Sophisticated peak-based fraction collection – working with up and down slope

Application

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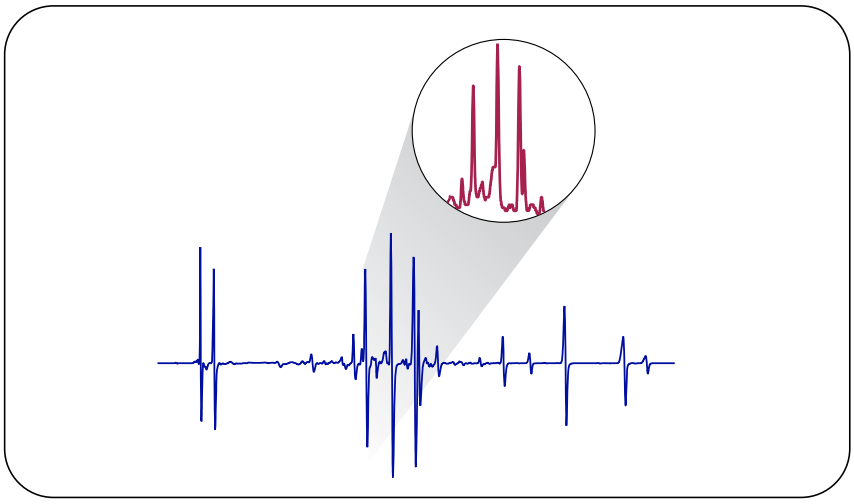

Abstract

With the Agilent 1100 Series purification system[1] fractionation of samples can be performed based on retention time windows, on peaks in a chromatographic signal, or on target masses. For many applications in drug discovery and development mass-based fraction collection is the method of choice, however, it can only be used if the mass of the target compound is known. If this is not the case, all compounds in a run have to be collected first and the desired substance has to be identified afterwards. Here peak-based fraction collection on a specific detector signal is the method of choice. In this Application Note we describe a more sophisticated approach to peak-based fraction collection by using the parameters up and down slope, upper threshold and maximum peak duration.

Agilent Technologies

Introduction

With the Agilent 1100 Series purification system it is possible to perform peak-based fraction collection using the signal from any Agilent UV detector, as well as from any Agilent non-UV detector[2] and any non-Agilent detector[3] with the Universal Interface Box (UIB). Regardless on which detector signal fractions are triggered the parameters threshold, up and down slope, upper threshold and maximum peak duration can be set to make the triggering decisions. In the fraction collection Working Mode *Threshold only* in the Agilent ChemStation a fraction is collected as soon as the signal rises above the defined threshold value, and collection is stopped when the signal falls back below the defined threshold value. For many applications peak-based fraction collection on threshold is sufficient, however for some applications it is necessary to use up and down slope, upper threshold and maximum peak duration to achieve the desired fraction collection performance.

In the first part of this Application Note we describe the parameters up and down slope, and how they are used to make triggering decisions. We also show the fraction preview tool of the Agilent ChemStation that helps to find the right parameter settings and allows the simulation of a fraction collection run. In the second part we show some applications and describe how the parameters up and down slope, upper threshold and maximum peak duration were applied for these applications. Throughout the Application Note we also provide tips and recommendations on how to use and set up proper values for the parameters influencing fraction collection.

Equipment

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

- Agilent 1100 Series quaternary-pump with degasser,
- Agilent 1100 Series well-plate autosampler,
- Agilent 1100 Series thermostatted column compartment ,
- Agilent 1100 Series diode-array detector, and
- Agilent 1100 Series fraction collector.

The system was controlled using the Agilent ChemStation (rev. A.10.02).

1. Fraction triggering decisions

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.
Tip:
Peak start and stop can only be triggered using up and down slope between the baseline and the inflexion points because at the inflexion points the slope reaches its positive or negative maximum. Also, always keep in mind that the slope maximum of the peaks strongly depends on the peak intensities.

Fraction triggering decision when using up and down slope
Figure 2 shows how the up and down slope set-up values are used to decide whether a peak start or stop is triggered. To trigger a peak start the slope simply has to rise above the value entered for up slope. As soon as a peak start is found the system expects to find a peak stop. To find a peak stop the slope must first fall below the negative value of the down slope entered and as soon as it rises again above the negative value the peak stop is triggered. While fraction triggering on slope-only is possible it can also be combined with the threshold using the *Working Mode Threshold/Slope* in the Agilent ChemStation. Figure 3 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.

