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Final SOP for HPLC based analysis of amino-acid-like algal toxins (cell bound and dissolved state) in natural waters

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1 Summary

The substance spectrum of new amino-acid-like toxins includes the following compounds:

- domoic acid,
- kainic acid, and
- ß-N-methylamino-L-alanine (BMAA)

The analytical approach considers the following steps:

- Preparation of aqueous standard solutions of amino-acid-like toxins - Analyte indentification and calibration with LC-MS/MS,
- Liquid phase derivatization and solid phase extraction of amino-acid like toxins in water samples,
- Extraction of cell-bound and protein-bound amino acid-like toxins and
- Conditions for analysis of amino-acid like toxins by high-performance liquid chromatography with MS/MS detection

Table S1: Calibration parameters of amino-acid-like toxins

Analyte- FMOC-derivative	Q1 mass $[M+H]^+$ (m/z)	Q3 mass production (m/z)	correlation coefficient	RSD [%]	LOD [µg/L]	LOQ [µg/L]	linear range [µg/L]
BMAA 2xFMOC	563	341	0.996	7.6	0.25	0.68	0.1-2.5
Kainic acid	436	214	0.998	5.0	0.16	0.59	0.1-3.0
Domoic acid	534	312	0.998	4.6	0.16	0.58	0.1-5.0
BMAA Σ Q3-area			0.996	7.7	0.78	2.2	1.0-25
2x FMOC	563	119					
1x FMOC	341	119					

LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation

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2 Introduction

The relevance of amino acid like algal toxins for drinking water quality cannot be assessed so far because the available analytical techniques are not efficient and sensitive enough.

The most relevant compounds which could influence the drinking water quality are ß-N-methylamino–L-alanin (BMAA), domoic acid and kainic acid.

In general all toxins occur cell bound. They can be released into the water by algal cell destruction. On the other hand, amino-acids are implemented in a protein scaffold which is water soluble as well. This is the reason that in case of BMAA three states are in discussion, the free dissolved BMAA (1), the dissolved protein-bound BMAA (2), and the cell bound BMAA (3).

However, in case of domoic and kainic acid the two states, cell bound and dissolved, are known.

The approach for trace analysis of BMAA and dissolved kainic and domoic acid is subdivided into the following steps:

- Preparation of aqueous standard solutions of amino-acid-like toxins* - Analyte indentification and calibration with LC-MS/MS,
- Liquid phase derivatization and solid phase extraction of amino-acid like toxins in water samples,
- Extraction of cell bound and protein-bound amino acid-like toxins and
- Conditions for analysis of amino-acid like toxins by high-performance liquid chromatography with MS/MS detection

3 Preparation of aqueous standard solutions of amino-acid-like toxins;

Analyte indentification and calibration with LC-MS/MS

3.1 Introduction

Calibration of HPLC systems with amino-acid-like toxins in the TECHNEAU project is based on the derivatization of aqueous samples with 9-Fluorenyl-methylchloroformiat (FMOC-Cl) and detection with triple quadrupole mass spectrometer.

3.2 Materials and special equipment

Domoic acid (DA) from Calbiochem (1mg)

Kainic acid (KA) from Calbiochem (10mg)

ß-N-methylamino-**L**-alanine (BMAA) from Calbiochem (10mg)

Homophenylalanine from Sigma-Aldrich (250mg), internal standard

L-Norvaline from Sigma-Aldrich (250mg), internal standard

9-Fluorenylmethylchloroformiat (FMOC-Cl) p.a. from Fluka

Acetonitrile (ACN) HPLC grade from VWR International

Methyl-tert-butylether (MTBE) HPLC grade from VWR International

Acetic acid, purris. , Fluka (Schwitzerland)

For 500mL Borate buffer 0.5M: solve 10g NaOH [40 g/mol] and add 15g boric acid [61 g/mol] in MilliQ- Plus water

Hydrochloric acid (HCl) 0.4M

Water purified to Milli-Q Plus quality

Glasbottles 1L and 250mL

Standard vessels 10mL, 25mL, 100mL

Borosilicate glass vials (micro reaction vials) with green/red top, 1mL, 2mL capacity

Borosilicate glass chromatographic vials (autosampler vials), e.g. from VWR International: 1.8mL clear glass with writing surface, and crimp caps with Si/PTFE septa.
Microliter syringes 5µL, 10µL, 25µL, 50µL, 1000µL

Electronic laboratory scale with precision of 0.0001g

HPLC Agilent1090 with Diode array detector (DAD) and fluorescence

detector HP 1046A (Agilent)

LC-MS/MS API 2000 triple-quadrupole mass spectrometer with electron

spray ion source (Fa. Applied Biosystems)

3.3 Preparation of standard solutions

3.3.1 FMOC-Cl stock solution; 3 g/L

Weigh 300mg FMOC-Cl in 100mL standard vessel; fill the vessel with acetonitrile up to the line measure.

Storage of the stock solution in freezer is possible about several months.

For daily use fill the stock solution in a 5mL vial with green/red cape, put in the fridge at 4°C to guarantee its stability about several weeks.

3.3.2 Internal standard stock solution; 100 ng/µL

Dissolve the internal standards (Homophenylalanine and L-Norvaline) in a mixture of acetonitrile/MilliQ-water (50:50, v/v).

Weigh 20mg of standard compound in 100mL standard vessel.

Fill the standard vessel up to 100mL with acetonitrile/MilliQ-water (50:50, v/v).

Store the stock solution at -18°C to guarantee its stability about several months.

For daily use give 1mL of each standard stock solution in a 2mL borosilicate glass vial (micro reaction vials) with green/red top. Put in the fridge at 4°C to guarantee its stability about several weeks.

3.3.3 Standard stock solution; 100 ng/µL

Dissolve the standards in a mixture of acetonitrile/MilliQ-water (50:50, v/v).

Weigh 1mg of domoic acid in a 10mL standard vessel.

Weigh 2.5mg of kainic acid in a 25mL standard vessel.

Weigh 2.5mg of BMAA in a 25mL standard vessel.

Fill each standard vessel up to fill line with acetonitrile/MilliQ-water (50:50, v/v).

Store the stock solution at -18°C to guarantee its stability about several months.

3.3.4 Standard stock solution; 10 ng/µL.

Give 0.1mL of each standard stock solution (see 3.3.3; domoic acid, kainic acid and BMAA) in one 1mL borosilicate glass vial (micro reaction vials) with green/red top.

Add 0.7mL of acetonitrile/MilliQ-water (50:50, v/v).

Put in the fridge at 4 °C to guarantee its stability about one week.

3.4 Preparation and identification of derivatized standards

3.4.1 Derivatization of single components with FMOC-Cl in an autosampler vial

Derivatives of target compounds are commercially not available. The following chapter describes their formation:

To 100mL Milli-Q-water add 2mL of acetonitrile and 1mL borate buffer. Adjust the solution to pH of 9.5 using HCl 0.4M.

Fill 5 autosampler vials each with 0.7mL of this solution.

Add 10 µg of standard solution in each vial (one standard in one vial), i.e.

50µL Homophenylalanine, 50µL Norvaline (see 3.3.2)

100µL Domoic acid, 100µL Kainic acid, 100µL BMAA (see 3.3.3)

Close the vials.

Add 5µL of the FMOC-Cl stock solution to each vial. Shake the vial for one minute. Add 5µL FMOC-Cl again and shake intensively.
Heat the vials to 30- 35°C for 10 to 15 minutes.

Stop the derivatization by adding 5 µL conc. acetic acid (pH = 4), add 0.2 mL acetonitrile. The FMOC derivatives (except the BMAA derivative) are used for substance dependent MS parameters.

3.4.2 BMAA- FMOC-derivatives

BMAA forms single and double derivatives. Figure 1 shows the chromatogram of different FMOC-derivatives. Table 1 contains chromatographic conditions. The fractions are indicated by no. 1 - 8. The fractions were separated and collected manually as shown in table 2.

Table 1: Suggested gradient programme for Macherey-Nagel C18AB 5 µm 250x3 mm i.D, injection volume 25 µL

Mobile phase A: acetonitrile + 0.1 % acetic acid

Mobile phase B: Milli-Q water / Acetonitrile (85:15, v/v) + 0.2 % acetic acid.

Time [min]	Mobile phase A [%]	Mobile phase B [%]	Flow rate [mL/min]
START	0	100	0.4
0.1	10	90	0.4
10.0	25	75	0.4
35.0	45	55	0.4
45.0	85	15	0.4
48.0	100	0	0.4
52.0	100	0	0.4
52.5	0	100	0.4
65	0	100	0.4

Table 2: BMAA fractions (Figure 1)

No.	Retention time [min]	Indication
1, 2	11.8; 12.8	BMAA–FMOC single-derivative
8	44.0	BMAA–FMOC double-derivative
4	22.1	FMOC-OH
3, 5, 6, 7	19.1; 28.9; 42.6; 43.4	Unknown peaks

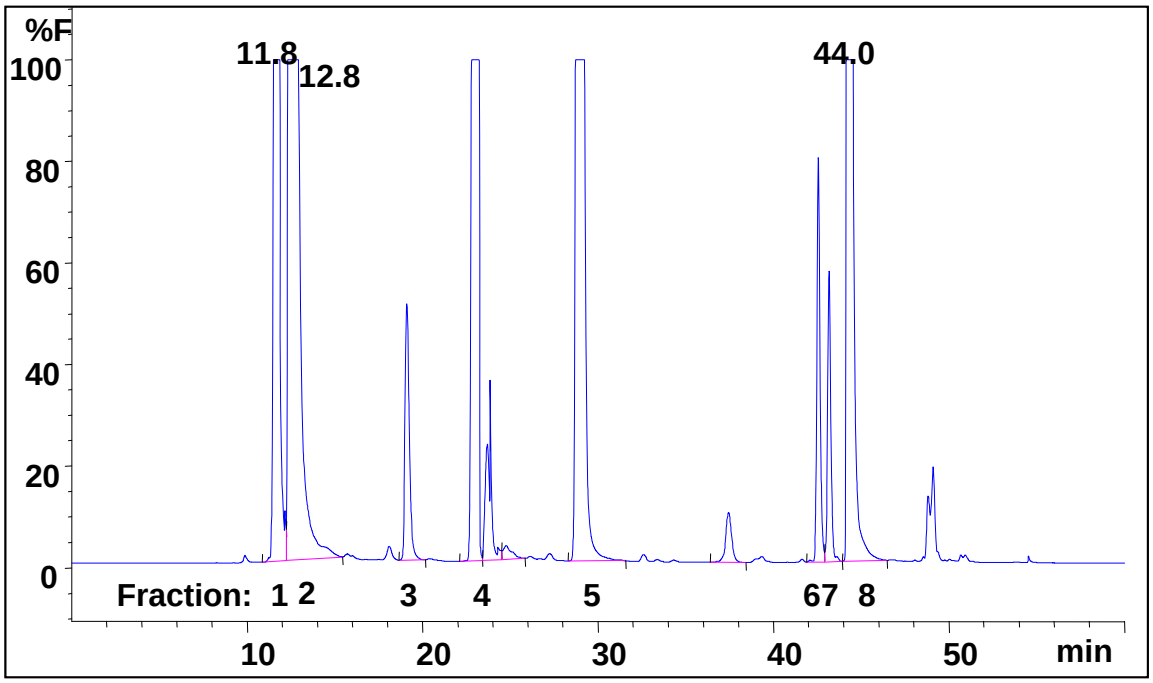


Figure 1: Chromatogram of several BMAA-FMOC-derivatives, fluorescence detection at EX280 nm / EM305 nm.

Fractions 1 und 2 for BMAA-single and fraction 8 for BMAA-double derivatives are collected.

3.4.3 Determination of substance dependent MS/MS-parameters

BMAA:

The determination of substance dependent MS-parameters for single fractions were done by direct injection. For the presented procedure the FMOC-derivatives of the target compounds should be used, respect. their fractions.

The use of manually compound optimization requires the knowledge of the molecule ion $[M+H]^+$ and the resulting product ions.

Figure 2 shows the fragmentation of the BMAA single derivative. The fragmentation of BMAA-FMOC-derivative m/z 341 follows the direction to the $[M+H]^+$ fragment of BMAA m/z 119 and fragment of BMAA m/z 102.

