

Agilent 1290 Infinity LC System

System Manual
and
Quick Reference



Notices

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In This Guide...

This manual covers the Agilent 1290 Infinity LC System

1 Introduction to Ultra-High Performance Liquid Chromatography

This chapter gives an introduction to the Agilent 1290 Infinity LC System and the underlying concepts.

2 The Agilent 1290 Infinity LC System – Product Description

This chapter discusses the features of the 1290 Infinity LC System.

3 Optimization of the Agilent 1290 Infinity LC System

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, stack configurations and preparing the system for operation.

5 Quick Start Guide

This chapter provides information on data acquisition and data analysis with the 1290 Infinity LC System.

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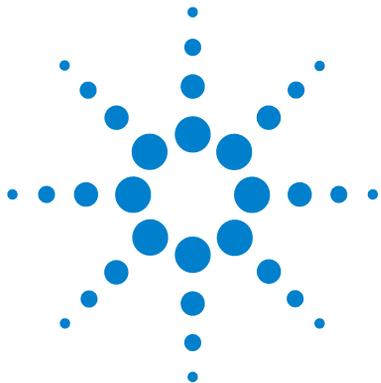
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This chapter gives an introduction to the Agilent 1290 Infinity LC System and the underlying concepts.



Theory of Using Smaller Particles in Liquid Chromatography

Introduction

In 2003 Agilent introduced the first commercially available porous silica columns with 1.8 μm particles. These were the first in a class of columns which has become known as ‘sub-two micron’ or STM columns. For use with the Agilent 1200 Series Rapid Resolution LC system, launched in 2006, these packing materials were introduced in ZORBAX RRHT columns capable of withstanding 600 bar pressure. In 2009 the range was extended to include RRHD columns capable of routine use at 1200 bar pressure to support the introduction of the Agilent 1290 Infinity LC System with its extensive operating power range up to 1200 bar pressure and 5 ml/min flow rate.

These sub-two micron (1.8 μm) 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, required for the separation of complex samples. Lower dispersion also means less dilution of the analyte peaks and consequent sensitivity gains, particularly for LC/MS.

The pressure needed to drive solvent through a column containing STM 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 that offer higher pressures than the 400 bar norm that was extant since the early days of HPLC. Ultra-High Performance (or Ultra-High Pressure) LC systems also offer low delay volumes and the fast data collection required for narrow peaks from fast or high resolution chromatography. The Agilent 1290 Infinity LC is a landmark in UHPLC as it is the first system that can completely cover and extend upon all the disparate performance ranges of UHPLCs pre-existing on the market.

The Theory

Separation efficiency in HPLC can be described by the van Deemter Equation (Figure 1 on page 9). This results from the plate-height model 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 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 1 on page 9 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.

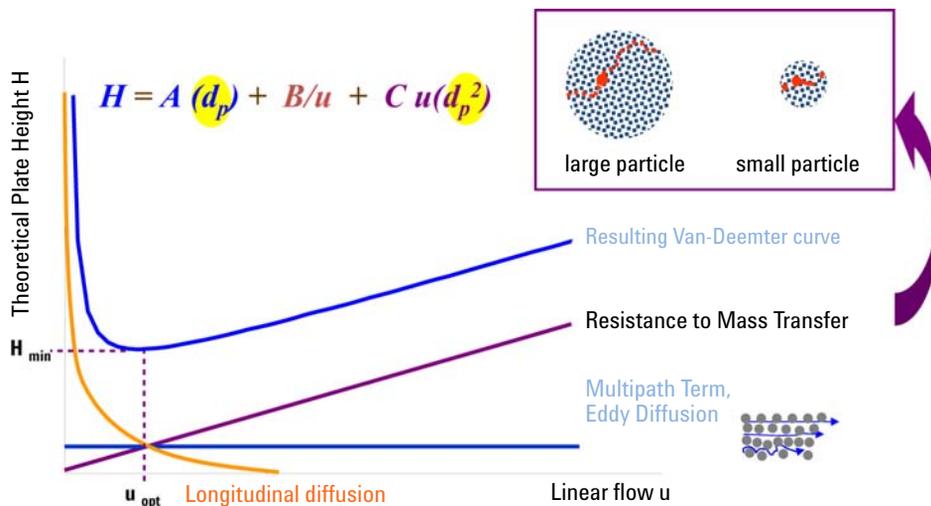


Figure 1 A hypothetical Van Deemter curve

The van Deemter plots in Figure 2 on page 10 show that reducing particle size increases efficiency. Switching from commonly used 3.5 μm and 5.0 μm particle sizes to 1.8 μm particles offers significant performance improvements. The 1.8 μm 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.

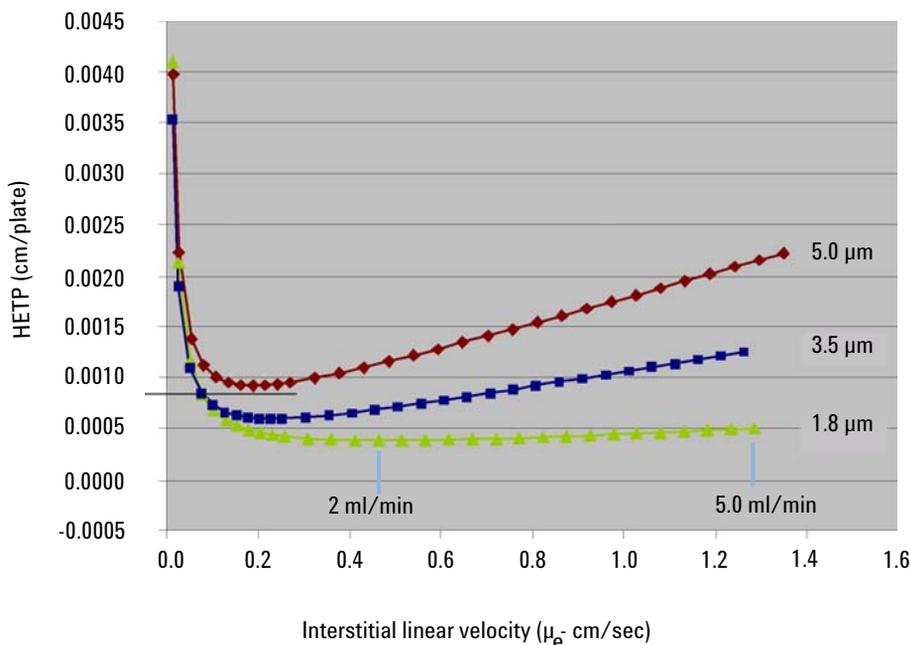


Figure 2 Van Deemter curve for different particle sizes

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.

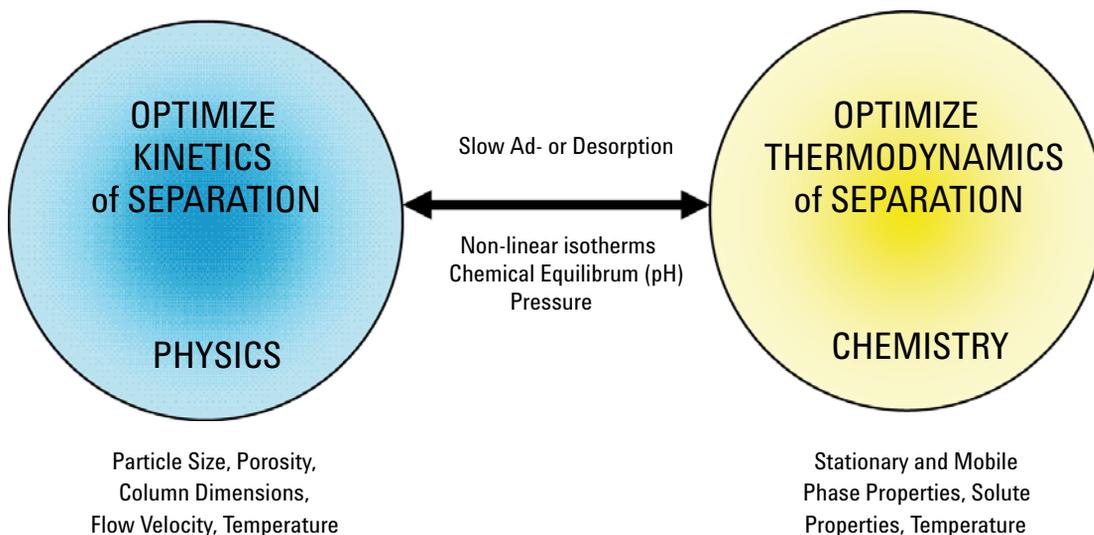


Figure 3 Selecting optimal conditions for HPLC

Resolution can be described as a function of three parameters:

- column efficiency or theoretical plates (N),
- selectivity (α),
- retention factor (k).

According to the resolution equation (Figure 4 on page 12), the selectivity has the biggest impact on resolution (Figure 5 on page 12). 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 4 Resolution equation

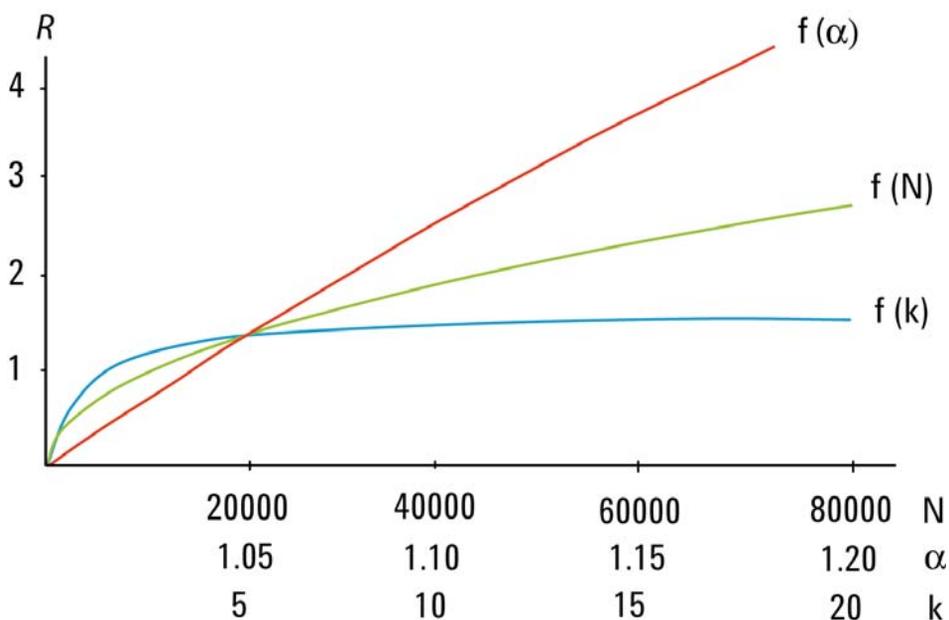


Figure 5 Effect of plate number, separation factor and retention factor on R

No matter whether the rapid resolution 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 already offered more than 140 ZORBAX 1.8 μm Rapid Resolution High Throughput (RRHT) columns (14 selectivity choices; 15 to 150 mm long; 2.1, 3.0 and 4.6 mm internal diameters) and with the launch of the Agilent 1290 Infinity LC the STM range is extended to include the Rapid Resolution High Definition (RRHD) 1200 bar columns. 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.

Many laboratories perform an extensive screening process to select the best combination of stationary phase, mobile phase and temperature for their separations. Agilent offers Method Development Solutions on both the 1200 Series and 1290 Infinity LC systems. These offer complete automation of this time consuming selection process – making method development and method transfer an easier and more reliable task.

ZORBAX 1.8 μm RRHD and RRHT columns use the same chemistry as ZORBAX columns with 3.5 and 5 μm particles. As a result, for any particular ZORBAX phase, the 5.0, 3.5 and 1.8 μm particles provide identical selectivity, which allows easy, fast and secure bidirectional method transfer between conventional LC, UHPLC and preparative LC.

Benefits of Columns Packed With Sub-2-micron Particles

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 1 on page 14).

Table 1 Time and cost savings over 700 runs

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

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

² 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. 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.

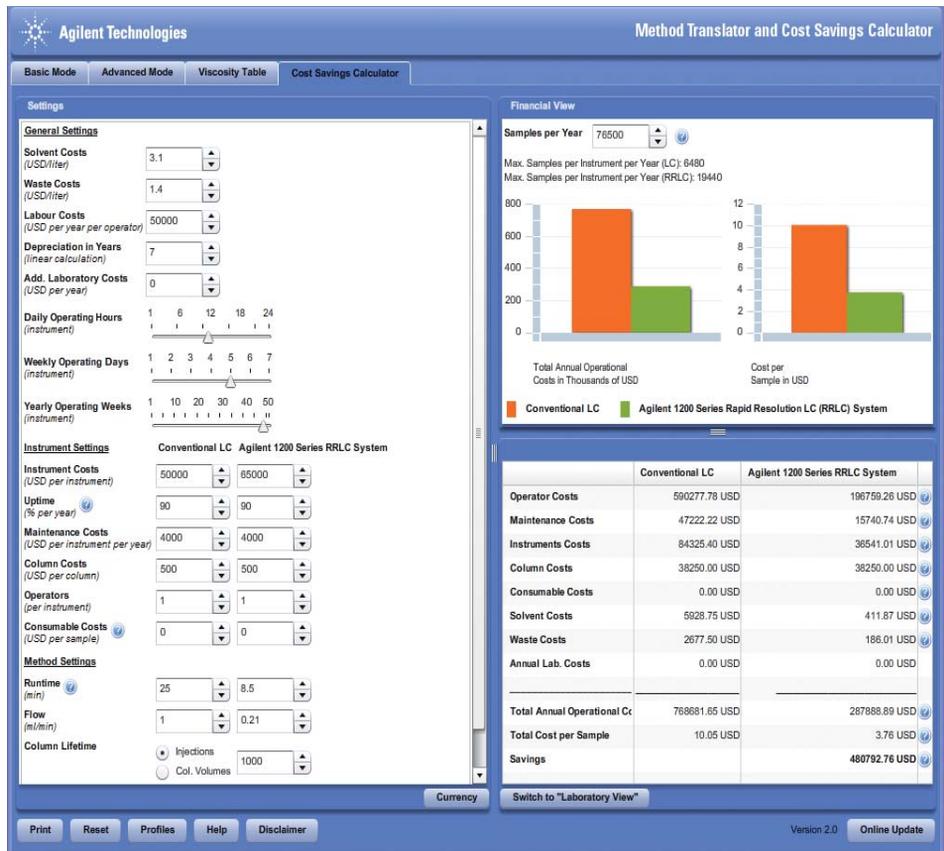


Figure 6 Cost savings calculator

1 Introduction to Ultra-High Performance Liquid Chromatography

Benefits of Columns Packed With Sub-2-micron Particles

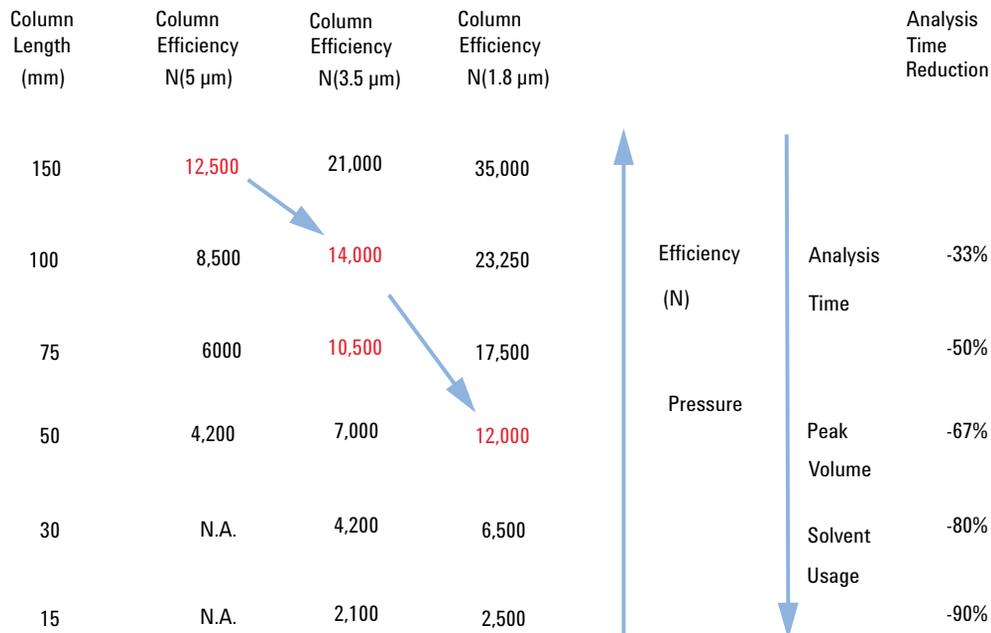


Figure 7 Relation between particle size, efficiency and analysis time

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.

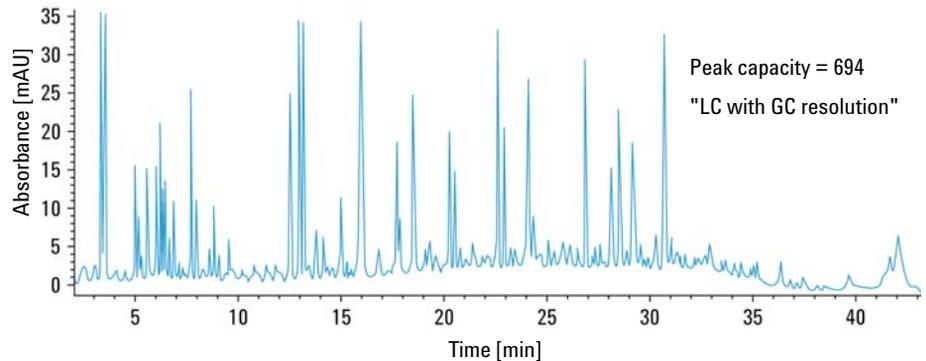


Figure 8 Peak capacities of more than 700 can be achieved using a ZORBAX RRHT SB-C18 column (2.1 x 150 mm, 1.8 μm) to analyze a tryptic digest of BSA

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.

An example of a gradient method transfer to a 2.1 mm i.d. STM column in which the separation has been speeded up is shown in [Figure 9](#) on page 19. The initial run on the 2.1 mm column was at a flow rate of 0.22 ml/min generating 380 bar pressure at a temperature setting of 37 °C with all peaks separated in 12.5 min (chromatogram not shown). The flow was increased to 0.66 ml/min and the gradient times adjusted downwards by a factor of three generating 1020 bar pressure with all peaks eluted in 4.2 min ([Figure 9](#) on page 19 above). This should give the same separation but a loss of resolution can be seen between peaks 7 and 8 and between 5 and the main peak and this is due to heating in the column changing the selectivity for these compounds. It was found that running the column thermostat at 5 °C lower was sufficient to offset the heating effect inside the column and restore the separation ([Figure 9](#) on page 19 below). The pressure increased to 1070 bar which also indicates that the in-column temperature was lower.

