Application Note

Small Molecule Pharma

Agilent

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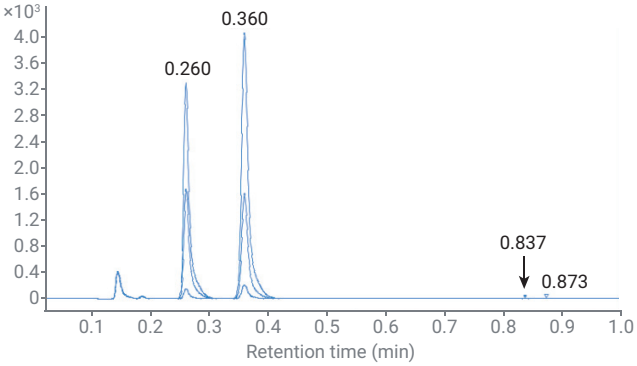
Monitoring of Trace Impurities Using the Agilent 1260 Infinity II Prime Online LC System with High Dynamic Range-DAD



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Abstract

This application note demonstrates the online monitoring of a small molecule reaction by means of the Agilent 1260 Infinity II Prime Online LC, including the Agilent High Dynamic Range - Diode Array Detection (HDR-DAD) Impurity Analyzer Solution. It will be demonstrated that the inclusion of the HDR-DAD offers a higher dynamic range for the detection of trace impurities, in combination with highly concentrated reaction educts and products, by means of two clustered Diode Array Detectors (DAD) with Max-Light cartridge flow cells of different length. Due to the enhanced dynamic range, it is possible to save time by only running a single injection for the simultaneous determination of highly concentrated compounds and trace compounds. This enables quantification of both highly concentrated and trace compounds without detector overload, by means of higher linear range.

Introduction

In the production process of small molecule active pharmaceutical ingredients (API), while the educts and main products of the reaction must be monitored, it is also of crucial importance to monitor the impurities, typically generated under chemical reaction conditions. Due to the fact that a large concentration of the main compound is present in parallel with a low-level impurity, it may be necessary to do the analysis twice. For instance, in UHPLC analysis with diode array detection, a detector cell with 10 mm path length is typically used. This requires separate injections to determine the high concentration compounds and the low-level impurities. For the low-level compounds, a larger injection volume is required, which typically generates a detector overload for the high concentration compounds. To overcome this obstacle, the HDR-DAD solution combines two DADs, one with a 60 mm high sensitivity Max-Light cell and a second one with a 3.7 mm Max-Light cell for highly abundant compounds. Both signals are combined and normalized to a 10 mm cell with a dynamic range up to 6,500 mAU. This is approximately three times higher than the available dynamic range for the Agilent 1290 Infinity II DAD equipped with a standard 10 mm DAD Max-Light cell.[1]

This application note demonstrates the advantage of using the Agilent High Dynamic Range Diode Array Detection (HDR-DAD) Impurity Analyzer solution in combination with the Agilent 1260 Infinity II Prime Online LC for the determination of highly concentrated reaction educts and products, together with occurring low-level impurities in a single analytical HPLC run, while keeping pace with the chemical reaction itself.

Experimental

Instrument:

- Agilent 1260 Infinity II Flexible Pump (G7104C)
- Agilent 1260 Infinity II Online Sample Manager Set (G3167AA): Agilent 1260 Infinity II Online Sample Manager (G3167A) clustered with external valve (part number 5067-6680) located at the Agilent 1290 Infinity Valve Drive (G1170A) and Agilent Online LC Monitoring Software
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- 2 × Agilent 1290 Infinity II Diode Array Detector (G7117B) with 60 mm Max-Light Cartridge Cell (G4212-60007) and 3.7 mm Max-Light Cartridge Cell (G4212-60032)
- Agilent High Dynamic Range DAD Solution Kit (G2199AA)

Column

Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 30 mm, 1.9 µm (part number 695775-302)

Software

- Agilent OpenLab CDS, version 2.6 or later
- Agilent Online LC Monitoring Software, version 1.0

Chemicals

p-Anisaldehyde, acetone, NaOH, formic acid

Additional material

- Agilent 96 deep-well plates, 1 mL, polypropylene (part number 5043-9305)
- Agilent sealing mat, 96 wells, round, preslitted, silicone (part number 5043-9317)

