

# Complementary Dual LC as a convenient alternative to multiple heart-cut 2D-LC for samples of medium complexity

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Keywords: Polyphenols, Dual LC, parallel LC, two-dimensional liquid chromatography, 2D-LC, 2D-HPLC, multi-heart-cut 2D-LC, Vanquish Duo UHPLC System for Dual LC, Vanquish Dual Split Sampler, Chromeleon CDS



## Benefits

- Complementary Dual LC provides a convenient alternative to 2D-LC workflows and has great potential for quantitative analysis of samples containing around 15–30 compounds.
- The Thermo Scientific™ Vanquish™ Duo UHPLC System for Dual LC facilitates the accomplishment of two parallel LC analyses in the same time as one analysis by a single-channel UHPLC instrument.
- In terms of quantification precision, LOQ, throughput, solvent consumption, and ease-of-use, the Vanquish Duo UHPLC System for Dual LC clearly outperforms multi-heart-cut 2D-LC.

## Goal

To illustrate the benefit of the Vanquish Duo UHPLC System for Dual LC to resolve semi-complex samples for quantitative analysis in direct comparison to multi-heart-cut 2D-LC approaches.

## Introduction

In general, the application of liquid chromatography (LC) aims for the unambiguous identification and quantification of several compounds in a certain sample. However, despite tremendous improvements in both liquid chromatography instrumentation and column technology, the analytical task sometimes just exceeds the capabilities of conventional one-dimensional LC (1D-LC). In particular, samples of increasing complexity (such as structurally similar compounds and samples with a large number of analytes) present an analytical challenge in terms of resolution power, efficiency, and selectivity. Thus, several more sophisticated techniques such as parallel LC<sup>1-4</sup>, serial column coupling<sup>5</sup>, column switching<sup>6</sup>, or

two-dimensional LC<sup>7</sup> (2D-LC) have gained increasing importance. Among these, parallel LC excels in ease of implementation in comparison to more complex instrument setups comprising switching valves, T-pieces, loops, etc. required for the latter three approaches, while parallel LC can be accomplished with convenient 1D-LC techniques. Although parallel LC (either in Dual or in Tandem mode) is often applied for a simple gain in throughput<sup>8-10</sup> its potential in enhancing sample resolution was already discovered for targeted<sup>1, 2</sup> as well as untargeted<sup>3, 4</sup> studies. Its utilization is particularly attractive in more routine applications (e.g., optical detection) where complete separation is required when no additional identification power is provided by a mass spectrometer (MS).<sup>2</sup> Orthogonality, in the context of 2D-LC, is understood as the independence of retention times ( $t_R$ ) obtained in both dimensions, resulting in a wide

separation of analytes over the theoretical 2D space.<sup>11</sup> Comprehensive 2D-LC provides the largest separation power due to the submission of the whole sample to each dimension and is usually applied to highly complex samples. In contrast, single or multiple heart-cutting 2D-LC is employed for semi-complex analyses, where only a single or few peaks are transferred from the first dimension (<sup>1</sup>D) eluate to the second dimension (<sup>2</sup>D) for further separation.<sup>7, 12</sup> However, Dual LC may provide a convenient alternative in solving analytical challenges. With few critical co-elutions for moderately complex samples, non-resolved peaks of one LC method can be separated by another one and vice versa.<sup>13</sup> As an example, the separation of a model mixture of 22 polyphenolic compounds from tea and olive oil analysis is presented by complementary Dual LC and multi-heart-cut 2D-LC.

## Experimental

**Table 1. Experimental details**

Chemicals	Part number
Deionized water, 18.2 MΩ/cm resistivity or higher	N/A
Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade	A955-212
Fisher Scientific™ Methanol, Optima™ LC/MS grade	A456-212
Fisher Scientific™ Formic Acid, Optima™ LC/MS grade	A117-50
Standards of 22 polyphenolic compounds (catechin, epicatechin, epicatechin gallate, gallic acid, tyrosol, homovanillyl alcohol, vanillic acid, syringic acid, caffeic acid, sinapic acid, ferulic acid, benzoic acid, <i>p</i> -coumaric acid, <i>o</i> -coumaric acid, pinoresinol, cinnamic acid, oleuropein and apigenin) were purchased from reputed vendors	
Sample handling	Part number
Vials (amber, 1.5 mL)	6000.0072
Thermo Scientific™ SUN-SRI™ 11 mm Orange Snap Caps PTFE/Sil	501 382
Instrumentation	Part number
<b>The following modules were set up as a Thermo Scientific Vanquish Duo UHPLC system for Dual LC</b>	
Vanquish System Base	VF-S02-A-02
2x Vanquish Binary Pump H	VH-P10-A-02
1x Vanquish Dual Split Sampler HT	VH-A40-A-02
1x Vanquish Column Compartment H	VH-C10-A-02
2x Vanquish Diode Array Detector FG	VF-D11-A-01
2x Flow cell, semi-micro, biocompatible, 7 mm	6083.0550
<b>The following parts were added for the re-configuration into a Multi-heart-cut 2D-LC system*</b>	
1x Vanquish Column Compartment H	VH-C10-A-02
1x Valve 2-p 6-p 150 MPa bio	6036.1560
2x Valve 6-p 7-p 150 MPa bio	6036.1570
4x Sample loop 200 µL Viper, ACC-3000	6830.2418

\* Only the left sampling unit of the Dual sampler was utilized; a common Vanquish Split Sampler HT with single injection path would be sufficient

## Sample preparation

Stock solutions of 22 single compounds were prepared in water/acetonitrile (50/50; v/v) or water/methanol (30/70; v/v). Mixed calibration standards (1, 5, 10, 25, 50 µg/mL) were prepared in methanol/water (25/75; v/v). Standards of 100, 150 and 200 µg/mL were simulated by injection of 2×, 3×, and 4× volume of the 50 µg/mL standard.

Method 0 gives an example for a single 1D-LC screening method. Method 1 and 2 were employed in parallel in the Dual LC approach. For the multi heart-cut 2D-LC approach Method 1 served as <sup>1</sup>D, Method 3 as <sup>2</sup>D. Fractions were collected in 200 µL loops and were transferred directly after the <sup>1</sup>D run was finished. Each injection of calibration standards was repeated three times.

## Chromatography Data System

System control and data analysis was performed with Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.9.

