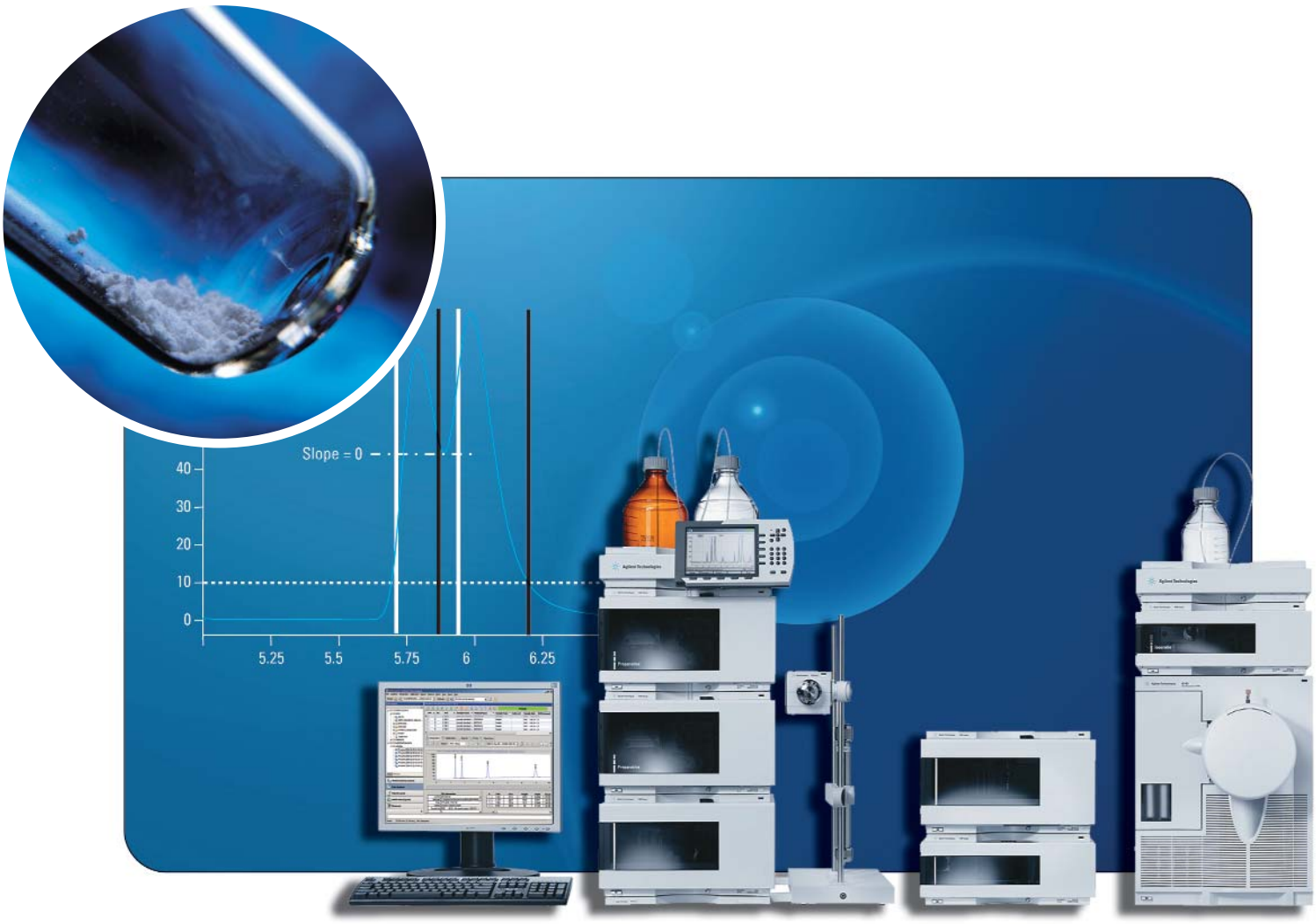
Solutions for Preparative HPLC

Application Compendium



Agilent Technologies

Contents

www.agilent.com/chem/purification

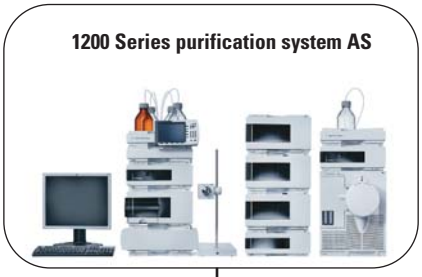
A convenient platform for a wide range of purification needs

This compendium is a collection of examples and applications based on studies performed on the Agilent 1200 and 1100 Series purification platforms. Discover the experiences of other professionals covering a variety of subjects, including: fractionation and re-analysis, peak- and mass-based fraction collection, as well as optimization of the purification system.

The compendium gives an overview of Agilent's Application Notes, for preparative HPLC. The applications performed on the Agilent 1100 Series system have since been verified using an Agilent 1200 Series LC system, which showed comparable or even better performance. The notes are presented in a condensed form, for more in-depth reading the full length versions can be downloaded from Agilents website at ***www.agilent.com/chem/purification.***

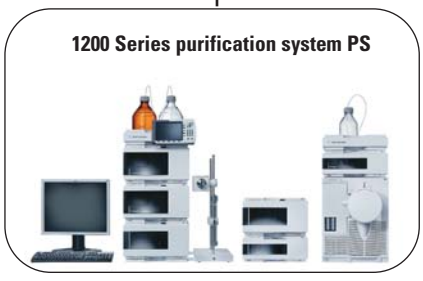
Advantages of the 1200 Series purification platform

- A choice of pumps, autosamplers, fraction collectors, detectors, columns and preparative flow cells to optimize each application for maximum recovery and purity
- Scaleable choices that let you match the purification process to sample size, ranging from µg to gram quantities
- Fraction collection based on time, peak and/or mass – an unique combination
- Easy Access software provides convenient walk-up access for non-expert users
- One-vendor solutions for all your equipment, supplies and support



The 1200 Series purification platform offers two basic systems for analytical and preparative scale applications:

- 1200 Series purification system AS (analytical scale) for flow rates below 5 ml/min
- 1200 Series purification system PS (preparative scale) for flow rates up to 100 ml/min



Isolation of impurities with preparative HPLC

Impurity profiling describes a group of analytical activities aimed at the detection, identification, structure elucidation and quantitative determination of organic and inorganic impurities in drugs. The first task in this process is the detection of all impurities. Even with the most sophisticated MS instruments, the complete structure elucidation of all compounds is often impossible. These compounds have to be isolated and purified and are then characterized by ^{1}H- and ^{13}C-NMR. The isolation and purification of a pair of impurities that could not be identified by MS is described in this Application Note.

Results and discussion

The impurities A and D in figure 1 could be identified by MS using an Ion Trap and TOF instrument. Impurities B and C, however, could not be characterized completely, and have therefore been isolated by preparative HPLC for further structure elucidation.

Method optimization

Since the sub-2 µm particle stationary phase used in the MS experiments is also available with 5-µm particle size as a preparative scale column, the method only had to be optimized for the resolution of impurities B and C and for shorter run times. The result for a sample enriched with impurities B and C is shown in figure 1.

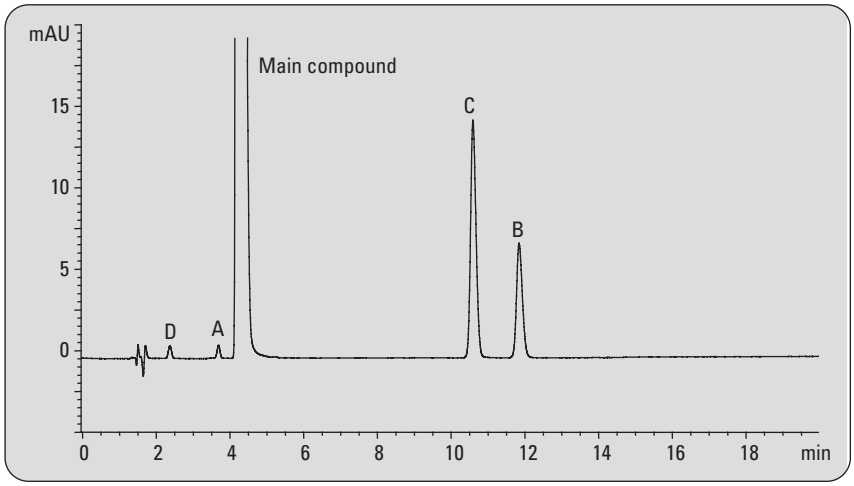


Figure 1
Optimized method for impurities C and B.

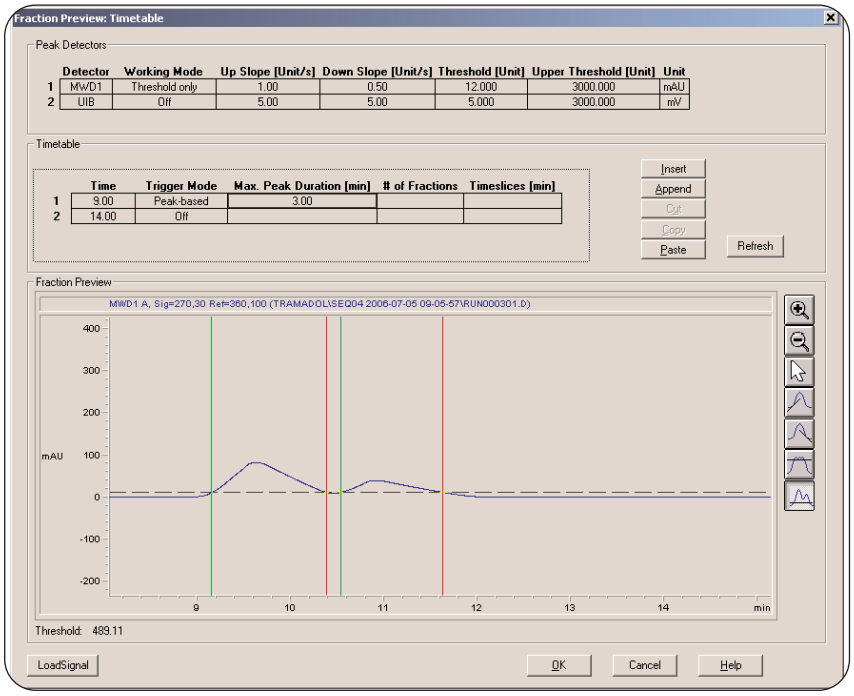


Figure 2
ChemStation *Fraction Preview*.

Purification parameters

To optimize the fraction collection parameters the *Fraction Preview* of the ChemStation was used. As shown in figure 2 the chromatogram from the preparative run was loaded and the fraction collection parameters like threshold, up slope, down slope and upper threshold were adjusted until the desired fraction collection performance was achieved. In this case a simple threshold-based collection within a time window (9 – 14 minutes) gave the best result. The result of the actual fraction collection run with the parameters optimized using *Fraction Preview* is shown in figure 3. The analysis of the combined fractions from ten consecutive purification runs is shown in figure 4. The structures as shown in figure 4 could be elucidated by NMR analysis (data not shown) after collecting sufficient pure material of impurities C and B.

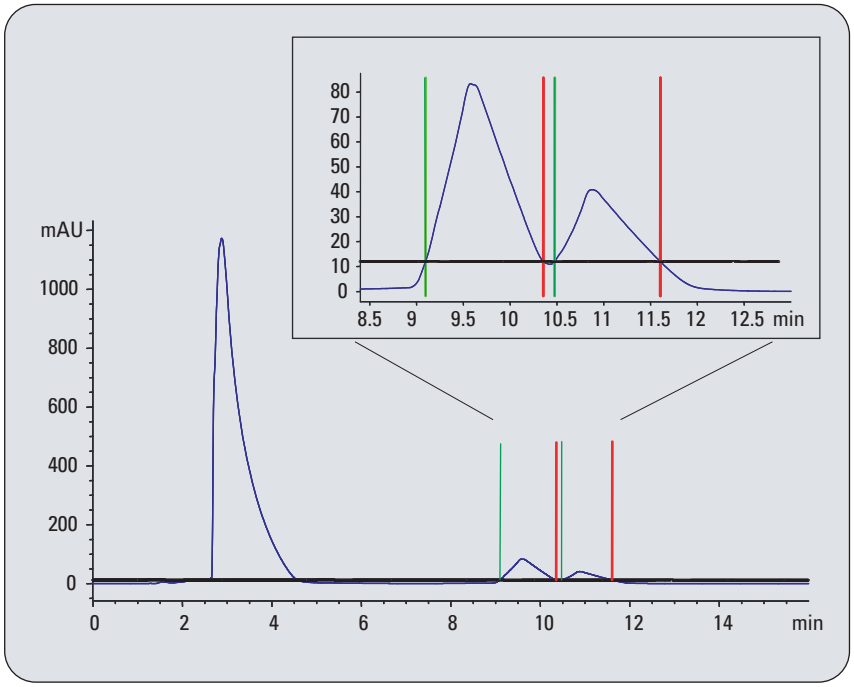


Figure 3
Result of fraction collection.

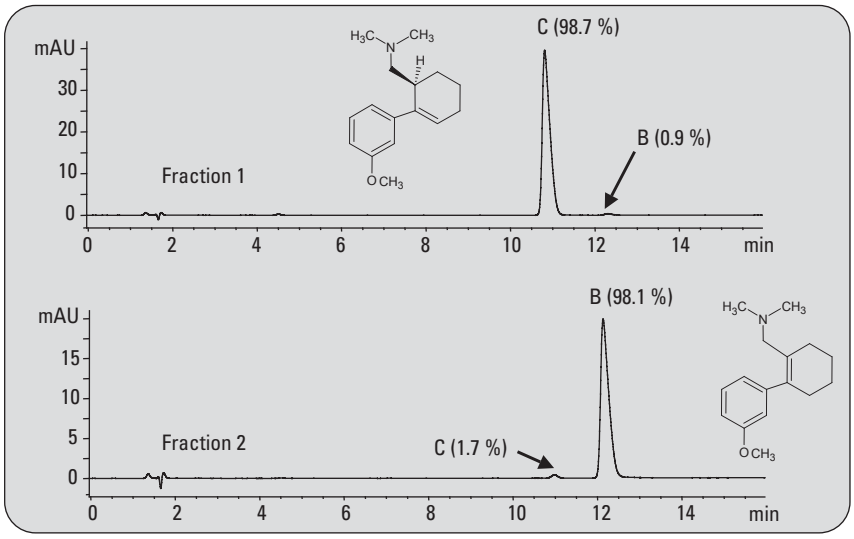


Figure 4
Identified impurities in the pharmaceutical drug synthesis of the main compound.

Conclusion

In this Application Note the isolation and purification of two impurities was illustrated, beginning with a high-resolution analytical method that was developed on an Agilent 1200 Series RRLC system. The transfer to a standard Agilent 1200 Series and to an Agilent 1200 Series preparative system was seamless because the stationary phase was available with sub-2 µm particles in analytical column size as well as in standard 5-µm particles in preparative column sizes. Therefore, the scale-up after the method optimization and loading experiments could be done directly and without any further method optimization on the preparative column.The excellent purity and recovery of collected fractions made the structure elucidation of the impurities an easy task.

Impurity Profiling with the Agilent 1200 Series LC/MS System – Part 2: Isolation of Impurities with Preparative HPLC, publication number 5989-5618EN.

Isolation and purification of radio-labeled drug metabolites from pre-clinical urine samples

In collaboration with Gordon Dear, GlaxoSmithKline, UK

The identification of metabolites of novel pharmaceutical drug candidates is a fundamental part of the drug development process. It plays an important role in early drug discovery lead optimization, leading to drug candidates with more favorable pharmacokinetic and disposition characteristics. Later in drug development the identification of drug metabolites, in test animals and later in humans, is required by the regulatory authorities in support of safety testing. During drug development drug metabolism studies are usually conducted with the radiolabeled drug candidate and therefore the identification of drug-related metabolites is facilitated using radiochemical detection. Metabolites can often be present at low concentrations in very complex matrices, such as urine, bile and plasma, making it necessary to isolate and purify metabolites for unequivocal identification, by techniques such as nuclear magnetic resonance (NMR) spectroscopy.

Results and discussion

Injection of a non-labeled standard of a GSK drug candidate

To test the chromatography system before the injection of a valuable biological sample an authentic standard of the drug candidate was injected from 10 mL of water (~1 µg/mL) using the injection pump. The retention time of the parent compound was 36.3 min as shown in figure 1.

Injection of a dog urine sample

Figure 2 shows the radiochemical detector (RD) and DAD trace following the injection of 10 mL dog urine sample containing radio-labeled drug candidate and its metabolites, which can easily be identified in the RD signal. To isolate the metabolites either time-based fraction collection using the RD as monitor or peak-based fraction collection based on the RD signal could be performed.

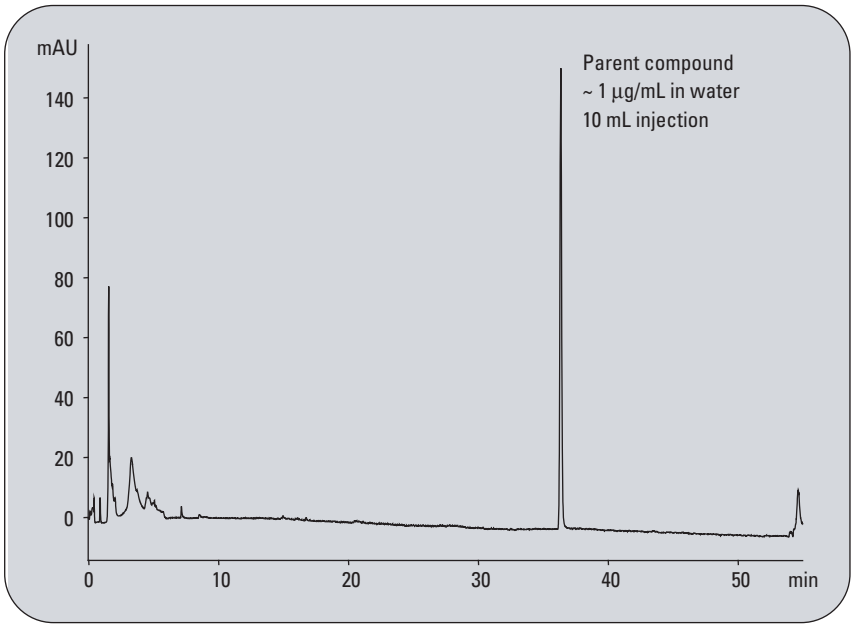


Figure 1
Injection of standard.

Peak-based fraction collection on the RD signal

To be able to trigger on the signal coming from the RD the signal output was connected to the Universal Interface Box (UIB), which was connected to the CAN network of the Agilent 1100 Series modules. To make sure no metabolite was lost even if it fails to trigger the fraction collector a recovery collector was configured into the system. The result of the peak-based fraction collection is shown in figure 2.

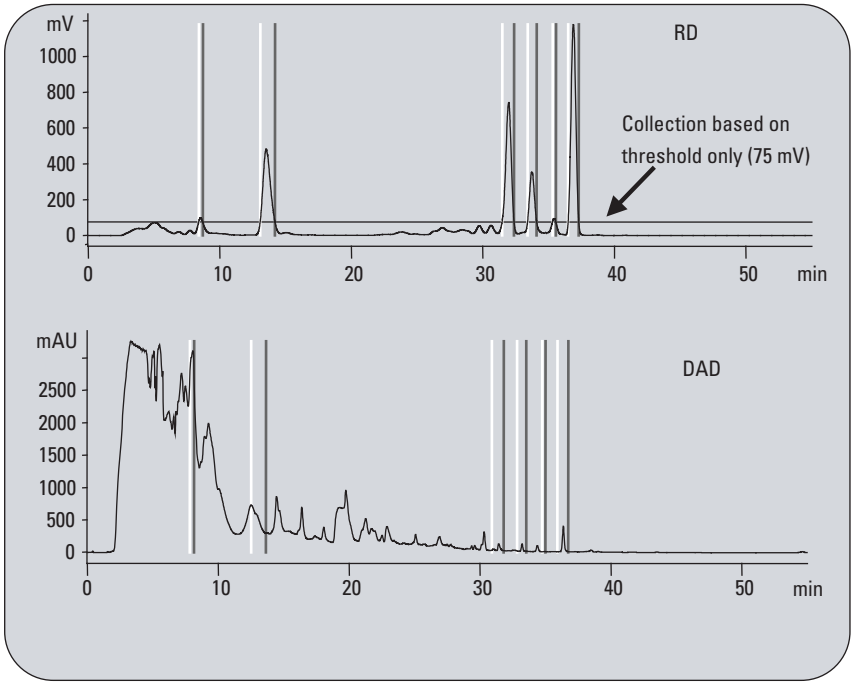


Figure 2
Peak-based fraction collection on the RD signal.

Conclusion

In this Application Note the set up and configuration of a purification system optimized for the isolation and purification of radiolabeled drug metabolites from typical DMPK samples, is described. The most important aspects of the configuration are the injection pump system enabling large injection volumes, and the possibility to identify the fractions containing the metabolites using a radiochemical detector. Furthermore, fraction collection strategies were shown using a real-life sample from a metabolism study carried out in a DMPK group at GlaxoSmithKline, Ware, UK.

"Isolation and Purification of Radiolabeled Drug Metabolites from Pre-clinical Urine Samples", publication number 5988-2088EN.

Isolation and purification of drug candidate metabolites from human urine samples

In collaboration with Gordon Dear, GlaxoSmithKline, UK

The identification of metabolites of novel pharmaceutical drug candidates is a fundamental part of the drug development process. It plays an important role in early drug discovery lead optimization, leading to drug candidates with more favorable pharmacokinetic and disposition characteristics. Later in drug development the identification of drug metabolites, in test animals and later in humans, is required by the regulatory authorities in support of safety testing. Investigation into the actual metabolism in humans is generally achieved through dosing the radiolabeled drug within a clinical study. However, preliminary human metabolism data can be gathered following administration of novel drug candidates to human volunteers, as part of phase I of the clinical program. This necessitates the need to isolate metabolites from large volumes of biological matrix, human urine in this instance, in order that unequivocal structures can be derived by techniques such as nuclear magnetic resonance (NMR) spectroscopy.

Injection of a standard of a GSK drug candidate

To test the chromatography system before the injection of a valuable biological sample an authentic standard of the drug candidate was injected from 50 mL of water (approx. 1 µg/mL) with the injection pump (figure 1).

Time-based fraction collection applied to a human urine sample

Figure 2 shows the results for time-based fraction collection of 50 mL of a human urine sample injected using the injection pump system with collection of a fraction every 0.5 min into 12 mL test tubes. All collected fractions are

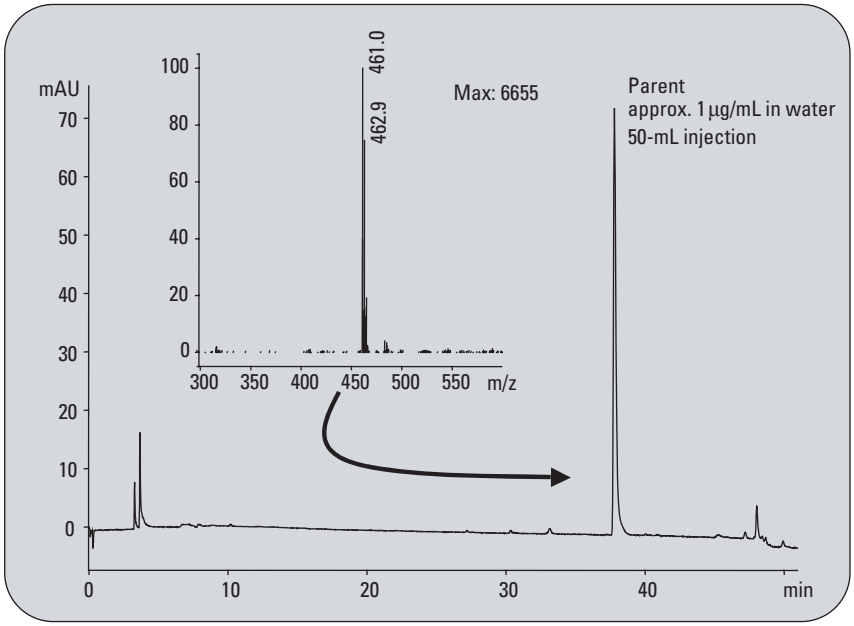


Figure 1
Chromatogram and MS spectrum (negative ionization mode) of drug candidate standard.

subsequently available for further structural analysis, using NMR or additional LC/MS.

Identification of fractions containing drug and its metabolites

Drug candidate and some of its metabolites with known masses could be identified in the MSD signal . The fractions containing the five compounds could easily be identified because the fraction tick marks in each signal are aligned using the delay time for the DAD and the MSD.

Conclusions

In this Application Note the injection of 50 mL of human urine using an injection pump system was described as well as the time-based fraction collection of drug candidate and its metabolites on an Agilent 1100 Series purification system. The result of the fraction collection was monitored with an MSD and 4 metabolites plus the parent compound could be found using the extracted ion chromatograms. The fractions containing the compounds could readily be identified, thereby providing semi-purified fractions for further analysis.

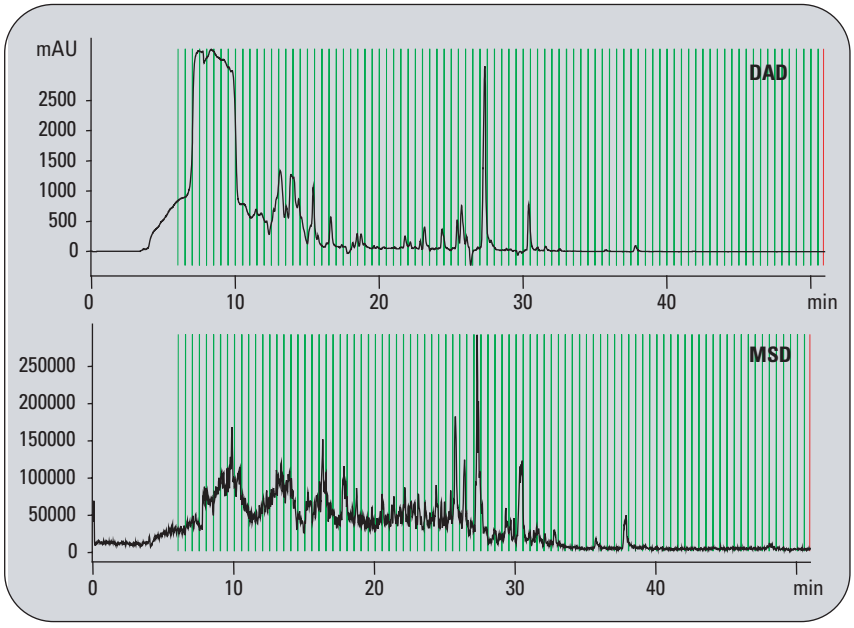


Figure 2
Time-based fraction collection on human urine sample.

"Isolation and Purification of Drug Candidate Metabolites from Human Urine Samples", publication number 5988-2089EN.

Purification of enantiomers

The stereoisomeric composition of drugs has become a key issue in today's drug development. Drug registration requires a pure drug enantiomer during the candidate selection phase to evaluate differences in efficacy, toxicity or pharmacokinetics of the two enantiomers. Due to the fact that only small amounts of potent drugs are required to carry out the corresponding experiments in early development, preparative high-performance liquid chromatography is a suitable tool to isolate the pure enantiomers from a racemic mixture. In this Application Note the separation of the racemate of a respiratory drug candidate in gram scale is described. The separation is carried out using the Agilent 1100 purification system.

In collaboration with Volkmar Körner, Boehringer Ingelheim, Germany

Results and Discussion

Analytical method

The analytical method to separate the enantiomers E1 and E2 was developed on a Chiralpak AD-H 5 µm column (4.6 x 150 mm). This method was also used later in the purification process to control the purity and enantiomeric excess of the fractions. Experiments were carried out to determine the maximum amount of sample, which can be loaded onto the analytical column.

Preparative method

Based on the results of the overloading experiments on the analytical column the scale-up to the preparative column was done. To increase the throughput the sample concentration was increased to 50 and to 200 mg/mL in ethanol, keeping the injection volume at 900 µL. The results of the runs are shown in figure 1.

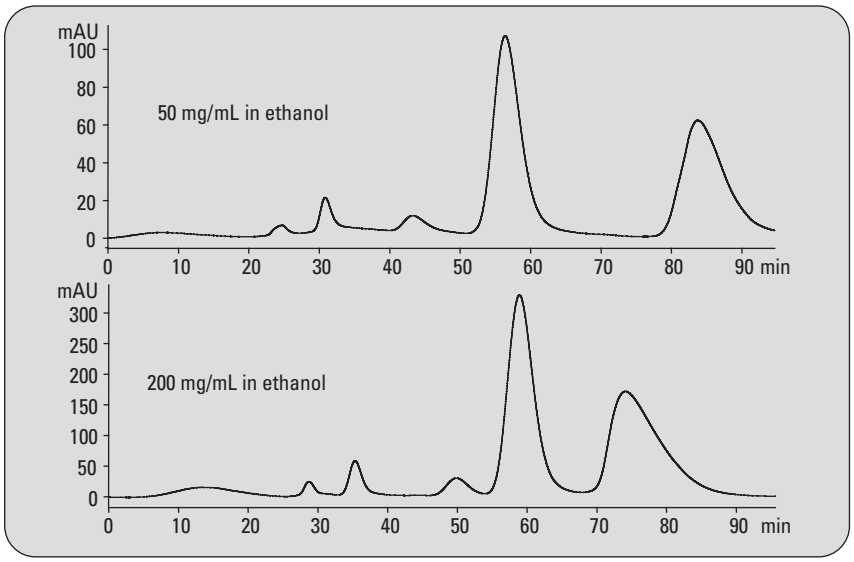


Figure 1
Increasing the sample amount loaded onto the column.