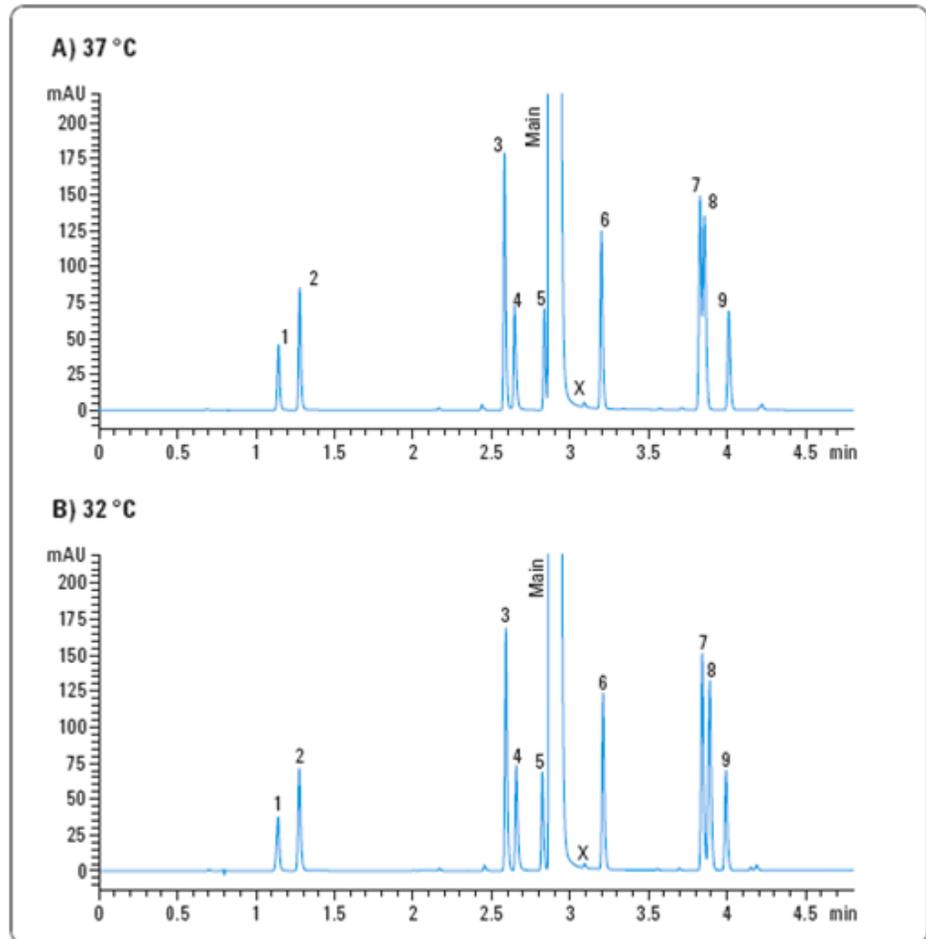
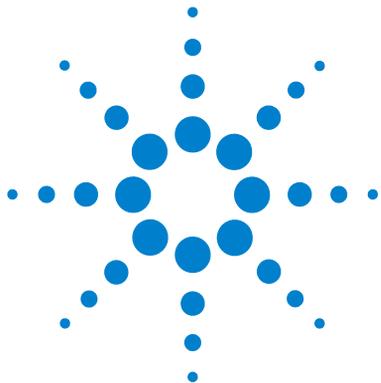


Figure 9 Influence of frictional heat generation on selectivity and effect of lowering column temperature

In summary, the use of sub-two-micron packing material offers benefits of increased efficiency, higher resolution and faster separations. The Agilent 1290 Infinity LC System and RRHD columns increase the separation space available and enable more of these benefits to be accessed. The features of the 1290 Infinity LC are discussed in [“The Agilent 1290 Infinity LC System – Product Description”](#) on page 21 and [“Optimization of the Agilent 1290 Infinity LC System”](#) on page 35 considers how to apply the theory and use these features to develop optimized separations.

1 Introduction to Ultra-High Performance Liquid Chromatography

Frictional Heating



2

The Agilent 1290 Infinity LC System – Product Description

New Features of the Agilent 1290 Infinity LC System 22

System Components 25

This chapter discusses the features of the 1290 Infinity LC System.



New Features of the Agilent 1290 Infinity LC System

The Agilent 1290 Infinity LC System is designed to offer the greatest flexibility for performing analytical liquid chromatography using all types of current and emergent column technologies. The 1290 Infinity LC has the widest range of operating parameters so that it can replicate method settings transferred in from any pre-existing analytical HPLC or UHPLC system available from any vendor. The Agilent 1290 Infinity LC System offers some radical new design concepts to achieve this objective and yet clearly shows the pedigree of well-engineered reliability that has made Agilent HPLC systems the most successful HPLC line available.

This system offers:

- Flow rates from 0.05 ml/min to 5 ml/min for conventional or fast chromatography with all analytical column diameters from 1 to 5 mm internal diameter and all types of column packing material.
- Pressure range up to 1200 bar (>17400 psi) allows for fast chromatography on short columns, high resolution on long columns using sub-two micron packing material and a wider choice of mobile phase viscosity.
- Ultra-low delay volumes for the fastest gradients with mass spectrometric detection or ultraviolet/visible light detection.
- Ability to operate any method transferred from another analytical HPLC or UHPLC system.
- Sophisticated pump control to deliver very low chromatographic noise and very low acoustic noise for better results and better working environment.
- Degasser and automatic purge valve integrated into pump module.
- Variable volume autosampler with reduced delay volume, reduced carryover and the option to operate as a fixed loop autosampler.
- New Flexible Cube module to add functionality to the autosampler such as back-flushing the needle seat and fixed loop operation.
- Thermostatted Column Compartment with enhanced usability and integrated valve solutions with a pressure range up to 1200 bar (17400 psi).
- Diode-array detector with greatly enhanced sensitivity and baseline stability using cartridge cell system with optofluidic waveguides.

- Data collection rates up to 160 Hz with full spectral information.
- New range of ZORBAX RRHD sub-two micron particle size columns for operation at pressures up to 1200 bar.

The most significant advance is the range of pressures and flow rates that the system can use. This operating envelope can be described as the power range (flow x pressure) of the instrument and is most easily understood in graphical form (Figure 10 on page 23). As can be seen from the diagram the power range for the 1290 Infinity pump allows 1200 bar operation up to 2 ml/min flow, reducing to 800 bar as flow increases to 5 ml/min. This encompasses the operating range envelopes of all pre-existing UHPLC systems on the market and makes possible the direct transfer of methods from any of these systems to the 1290 Infinity system.

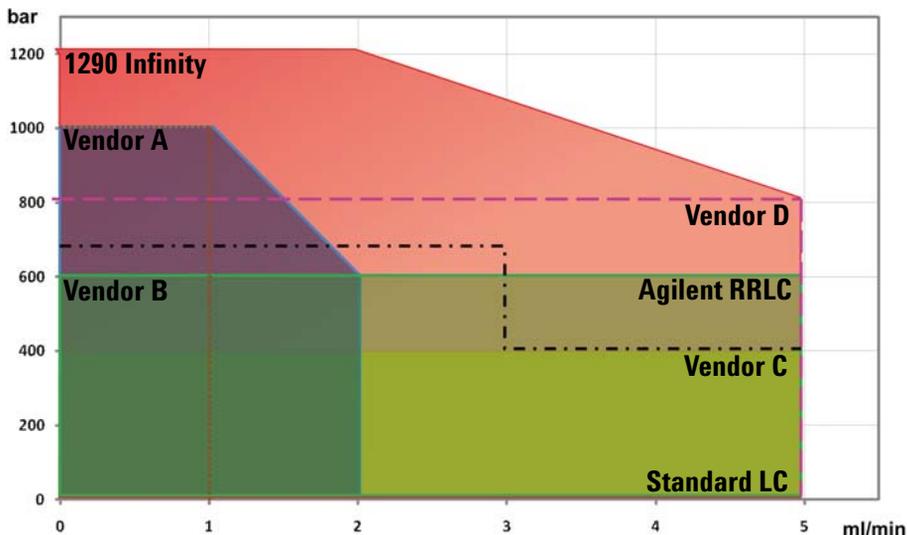


Figure 10 Power range of UHPLC systems (pressure x flow rate operating space)

2 The Agilent 1290 Infinity LC System – Product Description

New Features of the Agilent 1290 Infinity LC System

The pressure range offers the ability to work with the latest sub-two micron particles in long columns for high resolution and short columns for fast separation at increased flow rates. The flow rate range allows not only traditional methods to be used but also superficially porous (or pellicular) packing materials (for example, Poroshell) at high flow rates. These types of packing have recently gained new interest as an alternative approach to STM materials for high efficiency separations. The flow rate range allows the most suitable diameter column to be chosen for the separation whether it is 2 mm for low flow applications such as required by some MS systems or up to 5 mm id (typically 4.6 mm) for more traditional LC or increased loadability. The flow rate range also supports the latest research findings that show the benefit of using maximum flow rates to increase efficiency in gradient separations. (Refer to *Petersson et al., J.Sep.Sci, 31, 2346-2357, 2008, Maximizing peak capacity and separation speed in liquid chromatography*).

The new diode-array detector offers new levels of sensitivity combined with outstanding baseline characteristics and ease of use due to an innovative cell design.

The autosampler offers the well-established Agilent flow-through design for variable volume injection with low carryover upgraded for higher pressure, lower volume applications. A completely new module, the Flexible Cube, can be added on to the autosampler to offer fixed loop injection for the absolute lowest delay volume and other performance enhancements such as needle seat backflushing.

System Components

The Agilent 1290 Infinity Binary Pump

The Agilent 1290 Infinity Binary Pump contains new technology to overcome the problems of pumping LC solvents at ultra-high pressure and high flow rates: heavy duty drive motors on the pistons; new material for the pistons themselves not only to withstand the workload but also to actively transfer heat from the seals; microfluidic heat exchangers and the Jet Weaver, a microfluidic mixing device. The pump can deliver flow in the range of 0.05 - 5 ml/min at pressures up to 1200 bar.

The Agilent 1290 Infinity Binary Pump module contains two identical high pressure (1200 bar) pumps; a two-channel solvent degasser and four-channel inlet solvent selection valve, automatic purge valve and low-volume mixing device, the Jet Weaver, integrated into a single housing. The degasser increases flow stability, especially at low flow rates, and detector sensitivity.

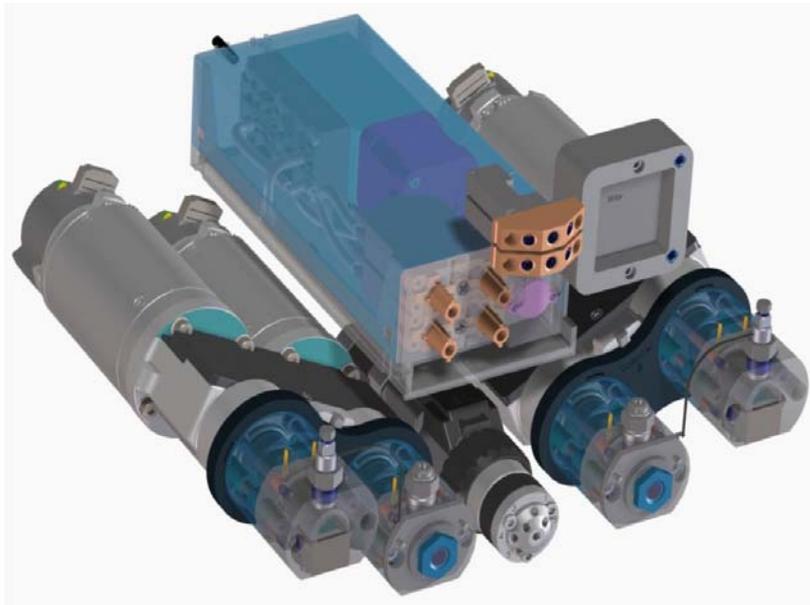


Figure 11 The Agilent 1290 Infinity Binary Pump

2 The Agilent 1290 Infinity LC System – Product Description

System Components

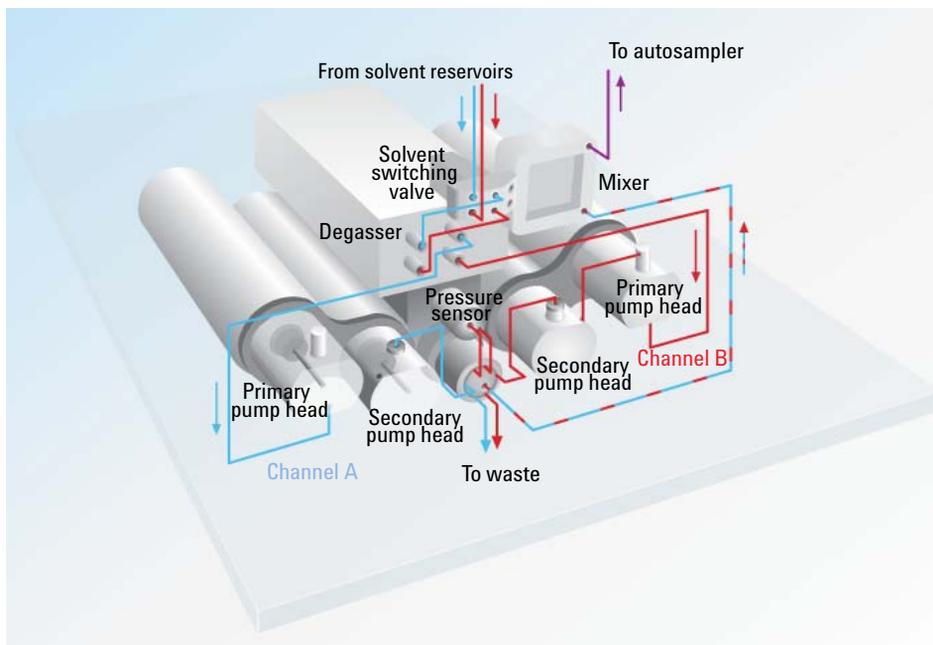


Figure 12 Parts identification and schematic of the 1290 Infinity Binary Pump

Each pump head is a dual piston in-series design utilizing novel firmware control and novel piston material, silicon carbide, which efficiently removes heat from the pump. The capillary joining the primary piston to the secondary piston has an integrated heat exchanger to remove the heat created at high pressure and flow. Each pump head has one passive inlet valve and one passive outlet valve on the primary piston chamber. Each piston is independently and precisely driven by a motor with 65000 steps giving 300 picolitres displacement per step.

The movement of the pistons is under intelligent control with a feedback loop to ensure that this active damping of pressure pulsation results in a ripple-free flow. The piston drive tunes itself for the compressibility characteristics of the solvent and the hydraulic characteristics of the system to maintain the ripple-free state. This in conjunction with the smooth-motion control, which reduces pressure pulsation caused by the movement of the piston, and the low-volume efficient mixing ensure that pump noise on UV traces is the lowest possible. A dedicated microprocessor in the pump takes care of the smooth motion control and optimization of the pistons' motion for real-time

optimization based on the static and dynamic parameters. In addition to the chromatographic performance these features makes the pump very quiet in operation.

When concentrated buffer solutions are used as mobile phase, the active seal wash option is available for use to extend the life of the pump seals.

A solvent selection valve allows binary mixtures (isocratic or gradient) to be formed from one of two solvents per channel. Binary gradients are created at the purge valve by high-pressure mixing of solvents from pump A and pump B. The purge valve allows the flow to be switched under software control to waste for purging new solvents through the pump head. A sensor is connected to the purge valve to monitor the system pressure.

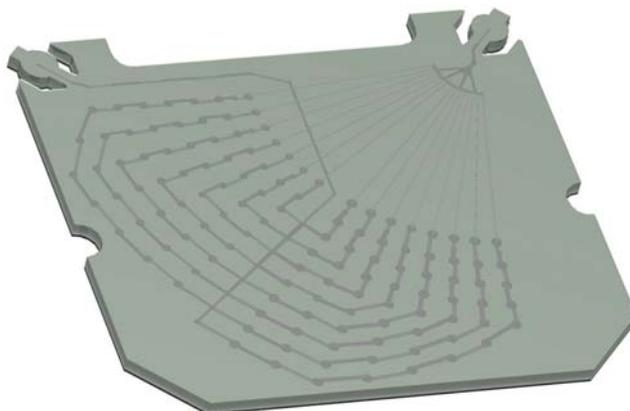


Figure 13 The Jet Weaver mixer

2 The Agilent 1290 Infinity LC System – Product Description

System Components

The flow path of the pump has been optimized for minimal delay of gradients and incorporates an innovative mixing system using microfluidics technology. The mixing device, known as the Jet Weaver, employs a network of multi-layer microfluidic channels (120 μm x 120 μm) to ensure the flow is thoroughly mixed. The Jet Weaver has two standard volumes: 35 μl for normal UV detection applications and 100 μl for demanding situations such as the use of TFA in UV detection. For MS detection it is often possible to work without the Jet Weaver and attain sufficient mixing using only the base 10 μl volume of the pump flow-path. Typical applications are high throughput methods with fast gradients on high resolution 2.1 mm columns.

The 1290 Infinity Binary pump comes ready for additional valve rails to be mounted on the right or left side of the pump. On these valve rails up to two additional 12-fold solvent selection valves can be attached. This allows a maximum of 26 solvents for binary gradients for analytical method development. A special “cluster driver” is available that makes the external solvent selection valves part of the pump user interface and allows very user-friendly and easy selection of the solvents by their names.

The Agilent 1290 Infinity Autosampler

The Agilent 1290 Infinity Autosampler offers the well-established Agilent flow-through design with variable volume injection and takes it to new levels of performance. New inert materials in the metering device seal and needle seat help to achieve extremely low carryover. The reduced hydraulic volume of the flow path is suited to faster gradients and the ability to use overlapped injections and automatic delay volume reduction (ADVR) contribute to faster cycle times and even faster gradient delivery to the column. The system draws exactly the set volume of sample solution without waste and achieves high reproducibility across the range from sub-microlitre to maximum injection volume of 40 μl . The standard installed injection capillary allows injections to 20 μl .

