Agilent 1290 Infinity 2D-LC Solution for Multiple Heart-Cutting

Technical Overview

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Abstract

This Technical Overview describes the Agilent 1290 Infinity 2D-LC solution for multiple heart-cutting, which significantly enhances separation performance compared to conventional 1D-LC as well as advancing targeted heart-cutting analysis to a higher level. Innovative interfacing technology facilitates parking of 1D aliquots while running 2D cycles. In this way, heart-cuts can be taken from multiple 1D peaks at high frequency, which provides detailed information on target components in complex samples. Features of the Agilent 1290 Infinity 2D-LC solution for multiple heart-cutting include:

- Superior separation performance to determine target compounds in complex samples

- Innovative technology for parking of multiple heart-cuts while analyzing in the second dimension

- Intuitive user assistance in method development as well as data analysis

- Automation that ensures reliability of results
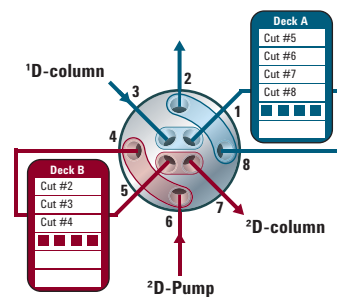
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Introduction

Two-dimensional liquid chromatography (2D-LC) has been demonstrated to greatly enhance separation performance compared to conventional 1D-LC. This is important when samples are complex. In principle, an online 2D-LC system comprises two single LC instruments that are connected through the 2D injector. A 2D-LC experiment starts with 1D-chromatography. Aliquots of the effluent from the 1D column are sampled, and then injected onto the 2D column for further analysis. Primarily, it is the difference in selectivity that governs the separation. In the ideal scenario, where the separation mechanism in the first dimension is orthogonal to the mechanism in the second dimension, the resolving powers are multiplied[1].

Alternating dual-loop interfaces have been used most commonly[2]. Figure 1 shows the Agilent 2-position/4-port duo-valve, which facilitates two symmetric flow paths and has been developed specifically for its application in 2D-LC. From a design perspective, this valve minimizes pressure fluctuations at the inlet of the 2D column (that inevitably occur upon switching), which extends column life substantially[3]. 2D-LC starts with the valve in position 1, where the 1D effluent passes through loop 1 (blue flow path). Switching the valve to position 2 injects the contents of loop 1 into the 2D cycle. Once this 2D separation has finished, the sample in loop 2 can be injected. This process is then repeated.
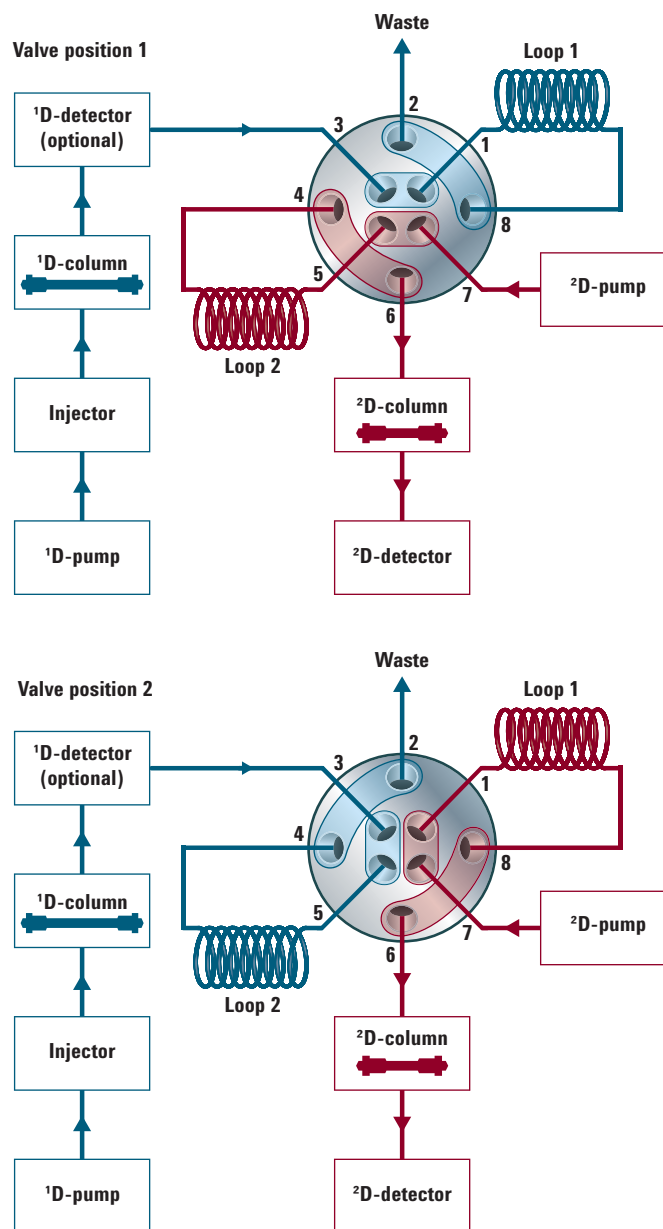

Figure 1. Schematic of 2D-LC system, showing first-dimension modules and flow path in blue, and second-dimension modules and flow path in red. The first and second dimensions are interlinked through an Agilent 2-position/4-port duo-valve. This dual-loop interface is shown in position-1 (top) and position-2 (bottom).

2D-LC can be divided into two major types of operation, comprehensive and heart-cutting. The application of each mode depends on the task.

Comprehensive 2D-LC is predominantly used to analyze unknown mixtures of high complexity as found in omic-type applications, natural products, and the analysis of biomolecules[4]. The task is to gather as much information as possible. So, every 1D peak eluting through the 1D column (typically the entire 1D effluent) is sampled and transferred to the second dimension. Sampling should occur frequently to avoid remixing of components that had been successfully separated in the first dimension[5]. These conditions entail a typical 2D-LC operation with fast gradients, short columns, and high flow rates in 2D, and reduced flows in 1D, because the duration of the 2D cycle is normally about equivalent to that of sampling.

