Explorative Solid-Phase Extraction (E-SPE) for Accelerated Microbial Natural Product Discovery, Dereplication, and Purification

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Microbial natural products (NP) cover a high chemical diversity, and in consequence extracts from microorganisms are often complex to analyze and purify. A distribution analysis of calculated pK_a values from the 34390 records in Antibase2008 revealed that within pH 2−11, 44% of all included compounds had an acidic functionality, 17% a basic functionality, and 9% both. This showed a great potential for using ion-exchange chromatography as an integral part of the separation procedure, orthogonal to the classic reversed-phase strategy. Thus, we investigated the use of an "explorative solid-phase extraction" (E-SPE) protocol using SAX, Oasis MAX, SCX, and LH-20 columns for targeted exploitation of chemical functionalities. E-SPE provides a minimum of fractions (15) for chemical and biological analyses and implicates development into a preparative scale methodology. Overall, this allows fast extract prioritization, easier dereplication, mapping of biological activities, and formulation of a purification strategy.

For the purification of natural products, specific chemical information on the target compounds prior to purification is vital for the success of the purification.[1,2] If no prior knowledge about the target compounds is available, a purification strategy is normally developed through trial and error or by standard fractionation procedures and often guided by one or more bioassays. Many natural product (NP) laboratories have developed internal strategies for standardized extract screening and purification,[3−5] while in other cases, only parts of the overall setup have been published as the techniques have probably evolved over many years and the rationale behind them has not been disclosed.[6]

For the most part, modern NP purification methods are based on reverse-phase (RP)[4,7] separations due to high capacity, recovery, reproducibility, and chromatographic resolution compared to most separation methods.[2] However, when faced with complex extracts containing many components, a purification strategy based solely on RP can lead to problems. This may involve the purity of the end products, the overall recovery from the extract, or at worst, permanent loss of activity due to instability or degradation. This is especially a problem with low yielding extracts typical of those arising from marine microorganisms.[8] For this type of extract, orthogonal purification strategies are required; classically, this can be achieved by combining RP with normal-phase chromatography on silica gel (low cost)[2] or alternatively using bonded phases such as polyethyleneimine[9,10] or diol.[11,12] Ion-exchange has been used less frequently[13−16] even though many natural products contain ionizable groups.

In contrast to preparative chemistry, matrix-dependent orthogonal solid phase extraction (SPE) purification strategies are widely used in trace analysis to complement the almost universal analytical RP-LC methods used.[17] This is essential to effectively remove coeluting interferences as well as the major constituents of the matrix. Mixed-mode sorbents,[18,19] multifunctional columns,[20] and especially ion-exchange SPE[21,22] are widely applied to ensure high and consistent recoveries.

Targeted exploitation of chemical functionalities in the work with novel compounds has not been used frequently prior to preparative isolations. Only two papers have described methods for the preliminary chemical characterization of NP extracts to be used as part of the development of purification strategies. Samuelsson et al. used Sephadex G25, anion- and cation-exchangers, and a set of solvents for liquid−liquid partitioning to investigate the size, charge, and polarity of the active constituents in aqueous plant extracts,[13] while Cardellina et al. used a combination of Sephadex G25 and RP resins.[23] Both groups used their methods to evaluate bioactive, aqueous extracts of high complexity. In addition, by using a bioactivity elution matrix Cardellina et al. were also able to use the method for dereplication and prioritization of extracts.[23]

In microbial NP extracts, multiple biosynthetic pathways are often represented,[21,24−28] resulting in complex extracts. To deal with this, a standard protocol capable of exploiting the different chemical functionalities is required. Thus, we have developed "explorative solid-phase extraction" (E-SPE) where a set of SPE columns with orthogonal selectivities are used to rapidly explore the optimum purification strategy on a small scale in the exploratory stage of the discovery process, which has the potential to be transferred to a preparative scale. Also, by supplying information on the presence of ionizable functional groups, the analytical (e.g., LC-MS) dereplication of candidates is facilitated. Here, we present the use of E-SPE as a standard procedure that is compatible with a broad series of bioassays and enables the use of a standardized screening method transferable between different organisms and bioactive NP targets. Overall, this allows a rational approach to the purification process that is independent of the experience and intuition of the chemist and integrates the process of extract prioritization, dereplication leading to the mapping of biological activities, and formulation of a purification strategy.

