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Final SOP for HPLC based analysis of cell bound and dissolved nodularin in natural waters

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1 Summary

This SOP describes the trace analysis of cell bound and dissolved nodularin in natural waters.

The analytical approach considers the following steps:

- Preparation of aqueous standard solutions of nodularin for HPLC calibration,
- Online Solid phase extraction of nodularin in water samples,
- Conditions for analysis of nodularin by high-performance liquid chromatography and mass detection.

Table S1: Calibration parameters of nodularin in dissolved and cell bound state

	Slope	Rel.devia-tion in % [1]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
dissolved	1.075	6.2	0.997	0.03	0.06	0.10	85
cell bound	1.247	4.1	0.998	0.05	0.10	0.19	89

Contents

2 Introduction

The SOP describes the trace analysis of cell bound and dissolved nodularin (NOD) in natural algal containing waters.

NOD is a cyclic nonribosomal peptide produced by the planktonic cyanobacterium *Nodularia spumigena*. This cyanobacterium forms blooms in brackish water bodies throughout the world. The late summer blooms of *Nodularia spumigena* are amongst the largest cyanobacterial mass occurrences in the world. Nodularin-R is a heptapeptide and contains several unusual non-proteinogenic amino acids such as methyldehydrobutyrine and the β-amino acid ADDA (-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-diene acid). Nodularin-R is a cyanotoxins and poses a health risk for wild and domestic animals as well as humans. Nodularin-R is a potent hepatotoxin and may cause serious damage to the liver.

The analytical approach considers the following steps:

- Preparation of aqueous standard solutions of nodularin for HPLC calibration,
- Online Solid phase extraction of nodularin in water samples,
- Conditions for analysis of nodularin by high-performance liquid chromatography and mass detection.

3 Preparation of aqueous standard solution

3.1 Materials

Nodularin (NOD) standard, Calbiochem or DHI (DK),

Acetonitrile HPLC grade,

Ammonia, ammoniumacetate, acetic acid, p.A.-quality, e.g. from Merck,

Heptafluorobutyric acid (HFBA), p.A.-quality, e.g. from Fluka,

Benzoyl-arginyl-4-amino-benzoic acid (BAAB) as internal Standard, e.g. from Fluka,

Water purified to 18.2 MOhm cm (e.g. Milli-Q Plus quality).

Clean borosilicate glass test tubes or vials with red/green switch, 1-5mL capacity (micro-reaction vessels).

Borosilicate glass chromatographic vials, e.g. from Merck Eurolab / VWR International: 1.5mL clear glass with writing surface, and crimp caps with Si/PTFE septa. For small sample volumes, polypropylene vials with 0.3mL glass insert.

3.2 Special equipment

Pipettes capable of accurately dispensing 0.5mL – 5mL,
Microlitersyringes: 1µL, 2µL, 5µL, 10µL, 50µL, 250µL,

Laboratory Scales: Precision scale (till 0,0001g),

Volumetric flask 50mL,

HPLC instrument, specifications according to the procedure of chapter 5 of this SOP.

3.3 Stock solutions

3.3.1 Stock solution of the internal standard with a concentration of 50ng/µL

Weigh 5mg BAAB in 100mL volumetric flask, add acetonitrile/MilliQ-water (50:50, v/v) till measuring line.
Give the stock in the ice (-18°C), it is stable for several months.
For daily use fill a 1mL vial with red/green cap, give it in ice (-18°C), it is stable for several weeks.

3.3.2 Stock solutions of standards till 100 ng/µL

The standard is delivered in a defined weigh per vial.
Add acetonitrile to the original vial and adjust a concentration of 100ng NOD / µL.
Transfer the acetonitrile/NOD mix in another vial (if the original is not suitable for longer storage), rinse carefully.
Give the standard in ice (-18°C), it is stable for several years.

3.3.3 Stock solutions of standards till 10 ng/µL

Give 0.1mL of the stock solution in a 1mL vial.
Add 0.9mL acetonitrile.
The stock is stable for several months (-18°C).

