Natural Products in Drug Discovery - Concepts and Approaches for Tracking Bioactivity

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Abstract: Effective methods for localization and characterization of bioactivity are a cornerstone for natural product based drug discovery efforts. Over the last decade, a wealth of new technologies and conceptual approaches for bioactivity screening have emerged. These developments are reviewed under the perspective of their applicability in the field of natural products discovery. The methods discussed here include bioautography, HPLC-based activity profiling, HPLC-based on-flow bioassays, assays based on capillary electrophoresis, molecular imprinted polymers, various MS- and NMR-based methods, biosensors, and chip-based technologies for affinity separation and expression profiling. Selected examples illustrate the potential and limitations of the different approaches for contemporary natural products lead discovery.

INTRODUCTION

Natural products have traditionally played a major role in drug discovery and still constitute a prolific source of novel lead compounds or pharmacophores for medicinal chemistry. About 40% of current ethical drugs are directly or indirectly derived from compounds of biogenic origin [1, 2]. Molecules originating from natural products leads are well represented in the worldwide 35 top selling prescription drugs with percentages ranging approximately from 20 to 40 % over the last five years. Substances like taxol, cyclosporine and the statins are cornerstones of modern pharmacotherapy. Despite increasing competition from combinatorial and classical compound libraries, there has been a steady introduction of natural product-derived drugs in the last years. According to a recent review on the role of natural product research in drug discovery [3], a total of 15 natural products or natural product derived drugs have been launched in either the United States, Europe or Japan in the years 2000-2003. They include essential medicines such as the anti-malaria drug arteeether (Artemotil®), galanthamine (Reminyl®) for the treatment of Alzheimer's disease, and pimecrolimus (Elidel®) for atopic dermatitis. The impact of natural products on the development pipelines of the pharmaceutical industry is unabated. As per December 2003, some 15 compounds were in phase III clinical trials or registration. Among these are representatives of novel compound classes such as the anticancer drug ixabepilone, a semisynthetic derivative of epothilone B produced by the myxobacterium *Sorangium cellulosum*, or ziconotide, a compound for the treatment of chronic pain which is the synthetic equivalent of ω-conotoxin found in the dart venom of the cone shell *Conus magus* [3].

The Potential of Natural Products

A striking feature of natural products accounting for their lasting importance in drug discovery is their unmet and still largely untapped structural diversity. In the postgenomic era with its increasing number of druggable targets, chemical diversity of screening pools is a key factor in the tremendous competition between pharmaceutical industries. In this respect, natural products remain a rather indispensable complement to synthetic compound collections. Natural products are sterically more complex and differ from synthetic compounds with respect to the statistical distribution of functionalities [4]. They cover a much larger volume of the chemical space and display a broader dispersion of structural and physicochemical properties than compounds issued from combinatorial synthesis [5]. Even though some academic groups and companies nowadays synthesise increasingly complex structures to match the chemical space occupied by natural products, a strategy referred to by Schreiber as "diversity oriented synthesis" (DOS) [6], about 40% of the chemical scaffolds found in natural products are still absent in today's medicinal chemistry [7]. Compared to synthetic molecules, a large proportion of natural products exhibit also more favourable ADME/T properties, despite the fact that they often do not satisfy "drug-likeness" criteria, such as Lipinski's Rule of Five [8]. Besides their potential as lead structures, natural products also provide attractive scaffolds for combinatorial synthesis and remain essential tools for the validation of new drug targets [9].

Challenges Faced by Industrial Natural Product Research

Despite the proven track record of natural products in drug discovery and their uncontested unique structural diversity, pharmaceutical companies have drastically scaled down or even terminated their activities in this field during the last decade [3]. Remaining operations have been quite often outsourced to biotech companies offering natural product related services such as libraries of pure compounds or semi-purified fractions. In industries where natural products remain in the screening program, they face increasing competition from other technologies such as combinatorial chemistry, drug modeling and virtual screening, for the allocation of drug discovery resources. The

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recent decline of interest observed in the pharmaceutical industry is, in part, due to the accelerated transformation of the drug discovery process during the last decade. Natural products have been facing major obstacles to fit into this new drug research environment. The advent of combinatorial chemistry and high throughput screening [10] in the 1990s enabled the testing of hundreds of thousands samples within a few weeks. This paradigm shift had major implications for natural product research. The classical and historically successful approach of screening crude or pre-purified extracts followed by several iterative steps of activity guided fractionation could not match with the short target cycle times in HTS, where testing capacities are only provided for a limited time window. A further complication is that natural product hits must go from mixture to pure compounds with enough time left for hit-to-lead assessment. Full structural information and accurate IC_{50} data are indispensable to compete with synthetic compounds in the lead selection phase. Hit clustering is gaining importance for establishing structure-activity relationship early in the lead selection process. Natural product hits are often observed as singletons, a fact that puts them at a disadvantage in comparison to compound families typically encountered in synthetic libraries.

A further issue with extract screening is the comparatively high number of false positives which are due to the common presence of compounds which display unspecific activities or interfere with the assay format. Tannins, for example, form tight complexes with metal ions and with a wide array of proteins and polysaccharides. This leads to false-positive result in most assays involving a purified protein. Detergent-like compounds have a tendency to disrupt membranes and produce misleading results in cell-based assays. Examples include widely occuring plant metabolites such as saponins, and fatty acids and panosialins, which are common in streptomycetes. Compounds such as polyenes and polyethers often display general cytotoxicity and may produce false results in cell-based assays. Strong metal chelators are susceptible to react with assay components, e.g. when nickel beads are used as linkers. UV quenchers such as the chlorophyll breakdown product phaeophorbide A, and autofluorescent compounds are prone to interfere with the readout in assays based on light measurement [11].

Confronted to unrealistic hit rates in HTS and a slow and labour-intensive deconvolution process, many pharmaceutical companies have switched from extract screening to prefractionated extracts or pure compounds libraries [12]. Large collections of compounds and semi-purified fractions have been generated using parallel fractionation and purification technology [12-15]. While these methods have the undeniable advantage of considerably reducing or even eliminating the time-consuming follow-up process, they also have some intrinsic drawbacks. Pure compound libraries will never be a full substitute for the huge structural diversity found in extracts. Trace components which are, in principle, as promising as major constituents, are likely not found in such collections. The splitting of an extract into a large number of fractions, on the other hand, leads to a considerable increase of the number of samples to be screened. This can be an issue when working with expensive assay formats or costly targets, such as recombinant proteins. A well-balanced combination of pure compounds, fractions and extracts, and a differential use thereof depending on the target and the screening format, appears to be the most promising approach.

The Discovery Process of Bioactive Natural Products

In recent years, natural product research has become a technology-driven process. The impact of HPLC-coupled spectroscopy has been tremendous. The concerted use of HPLC-DAD, -MS and -NMR has opened entirely new possibilities for the characterization of secondary metabolites in biological extracts. These techniques provide a wealth of structural information on-line with minute amounts of sample [16, 17]. Even absolute configuration of a molecule can be established using HPLC-CD [18] or HPLC-NMR after Mosher's ester derivatization [17]. With the more recent emergence of mass spectrometry-controlled preparative HPLC [19], compound purification has also become straightforward, provided the compounds exhibit sufficient chemical stability. Recent developments in NMR probe technology and higher magnetic fields [20], and miniaturization in X-ray crystallography make structural elucidation with sub-mg amounts becoming increasingly routine.

While analysis, purification and structure elucidation of natural products have experienced a technological breakthrough over the last decade, tracking bioactivity in complex matrices remains a highly challenging task. Extracts are complex mixtures. There is a continuing need for faster and more reliable methods to identify compounds that interact with therapeutic targets, with minimal interference from the multitude of chemicals present in the matrix. The classical process leading from a bioactive extract to a pharmacologically active pure constituents has always been a long and tedious process requiring substantial material amount and financial resources [21]. It consists of several consecutive steps of preparative chromatographic separation, whereby each fraction has to be submitted to suitable bioassays to track the activity ultimately to a defined pure compound. While this procedure has led to the successful isolation of many bioactive molecules, its weaknesses cannot be overlooked. Besides being slow and costly, the separation performance is poor, at least in the initial fractionation steps which are typically by open column chromatography. The loss of bioactivity in the course of the purification process is not uncommon, and there is little means for early dereplication of known or otherwise uninteresting compounds. The approach described above obviously no more matches the timelines and the workflow of modern drug discovery.

There is a compelling need for faster and more effective strategies, susceptible to be implemented in a high throughput environment. Probably the greatest challenge in this context is the judicious interfacing of chemistry and biology to correlate chemical analysis with biochemical data. The development of highly sensitive and miniaturized assays provides the technological basis for that purpose. Various innovative methodologies for the analysis of macromolecule-ligand interactions, and the on-line integration of

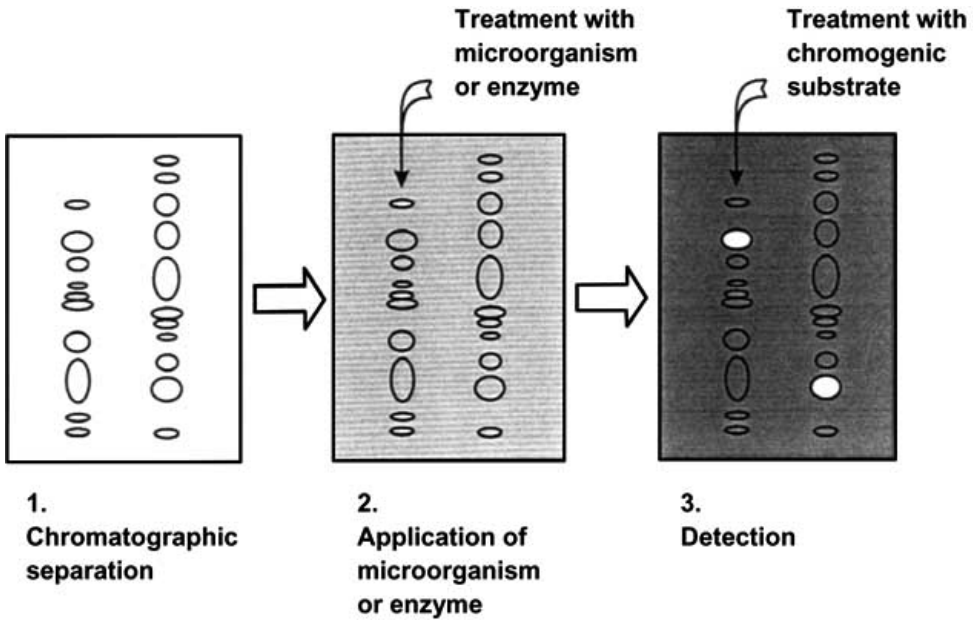


Fig. (1). Principle of bioautographic assays on TLC (reprinted from [23], with kind permission of Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart).

immunochemical and enzymatic methods with chemo-analytical systems have been recently implemented. New off-line strategies such as HPLC-based profiling, directly applicable to a broad range of mechanism-based and cellular assays, have become increasingly popular in the context of industrial natural product screening. The most significant developments in this field are presented below, together with a selection of representative examples.

BIOAUTOGRAPHY

Bioautography was the first and quite successful attempt to combine chemo-analytical and biological assay principles in a seamless manner. Early bioactivity-related detection methods for TLC were established in 1970s already [22]. Bioautography offered the possibility of directly tracking bioactive compounds in complex mixtures with minute amounts of material at hand. The principle of bioautographic assays is straightforward (Fig. **1**): A microorganism enzyme or biomolecule is applied onto a developed TLC plate. After suitable incubation, zones of inhibition may either become visible or may be visualized after a detection step involving enzymatic conversion of a chromogenic substrate [23]. Thanks to the relative simplicity of most assays and the possibility of parallel detection with various TLC staining reagents, bioautographic assays are of continuing popularity among natural product researchers.

At first, direct bioautographic assays were established for antifungal activity using spore forming fungi [22]. Due to their innocuous and simple handling in a standard phytochemical laboratory, plant pathogenic fungi, such as species of the genus *Cladosporium*, have been particularly popular. Later developments included assays for antibacterial [24] and anti-Candida [25] activities, whereby microbial reduction of tetrazolium salts and agar overlays were needed for visualization and maintenance of microbial viability. There is meanwhile a wealth of antimicrobial compounds, which have been detected and isolated upon TLC-bioautography (for a selection of recent examples see [26-29]). At the same time, new assay setups, such as 2D-TLC [30], continue to be reported and the list of organisms which have been used in combination with TLC is further expanding.

