PURIFICATION, DETECTION AND BIOLOGICAL EFFECTS OF CYANOBACTERIAL TOXINS

By

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DEDICATION

For my Mum and Dad

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ABSTRACT

Dense growths of cyanobacteria (blue-green algae) known as blooms commonly occur in fresh and brackish waters throughout the world. Cyanobacterial blooms present a considerable threat to water quality as many species produce toxins. These toxins have been implicated in the poisoning of humans and animals throughout the world, and there is a requirement for simple and effective methods for their detection. This study set out to investigate the detection of several cyanobacterial toxins, including a group of neurotoxins known as the saxitoxins. These toxins are also produced in marine environments by dinoflagellates and bacteria and can accumulate in edible bivalve shellfish. An alternative assay for the monitoring of these toxins was developed using the desert locust *Schistocerca gregaria*. The locust bioassay was used in conjunction with an established high performance liquid chromatography (HPLC) protocol to devise an extraction procedure for saxitoxins from cyanobacterial cells. It was also suitable for screening acid extracts of shellfish flesh for saxitoxins, and performed well in a large shellfish monitoring programme, indicating its potential as a replacement to the mouse bioassay.

The detection of another group of cyanobacterial toxins called the microcystins was also addressed. The routine monitoring of cyanobacterial cultures and natural bloom samples for microcystins relies on HPLC. However, the lack of purified standards has hindered accurate detection and quantification of many microcystin variants. Reversed-phase Flash chromatography was employed for the partial purification of microcystins from a laboratory culture of *Microcystis aeruginosa*. A technique was then developed to facilitate the separation of two closely eluting hydrophobic variants (microcystin-LW and -LF) using normal-phase flash chromatography. The resulting three step methodology provided a simple and inexpensive means of extracting and purifying microcystins for use as analytical standards.

Purified toxins were also employed to investigate the effects of microcystins on plants. Bioassay methods revealed the inhibitory effects of microcystins on plant growth and development. The accumulation of microcystins in exposed plants was then investigated. A simple plant model lacking roots was exposed to microcystin-LF and extracted using three different solvents to compare toxin recovery. HPLC analysis revealed that the most efficient extraction method was methanol, and indicated the presence of additional compounds possibly representing toxin metabolites. The uptake of microcystin-LR was then examined using a larger intact plant. Exposure to the toxin did not inhibit growth for up to 18 days, but had a marked effect on the roots, and caused plants to take up less medium than controls. The plants were then extracted in methanol and analysed by HPLC to determine whether toxin had accumulated. However, the co-elution of contaminants prevented the detection of microcystin-LR, highlighting the requirement for alternative clean-up methods for complex biological matrices.

Chapter 1

Introduction

1.1. The nature and occurrence of cyanobacteria.

Cyanobacteria are a diverse group of prokaryotic microalgae which can exist in a wide range of environments. These organisms possess chlorophyll *a*, and metabolise energy through oxygenic photosynthesis associated with photosystems I and II (Castenholz and Waterbury, 1989). Fossil records have verified the existence of cyanobacteria 3.3 to 3.5 billion years ago. As the first organisms capable of performing oxygenic photosynthesis, it is likely that they contributed to the oxygenation of the primitive atmosphere. It has been hypothesised that some of the earlier cyanobacterial cells may have been taken up by other microbes and lost their ability to function independently. This is widely believed to have been the first stage in the evolution of chloroplasts, the photosynthetic organelles in plants (Carmichael, 1994).

Cyanobacteria are also commonly known as 'blue-green algae', due to their algal-like morphology, and ability to photosynthesise. They were originally recognised by botanists over 200 years ago (Skulberg *et al.*, 1993), but their taxonomy has proven difficult, as they share a number of features with both green algae and bacteria (Mur, Skulberg, and Utkilen, 1999). Unlike eukaryotic algal cells, cyanobacteria do not possess nuclei, and their cell walls are composed of peptidoglycan and lipopolysaccaride layers rather than cellulose (Carmichael and Falconer, 1993). There has therefore been some confusion as to whether they should be classified according to the International Code of Botanical Nomenclature (ICBN) (Greuter *et al.*, 1994), or the International Code of Nomenclature of Bacteria (ICNB) (Sneath, 1992). Recent studies into the genetic characteristics of cyanobacteria have offered an alternative approach to their taxonomy (Castenholz and Waterbury, 1989). Phylogenetic classification has placed cyanobacteria within the group eubacteria as an individual class of organisms known as Cyanophyceae.

Cyanobacteria are commonly found in fresh, brackish, or salt water environments. Growth is influenced by variations in environmental factors including temperature,

light intensity, turbidity, and nutrient status. Many species possess cytoplasmic inclusions called gas vacuoles, which provide cells with buoyancy, and allow them to move vertically within the water column to positions where these factors are suitable for growth (Walsby, 1987). Cyanobacteria flourish during the summer months, in water bodies which are rich in nutrients, particularly nitrogen and phosphorous (Skulberg, Codd, and Carmichael, 1984). When optimum conditions prevail, such water bodies can support the formation of dense surface growths of cyanobacteria known as blooms. The occurrence of cyanobacterial blooms has increased due to the eutrophication of many aquatic environments with nutrients from agricultural effluent and human wastes (Bell and Codd, 1994). This has created a considerable water quality problem as certain species of bloom forming cyanobacteria are capable of producing toxins. Toxic cyanobacterial blooms may be directed towards the shoreline by the wind, where they accumulate as thick scums and present a risk to animals drinking from the water. The ingestion of water containing cyanobacterial toxins has caused illness and death in wild and domestic animals in many different parts of the world (Carmichael, 1992).

1.2. The cyanobacterial toxins.

Three groups of cyanobacterial toxins have been identified: hepatotoxins, neurotoxins, and lipopolysaccharide endotoxins. The hepatotoxins and neurotoxins are intracellular and are produced only by certain strains of cyanobacteria. High concentrations of these toxins can be released into the water body by lysed cells following the breakdown of a cyanobacterial bloom. Lipopolysaccharide endotoxins are found in the outer membrane of the cyanobacterial cell wall, and are common to all cyanobacteria.

1.2.1. The cyclic peptide hepatotoxins: microcystins and nodularins.

The most commonly occurring cyanobacterial toxins found in fresh and brackish waters are cyclic peptide hepatotoxins known as microcystins (Carmichael, 1994). They are known to be produced by certain strains of the cyanobacterial genera,

including *Microcystis, Anabaena, Oscillatoria (Planktothrix),* and *Nostoc* (Carmichael, 1992). Microcystins are monocyclic heptapeptides with a common structure containing three D-amino acids (alanine, erythro-β–methylaspartic acid and glutamic acid), two variable L-amino acids, and two unusual amino acids; N-methyldehydroalanine, and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Botes, Kruger and Viljoen, 1982). To date, over 60 microcystin variants have been characterised from naturally occurring bloom samples and isolated strains of cyanobacteria (Sivonen and Jones, 1999). Microcystins are named according to the two variable amino acids (Carmichael *et al.*, 1988 a and b), but are also distinguished by other structural features such as methylation and variations in the structure of Adda. For example, microcystin-LR contains the amino acids leucine (L), and arginine (R) at positions X and Z (Figure 1.1., Carmichael, 1997).

Particular strains of the brackish water cyanobacterium *Nodularia spumigena* are responsible for the production of another group of hepatoxins called nodularins (Rinehart *et al.*, 1988). These toxins are cyclic pentapeptides which are structurally related to the microcystins and have the same mode of toxicity (Namikoshi *et al.*, 1993). An analogue of nodularin has also been identified in the marine environment. Motuporin is identical to nodularin except that it contains the amino acid valine in place of arginine, making it more hydrophobic (DeSilva *et al.*, 1992). This toxin has been isolated from the marine sponge *Theonella swinhoei* which is known to form symbiotic relationships with cyanobacteria.

In mammals, intoxication with microcystins and nodularins results in acute liver damage, often leading to death (Beasley, *et al.*, 1989; Runnegar, Jackson, and Falconer, 1988). The cyclic peptide hepatotoxins are generally water soluble, and are therefore unable to enter into cells through hydrophobic cell membranes. Following ingestion, they are transported to the liver by bile-acid carriers located in hepatocytes and in the cells which line the small intestine (Runnegar, Gerdes, and Falconer, 1991; Falconer, Choice, and Hosja, 1992).

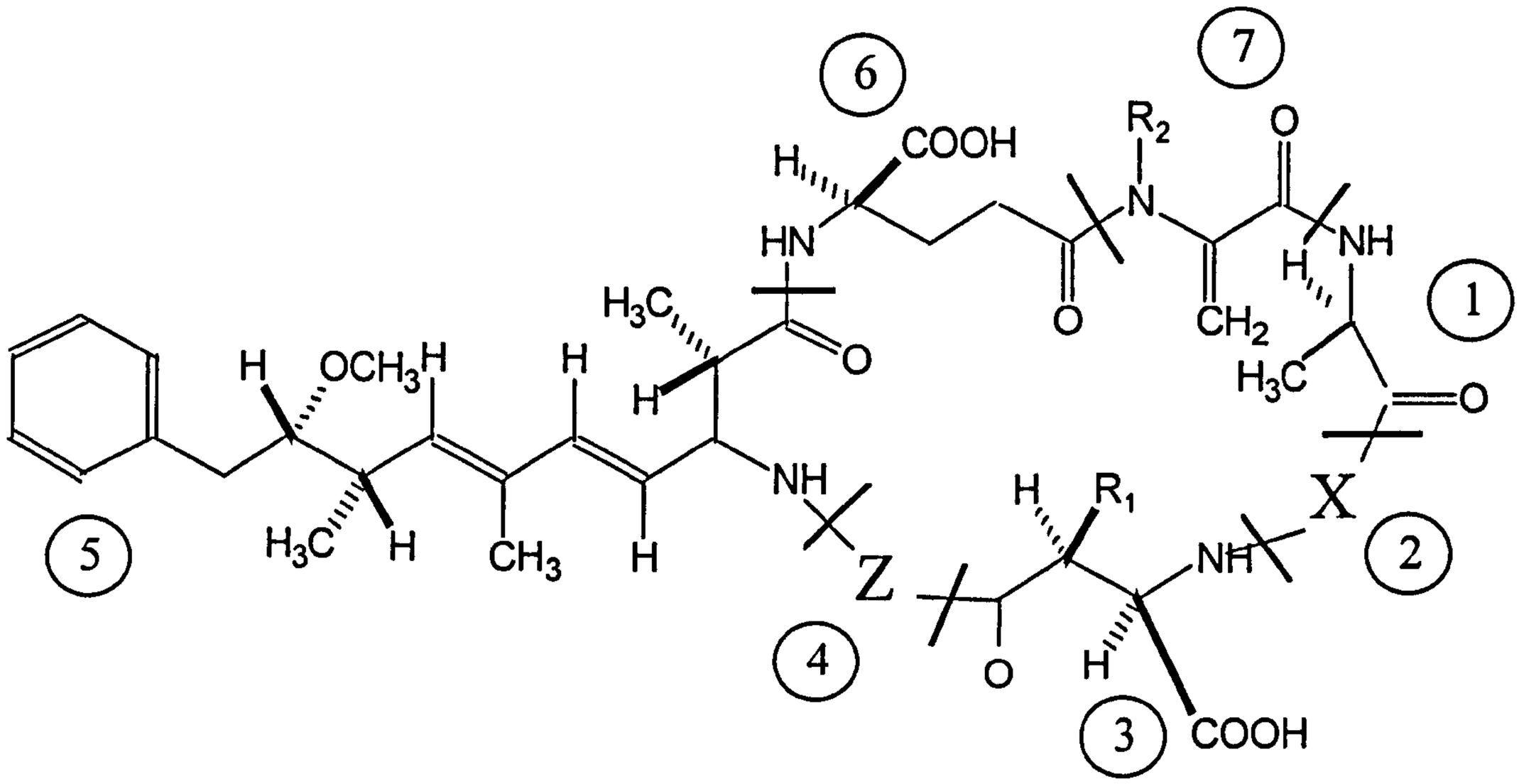


Figure 1.1. General structure of the cyclic heptapeptide hepatotoxins, microcystins, showing the most frequently found variations:

cyclo-(D-Ala^1 -X^2 -D-MeAsp^3 -Z^4 -Adda^5 -D-Glu^6 -Mdha^7).

X and Z = the 2 variable amino acids.

R_1 and R_2 = H (demethylmicrocystins) or CH_3.

D-MeAsp = D-erythro-β-methylaspartic acid.

Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

Mdha = N-methyldehydroalanine.

Microcystin and nodularin mediated toxicity is characterised by the disruption of cytoskeletal components in liver cells. This eventually causes hepatocytes to shrink and separate, allowing blood to accumulate in the liver (Carmichael, 1994). Animals which have ingested poisonous quantities of cyclic peptide hepatotoxins often display weakness, pallor of mucous membranes, diarrhoea, and vomiting (Carmichael, 1992). Depending on dose, intoxication can culminate in intrahepatic haemorrhage and hypovolaemic shock, leading to death within several hours or several days (Theiss *et al.*, 1988). Hepatotoxins have also been implicated in the fatalities of birds (Carmichael and Falconer, 1993) and fish (Kotak *et al.*, 1996). However, considerably less is known about the effects of the toxins in these animals. Takahashi and Kaya (1993) demonstrated that the target for microcystin intoxication in quails was not the liver, but the spleen. In fish, microcystin intoxication has been shown to cause hepatocyte damage, and it has been linked to netpen liver disease in Atlantic salmon (Andersen *et al.*, 1993).

The toxic effects of microcystins and nodularins are associated with the inhibition of protein phosphatases 1 and 2A (PP1 and PP2A; Eriksson *et al.*, 1990; Yoshizawa *et al.*, 1990). These enzymes are present in all eukaryotic cells where they control the activities of proteins by catalysing the dephosphorylation of serine and threonine residues (Cohen, 1989). In the liver, the inhibition of PP1 and PP2A by microcystins and nodularin leads to the excessive phosophorylation of cytoskeletal proteins by protein kinases, causing hepatocyte deformation (Eriksson *et al.*, 1990). Cell culture techniques have indicated that microcystin-LR inhibition of PP1and 2A causes similar cytoskeletal damage to rat fibroblasts and renal epithelial cells. However, these cell types were found to be approximately ten times less sensitive to the toxin than hepatocytes (Khan *et al.*, 1995). A recent study demonstrated symptoms typical of apoptosis (programmed cell death), in rat hepatocytes treated with 0.01-2 μM microcystin-LR. Human endothelial cells, epithelial cells and skin fibroblasts displayed similar morphological changes, but required toxin concentrations approximately one hundred times greater (McDermott *et al.*, 1998). The difference in sensitivity between hepatocytes and other cell types is probably due to uptake.

Microcystins may be transported into hepatocytes by bile acid transporters, while the toxin can only enter non-hepatocytes by diffusion (McDermott *et al.*, 1998).

Microcystins and nodularins have been shown to inhibit PP1 and PP2A with the same potency (Yoshizawa et al., 1990; Honkanen *et al.*, 1994). Structural studies have revealed that inhibition is mediated through interactions between the Adda-glutamate portion of the cyclic peptide toxins with a hydrophobic pore in the enzyme molecule (Goldberg *et al.*, 1995; Rudolph-Böhner, Mierke, and Moroder, 1994). The binding of microcystin to PP1 has been shown to be particularly stable due to covalent bonding between the methyl-dehydroalanine residue of the toxin and the sulphur atom of Cysteine-273 in the protein phosphatase molecule (Goldberg *et al.*, 1995; MacKintosh et al., 1995).

In addition to the acute toxic effects caused by high levels of cyanobacterial hepatotoxins, long term exposure to lower toxin concentrations also presents a considerable health risk. Microcystins and nodularins inhibit PP1 and PP2A in a similar manner to the diarrhetic shellfish toxin, okadaic acid, which is a potent tumour promoter (Suganuma *et al.*, 1988; MacKintosh and MacKintosh, 1994). Animal experiments have also indicated the tumour promoting activity of microcystins. Nishiwaki-Matsushima and co-workers (1992) demonstrated the liver tumour promoting activity of microcystin-LR in rats which had been pretreated with the tumour initiator diethylnitrosamine. Microcystin-LR has also been shown to induce neoplastic nodular formation in mouse liver without prior treatment with an initiator (Ito *et al.*, 1997). This effect was observed in mice which had been injected intraperitoneally with a sublethal dose of the toxin over a period of 28 weeks. However, nodule formation was not detected in mice which had received a higher dose of microcystin-LR administered orally (Ito *et al.*, 1997). Repeated exposure to cyanobacterial hepatotoxins has been implicated in the high frequencies of hepatocellular carcinoma which occur in south east China. Water bodies in this area are affected by an abundance of heavy cyanobacterial blooms. Studies conducted by Yu (1995), suggested that liver cancer mortalities were particularly high (100 per 100,000) in communities which acquired water from

eutrophic ponds and ditches likely to contain microcystin-producing cyanobacteria. In contrast, deaths resulting from liver cancer were considerably lower (approximately 10 per 100,000) in areas where water was extracted from deep wells.

1.2.2. The cytotoxic alkaloid cylindrospermopsin.

A hepatotoxin with a markedly different structure and mode of toxicity to the cyclic peptides has been identified in various tropical and subtropical water bodies. Cylindrospermopsin is a cyclic guanidine alkaloid which was originally associated with the cyanobacterium *Cylindrospermopsis raciborskii* in Australian waters (Hawkins *et al.*, 1997). However, the toxin has also been found in *Umezakia natans* in Japan (Harada *et al.*, 1994), and *Aphanizomenon ovalisporum* in Israel (Banker *et al.*, 1997). Unlike the microcystins and nodularins, cylindrospermopsin does not inhibit protein phosphatases 1 and 2A, and it appears to have a progressive effect on a number of other vital organs in addition to the liver (Ohtani, Moore, and Runnegar, 1992). Toxicity studies have revealed that mice administered cylindrospermopsin by intraperitoneal (i.p.) injection survive longer than those injected with the cyclic peptide hepatotoxins (Bell and Codd, 1996). Exposure to pure toxin has been shown to cause lipid accumulation and proliferation of the smooth endoplasmic reticulum in mouse hepatocytes (Terao *et al.*, 1994). However, i.p. injection of lysed cells of *C. raciborskii* has resulted in more widespread tissue damage in mice including necrosis to kidneys, adrenals, heart, lungs and spleen (Hawkins *et al.*, 1985).

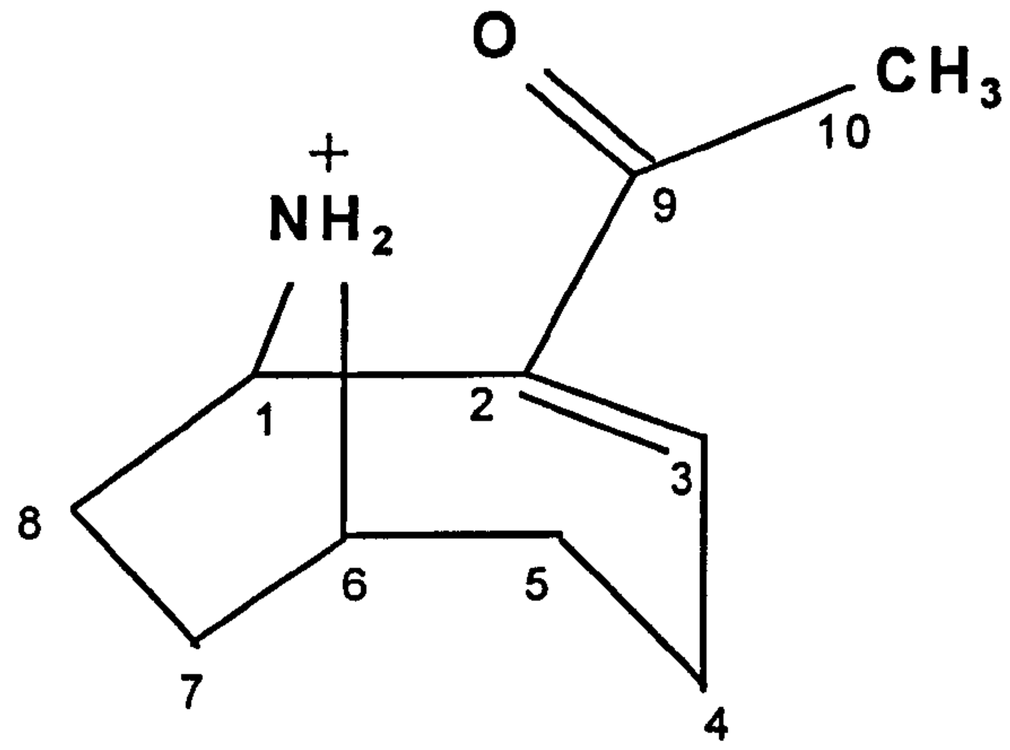
1.2.3. The neurotoxic alkaloids: anatoxins and saxitoxins.

The cyanobacterial neurotoxins are alkaloids associated with certain species of *Anabaena, Oscillatoria (Planktothrix), Aphanizomenon,* and *Trichodesmium* (Carmichael, 1997). Three classes of neurotoxins have been identified; anatoxin-a, anatoxin-a(s), and the saxitoxins.

Anatoxin-a was the first cyanobacterial toxin to be chemically characterised (Devlin *et al.*, 1977). It is a low molecular weight secondary amine with the structural formula 2-acetyl-9-azabicyclo(4-2-1) non-2-ene (Figure 1.2(a)). Anatoxin-a is a nicotinic (cholinergic) agonist which binds to neuronal nicotinic acetylcholine receptors. The toxin mimics the neurotransmitter acetylcholine by activating the flow of ions which induces muscle contraction. Muscle cells are normally allowed to return to their resting state following the degradation of acetylcholine by the enzyme acetylcholinesterase. However, anatoxin-a cannot be degraded by this mechanism, and therefore continues to act on muscle cells, causing them to become overstimulated. Exposure to sufficiently high levels of anatoxin-a causes muscle cells to cease functioning, leading to paralysis, respiratory arrest, and often death (Carmichael, 1997). The toxin has been found to be considerably more potent than either acetylcholine or nicotine in various central nervous system responses (Thomas *et al.*, 1993; Molloy *et al.*, 1995). Anatoxin-a is produced by members of the genus *Anabaena, Oscillatoria* and *Aphanizomenon*, and has been implicated in the poisoning of cattle, (Carmichael and Gorham, 1978), and dogs (Edwards *et al.*, 1992). A methylene homologue of anatoxin-a has been isolated from a strain of *Oscillatoria formosa.* Homoanatoxin-a possesses a propionyl group at C-2 in place of the acetyl group in anatoxin-a, and is slightly less toxic (Skulberg *et al.*, 1992).

Anatoxin-a(s) is an organophosphate which has been identified in *Anabaena flos-aquae* strain NRC 525-17 (Matsunaga et al., 1989), and in particular strains of *Anabaena lemmermannii* (Henriksen *et al.*, 1997). The toxin is a phosphate ester of a cyclic N-hydroxyguanine (Figure 1.2(b); Sivonen and Jones, 1999), and has a similar mode of action to synthetic organophosphate insecticides such as parathion and malathion (Carmichael, 1994). Anatoxin-a(s) inhibits the enzyme acetylcholinesterase irreversibly, and so interferes with the normal events which occur during muscle contraction. Unlike anatoxin-a, anatoxin-a(s) permits the binding of acetylcholine to its receptors. However, it prevents the neurotransmitter from being degraded by acetylcholinesterase, causing muscle cells to become overstimulated.

(a)



(b)

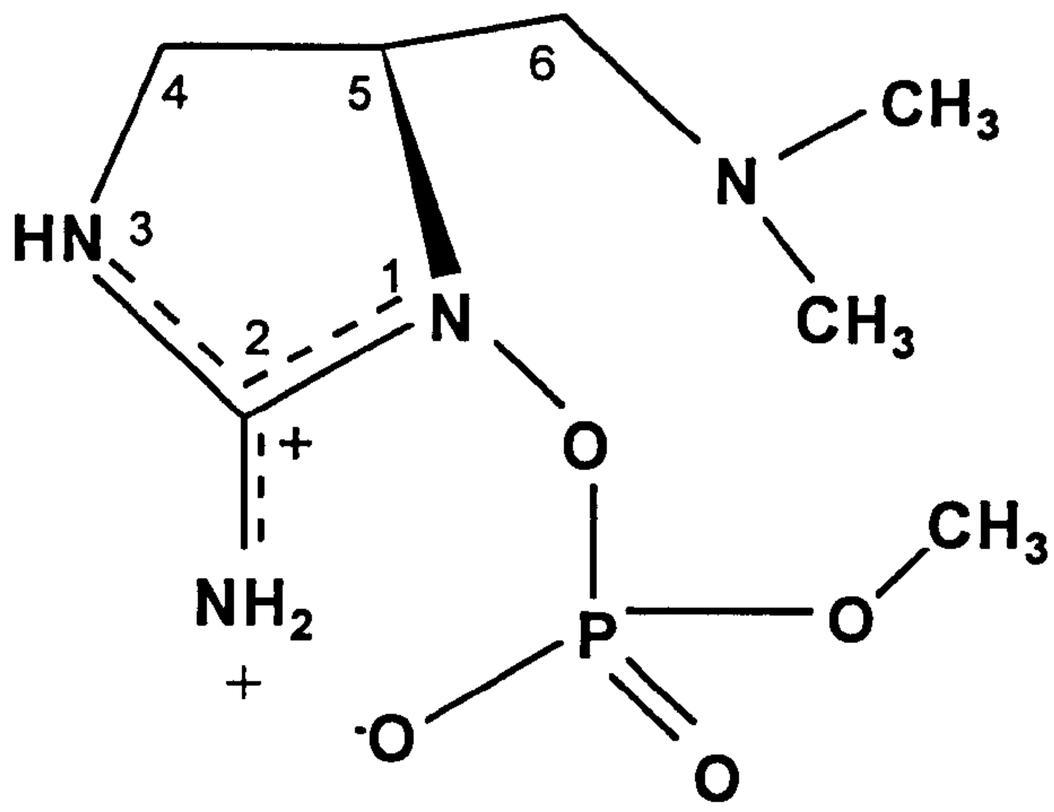


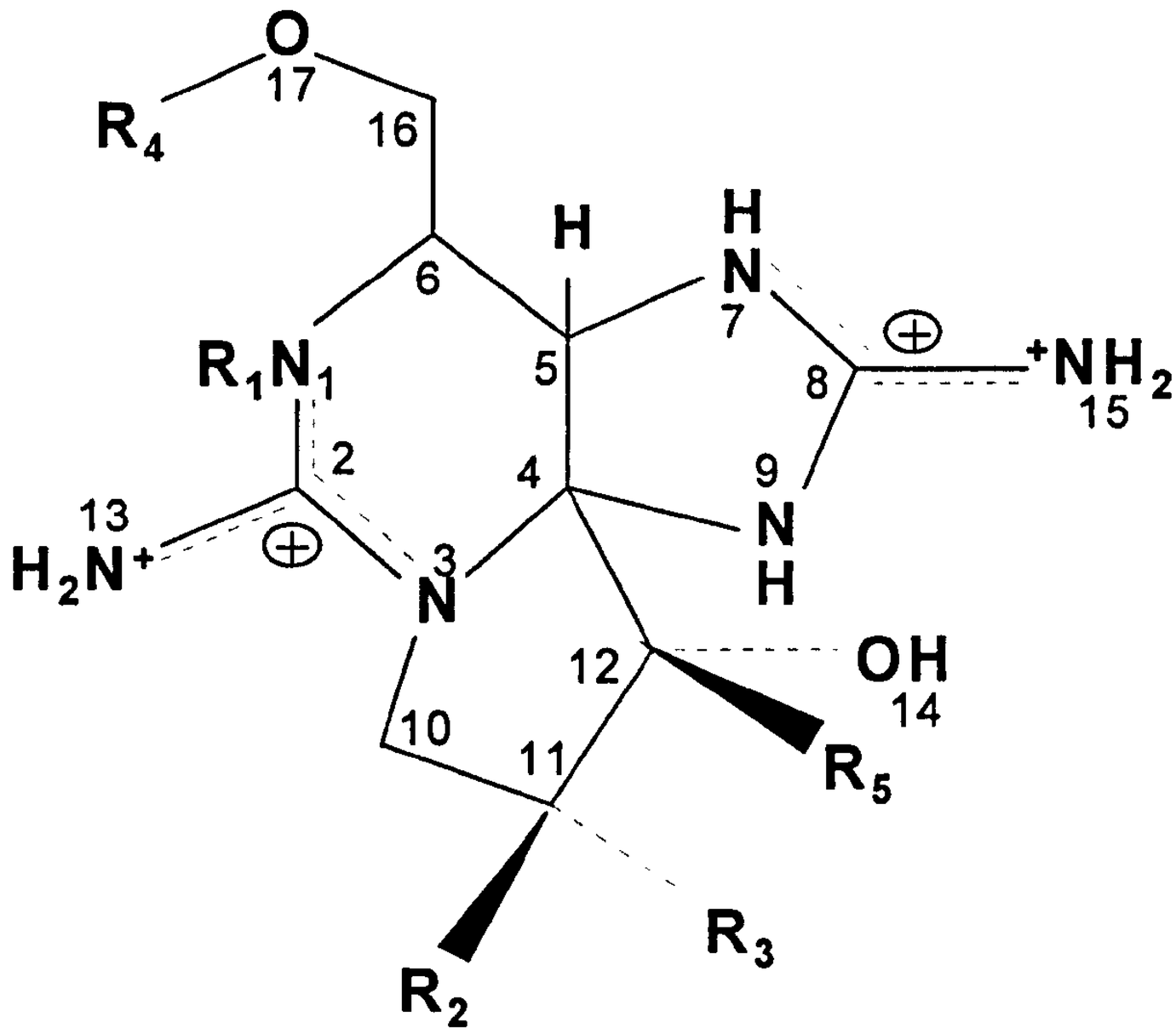
Figure 1.2. The chemical structures of cyanobacterial neurotoxins (a) anatoxin-a, and (b) anatoxin-a(s).

The clinical signs of anatoxin-a(s) intoxication are similar to those of anatoxin-a, but it can be diagnosed in exposed animals by the additional symptom of excessive salivation (Mahmood and Carmichael, 1986 a). Toxicity studies have shown anatoxin-a(s) to be approximately 10 times more potent than anatoxin-a (Carmichael, 1992). Ingestion of the toxin in bloom material containing *Ana. flos-aquae* and *Ana. lemmermannii* has been implicated in the deaths of dogs (Mahmood, Carmichael, and Pfahler, 1988), and birds (Onodera *et al.*, 1997) respectively.

Anatoxin-a(s) is believed to induce death through nicotinic effects on respiratory muscles in addition to muscarinic effects on the cardiovascular and respiratory systems. Atropine has proven successful in antagonising the muscarinic effects of the toxin in rats (Cook *et al.*, 1990), and has been investigated as a possible therapy for anatoxin-a(s) toxicosis (Hyde and Carmichael, 1991).

The third class of cyanobacterial neurotoxins are the saxitoxins. These toxins are also known as paralytic shellfish toxins (PST), as they were originally identified in the marine environment, where they are produced by dinoflagellates (Hashimoto and Noguchi, 1989) and certain bacteria (Ogata, Sato, and Kodama, 1989; Kodama *et al.*,1990 a and b). The saxitoxins are a family of over twenty structurally related tetrahydropurines. They can be divided into three broad groups according to structure (Figure 1.3.). The non-sulphonated saxitoxins are the most highly toxic of these compounds and include saxitoxin and its variant neosaxitoxin. The second group includes the gonyautoxins, which are singly sulphonated and are of intermediate toxicity. The least toxic group in the saxitoxin family are the N-sulphocarbamoyl-11-hydroxysulphate C-toxins (Shimizu, 1988; Oshima, 1995).

Saxitoxins act on nerve axon membranes in a similar manner to a structurally unrelated marine neurotoxin called tetrodotoxin (Kao and Walker, 1982). The toxins selectively block voltage-gated sodium channels and halt impulse propagation between neurones. This causes muscle cells to receive no stimulation and become paralysed (Reviewed by Kao, 1993).



TOXIN	R1	R2	R3	R4	R5
STX	H	H	H	$CONH_2$	OH
NEOSTX	OH	H	H	$CONH_2$	OH
GTX1	OH	H	OSO_3^-	$CONH_2$	OH
GTX2	H	H	OSO_3^-	$CONH_2$	OH
GTX3	H	OSO_3^-	H	$CONH_2$	OH
GTX4	OH	OSO_3^-	H	$CONH_2$	OH
GTX5	H	H	H	$CONHSO_3^-$	OH
GTX6	OH	H	H	$CONHSO_3^-$	OH
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH
dcSTX	H	H	H	H	OH
dcGTX2	H	H	OSO_3^-	H	OH
dcGTX3	H	OSO_3^-	H	H	OH

STX	Saxitoxin
NEOSTX	Neosaxitoxin
GTX	Gonyautoxins
C	C-toxins
dc	decarbamoyl

Figure 1.3. The structural variations of saxitoxins identified in marine and freshwater environments.

Humans are most commonly exposed to saxitoxins through the consumption of contaminated bivalve shellfish, such as mussels and clams, which ingest dinoflagellates indiscriminately and can accumulate the toxins. The ingestion of large quantities of saxitoxins in edible shellfish leads to an acute illness called paralytic shellfish poisoning (PSP). The symptoms of this illness can range from numbness or tingling in the mouth or lips, to paralysis and death from respiratory failure. There have been over 1000 reported cases of PSP in North and Central America since the turn of the century, and approximately 10% of these have resulted in death (Kuiper-Goodman, Falconer, and Fitzgerald, 1999).

Saxitoxin variants have also been identified in a number of freshwater cyanobacteria including *Aph. flos-aquae*, *Ana. circinalis*, *Lyngbya wollei*, and *C. raciborskii.* Strains of *Aph. flos-aquae* isolated from New Hampshire, USA (NH-1 and NH-5) have been found to contain predominantly neosaxitoxin and a small quantity of saxitoxin (Mahmood and Carmichael, 1986 b). Neosaxitoxin has also been identified in a Brazilian isolate of *C. raciborskii* (Lagos *et al.*, 1997). Australian isolates of *Ana. circinalis* have been shown to produce mostly C1 and C2, with lower levels of gonyautoxins 2 and 3 (Negri *et al.*, 1997). In a recent study, decarbamoyl saxitoxin variants (dcSTX, dcGTX2, and dcGTX3) were identified in *L. wollei*, along with six previously unknown analogues (Sivonen and Jones, 1999).

Incidences of human intoxication by saxitoxins in contaminated fresh water have not yet been documented. However, there have been several cases of livestock poisonings attributable to saxitoxin-containing cyanobacterial blooms (Mahmood and Carmichael, 1986 b; Negri, Jones, and Hindmarsh, 1995). One of the most neurotoxic cyanobacterial blooms in recent years occurred in the Darling river of Australia in 1991, which was implicated in the deaths of over 1000 cattle and sheep. This bloom was dominated by *Ana. circinalis* and it was later found to contain a number of saxitoxin variants including saxitoxin, gonyautoxins 2 and 3, and two C-toxins (Humpage *et al*, 1994).

1.2.4. Lipopolysaccaride endotoxins.

The third group of toxic compounds produced by cyanobacteria are the lipopolysaccharide endotoxins. Like other Gram-negative bacteria, cyanobacteria possess cell walls which are composed of lipopolysaccharides. These compounds are endotoxic and pyrogenic (fever-causing) agents (Weckesser and Drews, 1979), but are considerably less toxic than the cyanobacterial hepatotoxins and neurotoxins. Toxicity studies have also revealed that they are ten times less potent than the lipopolysaccharides of other bacterial pathogens such as *Salmonella* (Raziuddin, Siegelman, and Tornabene, 1983). Cyanobacterial lipopolysaccharides cause mainly skin irritations and allergic reactions, but they have also been implicated in gastrointestinal disorders (Keleti *et al.*, 1979).

1.3. Cyanobacterial toxins and human health.

The potential hazards presented by cyanobacterial toxins to human health have been evaluated using the information gathered from laboratory toxicity studies and epidemiological evidence of animal and human poisonings. Animal toxicity tests such as the mouse bioassay have proven useful in indicating the potency of the main groups of cyanobacterial toxins (Table 1.1). They have been used extensively to determine the mode of action of the different toxins and to demonstrate acute and chronic toxicity.

In many cases, evidence for the involvement of cyanobacterial toxins in outbreaks of human illness has been circumstantial. Epidemiological information of animal intoxications has therefore proved invaluable in determining the effects of exposure under natural conditions. The first scientific account of poisoning attributable to a cyanobacterial bloom was published in 1878 by George Francis. He described the fatalities of cattle, horses, sheep, pigs and dogs which had ingested cyanobacteria (later identified as *Nodularia spumigena*) after drinking from Lake Alexandrina, South Australia.

Francis confirmed that the ingestion of cyanobacteria was responsible for the mortalities by exposing a calf to bloom material sampled from the lake, which proved fatal. Since that time, there have been numerous accounts of animal poisonings which have been directly linked to the ingestion of toxic cyanobacteria.

Table 1.1. Comparison of the toxicities of a range of commonly occurring cyanobacterial toxins determined by mouse bioassay.

TOXIN	LD_{50} [a] (mouse bioassay)	REFERENCES[b]
MCYST-LR	25-150	1; 2; 3
(DMAdda^{5}) MCYST-LR	97	4
(Dha^{7}) MCYST-LR	250	4
MCYST-RR	111-650	1; 3; 4
MCYST-LY	91	3; 4
MCYST-LA	39	1
NODULARIN	50	5
CYLINDROSPERMOPSIN	2100 (24 hours); 200 (5-6 days)	6
ANATOXIN-A	375	7
ANATOXIN-A(S)	20	8
SAXITOXIN	10	9
NEOSAXITOXIN	10	9

MCYST: microcystin

a: Toxicity determined by intraperitoneal injection ($\mu g\ kg^{-1}$). LD_{50}: dose required to kill 50% of animals

b: 1. Stoner et al. (1989); 2. Fawell, James, and James(1993); 3. Watanabe *et al.* (1988); 4. Stotts *et al.* (1993); 5. Rinehart, Namikoshi, and Choi (1994); 6. Ohtani, Moore, and Runnegar (1992); 7. Fitzgeorge, Clark, and Keevil (1994); 8. Matsunaga *et al.* (1989); 9. Carmichael (1988).

The occurrence of cyanobacterial toxins in fresh water has been implicated in accounts of human illness from around the world. Many of the reported episodes of intoxication have involved exposure to the toxins through potable water

supplies. However, there are a number of exposure routes which may present a hazard to human health.

1.3.1. Exposure to cyanobacterial toxins in drinking water.

The presence of toxic cyanobacteria in fresh water supplies has been implicated in a number of episodes of human illness. One of the earliest reports described an outbreak of gastro-enteritis in towns situated beside the Ohio River in 1931. In the absence of pathogenic organisms, the outbreak was believed to have been due to a cyanobacterial bloom which had entered the water supply from a side branch of the river (Tisdale, 1931). A more severe outbreak occurred in 1979, involving a community which had obtained water from a dam on Palm island, off the north-eastern coast of Australia. Prior to the outbreak, copper sulphate was used to eradicate a dense cyanobacterial bloom which had tainted the water supply. Shortly afterwards, the community served by the dam experienced a severe outbreak of hepato-enteritis, which resulted in 139 children and 10 adults requiring hospital treatment (Blyth, 1980). The cyanobacterium *C. raciborskii* was later implicated as the causative agent (Hawkins *et al.*, 1985). Recently, a lethal epidemic of gastro-enteritis occurred in Brazil following the flooding of a newly constructed dam. Eighty-eight deaths were reported in areas supplied with drinking water from the dam, which was found to contain high concentrations of *Microcystis* and *Anabaena* (Teixera *et al.*, 1993).

1.3.2. Alternative routes of exposure.

The recreational use of water bodies contaminated with toxic cyanobacteria presents a further route for human exposure. There have been numerous reports of illness in swimmers following contact with cyanobacterial blooms. Skin contact with toxic bloom material has been associated with irritation and contact dermatitis (Codd, Bell, and Brooks, 1989). In some individuals, exposure to cyanobacteria may also cause allergic reactions such as hay-fever and asthma (Falconer, 1994).

A more severe skin condition has been experienced by swimmers after bathing in water containing the marine filamentous cyanobacterium *Lyngbya majuscula* (Grauer, 1961). The condition, known as 'swimmers' itch', causes inflammation, blistering, and desquamation of the skin. The accidental ingestion of toxic cyanobacteria during recreational water use has been implicated in episodes of more serious illness. One such incident occurred in 1989, when a heavy bloom of *M. aeruginosa* on Rudyard Lake, Staffordshire, UK was held responsible for the poisoning of army recruits who used the lake for canoeing exercises and swimming (Turner *et al.*, 1990). Symptoms including diarrhoea, vomiting, blistering of the mouth, and sore throats were experienced by individuals following contact with bloom material. Two of the soldiers were hospitalised after developing severe pneumonia-like symptoms attributed to the inhalation of a *Microcystis* toxin.

Another route of exposure resulted in possibly the most tragic episode of human intoxication to date. Following routine haemodialysis treatment, 86% of the patients attending a clinic in Caruaru, Brazil experienced visual disturbances, nausea and vomiting, and developed cholestatic liver disease. Microscopic examination of the liver tissue of affected patients revealed similar hepatocyte abnormalities to those observed in laboratory animals exposed to microcystins. Analysis of the carbon filter from the clinic's water treatment system later revealed the presence of several microcystins including microcystin-LR. Microcystins were also identified in blood sera and liver tissue from affected patients. The deaths of over 40 patients have now been attributed to the episode (Dunn, 1996; Jochimsen *et al.*, 1998).

The bioaccumulation of cyanobacterial toxins in foodstuffs may provide an alternative pathway for human exposure. Saxitoxins produced by dinoflagellates in the marine environment are known to be transferred to humans and animals via the consumption of shellfish which have accumulated the toxins. The transfer of cyanobacterial toxins to humans via the food chain may also be significant in freshwater environments.

Certain species of freshwater mussels have also been shown to accumulate saxitoxins from the cyanobacterium *Ana. circinalis* (Negri and Jones, 1995). The ability to accumulate microcystins has been demonstrated by a number of aquatic organisms. Microcystins have been detected in zooplankton (Watanabe, Kaya and Takamura, 1992), and crayfish (Liras *et al.*, 1998), indicating the possibility of transfer through the food chain. Studies have also shown the bioaccumulation of significant quantities of microcystins in freshwater mussels including *Anodonta cygnea L.* (Eriksson, Meriluoto and Lindholm, 1989), and *Mytilus galloprovincialis* (Vasconcelos, 1995). There is also some evidence for the concentration of microcystins in fish (Carbis *et al.*, 1997).

The use of water containing cyanobacterial toxins for the irrigation of crops may present a further health hazard for humans. A number of investigations have indicated that microcystins can be taken up by edible terrestrial plants. Exposure to microcystins has resulted in the inhibition of mustard seedling (*Sinapis alba* L.) growth (Kós *et al.*, 1995). One study demonstrated the uptake of radiolabelled microcystin-LR by *S. alba* at levels which exceeded recommended concentrations of the toxin in drinking water (Kurki-Helasmo and Meriluoto, 1998). The toxin was also found to inhibit photosynthesis in runner bean plants following the topical application of concentrations which have been detected in freshwater sources (Abe *et al.*, 1996). These findings are significant since food crops may be exposed to high levels of cyanobacterial toxins in water used for irrigation. While this is likely to have a considerable impact on crop productivity, it may also provide an additional vector for human exposure. Toxins may accumulate in edible plant tissues following uptake by the plant, or by concentration on external surfaces. The implications of these findings for human health are currently unclear, and further research is required to elucidate the fate of cyanobacterial toxins in food plants, and their possible transfer to humans and animals from exposed plant tissues.

1.4. Control and treatment of cyanobacterial blooms.

In order to minimise the risk of serious poisoning events, it has been necessary to monitor the occurrence of toxic cyanobacterial blooms, and the toxins which are produced. Surveys of cyanobacterial blooms occurring in different areas of the world have indicated that approximately half are toxic (Rapala *et al.*, 1994). An extensive monitoring programme carried out by the National Rivers Authority (UK) in 1989 showed that 68% of the cyanobacterial blooms investigated contained toxic species (Pearson *et al.*, 1990). A number of measures have been investigated for controlling the growth of toxic cyanobacteria in water supplies. The development of toxic cyanobacterial blooms may be regulated by addressing the factors which promote their growth and ability to produce toxins. Laboratory studies have demonstrated that the growth and toxin production of cyanobacteria is affected by a number of environmental parameters. These include temperature, light intensity, nutrient concentration, and pH. Studies have indicated that toxin production is generally optimum under conditions which are favourable for cyanobacterial growth. Concentrations of microcystins and anatoxin-a have been shown to be highest in cultures of *Microcystis* and *Anabaena* grown at temperatures between 18 and 25°C (Watanabe and Oishi, 1985; Rapala and Sivonen, 1998). Levels of phosphorous and nitrogen have also been shown to influence microcystin production by certain species (Kotak *et al.*, 1995; Utkilen and Gjølme, 1995; Sivonen, 1990). In one batch culture study, microcystin production by *M. aeruginosa* was highest at extremes of pH (Van der Westhuizen and Eloff, 1983).

In the environment, bloom formation may be prevented by limiting conditions which favour the growth of cyanobacteria. An important measure would be to minimise the input of nitrogen and phosphorous into water bodies from agricultural run-off and human waste. One successful growth limiting strategy has involved artificial aeration, which has been employed to mix and destratify water bodies. It also inhibits growth by limiting light availability (Visser *et al.*, 1996).

Cyanobacterial growth may also be influenced by the biomanipulation of aquatic food chains. These techniques employ measures to encourage the development of either zooplankton species which will consume cyanobacterial cells, or less harmful phytoplankton competitors. However, such strategies can be difficult to implement, and may prove expensive when applied on a large scale.

A short term, but more economical solution involves the use of algicides to destroy problematic cyanobacterial blooms. In the UK, barley straw has been employed to control bloom formation (Newman and Barrett, 1993). The phenolic compounds released into the water when the straw decomposes are believed to possess algicidal properties. However, this technique has had variable success. In one field trial, cyanobacterial growth remained unaffected in ponds treated with barley straw for a six month period (Cheng, Jose, and Mitrovic, 1995). The most extensively used algicide is copper sulphate, but its use can cause elevated levels of toxins to be released into the water. In a healthy bloom, the toxins are usually retained inside the cyanobacterial cells. Laboratory studies involving hepatotoxins and saxitoxins have shown that in actively growing cultures, less than 10-20% of the total toxin concentration is extracellular (Sivonen, 1990; Negri *et al.*, 1997). However, the breakdown of a bloom, either during senescence, or following the use of algicides such as copper sulphate, causes cyanobacterial cells to lyse. This leads to the release of high concentrations of toxins into the water column (Watanabe *et al.*, 1992 a; Kenefick *et al.*, 1993; Jones and Orr, 1994). The treatment of cyanobacterial blooms in fresh water reservoirs with copper sulphate and subsequent release of toxins has been implicated in several outbreaks of human illness.

1.5. Persistence and degradation of cyanobacterial toxins in water.

Most studies into the persistence of cyanobacterial toxins in fresh water have been concerned with microcystins. In living laboratory cultures, intracellular microcystin concentrations have been shown to be relatively stable (Sivonen, 1990). This also appears to be the case in natural environments.

Dried cyanobacterial crusts found on the shores of lakes have been found to contain high levels of microcystins after several months (Jones, Falconer, and Wilkins, 1995). Following their release, however, dissolved toxin may be subject to degradation. Tsuji *et al.* (1994) demonstrated that dissolved microcystins were rapidly decomposed in the presence of photosynthetic pigments and sunlight. Microcystins have also been shown to be broken down by aquatic bacteria identified in lake water and sediments. Cousins *et al.* (1996), showed in laboratory experiments that low concentrations (10 $\mu g\ l^{-1}$) of microcystin-LR were degraded by mixed bacterial populations present in reservoir water in less than one week. The biodegradation of microcystins has been shown to be preceded by a lag phase, during which there is little breakdown of toxin. Jones (1990) showed that microcystin-LR persisted in a range of natural surface waters for three days to three weeks. Once the degradation process had commenced, more than 95% of the toxin was broken down within 3-4 days. However, the efficiency of degradation appears to be dependent on the bacterial populations present in the water body. It has also been suggested that the degradation of microcystins in a water body may depend on its history of cyanobacterial growth (Jones and Orr, 1994; Lahti *et al.*, 1997). In one study, bacteria isolated from a river in Finland failed to degrade a variant of microcystin-RR after 90 days incubation with the toxin (Kiviranta *et al.*, 1991).

There have been fewer studies into the biodegradation of the cyanobacterial neurotoxins and cylindrospermopsin. Rapala *et al.* (1994), demonstrated the degradation of both microcystins and anatoxin-a in the presence of natural bacterial populations from lake water and sediments. In the laboratory, anatoxin-a from a non-axenic culture of *Ana. circinalis* was degraded by a strain of *Pseudomonas sp.* isolated from the growth medium (Kiviranta *et al.*, 1991). The biodegradation of saxitoxins has not yet been demonstrated in fresh water. Investigations carried out by Jones and Negri (1997), showed that saxitoxins extracted from *Ana. circinalis* persisted for over 90 days in river water.

1.6. Removal of cyanobacterial toxins by water treatment processes.

Several conventional water treatment processes have been investigated for their ability to remove cyanobacterial toxins from drinking water. Coagulation, flocculation, and sedimentation processes have proved unsuitable for toxin removal (Drikas, 1994; Rositano and Nicholson, 1994). One study demonstrated the removal of over 80% of the toxins present in *Microcystis*, 30-65% removal of toxins from *Oscillatoria*, and approximately 70% removal of anatoxin-a by slow sand filtration (Keijola *et al.*,1988). However, the dissolved toxin would not be expected to be removed through adsorption to the sand in the filter, but the removal of toxins in these studies was dependent on the activity of bacterial populations present. The most effective methods for toxin removal involve the use of activated carbon (Keijola *et al.*, 1988; Himberg *et al.*, 1989). Granular activated carbon filters have shown considerable promise when used in combination with ozonation (Drikas, 1994). However, these treatments are often expensive. The disinfection of water supplies using chlorine and chloramine has been investigated as a cheaper alternative. Nicholson, Rositano and Burch (1994) found that although the treatment of water with chloramine had little impact on toxin concentration, chlorine effectively destroyed microcystin-LR and nodularin below pH 8. The widespread use of chlorine may be limited, however, as relatively high levels (a chlorine residual of at least 0.5 mg ml^{-1} after 30 minutes contact time) were required for toxins to be completely destroyed. In natural water bodies with a high organic content, such high doses of chlorine may lead to the formation of organic halogens such as trihalomethane, which is a known carcinogen (Bellar, Lichtenberg and Kromer, 1974). Further research is also required to assess the biological effects of chlorinated microcystins.

Photocatalysis has recently been investigated for the detoxification of water contaminated with microcystins (Robertson *et al.*, 1997; Lawton *et al.*, 1999). The photocatalytic oxidation of microcystin-LR using a titanium dioxide catalyst has been demonstrated at extremely high toxin concentrations. This method shows great potential for the detoxification of microcystin-LR in water treatment

processes as it is economical and does not involve the use of potentially toxic reagents.

1.7. Methods for detecting and analysing cyanobacterial toxins in water.

The increased awareness of the hazards presented by cyanobacterial toxins in water supplies has led to the development of a diverse range of methods for their detection and identification. As microcystin-LR has been the most frequently cited cyanobacterial toxin in reports of human and animal intoxications (Carmichael, 1997), a guideline has recently been adopted by the World Health Organisation for the recommended level of the toxin in drinking water (1 μg l^{-1}, WHO, 1998). Specialised detection methods are employed to ensure that toxin levels in potable water do not exceed this guideline. The development of detection methods for cyanobacterial toxins is an ongoing area of research, and a variety of techniques are currently available, ranging from biological based assays to highly sensitive analytical techniques.

1.7.1. Biological based assays and detection methods.

Of the biological detection methods, the mouse bioassay is the longest established, and has been used extensively to determine bloom toxicity. The method relies on the use of laboratory bred mice (usually Male Swiss Albino), which are administered 0.1-1 ml of sterile cyanobacterial lysate by intraperitoneal injection. The mice are then observed for symptoms of intoxication for 24 hours, and sacrificed using an approved method (Falconer, 1993). If a sample is suspected to contain cylindrospermopsin, which is known to cause progressive symptoms, test animals are observed for 7 days. Quantification of toxicity is determined as the concentration of material (mg cell dry weight per kg mouse body weight) which is lethal to 50% of the animals tested (LD_{50}). In general, a sample is taken to be non-toxic at an LD_{50} value greater than 1000 mg cell dry weight per kg mouse body weight (Lawton *et al.*, 1994). The mouse bioassay can verify the overall toxicity of a sample, and observation of symptomology can

indicate the types of toxin which are present. For instance, the presence of neurotoxins in a sample is often indicated by the death of animals within a short period following injection (often within 15 minutes). Animal toxicity tests have provided valuable information on the mode of action of the different cyanobacterial toxins in mammals, and their comparative potencies. They have also been useful in confirming differences between toxin variants, and structural features which are necessary for toxicity. For example, studies have shown that the toxicity of microcystins requires a conserved D-glutamic acid (Stotts *et al.*, 1993), and Adda moiety (Harada *et al.*, 1990). Toxicity tests have also indicated that microcystin toxicity is dependent on the cyclic nature of the molecule (Rinehart, Namikoshi, and Choi, 1994). However, there are a number of problems associated with the mouse bioassay which have prompted the investigation of alternative detection methods. Although the assay provides a rapid measurement of toxicity, it lacks sensitivity. It is also non-specific since the presence of neurotoxins will mask the effects of the more slowly acting toxins, preventing the true toxin profile of the sample from being determined. The mouse bioassay has also suffered from increasing public opposition to the use of animals in toxicity testing, and it is now not permitted in certain countries (Falconer, 1993).

Alternative bioassay methods involving the use of invertebrates have been investigated for the detection of cyanobacterial toxins. Many of these methods may be carried out in basic laboratories as they require no specialised handling or equipment. A successful alternative to the mouse bioassay for the detection of hepatotoxins is the brine shrimp (*Artemia salina*) bioassay. Brine shrimp larvae are exposed to a dilution series of test sample (usually a methanolic extract of filtered cyanobacterial cells, diluted to 20% v/v with brine shrimp growth medium), in 96 well microtitre plates. After 18 hours at 25°C, toxicity is expressed as the LC_{50} value (concentration of sample which caused 50% mortality). The brine shrimp bioassay is simple to perform, inexpensive, and has been shown to compare favourably with both the mouse bioassay and high performance liquid chromatography (HPLC) for the detection of microcystins in bloom samples (Lawton *et al.*, 1994).

A bioassay employing *Daphnia* has also been investigated for the detection of microcystins. This method is cheap and has been found to correlate well with the mouse bioassay, but it has proved difficult to standardise due to varying sensitivities between species (Baird *et al.*, 1989).

Various insect species have been investigated as assay organisms for detecting cyanobacterial toxins. Microcystin-LR has been shown to be a powerful insecticide, with comparable potency to the insecticides rotenone, malathion and carbofuran (Delaney and Wilkins, 1995). This has been exploited in several bioassay techniques. Kiviranta *et al.* (1993) demonstrated the toxicity of a microcystin-RR variant to larval yellow fever mosquito (*Aedes aegypti*) by immersing the insect in a solution of the toxin. Adult female *Culex pipiens* mosquitoes have also been shown to be sensitive to microcystin-LR by intrathoracic injection (Turell and Middlebrook, 1988). Although these methods were capable of detecting low concentrations of microcystins, difficulties in handling the insects have prevented their widespread use. Swoboda *et al.* (1994) developed an alternative insect bioassay employing fruit flies (*Drosophila melanogaster*) for the detection of microcystins. This bioassay is more practical as the organisms may be easily maintained in most laboratories without specialised handling. It is also easier to carry out as toxin is administered orally, rather than by microinjection. The fruit flies are starved for 24 hours before being allowed to feed on filter disks spotted with sucrose solutions containing toxin. This assay was found to correlate well with the mouse bioassay for determining hepatotoxicity in cyanobacterial blooms. However, as it is unable to provide detailed information such as dose response it has only been recommended as an indicator of toxicity.

Adult house flies (*Musca domestica*) have been investigated as a replacement to the mouse in the detection of saxitoxins (Ross *et al.*, 1985). The house fly bioassay detected saxitoxins in extracts of contaminated shellfish tissue with comparable sensitivity to the mouse bioassay. However, the bioassay has not been widely implemented as it involves a microinjection technique which requires considerable expertise.

A number of bioassays involving bacteria have also been assessed for cyanobacterial toxin detection. These include bioassays which can indicate toxicity by the inhibition of bioluminescence and pigment production by certain bacteria. A commercially available bioassay system is the Microtox bioluminescence assay, which exploits the luminescence of *Photobacterium phosphoreum*. This assay involves the incubation of the bacterium with test solution prior to measuring the amount of light that is emitted by the organism. Toxicity is expressed as an EC_{50} value (Effective concentration causing a 50% reduction in emitted light). The simplicity and standardisation of the Microtox bioluminescence assay make it an ideal tool for the detection of cyanobacterial toxins. However, investigations involving both microcystins and anatoxin-a have demonstrated poor correlation between luminescence and toxin concentration (Lawton *et al.*, 1994; Lahti *et al.*, 1995). In a study which employed the Microtox bioassay to detect microcystin in cyanobacterial extracts, luminescence was completely inhibited by samples which did not contain any known cyanobacterial toxins (Campbell *et al.*, 1994).

The inhibition of bacterial pigment formation by cyanobacterial toxins has also been studied as a method for toxin detection. The production of the pigment prodigiosin by *Serratia marcescens* has been shown to be inhibited by both microcystins and saxitoxins (Dierstein, Kaiser, and Weckesser, 1989). However, although this assay gave a dose dependent response with extracts of *M. aeruginosa* and *Aph. flos-aquae*, it did not correlate with purified microcystin, suggesting that other inhibitory compounds were present in the extracts.

Cytotoxicity tests which employ mammalian cells have shown considerable promise for replacing the mouse bioassay. The hepatotoxic effects of microcystins have been exploited in several investigations using liver cells. Initial studies indicated a good correlation between microcystin concentration and hepatocyte damage by measuring the subsequent release of the enzyme lactate dehydrogenase (Aune and Berg, 1986). Recent investigations have indicated the potential of rat hepatocytes as a screening tool for microcystins. Heinze (1996), demonstrated a time-dependent loss of viability in rat hepatocytes incubated with

microcystin-LR or hepatotoxic bloom extracts. The assay also detected differences between microcystin variants comparable with results obtained from the mouse bioassay.

Mouse neuroblastoma cells have been successfully employed to detect the sodium channel-blocking saxitoxins (Gallacher and Birkbeck, 1992; Jellett *et al.*, 1992). Exposure to veratridine and ouabain results in the opening of sodium channels in mouse neuroblastoma cells, leading to an influx of sodium ions which causes the cells to lyse. The blocking of sodium channels by saxitoxins prevents the actions of the other two compounds, and allows cells to retain their morphology. Viable cells are then stained and measured to quantify toxicity. A test kit based on this assay is now available commercially, and it is currently being evaluated as a replacement to the mouse bioassay for the screening of shellfish extracts.

