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Review

Purification of microcystins

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Abstract

Microcystins are an increasingly important group of bioactive compounds produced by a number of mainly planktonic cyanobacteria. They are a family of cyclic heptapeptides that cause both acute and chronic toxicity. Purified microcystins are utilised in a range of research applications including toxicological and biochemical studies, development of detection systems and the investigation of water treatment strategies. The commercial availability of purified microcystins is still relatively limited and for many projects the cost of their purchase prohibitive. The purification of microcystins from both bloom material and laboratory cultures is reviewed including a discussion on extraction, separation, and the determination of purity and yield. © 2001 Published by Elsevier Science B.V.

Keywords: Reviews; Microcystins

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1. Introduction

Microcystins are a family of hepatotoxic peptides produced primarily by freshwater cyanobacteria (blue-green algae) belonging to the genera *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* (some species are now classified as *Planktothrix*). Their presence in water bodies has caused the death of wild and domestic animals worldwide [1], and more recently they have been implicated in human fatalities [2]. Their potential for causing both acute and chronic toxicity has prompted the need for extensive research into their detection, toxicology and removal from potable water, all of which requires the availability of purified microcystins.

Microcystins are cyclic heptapeptides that share a general structure (Fig. 1) containing γ-linked D-glutamic acid (D-Glu), D-alanine (D-Ala), β-linked D-*erythro*-β-methylaspartic acid (D-MeAsp), *N*-methyldehydroalanine (Mdha) and a unique C_{20}β-amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid (Adda). The other two L-amino acids are variable (denoted X and Z) and found in position 2 and 4 of the cyclic structure. The single letter abbreviation of the variable amino acids is used to distinguish different microcystins; for example, the most commonly occurring microcystin contains leucine and arginine in these positions and is therefore called microcystin-LR. Variation in these two amino acids accounts for many of the microcystin variants that have been characterised but other minor modifications such as demethylation increases the number of microcystin variants to at least 60 [1] (Table 1). It is unclear why so many microcystin variants are produced although some are only ever found at relatively low concentration and their characterisation depends on the extraction of substantial quantities of cyanobacterial cells e.g. in one publication 0.06 mg of purified microcystin was obtained from 60 g dry mass of cells (present at around 1 ppm) [3]. The structural similarity of these trace microcystins with the main microcystins produced by a cyanobacterium may suggest they are possible by-products or in some instances may be artefacts produced during purification.

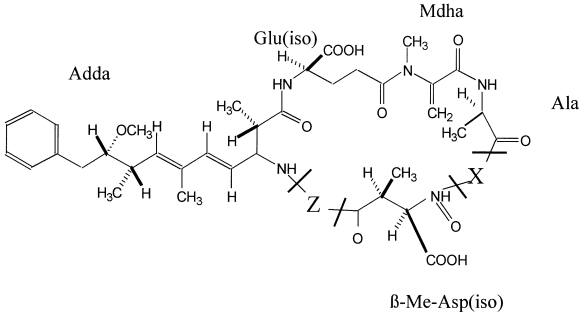

Fig. 1. General structure of microcystin where X and Z represent variable amino acids as presented in Table 1.

Microcystins are potent toxins due to their inhibition of the regulatory enzymes protein phosphatase 1 and 2A (PP1 and 2A) [4]. The use of microcystins in the study of these enzymes and related processes make them important biochemical tools further increasing the demand for purified microcystins. The available toxicity data indicates that many of the microcystin congeners vary little in toxicity demonstrating that most of the structural modifications have little influence on biological activity [5]. However, some modifications including structural changes in the Adda moiety and esterification of the glutamic acid does lead to dramatic reduction in toxicity [6,7]. This loss of toxicity has been shown to correlate with changes in the groups that are known to interact with sites on PP1 and 2A.

Toxic cyanobacterial isolates are known to produce between one and over a dozen microcystins in a single culture. The microcystin variants produced differ between genus, species and strain, but appear to remain reasonably constant for an individual isolate, although the relative proportion can change with different environmental conditions [8,9].

Microcystins were first purified by Botes et al. in 1982 [10] and since then many different approaches have been adopted. Typically cyanobacterial cells are extracted, the resultant extract concentrated and the microcystins purified by a range of sample separation techniques. However, the number of steps and methods employed vary greatly. To date there is no consensus on the most efficient approach to purifying microcystins although this may be due to the differing requirements of researchers. For example, where the aim is to purify substantial quantities of one or two key microcystins the approach will tend to be different from that employed to purify all mi-

Table 1
Microcystins characterised to-date indicating the molecular mass of each variant. Amino acids present in the two variable positions are given in italics with other modifications listed below the Z variable amino acid present in that microcystin[a]

Z variable amino acid *X*→ ↓	*Ala* (A)	*Arg* (R)	*Glu* (E)	*E (OMe)*	*Homo IsoL*	*Leu* (L)	*Methionine -S-oxide*	*Phe* (F)	*Try* (W)	*Tyr* (Y)	*Val* (V)
Alanine						909				959	
Aminoisobutyric acid						923					
Arginine	952	1037			1008	994	1028	1028	1067	1044, 1048^{t} 1058^{hY}	
D-Asp^{3},Dha^{7}		1009				966				1030^{hY}	
D-Asp^{3}		1023				980				1030, 1044^{hY}	
Dha^{7}		1023				980		1014, 1028^{hF}		1030, 1044^{hY}	
L-Ser^{7}		1041				998				1062^{hY}	
DMAdda^{5}						980					
D-Asp^{3}, D-Glu(OCH$_3$)^{6}						994					
(6*Z*)-Adda^{5}		1037				994					
D-Asp^{3}, ADMAdda^{5}						1008, 1022^{hR}					
D-Glu(OCH$_3$)^{6}						1008					
D-Asp^{3}, ADMAdda^{5},Dhb^{7}		1052				1009				1073^{hY}	
L-MeSer^{7}						1012					
D-Asp^{3},L-MeSer^{7}		1041									
ADMAdda^{5}						1022, 1036^{hR}					
D-Ser^{1}, ADMAdda^{5}						1038					
D-Glu-OC$_2$H$_3$(CH$_3$)OH^{6}						1052					
ADMAdda^{5},MeSer^{7}						1040					
N-Methyllanthionine						1115					
E(OMe)											
D-Asp^{3},Dha^{7}			969	983							
Dha^{7}			983	997							
L-Ser^{7}			1001	1015							
D-Asp^{3}, Ser^{7}				1001							
Leucine						951					
Methionine-S-oxide										1035	
Phenylalanine						985					971
Tryptophan						1024					
Tyrosine						1001					

[a] Abbreviations and superscripts: ^{h}, homo variant of the amino acid indicated by single letter; ^{t}, tetrahydrotyrosine; ADMAdda, *O*-Acetyl-*O*-demethylAdda; DMAdda, *O*-demethylAdda; (6*Z*)-Adda, stereoisomer of Adda at the Δ^{6} double bond; Dha, dehydroalanine; Dhb, dehydrobutyrine; MeSer, *N*-methylserine; E(OMe), glutamic acid methyl ester.

crocystins present in a sample which is required when characterising a cyanobacterial sample. It is therefore important to have a clear idea of which approach is required prior to beginning a purification.

This paper aims to review and evaluate the suitability of the methods available for the purification of microcystins and will also prove useful to those investigating nodularins, and related pentapeptides, although they will not be directly discussed here. It will include important associated issues such as selection and handling of starting material, the determination of product purity and microcystin quantification.

