Technical Overview

Pharma/Biopharma

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Trusted Answers

Accurate, Robust, and Accelerated Analytical LC Method Development with the Agilent InfinityLab LC Solutions

Authors

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Abstract

Agilent InfinityLab LC Solutions have advanced instruments, columns, and solutions for developing stability-indicating analytical methods that demonstrate the quality, safety, and efficacy of pharmaceutical products. A stability-indicating analytical method is important for the identification and quantification of components and impurities. This technical overview explains, step-by-step, the procedure for developing a stability-indicating method with the help of Agilent InfinityLab LC Solutions.

Introduction

Pharmaceutical products can have different types of impurities (organic and genotoxic) affecting their chemical, toxicological, and pharmacological properties. The identification and quantification of impurities require suitable analytical methods to determine the expiration date of drug products. If the analysis of impurities is not conducted using an appropriate analytical method, the quality of the product remains uncertain. Moreover, these analytical methods play an important role in establishing the expiration date, without which consumers are left unaware of the drug's safe consumption period. A stability-indicating analytical method is important for the identification and quantification of components and impurities to demonstrate the quality, safety, and efficacy of pharmaceutical products. The purpose of stability testing using a stability-indicating analytical method is to provide evidence of how the quality of a drug substance or drug product changes over time under various environmental conditions such as temperature, humidity, and light. Additionally, it aims to establish a retest period for the drug substance or a shelf life for the drug product, along with recommended storage conditions. InfinityLab LC Solutions offer advanced instruments, columns, and solutions for developing stability-indicating analytical methods to demonstrate the quality, safety, and efficacy of pharmaceutical products.

Step-by-step procedure for method development

Step 1: Survey the literature

Begin by conducting a literature survey to understand the solubility, analytical, and stability profiles of the drug substance or product.

Solubility profile: Gather solubility information in various solvents. These data are crucial for selecting appropriate diluents for preparing standard and test solutions.

Analytical profile: Clarify the absorption characteristics of the drug. This helps in choosing the right detector and determining the optimal wavelengths for analysis.

Degradation and metabolic pathways: Understand the degradation profile and metabolic pathways. This knowledge aids in developing methods to separate and estimate all possible impurities and degradants.

Stability profile: Study the stability profile of the drug substance or product. This information is essential for determining suitable storage conditions and adopting adequate precautions when handling drug solutions.

1220
Infinity II LC

Affordable efficiency

The Agilent 1220 Infinity II LC is an affordable, high-quality integrated LC system, putting you on the fast track to efficiency.

1260
Infinity II LC

Everyday efficiency in every way

The Agilent 1260 Infinity II LC is the trusted platform with the broadest instrument choice, taking you to the next level of efficiency.

1260
Infinity II Prime LC

The most convenient LC for routine analysis

The 1260 Infinity II Prime LC is the most capable and comfortable LC within the 1260 Infinity II LC portfolio.

1290
Infinity II LC

The benchmark in efficiency

The Agilent 1290 Infinity II LC embodies the next generation of LC, giving you the ultra-high performance to achieve maximum efficiency.

Figure 1. Agilent InfinityLab LC Series instruments.

Step 2: Determine the analytical techniques

HPLC Instrument

High-performance liquid chromatography (HPLC) is a routine tool for separating, identifying, and quantifying complex mixtures.

- **Separation mechanism:** Based on the affinity of compounds to the mobile phase (eluent) and the stationary phase.
- **Applications:** Extensively used in pharmaceutical, bioanalytical, food and beverage, clinical, forensic, environmental, and drug development laboratories.
- **Agilent InfinityLab:** Offers four different high-performance liquid chromatography models.
  - 1220 Infinity II LC
  - 1260 Infinity II LC
  - 1260 Infinity II Prime LC
  - 1290 Infinity II LC

Step 3: Select and optimize the mobile phase

The primary objective in selecting and optimizing the mobile phase is to achieve optimal separation of all impurities and degradants from each other and from the main peak. The following parameters should be considered when selecting and optimizing the mobile phase.

1. The buffer and its strength
2. The pH of the buffer or pH of the mobile phase
3. The mobile phase composition

1. The buffer and its strength

- Crucial for determining peak symmetries and separations. Various buffers, such as phosphate, acetate, triethylamine, trifluoracetic acid, formic acid, and ion pair reagents, can be used to achieve the required separations.
- Selected based on their pH and UV cutoff. It is important to use buffers with suitable strength to handle the injection load on the column, as inadequate strength can cause peak tailing.
- The strength of the buffer influences retention times, with molar strength being inversely proportional to retention times. Ideally, buffer strength should be between 0.005 and 0.20 M.
- The selection should always be done in combination with the selection of the organic phase composition in the mobile phase.
- Buffer strength can be increased to achieve the required separations, but care must be taken to avoid precipitation in the mobile phase.
- After selection, the buffer strength can be varied by approximately 10 to 15% to study the effect of variation.