Analysis of the combined collected fractions

The combined collected fractions were analyzed using the method as described in *Analytical method*. Both enantiomers could be isolated with very high enantiomeric excess as shown in figure 2.

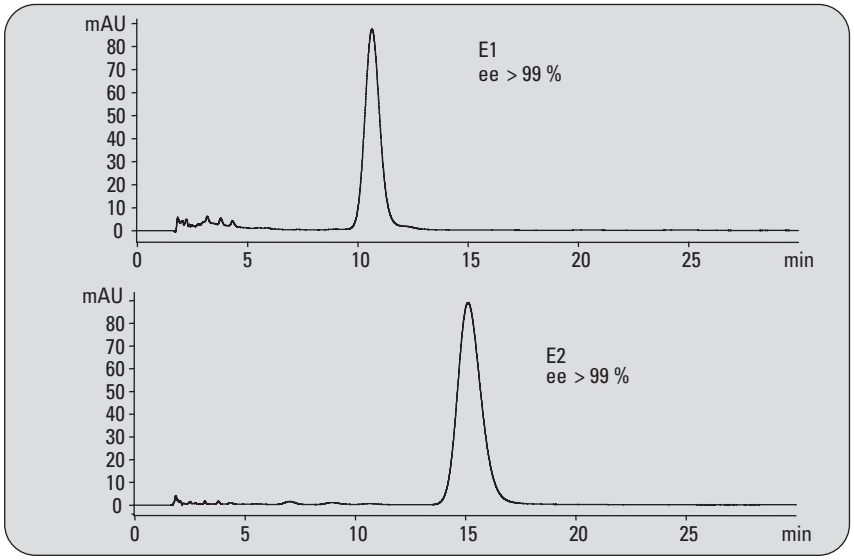


Figure 2
Re-analysis of the combined fractions.

Conclusion

In this Application Note the purification of two enantiomers of a respiratory drug candidate from Boehringer Ingelheim in Biberach, Germany was demonstrated using an Agilent 1100 Series purification system. The method was developed on an analytical column and scaled up to a preparative column after the loading experiments had been done. After adjusting the preparative method the purification was done in gram scale and the combined collected fractions were re-analyzed to determine the enantiomeric excess.

"Isolation and Purification of Enantiomers on the Agilent 1100 Series Purification System", publication number 5988-3396EN.

Mass-based purification of compound libraries

Compound libraries consist of structural analogues that need to be screened for their biological activity. However, although combinatorial chemistry simplifies the synthesis process in comparison to conventional synthesis chemistry, compounds still have to be purified from impurities and reaction by-products.

Equipment

The instrumental set-up comprises two flow paths. The main flow leads from the binary pump to the autosampler, the thermostatted column compartment, the diode-array detector and the active splitter before reaching the fraction collector. Since the mass selective detector (MSD) is a destructive detector and the flow rate of the main flow is too high to route it directly into the electrospray source, a make-up flow is sustained by the isocratic pump. This make-up flow leads from the isocratic pump to the active splitter before reaching the MSD. In order to facilitate mass detection the active splitter transports an aliquot of the main flow into the make-up flow which carries it into the MSD.

Results and discussion

Sample Preparation

To demonstrate mass-based fraction collection with the Agilent 1100 Series purification platform we generated a library comprising 17 related compounds that are all derivatives of phenothiazine (figure 1).

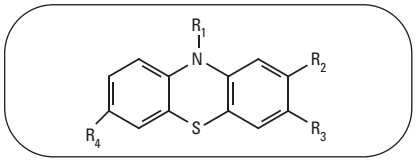


Figure 1
Structure of phenothiazine derivatives.

Mass-based fraction collection

In the software for each of the 17 mixtures the target mass for fraction collection was set-up. Fraction collection was triggered on the singly charged positive ion, see figure 2.

Conclusion

Fraction collection triggered by predefined masses is advantageous over conventional, less specific detectors:

- Only the compound of interest is collected, when this technique is applied in each run.
- It is not necessary to pick out target compounds out of a series of fractions collected during chromatographic runs.
- Agilent's patented fraction collection delay calibration ensures a reliable sample recovery.

Altogether, the Agilent 1100 Series offers a time-and resource-efficient purification platform for mass-based fraction collection.

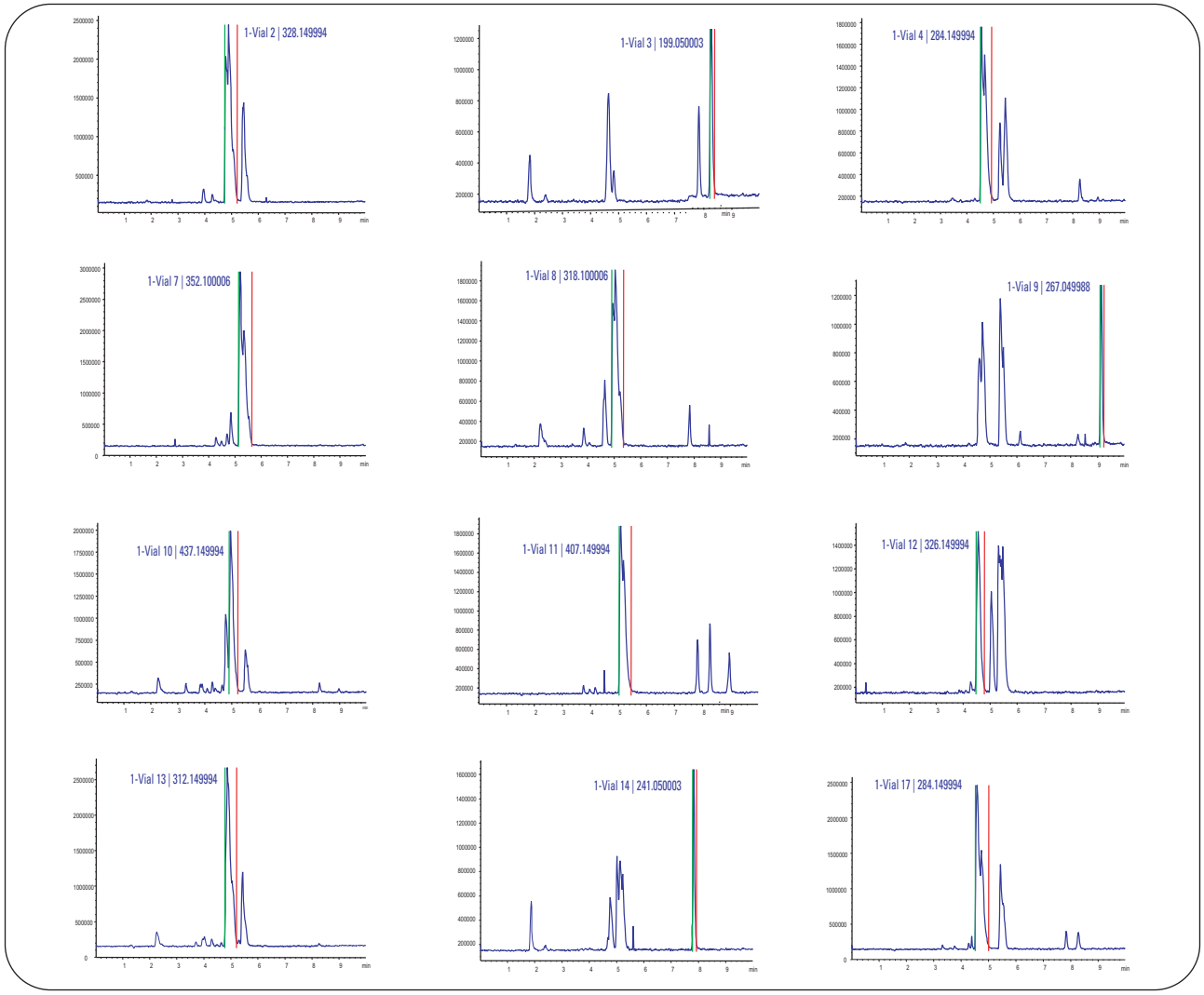


Figure 2
12 selected TIC chromatograms. The displayed tick marks show beginning and end of fraction collection.

"Mass-based Fraction Collection of Compound Libraries using the Agilent 1100 Series Purification System", publication number 5989-1656EN.

Purification of natural products

This is an example of the separation of formononetin and other phytoestrogens from red clover in analytical and preparative scale with the Agilent 1100 Series purification systems AS and PS.

Results and discussion

Volume overloading experiment

Since concentration overloading was not possible due to the fixed concentration of the red clover extract, volume overloading had to be done to isolate the compounds. Injecting up to 50 µL crude extract sample still lead to sufficient separation for analytical scale purification .

Isolation of phytoestrogens in analytical scale.

A common method to isolate compounds from complex natural extracts is fractionation by time slices. Because of the good separation achieved in the overloading experiments, peak-based fractionation was used for the red clover extract.

Isolation of higher amounts

To gain more phytoestrogen material, pooling of fractions from several runs was carried out. This means repetitive injections were performed from one sample vial and the resulting fractions were collected in the same function vials. 450 µL of sample was injected in nine 50-µL injections and the resulting fractions were pooled automatically. Reanalysis of the fractions showed good results which demonstates the excellent performance of the instrument and software (figure 1).

Another possibility to purify more material is to scale-up to a larger column. Based on the analytical scale column overloading experiment, scale-up calculations were done to inject 450 µL in one single injection. This was achieved on a 9.4 x 150 mm column at a flow rate of 7 mL/min. Since the Agilent 1100 Series well-plate autosampler AS can only be used up to a flow rate of 5 mL/min purification was transferred to a purification system PS.

Isolation of phytoestrogens in preparative scale

To purify even higher amounts of sample the method was further scaled up to a 21.2 x 150 mm column. At this scale it was possible to inject 2300 µL of sample in a single injection. The chromatogram is shown in figure 2. Re-analysis of fractions showed that scale-up was possible without losing any performance with regard to the purity of the fractions.

Conclusion

An analytical scale up method can be used to separate the compounds in a complex crude plant extract using the Agilent 1100 Series purification system AS. Based on this method an analytical scale preparative separation with peak-based fraction collection was carried out.

- To obtain more phytoestrogen material the pooling feature of the Agilent 1100 Series purification system was used.
- The purity of the compounds gained was determined by the reanalysis of the fractions.
- Based on the analytical scale results the method was scaled up.
- The purification was repeated on two different columns on the Agilent 1100 Series purification system PS to gain higher amounts of the desired compounds in single runs.

The results in preparative scale were comparable to the results achieved on the analytical scale system.

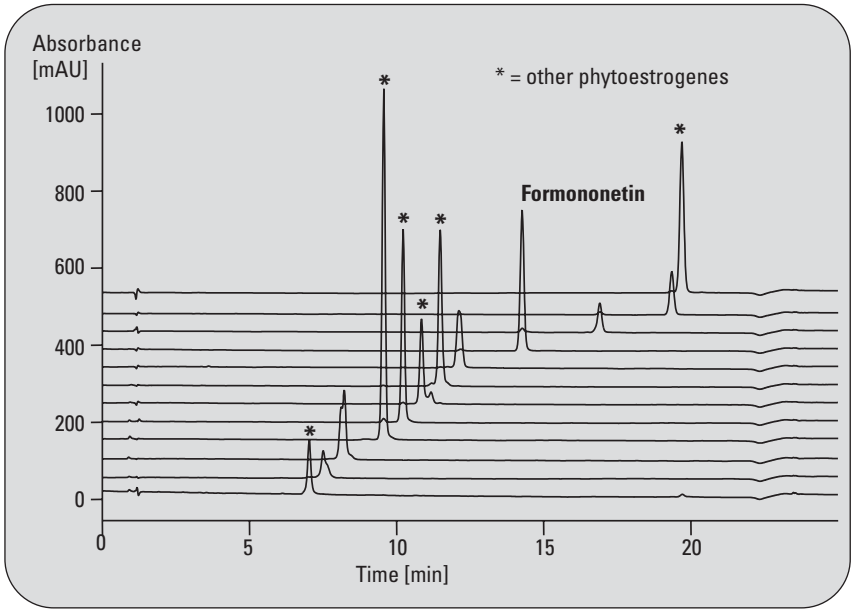


Figure 1
Re-analysis of fractions from pooling.

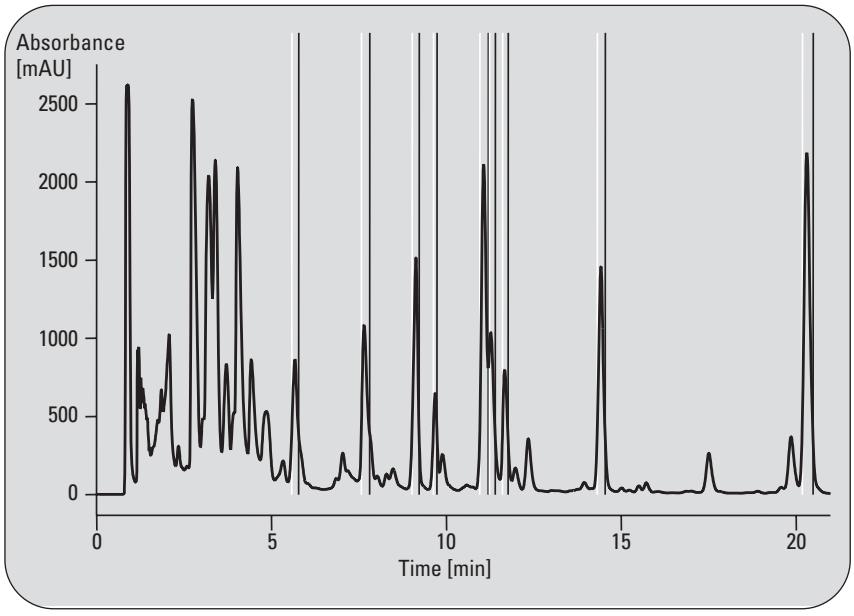


Figure 2
Fractionation on a 21.2-mm preparative column.

"Isolation of Formononetin and Other Phytoestrogens from Red Clover with the Agilent 1100 Series Purification System", publication number 5988-5747EN.

Protein purification and characterization

Protein yield and purity are important criteria for the success of a purification process. Besides efficient chromatographic conditions, well-established and reliable instruments also contribute to successful purifications. Due to its high resolving power, preparative reversed phase (RP) HPLC is often employed as a last polishing step in purification workflow for peptides and small hydrophilic proteins. Although the solvent conditions used in RP-HPLC are known to denature protein structure it can often be regained by adjusting favorable conditions especially for small proteins. The Agilent 1100 Series purification system represents an appropriate and well-established system for reversed phase chromatography.

Results and discussion

Only 4 µL of the pre-purified sample was used in a first analytical step using the Agilent 2100 bioanalyzer to determine whether and in what amounts the 56 kDa protein of interest was present. Figure 1 shows the electropherogram and gel-like image of the analysis. The peak in the electropherogram running at 28 seconds corresponds to a 58.6 kDa protein which has an abundance of 20 % of the total protein concentration and is also visible in the gel-like image. This clearly indicates the presence of the target protein in the sample.

The remaining material was subjected to a purification step using RP-HPLC. Figure 2 shows the chromatogram of the RP-HPLC purification run of the pre-purified sample with the Agilent 1100 purification system. The vertical lines indicate start and stop of fraction collection and the horizontal line visualizes the threshold value for fraction triggering. Further, the corresponding vials in the fraction collector are denoted for each fraction.

To identify the fraction that contains the 56 kDa target protein all three fractions were lyophilized, solubilized in PBS buffer and analyzed with the Agilent 2100 bioanalyzer. Figure 3a shows the electropherograms of the starting material and the three fractions. The corresponding gel-like image is displayed in Figure 3b. The data of the electropherograms and the corresponding gel-like image clearly show that the 56 kDa target protein was purified in fraction 2. Figure 4a shows the RP-HPLC re-analysis of fraction 2. Due to a precise delay volume calibration the chromatogram of the re-analysis shows a single, symmetrical peak at about 11.5 minutes. Although this result suggests a pure protein, the electropherogram of fraction 2 (figure 4b) still shows a minor impurity around 20 kDa. The purity of the protein determined by the 2100 bioanalyzer software was 76 % with a relative concentration of that protein of 456 µg/mL. This result can be easily understood, knowing that the Agilent 2100 bioanalyzer separates proteins by a method that is orthogonal to RP-HPLC. RP-HPLC is known to separate molecules due to differences in hydrophobicity, whereas the 2100 bioanalyzer detects differences in protein size. For this reason the impurities seen in figure 4b could be revealed. In the RP-HPLC chromatogram the peak comprises two different co-eluting proteins. To yield a pure protein a further purification step with a complementary method such as ion exchange chromatography or gel filtration would be necessary.

Conclusion

If sample purity is a crucial issue it should not only be determined by a single analytical method. For this reason Agilent offers solutions – the 1100 Series purification system and the 2100 bioanalyzer – which together ensure efficient and reliable protein purification and characterization.

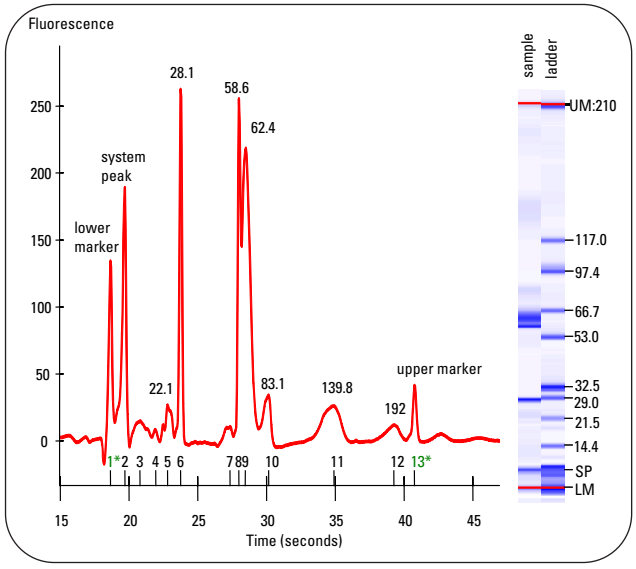


Figure 1
Analysis of the pre-purified protein sample on the Agilent 2100 bioanalyzer using the Protein 200 Plus assay. The electropherogram and gel-like image are shown. The numbers indicate the molecular weight in kDa. The 56 kDa protein of interest runs at a migration time of 28 s corresponding to 58.6 kDa.

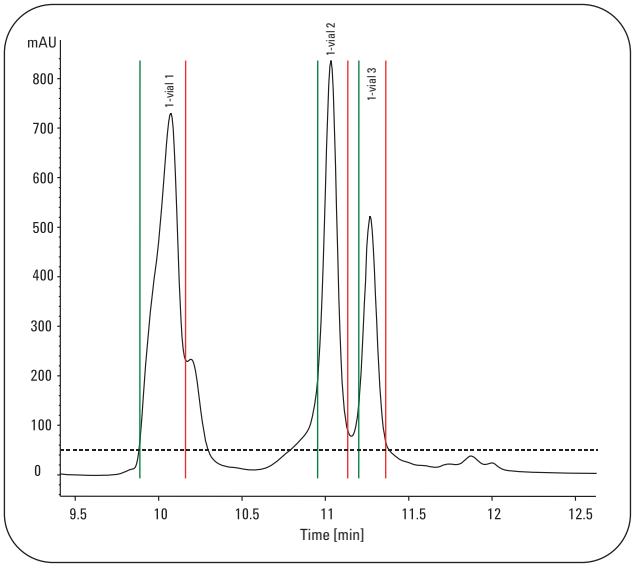


Figure 2
Chromatogram of the purification of the pre-purified protein sample. The vertical lines indicate start and stop of fraction collection, respectively. The horizontal line indicates the threshold value for peak triggering of the 280 nm UV-signal. Additionally, the numbers denote the positions of the collected fractions in the fraction collector.

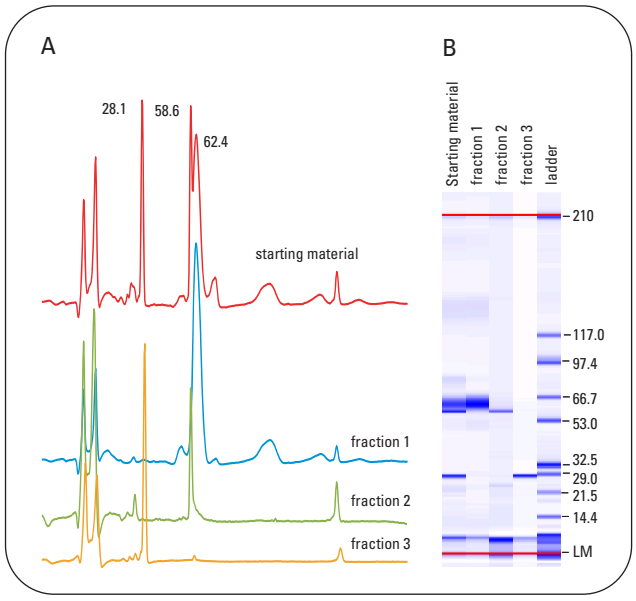


Figure 3a/b
Analysis of the three fractions generated with the purification system on the Agilent 2100 bioanalyzer using the Protein 200 Plus assay. The electropherograms (A) and gel-like image (B) are shown. The numbers indicate the molecular weight in kDa. The 56 kDa protein of interest was located in fraction 2.

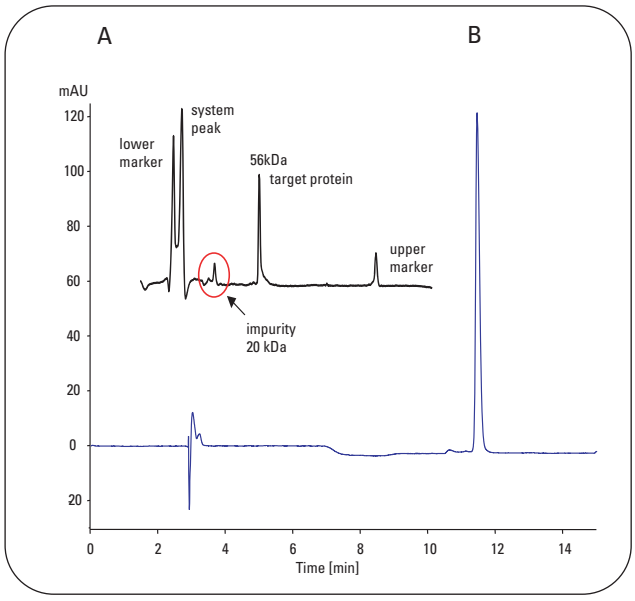


Figure 4a/b
Chromamtogram (A) and electropherogram (B) of fraction 2. Although the RP-HPLC suggests a pure protein, the electropherogram still reveals a minor impurity around 20 kDa.

"Protein Purification and Characterization Using the Agilent 1100 Series Purification System and the 2100 Bioanalyzer", publication number 5988-8630EN.

Visit our website at **www.agilent.com/chem/purification** to download the detailed Application Note.

Mass-based fraction collection with the Agilent 6110/6120 Quadrupole LC/MS system

The Agilent 1200 Series purification system is a versatile tool for purification of small and large compound libraries in medicinal and high throughput synthesis, resulting in fractions with high purity and excellent recovery for the target compounds. The Agilent 6110/6120 Series MSD is the next-generation single quadrupole instrument, which allows easy and reliable mass-based fraction collection but also more sophisticated purification tasks like working with the Multimode Source or using positive/negative polarity switching.

Results and discussion

Mass-based fraction collection with Multimode Source

The Agilent 6120 MSD is required to operate the Agilent Multimode Source. It enables simultaneous electrospray and atmospheric pressure chemical ionization. Ions will be generated and fractions will be triggered if the compound ionizes either in ESI or in APCI. The result of a mass-based fraction collection run is shown in figure 1

Mass-based fraction collection with Multimode Source and positive/negative polarity switching

Using the Agilent 6120 MSD, it is also possible to perform positive/negative switching within one run. Therefore, ions will be generated if the target compound

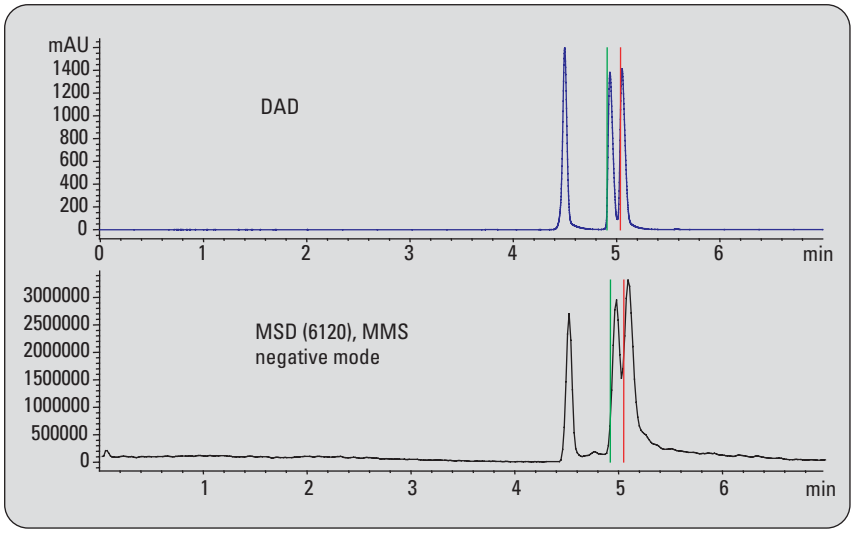


Figure 1
Result of mass-based fraction collection, Multimode Source, negative ionization mode.

ionizes either in ESI or APCI in either positive or negative mode. To trigger fractions the adducts for the $[M+H]^+$ and the $[M-H]^-$ ions are typically specified in the method. The results of a mass-based fraction collection experiment is shown in figure 2.

Conclusions

In this Application Note mass-based fraction collection with the Agilent 6110/6120 MSD was shown using different ionization techniques such as ESI and APCI in negative and positive ionization modes. For easy and reliable mass-based fraction collection the Agilent 6110 MSD can be used either with ESI or APCI source in either positive or negative ionization mode. The Agilent 6120 MSD is required to operate the Multimode Source with simultaneous ESI and APCI or if positive/negative ionization is required in a single run.

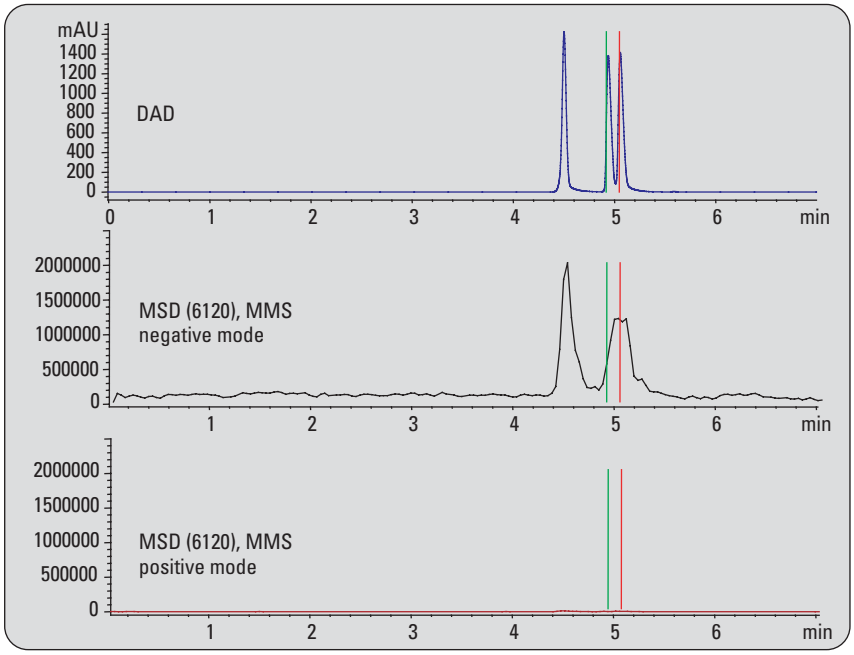


Figure 2
Result of mass-based fraction collection, Multimode Source, negative and positive ionization mode.

"High Performance Mass-based Fraction Collection with the Agilent 6110/6120 Quadrupole LC/MS System Equipped with Multimode Source", publication number 5989-5673EN.

Optimal configuration of a mass-based purification system

In collaboration with Martin Fuhr, Judith Steffens, Grünenthal GmbH, Germany

Since the molecular mass of compounds from medicinal or high throughput synthesis is known by the synthetic chemists, preparative HPLC with mass-based fraction collection is usually the purification method of choice.
An important part of a mass-based fraction collection system is the flow splitter which also has a tremendous influence on the purity of the collected fractions. The influence of the splitter design and the correct configuration of the flow splitter in the system are discussed in this Application Note.

