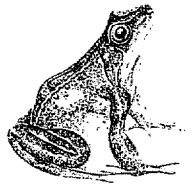
WRc

Microcystin Analysis - Supervision of and results from performance tests

Final Report to the Drinking Water Inspectorate

762



DoE/DWI 4108
FEBRUARY 1996

MICROCYSTIN ANALYSIS - SUPERVISION OF AND RESULTS FROM PERFORMANCE TESTS

Final Report to the Department of Environment

Report No: DoE/DWI 4108

February 1996

Authors: H A James, M J Gardner, J E Ravenscroft and A Sutton

Contract Manager: H A James

Contract No: 09844-0

DoE Reference No: Consultancy

Contract Duration: October 1995 - February 1996

This report has the following distribution:

DoE Nominated Officer - <> copies
Authors

Any enquiries relating to this report should be referred to the Contract Manager at the following address:

WRc plc, Henley Road, Medmenham, Marlow, Buckinghamshire SL7 2HD.
Telephone: 01491 571 531

EXECUTIVE SUMMARY

A method for the determination of several microcystins and nodularin (algal toxins), which was recently published, has been performance tested.

Prior to the performance testing it was necessary to draft the method in the format of the Methods for the Examination of Waters and Associated Materials (MEWAM; SCA Blue Book) series, check the stability of the toxins in water and provide an experimental design for the testing which complied with the normal recommendations provided by the SCA.

Three of the laboratories participating in the performance testing (all from the water supply industry) were chosen following a competitive tendering process and the fourth laboratory was a National rivers Authority (NRA) laboratory.

Following comments from the participating laboratories, and with the agreement of the Drinking Water Inspectorate (DWI), the original method drafted in Blue Book format was modified to reflect current water industry analytical practice.

The performance achieved by three laboratories suggests that:

- the method appears to be applicable over a range between 1 and 10 $\mu g\ l^{-1}$.
- the total standard deviation of the results should not exceed 0.5 µg l-1 or 10%, whichever is the greater, depending on the determinand concentration in the sample.
- the bias should not exceed 1.0 $\mu g\ l^{-1}$ or 20%, whichever is the greater, depending on the determinand concentration in the sample.

However the markedly poorer performance found by one participating laboratory indicates that the technique may not be robust, and that the potential performance indicated above may not necessarily be achieved by all users of the method.

The following should be taken into account when evaluating the results of the performance testing of the method:

- the test was set up and carried out over a relatively short timescale but there is little evidence that performance improved in the later batches of the test; this suggests that experienced laboratories can set up the technique without a great deal of preparation.
- the procedure tested was modified from that in the original published paper; from comments recieved it is likely that further modifications would be incorporated for use in routine analysis.

CONTENTS Page

APPENDICES

LIST OF TABLES

1. INTRODUCTION

Microcystins are cyclic heptapeptide hepatotoxins produced by certain strains of various species of blue-green algae (cyanobacteria). Over fifty have now been characterised worldwide. All contain an unusual β-amino acid (3-amino-9-methoxy-10-phenyl-2,3,8,-trimethyldeca-4,6-dienoic acid), although this moiety may be modified (e.g. geometrical isomerism at the C=C double bonds, or replacement of the C_9-methoxy group by an acetoxy group) in some microcystins.

One of the more toxic and widely occurring microcystins in the UK is microcystin-LR. A method for its determination in water was developed some years ago (James & James 1991), and following performance testing (James 1993), it was adopted as a method in the Methods for the Examination of Waters and Associated Materials (MEWAM) series of publications (commonly referred to as "Blue Books") (HMSO 1994).

A method for the determination of several microcystins was recently published (Lawton *et al.* 1994), and some performance data presented. Given the need for a multi-microcystins method, the Drinking Water Inspectorate (DWI) invited tenders for a consultancy contract to supervise inter-laboratory performance tests of this method in order to more fully evaluate it prior to considering whether it should be adopted as a MEWAM method.

The laboratories participating in the performance testing needed unambiguous instructions regarding the handling, storage, spiking, sub-sampling and analysis of samples, and in order to facilitate this the paper published by Lawton *et al.* needed drafting in MEWAM format; this task was part of the project specifications. In order to establish how best to undertake the performance testing, it was also necessary to check the stability in water of the various microcystins to be used in the testing.

Following a competitive tendering process, WRc were awarded a contract to undertake the work.

2. OBJECTIVES

The objectives of this work were as specified in the contract brief supplied, and were as follows:

- to redraft the method published in the "Analyst", July 1994, Volume 119 pp. 1525-1529, entitled "Extraction and high performance liquid chromatographic method for the determination of microcystins in raw and treated waters", by L.A. Lawton, C. Edwards and G.A. Codd, into MEWAM format;

- to establish that the stability of samples was satisfactory for distribution and analysis over a ten day period;

- to develop a protocol capable of establishing limits of deviation and detection for the HPLC analytical system for microcystin-LR, microcystin-RR, microcystin-LY, microcystin-LW, microcystin-LF and nodularin;

- to evaluate the results of the collaborative trial, to collate the estimates of limits of detection and deviation and to assess whether the performance of the method is satisfactory.

Following the award of the contract to undertake this work, discussions took place between WRc and DWI regarding the timescale of the second objective (i.e. stability testing over a ten day period), as WRc considered that none of the laboratories participating in the performance testing could complete the necessary work within a ten day period. There was also concern that a ten day period would allow insufficient time for an acclimatisation period, which often precedes biodegradation (in the case of microcystin-LR, one study (Jones and Orr 1994) suggested that this could be 8-9 days), and subsequent biodegradation to become detectable; work previously undertaken by WRc (Bealing *et al.* 1993) indicated that microcystin-LR had a half-life of 4-5 days in reservoir water.

It was agreed that the stability tests would extend over sixteen days.

It also proved impossible to fully comply with the third objective, as only three microcystins (-LR, -RR and -YR) and nodularin are currently commercially available. Other potential suppliers were approached, but no additional microcystins were available in sufficient quantities (5-10 mg) for the inter-laboratory performance testing. With the agreement of DWI, the performance testing was restricted to the toxins which were commercially available.

3. STABILITY TESTING

Prior to undertaking the stability testing, the method to be used for monitoring the concentrations of the microcystins and nodularin was applied on several occasions, to ensure that analytical personnel were accustomed to its use and that reproducible results could be obtained.

Following the successful completion of this preliminary work, an experiment was designed to investigate the stability of these compounds. This involved spiking reservoir water samples (sample volume 1 litre; four replicates) with microcystin-RR, microcystin-LR, microcystin-YR and nodularin (so that the concentration of each of the toxins was 10 $\mu g\ l^{-1}$) on five occasions over a sixteen day period (days 0, 5, 8, 12 and 16). On day 16, all samples were analysed.

The concentrations of each of the toxins found in the samples for days 0 through 16 are given in Table 3.1, and it can be seen that after 5 days the levels of all were significantly lower, compared to the initial levels.

