



Deliverable 3.2.5

*Interim SOP for HPLC based analysis
of new algal toxins (dissolved state) in
natural waters*

TECHNEAU

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

Table S1: Calibration parameters of saxitoxins in dissolved state

Compound	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

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2 Introduction

Besides the 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 the saxitoxins occur cell bound. On the other hand, they can be released in to the 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,
- 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, 5 ml 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 10 μ L, 25 μ L, 50 μ L, 100 μ L, 250 μ L

Measuring cylinder 100 mL

Electronic laboratory scale with precision of 0.0001 g

3.2 Standard solutions

3.2.1 Concentration of certified calibration solutions

STX 65 \pm 3 μ M in 3mM hydrochloric acid
Lot#20020227

NEO 65 \pm 2 μ M in 3mM hydrochloric acid
Lot#20060419

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

3.2.2 Internal standard stock solution, concentration 50ng/µL

- 5mg BAAB will be dissolved in a 100ml measuring cylinder in a mixture of 50/50 v acetonitrile/Milli-Q-wate,
- For daily use fill 3 mL in a 3 mL 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 0.5 mL. 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 0.3 mL glass inserts are used. The total volume of each calibration solution is 0.1 mL/vial. This volume is sufficient for 3 till 5 injections with 10 µL injection volume.

Table 2: Standard solutions for calibration curves

Conc. in vial in $\mu\text{g/mL}$	Abs. conc. pro injektion in $\text{ng}/10\mu\text{L}$	Stock solution $5\text{ng}/\mu\text{L}$ addition in μL	IS BAAB $50\text{ng}/\mu\text{L}$ 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 SOP_TCHNEAU_SNGTX_2007_03

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

$$y = mx + b$$

$$y = \text{ng analyte per injection}$$

$$x = \text{peak area}$$

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.

Repeat the calibration as necessary, typically after 3 or 6 weeks.

Table 3: Correlation coefficients for 2- 25ng/10µL/inj.volume

Compound	Correlation coefficient
Saxitoxin	0.997
Neosaxitoxin	0.990
GTX 2/3	0.997
GTX1/4	0.995

