Combined Use of Extract Libraries and HPLC-Based Activity Profiling for Lead Discovery: Potential, Challenges, and Practical Considerations

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- extract library
- database
- microfractionation
- HPLC-based activity profiling

Abstract

A 96-well format plant extract library and a tailored technology platform have been set up for the discovery of new natural product lead compounds. The considerable advantages of the library approach are discussed. Key considerations such as sample generation, logistics, and data management are addressed. The potential of a HPLC-based profiling approach combining off-line bioassays with in-line and off-line spectroscopy (including HR-ESIMS and microprobe NMR) for tracking bioactivity is demonstrated with a selection of examples encompassing different types of bioassay formats. The information generated by this approach with regards to hit prioritization and preliminary structure activity-relationships is discussed. Practical aspects, such as validation of profiling protocols, the amounts of extracts to be applied, and the re-dissolution of fractions are addressed. Such information is intended for scientists aiming at implementing library-based discovery platforms in their own laboratory.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

The unique contribution of natural products to drug discovery is undisputed. Approximately 35% of the new chemical entities approved as drugs over the past 30 years are directly derived from natural products [1]. However, despite this impressive track record, we have witnessed over the past two decades in the pharmaceutical industry a steady decline of interest for natural products. Various factors have contributed to this apparently paradoxical situation. Emerging technologies may have appeared at one time more promising and "easy" than natural products, which are often perceived as "old-fashioned" at the top management level. However, there have also been more objective reasons for this decline. With the advent of high throughput screening (HTS) in the 1990s, natural product research struggled to find its place in the new drug discovery environment. The classical approach relying on several iterative steps of bioactivity-guided fractionation could not keep pace with the fast turnaround and the tight deadline of HTS-based screening programs [2].

At the same time, the technological advances in HTS, in particular with respect to automation and data management, and significant developments in chromatography and spectroscopy have opened entirely new possibilities for the investigation of natural product extracts. In a time where most major pharmaceutical companies have discontinued their natural product screening activities, these new opportunities have been recognized by biotech companies, and by an increasing number of academic research groups. Switching to a database format for extract handling enables efficient library management and smooth data transfer to screening facilities. The use of new strategies combining, in a more or less seamless manner, HPLC microfractionation with spectroscopic and bioactivity data enables prioritization of hits and identification of bioactive constituents at an early stage [2].

In this context, we set up a tailored technology platform for library-based lead discovery. Its core features are an extract library in 96-well format combined with a customized database, automated liquid handling, HPLC-based tracking of bioactivity, on-line (DAD and ESI- and APCI-MS, including HR-MS) and off-line (microprobe NMR) spectroscopy for structure elucidation. We describe here the different components of this platform and discuss the potential of our workflow

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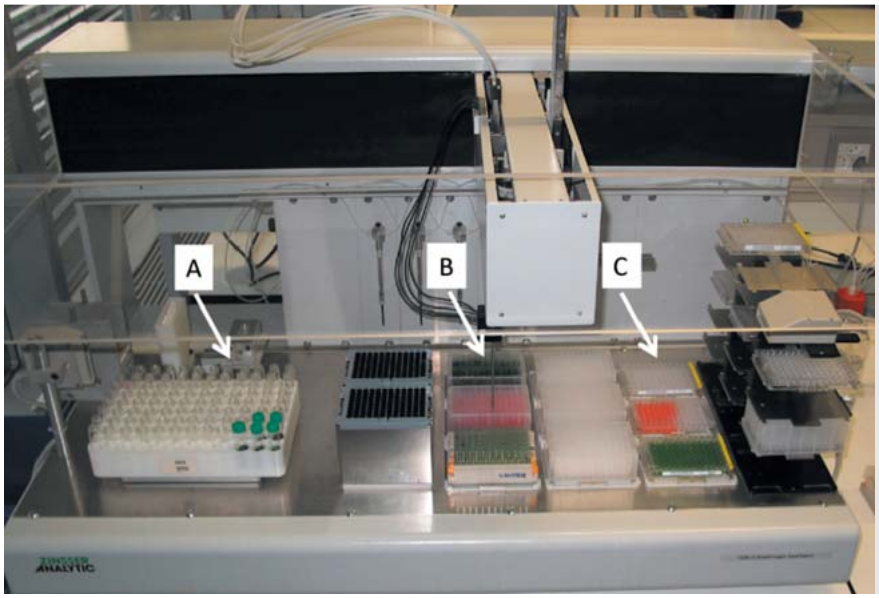

Fig. 1 Robotic liquid handling station for dissolution and dispensing of extracts. **A** 96-position rack with vials containing extracts to be dissolved. **B** Racked microtubes for storage. **C** Daughter microplates for screening. (Color figure available online only.)

with a selection of applications. When doing so, particular attention is paid to practical aspects with the aim of providing useful practical hints to the reader, but also drawing the attention to potential pitfalls.

Generating a Library

With the miniaturization of assays and the generalization of the microplate format in biological laboratories, sample libraries have become the standard for industrial screening programs. A library can be defined as a collection of compounds/extracts stored in a standardized (usually microplate) format and readily deliverable to screening assays. The information associated with each sample is stored in a database. Working with libraries enables a considerable simplification of sample and data handling, and most process steps can be automatized. Several biotech companies have established natural products libraries consisting of extracts, fractions, or purified compounds. Meanwhile, some academic groups have also recognized the potential of this approach. For example, Quinn and coworkers at Griffith University generated a library of 814 pure compounds from which 84% had no Lipinski violation. They also prepared large libraries of "lead-like enhanced extracts" and "lead-like enhanced fractions" from plants and marine invertebrates [3]. Philip Crews and coworkers at the University of California established a library consisting of extracts from marine sponges, tropical plants, and microbial culture broths [4]. Sub-libraries are selectively prepared from active extracts with the aid of automated HPLC-MS-UV-ELSD fractionation. The Ireland group at the University of Utah used a desalting step on HP20S followed by HPLC-MS separation to generate a library of pre-fractionated extracts from marine invertebrates [5]. While academic libraries are typically smaller than industrial compound/extract collections and the degree automation is significantly lower, their generation and management follow the same principles.

