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Final SOP for HPLC based analysis of Saxitoxins (cell bound and dissolved state) in natural waters

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1 Summary

The substance spectrum of new toxins includes the following compounds:

- saxitoxin (SAX),
- neosaxitoxin (NEO),
- gonyautoxin-I (GTX-I),
- gonyautoxin-II (GTX-II),
- gonyautoxin-III (GTX-III),
- gonyautoxin-IV (GTX-IV),

The analytical approach considers the following steps:

- Preparation of aqueous standard solutions of saxitoxins and for HPLC calibration,
- Online Solid phase extraction of saxitoxins in water samples using a combination of pre-concentration columns,
- Extraction of cell bound saxitoxins,
- Conditions for saxitoxin analysis by high-performance liquid chromatography and mass detection.

Table S1: Calibration parameters of saxitoxins in cell bound state

Com-pound	Slope	Rel.deviation in % [1]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
SAX	0.365	4.4	0.998	0.05	0.09	0.18	83
NEO	0.049	7.1	0.996	0.09	0.18	0.31	79
GTX 2/3	0.214	4.8	0.998	0.06	0.12	0.22	87
GTX 1/4	0.233	7.2	0.996	0.09	0.18	0.31	85

Table S2: Calibration parameters of saxitoxins in dissolved state

Com-pound	Slope	Rel.deviation in % [1]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
SAX	0.423	8.4	0.994	0.03	0.07	0.12	70
NEO	0.087	21.4	0.965	0.09	0.18	0.30	60
GTX 2/3	0.253	7.1	0.996	0.03	0.07	0.12	86
GTX 1/4	0.298	10.1	0.992	0.04	0.08	0.14	72

Contents

2 Introduction

Besides microcystins which are the most common cyanotoxins world wide, a new group of toxins, the so called *saxitoxins* became more relevant in aqueous medium in recent years. Up to now about eighteen (18) different structures are known. Saxitoxins are neurotoxins and can be formed by marine dinoflagellates and fresh water blue green algae. The importance of saxitoxins is in relation to red tide in shellfish and causes the paralytic shellfish poisoning (PSP). In general saxitoxins occur cell bound. On the other hand, they can be released into water by algal cell destruction. The molecular weight of saxitoxins lies between 300 and 400 a.u. Their structures are characterized by amino and sulfonic acid groups. This is the reason of a very high polarity which makes their extraction from the water phase quite difficult.

The approach for trace analysis of dissolved saxitoxins is subdivided into the following steps:

- Preparation of aqueous standard solutions of saxitoxins and for HPLC calibration,
- Online Solid phase extraction of saxitoxins in water samples using a combination of pre-concentration columns,
- Extraction of cell bound saxitoxins,
- Conditions for analysis saxitoxins by high-performance liquid chromatography and mass detection.

3 Preparation of aqueous standard solutions

3.1 General

For preparation of stock solutions certified reference standards were used.

3.1.1 Materials

Saxitoxin (STX) from NRC Canada

Neosaxitoxin (NEO) from NRC Canada

Gonyautoxin-1 & -4 (GTX1/4) from NRC Canada

Gonyautoxin-2 & -3 (GTX2/3) from NRC Canada

Benzoyl-L-argenyl-4-amino-benzoic acid (BAAB) from Fluka

Acetonitrile HPLC grade from VWR International

Water purified to Milli-Q Plus quality

Standard vessels 10mL – 100mL

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

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

3.1.2 Special equipment

Microlitersyrings 10µL, 25µL, 50µL,100µL, 250µL

Measuring cylinder 100mL

Electronic laboratory scale with precision of 0.0001g

3.2 Standard solutions

3.2.1 Concentration of certified calibration solutions

STX 65±3 µM in 3mM hydrochloric acid
Lot#20020227
NEO 65±2 µM in 3mM hydrochloric acid
Lot#20060419

GTX1 106±5µM; GTX4 35±2µM in 3mM hydrochloric acid Lot#20020711
GTX2 118±6µM; GTX3 39±2µM in 3mM hydrochloric acid Lot#20020613

3.2.2 Internal standard stock solution, concentration 50 ng/µL

- 5mg BAAB will be dissolved in a 100ml measuring cylinder in a mixture of acetonitrile/Milli-Q-water (50:50, v/v)

- For daily use fill 3mL in a 3mL vial with traffic light cap.

3.2.3 Standard stock solution; concentration 5 ng/µL

The following standard mix is used for daily control. The total need of the standard solution is 0.5mL. Put in the fridge at 4°C to guarantee its stability about 4 weeks.