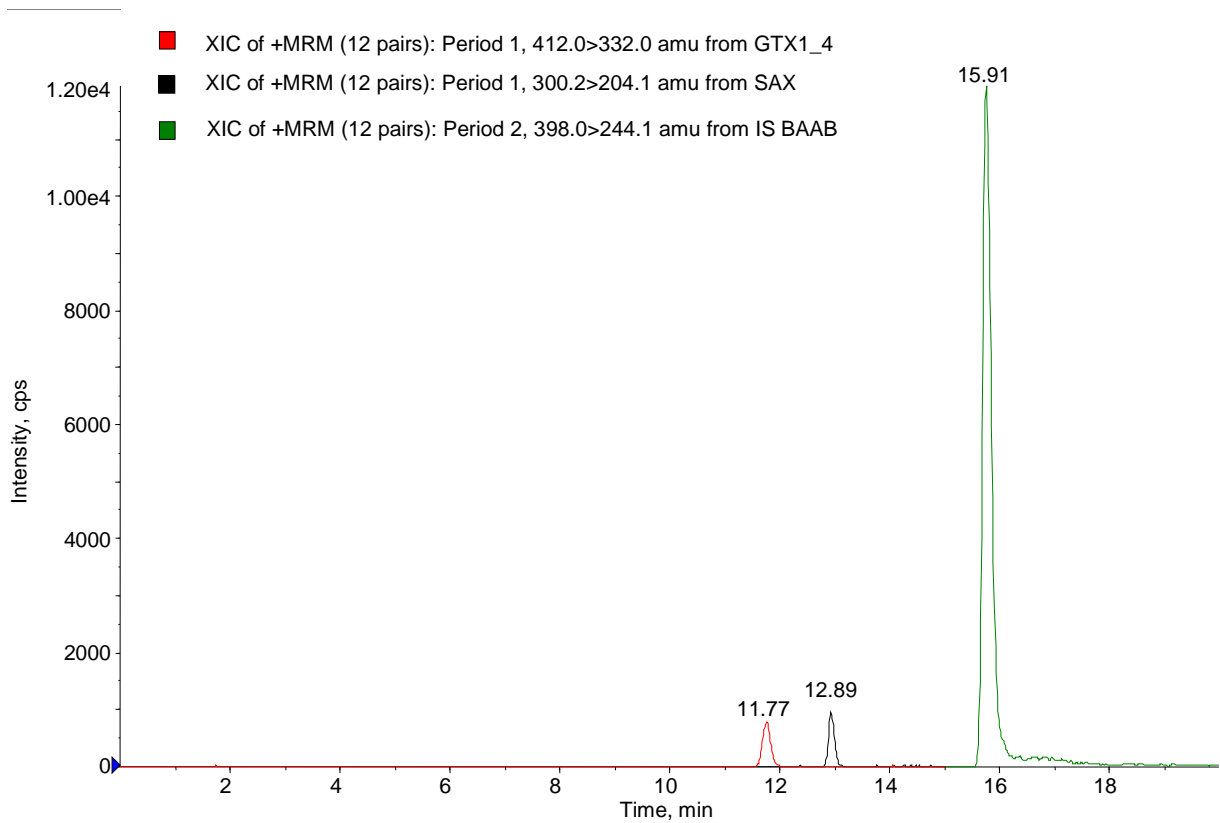


Figure 1: Standard extracted ion chromatogram of a pure 100% calibration for GTX1/4, SAX und BAAB (IS)

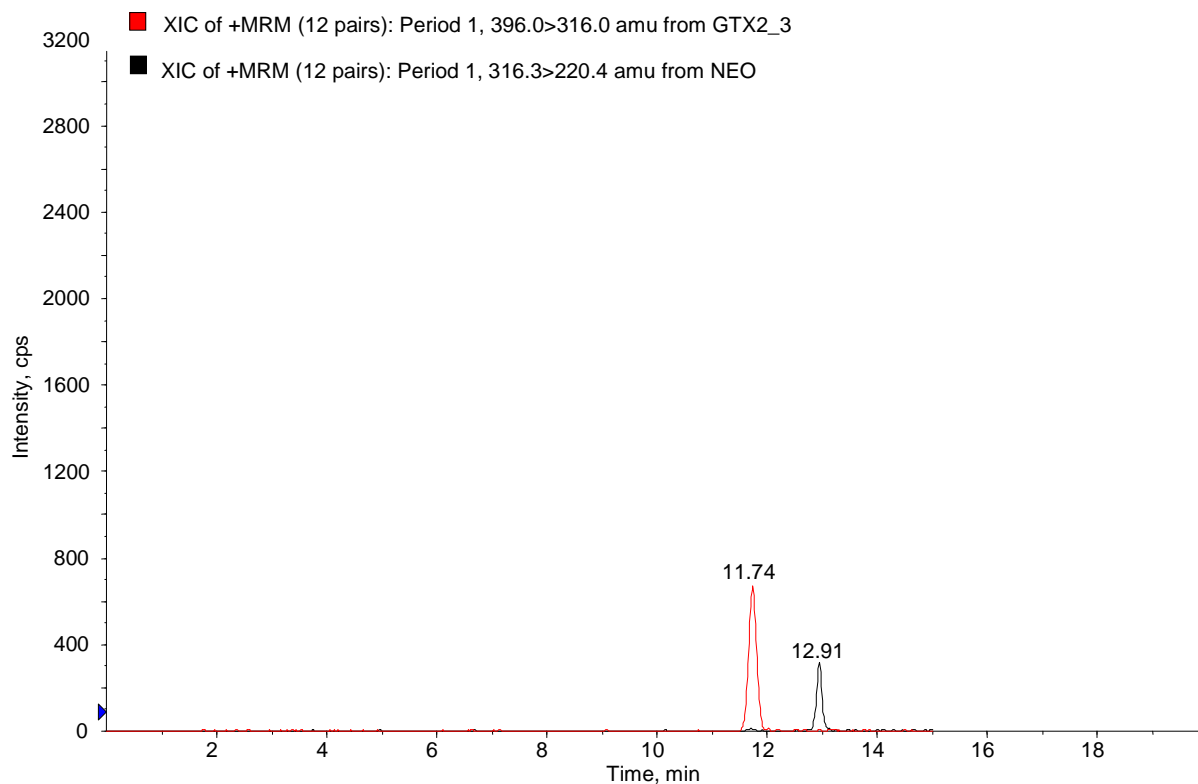


Figure 2: Standard extracted ion chromatogram of a pure 100% calibration for GTX2/3 and NEO