Table 3.1 Results of stability testing of microcystins-LR, -RR and -YR and nodularin in reservoir water; initial concentration of each toxin 10 $\mu g\ l^{-1}$

	Microcystin-LR	Microcystin-RR	Microcystin-YR	Nodularin
After 0 days	8.89	9.49	8.22	9.63
	9.19	11.39	11.12	10.57
	9.14	11.80	7.70	11.99
	9.86	10.89	9.12	10.69
Mean	*9.27*	*10.89*	*9.04*	*10.72*
After 5 days	5.51	5.83	4.78	5.79
	5.94	6.17	5.06	6.16
	7.34	9.00	5.14	7.79
	6.84	9.38	5.27	8.39
Mean	*6.41*	*7.60*	*5.06*	*7.03*

Table 3.1 (continued)

	Microcystin-LR	Microcystin-RR	Microcystin-YR	Nodularin
After 8 days	0.13	0.74	0.00	2.58
	1.00	0.43	0.30	0.23
	0.17	0.42	0.00	0.11
	0.29	0.90	0.00	0.00
Mean	*0.41*	*0.24*	*0.15*	*1.24*
After 12 days	0.00	0.87	0.18	1.24
	0.54	0.36	0.45	0.20
	0.49	1.02	0.35	0.09
	0.62	0.00	0.45	0.19
Mean	*0.62*	*0.56*	*0.36*	*0.43*
After 16 days	0.92	1.18	0.39	0.00
	0.45	0.42	0.49	0.18
	1.95	1.52	0.12	0.61
	0.75	1.03	0.00	0.00
Mean	*1.02*	*1.04*	*0.25*	*0.20*

After 12 days the concentrations had dropped to less than 90% of the original values. It was therefore obvious that for the performance testing exercise it would not be possible to circulate spiked samples for anlysis; the participating laboratories would need to spike the water samples to be analysed immediately prior to the commencement of the analysis.

4. RE-DRAFTING OF PUBLISHED METHOD INTO SCA "BLUE BOOK" FORMAT

The published method (Lawton, Edwards and Codd 1994) Extraction and high performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst **119** 1525-1529) was drafted into SCA "Blue Book" format, and copies of the draft sent to the DWI, the laboratories participating in the performance testing and two of the authors of the published paper. The latter were satisfied that this initial draft was an accurate reflection of their method.

However, there was immediately some concern expressed by the participating laboratories regarding the volume of work involved and the time which was available to carry this out. This was based on the requirement of the method to analyse each sample in duplicate, with and without standard addition of an internal standard i.e. four extractions and HPLC analyses were required to produce a result for each sample. Currently the SCA requirement for performance testing involves the analysis of up to 154 samples (see Section 5), which would lead to over 600 extractions and HPLC analyses and many additional HPLC analyses (up to 200) to produce calibration curves and for calibration checks.

Representations were made to the DWI regarding the experimental design implicit in the published method. It was pointed out that no exisitng SCA "Blue Book" methods

i) utilise standard addition as a means of quantification, or

ii) involve duplicate analyses.

It was also considered that even if the method as drafted was found to be satisfactory, following performance testing, it was unlikely to be widely used because of its complexity. Following discussions between the DWI and the laboratories it was agreed that the method should be amended to reflect current water industry practice.

The method was therefore redrafted by WRc to reflect these changes, and approved by DWI prior to distribution to the participating laboratories some time before the performance testing commenced. This final draft is given in Appendix A of this report.

5. EXPERIMENTAL DESIGN FOR PERFORMANCE TESTING

The experimental design for the performance testing was intended to ensure that the method was tested to the currently accepted SCA standard. The instructions sent to the participating laboratories are given in full in Appendix B, and are summarised below.

The analytical range of primary interest was considered to be between 0 and 10 $\mu g\ l^{-1}$ (a gudeline level for drinking water of 1 $\mu g\ l^{-1}$ has been suggested for microcystin-LR (Fawell *et al.* 1993)), and the aim of the performance testing was to assess the precision and some sources of bias for the determination of the specified toxins in raw and drinking waters. It was emphasised to the participating laboratories that they should adhere to the exact details of the method, otherwise the whole exercise would be rendered meaningless.

As the stability testing undertaken by WRc had demonstrated that the toxins were not stable in raw water for a sufficiently long period for spiked water samples to be circulated and analysed, it was necessary for the participating laboratories to spike water samples immediately prior to analysis. In order to remove any variation due to different laboratories using toxins from different suppliers (or different batches of toxins) it was agreed that WRc would make up a bulk solution containing appropriate concentrations of each toxin, and that the participating laboratories would be supplied with aliquots of this solution to spike the water samples. These were delivered by WRc personnel to the participating laboratories.

Bulk samples of the waters to be used for the performance testing were collected by WRc staff. The raw water was obained from the River Goyt (above the Goyt Valley reservoir) in Derbyshire, which is a soft upland water. The treated water was obtained from Farmoor water treatment works; this water is hard and lowland river-derived. Each of these bulk samples was filtered through a 0.2 μm filter (which , in effect, sterilised the sample), thoroughly mixed in bulk and dispensed into 2.3 l Duran glass screw-topped bottles. The screw caps (red 'Resinol') were fitted with PTFE-faced liners. Participating laboratories were provided with a sufficient volume of each water for the performance testing and an allowance made for mishaps or breakages.

The performance testing involved analysing the following samples:

- a blank-spiked (0.5 $\mu g\ l^{-1}$) deionised water
- a low-spiked (2.0 $\mu g\ l^{-1}$) raw water
- a high-spiked (8.0 $\mu g\ l^{-1}$) raw water
- a low-spiked (2.0 $\mu g\ l^{-1}$) treated water
- a high-spiked (8.0 $\mu g\ l^{-1}$) treated water

- a low-spiked (1.0 $\mu g\ l^{-1}$) deionised water
- a high-spiked (9.0 $\mu g\ l^{-1}$) deionised water

Each participating laboratory was instructed to carry out two replicate determinations, in random order, of each of the above test samples, in each of eleven batches of analysis.

6. ASSESSMENT OF RESULTS FROM PERFORMANCE TESTING

Whenever a new analytical method is developed, it is important that potential users are provided with an indication of the standard of performance which can be achieved (or expected). However, there is no widely agreed means of providing such information. The process of method validation, is not clearly defined and can be the subject of misunderstanding. The main points concerning method validation and the approach to method testing recommended by the DOE Standing Committee of Analysts (SCA) are summarised below.

It is important in any consideration of method validation to dismiss at the outset the widely held misconception that an analytical method has an associated "performance" - i.e. a standard of accuracy for the results which are produced when the method is used. This false idea has lead to approaches to method validation using ring-tests and other interlaboratory exercises (e.g. collaborative trials). Unless such tests are designed with great care and the participants are carefully vetted for experience, the results tend to reflect a combination of the capability of the chosen analytical technique and the competence and facilities of the laboratories chosen for the test. The key point concerning method validation is that a method is a set of instructions for the analyst; it does not possess an inherent performance, only the potential to be used to achieve certain standards of accuracy.