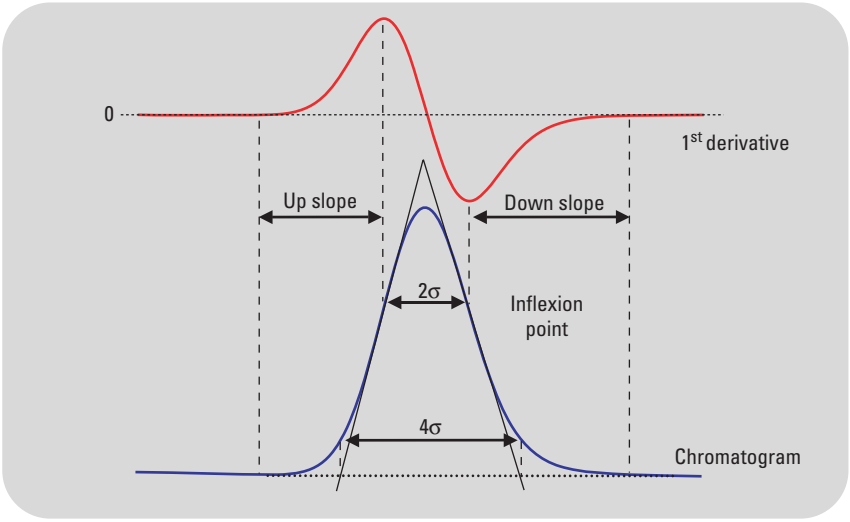

Figure 1
The slope is the first derivative of a peak

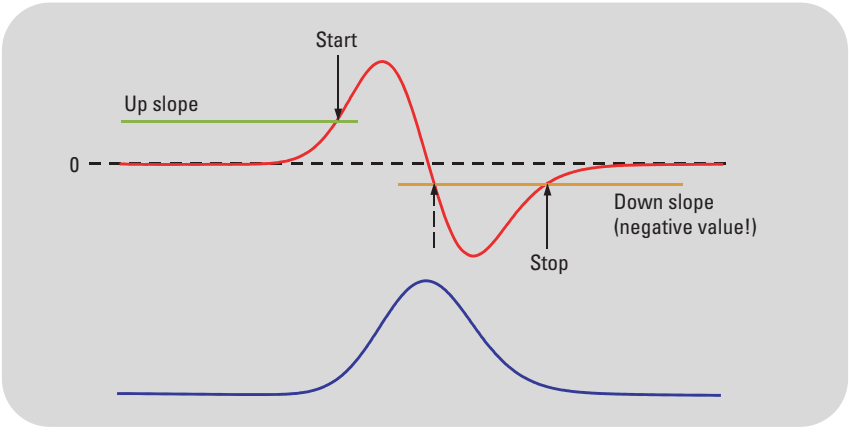

Figure 2
Using up and down slope values for fraction triggering

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.

