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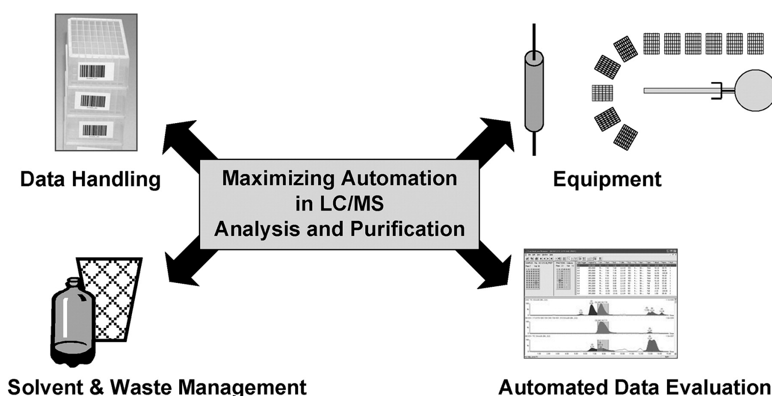

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Maximizing Automation in LC/MS High-Throughput Analysis and Purification

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Here, we describe a system for LC/MS-based analysis and purification of compounds aiming at the minimization of manual interference in the overall process. Key elements of the concept are automated identification of the target compounds, automated assignment of optimized preparative gradients for purification of the target compounds, and automated purity assessment of fractions with subsequent pooling of validated product fractions. Additional support is provided by an automated solvent and waste management system. One person can easily process 100−200 compounds on a 150-mg scale per day on that system, while still the maximization of purity and yield after purification is guaranteed. Reduced demands with respect to purity or yield can lead to significantly higher throughput numbers.

Introduction

The number of compounds that are purified or at least analyzed prior to their submission to biological tests is increasing steadily within the pharma community, since insufficient compound quality has often led to misleading false positives and negatives in biological assays.[1] Simultaneously, the number of desired pure compounds from combinatorial chemistry groups to support high-throughput screening procedures or MedChem projects is increasing as well, thereby posing enormous challenges to the respective purification units. In recent years, LC/MS has become the method of choice to analyze or purify compounds in high-throughput mode, as can be seen from the steadily increasing number of publications in this field.[2−22] Our HPLC/MS laboratory was designed from scratch in the context of the concomitant establishment of an automated MedChem department. Restrictions in staffing capacity (1 person) suggested that we utilize as much automation as possible to fulfill the requirements of purifying and analyzing 100+ compounds/day. In-house philosophy is to deliver significant amounts of novel compounds to the compound collection facility (10+ mg or 20+ μmol), which then allows not only the generation of daughter plates for screening excercises, but also the storage of a portion of the material as solids. Since the experience of others and ourselves has shown that impure compounds can be quite troublesome in the hit validation process,[1] it was decided to set a threshold of 85% (UV) purity for stored compounds. For various reasons, crude products synthesized in a library format may contain significant impurities, making a purification process mandatory. One reason is that the strategic goal to also synthesize more complex structures via high-throughput chemistry utilizing "demanding chemistry" sometimes leads to compounds of lower purity. In addition, a computer-aided library design step often top-prioritizes building blocks which are only of intermediate reactivity. The desired system, as a consequence, has to deliver the compounds not only in sufficient purity, but also with the maximum possible yield, minimizing the amount of impure product fractions.

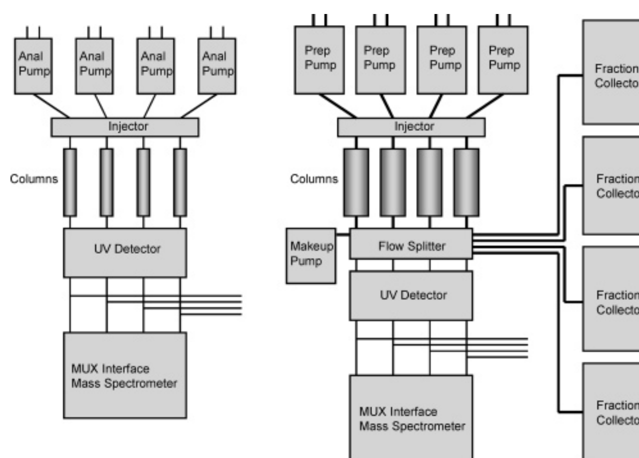

Figure 1. Layout of the purification factory with four-channel analytical and four-channel prep system.