With the growing interest into natural antioxidants, assays using stable radicals such as DPPH as a detection reagent for radical scavengers were established. Although this test cannot be considered as a bioautographic assay in a strict sense, the link of chemical and biological properties is obvious and the assay predictive in the search of antioxidants. The DPPH assay was first proposed for the screening of antioxidants in marine bacteria from fish and shellfish [31]. It has become increasingly popular among phytochemists and is widely used for the detection of radical scavengers in plant [29, 32-36] and fungal [37, 38] extracts. The assay consists of spraying a methanolic solution of the purple coloured DPPH radical onto a developed TLC plate. Upon reduction, the compound turns yellow, and active compounds appear as yellows spots against a purple background. Another simple approach which has been used for the detection of antioxidants on TLC plates relies on the decolouration of β-carotene, a compound undergoing bleaching in the presence of air and light [33, 36, 39]. In this assay, substances with antioxidative properties appear as orange spots against a colourless background. After compound purification, the activity can be quantified in a corresponding solution assay using the water soluble carotenoid crocin, readily available from saffron [40].

Recently, attempts have been made to visualize natural products interactions with biomolecules, such as enzymes and nucleic acids, on TLC. Representative examples of such assays are TLC screens for new acetylcholinesterase inhibitors, as potential drugs in the treatment of Alzheimer's disease. Two assay protocols involving different detection methods have been reported. In one of them [41], the TLC plate is sprayed with the enzyme, acetylthiocholine as a

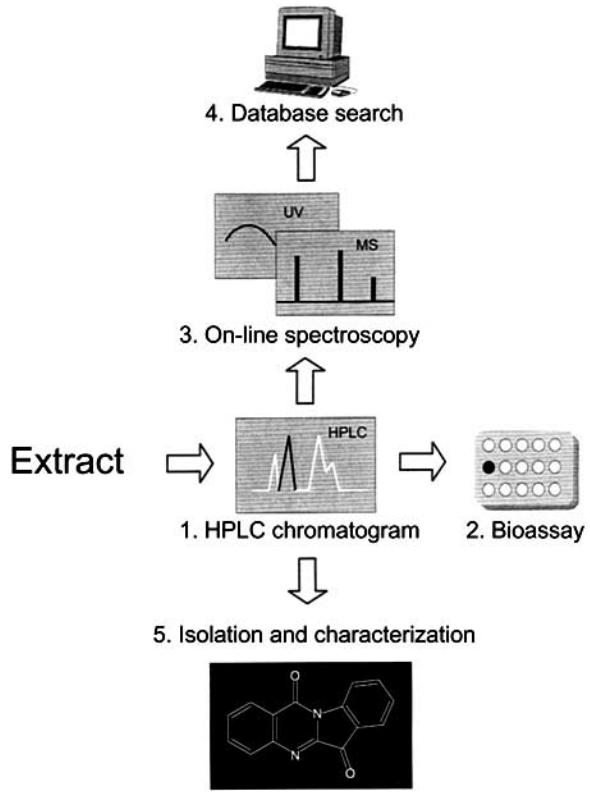


Fig. (2). Principle of HPLC-based activity profiling (reprinted from [23] with kind permission of Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart).

substrate, and Ellman's reagent. The enzyme hydrolyses acetylthiocholine resulting in thiocholine which reacts with the Ellman's reagent. The plate stains yellows and active spots appear as white spots. In an alternative method which has been applied to both actetylcholinesterase and butylcholinesterase, naphthyl acetate is used as a substrate and fast blue salt B as a detection reagent. Inhibitors of cholinesterases produce white spots on the background which is stained purple by the diazonium dye [42].

An interesting variant of bioautography has been developed for visualizing the binding properties of secondary metabolites to biomacromolecules [43, 44]. Binding can be detected via the differential chromatographic mobility of a compound with and without the presence of a target macromolecule. The method has been used in particular to detect interaction with DNA revealed by a significant decrease of the Rf value on the TLC plate.

While bioautography is inexpensive and can be set up in almost every laboratory, its applicability remains limited by mainly two factors: The restricted number of relevant biological targets, which can be developed into an assay of this format and the lack of quantitative data. As a further drawback, there is no simple correlation with structural information provided by modern LC-coupled spectroscopic techniques. Dereplication of active compounds is thus not straightforward.

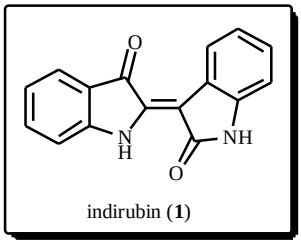
HPLC-BASED ACTIVITY PROFILING

Over the last decade, the focus in natural product analysis has shifted towards HPLC. The on-line coupling of HPLC with powerful spectroscopic methods provides a wealth of structural information from minute sample amounts without the need for tedious preparative isolation. Preparative HPLC-MS is nowadays ubiquitous as a purification method in the pharmaceutical industry. The advent of high throughput purification platforms combining UV and MS triggering modes has let to an unprecedented level of automation in natural products isolation.

With the development of microtitre based biossays, the great potential of HPLC micro-fractionation became apparent. Bioactivity can be tracked in complex mixtures without isolation of compounds and correlated with spectroscopic information available on-line. The principle of the approach is shown in Fig. **2** [45]: An extract is separated by analytical gradient HPLC. Via a T-split, a portion of the effluent is fractionated in the 96-well format, while the other part serves for the on-line spectroscopic characterization of the eluted peaks. After drying, the fractions are redissolved in a small amount of a suitable solvent, typically DMSO, and assayed for bioactivity. The activity profile is then matched with the HPLC chromatogram and the spectroscopic data. A targeted preparative isolation is carried out if the active principles are deemed of sufficient interest. While the potential of this approach has been first realized in the pharmaceutical industry, similar procedures have subsequently been implemented in academic research [46 - 49].

HPLC-Based Profiling in Medicinal Plant Research

The identification of the cyclooxygenase-2 (COX-2) inhibitory principles in *Isatis tinctoria* L. (Brassicaceae) illustrates the potential of this approach in medicinal plant research [47]. In the search for the anti-inflammatory principles in lipophilic leaf extracts of this traditional dye and medicinal plant, a pronounced COX-2 inhibitory activity had been detected in a cell-based assay with Mono Mac 6 cells. Subsequently, the extract was submitted to activity profiling for rapid identification of the active constituents. The HPLC profiles, fractionation steps and COX-2 inhibition of individual fractions are shown in Fig. **3**. In a first step, 11 fractions were taken at 8 min intervals. The inhibition profile revealed that virtually all activity of the extract was located in fraction 4. In a second round, the time window of 24-32 min was assayed at higher resolution. The COX-2 inhibitory principle was concentrated in fraction Tf 25-26 min. Finally, the active compound could be located in the shoulder at 25.0 min preceding the peak at 25.5 min. On the basis of ESI-MS and UV-vis data recorded on-line, the major peak was identified as indirubin (**1**), whereas the active compound was the indoloquinazoline alkaloid tryptanthrin (**2**) (Fig. **3**). The compound was subsequently characterized as a potent dual inhibitor of COX-2 (IC_{50} in Mono Mac 6 cells 0.037 μM)



indirubin (**1**)

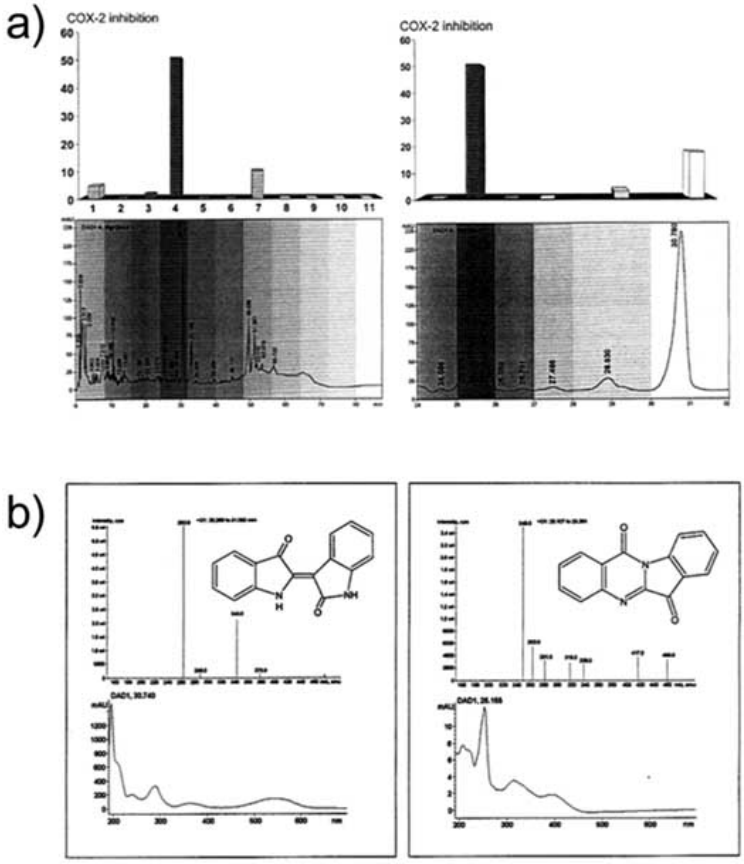


Fig. (3). Activity profiling of *Isatis tinctoria*. a) HPLC fingerprint and COX-2 inhibitory activity of a lipophilic extract. Left: full chromatogramm (0-60 min); Right: Expanded view of fraction IV (time window 24-32 min) (reprinted from [47] with kind permission of Georg Thieme Verlag KG). b) UV-vis and ESI-MS spectra of tryptanthrin (peak at 25.1 min; left) and indirubin (peak at 30.8 min; right) (reprinted from [23] with kind permission of Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart).

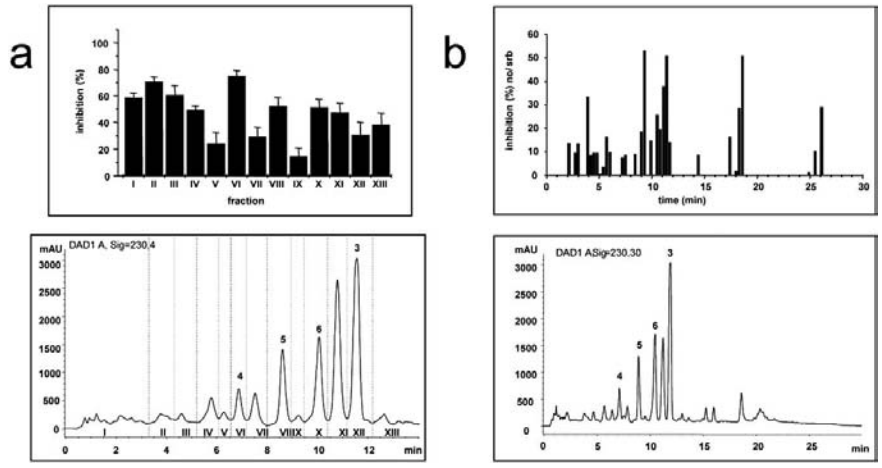
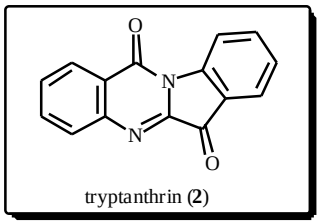


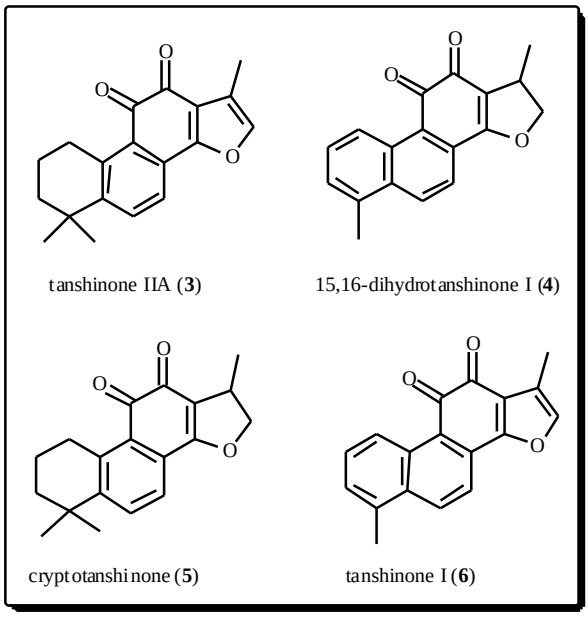
Fig. (4). Activity profile for inhibitors of MAO A (a) and iNOS induction (b) in *S. milthiorrhiza* extract. The HPLC fingerprints of the dichloromethane extract were recorded at 230 nm. (reprinted from [49] with kind permission of Georg Thieme Verlag KG).

and 5-lipoxygenase (IC_{50} in human granulocytes 0.15 μM) [48]. The entire profiling was carried out with injections corresponding to 200 μg of extract, an amount sufficient for assaying each fraction in triplicate.