Results and Discussion

To determine the incidence of microbial natural products with charged functionalities, a batch calculation using the Advanced Chemistry Development pK_a suite was conducted on all records in AntiBase 2008,[29] resulting in 34390 valid records. This revealed (Figure 1) that 52% of all compounds reported had an ionizable functionality (within pH range 2−11, see Figure 2). This confirmed the potential for incorporating ion-exchange chromatography as an integral part of the separation procedure. Three different ion-exchangers were included in the overall setup. An approach based on the cation exchange of amines was perceived to be the most discriminatory prospect followed by anion exchangers for carboxylic

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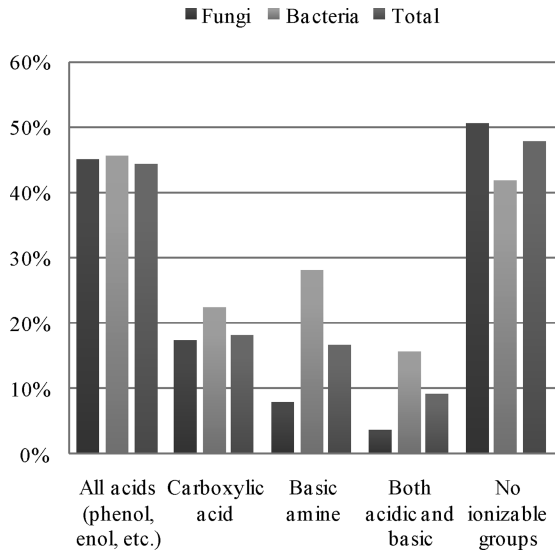


Figure 1. Distribution of microbial natural products in AntiBase 2008 with charged functionalities within pH range 2−11, permanently charged groups such as sulphates, phosphates, and tertiary amines as well as potential tautomeric forms excluded. Statistics are based on theoretical pK_a values calculated using Advanced Chemistry Development pK_a suite.

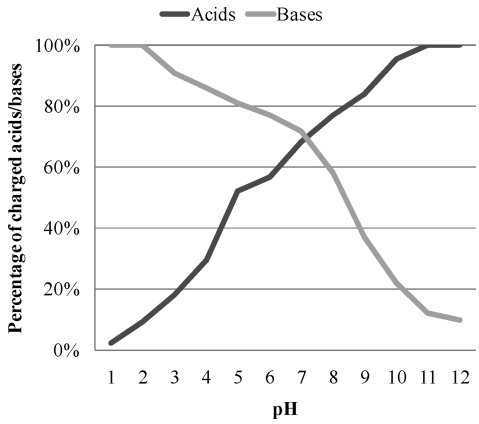


Figure 2. pK_a Cumulative distribution of the ionic form of acids and bases in Antibase 2008 at a given pH value, based on their calculated pK_a values. Theoretical pK_a values calculated using Advanced Chemistry Development pK_a suite. The statistics includes permanently charged groups such as sulphates, phosphates, and tertiary amines but excludes potential tautomeric forms.

acids. Thus, strong anion- and cation-exchangers (SAX and SCX) were included to reveal the presence of carboxylic acids and amines. A mixed-mode polymeric RP anion-exchanger (Oasis MAX) with a poly(divinylbenzene-co-vinylpyrrolidone) backbone was included as it has been shown not only to retain acidic compounds with a carboxylate functionality but also weaker anions of phenols and tautomeric enols; due to the polar polymeric backbone, it can retain these close to the ionic groups.[18,19] On the basis of observations from analysis of fungal metabolites in food and feed samples, it was speculated that enols, phenols, and other compounds with acidic functionalities could be differentiated from carboxylic acids by comparing SAX and MAX elution patterns. In addition, the MAX column could also provide information about the relative polarity of the compounds in the extract, thereby keeping down the number of columns and fractions as an RP column was now not needed. A Sephadex LH-20 column (polymeric cross-linked dextran gel for size-exclusion) was included to give information about the relative size of the compounds. These four orthogonal columns provided a rational compromise to obtain the necessary information on size,

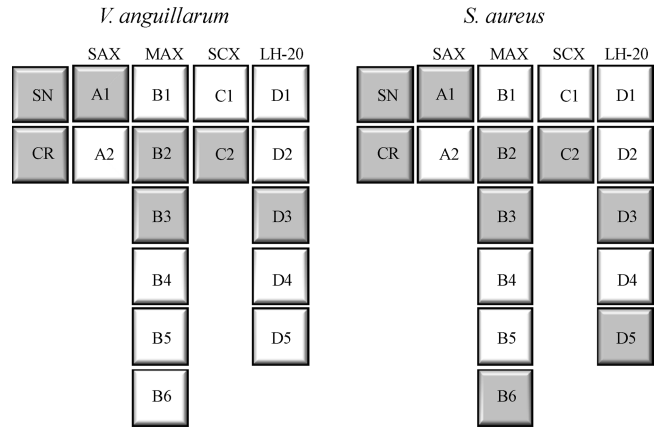


Figure 3. E-SPE profiles of the ethanolic extract of *P. luteoviolacea* obtained in well-diffusion assays with *V. anguillarum* and *S. aureus.* The matrix represents fractions from the four SPE columns as well as their corresponding supernatant (SN) and crude extract (CR). Gray = active; white = no activity observed. SAX fractions: A1 = unretained bases and neutrals, A2 = retained acids. MAX fractions: B1/B2/B3 = polar/medium polar/apolar unretained bases and neutrals, B4/B5/B6 = polar/medium polar/apolar acidics. SCX fractions: C1 = unretained acids and neutrals, C2 = retained bases. LH-20 fractions: D1−D5 = fractions of decreasing molecular size (band-based). Violacein identified in fractions A1, B6, C2, and D5. Potential new bioactive identified in fractions A1, B2, B3, C2, and D3.

