Supplemental Material

An all-D amphipathic undecapeptide shows promising activity against colistin-resistant strains of *Acinetobacter baumannii* and a dual mode of action

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Content:

Peptide characterization data

BP100: purity = 98.7%; t_R = 14.7 min; calculated *m/z* for $C_{72}H_{126}N_{17}O_{12}$ $[M+H]^+$ = 1420.97; experimental *m/z* = 1420.72

BP143: purity = 95.5%; t_R = 15.0 min; calculated *m/z* for $C_{72}H_{126}N_{17}O_{12}$ $[M+H]^+$ = 1420.97; experimental *m/z* = 1420.70

BP157: purity = 99.0%; t_R = 14.4 min; calculated *m/z* for $C_{72}H_{126}N_{17}O_{12}$ $[M+H]^+$ = 1420.97; experimental *m/z* = 1420.58

RW-BP100: purity = 100%; t_R = 15.7 min; calculated *m/z* for $C_{74}H_{127}N_{28}O_{11}$ $[M+H]^+$ = 1584.02; experimental *m/z* = 1584.02

BP201: purity = 99.0%; t_R = 14.8 min; calculated *m/z* for $C_{72}H_{126}N_{21}O_{12}$ $[M+H]^+$ = 1476.98; experimental *m/z* = 1476.59

BP202: purity = 99.0%; t_R = 14.8 min; calculated *m/z* for $C_{72}H_{126}N_{21}O_{12}$ $[M+H]^+$ = 1476.98; experimental *m/z* = 1476.59

BP203: purity = 100%; t_R = 15.0 min; calculated *m/z* for $C_{72}H_{126}N_{19}O_{12}$ $[M+H]^+$ = 1448.98; experimental *m/z* = 1448.76

BP204: purity = 99.9%; t_R = 14.7 min; calculated *m/z* for $C_{72}H_{126}N_{23}O_{12}$ $[M+H]^+$ = 1504.99; experimental *m/z* = 1504.43

BP205: purity = 95.2%; t_R = 14.6 min; calculated *m/z* for $C_{72}H_{126}N_{21}O_{12}$ $[M+H]^+$ = 1476.98; experimental *m/z* = 1476.86

BP206: purity = 98.5%; t_R = 15.0 min; calculated *m/z* for $C_{72}H_{126}N_{21}O_{12}$ $[M+H]^+$ = 1476.98; experimental *m/z* = 1476.33

BP207: purity = 99.3%; t_R = 14.5 min; calculated *m/z* for $C_{72}H_{126}N_{17}O_{12}$ $[M+H]^+$ = 1420.97; experimental *m/z* = 1420.46

BP208: purity = 98.4%; t_R = 14.2 min; calculated *m/z* for $C_{72}H_{126}N_{17}O_{12}$ $[M+H]^+$ = 1420.97; experimental *m/z* = 1420.46

BP209: purity = 99.9%; t_R = 14.7 min; calculated *m/z* for $C_{76}H_{128}N_{27}O_{12}$ $[M+H]^+$ = 1404.97; experimental *m/z* = 1404.55

BP210: purity = 95.1%; t_R = 15.7 min; calculated *m/z* for $C_{76}H_{128}N_{27}O_{12}$ $[M+H]^+$ = 1611.02; experimental *m/z* = 1610.70

BP211: purity = 99.1%; t_R = 15.0 min; calculated *m/z* for $C_{72}H_{126}N_{19}O_{12}$ $[M+H]^+$ = 1448.98; experimental *m/z* = 1448.51

BP212: purity = 100%; t_R = 15.7 min; calculated *m/z* for $C_{76}H_{128}N_{17}O_{12}$ $[M+H]^+$ = 1470.99; experimental *m/z* = 1470.25

BP213 purity = 96.3%; t_R = 14.4 min; calculated *m/z* for $C_{72}H_{126}N_{19}O_{12}$ $[M+H]^+$ = 1448.98; experimental *m/z* = 1448.51

BP214 purity = 96.8%; t_R = 14.6 min; calculated *m/z* for $C_{72}H_{126}N_{19}O_{12}$ $[M+H]^+$ = 1448.98; experimental *m/z* = 1448.51

Reverse-phase HPLC Setup

For all experiments:

- **Buffer A:** 99.9% Water, 0.1% TFA
- **Buffer B:** 89.95% MeCN, 9.95% Water, 0.1% TFA

Analytical HPLC

- **Pump:** Waters™ 600 Pump
- **Detector:** Waters™ 2996 Photodiode Array Detector
- **Column:** Waters™ Symmetry™ C18, 4.6 x 250 mm, 5 µm

Gradient:

Time (min)	Flow (ml/min)	Buffer A	Buffer B
0.0	1.50	100%	0%
20.0	1.50	30%	70%
22.0	1.50	0%	100%
27.0	1.50	0%	100%
30.0	1.50	100%	0%

Equilibration delay between injections (both methods): 9 min with 100% Buffer A, 1.50 ml/min.The ApexTrack™ algorithm has been used for peak integration. Peaks having an area <0.01% were not included in the purity calculations. Spectra were processed as follows: the raw data were exported using the Waters Empower 3 software and imported in Microsoft Excel. The baseline (blank) was subtracted and negative absorbance values were corrected to 0 (zero).

Preparative HPLC

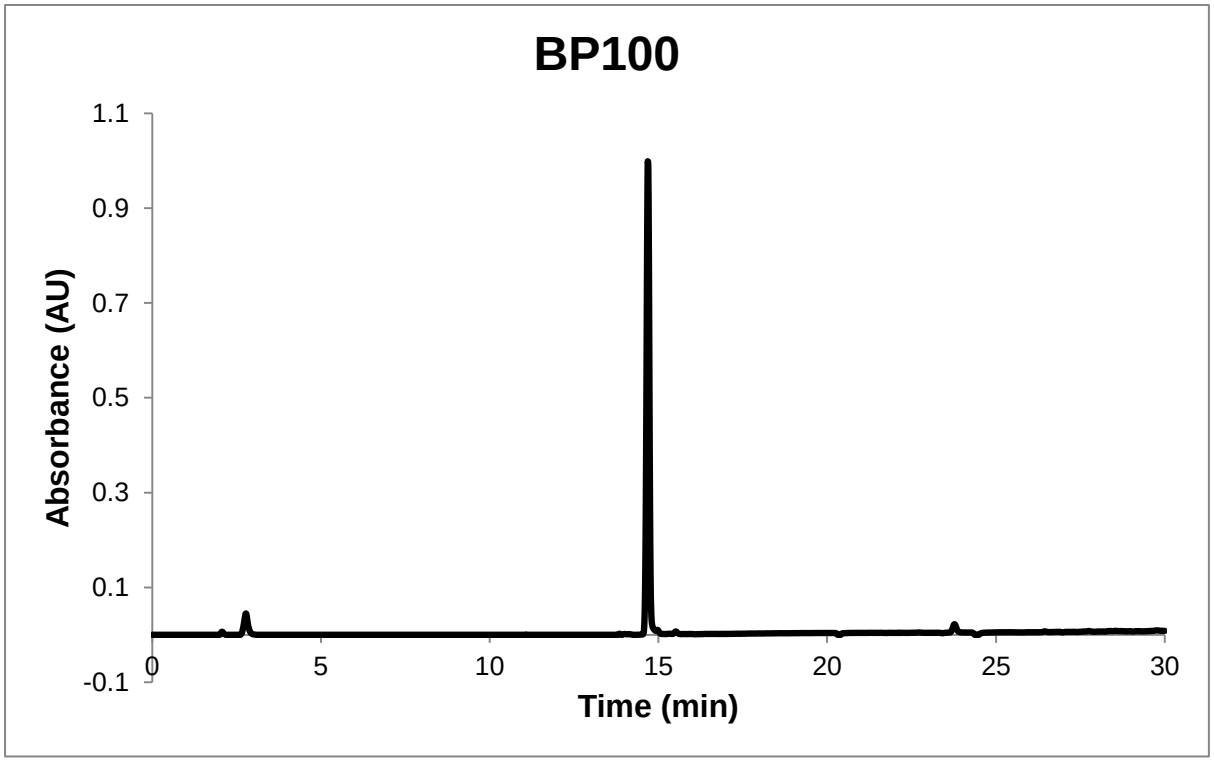
- Pump: Waters™ 600 Pump
- Detector: Waters™ 996 Photodiode Array Detector
- Column: Waters™ XBridge™ BEH130 C18, 10 x 250 mm, 5 µm

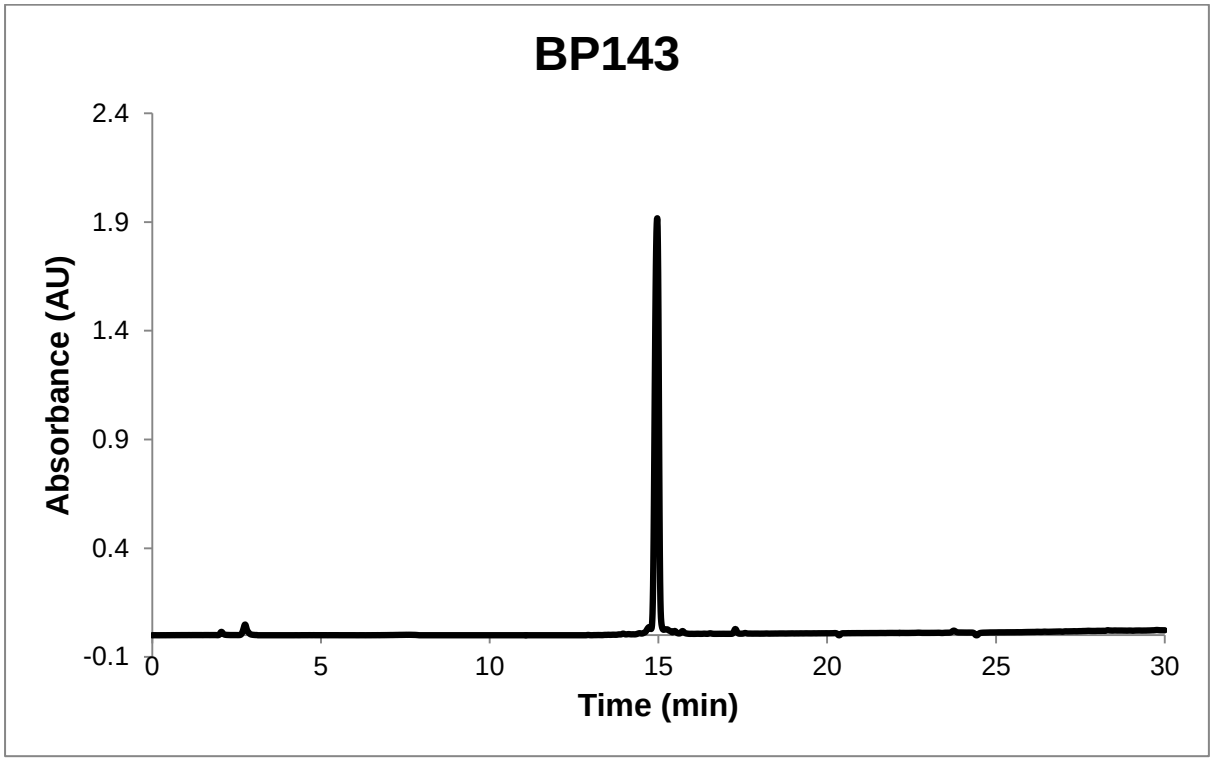
Gradient:

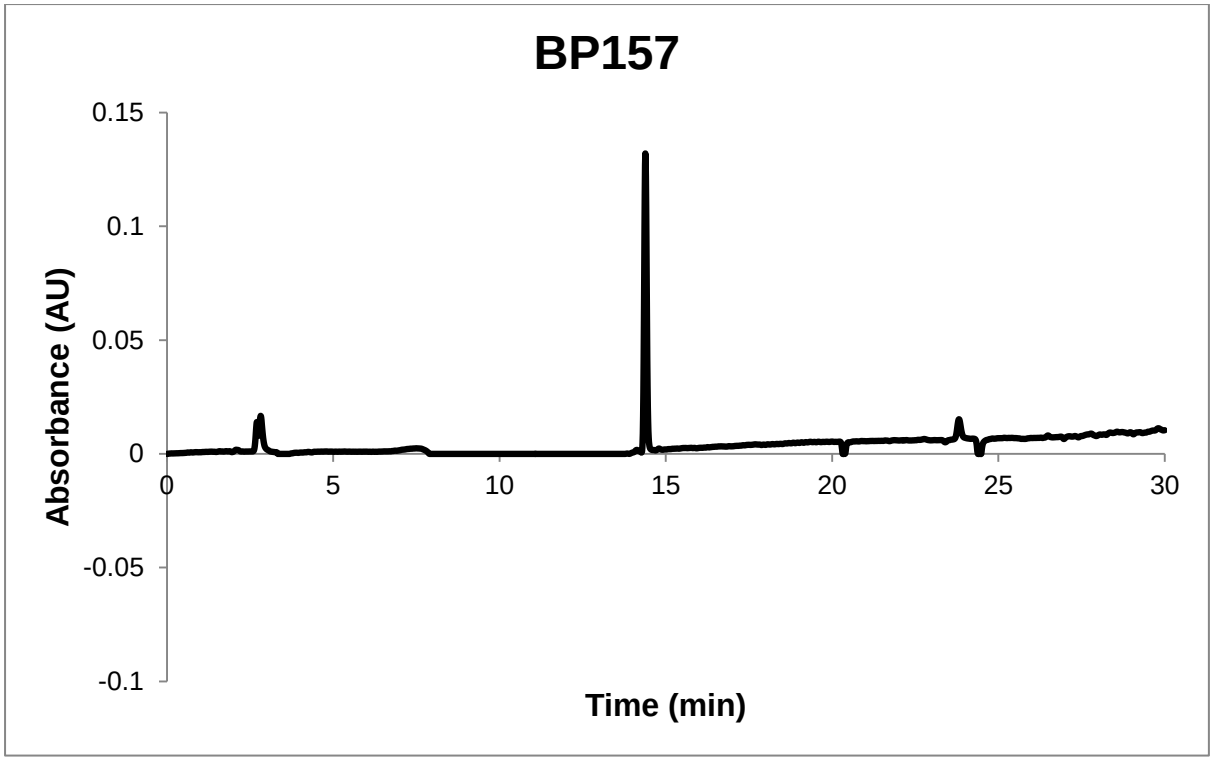
Time (min)	Flow (ml/min)	Buffer A	Buffer B
0.0	4.00	100%	0%
1.0	6.00	100%	0%
3.0	8.50	100%	0%
5.0	8.50	90%	10%
20.0	8.50	45%	55%
26.0	9.00	0%	100%
27.0	9.00	0%	100%
30.0	9.00	100%	0%
39.0	9.00	100%	0%