2. Starting material

Prior to commencing purification of microcystins it is advisable to perform preliminary HPLC analysis

to determine the quantity of microcystins, the number of variants and their potential identity. This both assists in determining the efficiency of the purification and predicts the potential yield of variants, preventing wasted effort in purifying samples containing little or no microcystins.

There are two main sources of material used for the purification of microcystins, namely material collected from the field or laboratory grown cultures. Both sources have their own related advantages and disadvantages. Material collected from water bodies has the benefit of often being available in substantial quantities particularly when a dense bloom has formed, and is usually easily accessible since scum typically accumulates on the shoreline. In this case large quantities can be directly collected and require no further sample concentration. However, in many cases the cyanobacteria can remain suspended in the water column and concentration, which can be achieved using an appropriate plankton net [11], has to be carried out. Where concentration is required it will limit the amount of material that can practically be harvested since this tends to be a rather time consuming process. The main advantage of collecting bloom material is that there is no initial investment of time required for the preparation of bulk culturing materials and generally the biomass that can be harvested is much greater than could be produced by laboratory culture methods. The use of field samples is widely reported in the characterisation of microcystins and in some cases it is only the extraction of substantial quantities (~100–600 g dry mass) [12,13] which has enabled the purification and identification of microcystins that are present in trace amounts. However, there are a number of drawbacks associated with the use of bloom material. Firstly, samples can be more complex than those produced in the laboratory since they are rarely composed of only cyanobacteria, let alone a single species. This leads to difficulty in determining which organism is responsible for the production of microcystins in the sample. Commonly this will be attributed to the cyanobacterial species that is dominant in the sample, however, organisms present in relatively small amounts may contribute to the overall diversity of microcystin variants detected. Furthermore, field samples are finite and it is rarely possible to return to a location and collect identical material for further extraction.

In contrast, cultured cyanobacteria will tend to be highly reproducible, with isolates producing the same microcystins at predicable concentrations. However, some isolates have been known to lose their ability to produce the toxins although this is rarely reported in the literature. Laboratory cultures are usually well characterised, containing a single cyanobacterium identified to species level, and in some cases axenic (bacterial-free) cultures are available. Many are maintained in culture collections (e.g. Pasteur Culture Collection, France; Niva, Norway; Nies, Japan) thus providing a reliable and renewable source. Extracts from laboratory-harvested cells tend to be less complex and can make the purification of microcystins simpler. For example, Fig. 2 compares a partially purified extract of two hydrophobic microcystins, microcystin-LW and -LF from both a bloom and laboratory isolate of *Microcystis aeruginosa*. Many components are seen to be present in the

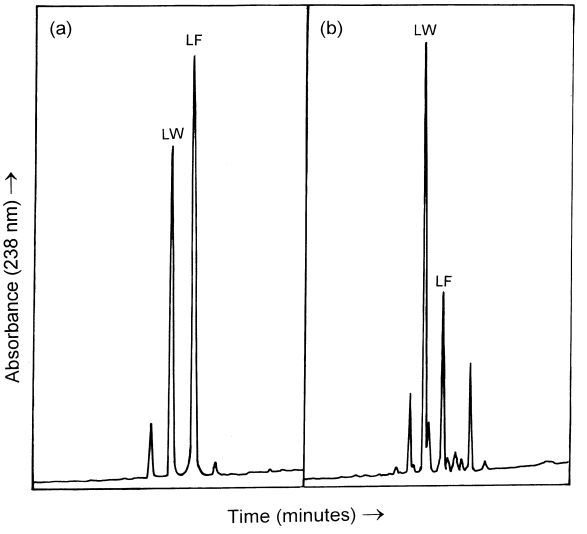

Fig. 2. Comparison of the separation of microcystin-LW and microcytsin-LF in (a) extract from *Microcystis aeruginosa* PCC 7820 and (b) extract of bloom material from Rutland Water (Leicestershire) in 1989. Both samples were extracted in 100% methanol, diluted to 20% aqueous methanol and applied to a C_{18} cartridge. The chromatograms shown are of the fraction eluted by 60% methanol from step gradient using 10% increments between 0 and 100%.

bloom extract making simple purification by normal-phase flash chromatography impossible, whereas the microcystins in the cultured sample were readily purified using this technique [14]. The main disadvantage in using cultured cyanobacteria is the effort involved in producing sizeable quantities (tens of grams dry mass) of cells. Few papers report the actual yield of cells obtained in mass culture (typically 8–12 l batch cultures). Botes et al. [10] obtained a yield of around 1 g from 12 l while other researchers have reported 1–2 g from 10-l cultures [13]. The ease of obtaining, for example, over 300 g of cell from 7 l of scum collected in the field [15] compared to the effort required to produce similar quantities in the laboratory highlights the reason a great number of researchers rely on bloom material. One other factor that must be kept in mind when considering the use of cultures is the time required to harvest relatively dilute suspensions of cells. Lower volumes ($<$10 l) can be harvested by batch centrifugation although cells that are gas vacuolate may present a problem. Larger volumes require either continuous centrifugation or tangential flow filtration.

Once harvested, cells both collected in the field or grown in the laboratory usually need to be stored prior to extraction. Freeze-drying is commonly carried out with subsequent storage at -20°C. This method is useful as it allows a known mass of cells to be extracted and readily enables the toxin yield to be related to mass of cells extracted. This approach also makes it easy to work with reproducible subsamples over a period of time. However, freeze-drying can be time consuming especially where large amounts of cells are involved and there may be safety implications in handling dried material. Freeze-dried cells can easily become airborne during handling and since nasal inhalation has been identified as a potentially hazardous route of exposure [16] great care must be taken. Therefore, where a predetermined mass is not required, direct freezing of harvested cells is simplest and if necessary the mass extracted can easily be determined by removing and drying a small subsample. Freezing of wet samples may also be beneficial in the subsequent extraction of the cells, as it will enhance cell lysis [17] and the release of microcystins from within the cells. One of the only other methods used in the storage of cyanobacteria cells is air-drying where cells are either placed in the sun or in a drying oven. This is usually only carried out where freeze-drying is unavailable and cells need to be stored without a freezer. This method should be used with caution since it has been observed that elevated temperatures may affect microcystins [18] and UV light may alter or degrade them [19].

3. Extraction

3.1. Solvent extraction

Extraction solvents used in the purification of microcystins vary greatly and as yet there appears to be little agreement as to which is the most appropriate. This may be because the range of microcystin variants can differ between samples and the behaviour of the different toxins is not uniform. However, there is a need for a reliable procedure that will extract all microcystins present in a sample to allow the total microcystin content to be evaluated. After that the choice of media will depend on the requirements of the purification. Since a wide number of solvents have been utilised, often with no published report of their efficiency, only those frequently used and/or found to be satisfactory will be discussed.

