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E-SPE : Explorative Solid-Phase-Extraction for Accelerated Natural Product Discovery and Purification

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Background

Many NP labs have developed internal strategies for standardised extract screening and purification, only publishing parts of the overall setup[1,2]. Most modern NP purification methods are based on reverse-phase (RP) due to its versatility[3,4,5]. However facing complex extracts with many components, a simple RP strategy can lead to poor recoveries. This is especially a problem when dealing with low-output extracts, e.g. from the marine environment[6]. For these types of extracts, orthogonal purification strategies are requisite[7].

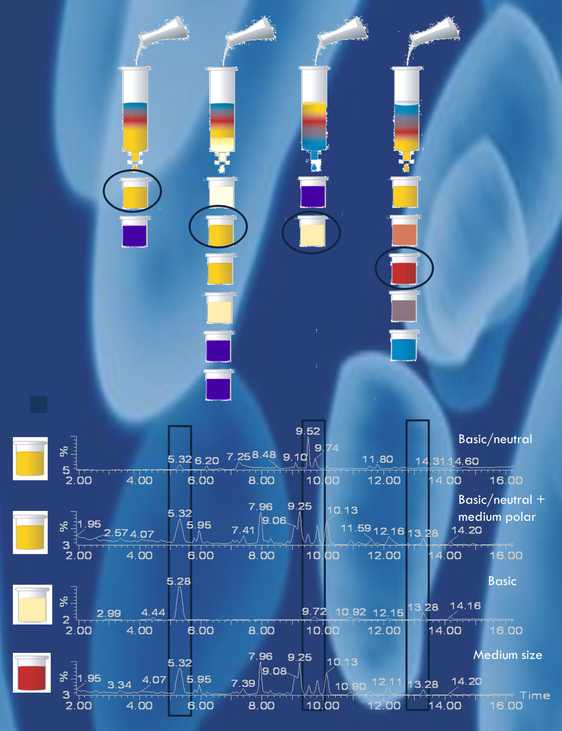
So, we have developed a so-called Explorative Solid-Phase Extraction (E-SPE) method, which in a fast and easy way will indicate the optimum purification strategy on a small scale in the exploratory stage of the discovery process. This will allow a more rational approach to the purification process.

Method setup

To obtain maximum complementarity, four different columns were selected. Together they provide information on size, charge and polarity of the active components needed to develop a purification strategy.

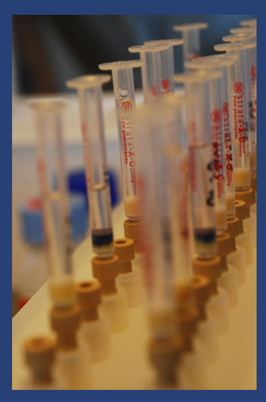
- Strong anion-exchanger (SAX):
  Presence of carboxylic acids
- Mixed-mode (RP) anion-exchanger (MAX):
  Presence of acids, enols and phenols
  Relative polarity (25, 60, 100% AcCN)
- Strong cation-exchanger (SCX):
  Presence of basic amines
- LH-20 for size-exclusion:
  Relative size

A total of 15 fractions for bioassay is generated from each extract (1 agar plate or 50 mL of liquid culture).



Each of the active fractions are subjected to comparative dereplication by LC-HRMS. The MS chromatograms for all active fractions are compared, and the MS spectra are extracted for all peaks in common. The accurate mass is then used as query in a database search (AntiBase, AntiMarin or similar). This provides a full list of potential candidates as well as their likelihood to be novel.

Method validation

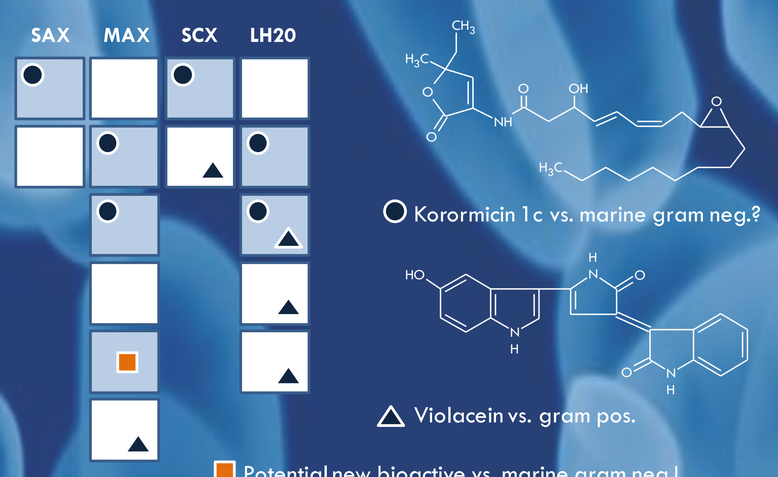


The E-SPE method was tested on 25 different marine bacteria with antibacterial activities such as growth and quorum sensing inhibition. A few well-researched filamentous fungi were included as further validation. Each organism was tested in triplicates to ensure reproducibility.