This paper describes how we solved the various bottlenecks in the process of LC/MS analysis and purification utilizing extensive hardware automation and software programming. The establishment of the system was realized in a joint project with Waters, which took over the translation of our requirements into the MassLynx software and FractionLynx application manager. This software is now commercially available.

Experimental Details

Instrumentation. The whole setup of the system is shown schematically in Figures 1−3. It consists of an analytical four-channel multiplexed electrospray (MUX) LC/MS system,[4] a preparative four-channel MUX LC/MS system (Figure 1), a liquid-handling device with attached microtiter plate

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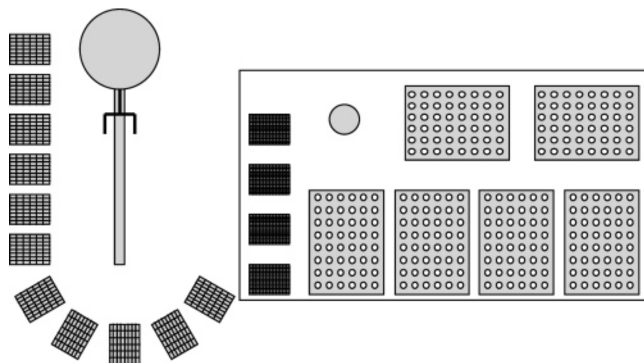

Figure 2. Layout of the fraction pooling station.

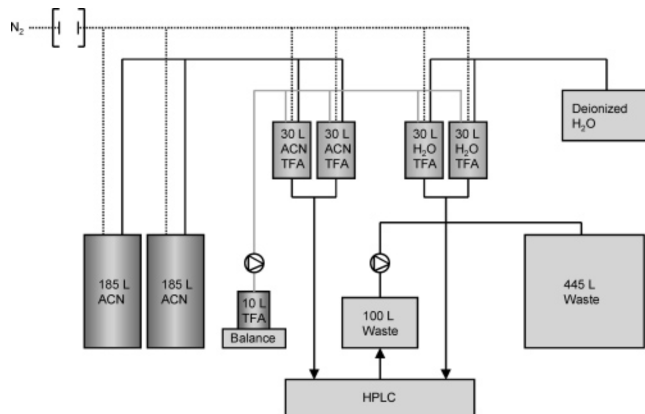

Figure 3. Layout of the solvent and waste management system.

(MTP) hotel for fraction pooling (Figure 2), and an automated solvent and waste management system (Figure 3).

The analytical LC/MS consists of a CTC Pal four-way injector, four Waters 1525 pumps, a Waters 2488 multichannel UV detector and a ZQ 2000 single quad MS detector with four-channel MUX interface and Valco flowsplitters. The preparative system is composed of a CTC Pal four-way injector, four Waters 2525 pumps, a Waters 2488 multichannel UV detector, a ZQ 2000 single-quad MS detector with four-channel MUX interface and Valco flowsplitters, an LC packings accurate 1:1000 four-channel flowsplitter, a Waters 515 makeup pump, and four Waters 2757 fraction collectors. Each fraction collector can accommodate 15 MTPs. For fraction pooling, a Hamilton Microlab 4200 pipetting platform and an attached Hamilton Microlab Swap MTP 420 handling robot with plate stack depot are used. The plate hotel holds up to 60 MTPs. The solvent and waste management system was built by Hecht, Bodnegg and is composed of a Membrapure Aquintus deionizing water unit; two 185-L barrels for acetonitrile (ACN); a 10-L modifier container (here for trifluoroacetic acid (TFA)); an LMI Memdos piston stroke pump for dosing of modifier; a Mettler balance for modifier consumption monitoring; two 30-L containers for buffered ACN; two 30-L containers for buffered water; a 100-L container for intermediate waste; a 445-L KTC container for final waste collection; a Flux pump for transfer of waste from the 100-L waste to the KTC; various lines of copper, stainless steel, and Teflon; Bürkert valves; Liquifant-M liquid level detectors; and a Siemens Simatic OP 7 control unit. A Frog leak detector that is installed around the whole HPLC equipment allows for safe 24 h/day operation.