Early publications [10,20] on the purification of microcystins describe the use of 0.1 *M* ammonium hydrogencarbonate (pH 8.4), and in 1982 Van der Westhuizen and Eloff [21] reported their findings on the suitability of a range of different extraction media. They concluded from their study that water at pH 10 gave the best recoveries although they also reported higher toxicity in extracts using Triton X-100 (0.1–1%) but were concerned about the possibility of Triton contributing to the toxicity of the samples. Furthermore, they reported that at low pH toxin recovery is poor which is consistent with much later findings [22,23]. However, the analysis carried out by Van der Westhuizen and Eloff relied on the mouse bioassay and the microcystin variants present were not known. Despite the publication of their findings, high pH media were not generally em-

ployed for the extraction of microcystins. Instead, when the use of butan-1-ol–methanol–water (5:20:75) was described by Siegelman et al. in 1984 [24], it was widely adopted by many researchers and is still used to date [25,26]. Although this media has been extensively employed there are few reports commenting on its efficiency. One study reported satisfactory extraction of a range of microcystins (microcystin-LR, -LY, -LW, LF) although methanol was found to extract slightly more of each variant [22]. One of the drawbacks of using this mixture of butanol, methanol and water was that it coextracted many compounds resulting in a complex sample. This method also advocated lengthy high-speed centrifugation and solvent removal prior to solid-phase extraction. Therefore when Harada et al. [27] published the use of 5% acetic acid (pH 2.7) it was increasingly employed probably due to the simplicity of the extraction procedure and subsequent ease of processing. Acetic acid extracts the blue water-soluble phycocyanins but does not extract many of the pigments (including green chlorophyll a) that often make purification difficult. When extracts are applied to C_{18}, the typical step after solvent extraction, the phycocyanins are not retained enabling a much cleaner microcystin-containing sample to be eluted. Other advantages that prompted the widespread use of this method are that no rotary evaporation step is required and acidic conditions enhance pellet formation during centrifugation therefore bench centrifuges could be used. Comparison of the suitability of acetic acid for the extraction of microcystins indicates that it is slightly less efficient than butanol–methanol–water in extracting microcystin-LR (15% less) however the same study found the recovery of other microcystins (-LY, -LW, -LF) was reduced by between 75 and 89% [22]. Extraction of microcystin-RR has also been evaluated, indicating that recovery of this toxin was slightly less (~10% lower) in acetic acid than the other solvents used (water, methanol and 70% aqueous methanol) [28]. Reduced recovery of microcystins in low pH solvents had been demonstrated a number of years ago [21] but it is only in the last year or so that data has become available that may explain this. Until recently little work had been published on the physicochemical properties of microcystins, however in 1999 De Maagd et al. [23] reported the changing solubility of microcystin-LR over a range of pH. They investigated the octanol–water distribution ratio (the logarithmic value D_{ow}) of microcystin-LR as a function of pH and found it to be 2.18 at pH 1 and -1.76 at pH 10. This indicates that, as would be expected, microcystin-LR becomes increasingly hydrophobic with the lowering of pH making it less soluble in acidic aqueous extraction media. However, since acetic acid does provide a straightforward extraction procedure and gives reasonable recovery of a number of microcystins that are often purified for commercial and research purposes it can still be recommended in some instances.

Other media that are commonly used and have been evaluated for a number of microcystins are methanol, water, and mixtures of the two. In one study, a laboratory culture known to produce microcystin-RR was extracted using 100% methanol, 70% aqueous methanol, 100% water and 5% aqueous acetic acid. Analysis indicated that methanol, water and 70% aqueous methanol performed equally well but acetic acid extracted slightly less than the other three solvents [28]. Additionally, Fastner et al. [29] found the suitability of methanol, water and 75% methanol varied between samples. In some samples no variation was observed between different solvents but in others, especially in field samples, extraction in 100% methanol sometimes gave total toxin recoveries that were 50% less than that observed with water or 75% methanol. The samples under investigation contained a range of microcystins and the authors suggested that some samples gave poor extraction efficiency due to low recovery of microcystin-RR. This is also consistent with the work of Wirsing et al. [30] who reported that less than 25% of microcystin-RR was extracted by methanol from a bloom sample and none of the microcystin-YR. Ward et al. [31] confirm the disadvantages of using 100% methanol with only approximately 50% of the total microcystins being extracted but they also reported poor extraction efficiency in water and low methanol concentrations. The best extraction was observed at 50–80% methanol with 70% aqueous methanol selected as the most suitable.

A number of authors have also investigated sequential extraction where samples were first extracted in methanol then the cell pellet reextracted in

water. Between 30 and 50% of the total microcystins in the sample were present in the second extraction (water) indicating the shortfall in the use of 100% methanol. Other workers attempting to maximise microcystin yield during purification have performed an initial extraction in 5% acetic acid followed by 100% methanol [32,33]. This proved satisfactory for the recovery of hydrophobic microcystins that were poorly extracted by the 5% acetic acid alone [32]. A further example of the usefulness of sequential extraction was demonstrated by Harada et al. when isolating microcystins from Anabaena. They used acetic acid–ethanol for the initial extraction (three times), yielding 170 mg crude toxin, followed by extraction of the pellet with 5% acetic acid to give an additional 192 mg crude microcystins [52].

Many of the methods evaluated for microcystin extraction have been carried out primarily for the quantification of the toxins present, hence the aim is to extract all variants equally well. This will be appropriate where the objective is to purify and characterise all microcystins present. However, it may often be the objective to purify one or a few specific toxins, then the media that provides the best yield of these microcystins should be employed. Clearly there is still scope to further investigate the extraction solvents and it appears that pH is probably an important factor that has been overlooked in the past. Findings to date do indicate that the selection of solvent(s) will vary depending on the sample and microcystins present therefore it is advisable that researchers evaluate solvent suitability related to their starting material prior to commencing purification. This is demonstrated in Fig. 3, where a small quantity (50 mg) of freeze-dried cultured cells has been extracted in a range of different solvents to

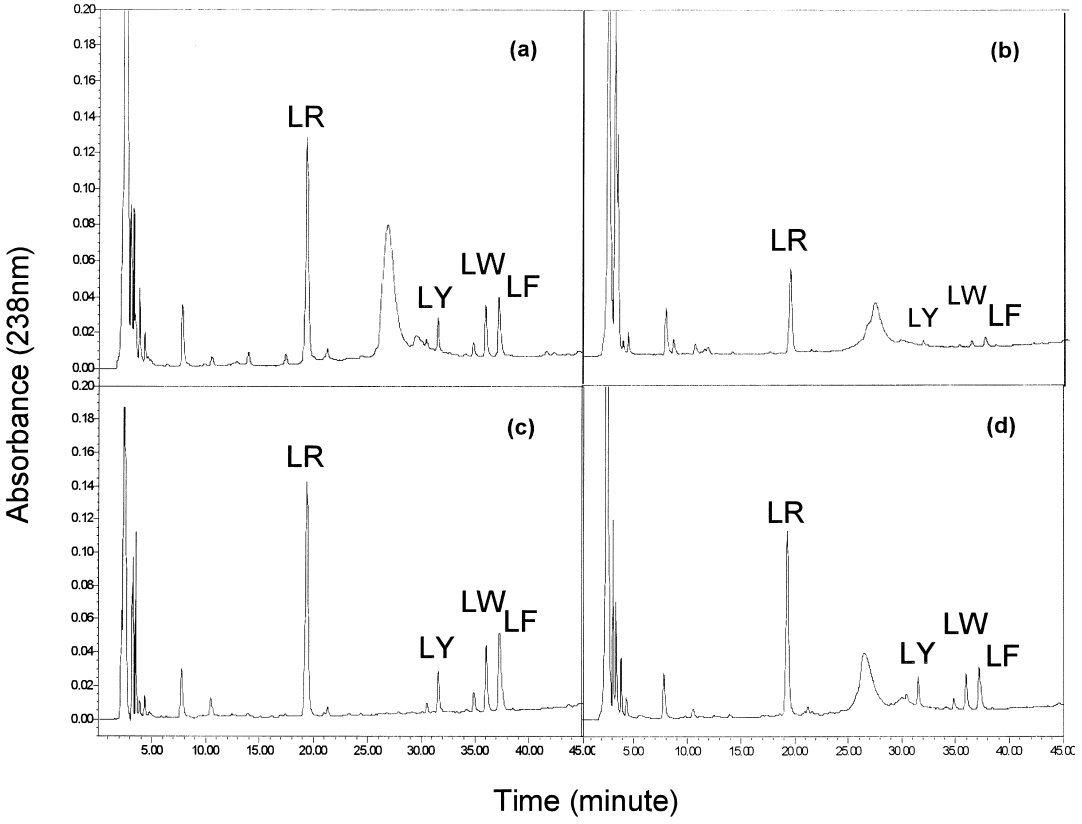

Fig. 3. Extraction of freeze-dried cells (50 mg in 1 ml solvent for 1 h) of *Microcystis aeruginosa* PCC 7820 in (a) water, (b) 5% acetic acid, (c) methanol and (d) butanol–methanol–water (1:4:15).

