Agilent InfinityLab Purification Solutions

Method Developer's Quick Start Guide

Agilent Technologies

Notices

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In This Book

This manual contains instructions for Method Developers on how to use the Automated Purification software.

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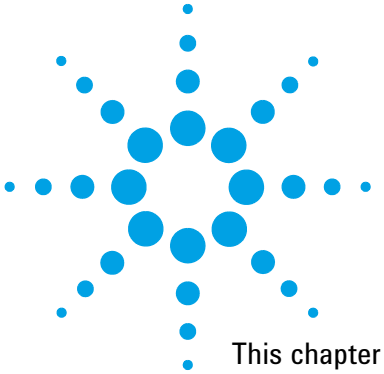


1 The Role of the Method Developer

This chapter describes the role of the user of the Automated Purification Software in Expert mode.

Purification software Method Developers work in *Expert* mode. They are responsible for establishing the software work environment such that Operators (working in *Easy Prep* mode) can use the purification software to purify their samples.

This comprises the following tasks:

- Creating the (analytical and) preparative base methods that are used in the instrument runs (see "Preparing Default Purification Methods" on page 9).
- Creating the analytical and preparative system parameter sets for all analytical and preparative instruments and operating conditions that are used by the Operators (see "Setting Up an Analytical and Preparative System" on page 29). These systems should be named such that Operators can easily identify their system. To retrieve system parameters, a set of calibration procedures has to be executed, see "Calibration Procedures" on page 49.
- Creating a set of purification task templates that are optimized for purifying the sample types that are provided by the Operators (see "Setting Up and Running a Purification Task" on page 35). We recommend that you provide a task template for each combination of analytical and preparative system that applies. Operators are expected to create their purification tasks based on a Method Developer's task template that matches their system combination and separation needs.
- Creating a system suitability test task (see "System Suitability Tests" on page 40) to verify that the instrument is still operating as expected. System suitability tests can be executed by operators and method developers. However, only Method Developers can create new kinds of system suitability test, and only Method Developers can accept a failed system suitability test to release a blocked instrument.

- Instructing Operators on the work environment and how to set up and run purification tasks.
- Fixing problems (for example, adjusting parameters or target identification) of Operators' tasks, to which the Operators do not have access.

Method Developers have full access to all features of the purification software and to the ChemStation while working within the main purification software user interface (the purification task screen).

2
Preparing Default Purification Methods

This chapter describes the three default Purification methods that can be used as a basis for the development of specific Purification methods.

This description summarizes the recommended purification method settings for purification-related modules. Most of these method settings can be freely modified according to your needs, with limitations noted in "Critical Method Parameters" on page 26. For the alternative modules, which are not listed here, use the applicable settings of the default modules.

Start with a new method:

- **Method > New Method**.

Methods settings are accessible from **Instrument > Setup Instrument Method** or **Method > Edit Entire Method**.

NOTE For the Automated Purification Software, it is important to mark the **Save Method with Data** check box in **Method > Runtime Check List**.

NOTE Switch off all pumps while you are setting up the methods.

NOTE It is highly recommended that the pumps are not switched off in a pre- or post-run command of the method.

NOTE Methods rely on the instrument configuration and driver revision. If you change the instrument configuration or upgrade the driver revision for which you originally set up your purification (base) method, you must at least save the method for the changed instrument configuration under a new name. However, we recommend that, for a changed instrument configuration or upgraded driver revision, you create a new method from scratch. Otherwise, method resolution will try to adapt the methods from the old configuration/driver revision with every method load (that is, for every sample run), leading to a method changed state, and preventing the run going forward.

When creating a new task based on a task template with a different instrument configuration, make sure that you choose an appropriate preparative base method for the current instrument configuration and driver revision.

We recommend that you choose different tasks root folders for different instrument configurations so that you do not mix different configurations in the same tasks root folder.

General Method Settings

In all modules, set the **Stoptime** to **As Injector/No Limit** (infinite run time). The actual stoptime will be specified when the method is used.

Isocratic Pump (G1310B)

Make-up pump for splitter used with separated preparative MSD instrument.

Stoptime	Select **As Injector/No limit**
Pressure Limits	Consider Active Splitter pressure limit (69 bar).

Quaternary Pump (G1311A/B/C, G7111B, G7104A) or binary pump (G7120A) with internal solvent selection valve

Make-up pump for splitter in LC/MSD instrument; and analytical pump for combined analytical and preparative instrument.

Stoptime	Select **As Injector/No limit**
Pressure Limits	• Because of the 400-bar pressure limit of the preparative system, set the upper limit for analytical scouting runs to 400 bar or lower. • Consider Active Splitter pressure limit (69 bar) when used as make-up pump.

Pump Valve Cluster (PVC)

Make-up pump for splitter in LC/MSD instrument, and analytical pump for combined analytical and preparative instrument. Use the same stop time and pressure limits as for the quaternary pump. Make sure that the selected solvent(s) from the clustered channel(s) is/are the one(s) that you want to use for your analytical run or make-up.

Prep Pump Cluster (G1361A and G1391A)

Solvent description texts	• Channel A: Water • Channel B: Acetonitrile
Solvent B	2 %
Pressure Limits	Set **Max.** limit. Find the pressure limit of the used columns (for example, 21.2 mm guard system seals finger-tight up to 135 bar; 21.2 mm prep column without the guard up to 350 bar).
Stoptime	Select **As Injector/No limit**
Advanced Channel A	Set **Compressibility** to 46 (Water)
Advanced Channel B	Set **Compressibility** to 115 (Acetonitrile)

Prep Pump (G7161A)

Solvents	**A** Select **Aqueous** and type the description text `Water`. **B** Mark the check box, enter `2 %`, select **Acetonitrile**, and type description text `Acetonitrile`.
Pressure Limits	Set **Max limit**: find the pressure limit of the columns used (for example, 21.2 mm guard system seals finger-tight up to 135 bar; 21.2 mm prep column without the guard up to 350 bar).
Advanced	In **Compressibility**, keep the check box **Use Solvent Types** marked

Prep Pump (G7161B)

Solvents	**A** Select solvent line 1 or 2, select **Aqueous** and type the description text `Water`. **B** Mark the check box, select solvent line 1 or 2, enter `2 %`, select **Acetonitrile**, and type description text `Acetonitrile`.
Pressure Limits	Set **Max limit**: find the pressure limit of the columns used (for example, 21.2 mm guard system seals finger-tight up to 135 bar; 21.2 mm prep column without the guard up to 350 bar).
Advanced	In **Compressibility**, keep the check box **Use Solvent Types** marked

Dual Loop Autosampler (G2258A)

Needle Wash	**Enable Needle Wash** with **Mode: Flush Port** and **Time**15 s (to avoid contamination of injection seat, do not use less than 10 s). The wash procedure requires needle wash solution.
Stoptime	Select **As Pump/No limit**
Advanced	In **Auxiliary**, mark **Vial/Well bottom sensing** only if the vessels used are suitable for this function, such as flat-bottomed glass vials; see the Dual Loop Autosampler manual for more information.

Prep Sampler (G7157A)

Needle wash	Set **Time**15 s. The wash procedure requires needle wash solution.
Stoptime	Select **As Pump/No Limit**.

Prep Sampler or Prep Sampler ASC (G7169B)

Important: Make sure that analytical (if used) and preparative pumps are properly linked (**Instrument > More Prep. Sampler > Control**)

Valve 2/10 (G1170A and G4730A)

Selection of a flow path with a certain column.

Position	Select **Use valve position**
Position switch at end of run	Select **Do not switch**
Position Names	Enter description of preparative and analytical positions. For the combined setup, set Position 1: Analytical, Position 2: Preparative.

Other Valves

Position	Select **Use valve position** (if applicable)

UV (DAD, MWD,VWD)

Wavelength	**DAD and MWD** Set **Signal AWavelength** to 263 nm, **Bandwidth**: 4 nm, clear **Reference** wavelength. **VWD** Set **Signal** to 263 nm.
Peakwidth	**DAD and MWD** 20 Hz (> 0.013 min) **VWD** 40 Hz (> 0.013 min)
Advanced > Spectrum (optional for DAD)	**Store**: **All**, **Range** from: 200 nm to 400 nm, **Step**: 2 nm
Autobalance	Mark **Prerun**.
Lamps on required for acquisition (DAD and MWD)	Mark **UV Lamp**.
Miscellaneous VWD	Mark **Lamps on required for acquisition**.
Stoptime	Select **As Pump/Injector**

UIB II (G1390B)

- If an auxiliary detector is connected via the UIB II box, set all related parameters in the UIB II method.
- If there is no detector connected via UIB II box, clear both **Analog In** check boxes in **Advanced > Analog In Port Settings**
- Set **Stoptime** to **As Pump/Injector**

Fraction Collectors (G1364B, G1364E, G7159B, G7166A and FCC*)

* FCC is a Fraction Collector Cluster of two or more modules.

- *G1364E, G7159B, G7166A and FCC*:
  - Set **Fraction Collection** to **Disabled**
  - If available, set **Recovery Collection** to **Disabled**
- Set **Stoptime** to **As Pump/Injector**

Flow Modulator (G7170B)

Splitting Operation	Select **Splitting disabled**.

MSD

Access by **Method > Edit entire method** or by right click on MSD icon

Set Up MSD Signals	**General** **Peakwidth**: 0.1 min (this defines the MSD data rate; make sure that it is sufficient). **Active Signals**: Mark 1 and 2 signals and name them, for example, `Positive` and `Negative` **MSD Signal Settings** **Signal**: 1, **Mode**: Scan, **Polarity**: Positive, **Mass range**: 125 – 725, **Step size**: 0.1 **Signal**: 2, **Mode**: Scan, **Polarity**: Negative, **Mass range**: 125 – 725, **Step size**: 0.1
MSD type-specific settings	• *MSD G6120B/G6125B* If the actual cycle time is above 0.9 s, consider decreasing it by using only one scanning polarity (lower cycle time will require less delay coil volume): ◦ **General** > **Active Signals** > clear Signal 2 ◦ **MSD Signal Settings** > **Signal 1** > specify polarity • *MSD G6130B/G6135B* **General** > **Peakwidth** > 0.07 min

Save the method as
`General_Purification`

- **Method > Save Method as**

Analytical Method Settings

Only for the combined analytical and preparative system.

Load the general settings of the purification method:
General_Purification("General Method Settings" on page 11)

Save the method as
`Analytical_Purification`:

- **Method > Save Method As**.

Quaternary Pump (G1311A/B/C, G7111B, G7104A) or binary pump (G7120A) with internal solvent selection valve

Define a for pump channels A/A1 and B/B1 or allow the Automated Purification Software write the generic gradient on the page.

Flow	1.0 mL/min
Advanced	Set **Maximum Flow Gradient** to about 10 times the target flow.

Prep Pump Cluster (G1361A and G1391A)

Flow	0 mL/min

Prep. Pump (G7161A)

Flow	0 mL/min

Prep. Pump (G7161B)

Flow	0 mL/min

Dual Loop Autosampler (G2258A)

Injection Loop	Lower
Injection mode	Depends on installed loop volume and injection volume. • Loop equal to injected volume: set **Full loop with overfill factor** 5, disable **Plug Settings** • Loop larger than injected volume: set **Partial loop filling** and set **Plug Settings** to maximum Plug volume: ◦ Mark **Draw Plug before and after the sample** ◦ **Plug Volume**: set the volume later in the method based on injection volume: Plug volume = ½[(Loop volume) – (Injection volume)] ◦ **Draw Plug from**: select **Location**, specify vial location **NOTE** The application of the Plug removes up to 27 µL of the purge solvent from the lower loop that is introduced by the seat capillary in front of the loop, which is purged after each run. The purge solvent has typically high elution strength by design; therefore, the removal of this solvent from the lower injection loop by the Plug feature is highly recommended.
Injector Cleaning	**Rinse volume** to 15
Advanced	**Draw speed** and **Eject speed** to 1000.0 µL/min.

Prep Sampler or Prep Sampler ASC (G7169B)

Operation Mode	Select **Analytical**
Method Presets	(After **Operation Mode** is set to **Analytical**) Click **Select Method Presets** and select **Non-polar Sample Matrix**
Stoptime	Select **As Pump/No limit**
Plug Settings	In the **Plug Solvent** column, specify the **Location** of the plug vessel containing 25 % Methanol

Advanced	In **Needle Height Position**, mark the **Use Vial/Well Bottom Sensing** check box
Injection Path Cleaning	Set up the **Multi-wash** table: • Keep the default 30 sec **Time** for solvent **S2** • Clear the **Draw Port** check boxes for **S1** and **S2** solvents (recommendation for Plug settings 2 and 3). Recommended **S2** solvent is acetonitrile. For standard operation, solvent **S1** (the draw solvent) should be water.

Valve 2/10 (G1170A and G4730A)

Position	**Use valve position**: set analytical position

UIB II (G1390B)

Expected ERI Mode	No mode check

If the Active Splitter is connected to the external contacts of the UIB II:

- **Timetable**: Empty
- **External Contacts**: **Contact A**: mark **Closed**

Save the method changes:

- **Method > Save Method**.

Preparative Method Settings

Load the general settings of the purification method:
General_Purification("General Method Settings" on page 11)

Save the method as
`Prep_Purification`:

- **Method > Save Method As**.

Isocratic Pump (G1310B)

Separated preparative MSD system – make-up pump

Flow	1.5 mL/min
Advanced	Set **Maximum Flow Gradient** to about 10 times the target flow.

Quaternary Pump (G1311A/B/C, G7111B, G7104A) or binary pump (G7120A) with internal solvent selection valve

Combined analytical and preparative MSD system – as make-up pump:

Channel D	100 %
Flow	1.5 mL/min
Advanced	Set **Maximum Flow Gradient** to about 10 times the target flow.

Prep Pump and Prep Pump Cluster (G7161A/B, G1361A and G1391A)

Flow	20 mL/min (scale-up of 4.6 × 50 mm to 21.2 × 50 mm columns with 5 µm particle size).
Advanced Channel A and B	In **Maximum Flow Gradient**, set both **Flow ramp up** and **Flow ramp down** to about 10 times the target preparative flow.

Dual Loop Autosampler (G2258A)

Injection Loop	Upper
Injection mode	Partial Loop Filling
Injector Cleaning	**Rinse volume**: 1 (increase to 5 for injections below 200 µL)
Advanced	**Draw speed** and **Eject speed**: 8000.0 µL/min (decrease to 3000.0 for injections below 200 µL)

Prep Sampler (G7157A)

Advanced	Set **Draw Speed** to 3000.00 µL/min.

Prep Sampler or Prep Sampler ASC (G7169B)

Operation Mode	Select **Preparative**
Method Presets	(After **Operation Mode** is set to **Preparative**) Click **Select Method Presets** and select **Saturated Non-polar Sample Matrix**
Stoptime	Select **As Pump/No limit**
Plug Settings	In the **Plug Solvent** column, specify the **Location** of the plug vessel containing 100 % Methanol
Advanced	In **Needle Height Position**, mark the **Use Vial/Well Bottom Sensing** check box
njection Path Cleaning	Set up the **Multi-wash** table: • Keep the default 30 sec **Time** for solvent **S2** • Clear the **Draw Port** check boxes for **S1** and **S2** solvents (recommendation for Plug settings 2 and 3). Recommended **S2** solvent is acetonitrile. For standard operation, solvent **S1** (the draw solvent) should be water.

