Application Note

Biopharma/Pharma

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Online Reaction Monitoring by the Agilent InfinityLab Online LC Solutions

Aspirin hydrolysis with pH value-dependent reaction speed

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Abstract

The objective of this application note is to demonstrate the online monitoring of a small molecule reaction by means of the Agilent InfinityLab Online LC Solutions. The highly precise sampling enables quantitative monitoring of the reaction and accurate determination of the concentration of educts and products in the reaction vessel. The capabilities of the InfinityLab Online LC Solutions to sample and dilute/quench prior to injection or to inject directly for highest speed will be demonstrated for optimized reaction monitoring.

Introduction

In modern production of small molecule pharmaceuticals and biopharmaceuticals, the reaction has to be closely monitored and potentially even controlled by, for instance, online reaction monitoring analytics. Therefore, it can be helpful to connect an UHPLC instrument by a sampling device to the reaction vessel. The InfinityLab Online LC Solutions offers a combined UHPLC and integrated reactor sampling interface for automated reaction sample analysis with the Agilent 1260 Infinity II Online Sample Manager. This device enables drawing of samples from a reactor and dilution/quenching prior to an injection.

This application note demonstrates the use of the InfinityLab Online LC Solutions for monitoring of reactions with different reaction speeds, requiring high sampling speeds for sampling and quenching as well as direct injection for fastest results. As a model reaction, the pH-dependent hydrolysis of acetylsalicylic acid (Aspirin) to salicylic acid has been used.

Experimental

Instrument

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1260 Infinity II Online Sample Manager Set (G3167AA): Agilent 1260 Infinity II Online Sample Manager (G3167A) clustered with external valve (5067-6680) located at the Agilent 1290 Infinity Valve Drive (G1170A) and Agilent Online LC Monitoring Software
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Diode Array Detector (G7115A)

Instrument setup (Figure 1)

The InfinityLab Online LC Solutions feature the Agilent 1260 Infinity II Online Sample Manager. This module is based on the Agilent 1260 Infinity II Multisampler housing, but uses new proprietary technology such as two highly synchronized valves, one inside the module and an additional one outside.[1] The newly developed valve concept with special switching modes enables Agilent Feed Injection and classical flow-through injection. The design of ports and grooves allows direct drawing and injection from a reaction vessel as well as sample handling like dilution and quenching prior to injection. The 1260 Online Sample Manager is connected to the reaction vessel by a separate, independent pump unit, providing a stream of solvent from the reactor. For lowest delay volume, the reactor is connected directly to the pump head by a 0.8 mm id PTFE tubing. The complete sampling and injection processes are controlled by the dedicated Agilent Online LC Monitoring Software, which integrates seamlessly with the Agilent OpenLab CDS.

Agilent Online LC Monitoring Software setup

The Online LC Monitoring Software comprises three sections: Configuration, Experiment Setup, and Experiment Run. In Configuration, the connected CDS and instruments will be chosen and displayed. In Experiment Setup, the analytical method will be combined with sample handling, scheduling, and limits (Figure 2). As limits the area percentage, the concentration or the corrected concentration of a reaction compound can be used. If the limits are crossed in the course of the reaction, a warning will be displayed. In the Experiment Run section, the vials for sampling, calibration, quality controls, and blanks can be chosen, and the experiment can be started. The results of the experiment will also be displayed here.

In the Samples tab of the **Experiment Setup**, the sampling and analysis methods can be selected and combined (Figure 3). In addition, controls like recalibrations, QC samples, and blanks can be configured. For the setup of direct injections, the source of the sample either from the reactor

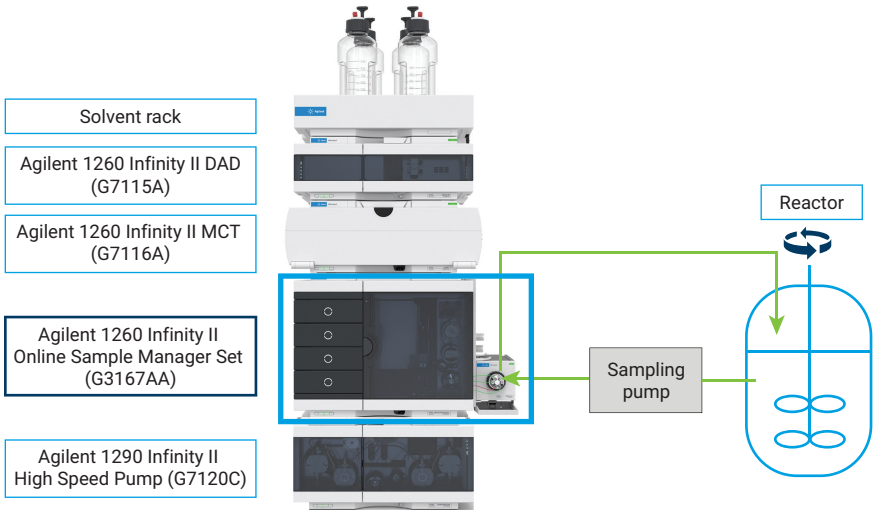


Figure 1. Schematic of an exemplary instrumental setup including sampling pump and reactor. Connection reactor to pump: 0.8 mm id PTFE tubing (p/n 5041-2191), ferrule (p/n 5022-2154), and PTFE nuts (p/n 5022-2158). Connection pump to sampling interface: SST capillary 0.17 mm id, 900 mm length (p/n 5500-1217). Connection sampling interface to reactor: 0.8 mm id PTFE tubing, fittings, and ferrule (p/n 5065-4454).

stream or from a vial can be selected and combined with the analytical setting (Figure 3A). For the setup of samplings from the reactor stream to a vial, the dilution factor and the target volume can be provided, and the required sample volume will be calculated automatically (Figure 3B). The diluted/quenched sample could be either retained for later analysis or analyzed immediately by combining with an analysis method. The Pure to vial setting allows sampling of the undiluted reactor stream sample to a vial with or without analysis, accordingly.

