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Review

High-performance liquid chromatographic–electrospray ionization mass spectrometric analyses for the integration of natural products with modern high-throughput screening

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Abstract

Within the pharmaceutical industry, significant resources have been applied to the identification of new drug compound leads through the use of high-throughput screening (HTS). To meet the demand for rapid analytical characterization of biologically active samples identified by HTS, the technique of high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) has been utilized, and the application of this technique specifically for the integration of natural product sample mixtures into modern HTS is reviewed. The high resolution provided by reversed-phase HPLC coupled with the gentle and relatively universal ionization facilitated by the electrospray process has had significant impact upon a variety of procedures associated with the HTS of natural products, including extract sample diversity evaluation, dereplication, structure elucidation, preparative isolation, and affinity-based biological activity evaluation.

Keywords: Reviews; High-throughput screening; Natural products

Contents

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1. Introduction

1.1. High-throughput screening

During the latter part of this decade, the drug discovery model employed within the pharmaceutical industry has evolved into a search for new biochemical targets, cloning and expression, the development of assays, and the high throughput screening (HTS) of as many compounds as possible to find structures for development as drug candidates. Currently much attention has been focused upon increasing the speed and efficiency of HTS. Time periods of approximately 1–12 months have been typically required for the screening of 10^4–10^6 compounds [1], although new technologies such as the use of high-density (2400–9600 well) arrays may facilitate the screening of even the largest compound libraries in as few as 100–400 plates, requiring only days or weeks [2].

1.2. Natural products as pharmaceuticals

The success of an HTS program may be considered to be dependent upon several key factors, one of which is the chemical diversity of the compound libraries which are submitted for screening. Compound libraries for HTS are provided by several sources, including traditional synthetic organic compound collections, combinatorial chemistry, and natural products. Of these three, natural products (i.e. samples of biological origin) are believed to present the greatest source of chemical diversity available on the earth. Synthetic organic techniques such as combinatorial chemistry can in many cases be considered as complementary tools for the facilitation of natural product drug discovery and development via structure-activity relationship (SAR) investigations. Natural products fall into several different categories: steroids from marine animal, plant, and fungal sources, alkaloids from plants and some bacteria, proteins, amino acids, and antibiotics from microbes, pigments from microbes and plants, pyrimidines and purines from microbes, and terpenes, carbohydrates, fats, macromolecular products, and miscellaneous compounds from all sources, including terrestrial animals. Although natural products gained prominence through antibiotics earlier in this century, today they have been developed for a variety of medicinal uses such as immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigrane agents, herbicides, antiparasitic agents and ruminant growth promoters, and bioinsecticides [3]. Natural products are also among the most important anticancer agents, where over 60% of approved and pre-New Drug Application candidates are either natural products or synthetic molecules based upon natural product molecular scaffolds. Although almost half of the best-selling pharmaceuticals are natural or closely related to natural products, there remains tremendous potential for the identification of new medicinal compounds from these sources, since it has been estimated that only a small percentage of compounds from biological sources have been investigated for this purpose.

1.3. Natural product drug discovery processes

Historically, the ‘golden age’ of natural products occurred between approximately 1950 through 1970, and during this time the successful isolation of potent antibiotic compounds was routinely achieved through a sequential process. After the presence of biological activity was identified in a microbial fermentation or plant extract, the isolation of sufficient quantities of the component of interest was achieved through biological assay-guided solvent/solvent extraction or liquid chromatography. Following successful purification, the structure of the biologically active substance was determined through chemical and spectral characterization techniques. About 20 years ago, however, the difficulty and high cost of isolating novel structures with new modes of action became apparent, and focus within the pharmaceutical industry began to shift toward molecules such as recombinant peptides and growth hormones, antibody-based ‘magic bullets’, and transgenic plants and animals. Nevertheless, during this current era of high-throughput drug discovery when many new targets for therapeutic intervention have been identified, attention has returned to natural products because of the high value now being placed upon the chemical diversity which biological sources can provide. The modern natural products drug discovery process has evolved into a series of five steps which are diagrammed in Fig. 1. As described earlier, significant emphasis has been placed upon optimization of the