4 Online Solid phase 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 must 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. Additionally 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 500 mL, which is a general procedure for microcystins, lies in the average near 10% only. So, standard materials can be saved by online techniques.

4.1.1 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 5 μ L, 100 μ L, 500 μ L

4.1.2 Equipment

Magnetic stirrer

pH-meter z.B. Fa. WTW (Germany)

Vacuum filtration unit Fa. Satorius (Germany)

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

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

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

4.2 Procedure

4.2.1 Sample filtration

Water samples, 500 mL, can be stored 2 days in maximum at 4°C. 250 mL 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 20 mL glass vial - 18°C for further analysis of cell bound toxins. Filtrates which cannot be analyzed immediately are stored at -18°C also.

4.2.2 Sample adjustment

Frozen samples will be thawed at room temperature. Take from the sample 100 mL and give it in a 250 mL flask. In the case of fresh water samples add 2 g 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 μ L of the internal standard to the sample. The sample is ready for online concentration.

4.2.3 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).

The flushing is carried out with 10 mL sample, 10 mL Milli-Q and 5 mL acetonitrile. In order to avoid memory effects this step should be done before each sample concentration. The flow scheme is shown in Figure 1.

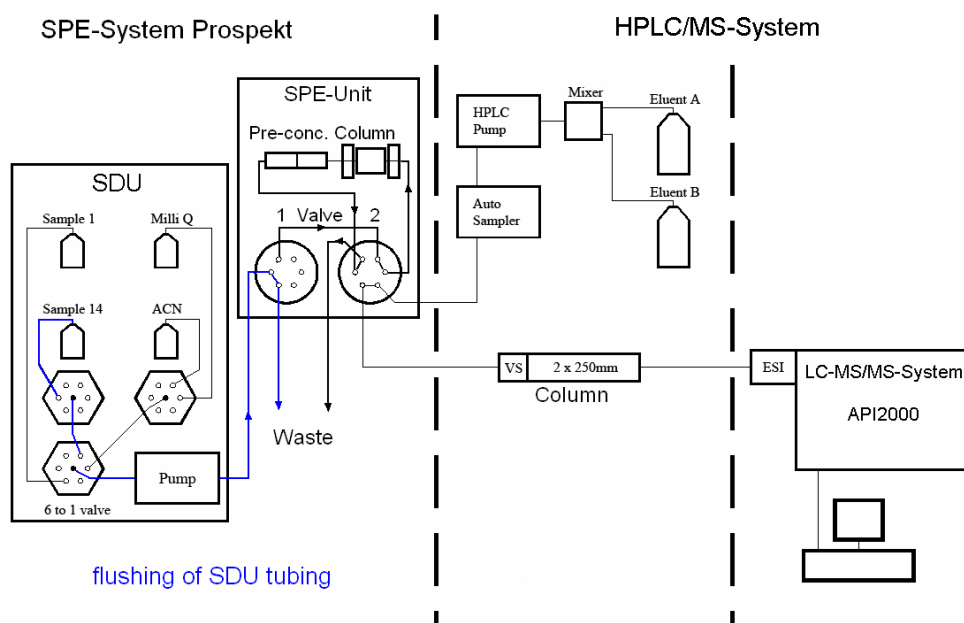


Figure 1: Flow scheme of flushing of the pre-concentration system with sample and solvent

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 for this SOP the flow is illustrated in Figure 2.