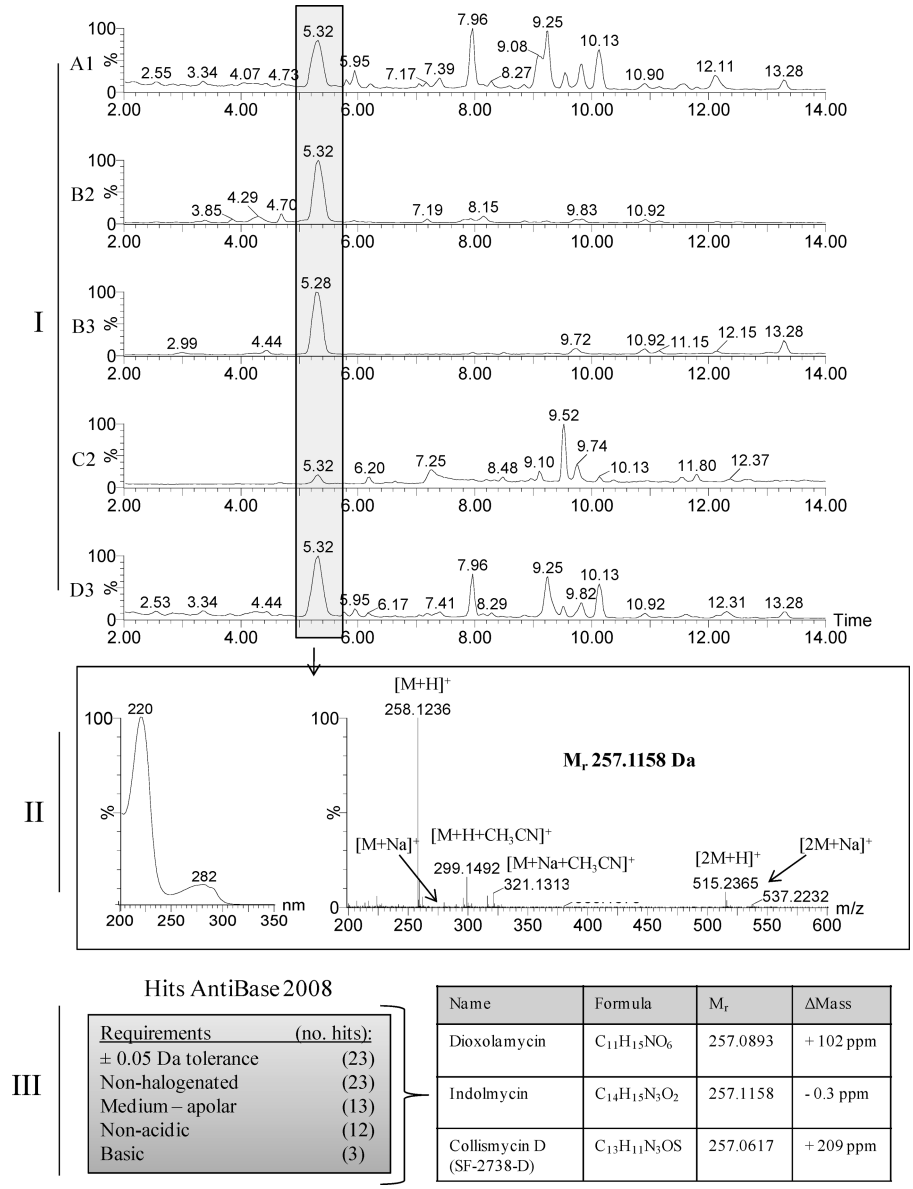
charge, and polarity of the extract components while still generating the minimal number of fractions.

Beause the goal was to develop E-SPE as a standard operating procedure when dealing with new microbial extracts, the procedure was tested on both fungal and bacterial extracts, including extracts of marine bacterial origin. Plant extracts were not included, as an existing range of standardized prefractionation or partitioning steps are established and targeted to separate the major compound classes present in plants such as chlorophylls, polyphenolics, tannins, and saponins.[30−32]

Typically 0.5−2 mg dry extract aliquots were applied to each column. The specific extraction procedure varied with the type of organism and matrix. For example, media with high salt content should be freeze-dried and redissolved in EtOH prior to ion-exchange,[33] as the ions from the media will impair the binding (salting-out) of possible target compounds on the SAX and SCX columns.

A total of 15 fractions were generated from each extract and submitted for biological testing with part of the crude extract/culture supernatant and recombined fractions from each of the columns to reveal potential instability issues or synergy effects. To track false positives, a blank medium sample was subjected to the same extraction and fractionation procedures as the cultures.