Prior to establishing an extract library, several aspects need to be considered. A key issue, for example, is the choice of a suitable extraction procedure, and the degree of pre-purification or fractionation to be applied to the samples. Highly pre-fractionated samples are less complex and can be more suitable for screening, since they cause less assay interferences. This increases the chance for detecting minor active constituents present only in low concentration in the crude extract. On the other hand, these advantages have to be balanced against the rising costs for the generation and screening of an increased number of samples which also may exceed the capacity of low- to middle throughput assays. Another critical aspect to consider is the sample concentration. It should not exceed the solubility of most extracts but should, at the same time, be high enough to keep the final solvent concentration in the bioassays as low as possible. Additional logistical considerations include the selection of appropriate storage plates, storage conditions, and the data management system enabling unequivocal sample tracking and smooth data transfer.

Below we briefly describe the generation, storage and handling of an extract library that has been built by our group over the last decade. The focus is on the technical aspects in order to provide the reader with useful practical information and to draw his attention to critical issues to be considered for planning and establishing a natural product library.

Our library includes at the moment more than 3500 extracts, mostly of plant origin, and is growing by about 500 new samples per year. For sample generation, few grams of plant material are extracted by three solvents of increasing polarity, typically petroleum ether (or dichloromethane), followed by ethyl acetate and methanol. This allows a preliminary fractionation of the plant constituents (lipophilic, intermediate, polar) but keeps, at the same time, the number of samples manageable in an academic setting. The extraction is performed by pressurized liquid extraction (accelerated solvent extraction) on an ASE 200 instrument (Dionex), enabling efficient extraction under standardized conditions. For fatty or sticky samples, the powdered plant material is mixed with a few grams of Kieselgur prior to extraction. After extraction, the extracts are dried using a parallel vortex evaporator (Multivapor, Büchi). Approximately 50 mg of the dried extracts are then exactly weighted into 8 mL glass vials which are then placed at a predefined position in a 96-position vial rack (**Fig. 1**).

The dissolution of extracts in DMSO, at a concentration of 10 mg/mL, and the subsequent transfer to the storage plates are carried

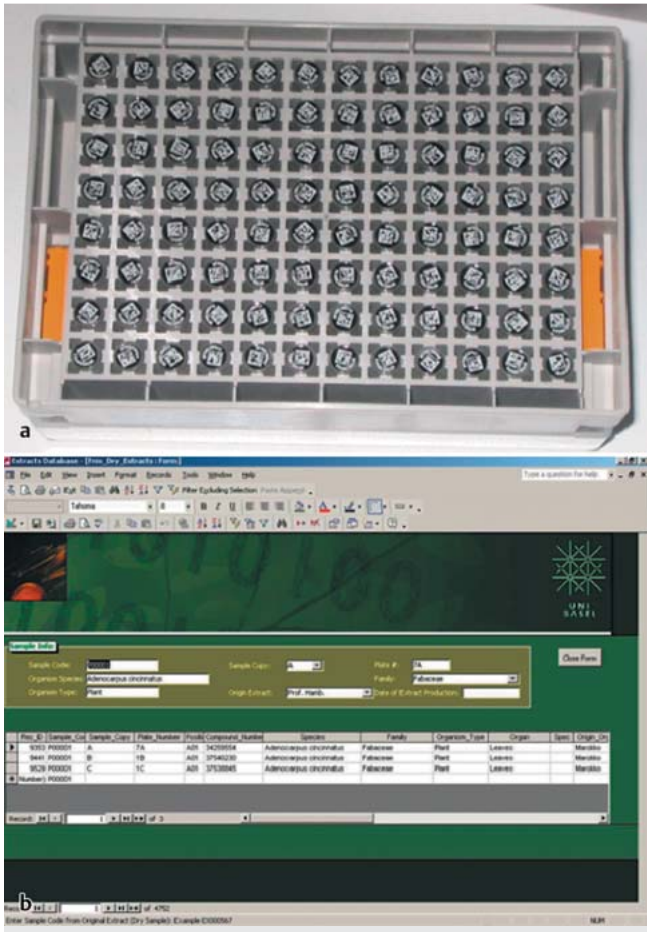

Fig. 2 Barcoded tubes used for the extract library (**a**), and screenshot of database for sample management (**b**). (Color figure available online only.)

out by a robotic system (Moss, Zinsser Analytic). The volume of DMSO to be pipetted is calculated according to the exact weight for each sample, and the values are entered into the dispenser software (WinLissy, Zinsser Analytic). Dissolution of poorly soluble extracts usually requires some manual handling such as ultrasonication before the sample solutions can be transferred into plates. Extracts are stored in 1.8 mL racked microtubes in 96-well format (Matrix, Thermo). The microtubes are 2D-barcoded at their bottom (**Fig. 2a**). This ensures unambiguous sample tracking even if microtubes have been removed from a plate. The last column of each plate (A12-H12) is kept free for controls in screening assays. Three identical copies of mother plates (A, B, and C) are generated and stored at −80 °C. One copy is used for ongoing screening and profiling activities, while the other copies serve as long-term backup. A critical issue with such libraries is the hygroscopicity of DMSO. Water in DMSO not only modifies the concentration of extracts but also negatively impacts the stability of secondary metabolites [6]. For that reason, it is judicious to (i) prepare several copies of mother plates to ensure long-term access to pristine samples, and (ii) to generate, whenever possible, daughter plates for more than one screening project at the same time, in order to keep the number of thaw/freeze cycles of mother plates to a minimum.

Extract information is stored in a customized Microsoft Access-based database (**Fig. 2b**). Key information includes the species and organ, the origin of the material, the type of extract, and the date of extract preparation. In addition, a sample code is given to each extract, and the position of the sample in the library (plate number and position on the plate (plate maps)) is defined. In a first step, this information is assembled for each plate in an MS Excel table, by filling in a template sheet (**Fig. 1S**, Supporting Information) containing predefined sampled codes and plate positions, and the corresponding extract metadata. These data can be provided in an import-ready state by the sample supplier if extracts are provided by a collaborating partner. In a separate process, the tube numbers (barcodes) of an entire plate are scanned with VisionMate 96 software (Matrix Thermo) and stored as a text file (**Fig. 2S**, Supporting Information). The Excel file containing the extract data, and the text file with the tube numbers are then automatically combined and imported into the database using a macro command. A message is delivered indicating whether the import was successful. Samples already contained in the database are automatically recognized to avoid duplicate records. This is important, e.g., if the data import needs to be repeated for a plate in which not all positions were filled initially. The automatic batch-based import process efficiently prevents any erroneous sample assignment when feeding the database with new data.

When samples are to be delivered to a screening project, the library is replicated into 96-well daughter plates. The plate type to be used is selected according to the specific requirements of the assay. After dispensing, the plates are sealed. Special attention should be paid to seal integrity of the adhesive foil over the required temperature range, since plates are typically shipped on dry ice. Plates are then transferred to the screening laboratory, together with the corresponding sample information which is extracted from the database as an Excel file.

