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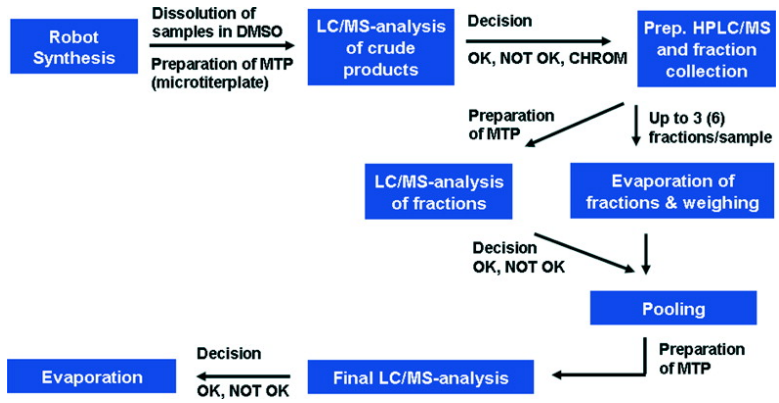

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Automated Modular Preparative HPLC-MS Purification Laboratory with Enhanced Efficiency

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Automated parallel synthesis as tool to increase productivity in chemical synthesis is well-established. However, even more time-consuming than the synthesis process is the following purification of the resulting crude products. To enhance efficiency of the lead optimization process at Bayer CropScience, a high-throughput HPLC/MS-laboratory for the purification of up to 48 crude products per day in the range of 200−400 mg each in one injection per sample has been set up. The use of Covaris technology for HPLC sample preparation, automated aliquotation during fractionation, and a novel evaporation process by combination with freeze-drying are new key technologies applied successfully for the first time in this purification unit facilitating to achieve the targeted efficiency. The whole process is supported by a specially designed IT-landscape covering each step of the workflow. Both the technical instruments used within the laboratory and the workflow and IT platform are described in this article.

Introduction

While parallel synthesis techniques to increase productivity in the early discovery process of hit or lead finding are well-established in the pharma and agrochemical industry for some years now,[1] there are only a few examples for the use of such technology in the later stage of lead optimization.

However, experiences at Bayer CropScience in Monheim have shown that the time needed for the optimization of new potential crop protection agents can be significantly reduced using automated solution-phase synthesis. A special synthesis laboratory was built for this purpose consisting of two fully automated robotic systems that serve as well-accepted support units for lead and research projects in all three indications (herbicides, fungicides, and insecticides).[2] Full automation and "around-the-clock" operation leads to a clearly increased productivity compared to classical laboratories. All traditional laboratory procedures like mixing, heating and cooling, filtration, liquid−liquid extraction, drying, and evaporation can be performed by the robots, thus allowing for an easy adaption of synthesis protocols from classical laboratories. Manipulations under inert gas atmosphere are also feasible. Purities are analyzed online by HPLC and offline by HPLC/MS, and quantities are determined by weighing directly by the system. All data is managed by an internal oracle database application called AutoChemDataBase (ACDB).

The robotic systems are especially designed in size for the requirements of later stage lead optimization in crop protection research, where smaller, focused libraries (∼20−200 compounds) and rather large amounts of active ingredient (50−150 mg) are needed. Such quantities are necessary because the lead optimization of new agrochemicals relies heavily on whole organism screening (against fungi, insects, or weeds) as inhibition on the target level cannot always be used to predict biological activity on the whole organism level.[3,4]

One robotic unit can produce up to 48 compounds in parallel per day in the required scale. In the past few years, by continuous optimization of the system, the number of reactions performed in the robot synthesis laboratory has risen to more than 6000 per year.

Until recently, the resulting crude products were purified by classical preparative HPLC in a laborious process with high expenditure of time and personnel rendering purification the time-limiting step in the process because it is also often observed in classical organic synthesis.[5] Thus, to be able to fully leverage the benefits of robot synthesis, there was a clear demand for a modern, automated purification process to accompany the existing highly efficient robot synthesis and workup process.

However, while high-throughput HPLC/MS purification of compound libraries for early research with up to 50 mg of crude product each is well-established,[6] there is little precedence for automated purification of larger scale libraries with quantities of >200 mg crude reaction mixture.[7] Thus, in this article, we want to describe our efforts at Bayer CropScience in Monheim resulting in the successful setup of a new, state-of-the-art HPLC/MS laboratory for the purification of batches of up to 48 crude reaction mixtures in the scale of 200−400 mg per day, allowing isolation of up to two products per reaction (two separate isomers of the same target molecule or target molecule plus byproduct).