In the Schedule tab, the selected rule-based events, like a shutdown at the end of analysis, will be displayed and the time-based monitoring events can be setup in a table (Figure 4). In the time-based setup section, the previously configured experiments, like direct injection, vial sampling, QC samples, blanks, and recalibrations can be selected. For each line, a start time will be given together with an interval and a count. The end time will be calculated automatically. In the preview, all time-based events will be listed and can be checked for any time conflicts.

In the Limits tab, a limit for lower and higher concentration or area percent can be set. If these limits are hit, a message will be displayed in the results (see Experimental Run section in the Results and discussion section).

Software

- Agilent OpenLab CDS 2.6 or later version
- Agilent Online LC Monitoring Software, version 1.0.1

Columns

Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (part number 959757-902)

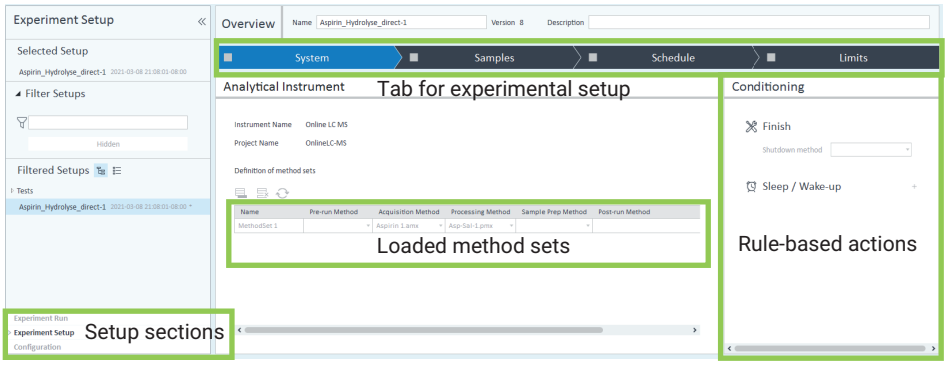


Figure 2. Agilent Online LC Monitoring Software in **Experiment Setup**, showing the system setup. Methods can be chosen and combined to a method set. Rule-based actions, as well as sample preparation methods, can be defined.

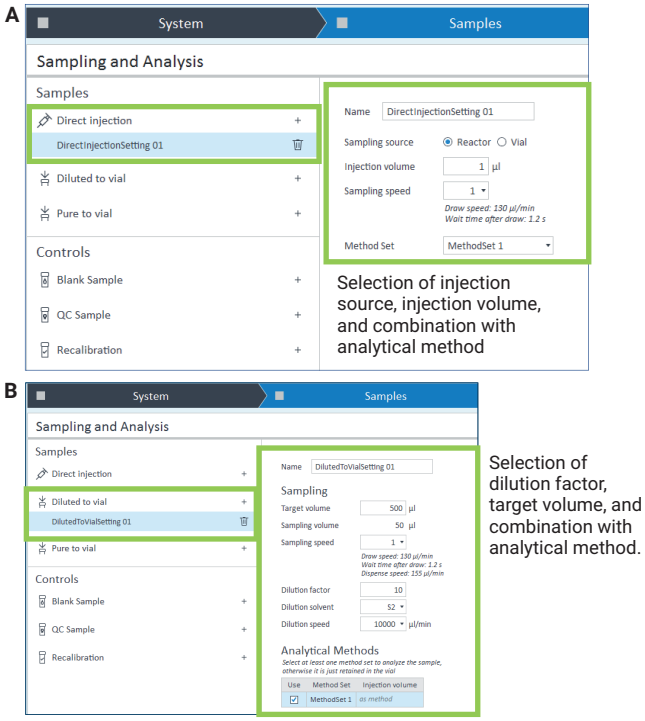


Figure 3. A) Settings for direct injection from reactor stream or vial. B) Settings for dilution/quenching of reactor stream sample with or without injection.

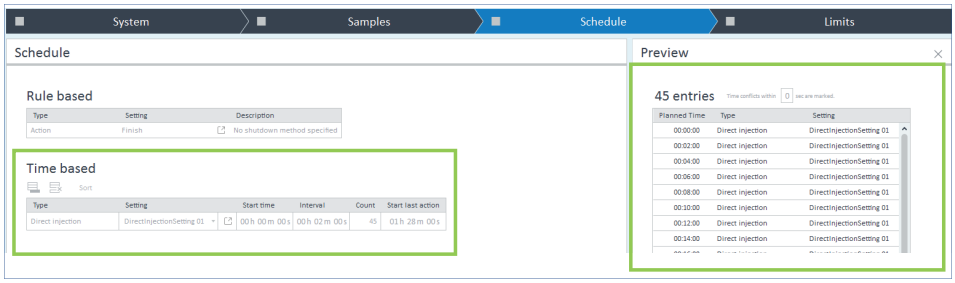


Figure 4. Schedule tab in the **Experiment Setup**. Multiple combinations of time-based events can be setup and a preview can be used to identify and resolve timing conflicts.