Setting Up HPLC-Based Activity Profiling

When searching for new lead compounds in natural product extracts, the efficient tracking of active constituents probably still remains the single most challenging step. The classical bioactivity-guided isolation strategy involving several iterative steps of purification and biological testing is time consuming and requires large amounts of material [7]. While it has been successfully applied to the discovery of bioactive natural products in the past, it is no more compatible with the timelines of modern drug research. In addition, loss of activity along the isolation procedure, and repeated isolation of the same compounds are not unusual. With the technological advances in chromatography and spectroscopy over the last two decades, more efficient alternative approaches have been developed to track bioactivity in complex samples. They include affinity-based methods such as pulsed ultrafiltration mass spectrometry and HPLC-based approaches. (For a recent review see [8]). Among the latter, HPLC-based activity profiling has been developed as a highly versatile strategy to accelerate deconvolution of active extracts. The principle of this approach consists in the separation of bioactive extracts by analytical or semi-preparative HPLC. UV and MS data are recorded on-line and, in parallel, fractions are collected into microplates or deep-well plates, via a T-split of the column effluent. The fractions are dried, re-dissolved in a small amount of a suitable solvent and assayed for bioactivity. The chromatogram and the activity profile are then matched to identify active peaks (**Fig. 3**). On-line spectroscopic information in combination with

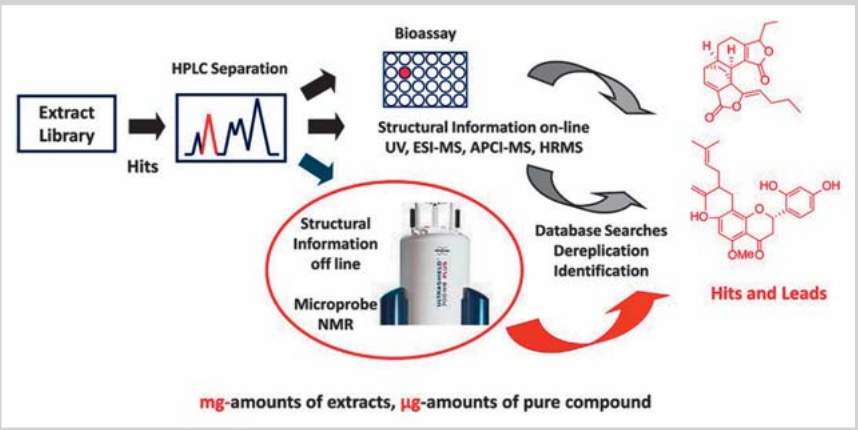

Fig. 3 Concept of our platform for HPLC-based activity profiling of bioactive extracts. Reprinted with permission from [8]. (Color figure available online only.)

database searches can be used to dereplicate known compounds and pinpoint potentially new molecules.

This microfractionation step can be performed with the bioactive sample that is stored in the library, since mg amounts of extracts are sufficient for the purpose. Hence, there is no need for time-consuming refermentation or recollection/extraction of samples, which typically leads to unsatisfactory reproducibility of initial activity data. When needed, subsequent preparative purification can be performed using a peak-guided strategy. The scale-up of the separation is straightforward, since the chromatographic conditions can be easily transposed to preparative HPLC.

UV and MS data recorded online can be used for early dereplication and tentative identification of bioactive compounds. High resolution MS data that can be usually obtained on a time-of-flight (TOF) mass spectrometer are particularly useful, since molecular formula of compounds can be derived from them. These data can be used for a narrowed search in the Dictionary of Natural Products [9] or, after calculation of a restricted number of possible molecular formulas, for a search in Chemical Abstracts Service (CAS) Registry via SciFinder. NMR data for fractions or peaks are typically recorded off-line using microprobe NMR technology. Compared to on-line setups, off-line NMR with disposable tubes has several attractive features for profiling and was, therefore, adopted for our platform. The system operation is straightforward and does not require delicate adjustments. The collected HPLC peaks can be processed in parallel, and an NMR autosampler allows unattended measurement of ^{1}H NMR spectra which serve as a basis for more advanced 1D and 2D experiments. 2D experiments are typically ^{1}H detected and, thus, can be performed with the same sample. Microtubes can be stored for a certain time, and time-consuming experiments can be performed at a later moment [10]. The configuration is, therefore, well adapted to academic settings. In our case we operate a 500 MHz NMR instrument in our group in an open-access mode. Our standard configuration with a room-temperature 1 mm probe has proven to be extremely robust, and it has been sufficiently sensitive for most applications. If highest sensitivity is needed, we have access to a 600 MHz instrument equipped with a 1.7 mm cryoprobe.

For a successful application of HPLC-based activity profiling, some aspects need to be carefully considered: The choice of the column diameter to be used depends on the degree of miniaturization and sensitivity of the bioassay. An analytical HPLC column can be used for most cellular and biochemical assays since they require only minute amounts of sample. For most cases, we use analytical HPLC columns (3 mm i.d.) and typically inject 300 µg of extract (corresponding to 30 µL of DMSO solution). For more complex pharmacological assays, the separation has to be performed on a semi-preparative scale. For assays in *Xenopus* oocytes (e.g., $GABA_A$ and human ether-a-go-go related gene (hERG) assays) separations of 5 mg of extract are carried out on semipreparative columns (i.d. 10 mm). When working with analytical HPLC columns and flow-rates below 1 mL/min, the delay between detector and fraction collector is a critical issue. Also, post column diffusion can significantly lower the resolution achieved, and hence, affect the quality of activity profiles. With conventional HPLC instrumentation, fractions as small as 1 min can be reasonably collected, provided that Teflon tubing connecting the HPLC detector to the fraction collector is as short as possible and of a suitably small inner diameter. For our applications, we use a Gilson FC 204 fraction collector and tubing with 0.3 mm i.d. Fraction times shorter than 1 min are only meaningful with microfluidic fractionation devices.