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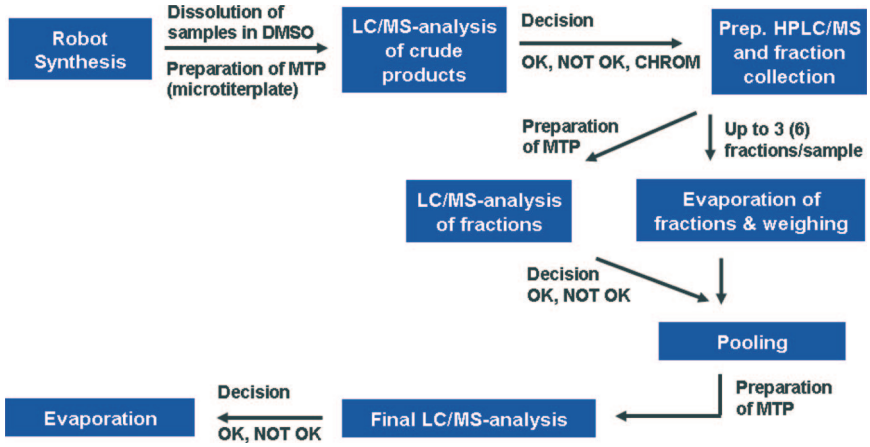
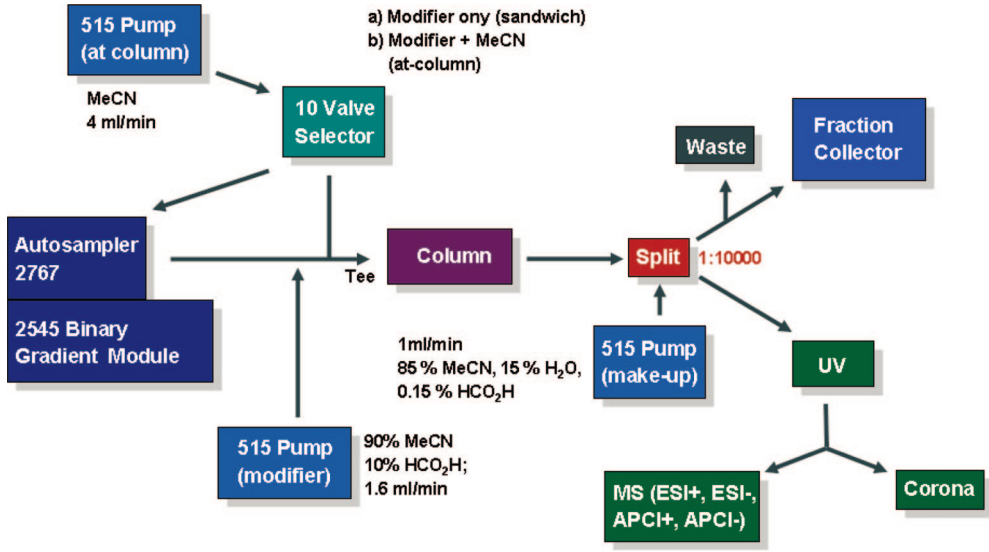
Scheme 1. Definition of Purification Process



Scheme 2. Preparative HPLC/MS Unit



Our plans for the new laboratory were based on the following key ideas regarded as essential to guarantee a time- and personnel-efficient process:

- Implementation within the existing robot synthesis laboratory to minimize interfaces and to allow flexible shift of personnel between synthesis and purification.
- Use of a batch-oriented, "around-the-clock" process to avoid any individual treatment of samples and to allow maximum utilization of instrument capacity.
- Automation of time-consuming manual steps like sample dissolution, liquid handling, weighing, labeling, and data management and avoidance of any unnecessary liquid handling step.
- Use of HPLC/MS technology with corresponding analytical and preparative systems to ensure easy transfer of methods between the systems.
- Optimization of prep HPLC/MS purification as integral part of the chemical method development process prior to library synthesis.
- Availability of an integrated IT platform to facilitate safe and easy data handling and storage during all steps of the process.
- Use of standardized vessels and racks within all purification laboratories at Bayer CropScience whenever possible to ensure availability of back-up instruments in case of breakdowns. In addition, it soon became evident that automation of individual steps only instead of having one central robot was favored because this allows visual control after each step of the whole process.

Scheme 1 illustrates the purification process that we intended to use in the new laboratory.

After this planning phase, we not only had to find suitable hardware but also an appropriate IT platform had to be programmed to map the whole process.