2. The pH of the buffer or pH of the mobile phase

pH plays an important role in achieving chromatographic separation as it affects the elution properties by controlling the ionization characteristics. In reversed-phase HPLC, the retention of analytes is related to their hydrophobicity; the more hydrophobic the analyte, the longer it is retained.

- **Effect of pH on acids:** With increasing pH, acids lose a proton and become ionized, making them less hydrophobic and decreasing their retention.
- **Effect of pH on bases:** With decreasing pH, bases gain a proton and become ionized, making them less hydrophobic and decreasing their retention time.

It is important to maintain the pH of the mobile phase between 2.0 and 8.0, as most columns cannot withstand pH levels outside of this range. Below pH 2.0, siloxane linkages are cleaved, while above pH 8.0, silica may dissolve. Agilent offers a wide range of pH columns that can withstand values even above pH 8.0.

The pH of the buffer that provides separation of all individual impurities and degradants from each other and from the main peak should be selected. The pH can be varied by approximately ±0.2 from the selected value to study the effect of variation.

3. Mobile phase composition

In reversed-phase chromatography, the separation is mainly controlled by the hydrophobic interactions between drug molecules and the alkyl chains on the column packing materials. Optimal separations can be achieved by choosing the appropriate mobile phase composition. Acetonitrile and methanol are the best organic solvents, with tetrahydrofuran (THF) being used occasionally. These three solvents are widely used to control selectivity and separations. However, THF has some disadvantages, such as higher UV absorbance, reactivity with oxygen, and slower column equilibration. Despite these drawbacks, THF can provide unique selectivity for closely eluting peaks.

- **Order of polarity:** Methanol > acetonitrile > ethanol > THF > propanol
- **Order of solvent strength:** Propanol > THF > ethanol > acetonitrile > methanol

Step 4: Selection of column

Choose the bonding phase based on the polarity of the molecule. Select a bonding phase with similar polarity as that of analytes. For reversed-phase chromatography, a wide variety of columns are available covering a range of polarity by cross-linking the Si-OH groups with alkyl chains (C6, C8, C18, nitrile, phenyl, and amino groups). The following parameters should be considered when choosing a column for the separation of impurities and degradants.

- Length and diameter of the column
- Packing material
- Shape of the particles
- Size of the particles
- Percentage of carbon loading
- Pore volume
- Surface area
- End capping