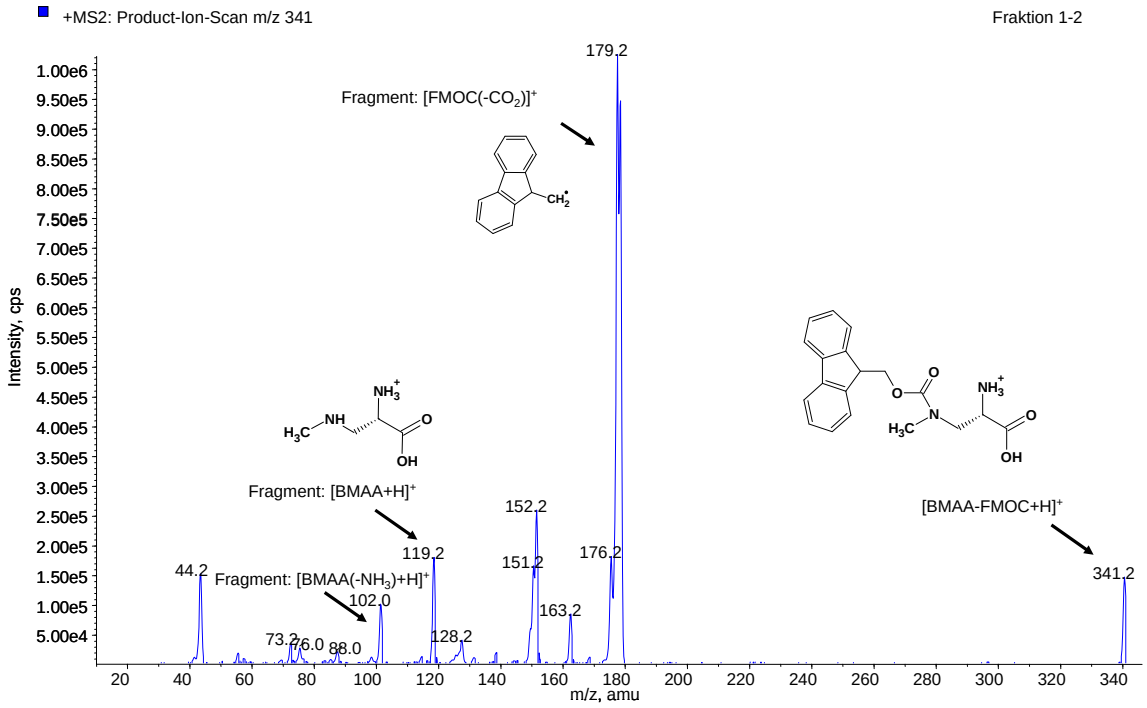


Figure 2: Product ion scan of fragmentation of BMAA-FMOC-derivatives

The fragmentation of the BMAA double derivative, m/z 563, is shown in Figure 3. The fragmentation follows the line m/z 341 (single derivative) to $[M+H]^+$ fragment of BMAA with m/z 119.

For quantification in lower ranges (till 2.5 µg/L) the double derivative fragment m/z 563 → 341 is suitable whereas in the range from 2.5 till 25 µg/L both fragments, means also the single derivative m/z 341 → 119 can be used.

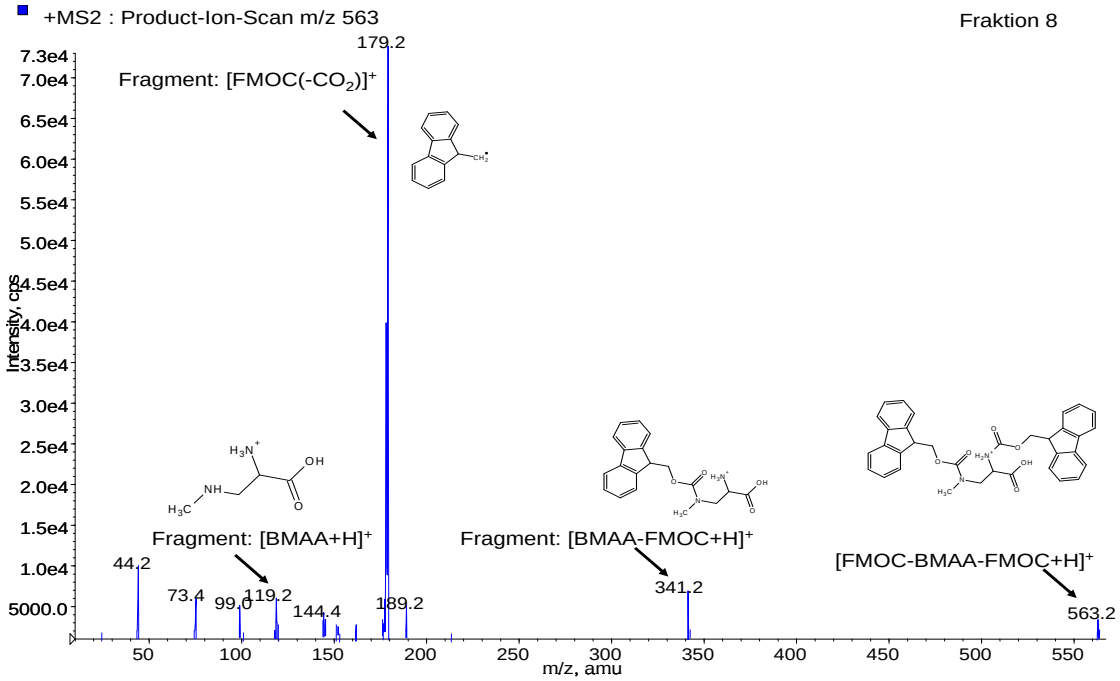


Figure 3: Product ion scan of fragmentation of BMAA-FMOC-double derivative

Domoic (DA) and kainic acid (KA):

The fragmentation of the DA-FMOC-derivatives, m/z 534, is shown in Figure 4. The $[M+H]^+$ fragment with m/z 312 and the fragment m/z 266 are characteristic.

In Figure 5 the fragmentation of kainic acid is shown. The KA-FMOC-derivative m/z 436, is fragmented to $[M+H]^+$ with m/z 214 and to m/z 168.

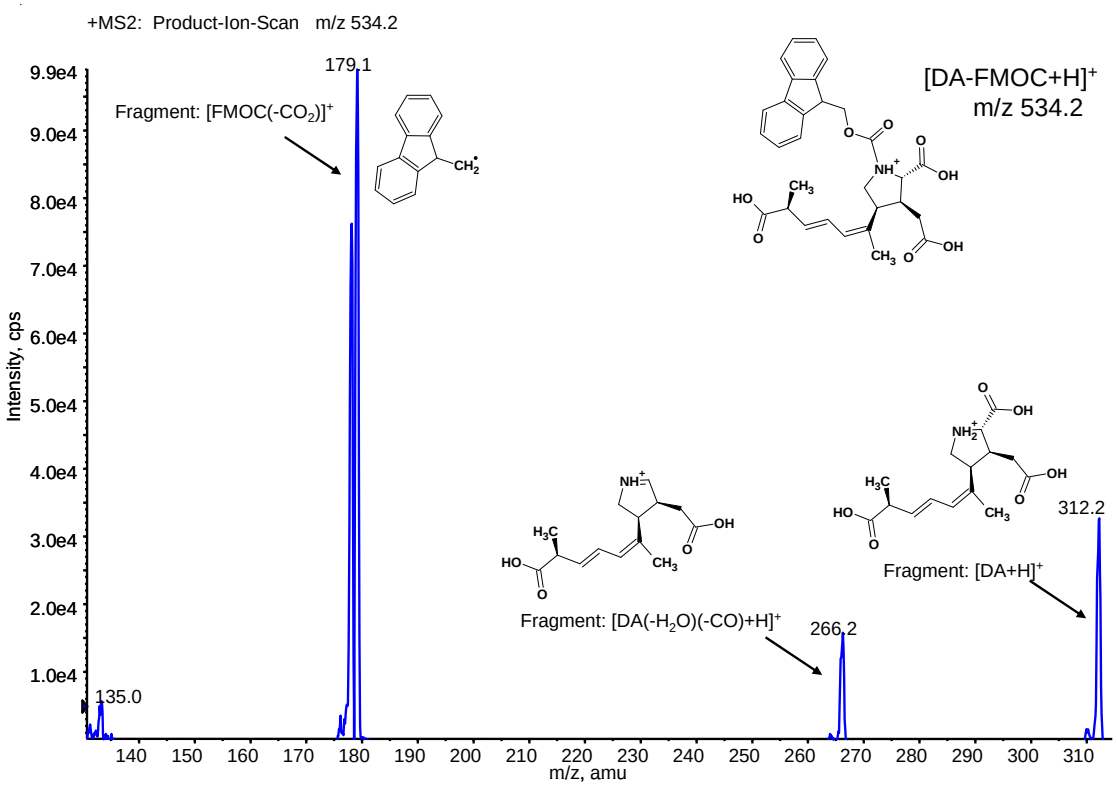


Figure 4: Product ion scan of fragmentation of DA-FMOC-derivative

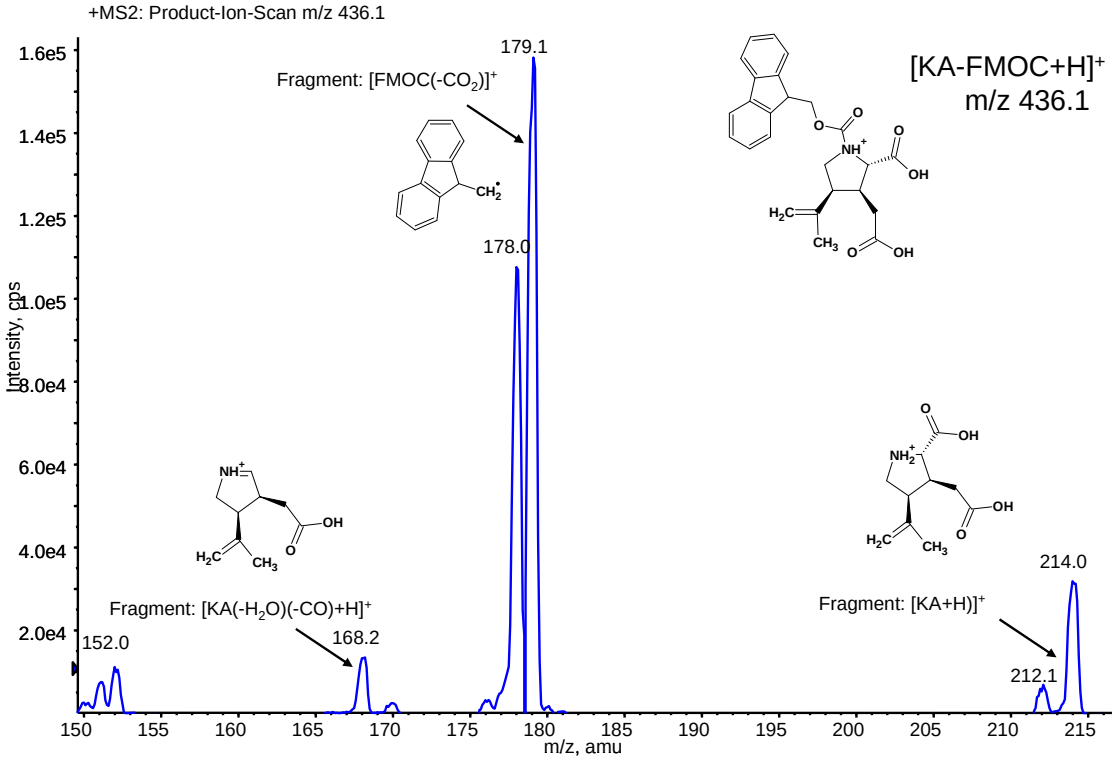


Figure 5: Product ion scan of fragmentation of KA-FMOC-derivative

Homophenylalanine (HPhA)– internal standard:

The fragmentation of HPhA-FMOC-derivative, m/z 402, follows the direction to $[M+H]^+$ fragment of HPhA with m/z 180 and to an intensive fragment of homophenylalanine with m/z 91 (see figure 6).