Table 1: Composition of daily standard

Standard	Conc. Standard in µM	Conc. Standard in µg/mL	Use x µL for Conc. 5ng/µL
Hydrochloric acid	3mM		166.7µL
STX	65	19.5	128µL
NEO	65	20.5	122µL
GTX1&4	ca. 141	58	43.1µL
GTX 2&3	ca. 157	62.1	40.2µL

3.3 Preparation of standard curves for saxitoxins

For each concentration autosampler polypropylen vials with 0.3mL glass inserts are used. The total volume of each calibration solution is 0.1mL/vial. This volume is sufficient for 3 till 5 injections with 10µL injection volume.

Table 2: Standard solutions for calibration curves

Conc. in vial in µg/mL	Abs. conc. pro injektion in ng/10µL	Stock solution 5ng/µL addition in µL	IS BAAB 50ng/µL addition in µL	MilliQ-water addition in µL
-	-	-	10	90
0.1	1.0	2	10	88
0.2	2	4	10	86
0.3	3	6	10	84
0.5	5.0	10	10	80
0.75	7.5	15	10	75
1	10	20	10	70
1.5	15	30	10	60
2	20	40	10	50
2.5	25	50	10	40

HPLC and mass spectrometry: see chapter 5

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

$y = mx + b$

y = ng analyte per injection
x = peak area

The slope of the calibration line, 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 3: Correlation coefficients for 2- 25ng/10µL/inj.volume

Compound	Correlation coefficient
Saxitoxin	0.997
Neosaxitoxin	0.994
GTX 2/3	0.997
GTX1/4	0.995

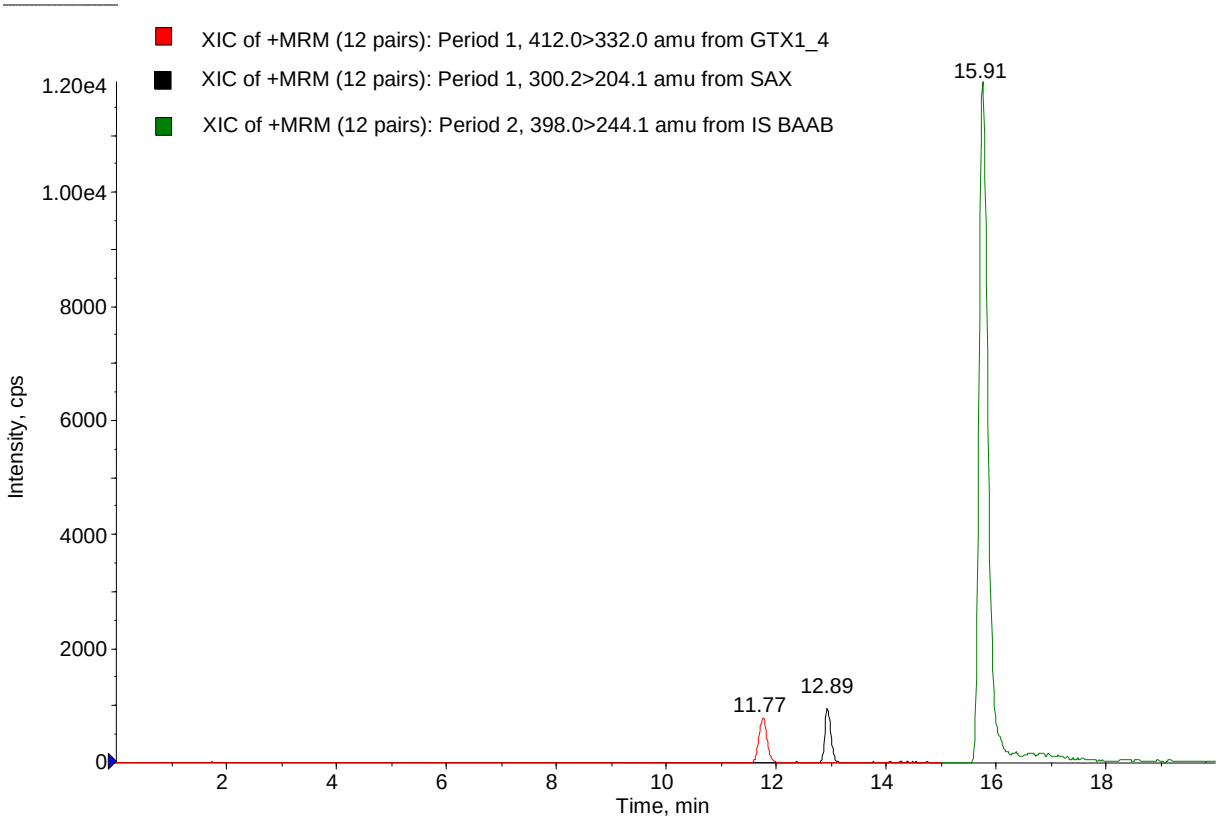

Figure 1: Standard extracted ion chromatogram of a pure 100% calibration for GTX1/4, SAX und BAAB (IS)

