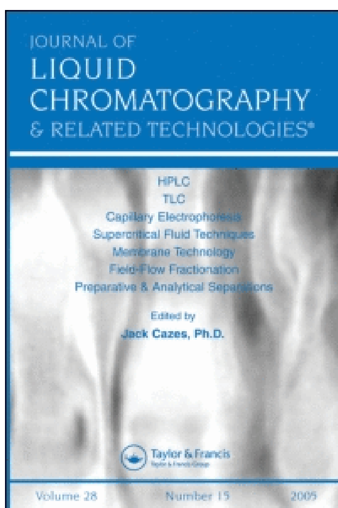
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



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-RR, LR, RR AND YR IN WATER USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Victor 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: 20 December 2000

To cite this Article Hormazábal, Victor , Østensvik, Øyvin , Underdal, Bjarne and Skulberg, Olav M.(2000) 'SIMULTANEOUS DETERMINATION OF THE CYANOTOXINS ANATOXIN A, MICROCYSTIN DESMETHYL-3-RR, LR, RR AND YR IN WATER USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY', Journal of Liquid Chromatography & Related Technologies, 23: 20, 3155 — 3164
To link to this Article: DOI: 10.1081/JLC-100102375
URL: http://dx.doi.org/10.1081/JLC-100102375

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(20), 3155–3164 (2000)

SIMULTANEOUS DETERMINATION OF THE CYANOTOXINS ANATOXIN A, MICROCYSTIN DESMETHYL-3-RR, LR, RR AND YR IN WATER 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: 09:40 24 January 2011

ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method for the determination of anatoxin-a, microcystin desmethyl-3-RR, LR, RR and YR in water, is described. The method involves no sample pre-filtration to separate cyanobacteria from the water. The water sample was added to sodium 1-heptanesulfonate and cleaned-up using LMS solid phase extraction columns. The method is simple, specific, and requires small quantities of chemical reagents. The lower limits of quantification were 0.4 µg/L for anatoxin-a, microcystin desmethyl-3-RR and RR, and 0.5 µg/L for LR and YR.

INTRODUCTION

Toxic cyanobacterial blooms are described from most parts of the world, and occur in inland, brackish, and marine waters.[1,2] Exposure to cyanotoxins

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

may be detrimental to both animal and human health. Two groups of cyanotoxins, neurotoxins and hepatotoxins, have caused several incidents of acute death of domestic animals and wildlife.[3] Small concentrations of hepatotoxins in drinking water, combined with daily intake, may be a risk factor in long-term health. Currently, WHO has proposed a preliminary guideline of 1 μg/L for microcystin-LR in drinking water.[4] Consequently, there is need for simple and efficient methods for the detection and quantification of the relevant cyanotoxins in water.

Several analytical methods based upon HPLC for the detection of microcystins in water samples have been described,[5,6] 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 the total quantification of both intracellular and extracellular anatoxin-a (ATX) and microcystin desmethyl-3-RR (D-3), LR, RR, and YR.

In an earlier paper we have described a LC-MS method for the determination of ATX, D-3, LR, RR, and YR in fish muscle.[7]

The purpose of the present study was to develop an appropriate and specific method for the determination of five cyanotoxins in water.

Downloaded At: 09:40 24 January 2011

EXPERIMENTAL

Materials

Algal suspensions were prepared and used as reference material, and for spiking with a selection of cyanotoxins to conduct the recovery experiments. The algal test sample was made by mixing aliquots of algal cultures of six common phytoplankton species covering the major systematic categories – except cyanobacteria – present in inland waters. This sample mimics the phytoplankton composition in eutrophic waters during water bloom conditions.

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,[8] and grown under controlled conditions.[9] 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, then further, with methanol-water (8+2). Microcystin-YR was dissolved in 1.5 mL ethanol and further with methanol-water (8+2). Working solu-

tions of 0.1 μg/mL (mixed standards) were prepared by dilution with water. The standard stock solutions were stored at -20°C. The working standards were stored in a refrigerator (+ 4°C).

Solution A, consisting of 0.065 M 1-heptanesulfonic acid (sodium salt) (Avocado Research Chemicals, England) and 0.02 M di-sodium hydrogenphosphate (Ferax, Germany), was made by dissolving 13.15 g/L heptane sulfonate and 3.6 g/L di-sodium hydrogenphosphate in approx. 750 mL of water, making 1 litre of solution. The pH was then adjusted to c. 6.2 with 3 M phosphoric acid and then to 6.0 with 0.5 M phosphoric acid, and the solution was diluted with water to 1 litre and, finally, the pH was adjusted to 6.0 with 0.5 M phosphoric acid.