**Table 2. Columns**

Columns	Part number
<b>The following columns were screened</b>	
Thermo Scientific™ Hypersil GOLD™ C18 selectivity, 2.1 × 100 mm, 1.9 µm	25002-102130
Thermo Scientific™ Accucore™ PFP, 3 × 50 mm, 2.6 µm	17426-053030
Thermo Scientific™ Acclaim™ Vanquish™ PolarAdvantage II, 2.1 × 150 mm, 2.2 µm	071401-V
Thermo Scientific™ Acclaim™ Phenyl-1, 3 × 100 mm, 3 µm	74693
Thermo Scientific™ Hypersil GOLD™ aQ Polar Endcapped C18, 2.1 × 100 mm, 1.9 µm	25302-102130
Thermo Scientific™ Accucore™ Polar Premium, 2.1 × 100 mm, 2.6 µm	28026-102130

**Table 3. Chromatographic conditions**

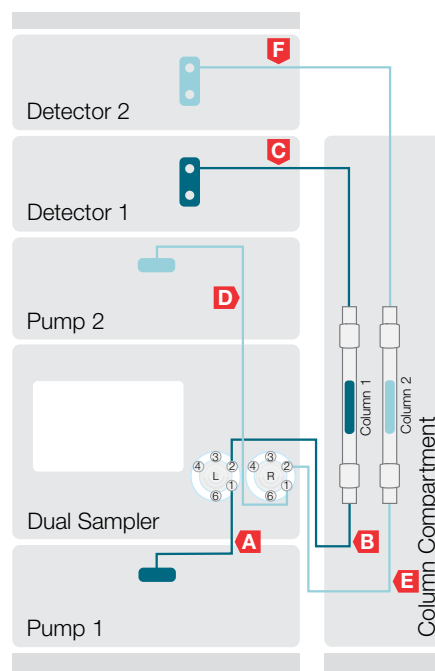
Description	Method 0			Method 1			Method 2			Method 3		
Column	Hypersil GOLD aQ, 1.9 μm, 2.1 × 100 mm			Accucore Polar Premium, 2.6 μm, 2.1 × 100 mm			Hypersil GOLD aQ, 1.9 μm, 2.1 × 100 mm			Acclaim Phenyl-1, 3 μm, 3 × 100 mm		
Eluents	A–0.1% formic acid in water B–0.1% formic acid in methanol											
Gradient	min	%A	%B	min	%A	%B	min	%A	%B	min	%A	%B
	0	95	5	0	97	3	0	90	10	0	95	5
	10	0	100	2.5	97	3	1	90	10	3.5	40	60
	11	0	100	8.83	40	60	4.75	65	35	3.9	0	100
	11.1	95	5	10	0	100	7.35	35	65	4.3	0	100
	17	95	5	11	0	100	8	0	100	4.35	95	5
				11.1	97	3	8.5	0	100	5.85	95	5
				14.1	97	3	8.6	90	10			
						11.6	90	10				
Flow rate	400 μL/min			400 μL/min			400 μL/min			1.3 mL/min		
Col. temp.	40 °C			40 °C			40 °C			50 °C		
Inj. vol.	1 μL			1 μL			1 μL			up to 150 μL		
Detection	260 nm, 280 nm, 20 Hz, 0.2 s resp. time											

## Results and discussion

### Method development

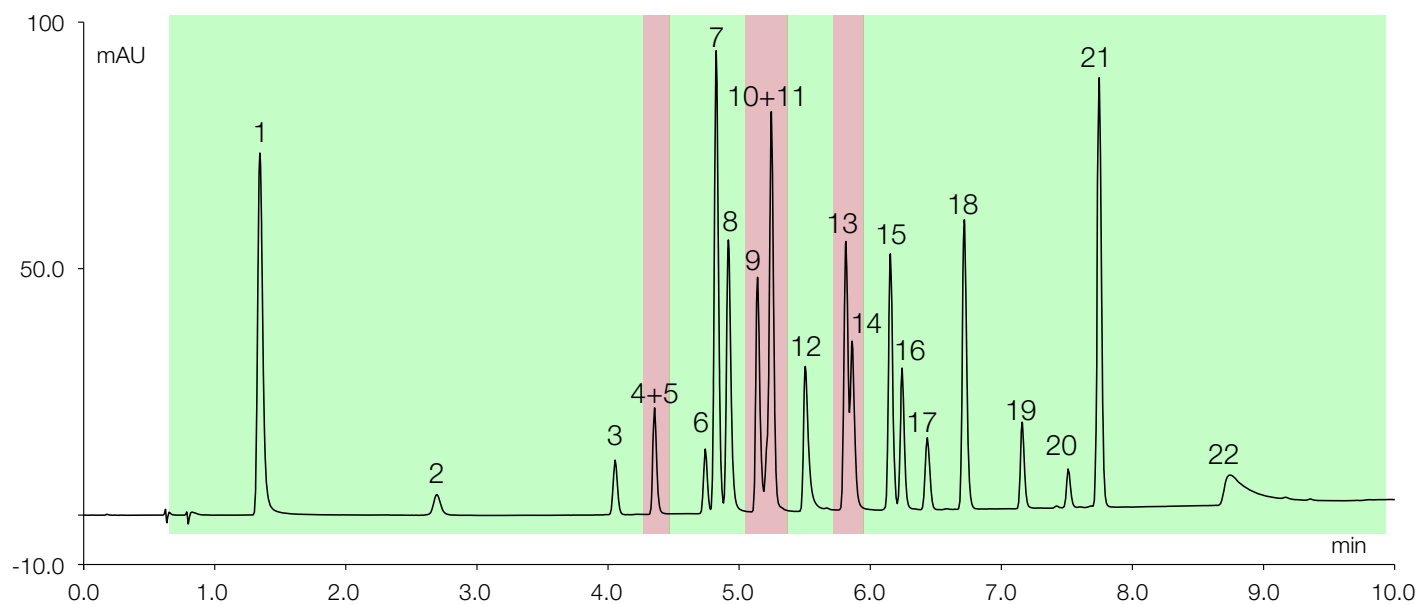
The instrumentation setup of the Vanquish Duo UHPLC System for Dual LC is displayed in Figure 1, comprising two independent 1D-LC flow paths in one stack. The empirical search for proper LC conditions in Dual LC started with a screening of the six chromatographic columns listed above for the separation of the 22 test compounds with generic gradients from 95% mobile phase A (0.1% formic acid in water) to 100% mobile phase B (0.1% formic acid in methanol or acetonitrile). All single 1D-LC methods resulted in more or less severe co-elution of some test compounds. The overall best separation was achieved with the Hypersil GOLD aQ column (Method 0) and resulted in 20 peaks with 16 at a resolution  $R_s \geq 1.5$  shown in Figure 2. For further method development, the most promising column pair had to be selected. Similar to method development in 2D-LC, the selection of the chromatographic setup is a critical step for Dual LC as well. However, in contrast to 2D-LC, here the key is not in finding the most orthogonal/dissimilar columns and conditions but in finding conditions that give complementary separations, i.e. compounds that are overlapped in one method need to be singlet peaks (specified for example by  $R_s \geq 1.5$ ) with the other one.<sup>1,3</sup> The same proved true in the current case: the most orthogonal column pair from the screening experiments were the Hypersil GOLD C18 and Acclaim Phenyl-1 columns with a methanol gradient each, indicated by the lowest correlation coefficient ( $r^2=0.7444$ ) of the retention times.\* However, that column combination was inapplicable for complementary Dual LC due to several peak pairs which partially or completely co-eluted on both columns ( $R_s < 1.5$ ). The most promising column pair regarding complementary separation was Hypersil GOLD aQ and Accucore Polar Premium columns with methanol gradients. It exhibited a lower orthogonality ( $r^2=0.9224$ ) but indicated a good chance to separate each compound with at least one column. After gradient optimization (Method 1 and 2) correlation was still high ( $r^2=0.9008$ ) but all compounds were separated with  $R_s \geq 1.5$  with at least one method (Figure 3). The chromatograms are depicted in Figure 4.