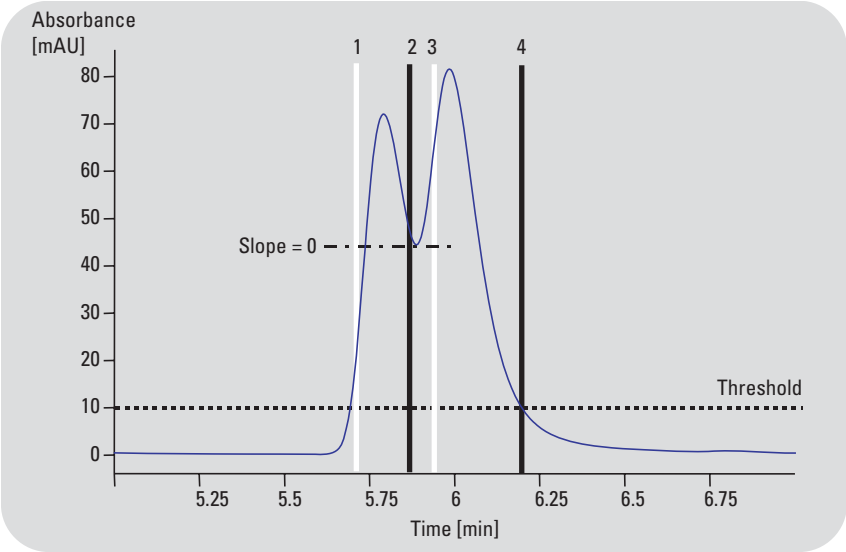

Figure 3
Fraction triggering decisions

Upper threshold

In preparative HPLC, where highly concentrated samples are usually injected onto the columns, the detector can easily be overloaded. When the peaks in the detector signal go into saturation the plateau at the top of the peaks looks rather flat on first sight but is quite noisy, due to electronic noise, when zoomed in (figure 4). When triggering on slope only, or on slope and threshold in combination the electronic noise at the peak apex can lead to the triggering of many unwanted fractions. To avoid this the parameter upper threshold can be set in the Agilent ChemStation. When the detector signal rises above the upper threshold, which must be set higher than the threshold, triggering decisions based on up and down slope are ignored. As soon as the signal falls back below the upper threshold the slope trigger is switched back on.

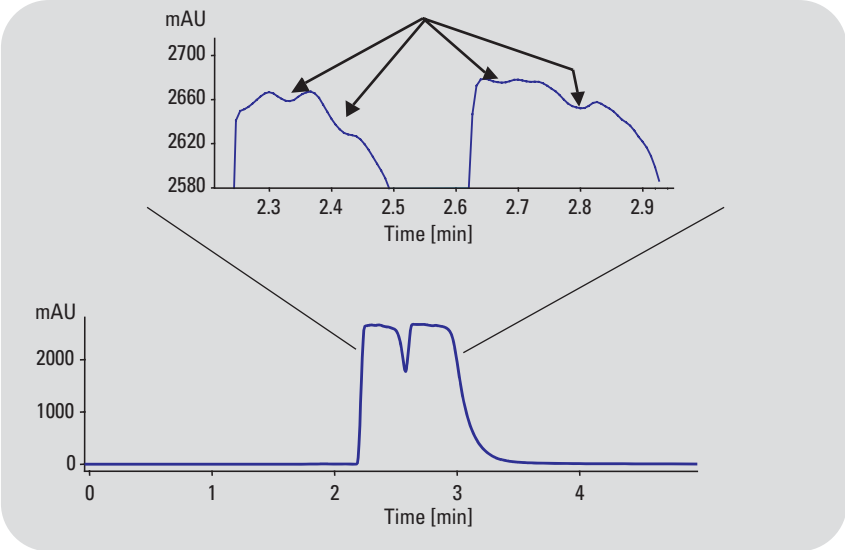

Figure 4
Electronic noise of a peak in detector saturation

Maximum peak duration

The parameter *Maximum Peak Duration* in the ChemStation defines the maximum length of a fraction. If a peak start but no peak stop criterion was found and the maximum peak duration is exceeded the fraction will be

terminated. Since the fraction triggering parameters are activated again immediately after the maximum peak duration was exceeded this feature should not be used when triggering on threshold only. If the end of the fraction could not be found when collecting on threshold only indicates, that the signal did not fall back below the specified threshold. If the maximum peak duration terminates the fraction but the signal is still above the threshold means, that the collection of the next fraction starts immediately. To avoid this it is recommended to set *Maximum Peak Duration* to a relatively high value for fraction collection on threshold only.

Tip:
When Threshold only was selected as Working Mode for fraction collection the Maximum Peak Duration should be set to a high value, for example the run time.