Discussion

1. Technical Instruments. After a comprehensive market survey, the following instruments were chosen as best fitting our requirements:

- Waters analytical UPLC/MS and preparative HPLC/MS system
- Labomatic fraction collector LH-3000 with a second arm for taking of aliquots into microtiter plates (MTPs) during fractionation (interface to the Waters preparative HPLC/MS-system programmed by Labomatic)

- Sias Xantus liquidhandler with an integrated Covaris unit for automated sample dissolution by focused ultrasound built by Synchron
- Zirbus zentrifugal evaporators for removal of solvents
- Mettler Toledo weighing robot with barcode scanner
- Laboratory furniture from Bense Laborbau
- Pure water supply from Millipore

a. Analytical UPLC/MS. For analytical control of crude product mixtures, fractions after preparative HPLC/MS, and final products after pooling, an analytical Waters Acquity UPLC/MS system was used, including a binary solvent manager, a sample organizer for the UPLC/MS system increasing the sample capacity up to 10 MTPs, and a column manager for fast heating and cooling and changing of columns.

To meet multiple detection requirements the following detectors were chosen: an Acquity photodiode array detector for a wavelength range of $\lambda = 190$ nm $-$ 500 nm with up to 80 measurements/second, a Waters SQD mass detector to measure ESI+, ESI−, APCI+, APCI− at the same time, and a Corona CAD from ESA used as additional detection system.

The run times we use with the UPLC/MS system are in the range of 3 min per measurement, the columns utilized are Zorbax Eclipse Plus C18 4.6 × 50 mm, 1.8 μm, working at a pressure of 600 bar (~8700 psi). By harmonization of the columns used within the different analytical departments at Bayer CropScience, comparability of results is ensured. Thus, purities deviate from the ones measured in the analytical department by less than 5% and logP values by less than 0.1 unit.

Visualization of results is provided by Waters OpenLynx or FractionLynx, and customers can check final analytics of their products via SDMS, which is a server based application provided by Waters for the storage of analytics results.

b. Preparative HPLC/MS. The preparative HPLC/MS system is used for purification of up to 400 mg crude product in one injection. A Waters 2545 Binary Gradient Module serves as solvent handling unit allowing flow rates between 0.5 and 150 mL/min and up to 6000 psi pressure. Usually our separations are performed at a flow rate of 80 mL/min. Thus, run times of nearly 20 min lead to a time demand of approximately 16 h for a series of 48 separations.

A Waters 2767 Injector-collector with custom-made 16 × 24 cm racks for 24 of our 30 mL robot synthesis vials allows injection volumes from 5 μL up to 10 mL in full loop or partial loop injection mode. Waters Autopurify software can be applied for easy choice of gradients from UPLC data. Nevertheless, we have also established a few standard gradients within the laboratory that work well with a wide range of substance classes and are mainly used to avoid precipitation on the column (or precolumn) or shutdowns of the system caused by overpressure, which is a prerequisite to run a robust system.[8]

The detector system consists of a Waters 2487 Dual λ Absorbance UV/vis Detector, a Waters 3100 single quadrupole mass detector for mass-directed purification applications, and a Corona CAD from ESA used as additional detection system. Fraction collection is usually triggered by the mass signal, which has become a popular tool during the past decade,[9] and since the detection system is identical to the one used in the UPLC/MS-system, collection of all compounds seen in the analytical spectrum is guaranteed.

We obtain best results during purifications in the envisioned quantity range using either Waters SunFire Prep C18, OBD 50 × 100 mm, 5 μm columns with SunFire Prep C18, OBD 19 × 10 mm, 5 μm precolumns or Phenomenex Axia Luna 10 μm C18(2) 100A, 50 × 100 mm columns with Security Guard PREP Cartridges C18, 15 × 30 mm. The injection volume used is mostly 3 mL for sandwich injection or 5 mL for at-column injection.[8,10] The method of choice depends on solubility and polarity of the compound class to be purified. In case of low solubility in DMSO, at-column dilution is preferred because a higher solvent volume for solubilization can be used. If we deal with less polar compound classes, we do sandwich injection.

c. Fraction Collection. According to our previous experiences with HPLC/MS purifications of compounds in the scale of up to 400 mg crude product, we decided that the collection of up to three fractions with up to 80 mL each should be sufficient per sample. For the eventual collection of two products per sample (two separate isomers of the same target molecule or target molecule plus byproduct), up to six fraction vials each were provided. Thus, for batches of 48 samples, a fraction collector was needed that could accommodate up to 288 fraction vials. According to our market analysis, a fraction collector of this size was only available from Labomatic or CTC Analytics. We decided to choose a Labomatic LH-3000 fraction collector because Waters and Labomatic agreed upon collaboration to ensure communication with the Waters MassLynx software via a compiler program written by Labomatic. Because of this emulation, the LH-3000 is treated by MassLynx like a standard fraction collector. When MassLynx software is updated, Waters guarantees the provision of necessary source codes to Labomatic for eventual adjustments of the compiler program.

