Determination of microcystin-LR in drinking waters by HPLC 1994

Methods for the Examination of Waters and Associated Materials

This method was developed as part of a wider research program into algal toxin analysis and was co-funded by the Foundation for Water Research and National Rivers Authority. The evaluation of the performance testing of the method was funded jointly by the Department of the Environment and Foundation for Water Research. The results of the full performance testing are shown in Table 1.

Chromatographic methods are very sensitive to minor physical and chemical variations in the quality of materials and apparatus used. The method in this booklet reports the materials actually used in the evaluation tests, but in no way endorses these materials as being superior to other similar materials. Equivalent materials are acceptable and it should be understood that the performance characteristics of the method may differ with other materials used. It is left to users to evaluate these methods in their own laboratories.

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In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

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1.0 General principles of sampling and accuracy of results
2.0 Microbiological methods
3.0 Empirical and physical methods
4.0 Metals and metalloids
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Every effort is made to avoid errors appearing in the published text. If however, any are found, please notify the Secretary.

Dr D WESTWOOD
Secretary

27 December 1993

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with The Health and Safety at Work etc Act 1974 and any regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 1988 SI 1988/1657. Where particular or exceptional hazards exist in carrying out the procedures described in this booklet then specific attention is noted. Numerous publications are available giving practical details on first aid and laboratory safety and these should be consulted and be readily accessible to all analysts. Amongst such publications are those produced by the Royal Society of Chemistry, namely 'Safe Practices in Chemical Laboratories' and 'Hazards in the Chemical Laboratory, 5th edition, 1992'; by Member Societies of the Microbiological Consultative Committee, 'Guidelines for Microbiological Safety, 1986, Portland Press, Colchester'; and by the Public Health Laboratory Service 'Safety Precautions, Notes for Guidance'. Another useful publication is produced by the Department of Health entitled 'Good Laboratory Practice'.

Determination of microcystin-LR in drinking waters by HPLC

Introduction

The cyclic heptapeptide hepatotoxins produced by various genera of cyanobacteria (blue-green algae) are collectively known as microcystins. They are potent mammalian toxins, and there is concern regarding their potential presence in both raw waters and drinking waters, particularly when significant numbers of blue-green algal cells are present in raw water storage reservoirs.

The chemical structure of the first microcystin to be fully identified was determined only relatively recently (Ref 1), and reports of new microcystins are still appearing (Refs 2 and 3). More than 50 different microcystin variants have now been identified and microcystin-LR is generally considered to be the most commonly occurring. It is also considered to be one of the most toxic.

This method was developed to detect and quantify microcystin-LR dissolved in drinking waters and reservoir waters. The method described in this publication is only suitable for microcystin-LR soluble in drinking water and cannot be used to determine the 'total' microcystin-LR content (dissolved plus intra-cellular) of environmental samples containing algal cells, although with suitable modifications, for example lysis of algal cells prior to analysis, it is possible to do so. The method has been applied to samples containing more than 10^6 algal cells per mL, but care should be taken to ensure that the filtration procedure does not 'release' intra-cellular toxins. Experience has shown that it is necessary to dilute or pre-treat samples containing high numbers of algal cells to allow filtration to remove them. The limit of detection is, in these circumstances, increased (by the dilution factor), and the performance characteristics of the method, when modified to allow measurement of 'total' microcystin-LR, or when used following dilution to facilitate filtration, have not yet been established.

1 Performance characteristics of the method

1.1 Substances determined

Soluble microcystin-LR (see Figure 1).

1.2 Type of sample

Drinking waters. Raw waters containing algal cells will require pre-treatment.

1.3 Basis of the method

Following a clean-up step, microcystin-LR is extracted from a sample using a solid phase extraction procedure involving a weak cation exchange phase.

The extract is concentrated and analysed using reversed-phase high performance liquid chromatography with ultraviolet detection.

Nodularin, a cyclic pentapeptide hepatotoxin produced by the brackish water blue-green alga *Nodularia spumigena* is used as an internal standard.

1.4 Range of application

The method has been tested for the range 0–10 μgL^{-1}.

1.5 Calibration curve

Linear over the range of application.

1.6 Standard deviation

See Table 1.

