Metabolomics (2011) 7:485–499
DOI 10.1007/s11306-010-0265-0

ORIGINAL ARTICLE

Investigation of the effect of exposure to non cytotoxic amounts of microcystins

Grace Birungi · Sam Fong Yau Li

Received: 12 July 2010 / Accepted: 19 November 2010 / Published online: 12 December 2010

Abstract This paper describes a metabolomic approach for investigation of the potential effect of exposure of humans to low amounts of microcystins using HepG2 cell line. Microcystins are hepatotoxins produced by cyanobacteria (blue-green algae) which occur in water bodies with high eutrophication especially those with a slow flow rate or those that are stagnant in warm climates. Mammalian exposure to these compounds has been associated with deleterious effects and in high dosage cases, deaths of animals has been reported. The metabolic profile of HepG2 cells is closely related to that of hepatocytes and therefore serves as a good model due to their human origin. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and direct injection mass spectrometry (DIMS) were used to analyse media extracts from the cells and data obtained was reduced by chemometric methods. The use of principal component analysis (PCA) enabled achievement of a visual distinction between the metabolic profiles of samples exposed to microcystins, control samples (unexposed), and those which were exposed to acetaminophen (positive control). A profile of media components showed that some components in the samples exposed to microcystins increased compared to those in control samples. They included amino acids, organic acids, lipids, some purines and pyrimidines. In general exposure to low concentration of microcystin was found to interfere with amino acid metabolism, carbohydrate metabolism, lipid metabolism and nucleic acids metabolism. Furthermore, low concentration of microcystins did not result in significant cell death; rather the cells continued to proliferate.

G. Birungi · S. F. Y. Li (✉)
Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore
e-mail: chmlifys@nus.edu.sg

Keywords Metabolomics · Chronic exposure to microcystins · HepG2 · ^{1}H NMR · Direct injection MS (DIMS)

1 Introduction

Microcystins are cyclic hepatotoxic compounds produced by blue green algae (also known as cyanobacteria). They inhibit serine/threonine phosphatases 1 and 2A (Towner et al. 2002a), which leads to protein phosphorylation and cause cytoskeletal damage, necrosis and haemorrhage in the liver (Towner et al. 2002a; Bouaicha and Maatouk 2004; Dawson 1998; Toivola and Eriksson 1999).

Microcystins were reported to be responsible for fatalities of animals and humans (Azevedo et al. 2001; Jochimsen et al. 1998). Epidemiological studies have suggested that microcystins are one of the risk factors for the high incidence of primary liver cancer in certain areas where water supplies had a common occurrence of blue green algae (Zegura et al. 2003) and more recently microcystins were detected in sera of a chronically exposed human population (Chen et al. 2009) which demonstrates a health risk of chronic exposure.

Furthermore, microcystin LR has been implicated in causing DNA strand breaks (Nong et al. 2007; Zegura et al. 2003; Zegura et al. 2006; Zegura et al. 2008), it has been reported to induce oxidative DNA damage in human hepatoma cell line HepG2; microcystin-LR in low concentration (nM) caused a collapse of actin filaments in human primary hepatocytes (Batista et al. 2003) and microcystin LR and nodularin were reported to induce time dependent intracellular glutathione alteration (Zegura et al. 2006; Bouaicha and Maatouk 2004), production of reactive

oxygen species and to cause lipid peroxidation in rats (Bouaicha and Maatouk 2004; Maatouk et al. 2004) which are characteristic of oxidative stress. Oxidative lipid metabolism following acute hepatotoxicity (Towner et al. 2002a, b), induction of apoptosis and nephrotoxicity (Milutinovic et al. 2003) have also been reported. It is therefore evident that exposure to a high concentration of microcystins is hazardous.

In the case of humans, acute exposure is not common because compared to other animals 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 sea food or by exposure through recreational activities or even work related exposure. Therefore, since the exposure is not so obvious, there is a risk of continued exposure. While the amount of toxin may be low, continued chronic exposure may be hazardous. In this research, we studied the effect of exposure to lower concentrations of microcystins in order to understand the effect of chronic exposure to microcystins on humans.

Awareness of the toxic effects of microcystins led to the establishment of a drinking water guideline of 1 μg/l (World Health Organization 2006). Most of the investigations of microcystin toxicity in literature were obtained at higher doses and studies of effects at a low dosage exposure over a long term (chronic toxicity) have mainly focused on gene expression and DNA damage using whole animals or rat cells, for example it was reported that microcystin LR in low amounts (2–20 μg/l) altered the profile of proteins in zebra fish affecting proteins involved in cytoskeleton assembly, macromolecule metabolism, oxidative stress and signal transduction (Wang et al. 2010). In our study we investigated whether exposure to low concentrations of microcystins would be as hazardous and if it may eventually lead to liver injury which would be a health risk. We used a metabolomic approach to investigate the effects of exposure to microcystins LR, RR and YR (Fig. 1) at concentrations that do not cause cytotoxicity, on the metabolome of HepG2 cells.

The HepG2 cell line was used because microcystins are liver specific toxins. They are relatively large molecules ~1000 Da and have a polar moiety from the carboxyl and amido groups even though they exhibit a partial hydrophobic character due to the Adda moiety (Rivasseau et al. 1998). Their transport into the liver is facilitated by the bile acid transport system. Hepatocytes are rich in organic anion transport peptides which are responsible for active uptake of microcystins from blood (Paskova et al. 2008). Furthermore, the liver is a target to chemical or drug induced toxicity due to its involvement in active metabolism. The HepG2 cell line therefore provides a good model for this investigation due to their human origin and because

