepatocellular damage among individuals chronically exposed to microcystins ²⁸ , in fact chronic exposure has been linked to development of primary liver cancer ⁶⁹. Several studies have been carried out *in vivo* using whole animals but due to ethical issues, there is a growing tendency to use cell lines to investigate toxic effects *in vitro*.

Microcystins were found to induce apoptosis in several mammalian cells but hepatocytes were severely affected with primary hepatocytes being the most sensitive ⁷³. Primary hepatocytes usually lose the organic anion transporting peptides during isolation and culturing and therefore lose responsiveness to microcystins ⁷⁴, HepG2 (human hepatocellular carcinoma) can be used as an alternative because they have been demonstrated to uptake microcystins even at low concentrations ^{24, 75, 76} and in addition to their human origin they retain several enzymes used in the metabolism of xenobiotics such as cytochrome P450 oxidases and glutathione-s-transferases.

Microcystins inhibit phosphatases PP1 and PP2A in both acute and chronic exposures ⁶⁹ however, the effects of microcystins are time and concentration dependent ⁷⁰. Cytoskeletal damage, protein hyperphosphorylation, apoptosis and cell blebbing all leading to cell death have been reported to occur shortly after exposure to high amounts of microcystins in hepatocytes and other cells ^{32, 70, 73, 77}. Exposure to high amounts of microcystin inhibits the protein phosphatases affecting the cell's homeostasis by disrupting the functions of PP1 and PP2A as well as their protein substrates ^{69, 70}. The main pathways regulated by phosphorylation and therefore affected by the activities of PP1 and PP2A are affected by

regulation of protein activity, examples include: cell signaling, gene expression, DNA repair and mitosis ⁷⁰. These pathways are affected through phosphorylation of signaling proteins which can result into uncontrolled cell proliferation leading to cancer or apoptosis and cell death. Loss of protein kinase activity of DNA-dependent protein kinase (DNA-PK) is induced by phosphorylation of proteins and this affects DNA repair. Calcium-calmodulin-dependent multifunctional protein kinase II (CaMKII) can be activated by Ca^{2+} /calmodulin, autophosphorylation and limited proteolysis. Exposure to microcystins activates CaMKII and this activation of CaMKII is responsible for regulation of formation of reactive oxygen species (ROS) and phosphorylation of proteins. Inhibition of PP1 and PP2A activates CaMKII which subsequently initiates apoptosis ⁷¹. Furthermore, inhibition of PP1 and PP2A affects the nuclear phosphoprotein P⁵³ which is induced in response to cellular stress and plays a role in DNA repair, apoptosis and tumor suppression pathways ^{69, 70}.

Exposure to low amounts of microcystins on the other hand down-regulates the effects of PP1 and PP2A and leads to an increase in the activity of survival proteins: diacylglycerol (DAG), protein kinase C (PKC) and mitogen activated protein kinases (MAPK) of the kinase pathway; and down regulation of death proteins and damaged cells therefore can survive. Down regulation of PP1 and PP2A also produces a low amount of ROS. A low amount of ROS promotes DNA strand breaks and oxidation of purines and pyrimidines resulting into enhanced survival of damaged cells ⁷¹.

1.5.5 Metabolomics and Metabonomics Approach in the Study of Microcystin Toxicity

The “metabolomic” approach was used to investigate the effect of chronic exposure to microcystins. In the case of humans, acute exposure is not common because compared to other animals for which may drink algae contaminated water in large quantities, humans can be exposed to low amounts by ingesting contaminated water (but they drink less and in cases where the algae/toxins are not obvious to the naked eye) or raw seafood or by exposure through swimming or even work related exposure. Therefore, since the exposure is not so obvious, there is a risk of unknowingly being exposed to microcystins in large or small amounts over a long term leading to chronic exposure. While the amount of toxin may be low, continued chronic exposure may be hazardous.

In this study, we investigated the effect of exposure of a human cell line (HepG2) to lower concentrations of microcystins in order to understand the effect of chronic exposure to microcystins on humans because that is the most probable risk to humans. Besides, a lot of research works have been reported for acute exposure in mammals such as rats for whole organisms as well as their hepatocytes and there are only a few works about chronic exposure and even these focused on proteomic and transcriptomics approaches.

The HepG2 cell line was used because microcystins are liver specific toxins. They are relatively large molecules, have a partial hydrophobic character but also exhibit a polar character due to the presence of amido and carboxylic groups⁸ and their transport to the liver is facilitated by the bile acid transport system. In the ileum, microcystins are rapidly absorbed and transported through the iliac and portal vein to the liver. Hepatocytes are rich in organic anion transport proteins which are responsible for active uptake of microcystins from blood

⁶⁸. Furthermore, the liver is a target for xenobiotics and chemical or drug induced toxicity due to its involvement in active metabolism. HepG2 cells retain activities of various phase 1 and phase 2 drug metabolising enzymes that participate in activation and detoxification of xenobiotics and they have been reported to assimilate microcystin LR ³⁵. The HepG2 cell line therefore provides a good model for investigation of effect of exposure to microcystins in humans due to their human origin and because they retain many of the specialized liver functions and metabolizing enzyme activities present in human hepatocytes ⁷⁸.

CHAPTER 2 –DEVELOPMENT OF METHODS OF ANALYSIS

2.0 Development of CE Methods for the Determination of Cyanotoxins

Capillary electrophoresis is a family of techniques which can be modified to suit separation of compounds with different chemistries, the simplest of them CZE can separate compounds of different polarities, where analytes are hydrophobic MEKC can be used and in cases of a range of polarities MEEKC can be used. These techniques were used as shown the sections that follow.

2.1 CZE and MEKC Methods for Determination of Microcystins LR, RR, YR and Nodularin.

In the first phase of method development, capillary zone electrophoresis (CZE) and micellar electrokinetic electrochromatography (MEKC) methods were developed for application to three microcystins that have similar chemical properties namely: LR, RR and YR and a related hepatotoxin nodularin. These microcystins are also the most frequently reported to occur in environmental waters³.

2.2 CZE and MEKC Materials and Methods.

2.2.1. Experimental

Capillary electrophoresis was performed on a portable CE-P2 system (CE Resources, Singapore) equipped with a UV-Vis detector (Shimadzu SPD 10A). Detection was at 238 nm ($\lambda^{\max}$ for microcystin). Fused silica capillaries of 50 μm ID (Polymicro technologies) of 65 cm total length (55 cm effective length) were used and were conditioned by flushing with aqueous NaOH (1M) for 30 minutes, followed by deionised water for 30 minutes and finally BGE for 30 minutes. In subsequent use the capillary was flushed with NaOH (1M) followed by water and BGE for 20 minutes each before commencing experiments each morning, and the capillary was rinsed with BGE between runs. The sample was injected hydrodynamically at 0.3 psi for 10-15 seconds and voltage applied was + 20 to + 25 KV or – 20 KV for the CZE and MEKC modes respectively. Data was acquired and analyzed using Clarity chromatography software from Data Apex Clarity (Prague, The Czech Republic).

2.2.2 Chemicals

Cylindrospermopsin, microcystins LA, LF, LR, LW, LY, RR, YR, and nodularin were obtained from Alexis biochemicals (Switzerland). Their structures are shown in figure 2.1. Formic acid and sodium hydroxide were from Fluka (Singapore). Sodium tetraborate was from Merck, cetyltrimethylammonium bromide (CTAB), ethylene glycol, octane and sodium dodecyl sulphate (SDS) were from Sigma Aldrich (Singapore). Phosphoric acid was supplied by Riedel-de Haen (Germany) and methanol was obtained from Tedia (Tritech Scientific Pte Ltd, Singapore). Butan-1-ol used was from Univar. C₁₈ octadecyl silica (ODS) solid phase

extraction (SPE) column, 500 mg (Whatman) and SPE manifold from Supelco (Sigma Aldrich- Singapore) were used for sample preparation.

2.2.3 Sample Preparation

Standard solutions of 25-100 µg/mL (25-100 ppm) concentration were prepared by dissolving 25-100 µg of each individual standard in deionised water (1 mL). For the test samples, known amounts of the standard solution were spiked in tap water or reservoir water. This was followed by extraction using a C₁₈ (octadecyl silica) SPE column aided by an SPE manifold for nodularin and microcystins LR, RR and YR.

Real water samples (1 litre each) made up of surface water (collected at 4 metres deep in the water reservoir) were collected from Kranji water reservoir (Singapore). An aliquot of the real sample (50 - 500 mL) was spiked with microcystin standards and extracted. Another aliquot of equivalent volume was extracted using the same procedure as the spiked sample and the two were compared.

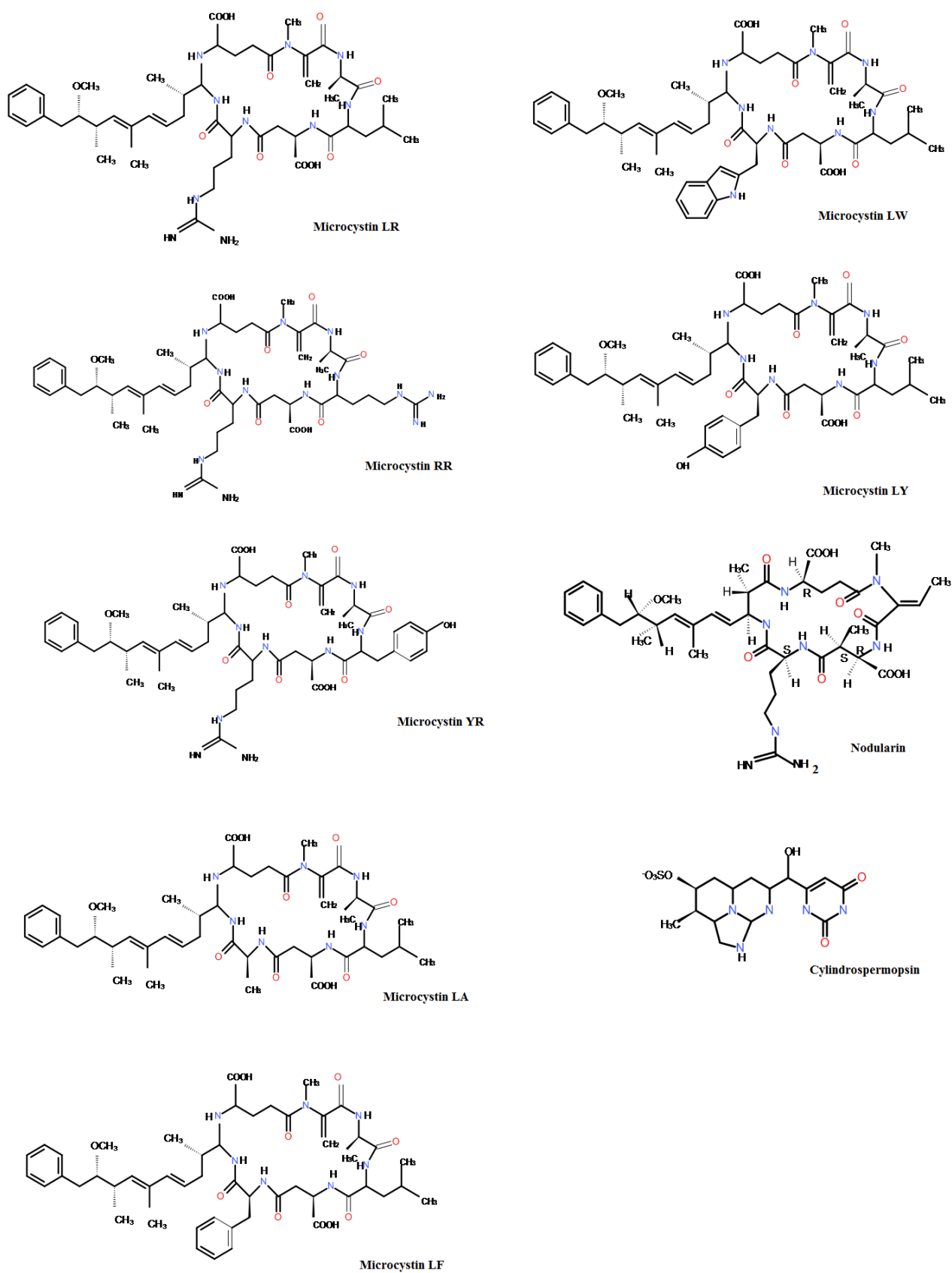


Figure 2. 1 Structures of cyanotoxins investigated in the study

2.2.4 Selection of Background Electrolyte and Detection Mode

Glycine, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), phosphoric acid and formic acid were investigated as possible background electrolytes for use in CZE separation mode using a capacitively coupled contactless conductivity detector (C4D) and a UV detector at the wavelength of 238 which is $\lambda^{\max}$ due to the Adda moiety in microcystins.

2.2.5 Extraction of Nodularin and Microcystins LR, RR and YR

Sometimes the water sample under test may contain other impurities. Furthermore the WHO guideline for microcystin in drinking water is 1 µg per litre^{4, 16, 79}. Solid phase extraction can be used to remove some interference and to concentrate the sample to an amount that can be detected by UV using the methods investigated here.

The SPE column used was activated by 10 ml of HPLC grade methanol (100 %,) followed by deionised water (10 mL). The test sample solution was prepared by spiking 5 - 50 µL each of the 100 ppm standard solution of microcystins LR, RR, YR and nodularin into 50 - 500 mL of tap water or reservoir water sample. This was extracted using C₁₈ SPE column. Finally, the test sample was eluted with methanol (5 mL) and concentrated by evaporation in a water bath. The test solution was made by reconstituting the sample with deionised water (100-500 µL).

2.3. Results and discussion

2.3.1 Detector and Background Electrolyte (BGE) Choices

2.3.1.1 Detector

Test samples were run using a capacitively coupled contactless conductivity detector (C4D) and a UV-vis detector to investigate the possibility of use for either detection method. The use of C4D detector did not yield satisfactory results as the peaks were not reproducible as seen in figure 2.2 below perhaps due to poor sensitivity of the detector used. The use of UV detection provided better peak reproducibility and was therefore used in subsequent method development.

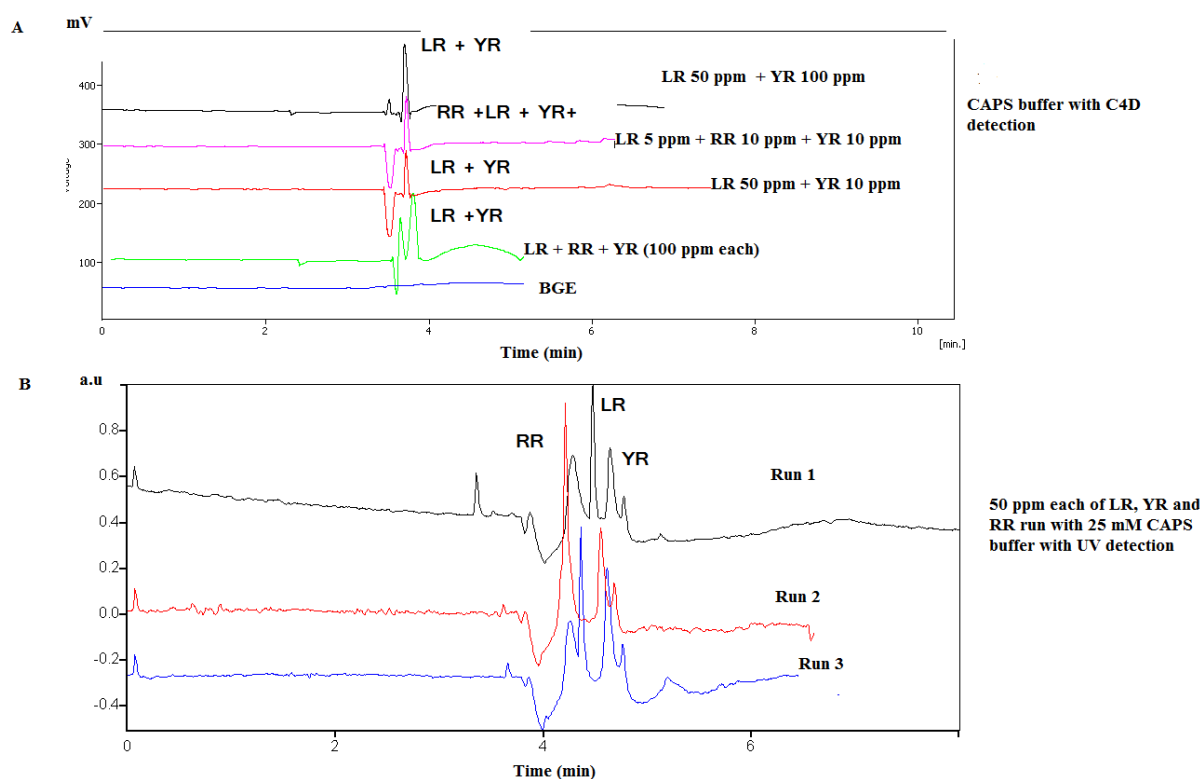


Figure 2. 2 Electropherograms obtained with C4D detection (A) and with UV detection (B).

2.3.1.2 Background Electrolyte (BGE)

CAPS buffer as a BGE resulted in three main peaks for the four analytes. Even though the peaks were reproducible, they were broad. Therefore, formic acid (1 M) was investigated as a BGE using UV detection. The choice of an acidic BGE was influenced by the properties of the analytes. Microcystins LR, RR and YR all have pKa of less than 3.5, so above this pH, they will ionise and become positively charged⁸, if the BGE pH is above 3, the silanol wall in the fused silica capillary would be negatively charged. These two factors encourage analyte binding onto the capillary wall reducing efficiency of the CZE method for quantitation.

Good separation was obtained and the peaks were reproducible when formic acid (FA) was used as a BGE. Therefore, for subsequent separations, formic acid was used as the background electrolyte (BGE).

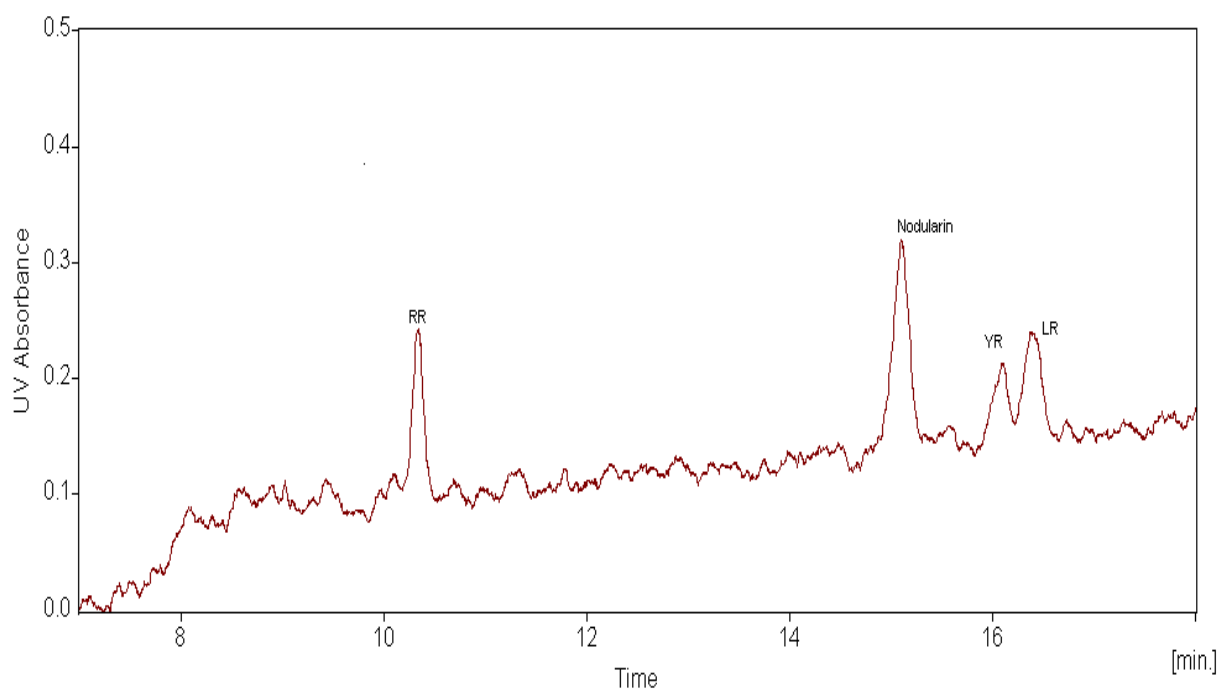


Figure 2. 3 CZE electropherogram of microcystins LR, RR, YR and nodularin. Analytes were 5 µg /mL each; separation was at +25 KV, BGE 1M HCOOH.

2.3.2 CZE Separation Conditions

Formic acid (1 M) was used as a BGE in the CZE mode at a voltage of + 25 KV. The microcystins were very well resolved. No special pre-treatment or conditioning of the capillary was necessary and the hepatotoxins could be detected at low $\mu\text{g/mL}$ concentration levels. (Figure 2.3 and table 3.)

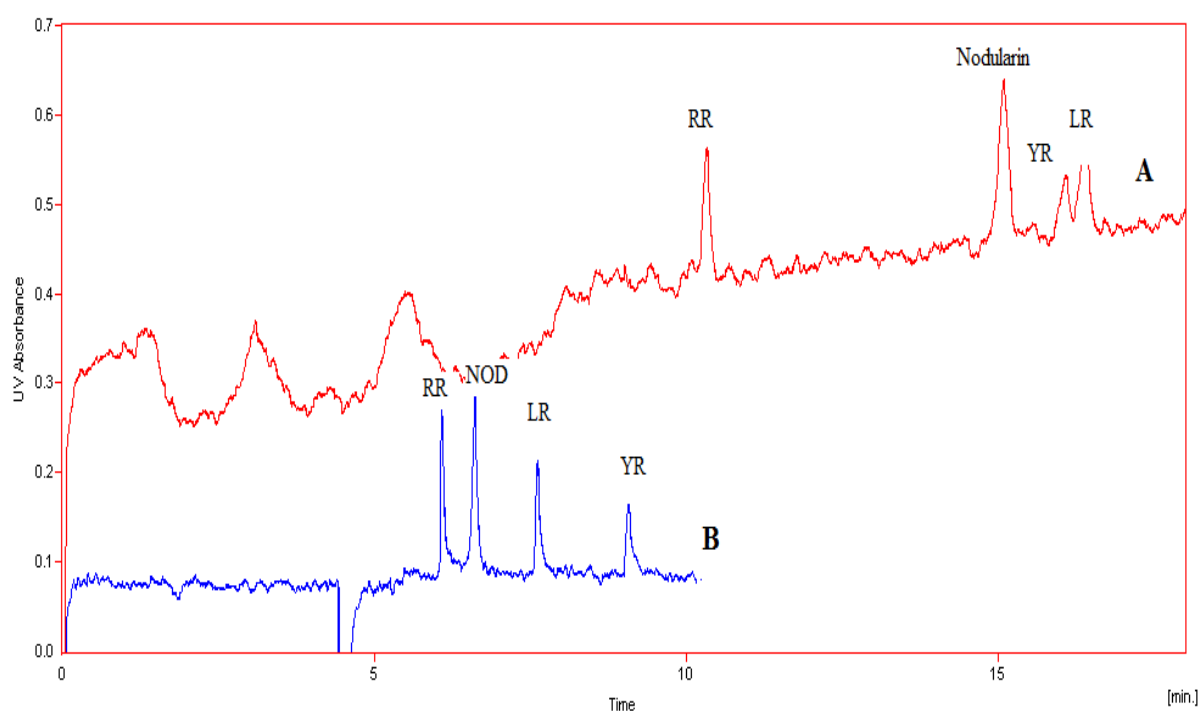


Figure 2. 4. Electropherograms of microcystin RR, nodularin, microcystin YR and microcystin LR. A) CZE - BGE was formic acid (1 M) and voltage was +25 KV. B) MEKC - BGE was made up of formic acid (1 M), CTAB (0.1 %) and ethylene glycol (0.1 %). Voltage was - 20 KV. The concentration of each of the hepatotoxins was 5 $\mu\text{g/mL}$, UV detection at 238 nm.

The elution time in CZE mode was slightly long (~ 17 min) compared to the elution time in the MEKC mode. This is in agreement with other research works^{11, 18, 19, 80} which show that the microcystins migrate slowly in acidic pH. The use of formic acid as a BGE was first reported by Bateman et al⁸⁰ in the analysis of microcystin LR. In this study we investigated

simultaneous determination and quantification of three related microcystins LR (RMM 995.17), RR (RMM 1038.2) and YR (RMM 1045.19) and a related hepatotoxin nodularin. Phosphoric acid used as a BGE behaved similarly even though the retention time was slightly longer and peaks were broader as shown in figure 2.5 A.

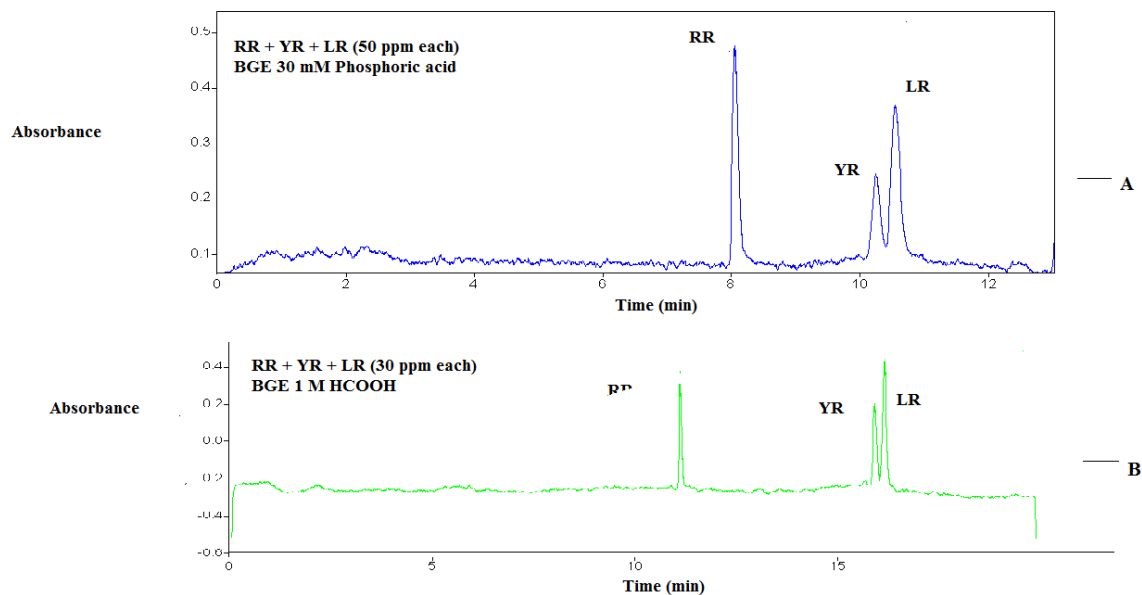


Figure 2. 5. CZE Electropherograms obtained using: A - 50 µg/mL each of microcystins RR, YR and LR with phosphoric acid (0.1 M), B - 30 µg/mL each of the microcystins with formic acid (1 M) as BGE

At acidic pH, microcystin RR has two positive charges (from arginine) and therefore will migrate towards the cathode faster. Moreover arginine is very hydrophilic and so solubility in polar solvents is high. Microcystins LR and YR have a single positive charge but the amino acid leucine is more hydrophobic than tyrosine. Therefore YR migrates slightly faster and hence they can be separated. Nodularin has a single positive charge as well and migrates faster than microcystins LR and YR since it is smaller.

2.3.3 MEKC Separation

For MEKC, separation was achieved at a voltage of – 20 KV. The BGE used was composed of formic acid (1 M), cetyl trimethyl ammonium bromide (CTAB) (0.1 %) and ethylene glycol (0.1 %). Figure 2.3 B shows the results obtained. The migration order between microcystins LR and YR reversed and MEKC gave a very good resolution between the analytes (4.3 between RR and nodularin, 7.1 between nodularin and LR; and 10.2, between LR and YR) as well as a shorter total run time.

The use of both CZE and MEKC is applicable for confirmation of the results and can be useful especially if the peak of the analyte overlaps with that from matrix component ¹⁸ in cases where no sample pre-treatment is done or where sample pre-concentration does not include removal of interfering substances. The CZE method and the MEKC method were evaluated in their analytical performance and the data is shown in Table 2.1.

2.3.4 Comparison of the CZE and MEKC Methods.

Both techniques offer a good separation. Good resolution (4.3 between RR and nodularin, 7.1 between nodularin and LR; and 10.2, between LR and YR) and faster migration times were obtained using the MEKC technique (refer to table 3 parts A and B). However in both cases the resolution obtained is acceptable (18.7 between RR and nodularin, 3.5 between nodularin and YR; 0.95 between YR and LR for CZE) and the detection limits were in the same range.

2.3.5 Pre-concentration

The low volumes injected in CE separations are said to be partly responsible for high detection limits. Furthermore in these methods developed the limits of detection (LOD) obtained were higher than the recommendations from WHO drinking water guidelines of microcystin LR 1 µg per litre ¹. Therefore pre-concentration techniques were used to improve the LOD.

Table 2. 1 Analytical Performance of CZE and MEKC methods

	Migration time (minutes)	% RSD in migration time	LOD 3σ (µg/mL)	% RSD in peak area	R²
Part A. CZE*					
Microcystin RR	10.79 13.88**	0.42 1.36**	3.40	4.61	0.978
Nodularin	19.45**	1.81**	0.78	2.59	0.991
Microcystin YR	14.85 20.58**	0.53 1.36**	4.82	2.89	0.995
Microcystin LR	15.05 20.96**	0.58 1.36**	4.73	8.80	0.992
Part B. MEKC ^a					
Microcystin RR	6.13	0.62	1.40	6.44	0.995
Nodularin	6.63	0.63	1.03	5.84	0.999
Microcystin LR	7.60	0.75	2.70	2.26	0.988
Microcystin YR	9.09	0.85	3.20	2.70	0.997
Part C. CZE of a real sample spiked with RR, YR and LR					
			(µg/L)		
Microcystin RR	13.43**	2.06**	0.09	9.91	
Microcystin YR	21.16**	3.23**	0.08	11.70	
Microcystin LR	21.61**	3.31**	0.11	9.96	

* CZE at + 25 KV without stacking

** Stacking with the aid of methanol at + 20 KV

a MEKC experiments were done at – 20 KV, no stacking

2.3.4.1 Online Pre-concentration by Sample Stacking.

This technique is usually achieved through by increasing the amount of sample injected. Such techniques include large volume injection also known as large volume sample stacking.

The small sample volumes injected in CE are partly responsible for low sensitivity. Therefore a larger volume was injected. Optimum increases in area and height were obtained after injection of sample for 60 seconds; however the resolution between the analytes became worse with increasing time as shown in figure 2.6. Therefore a trade off was made at injection of 40 seconds to take advantage of large volume injection without compromising resolution.

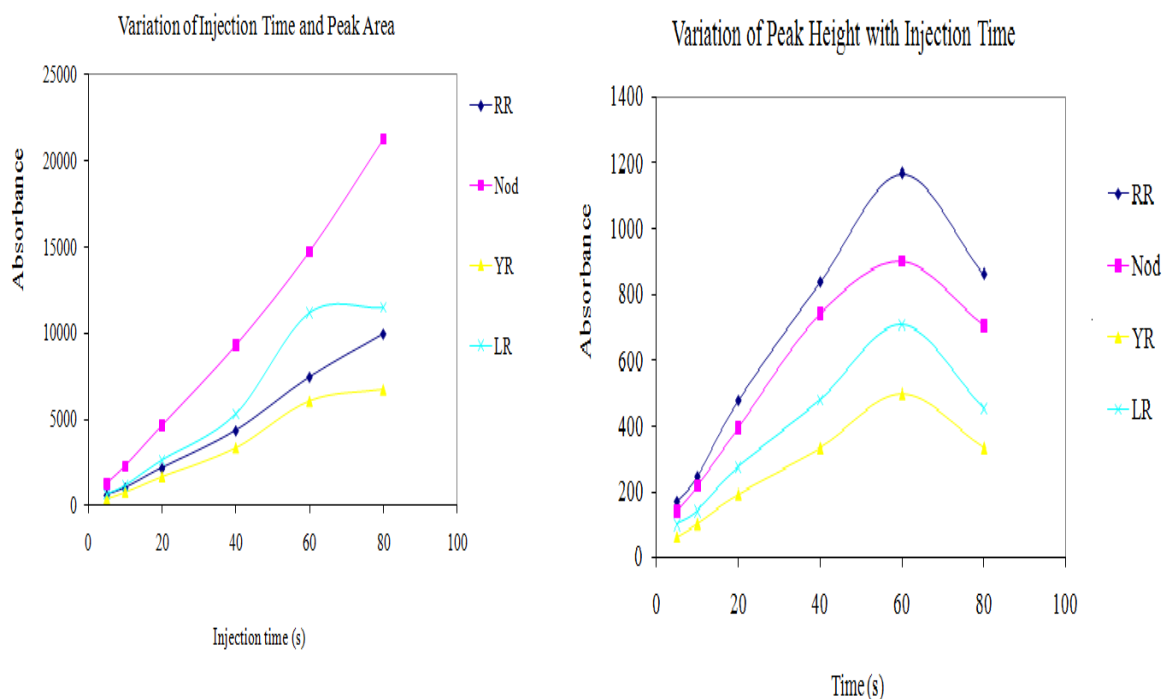


Figure 2. 6. Variation of injection time with peak area (A) and peak height (B)

Stacking with the aid of solvents has been reported to improve on the sensitivity. Therefore, the attempt to use various solvents: water, methanol and acetone was studied for use as the

solvent in the plug to optimize the stacking efficiency. Injection times (volume) for the sample and for solvent were also investigated. Optimum results were obtained by injecting methanol in the solvent plug for 5 seconds followed by the sample at 0.3 psi for 40 seconds as shown in figure 2.7 which show optimum organic solvent injection below.

