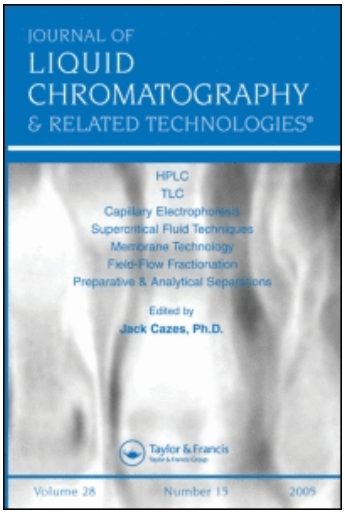
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

SIMULTANEOUS DETERMINATION OF THE CYANOTOXINS ANATOXIN A, MICROCYSTIN DESMETHYL-3, LR, RR, AND YR IN FISH MUSCLE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Víctor Hormazábal[a]; Øyvin Østensvik[a]; Bjarne Underdal[a]; Olav M. Skulberg[b]
[a] Division of Food Hygiene, The Norwegian School of Veterinary Science, Oslo 1, Norway [b] Norwegian Institute for Water Research, Oslo, Norway

Online publication date: 01 October 2000

To cite this Article Hormazábal, Víctor , Østensvik, Øyvin , Underdal, Bjarne and Skulberg, Olav M.(2000) 'SIMULTANEOUS DETERMINATION OF THE CYANOTOXINS ANATOXIN A, MICROCYSTIN DESMETHYL-3, LR, RR, AND YR IN FISH MUSCLE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY', Journal of Liquid Chromatography & Related Technologies, 23: 2, 185 — 196
To link to this Article: DOI: 10.1081/JLC-100101444
URL: http://dx.doi.org/10.1081/JLC-100101444

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 23(2), 185–196 (2000)

SIMULTANEOUS DETERMINATION OF THE CYANOTOXINS ANATOXIN A, MICROCYSTIN DESMETHYL-3, LR, RR, AND YR IN FISH MUSCLE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Víctor Hormazábal,[1] Øyvin Østensvik,[1] Bjarne Underdal,[1] Olav M. Skulberg[2]

[1] Division of Food Hygiene
The Norwegian School of Veterinary Science
P.O. Box 8146-Dep.
N-0033 Oslo 1, Norway

[2] Norwegian Institute for Water Research
P.O. Box 173 Kjelsås
N-0411 Oslo, Norway

Downloaded At: 10:08 24 January 2011

ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method for the determination of anatoxin-a, microcystin desmethyl-3, LR, RR, and YR in fish muscle, is described. A salmon muscle sample was extracted with a mixture of methanol-water and acetone. The organic layer was evaporated and cleaned-up using LMS solid phase extraction columns. The method is simple, specific, and requires only small quantities of chemical reagents. The lower limits of quantification were 15, 2, 10, 1, and 10 ng/g for anatoksin-a, microcystin desmethyl-3, LR, RR, and YR, respectively.

Copyright © 2000 by Marcel Dekker, Inc. www.dekker.com

INTRODUCTION

Toxic cyanobacterial blooms occur worldwide in inland, brackish, and marine waters.[1,2] Of the cyanotoxins, two groups, the neurotoxins and hepatotoxins, are mainly responsible for deaths of domestic animals and wildlife.[3] There is evidence indicating accumulation of cyanotoxins in mussels and fish.[4]

More extended use of water resources will increase the human exposure to cyanotoxins. As their presence thus represents a potential health risk, there is need for simple and efficient methods for the detection and quantification of these toxins.

Several analytical methods based upon HPLC for the detection of microcystins in water samples have been described,[4,5] as well as a combined HPLC-mass spectrometry method to confirm the identity of microcystins.[6] However, none of these methods appear to be applicable to fish muscle samples.

The purpose of the present study was to develop a rapid, simple, and specific method for the determination of anatoxin-a (ATX), microcystin desmethyl-3 (D-3), LR, RR, and YR in fish muscle.

Downloaded At: 10:08 24 January 2011

EXPERIMENTAL

Samples of fish muscle (*Salmo salar* L.) were used as control material and also for spiking with a toxin-mixture to conduct the recovery experiments.