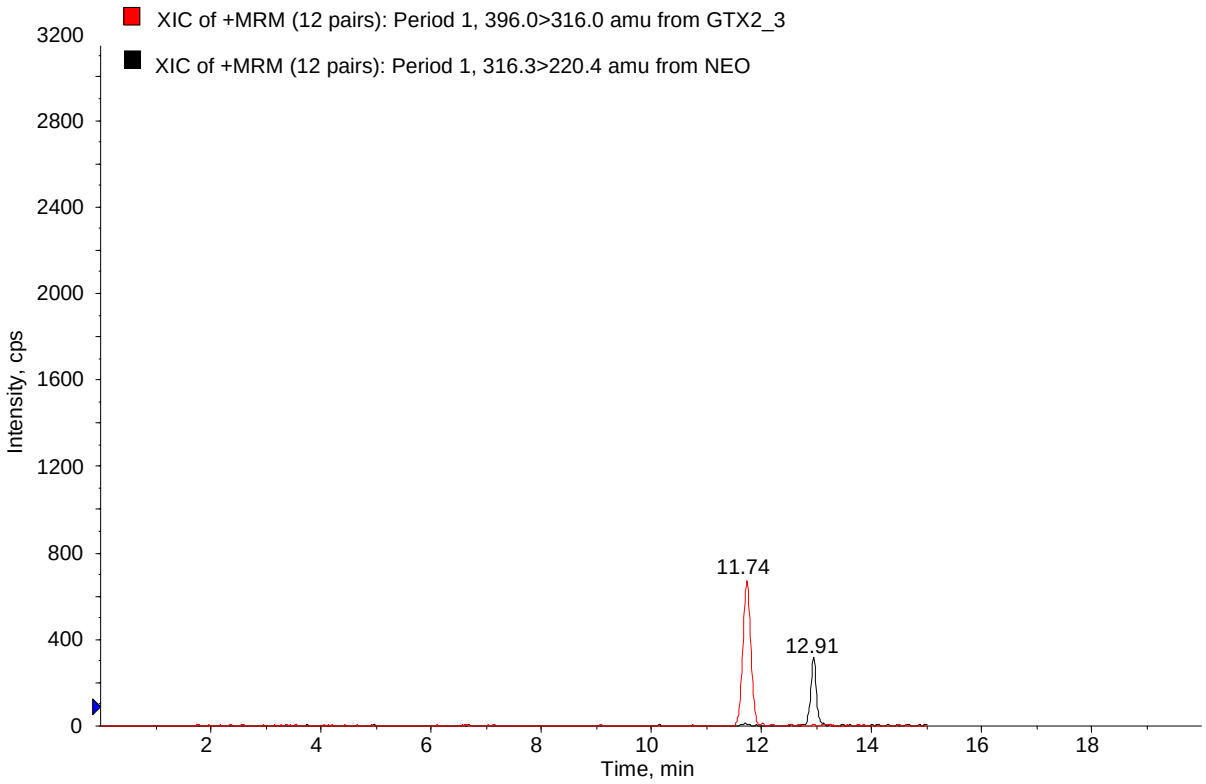

Figure 2: **Standard extracted ion chromatogramm of a pure 100% calibration for GTX2/3 and NEO**

4 Extraction of saxitoxins

4.1 General

Certified saxitoxin standards are available world wide via the National Research Council (NRC) Canada. The quantity of this material is very limited and expensive.
The high polarity of those structures makes their pre-concentration from aqueous phase very difficult. A combination of different pre-column materials should be used. Such a system works only by maintenance of a relevant pressure during the concentration step. This pressure can be maintained by HPLC-pumps only.
Therefore, for the method described in this SOP, a commercially on-line concentration system was used. This equipment guaranteed the constancy of pressure needed. Additional the online equipment makes it possible to use lower sample volumes for pre-concentration, because 100% of the concentrated sample passes the analytical column. In comparison to that the injection of toxins obtained via on-site pre-concentration of 500mL, which is a general procedure for microcystins, lies in the average near 10% only.
So, standard materials can be saved by online techniques.

4.2 Online solid phase extraction of dissolved saxitoxins

4.2.1 Materials

Acetonitrile HPLC grade, VWR International

Water purified (Milli-Q Plus quality)

Sodium hydroxide in Milli-Q 0.1M

Hydrochloric acid 0.1M

Phosphate buffer solution 0.25M pH5.5

Sodium chloride, p.A.