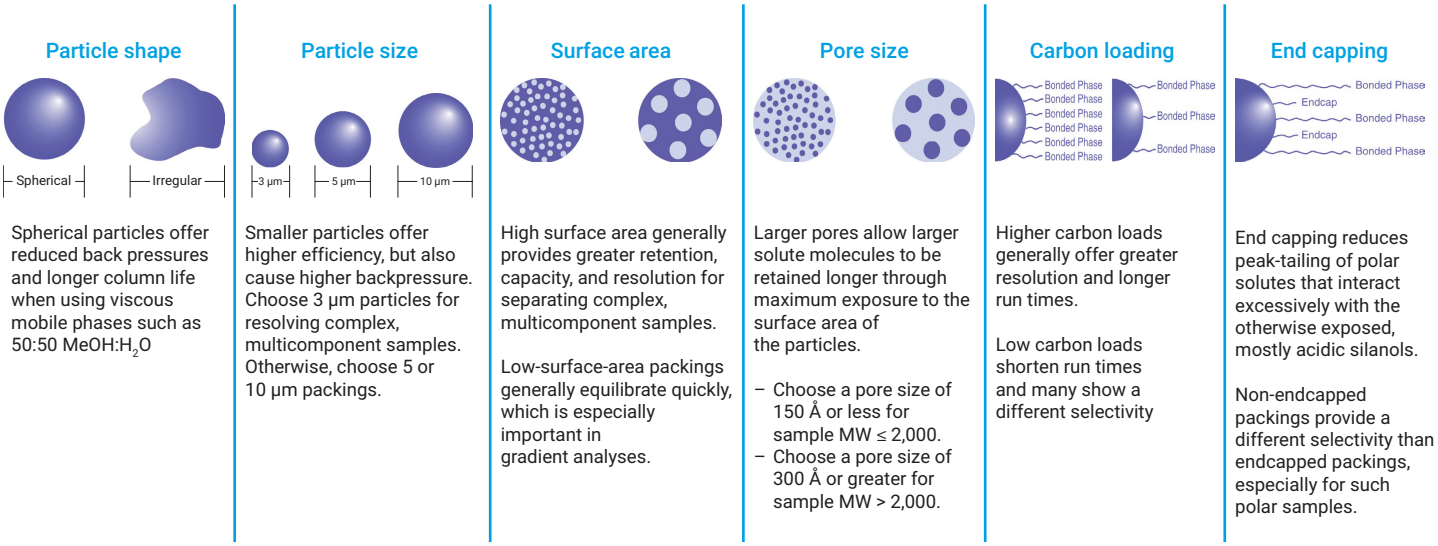


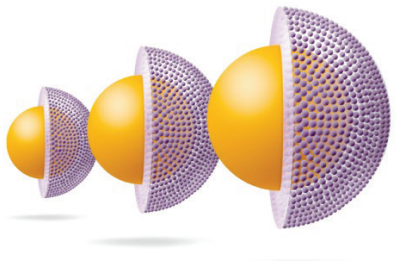
Figure 2. Parameters to consider when choosing the column.

Agilent InfinityLab Poroshell 120 columns are based on superficially porous particle technology, featuring a solid silica core and a porous outer layer. Compared to traditional totally porous particles of the same (or similar) size, Poroshell particles deliver higher chromatographic efficiencies and enable fast, high-resolution separations.

InfinityLab Poroshell 120 columns are available in three particle sizes to fit all your separation needs. These particles provide several advantages over totally porous particles.

- **Uniform particles:** Smooth surfaces ensure smaller particle size distributions.
- **Short diffusion path:** Analytes have a shorter diffusion path in and out of the porous layer.
- **Narrow particle size distribution:** Allows for more uniform packing of column particles.

With 18 different chemistries, these columns offer a range of selectivity, making method development fast and easy (Table 1).



1.9 µm	Highest UHPLC performance
2.7 µm	UHPLC performance at lower pressures
4 µm	Improved HPLC performance

System	Maximum Pressure (Bar)	Typical LC Instrument
UHPLC (Very Low Dispersion)	600 to 1,000+	Agilent 1290 Infinity II
UHPLC (Moderate-Low Dispersion)	600 to 1,000	– Agilent 1260 Infinity II – Agilent 1260 Infinity II Prime
HPLC	400 to 600	Agilent 1220 Infinity II

Figure 3. Agilent InfinityLab Poroshell 120 columns.

Table 1. Agilent column chemistries by application.

Best All Around	Best for Low-pH Mobile Phases	Best for High-pH Mobile Phases	Best for Polar Compounds (HILIC)	Best for Alternative Selectivity	Best for Chiral Separations
– **EC-C18** 1.9, 2.7, 4 µm – **EC-C8** 1.9, 2.7, 4 µm	– **SB-C18** 1.9, 2.7, 4 µm – **SB-C8** 2.7 µm	– **HPH-C18** 1.9, 2.7, 4 µm – **HPH-C8** 2.7, 4 µm	– **HILIC** 1.9, 2.7, 4 µm – **HILIC-Z** 1.9, 2.7, 4 µm – **HILIC-OH5** 2.7 µm	– **Bonus-RP** 2.7 µm – **PFP** 1.9, 2.7, 4 µm – **Phenyl-Hexyl** 1.9, 2.7, 4 µm – **SB-Aq** 2.7 µm – **EC-CN** 2.7 µm	– **Chiral-T** 2.7 µm – **Chiral-V** 2.7 µm – **Chiral-CD** 2.7 µm – **Chiral-CF** 2.7 µm

Step 5: Selection of the solvent delivery system

Chromatographic separations with a single eluent are always preferable. In isocratic elution mode, the mobile phase is mixed and pumped together as a single eluent. However, gradient elution is a powerful tool for achieving separation between closely eluting compounds having widely differing polarities. The important feature of gradient elution is the ability to change the polarity and ionic strength of the mobile phase during the run.

While running a gradient, two mobile phases are introduced into the column in two different ways.

- **Low-pressure gradient:** Mobile phases are mixed at the predetermined ratios, then pumped using a single pump.
- **High-pressure gradient:** Mobile phases are pumped at different flow rates to achieve the required composition, then mixed in a chamber before being introduced into the column.