Results and discussion

Splitter design

The most commercially available flow splitters are passive splitters (figure 1a). The split is achieved by using tubing of various lengths and diameters, resulting in different back-pressures. The Agilent active splitter (figure 1b) has an unique working principle: a rapidly switching valve transfers a certain volume of mobile phase actively from the main flow into the make-up flow. The split ratio is determined by the switching frequency of the valve.

Configuration 1: UV detector in front of splitter

This configuration (figure 2) is only possible with the active splitter because the passive splitter creates a back pressure too high

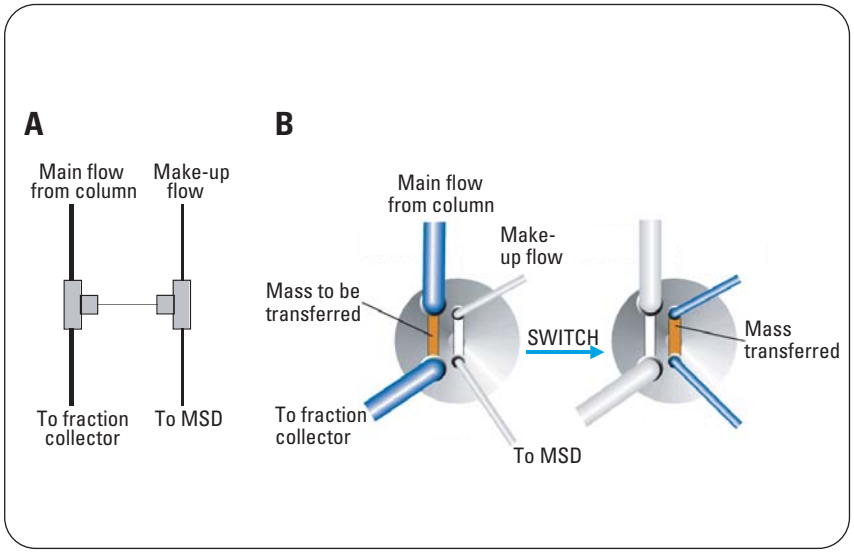


Figure 1
A) Passive splitter B) Active splitter.

for the flow cell of the UV detector. The purity results for UV, MS and combined fraction collection are shown in table 1.

Configuration 2:
UV detector between splitter and MSD
Experiments using configuration 2 were done with the active and passive splitters. The results are shown in table 2. The purity results achieved with the passive splitter are always slightly lower than with the active splitter. Furthermore purities are lower than with configuration 1, especially when using mass-based fraction collection.

Configuration 3:
Second splitter to UV and MS detector
In this configuration the flow coming from the active or the passive splitter was split again to the UV and to the MS detector using a tee. The split ratio achieved by the back pressures of the detectors was not determined. Experiments using configuration 3 were done with the active and with the passive splitter. The results are shown in table 3.

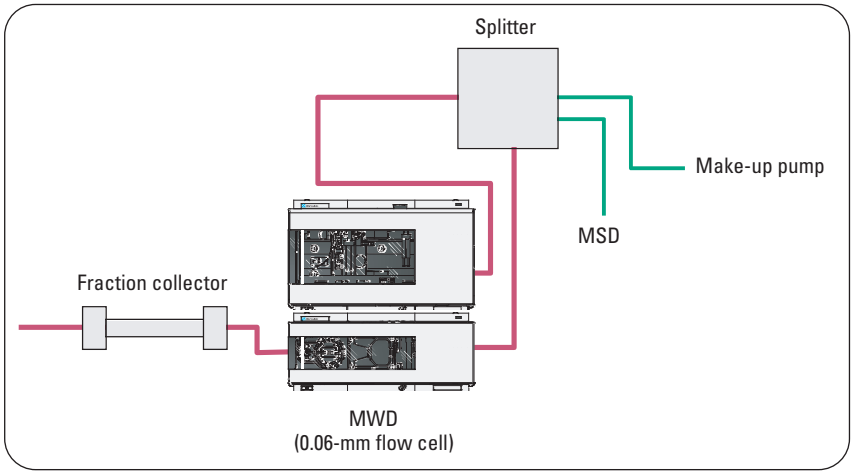


Figure 2
Configuration 1: UV detector in front of splitter.

Splitter	Collection	Purity nimodipin
Active	UV	99.4 %
	MS	92.1 %
	UV AND MS	98.8 %

Table 1
Purity results configuration 1.

Splitter	Collection	Purity nimodipin
Active	UV	98.4 %
	MS	85.4 %
	UV and MS	94.5 %
Passive	UV	97.3 %
	MS	81.0 %
	UV and MS	93.0 %

Table 2
Purity results configuration 2.

Splitter	Collection	Purity nimodipin
Active	UV	98.3 %
	MS	89.2 %
	UV and MS	95.2 %
Passive	UV	98.2 %
	MS	83.1 %
	UV and MS	94.2 %

Table 3
Purity results configuration 3.

Conclusion

The configuration which provides the best purity results, especially for mass-based fraction collection, places the splitter directly after the UV detector. However, this configuration can only be set up using the active splitter because the back pressure introduced with the passive splitter is too high for the UV flow cell. The results of the purity experiments are summarized in figure 3.

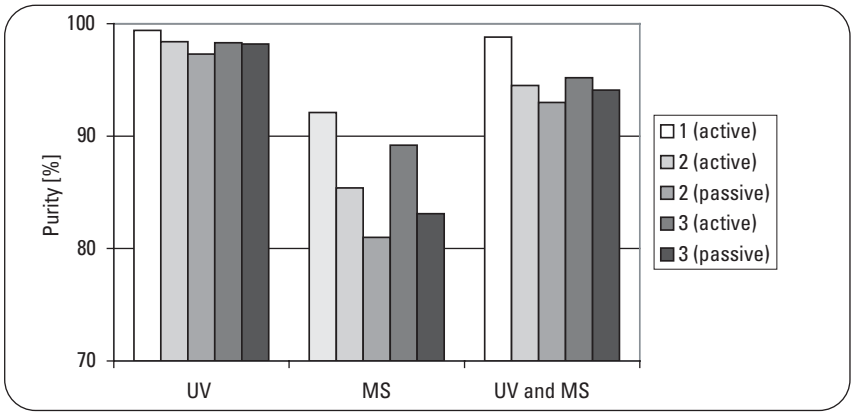


Figure 3
Results of purification experiments.

"Configuring a Mass-based Fraction Collection System for Highest Purity", publication number 5989-4845EN.

Mass-based fraction collection at high flow rates

Combinatorial compound libraries for drug discovery are routinely purified by automated preparative LCMS-systems. Samples of 30 to 50 mg compound mixture are separated on 20 mm diameter columns at flow rates of 20 to 35 mL/min using mass-based fraction collection. Until now purification of several grams of starting material needed for the synthesis of compound libraries had to rely on classical purification methods like crystallization or manual flash chromatography on silica gel. In this Application Note we demonstrate the operation of the Agilent 1100 Series purification system in mass-based fraction collection mode at flow rates of 100 mL/min for the purification of up to 4 grams of a crude product in a single run on 50 mm diameter columns.

Results and discussion

System configuration for mass-based fraction collection at 100 mL/min

To achieve the high flow rate of 100 mL/min 0.7-mm i.d. capillaries were used to connect the preparative pumps to the dual-loop autosampler and the autosampler to the column. The 0.8-mm i.d. capillary shipped with the 0.06 mm pathlength preparative flow cell was used to connect the column to the UV detector. A 10-mL capillary was installed between the active splitter and the fraction collector because a time delay of about five seconds between MSD and fraction collector is required. A 10-mL sample loop was installed in the dual-loop autosampler for the injection of larger sample volumes.

The analytical scale fraction collector was modified with the 0.8-mm i.d. preparative tubing kit (G1364-68711) and with the high flow needle (G1364-87202) and was equipped with the 40-funnel tray (G1364-84512) for the collection of high volumes.

Purification of gram amounts
Test mix A (1333 mg of each compound in 12000 µL of DMSO) was injected onto the 50 x 200 mm column packed with Agilent Prep-C18 material for the purification of approximately 4 grams. Nimodipin was collected based on the trigger mass of 441 because the compound showed a strong sodium adduct (418 plus the expected adduct $[M+Na]^+$ results in a trigger mass of 441) as shown in figure 1.

Re-analysis of the collected fraction presented a purity (based on area percent in the UV signal) of 96.2 % for nimodipin as shown in figure 2.

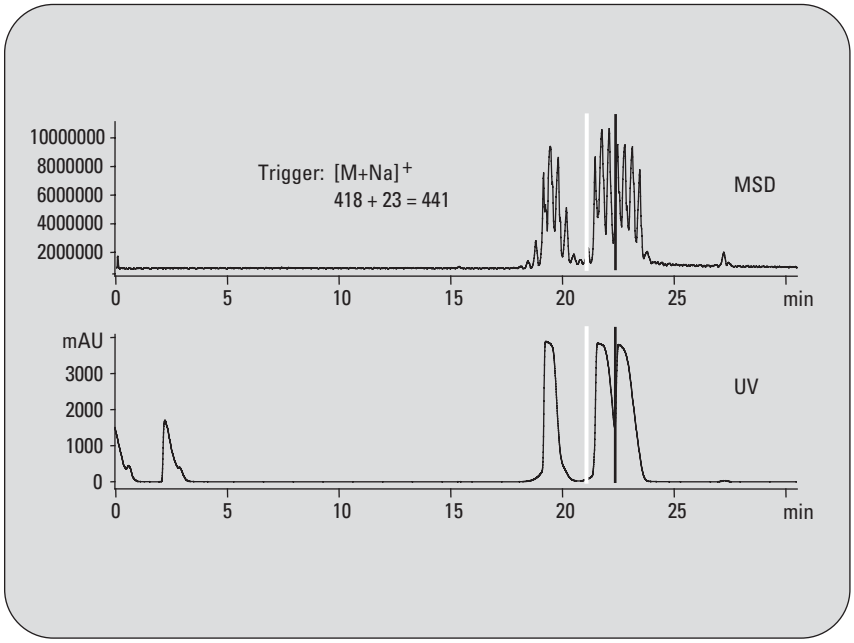


Figure 1
Result of mass-based fraction collection.

Conclusion

In this Application Note the mass-based purification of low gram amounts of compounds on a 50-mm i.d. column operated at 100 mL/min was demonstrated. Compounds could be isolated in high purity even for non-baseline separated peaks with some minor system modifications. The UV signals could no longer be separated even using a preparative flow cell with a very short pathlength.

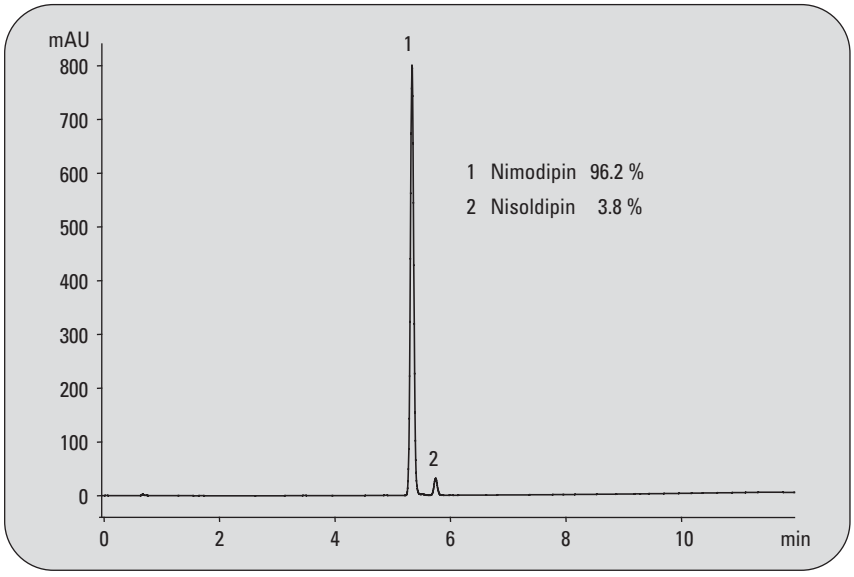


Figure 2
Re-analysis of the collected fraction.

"Mass-based Fraction Collection at High Flow Rates for the Purification of Compounds in the Lower Gram Scale", publication number 5989-2469EN.

Optimizing mass-based fraction collection

Fraction collection can be performed with modern purification systems based on retention time windows, signals from a detector or on target masses using an MSD. The MSD offers the additional advantage of higher selectivity that leads to a lower number of collected fractions and the confidence that the fraction contains the desired target compound.

In this Application Note different fraction trigger techniques for peak-based and mass-based fraction collection are compared with respect to purity and recovery of the desired product and the advantages and disadvantages of each trigger technique are explained.

Results and discussion

Mass-based fraction collection

A system for mass-based fraction collection must contain a flow splitter that divides the flow coming from the column to the fraction collector and to the MSD. Depending on the design the splitter leads to more or less peak broadening in the MSD. Another source of peak broadening is the MSD itself. Since the MSD is a concentration-dependent detector built for highest sensitivity it is always overloaded if a highly concentrated, preparative sample is applied to the system.

Fraction collection on the logical AND combination of the UV and MSD signal

The best purification result with respect to purity and recovery by maintaining the selectivity of the MSD can be achieved by a logical AND combination of the UV and the MSD signal as shown in figure 1. A fraction is only collected if the triggering criteria of the UV as well as of the MSD are met, which indicates that no peak in the UV signal will be collected as long as the specified target mass is not present.

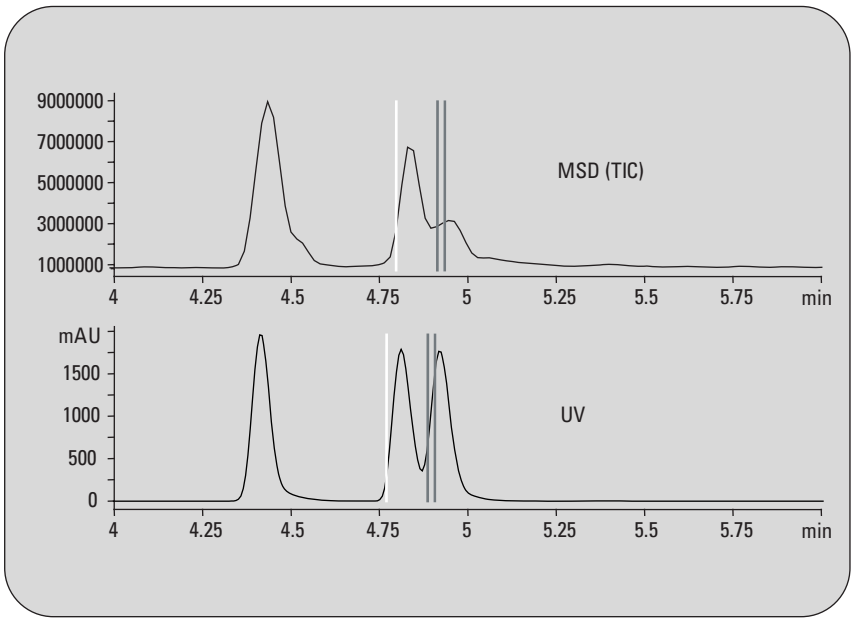


Figure 1
Result of fraction collection on the logical AND connection of UV and MSD.

Depending on the resolution of the two compounds one or two fractions are collected. As a result most of the target compound is present in the first fraction in high purity and some additional compound is collected in the second fraction together with some of the impurity. Re-analysis of fractions 1 and 2 (figures 2A and 2B) showed that fraction 1 contained 9.76 mg nimodipin, which is equivalent to 92.5 % recovery with a purity of 95.8 %. Fraction 2 contained another 0.01 mg of nimodipin, which results in an overall recovery of 92.6 %.

Conclusion

The advantages and disadvantages of the different trigger modes are summarized in table 1.
Peak-based fraction collection, for example on a UV detector, offers the best purification results in respect to purity and recovery if the detector is set up in the flow path directly after the column without any flow splitter. Using an MSD adds selectivity to the system but decreases the purity of collected fractions due to peak broadening introduced by the flow splitter and the MSD itself. The best result can be achieved by combining the selectivity of the MSD with the good peak-shape obtained with the UV detector using a logical AND combination. This can only be achieved by using a flow splitter that adds no backpressure to the system to make it possible to place the UV detector directly after the separation column and not after the splitter.

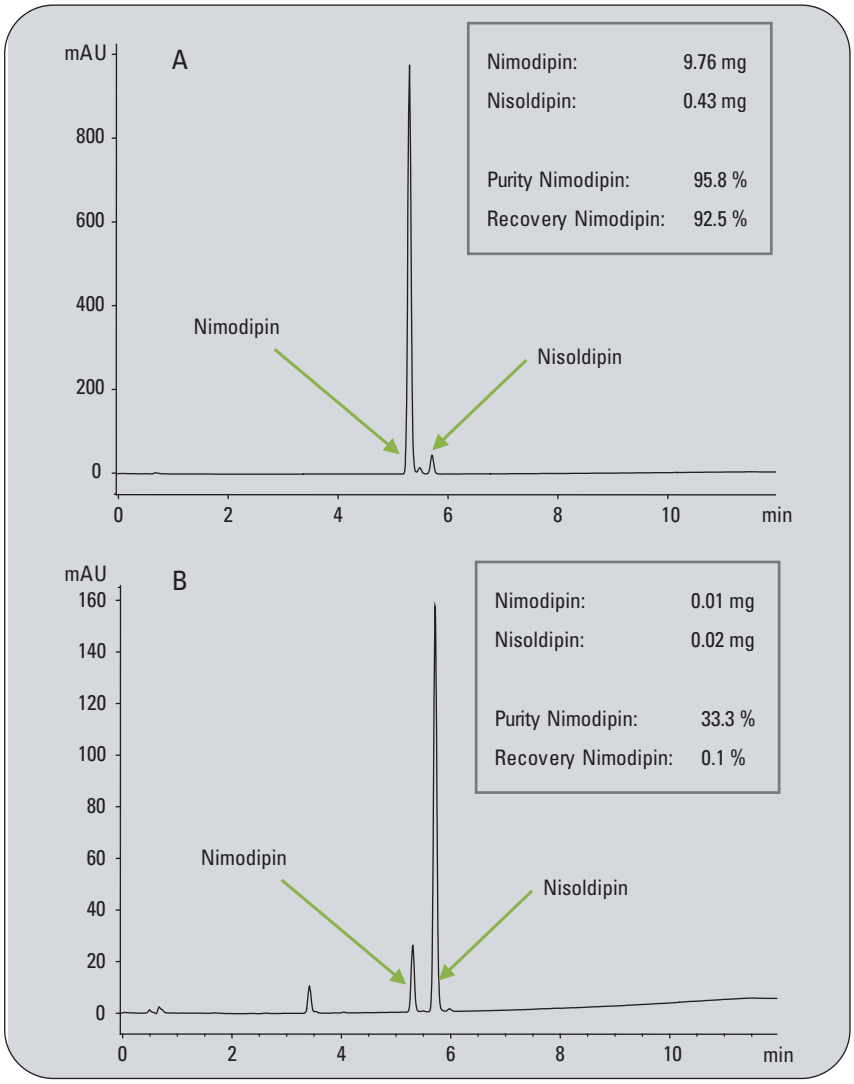


Figure 2
A) Re-analysis of fraction 1, B) Re-analysis of fraction 2.

Trigger mode	Advantages	Disadvantages)
Peak-based	• Best purity and recovery	• Collection of many unwanted fractions • Fraction containing target compound must be identified
Mass-based	• Good selectivity • Good recovery	• Low purity fractions if closely eluting impurity
Mass-based on two masses	• Better purity than simple mass-based fraction collection	• Separation of isomers not possible • Complete compound loss if target and impurity co-elute • Parameters (e.g. threshold) have to be estimated from analytical run
Logical-AND-combination of UV and MSD	• Best purity results for mass-based fraction collection • Generic approach • Isomers can be isolated if separated on column	• UV detector must be placed directly after the column • Requires flow splitter that generates no back-pressure

Table 1
Comparison of fraction trigger modes.

"Optimizing Mass-based Fraction Collection for Highest Purity using the *Boolean* Logical -AND- Combination with the UV Signal", publication number 5988-2014EN.

Optimum performance with the Agilent dual-loop autosampler

The injection principle of the dual-loop autosampler (DLA) is different to that of other Agilent 1100 Series autosamplers. In the DLA the sample is drawn into a buffer loop and then transferred into a sample loop using a low-pressure metering device. Then the sample loop is switched into the high-pressure flow path. This so-called fixed loop injection principle allows two modes of operation:

- partial loop fill, where a sample volume smaller than the sample loop volume is injected without loosing any sample, and
- complete loop fill, with high precision which sacrifices most of the drawn sample volume.

Partial loop fill is the method of choice for preparative work while complete loop fill is used for analytical tasks.

Operating principle

Complete and partial loop fill

The fixed-loop concept of the DLA allows two modes of operation – complete and partial loop fill. When high injection precision is required complete loop fill must be used, which means that a sample volume higher than the sample loop volume must be drawn from the sample container. If injection precision is not crucial and it is important that no sample is wasted, partial loop fill is the method of choice. A sample volume smaller than the sample loop volume is drawn from the sample container and ejected into the loop (figure 1). Summary of the advantages and disadvantages of complete and partial loop fill:

- Complete loop fill
  - Loop must be overfilled (by factor 3 to 5), most of the sample goes to waste
  - High precision

→ Ideal for analytical tasks

- Partial loop fill
  - Complete sample is injected onto the column
  - Lower precision (depends on sample loop size, sample volume, draw speed, etc.)

→ Ideal for preparative tasks.

Performance

1. Complete loop fill

Complete loop fill is the method of choice for highest injection precision which is required for analytical HPLC. Using the minimum overfill factor of 1 will not lead to accurate and precise results. In figure 2 the peak area is shown for different overfill factors from 1 to 7 (50-µL sample loop). It can be seen that the peak area strongly increases up to an overfill factor of 3 to 5. This means that with overfill factors below 3 the sample loop is not completely filled.

2. Partial loop fill

The objective of the injection in preparative HPLC is to apply the entire sample drawn from the sample container to the column while injection precision is only of minor interest.

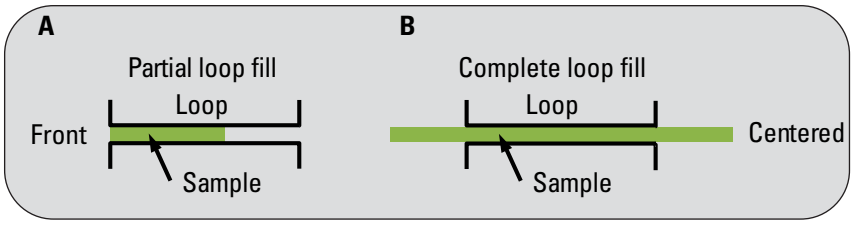


Figure 1
Partial and complete loop fill.

Sample loop fill factor

Figure 3 shows the result of several injections with different injection volumes using the same sample loop (500 µL). The peak area increases linearly until the loop is filled approximately up to 50 %, marked by the dotted line. This means that in order to minimize the sample loss the maximum injection volume should not be more than 50 % of the sample loop volume.

Draw and eject speed

The draw and inject speed have no influence on area precision, but there is an influence on the recovery as shown in figure 4.

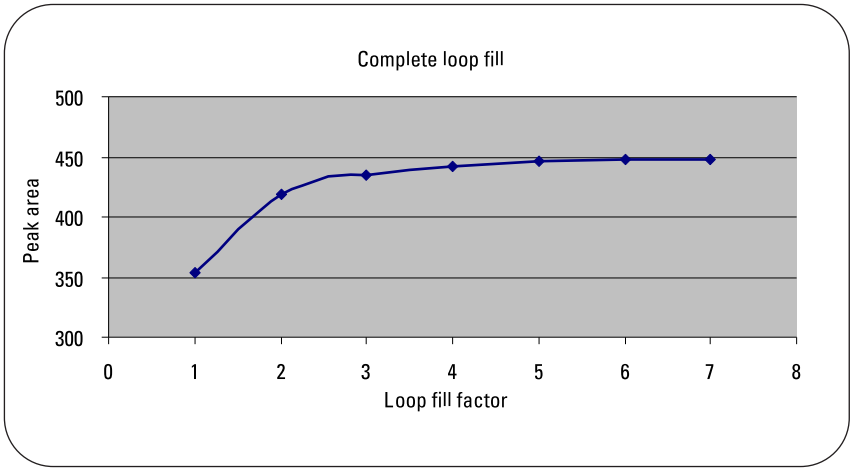


Figure 2
Peak area for different overfill factors.

Conclusion

The following should be kept in mind to achieve the best performance of the Agilent 1100 Series dual-loop autosampler:

Complete loop fill

- For the highest area precision and best analytical results the complete loop fill method must be used.
- To achieve the best injection accuracy and precision the overfill factor should be between 3 to 5.

Partial loop fill

- Partial loop fill must be used for preparative work, where the entire sample drawn from the sample container must be applied to the column.
- For best injection accuracy and highest sample recovery the sample loop should not be filled with more than 50 % of the sample loop volume.

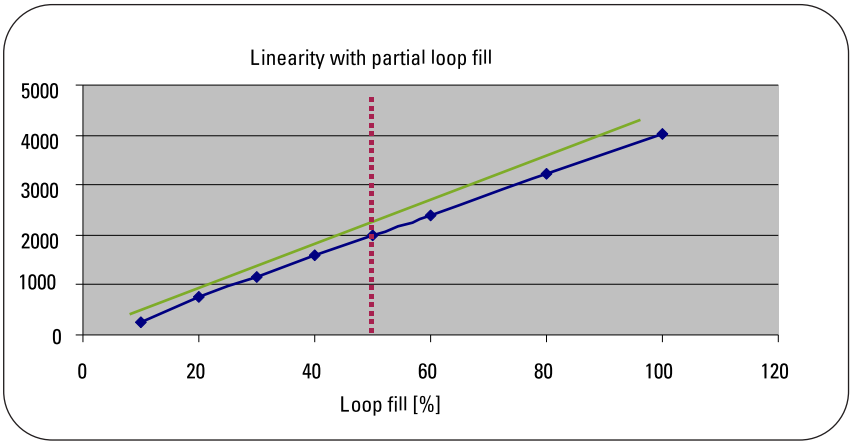


Figure 3
Linearity of peak area for partial loop fill.

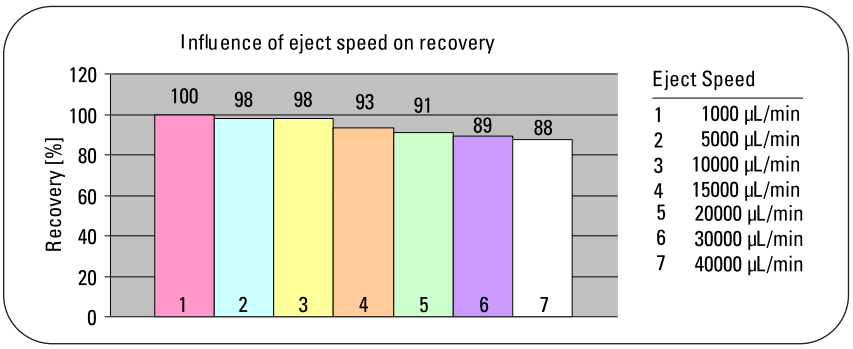


Figure 4
Partial loop fill performance – influence of draw/eject speed.

"The Agilent 1100 Series Dual-loop Autosampler PS – Optimum Performance when Injecting Large Sample Volumes", publication number 5989-1714EN.

Multiple injections in an isocratic purification experiment

Preparative HPLC is currently the method of choice for the purification of compounds in drug discovery. Large numbers of crude samples are purified using generic methods with short and steep gradients of about 10 minutes. While most compounds can be isolated using these methods, the isolation and purification of stereoisomers or diastereomers requires much longer run times, either under isocratic conditions or with very shallow gradients. In this Application Note we will demonstrate how sample throughput can be increased by injecting the sample several times within one run, saving precious time and mobile phase.

Results and discussion

Multiple injections

It is possible to inject additional sample onto the column after a certain time as the first isomere elutes after about 25 minutes and there are no other impurities in the sample. This duration is determined by the time difference between the start of the first peak and the end of the second peak, which is approximately 7 minutes. The result for five consecutive injections is shown in figure 1.

The overall run time for five injections was 70 minutes with a mobile phase consumption of 1470 mL. Compared to five single injections (175 minutes, 3675 mL), this equals a time and mobile phase saving of about 60 %.