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

20mL Vial with screw cap

Pipette 1-2mL

Glass bottles 250mL, 500mL

Measuring cylinder 100mL

Microlitersyringe 5µL, 100µL, 500µL

4.2.2 Equipment

Magnetic stirrer

pH-meter Fa. WTW (Germany)

Vacuum filtration unit Fa. Satorius (Germany)

Agilent HPLC 1090 online-SPE- System Prospekt-SDU Fa.Spark (Netherlands)*

LC/MS/MS API 2000 Fa. Sciex Applied Biosystems

* use of other commercial available on-line SPE-systems is possible

4.2.3 Procedure

4.2.3.1 Sample filtration

Water samples, 500mL, can be stored 2 days in maximum at 4°C. 250mL 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.

4.2.3.2 Sample adjustment

Frozen samples will be thawed at room temperature. Take 100mL from the sample and give it in a 250mL flask. In case of fresh water samples add 2g sodium chloride p.A.. After addition of 1 mL phosphate buffer adjust the pH-value with 0.1 M HCl or 0.1 M NaOH to the range between 5.3 and 5.5.
Add 5 µL of the internal standard to the sample. The sample is ready for online concentration.

4.2.3.3 Online- concentration

Adjustment of the equipment:

With the SPE-system the columns can be flushed and conditioned. The autosampler has space for 14 samples. The ports 1 till 14 are for samples, port 15 and 16 for conditioning solvents (Milli-Q, acetonitrile).
Flushing is carried out with 10mL sample, 10mL Milli-Q and 5mL acetonitrile. In order to avoid memory effects this step should be done before each sample concentration. The flow scheme is shown in Figure 1.

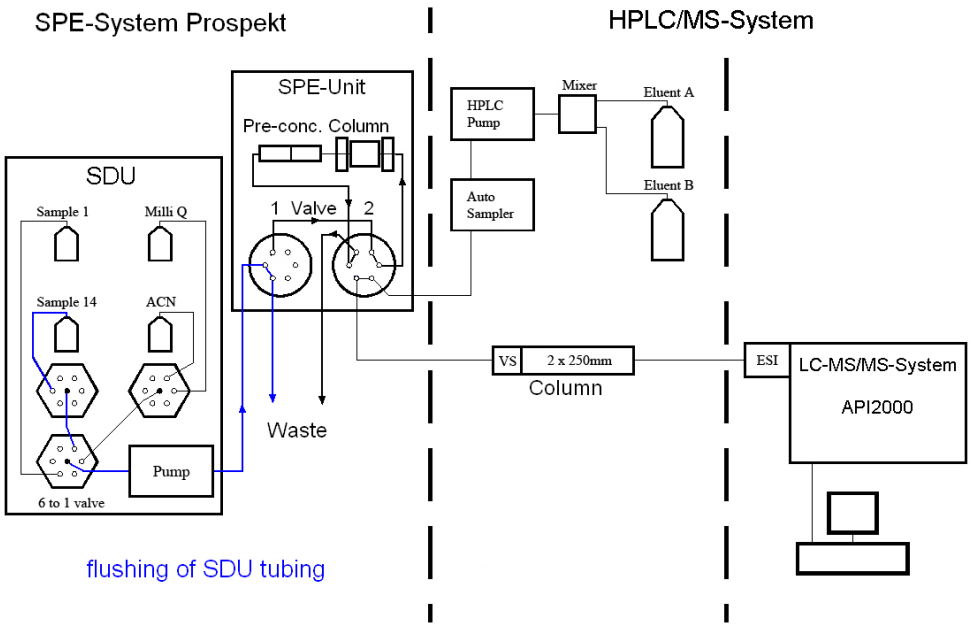

Figure 1: Flow scheme of flushing of the pre-concentration system with sample and solvent

Conditioning of concentration columns:

The solid phase system consists of a combination of an online extraction cartridge strata-x 20x2mm Fa. Phenomenex and a guard column 20x2mm filled with Hypercarb Fa. ThermoHypersil-Keystone.

Pre-Concentration of samples:

The concentration of samples is given by the software of the concentrator and the HPLC system used. For the equipment used in this SOP the flow is illustrated in Figure 2.