1.7 Limit of detection	Nominally 0.5 μgL^{-1}, based on standard deviations of low level spikes.
1.8 Sensitivity	Not determined. Quantification is based on the response and ratio of microcystin-LR to the internal standard.
1.9 Bias	See Table 1. A small positive bias may be apparent for low levels.
1.10 Interferences	See Section 3.
1.11 Time required for analysis	Six hours to obtain a result from receipt of a sample, assuming all reagents are prepared and appropriate apparatus is available. Fourteen man-hours to analyse a batch of eight samples including all preparation time.

2 Principle

The sample is spiked with the internal standard, nodularin, and filtered to remove any low numbers of algal cells that may be present. The filtered sample is passed through a solid phase cartridge (aminopropyl) to remove any interferences, and then through a weak cation exchange (carboxylic acid, CBA) solid phase cartridge. The CBA cartridge is washed to remove interferences and microcystin-LR and nodularin are eluted using 2% trifluoroacetic acid in methanol. This extract is reduced to dryness and the residue redissolved in the mobile phase used for the HPLC analysis.

Microcystin-LR is determined using reversed-phase HPLC with ultraviolet detection, and quantified by comparison of its response with that of the internal standard.

3 Interferences

Any compounds that are not removed during the preliminary clean-up step, and are adsorbed by the CBA cartridge, and are not removed by the washing steps, and are desorbed from the CBA cartridge by the methanolic trifluoroacetic acid and which co-elute with either nodularin or microcystin-LR will interfere with the method.

Many microcystin congeners are known, but only microcystin-LR and microcystin-RR are at present commercially available, and it has not been established whether other toxins interfere with this method. The analysis of a sample which has not been spiked with the internal standard should be undertaken to establish whether nodularin itself is already present in the sample.

4 Hazards

Microcystin-LR and nodularin are potent mammalian toxins, and although no human fatalities have been recorded, numerous cases of animal deaths have been reported worldwide following ingestion of large quantities of blue-green algal cells. As microcystin-LR is an inhibitor of protein phosphatases 1 and 2A, it is possible that it is also a tumour promoter. Appropriate precautions should therefore be taken when handling pure microcystin-LR and nodularin and standard solutions and samples.

Several of the reagents used are potentially hazardous. Methanol is toxic and flammable, trifluoroacetic acid is toxic and corrosive and solutions in methanol should be made up in a well ventilated fume cupboard. Monochloroacetic acid, hydrochloric acid and sodium hydroxide are toxic and corrosive.

5 Reagents

5.1 Microcystin-LR and nodularin.

Microcystin-LR is available from several sources. The material used for the performance testing, and the internal standard, nodularin, can be obtained in septum-sealed vials containing 500 ± 10 μg. Checks should be undertaken to ascertain the comparative purity of samples from various suppliers prior to using as a standard. These checks may include, for example HPLC analysis with UV detection, and comparison of peak heights or areas obtained from apparently equal amounts of microcystin-LR from two or more different sources.

Vials of microcystin-LR and nodularin should be stored in a freezer at a temperature of approximately −18°C.

5.1.1 Standard solutions. Add 500 ± 5 μL of methanol to a septum-sealed vial containing 500 μg of microcystin-LR to give a concentration of 1 $\mu g \mu L^{-1}$. Appropriate dilution of aliquots of this solution with methanol provides suitable spiking solutions; for example dilute 50 μL to 10 mL to give a 5 $ng\mu L^{-1}$ solution which is suitable for low level spiking. Dilution of 200 μL to 4 mL provides a 50 $ng\mu L^{-1}$ solution suitable for high level spiking.

5.1.2 Internal Standard Solution. Add 500 ± 5 μL of methanol to a septum-sealed vial containing 500 μg of nodularin to give a concentration of 1 $\mu g \mu L^{-1}$. Appropriate dilution of aliquots of this solution with methanol provides suitable spiking solutions; for example dilute 100 μL to 10 mL to give a 10 $ng\mu L^{-1}$ solution. Add 25 μL of this diluted solution to 250 mL of sample to give a spiking level of 1 μgL^{-1}.

All solutions should be stored in a freezer (−18°C) when not in use, and checked periodically to ensure that their concentrations remain constant.

5.2 Solvents and reagents.

The methanol used for the HPLC eluent should be of HPLC grade, and double-distilled deionised water should be used for conditioning the solid phase cartridges and for making up the various wash solutions and the aqueous portion of the HPLC eluent.

All other reagents are of analytical grade quality.

5.2.1 1M Sodium hydroxide. Dissolve 40 ± 1 g of sodium hydroxide in 800 mL of water. Cool and dilute to 1000 ± 10 mL with water. Mix well.