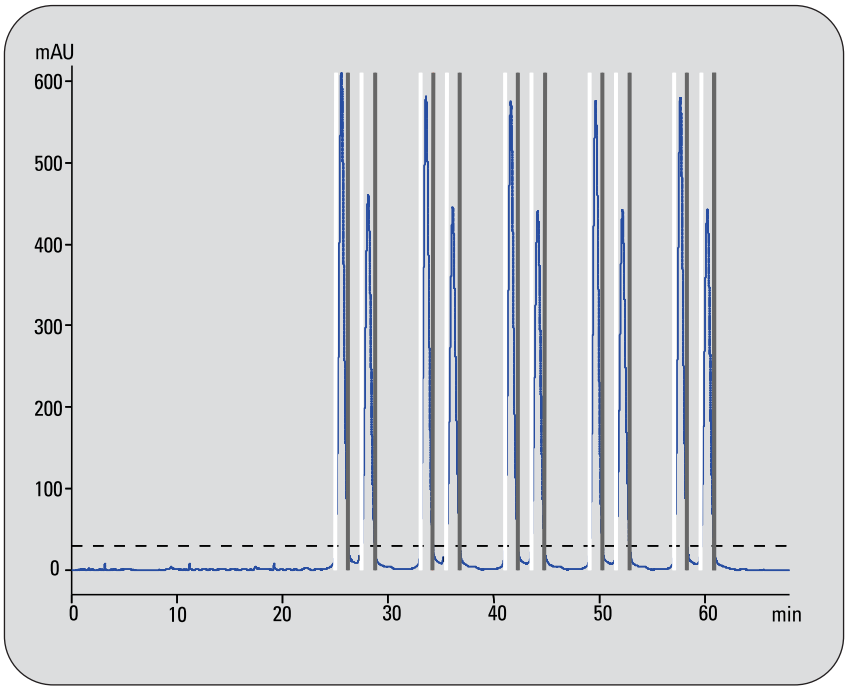


Figure 1
Multiple injections.

Injector program

The injector program for multiple consists of 10 lines, which include washing the needle's surface and flushing the needle, needle seat, needle seat capillary and valve after ejecting the sample into the sample loop. Copies of these ten commands have to be made, depending on the number of injections done in a run.

Limits and restrictions

- The mobile phase must be used as the sample solvent.
- The sample loop used for multiple injections has to be set up in the *Set up Injector* window under *Use Loop*.
- The number of lines in the injector program is limited to 60 steps.
- To lower the number of injector program lines the REPEAT/END REPEAT command can be used.

Conclusion

Performing multiple injections in a single isocratic run can save precious time and mobile phase for the separation and purification of isomers or enatiomers. With the Agilent 1100 Series purification system, multiple injections can be easily performed by setting up an injector program.

"Performing Multiple Injections in an Isocratic Purification Experiment using the Agilent 1100 Series Purification System", publication number 5989-1651EN.

Combined system for analytical and preparative work

A combined system for analytical and preparative work is always a compromise with regard to performance of the hardware, such as, the inner diameter of the capillaries. In this Application Note we will illustrate the setup of a system with the best possible configuration.

Results and discussion

Basic ideas

Mode selection

The analytical or preparative mode is chosen by selecting a method. There is at least one method for analytical work and one for preparative work. The selection valve is used to switch between analytical and preparative mode guiding the flow either from the quaternary or from the preparative pump through the dual-loop autosampler. In the preparative mode the quaternary pump is used as a make-up pump.

Active splitter

During a preparative session the splitter is switched on and the quaternary pump is used to deliver the make-up flow. During an analytical session the splitter is switched off. The flow from the quaternary pump is directed through the dual-loop autosampler, the analytical column, diode array detector, and the make-up flow section of the Agilent active splitter to the MSD.

System plumbing

The system plumbing is shown in figure 1. The only large i.d. capillaries (0.5-mm i.d) that are used for analytical work are the ones connecting the dual-loop autosampler to the selection valve. All other capillaries used in the analytical flow path have a small i.d. (0.18-mm).

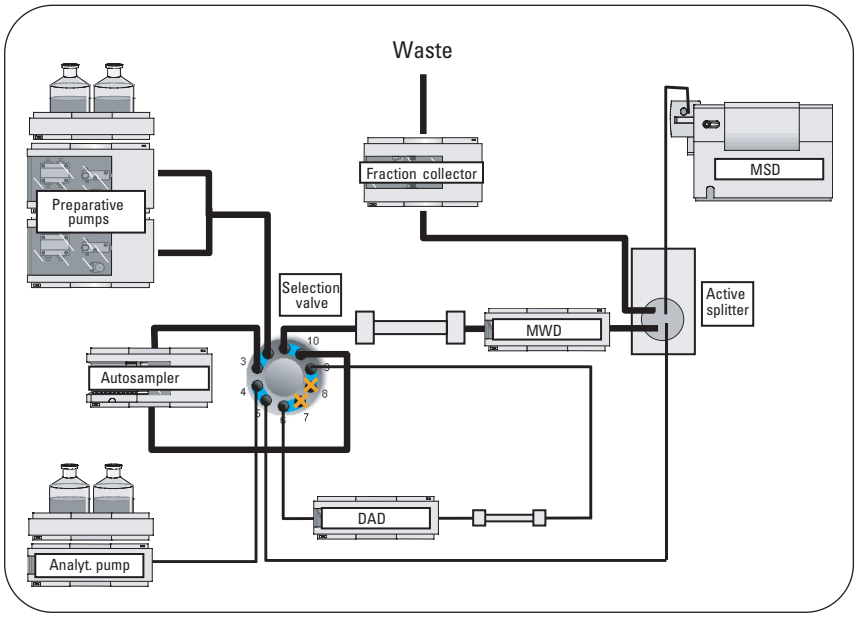


Figure 1
System plumbing diagram.

Dual-loop autosampler

The dual-loop autosampler contains two injection loops. For preparative work a 2000-µL was configured and the partial loop fill technique was used. For analytical work, a 50-µL loop was used either in partial or complete loop fill mode, depending on the concentration of the analytical sample.

Application example

The following sample applications were performed on a system setup and configured as described earlier. Fractions were collected based on a combination of UV-based (slope only, up slope 150 mAU/s, down slope 150 mAU/s) and mass-based fraction collection (threshold only, 150000 counts) using a logical AND connection for the preparative run. The results are shown in figure 2.

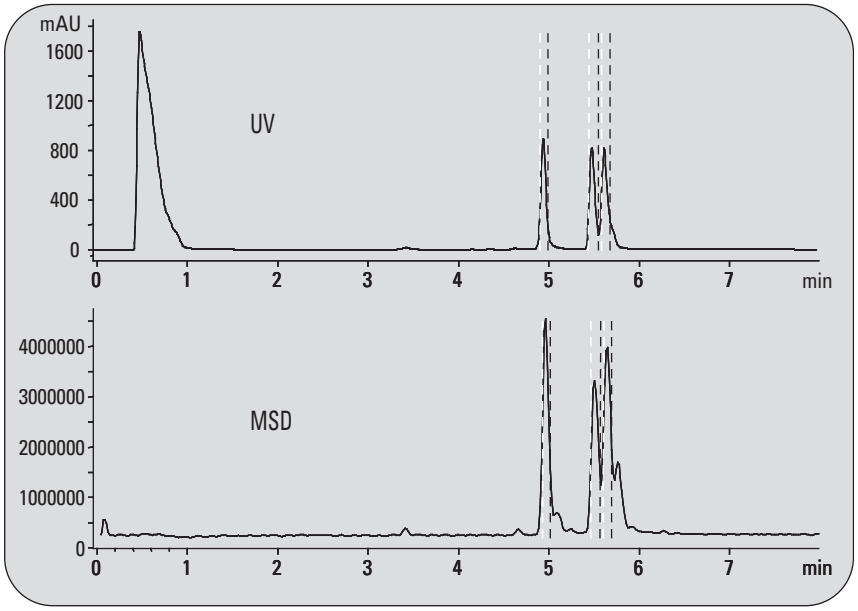


Figure 2
Application example.

Conclusion

In this Application Note the configuration, setup and operation of a system suitable for preparative and analytical runs is described. The mode used is determined by applying a specific method to the sample. Due to the generic approach the system can be used for ChemStation-only operation (ChemStation rev. A.10.02), with the Purification software as well as with EasyAccess Plus. Nevertheless, a combined system for analytical and preparative work is always a compromise with regard to flow paths. The system described in this Application Note was set up with the best possible configuration.

"An Optimized Agilent 1100 Series System for Analytical and Preparative Work", publication number 5988-9649EN.

Injection pump system

In collaboration with Graham Foster, Richard O'Hanlon, GlaxoSmithKline, UK

Depending on the solubility of the sample compound, sample injection volumes can vary from a few microliters up to several liters. While small sample volumes up to 5 mL can easily be injected using an autosampler, larger sample volumes are usually injected using an injection pump. If a high-pressure injection pump is used, the sample can be pumped directly onto the column. Such a purification system, equipped with an Agilent 1100 Series isocratic pump as high-pressure injection pump, is described in this Application Note.

Results and discussion

System configuration

The configuration of the system is shown in figure 1. A 2-position/6-port valve is used to switch between the preparative pumps, which generate the gradient for the purification run and the injection pump. The complete purification run consists of three steps:

1. Initially the injection valve is in position 1, in which the flow from the gradient pump goes to the column and the flow of the injection pump goes to waste.
2. When the run is started the injection valve switches to position 2 where the flow from the injection pump goes to the column. The injection pump transfers the sample onto the column.
3. After the sample is applied to the column the injection valve switches back to position 1 and the gradient is started. While the gradient is applied to the column, the injection pump and the injection valve are washed with an appropriate solvent.

Sample injection system

The sample injection system consists of the injection pump and a 12-position/13-port valve where the outlet position of the valve is connected to the pump. Several containers with solvent of the gradient starting conditions (solvent A), the sample and wash solvent are connected in the following order to the inlet positions (figure 2):

- Position 1: Initial position, solvent A (gradient start composition)
- Position 2: Sample
- Position 3: Solvent A to flush sample through the valve and the injection pump completely onto column

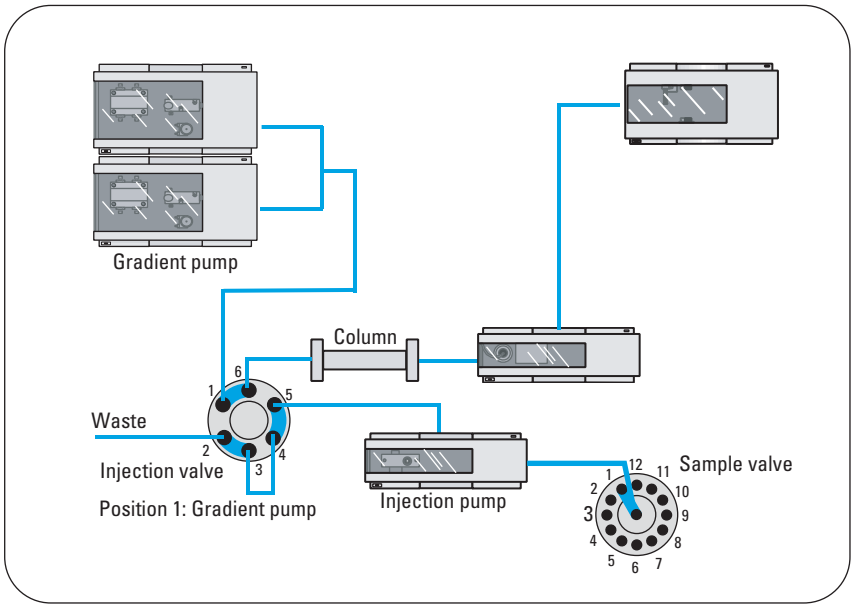


Figure 1
System configuration.

- Position 4: Wash solvent to rinse injection valve and injection pump
- Position 5: Solvent A to remove wash solvent from injection pump and injection valve.

Timing of pumps and valves

The workflow described under "Sample injection system" is executed by setting up timetables for the gradient pump, injection pump, injection valve and sample valve.

System scope and limitations

- The system is operated by the Agilent ChemStation.
- Fractions can be collected based on time or peak using the ChemStation sequence. Mass-based fraction collection is only possible if a single run (*Run Method* task) is set up.
- The injection cycle is controlled by gradient pump, injection pump, injection valve and sample valve timetables.
- The delay volume calibration procedure is not available.
- The maximum flow rate of the isocratic pump is 10 mL/min.
- For highly concentrated samples it is recommended to remove the PTFE frit from the purge valve of the isocratic pump (procedure described in the isocratic pump manual, part number G1310-90003).

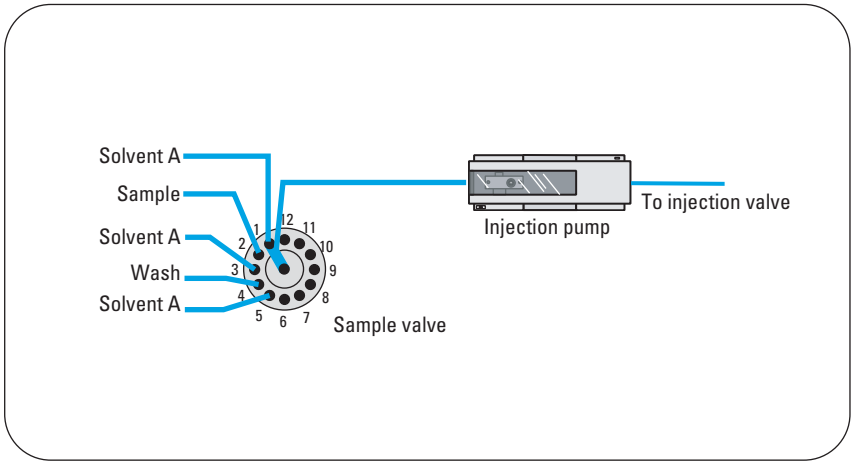


Figure 2
Sample injection system.

Conclusion

In this Application Note we showed the setup and configuration of an Agilent 1100 Series purification system with an isocratic pump and a simple valve solution for sample injection. The method setup using timetables for the pumps and valves were described

"Injection of Large Sample Volumes using the Agilent 1100 Series Purification System with an Injection Pump", publication number 5989-0029EN.

Recovery collection

Regardless of the triggering mechanism it is sometimes desirable to collect not only the compounds of interest but also everything else from the sample in a dedicated vessel the, so-called, recovery location (figure 1). The recovery solution can be dried down, re-dissolved and re-injected to recover anything of interest that was in the sample but was missed in the first purification run.

Discussion

Recovery collection – Why?
The recovery solution can be used to recover anything from the sample that was not triggered as a fraction in the first purification run. But it is also a safety feature to recover, for example, the target compound if the purification system did not collect fractions as expected.

Recovery collection – How?
Collection of the recovery solution must be done from the waste line of the fraction collector. If more than one fraction collector is configured in the system the waste lines must be combined before going to the recovery collection.

Recovery collection with the Agilent 1100 Series purification system
With the Agilent 1100 Series purification system recovery collection can be done in three different ways:

- 12-position/13-port valve in the fraction collector waste line
  - 12 recovery locations
  - Basic recovery location tracking by software
- Third-party fraction collector and BCD board
  - Number of recovery locations depends on fraction collector
  - No recovery location tracking by software
- Agilent 1100 Series fraction collector
  - Up to 120 recovery locations with funnel tray
  - Complete recovery location tracking

12-position/13-port valve
The waste line of the fraction collector is connected to the inlet position of the valve and the twelve outlet positions are connected to the recovery containers. For each injection the valve is automatically switched to the next position.

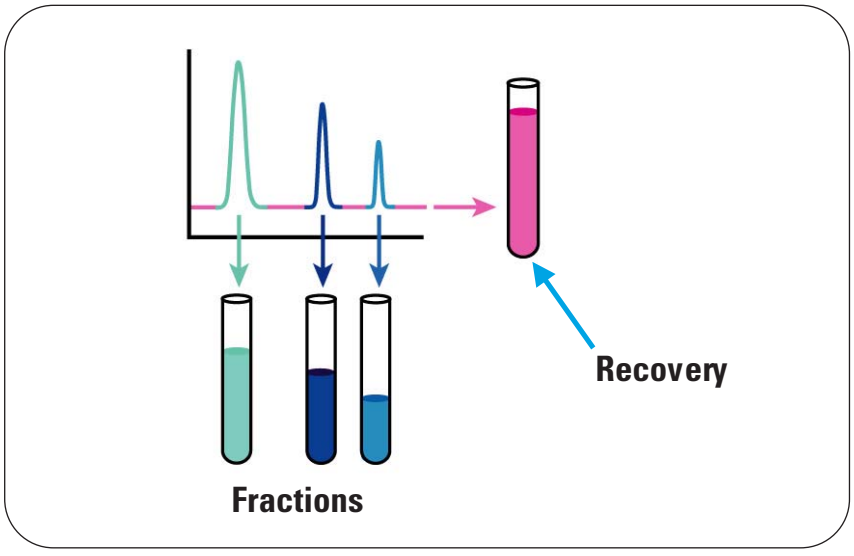


Figure 1

Third-party fraction collector and BCD board

The BCD board, which can be installed in the autosampler, provides a BCD output for the bottle number of the autosampler and four external relay contacts. General-purpose cables are available to connect the BCD output and the external contacts to external devices. The four contacts can be closed and opened using an injector program in the ChemStation to move the third-party fraction collector to the next position after an injection or to switch the diverter valve.

Agilent 1100 Series fraction collector

With ChemStation rev. A.10.01 or higher it is possible to configure an additional Agilent 1100 Series fraction collector in the system for recovery collection. In this fraction collector all available trays for vials, well-plates and test tubes as well as the new funnel tray can be used. Each funnel tray consists of 40 funnels with tubing connected to the funnels (figure 2). This tubing can be placed in any vessel or container, for example, large glass bottles. Up to three funnel trays can be installed in the fraction collector, which gives up to 120 recovery locations as maximum.

Figure 2
Funnel trays installed in a fraction collector.

Conclusion

The Agilent 1100 Series purification system offers three possibilities for recovery collection. The easiest way is to connect a 12-position/13-port valve into the waste line of the fraction collector.

A third-party fraction collector can also be configured into the system controlled by the ChemStation injector program via external contacts of a BCD board.

The most sophisticated approach is an additional Agilent 1100 Series fraction collector configured as recovery collector in the Agilent ChemStation. In addition to vials, well-plates or tubes the new funnel tray offers the possibility to collect virtually unlimited recovery volumes.

"Recovery Collection with the Agilent 1100 Series Purification System", publication number 5988-9650EN.

Mass-based fraction collection at higher flow rates

Nifedipin, nimodipin and nisoldipin are antianginal drugs with 1,4-dihydropyridine structure. They are used as calcium antagonists in cases of hypertonia, cardiac disrythmia and angina pectoris. The isolation of these three compounds from a mixture is an example application for purification of pharmaceutical drugs. The goal of the application was to purify 20 mg of each compound in a single run and to get three fractions containing only the desired substances.

Results and discussion

System set-up and configuration

The system was configured to operate a generic method at a flow rate of 25 mL/min on a 21.2 x 50 mm Zorbax SB-C18 column. The flow coming from the column was split after the UV detector – the main flow going to the fraction collector and the split flow going to the MSD. Since the samples were dissolved in DMSO the method was set-up to make sure the DMSO elutes before any compound of interest. Therefore, water/acetonitrile 90:10 was pumped through the column for two minutes before the gradient was started. To avoid contamination of the MSD with DMSO the stream selection valve was switched to the waste position for the first 2 minutes of the run.

Mass-based fraction collection

The molecular masses of the three compounds are 346.34 for nifedipin, 418.45 for nimodipin and 388.42 for nisoldipin. All three compounds show strong fragmentation in positive ionization mode. Therefore, nimodipin was not triggered on the molecular mass of 418 but on the dominant fragment mass of 342. Figure 1 shows the result of the mass-based fraction collection for triggering on the $[M+H]^+$ - ion.

Re-analysis of fractions

To check the performance of the purification run the collected fractions were re-analyzed on an analytical HPLC system using a method that was calibrated with pure standards before. The chromatograms of the fractions are shown in figure 2. The analytical results are summarized in table 1. The injected amounts of nifedipin, nimodipin and nisoldipin were 19.43 mg, 19.05 mg and 18.92 mg, respectively.

Conclusion

The three drugs have been purified by mass-based fraction collection at a flow rate of 25 mL/min. Even with a highly overloaded column the re-analysis of the collected fractions showed that about 20 mg of each compound could be purified in a single run with high recovery and a purity of 90 % or more. This result proves the excellent performance of the Agilent 1100 Series purification system equipped with MSD at higher flow rates.

	Nifedipin] [mg]	Nimodipin [mg]	Nisoldipin [mg]		
Fraction 1	18.90	0.11	0.16	Purity Nifedipin	98.6 %
Fraction 2	0.29	17.66	0.77	Purity Nimodipin	94.4 %
Fraction 3	0.49	1.66	18.36	Purity Nisoldipin	89.5 %
Recovery [mg]	19.68	19.43	19.29		
Recovery [%]	101.3	102.0	101.9		

Table 1
Results of fraction re-analysis.

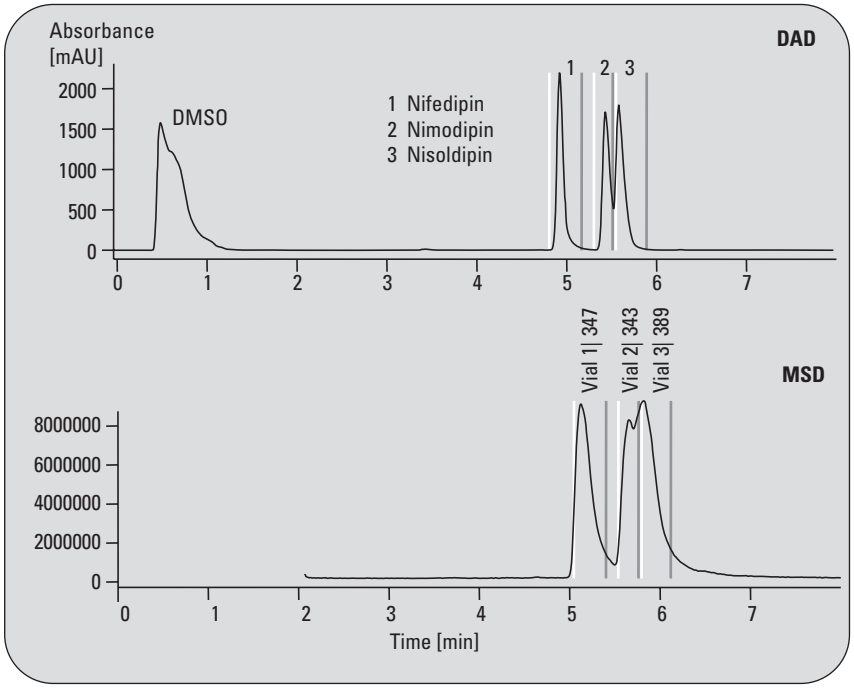


Figure 1
Result of mass-based fraction collection.

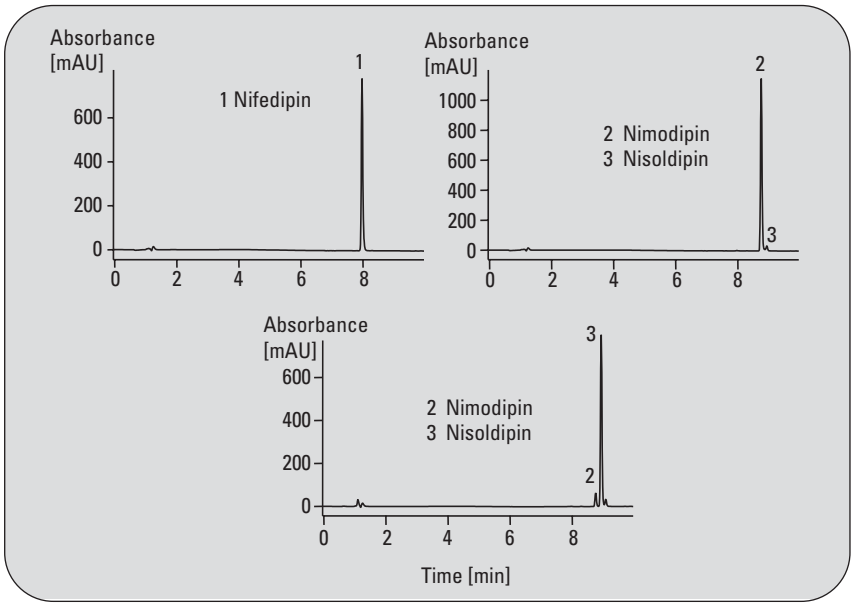


Figure 2
Re-analysis of fractions.

"Purification of Pharmaceutical Drugs by Mass-based Fraction Collection at Higher Flow Rates", publication number 5988-7113EN.

Optimization of mass-based fraction collection

With the growing trend to higher-purity compounds, synthetic chemists typically want to concentrate on organic synthesis issues and minimize the time spent on the analysis and purification of their synthesis products. The Agilent active splitter and delay sensor are an integral part of the Agilent purification/analysis platform and provide users with powerful purification flexibility while maximizing efficiency and ease of use.

Results and discussion

Improving purification productivity

The application area where the active splitter and delay sensor are most powerful is in early drug discovery in the pharmaceutical industry. With the pressure to prepare large numbers of pure compounds, synthetic-organic and medicinal chemists are searching for tools to improve productivity. The primary objectives are to:

- Collect a pure sample of synthesized products of known formula or molecular weight
- Collect microgram to milligram component amounts
- Minimize the time spent on analysis and purification of new compounds
- Reduce the cost, time, effort, knowledge, and skill required to collect the sample
- Increase chemist and lab efficiency

Figure 1 is a diagram of the system used to achieve these objectives.

Active splitter for flexible operation

A very key component of this system is the Agilent active splitter. This splitter functions by taking small aliquots of the HPLC flow stream and transferring them to a mass spectrometer flow stream. This process is shown in Figure 2.

Key features of the active splitter are:

- Both the aliquot size and switch rate can be varied to adjust split ratio for each method employed. In addition, the active splitter software automatically starts and stops the valve as directed by each method.
- Different methods can have different splits without replumbing
- No inherent delay added, simplifying chromatography
- Minimal backpressure prolonging useful life of system components
- Software control of active splitter parameters and early maintenance feedback (EMF) to help schedule seal replacement

Delay sensor improves systems accuracy and ease of setup

Another key feature that makes the use of this system easier and more reliable is the process by which volume delays are determined. In order to avoid potential loss of valuable fractions, it is essential in mass-based systems that the peaks of interest arrive at the triggering detector(s) before they arrive at the fraction collector. This requires that tubing is in place to provide a reproducible delay from the time each detector detects a peak to the time it arrives at the fraction collector. To more easily accomplish this, the fraction collector has a built-in delay sensor (FDS). The dispensing arm positions itself over the sensor and the firmware can precisely calculate the delay volume between any detector and the fraction collector.

Key benefits of the delay sensor include:

- Accurate calculation of the time delay between peak detection and fraction collection
- Automatically saves correct delay values with the method and fraction collector configuration

For the LC/MSD, the delay sensor calculates the time between LC/MSD detection of a peak and when the peak arrives at the fraction collector. Unlike for UV detectors, this delay is impacted by changes in the make-up pump flow as well as HPLC pump flow. Therefore, this delay needs to be recalibrated for each method flow. This is done using a sample containing a dye and caffeine which are injected together with no chromatographic column. The UV detector and FDS respond to the dye, while the caffeine ion at m/z 195 is monitored by the LC/MSD. The delay sensor data is monitored and saved just like any other 2D detector.

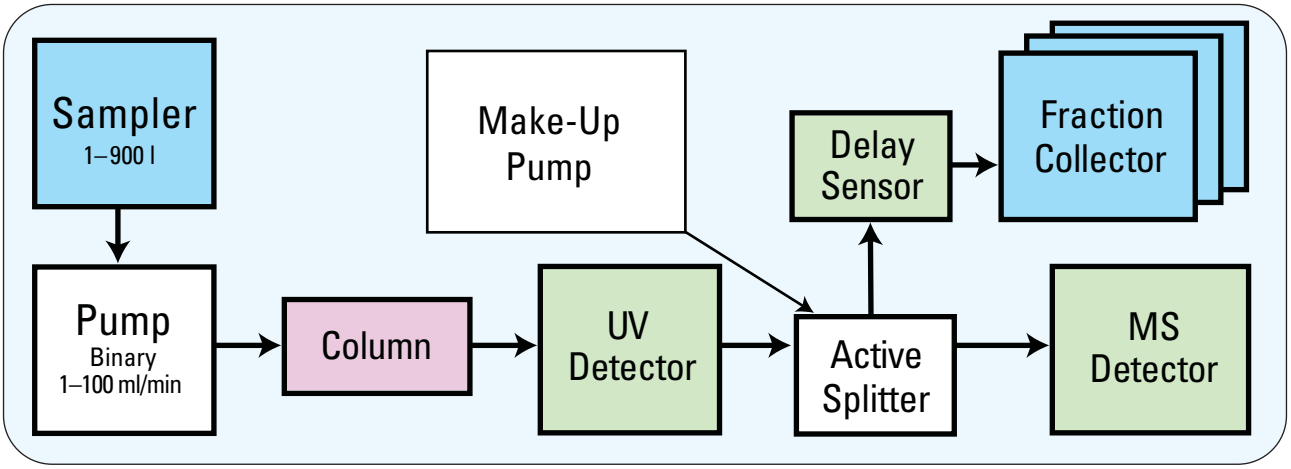


Figure 1
Purification flow diagram.

Conclusion

The Agilent active splitter and delay sensor can greatly improve the efficiency of laboratories doing compound analysis and purification. Active splitting provides more flexibility in optimizing analysis and collection of a wide variety of compounds from different matrices. The delay sensor simplifies system setup while insuring valuable fractions are properly collected.