LC/MS Conditions. Our standard eluents on both systems are 0.1% TFA in ACN and 0.1% TFA in water. On the analytical system, we routinely use 4.0 × 125 mm Purospher Star C-18, 5-μm particle-size columns with 3.9 × 10 mm X-Terra MS C18 10-μm particle-size guard columns. The standard gradient employed is 5−95% in 5 min, followed by a hold of 1 min at 95%, then return to 5% within 0.25 min and reequilibrate at 5% for 1.25 min, all at a flow rate of 2 mL/min. A 10% portion of the analytical flow is transferred after the UV detector to the ZQ MS. The sampling rate is 2/s for each channel, with a standard cone voltage of 30 V. Usually, only positive-mode spectra are recorded. A typical injection volume for analysis of crudes is 5 μL, which often is equal to ~50 to 100 μg. On the prep system, 25 × 125 Purospher Star C18, 5-μm particle-size columns are used with 19 × 10 mm X-Terra MS C18 10-μm particle-size guard columns. We have two different schemes for purification, with optimized gradients depending on the purity challenge posed by the respective library. The pattern of the optimized gradients for standard separations is x% for 2 min, then x to $(x + 40)$% in 9 min, then go to 95% within 0.5 min and hold at 95% for 3.5 min. Currently, we use four different optimized preparative gradients, with x being 5, 15, 25, 35% (combined UV/MS trigger) and two generic gradients with UV or MS-only trigger. The flow rate is always 25 mL/min. After the columns, a 1:1000 LC packings flow splitter allows transfer of a small portion of the eluent into the UV detector and, subsequently, a 10% portion into the ZQ MS. The sampling rate is 2/s for each channel, with a standard cone voltage of 25 V. Usually, only positive mode spectra are recorded. Typical amounts for purification range between 50 and 150 mg, which can be dissolved in up to 5 mL of solvent. We routinely use TFA modifier for all our compounds for analysis and purification, although polarities and functional groups of compounds in a library format can vary significantly. Practical experience has shown that the application of the optimized gradient procedure still allows successful purification of more than 95% of samples. For basic compounds, we found it helpful to add modifier to the dissolved samples prior to analysis and purification, since otherwise, sometimes smearing is observed.

Results and Discussion

The request to maximize automation meant not only to address the hardware analysis and purification challenge, but also to tackle the informatics problem as a key element in the concept, as well. Waters R&D and Schering IT worked here as a team to allow data exchange in the informatics environment. The whole automation process relies heavily on the molecular weight of the target compounds as the key identifier. Usually, the compounds are delivered batchwise on one or more 6-mL 48-well MTPs, although many other formats are possible, as well. The whole process is depicted in Figure 4 and will be detailed in the following section.

Registration. Each batch of compounds (library) is delivered with an Excel file. The Excel file name corresponds to the library name and is attached to the MTPs via a barcode label. The file itself contains the identifier (protocol number, sample name) and the characteristic molecular formula for each library member. The automation process starts out on

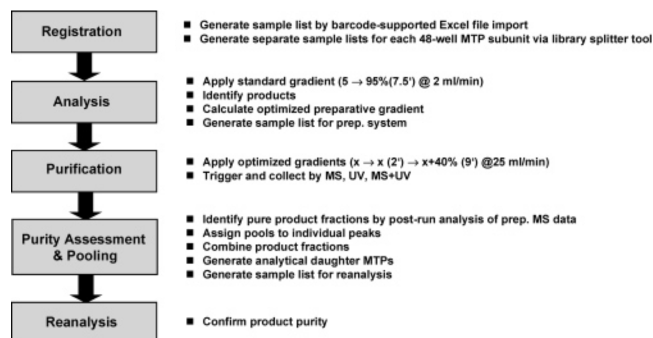

Figure 4. Overall workflow for compound analysis and purification.

the analytical system by scanning the MTP barcodes of a library. This accesses the corresponding Excel file on the server and from that automatically generates a sample list of compounds to be analyzed with their characteristic molecular weight. No "copy and paste procedures" are necessary. Since libraries are often larger than 48 members, which is the maximum capacity of one MTP, a library splitter software tool breaks the library in pieces of 48 to be loaded on the system, which allows for error-free sample logging. It is possible, through the flexibility of the software, to search for up to three target compounds (= masses) per library member. This function is rarely used, but it does give the ability to isolate starting materials, interesting side products, etc., if the need arises.