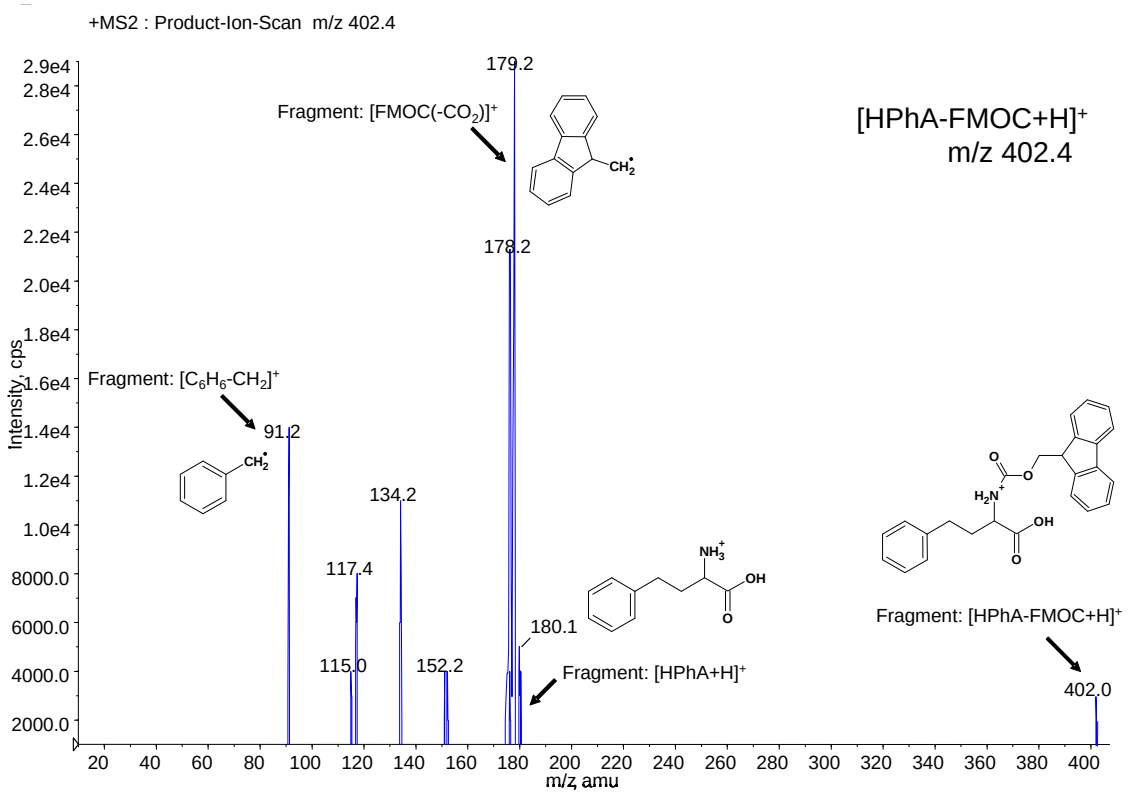


Figure 6: Product ion scan of fragmentation of HPhA-FMOC-derivative

3.5 Determination of the recovery of the analytes according to the whole procedure

The recovery is calculated by the results obtained with 100% standard derivatives and analytes derivatized in water.

3.5.1 100% standard derivatives

Because of the non availability of commercially FMOC standard derivatives the recovery has to be determined by indirect procedure.

For this procedure the compounds are derivatized in an autosampler-vial. These derivatives are used as 100% standards.

Dilute the internal standard stock solution (see 3.3.2) 1:10.

Add 100mL Milli-Q water with 2mL acetonitrile and 2mL borate buffer. Adjust the solution to pH 9.5 using HCl 0.4M.

Fill 10 autosampler vials with 0.7mL each.

Add the standard solution according to table 3:

Table 3: Standard solutions

No.	concentration analyte in vial [ng/mL]	stock solution (10 ng/µL), see 3.3 addition [µL]	int. standard solution (10 ng/µL), see 3.2 addition [µL]
1	blank	-	-
2	20	2	2
3	30	3	3
4	50	5	5
5	70	7	7
6	100	10	10
7	150	15	15
8	250	25	25
9	350	35	35
10	500	50	50

Close the vials.

Heat the vials to 30°C.

Add 10µL of the FMOC-Cl stock solution to each sample.

Shake the vials for one minute.

Add 5µL FMOC-Cl again and shake intensively.

Heat the vials to 30- 35°C for 10 to 15 minutes.

Stop the derivatization by adding 5µL conc. acetic acid (pH = 4), add 0.2mL acetonitrile.

Add ACN till a total volume of 1mL per vial after derivatization (see table 4) is reached.

Table 4: Spiking of ACN before analysis

No.	concentration analyte in vial [ng/mL]	after derivatization ACN addition [µL]
1	blank	285
2	20	280
3	30	280
4	50	275
5	70	275
6	100	265
7	150	255
8	250	235
9	350	215
10	500	185

These 10 derivatives are analyzed by chapter 5.
(Consider the injection volume of 25µL).
In case of calibration curves of 100% derivatives (see table 5) the linear correlation coefficient must be higher than 0.995.

Table 5: Concentration of 100% derivatives in the case of 25µL injection

No.	concentration analyte in vial [ng/mL]	abs. concentration analyte per 25 µL injection [ng]
1	blank	0.0
2	20	0.5
3	30	0.75
4	50	1.25
5	70	1.75
6	100	2.5
7	150	3.75
8	250	6.25
9	350	8.75
10	500	12.5

3.5.2 Standard derivatives over the whole procedure

Add 1000mL Milli-Q water to 1L glass bottle.

Add 10mL 0.5M borate buffer, adjust the pH to 9.5 with 0.4M hydrochloric acid.

Fill 10 glass bottles (250mL) with 100mL each.

Table 6: Calibration points of standard derivatives

No.	for analyte concentration in 100 mL water [ng]	stock solution (10 ng/µL) addition [µL]	int. standard solution (10 ng/µL) addition [µL]
1	blank	-	-
2	10	1	1
3	15	1.5	1.5
4	25	2.5	2.5
5	35	3.5	3.5
6	50	5	5
7	75	7.5	7.5
8	125	12.5	12.5
9	175	17.5	17.5
10	250	25	25

Use the stock and int. standard solution as described in table 6 to get 10 calibration points with concentrations between 0 and 250 ng per 100 mL water sample.

The further sample preparation is described in chapter 4.2.4, beginning with warming up the samples to 30- 35°C.

Chapter 4.2.5 of this SOP describes the solid phase extraction. The 10 samples have to be analyzed according to the instructions in chapter 5

In case of calibration curves of 100% derivatives (see table 6) the linear correlation coefficient must be higher than 0.990.

The concentrations of analytes which should be derivatized in water phase are given in table 7.

Table 7: Calibration points of in water derivatized analytes, 10µL injection volume

No	for analyte concentration in 100 mL water [ng]	abs. analyte concentration per 10 µL injection [ng]
1	blank	0.0
2	10	0.5
3	15	0.75
4	25	1.25
5	35	1.75
6	50	2.5
7	75	3.75
8	125	6.25
9	175	8.75
10	250	12.5

3.5.3 Recovery

The recovery is calculated by the results obtained with 100 % standard derivatives and analytes derivatized in water. Both types of samples have to be pre-concentrated by solid phase extraction (see chapter 4).

In table 8 the recovery results obtained are given as an example. Figure 8 shows two types of calibration curves.

Table 8: Recoveries of compounds

Compound	**Q1 mass $[M+H]^+$ (m/z)**	**Q3 mass Production (m/z)**	**Recovery [%]**
IS Homophenylalanine	402	91	79.4
BMAA	563	341	61.5
Domoinic acid	534	312	82.0
Kainic acid	436	214	76.1

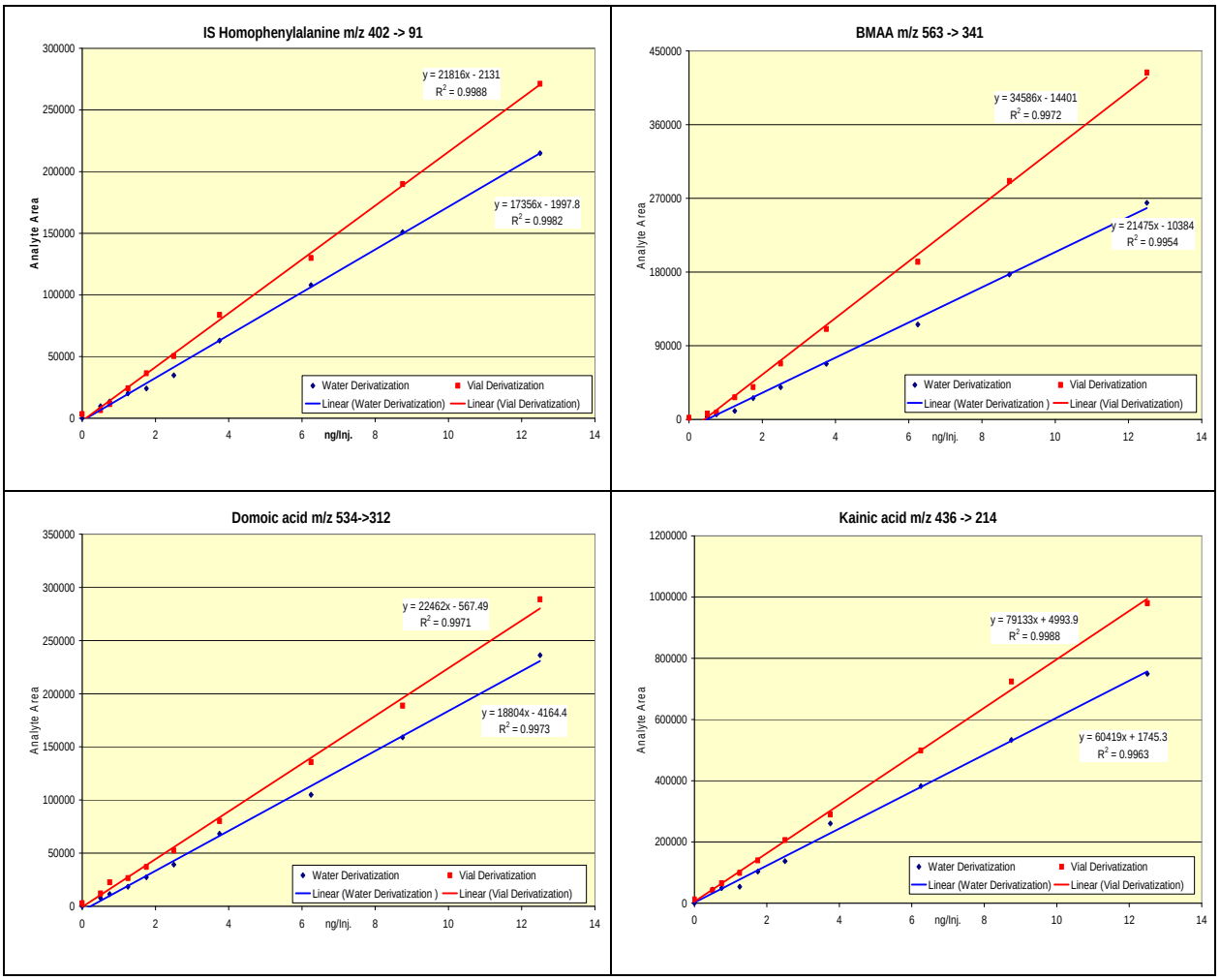


Figure 8: **Calibration curves (0-12.5 ng/Inj.) for determination of recoveries of derivatized analytes.**

red curve: 100% derivatization in vial,
blue curve: derivatization of analytes in water, solid phase extraction (whole procedure).

3.6 Preparation of standard curves for domoic acid, kainic acid, and BMAA

3.6.1 Standard curves

Base of standard calibration curves is the peak area. The area should be linear to the concentration of the analytes. Additional there is a dependency existing between peak area and LC-MS parameters (equipment specific).

In case of using derivatization techniques the use of internal standards (e.g. homophenylalanine) is urgent. Two standards, norvaline and homophenylalanin are available. We used homophenlyalanin with m/z 402 → 91.