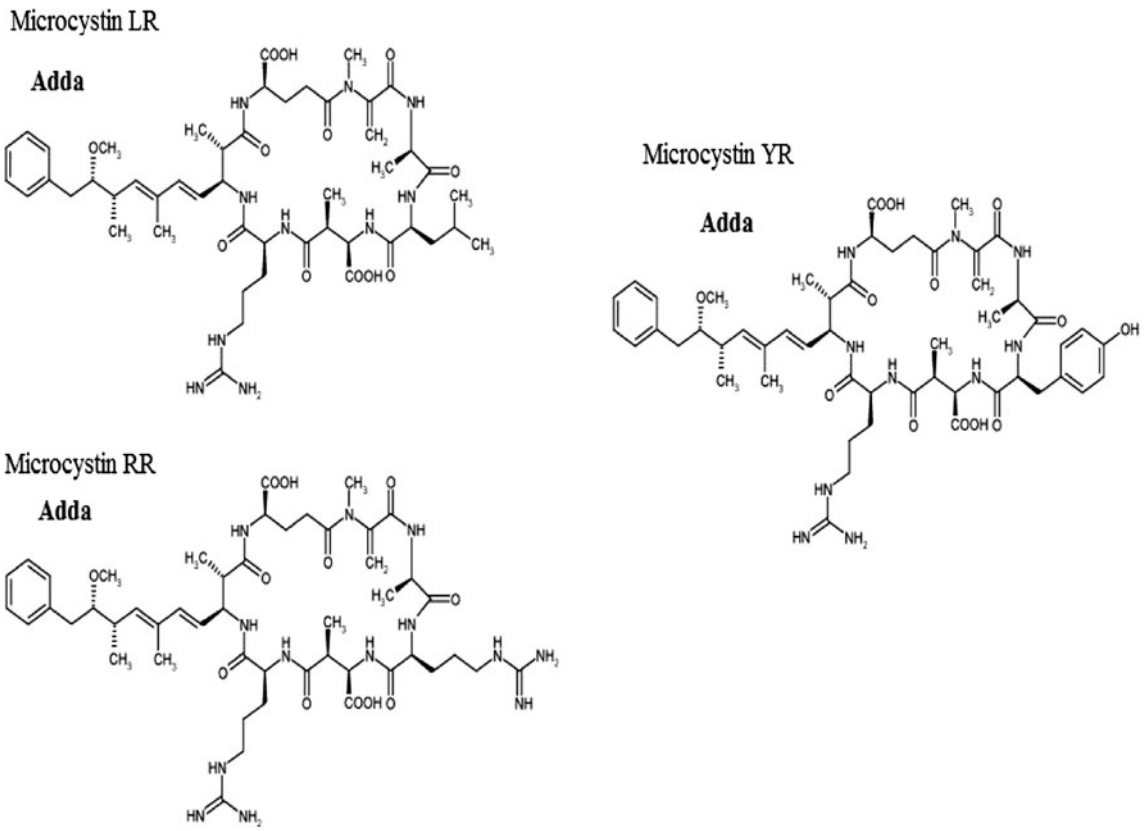

Fig. 1 Structures of microcystins used in the study

they retain many of the specialized liver functions and metabolizing enzyme activities present in human hepatocytes (Miccheli et al. 2006).

Bio-analytical 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). These techniques are powerful and generate a wealth of information about the different responses of a system to external factors but they are expensive, labour intensive (Nicholson et al. 1999) and they do not take into consideration the dynamic metabolic status of the whole organism. In our study the metabolomic approach was used because metabolites have a direct effect on the phenotype and by profiling all metabolites, it is possible to obtain unbiased marker(s) of toxicity compared to conventional approaches of investigation of toxicity such as analysis of enzyme activity (Wang et al. 2010). Metabolomics is generally aimed at generating an endogenous metabolite profile, a unique finger print of the cellular processes that occur in a biological system (Nicholson et al. 1999). It provides many opportunities to investigate and understand changes caused by external stimuli (Clarke and Haselden 2008).

Techniques mainly used in metabolomics include: proton nuclear magnetic resonance spectroscopy (^{1}H 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 (Lindon and Nicholson 2008; Tomita et al. 2005).

In our study we used ^{1}H NMR and DIMS because ^{1}H NMR requires minimal sample preparation, has a wide application (i.e. can be used as long as the compound has a proton) with high analytical precision in a short time, and has a quantification ability because peak area/intensity is directly proportional to concentration and it is non destructive of the sample (Lindon and Nicholson 2008; Goodacre and Harrigan 2003). DIMS on the other hand provides information of the specific molecular masses and mass to charge ratios of the compounds. It is a high-throughput technique and takes a short overall analysis time per sample. The short analysis time increases inter-sample reproducibility and improves the accuracy in subsequent cluster analysis (Dettmer et al. 2007).

2 Materials and methods

2.1 Materials

Microcystins LR, RR and YR were obtained from Alexis Biochemicals (Switzerland), HepG2 Cell line was kindly provided by Professor Hanry Yu of department of physiology NUS. Dulbecco's modified eagles medium (DMEM) was from Invitrogen (Singapore). Foetal 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). $CDCl_3$ was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), D_2O with 0.05% (wt) 3-(trimethylsilylpropionic-2,2,3,3,-d_4 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).

2.2 Instrumentation

HepG2 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 ^{1}H NMR experiments while an Agilent 1200 series system equipped with an Agilent 6410 Triple Quad mass spectrometer was used in direct injection mass spectrometry.

2.3 Cell culture

HepG2 Cells were cultured in 25 cm^3 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% CO_2. Whenever cells reached ~90% 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 1 min to allow detachment. After all cells detached, they were reconstituted with medium and split into the required portions for the next seeding stage.

2.4 Assessment of cell viability

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 h to determine the lethal concentration for 50% (LC_{50}) of the cells. After 24 h, 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 resuspended 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 analyzed using flow cytometry.

2.5 Sample preparation

Media (1 ml) collected from the cell wells after 6, 24 and 48 h were extracted using the Bligh-Dyer extraction technique as modified by Miccheli et al. (2006). 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 min at 4°C. The extract was separated into the aqueous and organic portions which were freeze dried to remove excess solvent. The dry extracts from the media were kept in the freezer at −20°C until analysis and were analysed separately.

2.6 ^{1}H NMR analysis

The aqueous portion was reconstituted with deuterated water (D_2O buffered with sodium phosphate at pH 7), while the organic portion was reconstituted with deuterated chloroform (0.7 ml each), before analysis by ^{1}H NMR. Samples were analyzed on a Bruker Ultrashield spectrometer to obtain one-dimensional ^{1}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 s 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 ^{1}H NMR spectra were manually processed using TOPSPIN software (Bruker Biospin). Peak areas were used for quantification and they were identified based on literature (Nicholson et al. 1995; Miccheli et al. 2006; Foxall et al. 1993). Results were analysed by Principal component analysis (PCA) using SIMCA P^{+} software.

2.7 Analysis by direct injection mass spectrometry (DIMS)

DIMS analysis of all samples were performed on Agilent 1200 series system composed 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 min.

2.8 Data analysis

^{1}H 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 area data was consolidated in Excel. MS data acquired using Agilent Mass Hunter Qualitative Analysis software was extracted into mass spectra in the scan range of mass-to-charge ratios from 70 to 1000 and the relative intensities of the peaks were also consolidated in Excel. These data were analyzed by Principal component analysis (PCA) using SIMCA P_{+} software (Umetrics, Umea, Sweden). Component 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 be of statistical significance.

3 Results and discussion

3.1 Assessment of cell viability

At lower concentrations of microcystins exposed cells did not exhibit structural differences from those that were not exposed to microcystins when observed under a light microscope. Propidium iodide (PI) and thiazole orange (TO) were used to assess the cytotoxic concentration of microcystins. Cells incubated for 24 h 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. From these results, it was obvious that the rate of cell membrane damage increased with increasing concentration of the toxin. The percentage of viable cells was plotted against concentration of the toxins as shown in Fig. 2 below.