The assay results were organized in a bioactivity matrix similar to that used by Cardellina et al.[23] (Figure 3). Visual interpretation of this matrix made it possible to map the active components by comparing the results from different assays. Active and nonactive fractions were subjected to comparative dereplication by LC-HRMS (Figure 4) by cross-referencing MS and UV peaks between fractions. Peaks only appearing in active fractions and not in nonactive fractions are potential candidates for the observed bioactivity. Afterward, the corresponding MS and UV spectra were extracted, adduct patterns established, and the accurate mass data determined using reported analyses.[34] The accurate mass was used as query in a database search (AntiBase, AntiMarin, or similar). The resulting candidates were assessed based on their: (i) mass accuracy and isotope pattern, (ii) match between the acquired UV spectrum and the reported UV data, (iii) observed retention time compared to structure, LogD, molecular size etc., (iv) taxonomic

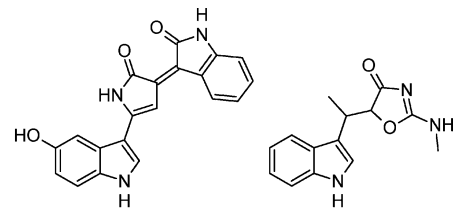


Name	Formula	M_r	ΔMass
Dioxolamycin	$C_{11}H_{15}NO_6$	257.0893	+ 102 ppm
Indolmycin	$C_{14}H_{15}N_3O_2$	257.1158	- 0.3 ppm
Collismycin D (SF-2738-D)	$C_{13}H_{11}N_3OS$	257.0617	+ 209 ppm

Figure 4. Comparative dereplication of E-SPE fractions from *P. luteoviolacea* active against *V. anguillarum*. (I) LC-MS total ion chromatograms (ESI^+) of active fractions A1, B2, B3, C2, and D3. (II) Extracted UV and ESI^+ MS spectra from common peak with retention time RT = 5.32 min, providing an accurate mass (monoisotopic) M_m of 257.1158 Da. (III) Candidates from AntiBase 2008 that satisfy all functional criteria elucidated by E-SPE. Total number of hits noted in parentheses.

data, (v) match between charged functionalities and ion-exchange properties, and (vi) ESI^- and ESI^+ adduct patterns and the strongest ionization mode. The final list of possible targets indicated the likelihood of the candidates being novel structures. When the potential targets were identified, it was possible to develop a suitable procedure for purification, preferably based on selective ion-exchange properties. As final validation before scaling up, the chosen optimized combination of columns was tested in series while following the concentration of bioactivity.

E-SPE has been introduced as a standard screening procedure in our lab for the evaluation of new extracts. Many extracts have been screened, and herein we present two cases, one bacterial and one fungal, which represent some of the experience we have gained through using this protocol. To validate our method, we applied E-SPE with an optimized fractionation protocol to an extract of the marine bacterium *Pseudoalteromonas luteoviolacea* grown in a rich medium containing 3% sea salts. *P. luteoviolacea* is a known producer of several antibacterial compounds.[35−41] Under the conditions used, it generated a highly complex extract which was further complicated by high levels of leftover media components. This made full dereplication on the data from the crude extract a difficult task. The ethanolic extract was dominated by the purple pigment, violacein, which has a broad range of antibacterial activities, especially on Gram positive bacteria.[36,42,43] E-SPE bioactivity profiles (Figure 3) were obtained for inhibitory activity against the Gram-negative bacterium *Vibrio anguillarum* and the Gram-positive bacterium *Staphyloccocus aureus.*



Violacein Indolmycin

Comparison of the three profiles showed the presence of at least two bioactive compounds in the extract. As expected, violacein was

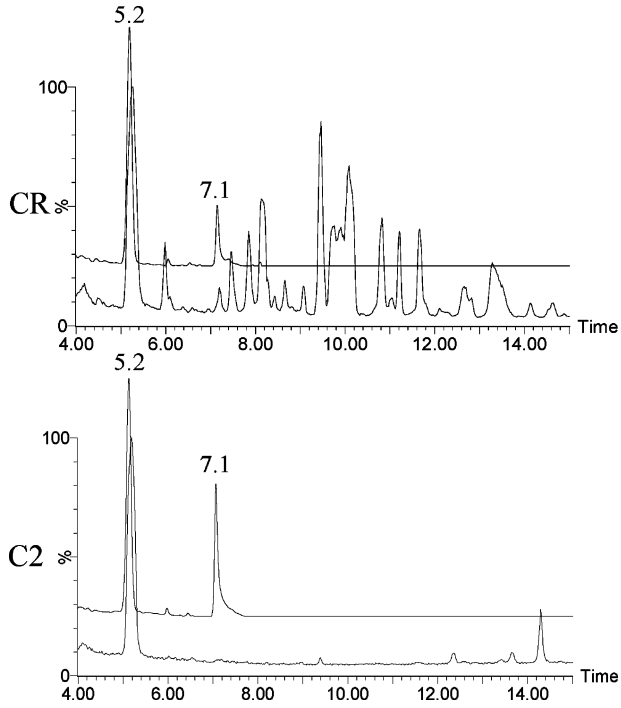


Figure 5. LC-DAD-MS chromatograms with UV (200−700 nm) above total ion chromatogram (*m/z* 100−900 Da) of *P. luteoviolacea* crude (CR) extract (top) and retained (C2) SCX fraction (bottom). Indolmycin (RT 5.2) and violacein (RT 7.1) are selectively retained on a strong cation-exchanger.

identified by LC-HRMS in fractions A1, B6, C2, and D5 that were active against *S. aureus*. This distribution was consistent with the functional groups present in the structure.

