

# Agilent 1260 Infinity Binary LC

System User Guide



**Agilent Technologies**

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## In This Guide...

This manual covers the Agilent 1260 Infinity Binary LC.

### **1 The Agilent 1260 Infinity Binary LC - Product Description**

This chapter discusses the features of the 1260 Infinity Binary LC.

### **2 Introduction**

This chapter gives an introduction to the Agilent 1260 Infinity Binary LC and the underlying concepts.

### **3 Optimization of the Agilent 1260 Infinity Binary LC**

This chapter considers how to apply the theory and use the features of the LC system to develop optimized separations.

### **4 System Setup and Installation**

This chapter includes information on software installation, installation of the modules, and preparing the system for operation.

### **5 Quick Start Guide**

This chapter provides information on data acquisition and data analysis with the 1260 Infinity Binary LC.

### **6 Appendix**

This chapter provides additional information on safety, legal and web and about setting up a method.

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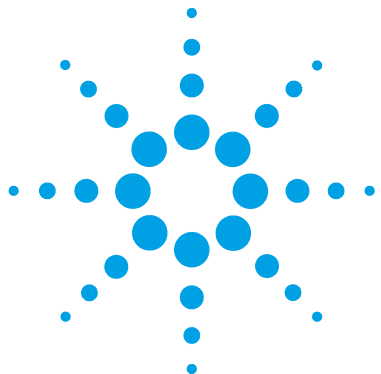
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This chapter discusses the features of the 1260 Infinity Binary LC.



# Features of the Agilent 1260 Infinity Binary LC

The design concept of the 1260 Infinity Binary LC is to provide a liquid chromatograph offering ultra fast and high resolution separation capability and yet which retains full functionality for standard HPLC applications. Therefore, it provides full backwards compatibility for your established HPLC and UHPLC methods. The use of sub-two micron (STM) particles means that for high flowrates or long columns additional pressure is required to drive the mobile phase through the column. The flowpath of the 1260 Infinity Binary LC is optimized to produce minimal backpressure, and ZORBAX RRHT columns have an engineered particle size distribution that produces significantly less backpressure than other STM columns.

The design features and benefits of the Agilent 1260 Infinity Binary LC are:

- The configurable delay volume down to 120  $\mu\text{L}$  in the 1260 Infinity Binary Pump combined with a flow range from 0.05 up to 5 mL/min at pressures up to 600 bar provides universal applicability from narrow bore (2.1 mm ID) to standard bore (4.6 mm ID) columns, matching the needs for both LC and LC/MS.
- The standard delay volume configuration of the 1260 Infinity Binary Pump allows you to run not only UHPLC but also conventional HPLC methods without compromising performance or changing chromatographic patterns.
- The next generation flow-through design of the 1260 Infinity High Performance Autosampler achieves highest precision for a wide range of injection volumes (from 0.1 up to 100  $\mu\text{L}$ ) without changing sample loops. It is designed for high sample throughput, low carryover, and fast injection cycles.
- High temperature, up to 100  $^{\circ}\text{C}$  on certain columns, allows more selectivity flexibility and reduces solvent viscosity to allow even faster separation.
- In the 1290 Infinity Thermostatted Column Compartment, different heater (1.6  $\mu\text{L}$ ) and cooling elements (1.5  $\mu\text{L}$ ) for low extra-column volume can be installed. The temperature is adjustable from 10  $^{\circ}\text{C}$  below ambient up to 100  $^{\circ}\text{C}$ .



- The new pull-out valve drive design and user-exchangeable Quick-Change valves in the 1290 Infinity Thermostatted Column Compartment boosts usability and paves the way for ultra high-throughput, multi-method and automated method development solutions.
- A low dispersion tubing kit and low volume flow cells minimize peak dispersion for narrow bore columns.
- Baseline robustness and fast spectral acquisition at data rates up to 80 Hz through the new optical design of the 1260 Infinity Diode Array Detector .
- Different UV detector flow cells for use with 2.1, 3.0 and 4.6 mm inner diameter columns are available, including the revolutionary Agilent Max-Light cartridge flow cell with 60 mm optical path length (typical noise:  $<\pm 0.6 \mu\text{AU}/\text{cm}$ ) for ultra sensitivity in detection.

A stepwise upgrade from 1100 Series or 1200 Series to Agilent 1260 Infinity Binary LC is possible; for example a 1100 Series Detector or 1200 Series Column Compartment can be further used in combination with a 1260 Infinity Binary Pump.

## **System Components**

Numerous system configurations of the 1260 Infinity Binary LC are possible, tailored to the needs of your individual application requirements. A few configurations are described in more detail in this manual (see [“System Setup and Installation”](#) on page 67). The modules that are described in the following sections are typical components of a 1260 Infinity Binary LC. In addition to these core components, individual solutions are available for specific applications (some of which are described in the Chapter "Optimization").

## 1260 Infinity Binary Pump (G1312B)

The binary pump comprises two identical pumps integrated into one housing. Binary gradients are created by high-pressure mixing. Pulse damper and mixer can be bypassed for low flowrate applications or whenever a minimal transient volume is desirable. Typical applications are high throughput methods with fast gradients on high resolution 2.1 mm columns. The pump is capable of delivering flow in the range of 0.1 – 5 mL/min against up to 600 bar. A solvent selection valve (optional) allows to form binary mixtures (isocratic or gradient) from one of two solvents per channel. Active seal wash (optional) is available for use with concentrated buffer solutions.

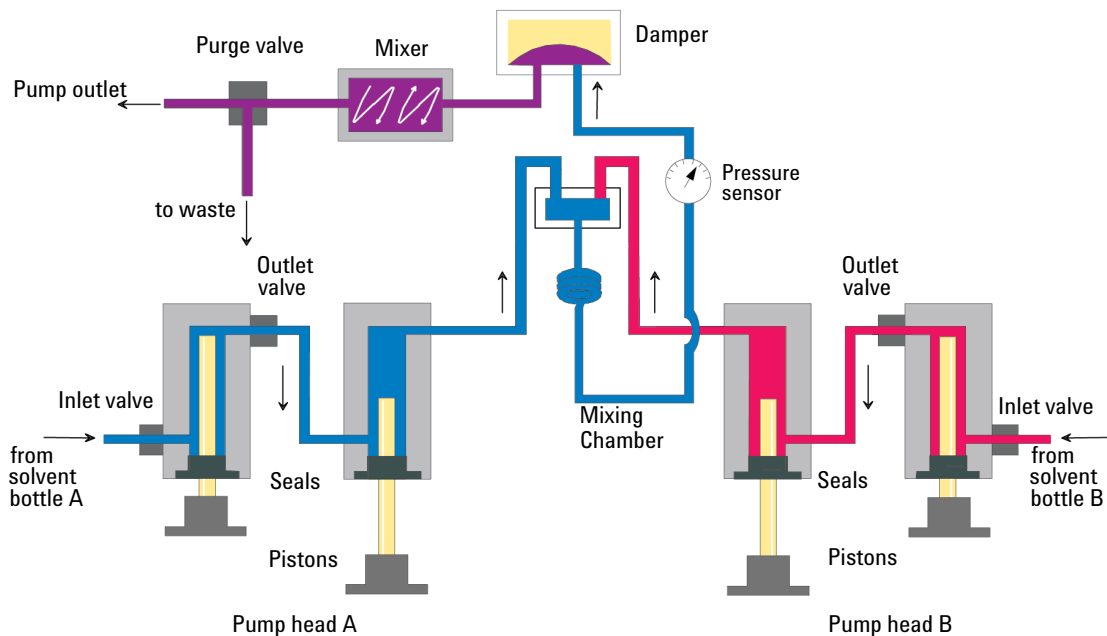
### Principle of operation

The binary pump is based on a two-channel, dual-piston in-series design which comprises all essential functions that a solvent delivery system has to fulfill. Metering of solvent and delivery to the high-pressure side are performed by two pump assemblies which can generate pressure up to 600 bar.

Each channel comprises a pump assembly including pump drive, pump head, active inlet valve with replaceable cartridge, and outlet valve. The two channels are fed into a low-volume mixing chamber which is connected via a restriction capillary coil to a damping unit and a mixer. A pressure sensor monitors the pump pressure. A purge valve with integrated PTFE frit is fitted to the pump outlet for convenient priming of the pumping system.

# 1 The Agilent 1260 Infinity Binary LC - Product Description

## System Components



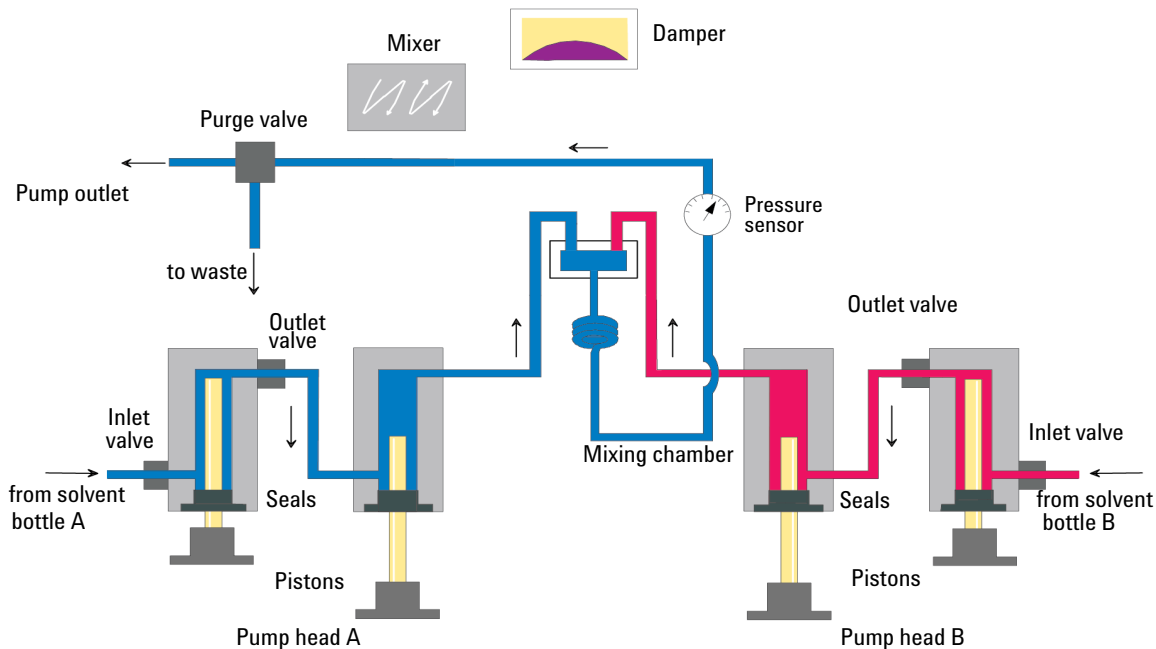
**Figure 1** The Hydraulic Path of the Binary Pump with Damper and Mixer

Damper and mixer can be bypassed for lowest delay volume of the binary pump. This configuration is recommended for low flow rate applications with steep gradients.

Figure 2 on page 13 illustrates the flow path in low delay volume mode. For instructions on how to change between the two configurations, see the G1312B Binary Pump User Manual.

**NOTE**

Bypassing the mixer while the damper remains in line is not a supported configuration and may lead to undesired behavior of the binary pump.

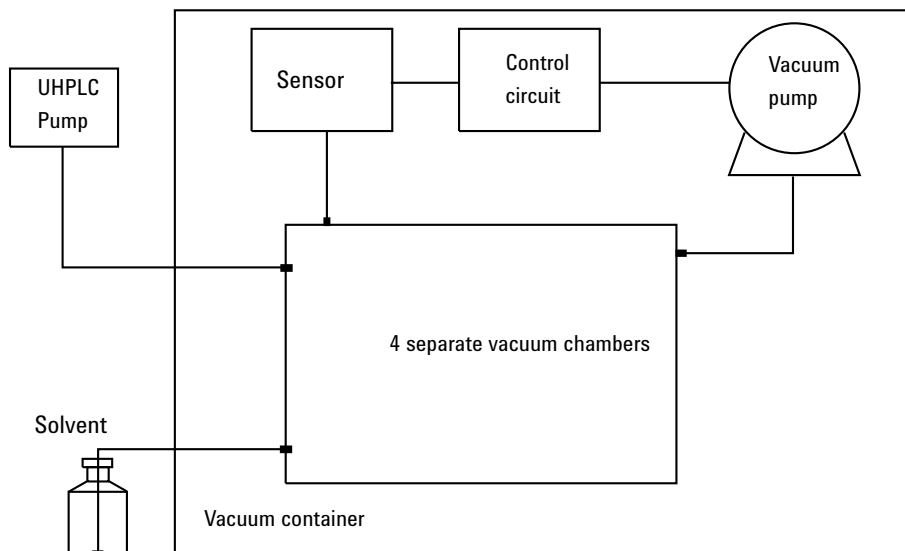


**Figure 2** The Hydraulic Path of the Binary Pump with Bypassed Damper and Mixer

## 1260 Infinity High Performance Degasser (G4225A)

The Agilent 1260 Infinity High Performance Degasser, model G4225A, comprises four separate vacuum chambers with semipermeable tubings, a vacuum pump and control assembly. When the vacuum degasser is switched on, the control assembly turns on the vacuum pump, which generates a low pressure in the vacuum chambers. The pressure is measured by a pressure sensor. The vacuum degasser maintains the low pressure by a controlled leak in the air inlet filter and a regulation of the vacuum pump using the pressure sensor.

The LC pump draws the solvents from their bottles through the semipermeable tubes of the vacuum chambers. When solvents pass through the vacuum chambers any dissolved gas in the solvents permeates through the tubings into the vacuum chambers. The solvents will be degassed when leaving the outlets of the vacuum degasser.



**Figure 3** Overview (only one of the four solvent channels is shown)

## 1260 Infinity High Performance Autosampler (G1367E)

### Features

The 1260 Infinity High Performance Autosampler features an increased pressure range (up to 600 bar) enabling the use of today's column technology (sub-two-micron narrow bore columns) with the Agilent 1260 Infinity Binary LC. Increased robustness is achieved by optimized new parts, high speed with lowest carry-over by flow through design, increased sample injection speed for high sample throughput, increased productivity by using overlapped injection mode, and flexible and convenient sample handling with different types of sample containers, such as vials and well plates. Using 384-well plates allows you to process up to 768 samples unattended.

### Autosampler Principle

The movements of the autosampler components during the sampling sequence are monitored continuously by the autosampler processor. The processor defines specific time windows and mechanical ranges for each movement. If a specific step of the sampling sequence is not completed successfully, an error message is generated. Solvent is bypassed from the autosampler by the injection valve during the sampling sequence. The needle moves to the desired sample position and is lowered into the sample liquid in the sample to allow the metering device to draw up the desired volume by moving its plunger back a certain distance. The needle is then raised again and moved onto the seat to close the sample loop. Sample is applied to the column when the injection valve returns to the mainpass position at the end of the sampling sequence.

The standard sampling sequence occurs in the following order:

- 1 The injection valve switches to the bypass position.
- 2 The plunger of the metering device moves to the initialization position.
- 3 The needle lock moves up.
- 4 The needle moves to the desired sample vial (or well plate) position.
- 5 The needle lowers into the sample vial (or well plate).
- 6 The metering device draws the preset sample volume.
- 7 The needle lifts out of the sample vial (or well plate).
- 8 The needle is then moved onto the seat to close the sample loop.
- 9 The needle lock moves down.
- 10 The injection cycle is completed when the injection valve switches to the mainpass position.

If needle wash is required it will be done between step 7 and 8.

## **Injection Sequence**

Before the start of the injection sequence, and during an analysis, the injection valve is in the mainpass position. In this position, the mobile phase flows through the autosampler metering device, sample loop, and needle, ensuring all parts in contact with sample are flushed during the run, thus minimizing carry-over.

When the sampling sequence begins, the valve unit switches to the bypass position. Solvent from the pump enters the valve unit at port 1, and flows directly to the column through port 6.

The final step of the sampling sequence is the inject-and-run step. The six-port valve is switched to the main-pass position, and directs the flow back through the sample loop, which now contains a certain amount of sample. The solvent flow transports the sample onto the column, and separation begins. This is the beginning of a run within an analysis. In this stage, all major performance-influencing hardware is flushed internally by the solvent flow. For standard applications, no additional flushing procedure is required.

## **Flush the Needle**

Before injection and to reduce the carry-over for very sensitive analysis, the outside of the needle can be washed in a flush port that is located behind the injector port on the sampling unit. As soon as the needle is on the flush port a peristaltic pump delivers some solvent during a defined time to clean the outside of the needle. At the end of this process the needle returns to the injection port.



## 1290 Infinity Thermostatted Column Compartment (G1316C)

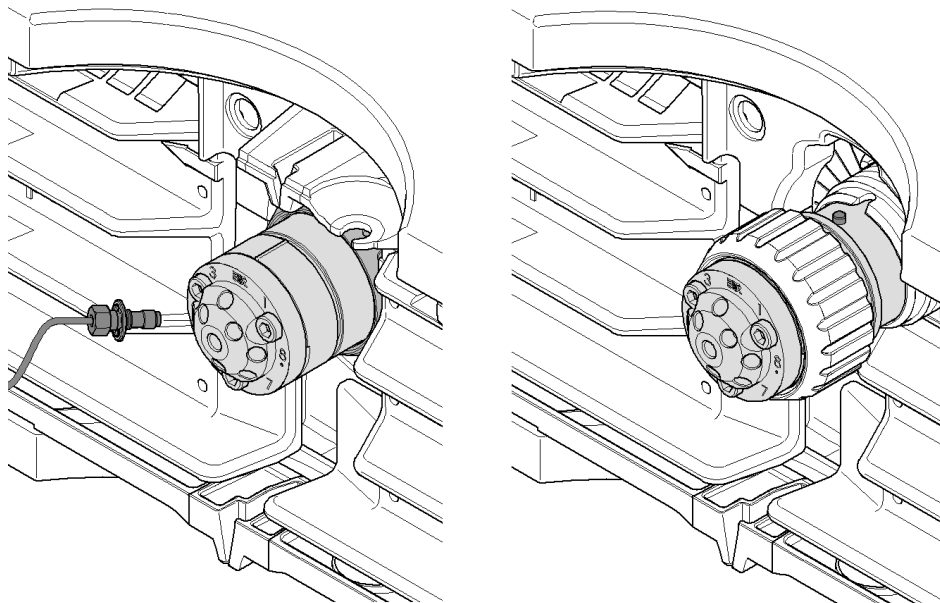
The Agilent 1290 Infinity Thermostatted Column Compartment (TCC) controls the temperature between 10 °C below ambient and up to 100 °C at 2.5 ml/min and 80 °C at up to 5 ml/min, respectively. The temperature stability specification is  $\pm 0.05$  °C and the accuracy specification  $\pm 0.5$  °C (with calibration)<sup>1</sup>. This is achieved by a combination of conduction from contact with the thermostat vanes, still-air temperature in the column environment and most importantly by pre-heating (or cooling) the mobile phase by passing it through a heat exchanger before entering the column. There are two independent temperature zones in each TCC which can work together for long columns up to 300 mm length or work at different temperatures for short columns of 100 mm length or less.

The module comes with a 1.6  $\mu$ l low dispersion heat exchanger and each valve kit contains additional low dispersion heat exchangers for each column. The low dispersion heat exchangers, up to 4, can be mounted flexibly inside the TCC. For conventional HPLC operation, 3  $\mu$ l and 6  $\mu$ l built-in heat exchangers are also available.

Each TCC can accommodate one internal valve drive to facilitate valve switching applications from simple switching between two columns to alternating column regeneration, sample preparation or column back-flushing. Each valve head comes as a complete kit containing all required capillaries, additional low dispersion heat exchangers and other parts.

The switching valves have exceptional ease-of-use and flexibility when making connections to the valve: When pressed, the drive unit of the Quick Change Valve slides forward for easy access (see [Figure 4](#) on page 18 left). Alternative valve heads can be interchanged by the user on the drive mechanism for different applications (see [Figure 4](#) on page 18 right). Note the RFID tag on top of the valve head.

<sup>1</sup> All specifications are valid for distilled water at ambient temperature of 25 °C, setpoint at 40 °C and a flow range from 0.2 to 5 ml/min.



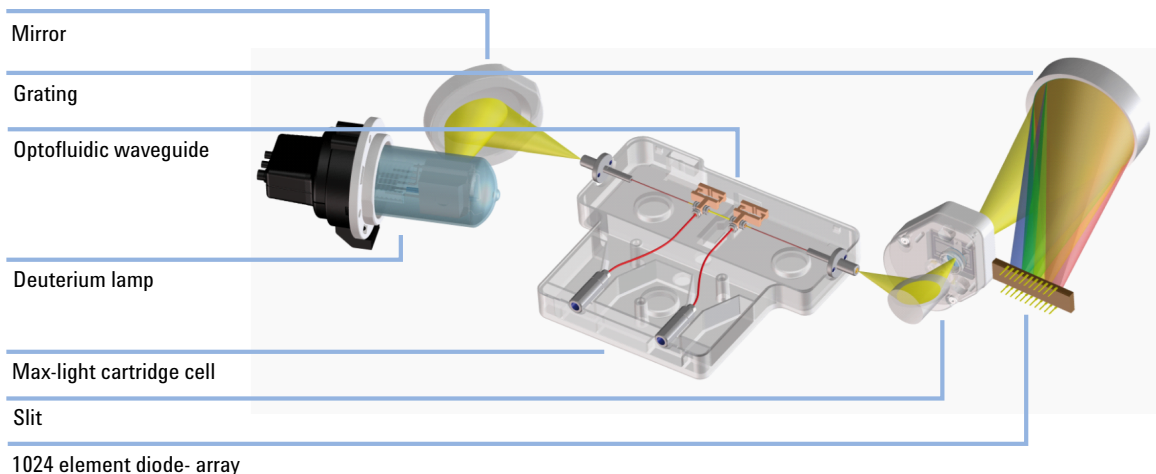
**Figure 4** Quick change valve in TCC

Up to three TCC can be “clustered” to allow advanced applications such as switching between eight columns for automated method development or to make additional columns available for different applications. Thus, the column to be used becomes a simple method parameter. This requires two 8 position/9 port valve heads, one each in two of the TCCs. Clustered TCC are represented by the software as one unit with one interface for ease of operation.

Further improvements compared to earlier designs include better thermal insulation, better capillary guides and a “door open” sensor so that methods can define that the door must be closed – especially useful for low or high-temperature methods.

## 1260 Infinity Diode Array Detector (G4212B)

The 1260 Infinity Diode-Array Detector (DAD) is a new optical design using a cartridge cell with optofluidic waveguide technology, offering high sensitivity with low dispersion, a wide linear range, and a very stable baseline for standard or ultra-fast LC applications. The Agilent Max-Light cartridge cell dramatically increases the light transmission by utilizing the principle of total internal reflection along a non-coated fused silica capillary, achieving a new level of sensitivity without sacrificing resolution through cell volume dispersion effects. This design minimizes baseline perturbations that are caused by refractive index or thermal effects and results in more reliable integration of peak areas.



**Figure 5** The light path through the DAD

The module also incorporates electronic temperature control to further enhance the resistance to ambient temperature effects. Although the hydraulic volume of the Max-Light cartridge cell is very small, the path length is a standard 10 mm. However, for even higher sensitivity the alternative Agilent Max-Light high sensitivity cell is available with a path length of 60 mm. Cells are easily exchanged by sliding them in or out of the cell holder and they are auto-aligned in the optical bench. The DAD light source is a deuterium lamp and the operating wavelength range that is covered is 190 to 640 nm. This is detected by a diode-array comprising 1024 diodes. The entrance to the spectrograph is through a fixed optical slit of 4 nm.

## 1 The Agilent 1260 Infinity Binary LC - Product Description

### System Components

The chromatographic signals are extracted from the diode-array data within the firmware of the module. Up to eight individual signals can be defined. Each of them comprises a signal wavelength, a diode-bunching bandwidth and - if required - a reference wavelength and bandwidth. Signals can be output at 80 Hz (80 data points/second) for accurate recording of the fastest (narrowest) chromatographic peaks. At the same time, the module can also output full-range spectra to the data system at the same rate of 80 Hz.

For regulated laboratories, it is important that all the method parameters are recorded. The 1260 Infinity DAD not only records the instrument setpoints but also has RFID tags (radio-frequency identification tags) incorporated into the lamp and flow cell cartridge so that the identity and variables of these important components are also recorded by the system.