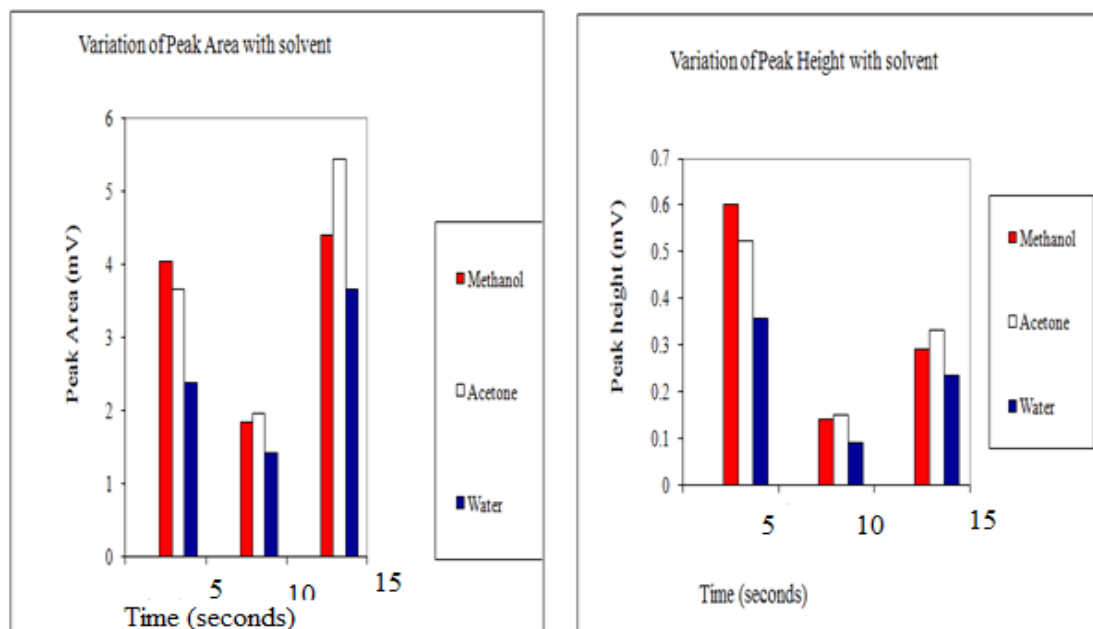
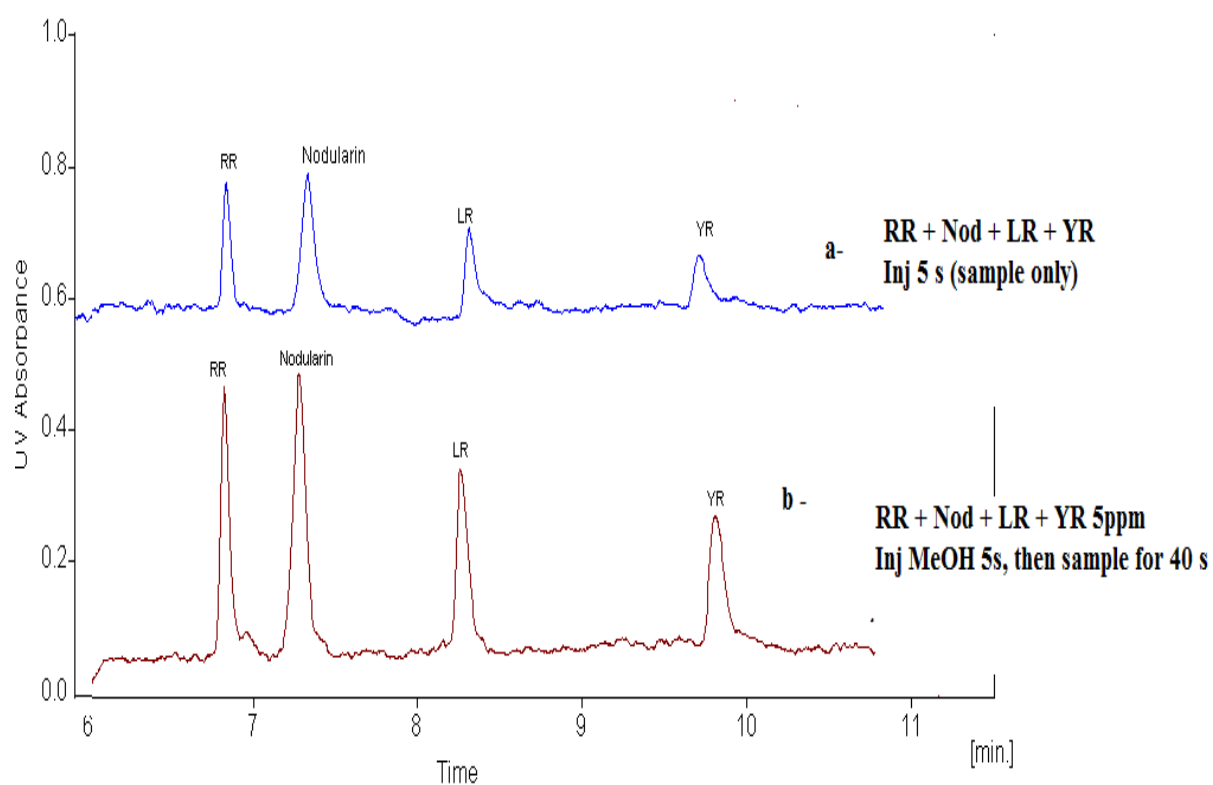


Figure 2. 7. Investigation of effect of solvent on peak area (A) and peak height (B)

Using methanol as an aid during stacking resulted in peak heights for the analytes increasing 3.9 times for RR, 4.8 times for YR and 2 times for LR in CZE and at least twice in MEKC. Table 2.2 and the electropherograms in figure 2.7 demonstrate that there was a slight sensitivity improvement.

Table 2. 2 Stacking with the aid of methanol in the CZE mode

Condition	Microcystin RR Peak height (mV)	Microcystin YR Peak height (mV)	Microcystin LR Peak height (mV)
Injection of sample for 10 s	0.154	0.031	0.163
Injection of sample for 40 s	0.411	0.131	0.292
Injection of methanol for 5 seconds followed by sample for 40 s	0.601	0.149	0.330
Enhancement	3.903	4.806	2.035

**Figure 2. 8 Stacking with the aid of methanol**

2.3.5.2 Offline Sample Pre-concentration with the Aid of Solid Phase Extraction (SPE)

Off line concentration was achieved with the aid of solid phase extraction. Water samples spiked with nodularin and microcystins LR, RR and YR were extracted and eluted as described in the experimental section. When a known amount of standard was spiked into a known volume of water and extracted, recoveries were 90.3 % (RR), 101.5 % (nodularin), 90.6 % (YR), and 88.2 % (LR).

The use of SPE enabled cleanup and pre-concentration of a real sample to achieve a 100-fold concentration factor. Detection limits after SPE of the real sample spiked with microcystins were 0.090 mg/L (RR), 0.076 mg/L (YR), and 0.110 mg/L (LR), with percentage RSD values of 9.9 – 11.7 % for peak area and 2.2 – 3.3 % for migration time. This method therefore can be used to determine microcystins at a level acceptable to the WHO drinking water guidelines.

2.3.6 Tap Water Analysis

Tap water was spiked with microcystins RR, LR and nodularin (5 µg/mL each), and the sample was analyzed by CZE with stacking at + 20 KV. The results are shown in the electropherogram in figure 2.8 A. The use of CZE and MEKC is particularly useful for cases where no sample cleanup is carried out. This is because the different separation mechanism involved can allow for identification of peaks in case of the matrix peak overlapping with the microcystin peaks. When sample cleanup is done, either CZE or MEKC can be used as a stand-alone technique in the determination of the microcystins.

2.3.7 Reservoir Water Sample Analysis

A reservoir water sample (50 mL) was extracted as described in the experimental section and eluted with methanol (5 mL). The sample was evaporated in a water bath and the final test solution was reconstituted with deionised water (500 μ L). To another aliquot of the reservoir water sample (50 mL), microcystin standards RR, LR and YR (50 μ L each of the 100 μ g/mL) were added. This sample was also extracted, eluted and reconstituted as the first sample. Microcystins LR, RR and YR were not detected in the reservoir water sample. Good reproducibility was obtained for the spiked sample as shown in table 3 part c. Figure 2.8 B shows the electropherograms obtained.

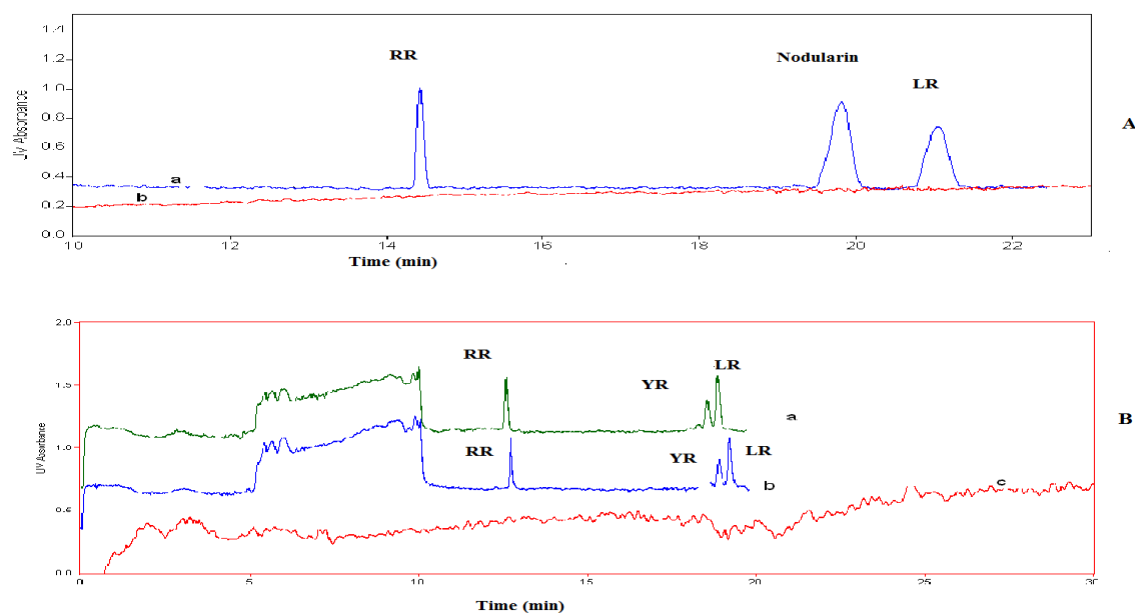


Figure 2. 9. CZE of real samples: A - tap water: (a) spiked with 5 μ g/mL each of microcystins LR, RR and nodularin after stacking with the aid of methanol, (b) tap water that was not spiked. B - reservoir water sample (a) spiked with microcystins LR, RR and YR (10 μ g/mL each), (b) a repeat run of the spiked sample and (c) reservoir water sample which was not spiked.

CHAPTER 3 – DEVELOPMENT OF MEEC

3.0 MEEC Principle

In MEEC, separation is based on differences in hydrophobicities of the analytes. Analytes partition between the microemulsion (oil) and the mainly aqueous phase in background electrolyte. For charged analytes like in this case, additional selectivity due to the electrophoretic behaviour of the analytes is achieved. The oil in water emulsion is stabilised by a surfactant at a concentration above its critical micellar concentration (cmc) as well as a cosurfactant usually a short chain alcohol that reduces electrostatic repulsion between surfactant molecules in effect reducing surface tension and stabilising the microemulsion ⁸¹.

Figure 3.1 below is a schematic of the MEEC principle of separation.

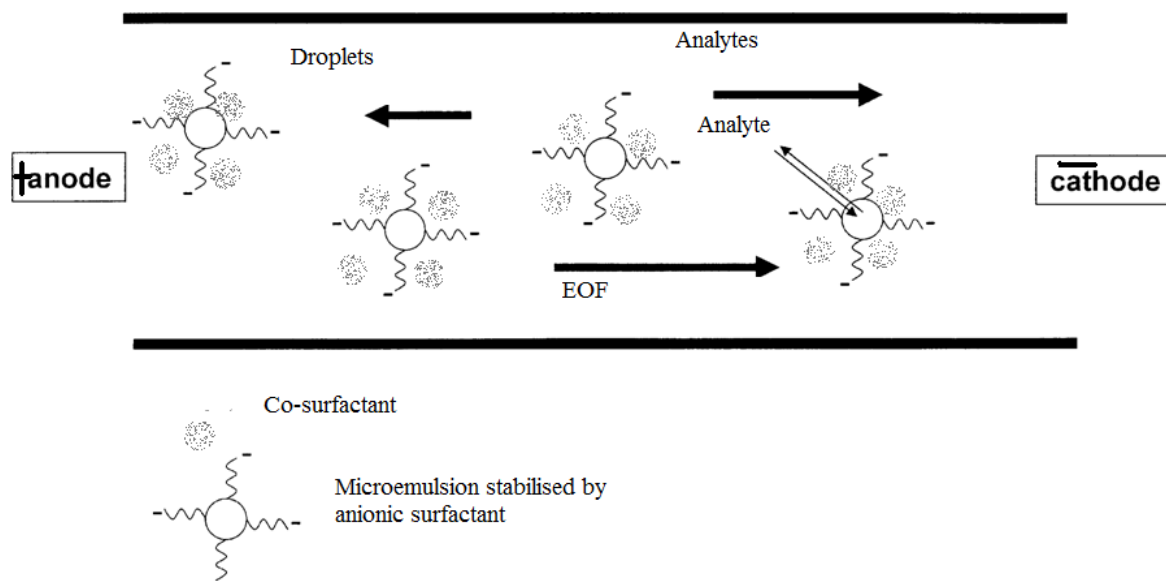


Figure 3. 1: Scheme showing the principle of MEEC

3.1 Development of Microemulsion Electrokinetic Chromatography (MEEC) Method for the Determination of Cyanotoxins in Water.

MEEC is a CE technique commonly used for simultaneous separation of both aqueous and water insoluble analytes. The presence of oil and a co-surfactant in the MEEC BGE results in good selectivity by this method. Hydrophobic analytes can be solubilised⁸¹, at the same time water soluble components will be solubilised by the aqueous portion of the BGE. This technique is therefore particularly useful in simultaneous separation of analytes with a range of hydrophobicities. Compared to MEKC, MEEC offers better selectivity for hydrophobic compounds because hydrophobic analytes are trapped in the micelle in MEKC and the fraction of hydrophobic analytes in the aqueous phase is very low, so they will tend to migrate closely, in MEEC on the other hand the amount of hydrophobic analytes in the aqueous phase is quite significant⁸² and the better partition between the oil phase and aqueous phase result in better resolution. The analytes under investigation have a range of polarities ranging for the polar cylindrospermopsin to more hydrophobic microcystins such as LF and LA (based on the amino acids at positions X and Z), therefore MEEC may offer better separation.

In the CZE method developed in this study, more hydrophobic microcystins took a long time to elute for example microcystin LA did not elute after 40 minutes of CZE separation using formic acid as a BGE. When sodium tetraborate was used in CZE, resolution between analytes was not good. MEKC resulted in better separation and all the analytes eluted in the positive polarity mode, but baseline separation between the late eluting components was not achieved, therefore we used MEEC and baseline separation for all the analytes considered in this study was achieved. Due to instrument limitations at the time, negative polarity for the MEEC mode was not investigated.

For MEEC separation a bigger number of hepatotoxins were studied. Cyclic heptapeptides (microcystins LA, LF, LR, LW, LY, RR and YR) were separated in the presence of a related pentapeptide nodularin and the alkaloid hepatotoxin cylindrospermopsin.

Apart from the regular factors that affect a CE separation such as absorption wavelength used for detection, capillary length, background electrolyte concentration and voltage, other factors affecting selectivity in MEEC are the type of surfactant and co-surfactant ⁴⁸. Selectivity can be changed using non-ionic surfactants or zwitterionic surfactants but the most important factor which affects selectivity is the nature of the co-surfactant (low molecular weight alcohol) ⁴⁸. Therefore, neutral surfactants and different co-surfactants were studied to understand their effects on selectivity.

3.2 MEEC Method Development

3.2 .1 Effect of Absorption Wavelength

The $\lambda^{\max}$ for microcystins due to the Adda moiety is 238 nm and optimum detection is at 220 – 250 nm while that of cylindrospermopsin is 260 – 290 nm ¹¹. Cylindrospermopsin and Microcystin LA were studied at 230, 238 and 250 nm (refer to figure 3.2)(because the absorbance of microcystins beyond 250 nm is weak) to find an optimum in terms of peak height and area. The optimum peak height was obtained at 230 nm; therefore, samples were run at this wave-length.

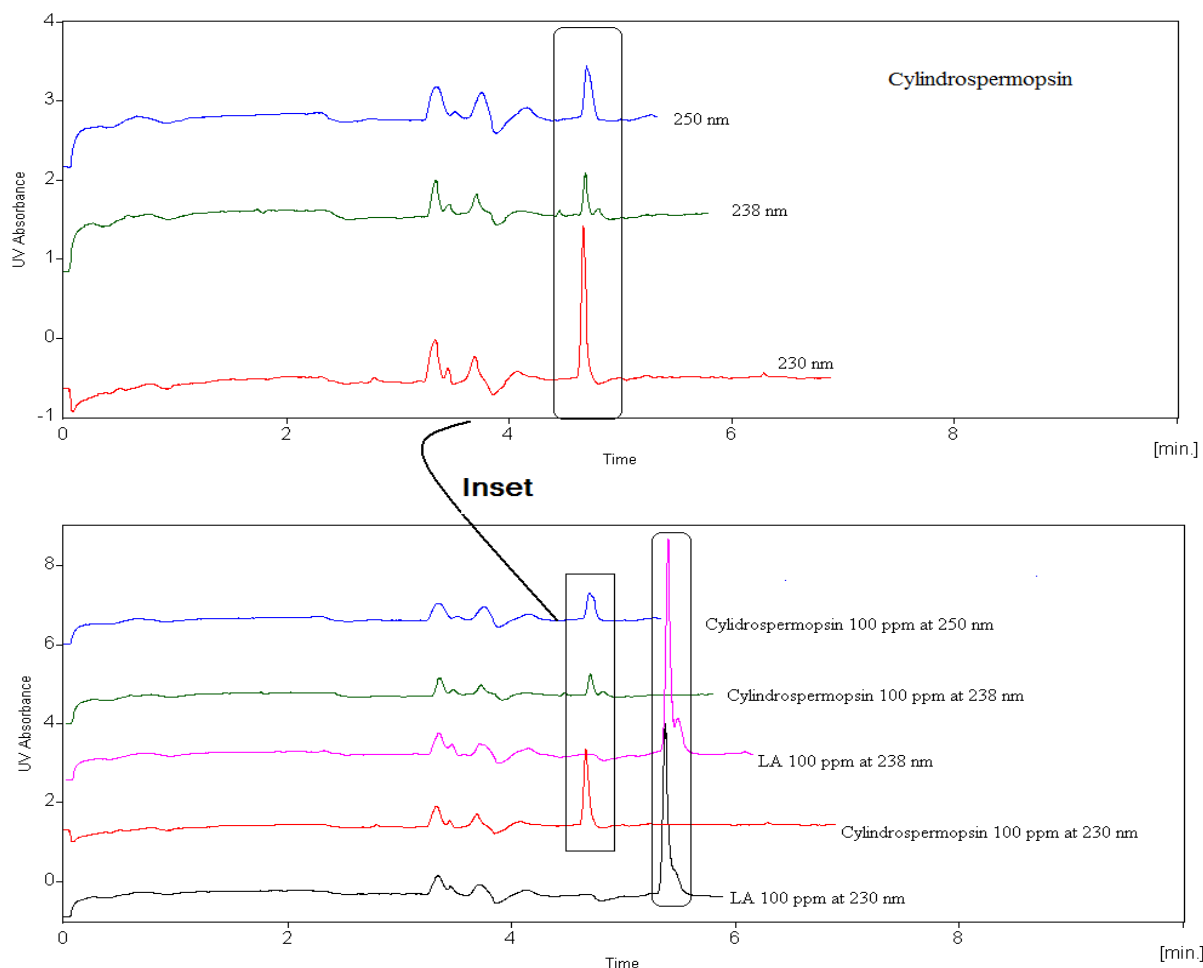


Figure 3. 2 Effect of wavelength on peak height

3.2.2 Effect of Background Electrolyte Concentration

The effect of BGE concentration was studied for sodium tetra borate in the range 5 - 30 mM. Good separation was obtained using 20 mM BGE as shown in the figure 3.3 below. This was because at a higher concentration of BGE, the increase in ionic concentration reduces the speed of the sample ions leading to longer elution times for the analyte. Considering the separation and the total analysis time, 20 mM sodium tetra borate was used in subsequent separations.

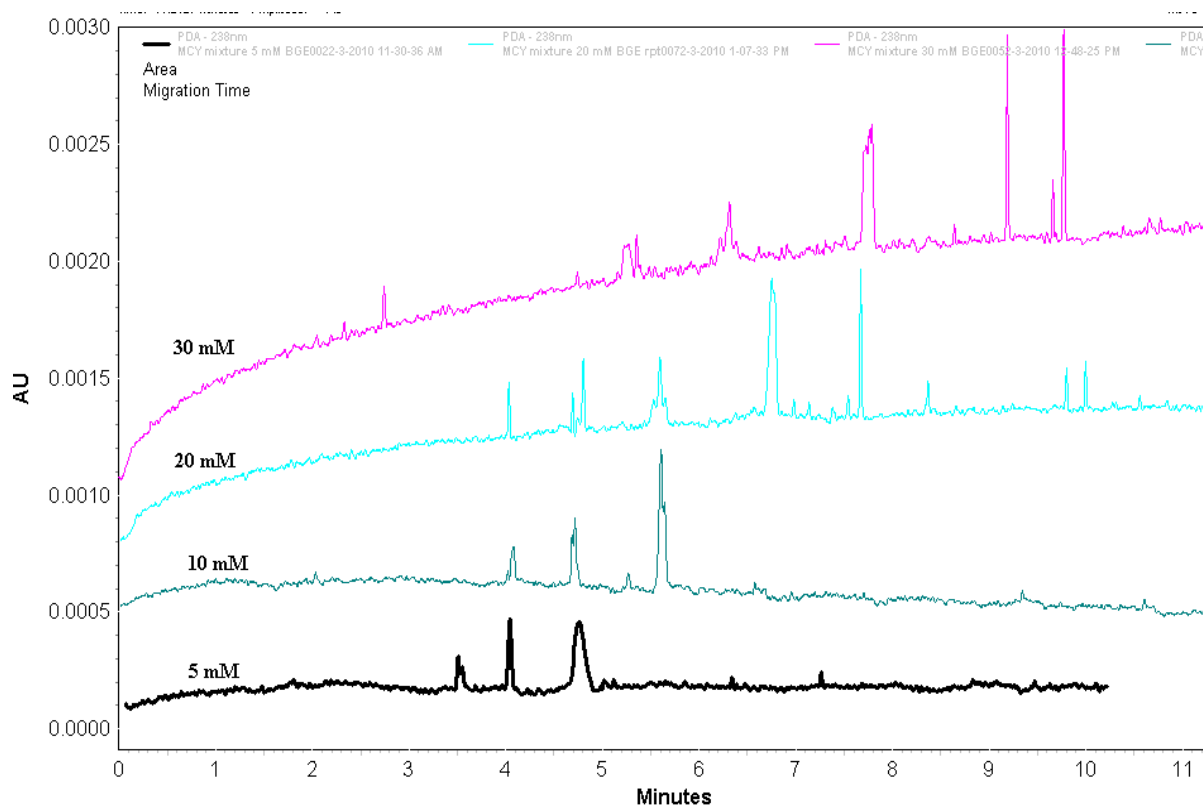


Figure 3.3 Effect of concentration of the BGE

3.2.3 Effect of Surfactant

Neutral surfactants unlike ionic surfactants can be used in larger quantities without too much increase in the electric current and therefore polyethylene glycol was investigated. However the analytes co-eluted and the electropherogram was noisy as shown in the figure 3.4. The use of cetyltrimethylammonium bromide (CTAB) here was not of any advantage because positive polarity was used and in presence of CTAB, the EOF is reversed and thus analytes may take a very long time to reach the detector. In this case no peaks were observed after 40 minutes of analysis. Therefore, sodium dodecyl sulphate (SDS) was used as the surfactant because it gave the best resolution in the shortest analysis time.

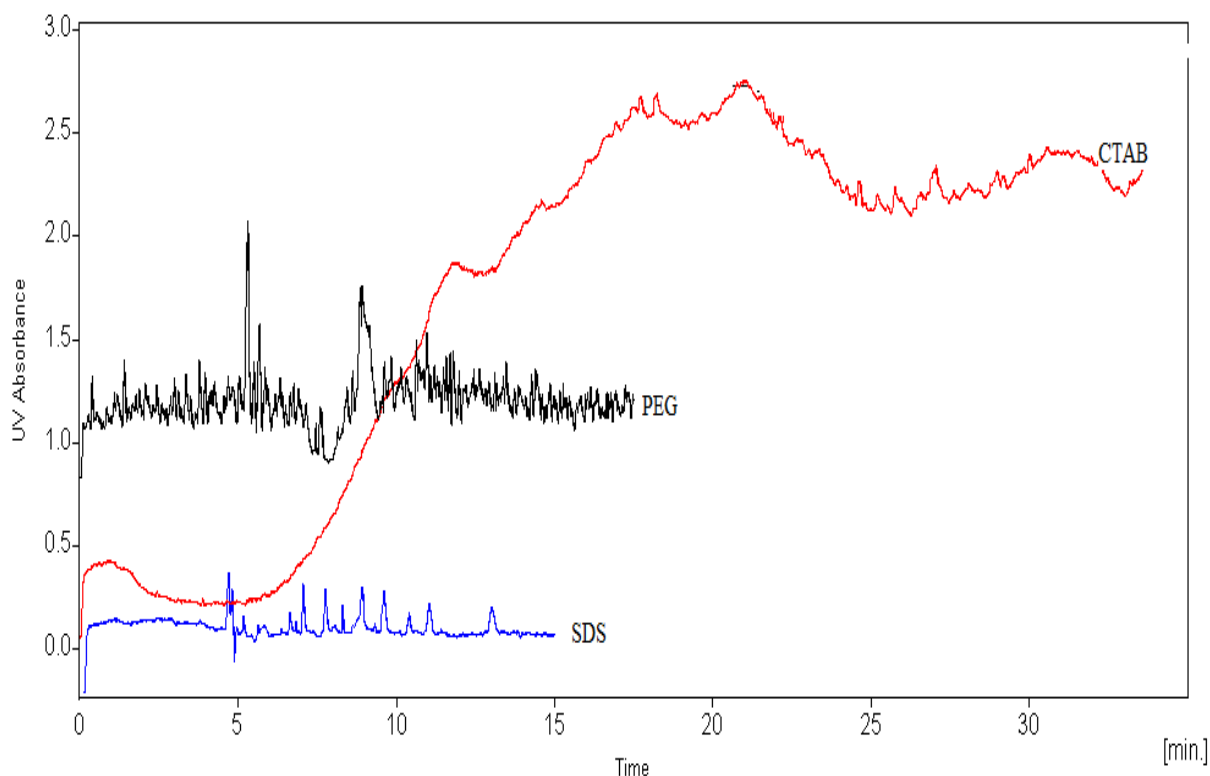


Figure 3. 4 Effect of surfactant

The effect of percentage SDS was investigated and the electropherograms obtained are as shown in figure 3.5. Increase in the amount of SDS resulted in longer elution times perhaps due to increased BGE viscosity and also resulted in increased current due to increased ionic concentration. At 3 % SDS composition, peaks were better resolved and the total run time was not as long as when 5 % SDS was used. Therefore 3% SDS was used in the BGE composition based on favourable current amount and elution time, and in subsequent experiments, the BGE was composed of 20mM sodium tetraborate (95 %), SDS (3 %), butan-1-ol (1.5 %) and octane (0.5 %). Further improvement in peak resolution was achieved by adjusting the pH to 10. 2. At this pH all the 9 compounds were well resolved (figure 3.6).

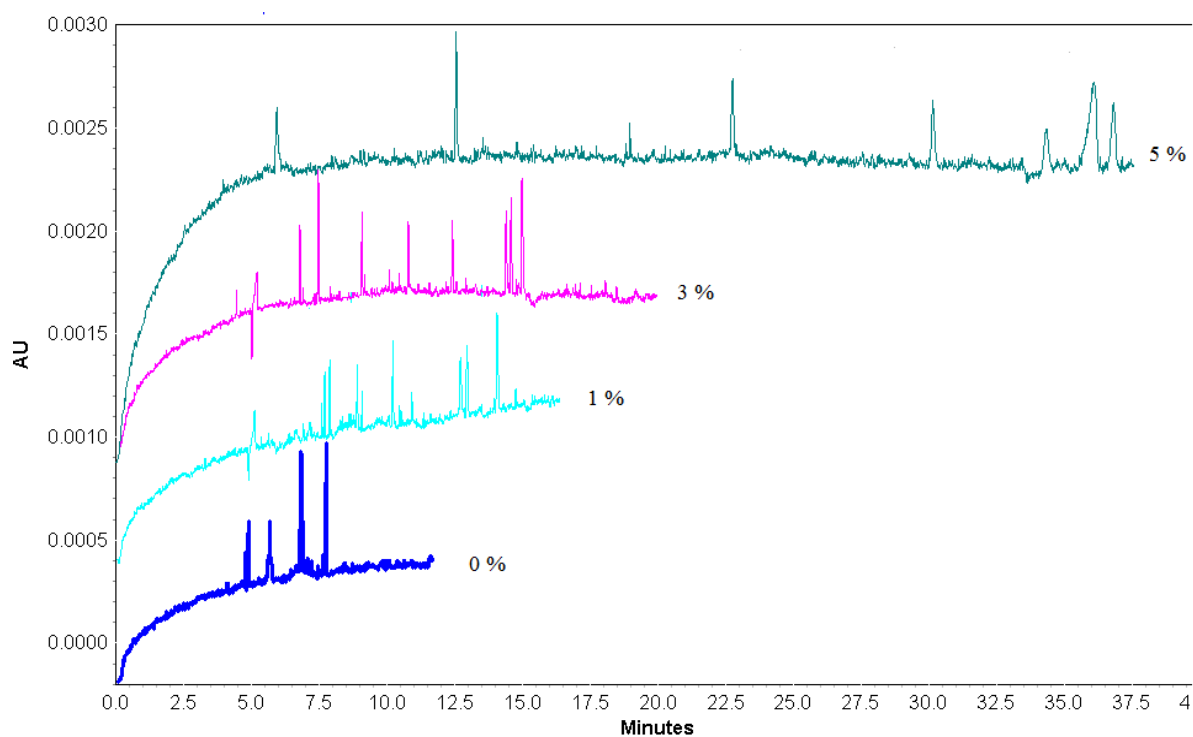


Figure 3. 5 Effect of SDS percentage

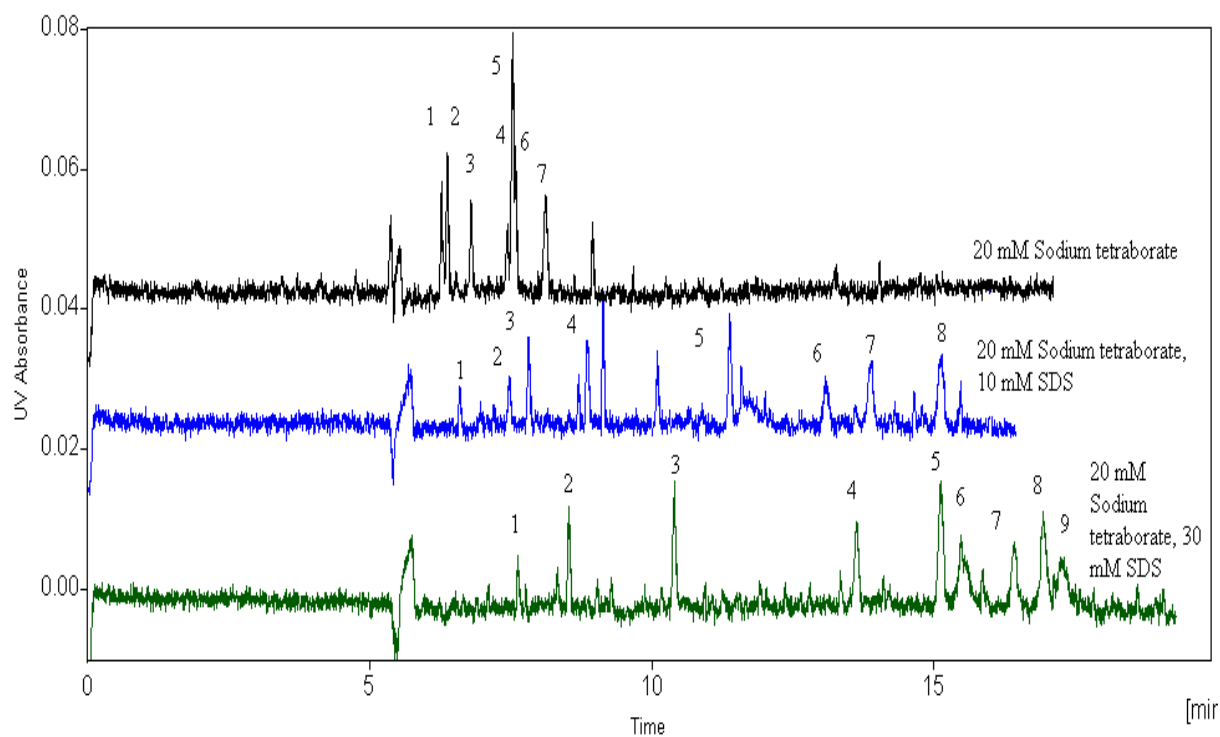


Figure 3. 6. Effect of SDS on resolution at pH 10.2

Better resolution might have been achieved by increasing the amount of SDS in the mixture but this led to an increase in current. Therefore an organic phase (oil) was included in the buffer composition. The addition of oil and therefore changing the separation mode from micellar electrochromatography (MEKC) to microemulsion electrochromatography (MEEC) improved the resolution between the last 2 peaks (8 and 9) (figure 3.7) to achieve baseline separation.

Octane and ethyl acetate were investigated for use as the oil phase. Both gave similar results as well as selectivity and the order of elution was the same so either could be used as the oil for this separation.

3.2.4 Effect of Co-surfactant

Co-surfactants methanol, propan-2-ol, butan-1-ol and acetonitrile were investigated. Methanol, propan-2-ol and acetonitrile increased the total run time slightly and for methanol some analytes co-eluted. The use of co-surfactant butan-1-ol in the MEEC buffer system resulted in good resolution and a shorter analysis time and was therefore used for BGE compositions. Results obtained are shown in figure 3.7. A buffer system containing 20 mM sodium tetraborate with SDS (3 % or 30 mM), butan -1-ol (1.5 %) and octane (0.5 %) at pH 10.2 was prepared for use in the MEEC method and the method was evaluated as shown in table 3.1.

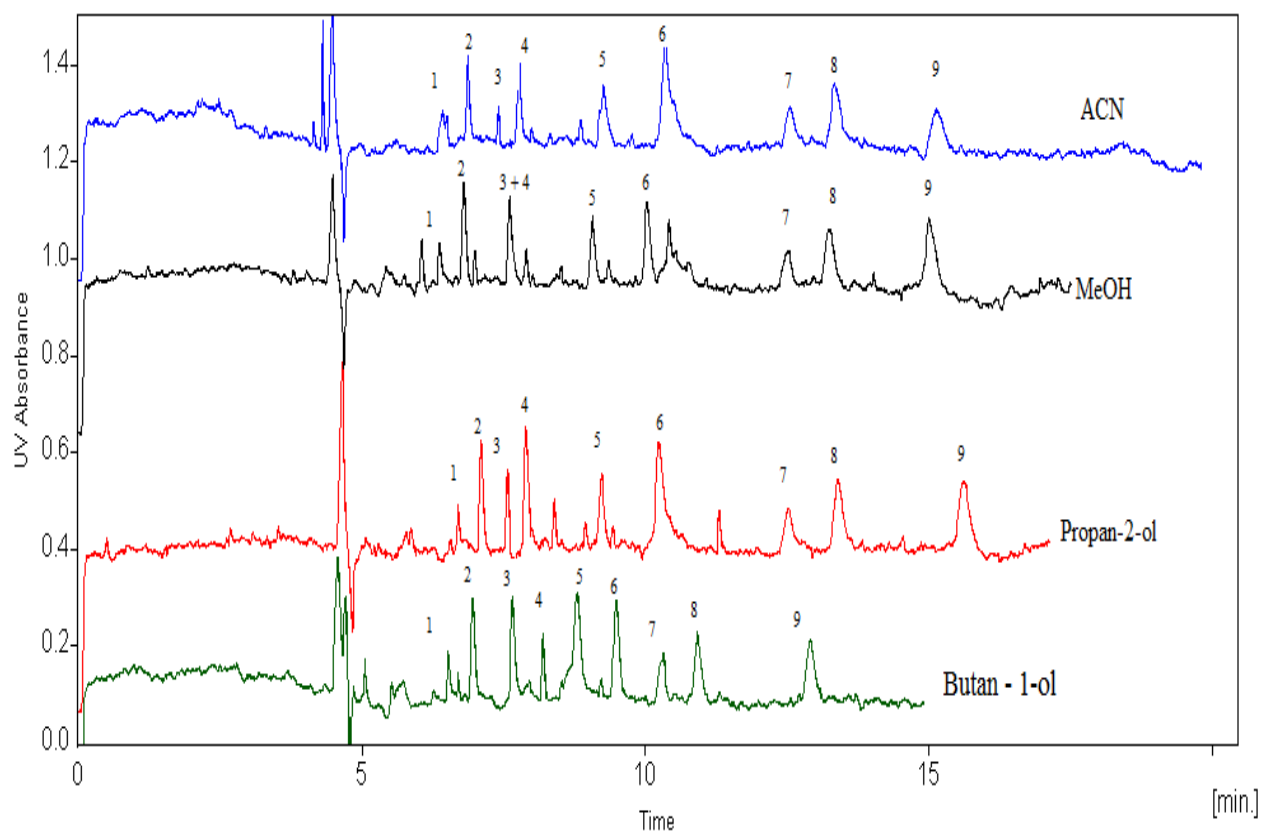


Figure 3. 7. Effect of co-surfactant

Table 3. 1 Evaluation of the MEEC method

Analyte	Migration time (minutes)	% RSD Migration Time	LOD (3 σ) (ppm)	%RSD Peak Area	R ²
Cylindrospermopsin	6.332	1.080	2.237	6.306	0.998
Microcystin LA	7.498	1.317	2.921	3.268	0.983
Microcystin LY	9.691	2.013	3.056	10.961	0.999
Microcystin LF	12.555	1.957	4.124	4.952	0.981
Microcystin LW	13.049	2.605	1.636	7.452	0.929
Nodularin	13.901	2.284	3.370	4.890	0.982
Microcystin YR	14.715	2.405	4.242	7.907	0.999
Microcystin LR	15.266	2.247	3.636	9.600	0.984
Microcystin RR	15.814	2.192	1.060	9.147	0.990

3.3 Pre-concentration

3.3.1 Online Pre-concentration Using a Salt

The use of salt in sample during stacking has been reported to improve sensitivity⁵⁵, so the test samples were dissolved in NaCl at different concentrations in an attempt to improve the peak height and sensitivity. As seen from figure 3.8 below, peak heights increased for the more polar components in the sample as seen in region (A). While the peak heights increased slightly for the hydrophobic components (B), the biggest limitation was degrading resolution and peak broadening with salt concentration (visual observation). The method was therefore not used.