\* Although  $r^2$  is not a good measure for orthogonality in general, it is a simple and convenient metric to compare 2D peak distributions, which are characterized by an evident linear correlation and clustering of data points along the upward diagonal of the 2D space.<sup>11</sup> Such correlations, for example, are frequently seen for combinations of reversed-phase (RP) conditions as in the current study.



#	Capillary
a	0.1 × 550 mm (P/N 6042.2360)
b	Active preheater 0.1 × 380 mm (P/N 6732.0110)
c	Post-column cooler 0.1 × 590 mm (P/N 6732.0520)
d	0.1 × 550 mm (P/N 6042.2360)
e	Active preheater 0.1 × 380 mm (P/N 6732.0110)
f	Post-column cooler 0.1 × 590 mm (P/N 6732.0520)

**Figure 1. Instrumental setup of the Vanquish Duo UHPLC system for Dual LC**



- |                         |                             |                             |                   |
|-------------------------|-----------------------------|-----------------------------|-------------------|
| 1. Gallic acid          | 7. Vanillic acid            | 13. <i>p</i> -Coumaric acid | 19. Oleuropein    |
| 2. Galocatechin         | 8. Caffeic acid             | 14. Epicatechin gallate     | 20. Pinoresinol   |
| 3. Tyrosol              | 9. Epigallocatechin gallate | 15. Ferulic acid            | 21. Cinnamic acid |
| 4. Epigallocatechin     | 10. Epicatechin             | 16. Sinapic acid            | 22. Apigenin      |
| 5. Catechin             | 11. Syringic acid           | 17. Benzoic acid            |                   |
| 6. Homovanillyl alcohol | 12. Gallocatechin gallate   | 18. <i>o</i> -Coumaric acid |                   |

Figure 2. Best single 1D-LC separation achieved with Method 0; green color marks full resolution ( $R_s \geq 1.5$ ), red color marks peak overlap ( $R_s < 1.5$ )

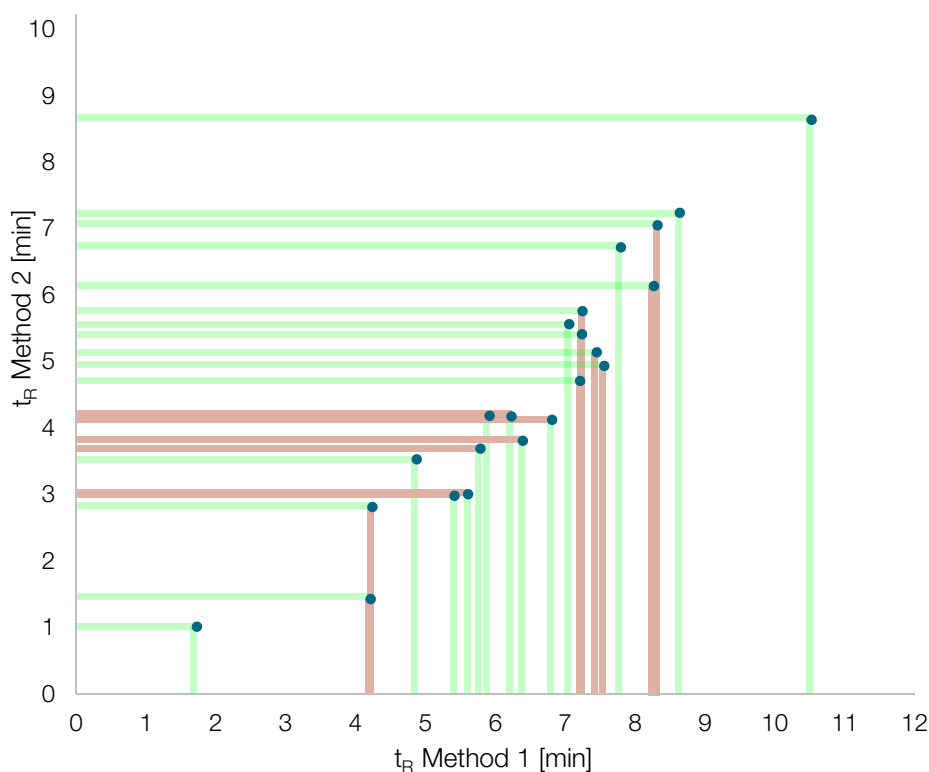
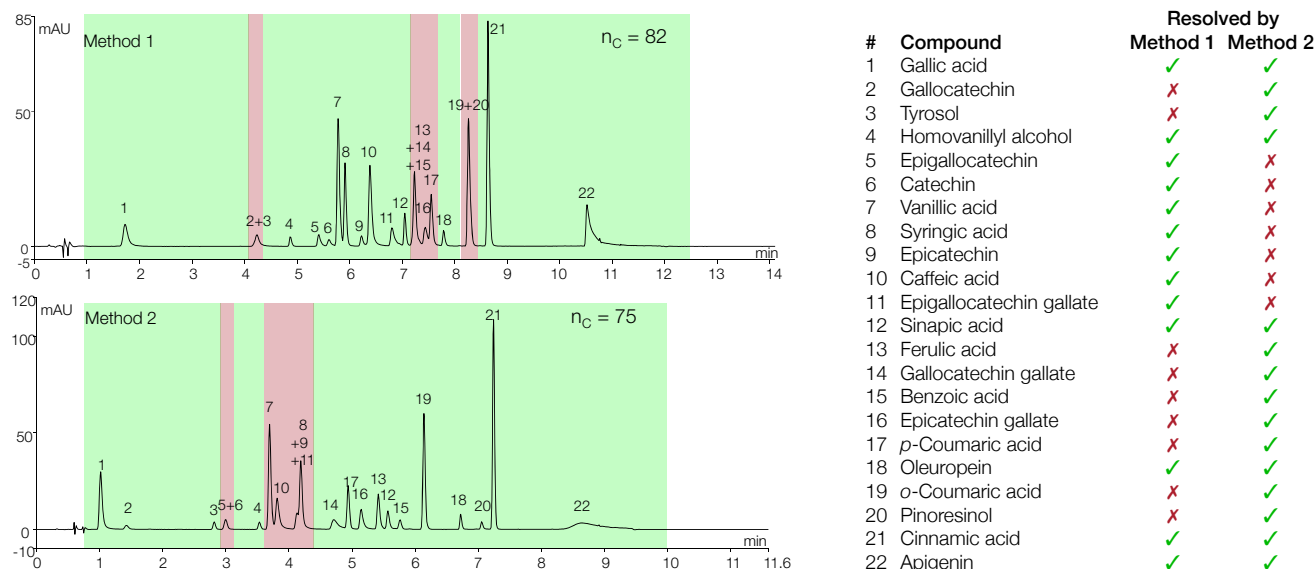


