# Detection of impurities by heart cutting using the Agilent 1290 Infinity 2D-LC Solution

## Application Note

Pharmaceutical and Chemical


## Author

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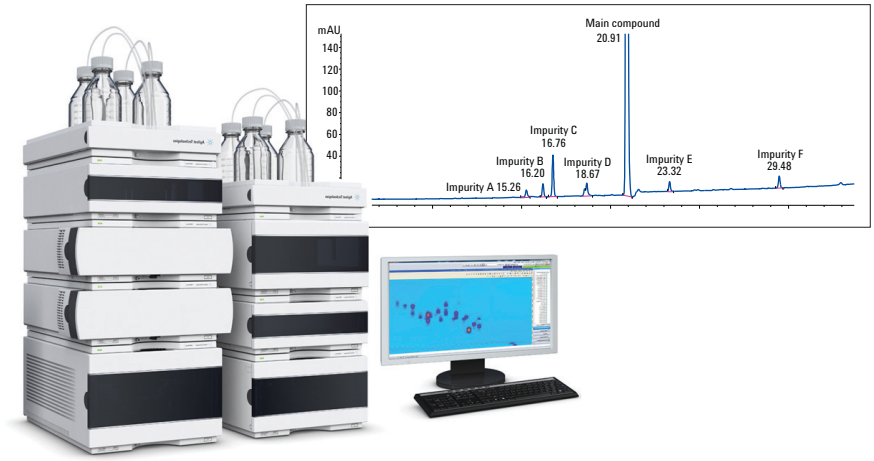



## Abstract

This Application Note demonstrates the capability of the Agilent 1290 Infinity 2D-LC Solution for heart-cutting applications. As an example, a minor impurity is resolved by heart cutting from a co-eluting main compound. The minor impurity, which is isolated on a second-dimension column after heart cutting, is verified by additional spiking and statistical evaluation of the separation performance.


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## Introduction

The analysis of impurities in pharmaceutical compounds and fine chemicals is an important part of the development and production process in pharmaceutical and chemical industries. Due to the fact that most of the impurities are structurally similar to the main compound it can be very difficult to separate them chromatographically. The impurities can co-elute with each other or with the main compound. In the worst case, separation could be impossible with the chosen stationary phase-eluent combinations.

A solution to this challenge is provided by the Agilent 1290 Infinity 2D-LC Solution, which has the capability to do not only comprehensive 2D-LC (LC×LC) but also heart-cutting 2D-LC (LC-LC). In a heart-cutting experiment, a defined amount of the eluent from the first-dimension column is eluted into a loop capillary and then transferred online by a switching valve to the second-dimension column. On this second column, it is possible to separate the co-eluting compounds from the first-dimension column because stationary phase-eluent combinations with different selectivities and longer, adapted gradients can be used.

This Application Note demonstrates the capability of the Agilent 1290 Infinity 2D-LC Solution for heart-cutting applications. As an example, a minor impurity is resolved by heart cutting from a co-eluting main compound. The minor impurity, which is isolated on a second-column dimension after heart cutting, is verified by additional spiking and statistical evaluation of the separation performance.

## Experimental

### Equipment

The Agilent 1290 Infinity 2D-LC Solution consisted of the following modules:

- Two Agilent 1290 Infinity Pumps (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with thermostat (G1330A)
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC) (G1316C) with 2-position/4-port-duo valve (G4236A) for 2D-LC
- Two 1290 Infinity Diode Array Detectors (DAD) (G4212A) with 10-mm Max-Light flow cell (G4212-60008) and 60-mm Max-Light flow cell (G4212-60007). One DAD acquires the chromatogram after the first-dimension column before the eluent enters the loop capillary. The second DAD acquires the chromatogram after the second-dimension column, the chromatogram shows the separated compounds cut out of the first-dimension eluent. Figure 1 shows the instrument set up of the Agilent 1290 Infinity 2D-LC Solution for heart-cutting applications