5.2.2 4M Hydrochloric acid. Dilute 360 ± 5 mL of hydrochloric acid (d_{20} 1.18) with water and make up to 1000 ± 10 mL with water. Mix well.

5.2.3 Trifluoroacetic acid 1% v/v in water. Dilute 10 ± 0.5 mL of trifluoroacetic acid in water and make up to 1000 ± 10 mL with water. Mix well.

5.2.4 Trifluoroacetic acid 2% v/v in methanol. Dilute 20 ± 1 mL of trifluoroacetic acid in methanol and make up to 1000 ± 10 mL with methanol.

5.2.5 Methanol 30% v/v in water. Dilute 300 ± 10 mL methanol with 700 ± 10 mL water. Mix well.

5.3 Solid phase cartridges.

The solid phase cartridges used for the method development and performance testing were 2 g Bond Elut^R NH_2 aminopropyl (for the clean-up step) and 2 g Bond Elut^R CBA carboxylic acid (for the extraction). Similar cartridges from other manufacturers may also be suitable, but should be evaluated. At no time should the cartridges be allowed to dry out either during conditioning or elution of the samples. The performance of the method can vary considerably with different batches of cartridge materials and supplier. It is important that different batches are performance tested before they are used for routine purposes.

5.4 HPLC eluent.65:35 v/v methanol: monochloroacetate buffer (pH 2.5) containing 0.01M sodium octyl sulphonate.

The monochloroacetate buffer consists of 9.5 gL^{-1} monochloroacetic acid, 20 gL^{-1} sodium hydroxide and 0.2 gL^{-1} disodium ethylenediaminetetraacetic acid. The sodium octyl sulphonate (2.32 gL^{-1} of total eluent) should be added to the monochloroacetate buffer prior to mixing with the methanol, otherwise it will prove difficult to dissolve. The HPLC eluent should be pre-mixed and filtered if necessary as this avoids any potential problems which might occur with high pressure mixing, such as precipitation from the buffer solution. The eluent should also be vacuum de-gassed prior to use. The mobile phase composition may be modified slightly, if necessary, to affect satisfactory resolution, depending on the particular column used.

6 Apparatus

6.1 Syringes and volumetric flasks.

A range of, for example 25 μL and 1 mL glass syringes and 5 mL and 10 mL volumetric flasks should be available in order to prepare standard solutions.

6.2 Sampling bottles.

Sampling bottles should be made of glass. Plastic screw tops are suitable provided they are fitted with PTFE or PTFE-faced liners. Alternatively, ground-glass stoppered glass bottles may be used. Bottles with a capacity of 500 mL allow the collection of duplicate 250 mL samples. Prior to use, the bottles should be cleaned using a suitable proprietary cleaning agent (for example Decon 90), acid washed (4M hydrochloric acid) and rinsed thoroughly with deionised or distilled water.

6.3 Filtering equipment.

An all-glass filter apparatus, for example all-glass 47 mm filter holder fitted with a GF/F (0.7 μm) filter disc or equivalent, attached to a ground glass joint filter flask (1 litre), should be used to remove particulate matter, including small numbers of algal cells and cell debris, from the samples. This prevents problems arising from partial or complete blockage of the solid phase cartridges during the clean-up or extraction stages.

6.4 Extraction apparatus.

Various apparatus can be used for the extraction step of the analysis. An extraction rig, for example that shown in Figure 2 allows several extractions to be undertaken simultaneously, and may be constructed using equipment available from general laboratory suppliers and suppliers of solid phase cartridges. Alternatively, suitable solid phase extraction rigs which can handle varying sample volumes are commercially available.

6.5 Extract concentration equipment.

Although it is possible to reduce the initially produced extract to dryness using nitrogen blow-down at approximately 50°C, this may take some time. The use of concentration equipment such as a Turbo-Vap^{R} (Zymark) concentrator allows concentration from 10 mL to about 0.5 mL in approximately 45 minutes. Nitrogen blow-down can then be used for the final concentration step. Other equivalent evaporator systems may also be used.

6.6 HPLC system.

Any suitable HPLC system with a variable wavelength UV detector operating at 240 nm can be used; (the detector may be of the diode array type). As the chromatographic resolution is markedly influenced by temperature, a column temperature controller capable of maintaining a stable (fixed) temperature in the range 20–30°C is required. The use of a diode array detector will enhance identification and peak purity assessment.