## 1200 Infinity Series Quick Change Valves

Agilent 1200 Infinity Quick Change Valves support a variety of challenging valve applications. Each valve head comes as a complete kit containing all required capillaries, additional low dispersion heat exchangers and other parts.

Some typical applications for Quick Change Valves are:

- Dual column selection
- Sample enrichment
- Sample clean-up
- Alternating column regeneration
- Special applications, such as method development or 2D-LC

For detailed descriptions of these applications, please refer to the Valve Solution User Guide.

## Specifications

The modular design of the 1260 Infinity Binary LC allows you to configure a system that exactly meets your individual application requirements. This individual configuration can be different from the standard configuration which is described in this System User Guide.

The physical and performance specifications of the 1260 Infinity Binary Pump are shown below. Information on the specifications of other modules in your system can be found in the respective module user manuals.

## Physical Specifications 1260 Infinity Binary Pump (G1312B)

**Table 1** Physical Specifications

Type	Specification	Comments
Weight	15.5 kg (34 lbs)	
Dimensions (height × width × depth)	180 x 345 x 435 mm (7 x 13.5 x 17 inches)	
Line voltage	100 – 240 VAC, ± 10 %	Wide-ranging capability
Line frequency	50 or 60 Hz, ± 5 %	
Power consumption	220 VA, 74 W / 253 BTU	Maximum
Ambient operating temperature	4–55 °C (39–131 °F)	
Ambient non-operating temperature	-40 – 70 °C (-40 – 158 °F)	
Humidity	< 95 % r.h. at 40 °C (104 °F)	Non-condensing
Operating altitude	Up to 2000 m (6562 ft)	
Non-operating altitude	Up to 4600 m (15091 ft)	For storing the module
Safety standards: IEC, CSA, UL	Installation category II, Pollution degree 2	For indoor use only.

## Performance Specifications

**Table 2** Performance Specifications of the Agilent 1260 Infinity Binary Pump (G1312B)

Type	Specification	Comments
Hydraulic system	Two dual piston in series pumps with servo-controlled variable stroke drive, power transmission by gears and ball screws, floating pistons	
Settable flow range	Set points 0.001 – 5 mL/min, in 0.001 mL/min increments	
Flow range	0.05 – 5.0 mL/min	
Flow precision	≤0.07 % RSD or ≤0.02 min SD, whatever is greater	based on retention time at constant room temperature
Flow accuracy	± 1 % or 10 µL/min, what ever is greater	pumping degassed H <sub>2</sub> O at 10 MPa (100 bar)
Pressure operating range	Operating range 0 – 60 MPa (0 – 600 bar, 0 – 8700 psi) up to 5 mL/min	
Pressure pulsation	< 2 % amplitude (typically < 1.3 %), or < 0.3 MPa (3 bar), whatever is greater, at 1 mL/min isopropanol, at all pressures > 1 MPa (10 bar, 147 psi) <i>Low delay volume configuration:</i> < 5 % amplitude (typically < 2 %)	
Compressibility compensation	Pre-defined, based on mobile phase compressibility	
Recommended pH range	1.0 – 12.5, solvents with pH < 2.3 should not contain acids which attack stainless steel	
Gradient formation	High-pressure binary mixing	
Delay volume	<i>Standard delay volume configuration:</i> 600 – 800 µL, (includes 400 µL mixer), dependent on back pressure <i>Low delay volume configuration:</i> 120 µL	measured with water at 1 mL/min (water/caffeine tracer)
Composition range	settable range: 0 – 100 % recommended range: 1 – 99 % or 5 µL/min per channel, whatever is greater	



**Table 2** Performance Specifications of the Agilent 1260 Infinity Binary Pump (G1312B)

Type	Specification	Comments
Composition precision	< 0.15 % RSD or < 0.04 min SD whatever is greater	at 0.2 and 1 mL/min; based on retention time at constant room temperature
Composition accuracy	± 0.35 % absolute, at 2 mL/min, at 10 MPa (100 bar)	(water/cafeine tracer)
Control	Agilent control software (e.g. ChemStation, EZChrom, OL, MassHunter)	
Local control	Agilent Instant Pilot	Revision B.02.00 or above
Analog output	For pressure monitoring, 1.33 mV/bar, one output	
Communications	Controller-area network (CAN), RS-232C, APG Remote: ready, start, stop and shut-down signals, LAN optional	
Safety and maintenance	Extensive support for troubleshooting and maintenance is provided by the Instant Pilot, Agilent Lab Advisor, and the Chromatography Data System. Safety-related features are leak detection, safe leak handling, leak output signal for shutdown of pumping system, and low voltages in major maintenance areas.	
GLP features	Early maintenance feedback (EMF) for continuous tracking of instrument usage in terms of seal wear and volume of pumped mobile phase with pre-defined and user settable limits and feedback messages. Electronic records of maintenance and errors	
Housing	All materials are recyclable	

**NOTE**

For use with flow rates below 500 µl/min or for use without damper and mixer a vacuum degasser is required.

All specification measurements are done with degassed solvents.

## **1 The Agilent 1260 Infinity Binary LC - Product Description Specifications**



## 2 Introduction

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Frictional Heating 37

This chapter gives an introduction to the Agilent 1260 Infinity Binary LC and the underlying concepts.



# Theory of Using Smaller Particles in Liquid Chromatography

## Introduction

In 2003, Agilent introduced the first commercially available, totally porous silica columns with 1.8  $\mu\text{m}$  particles.

In combination with the Agilent 1260 Infinity Binary LC the sub-two micron (1.8  $\mu\text{m}$ ) particle size columns can be used in pursuit of two main objectives:

### 1 Faster Chromatography

Short columns with sub-two-micron particles offer the opportunity to dramatically reduce analysis time by increasing the flow rate without losing separation performance.

### 2 Higher Resolution

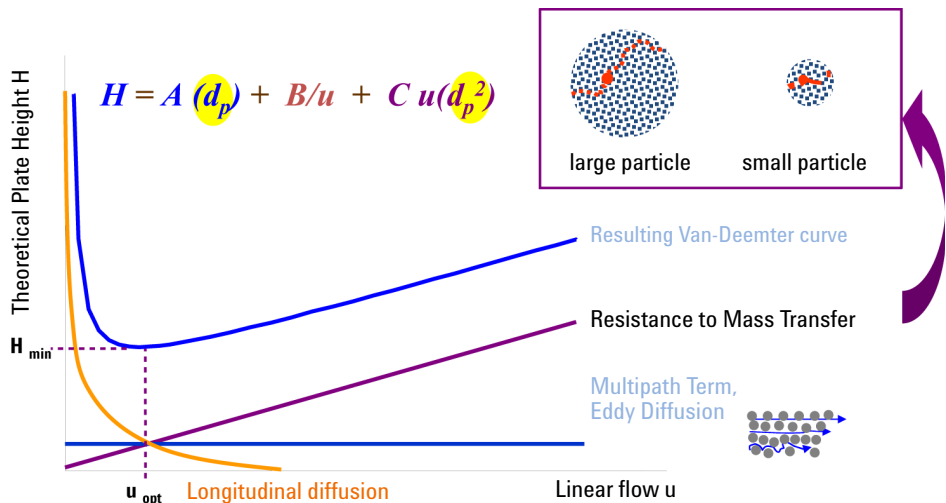
Long columns with sub-two-micron particles provide higher efficiency and therefore higher resolution, which is required for the separation of complex samples.

The pressure that is needed to drive solvent through a column containing STM (sub-two micron) particles rises rapidly as flow rate is increased for faster separations and very rapidly as the length of the column increases for more resolution. Thus the acceptance of STM columns has been synonymous with the development of UHPLC systems – that is HPLC systems offering higher pressures than the 400 bar norm that was extant since the early days of HPLC.

Today, Agilent offers the 1290 Infinity LC for highest UHPLC requirements with pressures up to 1200 bar.

## The Theory

Separation efficiency in HPLC can be described by the van Deemter equation (Figure 6 on page 29). This results from the plate-height model that is used to measure the dispersion of analytes as they move down the column.  $H$  is the Height Equivalent to a Theoretical Plate (sometimes HETP),  $d_p$  is the particle size of the column packing material,  $u_0$  is the linear velocity of the mobile phase and  $A$ ,  $B$  and  $C$  are constants that are related to the different dispersive forces. The  $A$  term relates to eddy diffusion or multiple flow paths through the column;  $B$  relates to molecular diffusion along the column axis (longitudinal);  $C$  relates to mass transfer of the analyte between the mobile and stationary phases. The separation is at its most efficient when  $H$  is at a minimum. The effect of each individual term and the combined equation are shown in Figure 6 on page 29 where the plate height is plotted against the linear flow rate through the column. This type of plot is known as a Van Deemter Curve and is used to determine the optimum flow rate (minimum point of the curve) for best efficiency of separation for a column.



**Figure 6** A hypothetical Van Deemter curve

The van Deemter plots in Figure 7 on page 30 show that reducing particle size increases efficiency. Switching from commonly used 3.5  $\mu\text{m}$  and 5.0  $\mu\text{m}$  particle sizes to 1.8  $\mu\text{m}$  particles offers significant performance improvements. The 1.8  $\mu\text{m}$  particles give two to three times lower plate height values and proportionately higher efficiencies. This allows a shorter column to be used without sacrificing resolution and hence the analysis time is also reduced by a factor of two to three. The increased efficiency is derived to a large extent from the reduction in multiple flow paths as a result of the smaller particles - this leads to a smaller A term (eddy diffusion). In addition, smaller particles mean shorter mass transfer times, reducing the C term, and it can be seen that the overall effect is a much reduced loss of efficiency as the flow rate increases (the slope of the line is reduced). This means that the separation on smaller particles can be further accelerated by increasing the flow rates without significantly reducing efficiency.

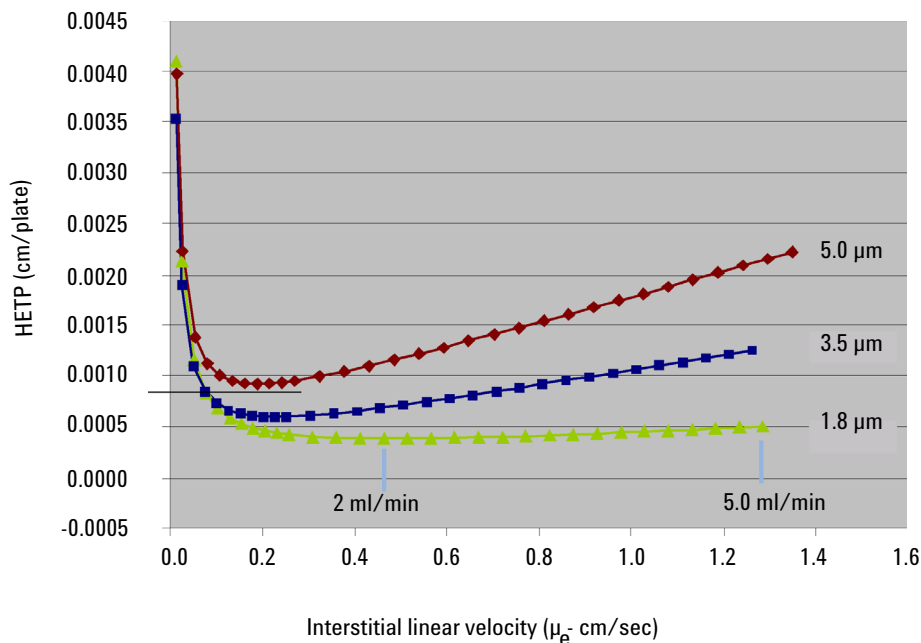
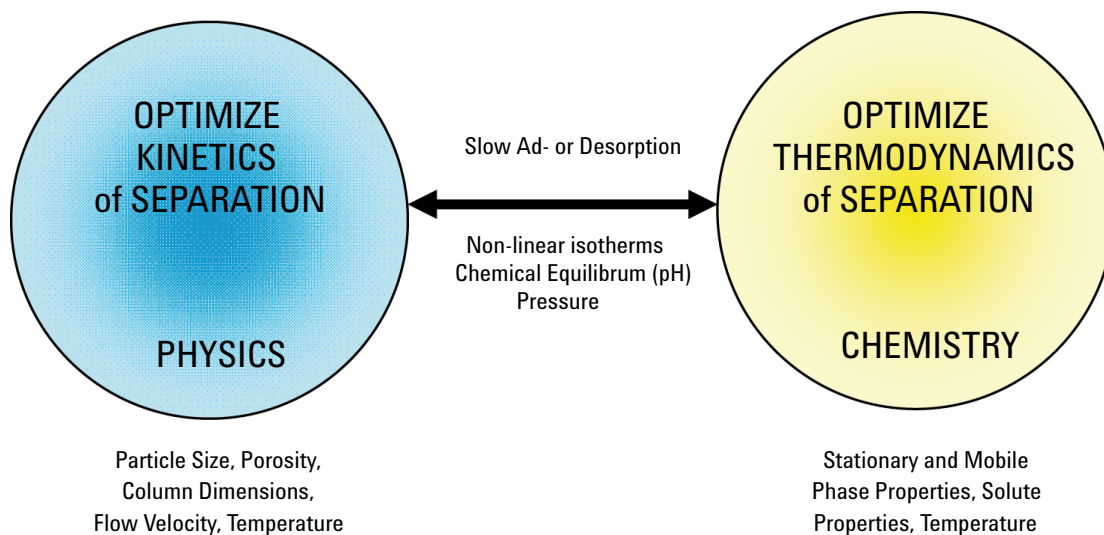


Figure 7 Van Deemter curve for different particle sizes

A chromatographic separation can be optimized based on physical parameters of the HPLC column such as particle size, pore size, morphology of the particles, the length and diameter of the column, the solvent velocity, and the temperature. In addition, the thermodynamics of a separation can be considered and the properties of the solute and the stationary and mobile phases (percentage of organic solvent, ion strength, and pH) can be manipulated to achieve the shortest possible retention and highest selectivity.



**Figure 8** Selecting optimal conditions for HPLC

## 2 Introduction

### Theory of Using Smaller Particles in Liquid Chromatography

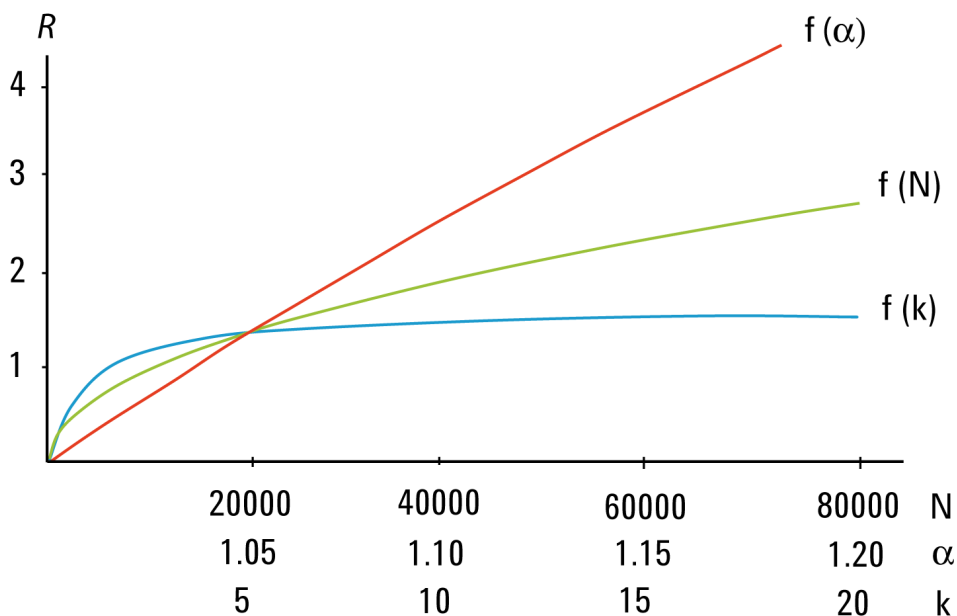
Resolution can be described as a function of three parameters:

- column efficiency or theoretical plates (N),
- selectivity ( $\alpha$ ),
- retention factor (k).

According to the resolution equation ( [Figure 9](#) on page 32 ), the selectivity has the biggest impact on resolution ( [Figure 10](#) on page 32). This means that the selection of appropriate mobile and stationary phase properties and temperature is critical in achieving a successful separation.

$$R_s = \frac{\sqrt{N}}{4} \cdot \left[ \frac{\alpha - 1}{\alpha} \right] \cdot \left[ \frac{k_2'}{k_2' + 1} \right]$$

**Figure 9** Resolution equation



**Figure 10** Effect of plate number, separation factor and retention factor on R



No matter whether the UHPLC separation method is being newly developed or simply transferred from an existing conventional method, it is clearly beneficial to have a wide choice of stationary phase chemistries available in a range of column formats.

Agilent offers more than 140 ZORBAX 1.8  $\mu\text{m}$  Rapid Resolution High Throughput (RRHT) columns (14 selectivity choices; 15 to 150 mm long; 2.1, 3.0 and 4.6 mm internal diameters).

Additionally to the ZORBAX columns, PoroShell columns with nine selectivity choices are available for use with the Agilent 1260 Infinity Binary LC.

This enables the optimum stationary phase to be selected so that the selectivity is maximized. The resolution, flow rate, and analysis time can be optimized by selecting the appropriate column length and diameter and operation with longer STM columns has become more accessible than ever before.

PoroShell columns are so-called superficially porous particle (SPP) columns. In contrast to totally porous silica columns, these SPP columns have a solid core (1.7  $\mu\text{m}$  in diameter) and a porous silica layer (0.5  $\mu\text{m}$  thickness) surrounding it. Speed and resolution of PoroShell columns are comparable to sub-two micron columns with up to 50 % less backpressure. PoroShell columns have enjoyed a recent resurgence in smaller particle sizes than the older 'pellicular' particle columns. The current interest in this technology is driven by its re-introduction in smaller particle sizes, such as the sub 3 micron sizes, for use in typical small molecule reversed phase separations.

Many laboratories perform an extensive screening process to select the best combination of stationary phase, mobile phase, and temperature for their separations. The Agilent 1200 Infinity Series Multi-Method Development Solution facilitates complete automation of this time-consuming selection process – making method development and method transfer an easy and reliable task.

ZORBAX 1.8  $\mu\text{m}$  RRHT columns use the same chemistry as ZORBAX columns with 3.5 and 5  $\mu\text{m}$  particles. As a result, for any particular ZORBAX phase, the 5.0, 3.5 and 1.8  $\mu\text{m}$  particles provide identical selectivity, which allows easy, fast, and secure bidirectional method transfer between conventional LC and UHPLC.

## Benefits of small particle size columns

### Faster Chromatography

There are several advantages of having shorter run times. High Throughput labs now have higher capacity and can analyze more samples in less time. More samples in less time also means lower costs. For example, by reducing the analysis time from 20 min per sample to 5 min, the cost for 700 samples is reduced by 79 % (Table 3 on page 34).

**Table 3** Time and cost savings over 700 runs

Cycle time	20 min cycle time	5 min cycle time
Runs	700	700
Approx. costs/analysis <sup>1</sup>	\$ 10.58	\$ 2.24
Approx. cost/700runs <sup>1</sup>	\$ 7400	\$ 1570
Cost savings	-	\$ 5830
Time <sup>2</sup>	10 days	2.5 days

<sup>1</sup> solvents = \$ 27/l, disposal = \$ 2/l, labor = \$ 30/h

<sup>2</sup> 24 hours/day

The Agilent cost savings calculator provides an easy way to calculate the cost savings by switching from conventional HPLC to UHPLC using 1.8 µm particle size columns. This calculator is available on the Agilent Technologies web site along with a method translator calculator – [www.chem.agilent.com](http://www.chem.agilent.com). The results are presented graphically and in tabular form.

Shorter run times also deliver faster answers. This is important in process control and rapid release testing. Instead of waiting hours to release a single batch of a drug, all the system suitability, calibration and sample analysis can now be done in less than an hour. Rapid answers are also important for synthetic chemists using open access LC/MS systems for compound confirmation and reaction control. Shorter run times can also accelerate the method development process significantly.

## Benefits of small particle size columns

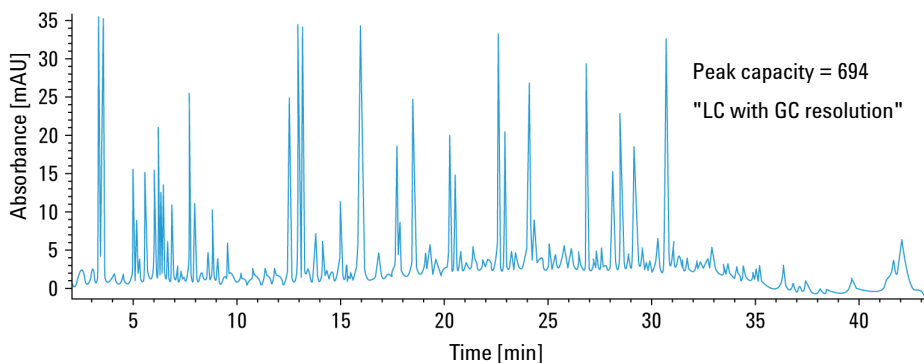
Column Length (mm)	Column Efficiency N(5 $\mu\text{m}$ )	Column Efficiency N(3.5 $\mu\text{m}$ )	Column Efficiency N(1.8 $\mu\text{m}$ )		Analysis Time Reduction	
150	12,500	21,000	35,000			
100	8,500	14,000	23,250		Analysis Time	-33%
75	6,000	10,500	17,500		Peak Volume	-67%
50	4,200	7,000	12,000		Solvent Usage	-80%
30	N.A.	4,200	6,500			
15	N.A.	2,100	2,500			

**Figure 11** Relation between particle size, efficiency and analysis time

Analysis time can be shortened without sacrificing column efficiency by optimizing particle size and pressure.

## Higher Resolution

Long columns packed with smaller particles result in higher efficiency and higher resolution. This is important for analysis of complex samples from metabolomics or proteomics studies. Also, applications such as impurity profiling can benefit from higher separation power. Even the LC/MS analysis of drugs in biological fluids can benefit from the higher peak capacity, because of the reduced interference from ion suppression. In general, higher separation power provides more confidence in the analytical results.



**Figure 12** Peak capacities of more than 700 can be achieved using a ZORBAX RRHT SB-C18 column (2.1 x 150 mm, 1.8  $\mu\text{m}$ ) to analyze a tryptic digest of BSA

## Frictional Heating

Forcing mobile phase through the column at higher pressure and higher flow rates generates heat. The resulting temperature gradients (radial and longitudinal) can have an impact on the column efficiency.

$$\text{Power} = F * p$$

where F is the flow rate and p is the pressure.

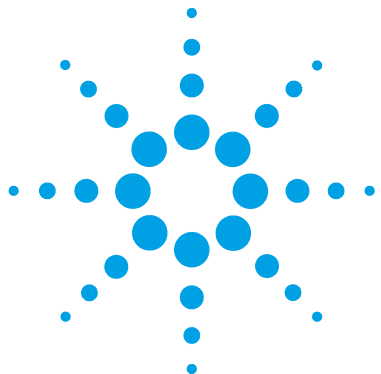
Powerful column thermostating (for example, using a water bath) generates a strong radial temperature gradient, which leads to significant loss in column efficiency. Still-air column thermostating reduces the radial temperature gradient and therefore reduces the efficiency losses, but a higher column outlet temperature has to be accepted. The raised temperature may have an effect on selectivity. At lower back-pressure, performance losses due to frictional heat are minimized so that 4.6 or 3 mm inner diameter sub-2-micron columns still deliver superior efficiencies compared with the respective 2.1 mm inner diameter columns.

In summary, the use of sub-two-micron packing material offers benefits of increased efficiency, higher resolution and faster separations.

The features of the Agilent 1260 Infinity Binary LC are discussed in the chapter *Product Description*. The chapter *Optimization* considers how to apply the theory and use these features to develop optimized separations.

## **2 Introduction**

### **Frictional Heating**



### 3 Optimization of the Agilent 1260 Infinity Binary LC

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This chapter considers how to apply the theory and use the features of the LC system to develop optimized separations.



## How to Configure the Optimum Delay Volume

### Delay Volume and Extra-Column Volume

The *delay volume* is defined as the system volume between the point of mixing in the pump and the top of the column.