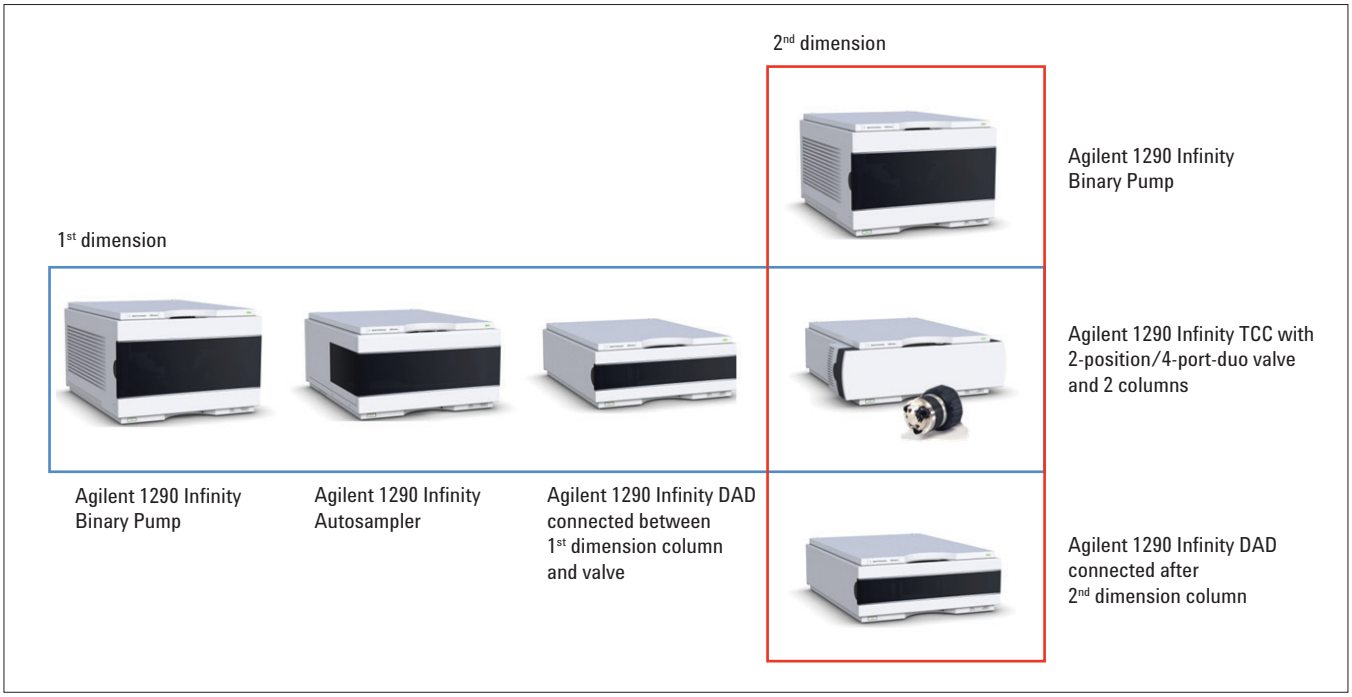


**Figure 1**
**Instrument set up for heart cutting 2D-LC applications (LC-LC). In this configuration, the 1st and 2nd dimension pumps are identical. Typically, the 2nd dimension pump must be a 1290 Infinity pump to deliver fast gradients to the 2nd dimension column. The first dimension pump is flexible and could also be a 1260/1290 Infinity quaternary pump or a 1260 Infinity binary pump.**

## Software

Agilent OpenLAB CDS ChemStation Rev. C01.03 with 2D-LC add-on software.