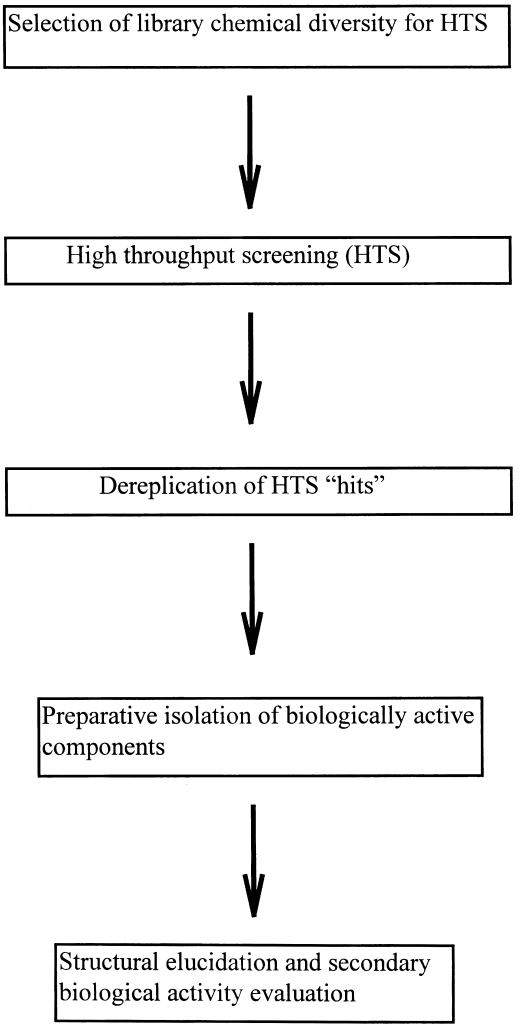

Fig. 1. A flowchart overview of a drug discovery program utilizing natural product samples and high throughput screening.

diversity (usually biological diversity of the source organisms) of sample libraries to be submitted for HTS. Following HTS, 'hits' (biologically active samples) are 'dereplicated', a term which refers to the identification of compounds or classes of compounds that have already been discovered and reported in the literature to have activities identical or similar to those observed in the extracts of interest. Novel 'hits' can subsequently be forwarded for isolation of mg quantities for the facilitation of both secondary biological activity evaluation and structure elucidation via techniques such as nuclear magnetic resonance (NMR) and infrared absorbance (IR) spectroscopy. While the traditional natural product drug discovery process could require months or even years, in the current highly competitive pharmaceutical industry environment represented by HTS in which speed-to-decision is critical, time periods consisting of only days or weeks are available, and access to analytical methodologies which may support such rapid evaluations is crucial.

1.4. Analytical characterization of natural product extracts

The inherent diversity of natural product extracts has not only made their evaluation for biological activity attractive, but has also presented significant challenges for separation and detection techniques to enable rapid characterization of the biologically active component present in the screened mixture. Separation techniques such as paper chromatography and thin-layer chromatography represented early attempts at parallel evaluation of screen hits, but these methods were severely limited by throughput and the amount of information they could provide. Gas chromatography (GC) is a powerful separation technique that has been utilized since the 1960s for the analysis of volatile natural products or derivatives, and has been especially effective following the successful interface with a mass spectrometer detection system in the GC–MS configuration. However, the role that has recently been played by high-performance liquid chromatography (HPLC) for work of this nature has been invaluable, considering that approximately 80% of all known natural compounds are nonvolatile or thermally unstable and therefore incompatible with GC [4]. When coupled with photodiode-array UV–Vis absorbance detection, HPLC began to serve as a powerful tool for the rapid characterization of natural product extracts [5]. In addition to HPLC, a second technological revolution which has had tremendous impact upon the analysis of natural products has been the development of electrospray ionization mass spectrometry (ESI–MS), a method which has rapidly evolved to powerfully complement other HPLC detection systems. In contrast to earlier MS techniques such as electron ionization (EI) which were applicable only to thermally stable, low MW volatile compounds, virtually

any 'ion' in solution (i.e. solution components ranging in size from inorganic salts to large proteins and other macromolecules such as nucleic acids) is amenable to analysis with ESI–MS (see Table 1 for a comparison of ESI with other MS techniques). The gentleness of the ESI process has also extended the polarity limit of analysis with mass spectrometry far beyond any boundary associated with earlier technology. Another very significant advantage of ESI–MS over other 'soft ionization' techniques such as fast atom bombardment (FAB) and matrix assisted laser desorption ionization (MALDI) is its compatibility with liquid chromatography. As a result, the interface of HPLC with ESI–MS has provided a method which has effectively facilitated the integration of complex mixtures with the rapid drug discovery processes affiliated with HTS.

The goal of a contemporary natural products HTS program is to rapidly identify novel biologically active chemical structures which can be developed as pharmaceutical compounds, an objective highly dependent upon analytical chemistry methodology. This review focuses on the influence HPLC–ESI-MS has had upon the effective integration of natural products with HTS and drug discovery, and begins with a survey of the application of this analytical technique for general natural product extract analysis followed by a review of its impact upon each of the sequential steps within a natural products drug discovery program (see Fig. 1).