Analysis. Analysis of crude products is performed for several reasons: First, it gives an overall impression on how well the synthesis of a given library worked, and it reveals if some compounds exhibit problematic behavior on an LC/MS system (no UV, extreme polarities, low MS ionization, strong fragmentation). Furthermore, it allows automated calculation of UV purities and automated calculation of optimized gradients for purification. For analysis, a standard gradient of 5−95% ACN is employed, by which more than 95% of our current samples are correctly analyzed. After analysis, the target compounds are automatically identified by their target mass. For automated calculation of UV purity, the retention time (RT) of the maximum of the extracted ion chromatogram (EIC or mass trace) is used for integration of the corresponding peak in the UV trace.

Purification becomes more and more of a challenge when compound amounts increase and crude product purities decrease. Consequently, several groups have elaborated on the issue of how to obtain compound-specific shallow gradients in combination with an appropriate fraction trigger for better and faster purification results. Whereas some groups rely on theoretical predictions applying relevant descriptors,[23] others follow a more practical approach and utilize information from analytical screens to assign an optimized purification protocol. We essentially use this latter procedure, which has recently been described by two groups[14,19,22] and, therefore, will not be discussed in detail here. In short, the retention time of the product in a fast analytical run with a genereric gradient is used to assign an optimized shallow preparative gradient. We have used this procedure for several years now in our lab using Excel macros; however, through the establishment of our new platform, this step has now become fully automated. It is emphasized that the design and the assignment of our optimized preparative gradients results purely from empirical correlations using large sample sets and does not require any common conditions (e.g., slope) for the analytical and preparative runs.[2,3,24,25] Since the RT is very critical, however, we decided to install our analytical four-channel MUX system with four individual pumps. Other systems utilize only one pump with a subsequent four- or eight-way split,[12,19] but we felt the danger of incorrect RTs resulting from columns with different back-pressures to be too high.[26]

Purification. The subsequent purification step is initiated by again scanning the barcode of the MTPs of the library on the preparative system, which is located directly adjacent. This automatically retrieves the appropriate sample list with assigned optimized gradients that was generated as a result of the analytical screen. Still, the software does allow the user to overwrite the purification recommendations (e.g., change the gradient) through an interactive browser. To minimize inefficient use of the system, compounds of very low purity can automatically be excluded from purification by user-defined purity thresholds. Equally, pure compounds can be further processed without intermediate purification. Up to six individual gradients of any kind, but of course, with the same overall duration time, can currently be applied. We routinely use gradients of the type x to x to $(x + 40)$. Limited solubility of compounds sometimes forces us to dissolve them in up to 5 mL of DMSO. The initial isocratic conditions are used to wash away the sample solvent, thereby minimizing the danger of breakthrough. The initial isocratic conditions also correlate with the polarity of the compounds and thereby reduce the risk of column clogging. On the preparative four-channel MUX system, four independent pumps are mandatory for the optimized gradient procedure. The fractionation usually occurs by a combined UV-MS trigger, since this dramatically reduces the number of collected fractions, as compared to only UV or only MS.[27] Still, the capacity of the fraction collectors allows use of single triggers, as well. We use barcoded and preregistered 4-mL 48-well MTPs for fraction collection. For a batch run of 96 samples, on average, a total of 7.5 × 4 mL fraction volume is possible. We would like to emphasize, that we do not aim to collect the pure compounds in one single fraction tube, since our experience has shown that this does not work reliably enough for all samples, independent of the trigger combination in use. Possible reasons can be, for instance, distorted or fuzzy peak shapes in UV or MS spectra, smearing of the target mass through the chromatographic run, or failed chromatographic separation of byproducts.[9] Instead, we collect several fractions per compound, which are subsequently assessed and pooled.

Purity Assessment and Pooling. A critical step in the overall process is the automated identification of product fractions which pass the purity criteria and are used for pooling. An often employed procedure for purity assessment of fractions is to integrate the corresponding MS spectrum, considering the molecular weight of the target compound and typical associated masses, such as sodium adducts or binary aggregates.[28−30] The purity threshold for these