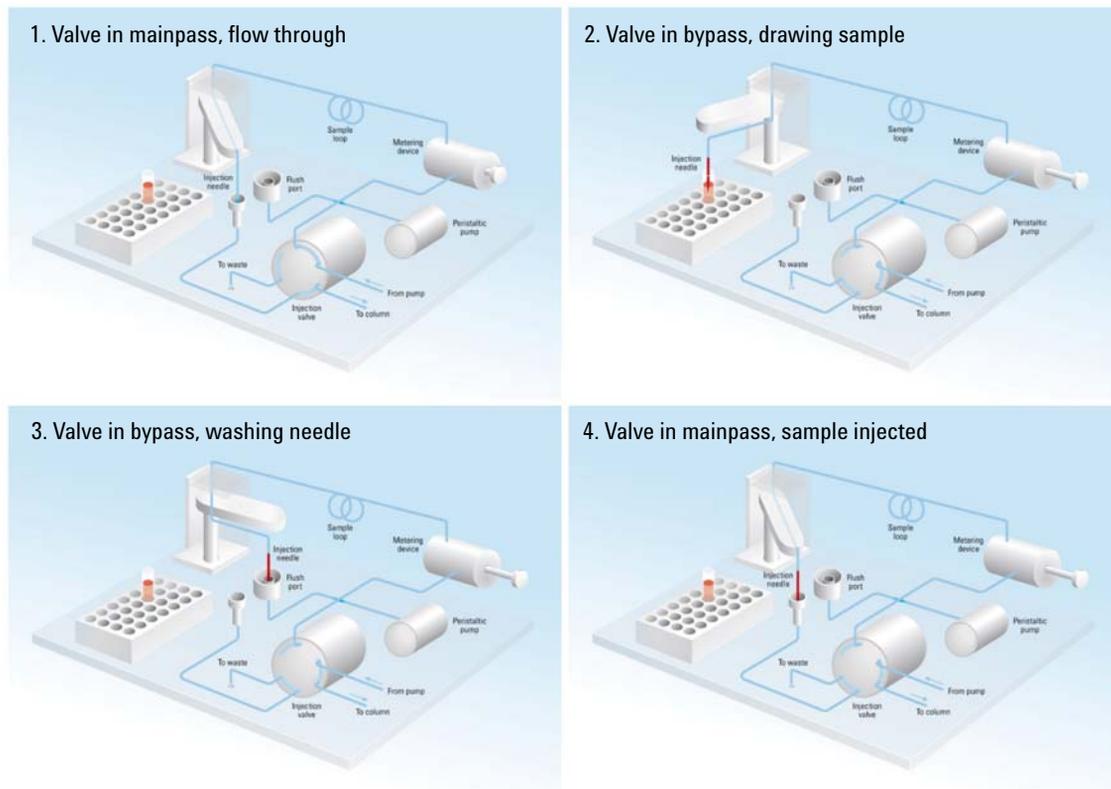


Figure 14 Schematic of injection steps in 1290 Infinity Autosampler

2 The Agilent 1290 Infinity LC System – Product Description

System Components

A totally new optional add-on module, the Flexible Cube, works seamlessly with the autosampler to provide additional capabilities. With the addition of the new Flexible Cube module, which comprises a 500 µl syringe pump, one low pressure valve and two high pressure switching valves further options become possible. For instance, the flow-through injection system can be replaced by a fixed injection loop using the syringe pump and valving of the Flexible Cube to fill the sample loop. The advantage of this configuration is that it eliminates the delay volume from the autosampler and so may be preferred in some fast-gradient high-throughput situations. The trade-off is that the flexibility of variable volume injection is turned off and some sample is wasted in flushing the loop. Other tasks such as automatically back-flushing the injection needle seat after injection are possible with the Flexible Cube giving further confidence that carryover of difficult compounds or blockages caused by dirty samples can be routinely avoided.

The autosampler sample rack has 10 fixed positions for 2 ml vials and two removable trays, which may or may not be identical, selected from:

- Tray for 2 ml vials with 54 positions
- Microtitre plate with 96 well positions (various heights configurable)
- Microtitre plate with 384 well positions (various heights configurable)

If required the autosampler can be thermostatted from 4 °C to 40 °C by the addition of the autosampler temperature control module.

The Agilent 1290 Infinity Thermostatted Column Compartment

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 ± 0.05 °C and the accuracy specification ± 0.5 °C (with calibration)¹. 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 μ 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 μ l and 6 μ 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 automatic 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 15](#) on page 32 left). Alternative valve heads can be interchanged by the user on the drive mechanism for different applications (see [Figure 15](#) on page 32 right). Note the RFID tag on top of valve head.

¹ 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.

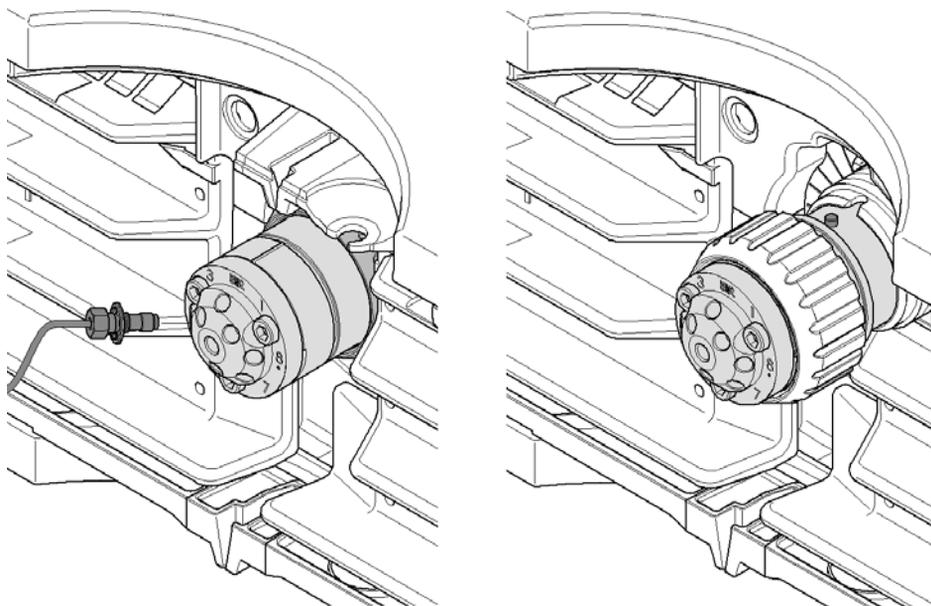


Figure 15 Quick change valve in TCC

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.

The 1290 Infinity Diode-Array Detector

The 1290 Infinity Diode-Array Detector 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 caused by refractive index or thermal effects and results in more reliable integration of peak areas.

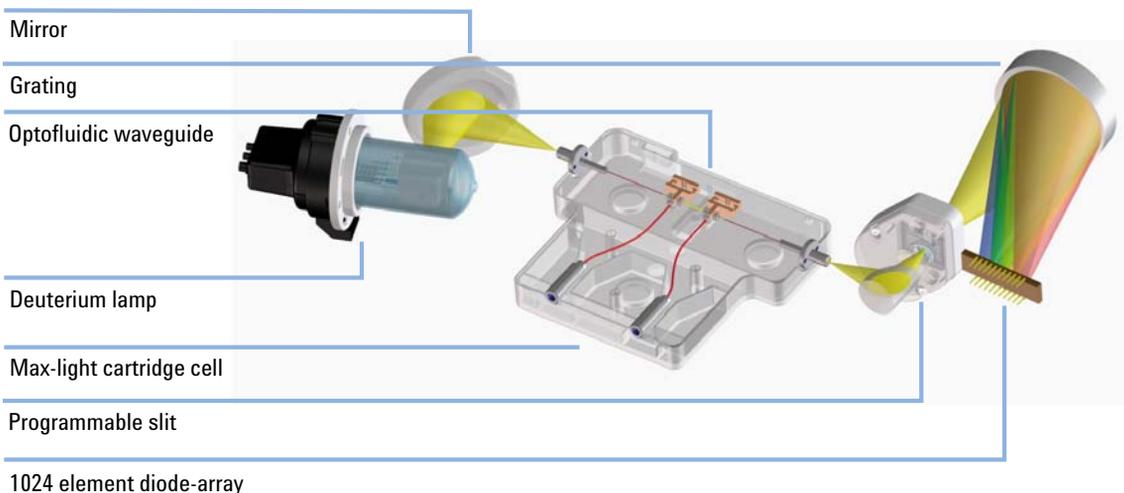


Figure 16 The light path through the 1290 Infinity Diode-Array Detector

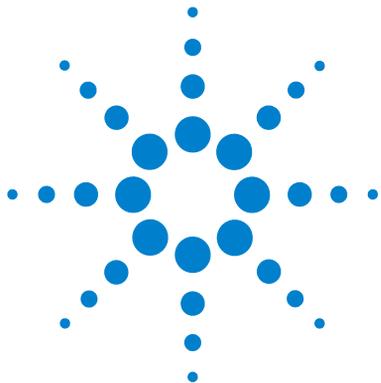
2 The Agilent 1290 Infinity LC System – Product Description

System Components

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 covered is 190 to 640 nm. This is detected by a diode-array comprising 1024 diodes. The entrance to the spectrograph is through a programmable optical slit which can give spectral resolution from 1 to 8 nm. This is generally operated in the middle of the range but for optimization can be closed down to 1nm for high spectral resolution (rarely required in liquid phase UV spectra) or opened up to 8 nm for maximum light transmission and minimum noise in the signal.

The chromatographic signals are extracted from the diode-array data within the firmware of the module. Up to 8 individual signals can be defined, each comprising a signal wavelength, a diode-bunching bandwidth and, if required, a reference wavelength and bandwidth. Signals can be output at up to 160 Hz (160 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 160 Hz.

For regulated laboratories it is important that all the method parameters are recorded. The 1290 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.



3 Optimization of the Agilent 1290 Infinity LC System

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



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 (e.g., 2.1 mm i.d.) as often used with mass spectrometric detection.

The delay volume in a system includes the volume in the pump from the point of mixing, connections between pump and autosampler, volume of the flow path through the autosampler and connections between autosampler and column.

As an example, in HPLC methods using 5 μm 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 μl and using a 2.1 mm column there would be an initial “hidden” isocratic segment of 5 min whereas on a system with 600 μl 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 μm 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 1290 Infinity LC System due to the low delay volume of the pump flow path, low volume of the Jet Weaver mixer 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 1290 Infinity LC System 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.

How to Configure the Optimum Delay Volume

Table 2 on page 38 and Table 3 on page 39 show the component volumes which contribute to system delay volume in the Agilent 1290 Infinity LC System. In the standard configuration with the Agilent 1290 Infinity Binary Pump, with the Jet Weaver mixer, the 1290 Infinity Autosampler and Thermostatted Column Compartment the system delay volume is about 125 μl . This standard delay volume is suitable for the majority of applications. For example, a fast separation on a 50 mm x 2.1 mm column packed with sub-two-micron particles at a moderate flow rate of 0.6 ml/min will result in a typical gradient delay time of about 0.2 min which is generally acceptable with gradient times of two to three minutes (see Table 4 on page 39). It is often useful to consider the flow rate in terms of column volumes and it can be seen from Table 5 on page 40 that with this column at 0.6 ml/min about 6 column volumes per minute flow through the system and the delay volume is about 1.2 times the column volume.

Table 2 Delay volumes of 1290 Infinity LC modules

Components	Delay Volume (μl)
Binary Pump	10
Jet Weaver mixer (standard)	35
Binary Pump + Jet Weaver	45
Autosampler (fixed 5 μl loop)	5
Autosampler (standard, variable volume)	80
Column Compartment low-dispersion heat exchanger	1.6
Connection tubing, 0.12 mm i.d., per 100 mm	1.1

Table 3 Delay volumes of 1290 Infinity system configurations

System Configurations ¹	Delay Volume (µl)
Binary Pump + Fixed Loop Autosampler (MS only)	20
Binary Pump + Jet Weaver + Fixed Loop	55
Binary Pump + standard Autosampler (MS only)	90
Binary Pump + Jet Weaver + Autosampler	125

¹ added 5 µl to allow for connections in system configurations

Table 4 System delay times for gradient to reach head of column

Flow Rate (ml/min)	System Delay Volume (microliters)			
	20	55	90	125
	Delay Times (minutes)			
0.2	0.10	0.28	0.43	0.60
0.4	0.05	0.14	0.21	0.30
0.6	0.03	0.09	0.14	0.20
0.8	0.03	0.07	0.11	0.15
1.0	0.02	0.06	0.09	0.12
1.5	0.01	0.04	0.06	0.08
2.0	0.01	0.03	0.04	0.06
3.0	0.01	0.02	0.03	0.04
4.0	0.01	0.01	0.02	0.03
5.0	0.00	0.01	0.02	0.02

3 Optimization of the Agilent 1290 Infinity LC System

How to Configure the Optimum Delay Volume

Table 5 Approximate volume of liquid in typical column dimensions assuming that porosity = 0.6

Column Diameter (mm)	Column Length (mm)				
	30	50	100	150	250
	Column Volume - Liquid Phase (microliters)				
2.1	62	104	208	312	520
3.0	127	212	424	636	1060
4.0	226	377	754	1131	1885
4.6	299	499	997	1496	2493

For very fast gradients over 0.5 min the delay volume of the system can be easily reduced without changing the physical configuration of the system. The change is achieved by changing the behavior of the autosampler.

The 80 µl delay volume of the Agilent 1290 Infinity Autosampler is due to the flow path from the injection valve through the metering device, needle, needle seat and connecting capillaries back to the injection valve (see [Figure 14](#) on page 29). To make an injection the valve switches from mainpass to bypass so that the metering device can draw the sample into the needle capillary. The injection is made when the valve switches back to mainpass and the sample is flushed onto the column. The valve remains in this position during analysis so that the autosampler is continually flushed and hence the gradient has to flow through this delay volume to reach the column. This can be eliminated by switching the injection valve from mainpass to bypass after the injection has been made and the injected sample has been flushed onto the column. In practice this can be done a few seconds after injection and is activated by selecting the “Automatic Delay Volume Reduction” (ADVR) function in the autosampler setup menu. The Flush-out Factor (typically 5 times injection volume) ensures that enough time is allowed to flush the sample out of the injector before switching to bypass. This effectively reduces the system delay volume from 125 µl to 50 µl.

When using ADVR it should be noted that the gradient has already started at the pump at the instant of injection. The question should be asked whether the gradient has already reached the autosampler, in which case a small step in the gradient will result. This happens when the delay volume is less than the flush-out volume and is not necessarily a problem but may be a factor to be considered in a method transfer. With a flush-out factor of 5 and an injection volume of 10 μl , the autosampler will allow 50 μl to pass through before switching to bypass which, with a delay volume of 50 μl , means the gradient just reached the injection valve. Smaller injection volumes will have no effect but for larger injection volumes this will introduce a small step in the gradient. The flow rate in use will also have an impact on the decision to use ADVR or not. At 0.2 ml/min the delay time saved is 21 seconds while at 1.0 ml/min it is 4 seconds.

The ADVR function is unlikely to be suitable for applications involving compounds which are known to cause carry-over problems.

To minimize peak dispersion and delay volume in the Thermostatted Column Compartment the low dispersion heat exchanger must be installed. The low dispersion heat exchanger is part of the capillary kits recommended for low dispersion applications. The common capillary kit also includes narrow 0.12 mm i.d. capillaries. The built-in 3 μl and 6 μl heat exchangers are for backwards compatibility and should only be used in case a conventional method needs to be run on the system even though in this case also the low dispersion heat exchanger could be used.

To maintain resolution in the Agilent 1290 Infinity Diode-array Detector the Max-Light cartridge cell has a low dispersion volume (σ volume 1.0 μl) and no further volume optimization is required. In situations where the alternative Agilent 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.

For pump operation it is recommended to set the correct solvent in the pump setup screen. Even though the intelligent control will automatically tune the pressure ripple to a minimum the solvent compressibility can have an effect on maintaining absolutely correct flow rate at high pressure. 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 1290 Infinity LC System

How to Configure the Optimum Delay Volume

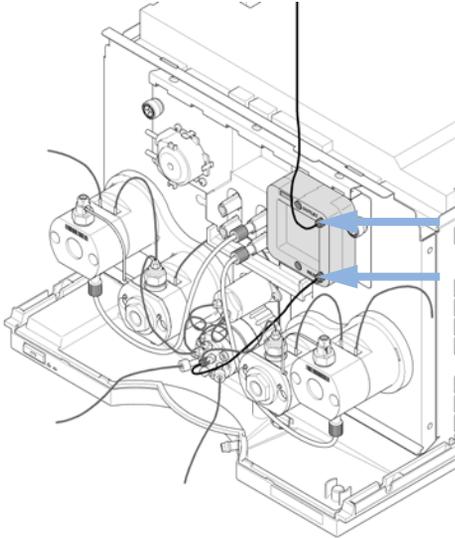
The physical delay volume of the pump is primarily dependent on the use of the Jet Weaver mixer. For UV detection the Jet Weaver should always be used but for mass spectrometric detection the user can decide to bypass the Jet Weaver removing 35 μl from the delay volume. This only makes sense for ultra-fast gradient operation (less than 0.5 min) or for use with very small volume columns. Refer to [Table 4](#) on page 39 for the effect on the delay time in the system. If the Jet Weaver is bypassed the connection tubing to the autosampler is routed directly from the purge valve. Ensure that the Jet Weaver has been flushed with solvent containing no buffers or other additives before disconnecting it.

Sometimes it may be advisable to increase the delay volume in the pump. Specifically this can be the case when UV detection is employed and a strongly UV-absorbing compound has been added to the mobile phase. This can have the effect of emphasizing any pump noise and the most common example is the use of trifluoro acetic acid (TFA) in the analysis of proteins and peptides. The effect can be mitigated by increasing the mixer volume. The Jet Weaver mixer has two alternative volumes in the same unit. The switch from the lower volume, 35 μl , to the higher volume, 100 μl , is done by de-installing it, turning it around from front to back and re-installing it. The mixing volume (and hence delay volume) is increased by 65 μl and the baseline performance with additives like TFA will be improved. The configuration of the Jet Weaver is logged automatically by an attached RFID tag.

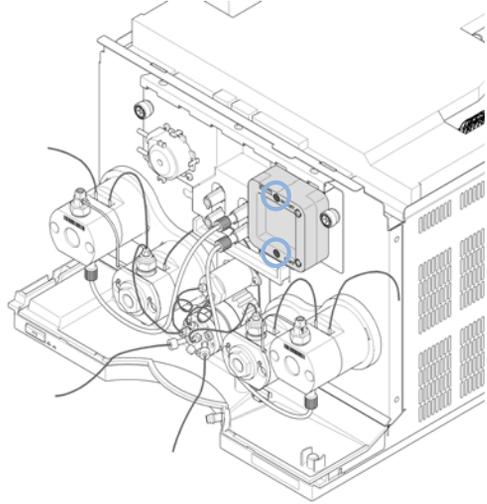
The procedure is illustrated in [“Replacing the Jet Weaver”](#) on page 43.

Replacing the Jet Weaver

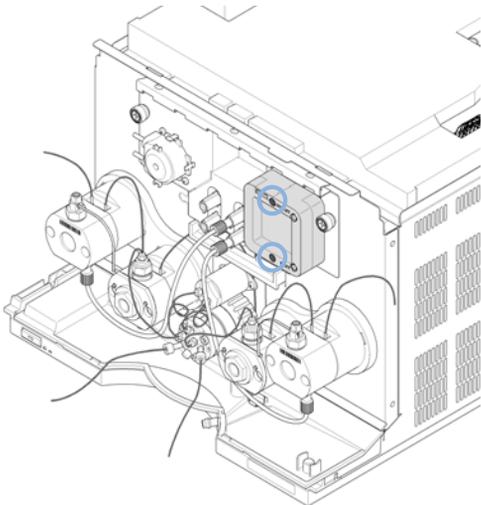
1 Remove capillary connections from the Jet Weaver.