Valve 2/10 (G1170A and G4730A)

Position	**Use valve position**: set the preparative position

UV (DAD, MWD, VWD)

- For instruments with the UV detector in the make-up flow of the Flow Modulator (G7170B) or Agilent active splitter (G1968D), or if using the slope-based collection mode, set **Peakwidth** to:
  - for DAD and MWD: > 0.1 min (2 s response time) (2.5 Hz)
  - for VWD: > 0.2 min (4 s response time) (2.5 Hz)

UIB II (G1390B)

If an MSD is configured:

- G1364B: **Expected ERI Mode**: MSD
- G7159B: No mode check

If the Agilent Active Splitter (G1968D) is connected to the external contacts of the UIB II:

- **Timetable**:
  - 0, Change contacts, Contact A state: Closed
  - 0.01, Change contacts, Contact A state: Open.
- **External Contacts**: **Contact A**: mark **Closed**

NOTE If the active splitter is activated by a contact other than contact A, use this contact instead.

Fraction Collector (G1364B and its cluster unit)

Fraction Trigger Mode	Peak Based, max. peak duration: 1 min
Peak Detector	G1390B (UIB II) **Mode**: Off (if no auxiliary detector is connected to UIB II box) **Mode**: On (if auxiliary detector is connected to UIB II box) UV detector **Mode**: Set one of modes and related values **Use MSD for mass-based Fraction Collection** Mark this check box for mass-based collection. **Fraction is collected when a peak is detected by** Select **all peak detectors**

In the module **Configuration**, verify that the preparative pump is linked.

Fraction Collector (G1364E, G7159B, G7166A and cluster unit)

Fraction Collection	Select **Enabled**.
Recovery Collection (if used)	Select **Enabled**.
Peak Triggers	**Use** Mark the check box **Peak Detection Mode** Select a peak detection mode and set the related values. **Use MSD for mass-based Fraction Collection** Mark this check box for mass-based collection.
Trigger Combinations	Select **AND**.

Verify that the preparative pump is linked to the fraction collector (**Instrument > More Fraction Collector > Modify > Linked Pump**).

Flow Modulator (G7170B)

Splitting Operation	Select **Splitting enabled**.
Intended Pump Flow	Set **Main Flow** and **Makeup Flow**.
Split Mode	Select the target split mode.

MSD

Access by **Method > Edit entire method** or by right click on MSD icon

Fraction Collection	**FC Mode** Use sample target masses **MS Signals** Mark active signals (Signal 1, and Signal 2 if used) **Positive adducts** Mark **M+H(1)** if positive polarity is active **Negative adducts** Mark **M-H(1)** if negative polarity is active
Fraction Collection Parameters	**Max Peak Width (min)** Consider changing the **Max Peak Width** for peak-based fraction collection of larger peaks. If a target peak is larger than this value, the fraction collection will be split into several fraction locations.

Save the method changes:

- **Method > Save Method**.

Agilent Active Splitter (G1968D/E/F)

To start the Agilent active splitter automatically with the preparative method:

- Set the external contacts and related timetable as in the UIB II method settings.
- Set the Agilent active splitter to **Local** (communication with External Contacts).
- Set the split ratio of the splitter manually (refer to the *MRA Operating Manual*).

Critical Method Parameters

In some situations, incorrect settings of the following parameters can cause incorrect functioning of the Automated Purification Software, incorrect fraction collection, or damage or contamination to some parts of the system. Therefore, if the purification system does not work correctly, ensure that the following parameters are set correctly.

The following list is intended for Agilent 1260 Infinity instruments based on G2258A autosampler, G1361A preparative pumps and G1364B fraction collectors:

Configuration

- Fraction collector: **Linked pump** > select preparative pump

General default method

- Pump: **Pressure limits** (to avoid column damage)
- Autosampler: **Needle wash** (to avoid autosampler seat contamination)
- Autosampler: **Vial/Well Bottom Sensing** (use only with suitable sample wells or vials)
- Valve: **Position** > **Use valve position** (necessary for the Automated Purification Software)
- Valve: **Position switch at end of run** > **Do not switch** (to avoid system overpressure due to flow path switch on the 2/10 valve)
- UV Detector: **Signals** > **Set Signal A** (set a wavelength suitable for analyzed compounds)
- MSD: **Set Up MSD Signals** > **General** > mark **Active Signals** (to collect MSD data)
- MSD: **MSD Signal Settings** > **Mode** > **Scan** (necessary for the Automated Purification Software)

Analytical default method (combined system)

- Valve 2/10 > **Position** > **Use valve position** > select correct analytical position
- Iso. Pump: **Flow** > 0 mL/min (make-up not used in analytical; flow can cause leak sensor error)
- UIB II: **Expected ERI Mode** > **No mode check** (for use with MSD, to avoid not ready state in some cases)
- Autosampler Plug: Plug solvent should contain only minimum amount of organic solvent to avoid unwanted partial elution of the sample on the column causing peak distortion and/or elution time variability

Preparative default method (Automated Purification Software base method)

- Valve 2/10: **Position** > **Use valve position** > select correct preparative position
- Iso. Pump (with splitter): **Flow** > target make-up flow must be set before use
- UIB II (with MSD): **Expected ERI Mode** > **MSD** (for MS based fraction collection)
- UIB II (with G1968D/E/F active splitter): **Timetable** > set for correct active splitter operation
- UIB II (with G1968D/E/F active splitter): **External contacts** > set for correct active splitter operation
- Set up active splitter split ratio and operation mode to *Local*

Note that some points also apply to stand-alone analytical systems.

3
Setting Up an Analytical and Preparative System

This chapter gives step-by-step instructions on setting up the analytical system and the preparative system in the Automated Purification software.

In order to perform purification runs, an analytical and a preparative system need to be defined. An analytical system is either part of a separate analytical instrument or part of a combined analytical and preparative instrument. Here, the meaning of a *system* is the relevant parameters in the purification workflow, which comprise both instrument configuration and operating parameters. These parameters are mandatory for the automatic purification run calculations.

Setting up an analytical system

You can either create a new analytical system from scratch or, if you have already created a similar system, set up a clone of an existing system.

1 In the Chemstation **Method and Run Control** view, open the **Purification** menu and select **Systems**.

The **Setup Systems Parameters** dialog box is displayed. The analytical system parameters are displayed in the left panel; the preparative system parameters are displayed in the right panel. These instructions are for the analytical system parameters in the left panel.

2 If no analytical system has already been set up, click **Add System** and provide a name and analytical flow rate.

NOTE Make sure that you choose a system name that helps you and other operators identify your system among others; for example, use the laboratory and instrument name/number and key operating parameters within the system name.

OR

If at least one analytical system has already been set up, select one that corresponds closely with your new system and click **Add System**.

A new analytical system is created or cloned.

3 In the **Pumps & Detectors** tab:

- Select the system type to which your analytical parameters belong. If they are part of a combined analytical and preparative instrument, then your choices for pumps and detectors are based on the current instrument configuration. If your analytical system parameters describe a separate analytical instrument, then you need to choose one method from that instrument. Your choices for pumps and detectors are then based on the instrument configuration as read from that example method. As such, any method parameters are ignored; only the configured pumps and detectors are used from that method.
- In the **Pumps** section, if there is more than one pump in the instrument, select the analytical pump delivering the main flow. Specify the pump channels for organic solvent and water for the selected pump. The analytical flow rate needs to be set according to your planned analytical

runs. The preparative flow rate can be calculated from the analytical flow considering the different column geometry in order to maintain the same chromatographic conditions. It is also possible to adapt the analytical flow rate according to a given preparative flow rate from the selected preparative system in the right panel of the **Setup Systems Parameters** dialog box.

- In the **Detectors** section, select the relevant detectors from the available detectors in the instrument configuration. The system schematics graphic corresponds to the selected detectors.

4 In the **Sampler** tab:

- The tab is present only for flow-through samplers such as the G7157A 1260 Infinity II Prep Autosampler. There, the volumes of the sample loop and seat capillary contribute to the time that the sample needs to reach the column. If the analytical system is separate from the preparative system, such volumes are read from the method as specified in the **Pumps & Detectors** tab. For combined instruments, the volumes must be imported from the current instrument configuration. Previously created analytical system settings may refer to a different sample loop or seat capillary and then will be displayed with a warning sign.

5 In the **Delay Volumes** tab:

- Select the tubing by selecting a system type, and provide the initial pressure.

  If you select a custom system type, no initial pressure is required, but you need to determine the tubing volumes. For details about determining delay volumes and times, see Characterizing the delay volumes ("Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector" on page 52).

- Specify the delay times of an MSD or an auxiliary detector to the UV detector, if such detectors are selected in the **Pumps & Detectors** tab.

6 Switch to the **Column** tab and provide details of the analytical column.

7 Click **Save System** to save the analytical system parameters.

Setting up a preparative system

The process of setting up a preparative system is very similar to that for setting up the analytical system, but since the Automated Purification software is installed on the preparative system, it is able to determine the current instrument configuration and show a warning if the system does not match the instrument configuration.

1 In the Chemstation **Method and Run Control** view, open the **Purification** menu and select **Systems**.

The **Setup Systems Parameters** dialog box is displayed. The preparative system parameters are displayed in the right panel; the analytical system parameters are displayed in the left panel. These instructions are for the preparative system parameters in the right panel.

2 Click **Add System** and provide a name.

NOTE Make sure that you choose a system name that helps you and other operators identify your system among others; for example, use the laboratory and instrument name/number and key operating parameters within the system name.

You can also clone an existing preparative system.

3 In the **Pumps & Detectors** tab:

- The settings are based on the available modules in the current instrument configuration. This is either a preparative-only or a combined analytical and preparative instrument.
- In the **Pumps** section, if there is more than one pump in the instrument, select your preparative pump for the main flow. Specify the pump channels for organic solvent and water for the selected pump. The preparative flow rate needs to be set according to your planned preparative runs. The preparative flow rate can be set as calculated from the analytical flow (of the selected analytical system in the left panel) considering the different column geometry in order to maintain same chromatographic conditions. If a second pump is configured in the instrument and an MSD or auxiliary detector is set up for the preparative system, you can also select a make-up pump, which delivers the flow to the MSD or auxiliary detector.

- In the **Detectors** section, select the relevant detectors from the available detectors in the instrument configuration. The system schematics graphic corresponds to the selected detectors.

4 In the **Sampler** tab

- The tab is present only for flow-through samplers such as the G7157A 1260 Infinity II Prep Autosampler. There, the volumes of the sample loop and seat capillary contribute to the time that the sample needs to reach the column. The volumes must be imported from the current preparative instrument configuration. Previously created preparative system settings may refer to a different sample loop or seat capillary and then are displayed with a warning sign.

5 In the **Delay Volumes** tab:

- Select the tubing by selecting a system type.

  If you select a custom system type, you need to determine the tubing volumes. For details about determining delay volumes and times, see Characterizing the delay volumes and Fraction collector delay time and volume calibration ("Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector" on page 52, "System Configuration and Delay Time Calibration" on page 78).

- Specify the additional detectors that are used. If an MSD is part of the system, specify the delay time between the UV detector and the MSD, and click **Do calibration run** to determine the delay time between the UV detector, fraction collector and MSD.

6 Switch to the **Column** tab and provide details of the preparative column.

7 Click **Save System** to save the preparative system parameters.

4 Setting Up and Running a Purification Task

This chapter gives instructions for the most important steps for setting up and running a Purification Task.

Setting up a purification task

The purification task holds the complete data of the purification job, including the analytical and preparative systems, the analytical and preparative run conditions and parameters, and the analytical and preparative results once they are available.

Purification tasks are managed in the purification task screen, which is thus the center for the execution of all purification work:

- configuration of the purification job (samples and operating setup)
- submission of the analytical and preparative runs (including progress indication and stop)
- review and modification of the target identification from the analytical run
- review and export of the purification results (collected fractions)

1 In the ChemStation **Method and Run Control** view, open the **Purification** menu and select **Tasks**.

  The purification task screen is shown, displaying in its upper region a list of all tasks in the selected task folder. Details of the selected task (setup and result info) are displayed in the lower region. By default, the configuration tab with the **Select System** page of the selected task is displayed.

After launching the **Tasks** screen or logging in, you are in the **User task** view by default. This view displays the tasks in your default user tasks root folder. Administrators and Method Developers can modify the user tasks root folder using the **Administration** dialog box. However, you can choose a different root folder or navigate to another user's folder in the **Purification task parameters root folder** text-box at the top of the tasks list. As a Method Developer you can modify and submit other users' tasks. Operators can modify and submit only their own tasks.

Configuring a new task

A new empty task can include information from an analytical run, which is either specified to run on a combined analytical and preparative instrument or which has been run on a separate analytical instrument. This is an analytical-to-preparative workflow task. It includes the upscaling of analytical conditions to the preparative instrument. A preparative-only (workflow) task does not include an analytical run and therefore has no upscaling. It defines a preparative run on the preparative instrument only. System suitability tasks are discussed in "System Suitability Tests" on page 40.

The following description is for an analytical-to-preparative task, which requires the completion of all configuration screens.

1 Click **Add Task** to create a new default purification task, template-based purification task or a copy/clone of the currently selected purification task. Provide a name for your new purification task.

2 Verify that the **Analytical System** matches the system providing the analytical run, and that the **Preparative System** corresponds to the current preparative instrument configuration.

   The current instrument configuration of the preparative system is displayed in the table.

   If one of the systems does not match, choose another template to create your task. If there is no matching template, create a new task with matching systems and save it as a template.

3 Select each of the subpages, **Pumps & Detectors**, **Sampler**, **Delay Volumes** and **Columns**, and verify that the parameters are correct. If they are not, select a suitable system.

4 Select the **Ion Species** page and select the ion species of interest for the analytical run. If necessary, select the charge states. For the preparative run, select what to do with the ion species.

5 Select the **Analytical Run** page and specify the source of the analytical run:

   - To run an analytical scouting sequence on a combined analytical and preparative system, select an existing ChemStation sequence or prepare a sequence from scratch. Select the path and name of an existing

ChemStation sequence in the text input field or click **Edit sequence** to start editing a sequence from scratch or modifying a selected sequence. You can also import a sequence as a text (CSV or TXT) file.

- To use analytical results that have already been acquired on a stand-alone system, specify the location of the source files (result set or single sample results) in the text input field.

  Select data files using the **Process?** check box in the sequence table.

6 Select the **Scale Up** page and verify that the **Analytical Signal Correlation** and **Purification of Target** parameters are correct. Make any necessary changes.

In the **Gradient** tab of the **Scale Up** page, the gradient profile can be adjusted. The default settings should deliver reasonable purification results. The software design allows an experienced Method Developer to optimize the default settings to obtain the best purification results.

NOTE Any changes of the gradient profile in the task configuration page are applied to all samples of the task. The parameters are maintained when you save the task as a template, and use the template to create new tasks.

7 Select each of the subpages, **Integration** and **Gradient**, and verify that the parameters are correct. Make any necessary changes.

8 Select the **Preparative Run** page and specify a **Preparative base method**. If you want to review the analytical results before starting the preparative run, mark the **Review analytical results before purification** check box.

NOTE

Mark this check box, if:

- you want to review the automatic target identification of individual samples and eventually correct it by manual target assignment or by changing the target identification parameters for all samples (in the **Task Configuration** tab) or for individual samples (in the **Analytical Results** tab)
- you want to modify integration parameters, gradient profiles, or target masses for individual samples
- you want to revise the automatic decision of samples to purify
- you want to adjust fraction collection thresholds or fraction collection conditions for specific targets
- you are not using an MSD for target identification

Clearing the check box starts the purification step automatically when the target compounds have been identified.

9 Enter the injection volumes and vial locations for the preparative run into the table.

You can also use the **Location Mappings** subpage for this task, or to make any adjustments.

10 Select each of the subpages, **Fraction Collection** and **Location Mappings**, verify that the parameters are correct, and make any necessary adjustments.