## Columns

1st dimension: Agilent ZORBAX RRHD Eclipse Plus, C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)

2nd dimension: Agilent ZORBAX RRHD Eclipse Plus, Phenyl-Hexyl, 3.0 × 50 mm, 1.8 µm (p/n 959757-312)

## Method

### 1st dimension Pump

| | |
|---|---|
| Solvent A: | Water + 0.1% formic acid |
| Solvent B: | Acetonitrile + 0.1% formic acid |
| Flow rate: | 0.2 mL/min |
| Gradient: | 0 min 5% B – 30 min 95% B |
| Stop time: | 35 minutes |
| Post time: | 15 minutes |

### 2nd dimension Pump

| | |
|---|---|
| Solvent A: | Water + 0.1% formic acid |
| Solvent B: | Methanol + 0.1% formic acid |
| Flow rate: | 3 mL/min |
| Gradient: | 0 min - 5% B, 10 min - 15% B |

### Thermostatted Column Compartment

- 1st dimension column on the left side at 25 °C.
- 2nd dimension column on the right side at 60 °C.
- One 80-µL loop and a short cut capillary are connected to the 2-position/4-port-duo valve. The valve is switched automatically at the time point determined by the part of the main chromatogram that should be cut from the first-dimension column onto the second-dimension column. After a determined period of time, the loop capillary is switched back in front of the second-dimension column for analysis of the content of the loop capillary (Figure 2).

### Autosampler

| | |
|---|---|
| Injection volume: | 3 µL |
| Sample temperature: | 8 °C |
| Needle wash: | 6 seconds in methanol |

### 1st dimension DAD

| | |
|---|---|
| Wavelength: | 254/4 nm, Ref. 360/16 nm |
| Slit: | 4 nm |
| Data rate: | 20 Hz |
| Flow cell: | 10-mm Max-Light flow cell |

### 2nd dimension DAD

| | |
|---|---|
| Wavelength: | 254/4 nm, Ref. 360/16 nm |
| Slit: | 4 nm |
| Data rate: | 20 Hz |
| Flow cell: | 60-mm Max-Light flow cell |

## Chemicals

All solvents used were LC grade. Acetonitrile and methanol were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak).

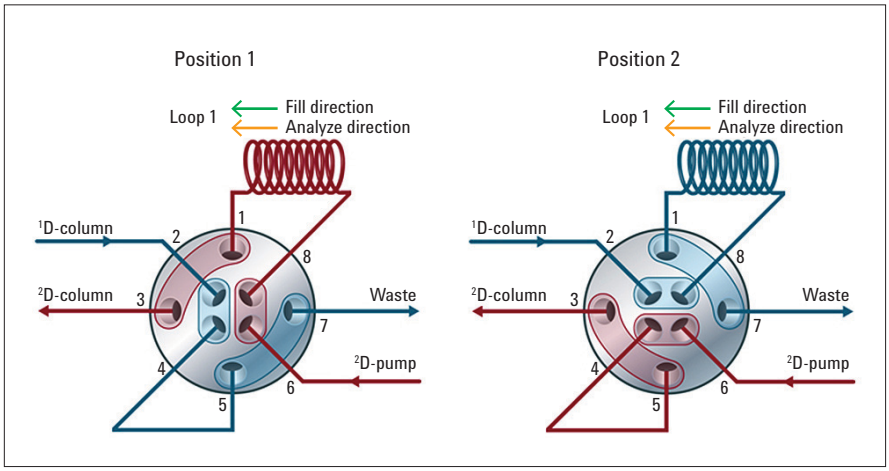


**Figure 2**
**Plumbing diagram of the 2-position/4-port-duo valve for heart cutting applications.**
**Position 1: The main separation is done in the first-dimension column. The eluent goes to the 1st DAD, through the short cut capillary (between ports 4 and 5) and to waste until the region of interest appears.**
**Position 2: The loop is switched behind the first-dimension column and first-dimension DAD and loaded with the interesting part of the first-dimension chromatogram. Then, after a defined time, it is switched back to position 1 for analysis of the content of the loop capillary on the second-dimension column.**

## Results and discussion

During the development of the production process for a pharmaceutical drug or fine chemical substance, much effort is spend on the analysis and determination of the impurity profile to identify them easily in the subsequent large scale production. To detect low-level impurities, a large amount of the compound is injected to separate chromatographically. The impurities can be seen as tiny peaks beside a large peak for the main compound (Figure 3). In the example shown, a pharmaceutical/chemical compound from a concentrated solution is injected (2.5 mg/mL). Possible impurities are A to F, and it might also be possible that another impurity co-elutes with the main compound.