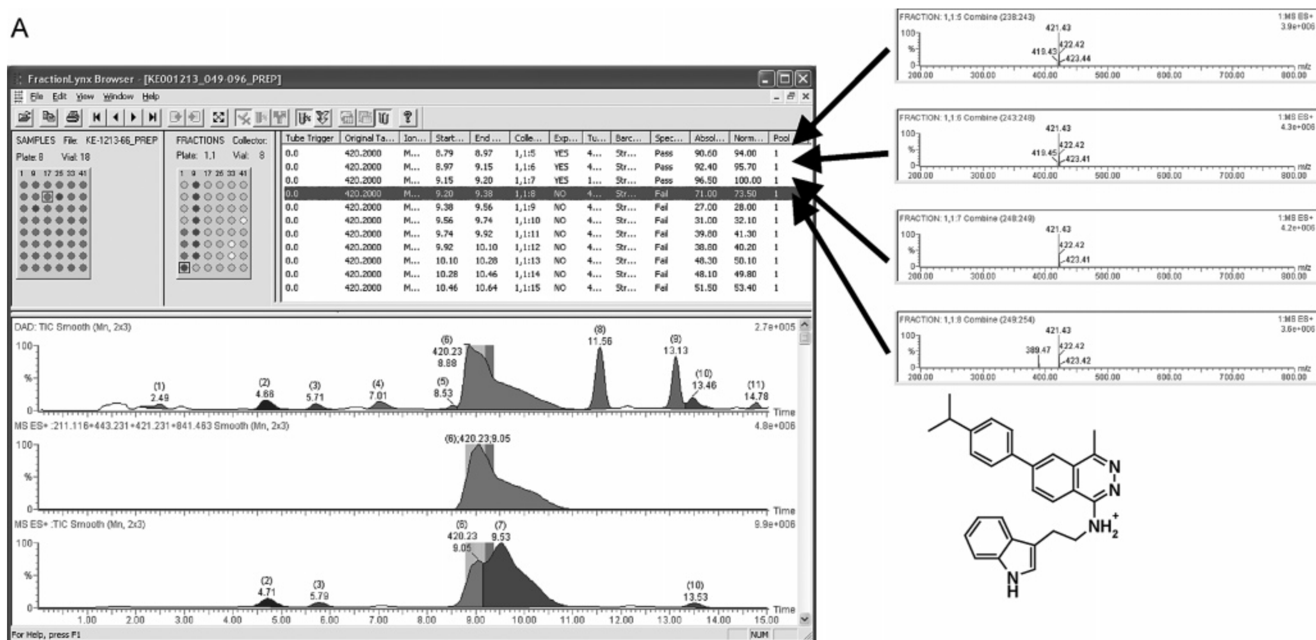

Figure 5. Purity assessment of fractions by postrun analysis of prep. LC/MS data. An example for a stable (A) and an easily fragmenting compound (B) is given.

masses is then set to a relatively high value. Although this procedure is working well for compounds which do not fragment in the mass spectrometer, for fragile structures, it results in dramatic compound loss due to misinterpretation of fragments as impurities in the mass spectrum. Since fragmentation is very difficult to predict, to the best of our knowledge, a general automated solution to this problem has not been achieved so far. Our approach is to define a reference MS spectrum that is used to obtain the characteristic achievable MS purity of each compound and to identify possible characteristic fragments. In our routine, apart from the standard adducts, such as M + H or M + Na, a user-definable number of the most prominent peaks in the reference MS are identified and taken into account for