The initial microfractionation is typically time-based, with a standard gradient ranging from 5 to 100% acetonitrile. Highly complex extracts may require two consecutive steps of profiling, where the first fractionation is followed by a second microfractionation performed in a peak-based collection mode. For that, the separation of peaks in the active time-window is optimized. Whenever possible, the profiling protocol should be validated for a specific bioassay. This can be typically done by injecting pure active substances, or by spiking an inactive extract with a defined amount of a known active compound. As an example of such a validation exercise we refer to the profiling for new $GABA_A$ receptor modulators in plant extracts [11]. An EtOAc extract of *Notopterygium incisum* (essentially inactive in the initial extract screening) was submitted to semipreparative HPLC separation. Subsequent testing of the 90 sec microfractions confirmed the lack of activity. The extract (3 mg) was then spiked with 500 µg (corresponding to 300 µM in the bioassay) of the known $GABA_A$ receptor modulator magnolol and again submitted to microfractionation. The activity was found in Fr. 11 and was similar to that obtained with 300 µM of pure magnolol (**Fig. 4**). When available, extracts containing known active compounds can be also used for validation purposes. In this specific case, we used a valerian extract (*Valeriana officinalis*) which is known to contain the $GABA_A$ receptor modulator valerenic acid.

Likewise, we validated a protocol for HPLC–based activity profiling for antiprotozoal activity [12]. Pure artemisinin (9.6 ng) and melarsoprol (14.4 ng) were injected for activity profiling against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*, re-

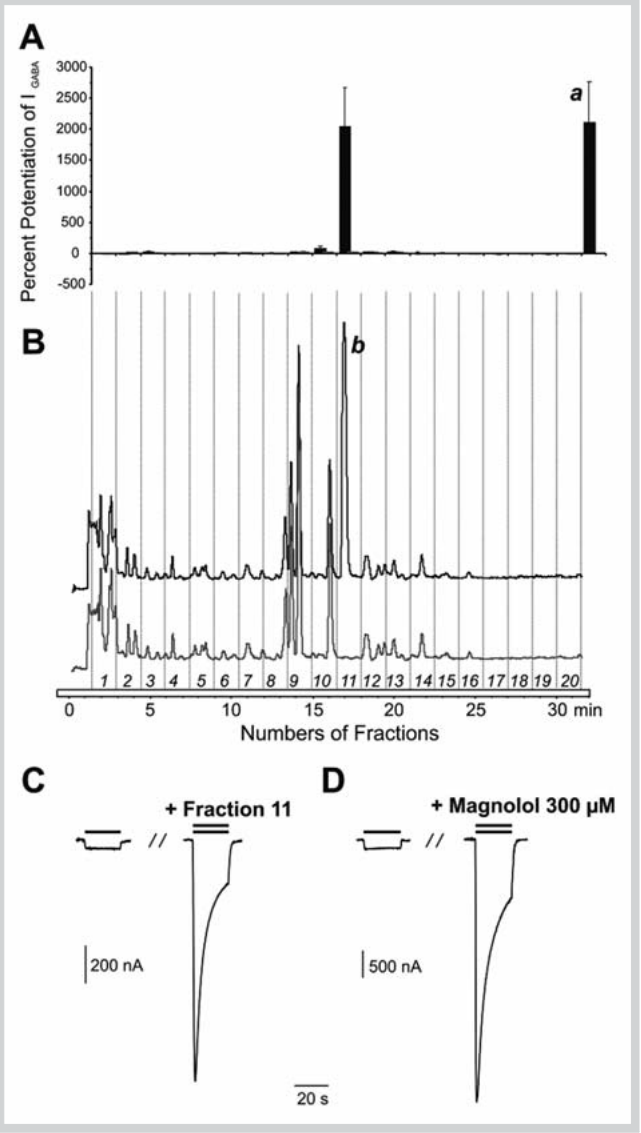

Fig. 4 HPLC-based activity profiling of EtOAc extract from the roots of *Notopterygium incisum* and magnolol for $GABA_A$ ligand activity. **A** The bar graph represent potentiation of I_{GABA} in % by the fractions collected from a single injection of the original extract of *N. incisum* (3 mg) (grey bars) and the extract spiked with magnolol (500 µg) (black bars). Magnolol, which was collected from an injection of the same amount under the same chromatographic conditions, was used as control (*a*) for GABAergic activity. **B** HPLC chromatograms of extract without (bottom), and with spiked magnolol (*b*) eluting at 16.77 min (top). Lines indicate microfractions (90 sec each) that were collected for bioassay. **C** Representative current traces recorded from oocytes expressing $GABA_A$ receptors composed of α_1, β_2 and γ_{2S} subunits in the presence of control GABA (EC_{3-10}, single bar), and traces recorded during co-application of GABA EC_{3-10} and fraction 11 (left column, double bar) or magnolol (300 µM, right column, double bar). Reproduced with permission from [11].

spectively. The amounts were selected such that the lower of the two final concentrations equaled the IC_{50} of the compounds in the respective assay. Both positive controls appeared as clear activity peaks matching the HPLC peak of the compounds. As a next step, an EtOAc extract of *Artemisia annua*, the source plant of artemisinin, was profiled. Again, the peak of activity fitted well with the HPLC peak of artemisinin.

Re-dissolution of the fractions after removal of the HPLC eluent is a particularly critical step. DMSO is typically used because of its good solubilizing properties and its compatibility with bioassays. When a mixture of DMSO and water has to be used, the sample should be dissolved first in pure DMSO and the water added afterwards. Moreover, the plates should be sonicated or vigorously shaken for at least 30 min. The solvent volume required to obtain a concentration of the active constituents in the fraction(s) similar to that found in the original extract can be estimated, based on the assumptions that (i) a particular compound is eluted in one or two 1 min-fractions and (ii) a 50% loss of compound occurs during chromatography and re-dissolution. The latter value is an empirical estimate from several years of practical experience with various types of active compounds. Thus, a concentration of an active compound similar to that in the extract will be obtained for a fraction when half of the injection volume is used for its re-dissolution.

Information to Be Obtained from Profiling

The main purpose of HPLC-based activity profiling is an efficient tracking of active compounds in an extract. When working on a semi-preparative scale, typically with 10–20 mg of extract, the bioactive constituents can be quantified in fractions at the microgram scale by qNMR [13], so that even quantitative bioactivity data such as IC_{50} values can be obtained at an early stage. In most cases, however, a quantitative approach is not possible (i.e., when µg of extracts are separated by analytical HPLC) or pursued (normally, concentration-response curves are recorded later with pure compounds). Therefore, the distribution of the activity in the different fractions and not the potency is in the foreground. It should be noted that this point is not always clear for colleagues working in screening laboratories who are typically used to work with defined amounts of material. Also, they sometimes do not understand that activity profiling is primarily a means for tracking activity, and they erroneously assume that the HPLC-microfractions will somehow undergo further purification.