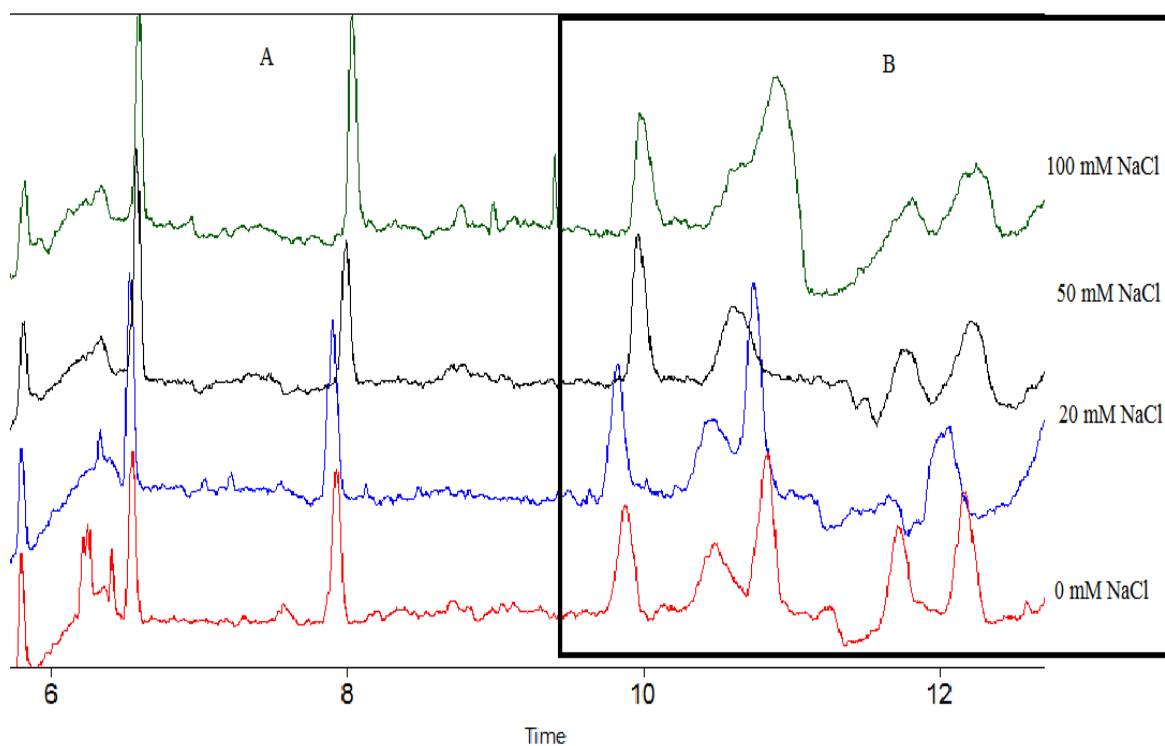


Figure 3. 8: Salt stacking (salt in sample)

3.3.2 Online Pre-concentration with the Aid of a Water Plug

An alternative stacking mode is the use of large injection volume stacking. Injection time determines the volume of sample introduced in a capillary in hydrodynamic sampling⁴¹. Peak area increase is normally linear although the increase in peak height may not be linear. While increased injection time and therefore increased volume of sample injected leads to an increase in peak area and height, separation efficiency reduces with time and in effect the resolution between closely eluting peaks declines. This was demonstrated in the use of large volume sample injection to optimise sensitivity as shown in the figure 3.9 and table 3.2.

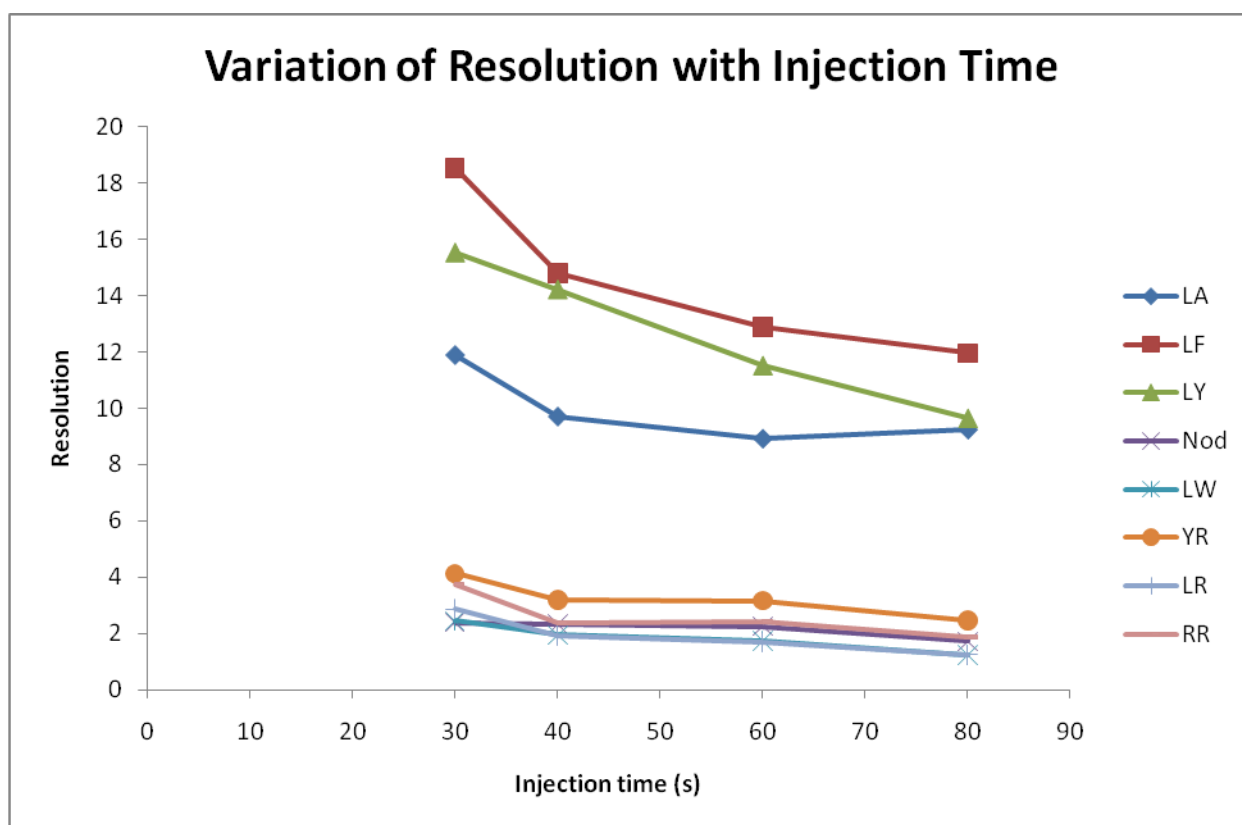


Figure 3. 9: Variation of resolution with sample injection time

Table 3. 2 Performance of the MEE method with large volume stacking

Analyte	10 S Peak Area	80 S peak Area	Number of times Area Increased	10 S Peak height	80 S Peak height	Number of times height increased
CYN	0.254	1.125	4.429	0.061	0.243	3.988
LA	0.444	2.962	6.671	0.147	0.552	3.755
LF	1.174	4.094	3.487	0.292	0.473	1.619
LY	0.524	5.071	9.677	0.104	0.366	3.514
Nod	0.442	5.492	12.425	0.038	0.206	5.414
LW	0.861	6.705	7.787	0.124	0.364	2.933
YR	0.603	3.59	5.953	0.083	0.221	2.659
LR	0.708	5.537	7.821	0.102	0.339	3.326
RR	0.737	6.798	9.224	0.100	0.452	4.520
Analyte	Retention Time %RSD	Peak Area% RSD	Peak height %RSD	R² Peak height	R² Peak Area	LOD 3σ (ppm)
CYN	0.545	16.903	10.58	0.930	0.975	0.158
LA	0.816	8.835	20.09	0.996	0.99	0.422
LF	1.648	0.932	4.608	0.999	0.971	1.283
LY	2.029	4.909	11.118	0.992	0.994	0.376
Nod	2.422	5.513	8.557	0.989	0.999	0.329
LW	2.317	10.28	6.062	0.904	0.998	0.276
YR	2.434	13.163	12.773	0.972	0.995	3.095
LR	2.343	8.826	15.977	0.950	0.999	0.308
RR	2.232	8.833	2.676	0.994	0.999	0.493

Peak areas increased at least 3 times and peak heights increased at least 2.6 times when large volumes were injected. As mentioned previously sample injection for long time results in poor resolution, so a short solvent plug was injected before the sample to form a different conductivity plug within the capillary and this improved resolution. The highest peak areas and heights were achieved with injection of water for 5 seconds followed by the sample injection for 80 seconds. The use of other solvents like methanol and acetonitrile in the plug resulted in poor resolution for the more hydrophobic late eluting microcystins as demonstrated in figure 3.10, therefore water was used in the solvent plug.

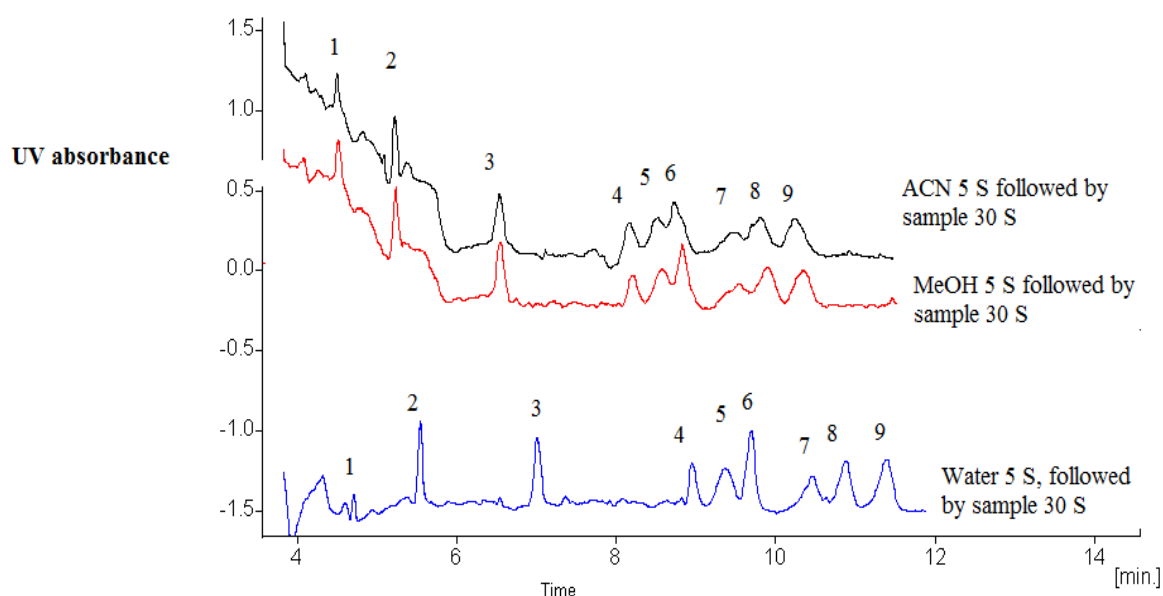


Figure 3. 10: Stacking using different solvent plugs.

The use of ethyl acetate as the oil in the buffer resulted in better resolution of peaks during large volume injection stacking (figure 3.11 and 3.12). Therefore during stacking mode ethyl acetate was used instead of octane (figure 3.12). This could be because ethyl acetate has a lower surface tension than octane; in fact its use resulted in lower current compared to when octane was used.

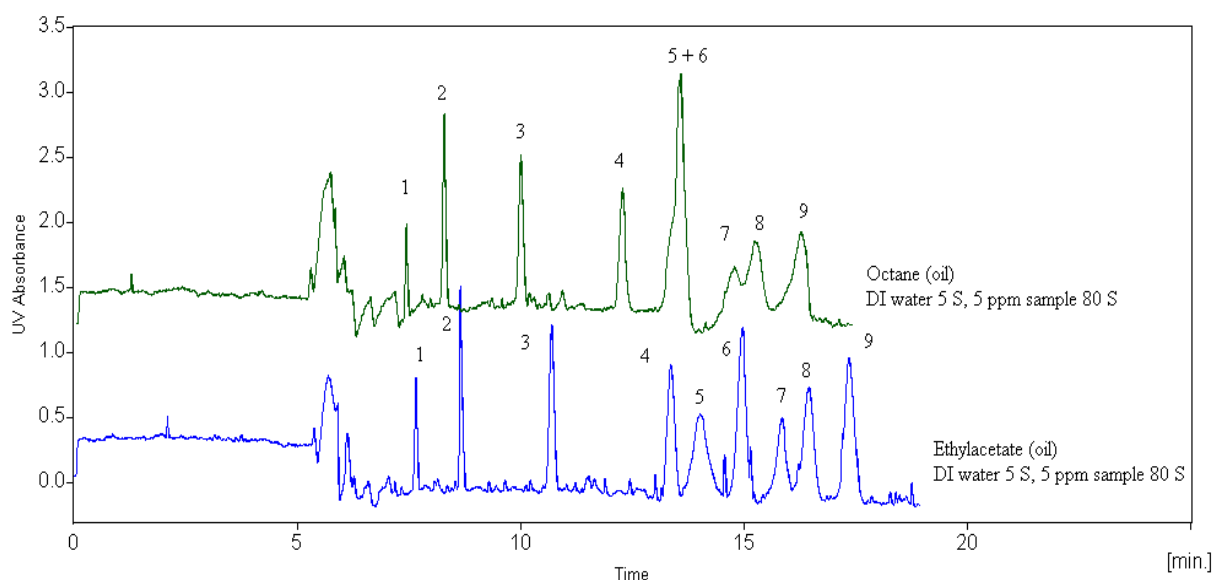


Figure 3. 11: Comparison of octane and ethyl acetate in BGE for stacking

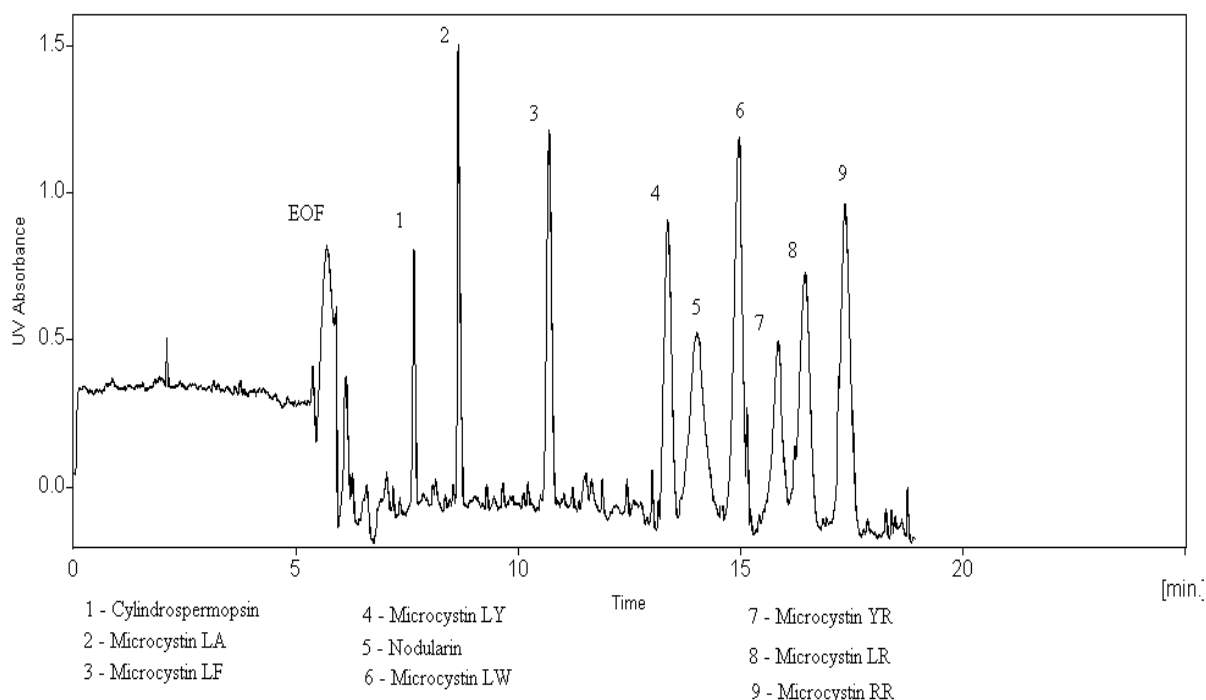


Figure 3. 12: Electropherogram of the 9 hepatotoxins after large volume injection with stacking. Analytes were at 5 μ g per mL, separation was performed at +20 KV.

Use of a high conductivity solution in the plug was investigated by injecting 0.1 M HCl for 5 seconds followed by the sample for 80 seconds. The results were similar to those obtained using water in the plug. Resolution of the peaks was better than when NaCl was used for pre-concentration. This may be due the fact that in both cases the methods make use of the differences in conductivity as well as pH. Therefore a low pH (2) did not result in a significant difference.

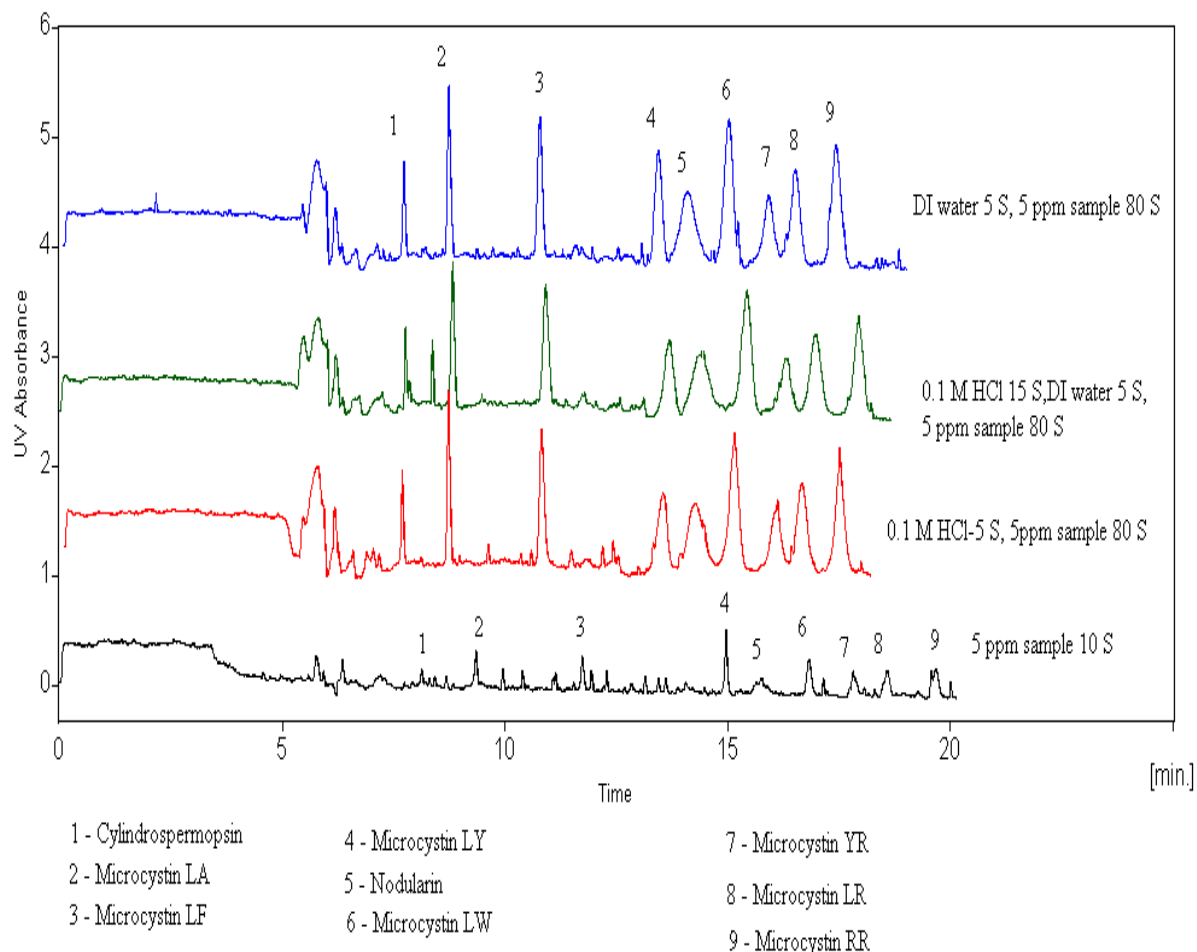


Figure 3. 13: Electropherogram of 5 μ g per mL of standards with and without stacking

3.4 Real Sample Analysis

Real samples can be analysed after pre-concentration to achieve the 1 μ g per litre detection limit as set by WHO for drinking water guidelines. There are several ways of pre-concentration such as the use of solid phase extraction (SPE) or other solvent evaporation techniques like rotor evaporation and freeze drying. In this method freeze drying was used because it applicable to a wide range of analytes, does not compromise the composition and

is less tedious. Compared to SPE there is no need for specific sorbents based on different polarities of the analytes.

A real sample of water collected from Kranji reservoir (Singapore) as explained in the experimental section in chapter 2, was spiked with microcystin standards at a concentration of 2.5 ppm. Samples were freeze dried and reconstituted with deionised water. Another sample collected in the same way was also freeze dried and reconstituted with deionised water. Test samples were analysed using large volume injection stacking technique. Microcystins investigated here were not detected in the reservoir water sample analysed. This is as shown in figure 3.14 below.

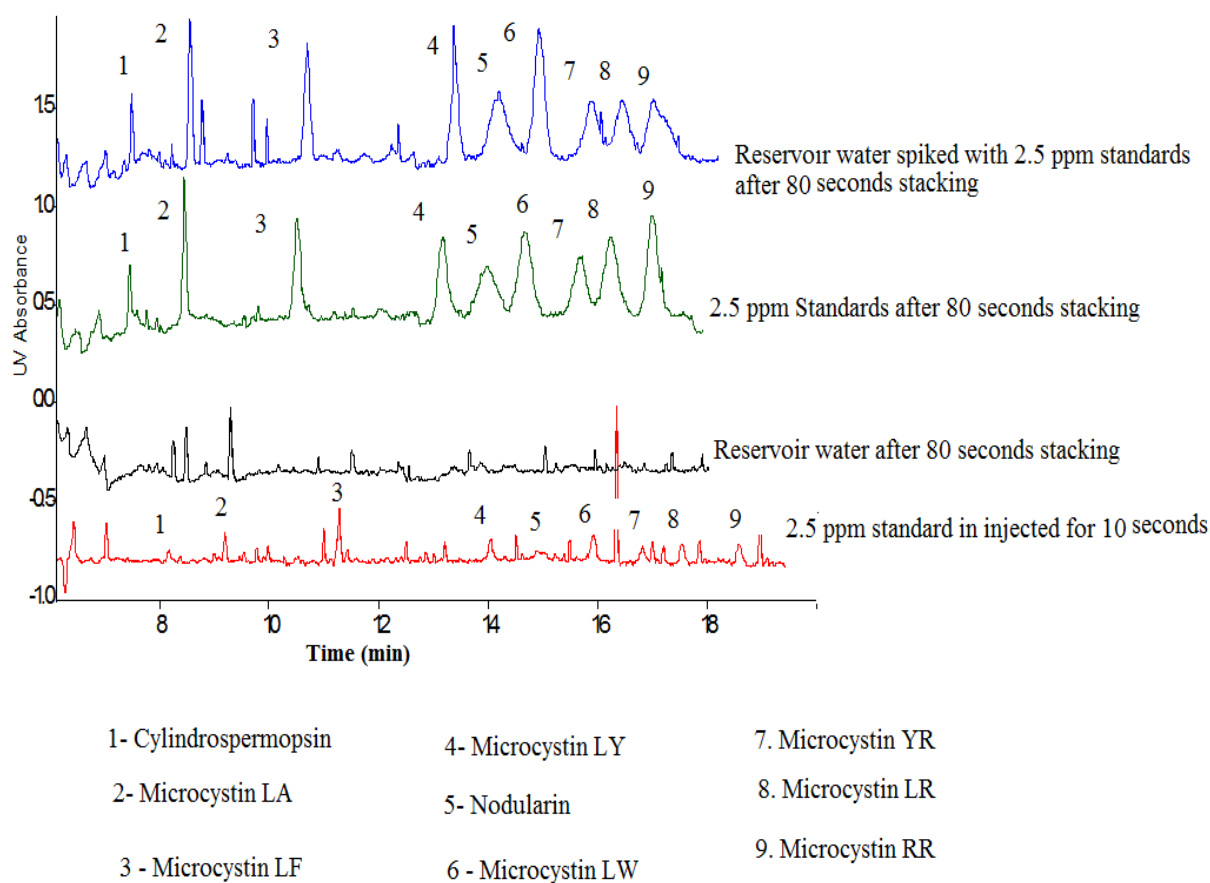


Figure 3. 14: Real sample analysis

3.5 CZE, MEKC and MEEC Comparison

CZE - The method developed was very simple. The capillary and BGE did not require any modification since the separation was at low pH using formic acid as BGE. For those analytes which eluted in less than 40 minutes, the resolution between peaks was good (refer to chapter 2). However, the total analysis time was long and the more hydrophobic microcystins like microcystin LA took a long time to elute.

MEKC - This method offered better resolution compared to CZE. When negative polarity with reversal of EOF was employed, total analysis time was less than 10 minutes for the analytes considered (chapter 2 figure 2.3). In the positive polarity mode, the resolution between microcystins RR and LR was not very good even though it was acceptable (figure 3.7).

MEEC- This technique offered the best resolution for all the 9 analytes studied, however analysis time was long (~20 minutes) compared to the CZE and MEKC modes. But even then it was generally shorter than conventional HPLC techniques which take 20 – 45 minutes¹³. It is possible to achieve a shorter analysis time by use of reverse polarity but this was not investigated due to instrument and time limitation at the time.

Generally the methods developed here achieved detection limits in ranges comparable to conventional HPLC-UV methods and together with off line and online pre-concentration techniques the LOD improved by at least 10 times. They are applicable for use in a concentration range acceptable by WHO drinking water guidelines. Furthermore separation

between the different microcystin analogues was achieved using MEEC with higher resolution and shorter analysis time compared to conventional HPLC-UV methods.

3.6 CE (CZE, MEKC, MEEC) Compared to HPLC

Methods for determination of microcystins in water have continued to develop. The lowest detection limits so far can be achieved using mass spectrometry detection often with LC or CE. During our method development we used LC-MS to validate our results obtained using CE-UV. Microcystin LR, microcystin RR and microcystin YR were analysed using an Agilent 1100 series liquid chromatography equipped with electrospray ionisation source (LC/ESI-MS) system (Waldbronn, Germany). It was made up of a binary gradient pump, auto sampler, column oven, diode-array detector and a LCQ-duo ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The LCQ mass spectrometer was operated with the capillary temperature at 270 °C, sheath gas at 80 (arbitrary units) and auxiliary gas at 20 (arbitrary units). The electrospray voltage was set at 4.5 kV, the capillary voltage at 10V and lens tube offset at 0V. Mass spectra were recorded from m/z 500 –1500.

The mobile phase used was (A) water with formic acid (0.1 %) and (B) acetonitrile with formic acid (0.1 %). A gradient elution starting at 100 % A for 5 min, then 95 % A and 5 % B was used. The oven temperature was set at 40 °C and the flow rate was 1.5 mL/min. For all experiments, volumes of 5 µL of standards and samples were injected. The column used for the separation was a reversed-phase 3 µm, C₁₈ (100 × 2.0 mm) Luna column. (Phenomenex, USA). The ESI-MS spectra were acquired in the positive and negative ion mode. The instrument was operated in selected reaction monitoring (SRM) mode. The heated capillary temperature was maintained at 350 °C, the drying gas and nebulizer nitrogen gas flow rates

were 10 L/min and 50 psi, respectively and the number of average scans was 5. The three microcystins LR, RR and YR were separated as shown in the chromatogram and spectra below (figure 3.15) and the results obtained are as shown in table 3.3.

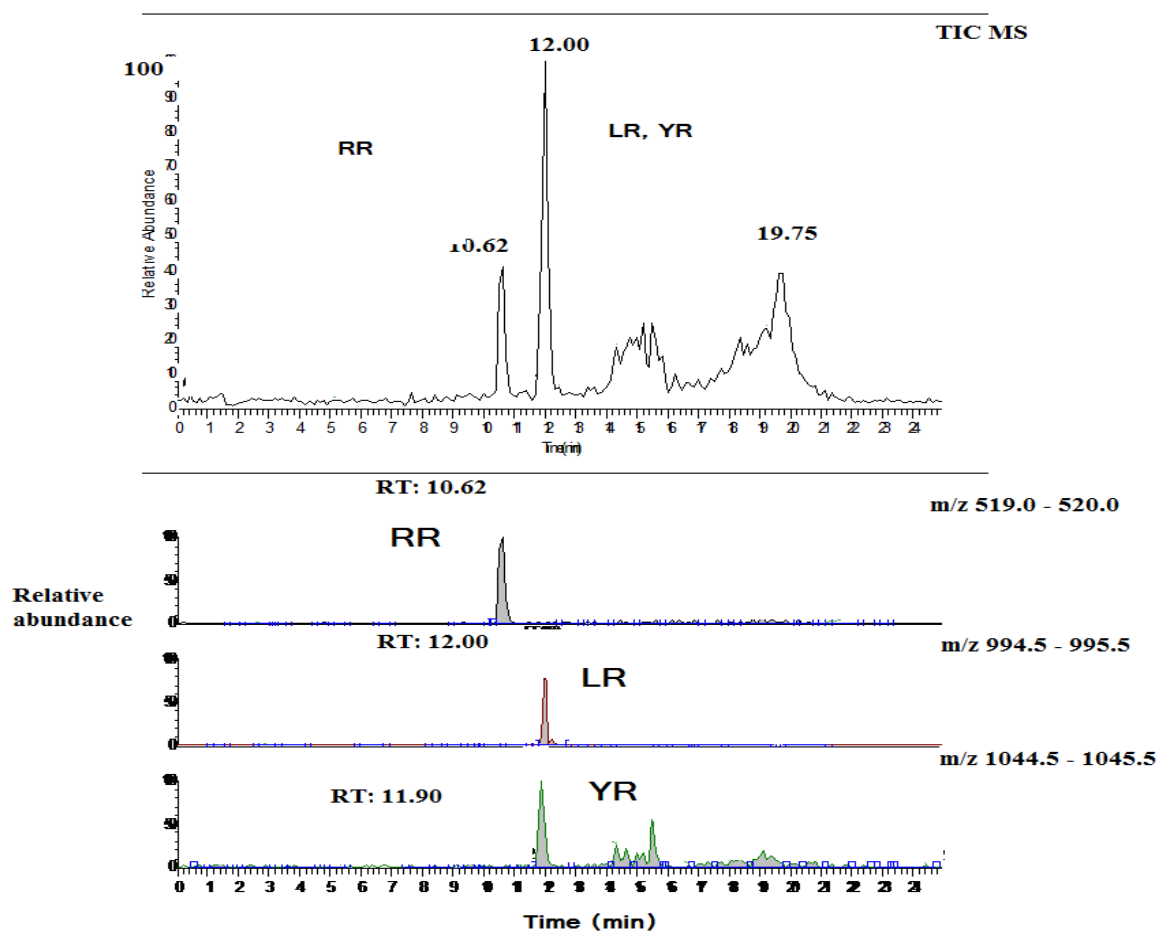


Figure 3. 15. Spectrum of 20 ppm each of microcystins LR, RR and YR.

Table 3. 3 LC-MS performance

Microcystin	LOD $\mu\text{g/L}$ (3σ)	%RSD intensity (inter day (n = 6))	%RSD Intensity-Intraday(n=3)	R^2
LR	0.610	6.950	9.589	0.988
RR	0.3054	3.690	8.547	0.999
YR	0.756	5.637	6.602	0.993

While it was possible to achieve separation in less than 10 minutes using CE (MEKC), the earliest eluting analyte in LC was after 10 minutes. The detection limits for HPLC-UV and CE-UV were comparable, but MS gave superior results with LOD below the WHO drinking water guideline. Separation between microcystins LR and YR was not achieved using LC-MS but they were identified using SRM. Furthermore, literature reports have shown better resolution between microcystin analogues by CE compared to LC and the possibility of a faster separation by CE coupled with good resolution was demonstrated in our methods developed here. A comparison of CE and LC is shown in table 3.4 below.

Table 3. 4 Summary of a comparison between CE and LC-MS

Property	CE	LC-MS
Total analysis time	Faster when reverse polarity is used	Generally take longer
Sensitivity	Low, limited by UV detection. Can be improved by pre-concentration techniques or use of MS for detection when the current challenges in CE-MS interfaces are overcome	Very sensitive due to MS
Resolution	Higher resolution. All the analytes investigated clearly separated	Resolution was not very good. The use of SRM mode helped.
Solvent consumption	Low amounts (nanolitre consumption)	Very high (1.5 ml/min for every 20 min run time)

On this basis, CE separation with MS detection should offer better results in resolution than LC-MS.

The analytical methods developed in this study demonstrated a potential for application in real samples because there was generally good resolution between microcystin peaks. Real samples spiked with standards were analysed and there was no visible matrix interference, therefore the results were satisfactory.

CHAPTER 4 – METABOLOMICS APPROACH TO INVESTIGATION OF MICROCYSTIN TOXICITY

4.0. Development of Metabolomics Approaches for the Investigation of the Effect of Exposure of a Human Cell Line to Non Cytotoxic Amounts of Microcystins.

“Metabolomic” approaches were used to investigate the effect of exposure of humans to low amounts of cyanotoxins. In humans exposure is usually at low concentrations that are not easily visible for example in food or dermal exposure. The purpose of this investigation was to determine if exposure to low amounts of microcystins is hazardous and what their effects on the metabolome are. A model cell line HepG2 was used in this investigation. “metabolomics” was used because the metabolome is more sensitive to perturbations compared to the transcriptome or the proteome, the activities of the different pathways are reflected more accurately in the concentrations of the metabolites compared to the concentrations of the relevant mRNAs (transcriptome) or enzymes (proteome) involved in the pathways; and changes in the metabolome are more amplified in comparison⁸³.

Furthermore, metabolites are usually the same in every organism that contains them thus generic techniques can be used in different biosystems. Based on these reasons, the use of metabolic profiling should provide superior results compared to proteomics or transcriptomics. It is also a cheaper and high throughput technique and that is why this technique was used instead of protein profiles or nucleic acid profiles.

In metabolomics, the aim is to profile and quantify all the small molecular weight metabolites in the sample in order to understand how metabolites and their interactions influence the

phenotype^{83, 84}. This implies profiling all the intracellular and extracellular metabolites. The main challenge in measurement of intracellular metabolites is the rapid turnover of intracellular metabolites so it requires a rapid quenching technique and it is also time consuming^{83, 85}. While intracellular and extracellular metabolites can play different roles⁸⁴ metabolites which are secreted, excreted or not taken up by the cells clearly reflect the cellular metabolic activity⁸³. Analysis of the medium also referred to as “footprinting” can be used to study the metabolic activity of an organism instead of analysing the intracellular metabolism because the components in the medium are a reflection of the organism’s metabolic activity. Furthermore, the turnover time of metabolites in the medium is not as fast as for intracellular components therefore the need for rapid quenching can be avoided^{83, 85}.

In this study, we monitored extracellular metabolites by analysis of the medium and as such employed “footprinting” technique as opposed to “fingerprinting” in order to study the metabolic activity of HepG2 cells exposed to microcystins, while avoiding errors that may result from the failure to achieve rapid quenching, besides it would be easier to analyse a biomarker from extracellular fluids like serum or blood instead of from cells or tissue which would be considered to be more invasive than the former.

4. 1 Methodology and Experimental

4.1.1 Materials

HepG2 Cell line was kindly provided by Professor Hanry Yu of Department of Physiology, National University of Singapore. Dulbecco's modified eagles medium (DMEM) was from Invitrogen (Singapore). Fetal bovine serum (FBS) and trypsin - EDTA were from GIBCO (Auckland, NZ). Phosphate buffered saline (PBS) was from 1st Base (Singapore). HPLC grade chloroform and methanol were obtained from Tedia (Fairfield, OH, USA). CDCl₃ was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), D₂O with 0.05 % (wt) 3-(trimethylsilyl)propionic-2, 2, 3, 3,-d₄ acid, sodium salt, TSP), propidium iodide (PI) and thiazole orange (TO) were from Sigma Aldrich. Deionised water was obtained from a Millipore Milli Q purification system (Bedford, MA, USA).