Figure 3. Concept of complementary Dual LC: blue dots represent the test compounds with  $t_R$  by Method 1 and 2; clustering along the diagonal proves strong correlation; green lines mark resolution  $R_s \geq 1.5$  in the respective method, red lines mark peak overlap; each dot is tagged with at least one green line

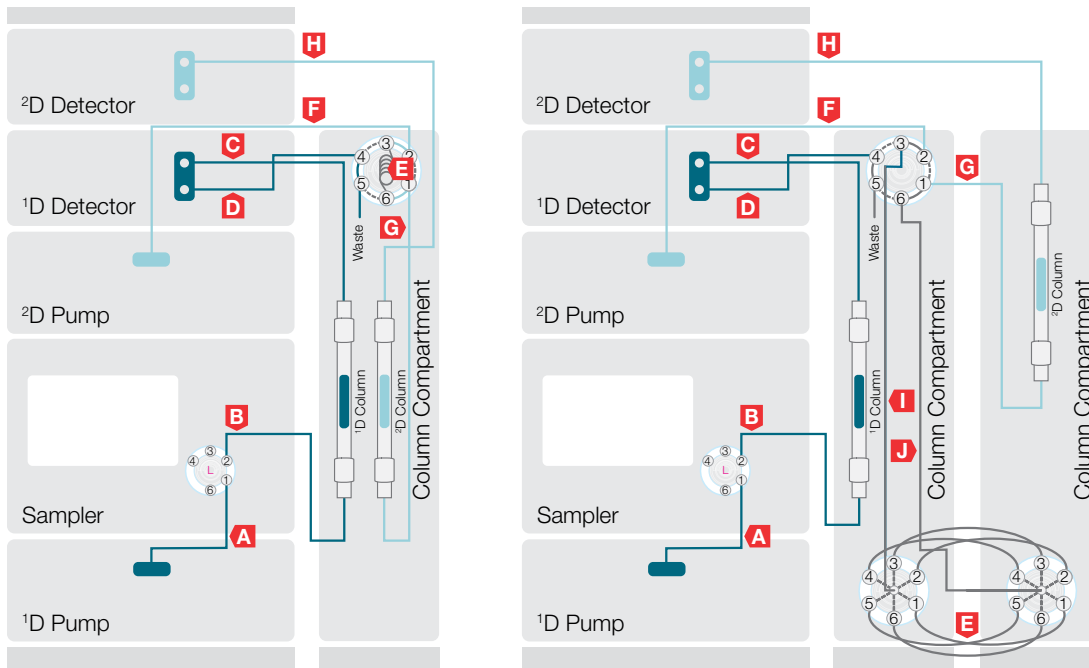


**Figure 4. Complementary Dual LC of the 22 test compounds (25 µg/mL, 260 nm); green color marks full resolution ( $R_s \geq 1.5$ ), red color marks peak overlap ( $R_s < 1.5$ )**

To set up the 2D-LC instrument a conventional loop-based online configuration was selected, meaning that the interface, which transfers fractions of the  $^1D$  eluate to the  $^2D$  separation, consists of switching valves and storage loops. For single heart-cut 2D-LC one 6-port/2-position valve and one loop is installed (Figure 5 left). If multiple heart-cuts are required the setup can be upgraded by two additional 7-port/6-position selection valves equipped with up to six loops (Figure 5 right). The  $^1D$  pump and column are in-line with the injector, the two-position valve controls if the  $^1D$  eluate is going to waste or directed to the loop interface. The target fractions are placed in the respective loops before the  $^2D$  flow path is switched in-line and successively processes each loop with the  $^2D$  analysis. The individual steps are illustrated in Figure 6.

As all 1D-LC screenings exhibited more than one zone of overlapping peaks, multiple heart-cuts were required for the current analysis, targeting a quantitative determination by UV detection. The columns selected from the given set were the Accucore Polar Premium and the Acclaim Phenyl-1 with methanol gradients. Although this pair had a quite high  $r^2=0.9343$  the selection was made due to the following four reasons: (1) for better comparability one of the methods from the Dual LC approach should be used as the  $^1D$ ; (2) a column of wider inner diameter should be used in the  $^2D$  due to the high fraction volumes to be transferred. These result from cuts over the complete  $^1D$  peak widths for a reliable quantification;<sup>7</sup> (3) a more retentive column is preferred in the  $^2D$  to counteract solvent mismatch effects caused by the elution power of the  $^1D$  solvent plug, if no solvent modulation is performed; (4) the  $^2D$  has to provide

full separation of the critical peak pairs of the  $^1D$ . The selected column pair met all the criteria. Method 1 was set as  $^1D$  and a fast gradient that resolved all critical peak pairs was developed for the  $^2D$  (Method 3). The areas marked red in Figure 4 for Method 1 were transferred in four fractions into the storage loops (the coeluting peaks 13/14/15 and 16/17 were split in two fractions to maintain the inherent resolution and to limit the fraction volume size). As only four loops were required the 2D-LC method was setup with the 2-position valve in position 1\_2 for the complete  $^1D$  run time. Loops 1–4 were dedicated for fraction storage and loop 5 was set as default and flushed when no cut was intended. With that technique less pressure shocks affected the  $^1D$ . Furthermore, the cut delay was optimized to compensate the delay between  $^1D$  detection and arrival at the selection valve. In order to obtain valid quantitative results accurate peak cutting is of high importance. The capillaries d and i in Figure 5 provided a calculated volume of around 8.6 µL, corresponding to ~0.02 min delay at a flow rate of 400 µL/min. The optimal cut delay of 0.02 min was confirmed by experimental variations from 0.00 to 0.05 min. The resulting  $^1D$  and  $^2D$  chromatograms are displayed in Figure 7, demonstrating the full resolution of the 22 test compounds. An adequate multi-heart-cut 2D-LC method was set up with the selected column pair. However, the effect of transferring high fraction volumes (90–140 µL) with a substantial amount of organic solvent is still visible in the  $^2D$  for compound 17. Due to the high elution power of the fraction solvent, the focusing at the head of the  $^2D$  column can be impaired in RP-RP combinations, even if the more retentive column is used in the  $^2D$  as generally recommended.