3.4 Preparation of the standard curve for nodularin

The following standard curve can be applied for samples containing 1 – 100ng nodularin / 10µL injection.

Mix 25 volumes of acetonitrile and 75 volumes of water to get 25% acetonitrile.
Prepare a dilution series of nodularin.
Use polypropylene vials with 0.3mL glass insert for small sample volumes (total 0.1mL), add 10µL of internal standard (BAAB).

Table 1: Preparation of the nodularin standard curve

concentration in Vial [ng/100µL]	absolute concentration per injektion [ng/10µL]	stock 10ng/µL dosage [µL]	stock 100ng/µL dosage [µL]	dosage of 25%- ACN in Vial [µL]
blind	0	-	-	90
10	1	2	-	86
30	3	3	-	84
50	5	5	-	80
100	10	10	-	70
200	20	-	2	86
300	30	-	3	84
500	50	-	5	80
750	75	-	7.5	75
1000	100	-	10	70

Analyse the samples on the HPLC system in duplicate injections.

Calculate the linear regression for your calibration curve using e.g. calculator or Microsoft Excel software.

$y = mx + b$

y = ng nodularin per injection
x = peak area

The slope of the calibration curve, m, gives the response factor, which is characteristic for your specific chromatographic conditions. The y-axis intercept, b, should be negligible (typically below 0.3) and the correlation coefficient, R^2, should approach 1.

Check the HPLC-MS-Systems regularly by injection of known samples and repeat the calibration as necessary, typically after 1 or 2 months (depending on the amount of samples, solvents used etc).

Table 2: Correlation coefficients for 1- 100ng/10µL inj.volume

Compound	Correlation coefficient
Nodularin	0.999

References:

Pietsch, J., S. Fichtner, S., Imhof, L. Schmidt, W. and Brauch, H.-J.: Simultaneous determination of cyanobacterial hepato- and neurotoxins in water samples by ion-pair supported enrichment and HPLC-ESI-MS-MS. Chromatographia, 54(5,6) 339-344) 2001.

Meriluoto, J., Codd, G.A., 2005. TOXIC. Cyanobacterial Monitoring and Cyanotoxin Analysis, ABO Akademi Finland, ISBN 951-765-259-3.

4 Solid phase extraction of nodularin (NOD) in water samples

4.1 General

Solid phase extraction of nodularin from natural waters is typically performed on C_{18} silica cartridges using the ion pair agent heptafluorobutyric acid (HFBA) [e.g. Pietsch, et al., 2001].

4.2 Solid phase extraction

4.2.1 Materials

Use analytical reagent grade reagent if not indicated otherwise.

Glass-fibre filters (typically Whatman (Maidstone, UK) GF/C), diameter 25-47 mm,

Heptafluorobutyric acid (HFBA), p.A.-quality, e.g. from Fluka,

Ammonium hydroxide (NH_4OH),

Hydrochloric acid (HCl),

Internal Standard BAAB 50ng/µL (see SOP chapter 3.3.1),

Isolute C18(EC) solid phase extraction columns, size 1g sorbent in 6mL reservoir,

Nitrogen, 99.999%.

Borosilicate glass vials (micro reaction vessels) with green/red top, 1mL, 3mL, 5mL capacity.

Borosilicate glass chromatographic vials, e.g. from Merck Eurolab / VWR International: 1.5mL clear glass with writing surface, and crimp caps with Si/PTFE septa. For small sample volumes, polypropylene vials with 0.3mL glass insert.

4.2.2 Special equipment

Vacuum manifold, preferably transparent, equipped with stopcocks, vacuum source and vacuum control,

pH meter,

Filtration unit for 500mL volume,

Evapuration unit, Fa. Barkey (Germany).

4.2.3 Solutions

Acetonitrile, methanole HPLC grade,

Water purified to 18.2 MOhm cm (e.g. Milli-Q Plus quality),

Heptafluorobutyric acid (HFBA),

Acetate buffer 1M.