The Labomatic fraction collector is built of a LABOMAT LH-3000 basis module together with a LABOCOL FS-3000 fraction collector module for up to 12 racks with 24 fraction vials (for up to 80 mL solvent volume) each and a LABOCOL AS/AL-3000 liquid handling module. This combined autosampler/aliquotation module provides the possibility to take aliquots for fraction analytics directly in up to three 96-well MTPs. Like this HPLC/MS analysis of the fractions taken can be started immediately after the purification of a batch of samples without any additional transfer of the fraction racks to a liquid handler. To circumvent persisting gradients in the fraction vials, all aliquotation steps were programmed in the following way: The aliquotation needle takes an aliquot, goes back into the respective fraction vial again and rinses the aliquot out to have better mixing within the vial. Afterward, the aliquot for analysis is taken.

d. Liquid Handling and Dissolution of Compounds. All liquid handling steps within the purification process (dissolution of samples prior to preparative HPLC/MS-purification and subsequent dissolution and pooling of dried-down fractions into the final target vials for compound storage) are done on two

Sias Xantus 200/200 liquid handling systems provided by Synchron. Taking of aliquots from the fraction vials into MTPs can also be done using these liquid handlers as back-up systems for the Labomatic LH-3000. The Sias Xantus 200/200 can accommodate up to 12 source or target racks (16 × 24 cm), giving space for 144 fraction vials, 48 target vials, and an MTP for analytical HPLC/MS control of the final test compounds before sending them to the compound storage. It was built with two robotic arms, one arm equipped with four piercing and one spraying needle and the second arm with a gripper. The Sias X-AP software gives a trained user the opportunity to easily develop own pipetting programs by "drag and drop" definition of operations and allows import and export of pipetting lists as xls-files, csv-files, and txt-files.

A Covaris S2 unit generating focused ultrasound directly within the sample is integrated into the Sias liquid handler for fast compound dissolution.[11,12] After samples are mixed in the Covaris unit, the glass vials are dried by a sponge station to avoid the danger of cross-contamination of other samples with water droplets from the ultrasound bath.

The novel Covaris technology of so-called adaptive focused acoustics works by sending acoustic energy wave packets from a dish-shaped transducer that converges and focuses the energy to a small and localized area. At the focal point, the energy density may be controllably focused into the sample. Operating at shorter wavelengths than standard sonication processes allows peak energy density directly within the sample. While standard sonication processes work with wavelengths of ten's of centimeters the Covaris process operates at wavelengths of approximately 1 mm. These shorter wavelengths allow us to avoid typical problems of standard sonication processes like energy scattering, reflection by the sample particles, and in many instances, "hot spots" that may damage the sample. In contrast to sonotrodes that have to be placed directly inside the sample, the Covaris process enables mechanical energy to be applied to a sample without direct contact. Furthermore, no heating of the sample takes place, as would happen with standard sonication processes. All these features support an easy automation of this technology, which makes it very attractive for our purposes.

Adaptive focused acoustics have already proven to be beneficial to numerous applications like RNA extraction, liquid membrane vesicle preparation, compound formulation, and of course, compound dissolution.[13] However, to the best of our knowledge, this is the first combination of a liquid-handling unit and Covaris ultrasound technology for high-throughput HPLC sample preparation.

e. Evaporation Process. Efficient solvent removal is another crucial point for the throughput of a purification laboratory as this is one of the rate-determining steps of the whole process.[14] In general, solvents can be removed either using freeze-dryers or centrifugal evaporators. Freeze-drying is known to be a high-throughput technology because of the possible continuous processing, that allows for careful treatment of samples without significant danger of evaporation of volatile compounds, and usually leads to powdery solids that are easy to handle in consecutive liquid handling steps. On the other hand, freeze-drying is a rather slow process. In contrast, solvent removal in centrifugal evaporators is a much faster process, but it implies the risk of spilling if the pressure is lowered too fast and very often leads to viscous oils or glassy materials, which are difficult to dissolve again.

Table 1. Evaporation Program for Acetonitrile/Water

	MeCN/water			
step	pressure (mbar)	temp of walls (°C)	temp limit of product (°C)	temp of irradiation (°C)
1	300	50	70	250
2	200	50	70	250
3	40	50	70	250
4	20	80	70	250
5	1	80	70	250
"freeze- drying"				
6	1	80	70	250

The usual solvents to be removed within our laboratory are either up to 80 mL of acetonitrile/water mixtures per fraction after preparative chromatography or 5−15 mL of DMSO per vial after the pooling step. Investigations soon revealed that a freeze-drying process with these quantities would last at least 48 h, which was considered too long for the desired throughput.