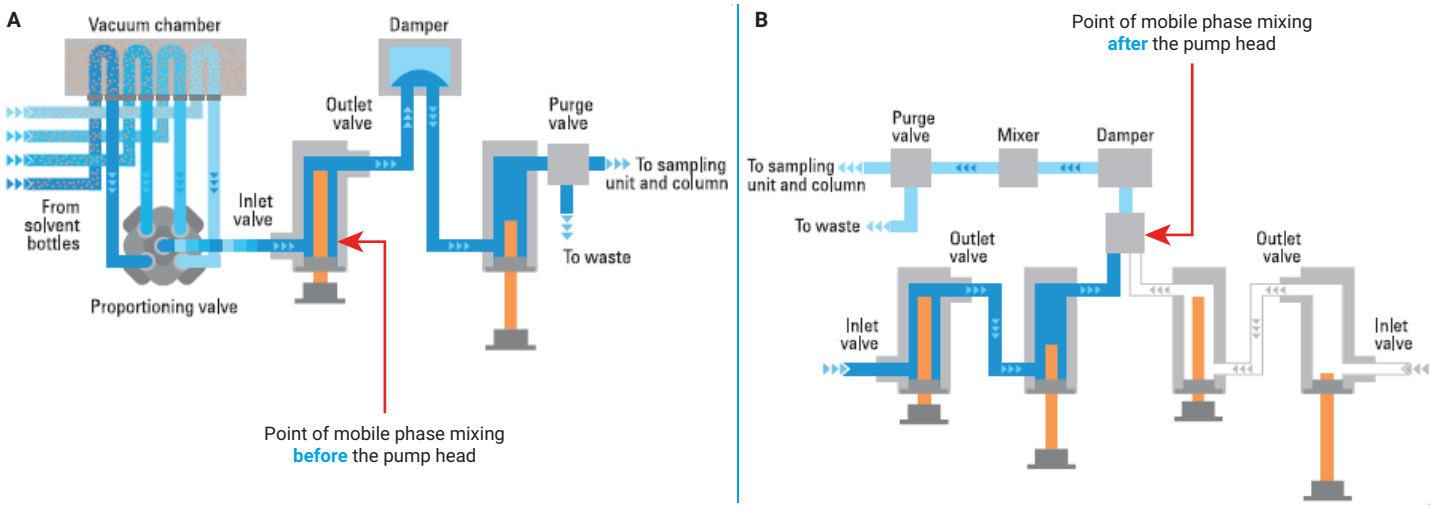


Figure 4. Differences between Agilent Binary (A) and Quaternary pumps (B).

Table 2. Differences between Agilent binary and quaternary pumps.

Parameter	Agilent 1290 Infinity II High-Speed Pump	Agilent 1290/1260 Infinity II Flexible Pump
Power Range	1,300 Bar at 2 mL/min flow, ramping down to 800 bar at 5 mL/min	– 1290: 1,300 bar at 2 mL/min flow, ramping down to 800 bar at 5 mL/min – 1260: 800 bar at 5 mL/min flow
Full HPLC Method Compatibility	Unmatched ISET reproducibility	– ISET: For seamless method transfer, regardless of the brand – Blend Assist: Buffer-blending, installed in the drivers
Smartest Pump Intelligence	Active damping, automatic leak correction, and pump resolution	Active damping, automatic leak correction, and pump resolution
Built-in Technology	Jet Weaver, SSV, Degasser, and Purge Valve	Inlet Weaver, multipurpose valve, in-line filter, switchable (and optional) Jet Weaver
Delay Volume	Lowest delay volume for fastest gradients (45 or 10 µL)	Lowest quaternary pump delay volume: ≤ 350 µL

Table 3. Agilent 1260 Infinity II pump comparisons.

Agilent 1260 Infinity II Binary Pump	Agilent 1260 Infinity II Isocratic Pump	Agilent 1260 Infinity II Quaternary Pump
Power range: 600 bar at 5 mL/min flow Lowest delay volume: down to 120 µL	Power range: flow rate 5 mL/min up to 600 bar, 10 mL/min up to 200 bar	Flow rate: 5 mL/min up to 600 bar, 10 mL/min up to 200 bar Buffer blending: up to quaternary solvent blending