NOTE

Parameters that have been changed in the **Task Configuration** page are applied as global settings for all samples from a task. They are also applied in all further tasks that you create if you save and use this task as a template.

System Suitability Tests

The system suitability tests are specific tasks of the preparative-only workflow. They are used to purge the autosampler, flush the instrument and submit a run of at least one known standard sample in order to verify that the compounds in the sample are still eluting within the expected retention time range and that their ion masses can still be detected. As such, the system suitability tasks verify that the preparative instrument is (still) ready to run. We recommend that you submit such task every morning before starting purification. A system suitability task run should be repeated if you feel that the instrument is not operating as it should. If a system suitability test fails, then no regular tasks can be submitted until you fix the instrument and a repeated system suitability run confirms the system readiness. As a method developer, you can also classify a failed system suitability test run as *accepted* to release the instrument for further task runs if you judge that the instrument is capable of performing the required purification tasks.

System suitability test tasks are created and managed in the **SST Tasks** view, which you access by clicking the **SST Tasks** button in the main toolbar of the **Tasks** screen. A Method Developer must create at least one SST task where the acceptance criteria are defined. An SST task can be executed by any user. However, Operators can only clone existing SST tasks to submit a new SST task execution. Operators cannot create new SST tasks from scratch (to define new acceptance criteria). The root task folder for SST tasks is specified in the **Administration** dialog box, which Administrators and Method Developers can access from the main toolbar of the **Tasks** screen. Operators do not have access. Method Developers should clean-up, archive or choose a different SST tasks root folder from time to time to prevent the folder becoming too big, which would affect the task reading time.

NOTE

We recommend that you run System Suitability Test (SST) tasks with a preceding flush run. Make sure that you use a base method with mass-based fraction collection switched *off* (in the method settings for both the fraction collector and the MSD) for the flush run, because no target mass is used for the flush run.

Running a task

1 Click  **Run** in the top toolbar of the **Task** dialog box to start the purification run.

If the analytical sequence has not yet been run, it will be submitted to the ChemStation run queue.

If the analytical run is already available, the process will start with the evaluation of the analytical results.

The buttons in the top toolbar also allow you to stop a run in progress or unschedule a scheduled run that has not yet started. If the run queue is paused (for example, after a stop) you can resume it using the  **Resume** item in the **Task** toolbar. Note that the  items can be helpful if the run gets stuck or lost for any reason.

The run progress is displayed in the bottom line of the **Task** screen. Run state events and important processing events are displayed in the **Logbook** tab.

You can log off while the run is proceeding and you or another user can log in and create and/or submit other tasks. While no user is logged in, the Purification Software is locked (in sync with ChemStation) and switched to the **Scheduled Tasks** view, which lists the running and all scheduled tasks from all users with their remaining times. At any time while you are logged in, you can switch to the **Scheduled Tasks** view to see the purification tasks run queue.

Processing partial data and cloning tasks

You are dealing with partial data under the following conditions:

- You stopped your analytical or preparative run, or your run was stopped due to an instrument error (such as solvent bottles running empty). In such a case, some of the planned samples were executed but others were missed.
- You have a large set of analytical data from a separate analytical instrument, but you want only a subset of it to be purified in one task. For example, your preparative plate does not have the capacity to hold all samples.

Task cloning (or copying) means creating a new task with the same settings and analytical data as another task. The preparative results are not cloned.

1 Select the task to be cloned in the task list of your purification task dialog box.

2 create a new task as a clone of the selected task by choosing **Add clone of currently selected task** in the **Add Purification Task** dialog box.

If you want to repeat a (previous) system suitability test run, you can clone the system suitability test task and let it run in **SST Tasks** view. This way, operator users can also repeat system suitability task runs. Operator users cannot create a new system suitability task from scratch.

If your analytical run was stopped or aborted, the successfully processed samples remain as analytical result data with the task. Purification proceeds with those samples only. If you want to process the remaining/missed samples from such a task:

1 Create a clone of the incomplete task.

2 In the new cloned task, go to the **Analytical Run** page of the **Task Configuration** and click **Revert to sequence**.

This restores your original sequence.

3 Click **Edit Sequence** and remove the samples that already ran successfully.

4 Click **Accept changes** to save your modified sequence with the task.

If your preparative run was stopped or aborted, the successfully processed samples are saved with the task, which is now in a *completed* state, and no further modifications can be made. You can proceed to reviewing your

purification results and export fractions as described in "Reviewing purification results" on page 45. To process the missed samples of the purification run:

1 Create a clone of the partially processed task.

2 In the new cloned task, go to the **Analytical Results** tab and select the samples to be purified by marking the check boxes in the **Purify?** column.

3 Re-submit the purification run for the selected (missed) samples.

If you want to process only a subset of the samples from your available analytical data, select the analytical data (result set folder or folder of your single sample results) in the **Analytical Run** page of **Task Configuration** of a new task.

1 Select those samples that you would like to be evaluated and purified in the **Process?** column of your analytical samples table.

2 Create a new task as a clone of your previous task to run another subset of samples from your analytical data and select the next subset of samples.

Make sure that all tasks that refer to the same analytical data are stored in the same tasks root folder, because the analytical results are copied into the same root folder.

This avoids unnecessary copies of your analytical data. For example, the default tasks root folder is C:\Users\Public\Documents\ChemStation\1\Purify\Tasks. The analytical results are then stored in C:\Users\Public\Documents\ChemStation\1\Purify\Tasks\AnalyticalResults.

If you have set up user- and/or time-specific sub-folders as task root folders in the **Administration** dialog box, the analytical results are copied into each such folder if, for example, multiple users work for several days on the same analytical result data, while you have user-name-specific folders and daily time task root folders.

Reviewing analytical results

1 If you have selected to review the analytical results before starting the preparative run, the purification run stops after the evaluation of the analytical results. Select the **Analytical Results** tab to review the results.

You can make any adjustments to the scale-up parameters and preparative run conditions before continuing with the purification step.

In the **Analytical Results** tab, the identified target compound and the calculated gradient profile are visualized. Spectral data of each peak is displayed in the **Spectra** tab. Scale-up, integration and fraction collector thresholds can be adjusted for each sample individually. Target masses or formulas can be corrected.

NOTE All changes in the **Analytical Results** tab apply to the selected sample, so you must step through all samples that you want to review or for which you want to change settings. If settings have to be adjusted for all samples, return to the **Task Configuration** tab to change the global settings, then restart the evaluation of the analytical results.

2 When you are satisfied with the results, click  **Run** in the top toolbar of the **Task** dialog box to start the preparative run.

3 When the preparative run is finished, select the **Preparative Results** tab to review the results.

Reviewing purification results

The purification results can be reviewed either in the **Preparative Results** tab or in the stand-alone **Preparative Results** window. The functionality of both review methods is the same, but the **Preparative Results** window does not contain task-specific information such as sample purity or target formula.

1 To display the purification results, click the **Preparative Results** tab.

OR

Open the **Purification** menu in the Chemstation **Data Analysis** view and select **Results**.

The purification results are shown in a window with four sections:

- Upper left: the samples list. You can choose to view the samples either as a tabular display or as a graphical representation of the autosampler tray.
- Upper right: the fractions collected. You can choose to view the fractions either as a tabular display or as a graphical representation of the fraction collector.
- Lower left: the signals display, which shows all collected chromatograms from the selected sample. The peaks are annotated with start and end ticks and retention times, and the collected fractions are denoted by colored bands.
- Lower right: the spectra display, which shows the spectra for the selected fraction.

The **Preparative Results** tab displays the location of the injected samples in the autosampler and the location of the collected fractions in the fraction collector. It indicates the collected fractions in the chromatograms and the corresponding spectral data that have been acquired. The display of spectral data is an interactive process: clicking on a peak of the chromatogram displays the spectral data, or clicking on a collected fraction displays the spectral data and the chromatographic information. Select fractions to export by CTRL-clicking fractions in the graphics or the table. Selected fractions can be exported as a re-analysis sequence file or a liquid handler pooling file by clicking  in the Purification Task toolbar.

The fraction locations and containers/trays/plates might not match the sampler containers/trays/plates for a re-analysis sequence. In this case, you may want to choose the **Convert fractions to parking lot locations** option in the **Export Selected Fractions** dialog box, which converts your fraction locations to the PLOT format. This allows you to re-configure your containers in the sampler using the parking lot of the **Sampler Entry** tab of the ChemStation **Method And Run Control** view.

Task templates

Save a template from a task that provides optimum parameter settings. A new task can then easily be created from a template with pre-populated settings. As a Method Developer, you should provide task templates for any combinations of analytical and preparative system settings. Operators can then simply create new tasks by using the template that suits their instrument combination. Choose a template description that helps to identify the appropriate template. Templates do not contain sample lists and result data, but the first sample of a task defines default settings in a template, which are then used to pre-populate sample parameters in a new task created from that template. This is a pre-defined injection volume and method. Templates can be saved only by Method Developers; Operators cannot save templates.

5
Calibration Procedures

This chapter gives step-by-step instructions for the important calibration procedures for the Purification system.

Before starting the calibration procedure(s), check that all prerequisites are available (solvents, samples and others). The list is placed in the beginning of each document.

The calibration procedures are a set of measurements and calculations that calibrate all required parameters for the Automated Purification Software. The work-flow is as follows:

1 Create default methods, see "Preparing Default Purification Methods" on page 9

"Critical Method Parameters" on page 26 summarizes parameters of the purification LC system that are critical for correct function of the LC system and Automated Purification Software.

Set up default methods for the purification instrument:

- General
- Analytical (only for combined analytical and preparative instrument)
- Preparative
- Agilent Active Splitter

2 Determine instrument tubing volumes (Mixing point to UV and Column to UV), see Characterizing the tubing volumes("Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector" on page 52)

Characterization of tubing volumes between the mixing point-to-UV and the column-to-UV that include volumes of tubing and some modules. For the *standard analytical system*, it is recommended to calculate the Mixing point-to-UV detector tubing volume if the void volumes of the modules are available. For the *purification system*, it is recommended to perform both measurement and calculation of the instrument Mixing point-to-UV detector tubing volume. Comparison of these two values can serve as a test for the correct tubing set-up, and that it is free of air bubbles; if the deviation is significant (more than about 10 %), check the tubing connection and/or flush the tubing with a high flow rate to remove air bubbles (typically in the mixer).

If you are using custom tubing systems:

- Calculate the Column-to-UV detector tubing volume for:
  - Analytical flow path (combined or standard analytical system)
  - Preparative flow path
- Calculate Mixing point-to-UV detector tubing volume for:
  - Analytical flow path (combined or standard analytical system)
  - Preparative flow path

- Measure the Mixing point-to-UV detector tubing volume for:
  - Preparative flow path (bypass the splitter, if you are using the combined system)

NOTE Do not forget to remove the column from the flow path.

3 Determine the column void volume, see "Characterizing the column volume" on page 71

Determination of the column Porosity parameter. In the Automated Purification Software, the Porosity equals the column void volume divided by the column geometric volume. A typical value for 4.6 mm and 21.2 mm ID ZORBAX StableBond (SB) C18 columns is 50 %.

4 Determine the delays for the analytical system and measure the fraction collector delays for the preparative system, see "System Configuration and Delay Time Calibration" on page 78

- Configure the purification systems in the Automated Purification Software.
- Determine the delay time for the Analytical systems.
- Calibrate the delay time for the Preparative systems.

5 Check-out sample test in Automated Purification Software, see "Checkout Procedure" on page 91

a Acquire analytical run(s) in a ChemStation sequence.

b Set up and measure a focused gradient in the Automated Purification Software for separated analytical and preparative UV-only instrument.

c Set up and measure a focused gradient in the Automated Purification Software for combined and separated analytical and preparative MSD instruments.

The use of the respective capillary must be considered in all tubing volume calculations. If you have a custom tubing setup, use the tables as templates.

Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector

Chemicals (needed only if values are measured; all solvents degassed)

- Preparative or combined system (measurement recommended):
  - Solvent A: Water
  - Solvent B: 1% acetone in water as a UV tracer
  - Solvents can contain additives.

The *Mixing point to UV detector* tubing volume is used to correct the HPLC elution times for the dead volumes of the tubing and a mixer. The *Column to UV detector* tubing volume is the dead volume of the tubing between the end of the column and the UV cell. The difference between these tubing volumes is called the dwell volume, which serves as a correction of the gradient-related calculations for the delay of the gradient to the column. It causes a delay of the programmed gradient to the column, and it negatively influences the performance of gradient-based separations. Therefore, it is an important parameter for the calculation and optimization of generic and focused gradients. The *Sampler valve to column tubing* volume is the volume between the sampler valve outlet port and the column inlet. It expresses the delay of the sample to the column.

Tubing Volumes for Predefined Systems

The **Volume Sampler valve to Column**, **Volume Column to UV detector** and **Volume Mixing point to UV detector** for predefined systems can be selected for each analytical and preparative system from the **Tubing** drop-down list in the in the **Delay Volumes** tab of the **Analytical Systems** or **Preparative Systems** dialog box. Predefined systems are several default supported configurations defined in the *M8368-90302 System User Guide for the 1260 Infinity Purification System* and Agilent Infinity II preparative capillary kit documentation (Technical Notes G7161-90201, G7161-90202 and G7161-90203), which have modules connected using the following capillary kits:

- For the Agilent 1260 Infinity Systems

  - *Preparative UV-only* system:

    5067-6175 Capillary Kit for UV-based Systems
  - *Combined UV/MSD* and *Preparative UV/MSD* systems:

    5067-6176 Capillary Kit for Mass-based Systems
- Agilent 1260 Infinity II and Agilent 1290 Infinity II Systems
  - *Preparative UV-only* system:

    System capillary kits: 5067-7015, 5067-7016, 5067-7017 or 5067-7018
  - *Autoscale Preparative UV-only* or *UV/MSD* system

    Upgrade System Cap. Kit Autoscale 5067-7019
  - *Preparative UV/MSD* or *Autoscale UV/MSD* system

    Upgrade System Cap. Kit MS-based 5067-7023.

For the combined preparative and analytical MSD default setup, the tubing volumes of the analytical flow path are dependent on pressure, solvent type and composition. This is due to the presence of the dampener in the quaternary pump G1311A/B/C and G7111A/B used with this system. Therefore, by selecting one of the predefined combined systems, an **Initial pressure** field is provided in the **Delay Volumes** tab of the **Analytical Systems** dialog box. The **Volume mixing point to UV detector** tubing volume is updated based on the value of **Initial pressure**.

Table 1 List of predefined Agilent 1260 Infinity combined systems in the Analytical Systems dialog box

Setup	Description
Combined UV/MSD, G1311B, ACN Combined UV/MSD, G1311B, MeOH	Analytical flow path of the default combined UV/MSD analytical-to-preparative instrument setup with G1311B quaternary pump and 50 µL loop in the dual-loop autosampler (G2258A). Acetonitrile or methanol as solvent for the pump channel B.
Combined UV/MSD, G1311A/C, ACN Combined UV/MSD, G1311A/C, MeOH	Analytical flow path of the default combined UV/MSD analytical-to-preparative instrument setup with G1311A or G1311C quaternary pump and 50 µL loop in the dual-loop autosampler (G2258A). Acetonitrile or methanol as solvent for the pump channel B.

Table 2 List of predefined Agilent Infinity MSD-based systems in the Preparative Systems dialog box

Setup	Description
Combined UV/MSD	Preparative flow path for the default combined UV/MSD analytical-to-preparative instrument setup with 3 mm preparative UV cell and 5 mL loop in the dual-loop autosampler (G2258A).
Preparative UV/MSD default setup; 3 mm UV cell	Preparative UV/MSD instrument setup with 3 mm preparative UV cell and 5 mL loop in the dual-loop autosampler (G2258A).