The effects of saxitoxins on nerve membranes have also been exploited to develop bioassay techniques. A neuroreceptor binding assay was developed by Davio and Fontelo (1984) in which the binding of saxitoxins to rat brain membranes was determined by the displacement of radiolabelled saxitoxin. This method was shown to correlate well with the mouse bioassay for the screening of shellfish samples (Cembella *et al.*, 1995). More recently, a channel biosensor was constructed from a Na^+ electrode covered with a frog bladder membrane (Cheun *et al.*, 1998). By measuring the active transport of sodium ions across the membrane, levels of saxitoxin could be quantified. The assay also showed a good correlation with the mouse bioassay for the quantification of saxitoxin, neosaxitoxin, and four GTX toxins in shellfish extracts. Although these methods have proved sensitive, their widespread utility may be prevented for ethical reasons, as they depend on the use of animal tissue.

Various techniques have also been developed for the immunological detection of cyanobacterial toxins. Both polyclonal and monoclonal antibodies have been raised against microcystins, and these have been employed for detecting the toxin using Enzyme-Linked Immuno Sorbent Assay (ELISA) techniques. Preliminary studies were carried out by Kfir, Johannsen, and Botes (1986), who raised monoclonal antibodies specific for microcystin-LA. Polyclonal antibodies which were raised against microcystins isolated from *M. aeruginosa* were employed in an ELISA technique and successfully detected the toxins in cyanobacterial extracts (Brooks and Codd, 1988). In another study, polyclonal antiserum was raised in rabbits against bovine serum albumin conjugated with microcystin-LR (Chu *et al.*, 1989). When used in an ELISA method, the antibodies cross-reacted well with microcystin -RR, -YR and nodularin. This bioassay has since been shown to detect microcystin concentrations of 0.2 $\mu g\ l^{-1}$ in water samples (Chu, Huang, and Wei, 1990). Monoclonal antibodies developed by Nagata *et al.* (1995) have recently been employed in a more sensitive ELISA technique. With a detection limit of 0.05 $\mu g\ l^{-1}$, this method has provided a means of analysing drinking water for low levels of microcystins which may contribute to the occurrence of cancer (Ueno *et al.*, 1996).

Variations of ELISA have also been investigated for the detection of saxitoxins (Chu and Fan, 1985; Usleber, Schneider, and Terplan, 1991). These techniques can be useful in the screening of shellfish samples, but they suffer from poor cross-reactivity with other saxitoxin variants, including neosaxitoxin. The failure of these methods to detect saxitoxin variants therefore renders them unsuitable for the screening of samples (particularly neurotoxic cyanobacterial extracts), which do not contain saxitoxin but have higher concentrations of the variant compounds.

Detection methods have also been developed which exploit the biochemical activities of certain toxins. The detection of microcystins has been facilitated by exploiting the ability of the toxins to inhibit protein phosphatases. Protein phosphatase assays involve measuring the inhibitory effect of microcystins on the

release of phosphate from phosphorylated protein substrates (Bell and Codd, 1996). Phosphate release may be quantified using ^{32}P-labelled substrates (MacKintosh *et al.*, 1990; Holmes, 1991), or the chromogenic substrate *p*-nitrophenol phosphate (An and Carmichael, 1994; Ash *et al.*, 1995). Both types of assay have proved successful in determining sample toxicity, but the use of radioactive compounds may not be feasible in all laboratories. Although the colorimetric protein phosphatase assay is slightly less sensitive than the radioactive assay, it has correlated well with HPLC for the detection of microcystins in cyanobacterial samples (Ward *et al.*, 1997).

Detection of the acetylcholinesterase inhibitor, anatoxin-a(s) may also be carried out using a colorimetric enzyme assay (Mahmood and Carmichael, 1987). This method provides a sensitive alternative to the mouse bioassay for the determination of anatoxin-a(s) in samples, but is not specific for the toxin, as it may detect organophosphate pesticides which have the same mode of action.

1.7.2. Analytical techniques.

A number of analytical techniques may be employed for the detection of cyanobacterial toxins. These methods are particularly useful as they are both quantitative and qualitative. The most commonly used analytical method is high performance liquid chromatography (HPLC). Reversed phase HPLC in conjuction with photodiode array (PDA) detection has been used very successfully for the detection of microcystins. Prior to toxin detection by HPLC, microcystins can be extracted from cyanobacterial cells in a number of solvents, including 5% acetic acid, methanol, acidified methanol, or butanol: methanol: water (5:20:75) (Harada, 1996). Methanol has been shown to be the most efficient solvent for extracting the different microcystin variants which may be present in a sample (Lawton, Edwards and Codd, 1994). The use of methanol also allows samples to be concentrated by evaporation, thus increasing the likelihood of detection.

One of the most commonly used HPLC methods for microcystins involves the separation of the toxins on a C_{18} silica column using a gradient of water and acetonitrile, both containing 0.05% trifluoroacetic acid (TFA). Microcystins are then detected by UV at 238 nm and identified by their characteristic absorption profiles between 200 and 300 nm (Lawton, Edwards, and Codd, 1994). The detection of cylindrospermopsin may also be facilitated using reversed phase HPLC with photodiode array detection (Hawkins *et al.*, 1997). A methanol gradient is applied to separate aqueous acetic acid extracts, and detection of cylindrospermopsin is achieved at 262 nm. HPLC has also proved a useful tool in the detection of anatoxin-a in cyanobacterial samples (Harada *et al.*, 1989; Edwards *et al.*, 1992). Reversed phase columns can be used with a number of different mobile phases for the analysis of anatoxin-a. The toxin is identified by its characteristic UV absorbance at 227 nm.

HPLC techniques have been used extensively for the determination of saxitoxins. Although these techniques have been optimised for the detection of the toxins in shellfish samples, they may also be used for toxin detection in other sample matrices including cyanobacterial extracts. The HPLC methods involve the detection of fluorescent saxitoxin analogues, which are formed following the oxidation of samples. This reaction may be carried out either before (prechromatographic) or after (postchromatographic) samples are injected onto the column.

Lawrence *et al.* (1995) has optimised an HPLC method involving the prechromatographic oxidation of shellfish samples using either peroxide or periodate. Although no single set of oxidation conditions is optimum for all of the saxitoxins (Lawrence *et al.*, 1991; Lawrence and Menard, 1991), periodate has been used most extensively as it enables the detection of a wider range of variants. Following sample oxidation, the toxins are separated on a reversed phase column and detected using a fluorescence detector set at 330 nm (excitation) and 400 nm (emission). This method has been used successfully in the analysis of shellfish extracts for a range of saxitoxin variants. A modified

version of the methods described by Lawrence *et al.* (1991; 1995) has also proved useful in identifying the most efficient method for extracting saxitoxins from the cyanobacterium *Aph. flos-aquae* (McElhiney *et al.*, 1998).

The HPLC method for saxitoxin detection which has gained most favour is HPLC with on-line postcolumn oxidation and fluorescence detection (Oshima, 1995). In this technique toxins are separated on a C_8 column and oxidised with periodate in a heated reaction coil following their elution. Acetic acid is then delivered to the sample, and the resulting fluorescent compounds are detected as described for the precolumn method. The requirement for three HPLC pumps to deliver sample, oxidant and acidifier can make this technique expensive. It can also be labour intensive, as three different mobile phases are needed to facilitate the detection of all of the saxitoxin variants. Nevertheless, it is reproducible, and has provided good separation of saxitoxin variants in both dinoflagellate and shellfish matrices. It is also more suitable for the analysis of large numbers of samples than the precolumn method as it is easier to automate.

Capillary electrophoresis (CE) has shown some promise as a detection method for saxitoxins. This analytical technique involves the separation of the toxins according to differences in their molecular size and charge (Bell and Codd, 1996). CE combined with ion-spray mass spectrometry has been investigated for the detection of saxitoxins in both dinoflagellate and shellfish extracts (Thibault, Laycock, and Pleasance, 1991). However, as only a small volume of sample (less than 10 nl) can be injected onto the system, analysis using CE has given poor detection limits (Pleasance, Thibault, and Kelly, 1992; Locke and Thibault, 1994).

The identity of the cyanobacterial toxins present in a sample may be confirmed using mass spectrometry. The separation and identification of microcystins may be carried out simultaneously using liquid chromatography/mass spectrometry (LC/MS) (Kondo *et al.*, 1992 a; Edwards *et al.*, 1993). LC/MS with fast atom bombardment ionisation (Frit-FAB) has been used with considerable success to identify microcystins from different mixtures of compounds in bloom samples

(Kondo *et al.*, 1992 a). The method was recently optimised to allow the detection of nanogram levels of microcystins in water and biological samples (Kondo *et al.*, 1995, 1996). Mass spectrometry has been used in conjunction with gas chromatography (GC/MS) for the identification of anatoxin-a in bloom and water samples (Himberg, 1989; Sivonen *et al.*, 1989), and in the stomach contents of dogs which had ingested the toxin (Edwards *et al.*, 1992).

1.8. Aims of the thesis.

Although there are a wide variety of methods currently available for the detection of cyanobacterial toxins, all have associated disadvantages which prevent their utility in many laboratories. To date, none of the biological based assays developed have been validated sufficiently to replace the mouse bioassay. In particular, the detection of saxitoxins is still largely dependent on the mouse bioassay despite the considerable research efforts which have been made to find a replacement. A number of drawbacks have been associated with the mouse bioassay, including considerable interlaboratory variation, which has been observed during trials involving highly toxic shellfish samples (Park *et al.*, 1986).

Many of the detection methods developed for saxitoxins have also been optimised to deal with shellfish and dinoflagellate extracts. Although methods for the extraction of saxitoxins from these sample matrices have been explored, there is little information on the recovery of the toxins from cyanobacterial cells. It was therefore important to explore the suitability of existing extraction methods for the recovery of saxitoxins. In this study, the recovery of saxitoxins from a laboratory culture of *Aph. flos-aquae* was assessed using four different media. HPLC with precolumn oxidation and fluorescence detection was employed to identify saxitoxin variants, and to determine the amounts of toxin extracted.

The requirement for an alternative to the mouse bioassay for assessing sample toxicity was then addressed by developing a bioassay using desert locusts (*Schistocerca gregaria*). Pure toxin standards were employed to ascertain

the suitability of the locust for the detection of saxitoxins, and to establish assay parameters. The assay was then applied to verify the toxicities of *Aph. flos-aquae* extracts previously analysed by HPLC (McElhiney *et al.*, 1998). Methodology developed for saxitoxin extraction and detection was then applied to determine the toxicities of natural cyanobacterial bloom samples identified as neurotoxic by mouse bioassay.

The locust bioassay was then investigated for the detection of saxitoxins in shellfish extracts. Preliminary studies were carried out to assess the compatibility of the assay with the standardised extraction method for shellfish, and its ability to detect alert levels of saxitoxins in extracts. The optimised bioassay was finally employed in a large shellfish monitoring programme in conjunction with the mouse bioassay and the results compared (McElhiney *et al.*, 1997).

The detection of cyanobacterial toxins using analytical techniques such as HPLC is often hindered due to the lack of toxin standards. This has been a limiting factor in the analysis of microcystins and saxitoxins in particular, due to the number of variant compounds which exist. As microcystins are the most commonly occurring cyanobacterial toxins, considerable research has been focused on methods for purifying the toxins from cyanobacteria. Several purification methods have been developed, but most are labour intensive, and are capable of purifying only small quantities of microcystins. Furthermore, many of the commonly used techniques have proven unsuitable for the extraction and purification of more hydrophobic toxins. It is important that a wider range of purified microcystins are made available, in order that the detection of variants can be improved, and their biological activity elucidated. A large scale purification method was employed for recovering microcystins from laboratory grown cultures of *M. aeruginosa*. The method provided a rapid means of obtaining microcystin-LR at sufficient purity for use in subsequent laboratory studies.

This study also set out to address previous difficulties experienced in the purification of two relatively hydrophobic microcystins, -LW and -LF, which are produced by laboratory strains of *M. aeruginosa* and are commonly identified in natural bloom samples (Lawton, McElhiney and Edwards, 1999). The effects of purified microcystin variants on the growth and development of plants were then examined using bioassay techniques. Although the biological effects of microcystins have been established in animals, considerably less information is available on the impact of the toxins on vegetation. This is significant, since crop plants may be exposed to levels of these toxins in irrigation water. High levels of cyanobacterial toxins may be present in water supplies used for the irrigation of crop plants, and may have an adverse effect on productivity (McElhiney, Lawton and Leifert, submitted).

The accumulation of the toxins in edible plant material may also provide a further route of exposure for humans. The uptake and fate of microcystin in plant tissues was explored using two plant models. The biological effects of long-term microcystin-LR exposure were also assessed by exposing growing runner beans to the toxin in a liquid growth medium. Finally, an extraction method was devised for recovering the toxin from plant tissues. The recovery of toxin from extracted plant material was then assessed using HPLC and an invertebrate bioassay (McElhiney, Lawton and Leifert, 1998).

Chapter 2

Development of extraction and detection methods for saxitoxins from cyanobacteria and shellfish

2.1. Introduction.

The saxitoxins, also known as paralytic shellfish toxins (PST) are a family of over twenty structurally related neurotoxins produced by marine dinoflagellates (Hashimoto and Noguchi, 1989), freshwater cyanobacteria (Mahmood and Carmichael, 1986 b), and marine bacteria (Ogata, Sato, and Kodama, 1989; Kodama *et al.*,1990 a and b). The toxins selectively block sodium channels and halt the propagation between neurones, causing muscle cells to become paralysed (Reviewed by Kao, 1993).

Humans are most commonly exposed to saxitoxins through the consumption of contaminated bivalve shellfish, such as mussels and clams, which ingest dinoflagellates indiscriminately and accumulate the toxins. There have also been reported cases of livestock poisoning attributable to saxitoxin-containing cyanobacterial blooms (*Aphanizomenon flos-aquae* and *Anabaena circinalis*) in freshwater sources (Mahmood and Carmichael, 1986 b; Negri, Jones, and Hindmarsh,1995). One of the most notable occurrences in recent years was an extensive cyanobacterial bloom in the Murray-Darling river basin of Australia which was found to contain a number of saxitoxin variants (Humpage *et al.*, 1994). Increased eutrophication of fresh waters due to intensive agricultural practices and sewage runoff has led to the widespread occurrence of nuisance cyanobacterial blooms. With a high proportion of blooms being found to be toxic, and the increasing awareness of these compounds in the freshwater environment there is a need for more extensive monitoring.

Monitoring programmes have been in place for many years in the marine environment. In most countries where paralytic shellfish poisoning is a threat, large scale shellfish screening programmes are undertaken to minimise the risk to human health. Currently, the only authorised method for the detection of saxitoxins in shellfish is the mouse bioassay, which was first developed by Sommer and Meyer in 1937. It has since been standardised (AOAC, 1990), and is now used in monitoring programmes worldwide. This method involves the

extraction of 100 g shellfish flesh in 100 ml of 0.1 N HCl for 5 minutes at 90°C. Once the extract has cooled, 1 ml is injected intraperitoneally into a 19-21 g mouse, and the time of death is recorded. The toxicity of the extract is determined in mouse units, which can then be converted to μg saxitoxin equivalents/100 g shellfish tissue. The harvesting of shellfish for human consumption is generally prohibited when toxin concentration reaches 80 μg saxitoxin equivalents/100 g shellfish tissue (Fernandez and Cembella, 1995). To date, there are no such approved levels of saxitoxins in freshwater.

Although the mouse bioassay provides a rapid quantification of toxicity with a minimum of expertise, there are a number of disadvantages associated with it, including poor reproducibility (Park *et al.*, 1986), and interferences from sample constituents. For example, high salt concentrations (particularly Na^+ ions), can cause a decrease in the toxicity of extracts by mouse bioassay (Schantz *et al.*, 1958). Elevated levels of zinc in extracts can also lead to false toxicity determinations by inducing symptoms in mice that are similar to those caused by saxitoxin intoxication (McCulloch *et al.*, 1989). These problems, coupled with increasing public pressure on the use of mammals in screening programmes, have prompted research into alternative methods of detection and quantification. There are a wide variety of techniques under investigation, ranging from sophisticated instrument based analytical techniques to biological assays. Of the instrument based techniques, High Performance Liquid Chromatography (HPLC) has gained most favour. A number of methods for the analysis of paralytic shellfish toxins by HPLC have been optimised (Lawrence, Menard, and Cleroux, 1995; Oshima, 1995), and these have been used in many monitoring programmes in conjunction with the mouse bioassay. Although HPLC is sensitive, it is only cost effective in large scale monitoring, and requires considerable skill. The widespread use of analytical techniques has also been hindered by the lack of toxin standards.

More recently developed bioassays, including tissue culture assay (Kogure *et al.*, 1988; Jellett *et al.*, 1992), and various immunological techniques (Chu and Fan, 1985; Smith and Kitts, 1994; Kralovec *et al.*, 1996) have proved highly sensitive, but can be laborious and difficult to implement in many laboratories. Immunoassays such as ELISA have also suffered from variable cross reactivities with different saxitoxin variants (Usleber, 1991). Investigations into the properties of the voltage gated sodium channel has led to the development of several neuroreceptor binding assays (Trainer, Baden, and Catterall, 1995; Cheun *et al.*, 1998). These techniques are also capable of detecting the structurally unrelated tetrodotoxins, another group of marine toxins which compete with saxitoxin for the same binding on the sodium channel (Kodama *et al.*, 1996). A saxitoxin specific assay has recently been reported employing saxiphilin; a transferrin isolated from the haemolymph of centipedes, which binds to saxitoxins but not to tetrodotoxins (Negri and Llewellyn, 1998).

Further investigation is required into the development of accurate screening techniques which may be carried out with a minimum of expense and skill. Saxitoxins pose a threat to the health of populations world-wide, and it is important that they can be monitored with a minimum of cost. An inexpensive alternative to the mouse bioassay may be the use of a suitable insect species as the test organism. Insects have been employed as screening tools for a range of natural neurotoxins. Toxins found in arthropod venoms have been detected by assays employing either the cricket *Gryllus bimaculatus* or the fruit fly *Drosophila melanogaster* (Escoubas, Palma and Nakajima, 1995), and the mosquito *Aedes aegypti* has been used to detect ciguatoxin (Chungue, Bagnis and Parc, 1984). An alternative bioassay system for PST was developed which involved the common housefly (*Musca domestica*) in place of the mouse as the test organism (Ross, Siger, and Abbott, 1985). The housefly bioassay is a sensitive method for the detection of PST, but the microinjection (1.5 μl) technique involved is difficult to perform in basic laboratories. The bioassay also requires that insects are anaesthetised prior to injection, which may have some impact on results.

This chapter describes the development of an alternative bioassay technique for the detection of saxitoxin and its variant neosaxitoxin in cyanobacterial and shellfish extracts involving the use of the desert locust (*Schistocerca gregaria)* in the screening of samples. The locust was found to be an appropriate model for the detection of saxitoxins following preliminary studies in which the insects were tested with a range of toxins purified or extracted from cyanobacterial cells. The locust bioassay was used in conjunction with HPLC to develop a processing method for the extraction of saxitoxin and neosaxitoxin from cyanobacterial cells. Samples taken from a river contaminated by a cyanobacterial bloom found to be neurotoxic by mouse bioassay were then processed using the optimum extraction method and their toxicities determined by locust bioassay. HPLC was then used to confirm the presence of saxitoxins in the samples. Finally, acid extracts of toxic mussel tissue were assessed by locust bioassay, and the results compared to mouse bioassay data.

2.2. Materials and methods.

2.2.1. Toxins and cyanobacterial cultures.

Saxitoxin diacetate (1 μmol in 1 ml AcOH), and anatoxin-a were purchased from Calbiochem (Novabiochem Corporation, LaJolla, CA, USA). Saxitoxin, neosaxitoxin, and gonyautoxin mixtures ($GTX_{2/3}$ and $GTX_{1/4}$) were purchased in kit form from the National Research Council, Canada. Microcystin-LR was purified from bloom material of *Microcystis* sp. as previously described (Edwards *et al.*, 1996 b). Toxins were diluted in a simple saline solution unless stated otherwise. Saline solution was prepared by dissolving 7.5 g sodium chloride and 0.37 g potassium chloride (AnalR, BDH, Poole, UK) in 1000 ml glass distilled water, corrected to pH 3.3 using 1M HCl (AnalR, BDH). Cultures of the cyanobacteria *Aph. flos-aquae* NH-5a; known to produce saxitoxin and its variant neosaxitoxin (Mahmood and Carmichael, 1986 b) were provided by W.W. Carmichael. *M. aeruginosa* CYA 43 (NIVA, Oslo, Norway), a non-toxic species, and *Ana. flos-aquae* NRC 525-17, known to produce the acetylcholinesterase inhibitor anatoxin a(s) (Mahmood and Carmichael, 1987) were grown in BG-11 medium (*Stanier et al.*, 1971) plus nitrate (8.8 mM $NaNO_3$). All cultures were grown in 4-8 litre flasks for one month under continuous illumination (20 μmol m^{-2} s^{-1}) by cool white fluorescent tubes (36 W), and sparged with sterile air throughout. Growth temperature ranged between 21 and 29°C. Cells were harvested by filtering onto GF/C filter disks (110 mm, Whatman International Ltd, Maidstone, UK), to a dry weight of 100 mg. All dried cells and toxins were stored at -20°C until required.

BG-11 plus nitrate

$NaNO_3$	0.750 g l^{-1}
K_2HPO_4	0.040 g l^{-1}
$MgSO_4.7H_2O$	0.075 g $^{-1}$
Na_2CO_3	0.020 g l^{-1}
$CaCl_2.2H_2O$	0.036 g l^{-1}
EDTA	0.001 g l^{-1}
$FeSO_4.7H_2O$	0.006 g l^{-1}
Citric acid	0.006 g l^{-1}
Trace element solution	1 ml l^{-1}

Trace element solution

H_3BO_3	2.680 g l^{-1}
$MnCl_2.H_2O$	1.810 g l^{-1}
$ZnSo_4.7H_2O$	0.222 g l^{-1}
$Na_2MoO_4.2H_2O$	0.390 g l^{-1}
Cu $(NO_3)_2.6H_2O$	0.079 g l^{-1}
Co $(NO_3)_2.6H_2O$	0.049 g l^{-1}

2.2.2. Detection of saxitoxins by prechromatographic oxidation followed by HPLC with fluorescence detection.

Samples were analysed according to a modified version of the prechromatographic periodate oxidation methods described by Lawrence *et al.* (1991) and Lawrence and Menard (1991). All chemicals used in HPLC analyses were prepared using Milli-Q water (Millipore, Watford, UK). Oxidant was prepared by mixing 1 ml of 0.03 M periodic acid (Sigma-Aldrich, Poole, Dorset, UK) with 1 ml of 0.03 M ammonium formate and 1 ml of 0.03 M disodium hydrogen phosphate (AnalR).

The mixture was prepared daily, and corrected to pH 8 with approximately 50 μl of 1 M sodium hydroxide. Samples were derivatised manually by mixing 25 μl with 125 μl of oxidant for 3 minutes, followed by acidification with 5 μl glacial acetic acid (HiPerSolv, BDH, Poole, UK) for 2 minutes prior to injection. For the verification of neosaxitoxin in samples, oxidation was carried out using peroxide. Sample (15 μl) was mixed with 10 μl of 10% aqueous hydrogen peroxide (AnalR, BDH), followed by 50 μl of 1 M sodium hydroxide (Fisher Scientific). The solution was allowed to react at room temperature for 2 minutes then vortexed thoroughly with 5 μl glacial acetic acid prior to injection. HPLC analysis was carried out using a Waters 600E multisolvent delivery system with a Linear Fluor LC304 fluorescence detector. A Waters Symmetry C_{18} column (150 x 4.6 mm I.D.; 5 μm particle size) was used in all analyses and the injection volume was 25 μl. The mobile phase consisted of (A) 0.02 M potassium dihydrogen phosphate; and (B) acetonitrile (Rathburn, Walkerburn, U.K.). Solvent (B) was linearly increased from 0 to 20% over 30 minutes at a flow rate of 1 ml min^{-1}. The column was then returned to 0% B over 10 minutes and equilibrated at the starting conditions for a further 10 minutes between injections. Fluorescence was detected with excitation at 332 nm and emission at 390 nm. A calibration curve for saxitoxin was constructed using this method in the range of 0-100 ng injected onto the column.

2.2.3. Stability of saxitoxin derivatives.

In order to facilitate the analysis of large numbers of samples using the HPLC method described in 2.2.2., an experiment was carried out to determine the stability of manually oxidised samples over time. Ten samples of saxitoxin (5 μg ml^{-1}) were oxidised using periodate as described, and placed in a Waters 717 plus autosampler for injection. The autosampler was kept at room temperature throughout analyses. The stability of saxitoxin derivatives was determined from 0 to 450 minutes at 50 minute intervals (the duration of one HPLC run).

2.2.4. Detection of saxitoxins by HPLC with post-column oxidation and fluorescence detection.

An alternative method employed to analyse large numbers of samples was developed from the method of Oshima (1995). This technique involves post column oxidation of samples followed by fluorescence detection. Three different mobile phases are used to separate the different classes of PSP toxins. All chemicals were AnalR grade and obtained from Fisher Scientific (Leistershire, UK) unless stated otherwise. Analyses were carried out using a Waters 600E multisolvent delivery system to deliver mobile phase at a flow rate of 0.8 ml min^{-1}, and 2 Bromma LKB 2150 HPLC pumps set at a flow rate of 0.4 ml min^{-1}, to deliver oxidant and acidifer. Samples were injected by a Waters 717 plus autosampler, and separated using a C_8 Inertsil column (150 x 4.6 mm I.D.; particle size 5 μm). Sample oxidation was carried out in 10 m Teflon tubing (0.5 mm I.D.) in a dry oven (80 °C). Detection was achieved using a Linear Fluor LC304 fluorescence detector. Samples were analysed using the appropriate mobile phases described by Oshima, for the separation of saxitoxin and neosaxitoxin, and gonyautoxins 1, 2, 3, and 4. Reagents used for the preparation of mobile phase solutions were; sodium 1-heptanesulphonate (Sigma, Dorset, UK), orthophosphoric acid (85%), ammonium hydroxide, and acetonitrile. Solutions were prepared using Milli-Q water. The mobile phase for the separation of saxitoxin and neosaxitoxin was prepared by dissolving 0.41 g sodium 1-heptanesulphonate in 800 ml water, adding 3.48 ml phosphoric acid (85%) and adjusting the solution to pH 7.1 with ammonium hydroxide. Finally, 30 ml of acetonitrile was added, and the solution made up to a final volume of 1000 ml with Milli-Q water. The mobile phase for the separation of GTX toxins was prepared in the same way except the volume of phosphoric acid added was 1.16 ml, and no acetonitrile was added. Oxidising reagent was prepared by dissolving 11.4 g of dipotassium hydrogen phosphate in 800 ml water, and adding 1.6 g of periodic acid. The pH of the solution was then adjusted to pH 9 with 1 M potassium hydroxide and made up to 1000 ml with water. To prepare acidifying reagent, 15 ml glacial acetic acid was diluted to 250 ml with filtered Milli-Q water.

Mobile phase, oxidising reagent, and acidifying reagent were all filtered using 0.5 μm PTFE millipore filters, and degassed prior to use. The column was washed in 25% aqueous acetonitrile for 5 minutes before priming the pump with mobile phase. When a column flow rate of 0.8 ml min^{-1} was established, and the oxidiser and acidifier pumps set at 0.4 ml min^{-1}, 20 μl of saxitoxin standard diluted in saline was injected until retention times became stable prior to sample analyses. The run time for each analysis was 30 minutes. The retention times for pure toxin standards were recorded for identification in samples. A calibration curve for saxitoxin was constructed using this method in the range of 0-1000 ng on the column.

2.2.5. Determination of the optimum extraction method for saxitoxin and neosaxitoxin from cyanobacterial cells.

Filter disks with 100 mg dry weight *Aph. flos-aquae* NH-5a were extracted by four different methods and the amount of saxitoxin recovered by each method determined by HPLC. Three 30 minute extractions were carried out in 20 ml of either water, water + 0.01% (v/v) trifluoroacetic acid (TFA, Fisher scientific), methanol (Rathburn), or methanol + 0.01% (v/v) TFA. The extracts obtained after each 30 minutes were pooled and rotary evaporated to dryness at 45 °C to obtain a concentrated sample which was redissolved in 1 ml of saline, giving a final concentration equivalent to 100 mg cells ml^{-1}. Each extraction was carried out in triplicate. Samples were immediately analysed for the presence of saxitoxins by prechromatographic oxidation followed by HPLC as described in 2.2.2.

2.2.6. Development of a bioassay for the detection of saxitoxins employing the desert locust (*Schistocerca gregaria*).

Desert locusts were purchased from Blades Biological (Kent, UK). All insects were kept in the laboratory for a maximum of 48 hours prior to use and were maintained on a diet of assorted plant material (e.g. washed lettuce) until required.

Male nymphs of 0.9-1.2 g (early fifth instar) were used in all experiments. The locust bioassay was developed as described by McElhiney *et al.* (1998). Locusts were injected along the abdomen between the 2nd and 3rd segments, with 10 μl of test solution, using a 25 μl gas-tight syringe (Hamilton, Nevada, USA). In initial studies, three cyanobacterial toxins were tested by locust bioassay. The hepatotoxin microcystin-LR, the neurotoxic alkaloid anatoxin-a, and saxitoxin were tested at a concentration of 100 μg ml^{-1}. A saline extract of *Ana. flos-aquae* NRC 525-17 (50 mg ml^{-1}) was also assayed. The sensitivity of the locust bioassay to saxitoxin was determined by preparing a dilution series, (100, 50, 20, 10, 5, 2.5, 1, and 0 μg ml^{-1}) in saline. For each concentration of saxitoxin, 3 sets of 5 locusts were injected. A set of controls were injected with 10 μl of saline only. Following injection, the insects were each placed in a clear plastic container and effects of intoxication were determined by direct observation of paralysis symptoms (locusts dead or unable to self-right when placed on the back) after injection. All injected locusts were then weighed, and saxitoxin concentration converted to ng/g locust. Results were expressed as the percentage of insects paralysed for each concentration assayed at 30, 60, and 90 minutes after injection. These values were then plotted in a graph, and used to determine the ED_{50} values (concentration of saxitoxin to cause 50% of locusts injected to become paralysed) for saxitoxin at each of the assay end points. The ED_{50} value for authentic neosaxitoxin was also determined by locust bioassay using an end-point of 90 minutes.

2.2.7. Use of the locust bioassay to confirm the optimum extraction method for saxitoxin and neosaxitoxin from cyanobacterial cells.

The toxicities of the extracts prepared as described in 2.2.5. were assessed by observation of paralysis symptoms in locusts following a 10 μl injection. One locust was injected for each of the extracts prepared using each method.

The effects of methanol and TFA on toxicity determinations by locust bioassay were also assessed using the non-toxic cyanobacterial strain *M. aeruginosa* CYA 43. Triplicate filters with a dry weight of 100 mg of cells were extracted in methanol + TFA as described.

The effect of sample processing on the toxicity of extracts was determined by spiking extracts with saxitoxin to a concentration of 20 $\mu g ml^{-1}$ before and after rotary evaporation, and the toxicity compared by observation of paralysis symptoms in locusts. Unspiked cells were processed and assessed in the same way in order to determine whether any toxic effects were being carried over into samples from the solvent or TFA.

2.2.8. Analysis of natural cyanobacterial samples and laboratory isolates by locust bioassay and HPLC.

In the late summer of 1995, an extensive cyanobacterial bloom was reported in the river Great Ouse. The cyanobacterial species responsible for this bloom was initially identified as *Aph. flos-aquae* (Environment Agency). However, further investigation indicated that the species was more likely to be *Oscillatoria agardhii*, due to the lack of heterocysts and minimal scum formation after 3 days in culture. Following initial sampling by the Environment Agency, the toxicity of the bloom was determined by mouse bioassay (at the University of Dundee), and it was found to be neurotoxic, causing similar symptoms to those associated with saxitoxin intoxication. The minimum lethal dose was found to be 81 mg dry weight of bloom material per kg body weight, indicating that the bloom was of high toxicity. Samples were taken at 3 sites on the river where warning notices had been erected; St. Neots, Roxton, and Great Barford. Of these 3 sites, the bloom was most dense at Great Barford. On the 10th of August, two litre volumes of water taken from the Great Barford site were filtered onto Whatman GF/C filter disks and dried (no dry weights were recorded).

On the 22nd of August, further samples were taken at each of the sites. On this occasion, 2 litres of water sampled from each of the 3 sites were filtered onto pre-weighed GF/C filter disks, dried, and the dry weights recorded. The processing and analysis of each of these samples is outlined in Table 2.1.

Table 2.1. Extraction and analysis of cyanobacterial samples obtained from 3 sites on the river Great Ouse in August 1995.

Sample Number	Site	Sampling date	Extraction	Concentration (mg cells ml^{-1})	Analysis
1	Great Barford	10/8/95	Methanol	not known	HPLC (precolumn), locust bioassay
2	Great Barford	22/8/95	Methanol	120	locust bioassay
3	Great Barford	22/8/95	Methanol + TFA	100	HPLC (postcolumn), locust bioassay
4	St. Neots	22/8/95	Methanol + TFA	100	HPLC (postcolumn), locust bioassay
5	Roxton	22/8/95	Methanol + TFA	100	HPLC (postcolumn), locust bioassay

Initial screening of samples by locust bioassay were carried out prior to studies which determined the most efficient extraction method for saxitoxins from cyanobacterial samples. Two filters prepared from samples taken at the Great Barford site on the 10th of August were subjected to three 30 minute extractions in 20 ml methanol, rotary evaporated to dryness, and resuspended in 0.5 ml saline (sample 1). Two filters prepared from samples taken from the same site on the 22nd of August (60 mg cells per filter), were processed in the same way, giving a final concentration of 120 mg cells ml^{-1} (sample 2). Filters obtained from each of the three sampling sites on the 22nd of August were then extracted in 20 ml methanol + 0.01% TFA for 3 x 30 minutes, dried, and resuspended in saline to a concentration of 100 mg cells ml^{-1} (samples 3-5). All samples were assessed by locust bioassay as described in 2.2.6.

The methanol extract prepared using filters obtained from the bloom sample taken at the Great Barford site on the 10th of August (sample 1) was analysed using prechromatographic oxidation followed by HPLC as described in 2.2.2. Fluorescence was monitored using a Waters 474 scanning fluorescence detector. The sample was oxidised using both peroxide and periodate, and analysed by HPLC. An underivatised sample of the extract was also analysed. Methanol + TFA extracts prepared from filters obtained from each of the 3 sites on the 22nd of August (samples 3-5), were analysed for saxitoxin, neosaxitoxin, and gonyautoxins 1, 2, 3, and 4 using HPLC with postcolumn oxidation as described in 2.2.4.

Water samples taken at the Great Barford site on the 10th of August were also used to inoculate 50 ml of BG-11 liquid medium plus and minus nitrate containing 25 $\mu g\ ml^{-1}$ cyclohexamide (Sigma) to inhibit the growth of eukaryotes, e.g. green algae. Aliquots (100 μl) were also spread aseptically onto BG-11 agar plates (plus and minus nitrate), also containing cyclohexamide. Plates and liquid cultures were both subcultured monthly. After 10 months, 11 isolates taken from agar plates were transferred to 100 ml liquid BG-11 plus nitrate. These were allowed to grow for 8 weeks, and then filtered onto pre-weighed GF/C filter disks. In order to assess whether toxic species had been isolated from the Great Barford site, filters containing each of the 11 isolates were extracted in methanol + TFA, and resuspended in saline to a concentration of 100 mg cells ml^{-1}. These samples were then assessed by locust bioassay, and analysed using HPLC with post column oxidation for saxitoxin, neosaxitoxin, and gonyautoxins 1, 2, 3, and 4.

2.2.9. Analysis of cyanobacterial samples from the river Great Ouse for the presence of the neurotoxins anatoxin-a, and anatoxin-a(s).

One filter prepared from sample taken at the Great Barford site on the 10th of August was extracted in 5 ml of methanol + 0.01% TFA for one hour. This sample was analysed for the presence of anatoxin-a using the method described by Edwards *et al.* (1992). HPLC apparatus consisted of a Waters 600E multisolvent delivery system with a Waters 996 photodiode array detector (Waters Corp., Milford, MA). The sample was separated on a Waters Symmetry C_{18} column (250 x 4.6 mm I.D., 5 μm particle size) and the injection volume was 25 μl. The mobile phase consisted of (A) 0.02 M potassium dihydrogen phosphate; corrected to pH 2.5 using concentrated hydrochloric acid (BDH), and (B) acetonitrile (Rathburn). Solvent (B) was linearly increased from 0 to 20% over 30 minutes at a flow rate of 1 ml min^{-1}. The column was then returned to 0% B over 10 minutes and equilibrated at the starting conditions for 5 minutes between injections. Absorbance was monitored from 200 to 300 nm since the absorbance maximum of anatoxin-a is 227 nm. Identification of anatoxin-a in the bloom extract was verified following injection of authentic toxin (10 μg ml^{-1}).

For the determination of the acetylcholinesterase inhibitor anatoxin-a(s) in the Great Barford sample, 1 filter prepared from sample taken on the 10th of August was extracted in 5 ml water for 1 hour. The presence of anatoxin-a(s) was determined using a colorimetric acetylcholinesterase inhibition assay. This assay is based on the hydrolysis of acetylcholine by the enzyme acetylcholinesterase to give acetic acid and choline. The acid-base indicator m-nitrophenol is used to detect the release of acetic acid by a colour change from yellow to clear. The presence of anatoxin-a(s) in a sample will prevent this reaction from taking place. All chemicals used in this assay were supplied by Sigma unless stated otherwise. Acetylcholinesterase (Type VI-S, from electric eel, 530 units/mg protein), was prepared in a stock solution of 115.75 units ml^{-1} in Milli-Q water, and stored at -40°C until required.

m-Nitrophenol (0.75 g) was dissolved in 100 ml of 50 mM phosphate buffer (3.9 g sodium dihydrogen phosphate (AnalR, BDH) dissolved in 500 ml Milli-Q water), corrected to pH 7.8 using 1M sodium chloride (AnalR, BDH). Acetylcholine chloride solution was prepared by dissolving 750 mg in 2.5 ml Milli-Q water. The assay was carried out by mixing 50 µl of sodium chloride with 15 µl acetylcholinesterase in a 1.5 ml microcentrifuge tube (Eppendorf, UK.). Extract (750 µl) was added, and allowed to react with the enzyme for 30 seconds before adding 400 µl m-nitrophenol. Following the addition of 50 µl acetylcholine chloride solution, the mixture was incubated at 25°C for 60 minutes. Finally, the absorbance of the mixture was read at 420 nm using a Novaspec spectrophotometer.

Positive and negative controls with which to compare the Great Barford extract were prepared by extracting GF/C filter disks with 100 mg of *Ana. flos-aquae* NRC 525-17 and the non-toxic strain *M. aeruginosa* CYA 43 in 100 ml water for 1 hour; giving a concentration of 1 mg cells ml^{-1}. The samples were then assayed as described above. The absorbance recorded for each sample after 60 minutes was compared to that of a control mixture in which enzyme was replaced by water. Each sample was blanked against an equivalent concentration of extract minus enzyme and substrate (750 µl extract + 515 µl water). The extent of inhibition was determined by subtracting the absorbance of the test sample from that of the control mixture.

2.2.10. Isolation of toxic components from Great Barford extract.

An extract of filtered bloom material taken at the Great Barford site on the 22nd August was prepared by extracting filters in methanol + 0.01% TFA for 3 x 30 minutes, followed by rotary evaporation, and resuspension of the sample in saline to a final concentration of 100 mg cells ml^{-1}. The toxicity of this extract was assessed by locust bioassay and found to be toxic (paralysis in 10 minutes). The sample was then separated using reverse phase HPLC, in order to determine whether the toxic components present in the sample could be isolated.

The HPLC system consisted of a Waters 600E multisolvent delivery system with a Waters 996 Photodiode Array Detector. Sample injection was performed by a Waters 717 plus autosampler, and separation was carried out using a Waters Symmetry C_{18} column (250 x 4.6 mm I.D.). Detection was carried out in the range 200-300 nm. Eight 50 µl volumes of Great Barford extract were injected onto the column, and the sample constituents were eluted using a gradient of 0-100% methanol over 30 minutes, with a flow rate of 1 ml min^{-1}. After each run, the column was returned to 0% methanol over 10 minutes, and equilibrated at the starting conditions for a further 10 minutes. Fractions were collected every minute using a Waters Fraction Collector. Fractions collected each minute for the 8 subsequent runs were combined. Aliquots (4 ml) of fractions collected every 3 minutes were pooled, rotary evaporated to dryness, and resuspended in 1 ml methanol. Each of the ten pooled extracts was dried again under nitrogen, resuspended in a final volume of 100 µl saline, and their toxicity determined by locust bioassay as described.

2.2.11. Compatibility of the locust bioassay with the screening of shellfish flesh for saxitoxins.

An experiment was carried out to determine the compatibility of the locust bioassay with the standardised procedure for extracting saxitoxins from shellfish flesh which involves boiling samples in 0.1 M HCl.

Non-toxic mussel flesh was homogenised and divided into 0.5 g aliquots in 1.5 ml microcentrifuge tubes. Three aliquots were then mixed with 15 µg saxitoxin in 50 µl saline. Extraction in HCl was carried out by mixing each sample with 0.5 ml of 0.1 M HCl, and holding each in a beaker of boiling water for 5 minutes. Each sample was then centrifuged for 3 minutes, and the supernatant removed. This process was repeated using 3 mussel flesh aliquots which had not been mixed with saxitoxin. Each sample (10 µl) was injected into a locust and the toxicity determined by observation of paralysis symptoms at 90 minutes. The toxicity of HCl was also assessed by injecting 3 locusts with 10 µl acid only.

2.2.12. Toxicity determinations in natural mussel samples using the locust bioassay.

The toxicities of 9 natural mussel samples extracted in 0.1 M HCl determined by standard mouse bioassay were re-assessed by locust bioassay. The toxicity of each sample was then scored positive or negative by observation of paralysis symptoms for up to 90 minutes post-injection. For each sample, 3 locusts were injected. All samples which scored negative by the locust bioassay were then concentrated 10 fold by drying 150 µl under nitrogen at 45 °C, dissolving in 150 µl methanol, drying again and resuspending in a final volume of 15 µl saline. Concentrated samples were then assayed again in triplicate as before.

2.2.13. Use of the locust bioassay in a shellfish monitoring programme.

The locust bioassay was used in further trials in a large scale monitoring programme undertaken by The Marine Laboratory, Torry, Aberdeen, involving 3 groups of toxic and non-toxic mussel extracts. The method adopted in this screening programme is outlined in Figure 2.1. Samples were scored 1-3 according to toxicity, and the results compared with those obtained by mouse bioassay.

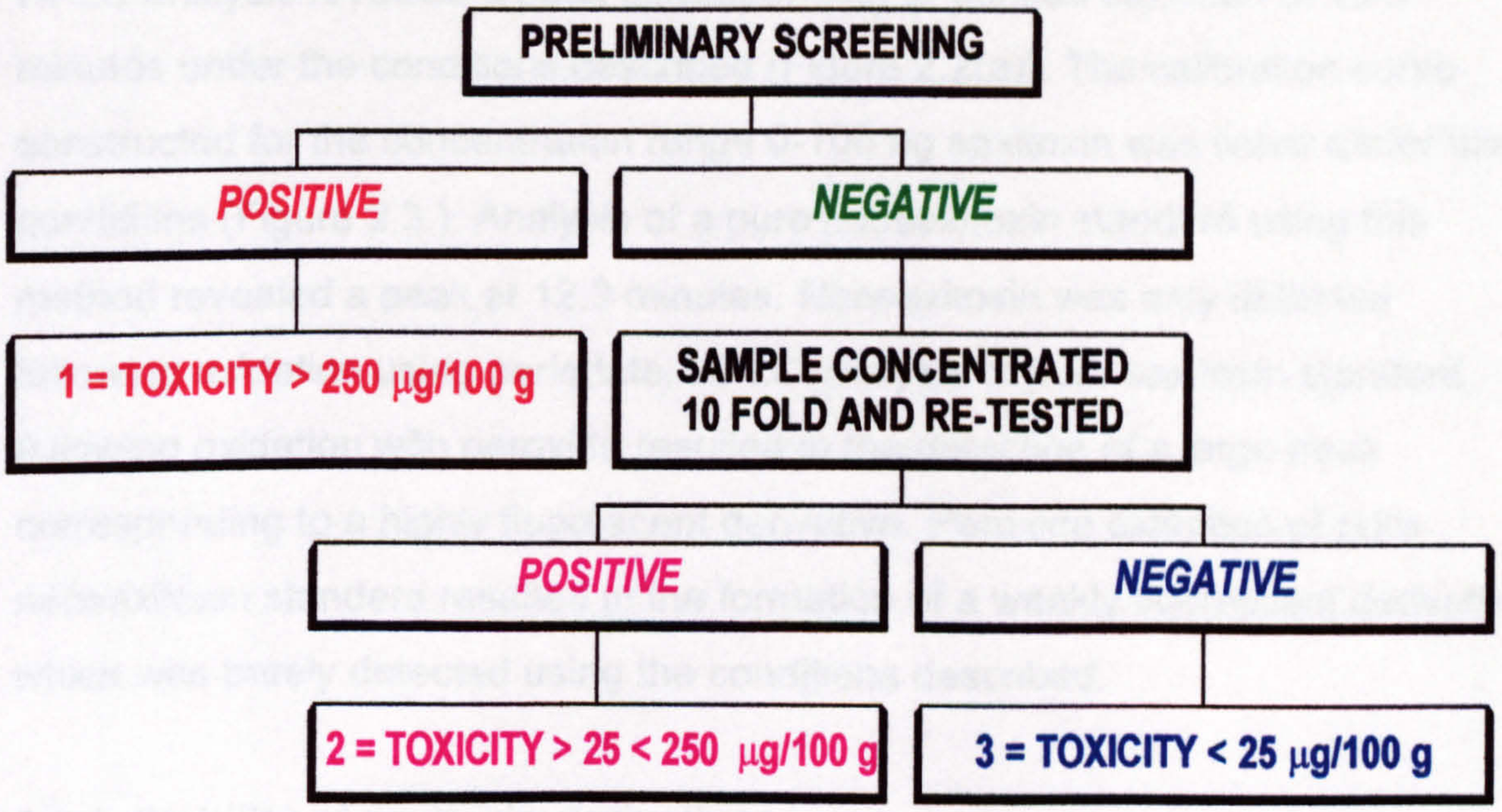


Figure 2.1. Toxicity assessment of shellfish extracts by locust bioassay.

2.3. Results.

2.3.2. Detection of saxitoxins by prechromatographic oxidation followed by HPLC with fluorescence detection.

HPLC analysis revealed a peak corresponding to purified saxitoxin at 15.5 minutes under the conditions described (Figure 2.2(a)). The calibration curve constructed for the concentration range 0-100 ng saxitoxin was linear under these conditions (Figure 2.3.). Analysis of a pure neosaxitoxin standard using this method revealed a peak at 12.3 minutes. Neosaxitoxin was only detected following oxidation using periodate. HPLC analysis of pure saxitoxin standard following oxidation with peroxide resulted in the detection of a large peak corresponding to a highly fluorescent derivative. Peroxide oxidation of pure neosaxitoxin standard resulted in the formation of a weakly fluorescent derivative which was barely detected using the conditions described.

2.3.3. Stability of saxitoxin derivatives.

HPLC analysis of ten identical saxitoxin samples (5 $\mu g\ ml^{-1}$) oxidised using periodate revealed that the peak areas of oxidised samples decreased markedly over the time period assessed (Figure 2.4.). The peak area of 5 $\mu g\ ml^{-1}$ saxitoxin injected immediately after periodate oxidation resulted in a peak area of 1.02 x 10^7. Peak area remained relatively constant for the first 150 minutes, but began to decrease sharply when the time of injection increased beyond 200 minutes. When the delay between oxidation and injection reached 450 minutes, peak area was reduced to 3.06 x 10^6; almost one third of the original value, indicating that periodate derivatives of saxitoxin were unstable under the conditions described.

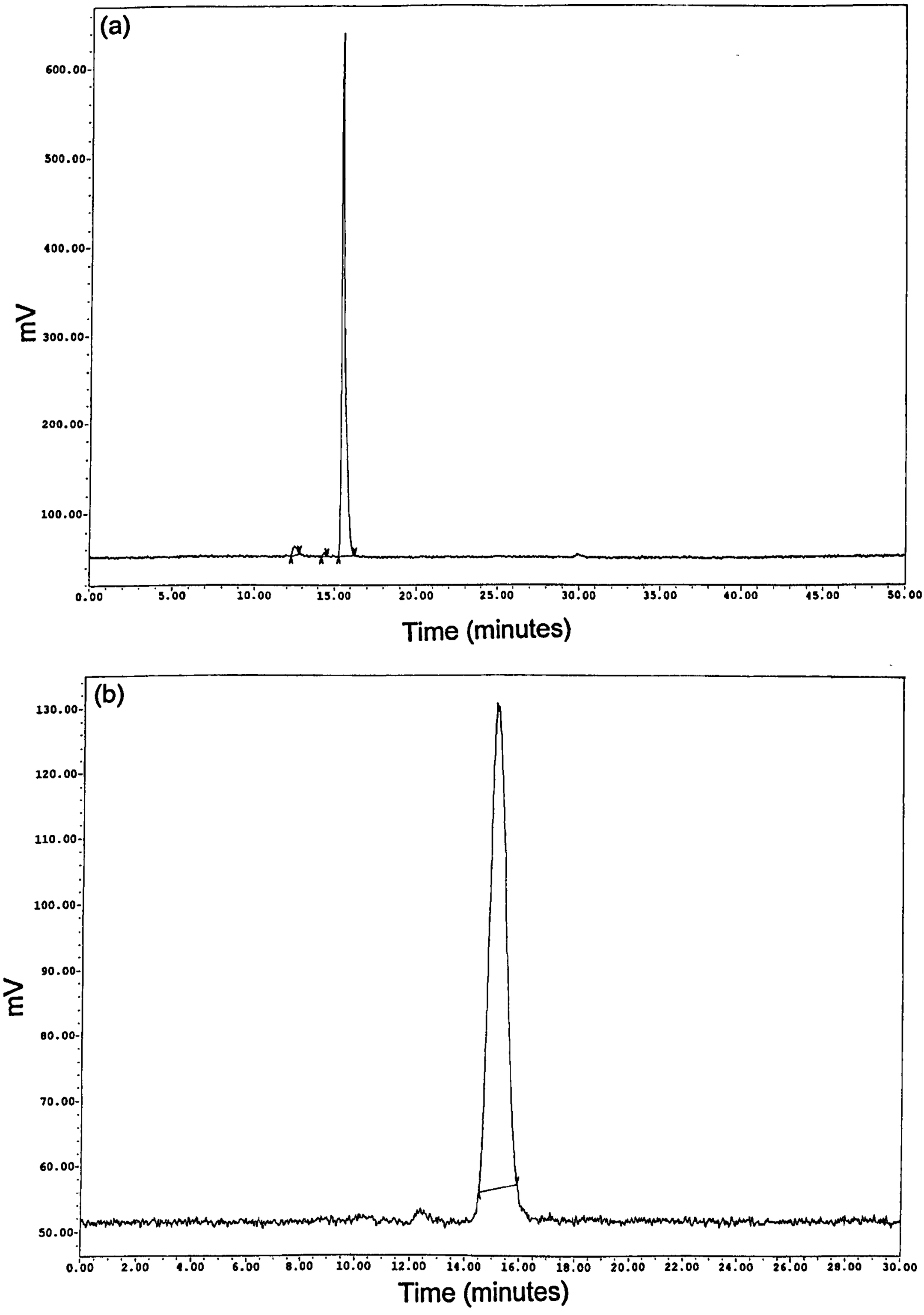


Figure 2.2. Chromatograms of authentic saxitoxin (5 $\mu g ml^{-1}$) analysed by (a) precolumn oxidation followed by HPLC analysis as described in 2.2.2., and (b) HPLC analysis followed by postcolumn oxidation as described in 2.2.4.

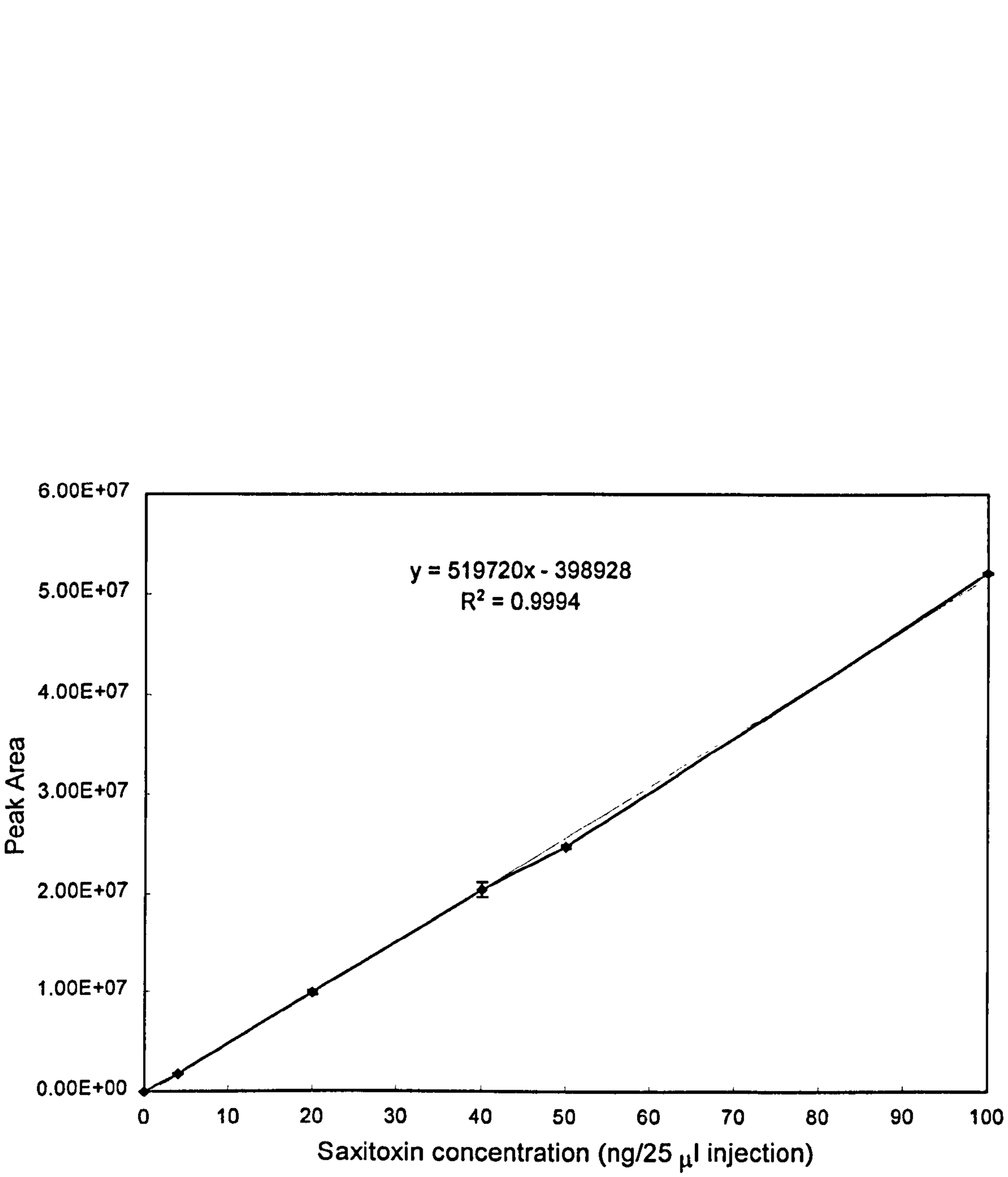


Figure 2.3. Calibration curve for pure saxitoxin using precolumn derivatisation followed by HPLC analysis with fluorescence detection constructed in the range 0-100 ng per 25 µl injection. Samples were derivatised using periodate prior to injection. Data plotted are the mean of 2 replicates and bars indicate sample standard deviation.

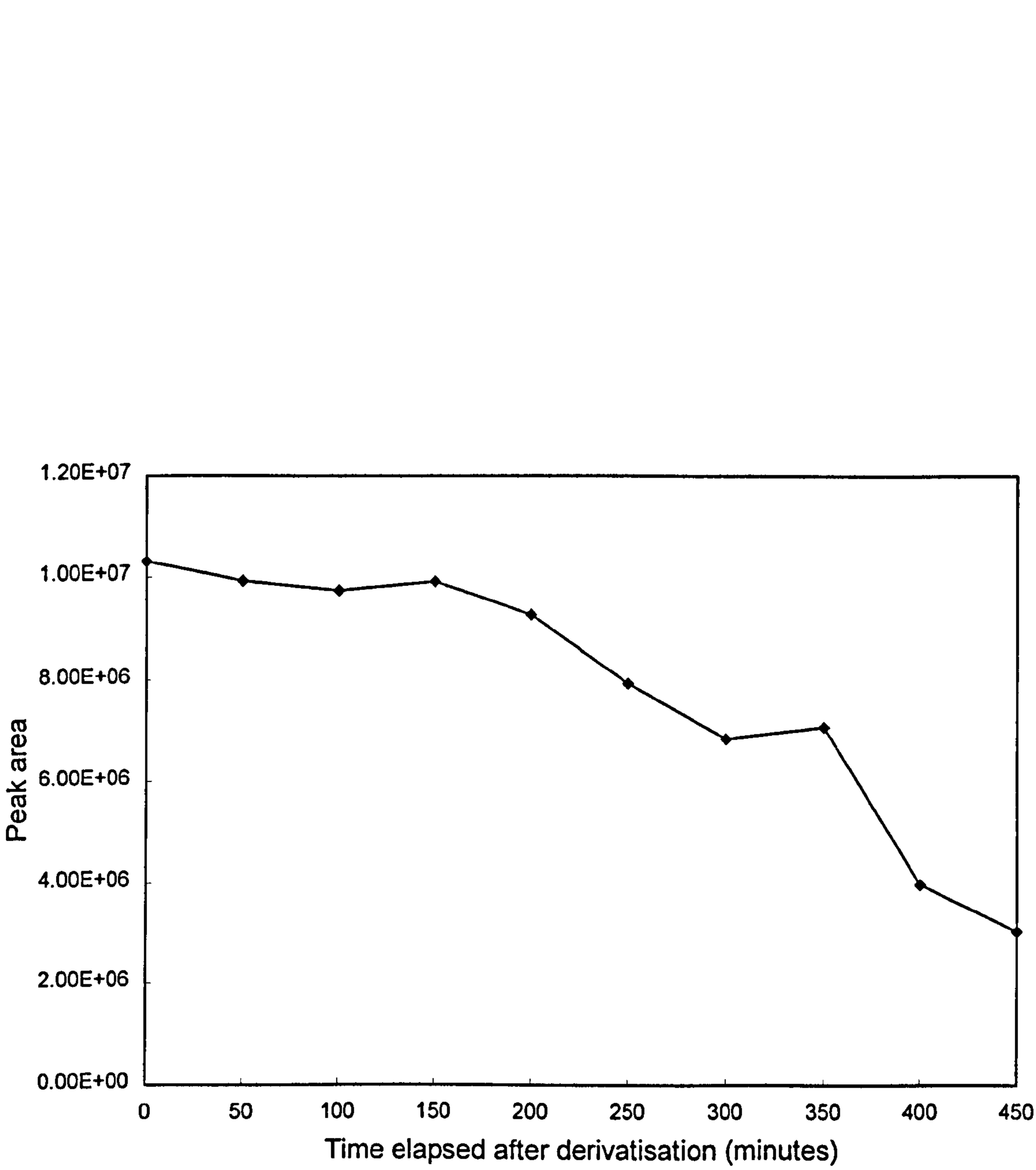


Figure 2.4. Stability of saxitoxin derivatives following oxidation of pure toxin (5 μg ml^{-1}) with periodate.