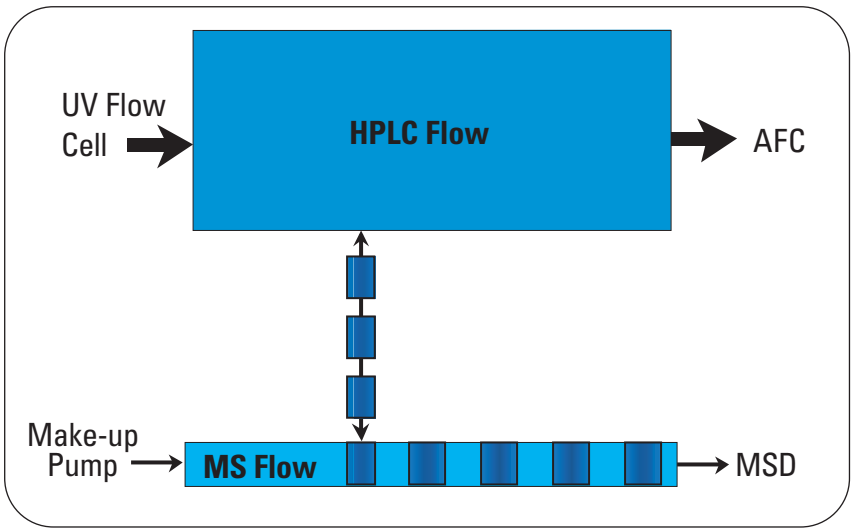


Figure 2
Operation of the active splitter.

"Optimizing Fraction Collection with LC/MSD Systems Using an Active Splitter and Delay Sensor", publication number 5988-7610EN.

Optimization of fraction collection in general

Ideally, the analyte composition that elutes as a fraction from the fraction collector needle tip corresponds in its composition exactly to the detector signal, that is, if an additional chromatogram would be recorded at the fraction collector needle tip, it would be identical to the chromatogram measured by a detector that is located after the column. However, in order to match this requirement the following effects that impact compound recovery and collection reliability have to be considered: delay volume, dispersion and system response. It is outlined how the innovative Agilent 1100 Series purification platform design takes into account the influence of these effects on the chromatographic results.

Results and discussion

Delay volume calibration

The delay time is referred to as the time it takes for an analyte molecule to migrate from the detector cell to the fraction collector. In order to trigger start and stop of fraction collection precisely the delay time has to be determined. Later on, this delay time can easily be converted to the flow rate independent delay volume. Conventionally, the delay time is determined by injecting a dye and stopping the time until the dye appears at the fraction collector needle tip. Measuring the delay time in such a manner is not only laborious but also imprecise. Therefore, Agilent 1100 Series purification systems comprise an innovative delay volume calibration functionality. This patented feature performs the measurement of the delay volume, fully automatically and precisely.

In addition to the UV detector, a second detector, the so-called delay sensor, is integrated into the fraction collector. Whenever a delay calibrant is injected into the flow path both detectors record a signal. The time-delay between the two signals (minus the migration time between diverter valve and fraction delay sensor that is calculated internally) is the delay time. Depending on the flow rate used for the calibration procedure the exact delay volume is automatically calculated by the system and stored in the fraction collector memory. The precise delay time can now be calculated by the system for every flow rate. Re-calibration is not necessary.

Dispersion

An often-overlooked phenomenon that impacts the compound distribution during migration from the detector to the fraction collector is dispersion. Dispersion equates with peak broadening and therefore impairs chromatographic resolution tremendously. According to the Aris-Taylor equation, band broadening is directly proportional to flow rate and tubing length, but proportional to the fourth power of the tubing inner diameter. This effect is impressively shown in figure 1. Consequently, connecting the detector with the fraction collector by tubing with an inappropriate i.d. will lead to bad purification results, such as a poor recovery or even a remixing of compounds. Therefore, a non-specific fraction collector designed for a broad flow range might give acceptable results at high flow rates (column i.d. larger than 25 mm) but will sacrifice progressively recovery and purity when approaching low flow rates (column with i.d. smaller than 9 mm). Agilent therefore offers fraction collectors especially tuned for various purification scales. Each fraction collector type has been manufactured to provide optimal performance at dedicated flow rate ranges and column i.d. for highest compound purity and recovery.

System-integrated intelligence

System-integrated intelligence not only allows users a tailored modular system set-up from the wide choice of Agilent 1100 Series modules but also accomplishes real-time data processing. Real-time data processing is of particular importance for instantaneous fraction collection and safe and reliable system operation even in the case of PC-power or network breakdown. In order to maintain such a flexible and safe system

operation all Agilent 1100 Series modules communicate via a Controller Area Network (CAN). The outcome of this is fully PC-independent system operation. Fraction triggering still proceeds in real-time even if the communication between system and PC is disturbed by heavy network traffic, a busy CPU or gets totally lost. Because of this fractions are collected precisely as indicated in the corresponding chromatogram. Besides CAN connection each Agilent 1100 Series module bears its own intelligence that starts becoming active as soon as the system receives a task from the PC. The PC just represents the interface between user and instrument. Its functionality is reduced to monitoring and evaluating experimental results.

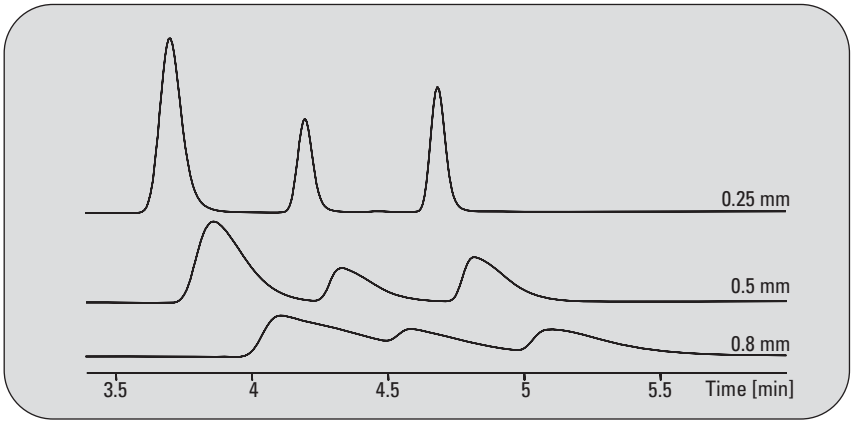


Figure 1
Impact of tubing i.d. on chromatographic resolution. Sample volume, tubing length and flow rate remained constant during the experiments.

Conclusion

Chromatographic purification systems have a crucial impact on purity and recovery of the target compounds. Generally re-analyses of the purified samples are performed in order to confirm purity of these compounds. Users' experiences have shown that the composition of the purified samples is usually different from what the corresponding chromatograms suggest. The eluent flow is susceptible to distrubances during its migration from the detector to the fraction collector that may tremendously impact the compositions of the collected fractions. Some of the innovative features of the 1100 Series purification systems are:

- Nearly unperturbed analyte transport to the fraction device.
- A patented delay volume calibration guarantees a convenient and precise determination of the delay volume between detector and fraction collector, which is of significant importance for high purities and recoveries.
- A sophisticated flow path design keeps dispersion low and therefore contributes to high chromatographic resolution without remixing of previously separated peaks.
- Agilent's system-integrated intelligence principle provides real-time data processing for fast, reliable and precise fraction collection.

Altogether, Agilent provides high performance purification systems for a broad application range and proven recoveries of nearly 100 %.

"Innovative Fraction Collection with the Agilent 1100 Series Purification Platform", publication number 5988-9250EN.

System optimization for highest recovery

Agilent's 1100 Series fraction colectors are designed for minimum delay volumes offering highest fraction collection performance, which is especially important for purification at low flow rates. Figure 1 shows a schematic drawing of the fraction collector with its two delay volumes V_{D1} and V_{D2}.

When a peak is detected the fraction collector must wait until the peak has traveled from the detector flow cell to the diverter valve before switching to collect the fraction. Therefore, the delay time t_{D1} is added to the start time t_0 of the peak. To make sure that all of the peak is collected the fraction collector switches back to waste at the end time of the peak t_E plus the delay times t_{D1} and t_{D2}. Then the end of the peak has reached the tip of the fraction collection needle.

Results and discussion

Influence of delay volume V_{D1} on recovery

When a peak travels through a capillary, dispersion occurs due to different velocities of the mobile phase over the cross-section of the capillary. This is caused by interaction of the mobile phase with the capillary wall. Therefore, dispersion depends on the length and the inner diameter of the capillary. Figure 2 shows the dispersion of a peak travelling from the detector to the fraction collector with increasing delay volume V_{D1}. To increase V_{D1} the standard capillary of the fraction collector (0.25 mm id) was enhanced with capillaries of different lengths but with the same inner diameter.

Triggering of fractions is based on the detector signal, which means the width of the fraction is determined by the width of the peak. When the peak reaches the fraction collector it is broadened due to dispersion, however the diverter valve switches to the collect position only for the time window determined by the peak width in the detector. This results in a loss of compound at the beginning and at end of the peak. The higher the delay volume V_{D1}, the more compound is lost. The Agilent 1100 Series fraction collector minimizes this effect, as it was designed for smallest delay volume V_{D1} that can further be minimized by cutting the inlet capillary of the fraction collector after setting up the 1100 Series stack.

Influence of delay volume V_{D1} on resolution

The influence of dispersion on the re-mixing of peaks that were separated on a column is also an important aspect. A parameter to measure the separation of two peaks is the resolution calculated using the 5-sigma method. This method was selected because it accounts for the peak width at 5 sigma height (4.4 % of peak height), which is close to the baseline of the peak.

To show the influence of enhanced delay volume V_{D1} on the resolution, two peaks were measured in the detector and also in the fraction collector. The delay volume was increased by adding tubing with two different inner diameters (0.25 mm and 0.8 mm). Then the resolution of the peaks in the fraction collector and the detector was compared to give the relative resolution. Figure 3 shows that the relative resolution decreases

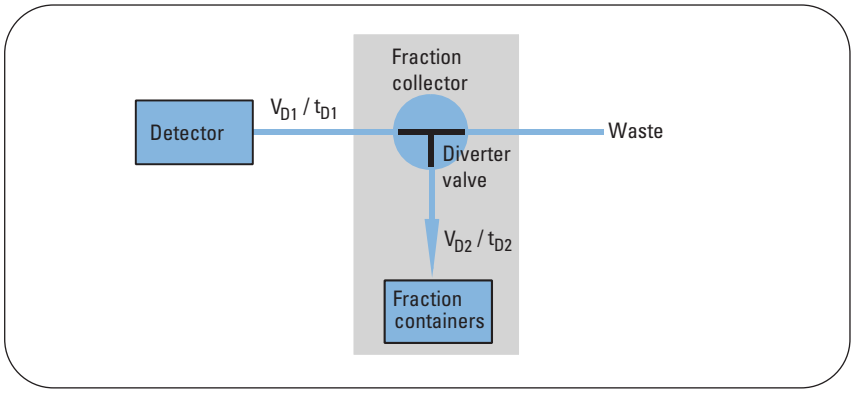


Figure 1
Schematic drawing of a fraction collector for a UV-based purification system.

with increasing delay volume V_{D1}. It also shows that the relative resolution for the same delay volume greatly decreases with increasing inner diameter of the capillary. This is due to the influence of the capillary cross-section on the re-solution in which the radius is squared.

Conclusion

In this Technical Note we showed the influence of the different delay volumes, detector signal delays and how the delay volume can be measured automatically.

Different delay volumes are used to trigger peaks for maximum recovery. The delay volume V_{D1} was minimized to avoid low recovery or re-mixing of the separated peaks due to dispersion

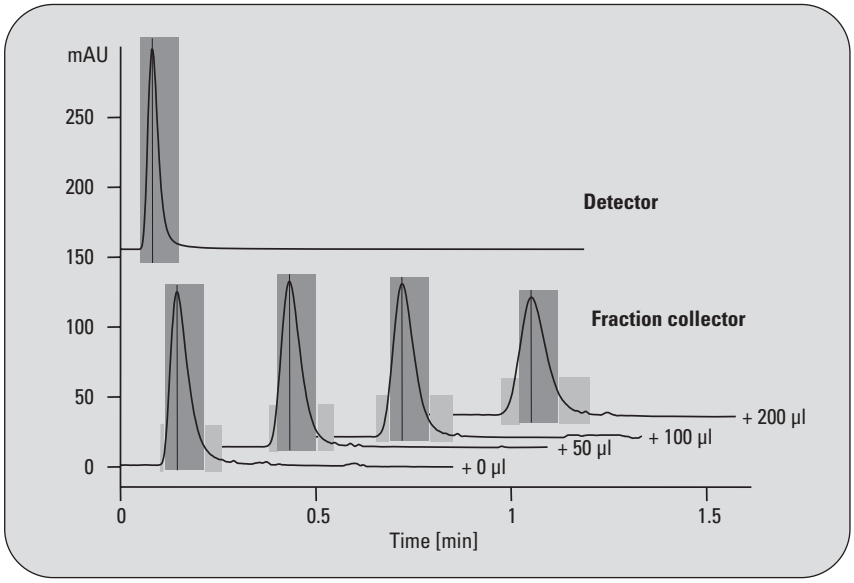


Figure 2
Increasing dispersion with incrreasing capillary volume.

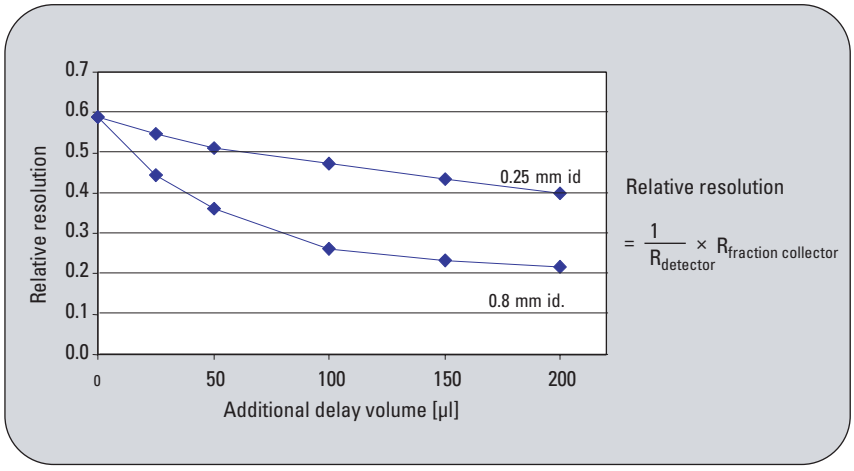


Figure 3
Relative resolution for two capillaries with different inner diameters.

"Peak-based Fraction Collection with the Agilent 1100 Series Purification System AS – Influence of Delay Volumes on Recovery", publication number 5988-5746EN.

Peak-based fraction collection on a third-party detector signal

The evaporative light scattering detector (ELSD) is a detector commonly used in the pharmaceutical industry. It can detect compounds with no chromophoric group and the detector response depends only on the amount of eluted compound. Therefore, it is ideal for purity checks.

The system setup described allows the integration of an ELSD as well as other non-Agilent detectors into an 1100 Series purification system. As an example the separation of four glucocorticoid standards is shown. The collected fractions were re-analyzed to demonstrate the purification performance of the system.

Equipment

System setup and configuration

Since the ELSD is a destructive detector it was set up in the flow path using a splitter (Agilent 1:10/1:20 splitter). The main flow went to the fraction collector, the split flow to the ELSD (figure 1).

To trigger fractions on the ELSD signal the analog output of the detector was connected to a universal interface box (UIB). The UIB was connected to the 1100 Series modules via a CAN cable (figure 2). Any other non-Agilent detector with an analog output can be set up in this way for peak-based fraction collection with the Agilent 1100 Series purification system.

Delay volume calibration for the ELSD

For proper collection of fractions the delay time between the ELSD and the diverter valve of the fraction collector must be measured. This can be done using the Agilent fraction delay sensor built in the fraction collector. The delay calibration procedure is described in the Agilent 1100 Series Purification System User's Guide. The standard delay calibrant (G1946-85020) and the standard delay calibration method of the ChemStation can be used. Since it is absolutely necessary that the desired compound in the split flow arrives at the ELSD before reaching the fraction collector in the main flow, the delay calibration must give delay volume with a positive value. If a negative delay volume is measured the compound would reach the fraction collector before it arrives in the ELSD and would therefore go to waste before it can be triggered. To avoid negative delay volumes the flow connection between the splitter and the ELSD must be kept as short as possible and a capillary with a low internal diameter should be used. If it is not possible to shorten further the capillary leading to the ELSD, the capillary going from the splitter to the fraction collector can be enlarged. However, this would increase dispersion which would result in decreasing fraction collection performance, and should therefore be avoided.

Results and discussion

Fraction collection triggered on the ELSD signal

To trigger fraction collection on the ELSD signal the UIB must be selected as *Peak Detector* in the *Setup Fraction Collector* window in the ChemStation. The parameters *Up Slope*, *Down Slope* and *Threshold* can be selected as for any UV detector. To isolate glucocorticoid standards the values for up and down slope were removed from the table to trigger on threshold only at 70 mV. Figure 3 shows the DAD, dual-channel A/D interface and UIB signal of the glucocorticoid standards — vertical lines mark the collected fractions.

To confirm the purification results the fractions were re-analyzed on the same system without using the splitter. The results clearly show that the four compounds could be separated without any impurities. This confirms the excellent performance of the Agilent 1100 Series purification system with the non-Agilent ELS detector.

Conclusion

In this example a Sedere Sedex Model 75 ELS detector was used for triggering peak-based fraction collection. It is however possible to set up any other non-Agilent detector in the same way as long as it has an analog signal output. As an example we showed the separation of four glucocorticoid standards in the anlytical scale preparative HPLC.

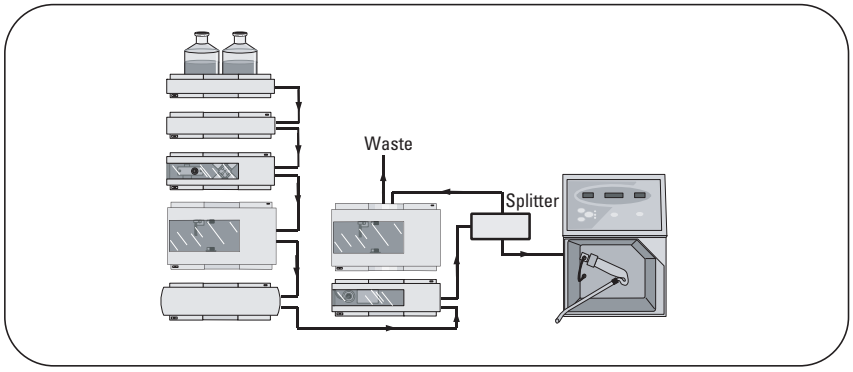


Figure 1
Setup of the ELSD in the flow path using a splitter.

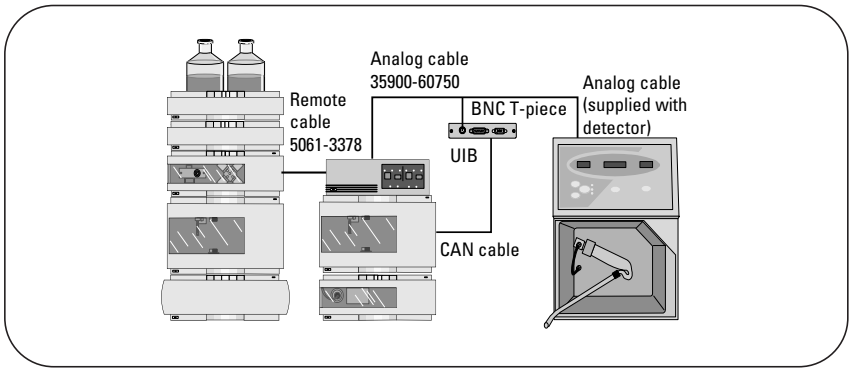


Figure 2
Electronic connections using a UIB and dual-channel A/D interface box.

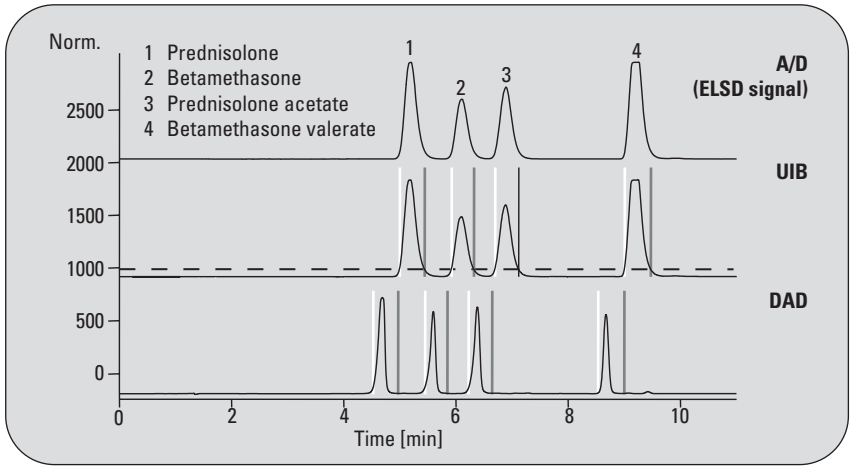


Figure 3
Peak-based fraction collection on the ELSD signal

"Peak-based Fraction Collection Using an Evaporative Light Scattering Detector with the Agilent 1100 Series Purification System", publication number 5988-5816EN.

Method scale-up and scale-up calculations

During drug discovery and development compounds have to be purified for activity testing. Due to the rise of high throughput synthesis and high throughput screening, traditional low throughput purification techniques, such as preparative TLC or crystallization, can create a bottleneck in a synthesis laboratory. A modern and completely automated technique for compound purification is preparative HPLC. In the early stages of drug discovery a large number of compounds are usually synthesized in minute amounts. Therefore, purification can be carried out on columns of 5 mm id or less, which is called analytical scale preparative HPLC. In the later stages of drug discovery and development the compound amount to purify and, therefore, the column id increases.

Agilent offers for its 1100 Series purification system PS preparative pumps, which deliver a flow of up to 100 mL/min at a maximum backpressure of 400 bar. The pumps and the system were developed for high flow rates, however, it is also possible to use the system at flow rates below 5 mL/min due to the excellent performance of the Agilent 1100 Series preparative pumps.

In this application we show a gradient analysis of a model sample on a 3 mm id column at a flow rate of 0.35 mL/min as well as on a 50 mm id column at a flow rate of 100 mL/min without changing the configuration of the Agilent 1100 Series purification system PS.

Results and discussion

Analytical scale method development

The method to analyze the three model compounds was developed on an Agilent 1100 Series system including two Agilent 1100 Series preparative pumps to form the required gradient. A 3.0-mm id ZORBAX SB-C18 column was used at a flow rate of 0.35 mL/min. The peaks in figure 1 show a good resolution and peak shape, which confirms the good performance of the Agilent 1100 Series preparative pump even at low flow rates.

Scale-up calculations

The complete scale-up procedure was calculated according to the equations in figure 2. To calculate the flow rates using the upper equation the 3.0 mm id column with a flow rate of 0.35 mL/min was chosen as the starting point. The lower equation was used to calculate the injected sample volume.
Due to higher flow rates and applied sample amounts it was necessary to change from the 3-mm pathlength SST preparative flow cell to a 0.3-mm pathlength quartz cell for the larger columns. This causes the tenfold increase in the calculation of the injection volume. The calculated flow rates and injection volumes for all columns and flow cells are summarized in table 1.

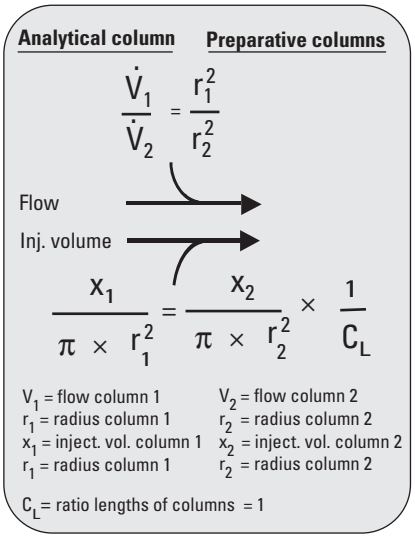


Figure 2
Equations used for scale-up calculations.

Analytical and preparative scale purification

Analytical and preparative scale runs were performed on four different columns (4.6 mm id, 9.4 mm id, 21.2 mm id and 50 mm id) using the flow rates and injection volumes as outlined in table 1. The resulting chromatograms in figure 3 show two things – first, that the Agilent 1100 Series preparative pump performs very well over a wide flow rate range. And, second, that it is possible to scale-up on the ZORBAX columns without losing resolution, which is very important for a fast scale-up process without any method re-development.

Conclusion

The Agilent 1100 Series preparative pump shows an excellent performance over a wide flow rate range from 0.35–100 mL/min.

- This pump is fully compatible with the ChemStation's OQ/PV and EMF (Early Maintenance Feedback) features.
- It is possible to scale-up an application from a ZORBAX column of 3 mm id to one with 50 mm id without any loss of resolution. This increases throughput by reducing the time required for re-developing or adjusting the method.

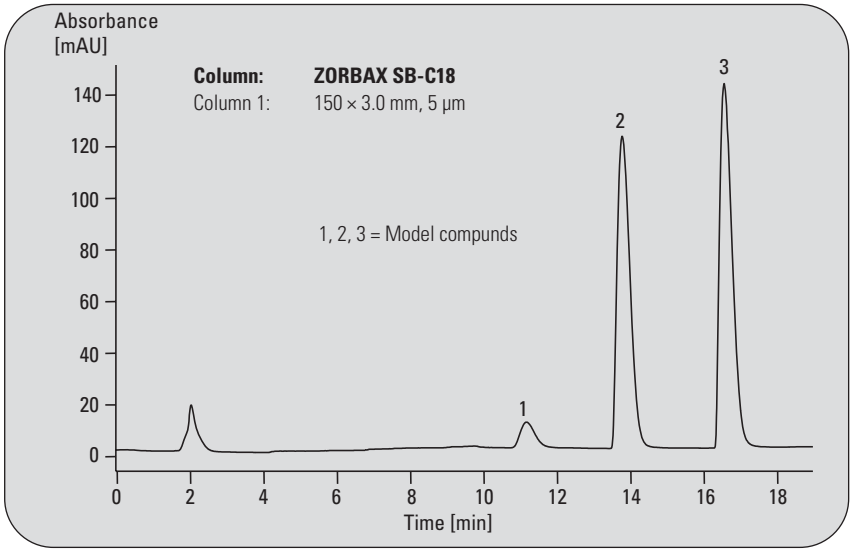


Figure 1
Chromatogram at 0.35 mL/min flow rate.

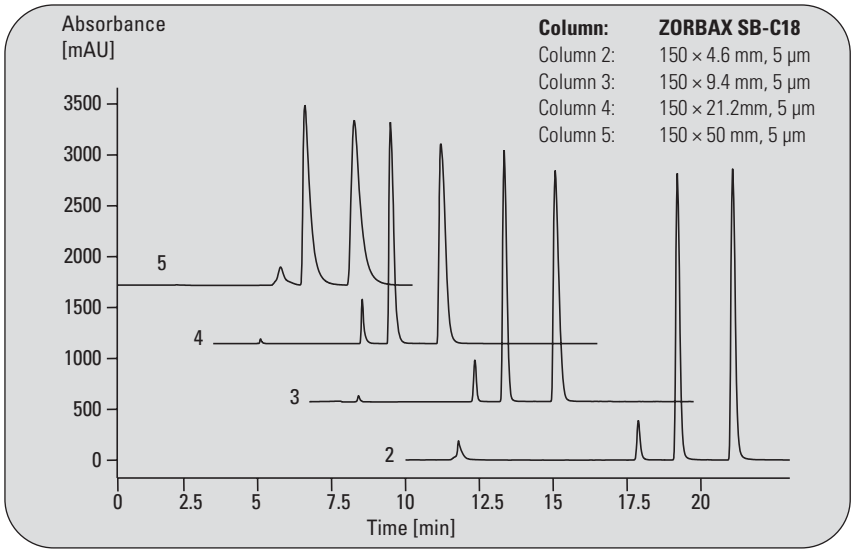


Figure 3
Results of scale-up calculations.

Column	Dimension	Flow	Injection volume	Detector cell
1 (figure 1)	150 x 3.0 mm	0.35 mL/min	0.8 µl	3 mm SST
2 (figure 3)	150 x 4.6 mm	0.85 mL/min	2.0 µl	3 mm SST
3 (figure 3)	150 x 9.4 mm	3.5 mL/min	80 µl (8 µl x 10)	0.3 mm quartz
4 (figure 3)	150 x 21.2 mm	18 mL/min	400 µl (40 µl x 10)	0.3 mm quartz
5 (figure 3)	150 x 50 mm	100 mL/min	2200 µl (220 µl x 10)	0.3 mm quartz

Table 1
Flow rates,injection volumes and detector cells for different column sizes.

"Method scale-up from analytical to preparative scale with the Agilent 1100 Series purification system PS", publication number 5988-6979EN.

Visit our website at **www.agilent.com/chem/purification** to download the detailed Application Note.