A second bioactive compound was observed in both assays, with a recognizable activity pattern of fractions A1, B2, B3, C2, and D3. On the basis of its distribution in E-SPE fractions, the compound was expected to be nonacidic, medium to apolar, medium sized with a basic functionality. By comparative dereplication (Figure 4), an accurate mass of 257.1157 Da was elucidated. No previously reported compounds from the genus *Pseudoalteromonas* or related genera were found to possess these properties. However, indolmycin,[44,45] a known antibacterial from *Streptomyces*, was found to be a likely candidate, as determined by a search in AntiBase. Indolmycin has not previously been reported to be produced by Gram negative bacteria. Therefore, for absolute identification of the compound, a large-scale extract was made and subjected to cation-exchange (Figure 5) followed by size-exclusion as indicated by the E-SPE profile (Figure 3). This led to the isolation of both violacein and indolmycin. Both structures were verified by NMR spectroscopy and matched with previously published data.[46−48] The finding of these two compounds was consistent with the original E-SPE bioactivity matrix.

As part of the method validation, the E-SPE protocol was also tested on a *Penicillium roqueforti* extract. Like the *Pseudoalteromonas luteoviolacea*, *P. roqueforti* yielded a complex extract with a diverse range of metabolites.[49,50] This species has been thoroughly investigated for metabolite production because it is a very common contaminant of food and feed as well as a starter culture for blue cheese. As *P. roqueforti* is prolific in production of organic acids, phenols, and enols, the two anion-exchangers were suitable for the early fractionation of extracts from this fungus.[51] The mixed-mode anion exchanger (MAX) selectively retained all acids, including acidic enols like the andrastins (pK_a 4.5). Comparing the two fractions eluted with 60% organic, unretained bases/neutrals (B2) and acids (B5), respectively, it was clear that the MAX column as a first step of purification markedly simplified the extract (Figure 6). The peaks across the chromatogram were essentially bisected between B2 and B5 with no overlaps between the compounds in the nonacidic and acidic fractions (Figure 6). The MAX separation revealed a series of potential new compounds masked under the peaks of the major metabolites, roquefortine C and mycophenolic acid, which dominate the chromatogram of the crude fraction before partitioning. These new compounds could then be purified using an RP strategy with much improved recoveries, potentially after an SCX step to remove alkaloids like the roquefortines. This underlined the usefulness of employing an orthogonal purification strategy.

The E-SPE approach offers several advantages when dealing with complex extracts. First of all, E-SPE enables the formulation of a purification strategy based on small amounts of crude extract. Then, by using a four-column strategy, it is possible to design preparative purification steps that selectively retain the target candidate (and related compounds) or remove unwanted components. Furthermore, when working with bioactive extracts, it is possible to test the first steps and track the concentration of bioactivity by putting the columns in series. This procedure can be directly translated into a larger scale purification process with high reproducibility as was demonstrated with *P. luteoviolacea*. These aspects of the E-SPE approach are considered in more detail under the following headings.

Dereplication. Successful dereplication of natural product extracts is imperative in the discovery process but can be time-consuming because it requires assessment of the candidates and retrieval of the papers describing all candidates.[24,52] E-SPE accelerates dereplication by reducing the overall number of peaks to be identified and many of the potential candidates in a database search can be eliminated directly based on their ion-exchange properties. E-SPE and *comparative* dereplication makes it, in simple cases, possible to carry out target-guided isolation rather than bioguided fractionation, thereby reducing the need for bioassay support during the isolation process.[53] Further advantages can be gained by using an automatic comparison of chromatograms, e.g., multivariate tools based on principal component analysis (PCA) within the software packages of the major MS vendors. By using E-SPE, it is possible to exploit the sensitivity of biological assays to extract information about the active components and the chemical functionalities. This is of particular importance when working with natural products that are poorly ionized in all MS modes and/or for minor components present below the detection threshold.

Mapping Biological Activities. E-SPE serves as a valuable prefractionation step before biological testing. Bugni et al.[4,54] and Appleton et al.[55] demonstrated that prefractionation can extensively reveal masked candidates in a bioassay (with up to 80% of the candidates being masked in the original extract) and reduce false positives.