Solvents and chemicals

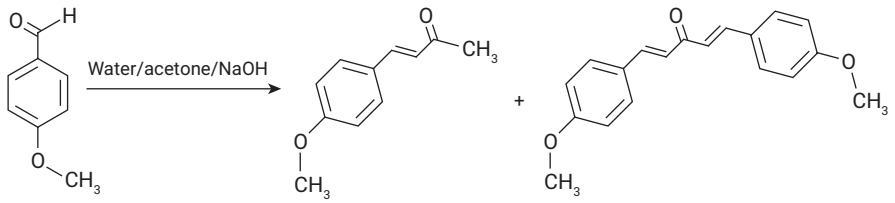
- All solvents were purchased from Merck, Germany
- Chemicals were purchased from VWR, Germany
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Analytical method

Parameter	Value
Solvents	A) Water + 0.1% formic acid (FA) B) Acetonitrile (ACN) + 0.1% FA
Analytical Flow Rate	1.3 mL/min
Gradient	40% B to 90% B in 0.85 min, stop time: 1.0 min
Column Temperature	45 °C
Agilent Feed Injection (Automatic)	80% of analytical flow rate
Flush Out Solvent	Water:ACN 9:1 + 0.1% FA (S2)
Flush Out Volume	Automatic
Injection Volume	1 µL
Needle Wash	3 s, water:ACN 1:1 + 0.1% FA (S1)
Sampling	See sampling methods for sampling to vial
Diode Array Detector	A) 290 ±4 nm, Ref.: off B) 360 ±4 nm, Ref.: off, 40 Hz data rate
Sampling to Vial (Dilutions)	
Sampling from reactor to deep-well plate sealed with silicon mats	
Target Volume	500 µL
Dilution Factor	10
Sample Volume	50 µL
Draw Speed	Setting 2 (draw speed: 100 µL/min, wait time: 3.6 s, dispense speed: 130 µL/min (ejection of sample into well before dilution))
Dilution Solvent	S2
Dilution Eject Speed	10,000 µL/min (after sample ejection for mixing)
Schedule	Interval: 3 min, run time: 90 min
Sample Delivery Pump	
Pump Used	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	
Reaction Conditions	
Educt	*p*-Anisaldehyde, 1 mL
Solvent	100 mL acetone:water 2:1 (v:v)
Stirring at room temperature	
Reaction Start	Add 100 µL NaOH 50% in water (w/w)

Results and discussion

As an example for a small molecule reaction, an aldol condensation of *p*-anisaldehyde and acetone was used.[2] This reaction produces a main product, E-anisylidene acetone, and a low-level impurity of a second condensation with acetone (Figure 1).



p-Anisaldehyde E-Anisylidene acetone Byproduct

Figure 1. Aldol condensation reaction of *p*-anisaldehyde and acetone to the main product E-anisylidene acetone, and a byproduct formed by a second aldol condensation.

For this reaction, the educt and main product were easily monitored by means of a DAD equipped with a standard 10 mm path length Max-Light flow cell.[3] In previous works, an Agilent LC/MSD iQ was used for the identification of low-level impurities.[4] Due to the higher sensitivity and mass selectivity, this is the optimum configuration for the development phase of the reaction and optimization of reaction conditions. However, under production conditions, the HDR-DAD solution is more economical, easier to handle, and already-confirmed low-level byproducts can be monitored in a single run by the higher dynamic range, together with the main compounds.

The HDR-DAD cluster comprises two DADs with flow cells of 60 mm and 3.7 mm path length. The DAD equipped with the long path length cell is capable of detecting the low-level impurities, while the high abundant compound exceeds the linear dynamic range of the detector. On the other hand, the DAD equipped with the short path length flow cell is capable of measuring the high abundant compounds within the linear dynamic range (Figure 2).