Table 3 List of predefined Agilent Infinity UV-only systems in the Preparative Systems dialog box

Setup	Description
Preparative UV-only default setup; 3 mm UV cell	Preparative UV instrument setup with 3 mm preparative UV cell and 5 mL loop in the dual-loop autosampler (G2258A).

Agilent 1260 Infinity II Preparative default systems

There is a wide selection of default preparative systems available for the following instrument/capillary kit combinations:

- Contains a G7161A or G7161B pump
- Contains a G7157A autosampler
- The autosampler may optionally contain a 5 mL extension loop (G7157-68711)
- The capillary kit for the default setups is used: 5067-6858, 5067-6859, 5067-6860 or 5067-6809 or 5067-7015, 5067-7016, 5067-7017 or 5067-7018
- Contains a DAD (G7115A) or MWD (G7165A) with appropriate cell inlet tubing for the desired flow rate, or contains a VWD (G7114A)
  - one of the following preparative cells is used: G7115-60001, G7115-60002, G7115-60003, G1314-60023, G1314-60024, G1314-60025, G7114-60024
- Optionally contains an MSD (G6125B or G6135B) connected using the default MS upgrade capillary kit, 5067-6861 or 5067-7023
- Optionally contains a flow modulator (G7170B) and make-up pump (G7110A/B or G7111A/B)

NOTE The specifications for standard systems are given in Technical Note *G7161-90202 InfinityLab Purification, Capillary Kits*

Agilent 1290 Infinity II default systems

Preparative-only and Autoscale systems predefined default configurations are available for the following instrument and capillary kit combinations:

- Contains a G7161A/B pump
- Contains a G7158B autosampler with different loops (2 mL – 20 mL, 5068-0334, 5068-0335, 5068-0336, 5068-0350, 5068-0351)
- For the autoscale system, the Upgrade System Cap. Kit Autoscale 5067-7019 is used
- The capillary kit for the default preparative setups is used: 5067-7015, 5067-7016, 5067-7017 or 5067-7018
- Contains a DAD (G7115A) or MWD (G7165A) with appropriate cell and cell inlet tubing for the desired flow rate, or contains a VWD (G7114A)
  - one of the following analytical cells is used: G1315-60022, G1315-60025, G1314-60182, G5615-60022
  - one of the following preparative cells is used: G7115-60001, G7115-60002, G7115-60003, G1314-60023, G1314-60024, G1314-60025, G7114-60024
- Optionally contains an MSD (G6125B or G6135B) connected using the default Upgrade System Cap. Kit MS-based Prep LC, 5067-7023
- Optionally contains a flow modulator (G7170B) and make up/analytical pump (G7110A/B or G7111A/B)
- Optionally contains the system valve (G1170A) with the 2/14 valve pod (5320-0002)

NOTE The predefined volumes for instrument configurations are given in the following:

- The specifications for standard systems given in Technical Notes *G7161-90201* and *G7161-90202 InfinityLab Purification, Capillary Kits,*
- The specifications for Autoscale systems given in Technical Note *G7161-90203 InfinityLab Autoscale System Upgrade Kit*.

Default capillaries, UV cells and sample loops are listed in the tables in "Calculation of tubing volume: Column to UV detector" on page 56 and "Calculation of tubing volume: Mixing point to UV detector" on page 58. The

capillaries are delivered in the respective capillary kits, which are flow-dependent

- 4 – 8 mL/min (P/N 5067-7015 or 5067-6858)
- 15 – 40 mL/min (P/N 5067-7016 or 5067-6859)
- 40 – 80 mL/min (P/N 5067-7017 or 5067-6860))
- 80 – 200 mL/min (P/N 5067-7018 or 5067-6809)

Capillaries are also delivered in the InfinityLab Autoscale Syatem Upgrade Kit (5067-7019) and the InfinityLab Upgrade Capillary Kit for MS-based Fraction Collection (5067-8023 or 5067-6861).

Calculation of tubing volume: Sampler valve to column

This is the volume between the autosampler outlet (that is, the outlet port of the autosampler valve) and the column inlet. If the G7157A extension loop is installed, it is not included in this volume.

Calculation of tubing volume: Column to UV detector

The tables provided below give some examples of predefined capillaries, UV cells and sampler loop configurations, and can be used to calculate custom setups. The *Volume column to UV detector* tubing volume consists of all tubing between the column and UV detector cell (including the UV cell inlet tubing).

For a standard analytical system the **Volume column to UV detector** typically consists of the following volumes:

- Column to UV detector capillary
- Inlet capillary of the UV detector cell
- Dead volume of the UV detector cell

Table 4 Volume in µL per mm of some stainless steel and PEEK capillaries

Stainless Steel		PEEK	
ID [mm]	**µL/mm**	**ID [mm]**	**µL/mm**
0.12	0.0113	0.1 (0.004")	0.0081
0.17	0.0227	0.13 (0.005")	0.0127
0.25	0.0491	0.18 (0.007")	0.0248
0.5	0.196	0.25 (0.01")	0.0507
0.7	0.385	0.51 (0.02")	0.203
0.94	0.694	0.76 (0.03")	0.456
1.0	0.785		

Example calculation of the *Column to UV detector* delay volume of predefined Agilent 1260 Infinity purification systems:

Table 5 UV/MSD-based combined analytical and preparative Agilent 1260 Infinity LC system

Analytical	**Volume, mL**	**Preparative with bypassed splitter**	**Volume, mL**
Column to valve (0.17 × 400 mm)	0.009	Column to splitter (0.5 × 600 mm)	0.118
Valve to UV (0.17 × 400 mm)	0.009	Splitter bleed bypass (2 × 0.25 × 80 mm)	0.008
UV inlet tubing for 3 mm Prep Flow Cell (0.5 × 250 mm)	0.050	Splitter to valve (0.18 × 1400 mm)	0.034
		Valve to UV (0.17 × 400 mm)	0.009
		UV inlet tubing for 3 mm Prep Flow Cell (0.5 × 250 mm)	0.050
Total	*0.068*	*Total*	*0.219*

Table 6 UV/MSD-based system with UV detector in front of splitter or UV-based preparative 1260 LC system

Preparative with bypassed splitter	Volume, mL
Column to UV (0.5 × 600 mm)	0.118
UV inlet tubing for 3 mm Prep Flow Cell	0.050[1]
Total	*0.168*

[1] Note that the inlet tubing of 0.06 mm (or 0.3 mm) preparative UV cell does not have a fixed length (the tubing 0.5x800 or 0.8x2000 mm provided should be cut before use). UV inlet tubing cell volume has to be adjusted in the table.

Calculation of tubing volume: Mixing point to UV detector

The tables provided in these sections describe the predefined capillaries, UV cells and sampler loop configurations, and can be used to calculate custom setups. An approximate value of the *Mixing point to UV detector* tubing volume can be calculated if the volumes of all modules and capillaries are known:

Table 7 Volume in µL per mm of some stainless steel and PEEK capillaries

Stainless Steel		PEEK	
ID [mm]	**µL/mm**	**ID [mm]**	**µL/mm**
0.12	0.0113	0.1 (0.004")	0.0081
0.17	0.0227	0.13 (0.005")	0.0127
0.25	0.0491	0.18 (0.007")	0.0248
0.5	0.196	0.25 (0.01")	0.0507
0.7	0.385	0.51 (0.02")	0.203
0.94	0.694	0.76 (0.03")	0.456
1.0	0.785		

Standard Analytical System

Due to the damper used in the 1100 and 1200 series pumps (G1311A/B and G1312A/B), the pump's dead volume is dependent on pressure, solvent type and composition. The following table summarizes the volumes of some 1100, 1200, 1260 and 1290 pumps and autosamplers with their optional set-ups. All listed autosamplers except the G2258A and G7158B have their metering devices in the main flow path; therefore, their volumes vary with the sample injection volume.

The calculation of the *Mixing point to UV detector* tubing volume consists of the following volumes:

- Pump (including mixer, if installed)
- Autosampler (Injector)
- Capillaries
  - Pump to autosampler
  - Autosampler to column
  - If used, heat exchange capillary in the thermostat of the column compartment (TCC)
  - Column to detector
  - Inlet capillary of the UV detector cell
  - Dead volume of the UV detector cell

Dead volumes of Agilent pumps

The dead volume of some modules is dependent on pressure and solvent; therefore, the table contains a calculation of the dead volumes for Water/Acetonitrile and Water/Methanol gradients using the initial pump pressure (p_0, bar) at the beginning of the gradient.

Pump	Volume, mL Water/ACN (p_0, bar)	Volume, mL Water/MeOH (p_0, bar)
G1311A/C G7111A 1120 Compact LC 1220 VL Compact LC	$p_0/2000 + 0.76$	$p_0/4000 + 0.82$
G1311B G7111B 1220 Compact LC	$p_0/2760 + 0.742$	$p_0/6060 + 0.787$

Pump	Volume, mL Water/ACN (p_0, bar)	Volume, mL Water/MeOH (p_0, bar)
G5611A	p_0/2860 + 0.725	p_0/2860 + 0.725
G1312A/C	p_0/3330 + 0.76	0.865
G1312B G7112B	No mixer: p_0/20000 + 0.205 Mixer: p_0/4000 + 0.77	No mixer: 0.225 Mixer: p_0/6670 + 0.83
G4204A G7104A/C	No mixer: 0.46 V380: 0.85	No mixer: 0.46 V380: 0.85
G4220A/B G7120A	No mixer: 0.033 V35: 0.075 V100: 0.16 V380: 0.39	No mixer: 0.033 V35: 0.075 V100: 0.16 V380: 0.39
G7161A	No mixer: 0.64 With mixer: 0.97	No mixer: 0.64 With mixer: 0.97
G7161B Two pressure sensors	No mixer: 0.14 With mixer: 0.47	No mixer: 0.14 With mixer: 0.47
G7161B One pressure sensors	No mixer: 0.64 With mixer: 0.97	No mixer: 0.64 With mixer: 0.97

Dead volumes of Agilent autosamplers

Because of the flow-through design, the injected volume must be added to the module's dead volume.

Autosampler	Syringe: Volume, mL
G7129A/B/C G7167A/B	40 µL: 0.04 + injected volume 100 µL: 0.062 + injected volume 900 µL: 0.177 + injected volume
G4226A	20 µL: 0.08 + injected volume 40 µL: 0.118 + injected volume 100 µL: 0.3 + injected volume
G1377A	8 µL: 0.048 + injected volume 40 µL: 0.142 + injected volume

Autosampler	Syringe: Volume, mL
G1367E	40 µL: 0.118 + injected volume 100 µL: 0.3 + injected volume
G1367D	40 µL: 0.142 + injected volume 100 µL: 0.3 + injected volume
G1313A G1329A/B G1367A/B/C G5667A 1120 Compact LC 1220 VL Compact LC 1220 Compact LC	100 µL: 0.3 + injected volume
G5668A	100 µL: 0.062 + injected volume
G7158B	5068-0348: 424 µL (Analytical loop + valve) 5068-0334 : 5577 µL (Preparative loop + valve) 5068-0335 : 11022 µL (Preparative loop + valve) 5068-0336 : 21908 µL (Preparative loop + valve) 5068-0350 : 2117 µL (Preparative loop + valve) 5068-0351 : 5256 µL (Preparative loop + valve)

Example of the calculation of the Mixing point to UV detector delay tubing volume

Calculation for a custom Agilent 1290 Infinity II binary system in a set-up using a thermostat capillary in the TCC module:

Part	Description	Volume, mL
Pump	Agilent 1290 Infinity Binary Pump with V100 mixer	0.160
Capillary	Pump to autosampler (0.17 × 200 mm)	0.005
Autosampler	Agilent 1290 Infinity Autosampler with 20 µL loop	0.080 + injected volume
Capillary	Autosampler to TCC (0.12 × 340 mm)	0.004
Capillary	TCC to column (0.12 × 150 mm)	0.002
Thermostat	Thermostat (3 µL)	0.003
Capillary	Column to UV detector (0.12 × 280 mm)	0.003

Part	Description	Volume, mL
UV Cell	Max-Light Cartridge, 10 mm cell	0.001
Total		*0.258 + injected volume*

Preparative System

The *Mixing point to UV detector* tubing volume of the preparative system or combined analytical and preparative system is calculated between the T-connection that connects the preparative pumps (for example, G1361A) or channels in a pump (for example, G7161A/B) and the UV detector cell. The calculation of the *Mixing point to UV detector* tubing volume comprises two steps:

1 Calculate *Mixing point to column* delay volume
   - Preparative flow path:
     - Include all capillary volumes in the flow path
     - Pump (see Table 11 on page 65)
     - Autosampler (see Table 11 on page 65)
     - UV detector cell if it is in the preparative flow path
   - Analytical flow path of combined instrument:
     - Include all capillary volumes in the flow path
     - Analytical pump (see the tables in Standard Analytical Systems)(“Standard Analytical System” on page 59)
     - Autosampler (see the tables in Standard Analytical Systems)(“Standard Analytical System” on page 59)
     - UV detector cell
2 Calculate the final *Mixing point to UV detector tubing volume* as the sum of:
   - Mixing point to column tubing volume (sdee above)
   - Column to UV detector tubing volume (see “Calculation of tubing volume: Column to UV detector” on page 56)

Example for the Agilent 1260 Infinity systems:

1 Calculate *Mixing point to column* delay volume

Table 8 Preparative flow path of combined instrument:

Item	Volume, mL
T-connection (preparative pumps)	0.002
T to mixer (0.6 × 40 mm)	0.011
Mixer (79835-87330)	0.75
Mixer to 2/10 valve (0.5 × 800 mm)	0.156
Valve to autosampler (0.5 × 600 mm)	0.117
Dual loop autosampler(G2258A), 5 mL loop	5.13
Autosampler to 2/10 valve (0.5 × 600 mm)	0.117
Valve to column (0.5 × 600 mm)	0.117
Total Preparative (mixing point to column)	*6.40*

Table 9 Analytical flow path of combined instrument:

Item	Volume, mL
Quat. Pump G1311B, Water/ACN gradient[1]	$p_0/2760 + 0.742$
Quat. pump to 2/10 valve (0.17 × 700)	0.016
Valve to autosampler (0.5 × 600 mm)	0.118
Dual loop autosampler (G2258A), 50 µL loop	0.05
Autosampler to 2/10 valve (0.5 × 600 mm)	0.118
Valve to column (0.17 × 400 mm)	0.009
Total Analytical (mixing point to column)	*$p_0/2760 + 1.053$*

[1] For different pump use volumes from tables in Standard analytical system section.

Table 10 UV/MSD-based instrument with UV detector in front of splitter (that is, in preparative flow) or UV-based preparative instrument

Item	Volume, mL
T-connection	0.002
Capillary to mixer (0.6 × 40 mm)	0.011
Mixer (79835-87330)	0.75
Capillary to autosampler (0.6 × 400 mm)	0.113
Dual-loop autosampler (G2258A), 5 mL loop	5.13
Capillary to Column (0.5 × 800 mm)	0.156
Total Preparative (mixing point to column):	*6.162*

2 Calculate the final *Mixing point to UV detector tubing volume* as the sum of:

- *Mixing point to column tubing volume*: step 1
- *Column to UV detector tubing volume*: see "Calculation of tubing volume: Column to UV detector" on page 56

NOTE

Because of piece-to-piece variations noted in preparative tubing, the calculated *Mixing point to UV detector tubing volume* can deviate from the actual value, typically by 5 – 10 % . For this reason, consider measuring this value for the preparative flow path experimentally as described in "Experimental measurement of delay volume: Mixing point to UV detector" on page 65 to minimize the error.