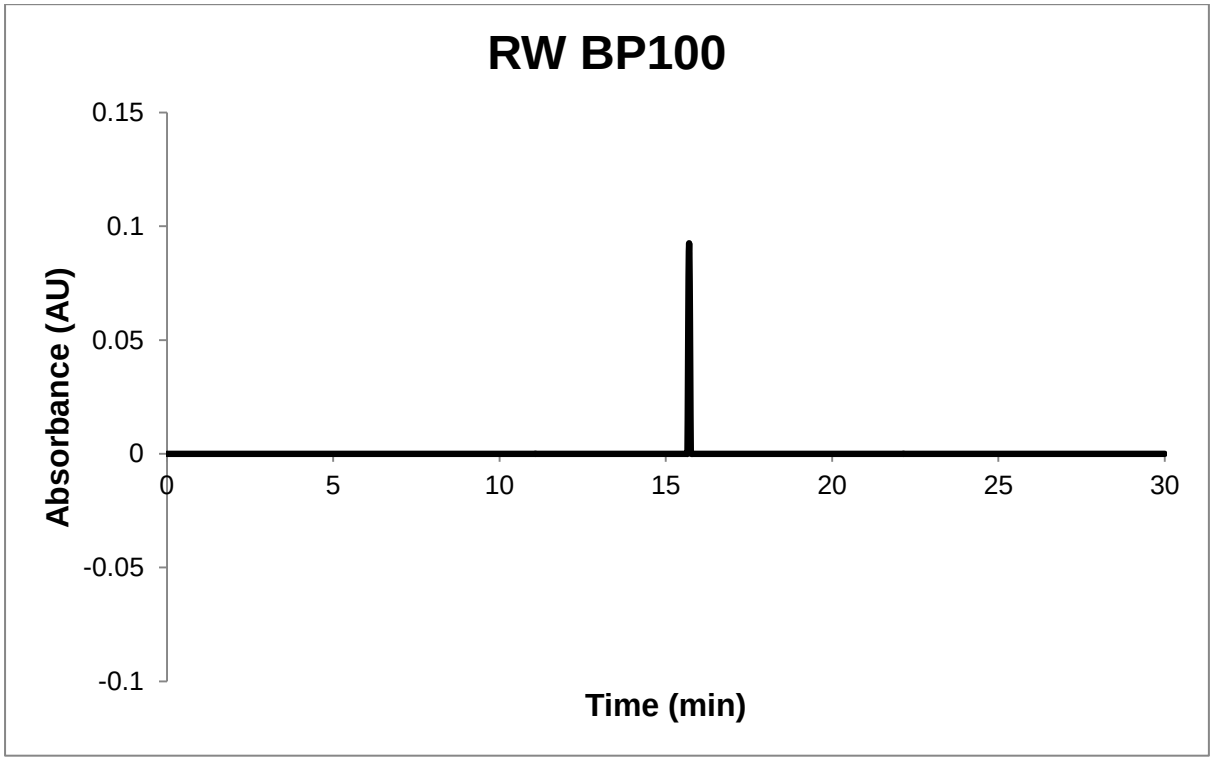
Purified fractions were collected manually.

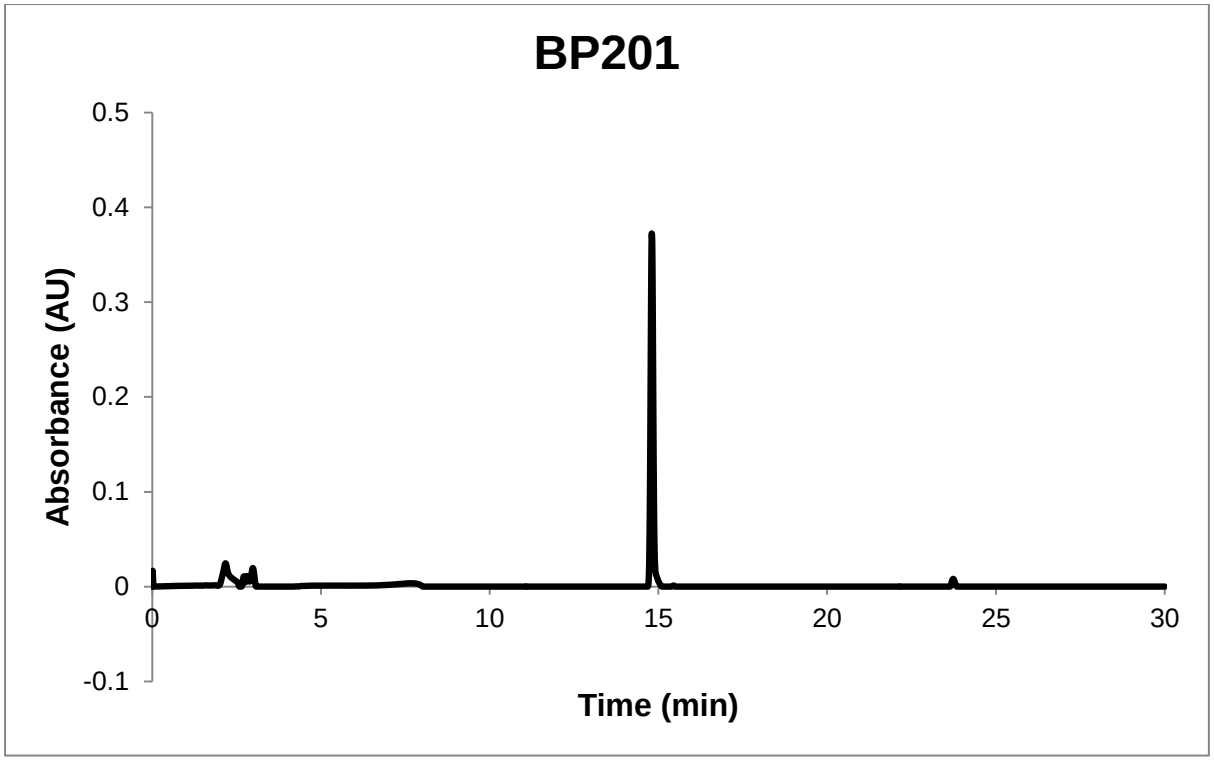
Analytical Chromatograms

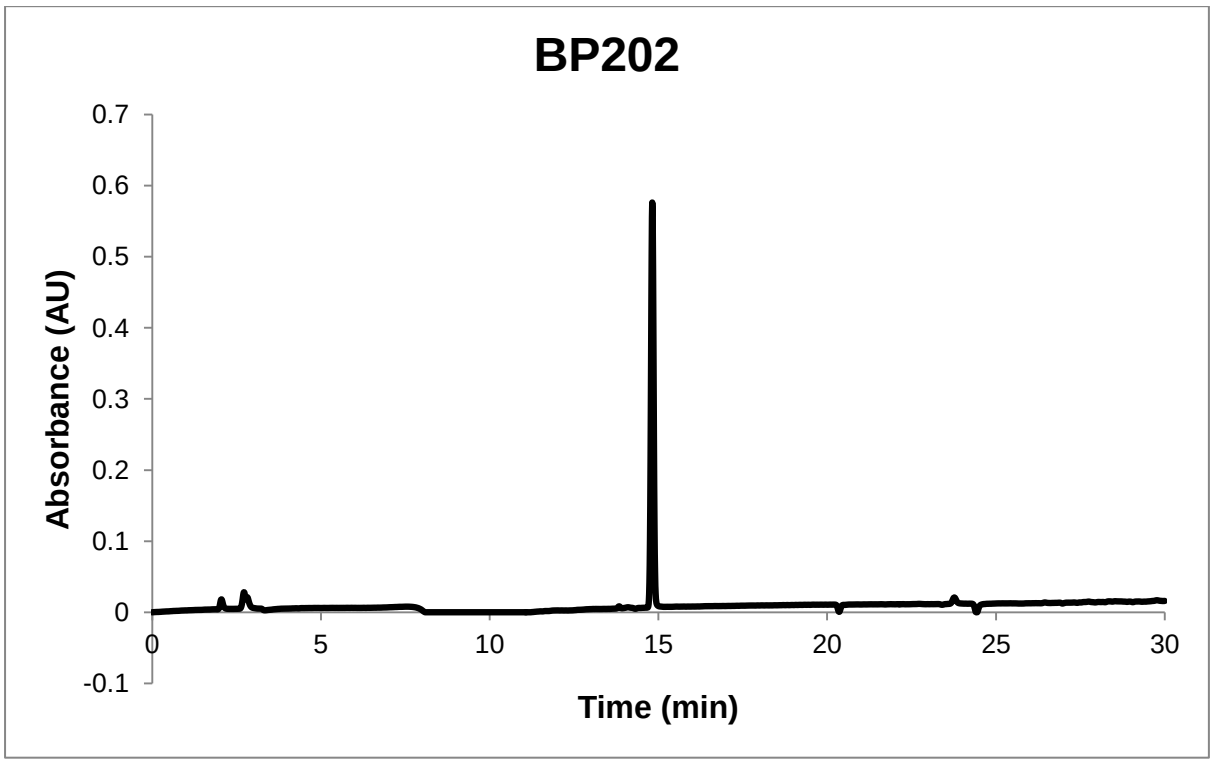


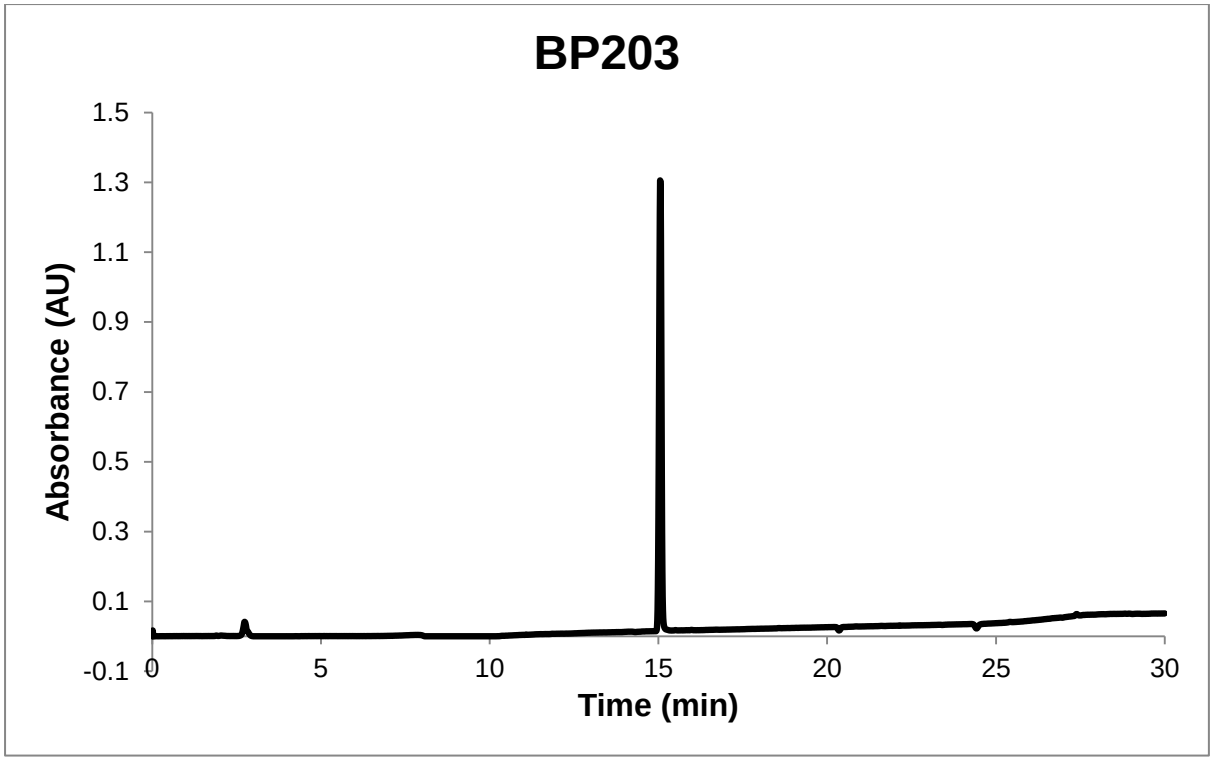


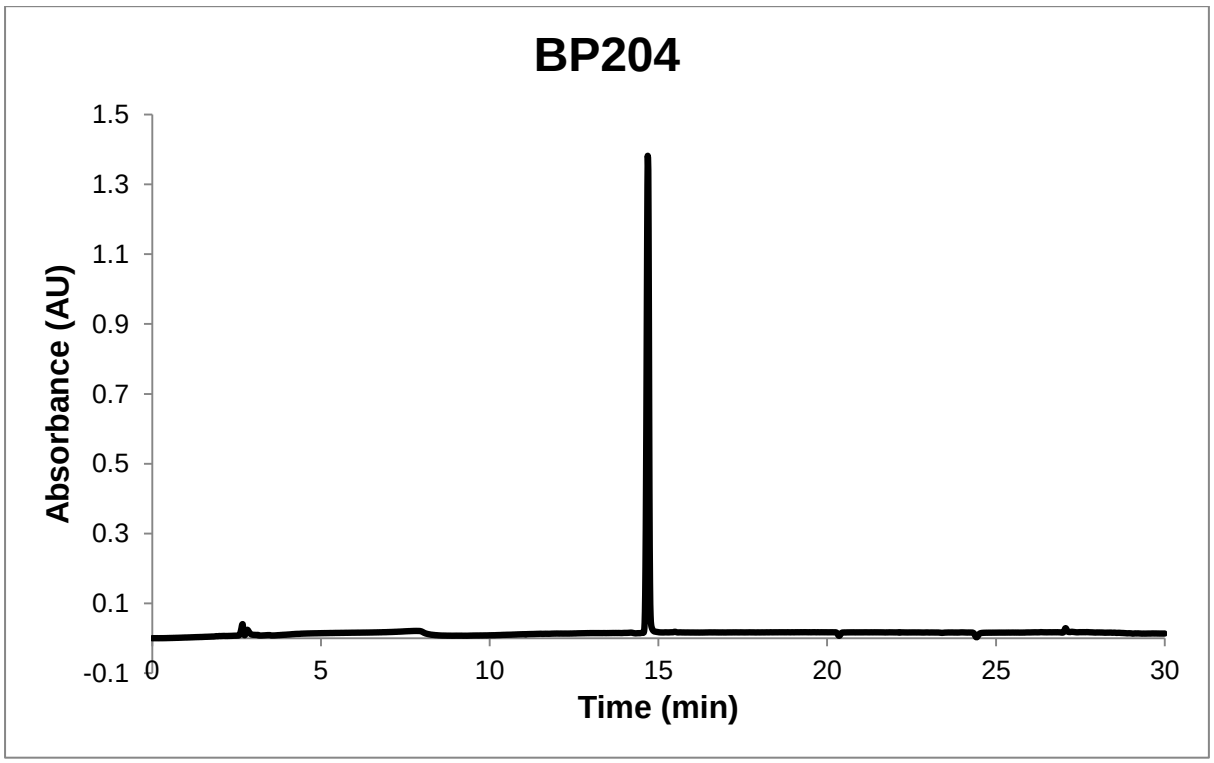

BP157
0.15
0.1
0.05
0
-0.05
-0.1
Absorbance (AU)
0
5
10
15
20
25
30
Time (min)