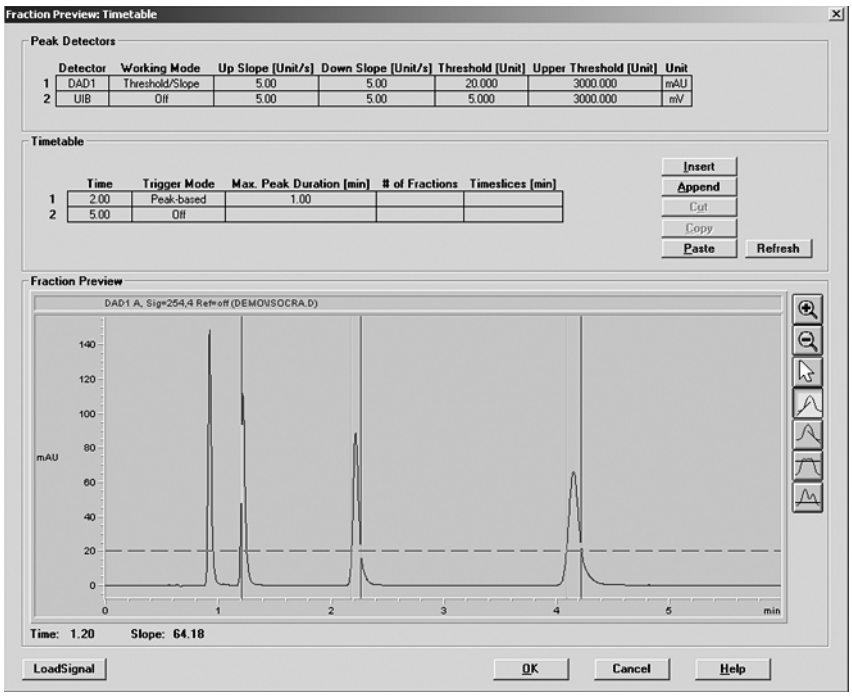

Figure 5
Fraction preview tool

Fraction preview tool

The fraction preview tool (figure 5) can be used to determine the optimal fraction collection parameters for repetitive purification runs and to simulate fraction collection using these parameters. After loading a signal the fraction collection parameters threshold, up and down slope, upper threshold and maximum peak duration can be applied to the signal and the resulting fraction collection is displayed. With the buttons on the right hand side of the screen, several cursors for up slope, down slope, upper threshold and threshold (from top to bottom) can be activated and moved through the chromatogram (black line at 1.20 min). When moving the cursor through the chromatogram the value of the selected parameter is also displayed at the bottom of the window. When the cursor is set to a specific point in the chromatogram a left-click with the mouse copies the value at this point into the parameter section of the peak detector. While the fraction preview tool can be used to optimize fraction collection parameter settings for repetitive purification runs it can also be used to get familiar with the fraction collection parameters threshold, up and down slope and upper threshold.

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 6 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. In many purification applications, especially in the drug discovery phase, this is not the case. Due to the large number of samples a generic method has to be set up. Optimum

settings would allow the desired peaks to be collected without too many unwanted fractions. How well this is done depends entirely on the experience of the user.
Tip:
There are no generic settings for up and down slope that can be applied for all fractionation problems. When doing repetitive purification runs of the same sample the fraction preview tool can be used to adjust slope settings.

Figure 7 shows the fractionation of a real-life sample. With different slope settings either only the biggest peaks (figure 7b) were collected or smaller peaks were collected as well (figure 7a). Since the peaks were not baseline-separated, fraction collection based on threshold only would not have been successful.
Tip:
Reliable fraction collection using up and down slope cannot be expected with slope settings less then approximately 10 mAU/s. At lower settings slight changes in the chromatography lead to results that are not reproducible. For chromatograms showing a higher level of noise even higher slope settings have to be used to achieve reproducible fraction collection

Very often peaks show tailing in chromatographic runs, especially if the column is overloaded as often done in preparative HPLC. In this case the slope value at the inflexion point at the peak front is higher than the negative slope value at the inflexion point at the