For the generation of the High Dynamic Range (HDR) signal, the two DADs are combined electronically and normalized to 10 mm path length (Figure 3). With this signal, it is possible to quantify the high-abundance compounds due to the enhanced linear dynamic range up to approximately 6,500 mAU. In parallel, the low-abundance compounds are also present in the HDR signal. The declining educt *p*-anisaldehyde elutes at 0.26 minutes, and the increasing product E-anisylidene acetone elutes at 0.36 minutes. The low-level byproduct of a second aldol condensation reaction (already identified as described in an earlier application note) eluted at 0.837 minutes.[4]

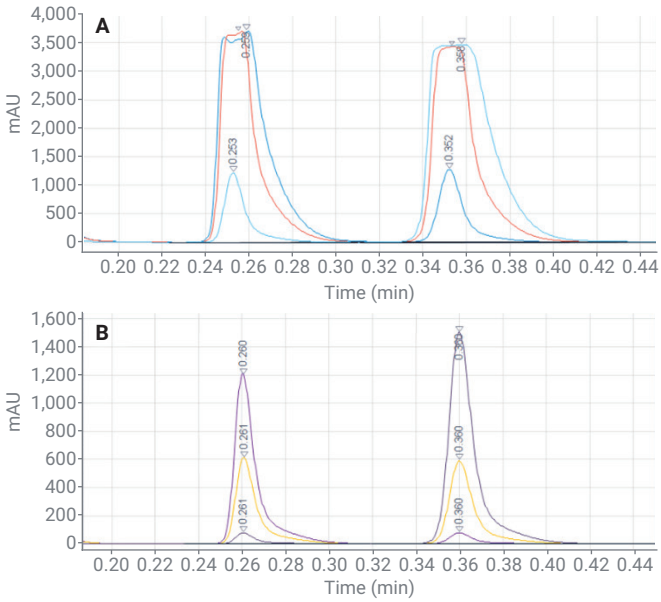


Figure 2. (A) Signal of the DAD equipped with the long path (60 mm) length flow cell. High abundant compounds exceed the dynamic range and cannot be quantified. Lower abundant compounds are detectable. (B) Signal of the DAD equipped with the short path (3.7 mm) length flow cell. The high abundant compounds do not exceed the linear dynamic range of the DAD, and quantification is possible.

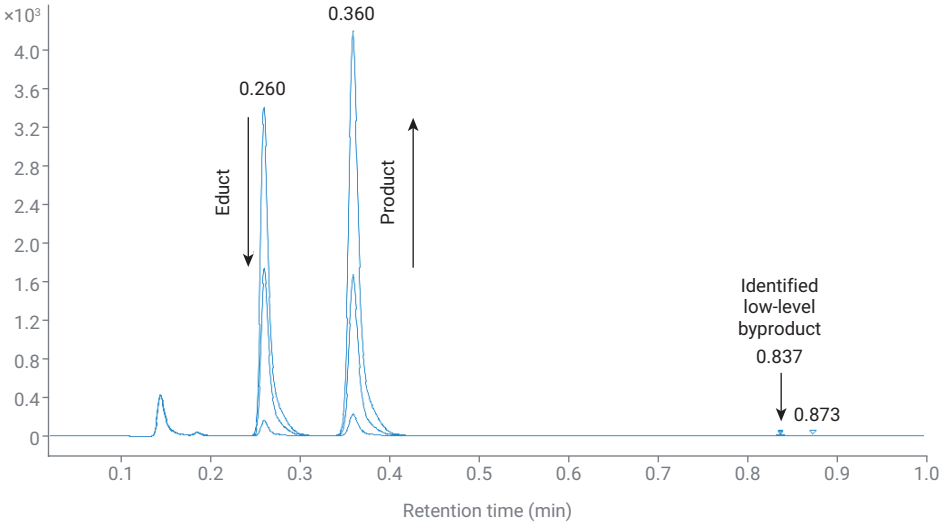


Figure 3. This figure shows the high dynamic range signal (290 nm) of combined long and short path length flow cells. The combined signal provides enlarged dynamic range for the determination of the high abundant main compounds as well as enough sensitivity for the detection of the low-level impurities.