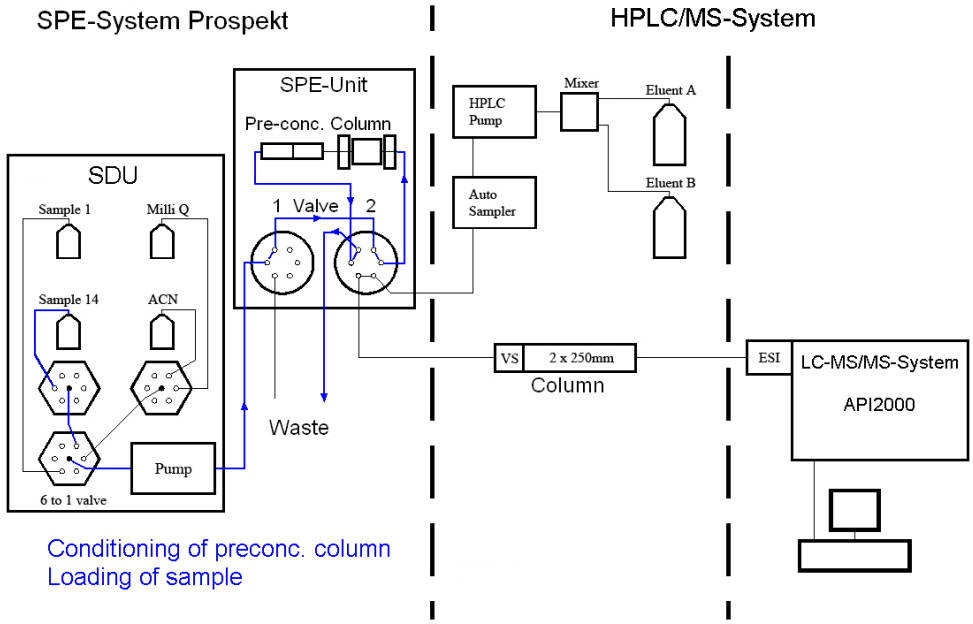

Figure 2: Flow scheme of sample concentration

Software for concentration:

The software consists of two time programs:

1. The sample preparation program (responsible for conditioning, concentration and flushing)

and

2. The sample transfer program (responsible for elution of the sample and clean up of the concentration column).

The chronological coordination of all steps is done by the HPLC 1090 software. The concentration and the analysis by LC/MS/MS are running in parallel (see Figure 3).

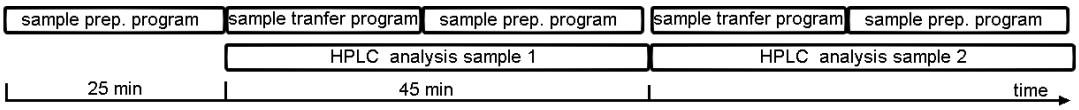

Figure 3: Chronological coordination of "concentration" and "analysis"

In Tables 1 and 2 the detailed time regime is illustrated.

Table 1: Timetable of the sample transfer program

time	Set at:	function	comment
0:00 min	Switch valve 1	Valve to Elution and Start Chromatograph	start of analysis
12:00 min	Switch vale 2 Channel 16	Valve to Waste Switch to Solvent channel acetonitrile	
12:01 min	Flow Rate 2mL	Start Pump SDU	Clean up of capillaries with ACN
14:01 min	Switch Valve 1	Valve to Purge	Clean up of concen-tration column with ACN
14:05 min	Flow Rate 2.5mL	Pump flow rate 2.5mL/min	
15:30 min	Flow Rate 0mL	Stop pump SDU	
15:38min	Switch valve 2	Valve to waste	
15:40 min		End of timetable	

Table 2: Timetable sample preparation program

time	Set at:	function	comment
0:00 min	Switch valve 2 Channel 16 Flow Rate 2.5mL	Valve to preconc. column Channel with Acetonitrile Start Pump SDU	Conditioning of concentration column with ACN
2:00min	Channel 15	Switch to Solvent channel MilliQ-water	Conditioning of concentration column with MilliQ-water
8:00min	Switch valve 2	Valve to waste	
	Channel 1-14 Flow rate 5mL	Choice of sample Pump flow rate 5mL/min	Clean up of capillaries with sample
10:00 min	Flow Rate 2.0mL Switch valve 2	Pump flow rate 2.0mL/min Valve to preconc. column	Concentration of sample
25:00min	Switch valve 2 Channel 15 Flow Rate 5mL	Valve to waste Switch to MilliQ-water Pump flow rate 5mL/min	Clean up of capillaries with MilliQ-water

time	Set at:	function	comment
26.00min	Flow Rate 2.5mL Switch valve 2	Pump flow rate 2.5mL/min Valve to preconc. column	Clean up of pre-concentration column with Milli Q-water
27:30min	Flow Rate 0mL	Stop pump	
27:31min		End of timetable	

4.3 Extraction of cell bound saxitoxins by ultrasonication and shaking

The filters frozen near -18°C (see chapter 4.2.3.1) will be used. The filters are stored in 20mL glass vial with screw cap. For toxin extraction a mixture of methanol/MilliQ (1:1, 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.