2. Reversed-phase HPLC–ESI-MS and general natural product extract analyses

HPLC–ESI-MS represents the combination of a high resolution separation system with a powerful detection/characterization technique. Reversed-phase chromatography is the separation method which has been utilized for the vast majority of HPLC–ESI-MS analyses. The hydrophobicity-based separation mechanism of reversed-phase has provided the resolution of a large variety of chemical entities, and the employment of volatile buffers and aqueous/organic mobile phases which support the formation of ions in solution has enabled reversed-phase to be highly compatible with ESI–MS. The simplicity of the ESI design has enabled it to serve as a universal tool for LC–MS interfacing. ESI is provided by a hollow needle through which effluent flows, and an electric field at the tip of the needle produces a cone-shaped meniscus from which a spray of highly charged droplets emerges, with subsequent evaporation of the droplets resulting in the formation of ions which can be measured through the use of a variety of detection systems such as quadrupole, ion trap, and time-of-flight [6]. Routine instrument operation and data collection and manipulation can be achieved through the use of commercially available software such as MassLynx (Micromass, Cheshire, UK) or Xcalibur (Finnigan Corp., San Jose, CA, USA). Depending upon the complexity of the sample to be analyzed, LC–ESI-MS run times typically range between 5–90 min per injection, and therefore the technique is capable of facilitating high throughput when the instrumentation is configured with an autosampler and an automated HPLC pumping system, a critical requirement within the pharmaceutical industry.

HPLC–ESI-MS has been successfully applied to the determination of compounds present in material from a variety of natural product sources. The preparation of natural product samples for analysis has typically consisted of the extraction of secondary metabolites from the biological material via solid–liquid or liquid–liquid extraction using an organic solvent such as methanol which may be either directly injected or diluted with aqueous prior to injection. He et al. [7] employed the technique in conjunction with photodiode-array detection to analyze the phytochemical constituents of an extract of sour orange (*Citrus aurantrium*), and after identification of eight flavonoids found that their methodology provided sufficient sensitivity for trace analysis at concentrations in the range of 1–10 ng. A novel, rapid determination procedure for acetogenins in crude plant extracts by HPLC–ESI-MS was developed by Gu et al. [8]. This method proved useful for the detection of both previously reported and novel molecules during a search for bioactive compounds, and facilitated focused identification of selected compounds targeted for re-isolation and biological evaluation. A comparison of ESI, atmospheric pressure chemical ionization (APCI), thermospray (TSP), and continuous flow-fast atom bombardment (CF–FAB) MS interface techniques for the

Table 1
A comparison of mass spectrometry ionization techniques

MS Ionization technique	Advantages		Disadvantages	
Electron Ionization (EI)	·	High (picomole) sensitivity	·	Limited mass range due to
	·	Availability of large computer databases of		requirement
	·	fragmentation patterns for indentification of	·	Possible sample decomposition by thermal
		unknowns		desorption prior to vaporization
	·	Adaptable to liquid chromatography via	·	High fragmentation, often resulting in no
		particle beam interface		observable molecular ion
Fast Atom Bombardment (FAB)	·	Practical mass range up to 7000 Da	·	Low (nanomole) sensitivity
	·	'Soft ionization' technique, the molecular ion	·	Requirement of solubility of sample in matrix
		is obtainable with little fragmentation	·	High background matrix peaks
Matrix Assisted Laser Desorption/Ionization	·	Practical mass range up to 300 000 Da	·	Low resolution
(MALDI)	·	High (femtomole) sensitivity	·	Matrix background which can be a problem for
	·	'Soft ionization' technique		compounds with MW less than 1000 Da
	·	Tolerance of salts in millimolar concentrations	·	Possibility of sample photodegradation
Thermospray Ionization (TSP)	·	Practical mass range up to 2000 Da	·	Vaporization and ionization conditions can be
	·	'Soft ionization' technique		very sensitive, depending on compound
	·	Adaptable to liquid chromatography		structure
			·	Possible sample decomposition by thermal
				desorption prior to vaporization
Atmospheric Pressure Chemical Ionization (APCI)	·	Practical mass range up to 2000 Da	·	Sensitivity may be highly variable from
	·	High (femtomole) sensitivity		compound to compound
	·	'Soft ionization' technique	·	Possible sample decomposition by thermal
	·	Easily adaptable to liquid chromatography		desorption prior to vaporization
Electrospray Ionization (EI)	·	Practical mass range up to 70,000 Da	·	Relatively low salt tolerance
	·	High (femtomole) sensitivity	·	The purity of the sample entering the
	·	'Soft ionization' technique		ionization source is important
	·	Easily adaptable to liquid chromatography		