Samples and dilution for calibration

- **Educt:** acetylsalicylic acid, product: salicylic acid
- **Stock solution of 1 g/L acetylsalicylic acid and 1 g/L salicylic acid:** 100 mg each dissolved in 10 mL of ethanol (EtOH), filled up with water to 100 mL
- **Dilution series:** 1000, 200, 100, 20, 10 mg/L

Sample delivery pump

- **Pump:** Agilent 1260 Infinity II Isocratic Pump (G7110B)
- **Flow rate:** 5 mL/min
- Solvent stream from reaction vessel to Online Sample Manager reactor interface and back to reaction vessel (see also instrument setup)

Setup of the experiment in the Online LC Monitoring Software

- Direct injection
  - **Direct sampling from reactor:** 1 µL (as method)
  - **Sampling speed setting:** 1
  - **Schedule:** interval: 2 minutes, run time 90 minutes
- Dilution to vial
  - **Dilution factor:** 1:10, dilution solvent (S2): water + 10% ACN + 0.1% FA
  - **Target volume:** 500 µL
    Sample volume: 50 µL
  - **Sampling speed setting:** 1
  - **Dilution eject speed:** 10,000 µL/min
  - Agilent InfinityLab deep-well plates, 31 mm, 1 mL (part number 5042-6454) for sampling
  - Agilent InfinityLab silicone sealing mats for well plates (part number 5043-9317)
  - **Schedule:** interval: 5 minutes, run time 180 minutes

Chromatographic method in OpenLab CDS 2.6

Parameter	Value
Flow Rate	A) Water + 0.1% FA, B) ACN + 0.1% FA, 0.7 mL/min
Isocratic Conditions	35% B, stop time 1.0 min
Injection Volume	1 µL
Needle Wash	3 s, wash solvent (S1): water + 50% ACN + 0.1% FA
Feed Injection	Feed speed: adaptive 80% of flow rate Overfeed volume: automatically calculated dependent on injection volume Overfeed solvent (S2): water + 10% ACN + 0.1% FA
Column Temperature	45 °C
DAD	230/4 nm, ref. 360/100 nm, data rate 20 Hz

Data processing method in OpenLab CDS data analysis

Parameter	Value
Integration	
Off at 0.001 min, on at 0.55 min, off at 0.95 min	
Area Reject	15.00
Height Reject	1.70
Peak Width	0.02
Area% Reject	0.00
Slope Sensitivity	5.0
Shoulders Mode	Off
Identification	
Acetylsalicylic Acid	Signal DAD1A, RT 0.638 min, window 0.1 min
Salicylic Acid	Signal DAD1A, RT 0.797 min, window 0.1 min
Calibration	
Concentration Unit	mg/L
Response	Area
Weighting	None
Curve model	Linear, ignore origin
Levels	1,000, 200, 100, 20, 10 mg/L

Reaction setup

A solution of 100 mg Aspirin in 10 mL of EtOH was added very quickly with a syringe under intense stirring by a magnetic bar to 90 mL of glycine buffer at the chosen pH value.

Chemicals

Acetylsalicylic acid, salicylic acid, glycine, NaCl, NaOH, HCl, EtOH

Buffer

- **Solutions:**
  1) 0.1 M glycine + 0.1 M NaCl in 1 L of water
  2) 0.1 M NaOH
- **pH 11:** 52 mL of solution 1 + 48 mL of solution 2
- **pH 12:** 45 mL of solution 1 + 55 mL of solution 2
- pH was adjusted by either 0.1 M NaOH or 0.1 M HCl

Solvents and chemicals

- All solvents were purchased from Merck, Germany.
- Chemicals were purchased from VWR, Germany.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane pointofuse cartridge (Millipak).

Results and discussion

The hydrolysis of acetylsalicylic acid (Aspirin) was chosen as a model reaction to demonstrate the capabilities of the InfinityLab Online LC Solutions compromising the 1260 Infinity II Online Sample Manager and the Online LC Monitoring Software (Figure 5). The speed of this reaction can be influenced by the pH value of the applied buffer solution for hydrolysis. As such, different capabilities of sampling, sample handling, and speed could be demonstrated using this model reaction.

Prior to the chemical experiment, a method for fast separation of acetylsalicylic acid and salicylic acid was developed (see Experimental). This method was used to generate a data processing method with calibration for both compounds to quantify the course of the chemical reaction (Figure 6).

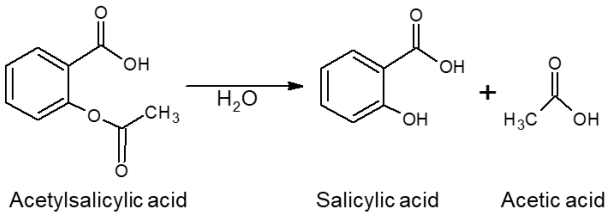


Figure 5. Hydrolysis of acetylsalicylic acid (Aspirin) to salicylic acid and acetic acid.