For the detection of this possible impurity, a heart-cutting experiment gives the best access to co-eluting impurities. Therefore, a part of the main peak is captured in a loop capillary and transferred to the second-dimension column that has a different selectivity than the first-dimension column. Additionally, a shallower gradient can be applied on this particular separation (Figure 4). The first piece cut out of the first dimension was between 20.75–21.00 minutes. This was the first part of the main compound peak. In the separation on the second dimension, the signal of the main compound and another small impurity can be seen.

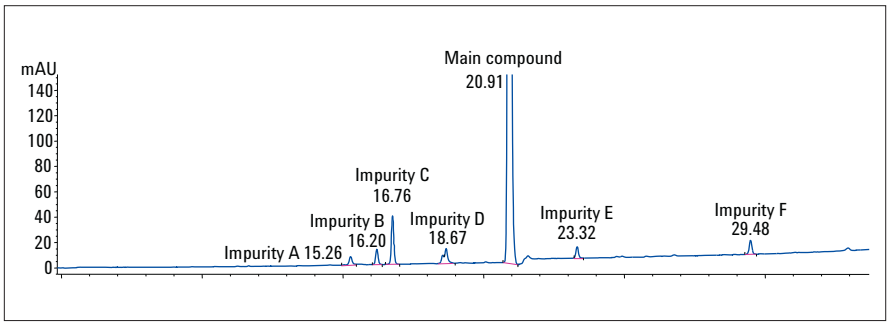


**Figure 3**
**Main compound and detected impurities A to F.**

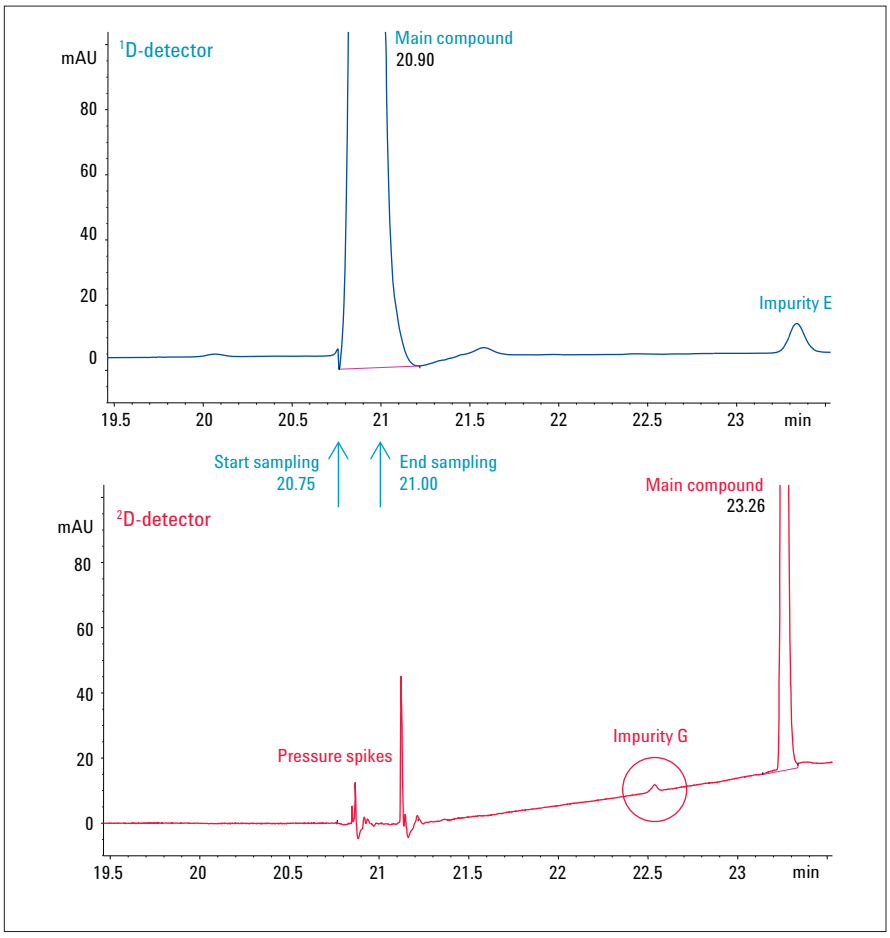


**Figure 4**
**Main chromatogram (blue) and chromatogram of heart cut (red). Heart cut from the main peak between 20.75-21.00 minutes. The main compound elutes at 23.26 minutes from the second-dimension column. Pressure spikes caused by valve switching at start and end of sampling. Delay caused by delay volume plus column volume.