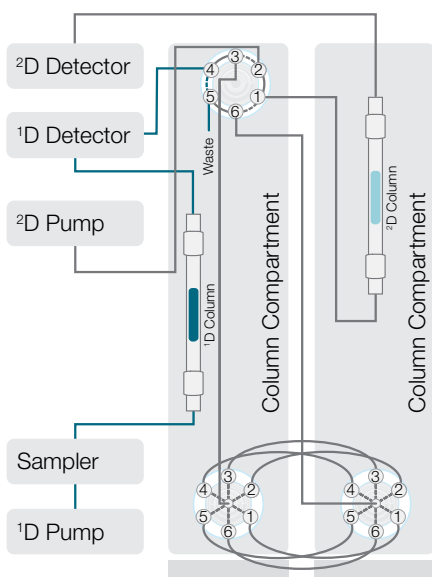


#	Capillary
a	0.1 × 350 mm (P/N 6042.2340)
b	active preheater 0.1 × 380 mm (P/N 6732.0110)
c	post-column cooler 0.1 × 590 mm (P/N 6732.0520)
d	0.1 × 350 mm (P/N 6042.2340)
e	loops; size depends on the application; i.e.: sample loop 200 $\mu$ L (P/N 6830.2418) sample loop 20 $\mu$ L (P/N 6826.2420)

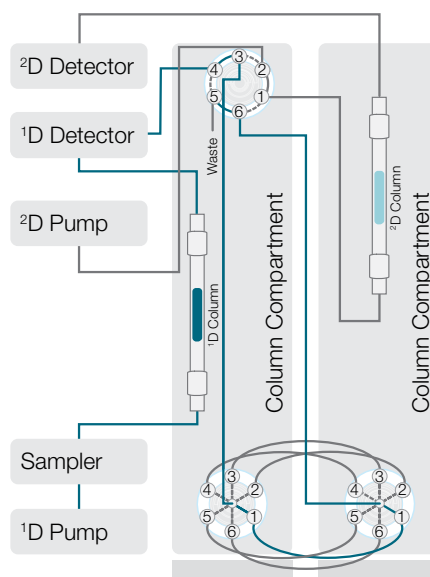
#	Capillary
f	0.1 × 450 mm (P/N 6042.2350)
g	active preheater 0.1 × 610 mm (P/N 6732.0150)
h	post-column cooler 0.1 × 590 mm (P/N 6732.0520)
i	0.1 × 650 mm (P/N 6042.2370)
j	0.1 × 650 mm (P/N 6042.2370)

Figure 5. Loop-based single- (left) and multi- (right) heart-cut online 2D-LC setups with the Vanquish platform

1D analysis / 2D idle



heart-cut



2D analysis

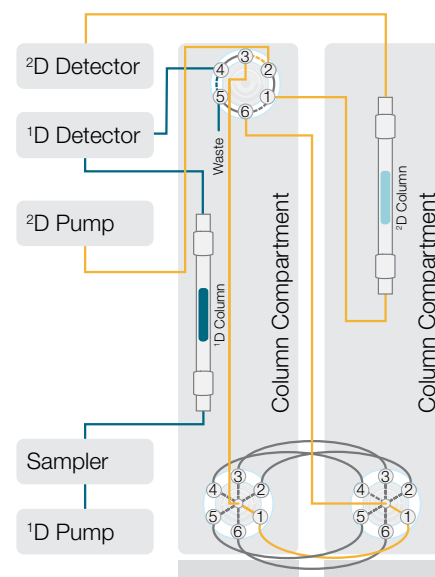
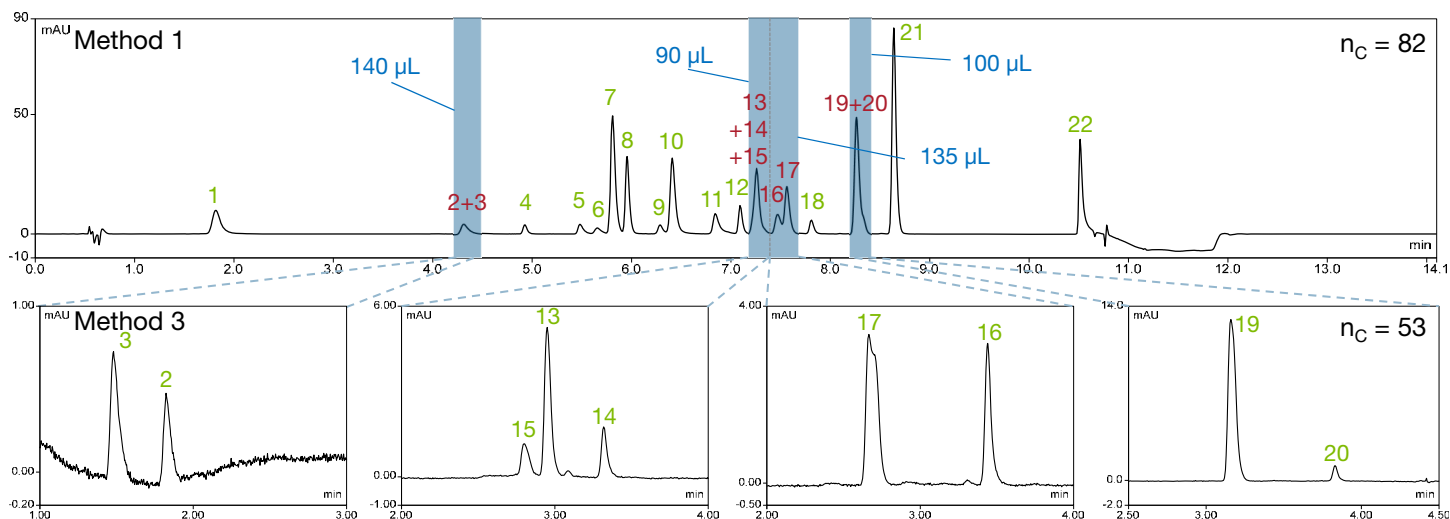


Figure 6. Flow schemes of the different steps in multi-heart-cut 2D-LC (example for loop 1)