All chemicals and solvents were of analytical or HPLC grade. ATX, LR, RR, and YR were supplied by Calbiochem (USA). D-3 was obtained from a clone culture of the cyanobacterium *Planktothrix prolifica* (Gom.) Anagn. et Kom. (NIVA-CYA 98).

The strain was isolated from a bloom sample collected in Lake Steinsfjorden, Norway,[7] and grown under controlled conditions.[8] The toxin content was related to a defined D-3 standard obtained from the National Institute of Public Health, Norway.

Stock standard solutions (10 μg/mL) of ATX, D-3, RR, and LR were prepared by dissolving the compounds in 1.5 mL water and further with methanol-water (8+2). Microcystin-YR was dissolved in 1.5 mL ethanol and further with methanol-water (8+2). Working solutions of 1 μg/mL (mixed standards) were prepared by dilution with methanol - water (8 + 2). The standard solutions were stored at -20°C. Solid phase extraction (SPE) columns Bond Elut (1cc 25 mg) LMS, were purchased from Varian (Habor City, CA, USA).

Table 1

Mobile Phase Operating Conditions

Step	Time (min)	Flow (μL/min)	Solution A (%)	Solution B (%)
1	3	800	81	19
2	9	800	17	83
3	2	600	81	19
4	2	900	81	19
5	4	800	81	19

Downloaded At: 10:08 24 January 2011

Chromatographic Conditions

The analyses were performed using a Perkin Elmer HPLC-MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler. The acquired data were entered into a Model 8500 Apple Power Macintosh and processed with either Multiview 1.3 or MacQuan 1.5 software packages (Perkin Elmer), for spectral information data processing and quantification data processing, respectively. An API 100 LC-MS system (PE SCIEX) single quadruple mass spectrometer with a standard API-Ion Spray ionization source, was employed for this study. The LC-MS was set to collect multiple single-ion data in positive ion mode for the ions at *m/z* 166, 512.9, 995.5, 519.8, and 1045.5 for ATX, D-3, LR, RR, and YR, respectively. The entrance electrode voltages were adjusted to provide optimum overall intensities for the five molecular ions. The analytical column (stainless steel, 250 x 4.6 mm I.D.) was packed with 5 μm, 100 Å particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA). The mobile phase consisted of a mixture of two solutions, A and B (Table 1). Solution A consisted of 0.1 % formic acid in water (999 mL water+1 mL formic acid) while solution B was methanol.

The LC eluent was split post-column approximately 1:20 so that c. 50 μL flowed into the Ion-Spray ion source.

Sample Pretreatment

Volumes of 1 mL methanol-water (8+2) or standard, and 4 mL acetone were added to 3 g fish muscle. The mixture was homogenized for approximately 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Junkel, Germany). After centrifugation for approximately 5 min. (5000 rpm), 4 mL of the supernatant (corresponding to 1.5 g sample) was pipetted into a conical, graduated glass-stoppered centrifuge tube, and evaporated to between 0.9 and 1mL under a stream of air, using a Reacti-Term heating module at 40°C and a Reacti Vap

evaporating unit (Pierce, USA).Two mL hexane was added and the mixture was shaken for 5 sec. After centrifugation (2 min. for 3500 rpm) the hexane was discharged, and 2 mL hexane was added to the remaining water phase. This mixture was shaken for 5 sec. After centrifugation, the hexane layer was discharged and the water layer was evaporated to a final volume of about 0.6 mL. Fifty µL methanol and 1.5 mL water were added to the water residue and blended. The water based sample was loaded onto a conditioned LMS column.

