Optimizing mass-based fraction collection for highest purity using the *Boolean* logical -AND- combination with the UV signal

Application Note

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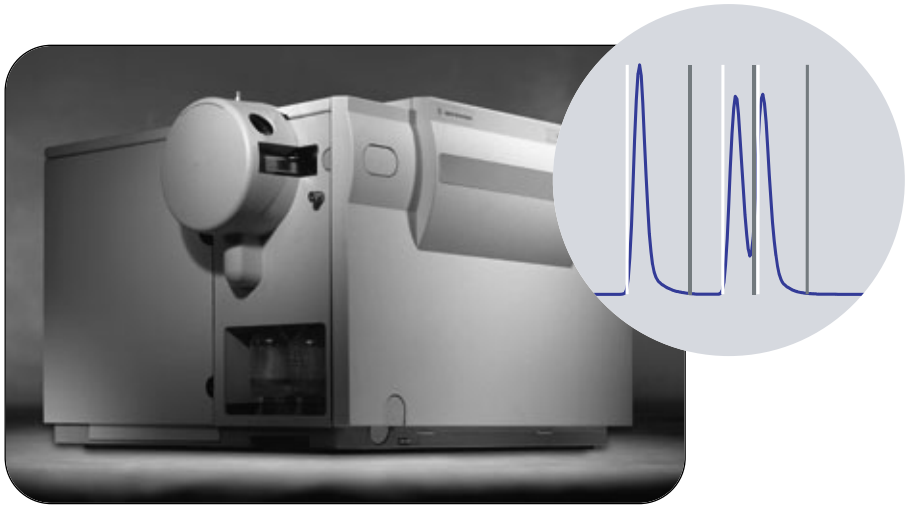

Abstract

Mass-based fraction collection is the method of choice for fraction triggering in a high-throughput purification environment. Its high selectivity assures that only fractions containing the target mass are collected, which saves valuable fraction collector space and allows the purification of more samples before manual interference by the system operator is required. On the other hand, the set up of a mass-based purification system is more complex and requires a flow splitter because of the destructive detection mechanism of the mass-selective detector (MSD). In this Application Note different fraction triggering techniques using an MSD are described and compared to the fraction purity and sample recovery results of peak-based fraction collection using a UV detector. The advantages and disadvantages of the different techniques are explained and the optimal technique, the combination of UV and mass-based fraction collection using a logical -AND- combination, is described.

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Introduction

Purity, recovery and throughput are the important parameters in preparative HPLC[1]. Since it is not possible to optimize the chromatographic method regarding all three parameters the most important parameter for each application must be identified. In drug discovery the synthesized and purified compounds have to be submitted to activity screening. Therefore, purity is the most important parameter not only to ensure that the measured activity really originates from the target compound but also to be able to generate the right concentration of the sample submitted for screening. Fraction collection can be performed with modern purification systems[2] based on retention time windows, signals from a detector or on target masses using an MSD. As time-based fraction collection is often only used as a first fractionation step for complex samples, peak and mass-based fraction collection can be used to isolate single peaks, which results in pure compounds. 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.

Equipment

The experiments were performed on an Agilent 1100 Series purification system containing the following modules:

- Two Agilent 1100 Series preparative pumps
- Agilent 1100 Series dual-loop autosampler PS (1000-µL loop)
- Agilent 1100 Series column organizer
- Agilent 1100 Series multi wavelength detector (flow cell: 0.06-mm path length)
- Agilent 1100 Series fraction collectors PS
- Agilent 1100 Series MSD
- Agilent 1100 Series isocratic pump (make-up pump)
- Active splitter
- Agilent 1100 Series UIB

The system was controlled using the Agilent ChemStation (rev. B.01.01).