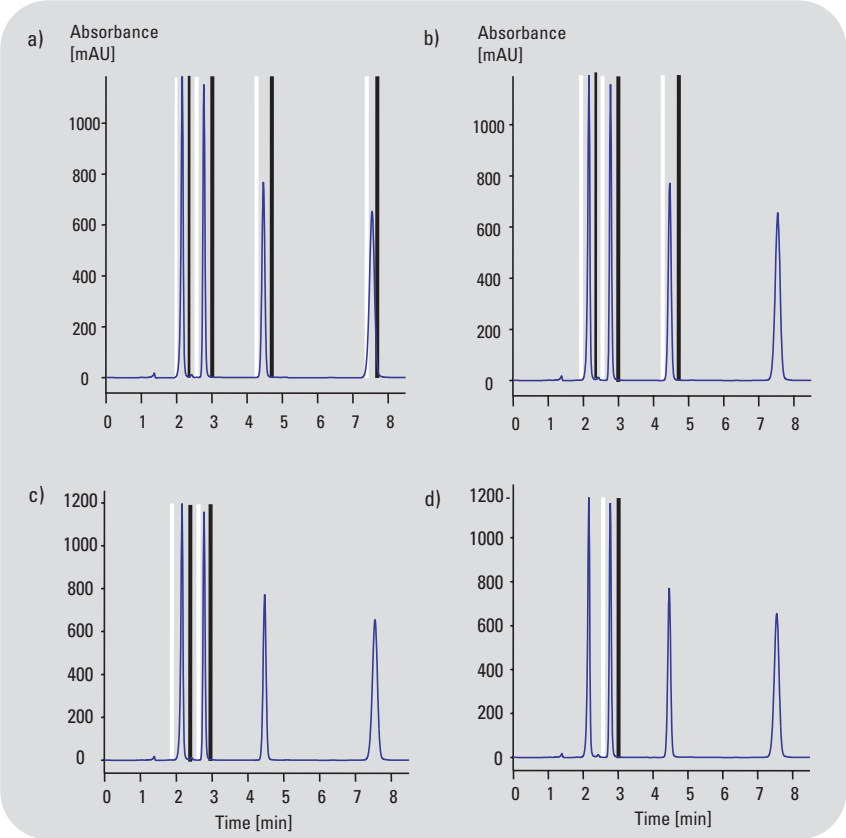

Figure 6
a) Up and down slope: 30 mAU/s, b) Up and down slope: 120 mAU/s
c) Up and down slope: 250 mAU/s, d) Up and down slope: 400 mAU/s

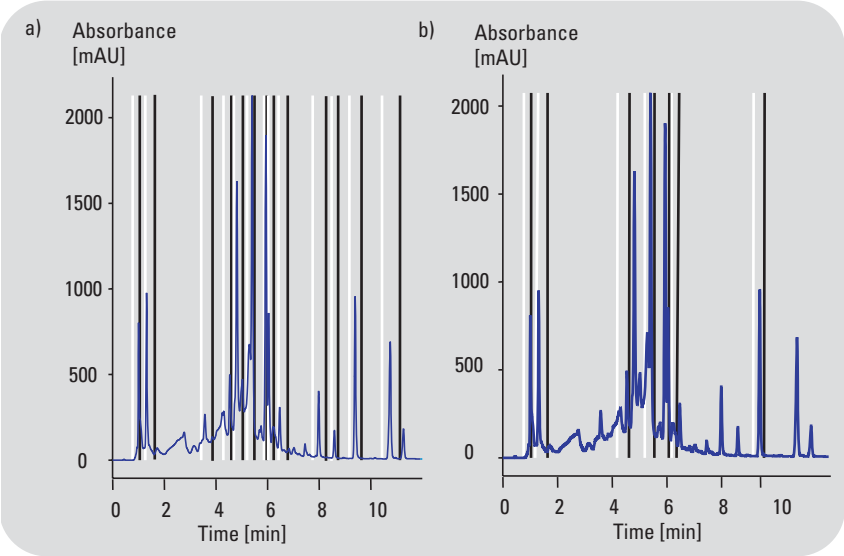

Figure 7
a) Up and down slope: 50 mAU/s, b) Up and down slope: 200 mAU/s

peak tail. This means that with the same values for up and down slope it can happen that a start criteria for a peak was found but no stop criterion (figure 8a). Using a lower value for down slope than for up slope led to a proper collection of the two peaks at about 6.5 and 8 minutes (figure 8b).

Tip:

The down slope value should be set to a lower value then the up slope value. Most peaks are tailing and with identical up and down slope values a peak stop may not be found.