2.3.4. Detection of saxitoxins by HPLC with post-column oxidation and fluorescence detection.

HPLC separation followed by post column oxidation of pure saxitoxin standard revealed a peak corresponding to saxitoxin at 15.8 minutes (Figure 2.2.(b)).The retention time of neosaxitoxin using this method was 11.2 minutes. Analysis of a mixture of the toxins GTX 2 and 3 revealed peaks corresponding to GTX 3 at 10.1 minutes, and GTX 2 at 11.7 minutes. Peaks corresponding to GTX 4 and 1 were observed at 7.3 and 8.4 minutes respectively. The calibration curve constructed for pure saxitoxin using this method was found to be linear in the range 0-1000 ng per 20 μl injection (Figure 2.5).

2.3.5. Determination of the optimum extraction method for saxitoxin and neosaxitoxin from cyanobacterial cells.

When extracts of *Aph. flos-aquae* NH-5a were analysed using precolumn oxidation followed by HPLC two major peaks were observed (Figure 2.6.(a)). A peak at 14.9 minutes (peak 2) was identified as saxitoxin by comparison with the chromatographic data for pure toxin. Analysis of the extracts also revealed an earlier peak with a retention time of 12.2 minutes (peak 1). This peak was confirmed as neosaxitoxin by oxidising the sample using peroxide instead of periodate. Figure 2.6.(b) shows the chromatogram obtained following analysis of a methanol + 0.01% TFA extract of *Aph. flos-aquae* NH-5a following peroxide oxidation. The chromatogram shows that while saxitoxin yields a highly fluorescent compound following peroxide oxidation (peak 2), the derivative formed by the oxidation of neosaxitoxin by peroxide is only weakly fluorescent (peak 1).

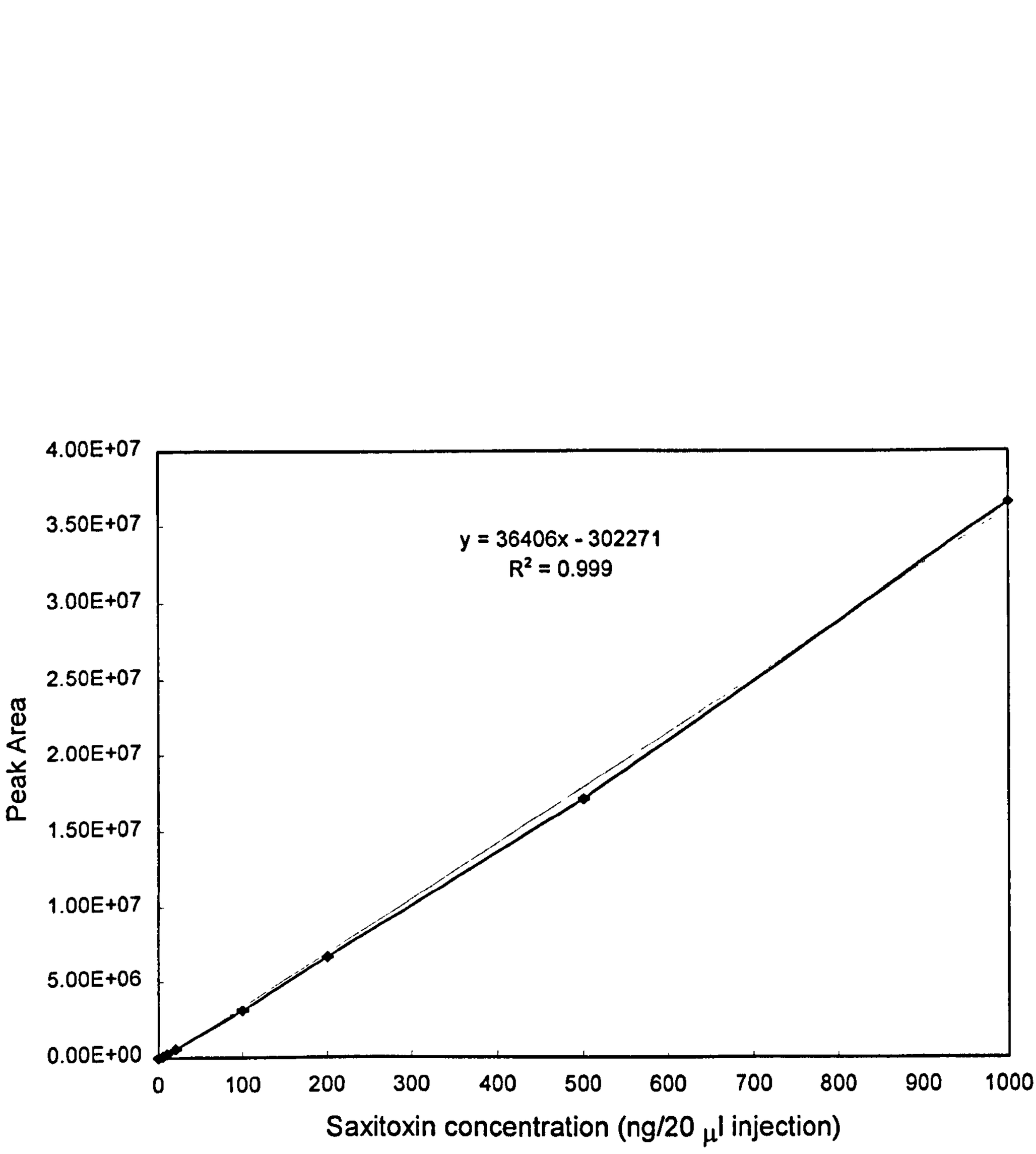


Figure 2.5. Calibration curve for pure saxitoxin using HPLC separation followed by postcolumn derivatisation and fluorescence detection constructed in the range 0-1000 ng per 20 µl injection. Data plotted are the mean of 2 replicates and bars indicate sample standard deviation.

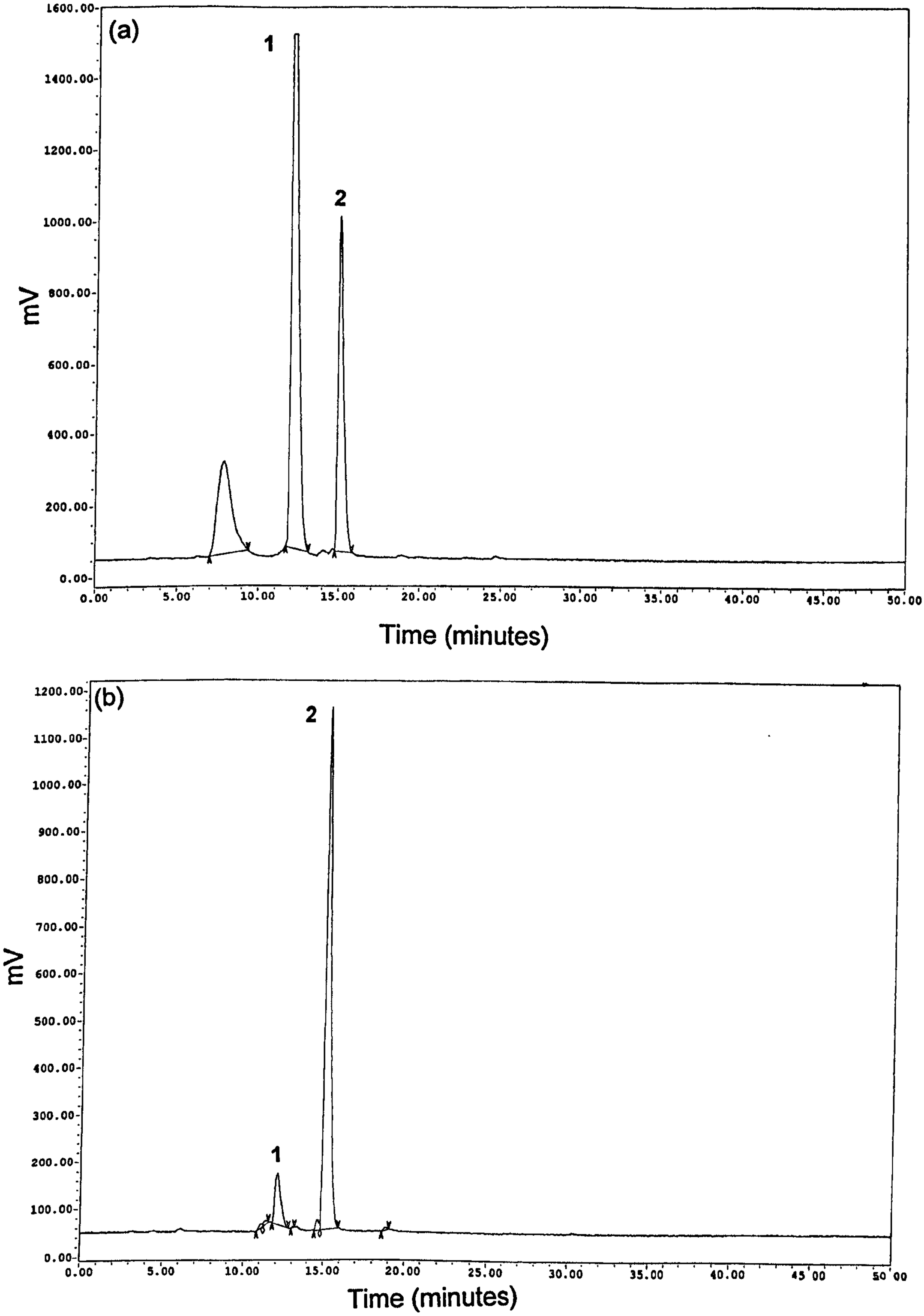


Figure 2.6. HPLC chromatograms of *Aph. flos-aquae* NH-5a extracted in methanol + 0.01% TFA and analysed following precolumn oxidation with (a) periodate, and (b) peroxide. Peaks correspond to 1. Neosaxitoxin, and 2. Saxitoxin.

The saxitoxin concentrations of each of the four extractions of *Aph. flos-aquae* in water, water + 0.01% TFA, methanol, and methanol + 0.01% TFA were determined by HPLC and quantified using the calibration curve shown in Figure 2.3. The curve was also used to estimate the concentration of neosaxitoxin as a saxitoxin equivalent. Figure 2.7. shows the amounts of saxitoxin and neosaxitoxin extracted by each of the methods and quantified using precolumn oxidation with HPLC analysis. The results indicate that the highest toxin concentrations were detected in the acidified extracts and the lowest in the methanol extract. The most successful method assessed for the extraction of saxitoxins from *Aph. flos-aquae* NH-5a was found to be methanol + 0.01% TFA, which extracted around 0.17 μg saxitoxin and 0.26 μg neosaxitoxin per mg of cells. The graph shows that although there was no appreciable difference in the amounts of saxitoxin detected in the water + TFA and methanol + TFA extractions, methanol + TFA extracted a greater amount of neosaxitoxin from cells. A methanol + 0.01% TFA extract of *Aph. flos-aquae* was also analysed using HPLC analysis followed by postcolumn oxidation. The extract chromatographed poorly using this HPLC method compared to analysis following precolumn oxidation (Figure 2.8.). Retention times of the main 2 peaks were 11.7 and 16.9 minutes respectively. These were identified as saxitoxin and neosaxitoxin by spiking the extracts with 5 μg ml^{-1} of authentic saxitoxin and neosaxitoxin (results not shown).

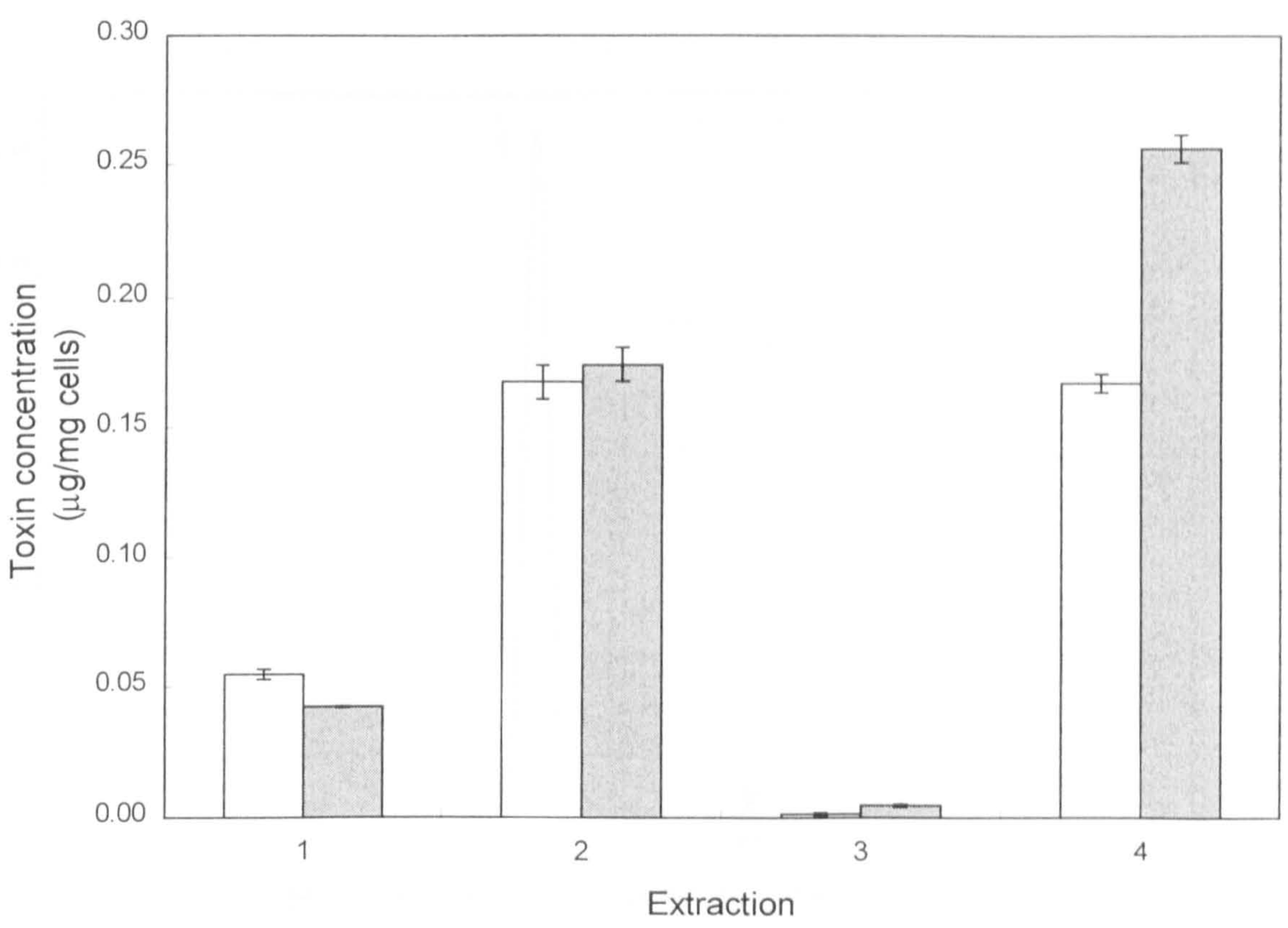


Figure 2.7. Comparison of extraction methods for the recovery of saxitoxin (white) and neosaxitoxin (black) from *Aphanizomenon flos aquae* NH-5a quantified by pre-column oxidation followed by HPLC analysis. Extraction media: 1 - Water; 2 - Water + 0.01% TFA; 3 - Methanol; 4 - Methanol + 0.01% TFA. Data plotted are the mean of 3 replicates and bars indicate sample standard deviation.

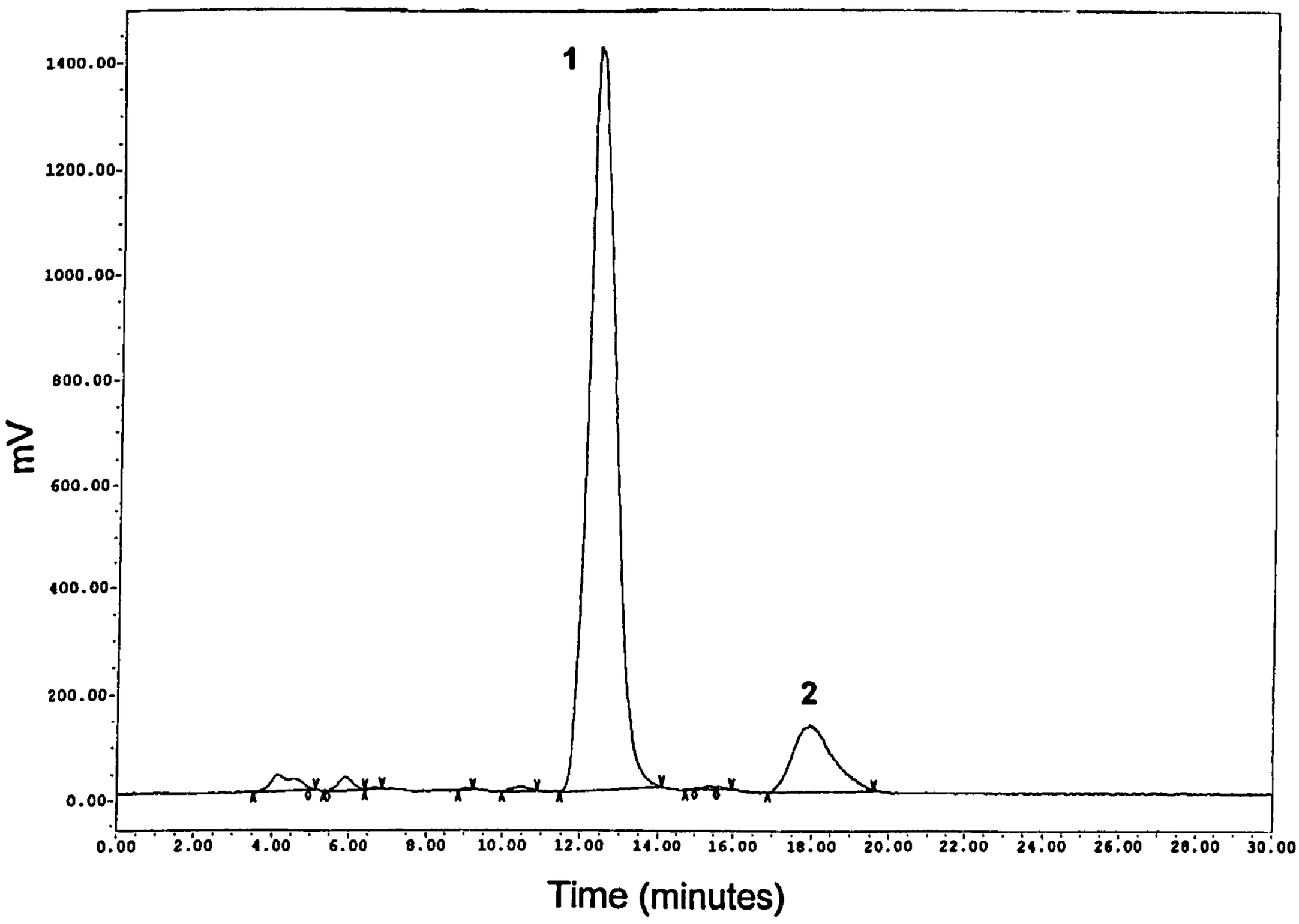


Figure 2.8. Chromatogram of *Aph. flos-aquae* NH-5a extracted in methanol + 0.01% TFA as described and analysed by HPLC followed by postcolumn oxidation. Peaks correspond to 1. Neosaxitoxin, and 2. Saxitoxin.

2.3.6. Development of a bioassay for the detection of saxitoxins employing the desert locust (*Schistocerca gregaria*).

In initial trials with the three cyanobacterial toxins tested, only saxitoxin caused intoxication in locusts. At an end-point of 90 minutes, no detrimental effects were observed in locusts injected with 100 μg ml^{-1} microcystin-LR, 100 μg ml^{-1} anatoxin-a, or a saline extract of *Ana. flos aquae* NRC 525-17 (50 mg ml^{-1}). At a concentration of 100 μg ml^{-1}, saxitoxin caused paralysis in under 10 minutes post injection. Paralysis symptoms were initiated by the loss of control of the hind legs, followed by a dragging of the abdomen and the inability of the insects to jump or climb. Paralysis was judged when the insect was dead or unable to self-right when placed on the back. No paralysis symptoms were observed in the control set of locusts which had been injected with saline only. Injection with saline caused no detrimental effects for the duration of the assay, and for up to 48 hours afterwards.

ED_{50} values for saxitoxin were determined by plotting the percentage of insects paralysed against saxitoxin concentration expressed in ng/g insect, 30, 60, and 90 minutes following injection (Figure 2.9.). The time at which paralysis occurred was found to be dependent on concentration, with the ED_{50} value decreasing as the end-point of the assay was increased. At an end-point of 30 minutes, the ED_{50} value was 225 ng g^{-1}, and at 60 minutes 32 ng g^{-1}. The sensitivity of the assay increased a further four times between 60 and 90 minutes to 8 ng g^{-1}. For future assays, the 90 minute end-point was selected, since this provided the most sensitive measurement of toxicity. The detection limit of the assay was determined as 2.5 μg ml^{-1} (approximately 20 ng g^{-1} insect), since this was the lowest saxitoxin concentration which caused paralysis symptoms to occur in 100% of locusts injected after 90 minutes. Using this end-point, the ED_{50} value for pure neosaxitoxin was determined as 12 ng g^{-1}.

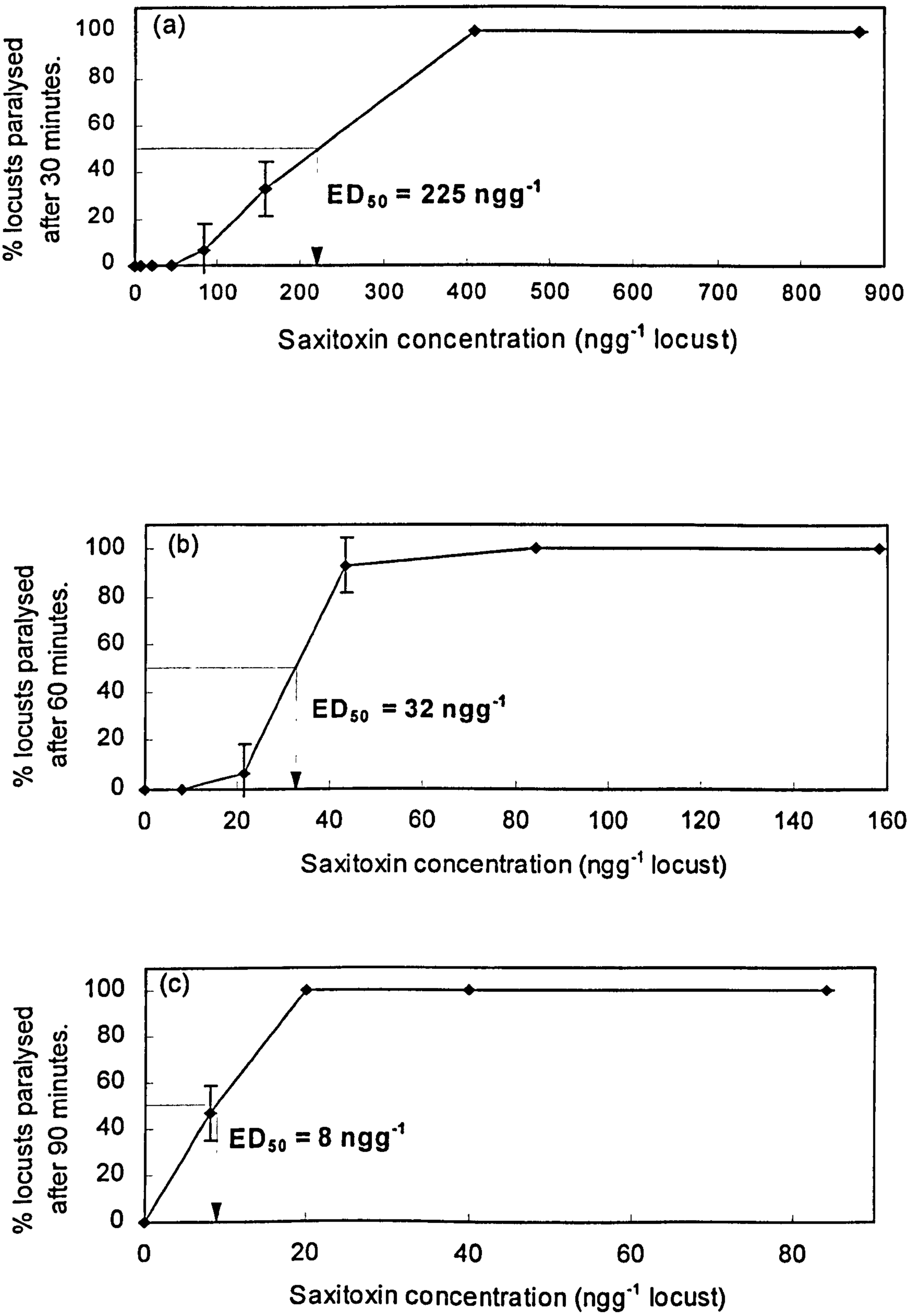


Figure 2.9. ED_{50} values calculated for authentic saxitoxin by locust bioassay at (a) 30 minutes, (b) 60 minutes, and (c) 90 minutes following injection. Data plotted are the mean of 15 replicates and bars indicate sample standard deviation.

2.3.7. Use of the locust bioassay to confirm the optimum extraction method for saxitoxin and neosaxitoxin from cyanobacterial cells.

When each of the four extracts of *Aph. flos-aquae* were tested by locust bioassay, acidified extracts appeared to be the most toxic, particularly the methanol + 0.01% TFA extract, which caused paralysis in 8.3 minutes (± 2.9 minutes) post injection. Injection with the water + 0.01% TFA extract caused paralysis to occur at 13.3 minutes (± 2.9 minutes). Water extracts caused paralysis to occur in 21.7 minutes (± 2.9 minutes) following injection. Methanol extracts were the least toxic to locusts and did not cause paralysis until 101.7 minutes (± 7.6 minutes), beyond the designated end-point of 90 minutes.

Dried non-toxic cyanobacterial cells (*M. aeruginosa* CYA 43) extracted with methanol + TFA spiked with 20 μg ml^{-1} saxitoxin prior to rotary evaporation caused paralysis in locusts at the same time (30.0 minutes ± 2.1 minutes), as those which had been spiked after rotary evaporation (30.0 minutes ± 2.2 minutes). This time also compared well to the time at which paralysis occured in locusts injected with 20 μg ml^{-1} of the authentic toxin. Unspiked cells processed in the same way had no toxic effect on locusts. Extraction of cells in methanol + TFA followed by rotary evaporation and resuspension in saline was therefore considered suitable for the processing of cyanobacterial samples for toxicity determinations by locust bioassay.

2.3.8. Analysis of natural cyanobacterial samples and laboratory isolates by locust bioassay and HPLC.

Following sub-culturing over 3 months, one filamentous cyanobacterial species predominated in both solid and liquid medium. Growth was successful in both solid and liquid BG11 containing nitrate. The cyanobacteria grew only for a short time (up to 1 month) in medium which lacked nitrate.

When examined microscopically, the filaments appeared long and thin, and lacked heterocysts, even in isolates growing in medium without nitrate. Microscopic comparison of the isolated cyanobacteria with 2 other filamentous species, *Aph. flos-aquae* NH-5a, and *Oscillatoria agardhii* revealed no similarities with either (Figure 2.10.).

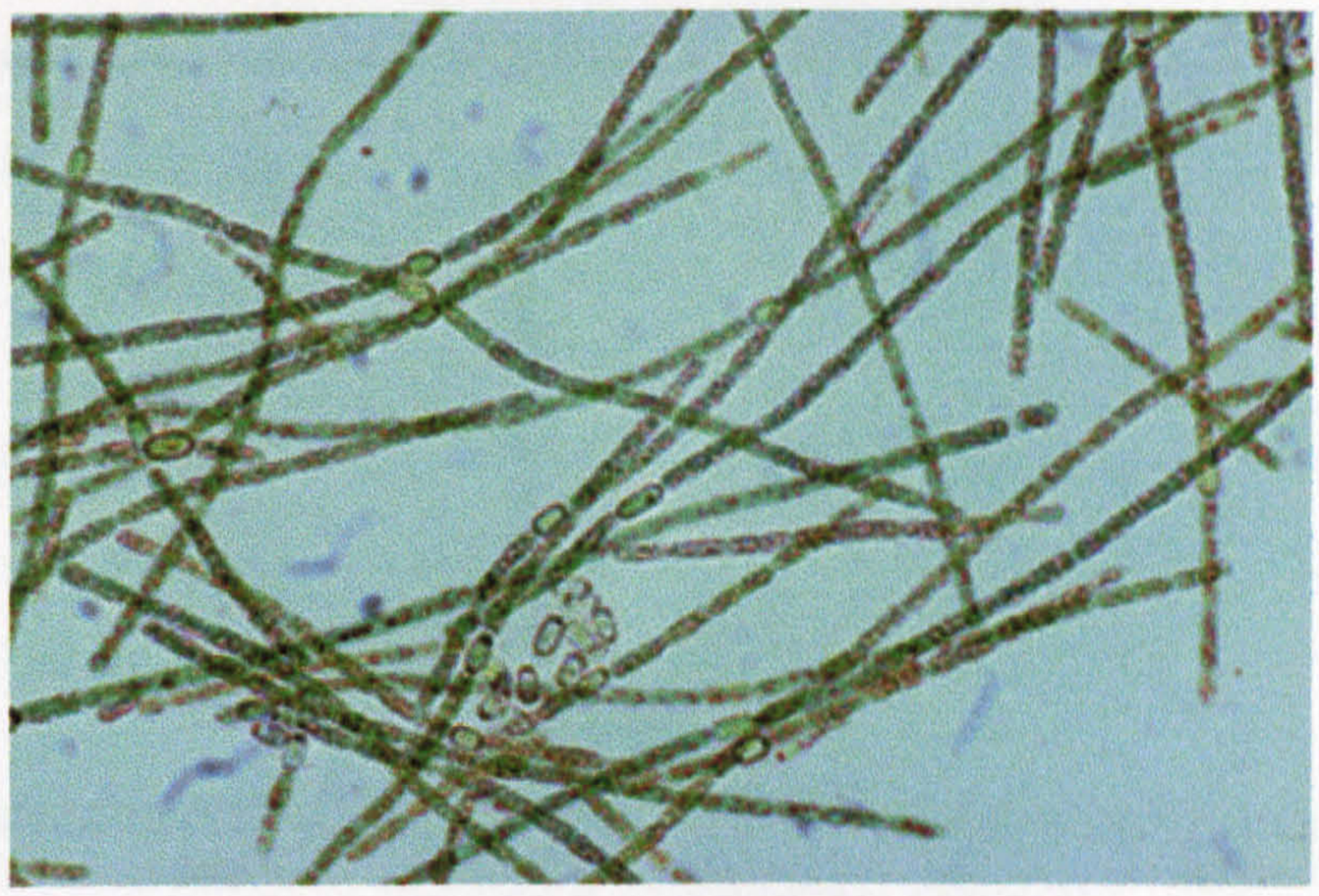
In initial screening of the methanolic extract prepared from filters obtained from the Great Barford site on the 10th of August (Table 2.1., sample 1), paralysis occurred in locusts in 30 minutes (± 2.3 minutes). Methanolic extract prepared from samples taken from the same site on the 22nd of August (sample 2, 120 mg cells ml^{-1}), did not cause paralysis symptoms to occur until beyond the 90 minute end-point of the assay.

Samples which were obtained from all 3 sites on the 22nd of August, and subjected to extraction in methanol + 0.01%TFA (samples 3-5, 100 mg cells ml^{-1}), were all toxic to locusts. The most toxic of these extracts were those prepared from filtered samples taken at Great Barford and Roxton, both of which caused paralysis in locusts in under 10 minutes. The extract of the St Neots sample caused paralysis to occur in 20 minutes (± 1.2 minutes).

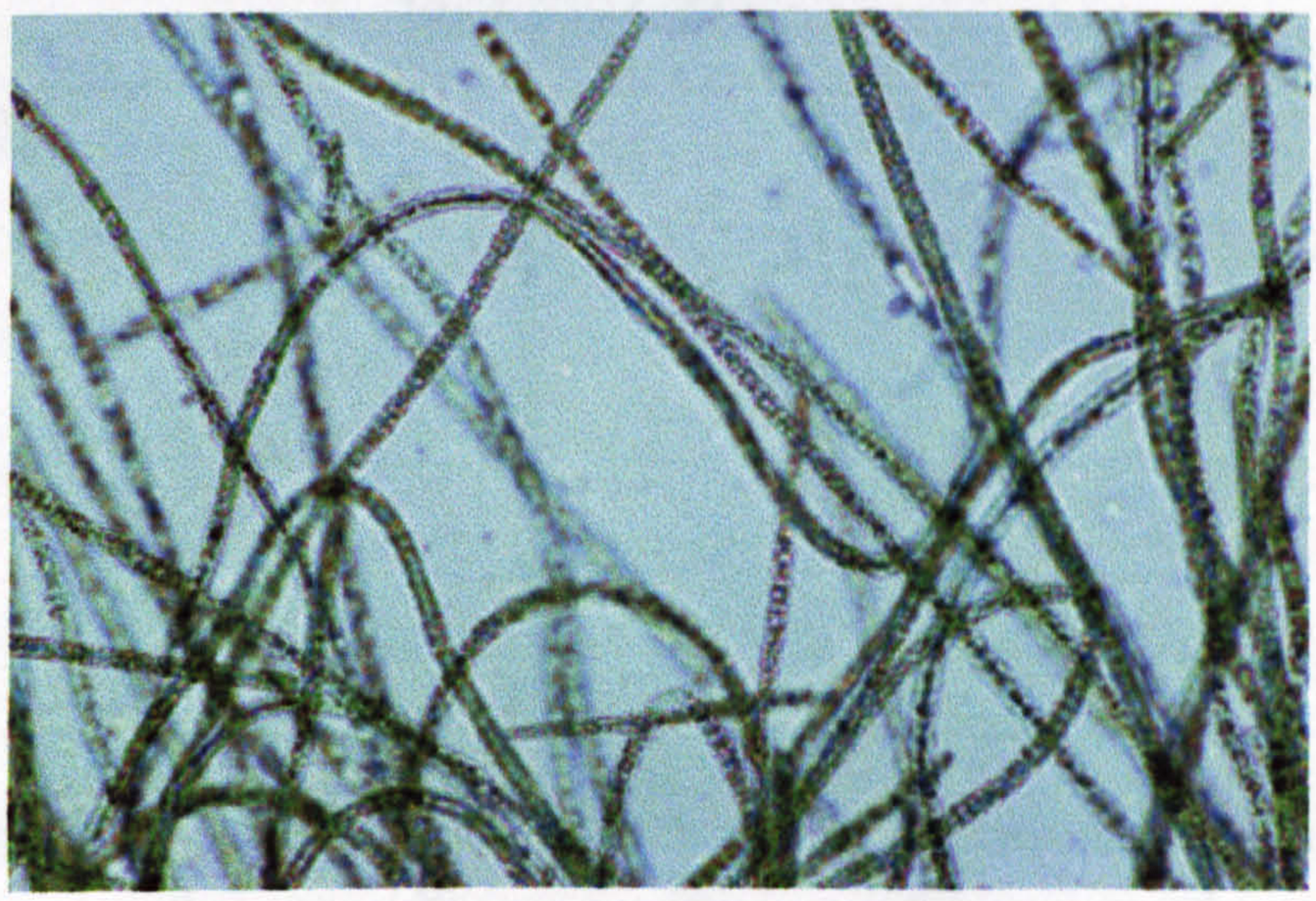
Saxitoxin and neosaxitoxin were not detected in sample 1 by prechromatographic oxidation followed by HPLC. Chromatograms of samples oxidised using peroxide showed no difference to those oxidised using periodate. Analysis of unoxidised sample indicated that the peaks obtained corresponded to naturally fluorescent compounds in the sample (Figure 2.11.).

Saxitoxin, neosaxitoxin and Gonyautoxins 1-4 were not detected by HPLC followed by post column oxidation and fluorescence detection in methanol + TFA extracts prepared from filtered samples taken at Great Barford, Roxton or St Neots (samples 3-5), or in any of the 11 laboratory isolates at a concentration of 100 mg cells ml^{-1}.

(a)



(b)



(c)

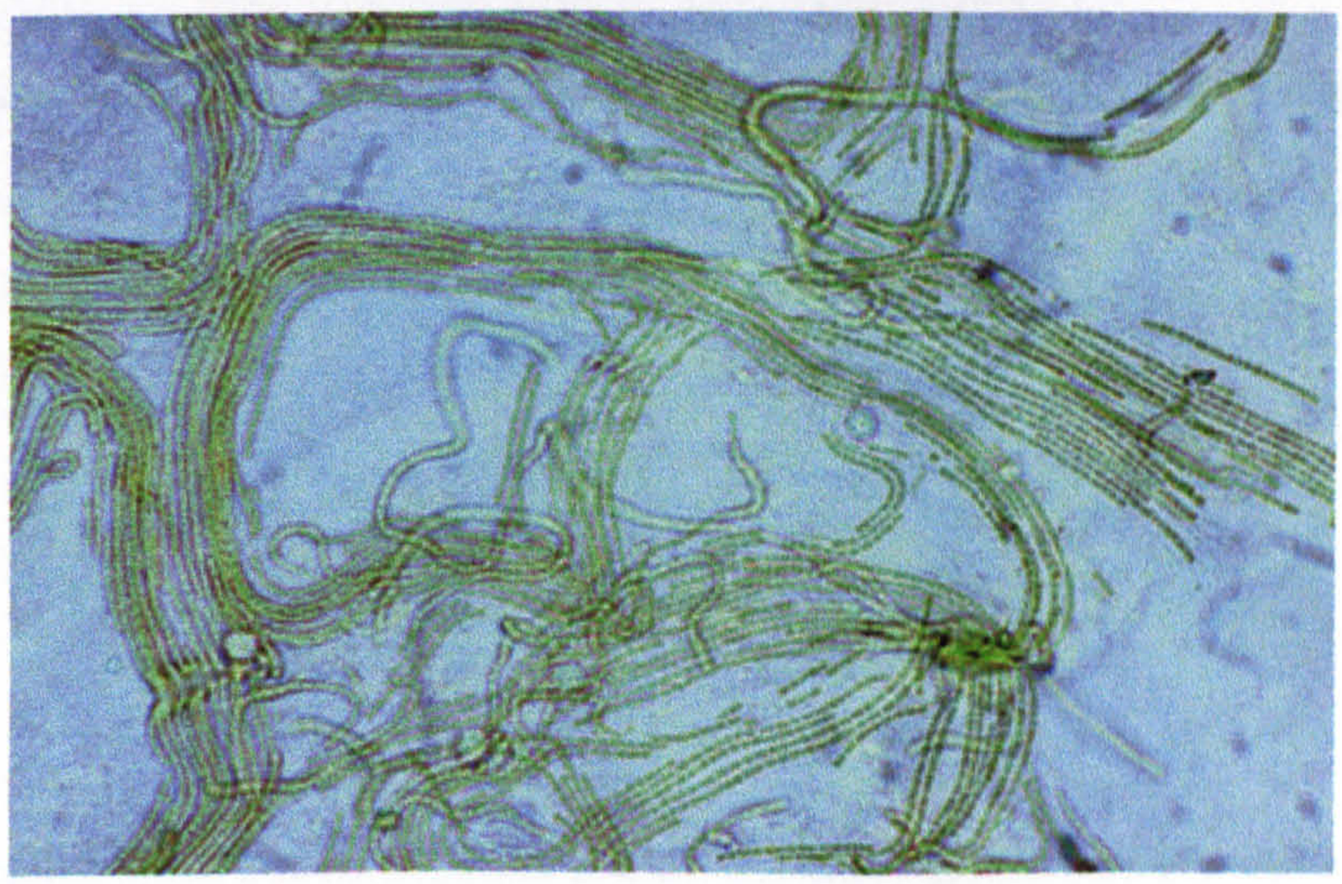


Figure 2.10. Photographs (x 270 magnification) showing microscopic examination of (a) *Aphanizomenon flos-aquae* NH-5a, (b) *Oscillatoria agardhii*, and (c) filamentous cyanobacteria isolated from bloom material collected at the Great Barford site of the River Great Ouse in August 1995.

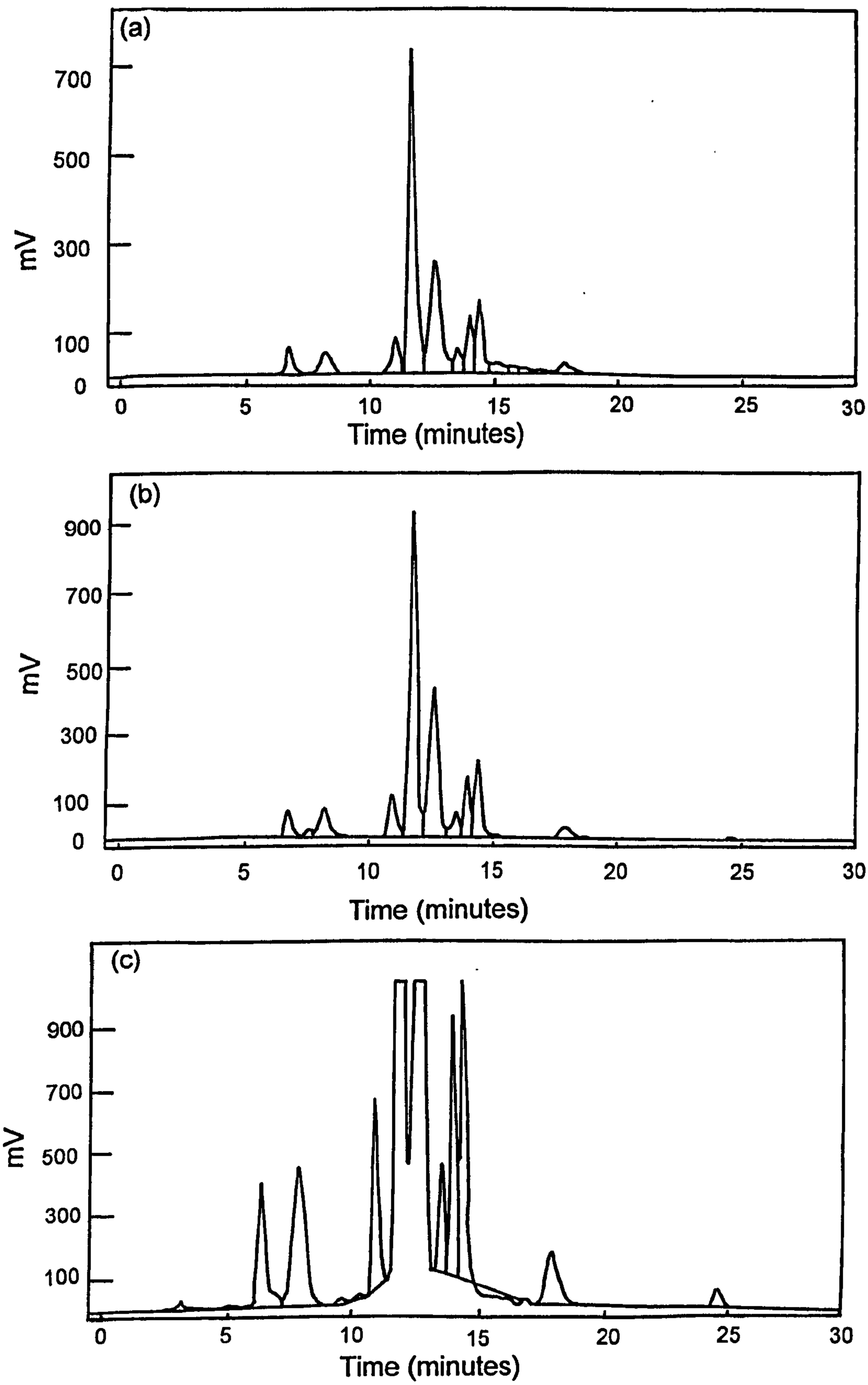


Figure 2.11. HPLC chromatograms of bloom material sampled at the Great Barford site extracted in methanol as described and analysed following precolumn oxidation with (a) periodate, and (b) peroxide. Chromatogram (c) shows the HPLC profile of the same sample without oxidation.

2.3.9. Analysis of cyanobacterial samples from the river Great Ouse for the presence of the neurotoxins anatoxin-a, and anatoxin-a(s).

HPLC analysis of pure anatoxin-a standard revealed a peak at 12.1 minutes. The toxin was not detected in the methanol + TFA extract of filtered material sampled on the 10th of August at the Great Barford site.

The colorimetric acetylcholinesterase inhibition assay showed that the colour change indicating cholinesterase activity was inhibited in the presence of the aqueous extract of *Ana. flos-aquae* NRC 525-17, but not in the presence of the extracts of *M. aeruginosa* CYA 43, or in the aqueous extract of the Great Barford sample (Table 2.2.). These results indicate that the cholinesterase inhibitor anatoxin-a(s) was not present in the Great Barford bloom sample.

2.3.10. Isolation of toxic components from Great Barford extract.

HPLC analysis with photodiode array detection revealed a cluster of sharp peaks between 2 and 20 min (Figure 2.12.). The spectra obtained for these peaks by photodiode array detection (200-300 nm) were uncharacteristic of any of the commonly occurring cyanobacterial toxins. None of the pooled fractions caused paralysis to occur in locusts at the 90 minute end-point of the assay, indicating that toxic components in the Great Barford extract had not been isolated using this method.

Table 2.2. Cholinesterase inhibition assay for the presence of anatoxin-a(s) in cyanobacterial extracts.

Sample	**Absorbance @ 420 nm (control)**	**Absorbance @ 420 nm (+ enzyme)**	**Δ Absorbance**	**Inhibition**
Ana. flos-aquae	1.775	1.772	0.003	*YES*
M.aeruginosa CYA 43	1.967	0.741	1.226	NO
Great Barford extract	1.834	0.701	1.133	NO

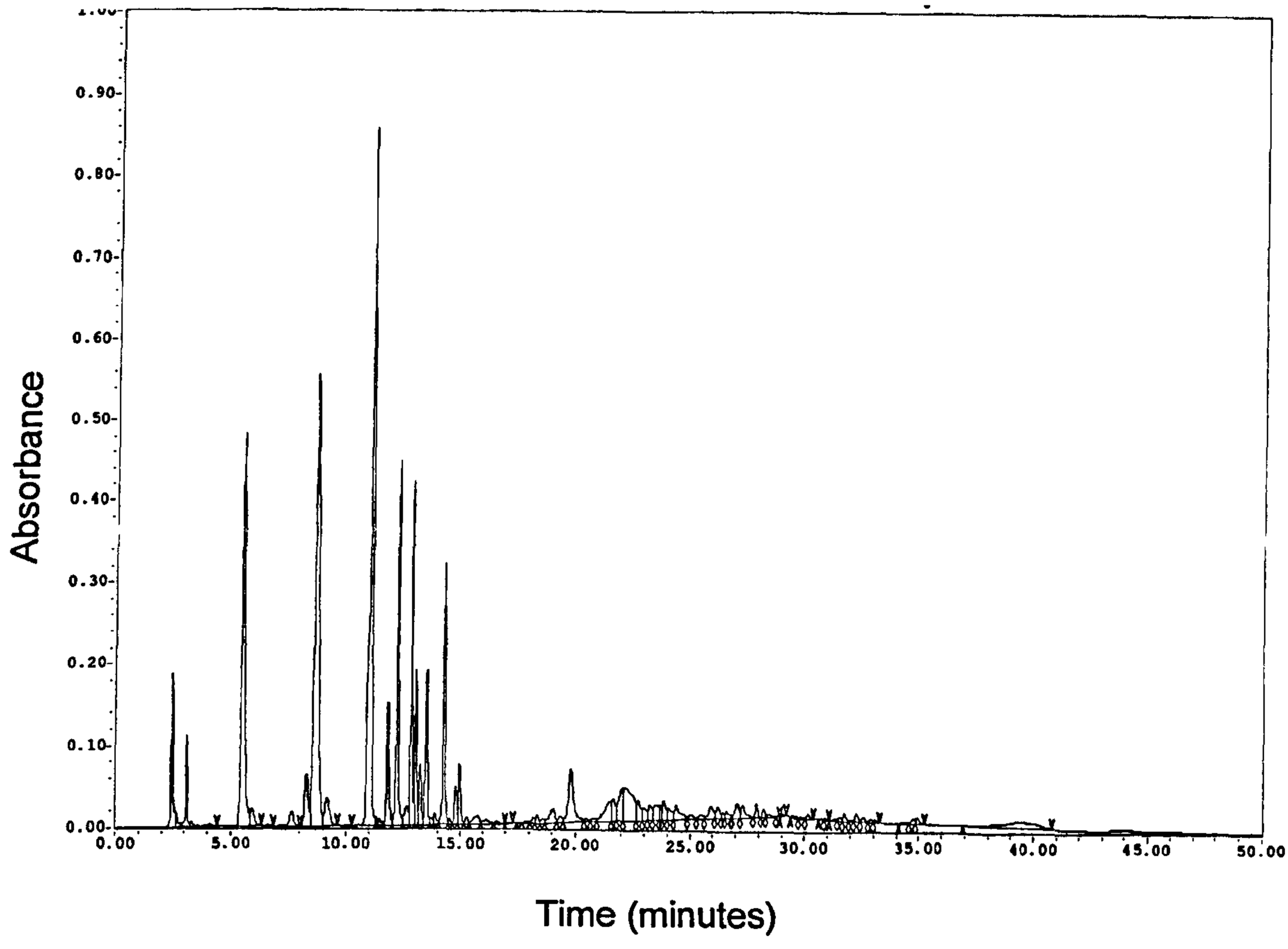


Figure 2.12. HPLC analysis with photodiode array detection of the methanol + 0.01% TFA extract of bloom material sampled at the Great Barford site as described in 2.2.10.

2.3.11. Compatibility of the locust bioassay with the screening of shellfish flesh for saxitoxins.

Mussel flesh samples spiked with 15 μg saxitoxin and extracted in 0.1 N HCl caused paralysis symptoms in locusts 15-20 minutes post injection. This time corresponded to the onset of paralysis observed in trials with pure saxitoxin at the same concentration. Injection of locusts with either HCl alone or extracts of unspiked mussel tissue had no adverse effects for up to 48 hours. These results indicated that HCl extracts of mussel flesh were suitable for screening by locust bioassay.

2.3.12. Toxicity determinations in natural mussel samples using the locust bioassay.

The locust bioassay results for nine natural mussel extracts of varying toxicity are shown in Table 2.3. The toxicity of each of the samples was determined by standard mouse bioassay and is expressed as μg toxin/100 g flesh. At the 90 minute end-point, the locust bioassay detected toxicity only in samples which were found to be highly toxic by mouse bioassay (555 - 915 μg/100 g). When the samples which had originally scored negative were concentrated 10-fold and tested again, all caused paralysis to occur in less than 90 minutes, with the exception of the one sample which was also non-toxic by mouse bioassay. This sample was scored negative at the end-point of the assay. Following the concentration step, samples with original concentrations of 159 and 131 μg/100g caused paralysis in under 5 minutes while the sample with an original concentration of 38 μg/100g caused paralysis at 18.3 min (± 2.9 min).

Table 2.3. Toxicity of natural mussel extracts determined by mouse and locust bioassays (+ by locust bioassay indicates the occurrence of paralysis before a 90 minute end-point).

Sample	Toxicity by mouse bioassay (μg STX/100g flesh)	Toxicity by locust bioassay
1	915	+
2	685	+
3	634	+
4	612	+
5	555	+
6	159	-
7	131	-
8	38	-
9	0	-

2.3.13. Use of the locust bioassay in a shellfish monitoring programme.

The locust bioassay was then used to screen 3 groups of mussel extracts in conjunction with the mouse bioassay as part of a large monitoring programme carried out by the Marine Laboratory, Torry, Aberdeen. The detection limit of the assay allowed a saxitoxin concentration of 250 μg/100 g of mussel flesh (2.5 μg ml^{-1}) to be detected following initial screening, and the concentration step allowed the detection of 25 μg/100g. The extracts were scored 1-3 according to toxicity, and the results compared to mouse bioassay data. The toxicity determinations of these samples by mouse and locust bioassay are shown in Tables 2.4.-2.6., and compared in a bar chart in Figure 2.13. Samples which were under or over estimated for toxicity by the locust bioassay are highlighted. The results show that toxicity determinations by locust bioassay compared well with those by mouse bioassay. Only 6% of samples were under estimated for toxicity by locust bioassay compared with mouse bioassay, while 8% were over estimated for toxicity.

Tables 2.4.-2.6. Assessment of toxicity in 3 groups of mussel extracts by mouse and locust bioassays as described in 2.2.13.

Key -

Under estimation of toxicity

Over estimation of toxicity

Toxicity rating-

1 - > 250 μg/100 g

2 - > 25 < 250 μg/100g

3 - < 25 μg/100 g

N.D. - not detected

GROUP 1

SAMPLE	TOXICITY BY LOCUST BIOASSAY	TOXICITY BY MOUSE BIOASSAY (μg per100 g)
S400	2	2 (68)
S390	2	2 (77)
S387	2	2 (168)
S638	3	3 (ND)
S105	2	2 (48)
S531	2	2 (48)
S455	2	2 (32)
S551	3	3 (ND)
S520	3	3 (ND)
S593	**3**	**2 (56)**
S104	2	2 (58)
S299	2	2 (52)
S457	2	2 (37)
S369	2	2 (149)
S635	2	2 (28)
S543	2	2 (32)
S338	3	3 (ND)
S354	3	3 (ND)
S414	2	2 (152)
S583	1	1 (291)
S453	2	2 (216)
S454	3	3 (ND)
P91	3	3 (ND)
S536	2	2 (187)
S174	1	1 (849)
S129	2	2 (69)
S533	3	3 (ND)
S490	2	2 (50)
S486+367	2	2 (115)
S323	**1**	**2 (194)**

GROUP 2

SAMPLE	TOXICITY BY LOCUST BIOASSAY	TOXICITY BY MOUSE BIOASSAY (μg/100 g)
S467	2	2 (81)
S241	2	3 (ND)
S235	2	2 (60)
S254	2	2 (45)
S471	2	2 (80)
S21	2	2 (35)
S547	3	3 (ND)
S255	2	2 (35)
S55	2	3 (ND)
S2	3	3 (ND)
S466	2	2 (43)
S345	2	2 (86)
S444	2	2 (79)
S591	2	2 (49)
S592	2	2 (40)
S641	1	1 (388)
S589	2	2 (80)
S209	1	2 (215)
S475	1	1 (1247)
S528	1	1 (808)
S386	1	1 (325)
S462	2	2 (241)
S412	2	2 (185)
S300	2	1 (295)
S508	2	2 (180)
S297	2	2 (128)
S492	2	2 (81)

GROUP 3

SAMPLE	TOXICITY BY LOCUST BIOASSAY	TOXICITY BY MOUSE BIOASSAY (μg/100 g)
S364	2	2 (69)
S628	2	1 (359)
S347	2	2 (96)
S590	3	3 (ND)
S169	2	2 (83)
S529	2	3 (ND)
S639	2	2 (142)
S472	3	3 (ND)
S468	1	1 (668)
S464	1	2 (116)
S4	3	3 (ND)
S584	3	3 (ND)
S418	2	2 (197)
S627	2	2 (62)
S554	2	2 (39)
S378	2	2 (71)
S401	2	2 (32)
S575	2	2 (68)
P88	1	2 (138)
S407	2	2 (51)
S321	3	2 (33)
S240	3	3 (ND)
S210	2	2 (32)
S684	2	2 (58)
S481	2	2 (81)
S563	2	2 (84)
S172	3	3 (ND)
S527	1	1 (251)
S127	3	2 (35)
S405	2	2 (109)
P92(POOLED)	2	2 (84)

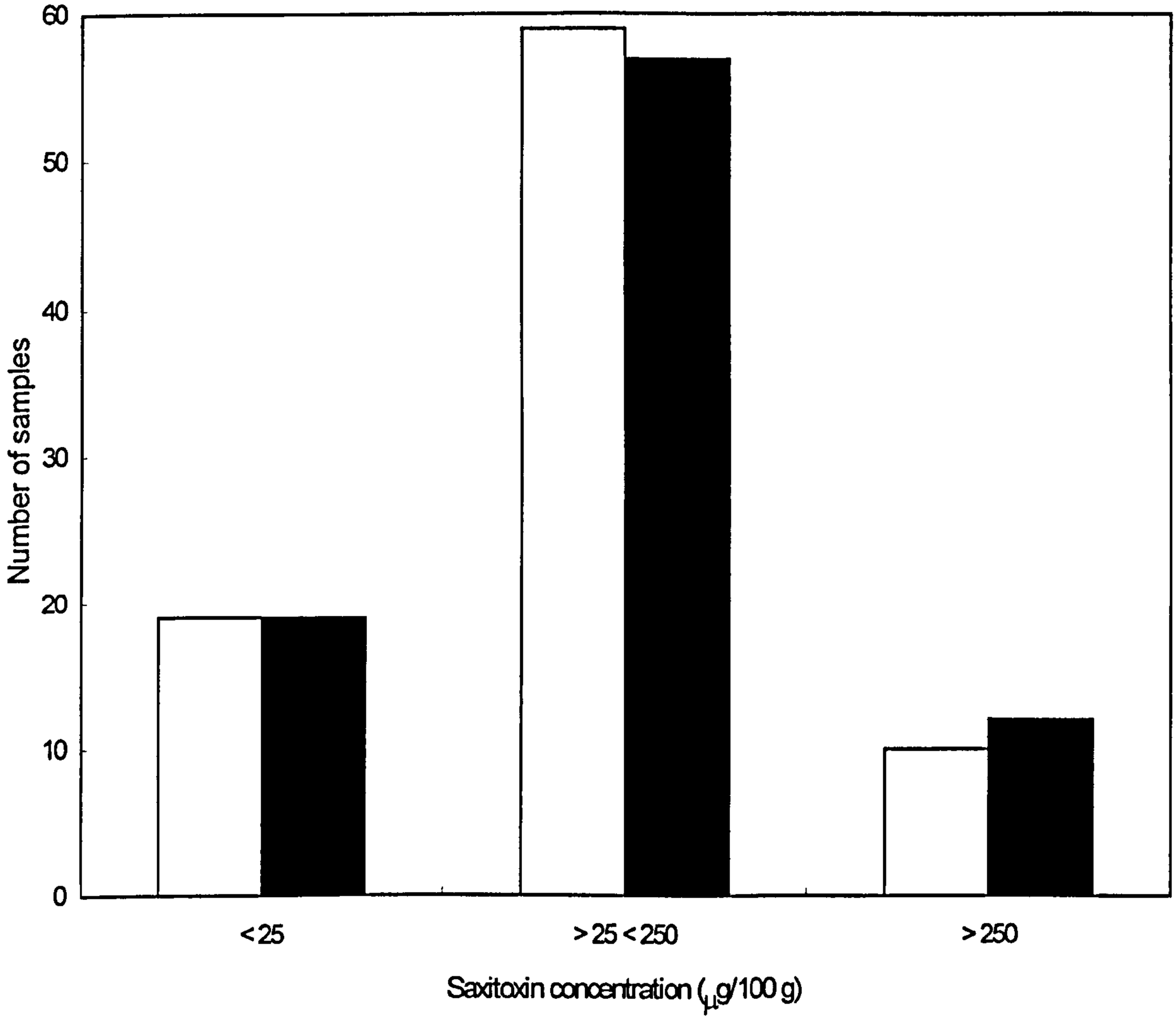


Figure 2.13. Toxicity determinations of mussel extracts determined by mouse bioassay (white columns), and locust bioassay (black columns).