For microcystin concentrations in the range 1–100 ng/ml, cell death was less than 50% even though the number of cell death increased with microcystin concentration and there were no significant physical differences in the cell structure as observed under an inverted light microscope. This is in agreement with literature reports (Zegura et al. 2003; van Apeldoorn et al. 2007), and this concentration range was used in the investigation of sub lethal toxicity in this study. Microcystins were spiked into the media into which cells were cultured at concentration range 1–100 ng/ml and aliquots of 1 ml of media were taken out after 6, 24 and 48 h

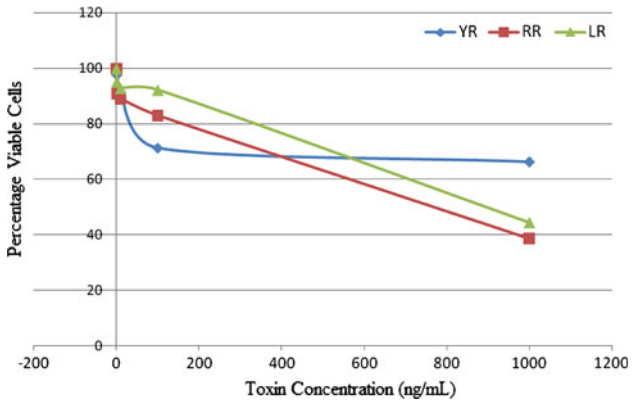

Fig. 2 Variation of percentage of viable cells with concentration of microcystin in ng/ml

and were later extracted and analyzed by ^{1}H NMR and DIMS.

3.2 ^{1}H NMR and DIMS results

There were obvious differences in the spectra obtained for aqueous samples and organic samples in both ^{1}H NMR and MS results. Furthermore the positive mode and negative mode MS spectra were also different as expected. Due to the large number of peaks in ^{1}H NMR spectra and the large number of 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 foot printing and a list of components was generated for the purpose of component profiling.

3.3 Principal component analysis

3.3.1 ^{1}H NMR

For aqueous samples, negative control samples − (black), clearly separated from samples exposed to microcystin LR (blue), and from positive control samples + (red) which were exposed to acetaminophen (Fig. 3a). Samples exposed to microcystins RR (pink and purple) and YR (green) did not clearly separate from the control as shown in Fig. 3b and c.

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 (Fig. 4), but 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. For samples exposed to microcystin LR, they moved farther from the control as concentration increased (Figs. 3, 4).

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 Fig. 5. The overlap may be attributed to the fact that the three microcystin exhibit similar properties and undergo a similar pathway for detoxification.

Organic samples exposed to microcystins clearly separated from the negative control as shown in Fig. 6a and b. Figure 6b shows two sets of data obtained when two sets of cells were cultured in a mixture (LR, RR and YR). Both separated from the unexposed negative control.

3.3.2 DIMS

Points in the clusters obtained from DIMS aqueous data were closer together (Fig. 7) compared to the ^{1}H NMR data (Fig. 3). There was good clustering and clear separation of samples exposed to microcystin LR, negative control and positive control (Fig. 8). Samples exposed to microcystin YR did not clearly separate from the negative control, but separated from the positive control (Fig. 7b). The same situation was observed for microcystin RR (PCA plot not shown). A compilation of the data showed a clear separation of samples exposed to microcystin LR and positive control which separated from the negative control that was slightly overlapping with samples exposed to RR and YR (Fig. 7c).

Organic samples all separated from the negative control (Fig. 8). Samples exposed to a single microcystin (LR, RR or YR) separated from those exposed to a mixture. This could be because the effect of the mixture was more severe than for a single toxin. Figure 9 shows PCA plots obtained after DIMS in the negative mode. In both positive mode (Fig. 8) and negative mode (Fig. 9) visual distinction of the samples was achieved.

3.4 Data analysis

Peak lists were generated using SIMCA P^{+} and subjected to the student *T*-test. Data were presented as mean ± standard deviation and several components were found to have increased and others decreased in samples exposed to microcystins compared to control samples. Some of the metabolites are shown in Table 1. Details of the other changes in relative amounts of metabolites and their variations with time and concentration are shown in the supplementary data.

3.5 Discussion

The main target of microcystin toxicity is the liver and the brain has also been reported as a target. Microcystins are

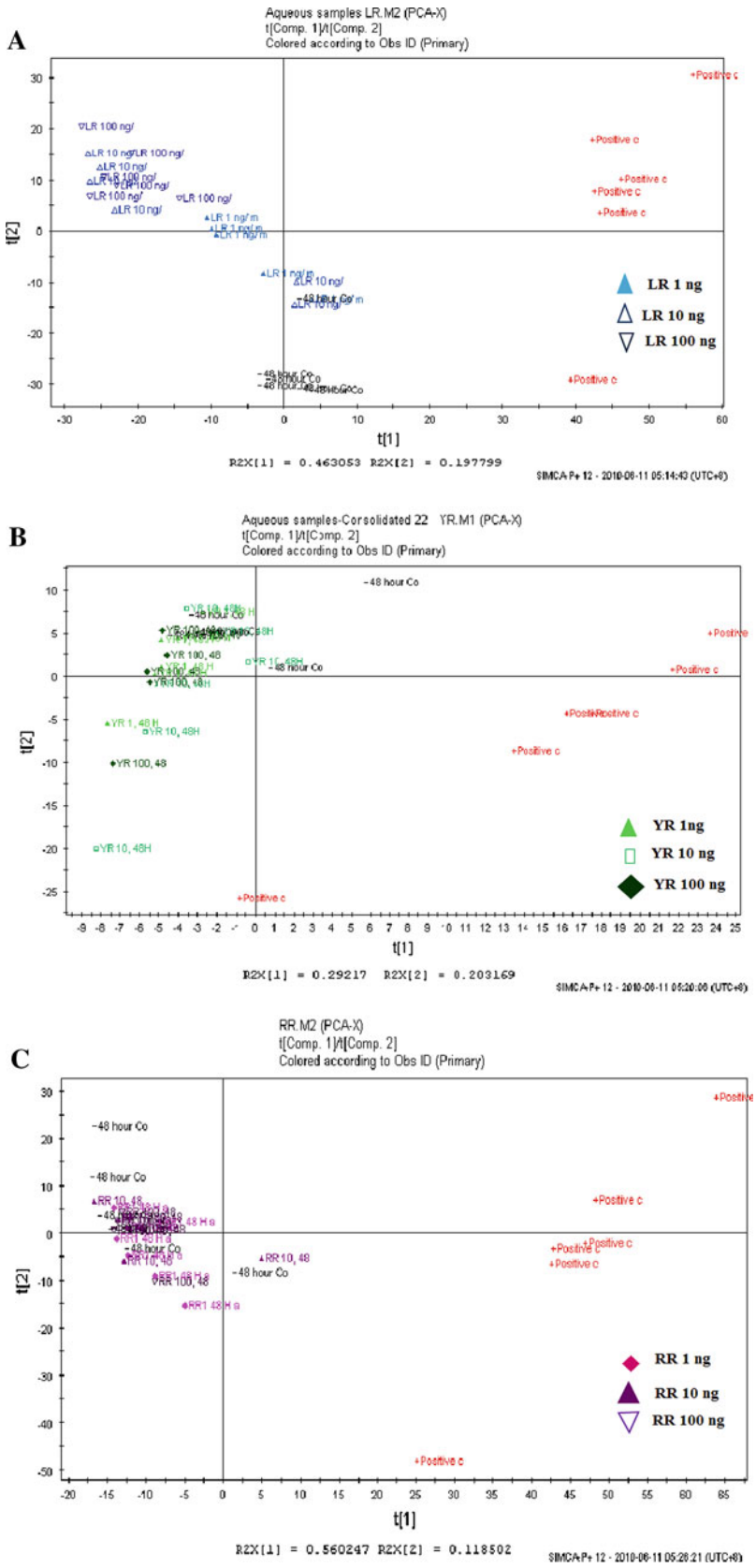

Fig. 3 PCA plots for individual microcystin as obtained from NMR data. **a** microcystin LR (*blue triangles*), **b** microcystin YR (*green triangle*, *diamond* and *square shapes*) and **c** is microcystin RR (*purple diamond* and *triangle shapes*). The concentration of microcystins was in ng/ml in all PCA plots. + (*Red*)—positive control and − (*black*) represents negative control. The symbols on the right of each PCA plot show the specific microcystin concentrations in ng/ml (color figure online)