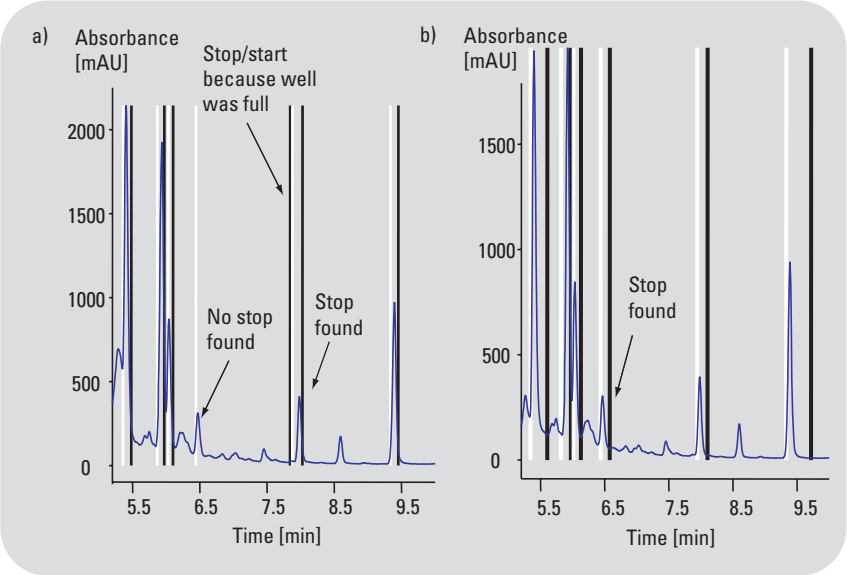

Figure 8
a) Up and down slope: 100 mAU/s, b) Up slope: 100 mAU/s, down slope 1 mAU/s

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[4]. One is peak-based fraction collection using up and down slope. Since the slope in the minimum between the peaks equals zero the peaks can be split into two fractions even if the signal does not fall below the threshold value by using a minimum down slope value of 1 mAU/s or even lower (figure 9). This means the peak stop criterion is found almost at the valley point and the start of the next fraction is found immediately afterwards. However, it takes some time for the fraction collection needle to move to the next position, which leads to the gap between the stop and start tick marks in figure 9. As a result fraction one contains some amount of the second compound while in the second fraction some

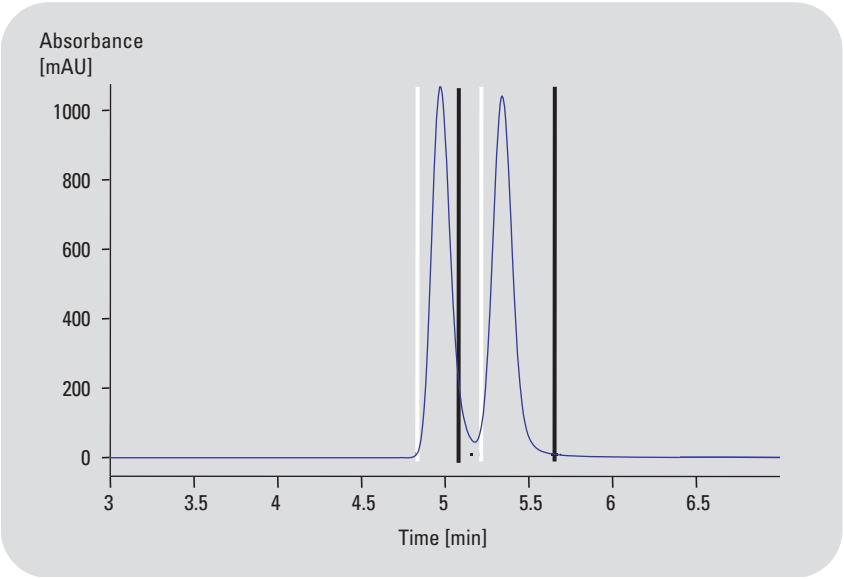

Figure 9
Up and down slope: 1 mAU/s,

of compound two was missed. This can be avoided by using higher settings for up and down slope.
Tip:
Basically two peaks can be separated by using the minimum slope setting of 0.01 mAU/s. (The gap between the start and stop tick marks is due to the movement of the fraction collection needle to the next position.) Higher slope settings, e.g. 10 mAU/s, give better fractionation because the part of the chromatogram where the peaks overlap is not collected in either of the two fractions.