Preparative pump performance from 1 to 100 mL/min

The Agilent 1100 Series preparative pump is an isocratic high performance pump with two parallel pistons. It offers flow rates up to 100 mL/min at 400 bar without the need to change pump heads. Two pumps are connected via a low-volume mixing device to give a high-pressure gradient mixing system with an internal delay volume of only about 0.7 mL. Additional features of the pump include an automated seal wash using a peristaltic pump and an automated electro-magnetic purge valve. Although the pumping system is designed for flow rates up to 100 mL/min it also shows very good performance at lower flow rates.

Note:
All experiments were carried out on a system that is used for the daily work in Agilent's laboratory. Depending on the working hours of your system the results for the tests may vary slightly.

Results

1. Flow precision – isocratic

Flow precision is important for run-to-run precision of retention times. Since the pump flow is controlled by the compensation algorithms of the preparative pump firmware the flow precision is backpressure dependent.

Flow precision test set-up

- The flow precision was measured by collecting the solvent (water) over 5 minutes. The collected volume was determined by weighing the collected water and dividing the amount by the density.
- The test was performed at a flow rate of 25 mL/min.
- The precision was measured at three different backpressures (< 100 bar, > 100 and < 200 bar, > 200 bar).
- Five runs were performed at each pressure to determine the precision.
- Restriction capillaries were used instead of a chromatographic column for all experiments.

Results of flow precision test

Figure 1 shows the results for the isocratic flow precision tests. The relative standard deviation of the flow precision is marked. The relative standard deviation is below 0.3 % for the different backpressures.

2. Flow precision – gradient

Flow precision test set-up

- The flow precision was measured by collecting the solvent (water, methanol) over seven minutes. The collected volume was measured.
- The test was performed at a flow rate of 10 mL/min.
- Gradient: 100 % water for 1 min, 100 % water to 100 % MeOH in 5 min, 100 % MeOH for 1 min.
- The precision was measured at three different backpressure ranges.
- Five runs were performed at each pressure to determine the precision.
- Restriction capillaries were used for all experiments instead of a chromatographic column.

Results of flow precision test

Figure 2 shows the results of the gradient flow precision tests. The relative standard deviation of the flow precision, shown with the black error bars, is below 0.4 % for the different backpressure settings.

Conclusion

- The performance of the 1100 Series preparative pump has been shown over the flow range of 0.35 up to 100 ml/min.
- The performance below 5 ml/min is almost as good as for an analytical pump, for example, the 1100 Series quaternary pump.
- Capillaries, especially those with a larger inner diameter required for higher flow rates, will decrease overall sytem performance.

Therefore Agilent Technologies offers dedicated systems from capillary to preparative scale rather than a single system covering the complete flow range. This ensures best performance for a specific application rather than a low performance over a wide application range.

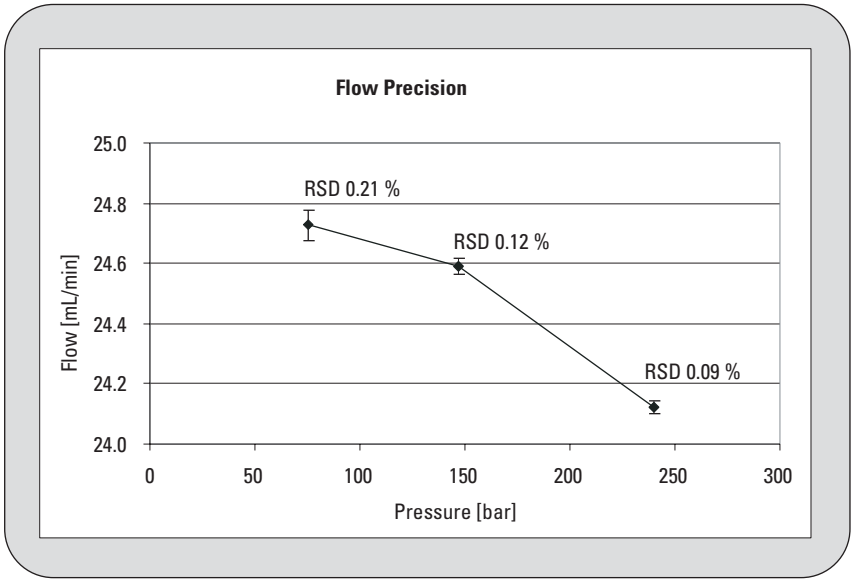


Figure 1
Relative standard deviation of the isocratic flow precision at different backpressure settings.

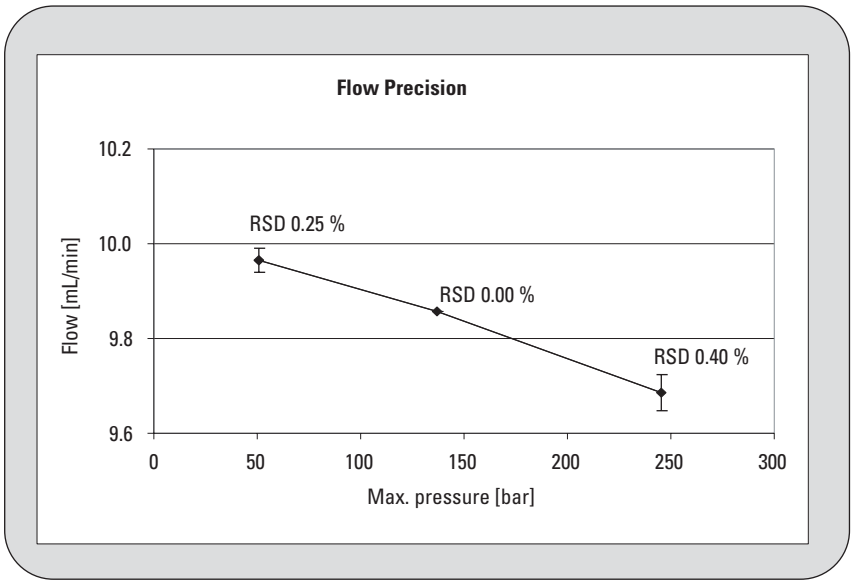


Figure 2
Relative standard deviation of the gradient flow precision at different backpressure settings.

"Performance Characteristics of the Agilent 1100 Series Preparative Pump", publication number 5988-7110EN.

Remote data review for enhanced lab productivity

In the modern drug discovery laboratory there is a tremendous growth in the number of new drug compounds to be analyzed and purified. Analysis and purification is often accomplished by the synthetic organic and medicinal chemists “walking up” to a central LC/MS system to submit the samples and then returning to their labs to generate more syntheses. This has greatly increased the need for chemists to be able to review the data from their own PCs in their own labs without returning to the central LC/MS lab.

The new Agilent ChemStation data browser makes remote data review easy and efficient. This is accomplished by generating an intermediate file (called a .AEV file) on the system acquiring the original data and then making this .AEV file available to remote PCs through a server or e-mail. Although targeted at drug discovery, the browser is applicable to any laboratory needing to review data files generated on an LC or LC/MS ChemStation data system.

Primary functionality of the data browser

The main use of the browser in the drug discovery laboratory is to answer the following question as quickly as possible for a large number of samples:

Did I make the expected compound and, if so, at approximately what purity level?

The main screen is comprised of a series of views. What is

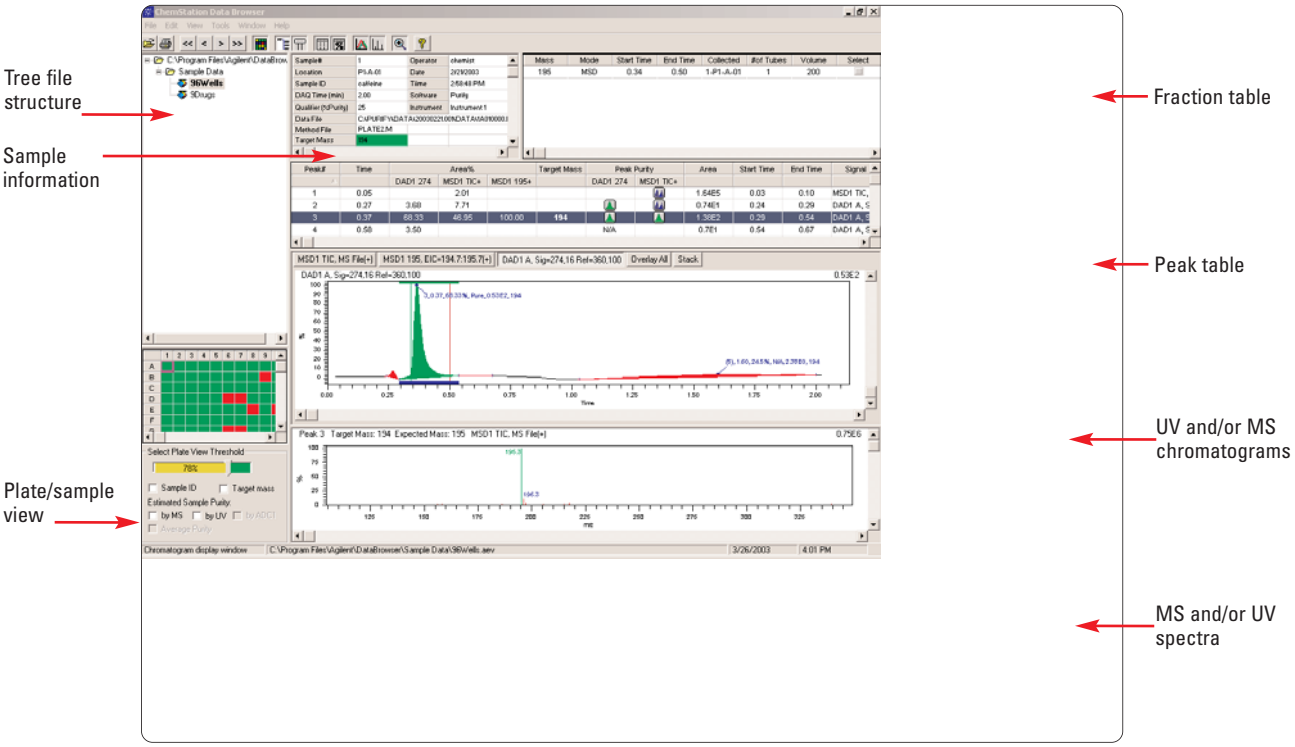


Figure 1
Main screen of ChemStation data browser.

shown is very dependent on the application and is customizable by the user. Figure 1 shows a typical configuration of views for a drug discovery lab reviewing microtiter plates containing target compound reaction mixtures. In plate view in the lower left corner of this screen, the user can see at a glance the graphical representation of the samples in a microtiter plate or the vial positions available with other 1100 Series autosampler trays such as the 100-position tray.

In Figure 1, the green wells indicate the target mass was found and the red wells indicate the target mass was not found based on the specified threshold. To see more specific sample information such as UV and MS chromatograms, the user can simply click on a well. If purification was done on the sample, then fraction information is presented in a fraction table.

Following are key questions that the data browser can answer quickly, helping to insure efficiency and that the proper decisions are made for the next steps of drug development.

In addition the browser can be used to:

- provide information on the purity of the peak (number of co-eluting componets
- review all the data from a plate view all at one time
- compare multiple samples in separate windows on the screen at one time
- provide flexible reports customized by the user.

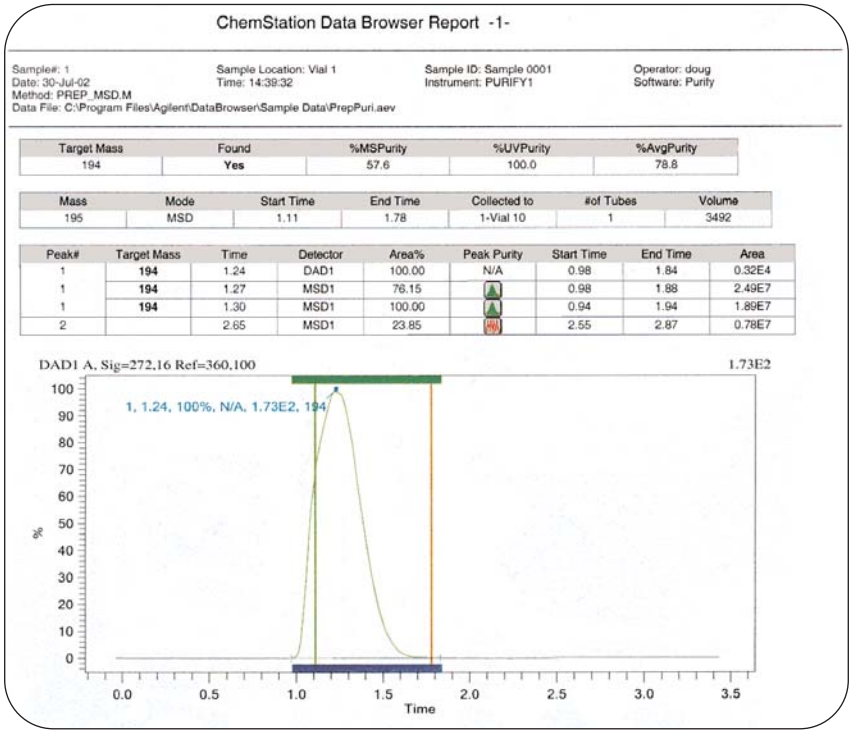


Figure 2
Example of a customizable report from the data browser software.

Reporting

Reporting of data is another important function for drug discovery. With the flexibility of the ChemStation data browser the user can optimize this capability by choosing which elements to include in a given report. Figure 2 is an example of a report showing the key elements for confirming the presence of the target compounds in drug discovery.

"Enhancing Lab Productivity with the Agilent ChemStation Data Browser", publication number 5988-8490EN.

Generic gradients of 5 – 95 % organic mobile phase are frequently used for purification runs, which do not always lead to sufficient resolution for the target compound. To increase the resolution an optimized method with a shallower gradient around the elution composition of the target compound could be used. The preparative method is selected according to a retention time window, in which the compound elutes in the pre-preparative analytical run.

Creating an optimized preparative method set based on a pre-preparative analytical run

Results and discussion

To produce a method set for retention time windows, the retention times of several standard compounds were measured using an analytical method. A diagram was generated showing the relation of retention time and true elution composition for these standards. The elution composition of any compound can be extracted or calculated from this diagram by knowing its retention time in the analytical run. The same approach can be taken for the initial and final gradient composition for retention time windows. As the elution composition for eight Agilent standard is provided in this Application Note, the generation of a preparative method set involves four easy steps:

1. Run the Isocratic Test Sample and the Electrospray LC Demo Sample with the analytical method.
2. Generate a diagram with the measured retention times and the known gradient elution compositions.
3. Calculate or extract the initial and final gradient composition for the optimized preparative methods from the graph.
4. Set up the dedicated gradient for each preparative method.

Purification example
To purify the target compound sulfa 2, the sample was applied to a preparative column using a generic gradient and an optimized gradient. The results are shown in figure 1 (A,B).

The target compound partly co-elutes with the previous peak using the generic gradient, however, baseline separation could be achieved with the optimized method. The resolution between the previous peak and sulfa 2 is more than doubled, the resolution between sulfa 2 and the following peak increases by about 50 %.

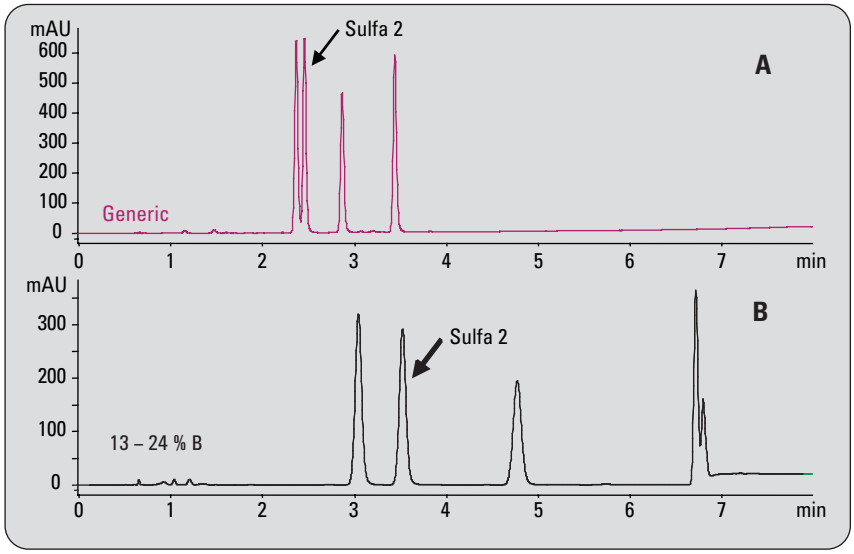


Figure 1
Preparative run with generic (A) and optimized gradient (B).

Conclusion

In this Application Note the generation of a small set of optimized preparative methods based on retention time windows in the pre-preparative run was explained. By using the Isocratic Test Sample and the Electrospray LC Demo Sample the complete process was done in four easy steps taking less than one hour to accomplish. The improved resolution of or near the target compound using optimized gradients was presented in an application example.

"Creating an Optimized Preparative Method Set Based On a Pre-preparative Analytical Run", publication number 5989-4844EN.

Development of a compound purification strategy for a medicinal chemistry group

In collaboration with Darryl McConnell, Georg Egger, Boehringer Ingelheim, Austria

In this Application Note we describe the development of a purification strategy for a medicinal chemistry group. The goals for the development of the purification strategy were:

- a simple, walk-up workflow,
- a set of guidelines for sample preparation and method selection for the chemists,
- purification of problematic samples - insoluble samples, polar samples and samples that show poor ionization,
- one standard method suitable for 80 - 90 % of the daily samples,
- a small set of generic purification methods.

Results and discussion

Workflow

As the chemists were familiar with the ChemStation software, the decision was made to use the ChemStation software for purification without the Agilent Purification software. The workflow was designed similar to the analytical workflow. The only differences between the preparative and analytical runs are:

- a fraction start location can be entered optionally (if no position is entered, the next free position in the fraction collector is automatically used),
- if the sample volume to be injected differs from the default sample volume (500 µL), it must be entered.

Injection of insoluble compounds

For the injection of insoluble compounds into the mobile phase at the gradient starting conditions, two injection techniques are possible: organic-phase injection or sandwich injection.

Purification of polar compounds

A dedicated method was set up for polar compounds. In order to avoid co-elution of polar compounds with the DMSO peak the following precautions were made:

- the gradient starting conditions were set to 5 % acetonitrile (10 % was used for the standard method)
- an initial hold phase for the gradient of 2.5 minutes was adopted
- gradient end conditions were set to 50 % acetonitrile (95 % was used for the standard method).

Applications

In this section the purification of a real-life crude reaction mixture is shown. The crude reaction mixture was analyzed on an Agilent 1100 Series analytical system, which showed that the desired product existed as a mixture of cis and trans isomers. The analytical chromatogram is shown in figure 1.

In order to separate the two isomers, the crude product was purified again, this time using the *AND* combination of the UV and MS signal. As shown in figure 2, by using this method the isomers were successfully collected in two separate fractions.

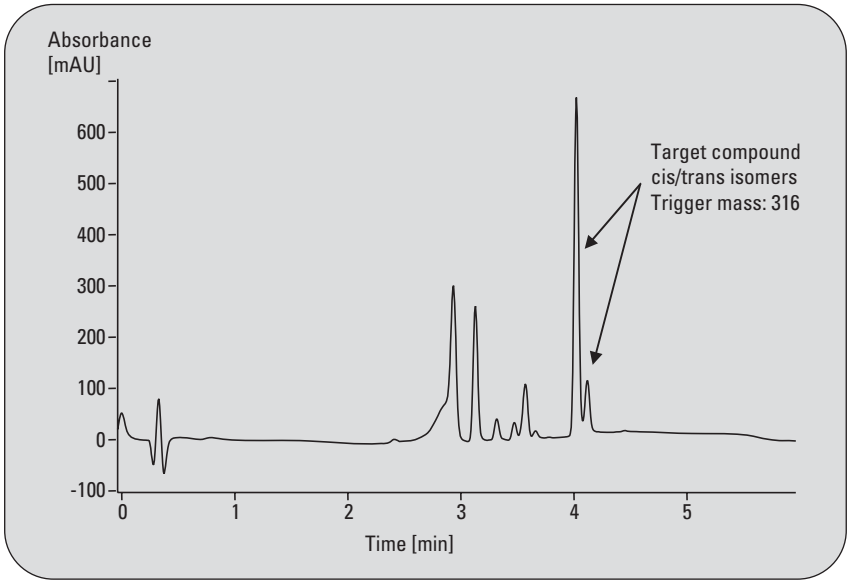


Figure 1
Analytical chromatogram of crude reaction mixture.

Conclusion

In this Application Note we showed the development of a purification strategy for a medicinal chemistry group. A simple, walk-up workflow for preparative HPLC-MS purification was established. A set of five pre-defined methods were generated for different sample types, with a standard method suitable for 80–90 % of the daily samples. Further methods were set up for insoluble samples, polar samples and for samples that show poor ionization. An additional method was established using the AND combination of the UV and MS signal.

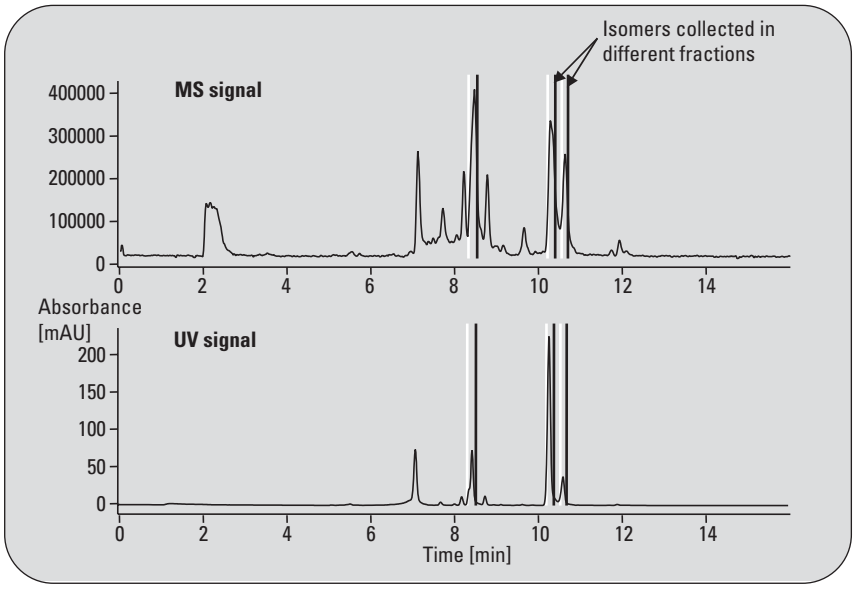


Figure 2
Fraction collection using the AND combination of UV and MS signal.

"Development of a Compound Purification Strategy for a Medicinal Chemistry Group", publication number 5989-0055EN.

The following three fraction collection strategies are leading to either many or only a single fraction, with complete or low recovery of the injected sample. However, in all three cases, there is no need for fraction re-analysis to identify the desired target compound. This is achieved by connecting an 1100 Series mass selective detector to the Agilent 1100 Series purification system.

Results and discussion

In this Application Note we show the following three purification strategies:

1. Time-based fraction collection monitored with an MSD
2. Peak-based fraction collection monitored with an MSD
3. Mass-based fraction collection

1. Time-based fraction collection monitored with the MSD

Fractions were collected using time-based fraction collection, which guarantees that almost nothing of the injected sample is lost.

Fractions were collected based on time, collecting 80 fractions between 2 and 7.5 min and resulting in the chromatograms shown in figure 1. The vertical lines show the start tick-marks of the collected fractions.

Compound purification on a system equipped with mass-selective detector

To identify the fractions containing the desired compound (target mass 241 amu) the extracted ion chromatogram (EIC) of the mass 242 ($[M+H]^+$) was overlaid with the total ion chromatogram (TIC). Figure 2 shows the fractions containing the target mass in the well-plate positions B-02 to B-06.

2. Peak-based fraction collection monitored with an MSD

Using the second strategy, the fractions were collected based on the peaks in the UV signal with a threshold setting of 50 mAU. This ensures that the main peaks of the sample are collected but the number of collected fractions is dramatically reduced compared to time-based fraction collection.

To identify the fractions containing the desired compound the EIC of the mass 242 ($[M+H]^+$) was again overlaid with the TIC.

3. Mass-based fraction collection

When using mass-based fraction collection a fraction is only triggered when a peak in the MSD contains the desired target mass and the EIC of this target mass exceeds the specified threshold. This ensures that in most cases only a single fraction per sample run containing the desired compound is collected. The disadvantage is that most of the injected sample is not recovered. The result of the mass-based fraction collection is shown in figure 3.

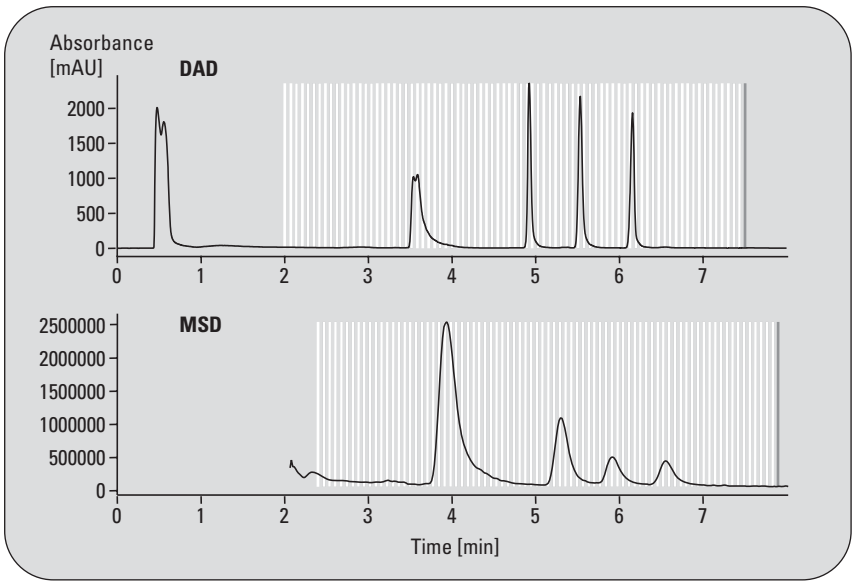


Figure 1
Result of time-based fraction collection.

As figure 3 shows, only one fraction containing the target mass was collected in vial number 1, therefore the number of collected fractions decreased to 1, however the sample loss increased to 95 %.

Conclusion

While an MSD is not required for time- or peak-based fraction collection, it offers the advantage that tedious re-analysis of fractions to determine the ones containing the target compound is unnecessary. The desired fractions can easily be identified by overlaying the TIC with the EIC of the target mass.

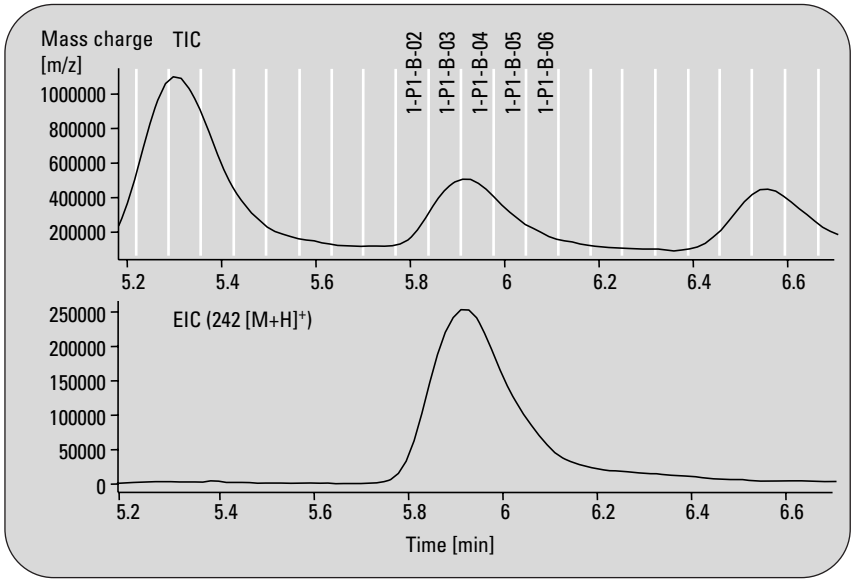


Figure 2
Overlaid TIC and EIC to identify the desired fractions.

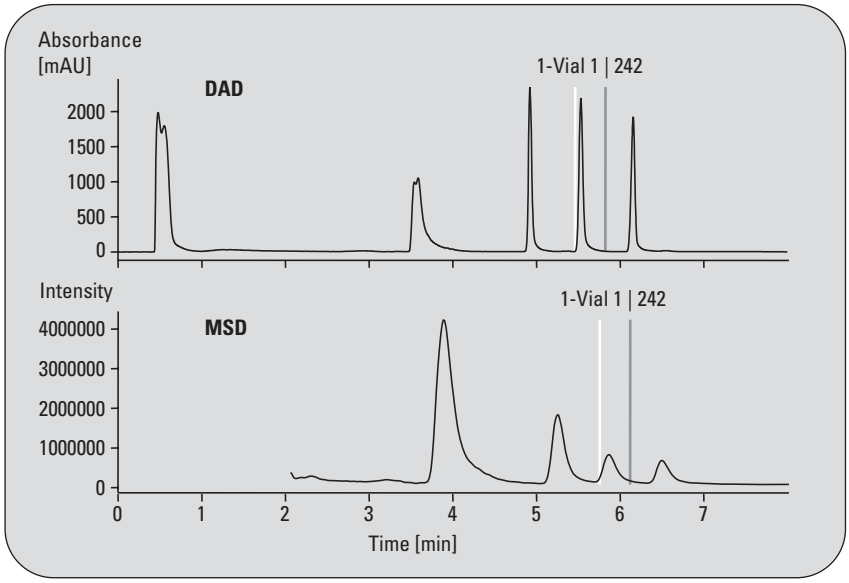


Figure 3
Result of mass-based fraction collection.