**Figure 7.** Multi-heart-cut 2D-LC of the 22 test compounds (25 µg/mL, 260 nm); green numbers mark full resolution ( $R_s \geq 1.5$ ), red numbers mark peak overlap ( $R_s < 1.5$ ), blue numbers indicate the transferred fraction volumes; peak assignment as in Figure 4

### Performance comparison

To compare both techniques, calibration standards from 1 to 200 µg/mL were injected three times respectively. Table 1 summarizes the results on calibration linearity, limits of quantification (LOQ), retention and area precision for each test compound, Figure 8 visualizes some averaged aspects. Equivalent calibration linearities and retention time precisions were seen for both approaches. Area precisions and sensitivities were also very similar for all the compounds, which could be separated in the  $^1D$  in 2D-LC. However, if a compound was transferred to the  $^2D$  its LOQ and area standard deviation were significantly higher compared to the Dual LC approach. This is mainly due to the dilution effect when peaks are cut and transferred to a column of wider diameter and higher flow rate. Thus, the average sensitivity and area precision of the current 2D-LC method were inferior to the complementary Dual LC methods. It should be mentioned here that effective techniques to improve the detection sensitivity in 2D-LC have already been established elsewhere but would require changes in the instrumental setup, e.g., for the decrease of solvent strength and improved focusing.<sup>7</sup>

The analysis time of the dual methods run in parallel was equivalent to a single 1D-LC analysis and thus distinctly shorter than under 2D-LC conditions. The solvent consumption increased more than 10-fold with the multi-heart-cut setup due to the longer run time and high flow rate in the  $^2D$ . Sample consumption was doubled with

Dual LC though, because of a duplicate injection. The peak capacity ( $n_c$ ) of a method is a property that gives the number of theoretically separable peaks and is usually calculated by dividing the gradient window by the average peak width. The  $n_c$  values of the tested workflows are indicated in Figure 4, Figure 7 and Figure 8. The repetition of the  $^2D$  causes an increased peak capacity of the 2D-LC. However, the produced excess is hardly utilized, causing the peak production rate (number of peaks divided by overall analysis time) being markedly lower than for Dual LC (Figure 8). Thus, the Dual LC approach significantly improved the productivity of the analysis.

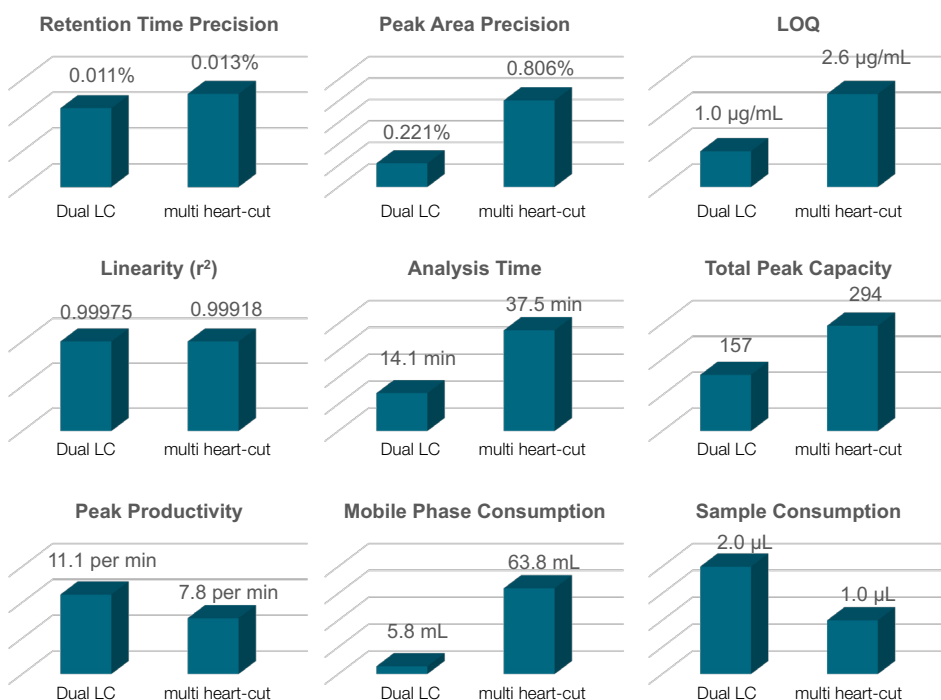
Overall, the Dual LC approach clearly outperformed the multi-heart-cut 2D-LC approach in terms of quantification precision, LOQ, throughput, time, and solvent consumption for the current test case, substantiating parallel complementary Dual LC as a convenient alternative for the separation of semi-complex samples. One further strong point is its simple implementation as common 1D-LC is applied and no advanced instrument and method setup is required. Nevertheless, it is worth mentioning that Dual LC is not presented as a general substitute for heart-cut 2D-LC techniques. For instance, applications that utilize heartcutting for the removal of complex matrix components or high salt contents from the  $^1D$  mobile phase cannot be easily replaced by dual 1D-LC.



**Table 4. Comparison of chromatographic performance in Dual LC and multi-heart-cut 2D-LC mode;  $\lambda$ =detection wavelength,  $r^2$ =determination coefficient of linear calibration curve, LOQ=limit of quantification, %RSD=relative standard deviation**