Column:	Agilent Prep C-18 21.2 x 50 mm, 5 µm
Mobile phases:	water = A acetonitrile = B
Gradient:	at 0 min 10 % B at 2 min 10 % B at 5 min 95 % B at 7 min 95 % B
Stop time:	7 min
Post time:	5 min
Flow:	25 mL/min
Injection:	500 µL, sandwiched with 2 x 50 µL DMSO
Column temp.:	ambient
UV detector:	DAD 220 nm/8 (ref. off) prep. flow cell (0.06 mm path length)

Results and discussion

Peak-based fraction collection

The configuration and set up of a system for peak-based fraction collection on the signal from a UV detector is easy. The flow from the preparative column can be directed through the detector to the fraction collector without using a flow splitter as the UV detector is a non-destructive detector and flow cells for higher flow rates are available. Capillary connections can be kept short, which signifies that peak broadening due to dispersion is minimal. Therefore, the peak shape after the column, in the detector and at the fraction collector is almost identical. In figure 1 peak-based fraction collection on the signal from a UV detector is shown, triggering on slope only (up- and down slope 5 mAU/s, between 4 and 6 minutes). The sample consisted of three compounds: nifedipin,

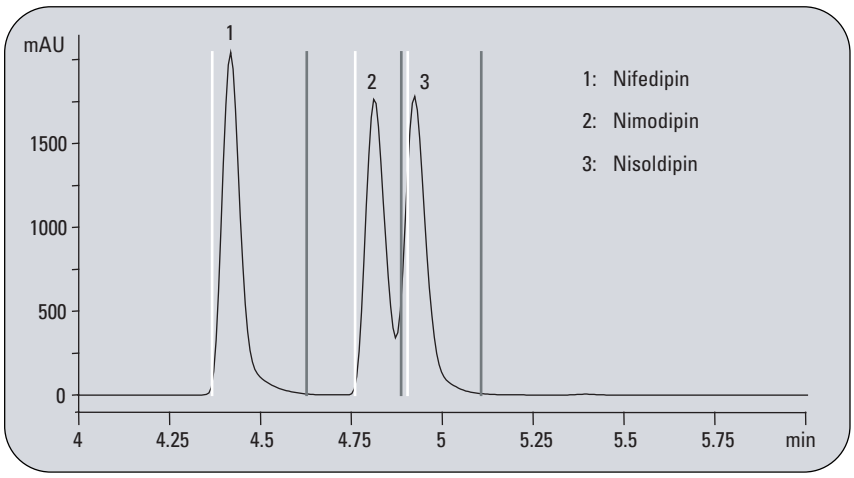

Figure 1
Result of peak-based fraction collection triggered on slope only

nimodipin and nisoldipin with a concentration of about 20 mg each in 1 mL DMSO. Re-analysis of fractions 2 and 3 (figures 2A and 2B) showed that fraction 2 contained 9.64 mg of nimodipin, which equals 91.4 % recovery with a purity of 96.8 %. Fraction 3 contained another 0.41 mg of nimodipin, which results in an overall recovery of 95.3 %. Peak-based fraction collection almost always leads to fractions as pure as the chromatography allows. The problem is that all compounds in a run are collected if their signal meets the triggering criteria. A more selective fraction collection trigger technique is desirable as the space in the fraction collector is always a limiting factor regardless of the fraction collector size.