The purpose of heart-cutting 2D-LC is different from comprehensive chromatography in that it aims to determine only one, or a few target compounds, characteristically in samples of high complexity. As shown in Figure 2, only a distinct fraction of the 1D peak is taken for reanalysis in the second dimension. This breaks the link between sampling time and 2D cycle so that both dimensions can operate under optimal conditions. The use of longer 2D gradients and columns with higher separation efficiency often leads to improved chromatography compared to comprehensive mode, and it is not necessary to reduce flow rates in the first dimension[6]. Nevertheless, such a gain in chromatographic quality has a price, which, in this case, is the potential loss of information. For instance, the schematic in Figure 2 indicates the omission of the gray, orange, and blue 1D peaks during 2D cycles of cut 1 and cut 2, respectively. The inset shows that there are more compounds underneath the green peak.

Multiple heart-cutting 2D-LC

This Technical Overview describes the Agilent multiple heart-cutting 2D-LC solution. It prevents data loss by parking more cuts while running the 2D cycle. This is illustrated in Figure 3. Heart-cuts are taken from every 1D peak and higher frequent sampling provides for detailed examination of the green peak.
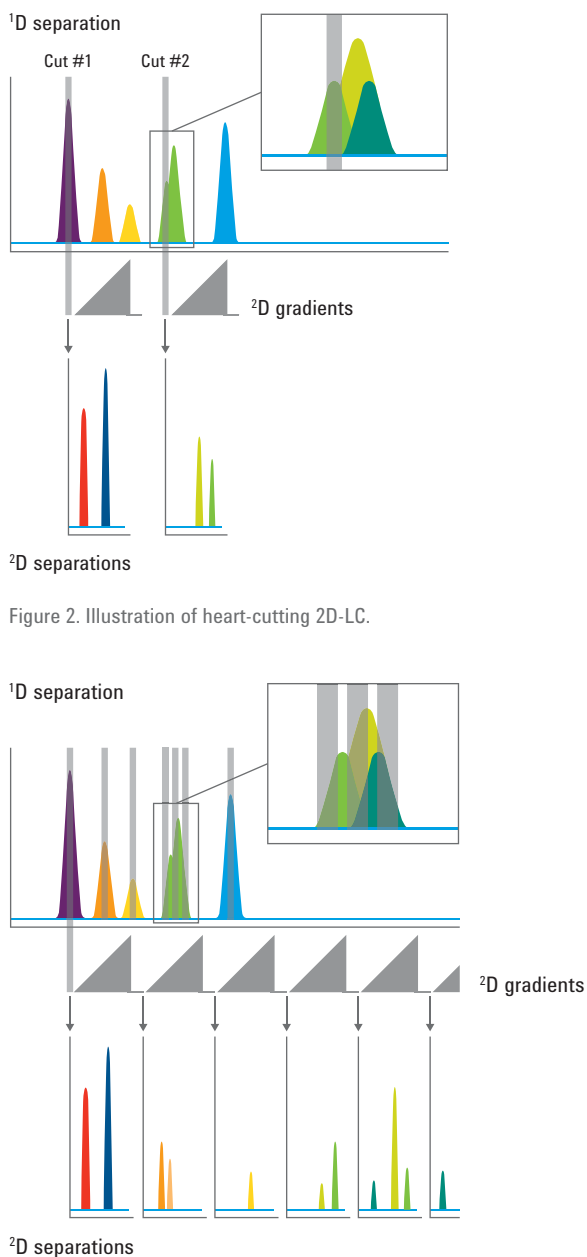

Figure 2. Illustration of heart-cutting 2D-LC.

Figure 3. Illustration of multiple heart-cutting 2D-LC.

New interface technology supports the parking concept. One configuration is shown in Figure 4. Parking decks, each holding six sampling loops, replace the sampling loops of the dual-loop interface. The multiple heart-cut interface offers 12 loop positions.

Example applications of 2D-LC demonstrate:

• 2D-LC software that markedly facilitates method setup and data evaluation

• The routine for detailed determination of multiple targeted compounds

• Features to ensure repeatability and correctness of results

• 2D-LC quantification performance

Method Setup and Experimental

Data were acquired using an Agilent 1290 Infinity 2D-LC solution operated through Agilent OpenLAB CDS ChemStation Edition, version C.01.07.

Samples were dilutions of the 2D-LC checkout sample (p/n 5190-6895), containing 16 small molecular weight compounds each at a concentration of 1 mg/mL: atrazine, chlorotoluron, desethylatrazine, desethylterbuthylazine, diuron, hexazinone, linuron, metazachlor, methabenzthiazuron, metobromuron, metoxuron, nifedipine, nimodipine, prometryn, sebuthylazine, and terbuthylazine. Dilutions were prepared freshly before analysis in 1D mobile phase (A:B, 80:20, v/v).

First dimension

The first-dimension system included:

• Agilent 1290 Infinity Quaternary (G4204A) or Binary (G4220A) Pump

• Agilent 1290 Infinity Autosampler (G4226A)

• Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

• Agilent 1290 Infinity Diode Array Detector (G4212A) equipped with 10-mm flow cell (G1315-60022)

The detector tracked the 254-nm UV trace at a 5-Hz acquisition rate. An Agilent ZORBAX SB C18, 100 × 2.1 mm, 1.8 µm column at 40 °C was used. Solvent A was 0.2 % formic acid in water and solvent B was methanol (MeOH). Gradients were from 20 to 100 %B in 50 minutes at a flow rate of 600 µL/min.