HPLC-based activity profiling is not restricted to the mere identification of bioactive peaks, but provides a wealth of additional and highly useful information. First, activity profiles are extremely important for prioritization of active extracts. Screens quite often deliver a large number of hits exceeding the capacities for a preparative follow-up. Especially in industrial settings, a work-up of all active extracts is typically impossible within the timeframe allocated to follow-up activities in the lead discovery phase, which is usually restricted to as less as a few weeks. With the activity profile at hand, isolation capacities can be dedicated to extracts where bioactivity correlates with discrete chromatographic peaks. Low priorities are assigned to extracts for which activity cannot be recovered after fractionation or is dispersed over a broad time window. HPLC-based activity profiling is also an efficient tool for the dereplication of active extracts. Microbial libraries, in particular, often contain samples of similar composition originating from cognate strains or extracts with commonly occurring bioactive compounds. For polar plant extracts, the presence of tannins typically results in unspecific activity, in particular with biochemical assays. In tannin-containing extracts, activity due to tannins is characterized by a wide activity window correlating with broad humps and unresolved peaks in the HPLC chromatogram. Such extracts can be excluded from follow-up or

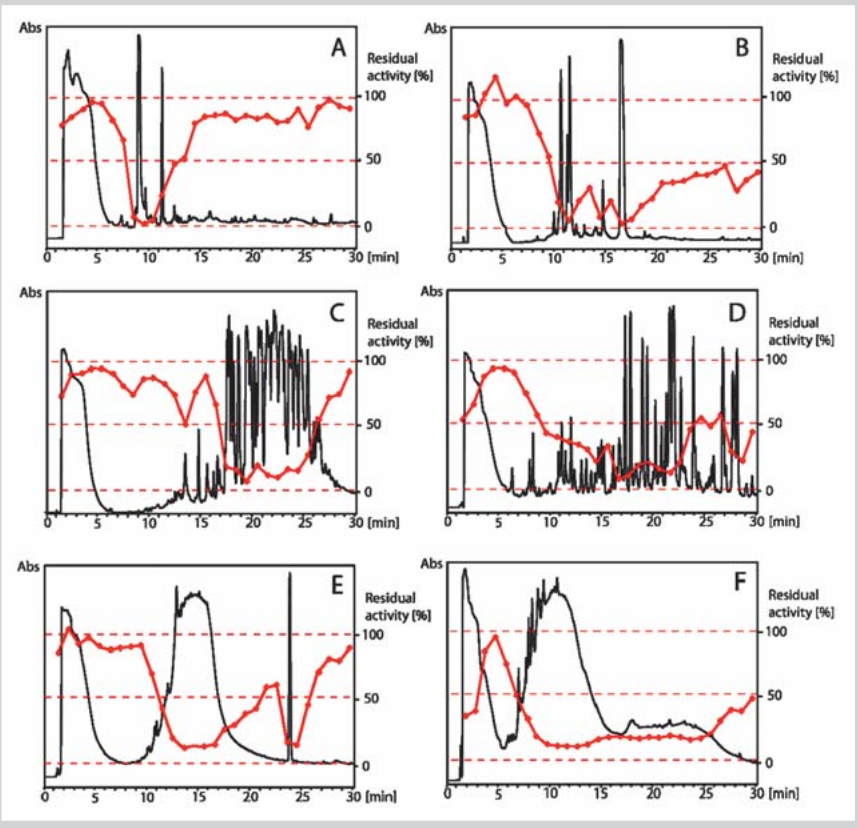

Fig. 5 Representative examples from HPLC-based activity profiling for DYRK1A kinase. *Peganum harmala* (aerial parts) CH_2Cl_2 extract (**A**); *Cuscuta chinensis* (seeds) CH_2Cl_2 extract (**B**); *Larrea tridentata* (aerial parts) CH_2Cl_2 extract (**C**); *Cistus monspeliensis* (aerial parts) EtOAc extract (**D**); *Cassia nigricans* (twigs and leaves) MeOH extract (**E**); *Xymenia americana* (roots) MeOH extract (**F**). Conditions: SunFire C_{18} column (3 × 150 mm i. d., 3.5 µm); 5–100 % MeCN/0.1 % aqueous formic acid in 30 min, 0.5 mL/min; time-based fractionation; detection: 210–700 nm, maxplot. DYRK1A kinase activity (red) is shown as percentage of maximal kinase activity in the absence of fractions. Reproduced with permission from [15]. (Color figure available online only.)

possibly retested after removal of tannins by filtration of the sample over a polyamide cartridge [14].

This strategy has been followed in a project aimed at the discovery of new inhibitors of the DYRK1A kinase. A total of 25 extracts were found to be active in this enzyme-based assay (inhibitory activity > 50 % at 10 µg/mL). However, based on the activity profiles, only seven extracts were selected for follow-up investigations. In these extracts, activity was nicely correlated with discrete HPLC peaks. Some typical cases of HPLC and corresponding activity profiles are shown in (**Fig. 5**) [15]. The extracts of *Peganum harmala* (**Fig. 5A**) and *Cuscuta chinensis* (**Fig. 5B**) showed simple chromatograms correlating with peaks of activity, and the active compounds were rapidly identified as the beta-carboline alkaloid harmine and flavonoids, respectively. The chromatograms of *Cistus monspeliensis* (**Fig. 5C**) and *Larrea tridentata* (**Fig. 5D**) were very complex. The major active compounds in *Larrea* were readily purified and identified as flavonoids, but the highly complex extract of *Cistus* was not further pursued given that on-line spectroscopic data clearly indicated that the compounds in the active time window were also flavonoids. The chromatograms of *Cassia nigricans* (**Fig. 5E**) and *Xymenia americana* (**Fig. 5F**) showed unresolved humps and broad distribution of activity that were most likely due to tannins. The *Xymenia* extract was excluded from a further follow-up, but the sharp HPLC peak at t_R 22 min in the *Cassia* extract was identified as emodine, while the early eluting zone of activity was not pursued further. It should be noted that the entire profiling and structural characterization of compounds in this project was completed in less than five months.

Tannins are a likely interference in many bioassays and not only in biochemicals screens with purified proteins. For example, in a screening for hERG channel inhibitors with a functional assay in *Xenopus* oocytes, the extracts of plants such as guarana (*Paullinia cupana*), cinnamon (*Cinnamomum zeylanicum*), and nutmeg (*Myristica fragrans*) showed significant inhibition. Again, the HPLC-based activity profiles suggested that the activity was likely due to presence of tannins. This was confirmed by removal of tannins over polyamide cartridges and re-profiling of the tannin-depleted samples [16].