Cases where a single compound is responsible for a particular pharmacological activity of a plant extract are rare. Typically, it is rather the sum of activities of structurally related compounds which contribute, to varying degrees, to



tryptanthrin (**2**)



the overall effect. A typical example is shown in Fig. **4** with the tracking of the inhibitory activity of a lipophilic extract of *Salvia miltiorrhiza* Bunge (Lamiaceae) on recombinant monoamine oxidase (MAO) A and inducible NO synthase (iNOS) [49]. *S. miltiorrhiza*, known as "Danshen", is a renowned Chinese medicinal plant chiefly used in the treatment of various cardiovascular disorders and in some infectious and inflammatory diseases. MAO A activity was assayed with a kinetic measurement of the conversion of kynuramine to 4-hydroxyquinoline [50]. Correlation of the activity profiles with the HPLC fingerprint strongly pointed towards the tanshinone-type diterpenoids as compounds with dual inhibitory properties for MAO A and iNOS induction. Targeted preparative purification afforded tanshinone IIA (**3**), 15,16-dihydrotanshinone I (**4**), cryptotanshinone (**5**) and tanshinone I (**6**). Dihydrotanshinone I was in both assays the most potent compound (MAO A: IC_{50} 23 μM; iNOS: IC_{50} 2.4 μM) while tanshinone IIA showed only marginal activity. The robustness of the kinetic assay for the detection of MAO A inhibition in the presence of potentially interfering compounds was established by spiking crude plant extracts, selected to contain diverse compounds exhibiting strong UV/Vis absorption and/or fluorescence, with small amounts of known inhibitors [50].

HPLC-Based Profiling in Industrial Environment

HPLC-based activity profiling has been widely used in natural product HTS programs to prioritize hits and guide isolation work. Prioritization is of utmost importance in a HTS environment, since screens not seldom deliver a large number of hits exceeding by far the isolation capacities. A complete work-up of all active extracts is 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. As a major asset, the microfractionation step can be performed with the bioactive sample stored in the library, without need of macroscopic isolation nor time-consuming refermentation or recollection activities and the associated issues of unsatisfactory reproducibility. With the activity profile at hand, isolation capacities can be dedicated to extracts where bioactivity correlates with chromatographic peaks. Preparative purification can be performed using a straightforward peak-guided strategy. Low priorities are assigned to extracts when activity cannot be recovered after fractionation or appears dispersed over a broad time window. The scale-up of the separation is straightforward, since the chromatographic system can be easily transposed to preparative HPLC.

Examples of HPLC-based activity profiling in the context of industrial HTS programs have been recently reported. The application of this approach in a screening program for novel glucagon receptor antagonists demonstrates its potential for early dereplication. In this project, an extract of *Streptomyces* sp. strongly inhibited glucagon induced cAMP elevation. Semipreparative gradient HPLC separation of 30 μl of extract into 30 one-minute fractions and subsequent testing of each fraction enabled the activity to be attributed to a main peak at 16.1 min. (Fig. **5**). HPLC-UV-MS analysis revealed a UV-spectrum similar to that of tryptophan and a MW of 2036 amu. Since no record corresponding to these data could be found in the literature, purification of the compound was undertaken on a preparative scale. The compound, a new bicyclic peptide (**7**), exhibited potent and selective antagonist activity towards the human glucagon receptor in a functional assay (IC_{50} 0.44 μM) [51]. At the same time, micro-scale fractionation reliably filtered out extracts in which the inhibitory activity could be assigned to known compounds of little interest, such as cephalochromin (**8**) or resistomycin (**9**).

The same strategy was successfully applied to the detection and isolation of a series of terphenylquinones as part of a HTS-supported lead finding process where more than 80'000 natural product extracts of plant and microbial origin were tested. Among other hits, an extract of a culture of the fungus *Stillbella* sp. showed a remarkable inhibition of the tyrosine kinase src, the prototype member of the src family of kinases involved in several signaling pathways**.** Comparative analysis of the LC-MS-UV dataset and the bioactivity plot of the fractionated extract indicated that the activity of this extract was based on a group of hydroxyquinone derivatives (**10**-**13**). Targeted purification of the active compounds was subsequently achieved by mass spectrometry-controlled preparative HPLC using ESIMS. The most potent compound, the trihydroxyphenyl derivative **12**, inhibits the tyrosine kinase activity of human src with an IC_{50} of 3.9 μM [52].

A similar approach was also applied in the course of a screening program for new CDK5 inhibitors. CDK5 is a cyclin dependent kinase playing a crucial role in the development of the central nervous system and the regulation of neuronal signal transduction. A lipophilic extract of the stems and leaves of the shrub *Clausena excavata* Burm. (Rutaceae) was selected upon HPLC-based activity profiling. Activity profile and spectroscopic data strongly pointed towards a compound with a UV spectrum characteristic of a 9H-carbazole chromophor. Peak-guided isolation afforded a new alkaloid (**14**) with potent antagonist activity (IC_{50} 0.51 μM) [53].

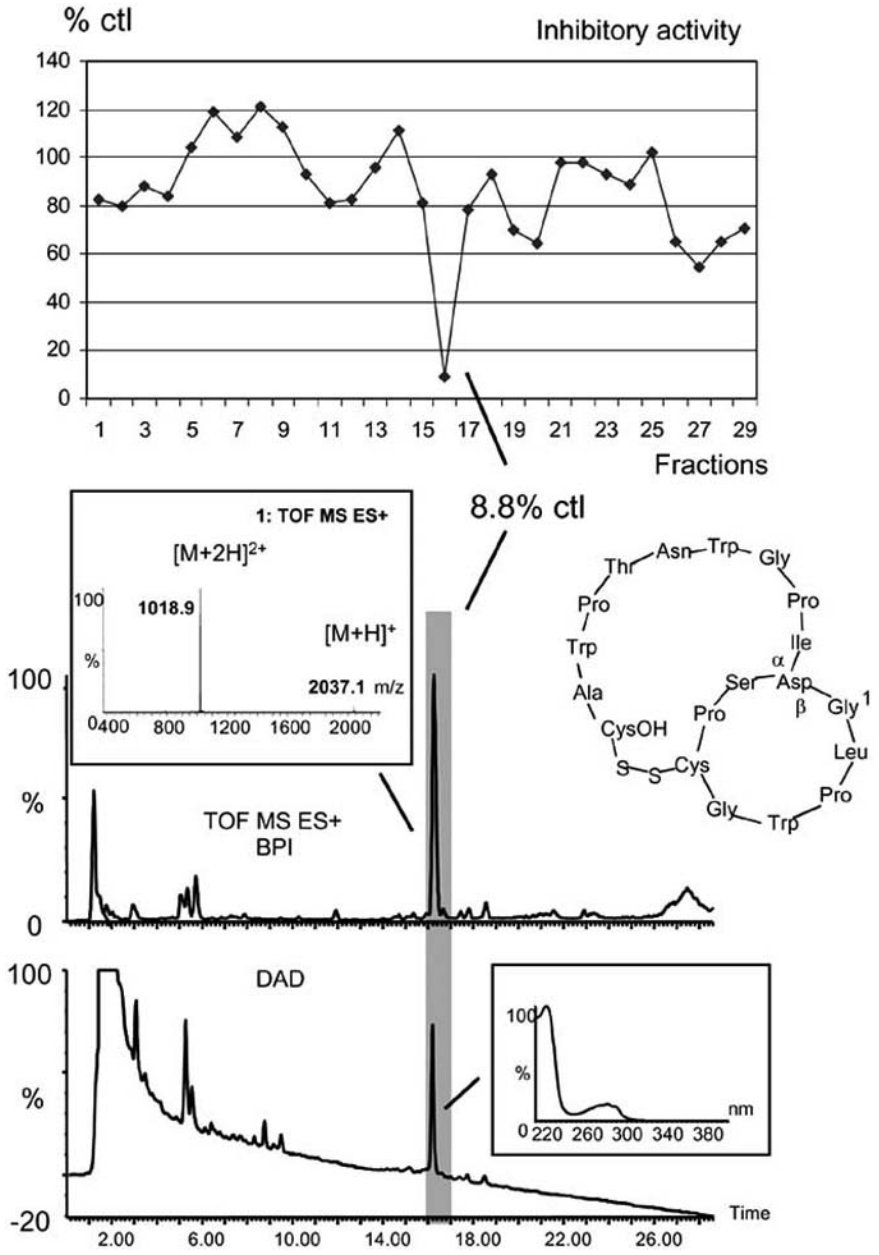
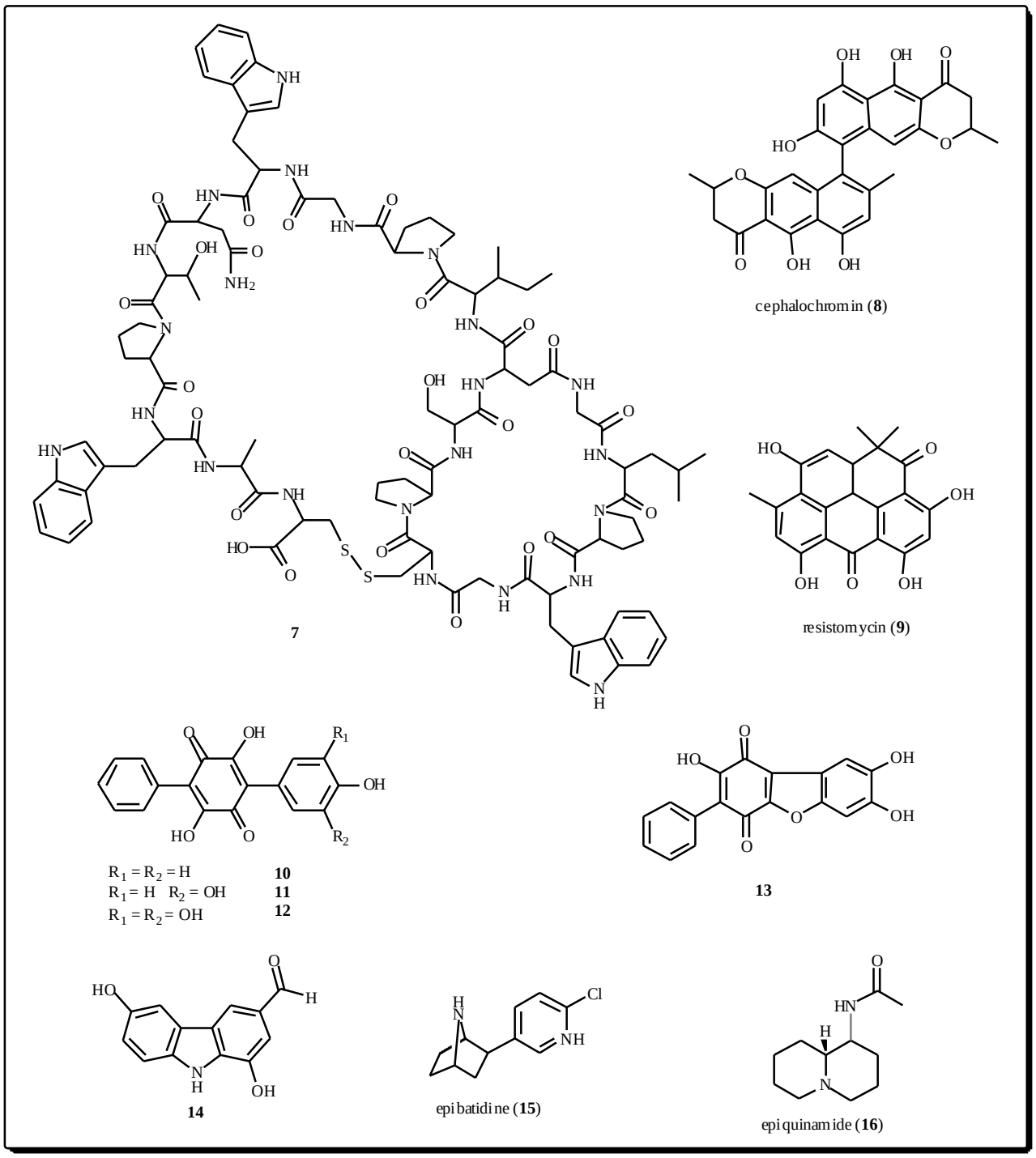


Fig. (5). Activity profiling for glucagon receptor inhibitory activity in a streptomycete extract: Activity plot, HPLC/MS/UV analysis and structure of the bioactive constituent.