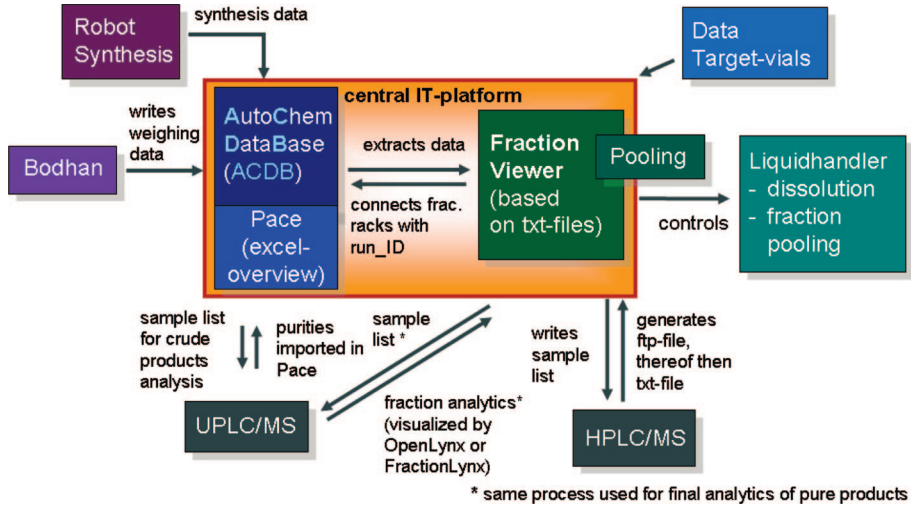
Thus, evaporation is now performed in two vacuum centrifugal evaporators ZT-H6 from Zirbus with space for 144 fraction or target vials. The rack holders have been built according to our specification, so that both fraction and target racks can be transferred directly into the evaporators. The condensator of each centrifuge has a volume of 17 L, perfectly matching our needs of maximum 11.5 L (144 × 80 mL) solvent to be removed. All chamber walls including the door are heated and 6 infrared radiators are positioned above the racks. A Leybold vacuum pump TrivacD25B is used to reduce the pressure within the chamber to 1 mbar within 20 min.

We use a newly developed protocol combining first a classical centrifugal evaporation process to distill off the major quantity of the solvent mixture and afterward a short freeze-drying process, which offers the benefits of both technologies, a relatively fast process which is nevertheless delivering powdery solids as final products.

The run time of a typical evaporation cycle in our laboratory is approximately 17 h. Thus, solvent evaporation can easily take place overnight. Usually, the process consists of 14 h of evaporation at different pressure and temperature stages (see Table 1), 1 h during which the frozen solvent is removed automatically by heating the condensing vessel and applying nitrogen pressure and 2 h of "freeze drying" with the last two steps making the difference compared to a classical evaporation process.

The freeze-drying step is not freeze-drying in a classical way. Actually, at first glance it might seem contradictory that freeze-drying is done while heating the chamber walls to 80 °C, which is necessary to avoid condensation of solvent on the walls, and additionally irradiating the samples with infrared light to support complete solvent removal. However, despite this heating, cooling the condenser to −70 °C, together with the full vacuum applied leads to measured temperatures around 5 °C within the chamber, which is low

Scheme 3. Data Management Throughout the Laboratory Workflow



enough to obtain powdery solids. During this last step of the evaporation process the samples are spinning, too.

When discussing the different temperatures, it should be kept in mind that vacuum is a very poor heat transporter. The temperature within the sample vials during the evaporation phase is limited to 70 °C by means of a temperature controlled reference sample.

Safe handling of the system is guaranteed by inertization with nitrogen before starting the centrifugal dryers and also after each run. This avoids explosive acetonitrile/oxygen mixtures within the chamber, which is especially important because of the infrared irradiators. In case of any unexpected stop of the evaporation program, inertization also takes place automatically. In addition to the safety aspect, oxidation of the substances can thus be inhibited.