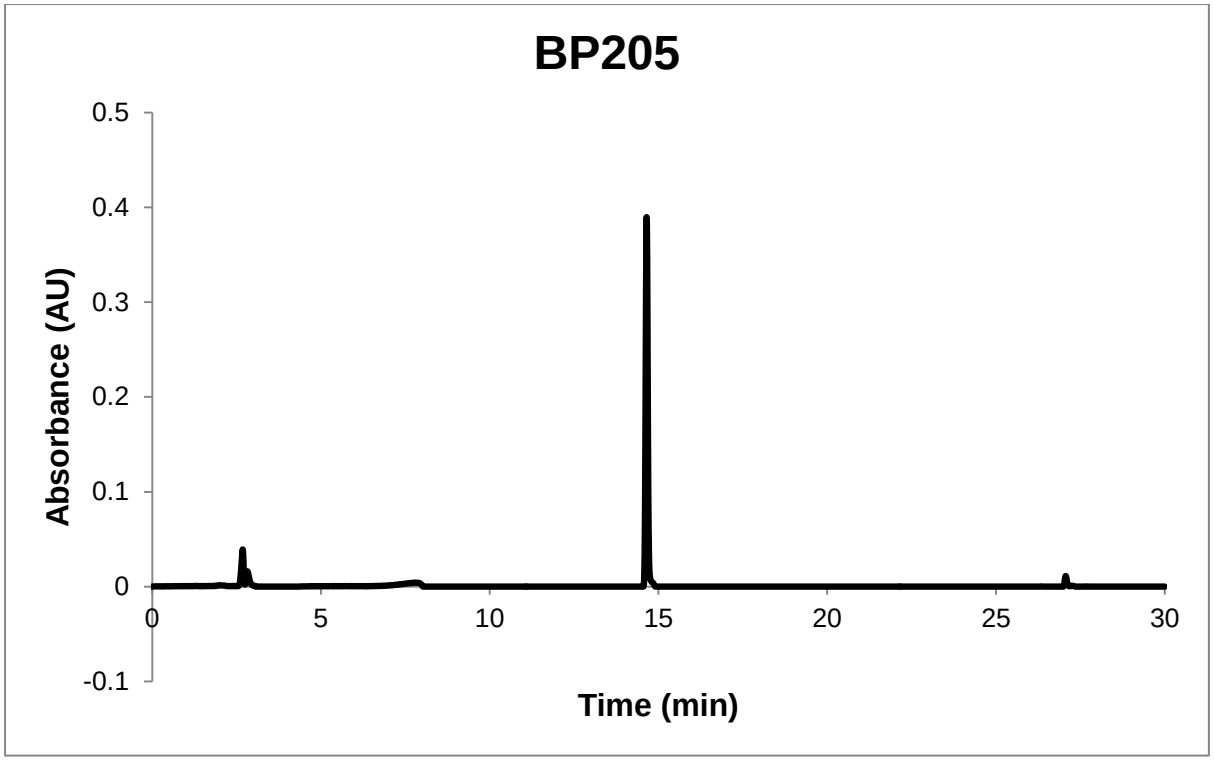

RW BP100
0.15
0.1
0.05
0
-0.05
-0.1
Absorbance (AU)
0
5
10
15
20
25
30
Time (min)

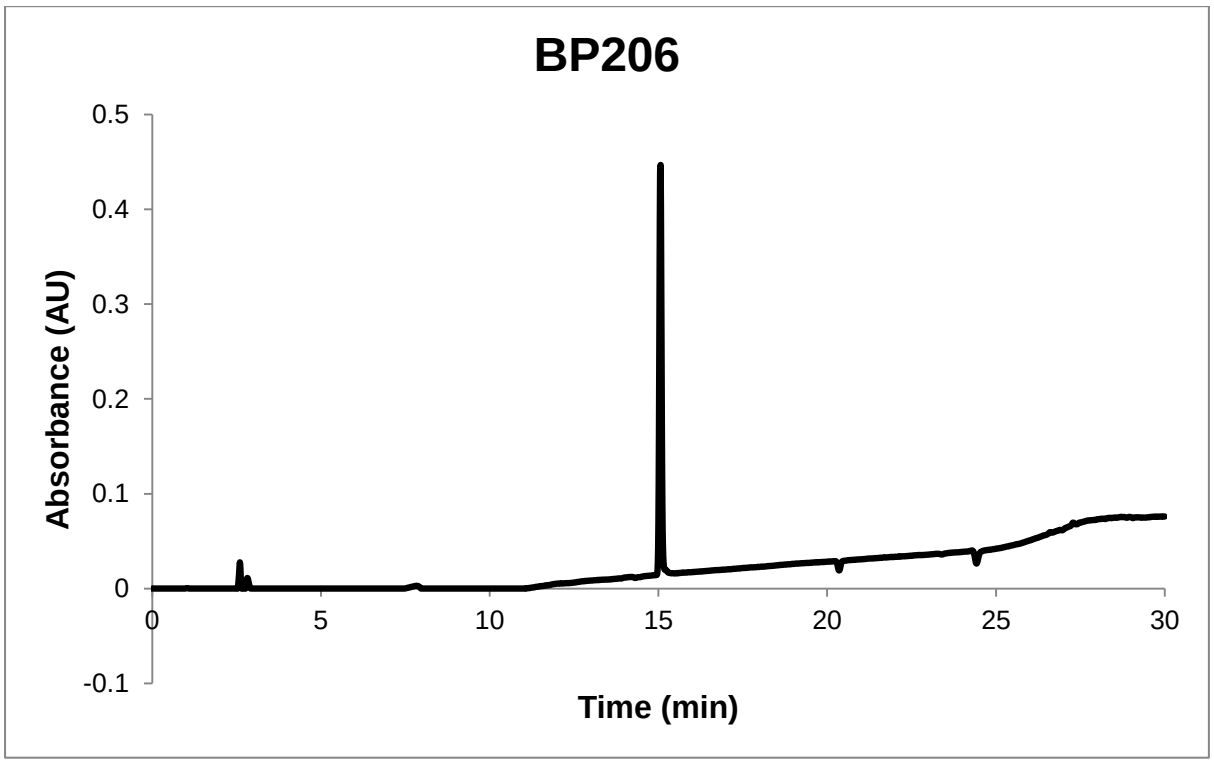

BP201
Absorbance (AU)
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)

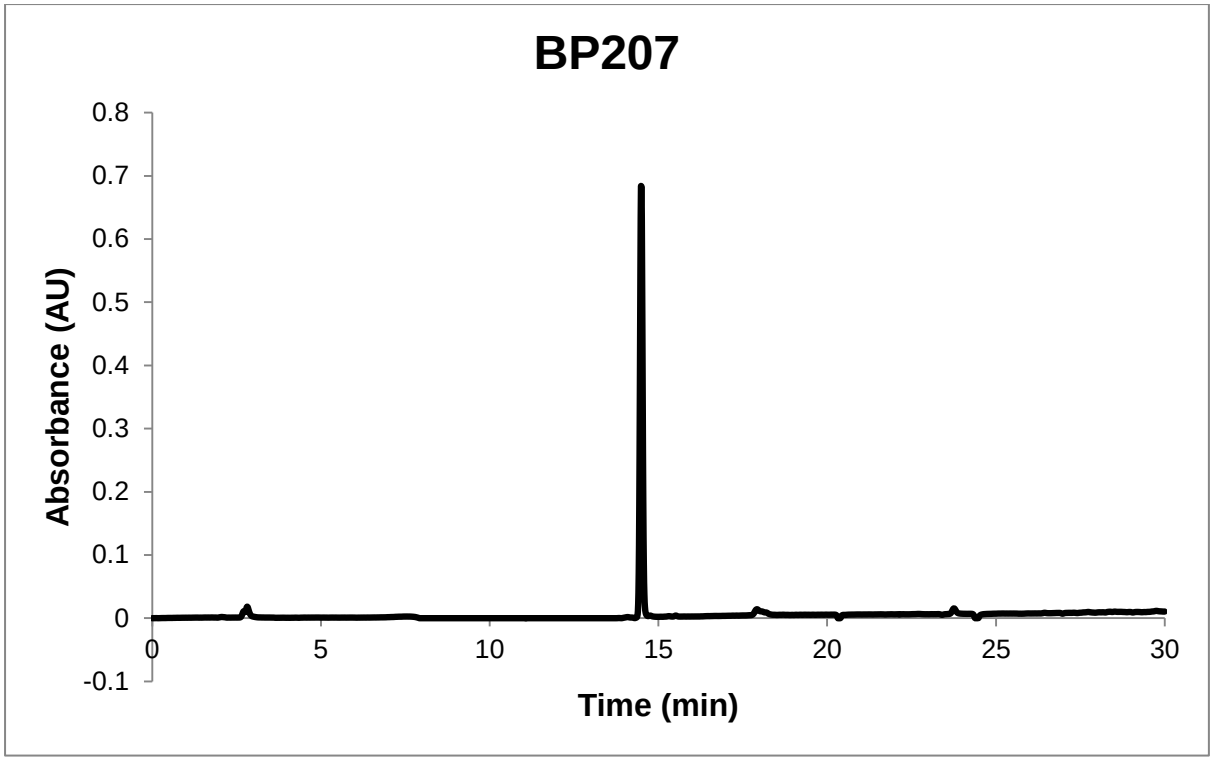

BP202
Absorbance (AU)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)

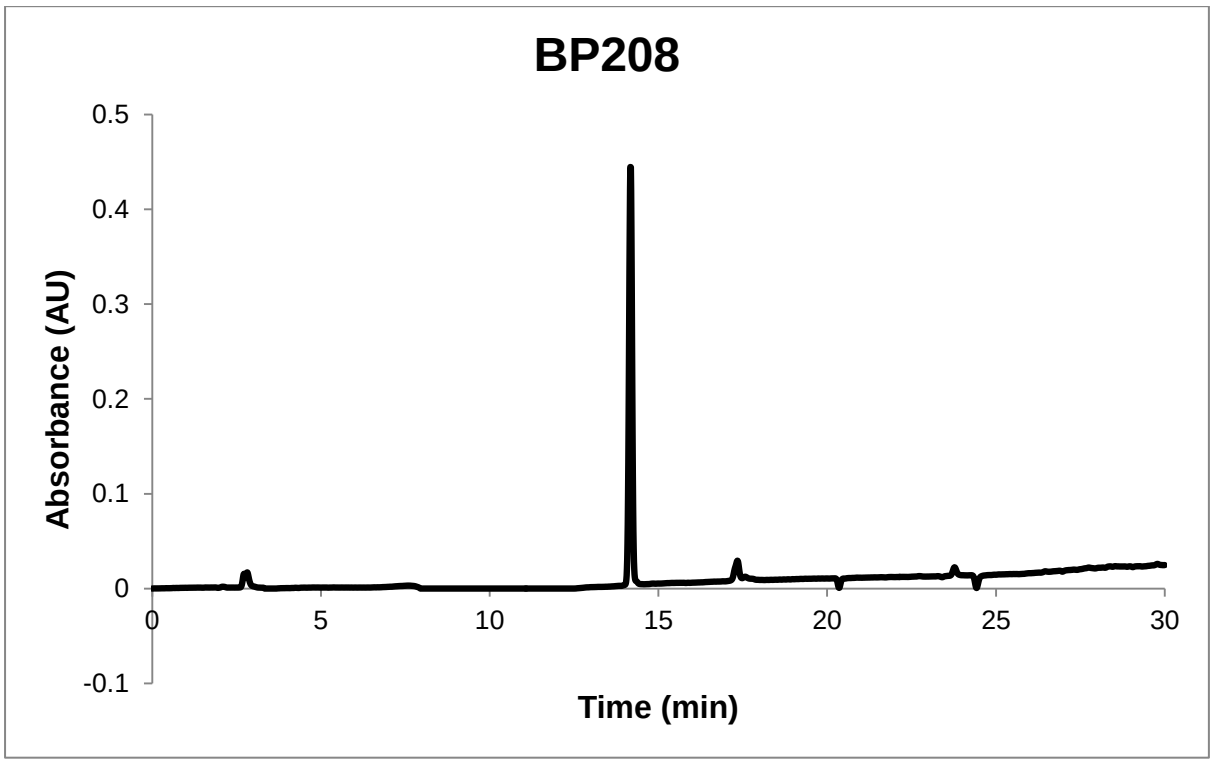

BP203
Absorbance (AU)
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
10
15
20
25
30
Time (min)

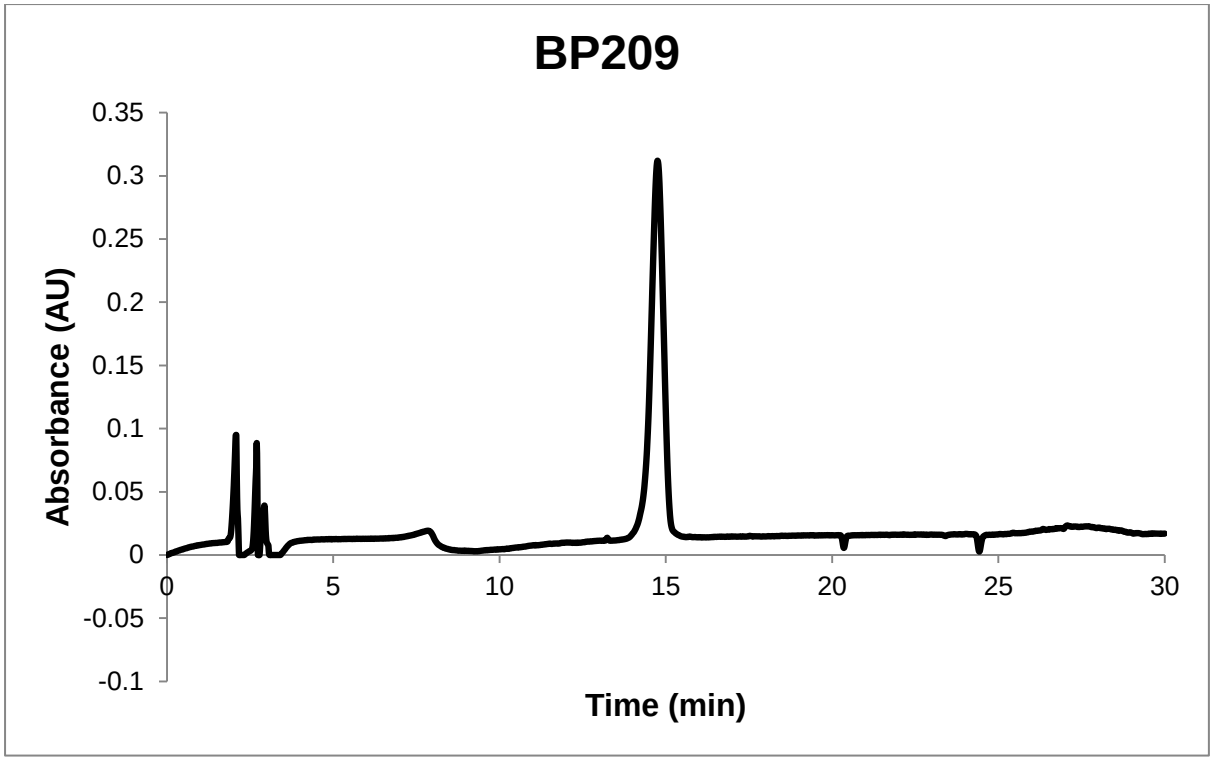

BP204
Absorbance (AU)
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
10
15
20
25
30
Time (min)