Because of the presence of the damper and degasser, it is not recommended that this value is measured experimentally for any analytical system.

Table 11 Dead volumes of some Agilent preparative modules.

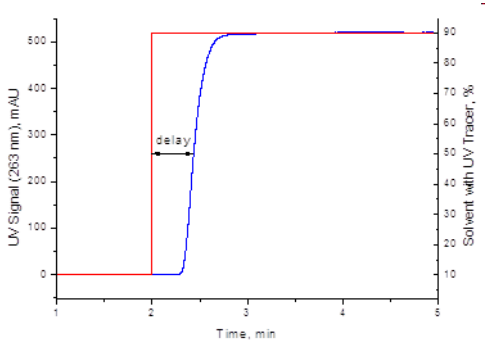
Module	Module Volume, mL
G7157A Autosampler	1.73 + injected volume 5 mL Extension loop: 6.86 + injected volume
G7158B Sampler	424 µL (Analytical loop + valve) 5068-0334: 5577 µL (Preparative loop + valve) 5068-0335: 11022 µL (Preparative loop + valve) 5068-0336: 21908 µL (Preparative loop + valve) 5068-0350: 2117 µL (Preparative loop + valve) 5068-0351: 5256 µL (Preparative loop + valve)
G7161A Pump	No Mixer: 0.64 With Mixer: 0.97
G7161B Pump Two pressure sensors	No Mixer: 0.14 With Mixer: 0.47
G7161B Pump One pressure sensor	No Mixer: 0.64 With Mixer: 0.97
G7115A DAD G7165A MWD	Inlet capillary and cell body: • 4 – 8 mL/min: 0.02 • 15 – 40 mL/min: 0.05 • 40 – 80 mL/min: 0.07 • 80 – 200 mL/min: 0.17
G7114A VWD	Inlet capillary and cell body: 0.06

Experimental measurement of delay volume: Mixing point to UV detector

Because of the presence of the damper and degasser in analytical pumps, it is not recommended that this value is measured by this procedure for any analytical system. Use calculation based on the known volumes of individual modules and capillaries as described in "Calculation of tubing volume: Mixing point to UV detector" on page 58.

Experimental characterization of the *Mixing point to UV detector* tubing volume is based on a step response of acetone as a UV tracer that is added to solvent B. The LC system without a column is first preconditioned to a starting percentage of solvent B and, during a run, a step change of % B is

programmed in the pump timetable. The time offset between the recorded and programmed positions of the tracer's step in the UV signal is the *Mixing point to UV detector* tubing volume, as shown in the following figure:



The figure shows an example of the measurement of *Mixing point to UV detector tubing volume* by step boundary using 1% acetone as UV tracer. The programmed profile of solvent B (is shown in red; the UV profile of the tracer is shown in blue. Instrument in analytical flow path of combined analytical and preparative system with 10 µL sample loop in flow path; flow 2 mL/min. Note that the current procedure uses a step of 40 – 60 % B.

Preparative system

NOTE

For the analytical flow path of the combined analytical and preparative instrument, use the calculation described in "Calculation of tubing volume: Mixing point to UV detector" on page 58.

1. Switch off all pumps.
2. Prepare the flow path:
   - **a** Replace the column by a union.
   - **b** If the flow path includes a splitter with a UV detector after it in the make-up flow, bypass the splitter by connecting the following tubing with a union:
     - HPLC stream inlet tubing from the preparative pump.
     - Make-up outlet tubing to the UV detector.
   - **c** Recommended: place a back-pressure regulator (2 – 7 bar, 40 – 100 psi) after the UV detector.

3 Prepare the solvents (solvents can contain additives):

   a Solvent A: water

   b Solvent B:

      - 1 % acetone in water as a UV tracer for 3 mm and 10 mm UV cells.
      - 10 % acetone in water as a UV tracer for 0.3 mm and 0.06 mm UV cells.

   c Degas both bottles in an ultrasonic bath for 10 min if the back-pressure regulator is not used.

   d Purge the solvent lines with the new solvents.

4 Set up a method as follows:

   - Use the *General_Purification* method settings as described in Default Purification Method ("Preparing Default Purification Methods" on page 9).
   - Save the Method as **`Prep_Mixing_Point_To_UV`**.
   - Set **Stoptime** to **No Limit** for all modules (infinite run time).
   - For the combined instrument, set the method to the preparative flow path.
   - Dual-loop autosampler if used:
      - Set the Injection loop: **Upper**
      - Make sure that the upper loop is in the main pass. Right-click on the autosampler diagram and check if the command **Switch Valve to Upper or Lower Loop** refers to the lower loop; if not, change to the upper loop using this command.
   - Set the UV detector wavelength to 263 nm with 4 nm bandwidth and without a reference.
   - Prep Pump:
      - Set the flow to 2 mL/min.
      - Set the solvent composition to 40 % B.
      - In the **Advanced Channel A** section, set the **Compressibility** to 46.
      - In the **Advanced Channel B** section, set the **Compressibility** to 46.
      - Set following pump time table:

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	60.0	40.0	2.000	200.00
1.99	60.0	40.0	---	---
2.00	40.0	60.0	---	---

- Save the method changes.

5 Display the 263 nm UV profile (signal A) in the **Online Plot**.

6 Switch on all modules.

7 Equilibrate the system with 40 % solvent B at 2 mL/min for 5 min.

8 If the pressure is below 20 bar, place a restriction capillary of known volume (0.12 × 2000 mm) in the position of the column and equilibrate the system again.

9 Open the **Sample Info** dialog box, clear **Vial/Location** (blank run), enter a run name and click **Run Method**.

10 Stop the run 2 min after the acetone UV signal has reached maximum absorption.

11 Evaluate the collected data (time in min and flow in mL/min):

a Determine the elution time of acetone (t_1) at one half of its UV signal height. See Determining the elution time of acetone for more details ("Determining the elution time of acetone" on page 69).

b Calculate the **Volume mixing point to UV detector** ($t_{MIX/UV}$) as the difference between the programmed and the actual elution times (subtract 2 min in this case) and multiply the result by the flow rate (2 mL/min): $V_{MIX/UV} = 2 \times (t_1 - 2)$.

c Subtract the volume of the restriction capillary, if used (0.12 × 2000 mm is 0.023 mL).

12 Run the procedure twice, and check if the determined volumes differ by less than 5 %. If not, repeat the procedure until the values are reproducible.

NOTE The reason for temporal irreproducibility of the measured volume can be, for example, the presence of air bubbles in the system (especially in the mixer or pump pistons) that can happen in a new or modified system, or when solvent bottles were exchanged. To improve performance, purge the pumps with a high flow, remove the restriction capillary (if present) and then set an acceptably high flow (respecting the UV cell pressure limit) to the whole system for a few minutes to flush out potential bubbles.

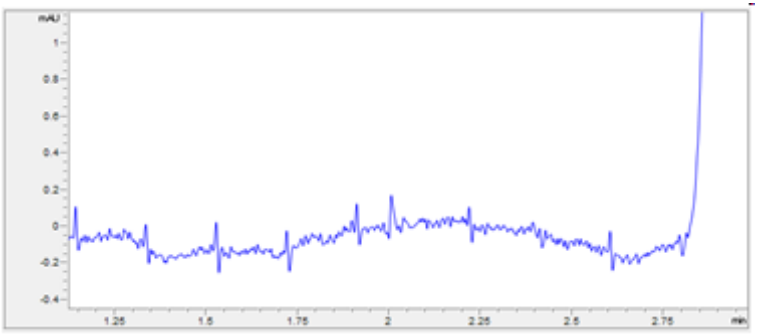
13 Repeat the procedure for the other flow path.

Determining the elution time of acetone

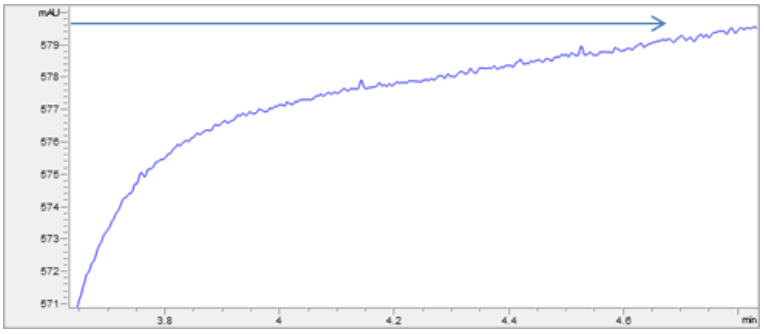
1 Open the appropriate data file with the recorded acetone UV trace (263 nm without reference).

2 Determine the absorbance at half the acetone UV signal height ($A_{1/2}$) with precision of about 0.1 mAU:

- Zoom in to the baseline before the elution of acetone and note this absorbance as A_1.



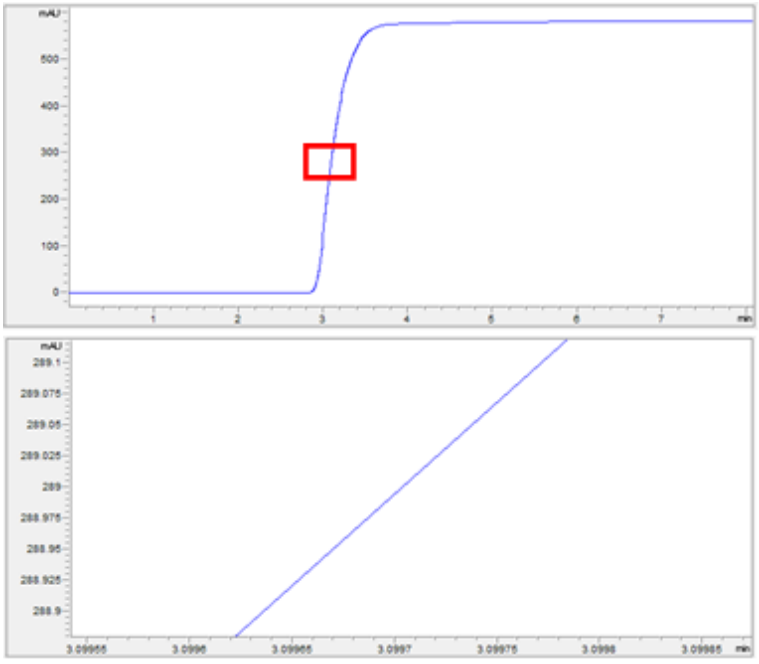
- Zoom in to the signal after the elution of acetone and note the absorbance approximately 1 min after the signal rises as A_2.



NOTE The signal after the elution of acetone is typically not completely stable, so it is sufficient to have only an approximate reading.

- Calculate $A_{1/2} = (A_1 + A_2)/2$

3 Zoom in several times to the curve around $A_{1/2}$ and note the elution time with a precision of three decimal places.



Characterizing the column volume

Chemicals

Both standard analytical system and preparative or combined system:

- Solvent A: water (optionally with 0.1 % formic acid)
- Solvent B: acetonitrile (optionally with 0.1 % formic acid)
- For G2258A: Needle and purge wash solution (degas in ultrasonic bath): 80 % acetonitrile or another suitable solution

Sample

- Thiourea or uracil in 75 % acetonitrile (in water); concentration based on the UV detector flow cell used. Read the MSDS data sheet for thiourea before preparing the sample from a powder.
- Recommended: For best column performance and lifetime, filter the sample before use with a regenerated cellulose syringe filter (5190-5108)

The characterization of the column volume is based on the elution of a non-retained compound (marker), thiourea or uracil. In the first step, the column is replaced by a zero-dead-volume connection, and the marker is eluted using as low a flow as possible with reliable performance. The retention time in this setup gives the volume of the tubing between the points of injection and detection. In the second step, the target column is installed and equilibrated with 75 % acetonitrile. The difference between the retention volumes of the marker in the setup with and without the column gives the target column volume. For best results, measure column volumes on a standard analytical LC system if available.

Characterization of the column volume using thiourea or uracil was optimized for ZORBAX StableBond C18 columns. A different type of stationary phase may require adjustment to the solvent composition or a different marker.

Column Volume: Standard Analytical System

1 Prepare a sample vial containing thiourea or uracil at the following concentration, and place it in the autosampler:

- 3 mm flow cell: 0.5 mM thiourea or uracil in 75 % acetonitrile.
- 10 mm flow cell: 0.3 mM thiourea or uracil in 75 % acetonitrile.
- 60 mm flow cell: 0.04 mM thiourea or uracil in 75 % acetonitrile.

2 Recommended: Filter the sample before use with the regenerated cellulose syringe filter.

3 Replace the column by a zero-dead-volume union.

4 Prepare the solvents:

- Solvent A: water (optionally with 0.1% formic acid).
- Solvent B: acetonitrile (optionally with 0.1% formic acid).
- Needle wash solution: 80 % acetonitrile or other suitable solution.
- Purge the solvent lines with the new solvents if not yet done.

5 Set up the method:

- Set the **StopTime** to **No Limit** in all modules (infinite run time).
- Pump:
  - Set **Solvents**: 75 % B.
  - Clear the **TimeTable**.
- Autosampler: Set the injection volume to 1 µL.
- UV detector:
  - Thiourea: Set Signal A wavelength to 242 nm with 4 nm bandwidth and without a reference.
  - Uracil: Set Signal A wavelength to 260 nm with 4 nm bandwidth and without a reference.

6 Display the relevant UV wavelength profile (242 nm or 260 nm) in the **Online Plot**.

7 Equilibrate the system with 75 % B at 1 mL/min (if possible) for 2 min.

8 Set the flow to 0.2 mL/min.

NOTE Use a restriction capillary of known volume to maintain the backpressure above 20 bar if necessary.

9 Open the **Sample Info** dialog box.

- Enter the **Vial/Location** of the sample vial
- Enter a run name.
- Run the method.

10 Stop the run after the delay marker peak has been detected.

11 Repeat twice.

12 Install the target column.

13 Equilibrate the column until the pressure and UV signal are stable.

14 Set a suitable flow in the range 0.2 – 4 mL/min in a way that the marker elutes at 1 min or later. Since the expected column volume is around one half of a geometric column volume (cross-sectional area multiplied by length), set the flow to around one half of a geometric column volume in mL (for example, the geometric volume of a 4.6 × 50 mm column is 3.14 × 4.6 × 4.6 × 50 ÷ 4000 = 0.83 mL, which suggests a flow rate of 1/2 × 0.83 mL / 1 min = 0.4 mL/min).

15 Adapt the injection volume to the applied flow (and column volume):

- Flow 0.2 – 0.5 mL/min: inject 1 µL.
- Flow 0.5 – 1.0 mL/min: inject 2 µL.
- Flow 1 – 2 mL/min: inject 5 µL.
- Flow > 2 mL/min inject 10 µL.

16 Note the applied flow rate and injection volume.

17 Open the **Sample Info** window, update the run name and run the method.

18 Stop the run after the delay marker peak has been detected.

19 Repeat twice; it is sufficient to measure runs longer than 5 min once or twice.

20 Evaluate the data:

a Read the elution time of all peaks at the apex.

b The elution volume without the column:

- Calculate the average elution time of data without the column.
- Multiply it by the applied flow rate.
- Subtract the volume of the restriction capillary if used and one half of the injection volume.

c The final column volume:

- Calculate the average elution time with the column.

- Multiply it by the applied flow rate.
- Subtract the elution volume without the column and one half of the injection volume.

Combined Analytical and Preparative System

NOTE Do not use the combined preparative system to determine the column volume of columns with ID smaller than 4.6 mm (column volume too low) and particle sizes below 3.5 µm (back pressure too high).