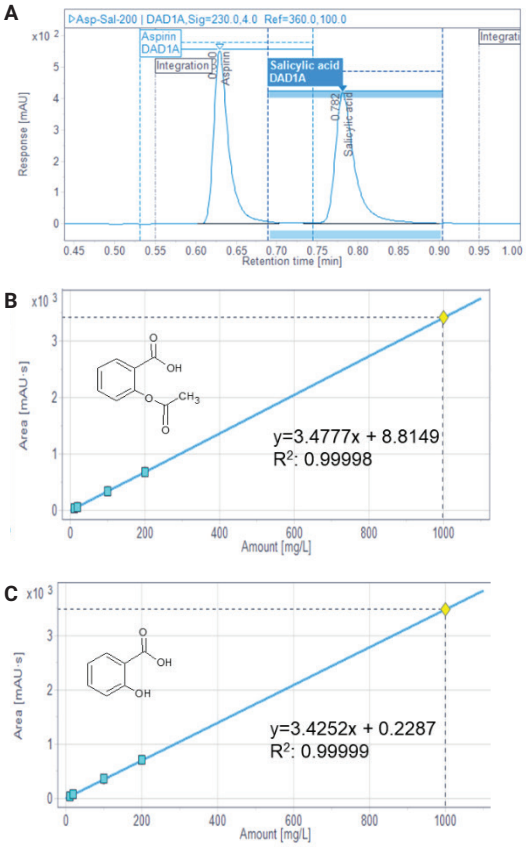


Figure 6. Calibration of acetylsalicylic acid and salicylic acid to generate a data processing method. (A) Fast separation of acetylsalicylic acid and salicylic acid and integration ranges (200 mg/L). (B) Calibration curve acetylsalicylic acid (Aspirin), R^2: 0.99998. (C) Calibration curve of salicylic acid, R^2: 0.99999.

Hydrolysis of acetylsalicylic acid at pH 11

To set up the experiment, 90 mL of glycine buffer at pH 11 was filled in a flask and 100 mg acetylsalicylic acid, dissolved in 10 mL of ethanol, was added quickly under continuous stirring. The flask was connected to the pump to deliver a constant stream of reaction solution at a high flow rate to the reactor interface of the Online Sample Manager. Before the experiment starts, the positions for sampling have to be defined. Additional positions, like for QCs, blanks, and calibrants, could be defined if necessary (Figure 7). After starting the experiment from the Online LC Monitoring Software, the samples were drawn every 5 minutes for dilution and subsequent analysis.

The current status of the individual samples with all information is displayed in the table shown in the **Execution** tab (Figure 8). This table will be populated during the experiment run with real time status information. From this screen, it is also possible to influence the experiment on the fly with changes to samples, methods, and schedule. As can be seen in Figure 8, the data were also reprocessed after acquisition due to the declining peak of acetyl salicylic acid receding below the area reject limit in the data processing method.