evaluate the most suitable method for a larger scale extraction. Water and methanol extracted all the microcystins well, although there were additional contaminants in the water extract that would most likely interfere with subsequent purification steps. Acetic acid was shown to be the least efficient extraction solvent, recovering significantly less microcystin-LR and only trace amounts of the more hydrophobic microcystins, a result of a change in solubility due to protonation of the carboxylic groups. Butanol–methanol–water, extracted all the microcystins but to a lesser degree than water or methanol, as with water, large amounts of interfering compounds were also extracted.

3.2. Physical parameters

The treatment of the samples during extraction also needs to be considered. In the literature this ranges from initial mixing and, allowing to stand for a given time, continuous stirring or shaking, and sonication. There is little conclusive evidence regarding which approach performs best although sonication is used in a significant number of papers but published comparisons suggest its use has little effect on yield. Another point to consider is the length of time and number of sequential extractions carried out. Many published methods reextract with the same solvent a number of times then pool the extract. A total of three extractions appears to be the most common [12,34] and it has been confirmed that this should be sufficient to exhaustively extract microcystins from cells [18,22] as only a few percent of the total microcystins present were extracted in the third step. However, the volume of extraction media to mass of cells may influence this although there are no reports of optimum or maximum ratio being determined. One of the most common ratios reported is around 10 g per 200 ml of media with the highest being over 10 g per 100 ml [13] and 10 g per 1000 ml on the lower side [35]. Furthermore, the total quantity of cells extracted in one batch will influence the number of microcystins detected [36,37] since it will be considerably easier to detect and purify microcystins present in trace amounts when a high biomass is used.

Extraction duration is also highly variable ranging from a few minutes to 15 h per extraction, this equates to almost 3 days for one step of the purification [37]. The extraction time will greatly influence overall processing time and also extended extractions may influence stability or promote modification. Again there is little reported in the literature on the evaluation of the most suitable time. One study compared 5-, 30- and 60-min extractions and found that an extraction period of 1 h was best although 30-min extractions were only slightly less efficient, recovering around 85–97% of the microcystins [18]. It would appear that lengthy extractions are unnecessary as seen in Fig. 4, which follows the release of microcystin-LR in 5% acetic acid over time. These findings suggest that relatively short extraction periods are sufficient with the most commonly reported extraction period of 1 h providing acceptable results.

Finally, the temperature that extractions are performed at range from around 4°C to room temperature [38] although it is generally felt that microcystins are sufficiently stable for their extraction to be performed without the need for cooling. However, where extended extraction times are used e.g. overnight, it would be wise to maintain a reduced temperature. Recently, several publications have evaluated the use of elevated temperatures. Wirsing et al. [30] found no difference between extraction in 5% acetic acid at 20 and 40°C. Metcalf

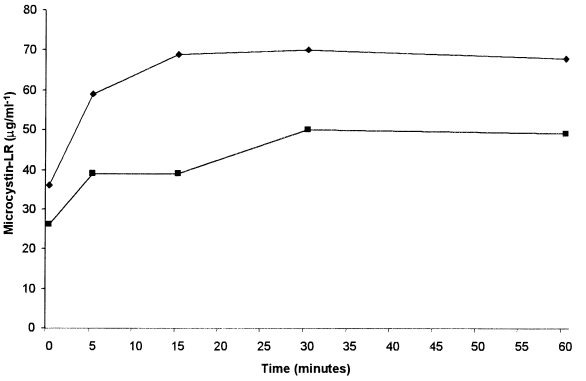

Fig. 4. Extraction of microcystin-LR from lyophilized cells of *Microcystis aeruginosa* PCC 7813 using 5% acetic acid over 1 h. Concentration of microcystin extracted was measured using reversed-phase analytical HPLC in the first (♦) and second (■) extraction.

and Codd [39] explored the use of a boiling water-bath and microwave oven, and found both resulted in rapid (>10 min) and efficient extraction of microcystins with no recorded degradation. However, this was evaluated on the very small cell mass since it was developed primarily as a routine quantification method therefore further tests would have to be performed before it can be recommended for purification scale.

4. Sample concentration

Initial extraction of cyanobacterial cells typically results in large volumes of solvent containing relatively dilute amounts of microcystins which require concentration prior to purification. Several methods have been commonly adopted mainly evaporation and solid-phase extraction. Evaporation is usually carried out using a rotary evaporator at 40°C [22]. In some instances samples are completely dried then resuspended in a suitable solvent followed by centrifugation or filtering to remove particulates before separation is performed [40]. Other methods report the use of rotary evaporation to reduce the volume of the sample and remove some organic solvents (e.g. methanol) prior to solid-phase extraction [26,41]. Both air and nitrogen are also commonly used to dry or reduce the volume of extracts [22,25,42] in a similar manner to rotary evaporation.

C_{18} solid-phase extraction is probably the most commonly used concentration step. Extracts prepared in acetic acid can be directly applied to C_{18} cartridges then eluted with methanol or aqueous methanol (typically around 70%), which can be more selective, leaving a proportion of contaminants on the cartridge. The concentration of extracts on C_{18} can also form the first stage of sample separation if the adsorbed toxin sample is eluted in a selective manner e.g. methanol step gradient. Extracts containing a high proportion of organic solvents need to be modified prior to loading. The most common approach is to reduce the solvent concentration through evaporation although the same affect can be achieved by dilution with water. Both effectively reduce the solvent concentration sufficiently to enable the C_{18} to retain microcystins.

5. Separation

The first *Microcystis* toxins were purified by a combination of dialysis, solvent extraction and column chromatography using DEAE–Sephadex A-25 [43], but it took until 1982 before sufficient pure material was obtained for complete structural analysis [10]. Since then, a wide range of chromatographic techniques has been used to purify large numbers and sometimes large quantities of microcystin variants. In the literature there are no reports of a successful purification using a single procedure, all methods described include numerous purification steps. The number of chromatographic steps may be as little as two or as many as seven, with repetition essential for some steps to process all the extract. This section of the review outlines the separation techniques that have been used along with practical considerations.

5.1. Size exclusion

Botes et al. [10] used size exclusion as the first chromatography step of a two-step procedure giving two pure microcystins. An extract was applied to a Sephadex G-50 column (450 cm×38 mm I.D.) and eluted with 0.1 *M* ammonium hydrogencarbonate solution resulting in a simplified toxic fraction which was further purified by ion-exchange using a DEAE–cellulose column.

Many workers have used size exclusion, usually Sephadex LH-20 from Pharmacia, as an initial separation technique that will allow the removal of pigments and large interfering molecules. This stationary phase is particularly useful as it can be used with organic solvents, facilitating normal-phase partition chromatography as well as size exclusion. Sephadex LH-20 has been widely used for the fractionation of lipids, fatty acids, hormones, vitamins and other small biomolecules. Although there is very little detailed information on the methods used in the literature, Lee et al. [44] recently used a 55×3 cm column for initial clean-up, where the microcystin containing fraction was eluted with methanol at a flow-rate of 13 ml/min. Fractions were analysed by TLC, and those absorbing UV were combined and further purified using normal-phase

flash chromatography, followed by repetitive injections on an analytical reversed-phase column.