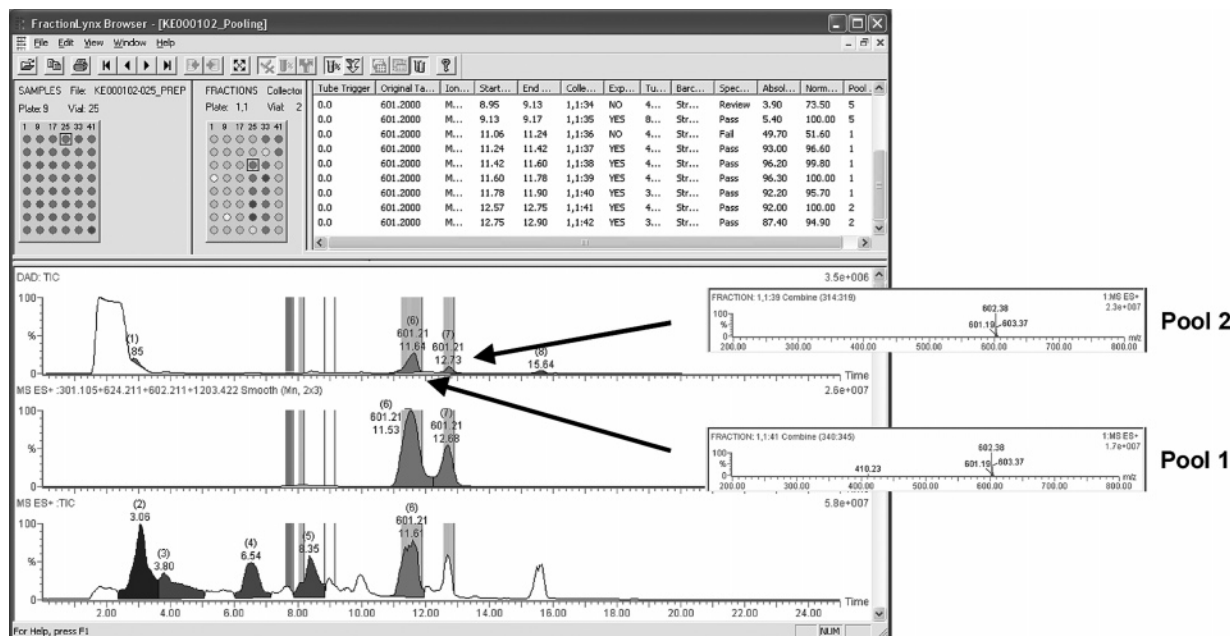

Figure 6. Pool assignment of isomers. Pool 1 is the peak corresponding to the maximum in the EIC. Pool 2 corresponds to the second highest peak.

integration. These additional peaks, which have to be of lower molecular weight than the target mass, are basically per definition considered as compound-specific fragments. For compounds with high fragmentation potential, MS purities can be converted from low values to numbers approaching 100%. A subsequent normalization step gives purities of fraction tubes relative to the reference MS. All fractions which are in the range of this reference purity or better are then selected for pooling. To obtain the reference MS, we ended up in a compromise, which we found gives excellent results for almost all samples and eliminates the problem of compound loss due to false-negative fractions. Per definition, the reference MS spectrum is the MS spectrum of the maximum of the mass trace of the target compound. Figure 5A depicts an example for an MS-stable compound. Practically no fragmentation occurs. Consequently, the absolute MS purities are close to 100%. Purity assessment detects "pass" fraction tubes for tubes 1,1:5 to 1,1:7, while tubes 1,1:8−1,1:15 "fail" the assessment.

In Figure 5B, an MS-fragile compound is shown. The algorithm recognizes the 278 mass as a compound fragment and considers it for assessment. Consequently, the absolute purities of the "pass" fraction tubes are relatively low, while the normalized purities again approach the 100% value. Fraction tubes 2,1:14 to 2,1:19 are assessed as pure, whereas, for instance, fraction 2,1:13 does not pass. The approach does not work correctly in cases when this reference MS spectrum contains the masses of real impurities; that means when the chromatographic separation had failed and the corresponding fraction is not pure. However, since the reference MS spectrum corresponds to the maximum sample amount during elution from the column, this means that the whole chromatographic separation was not successful, and it is very likely that only little, if any, pure compound could be obtained using a manual pooling procedure.

Using this algorithm, the software automatically decides which fraction tubes to pool and how to pool them. All fractions which belong to one peak in the processed and smoothened EIC and have sufficient purity are put in one pool. Isomers or even impurities of unknown structure, which for various reasons contain the target mass in the MS, may cause several peaks in the EIC. In these cases, pool numbering occurs according to decreasing peak heights; i.e., pool 1 is always assigned to the highest peak. This reflects our experience that for single-target products, in more than 99% of cases, pools are assigned correctly by that. Isomers pose an additional challenge on an automated purification and pooling system, since various scenarios are conceivable which require different processing. In cases that almost all isomers of a library can be separated by the system and the structure belonging to each peak is known, one probably wants to treat those as separate pools, as has been recently communicated.[10] As shown in Figure 6, pool 1 and pool 2 of a compound are assigned to the two highest peaks in the EIC, reflecting the two expected isomers. The software detects a few more "pools" containing the target mass, which are ignored, however, in the subsequent pooling step.

When a significant portion in a library with isomers does not resolve or the precise structures cannot be assigned to the peaks, it is probably better to combine those in one pool. Through the flexibility of the software, it is possible to combine pools on request, thereby allowing the application of the most appropriate pooling technique. Although barely used, the interactive browser again allows the user to overwrite the software decisions.