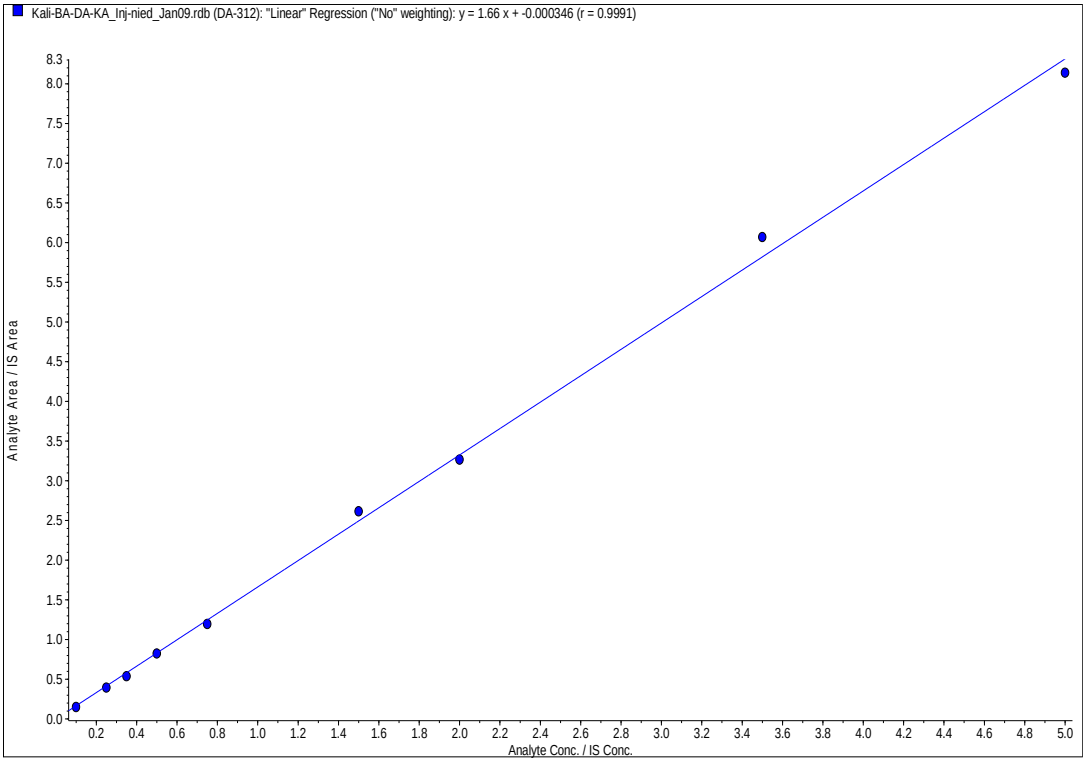


Figure 9: Calibration curve of domoic acid FMOC-derivative m/z 534.2 → 312.1

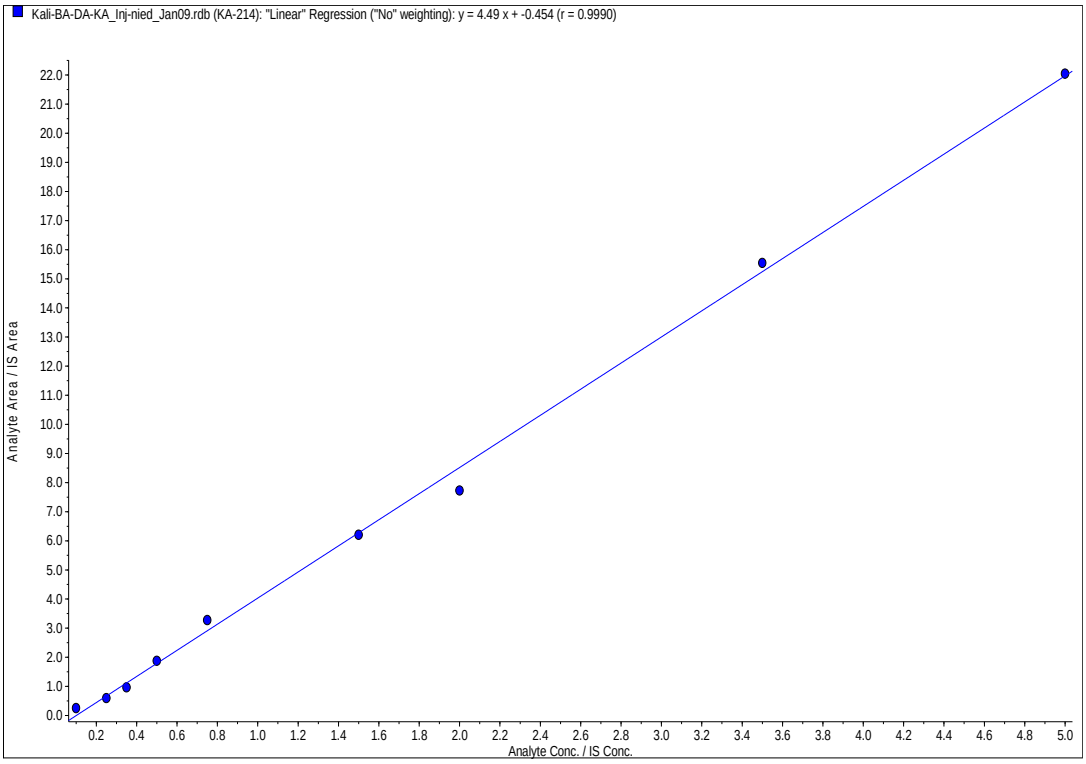


Figure 10: Calibration curve of kainic acid FMOC-derivative m/z 436.1 → 214.0

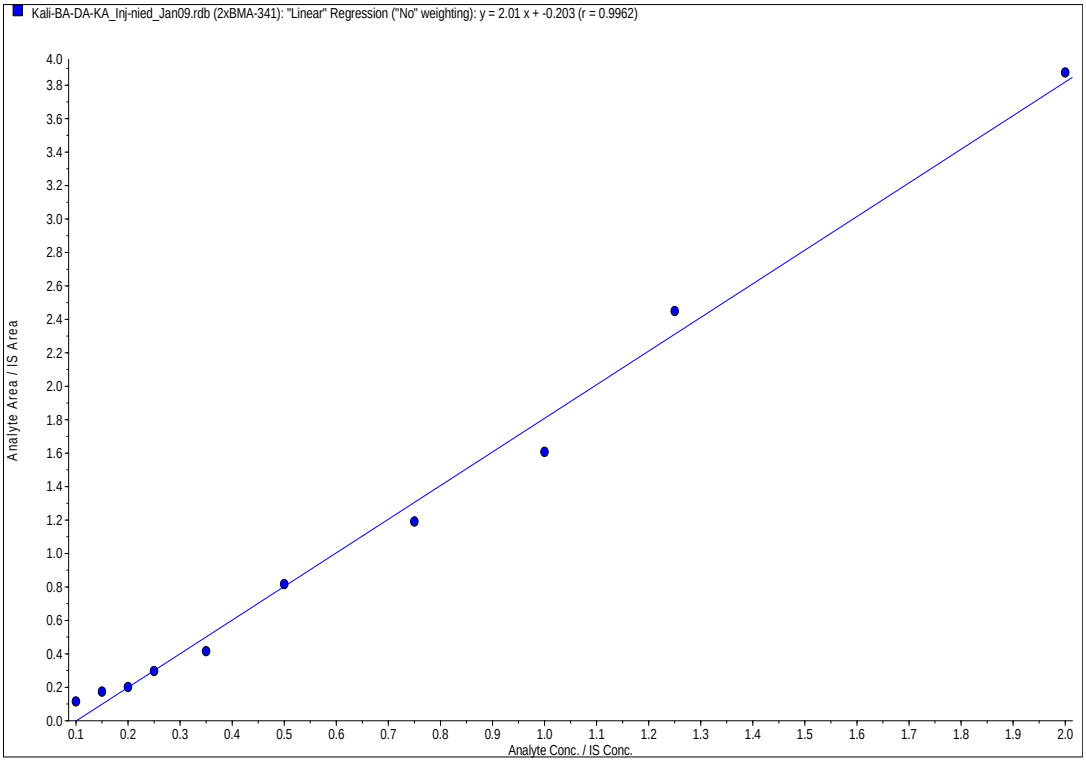


Figure 11: Calibration curve of BMAA-FMOC-double derivative m/z 563.2 → 341.1

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

$$y/a = m*x/c + b$$

y = peak area analyte
a = peak area internal standard
x = ng analyte per injection
c = ng internal standard per injection
m= slope
b = y-axis intercept

The correlation coefficient, R^2, should be better than 0.995

3.6.2 Parameter for standard curves

BMAA forms two, a single and a double FMOC-derivative.

In the range of lower concentrations from 0.1 to 2.5 µg/L (peak area m/z 563 → 341) the double derivative is characteristic under the applied conditions (about 90 %).

In the case of higher concentrations a mixed derivative occurs. In this case the sum of both peak areas m/z 341 → 119 and m/z 563 → 119 should be used for calibration. Under these conditions the calibration is linear up to 25 µg/L.

Under consideration of the applied conditions the BMAA curve is valid for all BMAA modifications (i: dissolved, ii: protein associated-dissolved, iii: protein associated-cell bound).

The calibration curves for domoic acid and kainic acid are valid for the dissolved state.

Table 9: Retention time

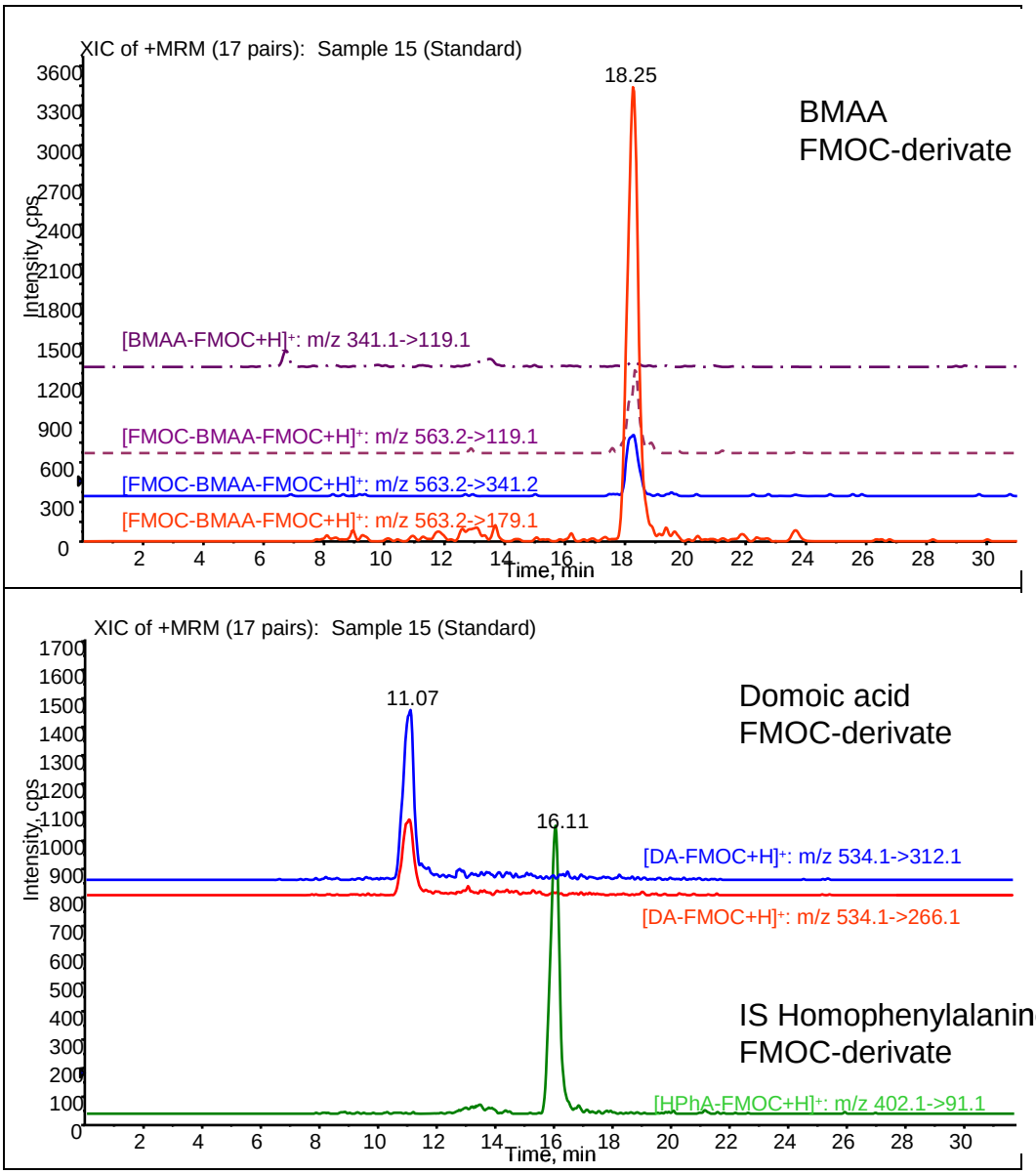
Analyte-FMOC-derivative	Q1 mass $[M+H]^+$ (m/z)	Q3 mass product ion (m/z)	retention time [min]
IS Homophenyl-alanine-FMOC	402	91	16.1
BMAA 2xFMOC	563	341	18.2
Kainic acid	436	214	11.7
Domoic acid	534	312	11.1

Table 10: Validation parameters of the calibration

Analyte-FMOC-derivative	Q1 mass $[M+H]^+$ (m/z)	Q3 mass production (m/z)	correlation coefficient	RSD [%]	LOD [µg/L]	LOQ [µg/L]	linear range [µg/L]
BMAA 2xFMOC	563	341	0.996	7.6	0.25	0.68	0.1-2.5
Kainic acid	436	214	0.998	5.0	0.16	0.59	0.1-3.0
Domoic acid	534	312	0.998	4.6	0.16	0.58	0.1-5.0
BMAA Σ Q3-area			0.996	7.7	0.78	2.2	1.0-25
2x FMOC	563	119					
1x FMOC	341	119					

LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation

Annex


XIC of +MRM (17 pairs): Sample 15 (Standard)
18.25
BMAA
FMOC-derivate
Intensity, cps
[BMAA-FMOC+H]+: m/z 341.1->119.1
[FMOC-BMAA-FMOC+H]+: m/z 563.2->119.1
[FMOC-BMAA-FMOC+H]+: m/z 563.2->341.2
[FMOC-BMAA-FMOC+H]+: m/z 563.2->179.1
Time, min
XIC of +MRM (17 pairs): Sample 15 (Standard)
11.07
16.11
Domoic acid
FMOC-derivate
[DA-FMOC+H]+: m/z 534.1->312.1
[DA-FMOC+H]+: m/z 534.1->266.1
IS Homophenylalanine
FMOC-derivate
[HPhA-FMOC+H]+: m/z 402.1->91.1
Intensity, cps
Time, min

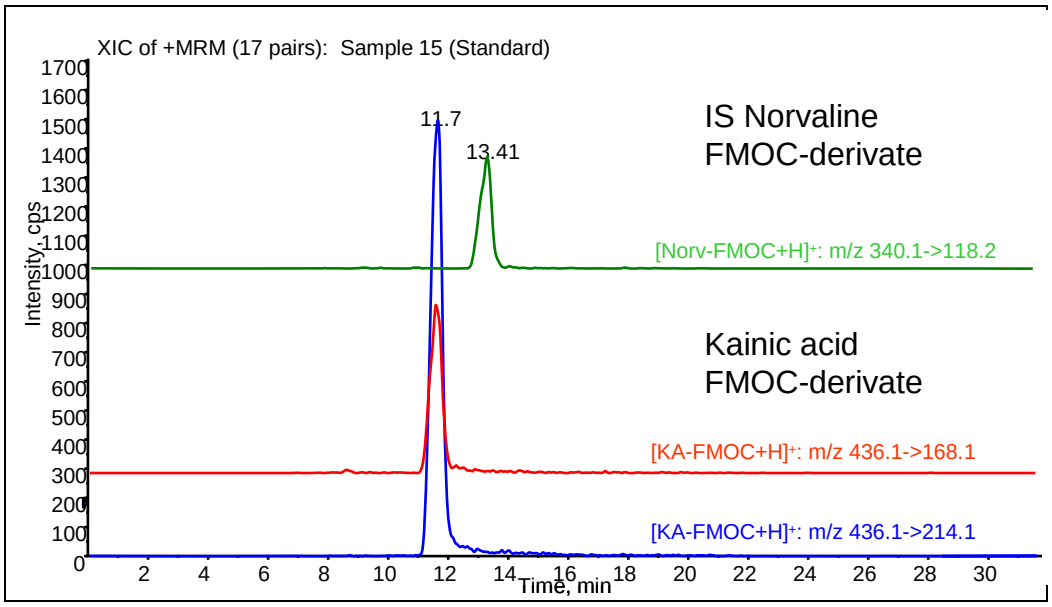


Figure A1: Extracted ion chromatogram (XIC)

Extracted ion chromatogram (XIC) of the mass signal of the analytes

red curve: ion chromatogram for identification
blue curve: ion chromatogram for quantification
green curve: ion chromatogram of the internal standard homo-phenylalanine
broken curve: ion chromatogram for quantification of BMAA higher concentration (1- 25 µg/L)

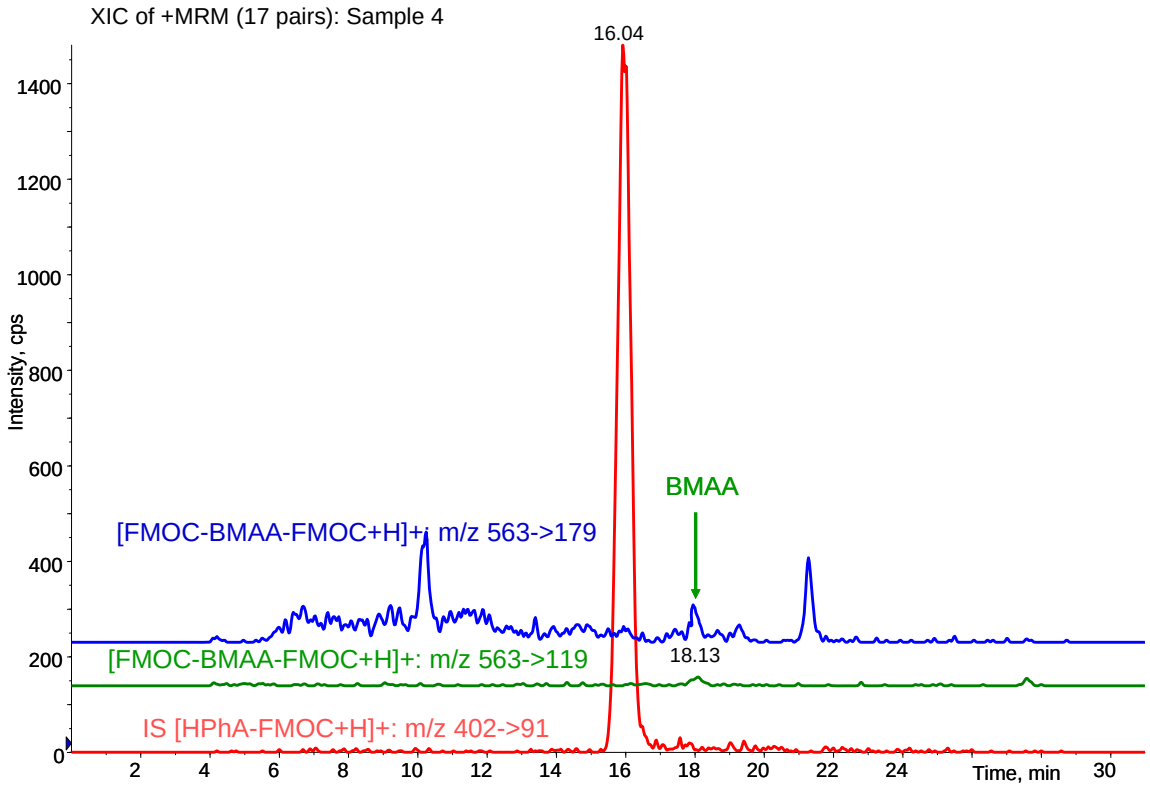


Figure A2: **Extracted ion chromatogram (XIC) of amino-acid-like toxins** Multiple Reaction Monitoring positive mode (+MRM) protein-bounded BMAA (Ret.time 18.13min) of a real water sample

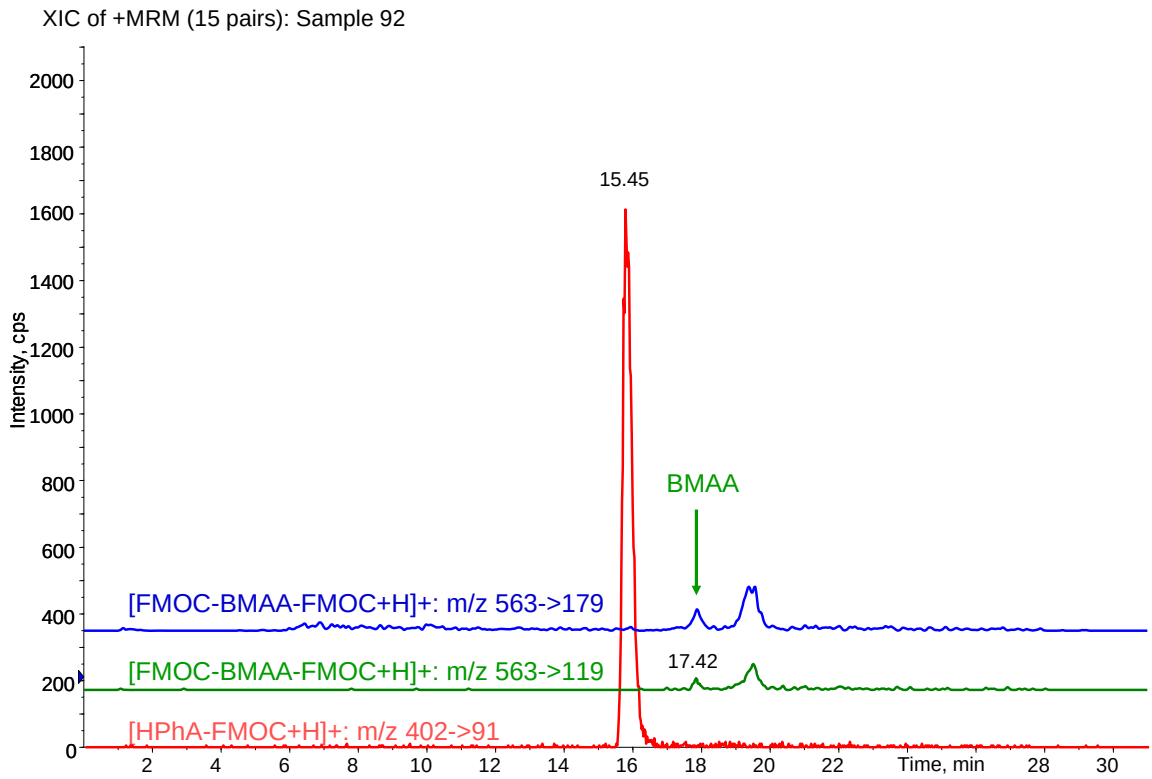
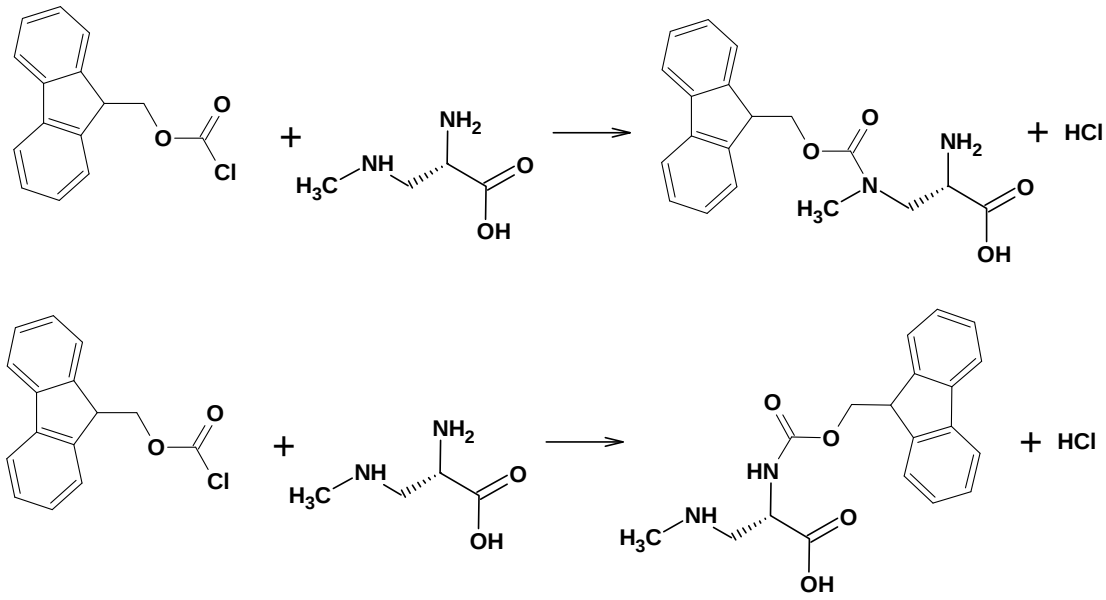


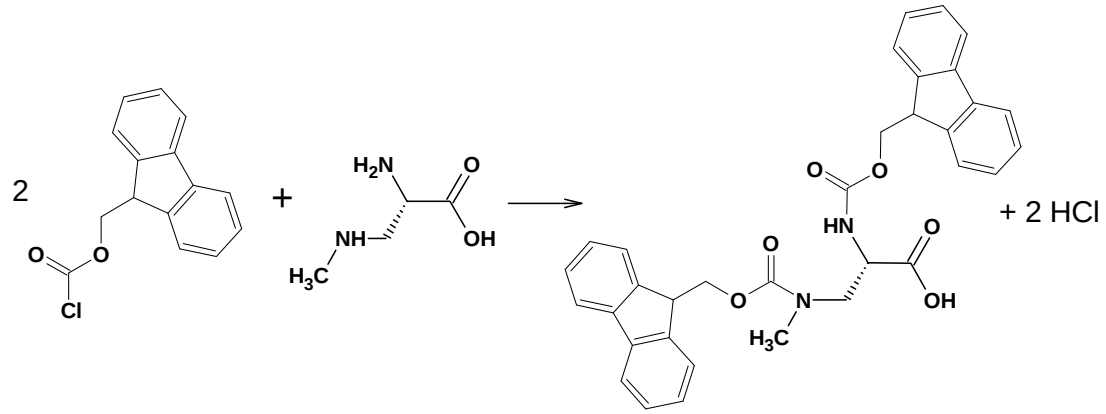
Figure A3: **Extracted ion chromatogram (XIC) of amino-acid-like toxins** Multiple Reaction Monitoring positive mode (+MRM) solved BMAA (Ret.time 17.42min) of a real water sample