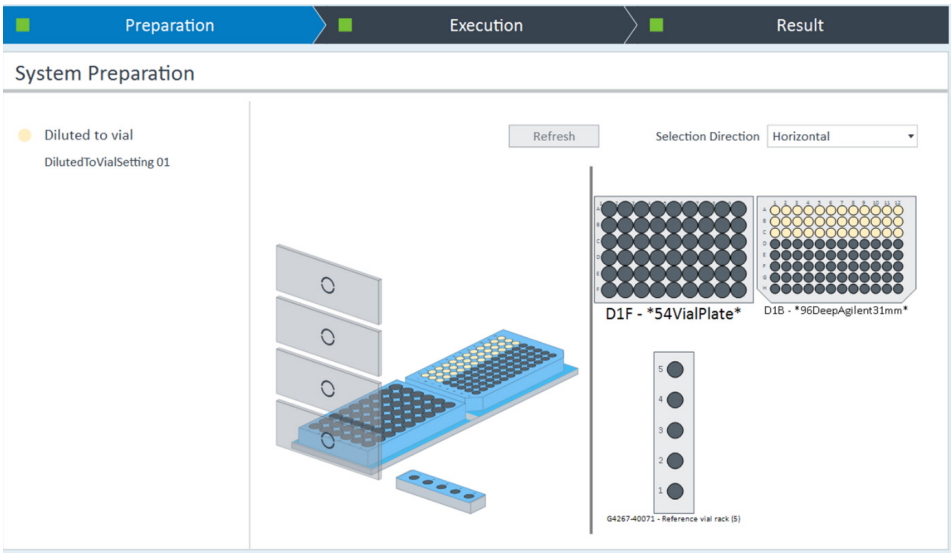
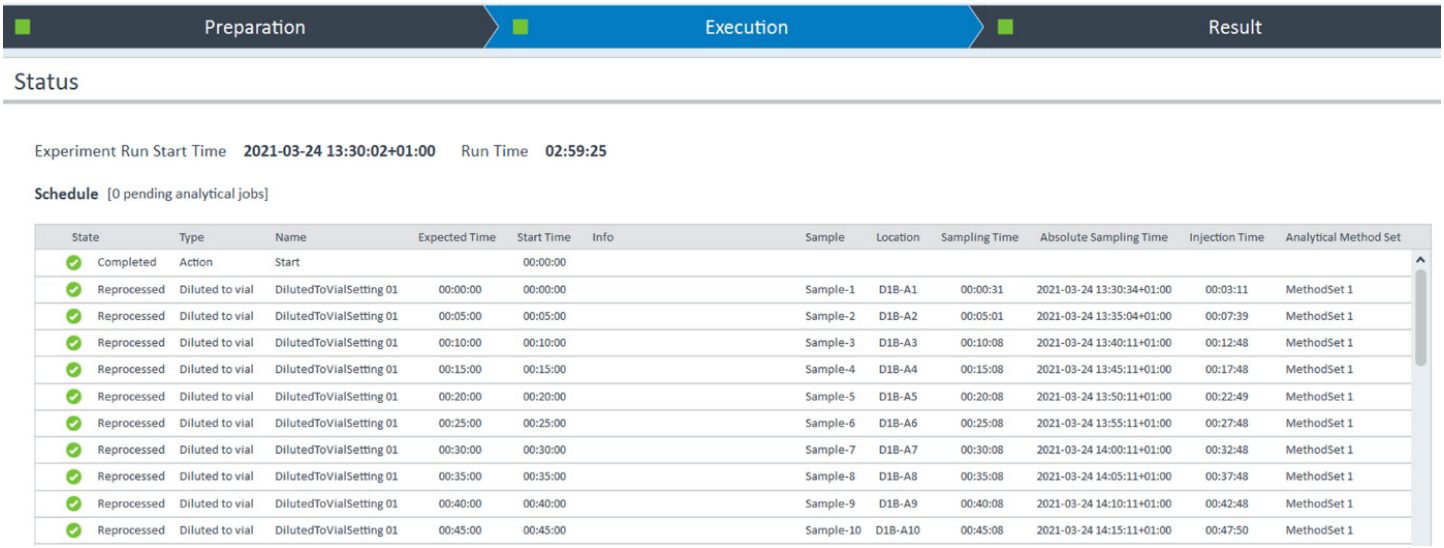


Figure 7. Preparation screen of the experiment showing chosen sampling positions. Additionally, necessary positions for QCs, blanks, and calibrants can be defined here.



State	Type	Name	Expected Time	Start Time	Info	Sample	Location	Sampling Time	Absolute Sampling Time	Injection Time	Analytical Method Set
Completed	Action	Start		00:00:00							
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:00:00	00:00:00		Sample-1	D1B-A1	00:00:31	2021-03-24 13:30:34+01:00	00:03:11	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:05:00	00:05:00		Sample-2	D1B-A2	00:05:01	2021-03-24 13:35:04+01:00	00:07:39	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:10:00	00:10:00		Sample-3	D1B-A3	00:10:08	2021-03-24 13:40:11+01:00	00:12:48	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:15:00	00:15:00		Sample-4	D1B-A4	00:15:08	2021-03-24 13:45:11+01:00	00:17:48	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:20:00	00:20:00		Sample-5	D1B-A5	00:20:08	2021-03-24 13:50:11+01:00	00:22:49	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:25:00	00:25:00		Sample-6	D1B-A6	00:25:08	2021-03-24 13:55:11+01:00	00:27:48	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:30:00	00:30:00		Sample-7	D1B-A7	00:30:08	2021-03-24 14:00:11+01:00	00:32:48	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:35:00	00:35:00		Sample-8	D1B-A8	00:35:08	2021-03-24 14:05:11+01:00	00:37:48	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:40:00	00:40:00		Sample-9	D1B-A9	00:40:08	2021-03-24 14:10:11+01:00	00:42:48	MethodSet 1
Reprocessed	Diluted to vial	DilutedToVialSetting 01	00:45:00	00:45:00		Sample-10	D1B-A10	00:45:08	2021-03-24 14:15:11+01:00	00:47:50	MethodSet 1

Figure 8. Execution table with information including sampling and analysis time, applied methods, and sampling position.

During the run, the progressing results can be watched in real-time within the Result tab's data visualization tools, such as the trending plot of the Online LC Monitoring Software. The final result can be shown in the **Experiment Run** tabs, too (Figure 9). Here, interactively linked graphics and tables will be displayed. A configurable table will summarize the results of selected samples of the reaction course. The table shows e.g. retention times, peak area, area percent, concentration, or corrected concentration, taking the sampling dilution into account (Figure 9A). Exceeded or undershot limits will be marked. And an overlay of the chromatograms of decreasing educts and increasing products can be shown (Figure 9B).

The selected samples in Figure 9 show that the concentration of the reactants is equal for sample 10 after approximately 45 minutes and the concentration of acetylsalicylic acid is below 10% of the initial concentration after 145 minutes.

