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Internal Standard and Flow Correction with Agilent WinGPC Software

Introduction

In GPC/SEC, a constant flow rate during measurement is one of the most important prerequisites for reproducible chromatograms and accurate results. Fortunately, statistical fluctuations in flow rate do not greatly affect results: an approximately 1% statistical variation in flow rate causes around 1% deviant molar mass averages.[1] However, if the pump flow rate is constantly higher or lower than the setpoint, the effects are much more pronounced: an approximately 1% higher or lower flow rate results in around ±20% error in M_n and M_w.[1]

GPC/SEC pumps are designed to minimize flow rate fluctuations. However, environmental temperature can influence the density, and therefore, the transport characteristics of the eluent. For this reason, measurement conditions can differ depending on the time of day or season, even when all method parameters are kept strictly constant. Agilent WinGPC Software features automatic flow correction with an internal standard or flow marker, which is a powerful tool to compensate for the negative effects of constantly fluctuating flow rates.

Experimental

Sample preparation with a suitable internal standard

To perform automatic flow correction, a narrowly distributed peak is required that always elutes at the same volume and does not disturb the sample chromatogram (Figure 1). For this purpose, a monodisperse, low molecular weight substance is added to the sample. For organic eluents, butylhydroxytoluene (BHT) or toluene can be added to the eluent stock the samples are dissolved in. Ethylene glycol, isopropanol, and methanol are suitable flow markers for aqueous eluents.
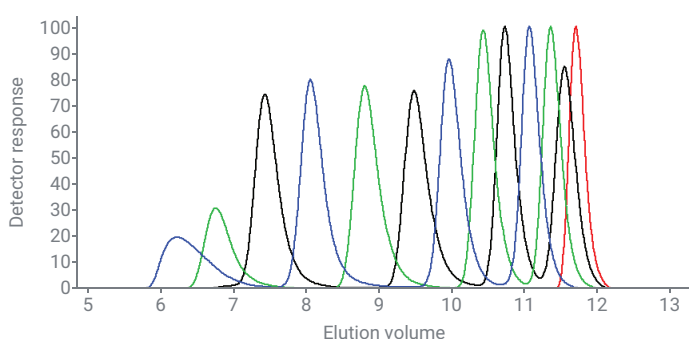

Figure 1. Overlay of three Agilent ReadyCal standards (blue, green, black) and the flow marker (butylated hydroxytoluene, red)

In more complex chromatograms, it is sometimes difficult to clearly identify the internal standard. For certainty, the eluent, including the flow marker, can be injected and run alone as a blank run. This allows the user to overlay the blank run with subsequent runs, ensuring accurate flow marker selection. The internal standards will always elute at the same position in all chromatograms and can easily be found and defined in Overlay mode. Salt and system peaks can be overlaid with other salt and system peaks. Due to this potential variability, it is not recommended to use salt or system peaks as flow markers.

Defining and adding the internal standard position

First, the exact position of the internal standard must be defined. Set the baseline in the raw data chromatogram, apply the integration limits in the elugram, and find the peak maximum (in the Elution Axis Context menu, click the right mouse button and then select **Find maximum**).

To set the baseline and the integration limits at the same time, click **Options**, **Analysis**, and **Quick Analysis** in the Raw Data window menu.

Every injection in the current login can be processed in one step by choosing All injects as the relevant injection input (choose **Method** and then **Options** in the Method window menu).

The internal standard is stored together with the calibration curve. To add the internal standard position and name, select **Calibration**, then **Parameters** from the Calibration window menu (Figure 2). Enter the previously found position (the default is 50 mL), confirm by clicking **OK**, and save the calibration curve.

Figure 2. The Calibrations Settings window, where the position and name of the internal standard can be defined

Applying the internal standard to the measurements

To transfer the flow marker from calibration parameters to the chromatograms, load the calibration file to the measurements again. The message "Attention! The new calibration uses a different internal standard!" shows that WinGPC has noticed the changes and can be confirmed. In the chromatograms, a dark green triangle will now appear at the defined position.
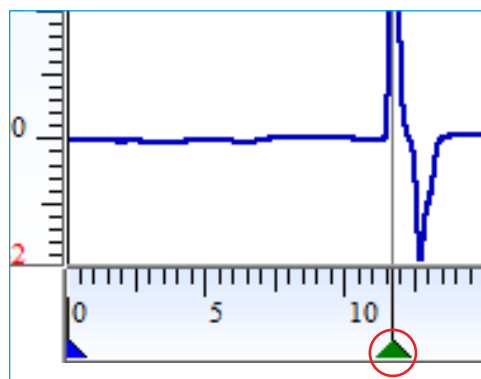

Figure 3. A dark green triangle marks the position of the internal standard in the chromatogram