"Compound Purification Using the Agilent 1100 Series Purification System with a Mass-selective Detector", publication number 5988-7830EN.

Purification of compounds from non-baseline separated peaks

Four separation strategies are subsequently described:

1. Peak-based fraction collection with high threshold setting
2. Peak-based fraction collection using up- and down-slope
3. Peak-based fraction collection with time slicing of the peak
4. Mass-based fraction collection

Results and discussion

1. Peak-based fraction collection with high threshold setting

The easiest way of doing peak-based fraction collection is the collection on threshold only. This means a fraction is collected as soon as the detector signal exceeds a predefined value, and the collection ends when the signal falls below the specified threshold. This kind of peak-based fraction collection is sufficient for most purification applications. Non-baseline separated peaks can also be separated but the threshold must be set to a rather high value. This leads to a cut-off of some of the compound at the beginning and the end of the peaks, which decreases the recovery.

2. Peak-based fraction collection using up- and down-slope

To avoid compound loss at the beginning and the end of the peak the Agilent ChemStation and Purification/HighThruput software offers two additional parameters for peak-based fraction collection—up-slope and down-slope. When these parameters are set, a peak start is triggered if both parameters, up-slope and threshold, are exceeded. A peak stop is triggered if the signal meets one criteria, i.e. if it either falls below the threshold or below the specified down-slope value. As a result the threshold can be set to a rather low value, which allows to separate two non-baseline separated peaks into two fractions without losing compound at the peak start and end.

3. Peak-based fraction collection with time slicing of the peak

Peak-based fraction collection using up- and down-slope gives good recovery and purity as shown above. But if the overlapping peaks are very broad and therefore the overlapping area is large the two collected fractions are significantly impure. In this case a better strategy is to trigger the start of the fraction collection based on the signal but then cut the two peaks into several slices by time-based fraction collection. To accomplish this the *Max. fill volume per location* feature in the *Auxiliary* section of the *Setup fraction collector* window of the Agilent Chemstation software can be used. With this feature the pre-configured fill volume of a fraction container can be changed within the method.

In the example shown in figure 2 deep-well plates were used as fraction containers and a fill volume of 2 mL was specified. Since the used flow rate was 20 mL/min a well fills up every six seconds. With the overall peak width of the two peaks of about 45 seconds the peaks are spread over eight wells. To avoid the loss of compounds when the fraction collection needle moves to the next collection location *Continuos flow* was configured as *Collection Mode*. In this mode, which is only configurable if well-plates are

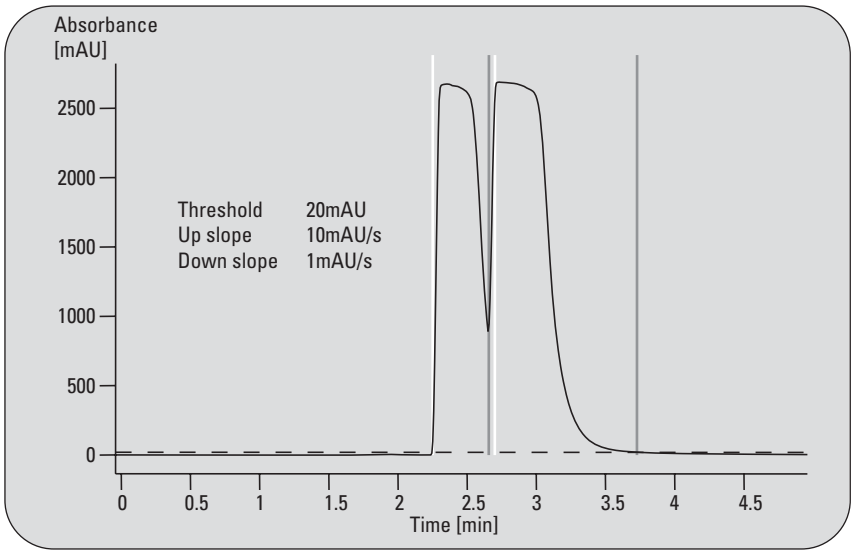


Figure 1
Peak-based fraction collection using up- and down-slope.

used as fraction containers, the diverter valve does not switch to the waste position when the collection needle moves to the next position. The results of the fraction collection are shown in figure 2.

4. Mass-based fraction collection
Another possibility to purify compounds from non-baseline separated peaks is mass-based fraction collection. Since triggering is done on the target mass with the highest abundance a second fraction is started as soon as the target mass of the second peak becomes the dominant ion in the MS spectrum. The result of the fraction collection is shown in figure 3. Re-analysis of the collected fractions gave similar results for recovery and purity as for peak-based fraction collection using up- and down-slope.

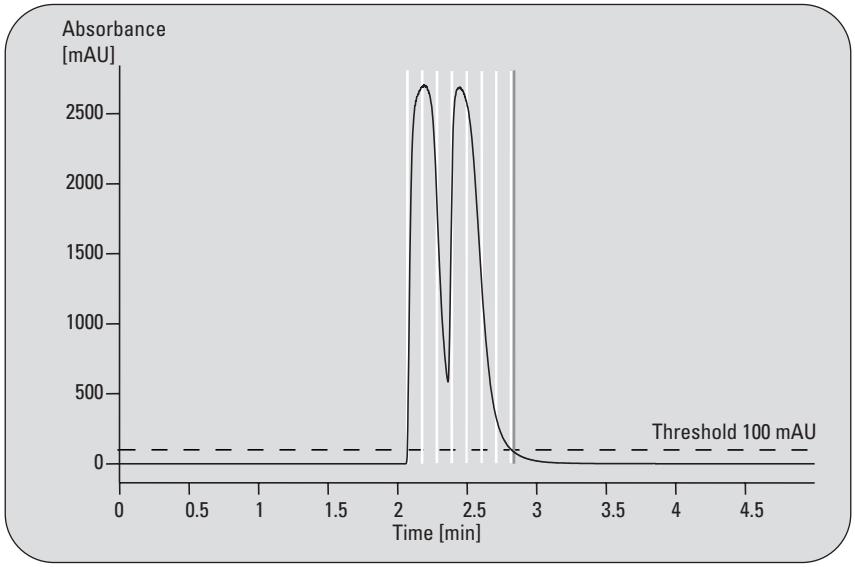


Figure 2
Peak-based fraction collection with time slicing of the peak.

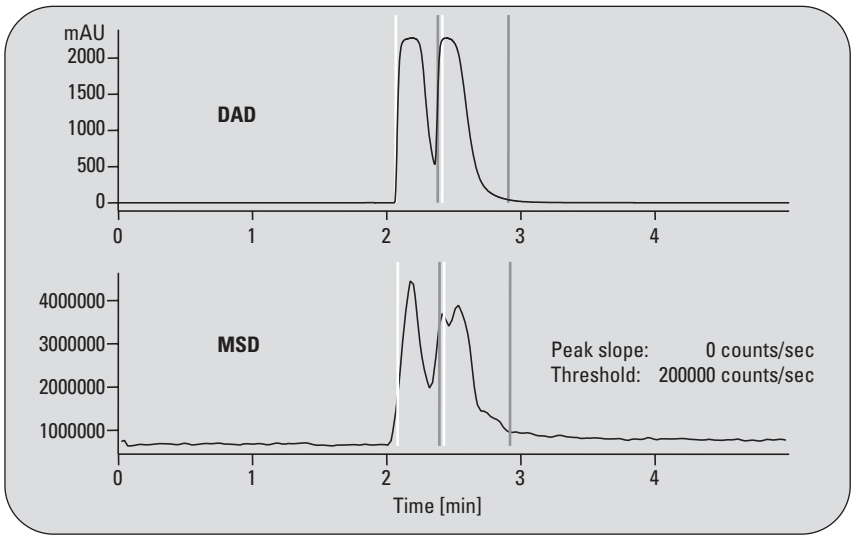


Figure 3
Mass-based fraction collection.

Conclusion

Peak-based fraction collection on threshold only is the easiest way to separate the two compounds but the threshold has to be set high, which can lead to compound loss at the beginning and end of the peaks. This can be avoided by using the up-and down-slope features of the ChemStation software. If the peaks are broad and are overlapping over a broad area the peak-based fraction collection with time slicing of the peaks can be used. This requires some manual calculations regarding the proper fraction fill volume and will lead to a higher number of fractions. If a purification system equipped with an MSD is available mass-based fraction collection can be performed, which also leads to only two fractions.

"Strategies for Purification of Compounds from Non-baseline Separated Peaks", publication number 5988-7460EN.

Usage of up and down slope in peak-based fraction collection

What is up and down slope? How is it used to make triggering decisions? What kind of applications require up and downslope?

Results and discussion

1. Fraction triggering decisions based on "up and down slope"

What is "up and down slope"?

The slope is the first derivative of the signal as shown in figure 1. The slope value equals zero at the baseline, rises to a maximum value at the first inflexion point, becomes zero at the peak apex, falls to a negative maximum at the second inflexion point and becomes zero again at the baseline after the peak.

Fraction triggering decision when using up and down slope

Figure 2 shows a chromatogram with the start and stop tick marks for the collected fractions. The start of a fraction is triggered when the set up values for threshold and up slope are exceeded (tick mark 1). A peak stop is triggered when the signal falls either below the threshold or meets the down slope criteria. At tick mark 2 the fraction was stopped since the down slope criteria was met because the slope equals zero at the local minimum. The threshold was still exceeded at this point. At tick mark 3 the up slope is again exceeded and the threshold is still above the set value. Finally at tick mark 4 the signal fell below the threshold and therefore, regardless of whether the down slope criteria was met or not, fractionation was stopped.

2. Applications

Separation of steep and shallow peaks

By using up and down slope it is possible to separate steep peaks from shallow peaks. Figure 3 shows that it is easy to separate steeper peaks from more shallow peaks. To to do this the up and down slope values have to be known before starting fraction collection. This is only possible if a pilot run is available.

Separation of non-baseline separated peaks

If two peaks are not baseline-separated there are a few strategies to collect the two compounds as separate fractions, usually with good recovery and purity. One is peak-based fraction collection using up and down slope. Since

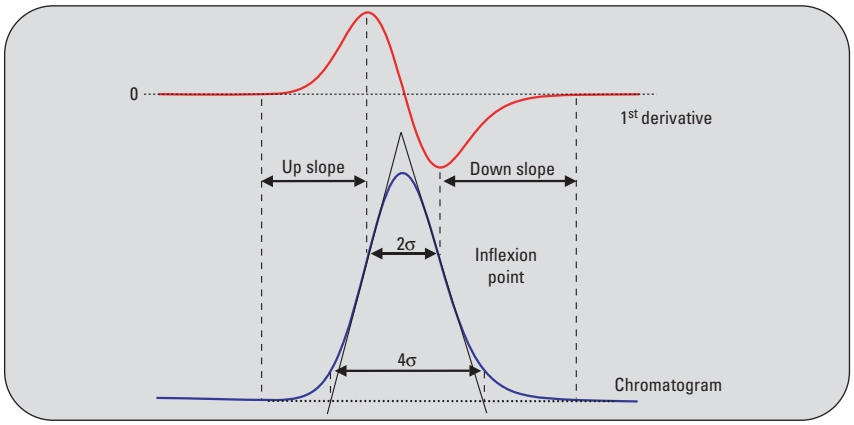


Figure 1
The slope is the first derivative of a peak.

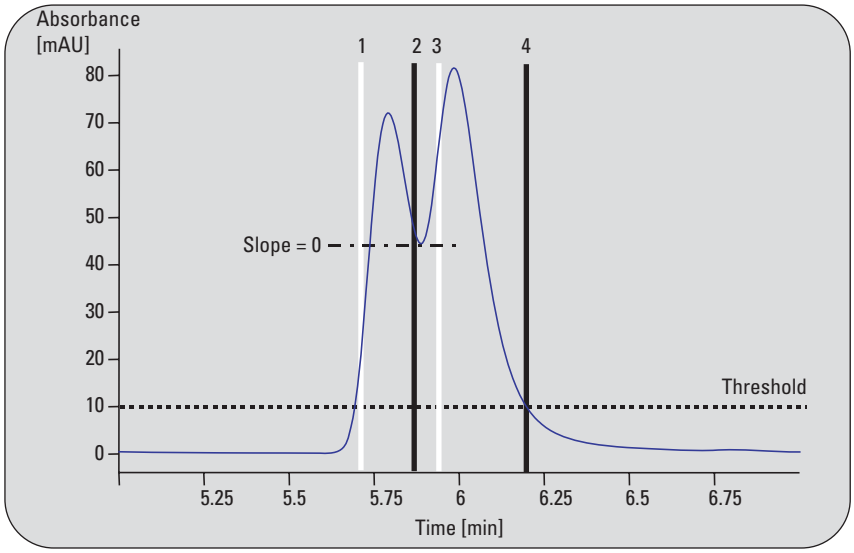


Figure 2
Fraction triggering decisions.

the slope in the minimum between the peaks equals zero the peaks can be split in two fractions even if the signal does not fall below the threshold value.

Purification of compounds in chromatograms with drifting baseline

In a chromatogram with drifting baseline peak-based fraction collection on threshold only is not suitable. If the baseline drifts upwards everything above the threshold is collected as a fraction. If the baseline drifts downward only small portions of the peaks, if at all, are collected. Therefore, proper values for up and down slope have to be applied. Figure 4 shows the result of a peak-based fraction collection in a chromatogram with rising baseline.

Conclusion

Up and down slope can be used to trigger fractions in peak-based fraction collection. There is an clear advantage in being able to specify two parameters, rather than only a single slope parameter. For many applications peak-based fraction collection based on threshold only is sufficient, however, we also showed some application examples that also require up and down slope.

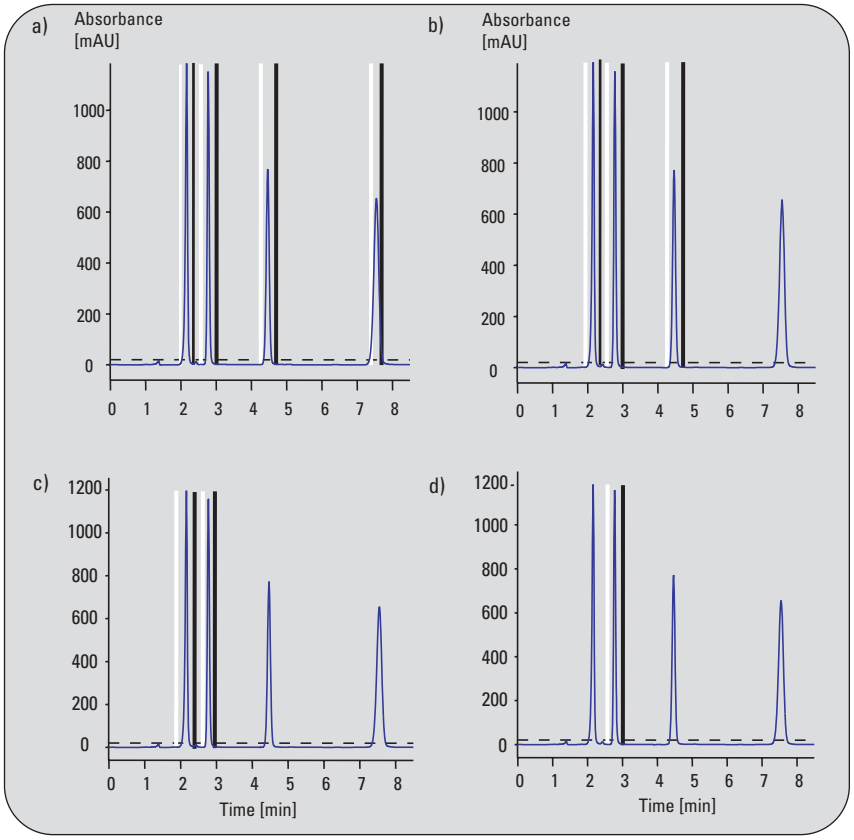


Figure 3
a) Up and down slope: 30 mAU/s, b) Up and down slope: 120 mAU/s
c) Up and down slope: 250 mAU/s, d) Up and down slope: 400 mAU/s.

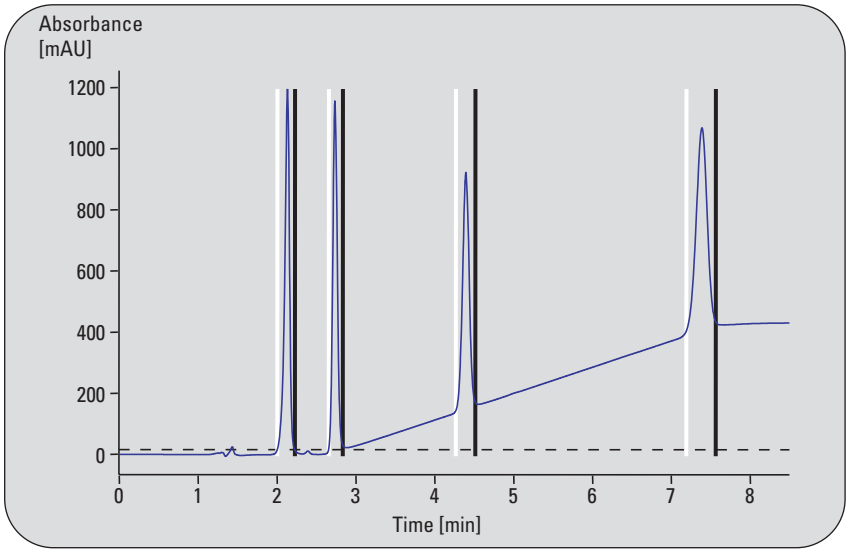


Figure 4
Threshold settings: 15 mAU, up and down slope: 15 mAU/s.

"Sophisticated Peak-based Fraction Collection – Working with Up and Down Slope", publication number 5989-0511EN.

Why it is better to perform the re-analysis for purity checks not directly after fraction collection but at a later stage in the sample workflow on a dedicated HPLC system? This observations are based on experiences of users of the 1100 Series purification systems.

Results and discussion

Typical workflow

The typical workflow in drug discovery but also in other industries searching for active compounds, for example Crop Science, can be described in six steps:

1. Submission of the impure sample by the chemist.
2. Purification run on the preparative HPLC system.
3. Fraction collection.
4. Solvent evaporation and weighing of the compound.
5. Re-dissolving the pure compound to a certain concentration.
6. Submission of a small portion of the solution for activity testing.

At a certain point in this workflow it has to be checked that the compound submitted for activity testing (step 6) has the required purity, which must usually be higher then 90 – 95 %.

Possible errors when doing automated fraction re-analysis

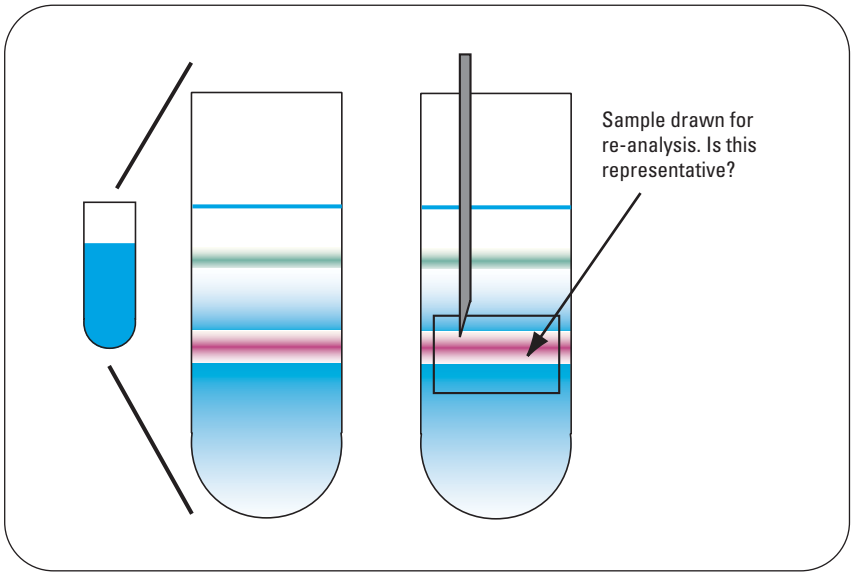


Figure 1
Error due to concentration gradient.

1. Concentration gradient

The target compound elutes from the column over a certain time leading to a concentration gradient in the fraction. The concentration gradient can be seen in the fraction container (figure 1). If a small volume is drawn directly from the tube for re-analysis this sample is certainly not representative for the sample submitted for activity testing.

2. Crystallization

For good solubility, samples are very often submitted for purification not in the solvents used as mobile phase but in strong solvents like DMSO or DMF. However, the mobile phase used for reversed phase chromatography is usually water and acetonitrile or methanol. Depending on the injected sample amount and solubility the target compound starts to crystallize out of the mobile phase. If a sample volume for the purity check is drawn from the mother liquid this sample is again not representative for the sample going for activity testing.

3. Decomposition during evaporation
After purification the mobile phase of the fraction containing the target compound has to be evaporated. This can be done either in a vacuum centrifuge or by heating and flushing with nitrogen. After weighing the residue it has to be re-dissolved to a certain concentration using an appropriate solvent, for example, DSMO or DMF. If the purity check of the target compound was done directly from the fraction container a possible decomposition of the target compound during the evaporation process would not be recognized. In the worst case it could happen that a highly active compound could be missed.

Which is the right step to perform the purity re-analysis?
The three problems described in the previous section do not occur when the reanalysis is not performed directly after fraction collection but at a later step in the workflow. Since a portion of the re-dissolved compound goes for activity testing (step 6) a second portion of the same solution could be used for analysis and purity testing. This makes absolutely sure that a representative sample of the compound going for activity testing is re-analyzed.

Conclusion

Automated fraction re-analysis would be another step towards a completely automated purification solution. It is shown that:

- the gain in automation sacrifices the reliability of the measured results
- the measured purity can lead to wrong results due to the concentration gradient and possible crystallization in the fraction container but also that an active compound could be completely missed due to decomposition during evaporation.

All these disadvantages can be overcome if the analysis is performed at a later step of the sample purification and activity testing process on a dedicated analytical HPLC system.

"Automated Fraction Re-analysis – Does it Really Make Sense?",
publication number 5988-8653EN.

In the following two approaches are described to increase the column loading – the sandwich injection and the organic-phase injection.

Results and discussion

Sandwich injection

For the sandwich injection the sample is injected between two plugs of the sample solvent. If, for example, the compound is dissolved in DMSO a plug of DMSO is placed before and after the sample (figure 1). When the sandwich starts to mix with the mobile phase the mixing begins from the end of the plugs where the sample concentration is zero and therefore no precipitation can occur. With the Agilent ChemStation the sandwich injection can be done easily using an injector program.

Advantages of sandwich injection

- The sample does not come in contact with the mobile phase until it reaches the column. Therefore no precipitation in the critical part of the flow path can occur.
- No hardware re-configuration required. Sandwich injection can be done on a standard system using an injector program

Injection of high-concentration samples

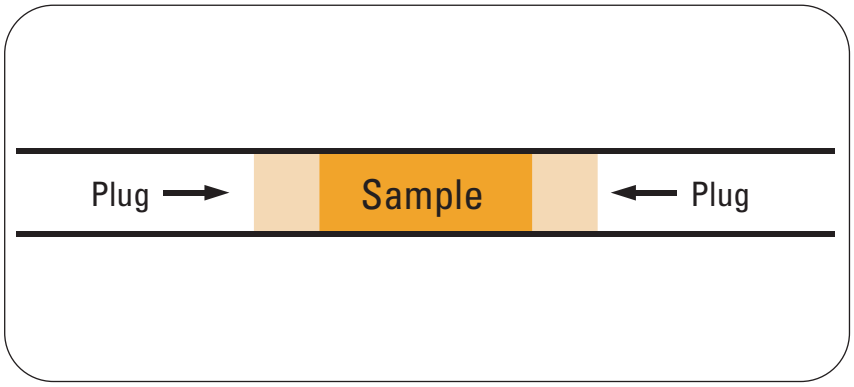


Figure 1
Sandwich injection.

Disadvantages of sandwich injection

- The sample must be retained on the column in a rather narrow band. The strong sample and plug solvent leads to a significant band broadening of the sample on the column head.
- If the ratio of plug and sample volume compared to the column volume is too large some of the compound can elute with the solvent front as column breakthrough.
- The plug and sample solvent might disturb the equilibrium of the stationary phase.

Organic-phase injection

Organic-phase injection requires re-plumbing of the preparative pumps and the injector. The idea is that the injector is connected into the flow path right after the pump delivering the organic solvent prior to the mixing point of the aqueous and organic mobile phase (figure 2). This means the sample has only contact to the organic phase until it reaches the mixing tee and the pre-column. Therefore precipitation can not occur in the critical part of the flow path, which is the switching valve of the autosampler. The mixing tee and the pre-column must be close together so that the sample has moved already to the head of the pre-column before precipitation occurs.

Advantages of organic-phase injection

- No strong sample solvent is required, which could lead to additional peak broadening or column breakthrough.
- Since the Agilent 1100 Series preparative pump in the gradient version consists of two physical pumps the gradient programming is very easy. It can be set up in the same way as for the standard configuration, no flow gradient programming is necessary.

Disadvantages of organic-phase injection

- Although the autosampler valve is the critical part for sample precipitation in the flow path clogging can still occur in the mixing tee or on the head of the pre-column. While precipitation on the column head usually leads only to an increased pressure clogging in the mixing tee is critical.
- Setting up the purification system for organic-phase injection requires re-plumbing of the system. This means it is not possible to perform organic-phase and standard injection on the same system without hardware changes.

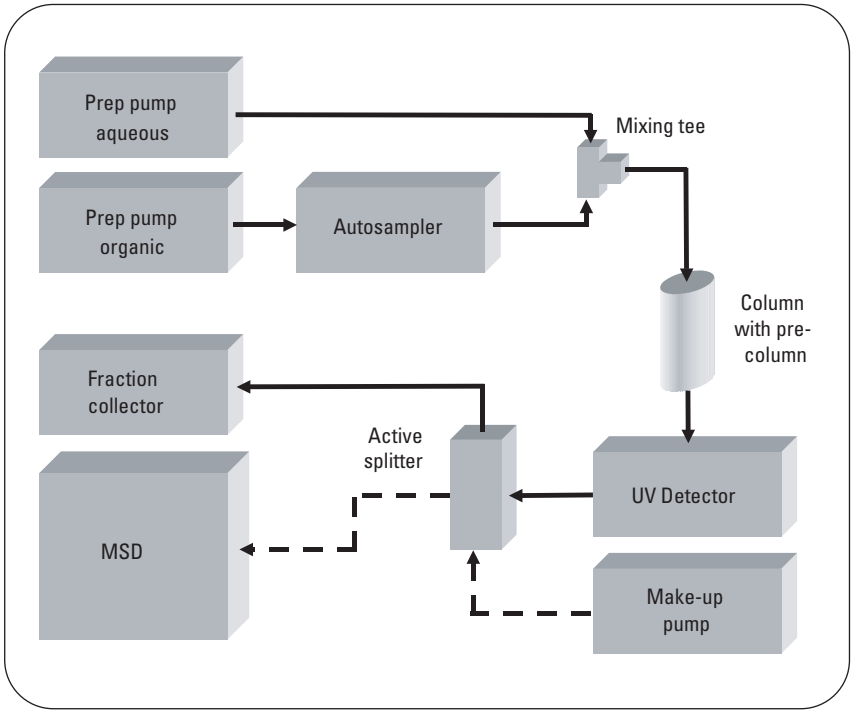


Figure 2
Organic phase injection.

Conclusion

- Using the sandwich injection the sample is placed between two plugs of pure solvent to avoid mixing of the sample with the mobile phase of the gradient starting conditions. This can be achieved easily by using an injector program in the Agilent ChemStation.
- When doing organic-phase injection the autosampler is connected into the flow path of the organic mobile phase prior to the mixing point with the aqueous mobile phase. This means mixing and precipitation does not occur in the autosampler valve but very close to the pre-column head where it does not cause problems.

"Injection of High-Concentration Samples with the Agilent 1100 Series Purification System", publication number 5988-8654EN.

Walk-up access for increased productivity

With the explosive growth of synthetic organic chemistry over the past several years, there is corresponding demand for easy to use, robust, rapid turnaround systems to analyze and confirm the molecular weight of the tremendous numbers of new compounds. Synthetic chemists typically want to concentrate on organic synthesis issues and want to minimize the time spent on the analysis of their compounds.
The Agilent LC/MS Easy Access software described here allows users to simply "walk up" with their samples, input simple sample information, choose from a list of methods, position the samples as directed by the system, and then return to their labs and wait for an e-mail of the results.