**

In the following injection of the sample, the second part of the main peak was cut out between 20.95–21.20 minutes and transferred to the second-dimension column. Here, the additional impurity H could be separated from the main peak with a retention time at 23.24 minutes (Figure 5). To verify the process impurity H, the sample solution was spiked with increasing amounts of synthesized probable impurity H (double, three times, and four times the initial amount of impurity H in the sample). The following heart-cut experiments show that the peak of impurity compound H increases by the given factor of the added amount and, hence, proves the identity of the separated peak as impurity H (Figure 6).

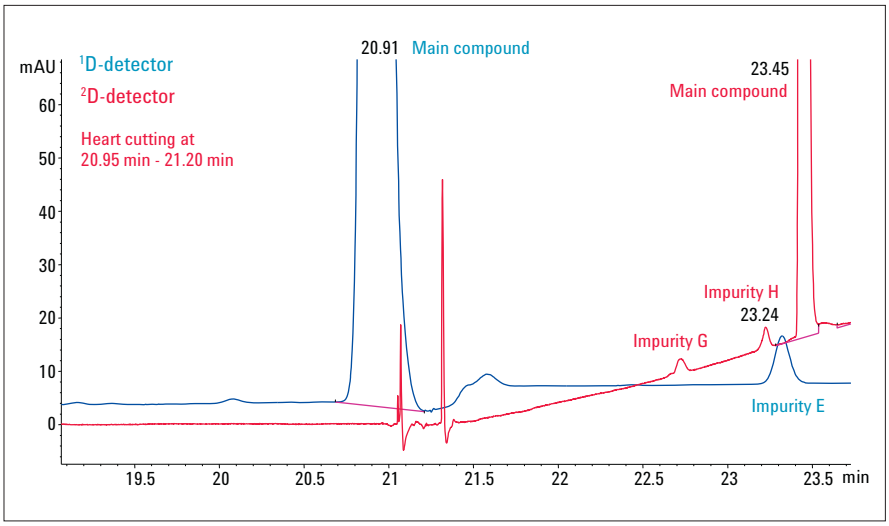


**Figure 5**
**Main chromatogram (blue) with overlaid chromatogram of heart cut (red). Heart cut from the main peak between 20.95–21.20 minutes. Impurity H separates in the chromatogram of the second dimension with retention time at 23.24 minutes the peak of the main compound elutes at 23.45 minutes (different retention time compared to Figure 4 is due to the later heart cut event).**