2.4. Discussion.

These investigations have identified a suitable method for extracting saxitoxins from cyanobacterial cells for detection by HPLC and locust bioassay. Results have also shown that the desert locust may be a suitable alternative to the mouse for the screening of cyanobacterial and shellfish extracts for saxitoxins. The locust bioassay is inexpensive, and may be carried out in most laboratories with a minimum of expertise.

A considerable amount of research into the detection of saxitoxins has been devoted to finding an appropriate replacement to the mouse bioassay. Analytical methods such as HPLC have a number of advantages over bioassay techniques as they are able to identify different saxitoxins which may be present in a sample in addition to measuring toxicity. HPLC has therefore provided an invaluable tool to researchers studying the diversity and chemical behaviour of saxitoxins. However, the problems associated with HPLC have prevented its widespread implementation in monitoring programmes concerned with the protection of public health. Such programmes often require only a measurement of overall toxicity in order that poisonings may be prevented. The locust bioassay provided a simple and effective means for detecting the overall toxicity of samples in comparison with the HPLC techniques described in this chapter, which proved both expensive and time consuming.

Of the two HPLC methods used in this study, the method of Lawrence *et al.* (1991) involving precolumn oxidation of samples provided the most sensitive detection of saxitoxin and neosaxitoxin. It was determined that the lowest concentration of saxitoxin which could be detected with sufficient accuracy was equivalent to 1 ng on the column (results not shown). The analysis of saxitoxins by this technique has since been optimised by modifying the mobile phase composition to improve peak shape and reproducibility, and changing sample pH to 8 prior to oxidation to minimise sample matrix effects in shellfish extracts (Lawrence and Menard, 1991, and Lawrence, Menard, and Cleroux, 1995).

However, the earlier method was simple to perform, and provided sufficient sensitivity for identifying the optimum method for extracting saxitoxin and neosaxitoxin from dried cells of *Aph. flos-aquae* NH-5a. Analysis of samples using this technique resulted in the detection of sharp, defined peaks for both toxins with reasonable reproducibility. Neosaxitoxin could also be easily identified in samples by comparing the chromatograms obtained following periodate oxidation with those following oxidation with peroxide. Analysis of samples using HPLC with post column oxidation of samples was found to be considerably less sensitive and resulted in the detection of broader, tailing peaks. This may be observed by comparing the chromatograms obtained following analysis of 5 $\mu g\ ml^{-1}$ authentic saxitoxin by both techniques (Figure 2.2.). The toxin profiles of cyanobacterial extracts were also examined using the post-column method (Figure 2.8.) The retention time of saxitoxin was over 1 minute later in analyses of extracts compared with those of pure saxitoxin standard, and the identity of the toxins had to be confirmed by spiking the extracts with purified toxins. The variation in retention times may have been due to insufficient equilibration of the column and reaction tubing between injections. The main advantages of the precolumn method were that it was easier to set up and required less time for column equilibration. However, the method was unsuitable for the analysis of a large number of samples. Lawrence, Menard, and Cleroux (1995) reported that manually oxidised samples could be analysed by HPLC for up to 12 hours without any loss in sensitivity. In this study, pre-column saxitoxin derivatives were found to be reasonably stable for only 150 minutes under the conditions described (Figure 2.4.). For the accurate analysis of extracts investigated in this study, oxidation reactions were carried out immediately prior to injection. The reasons for the instability of saxitoxin derivatives are unclear at present, but they may have been due to the temperature at which the autosampler was maintained during analyses. Lawrence and co-workers used a refrigerated autosampler whereas the autosampler employed in this study remained at room temperature (25 °C) for the duration of the experiment.

Attempts to programme the Waters 717 plus autosampler to carry out the oxidation steps prior to each injection were also unsuccessful, meaning that each sample had to be oxidised and injected individually. HPLC with post column oxidation was therefore more suitable for the analysis of large numbers of samples because the oxidation reaction occurs immediately after injection and separation, allowing samples to be analysed in succession. This method was therefore adopted to determine the presence of saxitoxins in the natural bloom samples obtained from the 3 sites on the river Great Ouse, and in the cyanobacteria isolated from the Great Barford site. HPLC with post column oxidation was found to be considerably less sensitive than the precolumn method, with a detection limit equivalent to approximately 5 ng on the column. However, it would have been capable of detecting the amounts of toxin associated with the symptoms observed in locusts injected with extracts of original Great Barford bloom material.

The difficulties experienced with HPLC analysis of saxitoxins highlight the necessity for simpler, more amenable methods of detection. The limitations of the mouse bioassay, coupled with the ethical problems associated with its utility, has led to a number of studies which have exploited the effects of saxitoxins in invertebrates. The advantage of using insects in a bioassay of this nature is that they may be handled without specialist training, and may be maintained in the laboratory with a minimum of cost and expertise. Given that insect synaptosomes have been shown to contain approximately 95 saxitoxin binding sites per μm^2, compared to only 22 per μm^2 in rat brain (Dwivedy, 1990), the insect is an appropriate bioassay organism for saxitoxins.

The locust bioassay is particularly useful for the screening of cyanobacterial samples because it appears to be selective for the sodium channel blocking toxins. Locusts were not affected following injection with 100 $\mu g\ ml^{-1}$ of microcystin-LR and anatoxin-a, or a 50 $mgml^{-1}$ saline extract of *Ana. flos-aquae* NRC 525-17 containing anatoxin-a(s). It is not understood why these toxins failed to exert a response when injected into locusts.

A previous study into the toxicity of microcystin-LR in insects (Delaney and Wilkins, 1995), showed the toxin to be lethal to the housefly *Musca domestica*, the cabbage white butterfly *Pieris brassicae*, and the cotton leafworm *Spodoptera littoralis* following intrathoracic injection. However, these species are considerably smaller in size than the 5th instar *S. gregaria* employed in this study, which may account for their greater sensitivities to microcystin-LR. It is also possible that the effects of microcystin-LR toxicity are less acute in locusts than in the other species tested.

A further incentive for employing the locust bioassay is that the insects are a convenient size for handling and may be easily injected. The assay is more straighforward than the housefly bioassay proposed by Ross, Siger, and Abbott (1985), because the locust may be immobilised simply by holding the insect by its hind legs. The size of the housefly, on the other hand, necessitates immobilisation by exposure to a temperature of -20 °C prior to injection, which may have some impact on the results of the assay. Although the housefly bioassay is extremely sensitive, samples must be administered by microinjection, which may be impractical in many laboratories as considerable expertise is required. The 10 µl injection volume required for the locust bioassay may be administered without the need of specialised apparatus, and ease of handling also makes the assay safer to perform than the microinjection technique.

A major advantage of the locust bioassay is that it is compatible with both the standard extraction procedure for shellfish samples, and the optimum method investigated for the processing of dried cyanobacterial cells. The bioassay successfully detected toxicity in processed extracts of *Aph. flos-aquae* NH-5a without interferences from solvent, and identified the best extraction method for saxitoxin and neosaxitoxin used in this study. The HCl extraction of saxitoxins from shellfish flesh is well established, and the optimal conditions for extracting the toxins from dinoflagellates has also been investigated (Ravn *et al.*, 1995). However, little work has previously been done to determine the most suitable method for their extraction from cyanobacterial cells.

Previous reports have described the extraction of cyanobacterial material in 0.05 M acetic acid (Negri *et al.*, 1997), but the concentration of such samples is more complicated than with methanolic extracts. Methanol allows a significant degree of sample concentration, meaning that low concentrations of toxin can be detected. Processing cyanobacterial samples with methanol + TFA also provides an extract which can be analysed for the different types of toxins which may be present either in unialgal cultures or in natural bloom samples which may contain several cyanobacterial species. Methanol has proven successful in extracting the other main groups of cyanobacterial toxins and is particularly effective in the extraction of microcystins (Lawton, Edwards and Codd, 1994).

The comparison of extraction methods for saxitoxins from dried *Aph. flos aquae* cells showed that methanol + TFA extracts were the most toxic to locusts. HPLC analysis of these extracts showed that there was no difference in the amount of saxitoxin extracted from cells by water + TFA and methanol + TFA, but the recovery of neosaxitoxin appeared to be considerably enhanced when cells were extracted in acidified methanol. The higher concentration of neosaxitoxin in these extracts was therefore responsible for the more rapid onset of paralysis symptoms observed in locusts injected with methanol + TFA extracts. Extraction in methanol + TFA followed by locust bioassay provides a simple method for identifying the presence of neurotoxicity in cyanobacterial samples, which can then be further analysed by HPLC if the toxin profile is required.

Acidified methanol extracts of bloom material collected from the river Great Ouse on the 22nd of August were found to be toxic when extracted in methanol + TFA (sample 3) but not in methanol alone (sample 2). Sample 2; which was extracted in methanol (final concentration of 120 mg cells ml^{-1}) failed to cause paralysis in locusts at the 90 minute end-point of the assay. When the same material was extracted in methanol + TFA to a final concentration of 100 mg cells ml^{-1} (sample 3), paralysis occured in only 10 minutes. HPLC analysis involving both pre and post column oxidation failed to identify saxitoxin, neosaxitoxin, or gonyautoxins 1-4 analysed for in any of the extracts found toxic by locust bioassay.

Although saxitoxins were not detected in any of the samples by HPLC, locust bioassay results indicated that a toxic component causing similar symptoms to those observed with saxitoxin and neosaxitoxin was extracted by methanol + TFA, but not by methanol alone. Failure to isolate toxic fractions by HPLC means that the component responsible for causing paralysis in locusts remains unidentified. Further investigations were prevented due to the lack of original bloom sample available, and the inability to isolate the toxic cyanobacterial species in culture.

Comparison with mouse bioassay data showed that the locust bioassay was also suitable for the screening of HCl extracts of shellfish flesh. By standardised mouse bioassay, the acceptable regulatory limit for the human consumption of shellfish is 80 μg STX equivalent/100 g flesh (AOAC). Of the toxic mussel extracts tested, only highly toxic samples scored a positive result by locust bioassay. Initial screening by locust bioassay was unable to detect alert levels (40 μg ml^{-1}) of toxin in the samples tested, but a concentration step allowed such levels to be recognised. Sample concentration may also be carried out without increasing the concentration of HCl. By resuspending dried HCl extracts in methanol, drying and dissolving the sample in saline, the injection of locusts with high levels of HCl is prevented. This method is no less precise than the dilutions often required for the assay of highly toxic shellfish extracts by mouse bioassay. Further investigation will be required in order to ascertain the level of sensitivity which may be achieved by sample concentration. In these studies, the locust bioassay was able to detect down to 25 μg/100 g shellfish tissue, almost 2 times less than the minimum detection level of the mouse bioassay (Sommer and Meyer, 1937). Another advantage of employing the locust bioassay for the detection of paralytic shellfish toxins in shellfish is that a much smaller quantity of flesh is required to provide sufficient extract for the assay. Since the injection volume required is only 10 μl, sample may be injected into a larger number of insects, thus improving accuracy.

The use of the locust bioassay in a large shellfish screening programme indicated that it correlated well with the mouse bioassay, underestimating the toxicity in only

6% of the samples. Of all the samples which scored 3 by locust bioassay (<25 μg/100g), only one was found to contain greater than the alert level of saxitoxin by mouse bioassay (40μg/100 g). These results indicate the potential of the locust bioassay in the screening of shellfish samples, and with further validation, the method may prove to be a suitable replacement to the mouse bioassay in monitoring programmes of this nature.

In conclusion, the locust bioassay shows considerable promise as a method for detecting the presence of saxitoxins in different sample matrices. Toxicity levels in extracts of *Aph. flos-aquae* NH-5a confirmed by HPLC were correctly identified by the bioassay, without sample matrix effects. Although the assay is unable to determine the toxin profile of a sample, it is ideal for initial toxicity screening, and can save a great deal of time in indicating the presence of saxitoxins in samples, which can then be analysed in detail by techniques such as HPLC. The two HPLC methods used in this study were both extremely time consuming and labour intensive, and it was therefore not feasible to use them exclusively in the analysis of samples.

Chapter 3

Lab-scale purification of microcystin variants by normal and reversed-phase flash chromatography

3.1. Introduction.

The widespread occurrence of microcystins in water bodies has prompted a vast body of research into their detection and biological effects; therefore increasing the requirement for purified standards (Edwards *et al.*, 1996 a). Techniques for chemically synthesising these compounds have been investigated with some success (Namikoshi *et al.*, 1989; Zetterström *et al.*, 1995; Humphrey, Aggen, and Chamberlin, 1996), but these are still to be fully developed. Pure microcystins are therefore acquired by the separation and concentration of toxins extracted from cyanobacterial cells.

Over 60 microcystin variants have now been documented (Sivonen and Jones, 1999). Differences in variable L-amino acids, along with other minor chemical modifications to the basic microcystin structure can cause changes in the physico-chemical behaviour of different variants. For example, the presence of the amino acids tryptophan (W) and phenylalanine (F), make microcystins-LW and -LF relatively hydrophobic compared to most other microcystins. Previously, hydrophobic microcystins were often overlooked, as earlier methods for extraction were concerned with the recovery of the more polar variants, particularly microcystin-LR (Lawton *et al.,* 1995). Furthermore, many of the commonly used HPLC protocols fail to detect hydrophobic microcystins, as they do not elute over a broad enough range of polarity. This has resulted in the under reporting of these toxins in natural samples. Microcystin-LR is the most commonly occurring microcystin and has been studied extensively (Carmichael, 1992). However, much less is known of the more hydrophobic variants such as microcystins-LW and -LF. Hydrophobic microcystins have aroused considerable interest, as their chemical properties may allow them to be more cell permeable than microcystin-LR (Craig *et al.,* 1993). The ability of these toxins to enter cells may provide a means of identifying previously unknown substrates for protein phosphatases, and allow the role of these enzymes to be studied more closely in a variety of cell types. However, in order that the occurrence and biological effects of these variants may be studied in detail, methods for their purification require further investigation.

Microcystins-LW and -LF have been reported in a number of naturally occurring cyanobacteria and are produced in laboratory culture by several strains of *Microcystis aeruginosa* (Craig *et al.*, 1993; Azevedo *et al.*, 1994; Lawton *et al.*, 1995). However, the detection of these toxins has been hindered by the lack of purified standards. Hydrophobic microcystins have proven difficult to purify as they often occur in very low concentrations in cyanobacterial cells (Carmichael, 1997). They are also not fully recovered from cells using many of the commonly used extraction methods, which employ aqueous solvents (Lawton, Edwards, and Codd, 1994).

A number of different procedures have been developed for the extraction and purification of microcystins, but many involve numerous complicated stages (Krishnamurthy, Carmichael, and Sarver, 1986; Harada *et al.*, 1991; Namikoshi *et al.*, 1992). Lawton *et al.*, (1995) advocated the use of methanol for extracting microcystins from cyanobacterial cells. Extraction in methanol followed by concentration using 1 g C_{18} Sep-Pak cartridges not only provided a simple purification step for several microcystins, but also yielded considerably higher amounts of microcystins-LW and -LF than those which had been previously described. Recently, the purification of sub-gram quantities of microcystins was achieved following the concentration and preliminary purification of methanol extracts using reversed-phase flash chromatography (Edwards *et al.*, 1996 a, and 1996 b). This procedure facilitates the concentration of a large volume of aqueous methanolic cell extract onto a pre-packed C_{18} flash chromatography cartridge. Microcystins are then eluted from the cartridge using a step gradient from 0% to 100% (v/v) aqueous methanol. Cleaner fractions containing microcystins of similar polarity are obtained, which can then be separated further using preparative HPLC. Although a number of highly pure microcystin variants have been isolated in this way, the separation of microcystins-LW and -LF has proven difficult. Successful purification of these two toxins has only been achieved using closed-loop recycling, in which they are passed through the preparative column several times until separation is improved (Edwards *et al.*, 1996 b).

Although this method has been shown to provide good purity and yield, it relies on the availability of specialised HPLC equipment and requires considerable expertise.

This chapter presents an alternative approach to the purification of microcystin -LW and -LF (Lawton, McElhiney and Edwards, In press). The method described exploits the separation of these 2 variants by normal-phase chromatography, thus eliminating the need for preparative HPLC. Thin layer chromatography was first employed to identify a suitable mobile phase for separating mixtures of microcystin-LW and -LF. Conditions for purifying the two toxins were then optimised using small silica cartridges. The procedure was finally scaled up to facilitate the isolation of milligram quantities of both toxins following preliminary purification using reversed-phase flash chromatography. A methanolic extract of *M. aeruginosa* was concentrated on a reversed-phase (C_{18}) flash cartridge, which was then eluted using a step gradient from 0 to 100% methanol in water (v/v), in 10% increments. This allowed partial separation of the 4 main microcystins present in the extract. One of the fractions contained microcystin-LR of sufficient purity for use in the studies described elsewhere in this thesis. Another of the fractions collected contained a mixture of microcystin-LW and -LF which were then successfully purified using optimised normal phase chromatography on a simple pre-packed flash cartridge system.

3.2. Materials and methods.

3.2.1. Chemicals.

Chemicals were of analytical-reagent grade unless stated and obtained from Merck (Darmstadt, Germany), unless stated. HPLC-grade methanol, acetonitrile and dichloromethane (DCM) were obtained from Rathburn. Pure water was obtained from a Milli-Q system (Millipore). Microcystin standards (-LR and -LF) were purified from batch cultures of *M. aeruginosa* as previously described (Edwards *et al.*, 1996 b). Partially purified mixtures of microcystin -LW and -LF used in the method development were prepared in the same way using *M. aeruginosa* collected from a bloom which occurred at Rutland Water (Leicestershire, UK), in September 1989.

3.2.2. Cyanobacterial material.

Batch cultures (4-8 litres) of *Microcystis aeruginosa* PCC7820 were grown under the conditions decribed in chapter 2 (2.2.1.). Cells were harvested after approximately 5 weeks growth by tangential flow filtration (Pellicon-2; fitted with three 0.22 μm, type GVPP-V filters, Millipore) and stored as a wet pellet at -20°C until required.

3.2.3. Identification of the optimum solvent system for normal-phase separation of microcystin-LW and microcystin-LF using thin-layer chromatography (TLC).

Solvent optimisation was carried out initially using normal-phase thin layer chromatography (TLC) plates (5 x 10 cm, silica gel 60; Merck). A line was marked in pencil approximately 2 cm from the bottom of the plate for applying sample. Another line was marked approximately 2 cm from the top of the plate to indicate the solvent front for the measurement of R_f values.

The separation of microcystin-LW and -LF was evaluated in different mobile phases using a semi-purified mixture of both toxins, which was spotted onto the lower line using glass capillaries. Pure microcystin-LF was spotted for comparison, in order that the two toxins could be distinguished from the mixture. TLC plates were developed in a cylindrical glass chromatography tank which was filled up to approximately 1 cm with mobile phase, and covered with a watch glass. Mobile phase was allowed to run no further than the pre-marked line at the top of each plate. Components were visualised by developing TLC plates in a glass jar containing powdered silica mixed with approximately 10 g of iodine.

Separation of the two toxins was first assessed using a mobile phase consisting of either chloroform or DCM. When no separation was achieved using either of these solvents alone, varying amounts of a more polar solvent, (either ethanol or methanol) were added. Initial trials with different solvent mixtures revealed that the two toxins could be separated in mixtures of DCM and methanol. However, development of TLC plates revealed that tailing had occurred (the smearing of separated components in the mixture). This problem was overcome by adding a small amount of concentrated acetic acid to the mobile phase. The separation of microcystins -LW and -LF was assessed using different ratios of DCM, methanol, and acetic acid. Rf values were calculated (distance travelled by component/solvent front), and converted into column volumes (CV = 1/Rf), which represented the approximate number of column volumes of solvent required to elute each component in a packed column separation. In order to determine suitable separation conditions, the difference between predicted column volumes for each microcystin was determined (ΔCV). The ratio of DCM, methanol, and acetic acid which resulted in the highest ΔCV value was chosen as the optimum mobile phase for the separation of microcystin-LW and -LF.

3.2.4. Method development for normal-phase separation of microcystin-LW and -LF using Flash chromatography.

Using the solvent selected by TLC, the separation of microcystin-LW and -LF on pre-packed flash KP-Sil™ silica cartridges (15 x 1.2 cm I.D., 32-63 μm particle size, 60 Å pore size; Biotage) was assessed using a Biotage Flash 12i™ system (Biotage, a Division of Dyax Corporation, Charlottesville, VA, USA). This system is an upgrade of the Biotage Flash 40i™ system (Figure 3.1.) designed to facilitate relatively small sample loads. The system comprises a stainless steel solvent reservoir to which nitrogen pressure is applied to drive the solvent or sample through the pre-packed cartridge housed in an axial compression module. Axial compression compacts the bed of silica in the cartridge to prevent channeling and improve the flow of mobile phase. A flow diagram showing the set-up of this system is illustrated in Figure 3.2. Initial experiments were carried out using a partially purified mixture of microcystin-LW and -LF (prepared as described in 3.2.1.), which was divided into three equal portions (each containing 1.4 mg and 1.6 mg respectively), and dried under nitrogen. Separation was originally undertaken using the most suitable mobile phase identified by TLC (Table 3.4.). However, the use of an isocratic solvent system consisting of DCM:methanol:acetic acid (88:10:2) resulted in co-elution of the two toxins, necessitating the application of a step gradient. Mobile phase A was DCM and B contained methanol:acetic acid (10:2). The silica cartridge was conditioned with 200 ml (20 CV) of 95% A:5% B prior to sample loading. Nitrogen pressure (5-10 p.s.i.) was applied to the solvent reservoir to achieve a flow rate of 5 ml min^{-1}. One of the dried samples was dissolved in 2 ml DCM, and injected onto the cartridge through the injection port using a syringe. The cartridge was then eluted in 125 ml (12.5 CV) of 95% A:5% B, followed by 200 ml (20 CV) of 92% A:8% B and 50 ml (5 CV) of 90% A:10% B. Fractions (approximately 5 ml) were collected in glass vials and allowed to dry overnight in a fume cupboard, after which time the DCM had evaporated. Each concentrated fraction was then analysed by TLC using a mobile phase consisting of DCM:methanol:acetic acid (88:10:2). Crude starting material and pure microcystin-LF were also spotted onto

TLC plates in order to determine the expected R_f values of microcystin-LW and -LF under these conditions.

Following poor separation of microcystin-LW and -LF using the three elution steps described above, separation of the two toxins was evaluated using a longer step gradient. The toxin mixture was also found to be only partially soluble in DCM alone, causing the injection port to become blocked during sample loading. Therefore, the remaining sample was dissolved in 200 µl methanol, and then diluted with 2 ml DCM prior to loading. The sample was loaded onto a pre-conditioned silica cartridge as described above, and eluted using the gradient shown in Table 3.1. Fractions were allowed to stand uncovered in a fume cupboard overnight to allow the DCM to evaporate. Concentrated fractions were then analysed by TLC to determine the presence of microcystin-LW and -LF.

In order that the samples could be stored without compromising toxin stability, concentrated acetic acid was removed from fractions found to contain microcystin-LW and/or -LF using solid phase extraction. The fractions of interest were dissolved in methanol, then individually diluted with 20 ml Milli-Q water, mixed, and loaded onto an Isolute C_{18} (EC) cartridge (1 g, Isolute sorbent technology, Mid-Glamorgan, UK), which had been preconditioned with 20 ml methanol followed by 20 ml Milli-Q water. Toxins contained in each of the fractions were eluted from the cartridges with 5 ml methanol. The eluates were individually analysed by HPLC (3.2.5.), and the recoveries of microcystin-LW and -LF in each fraction determined by comparison to a pure microcystin-LR standard.

Figure 3.1. Biotage Flash 40i™ system comprised of a stainless steel solvent reservoir under pressure (approx. 20 p.s.i.) which drives solvent/sample through a cartridge (packed with appropriate stationary phase) housed in an axial compression module.

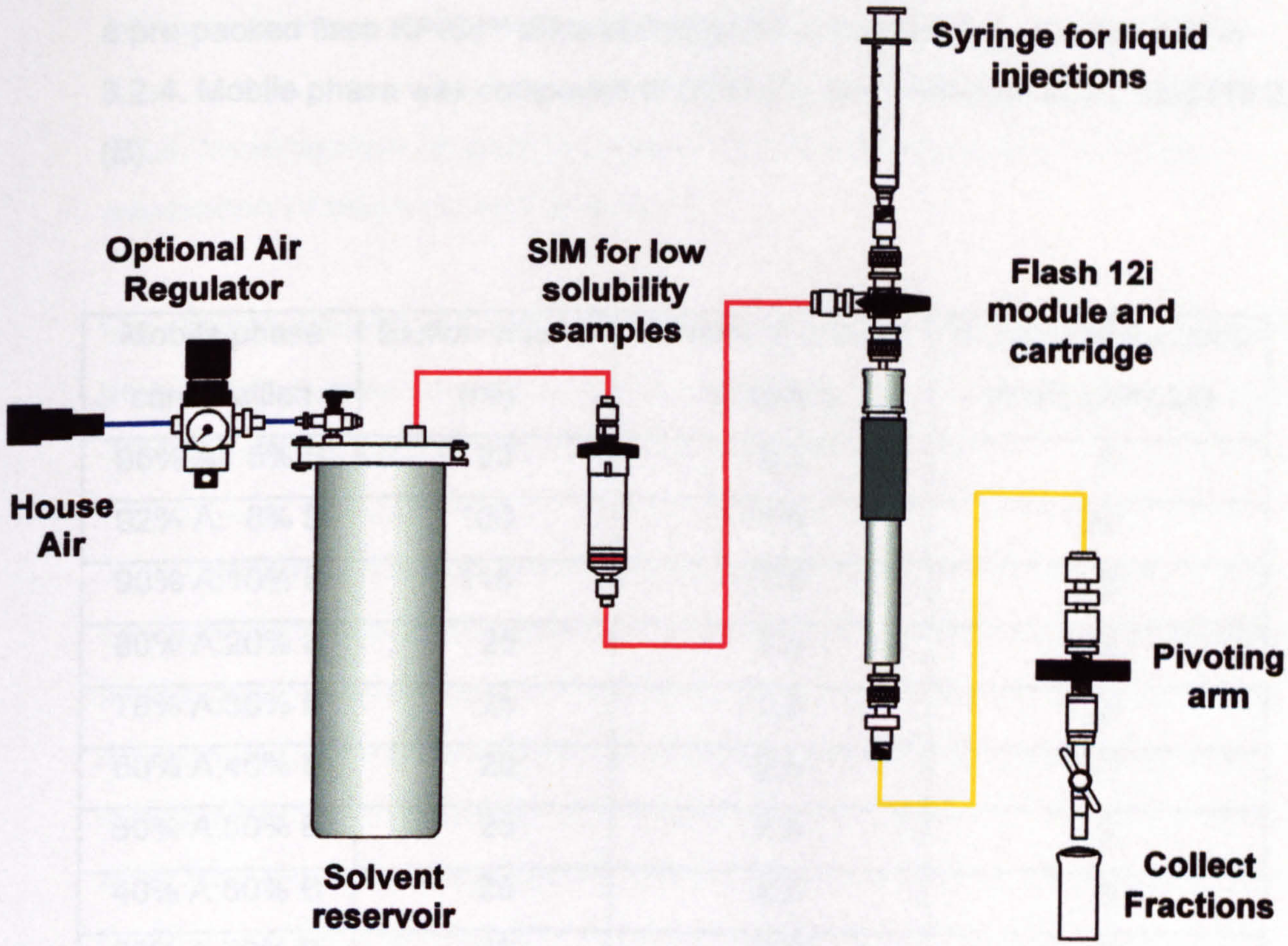


Figure 3.2. Schematic diagram showing the set-up of the Biotage Flash 12i™ system used in the purification of microcystin-LW and -LF.

Table 3.1. Gradient developed for the separation of microcystin -LW and -LF from a pre-packed flash KP-Sil™ silica cartridge (15 x 1.2 cm I.D.), as described in 3.2.4. Mobile phase was composed of DCM (A), and methanol:acetic acid (10:2) (B).

Mobile phase composition	Elution volume (ml)	Number of column volumes	Number of fractions (5 ml) collected
95% A: 5% B	25	2.5	5
92% A: 8% B	100	10.0	20
90% A:10% B	115	11.5	23
80% A:20% B	25	2.5	5
70% A:30% B	25	2.5	5
60% A:40% B	25	2.5	5
50% A:50% B	25	2.5	5
40% A:60% B	25	2.5	5
30% A:70% B	25	2.5	5
20% A:80% B	25	2.5	5
10% A:90% B	25	2.5	5
100% B	25	2.5	5

3.2.5. Analytical HPLC.

All fractions were analysed as described previously (Lawton, Edwards, and Codd, 1994). Toxin identification, quantification, and purity were determined by HPLC with high resolution diode array detection using a Waters 996 detector. Samples were separated on a Symmetry C_{18} column (250 x 4.6 mm I.D.; 5 μm particle size, Waters). Mobile phase consisted of water and acetonitrile both containing 0.05% trifluoroacetic acid (TFA). Separation was achieved using a linear gradient

starting at 30% (v/v) aqueous acetonitrile increasing to 35% over 5 minutes followed by an increase to 70% over the next 30 minutes at a flow rate of 1 ml min^{-1}. Detector resolution was set at 1.2 nm and data acquired from 200 to 300 nm with chromatograms monitored at 238 nm.

3.2.6. Investigation of toxin recoveries following normal-phase Flash separation of microcystin-LW and -LF.

Experiments were carried out in order to identify any stages during the separation process in which toxins may be lost, thus affecting the recoveries of microcystin -LW and -LF following normal-phase flash separation.

The stability of the two toxins in acidified mobile phase was first assessed, since it was routine to permit the evaporation of DCM from fractions at room temperature prior to analysis. Aliquots (2 x 70 µl) containing a mixture of microcystin-LW and microcystin-LF in methanol (approximately 40 µg and 35 µg respectively) were added to two glass test tubes containing 5 ml of 90% A:10% B. These solutions were then allowed to evaporate by letting them stand uncovered in a fume hood for 48 hours. Controls were also prepared by adding the same amount of toxin solution to two test tubes containing 5 ml methanol. After 48 hours, 1 ml of methanol was added to each tube, and mixed thoroughly to resuspend the toxins. Each solution was analysed by HPLC and the amounts of microcystin-LW and -LF determined as described.

A second experiment was carried out to determine the step gradient described in 3.2.4. (up to 100% methanol:acetic acid (10:2)) had eluted all of the toxins loaded onto the silica cartridge. A dried sample containing 2.5 mg microcystin-LW and 1.8 mg microcystin-LF was dissolved in 200 µl methanol, diluted with 2 ml DCM, and loaded onto a pre-conditioned silica cartridge as described. Toxins were eluted from the cartridge using the gradient shown in Table 3.1.

All eluate was collected as a single fraction which was rotary evaporated to dryness, and resuspended in 2 ml of methanol. The silica cartridge was then eluted with a further 100 ml of 100% B (methanol:acetic acid), in order to ensure the removal of any toxins which had remained bound to the cartridge. This sample was also rotary evaporated to dryness and resuspended in 2 ml of methanol. Both samples were analysed by HPLC, and the toxin recoveries determined as described.

The poor toxin recoveries obtained following previous separations suggested the possibility that microcystin-LW and -LF were being irreversibly bound on the silica cartridge. Strong, often irreversible sorbtion sites can arise in silica gels due to the presence of free silanol groups which can interact very strongly with analytes, preventing their elution (Thurman and Mills, 1998). In order to minimise irreversible binding of microcystin-LW and -LF, cartridges were washed in either 100% B (methanol:acetic acid), or 100% methanol prior to equilibration and sample loading. These solvents are considerably more polar than DCM, and can react with strong sorbtion sites, thus weakening the binding of the toxins to the silica gel.

Two identical samples each containing a dried mixture of microcystin-LW and -LF (1.5 mg and 1.4 mg respectively) were resuspended in 200 ml of methanol, and diluted with 2 ml of DCM. These toxin mixtures were separated on smaller silica cartridges than those used previously (7 x 1.2 cm I.D.), in order to minimise solvent consumption and save time. Cartridges were washed with 100 ml (20 CV) of 100% B, or 100% methanol, followed by 50 ml (10 CV) DCM (to ensure the removal of the more polar solvent mixture from the cartridge), prior to equilibrating with 50 ml (10 CV) of 95% A: 5% B, and loading the sample. Toxins were eluted using the gradient described in Table 3.1., except that the volumes and number of fractions (5 ml) collected at each stage were halved.

Separation of microcystin-LW and -LF was assessed by TLC. All of the collected fractions were then pooled, rotary evaporated to dryness, and resuspended in 2 ml methanol. Samples were analysed by HPLC and the total recoveries of both toxins determined.

3.2.7. Optimisation of separation conditions for normal-phase separation of microcystin-LW and -LF using Flash chromatography.

Final optimisation of separation conditions was carried out using a pre-packed flash KP-Sil™ silica cartridge (15 x 1.2 cm I.D.) which was conditioned by washing with methanol (200 ml) then DCM (100 ml). Mobile phase A and B were prepared as described in 3.2.6. The cartridge was equilibrated with initial mobile phase, 95% A:5% B (100 ml). For method optimisation, a partially purified sample containing 1.3 mg microcystin-LF and 1.5 mg microcystin-LW was resuspended in 200 µl of methanol and diluted with 2 ml of DCM prior to injection. The gradient used to separate the two toxins is shown in Table 3.2.

Table 3.2. Gradient applied for optimised separation of microcystin-LW and -LF from a pre-packed flash KP-Sil™ silica cartridge (15 x 1.2 cm I.D.), which had been pre-conditioned with methanol prior to sample loading. Mobile phase was composed of DCM (A), and methanol:acetic acid (10:2) (B).

Mobile phase composition	Elution volume (ml)	Number of column volumes	Number of fractions (5 ml) collected
95% A: 5% B	30	3.0	6
92% A: 8% B	100	10.0	20
90% A:10% B	120	12.0	24
80% A:20% B	30	3.0	6
70% A:30% B	30	3.0	6

Fractions (approximately 5 ml) were analysed by TLC. Fractions found to contain microcystin-LW and/or -LF were dried under nitrogen, and resuspended in 2 ml methanol. In order to assess toxin recoveries, fractions were grouped into those found to contain microcystin-LF only (group 1), mixtures of microcystin-LW and -LF (group 2), and microcystin-LW only (group 3). Fractions belonging to each group were combined, diluted to 20% methanol with Milli-Q water, and loaded onto pre-conditioned Isolute C_{18} cartridges. Each was eluted in 5 ml methanol, and analysed by HPLC. The amounts of microcystin in each of the groups was determined as described in 3.2.4.

3.2.8. Extraction of cyanobacterial cells and separation of microcystins using reversed- phase Flash chromatography.

The wet pellet of *M.aeruginosa* PCC7820 prepared in 3.2.2. was thawed and extracted in general grade methanol (2 l, Fisons, Loughborough, UK), for 30 minutes with regular stirring. Aliquots (3 x 1 ml) of the methanolic extract were placed in pre-weighed glass vials and dried in an oven at 80 °C. The vials were cooled in a dessicator and re-weighed to give the dry weight of cells per ml of extract. The extract was centrifuged at 1500 x g for 30 minutes, the supernatant decanted and the pellet re-extracted in an identical manner a further two times. The pooled supernatants were diluted with Milli-RO water to 20% methanol (v/v), and allowed to stand for 30 minutes, permitting enough time for precipitation to occur prior to removal by filtration (tangential flow). It has been found in the past that filtering immediately after dilution leads to cartridge blockage. A sample (1 ml) was removed from the diluted extract for HPLC analysis in order that toxin recovery could be determined following separation. Microcystins were concentrated using the Biotage Flash 40i™ system shown in Figure 3.1. A KP-C18-HS™ flash cartridge (15 x 4 cm I.D., 35-70 μm particle size, 60Å pore size) was conditioned with 1 litre of methanol followed by 1 litre of water before applying the aqueous extract. Nitrogen pressure (20-30 p.s.i.) was applied to the solvent reservoir to achieve a flow rate of 40 ml min^{-1}. Microcystins were eluted using a step gradient from 0 to 100% methanol in 10% increments (1 l per step)

with each step fraction collected separately and analysed by HPLC as described in 3.2.5. After use, the reversed-phase flash cartridge was cleaned with dichloromethane (DCM; 1 l) then flushed (1 l) and stored in methanol for future use.

3.2.9. Scale-up of normal-phase flash chromatography for the separation of microcystin-LW and -LF in partially purified reversed-phase flash fraction.

HPLC analysis of reversed-phase flash fractions obtained following the extraction described in 3.2.8. revealed that microcystin-LW and -LF eluted in 60% methanol (Figure 3.10.). This fraction had to be concentrated in a small volume of methanol and diluted with DCM before it could be loaded onto a normal-phase flash cartridge for final purification of the two toxins.

The fraction was diluted with 1 litre of water, loaded onto a pre-conditioned KP-C18-HS™ flash cartridge, and eluted in 500 ml of 100% methanol. This sample was then dried by rotary evaporation and resuspended in 7 ml of methanol. HPLC analysis revealed that no microcystin-LW or -LF was lost during either of these processing stages. To prepare this sample for application to the normal-phase flash cartridge, it was dried under nitrogen, resuspended in 1 ml of methanol and diluted with 10 ml of DCM. Sample load was determined by drying 50 µl of this sample (to remove DCM) and resuspending it in 1 ml of methanol (effecting a 1 in 20 dilution), followed by HPLC analysis. Thus, it was determined that the sample prepared for separation by normal-phase Flash chromatography was found to contain 9.3 mg microcystin-LW and 12.2 mg microcystin-LF. This indicated that approximately 3 mg of each toxin was lost during the preparation of the sample for application to the normal-phase flash cartridge.

A KP-Sil™ silica cartridge (15 x 4 cm I.D., 32-63 µm, 60 Å pore size; Biotage) was prepared by flushing with 20 CVs methanol (2400 ml), 10 CVs DCM (1200 ml) and 10 CVs of the initial mobile phase (95% A:5% B, 1200 ml). Nitrogen pressure (15 p.s.i.) was applied to achieve a flow rate of 50 ml min^{-1}.

The sample described above was injected onto the cartridge using a syringe (10 ml). Microcystins were then eluted using the gradient shown in Table 3.3.

Table 3.3. Gradient applied for the optimised scaled-up separation of microcystin -LW and -LF using a pre-packed flash KP-Sil™ silica cartridge (15 x 4 cm I.D.), as described in 3.2.9. Mobile phase was composed of DCM (A), and methanol:acetic acid (10:2) (B).

Mobile phase composition	Elution volume (ml)	Number of column volumes	Number of fractions (50 ml) collected
95% A: 5% B	360	3.0	7
92% A: 8% B	1200	10.0	24
90% A:10% B	2000	16.7	40

To facilitate rapid analysis of the fractions collected, aliquots (250 μl) were removed from each, dried, resuspended in 250 μl of methanol, and analysed by HPLC. The remaining fractions were left uncovered in a fume hood overnight to allow the DCM to evaporate. Fractions found to contain a single microcystin with purity (by HPLC) greater than 90% were pooled, diluted to 20% methanol (v/v) and passed through a KP-C18-HS™ flash cartridge (7.5 x 4 cm I.D., 35-70 μm particle size, 60Å pore size; Biotage, USA). The cartridge washed with water (500 ml) to ensure the removal of any residual acetic acid. Toxins were eluted from the cartridge in 500 ml of methanol and an aliquot (100 μl) removed and analysed by HPLC for the determination of toxin recoveries. Fractions in which the two toxins had co-eluted, and late fractions containing microcystin-LW at a purity of less than 90% were also pooled separately, and treated in the same way. The eluted toxins were then rotary evaporated to dryness prior to quantification by gravimetric analysis.

3.2.10. Final reversed-phase purification.

Gravimetric analysis and visual appearance suggested the presence of contaminants not revealed by HPLC. Each of the samples was therefore diluted with water (1 l), loaded onto a pre-conditioned KP-C18-HS™ flash cartridge (7.5 x 4 cm I.D., 35-70 μm particle size, 60Å pore size; Biotage, USA), and eluted by step gradient from 0 to 100% methanol in 10% increments (500 ml per step). Each increment was collected separately and analysed by HPLC. The fractions containing a single purified microcystin was rotary evaporated to dryness, resuspended in a small volume of methanol, dried and quantified gravimetrically.

3.3. Results.

3.3.3. Identification of optimum solvent system for normal-phase separation of microcystin-LW and microcystin-LF using thin-layer chromatography.

Solvent mixtures consisting of chloroform and methanol or ethanol proved unsuitable for the separation of microcystin-LW and -LF from the partially purified mixtures prepared in 3.2.1. The plates ran very quickly and when developed revealed poorly resolved smeared spots which were difficult to identify by comparison to analysis of purified microcystin-LF. The addition of acetic acid to mixtures containing chloroform caused both toxins to be eluted from the plate together.

The R_f and ΔCV values for microcystin-LW and -LF obtained following separation in each of the mixtures of DCM, methanol and acetic acid tested are shown in Table 3.4. The most suitable solvent system indicated by TLC was DCM:methanol:acetic acid (88:10:2). Rf values were 0.08 (12.7 CV) and 0.18 (5.4 CV) for microcystin-LW and -LF respectively. This gave a theoretical ΔCV of 7.3 which would indicate a potentially good separation of the two compounds when chromatographed on a silica cartridge.

Table 3.4. R_f and ΔCV values calculated for microcystin-LW and -LF separated from a partially purified mixture of the two toxins on silica gel TLC plates. Values were calculated after developing plates in different ratios of DCM, methanol and acetic acid. NS indicates no separation.

Ratio of DCM:Methanol:acetic acid	R_f -LW	CV -LW	R_f -LF	CV -LF	ΔCV
80:18: 2	0.73	1.40	0.84	1.20	0.20
81:18: 1	0.56	1.80	0.70	1.40	0.40
82:16: 2	0.55	1.80	0.64	1.60	0.20
85:10: 5	0.21	4.90	0.42	2.4	2.50
88:10: 2	***0.08***	***12.70***	***0.18***	***5.40***	***7.30***
90: 8: 2	NS	/	NS	/	/

3.3.4. Method development for normal-phase separation of microcystins-LW and -LF using Flash chromatography.

The optimum mobile phase determined by TLC was evaluated using small (15 x 1.2 cm I.D.) pre-packed flash cartridges. Direct transfer of the TLC method to the 12 mm column did not result in simple separation as predicted, thus it was necessary to employ a step gradient. Initial attempts to separate microcystins -LW and -LF by elution in 125 ml (12.5 CV) of 95% A:5% B, followed by 200 ml (20 CV) of 92% A:8% B, and 50 ml (5 C.V.) of 90% A:10% B were unsuccessful. Using this solvent system, microcystin-LF was not eluted from the cartridge until fractions 70-72 (90% A:10% B). Mixtures of microcystins-LW and -LF were identified in fractions 74 and 75, and pure microcystin-LW had not been eluted. However, this experiment did reveal that the microcystin-containing sample was not fully soluble in DCM alone, resulting in the blocking of the cartridge injection port with undissolved sample. This problem was overcome by resuspending the toxins in a small volume of methanol prior to a 10 fold dilution in DCM.

The step gradient shown in Table 3.1. resulted in adequate separation of microcystin-LW and -LF from the toxin mixture described in 3.2.4. TLC indicated that microcystin-LF eluted in fractions 34-45 (90% A:10% B). Microcystin-LW and -LF co-eluted in fractions 46-49 (90% A:10% B and 80% A:20% B); while pure microcystin-LW eluted in fractions 50-57 (80% A:20% B and 70% A:30% B). Although no microcystins were eluted in the first step (95% A:5% B) it was found to be essential to the initial retention and subsequent satisfactory separation of the microcystins. This step was also useful in the elution of pigments contained in the sample. The elution pattern determined by TLC for every second fraction collected over the period where microcystins were eluted from the cartridge is shown in Figure 3.3. HPLC analysis of individual fractions confirmed the presence of microcystin-LW and -LF in the fractions found to contain the toxins by TLC. Analysis of the waste eluent showed no breakthrough of either microcystin.

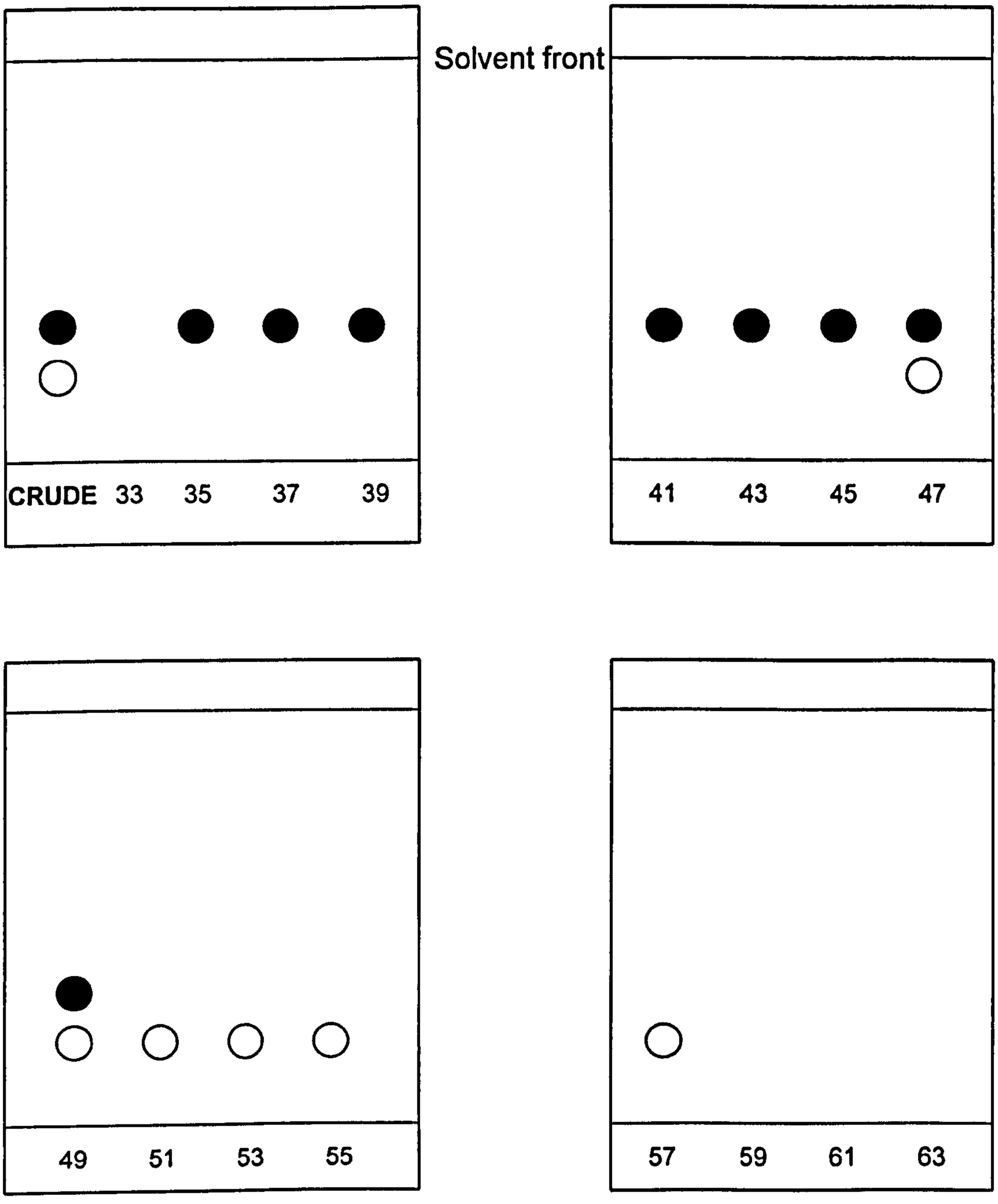


Figure 3.3. Illustration of the elution pattern indicated by TLC analysis of fractions collected following normal-phase Flash separation of a mixture of microcystin-LF (black spots) and microcystin-LW (white spots) as described in 3.2.4., using the gradient shown in Table 3.1.

The recoveries of microcystin-LW and -LF in each of the fractions were represented as a bar chart (Figure 3.4.). The graph shows that approximately 59% microcystin-LW, and 52% microcystin-LF was recovered from the cartridge following normal- phase Flash separation using the conditions described. Figure 3.5. illustrates the toxin purities of each of the fractions analysed. This graph shows that separation of microcystin-LW and -LF using the method outlined in 3.2.4. resulted in 8 fractions containing microcystin-LF at a purity greater than 90% (approximately 36% of the starting material). The purities of fractions containing microcystin-LW were considerably lower, with all of the fractions containing the toxin at a purity less than 90%. Fractions 51 and 52 (80% A:20% B) contained the most highly pure microcystin-LW, with purities of 82% and 79% respectively.

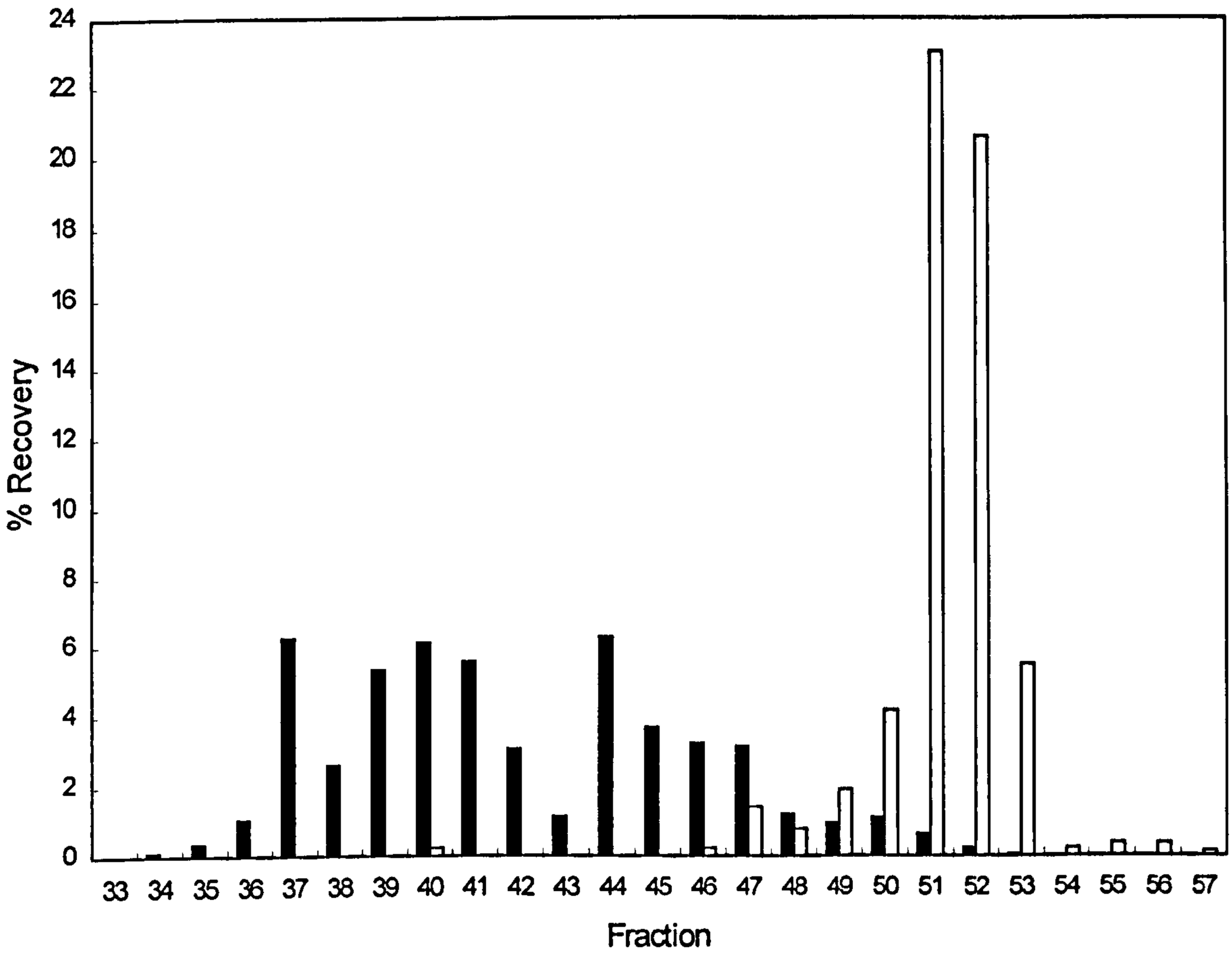


Fig. 3.4. Results of HPLC analysis of fractions collected following normal-phase Flash separation of a mixture of microcystin-LW and -LF as described in 3.2.4. The graph indicates recovery of microcystin-LF (black) and microcystin-LW (white) in each fraction.

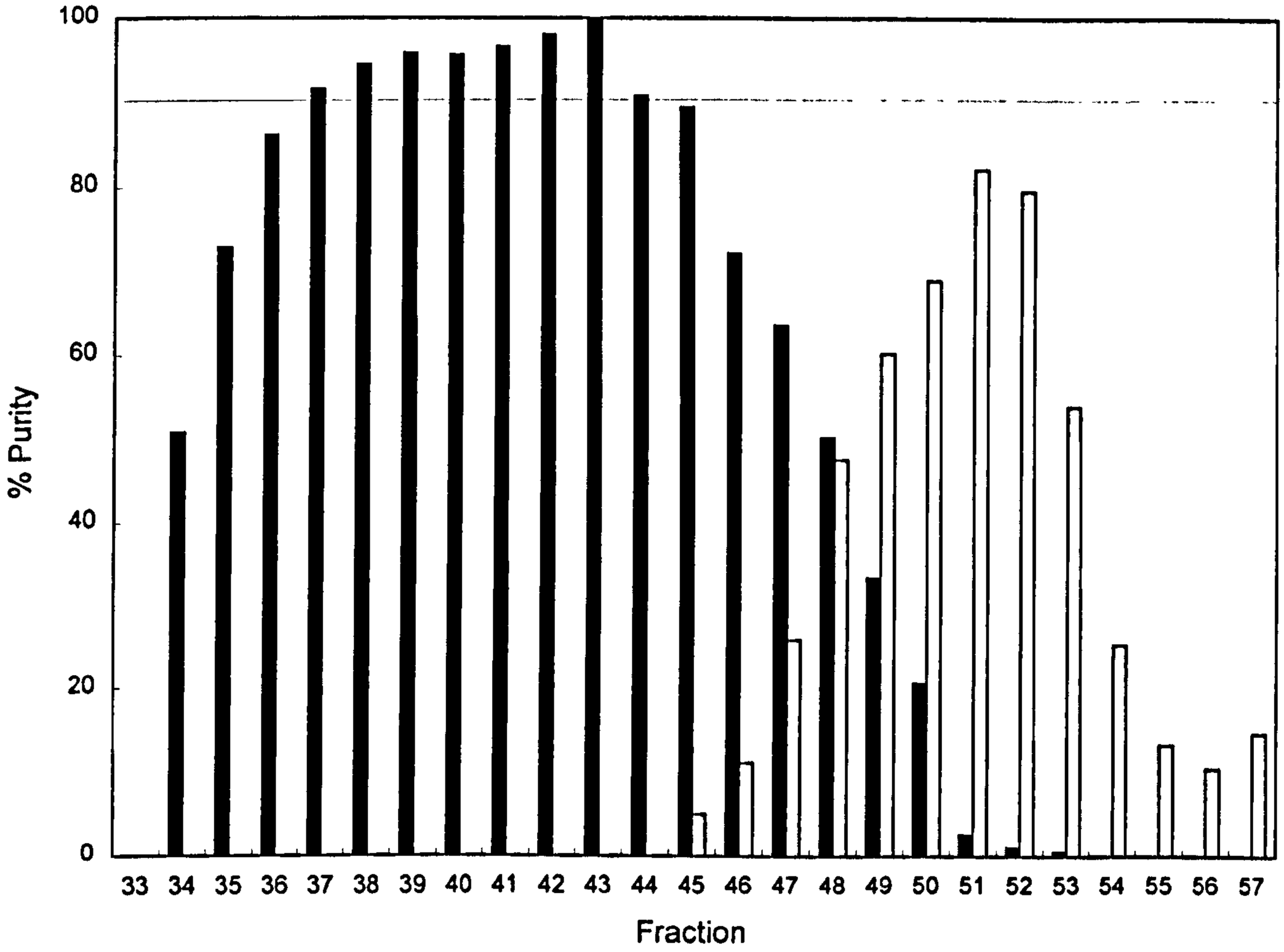


Fig. 3.5. Results of HPLC analysis of fractions collected following normal-phase Flash separation of a mixture of microcystin-LW and -LF as described in 3.2.4. The graph indicates the purity of microcystin-LF (black) and microcystin-LW (white) in each fraction. The line across indicates samples above 90% purity.

3.3.5. Investigation of toxin recoveries following normal phase Flash separation of microcystin-LW and -LF.

After 48 hours in a fume cupboard, toxin solutions which had been originally added to 5 ml of methanol or 5 ml of 90% A:10% B were resuspended in 1 ml methanol and analysed by HPLC. The amounts of microcystin-LW and -LF determined in the solution left in methanol were 38.73 μg (± 1.26 μg), and 34.63 μg (± 1.15 μg) respectively. The solution left in 5 ml of 90% A:10%B was found to contain 35.03 μg of microcystin-LW (± 0.09 μg), and 31.75 μg of microcystin-LF (± 0.33 μg) after 48 hours, indicating the loss of approximately 3 μg (approximately 10%) of each toxin.

The recoveries of microcystin-LW and -LF calculated in fractions separated from a sample containing 2.5 mg microcystin-LW and 1.8 mg microcystin-LF using the conditions described were found to be approximately 62% and 67% respectively. Washing the cartridge with a further 100 ml of 100% B resulted in the elution of 100 μg of microcystin-LW, and 70 μg of microcystin-LF. Although these amounts did not account for the remainder of the toxins loaded onto the cartridge, the results suggested that some of the toxins had remained bound to the silica following elution using the gradient shown in Table 3.1.

The separation and recoveries of microcystin-LW and -LF were examined following normal phase separation of a mixture of the toxins on smaller silica cartridges (7 x 1.2 cm I.D.), which had been washed with either 100 ml (20 CV) of 100% B (methanol:acetic acid), or 100 ml of methanol prior to sample loading. Washing the cartridge with 100% B (methanol:acetic acid (10:2)) resulted in poor separation of the 2 microcystins, with all of the toxins eluting in the 3rd fraction collected. However, toxin recoveries were much improved, with approximately 88% microcystin-LW and 89% microcystin-LF eluted from the cartridge. Preconditioning with methanol prior to sample loading resulted in better separation, causing toxins to elute between fractions 12 and 23 (92% A:8% B).

The toxin recoveries achieved using this method were also high at approximately 89% microcystin-LW, and 90% microcystin-LF.

3.3.6. Optimisation of separation conditions for normal phase separation of microcystin-LW and -LF using Flash chromatography.

The conditions described in section 3.2.7. provided good separation, with microcystin-LF eluted in fractions 21 to 29 (92% A:8% B and 90% A:10% B). Fractions 30 to 36 (90% A:10% B) contained a mixture of the two compounds while microcystin-LW was eluted in fractions 37 to 50 (90% A:10% B). The recoveries of microcystin-LW and -LF in each of the fraction groups are shown in Figure 3.6. The graph shows that preconditioning the cartridge with 100% methanol prior to sample loading resulted in the recovery of approximately 72% microcystin-LW, and 74% microcystin-LF of the total sample load (compared to 59% and 52% using the conditions described in 3.2.4.).