Preliminary structure-activity data are a further type of information which can be obtained by extending the profiling beyond the active compounds toward inactive, but structurally related molecules. This can be exemplified with the example of piperamides in *Piper nigrum*, which were characterized in the course of a screening for $GABA_A$ modulators [10] (**Fig. 6**). In a first step, bioactivity was profiled by time-based fractionation of 5 mg of extract using semi-preparative HPLC. After optimization of the chromatographic conditions, 10 mg of extract were separated, and a total of 30 peak-based fractions were collected (**Fig. 7**). A total of 13 peaks corresponding to piperamides were identified with the aid of HPLC-HRESIMS and DAD data, and by ^{1}H NMR spectra (128 scans) obtained with a 1 mm TXI probe. Structural information obtained during the profiling step for the compounds outside of the active region of the chromatogram was essential for deriving preliminary structure activity relationships. In the case of piperamides it became clear that the nature of the amide moiety, length of the linker between the aromatic ring and the amide were critical for activity, and that rigidity of the linker was positive. This information was then used for the development of piperine into the non-TRPV1 activating lead SCT-66 [17] (**Fig. 8**).

In most cases, HPLC-based activity profiling will be successful for tracking bioactive compounds. However, as for every strategy, there are some intrinsic limitations. When a particular assay is

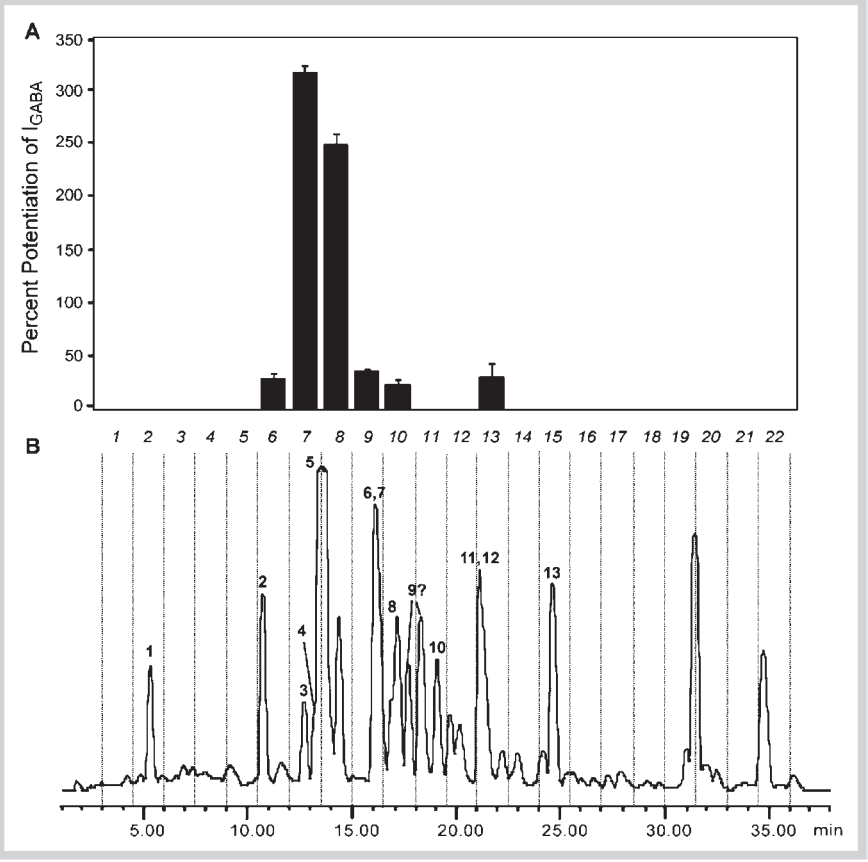

Fig. 6 HPLC based activity profiling of a black pepper (*Piper nigrum*) extract for $GABA_A$ receptor agonistic properties. **A** HPLC chromatogram (254 nm) of a semipreparative separation of 5 mg extract. Peak numbering corresponds to compounds **1**–**13** (see ◉ **Fig. 8**). The 22 collected time-based fractions, 90 seconds each, are indicated with dashed lines. **B** Potentiation of the GABA induced chloride current in *Xenopus* oocytes (IGABA) by each fraction is shown in **A**. Reprinted with permission from [10]. Copyright (2010) American Chemical Society.

relatively insensitive and requires milligram amounts of material, only large fractions can be collected, resulting in a poorly resolved activity profile. Also, compounds eluting with the solvent front or highly lipophilic substances which do not elute from the column under standard chromatographic conditions are likely to be missed. However, such compounds are of limited interest in drug discovery considering their unfavorable physicochemical properties. Finally, complex extracts containing strongly active constituents in minute amounts, or compounds that are difficult to detect with UV or MS are particularly challenging. In such cases, a first step of classical activity-based separation may be necessary before HPLC-based profiling can be successfully applied to the active and less complex fraction.

Selected Applications

An increasing number of examples demonstrate the potential of the HPLC-based activity profiling platform for the rapid isolation of bioactive natural products from complex extracts. In our research group, it has been successfully used for the follow-up of hits from various screening projects. Screens were based on various bioassay formats, such as whole organism assays (tropical parasitic diseases), cell-based antiviral (HIV), and functional assays (e.g., $GABA_A$ receptor modulation, hERG channel inhibition), and mechanistic screens (e.g., DYRK1A kinase) (◉ **Table 1**). For example, we were able to discover numerous new scaffolds for $GABA_A$ receptor modulators, by combining HPLC-profiling with a semiautomated two-microelectrode voltage clamp assay with *Xenopus* oocytes expressing $GABA_A$ channels [10,11,18–28]. Among these compounds, piperine demonstrated promising activity *in vivo* and was used as a starting point for the synthesis of the lead SCT-66 (◉ **Fig. 8**) with improved pharmacological properties [17]. Another line of research has been focussed on new antiprotozoal compounds against tropical parasites, and a structurally diverse series of active compounds were identified [12,29–40]. One of the promising compounds in this project was cynaropicrine which was identified as the antitrypanosomal constituent of an extract of *Centaurea salmantica* (◉ **Fig. 9**), and was subsequently isolated in a larger scale from artichoke leaves. Cynaropicrine was shown to be active against melarsoprol and pentamidine resistant strains, and to reduce parasitaemia in the *T. b. rhodesiense* mouse model [29]. In another example, an extract of *Abrus precatorius* exhibited activity against *P. falciparum, T. b. rhodesiense*, and *Leishmania donovani*. The activity could be tracked to a series of isoflavan quinones by differential profiling against the parasites [38,40]. In the search for antiviral agents, daphnetoxin was identified as a potent and selective HIV inhibitor in *Daphne gnidium* [41]. HPLC-based activity profiling is also useful to identify compounds with undesirable properties. For example, we recently established a profiling protocol for the identification of hERG channel inhibitors in herbal extracts [16, 42]. The hERG channel is a voltage-gated potassium channel expressed in the myocard and currently the most critical antitarget with respect to cardiac safety. We profiled a range of widely consumed herbal drugs and food plants with the aid of this approach. Earlier, we developed and successfully used this profiling approach for the identification of cyclooxygenase 2, 5-lipoxygenase, iNOS and monoamine oxidase inhibitors in plant extracts [43–46].