Based on the HDR-DAD signal, a trending plot of the area percentage of the educt, the product, and the byproduct was generated by the Online Monitoring Software. This plot can be reviewed on-the-fly using real-time analysis results (Figure 4). The second sample, drawn at 3 minutes, shows some conversion of the educt to product, but no byproduct. The byproduct appears for the first time in sample 6, drawn at 15 minutes reaction time. At the end of the reaction time of 90 minutes, the educt is nearly completely transferred to product, and the byproduct still stays at a very low level.

Table 1 outlines in detail the measured values of retention time, area, height, area percentage, and corrected amount (1:10 dilution) of the samples highlighted in Figure 4. The area percentage of educt and product is nearly equal in sample 6. This sample is also the first one which shows the byproduct at a very low level of 0.023 area percentage. Due to the fact that the UV response of educt and product are different, a relative single-point calibration curve was generated only for the educt *p*-anisaldehyde, measured by HDR-DAD with the enlarged dynamic linear range. For that purpose, the educt concentration in sample 1

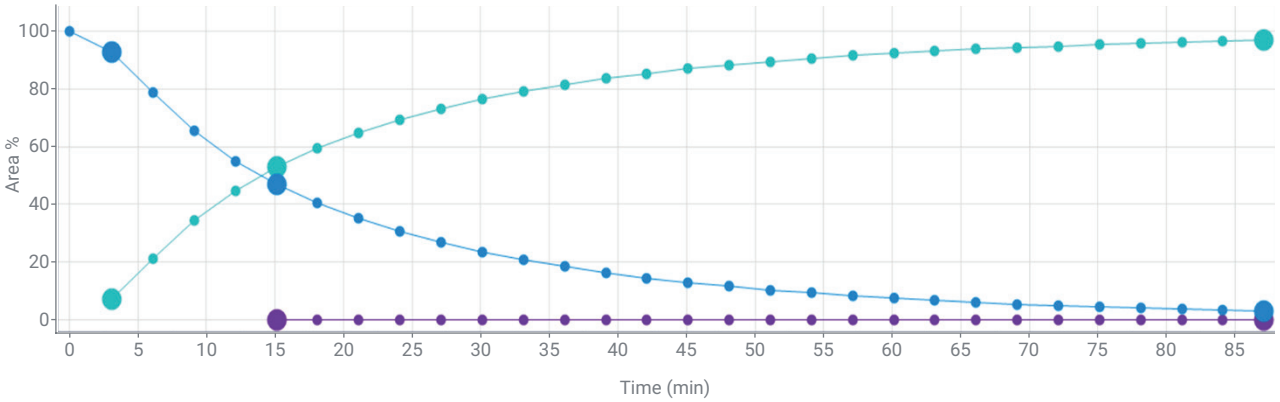


Figure 4. Trending plot of the area percentage of educt (blue), product (green), and byproduct (purple).

Table 1. Detailed measured values of retention time, area, height, area percentage, and corrected amount of samples 1, 2, 6, and 30.

Sample	Compound	RT (min)	Area %	Corr. Amount (%)	Area	Height
1	Anisaldehyde	0.260	100.000	100.000	4,712.930	6,320.723
2	Anisaldehyde	0.260	92.956	50.386	2,374.678	3,269.193
	Anisylidene acetone	0.360	7.044		179.961	211.639
6	Anisaldehyde	0.261	46.971	25.275	1,191.190	1,661.330
	Anisylidene acetone	0.360	52.913		1,341.879	1,606.135
	Byproduct	0.837	0.023		0.585	0.762
30	Anisaldehyde	0.261	3.005	2.284	107.655	149.913
	Anisylidene acetone	0.360	96.818		3,468.538	4,050.824
	Byproduct	0.837	0.122		4.354	5.719

was taken as 100% (Figure 5). Sample 2 shows a residual educt of 50.3% and sample 6 of 25.3%. As a lower limit, a notification level of 5% was introduced. This level was undershot in sample 22 and declined to 2.3% in sample 30, the end of the reaction time.

The data for the trending plot and calibration were acquired at wavelength of 290 nm because all compounds show a response at that wavelength. However, this is not the optimum wavelength for the detection of the byproduct. The optimum wavelength is at 360 nm.

Unfortunately, the educt does not show a response at that wavelength. Therefore, the wavelength of 360 nm was monitored at a second channel by the HDR-DAD. This gave better response with higher peaks for its detection (Figure 6).