Clean-up SPE-Column

The LMS column was conditioned with 1 mL methanol, followed by 1 mL water and 1 mL water - methanol (97+3). The aqueous extract was applied onto the column. Conditioning and application of the sample took place under gravity flow (dropwise rate). The column was washed at a flow rate of c. 1 mL/min (very slow vacuum) with 1 mL water, 1 mL water - methanol (97+3), and 0.5 mL water - methanol (90+10), and afterwards suctioned to dryness for 10 sec. at a vacuum of -10 in. Hg. using a Vac Master system from International Sorbent Technology. The column was then eluted with 2 x 1mL methanol - water (80+20). The eluate was collected in a graduated glass-stoppered tube and evaporated to between 0.3 and 0.4 mL, after which the volume was adjusted to 2 mL with water and blended. After centrifugation for 3 min (3500 rpm), aliquots of 50 µL were injected onto the LC/MS at intervals of 20 min. for the determination of ATX, D-3, LR, RR, and YR.

Downloaded At: 10:08 24 January 2011

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for ATX, D-3, LR, RR, and YR were determined by spiking muscle with standard solutions to yield 15, 20, 50, 75, and 100 ng/g for ATX, 5, 10, 20, 50, 75, and 100 ng/g for D-3 and RR, and 10, 20, 50, 75, and 100 ng/g for LR and YR, respectively. Duplicate samples were used. The recovery rates were determined by comparing analyses of spiked muscle with those of standard solutions. The linearity of the standard curves for ATX, D-3, LR, RR, and YR in muscle were calculated using peak area measurements.

RESULTS AND DISCUSSION

Chromatograms of clean muscle samples and of corresponding samples spiked with ATX, D-3, LR, RR and YR, are shown in Figures 1 to 5. The standard curves were linear in the investigated areas; 15-100 ng/g for ATX, D-3 and RR, 5-100 ng/g for LR, and 10-100 ng/g for YR.

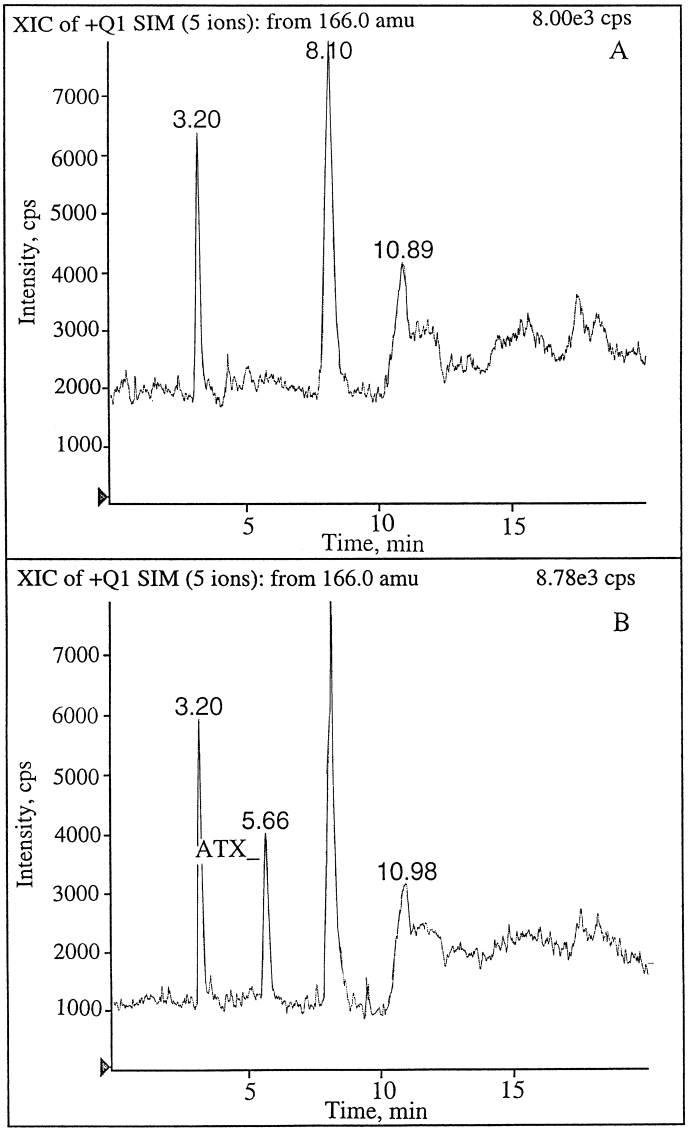


Figure 1. Chromatograms of extract from salmon muscle. A: Toxin-free muscle, B: muscle spiked with ATX (50 ng/g).