6.7 HPLC column.

The column used is a Spherisorb S3 ODS 1 (150 × 2 mm ID, 3 μm particle size). Other equivalent or similar columns may be used, following evaluation of their suitability.

6.8 HPLC data acquisition.

During performance testing it was noted that laboratories using PC-based computerised data handling systems were able to quantify microcystin-LR at lower levels than those using computing integrators. In the latter case, the lowest level of microcystin-LR that could be quantified in drinking water was 0.5 μgL^{-1}.

7 Sample collection and preservation

Samples should be collected in suitable glass bottles (see section 6.2). The bottles should be thoroughly rinsed with the water to be sampled prior to taking the same for analysis. Microcystin-LR has been shown to be biodegraded when stored in reservoir water for periods of 3–4 days; samples should therefore be analysed as soon as possible following collection. If storage is unavoidable, samples should be kept in a refrigerator at 4°C preferably after filtration.

8 Analytical procedure

CAUTION—before proceeding with the analysis, users should read the whole method carefully, paying particularly attention to section 4.

Step	Procedure	Notes
8.1	**Sample pre-treatment**	
	A sample volume of 250 ± 5 mL is used in the determination. Prior to extraction the pH of the sample should be checked, and if necessary adjusted to pH 7 ± 0.1, (note a). For pH adjustment, appropriate volumes of either 1M sodium hydroxide solution or 4M hydrochloric acid should be used.	(a) Narrow range pH papers are suitable for this purpose.
	Add 25 ± 0.2 μL of a 10 ngμL^{-1} solution of internal standard (5.1.2) below the surface of the sample and mix well, (note b).	(b) This gives an internal standard concentration of 1 μgL^{-1}.
	The sample is then vacuum-filtered through a GF/F filter (0.7 μm) or equivalent using an all glass filter apparatus, (see section 6.3).	
8.2	**Clean-up**	
	A 2 g Bond Elut^{R} aminopropyl cartridge is conditioned by passing through 30 ± 1 mL methanol, followed by 10 ± 0.5 mL water. Discard both eluates. A flow rate of 20 ± 5 mLmin^{-1} is appropriate for this conditioning step, (notes c and d).	(c) At no stage should the cartridges be allowed to dry out, either during the conditioning or the elution of the sample. (d) Several samples can be processed simultaneously (see section 6.4).
	Add 2.5 ± 0.2 mL of methanol to the filtered sample, mix well and then pass through the conditioned 2 g Bond Elut^{R} aminopropyl cartridge at a flow rate of 20 ± 2 mLmin^{-1}.	
	The eluate is retained, and the cartridge is discarded.	
8.3	**Extraction**	
	A 2 g Bond Elut^{R} CBA cartridge is conditioned by passing through 30 ± 1 mL methanol, followed by 10 ± 0.5 mL water at pH 7, (note e). Discard both eluates. The eluate from 8.2 is then passed through the conditioned CBA cartridge at a flow rate of 5 mLmin^{-1}, (notes f and g). Following complete passage of the eluate which is now discarded, the cartridge is sequentially washed with 5 ± 0.2 mL 1% trifluoroacetic acid (5.2.3), 10 ± 0.5 mL water and 5 ± 0.2 mL 30% methanol (5.2.5) and the eluates discarded. The cartridge is air-dried under vacuum for 1 minute. The nodularin and microcystin-LR are eluted from the cartridge using 10 ± 0.2 mL 2% trifluoroacetic acid (5.2.4), (note f).	(e) It may be necessary to adjust the water used in conditioning the CBA cartridge to a pH value of 7 ± 0.1. (f) The flow rate through this cartridge is critical—it should not exceed 5 mLmin^{-1}. Attempts to combine the clean-up and extraction steps, by directly coupling the aminopropyl and CBA cartridges, using a flow rate of approximately 5 mLmin^{-1}, have resulted in a deterioration in the performance of the method. (g) With suitable equipment, several samples can be processed simultaneously (see section 6.4).