Second dimension

The second-dimension system used an Agilent 1290 Infinity Binary Pump (G4220A), which was required to control the multiple heart-cutting 2D-LC workflow. An Agilent ZORBAX Bonus RP, 50 × 2.1 mm, 1.8 µm column at 40 °C was used. The Agilent 1290 Infinity Diode Array Detector (G4212A) equipped with a 10-mm (p/n G4212-60008) or a 60-mm (p/n G4212-60007) Max-Light cartridge cell, tracked the 254-nm UV trace at 80 Hz acquisition rate.
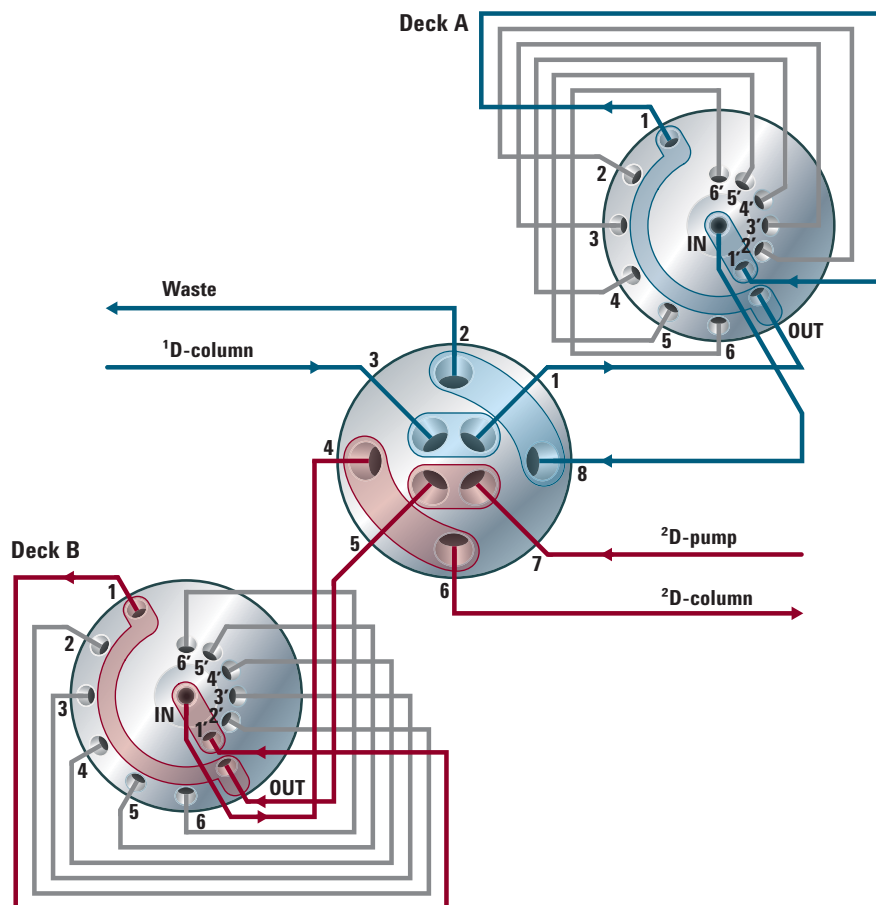

Figure 4. Interface with two parking decks (A and B) holding 12 sampling loops.

All experiments used the multiple heart-cutting 2D-LC interface as shown in Figure 4. The 6-position/14-port valves, which are included in the multiple heart-cutting 2D-LC upgrade kit (G4242A), are connected to the 2-position/4-port duo-valve (5067-4170). Each 6-position valve has a cluster of six pre-installed 40-µL sampling loops. This provides two parking decks (A and B) with 12 loop positions. Switching of the duo-valve places a deck in position for sampling or, alternatively, for 2D analysis. Switching the 6-position valves provides access to the discrete loop positions. The interface was operated in cocurrent mode (loop fill and loop flush in same flow direction). All valves were driven by universal valve drives (G1170A).

Solvent A was 0.2 % formic acid in water, and solvent B was acetonitrile. Data was acquired using a 2D cycle time of 1.75 minutes. A flow of 1 mL/min was used with an initial gradient from 10 to 60 %B in 1.25 minutes. A gradient shift was programmed to reach start conditions of 30 %B at 20 minutes. The 2D-LC analysis stop time was set to 30 minutes in the dialog box of the 2D binary pump. All other modules were set to: As injector/No limit. Setting the stop time in the dialog box of the 2D pump ensures processing of all cuts parked in the interface, even if processing exceeds the set stop time. The described 2D-specific settings are organized in the method setup dialog box shown in Figure 5. A more detailed description

is beyond the scope of this Technical Overview, and the interested reader is referred to the user manual[7].

Software Features and Operational Routine

Gradient preview
The gradient preview superimposes the 1D gradient (red trace) and the predicted 2D cycles (blue trace) as shown in panel G of Figure 5. The corresponding scale is the Y-axis at the left. A 2D gradient shift can be edited using this graphical interface by point values, which can be dragged to the desired location. Preview and tables (panels D and E) are interconnected so that any
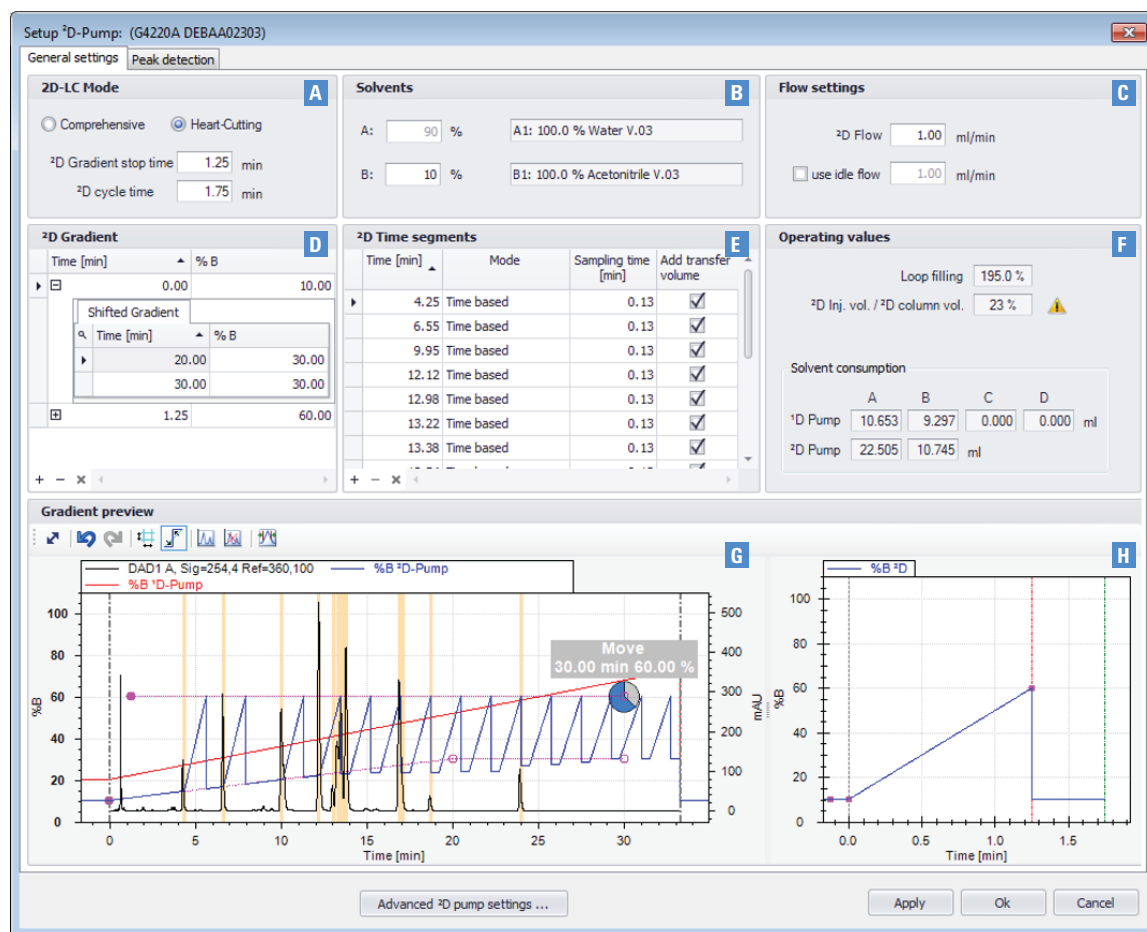