Columns packed with Toyopearl HW-40F have often been used as the final purification step due to its mixed mode action, similar to that of the Sephadex LH-20, i.e. a combination of partition–adsorption and size-exclusion chromatography [13,45]. The Toyopearl HW-40F is reported to be more efficient than LH-20 for separating individual microcystins [37].

5.2. Ion exchange

As with size exclusion, ion exchange has typically been used as a preliminary step in the purification of microcystins. Anion exchangers such as quaternary methylamine anion-exchange resins, have been used successfully to semipurify microcystin [46,47]. This has been employed on a small scale either in solid-phase extraction cartridges, or Pharmacia XK 16/10 columns packed with 5 g stationary phase and 0.2 *M* ammonium hydrogencarbonate in methanol–water (30:70, v/v) as the mobile phase. Martin et al. [35], also used this anion-exchange resin for rapid purification of microcystin-LR and nodularin. The separation was optimised at analytical scale and scaled-up eight fold to enable purification of 60 mg of crude toxin fraction. The use of the anion exchange was shown to be very effective for cleaning up, enabling simple and rapid final separation of microcystin-LR and [D-Asp^3]microcystin-LR by semipreparative, reversed-phase HPLC. Final purity of microcystin-LR was 95% by HPLC at 214 and 238 nm.

Botes in 1982 [10], used ion exchange for the final purification of several microcystins from natural bloom samples. A linear salt gradient of 0.005 to 1 *M* ammonium hydrogencarbonate on a DEAE cellulose column (50 cm×19 mm I.D.) was used to separate 2 g of semipure extract to give two microcystins. The same method was also adopted to purify four microcystins from another bloom extract.

5.3. Thin-layer chromatography

Most of the reports in the literature describe the use of analytical TLC or even high-performance analytical TLC (HPTLC) for final purification after column chromatography [48,49]. Final purification of three dehydrobutyrine containing microcystins from *Nostoc* was achieved by HPTLC after semipreparative chromatography [40]. Most reports use silica coated plates and solvents including chloroform, methanol, and water in varying proportions with the separated components visualised using short wave UV irradiation. Spots coeluting with known standards as well as unknowns are scraped off, the silica was extracted in methanol to remove the microcystin, with the silica removed by centrifugation or filtration. Al-Layal et al. [50] used HPTLC as the only technique for the purification of two peptide toxins from *Anabaena flos-aquae*. Crude extracts, from 1 g dried cells, were separated on silica gel 60 plates using methanol–chloroform (3:1). Peptides were detected using a scanning densitometer and the UV adsorbing spots were scraped off and extracted in water before toxicity testing and analysis of absorbance spectrum to identify microcystins.

Namikoshi used preparative TLC as an intermediate step for purifying some microcystins from fractions previously separated on a silica gel column [37]. Although the use of normal-phase analytical TLC is inexpensive and provides complimentary selectivity to other methods, it is only suitable for small quantities (i.e. micrograms not milligrams).

5.4. Flash/column chromatography

The majority of methods reported, especially where a large quantity of cells have been extracted, include an intermediate step following concentration, where glass columns are packed with silica or bonded silica, usually C_{18} and run under gravity or positive pressure. The most widely used silica, Kieselgel 60, has been used to separate micrograms to grams of crude microcystin extract. These methods are rarely optimised and there are very few reports on yields of microcystins based on initial composition of the extract.

Probably one of the largest scale extraction reported, 950 g cells in five batches, used a silica-gel column to partially purify the microcystins after pigments had been removed on the LH-20 column. The sample was separated on a column containing

150 g silica with a mobile phase of chloroform–methanol–water (26:15:3) to give crude microcystin-LR and a fraction containing fourteen other microcystins which were purified by repeated reversed-phase HPLC and silica gel TLC [51]. Lee et al. also employed a silica column but used a different solvent system, ethyl acetate–isopropanol–water (8:5:3), to obtain partially pure microcystin-LR, microcystin-RR and microcystin-RA [44]. The solvent system was optimised on TLC and scaled-up to a 3 cm diameter column, contain 42.5 g silica with a flow-rate of 36 ml/min. Final purification was achieved by repeated injections on reversed-phase analytical HPLC. Some researchers have employed very long columns (94 cm×1.1 cm I.D., ODS silica column) to obtain semipure microcystins, using a mobile phase of methanol–0.05 *M* sodium sulphate (1:1), necessitating an additional desalting step [52].The use of bonded silica, usually C_{18}, is also reported for initial separation of crude microcystin extracts e.g. microcystin-LA has been partially purified on a column containing 5 g YMC gel ODS using a simple step gradient where 60% aqueous methanol was used for final elution [53].

Prepacked, radially compressed cartridges packed with C_{18}, have enabled facile scale-up of microcystin purification, enabling extracts containing approximately 500 mg of microcystins to be partially purified in a single run [15]. The resulting fractions were greatly simplified and the concentration of many minor microcystins was increased making the task of further purification easier. The efficiency of this procedure is particularly well illustrated by the fact that the purity of microcystin-LR increased from 9% purity in the initial extract to 75% after this single step.

Reversed-phase flash chromatography has been found to be particularly suitable for the rapid simplification of multimicrocystin extracts. This is illustrated in Fig. 5 which shows the composition of microcystins in three fractions, from an extract of *Microcystis aeruginosa* PCC 7820, eluted from the reversed-phase flash cartridge using a simple step gradient from 0 to 100% methanol. Such concentration and clean-up steps enable easier subsequent purification steps with higher yields of microcystins [15].

Harada et al. [12] used a large reversed-phase column (800 mm×40 mm I.D.) for initial concentration and clean-up of an extract from 687 g of freeze-dried cells to yield 5.23 g of toxin containing fractions. A 4.2-g amount of this extract was then separated on a silica-gel column (450 mm×22 mm I.D.) using a mobile phase consisting of chloroform–methanol–water (65:25:5, lower layer) to yield 657 mg of crude microcystin-RR and 436 mg of crude microcystin-LR. The crude microcystin-RR was separated on a second silica column (235 mm×20 mm I.D.) using a solvent system of ethyl acetate–isopropanol–water (4:3:7, upper layer) to give 267 mg of semipure compound. The crude microcystin-LR was similarly chromatographed on a second silica gel column to give 228 mg of semipure material. Pure microcystin-RR (122 mg) was finally obtained from a second reversed-phase column using a solvent system of methanol–0.05 *M* sodium sulphate (6:4) along with 14 mg of a minor microcystin. This work is a good example of using the complimentary selectivities of normal and reversed-phase to obtain purified compounds. However, to obtain pure microcystin-LR and remove some minor compounds it was necessary to perform a further separation on preparative HPLC and final purification using Toyopearl HW-40F.