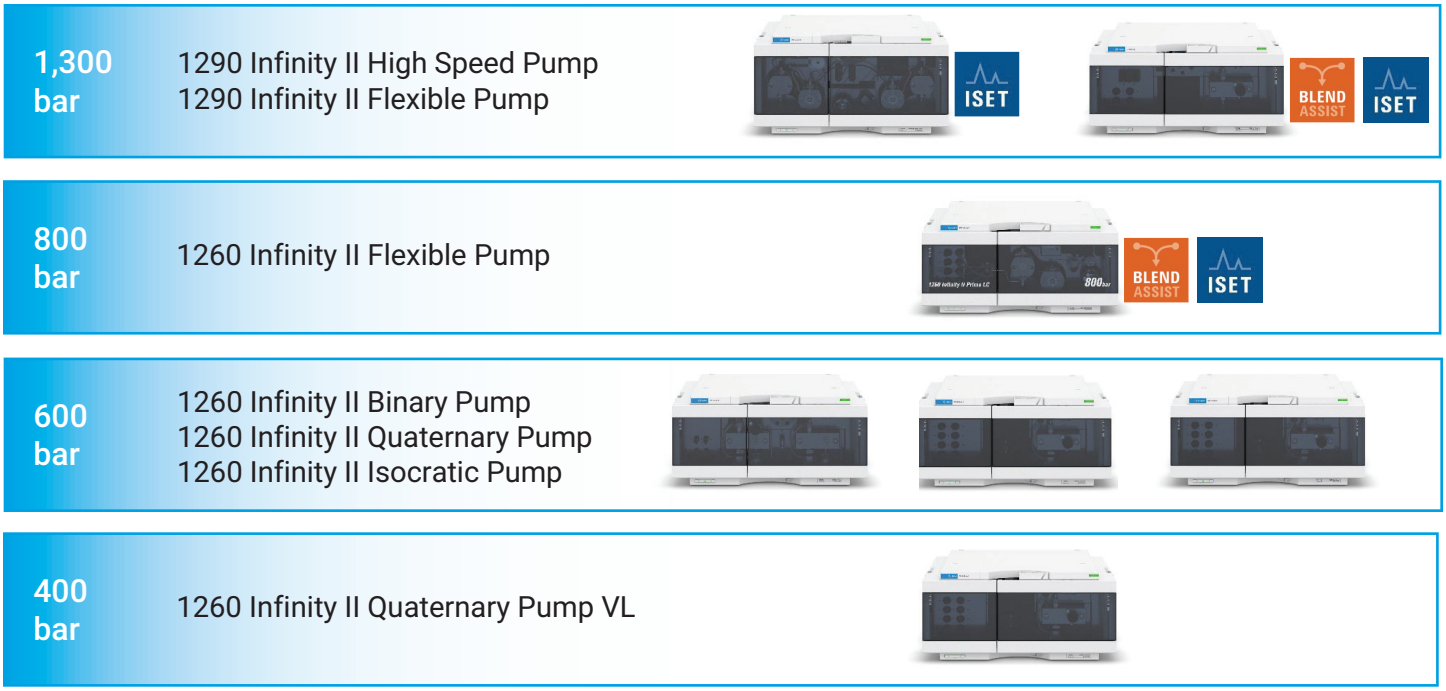


Figure 5. Agilent InfinityLab Series Pumps.

When optimizing the separation of impurities from an analyte, decide whether to use a low-pressure gradient or a high-pressure gradient. Low-pressure gradients can be added when pumping no more than 90% organic phase. Conversely, a high-pressure gradient is preferred when pumping more than 90% of the organic phase.

When optimizing the gradient, especially using low viscous solvents such as acetonitrile and phosphate buffers, it is recommended to mix in approximately 10% aqueous portion, preferably using the same buffer used in the mobile phase, to avoid pumping issues. Also, it is important to optimize the program for initialization after each run and before proceeding with the next injection.

Step 6: Selection of flow rate

The flow rate of the method is optimized based on the following data.

- Retention times
- Column backpressures
- Separation of impurities from the analyte peak
- Peak symmetries

The 1290 Infinity II High Speed Pump system has 1,300 bar at 2 mL/min flow, ramping down to 800 bar at 5 mL/min. Select the flow rate that gives the shortest retention times, good peak symmetries, lowest backpressure, and better separation of impurities from the active pharmaceutical ingredient (API) peak.