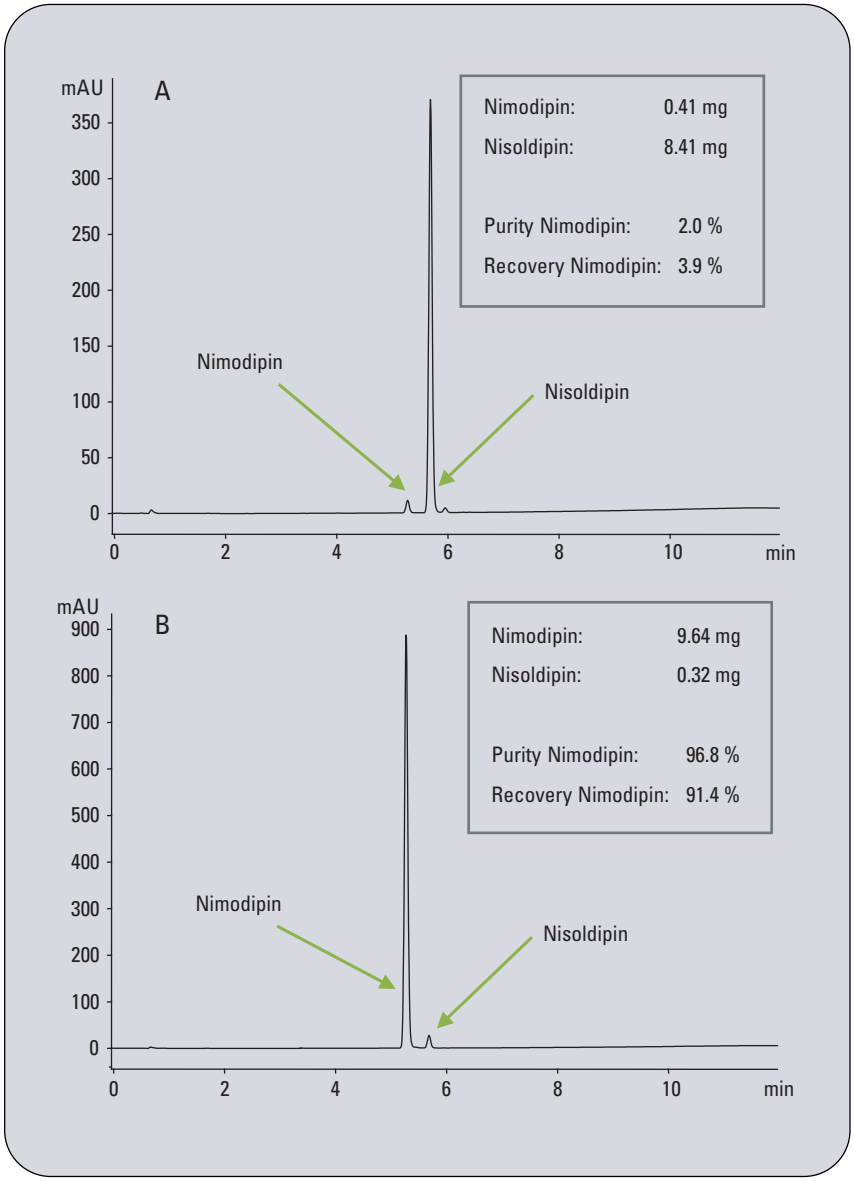

Figure 2
A) Re-analysis of fraction 2

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. A passive flow splitter, where the split is generated using a T-piece and tubing with different lengths and diameters, usually adds a lot of peak broadening to the system due to dispersion. Even the Agilent active splitter, where the split is generated by a rapidly switching valve system, leads to some peak broadening. 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. The result of mass-based fraction collection on the target mass of nimodipin (418 plus the expected adduct $[M+Na]^+$

results in a trigger mass of 441) is shown in figure 3. Faction collection was based on a threshold of 100000 counts.

Re-analysis (figure 4) showed that the collected fraction contained 10.08 mg of nimodipin, which is equivalent to 95.5 % recovery with a purity of only 87%.

Although fraction collection based on the mass of the target compound leads to good recovery results, the purity of the fraction can be low if there is a closely eluting impurity in the sample. The reason is the broad peak shape in the MSD signal due to the splitter and the overloading of the MSD as previously described.

Mass-based fraction collection on more than one mass

A better purification result with respect to fraction purity can be achieved by using not only the mass of the target compound but also the mass of the closely eluting impurity as trigger masses. If two target masses are entered into the ChemStation software, a new fraction is started as soon as a different trigger ion (the sum of the target mass plus the specified adduct) becomes the dominant ion. The result of the fraction collection on the target masses of nimodipin (418 plus the expected adduct $[M+Na]^+$ results in a trigger ion of 441) and the closely eluting nisoldipin (388 plus the expected adduct $[M+Na]^+$ results in a trigger ion of 411) is shown in figure 5. Fraction collection was based on a threshold of 100000 counts for each mass.

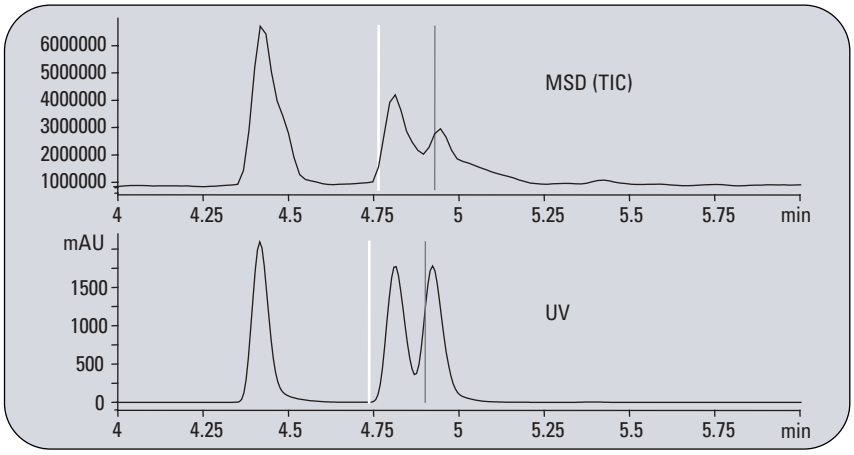

Figure 3
Result of mass-based fraction collection.