2 Remove the hex screws that fix the Jet Weaver to the pump housing.



3 Install the new Jet Weaver.



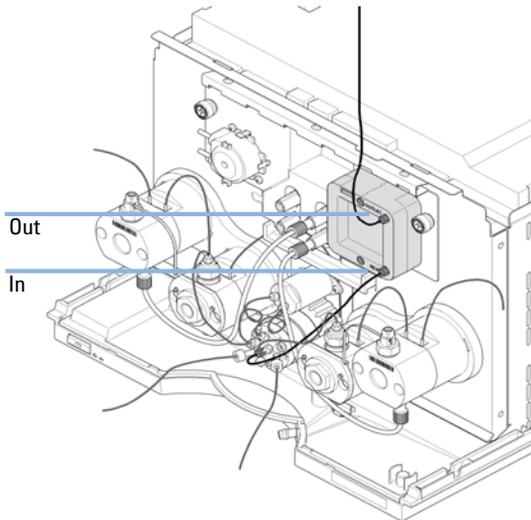
NOTE

The Jet Weaver has a front and a rear side with different internal volumes (35 / 100 μ l) that are optimized for a low delay volume or best mixing performance.

3 Optimization of the Agilent 1290 Infinity LC System

How to Configure the Optimum Delay Volume

4 Install the capillary connections.



The inlet at the bottom of the Jet Weaver is connected to the central port of the pump valve by a capillary (length 300 mm, 0.17 mm i.d.). The outlet at the top is connected to the autosampler.

How to Achieve Higher Injection Volumes

The standard configuration of the Agilent 1290 Infinity Autosampler includes a variable volume sample loop for up to 20 μl injections. The metering device can inject a maximum volume of 40 μl and the sample loop cartridge can be exchanged to allow this (refer to the *1290 Infinity Autosampler manual* for details). 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 big 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 μl but it is very dependent on the chemistry of the analyte and mobile phase as discussed above. A rough guide to maximum injection volume can be gained by looking at the volume of the column (see [Table 5](#) on page 40) – in a gradient separation injection volumes of about 5 % of the column volume might be achieved whilst maintaining good resolution and peak dispersion.

3 Optimization of the Agilent 1290 Infinity LC System

How to Achieve Higher Injection Volumes

One way to achieve larger injections is to use a trapping column selected by a switching valve to capture and concentrate the injection before switching it, i.e. injecting it, onto an analytical column, see [Figure 17](#) on page 46. The valve can be conveniently located in the Thermostatted Column Compartment.

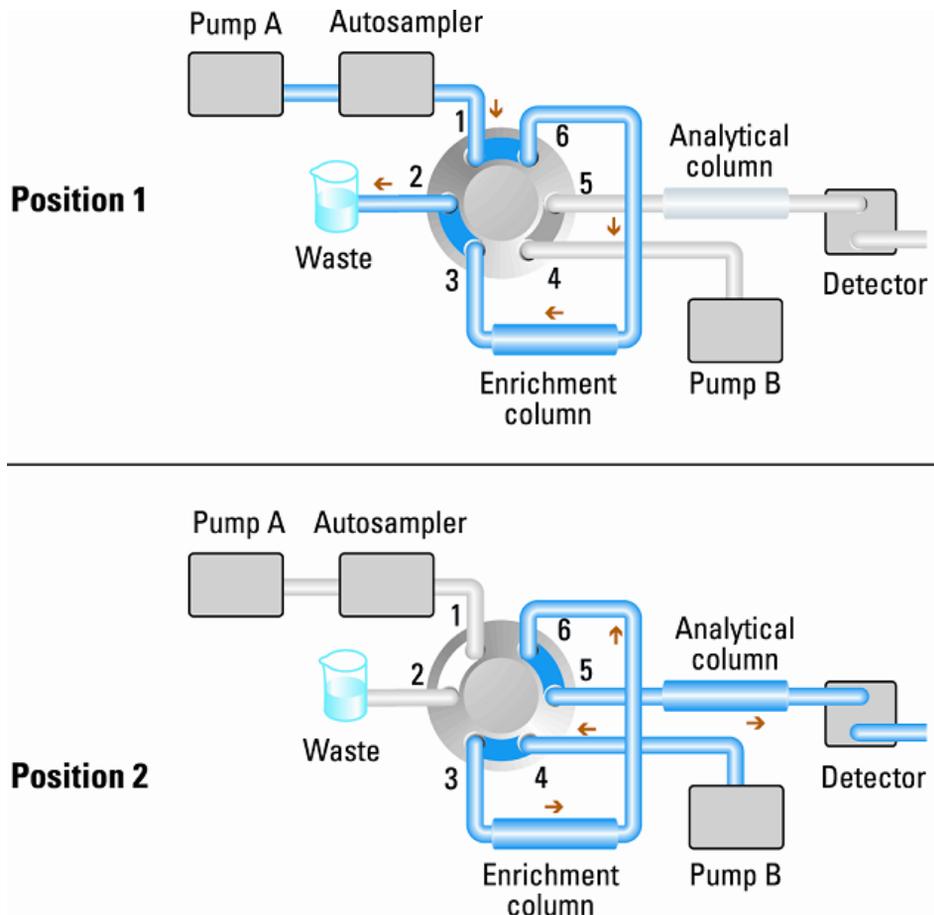


Figure 17 Sample enrichment

How to Achieve High Throughput

Some laboratories operate in a high throughput (HT) environment where the workload requires sequences of hundreds or even thousands of injections to be made to complete a body of work. In these situations it is highly desirable to minimize the cycle times as even a few seconds saved per injection will reduce the overall time to complete the work by a significant and useful amount. The key steps to achieving fast cycle times and high throughput operation are:

- Using fast separations
- Overlapping injections
- Minimizing equilibration time
- Alternating column regeneration

The first step to achieving high throughput operation is to ensure that the methods used have short cycle times, i.e., they are fast chromatography methods. The use of short columns with 1.8 μm particle size packing is ideal for this purpose because of the high efficiency available in a short column. If the methods involve isocratic separation this allows for the fastest cycle times because no column equilibration is required between runs. However, most often gradient methods are employed due to the range or complexity of the samples. In developing the method the gradient range should be kept to the minimum required to achieve the separation. In many “open access” systems gradients running from 5 % to 95 % organic solvent are used for the greatest flexibility to deal with a range of unknown compounds. In the high throughput situation it should be considered whether a shorter range will suffice on the basis that the expected range of compounds for analysis is reduced and this will not only allow the run time to be reduced but will also shorten the equilibration time between runs.

The cycle time is made up of a number of parts: Cycle time = injection + separation + equilibration + data processing.

With a large number of samples to process even a small reduction in cycle time can add up to a large reduction in the overall time to complete the work. For this reason the data processing can be taken off-line to allow the system to focus on running the samples and collecting data.

3 Optimization of the Agilent 1290 Infinity LC System

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 ChemStation 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. For sticky compounds it is recommend to do this during column equilibration, when the autosampler has seen the starting conditions of the next gradient run.

The column equilibration step can be a significant part of the cycle time. Typically the column needs flushing with three to five times the column volume to stabilize it ready for the next injection and this can be 50 % or more of the separation time in some applications. It is an essential process but can be taken out of the cycle time by using automated alternating column regeneration. For this a two-position ten-port, 1200 bar, valve head is required in the column compartment; a second analytical column, identical to the first; and a second pump is needed. As one column is being used in the separation run, the other column is being flushed with the starting composition of the mobile phase gradient and to start the next injection the newly re-equilibrated column is switched into the analytical flow path. The two columns then alternate in this way for the whole sequence of injections. The second pump is only required to flush an isocratic mixture through the column and so can be a simpler pump than the 1290 Infinity pumps. For instance a 1200 Series isocratic pump would be sufficient to perform this task. The setup is illustrated in [Figure 18](#) on page 49.

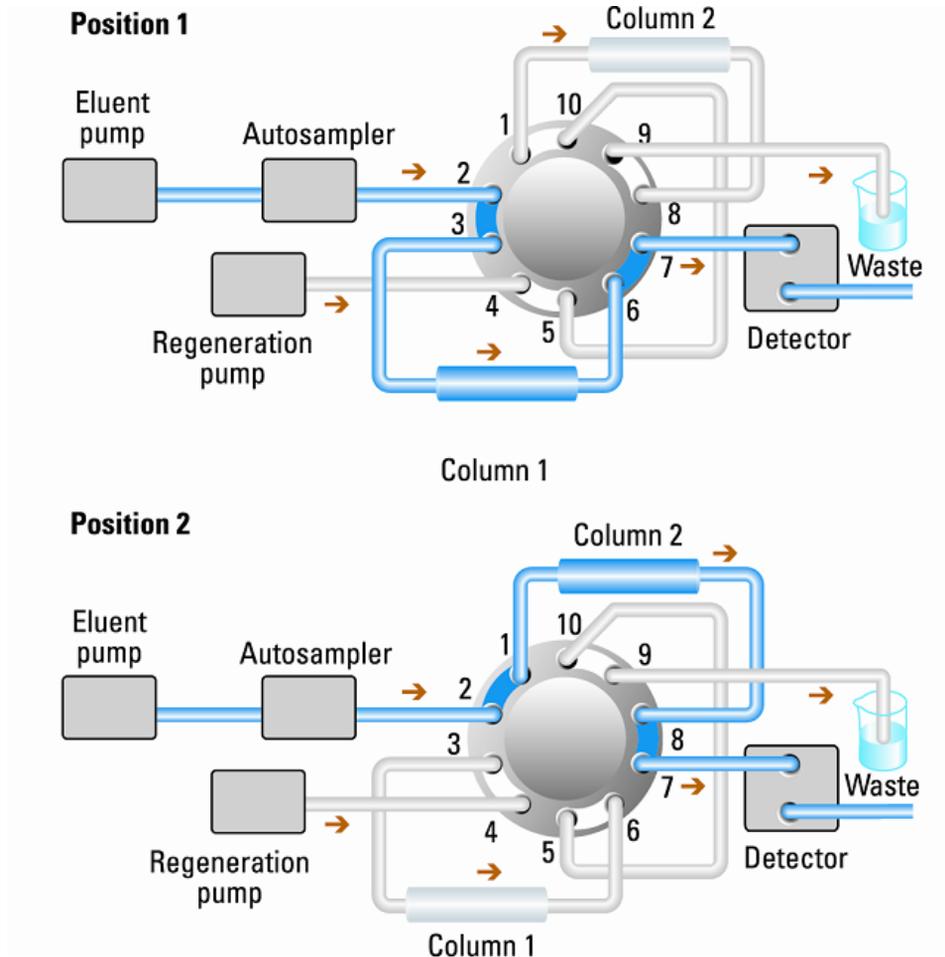


Figure 18 Alternating Column Regeneration

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 considers 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:

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

where

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

The term that has the most significant effect on resolution is the selectivity, α , 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 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 , and this 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. This is the reason that the 1290 Infinity LC system was designed to go to 1200 bar so that it can run sub-two-micron particles and column length can be increased to 100 mm or 150 mm. There are even examples of 100 mm and 150 mm columns linked to give 250 mm length. 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 and 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.

3 Optimization of the Agilent 1290 Infinity LC System

How to Achieve Higher Resolution

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. Recently published research has shown how a shorter STM column (at temperatures above 40 °C) can generate higher peak capacity than a longer STM column by virtue of running it faster. (Refer to *Petersson et al., J.Sep.Sci, 31, 2346-2357, 2008, Maximizing peak capacity and separation speed in liquid chromatography*).

Any reduction in extra-column volume will reduce dispersion and give better resolution. This is already optimized in the 1290 Infinity LC system 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.

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. This section considers how sensitivity is affected by:

- Pump mixer volume
- Narrower columns
- Detector flow cell
- Detector parameters

In addition, the discussion on detector parameters also mentions the related topics of selectivity and linearity.

Pump Mixer Volume

To achieve lowest baseline noise with UV detection, the standard delay volume configuration with the 35 μ l Jet Weaver is recommended for the 1290 Infinity pump module. This is applicable for almost all applications but when using TFA in the mobile phase, or other situations requiring more mixing, the larger volume side of the Jet Weaver mixer should be used to minimize mixing noise.

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.

How to Achieve Higher Sensitivity for Detector

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.

Flow Cell

The Max-Light cartridge flow cell has a standard 10 mm path length and is optimized for minimal volume and dispersion (σ volume 1.0 μl). It has high light transmission minimizing noise to reduce noise due to the optofluidic waveguide. It is suitable for use with a wide range of analytical columns from short narrow-bore columns to long standard diameter (4.6 mm) columns. Generally the peak dispersion volume (calculated from peak width x flow rate) should be greater than about 2 μl for this cell (for example 0.02 min x 200 $\mu\text{l}/\text{min}$ = 4 μl).

The Max-Light high sensitivity cell has a path length of 60 mm and this will give between three and five times increase in signal-to-noise values depending on the application conditions. The dispersion volume is fractionally increased compared to the standard cell.

Wavelength and Bandwidth

The detector measures absorbance simultaneously at wavelengths from 190 nm to 640 nm using diode-array detection. A UV-lamp provides good sensitivity over the whole wavelength range. The diode-array detector (DAD) can simultaneously compute and send to the data system up to eight chromatographic signals and the full-range spectra at every time point. A multi-wavelength detector (MWD) processes only the chromatographic signals. A UV chromatogram or signal is a plot of absorbance data versus time and is defined by its wavelength and bandwidth.

- The wavelength indicates the center of the detection band.
- The bandwidth defines the wavelength range over which the absorbance values are averaged to give the result at each time point.

For example, a signal at wavelength 250 nm with a bandwidth of 16 nm will be an average of the absorbance data from 242 nm to 258 nm. Additionally, a reference wavelength and reference bandwidth can be defined for each signal. The average absorbance from the reference bandwidth centered on the reference wavelength will be subtracted from its equivalent value at the signal wavelength to produce the output chromatogram.

The signal wavelength and bandwidth can be chosen so that they are optimized for:

- Broad band universal detection
- Narrow band selective detection
- Sensitivity for a specific analyte.

Broad band or universal detection works by having a wide bandwidth to detect any species with absorbance in that range. For example, to detect all absorbing molecules between 200 nm and 300 nm set a signal at 250 nm with a bandwidth of 100 nm. The disadvantage is that sensitivity will not be optimal for any one of those molecules. Narrow band or selective detection is used most often. The UV spectrum for a particular molecule is examined and an appropriate absorbance maximum is selected. If possible, the range where solvents absorb strongly should be avoided (below 220 nm for methanol, below 210 nm for acetonitrile). For example, in [Figure 19](#) on page 56, anisic acid has a suitable absorbance maximum at 252 nm. A narrow bandwidth of 4 nm to 12 nm generally gives good sensitivity and is specific for absorbance in a narrow range.

The narrow band can be optimized for sensitivity for a specific molecule. As the bandwidth is increased the signal is reduced but so is the noise and there will be an optimum for best S/N. As an approximate guide, this optimum is often close to the natural bandwidth at half-height of the absorption band in the UV spectrum. In the anisic acid example this is 30 nm.

The analytical wavelength is usually set at a wavelength maximum to increase sensitivity to that molecule. The detector is linear up to 2 AU and beyond for many applications. This offers a wide linear range for concentration. For high concentration analysis the concentration linear range can be extended by setting the wavelength to one with a lower absorbance such as a wavelength minimum or by taking a wider bandwidth which usually includes lower absorbance values. The use of wavelength maxima and minima for quantitation dates back to conventional UV detectors which because of mechanical tolerances in moving gratings needed to avoid steeply sloping

3 Optimization of the Agilent 1290 Infinity LC System

How to Achieve Higher Sensitivity

parts of the spectrum. Diode-array based detectors do not have this limitation but for reasons of convention maxima and minima are chosen in preference to other parts of the spectrum.

The reference bandwidth is normally set on a region of the UV spectrum in which the analyte has no absorbance. This is shown in the spectrum for anisic acid in Figure 19 on page 56. This spectrum is typical of many small molecules containing a UV chromophore. For best results the reference has been set so that it is a wide band as close to the signal wavelength as possible but on a zero absorbance region. Reference bandwidths of 60 nm to 100 nm are commonly used. The default reference is 360 nm with a bandwidth of 100 nm. A wide bandwidth is used because this reduces the noise in the reference signal (from statistical theory, the error, i.e. noise in this case, is reduced by the square root of the number of determinations). It is important that the reference bandwidth does not extend to a part of the spectrum that has some absorbance as this would then reduce the resulting signal and sensitivity would be reduced. The use of a reference wavelength can help to reduce drift or wander in the chromatogram caused by refractive index changes due to room temperature fluctuation or gradient operation. The effect of a reference signal can be easily tested by setting two otherwise identical signals, one with and one without a reference signal. If there is no part of the spectrum with zero absorbance then it will be better to have the reference signal turned off.

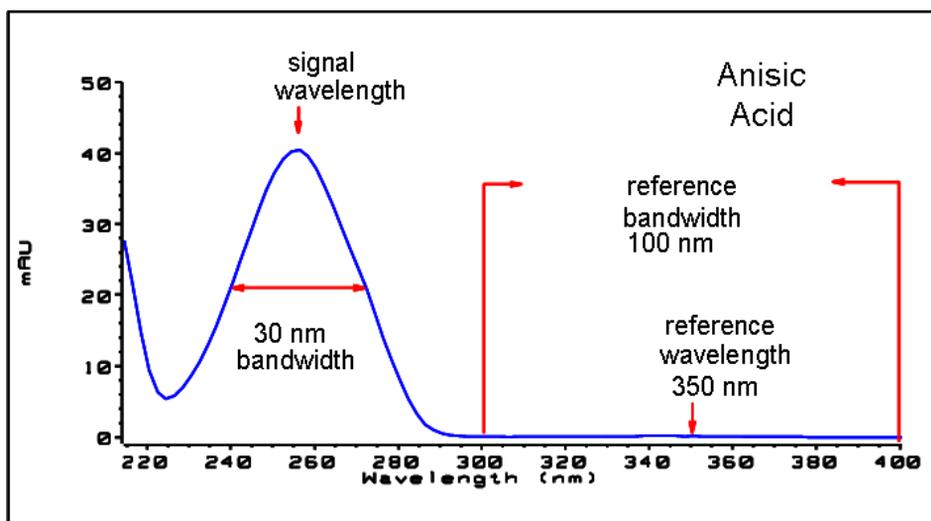


Figure 19 Spectrum of Anisic Acid

Slit Width

Light transmission into the spectrograph and the optical bandwidth are controlled by the variable aperture entrance slit. The default setting for the slit width is 4 nm which is appropriate for most applications as it gives good all-round performance. The performance characteristics affected are sensitivity, spectral resolution and linearity. Considering a particular wavelength entering the spectrograph, its light will effectively fall onto a small band of diodes, the width of which is proportional to the width of the entrance slit. The description of the slit as 4 nm describes this behavior – the light falls on the number of diodes that detect a bandwidth of 4 nm. It follows that the minimum optical resolution will be 4 nm and therefore the diode-array (or digital) bandwidth should be set to 4 nm or greater. For optimum sensitivity the 8 nm setting will allow most light in and will minimize noise but spectral resolution is at its lowest. This is not usually a problem with UV spectra as their natural bandwidths are usually greater than 25 nm without any fine structure. The optical bandwidth at 8 nm reduces the linearity range compared to 4 nm slit so it is important that a validated method always uses the slit width that was used for validation. For optimum spectral resolution the 1nm setting is best. This will enable fine structure such as in the benzene spectrum to be resolved (see [Figure 20](#) on page 57). Very few compounds display such fine detail in solution spectra. The light level will be lower so the signal will have more noise - the noise level depends on the wavelength and mobile phase solvents used.

