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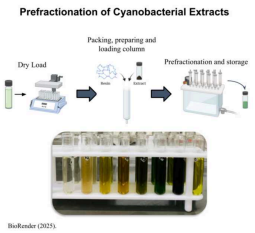
Prefractionating Cyanobacterial Extracts by Solid-Phase Extraction



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We use this protocol and it's working

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Abstract

This protocol presents a prefractionation method for cyanobacterial organic extracts by solid-phase extraction, using a isopropanol-water step gradient and HP20SS as stationary phase. Extract prefractionation allows for improved results in bioassays and metabolomic analyses.

Materials

Solvents and solutions:

- Dichloromethane;
- Methanol (100%);
- HPLC-grade water;
- Acetone;
- Ethyl Acetate;
- Isopropanol (IPA):water solutions (20%, 40%, 70%, 90%, and 100% IPA).

Resin:

- DIAION^{TM} HP20SS.

Equipment:

- SPE column with frits;
- Manifold and vaccum pump;
- 20 mL test tubes;
- Ultrasonic bath;
- 4 mL vials;
- N_2 flow (eqquiped with glass microfiber polypropylene filter, 0.3 µm);
- Vacuum concentrator (optional).

Abstract

1 Cyanobacteria are producers of chemically diverse secondary metabolites with pharmaceutical potential (Weiss *et al.*, 2025). The creation of extract and fraction libraries help systematize the process of natural product discovery (Wilson *et al.*, 2020). Most biological extracts do not allow for an efficient assessment of the bioactivity, often resulting in false positive or false negative results (Wagenaar, 2008). Prefractionation is a widely used method for separating mixtures aiming to reduce the chemical complexity of organic extracts (Stobaugh; Fague; Jorgenson, 2013). Separating extracts into a few prefractions increases the concentration of bioactive metabolites in the sample and allows for improved results in bioassays and metabolomic analyses (Clish, 2015; Rague *et al.*, 2018; Tu; Yan, 2012).

2 This protocol aims to present a prefractionation method for cyanobacterial organic extracts, using a isopropanol-water step gradient and HP20SS as the stationary phase. It was adapted from Bugni *et al.*, 2008 and Orjala *et al.*, 2011.

Materials and Equipment

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- 4 mL vials with caps;
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- Vacuum concentrator (optional).

Dry load preparation

4 Weigh the dry organic extract. A dry extract is shown in **Figure 1.** In a vial, dissolve the dry organic extract previously prepared from a dichloromethane-methanol 1:1 solution in the smallest possible amount of the same solvent. Use the ultrasonic bath, if needed.

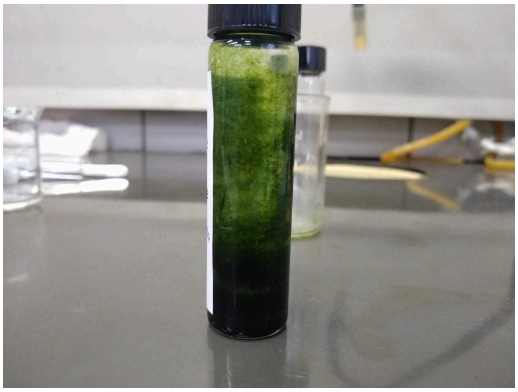

Figure 1: Cyanobacterial extract. Source: Authors (2023).

5 Add to the vial the same amount of chromatographic resin (HP20SS) as the initial dry extract. Allow the mixture to dry under a nitrogen (N_2) flow. Ensure that the flow is correctly regulated, as the resin is very powdery and can easily spread.

Prefractionation procedure

6 Packing the solid-phase extraction column:

6.1 - Place one of the frits at the bottom of the SPE column. Since new columns usually contain 2 frits at the bottom, remove one of them;

6.2 ▪ Add the HP20SS resin. Tap it a few times to ensure the resin is more evenly arranged on the frit. The amount of resin should be calculated according to the amount of dry extract. A minimum of 1 g of resin should be packed. In case >0,2 mg of dry extract, add 5-7 times the weight of the initial dry extract;

6.3 ▪ In this step, the resin tends to stick to the walls of the column. To avoid it, prepare a funnel with weighing paper, fold the bottom, and secure it with tape, so that it becomes a small cup. It must fit inside the SPE column. Weigh the HP20SS resin using the adapted cup and then fit it inside the column, upside down, as deep as possible (holding it with tweezers may help). Turning it over, releasing the resin directly at the bottom of the column;

6.4 ▪ Place the second frit. The frit should always be placed horizontally inside the column, trying to push any particles from the walls of the SPE column to its bottom, ensuring that the resin layer is as homogeneous as possible. Using a glass tube and pushing it from the edges, very slowly, helps with this process.

7 Lable the 20-mL test tubes and add them to rack inside the vacuum manifold.

8 Connect the packed SPE column to the manifold and wash it with 10 mL of ethyl acetate and 10 mL of IPA.

9 Equilibrate the packed column with 10 mL of water for HPLC (2x).

10 Add the dry load to the SPE column. Suggestion: use the weighing paper cup, following the same procedure described in step 6.3.

11 Place a third frit on top of the dry load with the same care described in step 6.4. The vacuum manifold and packed SPE column are shown in Figure 2.