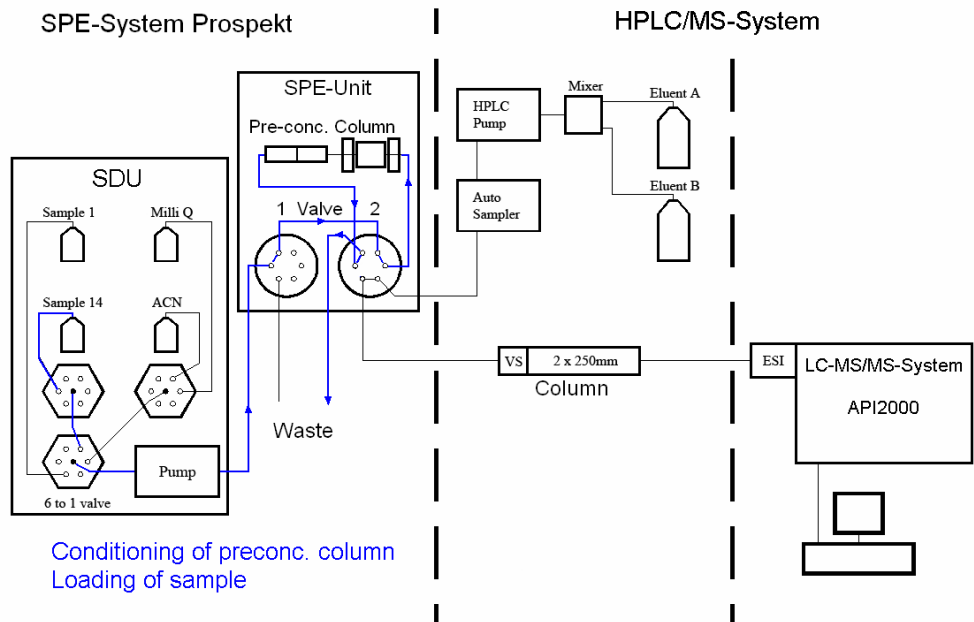


Figure 2: Flow scheme of sample concentration

Soft ware of 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).

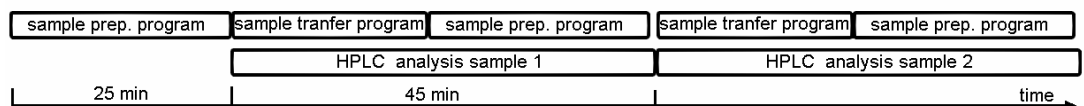


Figure 3: Chronological coordination of "concentration" and "analysis"

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 concentration 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 precon. 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 precon. column	Concentration of sample
25:00min	Switch valve 2 Channel 15	Valve to waste Switch to MilliQ-water	Clean up of capillaries with MilliQ-water

	Flow Rate 5mL	Pump flow rate 5mL/min	
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	

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.

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, Switzerland).

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

Ammonium hydroxide solution 25%, Fluka (Buchs, Switzerland).
Standard stock solution according SOP_Techneau_SNGTX_2007_1

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

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

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 SOP_TECHNEAU_SNGTX_2007_02.

Figure 1 shows the eluent flow during the step "analysis"