Often it is argued that evaporation of the solvent mixture after HPLC chromatography leads to high concentration of the acid used (usually formic acid or TFA) toward the end of the process, which may lead to decomposition of sensitive samples especially under the heating conditions used in centrifugal evaporators. Freeze-drying which is performed at far lower temperature is often regarded as milder and safer method. Therefore, we always perform a final LC/MS analysis of our compounds after the pooling step to guarantee that no decomposed compounds are sent to biological testing.

f. Weighing. All weighing steps during the purification process are done on a Bodhan Balance Automator BA-200 from Mettler Toledo, which allows automated weighing of empty and filled fraction and target vials. An integrated 1D and 2D barcode reading module is used for correct assignment of the data to the barcoded target vials used for compound storage at Bayer CropScience. In addition, we have installed "rack-holders" to avoid crashes by accidental moves of whole racks in case of canted vials. All data is written automatically into the internal database for parallel synthesis data management (AutoChemDataBase, ACDB, based on Oracle-db).

g. Laboratory Furniture. For integration of the purification unit into the robot synthesis laboratory area, some constructional changes were necessary. The former laboratory bench was removed, and a central unit existing of 10 fume hoods, in which all technical equipment except from the centrifugal evaporators was placed, replaced it. Opening of the fume hoods is possible by foot contact and automatic closing after 10 min takes place if the fixed sensors do not register any movement. Safety cabinets for solvent vessels were installed, as well as special soundproof cupboards for vacuum pumps. In addition, computer cupboards and drawers for laptops are part of the laboratory unit to avoid placement of computers and monitors inside the unit.

Not surprisingly a highly automated purification laboratory has special requirements concerning pure water and solvent supply as well as waste disposal. The pure water needed for analytical and preparative HPLC/MS chromatography is produced by a Millipore System consisting of an ELIX5 and a Milli-Q. Acetonitrile is supplied from 1000 L tanks outside the building. Automated waste disposal is ensured via waste containers with liquid level detection inside the unit from where the solvent is pumped to 1000 L tanks outside the building. The whole unit is equipped with liquid sensors on the surface leading to an automatic shut-down of the whole unit in case of leakages to avoid any potential danger of explosion during operation of the system at night or during the weekend.

2. Data Management. Consistent highly automated data management during all steps of the purification process is mandatory to ensure an efficient process and to avoid potential errors. Scheme 3 gives an overview on the IT systems we use to connect all different technical instruments throughout the robot synthesis and purification workflow.

As shown in the Scheme 3 two interconnected systems both programmed by Bayer Business Services serve as central IT-platform: (A) the AutoChemDataBase (ACDB) for storage of essentially all relevant compound data from parallel synthesis laboratories within Bayer CropScience with an Excel-based user-interface called Pace and (B) the Fraction Viewer enabling connection and communication with the different equipment of our purification unit using txt-files.

To illustrate the role of this fraction viewer let us follow the path of a sample through the whole purification process: A batch of crude products prepared by robot synthesis is

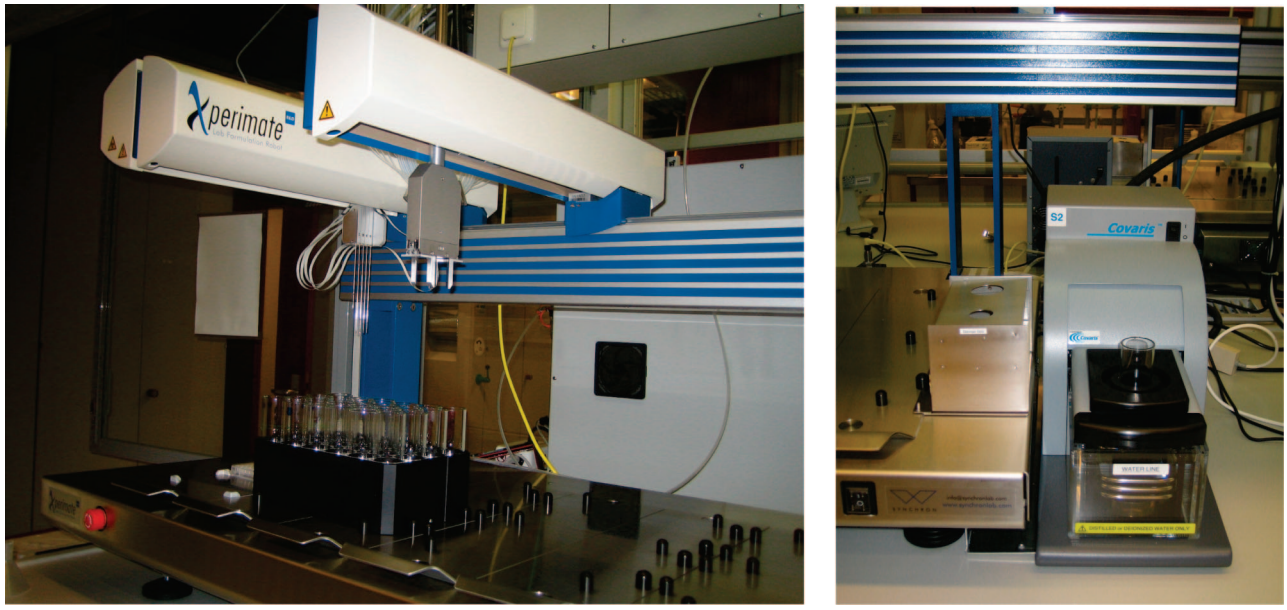


Figure 1. Synchron liquidhandling system with Covaris unit for sample dissolution.