Figure 5. 2D method setup dialog box. A) 2D-LC mode. B) Solvents, C) Flow settings. D) 2D-gradient. E) 2D time segments. F) Operating values. G) Gradient preview. H) Zoom of 2D-gradient at initial conditions.

graphical change is automatically reflected in the tables and *vice versa*. A 1D UV-chromatogram (black trace) can be uploaded, and can be from any previously acquired run. The matching UV scale is the Y-axis at the right. In this case, the chromatogram was obtained from a 1:10 dilution of the 2D-LC checkout sample, of which 2 μL were injected.

Multiple heart-cutting experiments can be performed in two operational modes, peak-based or time-based. In peak-based mode, heart-cuts are taken automatically for 2D analyses on 1D peak recognition. This requires the 1D detector that was marked as optional in Figure 1. The system is started for sampling when the detector signals the start of a peak. The trigger for this can be a baseline threshold (in mAU), slope (in mAU/s), or the combination of both. Sampling

stops when the trigger matches a second time (indicating the end of the peak), or when the sampling time (set in panel E of Figure 5) has elapsed. The software accounts for the delay between 1D peak detection (peak is located in the UV flow cell) and its arrival at sampling loop inlet.

Figure 6 shows magnified views of gradient previews (4 to 20 minutes). These experiments used a baseline threshold of 50 mAU for peak detection and a sampling time of 1 minute, which exceeded the peak width so that the 1D detector signal solely governs sampling. The green color shows the peak-based mode, and predicts the trigger interval for this uploaded chromatogram. Panel A shows the preview obtained with the multiple heart-cut interface and panel B the case if an ordinary dual-loop interface

were used. The software marks in red, heart-cuts that cannot be made.

In time-based mode, sampling is triggered by time events as specified by the user. The software assists in that it automatically generates a heart-cut table (panel E in Figure 5) based on the same criteria as in peak-based mode. The corresponding orange markings in the preview (panel G in Figure 5) designate the heart-cuts that will be made in this experiment. For further improvements of the separation, additional cuts were added manually in 1D regions where a number of peaks coeluted (at about 14 and 18 minutes). The preview in Figure 5 predicts fifteen 2D cycles. All features described markedly facilitate setup and editing of 2D-LC experimental conditions.
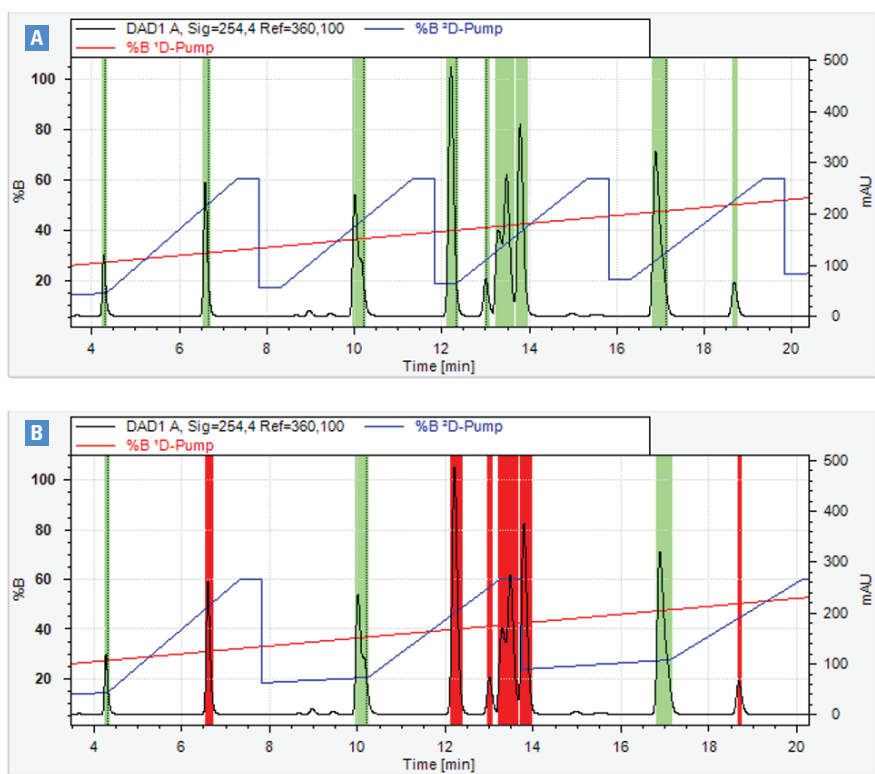

Figure 6. Gradient preview with coloration obtained in peak-based mode with A) multiple heart-cutting 2D-LC interface and B) dual-loop interface. The 2D cycle was set to 4 minutes for the purpose of illustration.