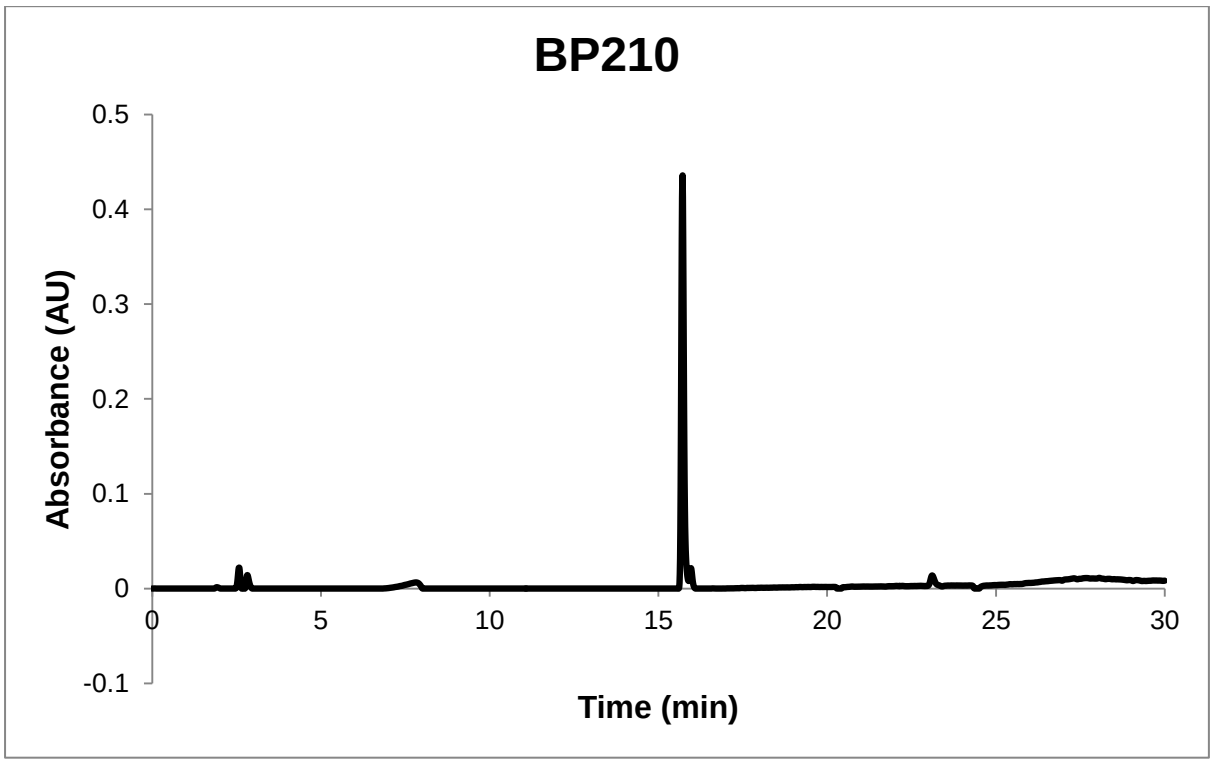

BP205
Absorbance (AU)
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)

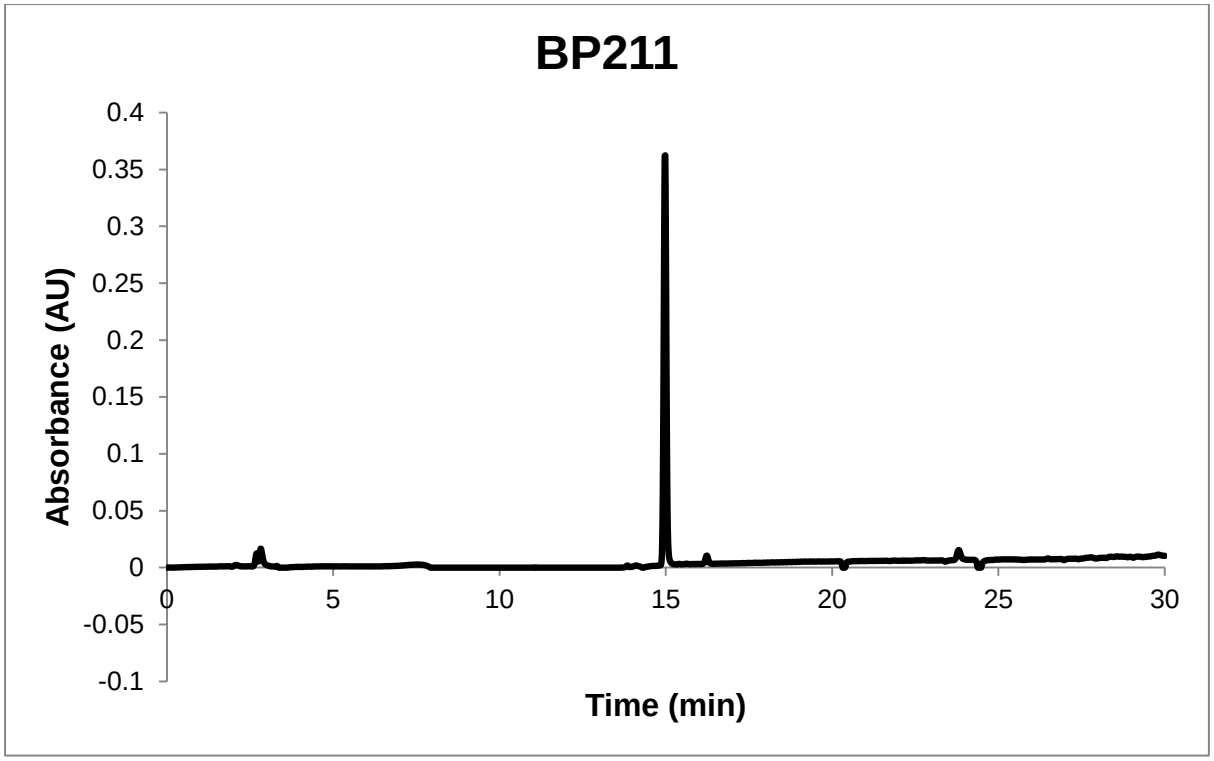

BP206
Absorbance (AU)
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)

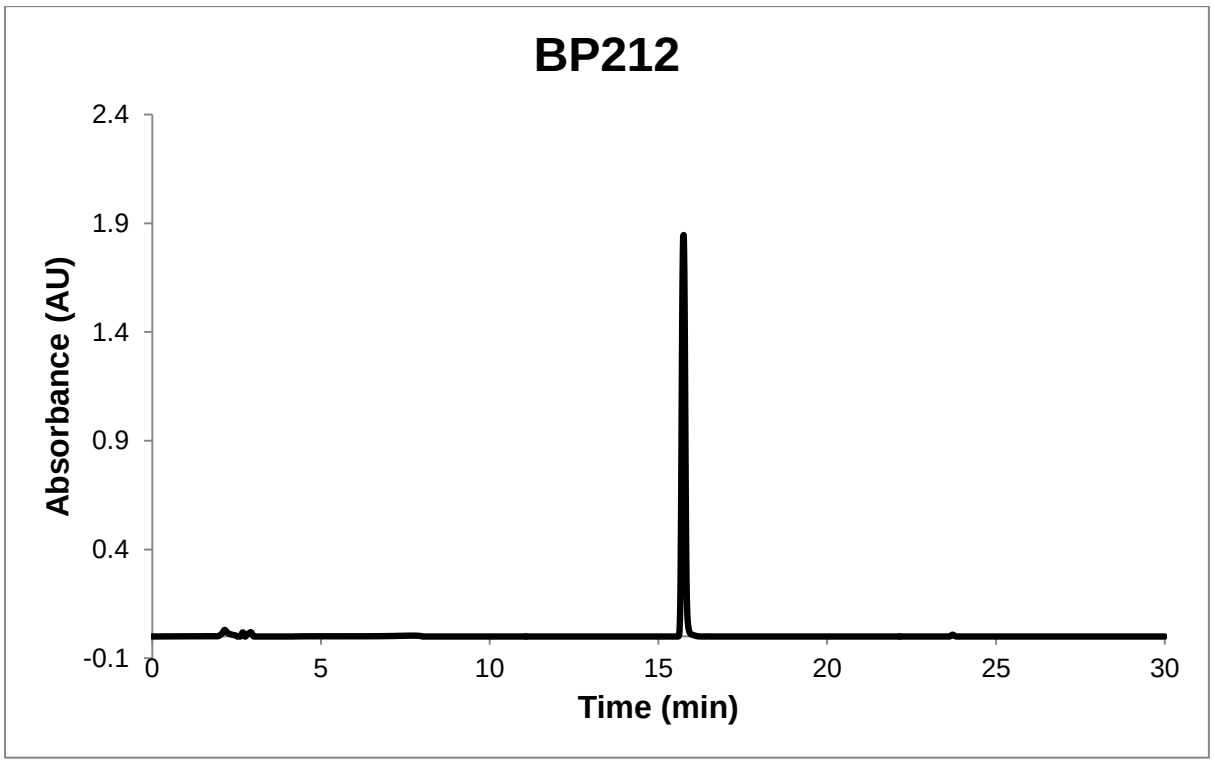

BP207
Absorbance (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)

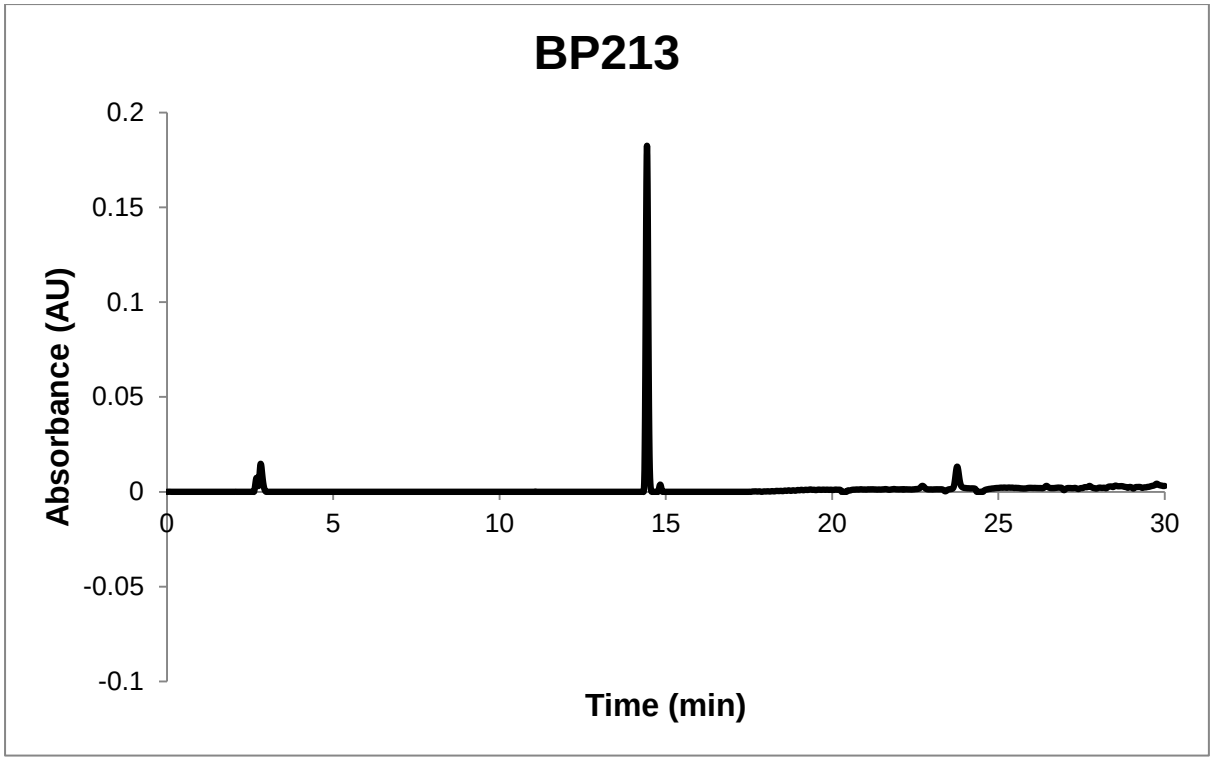

BP208
Absorbance (AU)
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)

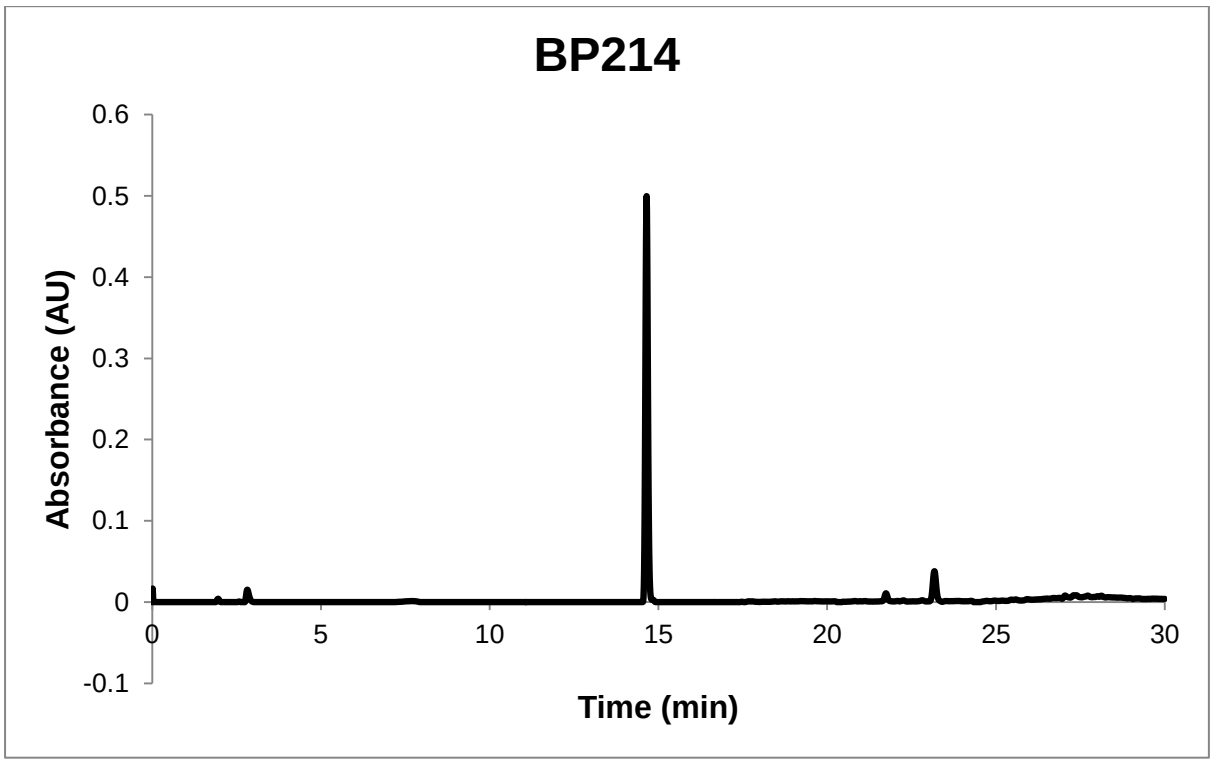

BP209
Absorbance (AU)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
0
5
10
15
20
25
30
Time (min)