Solid phase extraction (SPE) columns Bond Elut (1cc 25 mg) LMS, were purchased from Varian (Habor City, CA, USA). Bond Elut adaptors and reservoirs of 125 mL (Varian) were connected to the columns when large volumes were applied. Spin-X micro-centrifuge tube filter (0.22 μm nylon) was supplied by Costar (USA).

Downloaded At: 09:40 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 1.4 or MacQuan 1.6 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 Turbo-Ion Spray Inlet for the API LC-MS system was employed for this study. The turbo probe of the instrument was maintained at 150°C and the flow-rates of air for the probe was 6 L/min. 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, 520, and 1045.4 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, B and C (Table 1). Solution B consisted of 0.1% formic acid in water (999 mL water+1 mL formic acid) while solution C was methanol.

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

Table 1

Mobile Phase Operating Conditions

Step	Time Min	Flow µL/min	Solution B %	Solution C %
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: 09:40 24 January 2011

Sample Pretreatment

Volumes of 3 mL solution A were added to 15 mL algal suspension. The mixture was blended and loaded into a reservoir connected into a conditioned LMS column.

Clean-up SPE-Column

The LMS column was conditioned with 1 mL methanol, followed by 2 x 1 mL water. The column free volume was filled up with solution A and a 125 mL reservoir was attached. The water extract was applied into 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 2 x 250 µL water, 250 µL water - methanol (97+3), 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 placed in a freezer at -20°C for 30 min. After 10 min at room temperature, the column was eluted with 0.5 mL methanol-water (8+2) under gravity flow, and only at the beginning of the eluting process was it necessary to apply a little positive pressure with a syringe. After 5 min. the vacuum was set to -5 in. Hg. and then eluted with 3 x 1 mL methanol-water (8+2).

The eluate was collected in a graduated glass-stoppered tube and evaporated to between 0.5 and 0.6 mL, after which the volume was adjusted to 2 mL with water and blended. Approximately 500 µL of the water-based sample was filtered through a Spin-X centrifuge tube by centrifugation for 2 min. at 10000 rpm. (5600g). Aliquots of 50 µL were injected into the LC/MS at intervals of 20 min. for the determination of ATX, D-3, LR, RR, and YR.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for ATX, D-3, LR, RR, and YR were determined by spiking raw water with standard solutions to yield 0.4, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, and 30 ng/mL for ATX, D-3, and RR, and 0.5, 1, 2, 5, 10, 20, and 30 ng/mL for LR and YR. Duplicate samples were used. The recovery rates were determined by comparing analyses of spiked water with those of standard solutions. The linearity of the standard curves for ATX, and YR in raw water was calculated using peak area measurements. The linearity of the standard curves for D-3, LR, and RR in raw water were calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of defined algal suspensions and of corresponding samples spiked with ATX, D-3, LR, RR, and YR, are shown in Figures 1 to 3. The standard curves were linear in the investigated areas; 0.4-30 ng/mL for ATX, D-3, and RR, 0.5-30 ng/mL for LR and YR. The corresponding correlation coefficients were 0.999 for ATX, D3, LR, RR, and YR in raw water. The recovery and repeatability for ATX, D-3, LR, RR, and YR from raw water 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.[10]

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 water 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.

It is important to avoid letting the sorbent dry out in the LMS column during the conditioning step. When loading the sample to the column-reservoir some air bubbles could appear in the LMS column. These were removed by means of Pasteur pipettes.