A

Results

Sample		Method Set	Compound	Signal	RT (min)	Height	Area%	Corr. concentration	Area	Concentration
		MethodSet 1	<ALL>	DAD1A						
Sample-1		MethodSet 1	Aspirin	DAD1A	0.626	282.416	100.000	986.814 mg/L	350.428	98.681 mg/L
Sample-2		MethodSet 1	Aspirin	DAD1A	0.625	255.079	89.820	897.320 mg/L	319.337	89.732 mg/L
		MethodSet 1	Salicylic acid	DAD1A	0.778	18.309	10.180	103.216 mg/L	36.194	10.322 mg/L
Sample-10		MethodSet 1	Aspirin	DAD1A	0.627	116.590	49.319	402.762 mg/L	147.524	40.276 mg/L
		MethodSet 1	Salicylic acid	DAD1A	0.780	94.393	50.681	440.494 mg/L	151.596	44.049 mg/L
Sample-30		MethodSet 1	Aspirin	DAD1A	0.624	20.257	9.101	48.239 mg/L	24.361	4.824 mg/L
		MethodSet 1	Salicylic acid	DAD1A	0.777	151.258	90.899	708.503 mg/L	243.297	70.850 mg/L

B

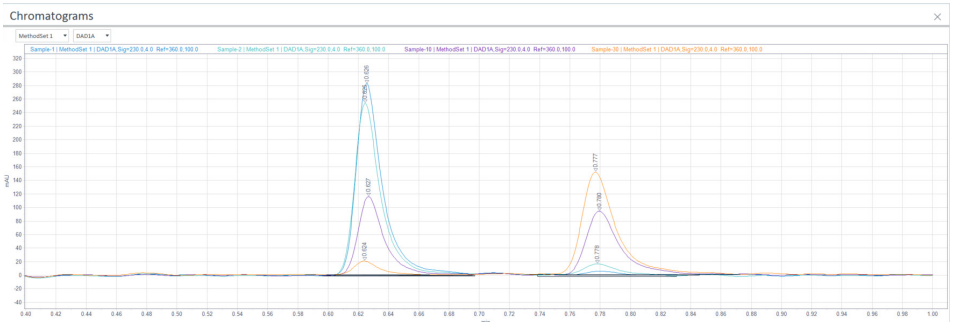


Figure 9. Visualization of the final results of the experiment in the **Result** tab. (A) Table of the results for highlighted reaction data points showing retention time, peak height, area, area percent, concentration, and corrected concentration. (B) Decreasing peaks of acetylsalicylic acid at 0.626 minutes and increasing peaks for salicylic acid at 0.777 minutes.

The results table shown in Figure 9B allows direct access by a one-click shortcut to the data displayed in OpenLab CDS data analysis (Figure 10). Sample 1 in Figure 10 displays the starting point of the reaction, where the turnover of the reaction has just started and the product of the reaction is under the limit of detection. The initial concentration of acetylsalicylic acid in the reaction vessel was 986.8 mg/L. Sample 2, which is drawn after a reaction time of 5 minutes, already shows some product of the reaction, 103.3 mg/L (10.18 area%) salicylic acid in the reaction vessel. Sample 10, drawn after 45 minutes, shows about equal concentration and peak area percent for acetylsalicylic acid and salicylic acid. Sampling point 30, drawn after 145 minutes, is the first to be flagged because the area percent of acetylsalicylic acid went under the defined limit of 10%. With the access to the data in OpenLab CDS data analysis, it is also possible to make changes to the data analysis method. For instance, integration limits could be changed to detect lower abundance peaks like impurities of initially formed products at the beginning of the reaction. The new method could be applied to the generated data in the Online LC Monitoring Software by its built-in reprocessing function, even when the experiment is still running.