1. Prepare a sample vial containing thiourea or uracil at the following concentration, and place it in the autosampler (if the sample cannot be dissolved in 75 % acetonitrile under the given conditions, use pure water):
   - 3 mm preparative flow cell: 0.5 mM thiourea or uracil in 75 % acetonitrile.
   - 0.3 mm preparative flow cell: 5 mM thiourea or uracil in 75 % acetonitrile.
   - 0.06 mm preparative flow cell: 25 mM thiourea or uracil in 75 % acetonitrile.
2. Recommended: Filter the sample before use with the regenerated cellulose syringe filter.
3. Replace the column by a zero-dead-volume union.
4. Prepare the solvents:
   - Solvent A: water (optionally with 0.1% formic acid).
   - Solvent B: acetonitrile (optionally with 0.1% formic acid).
   - Needle wash solution (degas in ultrasonic bath): 80 % acetonitrile or other suitable solution (methanol should not be used with dual-loop autosampler due to peristaltic pump tubing – check the dual-loop autosampler manual).
   - Purge the solvent lines with the new solvents if not yet done.
5. Set up the method:
   - Use the Analytical_Purification method settings (see “Preparing Default Purification Methods” on page 9 for details).

- Save the Method as `Column_Volume`.
- Set the **Stoptime** to **No Limit** for all modules (infinite run time).
- Pump:
  - Set **Solvents**: 75 % B.
  - Clear the **TimeTable**.
- Injection volume 2 µL (*important:* increase the volume if the marker signal is too low).
- If you are using the dual loop autosampler:
  - Select **Lower** injection loop.
  - Select **Partial loop filling**.
  - Use **Plugs** settings as described in "Analytical Method Settings" on page 17 of the *Default Purification Method*.
- UV detector:
  - Thiourea: Set the Signal A wavelength to 242 nm with 4 nm bandwidth and no reference.
  - Uracil: Set the Signal A wavelength to 260 nm with 4 nm bandwidth and no reference.
- Save the method changes.

6 Display the relevant UV wavelength profile (242 nm or 260 nm) in the **Online Plot**.

7 If you are using the dual loop autosampler:
- equilibrate the upper loop:
  - Switch the valve to the upper loop
    - Right-click on the autosampler diagram and click **Switch Valve to Upper Loop**, If the command is not available, the upper loop is already selected.
  - Apply a pump flow of 4 mL/min for 3 min.
  - Switch the valve to the lower loop.
- Wash the needle:
  - right-click on autosampler diagram
  - **Wash Needle > Flush Port**15 s
- Purge the needle:

- right-click on autosampler diagram
- **Start Purging** 3 times

8 Set the flow to 1 mL/min.

NOTE Use a restriction capillary of known volume to maintain the backpressure above 20 bar if necessary.

9 Open the **Sample Info** dialog box.

- Enter the **Vial/Location** of the sample vial
- Enter a run name.
- Run the method.

10 Stop the run after the delay marker peak has been detected.

11 Important: if the marker signal in the detector is too low, increase the injection volume or concentration of the sample.

12 Repeat twice.

13 Install the target column.

14 Equilibrate the column until the pressure and UV absorbance are stable.

NOTE Use 1 – 4 column volumes to flush the column with a flow of 1.5 – 4 mL/min depending on size of the column. For example

- for a column 4.6 × 50 mm, use 1.5 mL/min for 2 min
- for a column 4.6 × 150 mm, use 2 mL/min for 3 min
- for a column 21.2 × 50 mm, use 4 mL/min for 7 min
- for a column 21.2 × 150 mm, use 4 mL/min for 20 min

15 Set the flow and injection volume considering inner diameter and length of the column:

- 50 mm long analytical columns: flow 1 mL/min and injection volume 2 µL.
- Longer analytical columns and preparative columns below 12 mm mm internal diameter: flow 2 mL/min and injection volume 5 µL.
- Other preparative columns: flow 4 mL/min and injection volume 10 µL.

NOTE If the injection volume is close to or exceeds the volume of the sample loop, use Injection Mode **Full loop with overfill factor** and set the factor to 5.

16 Note the applied flow rate and injection volume.

17 Open the **Sample Info** window, update the run name and run the method.

18 Stop the run after the delay marker peak has been detected.

19 Important: if the marker signal in the detector is too low, increase the injection volume or concentration of the sample.

20 Repeat twice; it is sufficient to measure runs longer than 5 min once or twice.

21 Evaluate the data:

- Read the elution time of the peak at the apex.
- The elution volume without the column:
  - Calculate an average elution time of data without the column.
  - Multiply it by the applied flow rate.
  - Subtract the volume of the restriction capillary if used and one half of the injection volume.
- Final column volume
  - Calculate the average elution time with the column.
  - Multiply it by the applied flow rate.
  - Subtract the elution volume without the column and one half of the injection volume.

System Configuration and Delay Time Calibration

Chemicals

- Solvent A: water with 0.1 % formic acid
- Solvent B: acetonitrile with 0.1 % formic acid
- Make-up solvent: 80 % acetonitrile in water with 0.1 % formic acid
- For G2258A: Needle purge and wash solution (degas in ultrasonic bath): 80 % acetonitrile or another suitable solution

Sample

For G1364B

- Prep LC Standards #2 (5190-6887) containing blue dye thionine acetate. Dilute the sample with pure water based on purification instrument setup, and it is recommended that you filter the sample with the regenerated cellulose syringe filter (5190-5108).
  - Preparative UV or UV/MSD instrument with 3 mm UV cell:

    Dilute 20 times (1 mL = 50 µL sample + 950 µL water).
  - Preparative UV or UV/MSD instrument with 0.3 or 0.06 mm UV cell:

    Dilute 10 times (1 mL = 100 µL sample + 900 µL water).
  - Combined preparative instrument:

    Dilute five times (1 mL = 200 µL sample + 800 µL water).

For G7159B, G1364E and G7166A

- Agilent Delay and Checkout Calibrant (5190-8223) containing a mixture with Patent Blue VF dye. It is recommended that you filter the sample with the PTFE syringe filter (5190-5084).

The delay time calibration determines a delay of a signal between a fraction collector (FC) and a detector.

NOTE

If a flow splitter is used, then the delay calibration is valid only for the specific combination of preparative and make-up pump flows. If one of the flows is changed, or if tubing after the splitter is modified, then the delay calibration must be rerun. This is because the sample signal is split in to two independent flow paths, splitter to FC and splitter to detectors, that generally have different flows. If one or both flows are changed, then the resulting delays (UV to FC and UV to MSD) cannot simply be recalculated to the new conditions.

A similar approach applies for a system without a splitter: it is recommended to re-measure the delay when either the preparative flow or the delay tubing is changed. The main reason is a possible change of a hydrodynamic velocity profile in the tubing when the flow is changed significantly, or the material or shape of the tubing is modified.

The following example illustrates the change of the delay time in the UV-to-FC calibration when the preparative flow is changed and a splitter is used. It is assumed that the splitter-to-FC flow path volume is 5 mL, with a flow of 30 mL/min, and the splitter-to-UV detector flow path volume is 50 µL, with a flow of 1.5 mL/min. Under these conditions, sample arrives from the splitter to the FC in 10 s, and from the splitter to the UV detector in 2 s. The delay time is then 10 – 2 = 8 s.

Now, if the preparative flow is changed from 30 mL/min to 60 mL/min, the resulting delay is 5 – 2 = 3 s.

So, in this example, by increasing the preparative flow from 30 mL/min to 60 mL/min, the delay time is reduced from 8 s to 3 s. Also, there are combinations of preparative and make-up flows that cannot be used; for example, if the preparative flow of 60 mL/min (that is, a 5 s delay from splitter to FC) and a make-up flow of 0.5 mL/min (that is, a 6 s delay from splitter to detector) are used, the resulting delay time becomes negative.

Running conditions for calibration procedures

Calibration results are unique for each set of preparative and make-up flows, MSD cycle time, and tubing. If one or more of them changes, the procedure has to be repeated. Therefore, before starting the procedure decide the final set-up of:

- *Preparative flow*

The Automated Purification Software offers the function of a scale-up from the analytical to the preparative flow or back using parameters of analytical and preparative columns. Since the results of the FC delay calibration are required for the initial setup of the software, it is easier to do a manual flow scale-up using one of following equations:

- For columns of the same porosity:

$$F_{prep} = F_{anal} \frac{d_{prep}^2}{d_{anal}^2} \frac{d_{p,anal}}{d_{p,prep}}$$

- For columns of different porosity:

$$F_{prep} = F_{anal} \frac{V_{0,prep}}{V_{0,anal}} \frac{L_{anal}}{L_{prep}} \frac{d_{p,anal}}{d_{p,prep}}$$

where

F = flow

d = diameter

L = column length

V_0 = column void volume

subscripts: $_p$ is particle, $_{anal}$ is analytical and $_{prep}$ is preparative

Porosity, in the context of the Automated Purification Software, is ratio of the void column volume to the geometrical column volume.

- *Make-up flow*

  Tubing design is optimized for 1.5 mL/min make-up flow that is split approximately 1:5 by a T-junction in front of the MSD in order to have the final flow to the MSD close to 0.2 – 0.3 mL/min. Note that decreasing the make-up flow increases the MSD-to-FC delay; increasing it decreases the signal response in all detectors after the splitter.

- *MSD cycle time* if MSD is used

  The MSD cycle time is a decisive parameter in ensuring that the calibrated delays for UV to FC and UV to MSD are sufficient. For a default setting follow the *Peakwidth* recommendation in the "Preparing Default Purification Methods" on page 9, and consider further optimization of the value as suggested in "Insufficient MSD-to-FC delay time" on page 89.

Example of F_{prep} calculation for two columns of the same porosity:

- Analytical column 4.6 x 50 mm (d_{anal} = 4.6 mm) with $d_{p,anal}$ = 5 µm particle diameter

- Preparative column 21.2 x 50 mm (d_{prep} = 21.2 mm) with $d_{p,prep}$ = 5 µm particle diameter
- Analytical flow F_{anal} = 1 mL/min

The resulting scaled-up preparative flow $F_{prep} = 1 \times 21.2^2 / 4.6^2 \times 5 / 5 = 21$ mL/min.

Configuring the Analytical and Preparative Systems

In the **Purification** menu of the ChemStation, select **Systems** to open the **System Parameters** dialog box. The analytical system parameters are on the left of the divided window. Click **Add system** to add a new system, and set the following:

1 *List of systems*:
   - Name and description of the system

2 **Pumps and Detectors** tab:
   - Select the **System Type**
   - In the **Pumps** section:
     - Select the **Main Pump**
     - Select the analytical pump module and specify the solvent channels.
     - Set the analytical flow.
   - In the **Detectors** section:
     - Mark **MSD** and **Auxiliary detector** if used
     - Select UV detector and flow cell

3 **Delay Volumes** tab:
   - Tubing
     - Select the instrument setup:
       - *Custom Volumes:* If you are using a custom system, measure and/or calculate the tubing volumes as described in Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector ("Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector" on page 52).

- *Predefined Volumes:* Selecting a predefined system fills tubing volumes and the position of the UV detector in relation to the splitter if an MSD is used.
- For Predefined Volumes, enter the **Initial Pressure** The 1260 quaternary analytical pump has a dwell volume dependent on the actual pressure. In order to minimize any possible discrepancy, enter the initial pressure on the pump at the gradient start conditions.

- Delays
  - If the delays for Analytical systems are required for MSD and/or auxiliary detectors, follow the instructions in "Delay time determination for Analytical systems (MSD and/or auxiliary detectors)" on page 82

4 **Column** tab:
- Enter the name and parameters of the column.
- Void volume and porosity are recalculated to each other when edited using the diameter of the column (porosity is the ratio of void and geometrical column volume).

Delay time determination for Analytical systems (MSD and/or auxiliary detectors)

1 Acquire an analytical run in ChemStation as described in "Acquire an analytical run using the Chemstation sequence" on page 93.

2 Go to **Data Analysis** in ChemStation and load the acquired data.

3 Calculate the delays, for example, using the caffeine peak (the first major analyte peak) based on the elution times in all detectors used:
- Based on the extracted EIC 195 ion trace from MSD1 TIC signal calculate: Time UV detector to MSD = MSD1 EIC 195 signal – UV detector signal
- Time UV detector to auxiliary detector = UIB1 A (Analog In) – UV Detector signal

4 Enter the values into the respective fields in the **Analytical systems** dialog box.

Configuring the Preparative System Parameters

In the **Purification** menu of the ChemStation, select **Systems** to open the **System Parameters** dialog box. The preparative system parameters are on the right of the divided window. Click **Add system** to add a new system, and set the following:

1 *List of systems*:
   - Name and description of the system

2 **Pumps and Detectors** tab:
   - In the **Pumps** section:
     - Select the **Main Pump**.
     - Select the preparative pump module (cluster) and specify the solvent channels.
     - Set the preparative flow.
     - If a make-up splitter is used, mark **Use make-up pump** and select the pump module. Select the make-up solvent **Channel** and set the make-up **Flow.**
   - In the **Detectors** section:
     - Mark the **Use MSD** check box if the MSD is used to trigger fraction collection.
     - Mark the **Use auxiliary detector** check box if an auxiliary detector is used, and select the module.
     - Select the UV detector module that will be used for evaluation of the preparative UV signal. This detector can be located in the preparative flow path or in the make-up flow path, depending on the instrument configuration.
     - Select the flow cell.
     - Select the **Peak width** mode of the UV detector. The recommended frequency value is 2.5 Hz if the Agilent active make-up splitter, Agilent flow modulator or slope-based fraction triggering are used. Otherwise, 20 Hz is recommended.
     - Select the location of the UV detector based on its position in the flow path.

3 **Delay Volumes** tab:
   - Tubing

- Select the instrument setup:
  - *Custom Volumes:* If you are using a custom system, measure and/or calculate the tubing volumes as described in Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector ("Characterizing the tubing volumes: Mixing point to UV detector and Column to UV detector" on page 52).
  - *Predefined Volumes:* Selecting a predefined system fills tubing volumes and the position of the UV detector in relation to the splitter if an MSD is used.
- Delays and fraction collector calibration
  - Choose the **automated** option in **Fraction collector calibration** to perform a calibration run as described in "Delay time calibration for Preparative Systems" on page 85.
  - Delay fields are editable only if the **manual** option is selected. Also, if an MSD or auxiliary detectors are not set up, the related fields are not editable.
  - To enable the entry of known delays in Preparative systems, **Fraction collector calibration** must be set to **manual**.
  - If the **manual** option is selected and **Calculate delays to fraction collector** is marked, then **Time MSD to fraction collector** and **Time auxiliary detector to fraction collector** are calculated from other delays (UV to FC and UV to detector). If **Calculate delays to fraction collector** is cleared, then they can also be edited, and you must ensure integrity of entered delays (due to interconnection of UV to FC, UV to detector and detector to FC).
  - **Write delay times** stores the entered detector-to-fraction collector delay times to the instrument.

NOTE The **Write delay times** action is not mandatory but then the delay time values have to be written to the instrument at the beginning of every task and the original values are restored after the task is finished. This can significantly prolong the overall run time.

4 **Column** tab:

- Enter the name and parameters of the column.
- Void volume and porosity are recalculated to each other when edited using the diameter of the column (porosity is the ratio of void and geometrical column volume).

Delay time calibration for Preparative Systems

If a flow splitter is used, the calibrated values can be used only for runs with identical tubing and settings of preparative and make-up pump flow. In addition, a change of the data rate (that is, peakwidth for UV or cycle time for MSD), of the detector that is used for fraction collection, can cause previously measured delay times to be insufficient.