Downloaded At: 09:40 24 January 2011

Downloaded At: 09:40 24 January 2011

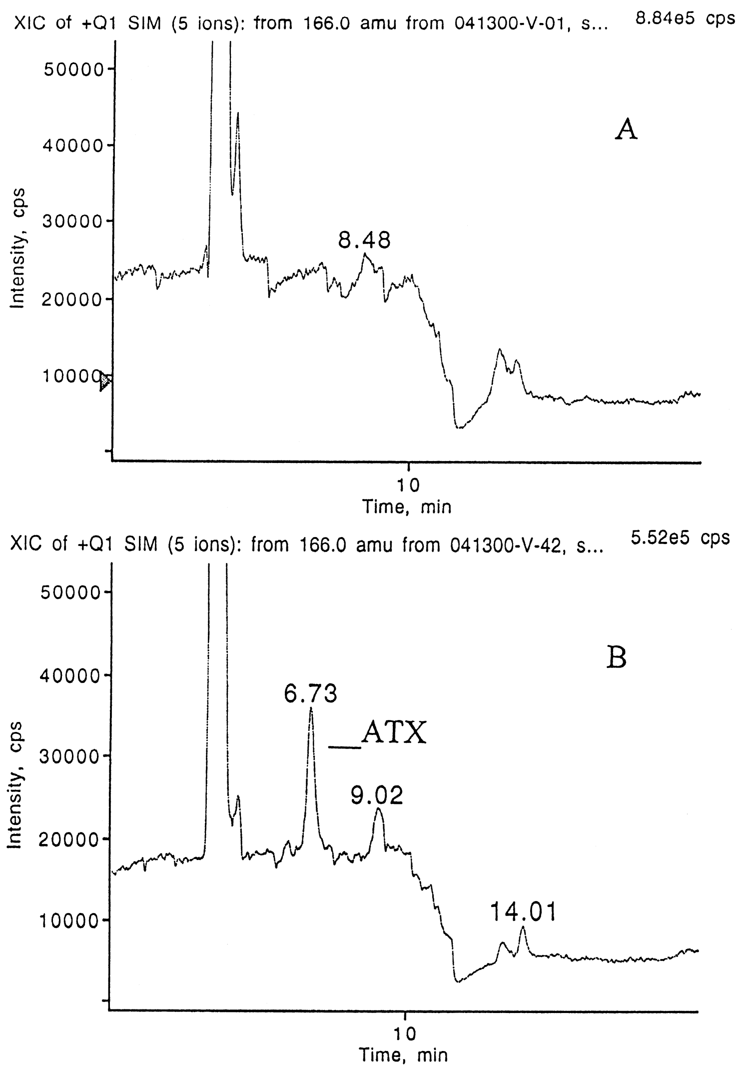


Figure 1. Chromatograms of algal suspension. A: Toxin-free water, B: water spiked with ATX (2 ng/mL).

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* 520, corresponding to the double charged ion, [M+2]2+, as they yield a stronger peak.

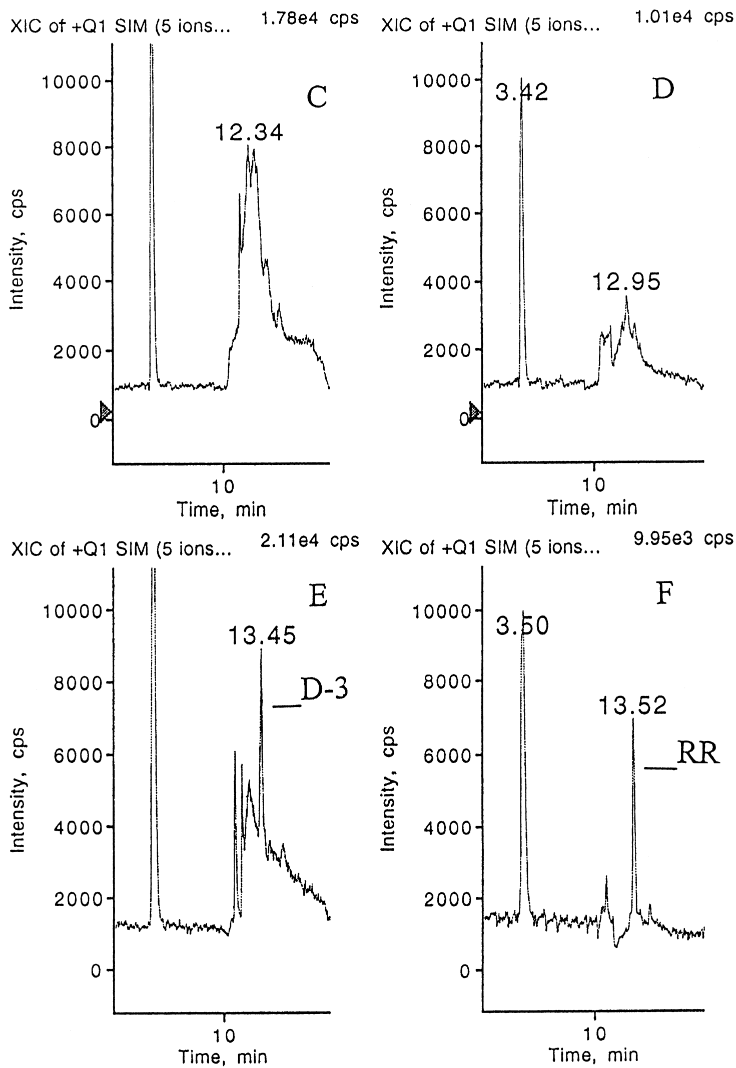


Figure 2. Chromatograms of extract from algal suspension. C: Toxin-free water for D-3, D: Toxin-free water for RR, E: water spiked with D-3 (2 ng/mL), F: water spiked with RR (2ng/mL).