Performance tests carried out by the laboratory which developed the method can give a falsely optimistic picture of the method's capability for a number of reasons. Firstly, the analysts at this laboratory are more familiar with the procedures which constitute the method than staff at other as yet inexperienced laboratories. Secondly, the developers of the method may also have got into the habit of carrying out some key aspects of the technique in a particularly favourable way (which may not be clearly specified in the written method). Finally, a research laboratory which develops new techniques will often not be in a position to test those techniques under the conditions of routine analysis - where the blend of analytical skills, the higher workload and application of automation might be relevant to the performance which is achieved. Hence it is important to test the capabilities of a new method by applying it under similar conditions to those under which it is likely to be used routinely.

The Standing Committee of Analysts has defined a programme for method validation which involves testing in approximately five experienced laboratories. Each laboratory incorporates the chosen method into its own analytical system and undertakes tests to estimate with adequate reliability the precision of analysis (the total standard deviation of results) across the analytical range and for a variety of sample types. The main types of systematic error are also examined. The standard of performance achieved in this type of test is interpreted in two principal ways. The estimates of total standard deviation and recovery obtained in an individual laboratory are clearly a guide to the accuracy of analytical data which might be produced by that laboratory's analytical system. The comparability of performance between the different participants is an important guide to

the robustness of the method. Poor overall comparability is usually taken to indicate that an important aspect of technique may not have been defined in sufficient detail - leading to significant differences in the way in which the method is used in the different laboratories.

This SCA approach to performance testing has been followed for these studies on the determination of algal toxins. Two minor modifications of the approach were used. For this work, participants carried out the programme of tests on the same samples. This did not give as wide a coverage of sample types as the conventional approach in which laboratories use their own samples. However, in this case, the use of identical samples made it possible to set up the test quickly and it allowed direct comparison to be made between the results of all participants. (Normally only the data for standard solutions are directly comparable.) The second deviation from the usual approach involved the use test samples prepared using determinand from the same source as that used in calibration. This limited the sources of bias which could be tested to those caused by the sample matrix - interferences.

The results from individual laboratories are summarised in Table 6.6-6.9, and the overall performance achieved in the tests (excluding one laboratory's results - see discussion below) has been used to provide a guide to the capability of the method for each of the individual determinands, which is given in Tables 6.2-6.5.

Estimates of the limits of detection achieved by the four participating laboratories are given in Table 6.1.

Table 6.1 Estimates of Limit of Detection ($\mu g\ l^{-1}$)

Laboratory	Microcystin-LR	Microcystin-RR	Microcystin-YR	Nodularin
1	0.3	0.6	0.6	0.4
2	0.4	0.5	0.5	0.4
3	2.0	2.8	2.1	0.9
4	0.3	0.3	0.4	0.3

These estimates of Limit of Detection have been obtained with 11 degrees of freedom. Given this level of reliability and assuming that the estimates obtained by the different laboratories were all of the same underlying limit of detection, differences of a factor of approximately 2x might be expected to occur by chance (p0.05). Hence three estimates in the table above do not differ significantly from one another (laboratories 1,2, and 4); the estimates from the fourth laboratory are clearly (and significantly) larger than the rest. A comparison of the performance achieved by the laboratories (Tables 6.6-6.9) reveals a similar picture - the precision and control over bias achieved by laboratory 3 is generally

poorer than, and distinct from, that achieved by the other three laboratories. This situation leads to two important conclusions:

i) it confirms the general principle referred to above that an analytical method does not possess an inherent performance. The accuracy of data produced depends on a collection of circumstances including the choice of method, the experience in its use, the equipment and time available, etc.

ii) it cannot be assumed that the generally satisfactory performance of the three laboratories (1,2, and 4) will be achieved by other users. The relatively poor performance of laboratory 3 is a valuable indication that the technique is not robust.

Thus, the data for method capability in Tables 6.2-6.5 represent the consensus performance exhibited by laboratories which appeared to have achieved good control over the main sources of error which might affect the method. That is, the performance data reflect "an achievable best case". They are clearly not an indication of the quality of data which could be produced by all potential users of the method.

Further investigation of the circumstances which might have contributed to the interlaboratory differences (and similarities) is recommended. Both methodological and organisational approaches should be examined.

Table 6.2 Summary performance data$^{\phi}$ for microcystin-LR (units - $\mu g\ l^{-1}$)

Determinand - Microcystin LR

Performance Characteristic	Typical Value	Range Observed in Initial Performance Tests
Limit of Detection	0.3	0.3-0.4
Total Standard Deviation of Results (rsd % in brackets) at determinand concn. of :		
1 µg/l *	0.15	0.10-0.21
2 µg/l +	0.3	0.15-0.36
8 µg/l +	1.0	0.70-1.73
9 µg/l *	0.9	0.69-1.04
Bias	No important bias for samples tested - recovery check recommended for real samples.	

* - for standard solutions in deionised water;+ - for samples of raw and potable water; ϕ - only data from laboratories 1,2 and 4 considered.

Table 6.3 Summary performance data$^{\phi}$ for microcystin-RR (units µg l^{-1})

Performance Characteristic	Typical Value	Range Observed in Initial Performance Tests
Limit of Detection	0.5	0.3-0.6
Total Standard Deviation of Results (rsd % in brackets) at determinand concn. of :		
1 µg/l *	0.3	0.19-0.98
2 µg/l +	0.3	0.23-0.44
8 µg/l +	1.0	0.77-1.35
9 µg/l *	1.0	0.80-1.51
Bias	No important bias for samples tested - recovery check recommended for real samples.	

* - for standard solutions in deionised water; + - for samples of raw and potable water; ϕ - only data from laboratories 1,2 and 4 considered.

Table 6.4 Summary performance data$^{\phi}$ for microcystin-YR (units µg l^{-1})

Performance Characteristic	Typical Value	Range Observed in Initial Performance Tests
Limit of Detection	0.5	0.4-0.6
Total Standard Deviation of Results (rsd % in brackets) at determinand concn. of :		
1 µg/l *	0.2	0.11-0.26
2 µg/l +	0.4	0.16-0.42
8 µg/l +	0.8	0.66-1.41
9 µg/l *	0.8	0.78-1.12
Bias	No important bias for samples tested - recovery check recommended for real samples.	

* - for standard solutions in deionised water; + - for samples of raw and potable water; ϕ - only data from laboratories 1,2 and 4 considered

Table 6.5 Summary performance data$^{\phi}$ for nodularin (units $\mu g\ l^{-1}$)

Performance Characteristic	Typical Value	Range Observed in Initial Performance Tests
Limit of Detection	0.4	0.3-0.4
Total Standard Deviation of Results (rsd % in brackets) at determinand concn. of :		
1 µg/l *	0.15	0.09-0.19
2 µg/l +	0.2	0.12-0.47
8 µg/l +	0.8	0.66-1.21
9 µg/l *	0.8	0..78-1.21
Bias	No important bias for samples tested - recovery check recommended for real samples.	