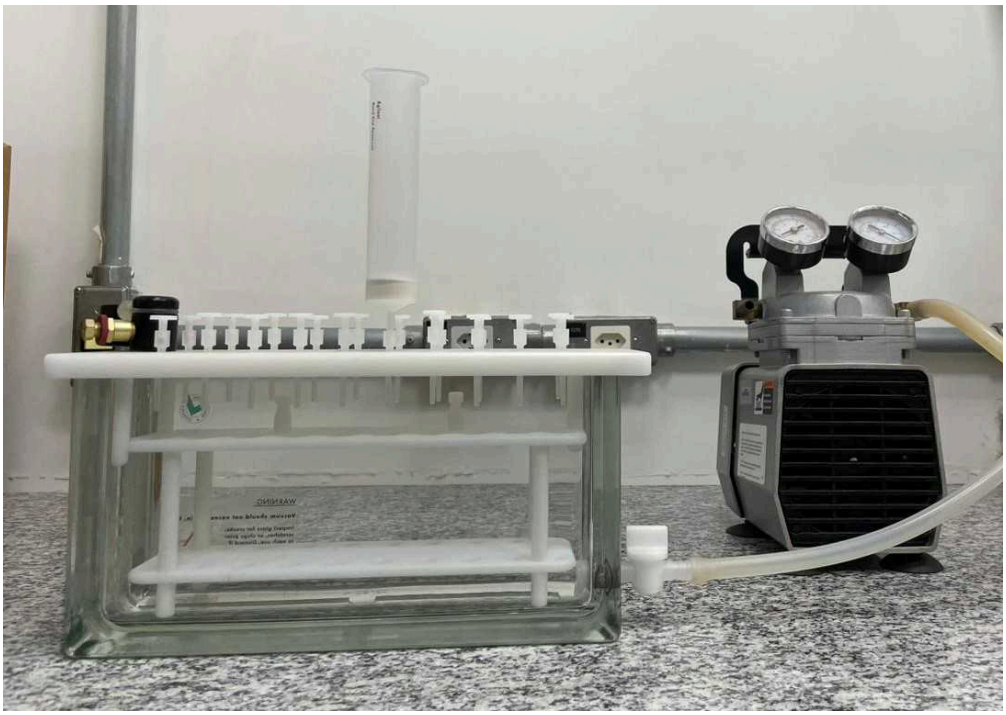

Figure 2: Vacuum manifold with a packed SPE column. Source: Authors (2024).

12 Use the IPA solutions to prefractionate the extract using the step-gradient shown in **Table 1**. The total volume of 20 mL per fraction should be distributed in 2 test tubes (2×10mL).

Table 1: Isopropanol (IPA) solutions for the fractionation method.

Fractions	IPA (%)	Volume (mL)
1	0*	20,0
2	20	20,0
3	40	20,0
4	70	20,0
5	90	20,0
6	100	20,0
Wash (7 and 8)	-	-

* HPLC-grade water 100%
Source: Authors (2023).

13 After the 6th fraction (F6), wash the column with 2×10 mL 100% ethyl acetate (7th fraction) followed by 2×10 mL of 100% acetone (8th fraction). An example of fractions obtained by this procedure is demonstrated in **Figure 3**.

Figure 3: Prefractionated cyanobacterial extract. Source: Authors (2023).

13.1 - Wash fractions usually contain a significant amount of chlorophyll and fatty acids. However, it is recommended to store them for potential future investigations, for example in cases where the extract displays biological activity, which is not detected in F1-F6.

14 Dry the fractions under N_2 flow or using a vacuum concentrator.

Resuspending fractions and calculating yields

15 Resuspend the dry fractions with a small volume of HPLC-grade methanol or the solvent solution used for producing the fraction. Use the ultrasonic bath.

16 Transfer the fractions from the tubes to labeled and pre-weighed 4 mL vials, as shown in **Figure 4.** Vials must be weighed without the caps.

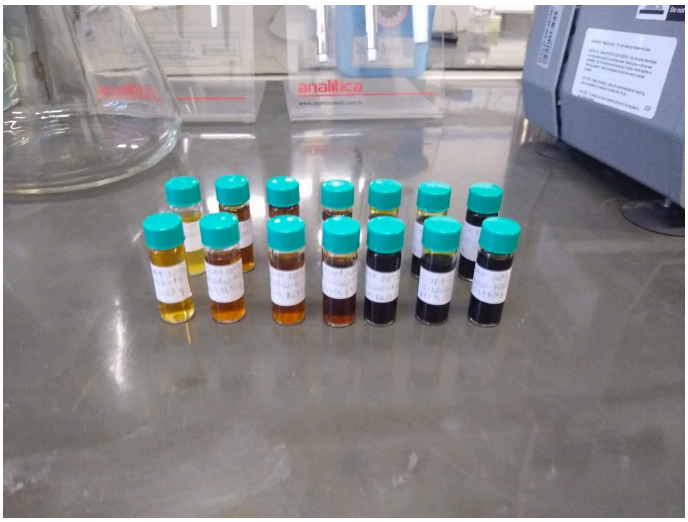

Figure 4: Fractions in 4 mL vials. Source: Authors (2023).

16.1 ▪ Fractions 7 and 8 (wash) may be stored in the same 4 mL vial.

17 Dry the fractions under N_2 flow or using a vacuum concentrator.

18 Weigh the fraction vials (without caps) and calculate the prefractionation yield (%): sum of fraction mass divided by the mass of the initial extract.

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