Compound	Dual LC					multi-heart-cut 2D-LC				
	method ( $\lambda$ [nm])	$r^2$	LOQ [ $\mu\text{g/mL}$ ]	$t_R$ %RSD*	area %RSD*	method ( $\lambda$ [nm])	$r^2$	LOQ [ $\mu\text{g/mL}$ ]	$t_R$ %RSD*	area %RSD*
Gallic acid	2 (280)	0.99995	0.3	0.045	0.347	1 (260)	0.99592	0.9	0.025	0.503
Gallocatechin	2 (260)	0.99972	3.3	0.032	0.272	3 (260)	0.99904	17.5	0.070	4.218
Tyrosol	2 (280)	0.99997	1.2	0.017	0.383	3 (280)	0.99883	6.2	0.002	0.843
Homovanillyl alcohol	1 (280)	0.99999	0.8	0.009	0.122	1 (280)	0.99999	0.8	0.000	0.410
Epigallocatechin	1 (260)	0.99802	1.9	0.009	0.517	1 (260)	0.99885	2.2	0.009	0.199
Catechin	1 (280)	0.99995	1.8	0.008	0.247	1 (280)	0.99996	1.6	0.000	0.325
Vanillic acid	1 (260)	0.99999	0.2	0.009	0.018	1 (260)	0.99999	0.2	0.000	0.120
Syringic acid	1 (280)	0.99997	0.3	0.008	0.026	1 (280)	0.99997	0.3	0.000	0.134
Epicatechin	1 (280)	0.99999	1.3	0.008	0.219	1 (280)	0.99999	1.2	0.000	0.164
Caffeic acid	1 (280)	0.99923	0.6	0.008	0.279	1 (280)	0.99949	0.4	0.000	0.270
Epigallocatechin gallate	1 (260)	0.99971	1.6	0.001	0.140	1 (260)	0.99987	1.5	0.007	0.185
Sinapic acid	1 (280)	0.99995	0.6	0.000	0.166	1 (280)	0.99994	0.6	0.000	0.018
Ferulic acid	2 (280)	0.99997	0.3	0.017	0.060	3 (280)	0.99993	1.0	0.016	0.737
Gallocatechin gallate	2 (280)	0.99956	2.1	0.036	0.156	3 (280)	0.99646	5.8	0.000	2.384
Benzoic acid	2 (260)	0.99997	1.7	0.001	0.368	3 (260)	0.99926	7.7	0.069	2.041
Epicatechin gallate	2 (280)	0.99997	0.8	0.010	0.247	3 (280)	0.99944	3.1	0.001	1.362
<i>p</i> -Coumaric acid	2 (280)	0.99994	0.2	0.009	0.070	3 (280)	0.99997	1.0	0.037	0.474
Oleuropein	1 (260)	0.99955	1.4	0.006	0.315	1 (260)	0.99984	1.7	0.006	0.511
<i>o</i> -Coumaric acid	2 (280)	0.99997	0.1	0.007	0.077	3 (280)	0.99988	0.5	0.015	0.564
Pinoresinol	2 (280)	0.99998	0.8	0.006	0.237	3 (280)	0.99997	2.2	0.013	0.561
Cinnamic acid	1 (280)	0.99997	0.1	0.000	0.041	1 (280)	0.99999	0.1	0.000	0.061
Apigenin	1 (260)	0.99907	1.4	0.004	0.546	1 (260)	0.99530	0.4	0.014	1.656
<b>average</b>		<b>0.99975</b>	<b>1.0</b>	<b>0.011</b>	<b>0.221</b>		<b>0.99918</b>	<b>2.6</b>	<b>0.013</b>	<b>0.806</b>

\* for three injections of 25  $\mu\text{g/mL}$  standard



**Figure 8. Comparison of some averaged performance aspects for Dual LC and multi-heart-cut 2D-LC**

## Conclusion

- For the separation and quantitative analysis of moderately complex samples (15–30 compounds), complementary Dual LC provides a convenient alternative to single- and multi-heart-cut 2D-LC approaches.
- In the current test case, multi-heart-cut 2D-LC was limited in comparison to the complementary Dual LC approach in terms of quantification precision, LOQ, throughput, solvent consumption and ease-of-use. Challenging aspects of 2D-LC methods resulting from peak cutting, fraction transfer and dilution can be avoided.
- Due to the availability of two independent flow paths, the Vanquish Duo UHPLC system for Dual LC is eminently suited for the parallel application of complementary Dual LC. It facilitates the accomplishment in the same time as a common single analysis.

## References

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## Appendix: How to setup a multi-heart-cut 2D-LC analysis with a Vanquish UHPLC instrument and the Chromeleon CDS

The basic concept of the method setup consists in the creation of two different instrument methods, one for the  $^1\text{D}$  and one for the  $^2\text{D}$ . The injection sequence then consists of blocks comprising one line for the  $^1\text{D}$  injection and 1–6 lines for the  $^2\text{D}$  analysis, depending on the number of fractions that were collected in the loops (see Figure A 1). The following step-by-step tutorial refers to the instrument setup shown in Figure 5 right.

### Instrument configuration:

- All involved modules are configured as one instrument.
- The two column compartments have to be added as two individual modules, but not in combined mode, to ensure that more than two valves can be configured.
- Make sure that double assignments of module and channel names are avoided, e.g., rename default “PumpModule” to “PumpModule\_1” and default “Pump\_Pressure” to “Pump\_Pressure\_1”. In the special case of two DADs used in one instrument to record UV spectra in both dimensions the renaming of the channel “3DFIELD” needs to be implemented by a certified service technician.

### Step 1: Create a sequence in Chromeleon and add a custom variable

- In the Chromeleon console ribbon, click “Create” → “Sequence” and follow the wizard as usual or copy a sequence.
- Right click into the sequence table header, select “Custom Columns” → “Insert Custom Variable”. In the wizard that opens click “Create a new custom variable”, select a name like “LoopD2”, type is “numeric”, check “allow empty values”, minimum is 1, maximum is 6, unit is empty, precision is 0 (see Figure A 2). A new column will appear in the sequence with the selected name in the header.
- The created custom variable facilitates a flexible selection of the target loop for the  $^2\text{D}$  with just one instrument method. Alternatively, one could create up to six separate instrument methods for each loop if no custom variable is used.

#	UV_VIS_1_1	Name	Type	Level	Position	*LoopD2	Volume [μl]	Instrument Method
1		1ppm	Calibration Standard	1	B:A2		1.00	D1_AccucorePolarPremium 100x2.1 1.9μm_0.4mL_cut+0.02
2	None	1ppm1	Blank		B:A2	1	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
3	None	1ppm2	Blank		B:A2	2	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
4	None	1ppm3	Blank		B:A2	3	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
5	None	1ppm4	Blank		B:A2	4	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
6		5ppm	Calibration Standard	2	B:A3		1.00	D1_AccucorePolarPremium 100x2.1 1.9μm_0.4mL_cut+0.02
7	None	5ppm1	Blank		B:A3	1	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
8	None	5ppm2	Blank		B:A3	2	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
9	None	5ppm3	Blank		B:A3	3	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
10	None	5ppm4	Blank		B:A3	4	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
11		10ppm	Calibration Standard	3	B:A4		1.00	D1_AccucorePolarPremium 100x2.1 1.9μm_0.4mL_cut+0.02
12	None	10ppm1	Blank		B:A4	1	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
13	None	10ppm2	Blank		B:A4	2	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
14	None	10ppm3	Blank		B:A4	3	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%
15	None	10ppm4	Blank		B:A4	4	1.00	D2_Acclaim Phenyl-I-1 100x3 3μm_5%

Figure A 1. Sequence in Chromeleon CDS for multi-heart-cut 2D-LC analysis comprising 3 blocks of  $^1\text{D}$  and  $^2\text{D}$  runs

Custom Variables Wizard

New Custom Variable

Create a new custom variable

Context: Injection

Name: LoopD2

Description: <Enter custom variable description>

Type: Numeric

☒ Allow empty values

Default: 6

Unit:

Minimum: 1

Precision: 0

Maximum: 6

Figure A 2. Custom variable wizard in Chromeleon CDS

## Step 2: Create 1D instrument method

- Instrument methods are created like usual in Chromeleon (follow the wizard, create gradients, detection parameters etc.).
- The 1D instrument method in addition contains the cutting events by the switching valves. As mentioned in the text above one can keep the 2-position valve (UpperValve\_1 in Figure A 3) in collecting position as long as one loop is not needed for fraction storage and can be flushed.
- The flushed loop is the default position for both selection valves (position 5 for LowerValve\_1 and LowerValve\_2 in Figure A 3). For each peak that is cut one switch to the respective storage position is entered to start the cut. To end the cut a switch back to the default position or to the next storage position is entered, if the next fraction follows directly (see Figure A 3).
- For proper cutting the delay between 1D detection and arrival at the loop should be considered.