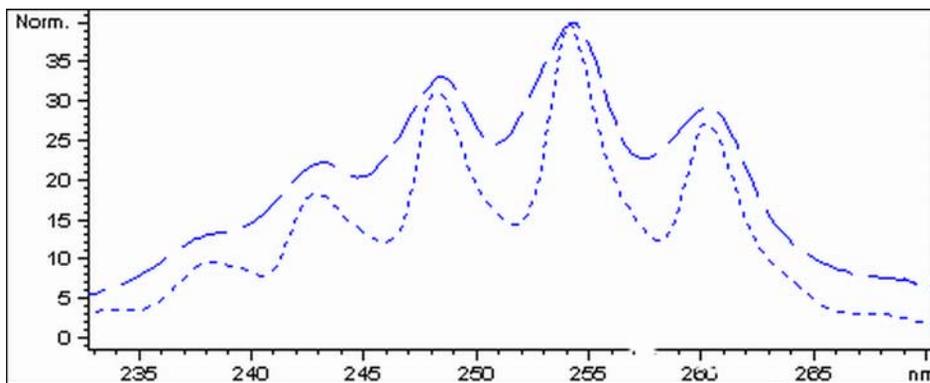


Figure 20 Benzene at 1 and 4 nm Slit Width (Principle)

3 Optimization of the Agilent 1290 Infinity LC System

How to Achieve Higher Sensitivity

The injection volume and the sample dissolution solvent are important in controlling dispersion. 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. To achieve this, the sample should be dissolved in a solvent composition of lower elution strength than the mobile phase. It may be possible to increase the injection volume to have a greater concentration of analyte on the column and hence increased peak height.

Refer to the comments in [“How to Achieve Higher Injection Volumes”](#) on page 45.

Peak Width, Response Time and Data Collection Rate

The peak width setting, response time and data rate in the detector are all linked. The available settings are shown in [Table 6](#) on page 59. It is important to set this correctly for optimum sensitivity and to preserve the resolution achieved in the separation.

The detector internally acquires data points faster than is needed for a chromatogram and processes them to produce the signal seen by the data system. Part of the processing reduces the data to an appropriate data rate which allows the chromatographic peaks to be accurately drawn. As with most analytical determinations groups of readings are effectively averaged to reduce error in the result. The detector bunches raw data points and produces the output signal data at the required data collection rate by an electronic filtering process. If the resulting data rate is too slow (over filtering) the peak heights will be reduced and the resolution between them reduced; too fast and the data is noisier than it need be to accurately profile narrow peaks.

The *peak width* setting in the detector allows the user to correctly set these parameters without needing any knowledge other than sight of the chromatogram integration results to see how wide the peaks are. The peak width setting should be set for the narrowest peak width observed in the chromatogram. If it is set too wide it will make the peaks appear lower in height and wider (and potentially less resolved) and if it is set too narrow it will increase the baseline noise unnecessarily. Essentially the software uses this value to set the *data collection rate* such that it collects enough data points over the narrowest peaks and it is aiming for 15 to 25 points across a peak. The 1290 Infinity DAD can collect at a maximum 160 Hz if required which would allow enough data points to be collected over a peak that is only 0.1 s wide. The *response time* setting is another way of indicating how this filtering is set. It is measured in seconds and is about one-third of the peak width value (which is measured in minutes). It effectively shows how quickly the plotted signal responds to a step change in the input signal.

Table 6 Peak Width — Response Time — Data Rate

Peak width at half height [min] ¹	Response [s]	Data rate [Hz]
< 0.0015625	0.015625	160
> 0.0015625	0.03125	160
> 0.003125	0.0625	80
> 0.00625	0.125	40
> 0.0125	0.25	20
> 0.025	0.5	10
> 0.05	1.0	5
> 0.1	2.0	2.5
> 0.2	4.0	1.25
> 0.4	8.0	0.625
> 0.85	16.0	0.3125

¹ Values in the User Interface may be rounded.

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 carry over 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 expressed as a percentage of the area in the previous active injection. The Agilent 1290 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.

The outside of the needle can be washed using a wash vial in a specific location or the needle can be washed using the flush port. If a wash vial in a tray location specified by the user is chosen then this vial should have no septum and should contain a solvent suitable for washing the sample from the needle. The septum is not used to avoid wiping contamination off the needle on the downstream only to re-apply it on the upstroke. The needle can be dipped into the vial multiple times. This will be effective in removing a small degree of carryover but for more effective washing of the outside of the needle use the flushport.

The flush port is located above and behind the needle seat and a peristaltic pump delivers the wash solvent. It has a volume of 0.68 ml and the peristaltic pump delivers 6 ml/min, which means the flush port volume is completely

refilled with fresh solvent in 7 s. If the flush port is selected, the user can set how long the outside of the needle is to be washed with fresh solvent. This may be as low as two or three seconds in routine situations where carryover is less of a problem and 10 to 20 s for more complete washing. It is recommended that washing the outside of the needle in the flush port should be standard procedure to avoid contaminating the needle seat. If the needle seat becomes contaminated it will have to be back-flushed, by manually changing the flow connections, to clean it. This is one of the tasks that can be automated using the Flexible Cube module.

The flush port and its solvent delivery pump and tubing should be regularly flushed to ensure the lowest carryover. For example, before using the system each day, prime the flush pump for three minutes with appropriate solvent.

When other measures have failed to eliminate carryover it might be that analyte is sticking inside the injector valve. The injector valve can be set to make additional switching movements to clean out the flow path in the valve if problems occur here with carryover. If the problem compounds need a high percentage of organic phase for elution, it is recommended to switch the injection valve at the high percentage of organic phase after the last peak has eluted. It is also recommended to switch the injection valve again after the initial conditions for the mobile phase have stabilized. This ensures that the bypass groove in the rotor seal of the valve contains the gradient start conditions, which is especially important for flow rates below 0.5 ml/min.

For samples where the outside of the needle cannot be cleaned sufficiently with water or alcohol from the flush pump use wash vials with an appropriate solvent. With an injector program several wash vials can be used for cleaning.

How to Prevent Column Blockages

As with any HPLC system, care must be taken to avoid partially or completely blocking the column or the system tubing through inadvertent use. Problems caused by material introduced to the system can generally be avoided by taking the following actions:

- Filter solvents
- Filter samples
- Replace mobile phases regularly
- Flush buffer salts from the system

An unavoidable source of particulates is the system itself. As with all HPLC systems, seals will wear out shedding material which will be trapped by frits in the system necessitating the routine replacement of these frits. 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 and from the instrument itself.

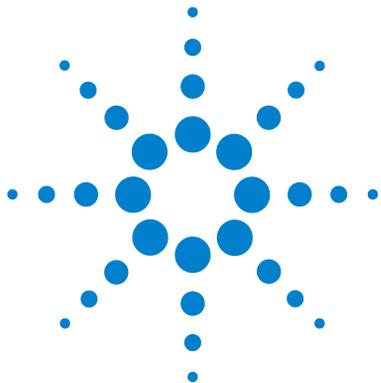
To ensure best results follow these simple usage guidelines:

- 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 one to two days. 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** Use the seal wash option with high concentration buffer mobile phases.
- 7** An in-line filter (1290 Infinity inline filter, diameter 2 mm, **part number: 5067-4638**) is recommended to catch particulates and extend column life.
- 8** Change the filter when the pressure increases by 10 %.

- 9 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.
- 10 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.
- 11 Once the pressure has stabilized, attach the column to the detector.
- 12 Equilibrate the column and detector with 10 column volumes of the mobile phase prior to use (see [Table 5](#) on page 40 for column volumes).
- 13 Avoid over-pressure. Check the pressure range of your gradient before starting any sequence.
- 14 If samples are dissolved in high organic content consider using an injection program to add a plug of weaker solvent before and after the sample aliquot to reduce the risk of precipitation in high concentration buffer mobile phase.

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How to Prevent Column Blockages



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This chapter includes information on software installation, stack configurations 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 *1290 Infinity Diode Array 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 upon the diagnostic functions that were formerly 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 Module

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

Optimizing the Stack Configuration

One Stack Configuration

Ensure optimum performance by installing the modules of the Agilent 1290 Infinity LC System in the following configuration (See [Figure 21](#) on page 68 and [Figure 22](#) on page 69). This configuration optimizes the flow path for minimum delay volume and minimizes the bench space required.

The Agilent 1290 Infinity Binary Pump should always be installed at the bottom of the stack.

4 System Setup and Installation

Installing the Module

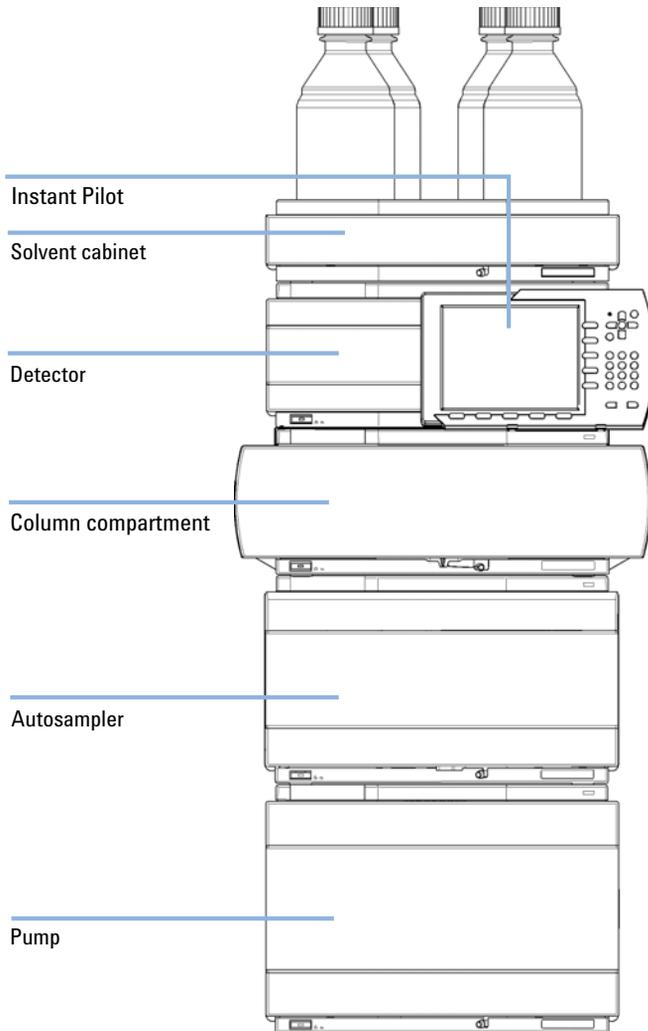


Figure 21 Recommended Stack Configuration (Front View)

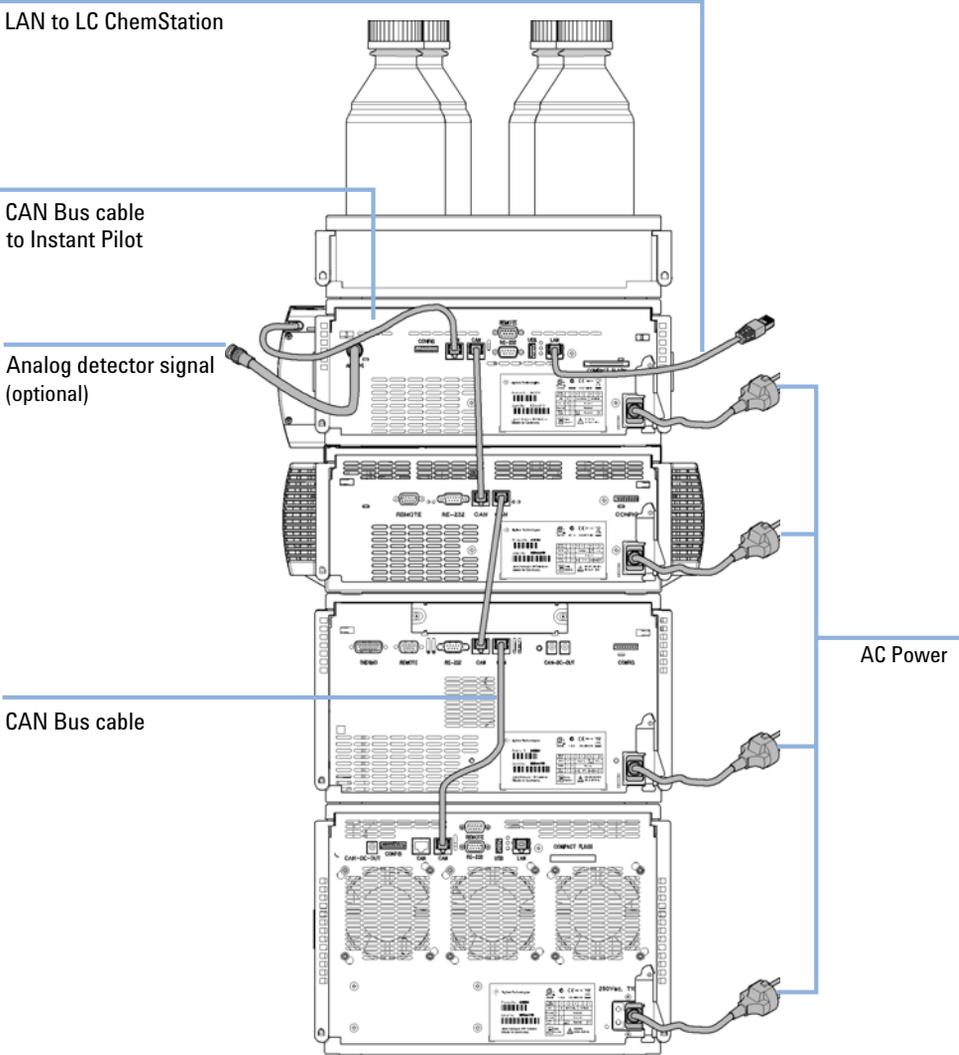


Figure 22 Recommended Stack Configuration (Rear View)

4 System Setup and Installation

Installing the Module

Two Stack Configuration

To avoid excessive height of the stack when the autosampler thermostat is added to the system it is recommended to form two stacks. Some users prefer the lower height of this arrangement even without the autosampler thermostat. A slightly longer capillary is required between the pump and autosampler. See [Figure 23](#) on page 70 and [Figure 24](#) on page 71).

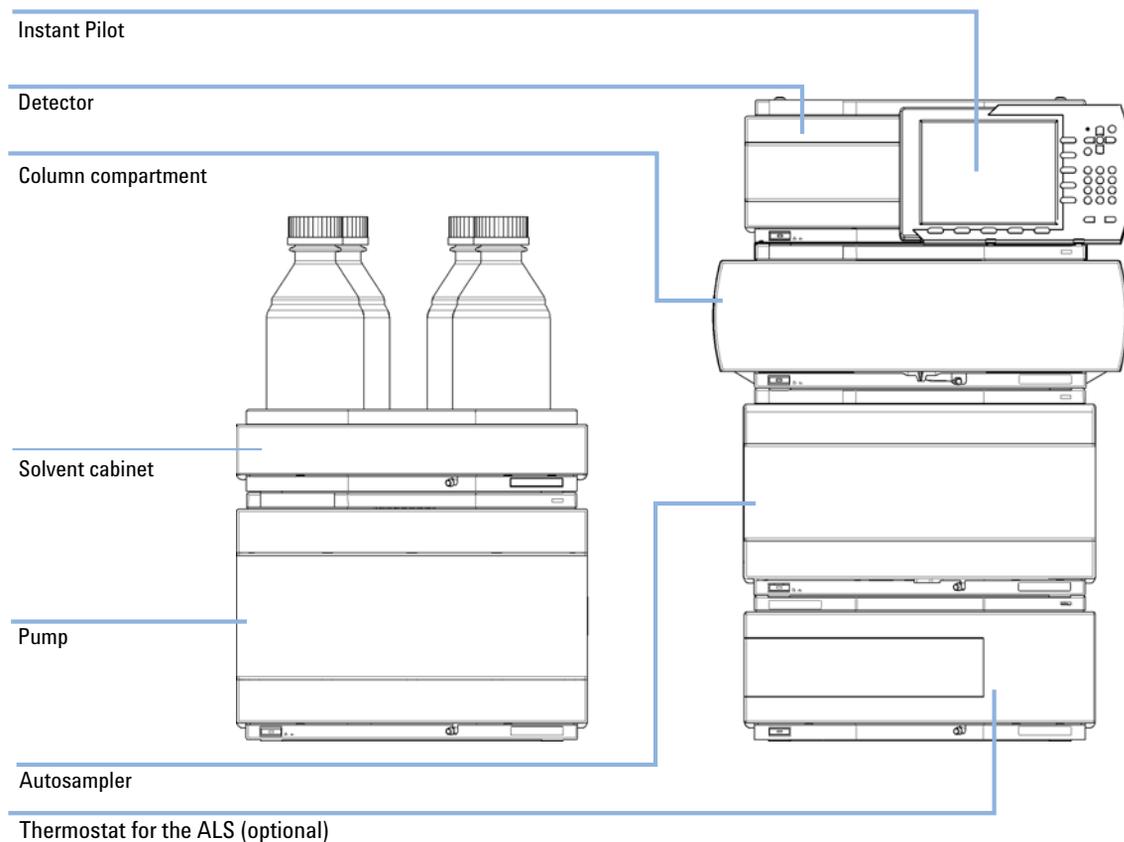


Figure 23 Recommended Two Stack Configuration (Front View)

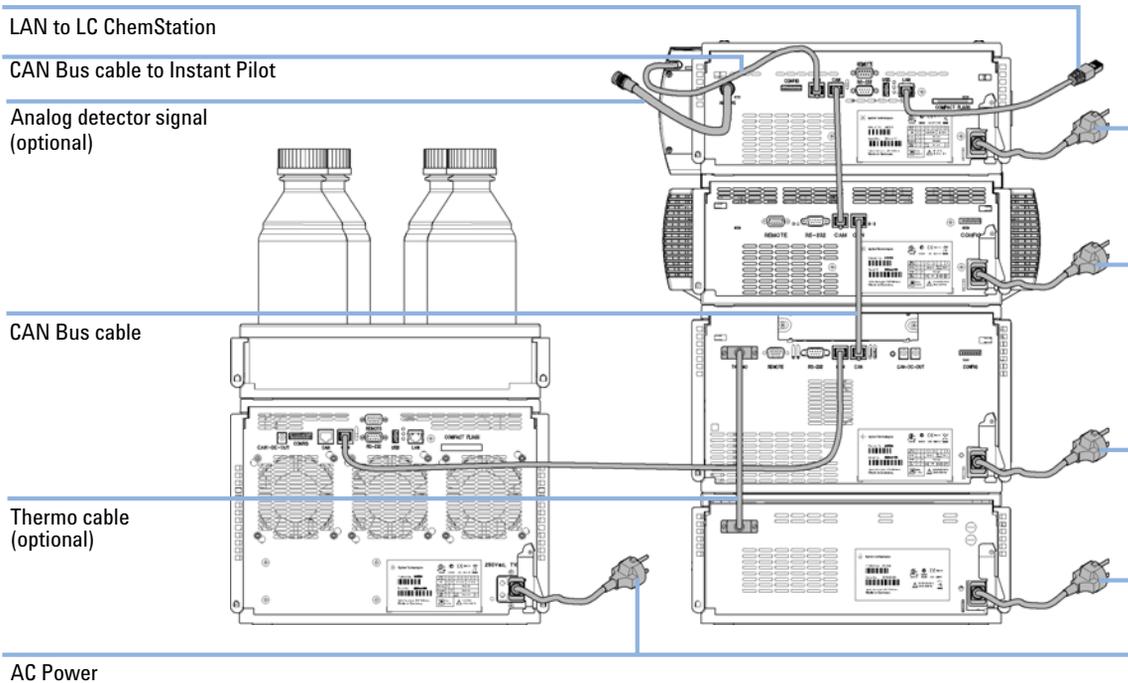


Figure 24 Recommended Two Stack Configuration (Rear View)

Priming the Pump

This procedure is required when...