Downloaded At: 10:08 24 January 2011

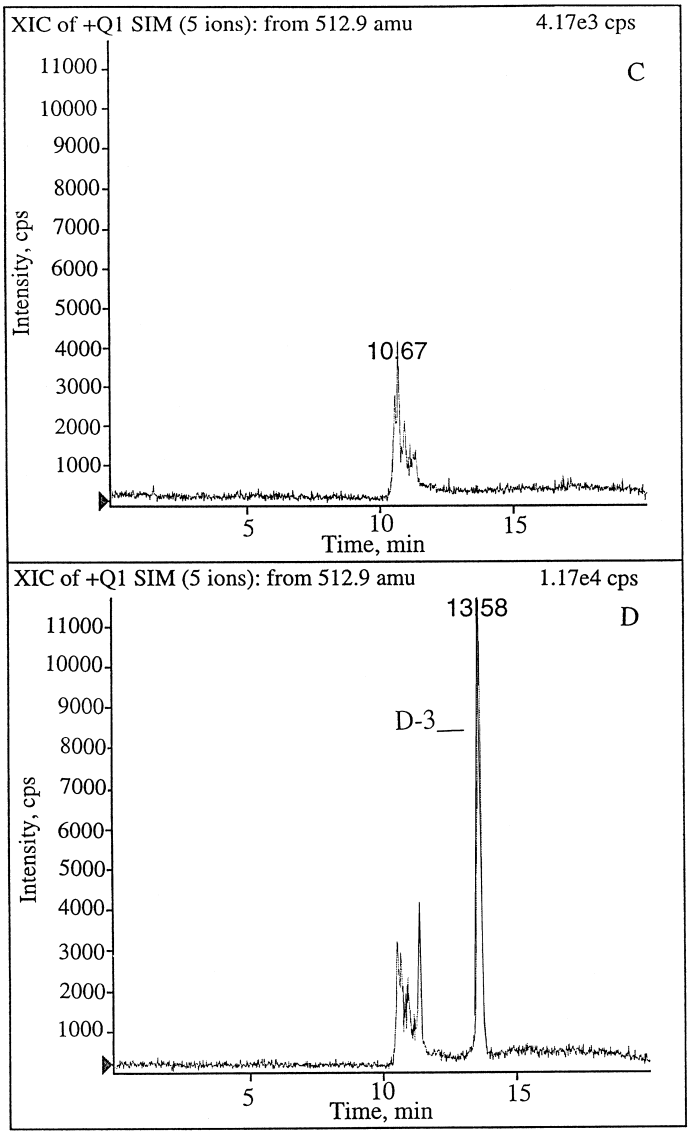


Figure 2. Chromatograms of extract from salmon muscle. C: Toxin-free muscle, D: muscle spiked with D-3 (50 ng/g).