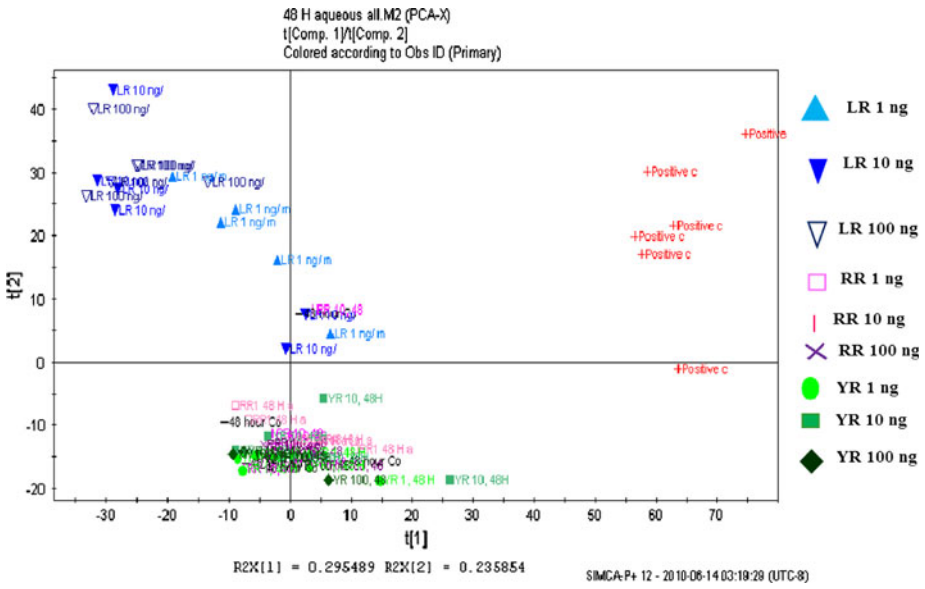

Fig. 4 PCA plot of aqueous samples of microcystins LR, RR and YR from NMR data after 48 h. + (*Red*)—positive control, − (*black*)—negative control. The legend on the right side of the box shows the specific samples (color figure online)

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 al. (2005) identified liver specific rat Oatp 1b2 and human OATP 1B1 and OATPB3 as mediators of microcystin LR uptake into hepatocytes.

The mechanism of microcystin toxicity involves inhibition of protein phosphatases 1 and 2A (PP1 and PP2A) (van Apeldoorn et al. 2007; Zegura et al. 2003; Towner et al. 2002a). 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 homeostasis (Towner et al. 2002a). 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 rates (Towner et al. 2002a). 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 metabolomic project shows that there were changes in the relative amounts of some components (mainly amino acids, some organic acids and lipids) in the samples exposed to microcystin compared to control samples.

3.5.1 Amino acids

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 for the synthesis of non essential amino acids. Nonessential amino acids proline, glutamine, glutamate, arginine, asparagine, aspartate, tyrosine and cysteine increased in samples exposed to microcystins compared to controls, while alanine and serine decreased.

Proline, glutamine and arginine are synthesised from glutamate (Gilbert 2001) which comes from α-ketoglutarate by transamination. In presence of oxaloacetate, glutamate is converted into aspartate. Aspartate can also be obtained from asparagine by hydrolysis. Asparagine is produced when glutamine reacts with aspartate. All these amino acids are interrelated in the arginine and proline metabolic pathway with the amino acid glutamine/glutamate playing a central role in their metabolic pathways. Therefore, their concentration in an organism will be dependent on the concentration of glutamine and glutamate as long as their catabolic pathways are not interfered with.

The increase in glutamate and glutamine is in contrast to the study of acute microcystin toxicity by Towner et al. (2002a) in which they reported a decrease in the levels of glutamine and glutamate. This could be because their study used a higher concentration (1 mg/ml) and investigated acute toxicity compared to our study where the highest concentration was 100 ng/ml. In fact an opposing response of liver cells to low and high concentration of microcystins was reported by Herfindal and Selheim (Herfindal and Selheim 2006). 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

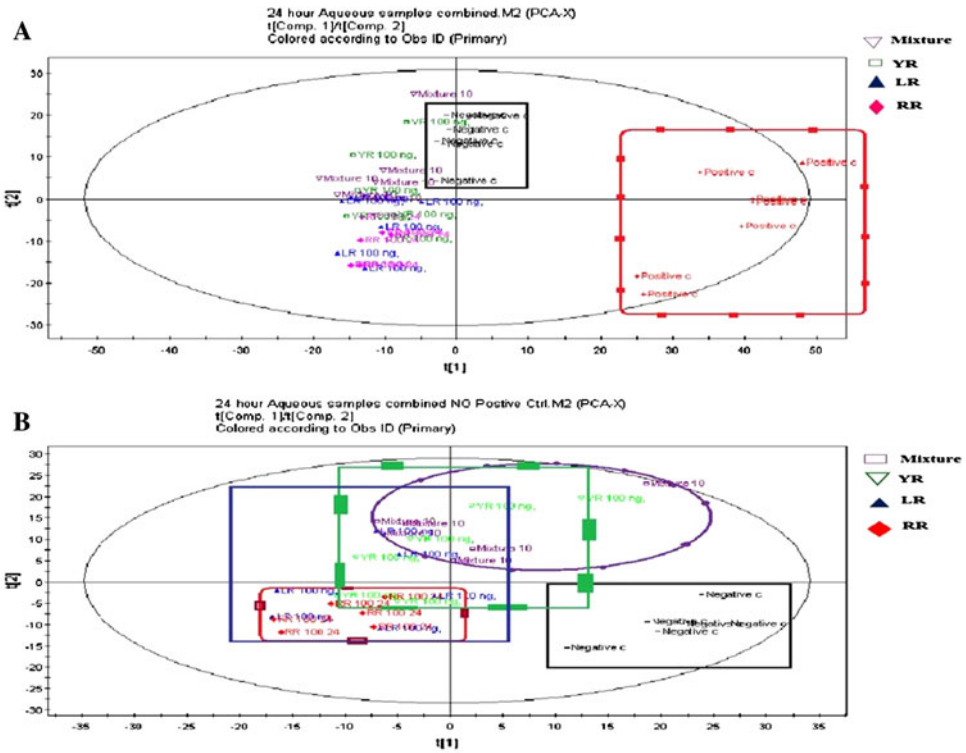

Fig. 5 PCA plots obtained from NMR data for microcystins LR, RR, YR and Mixture with positive control (**a**) and without positive control (**b**). + (*Red*)—positive control, − (*black*)—negative control. The legend on the right side of the box show the symbols used for the microcystins, the mixture and controls (color figure online)

damaged cells compared to higher amounts which inhibit the phosphatases leading to hyper phosphorylation, production of ROS, alteration of the cytoskeleton eventually leading to cell death (Herfindal and Selheim 2006).