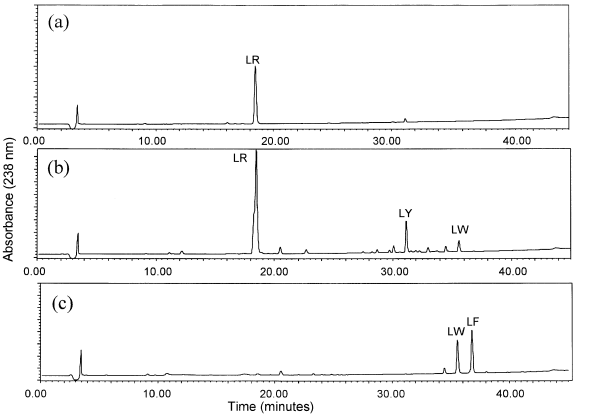

Fig. 5. Microcystins partially purified by step elution from reversed-phase flash chromatography (150 mm×40 mm I.D. column; elution volume 1 l). The fraction eluted with (a) 50% methanol contained microcystin-LR, (b) 60% methanol eluted some microcystin-LR, microcystin-LY and a trace of microcystin-LW and (c)70% gave a fraction containing microcystin-LW and microcystin-LF.

The combination of initial clean-up using reversed-phase flash chromatography, followed by normal-phase has also been adopted to provided a simple and cost effective method for obtaining microcystin-LW and microcystin-LF [14], two closely eluting hydrophobic microcystins which had only previously been purified by a complicated HPLC method [15]. The 60% aqueous fraction eluted from the reversed-phase step was shown to contain predominantly microcystin-LW and microcystin-LF. A small amount of this sample was separated using analytical TLC to determine a suitable solvent system before separation and load were optimised on a small column (15 cm×12 mm I.D.). Once acceptable purities and yields were obtained the method was scaled-up 10 fold. The fractions containing a microcystin with purity >90% by HPLC were pooled with a final reversed-phase flash chromatography polishing step giving 59% recovery of both microcystins with purity of 95%.

5.5. Preparative HPLC

Most methods described in the literature use 1 cm I.D. columns as these can easily be run on analytical systems, very few people have true preparative facilities and usually do not have the material to separate on them. Reversed-phase HPLC is widely used in the purification of microcystins with most purification strategies described containing at least one RP-HPLC step. More often than not this is used for the final polishing step.

C_{18} bonded to silica is the predominant stationary phase reported in the literature on the preparative chromatography of microcystins. The stationary phases were adopted from a broad number of manufacturers which generally implies variation in selectivity, thus a method that works successfully on one column may not give the same result on another. Namikoshi et al. [54] used preparative HPLC with a 300 mm×47 mm I.D. radially compressed cartridges packed with Bondapack C_{18} (55–105 μm) as an alternative to open column or flash chromatography in the early stages of purification. Although more expensive, this approach allows online detection thus removing the need to examine all the fractions to determine which ones contain the compounds of interest. Fractions obtained from this column were then further separated on a semipreparative column using a methanol gradient and final polishing was achieved on an analytical column. Cremer and coworkers successfully purified methylated and nonmethylated microcystin-LR homologues using a silica based stationary phase bonded to C_2 and C_{18} (15 μm particle size) packed into a PepRPC HR 16/10 column [46,47]. Gradients and ion pair reagents were investigated and the resolution was optimised using a Pharmacia fast-protein liquid chromatographic system. The best results were obtained using pentafluoropropionic acid as this enhanced retention on this column along with a shallow gradient.

Many publications describe the use of simple isocratic methods to separate fractions that have been partially purified by a previous chromatographic step, such as flash or ion exchange. This approach usually facilitates shorter, simpler methods along with higher loads. In work involving a complex natural bloom sample this approach yielded a greater number of pure microcystins than obtained by a gradient method where the extract had merely been concentrated, washed and eluted from the reversed-phase flash column. The latter method would have been satisfactory and simpler if the objective was to isolate the predominant component, microcystin-LR only [15,55]. Azevedo et al. [25] reported the use of a semipreparative HPLC (Bondapack C_{18}, 300 mm× 19 mm I.D., 15–20 μm particle size) using an extremely long gradient to purify two microcystins from a Brazilian isolate of *Microcystis aeroginosa*. The crude toxic extract eluted from a reversed-phase Bond Elut cartridge was separated on a Bondapak using a gradient which was isocratic (20 m*M* ammonium acetate– acetonitrile, 7:3) for 90 min followed by an increase to 50% acetonitrile over the next 50 min. Although this was an unusually lengthy gradient, it was essential for obtaining the more hydrophobic microcystin-LF [25].

A similar column was used in another successful approach for purifying large numbers of microcystin variants [3]. Extracts concentrated by solid-phase extraction were initially separated using an isocratic mobile phase consisting of acetonitrile–0.1% ammonium acetate (24:76). Fractions that were toxic by mouse bioassay were further purified on the same column using a solvent system of methanol–0.1%

phosphate buffer (pH 6.8). Fractions were separated using a gradient from 0 to 50% methanol over 25 or 35 min depending on the fraction. The use of different solvent systems resulted in the purification of microcystin-LR along with seven other pure microcystins, which were then fully characterised.

Three dehyrdrobutyrine containing microcystins were purified from a crude extract of cultured *Nostoc*, using a Mightysil RP-18 column (250 mm× 20 mm I.D.) with an isocratic mobile phase of 60% methanol containing 50 m*M* phosphate buffer [40]. In this study the analytical method, used to determine the microcystin composition of the sample, was the same as the preparative method. Final purity was again achieved by HPTLC. This method has also been employed successfully for the purification of two (*Z*)-dehydrobutyrine containing microcystins from a hepatotoxic bloom of *Oscillatoria agardhii* [33].

Microcystin-LR was also successfully purified from a culture extract, using a 47 mm I.D. column packed with ODS and a mobile phase of acetonitrile–0.02 *M* ammonium acetate at 30 ml/min [56]. Acetonitrile was removed from the toxic fraction, and this was reinjected and chromatographed under the same conditions. Final purification was achieved using a Toyopearl HW-40F column (25× 700 mm) with purity determined by HPLC and TLC. Although this is a useful approach for simple samples, there is a potential risk of contamination from more hydrophobic compounds on the second cycle of the method if there is no cleaning step between cycles.

Some researchers have employed techniques other than physicochemical detection to locate and track the purification of microcystins. Boland et al. [57] exploited the biochemical activity of microcystins through a protein phosphatase inhibition assay to determine which peaks were potential microcystins. Active fractions collected from the initial separation on Sephadex LH-20 were then separated on a Vydac C_{18} semipreparative column; fractions, which inhibited the enzyme, were finally purified by analytical HPLC. This method was modified by Craig et al. [58] who used an analytical column (250 mm× 4.6 mm I.D.) instead of a semipreparative column. Final purification of seven microcystins, including microcystin-LL for the first time, was achieved on a narrow bore Vydac C_{18} column. Each active fraction was separated on this using a mobile phase consisting of water plus 0.1% trifluoroacetic acid (TFA) and acetonitrile plus 0.1% TFA. A gradient from 0 to 35% acetonitrile in 20 min followed by an increase to 100% over the next 40 min provided a long gradient capable of separating microcystins of high, medium and low polarity.

Martin et al. [59], used a single analytical HPLC step for the purification of milligram quantities of 7-desmethylmicrocystin-RR from a bloom extract. Crude extract (200 mg) was processed by repeated injections on a 5 μm Superpac Prep-S ODS column (250 mm×4 mm I.D.) from Pharmacia. Analytical columns can be used for purification of small quantities of material and usually they are easily automated. One drawback of any system where large numbers of repetitive injections are necessary to process the sample, is the number of fractions generated. All fractions need to be analysed before they can be pooled, and this can be time consuming whereas a single run on a larger column is considerably more efficient.