Sampling/parking algorithm

The multiple heart-cutting algorithm reduces the number of valve switches to a minimum, but follows the rule to promptly process heart-cuts that are sampled or parked.

The first heart-cut is always sampled in loop 1 of deck A, and promptly submitted to its 2D cycle. As long as the time difference between consecutive cuts exceeds the 2D cycle time, there is no parking required, and the multiple heart-cutting interface works just like a dual-loop interface sampling in loop 1 of each deck. That is, only the duo-valve operates, the decks do not switch. In the analysis in Figure 5, this would be the case for cuts 1 to 4.

Panel A in Figure 7 shows an excerpt of the 1D chromatogram in Figure 5, representing 1D regions in which parking occurs. Under current experimental conditions (1D flow = 600 µL/min, loop size = 40 µL, sampling time = 0.13 minutes), the system was operated to overfill the loops twice as indicated by the trigger interval (orange markings). The shaded zone relates to the loop volume and is subsequently about half as wide as the orange marks. It predicts more exactly where the cut will be taken. Panels B and C in Figure 7 show snapshots of the 2D-LC online monitor. This is a part of the 2D-LC software, which traces the actual state of the interface during the 2D-LC experiment. Multiple heart-cut decks are displayed schematically, each with six slots representing the sampling loops. In these slots, the software displays cut numbers and the time of parking. Here, for reasons of illustration, only cut numbers are shown.

Panel B in Figure 7 reflects the situation corresponding to the point at about 14 minutes. During the 2D-run of cut 4 (loop 1, deck B), cuts 5 to 9 were parked in deck A (loops 1 to 5). Waiting for the processing of these cuts, deck A remains connected to the first dimension (blue path) and, therefore, needs a flow-through position. The waiting loop is always the loop one count greater than that where the last cut was parked.

In the current case, this is loop 6. Panel C in Figure 7 shows the situation where the duo-valve switched deck A into the 2D analysis position, currently processing cut 5, which was previously parked in loop 1. Deck B connected to

1D has parked cuts 10 to 13, and waits in position 5 until deck A has been totally emptied. After switching deck B back into 2D position, deck A once again offers its slots to 1D for sampling.
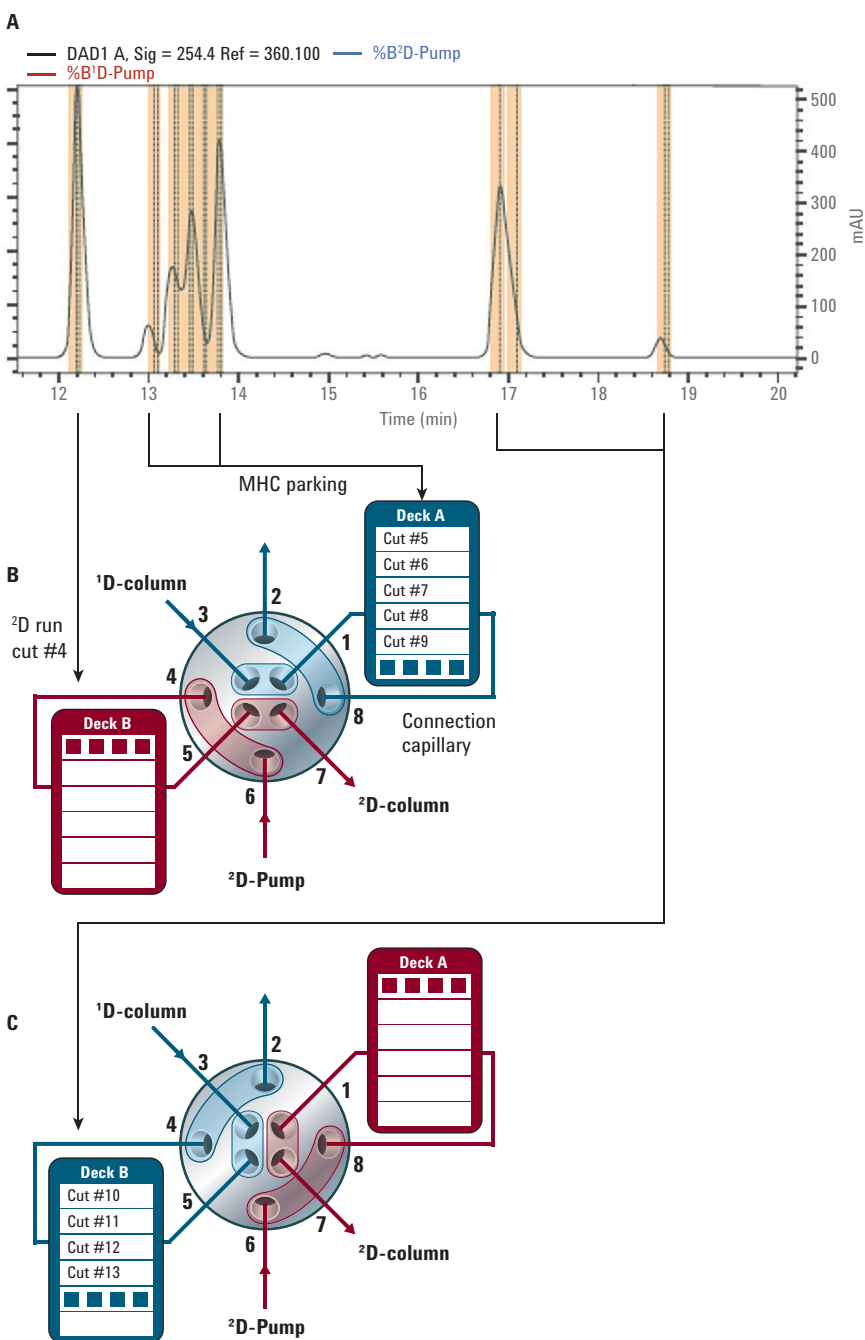

Figure 7. Schematic of multiple heart-cutting 2D-LC routine. A) Preview obtained in time-based mode. B and C) Online 2D-LC monitors, displayed here in counter-current mode.