- the pump is used for the first time
- whenever one or more of the inlet tubes contains air gaps or is dry for other reasons

The purpose of priming is to remove all air bubbles from the pump and its inlet tubes.

Propan-2-ol (alternative names: isopropanol, isopropyl alcohol, IPA) is the best solvent for priming the pump from dry and has the advantage of being miscible with most normal phase or reverse phase solvents (check a solvent miscibility table if unsure).

WARNING

Toxic and hazardous solvents

The handling of solvents and reagents can hold health risks.

- When opening capillary or tube fittings solvents may leak out.
- Please observe appropriate safety procedures (for example, 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

Avoid running the solvent reservoirs to emptiness by using the Bottle Filling monitor. Use a syringe to fill the solvent tubes, the pump will not pump air.

NOTE

Before starting the procedure ensure that all the solvent lines are connected to the pump as detailed in the pump installation manual, and that the waste tubes are safely positioned in a suitable waste solvent container. Ensure that the pump is controlled by computer software or the Instant Pilot controller and that the flow rate is set to zero.

- 1 Prepare channel A and pump head A for priming:
 - a Part fill each solvent reservoir with ca. 150 ml of HPLC grade propan-2-ol for priming the pump and place the sintered glass filter ends of the solvent tubing in the reservoirs.
 - b Disconnect the tubing entering the inlet check valve of pump head A. This is the outlet tubing from vacuum degasser channel A.
 - c Attach the priming syringe with threaded adaptor to the tubing.
 - d Slowly draw solvent through the tubing until all bubbles have been removed.
 - e Quickly remove the syringe and adaptor from the tubing and attach the tubing to the inlet check valve on pump head A.
- 2 Repeat previous step for solvent channel B and pump head B.
- 3 If a solvent selection valve (SSV) is installed in the pump, carry out the following steps for each of the remaining empty solvent tubes:
 - a Disconnect the empty tubing at the solvent select valve. Attach the priming syringe with threaded adaptor to the tubing.
 - b Slowly draw solvent through the tubing until all bubbles have been removed.
 - c Quickly remove the syringe and adaptor from the tubing and re-attach the tubing to the solvent selection valve.
- 4 In the ChemStation software, right-click on the pump section of the system diagram and then select **Prime On** from the context menu (do not confuse it with the **Purge On** command). Other controllers have a similar function.

The purge valve will now switch flow paths A and B to the waste position and will simultaneously prime both channels. The module draws solvent, at high speed with all four pump-drives simultaneously, and dispenses it to the waste position of the automatic purge valve. This is repeated 20 times and then the priming process ends. The purge valve switches the flow path back to the system.

It is recommended that each channel is now purged with 30 ml of propan-2-ol. Follow the procedure “[Purging the Pump](#)” on page 74.

Purging the Pump

Use this procedure...

- After the pump has been primed for the first time.
- When the pump is to be purged with fresh solvent before using the system, or when the solvent is to be exchanged for another.
- If the pump has been idle for a few hours or more (air may have diffused into the solvent lines and purging is recommended).
- If 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).

The inlet tubes to the pump should already be full of solvent. If the inlet tubes are partially or totally dry, follow the full priming procedure (see [“Priming the Pump”](#) on page 72).

The purge valve allows both pump heads to be connected to waste at the same time and to purge at their individual maximum flow rates of 5 ml/min, giving a total purge flow of 10 ml/min with 50/50 composition.

WARNING

Toxic and hazardous solvents

The handling of solvents and reagents can hold health risks.

- When opening capillary or tube fittings solvents may leak out.
 - Please observe appropriate safety procedures (for example, 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.
-

- 1 To access the setup page for controlling the purge valve, right-click on the pump section, and select **Control** from the context menu.

Alternatively, you can select **Instrument > More 1290 Infinity BinPump > Control**.

Control

Pump

On
 Off
 Standby

Seal Wash

Off
 Single Wash Duration min
 Periodic Period min
 on for min

Automatic Turn On

Turn on at Monday, July 06, 2009 11:00:00

Purge

On Duration min.
 Off Flow mL/min.
Composition A % B %

Prime

On
 Off

Ok Cancel Help

4 System Setup and Installation

Installing the Module

2 In the **Purge** section, set the following parameters:

- **Duration:** 6 min
- **Flow:** 10 ml/min
- **Composition B:** 50 %

Composition A will automatically assume 50 %. Leave the On/Off button set to **Off**. Click **OK** to exit.

3 Right-click on the pump section, and select **Purge On** from the context menu.

NOTE

Do not confuse **Purge On** with the next item **Prime On**.

The purge valve will now switch the flow path to the purge position, and will simultaneously purge both channels to waste at 5 ml/min each for 6 minutes duration. After the set duration, the purge flow will turn off, the valve will switch the flow path back to the system, and the flow rate and composition currently set in the method will resume. In this example, the method flow is still set to zero. The purge settings in the **Control** page from steps 1 and 2 remain configured so when the purge process is required again it can be started at step 3.

When the pump has been initially primed and purged with propan-2-ol, the solvents can be exchanged for the mobile phase solvents such as water and methanol. The purge procedure is repeated whenever a solvent is exchanged. Immediately following the purge, the solvent in the pump has not been degassed and so the system should be operated for at least 10 min so that the solvent is degassed.

Flow Connections Between Modules

When connecting the modules, always flush each capillary and the column with solvent before connecting to the next component in the flow path.

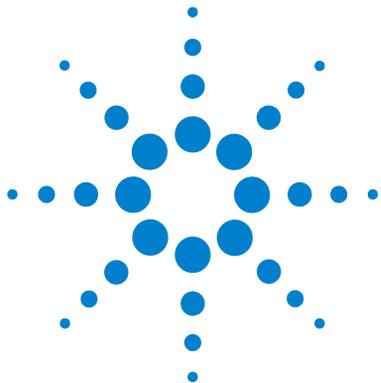
- 1 Connect the outlet from the Jet Weaver mixer to the autosampler using a 0.12 mm i.d. stainless steel flexible capillary (color code label is red). This should be connected to port #1 on the autosampler injection valve.
- 2 Connect a 0.12 mm i.d. stainless steel flexible capillary from port #6 on the autosampler injection valve to the thermostatted column compartment. Connect the capillary direct to the low dispersion volume heat exchanger for minimal delay volume or to the switching valve if installed.
- 3 Connect the outlet of the column to the inlet (marked CELL-IN, left-hand side connection) of the Max-Light Cartridge Cell in the 1290 Infinity detector
- 4 Connect the waste tubing to the outlet (marked CELL-OUT, right-hand side connection) of the Max-Light Cartridge Cell in the 1290 Infinity detector and place the outlet in an appropriate waste collection container.

Integration Into the Network

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

4 System Setup and Installation

Installing the Module



5 Quick Start Guide

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This chapter provides information on data acquisition and data analysis with the 1290 Infinity LC System.



About the Quick Start Guide

This chapter provides information on running the Agilent 1290 Infinity LC system. 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 column and checkout sample provided with the Agilent 1290 Infinity LC system. The example refers to ChemStation menus and commands, but identical functions are also available on the alternative control options including the Instant Pilot controller, EZChrom, and MassHunter software.

NOTE

The starting point assumes that the system has been installed, turned on and initially primed (see [“Priming the Pump”](#) on page 72). 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 ChemStation > Agilent ChemStation Instrument 1 Online**. As more than one instrument system may be connected to the computer, the number (1, 2, ...) indicates the system number.

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.

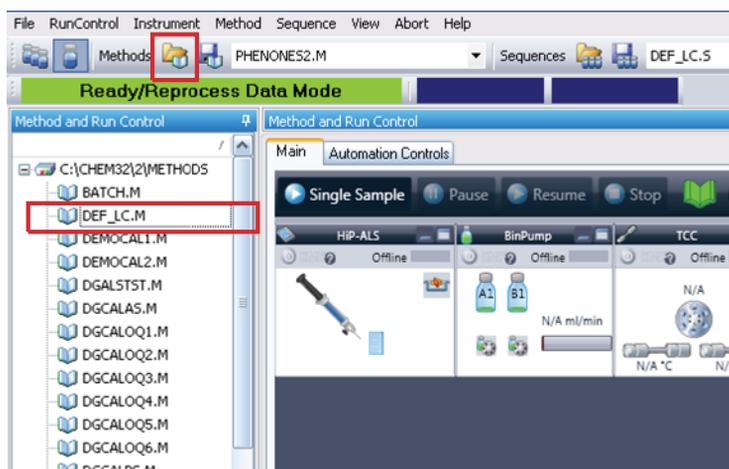
Loading the Default Method

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.



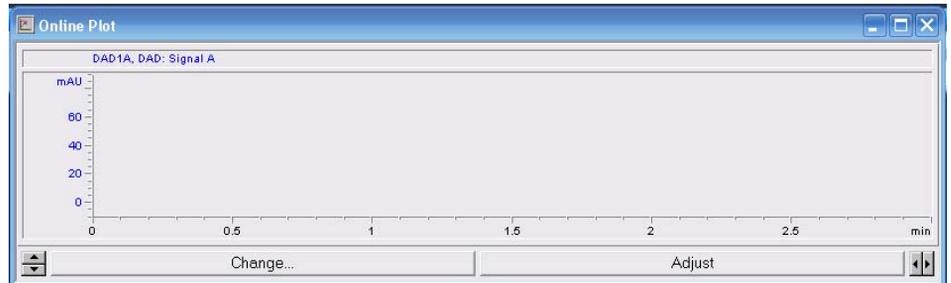
The default method (**DEF_LC.M**) has a set of default parameters which can then be modified to create a new method. For instance, the flow rate is set to zero, and the **Method Information** and **Method History** are blank.

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.

The screenshot shows the 'Edit Signal Plot' dialog box. It is divided into several sections:

- Available Signals:** A list box containing: PMP1B, BinPump: Pressure; PMP1C, BinPump: Flow; PMP1D, BinPump: Solvent Ratio A; PMP1E, BinPump: Solvent Ratio B; THM1A, TCC: Left Temperature; THM1B, TCC: Right Temperature; DAD1B, DAD: Signal B (highlighted); DAD1C, DAD: Signal C.
- Selected Signals:** A list box containing: DAD1A, DAD: Signal A.
- Buttons:** 'Add ->' and '<- Remove'.
- Window:** 'x-axis' set to 3 min; 'draw zero line' checkbox is unchecked.
- DAD1A, DAD: Signal A:** 'Type: acquired'; 'y-axis range: 100 mAU'; 'auto y-adjust' checkbox is unchecked; 'Offset: 10 %'.
- Fraction Collector:** 'Show fraction collection ticks' checkbox is unchecked.
- Method Settings:** 'Use method settings' checkbox is unchecked; 'Apply to Method' button.
- Bottom Buttons:** 'OK', 'Cancel', and 'Help'.

- 3 In the **Available Signals** box, highlight the required signal(s), and click **Add** to move them to the **Selected Signals** box.

5 Quick Start Guide

Preparing the system

- 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 “[Purging the Pump](#)” on page 74.

Data Acquisition in Method and Run Control View

Method Parameters for Test Mixture and ZORBAX RRHD Column

The 1290 Infinity LC System is supplied with a ZORBAX RRHD Eclipse Plus C18 1.8 μm , 2.1 mm x 50 mm, column and a test mixture containing phenones for use with this example procedure.

Phenones Test Mix (part number 5188-6529) contains 100 ng/ μl each of nine components dissolved in water / acetonitrile (65/35). The nine components are:

- Acetanilide
- Acetophenone
- Propiophenone
- Butyrophenone
- Benzophenone
- Valerophenone
- Hexanophenone
- Heptanophenone
- Octanophenone

The method parameters for separation of this test mixture are summarized in [Table 7](#) on page 87.

Table 7 Method parameters for first separation run

Module	Parameter	Setting
Pump	Solvent A	Water
	Solvent B	Acetonitrile
	Flow rate	0.4 ml/min
	Initial Composition	60 % A, 40 % B
	Gradient Timetable	At 4 minutes 20 % A, 80 % B
	Stop Time	5 minutes
Autosampler	Injection	1 µl
	Needle wash	Flush port, 6 s
TCC	Column	ZORBAX Eclipse Plus C18 1.8 µm, 2.1 mm x 50 mm i.d.
	Temperature	40 °C
Detector	Signal A	250 nm, bw 100 nm, ref 360 nm, bw 100 nm
	Peak width	0.025 min (10 Hz)
	Store Spectra	All

Setting Up the Method

This section shows how to quickly set the method conditions for an analysis using the test mixture conditions. For a more detailed explanation of all the available parameters see Appendix, “Setting Up a Method using Edit Entire Method” on page 105.

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. For the example separation discussed here, set the conditions listed in [Table 7](#) on page 87.

- 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 **1290 Infinity Binary Pump**, enter the following parameters:
 - Flow rate: 0.4 ml/min
 - Solvent A: Select **Water** from the compressibility drop-down list.
 - Solvent B: Select the check box to make Solvent B active.
 - %B: Initial value 40 %
 - Stop Time: 5 min
 - Max Pressure Limit: 1200 bar
 - b Click the + sign to open the **Timetable**.
 - c Add a line, select **Change Solvent Composition**, and set %B to 80 %
 - d Other parameters can remain at 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 **1290 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 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 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 **1290 Infinity DAD**, enter the following parameters:

- **Use Signal:** Turn all signals except **Signal A** off by clearing the check boxes.
- Signal A: 250 nm, bw 100 nm, ref 360 nm, bw 100 nm
- Peak width: 0.012 min (0.25 s Response, 20 Hz)

- b** In the **Advanced** section, set **Spectrum Store** to **All**.

- c** Other parameters can remain at 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...** to save the method with a new name.

The ChemStation will 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 test mix 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 method parameters that were used for the analysis in the data file as ACQ.M (acquisition method) so there is no doubt about the actual conditions used.

- 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 a **Subdirectory** name (optional), **Filename**, **Sample Location** (vial 1), **Sample Name** and any **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, click the **Start Single Sample** button above the system diagram.
- 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 [Figure 25](#) on page 91 – the time axis will be longer, however, as this was run under four times faster conditions in the next section.

Running the method faster

The first exercise ran at a pressure achievable in a standard system. Now the flow rate is increased and the gradient adjusted for a faster separation.

- 1 Edit the method conditions in the same way as in the previous section to make the following changes:
 - Flow rate: 1.6 ml/min
 - Gradient: Change the gradient so that the gradient slope is unchanged in terms of volume compared to the first run. Flow has been increased by 4x therefore reduce the gradient time by 4x; set the gradient time to 1 min.
 - Stop Time: 1.25 min
- 2 Save the method with a new name.
- 3 Ensure that in **Sample Info** a new filename is used.
- 4 When the baseline is stable and equilibrated, start the run using the **Start Single Sample** button.

The chromatogram should look similar to the one shown below, with a run time of about 1 min.

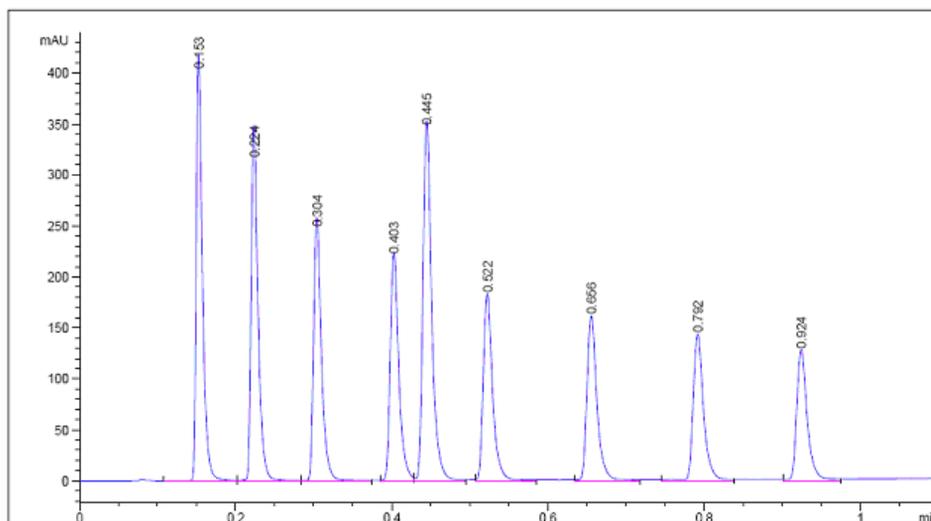


Figure 25 Example chromatogram of the phenones test mixture using Quick Start conditions

5 Quick Start Guide

Data Acquisition in Method and Run Control View

This separation is actually not optimized with these conditions and the user may like to gain further experience with running the system by trying to optimize the method further. Some changes that may help are:

- Reduce the concentration of the sample by diluting 1:10.
- Increase the range of the gradient.
- Increase the temperature.
- Examine the spectra of the peaks and select appropriate narrow band detection.