4.1.2 Instrumentation

Cells were incubated in an InCu saFe® incubator (Sanyo). Flow cytometry was performed on a Dako Cytomation Cyan LX and data was acquired and analyzed using Summit software version 4.3 from Dako Colorado Inc. A Bruker 500 MHz Ultra shield spectrometer was used in ¹H NMR experiments while an Agilent 1200 series system equipped with an Agilent 6410 triple quad mass spectrometer was used in direct injection mass spectrometry.

4.1.3 Cell Culture

HepG2 Cells were cultured in 25 cm³ flasks or 6 well plates. Dulbecco's modified eagles medium (DMEM) with 10 % foetal bovine serum (FBS) was used. The cells were incubated in a humidified incubator at 37 °C, 5 % CO₂. Whenever cells reached ~ 90 – 100 % confluence they were detached and split to be seeded in new flasks. Briefly, the method involved washing the cell layer two times using 3 ml of phosphate buffered saline (PBS 1×), followed by a rinse with 1 mL of trypsin (1×). Thereafter the cells were incubated for one minute to allow detachment. After all cells detached, they were reconstituted with fresh medium and split into the required portions for the next seeding stage.

4.1.4 Assessment of the Effect of Microcystins on Cell Viability

Cell viability assessment can be defined as the analysis of living or dead cells. It portrays the morphological, physicochemical and biological differences in the cells. In this study, flow cytometry was used to analyse the cells. Criteria used to characterize viability of cells include assessment of the size, shape and refringency of the cells; the ability to incorporate dyes and the ability to exclude dyes in relation to selective permeation⁸⁶.

For investigation of effects of xenobiotics on cells, the important parameter is the determination of the fraction of intact cells⁸⁶. Analysis of intact cells is commonly done with the aid of stains. Exclusion dyes such as trypan blue can be used to stain cells whose membrane is compromised. Healthy cells will exclude the dye and the cells can be differentiated visually and counted under an optical microscope. Fluorescent dyes such as

propidium iodide are also excluded by intact membranes and therefore have a wide application in flow cytometry. Vital fluorescent dyes accumulate in metabolically active cells and are sensitive to changes in the cell metabolism. Examples include Hoechst 33342 which stains DNA and can be used to sort living cells depending on their DNA content. A probe that stains all dead and living cells DNA when combined with propidium iodide or ethidium bromide for dead cell exclusion can be used for sorting viable cells. The combination of exclusion and vital dyes has been used to assess membrane integrity, mitochondrial function and cell viability⁸⁶.

The purpose of this assessment was to determine the concentration of microcystins that was cytotoxic. Therefore different concentrations (0.1 – 1000 ng/mL) of microcystins LR, RR and YR were incubated with the cells for 24 hours to determine the lethal concentration for 50 % (LC₅₀) of the cells. Since the main objective was to investigate sub lethal toxicity, it was necessary to use toxins at concentrations lower than the LC₅₀ for subsequent studies.

After 24 hours, cells were detached and stained using propidium iodide (PI) and thiazole orange (TO). The procedure involved detaching the cells by trypsin, followed by addition of DMEM (1 mL). The cell solution was then centrifuged and the spent media was poured away leaving behind a cell pellet. The cell pellet was re-suspended in phosphate buffered saline (PBS). To 500 µL of the cell suspension was added 5 µL of TO (42 µM) followed by 5 µL of PI (4.3 mM). The resulting solution was vortexed and analysed using flow cytometry. For cell viability assessment, 10,000 cells were considered for each sample.

PI is a DNA stain that works by exclusion i.e. the dye enters the cell only when the integrity of the membrane is compromised. Thiazole orange on the other hand stains the entire living

DNA in the cell; therefore by comparing the staining patterns by both dyes, live, damaged and dead cells can be identified.

4.1.5 Sample Preparation

Media (1mL) was extracted using the Bligh-Dyer extraction technique as modified by Miccheli et al ⁷⁸. To 1 mL of media, 3 mL of cold methanol: chloroform (2:1), followed by 1 mL of cold chloroform were added. The mixture was centrifuged at 8000 rpm for 20 minutes at 4 °C. The supernatant was separated into the aqueous and organic portions which were dried by blowing with gentle flow of nitrogen gas or by freeze drying. The dry extracts from the media were kept in the freezer at -20 °C until analysis and were analysed separately.

4.1.6 ¹H NMR Analysis

The aqueous portion was reconstituted with deuterated water (D₂O buffered with sodium phosphate at pH 7), while the organic portion was reconstituted with deuterated chloroform (0.7 mL each), before analysis by ¹H NMR. Samples were analyzed on a Bruker ultra shield spectrometer to obtain one-dimensional H NMR spectra at an observation frequency of 500 MHz. Aqueous samples were analyzed using a pre-saturation pulse sequence (relaxation decay -90° acquisition). The water resonance was eliminated by applying a secondary radio frequency irradiation during the relaxation delay of 2 s. Data was acquired at 300 K using 10330.6 Hz spectral width, into 64 K data points with an acquisition time of 1.586 seconds

and 64 scans. Organic extracts were also analyzed using a single 90° pulse, but with no solvent suppression and 64 scans were also collected.

The ^1H NMR spectra were manually processed using TOPSPIN software (Bruker Biospin). Relative peak intensities were used for quantification and they were identified based on literature ^{78, 87}. Results were analysed by principal component analysis (PCA) using SIMCA P⁺ software. Peak lists were generated using SIMCA P⁺ and they were tested for statistical significance using Student t-test and components of $P \leq 0.05$ were considered to have statistical significance.

4.1.7 Analysis by Direct Injection Mass Spectrometry (DIMS)

DIMS analysis of all samples were performed on Agilent 1200 series system made up of a degasser, binary pump, thermostatic auto-sampler and temperature-controlled column compartment. The LC was equipped with an Agilent 6410 triple quad mass spectrometer system with an electrospray ionization (ESI) source. The mobile phase used was (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid. All samples were run in full scan mode in the range 70 – 1000 mass to charge ratio values with an isocratic elution of 50 % each of A and B. The total run time for each sample was 3 minutes.

4.1.8 Data Analysis

^1H NMR peaks were picked and normalized using MestReNova software (Mestrelab research, Santiago de Compostela, Spain). Aqueous sample peaks were normalised to TSP (at a chemical shift of $\sigma = 0$) while organic sample peaks were normalised to chloroform (at $\sigma = 7.26$). Relative peak intensity data was consolidated in Excel and there after analyzed by principal component analysis (PCA) using SIMCA P⁺ software (Umetrics, Umea, Sweden).

MS data was acquired using Agilent Mass Hunter Qualitative Analysis software and then extracted into mass spectra in the range of mass-to-charge ratios from 70 to 1000. Relative intensities of the peaks were consolidated in Excel and analyzed by PCA using SIMCA P⁺.

In each case, six biological replicates were used and data was presented as mean $\pm$ standard deviation. To determine which components were significant a list of components was generated using SIMCA P⁺ software and they were subjected to a Student t-test and components with $p \leq 0.05$ were considered to be statistically significant.

4.2. Results

4.2.1 Assessment of Cell Viability

At lower concentrations of microcystins, exposed cells did not exhibit morphological differences from those that were not exposed to microcystins (as observed under an inverted optical microscope). Propidium iodide in combination with thiazole orange was used to assess the cytotoxic concentration of microcystins. Cells incubated for 24 hours in media

spiked with microcystins LR, RR and YR (0.1 – 1000 ng/mL) were stained with PI and TO and analyzed by flow cytometry. Sample results are as shown in figures 4.1 (PI only) and 4.2 (PI and TO).

From the results obtained after staining with PI only, it was obvious that the rate of cell membrane damage increased with increasing concentration of the toxin. However using only PI, only information about membrane integrity can be obtained, thus it is difficult to quantify the number of living or dead cells. Thiazole orange was therefore used in combination with PI to solve this problem.

The percentage of viable cells (as determined from quadrant R5 in figure 4.2) was plotted against concentration of the toxins as shown in figure 4 .3.

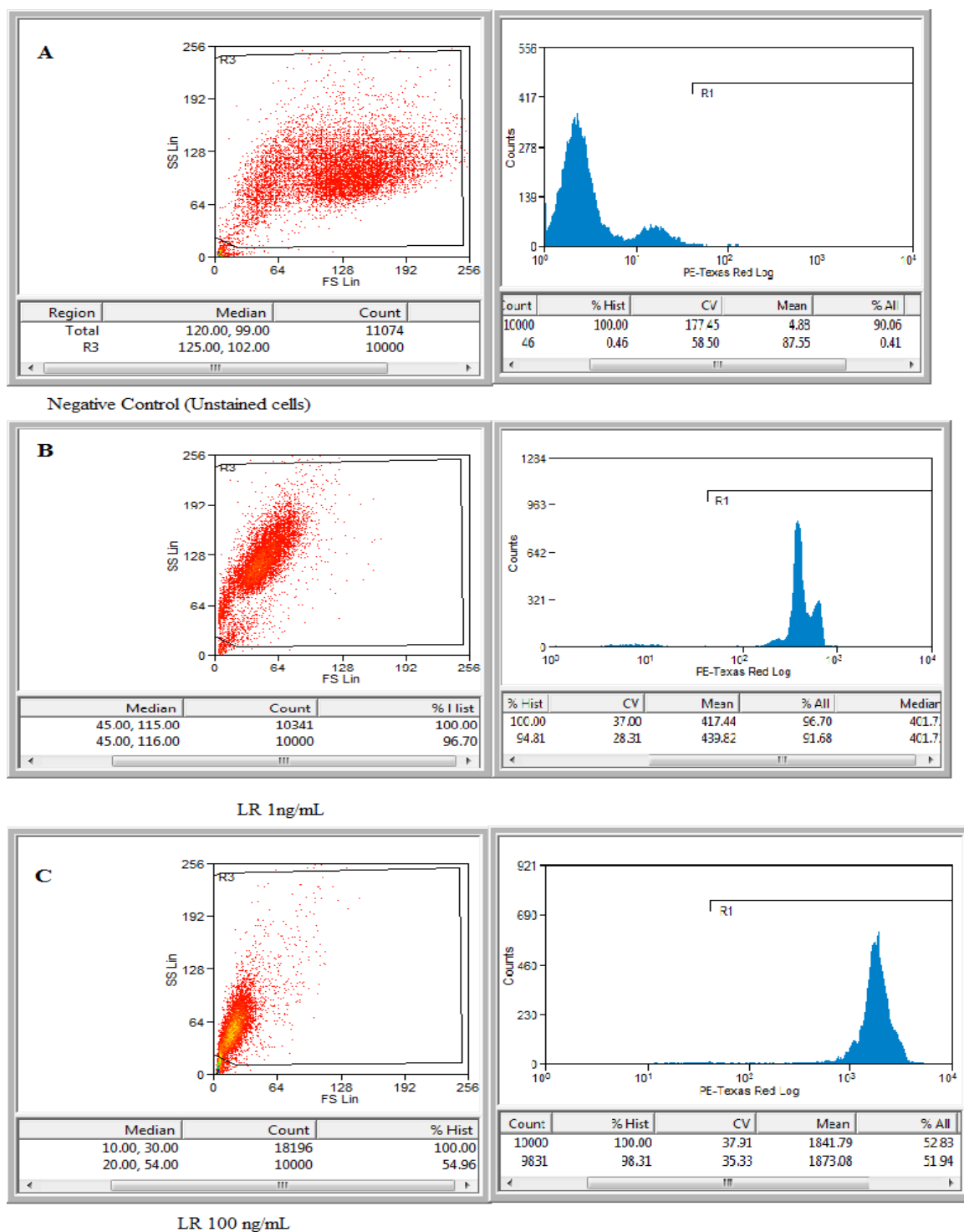


Figure 4. 1: Flow cytograms obtained using propidium iodide stain. A- Negative control, B- cells exposed to 1ng/mL LR, C- cells exposed to 100 ng/ mL LR.

SSlin represents the side scatter (related to inner cell contents) and FS lin represents the forward scatter (related to cell volume). PE- texas red log comp. is the axis for PI

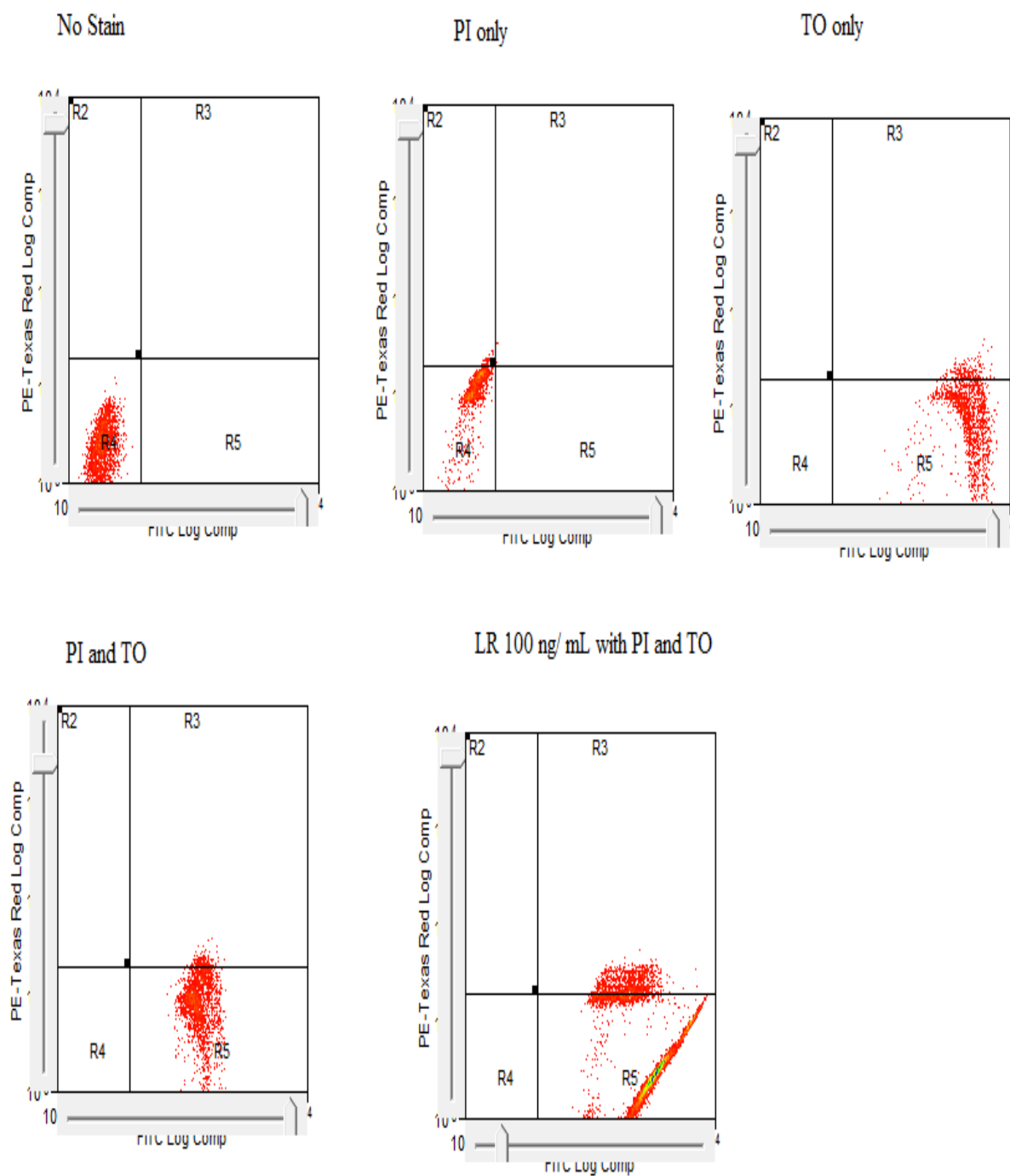


Figure 4. 2: Cytograms obtained using PI and TO

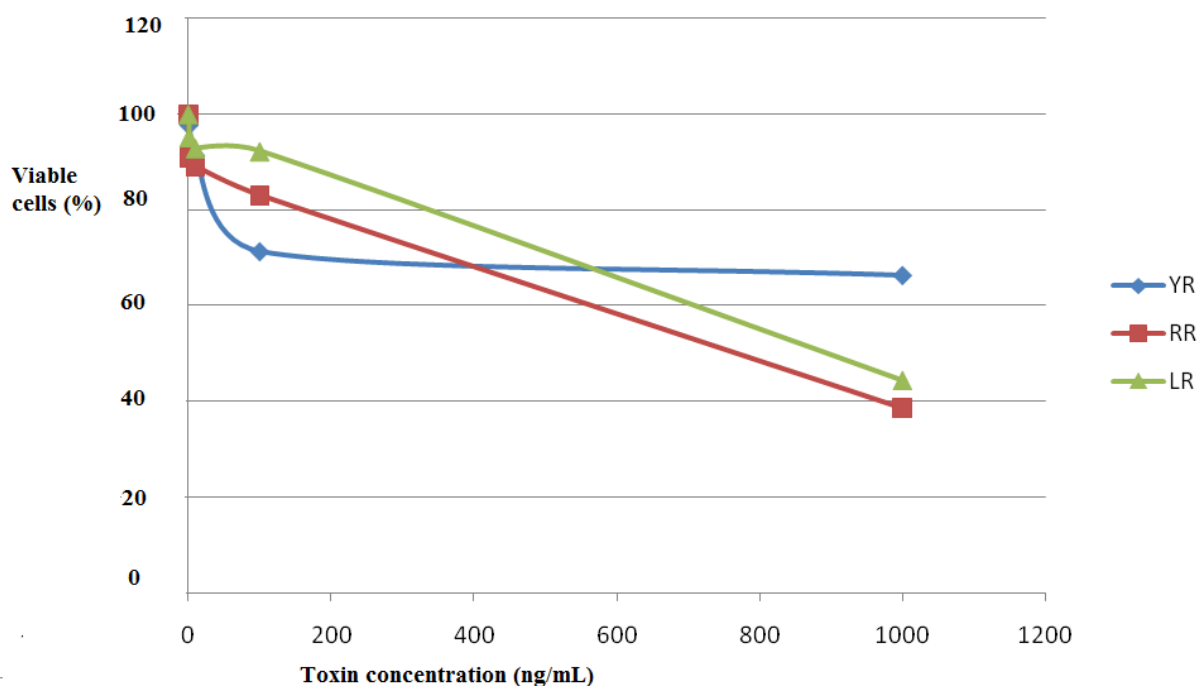


Figure 4. 3: Variation of percentage viable cells with concentration of microcystin in ng per mL

For microcystin concentrations in the range 1 – 100 ng/mL, cell death was less than 50% even though cell death increased with microcystin concentration and there were no significant physical differences in the cell structure. This is in agreement with literature reports ^{3, 24}, and this concentration range was used in the investigation of sub lethal toxicity in this research. Microcystins were spiked into the growth media (in which cells were cultured) at a concentration range 1 – 100 ng/mL. Subsequently 1 mL aliquots of media were withdrawn after 6, 24 and 48 hours and they were later extracted and analyzed by ¹H NMR and DIMS.

4.2.2 ^1H NMR Results

Figures 4.4 and 4.5 show sample ^1H NMR spectra of aqueous samples and organic samples respectively, obtained in ^1H NMR analysis.

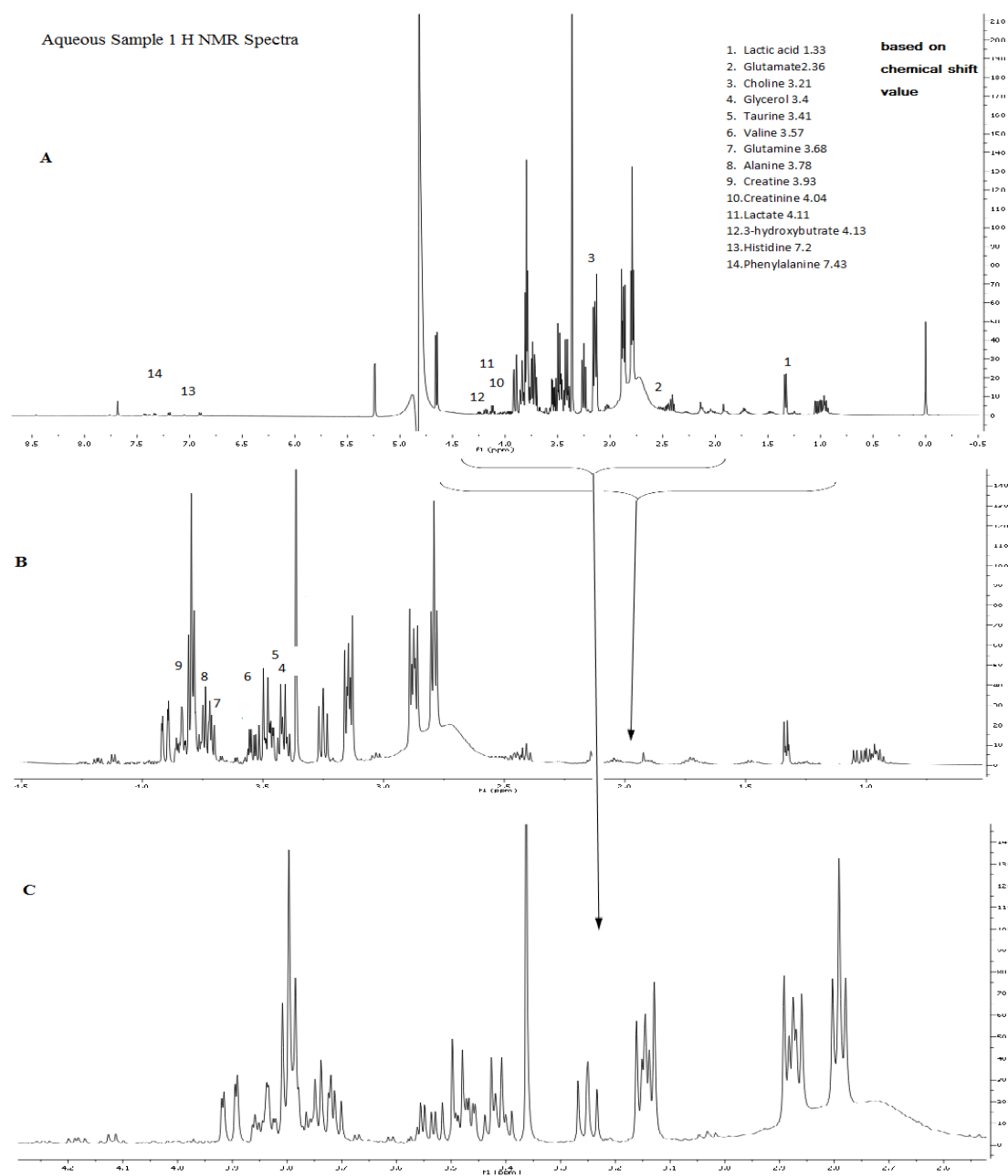


Figure 4. 4: Sample ^1H NMR spectra obtained for an aqueous sample. A- full spectra, B – magnification of region 1 – 4. 5, C- magnification of region $\sigma = 2$ - 4.5.

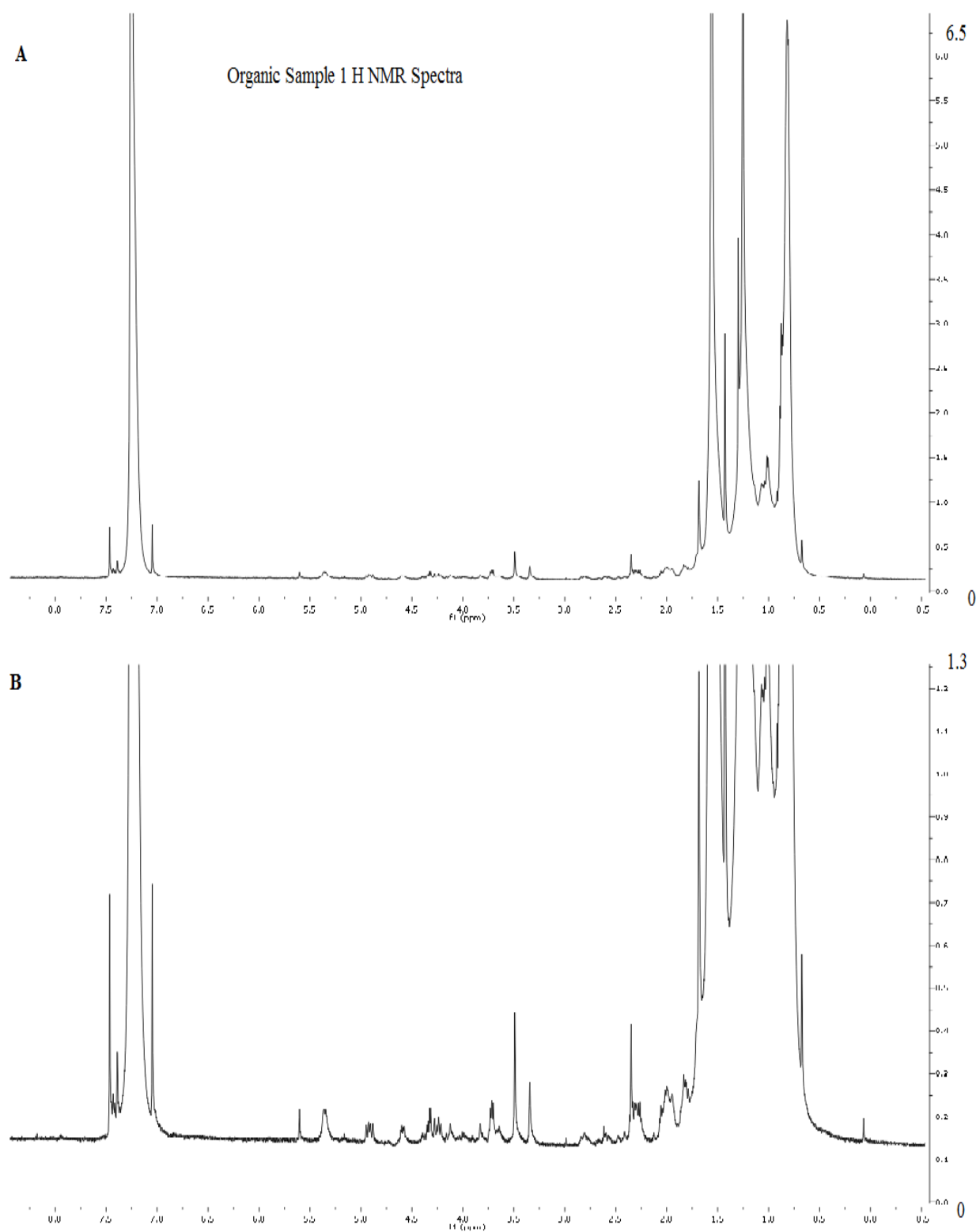
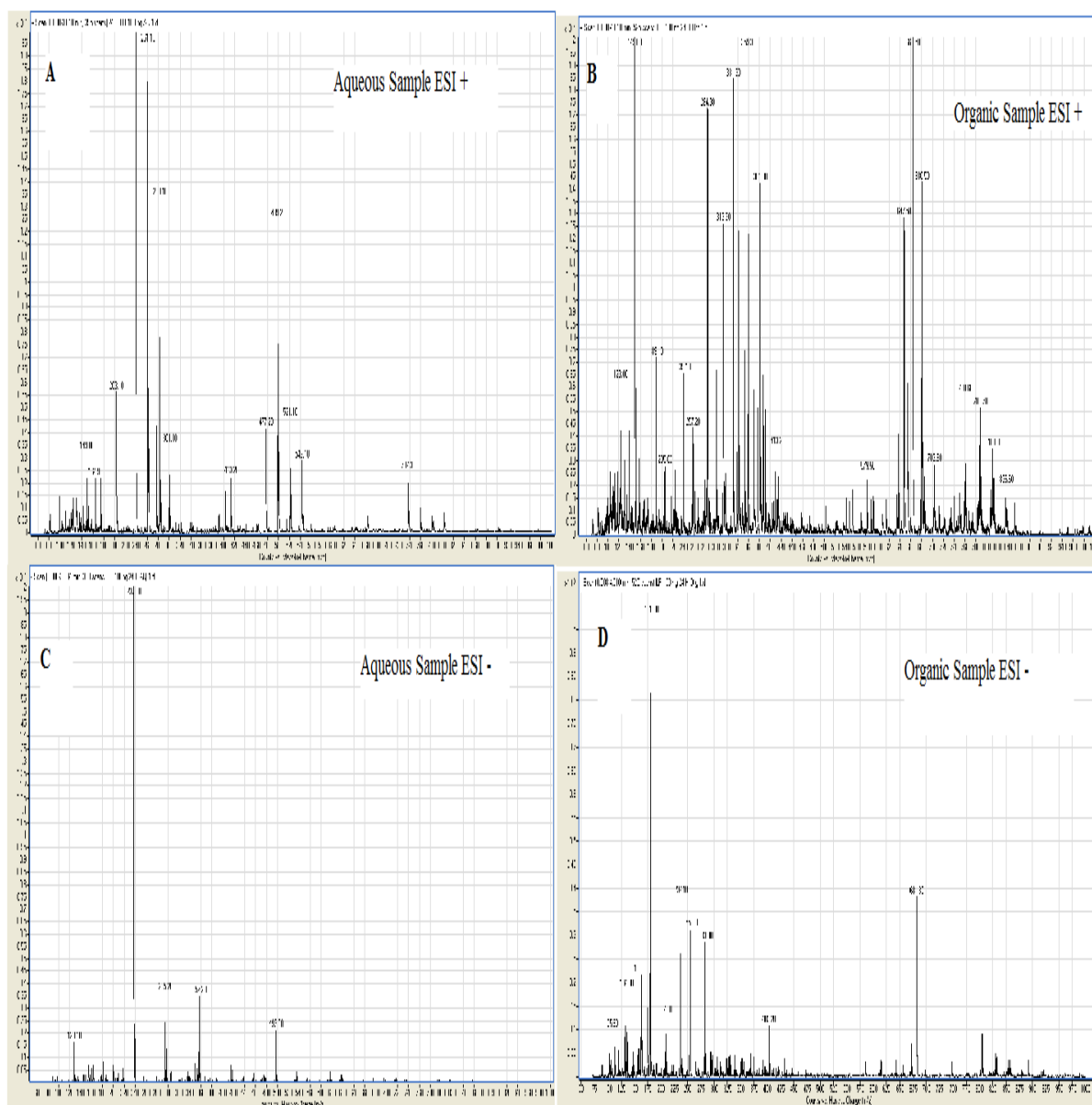


Figure 4. 5: Sample 1 ^1H NMR spectra obtained for an organic sample. B- Higher magnification of A.

4.2.3 MS Results

Figure 4.6 shows sample extracted spectra for aqueous and organic samples obtained from direct injection mass spectrometry (DIMS). There were obvious visual differences between the aqueous sample extracts and the organic extracts and as expected the positive and negative modes also produced different spectra.



4.2.4 Principal Component Analysis (PCA)

Due to the large number of peaks in ^1H NMR spectra and the large number of mass to charge ratio (mZ) values from MS data, the data were compiled in Excel and analyzed using principal component analysis (PCA). SIMCA P⁺ software was used to generate PCA plots for the purpose of footprinting and a list of components for the purpose of component profiling.

Figures 4.7 and 4.8 A show principal component analysis plots obtained from ^1H NMR data for aqueous samples after exposure to a single microcystin. Negative control samples (black), clearly separated from samples exposed to microcystin LR (Blue) and from positive (Red) control samples which were exposed to acetaminophen (figure 4.7 A). Samples exposed to YR (green) microcystin and RR (pink and purple) did not clearly separate from the negative controls but separated from the positive controls as shown in figures 4.7 B and 4.8 A. When the data for three microcystins was plotted together, samples exposed to microcystin LR (blue) clearly separated from the controls and from those exposed to microcystins RR and YR (figure 4.8 B), however, samples exposed to microcystins RR and YR overlapped and did not clearly separate from the negative control even though they separated from the positive control.

A plot of a mixture of LR, YR and RR together with the individual microcystins showed overlaps among samples exposed to microcystins, but the exposed samples clearly separated from the negative and positive controls as shown in figure 4.9. The overlap may be attributed to the fact that the three microcystin exhibit similar properties and undergo a similar pathway for detoxification.

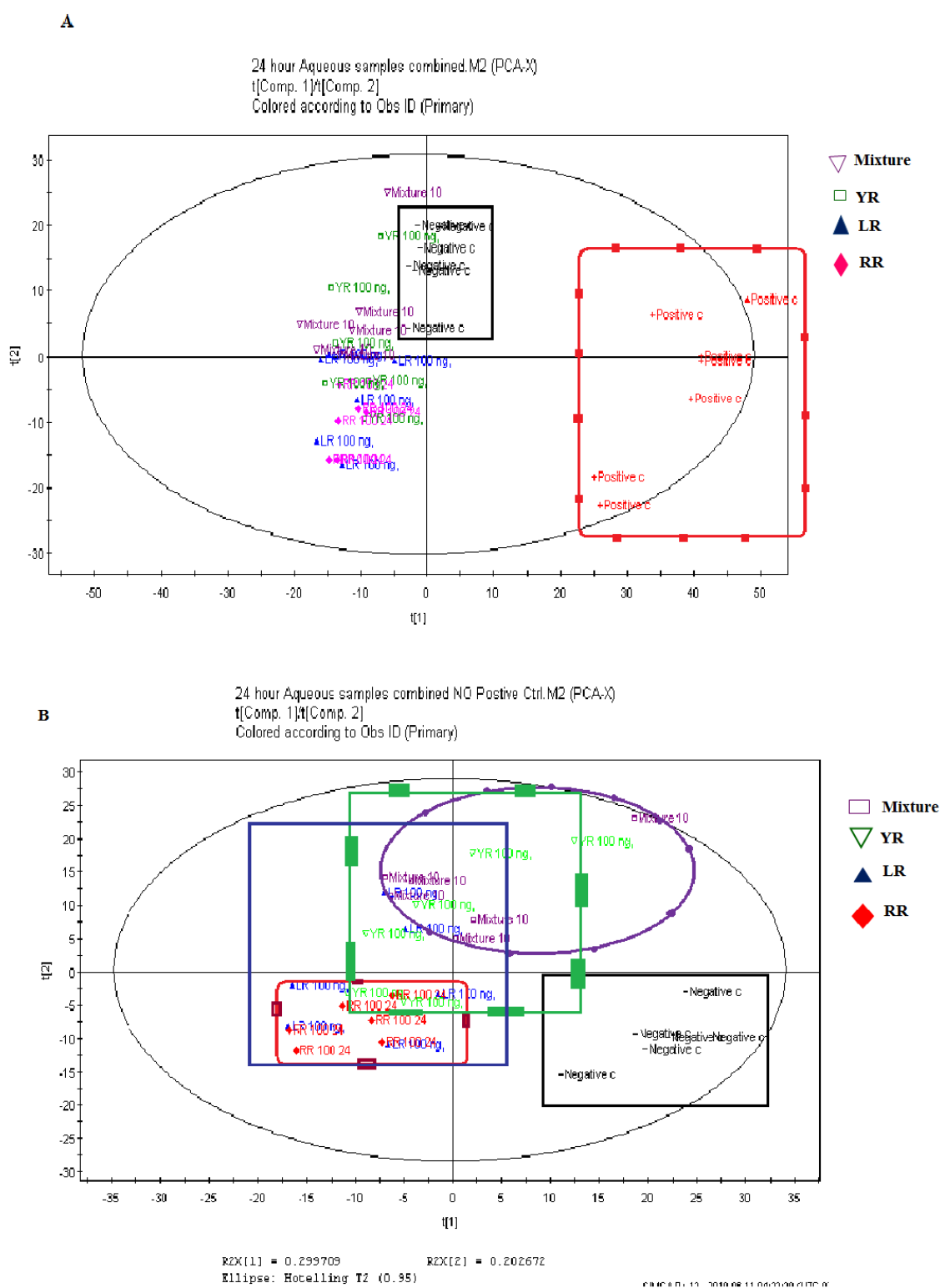


Figure 4. 9: PCA plots obtained from ^1H NMR data of aqueous samples for microcystins LR, RR, YR and mixture with positive control (A) and without positive control (B)

Organic samples exposed to microcystins clearly separated from the negative control as shown in figure 4.10 A. Figure 4.10 B shows two sets of data obtained when two sets of cells were cultured in a mixture (LR, RR and YR). Both separated from the negative control.