3.3.7. Extraction of cyanobacterial cells and separation of microcystins using reversed- phase Flash chromatography.

Determination of dry weight of 1 ml of methanolic cell extract revealed that the material extracted was equivalent to 30 g dry weight of cells. HPLC analysis of the aqueous cell extract prior to reversed-phase extraction revealed four main microcystins (Figure 3.7.), which were identified and quantified as microcystin-LR (82 mg), microcystin-LY (10 mg), microcystin-LW (17 mg) and microcystin-LF (16 mg). Analysis of the fractions eluted using a methanolic step gradient revealed that most of the microcystins were eluted in the 40 to 60% fractions (Figures 3.8.-3.10.). The percentage recovery and purity of each microcystin eluted in these fractions are shown in Table 3.5.

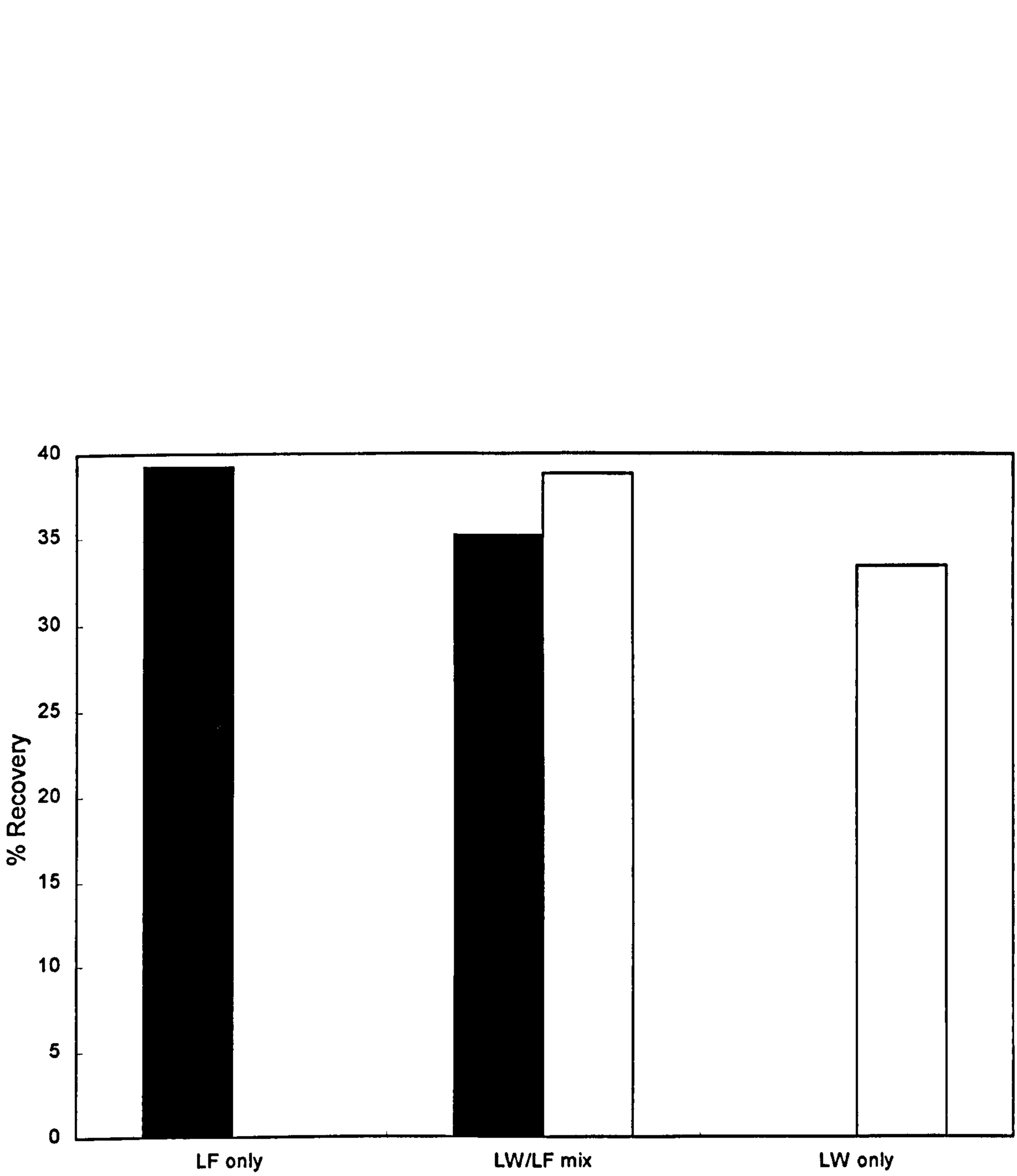


Figure 3.6. Recovery of microcystin-LW and -LF following optimised normal-phase flash separation as described in 3.2.8.

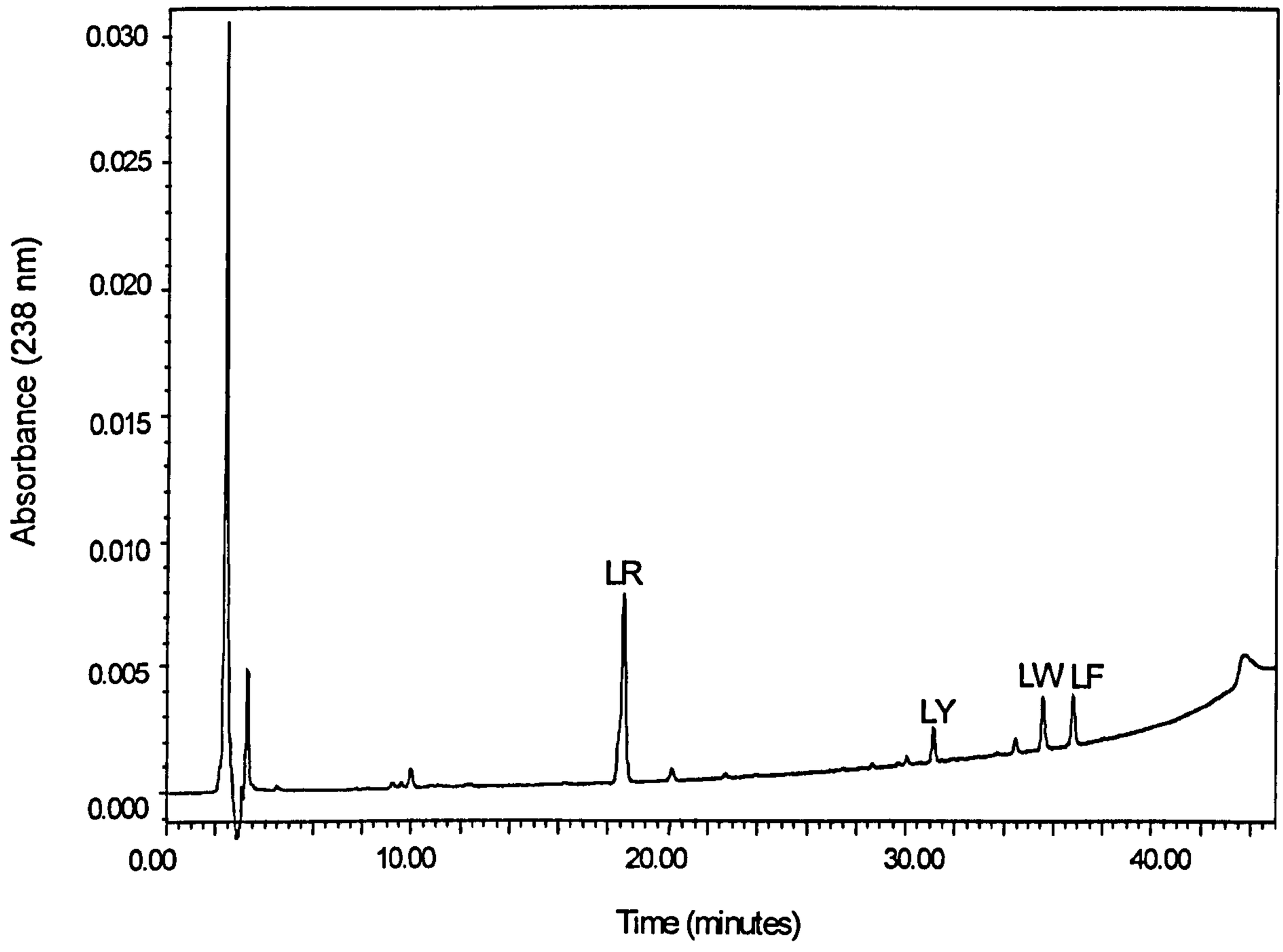


Fig. 3.7. Reversed-phase HPLC chromatogram of aqueous methanolic extract of *M. aeruginosa* PCC7820 prior to application to a C_{18} flash cartridge. Four main microcystins were present in the extract: microcystin-LR; microcystin-LY; microcystin-LW; and microcystin-LF.

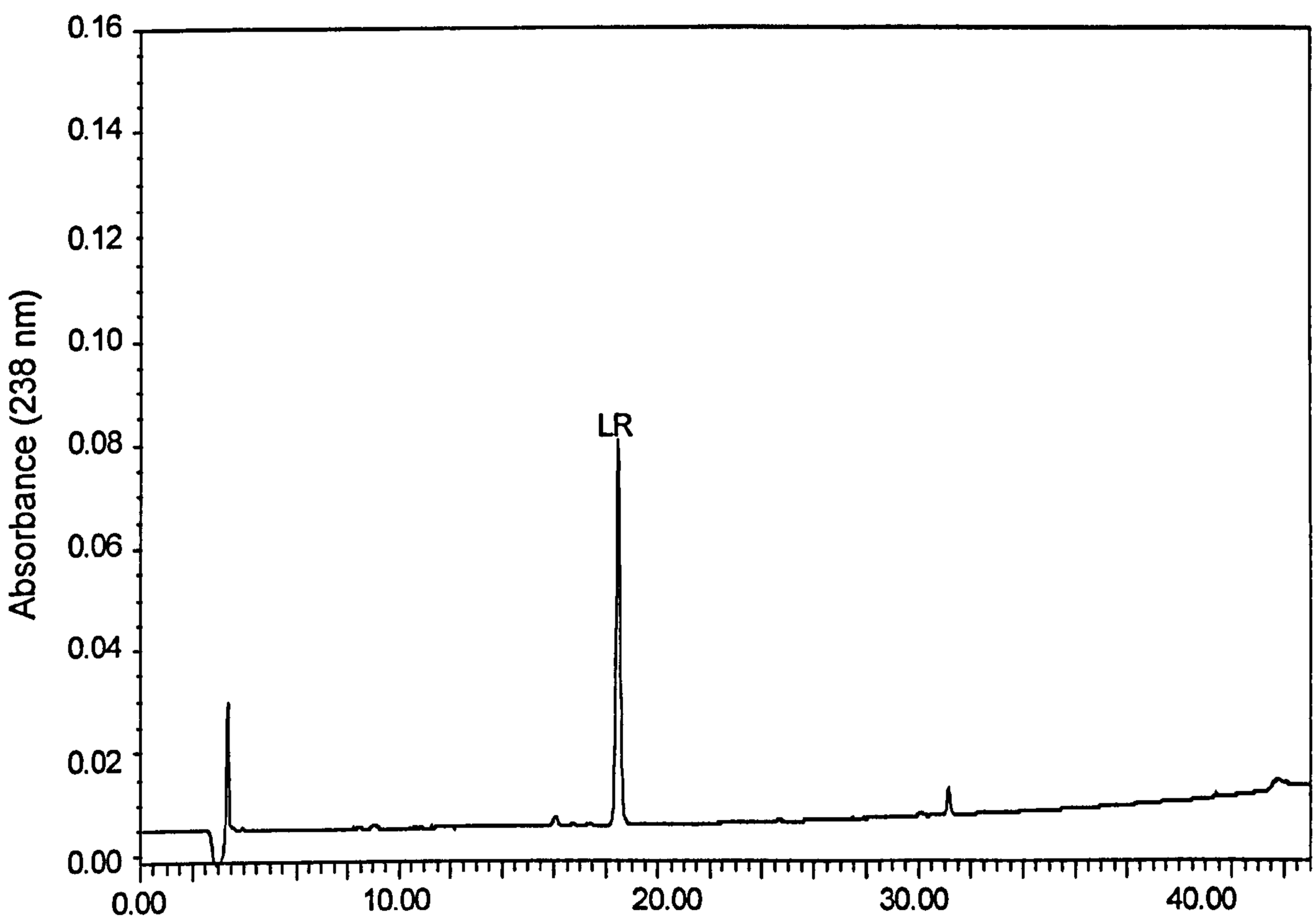


Figure 3.8. Reversed-phase HPLC chromatogram of microcystin-LR eluted from a KP-C18-HS™ flash cartridge with 40% aqueous methanol.

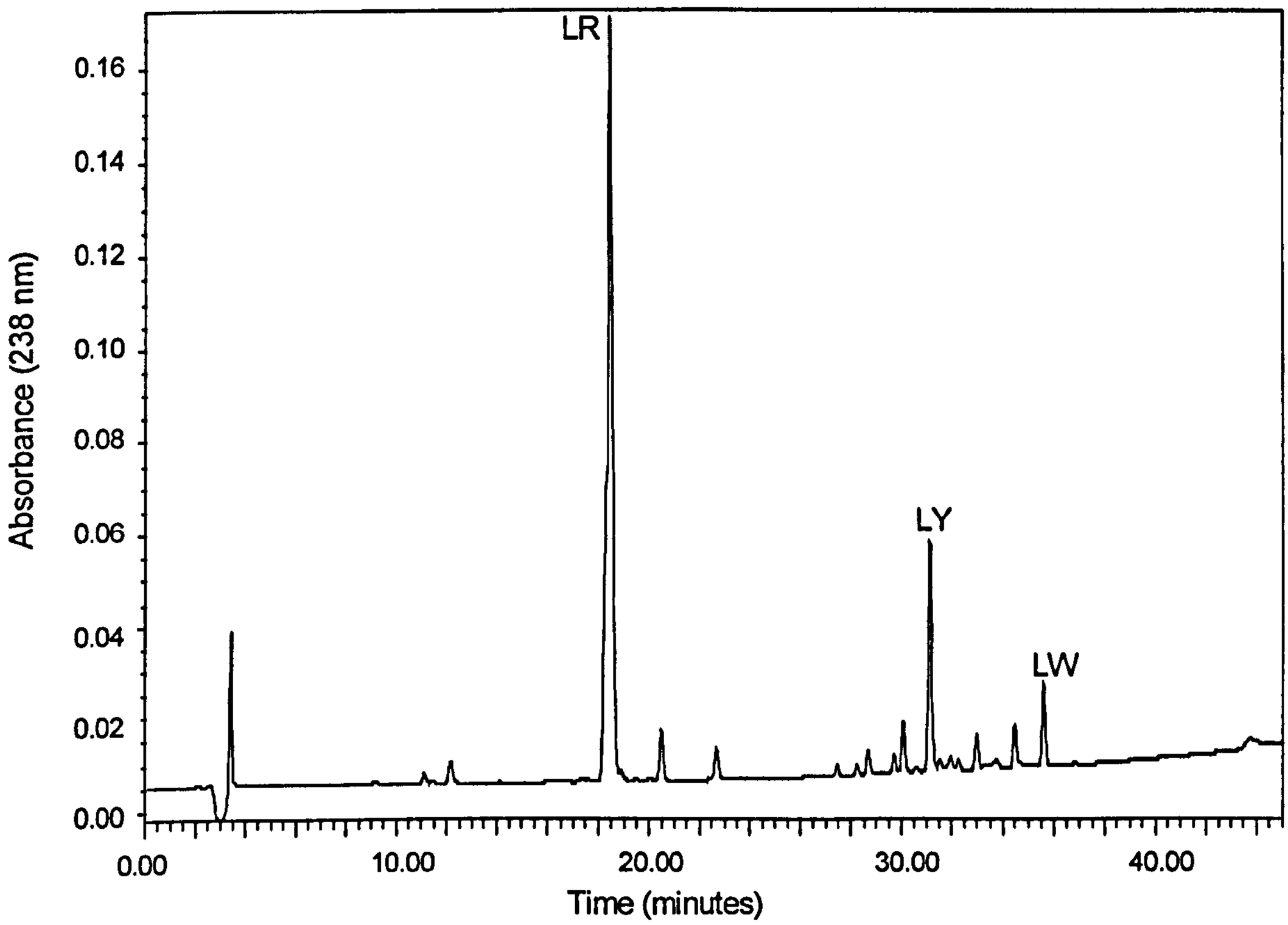


Figure 3.9. Reversed-phase HPLC chromatogram of microcystins-LR, -LY and -LW eluted from a KP-C18-HS™ flash cartridge with 50% aqueous methanol.

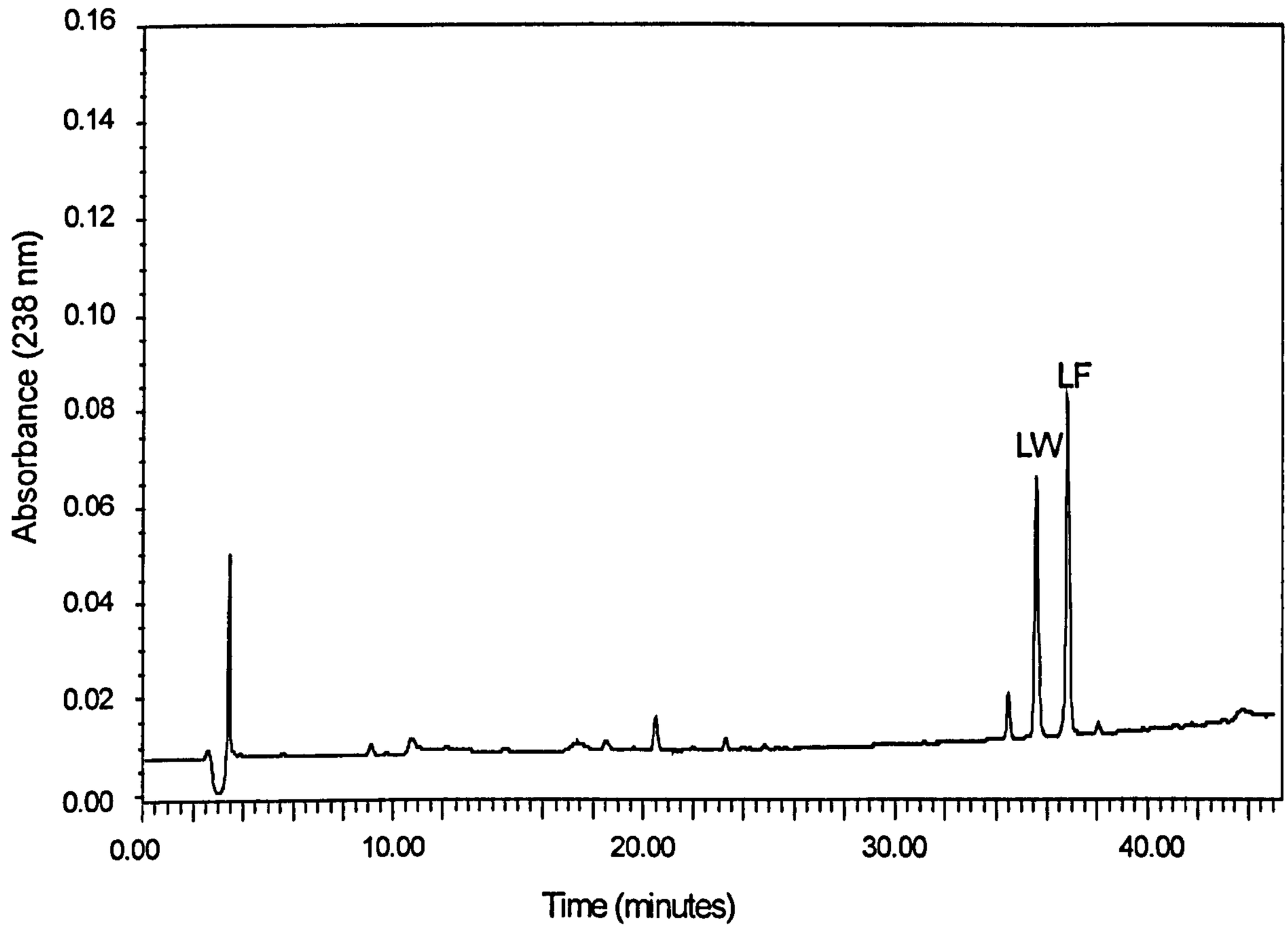


Fig. 3.10. Reversed-phase HPLC chromatogram of the two hydrophobic microcystins, microcystin-LW and -LF; eluted from a KP-C18-HS™ flash cartridge with 60% aqueous methanol.

Table 3.5. Recovery and purity of microcystins, determined by PDA-HPLC, from *Microcystis aeruginosa* PCC7820 following reversed-phase flash chromatography using an aqueous methanol step elution.

Methanol fraction (%)[a]	Microcystin variant	Amount of microcystin (mg)	% Recovery[b]	% Purity[c]
40%	LR	18	22	91
	LY	1	9	5
50%	LR	52	64	63
	LY	9	92	11
	LW	3	19	4
60%	LW	12	67	33
	LF	15	93	42

a - percentage of aqueous methanol used to elute that fraction.

b - percentage of microcystin recovered in that fraction relative to the total amount applied to the cartridge.

c - purity of microcystin variant eluted in that fraction by HPLC.

The fraction eluted in 40% methanol (Figure 3.8.) contained almost exclusively microcystin-LR (18 mg) at a purity of 91%. In the 50% fraction (Figure 3.9) microcystin-LR was found to be the predominant compound (52 mg) with microcystin-LY (9 mg) and microcystin-LW (3 mg) also present. A number of other minor peaks were also present, many of which were identified as microcystins by their characteristic UV spectra. The fraction eluted in 60% methanol (Figure 3.10.) was found to contain primarily microcystin-LW (12 mg) and microcystin-LF (15 mg). Analysis of waste eluent revealed that there was no breakthrough of microcystins following sample loading.

3.3.8. Scale-up of optimised normal-phase flash chromatography method.

The optimised method was used to purify microcystin-LW and -LF obtained following preliminary purification using reversed-phase flash as described in 3.2.3. Direct scale-up of the optimised method outlined in 3.2.8. was achieved using a 4 cm I.D. cartridge where volumes of solvent in the step gradient, volume of sample injected, flow rate and fraction size were increased accordingly. Due to limited amount of sample the load injected onto the 4 cm column was equivalent to 0.23 mg per gram of packing material compared to 0.35 mg per gram of packing material injected onto the 1.2 cm I.D. column. A similar separation was achieved (Figure 3.11.), although most toxin-containing fractions were eluted during the final step of the gradient (90% A:10% B). It can be seen (Figure 3.12.) that a significant number of fractions contained a single microcystin at a purity greater than 90%.

Reversed-phase flash was successfully used to ensure the removal of acid from the sample. HPLC analysis of the eluted pooled fractions revealed that 6.3 mg of pure microcystin-LW, and 9.7 mg of microcystin-LF were recovered with purity greater than 90%. The pooled fraction group in which microcystins-LW and -LF had co-eluted was found to contain 1.3 mg of microcystin-LW, and 1.5 mg of microcystin-LF. Later fractions containing microcystin-LW with purity less than 90% contained 1.1 mg of the toxin. Total toxin recoveries determined by HPLC were 8.7 mg of microcystin-LW, and 11.2 mg of microcystin-LF, which represent 93% and 92% respectively of the original toxin loads.

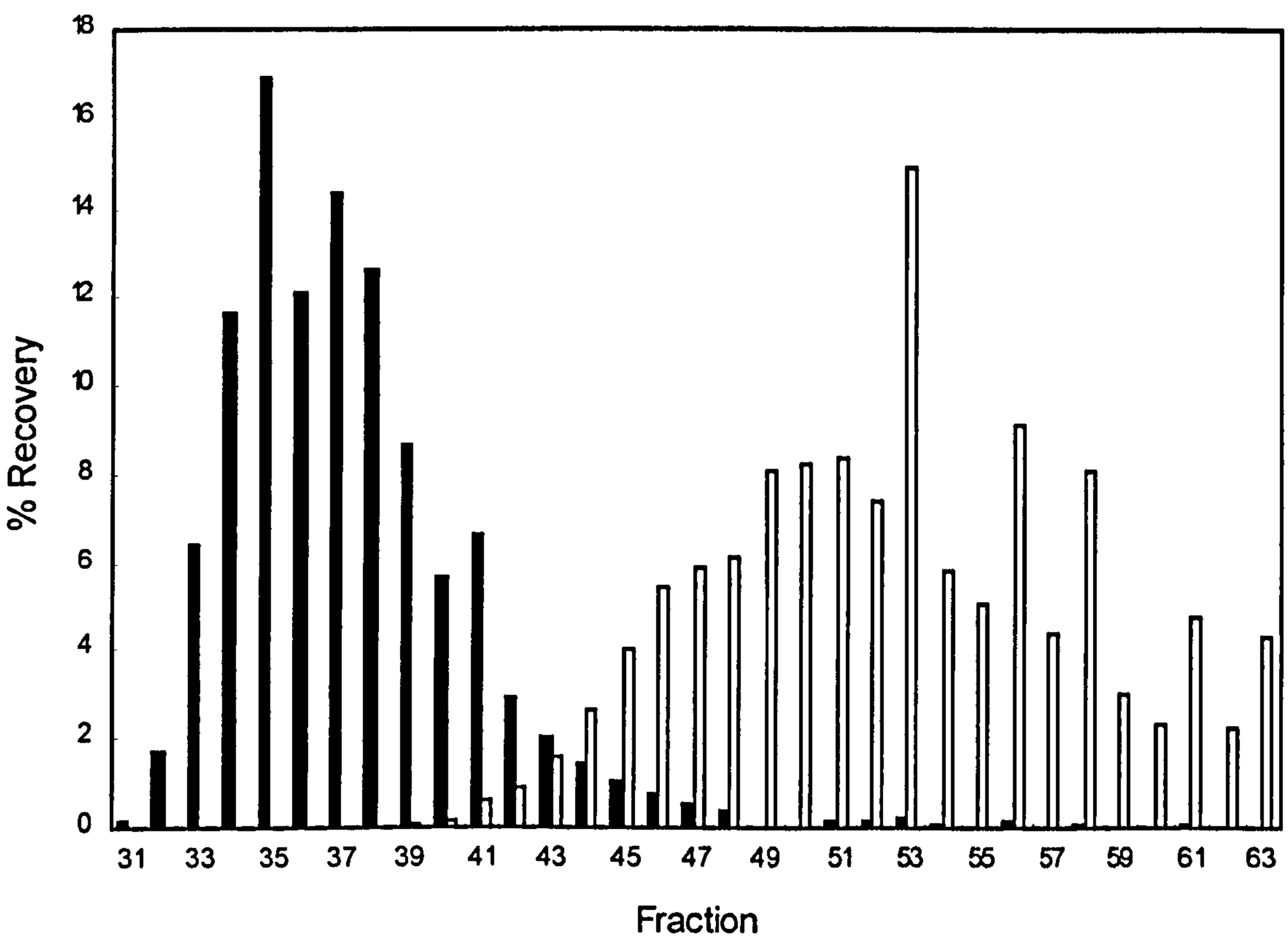


Fig. 3.11. Results of HPLC analysis of fractions collected following scaled-up normal-phase flash separation indicating recovery of microcystin-LF (black) and microcystin-LW (white) in each fraction.

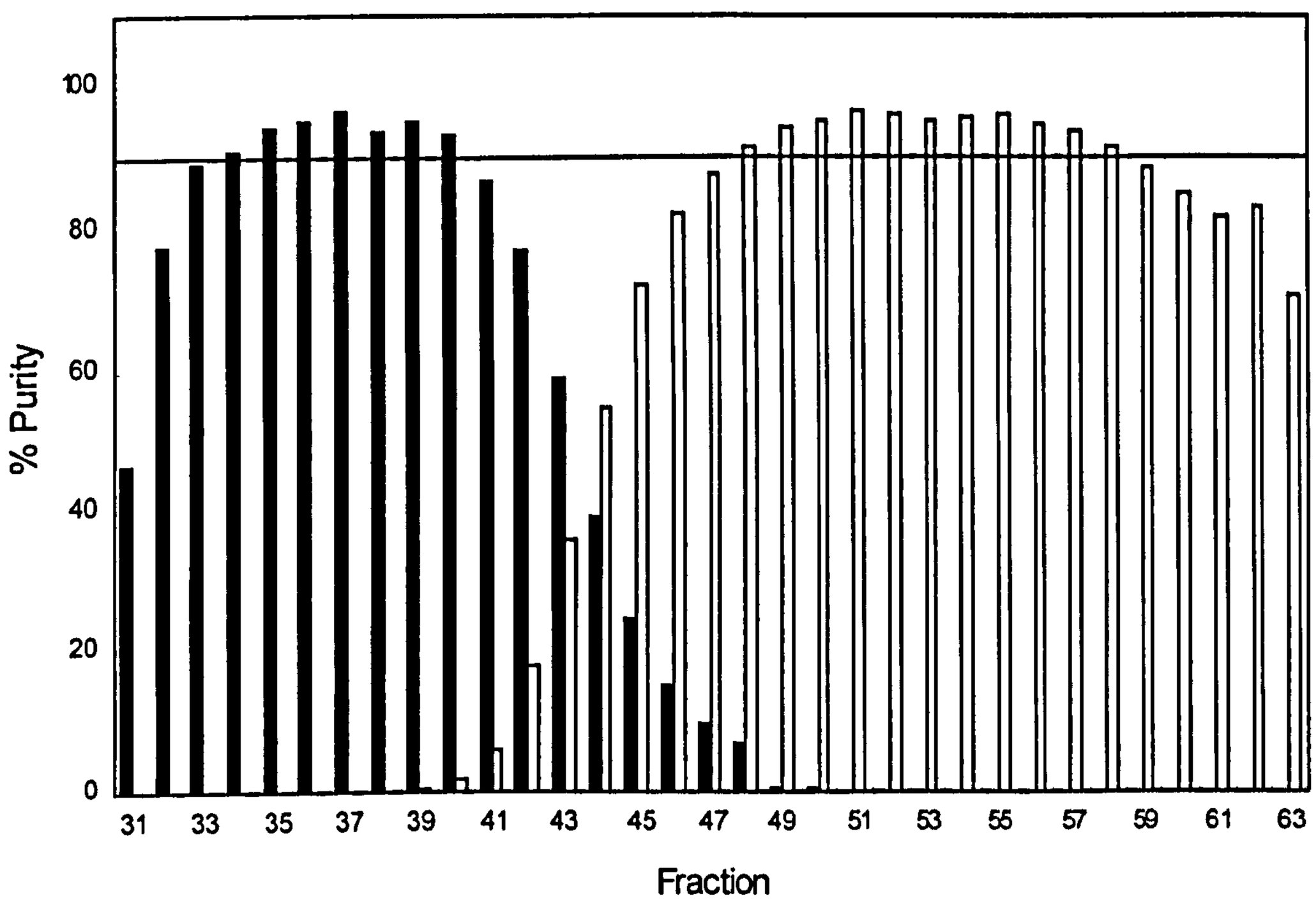


Fig. 3.12. Results of HPLC analysis of fractions collected following scaled-up normal-phase flash separation indicating the purity of microcystin-LF (black) and microcystin-LW (white) in each fraction. The line across indicates samples above 90% purity.

3.3.9. Final reversed-phase purification.

Gravimetric analysis revealed a dry weight for the purified compounds greater than that which was determined by HPLC. Furthermore, the dried sample was observed to have a green oily appearance where it is known that pure microcystins are white when dried. Detection at other wavelengths (214 and 254 nm) did not indicate the presence of any contaminants. The sample was re-applied to a C_{18} flash cartridge and eluted stepwise, successfully removing the contaminants. Final purity of both microcystins by HPLC was determined to be 95% (Figures 3.13. and 3.14.). When the samples were dried, a white powder remained, confirming that the microcystins were of high purity. The yield at this purity for microcystin-LW and -LF, determined by gravimetric analysis, was 5.5 mg and 7.2 mg respectively.

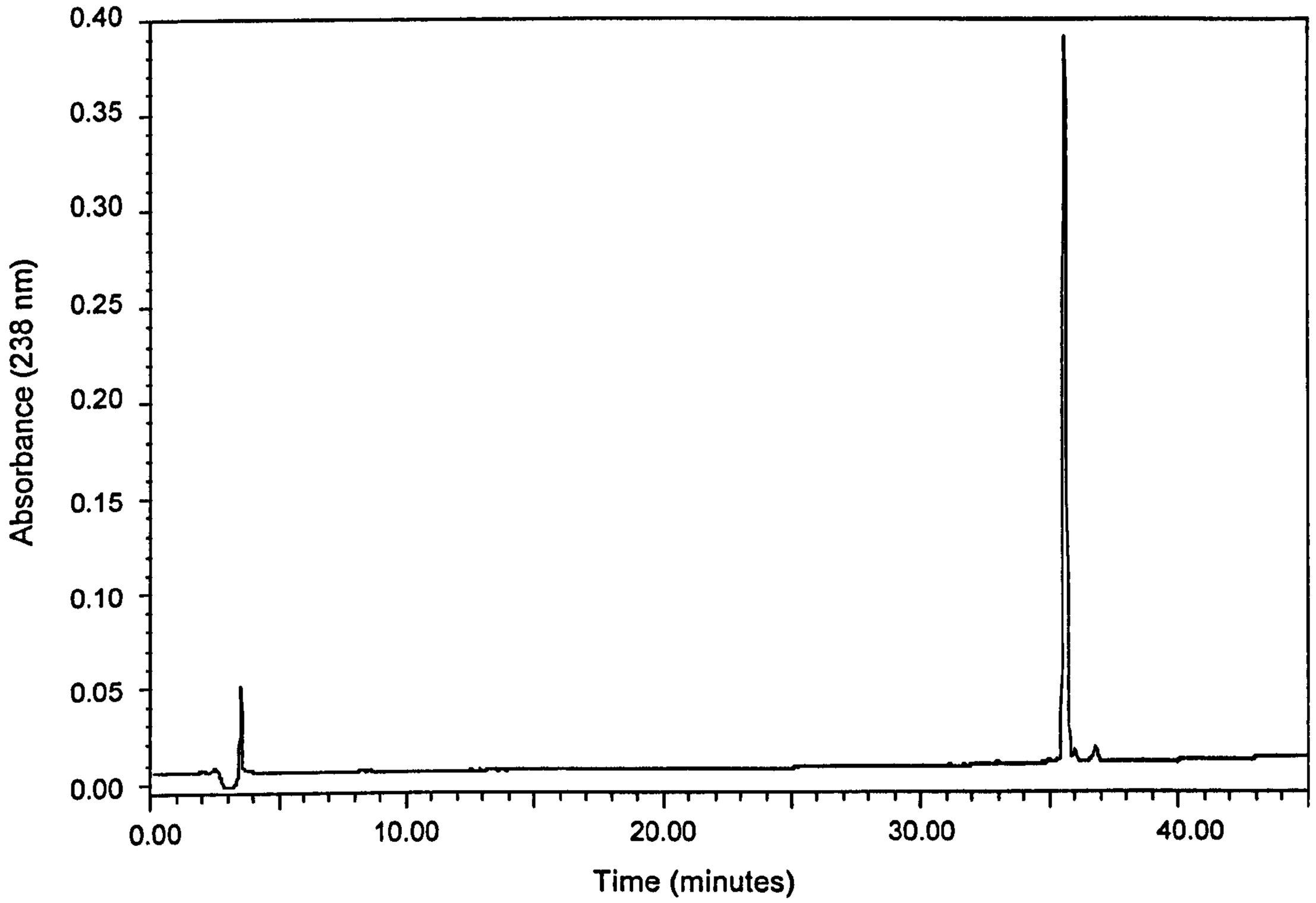


Figure 3.13. Reversed-phase HPLC chromatogram of purified microcystin-LW.

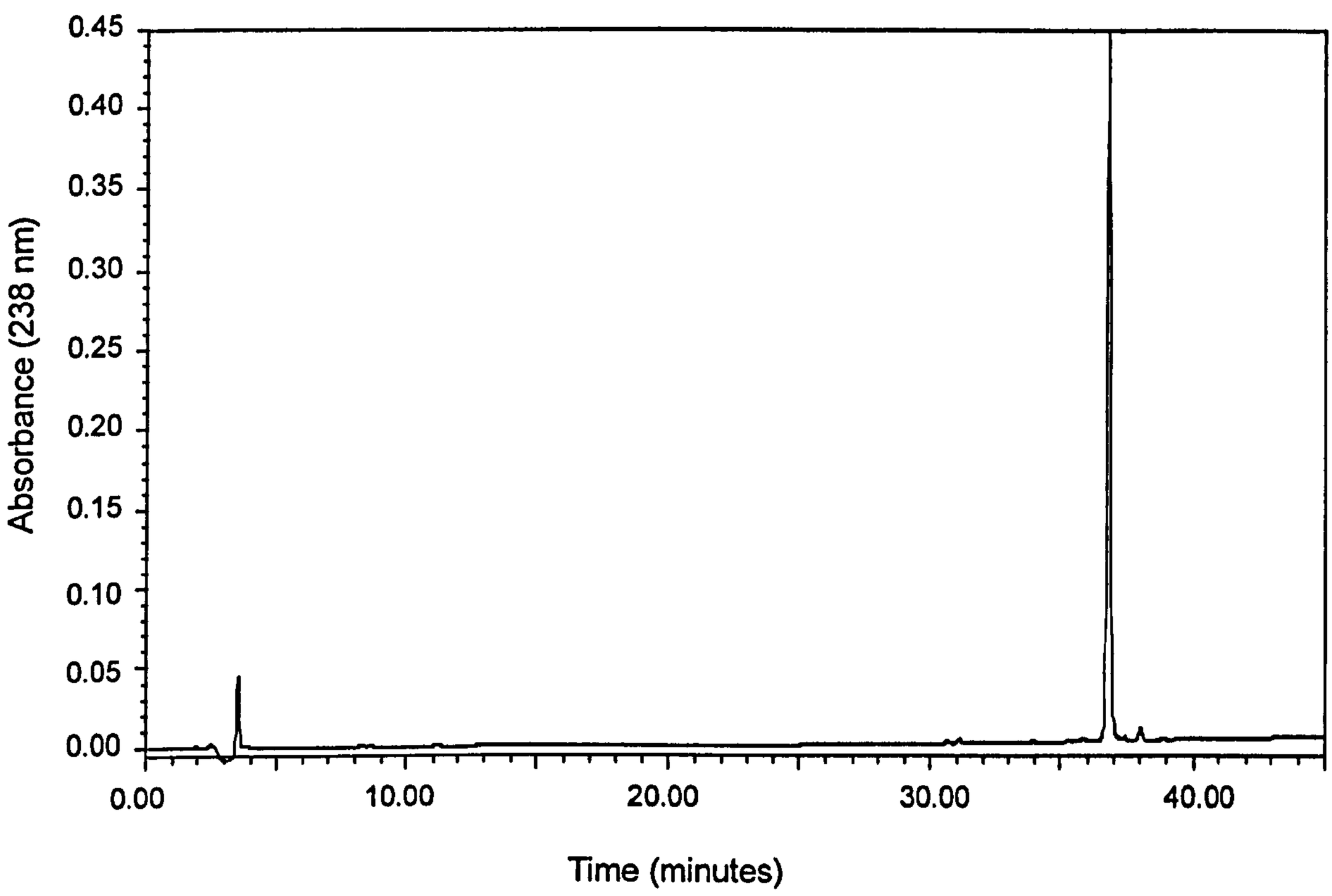


Fig. 3.14. Reversed-phase HPLC chromatogram purified microcystin-LF.

3.4. Discussion.

This chapter has described a simple and effective method for purifying microcystin -LW and -LF from cyanobacterial cells using normal and reversed-phase Flash chromatography. Since many of the commonly used extraction procedures fail to recover the more hydrophobic microcystins, the occurrence of these toxins is often under-reported (Lawton *et al.*, 1995). It is therefore important that they are available as purified standards in order that methods for their detection and subsequent reporting may be improved.

As previously reported (Edwards *et al.*, 1996 a and 1996 b), methanolic extraction followed by reversed-phase flash chromatography enables the concentration of microcystins from large quantities of cyanobacterial cells. This procedure is considerably more straightforward, and can provide much higher yields of microcystins than earlier methods of purification (Brooks and Codd, 1986; Poon *et al.*, 1987; Harada *et al.*, 1988; Sivonen *et al.*, 1992). Extraction of cells in methanol also ensures the recovery of more hydrophobic microcystin variants. The aqueous methanolic extract of *M. aeruginosa* PCC7820 prepared in this study was found to contain predominantly four microcystins (Figure 3.7.). Concentration on a C_{18} flash cartridge followed by elution in 0-100% aqueous methanol (v/v) provided a simple and effective method for the separation and partial purification of these toxins. Elution of the cartridge in 40% aqueous methanol resulted in a sample which contained microcystin-LR at a purity of 91% (Figure 3.8.). This sample was of sufficient purity for use in the plant studies described in chapters 4 and 5 of this thesis. The fraction which was eluted in 50% methanol (Figure 3.9.) contained the majority of the microcystin-LR, virtually all of the microcystin-LY, and a small amount of the microcystin-LW which was present in the original sample. Separation of the three microcystins in this fraction could be easily achieved by re-applying the fraction to the flash cartridge and eluting using the step gradient described. Gradient elution also successfully separated microcystin-LW and -LF from other microcystins and a large proportion of co-extracted contaminants present in the original sample.

Elution in 60% methanol provided a partially purified sample (Figure 3.10.) which could then be separated further using normal-phase flash cartridges.

The purification of microcystin-LW and -LF has previously presented difficulties for a number of reasons. Firstly, although analytical HPLC provides good separation (Lawton, Edwards, and Codd, 1994), it utilises TFA which has been found to greatly reduce recovery when applied to preparative separations, due to effects on the stability of the microcystins. Microcystin-LW has been found to be particularly unstable in solutions containing TFA. Secondly, when alternative mobile phases have been investigated, separation deteriorated rapidly with increased sample loading. This was successfully overcome using closed-loop recycling where eluent containing the two toxins was diverted back through the column two additional times to improve resolution (Edwards *et al.*, 1996 a). However, this method is both time consuming and costly, hence inducing the search for an alternative method.

The difficulties associated with purifying microcystin-LW and -LF on reversed-phase systems prompted investigations into their isolation on normal-phase silica gel. The separation of microcystins using thin layer chromatography was previously investigated by Poon *et al.* (1987). In their investigation, mixtures of microcystins were successfully separated on silica gel plates with methanol and DCM or chloroform. TLC was used in this study as a guide to a suitable solvent system for the separation of microcystin-LW and -LF, and indicated that they could be isolated on a larger scale using normal-phase flash cartridges. However, when this procedure was directly scaled-up using the 1.2 cm I.D. cartridge, the predicted separation was not achieved. This was not unexpected since modes of elution in TLC compared to LC are very different especially in the presence of polar modifiers such as methanol and acetic acid. This problem was overcome by applying a step gradient, which improved separation and resulted in fractions containing single toxins.

The 1.2 cm I.D. cartridges provided an ideal tool for rapid method optimisation with minimum consumption of sample and solvents. In initial attempts, however, poor recoveries of both toxins were obtained, with a considerable proportion of the sample load (over 40% of both toxins) remaining unaccounted for following analysis of fractions by HPLC. The low recoveries did not appear to be due to stability effect, as microcystin-LW and -LF were both found to be relatively stable in the concentrations of acetic acid present in the mobile phase used to elute the toxins, even after 48 hours. This suggested that the concentration of normal phase flash fractions prior to analysis by TLC had no impact on the recovery of either toxin. Nonetheless, fractions were subsequently processed immediately following elution from the silica cartridge in order to prevent possible solvent effects.

Further experiments revealed that both microcystins were strongly retained on the silica cartridge following gradient elution. Initially, it was not possible to elute the two toxins from the cartridge due to their strong affinities to the sorbent. As the toxin mixture was applied in high concentrations of DCM, which is very hydrophobic, microcystin-LW and -LF would have been very strongly sorbed by the silica. It is probable that some of the toxin remained irreversibly bound, due to the presence of free silanol groups which provide extremely strong sorbent sites (Thurman and Mills, 1998). Once bound, the toxins could therefore not be fully recovered, even following elution in 100% methanol. Recoveries of both toxins improved considerably when separation was carried out using a cartridge which had been washed in methanol prior to loading the sample. This would have deactivated the strongly sorbent silanol groups, thus reducing the retention of the microcystins.

The application of the optimised method to the separation of microcystin-LW and -LF from the simplified reverse phase flash fraction provided good separation of the two toxins. However, the original fraction obtained following the initial purification stage contained 12 mg of microcystin-LW, and 15 mg of microcystin-LF, while the amounts of each toxin detected in the sample which was loaded

onto the normal-phase cartridge were only 9.3 mg and 12.2 mg respectively. This would suggest that approximately 3 mg of each toxin was lost during the preparation of this sample for normal-phase separation. The reasons for the reduced sample load are unclear at present, but this problem could be avoided by diluting a larger volume of the original reverse phase flash fraction in DCM and applying the sample to the normal-phase cartridge via the solvent reservoir present on the Flash 40i ™ system. This would decrease the number of stages required between the reversed and normal-phase procedures thus saving time, while also minimising the risk of losing toxin.

The combination of normal and reversed-phase flash chromatography yielded 5.5 mg (59%) of microcystin-LW and 7.2 mg (59%) of microcystin-LF at a purity of 95% determined by analytical HPLC. Fractions collected at both stages which contained either mixtures of the two toxins or single toxins contaminated with other impurities were combined and retained for reprocessing. Although the method described involves a four stage process, it could be simplified to three stages by replacing the 100% methanol elution stage with an aqueous methanol step gradient during the acid removal step. This would allow the less clean material remaining after each purification to be separated relatively quickly with similar results.

In summary, two closely related, hydrophobic microcystins, which represent less than 0.1% of the cyanobacterial biomass, were concentrated and cleaned up from an aqueous extract using reversed-phase flash chromatography, separated by normal-phase flash chromatography followed by acid removal and final polishing on reversed-phase flash.

Chapter 4

Determinations of microcystin toxicity using plant bioassays

4.1. Introduction.

The majority of research into the toxic effects of microcystins has focused on their mode of action in humans and animals following exposure to water contaminated with toxic cyanobacteria. The acute hepatotoxic effects and tumour promoting capability of microcystins in mammals have been shown to be attributable to the inhibition of protein phosphatases 1 and 2A (PP1 and PP2A, Runnegar, Kong, and Berndt, 1993; Nishiwaki *et al.*, 1994). These enzymes have been implicated in the regulation of several important cellular processes in animals; including protein synthesis, cell differentiation, and metabolism (Cohen, 1989).

Reversible phosphorylation has also been shown to play an important role in the regulation of a number of important metabolic processes in plants (Budde and Chollet, 1988). Plant tissues have been found to contain high levels of PP1 and PP2A which share very similar characteristics with the same enzymes in animals (MacKintosh and Cohen, 1989). Furthermore, plant and mammalian forms of PP1 and PP2A can be inhibited by exactly the same mechanism (MacKintosh *et al.*, 1990).

The inhibition of PP1 and PP2A by microcystins and the resulting physiological effects have been studied in detail in animals, (Nishiwaki-Matsushima *et al.*, 1992; Carmichael & Falconer, 1993; Craig *et al.*, 1996), but there have been fewer investigations into the effects of microcystins on plants. In plant tissues, inhibitors of PP1 and PP2A have been used to demonstrate the involvement of these enzymes in a variety of molecular and physiological processes. For example, microcystin-LR, (as well as okadaic acid and calyculin A) was found to block the sucrose-inducible accumulation of mRNAs for proteins involved in the storage of starch (Takeda *et al.*, 1994). PP1 and PP2A have been implicated in the activation of enzymes involved in the fixation of CO_2, (Carter *et al.*, 1990) and light inducible sucrose synthesis (Siegl, MacKintosh and Stitt, 1990). The inhibition of protein phosphatase by okadaic acid has been shown to enhance the closure of stomatal pores by abscisic acid, thus limiting the loss of water via transpiration

(Esser, Liao, and Shroeder, 1997). The inhibition of PP1 by okadaic acid and calyculin A was also found to block light inducible chlorophyll accumulation and photosynthetic gene activation in etiolated maize leaves (Sheen, 1993). Another study demonstrated that the inactivation of the enzyme quinate dehydrogenase (QDH) purified from carrot cells was prevented, following inhibition of PP2A by microcystin-LR and okadaic acid (MacKintosh, Coggins, and Cohen, 1991). Most investigations into the effects of PP1 and PP2A inhibitors on plants have been carried out *in vitro*; using either material purified from plants, or excised plant tissues. However, there have been fewer studies concerned with the effects of microcystin intoxication on whole plant systems. Kós *et al.* (1995) reported the first investigations into microcystin toxicity on plant growth. They discovered a concentration dependent inhibition of the growth of mustard (*Sinapis alba*) seedlings by microcystin, and exploited this as an assay method for detecting the toxins. Crop plants may be exposed to the toxins via irrigation with water contaminated with toxic cyanobacteria. In one study, the implications of exposing intact, growing plants to microcystins via spray irrigation were assessed (Abe *et al.*, 1996). Results indicated that microcystin-LR inhibited photosynthesis in the primary leaves of *Phaseolus vulgaris* L., at environmentally relevent concentrations. Okadaic acid and calyculin-A were also found to change the shape of cortical cells and restrict the growth of root hairs in *Arabidopsis thaliana* L. seedlings (Smith *et al.*, 1994). The information gathered so far indicates serious implications for the productivity of crop plants irrigated with contaminated water, and highlights the need to determine the level of toxin which may be detrimental to crops in the environment.

This chapter describes the use of a plant tissue culture assay to assess the effects of microcystin-LR on the development of potato shoots. Potato shoot explants were cultured in solid growth medium containing different concentrations of microcystin-LR and the levels of toxicity required to inhibit growth and chlorophyll content were determined. The effects of microcystin-LR on shoot and leaf tissues, and root establishment were also recorded.

A previously described plant test involving the growth of white mustard seedlings (Kós *et al.*, 1995) was then employed to compare the toxicity of microcystin-LR with two other microcystin variants; microcystin-RR and microcystin-LF.

4.2. Materials and methods.

4.2.1. Plant material.

Sterile potato shoots (*Solanum tuberosum* L., 'Lady Rosetta' variety) were provided by H & L Akroyd (Dalcross, Inverness, Scotland, UK), and maintained on the media described below until required for assays. White mustard seeds (*Synapis alba* L., Suttons seeds Ltd., Torquay, UK) were sterilised in 5% hydrogen peroxide for 3 x 30 minutes and washed five times with sterile glass distilled water prior to use.

4.2.2. Toxins.

Initially, microcystin-LR, -RR, and -LF were provided by Dr Edwards, Biotage, UK, and had been purified by the method of Edwards et al. (1996 b) from cells of *Microcystis aeruginosa* harvested from a bloom at Rutland Water (Leicestershire, UK) in 1989. Further supplies of microcystin-LR and -LF were purified as described in Chapter 3, from a laboratory culture of *Microcystis aeruginosa* PCC7820. Bloom and culture material was stored at -20°C prior to purification.

4.2.3. Effect of microcystin-LR on the growth and development of potato shoot explants.

Murashige and Skoog basal salt mixture was prepared by dissolving 4.33 g of powdered medium (Sigma-Aldrich, Poole, Dorset, UK), in 1 litre of Milli-Q water (Millipore). The media was divided into 10 x 90 ml portions in media bottles, solidified with 1% agar (Sigma-Aldrich), and autoclaved for 15 min at 121°C. Stock solutions of purified toxin were diluted in Milli-Q water. Toxin solutions were then dispensed aseptically in a laminar flow cabinet into Pyrex bottles of cooled, molten media using 0.2 μm hollow fibre syringe filters (Dynagard™, Microgon Inc, Laguna Hills, California, USA) to give final microcystin-LR concentrations of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, and 5 μg ml^{-1}.

Aliquots (10 ml) were dispensed aseptically into screw-top sterile plastic universal tubes (25 ml, Labtech International Ltd., Uckfield, East Sussex, UK) and allowed to solidify. Shoot segments (15-20 mm) with 1-2 nodes were cut from 3-4 week old potato shoot tissue cultures, using flame sterilised scalpel blades. Each shoot segment was placed into a single universal bottle so that approximately 2 mm of the shoot base was pushed into the medium. All inoculations were carried out in a laminar flow cabinet. Plant cultures were grown in an incubator at 15 °C (± 2°C) under continuous cool white fluorescent light (60 $\mu E\ m^{-2}\ s^{-1}$) for a period of 16 days. After this time, each plant was scored for signs of chlorosis or necrosis on tissues. Plants with 0% chlorosis or necrosis to tissues were scored 0, while those exhibiting 0-25%, 25-50%, 50-75%, and 75-100% were scored 1-4 respectively. The fresh weight and shoot length of each plant was also determined. For analysis of chlorophyll content, each plant was extracted in 0.5 ml of 90% acetone in a mortar and pestle which was kept cool on ice. Following the first extraction, the homogenate was transferred to a microcentrifuge tube and centrifuged for 5 min at 10 000 x g. The pellet obtained was extracted twice in 0.5 ml of 90% acetone. All extractions were carried out in the dark. The supernatants obtained from each extraction were pooled and absorbance was measured at 664 nm and 647 nm using a Nova spec spectrophotometer (Nova spec, Pharmacia LKB Biotech, Cambridge, UK). The amounts of chlorophylls *a* and *b* (μg) per mg wet weight of cells was calculated using the equation :

Chl a $(mgl^{-1}) = 11.93\ (abs\ 664 - 1.93 \times Abs\ 647)$

Chl b $(mgl^{-1}) = 20.36\ (abs\ 647 - 5.5 \times Abs\ 664)$ (Geider & Osborne, 1992).

Fresh weight, shoot length, and chlorophyll data were subjected to one-way analysis of variance and individual means compared to the control by Tukey's honestly significant difference test ($p \leq 0.05$). The results of this test were used to determine the minimum concentration of microcystin causing phytotoxic effects.

4.2.4. Effects of 3 microcystin variants on the growth of mustard seedlings.

The toxicities of microcystin-LR, -RR, and -LF on the growth of *S. alba* were assessed using a modification of the method described by Kós *et al.*, (1995). Hoagland's No. 2 basal salt mixture (Sigma-Aldrich) was prepared by dissolving 1.63 g of powdered medium in 1 litre of Milli-Q water. The media was divided into 9 x 18 ml aliquots for each of the 3 toxins, solidified with 0.6% agar and autoclaved for 15 min at 121°C. Nine bottles were spiked aseptically with 2 ml of either microcystin-LR, -RR, or -LF to final concentrations of; 0, 0.1, 0.5, 1, 2.5, 5, 10, 25, and 50 $\mu g\ ml^{-1}$. All microcystin solutions were filter sterilised as described above prior to addition. Aliquots (2 ml) of each of the concentrations were dispensed aseptically into sterile plastic universal bottles and allowed to solidify. Five replicate bottles were prepared for each concentration of each microcystin variant under investigation. Three seeds were placed onto the surface of the media in each of the bottles, and incubated at 25 °C (± 2°C) under continuous cool white fluorescent light. Seedling length was measured after 7 days and the data subjected to one-way analysis of variance as before. The mean lengths were used to determine the concentration of each toxin to cause 50% inhibition of growth (GI_{50}).

4.3. Results.

4.3.3. Effect of microcystin-LR on the growth and development of potato shoot explants.

Following 16 days incubation, results indicated that growth was inhibited in the presence of microcystin-LR. Figure 4.1 shows the effect of microcystin-LR on the mean fresh weights and shoot lengths of potato shoot cultures after 16 days. Tukey's honestly significant difference test showed that the minimum concentration of microcystin-LR to inhibit fresh weight and shoot growth was 0.005 $\mu g\ ml^{-1}$. The growth of cultures decreased as the concentration of toxin increased, and at concentrations of between 0.5 and 5 $\mu g\ ml^{-1}$ no significant differences in fresh weight or shoot length were observed, indicating that there was no growth at these toxin concentrations. Intoxication with microcystin-LR also had an adverse affect on chlorophyll content (Figure 4.2.). Statistical analysis indicated that while toxin concentrations of 0.001-0.01 $\mu g\ ml^{-1}$ had no significant effect on the total chlorophyll content of cultures, those exposed to toxin levels of 0.05 up to 5 $\mu g\ ml^{-1}$ had significantly lower total chlorophyll content after 16 days than the control cultures.

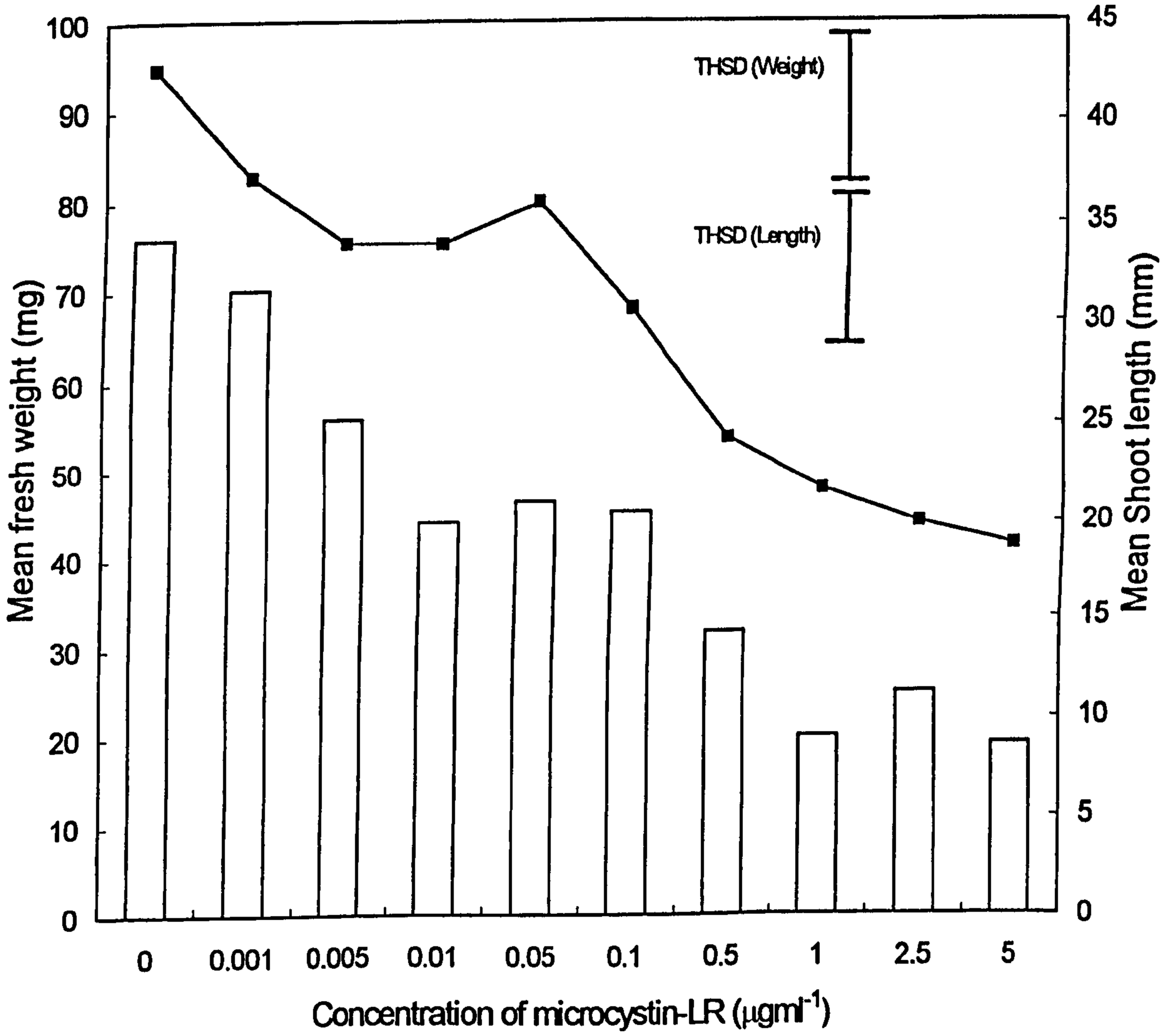


Figure 4.1. Effect of microcystin-LR concentration on the fresh weights (column) and shoot lengths (line) of potato shoot cultures after 16 days. Data plotted are the mean of 9 replicates. Bars indicate Tukey's Honestly Significant Difference (THSD, $p \leq 0.05$). The difference between two means is significant if it is greater than or equal to the value represented.