BP210
Absorbance (AU)
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
Time (min)


BP211
Absorbance (AU)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
0
5
10
15
20
25
30
Time (min)


BP212
Absorbance (AU)
2.4
1.9
1.4
0.9
0.4
-0.1
0
5
10
15
20
25
30
Time (min)


BP213
0.2
0.15
0.1
0.05
0
-0.05
-0.1
Absorbance (AU)
0
5
10
15
20
25
30
Time (min)

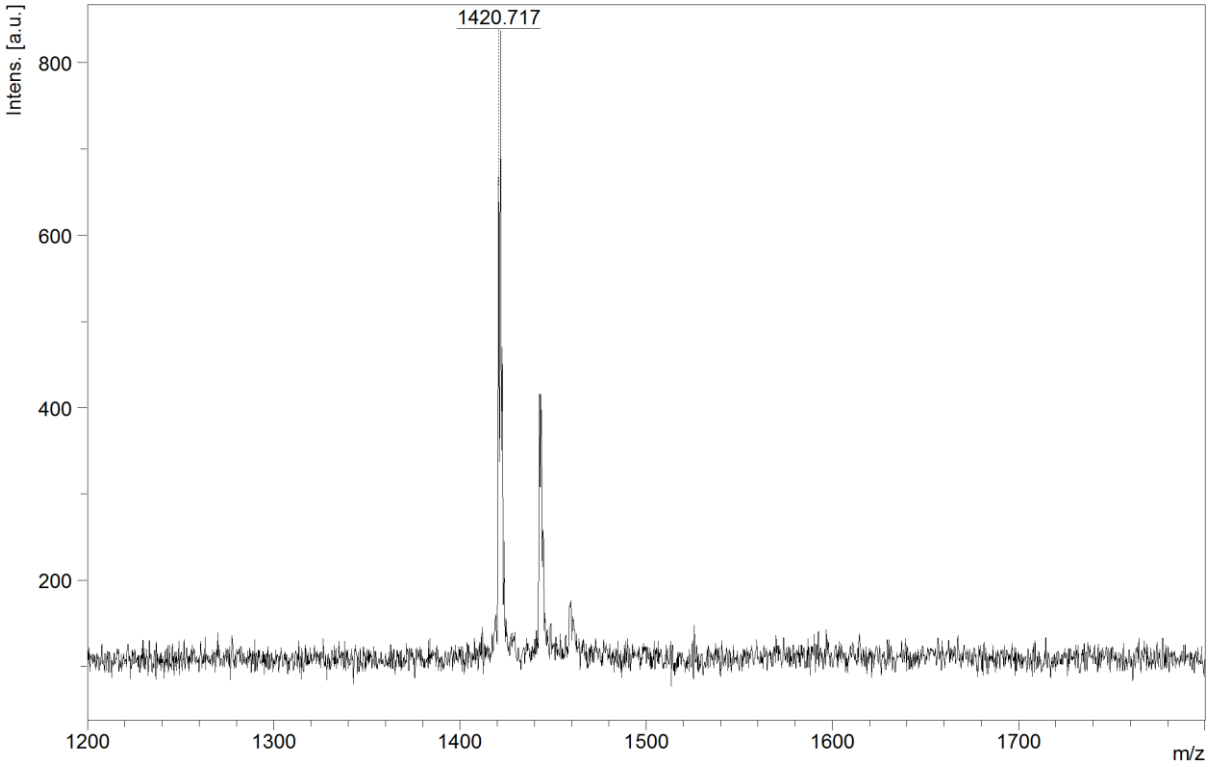

BP214
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Absorbance (AU)
0
5
10
15
20
25
30
Time (min)

MALDI-ToF-MS Setup

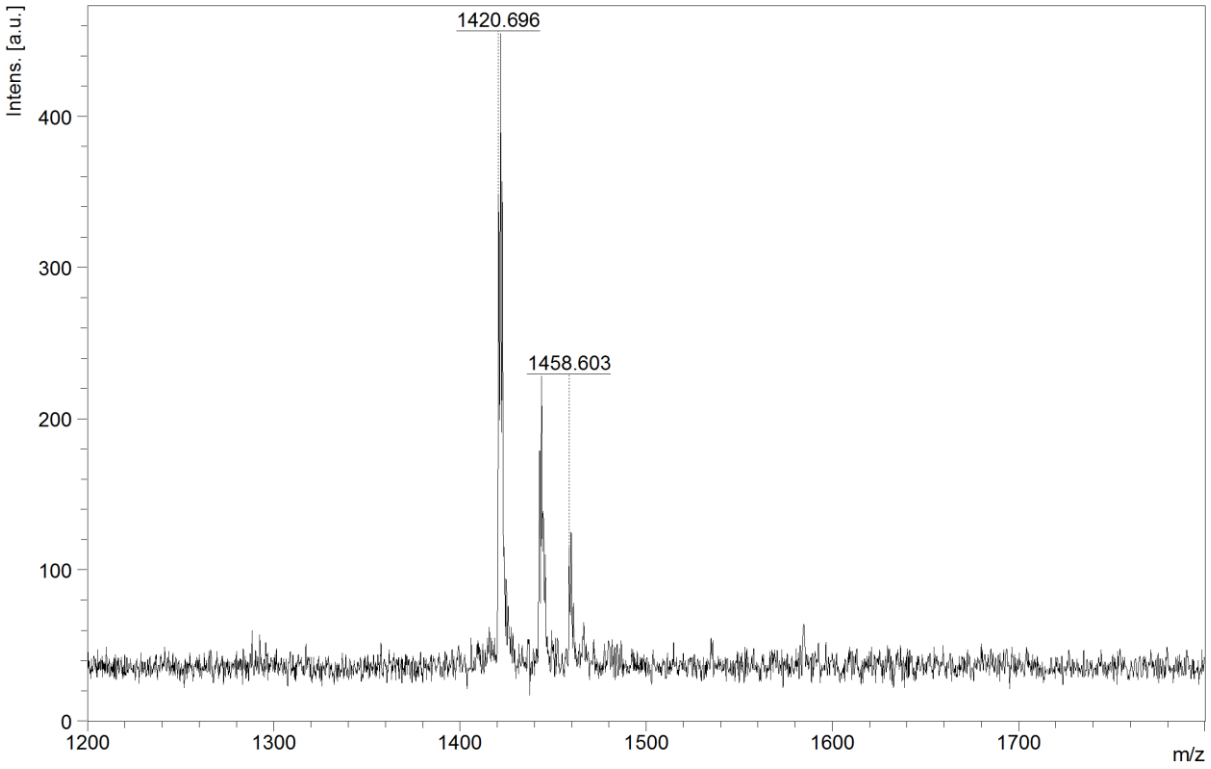
Instrument: Bruker Microflex™
Matrix: α-cyano-4-hydroxycinnamic acid in MeCN:H_2O:TFA (50:47.5:2.5) (10 mg/ml).

MALDI Spectra

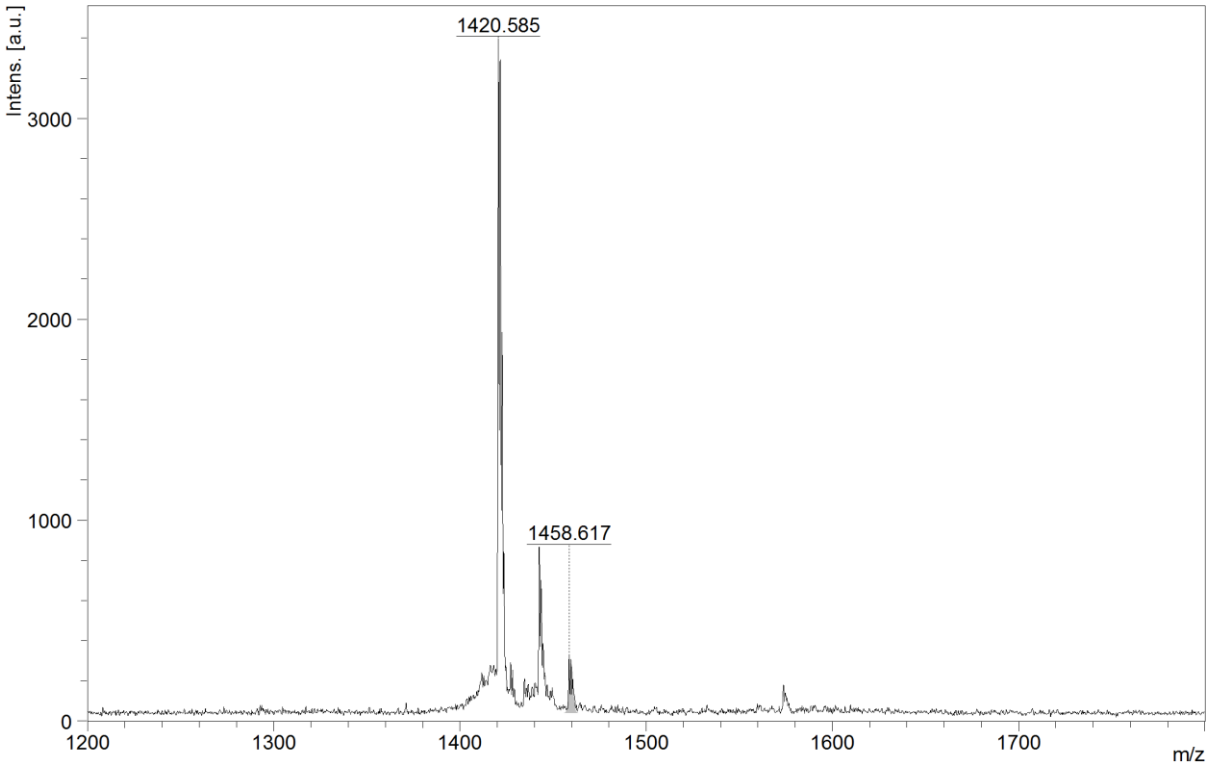
BP100



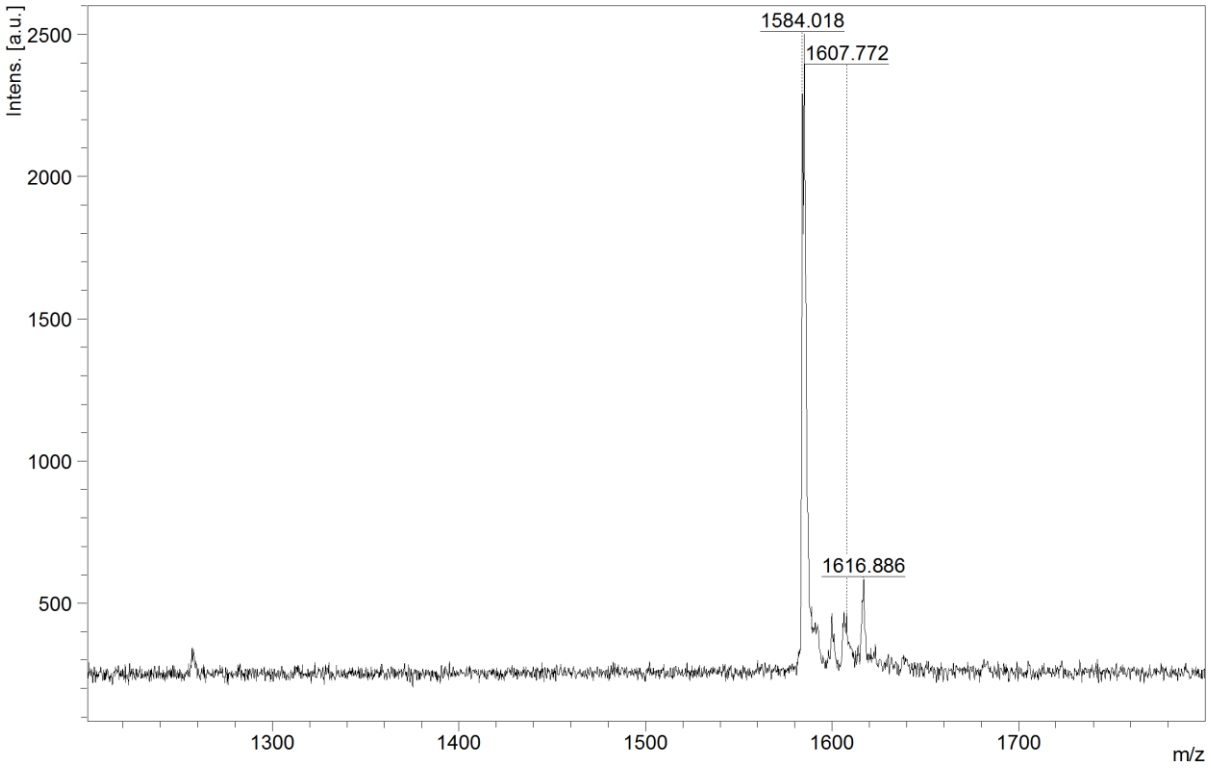
BP143


1420.696
1458.603
Intens. [a.u.]
400
300
200
100
0
1200
1300
1400
1500
1600
1700
m/z

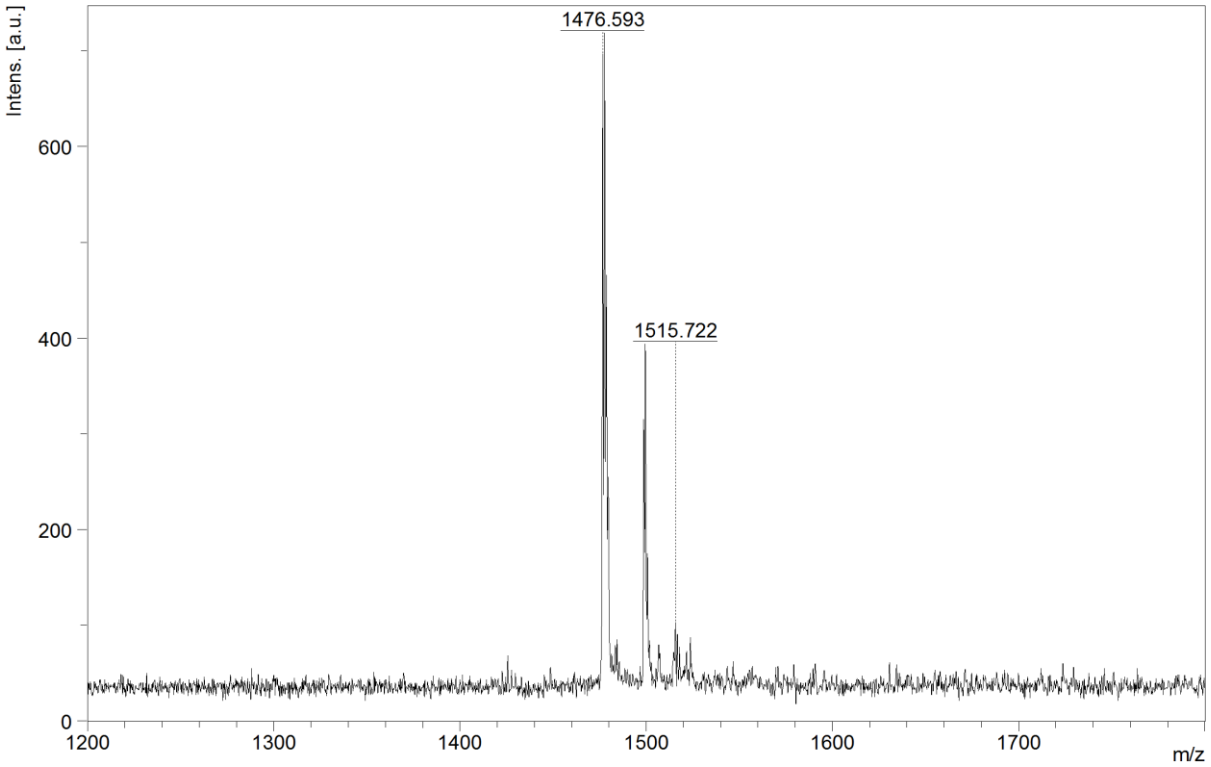
BP157


1420.585
1458.617
Intens. [a.u.]
3000
2000
1000
0
1200
1300
1400
1500
1600
1700
m/z