Agilent 1260 Infinity II Family			Agilent 1290 Infinity II Family	
1260 Infinity II Vialsampler	1260 Infinity II Vialsampler (Prime LC)	1260 Infinity II Multisampler (Prime LC)	1290 Infinity II Vialsampler	1290 Infinity II Multisampler
				
– Maximum 600 bar 0.1 to 100 µL (default) – Up to 132 vials (2 mL) or 36 vials (6 mL) – Flow-through design – 0.004% Carryover – Optional integration of Integrated Column Compartment (ICC) – Sample thermostat – Column Identification kit	– Maximum 800 bar 0.1 to 100 µL (default) – Up to 132 vials (2 mL) or 36 vials (6 mL) – Flow-through design – 0.004% Carryover – Optional integration of Integrated Column Compartment (ICC) – Sample thermostat – Column Identification kit	– Maximum 800 bar 0.1 to 100 µL (1,500 µL extended) – 16 Shallow wellplates up to 432 vials (2 mL) or 60 vials (6 mL) – Flow-through design – 0.003% Carryover and 0.0009% using Multi-wash option – Sample thermostat – Multi-wash – Dual-needle	– Maximum 1,300 bar 0.1 to 20 µL (100 µL extended) – Up to 132 vials (2 mL) or 36 vials (6 mL) – Flow-through design – 0.004% Carryover – Optional integration of Integrated Column Compartment (ICC) – Sample thermostat – Column Identification kit	– Maximum 1,300 bar 0.1 to 20 µL (100 µL extended) – 16 Shallow wellplates up to 432 vials (2 mL) or 60 vials (6 mL) – Flow-through design – 0.003% Carryover and 0.0009% using Multi-wash option – Sample thermostat – Multi-wash – Dual-needle

Figure 6. Agilent InfinityLab LC Autosampler.

Step 7: Selection of injection volume, carryover, and test concentration

The test concentration is typically chosen based on the response of the API. However, the test concentration should only be finalized after confirming that the API is completely extractable at the selected test concentration.

Set the test concentration to ensure the limit of quantitation (LOQ) of all impurities and that the analyte is below the reporting threshold. The reporting thresholds should be calculated based on the maximum daily dose. If the LOQs do not meet the requirements, increase either the test concentration or the injection volume to get the desired LOQ.

Step 8: Selection of column temperature

It is preferable to optimize the chromatographic conditions with a column temperature of 30 °C. However, if peak symmetry cannot be achieved with any combination of column and mobile phase, temperatures above 30 °C can be considered. Increasing the column temperature typically reduces peak asymmetry and retention times. When necessary, column temperatures between 30 and 80 °C can be used.

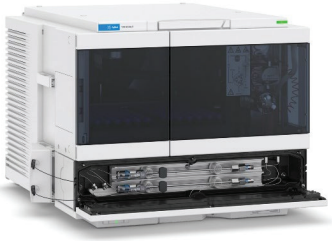
Agilent 1290 Infinity II Multicolumn Thermostat MCT

- Highest column capacity: up to eight columns
- Broad temperature range from 4 to 110 °C
- Column Identification kit for the identification and history tracking of up to eight columns
- Two temperature zones for better method development and optimized column lifetime

Agilent 1260 Infinity II Multicolumn Thermostat MCT

- Column capacity: up to four columns
- Broad temperature range from 10 to 85 °C
- Column Identification kit for the identification and history tracking of up to four columns
- Two temperature zones for better method development and optimized column lifetime

Agilent 1290 & 1260 Infinity II Integrated Column Compartment ICC



- Column capacity: up to two columns
- Broad temperature range from 5 to 80 °C
- Column Identification kit for the identification and history tracking of up to two columns
- One temperature zone for method development

Agilent InfinityLab Supplies for MCT

- InfinityLab Quick Connect Heat Exchangers
- InfinityLab Column Holder Clips

- InfinityLab Quick Connect Fittings (stable up to 1,300 bar)
- InfinityLab Quick Turn Fittings (finger-tight connection stable to 600 bar, up to 1,300 bar with a wrench)

InfinityLab Column Identification tag records column information and usage

Figure 7. Agilent InfinityLab LC column compartments.

Table 4. Types of column thermostat modules for the Agilent InfinityLab LC Series.