* - for standard solutions in deionised water; + - for samples of raw and potable water; ϕ - only data from laboratories 1,2 and 4 considered

The results from individual laboratories are summarised in Tables 6.6-6.9.

Table 6.6 Laboratory 1

Microcystin-LR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.51	2.29	8.50	2.16	8.76	1.02	9.16
s_t	0.13	0.36	1.43	0.41	1.73	0.21	1.04
rsd %	26	16	17	19	20	20	11
Bias %	2	14	6	8	10	2	2
bias - sig?	ns	s	ns	ns	ns	ns	ns

Microcystin-RR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.52	1.40	5.73	1.98	7.67	0.95	7.20
s_t	0.15	0.23	0.77	0.42	1.55	0.19	0.80
rsd %	28	17	14	21	20	20	11
Bias %	4	-35	-28	-1	-4	-5	-20
bias - sig?	ns	s	s	ns	ns	ns	s

Microcystin-YR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.58	2.57	8.88	2.24	8.99	1.15	9.08
s_t	0.14	0.42	1.70	0.38	1.34	0.20	1.48
rsd %	29	16	19	17	15	18	16
Bias %	16	28	11	12	12	15	1
bias - sig?	s	s	s	s	s	s	ns

Nodularin

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.52	1.97	7.67	2.06	8.10	0.99	8.59
s_t	0.08	0.24	1.15	0.47	1.41	0.16	1.21
rsd %	15	12	15	23	17	16	14
Bias %	4	-2	-4	3	1	0	7
bias - sig?	ns	ns	ns	ns	ns	ns	ns

s_t is the estimate of total standard deviation of results - obtained with 10 or more degrees of freedom
rsd % is the value for s_t expressed as a percentage of the sample concentration
Bias refers to the percentage error of the observed mean (x) with respect to the true concentration, µ ie (x - µ).100%/µ
Bias - sig? is an indication of the statistical significance of the observed bias at the 0.05 probability level - s = significant, ns = not significant.

Table 6.7 Laboratory 2

Microcystin-LR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.31	1.94	7.89	1.86	7.88	0.95	8.72
s_t	0.12	0.16	0.70	0.15	0.89	0.10	0.69
rsd %	24	9	9	8	11	11	8
Bias %	-38	-3	0	-7	0	-5	-3
bias - sig?	s	ns	ns	ns	ns	ns	ns

Microcystin-RR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.40	1.72	8.46	2.23	9.59	0.98	9.89
s_t	0.18	0.31	1.39	0.31	1.07	0.18	1.51
rsd %	35	18	16	14	11	18	15
Bias %	-20	-14	6	11	20	-2	10
bias - sig?	s	s	ns	s	s	ns	ns

Microcystin-YR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.38	1.78	7.83	1.90	8.10	1.03	8.69
s_t	0.13	0.17	0.74	0.16	0.99	0.11	0.74
rsd %	27	10	10	9	12	11	8
Bias %	-24	-6	-2	-5	1	3	-3
bias - sig?	s	s	ns	s	ns	ns	ns

Nodularin

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.33	1.92	8.34	2.05	8.27	1.01	8.49
s_t	0.12	0.17	0.66	0.12	0.69	0.09	0.78
rsd %	23	9	8	6	8	9	9
Bias %	-34	-4	4	2	3	1	-6
bias - sig?	s	ns	ns	ns	ns	ns	s

s_t is the estimate of total standard deviation of results - obtained with 10 or more degrees of freedom

rsd % is the value for s_t expressed as a percentage of the sample concentration

Bias refers to the percentage error of the observed mean (x) with respect to the true concentration, µ ie $(x - \mu).100\%/\mu$

Bias - sig? is an indication of the statistical significance of the observed bias at the 0.05 probability level - s = significant, ns = not significant.

Table 6.8 Laboratory 3

Microcystin-LR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	1.30	1.67	6.95	2.00	7.50	1.27	9.35
s_t	0.84	0.85	2.36	0.82	3.00	0.97	4.72
rsd %	168	42	29	41	38	97	52
Bias %	160	-17	-13	0	-6	27	4
bias - sig?	s	ns	ns	ns	ns	ns	ns

Microcystin-RR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	1.08	1.17	5.14	1.63	5.75	0.84	7.22
s_t	0.98	0.58	1.75	0.74	2.95	0.48	4.36
rsd %	196	29	34	37	37	48	48
Bias %	116	-41	-35	-19	-28	-16	-20
bias - sig?	ns	s	s	s	s	ns	ns

Microcystin-YR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	1.72	2.35	7.77	2.58	9.69	1.32	11.6
s_t	1.03	1.22	3.36	1.03	3.48	0.61	5.42
rsd %	206	61	42	51	43	61	60
Bias %	244	17	-3	29	21	32	29
bias - sig?	s	ns	ns	s	ns	s	ns

Nodularin

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	1.08	1.78	7.35	2.09	7.59	0.96	9.27
s_t	0.98	0.86	1.97	0.86	2.65	0.42	4.31
rsd %	196	43	26	43	33	42	48
Bias %	116	-6	-8	5	-5	-4	3
bias - sig?	s	ns	ns	ns	ns	ns	ns

s_t is the estimate of total standard deviation of results - obtained with 10 or more degrees of freedom
rsd % is the value for s_t expressed as a percentage of the sample concentration
Bias refers to the percentage error of the observed mean (x) with respect to the true concentration, µ ie $(x - \mu).100\%/\mu$
Bias - sig? is an indication of the statistical significance of the observed bias at the 0.05 probability level - s = significant, ns = not significant.

Table 6.9 Laboratory 4

Microcystin-LR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.66	2.07	7.91	2.01	7.90	1.13	9.21
s_t	0.11	0.29	0.87	0.35	0.94	0.17	0.90
rsd %	16	14	11	17	12	15	10
Bias %	32	3	-1	0	-1	13	2
bias - sig?	s	ns	ns	ns	ns	s	ns

Microcystin-RR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.67	1.98	7.67	2.03	7.93	1.19	9.30
s_t	0.11	0.29	0.78	0.44	0.92	0.23	0.97
rsd %	16	15	10	22	12	20	10
Bias %	34	-1	-4	2	-1	19	3
bias - sig?	s	ns	ns	ns	ns	s	ns

Microcystin-YR

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.69	2.63	8.42	2.01	7.96	1.25	9.31
s_t	0.13	0.41	0.92	0.42	0.89	0.26	1.07
rsd %	19	16	11	21	11	21	12
Bias %	39	32	5	1	-1	25	3
bias - sig?	s	s	s	ns	ns	s	ns

Nodularin

Test sample	Blank (0.5µg/l)	Raw Water (2µg/l)	Raw Water (8µg/l)	Treated Water (2µg/l)	Treated Water (8µg/l)	Standard Solution (1µg/l)	Standard Solution (9µg/l)
Observed Mean	0.68	2.16	8.02	2.07	7.99	1.16	9.33
s_t	0.09	0.29	0.83	0.18	0.66	0.19	0.78
rsd %	14	15	11	18	12	21	10
Bias %	36	16	0	4	0	16	4
bias - sig?	s	s	ns	ns	ns	s	ns

s_t is the estimate of total standard deviation of results - obtained with 10 or more degrees of freedom
rsd % is the value for s_t expressed as a percentage of the sample concentration
Bias refers to the percentage error of the observed mean (x) with respect to the true concentration, µ ie $(x - \mu).100\%/\mu$
Bias - sig? is an indication of the statistical significance of the observed bias at the 0.05 probability level - s = significant, ns = not significant.