Activating the flow correction

The easiest way to perform the flow correction is to select **Options**, **Analysis**, then **Quick Analysis** from the raw data window menu and click Correction (Figure 4). Negative peaks can be permitted as flow markers, and Internal standard only and baseline and integration limits can be toggled.
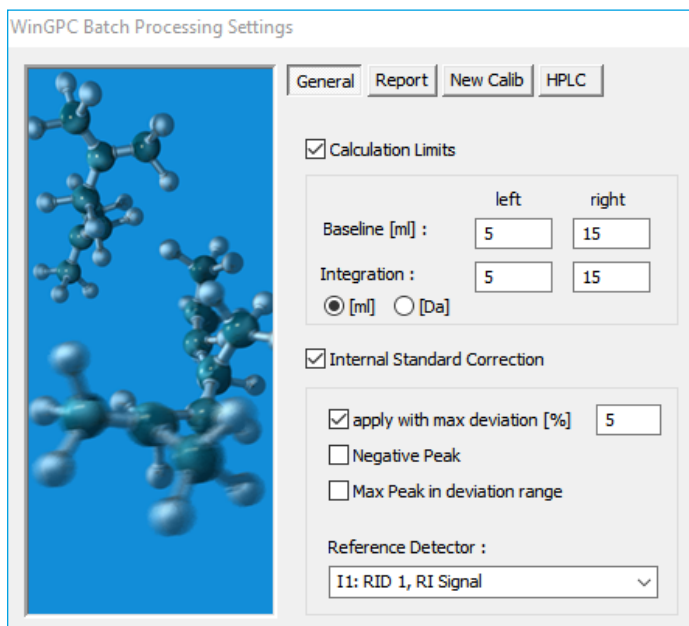

Figure 4. Quick Analysis window

The maximum allowable deviations can be set for the internal standard position in the chromatograms (the default is 5%). If the flow correction has been applied successfully, the dark green markers in the raw data chromatograms turn light green. When the flow rates vary too much or the maximum allowable deviations are defined too strictly, the internal standard might not be applied for some injections. The message "No changes will be applied to … samples!" will appear in this case. The markers in the relevant measurements will stay dark green. WinGPC also generates an entry in the session log (see Audit Trail: Session in the button bar).

Please make sure to apply the flow correction to all injections before evaluating calibration standards. Otherwise, a noncorrected calibration will be mixed with corrected measurements. To avoid this error, open a new calibration file, enter the internal standard's name and position in the calibration parameters, and save and load the calibration again to assign the internal standard to the measurements. From here, apply the flow correction, add the points to the calibration table, and calibrate the measurement as usual.

Flow correction can also be manually activated or deactivated, even for single injections, if actual inject is selected as the relevant injection input. Place the cursor below the desired peak, right click, and select Int. Standard search max. (or Int. Standard search min. if a negative peak was defined as flow marker). Int. Standard cancel deactivates the flow correction.

Demonstration of the effects of flow correction

Figure 5 provides an example of how a small fluctuation in the elution axis can drastically affect the molar mass averages.
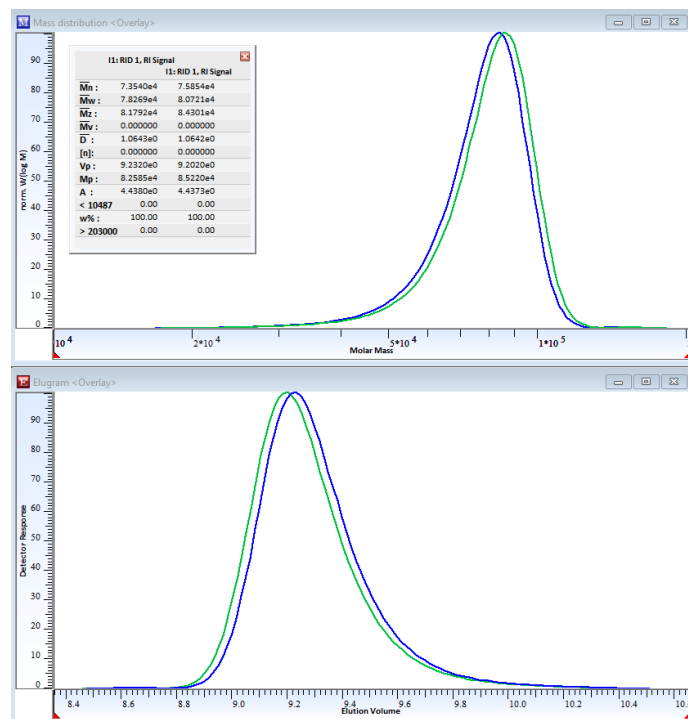

Figure 5. Comparison of elugrams (lower panel) and molar mass distributions (upper panel), without and with flow correction

In this example, the difference between the peak maxima before and after the flow correction is just 0.03 mL, a difference of around 0.2%. Because the linear scale elution volumes are transformed into the corresponding logarithmic scale molar masses, the molar mass averages would be expected to vary much more than 0.2%. Here, M_n and M_w both differ by approximately 3%.

Conclusion

As even slight fluctuations of the elution axis can result in drastic deviations of the molar mass distribution, automatic flow correction is highly recommended to improve reproducibility and accuracy of GPC/SEC measurements and evaluations. The automatic flow correction feature of Agilent WinGPC software effectively counteracts the negative effects of flow rate variation.

Reference

1. Yau, W. W. *et al.* Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography; Wiley-Interscience, **1979**.

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