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

5.1 General

The complete chromatographic separation of structural very similar compounds GTX 1 and GTX 4 as well as GTX 2 and GTX 3 is not possible. Additional, the fragmentation pattern of GTX 1 and 4 as well as GTX 2 and 3 is identical. Therefore the identification and quantification is only possible in form of the sum of GTX1/4 and GTX2/3 respectively. The MS/MS detection for this is necessary.

The described chromatographic conditions can be used for both, cell bound and dissolved toxins. For cell bound toxins 20 µL sample (see chapter 4.3) is injected using the gradient program in Table 1.

5.1.1 Materials

Acetonitrile HPLC quality grade,

Water purified to Millipore Milli-Q Plus quality

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

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

Ammonium hydroxide solution 25%, Fluka (Buchs, Schwitzerland).
Standard stock solution according in chapter 3

Column: Phenomenex Luna C18(2) 5µm 250x3mm i.D. Cat.Nr. 00G-4252-Y0

Guard-column: C18 4x2mm i.D. Cat.Nr. AJ0-4286, Fa. Phenomenex

Borosilicate glass chromatographic vials, e.g. from Merck Eurolab / VWR International: 1.5 mL clear glass

5.1.2

Special equipment

Agilent 1090 online/SPE System consists of HPLC 1090 und SPE-System Prospekt-SDU Fa.Spark (Netherlands)
LC/MS/MS API 2000 Fa. Sciex Applied Biosystems (Canada)

5.1.3 HPLC mobile phase

HPLC mobile phase component A: acetonitrile + 0.1% acetic acid
HPLC mobile phase component B: 95 % Milli-Q + 5 % acetonitrile + 0.1% acetic acid + 0.05% HFBA with Ammonium hydroxide solution, pH-value = 3.8 (replace every week).

5.2 Chromatography procedure for standards and real samples

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

Always use guard column (4 x 2mm, C18). Change guard column if the back-pressure rises or peak forms deteriorate.

Set column oven at room temperature.

The sample preparation and the software timing are described in chapter 4.

Figure 1 shows the eluent flow during the step "analysis" for dissolved toxins.

For cell bound toxins use HPLC autosampler only.

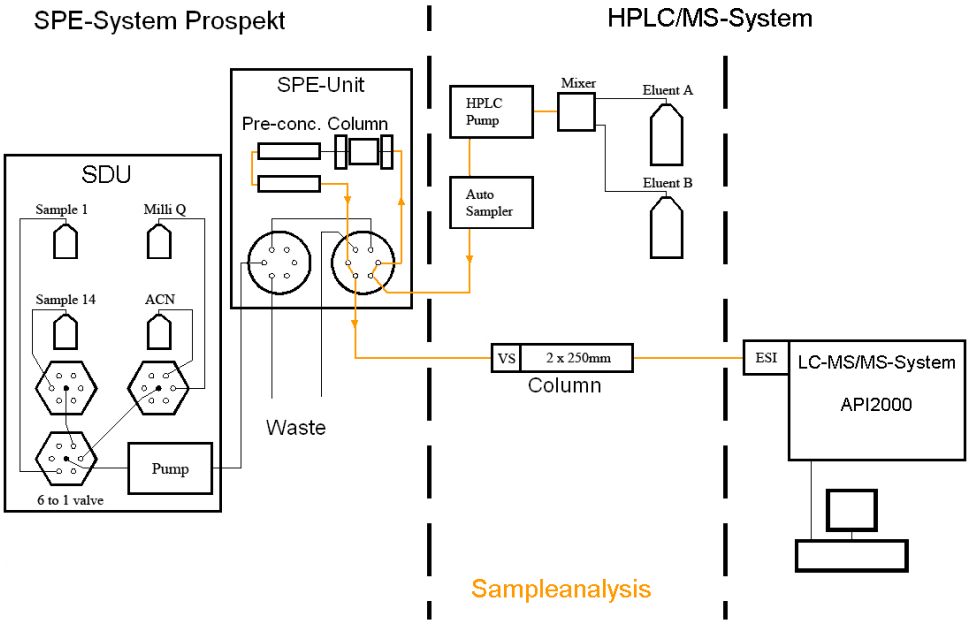

Figure 1: Flow scheme during the step „analysis“

Table 1: Gradient program for Phenomenex Luna C18(2) 5µm 250x3mm i.D. gradient at a flow rate of 0.2 ml min^{-1}, analysis cycle about 45 minutes

Time (min)	% A	% B
START	5	95
0.01	20	80
10	55	55
15	50	50
23	95	5
27	95	5
29	5	95
45	5	95

The identification and quantification of the saxitoxins is done by MS/MS-System API2000 in MRM (Multi Reaction Monitoring) – Mode.

All MS-parameters are focused for the system API2000 (see Tables 2 and 3). If other MS-systems are used all parameters have to be adjusted.