For proprietary reasons, only a limited number of industrial examples have been disclosed. Nonetheless, HPLC-based profiling has become a routine strategy in HTS programs for the deconvolution of bioactivity in complex mixtures. The high flexibility with respect to the assay format enables this approach to be used basically for every screen without a need for time-consuming method development.

Further Applications

Thanks to the miniaturized format, HPLC-based profiling is ideally suited when restricted availability of the bioactive sample is an issue. In a remarkable example, microfractionation combined with an off-line 96-well functional assay was used at the NIH for the investigation of nicotinic agonists in the skin of the Ecuadorian poison frog *Epipedobates tricolor*. The activity profile allowed most of the agonist activity to be attributed to the known alkaloid epibatidine (**15**). At the same time, a less intense activity peak, eluting before epibatidine, could be discriminated. A total of 250 µg of pure compound were subsequently isolated and the structure assigned to a new quinolizidine alkaloid, epiquinamide (**16**), representing a novel structural class of nicotinic agonists with β2 selectivity [54].



HPLC-based activity profiling has been also recently reported for the search of new radical scavengers and antibacterial compounds. Scavenging activity was detected with the stable DPPH radical, while antimicrobial properties were assayed against *Streptococcus pyrogenes*. After lyophilization, the chemical reagent and the bacterial suspension, respectively, were directly added to the microplate. UV absorbance of DPPH and turbidity resulting from bacterial growth were measured using a microplate reader [55].

HPLC-BASED ON-FLOW BIOASSAYS

A particularly remarkable line of development has been in the field of continuous-flow bioassays capable of detecting the desired biological activity directly in the HPLC effluent. Most of the work in this area has been carried out at the University of Amsterdam in close collaboration with the biotech company Kiadis (formerly Screentec) in Leiden. The numerous assay formats, which have been implemented, have been comprehensively reviewed by van Elswijk and Irth [56]. In this technology, complex mixtures such as

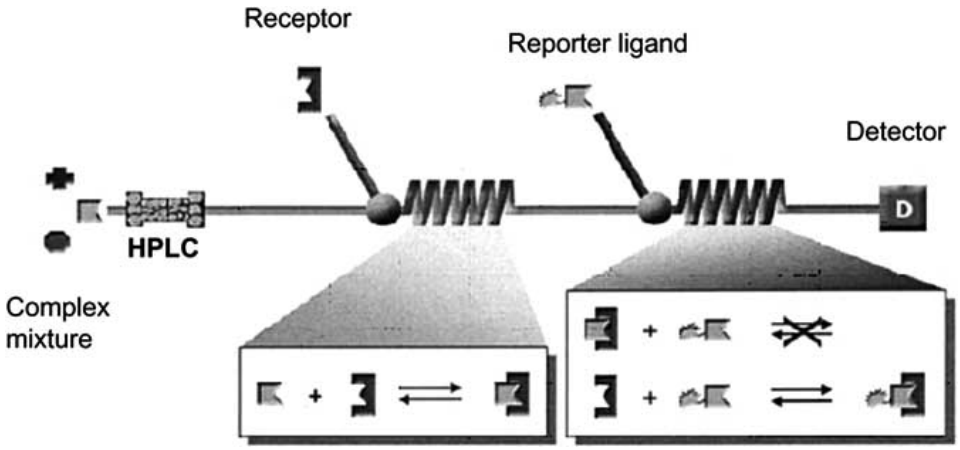


Fig. (6). Principle of an HPLC on-flow receptor assay (adapted from [56]; © 2003 Kluwer Academic Publishers, with kind permission of Springer Science and Business Media).

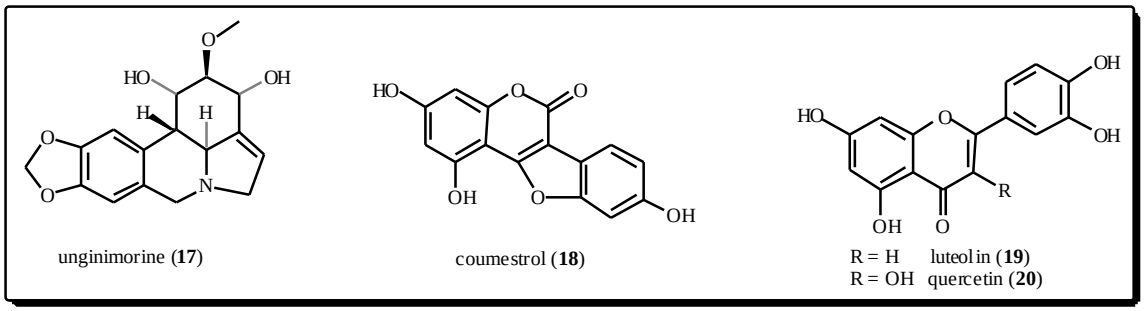
natural product extracts are separated using HPLC. A post-column biochemical assay determines the bioactivity of the effluent, while parallel mass spectrometry and diode array detection provide chemical characterization. Through additional coupling of evaporative light scattering detection (ELSD), quantitative information about the active components can be obtained. The greatest challenge of this technology is to find conditions which are compatible with both the HPLC separation and the biochemical assay. This on-line approach requires fast assays (typically 60-120 s) which match the timescale of HPLC chromatography. To increase the throughput, the LC-separation step can be omitted for primary screening and the assay performed in the flow injection mode. Active samples are subsequently re-analyzed with HPLC separation in the so-called high-resolution screening (HRS) mode.

A wide range of assay formats applicable to a large variety of protein targets have been developed. Basically, after separation by HPLC, a solution of the target is added and reacts with the analytes in a first reaction coil. A reporter ligand or substrate is subsequently added and interacts with the target in a second coil. Afterwards, the reaction products are analyzed using a UV, MS or fluorescence detector (Fig. **6**).

In one of the first on-flow applications, the Ellman's procedure was adapted for the detection of acetylcholine esterase inhibitors in natural product extracts [57]. The enzyme reaction product was detected by a UV-Vis detector at 405 nm. Presence of an inhibitor produced a negative peak. The potential of this method for screening purposes was demonstrated by the identification of ungiminorine (**17**), as a new acetylcholinesterase inhibitor in an extract of a *Narcissus* cultivar [58].

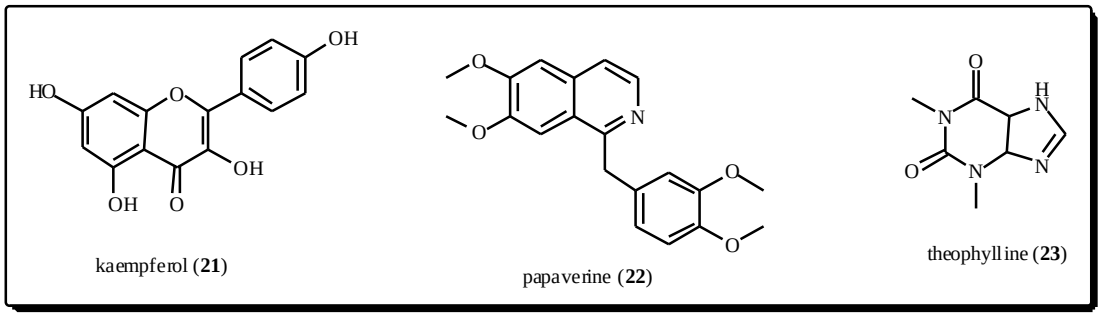
Fluorescence-Based Detection Systems

Current on-flow systems mostly use fluorescence-based readouts. In a common assay format, competition between binding compounds in the sample and a fluorescence labeled ligand is detected by means of a temporary fluorescence decrease in the effluent [56]. This approach has been used in particular for the detection of estrogen receptor α and β binding activity in plant extracts [59]. Coumestrol (**18**), a high affinity ligand for the oestrogen receptor was used as a reporter molecule. The binding of coumestrol to the receptor induces a blue shift and four times increase in fluorescence intensity. Luteolin (**19**), quercetin (**20**) and kaempferol (**21**) could be identified as active components of a pomegranate (*Punica granatum* L.; Punicaceae) pericarp extract [56]. An alternative procedure well suited for screening enzyme inhibitors is based on the measurement of substrate conversion. This assay format is applicable to various classes of enzymes, such as kinases, phosphatases, phosphodiesterases, and caspases. Presence of an inhibitor in the effluent produces a change in fluorescence intensity due to reduced substrate conversion. In the majority of cases, the substrate used does not exhibit high fluorescence prior to its conversion so that a negative response is obtained. An increase of fluorescence can be observed when the fluorescence of the substrate is decreased by the conversion. This assay format has been used for the screening of



phosphodiesterase (PDE) inhibitors [60]. This enzyme family hydrolyses cGMP and cAMP to GMP and AMP, respectively, and has attracted much attention recently since selective inhibitors have a therapeutic potential in diseases such as hypertension, asthma, heart failure and erectile dysfunction. The test system was based on the conversion of the fluorescent reporter substrate mant-cGMP into the less fluorescent mant-GMP. Active compounds were detected by means of an increase in fluorescence. The assay was validated by spiking extracts with the known PDE inhibitors papaverine (**22**) and theophylline (**23**).

Besides the research work carried out at the University of Amsterdam, there have been a few attempts to establish on-line detection systems for biologically relevant properties. Of particular interest is an on-line HPLC procedure for the detection of radical scavenging compounds in plant extracts. The method measures the reduction of the DPPH radical and can be run with mobile phase compositions ranging from 10 to 90% organic solvent in water or buffer. Reduction of the



In spite of their intrinsic selectivity, fluorescence-based assays suffer from background interferences which may decrease their sensitivity and lead to the assignment of false positives. Therefore, attempts have been made to switch to detection methods with increased selectivity, such as fluorescence resonance energy transfer detection (FRET). The suitability of FRET for on-flow systems has been demonstrated using the protease subtilisin as a model enzyme [61]. A further selectivity increase could be achieved with time-resolved FRET, which entirely suppresses the interferences from short lived fluorescence. A flow injection kinase assay using TR-FRET has been recently reported. The assay measures the phosphorylation of poly(GT)-biotin by the receptor tyrosine kinase EGFR [62]. While FRET fluorescence has been applied so far only to flow injection assays, work aiming at coupling this method to liquid chromatography is ongoing.

MS-Based Detection Systems

Mass spectrometry is an attractive alternative to fluorescence detection in on-flow assays. The principle consists of continuously monitoring a MS reporter molecule at its specific m/z. A major advantage over a fluorescence based measurement is that it does not require the synthesis and purification of labeled ligands [56]. As a further asset, this format is insensitive to natively fluorescent compounds which often cause problems in the screening of natural product extracts. The feasibility of MS based detection was demonstrated using streptavidin/biotin and anti-digoxigenin/digoxin competitition model assays. Subsequently, a substrate conversion based assay involving the detection of the reaction products by ESI-MS was described [63]. The assay was validated with the cysteine protease cathepsin B. It is based on the decrease of the MS signal corresponding to the reaction product in response to the presence of an inhibitor in the effluent. The reduction of the product signal can be readily detected using the single ion trace locked on the product mass. Operating the MS in the scan mode provides structural information about the compounds responsible for the inhibition.

radical is detected as a negative absorbance peak at 517 nm [64].

Full integration of the bioassay into the HPLC flow, providing both chemical and biological information in a single run, can be viewed as the ultimate achievement in interfacing bioactivity and chromatography. However, there are some limitations for on-line systems: Separation and bioassay conditions must be compatible, or rendered to be so, with the aid of make-up buffers. The considerable consumption of immunochemicals, receptors or enzymes may represent a major cost factor when operating such on-line assays. The resolution is typically lower than in an off-line configuration, since band broadening is induced by the post-column reaction. A further drawback in comparison to the off-line HPLC-based activity profiling is that on-line assays require substantial development and optimization for each target to be screened.