Tyrosine is produced by hydroxylation of the essential amino acid phenylalanine in the presence of phenylalanine hydroxylase and biopterin cofactor (Gilbert 2001). It is degraded to produce acetoacetate and fumerate. Wang et al. (2010) 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. In our study, tyrosine amino acid was found to have increased which could be due to end product inhibition due to interference in 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.

Cysteine is synthesised from the essential amino acid methionine by a series of reactions converting methionine to homocysteine which then reacts with serine to produce cytostathionine which breaks down into cysteine and releases α-ketoglutarate. It can be degraded to pyruvate by desulfration but the most important catabolic pathway is through oxidation to produce biosynthetic intermediates which are later oxidised to produce such compounds as taurine (Gilbert 2001). Metabolism of cysteine is an integral part of the adenosyltransferase S-adenosylmethionine (SAM) trans-sulfration pathway to glutathione and taurine. Therefore its increase implies a disruption of the SAM pathway.

Taurine is produced from cysteine and is catabolised through the bile acid metabolism pathway. Therefore increased cysteine amounts should have resulted 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.

Serine is produced through the glucose to hydroxypruvate pathway. It is a precursor to other amino acids such as glycine, cysteine and tryptophan as well as sphingolipids and folate (Gilbert 2001). 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 incomplete.

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. (2009) established a positive relationship between microcystin concentrations and serum enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) lactate dehydrogenase (LDH) and alkaline phospahatase (ALP). An increase in expression of these enzymes is associated with hepatocellular damage and tests

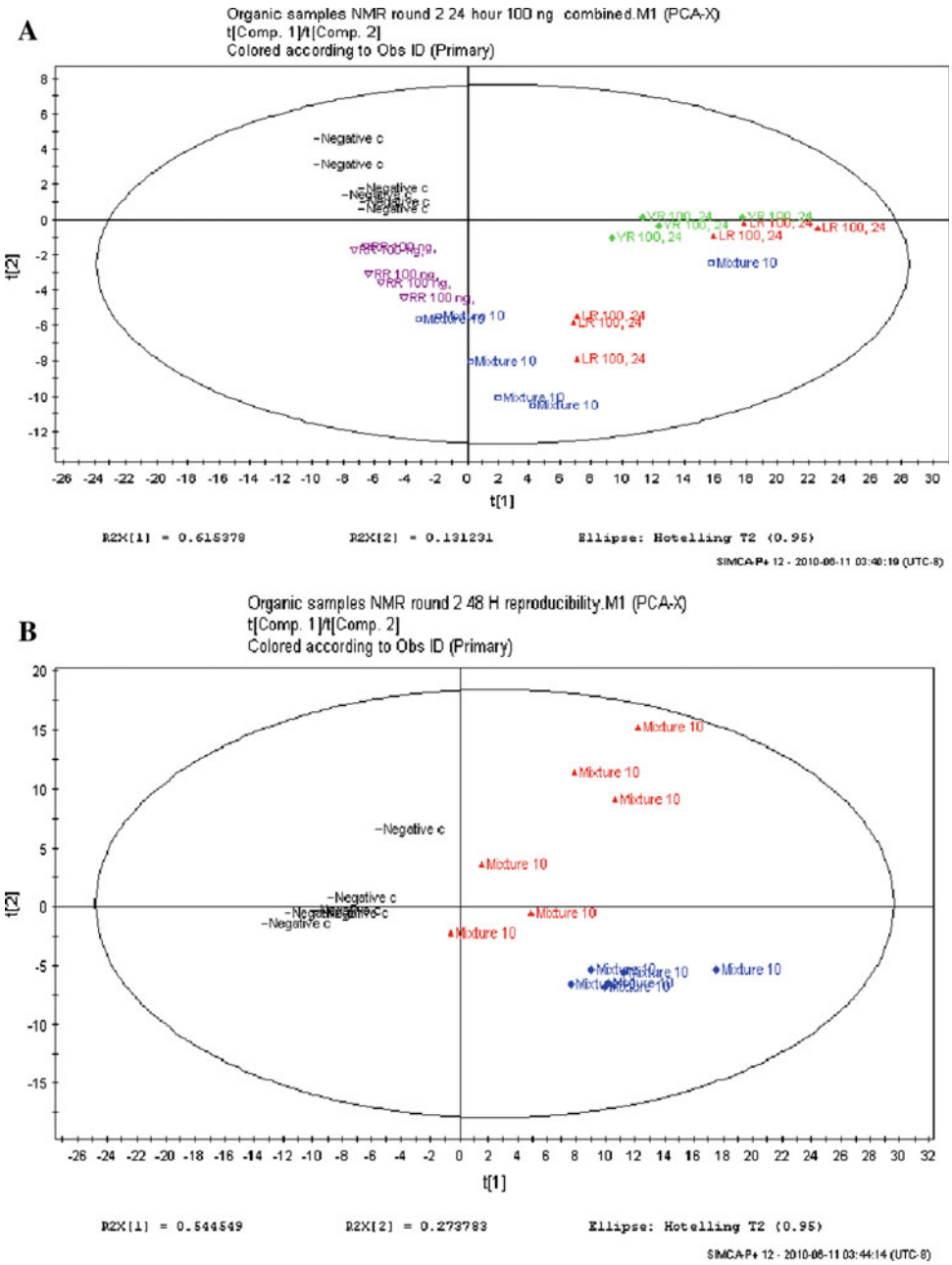

Fig. 6 Organic samples obtained from ^{1}H NMR data. **a** PCA plot of all the samples (*red*—LR, *purple*—RR, *green*—YR, *blue*—mixture) at a similar concentration of 100 ng/ml and negative control. **b** two sets of mixture at 100 ng/ml each and negative control (*red (triangles)*—set 1, *blue (diamond shapes)*—set 2, *black* (−) —negative control) (color figure online)

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.

$$\text{Glutamate} + \text{Pyruvate} \rightleftharpoons \alpha-\text{ketoglutarate} + \text{alanine}$$

The decrease in alanine in our study can be attributed to interference in substrate (pyruvate) production as well as over expression of ALT as suggested from literature (Chen et al. 2009)

3.5.2 *Organic acids*

Malonate, pyruvatoxime, succinic acid, beta hydroxybutrate, amino-tridecanoic acid increased, while glyoxylic acid, gylcolate, lactate, glyceric acid, 1-pyrroline-5-carboxylic acid and acetoacetate, decreased.