For more details on optimization, please refer to [“Optimization of the Agilent 1290 Infinity LC System”](#) on page 35.

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.

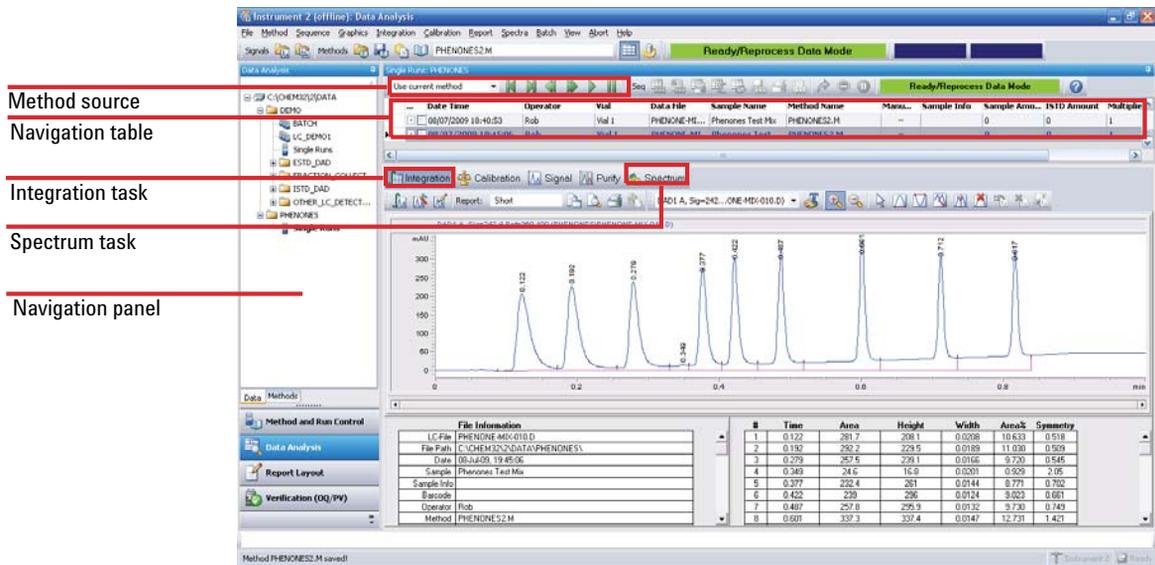


Figure 26 Data Analysis view

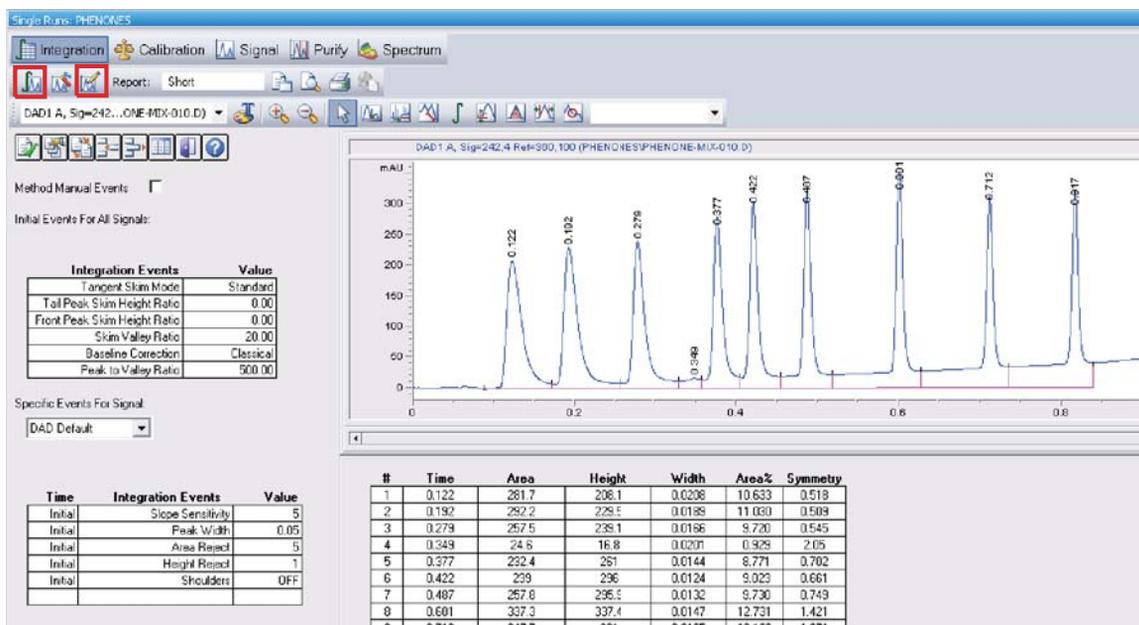
Data Analysis View

To open a chromatogram in the Data Analysis view:

- 1** Open the offline copy of the ChemStation.
- 2** Click Data Analysis in the bottom left of the screen (see [Figure 26](#) on page 93).
- 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.

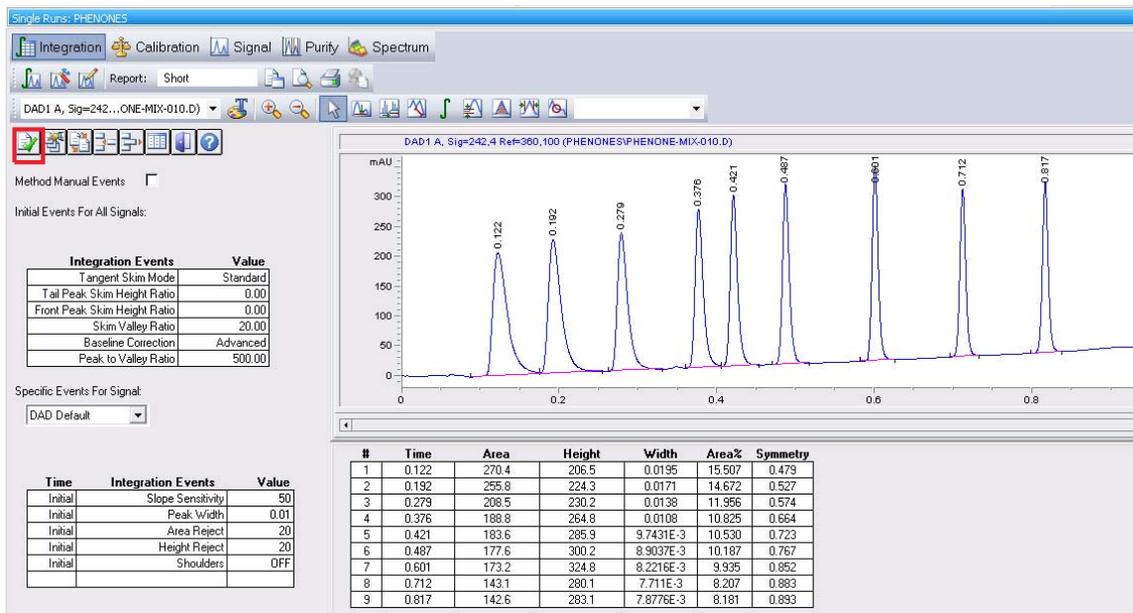
Integrating a Signal

- 1 Set the method source to **Use Current Method**.
- 2 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.



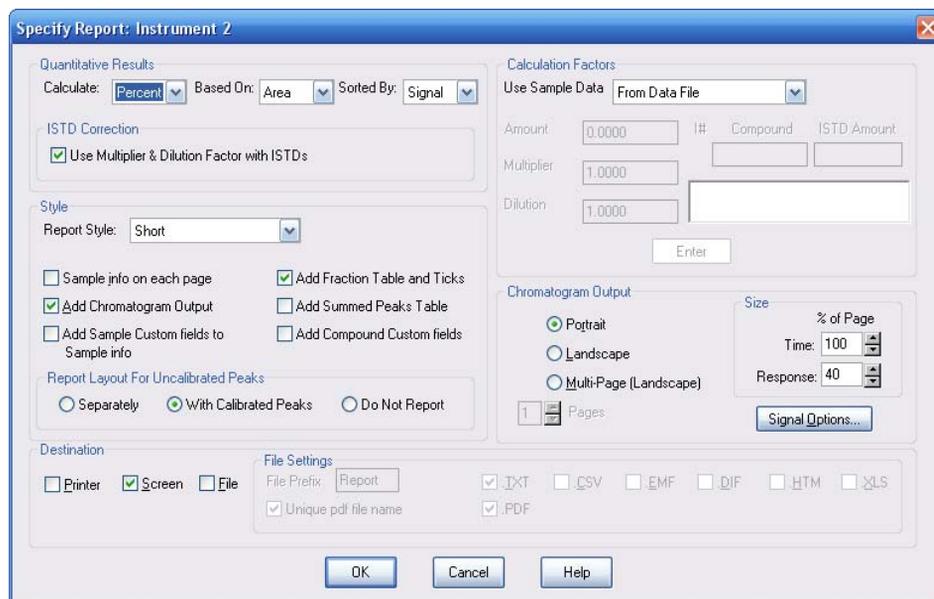
- 3 Click the **Set Integration Events Table** icon to open the table as shown.
- 4 Set **Baseline Correction** to **Advanced** for gradient runs.
- 5 Set **Slope Sensitivity** to 50. Higher numbers will integrate steeper peaks and ignore less steep peaks.
- 6 Set the **Peak Width** value to the narrowest peak of interest, approximately 0.01 in this case.
- 7 **Area Reject** and **Height Reject** can be set to reject the smallest peaks.
- 8 Click the **Integrate** icon to update the results using these new settings.

9 Exit from the Events Table using the green tick icon (see figure below).



Specify the Report

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

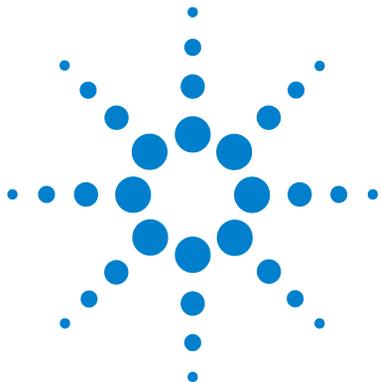


- 2 The default settings are acceptable to produce an Area Percent report on the screen.
- 3 In the **Destination** section, select **Printer** for a paper copy, and select **File** and **PDF** to obtain a useful PDF report file stored into the datafile (the data file with .D suffix is actually a directory, and the PDF report can be found there 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.

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Data Analysis



6 Appendix

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General 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 please observe appropriate safety procedures (e.g. 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 8 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

Flow Cell

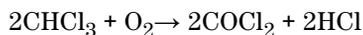
To protect optimal functionality of your flow-cell:

- Avoid the use of alkaline solutions (pH > 9.5) which can attack quartz and thus impair the optical properties of the flow cell.

Use of Solvents

Observe the following recommendations on the use of solvents.

- Brown glass ware can avoid growth of algae.
- Avoid the use of the following steel-corrosive solvents:
 - Solutions of alkali halides and their respective acids (for example, lithium iodide, potassium chloride, and so on),
 - High concentrations of inorganic acids like sulfuric acid and nitric acid, especially at higher temperatures (if your chromatography method allows, replace 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,
- Solvents containing strong complexing agents (e.g. EDTA),
- Mixtures of carbon tetrachloride with 2-propanol or THF.

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

<http://www.agilent.com>

Select Products/Chemical Analysis

It will provide also the latest firmware of the modules for download.

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 27 Check Method Sections to Edit

6 Appendix

Setting Up a Method using Edit Entire Method

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

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.

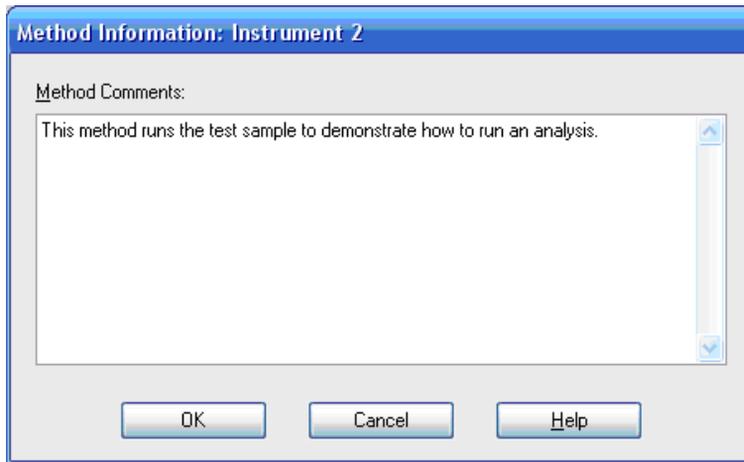


Figure 28 Method Information

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 (HiP-ALS)

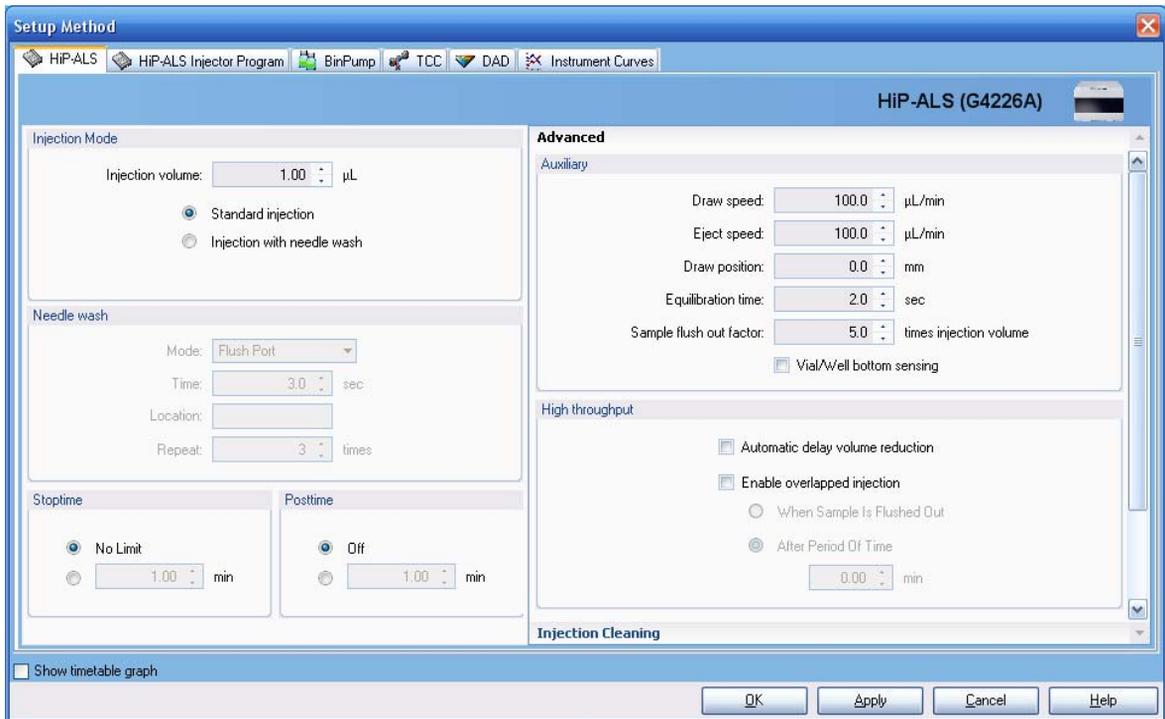


Figure 29 Setup Method screen –High Performance Autosampler tab

- **Injection Mode**

- **Injection volume** sets the volume to be injected (example 3 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 ($<x$ ml).
 - **Eject speed** is the speed of delivery from the needle.
 - **Draw position** is the vertical offset from the nominal injection position of 10 mm above the bottom of a vial. This is about half-way up a 2 ml vial so should be given a negative offset to take the sample from near the bottom of the vial, for example, a value of -7 mm would place the tip of the needle 3 mm above the bottom of the vial.
 - **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 1 mm. 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 μ 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

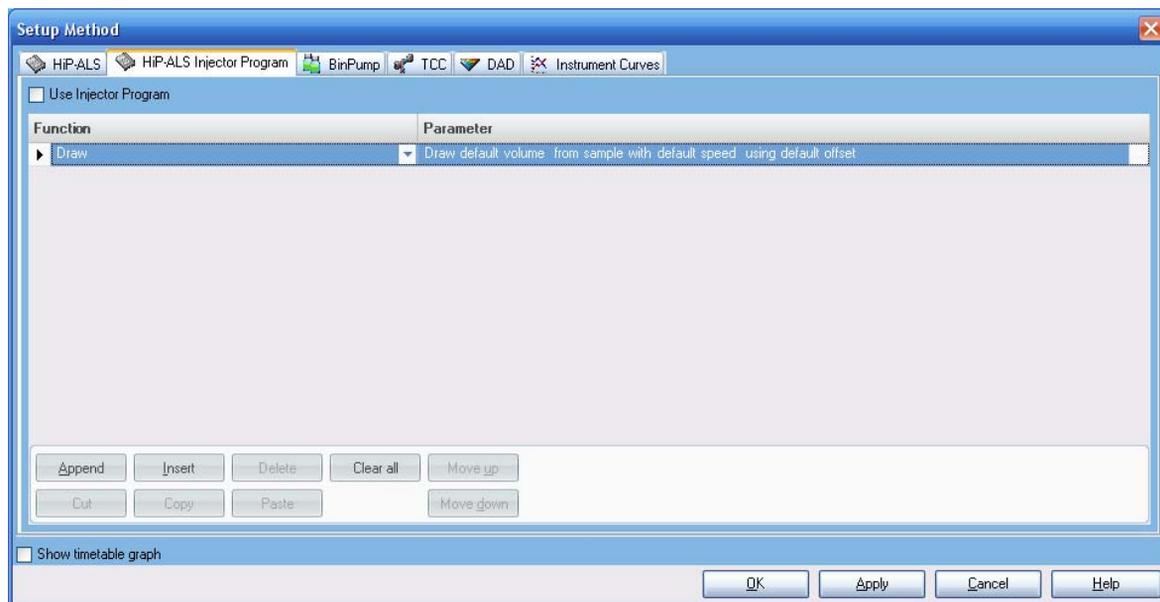


Figure 30 Setup Method screen - HiP Autosampler Injector Program tab

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 1290 Infinity Autosampler manual*.