CAPILLARY ELECTROPHORESIS-BASED ASSAYS

Capillary electrophoresis is a well-established technique for high resolution separation of compounds based on their charge-to-mass ratio and shape. Affinity capillary electrophoresis (ACE) detects the binding of small molecules to a labeled macromolecule (enzyme, receptor, nucleic acids) through a shift in electrophoretic mobility due to changes in surface charges or target conformation. The ACE technology has been recently optimized for the detection of bioactive compounds in complex mixtures such as natural product extracts [65]. An ACE screening platform has been commercialized by the biotech company Cetek Corporation (Marlborough, MA), which claims to have established validated assays for more than 150 pharmaceutical targets (CE Assay™). The basic principle is the detection of a shift in the capillary electrophoretic mobility of a fluorescently-labeled target in response to the binding of an active compound. The technology is well suited for the screening of natural product crude extracts, combinatorial libraries and

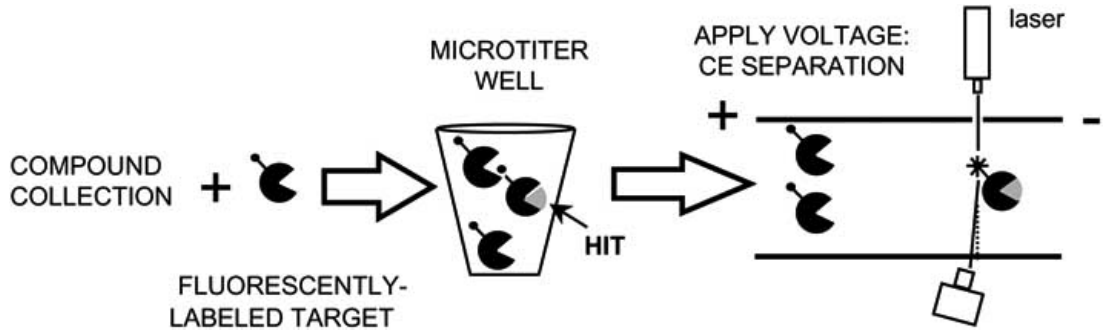
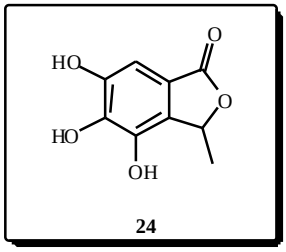


Fig. (7). Principle of a CE screening assay. Target-ligand complexes show different CE migration than unbound target.

compound collections. In this technology, a soluble macromolecular target is first incubated with the compounds to be tested. The ligand-target complexes are then electrophoretically separated and detected with laser-induced fluorescence (Fig. **7**). By modifying the separation conditions (run length, temperature, buffer composition), it is possible to discriminate between strong, moderate and weak binders. Since nuisance compounds migrate away from the labeled target, the methods allows the detection of specific ligands in the presence or interfering substances typically found in natural product extracts. In addition to measurement of functional activity, this method provides information on the binding affinity of ligands and appears well-suited for the investigation of protein-protein interactions. Automation of ACE to a throughput of approximately 1500 analysis per day by using multiple capillary channels has been reported. For subsequent characterization, ligands bound to the target can be purified using a collection reservoir [66]. In a very recent development, the setup of ACE on microchips has been achieved, enabling further miniaturization [67]. ACE has been in particular reported as a primary screening assay to discover binders to Akt1, a key component of biochemical pathways that control apoptosis. Screening of a crude natural extract library resulted in the detection and isolation of a new phthalide (**24**) from a strain of the fungus *Oidiodendron* [68].



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Capillary electrophoresis can be used for screening inhibitors in functional assays as well. CE is well suited for measuring enzymatic activity in minute biological samples. Assay protocols for a variety of enzymes including transferases, oxidoreductases, lyases and hydrolases have been reported [69]. Both off-line and on-line formats have been developed. CE enzymatic assays have been recently adapted for the detection of inhibitors in complex mixtures such as natural product extracts. The enzymatic reaction can be performed pre-column, post-column or on-line. In the pre-column format, the enzyme, the substrate and the compounds to be tested are first incubated. The products of the reaction are then injected into the CE column and the conversion of the substrate monitored using UV absorption or laser-induced fluorescence detection. In an on-line homogeneous version referred to as electrophoretic mediated microanalysis (EMMA) [69, 70], all the steps of the assay including mixing, separation and detection take place within the capillary. One of the greatest advantages of EMMA is that it provides fractionation of sample components before reaction with the substrate. In addition, the reaction products can be separated as well. The feasibility of EMMA technology as a screening method has been demonstrated by the detection of protein phosphatase inhibitors in natural product extracts. [71]. Briefly, the test extract and the substrate are dissolved in the CE running buffer. The enzyme is injected and allowed to equilibrate with the test extract. The voltage is switched off for a brief incubation time (15 s). When the voltage is switched on, the reaction products migrate to the detection window. In the electropherogram, the substrate is observed as a negative peak due to its partial conversion into the product, while the latter appears as a positive signal. Of particular interest is the fact that inhibition properties can be simultaneously assessed towards a mixture of related targets, since the enzymes have been electrophoretically separated in the initial step of EMMA.

MOLECULAR IMPRINTED POLYMERS (MIP)

Molecular imprinting, first introduced in 1972 by Wulff and Sarhan [72], produces materials with "antibody-like" selectivity [73, 74]. Molecular imprinted materials have found numerous applications. The technology is well established and imprinting of small, organic molecule has become almost routine [75]. The process of molecular imprinting consists of three successive steps (Fig **8**): First, a functional monomer and a target molecule used as a template form complexes or covalently react in solution. The imprint-monomers complexes are then fixed by cross-linking polymerization. Finally, the template is removed through extraction or hydrolysis. Vacant recognition sites of specific shape decorated with functional groups complementary to the original print molecule remain [73]. While covalent imprinting yields a higher density and more homogeneous population of binding sites than the non covalent approach, the latter technique is more flexible with respect to the choice of functional monomers and possible target molecules [75]. The numerous applications of molecular imprinting for screening compounds of biological origin have been recently

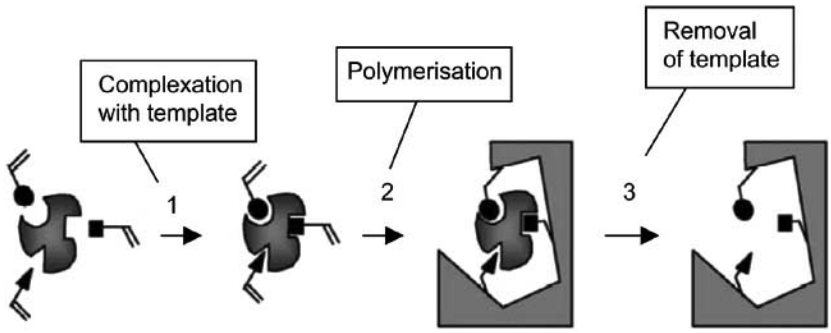
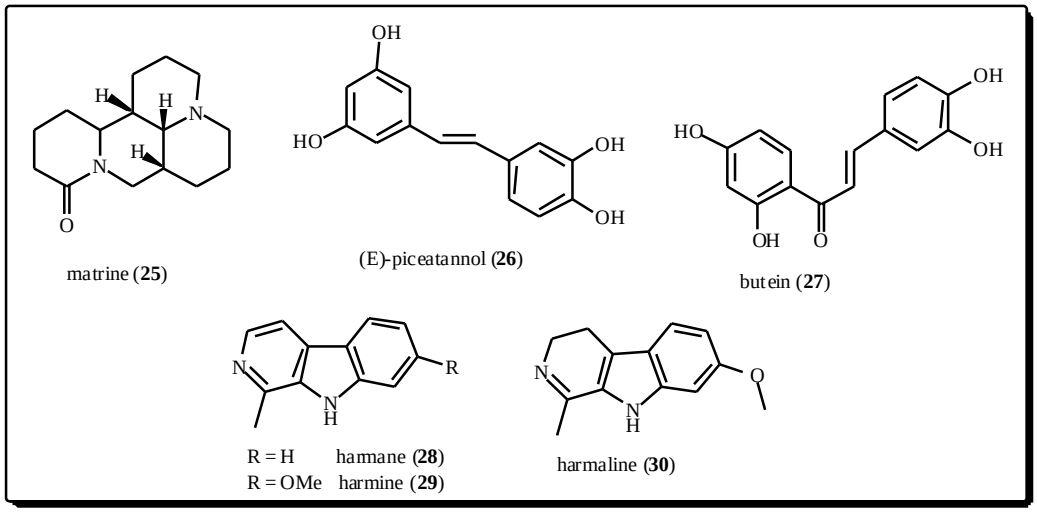


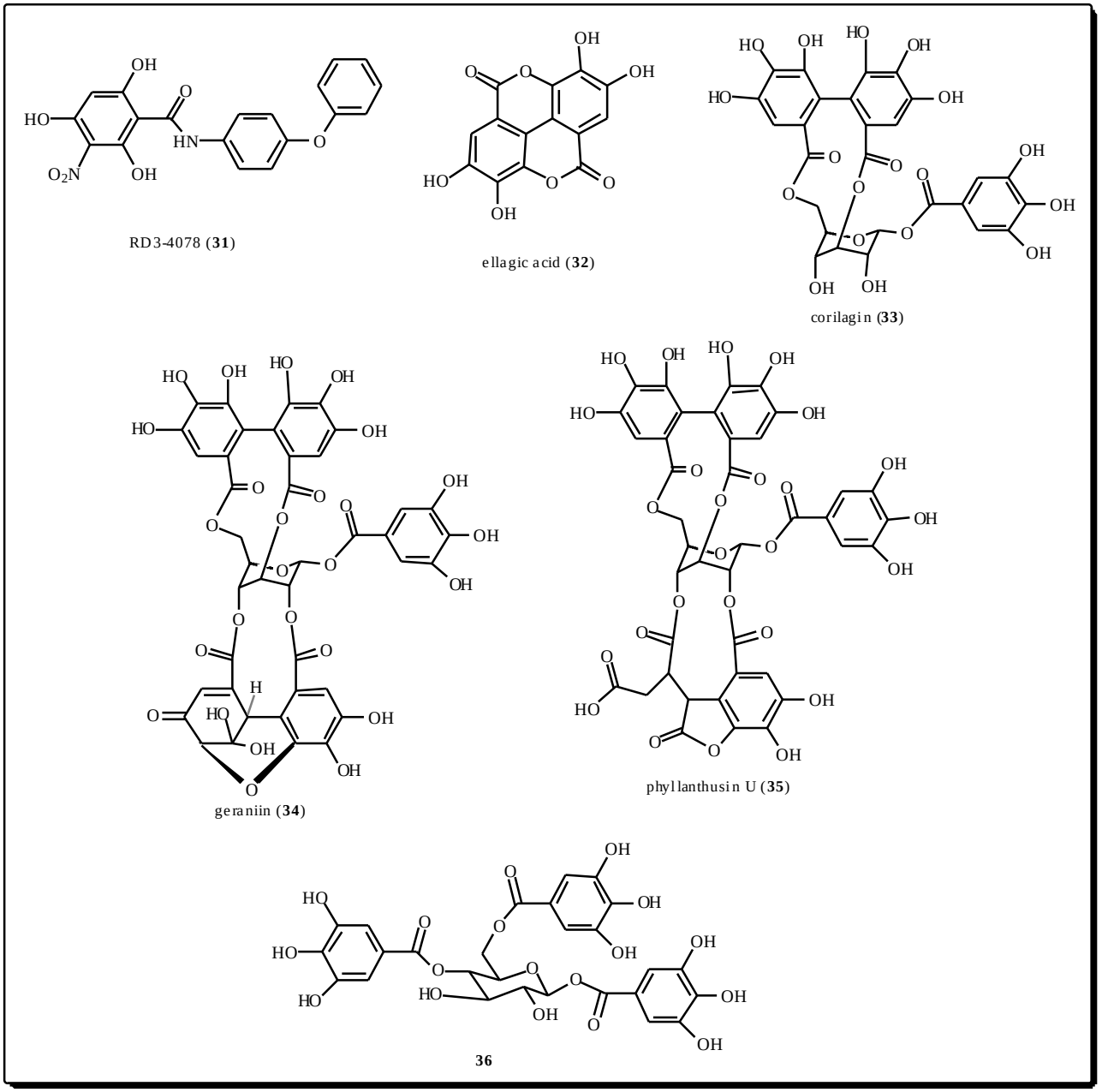
Fig. (8). Outline of the molecular imprinting process. (Adapted from [82]; © 2002 Kluwer Academic Publishers with kind permission of Springer Science and Business Media).