7. CONCLUSIONS

The performance of a method for the determination of four algal toxins in raw and potable waters has been assessed. It appears appropriate to apply the method over a working range between 1 and 10 µg/l.

Prior to performance testing it was demonstrated that none of the toxins were stable for longer than a few days in reservoir waters. This implies that analyses should be undertaken as soon as possible after sample collection.

No consistent picture for bias emerged from the tests. Any biases identified in the tests as significant were relatively small (though see comments below). The fact that significant bias could be detected, despite the use of an internally consistent calibration, probably reflects (a) the high power of the test to detect bias and (b) the fact that small biases can be introduced by slight inconsistencies in blank correction (i.e. use of a slightly inappropriate blank value) and through the process of internal standardisation.

The following accuracy requirements appear to be achievable using the method tested.

Precision: Total standard deviation of results should not exceed 0.5 µg/l or 10%, whichever is the greater, depending on the determinand concentration in the sample.

Systematic error: Bias should not exceed 1.0 µg/l or 20%, whichever is the greater, depending on the determinand concentration in the sample.

Achievement of these accuracy targets would correspond to compliance with a target that the maximum allowable error on a single analytical result should not be larger than 2 µg/l or 40%.

The design of the test complied with the normal recommendations provided by the SCA.

The following comments should be taken into account when evaluating the performance of the method:

a) the markedly poorer performance achieved by one participant in these tests indicates that there are aspects of the technique which may not be robust. From discussions with the laboratory concerned it emerged that experienced staff undertook the work. This implies that the potential performance indicated above may not necessarily be achieved by all users of the method.

b) the relatively short timescale over which the test was set up and carried out meant that the participating laboratories were afforded very little time to become familiar with the details of the method. Having made this point, there is little evidence that performance within a given laboratory improves in the later batches of the test - hence it appears that the technique can be set up without a great deal of preparation.

c) the procedure tested was modified from that in the original published paper. It is likely that the technique, if it were to be used in routine analysis, would be modified further. For example, one laboratory indicated an intention to use nodularin as an internal standard; another suggested changes to the HPLC column and gradient elution conditions. Such modifications are likely to made on the grounds of usability rather than on any marked improvements in performance which might be achieved.

d) this test was carried out using samples which were relatively "clean" compared with samples which might be taken at the time of an algal bloom. It was not possible to provide such samples at the time of year of the test, without an extensive period of pre-testing on the method of sample preparation and distribution. Thus there were no algal cells present in the test samples. Similarly, the tests samples did not contain the quantities of algal cell contents - potential interferents - which real samples might contain. This may have implication for performance for the analysis of real samples. It is recommended that further tests of spiking recovery should be undertaken on such real samples, as a check on systematic error.

REFERENCES

Bealing, D.J., Cousins, I. and James, H.A. (1993) An investigation of the Degradation of Microcystin-LR. FWR Report FR 0379.

Fawell, J.K., James, H.A. and James, C.P. (1993) Toxins from Blue-green Algae: Toxicological Assessment of Microcystin-LR and a Method for its Determination in Water. WRc Report FR 0359/1/DoE 3358/1.

HMSO (1994) Detrmination of Microcystin-LR in Drinking Waters by HPLC (1994). Methods for the Examination of Waters and Associated Materials. HMSO, London.

James, H.A. and James, C.P. (1991) Development of an Analytical Method for Blue-green Algal Toxins. FWR Report FR 0224.

James, H.A. (1993) Determination of Microcystin-LR in Drinking Waters and Reservoir Waters. WRc Report DoE3431/FR 0395.

Jones, G.J. and Orr, P.T. (1994) Release and Degradation of Microcystin Following Algicide Treatment of a *Microcystis aeruginosa* Bloom in a Recreational Lake, as Determined by HPLC and Protein Phosphatase Inhibition Assay. Water Research **28**, 871-876.

Lawton, L.A., Edwards, C. and Codd, G.A. (1994) Extraction and High Performance Liquid Chromatographic Method for the Determination of Microcystins in Raw and Treated Waters. The Analyst **119**, 1525-1529.

APPENDIX A

Determination of microcystins

Determination of microcystins and nodularin in waters by HPLC

Introduction

Cyanobacteria, also called blue-green algae, can produce various toxins. The cyclic heptapeptide hepatotoxins are collectively known as microcystins, and one of the cyclic pentapeptide hepatotoxins is called nodularin. These toxins are initially produced intra-cellularly, and may be released when algal cell walls rupture. As they are potent mammalian toxins, there is concern regarding their potential presence in both raw waters and drinking waters, particularly when significant numbers of cells are present in raw water storage reservoirs.

The method, as published (Ref. 1), describes the determination of microcystins and nodularin dissolved in raw and treated waters (extracellular toxins) and within algal cells (intracellular toxins). Only the determination of dissoed toxins is described in this publication as it is only this that has been performance tested.

1. Performance characteristics of the method

1.1 Substances determined	Soluble microcystins and nodularin
1.2 Type of sample	Raw and drinking waters
1.3 Basis of method	Samples are filtered to remove algal cells and extracted using solid phase extraction cartridges. Following concentration, the extracts are analysed using reversed phase high performance liquid chromatography (HPLC) with gradient elution and ultraviolet (UV) photodiode array detection.
1.4 Range of application	1-10 $\mu g\ l^{-1}$
1.5 Calibration curve	linear over the range 1-10 $\mu g\ l^{-1}$
1.6 Total standard deviation	microcystin-LR 10-21% at 1 μgL^{-1}; 8-12% at 9 μgL^{-1}. microcystin-RR 11-22 at 2 μgL^{-1} 9-17 at 8 μgL^{-1}.

	microcystin-YR 11-26% at 1μgL^{-1};
	9-12% at 9 μgL^{-1}.
	nodularin 6-23% at 2 μgL^{-1};
	8-15% at 8 μgL^{-1}.
1.7 Limit of detection	typically 0.5 $\mu g\ l^{-1}$
1.8 Sensitivity	not determined
1.9 Bias	in the performance test bias, did not exceed 1.0 $\mu g\ l^{-1}$ or 20%, whichever is the greater
1.10 Interferences	none detected (but see **9.3**)
1.11 Time required for analysis	Results may be obtained within 24 hours of the commencement of sample processing; depending on the availability of equipment, several samples may be extracted simultaneously and analysed sequentially so that several results may be available within this period.