While the parking process begins in loop 1 of a deck and proceeds in loops with increasing number, submission to 2D analyses occurs in the reverse order. The content of the loop that was used in the flow-through position is injected first into 2D. In this way, this loop and the capillaries connecting the deck and duo-valve are flushed. This is essential to ensure correctness of the results, as will be demonstrated in section *Flush cycle*.

Emptying backwards has significant advantages. For instance, the physical distance between the flow-through position and the next loop to run in 2D is the shortest. This entails a short time period for valve switching. Moreover, the continuous flow is directly led to the next 2D position without having to pass any other loop, which could lead to its contamination. 2D analysis in reverse chronological order requires intuitive data analysis tools.

Data analysis

Data evaluation of multiple heart-cutting is assisted by the 2D-LC Heart-Cut Viewer. Figure 8 shows results obtained from the time-based, multiple heart-cutting experiment. The viewer displays the 1D chromatogram (panel A), heart-cut table with sampling and parking details (panel B), the complete output of 2D (panel C), and an extracted view (panel D). Panels are cross-linked. The shades with cut number support the match of cut and 2D run. Blue highlights (panels A through C) indicate that heart-cuts 6 to 9 were selected. Corresponding 2D results are superimposed in panel D. This shows that three well-separated peaks were obtained, in contrast to the 1D region sampled. Yellow marks with description F represent the flush cycle.

Flush cycle

Whenever a parking event occurred, the 2D processing of a deck automatically started with a flush cycle (flush gradient). This was to clean the waiting loop and connection capillaries between the deck and duo-valve, which is vital to ensure the correctness of the result.

For instance, the first flush cycle in Figure 8 confirms its necessity. Its signal (panel C, at about 15 minutes) originates from the 1D effluent passing through the connection capillaries and loop 6 of deck A (panel B in Figure 7). When the switch of the duo-valve occurred, it positioned deck-A in the 2D process. This happened at 14.05 minutes and eventually led to the transfer of this 1D effluent onto the 2D column. Its content was from the rear of the 1D-peak eluting at that time (panel A in Figure 8). It did not belong to that of cut 9, whose sampling in loop 5 of deck A was finished about 0.2 minutes earlier (13.83 minutes, compare with panel B in Figure 8). Yet, without the flush gradient, at least the content of the connection capillaries would have been assigned to cut 9 erroneously. The use of flush cycles becomes more critical the greater the time difference between parking of the last heart-cut in a deck and the deck's submission to 2D.

It is possible to make incorrect assessments when a loop that is reused at a later phase in the analysis has not previously been cleaned completely. This is particularly severe when partial loop filling is used.
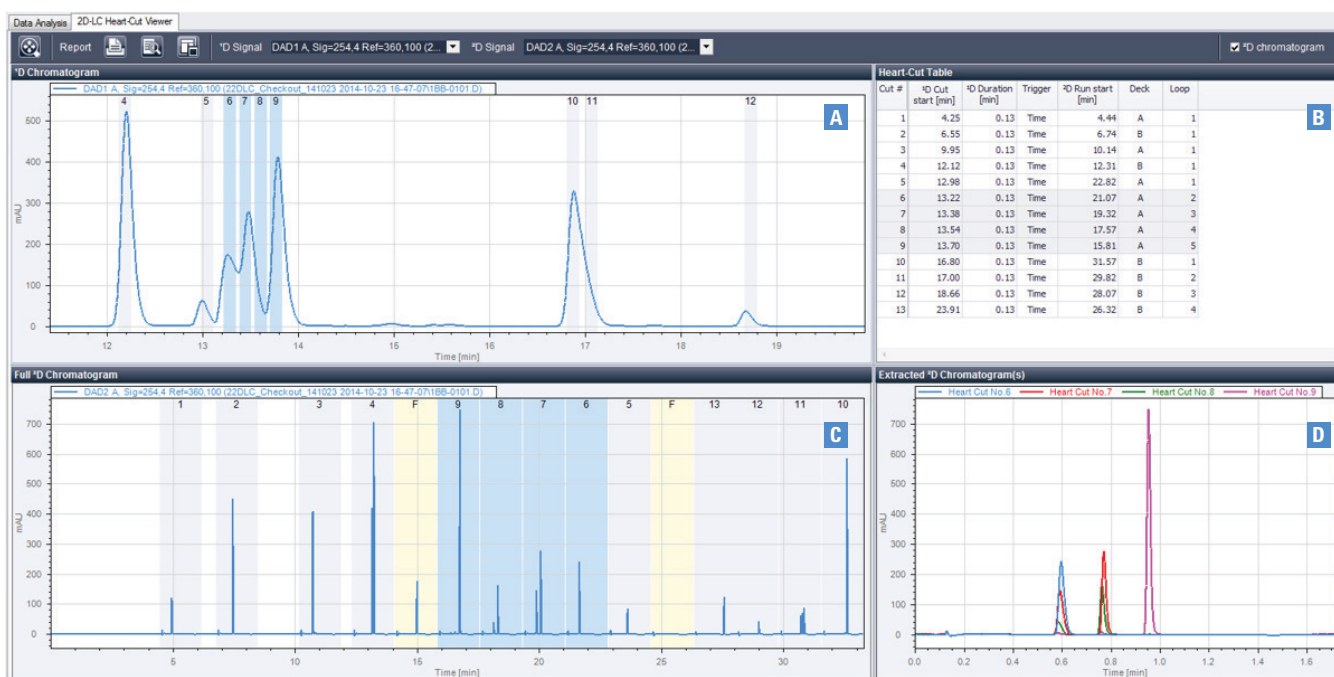

Figure 8. 2D-LC Heart-Cut Viewer.

Link of 2D gradient to the point of sampling

A consequence of multiple heart-cutting 2D-LC is that parking of a cut occurs at a different point in time than does its 2D analysis. An important measure implemented in the system is that it links the 2D condition to that specified in the method at the time of sampling. This makes the 2D condition used to analyze a given heart-cut independent of the number of cuts that have been analyzed prior to it. This is particularly important when multiple heart-cutting is used with shifted 2D gradients.