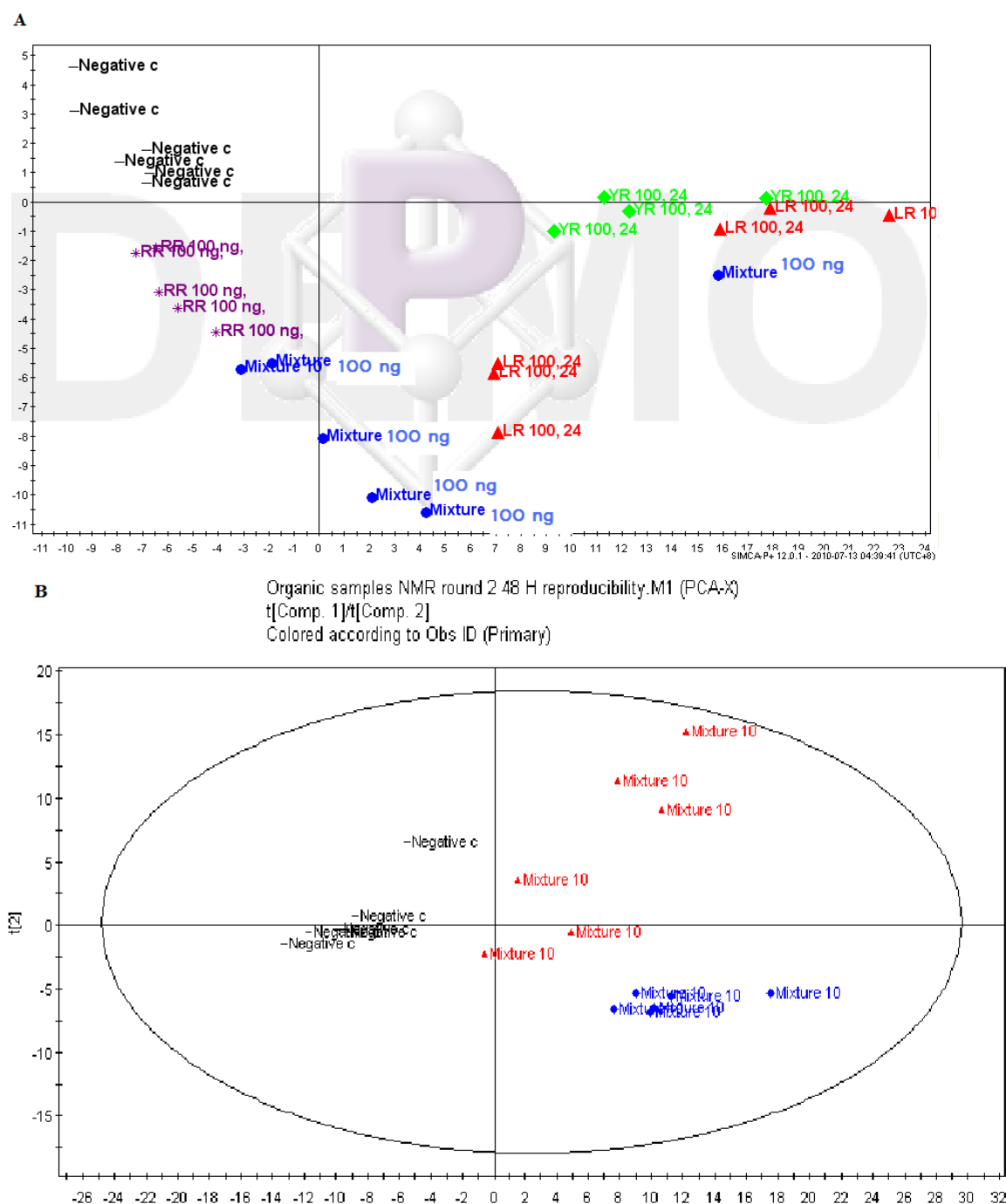


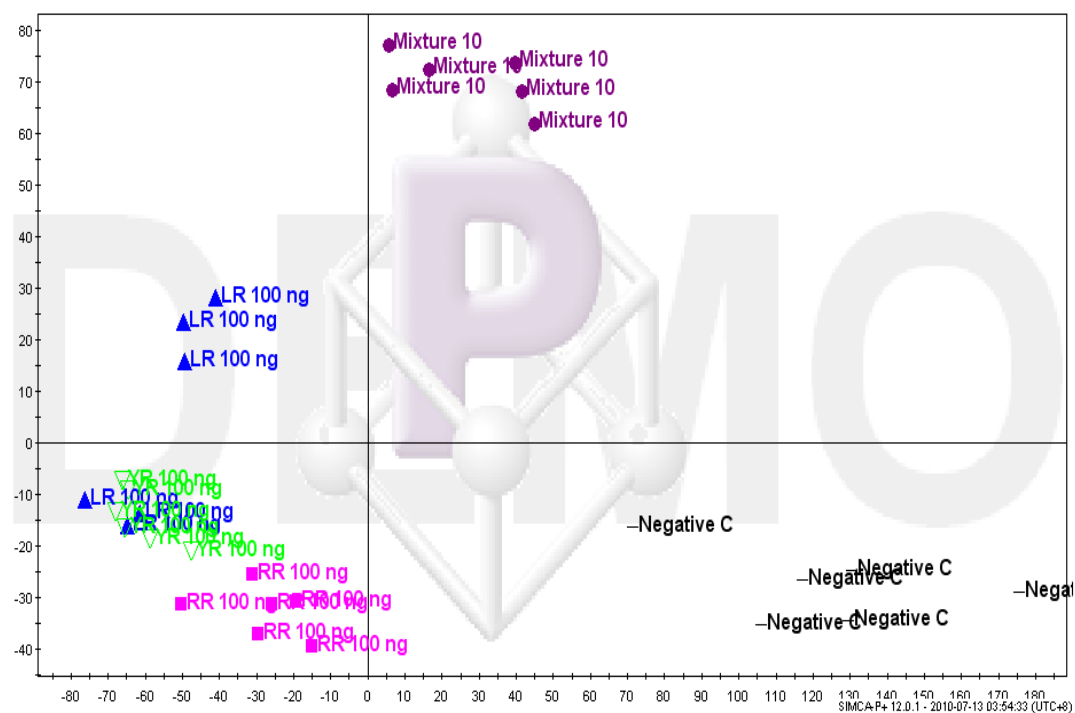
Figure 4. 10 PCA plots of organic samples obtained from ^1H NMR data. A – PCA plot of all the samples (LR, RR, YR, mixture) at a similar concentration of 100 ng per mL and negative control. B- Two sets of mixture at 100 ng/mL each and negative control.

Data points in the clusters obtained from DIMS aqueous data were closer together compared to those obtained from ^1H NMR data. There was good clustering and clear separation of samples exposed to microcystins, negative control and positive control (figure 4.11).

Organic samples also separated from the negative control. Samples exposed to a single microcystin (LR, RR or YR) separated from those exposed to a mixture (figure 4.12). This could be because the effect of the mixture was more severe than for a single toxin. Furthermore mass spectrometry detects compounds at lower concentration than ^1H NMR; this could be the reason why the samples separated better than in the results obtained using ^1H NMR in addition to better clustering achieved using DIMS. Figure 4.13 shows PCA plots obtained after DIMS in the negative mode. Visual distinction of the samples was achieved in both positive and negative modes of DIMS.

Figure 4. 11: PCA plots obtained from MS data for all aqueous samples of microcystins together with the mixture. A- after 24 hours B after 48 hours.

A



B

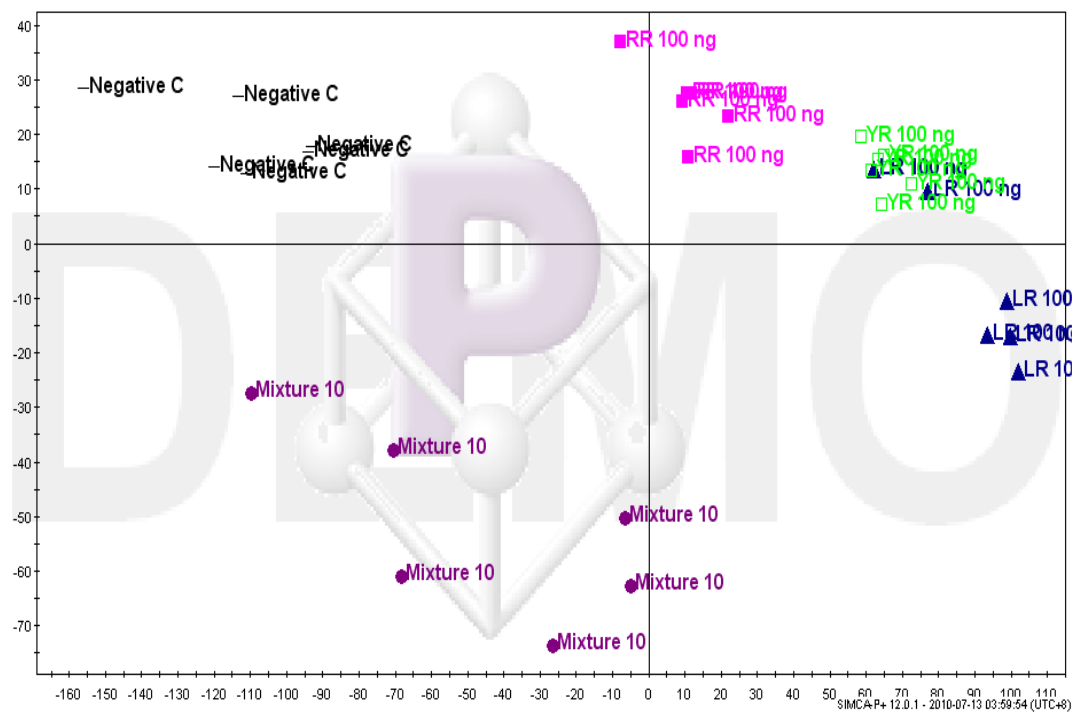


Figure 4. 12: PCA plots obtained from MS data for all Organic samples of microcystins together with the mixture. A- after 24 hours B after 48 hours.

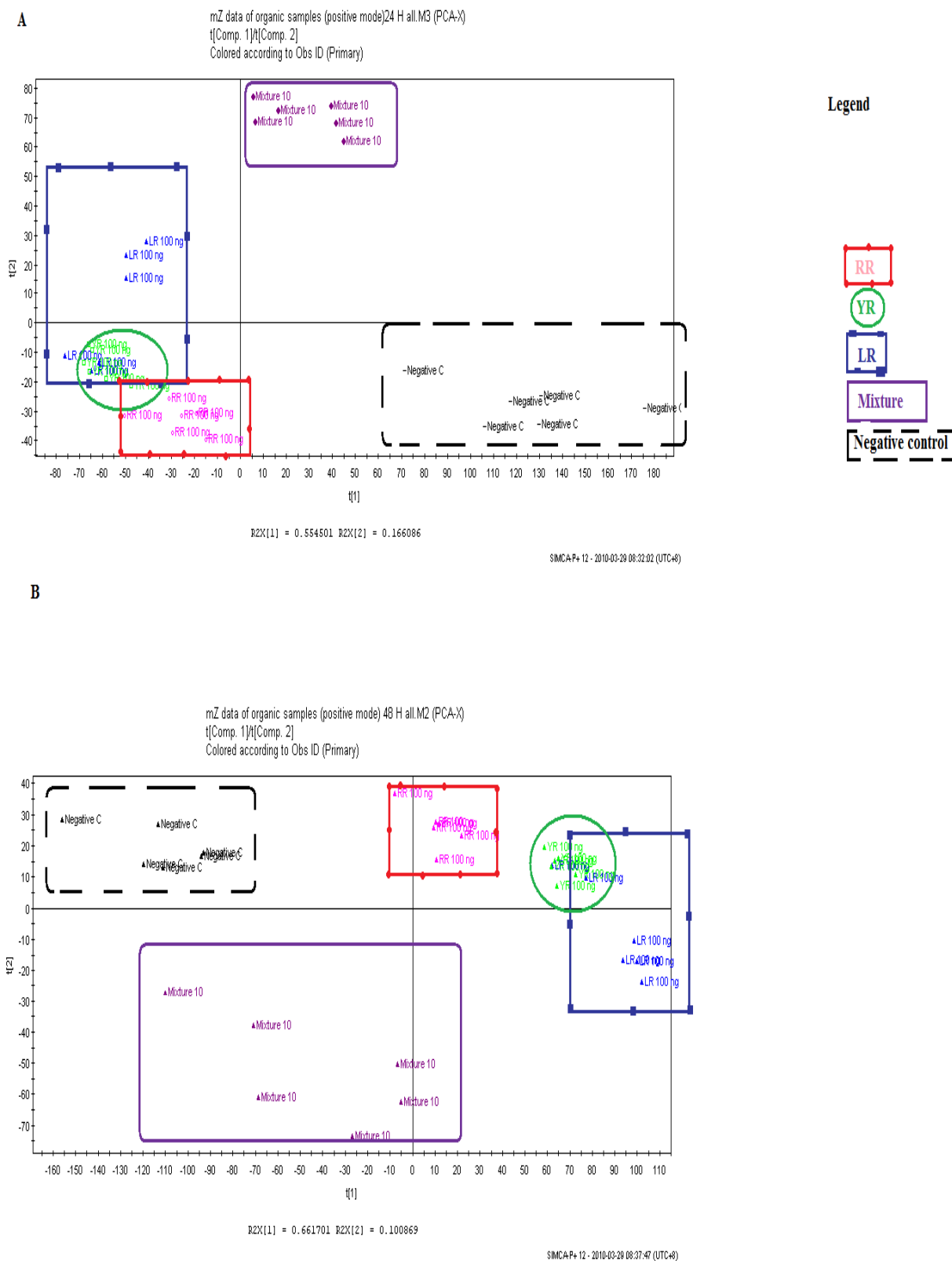


Figure 4. 13: PCA plots obtained for DIMS data from organic samples of microcystins LR, RR, YR, mixture and negative control in positive mode. A- samples after 24 hours, B- Samples after 48 hours.

4.3 Discussion

Exposure to microcystins has been reported in whole organisms for example exposure of Sprague Dawley rats to $500 \mu\text{g kg}^{-1}$ MC-LR by i.p. resulted in a loss of cell-to-cell contact, shrinkage, rounding, and disassociation of hepatocytes. Exposure has also been reported for cells for example MC-LR at 995.2 ng ml^{-1} induced formation of ROS in primary rat hepatocytes, which led to mitochondrial permeability transition cytoskeleton disruption and consequent hepatotoxicity⁶⁹.

The main target of microcystin toxicity is the liver and the brain has also been reported as a target, so microcystins are therefore hepatotoxic and neural toxic. This is attributed to the expression of organic anion transporters (OATP for human, Oatp for rat) by the liver and brain. Fischer et al⁸⁸ identified liver specific rat Oatp 1b2 and human OATP 1B1 and OATP B3 as mediators of microcystin LR uptake into hepatocytes.

The oral route is the main entrance of microcystins into the human body, microcystins are absorbed through the intestines. The majority of absorbed MC-LR (78%–88%) from gastrointestinal tract is resorbed by portal blood stream to liver where it penetrates into hepatocytes through a multi-specific bile acid transport system in liver. Detoxification of microcystins in the liver occurs by conjugation with glutathione via glutathione S-transferase, conjugation with cysteine or with oxidized ADDA diene. The three conjugates are excreted through biliary tract and found in urine, feces, and liver cytosolic fractions⁶⁹.

The toxicity mechanism of microcystin involves inhibition of protein phosphatases 1 and 2A (PP1 and PP2A)^{3, 24, 32}. These phosphatases play an important role in the signal transduction

pathways by mediating dephosphorylation of proteins. Phosphates regulate the proteins on which they are attached, the attachment and removal of phosphate mediated by kinases or phosphorylases and phosphatases, maintains cellular homeostasis³². Protein phosphatases 1 and 2A help in the maintenance of cellular homeostasis by participating in carbohydrate and lipid metabolism, signal transduction, maintenance of cytoskeletal structure, suppression of cell transformation and regulation of apoptosis and cell division rates³². Inhibition of PP1 and PP2 interferes with carbohydrate and lipid metabolism and its effect should be revealed by metabolite profiling.

The data resulting from this study shows that there were changes in the relative amounts of some components (mainly amino acids, some organic acids and lipids as shown in the following section) in the samples exposed to microcystin compared to control samples (refer to appendices 1 and 2). This is in agreement with research work by Wang et al²¹ on zebra fish exposed to low amount of microcystin LR. In their study, enzyme amylase, alpha 2A was inhibited while pyruvate kinase was prohibited by microcystin LR. This caused a dysfunction in carbohydrate metabolism. In our study, beta hydroxybutyrate a ketone body which is normally produced during fasting was found to have increased in samples exposed to microcystins compared to negative control samples implying disruption of the normal glycolysis (appendix 3) pathway. The metabolism of lipids, organic acids and amino acids was affected in our study and this is in agreement with research work by Wang et al²¹ which demonstrated perturbation of enzymes involved in lipid metabolism, organic acid metabolism and amino acid metabolism.

4.3.1 Amino acids

Proline, valine, threonine, cysteine, arginine, acetyl aspartate, tyrosine, phenylalanine, isoleucine/leucine, asparagine, glutamine, glutamate, histidine, 1-methyl histidine were found to increase in samples exposed to microcystins compared to the control samples; while amino acids alanine and serine decreased compared to the control (refer to appendix 4). This implies interference in the metabolism of these amino acids. Amino acid metabolism generally involves synthesis of nonessential amino acids. Essential amino acids are obtained from the diet of the organism.

Essential amino acids valine, threonine, phenylalanine, isoleucine/leucine, histidine increased. This implies interference in their catabolic pathways since normally that is the mechanism for their reduction. They are obtained from the diet and used to produce other biomolecules and in some cases they are used in the synthesis of non essential amino acids.

The main product from valine catabolism is propionyl-CoA, the glucogenic precursor of succinyl-CoA. Threonine is converted into propionyl-CoA through ketobutyrate, isoleucine catabolism produces propionyl-CoA and leucine is degraded to produce acetyl-CoA and acetoacetate through transamination, decarboxylation and several rearrangements ⁸⁹. For these glucogenic amino acids, increases could be linked to interruption of the tricarboxylic acid cycle (TCA) into which the products here should go to in a normal glycolysis process.

Phenylalanine is catabolised by conversion to tyrosine which is later degraded to fumarate and acetoacetate. Increase in phenylalanine could be due to interferences in the phenylalanine

to tyrosine pathway. Histidine is degraded to produce glutamate and one carbon pool; therefore its increase may be due to a dysfunction in the glutamate metabolism pathway.

Nonessential amino acids proline, cysteine, arginine, aspartate, tyrosine, asparagine, glutamate and glutamine increased, while alanine and serine decreased. Proline, glutamine and arginine are synthesised from glutamate⁸⁹ which comes from α -ketoglutarate by transamination, therefore when the amount of glutamate is high, amino acids proline and arginine which are derived from it are also likely to increase. In presence of oxaloacetate, glutamate is converted into aspartate by transamination. Asparagine is produced when glutamine reacts with aspartate. Aspartate can also be obtained from asparagine by hydrolysis as shown in figure 4.14.

Since syntheses of these amino acids are related by the common glutamine, their concentration in an organism will be interrelated. Increased production may be due to accumulation of α -ketoglutarate whose main source is the tricarboxylic acid cycle (TCA) if the cycle is interrupted and α -ketoglutarate is not converted to succinyl CoA. TCA cycle is a central part of aerobic metabolism as shown in the figure 4.15 below. Its main role is synthesis of ATP from, acetyl CoA and oxidative phosphorylation.

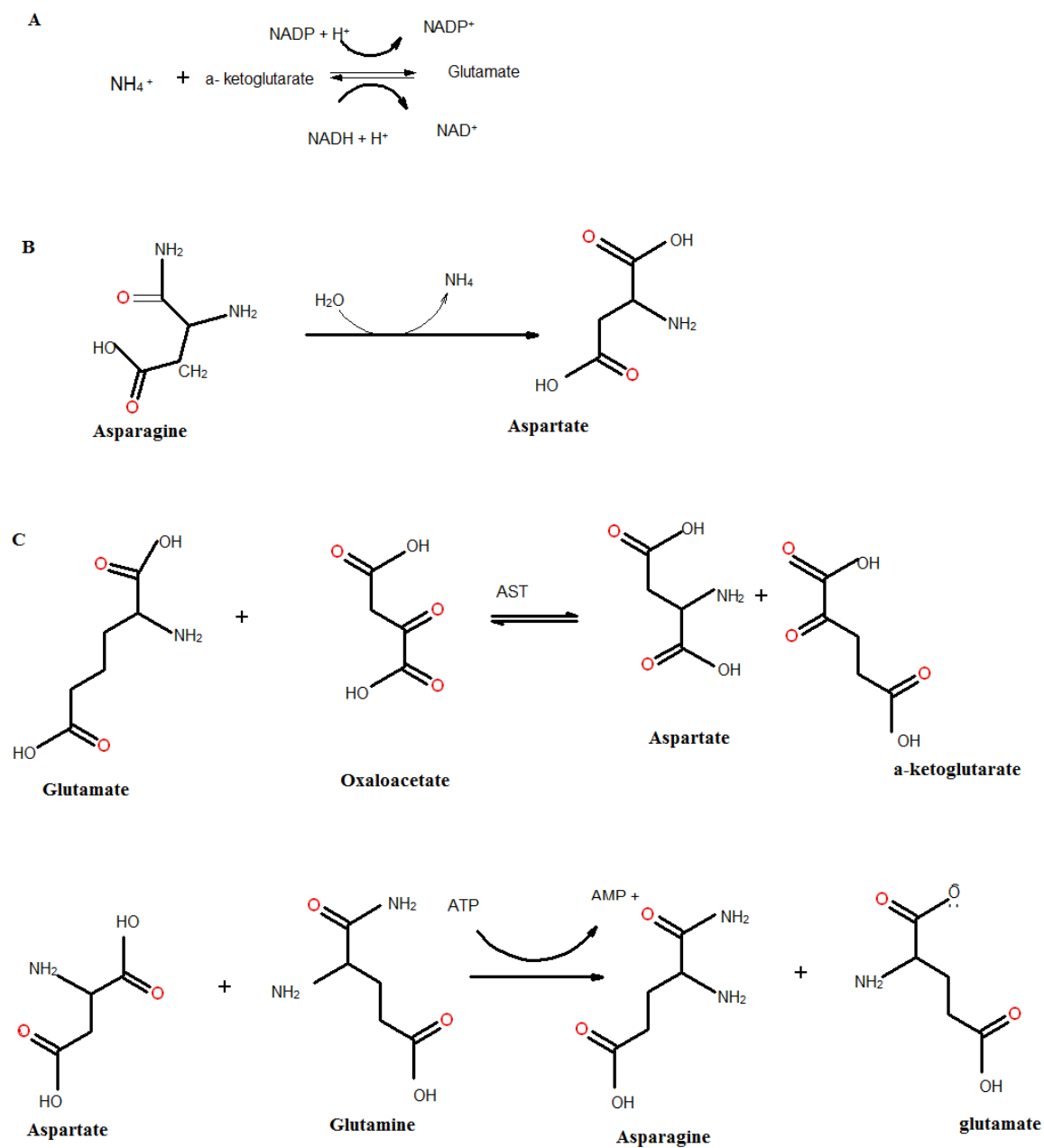


Figure 4. 14: Synthesis of glutamate (A) and its interrelation with aspartate (B) and asparagine (C & D)
 (from <http://themedicalbiochemistrypage.org/amino-acid-metabolism>)

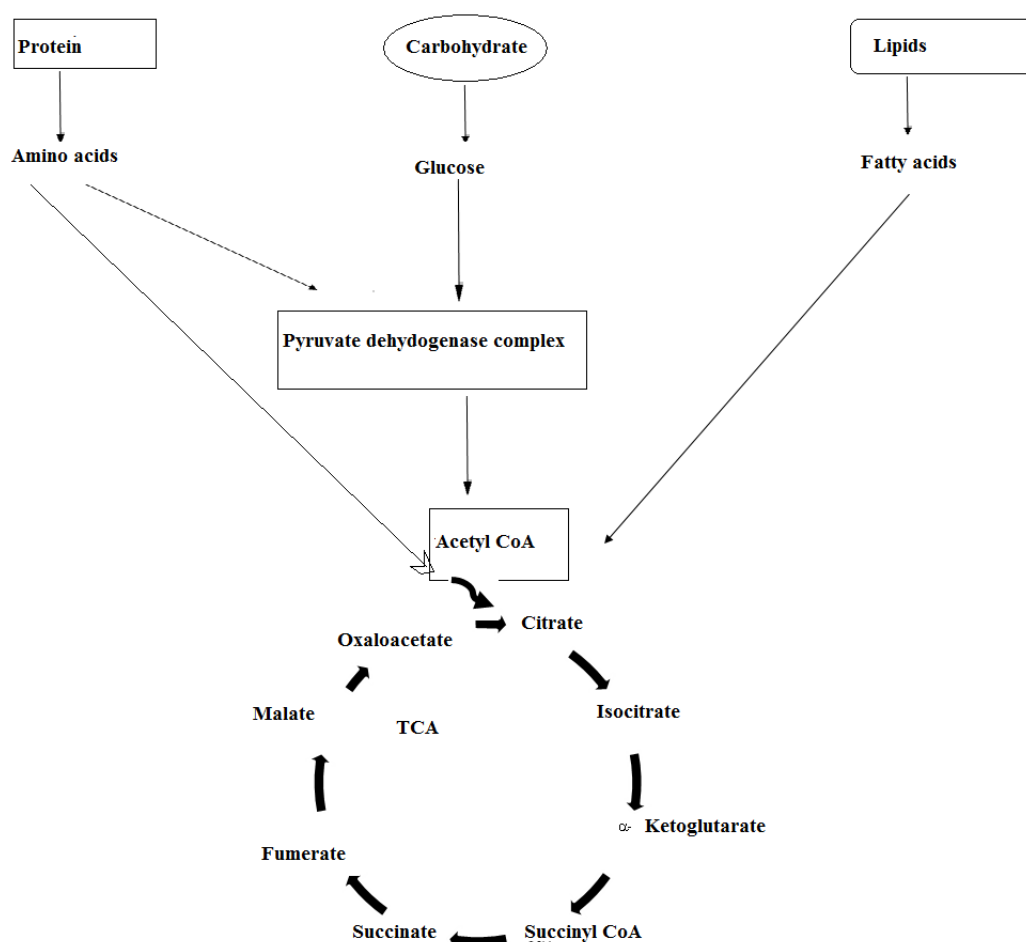


Figure 4. 15: Scheme showing the central role of the TCA cycle in metabolism

The TCA cycle produces α -ketoglutarate one of the first reactants in amino acid metabolism, therefore increase in those amino acids originating from the glutamate metabolism can be attributed to interference in the TCA cycle, in fact TCA cycle components like succinate were also found to have increased in samples exposed to microcystins.

Glutamine is the most significant nitrogen transporter between tissues and glutamate metabolism which is involved in almost all amino acid metabolism is mainly intracellular⁹⁰. In our study, we profiled extracellular metabolites therefore glutamine plays a central role in

balance of related amino acids proline, arginine, aspartate, glutamate as mentioned above. The Schemes below (figure 4.16) show the synthesis and utilisation of glutamine in the liver. Release of glutamine by the liver was reported in animals bearing tumours and this was attributed to a decreased glutamine degradation which shifts the balance from an uptake to its release. The utilisation or synthesis of glutamine is dependent on the rate of glutamine utilisation and its synthesis is relatively constant⁹⁰.

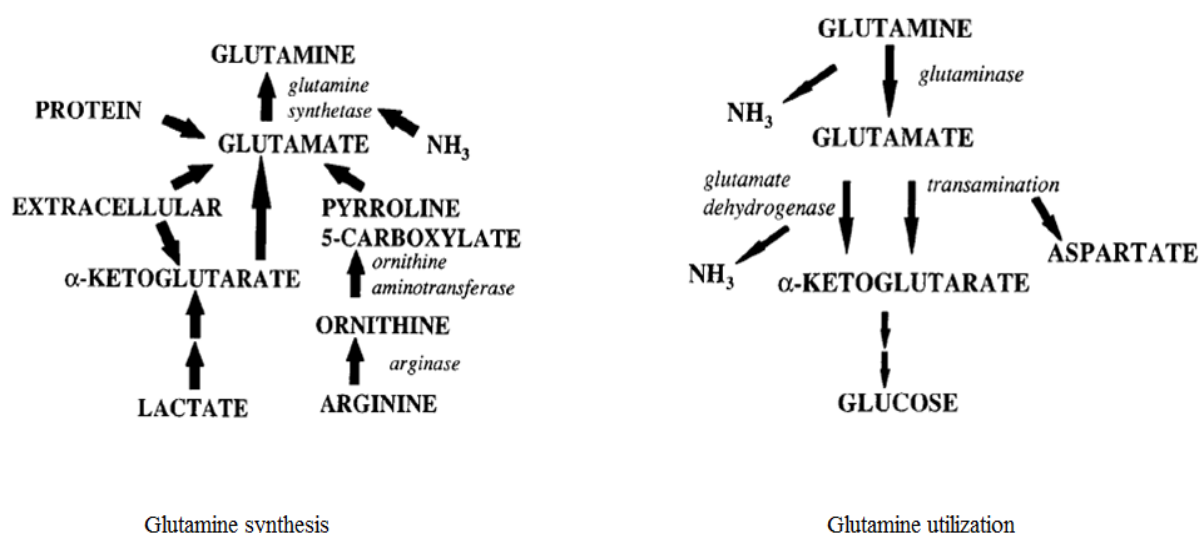


Figure 4. 16: Glutamine synthesis and utilization in the liver⁹⁰

The increase in glutamate and glutamine is in contrast to the study of acute microcystin toxicity by Towner et al³² in which they reported a decrease in the levels of glutamine and glutamate. This could be because in their study a higher concentration of microcystin (1mg/mL) was used and they investigated acute toxicity compared to our study where the highest concentration was 100 ng/mL and we investigated chronic toxicity. An opposing response of liver cells to low and high concentration of microcystins was reported by Herfindal and Selheim⁷¹. This was attributed to the different outcomes that result when phosphatases are inhibited by high microcystin dosage or when they are slightly down

regulated by low dosage. Low amounts of microcystins cause a slight down regulation of the protein phosphatases activity which alters some proteins leading to enhanced survival of damaged cells compared to higher amounts which inhibit the phosphatases leading to hyperphosphorylation, production of ROS, alteration of the cytoskeleton eventually leading to cell death ⁷¹.

Tyrosine is produced by hydroxylation of the essential amino acid phenylalanine in the presence of phenylalanine hydroxylase and biopterin cofactor ⁸⁹. The main source of tyrosine (phenylalanine) also increased possibly causing its increased production. Tyrosine is degraded to produce acetoacetate and fumarate ⁸⁹, in normal metabolism; it should therefore be degraded rather than accumulate. An increase signifies interference in its catabolism. Wang et al ²¹ reported that microcystin LR suppressed production of protein zgc:109929, which is a homolog of the fumaryl acetoacetate hydrolase (FAH) domain. FAH is the last enzyme of the tyrosine catabolic pathway which catalyzes the hydrolysis of fumaryl-acetoacetate into fumarate and acetoacetate. The increase in the amount of tyrosine in our study can therefore be attributed to disruption of its catabolic pathway. This would have an effect on phenylalanine metabolism too since its main catabolic pathway is production of tyrosine and in fact, phenylalanine was also found to increase in samples exposed to microcystins compared to controls. The increase in phenylalanine is probably due to end product inhibition i.e. It may no longer break down due to accumulation of tyrosine.

Cysteine is synthesised from the essential amino acid methionine ⁸⁹ by a series of reactions converting methionine to homocysteine which then reacts with serine to produce cystathionine which breaks down into cysteine and releases α -ketoglutarate. It can also be produced from amino acid serine through the homocysteine cycle of the glycine, serine and

threonine metabolism⁹¹. Cysteine is degraded to pyruvate by desulfuration but the most important catabolic pathway is through oxidation to produce biosynthetic intermediates which are later oxidised to produce such compounds as taurine. Cysteine is also used in the synthesis of tRNA. Metabolism of cysteine is an integral part of the adenosyltransferase S-adenosylmethionine (SAM) trans-sulfuration pathway to glutathione and taurine (figure 4.17). An increase in amount of cysteine could be due to disruption of this pathway or a disruption in the catabolism of products here such as taurine.

Taurine is produced from cysteine and is catabolised through the bile acid metabolism pathway, hence increased cysteine amounts should result in increased taurine production. In our study, the amount of taurine in samples exposed to microcystins was slightly less than in the control even though the trends were similar.

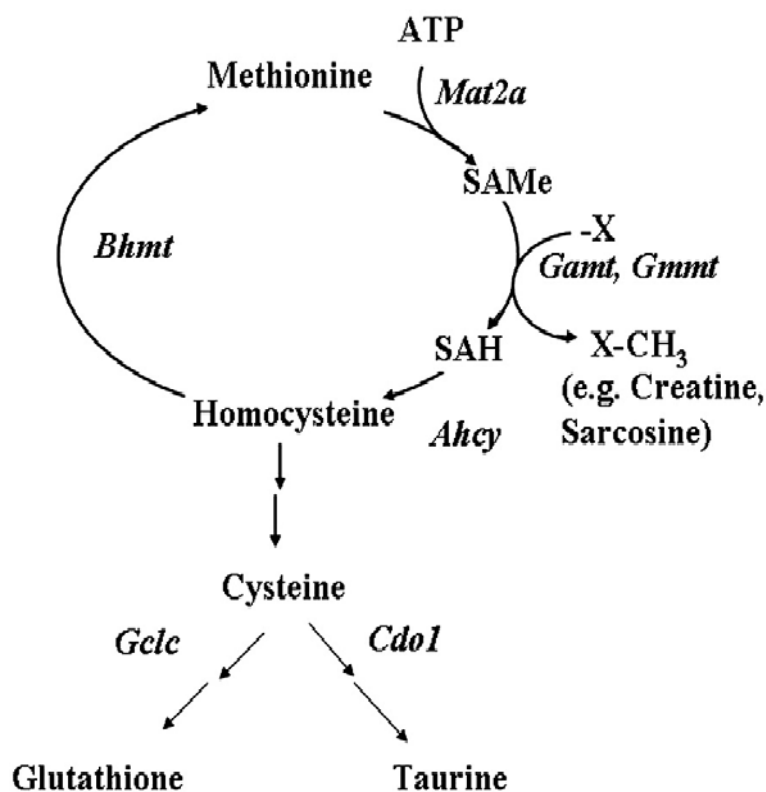
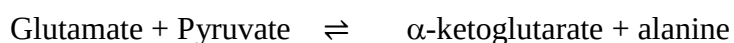


Figure 4. 17: Cystein synthesis, an intergal part of the trans-sulfuration pathway⁹²

Serine is produced through the glucose to hydroxypyruvate pathway. It is a precursor to other amino acids such as glycine, cysteine and tryptophan as well as sphingolipids and folate. A decrease may be due to low reactants, which does not cope with its rate of use and this would be the case if the pyruvate pathway is disrupted.

Alanine is synthesised from pyruvate by transamination and hence can be limited by the amount of pyruvate (substrate) present, the enzyme or end product inhibition. The implication is that interference in production of pyruvate will impact on the amount of alanine produced. Chen et al ²⁸ established a positive relationship between microcystin concentrations and serum enzymes alanine aminotransferase (ALT), aspartate amino transferase (AST) lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). A similar observation for ALT and AST was reported by Nishiwaki-Matsushima et al ⁷² and they noted that microcystin LR or YR concentration of 50 µg per kg or less did not induce a release of ALT and AST.

An increase in expression of these enzymes is associated with hepatocellular damage and tests of these enzyme expressions are commonly used to assess liver function. Since chronic exposure to low amount of microcystins led to increased expression of these enzymes, microcystin exposure can result in hepatotoxicity leading to liver damage even at low concentrations of the toxin. ALT catalyses the transfer of an amino group from alanine to α -ketoglutarate in the reversible transamination reaction to produce pyruvate and glutamate.

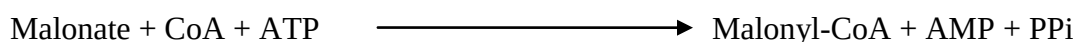


The decrease in alanine in this study can be attributed to interference in substrate (pyruvate) production as well as over expression of ALT as suggested from literature ²⁸.

4.3.2 Organic acids

The tricarboxylic acid cycle (figure 4.15) plays a central role in organic acid metabolism. Malonate, pyruvatoxime, succinic acid, beta hydroxybutrate, amino-tridecanoic acid increased, while glyoxylic acid, glycolate, lactate, glyceric acid, 1-pyrroline-5-carboxylic acid and acetoacetate, decreased. The variations in organic acid amounts are shown in appendix 5.