Downloaded At: 10:08 24 January 2011

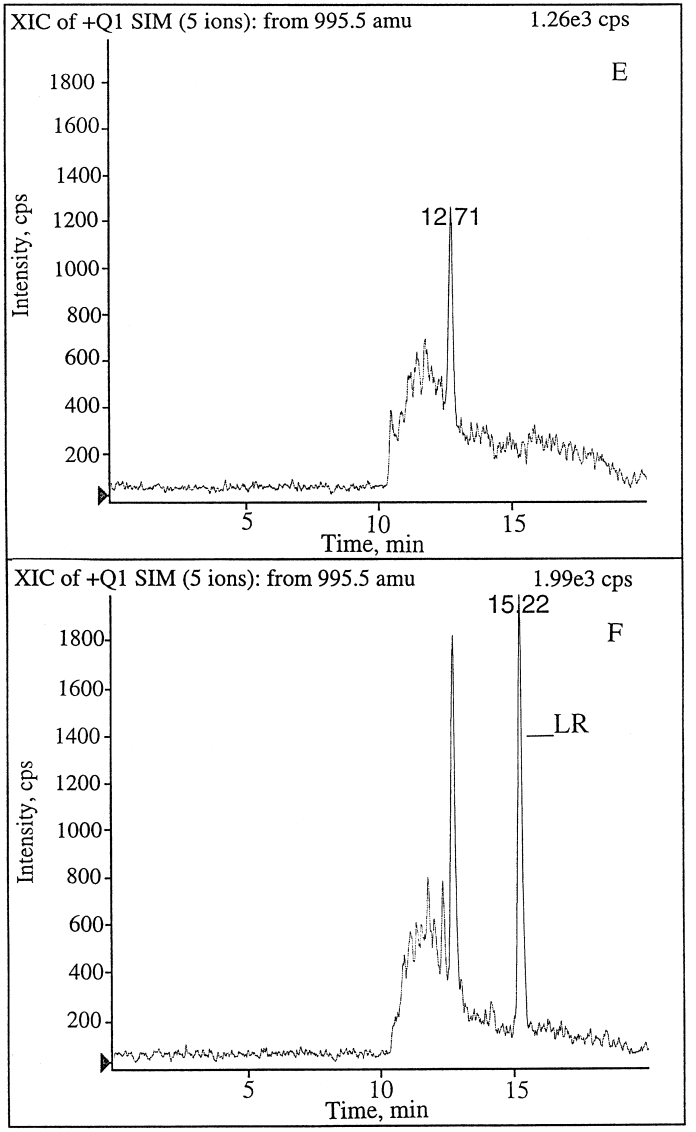


Figure 3. Chromatograms of extract from salmon muscle. E: Toxin-free muscle, F: muscle spiked with LR (50ng/g).

Downloaded At: 10:08 24 January 2011

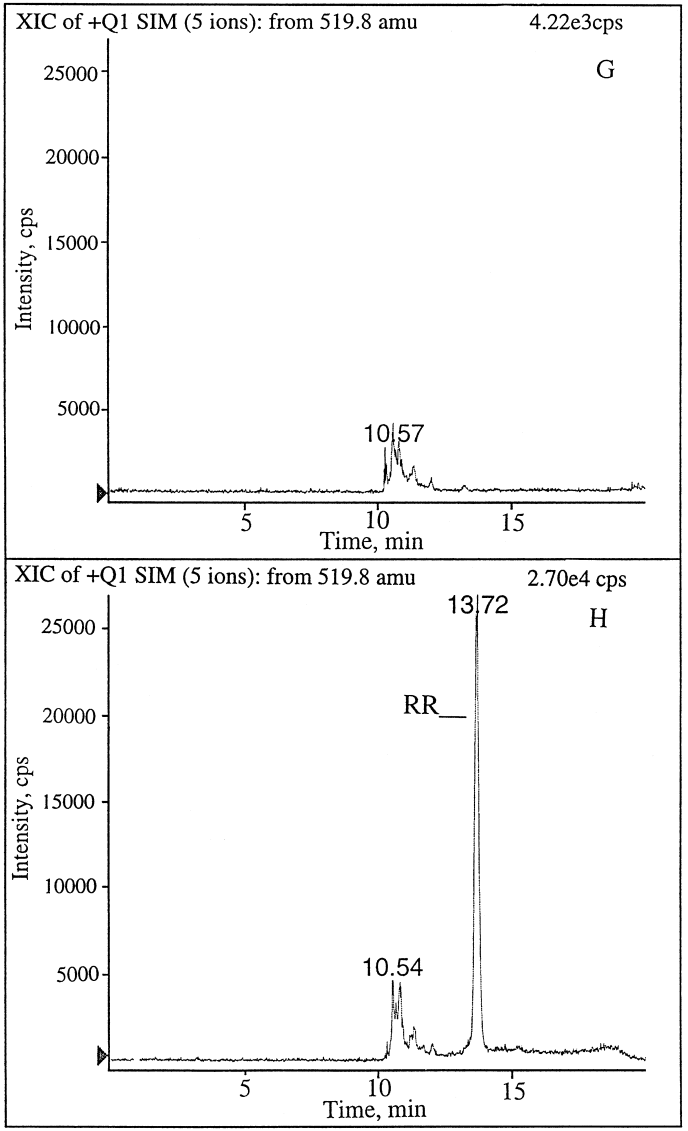


Figure 4. Chromatograms of extract from salmon muscle. G: Toxin-free muscle, H: muscle spiked with RR (50ng/g).