Step	Procedure	Notes
8.4	**Concentration of extract**	
	The final extract is reduced to dryness either using nitrogen (dry, purified) blow-down, or a Turbo-Vap^R concentrator, (note h) and the residue taken up in 100 ± 2 μL of the eluent used for the HPLC analysis, (section 5.4).	(h) the use of a Turbo-Vap^R concentrator is recommended as it allows several extracts to be simultaneously concentrated (to about 0.5 mL) relatively quickly. The remaining solvent can then be removed using nitrogen blow-down. If a Turbo-Vap^R concentrator is used, the extract is transferred to a suitable vial, the Turbo-Vap^R tube rinsed with methanol and the rinsing added to the vial prior to reduction to dryness using nitrogen blow-down.
8.5	**HPLC analysis**	
	Column: Spherisorb S3 ODS-1 (150 × 2 mm ID).	
	Eluent: 65% methanol—35% monochloroacetate buffer at pH 2.5. (See section 5.4).	
	Flow rate: 0.20 mLmin^{-1}.	
	Column temperature: 25–27°C, (note i).	(i) The appropriate column temperature which produces the desired chromatographic resolution for nodularin and microcystin-LR should be established for each column used.
	Injection volume: 2–10 μL, via an injection valve, (note j).	(j) An autosampler may be used.
	Detection: UV at 240 nm, (note k).	(k) A scanning photo-diode array detector may be used to aid confirmation of peak purities.
8.6	**Quantification**	
	The ratio of the peak heights obtained for microcystin-LR and nodularin are determined, and from the previously established calibrated curve, the concentration of dissolved microcystin-LR in the sample is determined.	

9 Calibration curve

An internal standard (see section 5.1.2) is used to compensate for the recovery of microcystin-LR which may be variable. A calibration curve is constructed by plotting the ratio of the response from the UV detector for varying amounts of microcystin-LR (see section 5.1.1) compared to a constant level of nodularin (rather than the absolute response for microcystin-LR), against the amount of microcystin-LR present.

It was noted during performance testing that the use of peak heights (rather than peak areas) provided a less variable result for the microcystin-LR: nodularin response ratio, and it is therefore recommended that peak heights are used to calculate this ratio.

For the range 0–10 μgL^{-1}, it is recommended that separate calibration curves are constructed for drinking water and reservoir water samples by spiking blank samples with microcystin-LR at levels of 0, 0.5, 1.0, 2.0, 5.0 and 10.0 μgL^{-1} respectively, and nodularin at a level of 1.0 μgL^{-1}. During performance testing, it was established that the calibration curve was linear over this range.

10 Sources of error

10.1 Contamination

Normal precautions should be taken to ensure that there is no cross-contamination between samples or extracts.

10.2 Solid phase cartridges

Some variation in the microcystin-LR:nodularin response ratio has been noted for different batches of CBA solid phase cartridges. It is recommended that new calibration curves be constructed for each batch of CBA cartridges. See also section 5.3.

10.3 Purity of standards

The purity of the microcystin-LR used to produce the calibration curve should be established. Suppliers should be able to provide this information.

10.4 Interfering substances

As previously noted, the presence of other microcystins in the sample may cause interference by co-elution. As very few microcystins are at present commercially available, it has not been possible to assess this potental problem.

10.5 Presence of algal cells in environmental samples

Experience suggests that for environmental samples containing large numbers of intact algal cells in suspension, a high proportion of microcystin-LR remains within the cell. This intra-cellular toxin is not determined by this method.

Cell lysis during sample storage or processing may lead to release of intra-cellular toxin into the water. This fraction will then be determined with the fraction already in solution and will result in an over-estimation of the level of microcystin-LR originally in solution at the time of sampling.

11 Checking the validity of results

Once the method has been put into routine operation, various factors may subsequently affect the accuracy of the analytical results. Some of these have been noted in section 10. It is recommended that at least one AQC sample is analysed in duplicate with each batch of samples.

12 References

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Table 1 Results from multi-laboratory performance testing*

	Drinking water							Reservoir water						
Laboratory	Level†	Mean†	S_w	S_b	S_t	RSD(%)	DoF	Level†	Mean†	S_w	S_b	S_t	RSD(%)	DoF
1	0.2	0.2277	0.1062	0.0000	0.1062	47	20	0.3	0.2941	0.1541	0.0000	0.1541	52	21
2	0.2	0.2531	0.0336	0.0258	0.0424	17	16	0.3	0.2480	0.0239	0.0365	0.0436	18	12
3	0.2	0.1256	0.0330	0.0643	0.0723	58	10	0.3	0.3472	0.0576	0.1121	0.1260	36	10
4	0.2	0.4230	0.0689	0.0123	0.0700	17	21	0.3	0.4236	0.0626	0.0000	0.0626	15	17
5	0.5	0.7971	0.0689	0.0586	0.0904	11	17	1.0	0.7535	0.1027	0.0747	0.1269	17	18
1	4.0	3.7464	0.3312	0.0616	0.3368	9	21	8.0	7.6591	0.6287	0.0000	0.6287	8	21
2	4.0	4.0476	0.4029	0.0000	0.4029	10	16	8.0	8.3089	0.8125	0.2489	0.8498	10	18
3	4.0	3.6565	0.5309	0.9793	1.1140	28	11	8.0	7.8225	0.8074	1.5710	1.7664	22	13
4	4.0	3.8036	0.2913	0.2510	0.3846	10	17	8.0	8.2795	0.2583	0.3346	0..4227	5	14
5	4.0	4.2605	0.6756	0.1804	0.6993	16	21	8.0	7.6298	1.9313	1.2372	2.2936	30	19