Malonate/malonic acid is a well known inhibitor of cellular respiration. It competes with succinate for the active site of succinate dehydrogenase, thereby inhibiting oxidation of succinate to fumarate in the tricarboxylic acid (TCA) cycle. Malonate occurs naturally in biological systems and is synthesised from oxaloacetate. It is metabolised to produce malonyl CoA in the presence of Malonyl-CoA Synthetase⁹³.



Succinic acid is produced from succinyl-CoA in the TCA cycle and converted to fumarate in the presence of succinate dehydrogenase. While malonate can inhibit oxidation of succinate by competing for succinate dehydrogenase active sites, Wang et al²¹ found that *suc1g2* protein which is responsible for regulating the formation of succinate and ATP from succinyl – CoA and ADP in the TCA cycle was enhanced by microcystin LR. Therefore, increase in succinate could be attributed to interference of the TCA cycle.

Glycolate and glyoxylate are oxalate precursors. The metabolism of glycolate and glyoxylate in HepG2 cells was studied by Baker et al⁹⁴ and they found that glycolate was taken up more

effectively by cells but glyoxylate was more efficiently converted to oxalate. In their study, glycolate in the media increased over time and glyoxylate was not detected, perhaps this could be because glyoxylate can be converted to glycine and glycolate. In our study both decreased. The decrease in glyoxylate in our study can be attributed to its conversion into glycolate and conversion into oxalate in the presence of lactate dehydrogenase (LDH). Exposure to microcystins has been associated with increased LDH ²⁸ which may be responsible for increased consumption of glyoxylate.

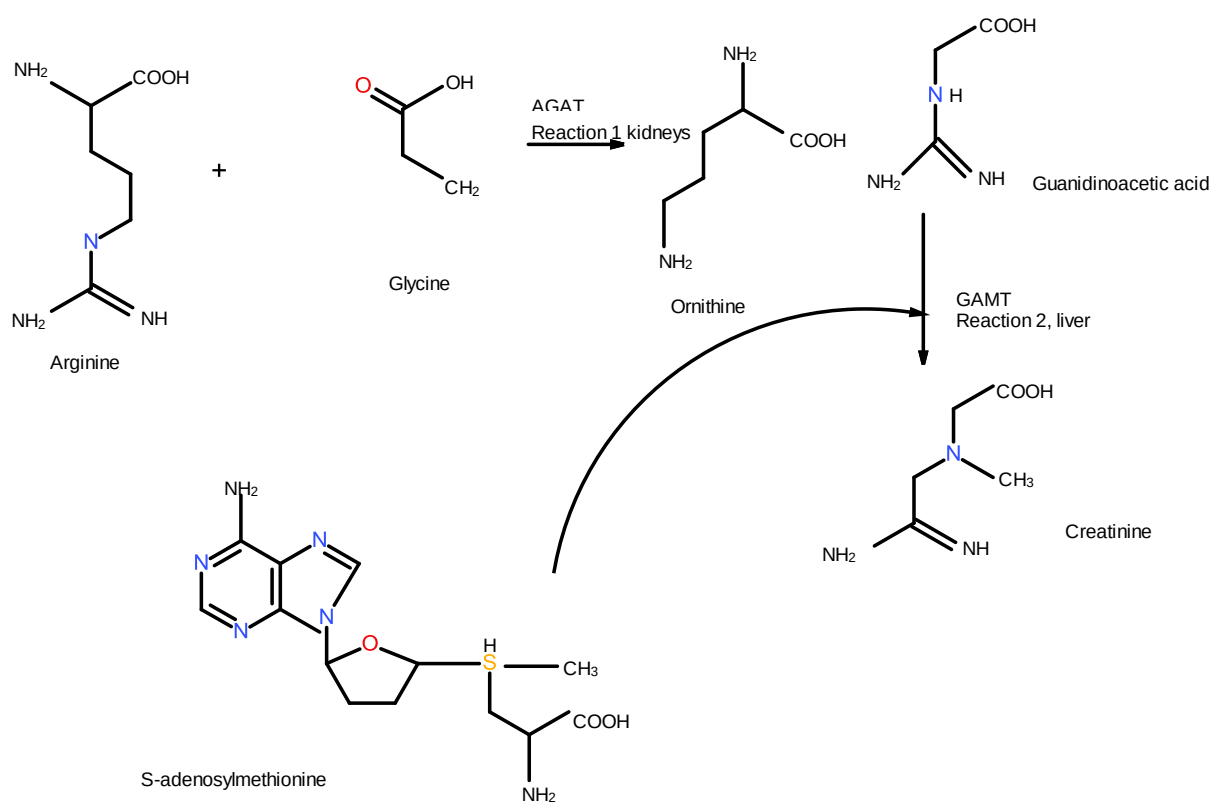
HepG2 cells rapidly uptake glycolate which is metabolized to produce glycine and the concentration of glycolate in the cells and the medium vary linearly ⁹⁴. A decrease as observed in our study can be attributed to decreased production perhaps associated with increased LDH which converts the reactant glyoxylate into oxalate.

Lactate was found to have decreased in our study. This decrease in lactic acid is in agreement with research work by Towner et al ³² even though a lower concentration was used in our study.

4.3.3 Lipids and Phospholipids

Phosphocreatine, 4-phospho-L-aspartate, inositol 1,3,4-trisphosphate, dihydrosphingosine, cytidine diphosphate (CDP), D-myo-inositol 1,4,5-trisphosphate, L-3,5-diiodotyrosine, allyl isothiocyanate, , octane, acetylcholine, dodecanamide , amino-tridecanoic acid, linoleic acid, decanoyl-L-carnitine, selenocystine, inosine 5'-monophosphate (IMP), cholesterol and 25-azacholesterol, increased while cyclic GMP and choline decreased (appendix 6).

Phosphocreatine is synthesised in the liver and transported to the muscle via the creatine phosphate shuttle. It is synthesised from amino acids arginine, glycine and methionine (figure 4.20). Phosphocreatine undergoes irreversible cyclization and dehydration to form creatinine (figure 4.21)⁹⁵. The increase in phosphocreatine is consistent with the increase in amino acid arginine observed and this has a direct relationship with the amount of creatine.



AGAT - Arginineglycine amidotransferase

GAMT - S-adenosyl - L - methionine N guanidinoacetate methyltransferase

Figure 4. 18 Synthesis of creatine

<http://www.creatinemonohydrate.net/illustrations-synthesis> (viewed 29 March 2011)

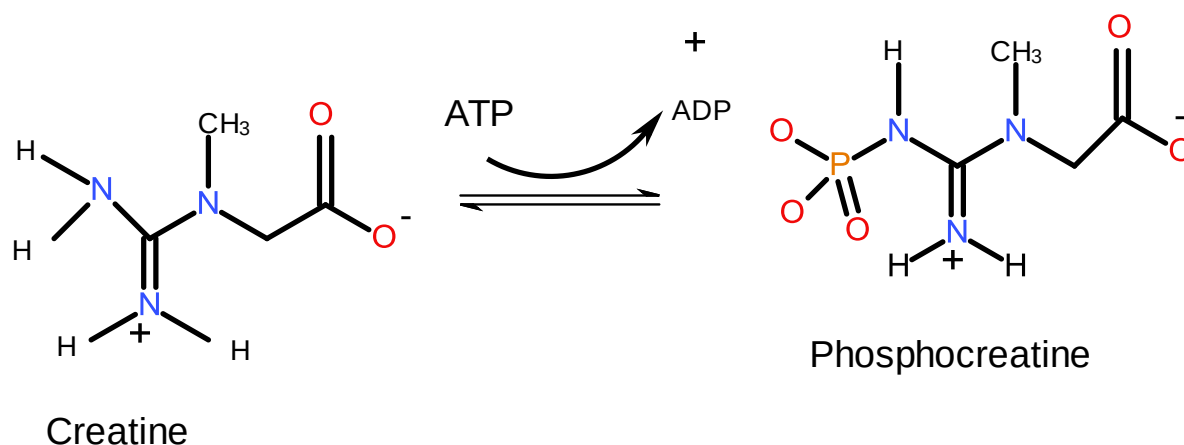


Figure 4. 19: Relationship between creatine and phosphocreatine

http://en.wikipedia.org/wiki/File:Creatine_kinase_rxn.png (Viewed 30 march 2011)

Creatine plays a vital role as phosphocreatine in regenerating adenosine triphosphate in skeletal muscle to energize muscle contraction. Creatine is phosphorylated to phosphocreatine in muscle in a reaction that is catalyzed by the enzyme creatine kinase. This enzyme is at the highest concentration in muscles and nerves.

Dihydrosphingosine (also known as sphinganine) is a blocker of post lysosomal cholesterol transport by inhibition of low-density lipoprotein-induced esterification of cholesterol and causes unesterified cholesterol to accumulate in perinuclear vesicles. It has been suggested that endogenous sphinganine may inhibit cholesterol transport in Niemann-Pick Type C (NPC) disease⁹⁶.

Inositol 1, 3, 4-trisphosphate is a specific regulator of cell signalling⁹⁷. It is a component of inositol phosphate metabolism (appendix 7) together with D-myo-Inositol 1, 4, 5-trisphosphate and inosine 5'-monophosphate (IMP). Inosine 5'-monophosphate (IMP), is also component of alanine and aspartate metabolism

Inositol phosphates play an important role in cellular functions, such as cell growth, apoptosis, cell migration, endocytosis, and cell differentiation. Inositol phosphates can be produced from glucose 6-phosphate in the presence of inositol-3-phosphate synthase 1 which catalyses conversion of glucose 6-phosphate to 1-myoinositol 1-phosphate^{98, 99}. This is later converted to myoinositol and enters the phosphatidylinositol phosphate metabolic pathway. The unphosphorylated inositol ring can be used to produce phosphoinositides through phosphatidylinositol phosphate metabolism. Inositol phosphates can also be produced through phosphatidylinositol phosphate metabolism which produces inositol 1, 4, 5 triphosphate and this joins the inositol phosphate pathway where it is converted to inositol 1, 3, 4, 5 tetra phosphate. The later can be converted to inositol 1, 3, 4-triphosphate by a kinase and a reverse reaction is facilitated by a phosphatase.

Phosphatidylinositol phosphates (also called phosphoinositides) are intracellular signalling lipids that regulate several signal transduction processes. They are important in cellular processes such as actin cytoskeletal reorganization, membrane transport, and cell proliferation. Phosphoinositides may also affect protein localization, aggregation, and activity by acting as secondary messengers⁹⁸.

An increase in phosphates can be attributed to inhibition of phosphatases. Literature^{100 - 101} reported the inhibition of protein phosphatases as well as disruption of the cytoskeletal structure of cells by microcystins, in our study, increased production of phosphates in the inosine phosphate and phosphatidylinositol phosphate pathways are in agreement with literature reports.

L-3,5-diiodotyrosine participates in protein synthesis and amino acid biosynthesis, selenocystine plays a role in protein synthesis, amino acid biosynthesis and it is a substrate for glutathione peroxidase 1. An increase in these components in samples exposed to microcystins compared to controls implies interference in protein synthesis due to exposure to microcystins.

Acetylcholine and choline are components of glycerophospholipid metabolism (appendix 8) and choline also participates in glycine, serine and threonine metabolism. Choline plays a physiological role in signalling, neural transmission and participates in the trans sulfuration pathway for the synthesis of S-adenosylmethionine (SAM) by providing the necessary methyl groups through its intermediate trimethylglycine. Choline was found to have decreased in samples exposed to microcystins compared to controls just like serine also a participant in the SAM path way, this implies that this pathway is affected by microcystin toxicity.

Decanoyl-L-carnitine plays a role in lipid catabolism, fatty acid transport, energy production and linoleic acid is an essential fatty acid while octane is a component of fatty acid metabolism. Cholesterol is a component of bile acid biosynthesis as well as a component of C21-Steroid hormone metabolism. 25-Azacholesterol is a component of hormones and membranes. Increases observed for these compounds suggest interruption in lipid metabolism and catabolism due to presence of the microcystins.

4.3.3.1 Fatty Acid Metabolism

Fatty acids are synthesised in the liver and adipose tissue in the cytoplasm. The main purpose of fatty acid synthesis is to produce fatty acids from acetyl-CoA. Acetyl-CoA is transported from the mitochondria to the cytoplasm via the citrate shuttle (figure 4.21).

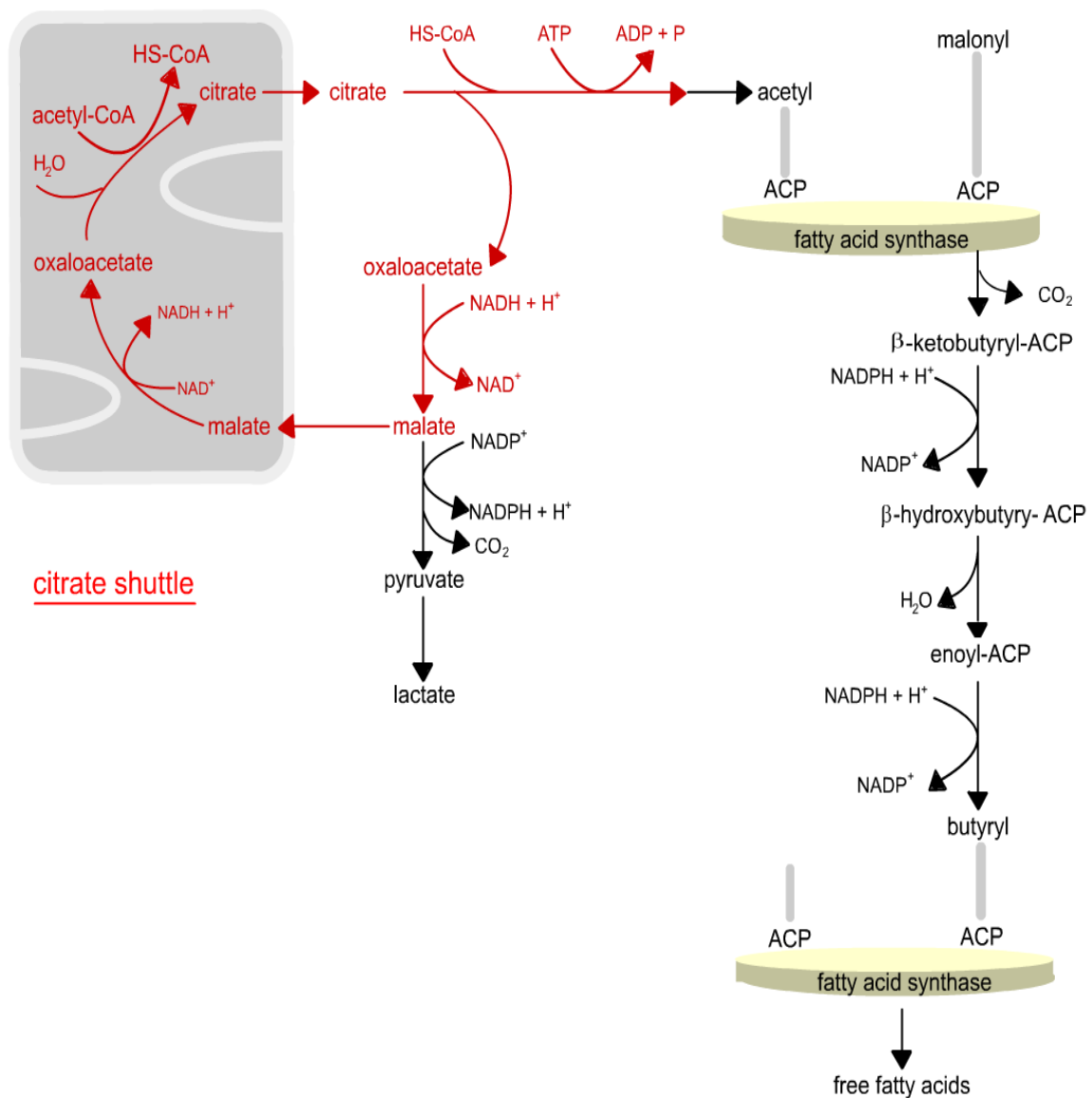


Figure 4. 20 Citrate shuttle and fatty acid synthesis

Taken from http://www.metabolic-database.com/html/citrate_shuttle_animation.html (viewed 30 March 2011)

4.3.4 Purines and Pyrimidines

Cyclic GMP, a component of purine metabolism (appendix 9) decreased and cytidine diphosphate (CDP) a component of pyrimidine metabolism increased. GMP is derived from guanosine triphosphate and normally catabolised to produce guanine and its decrease indicates interference in this pathway. Its role is mainly activation of protein kinases; therefore its decrease will lead to decreased phosphorylation of those proteins. CDP is converted to cytidine an essential component of RNA. In our study CDP increased implying a disruption in its conversion to cytidine for RNA synthesis. These results suggest that microcystins disrupt the metabolism of nucleic acids by interfering in purine and pyrimidine metabolism.

CHAPTER 5 – CONCLUSION

5.0 Conclusion

In this project, capillary electrophoresis techniques based on capillary zone electrophoresis, micellar electrokinetic chromatography and microemulsion electrokinetic chromatography were developed for use in determination of cyanotoxins in water. Furthermore, the application of metabolomics and metabonomics techniques to investigation of chronic microcystin toxicity was demonstrated. Distinction between samples exposed to microcystins and the controls was achieved and the technique of “footprinting” was successfully applied in the study.

5.1 Quantitative Analysis of Cyanotoxins Using Capillary Electrophoresis (CE)

CE techniques were used for separating and quantifying microcystins, nodularin and cylindrospermopsin in drinking water as well as reservoir water samples on a portable capillary electrophoresis system. Therefore the ability to use a portable instrument to determine cyanotoxins using the methods developed was demonstrated. The technique is very simple and can be readily extended to a wider range of microcystins. By using both CZE and MEKC the microcystins can be confirmed and pre-concentration enabled detection of the microcystins at lower concentrations in accordance with the WHO drinking water guidelines. MEEC demonstrated another option of determining a wider range of hepatotoxins. The method was applicable to analytes with a wide range of hydrophobicities and in combination

with absorbance spectra investigation; it is a powerful technique for determination of cyanotoxins. With the inherent advantages of capillary electrophoresis these methods can be used in monitoring of presence of microcystins in drinking water for water quality control.

The main limitation was detection limits that were higher than the WHO drinking water guideline of 1 µg/ litre. This is due to the short optical path of the detector and attempts were made to improve our detection limits through stacking. Stacking together with offline pre-concentration by freeze drying or solid phase extraction enabled us to achieve detection limits acceptable according to the WHO drinking water guidelines.

In the MEEC method, only positive polarity was used due to instrument limitation, the total analysis time in MEEC can be shortened by reversing the EOF with additives such as CTAB and use of negative polarity. Based on results obtained for MEKC, it is possible to achieve separation of the microcystins studied in a shorter time by the MEEC method. In future negative polarity can be used for the MEEC method to shorten the analysis time.

Furthermore, other detection methods can be investigated for example using laser induced fluorescence detection may improve detection limits, online derivitization can also be studied and the method can be applied to other cyanotoxins that lack strongly absorbing chromophores like saxitoxins.

5.2 Evaluation of Effects of Chronic Exposure of a Human cell Line to Microcystins

From this study, microcystins in concentrations lower than acute concentrations were found to have an effect on the metabolism of HepG2 cells as seen from the results of analysis of their metabolic finger/foot prints. Using the metabolomics approach, we demonstrated that exposure to microcystins even at non cytotoxic concentrations disrupts carbohydrate

metabolism, amino acid metabolism, the tricarboxylic acid cycle and fatty acid metabolism. Furthermore nucleic acid metabolism was also affected. In summary, exposure of HepG2 cells to non cytotoxic amounts of microcystins resulted in the following effects:

Hepatic glutamine and glutamate metabolism which is an integral part in amino acid metabolism was disrupted. As a result, the quantities of proline, arginine, asparagine and aspartate were also affected. Tyrosine catabolism was disrupted and this had an effect on phenylalanine amounts too. Microcystins affected the transulfuration pathway, the pyruvate pathway as well as protein synthesis. Pyrimidine and purine metabolism were affected as seen from a decrease in GMP and increased CDP. Therefore microcystins interfere in the synthesis of nucleic acids. The decrease on glycolate can be attributed to increased LDH which is often associated with exposure to microcystins. The later are also associated with inhibition of phosphatases and the increase in inositol phosphates observed in this study is in agreement with inhibition of phosphatases.

Furthermore we demonstrated that footprinting is very useful for distinguishing samples exposed to microcystins and those that were not exposed. By profiling the components in the media after exposure to microcystins, there were clear distinctions between samples exposed to microcystins, those exposed to acetaminophen (positive control) and control (negative control) samples. The metabolomic methods used here therefore were able to distinguish and to classify samples according to compound which they were exposed to. While it was not possible to differentiate between samples exposed to a specific microcystin, samples exposed to a single microcystin clearly separated from the control as well as from a mixture. This is attributed to the similar characteristics of microcystins LR, RR and YR which have similar

properties and undergo the same detoxification mechanism in the liver via formation of a glutathione complex.

Samples exposed to a mixture of the three microcystins at 100 ng per mL separated from those exposed to individual microcystins as well as those exposed to the mixture at a lower concentration of 1 – 10 ng/mL. This could be attributed to more severe effects of the mixture at a higher concentration (higher total concentration) compared to when the cells were exposed to a single microcystin or when the mixture was at a lower concentration.

It was observed that cells exposed to the microcystins at low concentrations in this study did not die rather they continually proliferated and were observed to have increased in number compared to the negative control cells (based on observation under a microscope) which could indicate a non controlled growth that can result into tumours. Therefore another type of cell could be investigated to confirm whether the proliferation was due to the cancerous nature of the HepG2 cell line or otherwise.

5.3 Future Work

In future, the mechanisms by which microcystins interfere with metabolic pathways can be investigated. For example, the enzymes that are affected by microcystin exposure can be investigated further to understand how exactly microcystins interfere with amino acid metabolism. Such studies could be applicable in search for an antidote for microcystin poisoning for example using compounds that compete with microcystins in their mechanism yet not as toxic. Understanding of the pathways and mechanisms involved can contribute to

understanding dose – response relationships and differential species sensitivity. Furthermore, understanding the mode of action can be applicable in risk assessments and provide a basis for extrapolation of the effects across species^{102, 103}.

Furthermore the metabolomics approach can be applied to study effects of exposure to other algal toxins such as cylindrospermopsin, saxitoxins and the lipopolysaccharides. This would help in identifying biomarkers of exposure to the different classes of toxins and understanding their mechanisms of action.

This metabolic approach can be used in combination with other omic approaches in further investigations, because the integration would help not only to understand the effects of exposure, but also be used to predict deleterious effects which are meaningful to risk assessment such as survival, growth, development and reproduction¹⁰².

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7.0 Publications

1. *G.Birungi, S.Y.F.Li*: Application of Capillary Zone Electrophoresis to Separation of Microcystins. SIIC-5/APCE 2007
2. *G. Birungi and S. F. Y. Li*: Determination of Microcystins and nodularin in Drinking Water Using Capillary Electrophoresis. IICS
3. *Grace Birungi, Sam Fong Yau Li*: Determination of Cyanobacterial Cyclic Peptide Hepatotoxins in Drinking Water using CE. *Electrophoresis* 2009, 30, 2737–2742
4. *Grace Birungi, Sam Fong Yau Li*: A Microemulsion Electrokinetic Chromatography (MEEC) Method for Analysis of Microcystins in Water, TWOWS Fourth General Assembly and International Conference, Beijing 27 – 30 June 2010
5. *Grace Birungi, Sam Fong Yau Li*: Investigation of the Effect of Exposure to non Cytotoxic Amounts of Microcystins, *Metabolomics* DOI 10.1007/s11306-010-0265-0.

8.0 Appendices

Appendix 1 Sample NMR Results

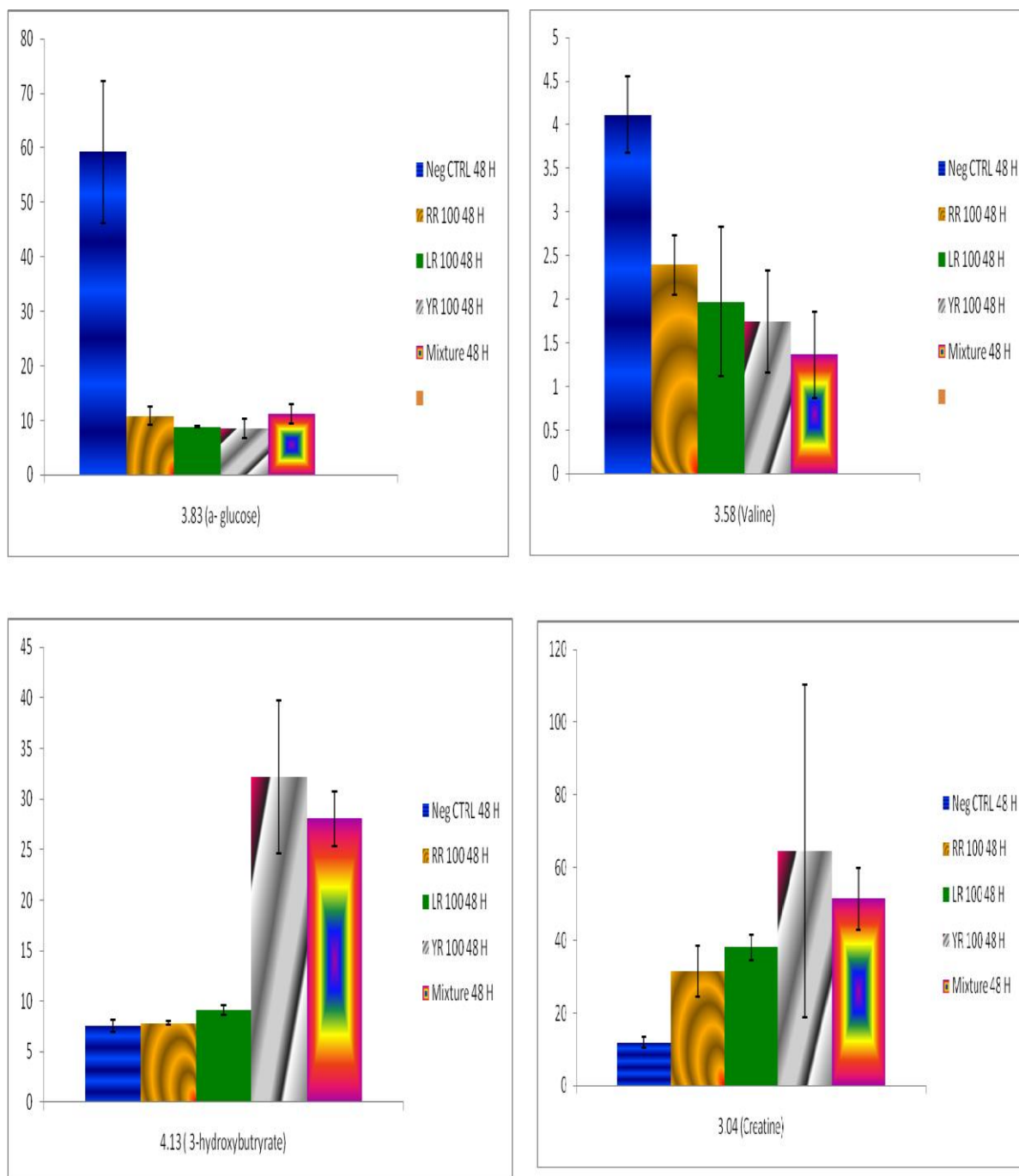
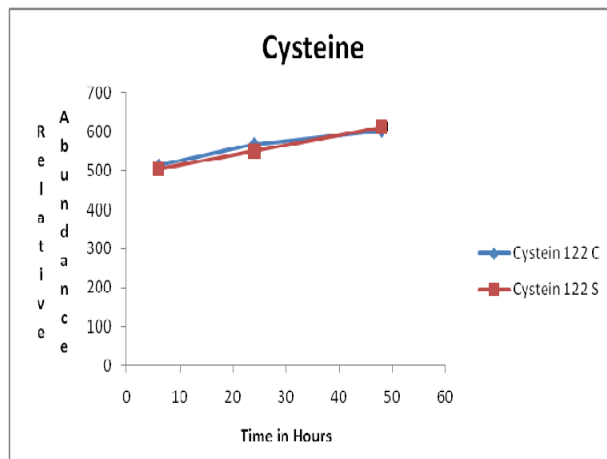
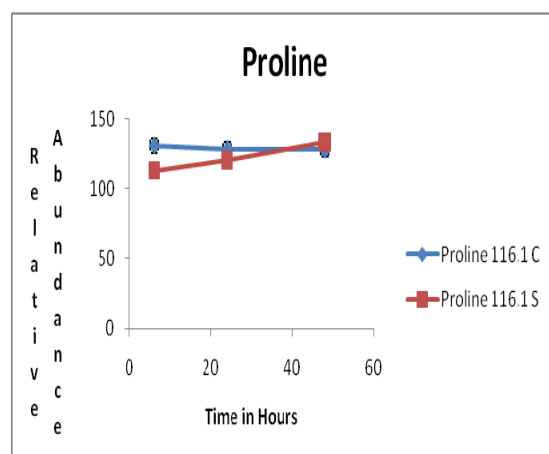
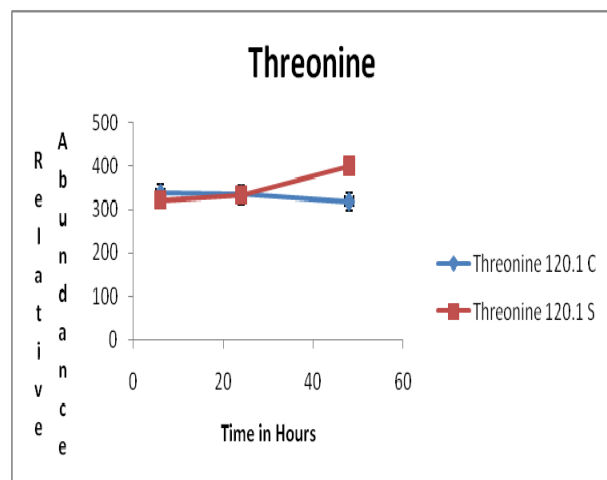
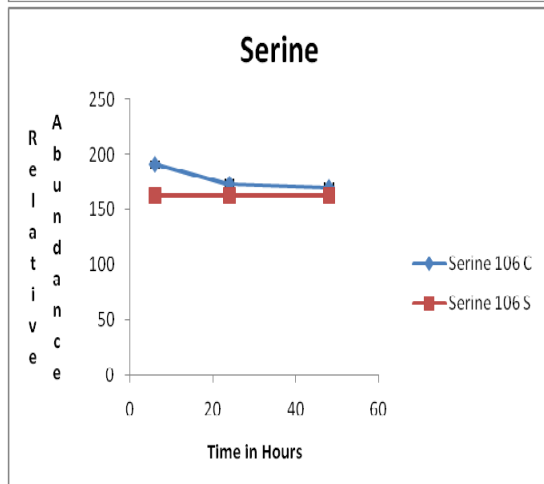
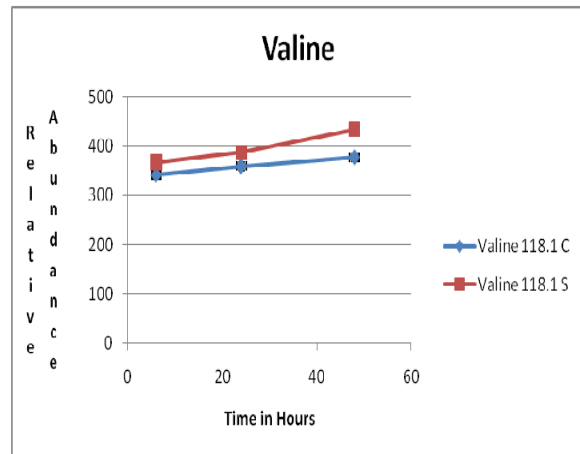
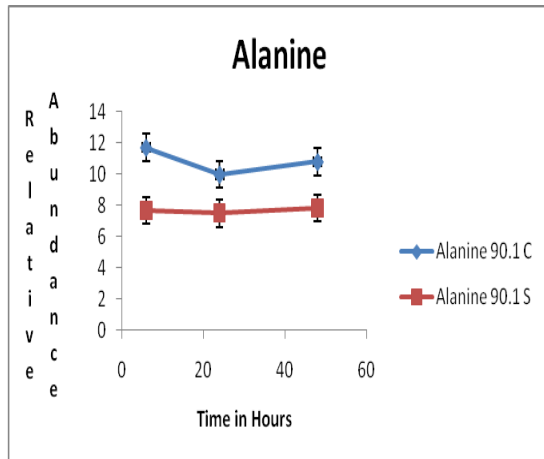
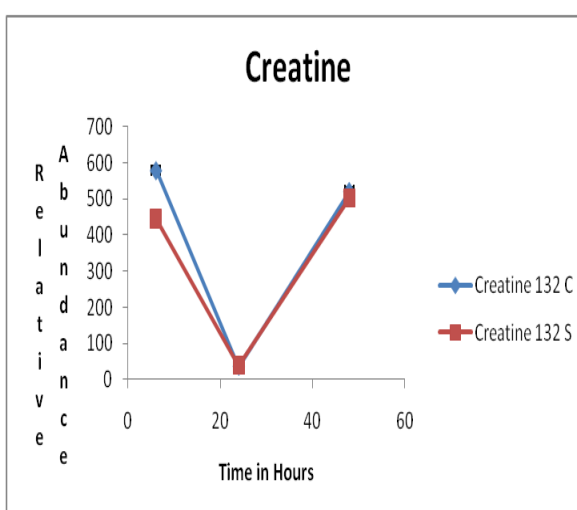
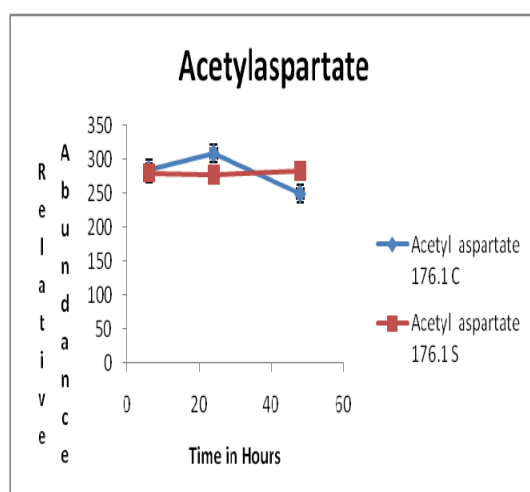
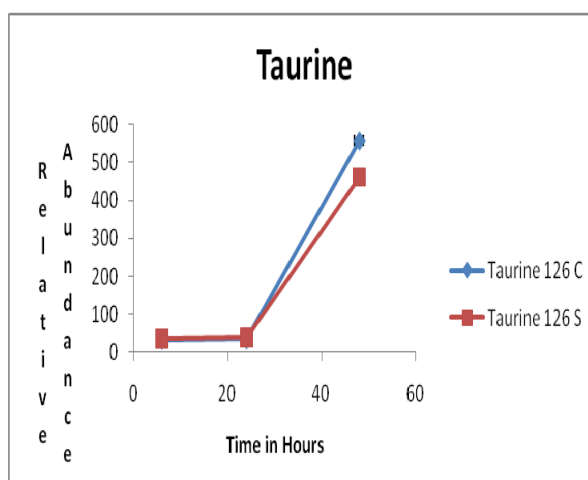
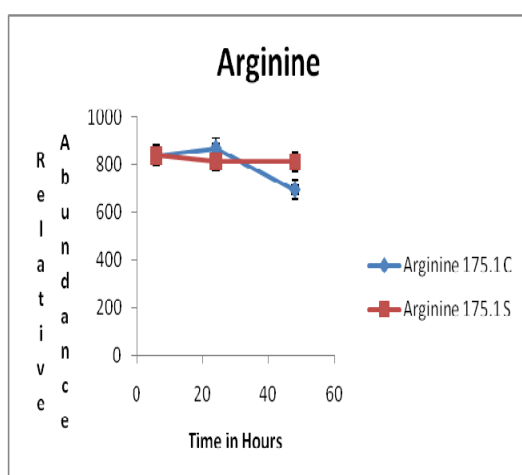
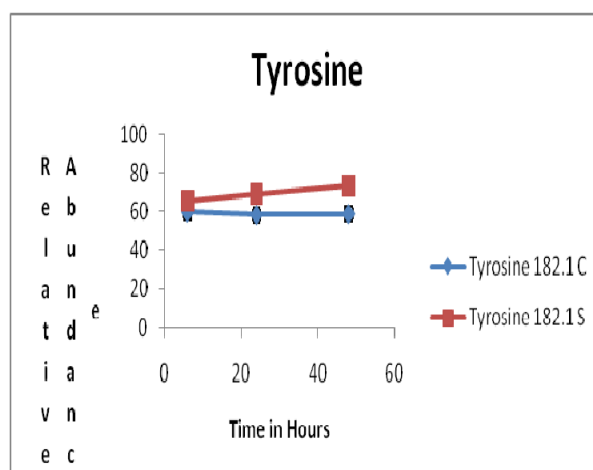
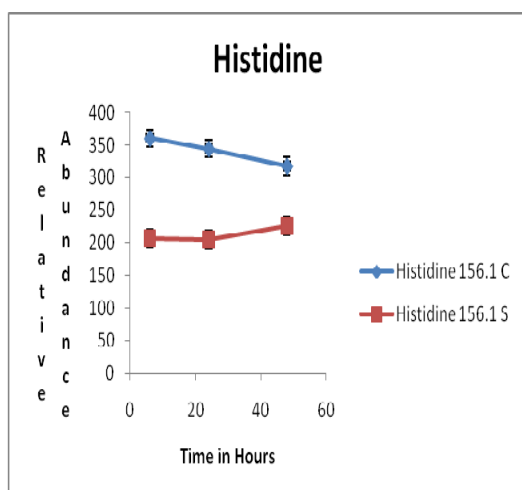
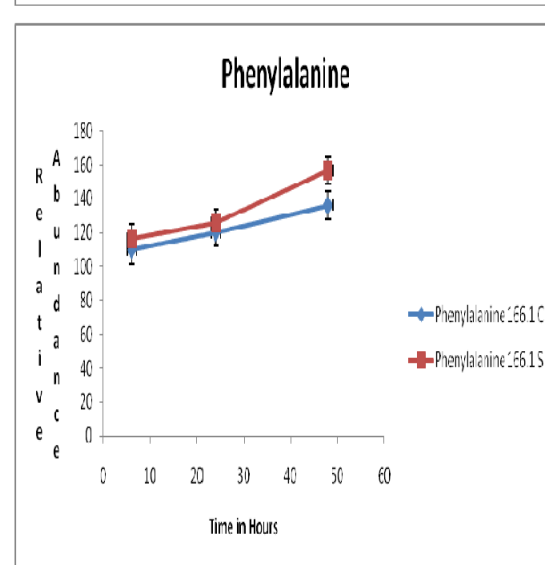
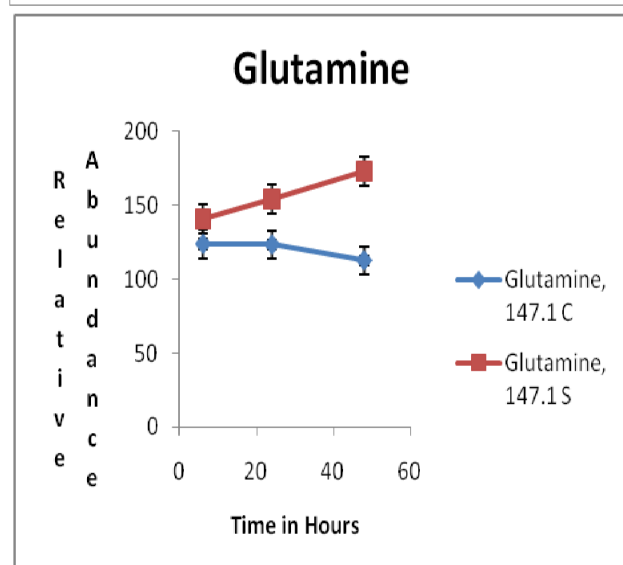
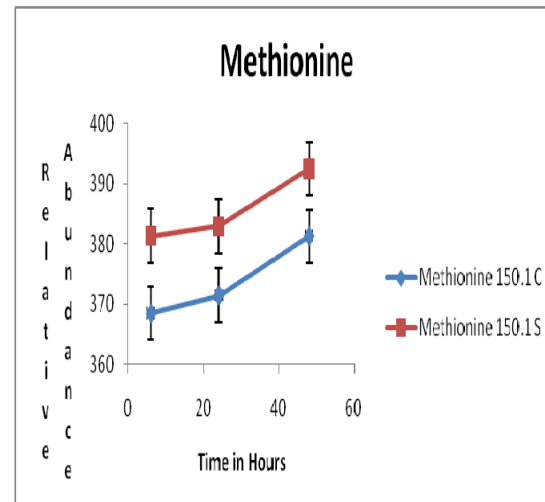
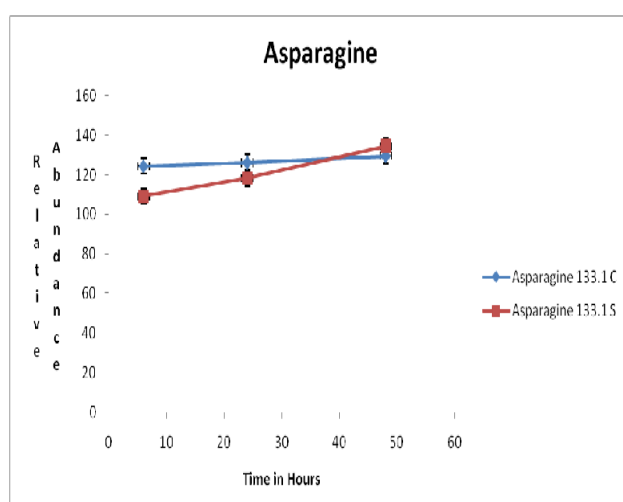
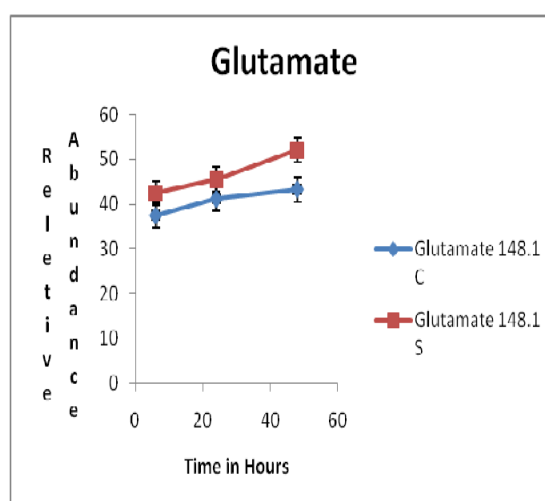
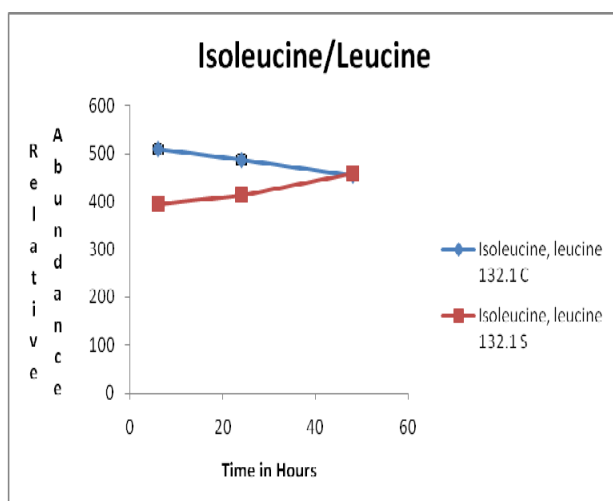