Parameters	Agilent 1290 Infinity II Multicolumn Thermostat MCT	Agilent 1260 Infinity II Multicolumn Thermostat MCT	Agilent 1260/1290 Infinity II Integrated Column Compartment ICC
Temperature Range	– 4 to 110 °C – Peltier: down to ambient – 20 °C	– 14 to 85 °C – Peltier: down to ambient – 10 °C	Ambient + 5 to 80 °C oven, no cooling
Number of Temperature Zones	2	2	1
Maximum Column Length	30 cm + Quick Connect fitting	30 cm + Quick Connect fitting	30 cm
Column Capacity	– 4 × 30 cm – 8 × 10 cm (supported by valves)	– 4 × 30 cm – 8 × 10 cm (not supported by valves)	2 × 30 cm (external valve drive needed)
Flexibility	Quick-Connect heat exchangers: – Standard flow (#062) – High flow (#063) – Low dispersion (#064)	Quick-Connect heat exchangers: – Standard flow (#062) – High flow ≥ 2.5 mL/min (#063)	Heat exchanger: – 3.0 µL (#063) – 6.0 µL (#066)

Step 9: Selection of detector type

Choosing the appropriate detector wavelength is a critical step when finalizing the analytical method for impurities and degradants. Inject the API and impurities standard solutions into the chromatographic system equipped with a diode array detector (DAD) and collect the spectra.

- Select the wavelength that provides the highest response for the analyte.
- Select the DAD detector based on the chromophores present in the compounds being separated.
- Select the initial wavelength by analyzing the UV spectra of the compounds using a UV-VIS spectrophotometer and verify the lambda maxima of the compounds using the absorption maxima based on the functional groups.
- Select other detectors, such as a refractive index (RI) or evaporative light scattering detector (ELSD), if the compounds lack chromophores.

For compounds containing a non-chromophoric group, which may produce non-chromophoric impurities upon cleavage, use both UV and other detectors such as RI and ELSD. Alternatively, non-chromophoric compounds can be analyzed by UV after derivatization. However, assess the suitability carefully, considering the functional groups involved in the derivatization reaction. This can be conveniently used for targeted known compounds, but is less favorable for unknowns unless it is confirmed that all impurities and degradants contain the functional group targeted for derivatization.

If the molecule of interest exhibits fluorescence properties, a fluorescence detector (FLD) is ideal for known compounds with available structural information. For unknown compounds, careful assessment is required to ensure that all possible impurities and degradants also possess fluorescence properties.

Molecules with the following properties typically exhibit fluorescence capabilities:

- Conjugated pi-electrons, especially in aromatic components
- Aliphatic and alicyclic compounds with carbonyl groups and compounds with highly conjugated double bonds
- Unsubstituted aromatic hydrocarbons with an increase in number of rings
- Inorganic metals that form complexes with ligands

Agilent offers different types of detectors, including variable wavelength (VWD), diode array/multiple wavelength (DAD/MWD), ELSD, FLD, and RI detectors.