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

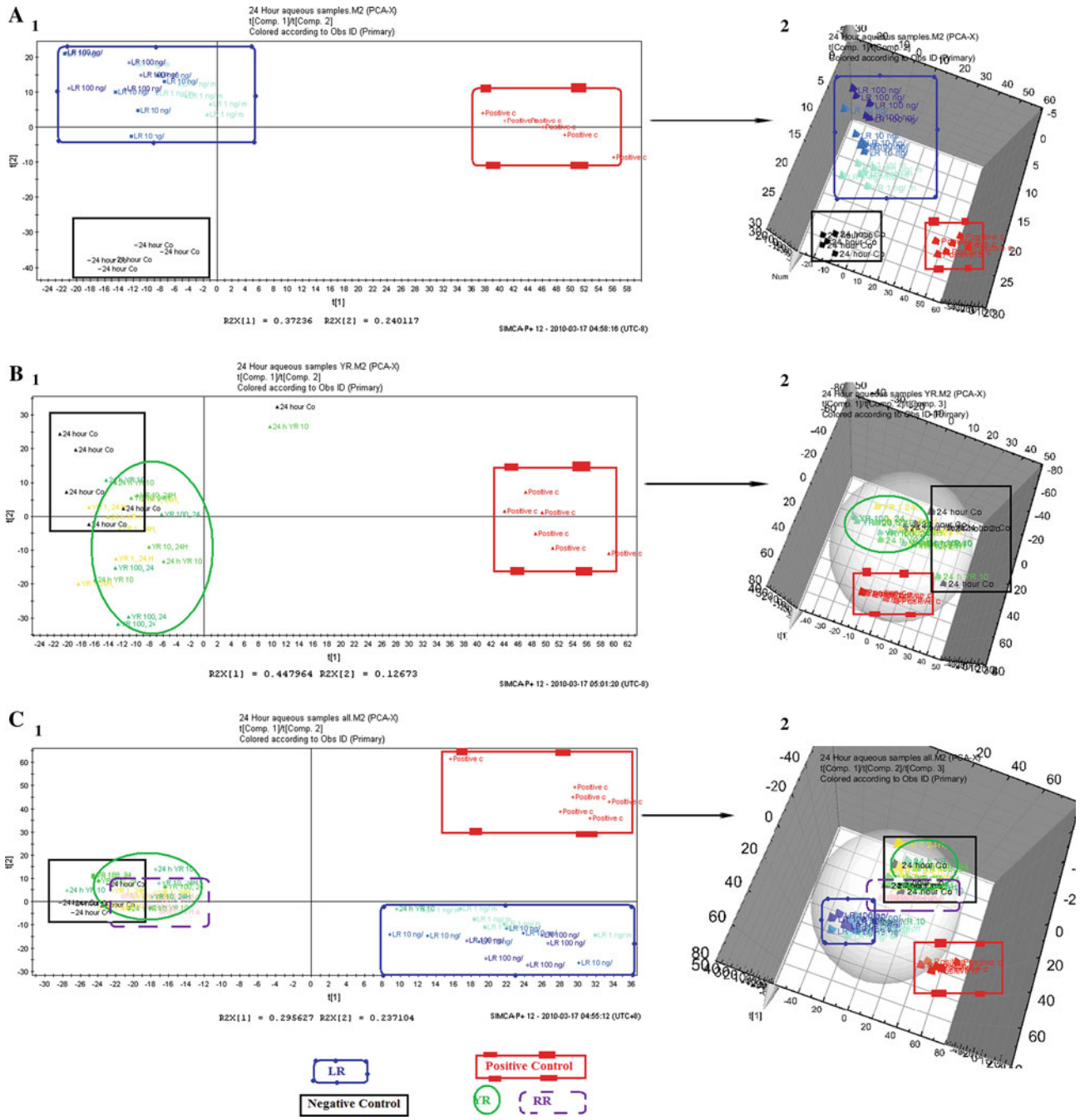

Fig. 7 PCA plots obtained from MS data for aqueous samples of microcystin LR in 2D and 3 D (**a**), microcystin YR (**b**) and the three microcystins LR, RR and YR together (**c**)

oxidation of succinate by competing for succinate dehydrogenase active sites, Wang et al. (2010) found that suclg2 protein which is responsible for regulating the formation of succinate and ATP from succinly—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 which plays a central role in organic acid metabolism.

Glycolate and glyoxylate are oxalate precursors. The metabolism of glycolate and glyoxylate in HepG2 cells was studied by Baker et al. (2004) 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