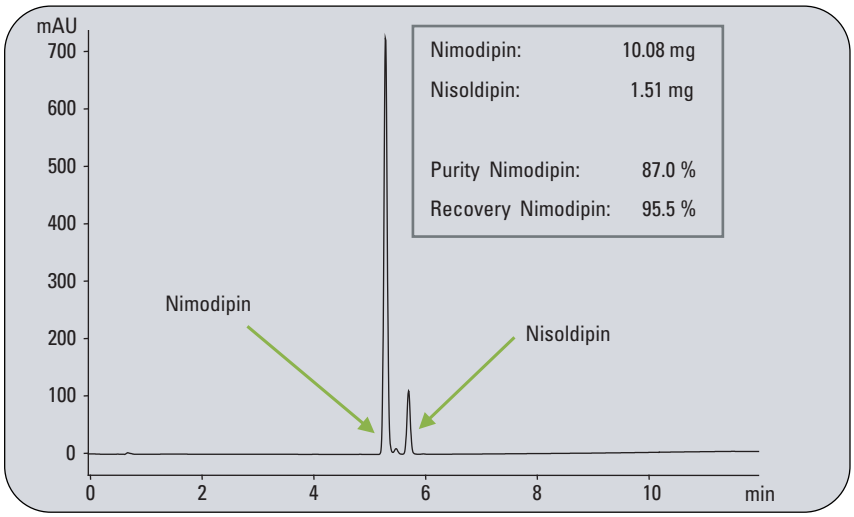

Figure 4
Re-analysis of the fraction using mass-based fraction collection.

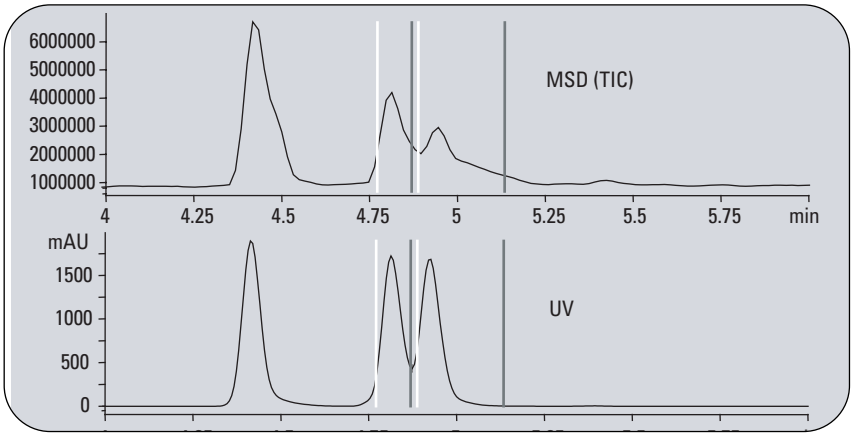

Figure 5
Result of mass-based fraction collection on two target masses

Re-analysis of fractions 1 and 2 (figures 6A and 6B) showed that fraction 1 contained 9.46 mg of nimodipin, which is equivalent to 89.7 % recovery with a purity of 91.5 %. Fraction 2 contained another 0.37 mg of nimodipin, which results in an overall recovery of 93.2 %. Fraction collection based on the target mass and the mass of the closely eluting impurity offers better fraction collection results regarding purity compared to mass-based fraction collection on the target mass only. The mass of the impurity is not know from the synthesis but can easily be extracted from the pre-preparative analytical run. Other purification system vendors offer software with the possibility of combining the mass of the target compound and the impurity with *Boolean* logic combinations. It would be possible, for example, to specify a collection of the target compound as long as the EIC of the trigger ion exceeds a specified threshold but only if the threshold of the EIC of the impurity is below a second specified threshold. As a result the second fraction in the chromatogram shown in figure 5 would not have been collected, which in turn would have lead to a lower recovery of only 89.7 %.