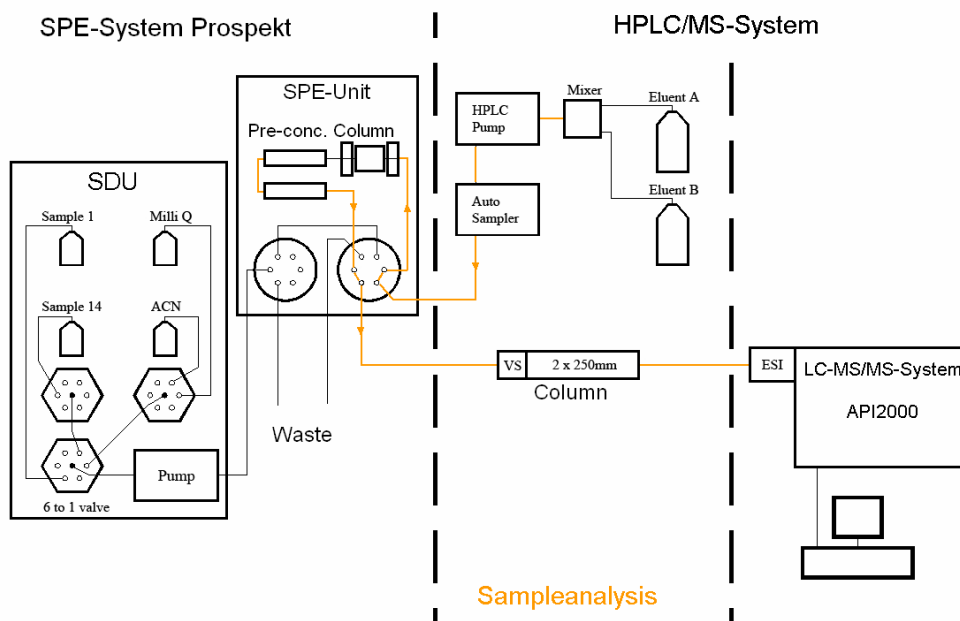


Figure 1. Flow scheme during the step „analysis“

Table 1: Gradient program for Phenomenex Luna C18(2) 5 μ m 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 adjusted.

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

Curtain gas (CUR)	30
Nebulizer gas (GS1)	60
Heater gas (GS2)	75
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

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

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

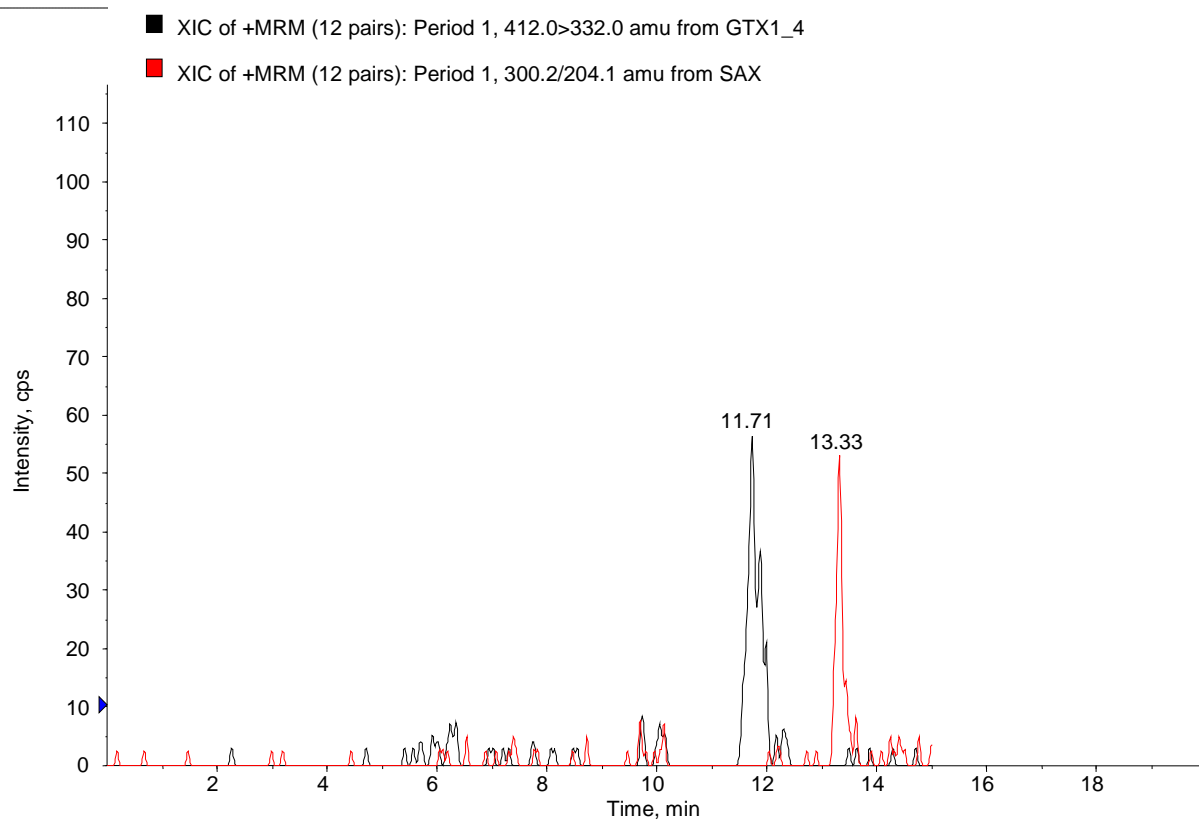


Figure 1: Standard chromatogram of GTX1/4, and SAX corresponding 0.1µg/L per substance in a real surface water matrix