2. Principle

Samples are filtered to remove algal cells. The filtrate is then extracted using C_{18} solid phase extraction cartridges. The cartridges are washed with aqueous methanol prior to elution with acidified methanol. The acidified methanol extracts are reduced to dryness, redissolved in methanol and again reduced to dryness. The residue is taken up in 70% aqueous methanol, centrifuged to remove solids if necessary, and a portion of the supernatant analyzed by reversed-phase high performance liquid chromatography with UV detection at 238 nm, using a photodiode array detector operating in the range 200-300 nm.

The UV spectra of peaks detected on the LC-UV chromatogram are produced and compared to typical microcystin UV spectra which fall into two groups, one group having an absorption maximum at 238-240 nm and the other group (tryptophan- containing microcystins) with a maximum at 222 nm. All of the peaks on the LC-UV chromatogram for which there is good correspondence between the spectra obtained and those from microcystin standards are assumed to be microcystins.

Quantification of microcystin responses is carried out using a standard curve based on the peak areas obtained for pure microcystin standards. Results are corrected for incomplete recoveries during the extraction process.

3. Interferences

Any compounds present in samples and extracted using C_{18} solid phase cartridges, which are unaffected by the concentration procedure, and which elute from the HPLC column under the same conditions as microcystins and have similar adsorption spectra to microcystins.

4. Hazards

All microcystins and nodularin are potent mammalian toxins and appropriate precautions should be taken when handling the pure toxins or standard solutions and samples.

Several of the reagents used are potentially hazardous. Methanol is toxic and flammable, trifluoroacetic acid is toxic and corrosive and should be handled in a well ventilated fume cupboard. Acetonitrile is toxic and flammable.

5. Microcystins and nodularin

Few microcystins are commercially available. Those that are, and nodularin, can be obtained in septum-sealed vials containing 500 +/- 10µg. Checks should be undertaken to ascertain the comparative purity of samples prior to use as standards. These checks may include amino acid analysis or HPLC analysis with UV detection. Vials of microcystins and nodularin should be stored in a freezer at a temperature of about -18°C.

5.1 Standard solutions

Add 500 +/- 5 µL of methanol to a septum-sealed vial containing 500 µg of the toxin, to give a concentration of 1 $\mu g \mu L^{-1}$. Appropriate dilution of aliquots of this solution with methanol provides suitable spiking solutions or standards suitable for injection onto the HPLC system.

All solutions should be stored in a freezer (at -18°C) when not in use and are stable for periods of up to one year. However, they should be checked prior to use to ensure that their concentrations remain unchanged.

5.2 Solvents and reagents

The methanol used for conditioning the solid phase cartridges, making up standard solutions and aqueous solutions containing various perentages of methanol should be HPLC grade. Double-distilled or distilled deionized water should be used for cartridge conditioning, for admixture with methanol for cartridge washing following the extraction

step, for admixture with methanol for suspension of the residues from the extract concentration step and for making up the HPLC eluent A. The acetonitrile used to make up HPLC eluent B should be HPLC grade.

All other reagents are of analytical grade quality.

5.2.1 10% Aqueous methanol. Dilute 100 +/- 2 mL of methanol in water and make up to 1000 +/- 20 mL with water. Mix well.

5.2.2 20% Aqueous methanol. Dilute 200 +/- 5 mL of methanol in water and make up to 1000 +/- 20 mL with water. Mix well.

5.2.3 30% Aqueous methanol. Dilute 300 +/- 5 mL of methanol in water and make up to 1000 +/- 20 mL with water. Mix well.

5.2.4 70% Aqueous methanol. Dilute 700 +/- 10 mL of methanol in water and make up to 1000 +/- 20 mL with water. Mix well.

5.2.5 Sodium thiosulphate solution. Dissolve 10.0 +/- 0.2 g sodium thiosulphate in water and make up to 1000 +/- 20 mL. Mix well.

5.2.6 Trifluoroacetic acid 10% v/v in water. Dilute 100 +/- 2 mL of trifluoroacetic acid in water and make up to 1000 mL. Mix well.

5.2.7 Trifluoroacetic acid 0.1% v/v in methanol. Dilute 1 +/- 0.02 mL of trifluoroacetic acid in methanol and make up to 1000 +/- 10 mL. Mix well.

5.3 Solid phase cartridges

The cartridges used for the performance testing are 1g Isolute C_{18} trifunctional, end-capped solid phase extraction cartridges (volume 3 mL). Similar cartridges from other manufacturers may also be suitable but should be evaluated. At no time during the conditioning process, or the extraction or washing stages, should the cartridges be allowed to dry out.

5.4 HPLC eluent

Two eluents, A and B, are used.

Eluent A, 0.05% v/v trifluoroacetic acid in water, is prepared by diluting 500 +/- 10 µL trifluoroacetic acid in water and making up to 1000 +/- 10 mL.

Eluent B, 0.05% v/v trifluoroacetic acid in acetonitrile, is prepared by diluting 500 +/- 10 µL trifluoroacetic acid in acetonitrile and making up to 1000 +/- 10 mL.

Both eluents should be degassed using helium prior to use.

6. Apparatus

6.1 Syringes and volumetric flasks

A range of glass syringes (e.g. between 10 µL and 1 mL) and low volume volumetric flasks (e.g. between 5 mL and 50 mL) should be available to carry out dilutions, to prepare standard solutions and to spike samples.

6.2 Sample 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 at least 500 mL are required for sampling. 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

Porcelain Buchner filter funnels 110mm diameter with 110 mm diameter GF/C filter discs (for example Whatman 1822-110) fitted to a 1 L Buchner flask with a Neoprene adapter ring funnel support are suitable. Alternatively, similar all-glass filtering equipment may be used. Water vacuum pumps provide a suitable vacuum.

6.4 Extraction apparatus

Various apparatus can be used. This may be manually operated or automated. Typically a vacuum manifold to which several solid phase extraction cartridges can be attached, is used. Each solid phase cartridge is fitted via a suitable connector to a reservoir tube (a reservoir volume of 6 mL is appropriate for the 1 g/3 mL cartridges used) and the reservoir connected to a 500 mL glass bottle containing the sample to be extracted via PTFE® tubing and suitable fittings.