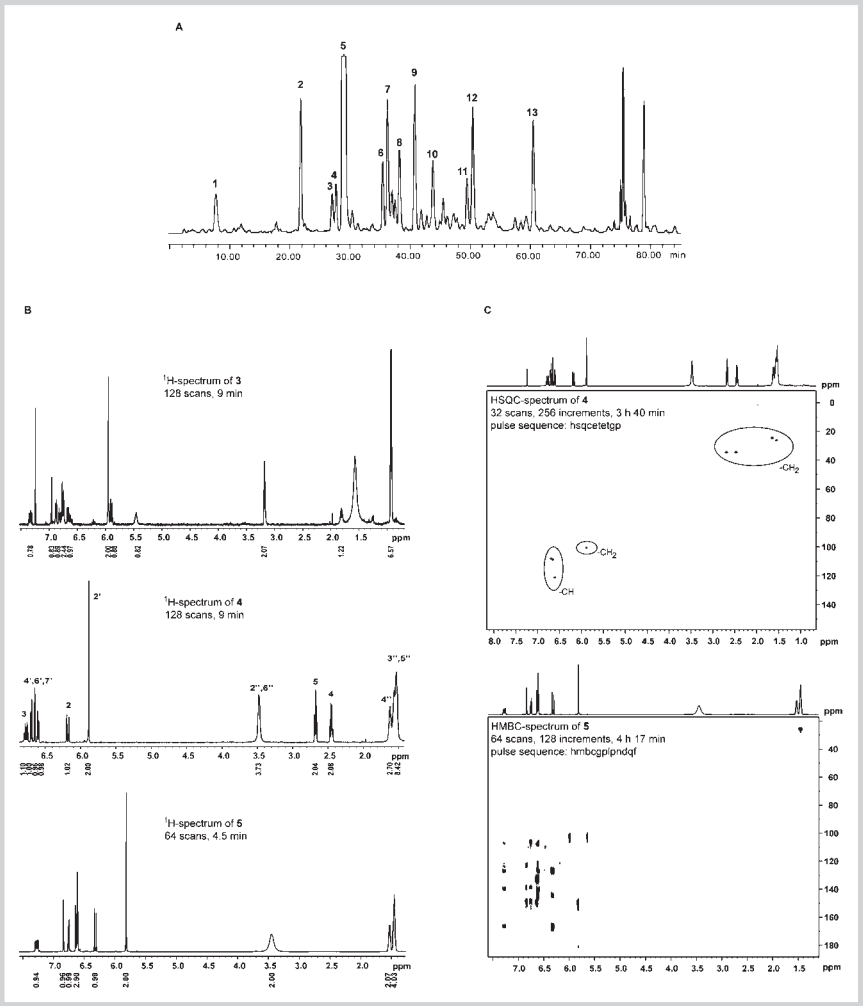

Fig. 7 Identification of piperamides in *Piper nigrum* extract. **A** Chromatogram (254 nm) of an optimized, semipreparative HPLC separation of the active *P. nigrum* extract (10 mg in 100 µL DMSO). A total of 30 peaks were collected for offline microprobe NMR. Peak labeling corresponds with the compounds **1–13** (see ◉ **Fig. 8**); **B** ^{1}H NMR spectra of selected compounds obtained from the separation above; **C** Spectra of two representative 2D NMR experiments. Reprinted with permission from [10]. Copyright (2010) American Chemical Society.

Table 1 Bioassays that have been used in HPLC-based activity profiling by our group.

Target/activity	Assay format and readout	Reference
Cyclooxygenase2	Cell based assay with stimulated Mono Mac 6 cells in 96 well format. Formation of 6-ketoPGF$_{1\alpha}$, readout with ELISA.	[43]
5-Lipoxygenase	Cell based assay in 96 well format. LTB$_4$ release from HL-60 cells, readout with EIA.	[44]
Inducible NO synthase	Cell-based assay in 96 well format with RAW 264.7 murine macrophages stimulated with LPS Determination of NO production with Griess reaction.	[46]
Monoamine oxidase-A	Kinetic assay with recombinant human MAO-A and kynuramine as substrate. Spectrophotometric measurement.	[45]
GABA$_A$ receptor modulation	Functional assay in *Xenopus* oocytes transiently expressing GABA$_A$ receptors of desired subunit composition. Semiautomated two microelectrode voltage clamp assay.	[11]
hERG channel	Functional assay in *Xenopus* oocytes transiently expressing hERG channel. Semiautomated two microelectrode voltage clamp assay.	[42]
Plasmodium falciparum *Trypanosoma brucei rhodesiense* *Leishmania donovani*	Whole parasite assays in 96 well format.	[12]
HIV inhibition	HIV replication assay in HEK293T cells, using CXCR4-tropic CCR-tropic viruses.	[41]
DYRK1A kinase	Biochemical assay with human recombinant DYRK1A.	[15]

Related HPLC-Based Configurations

Over the last two decades a number of innovative approaches have been described using HPLC-based methods for tracking bioactivity in complex matrices [8]. Configurations similar to ours have been established by some research groups. For example, Kautz and coworkers at the Northeastern University, Boston, established a platform in which HPLC microfractions are dissolved in deuterated solvent and loaded into a microcoil NMR probe. After NMR analysis, the samples are recovered and used for bioactivity testing [47]. Wolfender and coworkers at the University of Geneva have used semi-preparative HPLC-MS fractionation,