reviewed by Xu and Chen [73]. Molecular imprinted polymers (MIP) are well suited for a selective trapping of target molecules in complex matrices. Solid phase extraction (SPE) of various compounds from plant extracts or biological fluids has been reported. Typical examples include the preparative separation of matrine (**25**) from the Chinese medicinal plant *Sophora flavescens* Ait (Leguminosae) [76], and the selective extraction of flavonoids from *Gingko biloba* leaves [77]. A particularly interesting feature of molecular imprinting is that structural analoges of the template molecule often also fit into the imprinted cavities. Thus, a known inhibitor of a receptor or an enzyme can be used as template to screen for unknown structurally related inhibitors. In a representative example of this approach, quercetin (**20**), a known protein tyrosine kinase inhibitor was used as a template to search for inhibitors of the epidermal growth factor (EGFR) in *Caragana jubata* (Pall.) Poir. (Leguminosae), a traditional Tibetan medicine [78]. Two new inhibitors, (E)-piceatannol (**26**) (IC_{50} = 4.5 μM) and butein (**27**) (IC_{50} 10 μM) could be selectively separated from the EtOAc extract, and were shown to be the main active constituents of the extract. Interestingly, both compounds exhibited stronger activity than the template quercetin itself (IC_{50} = 15 μM). Molecular imprinted stationary phases have been used in analytical chromatographic and electrophoretic separation systems as well. A LC-MS system equipped with a MIP column imprinted with harmane (**28**) was used to identify harmine (**29**) and harmaline (**30**) as the antitumor components from the methanolic extract of *Peganum nigellastrum* Bunge (Zygophyllaceae) seeds [79]. In another example, LC separation on MIP phases enabled to selectively trap inhibitors of the hepatitis C virus NS3 protease in a crude extract of *Phyllanthus urinaria* L. (Euphorbiaceae). The known inhibitor RD3-4078 (**31**) was used as a template, and ellagic acid (**32**), corilagin (**33**), geraniin (**34**), phyllanthusiin U (**35**) and 1,3,6-tri-O-galloyl-β-D-glucose (**36**) were selectively separated and identified. These data were in good agreement with results obtained with frontal immunoaffinity chromatography (see below) [80]. In the form of thin films, MIPs are well suited for biosensor applications. In a recent example, a biomimetic covalently imprinted polymeric sensor with subpicomolar affinity has been developed for the δ-opioid G-protein coupled receptor agonist [D-Pen^2, D-Pen^5] enkephalin [81].

MS-BASED METHODS

A growing number of MS-based approaches taking advantage of the selectivity and high affinity of macromolecule-ligand interactions have been proposed as alternative screening methods for drug discovery. The developments in this field have been recently discussed in an excellent review which pays attention to both the technical aspects and the potential of the respective methods as tools





for drug discovery [83]. Basically, most MS-based methods expose a mixture of potential ligands to a target and use MS to detect binders and non-binding molecules by comparing their elution profiles from the compartment in which a target is immobilized or otherwise confined. Alternatively, pure spectroscopic methods directly detect ligand-macromolecule ions in the gas phase. A selection of the most promising procedures which have been recently implemented in drug discovery are presented below.

Pulsed Ultrafiltration Mass Spectrometry

Pulsed ultrafiltration mass spectrometry is a versatile MS-based approach originally developed by the group of van Breemen at the University of Illinois at Chicago. The method was initially established for the deconvolution of combinatorial libraries [84], but numerous applications have since demonstrated its potential for the screening of natural product extracts as well. Pulsed ultrafiltration MS can also be used for measuring ligand-receptor kinetic and thermodynamic binding parameters. Basically, a pulse containing the compounds to be analysed is injected into a flow-through chamber containing a macromolecular receptor. The chamber is equiped with an ultrafiltration membrane which retains macromolecules and receptor-ligand complexes but allows low molecular compounds to pass through. Once the unbound material has been washed away with water, the protein-ligand complexes are disrupted by addition of an organic modifier to the mobile phase or by a change in pH. Eluted compounds can be directly analysed by MS or, alternatively, trapped on an HPLC column to

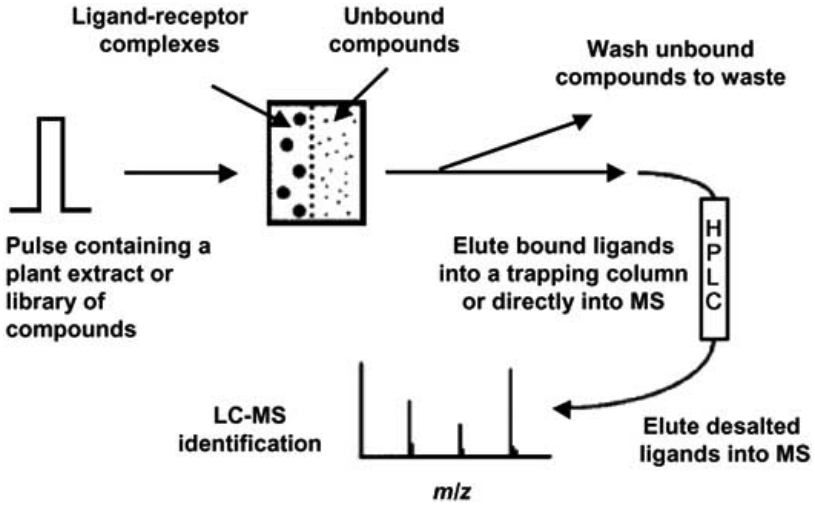
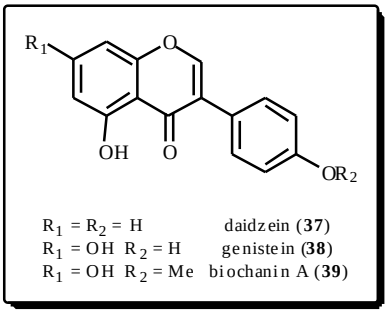


Fig. (9). Principle of pulsed ultrafiltration mass spectrometry (reprinted from [83]; © 2004 by Wiley Periodicals Inc., with permission of John Wiley & Sons, Inc.).

provide increased selectivity (Fig. **9**) [85]. By connecting multiple ultrafiltration chambers in parallel to the mass spectrometer, high throughput can be achieved. Compared to other techniques used for affinity selection, a major advantage of pulsed ultrafiltration is that it allows ligands and receptors to interact in solution. This prevents alteration of the binding characteristics, which may result from the covalent immobilization of a macromolecular target on a solid support.

Pulsed ultrafiltration MS has been used to identify and isolate ligands for various proteins including carbonic anhydrase [86], dihydrofolate reductase [87], cyclooxygenase-2 (COX-2) [88], estrogen receptor (ER) β and α [89]. Its potential for the investigation of botanical extracts is well illustrated by the detection and identification of the isoflavones daidzein (**37**), genistein (**38**) and bioachanin A (**39**) as ER β and α ligands in a chloroform extract of red clover (*Trifolium pratense* L., Leguminosae), a plant commonly used for the treatment of menopausal symptoms [89]. In this study, binding specificity was checked with a negative control in which the receptor was omitted in the ultrafiltration chamber. By a similar approach, linoleic acid was identified as an ER ligand in an extract from the fruits of chaste–berry (*Vitex agnus-castus* L. Verbenaceae), another traditional herbal remedy used for menstrual and hormonal disorders [90]. Pulsed ultrafiltration mass spectrometry also provides information on the relative binding affinity of ligands, as recently demonstrated with a series of estrogens and the ER α and ER β receptors [91].



Frontal Affinity Chromatography-Mass Spectrometry

The coupling of frontal affinity chromatography (FAC) to mass spectrometry by Schreimer and Hindsgaul [92] led the way to its utilization as a screening tool for receptor ligands and enzyme inhibitors [93]. In FAC, a receptor or an enzyme is immobilized on a suitable support material and packed in a column. A mixture containing potential ligands is continuously infused through the column (frontal mode), and the compounds are monitored in the effluent by mass spectrometry. Active compounds will bind to the column but eventually the capacity of the column will be exceeded, which results in the ligand breaking through at their infusion concentration. The elution delay observed compared to a reference compound such as methanol reflects the respective binding affinity of the ligands (Fig. **10**). Miniaturized FAC assays on PEEK capillary columns (column volume ca. 20 μl) have been established. The method has been first employed to screen oligosaccharide and peptide libraries for antibody, lectin and protease ligands. Later, chromatographic assays involving estrogen receptor β, sorbitol dehydrogenase, human α-thrombin, cholera toxin B subunit and β-galactosidase were established on microaffinity columns [94]. The applications of FAC/MS in drug discovery are continuously expanding. A FAC/MS kinase assay which enables simultaneous monitoring of binding at the ATP and substrate binding sites has been recently reported [95]. The assay opens new perspectives in the screening of kinase inhibitors.

Frontal immunoaffinity chromatography is a special application of FAC, which provides an alternative to the use of molecular imprinted polymers. Here, polyclonal antibodies are raised against a known inhibitor in order to mimic the binding properties of an enzyme or a receptor. After purification, the antibodies are immobilized on a suitable support and packed into a column. Frontal immunoaffinity chromatography has been used to characterize the binding properties of constituents in an extract of *Phyllantus urinaria* L. towards hepatitis C virus NS3 protease [80]. Brevifolin (**40**), brevifolin carboxylic acid (**41**), ellagic acid (**32**), corilagin (**33**) and phyllanthusin U (**35**) were identified as inhibitors. In first approximation, a

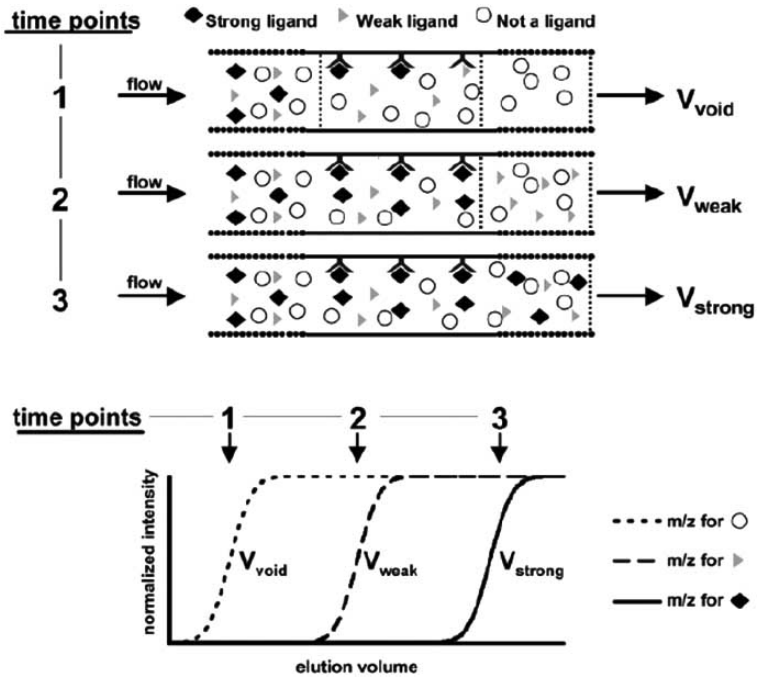
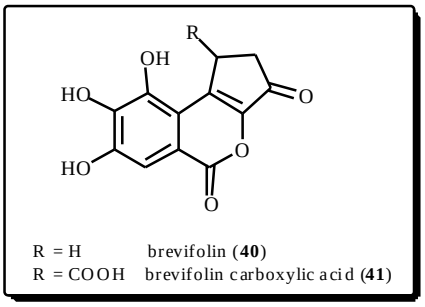


Fig. (10). Frontal affinity chromatography-MS. Panel A: Three stages of an experiment in which a mixture of three components with no, weak, and high affinity, respectively, is analyzed. Panel B: Elution profiles of the analytes. (reprinted from [83]; © 2004 by Wiley Periodicals Inc., with permission of John Wiley & Sons, Inc.).

linear relationship was found between the elution delay (frontal time) and the log(IC_{50}). This approach was also compared to MIP for the investigation of EGFR inhibitors in an extract of *Caragana jubata* (Pall.) Poir. (Leguminosae) [96]. MIP and polyclonal antibodies appear to be complementary techniques having both their own merits and limitations. Polyclonal antibodies require 10 to 100 times less template molecule to be produced. MIPs, on the other hand, possess superior chemical and physical stability, a fact that opens a broader range of possible applications.