6.5 Extract concentration equipment

Following collection of the sample extract (3 mL) into suitable tubes, the extract is reduced to dryness using a gentle stream of purified nitrogen gas. This process may be facilitated by heating the extracts to 45°C (for example using a hot block). The residue is resuspended in methanol (2 x 100 µL) and the suspensions transferred to a micro-centrifuge tube (capacity 1.5 mL) and reduced to dryness. Prior to HPLC analysis, the residue is resuspended in 70% v/v aqueous methanol (75 µL).

6.6 Centrifuge

If it proves necessary to centrifuge the concentrated extract prior to HPLC analysis (see section 8.4.1) a centrifuge suitable for use with the micro-centrifuge tubes referred to in 6.5 above is required.

6.7 HPLC system

Any suitable HPLC system capable of delivering a linear binary gradient at a flow rate of 1 $mLmin^{-1}$ and equipped with a diode array detector capable of operating in the range 200-300 nm can be used. It is advantageous if the system is also equipped with an autosampler and a computerised data handling system.

A column temperature controller is required to maintain the HPLC column at a temperature of 40°C.

6.8 HPLC column

The HPLC column used is a Waters μBondapak C_{18} column (300 x 3.9 mm i.d., 10 μm). As noted above (6.5) the column is operated at 40° +/- 0.5°C.

6.9 HPLC eluent gradient

A linear gradient is used, as described below.

	Time/min						
	0	10	40	42	44	46	55
Solvent A%*	70	65	30	0	0	70	70
Solvent B%*	30	35	70	100	100	30	30

* - for solvent composition see Section 5.4.

6.10 UV spectra of microcystins and nodularin

The UV spectra of the different microcystins can be classified into two groups. Most exhibit spectra with a maximum at 238 nm, but for those microcystins containing a tryptophan residue (i.e. those containing a W in the two letter suffix used to describe the amino acid residue variation at specific positions in the heptapeptide ring) the maximum is at 222 nm. Typical examples of UV spectra for these two groups are shown in Figure 1.

7. Sample collection and preservation

Samples should be collected in suitable glass bottles (see section 6.2) with a nominal capacity of at least 500 mL. The bottles should be thoroughly rinsed with the water to be sampled prior to taking the sample for analysis. As microcystins have been shown to be

biodegraded when stored in reservoir water at ambient temperatures for periods of a few days, samples should 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 particular attention to section 4.

8.1 Sample pre-treatment

A 500 mL portion of the sample (see section 7) is taken and filtered carefully through a GF/C filter disc (see section 6.3) using gentle suction to avoid rupture of either the filter disc or algal cells. To the filtered sample is added sodium thiosulphate solution (100 +/- 5 µL of a 10 g L^{-1} solution (see section 5.2.5)) to eliminate residual free chlorine (see note (a)), and the sample shaken vigorously and allowed to stand for 2-3 minutes. 5.0 +/- 0.2 mL of 10% v/v aqueous trifluoroacetic acid (see section 5.2.6) is then added to the sample and following thorough mixing it is vacuum filtered though a GF/C filter disc (see section 6.3) and stored temporarily in a 500 mL glass bottle. 5 mL methanol is then added prior to solid phase extraction.

Note (a): There is no requirement to add sodium thiosulfate to raw water samples.

8.2 Extraction

An Isolute C_{18} trifunctional end-capped solid phase extraction cartridge (see note (b)) is conditioned by passing 10 mL of methanol followed by 10 mL of water through it. Discard these eluates. Do not allow the cartridge to dry out. The water sample from 8.1 is then passed through the cartridge at a flow rate not exceeding 10 $mLmin^{-1}$. The cartridge is washed (i.e. the eluates are discarded) sequentially, firstly with 10.0 +/- 0.5 mL of 10% v/v aqueous methanol, then with 10.0 +/- 0.5 mL of 20% v/v aqueous methanol and finally with 10.0 +/- 0.5 mL of 30% v/v aqueous methanol (see section 5.2) before drawing air through the cartridge for 30 +/- 5 min. The cartridge is then eluted with 3.0 +/- 0.3 mL of 0.1% v/v trifluoroacetic acid in methanol (seed section 5.2.7). This extract is collected and concentrated as described in section 8.3 below.

Note (b): Specially designed vacuum manifold systems are available for solid phase extraction. Numerous cartridges may be fitted to many of these, so that several extractions can be carried out simultaneously. Fully automated equipment designed for solid phase extraction is also available and, provided it is suitably programmed, can be used for this extraction.

8.3 Concentration of extracts

The extract from 8.2 is transferred to a suitable tube or vial and placed in a hot block thermostatically maintained at 45 +/- 2°C and reduced to dryness using a gentle stream of dry purified nitrogen gas. The residue is re-suspended in 100 +/- 5 µL methanol and transferred to a micro-centrifuge tube (capacity 1.5 mL). A further 100 +/- 5µL of methanol is used to rinse the tube used for the nitrogen blow-down, and combined with the suspension in the micro-centrifuge tube. The methanol is then removed by nitrogen blow-down. If the HPLC analysis is to be undertaken immediately, the residue is re-suspended in 75 +/- 1 µL methanol; if the HPLC analysis does not immediately follow, the residue may be stored in a freezer (-18°C) and re-suspended in 75 +/- 1 µL methanol prior to analysis.

8.4 HPLC analysis

Column: µBondapak C18; 125 Å, 10 µm; 3.9 x 300 mm

Eluent: Linear gradient of 0.05% v/v trifluoroacetic acid in water (eluent A) and 0.05% v/v trifluoroacetic acid in acetonitrile (eluent B) (see sections 5.4 for eluent composition and 6.8 for the linear gradient).

Flow rate: 1.0 $mLmin^{-1}$

Column temperature: 40° +/- 0.5°C

Injection volume: 25 µL

Detection: Photodiode array detector operated over the range 200-300 nm. Microcystins containing a tryptophan residue (those microcystins with a W in the two-letter suffix) have an absorption maximum at 222 nm; all other known microcystins have an absorption maximum at 238 nm. This latter wavelength is also used for the detection of nodularin.

8.4.1 HPLC analysis of extracts

Following the transfer of the concentrated extract from 8.3 to a suitable vial, it is analysed under the conditions described above. Although manual injection can be used, it is more convenient to utilise an autosampler provided low-volume autosampler vials (or low-volume inserts for standard autosampler vials) are available.

If particulate matter is visible in the concentrated extract it is advisable to centrifuge it (whilst contained in the micro-centrifuge tubes described in section 8.3) prior to transferring it to the vial used for the HPLC analysis to prevent potential problems with blockages or increased back-pressures.

8.4.2 Calibration curve

Using standard solutions, a calibration curve for each of the microcystins to be determined, and nodularin, should be produced. This should cover the range 0 - 1500 ng of each toxin on column.

A five-point calibration curve should be constructed at the start of a series of HPLC runs. If this calibration is linear, for any series of HPLC runs involving in excess of twenty four runs this should be checked after every twenty four runs (or after every batch of HPLC runs, if this consists of less than 24 runs) by checking at least two of the calibration points to ensure that the response for each standard has not varied by more than +/- 20%. If the calibration is non-linear all of the calibration points should be checked to ensure that the response for each standard has not varied by more than +/- 20%.