The *extra-column volume* is defined as the volume between the injection point and the detection point, excluding the volume in the column.

#### Delay Volume

In gradient separations, this volume causes a delay between the mixture changing in the pump and that change reaching the column. The delay depends on the flow rate and the delay volume of the system. In effect, this means that in every HPLC system there is an additional isocratic segment in the gradient profile at the start of every run. Usually the gradient profile is reported in terms of the mixture settings at the pump and the delay volume is not quoted even though this will have an effect on the chromatography. This effect becomes more significant at low flow rates and small column volumes and can have a large impact on the transferability of gradient methods. It is important, therefore, for fast gradient separations to have small delay volumes, especially with narrow bore columns (that is, 2.1 mm i.d.) as often used with mass spectrometric detection.

As an example, in HPLC methods using 5  $\mu\text{m}$  packing material flow rates of 1 ml/min are typically used in a 4.6 mm i.d. column and about 0.2 ml/min in a 2.1 mm i.d. column (same linear velocity in the column). On a system with a typical delay volume of 1000  $\mu\text{l}$  and using a 2.1 mm column, there would be an initial “hidden” isocratic segment of 5 min whereas on a system with 600  $\mu\text{l}$  delay volume the delay would be 3 min. These delay volumes would be too high for run times of one or two minutes. With sub-two  $\mu\text{m}$  packings, the optimum flow rate (from the Van Deemter curve) is a little higher and so fast chromatography can use three to five times these flow rates yielding delay times of about one minute. However, the delay volume must be reduced further to achieve delay times which are a fraction of the intended run time. This is achieved with the Agilent 1260 Infinity Binary LC due to the low delay volume of the pump flow path and low-volume of the flow path through the autosampler.



## Extra-Column Volume

Extra-column volume is a source of peak dispersion that will reduce the resolution of the separation and so should be minimized. Smaller diameter columns require proportionally smaller extra-column volumes to keep peak dispersion at a minimum.

In a liquid chromatograph the extra-column volume will depend on the connection tubing between the autosampler, column, and detector; and on the volume of the flow cell in the detector. The extra-column volume is minimized with the Agilent 1260 Infinity Binary LC due to the narrow-bore (0.12 mm i.d.) tubing, the low-volume heat exchangers in the column compartment and the Max-Light cartridge cell in the detector.

## Delay Volumes in the Agilent 1260 Infinity Binary LC

Table 4 on page 42 and Table 5 on page 42 show the component volumes which contribute to system delay volume in the Agilent 1260 Infinity Binary LC System .

**Table 4** Delay volumes of the 1260 Infinity Binary LC modules

Components	Delay Volume (µL)
Binary Pump <sup>1</sup>	120
Binary Pump <sup>2</sup>	600 – 800
Low volume mixer	200
Mixer	400
Autosampler	270
Low dispersion heat exchanger	1.6
Built-in heat exchanger	3 and 6

<sup>1</sup> in low delay volume configuration with bypassed damper and mixer

<sup>2</sup> in standard delay volume configuration

**Table 5** Delay volumes of 1260 Infinity Binary LC configurations

System Configuration	Delay Volume (µL)
Low delay volume configuration	Pump: 120 Autosampler:
Medium delay volume configuration	Pump: 320
Standard delay volume configuration	Pump: 600 – 800

Switching between configurations can be done in two ways:

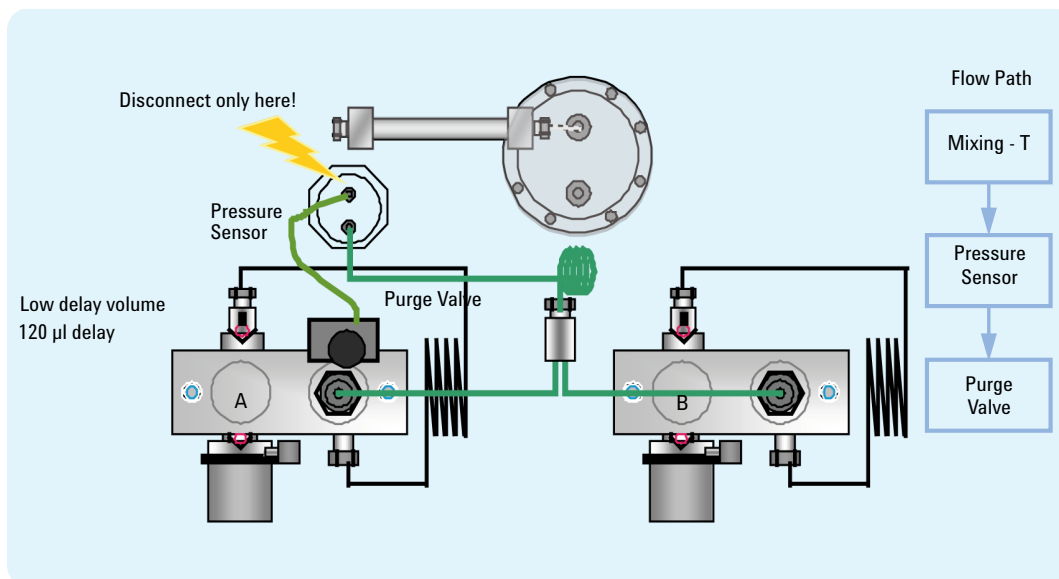
- manually, by disconnecting and reconnecting capillaries
- automatically, using a 600 bar 2PS/6PT valve (optional)

## Optimum Instrument Configuration for 2.1 mm i.d. Columns

### Low delay volume configuration to achieve shortest gradient delay for ultra-fast gradient separations

In the low delay volume configuration of the Agilent 1260 Infinity Binary Pump the damper and mixer are bypassed to reduce the pump delay volume to about 120  $\mu\text{L}$ . [Figure 13](#) on page 43 shows the flow path connections for this configuration. This provides the shortest gradient delay for ultra-fast gradient separations. In order to take full advantage of the electronic damping control which replaces the physical volume damping it is important to select the respective **Enhanced Solvent Compressibility** function in the auxiliary screen of the pump menu.

To minimize peak dispersion the low dispersion kit ( Low dispersion kit (G1316-68744)) must be installed. This kit includes short 0.12 mm i.d. capillaries and low dispersion heat exchangers (1.6  $\mu\text{L}$  and 1.5  $\mu\text{L}$ ) for the thermostatted column compartment. To maintain resolution in the UV detector, a low volume flow cell should be used. See [“Choosing a Flow Cell”](#) on page 63 for flow cell recommendations).



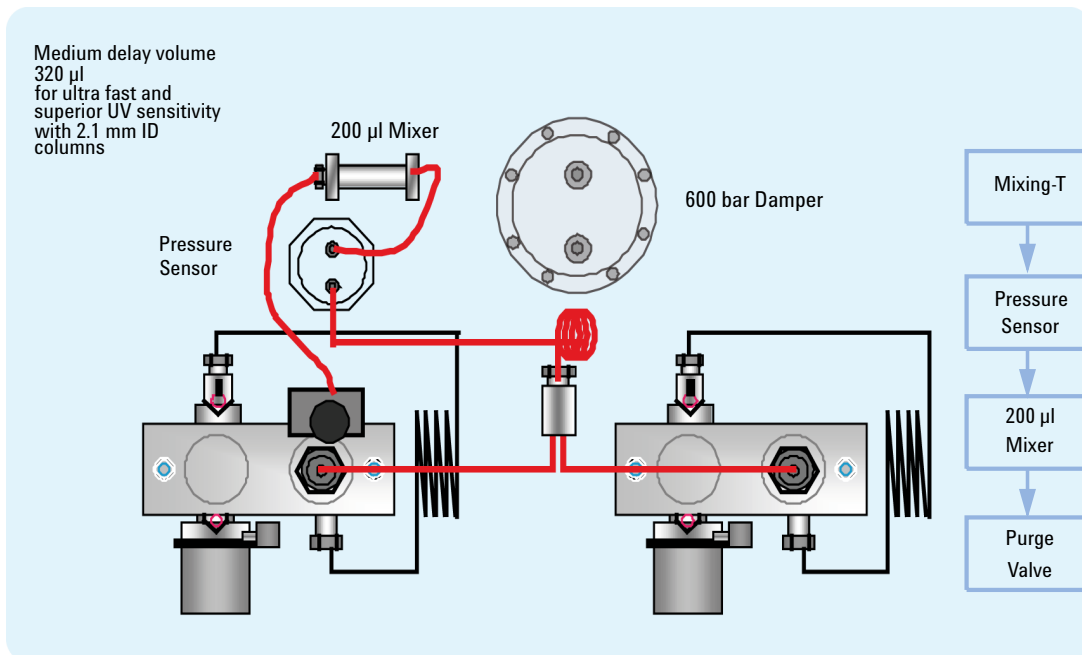
**Figure 13** Low delay configuration for 2.1 mm inner diameter columns

It is important to remember to set the correct parameter in the pump auxiliary screen. This ensures that the correct compressibility values are always applied for the mobile phases used. Calibration curves are available for most common solvents.

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Configure the Optimum Delay Volume

#### Medium delay volume configuration to achieve highest UV sensitivity

For high sensitivity UV applications an additional 200  $\mu$ L mixer ( Low volume mixer ( 200  $\mu$ L) (5067-1565)) can be installed to reduce any residual mixing noise. This small mixer gives the lowest UV baseline noise even under extreme gradient conditions. See [Figure 14](#) on page 44.

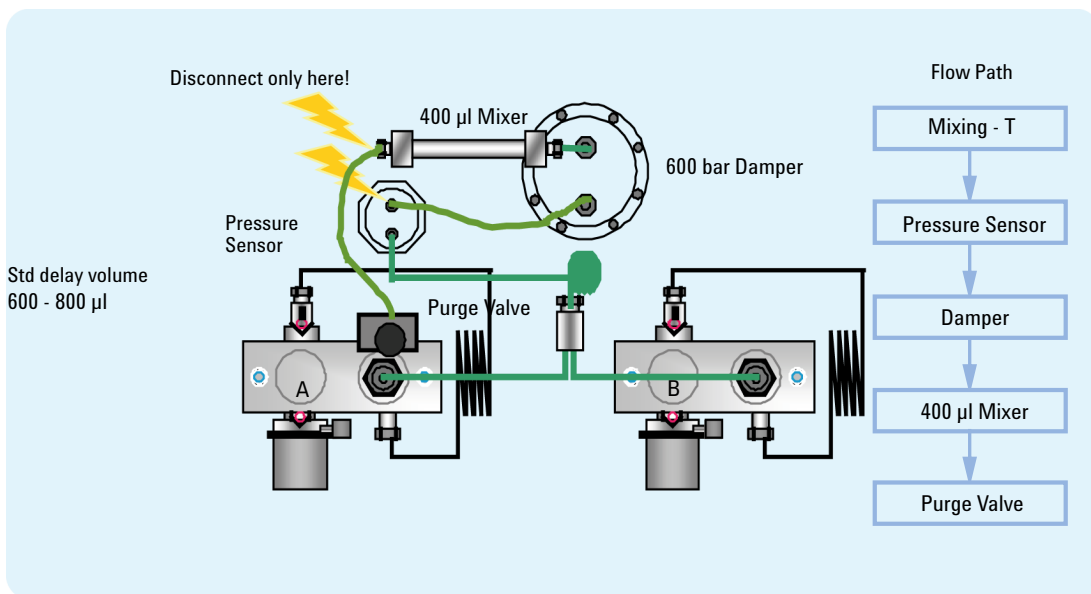


**Figure 14** Medium delay volume configuration for 2.1 mm ID columns with highest UV sensitivity

## Optimum Instrument Configuration for 3 and 4.6 mm i.d. Columns

### Standard delay volume configuration for highest UV sensitivity and direct method transferability

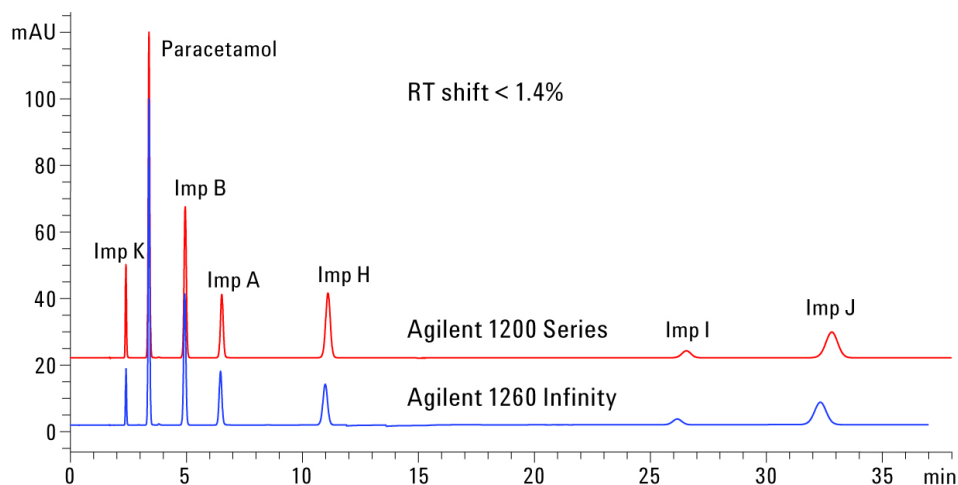
The relative column volumes for 3 mm and 4.6 mm inner diameter columns are about two and five times larger respectively than for the same length 2.1 mm i.d. columns, and the flow rates that are used are also proportionally higher. Therefore, the standard delay volume of the binary pump will not result in a significantly higher gradient delay.



**Figure 15** Standard delay volume configuration for 3 and 4.6 mm ID columns with highest UV sensitivity

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Configure the Optimum Delay Volume

The standard delay volume configuration is also the configuration which provides direct method transferability from the Agilent 1100 and 1200 Series LC system to the 1260 Infinity Binary LC or vice versa. Figure Figure 16 on page 46 shows two overlaid chromatograms of a method for analysis of paracetamol and related impurities. The method was transferred from an Agilent 1200 Series LC system to an Agilent 1260 Infinity Binary LC with the chromatographic conditions (column, mobile phase, pump settings, injection volume, column temperature, detector settings) left unchanged. As can be seen, a seamless method transfer is possible.



**Figure 16** Comparison Agilent 1200 Series and Agilent 1260 Infinity LC system on one Configuration

## How to Achieve Higher Injection Volumes

The standard configuration of the Agilent 1260 Infinity Autosampler can inject a maximum volume of 100  $\mu\text{L}$  with the standard loop capillary. To increase the injection volume, the Multidraw upgrade kit (G1313-68711) can be installed. With the kit, you can add a maximum of 400  $\mu\text{L}$  or 1400  $\mu\text{L}$  to the injection volume of your injector. The total volume is then 500  $\mu\text{L}$  or 1500  $\mu\text{L}$  for the 1260 Infinity Autosampler with 100  $\mu\text{L}$  analytical head. Note that the delay volume of your autosampler is extended when using the extended seat capillaries from the multi-draw kit. When calculating the delay volume of the autosampler you have to double the volume of the extended capillaries. The system delay volume due to the autosampler will increase accordingly.

Whenever a method is scaled down from a larger column to a smaller column it is important that the method translation makes an allowance for reducing the injection volume in proportion to the volume of the column to maintain the performance of the method. This is to keep the volume of the injection at the same percentage volume with respect to the column. This is particularly important if the injection solvent is stronger (more eluotropic) than the starting mobile phase and any increase will affect the separation particularly for early running peaks (low retention factor). In some cases, it is the cause of peak distortion and the general rule is to keep the injection solvent the same or weaker than the starting gradient composition. This has a bearing on whether, or by how much, the injection volume can be increased and the user should check for signs of increased dispersion (wider or more skewed peaks and reduced peak resolution) in trying to increase the injection size. If an injection is made in a weak solvent, then the volume can probably be increased further because the effect will be to concentrate the analyte on the head of the column at the start of the gradient. Conversely if the injection is in a stronger solvent than the starting mobile phase then increased injection volume will spread the band of analyte down the column ahead of the gradient resulting in peak dispersion and loss of resolution.

Perhaps the main consideration in determining injection volume is the diameter of the column as this will have a large impact on peak dispersion. Peak heights can be higher on a narrow column than with a larger injection on a wider column because there is less peak dispersion. With 2.1 mm i.d. columns, typical injection volumes might range up to 5 to 10  $\mu\text{L}$  but it is very dependent on the chemistry of the analyte and mobile phase as discussed above. In a gradient separation, injection volumes of about 5 % of the column volume might be achieved while maintaining good resolution and peak dispersion.

## How to Achieve Shorter Cycle Times

Shorter cycle times can be achieved by selecting a short column with good selectivity. The column dimensions are also determined by the detection system that is used. For UV detection, 3.0 mm inner diameter columns are ideal, because here the highest linear velocities can be obtained. With 4.6 mm inner diameter columns, high linear velocities can also be reached, but the maximum flow rate is limited to 5 mL/min.

The pump should be used in its standard delay volume configuration (see [Figure 13](#) on page 43) for 4.6 mm inner diameter and 3.0 mm inner diameter columns. For 2.1 mm inner diameter columns, the low delay volume configuration should be used. In addition, when using 2.1 mm inner diameter columns, the low dispersion kit should be installed to provide lowest extra-column volume. For highest UV sensitivity, it is recommended in addition to use the short mixer ( Low volume mixer ( 200 µL) (5067-1565)).

Chromatographic conditions strongly depend on the compounds that are to be analyzed, but some rules of thumb can be used to achieve short run times:

- The flow rates should be as high as possible, depending on the required resolution, back-pressure and the detection system used.
- Steep gradients should be used.
- High column temperatures are recommended to enable high flow rates to be used, and to shorten run time even further. Zorbax SB columns can be used up to 90 °C, at low pH.

### Alternating column regeneration

Even shorter cycle times can be achieved by using a column regeneration valve in combination with a regeneration pump. Using this setup, the regeneration of the column that is used previously takes place during the analysis on the second column. This shortens cycle time significantly.

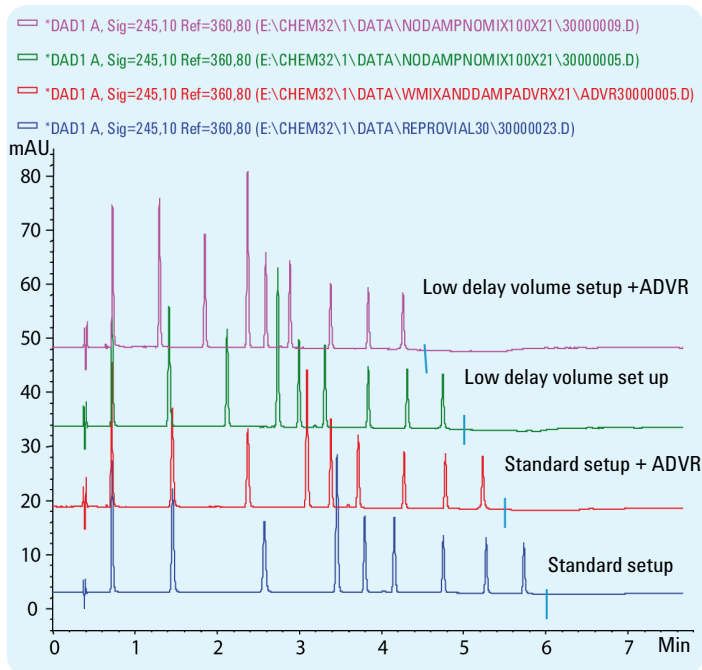
Using two columns, two pumps and one 2-position 10-port valve allows switching between these columns for shortest cycle times from injection to injection. Typically, columns of the same chemistry and the same batch provide a retention time precision that allows data processing using the same calibration table.

Please refer to the Valve Solution User Guide for a more detailed description of the alternating column regeneration.



## Automatic delay volume reduction (ADVR)

The Agilent 1260 Infinity High Performance Autosampler offers the possibility of performing overlapped injections (OI) and/or automatic delay volume reduction (ADVR). This means that the injection valve is switched out of the flow path after the sample has reached the top of the column. This reduces the delay volume significantly, see [Figure 17](#) on page 49.



**Figure 17** Reduction of the delay volume

The lower the flow rate, the greater the negative impact that can be expected from delay volume. In [Figure 17](#) on page 49, a 2.1 mm inner diameter column was used at a flow rate of 0.6 mL/min. The delay volume is reduced step by step from the lowest trace to the top trace. The influence on total run time, and the impact, especially on peak width and the heights of the first peaks, is obvious.

The drawback of overlapped injection and automatic delay volume reduction is that the autosampler is not in the flow path for the complete run time. For very sticky compounds this could lead to higher carry-over and/or compound discrimination.

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Achieve Shorter Cycle Times

Carry-over is the percentage of compound that remains in the parts of the instrument that come into contact with the sample, and is not flushed onto the column for analysis. It also means that this percentage is lost for quantitative measurement; it is discriminated. The carry-over can be measured by injecting pure solvent after the sample run is finished. Discrimination and carry-over can become even more important if the analyte compounds are non-polar and the start of the gradient contains a high percentage of water. In the worst case, the non-polar compound precipitates at the surface of contact. Small plugs of, for example, dimethylsulfoxide before and after the sample plug can help to minimize this problem.

For overlapped injection or automated delay volume reduction, the time before the injection valve is switched to the bypass mode should be increased using the flush out factor to 20. This extends the time during which the autosampler delay volume is flushed with mobile phase.

## How to Achieve High Throughput

The injection can be optimized for speed remembering that drawing the sample too fast can reduce the reproducibility. Marginal gains are to be made here as the sample volumes used tend towards the smaller end of the range in any case. A significant portion of the injection time is the time taken with the needle movements to and from the vial and into the flush port. These manipulations can be performed while the previous separation is running. This is known as "overlapped injection" and it can be easily turned on from the autosampler setup screen in the control software. The autosampler can be told to switch the flow through the autosampler to bypass after the injection has been made and then after, for example, 3 minutes into a 4 minutes run to start the process of aspirating the next sample and preparing for injection. This can typically save 0.5 to 1 minute per injection.

## How to Achieve Lowest Carry-over

Carryover is measured when residual peaks from a previous active-containing injection appear in a subsequent blank solvent injection. There will be carryover between active injections which may lead to erroneous results. The level of carryover is reported as the area of the peak in the blank solution that is expressed as a percentage of the area in the previous active injection. The Agilent 1260 Infinity Autosampler is optimized for lowest carryover by careful design of the flow path and use of materials in which sample adsorption is minimized. A carryover figure of 0.002 % should be achievable even when a triple quadrupole mass spectrometer is the detector. Operating settings of the autosampler allow the user to set appropriate parameters to minimize carryover in any application involving compounds liable to stick in the system.

The following functions of the autosampler can be used to minimize carryover:

- Internal needle wash
- External needle wash
- Needle seat backflush
- Injection valve cleaning

The flow path, including the inside of the needle, is continuously flushed in normal operation, providing good elimination of carryover for most situations. Automated delay volume reduction (ADVR) will reduce the delay volume but will also reduce the flushing of the autosampler and should not be used with analytes where carryover might be a problem.

To ensure lowest carry-over, consider the following recommendations:

- Always use the autosampler with the injection valve in mainpass position.
- Flush the exterior of the needle with an appropriate solvent using the flush port. The flush time should be a minimum of 10 s.
- If possible, reduce the draw speed to 10  $\mu\text{L}/\text{min}$ .
- Use Agilent capped 2 mL vials (Screw cap vial, 2 mL (5182-0556)).
- If the seat is contaminated, use an appropriate seat-flush procedure.
- Use flushing solvents that are capable of dissolving the sample compounds.
- Use acidic mobile phases for basic compounds.

### **Flushing and cleaning of the autosampler to achieve near zero carry-over**

During the injection routine, the sample loop, the inside of the needle, the seat capillary, and the main channel of the injection valve are in the flow path, and remain there throughout the duration of the run. This means that these parts are flushed continuously with mobile phase during the complete analysis. It is only during aspiration of the sample that the injection valve is switched out of the flow path. In this position, the pump effluent is led directly to the column. Prior to injection, the outside surfaces of the needle are washed with fresh solvent. This is achieved using the flush port of the autosampler, and prevents contamination of the needle seat. The flush port of the autosampler is refilled with fresh solvent by a peristaltic pump that is installed in the autosampler housing. The flush port has a volume of about 680  $\mu\text{L}$ , and the pump delivers 6 mL/min. Setting the wash time to 10 s means that the flush port volume is refilled more than once with fresh solvent, which is sufficient in most cases to clean the outside of the needle.