HPLC-MS of terpenes and polyphenol aglycones and glycosides in crude plant extracts has also been performed [9]. These studies demonstrated that TSP was suitable for the investigation of moderately polar compounds ranging up to 800 Da and for glycosides having not more than two sugar units, while CF–FAB, ESI, and APCI permitted the ionization of more polar compounds such as saponins bearing 1–8 sugar units. ESI and APCI facilitated the ionization of the broadest range of metabolites, but demonstrated an important selectivity for polyphenol aglycones and sensitivity to the use of HPLC mobile phase modifiers such as trifluoroacetic acid and ammonium acetate. Similar results were observed during a study of the HPLC–MS analyses of glycosidic carotenoids, where TSP was capable of providing ionization of compounds carrying up to three glucose units, but ESI provided the advantage of the ionization of even larger compounds [10]. HPLC–ESI-MS has also been employed for the determination of paclitaxel and related diterpenoids in a variety of plant extracts [11] and cavalactones and flavokavains in kava roots [12], as well as an investigation of the components responsible for aldose reductase activity in *Salvia* plant material [13].

Some natural compounds may be expected to present challenges for HPLC–ESI-MS analyses. For example, extremely polar molecules such as the amino sugar antibiotic nojirimycin and the beta-lactam antibiotic nocardicin C are not adequately retained on C18-based stationary phases, and have

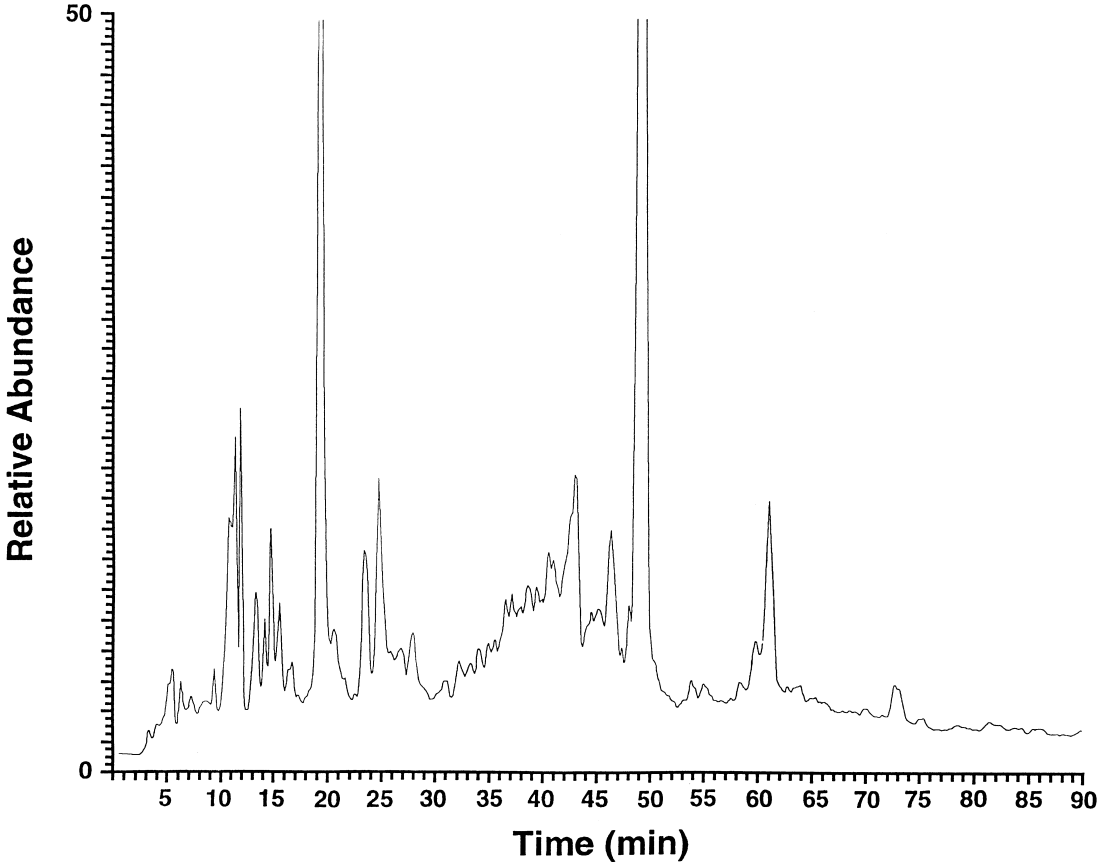

Fig. 2. HILIC–ESI-MS separation of the polar components (unretained by reversed phase solid phase extraction pre-treatment) of a fermentation extract, represented as a total ion chromatogram in positive ion ESI. The chromatography was obtained using a TSKGel Amide 80 packing, 6.5 ammonium acetate m*M* pH 5.5-buffered mobile phases, and a 90 min 10–40% aqueous gradient in acetonitrile. Reprinted from Ref. [16] with permission.