R = H brevifolin (**40**)
R = COOH brevifolin carboxylic acid (**41**)

Affinity Size Exclusion-Mass Spectrometry

Affinity size exclusion-mass spectrometry (SEC-MS) is an attractive approach requiring no immobilization or confinement of the macromolecular target. In this method, a target protein is incubated with a mixture of potential ligands. Target-bound ligands are subsequently separated from non-binding compounds using rapid size exclusion chromatography: The receptor with bound ligands is excluded from the column and the excess unbound ligands are eluted in the included volume bound to the targets. After the separation, an aliquot of the eluted receptor-ligand complex is trapped on a reversed phase cartridge, desalted and analysed using electrospray mass spectrometry. Under the conditions used for the elution off the cartridges, the complexes dissociate readily and the ligands can be identified from their mass spectrum [97]. The online coupling SEC with liquid chromatography-mass spectrometry has been later achieved to improve the sensitivity of the method [98]. The simplicity of SEC is attractive, as no immobilization of the target on a support is needed. A potential liability of the method is that the size-exclusion step is carried out under nonequilibrium conditions favoring protein/ligand complex dissociation. As a consequence, recovery efficiency can be low and strongly depends on the SEC isolation time. This aspect further limits the applicability of this method to compounds binding tightly to the protein target.

Multitarget Affinity/Specificity Screening

Multitarget affinity/specificity screening (MASS) is a pure MS method for investigating biomolecular interactions. It relies on the detection of ions from noncovalent complexes in the gas phase using mass spectrometry with very soft ionization methods such as electrospray [99]. This approach has been in particular used to measure binding constants between aminoglycoside antibiotics and the decoding region of the prokaryotic 16S rRNA. Multiplexing both targets and compound collections enabled the method to be run on a high throughput level. The applicability of this strategy for the screening of natural product extracts was exemplified by testing prefractionated streptomycete extracts for new inhibitors of bacterial protein synthesis [100]. Using both a specific (16S) and a non specific (16Sc) RNA construct it was possible to assess binding specificity.

NMR-BASED APPROACHES

Over the past few years NMR has emerged as a powerful method for identification of binding molecules directly from mixtures. Numerous approaches have been developed which selectively detect protein ligand interactions [101, 102] by observing either the resonances of the macromolecular target or the ligand. Binding of a small molecule to a protein or another macromolecular target alters the local chemical environment of the binding site and thereby causes changes of the NMR chemical shifts at the binding site. Protein ligand interaction can also be detected by means of intermolecular magnetization transfer or changes in sign of the nuclear Overhauser effect. NMR can be used to characterize both high- and low-affinity macromolecular complexes. One of the most common approach based on target resonance detection consists of analysing chemical shift changes in a ^{15}N-^{1}H HSQC spectrum of a ^{15}N-labeled target protein by comparing the spectra with and without a cocktails of low MW compounds [103]. The amide signals can be well assigned providing structural information on the binding site. Binding constants can be determined by measuring the changes in the 2D HSQC spectrum as function of ligand concentration. Limitations of this approach is that it is only suitable for small proteins (MW < 30 to 40 KDa) and requires large amount of uniformly ^{15}N-labeled protein [101]. On the other hand, methods based on ligand resonance detection have the intrinsic advantage that they typically do not require isotopic labelling of the protein. They represent a rapid means for ligand identification but have limited applicability for detecting strongly binding ligands (K_d < micromolar concentration) with slow dissociation rates, since the ligands are in high excess. They also do not provide information about the binding site. Ligand resonance observation methods can be divided into two main classes: (i) the detection of an altered hydrodynamic property of the molecule (diffusion- and relaxation-edited experiments, trNOE technique) or (ii) the transfer of magnetisation between the target and the ligand. Particularly attractive are saturation transfer difference (STD) NMR [104] and water-ligand observed via gradient spectroscopy (WaterLOGSY) [105, 106], since they require 10- to 1000-fold lower protein concentration than other methods. STD NMR relies on transfer of saturation from the protein to the ligand to detect binding. STD NMR spectroscopy of mixtures of potential ligands with as little as 1 nmol of protein yields spectra showing exclusively signals from molecules with binding affinity. In addition, the ligand binding epitope can be identified, since residues in direct contact to the protein show stronger signals [104]. WaterLOGSY is a related method which detects the transfer of magnetization from bulk water to the protein binding site and onto a bound ligand. In a recent example of NMR application in the field of natural product screening, STD-NMR has been used to investigate the binding of the flavonoid luteoline (**19**) and its 7-O-β-D-glucopyranoside to the nucleotide binding domain of the mouse multiple drug resistance transporter [107]. NMR approaches have been implemented for the identification of inhibitors in functional assays. A method named 3-FABS (three fluorine atoms for biochemical screening) uses ^{19}F NMR spectroscopy after substrate labelling with CF_3 for the detection of the starting and enzymatically modified substrates [108]. The method has been validated with the detection of AKT1 and trypsin inhibitors.

AFFINITY MICROCHIPS

Microfluidic systems have gained growing application in analytical processes and bioassays [109, 110]. In this context, there have been attempts to develop binding assays on microchip format. A patent application for microscale affinity separation on microchips has been recently filed by NEC Corporation (Japan) [111]. The device consists of a large number of columnar bodies. Their surfaces are coated with an adsorbent material on which a target molecule of a particular specificity is immobilized. Compounds to be tested are flushed through the system. After washing the flow path with a buffer to remove unbound material, a solvent is supplied to desorb the bound ligands, which can be collected for subsequent analysis. The setup is suited for assaying a broad range of macromolecule-macromolecule and macromolecule-ligand interactions such as protein-protein, protein-nucleic acid, nucleic acid-nucleic acid, enzyme-substrate, enzyme-inhibitor, carbohydrate-lectin and ligand-receptor interactions.

BIOSENSORS

Biosensors open new perspectives for the development of highly miniaturized on-flow assays. Biosensors are devices combining a biological component with a suitable physical transducer (Fig **11**). The biological portion consists of immobilized enzymes, receptors, antibodies or whole cells and determines the specificity of the sensor. Electrical devices, such as electrodes and semiconductors, or optical components such as fiber optics constitute the transducer elements of the biosensor [112]. In addition, a technique known as surface plasmon resonance (SPR) has recently received considerable attention for biosensing applications. SPR is a surface-based detection technology, which measures interaction between an analyte and a receptor through a change of the angle of minimum reflected light. SPR enables label-free, real-time monitoring of complex formation and dissociation [113]. Biosensors were initially developed for clinical diagnosis and meanwhile widely used for routine analysis in areas such as food technology (for a recent review see [114]), environmental analysis or fermentation process controlling. On the other hand, there have been comparatively fewer applications of biosensors for screening purposes. A number of enzyme-based sensors, usually specific for a particular class of compounds, have been designed for screening substances with particular chemical functionalities. A cystein-sulfoxide biosensor based on the enzyme alliinase and an ammonia gas electrode has been developed (Fig **12**). It is based on the conversion of cystein sulfoxides such as alliine into sulfenic and pyruvic acids and ammonia [115, 116]. The sensor had a detection limit of 0.5 mM alliine and could be operated over 400 h. It was used for the screening of *Allium* species from various origins for the presence of cysteine sulfoxides. An ammonia electrode was also employed in a sensor for cyanogenic compounds such as cyanogenic glucosides in plant extracts. The electrode was combined with the enzyme cyanidase which hydrolyses cyanide to formic acid and ammonia [117]. The enzyme cyanidase appears to be stable over years, and the detection limit was around 0.1 mM cyanide. Several

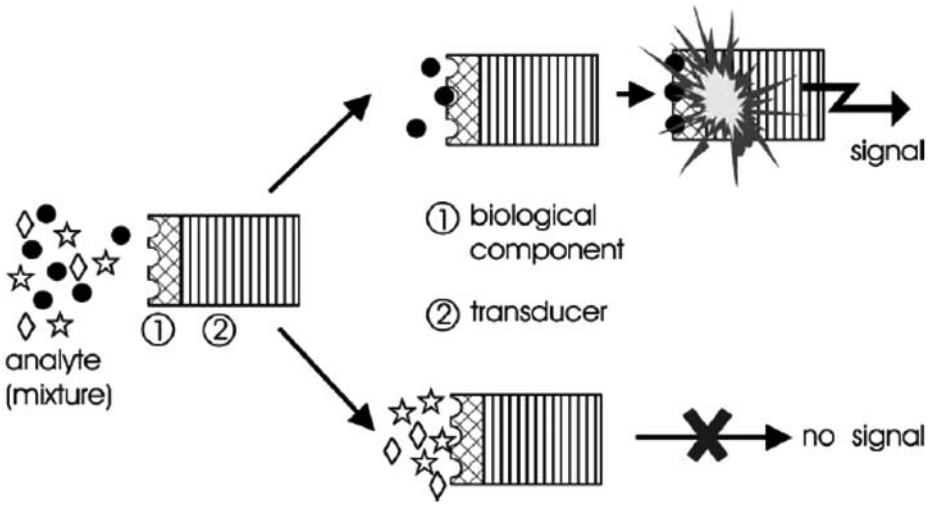


Fig. (11). Principle of function of a biosensor (reprinted from [112]; © Springer-Verlag 2002, with kind permission of Springer Science and Business Media).