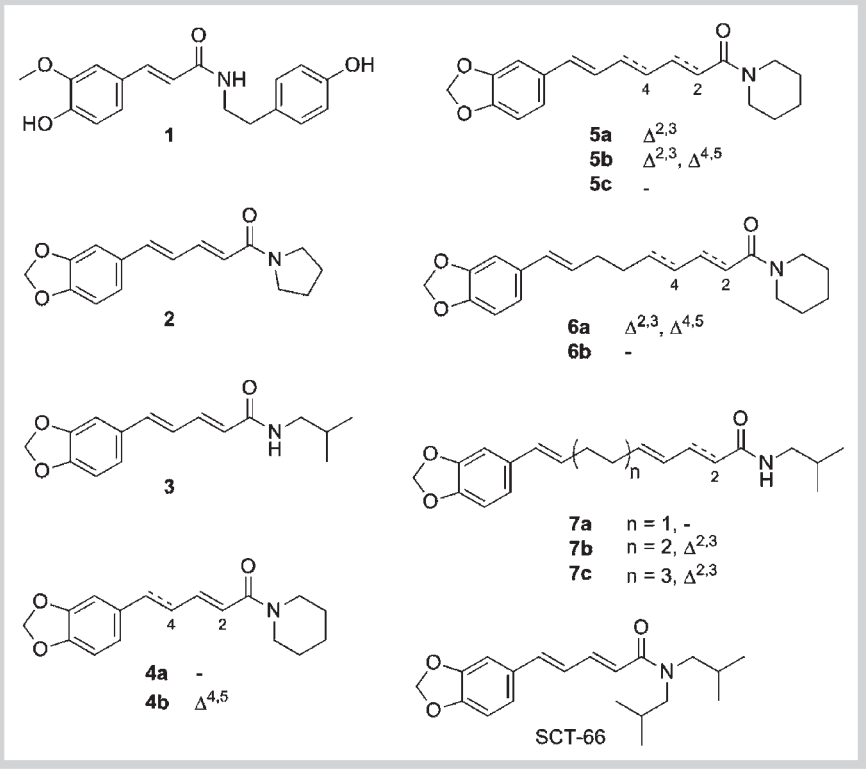

Fig. 8 Structures of piperamides identified in the *Piper nigrum* extract and of semisynthetic derivative SCT-66.

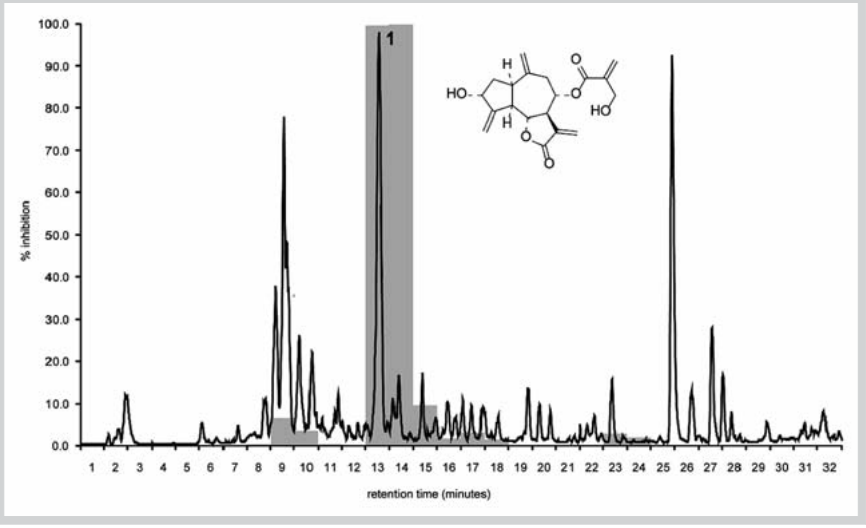

Fig. 9 HPLC-based activity profile of the dichloromethane extract of *Centaurea salmantica*. The grey bars represent the % inhibition of the 1-minute microfractions against *Trypanosoma brucei rhodesiense* compared to the control. The chromatogram in black shows the corresponding ESI-MS trace (*m/z* 150–1500) of the HPLC separation. The peak corresponding to cynaropicrin (**1**) eluted at t_R 13.3 min. Reproduced with permission from [29].

followed by capillary NMR and biological evaluation for the identification of fungitoxic metabolites occurring in confrontation zones of fungal strains [48]. Researchers at the University of California in Santa Cruz established a screening platform using a yeast halo assay in 384-well format to search for antifungal compounds. Active extracts were then fractionated by HPLC coupled to ELSD detection [49]. A remarkable line of research has been towards the development of fully integrated setups with on-flow post column assays [50, 51]. Most work in this area has been carried out by the group of Irth at the University of Amsterdam. A variety of assay formats using fluorescence-based readouts or mass spectrometric detection have been developed, which can be applied to a broad range of protein targets. A critical issue in these approaches is the diffusion occurring during the post-column reaction which negatively affects resolution. To circumvent this problem, Irth and coworkers have developed an at-line approach which combines elements from on-line configurations and off-line setups. In the at-line approach, biochemicals needed for the assay are still added on-line post-column, but the effluent is fractionated before incubation with the target [52]. Such a setting has been used, among others, for the discovery of protein kinase A inhibitors [51] and acetylcholine-binding protein (AChBP) ligands [53]. On-line and at-line configurations constitute impressive technical achievements, but the need for compatibility of chromatographic and bioassay conditions limits their applicability. Also, the large quantities of immunochemicals, proteins, and ligands consumed in such on-line arrangements represent a major cost factor. Finally, compared to off-line approaches, on-

line assays require considerable development and optimization for each target to be screened.

Conclusion

HPLC-based activity profiling is a powerful tool for the discovery of new bioactive scaffolds in natural product extracts. The approach is highly versatile and can be applied without much adaptation to a broad range of bioassays and various assay formats. In conjunction with on-line spectroscopy and off-line NMR microprobe technology, a wealth of information can be obtained, at a very early stage, on extract constituents and, in particular, on active compounds. This information greatly facilitates prioritization of samples for follow-up activities and the targeted preparative purification of compounds of interest. Using a 96-well format library in conjunction with a database allows smooth transfer of data and of extracts/fractions/compounds. In this paper, we focused on our discovery platform but also briefly described some similar configurations which have been implemented by other academic groups. Our aim was to provide the reader with useful information from our own experience. Practical and seemingly mundane aspects are of critical importance for the successful implementation of a natural product discovery platform but are rarely reported in the literature. While such considerations may be obvious for industrial researchers, this is not the case for many academic scientists. Hence, we hope that our paper will stimulate more colleagues in the field to establish similar platforms and approaches for their own research.

Supporting Information

Template file for extract metadata and text file with sample barcodes used for library generation (both as pdf) are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest

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