Because the steps of E-SPE are completely orthogonal, it allows access to potential new compounds and activities, for example, in cases where: (i) several compounds are responsible for the observed bioactivity, (ii) one compound is responsible for multiple bioactivities, i.e. privileged structures,[56,57] or (iii) if several compounds with different activities are present in the extract.

The potential differentiation was exemplified in the case of *P. luteoviolacea* (Figure 3); violacein was responsible for the antistaphyloccoccal effect observed, whereas indolmycin displayed multiple types of bioactivity (antistaphyloccoccal and antivibrio). The presence and bioactivity of indolmycin could easily have been overlooked in the absence of any kind of prefractionation.

Scaleup and Optimization. By using E-SPE as the first step in optimizing a small scale purification strategy, it is possible to investigate the success or failure of individual purification steps prior to working with a much larger extract. Because all the columns are readily accessible for scaleup, it is possible to directly transfer the strategy to preparative scale, as demonstrated for *P. luteoviolacea*. Sephadex LH-20 is probably the only column that cannot

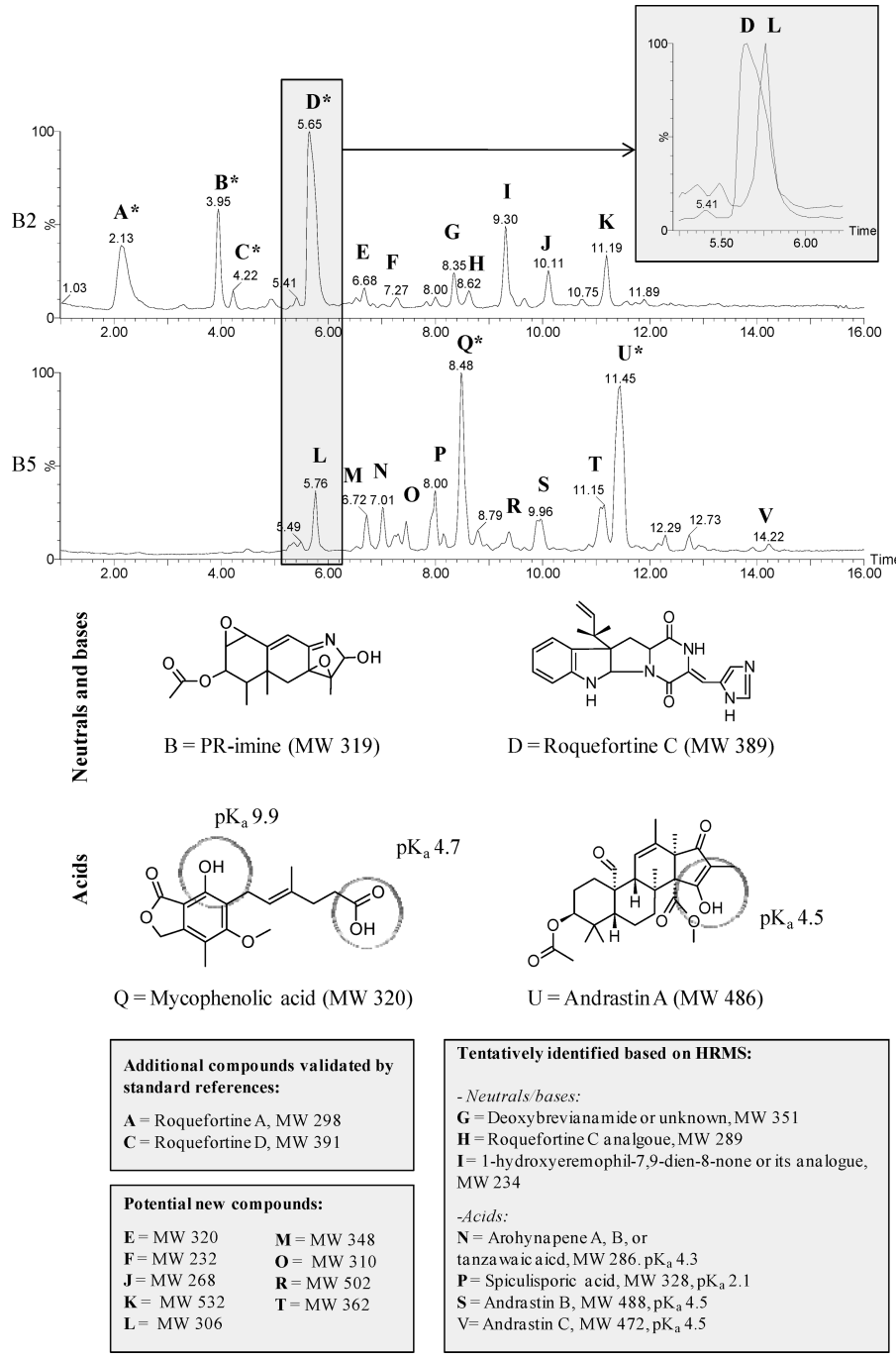


Figure 6. LC-MS total ion chromatogram (*m*/*z* 100−900) of *P. roqueforti* E-SPE fractions from MAX column. B2 = unretained neutrals and bases eluting with 60% MeOH; B5 = retained acids eluting with 60% MeOH and formic acid. Compounds with "*" are identified through comparison with available reference standards. The rest of the compounds were tentatively identified from AntiBase 2008 through E-SPE properties, UV spectrum, and accurate mass.

be directly translated into a large-scale method as the separation is dependent on the column dimensions, linear flow rate, loading of the sample, etc.[58] However, E-SPE still indicates whether this solvent and time-consuming chromatographic step would be worthwhile.