If the variation exceeds 20% in either of the above cases, a new five point calibration curve should be constructed and used for subsequent quantification.

8.4.4 Calculation of results

Quantification of microcystins and nodularin is carried out using a standard curve based on peak areas obtained using standards (see section 8.4.3 above), and a correction is applied to allow for incomplete recovery.

The recovery efficiency for each toxin is determined by from spiking experiments as follows. The spiking recovery should be determined for each batch of analyses.

A standard water sample (1 L +/- 10 mL) is spiked with a methanolic solution (20 +/- 0.5 µL) containing each toxin at a level of 100 +/- 2 µg mL^{-1} . It is then sub-divided into two 500 +/- 10 mL aliquots, each of which is extracted and analysed as described above. Another standard water sample (1 L +/- 10 mL) is spiked with a methanolic solution (80 +/- 2 µL) containing each toxin at a level of 100 +/- 2 µg mL^{-1}. It is then sub-divided into two 500 +/- 10 mL aliquots, each of which is analysed as described above.

A two point line is then plotted for each of the toxins analysed. The axes are the instrumental response obtained for solvent standards (from 8.4.2) and the instrumental response obtained for the recovery standards (the mean of the two duplicate analyses). The first point in each case should correspond to the response for 333 ng of the toxin and the mean response from the 2 µg L^{-1} recovery standards, and the second point should correspond to the response for 1333 ng of the toxin and the mean response from the 8 µg L^{-1} recovery standards. The slope of this line gives the recovery efficiency e.g. a slope of 1.00 would indicate a 100% recovery, whereas a slope of 0.75 would indicate a 75% recovery. It is possible that different recoveries efficiencies will be obtained for each toxin.

The recovery efficiency determined as above should be used to correct the analytical results for each batch of analyses.

9. Sources of error

9.1 Contamination

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

9.2 Purity of standards

Suppliers of standards should be contacted for this information.

9.3 Interfering substances

None detected during performance testing, but samples containing algal cells or constituents of algal cells not tested.

9.4 Presence of algal cells

As cyanobacterial toxins are contained within the cell walls of living cells, and are released when the cells are ruptured or senescent, cell lysis during sample storage or sample processing may lead to release of intra-cellular toxin into the water sample. This will then be determined with that already in solution (as a result of normal cell turnover) and will result in an over-estimation of the levels of toxins present in solution at the time of sampling.

10. 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 9. It is recommended that at least one AQC sample is analysed in duplicate with each batch of samples.

11. References

11.1 Lawton, L.A., Edwards, C. and Codd, G.A. 'Extraction and High-performance Liquid Chromatographic Method for the Determination of Microcystins in Raw and Treated Waters'. Analyst, 1994 **119** 1525-1530.

APPENDIX B

EXPERIMENTAL DESIGN FOR PERFORMANCE TESTING

EXPERIMENTAL DESIGN FOR TESTS OF THE PRECISION AND SOMESOURCES OF BIAS IN THE DETERMINATION OF FOUR ALGAL TOXINS IN RAW AND POTABLE WATER

1. INTRODUCTION

This experimental design describes the tests proposed for the assessment of the precision and some sources of bias for the determination of four algal toxins in raw and potable waters. The test is intended as an evaluation of the performance (and robustness) of a specific method, details of which have already been provided. The worksheet for the test results is attached.

The determinands of interest are microcystin LR, microcystin RR, microcystin YR and nodularin.

2. ACCURACY REQUIREMENTS TO APPLY IN THESE TESTS

The analytical range of primary interest has been established as between 0 and 10 µg/l.

The accuracy requirements against which the results of each participating laboratory will be assessed is that the error on a single analytical result should not be larger than 1µg/l or 20% of the determinand concentration of the sample, whichever is the greater.

Following the convention of dividing the tolerable error equally between random and systematic sources, this corresponds to targets of:

- maximum tolerable standard deviation of 0.25 µg/l or 5% of determinand concentration, whichever is the greater, and
- maximum tolerable bias of 0.5 µg/l or 10% of determinand concentration, whichever is the greater.

3. ANALYTICAL METHOD

Given the aim of this test - to investigate the performance achievable using a particular procedure, it is essential that participating laboratories adhere to the exact details of the method which has been circulated. It is accepted that there may be aspects of the procedure which you might wish to change (for example, to improve ease of use or throughput). Such modifications must on no account be made.

4. TESTS OF PRECISION AND BIAS.

4.1 Solutions Provided

Each participant is provided with the following:

- 20 x 2.3L Duran bottles of a filtered raw water - water R;
- 20 x 2.3L Duran bottles of a (filtered) treated water - water T;
- Approx. 10ml of a mixed standard solution of 4 algal toxins in methanol at a concentration of approximately 100 µg/ml (for each substance) - Standard X;

4.2 Samples to be Tested

The following seven samples are to be subjected to the familiar 2 replicate, 11 batch test regime:

- Sample A - a blank-spiked deionised water. This test sample should be prepared by addition of 5µl of Standard X to a 1L of the laboratory's deionised water.
- Sample B - a low-spiked raw water. This test sample should be prepared by the addition of 20 µl of Standard X to a 1L sample of Water R.
- Sample C - a high-spiked raw water. This test sample should be prepared by the addition of 80 µl of Standard X to a 1L sample of Water R.
- Sample D - a low-spiked treated water. This test sample should be prepared by the addition of 20 µl of Standard X to a 1L sample of Water T.
- Sample E - a high-spiked treated water. This test sample should be prepared by the addition of 80 µl of Standard X to a 1L sample of Water T.
- Sample F - a low spiked deionised water. This test sample should be prepared by addition of 10µl of Standard X to a 1L of the laboratory's deionised water.
- Sample G - a high spiked deionised water. This test sample should be prepared by addition of 90µl of Standard X to a 1L of the laboratory's deionised water.

Two replicate determinations should be made, in random order, on each of the above test samples, in each of **eleven** batches of analysis.

The 1L volume of each of these test samples corresponds to two 500 ml samples. These are required to carry out duplicate determinations for the performance test. Each 1L portion of test sample should be prepared freshly for each batch of analysis.

5. CALCULATIONS AND ADDITIONAL INFORMATION REQUIRED

The results of the test should be processed as described in the method. Results should be reported as concentration of each determinand in the spiked test samples (i.e. in the range 0 - 10 µg/l). The attached worksheet should be used. This should be returned to WRc before the contract deadline date of 31/1/95.

In addition, participants are asked to supply the following supporting information:

- all raw data for the tests, including HPLC chart traces, the retention time and peak identities and the peak area readings used to calculate results.

- other information necessary to convert raw data to analytical results, including HPLC calibration curves (and corresponding raw data) for each determinand and details of the periodic microcystin LR extraction recovery check (used as a correction for the standard curves).