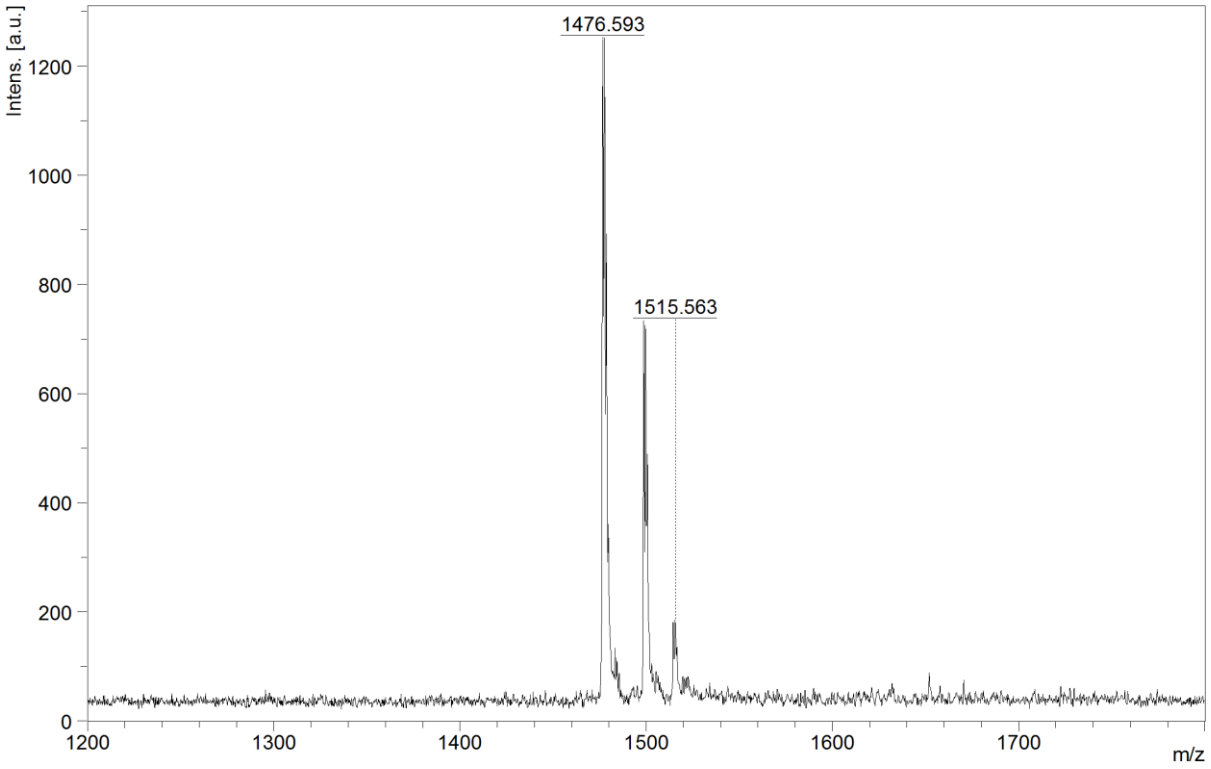
RW-BP100



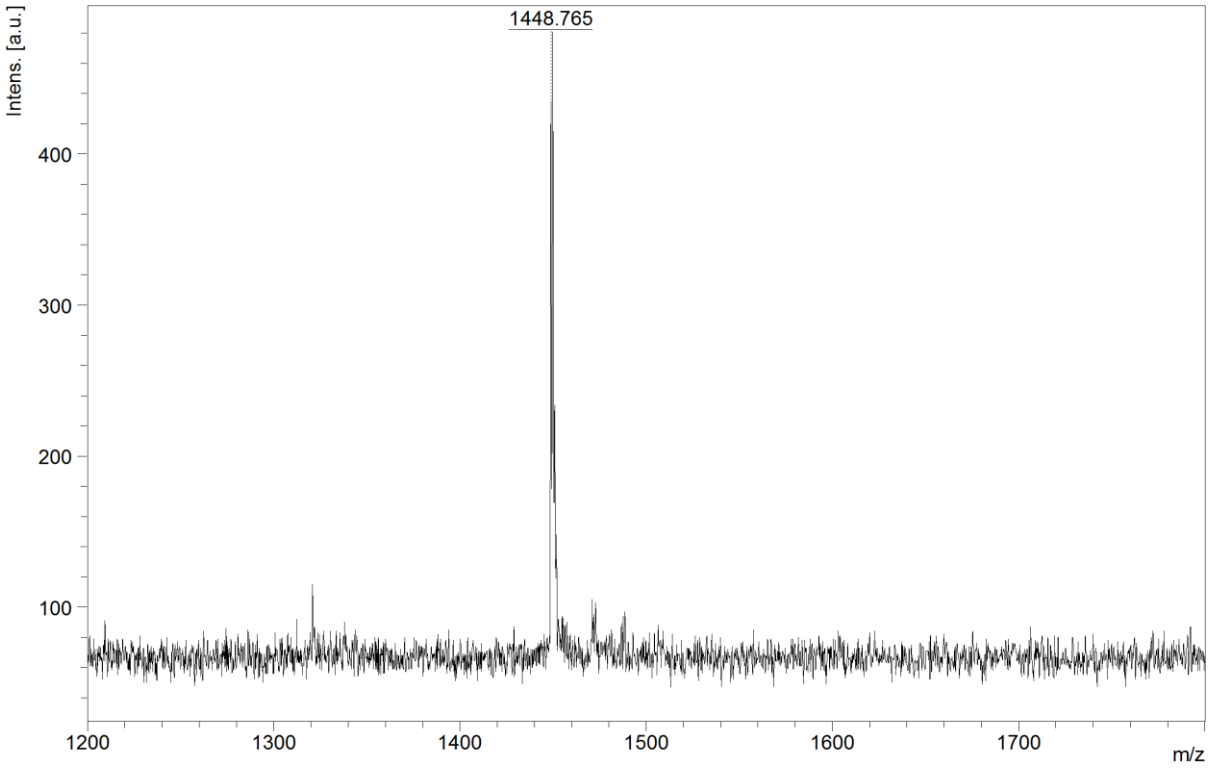
BP201



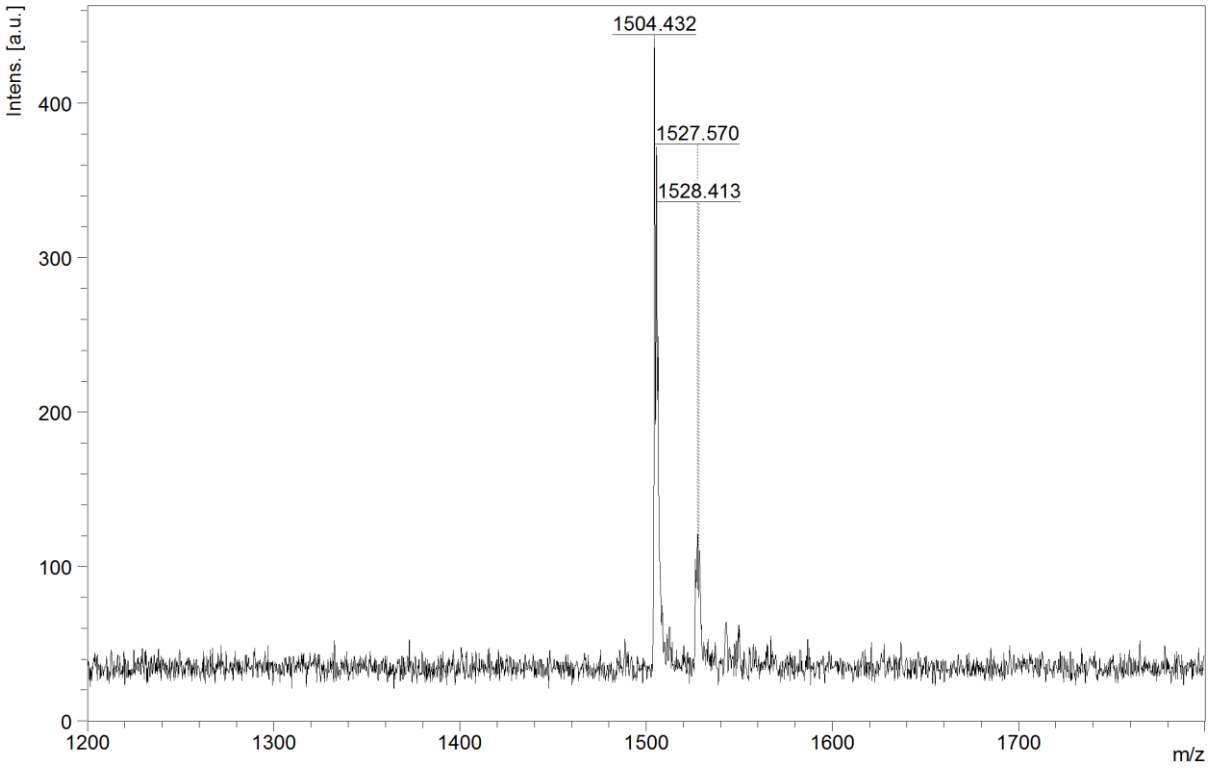
BP202


Intens. [a.u.]
1476.593
1515.563
0
200
400
600
800
1000
1200
1200
1300
1400
1500
1600
1700
m/z

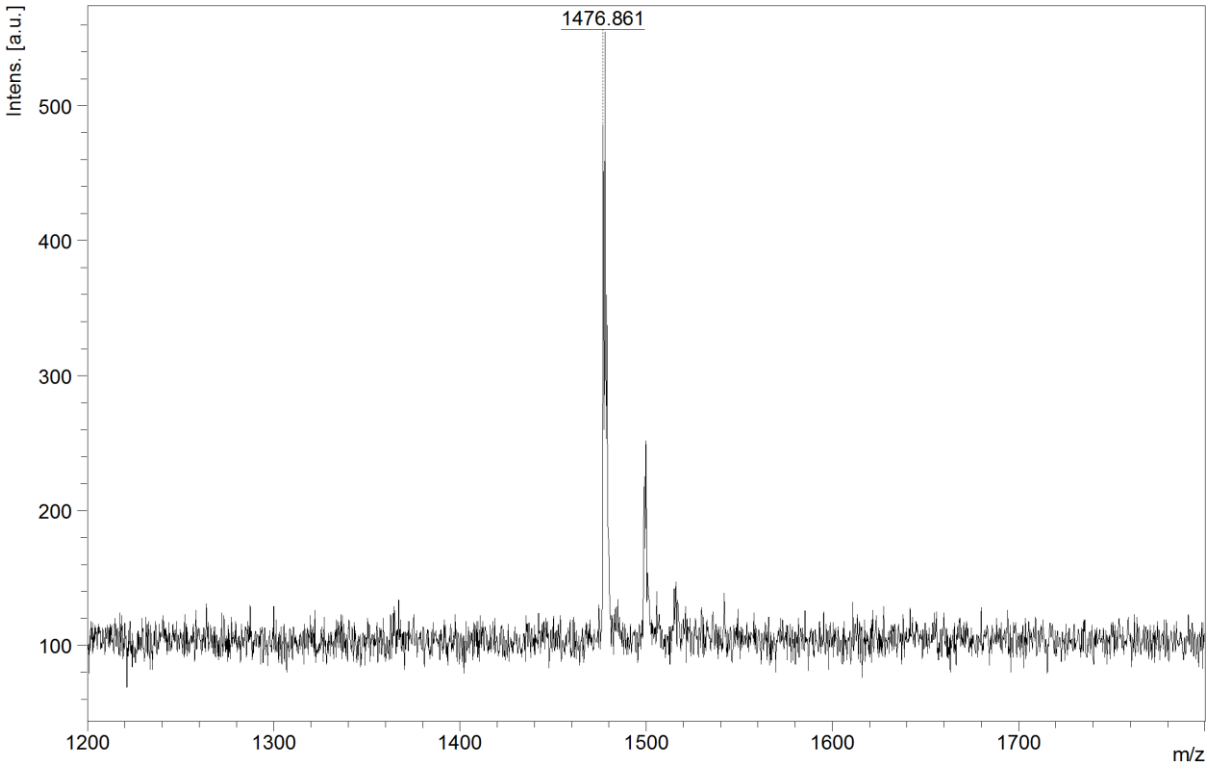
BP203


Intens. [a.u.]
1448.765
100
200
300
400
1200
1300
1400
1500
1600
1700
m/z

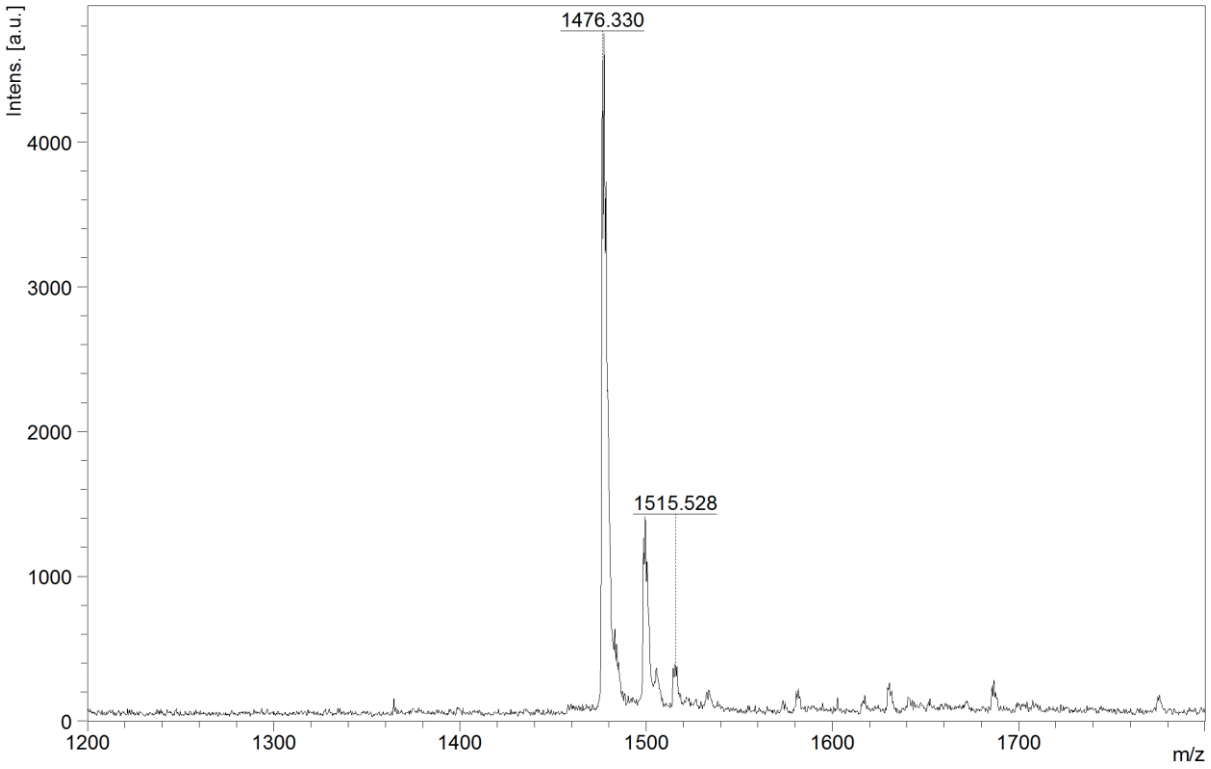
BP204


Intens. [a.u.]
1504.432
1527.570
1528.413
400
300
200
100
0
1200
1300
1400
1500
1600
1700
m/z