A single chromatographic, semipreparative step, using a 100 mm×25 mm I.D. column packed with Nova-Pak C_{18} was used to obtain milligram quantities of microcystins. Good separation was achieved using gradients of water and acetonitrile both containing 0.05% TFA [32]. The use of TFA would appear to be ideal in preparative systems, as it is volatile, and removes the need for a desalting stage. However, this has not been widely adopted probably due to problems experiences during the final concentration step. It has been observed that prolonged rotary evaporation in acetonitrile, water and TFA affects the stability of microcystins as indicated by the appearance of other compounds detected by HPLC. This is demonstrated in Fig. 6 which shows decomposition of microcystin-LR after rotary evaporation in the presence of acetonitrile. The initial sample prior to rotary evaporation (Fig. 6a) contained only microcystin-LR however, once the sample had been dried and resuspended in methanol a second compound was apparent which had the same UV absorbance spectra the parent microcystin (Fig. 6b).

A final point worthy of comment is the need for desalting, which is essential where fractions contain

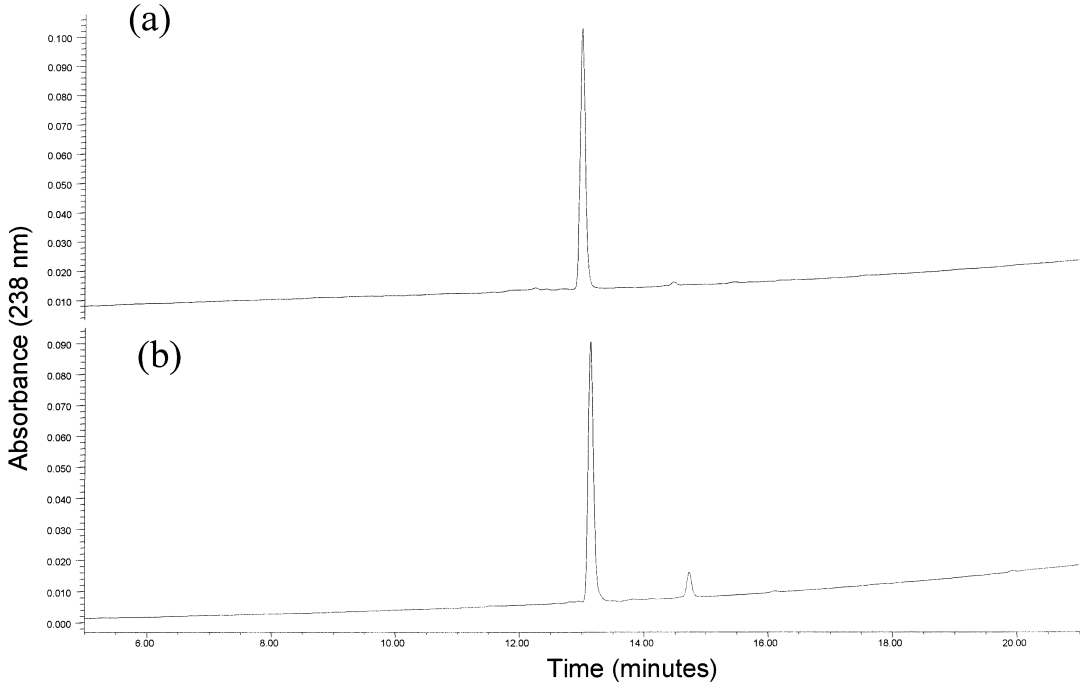

Fig. 6. Analysis of purified microcystin-LR (a) before rotary evaporation and (b) after rotary evaporation at 45°C in the presence of acetonitrile for approximately 1 h.

salts that were used in the mobile phase (e.g. ammonium acetate). This may be achieved by using the same column utilised during purification or another if resources are available. Typically, desalting is achieved by applying the purified sample to the column following dilution to ensure the microcystin is retained. The column is then washed with high purity water and the pure microcystin subsequently eluted in organic solvent, commonly methanol. Most publications describe the reuse of the column for this purpose, although it is often more practical to have a system with a small column, packed with inexpensive, large particle sized material. Another alternative is the use of solid-phase extraction cartridges that are only used once, ensuring that there is no risk of contamination from previous use [25,60].

6. Quantification and purity

Once purification is achieved it is necessary to both quantify the isolated microcystin and evaluate its purity. Analytical RP-HPLC with UV detection is the most common method used to assess both quantity and purity [61] although there are a number of important aspects to consider. Most researchers working in the field will have a set HPLC method that they use to evaluate all kinds of samples. It is important, however, to ensure that this will provide the required information about a purified sample. It is essential that the procedure employed allows sufficient separation permitting the detection of any component that may be copurified. Furthermore, most analytical methods for microcystins monitor the analytes at 238 nm, the λ_{max} of the majority microcystins. However, it is advisable to confirm purity of the isolated toxin by reviewing the chromatography at a less specific wavelength, for example, detection at 214 nm is useful to highlight the presence of a range of compounds with non-specific chromophores [35]. This is usually performed easily as many researchers routinely use photodiode array detection systems allowing data to be accessed

without performing separate analysis. Other methods of evaluating purity should also be considered including the use of TLC and LC–MS. TLC, as previously discussed, has been used for many years to separate and analyse microcystins but there are few published reports on its use in the detection of contaminants. Typically, TLC is visualised by UV, however, probably a less specific method such as iodine or phosphomolybdic acid will give a better indication of the presence of contaminants [34]. It also has the added advantage of being easily available to all researchers. In contrast, LC–MS although becoming increasingly common is not widely available but it does provide another means of evaluating sample purity. The application of LC–MS in the evaluation of microcystin purity is demonstrated in Fig. 7. The UV chromatogram, in this case microcystin-LY, indicates a pure compound yet the total ion chromatogram reveals purity of only 48% due to the presence of contaminants that obviously do not absorb UV. Ultimately it is recommended that wherever possible more than one method should be used to evaluate purity.

Accurate quantification of purified microcystins is often limited by the amount of material produced as this restricts the use of gravimetric analysis. Many reports indicate that sub-mg quantities have often been isolated necessitating quantification by alternative means. Where the exact amount of toxin is not required it may be sufficient to quantify by HPLC and determine the approximate amount using, for example, a microcystin-LR standard. Although, where accurate quantification is needed, the use of amino acid analysis can be considered [22] although this is rather time consuming. This analysis is often carried out to characterise the amino acid composition of a newly isolated microcystin [3,12,25] therefore, provided this is carried out in a quantitative manner, the same data can be used to determine the amount of microcystin purified.

On the other hand, many researchers are carrying out the purification of well-characterised microcystins e.g. microcystin-LR. Through comparison with commercially available microcystin-LR it is possible to confirm the identification and quantify by HPLC. However, it must be noted that although many of the commercially available purified microcystins are referred to, as standards in the literature none are actually sold as such. It is known that some suppliers will dispense 10% more than stated on the product while some researchers report receiving less microcystin that stated. This highlights the