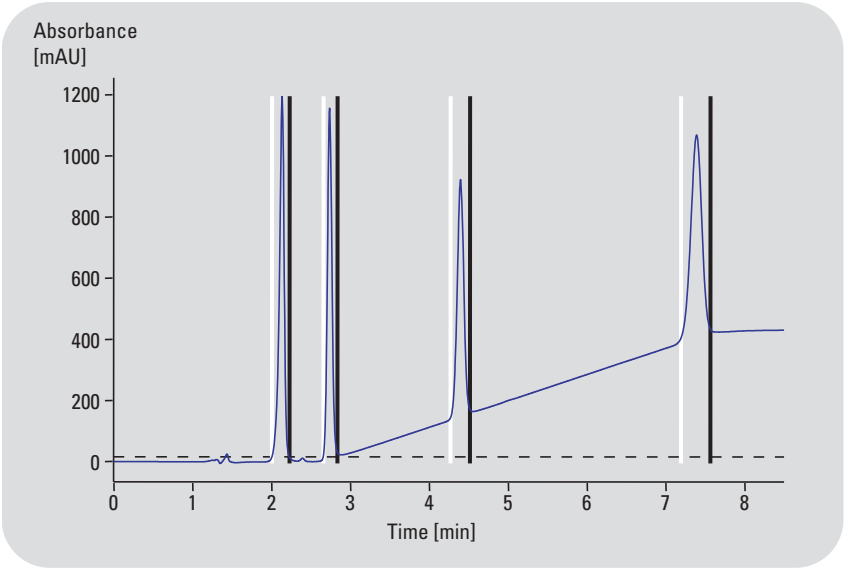

Figure 10
Threshold settings: 15 mAU, up and down slope: 15 mAU/s

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. The slope values of the drifting baseline can be measured in a blank run and calculated using the fraction preview tool described previously. Figure 10 shows the result of a peak-based fraction collection in a chromatogram with rising baseline.
Tip:
In a chromatogram with rising baseline the threshold is constantly exceeded. The peaks can only be separated from the baseline using proper slope settings. However, too much noise in the baseline can lead to unwanted triggering due to too many

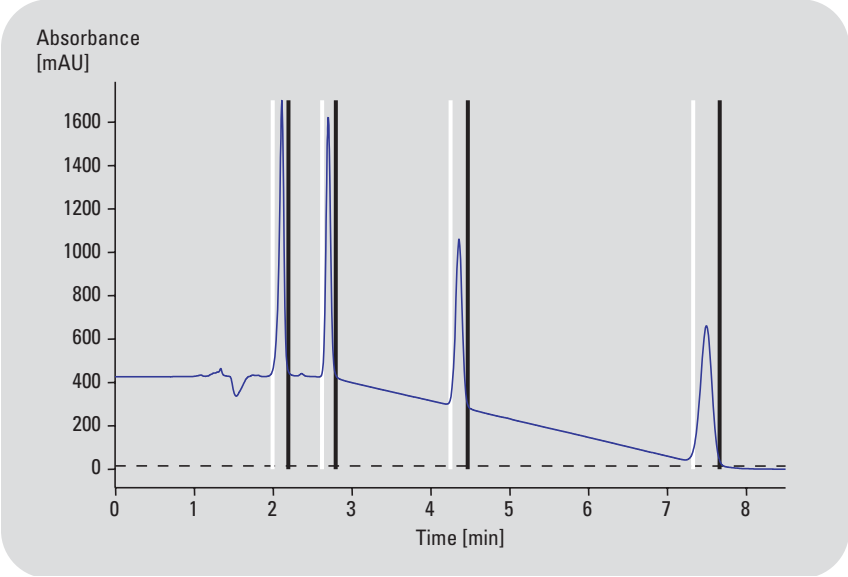

Figure 11
Threshold settings 15 mAU, up and down slope 15 mAU/s, detector balance during post-run

fractions. To avoid noise, the slope should not be set too low (> 10 mAU/s), the measurement wavelength should not be set too low (> 220 nm), an appropriate reference wavelength should be used, and the right compressibility settings for the solvents should be set up.

For a baseline that drifts downward, it is recommended to ensure that the signal does not fall below zero, by doing detector balance in the post-run instead of in the pre-run (figure 11). Also, setting up a reference wavelength often helps to reduce the baseline drift.
Tip:
Threshold cannot be set to negative values. To avoid that the baseline drifts to negative values the detector balance should be performed in the post-run.

Peaks in the detector saturation
In preparative HPLC highly concentrated samples are injected, which can lead to absorbances above the limit of the detector. The results are typical, box shaped peaks with a more or less flat peak apex. Due to electronic noise the plateau at the top of the peak is not completely flat but consists of several apexes and valleys. When triggering on slope each valley can trigger a peak stop and then, shortly after the valley another peak start. The fraction collection result for a chromatogram with high overloading of the detector old is shown in figure 12. Figure 13 shows the same purification run with setting the upper threshold to 2200 mAU. The two peaks are collected in two fractions, as expected.