4.2.4 Procedure

4.2.4.1 Sample filtration

Water samples, can be stored 2 days in maximum at 4°C.
500mL will be filtered by vacuum using a glass fibre filter (typically GF/Whatman). If the biovolume is too high more than one filter should be used.
The filter is stored in a 20mL glass vial – 18°C for further analysis of cell bound toxins. Filtrates which cannot be analyzed immediately are stored at - 18°C also.

4.2.4.2 Extraction of dissolved Nodularin

Frozen samples will be thawed at room temperature.
Add 130µL HFBA and 1mL 1M acetate buffer to 500mL sample, measure the pH of the water sample, and, if necessary, adjust to pH 5.1-5.2 with NH_4OH.
Add 7.5µL internal standard BAAB.

Condition the solid phase extraction cartridge, Isolute C18, 1g in a 6mL reservoir, with 5mL methanol and 5mL acetonitrile followed by 10mL of water.

Wait two hours in minimum. Do not let the cartridge dry during conditioning, sample application and wash.

Apply the sample at a flow rate not exceeding 10 mL/min (visible drops). Regulate the flow with vacuum pressure.

Dry the cartridge by drawing nitrogen through it for 30- 40min.

Elute nodularin with 3x 1mL of acetonitrile containing 0.1% acetic acid in 3mL borosilicate glass vials.

Evaporate the acetonitrile eluate at 40- 50°C using nitrogen carefully.

Resuspend the residue in 100µL of 25% acetonitrile and transfer this solution with a Pasteur pipette in the autosampler polypropylene vial with 0.3mL glass insert.

The analytical conditions are described in chapter 5.

4.2.4.3 Extraction of cell bound Nodularin

The filters frozen near -18°C (see chapter 4 2.4.2) will be used. The filters are stored in 20mL glass vial with screw cap. For toxin extraction a mixture of methanol/MilliQ (75:25, v:v) with 0.1 M acetic acid is used.
Add 5mL of the methanol/MilliQ-mixture in the vial and 15µL of the internal standard (BAAB). The sample extracted by ultrasonication about 15 minutes in a 1st phase. During a 2nd phase the extraction is continued by shaking about 12 h.

The liquid phase is transferred by a 5mL one way polypropylene syringe via a 25mm syringe filter (glass fiber/Nylon 0.45µm) in a 5mL micro reaction vessel. Put this vessel on a 45-50°C pre-heated plate and dry the sample by nitrogen stream completely. Take the dry residual in 0.2mL solution of acetonitrile/MilliQ (25:75, v:v) with 0.1% acetic acid. After sedimentation take the clear phase (min. 0.1mL) by a Pasteur pipette in the autosampler polypropylene vial with 0.3mL glass insert.

The analytical conditions are described in chapter 5.

References

Lawton, L.A., Edwards, C., Codd, G.A.: Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst (London) 119, 1525-1530 (1994).

Meriluoto, J., Codd, G.A., 2005. TOXIC. Cyanobacterial Monitoring and Cyanotoxin Analysis, ABO Akademi Finland, ISBN 951-765-259-3.

Pietsch, J., Fichtner, S., Imhof, L., Schmidt, W. and Brauch, H.-J.: Simultaneous determination of cyanobacterial hepato- and neurotoxins in water samples by ion pair supported enrichment and HPLC-ESI-MS-MS. Chromatographia, 54(5/6), 339-344(2001).

5 Analysis of nodularin (NOD) by high-performance-liquid chromatography with tandem mass spectrometry

5.1 General

Reversed-phase HPLC on C18 phases is a common choice for separating smaller peptides like the toxin nodularin, and the mobile phases for peptides often consist of acetonitrile gradients in the presence of perfluorinated alkyl carboxylic acids, usually trifluoroacetic acid (TFA).
The described chromatographic conditions can be used for both, cell bound and dissolved nodularin.