A problem with this approach is that isomers, which are quite often only poorly separated, cannot be collected in two fractions because they have the same target mass. Using the approach shown below with the logical combination of the UV and the MSD, this no longer present a problem as shown in a separate Application Note[3].

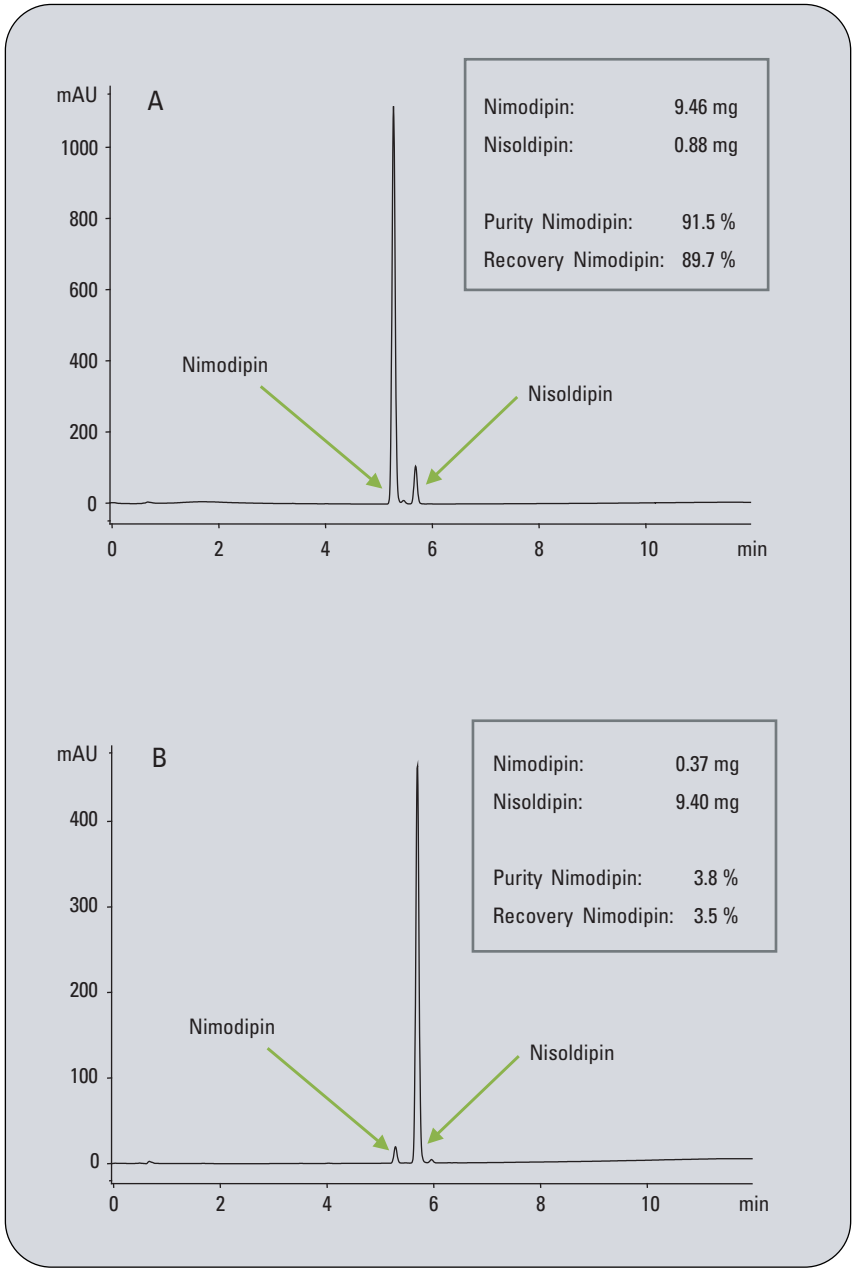

Figure 6
A) Re-analysis of fraction 1

Another problem can arise if the resolution between the target compound and the impurity from the analytical run cannot be achieved in the preparative run. As shown in figure 7 the amount of the collected fraction, and therefore the recovery, will decrease with decreasing resolution. Although the purity of the collected fraction is still sufficient for the screening assay at a certain point the recovery becomes too low. If the two peaks completely overlap, no fraction is collected and the valuable target compound goes to waste. In this case it would be better to collect the target compound together with the impurity and submit it into another purification run on a column with different selectivity, for example.