Reactions

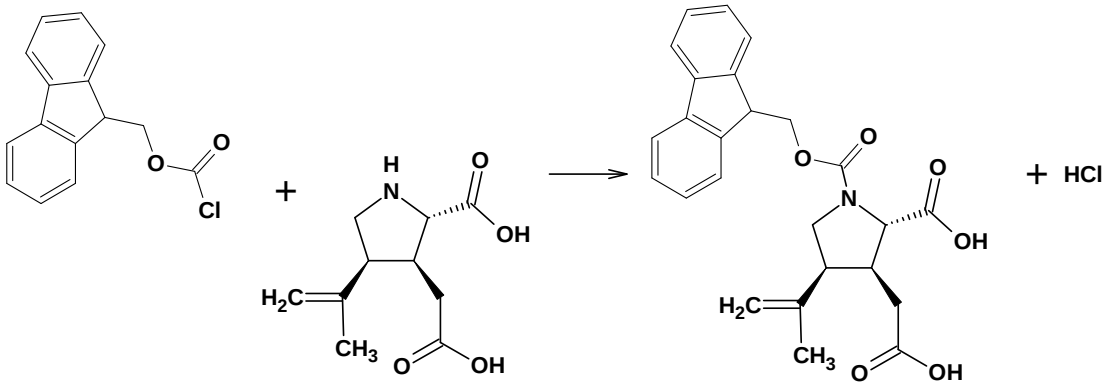
Reaction of BMAA with FMOC-Cl to the single derivative



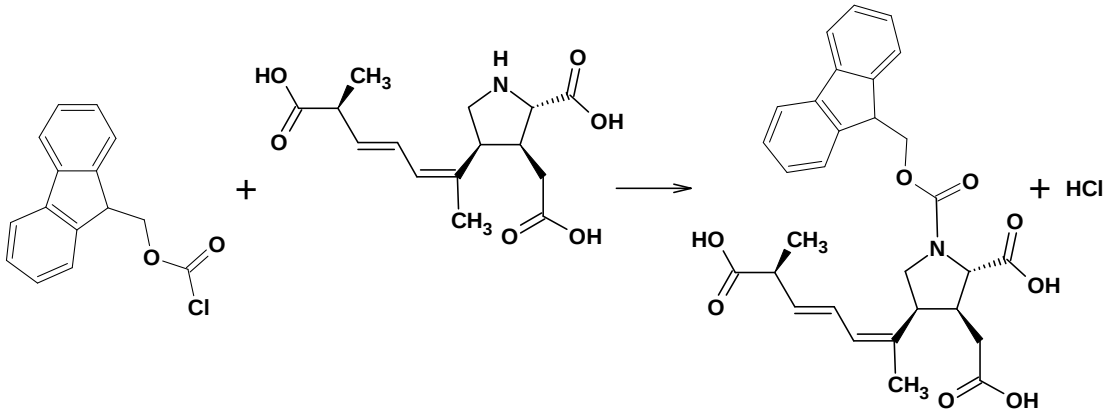
Reaction of BMAA with FMOC-Cl to the double derivative



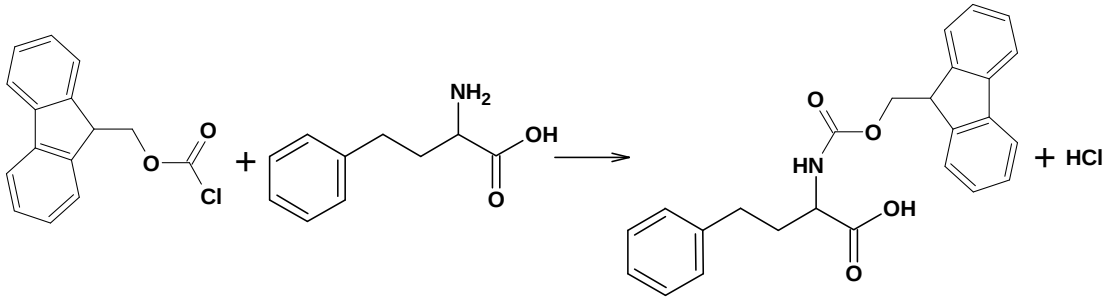
Reaction of kainic acid with FMOC-Cl



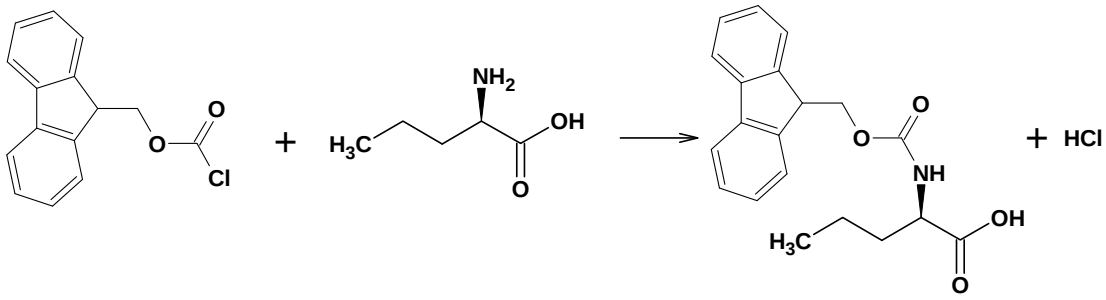
Reaction of domoic acid with FMOC-Cl



Reaction of homophenylalanine with FMOC-Cl



Reaction of norvaline with FMOC-Cl



4 Liquid phase derivatization and solid phase extraction of amino-acid like toxins in water samples

4.1 Introduction

This SOP covers the derivatization of dissolved BMAA, domoic acid and kainic acid before extraction.

The procedure is also valid for extraction (hydrolysis) of protein bound-dissolved BMAA and protein- and cell bound BMAA in a concentration range from 0.1 - 2.5 µg/L.

Additional the extraction of cell bound kainic and domoic acid is described.

Amino-acid like toxins are very polar and belong to the well water soluble molecules. Sample enrichment is necessary to reach relevant concentration levels for drinking water in the range of "ng/L". This is only possible by using derivatization methods if you work with this concentration range. One of the most common derivatization agents for such substances is 9-Fluorenyl-methylchloroformate (FMOC-Cl) [Pietsch, 1996] which has very intensive fluorescence emission at 305nm (Ex. 280nm). The derivatization with FMOC-Cl before extraction makes it possible to extract the analytes from aqueous medium better.

The BMAA molecule contains two aminogroups. Therefore, the formation of single- and double derivatives is possible and depends on the concentration of the reaction partners.

4.2 Experimental

4.2.1 Materials

Acetonitrile HPLC grade, VWR International

Methanole HPLC grade, VWR International

Hexane HPLC grade, VWR International

MTBE Methyl-tert.butylether HPLC grade, VWR International

Acetic acid p.A. , Fluka (Schwitzerland)

Hydrochloric acid (HCl) 6M purris., Fluka (Schwitzerland)

Water purified (Milli-Q Plus quality)

NaOH in Milli-Q 0.4M and 3M

HCl in Milli-Q 0.4M

0.5M borate buffer

Glass-fibre filters GF/C, typically Whatman (Maidstone, U.K.) diameter 47 - 50 mm

Glass bottles 250mL, 500mL

Silicon caps

Separation funnel 500mL

Microlitersyringes 10µL, 50µL, 500µL , 2.5mL

5mL micro reaction vessels, Supelco (USA)

Pasteurpipettes

2mL PP-microtubes, Fa. Brand (Germany)

0.3mL limited volume autosampler vials with screw caps, Phenomenex (USA)

Culture tubes with screw-cap, temperature-resistant till 180°C, Duran (Germany)

Magnetic stir bar, ca. 50mm length

3mL SPE-Tube C18 200mg, e.g. Fa. Phenomenex (USA)

Nitrogen 99.999%

Internal standard stock solution (100 ng/L)

(see chapter 3.3.3)

FMOC-Cl stock solution 3 g/L (see chapter 3.3.1)

4.2.2 Equipment

HP6 Magnetic stirrer (6 positions), Fa. H&P Labortechnik GmbH (Germany)

DR-3 Dry-Block, Techne Ltd. (U.K.)

Gas release equipment, Labortechnik Barkey (Germany)

Microwave MLS 1200, Fa. MLS GmbH (Germany)

Vacuum-filtration equipment, Fa. Satorius (Germany)

Sanoklav sterilisator, Fa. Wolf (Überkingen, Germany)

Zentrifugal vakuumconcentrator SpeedVac with cooling trap, Fa. Merck (Germany)

Drying cabinet, Fa. Binder (Germany)

Solid phase extractor, e.g. Autotrace, Fa. Dionex (USA)

HPLC 1090, Fa. Agilent (USA) with mass-spectrometer, e.g API 2000, Fa. Applied Biosystems (Canada)

4.2.3 Sample preparation

4.2.3.1 Sample filtration

Store the samples (500mL) at 4°C for two days in maximum. For longer time the sample has to be frozen (-18°C).

The determination of dissolved BMAA, DA, and KA as well as protein-bound (associated)-dissolved BMAA is made from this sample.

Filter 500mL sample through glassfiber GF/C Whatman under vacuum conditions. The filters have to be frozen (-18°C), if the determination of cell bound BMAA is not done immediately.

In the case of a higher algal density (e.g. filter clogging) 250mL should be enough.

The following sample volumes are needed:

100mL sample for analysis of free dissolved BMAA, Domoic acid and Kainic acid,

100mL sample for analysis of protein bound (associated)-dissolved BMAA,

The residual water is stored (use for repetition analysis).

4.2.3.2 Determination of protein bound (associated)-dissolved BMAA (soft hydrolysis)

Fill 100mL sample in a 250mL glass bottle.

Add 0.5mL 6M HCl

Cover the bottle with a silicon cap (not glass because of the heat).

The hydrolysis at 110°C takes 60 minutes.

After cooling the sample add 3M NaOH for neutralization.

The further procedure is described in 4.2.4.

4.2.3.3 Determination of cell bound BMAA (strong hydrolysis)

For hydrolysis add 1.5mL 6M HCl to the culture tubes with screw-cap, which contain the glass fibre filter.

Close the culture tube with the cap strongly.

The hydrolysis at 110°C takes 15h, shouldn't take longer.

After cooling give 1mL of the hydrolysis product in a 2mL glass tube.

Dry the sample in a vacuum evaporator.

Give the dry sample residue (take care that the whole sample is transferred) in 100mL chlorine free water in a 250mL glass bottle.

The derivatization is described in 4.2.4.

4.2.3.4 Extraction of cell bound kainic acid (KA) and domoic acid (DA)

500mL sample are filtered through glassfiber GF/C Whatmann under vacuum conditions. The filter with the biomass is stored in a 20mL glass vial with screw cap near -18°C. In cases of higher algal densities 250mL sample should be enough.

For extraction a mixture of methanole/MilliQ (1:1, v/v) with 0.1M acetic acid is used.

Add 5mL of this mixture in the vial with the frozen filter. Put the vial for 15 minutes in ultrasonic bath (maximum level of ultrasonication). The extraction is continued by shaking about 12h. 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.

1L MilliQ in a 1L borosilicate glass flask is spiked with 10mL borate buffer (0.5M). The pH-value is adjusted to pH=9.5 using 0.4M HCl. This is the water for derivatisation and can be used for about 8-9 samples.

The dry residual is dissolved in 3mL derivatization water three times. The solution is transferred by a Pasteur pipette in a 250mL borosilicate glass flask. This sample is adjusted to 100mL with derivatization water.

Add 5 µL of internal standard Homophenylalanine. Adjust the temperature to 35°C for derivatisation. The analysis follows the procedure of dissolved KA and DA.

The sample will be prepared now for derivatisation (see 4.2.4). Start with warming up the sample to 35°C.

Results:

The amount of cell bound toxins is calculated in µg/L water. That means, the amount of µg is extracted from the biomass on the filter which is separated from a specific water volume.

4.2.4 Derivatization of samples with FMOC-Cl

Transfer 100ml of water sample into a 250mL borosilicate glass flask.

Add 2mL borate buffer (0.5M).