Binary Pump (BinPump) tab

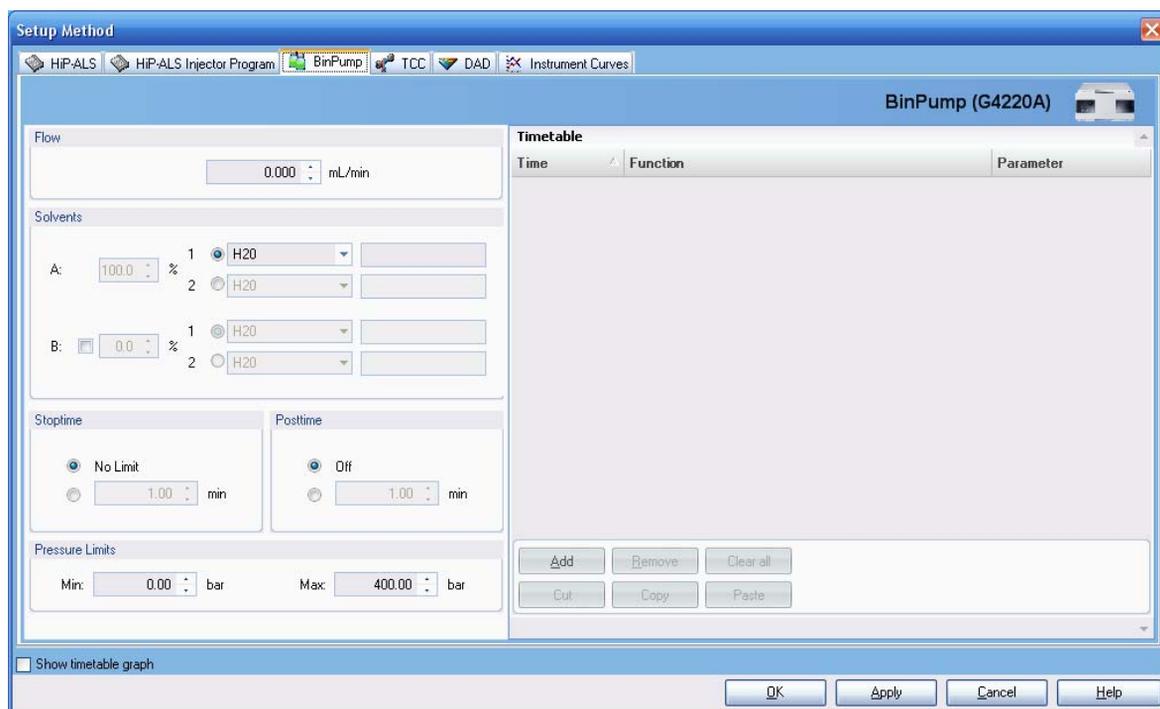


Figure 31 Setup Method screen – Binary Pump tab

- **Flow** sets the flow rate up to 5 ml/min. For the example separation 0.4 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 but 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 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 “[How to Configure the Optimum Delay Volume](#)” on page 38. A second text box allows a description of the mobile phase to be entered. If the solvent selection valve is installed in the pump then 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 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 methanol at 50 %. A will be 50 %.

- **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 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 setup a simple gradient for the example separation, first clear the timetable with the **Clear all** button if it is not already empty, add a line at 4.00 min to change composition to 90 %. The graphical display will show a linear gradient from 50 % B to 90 % B over 4 min. 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, that 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 1290 Infinity pump is 1200 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 reached and 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 RRHD columns are suitable for operation at 1200 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 bar is typical.

Thermostatted Column Compartment (TCC) tab

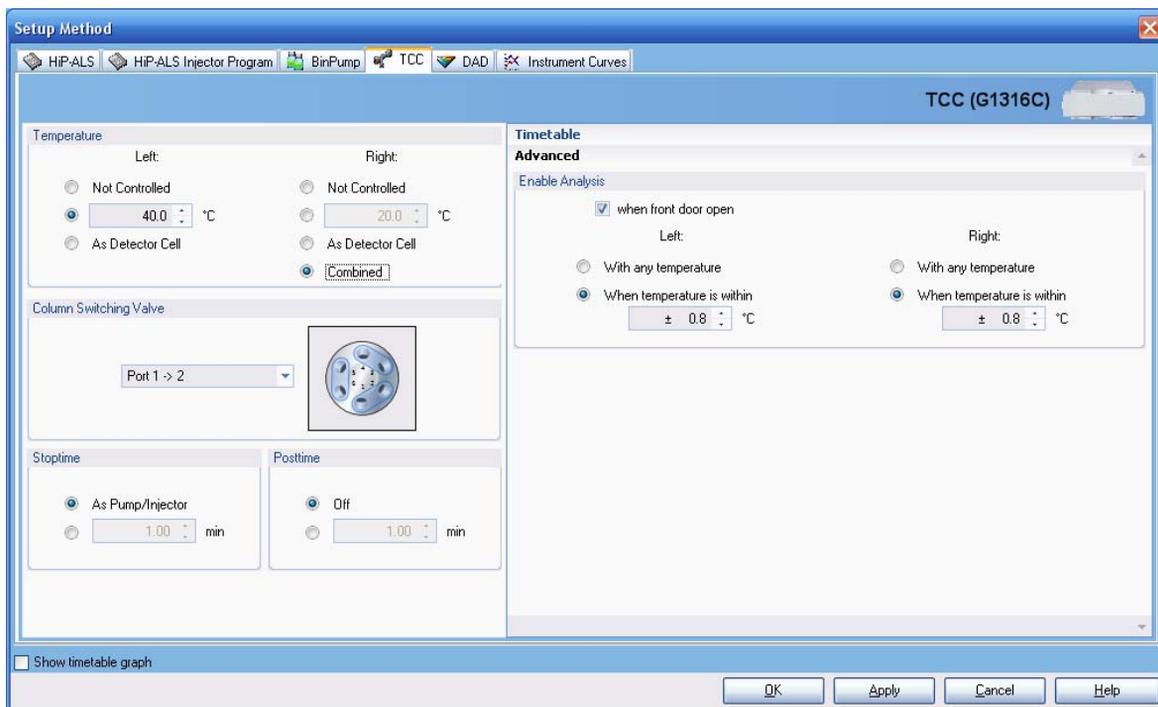


Figure 32 Setup Method screen – Thermostatted Column Compartment tab

- **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 1290 Infinity Detector.

The temperature of each zone can be set from $-5\text{ }^{\circ}\text{C}$ to $100\text{ }^{\circ}\text{C}$ and the user should check that the column is suitable for operation at that temperature. (Agilent ZORBAX RRHD and RRHT StableBond phases can be used at the higher end of the range). The temperature is controlled to $\pm 0.15\text{ }^{\circ}\text{C}$ down to $10\text{ }^{\circ}\text{C}$ below ambient although it should be noted that there are very few applications operating below $12\text{-}15\text{ }^{\circ}\text{C}$. 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 (DAD) tab

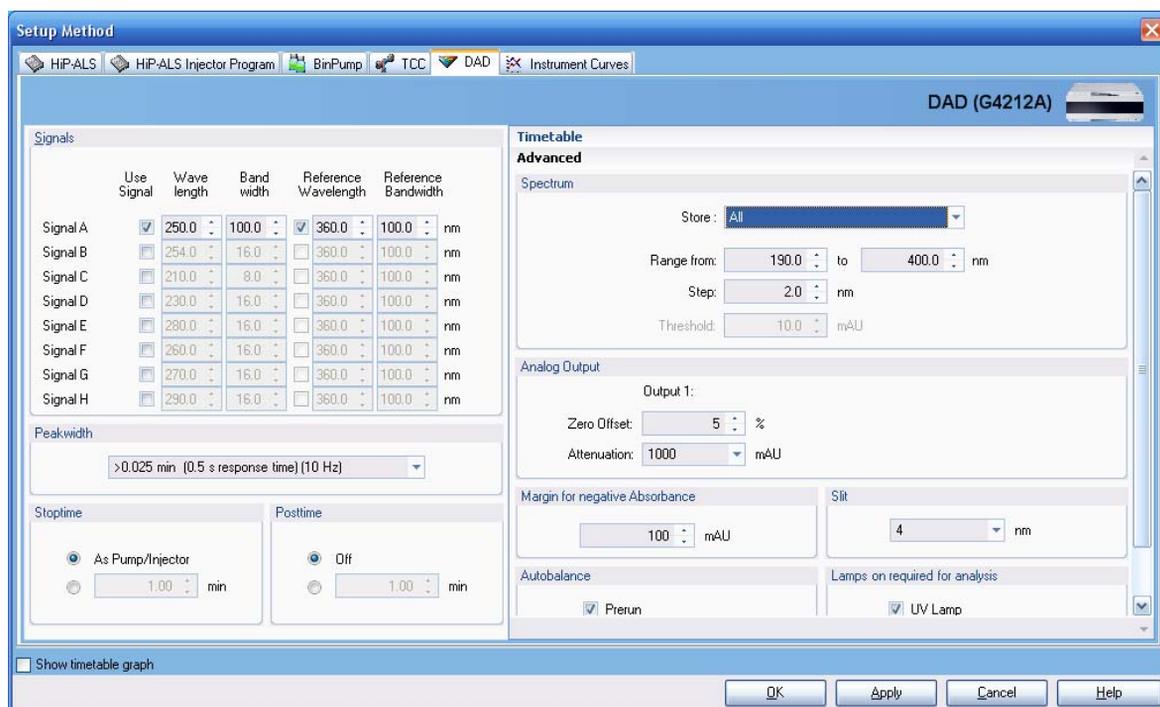


Figure 33 Setup Method screen – Diode-Array Detector tab

- **Signals:** Up to eight separate signals (chromatograms) can be recorded. To mark a signal for collection the **Use Signal** box is checked for that signal, the wavelength and bandwidth are defined, and if a reference signal is required that box is checked and defined.
 - **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.
- **Stop Time / Post Time** are set to **No Limit / Off** and these values are usually taken care of in the **Pump** tab. However, the detector module Stop Time can sometimes differ from the pump Stop Time when it is required that data analysis stops before the end of the run 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 so 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 Times to **As Pump**.

- **Timetable** operates in the same way as other modules, that is, 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 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 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 peak spectra are available for all the different wavelength signals.

- **Store** controls the spectral collection mode with the following options:
 - None** – no spectra stored,
 - Apex+Baselines** – 3 spectra taken at start, apex and end of peak,
 - Apex+Slopes+Baselines** – 5 spectra taken at 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 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 chosen as appropriate by the user. (This also reduces the number of data to be stored).
- **Step** – controls the interval (nm) of the data 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 1290 Infinity detector 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 that 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 - Slit**

The entrance slit to the spectrograph controls the spectral resolution and influences the baseline noise and sensitivity. The default setting is 4 nm, which is suitable for most applications. See [“How to Achieve Higher Sensitivity”](#) on page 53 for further discussion of this parameter.

- **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 analysis:** the 1290 Infinity DAD or MWD has one deuterium lamp and it needs to be on for analysis so this box should be checked.

Instrument Curves Tab

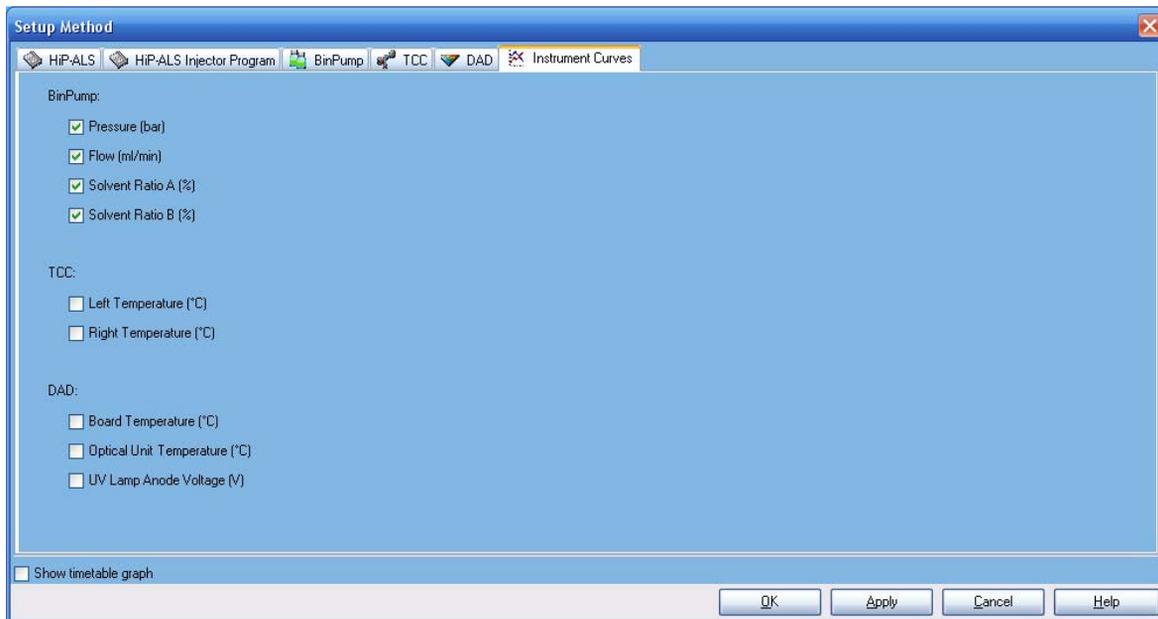


Figure 34 Setup Method screen – Instrument Curves tab

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 35 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.

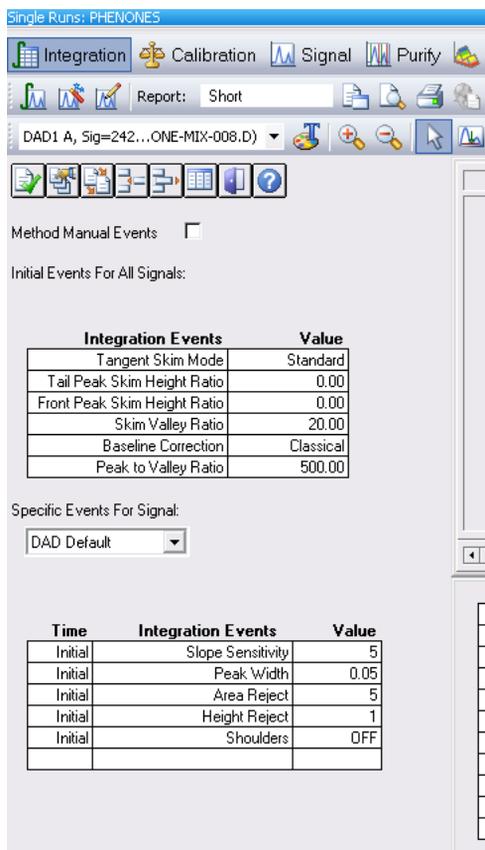


Figure 36 Edit Integration Events Screen

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.

Specify Report

Specify Report: Instrument 2

Quantitative Results
Calculate: Based On: Sorted By:

ISTD Correction
 Use Multiplier & Dilution Factor with ISTDs

Style
Report Style:

Sample info on each page Add Fraction Table and Ticks
 Add Chromatogram Output Add Summed Peaks Table
 Add Sample Custom fields to Sample info Add Compound Custom fields

Report Layout For Uncalibrated Peaks
 Separately With Calibrated Peaks Do Not Report

Calculation Factors
Use Sample Data:

Amount	#	Compound	ISTD Amount
<input type="text" value="0.0000"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Multiplier: <input type="text" value="1.0000"/>			
Dilution: <input type="text" value="1.0000"/>			

Chromatogram Output
 Portrait Landscape Multi-Page (Landscape)

Size: % of Page
Time:
Response:

Pages

Destination
 Printer Screen File

File Settings
File Prefix:
 Unique pdf file name

.TXT .CSV .EMF .DIF .HTM .XLS
 .PDF

Figure 37 Specify Report Screen

6 Appendix

Setting Up a Method using Edit Entire Method

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

- **Quantitative Results**
 - **Calculate:** Percentage
 - **Based on:** Area
 - **Sorted by:** Signal
- **Style**
 - **Report Style:** Short
 - **Add Chromatogram Output:** Checked
- **Chromatogram Output:** Portrait
- **Size:**
 - **Time axis** 100 % of page
 - **Response axis** 40 % of page
- **Destination**
 - **Printer:** Checked
 - **Screen:** Unchecked
 - **File:** Checked
- **File Settings:**
 - **PDF:** Checked
 - **Unique PDF Name:** Checked

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

Instrument Curves

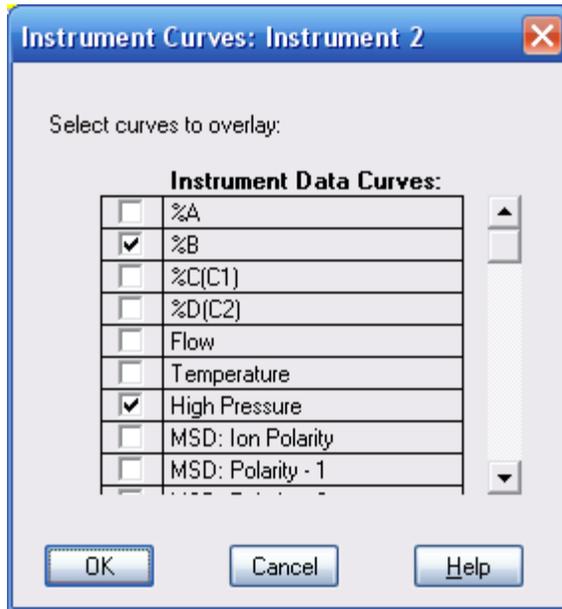


Figure 38 Instrument Curves screen

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.

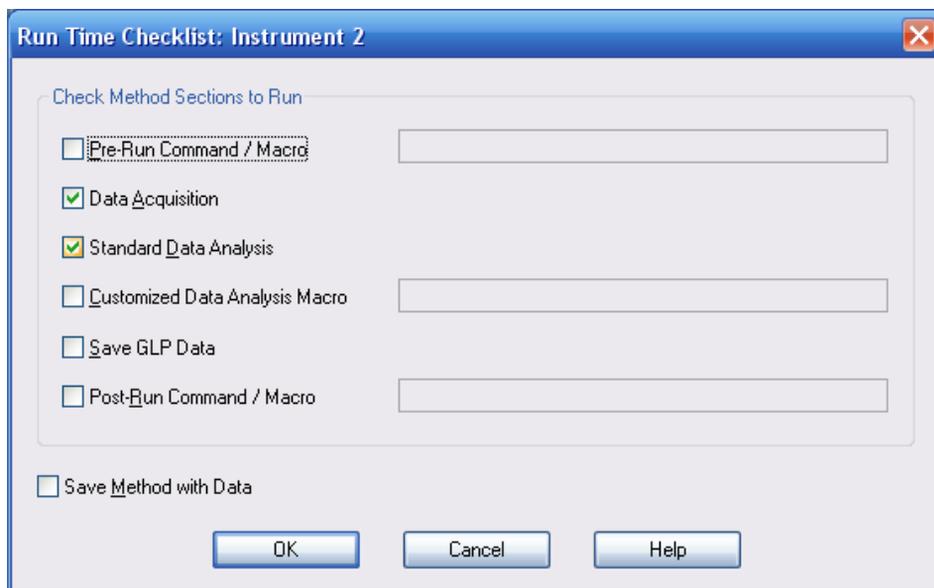


Figure 39 Run Time Checklist Screen

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 technical reference information about the Agilent 1290 Infinity LC System.

The manual describes the following:

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

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