* The laboratories taking part in the performance testing were presented with 11 batches of duplicate samples at the levels indicated.
The participating laboratories were: Lab Services (Yorkshire Environmental), AES Ltd (Northumbrian Water), The Water Quality Centre (Thames Water), Bath University (School of Pharmacy and Pharmacology) and WRc, though not necessarily in the order indicated.
S_w Within batch standard deviation.
S_b Between batch standard deviation.
S_t Total standard deviation.
RSD% Relative standard deviation (%).
DoF Degrees of freedom.
Units expressed in μgL^{-1} unless otherwise specified.

† Level indicates the level at which the samples were spiked; Mean indicates the mean level found.

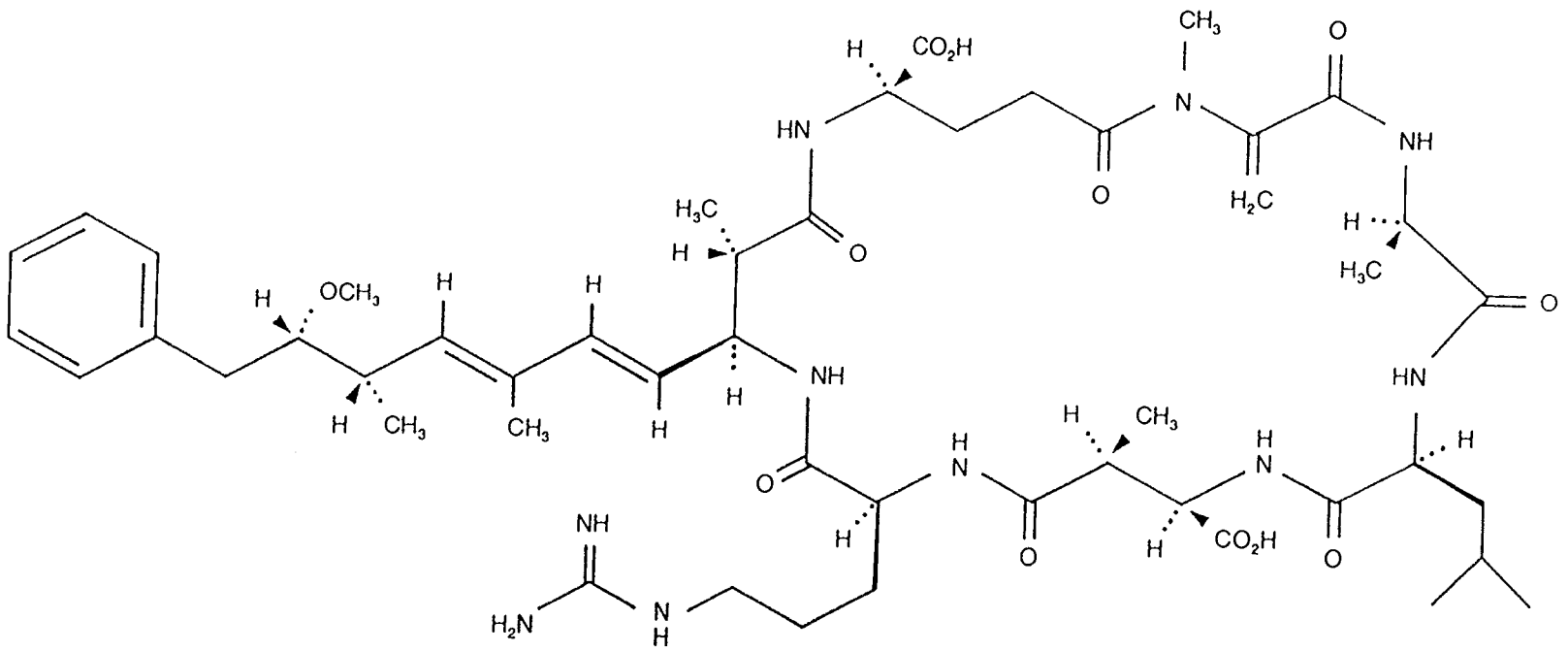

Figure 1 Structure of microcystin-LR

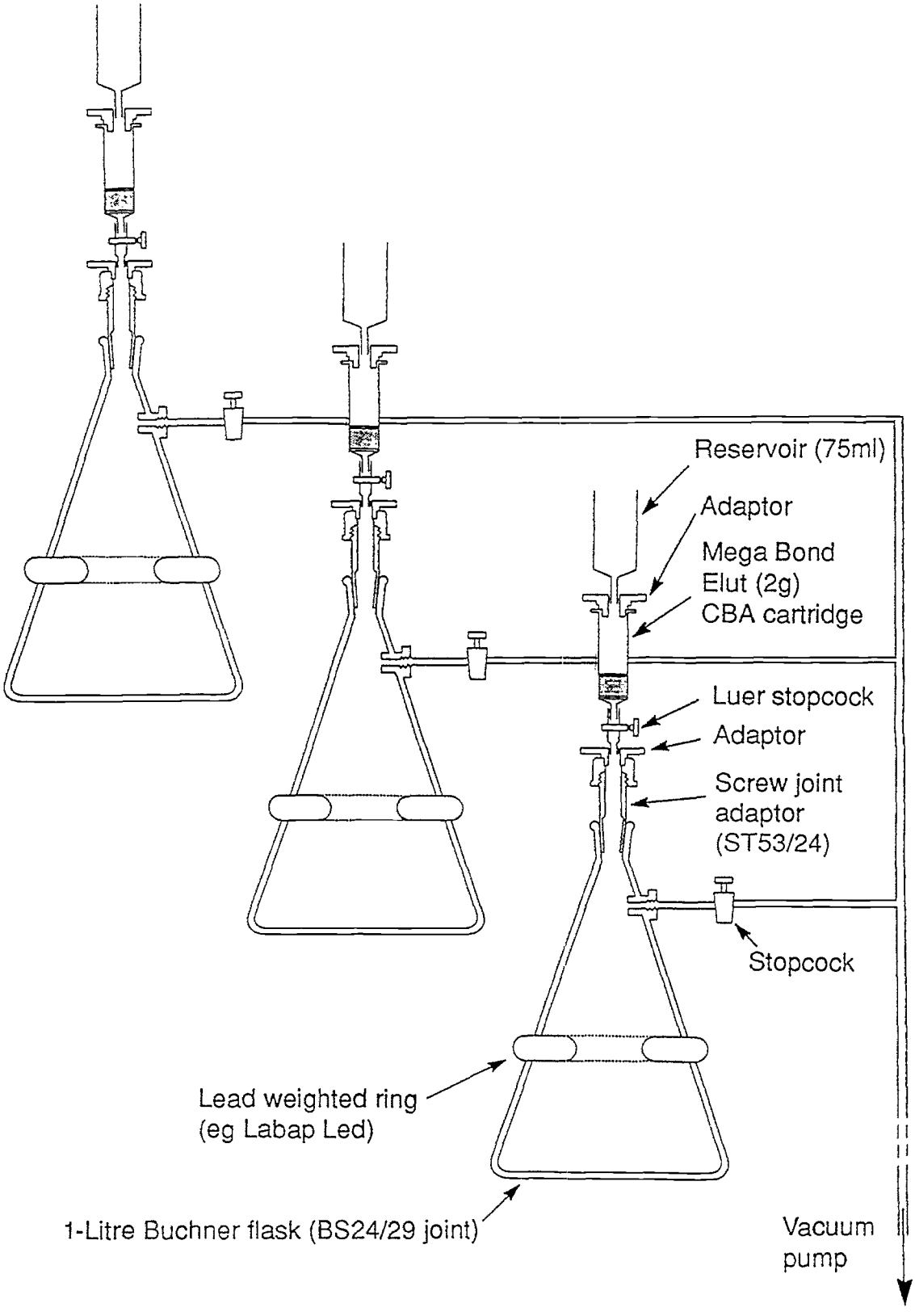

Figure 2 Example of an extraction rig

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