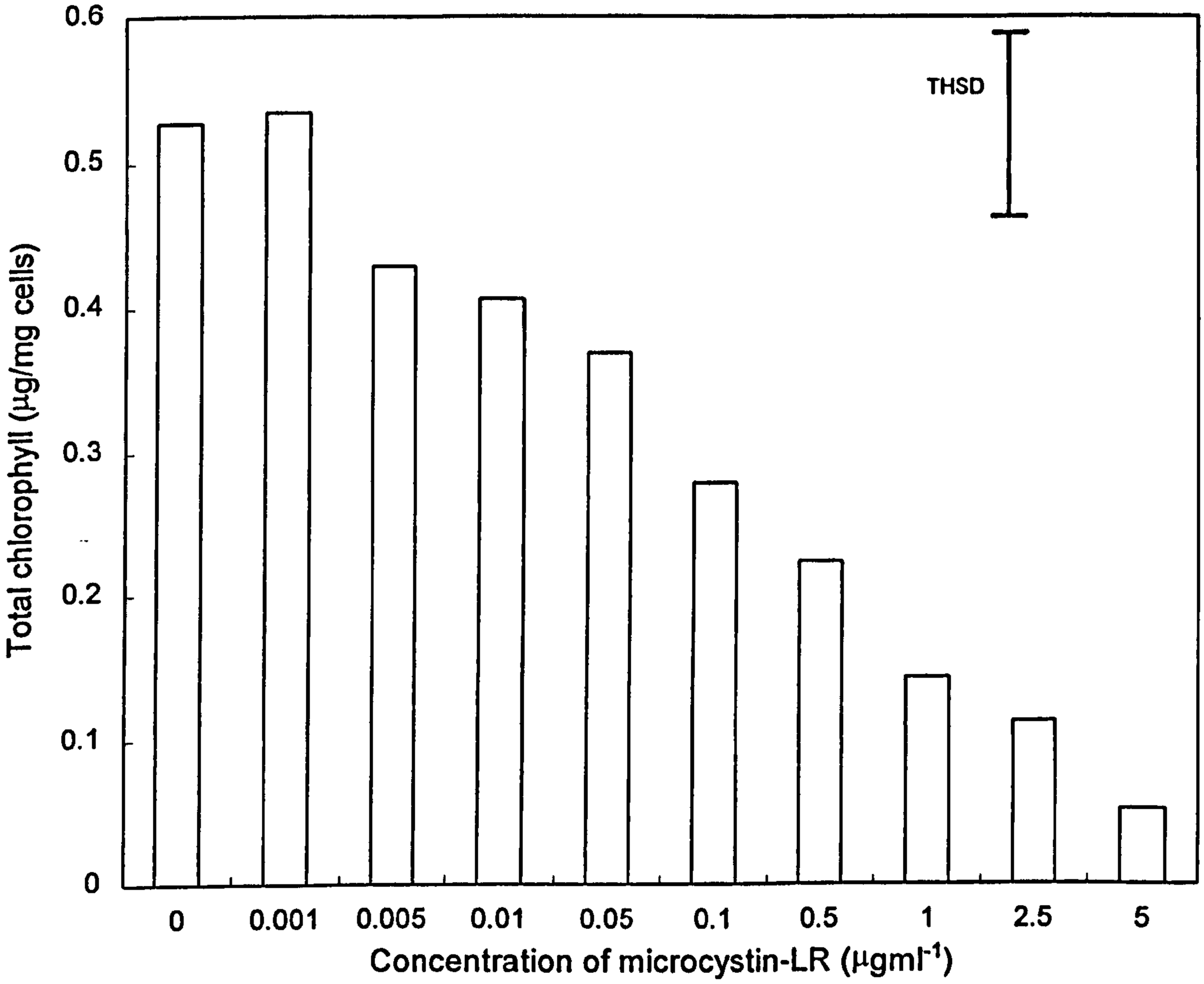


Figure 4.2. Effect of microcystin-LR concentration on the chlorophyll content of potato shoot cultures after 16 days. Data plotted are the mean of 9 replicates. Bar indicates Tukey's Honestly Significant Difference (THSD, $p \leq 0.05$). The difference between two means is significant if it is greater than or equal to the value represented.

Following 16 days incubation, the appearance of cultures was examined, and the proportion of chlorotic or necrotic tissue was determined for each shoot and compared with the control cultures (Figure 4.3.). At microcystin-LR concentrations of 0.05-5 $\mu g\ ml^{-1}$, shoots exhibited 50-100% necrosis (necrosis scores of 3-4) on shoot and leaf tissue. Figure 4.4. compares leaves removed from a control shoot (a) with that exposed to 1 $\mu g\ ml^{-1}$ microcystin-LR (b). It is clear that intoxication with microcystin-LR resulted in extensive loss of chlorophyll (chlorosis) and dark necrotic areas in leaf tissue. Closer observations of leaves taken from cultures exposed to 0.5 $\mu g\ ml^{-1}$ indicated that chlorotic/necrotic areas arose predominantly in the centre of the leaf (Figure 4.5.). Cultures exposed to lower toxin concentrations (0.001-0.01 $\mu g\ ml^{-1}$) generally exhibited less than 25% necrosis (necrosis scores of between 0-1) to shoot and leaf tissue. Examinations of cultures also revealed that intoxication had a detrimental effect on the development of roots (Figure 4.3.). Root development was evident in all replicate cultures grown in microcystin-LR concentrations from 0-0.05 $\mu g\ ml^{-1}$. However, as microcystin-LR concentration increased, the number of roots observed in cultures was found to decrease. Up to 3 roots were observed in untreated control cultures, and in cultures exposed to 0.001 and 0.005 $\mu g\ ml^{-1}$. This number decreased to 1-2 in cultures grown in microcystin-LR concentrations of 0.01-0.1 $\mu g\ ml^{-1}$. The number of replicate shoots exhibiting no root development increased at 0.5 $\mu g\ ml^{-1}$, and at microcystin-LR concentrations of 1-5 $\mu g\ ml^{-1}$ roots were absent in all replicate shoots.

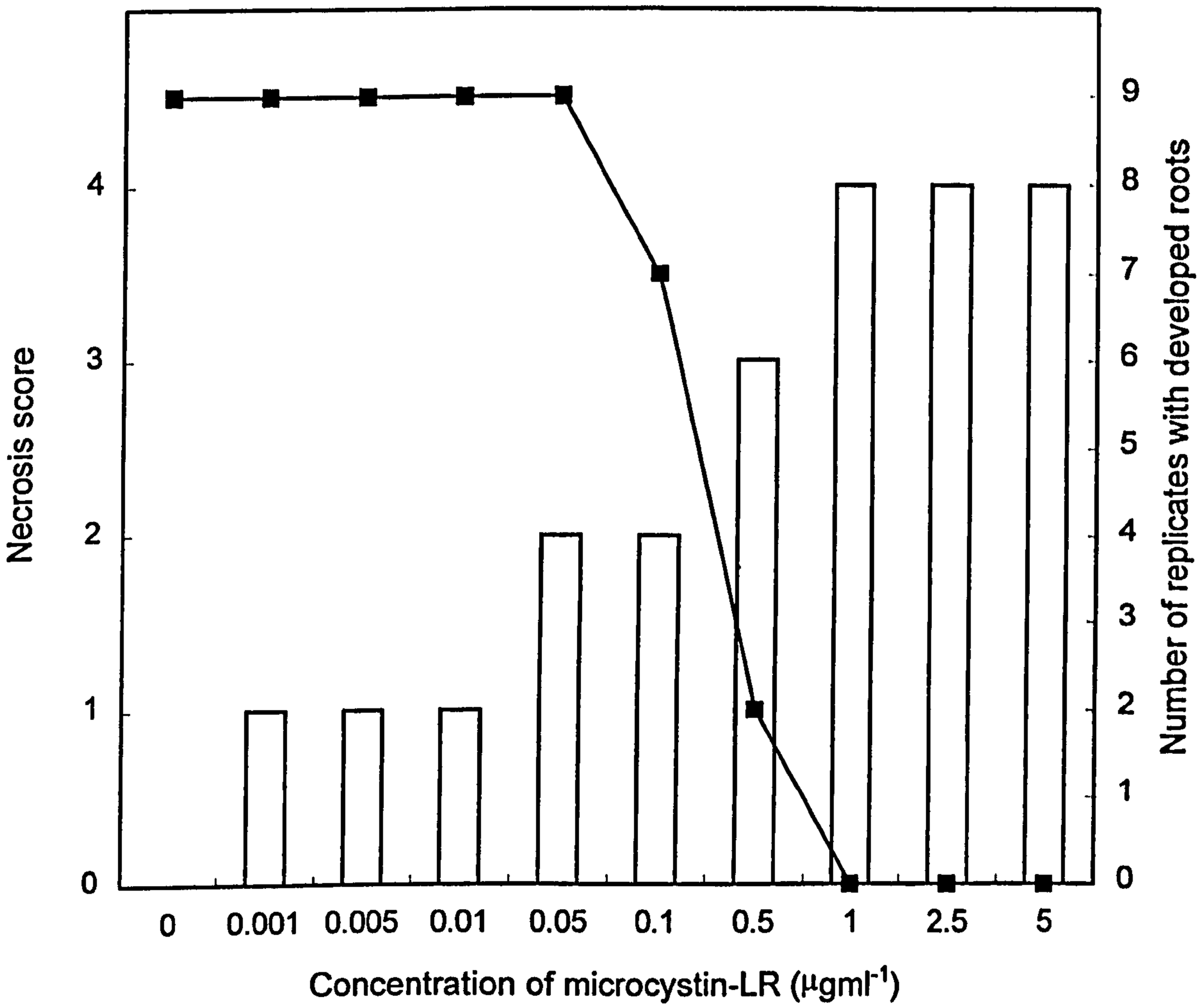


Figure 4.3. Effect of microcystin-LR on the visual appearance of potato shoot cultures after 16 days. The extent of necrosis on shoot and leaf tissue is represented by columns (score described in text); and the number of replicates exhibiting root development is shown as a line graph. Means are of 9 determinations.

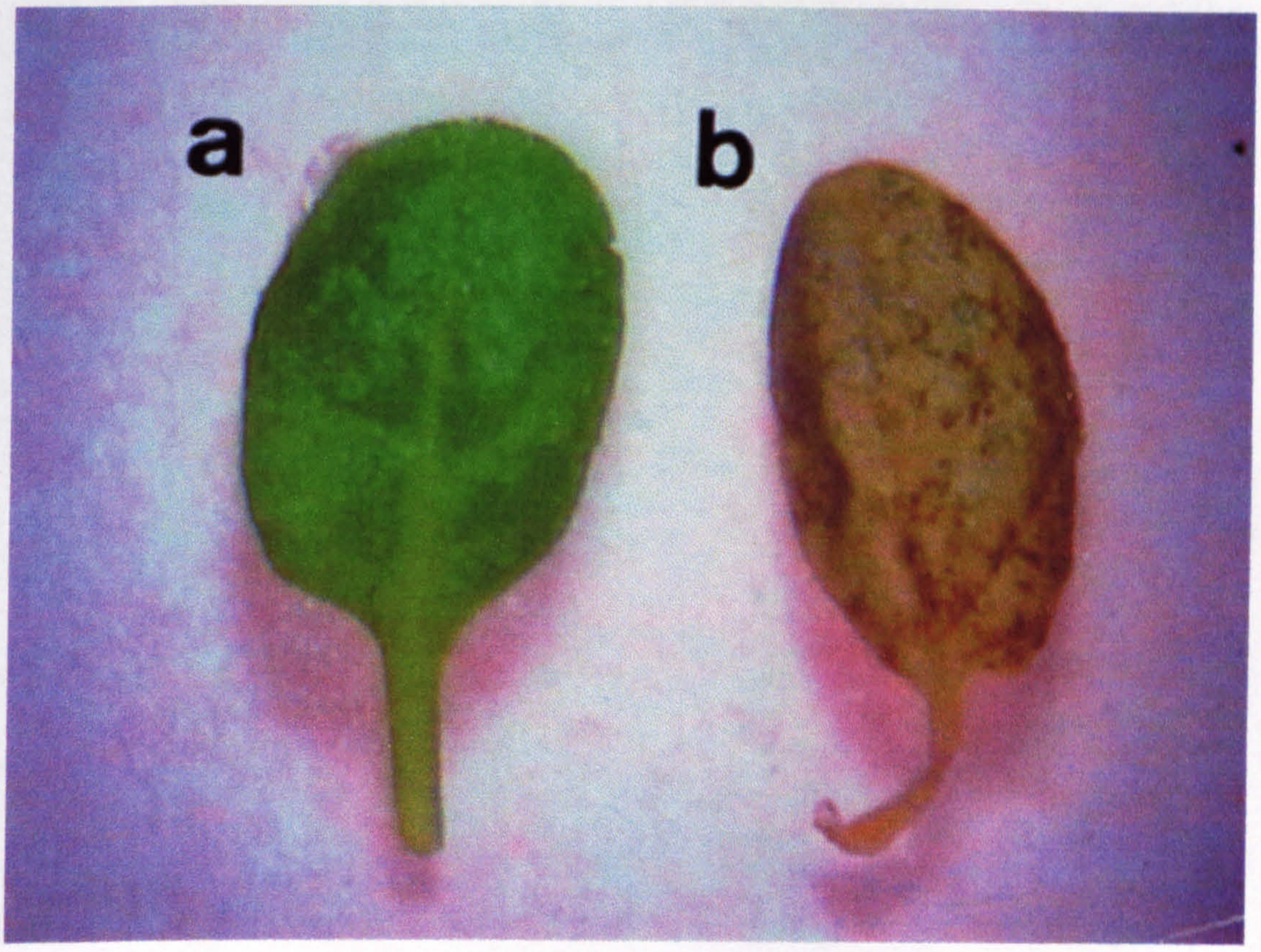


Figure 4.4. Necrosis on the leaves of potato shoot explants following exposure to microcystin-LR. The leaves shown were from explants grown in (a) 0 $\mu g ml^{-1}$ and (b) 1 $\mu g ml^{-1}$ microcystin-LR for 16 days. The extent of necrosis on each of these leaves is equivalent to (a) 0 (0% necrosis), and (b) 4 (75-100% necrosis) from the scoring system described in 4.2.3.

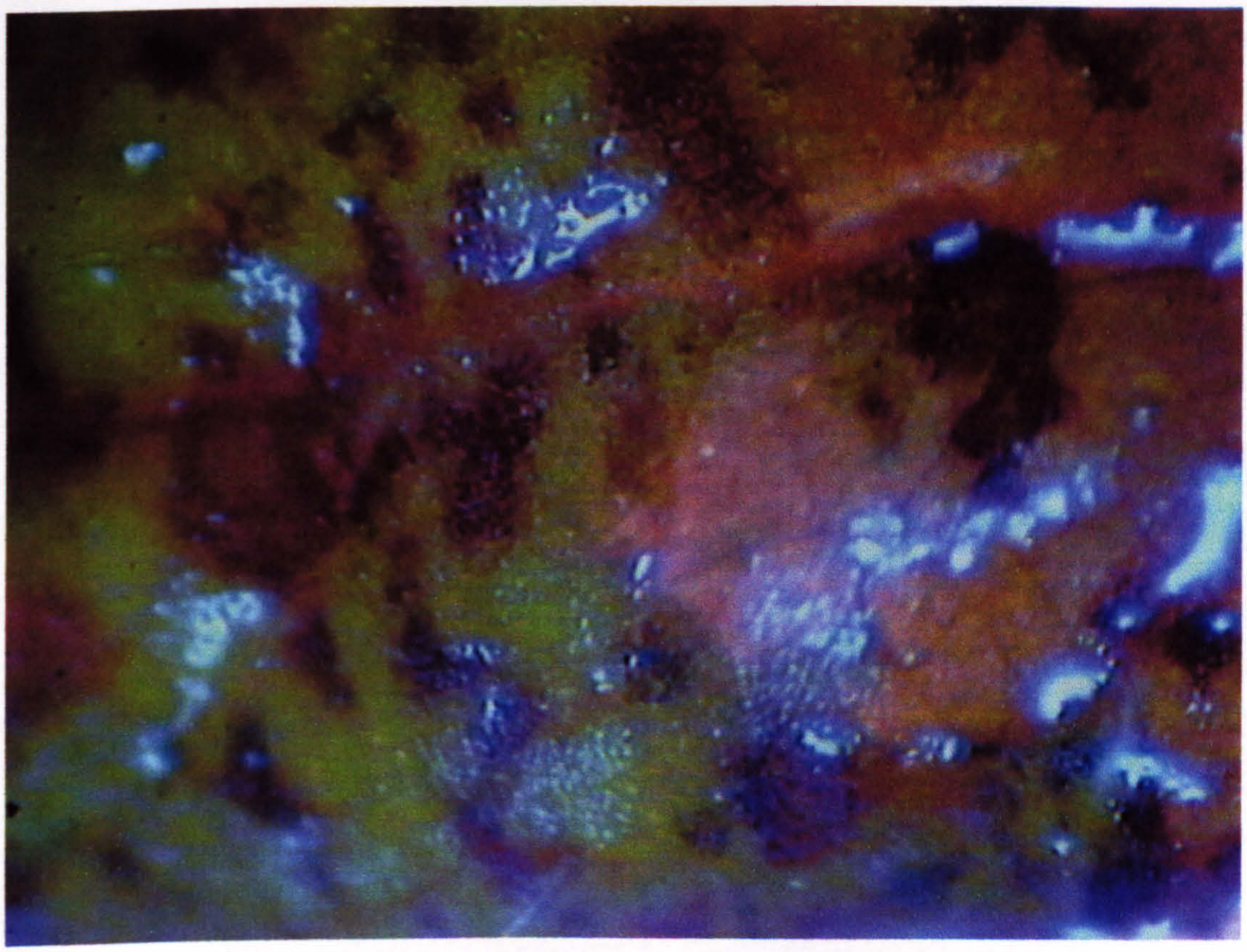


Figure 4.5. Necrotic areas on the leaf tissue of an explant grown in 0.5 μg ml^{-1} microcystin-LR. The photograph was taken from a microscopic image (x 20 magnification) of the centre of the leaf. The extent of necrosis on this leaf is equivalent to 3 (50-75% necrosis) from the scoring system described in 4.2.3.

4.3.4. Effects of 3 microcystin variants on the growth of mustard seedlings.

For each of the 3 toxins tested, there was significant concentration dependent inhibition of the growth of mustard seedlings. Figures 4.6.-4.8. illustrates the extent to which microcystin-LR, microcystin-RR, and microcystin-LF inhibited the growth of seedlings. The concentration of each microcystin which caused 50% growth inhibition (GI_{50}) was determined from the data. Microcystin-RR was found to be the most potent inhibitor of seedling growth, with a GI_{50} value of 1.6 $\mu g\ ml^{-1}$. Microcystin-LR was of similar toxicity with a value of 1.9 $\mu g\ ml^{-1}$, whereas microcystin-LF was found to be considerably less toxic, with a GI_{50} value of 7.7 $\mu g\ ml^{-1}$. Tukey's honestly significant difference test showed that microcystin-RR caused significant growth inhibition at a concentration of 0.1 $\mu g\ ml^{-1}$, and microcystin-LR at a concentration of 0.5 $\mu g\ ml^{-1}$ while microcystin-LF did not significantly inhibit the growth of seedlings until a concentration of 2.5 $\mu g\ ml^{-1}$.

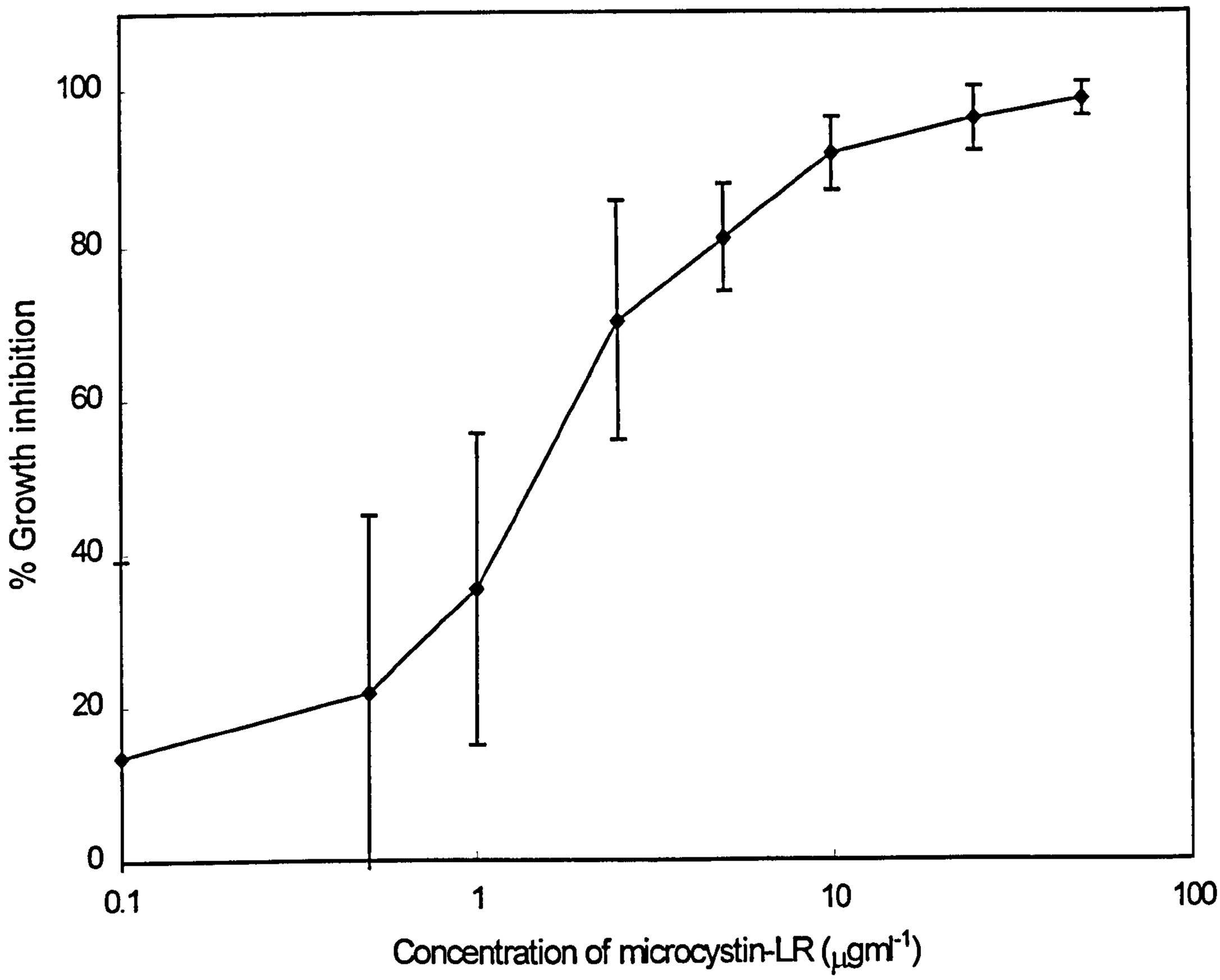


Figure 4.6. Inhibition of the growth of *Sinapis alba* seedlings by microcystin-LR. Data plotted are the means of 15 replicates, and bars indicate sample standard deviation.

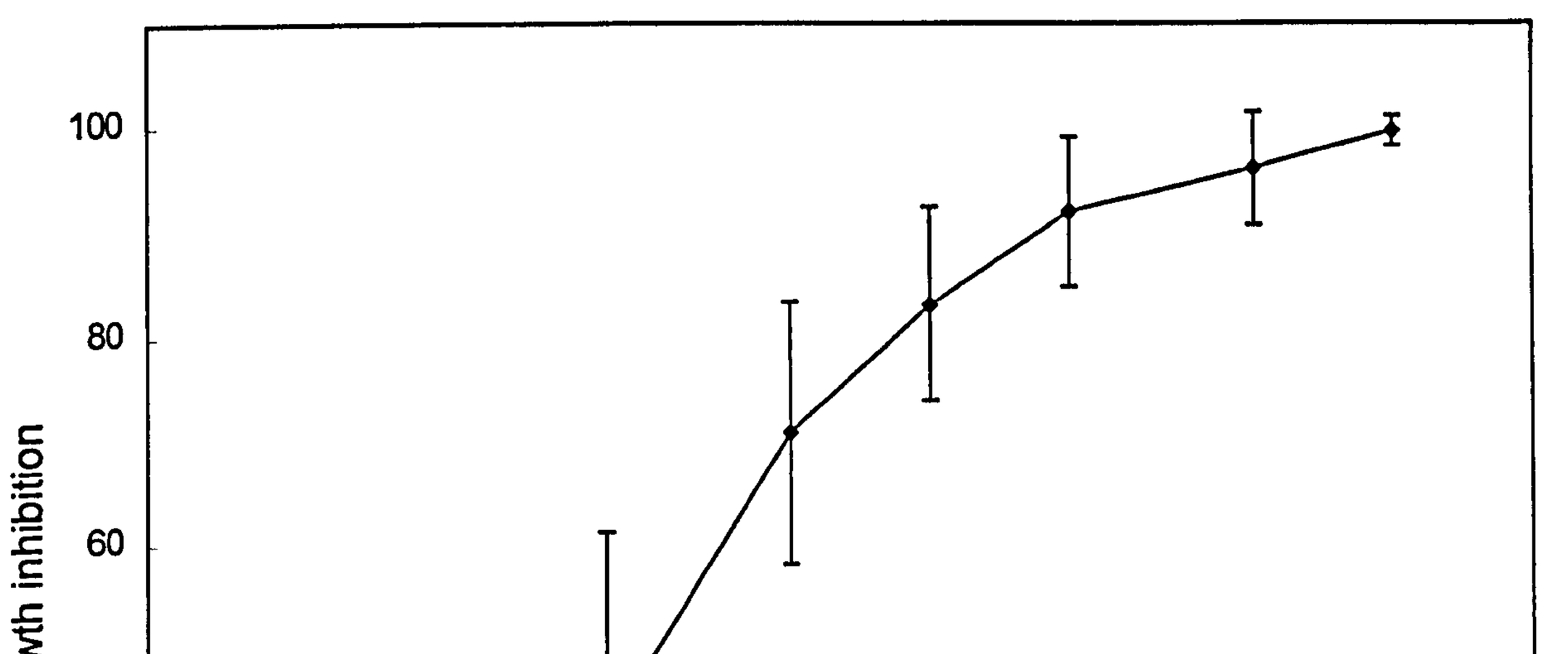


Figure 4.7. Inhibition of the growth of *Sinapis alba* seedlings by microcystin-RR. Data plotted are the means of 15 replicates, and bars indicate sample standard deviation.

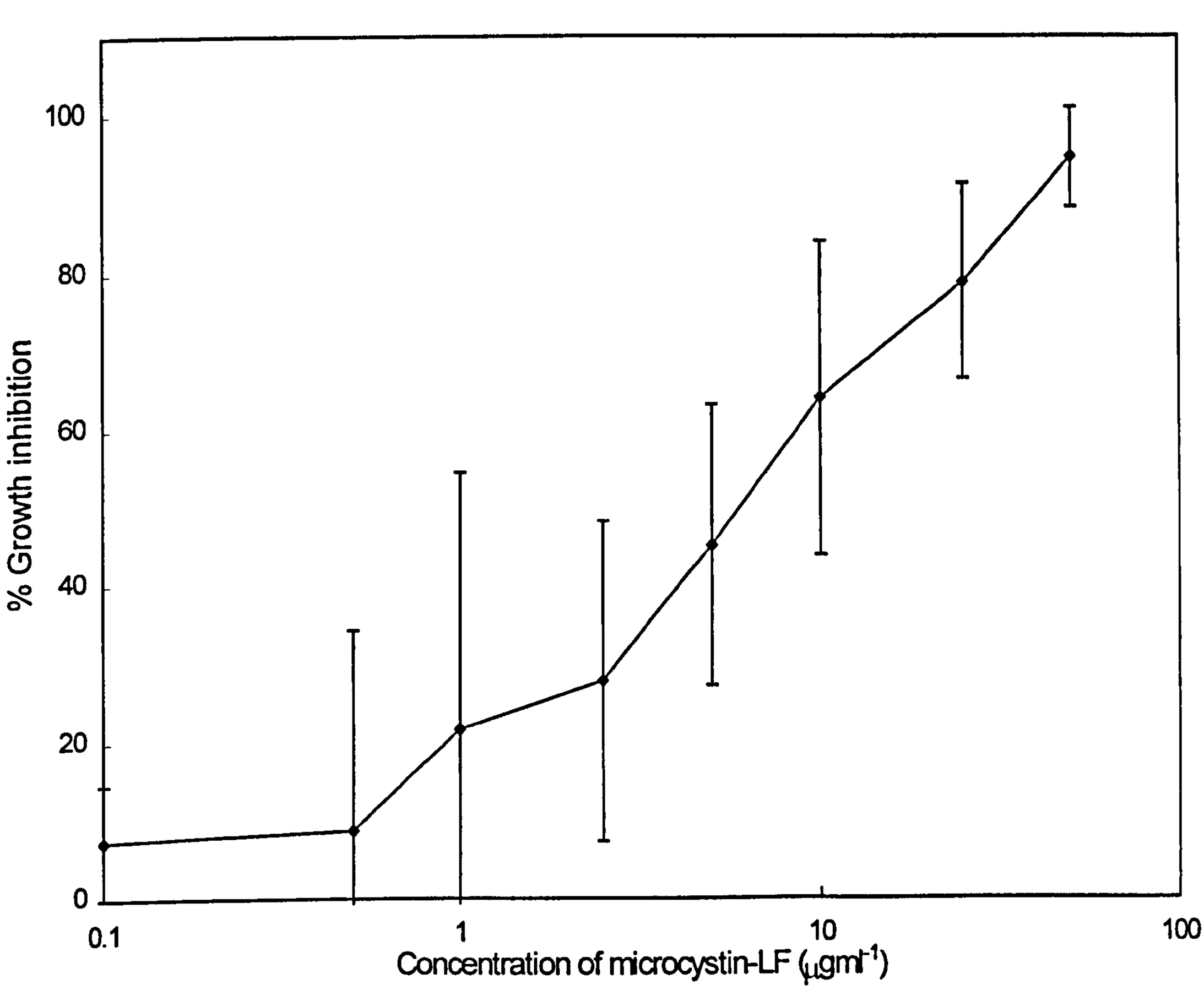


Figure 4.8. Inhibition of the growth of *Sinapis alba* seedlings by microcystin-LF. Data plotted are the means of 15 replicates, and bars indicate sample standard deviation.

4.4. Discussion.

This study has clearly shown that microcystins are inhibitors of growth and development in potato shoots and mustard seedlings under laboratory conditions. The findings suggest that exposure to microcystins via irrigation water contaminated with toxic cyanobacteria could pose a threat to the quality and yield of crop plants in the environment.

The growth and chlorophyll content of cultured potato shoot explants was shown to be significantly inhibited at microcystin-LR concentrations equivalent to 5-50 μg/litre microcystin-LR, which are representative of levels found in lake water during cyanobacterial blooms (McDermott *et al.*, 1995). Crop plants may be exposed to these toxin levels via irrigation water taken from a source which has experienced a bloom. In large scale irrigation systems, cyanobacterial growths can cause blockages in water outlets, and to prevent this water may be treated by filtration and chlorination prior to use (Keller and Bliesner, 1990). Problematic cyanobacterial growths may also be treated with algicides, the most common of which are copper based. Such treatments destroy blooms in the water body by causing cyanobacterial cells to lyse. However, following cell lysis, there is a subsequent release of toxins into the surrounding water (Jones and Orr, 1994), and the use of such treatments could lead to high toxin concentrations in water intended for irrigation use. The most commonly used irrigation techniques involve either sprinkle systems, in which water is applied to the external surface of the plant and the surrounding soil, or trickle systems, in which a small area of soil surrounding the plant is supplied with water. Plants may therefore be exposed to microcystins in contaminated water via uptake by the roots from the growth medium, or by deposition on the leaves and shoot tissue. The possible effects of spray irrigation practises on plant photosynthesis was investigated by Abe *et al.*, (1996). The authors demonstrated that photosynthesis was inhibited in the leaves of *P. vulgaris* following multiple topical applications of microcystin-LR at a concentration of 20 μg l^{-1}.

It was also observed that repeated exposure to lower concentrations of toxin inhibited photosynthesis to a greater extent than a single exposure to a higher concentration. In the present study, the exposure of potato shoots to microcystin-LR was more relevant to the supply of contaminated water to soil by trickle irrigation systems, or its use in the hydroponic cultivation of certain plants. Necrosis of 25-50% of tissues was apparent in cultures exposed to a microcystin-LR concentration of 0.05 $\mu g\ ml^{-1}$ (50 $\mu g\ l^{-1}$), and this was accompanied by a substantial reduction in biomass and chlorophyll content (Figures 4.1. and 4.2.). High levels of microcystins can occur in a water body following the destruction of a cyanobacterial bloom by the water treatments described above, or the ageing and breakdown of a bloom. Repeated irrigation using such water may therefore bring plants into contact with elevated levels of toxicity, inhibiting growth and causing damage to tissues.

The effects of microcystin-LR on potato shoots were observed under controlled and sterile conditions, therefore, the sensitivity observed may not be representative of the exposure experienced by plants in the environment. The differences between the uptake mechanisms of intact plants and excised shoots must also be considered. In whole plants growing in the environment, uptake is carried out by specialised root systems, whereas in cultures uptake may only occur through cells that are in contact with the medium. During the initial stages of culture, medium is taken up through the excised end of the shoot section. Prior to the development of roots, a callus forms in this area, and uptake occurs through undifferentiated callus cells (Leifert, Murphy, and Lumsden, 1995). Despite these differences, however, it was essential to employ aseptic techniques during the culture of potato shoot explants since microcystin-LR has been shown to undergo microbial degradation (Cousins *et al.*, 1996). Furthermore, sterile conditions ensured that the symptoms observed in treated shoots were attributable to the toxin. It is highly conceivable that the phytotoxic effects exhibited by potato shoots exposed to microcystin-LR were due to the inhibition of protein phosphatases. The toxin is known to be a potent inhibitor of protein phosphatases 1 and 2A, and these enzymes have been shown to play a vital role in mitotic growth events in

both animal and yeast cells (Kinoshita, Ohkura and Yanagida, 1990; Vandré and Wills, 1992). Statistical analysis revealed that the growth of potato shoot explants exposed to microcystin-LR concentrations of 0.5-5 $\mu g ml^{-1}$ was completely inhibited, indicating maximum growth inhibition within this range. The establishment of roots was also prevented in almost all replicate cultures at these concentrations. Smith *et al.* (1994), showed that the protein phosphatase inhibitors okadaic acid and calyculin-A inhibited the elongation of root hairs and root growth rates of *A. thaliana* seedlings. It is therefore likely that protein phosphatase inhibition by microcystin-LR was responsible for the lack of root development in potato shoots cultured in higher concentrations of the toxin. The inhibition of root growth would have impaired the uptake of nutrients from the surrounding medium. This may have contributed to the poor survival rate of potato shoot cultures at the higher concentrations of microcystin-LR.

Although microcystin-LR is often cited as the major toxin found in cyanobacterial bloom samples (Kotak *et al.*, 1993; Vezie *et al.*, 1997), cyanobacteria are capable of producing a number of microcystin variants simultaneously. Furthermore, many different strains of toxin producing cyanobacteria may be present in a single bloom sample, and a wide range of microcystins may be found (Jones, Falconer and Wilkins, 1995). The differences in toxicities between microcystin variants is well established in animals (Stoner *et al.*, 1989), but little is known about the potencies of different microcystins in plants. To date, plant studies have concentrated on the effects of microcystin-LR, but the effects of other naturally occurring microcystin variants on plant growth and development have not been explored. In this study, the effects of the microcystin-LR, -RR, and -LF were compared using a simple bioassay method devised by Kós *et al.* (1995). The results showed that microcystin-LR and microcystin-RR had a similar inhibitory effect on the growth of mustard seedlings (Figure 4.6. and 4.7.). However, the GI_{50} value for microcystin-LF was over 3 times higher (Figure 4.8.), suggesting it is less toxic to plants. These toxicities are markedly different from those reported in animals where microcystin-RR has consistently been found to be less toxic. By standardised mouse bioassay the LD_{50} of microcystin-RR is reported to be 12 times greater

than that of microcystin-LR (Botes *et al.*, 1982; Kusumi *et al.*, 1987). In animals the mode of action of microcystins in the liver is dependent on a bile-acid transport system (Runnegar, Gerdes and Falconer, 1991). In the mustard seed bioassay carried out in this study, toxin is present in the growth media at germination, which involves a rapid influx of water into the seed. This would account for the high toxicities of the more hydrophilic microcystin-LR and -RR, which would be carried rapidly into the seed with the influx of water. However, microcystin-LF is considerably less polar (Lawton *et al.*, 1995), which may have prevented it from being taken into the seed to the same extent as microcystin-LR or -RR. Figure 4.8. also shows that there is a more marked variation in the development of replicate seeds exposed to microcystin-LF compared to those exposed to microcystin-LR and microcystin-RR, particularly at lower concentrations. This may be accounted for by the lower solubility of microcystin-LF in the water based media, causing fluctuations in the amounts of the toxin taken up by the seeds.

In conclusion, these results have shown the effects of microcystin intoxication on two whole plant systems in the laboratory, and highlight the need for further research into the uptake and metabolism of these toxins in a variety of plant species. The difference in the toxicities of microcystin-LR, -RR, and -LF on the growth of mustard seeds is significant, and it is important that the effects of individual microcystin variants are determined, along with mixtures of toxins and natural bloom samples.

Chapter 5

Further investigations into the uptake of microcystins by plants

5.1. Introduction.

In chapter 4, exposure to microcystin was found to have an adverse effect on the development and growth of two terrestrial plant species. Although these studies were carried out under controlled laboratory conditions, toxic effects were observed at concentrations which have been found in water bodies following cyanobacterial blooms (McDermott *et al.*, 1995). These findings suggest that cyanobacterial toxins in water bodies may present a considerable risk to irrigated crops in the field.

The irrigation of crops makes the single largest demand for water worldwide (Table 5.1., Cook, 1998). Increasing pressures on food crop productivity have led to the gradual depletion of resources for irrigation water, particularly in warmer climates. As a result of this pressure, the quality of water used for irrigation may be compromised, causing plants to be increasingly susceptible to harmful water borne contaminants.

Table 5.1. Estimates of global water use in 1991(Cook, 1998).

Sector	**Water use ($km^3 yr^{-1}$)**
Domestic	100
Industrial	200
Cooling	225
Livestock	40
Irrigation	3300
Total	**3865**

Intensive agriculture also leads to the artificial enrichment of water bodies through run-off from fertilisers rich in nitrogen and phosphorous. The resulting eutrophic environments provide optimum growth conditions for bloom forming cyanobacteria. Lakes and rivers from which water is routinely used for irrigation have been found to experience cyanobacterial blooms (Abe *et al.*, 1996).

It is therefore probable that plants come into contact with toxins in this way. Furthermore, the repeated extraction of water from rivers and streams can introduce conditions which will promote the growth of cyanobacteria. An example is the Darling River in New South Wales, Australia, where diversions for irrigation have decreased flow rate and contributed to eutrophication, thus providing favourable conditions for cyanobacterial growth (Hötzel and Croome, 1994). Toxic cyanobacteria may also occur in smaller water bodies, such as farm dug outs (Kotak *et al.*, 1993), and dams from which water is often extracted for irrigation. In an unpublished communication (Brad Sherman, CSIRO, Australia), it was reported that poor growth and root callouses occurred in commercial rose crops grown both hydroponically and by trickle irrigation using dam water which had experienced a cyanobacterial bloom.

These findings may have far reaching consequences for both economic and health reasons. While exposure to microcystins will have a detrimental effect on the productivity of plants in the environment, the accumulation of these toxins in edible plant tissues may also provide a further pathway for human intoxication. Due to the tumour promoting capabilities of microcystins, a provisional guideline has been set for the level of microcystin-LR in drinking water (1 $\mu g l^{-1}$, WHO, 1998). The ingestion of microcystins through drinking water is thought to be the most common exposure route for humans. It is possible, however, that plants which have been irrigated using toxin-containing water may provide an alternative vector for human intoxication.

The uptake and fate of microcystins in terrestrial plants has not yet been adequately investigated. A recent investigation demontrated the uptake of radiolabelled microcystin-LR by aquatic plants suggesting that the toxins may accumulate in the aquatic food web (Pflugmacher *et al.*, 1998 a). In one laboratory study, a concentration dependent inhibition of protein phosphatase activity in the leaves and stems of mustard (*Sinapis alba* L.) seedlings by microcystin-RR was observed, indicating that the toxin was taken up through the plant into the edible tissues (Kurki-Helasmo and Meriluoto, 1998). Although the uptake of microcystins

by plants has been demonstrated, the toxicity of plant material following exposure has not been studied. There is also a lack of information on the fate of microcystins in plant tissues, and their metabolism. Experiments involving mice and rat liver have suggested that conjugation with glutathione contributes to the metabolic pathway involved in the detoxification of microcystins (Kondo *et al.*, 1996). Glutathione is a strong nucleophile found in high concentrations in most living cells and can form conjugates with certain metabolites mediating their detoxification (Monks *et al.*, 1990). Since glutathione has been found to play an important role in the metabolism of xenobiotics in plants (Sandermann, 1992), it is likely that similar conjugation reactions will occur in plant cells in response to microcystins.

This chapter describes the development of methodology for recovering microcystins from plant material following exposure to the toxins in growth medium. The most efficient method for extracting microcystins from plant tissues was determined using cut plants (*Dianthus sp.*) which had been left in water containing high concentrations of the toxin. HPLC was used to determine recoveries of free toxin in plant extracts, and to attempt to identify any additional compounds which could represent products of toxin metabolism.

The uptake of microcystin-LR was then observed using a larger intact plant, and accumulation of the toxin in plant tissues assessed following exposure to a lower toxin concentration over time. The effects of lower concentrations of microcystin-LR on the growth of runner beans (*Phaseolus vulgaris* L.) were observed over 18 days. Exposed plant material was extracted using the developed methodology and analysed for microcystins and related compounds using HPLC. The toxicities of bean plant extracts were finally assessed using a simple invertebrate bioassay.

5.2. Materials and methods.

5.2.1. Toxins.

Microcystins-LR and -LF were purified using normal and reverse phase flash chromatography as described in chapter 3. All toxin solutions were diluted using Milli-Q water unless stated otherwise.

5.2.2. Plant material.

Dianthus sp. (white spray carnations), were obtained from a local retailer. The stems of all flowers used in experiments were cut to 20 cm.

Runner bean seeds (*Phaseolus vulgaris* L., Suttons seeds Ltd) were rinsed three times in 70% ethanol, and soaked overnight in sterile distilled water prior to use. To obtain runner bean seedlings with straight radicals, soaked beans were individually placed on blotting paper which had previously been autoclaved for 15 minutes at 121°C, and dampened with sterile distilled water. The paper was then rolled up and placed in a sterile glass beaker containing a small volume of sterile distilled water. The beaker was then covered in aluminium foil and left at room temperature until germination had occurred.

5.2.3. Development of an extraction procedure for recovering microcystin from plant material.

To determine whether microcystin-LR could be recovered from plant material, cut *Dianthus sp.* were allowed to take up water containing microcystin-LR and extracted by three different methods. These plants provided a simple model for determining the recovery of microcystin from plant tissues exposed to a known amount of the toxin. The minimum time required for the vascular system of a cut plant to become saturated with aqueous solution was first assessed by leaving three stems with white flowers in a 10 ml measuring cylinder containing 3 ml of

blue food dye (Sharwoods, UK). After approximately two hours, the petals of each flower head had turned blue, indicating that saturation of the plant had occurred. This provided a means of estimating the amount of time it would take for a plant to become saturated with microcystin-LR when the dye was replaced with an aqueous solution of the toxin. An aqueous solution (3 ml) containing 30 μg ml^{-1} of microcystin-LR was then dispensed into six measuring cylinders (10 ml), and one plant placed in each. Aliquots (100 μl) were removed from each of the cylinders for HPLC analysis in order that toxin concentration could be determined accurately. Parafilm was wrapped around each plant at the open end of the cylinder to prevent evaporation. The plants were left in the solutions for 3 hours at room temperature, after which time it was predicted from the previous experiment using dye, that plant tissues should have become saturated with toxin. The plants were removed and residual toxin was rinsed from the base of each stem with a small volume of methanol. The volume of aqueous toxin solution taken up by each of the plants was then recorded in order that the amount of toxin present in plant tissues could be determined.

Three extraction methods were assessed to determine the recovery of microcystin-LR from plants exposed to the toxin. Two plants were each homogenised for 5 minutes in 20 ml of either methanol, methanol + 0.01% TFA, or butan-1-ol:methanol:water (5:20:75) using a food processor (Kenwood CH100 'Mini chopper', 150 ml capacity). Homogenised tissue was then transferred carefully from the food processor to a glass beaker, and allowed to extract for 30 minutes. A small volume of the extraction solvent was used to rinse the blades and container of the food processor to remove remaining plant material, and added to the extract. After 30 minutes, the extract was filtered, and the plant material extracted a further twice in 20 ml of the appropriate solvent. The three extracts were then pooled, rotary evaporated to dryness, and resuspended in 1 ml of methanol. Each extract was analysed by HPLC to determine the amounts of microcystin-LR recovered from plant tissues by each extraction method.

5.2.4. Determination of the recovery of microcystin-LF from plant material.

HPLC chromatograms of plant extracts prepared in 5.2.3. revealed a cluster of peaks with a similar retention time to the peak corresponding to microcystin-LR (Figure 5.2.). The presence of these peaks made the identification of microcystin-LR impossible, preventing the determination of toxin recoveries. However, the chromatograms also revealed the absence of less polar compounds eluting between 30 and 45 minutes. This suggested that a less polar microcystin would be more easily detected in plant extracts by HPLC. The comparison of extraction methods was therefore repeated by determining the recovery of microcystin-LF; a relatively hydrophobic microcystin. The retention time of microcystin-LF by analytical HPLC is around 36 minutes (chapter 3, Figure 3.14.). The chromatograms of plant extracts prepared in 5.2.3. did not indicate the presence of compounds eluting at this time, suggesting that microcystin-LF would be more easily quantified.

The procedure was further modified to enhance toxin detection by exposing plants to higher concentrations of microcystin-LF over a longer period. Six plants were placed in measuring cylinders containing 5 ml of an aqueous solution of microcystin-LF at a concentration of 100 $\mu g\ ml^{-1}$. Aliquots (100 μl) were removed from each of the solutions in order to confirm actual toxin concentration. Another six control plants were left in water only. The plants were allowed to stand in the test solutions overnight (approximately 15 hours). After this time the volume of toxin solution taken up by each plant was recorded, and two of the control plants and two which had been exposed to microcystin-LF were extracted using each of the extraction methods described in 5.2.3.

5.2.5. Synthesis of glutathione and cysteine conjugates of microcystin-LF.

HPLC analysis of methanolic extracts of plants which had been exposed to microcystin-LF revealed several peaks in addition to the toxin peak which were not detected in extracts of control plants (Figures 5.4-5.5). The retention times of

each of these peaks were considerably earlier than that of the toxin peak, indicating the presence of more polar compounds which may represent toxin metabolites. A major mechanism for the metabolism of microcystins in animals is mediated by conjugation with glutathione and cysteine (Kondo *et al.*, 1996). In order to determine whether this mechanism had occurred in these plants following exposure to microcystin-LF, glutathione and cysteine conjugates of microcystin-LF were prepared, and analysed by HPLC. The chromatographic data for each of the conjugates was then compared with that obtained for extracted plant material following exposure to microcystin-LF.

The method employed for chemically synthesising conjugates of microcystin-LF with glutathione and cysteine was modified from the procedure described by Kondo *et al.* (1992 b). A dried sample of microcystin-LF containing 2.6 mg of the toxin was dissolved in 25 ml of 5% potassium carbonate. Aliquots (10 ml) were transferred to 20 ml glass universal bottles, and placed on a magnetic stirrer. Glutathione (17 mg, Sigma) was dissolved in 5 ml potassium phosphate buffer (pH 6.5). This solution was added drop-wise over two hours to microcystin-LF solution in one of the universal bottles with constant stirring. The solution was acidified to pH 3 using 1 M HCl, and passed through an Isolute™ C_{18} cartridge, which had been preconditioned with 10 ml methanol followed by 10 ml water. The cartridge was then rinsed with 10 ml water, prior to eluting the conjugated toxin with 10 ml methanol. The cysteine conjugate of microcystin-LF was prepared by adding a solution containing cysteine (10 mg in 5 ml potassium phosphate buffer) to the other universal bottle containing 10 ml of the toxin solution in the same manner and repeating the procedure described above. Each of the conjugates were then analysed by HPLC.

5.2.6. Recovery of microcystin-LF from runner beans (*Phaseolus vulgaris L.*).

The cut plants employed in preliminary investigations provided a simple tool for determining whether microcystin could be recovered from plant material exposed

to the toxin. However, as these were incomplete plants, i.e., lacking a root system; the studies carried out did not truly represent the uptake of microcystin by intact growing plant systems. Further investigations were therefore carried out to determine the uptake of microcystin by growing runner beans exposed to the toxin via the roots. Instead of exposing the plants to a single high dose of microcystin, plants were continuously exposed to a lower toxin concentration (1μg ml^{-1}) over a longer period. The most efficient extraction procedure developed in 5.2.4 was then used to recover the toxin from the plants following exposure.

Six runner beans were germinated as described in 5.2.2. Using forceps, the germinated beans were then wrapped in cotton wool, and transferred to glass bottles containing 250 ml of sterile 1/4 strength Hoaglands No. 2 Basal Salt Mixture (4.08 g dissolved in 10 litres Milli-Q water, Sigma). Each seed was pushed into the neck of one bottle allowing the root to contact the medium. The bottles were then arranged in two rows along a trellis for support and allowed to grow for 7 days under continuous illumination (20 μmol m^{-2} s^{-1}) using cool white fluorescent tubes (36 W). Growth temperature ranged between 21 and 29 °C throughout experiments.

Toxin solution was prepared by dissolving 1 mg of microcystin-LF in 200 μl methanol and diluting with 800 μl water. This was dispensed into 400 ml of Hoaglands medium using hollow fibre syringe filters (Dynagard), and mixed. After 7 days, each plant had grown to 20-30 cm in length, and had developed roots. Medium was carefully removed from the bottles, leaving 150 ml in each. The volume in three of the bottles was made up to 250 ml by adding 100 ml of toxin solution, containing 250 μg of microcystin-LF. Aliquots (100 μl) were removed from each bottle of medium spiked with toxin to determine actual toxin concentrations. HPLC analysis revealed that the concentration of microcystin-LF in each of the bottles was 1.23 μg ml^{-1} (± 0.31μg ml^{-1}). To the remaining three bottles, 100 ml of unspiked Hoaglands medium was added. Each plant was then attatched to the trellis and allowed to grow for a further 7 days. After this time, each plant was removed, the roots rinsed thoroughly in methanol to remove residual toxin, and

dried on blotting paper. The volume of medium remaining in each of the bottles was then measured, in order that the amount of medium taken up by each plant could be determined. Aliquots (100 μl), were also removed from each of the bottles to determine whether toxin concentration remained stable after 7 days. The recovery of microcystin-LF from each plant was then assessed following extraction in methanol as described in 5.2.3. Each whole plant was homogenised in 50 ml of methanol using a food processor. The extract was then transferred to a glass beaker and extracted three times for 30 minutes. The extracts were combined, rotary evaporated to dryness, and resuspended in 1 ml of methanol. All samples were then analysed by HPLC, and the toxin recoveries determined as described.

5.2.7. Investigations into the uptake by and effects of microcystin-LR on runner beans.

Analysis of bean extracts prepared in 5.2.6. revealed peaks eluted throughout the chromatogram. These findings negated the advantage of using microcystin-LF in experiments. As described in chapter 3, the purification of microcystin-LF is time consuming compared with microcystin-LR, which can be purified in one step using reversed- phase Flash chromatography. Also, it is available in greater quantities as significantly more is produced by the batch culture compared to microcystin-LF (see Figure 3.7). Further studies into the uptake of microcystin by runner beans were therefore carried out using microcystin-LR.

An alternative experimental design was also employed in this study to allow for easier handling (Figures 5.1. and 5.2.). Germinated runner bean seeds were transferred using sterile forceps into modified plastic universal bottles (25 ml, Labtech) from which the bases had been removed. One seed was placed in a bottle with the root pointing downwards through the base. The plastic bottle was then attatched using parafilm to a sterile glass universal bottle containing 25 ml of Hoaglands medium. The bottles (60 in total) were then arranged in 4 rows against trellis and allowed to grow under continuous light. Figure 5.1. shows replicate

runner bean plants after 3 days growth in glass universal bottles containing Hoaglands medium. After 7 days, when the stems had grown to 20-30 cm, forty of the healthiest plants were selected for use in experiments. Hoaglands Basal Salts Mixture (1/4 strength, 1 litre) was dispensed into each of eight glass bottles. Each bottle of medium was then autoclaved at 121 °C for 15 minutes and allowed to cool. Microcystin-LR (approximately 1 mg dissolved in 200 μl methanol and diluted with 800 μl water) was added to four bottles of sterile medium using hollow fibre syringe filters (Dynaguard) and mixed. Aliquots (100 μl) were removed from each of the bottles in order that the actual toxin concentration could be determined by HPLC analysis. Thus, it was calculated that the mean toxin concentration was 1.12 μg ml^{-1} (± 0.21 μg ml^{-1}). Equal amounts (125 ml) of toxin solution were dispensed into 20 sterile glass bottles. Modified plastic universal bottles containing runner beans were transferred carefully from glass universals to each bottle containing toxic medium and secured using parafilm. Placing the beans in plastic universals allowed whole plants to be transferred easily to bottles without damaging the roots. Another 20 plants were transferred to bottles containing 125 ml Hoaglands medium which had not been spiked with microcystin-LR. The bottles were then arranged in 4 rows and attatched to trellis to prevent the stems from breaking (Figure 5.2.). To ensure that the roots were fully submerged at all times, the medium in each bottle was topped up during the experiment. This could be carried out without disturbing the plants by pouring fresh medium (either toxic or non-toxic) into the plastic universal and allowing it to drain into the bottle. The total volume of medium added to each bottle was recorded. Groups containing five plants growing in bottles containing toxic medium, and five in bottles containing medium lacking toxin were removed after 3, 6, 12, or 18 days. After each time period, plant roots were rinsed thoroughly with methanol to ensure the removal of residual toxin, and dried on blotting paper. The following parameters were then assessed for each plant:

- Stem length
- Number of leaves
- Presence of necrosis/chlorosis
- Volume of medium taken up

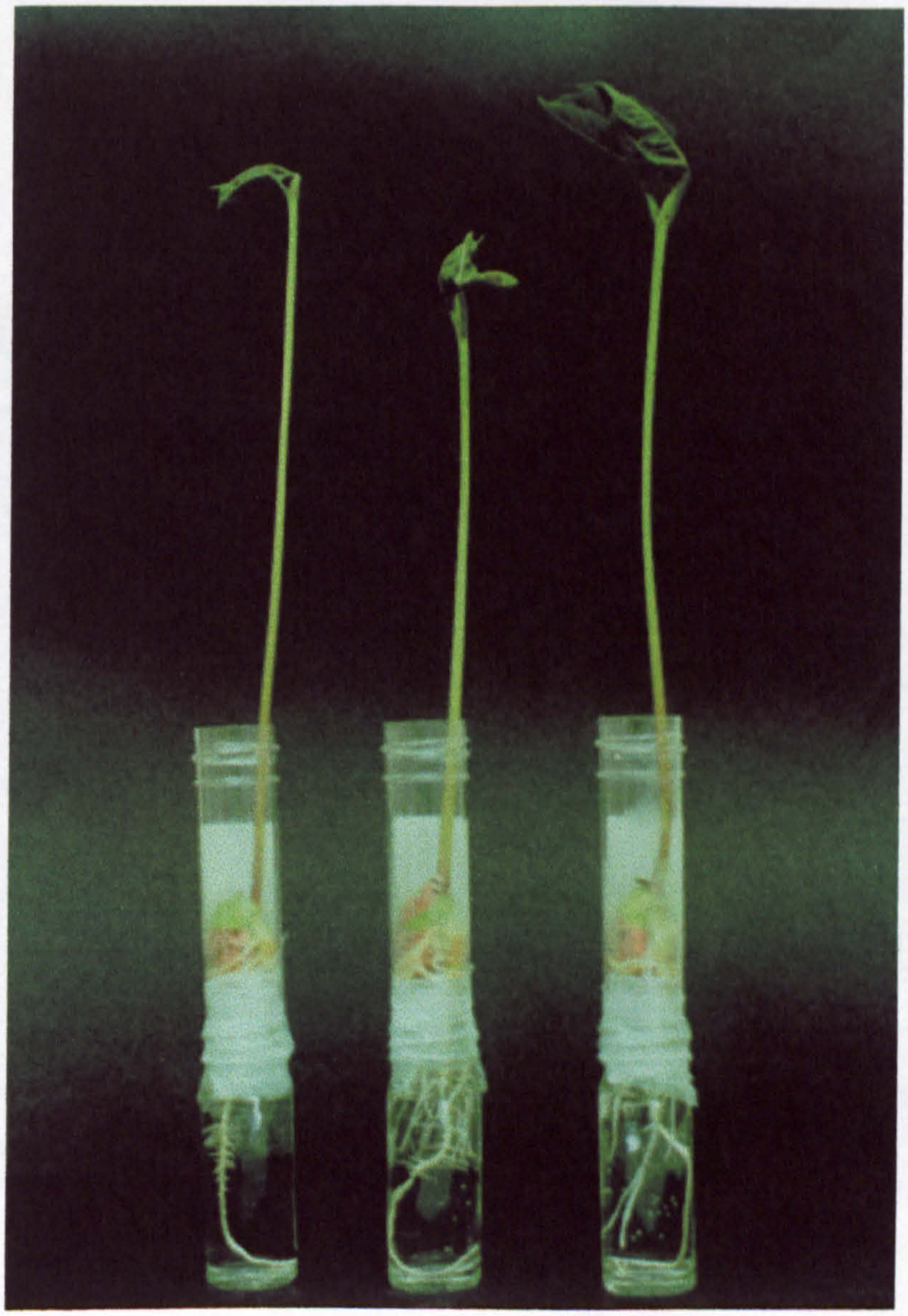


Figure 5.1. Experimental set-up designed to grow runner beans (*Phaseolus vulgaris*) under the conditions described in 5.2.7. Photograph shows replicate runner bean plants after 3 days growth in Hoaglands medium in glass universals.

Figure 5.2. Runner bean plants growing in glass bottles containing control or toxic medium.

Photographs were taken of plants removed at each time interval to compare the features of plants exposed to the toxin with those of plants grown in non-toxic medium.