## How to Achieve Higher Resolution

Increased resolution in a separation will improve the qualitative and quantitative data analysis, allow more peaks to be separated or offer further scope for speeding up the separation. This section explains how resolution can be increased by examining the following points:

- Optimize selectivity
- Smaller particle-size packing
- Longer columns
- Shallower gradients, faster flow
- Minimal extra-column volume
- Optimize injection solvent and volume
- Fast enough data collection

Resolution between two peaks is described by the resolution equation:

$$R_s = \frac{1}{4} \sqrt{N} \frac{(\alpha - 1)(k_2 + 1)}{\alpha k_2}$$

where

- $R_s$ =resolution,
- $N$ =plate count (measure of column efficiency),
- $\alpha$ =selectivity (between two peaks),
- $k_2$ =retention factor of second peak (formerly called capacity factor).

The term with the most significant effect on resolution is the selectivity,  $\alpha$ , and practically varying this term involves changing the type of stationary phase (C18, C8, phenyl, nitrile etc.), the mobile phase and temperature to maximize the selectivity differences between the solutes to be separated. This is a substantial piece of work which is best done with an automated method development system which allows a wide range of conditions on different columns and mobile phases to be assessed in an ordered scouting protocol. This section considers how to get higher resolution with any chosen stationary and mobile phases. If an automated method development system

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Achieve Higher Resolution

was used in the decision on phases, it is likely that short columns were used for fast analysis in each step of the scouting.

The resolution equation shows that the next most significant term is the plate count or efficiency,  $N$ , which can be optimized in a number of ways.  $N$  is inversely proportional to the particle size and directly proportional to the length of a column and so smaller particle size and a longer column will give a higher plate number. The pressure rises with the inverse square of the particle size and proportionally with the length of the column. Resolution increases with the square root of  $N$  so doubling the length of the column will increase resolution by a factor of 1.4. What is achievable depends on the viscosity of the mobile phase as this relates directly to the pressure. Methanol mixtures will generate more back pressure than acetonitrile mixtures. Acetonitrile is often preferred because peak shapes are better and narrower in addition to the lower viscosity but methanol generally yields better selectivity (certainly for small molecules less than about 500 Da). The viscosity can be reduced by increasing the temperature but it should be remembered that this can change the selectivity of the separation. Experiment will show if this leads to increase or decrease in selectivity. As flow and pressure are increased, it should be remembered that frictional heating inside the column will increase. That can lead to slightly increased dispersion and possibly a small selectivity change both of which could be seen as a reduction in resolution. The latter case might be offset by reducing the temperature of the thermostat by a few degrees and again experiment will reveal the answer.

The van Deemter curve shows that the optimum flow rate through an STM column is higher than for larger particles and is fairly flat as the flow rate increases. Typical, close to optimum, flow rates for STM columns are: 2 ml/min for 4.6 mm i.d.; and 0.4 ml/min for 2.1 mm i.d. columns.

In isocratic separations, increasing the retention factor,  $k$ , results in better resolution because the solute is retained longer. In gradient separations, the retention is described by  $k^*$  in the following equation:

$$k^* = \frac{t_G}{\Delta\%B} \cdot \frac{F}{V_m} \cdot \frac{100}{S}$$

where:

- $k^*$  = mean  $k$  value,
- $t_G$  = time length of gradient (or segment of gradient) (min),
- $F$  = flow (ml/min),
- $V_m$  = column delay volume,
- $\Delta\%B$  = change in fraction of solvent B during the gradient,
- $S$  = constant (ca. 4-5 for small molecules).

This shows that  $k$  and hence resolution can be increased by having a shallower gradient (2 to 5 %/min change is a guideline), higher flow rate and a smaller volume column. This equation also shows how to speed up an existing gradient – if the flow is doubled but the gradient time is halved,  $k^*$  remains constant and the separation looks the same but happens in half the time.

Any reduction in extra-column volume will reduce dispersion and give better resolution. This is already optimized in the 1260 Infinity Binary LC with narrow bore (0.12 mm i.d.) capillaries (check that the shortest length is used between column and detector) and the Max-light cartridge flow cell.

Finally, any gains in resolution must be preserved by having data collection which is fast enough to accurately profile the narrow peaks.

In summary, the following steps should be considered to increase resolution:

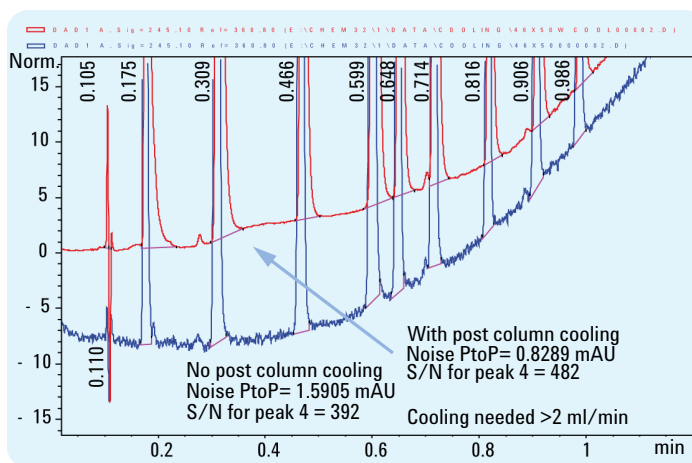
- The first step in improving resolution is always to test different stationary phases and to select the column with the best separation. This is the parameter that is of most importance for resolution.
- The second step is to use long columns or even coupled columns to increase the plate number.
- A third step is to shift peaks to higher retention factors. For  $k$  values of 5 to 10, the impact is significant. With higher  $k$  values, the effect is very low.

In practice, this means that longer columns with appropriate selectivity give better resolution.

## Optimum Instrument Configuration for High Resolution

### Column Compartment

The column compartment can be used in its standard version for 4.6 mm inner diameter columns. At flow rates above 2 mL/min, and temperatures above 60 °C, the column effluent should be cooled down to the temperature of the detector by using the 1.5 µL cooler/heater in the column compartment. This ensures lowest noise level with UV detectors, even at 5 mL/min and 80 °C (see [Figure 18](#) on page 56 ).



**Figure 18** Influence of post column cooling (PCC) on baseline noise

If 2.1 mm inner diameter columns are used at low flow rates, the small heating device should be used for lowest extra-column volume.



## Extra-column Volume

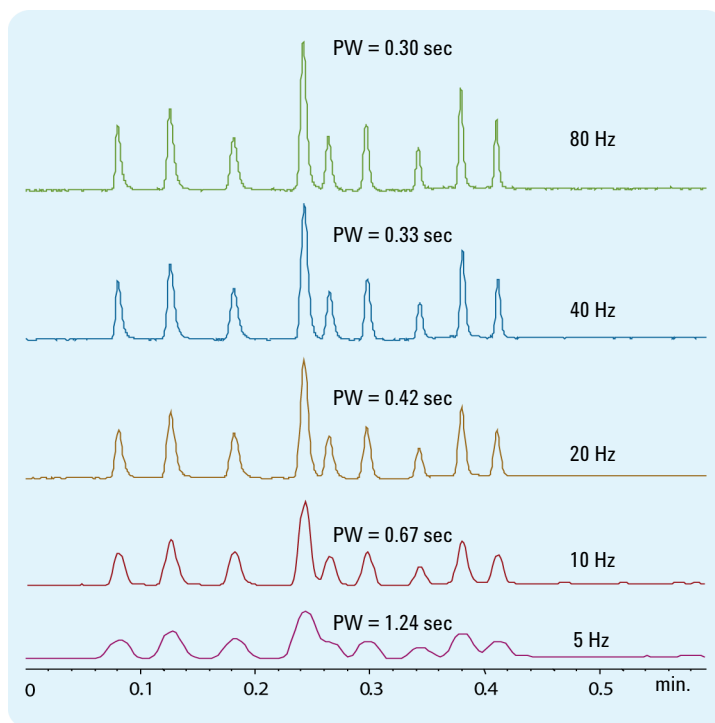
To maintain the high resolution achieved on the column, the extra-column volume, especially after the column, should be as low as possible.

- For 4.6 mm inner diameter columns the standard delay configuration should be used for the pump, see [Figure 15](#) on page 45.
- For 2.1 mm inner diameter columns, the low delay configuration should be used for the pump, and the low dispersion kit should be installed in the column compartment. For highest UV sensitivity, the short mixer is also recommended. See [Figure 13](#) on page 43 and [Figure 14](#) on page 44.
- The injection volume is also of importance, especially if the sample is dissolved in an organic solvent. In this case, the gradient should start with a low percentage of organic phase to focus the compounds at the top of the column. This avoids peaks dispersion due to the injection.
- The 10 mm Max-Light cartridge cell has a low dispersion volume ( $\sigma$  volume 1.0  $\mu$ L) and no further volume optimization is required.
- In situations where the alternative 60 mm Max-Light high sensitivity cell is used to get higher sensitivity the cell volume is optimized for the use with 3 mm and 4.6 mm inner diameter columns.

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Achieve Higher Resolution

#### Data Rate

The data rate setting for the UV detector must be selected appropriately. Selecting a data rate that is too low results in increased peak width and lower resolution, see [Figure 19](#) on page 58.



**Figure 19** Peak width vs. UV data Rate

[Table 6](#) on page 59 shows the dependency of peak width, resolution and peak capacity on the data rate. For fast and ultra-fast LC, high data rates are needed; otherwise, the separation that is achieved on the column is destroyed in the detector.

**Table 6** Relation between data rate and chromatographic performance

Data Rate	Peak Width	Resolution	Peak Capacity
80 Hz	0.300	2.25	60
40 Hz	0.329	2.05	55
20 Hz	0.416	1.71	45
10 Hz	0.666	1.17	29
5 Hz	1.236	0.67	16

80 Hz data rate compared with 20 Hz data rate results in improvements in:

- Peak Width:
  - 30 %
- Resolution:
  - + 30 %
- Peak capacity:
  - + 40 %
- Apparent column efficiency:
  - + 70 %

### Chromatographic Conditions

As already stated previously, chromatographic conditions depend on the compounds to be analyzed. But here also, some rules of thumb exist.

- Moderate flow rates should be used, but recent experiments have shown that elevated flow rates can also be advantageous for improved separation. For 4.6 mm inner diameter columns packed with sub-2-micron particles, a flow rate of 2 mL/min is recommended as a starting value. For 2.1 mm inner diameter sub-2-micron columns, 0.4 mL/min is a good starting point.
- Moderate gradients should be used, for example, 2 – 5 % gradient change per minute.
- The column temperature is an additional parameter for optimization. Temperature can influence a separation, and should not be overlooked.

## **How to Achieve Higher Sensitivity**

The sensitivity of a separation method is linked to the choice of stationary and mobile phases as good separation with narrow peaks and a stable baseline with minimal noise are desirable. The choice of instrument configuration will have an effect and a major impact is the setup of the detector.

## Optimum Instrument Configuration for High Sensitivity

### General Considerations

- To achieve lowest baseline noise, the standard delay volume configuration is recommended for the 1260 Infinity Binary Pump .
- Here also, the injection volume and the sample dissolution solvent are important. Care must be taken that the compounds are focused at the top of the column, to avoid peak dispersion due to the injection, which would cause a reduced peak height. In order to achieve this, the sample should be dissolved in a solvent composition of lower elution strength than the mobile phase.
- The column temperature should not be too low, to avoid long retention of the peaks on the column. This also creates peak dispersion and a lower peak height.
- The data rate of the UV detector should be selected according to the actual peak width. Higher than necessary data rates should be avoided because of higher noise levels.
- Available UV detectors are the Agilent 1260 Infinity Diode Array Detector (G4212B) and the Agilent 1260 Infinity Variable Wavelength Detector (G1314F), with data rates of 80 Hz and significantly lower noise and drift levels.
- “[Choosing a Flow Cell](#)” on page 63 gives an overview of the flow cells available with the G4212B DAD . For information on flow cells used with the G1314F VWD , please refer to the User Manual of the Agilent 1260 Infinity Variable Wavelength Detector .

### Chromatographic Conditions

For an optimum signal-to-noise ratio, it is of advantage if the peaks elute quickly.

- Elevated flow rates should be used to elute the peaks at low  $k'$  values.
- Faster gradients can also be applied to elute the peaks at low  $k'$  values.

## Columns

Sensitivity is specified as a signal-to-noise ratio (S/N) and hence the need to maximize peak height and minimize baseline noise. Any reduction in peak dispersion will help to maintain peak height and so extra-column volume should be minimized by use of short, narrow internal diameter, connection capillaries and correctly installed fittings. Using smaller inner diameter columns should result in higher peak height and is therefore ideal for applications with limited sample amounts. If the same sample amount can be injected on a smaller i.d. column, then the dilution due to column diameter will be less and the sensitivity will increase. For example, decreasing the column i.d. from 4.6 mm to 2.1 mm results in a theoretical gain in peak height of 4.7 times due to the decreased dilution in the column. For a mass spectrometer detector, the lower flow rates of narrow columns can result in higher ionization efficiencies and therefore higher sensitivity.

## Detector Settings

The detector has a number of parameters that are used to optimize performance. The following sections describe how the detector parameters affect performance characteristics:

- Flow cell affects sensitivity,
- Wavelength and bandwidth affect sensitivity, selectivity and linearity,
- Slit Width affects sensitivity, spectral resolution and linearity,
- Peak Width affects sensitivity and resolution.

## Choosing a Flow Cell

Several flavors of the Max-Light Cartridge Flow Cell are available, see (Table 7 on page 63).

**Table 7** Specifications for Max-Light Cartridge Flow Cells

Cartridge Cells	<ul style="list-style-type: none"> <li>• Max-Light Cartridge Cell ( 10 mm, V(<math>\sigma</math>) 1.0 <math>\mu</math>L) (G4212-60008)</li> <li>• Max-Light Cartridge Cell Bio-inert ( 10 mm, V(<math>\sigma</math>) 1.0 <math>\mu</math>L) (G5615-60018)</li> <li>• Max-Light Cartridge Cell ( 60 mm, V(<math>\sigma</math>) 4.0 <math>\mu</math>L) (G4212-60007)</li> <li>• Max-Light Cartridge Cell Bio-inert ( 60 mm, V(<math>\sigma</math>) 4.0 <math>\mu</math>L) (G5615-60017)</li> <li>• HDR Max-Light Cartridge Cell ( 3.7 mm, V(<math>\sigma</math>) 0.4 <math>\mu</math>L) (G4212-60032)</li> <li>• ULD Max-Light Cartridge Cell ( 10 mm, V(<math>\sigma</math>) 0.6 <math>\mu</math>L) (G4212-60038)</li> <li>• Max-Light Cartridge Test Cell (G4212-60011)</li> </ul>
Maximum pressure	70 bar (1015 psi) Maximum Operating Pressure (MOP) <sup>1</sup> 150 bar (2175 psi) Maximum Incidental Pressure (MIP) <sup>2</sup>
pH range	1.0-12.5 (solvent dependent)

<sup>1</sup> Maximum Operating Pressure (MOP): The maximum pressure at which the system can operate continuously under normal conditions.

<sup>2</sup> Maximum Incidental Pressure (MIP): The maximum pressure which the system can experience during a short time.

### Normal Applications

The Max-Light Cartridge Cell ( 10 mm, V(s) 1.0  $\mu$ L) (G4212-60008) covers a wide range of applications:

- all column diameter down to at least 2.1 mm ID or even less
- applications with peak dispersion (Peakwidth x flow) down to  $\sim 2 \mu$ L  
 [example: pw = 0.04 min at flow = 0.1 mL/min gives peak dispersion of  
 $0.04 \text{ min} \times 0.1 \text{ mL/min} = 0.004 \text{ mL} = 4 \mu\text{L}$ ]

### High Sensitivity

If higher sensitivity is necessary, the Max-Light Cartridge Cell ( 60 mm, V(s) 4.0  $\mu$ L) (G4212-60007) can be used. This cell enhances the detector by lowering the limit of detection (LOD) by a factor of about 3 (depending on the application).

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Achieve Higher Sensitivity

#### Ultra-Low Dispersion

The Max-Light Cartridge ULD cell can be used with the G4212A DAD and G4212B DAD . The cell is a requirement for the Ultra-Low Dispersion Kit solution which currently exists as 1290 Infinity Ultra-Low Dispersion Kit (5067-5189). The cell should be part of the ultra-low dispersion solution.

#### High Dynamic Range

The Max-Light Cartridge HDR cell can be used with the G4212A DAD and G4212B DAD . The cell is required as a part of the High Dynamic Range (HDR) solution which will be introduced March/April 2013.

#### NOTE

To protect the flow cell against overpressure (e.g. in systems with LC/MS) install Inline Pressure Relief Valve Kit (G4212-68001), see *Agilent 1200 Infinity Series Diode Array Detectors* manual.

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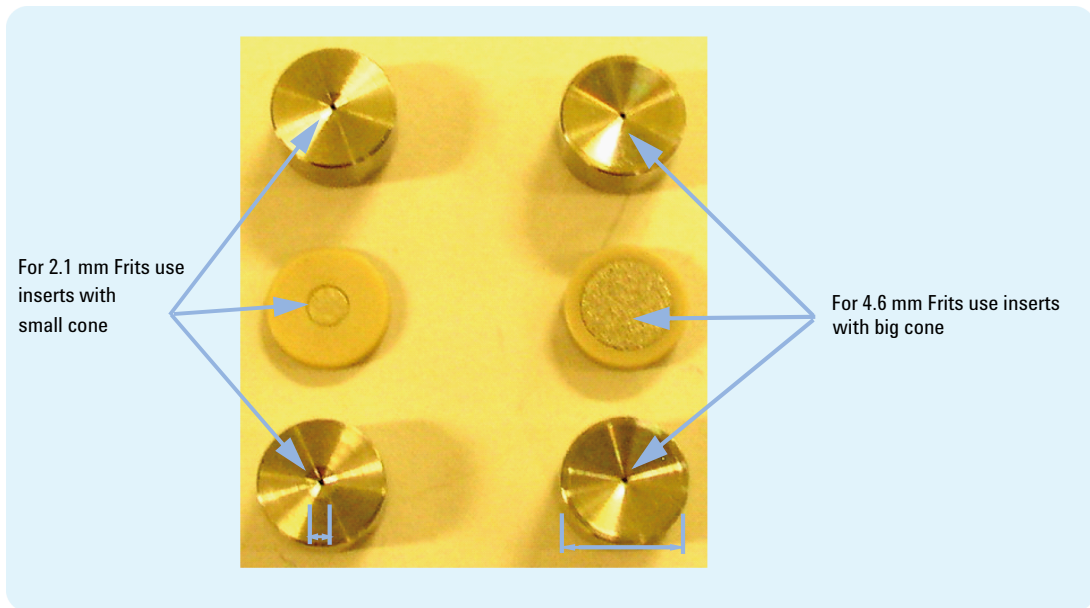
## How to Prevent Column Blockages

Columns packed with sub-2-micron particles also need frits with small pore size to prevent packing material being swept out. This immediately brings increased risk of blocking these frits with particulates from the sample, mobile phase or/and from the instrument itself. To protect the column, additional small filters ( [Figure 20](#) on page 66) can be used in front of the column. It is also recommended that the sample is thoroughly filtered or/and centrifuged, and that any kind of particulate matter in the mobile phases is avoided.

To ensure best results follow these simple usage guidance:

- 1** Install and run the column only in the flow direction marked on the column.
- 2** Use only solvents that are high quality, chromatography grade.
- 3** Filter all aqueous buffers and all samples through an appropriate 0.2 µm filter before use.
- 4** Replace bottles of mobile phase buffer every 24 – 48 h – do not add mobile phase to the bottle; always use a new bottle.
- 5** Do not use a high buffer salt mobile phase (> 50 mM) in combination with high acetonitrile concentrations due to possible precipitation.
- 6** An in-line filter is recommended to catch particulates and extend column life. Use the suitable 1260 Infinity In-line filter for your column: 1260 Infinity LC in-line filter, 2.1 mm, 0.2 µm pore size filter, max 600 bar, connecting capillary 70 x 0.12 mm SS (5067-1551) for 2.1 i.d. columns, 1260 Infinity LC in-line filter, 4.6 mm, 0.2 µm pore size filter, max 600 bar, connecting capillary 90 x 0.17 mm SS (5067-1553) for 4.6 mm or 3.0 mm i.d. columns. Change the filter when the pressure increases by 10 %.
- 7** Purge the pumps (the connections up to the column) of any buffer containing mobile phases and flush through 5 mL of solvent before attaching the column to the instrument.
- 8** Flush the column with compatible mobile phase starting slowly at 0.1 mL/min for a 2.1 mm inner diameter column, 0.2 mL/min for a 3.0 mm inner diameter column, and 0.4 mL/min for 4.6 mm inner diameter. Increase the flow rate to the desired flow over 5 minutes.
- 9** Once the pressure has stabilized, attach the column to the detector.
- 10** Equilibrate the column and detector with 10 column volumes of the mobile phase prior to use. (1 – 5 mL depending on column size.)
- 11** Avoid over-pressure. Check the pressure range of your gradient – which may be 100 – 130 bar or more – before starting any sequence.

### 3 Optimization of the Agilent 1260 Infinity Binary LC How to Prevent Column Blockages



**Figure 20** Protection for 4.6 and 2.1 mm id columns packed with 1.8  $\mu\text{m}$  particles, inlet frit with 0.2  $\mu\text{m}$  pore size



## 4 System Setup and Installation

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This chapter includes information on software installation, installation of the modules, and preparing the system for operation.



## **Installing Software**

### **Installing the Software Controller and Data System**

For details of installation procedures for the software, refer to the detector manual and the software manuals.

## Installing the Agilent Lab Advisor Software

For details of installation procedures for the Agilent Lab Advisor software, refer to the software documentation on the Lab Advisor DVD.

Agilent Lab Advisor replaces and extends the diagnostic functions that were formerly available only in the ChemStation software.

Agilent Lab Advisor is a Windows-based application that continuously monitors instruments in the lab in real time and increases productivity through automatic notification of maintenance and service needs with the use of advanced counters. This allows a problem to be fixed before it impacts results. The software includes an extensive suite of user information and documentation, a set of calculators and tools to help set up, calibrate, and maintain your instrument, and tests and diagnostic routines to verify proper performance. Agilent Lab Advisor also provides feedback and solutions for any instrument errors that may arise. The software will work with or without Agilent data systems.

The software monitors:

- LC module status
- Early Maintenance Feedback (to determine the need for upgrade or replacement)

In addition, the software:

- Automates useful tests,
- Attempts to identify supported LAN-based instruments that are powered on and connected to your PC or lab's network,
- Automatically suggests replacements parts and troubleshooting tasks for some common instrument problems.

## **Installing the Modules**

### **Installing the System Modules**

For details of installation procedures for the modules, refer to the individual module manuals. These manuals also contain information on specifications, maintenance and parts.

### **Integration Into the Network**

For network integration of your system refer to user manuals of your modules (chapter *LAN Configuration*).