required normal-phase chromatography on activated carbon for purification [14,15]. For compounds such as these, the technique of hydrophilic interaction chromatography (HILIC) has demonstrated successful application [16]. HILIC is a separation technique which utilizes a polar stationary phase (such as polyhydroxyethyl aspartamide [17]) and a hydrophobic mobile phase (typically 60–100% acetonitrile) together with an aqueous gradient to separate compounds by hydrophilic interactions. HILIC therefore employs a separation mechanism opposite to that of RP–HPLC, while still providing excellent compatibility with ESI–MS. Fig. 2 displays the HILIC–ESI-MS total positive ion chromatogram of a reversed phase solid phase extraction (SPE) effluent preparation of a fermentation broth sample, demonstrating the successful resolution and detection of the sample components which were not retained by the reversed phase SPE packing. Another problem which is somewhat rare but yet may be encountered during the HPLC–ESI-MS analysis of natural products is the absence of charged sites within neutral analytes as these molecules enter the gas phase inside the mass spectrometer inlet, resulting in an inability to detect these types of components by ESI–MS. In these cases, one may employ alternative ionization methods such as atmospheric pressure chemical ionization (APCI) which directly induce analyte ionization in the gas phase rather than rely upon existing ionization in the liquid phase prior to vaporization. For neutral, thermally unstable natural product compounds such as vitamins, selective tagging with charged reagents is a procedure which may be performed to facilitate ESI–MS detection [18].

3. Chemical diversity evaluation

As mentioned in the Introduction section, of crucial importance to the success of an HTS program is the chemical diversity of the compound libraries which are submitted for screening. Historically, natural product sources have been selected for HTS based on a variety of approaches such as collection geography, biological taxonomy, chemotaxonomy, or folk medicine, and have often provided chemical diversity for HTS simply through sheer number of samples screened as well as the intrinsic complexity of the extracts as mixtures. Recently, an efficient process to evaluate extracts for chemical diversity for the selection of samples for submission for HTS has been proposed by Julian et al. [19]. In this investigation, HPLC–ESI-MS was utilized to determine the presence of secondary metabolites through the generation of ion signal m/z vs. retention time plot images, and the data in these images were then subjected to statistical similarity measures for a quantitative determination of diversity within a sample set. Specifically, following collection, the three-dimensional HPLC data were converted into net CDF file format and then transferred to a UNIX workstation. Each ion contained in the data file (described by a retention time, mass, and intensity) was assigned to a location in a two-dimensional array, with retention time on one axis, mass on the other axis, and intensity stored in the array location. Following the filtering of noise, the center time and center mass (allowing for a width of ± 0.5 m/z) of ions identified using this procedure were computed. A quantitative measure of similarity between the complex mixtures represented by the LCMS data images was achieved through numerical treatment based upon the following relationship which represented a 'chemical space' distance between ion i in sample 1 and ion j in sample 2:

$$d_{ij} = \sqrt{(t_i - t_j/w_t)^2 + (m_i - m_j/w_m)^2}, \quad i = 1, \ldots, n_1, \quad j = 1, \ldots, n_2 \tag{1}$$

$$S_{ij} = \begin{cases} 1 - d_{ij} & \text{if } d_{ij} \leq 1 \\ 0 & \text{if } d_{ij} > 1 \end{cases} \tag{2}$$

where t_i is the chromatographic retention time of ion i, m_i is the mass-to-charge ratio (m/z) of ion i, w_t is a weight factor for the retention time dimension, w_m is a weight factor for the mass dimension, d_{ij} is a scaled Euclidean distance between ion i and ion j, n_1 is the number of ions identified in sample 1, n_2 is the number of ions identified in sample 2, and S_{ij} is the similarity (0–1) between ion i and ion j. Using an extension of this mathematical treatment, a set of 88 crude fungal extracts were analyzed and compared vs. replicate analyses of a control extract, and bar plots displaying the frequency of similarity values for these data are displayed in Fig. 3. In this diagram, a similarity value = 1.0 indicated identical samples, while similarity values approaching 0 sug-