Comparison of plants which had been grown in the presence of microcystin-LR to those grown in control medium revealed that exposure to the toxin had a marked effect on the plant roots (Figure 5.10.). In order to observe these effects more closely, root sections from contol and exposed plants were prepared for microscopic examination. This procedure was carried out by Mr K. MacKenzie at the Zoology department of the University of Aberdeen.

After 18 days, portions (approximately 2 mm^3) were removed from the root tips of a control plant and one which had been exposed to microcystin-LR for 18 days using a razor blade. All chemicals used in the preparation of root tissues for microscopic examination were obtained from TAAB laboratories (UK, Ltd). All steps were carried out at 4°C. The tissue was first fixed in 2.5% gluteraldehyde (prepared in 0.1M phosphate buffer, pH 7.4) for 24 hours, and then washed in three changes of phosphate buffer for 15 minutes. Dehydration was carried out in 70% (v/v) aqueous ethanol for 30 minutes, then in 90% ethanol for a further 30 minutes, before washing twice in 100% ethanol for 10 minutes. The tissue was then transferred to a 1:1 mixture of 100% ethanol:LRW (London Resin White) for 1 hour, then placed in LRW for 2 x 2 hours. After this time, it was transferred to fresh resin and left overnight. Embedding solution (1 ml of LRW mixed with 1.5 µl LRW accelerator) was then added to a polypropylene capsule until half full. The tissue was placed in the capsule, and covered with the remainder of the embedding solution. The capsule was finally sealed and placed on crushed ice for 2-3 hours to allow polymerisation. Longitudinal sections (1µM thickness) were cut from each embedded root tissue sample using a microtome and stained using an aqueous solution of 1% Toluidine blue plus 1% Borax (w/v). Stained tissue samples were then fixed onto microscope slides.

To analyse the amount of microcystin-LR present in plant tissues, each plant was extracted in methanol. Finally, the evaporated extracts were resuspended in 1 ml of methanol and analysed by HPLC. Aliquots of the remaining toxic medium in bottles left for 3 days and those left for 18 days were retained and analysed by HPLC. The toxin concentrations of these solutions were compared to the starting solution to determine whether concentration remained constant throughout the experiment.

5.2.8. Assessment of toxicity in runner bean extracts by brine shrimp (*Artemia salina*) bioassay.

Each of the runner bean extracts prepared in 5.2.7. were assayed using a simple invertebrate bioassay to indicate the toxicities of the plant material following exposure to microcystin-LR.

Aliquots (500 μl) of each of the plant extracts (both toxic and control) were dried under nitrogen and resuspended in 25 μl of methanol. Each sample was then diluted with 475 μl of brine shrimp medium (Harwig and Scott, 1971). This medium was prepared as a stock solution composed of; 300 g sodium chloride, 3 g of calcium chloride dihydrate, 15 g of magnesium chloride hexahydrate, 5 g of magnesium sulphate heptahydrate, 8 g of potassium chloride, 60 g of glycine, and 30 g of disodium glycerophosphate, dissolved in 1.25 litres of distilled water. Each chemical was dissolved separately in the order given to prevent precipitation. Brine shrimp cysts (100 mg, Sciento, Manchester, UK) were added to two flasks, each containing 20 ml of brine shrimp medium mixed with 140 ml of distilled water. These were incubated at 25°C for 48 hours, until the brine shrimps had hatched. The flasks were then placed in front of a bright light, which attracts the brine shrimps away from the unhatched cysts and cases, allowing them to be transferred using a plastic dropper to glass universal bottles containing fresh medium. The bottles were mixed carefully to obtain homogenous suspensions of brine shrimp larvae before transferring 100 μl to each of 40 wells of a 96 well Microtitre™ plate (Dynagard, Dynex Technologies).

Each of the prepared runner bean extracts was added to a well (containing approximately 20 brine shrimp larvae), and the plate was incubated overnight at 25°C. Using another plate, a calibration was prepared for microcystin-LR usingthe following concentrations; 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 12.5, 25, and 50 $\mu g\ ml^{-1}$. Each concentration of toxin was assayed in triplicate. Following incubation overnight, the dead brine shrimp were counted in each well using a microscope. Methanol (100 μl) was then added to each of the wells to kill the remaining living brine shrimp, and the total number in each well determined. The percentage of brine shrimp killed by each plant extract and by each concentration of microcystin-LR was then calculated.

5.3. Results.

5.3.3. Development of an extraction procedure for recovering microcystin from plant material.

After 3 hours, plants had taken up between 400 μl and 800 μl of toxin solution. HPLC analysis of plant extracts prepared in methanol, methanol +TFA, and butanol:methanol:water, revealed that microcystin-LR could not be detected. Figure 5.3. shows the HPLC chromatogram obtained following analysis of a methanol extract of a plant which had been exposed to microcystin-LR. Although this particular plant was calculated to have taken up 600 μl of toxin solution (equivalent to 18 μg of toxin), no microcystin-LR is detected. From the chromatogram, it is clear that the detection of toxin is impeded due to the co-elution of a number of other compounds between 15 and 20 minutes. Interferences from co-eluted compounds also prevented identification of microcystin-LR by spectral analysis. Peaks eluting at the retention time of microcystin-LR did not reveal any similarities to the UV spectra of pure toxin. However, chromatographic data did reveal an absence of compounds eluting between 30 and 45 minutes, implying that the recovery of a more hydrophobic toxin, such as microcystin-LF, would be more easily detected. It was also postulated that recovery could be further improved by increasing toxin concentration and exposure time.

5.3.4. Determination of the recovery of microcystin-LF from plant material.

Following overnight exposure to 100 μg ml^{-1} microcystin-LF, 2-2.6 ml of toxin solution had been taken up by each of the plants. HPLC analysis revealed that a peak corresponding to microcystin-LF could be clearly identified in all plant extracts which had been exposed to the toxin. Figure 5.4. shows the chromatograms obtained following HPLC analysis of methanolic extracts of carnations which had been left in water; or water plus 100 μg ml^{-1} microcystin-LF overnight. A peak corresponding to microcystin-LF was detected at 36 minutes

(Figure 5.4.(b)). HPLC analysis of methanolic extracts of carnations which had been exposed to microcystin-LF also revealed the presence of seven additional peaks which were not identified in the extracts of flowers which had been exposed to water only (Figure 5.4.). Comparison of spectral data revealed that these peaks were also identified in toxin-containing flowers which had been extracted in methanol + TFA (Figure 5.5.(b)), and butanol:methanol:water (Figure 5.6.(b)). HPLC analysis of non-toxic butanol:methanol:water extracts showed four of these peaks, but in much smaller amounts than in extracts of toxin-containing flowers (Figure 5.6.(a)). The retention times, peak areas, and maximum absorbance between 200 and 300 nm (λ_{max}) of the seven additional peaks identified in each extract are given in Table 5.1. The appearance of these peaks earlier in the chromatogram indicated that they represented compounds which were more polar than microcystin-LF, suggesting toxin metabolites.

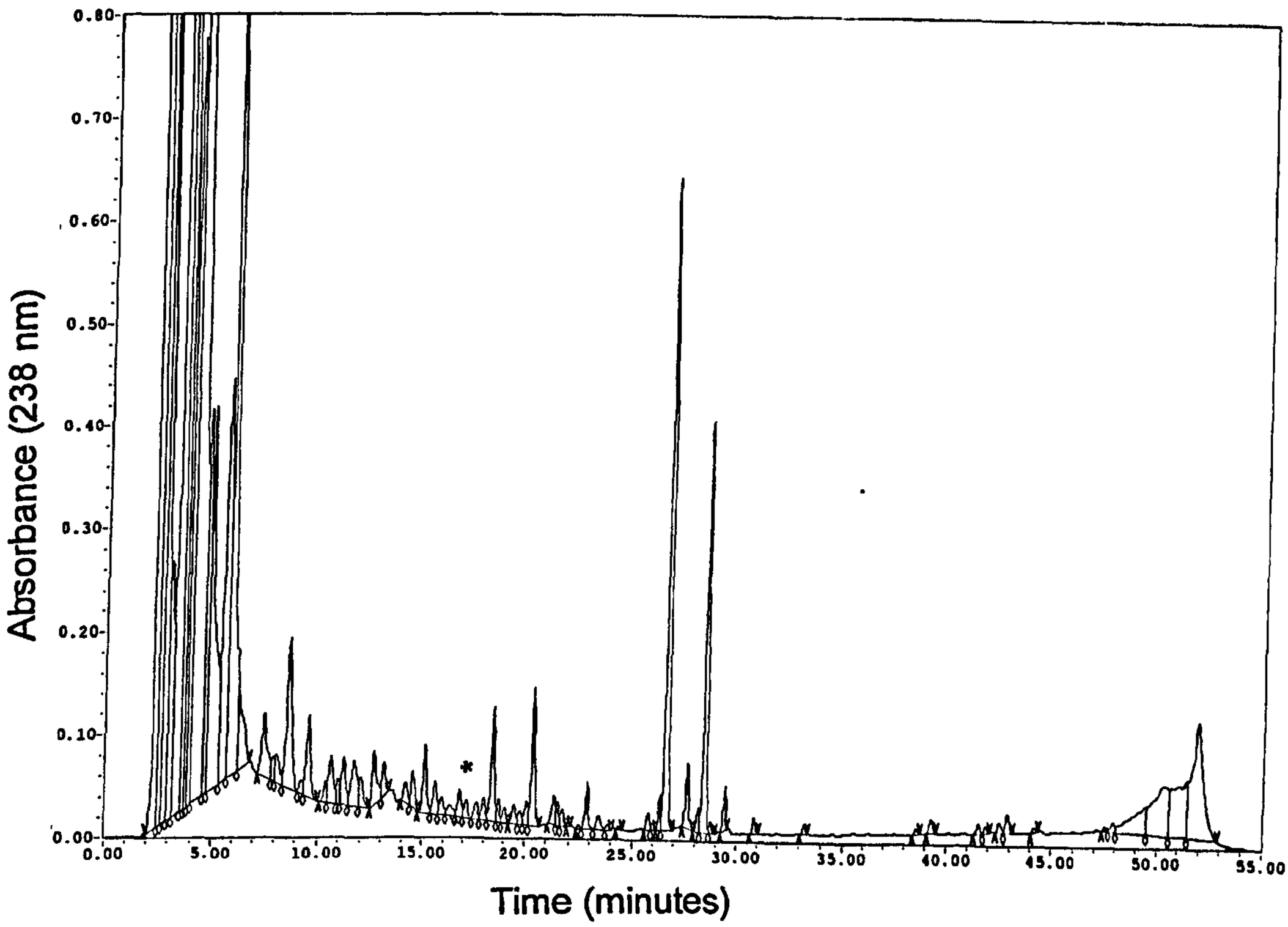
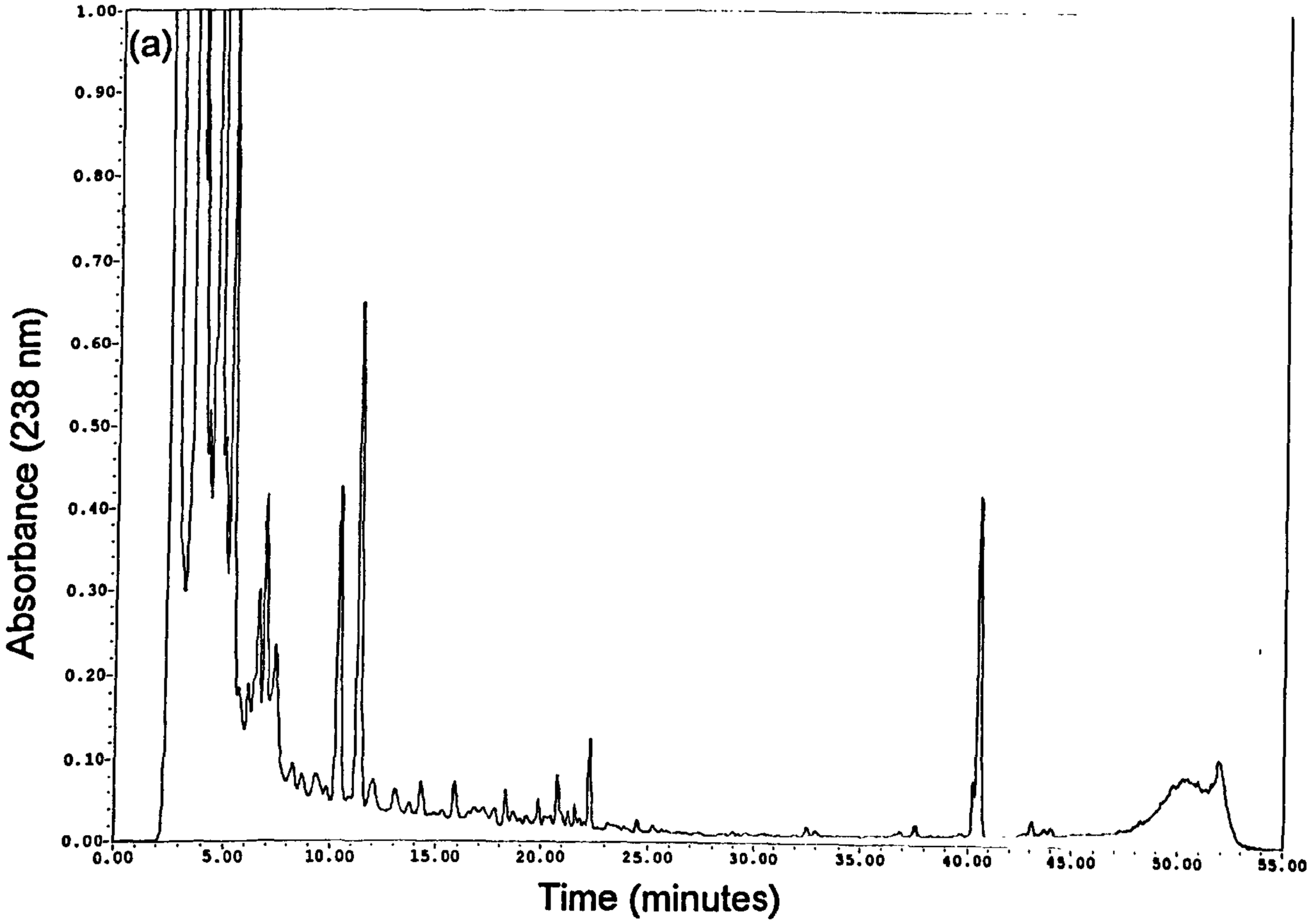


Figure 5.3. HPLC chromatogram obtained following analysis of a methanolic extract of a cut carnation which had been left in a solution containing 30 $\mu g ml^{-1}$ microcystin-LR for 3 hours. The expected retention time for the elution of microcystin-LR is labelled *.



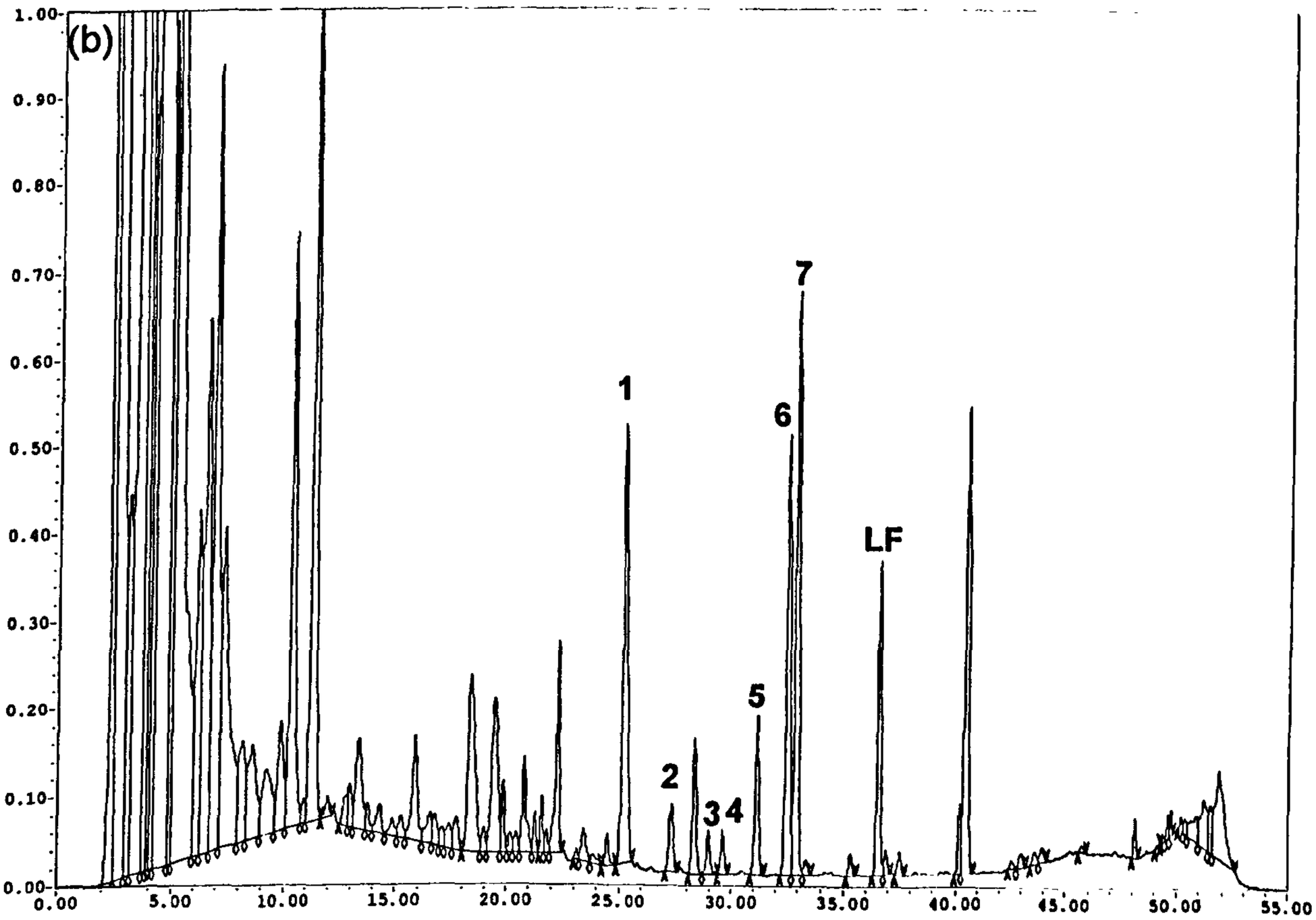


Figure 5.4. HPLC chromatogram obtained following analysis of a methanolic extract of a plant which had been left overnight in (a) water; and (b) 100 $\mu g ml^{-1}$ microcystin-LF. Additional peaks described in 5.3.4. are labelled 1-7.

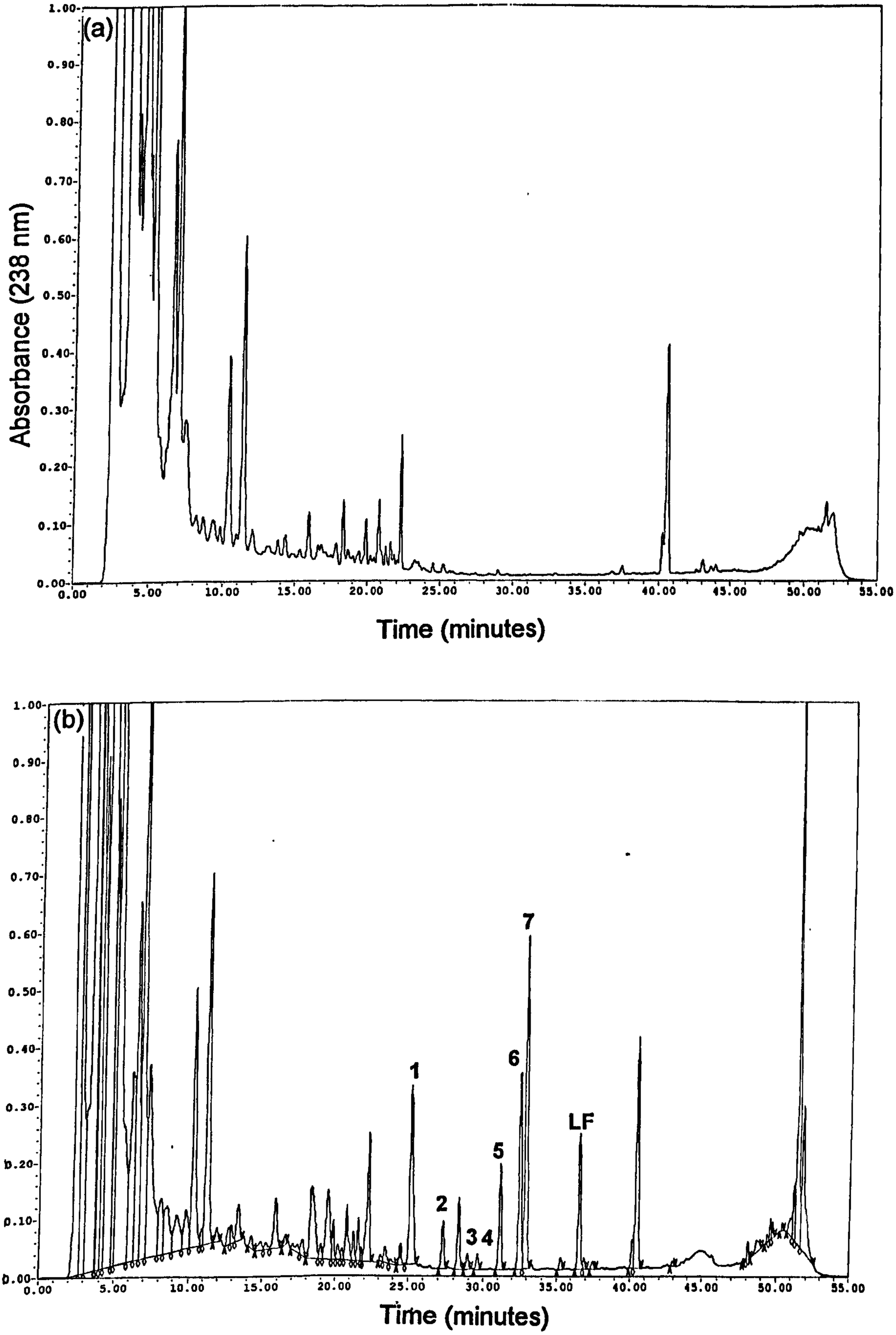


Figure 5.5. HPLC chromatogram obtained following analysis of a methanol +TFA extract of a plant which had been left overnight in (a) water; and (b) 100 $\mu g ml^{-1}$ microcystin-LF. Additional peaks described in 5.3.4. are labelled 1-7.

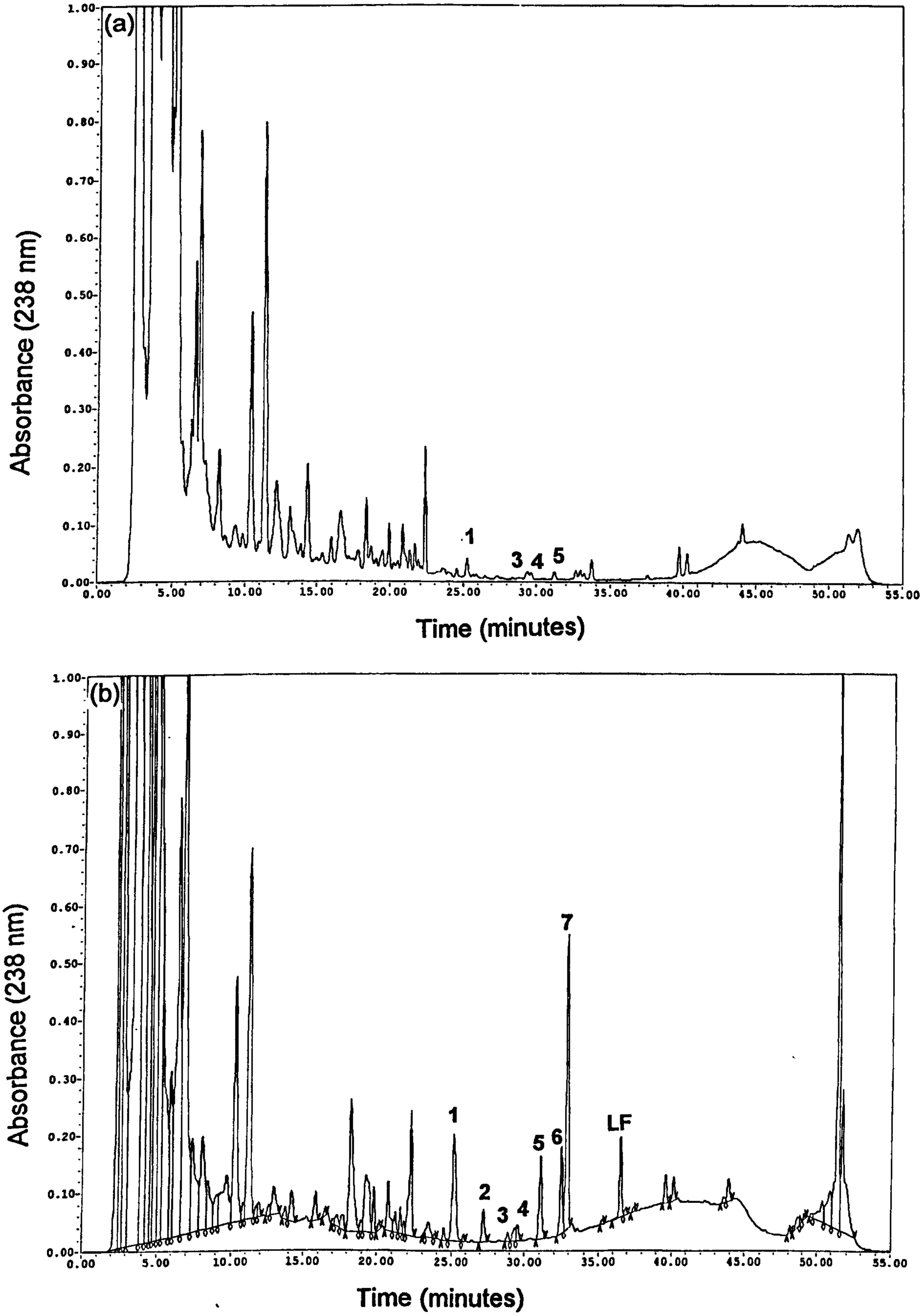


Figure 5.6. HPLC chromatogram obtained following analysis of a butanol:methanol:water extract of a plant which had been left overnight in (a) water; and (b) 100 $\mu g ml^{-1}$ microcystin-LF. Additional peaks described in 5.3.4. are labelled 1-7.

Table 5.1. Chromatographic data of additional peaks identified following HPLC analysis of extracts of flowers exposed to 100 μg ml^{-1} microcystin-LF overnight.

Peak	Retention time (minutes)	λ_{max} (200-300 nm)	Appearance in controls[a]
1	25.2	260.3	Butanol:methanol:water
2	27.3	252.1	n.d.
3	29.0	249.7	Butanol:methanol:water
4	29.6	252.1	Butanol:methanol:water
5	31.2	236.8	Butanol:methanol:water
6	32.5	254.4	n.d.
7	32.9	254.4	n.d.

n.d. - not detected

a - extraction media used in controls in which additional peaks were detected

The amount of microcystin-LF taken up by each plant was predicted by subtracting the volume of toxin solution remaining at the end of the experiment from the volume to which each plant was originally exposed. The amount of microcystin-LF recovered in each extract was then determined by HPLC analysis, and expressed as a percentage of the predicted amount. The recoveries of microcystin-LF from plant material using the three extraction methods studied are shown in Figure 5.7. The most successful method for extracting microcystin-LF was methanol, which extracted 37% of the toxin taken up. Methanol +TFA recovered 25%, while butanol:methanol:water (5:20:75) extracted only 17%.

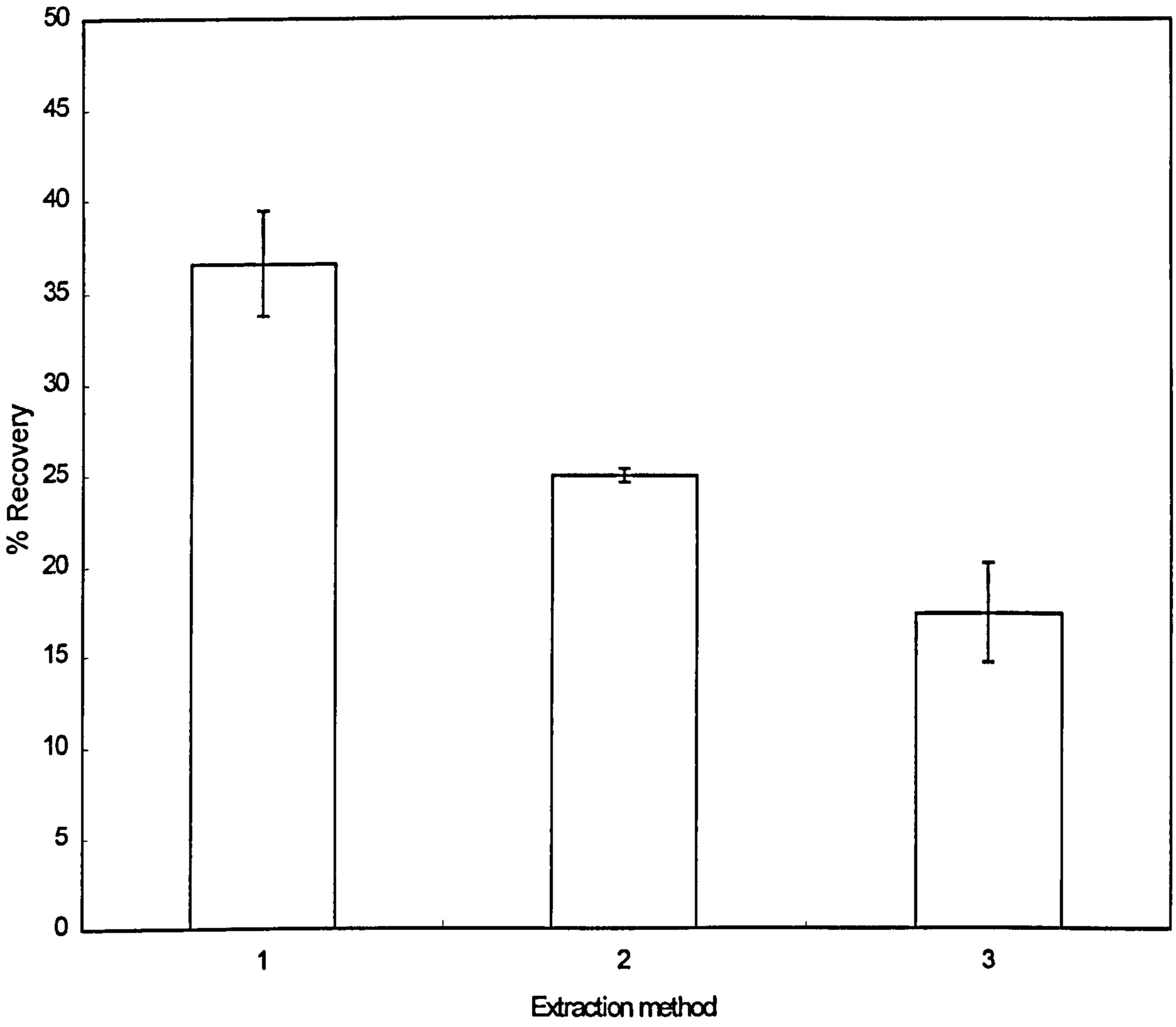


Figure 5.7. Comparison of extraction methods for recovering microcystin-LF from the tissues of carnations which had been exposed to 100 μg ml^{-1} of the toxin overnight. Recoveries were quantified by HPLC analysis of extracts and the toxin solutions at the beginning and the end of the experiment. Extraction media: 1 - Methanol, 2 - Methanol +0.01% TFA, 3 - Butan-1-ol:methanol:water (5:20:75). Data plotted are the mean of 2 replicates, and bars represent sample standard deviation.

5.3.5. Synthesis of glutathione and cysteine conjugates of microcystin-LF.

HPLC analysis of the glutathione conjugate of microcystin-LF revealed a peak at 26.9 minutes (Figure 5.8.(a)). The cysteine conjugate of microcystin-LF eluted at 27.7 minutes (Figure 5.8.(b)). The synthesised conjugates were not found to co-elute with any of the metabolites observed in toxin containing plant extracts. Spectral analysis revealed no similarities between the conjugates and the additional peaks identified in extracts of plants exposed to 100 $\mu g\ ml^{-1}$ microcystin-LF. Furthermore, the UV spectra (200-300 nm) of the two conjugates were identical to that of pure microcystin-LF (λ_{max} = 239.1 nm). These results indicated that the compounds described in 5.3.4. did not correspond to products of glutathione conjugation.

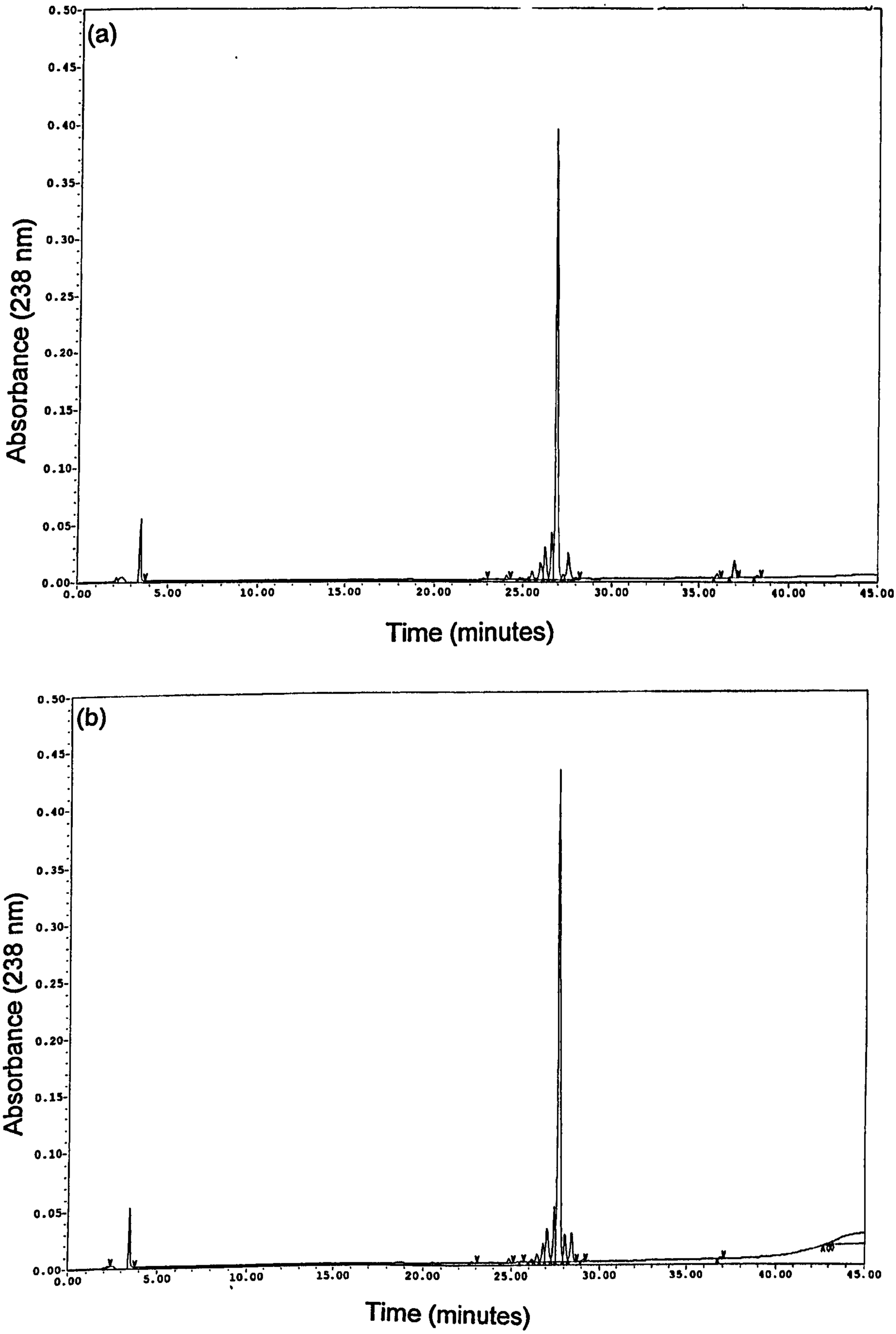


Figure 5.8. HPLC chromatogram obtained following analysis of (a) the glutathione, and (b) the cysteine conjugate of microcystin-LF.

5.3.6. Recovery of microcystin-LF from runner beans (*Phaseolus vulgaris L.*).

After 7 days, runner beans grown in the presence of microcystin-LF had taken up an average of 32.5 ml (± 19.7 ml) of medium, and had grown between 13 and 29 cm. Beans grown in medium lacking toxin had taken up 47.6 ml (± 8.5 ml) of medium and had grown between 19 and 32 cm. It was noted that the leaves of plants grown in the presence of microcystin-LF appeared slightly desiccated, and the roots were discoloured.

HPLC analysis of methanolic extracts of runner beans which had been exposed to microcystin-LF revealed that 4-10 μg of toxin was detected in extracts, but the co-elution of a number of other compounds with similar retention times hindered accurate determinations (Figure 5.9.). These findings suggested that there was no advantage to using microcystin-LF in future plant experiments.

5.3.7. Investigations into the uptake by and effects of microcystin-LR on runner beans.

The effect of microcystin-LR on the stem lengths of runner beans after 3, 6, 12, and 18 days is shown in Figure 5.10. The graph shows that although the growth of plants appeared to be inhibited in the presence of microcystin-LR after 3 days, stem length was not significantly affected after 6, 12, and 18 days exposure to the toxin.

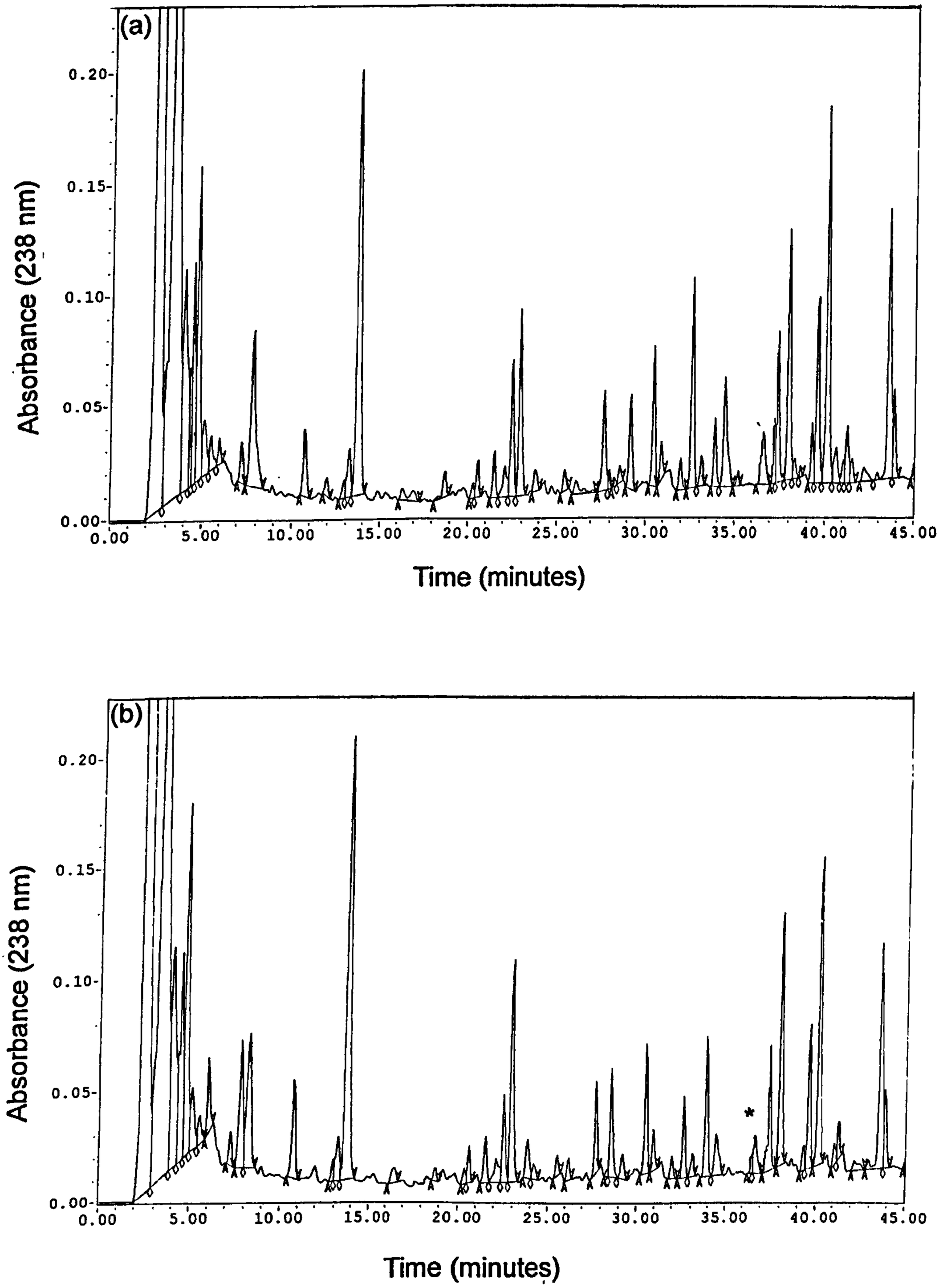


Figure 5.9. HPLC chromatograms obtained following analysis of a methanolic extract of a runner bean plant which had been grown in (a) control medium, and (b) medium containing 1μgml^{-1} microcystin-LF for 7 days. The expected retention time for the elution of microcystin-LF is labelled *.

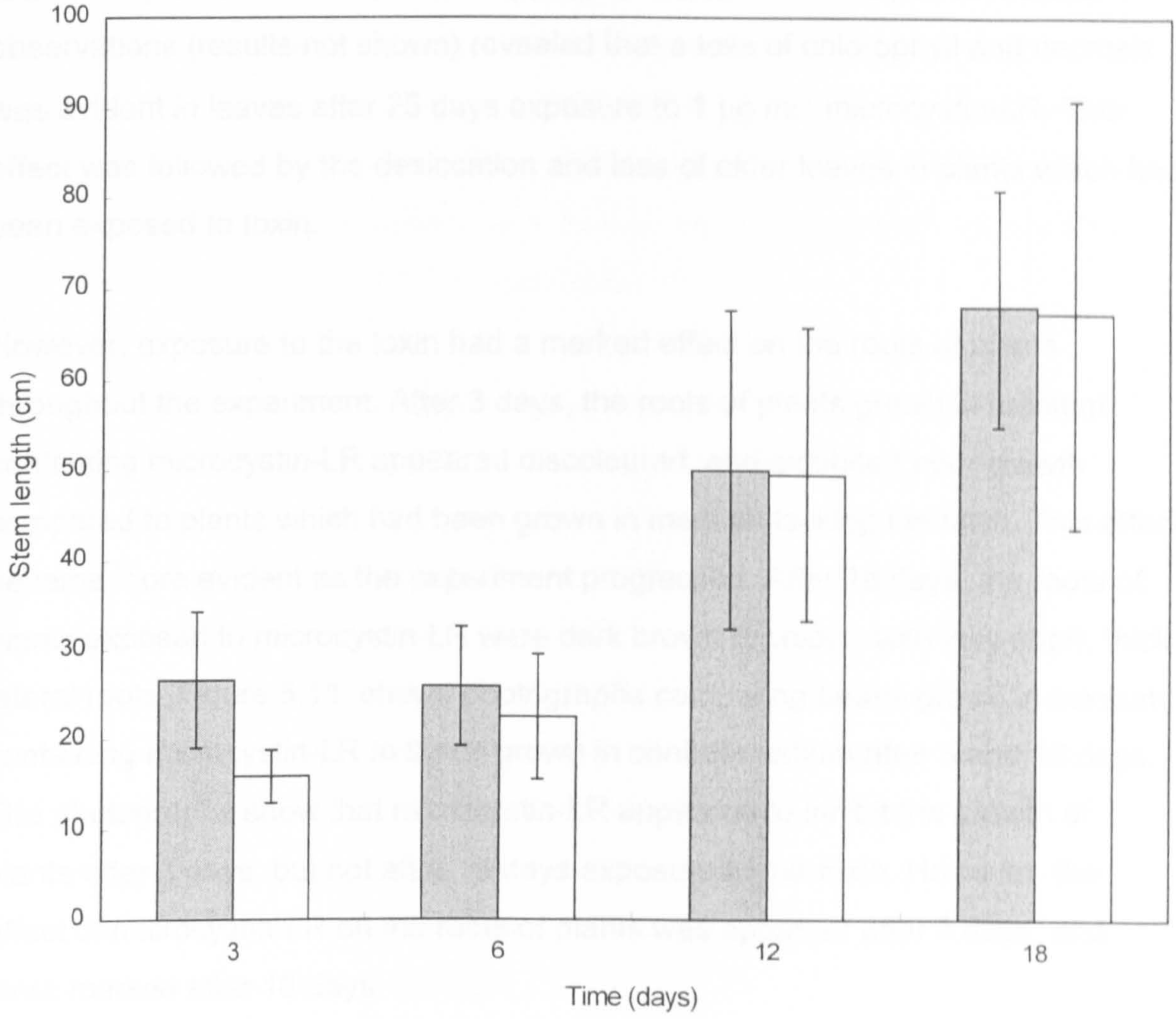


Figure 5.10. The growth of runner bean plants in Hoaglands 1/4 strength medium (shaded bars), and Hoaglands 1/4 strength medium containing 1.12 μg ml^{-1} microcystin-LR (white bars) after 3, 6, 12, and 18 days. Data plotted are the mean of 5 replicates and bars represent sample standard deviation.

Microcystin-LR did not appear to have a significant effect on the development of leaves in plants grown in medium containing the toxin, even after 18 days. Chlorotic and necrotic tissue was apparent on the stem and leaf tissues of only a few of the plants which had been exposed to the toxin for this period. Further observations (results not shown) revealed that a loss of chlorophyll and necrosis was evident in leaves after 25 days exposure to 1 $\mu g\ ml^{-1}$ microcystin-LR. This effect was followed by the desiccation and loss of older leaves in plants which had been exposed to toxin.

However, exposure to the toxin had a marked effect on the roots of plants throughout the experiment. After 3 days, the roots of plants grown in medium containing microcystin-LR appeared discoloured, and exhibited poor growth compared to plants which had been grown in medium lacking the toxin. This effect became more evident as the experiment progressed. After 18 days, the roots of plants exposed to microcystin-LR were dark brown in colour, with very short, thick lateral roots. Figure 5.11. shows photographs comparing beans grown in medium containing microcystin-LR to those grown in control medium after 3 and 18 days. The photographs show that microcystin-LR appeared to inhibit the growth of plants after 3 days, but not after 18 days exposure to the toxin. However, the effect of microcystin-LR on the roots of plants was apparent after 3 days, and more marked after 18 days.

Sections prepared from the root tips of control and exposed to microcystin-LR after 18 days were stained using toluidine blue and examined microscopically. Figure 5.12. shows longitudinal sections of control and exposed root tips. The photographs clearly show swelling in the root tip exposed to microcystin-LR. This appears to be have been due to effects on the morphology of root cells. In particular, cortical cells appear shorter and more rounded in exposed plant roots. This effect seems to be more pronounced further up the root tip in the zone of cell elongation. In this area, cells also appear to be pulled further apart from one another than cells in the same area of the control root tip. The cells also appear to be empty compared with cells in the control root tip.

The effects on root growth and morphology caused by exposure to microcystin-LR appeared to have an impact on the uptake of medium by runner bean plants over 18 days. Figure 5.13. shows the volume of medium which was taken up by plants grown in medium containing toxin compared with those grown in control medium over 18 days. The graph indicates that plants grown in the presence of microcystin-LR took up approximately 30% less medium after 18 days than those grown in control medium. Comparison of toxin concentration in medium at the start of the experiment and after 3 and 18 days by HPLC analysis revealed that toxin concentration remained constant throughout.

HPLC analysis detected microcystin-LR in a number of the extracts of exposed runner beans. Examination of the chromatograms of replicate plant extracts revealed no additional peaks in extracts prepared from plants which had been exposed to the toxin compared with extracts of control plants (Figure 5.14.). The amounts of toxin recovered from plants which had been exposed to the toxin could not be accurately calculated using HPLC, due to the co-elution of other components in the extracts (Figure 5.14.(b)). The calculation of toxin recoveries using peak area probably resulted in an over estimation of microcystin-LR in many of the extracts due to the co-elution of other impurities. The presence of these compounds also interfered with the spectral characteristics of the toxin peak, thus preventing its accurate identification.

5.3.8. Assessment of toxicity in runner bean extracts by brine shrimp (*Artemia salina*) bioassay.

Purified microcystin-LR was found to have an LD_{50} value of 6.35 $\mu g ml^{-1}$ using the concentration range described. However, the determination of the toxicities of extracts of runner beans which had been exposed to microcystin-LR was not possible, as extracts of control plants were also toxic to brine shrimp. This suggested that the effects of microcystin-LR were masked by additional toxic compounds extracted from the plants.

Figure 5.11. Photographs comparing runner beans grown in Hoaglands 1/4 strength medium only, and medium containing 1.12 $\mu g ml^{-1}$ microcystin-LR after 3 days and 18 days.

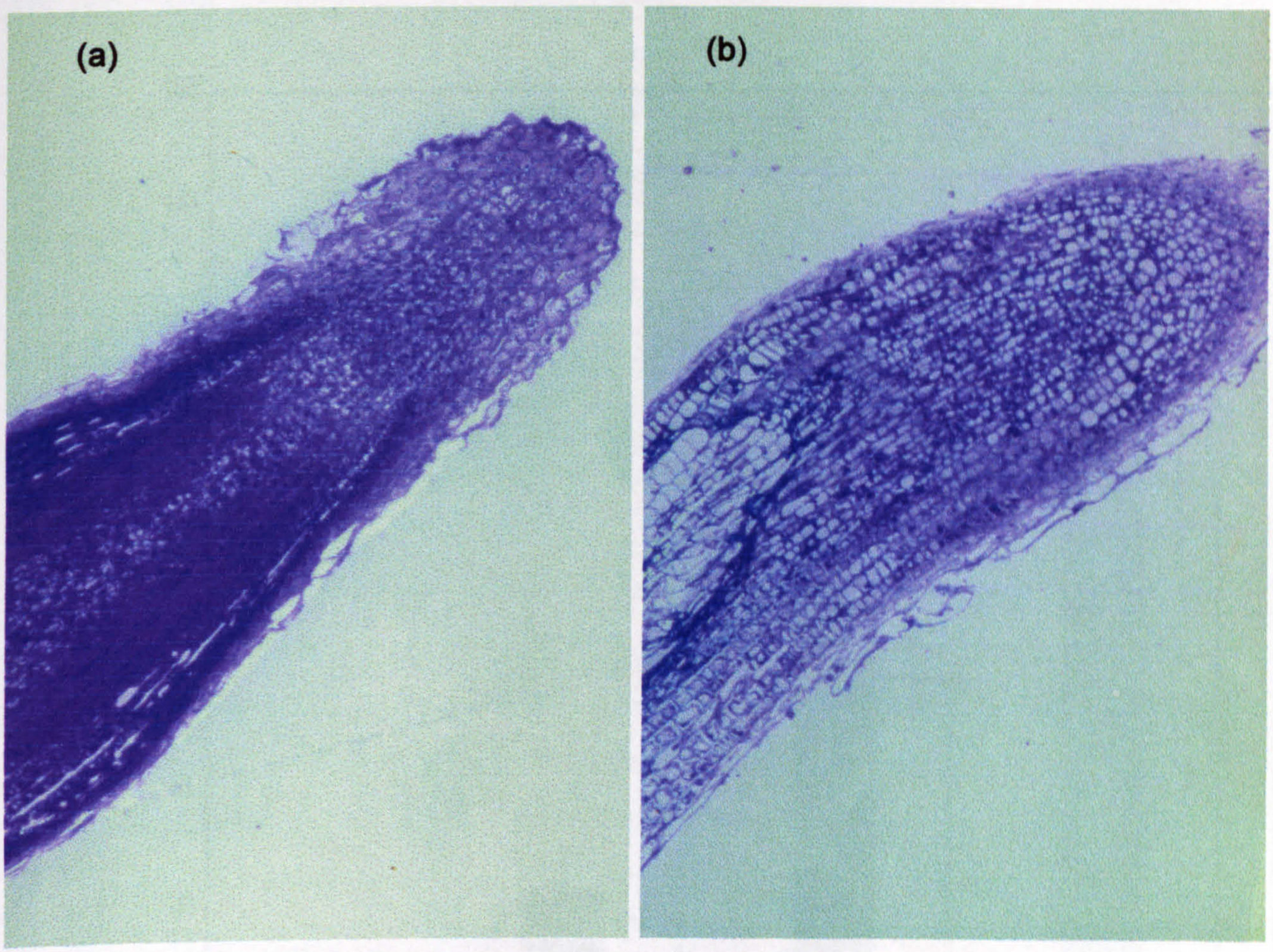


Figure 5.12. Microscopic examination (x 320) of longitudinal root tip sections prepared from (a) Untreated plants, and (b) Plants exposed to 1 $\mu g ml^{-1}$ microcystin-LR for 18 days.

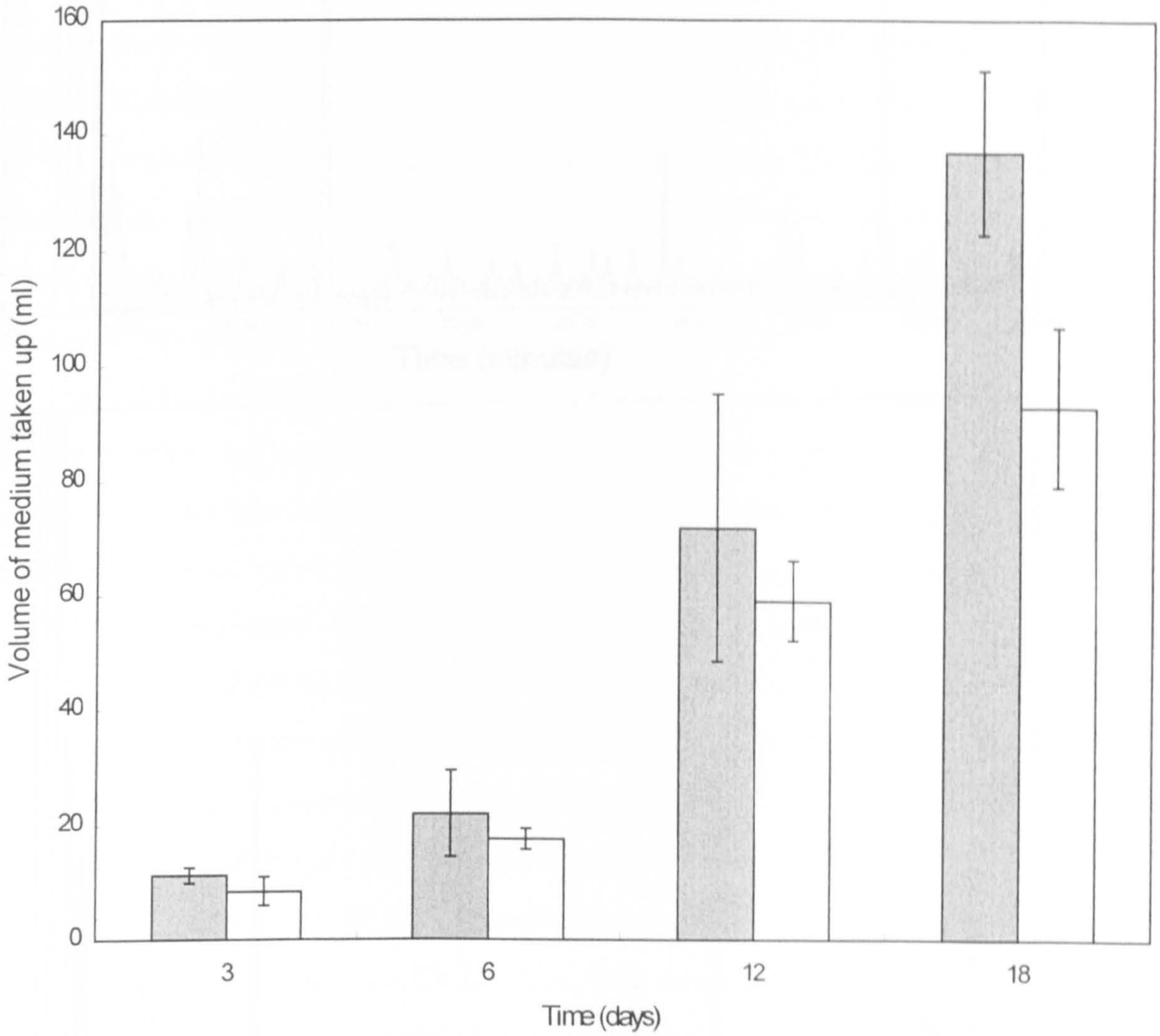


Figure 5.13. The volume of medium taken up by runner bean plants grown in Hoaglands 1/4 strength medium (shaded bars), and Hoaglands 1/4 strength medium containing 1.12 μg ml^{-1} microcystin-LR (white bars) after 3, 6, 12, and 18 days. Data plotted are the mean of 5 replicates and bars represent sample standard deviation.

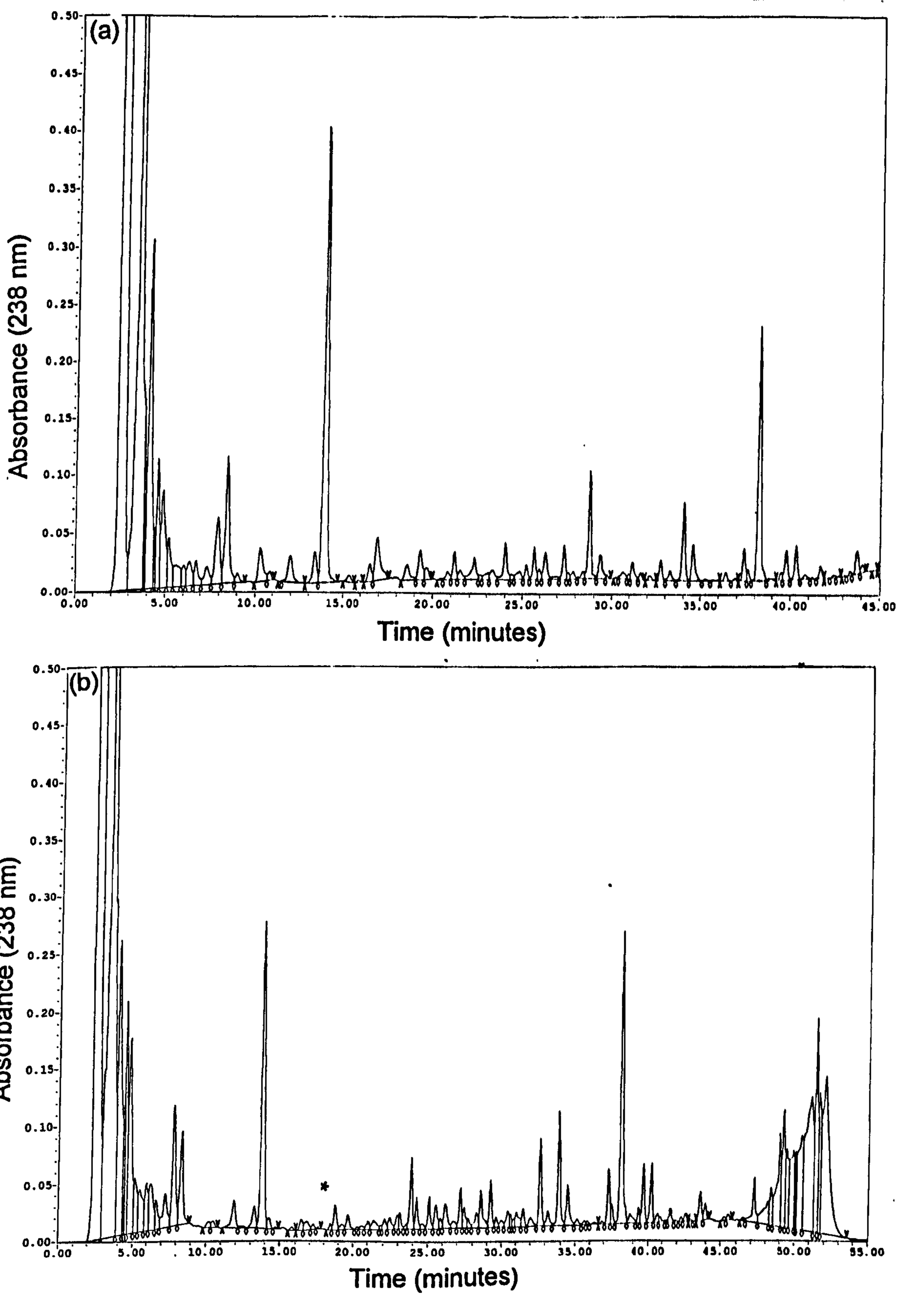


Figure 5.14. HPLC chromatograms obtained following analysis of a methanolic extract of a runner bean plant which had been grown in (a) control medium, and (b) medium containing microcystin-LR for 6 days. Chromatogram (a) shows a cluster of peaks between 15 and 20 minutes, preventing accurate quantification of microcystin-LR in (b). The expected retention time for the elution of microcystin-LR is labelled *.