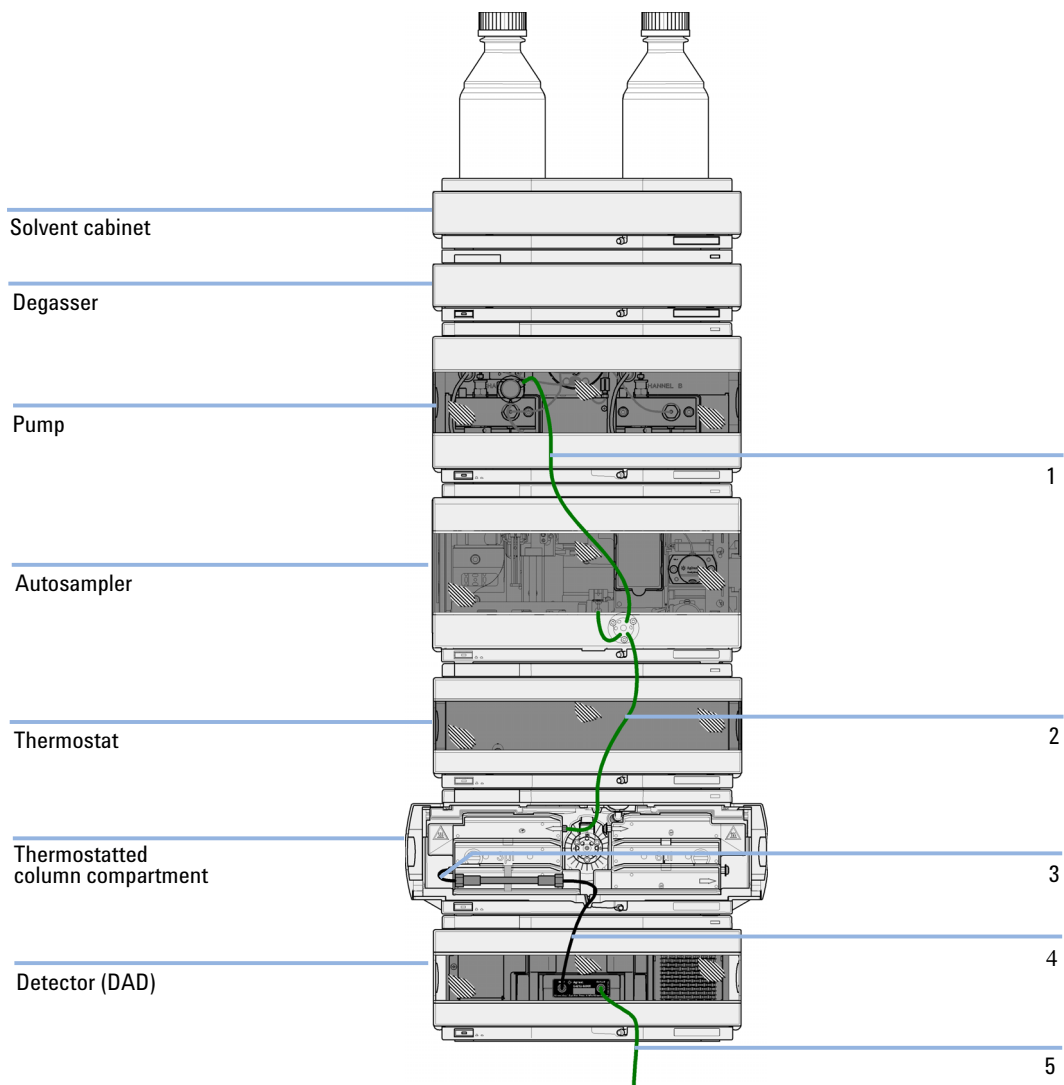
## Capillary and Tubing Connections in Flow Path

Depending on the system configuration, capillaries of different lengths and diameters are used. These are described below.

See module manuals for module-internal capillary and tubing connections.

### Connections for Standard Delay Volume Configuration

Figure 21 on page 72 shows capillary and tubing connections in the flow path for the standard delay volume configuration of the 1260 Infinity Binary LC.



**Figure 21** Capillary and tubing connections in flow path (standard delay volume configuration)



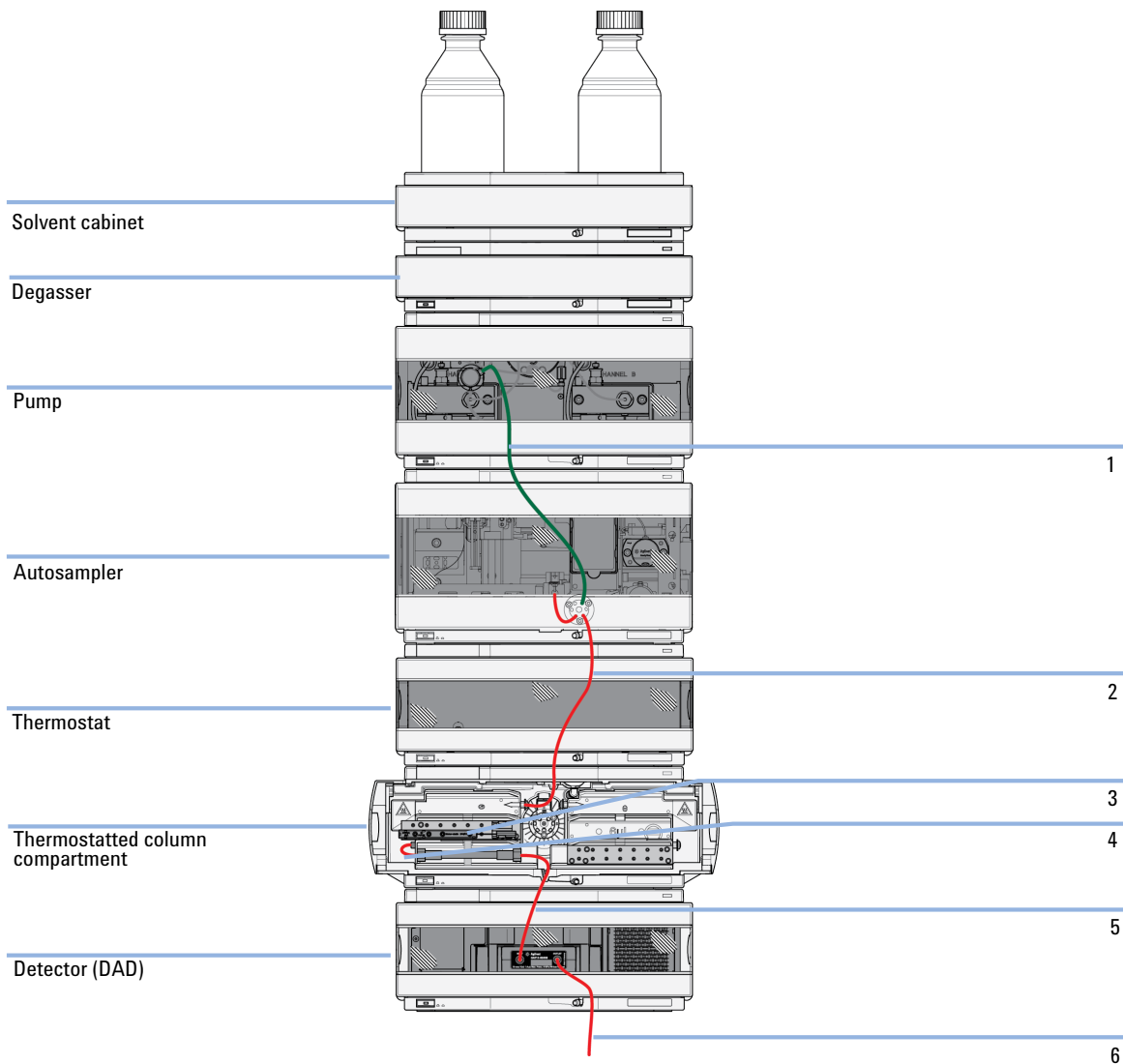
<b>Item</b>	<b>p/n</b>	<b>Description</b>
1	G1312-87303	Capillary ST 0.17 mm x 400 mm S/S (Pump to autosampler)
1	G1312-87304	Capillary ST 0.17 mm x 700 mm S/S (Pump to cooled Autosampler)
2	G1367-87304	Capillary ST 0.17 mm x 250 mm S/S (Autosampler to TCC)
2	01090-87306	Capillary ST 0.17 mm x 380 mm S/S (Cooled Autosampler to TCC)
3	G1316-87300	Capillary ST 0.17 x 90 mm S/S (TCC to Column)
4	G1315-87311	Capillary ST 0.17 mm x 380 mm S/S (Column to DAD)
5	5062-2462	Tube PTFE 0.8 mm x 2 m, re-order 5 m (DAD to waste)
5	5062-8535	Waste accessory kit (VWD to waste)

## 4 System Setup and Installation

### Installing the Modules

#### Connections for Medium and Low Delay Volume Configuration

Figure 22 on page 74 shows capillary and tubing connections in the flow path for medium and low delay volume configurations of the 1260 Infinity Binary LC.



**Figure 22** Capillary and tubing connections in flow path (medium and low delay volume configurations)

<b>Item</b>	<b>p/n</b>	<b>Description</b>
1	G1312-87303	Capillary ST 0.17 mm x 400 mm S/S (Pump to autosampler)
1	G1312-87304	Capillary ST 0.17 mm x 700 mm S/S (Pump to cooled Autosampler)
2	G1313-87304	Capillary ST 0.12 mm x 180 mm S/S (Autosampler to TCC)
2	01090-87610	Capillary ST 0.12 mmx 280 mm S/S (cooled Autosampler to TCC or to item 3 if installed)
3	G1316-80002	Heater long-up (0.12 mm i.d., 1.6 µL internal volume)
3	G1316-80003	Heater long-down (0.12 mm i.d., 1.6 µL internal volume)
4	01090-87611	Capillary ST 0.12 mmx 105 mm S/S (TCC to Column, not needed if item 3 is installed)
5	G1315-87312	Capillary ST 0.12 mm x 150 mm S/S (Column to DAD)
6	5062-2462	Tube PTFE 0.8 mm x 2 m, re-order 5 m (DAD to waste)

## Priming the System

### Initial Priming

**When** Before a degasser or solvent tubing can be used, it is necessary to prime the system. Isopropanol is recommended as priming solvent due to its miscibility with nearly all HPLC solvents and its excellent wetting properties.

Parts required	#	Description
	1	Isopropanol

**Preparations** Connect all modules hydraulically as described in the respective module manuals.  
Fill each solvent bottle with 100 mL isopropanol  
Switch the system on

#### WARNING

**When opening capillary or tube fittings, solvents may leak out.**

**The handling of toxic and hazardous solvents and reagents can carry health risks.**

→ Observe appropriate safety procedures (for example, wear goggles, safety gloves and protective clothing) as described in the material handling and safety data sheet supplied by the solvent vendor, especially when toxic or hazardous solvents are used.

#### NOTE

The purge tool of the Lab Advisor can be used to purge the pump automatically.

#### NOTE

If the pump is not able to draw in the solvent from the bottles, use a syringe to move the solvent manually through tubing and degasser.

#### NOTE

When priming the vacuum degasser with a syringe, the solvent is drawn through the degasser tubes very quickly. The solvent at the degasser outlet will therefore not be fully degassed. Pump for approximately 10 minutes at your desired flow rate before starting an analysis. This will allow the vacuum degasser to properly degas the solvent in the degasser tubes.

- 1** Open the purge valve of the pump
- 2** Set the flow rate to 5 mL/min.
- 3** Select channel A1
- 4** Turn the flow on
- 5** Observe if the solvent in the tubing of channel A1 is advancing towards the pump. If it isn't, disconnect the solvent tubing from the solvent selection valve, attach a syringe with a syringe adapter and pull the liquid through the degasser. Reattach the tubing to the solvent selection valve.
- 6** Pump 30 mL isopropanol to remove residual air bubbles.
- 7** Switch to the next solvent channel and repeat steps 5 and 6 until all channels have been purged.
- 8** Turn the flow off and close the purge valve.

## 4 System Setup and Installation

### Installing the Modules

#### Regular Priming

<b>When</b>	When the pumping system has been turned off for a certain time (for example, overnight) air will rediffuse into the solvent channel between the vacuum degasser and the pump. If solvents containing volatile components are left in the degasser without flow for a prolonged period, there will be a slight loss of the volatile components.
<b>Preparations</b>	Switch the system on

#### NOTE

The purge tool of the Lab Advisor can be used for automatically purging the pump.

- 1 Open the purge valve of your pump by turning it counterclockwise and set the flow rate to 5 mL/min.
- 2 Flush the vacuum degasser and all tubes with at least 10 mL of solvent.
- 3 Repeat step 1 and 2 for the other channel(s) of the pump.
- 4 Set the required composition and flow rate for your application and close the purge valve.
- 5 Pump for approximately 10 minutes before starting your application.

## Changing Solvents

**When** When the solvent of a channel is to be replaced by another solvent that is not compatible (solvents are immiscible or one solvent contains a buffer), it is necessary to follow the procedure below to prevent clogging of the pump by salt precipitation or residual liquid droplets in parts of the system.

Parts required	#	p/n	Description
	1		Purging solvent(s), see <a href="#">Table 8</a> on page 80
	1	5022-2184	Union ZDV

**Preparations** Remove the column and replace it by a ZDV fitting  
Prepare bottles with appropriate intermediate solvents (see [Table 8](#) on page 80)

- 1 If the channel is not filled with buffer, proceed to step 4.
- 2 Place the solvent intake filter into a bottle of water.
- 3 Flush the channel at a flow rate suitable for the installed tubing (typically 3 – 5 mL/min) for 10 min.
- 4 Modify the flow path of your system as required for your application.

### CAUTION

*Buffer salt of aqueous buffers may precipitate in residual isopropanol.*

Capillaries and filter may be clogged by precipitating salt.

- Flush solvent lines containing high concentration of salts first with water before introducing organic solvent.
- Do not perform steps 5 to 7 for channels running with aqueous buffer as solvent.

- 5 Replace the solvent bottle by a bottle of isopropanol.
- 6 Flush the channel at a flow rate suitable for the installed tubing (typically 3 – 5 mL/min) for 5 min.
- 7 Swap the bottle of isopropanol with a bottle of solvent for your application.
- 8 Repeat steps 1 to 7 for the other channel(s) of the pump.
- 9 Install the desired column, set the required composition and flow rate for your application and equilibrate the system for approx. 10 minutes prior to starting a run.

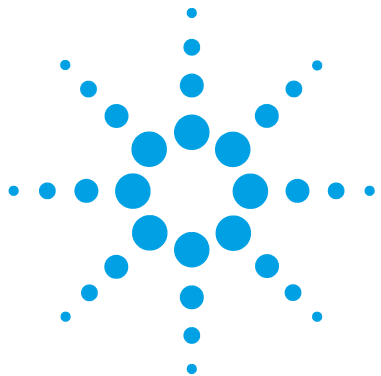
## 4 System Setup and Installation

### Installing the Modules

**Table 8** Choice of Priming Solvents for Different Purposes

Activity	Solvent	Comments
After an installation When switching between reverse phase and normal phase (both times)	Isopropanol Isopropanol	Best solvent to flush air out of the system Miscible with almost all solvents
After an installation	Ethanol or methanol	Alternative to isopropanol (second choice) if no isopropanol is available
To clean the system when using buffers After changing aqueous solvents	HPLC grade water HPLC grade water	Best solvent to re-dissolve buffer crystals Best solvent to re-dissolve buffer crystals
After the installation of normal phase seals (PE seals (pack of 2) (0905-1420))	Hexane + 5 % isopropanol	Good wetting properties





## 5 Quick Start Guide

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This chapter provides information on data acquisition and data analysis with the 1260 Infinity Binary LC.



## About the Quick Start Guide

This chapter provides information on running the Agilent 1260 Infinity Binary LC. It can be used as a guide to quickly running a first analysis after installation, serving both as a tutorial example and a check on the overall functioning of the system. It also includes more detailed information about method parameters.

This example demonstrates setting up and running an analysis using the columns and checkout sample that are provided with the Agilent 1260 Infinity Binary LC. The example refers to menus and commands in OpenLAB CDS ChemStation Edition, but identical functions are also available on the alternative control options such as OpenLAB CDS EZChrom Edition and MassHunter software.

In the following, the terms ChemStation and EZChrom always refer to Agilent OpenLAB CDS ChemStation Edition and Agilent OpenLAB CDS EZChrom Edition respectively.

### NOTE

The starting point assumes that the system has been installed, turned on and initially primed (see “[Initial Priming](#)” on page 76). The UV lamp should be turned on at least 30 minutes before starting any quantitative work.

---

## Preparing the System

### Turning the System ON

If the system is not already fully on with the software showing Ready status, follow these steps:

- 1 Turn on the computer system and wait for the Windows desktop to appear.
- 2 Turn on the electrical power to the LC modules using the button at the lower left of each module.

A green power on light will be visible in the center of the button.

- 3 Start the control software on the computer by clicking the icon (if configured). Alternatively, you can select **Start > All Programs > Agilent Technologies > OpenLAB > OpenLAB Control Panel**. Select the relevant instrument in the navigation pane under **Instruments**, and click **Launch online**.

The ChemStation software opens in the **Method and Run Control** view. The modules are initially in Standby mode and Not Ready status, except for the autosampler which immediately initializes and becomes Ready.

- 4 To switch on each module individually, right-click the relevant icon and select **Switch [module name] on** from the context menu.

Alternatively, you can turn on all modules simultaneously in the system by clicking the **System On/Off** button in the bottom right of the system diagram. The system status changes from *Not Ready* (yellow indication) to *Ready* (green indication) after a short delay as the setpoints are attained.

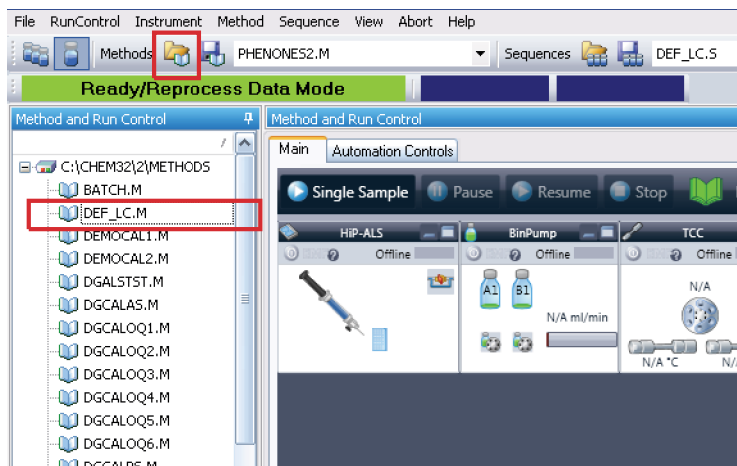
## Loading the Default Method

The ChemStation has a default method named **DEF\_LC.M** which is loaded at first execution or whenever a new blank method template is required. It contains default settings for all modules.

With this procedure, you load the method **DEF\_LC.M**. You can use it to set all parameters to default settings, or to get a blank method template before setting up a new method.

- 1 Go to **Method and Run Control** view of the ChemStation.
- 2 On the menu bar, select **Method > New Method...**, and select **DEF\_LC.M** from the context menu.

Alternatively, you can use the **Load Method** icon  under the menu bar, or double-click the method name **DEF\_LC.M** in the **Methods** tab of the Navigation Pane.



The default method (**DEF\_LC.M**) has a set of default parameters which can then be modified to create a new method. For instance, the flow rate is set to zero, and the **Method Information** and **Method History** are blank.

### NOTE

Note that this method can never be overwritten with new parameters. Hence clicking on **Save** will re-direct you into the **Save As...** function, so that you must enter a different method name.

## Configuring the Online Plot

- 1 If the **Online Plot** window is not visible: Click **View > Online Signals > Signal Window 1** to display the window.



- 2 To configure the desired signal(s) in the **Online Plot** window, click **Change....**

The **Edit Signal Plot** setup page opens.

## 5 Quick Start Guide

### Preparing the System

- 3 In the **Available Signals** box, highlight the required signal(s), and click **Add** to move them to the **Selected Signals** box.
- 4 To configure the individual settings for each signal, highlight the signal in the **Selected Signal** box and set the required values in the lower half of the page.

#### NOTE

In addition to the detector signals, parameter traces such as temperature and pressure can also be plotted. With **Apply to Method**, the settings in this page can be stored into the method.

---

The **Online Plot** window behaves like electronic chart paper, continuously recording the output from the detector(s) and other output parameters. The signals are drawn at the right of the window and move away to the left. Up to 90 min of past data is accessible. This is useful for checking the baseline and looking at previous injections. The X and Y-axis scales can be adjusted directly with the up/down buttons on each axis.

The **Adjust** button in the **Online Plot** window moves the current point on the selected signal to the zero line. The selected signal is indicated by the color of the Y-axis labels. A particular signal may be selected by clicking on the signal or by clicking on the relevant signal description at the top of the plot.

The **Balance** button zeroes all detectors when pressed.

#### NOTE

Changes made in the **Online Plot** page do not in any way affect the data stored into the individual data files.

---

## Purging the Pump

Purge the pump, if ...

- The pump has been primed for the first time.
- The pump is to be purged with fresh solvent before using the system, or when the solvent is to be exchanged for another.
- The pump has been idle for a few hours or more (air may have diffused into the solvent lines and purging is recommended).
- The solvent reservoirs are refilled, and the pump requires purging to fill the system with fresh solvent. If different solvents are to be used, ensure that the new solvent is miscible with the previous solvent and if necessary use an intermediate step with a co-miscible solvent (isopropanol is often a good choice, check with a solvent miscibility table).

For details on the purging procedure, refer to [“Regular Priming”](#) on page 78.

## Data Acquisition in Method and Run Control View

### Method Parameters for System Checkout with Isocratic Checkout Sample

For checkout of the 1260 Infinity Binary LC, a test run with an isocratic test mixture (Agilent isocratic checkout sample (01080-68704)) will be performed on the column ordered and delivered with your system.

Agilent isocratic checkout sample (01080-68704) contains the following components dissolved in methanol:

- dimethyl phthalate
- diethyl phthalate
- biphenyl
- o-terphenyl

The method parameters for separation of this test mixture are summarized in [Table 9](#) on page 89 for the standard delay volume configuration and in [Table 10](#) on page 90 for the low delay volume configuration.



**Table 9** Method parameters for first separation run - standard delay volume configuration

Module	Parameter	Setting
Pump	Solvent A	Water
	Solvent B	Acetonitrile
	Flow rate	4.0 mL/min
	Solvent Composition	40 % A, 60 % B
	Stop Time	1 min (1 min post time)
Autosampler	Injection	1 µL
Column Compartment	Column	<ul style="list-style-type: none"> <li>Column Eclipse Plus C18, 4.6 x 100 mm, 3.5 µm (959961-902)</li> <li>Column Poroshell 120 EC-C18, 3.0 x 50 mm, 2.7 µm (699975-302)</li> <li>Column Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm (699975-902)</li> </ul>
	Temperature	40 °C
Detector	Signal A	Wavelength: 250 nm, Bandwidth: 4 nm Reference Wavelength: 360 nm, Reference Bandwidth: 100 nm

## 5 Quick Start Guide

### Data Acquisition in Method and Run Control View

**Table 10** Method parameters for first separation run - low delay volume configuration

Module	Parameter	Setting
Pump	Solvent A	Water
	Solvent B	Acetonitrile
	Flow rate	0.75 mL/min
	Solvent Composition	40 % A, 60 % B
	Stop Time	1.2 min (1 min post time)
Autosampler	Injection	1 µL
Column Compartment	Column	Column: SB-C18, 4.6x 50 mm, 1.8 µm, 600 bar (827975-902)
	Temperature	40 °C
Detector	Signal	Wavelength: 250 nm, Bandwidth: 4 nm Reference Wavelength: 360 nm, Reference Bandwidth: 100 nm

Checkout specifications:

- four peaks are visible in the chromatogram
- **Intensity Test** – pass
- **Wavelength Calibration** ±1 nm

## Checkout Preparation

- 1 Fill solvent bottle A with HPLC grade Water. Fill solvent bottle B with HPLC grade Acetonitrile.
- 2 If installed, purge each channel of the on-line degasser using the supplied syringe.
- 3 Open the purge valve of the pump, and purge each channel at 5 mL/min.  
This should be sufficient to remove air in the system.
- 4 Install the supplied column into the column compartment.
- 5 Flush the flow-cell with HPLC-grade water for 5 min.
- 6 Run a Wavelength Calibration and Intensity Test where applicable.
- 7 Allow the system to equilibrate at the checkout conditions listed in [Table 9](#) on page 89 and [Table 10](#) on page 90 for 10 min.
- 8 Prepare a vial of the isocratic checkout sample.

## Setting Up the Method

This section shows how to quickly set the method conditions for an analysis.

**Prerequisites** The default method **DEF\_LC.M** has been loaded ready to prepare the new method. Now the key parameters can be edited to create the new method.

**1** To quickly access the **Method** page for each module, right-click in the system diagram for the module and select **Method...** from the context menu.

Each of the modules will be set up in this way.

**2** Right-click the pump area, and select **Method...** in the context menu.

**a** In the **Method** page for the **1260 Infinity Binary Pump**, enter the following parameters:

- Flow rate: 4.0 ml/min
- Solvent A: Select **Water** from the compressibility drop-down list.
- Solvent B: Select the check box to make Solvent B active.
- Set the value for %B to 60 %
- Stop Time: 1 min
- Post Time: 1 min

**b** Other parameters can remain at their default settings. Click **OK** to exit the window.

The changes are sent to the pump module.