FractionLynx tracks fractions through the barcodes on the preregistered fraction plates. The generated fraction pool/barcode plate list allows the easy operation of a separate liquid-handling device. To this end, the respective MTPs are physically transferred from the fraction collector to the MTP hotel attached to the liquid handler. Automated registration of the fraction MTPs opens the access of the liquid handler to the pooling list. In our case, all nominated product fractions are combined into a 50-mL Pyrex tube of a pretared,

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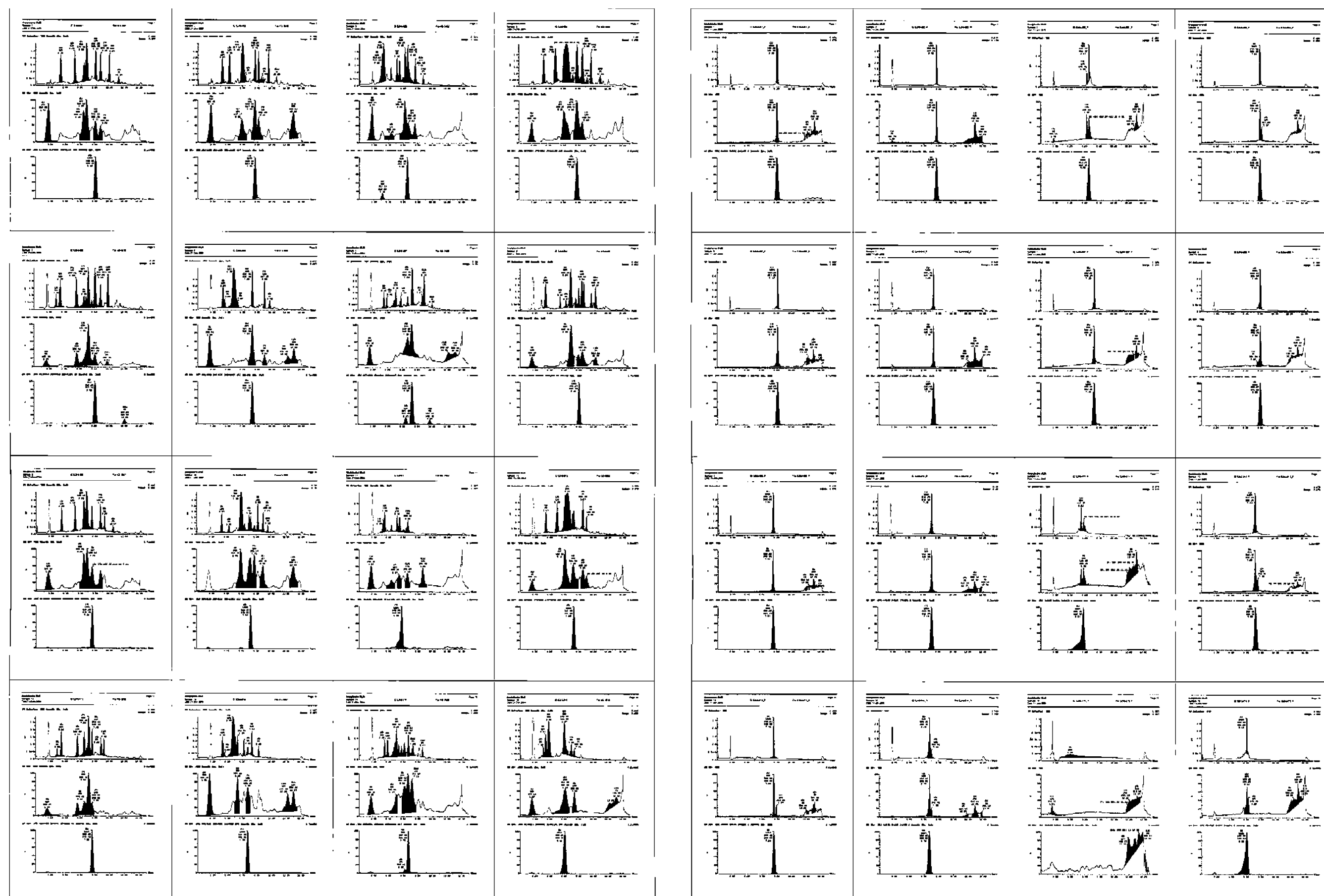

Figure 7. Thumbnail printouts of crude and purified products for quick manual review. UV, MS, and EIC traces are depicted for each sample.

barcoded, and preregistered 48-vial rack. Using this algorithm, an analysis of individual fraction tubes prior to pooling has become obsolete and, consequently, has significant impact on throughput. The subsequent reanalysis of each pooled compound mainly functions as a quality certificate, which is attached to each sample.