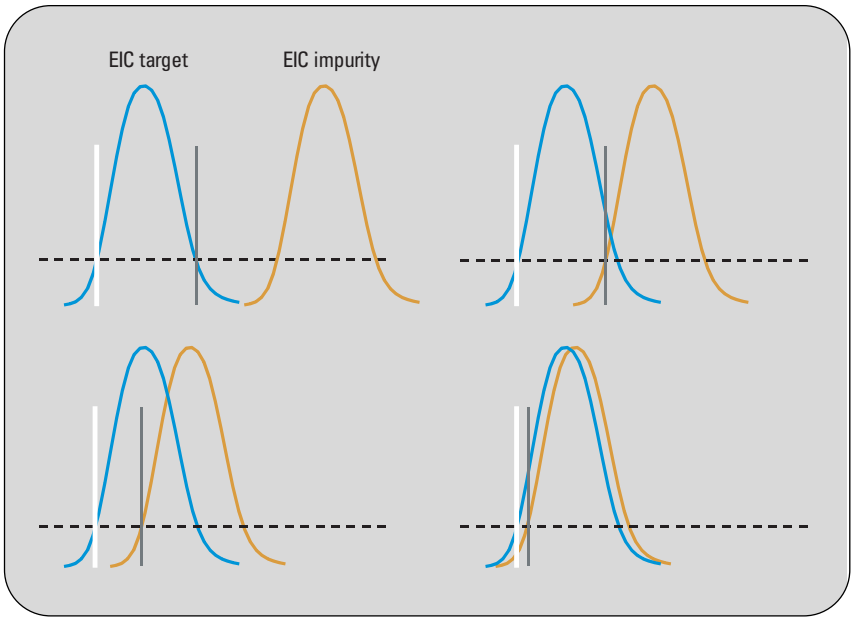

Figure 7
Decreasing recovery with decreasing resolution.

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 8. 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. In the experiment shown in figure 8 the MSD triggered fractions based on a threshold of 100000 counts for each mass, the UV detector triggered on slope only (5 mAU/s) to detect valleys between peaks.

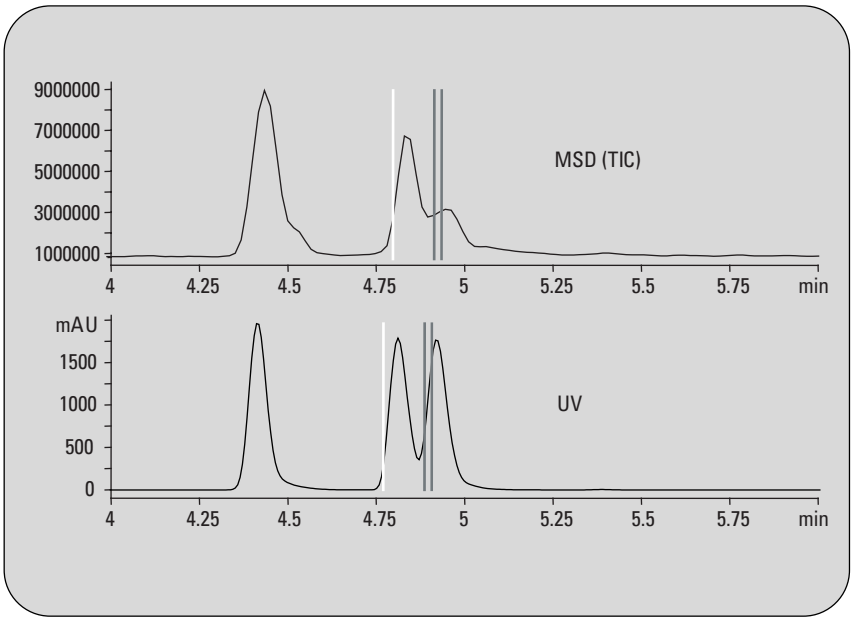

Figure 8
Result of fraction collection on the logical AND connection of UV and MSD.

Depending on the resolution of the two compounds one or two fractions are collected. If the second compound elutes closely to the first compound the UV detector triggers on the second peak while the mass of the first compound is still present in the MSD. 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 9A and 9B) 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 %. Fraction collection based on the logical-AND-combination of the UV and MSD signal gives the best results for purity and recovery. It combines the selectivity of mass-based fraction collection with the excellent performance of peak-based fraction collection on a signal showing the real peak shape without any peak broadening due to a flow splitter. Therefore it is important to place the UV detector into the flow path directly after the column and not after the splitter. This requires a flow splitter, which adds little backpressure to the system because the preparative 0.06 mm flow cell used in the experiments can only be used up to 20 bars.