**3** Right-click the autosampler area, and select **Method...** in the context menu.

**a** In the **Method** page for the **1260 Infinity Autosampler**, enter the following parameters:

- Injection volume: 1.0 µl
- Injection with Needle Wash
- Mode Flush Port, Time: 6 s

**b** Other parameters can remain at their default settings. Click **OK** to exit the window.

The changes are sent to the autosampler module.

- 4 Right-click the Thermostatted Column Compartment (TCC) area, and select **Method...** in the context menu.
  - a In the **Method** page for the **1290 Infinity TCC**, enter the following parameters:
    - Left Temperature 40 °C
    - Right Temperature Combined
  - b Other parameters can remain at their default settings. Click **OK** to exit the window.

The changes are sent to the TCC module.
- 5 Right-click the Diode-Array Detector area, and select **Method...** in the context menu.
  - a In the **Method** page for the **1260 Infinity DAD**, enter the following parameters:
    - **Use Signal:** Turn all signals except **Signal A** off by clearing the check boxes.
    - Signal A: 254 nm, bw 4 nm, ref 550 nm, bw 100 nm
    - Peak width: 0.02
  - b In the **Advanced** section, set **Spectrum Store** to **All**.
  - c Other parameters can remain at their default settings. Click **OK** to exit the window.

The changes are sent to the DAD module.
- 6 All the required module parameters have now been entered. Select **Method > Save Method As...** and enter **ISO-1.M** to save the method with a new name.



The ChemStation does not allow the method to be saved as **DEF\_LC.M** so that the default method template is not altered.
- 7 Allow the system to equilibrate for at least 10 min, and check that the baseline in the **Online Plot** is stable before starting the analysis.

## Running the Method for a Single Injection

This section shows how to run a single injection of the isocratic checkout sample using the conditions entered in the previous section.

Analyses on the ChemStation can be run in two modes:

- **Run Method** – single injections, for example, in interactive method development, using the parameter settings current at the time;
- **Run Sequence** – automated series of injections from multiple vials, possibly with multiple methods. For further details refer to the ChemStation manuals.

- 1 Click the **Select Run Method Task** icon  .
- 2 If required method conditions are not currently loaded, select **Method > Load Method** or the icon  under the menu bar to load them.

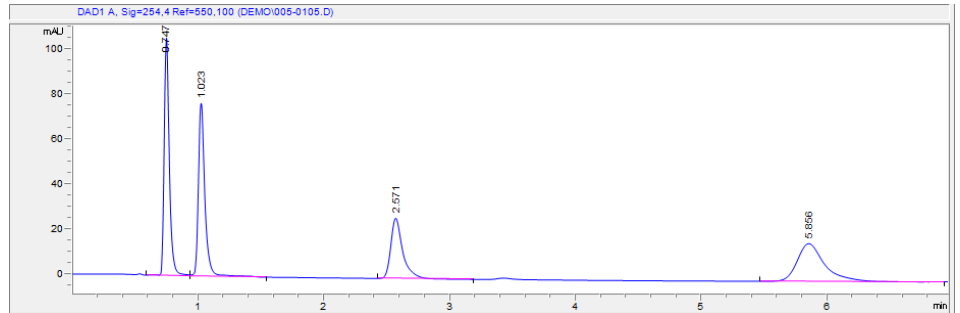
### NOTE

If changes have been made to a loaded method and not yet saved, this is indicated by a yellow asterisk on the method status icon. The injection can be made without first saving those parameters changes. The ChemStation always stores a copy of the acquisition parameters in the data file as ACQ.TXT to ensure the preservation of the original method parameters.

- 3 Place the sample vial in position 1 – this is the front position of the 10 x 2 ml vial positions on the right hand side of the sample tray.
- 4 Select **Run Control > Sample Info**, and enter the required sample parameters.  
For example:
  - **Subdirectory** (optional)
  - **Name Pattern**
  - **Vial/Location**
  - **Sample name**
  - **Comment**
- 5 If the system is already equilibrated and the baseline is stable, click **Run Method** in the **Sample Info** page to start the injection. Alternatively, click **OK**, and when ready, select **Run Control > Run Method**. The item will be added to the **Run Queue** and then automatically started from there.

- 6 The injection is made, and the chromatogram appears in the **Online Plot**. The data acquisition will stop when the **Stop Time** is reached.

The chromatogram should look similar to the one below:



# Data Analysis

A method in the ChemStation contains all the parameters for data acquisition (controlling the system) and data analysis (processing the data to give quantitative and qualitative results). This section looks briefly at integration and reports in data analysis so that the separations generated earlier in this chapter can be integrated and printed. For more detailed information on data analysis including the use of calibration for quantification refer to the ChemStation manual.

The screenshot shows the ChemStation Data Analysis software interface. Red boxes highlight the following components:

- Method source:** The top menu bar, specifically the 'Integration' menu.
- Navigation table:** A table listing various methods and their parameters, including columns for Date, Time, Operator, and Reference.
- Integration task:** The 'Integration' button in the toolbar.
- Spectrum task:** The 'Spectrum' button in the toolbar.
- Navigation panel:** The left-hand sidebar containing navigation options like 'Method and Run Control', 'Data Analysis', 'Review', 'Report Layout', and 'Verification (OOQ/PV)'.

The main window displays a chromatogram with several peaks and a table of integration results. The table below is a reproduction of the data shown in the screenshot.

#	Time	Area	Height	Width	Area%	Symmetry
1	0.747	301	153.9	0.0482	29.728	0.718
2	1.025	275.9	26.8	0.0269	27.207	0.616
3	3.371	136.2	26.9	0.0213	13.204	0.634
4	5.895	257.1	26.9	0.2255	25.401	0.676



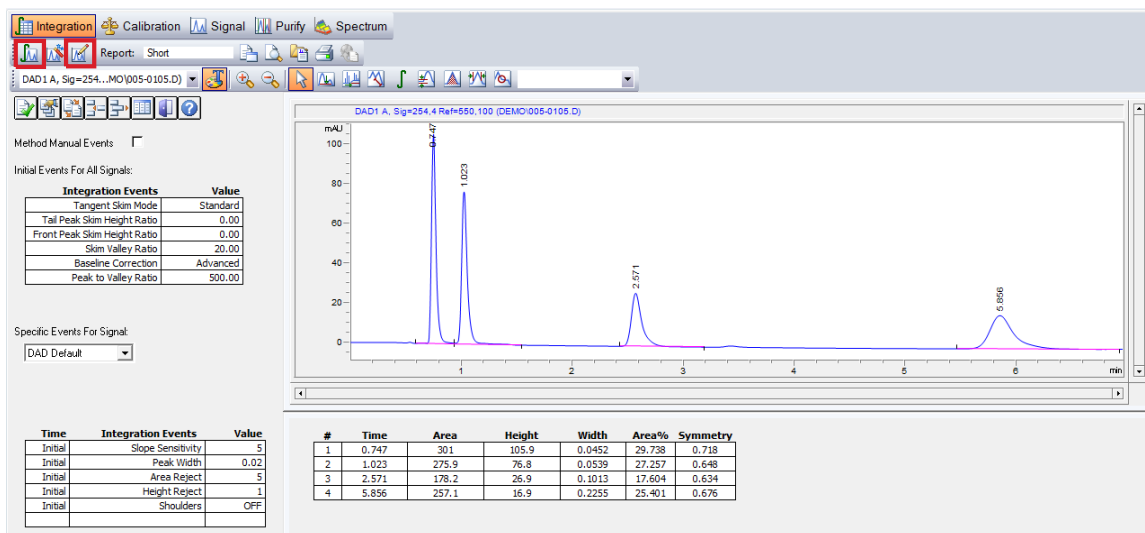
## Data Analysis View




To open a chromatogram in the **Data Analysis** view:

- 1 Launch an offline ChemStation.
- 2 Click **Data Analysis** in the bottom left of the screen.
- 3 In the Navigation Panel, find the data directory containing the data files. All the single injection data are represented as a subset called **Single Runs**. Double click **Single Runs** to load these data files into the Navigation Table.
- 4 Select a file in the Navigation Table, and double click it to load the chromatogram into the viewer.

## Integrating a Signal

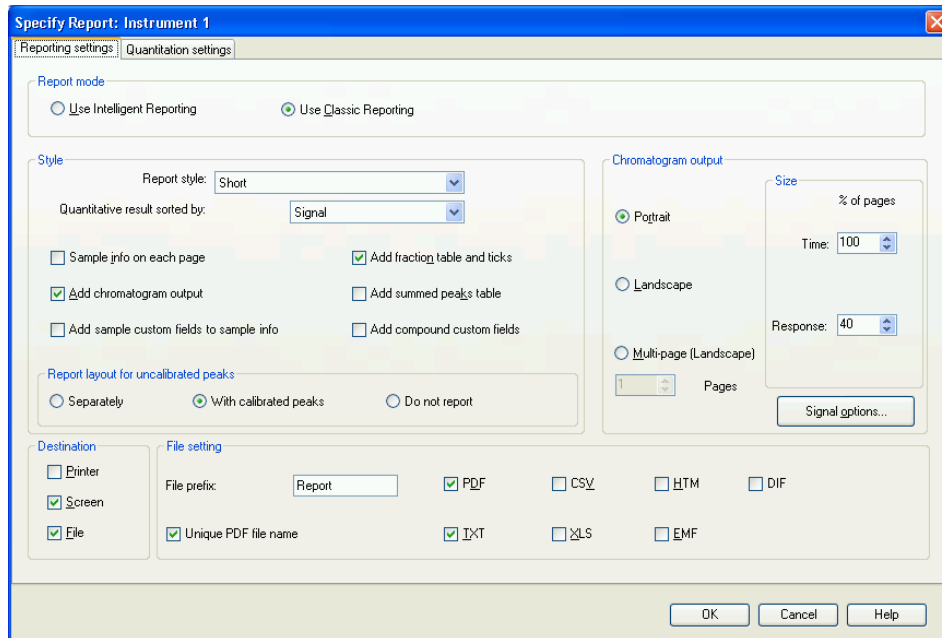
- 1 Select the Integration Task Tool (see figure below). The **Integrate** icon and the **Set Integration Events Table** icon are highlighted in the figure shown below.



- 2 Click the **Set Integration Events Table** icon  to open the table as shown.
- 3 Set **Baseline Correction** to **Advanced** for gradient runs.
- 4 Set **Slope Sensitivity** to 5.  
Higher numbers will integrate steeper peaks and ignore less steep peaks.
- 5 Set the **Peak Width** value to the narrowest peak of interest, approximately 0.02 in this case.
- 6 **Area Reject** and **Height Reject** can be set to reject the smallest peaks.
- 7 Click the **Integrate** icon  to update the results using these new settings.
- 8 Exit from the **Integration Events** table using the green tick icon .

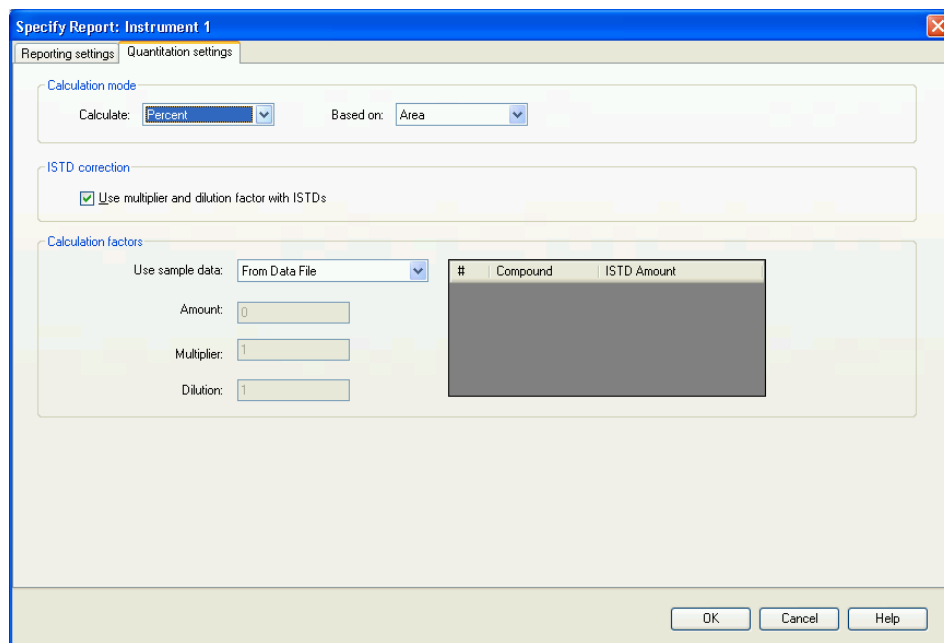
## Specify the Report

- 1 On the menu bar click **Report > Specify Report** to display the window shown in the figure below.



## 5 Quick Start Guide

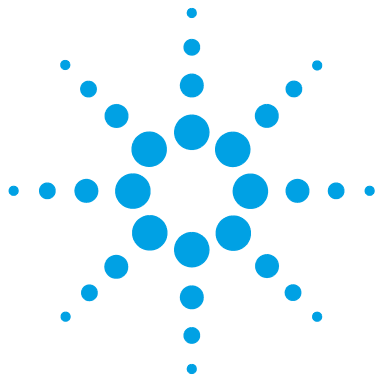
### Data Analysis



- 2 With the example settings shown in the figures above you can produce an Area Percent report on the screen.
- 3 In the **Destination** section, select **Printer** for a paper copy, and select **File** and **PDF** to obtain a useful PDF report file stored into the datafile (the data file with .D suffix is actually a directory. The report file can be viewed directly in ChemStation or it can be found in the directory using the normal Windows File Explorer).
- 4 Save the method once again to ensure the report settings are stored for future use by the method.

When the method is used again, these integration events and report settings will be used to produce the report.

This completes a brief look at using the Data Analysis part of the ChemStation software. Consult the ChemStation manuals and the online help system for further details about the powerful features of the ChemStation.



## 6 Appendix

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This chapter provides additional information on safety, legal and web and about setting up a method.



## Safety Information

### General Safety Information

The following general safety precautions must be observed during all phases of operation, service, and repair of this instrument. Failure to comply with these precautions or with specific warnings elsewhere in this manual violates safety standards of design, manufacture, and intended use of the instrument. Agilent Technologies assumes no liability for the customer's failure to comply with these requirements.

#### **WARNING**

**Ensure the proper usage of the equipment.**

**The protection provided by the equipment may be impaired.**

→ The operator of this instrument is advised to use the equipment in a manner as specified in this manual.

---

### Safety Standards

This is a Safety Class I instrument (provided with terminal for protective earthing) and has been manufactured and tested according to international safety standards.

## Operation

Before applying power, comply with the installation section. Additionally the following must be observed.

Do not remove instrument covers when operating. Before the instrument is switched on, all protective earth terminals, extension cords, auto-transformers, and devices connected to it must be connected to a protective earth via a ground socket. Any interruption of the protective earth grounding will cause a potential shock hazard that could result in serious personal injury. Whenever it is likely that the protection has been impaired, the instrument must be made inoperative and be secured against any intended operation.

Make sure that only fuses with the required rated current and of the specified type (normal blow, time delay, and so on) are used for replacement. The use of repaired fuses and the short-circuiting of fuse holders must be avoided.

Some adjustments described in the manual, are made with power supplied to the instrument, and protective covers removed. Energy available at many points may, if contacted, result in personal injury.

Any adjustment, maintenance, and repair of the opened instrument under voltage should be avoided whenever possible. When inevitable, this has to be carried out by a skilled person who is aware of the hazard involved. Do not attempt internal service or adjustment unless another person, capable of rendering first aid and resuscitation, is present. Do not replace components with power cable connected.

Do not operate the instrument in the presence of flammable gases or fumes. Operation of any electrical instrument in such an environment constitutes a definite safety hazard.






Do not install substitute parts or make any unauthorized modification to the instrument.

Capacitors inside the instrument may still be charged, even though the instrument has been disconnected from its source of supply. Dangerous voltages, capable of causing serious personal injury, are present in this instrument. Use extreme caution when handling, testing and adjusting.

When working with solvents, observe appropriate safety procedures (for example, goggles, safety gloves and protective clothing) as described in the material handling and safety data sheet by the solvent vendor, especially when toxic or hazardous solvents are used.

## Safety Symbols

Table 11 Safety Symbols

Symbol	Description
	The apparatus is marked with this symbol when the user should refer to the instruction manual in order to protect risk of harm to the operator and to protect the apparatus against damage.
	Indicates dangerous voltages.
	Indicates a protected ground terminal.
	Indicates eye damage may result from directly viewing the light produced by the deuterium lamp used in this product.
	The apparatus is marked with this symbol when hot surfaces are available and the user should not touch it when heated up.

### WARNING

#### A WARNING

alerts you to situations that could cause physical injury or death.

- Do not proceed beyond a warning until you have fully understood and met the indicated conditions.

### CAUTION

#### A CAUTION

alerts you to situations that could cause loss of data, or damage of equipment.

- Do not proceed beyond a caution until you have fully understood and met the indicated conditions.



## Solvent Information

Observe the following recommendations on the use of solvents.

- Follow recommendations for avoiding the growth of algae, see pump manuals.
- Small particles can permanently block capillaries and valves. Therefore, always filter solvents through 0.4 µm filters.
- Avoid or minimize the use of solvents that may corrode parts in the flow path. Consider specifications for the pH range given for different materials like flow cells, valve materials etc. and recommendations in subsequent sections.

## Material Information

Materials in the flow path are carefully selected based on Agilent's experiences in developing highest quality instruments for HPLC analysis over several decades. These materials exhibit excellent robustness under typical HPLC conditions. For any special conditions please consult the material information section or contact Agilent.

### Disclaimer

Subsequent data were collected from external resources and are meant as a reference. Agilent cannot guarantee the correctness and completeness of such information. Data is based on compatibility libraries, which are not specific for estimating the long-term life time under specific but highly variable conditions of UHPLC systems, solvents, solvent mixtures and samples. Information can also not be generalized due to catalytic effects of impurities like metal ions, complexing agents, oxygen etc. Apart from pure chemical corrosion, other effects like electro corrosion, electrostatic charging (especially for non-conductive organic solvents), swelling of polymer parts etc. need to be considered. Most data available refers to room temperature (typically 20 – 25 °C, 68 – 77 °F). If corrosion is possible, it usually accelerates at higher temperatures. If in doubt, please consult technical literature on chemical compatibility of materials.

## **PEEK**

PEEK (Polyether-Ether Ketones) combines excellent properties with regard to chemical resistance, mechanical and thermal stability. It is stable in a pH range of 1 to 12.5 and inert to many common solvents. There are several known incompatibilities with chemicals such as chloroform, methylene chloride, THF, DMSO > 1 %, strong acids (nitric acid > 10 %, sulphuric acid > 10 %, trichloroacetic acid, sulfonic acids), halogenes or aqueous halogene solutions, phenol and derivatives (cresols, salicylic acid etc.). When used above room temperature, PEEK is sensitive to bases and various organic solvents, which can cause it to swell.

## **Polyimide**

Agilent uses semi-crystalline polyimide for rotor seals in valves and needle seats in autosamplers. One supplier of polyimide is DuPont, which brands polyimide as Vespel, which is also used by Agilent.

Polyimide is stable in a pH range between 1 and 10 and in most organic solvents. It is incompatible with concentrated mineral acids (e.g. sulphuric acid), glacial acetic acid, DMSO and THF. It is also degraded by nucleophilic substances like ammonia (e.g. ammonium salts in basic conditions) or acetates.

## **Polyethylene (PE)**

Agilent uses UHMW (ultra-high molecular weight)-PE/PTFE blends for yellow piston and wash seals, which are used in 1290 Infinity pumps and for normal phase applications in 1260 Infinity pumps.

Polyethylene has a good stability for most common inorganic solvents including acids and bases in a pH range of 1 to 12.5. It is compatible to many organic solvents used in chromatographic systems like methanol, acetonitrile and isopropanol. It has limited stability with aliphatic, aromatic and halogenated hydrocarbons, THF, phenol and derivatives, concentrated acids and bases. For normal phase applications, the maximum pressure should be limited to 200 bar.

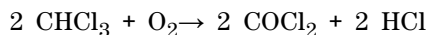
## Tantalum (Ta)

Tantalum is inert to most common HPLC solvents and almost all acids except fluoric acid and acids with free sulfur trioxide. It can be corroded by strong bases (e.g. hydroxide solutions > 10 %, diethylamine). It is not recommended for the use with fluoric acid and fluorides.

## Stainless Steel (SST)

Stainless steel is inert against many common solvents. It is stable in the presence of acids and bases in a pH range of 1 to 12.5. It can be corroded by acids below pH 2.3. It can also corrode in following solvents:

- Solutions of alkali halides, their respective acids (for example, lithium iodide, potassium chloride, and so on) and aqueous solutions of halogens.
- High concentrations of inorganic acids like nitric acid, sulfuric acid and organic solvents especially at higher temperatures (replace, if your chromatography method allows, by phosphoric acid or phosphate buffer which are less corrosive against stainless steel).
- Halogenated solvents or mixtures which form radicals and/or acids, for example:



This reaction, in which stainless steel probably acts as a catalyst, occurs quickly with dried chloroform if the drying process removes the stabilizing alcohol.

- Chromatographic grade ethers, which can contain peroxides (for example, THF, dioxane, di-isopropylether). Such ethers should be filtered through dry aluminium oxide which adsorbs the peroxides.
- Solutions of organic acids (acetic acid, formic acid, and so on) in organic solvents. For example, a 1 % solution of acetic acid in methanol will attack steel.
- Solutions containing strong complexing agents (for example, EDTA, ethylene diamine tetra-acetic acid).
- Mixtures of carbon tetrachloride with 2-propanol or THF.

### **Diamond-Like Carbon (DLC)**

Diamond-Like Carbon is inert to almost all common acids, bases and solvents. There are no documented incompatibilities for HPLC applications.

### **Fused silica and Quartz (SiO<sub>2</sub>)**

Fused silica is used in 1290 Infinity Flow Cells and capillaries. Quartz is used for classical flow cell windows. It is inert against all common solvents and acids except hydrofluoric acid and acidic solvents containing fluorides. It is corroded by strong bases and should not be used above pH 12 at room temperature. The corrosion of flow cell windows can negatively affect measurement results. For a pH greater than 12, the use of flow cells with sapphire windows is recommended.

### **Gold**

Gold is inert to all common HPLC solvents, acids and bases within the specified pH range. It can be corroded by complexing cyanides and concentrated acids like aqua regia.

### **Zirconium Oxide (ZrO<sub>2</sub>)**

Zirconium Oxide is inert to almost all common acids, bases and solvents. There are no documented incompatibilities for HPLC applications.

### **Platinum/Iridium**

Platinum/Iridium is inert to almost all common acids, bases and solvents. There are no documented incompatibilities for HPLC applications.

### **Fluorinated polymers (PTFE, PFA, FEP, FFKM)**

Fluorinated polymers like PTFE (polytetrafluorethylene), PFA (perfluoroalkoxy) and FEP (fluorinated ethylene propylene) are inert to almost all common acids, bases, and solvents. FFKM is perfluorinated rubber, which is also resistant to most chemicals. As an elastomer, it may swell in some organic solvents like halogenated hydrocarbons.

TFE/PDD copolymer tubings, which are used in all Agilent degassers except G1322A, are not compatible with fluorinated solvents like Freon, Fluorinert, or Vertrel. They have limited life time in the presence of Hexafluoroisopropanol (HFIP). To ensure the longest possible life with HFIP, it is best to dedicate a particular chamber to this solvent, not to switch solvents, and not to let dry out the chamber. For optimizing the life of the pressure sensor, do not leave HFIP in the chamber when the unit is off.

### **Sapphire, Ruby and Al<sub>2</sub>O<sub>3</sub>-based ceramics**

Sapphire, ruby and ceramics based on aluminum oxide Al<sub>2</sub>O<sub>3</sub> are inert to almost all common acids, bases and solvents. There are no documented incompatibilities for HPLC applications.

## **Agilent Technologies on Internet**

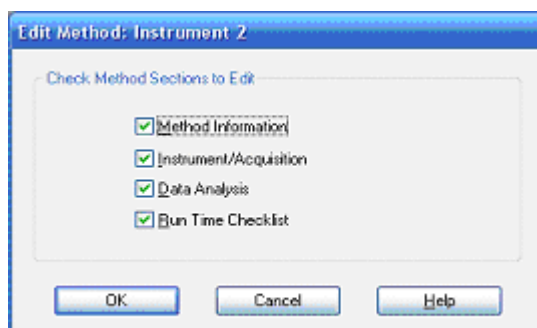
For the latest information on products and services visit our worldwide web site on the Internet at:

<http://www.agilent.com>

## Setting Up a Method using Edit Entire Method

A method in the ChemStation contains all the parameters for Data Acquisition (controlling the system) and Data Analysis (processing the data to give quantitative and qualitative results). The parameters are accessed through a series of screens which each focus on one module or function. These screens are accessed by clicking an icon on the graphical user interface (GUI) or through the menu bar with its drop-down menus. A new method can be created by loading and editing an existing method, or it can be created by loading and editing the blank template method **DEF\_LC.M**.