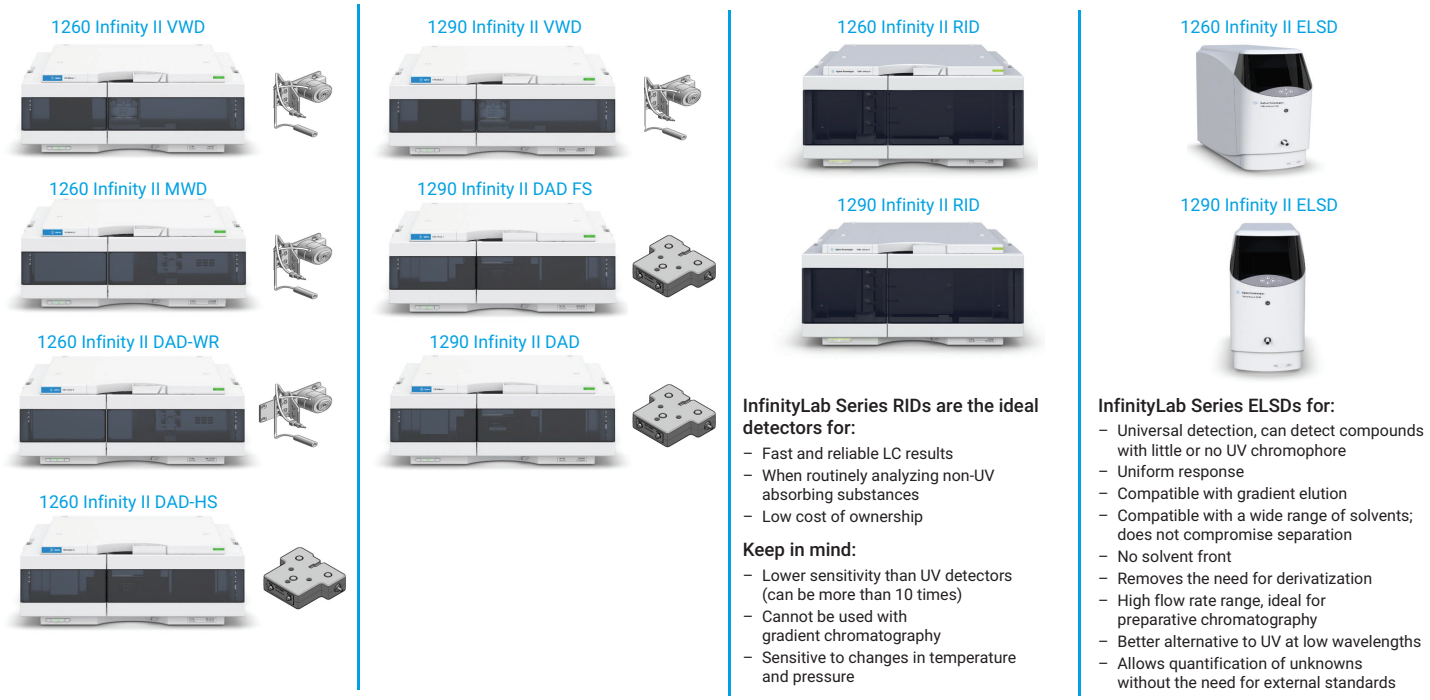


Figure 8. Agilent InfinityLab LC Series Detectors.

Agilent Infinity II LC UV detector portfolio

	VWD		MWD	DAD			
Product Number	G7114A	G7114B	G7165A	G7115A	G7117C	G7117A	G7117B
Description Short	1260 Infinity II VWD	1290 Infinity II VWD	1260 Infinity II MWD	1260 Infinity II DAD WR	1260 Infinity II DAD HS	1290 Infinity II DAD FS	1290 Infinity II DAD
Usage	For highest sensitivity in fast single- and dual λ-analysis	For highest sensitivity in ultra-fast single- and dual λ-analysis	For high sensitivity in fast multi-λ-analysis	For high sensitivity in fast multi-λ-analysis and high-resolution spectral analysis	For ultra-sensitivity in fast multi-λ-analysis and spectral analysis	For ultra-sensitivity in fast multi-λ-analysis and spectral analysis	For ultra-sensitivity in ultra-fast, multi-λ-analysis, and high-resolution spectral analysis
Data Rate	120 Hz 2.5 Hz (dual λ)	240 Hz 2.5 Hz (dual λ)	120 Hz	120 Hz	120 Hz	120 Hz	240 Hz
Noise	<± 2.5 µAU	<± 1.5 µAU	<± 7 µAU	<± 7 µAU	<± 0.6 µAU/cm* <± 3.0 µAU†	<± 0.6 µAU/cm* <± 3.0 µAU†	<± 0.6 µAU/cm* <± 3.0 µAU†
Drift	1×10^{-4} AU/h	1×10^{-4} AU/h	9×10^{-4} AU/h	9×10^{-4} AU/h	5×10^{-4} AU/h	5×10^{-4} AU/h	5×10^{-4} AU/h
Signals Maximum	2 Signals	2 Signals	8 Signals	– 8 Signals – Full spectra with variable slit	– 8 Signals – Full spectra with variable slit	– 8 Signals – Full spectra with variable slit	– 8 Signals – Full spectra with variable slit
Wavelength Range	190 to 600 nm	190 to 600 nm	190 to 950 nm	190 to 950 nm	190 to 640 nm	190 to 640 nm	190 to 640 nm
Linearity	> 2.5 AU (5%)	> 2.5 AU (5%)	> 2 AU (5%)	> 2 AU (5%)	> 2 AU (5%)	> 2 AU (5%)	> 2 AU (5%)
Flow Cell Type	Conventional	Conventional	Conventional	Conventional	Max-Light Cartridge Cell	Max-Light Cartridge Cell	Max-Light Cartridge Cell
Flow Cells	Analytical scale, Bio, preparative scale	Analytical scale, Bio, preparative scale	Analytical scale, Bio, preparative scale	Analytical scale, Bio, preparative scale	Analytical scale	Analytical scale	Analytical scale
Features	ETC, RFID	ETC, RFID	ETC, RFID	ETC, RFID	ETC, RFID	ETC, RFID, upgrade HDR	ETC, RFID, upgrade HDR

* Noise performance with 60 mm Max-Light cartridge flow cell
† Noise performance with 10 mm Max-Light cartridge flow cell
ETC = Electronic temperature control for complete optical unit
RFID = Radio frequency identification for lamps and flow cells
HDR = Upgradable to HDR-DAD (30x wider linear range)

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