The method was found to be widely applicable on both bacterial and fungal samples, however very dependent on the matrix. Especially for the marine samples, extensive desalting was necessary to ensure reproducibility. Further tests are necessary to investigate the robustness of the method as well.

Example - Pseudoalteromonas luteoviolaceae:

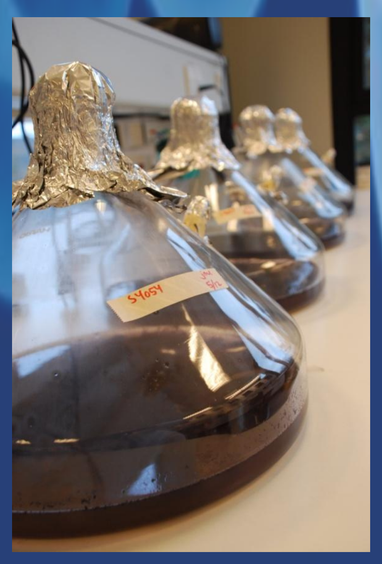
A marine bacterium representing a highly complex extract with multiple known antibacterials. Nonetheless, the E-SPE bioactivity profile against *Vibrio anguillarum* revealed the presence of a potential novel bioactive present in a fraction with no known bioactives. E-SPE also provided the information that this compound is medium polar with an acidic functionality other than COOH.



Above: E-SPE bioactivity profile for Ps. luteoviolaceae showing the distribution of active known and unknown compounds against gram-negative Vibrio aguillarum in a well-diffusion assay. Bioactivity profile shown as elution matrix from Cardellina et al. [1].

Method implementation

As a *proof-of-concept*, a purification strategy was developed based on the E-SPE activity profile for *Ps. luteoviolaceae* and carried out on extract from 8 L of culture.



Ps. luteoviolaceae

Broth extraction with XAD7/HP20

Unretained acidics and neutrals

Retained basic compounds

Unretained neutrals

Polar acidics

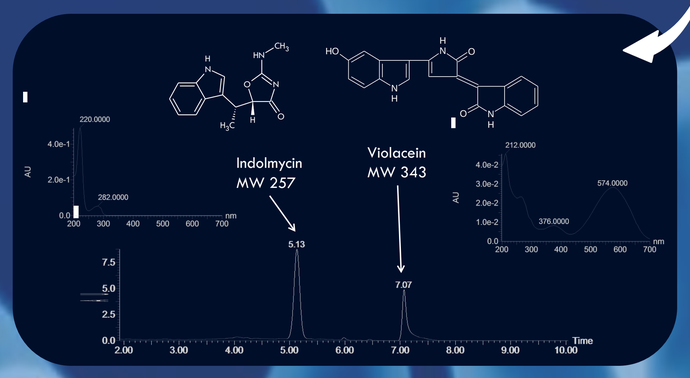
Medium polar acidics

Apolar acidics

Size-exclusion by LH-20:
Indolmycin
Violacein
Red pigment
Other yellow

C-18 separation:
Korormycin?
Total of 11 compounds awaiting final NMR confirmation

C-18 separation:
3-hydroxybutyric acid polymers
10 other compounds awaiting final NMR confirmation (incl. potential novel active phenol/enol)



Above: UV chromatogram of retained fraction from cation-exchange, further separated by LH-20 to give pure indolmycin and violacein (ID confirmed by CapNMR) as well as two minor, unknown pigments.

Each of the purified compounds were present in sub-milligram quantities and were therefore subjected to CapNMR for increased sensitivity. Besides a series of 3-butyric acid oligomers, the known antibacterial compound indolmycin was identified for the first time in a non-*Streptomyces* strain. We are currently awaiting final NMR confirmation of a series of unknown compounds as well as the rest of the active components.

Conclusions

E-SPE is a successful strategy to reveal novel, bioactive natural products from both fungal and bacterial sources.

The advantages of E-SPE are many:

- Finding a purification strategy on small amounts before engaging semi-preparative purification
- Identifying possible pit-falls (stability, synergy etc.)
- Reducing false positives (media components, salt etc.)
- Reducing number of candidates for dereplication (at CMB by LC-UV-HRMS)
- Unmasking potential candidates
- Enables target-guided isolation rather than bio-guided fractionation
- Standardising the analysis of samples (SOP)

Results obtained on a small scale (a single agar plate or 50 mL of culture) can readily be translated into bigger scale (200 plates, 10 L culture) for preparative results. The E-SPE strategy has proven it-self to be fast, easy and reproducible in use and has therefore been implemented as a standard screening procedure at CMB when dealing with new extracts.

References

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