## Step 3: Create 2D instrument method(s)

- Create the method with its gradient, detection parameters etc. as usual.
- For the 2D instrument method the 2-position valve (UpperValve\_1 in Figure A 4) disconnects the 1D from the loop interface and one loop is set in-line with the 2D flow path.

- For a flexible loop selection, the custom variable created in step 1 is utilized like in Figure A 4. In the script editor of the instrument method add two new commands for both selection valves into the “Inject” stage. The commands are “name of column compartment”. “name of valve”. CurrentPosition (type or select from the drop down list). The values refer to the custom variable that is entered into the sequence: System.Injection.CustomVariables.”name of custom variable”.
- Alternatively, the loop can be fixed for the selection valves. In that case a separate instrument method is required for each loop.

## Step 4: Insert injections to the sequence and run the sequence

- Per sample insert one 1D injection with the 1D instrument method and the respective number of lines with the 2D instrument method, depending on how many cuts are implemented in the 1D (Figure A 1).
- 2D lines need to be set as type “blank” to inhibit the sampler from injecting. If these injections for instance need to be utilized as calibration standards the type has to be changed after the runs are finished.
- If the custom variable is used, enter the respective loop positions into the column created in step 1. If no custom variable is used the different 2D instrument methods have to be selected accordingly in the sequence.
- It is also recommended to use separate processing methods for 1D and 2D runs.
- Start the sequence.

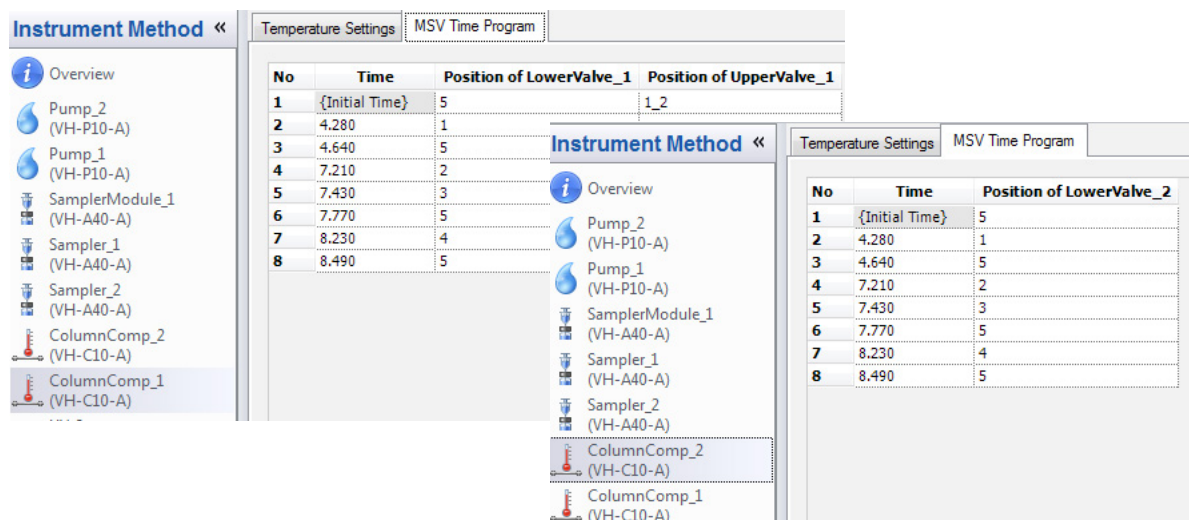


Figure A 3. Switching events of the 1D instrument method; 5 is the default position for the selection valves, 1–4 are storage positions

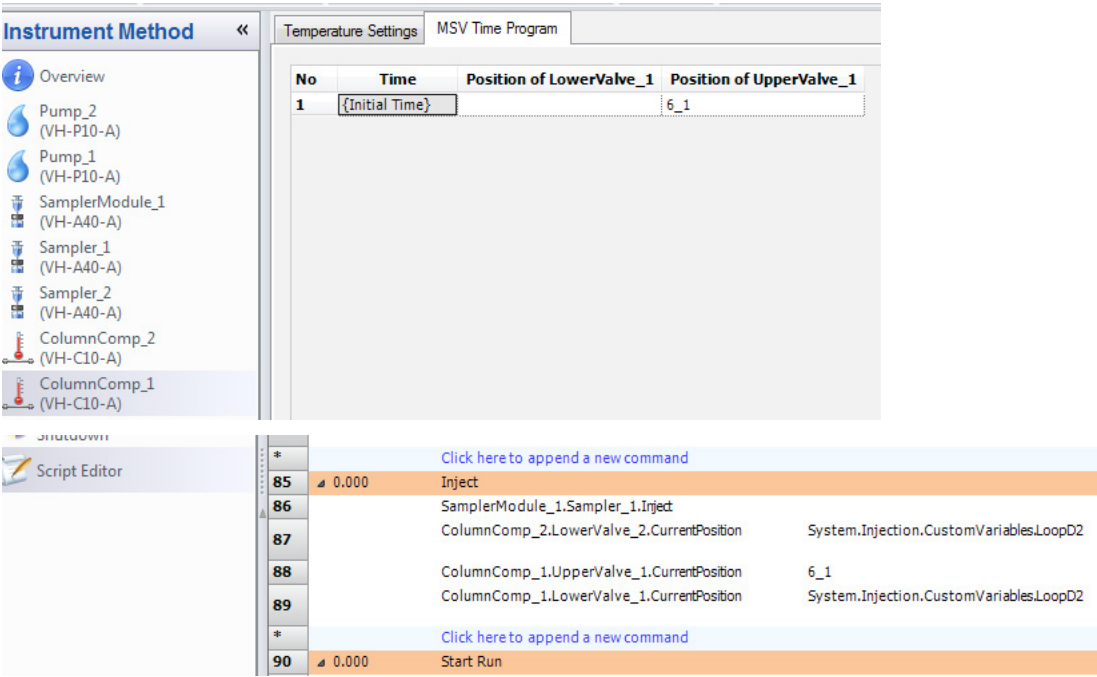


Figure A 4. Switching events of the 2D instrument method

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