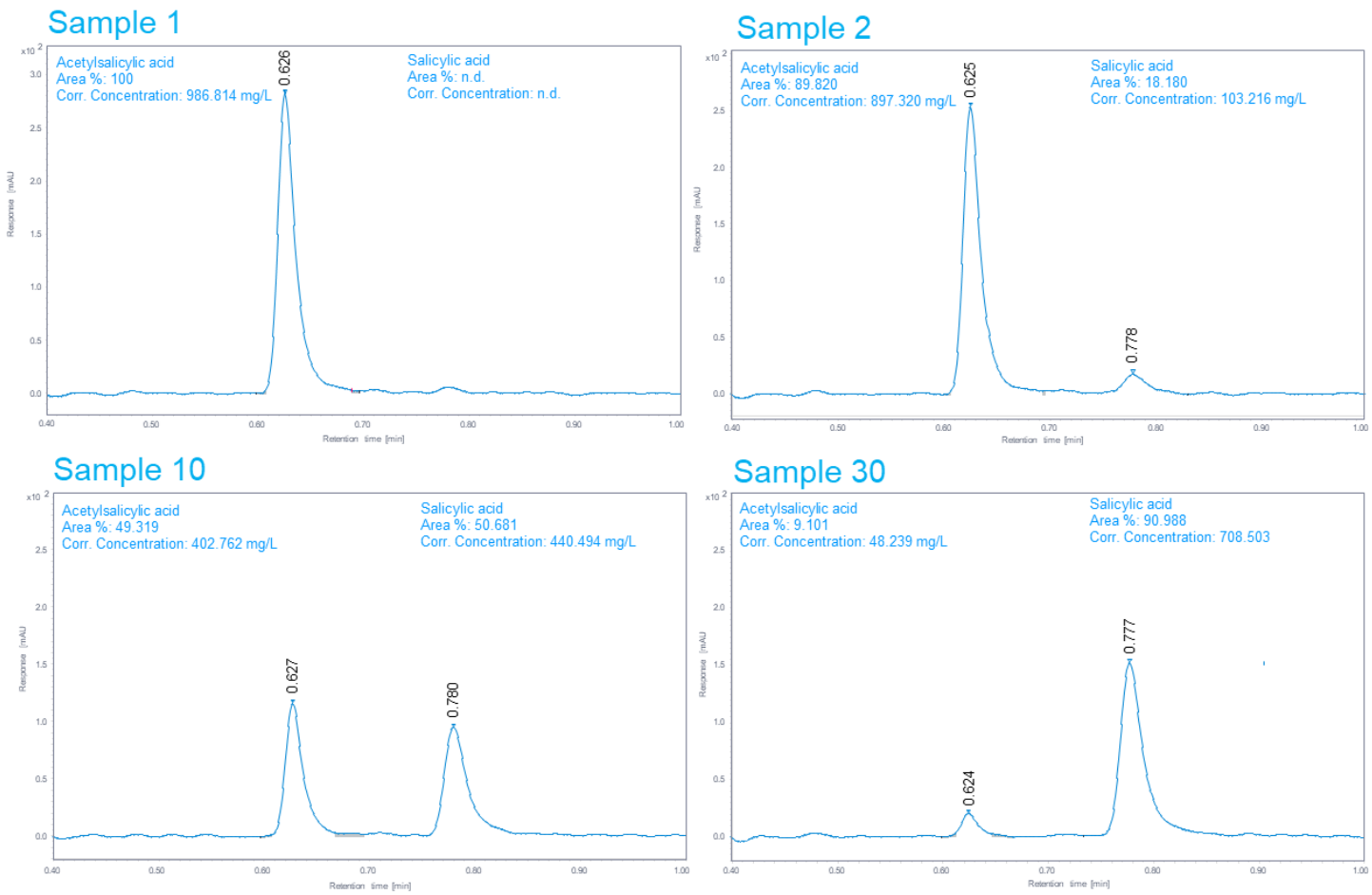


Figure 10. Data of individual runs displayed in Agilent OpenLab CDS data analysis. (Sample 1) First sample drawn at the start of the reaction showing only the educt. (Sample 2) Second sample drawn after 5 minutes beginning to show some product of the reaction. (Sample 10) Drawn 45 minutes after starting the reaction shows equal peak areas and concentrations. (Sample 30) The first sample to be flagged because the educt concentration went below the defined limit of 10 area%.

The Online Sample Manager makes it possible to apply different solvents for needle wash, wash of flow path, as well as for dilution and quenching. This enables stopping a reaction after sampling by dilution with a quenching solvent. In this example, the dilution was done with water + 10% ACN + 0.1% formic acid, which slows down the reaction (Figure 11). Only a slight degradation of Aspirin in the 1:10 dilution/quenching can be seen after 24 hours by a lower peak for acetylsalicylic acid and a higher peak for salicylic acid. This allows post experiment analysis or additional quality control by means of other analytical techniques.

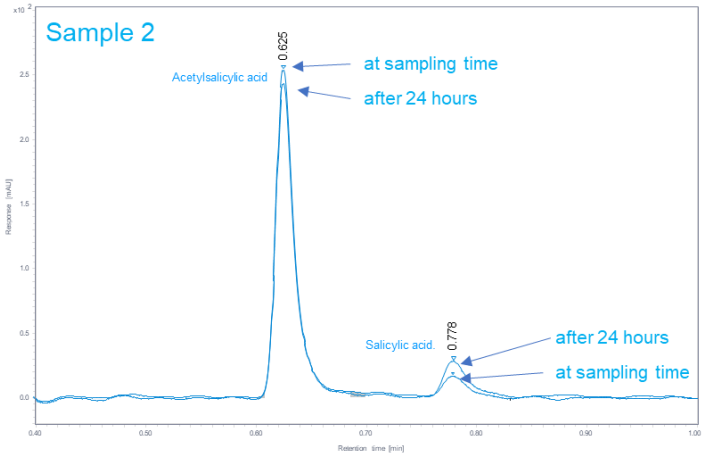


Figure 11. Overlay of diluted sample immediately after sampling and dilution/quenching and after 24 hours.

Hydrolysis of acetylsalicylic acid at pH 12

To demonstrate the capability of the Online LC to cope with fast reactions, the speed of the hydrolysis reaction of acetylsalicylic acid was increased by an increase of the pH value to 12. For monitoring of fast reactions, the sample was injected by direct drawing from the reactor stream into the needle without any mechanical movement, just by switching of the reactor interfacing valve. The chromatographic run time of 1 minute, together with all other processes, enabled a cycle time of 2 minutes per data point for the chosen method settings. The resulting trending plot (Figure 12A) shows a sample data point every second minute. The decreasing peak area curve of acetylsalicylic acid and the increasing peak area curve for salicylic acid cross each other after 20 minutes at equal area percent and concentration. After 78 minutes, the reaction is nearly complete, and the educt crosses the given limit of 10 area%. The results table (Figure 12B) shows details like retention time, peak area, peaks height, area percent, and concentration. The chromatograms (Figure 12C) display