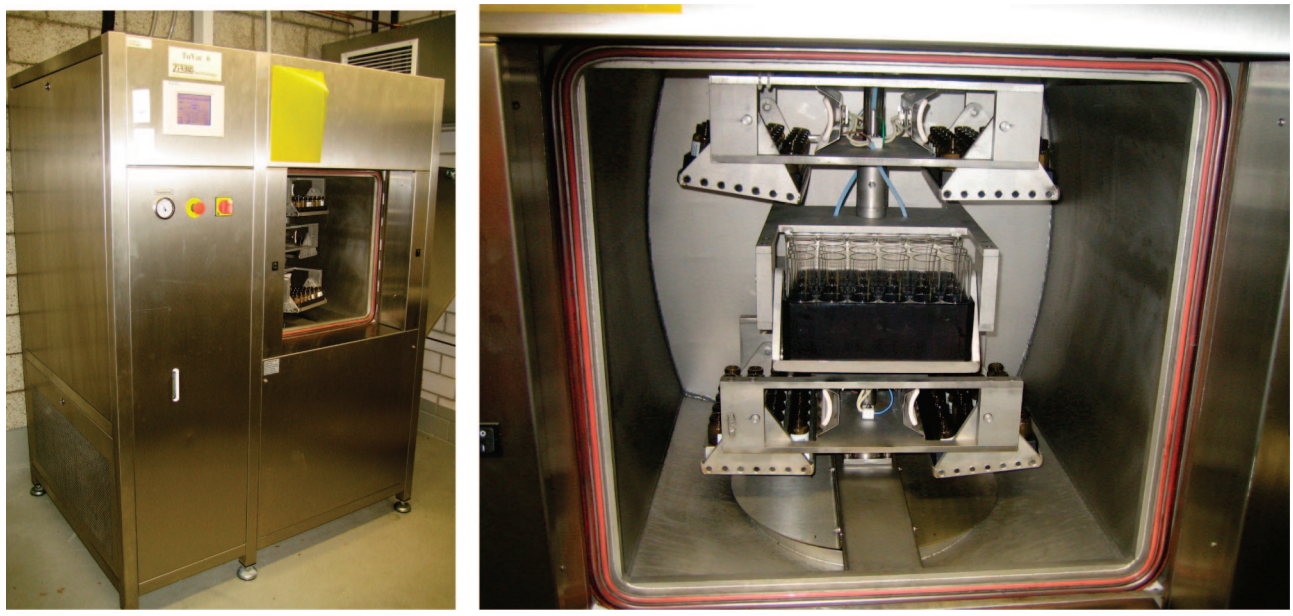


Figure 2. Zirbus centrifugal evaporator.

characterized by a run ID, and all synthesis data is imported into the ACDB. The sample lists for UPLC/MS-analysis of the crude products of the respective batch are then generated by Pace. After UPLC/MS analysis the purities of the crude products are imported into Pace. On the basis of these results a decision is made about whether a sample is already pure enough for biological testing, needs chromatographic purification, or is discarded without purification because it does not contain the desired product. In an independent step, all weighing data of the fraction and target vials assigned to this batch of crude products are determined by the weighing robot and imported into the ACDB. Next, the fraction viewer extracts the UPLC/MS analysis data from the ACDB and generates the sample list for preparative HPLC/MS purification in Waters MassLynx. Then sample dissolution prior to purification is initiated via the Fraction Viewer. After HPLC/MS purification of the whole batch is finished, a txt-file is generated and imported into the fraction viewer where now the assignment of the fraction racks to the run ID is performed, and a sample list for analytical fraction control is generated. Following the subsequent fraction analysis by the UPLC/MS, the results are transferred back into the Fraction Viewer. The solvent is removed in the centrifugal evaporators, and finally, the quantity of substance collected in the fraction vials is determined using the weighing robot again.

Before the reaction mixtures are pooled into the assigned target vials their barcodes are imported into the Fraction Viewer, which visualizes the robot vials, fraction vials, and target vials (ASV bottles) as shown in the Figure 3.