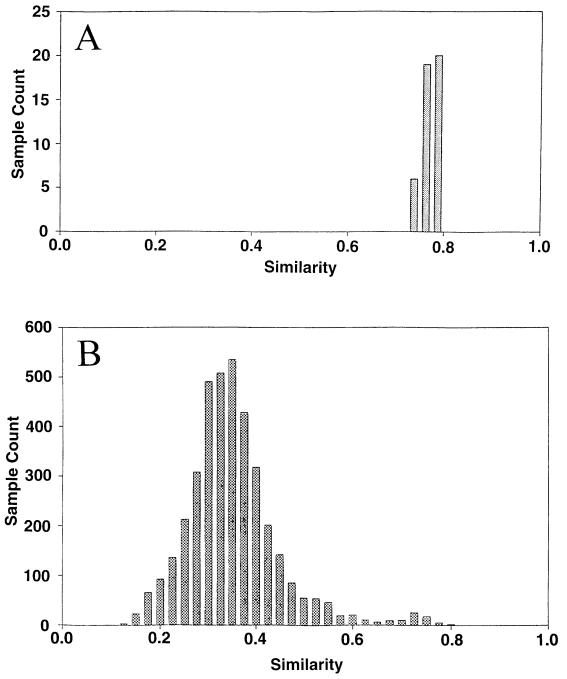

Fig. 3. (A) A histogram showing the number of samples (sample count) with a given similarity value measured for all pair-wise comparisons of the m/z vs. retention time HPLC–ESI-MS images of 10 control sample analyses. The mean of the distribution of similarities for all ten injections was 0.77 with a COV of 2%. (B) A histogram showing the number of samples with a given similarity value measured for all pair-wise comparisons of 88 fungal extracts. The distribution for fungal samples is much broader and has a lower average similarity than the control injections displayed in (A). Reprinted from Ref. [19] with permission.

gest high chemical diversity. In addition to the prioritization of extracts for HTS, other proposed uses for this technique included the evaluation of the diversity of natural product extracts relative to an existing collection for guidance in the acquisition of new extracts, as well as a tool for post-HTS prioritization of biologically active extracts through the identification of replicate 'hits' or undesirable compounds.

4. Dereplication

A second specific application of HPLC–ESI-MS natural product mixture analysis is the procedure known as dereplication (as defined in the Introduction section). The dereplication process has great impact since it focuses the application of limited isolation and analytical chemistry resources upon extracts that have a high probability of containing novel compounds or known compounds with novel modes of action. The rapid, precise, and efficient dereplication of natural products has become one of the key processes for maintaining samples from natural sources as a viable alternative to synthetic compounds in HTS programs. Historically, dereplication procedures have utilized separation techniques which evolved over time from paper chromatography to thin-layer chromatography to HPLC. The early methods were used successfully for the evaluation of extracts displaying antimicrobial activity, wherein the samples of interest were first separated and collected on a paper or silica support and then applied directly to agar in which the microorganism of interest was cultured [20]. Separated compounds appeared as zones of inhibition, and the chromatographic characteristics (retention, UV absorbance, etc.) of the components generating these zones were useful for comparison of samples to both other biologically active extracts as well as to known compounds. The instrumentation associated with today's state-of-the-art dereplication operation typically has consisted of an HPLC interfaced with photodiode array and MS detectors, a fraction collector, and in some cases an evaporative light scattering detector (ELSD) which may provide an on-line mass concentration determination (see Fig. 4). Photodiode

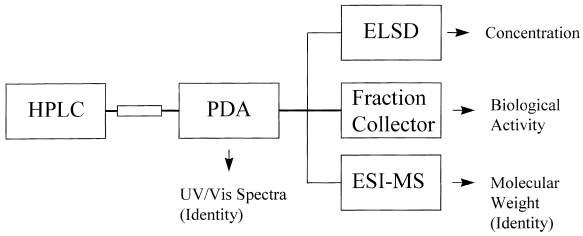

Fig. 4. Diagram of an instrumentation configuration consisting of a high-performance liquid chromatograph (HPLC), a photodiode-array detector (PDA), an evaporative light scattering detector (ELSD), a fraction collector, and an electrospray ionization mass spectrometer detector (ESI–MS), as employed for the dereplication of natural product mixture samples which have been identified as biologically active.

array and MS data are collected on-line during the separation, and after bioactivity evaluation of the collected fractions the biological signal can be correlated with the corresponding UV–Vis absorbance and MS information. In an early application of LC–MS for dereplication, TSP–MS had effectively been utilized to identify antitumor compounds in fermentation broth [21]. At a later date, however, the investigators discovered that TSP–MS was adequate for only about 50% of compound classes of interest and that ESI–MS was a much more universal HPLC–MS interface [22]. Others have reported similar findings, where HPLC–ESI-MS offered a significant advance over earlier techniques not only because of the increased stability and efficiency of ionization in the ESI source, but also because it was capable of directly correlating the biological activity observed during dereplication of *Eugenia jumbos* plant extracts to the molecular weight of an active flavonoid glycoside [23]. In this study, collision-induced dissociation (CID, see Section 5) fragmentation was also employed on-line for structural elucidation. In regard to the use of HPLC–ESI-MS for dereplication procedures, of crucial significance is the fact that this technique typically will provide the $[M+H]+$ or $[M-H]+$ ions from which the compound molecular weight can be directly inferred. Subsequently, the molecular weight information (together with UV–Vis absorbance data) can then be searched against commercially available databases of natural product information, such as the Chapman and Hall Dictionary of Natural Products [24] and NAPRALERT [25], for the identification or classification of the unknown compound [26]. These processes have revolutionized dereplication, since bioactive compounds can now be identified within a mixture, effectively avoiding the traditional requirement of the isolation of the components of interest from the mixture.