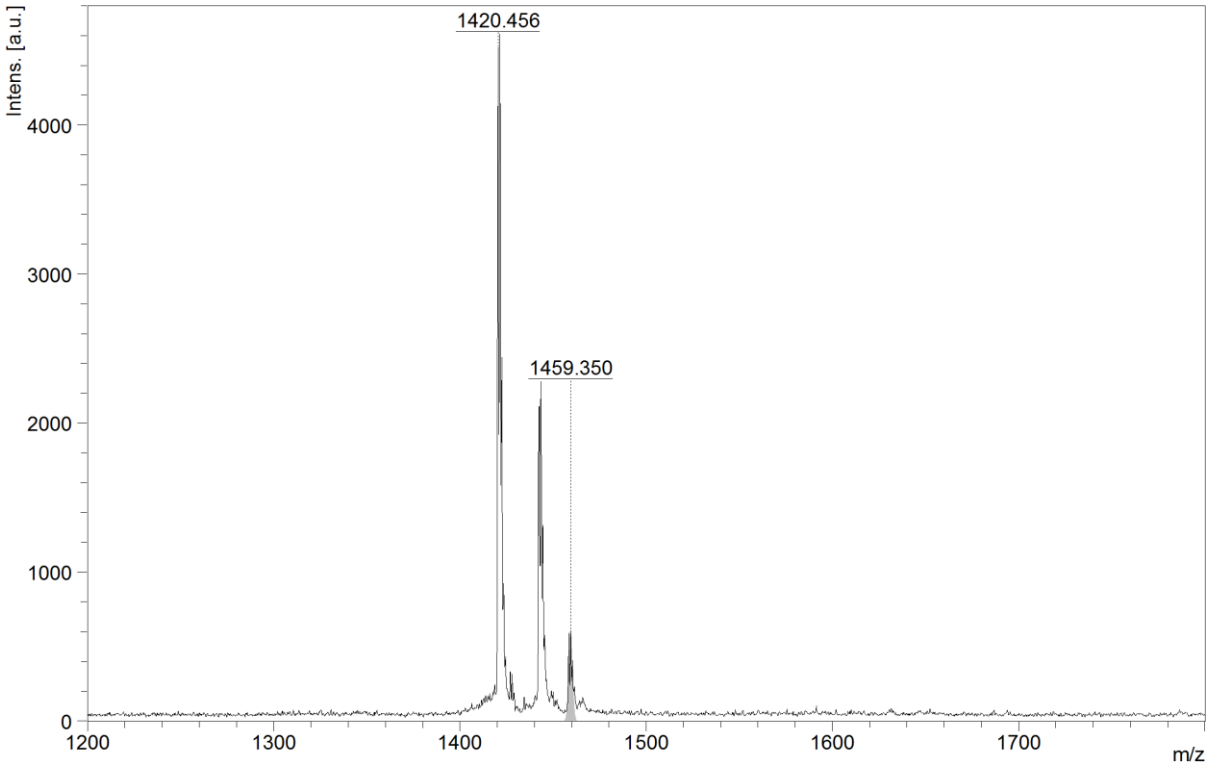
BP205


Intens. [a.u.]
1476.861
500
400
300
200
100
1200
1300
1400
1500
1600
1700
m/z

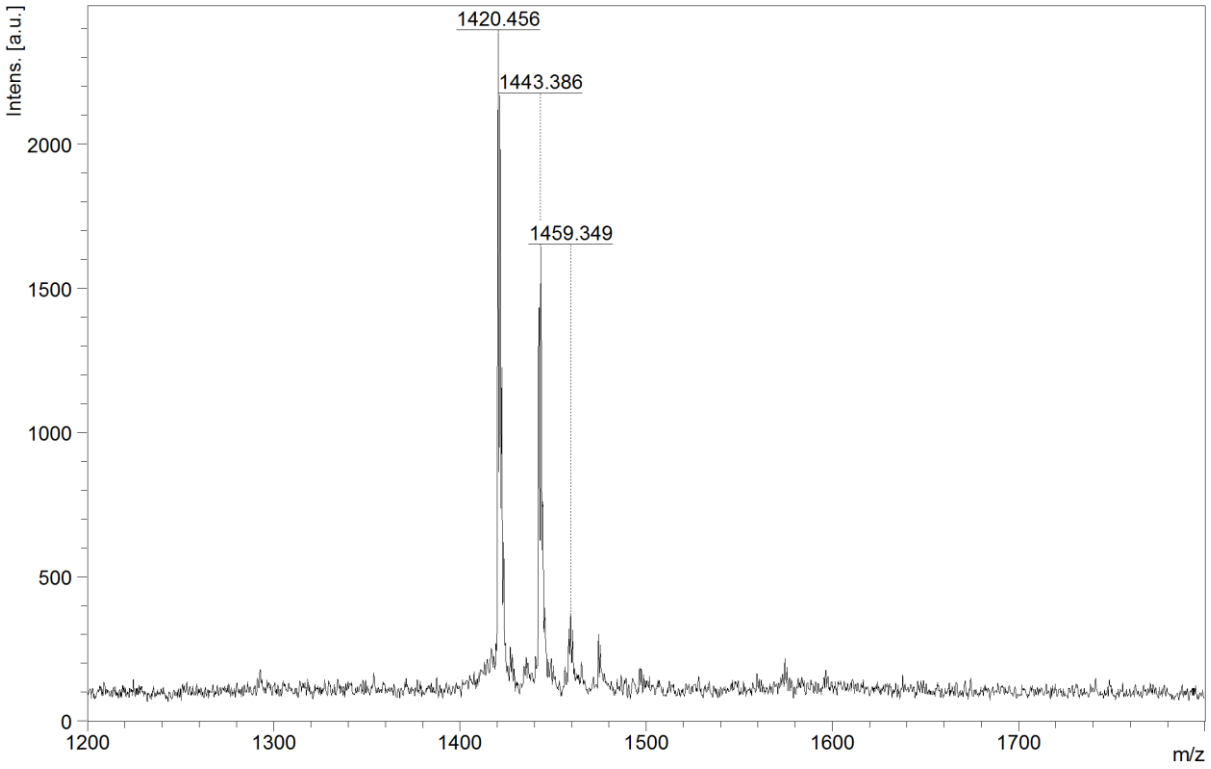
BP206


Intens. [a.u.]
1476.330
1515.528
4000
3000
2000
1000
0
1200
1300
1400
1500
1600
1700
m/z

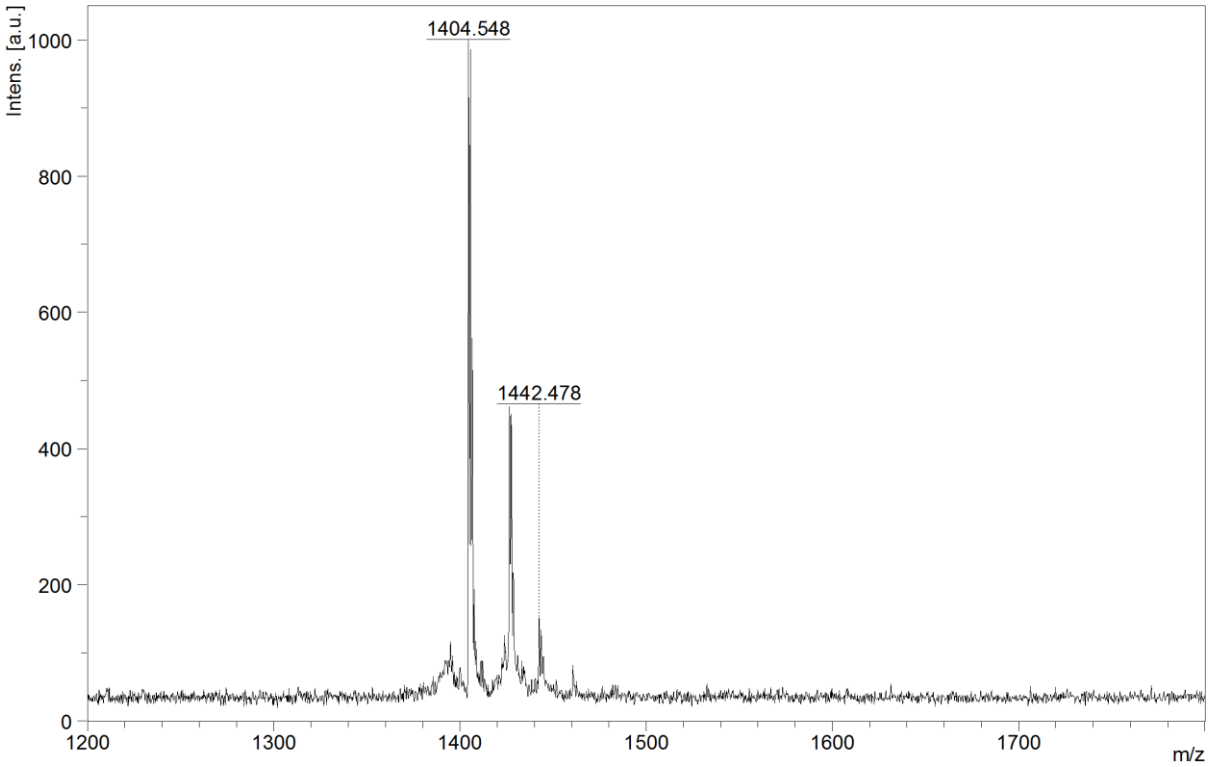
BP207


Intens. [a.u.]
1420.456
1459.350
4000
3000
2000
1000
0
1200
1300
1400
1500
1600
1700
m/z

BP208


Intens. [a.u.]
1420.456
1443.386
1459.349
2000
1500
1000
500
0
1200
1300
1400
1500
1600
1700
m/z

BP209


Intens. [a.u.]
1404.548
1442.478
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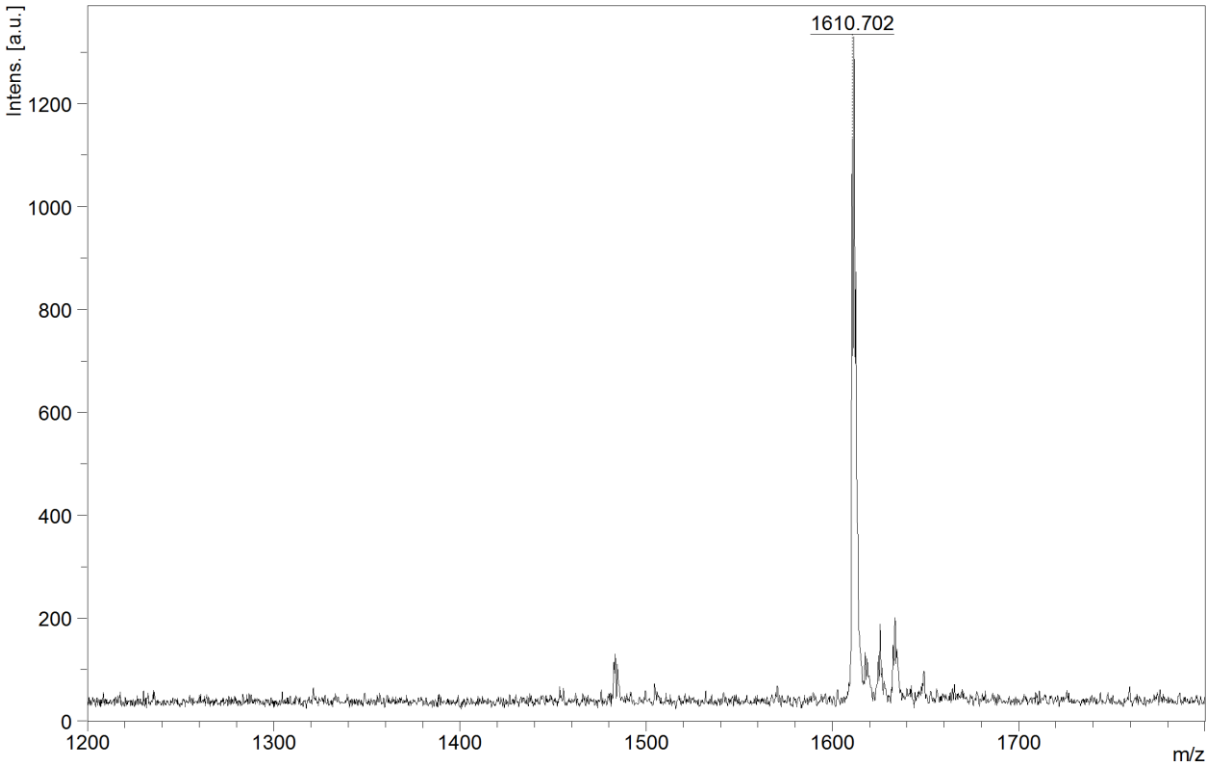
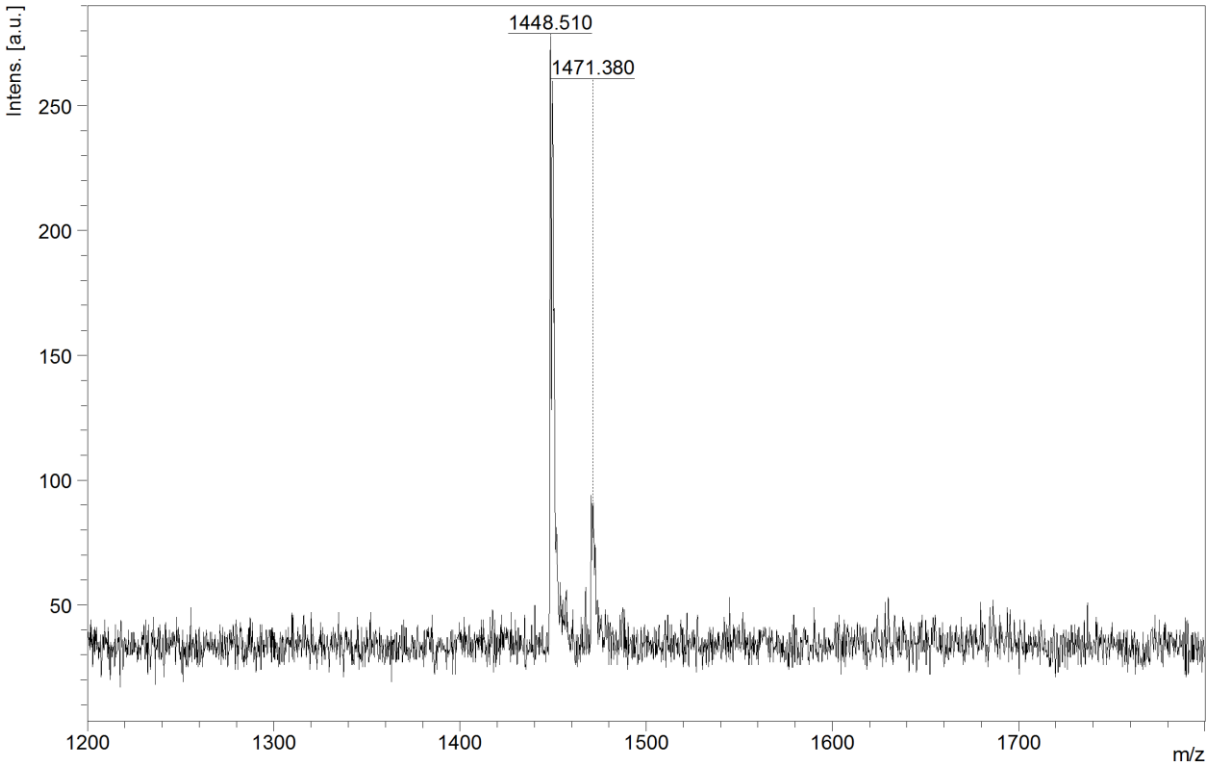
800
600
400
200
0
1200
1300
1400
1500
1600
1700
m/z