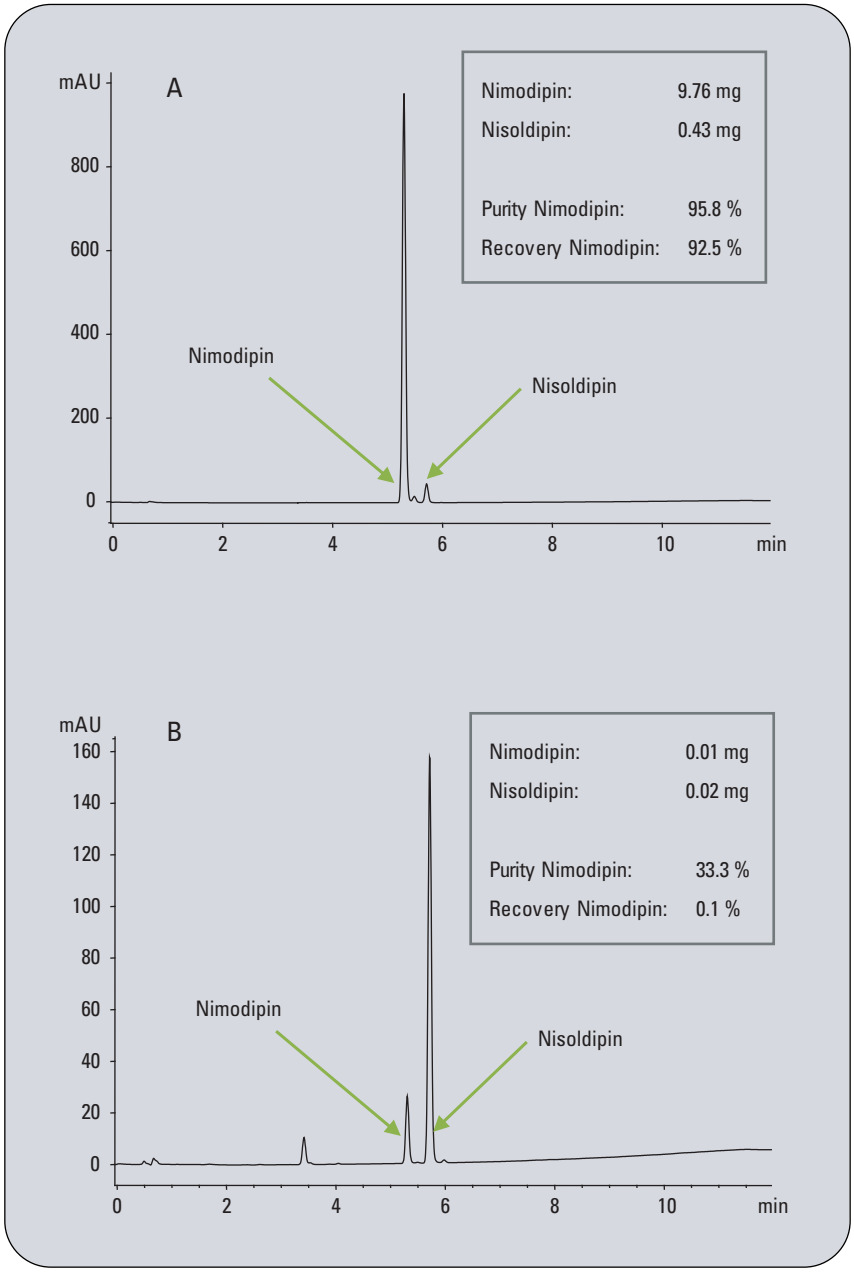

Figure 9
A) Re-analysis of fraction 1
B) Re-analysis of fraction 2

Since a passive flow splitter achieves the split with restrictive tubing it also generates too much backpressure for this configuration. However, this is not the case with the active splitter of the Agilent 1100 Series purification system. Fraction triggering on the logical AND combination of UV and MSD is much more generic than the combination of two masses. It is not necessary to estimate any parameters, such as the MS threshold for the impurity, from the analytical run, which could change in the preparative run. Also the separation of isomers that have the same target mass is not a problem. Further, exact co-elution of two compounds does not present a problem. The target compound would not go to waste but would be collected in a single fraction with the impurity as it elutes from the column.