5.2 Conditions for analysis by high-performance liquid chromatography and mass detection

5.2.1 Materials

Acetonitrile HPLC quality grade,

Water purified to 18.2 MOhm cm (e.g. Millipore Milli-Q Plus quality water),

Heptafluorobutyric acid (HFBA), protein sequence analysis grade, Fluka (Buchs, Schwitzerland),

Acetic acid HPLC grade, Fluka (Buchs, Schwitzerland),

Ammonium hydroxide solution 25%, Fluka (Buchs, Schwitzerland),

C_{18} endcapped HPLC column, e.g. Phenomenex Luna C18 endcapped, 3µm particles, 150 x 2mm I.D. and guard column C18 endcapped, 10µm particles, 20 x 2mm I.D. ,

Polypropylen autosampler vial with 0.3mL glass insert, e.g. from VWR and crimp caps with Si/PTFE septa,

Internal standard solution 50ng/µL BAAB N-Benzoyl-arginyl-4-amino-benzoic acid (BAAB).

5.2.2 Special equipment

High-performance liquid chromatograph system, e.g. Waters™ 600S controller, Waters™ 616 pump, Waters™ 717 auto sampler,

Chromatography analysis software Millennium 2.15,

Column oven, e.g. Techlab (Germany),

Triple-quadropole mass spectrometer equipped with electrospray ionization (ESI) API 2000, Fa. Sciex Applied Biosystems (Canada),

Chromatography analysis software.

5.2.3 HPLC mobile phase

Use brown glass boddles.

Eluent A: 480 mL Milli-Q-Wasser + 0.13 mL HFBA + 0.5 mL acetic acid. Adjust the pH-value with Ammonium hydroxide to 3.5. Add 20 mL acetonitrile.

Eluent A has to prepared weekly fresh.

Eluent B: 500mL acetonitrile + 0.5mL acetic acid.

5.2.4 Chromatography

The HPLC system should be set up as described in the manufacturers instructions including degassing, priming and changing columns.

Always use guard column. Change guard column if the back-pressure rises or peak forms deteriorate.

Set column oven at 36°C.

Chromatograph the samples and standards as per the recommended HPLC gradients (see below), use 10µL injections.

Compare retention times and spectra to standards.

Calculate the nodularin concentration according to the standard curve.

Table 1: **Suggested gradient programme for Phenomenex Luna C18 endcapped, 3µm particles, 150 x 2mm I.D., linear gradient at a flow rate of 0,2mL min^{-1}. Injection cycle about 45 minutes (the injection by the Waters 717 autosampler takes about 3 min).**

Time (min)	% A	% B
START	100	0
0.01	99	1
2.7	80	20
8	60	40
12	35	65
15	32	68
16	5	95
21	5	95
25	100	0

The quantification of nodularin is carried out in MRM-Mode (multiple reaction monitoring) of API 2000.

Conditions: Electrospray ionization (ESI) positive mode at 5200V

Heater gas pressure at 75psi; temperature 400 °C

Table 2: Parameters of detection by ESI-MS/MS API 2000 (CE: collision energy; DP: declustering potential; $[M+H]^+$: protonated molecule)

	CE (V)	DP (V)	$[M+H]^+$ (m/z)	Product-Ion (m/z)
Nodularin	80	115	825.4	135.1
IS BAAB	30	50	398	244.1
IS BAAB	30	50	398	216.1

In Table 3 and 4 the statistical evaluation of the method is summarized.

Table 3: Dissolved state: Calibration parameters of SPE of 500mL sample, injection volume 10µL, range: 0.1- 1.0µg/L, calculation via internal standard

	Slope	Rel.devia-tion in % [1]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
Nodularin	1.075	6.2	0.997	0.03	0.06	0.10	85

Table 4: Cell bound state: Calibration parameters of sample injection, injection volume 10µL, range: 0.1- 5.0µg/L, calculation via internal standard

	Slope	Rel.devia-tion in % [*]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
Nodularin	1.247	4.1	0.998	0.05	0.10	0.19	89

[*] DIN 38 402 part 51, Software: Perkin Elmer. Statistische Qualitätskontrolle analytischer Daten SQS 3.3 Microsoftversion, Produkt ID 00547-270-6402887-03-061, 1995.