5.4. Discussion.

The main aim of this study was to determine the fate of microcystin in plants exposed to the toxin, and to assess whether the toxin could be recovered from plant tissues following exposure. However, the method developed for extracting microcystins from plants had only limited success. Method development was carried out using cut plants, which provided a simple model system for assessing the recovery of toxin from plant tissue. Although the plants were not intact (i.e. lacked roots), they were capable of taking toxins up through the stem and into other tissues. This system allowed each plant to become saturated with an aqueous toxin solution in a relatively short time. It enabled the quantity of toxin taken up by each plant to be determined relatively accurately so that the most efficient method for extracting free toxin back out of the tissues could be elucidated. The three extraction methods investigated in this study were selected as they have been used previously to recover microcystins from cyanobacterial cells (Brooks and Codd, 1986; Lawton, Edwards, and Codd, 1994). However, due to the co-elution of other compounds extracted from the plant material, HPLC was unable to detect microcystin-LR in any of the extracts analysed. This problem was overcome in initial investigations using microcystin-LF, which was detected in extracts prepared from cut plants exposed to high concentrations ($100\ \mu g\ ml^{-1}$) of the toxin. This study indicated that methanol was the most efficient solvent for extracting microcystin-LF from plant material, but it resulted in the recovery of only 37% of the amount of toxin taken up by the plant. There may be a number of reasons why the total amount of toxin was not fully extracted using this method. Homogenising the fresh plant tissue in methanol may not have broken down the plant cell walls sufficiently to release all of the toxin present. However, it has been observed that toxin recoveries are not significantly enhanced in exposed plants which have been freeze-thawed prior to extraction (results not shown). This would have been expected to disrupt the plant cell walls, allowing more of the free toxin to be extracted. Alternatively, toxin may have been lost while transferring homogenised plant material from the food processor to a glass beaker for extraction. The risk of losing toxin during sample processing may have

been minimised by eliminating the requirement for the homogenisation step. In future experiments, plants could be cut into small pieces and dried to provide a sample which is easier to handle. This may also be accomplished by freezing the plants in liquid nitrogen prior to extraction. By carrying out this step, the plant material could be homogenised more easily using a mortar and pestle.

The relatively poor recovery of microcystins in plant extracts may also have been due to the transformation of the toxins to metabolites. In living cells, toxic compounds can be conjugated with glutathione by enzymes called glutathione transferases, which are widespread in both animals and plants (Ketterer, Coles, and Mayer, 1983). Glutathione conjugation has been identified as a major pathway for the metabolism of microcystins in animals (Runnegar *et al.*, 1987). Conjugates of microcystin -LR, -YR, and -RR with glutathione and cysteine have been chemically (non-enzymatically) synthesised (Kondo *et al.*, 1992 b). Analysis of these compounds by reversed phase HPLC revealed that they chromatographed earlier than the pure toxins, indicating their more polar nature. The compounds were also considerably less toxic than the pure toxins by mouse bioassay, suggesting that glutathione conjugation may play an important role in detoxification. In further studies, glutathione and cysteine conjugates of microcystin-LR and -RR were identified in the livers of mice and rats following injection of the toxins. The formation of these metabolites in liver cystosol was accompanied by a dose and exposure-time dependent depletion in glutathione pools (Kondo *et al.*, 1996).

A recent investigation into the effects of microcystin-LR on aquatic organisms suggested that a similar detoxification mechanism occurs in plants. Pflugmacher *et al.* (1998 b) demonstrated the *in vitro* conjugation of microcystin-LR with glutathione by glutathione transferases purified from the aquatic plant *Ceratophyllum demersum*. The authors concluded that conjugation with glutathione represented the first stage in the metabolism of microcystins in both animals and plants. In the present study, HPLC analysis of extracted plants which had been exposed to 100 $\mu g\ ml^{-1}$ of microcystin-LF detected a number of more

polar compounds which were not detected in extracts of control plants. Glutathione and cysteine conjugates of microcystin-LF were successfully synthesised using the method described by Kondo *et al.* (1992 b), and used to determine whether these compounds had been produced as a result of glutathione detoxification. HPLC analysis failed to identify any of these peaks as products of conjugation between microcystin-LF and either glutathione or cysteine. The retention time of peak 2 (27.3 minutes, Table 5.1.) was close to those of the glutathione and cysteine conjugates of microcystin-LF (26.9 minutes, and 27.7 minutes respectively). However, comparison of spectral data revealed that peak 2 was a different compound. Nevertheless, the relatively high concentrations of these more polar compounds detected in extracts of toxin-containing plant tissue do suggest that they represent products of toxin metabolism. Further characterisation of these compounds could be carried out using a more advanced analytical technique such as liquid chromatography/mass spectrometry (LC/MS).

The poor recovery of microcystin from plant extracts may also be accounted for by the binding of the toxin to protein phosphatases 1 and 2A, which are important regulatory enzymes in all eukaryotic cells (Cohen, 1989). One of the characteristics of microcystin toxicity is the covalent binding between the methyl dehydroalanine residue of the toxin and the protein phosphatase molecule (MacKintosh *et al.*, 1995). Plant protein phosphatases have been shown to be inhibited by microcystins to the same extent as animal forms of the enzymes (MacKintosh *et al.*, 1990). It is therefore likely that a significant amount of the microcystin taken up by cut *Dianthus sp.* in the present study formed an irreversible association with the protein phosphatases present in the tissues of the plant, therefore preventing its detection.

Investigations into the fate of microcystins in runner beans following uptake through the roots were also inconclusive. Determinations of toxin recoveries in methanolic extracts of *Phaseolus* tissues by HPLC and brine shrimp assay were unsuccessful, and it is therefore unclear whether the toxin had accumulated in these plants following uptake through the roots. The toxicities of extracts prepared

from plants which had been exposed to microcystin-LR could not be attributed to the toxin by brine shrimp assay, as the organisms were also killed by extracts prepared from non-exposed plants. The detection of microcystin-LR in these extracts may be facilitated in future studies using a more specific immunoassay technique, such as the competitive ELISA method devised by Ueno *et al.* (1996).

Determinations of the recoveries of both microcystin-LF and microcystin-LR from these extracts by HPLC were also impeded due to the co-elution of impurities. A clean up step prior to HPLC analysis would be required to eliminate the impurities responsible for masking the detection of microcystins, and allow toxin recoveries to be determined using this method. Sample clean up is commonly carried out using solid phase extraction, and octadecyl silanised (ODS) silica gel has been extensively used for this purpose. In this study, partial clean up of plant extracts was achieved by applying samples to Isolute C_{18} cartridges and eluting fractions in 0-100% (v/v) aqueous methanol. However, this procedure failed to separate microcystins from the compounds which had prevented their detection by HPLC due to similarities in retention (results not shown).

An alternative clean up stage to allow the detection of microcystins in complex plant extracts may be facilitated using antibodies. Kondo *et al.* (1996), employed an immunoaffinity purification method to enable HPLC analysis of microcystins-LR and -RR in mouse and rat livers. They found that conventional cleanup methods employing ODS silica gel and silica gel cartridges were insufficient for removing co-eluting impurities present in liver cytosol. The problem was successfully overcome using an immunoaffinity column prepared using an anti-microcystin-LR monoclonal antibody. The column selectively recognised microcystins-LR and -RR in cytosolic extracts, allowing contaminants to be eliminated from the samples.

In future studies, the requirement for sample clean up may be eliminated by exposing plants to radiolabelled microcystin. Measuring the radioactivity of plant extracts would allow microcystin uptake to be quantified more accurately. The detection of radioactivity in other compounds detected in exposed plant extracts

would also allow them to be confirmed as toxin metabolites. The uptake of radiolabelled microcystins has previously been studied in several smaller plant systems. Pflugmacher *et al.* (1998 a), used ^{14}C-microcystin-LR to study the uptake of the toxin by three aquatic macrophytes. Following 7 days exposure to 10 μg ml^{-1} radiolabelled microcystin, up to 1.75% of the applied radioactivity was recovered. By dividing the plants into root, leaf, and shoot tissue, the authors also found that the majority of the applied ^{14}C label was recovered from the leaves and shoots of the plants, whereas very little was recovered from the roots. The more effective uptake observed in the leaves and shoots of aquatic plants was believed to be due to the increased metabolic rate of these tissues compared with roots. However, in aquatic plants, these tissues are in constant contact with the toxins while the leaves and shoots of terrestrial plants will be exposed only intermittently if they are irrigated using spray systems. The main route of uptake for terrestrial plants is more likely to occur through the roots. There is a lack of information on the uptake of microcystins by plant roots. A study conducted by Kurki-Helasmo and Meriluoto (1998) demonstrated the uptake of radiolabelled microcystin-RR by mustard (*S. alba*) seedlings into leaf and shoot tissue, but did not ascertain the presence of toxin in the roots. However in the present study, the effects of toxin exposure appeared to be localised in the roots. For up to 18 days, exposure to microcystin-LR did not appear to have an adverse effect on the growth of plants, or on the pathology of leaf and shoot tissues. It is therefore unclear whether a significant amount of toxin was taken up into these tissues. This could be elucidated in future studies by exposing plants to radiolabelled toxin and separating the roots from stem and leaf tissue. The recovery of radiolabel could then be determined by extracting the tissues separately.

Exposure to microcystin-LR had a profound effect on the growth of *Phaseolus* roots. After 3 days exposure to the toxin, roots had developed a dark brown colour indicative of necrosis, and exhibited poor growth compared with control plants. These effects became more pronounced over the time period assessed (Figure 5.11.). The observed inhibitory effects of microcystin-LR on root growth indicate that exposure to the toxin may have a serious effect on plant stability. Roots are

important in anchoring the plant firmly in the soil and prevent it from being dislodged in adverse weather conditions. The inhibitory effects of microcystin-LR on the growth of *Phaseolus* roots were also accompanied by a reduction in the volume of medium taken up by plants. In the environment, reduced uptake of water and nutrients may also have a deleterious effect on plant survival, particularly during drought conditions.

Microscopic examination of the root tips of plants after 18 days exposure revealed that microcystin-LR had caused cortical cells to swell considerably (Figure 5.12.). The observed morphological changes were likely to be due to the inhibition of protein phosphatases 1 and 2A involved in the development of *Phaseolus* roots. These findings corresponded to those of Smith *et al.* (1994), who described similar changes in the cortical cell shape of *Arabidopsis* roots following exposure to the structurally unrelated protein phosphatase inhibitors okadaic acid and calyculin-A. Protein phosphatases 1 and 2A have been shown to play a vital role in cell development and particularly in the control of microtubule reorganisation during mitosis. The organisation of microtubules during mitotic growth events controls the direction of cell expansion by determining the direction of cellulose deposition in the cell wall (Shibaoka, 1991). In plant roots, the depolymerisation of microtubules following exposure to the chemicals colchicine or oryzalin inhibits cortical cell elongation and causes radial swelling (Morejohn, 1991). These effects have also been observed in both animal and plant cell cultures treated with the protein phosphatase inhibitor okadaic acid (Vandré and Wills, 1992; Hasezawa and Nagata, 1992).

In the present study, the pronounced rounded shape of cortical cells in *Phaseolus* roots exposed to microcystin-LR also indicated that cell contact had been disrupted. The cells also took up considerably less stain than those of control roots and appeared empty, suggesting that exposure to the toxin adversely affected the structural integrity of the cell wall. Although the mechanism for this effect is unclear at present, it may be due to a disturbance in the vesicular

transport of components required for cell wall expansion, which is believed to be mediated by protein phosphatase 2A (Davidson, McGowan, and Balch, 1992).

The results of this study have provided more detailed evidence of the damage caused to plants exposed to microcystins in the growth medium, and the possible ffects of this damage on the productivity of terrestrial plants in the environment. Although the main objectives of this investigation remain unresolved, the findings highlight the requirement for further research into the uptake of microcystins by plants. The extraction method developed to recover microcystins from plant material failed to establish their accumulation in plants exposed to environmentally relevent concentrations of the toxin. However, HPLC analysis did indicate the presence of the toxin in a number of extracts, and this could be confirmed in future studies using the clean up methods described. Determining the toxicities of these extracts using alternative bioassay techniques would be particularly important in assessing the possible human health hazards presented by microcystins in plant tissues.

Chapter 6

Conclusions

Growing awareness of the threat of cyanobacterial toxins to human health has increased the requirement for research into their detection and biological effects. The prevention of human intoxication is largely dependent on the monitoring of cyanobacterial toxins in water or foodstuffs. This was highlighted recently by the tragic incident in a Brazilian dialysis clinic, where the fatalities of at least 47 patients were attributed to the inadequate monitoring of the clinic's water supply for microcystins (Dunn, 1996, Jochimsen *et al.*, 1998).

A variety of methods are available for the detection and identification of cyanobacterial toxins in different sample types. The choice of method used can depend on the information required, and the facilities which are available. In the first instance, bioassay techniques may be employed to provide a rapid evaluation of the overall toxicity of a sample, and the potential hazards presented to human health. More detailed information concerning the different types and concentrations of toxins present may then be obtained using more sophisticated analytical techniques. Increased recognition of the incidence and health significance of cyanobacterial toxins has generated a need for methods which are accessible, sensitive and inexpensive. However, there are a number of disadvantages associated with many of the biological and analytical techniques currently available which have prohibited their use in many laboratories. This study set out to address problems associated with the detection of two groups of cyanobacterial toxins; the saxitoxins and the microcystins.

The routine monitoring of saxitoxins in shellfish and freshwater cyanobacteria relies to a great extent on the mouse bioassay, which has been authorised as an official method by the Association of Official Analytical Chemists (AOAC), for the screening of acid shellfish extracts. Alternative methods for saxitoxin detection have been difficult to implement on a large scale for a number of reasons. Analytical techniques such as HPLC are sensitive, but are expensive, and (as experienced during this study), laborious to set up. Alternative biological methods such as the housefly bioassay, tissue culture bioassay, receptor assays and immunological techniques areoften not feasible due to the requirement for

specialised facilities and expertise. The contamination of edible shellfish with saxitoxins presents a significant economic problem in many countries, and it is therefore important that monitoring programmes may be carried out with a minimum of cost and skill. This study has demonstrated that simple, rapid screening of saxitoxins in both shellfish and freshwater cyanobacterial samples may be facilitated using the locust bioassay. The assay provided a means of confirming the toxicities of extracts of *Aph. flos-aquae* which had been found to contain saxitoxin and neosaxitoxin by HPLC, and enabled an extraction and screening protocol to be devised for saxitoxin detection in cyanobacterial samples. Implementation of the assay in a large shellfish monitoring programme also indicated that its ability to detect alert levels of saxitoxins in acid shellfish extracts was comparable to the official protocol involving the mouse bioassay.

In recent years, increasing public pressure on the use of mammals in toxicity testing has led to the prohibition of the mouse bioassay in certain countries. This has prompted the development of more humane methods in screening programmes of this nature. Analytical techniques would appear to be most suitable for this purpose, but are not feasible in large-scale monitoring in many countries for economic reasons. Bioassay methods are often more practical, as they provide a simpler and cheaper means of detecting toxicity. Furthermore, the information derived using sophisticated analytical techniques is not essential when only a rapid indication of toxicity is required. In many cases, bioassays are also required to confirm the biological activity of a sample following analysis. This was highlighted in the screening of natural cyanobacterial bloom samples carried out during the present study. The locust bioassay indicated the presence of neurotoxic compounds in natural bloom samples in which cyanobacterial neurotoxins were not detected by HPLC.

The simplicity and low cost of the locust bioassay makes it an ideal screening method for saxitoxins. Another advantage of the assay is that it can be carried out without specialised facilities and may therefore be employed on site for rapid testing. It is hoped that it will undergo further evaluation in other laboratories to assess its suitability as a replacement to the mouse bioassay for saxitoxin screening.

In freshwater environments, the most commonly encountered cyanobacterial toxins are the microcystins (Lawton, Edwards and Codd, 1994). Evidence for the acute and chronic health effects which can occur as a result of exposure to microcystin-LR has led to the derivation of a guideline level of 1 $\mu g\ l^{-1}$ for this toxin in drinking water (WHO, 1998). Sensitive and reliable screening methods for drinking water are therefore required to ensure that microcystin concentrations do not exceed this guideline. The most widely used technique for the detection and quantification of microcystins is HPLC combined with photodiode array UV detection. The main advantage of this technique is that it is both quantitative and qualitative. Natural water samples may contain a number of different microcystins, and certain variants may be more abundant than microcystin-LR. In order that the true toxicity of a sample may be determined, it is vital that different microcystin variants can be identified. Many of the UV detectors which are currently available can identify microcystins very accurately as they are capable of detecting very small differences in absorbance. When used in conjunction with spectral matching software, the technique can enable a spectral library to be constructed for a range of microcystin variants. Natural samples may then be screened against the spectral library to identify the microcystins which are present. However, over 60 microcystin variants have now been identified from both laboratory cultures and natural bloom samples, the majority of which are not commercially available as standards. The lack of purified microcystins has consequently led to an under-reporting of many variants in natural water samples. Methodology developed in this study enabled the purification of microcystin-LW and -LF, which have been identified in several naturally occurring cyanobacteria, and laboratory strains of *Microcystis aeruginosa* (Azevedo *et al.*, 1994; Lawton *et al.*, 1995). The technique

described provided a rapid three step flash chromatography extraction and purification which eliminated the requirement for preparative HPLC. Normal-phase flash chromatography allowed microcystin-LW and -LF to be purified without specialised expertise, and may have potential as a simple purification method for a range of microcystin variants. It has since been observed that this method is also capable of separating microcystin-LR from a mixture also containing microcystin-LW and -LF, and will be investigated in future studies for the separation of other microcystins.

The use of normal-phase flash chromatography resulted in the separation of 59% of microcystin-LW and -LF from the original sample load, and granted a rapid means of reprocessing fractions which contained mixtures of the two toxins, or individual toxin fractions containing contaminants. The developed three stage methodology provided microcystin-LW and -LF at sufficient purity (95%) for analytical applications such as the construction of a spectral library. The purification of these standards will therefore enhance their detection in natural samples. A further outcome of this work is the commercial availability of microcystin-LW and -LF, which will enable their reporting in other laboratories, and contribute to the understanding of their biological effects.

The purification of microcystin-LR and microcystin-LF from a laboratory culture of *M. aeruginosa* allowed the effects of these toxins to be studied in plants. To date, the phytotoxic effects of microcystins have not been adequately investigated. However, as approximately 85% of the world's water is used for the irrigation of crops (Cook, 1998), it is likely that many terrestrial plants are exposed to cyanobacterial toxins. The impact of these toxins on crop productivity may have serious economic implications. It is also possible that the accumulation of toxins in food plants may provide a further route for human intoxication. In this study, the levels of microcystin-LR required to affect plant growth were assessed using a potato shoot culture assay.

The assay indicated that environmentally relevant concentrations of the toxin (5-50 μg l^{-1}) inhibited the growth and chlorophyll content of cultures, suggesting that exposure to microcystins may have a detrimental effect on crop plants in the environment. In this experiment, cultures were exposed to toxin in the growth medium, which is significant to the growth of terrestrial crops irrigated using trickle systems, and to the hydroponic cultivation of certain plants. With increasing pressures on crop production, the contamination of irrigation water with microcystins may pose a serious threat to current agricultural practises. These findings have indicated that stricter monitoring of freshwater sources used for irrigation is required in order that this hazard may be quantified.

A previously developed mustard seedling bioassay was employed to compare the phytotoxic effects of three microcystin variants. The assay revealed that the more polar toxins (microcystin-LR and -RR) had a greater inhibitory effect on seedling development than the relatively hydrophobic microcystin-LF. Microcystin-RR was found to be particularly toxic to seedlings, causing significant growth inhibition at a concentration of 0.1 μg ml^{-1}. However, microcystin-LR, which has been found to be considerably more toxic to animals, did not significantly inhibit the growth of mustard seedlings below 0.5 μg ml^{-1}. These findings are interesting, as they indicate differences in the biological effects of these toxins in animals and plants. The contrasting effects of the three variants on mustard seedlings also suggest that plant bioassays could prove useful in determining the structural features of microcystins which are required to cause toxic effects. In future studies, the toxicities of a wider range of microcystin variants will be assessed in order that this may be evaluated.

The uptake of microcystins by plants was studied in greater detail using a larger plant model. Investigations into the effects of microcystin-LR exposure on *P. vulgaris* indicated that toxicity was localised in the roots for up to 18 days. The observed inhibitory effects of the toxin on the growth of exposed plant roots and the accompanying cellular effects further highlight the possible impact of microcystins on crop productivity. The concentration of microcystin-LR employed

in this study was considerably greater than that which is normally observed in the environment. However, similar levels have been identified in lake water treated with algicides to eradicate cyanobacterial blooms (Jones and Orr, 1994). It is also possible that repeated irrigation could lead to an accumulation of the toxins in soil, causing roots to be exposed to high concentrations. The observations made during this study correspond to anecdotal descriptions of root damage observed in plants following exposure to water containing cyanobacteria. However, controlled field studies will be required in order that the implications of these findings for plants in the environment may be fully understood.

Methodology developed to recover microcystins from exposed plant tissues failed to ascertain whether accumulation of the toxin had occurred in *P. vulgaris*. It is therefore unclear from this study whether the consumption of contaminated plant material could present a threat to human health. However, there is considerable scope for improving the described extraction procedure for future investigations. Drying plant material prior to extraction in methanol has since been found provide a sample which was much easier to handle than the fresh plant tissue used previously. This method has been successfully employed in a field investigation to detect microcystins in plants which had been irrigated with water containing cyanobacteria. The quantification of microcystins in plant tissues using HPLC may be possible in the future by employing sample clean-up methods to remove interfering contaminants. It is also hoped that more accurate determinations of accumulated microcystins in plant material may be facilitated in subsequent studies using specific detection methods such as ELISA.

In conclusion, this study has provided valuable information concerning the detection and biological effects of cyanobacterial toxins. It has been shown that these toxins may pose a hazard to human health in both drinking water and through the food chain. The findings presented have indicated that the development of more amenable methods of detecting these compounds in a variety of sample matrices is required, in order that this may be fully explored.

Chapter 7

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DEVELOPMENT OF A BIOASSAY EMPLOYING THE DESERT LOCUST (*SCHISTOCERCA GREGARIA*) FOR THE DETECTION OF SAXITOXIN AND RELATED COMPOUNDS IN CYANOBACTERIA AND SHELLFISH

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Locusts were injected along the abdomen, between the second and third segments, with 10 μl of test solutions. In initial studies, three cyanobacterial toxins were tested at 100 μg/ml: microcystin-LR, anatoxin-a and saxitoxin (STX). Only STX was found to

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To assess extraction methods for cyanobacteria and determine compatibility with the bioassay, four extraction methods were investigated, with the efficiency of each assessed in locusts and by high-performance liquid chromatography (HPLC) (Lawrence *et al.*, 1991). Using *Aphanizomenon flos-aquae* NH-5a, a laboratory culture known to produce STX and neoSTX, extractions were carried out (3×30 min) in either water, water + 0.01% (v/v) trifluoroacetic acid (TFA), methanol, or methanol + 0.01% (v/v) TFA. The extracts were dried, then redissolved in 1 ml of saline. Acidified extracts were most toxic, particularly the methanol–TFA extract, which caused paralysis in 8.3 min (±2.9). The water–TFA extract caused paralysis to occur at 13.3 min (±2.9), and water alone also extracted a significant amount of toxin from cells, causing paralysis at 21.7 min (±2.9). The least successful method was methanol alone, which did not cause paralysis in locusts until 101.7 min (±7.6). HPLC results (Fig. 1) confirmed these findings, indicating that the highest toxin concentrations were in the acidified extracts and the lowest in the methanol alone. Greater toxicity found in the methanol–TFA extract appeared to be due to increased extraction of neoSTX. Extracts from non-toxic cells were processed to determine toxic effects from solvents and whether processing affected the stability of STX. There was no indication of toxicity carried over from solvents used and assessment of toxicity before and after processing indicated no change in toxicity.

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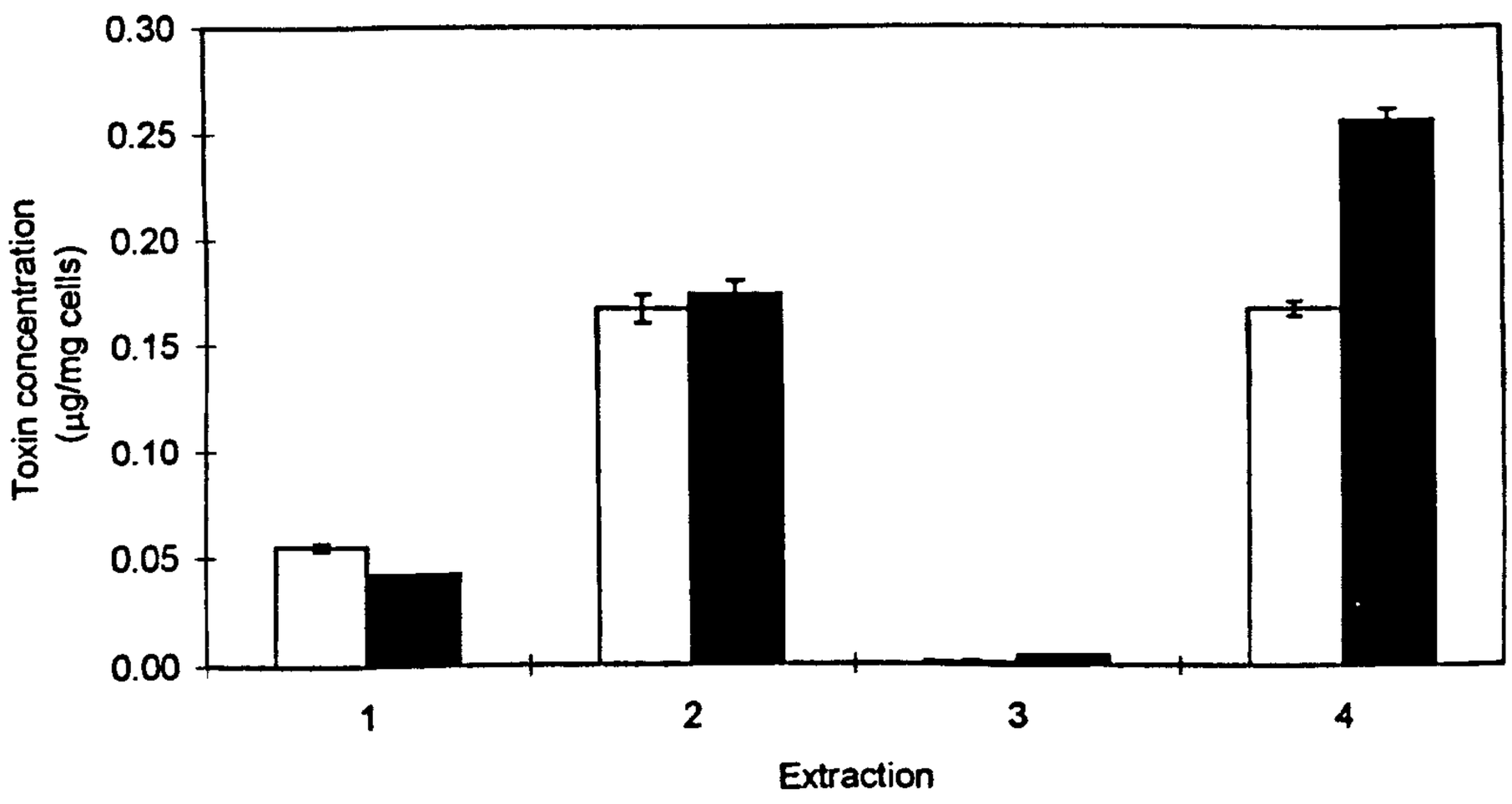

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These investigations demonstrate the potential of the desert locust as an alternative to the mouse for the screening of cyanobacterial and shellfish extracts for PST. The locust bioassay may be carried out in basic laboratories, it is inexpensive and it can provide a rapid estimation of toxicity with a minimum of expertise. A major advantage of the locust bioassay is that it is compatible with both the standard extraction procedure for shellfish samples and the optimum processing method for dried cyanobacterial cells. The assay is particularly useful for the screening of cyanobacterial samples because it appears to be selective for saxitoxins. The assay is more straightforward than the housefly bioassay proposed by Ross *et al.* (1985), because the locust is relatively large and may be immobilized simply by holding the insect by its hind legs. Although the housefly bioassay is extremely sensitive, samples must be administered by microinjection, which may be impractical in many laboratories as considerable expertise is required. The 10 μl injection volume required for the locust bioassay may be administered without the need of specialized apparatus, and ease of handling also makes the assay safer to perform than the microinjection technique.

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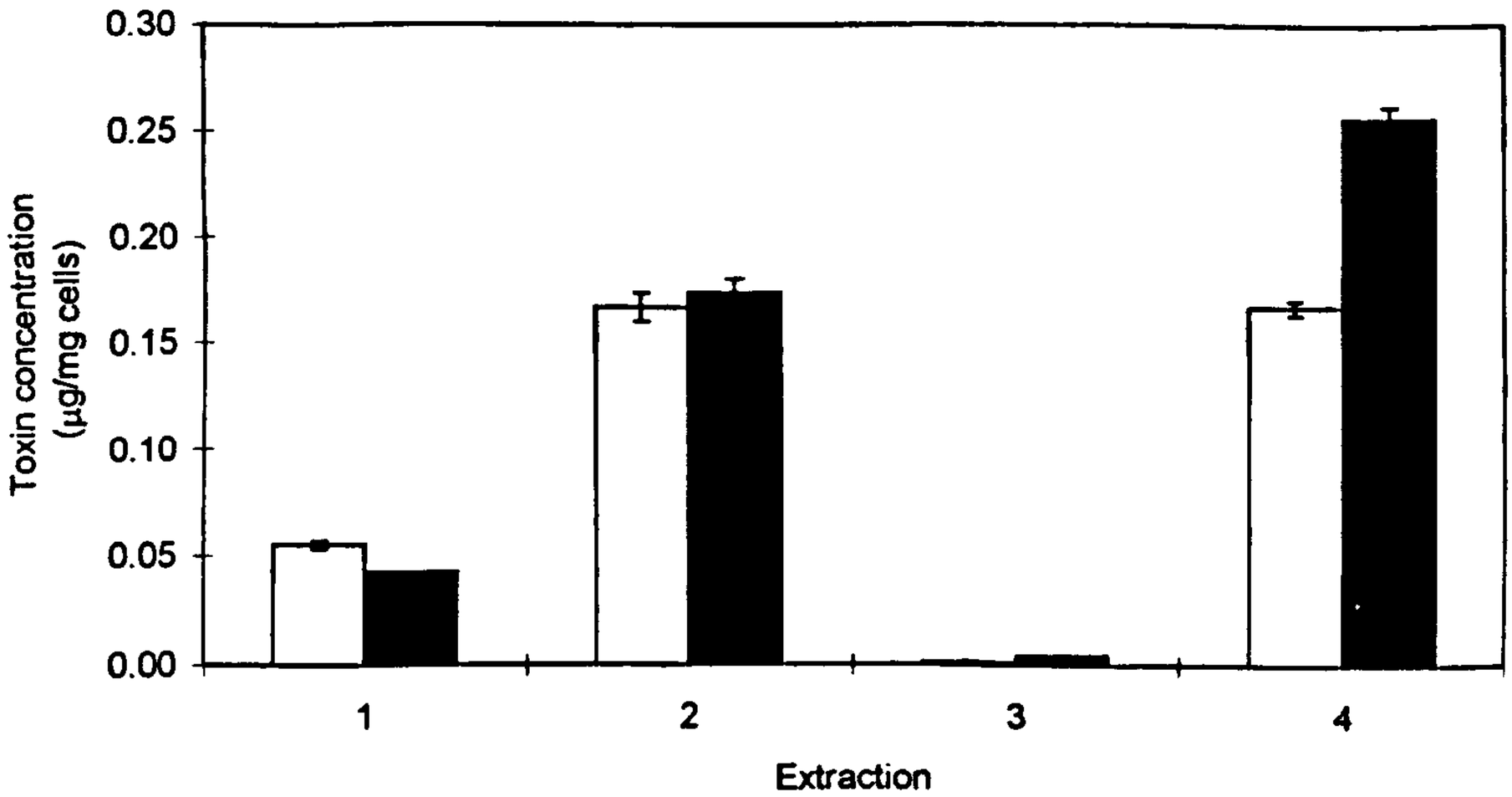

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Short communication

Purification of closely eluting hydrophobic microcystins (peptide cyanotoxins) by normal-phase and reversed-phase flash chromatography

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Purification of closely eluting hydrophobic microcystins (peptide cyanotoxins) by normal-phase and reversed-phase flash chromatography

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Abstract

Two closely eluting hydrophobic peptides, microcystin-LW and -LF had proved in the past to be particularly difficult to purify by reversed-phase HPLC. Initial extraction by reversed-phase flash chromatography provided a good method of concentrating microcystins and also partially purifying them, although the separation of closely eluting variants was not possible. The use of normal-phase flash chromatography after initial reversed-phase extraction was found to be a suitable method for achieving high purity and gave a good yield of both microcystins. However, a final reversed-phase flash step was necessary to eliminate trace contaminants. The method described provides a simple three step flash chromatography extraction and purification eliminating the need to use preparative HPLC.

Keywords: Flash chromatography; Microcystins; Peptides; Toxins

1. Introduction

Microcystins are a group of toxic, cyclic peptides which are produced by a number of genera of cyanobacteria (blue–green algae) including *Microcystis*, *Planktothrix* (*Oscillatoria*) and *Anabaena* [1]. These toxins have been implicated in frequent animal poisonings [2–4], and more recently in the fatality of a significant number of dialysis patients in Brazil [5,6].

Microcystins are cyclic heptapeptides named according to the single letter abbreviation for the variable amino acids which they contain hence those under investigation here, microcystin-LW and -LF both contain leucine (L), with tryptophan (W) and phenylalanine (F), respectively. Different amino acid substitutions and a number of minor chemical modifications yield a large family of related homologs. The microcystins studied here are relatively hydrophobic compared to most other microcystins which is due to the presence of the more hydrophobic amino acids, tryptophan and phenylalanine [4].

Microcystin-LW and -LF have been reported in a number of naturally occurring cyanobacteria and are produced in laboratory culture by several strains of *Microcystis aeruginosa* [4,7]. Many of the commonly used extraction methods utilise aqueous solvents which would not be suitable for recovering more

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hydrophobic microcystins [8,9]. Furthermore, the commonly used analytical high-performance liquid chromatography (HPLC) protocols fail to elute over a broad enough range of polarity, resulting in an under reporting of the occurrence of hydrophobic microcystins [10]. It is therefore important that purified hydrophobic microcystins are readily available to enable their detection and subsequent reporting.

Previously, we reported the use of reversed-phase flash chromatography as a suitable method for concentration and preliminary purification [11,12]. This facilitated simple isocratic preparative HPLC of several microcystins. However, successful purification of microcystin-LW and -LF was only achieved using closed-loop recycling. Although this method provided good purity and yield, it relies on the availability of specialised equipment and expertise. This paper describes the development of a normal-phase flash chromatography method for the purification of microcystin-LW and -LF after preliminary extraction and separation by reversed-phase flash chromatography. The method eliminates need for the relatively expensive, preparative HPLC and exploits the use of a simple pre-packed flash cartridge system.

2. Experimental

2.1. Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Merck, Darmstadt, Germany. HPLC-grade methanol, acetonitrile and dichloromethane (DCM) were obtained from Rathburn (Walkerburn, UK). Pure water was obtained from a Milli-Q system (Millipore, Watford, UK). Microcystin standards were purified from cultured cyanobacterial cells as previously described [11].

2.2. Cyanobacterial material

Batch cultures of *Microcystis aeruginosa* PCC7820 (Pasteur Culture Collection, Paris, France) were grown in BG-11 plus nitrate (8.8 m*M*) under continuous illumination and sparging with sterile air. Cells were harvested after approximately five weeks growth by tangential flow filtration (Pellicon-2; fitted with three 0.22 μm, type GVPP-V filters, Millipore) and stored as a wet pellet at −20°C until required.

2.3. Extraction of cyanobacterial cells

Wet pellet (equivalent to 30 g dry mass) was thawed and extracted as previously described. The aqueous extract was applied to a pre-conditioned KP-C_{18}-HS flash cartridge (15×4 cm I.D., 35–70 μm particle size, 60 Å pore size) using a Biotage Flash 40 system (Biotage, a Division of Dyax Corporation, Charlottesville, VA, USA) at a flow-rate of 40 ml/min. The microcystins were eluted using a step gradient from 0 to 100% methanol in 10% increments (1 l per step) with each step fraction collected separately and analysed by analytical HPLC. After use, reversed-phase flash cartridge was cleaned with DCM (1 l) then flushed (1 l) and stored in methanol for future use. It has been found that reversed-phase flash cartridge can be used a number of times depending on the sample and thoroughness of the cartridge cleaning.

2.4. Analytical HPLC

All fractions were monitored as described previously [9] with the following modifications. Quantification, identification and purity were determined by HPLC with high-resolution diode array detection using a Waters 996 detector. Samples were separated on a Symmetry C_{18} column (250×4.6 mm I.D.; 5 μm particle size; Waters). Detector resolution was set at 1.2 nm and data acquired from 200 to 300 nm.

2.5. Method development for normal-phase separation of microcystin-LW and -LF

Solvent optimisation was carried out initially using normal-phase thin-layer chromatography (TLC) (10×5 cm, silica gel 60; Merck). A range of solvents with different selectivities were evaluated and components were visualised by developing TLC plates in iodine. R_F values were calculated and converted into column volumes ($CV=1/R_F$) which represented the approximate number of column volumes of solvent required to elute each component. In order to determine suitable separation conditions, the difference

between predicted column volumes for each microcystin was determined (ΔCV).

Using the solvent selected by TLC, final optimisation of separation conditions was carried out using a pre-packed flash KP-Sil silica cartridge (15×1.2 cm I.D., 32–63 μm particle size, 60 Å pore size; Biotage) which was conditioned by washing with methanol (200 ml) then DCM (100 ml). Mobile phase A was DCM and B contained methanol–acetic acid (10:2). The cartridge was equilibrated with initial mobile phase, A–B (95:5) (100 ml). For method optimisation, a partially purified sample containing 1.3 mg microcystin-LF and 1.5 mg microcystin-LW was resuspended in a small volume of methanol then diluted in DCM prior to injection. A step gradient was performed as follows: three CVs (30 ml) A–B (95:5); 10 CVs A–B (92:8); 12 CVs A–B (90:10). Fractions (approximately 5 ml) were analysed by TLC (DCM–methanol–acetic acid, 88:10:2) and those containing microcystins were dried, resuspended in methanol, and analysed by HPLC.

2.6. Scale-up of normal-phase flash chromatography method

The fraction (60% aqueous methanol) eluted from the reversed-phase flash extraction and found to contain predominantly the two microcystins of interest (Table 1), was dried by rotary evaporation and the sample resuspended in a small volume of methanol. The KP-Sil silica cartridge (15×4 cm I.D., 32–63 μm, 60 Å pore size; Biotage) was prepared by flushing with methanol (2400 ml), DCM (1200 ml) and initial mobile phase (1200 ml) as described in Section 2.5. The sample (9.3 mg microcystin-LW and 12.2 mg microcystin-LF) was diluted in DCM prior to injection onto the prepared cartridge. Elution of the microcystins was carried out as before (Section 2.5) maintaining the number of CVs used to separate the toxins. Fractions (50 ml) were dried, resuspended in methanol and analysed by HPLC. Fractions containing a single microcystin with purity (by HPLC) greater than 90% were pooled, diluted to 20% (v/v) methanol and passed through a KP-C_{18}-HS flash cartridge (7.5×4 cm I.D., 35–70 μm particle size, 60 Å pore size; Biotage) and the cartridge washed with water (500 ml) to ensure the of removal any residual acetic acid. Each purified microcystin was eluted from the cartridge in methanol (500 ml) then rotary evaporated to dryness prior to quantification by gravimetric analysis.

Gravimetric analysis and visual appearance suggested the presence of contaminants not revealed by HPLC. Individual purified microcystins were therefore resuspended in methanol and the sample added to water (1 l). This was passed through a preconditioned C_{18} flash cartridge (7.5×4 cm I.D., 35–70 μm particle size, 60 Å pore size; Biotage) and the sample eluted by step gradient from 0 to 100% methanol in 10% increments (500 ml per step). Each increment was collected separately and analysed by HPLC. The fraction containing purified microcystin

Table 1
Recovery of microcystins, determined by HPLC–photodiode array detection, from *Microcystis aeruginosa* PCC7820 following reversed-phase flash chromatography using an aqueous methanol step elution

Methanol fraction (%)[a]	Microcystin variant	Amount of microcystin (mg)	Recovery (%)[b]
40	LR	18	22
	LY	1	9
50	LR	52	64
	LY	9	91
	LW	3	19
60	LW	12	67
	LF	15	93

[a] Percentage of aqueous methanol used to elute that fraction.
[b] Percentage of microcystin recovered in that fraction relative to total amount applied to the cartridge.

was rotary evaporated to dryness, resuspended in a small volume of methanol, dried and quantified gravimetrically.

3. Results

3.1. Solvent and reversed-phase extraction of microcystins

HPLC analysis of the cell extract prior to reversed-phase extraction revealed four main microcystins (Fig. 1) which were identified and quantified as microcystin-LR (82 mg), microcystin-LY (10 mg), microcystin-LW (17 mg) and microcystin-LF (16 mg). Analysis of the flash fractions eluted using a methanolic step gradient revealed that the microcystins were eluted in the 40 to 60% fractions (Table 1) with the fraction eluted in 60% methanol found to contain primarily microcystin-LW and -LF.

3.2. Method development of normal-phase separation of microcystin-LW and -LF

The most suitable solvent system indicated by TLC was DCM–methanol–acetic acid (88:10:2). The addition of acetic acid minimised tailing. R_F values were 0.08 and 0.18 for microcystin-LW and -LF, respectively. This gave a theoretical ΔCV of 7.3 which would indicate a potentially good separation when chromatographed on a column. This mobile phase was evaluated using small (15×1.2 cm I.D.) pre-packed flash cartridges. It was found that pre-washing of the cartridge with methanol followed by the mobile phase was necessary to prevent recovery problems.

Direct transfer of the TLC method to the 12 mm column did not result in the simple separation as predicted, thus it was necessary to use a step gradient. The conditions described (Section 2.5) provided the optimum separation and although no

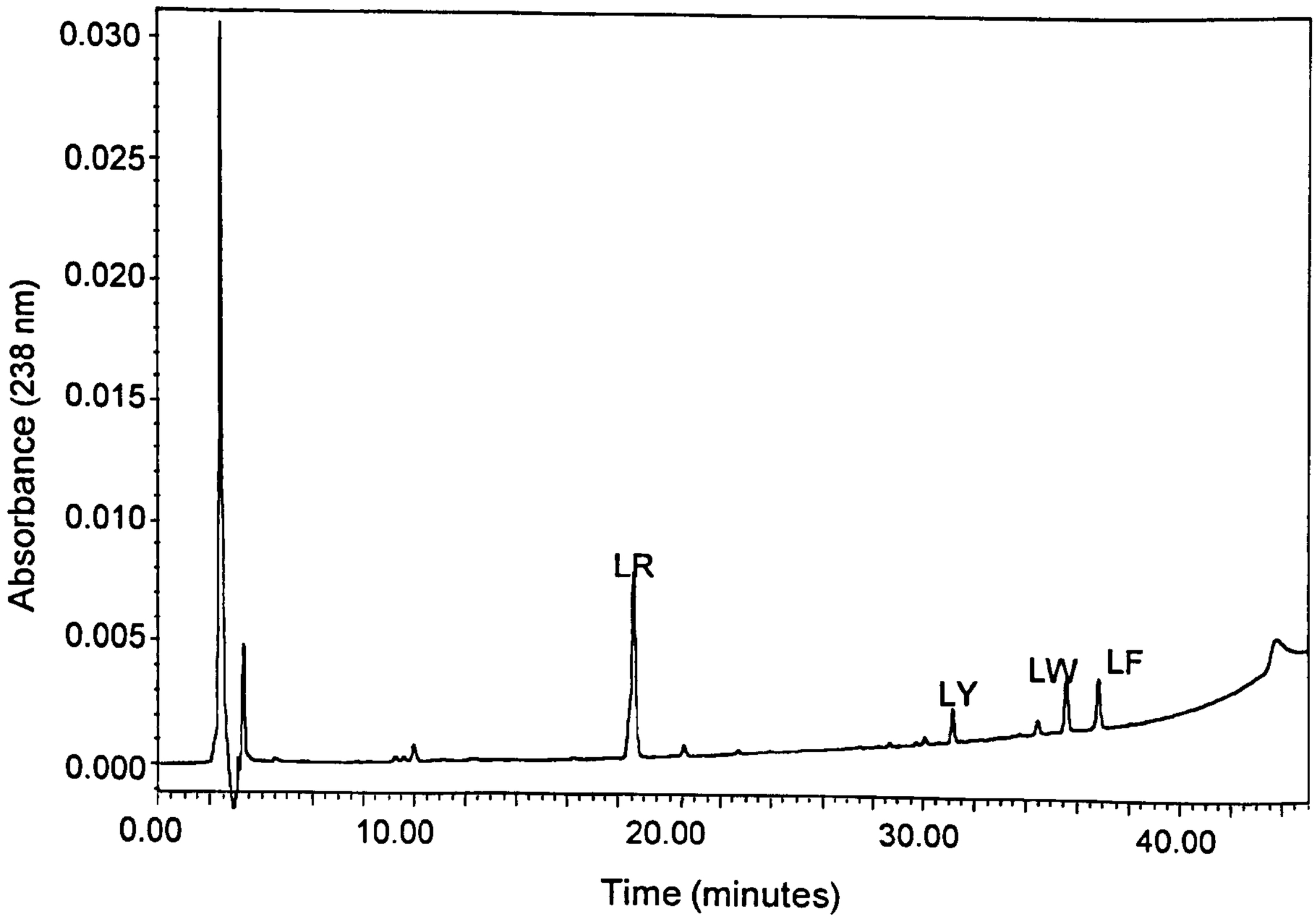

Fig. 1. Reversed-phase HPLC chromatogram of aqueous methanolic extract of *Microcystis aeruginosa* PCC7820 prior to application to C_{18} flash cartridge. Four main microcystins present in the extract: microcystin-LR; microcystin-LY; microcystin-LW; and microcystin-LF.

microcystins were eluted in the first step (A–B, 95:5) it was found to be essential to the initial retention and subsequent satisfactory separation of the microcystins.

3.3. Scale-up of normal-phase purification

The optimised method was used to purify microcystin-LW and -LF from the initial reversed-phase flash (Section 2.3). Direct scale-up was achieved using a 4 cm I.D. cartridge where volumes of solvent in the step gradient, volume of sample injected, flow-rate and fraction size were increased proportionally. Due to limited amount of sample, the load injected onto the 4 cm column was equivalent to 0.23 mg per gram of packing material compared to 0.35 mg injected onto the 1.2 cm I.D. column. A similar separation was achieved (Fig. 2a) although all toxin-containing fractions were eluted during the final step of the gradient (A–B, 90:10). It can be seen (Fig 2b) that a significant number of fractions contained a single microcystin at a purity greater that 90%.

3.4. Final reversed-phase purification

Reversed-phase flash was successfully used to ensure the removal of acid from the sample. Further analysis, however, revealed the sample still contained contaminants although these were successfully removed by re-applying the sample to a C_{18} flash cartridge and eluted stepwise, successfully removing the contaminants. Final purity of both microcystins by HPLC was determined to be 95% (Fig. 3), with the yield at this purity for microcystin-LW and -LF of 5.5 mg and 7.2 mg, respectively.

4. Conclusions

As previously reported [11], reversed-phase flash chromatography enabled sample concentration, and when combined with step elution, it provided an easy method for partial purification allowing simplification of subsequent chromatography. It was successfully used to separate the two microcystins of interest from both the other microcystins present in the sample and from a large proportion of the co-extracted contaminants.

The purification of microcystin-LW and -LF has previously presented difficulties for a number of reasons. Firstly, although analytical HPLC provides good separation [9], it utilises trifluoroacetic acid (TFA) which when applied to preparative separations, has been found to greatly reduce recovery due to effects on the stability of the microcystins. In particular, microcystin-LW has been observed to be particularly unstable in solutions containing TFA. Secondly, when alternative mobile phases have been investigated, separation deteriorated rapidly with increased sample loading. This was successfully overcome using closed-loop recycling where the toxins were passed through the column two additional times which enhanced resolution [11]. However, this requires specialised equipment and expertise, hence prompting the search for an alternative method.

Normal-phase flash chromatography was found to provide a suitable alternative. TLC provided a guide to a suitable solvent system, but did not result in the predicted separation when scaled-up to the 12 mm I.D. column. This was not unexpected since modes of elution in TLC compared to LC are very different especially in the presence of polar modifiers such as methanol and acetic acid. The 12 mm I.D. cartridges provided an ideal tool for rapid method optimisation with minimum consumption of sample and solvents. The optimised method was readily applied to a scaled-up purification. Although the mobile phase used was acidified (acetic acid), stability of the microcystins was not affected (unpublished data).

The combination of normal and reversed-phase flash chromatography yielded 5.5 mg (59%) of microcystin-LW and 7.2 mg (59%) of microcystin-LF at a purity of 95% determined by analytical HPLC. Less pure material was retained for reprocessing. Although a four stage process has been described in this paper, this could be simplified to three stages by replacing the 100% methanol elution stage with a aqueous methanol step gradient during the acid removal step.

In summary, two closely related, hydrophobic microcystins, which represent less than 0.1% of the cyanobacterial biomass, were concentrated and partially purified from an aqueous extract using re-

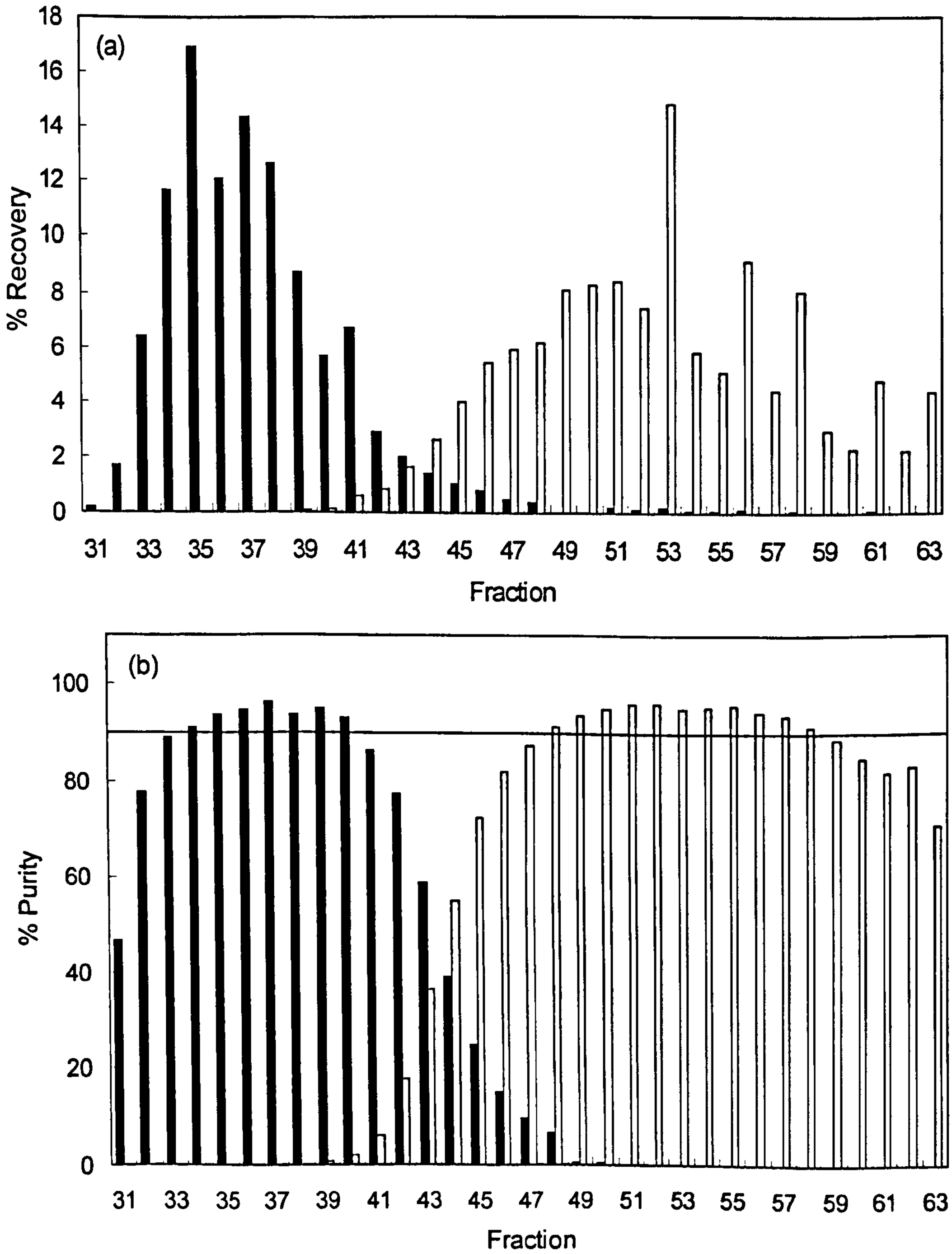

Fig. 2. Results of HPLC analysis of fractions collected from normal-phase flash separation indicating, (a) recovery and (b) purity o microcystin-LF (black) and microcystin-LW (white) in each fraction. The line across (b) indicates samples above 90% purity.

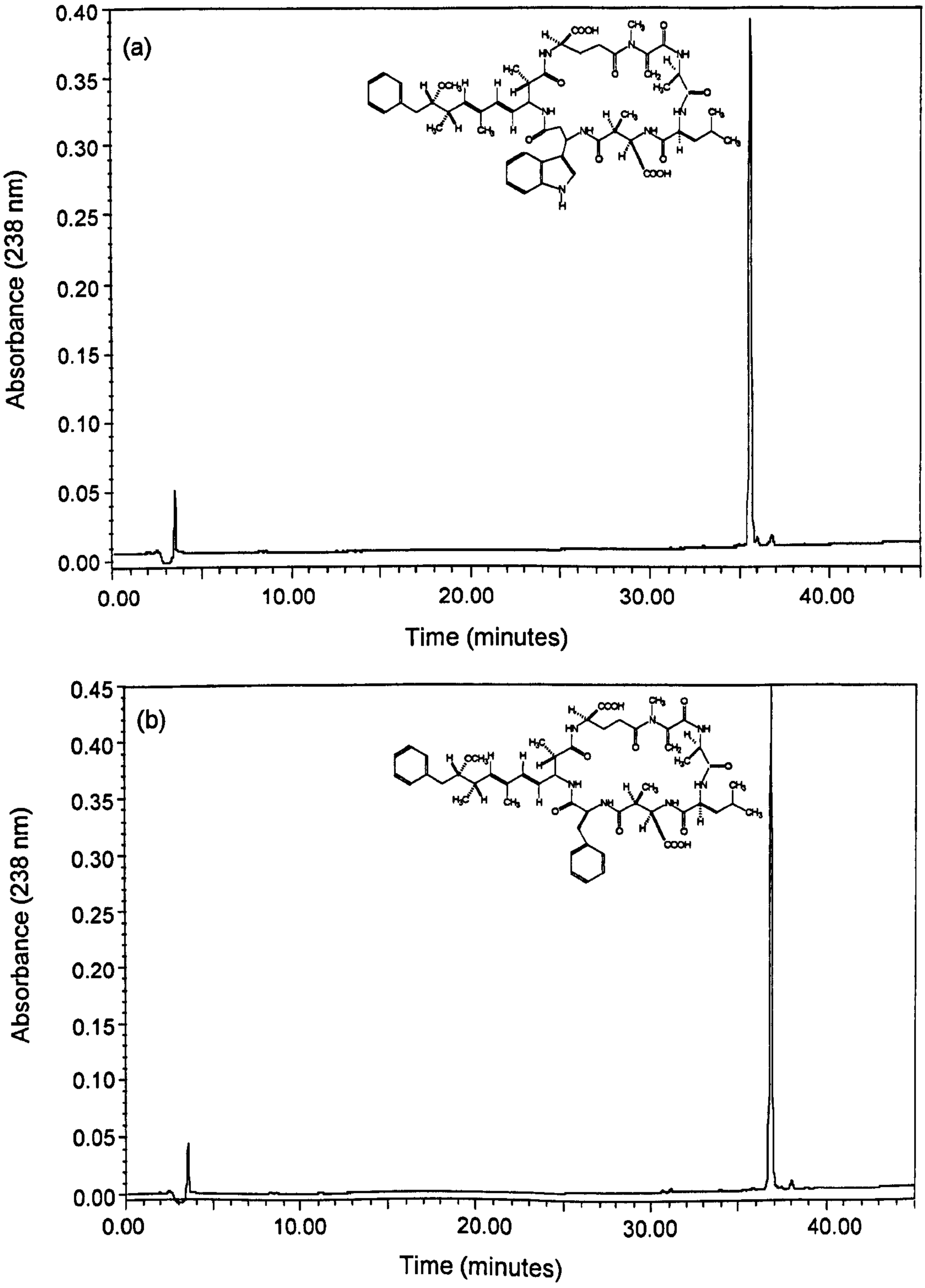

Fig. 3. Reversed-phase HPLC chromatograms of purified (a) microcystin-LW (1.93 μg injected) and (b) microcystin-LF (2.29 μg injected).

versed-phase flash chromatography. They were subsequently separated by normal-phase flash chromatography followed by acid removal and final polishing on reversed-phase flash to provide high purity microcystins.

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