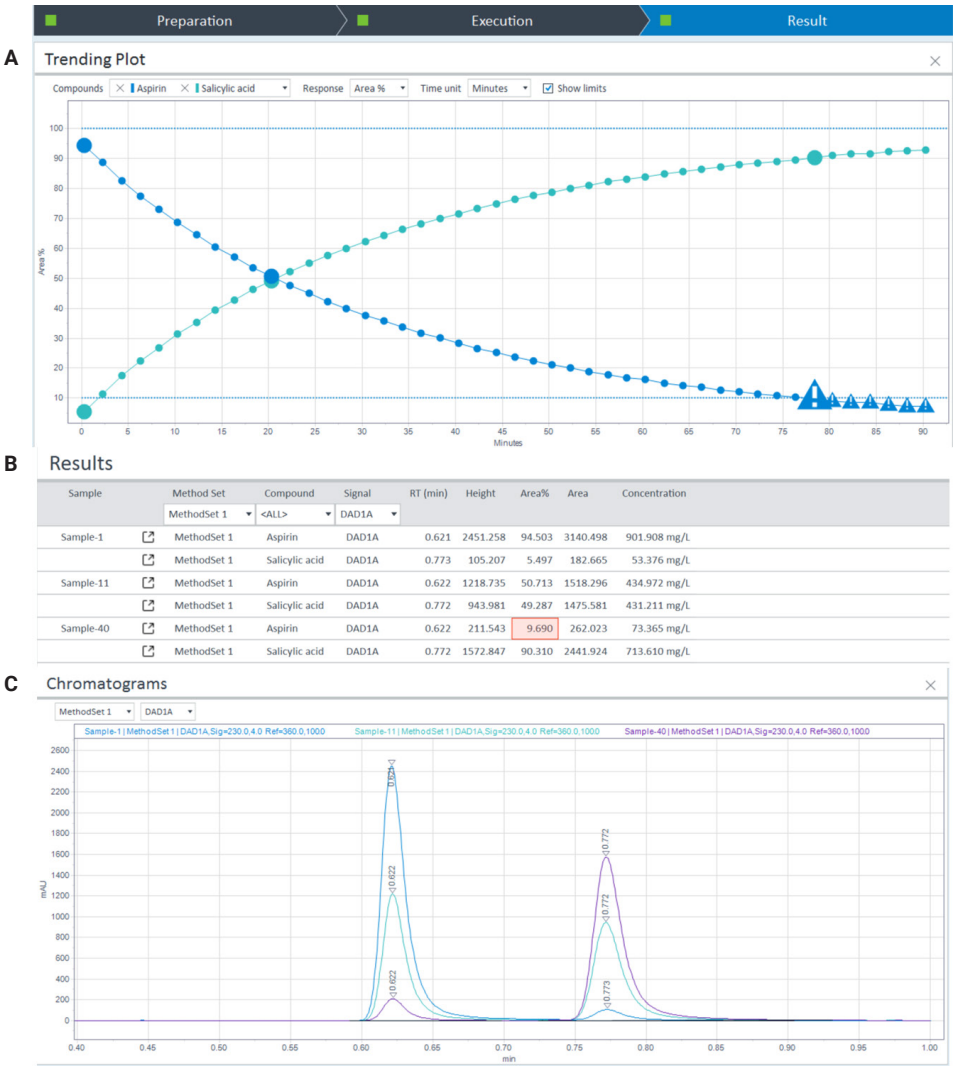


Figure 12. Visualization of the results of the experiment at fast reaction speed in the **Result** tab.
(A) Trending plot of the reactants acetylsalicylic acid and salicylic acid by peak area. (B) Table of the results for highlighted reaction data points showing area, area percent, and concentration. (C) Decreasing peaks of acetylsalicylic acid at 0.622 minutes and increasing peaks for salicylic acid at 0.772 minutes.

an overlay of increasing peaks of salicylic acid at 0.772 minutes and a decreasing peak of acetylsalicylic acid at 0.622 minutes. Initially, the reaction starts very fast, which results in product formation even in the first drawn sample (Figure 12B). During the reaction, the formation of product and the conversion of educt slows down. This could be taken into account by means of less frequent sampling, which could be achieved by introduction of additional time-based events (Figure 4). For instance, a sampling interval of two minutes up to 30 minutes, a sampling interval of 6 minutes up to 60 minutes, and a sampling interval of 10 minutes for rest of the reaction time. This flexibility follows the reaction, with higher speed during the starting phase where this is required, and slower sampling speed with the increased reaction turnover.

Conclusion

This application note demonstrates the use of the Agilent InfinityLab Online LC Solutions including the Agilent 1260 Infinity II Online Sample Manager and Agilent Online LC Monitoring Software to monitor even very fast reactions (e.g., in small molecule synthesis).

The reaction sample could be drawn directly from a reactor stream and diluted or quenched to stop the reaction for immediate or later analysis.

To cope with very fast reactions, it is possible to inject the sample directly after drawing from the reactor stream. This enables very short cycle times for monitoring fast reactions. The highly precise sample drawing and dilution/quenching allow accurate quantification of the reactants inherent in the reaction vessel for optimized gain of valuable products.

Reference

1. Performance Characteristics of the Agilent 1260 Infinity II Online Sample Manager. *Agilent Technologies technical overview*, publication number 5994-3529.

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