5. Structure elucidation by HPLC–ESI-MS ion fragmentation techniques

The utilization of HPLC with collision induced dissociation (CID) has opened further dimensions in the field of mixture analysis. CID refers to the process by which the translational energy of an ion accelerated toward a neutral target species (or 'collision gas', such as argon) is partitioned into internal energy, resulting in the decomposition of the incident ion into fragment ions. This process may be induced inside the ESI source, or within the mass analyzer region of the instrument to obtain MS–MS or MS^n spectra. Since the fragmentation achieved using these methods will be representative of analyte structure, these techniques can serve as effective tools for the direct on-line elucidation of the structure and/or classification of a variety of natural product compounds in complex matrices. Herderich et al. investigated the use of this technique for the characterization of heterocyclic aromatic amines, fumonisins, acylated glycoconjugates, and regioisomeric fatty acid hydroperoxides [27]. As an example, for the analysis of the heterocyclic aromatic amine compound PhIP, a triple quadrupole mass spectrometer was employed for the CID fragmentation of the m/z 225 molecular ion to form a product ion at m/z 210. This fragmentation represented the loss of a methyl group. In other studies, HPLC–ESI-MS–MS has been used for the identification of the compounds hypericin and pseudohypericin and their precursors via intense deprotonated species observed in the negative ion detection mode [28]. Characteristic MS–MS progeny ions of these naphthodianthrones in *Hypericum* extracts (St. John's wort) were obtained, and it was proposed that the method could be employed for the standardization of extracts as well as phytotherapeutic products. Post-HPLC off-line MS–MS analysis of fractions identified as biologically active has also been employed for the elucidation of compound structure during dereplication [29]. Fig. 5 displays a chromatogram of a bioactive natural product extract, the biological activity data associated with collected fractions, and the results of an MS–MS analysis of the 9.0 min fraction which was determined to contain the compound novobiocin (612 Da), the presence of which was confirmed through the appearance of the $M+H^+$ ion at 613 m/z and fragment ions at 189, 218, and 396 m/z.

Other MS–MS techniques which are useful for the analysis of natural products include precursor-ion ('parent-ion') and neutral loss scans. Precursor ion scanning may be employed for the identification of related compounds via the detection of a common fragment, while neutral loss scans can be utilized for

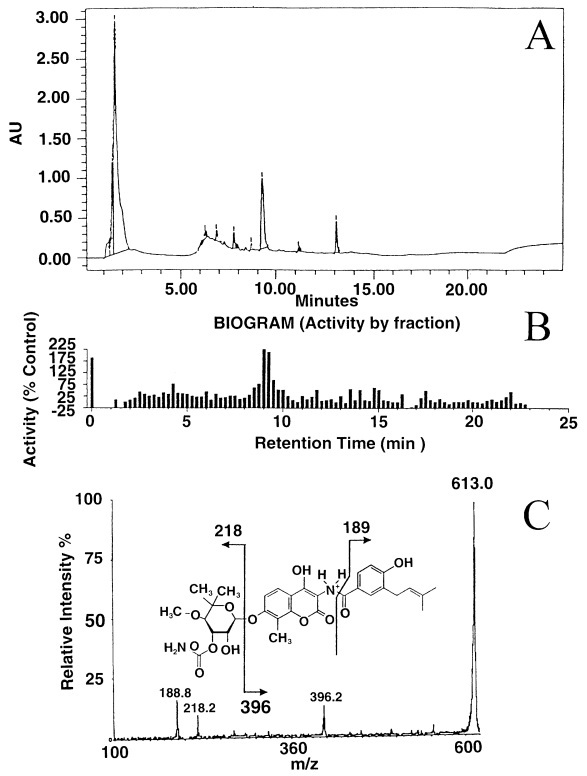

Fig. 5. (A) The UV–Vis chromatogram of a biologically active fermentation broth sample. (B) The biological assay signal plotted vs. fraction number for a series of fractions collected during the separation displayed in (C) The structure of novobiocin, identified within the bioactive fractions, is displayed with the fragments corresponding to the signals observed in its characteristic ESI–MS–MS spectrum. Reprinted from Ref. [29] with permission.

the determination of fragments corresponding to an uncharged mass. These procedures are typically performed through the use of triple quadrupole ion detection. For precursor-ion scanning, the third quadrupole is locked on the m/z of the fragment ion of interest while the first quadrupole is scanned for a larger mass ion containing that fragment. An application of this approach for natural product analysis has been reported by Blay et al., who performed HPLC–MS–MS precursor-ion scans for a fragment ion characteristic of the taxane nucleus to provide the necessary selectivity and sensitivity required for the identification of potentially active diterpenes in plant extracts [30]. For neutral loss scanning, the first and third quadrupoles are offset by a mass setting corresponding to an uncharged fragment of interest and then scanned simultaneously to detect the presence of ions containing that neutral mass. For example, the presence of sulphoconjugate structures of drug metabolites has been determined through the determination of a neutral loss of 80 Da [31].