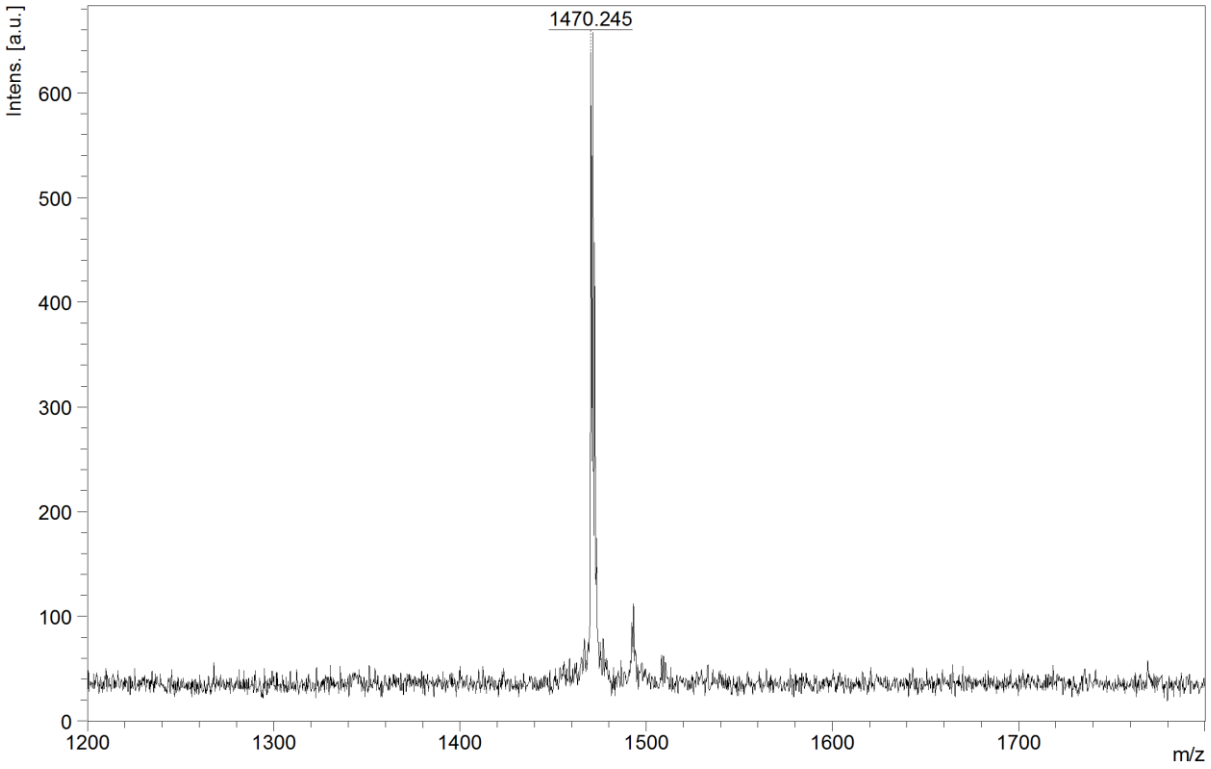
BP210


Intens. [a.u.]
1610.702
1200
1000
800
600
400
200
0
1200
1300
1400
1500
1600
1700
m/z

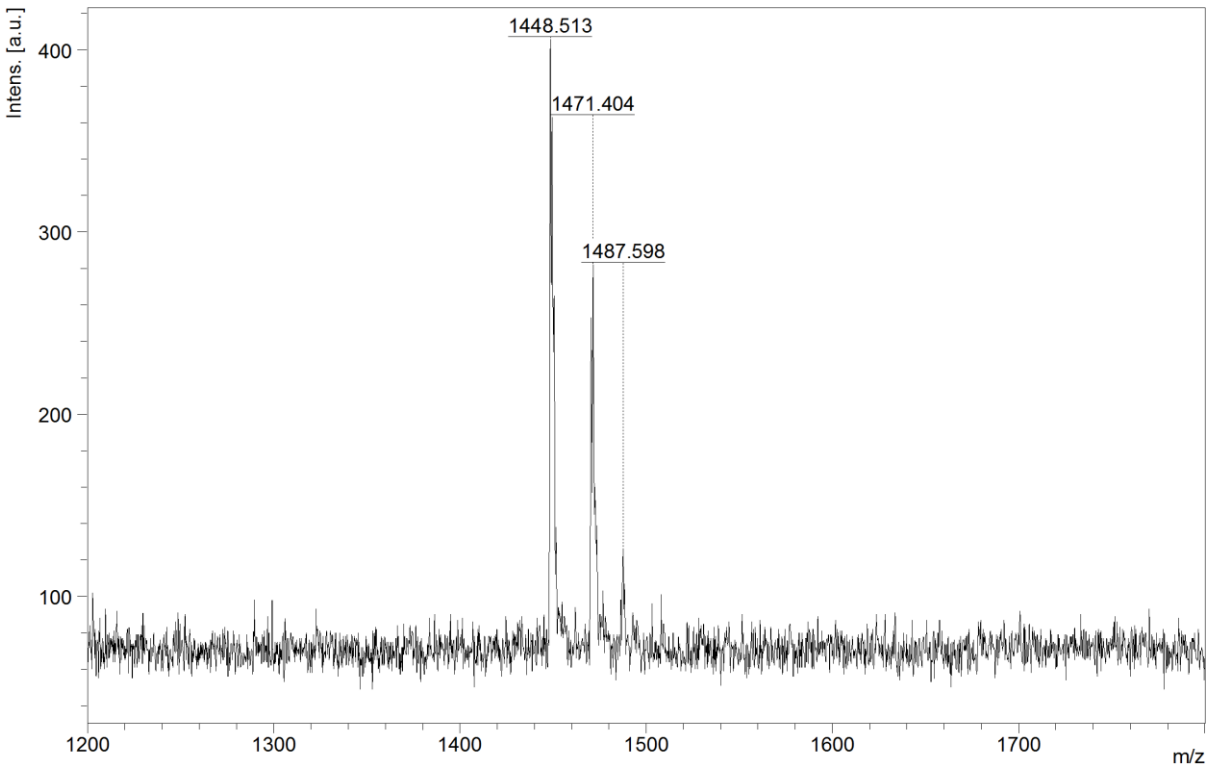
BP211


Intens. [a.u.]
1448.510
1471.380
250
200
150
100
50
1200
1300
1400
1500
1600
1700
m/z

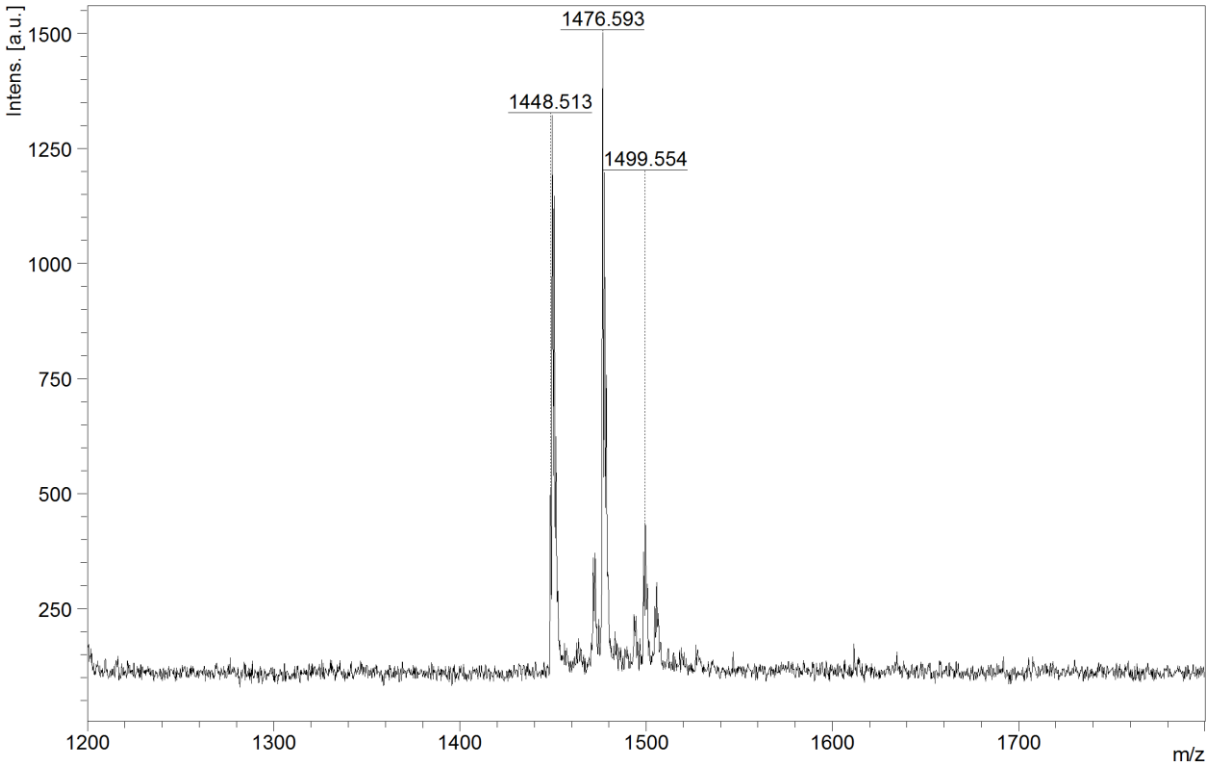
BP212


1470.245
Intens. [a.u.]
600
500
400
300
200
100
0
1200
1300
1400
1500
1600
1700
m/z

BP213


1448.513
1471.404
1487.598
Intens. [a.u.]
400
300
200
100
1200
1300
1400
1500
1600
1700
m/z

BP214


Intens. [a.u.]
1500
1250
1000
750
500
250
1476.593
1448.513
1499.554
1200
1300
1400
1500
1600
1700
m/z

Haemolysis Assays

The hemolytic process took place in a V-shaped polypropylene 96-well plate (NUNC Cat# 249946). Each plate accommodated 4 peptides to be tested in triplicate.

Preparation

Melittin precoating. The night before the test, 150 µl of melittin 5 µM in water were added to the positive control wells. The day of the test, the solution was discarded and the wells were washed 3 times with the same volume of PBS. Then 75 µl of a freshly-prepared 2.50 µM melittin solution in PBS were transferred to the same wells.

RBC suspension. Freshly-drawn human whole blood (type 0^+, 1 ml) in EDTA was mixed gently with 3 ml of PBS and then centrifuged. The supernatant was discarded and the washing procedure was repeated twice. A 0.5 % v/v RBC suspension was prepared by mixing 40 µl of RBC pellets with 8 ml of PBS.

Peptides: samples were dissolved in 500 µl of PBS.

Test procedure

150 µl of peptide solution were transferred to wells $A_{1\text{-}12}$ as follows:

- **Peptide 1:** $A_{1\text{-}3}$
- **Peptide 2:** $A_{4\text{-}6}$
- **Peptide 3:** $A_{7\text{-}9}$
- **Peptide 4:** $A_{10\text{-}12}$

75 µl of PBS were transferred to all the wells from B_1 to G_{12}, and to $H_{7\text{-}12}$. A dilution series was obtained by transferring 75 µl from row A to row B, mixing, then transferring 75 µl from row B to row C, etc.; 75 µl were discarded from row G after mixing.

75 µl of RBC suspension were added to each well and mixed with the peptide solution. The plate was covered with a Biorad Microseal film (Cat# MSF1001) and incubated at 37 ℃ for 1h. After such time the plate was centrifuged in order to precipitate the RBC, then 60 µl of supernatant from each well were transferred into a clear polystyrene flat-bottomed 96-well ELISA plate (Corning Costar Cat# 3370).

Absorbance at λ = 414 nm was determined with a Molecular Devices VersaMax spectrophotometer.

Interpretation

Results were normalized with respect of the averaged positive (100%) and negative (0%) controls. The formula applied is:

$$\%Hemolysis = \frac{Abs(sample) - Abs(NegAvg)}{Abs(PosAvg) - Abs(sample)} * 100$$

Abs(sample) = absorbance of the sample;
Abs(NegAvg) = averaged absorbance of the negative controls;
Abs(PosAvg) = averaged absorbance of the positive controls.

Finally, the three results for each peptide concentration were averaged. The EC_{50} was determined by plotting peptide concentration against hemolysis percentage and then reading the interception of the curve with the y-axis value for hemolysis = 50%. A noise level of ~4% was observed; for such reason hemolysis values inferior to twice the noise level are reported as "<8%".