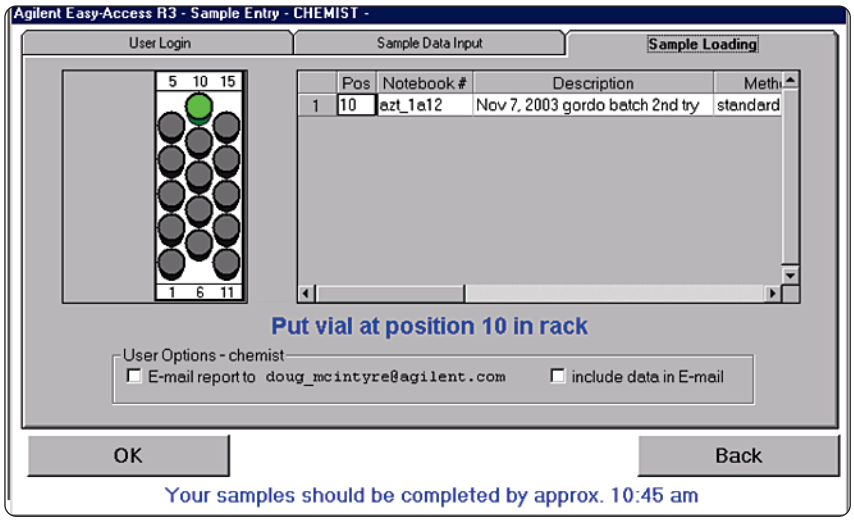


Figure 1
System directs user where to place samples and provides an approximate completion time.

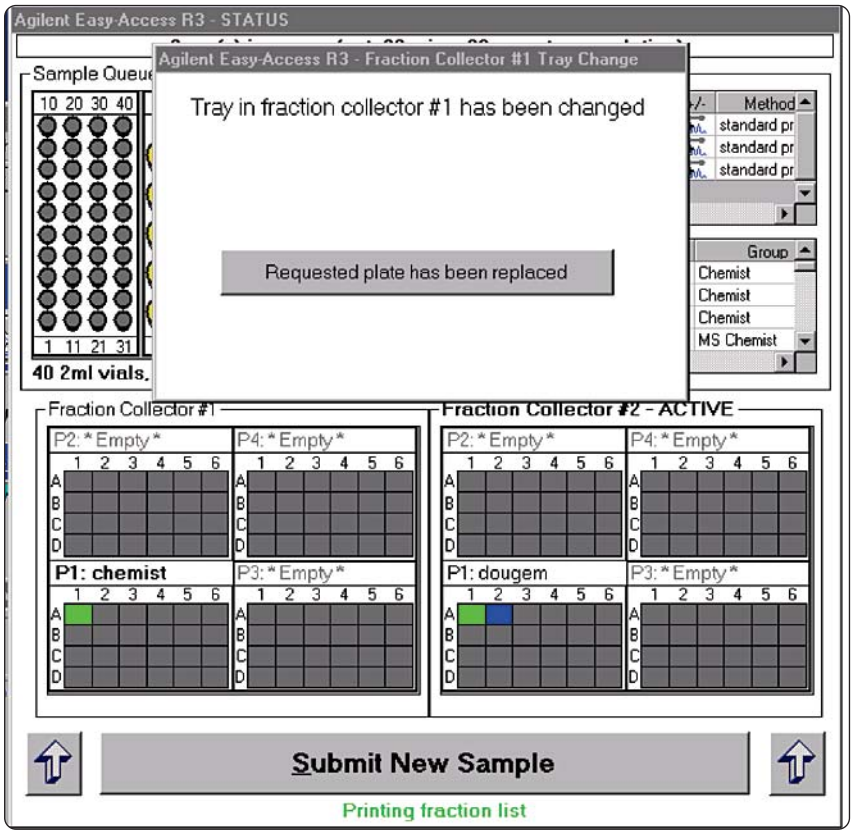


Figure 2
System prompts the user to replace plates after removing their fractions so that space is always available for next user.

Key features of LC/MS Easy Access software

- Emailed data can now be viewed with ChemStation data browser
- No need to reset fraction collector since fractions can be continually removed while system is running
- Very simple and easy sample submission and status checking
- Rapid confirmation of molecular weight and target ion presence
- Supports Agilent 1100 Series well plate sampler and automatic liquid sampler
- Automatic pre-equilibration on change of method
- Supports automated e-mailing of data and reports
- Flexible administrator tools to set user access, queue tracking and project management
- Multiple Instrument Networking

so instruments can share databases, eliminating the need to manage redundant configurations for each instrument and reducing administrative tasks

Results and discussion

After the user has logged in by supplying a password (optional security), described the samples, and assigned methods from a list, the system shows the locations in the sampler where the samples should be placed (Figure 1). Figure 2 shows the status screen which displays the overall status of the system and contains the following key information:

- Current samples and approximate time remaining in the queue
- The method currently running and name of last submitter
- Status of ChemStation, autosampler and UV/Vis lamp

System administration

The system administrator is responsible for overall management of the Easy Access system. Key capabilities include:

- User and group administration including optional passwords, method access and ChemStation availability
- Sample queue management including moving priority samples to the front of the queue
- Method management which defines the methods available to the users

Results sent by e-mail

The system can be directed to send the results of the analysis to selected users by e-mail. At right is an example of the information received. In addition, the email can also include attached reports showing which target masses were found, chromatograms and spectra.

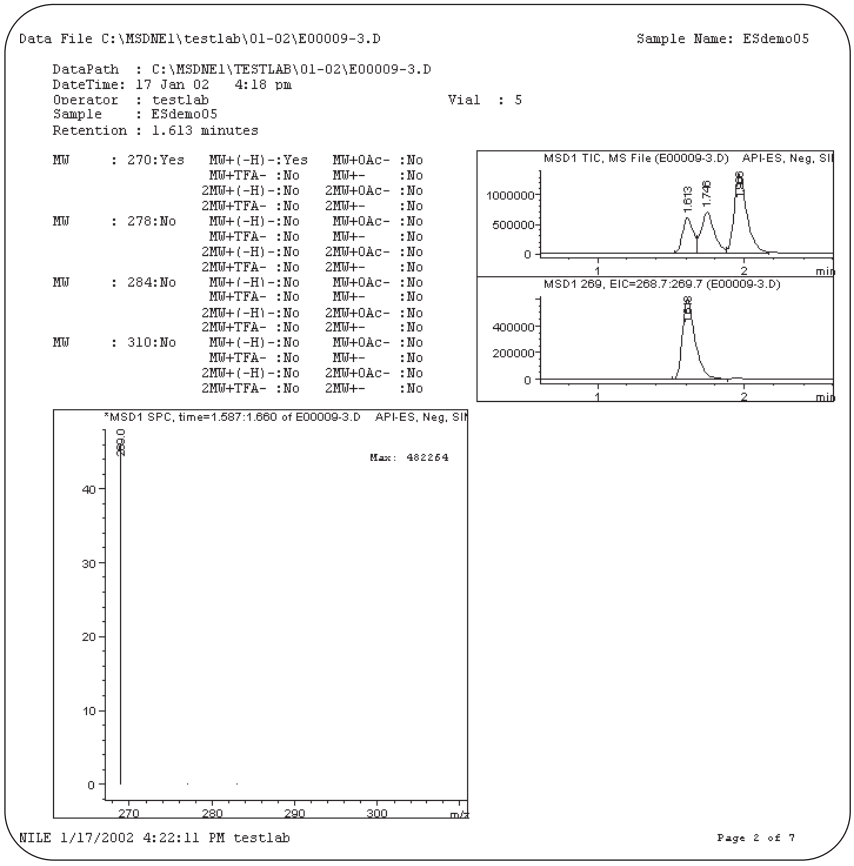


Figure 3
One page of a multipage report confirming the presence of a target mass at *m/z* 270.

```
Your sample run has been
completed.
Sample: ESdemo05'
Info: Sulfa Drugs'
Target masses: 270 278
284 310
Method: Loop2
Info: Loop SIM ES, multi
signal
Data file: E00009-3.D
==========================
A message generated by
the Agilent Easy-Access
system
```

Conclusions

LC/MS Easy Access is a very effective tool, helping synthetic organic chemists be more productive by confirming the identity of their compounds in a shorter period of time while requiring less analytical expertise.

"Increasing Productivity with LC/MS Easy Access Software", publication number 5988-5525EN.

Recycle chromatography

The separation of stereoisomers, diastereomers and especially enantiomeres is an important but challenging task in compound purification in the pharmaceutical industry. Very often only partial resolution of the compounds can be achieved. The usage of longer columns to get better resolution is usually restricted by the higher backpressure those columns generate. Therefore several recycling techniques were developed, to send the compounds over the same column several times until sufficient resolution is achieved. In this Application Note we demonstrate how recycle chromatography can be performed on the Agilent 1100 Series purification system.

Results and discussion

Operating principle

The operating principle of the recycle chromatography technique is to move the columns in the direction of the flow of the mobile phase (figure 1). After injecting the compounds, they elute from column 1 and are retained on column 2. While the compounds are further separated on column 2, column 1 is moved behind column 2 using a switching valve. After the compounds have eluted from column 2 and are retained on column 1, column 2 is moved behind column 1 and so on.

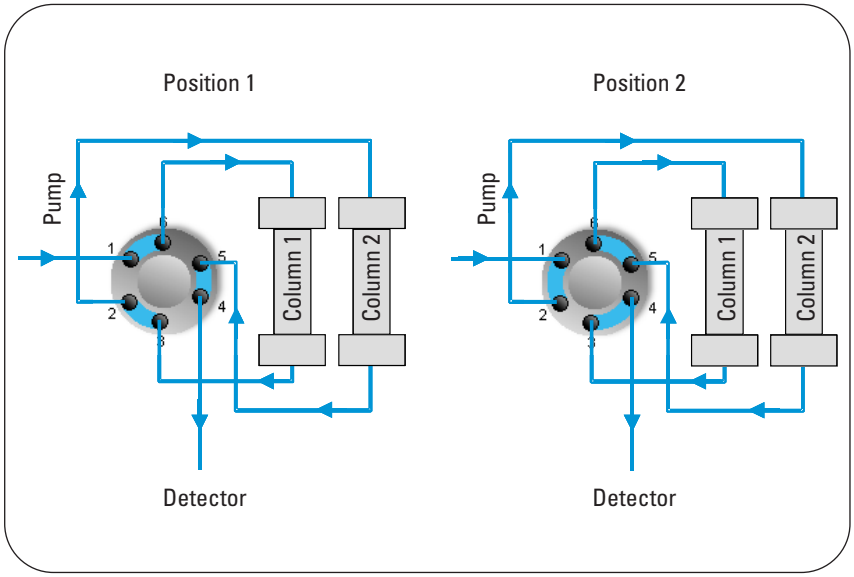


Figure 1
Operating principle of recycle chromatography.

Chromatographic results

The chromatographic results for an increasing number of virtual columns are shown in figure 2. As you can see the compounds are hardly separated using a single column, however baseline separation could be achieved using six virtual columns and four valve switches.

Limitations

- Recycle chromatography can only be used for the separation of compounds with similar retention times, for example enantiomeres, diastereomeres and stereoisomeres in isocratic runs. It is not suitable for gradient runs.
- The number of cycles is limited by the dwell time of the two compounds on one column.

Purification experiment

Fraction collection using the recycle chromatography technique with six virtual columns resulted in two fractions of 100 % purity as shown in figure 3.

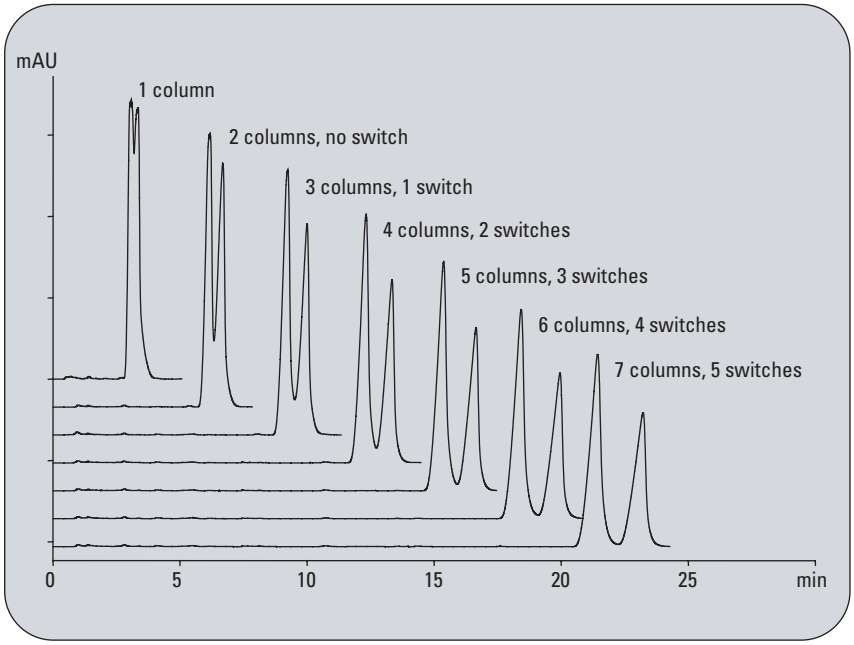


Figure 2
Resolution for various numbers of virtual columns.

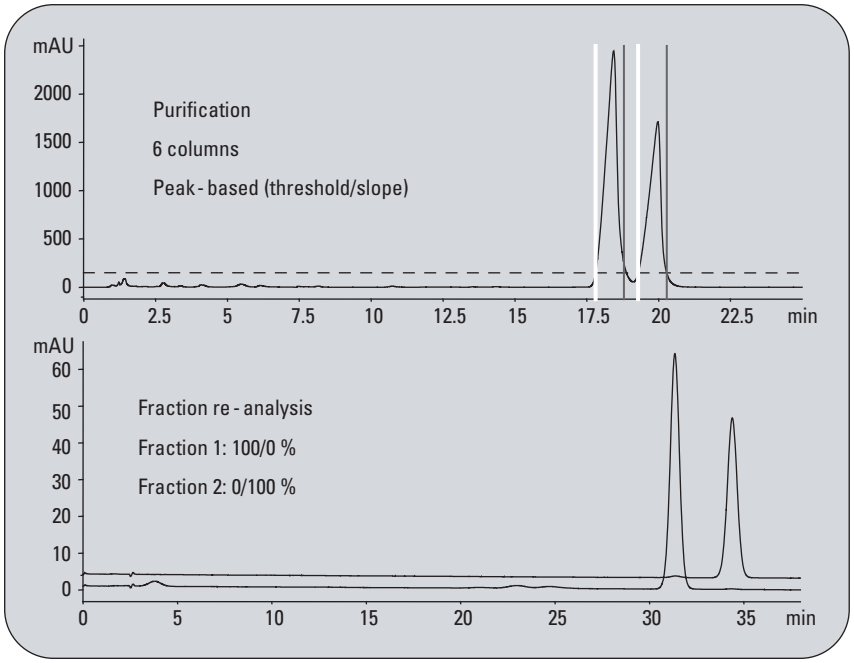


Figure 3
Peak-based fraction collection using recycle chromatography with six virtual columns.

Conclusions

In this Application Note we demonstrated how recycle chromatography can be performed on the Agilent 1100 Series purification system using a 2-position/6-port valve for the separation of anantiomeres, stereoisomeres and diastereomeres.

"Recycle Chromatography with the Agilent 1100 Series Purification System", publication number 5989-1693EN.

Alternating column regeneration in preparative HPLC

Alternating column regeneration in preparative scale HPLC can be performed using a 2-position/10-port valve.

The configuration of the valve, the capillary connections and the general method setup for the pumps are identical for both internal and external valves. Subsequently only the differences in the software are described.

Results and discussion

Valve set-up

The external 2-position/10-port valve is controlled from the *Setup Valve* window shown in figure 1. For automated column regeneration the box *Next position after run* must be checked. It sets *Position* automatically to *Use current*. This setting switches the valve automatically to the next column after a run is finished and this position is kept when the run starts. Further details, for example, configuration of the capillary connections can be found by selecting *Help*.

Sequential runs

Without column regeneration the purification run steps “draw & inject”, “gradient run”, “column wash” and “column equilibrate” are performed sequentially as shown in figure 2. This leads to an overall cycle time of 13 minutes for the method used in this Application Note.

Alternating column regeneration with isocratic regeneration pump

If an isocratic pump is used for alternating column regeneration the step “column equilibrate” can be done while the next purification run is already being performed on the second column (figure 3). To ensure that the volume between the pump and the valve is filled with mobile phase of the gradient starting composition, an additional rinse time has to be added after the column wash. The duration of this rinse time depends on the system setup, for example, on the delay volume of the autosampler. In the example in figure 3 the cycle time could be reduced to 10.5 minutes.

The following isocratic pumps can be used for column regeneration in a purification system:

- Agilent 1100 Series isocratic pump (max. flow rate 10 mL/min at 200 bar), and
- Agilent 1100 Series preparative pump (maximum flow rate 100 mL/min at 400 bar).

Alternating column regeneration with gradient regeneration pump

When using a gradient pump as regeneration pump the steps “column wash” and “column equilibrate” can be done while the next purification run is already being performed on the second column (figure 4). Then the rinse time has to be added directly after the gradient is finished.
The following gradient pumps can be used for column regeneration in a purification system:

- Agilent 1100 Series isocratic pump (max. flow rate 10 mL/min at 200 bar) with Agilent 1100 Series 12-position/13-port solvent selection valve, and
- Agilent 1100 Series quaternary pump (max. flow rate 10 mL/min at 200 bar).

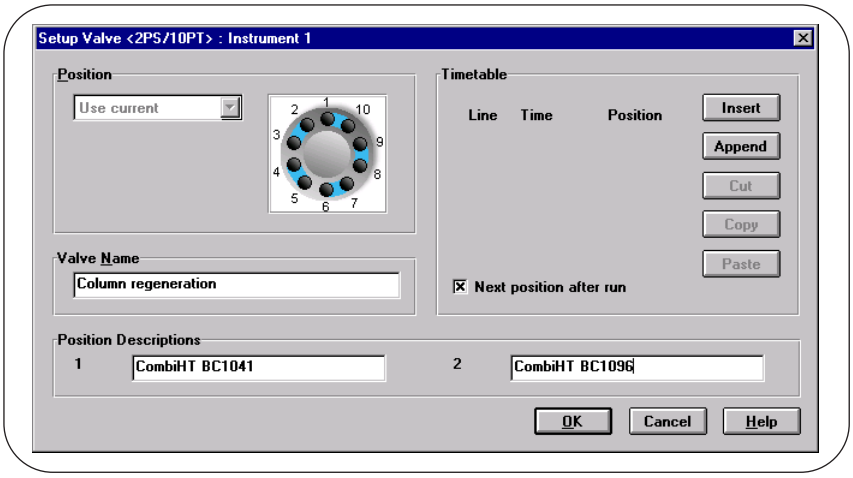


Figure 1
***Setup Valve* window.**

Alternating column regeneration with gradient regeneration pump and overlapped injection

With the overlapped injection feature of the Agilent 1100 Series preparative autosampler it is possible to draw the next sample while the previous purification run is still being performed. Especially in preparative HPLC where high sample volumes, possibly requiring multi-draw, have to be injected this can lead to an immense time saving (figure 5).

Conclusion

High-throughput purification runs can be performed using alternating column regeneration with an external Agilent 1100 Series 2-position/10-port valve. Depending on the type of regeneration pump either column wash, or column wash and column equilibrate can be done while the next purification run is already being performed on the second column. Furthermore, the possibility of overlapped injection with the autosampler reduces the cycle time, especially for preparative HPCL, where high injection volumes and therefore long injection times are common.

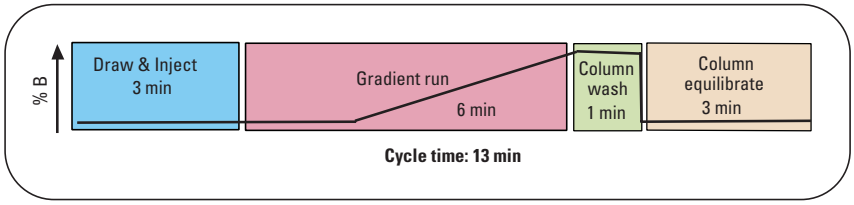


Figure 2
Sequential runs.

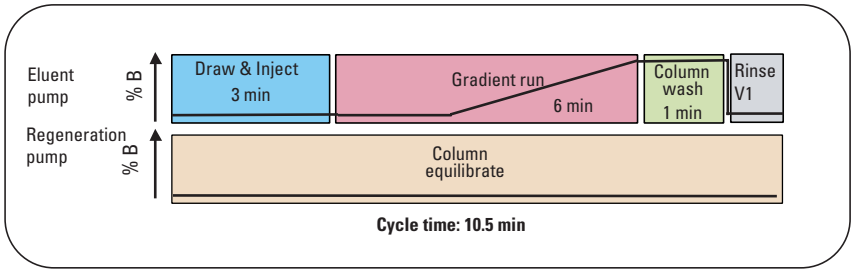


Figure 3
Alternating column regeneration with isocratic regeneration pump.

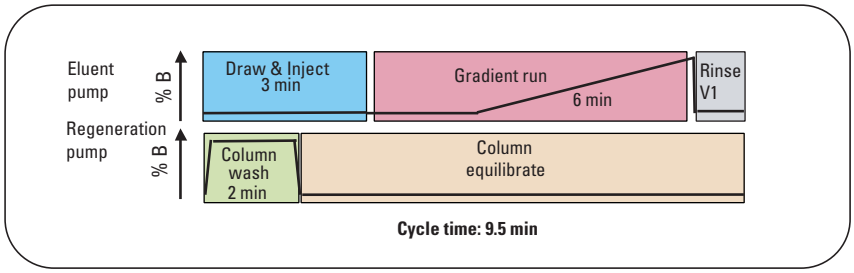


Figure 4
Alternating column regeneration with gradient regeneration pump

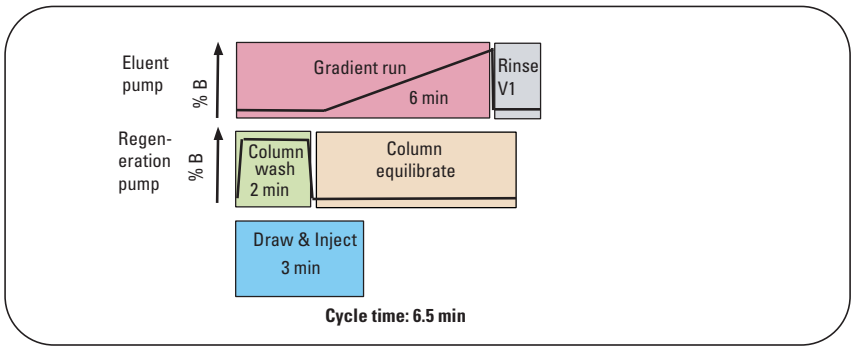


Figure 5
Alternating column regeneration with gradient regeneration pump and overlapped injection.

"Preparative High Throughput HPLC – Alternating Column Regeneration with the Agilent 1100 Series Valve Solutions", publication number 5988-8085EN.

Two application examples explain the use of the Agilent 1100 Series 12-position/13-port valve in a purification system. First, it is used for recovery collection where the remainder of a sample that is not collected as a fraction is collected in a dedicated container. If something goes wrong during the purification run, e.g. due to incorrect parameter settings, the sample does not go to waste but to a location where it can be recovered easily. Second, the valve can be used in a simple purification system for time-based fraction collection.

Results and discussion

Recovery collection

The ChemStation software offers the capability of recovery collection. This means that everything that is not collected as a fraction goes into a dedicated container where it can be easily recovered (figure 1). For recovery collection it is necessary to use the Agilent 1100 Series fraction collector AS with the funnel tray (G1364-84502). The needle of the 1100 Series fraction collector PS is too short to use the funnels.

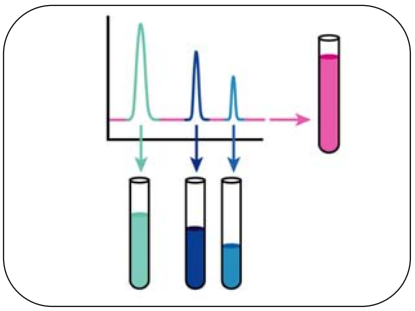


Figure 1
Recovery collection.

Recovery collection and time-based fraction collection

Recovery collection can also be done by setting up the 12-position/13-port valve into the waste tubing of the fraction collector for both fraction collectors AS or PS.

For recovery collection the *Next position after run* box must be checked. This automatically sets *Position* to *Use current*. With this setting the valve is automatically switched to the next recovery location after a run is finished and this position is kept when the next run starts. This means the recovery for sample one is collected into position one, for sample two into position two and so on. A prerequisite is that the valve is manually switched to position one before the first run is started. With the 12-position/13-port valve the recovery of up to twelve samples can be collected. If more recovery locations are needed, more 12-position/13-port valves could be attached to the outlets of the first valve. The only restriction is the maximum number of valves that can be attached to the system and the more complex method setup for each of the connected valves. Because the valve switches to the next position after the run is finished and before the post run is started, it is recommended to include the post run time, for example, for column equilibration into the run time. Therefore, the mobile phase coming from the column during column wash and equilibration is still collected into the recovery location of the sample to which it belongs and not yet into the recovery location of the next sample. Example methods are shown in tables 1a and 1b.

Time-based fraction collection

The Agilent 1100 Series 12-position/13-port valve can also be used as a simple fraction collector, connected to the detector instead of the Agilent 1100 Series fraction collector. Fractions can only be collected in time slices by switching the valve using the *Timetable* (figure 3). The last position is used as waste position to collect the mobile phase when no analysis is running. Since a fixed position is used as starting position in the method, the valve will switch back to this position after the run is finished. Therefore, all mobile phase coming from the column during equilibration, injection cycle, etc., will go to the waste position and will not dilute or contaminate any fraction. When using a method with these valve settings in a sequence it is possible to do pooling, even with injections from different sample vials. Time-based fraction collection with an Agilent 1100 Series 12-position/13-port valve works only with the ChemStation software — the Purification/HighThroughput software is neither required nor supported.

Conclusion

The 1100 Series 12-position/13-port valve together with the 1100 Series purification system can be used for:

- recovery collection when connected to the waste line of the fraction collector.
- time-based fraction collection in a simple purification system instead of a fraction collector.

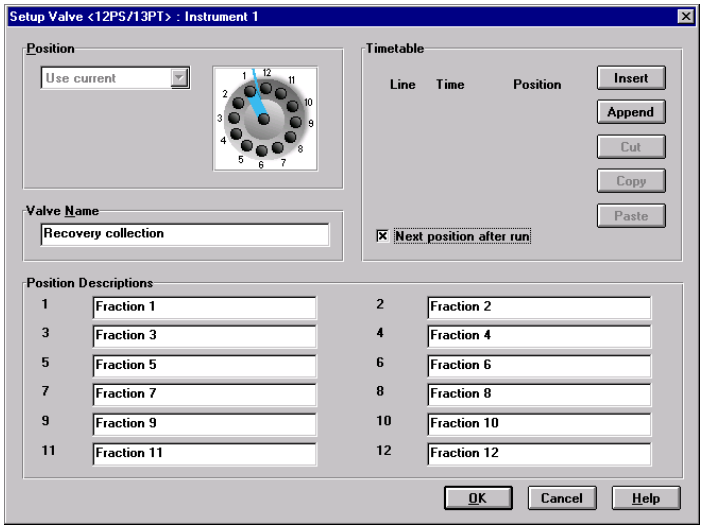


Figure 2
Setup Valve window.

With post time	
Gradient:	at 0 min 10 % B at 9 min 90 % B at 10 min 90 % B
Stop time:	10 min
Post time:	3 min

Table 1a
Gradient run with post time for equilibration

Without post time	
Gradient:	at 0 min 10 % B at 9 min 90 % B at 10 min 90 % B at 10.1 min 10 % B at 13 min 10 % B
Stop time:	13 min
Post time:	off

Table 1b
Equilibration included in run time

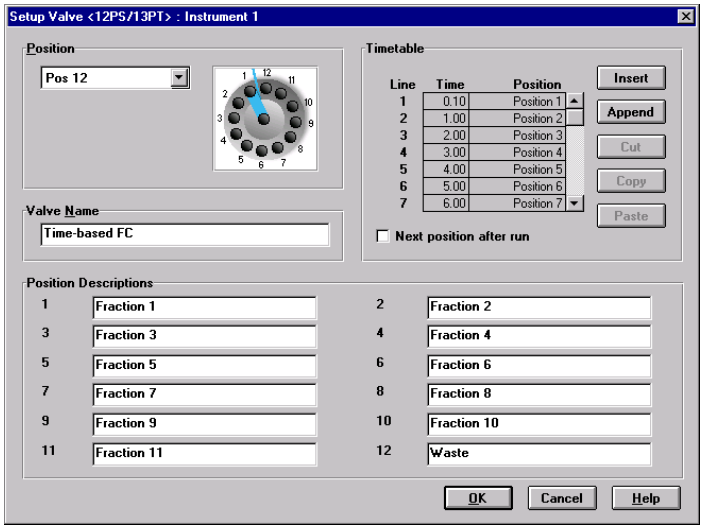


Figure 3
Time-based fraction collection with the 12-position/13-port valve.

"Recovery Collection and Time-based Fraction Collection – Preparative HPLC with the Agilent 1100 Series Valve Solutions", publication number 5988-8225EN.

Notes

Notes

www.agilent.com/purification

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