Conclusions

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. The drawback of peak-based fraction collection is the collection of many unwanted fractions and the necessity to identify the fraction containing the target compound afterwards. Using an MSD adds selectivity to the system but decreases the purity of collected fractions due to peak broadening

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.

introduced by the flow splitter and the MSD itself. This disadvantage cannot be overcome by using two masses, the mass of the target compound and the mass of a closely eluting impurity, for fraction triggering. 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. The comparison of purity and recovery results for the different trigger modes are shown in figure 10A and B.

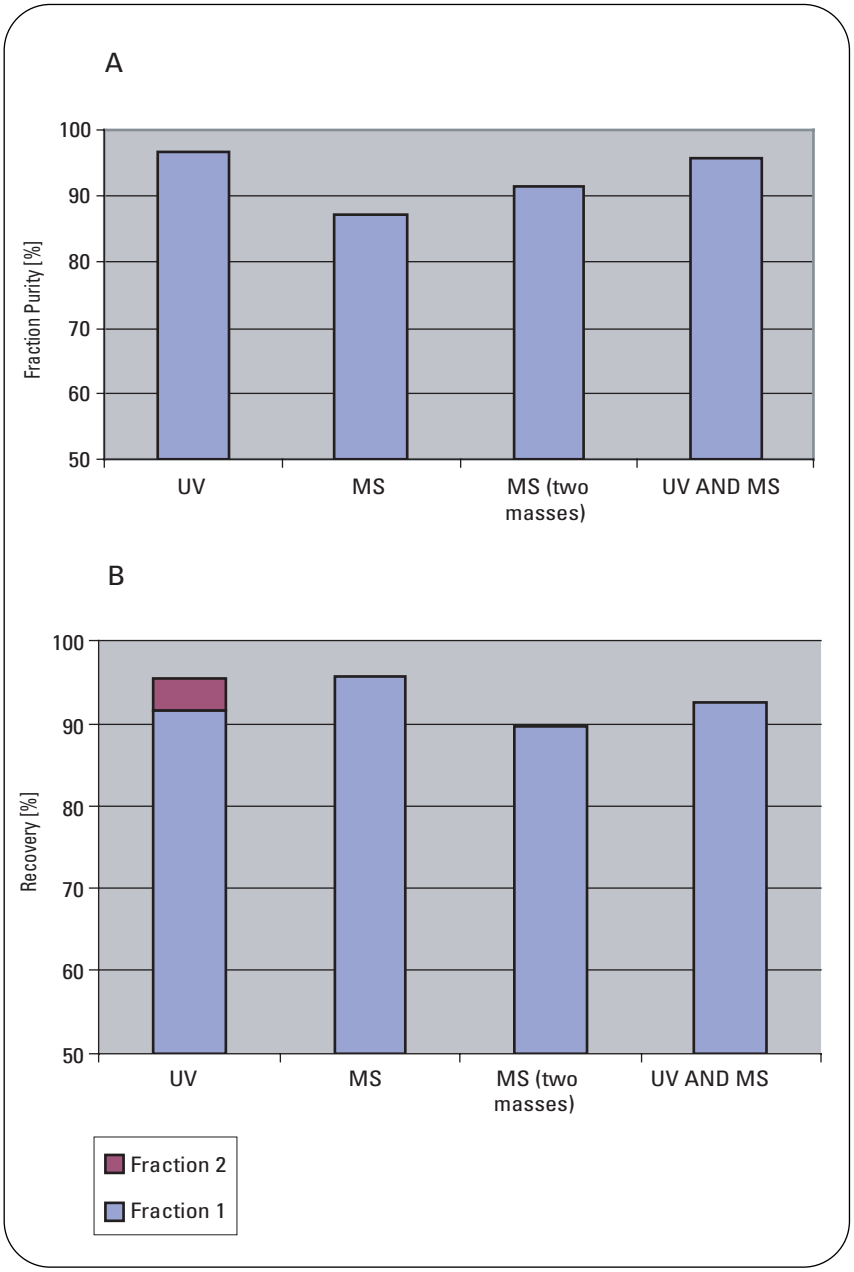

Figure 10
A) Comparison of purity results
B) Comparison of recovery results

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1.
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