Reanalysis. An aliquot of each product pool is subsequently taken to generate a daughter plate that is used for reanalysis/purity confirmation of the compounds using LC/MS. Here, the required sample list is generated by the liquid-handler software.

Although the automation usually works reliably, we do not want to hide that for safety reasons, we usually take a look at the analytical of the crudes prior to purification. Equally, prior to registration of compounds, a manual review of the analytical spectra of the purified compounds is performed. For rapid scanning of results, thumbnail printouts with 16 samples/page with UV, MS, and EIC traces for each sample work fine for us (Figure 7).

Solvent and Waste Management. In preparative, high-throughput HPLC laboratories, significant amounts of capacity are needed for preparation of solvents and removal of wastes. We therefore decided to design and develop an automated solvent and waste management system that requires minimum manual input for these aggravating tasks. The system is able to automatically prepare and deliver eluents from water and an organic solvent with an added modifier of choice and simultaneously removes all wastes from the HPLC systems. As eluents, we routinely use water/0.1% TFA and ACN/0.1% TFA. A schematic sketch of the system is shown in Figure 3. For description of components, please refer to the instrumentation section. The HPLC systems are supplied with eluent from the 30-L containers via hydrostatic pressure. When a container reaches the lower "empty" level, the system switches to the second stand-by container, which has been automatically refilled during the time it was not in use. The refill process is performed by filling a certain amount of solvent under the control of liquid-level detectors (either from the remote ACN barrel via nitrogen overpressure winding or from the Membrapure water unit via activation), adding modifier (TFA) by pumping a programmed amount of liquid and by mixing the solution via nitrogen bubbling. Similarly to the buffered eluents, the ACN barrels are used in alternate modes under the control of the software. When the barrel in use becomes empty, the system automatically switches to the second (full) barrel and sends out an e-mail request for a new ACN barrel. The empty barrel can be exchanged in the meantime without time pressure. The supply of TFA is monitored by a balance. Care had to be taken for the valves, especially for the delivery of pure TFA due to corrosion. We found Bürkert valves made from Teflon and steel the optimum choice.

Conclusion and Outlook

In conclusion, we have developed a high-throughput system for analysis and purification of compounds which

addresses all major bottlenecks within the process: Sample tracking, data transfer, generation of sample/worklists are all generated fully automated by one piece of software via barcode support. The automated determination of UV purity and assignment of optimized gradients for preparative HPLC/MS runs using the analytical prescreen allows the rapid and efficient purification of compound libraries. Even peptides that often represent difficult cases for purification can be purified on our system on a 150-mg scale. To allow even more compound specific gradients, we are considering changing the routine from an assignment of a number of fixed gradient programs to a more flexible one using an empirical mathematical correlation for gradient calculation. In our case, this means that the x values within the prep gradient are not limited to a number of predefined values, but instead, are calculated by the following formula: $x = [(\mathrm{RT} - y) \times 9\%\ \mathrm{min}^{-1} - z]\%$, with y being related to the dead volume of the system and z being an empirical number. The main difference and advantage is that this approach does allow many more compound-specific gradients for possibly better resolution and easy variation of desired retention times by changing the z value. Furthermore, to increase throughput, we plan to double the prep flow rates to 50 mL/min and simultaneously divide the gradient run times by 2. Initial experiments have shown that resolution is almost preserved under these conditions.

Purity assessment of product fractions allows cherry-picking of pure fraction tubes on the basis of an algorithm that calculates MS purities and considers fragmentation. The pooling algorithm allows the automated combination of pure product fractions and considers various scenarios for isomer pooling. Last, but not least, an automated solvent and waste management system has been implemented that enables us to run our system on a 24 h/day basis and has contributed significantly to solving our restrictions in manpower. To further expand analysis and purification capabilities, the purification factory has been extended to another analytical and preparative four-channel MUX system in the meantime, which has also been connected to our solvent and waste managment system.

Our future plans aim at incorporating four-channel ELS detectors to be able to purify non-UV compounds with combined MS triggers. In the optimal case, the ideal trigger combination will then be automatically picked by the software.

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