Preparation

1 Prepare the solvents:

- Solvent A: water with 0.1 % formic acid
- Solvent B: acetonitrile with 0.1 % formic acid
- Make-up solvent: 80 % acetonitrile in water with 0.1 % formic acid
- Make sure that a 20 psi back-pressure regulator (PN 5067-6840) is installed in the waste line behind the delay sensor of a collector.
- For G2258A: Needle purge and wash solution (degas in ultrasonic bath): 80 % acetonitrile or another suitable solution (methanol should not be used with dual-loop autosampler due to peristaltic pump tubing – check the dual-loop autosampler manual).

2 If not yet done, install the preparative column (recommended SB-C18 21.2 × 50 mm).

3 Prepare a sample:

G1364B:

- Dilute the Prep LC Standards #2 (5190-6887) sample with water, depending on the purification instrument setup, and filter the sample before use with the regenerated cellulose syringe filter (5190-5108):
  - Preparative UV or UV/MSD instrument with 3 mm UV cell: Dilute 20× (1 mL = 50 µL sample + 950 µL water).
  - Preparative UV or UV/MSD instrument with 0.3 mm or 0.06 mm UV cell: Dilute 10× (1 mL = 100 µL sample + 900 µL water).
  - Combined preparative instrument: Dilute 5× (1 mL = 200 µL sample + 800 µL water).

G7159B, G1364E and G7166A:

- Use undiluted Agilent Delay and Checkout Calibrant (5190-8223). It is recommended that you filter the sample with the PTFE syringe filter (5190-5084).

4 Place at least 1000 µL of diluted sample in a vial and place it in the injector.

5 For G1364B and G1364E: place an empty vial tray or well-plate tray in the fraction collector.

FC Delay Calibration

1 From the **Purification** menu in the Chemstation, open the **System Parameters** dialog box.

2 Select the preparative system configured in "Configuring the Preparative System Parameters" on page 83.

3 In the **Fraction collector calibration** section, select the **automated** mode and click **Do calibration run**.

4 In the **Setup fraction collector delay calibration** dialog box, enter the following:

- **Location of calibration sample**: the location of the *Prep LC Standards #2* vial.
- **Amount of calibration sample**: an injection volume of 80 mL. Adjust if necessary.
- **Calibration base method**: browse for the *Prep_Purification* method created in "Preparing Default Purification Methods" on page 9.
- If you are running the first calibration run, mark the **Clean sampler and equilibrate column before calibration** check box. For subsequent calibration runs, it is not necessary to repeat the cleaning.

5 Click **Start calibration** and then follow the instructions during the run.

6 When the calibration run is finished, click **Evaluate**.

7 In the **Delay calibration data evaluation** dialog box:

a Ensure that the peaks in the UV detector, fraction collector sensor signals and, if present, the MSD and auxiliary signals are integrated and identified (the integrated peak area is dark blue).

b MSD signal ion masses are predefined in the automatic evaluation procedure for each calibration sample. If multiple ion masses are expected, the procedure selects the most abundant one for the evaluation:

- G1364B with Prep LC Standards #2 (5190-6887):
  - Positive polarity (m/z): $[M+H]^+$ (228.1).
  - Negative polarity (m/z): $[M-H]^-$ (226.1).
- G7159B and G1364E with Agilent Delay and Checkout Calibrant (5190-8223):
  - Positive polarity (m/z): $[M+H]^+$ (545.2), $[M+2H]^{2+}$ (273.1), $[M+Na]^+$ (567.2) and $[M-H+2Na]^+$ (589.2).
  - Negative polarity (m/z): $[M-H]^-$ (543.2).
- If another ion mass is required for the evaluation under given conditions, select the relevant MSD signal in **Selected signal** and modify the **Ionic mass value**.

c If all acquired delay times are valid (no red error icon is shown next to the value), click **Accept** to write them into the respective boxes in the **Preparative systems** dialog box.

d If any of the acquired delay times are invalid, click on the adjacent error icon and follow the instructions provided.

e If the UV, MSD or fraction collector sensor signal is too low or too high, adjust the injection volume between 20 µL and 200 µL, or adjust the sample dilution. Repeat the calibration.

f If it is still not possible to calibrate all detector signals at once, then there is a possibility to run the calibration piecewise:

- It is necessary to obtain the detector-to-fraction collector delay for at least one detector signal in this window, which means that the fraction collector signal and one more signal must be capable of being evaluated. Note the correct delay value(s).
- Under identical flow conditions, and MSD and UV detector peakwidth settings, acquire the signal data in ChemStation with another suitable sample.
- Manually evaluate elution times, and from their differences together with already noted delay values, calculate all missing delay values.
- Enter the delay values manually in the **Delay Volumes** tab.

g It is recommended that you repeat the calibration at least once to avoid error from various sources such as bubbles in the instrument or its insufficient equilibration.

8 In the **Preparative systems** dialog box, click **Write delay times** to write the UV-to-fraction collector and auxiliary-to-fraction collector delays to the firmware of all fraction collectors.

This prevents a warning that the preparative system contains a different delay value than the collectors, and also avoids extra run time before and after the preparative sequence when the delay value needs to be written to the collectors.

Insufficient UV-to-FC delay time or MSD-to-FC delay time

Insufficient UV-to-FC delay time

The minimum required delay time for UV detector triggered collection is: 4/frequency + Response time. The term 4/frequency stands for four data points, which are needed in the slope-based collection.

There are several reasons why the UV-to-FC delay time may be too low:

- The UV detector data rate is too low. A low UV data rate, especially in combination with a high flow rate, may require a delay coil to ensure that the UV detector has enough time to evaluate the FC trigger signal.
- The volume of delay tubing in front of the fraction collector is too low.
- The make-up flow is too low for the applied preparative flow. There are limitations for combinations of preparative and make-up flows; see the example in "System Configuration and Delay Time Calibration" on page 78.
- The volume of tubing between the splitter and the detector(s) is too high.

There are several ways to increase the UV-to-FC delay time, but some of them can have a certain disadvantage (given in parentheses), so consider the most suitable option for the given situation:

- Increase the UV data rate (a higher data rate typically has higher noise, so in combination with the Slope mode for collection, the higher detector noise can cause a false FC start/stop signal if the noise is visible along a collected peak).
- Use a longer delay coil in front of the fraction collector (a higher coil volume causes more dispersion, so use coils with reduced dispersion rate such as knitted coils; also, a longer coil with the same inner diameter will increase back-pressure).
- Decrease the preparative flow (influences chromatographic resolution).

- Increase the make-up flow (increases the speed of the analytes when passing through the detectors, which typically results in narrower and/or smaller peaks, so consider whether or not the detector data rate is sufficient for the signal response).
- Decrease the volume of tubing between the splitter and the detector(s) in the make-up flow path.

After one or more solutions have been applied, repeat the fraction collector delay calibration from the beginning.

NOTE In general, do not use more than one delay coil kit to ensure that the pressure limit of the coils and other components earlier in the flow path of the system is not exceeded. If the required fraction collection delay is higher than can be satisfied with one delay coil kit, follow the recommendations above to reduce the required delay. If another delay kit is still necessary, make sure that the pressure limits are satisfied for all components in the flow path.

Insufficient MSD-to-FC delay time

The minimum required delay time for MSD triggered collection either 5 sec or 6 × (MSD cycle time), whichever is the higher.

The reasons for insufficient MSD-to-FC delay time could be the following:

- The volume of delay tubing in front of the FC is too low.
- The MSD cycle time is too high.
- The make-up flow is too low for the applied preparative flow. There are limitations to the combinations of preparative and make-up flows; see the example in "System Configuration and Delay Time Calibration" on page 78.
- The volume of tubing between the splitter and detector(s) is too high.

The following suggestions are options for increasing the MSD-to-FC delay time, but each of them has a certain disadvantage (given in parentheses) so consider the most suitable way for the given situation:

- Optimize the MSD cycle time in the MSD method (a cycle time higher than 0.83 sec will increase the required delay volume limit):
  - Use only one polarity if suitable (requires detailed knowledge of collected compounds).
  - Optimize peakwidth parameter: decrease it until MSD signal noise is still acceptable.

  - Decrease the MW range or increase the MW step size if suitable: typically, it will not directly decrease the cycle time but it allows further decreasing of the peakwidth parameter (a short MW range hides potential contaminants).
  - Consider using the MSD (ultra)fast mode, which enables a lower cycle time.
  - Performing MSD maintenance can also help in optimization of the noise level.
- Use a longer delay coil in front of the FC (a higher coil volume causes more dispersion, so use coils with a reduced dispersion rate such as knitted coils; also, a longer coil with the same inner diameter will increase back-pressure).
- Decrease the preparative flow (influences the chromatographic resolution).
- Increase the make-up flow (increases speed of the analytes when passing through detectors, which typically results in narrower and/or smaller peaks, so consider whether or not the detector data rate is sufficient for the signal response).
- Decrease the volume of tubing between the splitter and the detector(s).

After one or more solutions have been applied, repeat the fraction collector delay calibration from the beginning.

NOTE

In general, do not use more than one delay coil kit to ensure that the pressure limit of the coils and other components earlier in the flow path of the system is not exceeded. If the required fraction collection delay is higher than can be satisfied with one delay coil kit, follow the recommendations above to reduce the required delay. If another delay kit is still necessary, make sure that the pressure limits are satisfied for all components in the flow path.

6
Checkout Procedure

This chapter gives step-by-step instructions on how to carry out a checkout procedure to confirm the correct operation of your Purification system.

Chemicals

For all systems (stand-alone analytical, preparative or combined analytical and preparative):

- Solvent A: water with 0.1 % formic acid
- Solvent B: acetonitrile with 0.1 % formic acid
- Make-up solvent (used with splitter): 80 % acetonitrile in water with 0.1 % formic acid
- For G2258A: Needle purge and wash solution (degas in ultrasonic bath): 80 % acetonitrile or other suitable solution
- DMSO (dimethyl sulfoxide) for sample dilution

Columns

- Analytical (recommended): 4.6 × 50 mm, 5 µm (846975-902)
- Preparative (recommended): 21.2 × 50 mm, 5 µm (870050-902 with end fittings 820400-901)

Sample

- *Prep LC Standards #1* (5190-6886) containing check-out mixture. For the analytical run, dilute the sample with DMSO (or other compatible solvent) based on the UV cell length:
  - 3 mm UV cell (10 µL injection with dual-loop autosampler): Dilute 10× (1 mL = 100 µL sample + 900 µL solvent)
  - 10 mm UV cell (10 µL injection with dual-loop autosampler): Dilute 40× (1 mL = 25 µL sample + 975 µL solvent)

  - 10 mm UV cell (1 µL injection): Dilute 20× (1 mL = 50 µL sample + 950 µL solvent)
  - 60 mm UV cell (1 µL injection): Dilute 100× (1 mL = 10 µL sample + 990 µL solvent)
- *Plug vial solvent*:

  For G2258A: 2 % acetonitrile (or Solvent A or other compatible solvent). Refresh the solvent frequently to avoid bacterial growth and injection cross-contamination.

  For G7158B sampler, use plug solvent(s) as defined in its method.
- Recommended: For best column performance and lifetime, filter the sample in DMSO solvent before use with the PTFE syringe filter (5190-5084). Filter other sample solvents (water, acetonitrile) such as the plug solvent with the regenerated cellulose syringe filter (5190-5108). To select the most suitable filter type with the highest sample recovery, filter the sample solvent matrix without the sample first.

Acquire an analytical run using the Chemstation sequence

NOTE If the analytical run is to be acquired as a single run for the Automated Purification Software, it is important to mark the **Save Method with Data** check-box in **Method > Runtime Check List**. This applies to both the combined preparative instrument and the stand-alone analytical instrument.

1 Set up a generic gradient in the analytical method:

   a If using a combined analytical and preparative system:

      a Use the *Analytical_Purification* method setting as described in "Preparing Default Purification Methods" on page 9.

      b Save the method as `Analytical_Gradient`.

   b Enter the recommended generic gradient in the method:

   - **Pump** > **Solvents** > B: 2 %
   - **Pump** > **Timetable**:

     For an Agilent 1260 Infinity analytical run using a 4.6 × 50 mm, 5 µm column and a **Flow** of 1 mL/min:

Time (min)	A (%)	B (%)
0.00	98	2
0.50	98	2
5.30	2	98
6.40	2	98
6.50	98	2

Stoptime: 9.0 min

     For an Agilent 1290 Infinity II analytical run with a binary pump using a 2.1 × 50 mm, 1.8 µm column and a **Flow** of 0.6 mL/min:

Time (min)	A (%)	B (%)
0.00	98	2
0.17	98	2
1.83	2	98
2.20	2	98
2.30	98	2

Stoptime: 3.3 min

Set the recommended **Stoptime** and **Flow** as given above.

c Set the injection volume:

- Stand-alone analytical system: 1 µL injection (increase if necessary).
- Combined analytical and preparative system: 10 µL injection. If using the G2258A Dual-Loop Autosampler with a 10 µL injection loop, set **Full loop with overfill factor** 5; otherwise, for larger loops set **Partial loop filling** to 10 µL and set **Plug Settings**:

  a Mark **Plug Settings** > **Draw Plug before and after the sample**.

  b Set **Plug Settings** > **Plug Volume**: Enter the maximum plug volume: Plug volume = ½[(Loop volume) – (Injection volume)]

  That is, for a 10 µL injection to the 50 µL loop, set the plug volume to 20 µL.

  c Place plug solvent with 2 % acetonitrile (or Solvent A) into the autosampler. Use another solvent if necessary.

  d Enter the position of the plug vial in the autosampler method: **Plug Settings** > **Draw Plug from**.

d Save the Method.

2 Dilute the *Prep LC Standards #1* sample for analytical run with DMSO (or other compatible solvent) based on the system and UV cell length:

- Combined analytical and preparative system (for 10 µL injection):
  - 3 mm UV cell: dilute 10× (1 mL = 100 µL sample + 900 µL solvent).
  - 10 mm UV cell: dilute 40× (1 mL = 25 µL sample + 975 µL solvent).
- Stand-alone analytical system (for 1 µL injection):
  - 10 mm UV cell: dilute 20× (1 mL = 50 µL sample + 950 µL solvent).

- 60 mm UV cell: dilute 100× (1 mL = 10 µL sample + 990 µL solvent).

3 Place 1 mL filtered sample in the autosampler.

4 Create a new sequence: **Sequence > New Sequence Template**

5 Set **Sequence > Sequence Parameters** (for example, folder path, shutdown macro).

6 Complete the sequence table: **Sequence > Sequence Table**:

- **Location**: sample position.
- **Sample name**: Check-out sample
- **Method name**: select the analytical method (**Analytical_Gradient**)
- **Inj/Location**: 1
- Leave **Sample amount** field empty
- Click **OK**

7 **Sequence > Save Sequence Template As**`Checkout_Sample`

8 Display the 263 nm UV profile (signal A) in the **Online Plot**.

9 Turn on the pump.

10 For the G2258A Dual-Loop Autosampler:

a Equilibrate the upper loop:

a Switch the valve to the upper loop (right-click on the autosampler diagram and select **Switch Valve to Upper Loop**; if the command is not available, the upper loop is already selected).

b Apply a flow of 2 mL/min for 5 min

c Switch the valve to the lower loop

b Wash the needle (right-click on the autosampler diagram and select **Wash Needle > Flush Port > 15 s**)

c Purge the needle (right-click on the autosampler diagram and select **Start Purging > 3×**)

11 Set the target analytical flow (default is 1 mL/min for a 4.6 mm ID column and 0.6 mL/min for a 2.1 mm ID column).

12 Equilibrate the column until the pressure and UV signal are stable.

13 **RunControl > Run Sequence.**

14 Rerun the sample at least once and check the results of the sequence visually (peak shape, resolution). If the UV absorbance of five major peaks

is too low (below 100 mAU) or too high (above 1500 mAU), adjust the injection volume or sample concentration and measure the sample again.