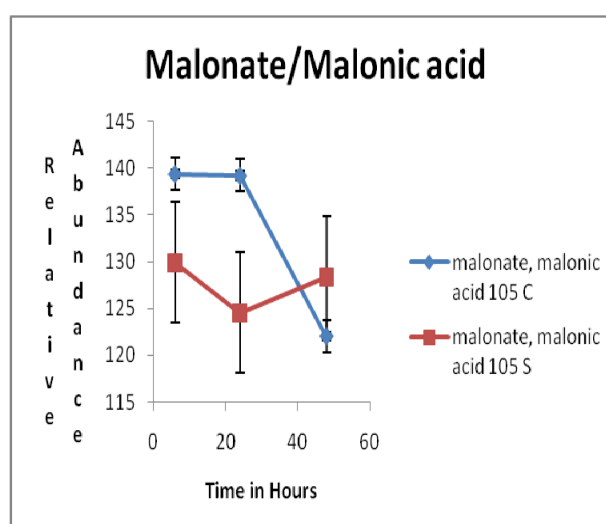
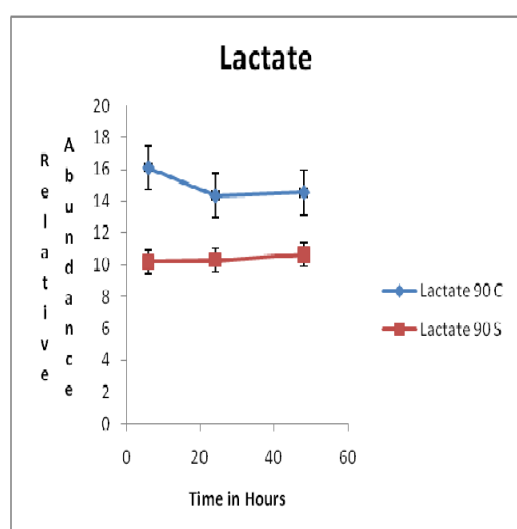
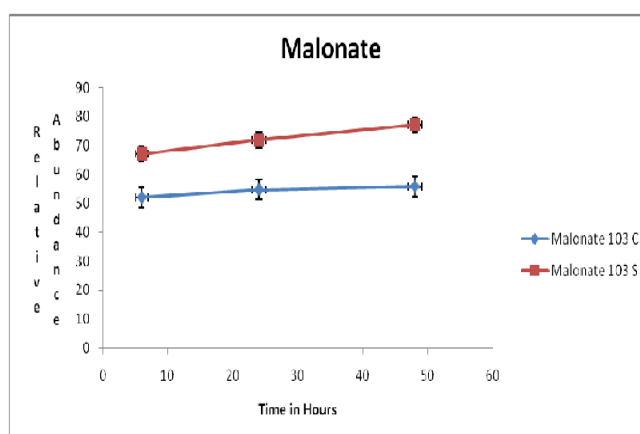
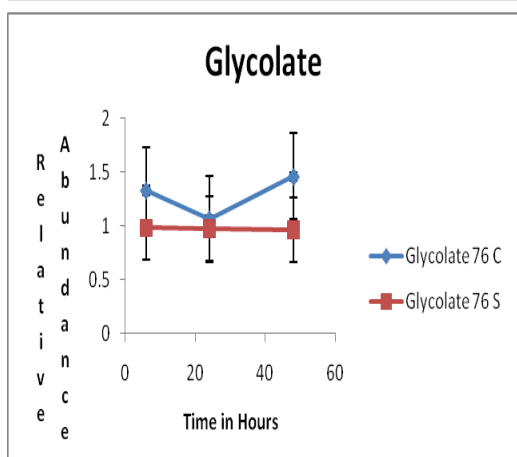
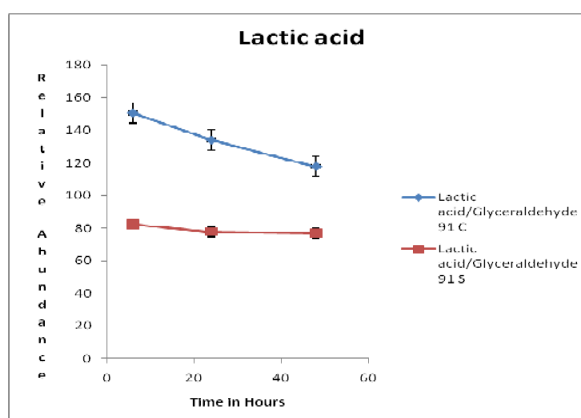
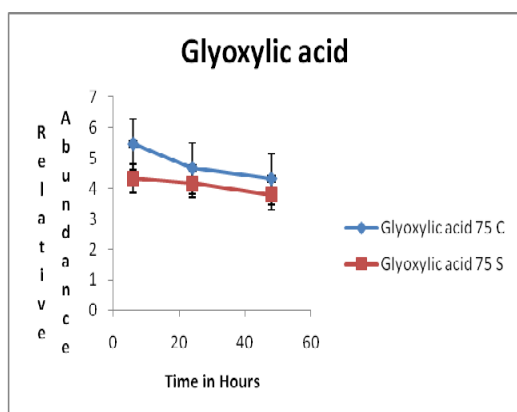
Figure 7. 1 Sample ^1H NMR data

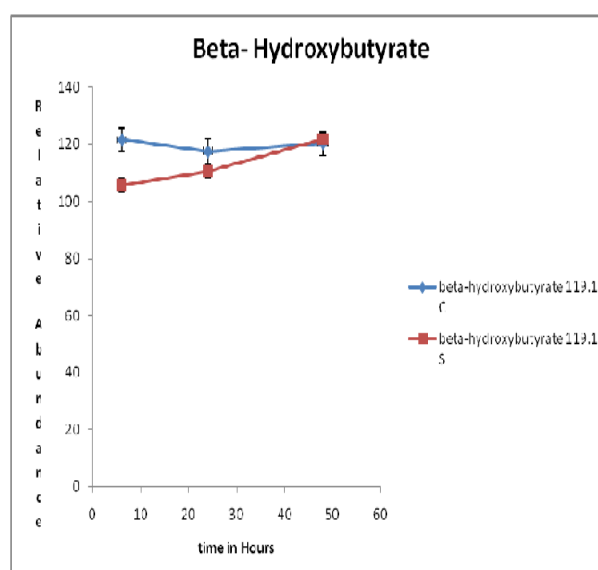
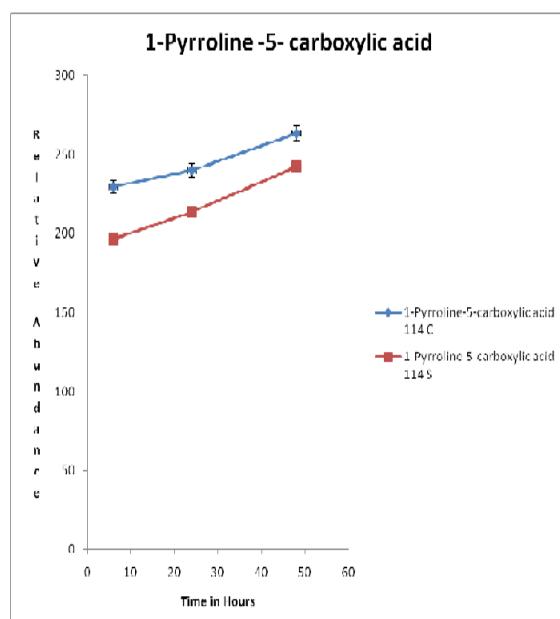
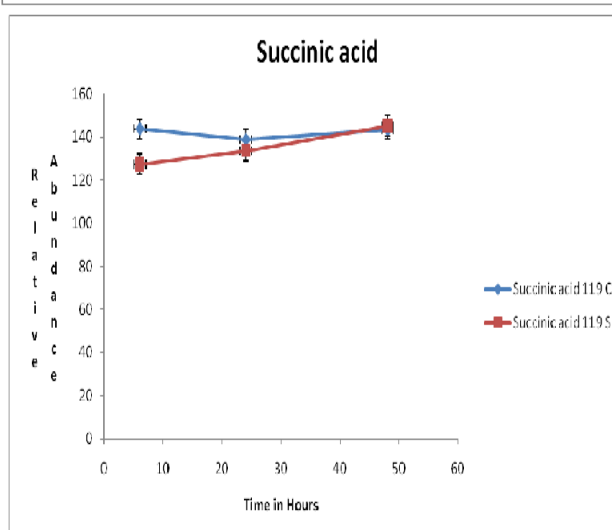
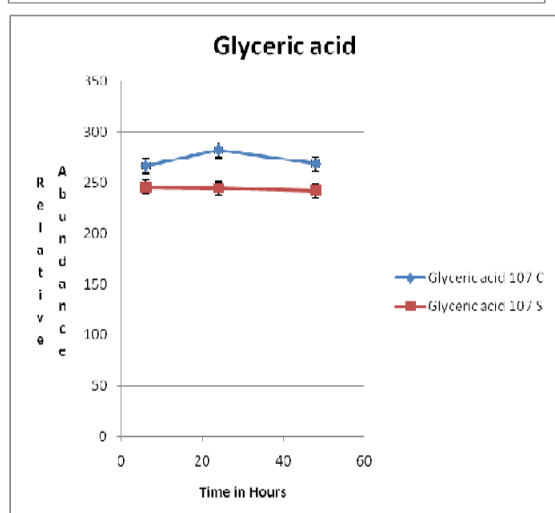
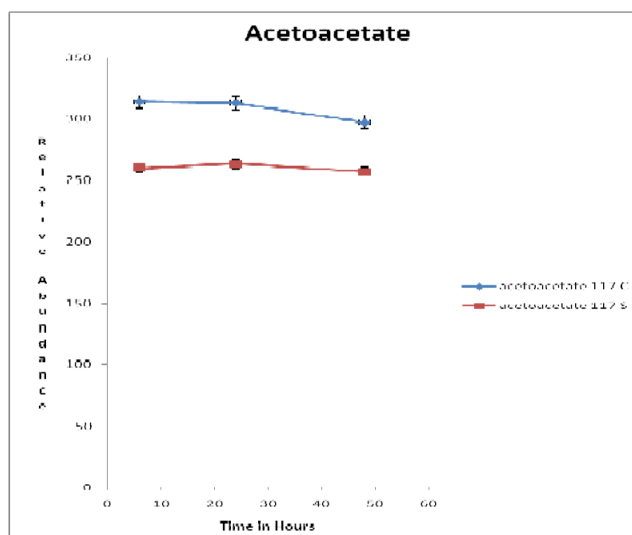
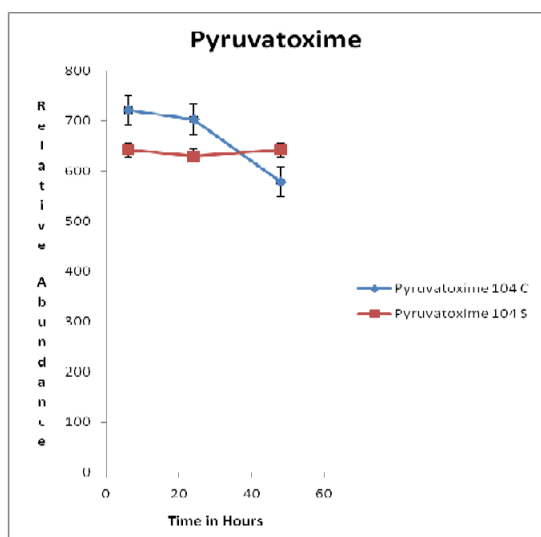
Appendix 2 Graphs showing Component Variations

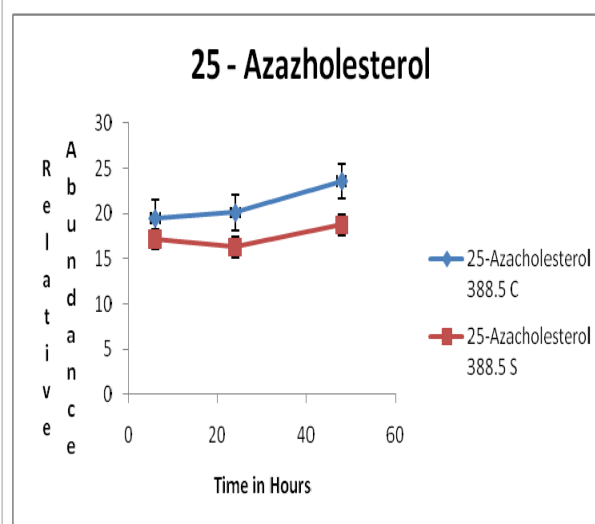
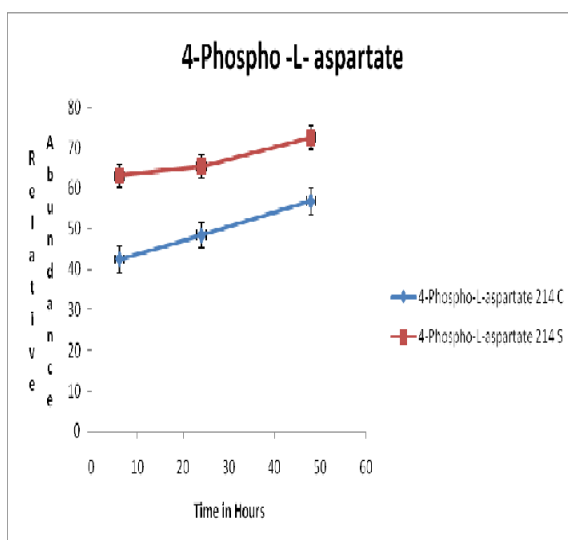
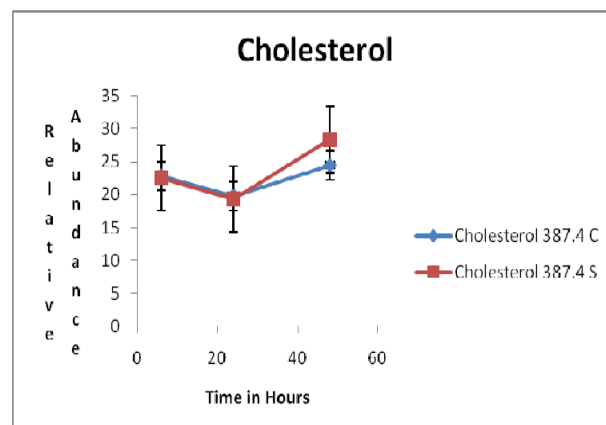
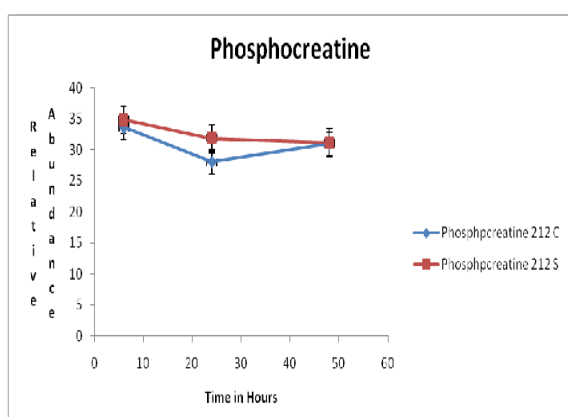
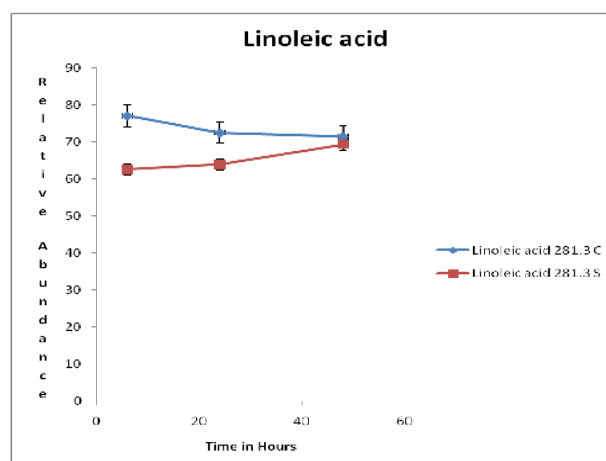
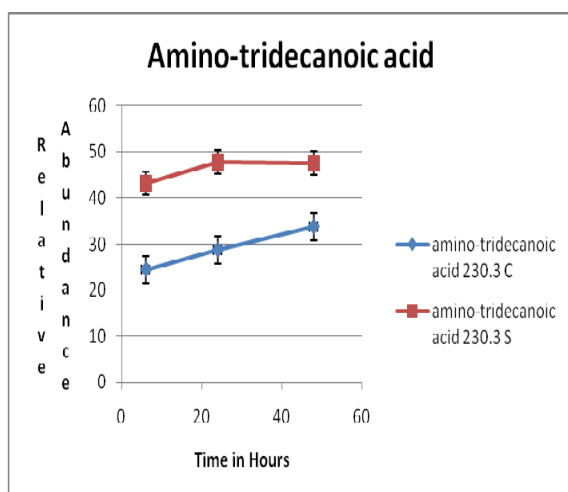


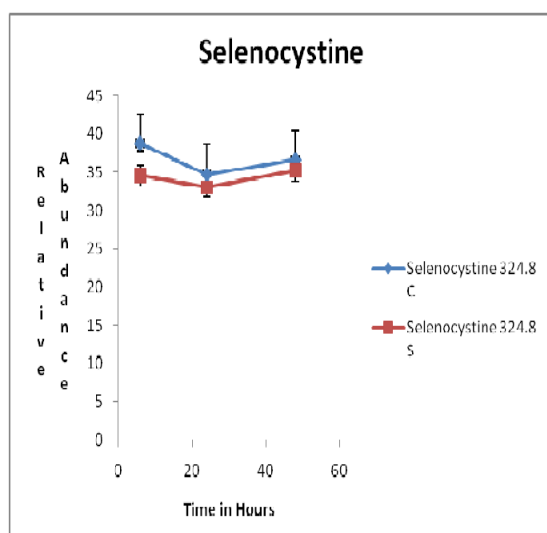
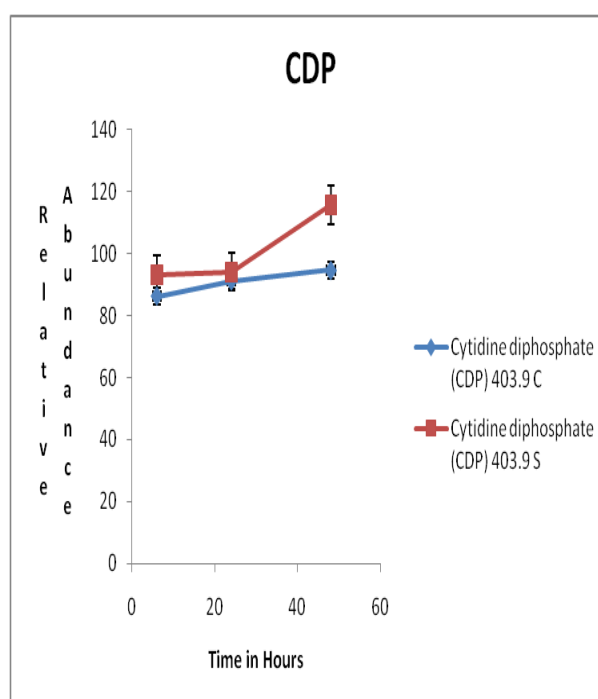
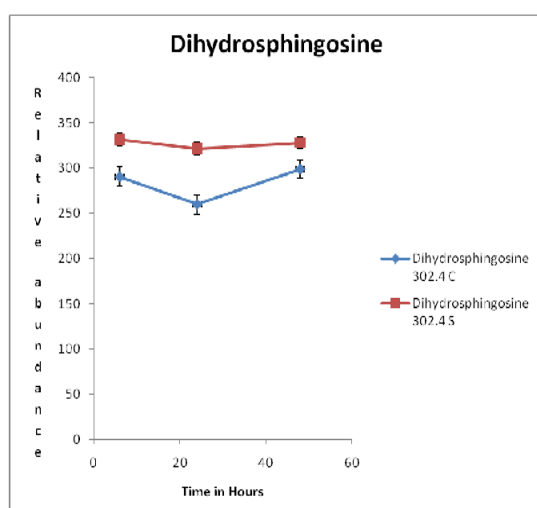
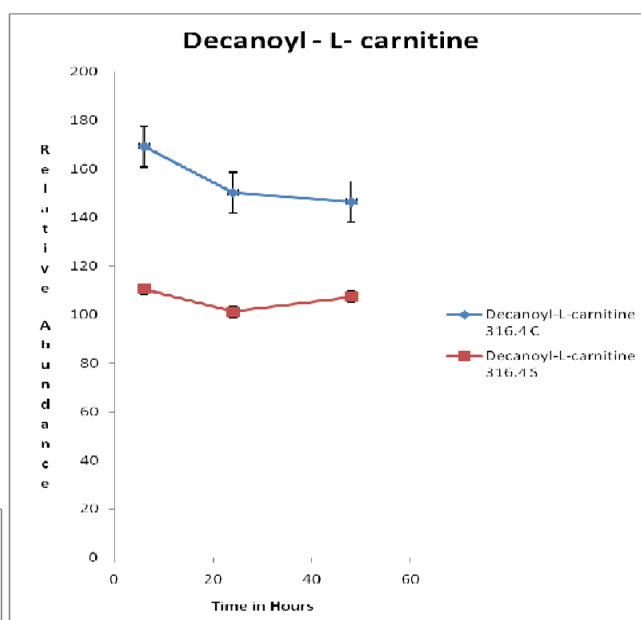
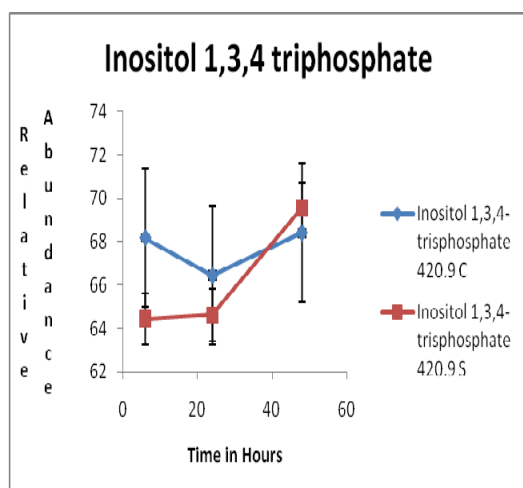


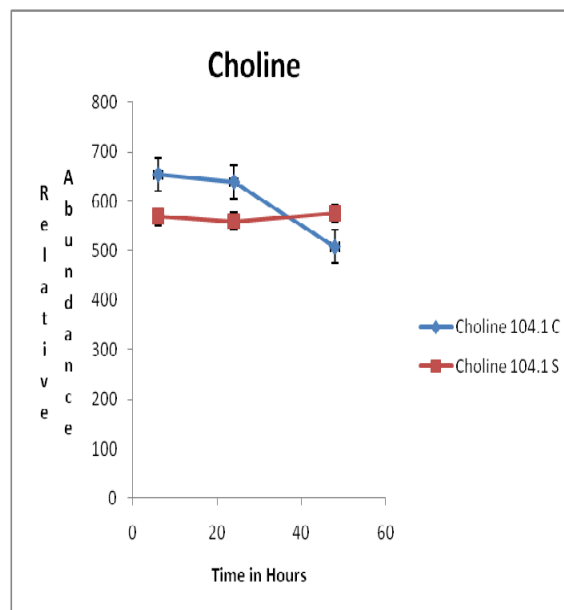
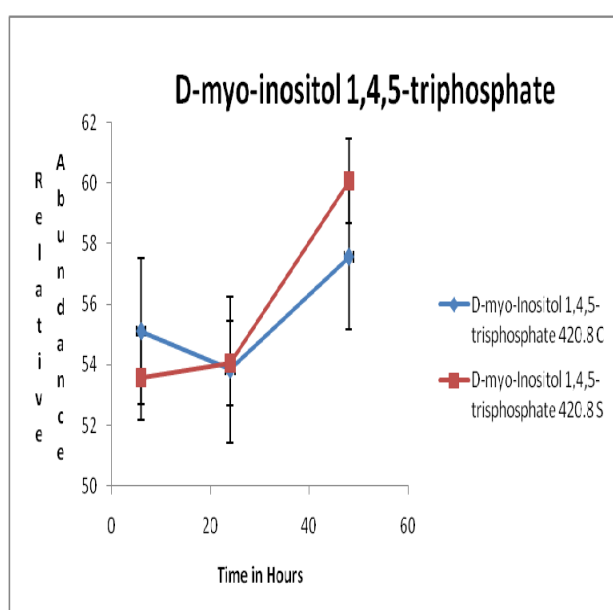
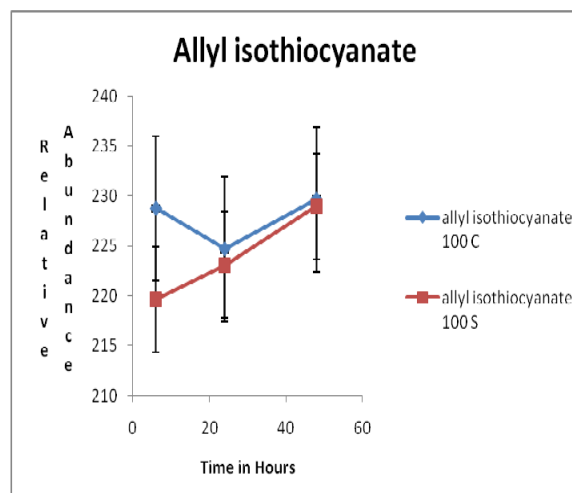
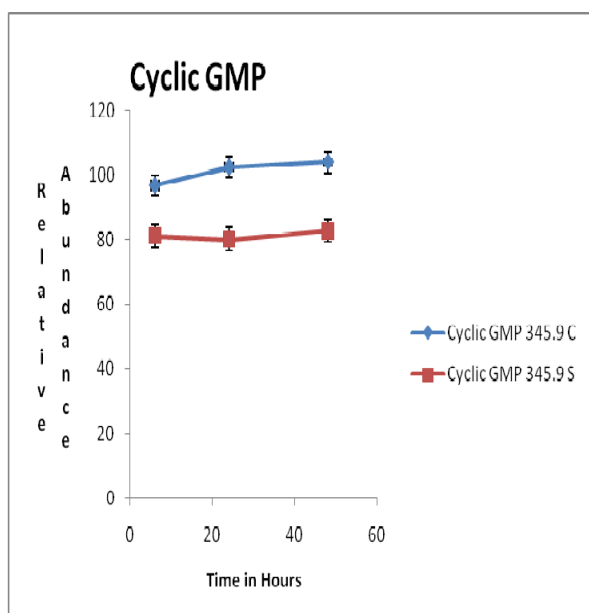


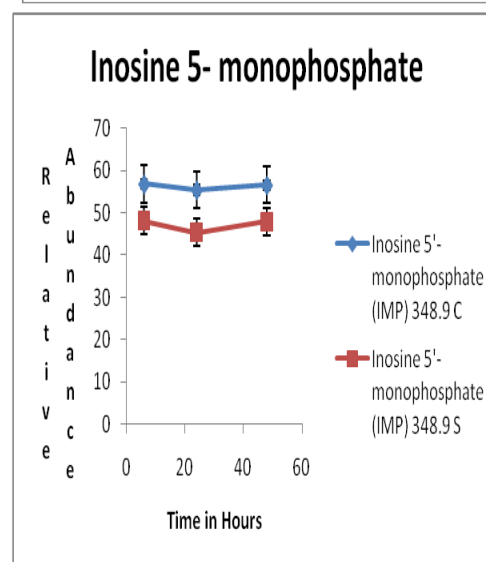
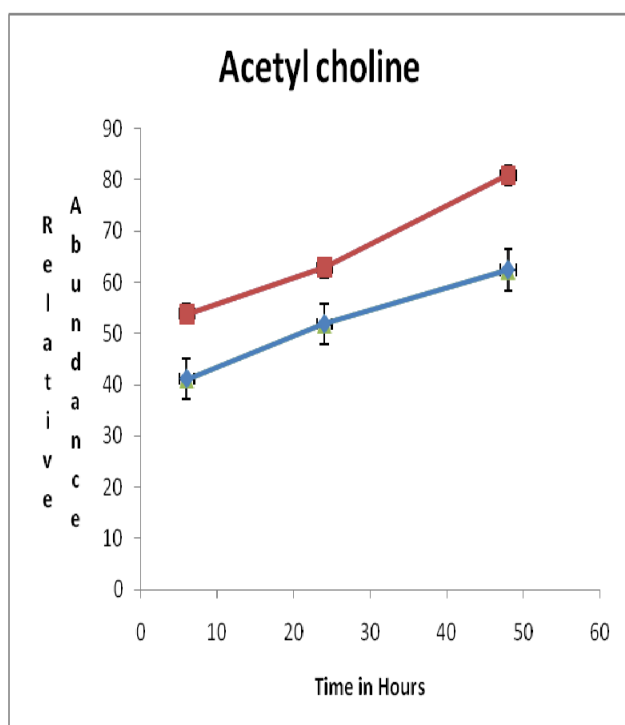
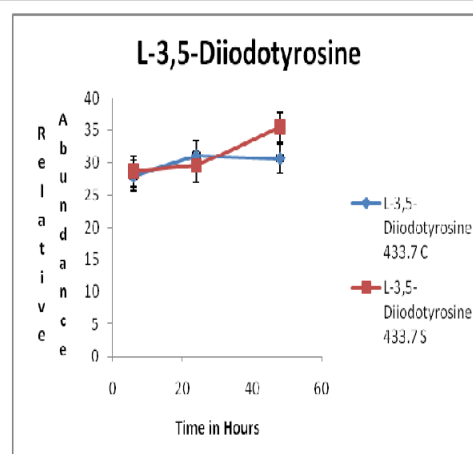
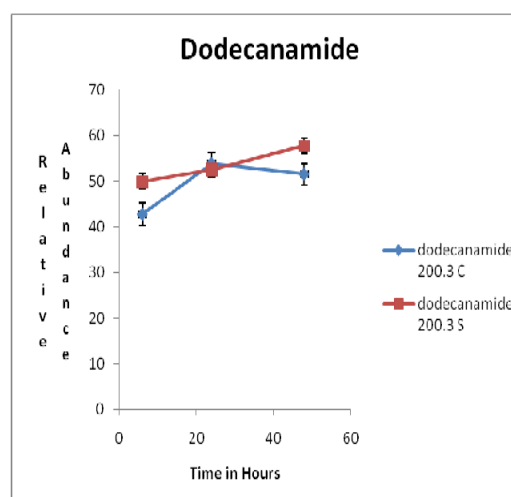
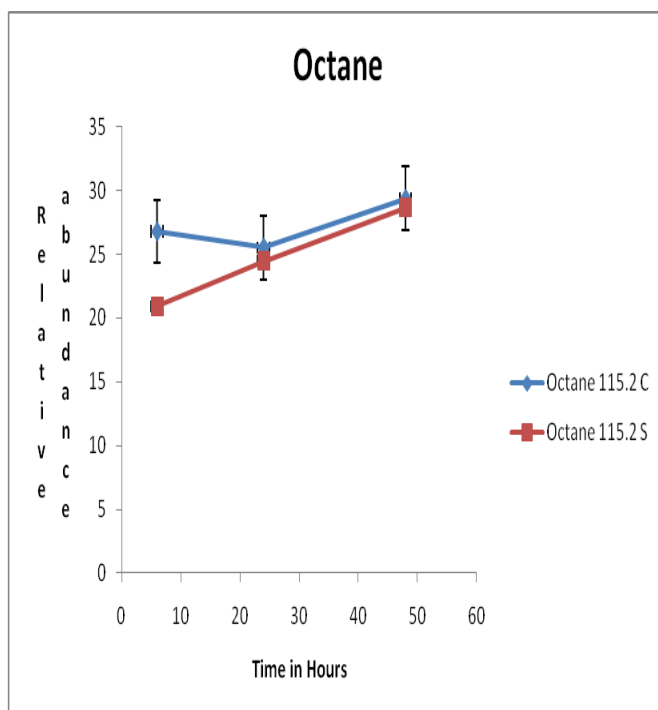












