

PEPCY PRACTICAL GUIDELINES

TITLE: Extraction and purification of anabaenopeptin-B.

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1. PURPOSE

To describe the large-scale extraction and purification of anabaenopeptin-B (ABPN-B) from cyanobacterial cells.

2. INTRODUCTION

Anabaenopeptins (ABPNs) are produced by many cyanobacterial genera including *Anabaena* and *Planktothrix*¹. Cyanobacterial biomass containing ABPN-B can be lyophilised and the ABPN-B extracted before purification by HPLC.

3. REQUIREMENTS

Materials

- ABPN-B containing cyanobacterial biomass (e.g. strain *Planktothrix* 2)
- Purified water (e.g. 18M Ω Millipore MilliQ or equivalent)
- HPLC grade: Acetonitrile, ACN (e.g. Rathburn)
 - Trifluoroacetic acid, TFA (e.g. Fisher)
 - Methanol (e.g. Rathburn)
- Analytical grade acetic acid
- Glass fibre filter disks (e.g. Whatman GF/C 47 to 70 mm diameter)
- Large C18 SPE cartridge (e.g. Phenomenex Strata 20 g Giga tube)
- C18 HPLC column (e.g. Phenomenex Luna 5 μ C18 (2), 250 x 10 mm)
- C18 SPE cartridges (e.g. Phenomenex Strata 1g)

Equipment

- Freezer (-20°C)
- Freeze-drier unit (e.g. Edwards Super Modulyo)
- Centrifuge and rotor with vessels to hold > 1L
- Magnetic stirrer
- Filtration apparatus and vacuum line
- Solid phase extraction (SPE) vacuum manifold with chamber and fittings (e.g. Phenomenex)
- Rotary evaporator
- HPLC (dual pump) with detector, associated equipment and fraction collector
- Software for the analysis of HPLC chromatograms

4. PROCEDURE

Solutions

5 % (v/v) aqueous acetic acid
10 % (v/v) aqueous methanol
40 % (v/v) aqueous methanol
50 % (v/v) aqueous methanol
MilliQ water + 0.1 % (v/v) TFA (HPLC solvent A)
ACN + 0.1 % (v/v) TFA (HPLC solvent B)

Sample preparation

Wet cyanobacterial biomass should be concentrated (e.g. filtration or centrifugation) lyophilised and the dry cells stored at -20°C.

Extraction

Dried cyanobacterial biomass (10 – 20 g dry weight) should be weighed into suitable vessels, 5 % acetic acid added (100 ml g⁻¹) and stirred vigorously for 30 min.

Centrifuge extracts (e.g. 30 min at 3000 rpm) and decant the supernatant into a separate container.

Re-extract the pellets a further twice with 5 % acetic acid as before, and pool all collected supernatants.

GF/C filter supernatants twice.

Connect large C18 cartridge to vacuum manifold and prime according to manufacturer's instructions.

Pass filtered extract through cartridge and keep waste.

Wash cartridge with MilliQ water and elute sequentially with volumes (according to manufacturer's instructions) of 10 %, 40 %, 50 % and 100 % methanol.

Analyse fractions for the presence of ABPN-B (PPG_UnivDun_001).

Pool those fractions containing ABPN-B, rotary evaporate in *vacuo* to remove methanol and lyophilise.

Purification

The HPLC solvents should be degassed, the system primed, the column oven set to 40°C and correct column with pre-column fitted all according to manufacturer's instructions.

Reconstitute the crude extract in 50 % methanol to give a concentration of 3 mg ml⁻¹ ABPN-B and clarify (e.g. centrifugation).

Set the detector to monitor chromatograms at 225nm.

Starting with 100 % HPLC solvent B gradually (over 30 to 45 min) increase the proportion of HPLC solvent A to reach starting conditions (below).

Elute an injection volume of 25 to 150 µl from the column using the following gradient:

Time	% HPLC solvent A	% HPLC solvent B
0 (starting conditions)	80	20
20	76	24
21	0	100
22	0	100
23	80	20
30	80	20

Note retention time of ABPN-B peak (refer to standard if necessary).
Set fraction collector to collect ABPN-B peak and other peaks of interest.
Collect fractions from repeated injections and dilute with MilliQ water so ACN < 10 %.
Prime C18 1g SPE cartridges (1 per fraction) according to manufacturer's instructions and concentrate each HPLC fraction.
Elute each cartridge with 100 % methanol and rotary evaporate to dryness *in vacuo*.
Check fraction purity and determine yield (PPG_UnivDun_001). Re-purify if necessary.

5. REFERENCE

1. Harada, K. (2004) Production of secondary metabolites by freshwater cyanobacteria. *Chem. Pharm. Bull.* **52**, 889 - 899