Add 5µL internal standard.

Adjust the pH to 9.5 using HCl (0.4M) or NaOH (0.4M)

Adjust the temperature to 35°C.

Put the samples immediately on the stirrer block and mix the sample intensively.

Add with a 500µL syringe 300µL of FMOC-Cl reagent (3.0 g/L in acetonitrile), 2-5 minutes intensive stirring, add further 200µL of FMOC-Cl reagent. After 30 minutes the reaction is complete and the sample is on room temperature.

Clean the sample by liquid extraction with 20mL Hexane/MTBE (3:2, v/v) to remove distracting nonpolar FMOC products.

Extract the sample 10 minutes while stirring intensively.

Separate the extract from the water phase (organic phase = waste) using the separation funnel.

Transfer the water phase back to the 250mL glass bottle.

Add 50- 80µL acidic acid to the water sample (pH: 5- 6.5). The sample is ready for solid phase extraction.

4.2.5 Solid phase extraction

Use 3mL SPE cartridges with C18 material.

The conditioning is carried out with 2 x 2mL methanol and 2 x 2mL Milli-Q Plus water.

Give the samples with 10 mL/min via the cartridges.

Rinse the cartridges with Milli-Q.

Dry the cartridges with nitrogen (30 minutes).

Elute the cartridges with 2 x 2mL methanol.

Give eluate in a 5mL micro reaction vessel.

Dry it completely with nitrogen (~40°C).

Dissolve the dry rest in 0.2mL ACN/water (30:70, v/v) and transfer the solution in a 0.3mL limited volume vial.

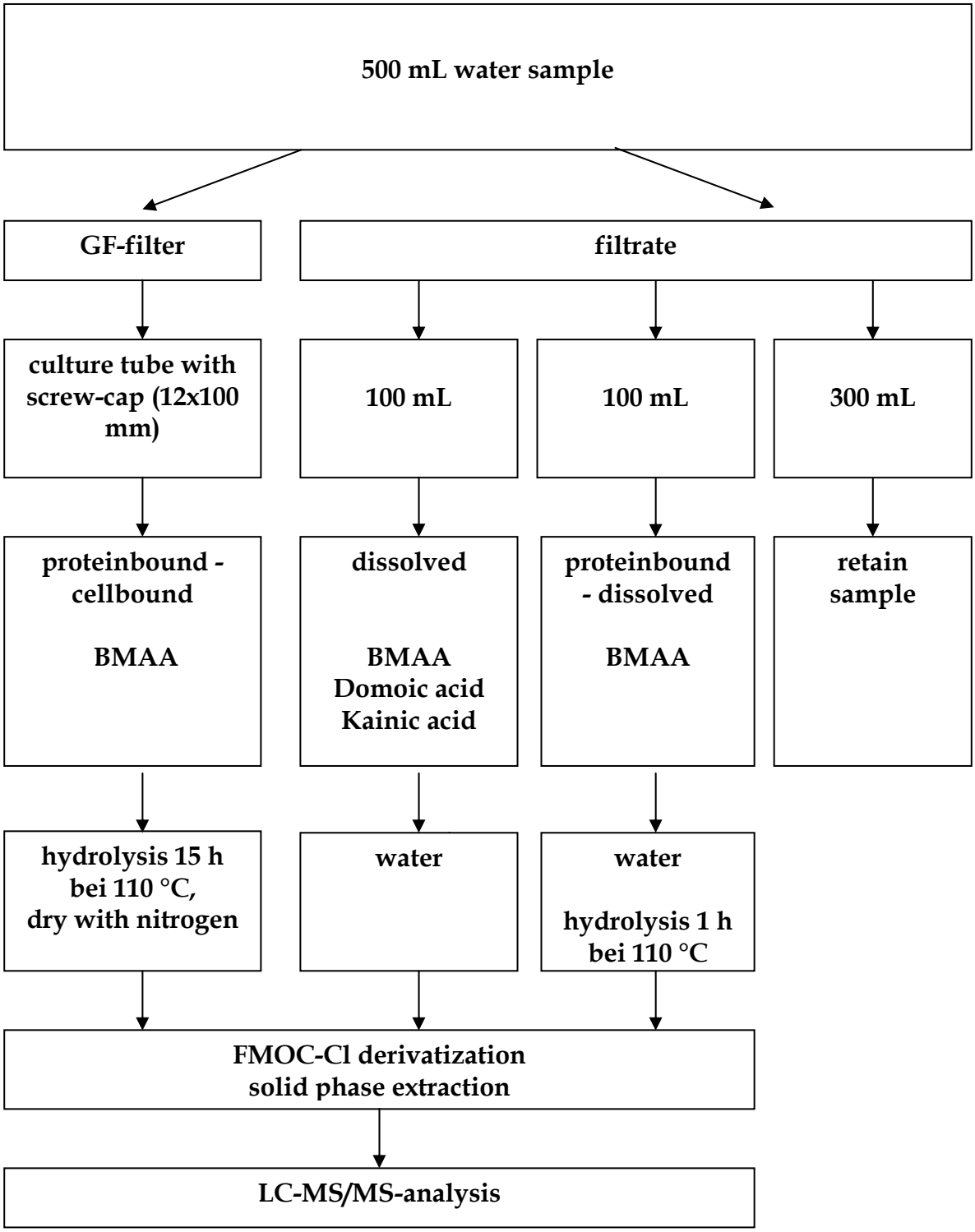
The maximum storage in the fridge should not exceed one day.

4.3 References

Pietsch, J., Hampel, S. Schmidt, W. , Brauch, H.-J. and Worch, E.: Determination of Aliphatic and Alicyclic Amines in Water by Gas- and Liquid Chromatography after Derivatization by Chloroformates. Fresenius J. Anal. Chem. 355, 164-173 (1996).

Annex:

Scheme of sample preparation



5 Conditions for analysis of amino-acid like toxins by high-performance liquid chromatography with MS/MS detection

5.1 Introduction

Reversed-phase HPLC on C18 phases is a common choice for separating smaller molecules, also if they are modified by derivatization with fluorophores [Milley, 1989; Pietsch, 1996].
The disadvantage of this sensitive procedure is that false positive results cannot be excluded totaly because of the high number of additional derivatized compounds.
Because of that fact the use of LC-MS/MS method is strongly recommended.

5.2 Experimental

5.2.1 Materials

Acetonitrile (ACN) HPLC grade from VWR International

Water purified to Millipore Milli-Q Plus quality

Acetic acid HPLC grade

Column: Macherey-Nagel C18AB 5µm 125x 2mm i.D.

Guard-column: 8x 3mm i.D.

0.3mL limited volume autosampler vials with screw caps, Phenomenex (USA)

5.2.2 Special Equipment

HPLC 1090 (Agilent, USA)

LC-MS/MS API2000 a triple-quadrupole mass-spectrometer with electron spray ion source (Fa. Applied Biosystems, Canada)

5.2.3 Chromatographic conditions

5.2.3.1 General 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 (8 x 3mm, C18). Change guard column if the back-pressure rises or peak forms deteriorate.

Set column oven at 40 °C.

Change the HPLC gradually up to starting conditions and start after equilibration.

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

5.2.3.2 HPLC mobile phase

Mobile phase component A: acetonitrile + 0.1% acetic acid
Mobile phase component B: MilliQ-water / Acetonitrile (85:15, v/v) + 0.2% acetic acid.

Table 1: gradient programme, injection cycle about 36 minutes

Gradient time [min]	% A	% B	Flow rate [mL/min]
START	10	90	0.2
2.0	30	70	0.2
10.0	55	45	0.2
20.0	82	18	0.2
20.1	100	0	0.2
24.5	100	0	0.2
25	10	90	0.2
36	10	90	0.2

The conditions can be adapted to specific laboratory situations. Nevertheless, the most possible separation of derivatized analytes from the FMOC-peak is helpful.

5.2.4 LC-MS/MS-parameters for detection and quantification

5.2.4.1 General procedure for standards and real samples

The analysis of derivatized amino acid like toxins is carried out with positive electrospray ionization.

The LC-MS/MS system should be set up and tuned as described in the manufacturer's instructions.

The regular cleaning of the turbo spray interface according to the instructions of the service is important.

5.2.4.2 Detection parameter API 2000

The parameters described in this SOP are only valid for API 2000.

For quantification of the analytes the positive MRM-Mode (Multi Reaction Monitoring) is used (see Table 2).

Table 2: source dependent parameters LC-MS/MS API 2000 positive electrospray ionization (ESI)

Curtain gas (CUR)	30
Nebulizer gas (GS1)	30
Heater gas (GS2)	40
Collision gas (CAD)	5
Temperature heater gas (TEM)	350 °C
Ion Spray voltage (IS)	+5200 V
Focusing potential (FP)	370
Entrance potential (EP)	10

The specific parameters are given in Table 3.

Table 3: compound dependent parameters LC-MS/MS API 2000

Analyt-FMOC-Derivative	Q1 mass $[M+H]^+$ (m/z)	Q3 mass Production (m/z)	Declustering potential (DP)	Cell entrance potential (CEP)	Collision energy (CE)
BMAA 1xFMOC	341.1	119.1	10	28	31
BMAA 1xFMOC	341.1	179.1	10	28	35
BMAA 2xFMOC	563.2	341.2	16	34	30
BMAA 2xFMOC	563.2	119.1	16	30	23
BMAA 2xFMOC	563.2	179.1	16	34	30
Domoic acid	534.1	312.1	16	33	21
Domoic acid	534.1	266.1	16	33	29
Domoic acid	534.1	179.1	16	33	24
Kainic acid	436.1	214.1	11	30	19
Kainic acid	436.1	168.1	11	30	29
Kainic acid	436.1	179.1	11	30	23
IS Norvaline	340.1	118.1	16	30	25
IS Norvaline	340.1	179.1	16	30	20
IS Homophenyl-alanine	402.2	91.1	16	20	67
IS Homophenyl-alanine	402.2	179.1	16	20	27

5.2.4.3 Analysis

For a correct identification and quantification of the analytes in minimum two mass paths $Q1[M+H]^+/Q3[Production]^+$ are necessary. The paths identify the FMOC derivative of the analyte via the FMOC-fragment (m/z 179) and the fragment ($[M+H^+]$) of the analyte.

Two internal standards are used for the whole procedure.

Table 4 shows the specific mass paths.

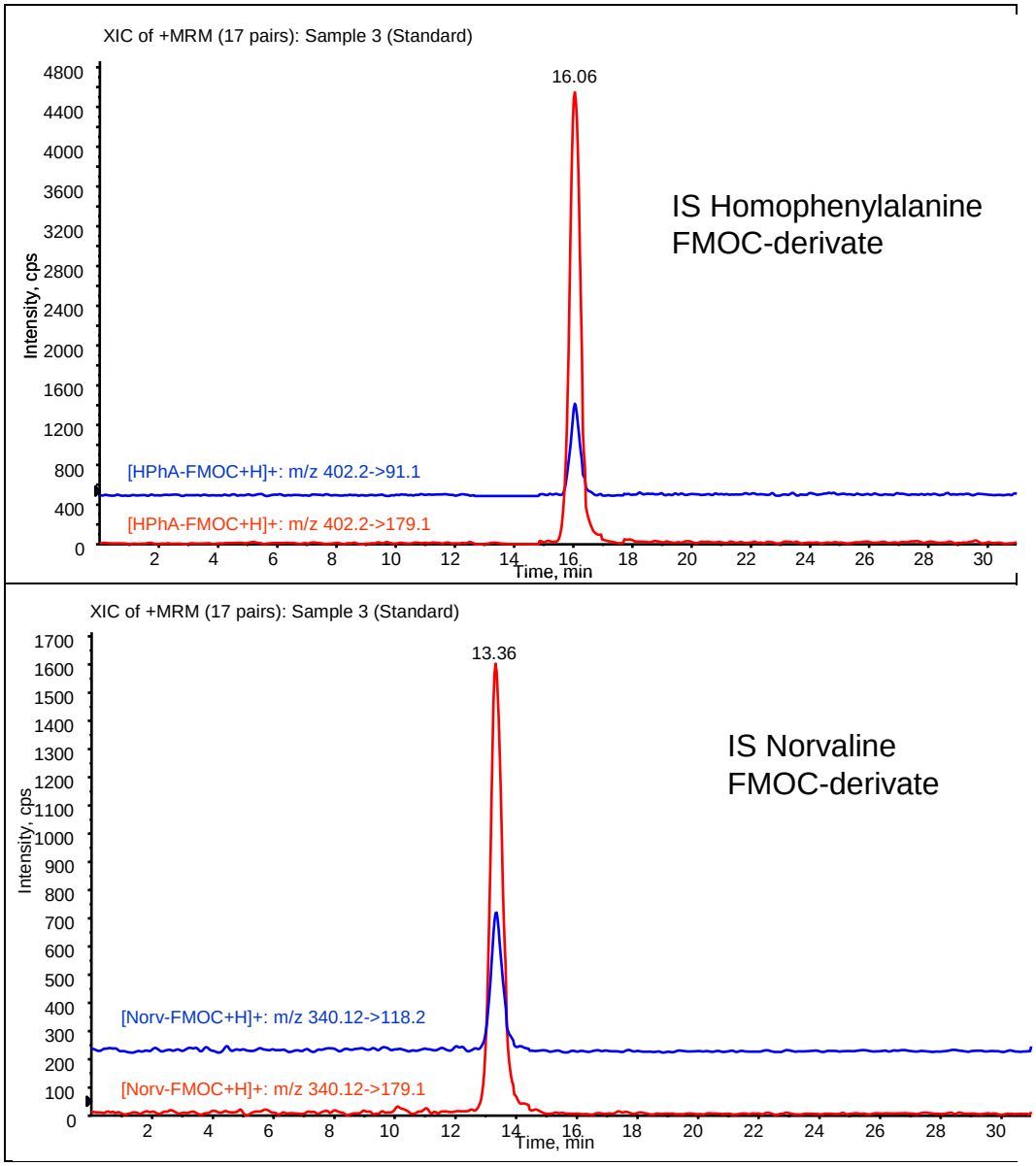
Table 4: Mass paths for quantification and identification