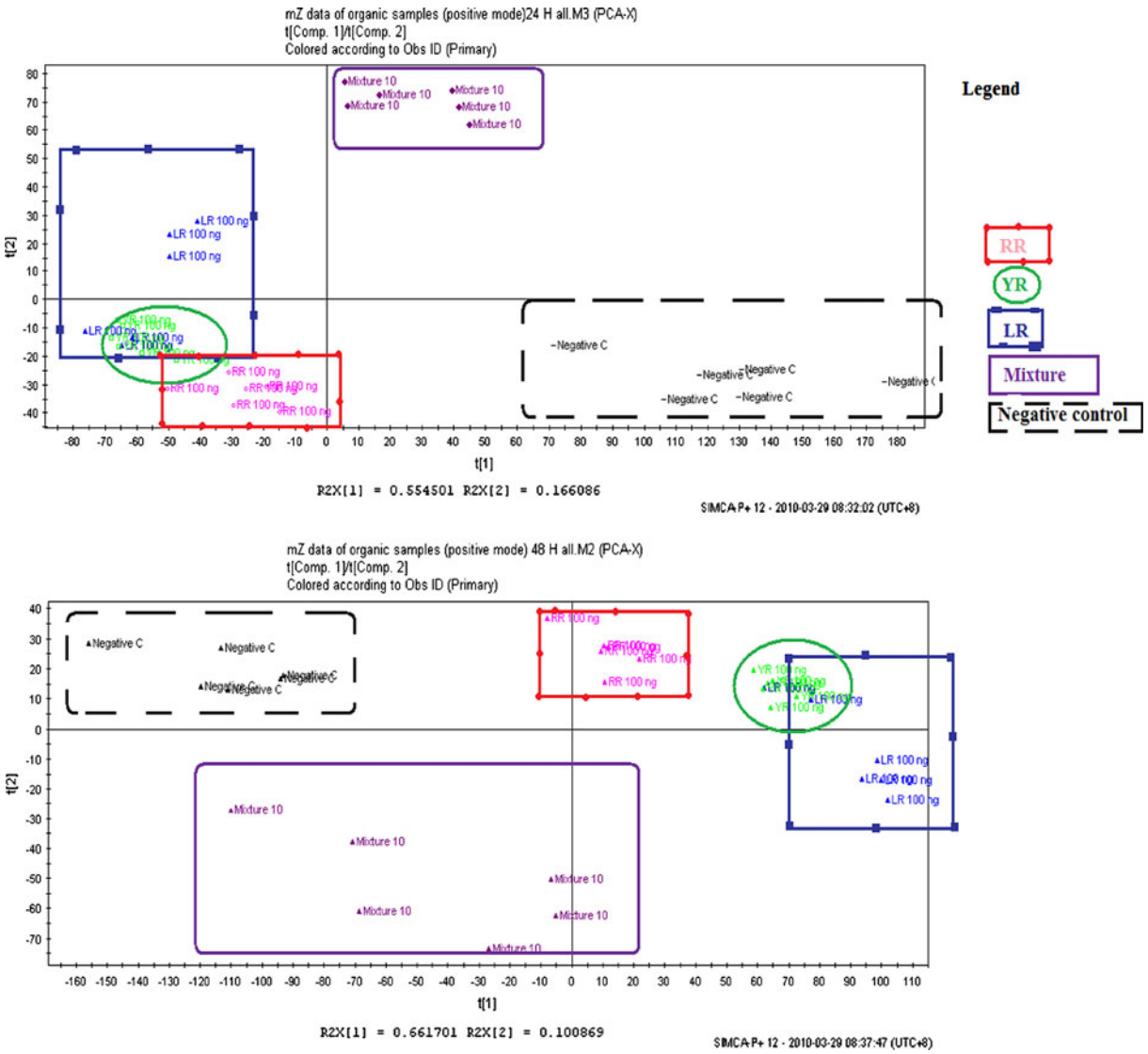

Fig. 8 PCA plots obtained for MS data from organic samples of microcystins LR, RR, YR, mixture and negative control

decreased. The decrease in glyoxylate in our study can be attributed to its conversion into glycolate and conversion into oxalate in presence of lactate dehydrogenase (LDH). Exposure to microcystins has been associated with increased LDH (Chen et al. 2009) 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 in the medium vary linearly (Baker et al. 2004). 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. (2002a) even though a lower concentration was used in our study.

3.5.3 Lipids

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.

Phosphocreatine is synthesised in the liver and transported to the muscle via the creatine phosphate shuttle. It is synthesised form amino acids arginine, glycine and methionine. Phosphocreatine undergoes irreversible cyclization and dehydration to form creatinine (Knox et al. 2010c). Creatine plays a vital role as phosphocreatine in regenerating adenosine triphosphate in skeletal muscle to

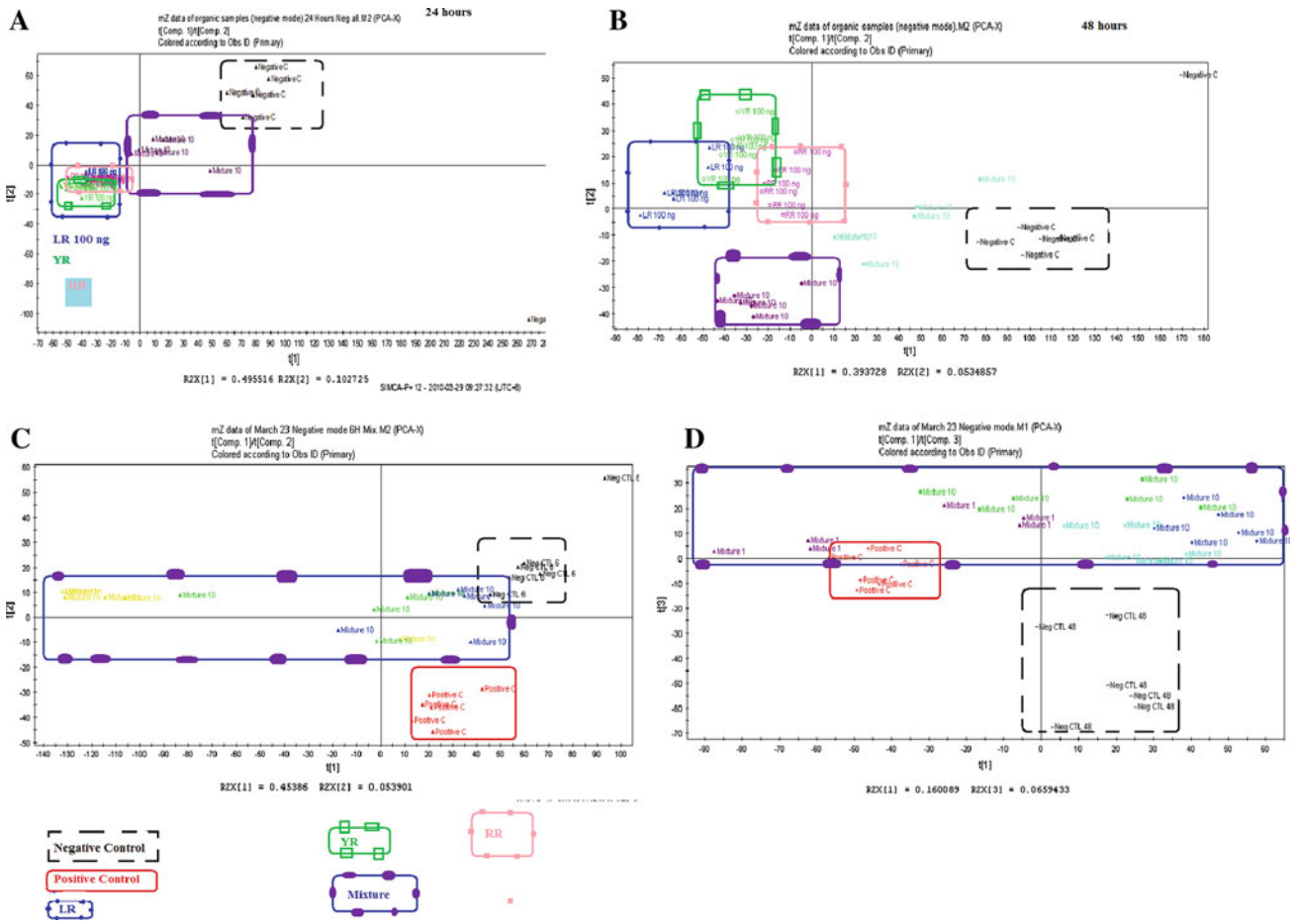

Fig. 9 PCA plots for samples analyzed by DIMS in negative mode. **a** All samples after 24 h, **b** all samples after 48 h, **c** mixture after 6 h and **d** mixture after 48 h

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 muscle and nerve. The increase in phosphocreatine is consistent with the increase in the amino acid arginine observed and this has a direct relationship with the amount of creatine.

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) disease (Knox et al. 2010b).