GLYCOLYSIS / GLUCONEOGENESIS

Starch and sucrose metabolism

α -D-Glucose-1P

α -D-Glucose

β -D-Glucose

D-Glucose (extracellular)

α -D-Glucose-6P

β -D-Glucose-6P

β -D-Fructose-6P

β -D-Fructose-1,6P₂

Glyceraldehyde-3P

Glycerone-P

Glycerate-1,3P₂

Glycerate-2,3P₂

Glycerate-3P

Glycerate-2P

Phosphoenolpyruvate

Pyruvate

L-Lactate

Ethanol

Acetaldehyde

Acetate

Acetyl-CoA

Oxaloacetate

Carbon fixation in photosynthetic organisms

Pentose phosphate pathway

Pyruvate metabolism

Propanoate metabolism

Enzymes (EC numbers):

- 2.7.1.41
- 3.1.3.10
- 5.4.2.2
- 2.7.1.69
- 2.7.1.1
- 2.7.1.2
- 2.7.1.63
- 5.1.3.3
- 5.1.3.15
- 5.3.1.9
- 3.1.3.11
- 2.7.1.11
- 4.1.2.13
- 5.3.1.1
- 1.2.1.12
- 1.2.1.59
- 5.4.2.4
- 1.2.7.5
- 1.2.7.6
- 1.2.1.9
- 2.7.2.3
- 3.1.3.13
- 5.4.2.1
- 4.2.1.11
- 2.7.1.40
- 1.1.1.27
- 1.1.1.1
- 1.1.1.2
- 1.1.99.8
- EutG
- 1.2.1.3
- 1.2.1.5
- 1.8.1.4
- 2.3.1.12
- 6.2.1.1
- 4.1.1.1
- 4.1.1.11
- 4.1.1.49
- 4.1.1.32
- 1.2.7.1

Metabolites:

- Arbutin (extracellular)
- Salicin (extracellular)
- Arbutin-6P
- Salicin-6P

00010 8/28/09
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Appendix 4 Amino acid Variation

Table 5. 1 Table showing Variation of Amino Acids (MS data) at 6 Hours

Amino acid	mZ	6H Neg AV	6H Mix 1 ng AV	6H mix 10 ngAV	6H Mix 100 ng AV
Alanine	90.1	11.702 ± 0.93	8.017 ± 0.83	7.445 ± 0.78	7.67 ± 0.88
Serine	106	190.723 ± 8.83	170.682 ± 4.46	165.89 ± 3.049	162.58 ± 2.19
Hypotaurine	110	573.572 ± 53.74	2248.767 ± 264.64	1699.33 ± 111.29	1419.843 ± 71.33
Creatinine	114.1	200.09 ± 1.99	173.05 ± 4.66	173.163 ± 5.41	167.993 ± 3.27
Proline	116.1	131.048 ± 6.82	117.987 ± 5.52	119.288 ± 4.7	113.073 ± 5.36
Valine	118.1	341.752 ± 7.68	360.738 ± 4.64	373.15 ± 6.93	366.763 ± 8.61
Threonine	120.1	337.587 ± 17.41	335.562 ± 31.68	332.273 ± 2.64	322.097 ± 20.14
Cystein	122	512.222 ± 13.69	517.545 ± 12.85	527.297 ± 15.1	503.203 ± 7.88
Taurine	126	32.938 ± 1.73	37.738 ± 1.08	36.287 ± 3.71	36.557 ± 1.36
Creatine	132	579.288 ± 19.97	455.512 ± 9.26	468.452 ± 10.76	444.682 ± 10.81
Isoleucine, leucine	132.1	509.215 ± 19.52	404.21 ± 12.05	416.815 ± 9.69	395.372 ± 10.44
Asparagine	133.1	124.375 ± 2.53	111.638 ± 1.49	115.645 ± 3.58	109.058 ± 3.91
Glutamine,	147.1	123.662 ± 6.85	141.773 ± 4.47	138.378 ± 3.93	140.63 ± 9.67
Glutamate	148.1	37.535 ± 2.97	43.187 ± 3.39	43.305 ± 2.69	42.423 ± 2.76
Methionine	150.1	368.505 ± 5.19	375.865 ± 9.18	384.943 ± 3.54	381.357 ± 4.42
Histidine	156.1	360.45 ± 10.97	228.633 ± 10.8	218.237 ± 5.71	206.52 ± 13.54
Phenylalanine	166.1	109.72 ± 5.79	126.628 ± 7.39	130.09 ± 3.98	125.807 ± 8.16
1-Methyl Histidine	170.1	120.792 ± 4.39	150.778 ± 2.96	155.775 ± 4.19	152.108 ± 4.17
Arginine	175.1	840.265 ± 54.71	952.49 ± 107.55	963.023 ± 13.1	837.93 ± 40.35
Acetyl aspartate	176.1	285.388 ± 14.89	286.463 ± 21.05	307.678 ± 7.92	279.763 ± 13.43
Tyrosine	182.1	59.675 ± 1.72	64.888 ± 3.09	64.452 ± 2.28	65.798 ± 3.74

Table 5. 2 Table showing Variations of Amino Acids (MS data) at 24 Hours

	m/Z	24 H Neg CtL AV	24 H Mix 1 ng AV	24 H Mix 10 ng AV	24 H Mix 100 ng AV
Alanine	90.1	9.978 ± 1.08	7.868 ± 1.11	7.703 ± 0.7	7.504 ± 0.68
Serine	106	173.382 ± 4.28	160.812 ± 2.74	165.073 ± 2.11	162.515 ± 4.11
Hypotaurine	110	477.123 ± 19.33	1233.268 ± 40.49	1119.655 ± 34.49	985.733 ± 33.2
Creatinine	114.1	205.818 ± 5.44	176.415 ± 2.78	174.907 ± 4.15	182.433 ± 11.8
Proline	116.1	128.375 ± 3.91	118.0717 ± 4.55	124.7 ± 4.61	120.538 ± 5.78
Valine	118.1	358.957 ± 10.7	372.35 ± 4.39	376.785 ± 4.6	386.372 ± 21.13
Threonine	120.1	335.09 ± 6.88	334.897 ± 12.64	347.007 ± 16.12	333.293 ± 33.71
Cystein	122	566.762 ± 10.49	545.212 ± 7.05	542.805 ± 5.06	550.707 ± 24.48
Taurine	126	34.087 ± 2.47	36.523 ± 1.85	38.588 ± 2.79	38.181 ± 2.04
Creatine	132	557.157 ± 8.92	453.775 ± 6.38	464.54 ± 9.97	461.021 ± 20.76
Isoleucine, leucine	132.1	486.562 ± 11.17	405.98 ± 4.43	416.348 ± 12.18	412.962 ± 20.94
Asparagine	133.1	126.17 ± 4.4	113.577 ± 6.32	118.773 ± 2.31	118.179 ± 5.8
Glutamine,	147.1	123.245 ± 1.79	146.142 ± 9.18	148.155 ± 4.93	154.307 ± 9.15
Glutamate	148.1	41.193 ± 2.21	46.047 ± 3.8	44.557 ± 2.42	45.465 ± 3.54
Methionine	150.1	371.497 ± 14.26	379.025 ± 5.19	381.702 ± 2.73	382.927 ± 5.11
Histidine	156.1	344.535 ± 12.74	204.832 ± 2.84	202.303 ± 6.48	205.128 ± 13.99
Phenylalanine	166.1	116.728 ± 3.84	130.492 ± 4.77	137.213 ± 5.97	136.199 ± 7.69
1-Methyl Histidine	170.1	133.225 ± 4.08	164.8 ± 4.37	167.342 ± 5.24	167.604 ± 9.97
Arginine	175.1	868.903 ± 27.89	877.563 ± 33.57	900.06 ± 25.93	813.123 ± 103.93
Acetyl aspartate	176.1	308.738 ± 5.028	287.115 ± 7.14	293.0517 ± 9.54	277.365 ± 17.48
Tyrosine	182.1	58.343 ± 4.06	65.71 ± 3.57	69.708 ± 3.79	69.132 ± 4.92

Table 5. 3 Table Showing Variation of Amino Acids (MS data) at 48 Hours

	m/Z	48 H Neg CtL AV	48 H Mix 1 ng AV	48 H Mix 10 ng AV	48 H Mix 100 ng AV
Alanine	90.1	10.788 ± 1.17	6.376 ± 1.34	6.71 ± 1.18	7.83 ± 1.68
Serine	106	170.352 ± 3.95	140.946 ± 7.02	140.042 ± 6.63	162.798 ± 8.53
Hypotaurine	110	425.943 ± 17.89	882.779 ± 24.79	872.985 ± 31.09	893.115 ± 38.2
Creatinine	114.1	224.858 ± 7.0	260.77 ± 10.64	253.067 ± 12.97	207.158 ± 7.92
Proline	116.1	127.977 ± 3.38	262.994 ± 5.58	263.085 ± 11.92	133.245 ± 35.67
Valine	118.1	377.282 ± 9.07	662.387 ± 15.23	665.223 ± 10.46	433.758 ± 66.6
Threonine	120.1	318.323 ± 18.17	953.723 ± 59.53	945.517 ± 85.44	399.836 ± 186.39
Cystein	122	601.423 ± 10.78	848.981 ± 41.17	831.108 ± 40.75	611.501 ± 79.64
Taurine	126	36.023 ± 3.13	54.973 ± 4.16	50.683 ± 4.95	39.218 ± 1.68
Creatine	132	521.477 ± 13.32	760.201 ± 30.24	755.062 ± 41.97	502.533 ± 76.85
Isoleucine, leucine	132.1	454.478 ± 10.72	749.089 ± 27.19	742.313 ± 41.56	459.006 ± 81.99
Asparagine	133.1	129.402 ± 2.78	197.316 ± 16.63	197.323 ± 14.19	134.343 ± 20.15
Glutamine,	147.1	112.622 ± 5.19	362.336 ± 18.54	363.753 ± 12.3	172.93 ± 42.29
Glutamate	148.1	43.303 ± 2.96	78.971 ± 7.73	76.555 ± 7.69	52.015 ± 9.98
Methionine	150.1	381.33 ± 11.2	345.517 ± 15.36	356.972 ± 9.89	392.513 ± 19.66
Histidine	156.1	317.888 ± 17.24	408.301 ± 14.75	416.823 ± 14.69	225.543 ± 45.99
Phenylalanine	166.1	120.095 ± 4.82	317.281 ± 23.13	330.702 ± 17.47	156.714 ± 54.51
1-Methyl Histidine	170.1	136.182 ± 5.43	382.441 ± 26.86	370.553 ± 25.64	188.864 ± 38.92
Arginine	175.1	693.678 ± 32.23	2392.936 ± 97.16	2402.477 ± 137.49	811.944 ± 542.71
Acetyl aspartate	176.1	249.303 ± 4.62	795.153 ± 38.83	819.173 ± 42.98	282.51 ± 172.05
Tyrosine	182.1	58.91 ± 2.52	129.5 ± 7.45	128.74 ± 6.35	73.402 ± 15.49

Appendix 5 Organic acids Table of Results

Table 5. 4 Relative Intensities of Organic acids (MS Data)

	m/Z	6H Neg	6H Mix 1 ng	6H mix 10 ng	6H Mix 100 ng	24 H Neg CtL	24 H Mix 1 ng	24 H Mix 10 ng	24 H Mix 100 ng	48 H Neg CtL	48 H Mix 1 ng	48 H Mix 10 ng	48 H Mix 100 ng
Glyoxylic acid	75	5.447 ± 0.83	3.855 ± 0.55	3.993 ± 0.64	4.327 ± 0.48	4.658 ± 0.60	4.303 ± 0.53	4.322 ± 0.90	4.18 ± 1.07	4.313 ± 0.55	3.030 ± 0.70	3.318 ± 0.64	3.789 ± 0.54
Glycolate	76	1.328 ± 0.40	1.048 ± 0.28	0.925 ± 0.31	0.982 ± 0.31	1.058 ± 0.31	0.953 ± 0.28	1.093 ± 0.16	0.971 ± 0.28	1.458 ± 0.19	1.307 ± 0.55	1.225 ± 0.33	0.964 ± 0.29
Lactate	90	16.11 ± 1.39	11.1367 ± 1.25	10.747 ± 1.52	10.188 ± 0.75	14.353 ± 1.15	10.613 ± 1.34	10.568 ± 0.87	10.298 ± 0.70	14.562 ± 1.56	8.189 ± 1.64	9.402 ± 1.20	10.648 ± 1.87
Lactic acid Glyceraldehyde	91	150.81 ± 6.20	88.595 ± 3.53	86.86 ± 3.52	82.397 ± 2.9	134.042 ± 5.25	80.057 ± 2.82	82.552 ± 1.72	77.789 ± 2.81	117.728 ± 5.81	66.961 ± 5.53	66.395 ± 4.13	77.017 ± 6.50
Malonate	103	52.08 ± 3.47	69.45 ± 3.43	71.11 ± 2.49	67.083 ± 2.68	54.725 ± 1.92	73.998 ± 3.25	78.043 ± 2.05	71.886 ± 5.16	55.770 ± 3.03	173.561 ± 10.93	175.118 ± 12.10	77.231 ± 20.86
Pyruvatoxime	104	721.692 ± 30.65	658.103 ± 15.62	689.762 ± 14.12	642.778 ± 13.55	703.438 ± 13.17	659.062 ± 15.49	667.607 ± 11.91	630.413 ± 24.49	579.120 ± 9.22	1009.327 ± 43.91	1000.790 ± 53.02	642.544 ± 120.59
malonate, malonic acid	105	139.398 ± 1.73	137.675 ± 2.81	137.775 ± 5.47	129.898 ± 6.45	139.218 ± 2.78	132.998 ± 4.56	135.297 ± 3.70	124.522 ± 4.36	122.022 ± 3.25	243.787 ± 7.52	236.595 ± 2.85	128.377 ± 26.83
Glyceric acid	107	266.525 ± 7.00	254.957 ± 6.44	248.077 ± 7.22	245.493 ± 7.01	281.988 ± 5.06	240.487 ± 4.48	247.107 ± 5.11	244.28 ± 8.63	268.587 ± 7.52	240.27 ± 11.42	238.237 ± 12.39	242.365 ± 3.37
1-Pyrroline-5-carboxylic acid	114	229.475 ± 4.35	202.055 ± 5.06	202.022 ± 6.40	196.498 ± 3.53	239.942 ± 4.06	206.528 ± 1.67	204.11 ± 4.18	213.537 ± 12.15	263.245 ± 9.18	293.344 ± 11.27	283.340 ± 14.57	242.290 ± 8.11
Acetoacetate	117	314.638 ± 5.82	276.193 ± 11.17	269.685 ± 8.11	259.973 ± 3.74	313.343 ± 4.15	259.602 ± 3.88	261.990 ± 5.60	263.487 ± 4.99	297.062 ± 8.11	223.88 ± 7.85	229.250 ± 4.45	257.373 ± 10.56
Succinic acid	119	143.758 ± 4.38	130.378 ± 4.52	131.472 ± 8.75	127.293 ± 4.73	138.765 ± 5.39	134.593 ± 10.69	134.662 ± 4.03	133.626 ± 10.15	143.312 ± 7.32	212.503 ± 15.08	205.190 ± 5.94	145.126 ± 19.49
β-hydroxybutyrate	119.1	121.647 ± 4.21	107.273 ± 3.87	109.803 ± 7.15	105.747 ± 2.37	117.448 ± 5.35	112.717 ± 9.79	112.765 ± 4.70	110.656 ± 6.96	120.307 ± 4.56	182.734 ± 9.02	178.810 ± 6.02	121.873 ± 15.03
Amino-tridecanoic acid	230.3	24.422 ± 3.02	39.777 ± 2.22	41.642 ± 1.84	43.188 ± 2.57	28.69 ± 3.00	44.503 ± 3.06	45.813 ± 3.52	47.736 ± 3.45	33.763 ± 1.63	62.367 ± 5.11	56.015 ± 8.61	47.591 ± 2.10

Appendix 6 Lipids Sample Results

Table 5. 5 Relative Intensities of Lipids and Phospholipids (MS Data) at 6 Hours

	m/Z	6H Neg AV	6H Mix 1 ng AV	6H mix 10 ngAV	6H Mix 100 ng AV
allyl isothiocyanate	100	228.743 ± 7.24	234.257 ± 4.40	222.187 ± 7.07	219.647 ± 5.35
Choline	104.1	653.193 ± 33.27	583.443 ± 14.52	616.752 ± 13.79	569.005 ± 18.00
Octane	115.2	26.785 ± 2.47	22.777 ± 1.59	22.412 ± 1.02	20.918 ± 0.38
Acetylcholine	146.1	41.150 ± 3.99	54.695 ± 3.48	56.027 ± 4.84	53.82 ± 1.96
dodecanamide	200.3	42.720 ± 2.44	50.768 ± 1.82	53.445 ± 2.44	49.827 ± 1.71
Phosphpcreatine	212	33.630 ± 1.94	36.743 ± 2.39	35.472 ± 1.79	34.835 ± 2.25
4-Phospho-L-aspartate	214	42.502 ± 3.17	63.075 ± 3.39	65.032 ± 4.06	63.133 ± 2.85
amino-tridecanoic acid	230.3	24.427 ± 3.03	39.777 ± 2.23	41.642 ± 1.84	43.188 ± 2.57
Linoleic acid	281.3	77.063 ± 2.92	67.548 ± 4.58	64.910 ± 1.49	62.620 ± 1.51
Dihydrosphingosine	302.4	290.238 ± 10.37	338.192 ± 9.18	331.063 ± 9.22	331.358 ± 6.90
Decanoyl-L-carnitine	316.4	169.287 ± 8.45	117.547 ± 3.47	112.620 ± 4.48	110.545 ± 2.30
Selenocystine	324.8	38.812 ± 3.85	36.823 ± 1.52	36.238 ± 1.83	34.602 ± 1.36
Cyclic GMP	345.9	96.888 ± 3.26	84.328 ± 7.045	82.788 ± 4.66	81.148 ± 3.58
Inosine 5'-monophosphate (IMP)	348.9	56.803 ± 4.36	49.840 ± 3.32	50.073 ± 3.27	48.133 ± 3.282
Inosine 5'-monophosphate (IMP)	349	63.258 ± 4.45	54.682 ± 3.65	53.702 ± 3.59	53.08 ± 3.53
Cholesterol	387.4	22.822 ± 2.22	24.092 ± 2.95	22.077 ± 3.05	22.547 ± 5.04
25-Azacholesterol	388.5	19.488 ± 1.98	18.407 ± 1.79	18.117 ± 3.05	17.152 ± 1.16
Cytidine diphosphate (CDP)	403.9	86.193 ± 2.65	94.715 ± 4.43	94.957 ± 3.42	93.123 ± 6.15
D-myo-Inositol 1,4,5-trisphosphate	420.8	55.102 ± 2.42	52.897 ± 3.14	54.430 ± 3.49	53.57 ± 1.39
Inositol 1,3,4-trisphosphate	420.9	68.173 ± 3.195	65.177 ± 3.48	64.092 ± 3.65	64.433 ± 1.20
L-3,5-Diiodotyrosine	433.7	28.00 ± 2.34	28.943 ± 2.29	32.345 ± 2.75	28.69 ± 2.40
L-3,5-Diiodotyrosine	433.8	37.220 ± 3.61	38.195 ± 2.59	42.407 ± 3.98	38.135 ± 2.58
L-3,5-Diiodotyrosine	433.9	46.107 ± 4.19	46.353 ± 3.43	51.163 ± 4.72	46.055 ± 3.53

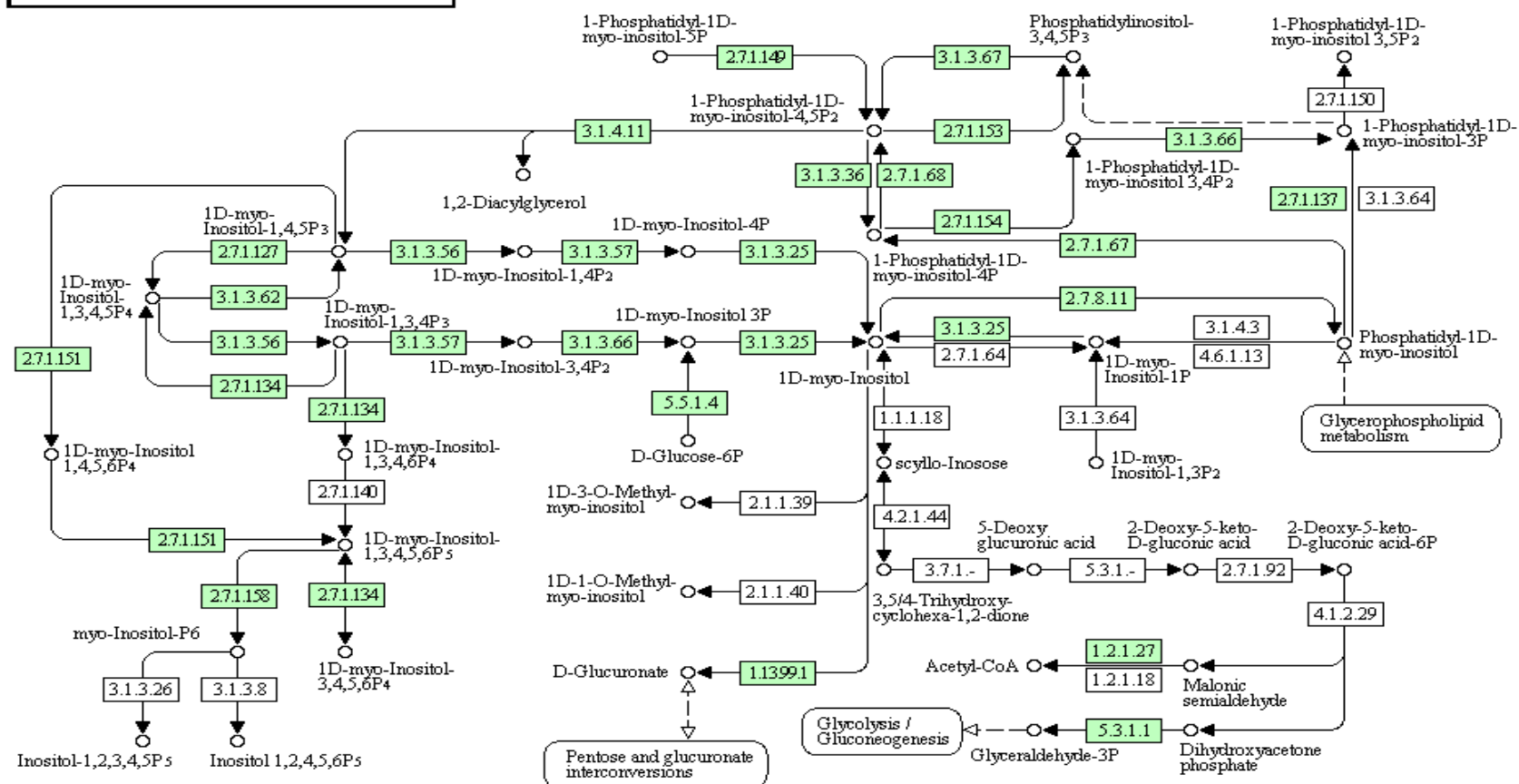
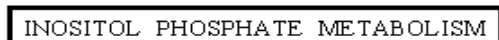
Table 5. 6 Relative Intensities of Lipids and Phospholipids at 24 Hours

	m/Z	24 H Neg CtL	24 H Mix 1 ng	24 H Mix 10 ng	24 H Mix 100 ng
allyl isothiocyanate	100	224.695 ± 5.33	221.632 ± 5.60	225.515 ± 4.60	223.078 ± 6.58
Choline	104.1	638.362 ± 12.66	585.78 ± 15.47	596.487 ± 14.33	559.412 ± 26.09
Octane	115.2	25.538 ± 1.21	22.688 ± 2.71	24.713 ± 1.66	24.464 ± 1.58
Acetylcholine	146.1	51.862 ± 4.42	58.117 ± 4.88	62.907 ± 2.20	62.856 ± 6.94
dodecanamide	200.3	53.85 ± 4.35	52.047 ± 3.41	52.163 ± 2.79	52.564 ± 4.23
Phosphocreatine	212	28.042 ± 2.06	33.973 ± 1.91	33.415 ± 2.27	31.868 ± 2.22
4-Phospho-L-aspartate	214	48.422 ± 3.38	65.098 ± 2.70	68.572 ± 3.23	65.371 ± 4.32
amino-tridecanoic acid	230.3	28.690 ± 3.01	44.503 ± 3.06	45.813 ± 3.52	47.736 ± 3.45
Linoleic acid	281.3	72.492 ± 3.80	66.22 ± 3.40	66.767 ± 5.33	63.916 ± 3.46
Dihydrosphingosine	302.4	259.913 ± 5.75	330.267 ± 4.70	327.905 ± 7.09	321.397 ± 8.25
Decanoyl-L-carnitine	316.4	150.197 ± 5.80	107.368 ± 3.78	109.247 ± 5.87	101.296 ± 9.25
Selenocystine	324.8	34.775 ± 2.02	34.918 ± 2.18	34.438 ± 1.23	33.108 ± 1.58
Cyclic GMP	345.9	102.450 ± 3.60	80.812 ± 4.50	81.163 ± 3.39	80.162 ± 5.81
Inosine 5'-monophosphate (IMP)	348.9	55.363 ± 2.19	49.01 ± 4.34	46.927 ± 1.52	45.336 ± 3.88
Inosine 5'-monophosphate (IMP)	349	58.727 ± 2.25	54.367 ± 5.53	51.565 ± 2.27	49.067 ± 4.42
Cholesterol	387.4	19.792 ± 2.67	23.137 ± 4.21	20.548 ± 2.59	19.268 ± 5.31
25-Azacholesterol	388.5	20.083 ± 1.02	17.173 ± 0.92	18.32 ± 1.73	16.288 ± 1.23
Cytidine diphosphate (CDP)	403.9	91.138 ± 4.89	89.612 ± 2.01	92.525 ± 4.43	94.0792 ± 8.09
D-myo-Inositol 1,4,5- trisphosphate	420.8	53.832 ± 3.34	54.09 ± 2.94	54.427 ± 4.43	54.053 ± 2.86
Inositol 1,3,4-trisphosphate	420.9	66.457 ± 3.90	64.387 ± 4.27	64.228 ± 4.59	64.623 ± 3.12
L-3,5-Diiodotyrosine	433.7	31.092 ± 2.23	28.662 ± 3.20	30.795 ± 2.26	29.502 ± 4.17
L-3,5-Diiodotyrosine	433.8	42.188 ± 2.01	38.583 ± 4.24	41.675 ± 3.58	39.607 ± 5.52
L-3,5-Diiodotyrosine	433.9	52.500 ± 2.78	48.077 ± 3.82	50.967 ± 4.35	48.39 ± 6.24

Table 5. 7 Relative Intensities of Lipids and Phospholipids at 48 Hours

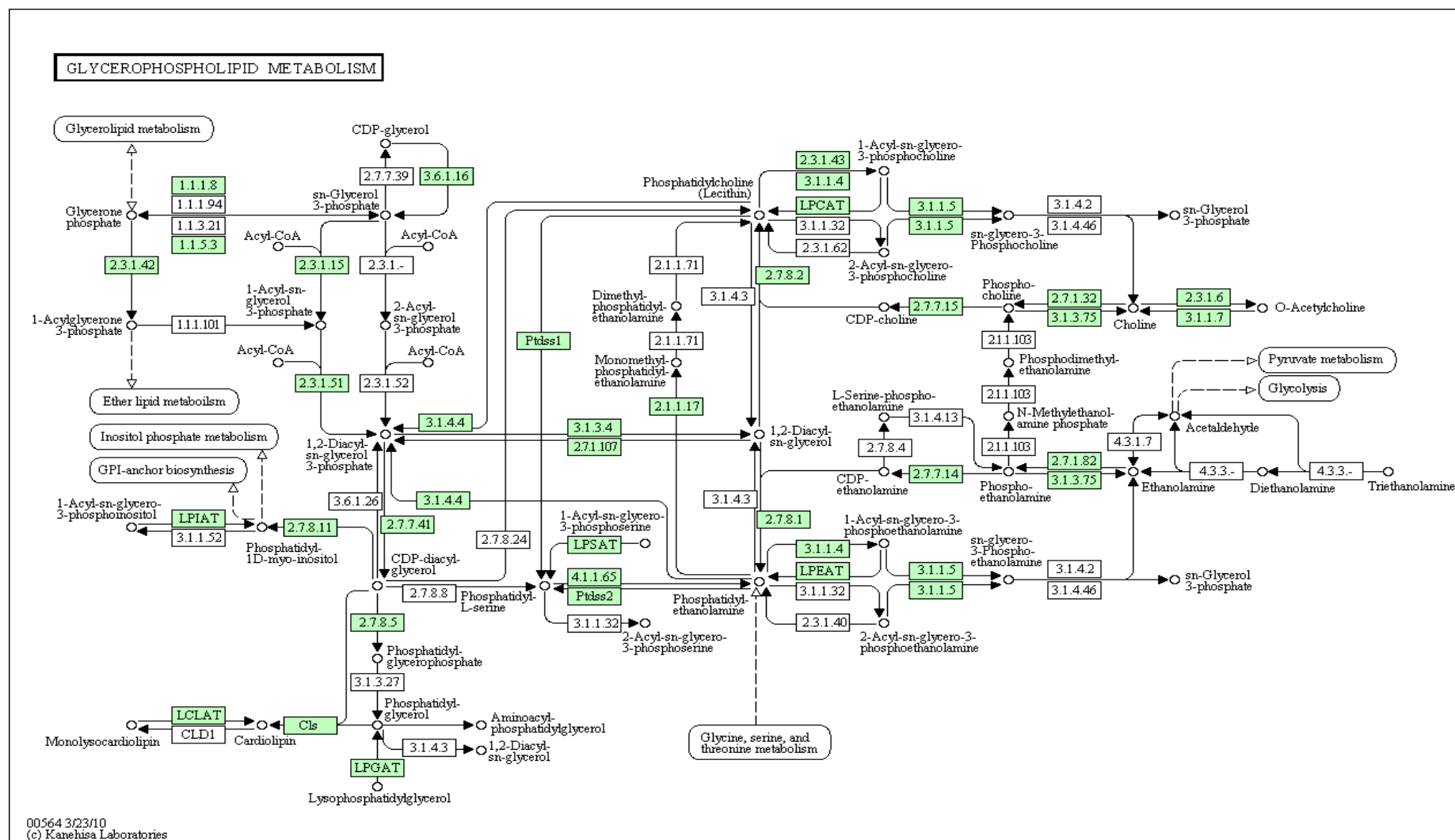
	m/Z	48 H Neg CtL	48 H Mix 1 ng	48 H Mix 10 ng	48 H Mix 100 ng
allyl isothiocyanate	100	229.648 ± 4.70	232.811 ± 7.43	234.042 ± 6.04	228.961 ± 6.83
Choline	104.1	507.815 ± 11.46	977.763 ± 46.16	968.800 ± 43.41	575.128 ± 132.63
Octane	115.2	29.357 ± 3.34	48.76 ± 3.537	47.510 ± 7.50	28.672 ± 3.39
Acetylcholine	146.1	62.405 ± 3.23	148.224 ± 5.58	143.003 ± 9.23	80.924 ± 8.07
dodecanamide	200.3	51.502 ± 2.69	121.29 ± 9.40	117.515 ± 9.32	57.703 ± 9.76
Phosphpcreatine	212	30.987 ± 2.15	43.517 ± 3.67	44.850 ± 3.90	31.119 ± 2.37
4-Phospho-L-aspartate	214	56.805 ± 3.33	114.999 ± 5.45	116.785 ± 5.51	72.518 ± 9.52
amino-tridecanoic acid	230.3	33.7633 ± 1.63	62.367 ± 5.11	56.015 ± 8.61	47.591 ± 2.10
Linoleic acid	281.3	71.468 ± 2.96	96.019 ± 10.21	92.142 ± 8.12	69.368 ± 6.28
Dihydrosphingosine	302.4	298.712 ± 14.09	410.924 ± 29.90	402.317 ± 38.81	327.847 ± 21.89
Decanoyl-L-carnitine	316.4	146.368 ± 8.56	151.757 ± 31.22	137.762 ± 21.70	107.352 ± 10.59
Selenocystine	324.8	36.692 ± 1.62	43.34 ± 2.64	40.768 ± 2.87	35.255 ± 3.59
Cyclic GMP	345.9	103.975 ± 4.09	68.263 ± 5.94	68.08 ± 4.88	82.737 ± 6.32
Inosine 5'-monophosphate (IMP)	348.9	56.518 ± 1.84	77.096 ± 7.05	71.365 ± 8.92	47.893 ± 9.60
Inosine 5'-monophosphate (IMP)	349	60.837 ± 1.90	85.646 ± 8.88	78.823 ± 8.96	51.914 ± 11.11
Cholesterol	387.4	24.455 ± 1.92	63.076 ± 15.85	52.265 ± 10.75	28.300 ± 18.85
25-Azacholesterol	388.5	23.555 ± 1.92	21.177 ± 2.96	23.217 ± 3.18	18.723 ± 2.40
Cytidine diphosphate (CDP)	403.9	94.615 ± 1.67	274.567 ± 6.73	279.537 ± 11.23	115.707 ± 55.87
D-myo-Inositol 1,4,5-trisphosphate	420.8	57.560 ± 3.49	85.593 ± 3.72	84.445 ± 5.27	60.073 ± 15.61
Inositol 1,3,4-trisphosphate	420.9	68.400 ± 3.11	96.52 ± 5.83	96.755 ± 5.72	69.553 ± 15.12
L-3,5-Diiodotyrosine	433.7	30.660 ± 1.70	66.724 ± 5.49	67.095 ± 6.05	35.465 ± 12.47
L-3,5-Diiodotyrosine	433.8	40.972 ± 2.40	94.917 ± 7.53	93.142 ± 6.18	48.768 ± 17.90
L-3,5-Diiodotyrosine	433.9	50.295 ± 2.73	115.334 ± 7.61	112.457 ± 7.56	59.805 ± 22.69

Appendix 7 Inositol Phosphate Metabolism



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Appendix 8 Glycerophospholipid Metabolism



Appendix 9 Pyrimidine Metabolism