Analyt-FMOC-Derivative	Q1 mass $[M+H]^+$ (m/z)	Q3 mass Production (m/z)	Use for quantification	Use for identification
BMAA 1xFMOC	341.1	119.1	xx	
BMAA 1xFMOC	341.1	179.1		x
BMAA 2xFMOC	563.2	341.2	x	
BMAA 2xFMOC	563.2	119.1	xx	
BMAA 2xFMOC	563.2	179.1		x
Domoic acid	534.1	312.1	x	
Domoic acid	534.1	266.1		x
Domoic acid	534.1	179.1		x
Kainic acid	436.1	214.1	x	
Kainic acid	436.1	168.1		x
Kainic acid	436.1	179.1		x
IS Norvaline	340.1	118.2	x	
IS Norvaline	340.1	179.1		x
IS Homophenyl-alanine	402.2	91.1	x	
IS Homophenyl-alanine	402.2	179.1		x

xx) for quantification of higher BMAA concentrations, the peak-areas of the single and the double derivates are used

5.2.4.4 Extracted ion chromatograms of the analytes

Figures 1 and 2 show exemplarily the extracted ion chromatograms (XIC) of the analytes



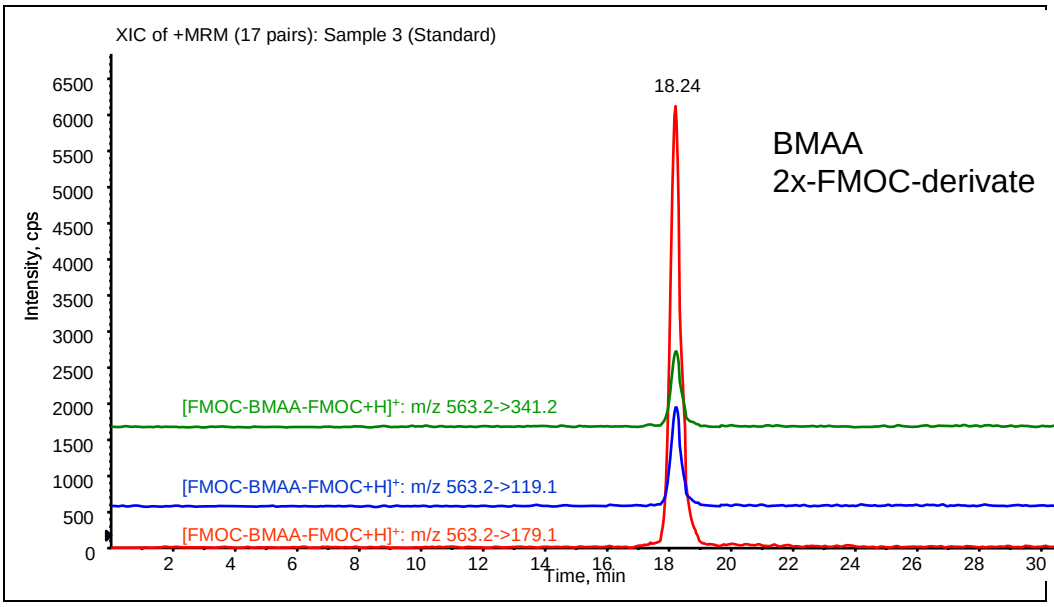
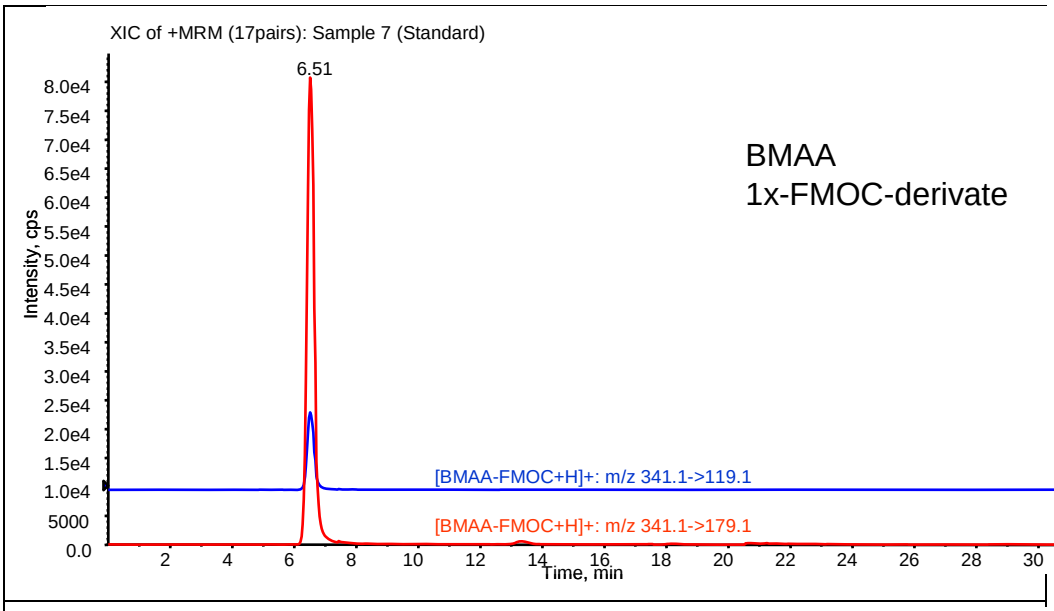
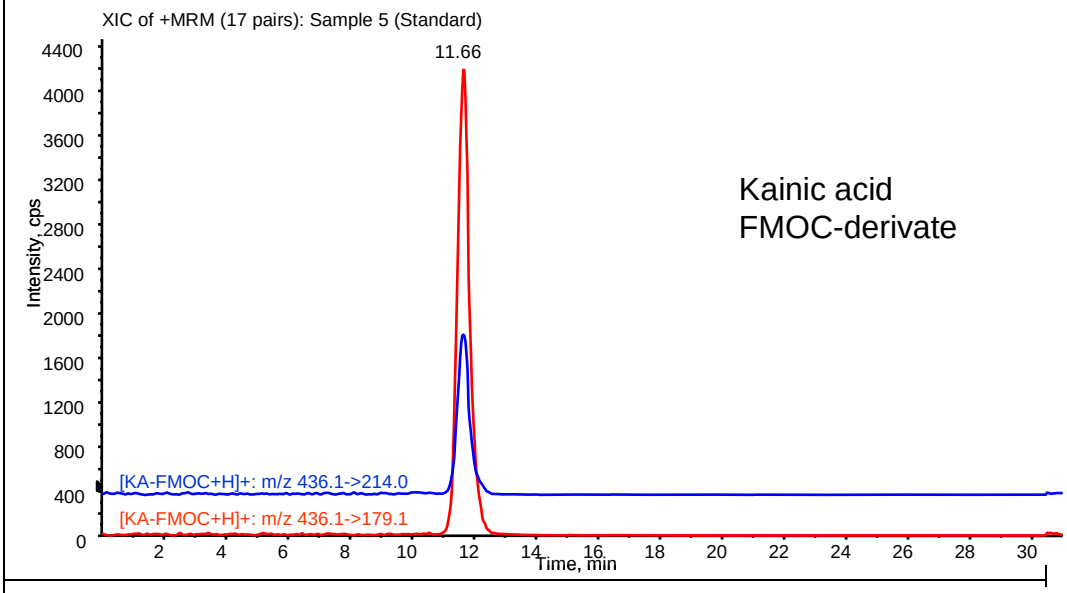


Figure 1: Extracted ion chromatogram (XIC) of amino-acid like toxins
Multiple Reaction Monitoring positive mode (+MRM).

The red mass path is used for identification.
The blue and green paths are used for quantification.


XIC of +MRM (17pairs): Sample 7 (Standard)
6.51
BMAA
1x-FMOC-derivate
[BMAA-FMOC+H]+: m/z 341.1->119.1
[BMAA-FMOC+H]+: m/z 341.1->179.1
Intensity, cps
Time, min


XIC of +MRM (17 pairs): Sample 5 (Standard)
11.66
Kainic acid
FMOC-derivate
[KA-FMOC+H]+: m/z 436.1->214.0
[KA-FMOC+H]+: m/z 436.1->179.1
Intensity, cps
Time, min

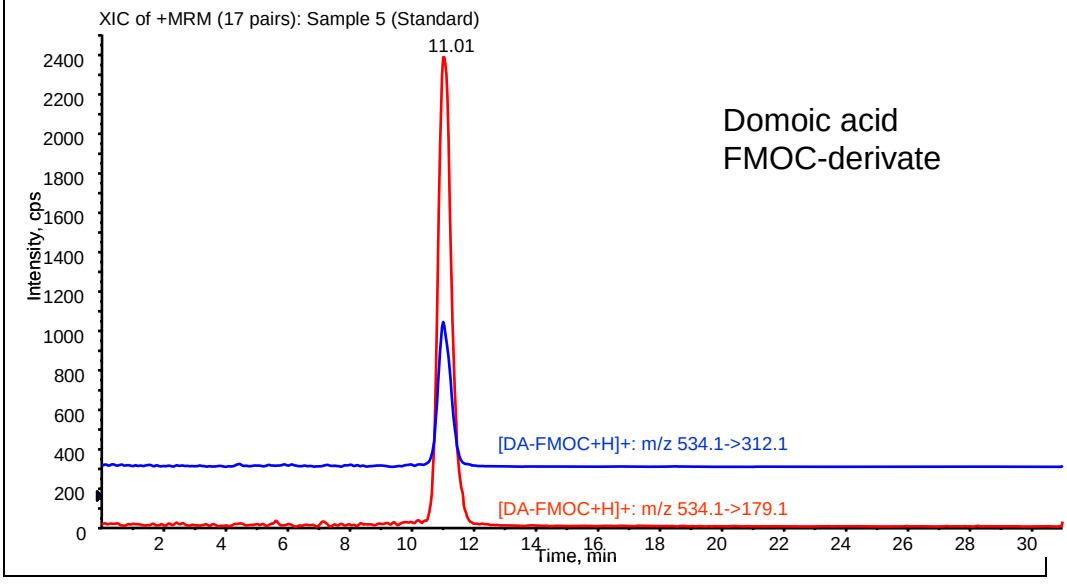

XIC of +MRM (17 pairs): Sample 5 (Standard)
11.01
Domoic acid
FMOC-derivate
[DA-FMOC+H]+: m/z 534.1->312.1
[DA-FMOC+H]+: m/z 534.1->179.1
Intensity, cps
Time, min

Figure 2: Extracted ion chromatogram (XIC) of amino-acid like toxins
Multiple Reaction Monitoring positive mode (+MRM)

5.3 References

Pietsch, J., Hampel, S. Schmidt, W., Brauch, H.-J. and Worch, E.: Determination of Aliphatic and Alicyclic Amines in Water by Gas- and Liquid Chromatography after Derivatization by Chloroformates. Fresenius J. Anal. Chem. 355, 164-173 (1996).

Milley, J.E., Bates, S.S., Bird, C.J., De Freitas, A.S.W. and Quilliam, M.A.: Trace determination of domoic acid in seawater and phytoplankton by high-performance liquid chromatography of the fluorenylmethoxycarbonyl (FMOC) derivative. Intern. J. Environ.Anal.Chem. 38, 351-368 (1989).