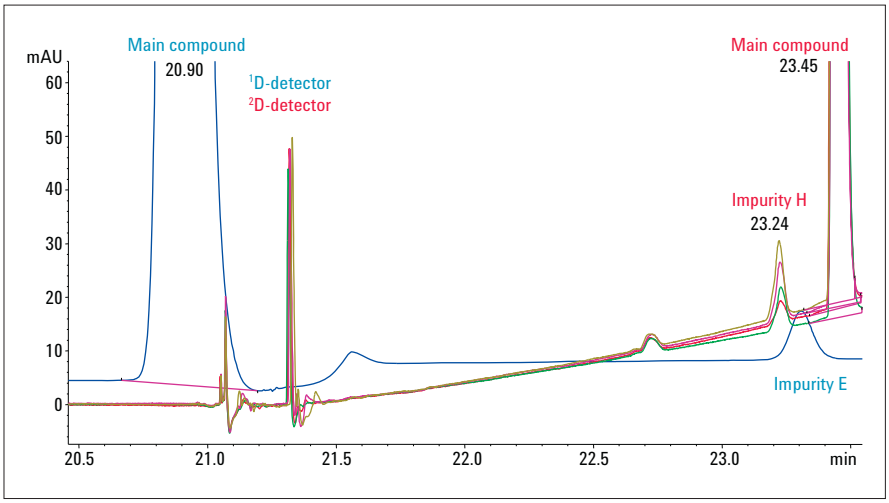


**Figure 6**
**Main chromatogram (blue) with overlaid chromatograms of heart cut (red). Heart cut from the main peak between 20.95–21.20 minutes. The sample was spiked with additional amounts of standard impurity H with increasing concentration.**

To determine the reliability of the heart-cutting process, a linearity of the transferred amounts and a statistical evaluation was done. The areas under the peaks obtained from the experiments with increased amount of H are displayed and the linearity coefficient was calculated (Figure 7). The displayed graph and linearity coefficient show that the heart-cutting process transfers reproducibly the same part of the first separation with increasing concentration of H. The statistical evaluation from 10 replicates with a retention time RSD of 0.015% and an area RSD of 3.53% shows good and reliable precision (Table 1).

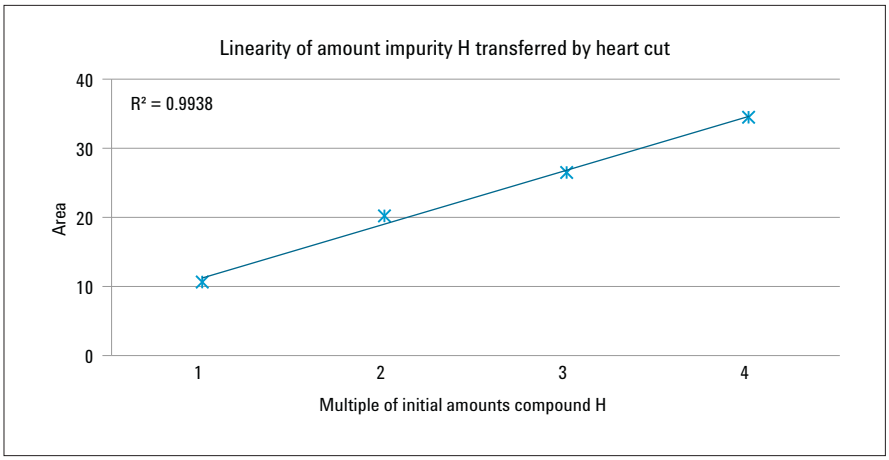


**Figure 7**
**Linearity of impurity H transferred form the first to the second column in the heart cut experiment by injection of sample spiked with increasing amounts.**

| | Retention time (min) | Area |
|---|---|---|
| Main | 23.22 | 11.13 |
| S.D. | 0.0037 | 0.3933 |
| RSD [%] | 0.015 | 3.53 |

**Table 1**
**Statistical evaluation of heart cut peak of impurity H (n = 10).**

## Conclusion

This Application Note demonstrates the capability of the Agilent 1290 Infinity 2D-LC Solution for heart cutting applications. The separation of a minor impurity from the co-eluting major compound is shown and verified. For the verification, multiple amounts of the initial concentration of the co-eluting impurity were spiked into the sample and separated. The linearity of the increasing peak areas of the impurity was determined showing a good linearity coefficient for a reliable cutting process. The precision of the retention time and peak area of the separated impurity were determined by multiple injection of the original sample, both values showed excellent precision.

**www.agilent.com/chem/infinity-2d-lc**


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