Downloaded At: 10:08 24 January 2011

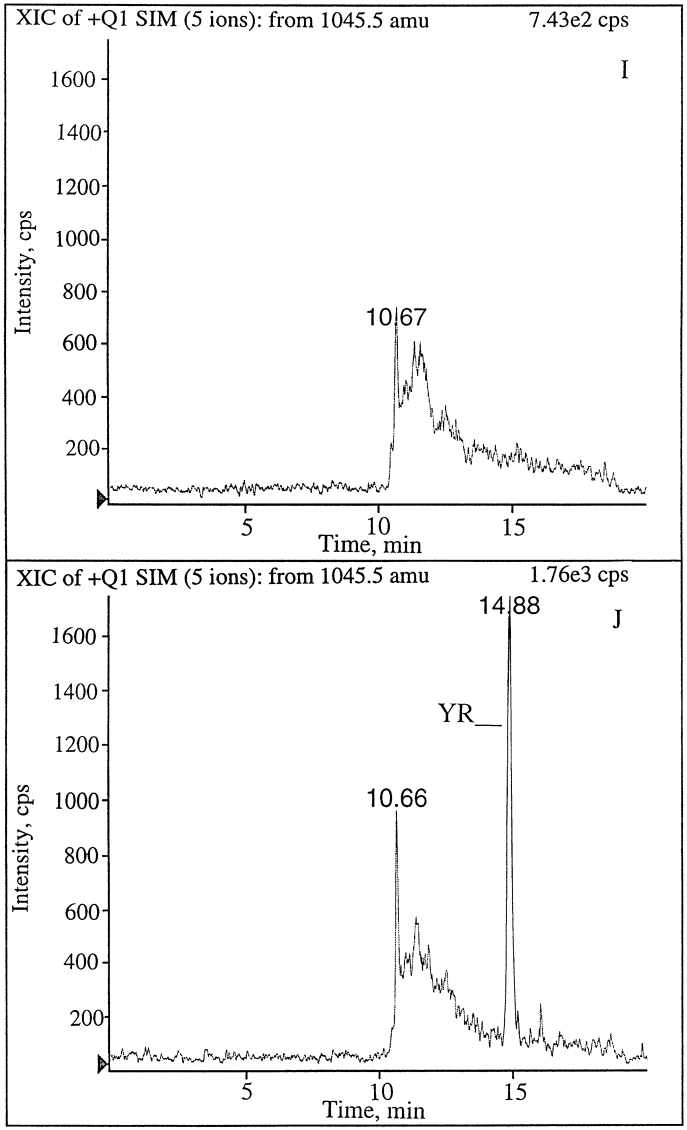


Figure 5. Chromatograms of extract from salmon muscle. I: Toxin-free muscle, J: muscle spiked with YR (50 ng/g).

Downloaded At: 10:08 24 January 2011

Table 2

Recovery and Repeatability for ATX, D-3, LR, RR, and YR from Spiked Samples of Salmon Muscle

		Amount	ATX		D-3		LR		RR		YR	
Sample	n	of Drug ng/g	SD (%)	Rec. (%)	SD (%)	Rec. (%)	SD (%)	Rec. (%)	SD (%)	Rec. (%)	SD (%)	Rec. (%)
Muscle 3g	8	20	2.4	73	1.3	81	3.6	88	2.3	85	1.8	96
	8	50	1.1	70	2.6	75	2.5	90	2.6	90	1.4	97

SD = standard deviation. Rec. = recovery.

Downloaded At: 10:08 24 January 2011

The corresponding correlation coefficients were 0.999 for ATX, D3, LR, RR, and YR in muscle. The recovery and repeatabilities for ATX, D-3, LR, RR, and YR from salmon muscle are shown in Table 2. The eluate from the SPE cartridge was evaporated prior to injection into the LC/MS, because 80% methanol in water will change the baseline resolution, especially for ATX. In contrast, more water in the final extract permits a larger volume to be injected into the column.[9]

The commercial RR standard is contaminated with small quantities of D-3, and the D-3 purified from the cyanobacteria *P. prolifica* is contaminated with a small quantity of RR. Consequently, for the determination of the limits of detection and quantification, we spiked muscle samples separately with D-3 and RR standards. This was necessary, since both have an almost equal retention time.