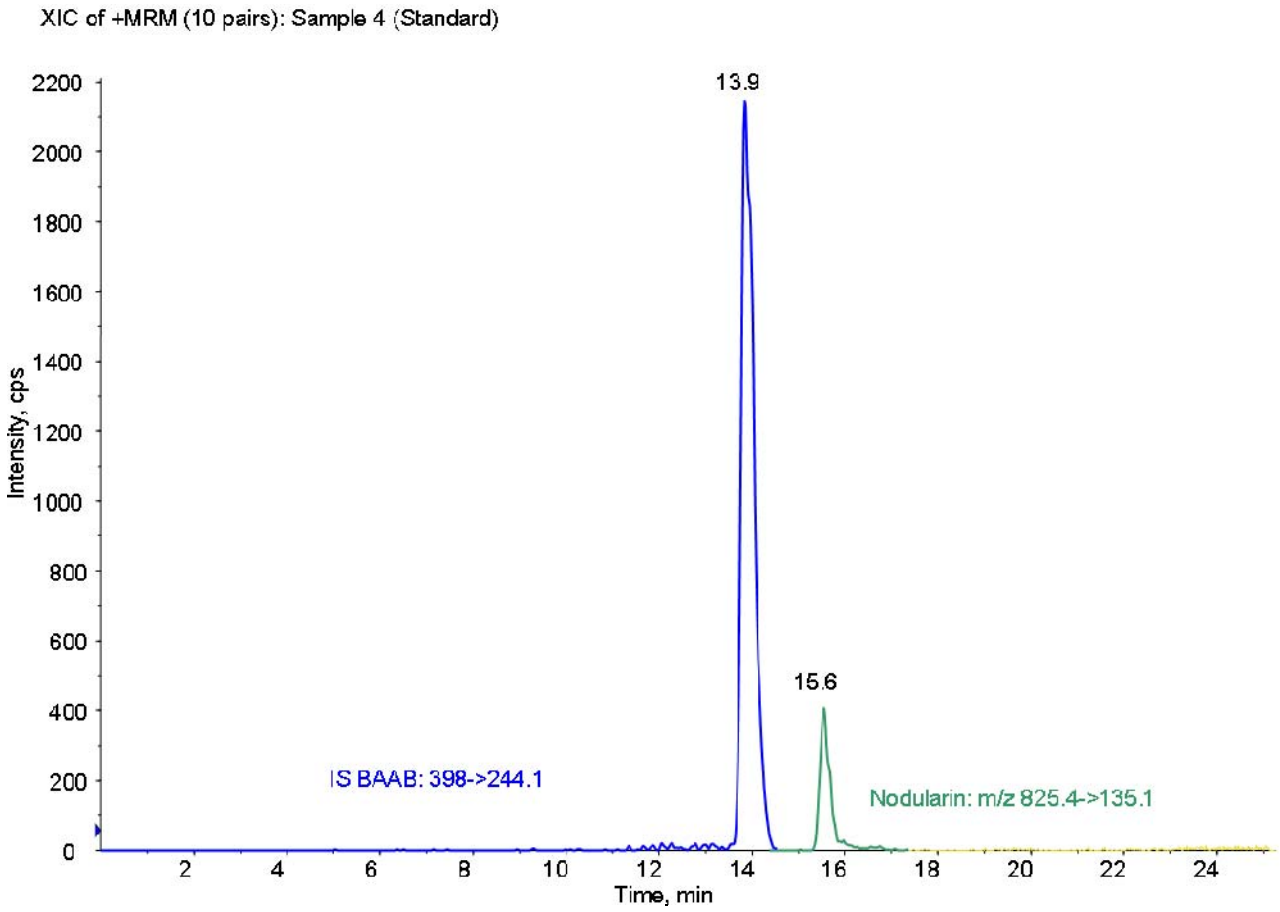

Figure 1: **Extracted ion chromatogramm (XIC) of nodularin and internal standard BAAB; Multiple Reaction Monitoring positive mode (+MRM)**

References

Pietsch, J., Fichtner, S., Imhof, L., Schmidt, W. and Brauch, H.-J.: Simultaneous determination of cyanobacterial hepato- and neurotoxins in water samples by ion pair supported enrichjment and HPLC-ESI-MS-MS. Chromatographia, 54(5/6), 339-344(2001).

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