It is important to note that this protocol is only the first step to determine the strategy being pursued. A second optimization experiment could be necessary when other columns or slight variations in the loading or elution of the compounds are to be evaluated. For example, if highly polar compounds are encountered, redissolving the extract in H_2O then loading on Strata-X or Oasis HLB columns may be a way to retain the bioactives. Compounds not retained under these conditions would need to be targeted by HILIC, Sephadex G-10, or other hydrophilic separation approaches.[59]

Optimization of the pH for binding acids and bases may be another important parameter to consider before applying the E-SPE strategy to a large-scale extract. This is of special importance when dealing with a pH labile target, or extracts containing a large fraction of charged compounds. Compounds such as (open lactone form) statins and homoserine lactones are selectively retained on Oasis MAX columns (80−100% recovery) at a 0.5 pH unit below the pK_a even though only 32% (= 10^{pK_a-pH}) theoretically should be charged. Thus, the selectivity and recovery of an ion-exchange step can be further improved by exploiting this dynamic equilibrium during ion-exchange.

Cost and Access. E-SPE saves time and resources when employed on complex extracts. The method represents an inex-

pensive and simple type of chemical screening that can also be used in laboratories with limited access to chemical equipment. The cost per extract is approximately 6−8 USD plus costs for analytical LC-DAD, LC-MS, LC-NMR, etc. When running SAX, MAX, and SCX cartridges, it is easy to run up to 14 extracts in parallel and these columns are readily accessible for automation in microtiter format, as whole plates, or plates with different sorbents that are commercially available. A lower number is recommended for Sephadex LH-20 due to a higher variability in flow rates through the small self-packed columns.

Even though E-SPE is fast, reproducible, and easy to use, there are a few issues to be aware of: There should be good correlation between the sensitivity of the assay used and the extract amounts to avoid false negatives, but more importantly, to track false positives, a blank medium sample should be run in parallel with the other E-SPE samples as one will also concentrate interfering media components.

Some knowledge of the sample matrix is necessary as high levels of salts and fats give rise to problems in the sample run and load, and potentially cause interference in the bioassays. This might require adjustments to the sample pretreatment by: (i) freeze-drying extracts and redissolving in EtOH, (ii) liquid−liquid partitioning, (iii) Sephadex G-10 size chromatography, (iv) $AgNO_3$ columns for chloride precipitation, or (v) ion precipitation with acetone.

While the SPE SCX and Oasis MAX columns both consistently retained basic and acidic compounds respectively, we found SAX columns to be less predictable, e.g., mycophenolic acid is not well retained on SAX (<10%) while moniliformin[60] and fumonisins are quantitatively retained.[21] Also, problems with loading capacity due to excessive amounts of organic acids in the crude extracts add to this unpredictability. We have observed this with *Aspergillus niger* extracts producing high levels of citric acid.

The original bioactivity of an extract can sometimes be lost upon fractionation. This may be due to instability under the conditions applied, e.g. acid/base, light, or reactive solvent, or it could be due to strong retention of the active compound on the selected column. For example, phosphates and sulphates will not be eluted by lowering pH on SAX/MAX columns but need salting out or the use of a weak anion-exchanger (WAX), while quaternary amines on SCX columns also need salting out or the use of a weak cation exchanger (WCX), e.g., a carboxylic acid (CBA) column.

A distribution analysis of calculated pK_a's from the 34390 records in Antibase2008 revealed that within pH 2−11, 44% of all the records had acidic, 17%, basic, and 9%, both functionalities. This shows great potential for using ion-exchange chromatography as an integral part of the separation procedure, which we pursued by E-SPE using SAX, Oasis MAX, SCX, and Sephadex LH-20 columns in a setup giving 15 fractions for biological evaluation. By employing orthogonal chromatographic methods, the E-SPE approach presents a consistent setup for accelerated discovery of novel compounds and offers faster dereplication and purification and uses fewer, more efficient steps. The setup can readily be tailor-made to suit individual laboratories with varying access to assays or equipment. We trust that the E-SPE protocol will encourage other NP groups to publish their methods and share their "tricks of the trade" with the community.

Experimental Section

General Experimental Procedures. Solvents and buffers were all HPLC or LC-MS grade. All aqueous solutions were prepared from H_2O obtained from a Milli-Q system from Millipore (Millerica, MA).