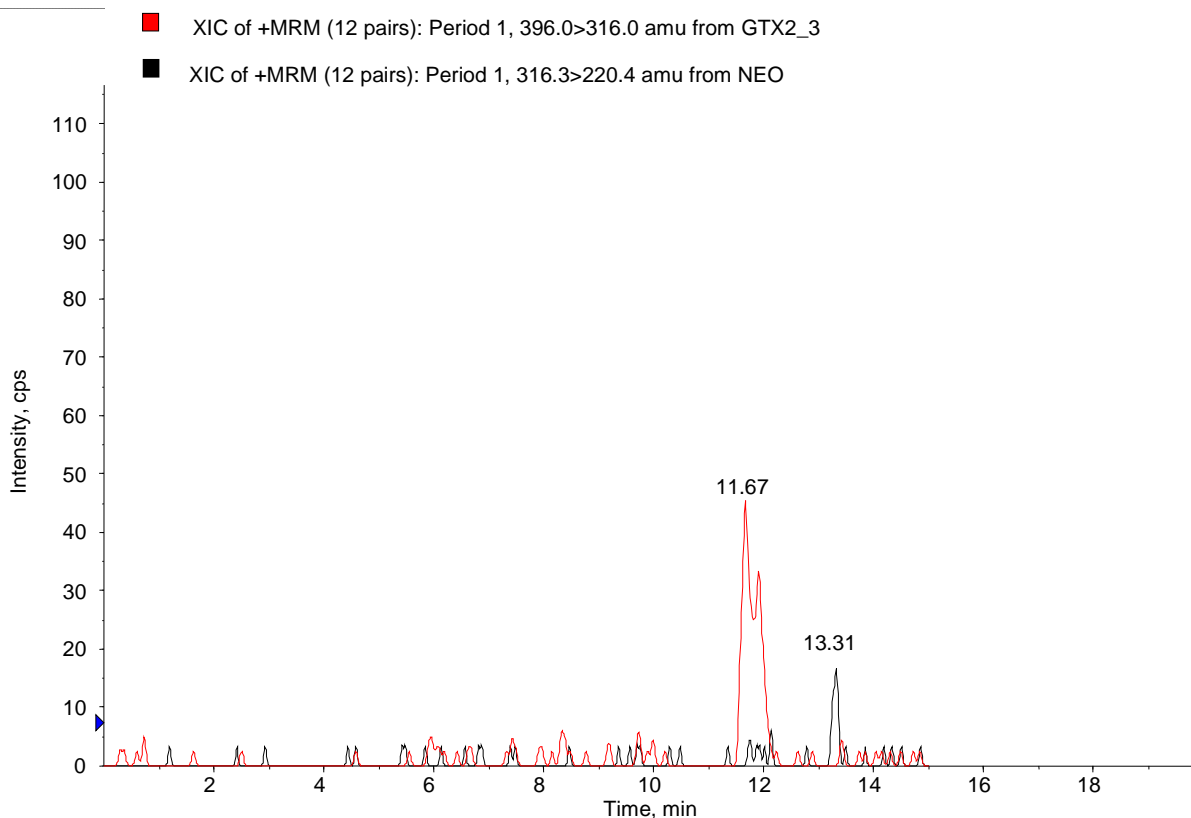


Figure 2: Standard chromatogram of GTX2/3 and NEO corresponding 0.1µg/L per substance in a real surface water matrix (a slide variation of retention time can be caused by matrix effects)

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

Compound	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

[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.