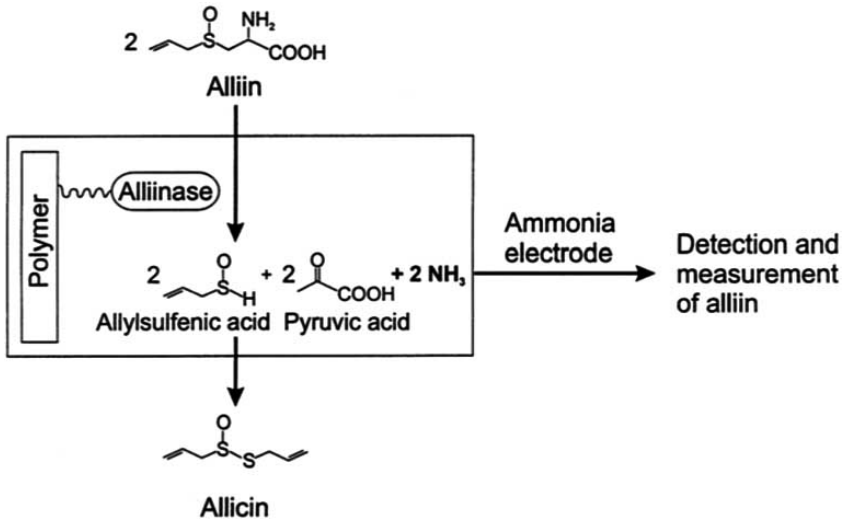


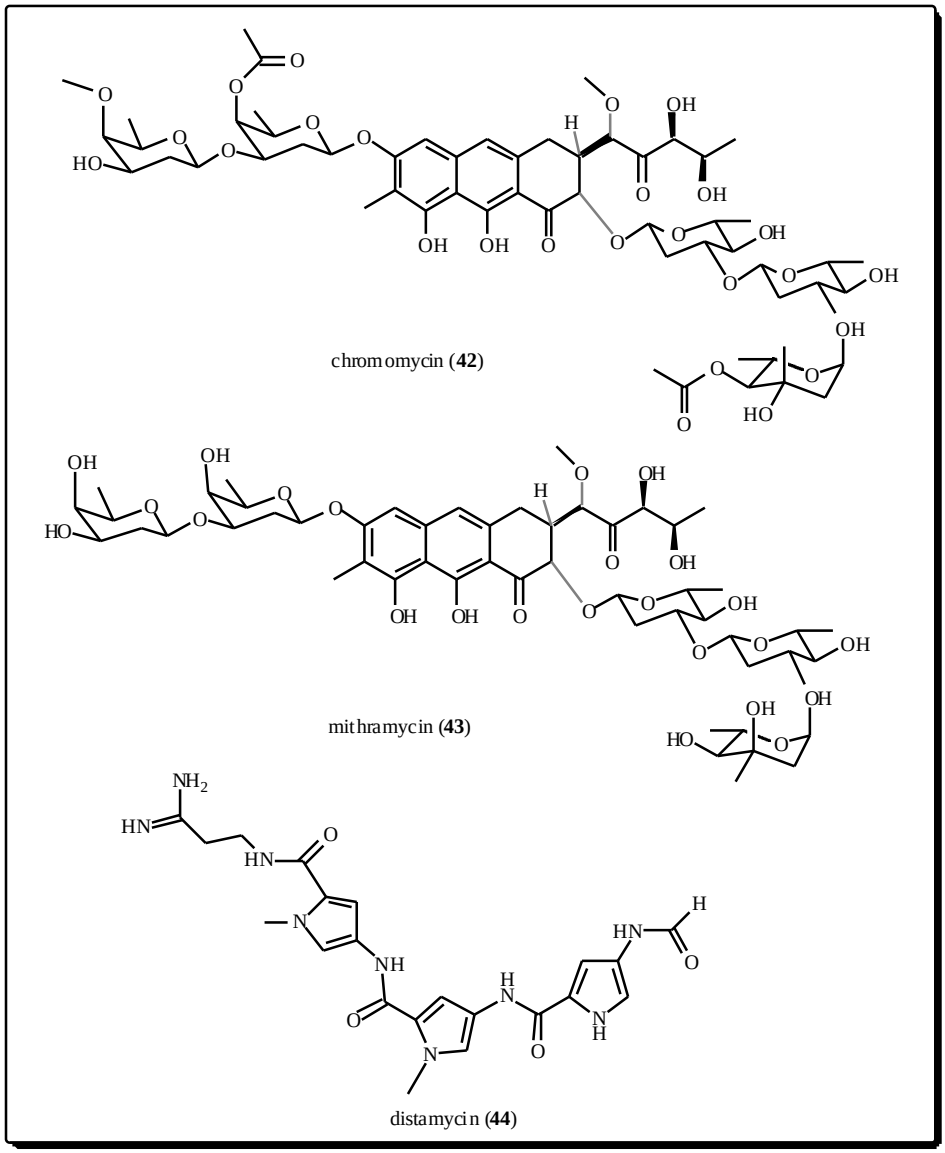
Fig. (12). Principle of an alliin biosensor (reprinted from [23] with kind permission of Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart).

optical sensors have been designed for the detection of natural products in complex matrices. Such sensors are based on antibodies (immunosensors) or molecular imprinted polymers (MIP), which specifically bind the analytes of interest. Fiber optics or wave guide devices are usually used as signal transducer. As a representative example, a biomimetic bulk acoustic wave sensor was fabricated on a MIP imprinted polymer for the detection of caffeine in human serum and urine [118].

Surface plasmon resonance and related approaches such as reflectometric interference spectroscopy (RIfS) appear to hold most promise for HTS applications. Such technologies have been used to establish various receptor assays [112]. For example, two assay formats have been set up for the detection of HIV-protease inhibitors [119]. In the first approach, the HIV protease was immobilized on a chip followed by exposure to the sample. Free binding sites of the enzyme were then blocked with a biotinylated substrate to which a biotin-specific antibody was coupled, giving the final sensor signal. Alternatively, a model inhibitor was immobilized on the chip and the sample containing potential HIV inhibitors incubated with the enzyme in solution. Free enzymes were then allowed to bind to the immobilized inhibitors.

A similar setup was used for the detection of thrombin inhibitors. Three different assay formats were established [120]. In the first two, thrombin, or alternatively the high-molecular-weight inhibitor hirudin were immobilized on the chips, and the assay was performed following the procedure described above. In a third alternative, thrombin was immobilized and the sample was premixed with hirudin (surface competition assay).

The suitability of the SPR technology for the investigation of molecular interactions between DNA and DNA binding substances has been also established [121]. Biotinylated single-stranded DNA with sequence mimicking the Sp1, NF-kB and TFIID binding sites of the long terminal repeat of HIV-1 were immobilized on chip surface precoated with streptavidin. The differential stabilities of the complexes formed between DNA and a series of model



compounds including chromomycin (**42**), mithramycin (**43**) and distamycin (**44**) could be characterized with respect to the nucleotide sequences.

Biosensing applications are not restricted to isolated to biomolecules. More complex systems, such as whole nerve cells have been attached on chip surfaces using poly-L-Lysine, special adhesive proteins or specific carbohydrates. Neuronal networks, membrane constituents and even entire tissues have been also immobilized on chip surface [112]. An increasing number of highly specific sensors consisting of cells and cell constituents arranged in complex arrays are being designed and biosensors are likely to play in future a growing role within screening technologies. While biosensors have been little used until now for the identification of new bioactive natural products in complex mixtures, it can be expected that the development of integrated microsystems for HPLC separation and biosensing detection [122] will open the way to assay formats providing both structural and bioactivity information in a single step.

EXPRESSION PROFILING

Functional genomics is an approach emerging from full genome sequencing which makes it possible to quantify mRNA transcripts on a genome-wide scale in cells and tissues. cDNA and oligonucleotide arrays were first proposed in the mid-1990 [123]. The steps involved with an cDNA array experiment are outlined in Fig. **13**. The fact that mRNA levels of thousands of genes can be simulaneously investigated in simple and complex biological samples has opened new avenues in the field of drug discovery. Current applications in pharmaceutical research include target identification and validation, compound profiling, and pharmacogenomic and toxicogenomic applications. The processing of single samples is currently leading the way to microarrays in the 96-well format enabling high throughput profiling against specific sets of genes of interest [124, 125]. Owing to the complexity of the protocols, measurements errors and data processing steps, early expression data sets suffered from poor reproducibility and agreement between

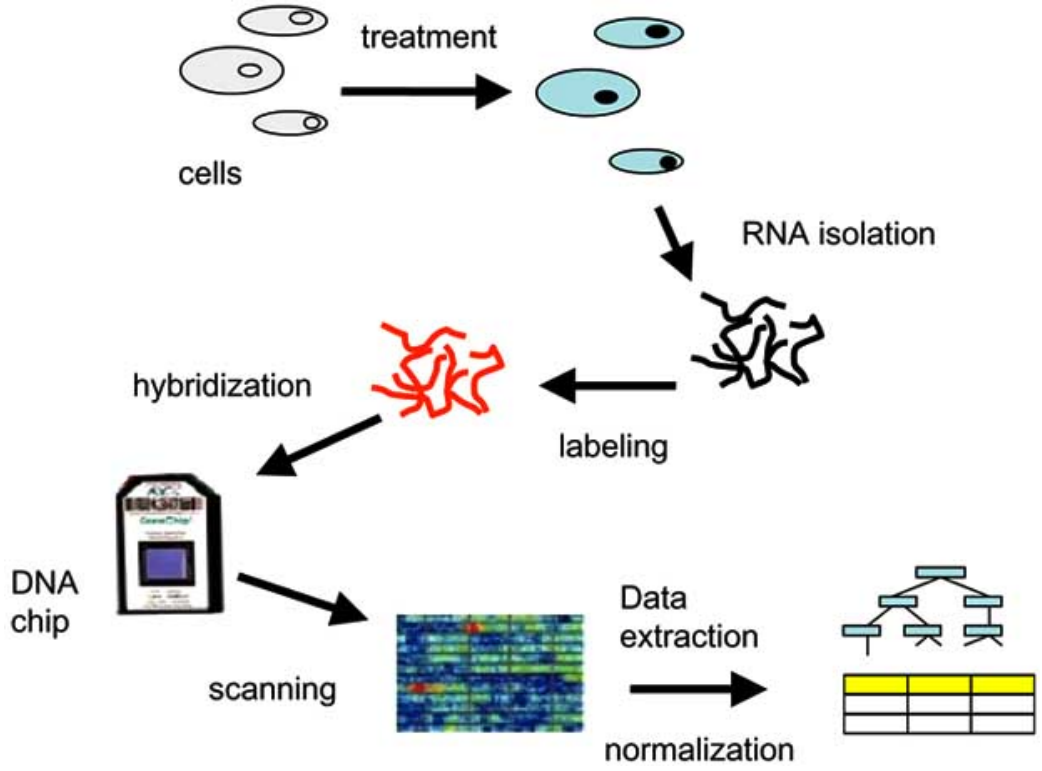


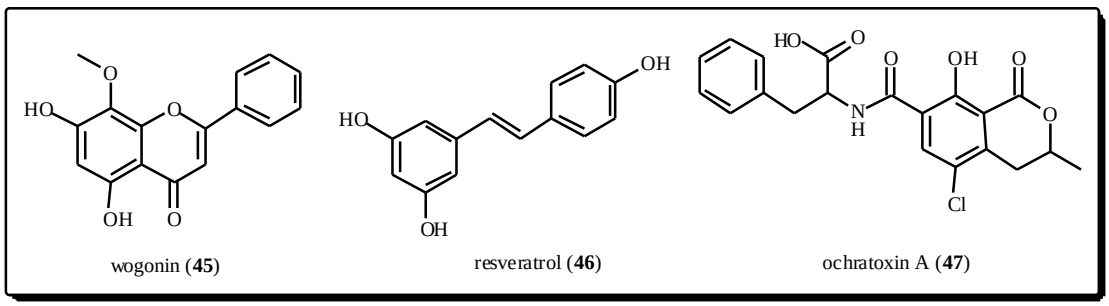
Fig. (13). Analysis of global gene expression profile.

platforms. Recent comparative studies support the validity of microarray experiments [123].

Microarrays have been successfully applied to the genome-scale analysis of gene expression profiles for selected natural products. In what can be considered as the seminal study in the field, the *in vivo* neuromodulatory effects of the standardized *Ginkgo* extract EGb761 were analyzed in the hippocamus and in cortical tissues with the aid of Affimetrix high-density arrays and confirmed by RT-PCR. Differential upregulation of genes was found in the two tissues, and some of these genes could be rationalized in the context of cognitive disorders and neurodegeneration. [126]. The fact that expression pattern can be studied *in vitro* and *in vivo* enables a global approach and a comprehensive and quantitative understanding of the effects, at the transcriptional level, of complex plant extracts, and a subsequent investigation of single molecules contributing to the overall effects [127, 128].

Further studies with natural products and plant extracts include the *in vivo* profiling the flavonoid wogonin (**45**) on inflammation-associated gene expression in the skin [129] and the profiling of *Centella asiatica* saponins in human fibroblast cells [130]. The modulation of expression pattern in bladder tumor cells by the isoflavonoid genistein (**38**) [131], and the resveratrol (**46**)-induced gene expression profile in ovarian cancer cells have been investigated [132]. Toxicogenomic applications of microarrays include the expression profiling of ochratoxin A (**47**), a potent carcinogen and nephrotoxin, with kidney tissues *in vitro* and *in vivo* [133]. These studies demonstrate the applicability of microarray technologies for the study of of extracts and pure natural products.

With the current implementation of 96-well microarrays, the use of expression profiling as a tool for activity-directed separation can be envisioned. A two-step approach can be conceived, whereby an initial genome-scale analysis of an extract would identify the genes of interest which would subsequently be used in a customized high-throughput microarray in the 96-well microtitre format to track the bioactivity. The interfacing with HPLC, in an approach



essentially similar to HPLC-based activity profiling described earlier in this review, should be technically feasible.

CONCLUSION

Some disappointing experiences with natural product extracts in modern screening formats, time consuming and expensive follow-up of hits, problems with re-supply and reproducibility have contributed to the misconception of natural products drug discovery being old-fashioned, too slow, too expensive and too complex. Over the last decade, natural product research has evolved into a technology-intensive activity. Judicious combinations of various technologies allow natural products to be quite competitive with synthetic compounds as a source for new lead compounds. The unique diversity of natural products and their successful track record is fully recognized in the pharmaceutical industry. However, continuing improvements are needed if natural products research should keep pace with the ongoing changes in drug discovery, in particular with the unbroken trend towards miniaturization and higher throughput. New advances in HPLC separation, such as ultra high pressure liquid chromatography [134] and HPLC-Chip/MS technology [135] hold promise of increased speed, resolution and sensitivity. IT-platforms combining, in a single database, spectroscopy data of various formats [136] are expected to increase automation and throughput in compound dereplication. In this context, a seamless correlation of bioactivity with chemo-analytical processes is likely to be, more than ever, a key factor for successful natural product screening. Innovative approaches, which have emerged recently to meet this goal have been presented above, and sustained efforts to establish new assay formats with improved selectivity are in progress. As a result, natural product research should continue to be in the future an active contributing force in the quest for new innovative drugs.

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