Samples were analyzed using an Agilent 1100 HPLC system with a diode array detector (Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. A Phenomenex (Phenomenex, Torrance, CA) Luna II C_{18} column (50 mm ×2 mm, 3 μm) was used for separation, applying an MeCN−H_2O 0.3 mL min^{-1} gradient (15−100%) over 20 min at 40 °C. Both MeCN (LC-MS grade) and H_2O were buffered with 20 mM formic acid (LC-MS grade).

Large-scale cation SPE purification of the *P. luteoviolacea* extract was performed on an Isolera (Biotage, Uppsala, Sweden) automated flash system. Details are given in the Supporting Information.

NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 MHz) equipped with a Protasis CapNMR capillary probe, using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 3.3 and δ_C 49.3 ppm for methanol-d_4).

Bioassays were performed using standard protocols.[61]

Theoretical pK_a calculations of Antibase2008 were made by converting a ChemFinder version of Antibase2008 to an sdf file using ChemFinder (Cambridgesoft, Cambridge, UK) and then importing the sdf file into ACD ChemFolder (Advanced Chemistry Development, ACD, Toronto, Canada) and then batch calculating the pK_a values using the ACD 2008 pK_a suite.

Biological Material. The marine strain included in the study, *Pseudoalteromonas luteoviolacea*, was selected due to its ability to antagonize the fish pathogenic bacteria, *Vibrio anguillarum*.[62] *P. luteociolaceum* was isolated from seaweed (latitude: 2.9817 N, longitude: −86.6892) and identified based on phenotypic tests such as Gram reaction, cell shape, motility, and glucose metabolism, as well as 16S rRNA gene sequence homology. The bacterium was routinely cultured in Marine Broth (Difco 2216). Three-day static cultures (25 °C) were used for extractions.

The fungus used was from the IBT collection at the Center for Microbial Biotechnology, Denmark, and authenticated by Prof. J. C. Frisvad. *Penicillium roqueforti* (IBT 16404) was grown on one plate of CYA agar for 7 days (25 °C, dark).

Sample Preparation. Bacterial cultures (25 mL) were freeze-dried and extracted EtOH:H_2O (96:4 v/v, 2 × 10 mL) for partial desalting (no sonication; 2 × 12 h), filtered, pooled, and evaporated to dryness with N_2 flow. The agar plate containing the fungal culture was homogenized using a stomacher and extracted directly with 20 mL CH_2Cl_2:EtOAc:MeOH (3:2:1 v/v/v) and then a 50:50 (v/v) mixture of MeCN:H_2O (20 mL). Extracts were filtered, pooled, and evaporated to dryness with N_2 flow.

E-SPE Procedures. All extracts were redissolved in 400 μL of the loading solution prior to running each column, except for Sephadex LH-20 where 100 μL of MeOH was used.

The strong anion-exchange step was performed using Strata-SAX columns (Phenomenex, 100 mg/1 mL), using MeOH:H_2O (70%) for extract loading and wash, and MeOH (1% formic acid or 0.5 M K_3PO_4) for elution, collecting two fractions (A1 = unretained and A2 = retained).

Mixed-mode reverse-phase anion-exchange was performed on an Oasis MAX column (Waters, Milford, MA, 30 mg/1 mL, 30 μm). A MeOH:H_2O solution (25%) with NH_4OH (2%) was used to lock and load the column. A series of MeOH:H_2O solutions (25%, 60%, and 100%) were used for eluting nonacidic compounds, and an equal series of MeOH:H_2O solutions (25%, 60%, and 100%) acidified with formic acid (1%) for eluting acidic compounds. For the MAX column, a total of six fractions were collected, i.e., B1, B2, and B3 = unretained polar/25% MeOH, medium polar/60% MeOH, and apolar/100% MeOH; B4, B5, and B6 = retained polar/25% MeOH + 1% formic acid, medium polar/60% MeOH + 1% formic acid, and apolar/100% MeOH + 1% formic acid.

The strong cation-exchange was performed on Strata SCX (Phenomenex, 100 mg/1 mL, 33 μm) using MeOH:H_2O (70%) for loading and wash, and MeOH with NH_4OH (2%) for elution, collecting two fractions (C1 = unretained and C2 = retained).

For size exclusion, Sephadex LH-20 (GE Healthcare, Hillerød, Denmark) was swelled in MeOH and wet packed in syringes (1 mL) (25 mg/1 mL, 27−163 μm) equipped with a frit, top and bottom. For each extract (100 μL) a total of five fractions were collected, either band-based collection for colored extracts or time-based (1:0.5:1:2:4 mL), respectively.

For all four columns, a blank medium sample corresponding to the media used for cultivation was subjected to the same fractionation and represented a negative control.

Detailed protocol can be found in the Supporting Information.

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Supporting Information Available: Experimental details on the purification of indolmycin and violacein, and the E-SPE protocol is available free of charge via the Internet at http://pubs.acs.org.

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