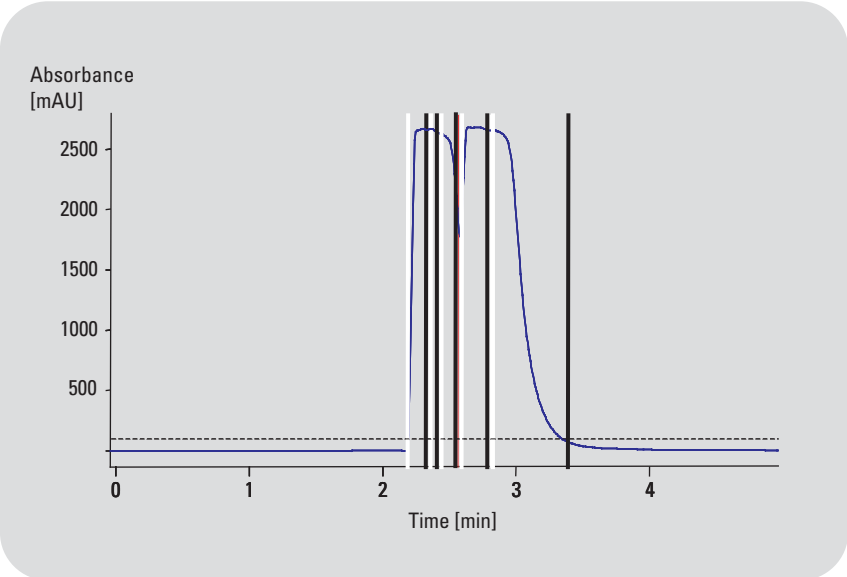

Figure 12
Multiple fractions for peaks in detector saturation

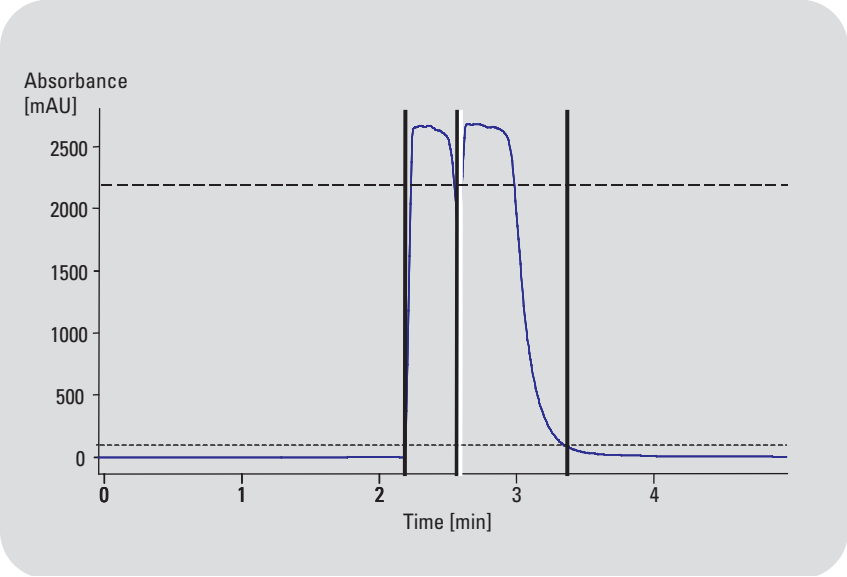

Figure 13
Usage of upper threshold

Tip:
To avoid the triggering of unwanted fractions when peaks go into the detector saturation the upper threshold can be set for every purification run. Setting it to 2200 mAU is a good value for the Agilent 1100 Series diode array or multiple wavelength detector.

Conclusion

In this Application Note we explained what up and down slope is and how it is used to trigger fractions in peak-based fraction collection. We also showed the advantages of being able to specify two parameters, up and down slope, 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. Examples are separation of steep and shallow peaks, separation of compounds showing non-baseline separated peaks and purification of compounds from chromatograms with drifting baseline. We also explained how the triggering of unwanted fractions for a peak in the detector saturation can be avoided using the upper threshold parameter. To simulate a fraction collection run or to get familiar with parameter settings for threshold, up and down slope, upper threshold and the maximum peak duration the fraction preview tool was described. For those and all other applications requiring additional peak detection criteria up and down slope offers the possibility of sophisticated peak-based fraction collection with the Agilent 1100 Series purification system.

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