To change only a few parameters, you can go directly to the relevant setup pages for the parameters to be altered. Less experienced users may find it easier to use the **Edit Entire Method** function as this automatically steps through the pages. This is accessed through the menu **Method > Edit Entire Method**, and opens the **Check Method Sections to Edit** dialog:



**Figure 23** Check Method Sections to Edit

This dialog summarizes the sections that will be viewed, and offers an opportunity to bypass certain parts by deselecting them.

## 6 Appendix

### Setting Up a Method using Edit Entire Method

Depending on the selected parts, the function sequentially shows several screens:

- **Method Information** comprises a text description about the method.
- **Instrument/Acquisition** comprises:
  - injector parameters,
  - pump parameters,
  - oven parameters,
  - detector parameters, and
  - instrument curves.
- **Data Analysis** comprises:
  - signal details,
  - integration parameters, and
  - reporting parameters.
- **Run Time Checklist** comprises the parts of the method that will be executed.

#### NOTE

During **Edit Entire Method**, clicking **OK** will close the current entry screen and move on to the next screen. It is a one-way process.

If you inadvertently pressed **OK** before all entries have been made, then use **Cancel** and restart **Edit Entire Method**. Alternatively, carry on and return to the unfinished screen at the end. Clicking **Cancel** offers a button to **Skip** the remaining screens.

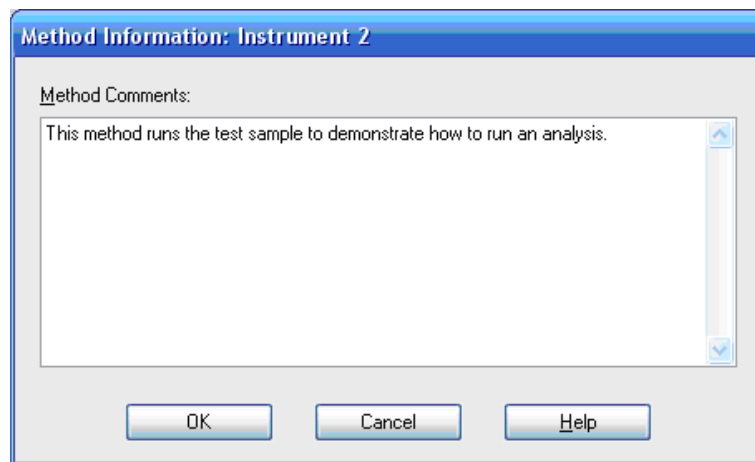
---



## Method Information

The **Method Information** screen can also be directly accessed through the menu **Method > Method Information** or by right-clicking on the graphical user interface.

This box allows information about the method to be entered. This information will be displayed above the system diagram on the **Method and Run Control** screen whenever this method is loaded and resident in memory.



**Figure 24** Method Information

## Instrument/Acquisition

### Setup Instrument Method

The **Setup Method** screen can be directly accessed through the menu **Instrument > Setup Instrument Method...**, or by right-clicking on the graphical user interface on any module icon and then selecting **Method...** in the context menu. This next stage in **Edit Entire Method** is the **Setup Method** screen with six tab dividers for different modules or functions .

The tabs are:

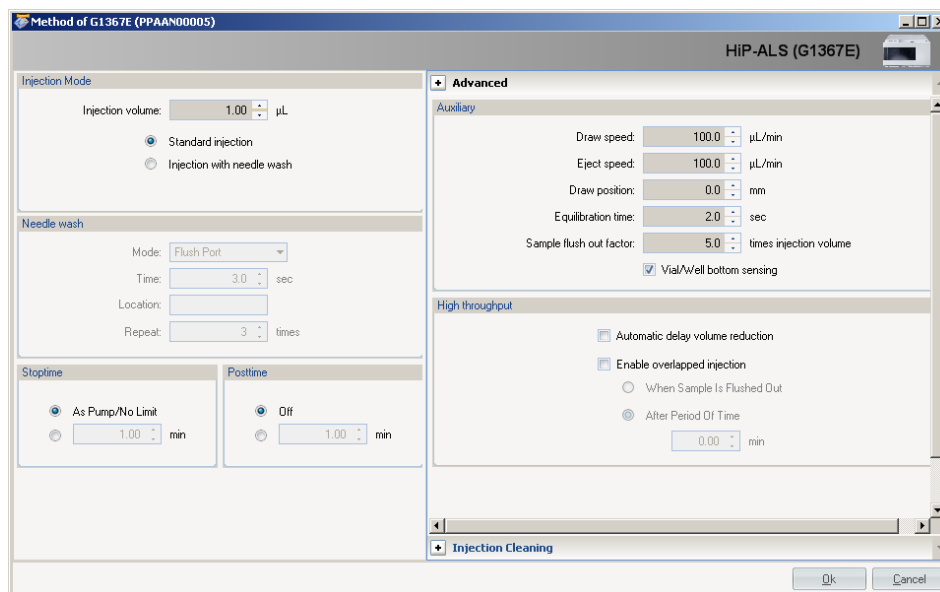
- High Performance Autosampler (**HiP-ALS**)
- **HiP-ALS Injector Program**
- Binary Pump (**BinPump**)
- Thermostatted Column Compartment (**TCC**)
- Diode-array Detector (**DAD**)
- **Instrument Curves**

To move between the tabs, click on the tab name at the top of the screen. When parameter changes have been entered they can be immediately sent to the instrument by clicking **Apply** or when all tabs have been completed clicking **OK** will send all parameters to the modules, close the screen and move on to the next stage.

The parameter entry tabs look similar in all the control programs (ChemStation, EZChrom, MassHunter et al.) due to the Agilent concept of common RC.Net drivers for the instrument modules.

To run the example separation, as with most methods, it is not necessary to change every available parameter but for completeness they are described in the next sections.

## Autosampler tab



**Figure 25** Setup Method screen – High Performance Autosampler tab

- **Injection Mode**
  - **Injection volume** sets the volume to be injected (example 1 µL),
  - **Standard injection** indicates that no external needle wash is done,
  - **Injection with needle wash** is used to reduce potential carryover. This is the recommended option and is configured in the next entry.
- **Needle Wash** – if selected above.
  - **Mode** determines how the outside of the needle will be rinsed: either actively in the **Flush Port** or by dipping into a specified **Wash Vial**.
  - **Time** in seconds the peristaltic pump connected to the flush port will pump the rinse solvent. It then pumps for a further 15 s to clear the flush port.
  - **Location** determines which vial or well plate will be used if **Wash Vial** was selected.

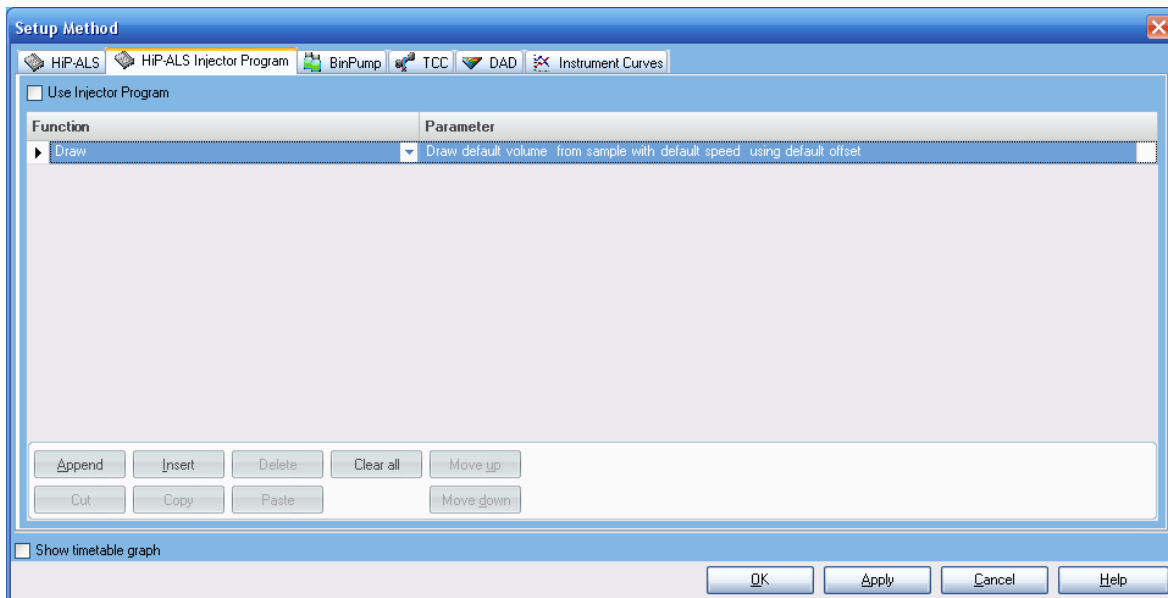
### NOTE

Vials should not have a septum i.e., they should be open to avoid transfer of carryover material on the septum.

- **Repeat** determines, if Wash Vial was selected, how many times the needle dips into the vial (default 3, maximum 5).
- **Stop Time / Post Time** are set to **No Limit / Off** and these values are taken care of in the pump tab.
- **Advanced - Auxiliary**
  - **Draw speed** is the speed that the sample is pulled into the needle. Default value is 100  $\mu\text{L}/\text{min}$ . It should be slowed down for viscous samples or for greater precision with small sample volumes ( $<2 \mu\text{L}$ ).
  - **Eject speed** is the speed of delivery from the needle.
  - **Draw position** is the vertical offset from the nominal injection position above the bottom of a vial. Usually a draw offset of 0 would be sufficient to take the sample from near the bottom of the vial. With the HiP ALS, for example, a value of 0 would place the tip of the needle 1 mm above the bottom of a wellplate.
  - **Equilibration time** is a delay time between drawing the sample and moving the needle.
  - **Sample flush out factor** determines how long the autosampler would wait after injection before allowing the valve to turn to bypass. This ensures that the sample zone has cleared from the needle, seat, and injection valve. Default value is 5.
  - **Vial/Well bottom sensing** is an alternative to using draw position offset. The needle slowly moves down until it touches the bottom of the vial or well and then lifts up to the preset draw offset position. This is a versatile way of ensuring the needle is close to the bottom of the vial but it takes slightly longer to complete the injection and should not be used if there are particulates at the bottom of the vial which might block the needle.

- **Advanced - High Throughput**
  - **Automatic delay volume reduction (ADVR)** switches the injection valve from mainpass to bypass after the injection has taken place and a volume defined by the sample flush out factor has swept through the injector. This reduces the delay volume of the system by about 70  $\mu\text{l}$  and allows the gradient changes to reach the column sooner.
  - **Enable overlapped injection** also switches the injection valve from mainpass to bypass after the injection has taken place either after the sample has flushed out of the injector or at some specified later time in the run. The injector will then aspirate the next sample in readiness for the next injection thus reducing the overall cycle time and increasing the sample throughput.
- **Injection Valve Cleaning**
  - **Injector Cleaning** allows the injection system to be flushed with solvent.
  - **Injection Valve Cleaning** allows the valve to be switched at setpoints during the run to minimize carryover when problem compounds are injected.

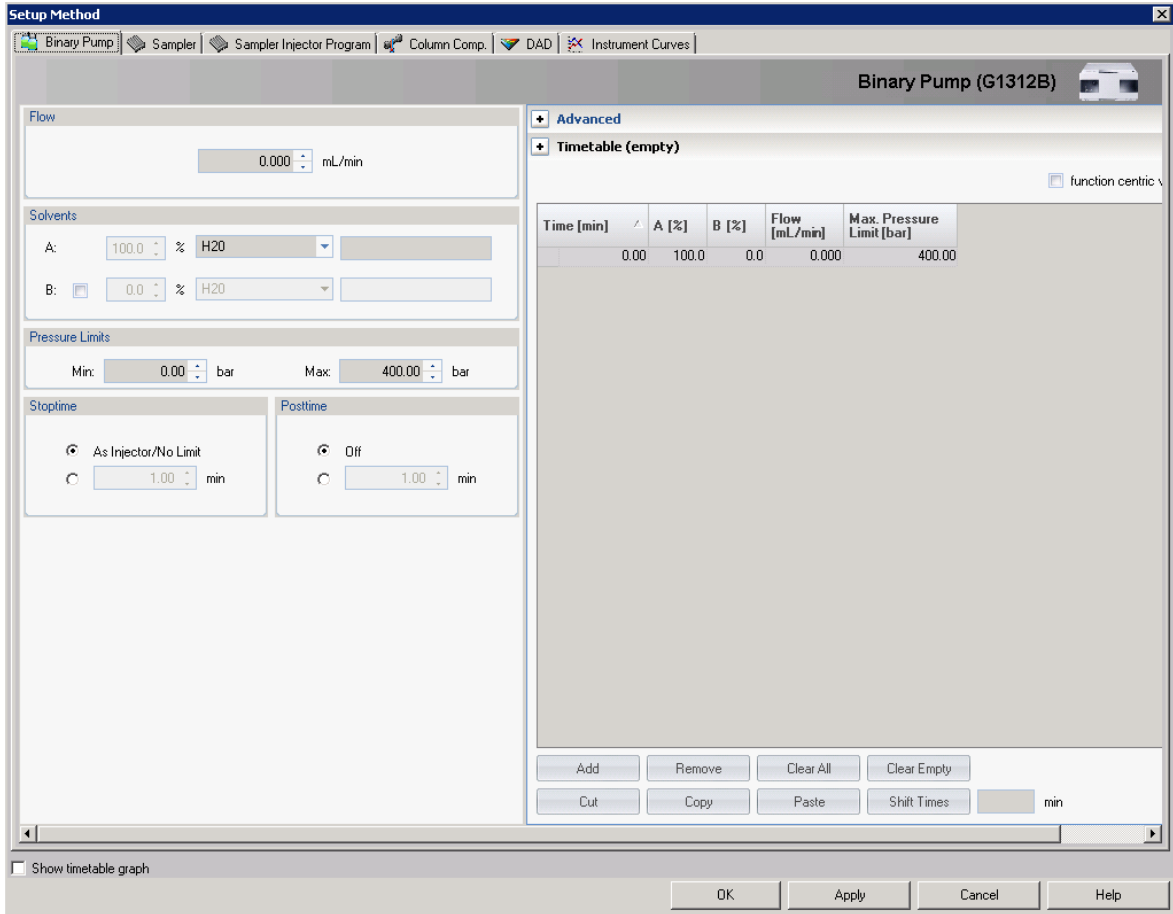
## High Performance Autosampler (HiP-ALS Injector Program) Tab



**Figure 26** Setup Method screen - HiP Autosampler Injector Program tab

This allows specialized injection procedures to be constructed which involve the manipulation of aliquots from multiple vials as, for instance, in pre-column derivatization. Reagent chemicals are automatically mixed with the sample to enhance detectability or sensitivity. A commonly used example is the derivatization of amino acids with OPA and FMOC reagents. For details, please refer to the *Agilent 1260 Infinity High Performance Autosampler manual*.

## Binary Pump Tab



**Figure 27** Setup Method screen – Binary Pump tab

- **Flow** sets the flow rate up to 5 mL/min. For the example separation 4 mL/min is used. If the back pressure briefly reaches the maximum pressure setting, the flow will be reduced for a few seconds to lower the pressure. If the pressure continues to be limited in this way, an error condition will be created and the flow will be stopped.

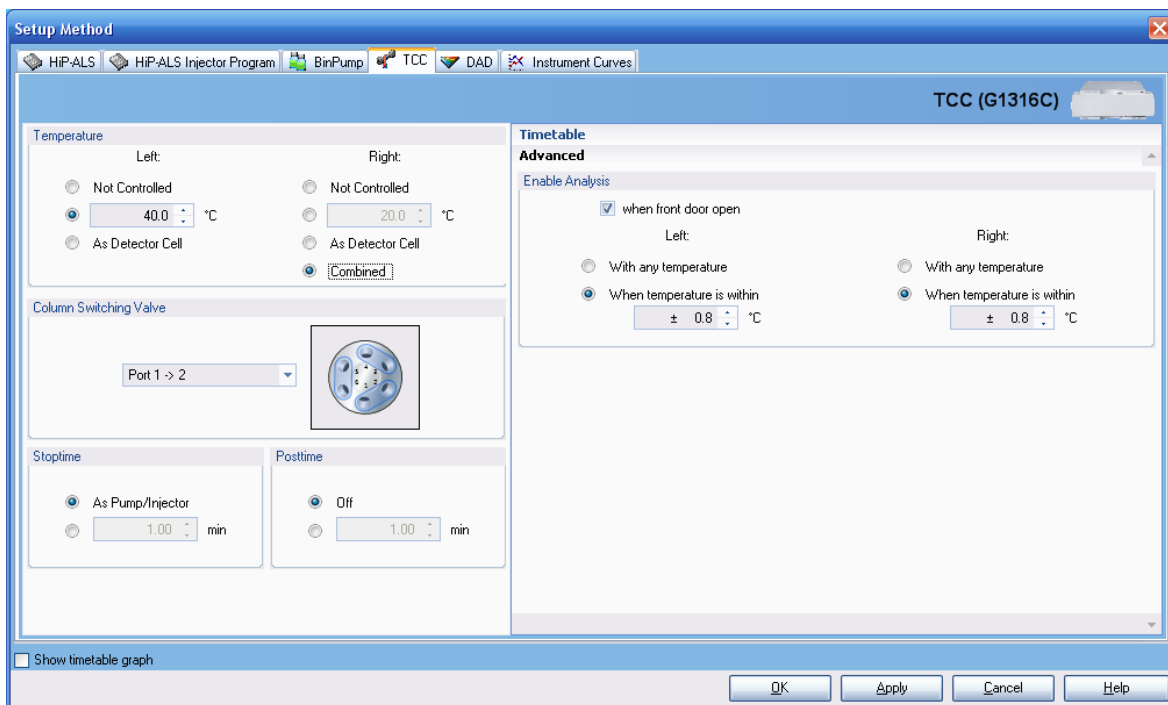
- **Solvents** defines the mobile phases that are available and the percentage proportions that are pumped on the two channels, A and B. On each channel, a drop-down box allows the selection of a solvent from a list so that the pump control uses the optimum compressibility settings. This will optimize the flow characteristics as described in “[Optimum Instrument Configuration for 2.1 mm i.d. Columns](#)” on page 43. A second text box allows a description of the mobile phase to be entered. If the solvent selection valve is installed in the pump, each channel has two solvent options and the correct option for the method is selected with the radio button to the left of the solvent description. The pump will form binary mixtures of the selected A and B channels, for example A2 and B1; it is not possible to mix A1 with A2 or B1 with B2. The value that is entered for the proportions of A and B defines the composition of an isocratic method or they define the starting conditions of a gradient method and the equilibration conditions between gradient runs. Only the value of B is entered, A will then update to show 100 % minus B when the cursor is moved. For the example separation, set A to water and B to acetonitrile at 60 %. A will be 40 %.
- **Timetable** details the changes that should occur during the run to the percentage composition of A and B in the mobile phase or, if required, to the flow rate and maximum pressure allowed. The timetable makes linear changes to the parameters between the given setpoints. The settings that are made elsewhere on this screen act as the initial conditions and will only change if an entry is made in the timetable. For example, if the flow is constant during the run then there is no need to make an entry about the flow in the timetable. To make an entry in the timetable click the **Add** button to add a line to the timetable; enter the time of the setpoint, select the type of entry from the drop-down list (composition, flow, pressure) and click the **Parameter** box to reveal the entry box for the value. If timetable entries are made out of logical sequence, the entries are automatically re-ordered into time order. Timetable lines can be edited directly and the **Cut**, **Paste**, and **Remove** buttons can be used to add and remove lines. Multiple lines can be added to give a series of linear gradient segments to create any gradient profile. To set up a simple gradient, first clear the timetable with the **Clear all** button if it is not already empty, then add a line and enter the time and solvent composition required. If a step gradient is required, it can be formed by making two entries with the ‘before step’ and ‘after step’ settings separated by 0.01 min. This is often used to rapidly elute strongly retained peaks from a column near the end of a run by increasing the stronger solvent and/or the flow rate in a step gradient; for example, the % B might be stepped up from 75 % to 95 %. It is not necessary to enter



the settings for time 0.00 min into the timetable, these values are picked up from other setpoints on this screen. However, some users like to see a 'complete' list in the timetable and make an entry for 0.00 min. There is no problem with this but if the initial conditions are ever changed then the new settings must be entered in both the timetable and the setpoints in the **Solvents** section of the screen.

- **Show timetable graph** displays the timetable changes graphically when the box is ticked.
- **Stop Time** defines the overall time for the separation or run and is sometimes referred to as the 'Run Time' by some users. This is the time (counted in minutes since the injection was made) when the run ends, which means that data acquisition will stop, the flow, composition, and other system settings will revert to the initial values for the method and the system will become available to make the next injection. It should always be at least as long as the last entry in the timetable or else the run will stop and revert to starting conditions before the timetable events are complete. **Stop Time** can be set to **No Limit** in which case the user must manually stop the run. While all the system modules have **Stop Time** parameters, the **Stop Time** in the pump is considered to be the master and the other modules will usually be set to follow this value.
- **Post Time** defines a countdown period after the end of a run during which the next injection is prevented. It allows time for the system to re-equilibrate after a gradient run. For an isocratic method it can be set to **Off**. For a gradient method, the value can be determined experimentally by observing the baseline behavior but typically will need time for the delay volume of the system plus at least three to five column volumes to be flushed through the system.
- **Pressure Limits** control the behavior of the pump regarding pressure. The maximum pressure of the 1260 Infinity Binary Pump is 600 bar but some columns can only withstand lower pressure and by setting this value here the column will be protected. The pump will generate an error condition if this pressure is exceeded. Any run in progress will be stopped and the pump switched to standby mode with no flow. Information about the maximum pressure for a particular column is supplied with the column. Agilent ZORBAX RRHT columns are suitable for operation at 600 bar. The Low Pressure limit is 'Off' when the value is zero but at any other value the pump will generate an error if the pressure goes below this value during operation. This is used as an alternative safeguard when the column is not inside a module with a leak sensor or in case the system is pumped dry. A value of 10 to 20 bar for minimum pressure limit is typical.

## Thermostatted Column Compartment (TCC) tab



**Figure 28** Setup Method screen – Thermostatted Column Compartment tab

- **Temperature** defines the temperature of the left and right-hand side column holders which can be independently controlled or linked together by clicking the **Combined** radio button on. When combined the settings for the left side control both sections and this is certainly necessary when the column is longer than 15 cm and needs to be supported by both sections. The two sides can be operated separately when it is required to have two columns operating at different temperatures – this can be implemented when a column switching valve is also installed to switch between them. Another use of separate temperature zones is when the column is operated at a high temperature (e.g. above 60 °C) on one side and the heat exchanger on the other side is used to cool the eluent before it enters the detector thereby reducing noise due to thermal effects in the flow cell. Selecting the **As Detector Cell** option will automatically read the temperature from the cell in the detector.

The temperature of each zone can be set from -5 °C to 100 °C and the user should check that the column is suitable for operation at that temperature. (Agilent ZORBAX RRHT StableBond phases can be used at the higher end of the range). The temperature is controlled to  $\pm 0.15$  °C down to 10 °C below ambient although it should be noted that there are very few applications operating below 12-15 °C. Avoid using the TCC at such low temperature that condensation of water from humid air occurs as this will trigger the leak sensor.