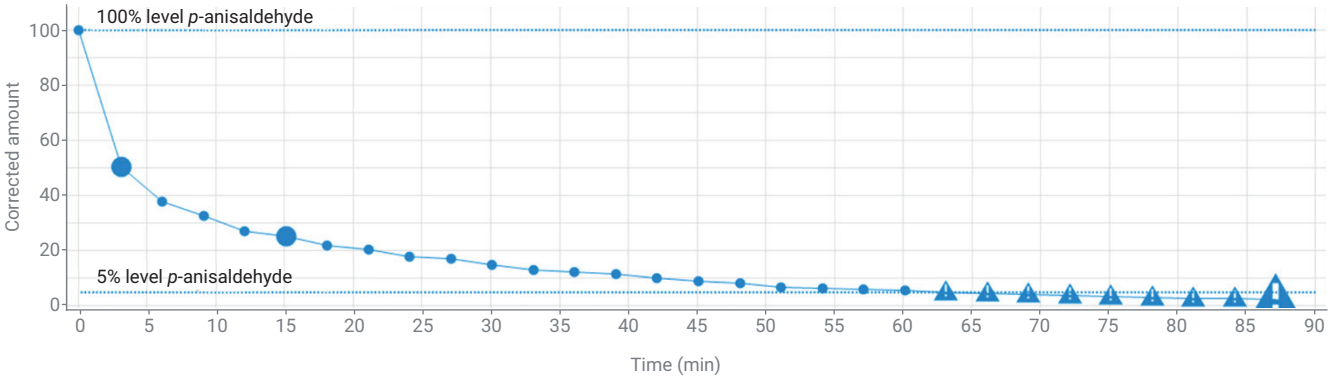


Figure 5. This figure shows relative quantification of *p*-anisaldehyde by means of HDR-DAD. Values under the lower threshold are marked with a triangle and exclamation mark.

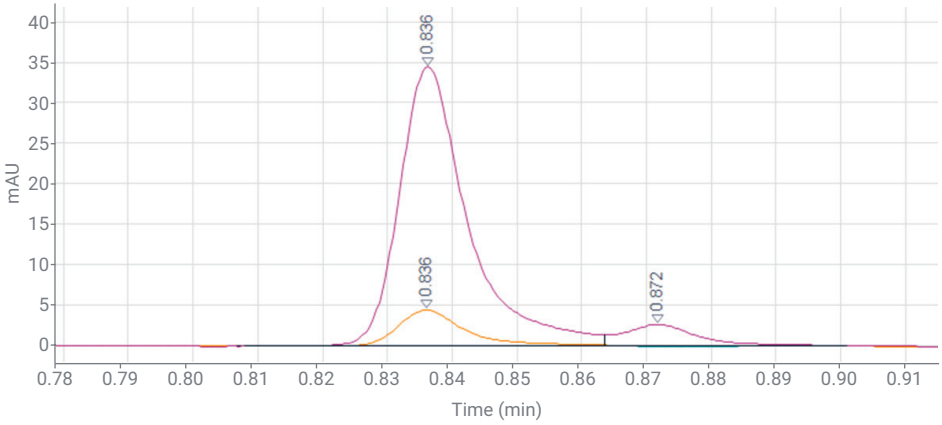


Figure 6. This graph depicts an overlay of the peak of the byproduct of the aldol reaction from a double condensation. Sample 6 (orange) and sample 30 (purple) at the beginning and end of the reaction time, respectively (360 nm).

Conclusion

This application note demonstrates the capability of the Agilent equipped with an HDR-DAD to detect low-level impurities in a small molecule reaction simultaneously with the highly concentrated educt and product by means of the enhanced dynamic range in a single run. Due to the enhanced dynamic range, it is possible to save time by doing only one injection for determination of highly concentrated compounds and trace compounds. Absolute or relative quantification of highly concentrated compounds by means of the higher linear dynamic range is possible.

References

1. Impurity Testing of Fixed-Dose Combination Drugs Using the Agilent 1290 Infinity II HDR-DAD Impurity Analyzer Solution. *Agilent Technologies application note*, publication number 5991-5743EN, **2015**.
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