Then, the fractions are assigned to the original robot synthesis sample ID. For each fraction, the user manually checks the LC/MS control analytics using Waters OpenLynx or FractionLynx and decides whether a fraction fulfills our purity criteria and is transferred into a target vial for biological testing. In some cases, the quantity of the

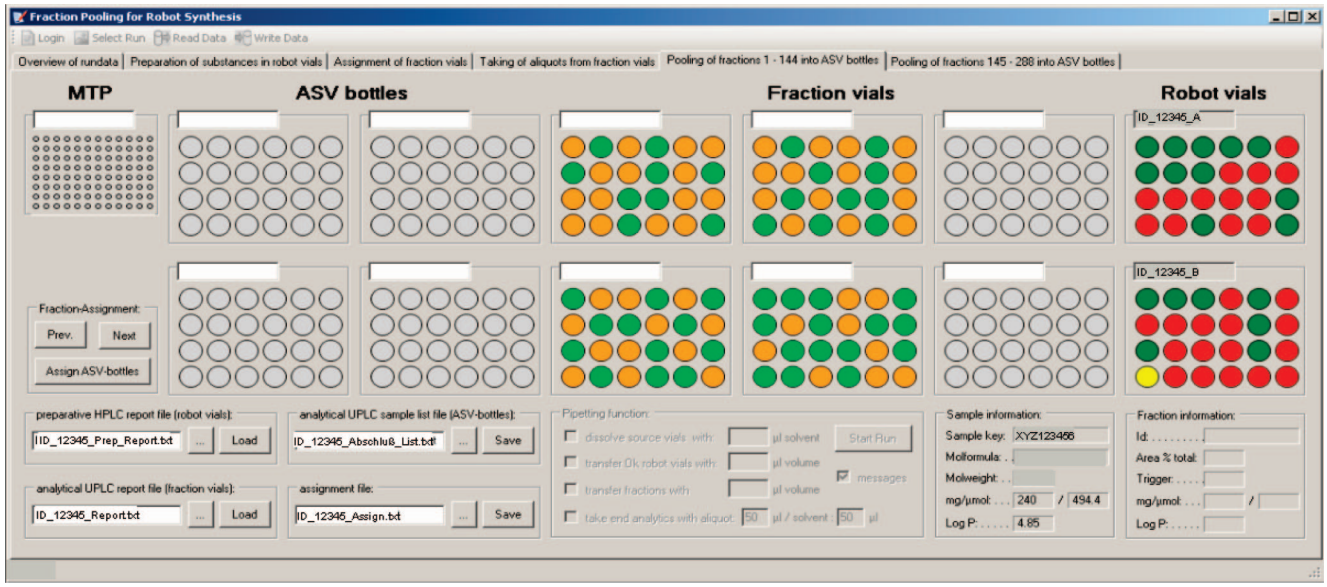


Figure 3. Fraction viewer.

respective fraction is considered as a secondary decision criteria. After this manual assignment is finished the Fraction Viewer starts the pooling process with the Sias liquid handler including subsequent sampling for final control analytics. Samples that were pure enough for biological testing (usually >90% purity) prior to purification are transferred directly from the robot vials into the target vials within the same working step. Evaporation of the target vials and determination of the final purities by LC/MS complete the purification process.

For legally admissible documentation, files and data are stored in defined folders or databases and a final report is printed out and handed over to the customer together with the final analytics.

Conclusion

In conclusion, we have set up a state-of-the-art high-throughput purification unit for robot synthesis products that helps to further increase the productivity and efficiency of the whole process of automated synthesis and purification. With this new purification unit we are able to purify up to 48 crude products per day in a scale of 200−400 mg crude product using just one injection per sample. Thus, the overall time needed from starting a synthesis of a batch of 48 compounds using our robotic system until its finalization by handing over the patent documentation to the customer could be reduced to less than half of the original time needed when our purification process was not yet automated.

These improvements were made possible by successful application of several new technologies to high-throughput purification for the first time, for example, use of Covaris technology for automated sample dissolution and implementation of Zirbus centrifugal evaporators combining the advantages of standard evaporation and freeze-drying processes. This modern hardware system is perfectly accompanied by a highly efficient, interconnected IT-landscape covering the whole synthesis and purification process.

Integration of the purification unit within the robot synthesis laboratory to minimize interfaces and allow for a flexible allocation of staff between synthesis and purification was another important success factor supporting the use of automated synthesis and purification in the lead optimization process to increase efficiency of Bayer CropScience research process.

Despite having setup a fully operable state-of-the-art purification laboratory, further improvements to constantly increase the efficiency of the whole process will still be in our focus for the future.

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