15 When the sequence is finished, turn off the pump(s).

UV-only Workflow: Focused gradient in the Purification software

This procedure is the analytical-to-preparative workflow for the standalone UV-only preparative instrument.

1 Place at least 1.5 mL of filtered undiluted *Prep LC Standards #1* sample in the autosampler.

2 For the G2258A Dual-Loop Autosampler:

   a Switch the valve to the upper loop (right-click on the autosampler diagram and select **Switch Valve to Upper Loop**; if the command is not available, the upper loop is already selected).

   b If the autosampler has been idle for more than one hour:

   - Wash the needle (right-click on the autosampler diagram and select **Wash Needle > Flush Port > 15 s**)
   - Purge the needle (right-click on the autosampler diagram and select **Start Purging > 3×**)

3 Precondition the preparative column if it has not been used recently:

   a Set the preparative flow and set 95 % of solvent B (default flow: 20 mL/min for 21.2 × 50 mm column and 1 mL/min analytical run flow).

   b Turn on all pumps.

   c Apply 95 % of solvent B for approx. 1 min.

   d Apply 2 % of solvent B until UV signal and pressure are stable.

   e Turn off all pumps.

4 In the ChemStation, open the **Tasks** dialog box from the **Purification** menu.

5 Create a new task:

   - Click **Add Task**.
   - Select **Add empty Analytical to preparative task**.

6 In the **Task Configuration** tab:

   - **Select System**
     - Select the analytical and preparative systems.
     - Review the analytical and preparative flows in the **Pumps and Detectors** tab if the target flows are set.

- **Analytical Run**
  - Click **Browse** and select the acquired analytical sequence (**Checkout_Sample**)
- **Preparative Run**
  - Select the **Preparative base method** (**Prep_Purification**)
  - Keep **Review analytical results before purification** marked.
  - Set the preparative vial **Location**.
  - Set an appropriate **Inj. volume** (for example, 200 µL).
- **Fraction collection** tab (in **Preparative Run**)
  - UV detector: select **Trigger mode** > **Threshold**.

7 Click **Run**.

8 Set the parameters in **Analytical Results**:

- Set a suitable threshold in the **Fraction collection** tab:
  - UV detector: set **Threshold** to 5 or adjust the value to achieve a threshold of about 5 % of the peak height in the preparative run.

9 Review the **Analytical Results** (check the settings and the target peak):

- Select the first major peak (caffeine) in the chromatogram and click **Assign As Target** above the chromatogram.

10 Click **Run** again to submit the preparative task to the ChemStation run queue.

11 When the preparative task is completed, check in **Preparative Results** that the correct peak was focused and that it is among collected fractions.

12 Create a new task:

- Click **Add Task**.
- Click **Clone currently selected task**.

13 Review the **Analytical Results**:

- Select the third major peak (Ethyl 4-hydroxybenzoate) in the chromatogram and click **Assign As Target** above the chromatogram.

14 Click **Run**.

15 When the preparative task is completed, check in **Preparative Results** that the correct peak was focused and that it is among collected fractions.

UV/MS Workflow: Focused gradient in Purification software

This procedure is the analytical-to-preparative workflow for the combined analytical and preparative UV/MSD instrument and for standalone preparative UV/MSD instrument.

1. Place at least 1.5 mL of filtered undiluted *Prep LC Standards #1* sample in the autosampler.
2. For the G2258A Dual-Loop Autosampler:
   a. Switch the valve to the upper loop (right-click on the autosampler diagram and select **Switch Valve to Upper Loop**; if the command is not available, the upper loop is already selected).
   b. If the autosampler has been idle for more than one hour:
      - Wash the needle (right-click on the autosampler diagram and select **Wash Needle > Flush Port > 15 s**)
      - Purge the needle (right-click on the autosampler diagram and select **Start Purging > 3×**)
3. Precondition the preparative column if it has not been used recently:
   a. Set the preparative flow and set 95 % of solvent B (default flow: 20 mL/min for 21.2 × 50 mm column and 1 mL/min analytical run flow).
   b. Turn on all pumps.
   c. Apply 95 % of solvent B for approx. 1 min.
   d. Apply 2 % of solvent B until UV signal and pressure are stable.
   e. Turn off all pumps.
4. In the ChemStation, open the **Tasks** dialog box from the **Purification** menu.
5. Create a new task:
   - Click **Add Task**.
   - Select **Add empty Analytical to preparative task**.
6. Select the **Task Configuration** tab.
   - **Select System**
     - Select the analytical and preparative systems from "Configuring the Analytical and Preparative Systems" on page 81.

- Review the analytical and preparative flows in the **Pumps and Detectors** tab if the target flows are set.

- **Ion Species**
  - Analytical run: mark **+H** and **–H** species for the analytical run.
  - Preparative run: mark **Use best performing ion from analytical run**.
- **Analytical Run**
  - Click **Browse** and select the acquired analytical sequence (**Checkout_Sample**)
- **Preparative Run**
  - Select the **Preparative base method** (**Prep_Purification**)
  - Keep **Review analytical results before purification** marked.
  - Set the preparative vial **Location**.
  - Set an appropriate **Inj. volume** (for example, 200 µL).
- **Fraction collection** tab (in **Preparative Run**)
  - Select **Trigger mode** > **Threshold** for both MSD and UV detectors.

7 Click **Run**.

8 Set the parameters in **Analytical Results**:

- Enter a **Target mass** of 166.1 (Ethyl 4-hydroxybenzoate).
- Click **Re-evaluation** to apply the change.
- Set suitable thresholds in the **Fraction collection** tab:
  - MSD detector (if available):
    - Display MSD ion species curve in the graph.
    - Zoom to baseline and determine its highest level.
    - Set the **Threshold** value to five times the determined analytical baseline level or 2000, whichever is higher.
  - UV detector: set **Threshold** to 5 or adjust the value to achieve a threshold of about 5 % of the peak height in the preparative run.

9 Review the **Analytical Results** (check the settings and the target peak):

- MS-based target identification:
  - Check in the chromatogram that the correct peak was selected: Ethyl 4-hydroxybenzoate (166.1) is the third major peak of the mixture to elute.

- Also check that the ethyl 4-hydroxybenzoate peak was identified in both the UV detector and the MSD target ion species. If the UV and MSD are not well aligned, then the problem can be in UV/MSD delay value or integration parameters. If the UV signal is saturated, the integration parameters may need to be changed.

10 Click  **Run** again to submit the preparative task to the ChemStation run queue.

11 When the preparative task is completed, check in **Preparative Results** that the correct peak was collected.

12 Create a new task:

- Click **Add Task**.
- Click **Clone currently selected task**.

13 Set the parameters in **Analytical Results**:

- Enter the **Target mass** of 194.1 (Caffeine)
- **Scale-Up** tab: set **Minimal target candidate peak height in EIC** to 200000.
- Click **Re-evaluation** to apply the change.
- Set suitable thresholds in the **Fraction collection** tab:
  - MSD detector:
    - Display MSD ion species curve in the graph.
    - Zoom to baseline and determine its highest level.
    - Set the **Threshold** value to five times the determined analytical baseline level or 2000, whichever is higher.
  - UV detector: set **Threshold** to 5 or adjust the value to achieve a threshold of about 5 % of the peak height in the preparative run.

14 Review the **Analytical Results** (check the settings and the target peak):

- Check in the chromatogram that the correct peak was selected: caffeine (194.1) is the first major peak of the mixture to elute. Note that caffeine, as a complexing agent, can create ghost peaks in the MSD signal of caffeine on positions of other mixture compounds. For this purpose, the **Minimal target candidate peak height in EIC** default value was increased. Change the value if necessary.
- Also check that the caffeine peak was identified in both the UV detector and the MSD target ion species. If the UV and MSD are not well aligned, then the problem can be in UV/MSD delay value or integration

parameters. If the UV signal is saturated, the integration parameters may need to be changed.

15 Click  **Run** again to submit the preparative task to the ChemStation run queue.

16 When the preparative task is completed, check in **Preparative Results** that the correct peak was focused and collected.

The table below lists the compounds in the *Prep LC Standards #1* sample, their most abundant isotopic masses, formulas and a typical ion species for MS-based fraction collection:

Compound	Most abundant isotopic mass	Formula	Typical Ion species
Caffeine	194.1	$C_8H_{10}N_4O_2$	M+H
Methyl 4-hydroxybenzoate	152.0	$C_8H_8O_3$	M-H
Ethyl 4-hydroxybenzoate	166.1	$C_9H_{10}O_3$	M-H
Propyl 4-hydroxybenzoate	180.1	$C_{10}H_{12}O_3$	M-H
Benzyl 4-hydroxybenzoate	228.1	$C_{14}H_{12}O_3$	M-H

7 Generating Support Information

This chapter describes how to generate information to help Agilent support in troublshooting.

In the event of an unexpected error, the Automated Purification Software can generate a support information file containing all information necessary for Agilent support to troubleshoot the problem. You can generate the support file from two places:

- **Generate Agilent Support Information** on the **Miscellaneous** page of the **Administration** dialog box
- **Generate Support Information** on the Purification Software's **About** box

On clicking either button, the Automated Purification Software shows a dialog box that allows you to select a task for inclusion in the support file. Task folders anywhere in the file system can be selected, which allows you to include a task run by a different user. When a task is selected, the Automated Purification Software automatically collects all information connected to the task, including methods and results outside the task folder. If you clear the **include a task in the support file** check box, a support information file without a task is generated.

On successful generation, the support information file is stored in the current instrument's TEMP folder (usually located at C:\ProgramData\Agilent Technologies\ChemStation\<instrument number>\TEMP). You may send this file to Agilent support to assist us in helping you with your problem. The Automated Purification Software does not send any information to Agilent on its own.

NOTE

Support files that you submit could contain personally identifiable information such as user names. All personal information is treated in accordance with Agilent's Privacy Statement, available at https://www.agilent.com/home/privacy-policy. If you have questions regarding Agilent's Privacy Statement, please contact our Data Protection Officer at data-protection.officer@agilent.com.

Glossary

Administrator	User who sets up the users of the Automated Purification Software as Operators or Method Developers in the Agilent OpenLab Control Panel.
Agilent 1260 Infinity	Instrument configuration based on the Agilent 1260 Infinity Dual-Loop Autosampler G2258A and other Agilent 1260 Infinity modules Specifications for standard and autoscale systems are given in *M8368-90302 System User Guide for the 1260 Infinity Purification System*.
Agilent 1260 Infinity II	Instrument configurations based on the Agilent1260 Infinity II Preparative Autosampler G7157A and other Agilent 1260/1290 Infinity II modules Specifications for standard systems are given in Technical Note *G7161-90202 InfinityLab PurificationCapillary Kits*.
Agilent 1290 Infinity II	Instrument configurations based on the Agilent1290 Infinity II Preparative Open-Bed Sampler/Collector G7158B and other Agilent 1260/1290 Infinity II modules Specifications for standard systems are given in Technical Note *G7161-90202 InfinityLab PurificationCapillary Kits*. Specifications for autoscale systems are given in Technical Note *G7161-90203 InfinityLab Autoscale SystemUpgrade Kit*.
Analytical run	The analytical (scouting) run scans your samples to find out if your expected target compound is present, and if it exists in a quantity sufficient to proceed with a purification run. Analytical runs operate with low flow rates, low injection volumes and small column diameters.
Analytical system parameters	Parameters of the analytical instrument and its operation mode that are used in the up-scaling process of analytical-to-preparative runs. Such parameters are maintained in the analytical systems parameters dialog box. Different parameter sets can belong to the same physical instrument, distinguished by different flows, tubing or columns. The analytical system can be part of the analytical flow path of a combined analytical and preparative instrument, or part of a separate physical analytical-only instrument. The analytical system is used for the *analytical run*
ChemStation	OpenLab CDS ChemStation
Easy Prep mode	Automated Purification Software work mode that focuses on the key data to set up and run purification tasks. ChemStation access is blocked to prevent unintended interaction.
Expert mode	Automated Purification Software work mode that grants full access to all features and functions of the software.
Method Developer	User who provides the work environment (methods, procedures, systems, master tasks) for Operators. The Method Developer works in *Expert* mode.
Operator	User who operates an analytical and/or preparative instrument using predefined methods and procedures. The Operator works in *Easy Prep* mode.
Preparative system parameters	Parameters of the preparative instrument and its operation mode that are used in the up-scaling process of analytical-to-preparative runs. Such parameters are maintained in the preparative systems parameters dialog box. Different parameter sets can belong to the same physical instrument, distinguish by different flows,

	tubing or columns. The preparative system can be part of the preparative flow path of a combined analytical and preparative instrument, or part of a separate physical preparative-only instrument. The preparative system is used for the *preparative run*.
Purification run	Also known as *Preparative run* Run to separate the target compound from the remainder in your sample and collect the fraction of the target compound. Purification/Preparative runs operate with high flow rates, high injection volumes and large column diameters.
Purification task	Entity in the purification software that describes all settings of a purification experiment, including its results once the experiment has completed.
System	A *system* within the purification software means a certain set of instrument configuration and operation parameters that describe either the analytical or preparative instrument and run. Such a parameter set provides the relevant settings in the analytical-to-preparative scale-up process.
System suitability test	Specific preparative-only workflow task, used to purge the auto-sampler, flush the instrument and verify that the compounds in a standard sample are separated as expected. Regular purification tasks can be submitted to run only if the last system suitability test was *passed* or *accepted* by a method developer.
Tasks root folder	Folder on disk where the Purification tasks are saved. Default folder for instrument 1: C:\Users\Public\Documents\ChemStation\1\Purify\Tasks Different tasks root folders can be defined in the Purify folder; for example, if a default tasks root folder with user's name or time stamp is defined in the **Administration** dialog box. A valid tasks root folder must be selected in the **Purification Tasks** dialog box to create and submit Purification tasks. When the **Purification Tasks** page is opened, the **User tasks** view is shown and the default tasks root folder is displayed and is editable. The SST tasks root folder is a tasks root folder containing System Suitability Test tasks only. It is editable in the **Administration** dialog box only by an advanced user. Default folder for instrument 1: C:\Users\Public\Documents\ChemStation\1\Purify\SST
Template	Template created from an already defined Purification task, used for creating a new Purification task. A template includes all settings of a task (such as analytical and preparative system settings, ion species, gradient and scale-up parameters, fraction collection settings) but does not contain any run data (result set, chromatograms, spectra). A template does not include analytical or preparative sequence. However, analytical and preparative sequence fields are pre-populated according to the task used to create the template. Templates can be created and modified by Method Developers only.
Threshold Calibration Setting	Parameter set created from a completed analytical-to-preparative purification task, for which a target peak was found. It is used to automatically calculate threshold values in analytical-to-preparative purification tasks: — The user specifies calibration threshold values for each type of peak detector (UV, MS and Auxiliary) using the measured preparative chromatograms. — Using the specified threshold values, the software calculates scaling factors between the analytical system and the preparative system for the specific set of parameters. — The threshold calibration setting can then be used in a new purification task with the same specific set of parameters.

— The threshold values are automatically calculated using the analytical results of the task and the scaling factors calculated in the threshold calibration setting.
Threshold calibration settings can be created and deleted in the **Threshold Calibration** view.

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In This Book

This manual contains information for Method Developers of the Automated Purification software.

The manual describes the following:

- the role of the Method Developer
- how to set up default Purification methods
- how to set up the analytical and preparative systems
- how to set up a Purification task and review the results
- how to calibrate the system and run a checkout sample

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