6. Mass-guided isolation

Rapid processes are required for post-HTS ‘hit’ characterization, at which point milligram quantities of the compound of interest must typically be isolated for further biological evaluation as well as complete structure elucidation. To meet these needs, fully automated preparative HPLC–ESI-MS systems have been developed. In one report which focused upon the isolation of synthetic compounds from combinatorial chemistry-derived mixtures, multi-milligram quantities of target compound were purified by reversed phase chromatography and an ESI mass spectrometer was used to initiate fraction collection following the detection of target compounds as specific ion signals reached pre-set threshold intensities [32]. This one-sample-one-fraction format facilitated the purification of large numbers of compounds without the need for excessively large fraction collector beds, and more importantly eliminated the need for post-fraction collection analysis for the determination of the presence of the target compound for fraction pooling. The ability to perform mass-guided fractionation has been incorporated into several commercially available ESI mass spectrometers, and should serve as an important tool for the rapid isolation of bioactive compounds from natural product sources.

7. Affinity-based biological activity evaluation

HPLC–ESI-MS has also found application within several recently developed affinity-based binding determination methodologies. Although the throughput of these procedures is not yet competitive with that of microtiter plate-based HTS, this methodology offers current value for secondary biological evaluation of molecules identified by HTS. The applications of interest have consisted of the incubation of target biomolecules with mixtures of potential lig-

ands, followed by the removal of unbound ligand and then the subsequent release and analysis of bound ligands. Multidimensional automated chromatographic techniques coupled to ESI-MS have facilitated the compound selection process and have provided maximum characterization information within a single analysis. Size-exclusion chromatography (SEC) [33,34] and immuno-affinity chromatography [35] have been employed for the separation of protein-ligand complexes from unbound ligand. Following dissociation from the protein, the previously bound ligands can be identified through the use of reversed phase HPLC–ESI-MS. A limitation of these approaches includes restrictions of the application to tight-binding protein-ligand interactions, since the time required by the SEC or immuno-affinity separations (5–10 min) can facilitate the evaluation of only compounds with relatively slow kinetic rates of dissociation. Also, because this technique represents a binding assay and not a functional determination, the specificity of binding must be determined through the use of competitive experiments with a ligand of known affinity. However, the utility of on-line mass spectrometry as an alternative to serial chromatographic isolation may offer advantages in regard to the reduction of the time required for binding evaluation, ligand purification, characterization, and identification for the post-HTS evaluation of complex mixtures (such as natural product extracts) of interest.

8. Conclusions

The availability of HPLC–ESI-MS has been invaluable for integration of natural product extract complex mixtures into the rapid drug discovery programs within today's highly competitive pharmaceutical industry. Future advances of the analytical methodology will be dependent upon the development of both chromatographic separation technology as well as mass spectral detection capabilities. For example, separation techniques such as HILIC (described in Section 2) which are complementary to reversed phase HPLC may further increase the diversity of samples available for HTS. In regard to developments in mass spectrometry, recent significant improvements in instrumentation have facilitated the commercialization of time-of-flight (TOF) analyzers for accurate-mass and accurate-mass tandem mass spectrometry. TOF or quadrupole-TOF (Q-TOF) detectors may be interfaced with HPLC–ESI systems to provide ppm-level mass accuracy which can enable the determination of the empirical chemical formula of an unknown compound [36], in essence providing a powerful tool which may be useful for the rapid identification of natural product mixture components for applications such as dereplication. Recent progress in the field of nuclear magnetic resonance (NMR) spectroscopy has resulted in new efforts to couple this detection method with liquid chromatography and HPLC-MS, and several HPLC-NMR probes are now commercially available. Although sensitivity issues have been found to limit the application of on-line HPLC-NMR to only the major components present in natural product extracts [37], the potential power of this technique for the rapid structure determination of components in mixtures may make it a strategically important analytical tool in the near future. These emerging analytical chemistry strategies, together with new concepts for screening sample analyses such as chemical diversity determination (see Section 3), will continue to assist all aspects of modern natural product drug discovery programs in keeping pace with the advancements of HTS.

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