In this way, method setup is considerably simplified, and repeatability ensured. One example is the case where cuts are added to an existing multiple heart-cutting method that uses a 2D gradient shift. Due to the link (2D gradient to sampling from 1D), no reprogramming is required. Such an addition of cuts can also occur unexpectedly, for example due to an impurity detected in peak-based mode.

The measure (linking 2D gradient to sampling time) is particularly useful when high-frequency sampling is performed across densely populated 1D regions. For instance, the 2D gradient in Figure 5 was programmed to shift initial gradient conditions in 20 minutes from 10 to 30 % acetonitrile, where it remains. Designated by the purple line, panel D indicates the departure of this shift from gradient traces (blue). This is correct because cuts 6 to 9 have been taken between 13.35 and 13.83 minutes,

as shown in Figure 8. According to the method, the anticipated 2D gradient begins at 23.35 and 23.83 % acetonitrile, respectively. Based on real 2D time, 2D gradients would have initiated at conditions corresponding to the start of the respective 2D analysis. These would have been very different. For example, for cuts 6 and 9, 30.00 % (at 21.07 minutes) and 25.81 % (at 15.81 minutes), respectively, which would have shown in the elution profiles. In contrast, the overlay of 2D chromatograms in Figure 8 (panel D) enables comparison of retention times of associated components obtained from cuts 6 to 9.

Targeted Quantification

System linearity and sensitivity

Linearity experiments used dilutions of the 2D-LC checkout sample in the range 0.5 to 1,000 µg/mL, of which 2-µL volumes were injected at least in duplicate. Two 1D regions were sampled. This is shown in panel A of Figure 9 with the intention to quantify C1 and C2 coeluting in region 1, and accordingly C7 and C8, which also partially coelute with C6 in region 2. The interface was operated in partial loop fill mode with a sampling time of 0.06 minutes per cut (this equals 90 % loop filling at current 1D conditions) using time-based sampling.

Panel B in Figure 9 shows 2D chromatograms of cut 3 and cut 4 of region 1 and accordingly cut 7 and cut 9 of region 2 acquired at the lowest 0.5 µg/mL level. Prominent peaks

were obtained in 2D. These were all well separated, which allowed for the integration of the single components. The peak heights exceeded those in 1D, despite heart-cutting. For instance, the 1D peak (C1 and C2), from which cut 4 was taken, has a peak height of about 1 mAU. The corresponding 2D run divided the signal into two peaks (C1 and C2), with heights of 3 and 4.5 mAU, respectively. The reason for this increased absorption was primarily due to the use of a 60-mm UV cell in the 2D detector. Different gradient slopes, solvents, and type of detector could have played a role, too.

Panel C in Figure 9 shows log plots of peak area against concentration. The logarithmic scale better exhibits the linearity at the lower end. The top chart is from 1D results obtained when integrating the peak (C1 and C2) of region 1. It shows linearity across the entire concentration range investigated with R^2 values greater than 0.999. The chart at the bottom is representative of 2D results and was generated from cycles of cut 4 (of region 1) and cut 9 (of region 2). Calibration plots, normalized to the area of the largest peak, are superimposed. The two highest levels exceeded the linear range of the detector, but good linearity was measured down to 0.5 µg/mL with R^2 greater than 0.999 for all four compounds (C1, C2, C7, and C8). System linearity and sensitivity of 2D demonstrated here are important criteria for quantification experiments, as well as repeatability.
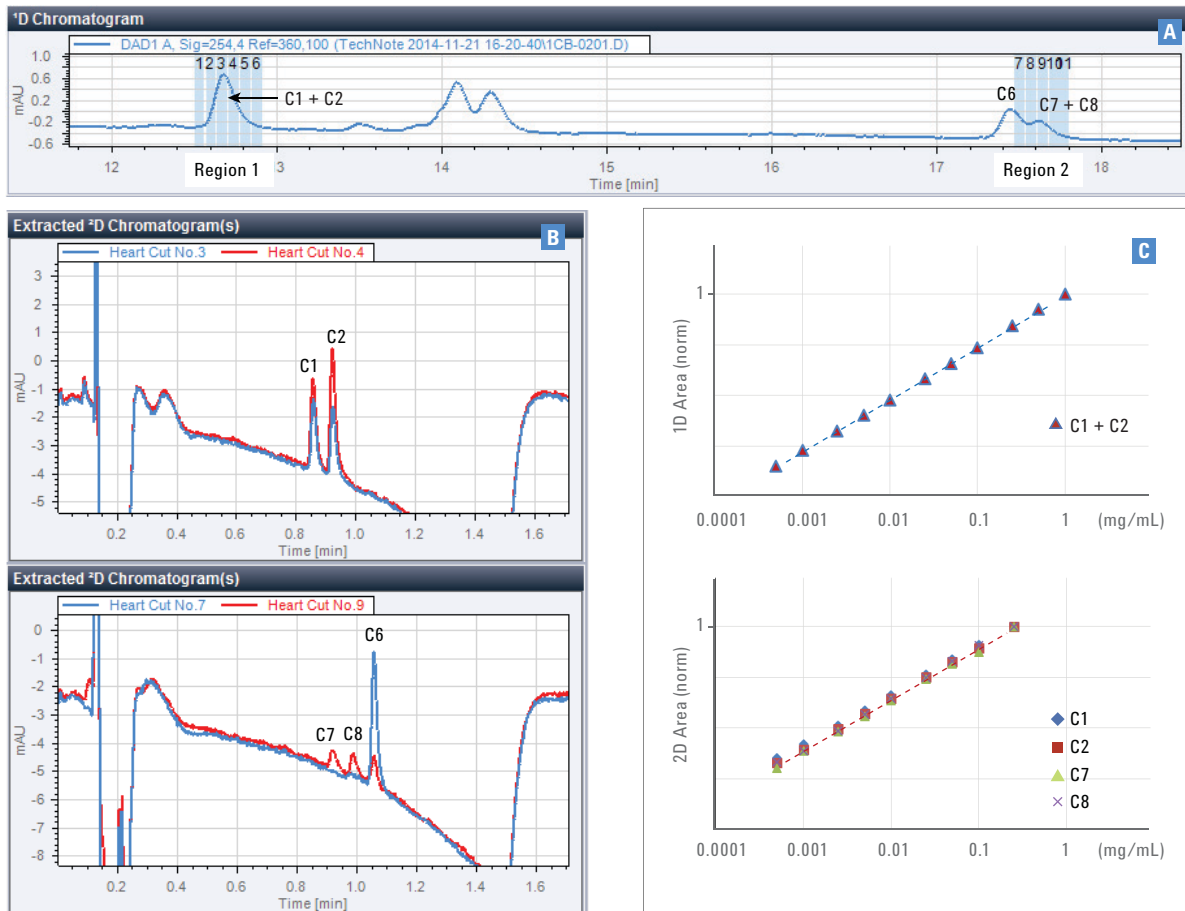
Figure 9. 2D-LC results at 0.5 µg/mL level. A) 1D chromatogram with cutting scheme, 1D used an Agilent 1290 Infinity Quaternary Pump. B) 2D chromatograms of representative cuts overlaid. C) Calibration (log/log) plots of peak area (normalized to area obtained for largest concentration) against concentration in mg/mL.