Table 2: Source dependent parameters for API2000 with Electron spray interface (ESI)

Curtain gas (CUR)	25
Nebulizer gas (GS1)	30
Heater gas (GS2)	50
Collision gas (CAD)	3
Temperature heater gas (TEM)	350°C
IonenSpray voltage (IS)	+5200 V
Focusing Potential (FP)	350
Entrance Potential (EP)	10

Table 3: Compound dependent parameters LC/MS/MS API2000

Analyte	Q1 mass m/z	Q3 mass m/z	Declustering potential (DP)	Cell entrance potential (CEP)	Collision energy (CE)
STX	300.2	204.1	30	30	35
STX	300.2	138.1	40	30	40
NEO	316.3	220.4	30	30	25
NEO	316.3	238.3	20	30	20
GTX1/4	412	332	20	30	20
GTX2/3	396	316	20	30	20
IS BAAB	398	244.1	50	19	28
IS BAAB	398	216.1	50	19	33

Q1 = $[M+H]^+$: protonated molecule; Q3 = Product ion

In Figures 2 and 3 standard chromatograms corresponding to 0.1 µg/L for each are shown exemplarily.

In Table 4 and 5 the statistical evaluation of the method is summarized.

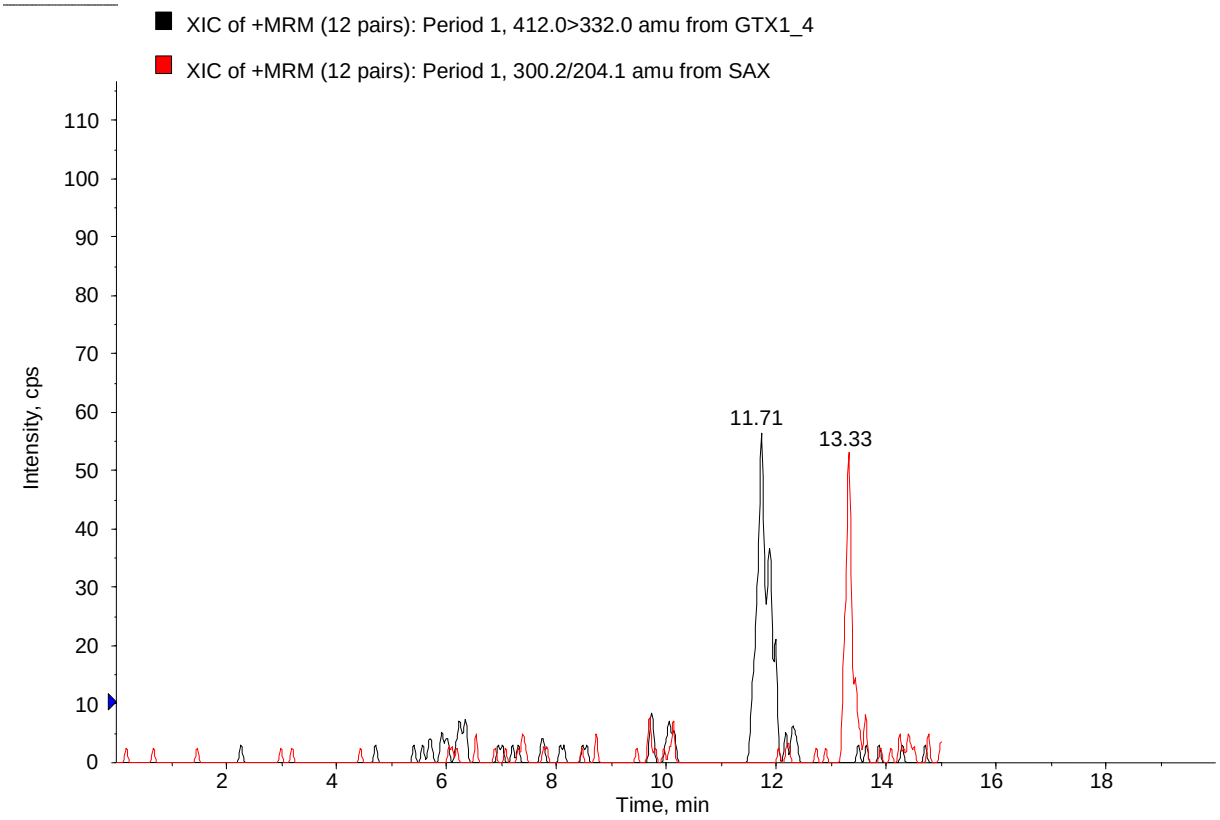

Figure 2: **Standard chromatogram of GTX1/4, and SAX corresponding 0.1µg/L per substance in a real surface water matrix**