Inositol 1,3,4-trisphosphate is a specific regulator of cell signalling (Knox et al. 2010a). It is a component of inositol phosphate metabolism 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. They can be produced from glucose 6-phosphate in the presence of inositol-3-phosphate synthase 1 which catalyses conversion of to glucose 6-phosphate to1-myo-inositol 1-phosphate (Abel and Shears 2001). This is later converted to myo-inositol and enters the phosphaditylinositol 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 and is converted to inositol 1,3,4,5 tetraphosphate. The later can be converted to inositol 1,3,4-trisphosphate 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,

Table 1 Summary of variation of metabolites in samples exposed to microcystins relative to the controls

Metabolite	m/Z	Relative peak intensities			
		Negative control (24 h)	Sample (24 h)	Negative control(48 h)	Sample (48 h)
Valine ↑	118.1	358.957 ± 10.7	386.372 ± 21.13	377.282 ± 9.07	433.758 ± 66.6
Glutamine ↑	147.1	123.245 ± 1.79	154.307 ± 9.15	112.622 ± 5.19	172.93 ± 42.29
Glutamate ↑	148.1	41.193 ± 2.21	45.465 ± 3.54	43.303 ± 2.96	52.015 ± 9.98
Phenylalanine↑	166.1	116.728 ± 3.84	136.199 ± 7.69	120.095 ± 4.82	156.714 ± 54.51
Tyrosine ↑	182.1	58.343 ± 4.06	69.132 ± 4.92	58.91 ± 2.52	73.402 ± 15.49
Alanine ↓	90.1	9.978 ± 1.08	7.504 ± 0.68	10.788 ± 1.17	7.83 ± 1.68
Serine ↓	106	173.382 ± 4.28	162.515 ± 4.11	170.352 ± 3.95	162.798 ± 8.53
Acetylcholine ↑	146.1	51.862 ± 4.42	62.856 ± 6.94	62.405 ± 3.23	80.924 ± 8.07
Dodecanamide ↑	200.3			51.502 ± 2.69	57.703 ± 9.76
Phosphocreatine ↑	212	28.042 ± 2.06	31.868 ± 2.22	30.987 ± 2.15	31.119 ± 2.37
4-Phospho-L-aspartate ↑	214	48.422 ± 3.38	65.371 ± 4.32	56.805 ± 3.33	72.518 ± 9.52
Amino-tridecanoic acid ↑	230.3	28.690 ± 3.01	47.736 ± 3.45	33.7633 ± 1.63	47.591 ± 2.10
Dihydrosphingosine ↑	302.4	259.913 ± 5.75	321.397 ± 8.25	298.712 ± 14.09	327.847 ± 21.89
Cholesterol ↑	387.4			24.455 ± 1.92	28.300 ± 18.85
Cytidine diphosphate (CDP) ↑	403.9	91.138 ± 4.89	94.0792 ± 8.09	94.615 ± 1.67	115.707 ± 55.87
D-myo-Inositol 1,4,5-trisphosphate ↑	420.8	53.832 ± 3.34	54.053 ± 2.86	57.560 ± 3.49	60.073 ± 15.61
Inositol 1,3,4-trisphosphate ↑	420.9			68.400 ± 3.11	69.553 ± 15.12
L-3,5-Diiodotyrosine ↑	433.7			30.660 ± 1.70	35.465 ± 12.47
Cyclic GMP ↓	345.9	102.450 ± 3.60	80.162 ± 5.81	103.975 ± 4.09	82.737 ± 6.32
Aminotridecanoic acid ↑	230.3	28.69 ± 3.00	47.736 ± 3.45	33.763 ± 1.63	47.591 ± 2.10
Glyceric acid ↓	107	281.988 ± 5.06	244.28 ± 8.63	268.587 ± 7.52	242.365 ± 3.37
1-Pyrroline-5-carboxylic acid ↓	114	239.942 ± 4.06	213.537 ± 12.15	263.245 ± 9.18	242.290 ± 8.11
Acetoacetate ↓	117	313.343 ± 4.15	263.487 ± 4.99	297.062 ± 8.11	257.373 ± 10.56
Glyoxylic acid ↓	75	4.658 ± 0.60	4.18 ± 1.07	4.313 ± 0.55	3.789 ± 0.54
Glycolate ↓	76	1.058 ± 0.31	0.971 ± 0.28	1.458 ± 0.19	0.964 ± 0.29
Lactate ↓	90	14.353 ± 1.15	10.298 ± 0.70	14.562 ± 1.56	10.648 ± 1.87
Lactic acid ↓	91	134.042 ± 5.25	77.789 ± 2.81	117.728 ± 5.81	77.017 ± 6.50

Relative intensities of metabolites. ↑ Metabolite which increased in samples exposed to microcystins compared to the control. ↓ Metabolite which decreased in samples exposed to microcystins compared to control. Values are represented as mean ± SD (standard deviation). Significant difference between control and samples exposed to microcystins was based on two-tailed student's *t*-test where $P \leq 0.05$

aggregation, and activity by acting as secondary messengers (Abel and Shears 2001). An increase in phosphates can be attributed to inhibition of phosphatases. Literature (Ding et al. 1998; Toivola and Eriksson 1999) 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 these 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. Acetylcholine and choline are components of glycerophospholipid metabolism 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.

3.5.4 Purines and pyrimidines

Cyclic GMP, a component of purine metabolism 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. In general these results suggest that microcystin disrupt the metabolism of nucleic acid by interfering in purine and pyrimidine metabolism.

4 Conclusion

Microcystins in concentrations that are not cytotoxic affected the metabolism of HepG2 cells as seen from the results of their metabolic finger/foot prints. There were clear distinctions between samples exposed to microcystins, those exposed to acetaminophen (positive control) and control (negative control) samples. Samples exposed to a single microcystin clearly separated from the control as well as from a mixture. It was not possible to differentiate between samples exposed to a specific microcystin, probably due to the similar characteristics of microcystins LR, RR and YR. Samples exposed to a mixture (LR,RR and YR together) separated from those exposed to individual microcystins and from those exposed to the mixture at a lower concentration (Fig. 9b). This could be attributed to more severe effects of the mixture at a higher total toxin concentration compared to when the cells were exposed to a single microcystin or to a lower mixture concentration.

It was evident that exposure to non cytotoxic concentrations of microcystins disrupts the metabolisms of pyruvate, glutamate, glycerophospholipids and affects the trans sulfuration pathway. Cells exposed to the microcystins at low concentrations did not die rather they continually proliferated which indicates a possibility of uncontrolled growth that can result into tumours.

Acknowledgments We acknowledge financial support from the organization of women in science fellowship (TWOWS–TWAS), the National University of Singapore, National Research Foundation and Economic Development Board (SPORE, COY-15-EWI-RCFSA/N197-1), Ministry of Education (R-143-000-382-112, and R-143-000-416-232, and R-143-000-441-112), A-STAR (SERC PSF 052 101 0044), Ministry of Defense (R-143-000-416-646) and Environment and Water Industry Development Council (0601-IRIS-093-08). We would also like to thank the Medicinal Chemistry Program of NUS for equipment support, and Agilent Technologies for the generous loan of the LC-MS QQQ system, and Professor Hanry Yu and his staff and students for provision of the cell line used in this study.

Conflict of interest The authors declare no conflict of interest.

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