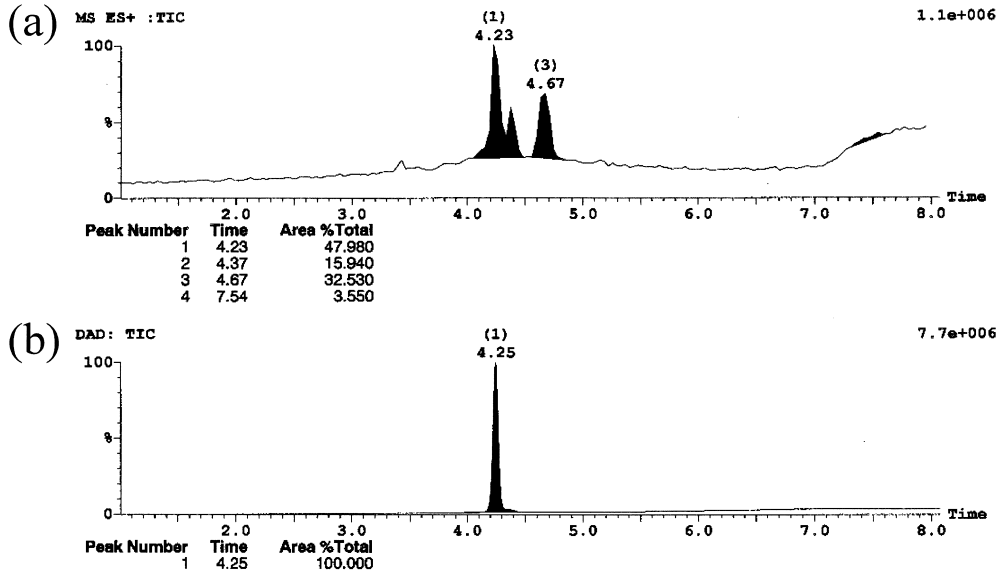

Fig. 7. Analysis of a fraction containing microcystin-LY by HPLC with (a) positive ion electrospray and (b) diode array detection.

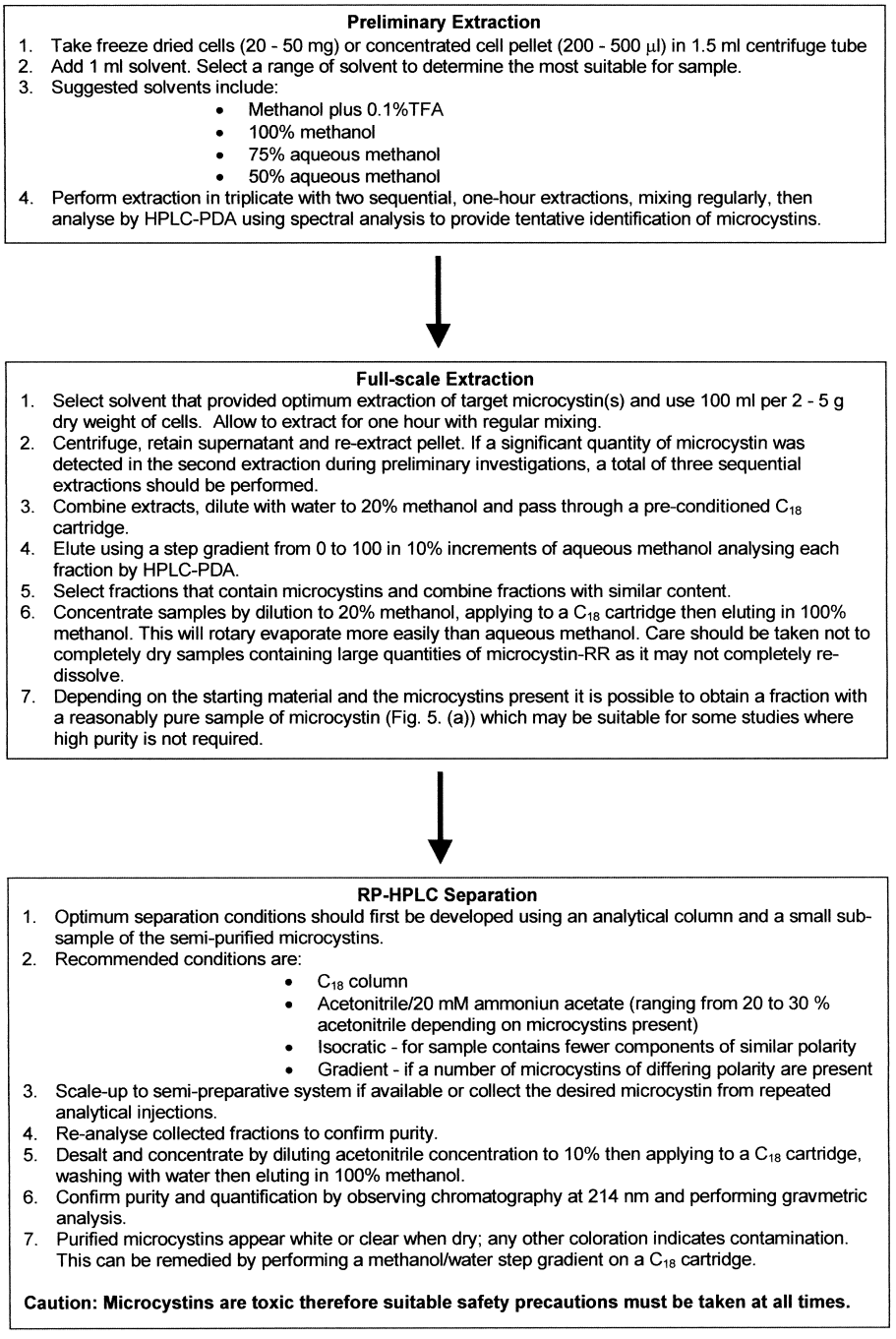

Fig. 8. Recommended strategy for the purification of microcystins.

requirement for certified microcystin standards to enable accurate quantification.

The molar absorptivity for a number of purified microcystins has appeared in the literature [62] and in theory can provide a very simple method of quantifying a solution of purified toxin. However, it is felt that further studies need to be carried out before molar absorptivity can be used with confidence.

Gravimetric measurement is encouraged and can be performed with reasonable confidence where the amount of purified microcystin exceeds a few milligrams [14]. Where larger quantities are available, the masses of replicate aliquots can be determined allowing increased confidence in the values obtained. Of course, accurate determination will be dependent on purity of the sample although the use of gravimetric analysis has been demonstrated to be useful in revealing the presence of contaminants that were not apparent through HPLC alone [14]. This can come about either where the anticipated mass is significantly lower than that obtained, indicating contaminants are contributing to the total mass, or where the visual appearance of the dried sample, e.g. yellow or green colouration where purified microcystin is expected to be white, indicates impurities are present.

Finally, there are some other methods that, although not specifically reported as techniques used to quantify purified microcystins, are worthy of consideration. For example, quantification of the oxidised product from the Adda side chain [3-methoxy-2-methyl-4-phenylbutyric acid([MMPB)] permits accurate determination of the total amount of microcystin present [63,64]. Further discussion of the analysis of microcystins using chromatographic methods can be found elsewhere [65].

7. Conclusion

It is clear that the most common approaches to microcystin purification, include a solvent extraction followed by concentration and sometimes clean-up step on C_{18} which may be packed in a glass column or prepacked in solid-phase extraction cartridges or specialised chromatography columns. HPLC is particularly popular as the main method of separation although few are employing large-scale preparative systems. This is probably because of the prohibitive cost to most investigators along with limited availability of starting material.

Analysis of the literature reveals that there are very few published examples where a coherent approach has been adopted for the purification of microcystins, i.e. small-scale studies initially applied to optimise a method followed by scale-up, thus maximising yields of an often scarce resource. It is possible that much of this work is performed but omitted from final reports. Researchers must be encouraged to comment in publication on the unsuitability of methods they have evaluated along with reporting the method finally employed as this will generally be of benefit particularly to those new to the field.

Finally, it is recommended that prior to commencing a full-scale purification of microcystins it is advisable to first determine the initial microcystin composition and then ascertain the aim of the purification. An outline providing a recommended purification strategy is shown in Fig. 8. In all instances where practical, the optimum procedure should be developed on the microscale before the whole sample is committed to the lengthy extraction and clean-up process.

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