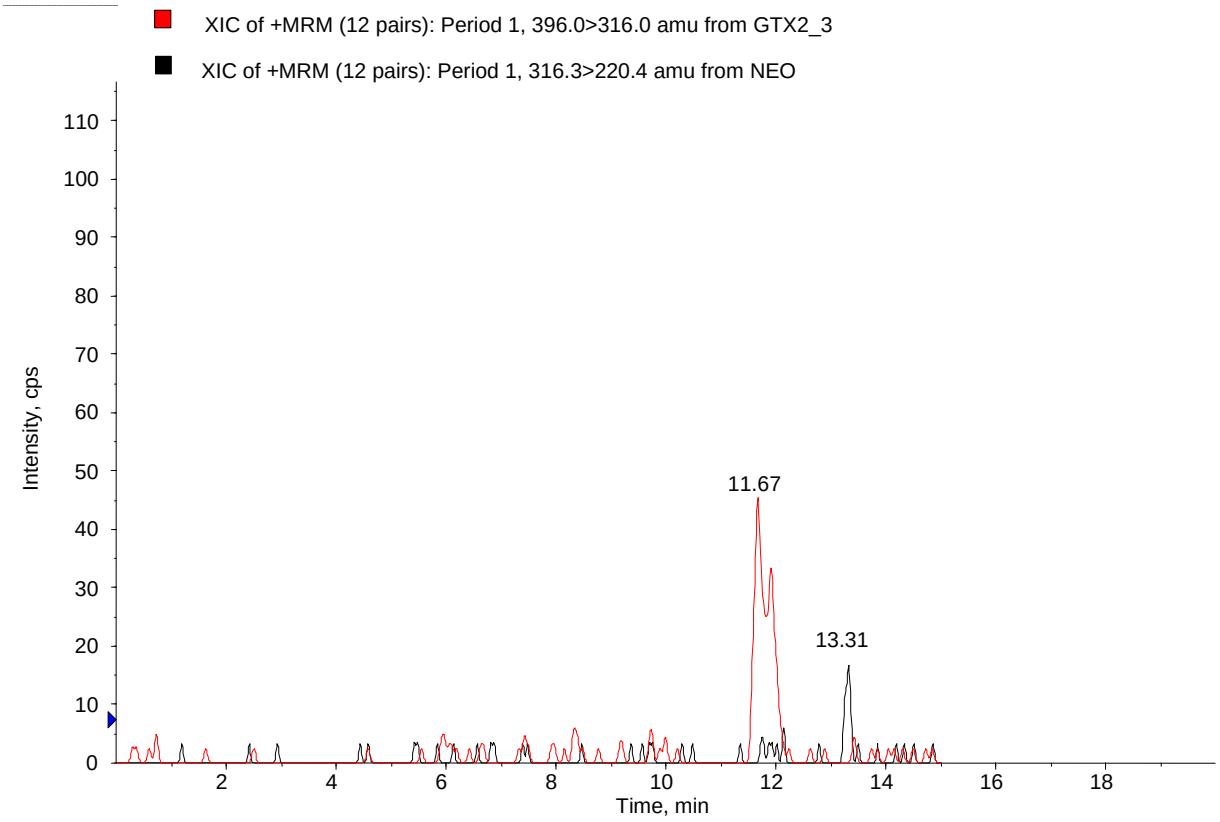

Figure 3: **Standard chromatogram of GTX2/3 and NEO corresponding 0.1µg/L per substance in a real surface water matrix** (a slide variation of retention time can be caused by matrix effects)

Table 4: **Dissolved state: Calibration parameters of online-SPE of 30mL sample, range: 0.1- 0.5µg/L, calculation via internal standard**

Com-pound	Slope	Rel.deviation in % [1]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
SAX	0.423	8.4	0.994	0.03	0.07	0.12	70
NEO	0.087	21.4	0.965	0.09	0.18	0.3	60
GTX 2/3	0.253	7.1	0.996	0.03	0.07	0.12	86
GTX 1/4	0.298	10.1	0.992	0.04	0.08	0.14	72

Table 5: Cell bound state: Calibration parameters of sample injection, injection volume 20µL, range: 0.1- 2.5µg/L, calculation via internal standard

Com-pound	Slope	Rel.deviation in % [1]	Correlation coefficient	Limit of detection in µg/L	Limit of registration in µg/L	Limit of determination in µg/L	Recov. in %
SAX	0.365	4.4	0.998	0.05	0.09	0.18	83
NEO	0.049	7.1	0.996	0.09	0.18	0.31	79
GTX 2/3	0.214	4.8	0.998	0.06	0.12	0.22	87
GTX 1/4	0.233	7.2	0.996	0.09	0.18	0.31	85

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