Repeatability

The repeatability of the 2D-analysis was determined from 20 consecutive 1-µL injections of a 1:10 dilution of the 2D-LC checkout sample. Panel A in Figure 10 shows 1D chromatograms with cutting scheme, and panel B shows 2D chromatograms of cuts 5 to 9 overlaid.

The separation again was always superior in 2D, and the improved sensitivity, compared with 1D, agrees with the findings reported above. Cuts 5 to 9 comprised five components marked in Figure 10 as C1 to C5. Table 1 gives precision data (%RSD) calculated for peak areas, which normally were well below 10 %. Component C4 in cut 6 showed a few outliers. Close inspection revealed that when the area value in cut 6 was low, it was higher in cuts 7 and 8. It was difficult to attribute this to any particular reason, but similar findings were made in the determination of additives in polymers, where even a very small retention shift in 1D caused an area variation[8]. This can occur because cuts are often made at the flank of the peak. One way to compensate for this is to sum peak areas of each component across the multiple cuts to give the *total area*. For example, the first injection in the current experiment gave areas for C4 of 420, 794, and 53 mAU, respectively, in cuts 6, 7, and 8, resulting in a total area of 1,267 mAU. Table 1 demonstrates that RSD values improved when using the total 2D peak area for calculation with 1.6, 0.5, and 2.2 % for components C3, C4, and C5, respectively.
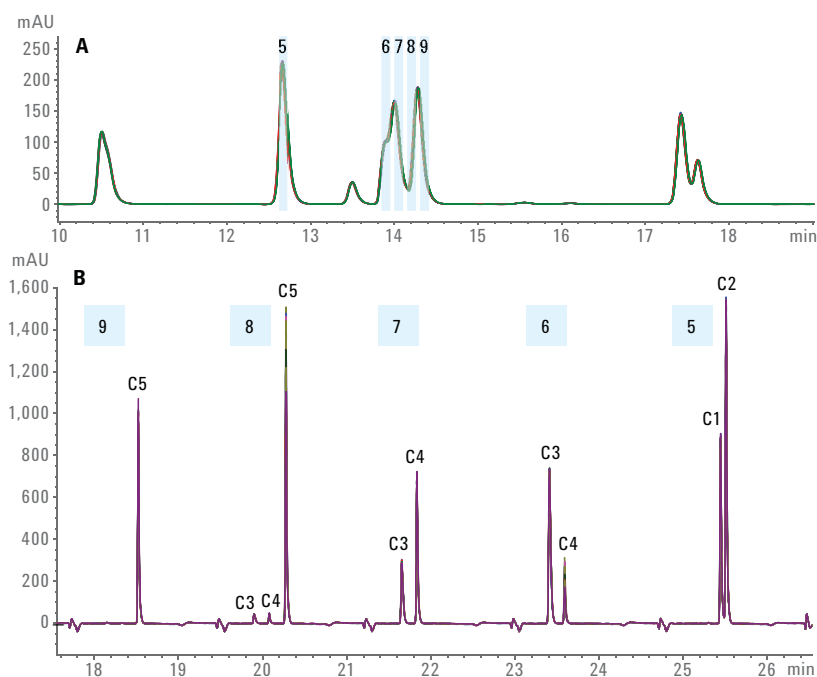

Figure 10. Overlay of 20 consecutive 1-µL injections of 2D-LC checkout sample. A) 1D results with cutting scheme. B) 2D results for cuts 5 to 9.

Table 1. Precision data calculated for 2D results obtained from 20 consecutive 2D-LC runs.

	C1	C2	C3				C4				C5		
Cut no.	5	5	6	7	8	Total	6	7	8	Total	8	9	Total
Average area	1,121	1,995	1,344	524	77	1,945	307	900	60	1,267	1,592	1,142	2,734
%RSD	2.8	1.9	0.8	3.5	4.7	1.6	16.1	5.4	8.0	0.5	8.7	6.9	2.2

Conclusion

This study demonstrates that the Agilent 1290 Infinity 2D-LC solution for multiple heart-cutting greatly enhances separation performance compared to conventional 1D-LC, as well as advancing targeted heart-cutting analysis to another level.

Interface technology permits parking of 1D aliquots while running 2D cycles simultaneously. In this way, heart-cuts can be taken from multiple 1D peaks at high frequency, which provides detailed information on target components in complex samples. This concept has been demonstrated as highly beneficial, for instance, in the analysis of natural products, pharmaceuticals, and polymer samples[8-15]. Decoupling of 2D from the 1D time-scale enables the operation of both dimensions under more optimal conditions.

Intuitive software significantly facilitates operation of multiple heart-cutting 2D-LC by assisting the setup of methods and the assessment of 2D information. Automated features that ensure repeatability and the correctness of the result move 2D-LC one step closer to routine quantification of target compounds at low detection levels.

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