- **Column Switching Valve** is an active option only when a valve is fitted between the column holders. Three types of valve are available:
  - 2-position 6-port – used to switch between 2 columns
  - 2-position 10-port – used for alternating column regeneration
  - 8-position 9-port – used for multiple column selection in MDS

## Diode Array Detector Tab

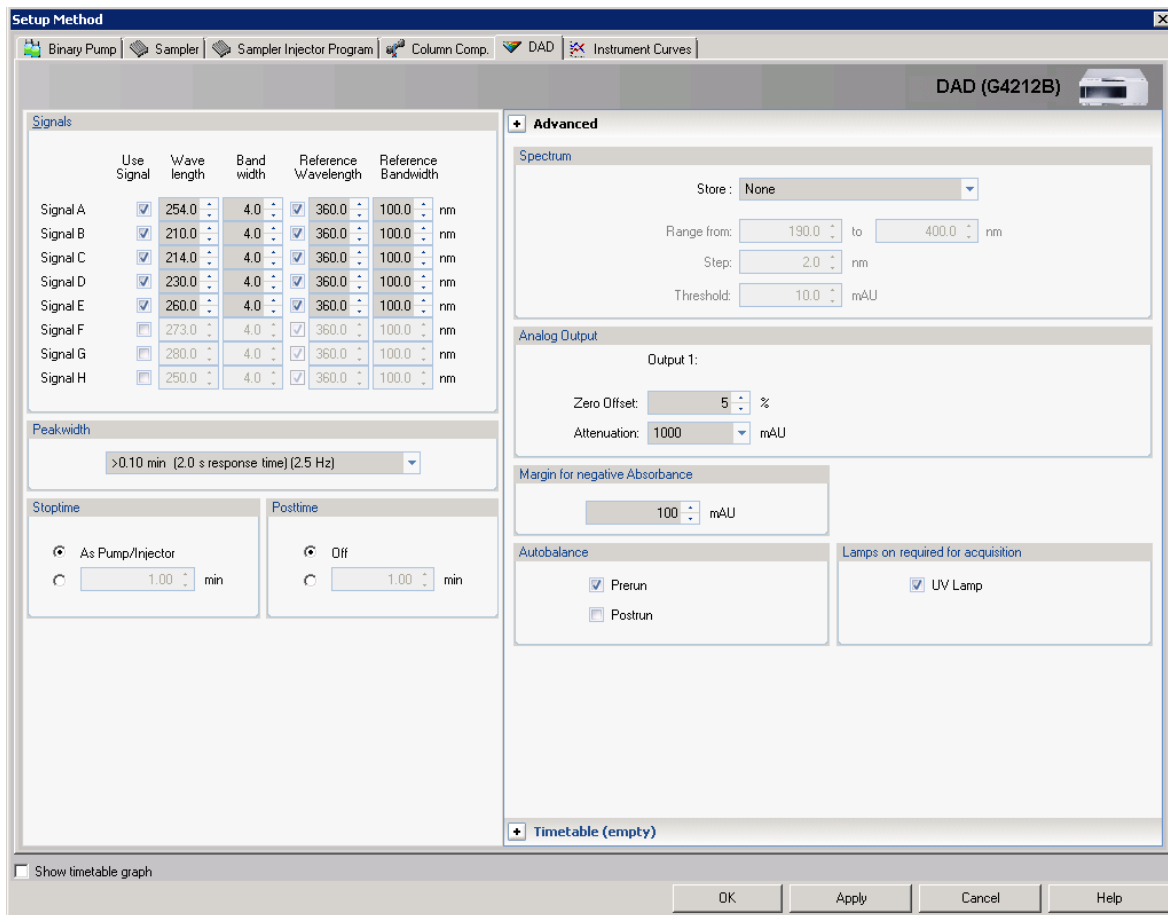


Figure 29 Setup Method screen – Diode Array Detector tab

- **Signals:** Up to eight separate signals (chromatograms) can be recorded. To mark a signal for collection, check the **Use Signal** box for that signal, define the wavelength and bandwidth, and if a reference signal is required also check and define that box.
  - **Wavelength** sets the central wavelength (nm) of the signal,
  - **Bandwidth** sets the width (nm) of the signal,

- **Reference Wavelength** sets the central wavelength (nm) of the reference band which is subtracted from the analytical signal,
- **Reference Bandwidth** sets the width (nm) of the reference band.
- **Peakwidth** sets the data collection rate and signal filtering.
- **Stoptime / Posttime** are set to **As Pump/Injector** and **Off** and these values are usually taken care of in the **Pump** tab. However, a detector stop time that is different from the pump stop time can be set if it is required to stop data analysis before the end of the run which is defined in the pump. This can be the case when a gradient equilibration ramp has been set at the end of the gradient. For instance, the %B may rise to 95 % at 10 min and it is expected that all peaks have eluted from the column. The run is essentially at an end but an extra gradient segment is added which takes %B back down to the initial value over two minutes to gently start the re-equilibration of the column. No useful data is expected during this downwards ramp so the detector stop time is set to 10 min and data collection stops while the pump stop time is 12 min to allow the downwards ramp to complete. This is a matter of user choice and some accept that the final minutes of the chromatogram cannot contain useful data but record it anyway to avoid the inconvenience of having a different stop time for the detector. The detector stop time does not override the pump and cause the run to end as would an earlier stop time in any other module hence the convenience of setting stop time to **As Pump/Injector**.
- **Timetable** operates in the same way as other modules: add a line, select the function to be changed and enter the new values for that function. The changes for the detector will take place immediately at the specified time. The following functions can be changed during the run:
  - **Balance**
  - **Change Signal**
  - **Change Threshold**
  - **Change Peak –detector Peakwidth**
  - **Change Spectra Acquisition Mode**
  - **Change Contacts**

- **Advanced - Spectrum**

Spectra can be saved during the run on a continuous or peak-controlled basis (this applies to the ChemStation software. Some software packages, e.g. EZChrom, only support continuous collection of all spectra and the peak-controlled options do not appear). Spectral collection and signal collection are independent operations that are performed by the detector firmware and are not dependent on the computer software extracting data from the 3D data matrix. The rate at which spectra are taken is determined by the **Peakwidth** setting and eight spectra are taken during the time that is specified in **Peakwidth**. The firmware does peak detection on signal A only to determine when peak-controlled spectra should be saved. For multiple signals, it may be necessary to have signal A set as a broadband detector to ensure that peak spectra are available for all the different wavelength signals.

- **Store** controls the spectral collection mode with the following options:
  - None** – no spectra are stored,
  - Apex+Baselines** – three spectra are taken at the start, apex, and end of peak,
  - Apex+Slopes+Baselines** – five spectra are taken at the start, upslope, apex, downslope, and end of peak,
  - All in Peak** – all the available spectra within a peak are stored,
  - All** – all spectra throughout the run are stored,
  - Every 2nd Spectrum** – stores only alternate spectra that are acquired throughout the run.
- **Range** – Spectra can be saved across the full range of the detector, 190 nm to 640 nm, or any reduced range that is chosen as appropriate by the user. (This also reduces the number of data to be stored).
- **Step**– controls the interval (nm) of the data that are stored in a spectrum and hence affects the spectral resolution seen. The default setting of 2 nm is a good choice for most applications.
- **Threshold** – determines that spectra are not stored for peak heights below this value (mAU).

- **Advanced - Analog Output**

The 1260 Infinity DAD has one analog signal output connector for use with data systems that do not accept a digital input. The following may be set:

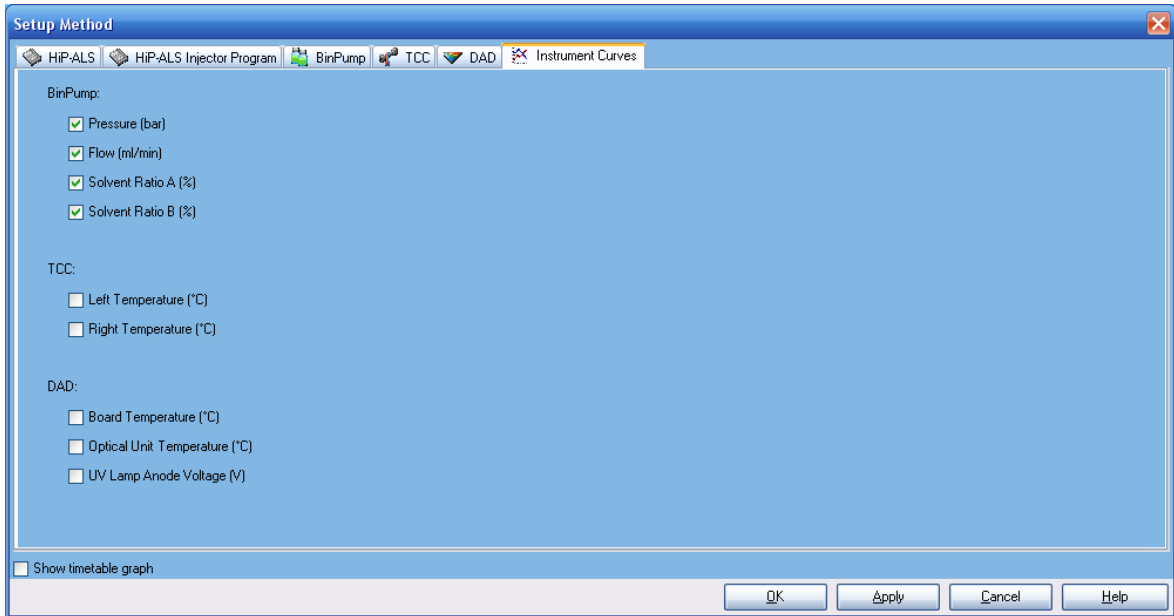
- **Zero Offset** places the zero level at a set percentage of the output signal hence allowing some scope for negative drift,
- **Attenuation** scales the set absorbance to the full output.

- **Advanced - Margin for Negative Absorbance**

The default setting is 100 mAU, which means the detector has sufficient dynamic range, considering where the zero level was set, to measure down to this value. To measure larger negative peaks or follow a baseline with a strong negative drift, the value needs to be adjusted downwards to prevent the signal flat-lining at the bottom of the range. However, it should not be changed without good reason, as making it more negative will increase baseline noise and reduce the range available for measuring positive peaks.

- **Advanced - Autobalance** sets the absorbance level to zero at all wavelengths (that is, balances all points on the spectrum to zero) and hence also zeroes the baseline signal. **Prerun** is selected to balance just before the start of the run, which is the normal situation. Sometimes **Postrun** is alternatively selected to balance at the end of the run after the postrun time has expired. For example, if the signal always shows a negative drift and the user prefers the run to end at zero absorbance, then this will set the correct zero level for the following run. It will not retrospectively change the run at the end of which it performed the balance.
- **Advanced - Lamps on required for acquisition:** the 1260 Infinity DAD has one UV lamp and it needs to be on for analysis so this box should be checked.

## Instrument Curves Tab



**Figure 30** Setup Method screen – Instrument Curves tab

The instrument curves tab allows monitored data streams other than detector signals to be stored with the data by checking the relevant box. These are primarily used for diagnostic purposes. They are:

- Pump:
  - Pressure
  - Flow
  - A/B Composition – can be useful for overlaying the gradient profile on a chromatogram.
- Thermostatted Column Compartment:
  - Left/Right temperature
- Detector:
  - Board temperature
  - Optical unit temperature
  - UV lamp anode voltage



## Data Analysis

### Signal Details

The **Signal Details** screen can also be directly accessed in **Method and Run Control** view: right-click on the graphical user interface on the **Calibration** icon, and then select **Signal Details** in the context menu. In the Data Analysis view, it can be accessed through the menu **Calibration > Signal Details**.

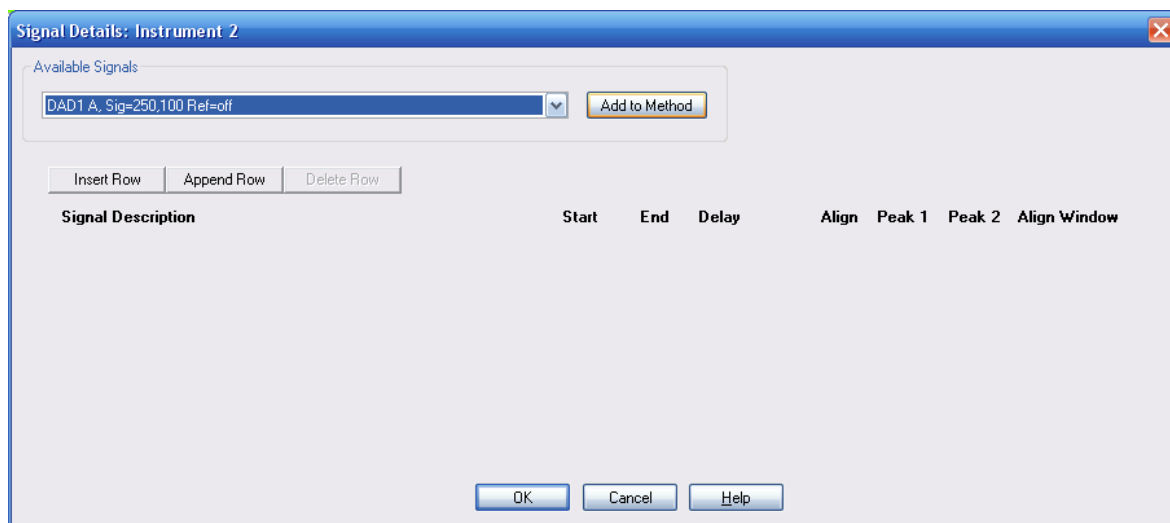
The **Signal Details** screen is the next step in **Edit Entire Method** and it tells Data Analysis which of the acquired signals to process. The drop-down box lists the available signals, including the analytical signals defined in the detector settings, and recorded parameters such as temperature, flow, composition, pressure and diagnostic traces. Select a signal and click **Add to Method** to transfer it to the **Signal Details** table shown in the lower part of the screen. You can select any or all of the acquired detector signals for processing. If no signals are selected, the table is empty; in this situation, ChemStation will default to processing all of the acquired detector signals.

Sometimes the user might edit an existing method to create a new method and is faced with a **Parameter Mismatch** error when attempting to run the method. What has happened is that in the old method the **Signal Details** contained a specified signal, for example 250 nm with 8 nm bandwidth, and this is changed in the new method to 254 nm/12 nm for example. The **Signal Details** table still contains the original details and is being told to process a signal which is no longer acquired. Highlighting the old signal in the table and using the **Delete Row** button will correct the problem.

If a system uses multiple detectors such as the diode-array detector and a mass spectrometer, the **Signal Description** lines allow delay times for the downstream detector to be entered so that the software can align the peaks from the different detectors.

## 6 Appendix

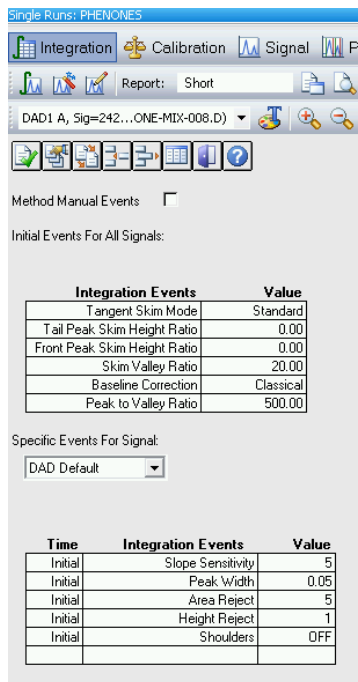
### Setting Up a Method using Edit Entire Method



**Figure 31** Signal details

## Edit Integration Events

The **Edit Integration Events** screen can also be directly accessed in **Method and Run Control** view by right-clicking on the graphical user interface on the Integration Events icon and then clicking **Edit Integration Events** in the context menu. In the **Data Analysis** view it can be accessed through the menu **Integration > Integration Events...** or the **Edit Integration Events** task icon.



**Figure 32** Edit Integration Events screen

Integration, calibration and reporting are the data analysis part of the method. Integration parameters and the calibration table are easier to setup once data have been acquired and the data are being reviewed in Data Analysis view. The integration events can be optimized at that time and default settings are often used for the initial acquisition runs.

The **Edit Integration Events** screen has two tables:

- **Initial Events For All Signals** contains events (integration parameters) that apply to all signals acquired with the method,
- **Specific Events For Signal** contains events which are specific for one type of detector or specific to different signals from the same detector.

Key parameters in this table are:

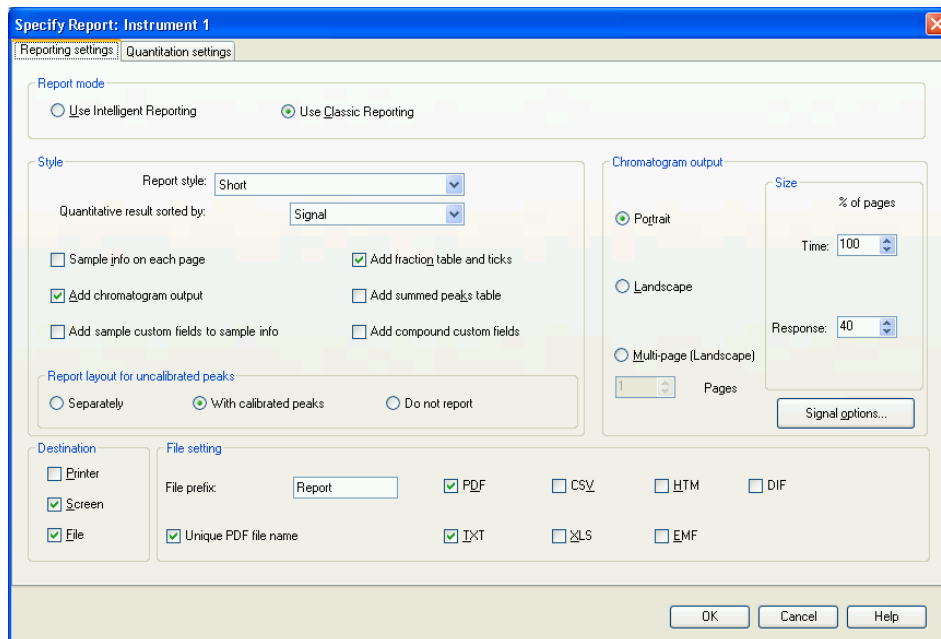
- **Slope Sensitivity** represents the slope and curvature of the baseline needed to mark the start and end of a peak;
- **Peak Width:** the width at half-height of the narrowest peak of interest should be entered. This helps the integrator to distinguish between noise and very small peaks;
- **Area Reject / Height Reject** values that control rejection from the results of peaks whose area or height falls below these values;
- **Integration OFF/ON** suppresses integration between set limits. Almost always used to inhibit integration in the region from injection to solvent front or unretained peak marker.

Lines such as **Integration OFF/ON** are added to the table using the icons at the top of the window.

Click **OK** to exit and the next screen in the Edit Entire Method process will open.

## Specify Report

The **Specify Report** screen can also be directly accessed in **Method and Run Control** view by right-clicking on the graphical user interface on the Report icon and then selecting **Specify Report** in the context menu. In the **Data Analysis** view it can be accessed through the menu **Report > Specify Report** or the Specify Report task icon.



**Figure 33** Specify Report Screen

To setup a simple area% report with Classic Reporting, which prints to the printer and to a PDF file, enter the following settings in these sections of the Specify Report screen:

On the **Reporting settings** tab:

- **Report mode:** Use Classic Reporting
- **Style**
  - **Report Style:** Short
  - **Quantitative results sorted by:** Signal
  - **Add Chromatogram Output:** Checked

## 6 Appendix

### Setting Up a Method using Edit Entire Method

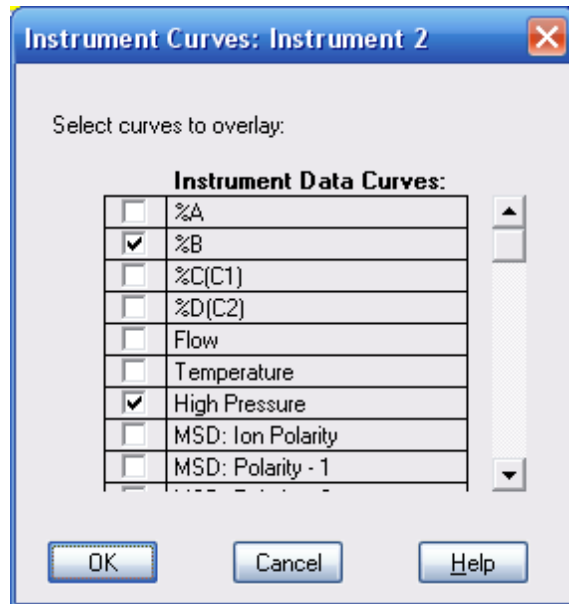
- **Chromatogram Output:** Portrait
- **Size:**
  - **Time** axis 100 % of page
  - **Response** axis 40 % of page
- **Destination**
  - **Printer:** Checked
  - **Screen:** Unchecked
  - **File:** Checked
- **File Setting:**
  - **PDF:** Checked
  - **Unique PDF file name:** Checked

On the **Quantitation settings** tab:

- **Calculation mode**
  - **Calculate:** Percent
  - **Based on:** Area

Click **OK** to exit and the next screen in the Edit Entire Method process will open.

## Instrument Curves

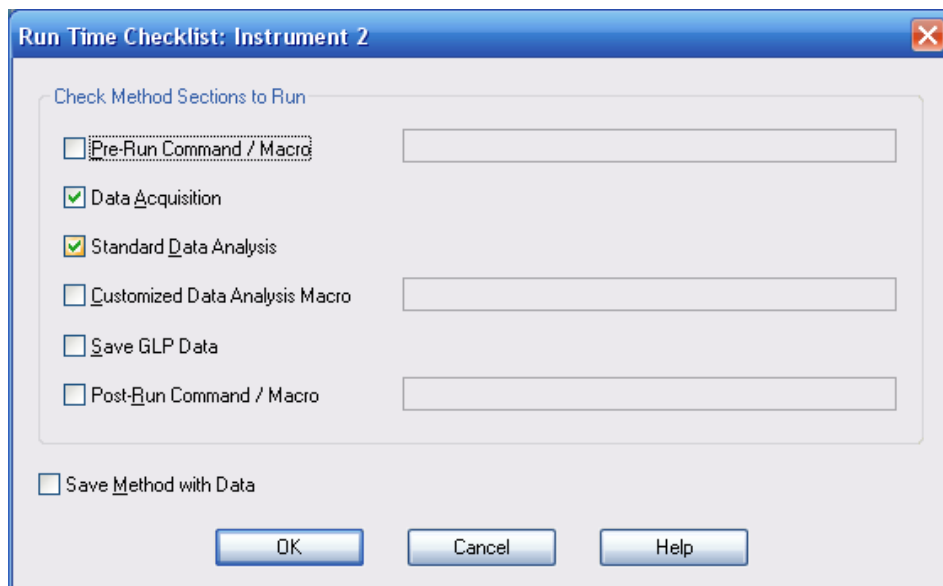


**Figure 34** Instrument Curves screen

The **Instrument Curves** checkboxes allow these recorded parameters to be overlaid as a graph on the chromatogram.

## Run Time Checklist

The **Run Time Checklist** can also be directly accessed through the menu **Method > Run Time Checklist...** or by clicking on the **Run Time Checklist** icon at the top right of the screen.



**Figure 35** Run Time Checklist Screen

The **Run Time Checklist** selects whether the method should run both data acquisition and data analysis and also offers an opportunity to link macro commands or programs into the work flow at various points. In most cases the **Data Acquisition** and **Standard Data Analysis** check-boxes are checked. If no data analysis is required, for example, in a series of method development runs, **Standard Data Analysis** can be unchecked so that no report is produced and the data can be visually assessed later in the **Data Analysis** view.

To link in a macro program at one of the access points the relevant box is checked and the name of the macro is typed in the text box to the right. The software looks for the macro in the C:\Chem32\Core directory; include the path if it is located elsewhere.



The access points in the work flow of the method are:

- **Pre-Run Command / Macro**
- **Customized Data Analysis Macro**
- **Post-Run Command / Macro**

**Save Method with Data** saves a copy of the method in the data file and names it RUN.M. This is not needed if the ChemStation is operated in the usual configuration as the software always saves the method in the data file (all versions since B.02.01). Only if the ChemStation has been configured such that **Unique Sequence Folder Creation** is turned off and hence methods are not routinely copied into the data file, this option will be relevant.

As this is the final screen in the process, clicking **OK** will exit from **Run Time Checklist** and from the **Edit Entire Method** process. The method should now be saved into the master methods directory, by default C:\Chem32\1\Methods, using **File > Save As > Method** or **Method Menu > Save Method As**.

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## In This Book

This manual contains information about the Agilent 1260 Infinity Binary LC.

The manual describes the following:

- Product description,
- introduction,
- specifications,
- system optimization,
- system setup and installation,
- quick start guide.

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