In many laboratories, a stream of nitrogen is used to evaporate microcystin samples to dryness. We compared nitrogen and air produced from a central air compressor (generator) for evaporating the samples ATX, D-3, LR, RR, and YR from muscle. No differences were found.

The molecular weights of D-3 and RR are *m/z* 1023 and 1038, respectively. The precision, recovery, linearity, detection, and quantification limits for D-3 and RR were validated from ion *m/z* 512.9 and *m/z* 519.8, corresponding to the double charged ion, M+22+, as they yield a stronger peak.

The limits of detection were close to 7, 1, 5, 0.5, and 5 ng/g and the limits of quantification 15, 2, 10, 1, and 10 ng/g for ATX, D-3, LR, RR, and YR in salmon muscle, respectively. The detection limit of the assay was calculated to be three times the baseline noise from a drug-free muscle. The method presented in this paper is selective, robust, sensitive, and accurate.

The detection limit of the assay depends mainly on the sensitivity of the LC/MS. This in turn could be influenced by such factors as the position of the ion spray inlet, the position of the silica tubing on the sprayer nozzle, the position of the electrode tube in relation to the nebulizer tubing, the composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

During the analysis of microcystin and ATX, API-100 maintained a stable sensitivity for more than three weeks without interruption. Afterwards, the curtain plate of API-100 was cleaned with methanol-water (1+1) with a paper with good absorption capacity, and the sprayer nozzle was cleaned by squirting a few μL of water, methanol, and again water. The instrument can then be used for further analysis. No adjustments were necessary, not even if the ion source housing had been removed to clean the curtain plate, an operation that took a few minutes.

The advantage of the LC-MS technique lies in the combination of the separation capabilities of HPLC with the power of MS as an identification and confirmation method with high sensitivity, selectivity, and quantitative capability. Quantification using selected ion monitoring has high selectivity, sensitivity and a broad dynamic range. While conventional HPLC methods may require long complex separations, the LC-MS method requires only a simple clean-up procedure and no derivatization. LC-MS should be the method of choice despite the requirement of a more specialised technique.[10]

Downloaded At: 10:08 24 January 2011

ACKNOWLEDGMENT

We are grateful to Dr. Hans Utkilen, National Institute of Public Health for providing the D-3 standard. The National Food Control Authority is thanked for financial support.

REFERENCES

1. S. G. Bell, G. A. Codd, Reviews in Medical Microbiology, **5**, 256- 264 (1994).

2. W. W. Carmichael, Scientific American, **270**, 64-72 (1994).

3. W. W. Carmichael, Journal of Applied Bacteriology, **72**, 445-459 (1992).

4. G. A. Codd, C. J. Ward, S. G. Bell, Archives of Toxicology Supplement, **19**, 399-410 (1996).

5. L. A. Lawton, C. Edwards, G. A. Codd, Analyst, **119**, 1525-1530 (1994).

6. C. Edwards, L. A. Lawton, K. A. Beattie, G. A. Codd, Rapid Communication in Mass Spectrometry, **7**, 714-721 (1993).

7. O. M. Skulberg, Norwegian Institute for Water Research, ISBN 82-577-3488-8, 1-42 (1997).

8. R. Skulberg, O. M. Skulberg, Norwegian Institute for Water Research, ISBN 82-577-1743-6, 1-32 (1990).

9. D. U. Neue, E. Serowik, Waters Column, **5 (2)** (1996).

10. I. Chorus, J. Bartram, eds., **Toxic Cyanobacteria in Water. A Guide to Their Public Health Consequences, Monitoring, and Management,** WHO, E & EN Spon, London, UK, 1999.

Received May 20, 1999
Accepted June 5, 1999
Manuscript 5080

Downloaded At: 10:08 24 January 2011