Downloaded At: 09:40 24 January 2011

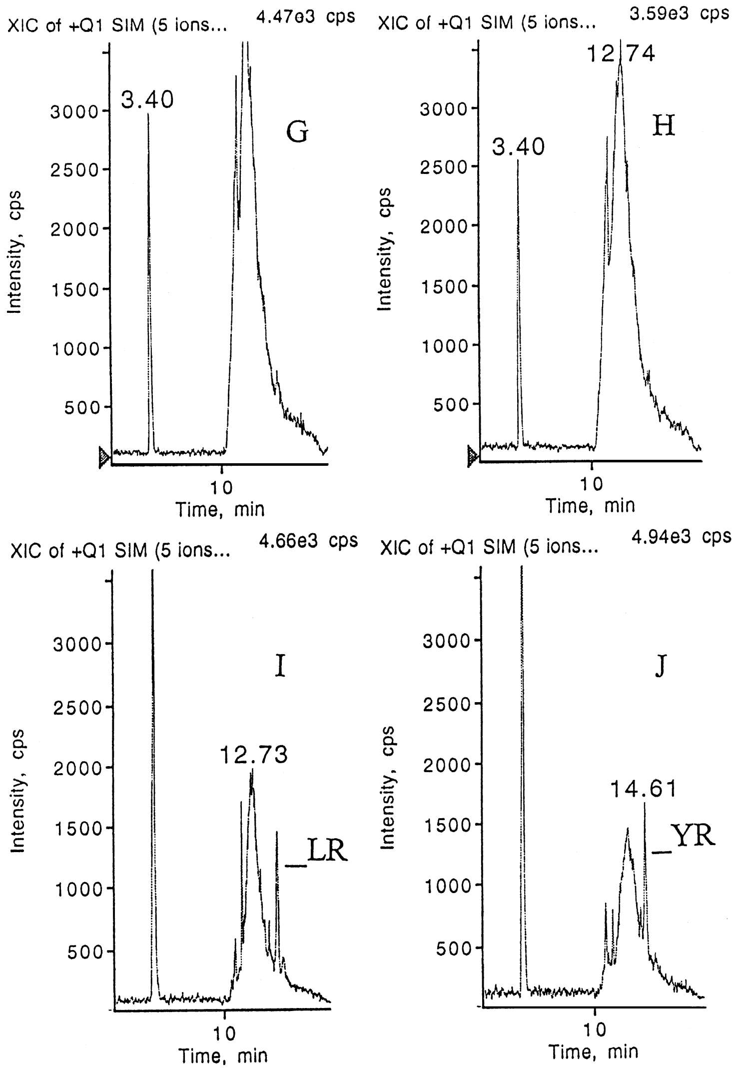


Figure 3. Chromatograms of extract from algal suspension. G: Toxin-free water for LR, H: Toxin free water for YR, I: water spiked with LR (2 ng/mL), J: water spiked with YR (2 ng/mL).

Downloaded At: 09:40 24 January 2011

Table 2

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

Sample	n	Cyanotoxin Added ng/mL	ATX SD*	ATX Rec	D-3 SD	D-3 Rec	LR SD	LR Rec	RR SD	RR Rec	YR SD	YR Rec
Water 15 mL	8	1	1.8	85	1.8	87	1.3	86	2.0	86	2.6	93
	8	10	1.6	84	1.6	89	0.6	89	1.9	89	1.4	93

The limits of detection were close to 0.2 ng/mL for ATX, D-3, and RR, and 0.25 ng/mL for LR and YR. The limits of quantification were 0.4 ng/mL for ATX, D-3, and RR and for LR and YR were 0.5 ng/mL, respectively.

The detection limit of the assay was calculated to be three times the baseline noise from toxin-free water. 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 composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

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. The MS step has high sensitivity, selectivity, and quantitative capability. Quantification using selected ion monitoring has high selectivity, sensitivity, and a broad dynamic range. While conventional HPLC methods require labour- and time-consuming preparation of the samples, 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.[4]

ACKNOWLEDGMENT

We are grateful to Dr. Hans Utkilen, National Institute of Public Health, for providing the D-3 standard.

Downloaded At: 09:40 24 January 2011

REFERENCES

1. S. G. Bell, G. A. Codd, Rev. Med. Microbiol., **5**, 256-264 (1994).

2. W. W. Carmichael, Sci. Am., **270**, 64-72 (1994).

3. W. W. Carmichael, J. Appl. Bacteriol., **72**, 445-459 (1992).

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

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 Comm. Mass Spectr., **Vol. 7**, 714-721 (1993).

7. V. Hormazábal, ∅. ∅stensvik, B. Underdal, O. Skulberg, J. Liq. Chrom. & Rel. Technol., **23(2)**, 185-196 (2000).

8. O. M. Skulberg, Norwegian Institute for Water Research, ISBN 82-577-3488-8, 1-42 (1998).

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

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

Received June 10, 2000
Accepted July 4, 2000
Author's Revisions August 7, 2000
Manuscript 5321

Downloaded At: 09:40 24 January 2011