Technical Overview

Assuring the Integrity of Biologicals in High-Intensity UV Detectors

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Introduction

The demand for more sensitive UV detectors to lower detection limits further comes with a drawback. Higher sensitivity often means higher intensity of the light that analytes are exposed to. For some analytes that are vulnerable to the energy of the incoming UV light, this might cause degradation. One mechanism is the UV-mediated oxidation of some amino acids (Met, Trp, Cys, and Tyr) in peptides and proteins.[1] The degradation products are often not visible in the detector in which the degradation occurs, potentially leading to analytical artifacts. But if any further analysis method is connected to the system, for example a second dimension in HPLC or a mass detection, results of the degradation could be visible.

If degradation effects are visible in a second dimension or a mass detector, the only possible solution is to switch off the UV detection or to exchange the detector with another with lower light intensity. However, switching off the detection reduces the information content acquired by a run or makes it impossible to use peak-triggered sampling, for example.

With the Agilent InfinityLab Max-Light Cartridge Cell LSS (G7117-60020) for light-sensitive samples, there is an easy way to reduce the light intensity impacting on the sensitive analytes while retaining the analytical results of the UV detector. On the light entrance side of the flow cell, a small aperture can be applied to reduce the light intensity by a factor of 10. Without the aperture, the cell is identical in build and performance to the standard InfinityLab Max-Light Cartridge Cell 10 mm.

This technical overview investigates how this reduction of the light intensity influences possible photodegradation and performance of the system with a light-sensitive sample and mass detection, as well as having a look at the performance parameters comparing the cell with and without an installed aperture.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with the standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II DAD (G7117B), equipped with a biocompatible Max-Light Cartridge Cell LSS, 10 mm

The following alternatives were used for UV detection:

- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with either a Bio Standard Flow Cell, 10 mm, or a Bio Micro Flow Cell, 3 mm
- Agilent 1260 Infinity II Diode Array Detector (G7115A) with Bio-Inert Flow cell, 10 mm (G5615-60022)

Mass detection was performed on an:

- Agilent 6545XT AdvanceBio LC/Q-TOF

Software

- Agilent OpenLab CDS Version 2.5 or later versions
- Agilent MassHunter 10.0

Columns

- UV photodegradation into MS—Agilent PLRP-S 300 Å, 50 × 2.1 mm, 3 µm (PL1912-1301)
- UV sensitivity comparisons—Agilent AdvanceBio SEC 300 Å, 50 × 4.6 mm, 2.7 µm (PL1580-1301)

Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Acetonitrile, methanol, formic acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, and sodium chloride were obtained from VWR International, Darmstadt, Germany.

Samples

- NIST standard humanized monoclonal antibody was obtained from Sigma Aldrich (NIST8671)
- Herceptin (Trastuzumab) was obtained from Roche (Basel, Switzerland).
- The lyophilized powders were reconstituted in PBS at pH 7.4.

Methods

1. Reversed-phase method with MS detection

Parameter	Value
Column	Agilent PLRP-S 300 Å, 50 × 2.1 mm, 3 µm (PL1912-1301)
Solvent	A) Water + 0.1% formic acid B) Acetonitrile (ACN) + 0.1% formic acid
Gradient	Time (min) A B 0 85% 15% 0.5 85% 15% 10 30% 70% 10.1 85% 15% Stop time: 10.1 minutes Post time: Off
Flow Rate	0.5 mL/min
Column Temperature	60 °C
UV-Detection	280 nm (bandwidth 4 nm; reference OFF) 2.5 Hz
Injection	Injection volume: 1 µL of a 1 mg/mL NIST mAb solution Sample temperature: 10 °C
MS Detection	Acquisition mode: MS1 Minimum range: *m/z* 500 Maximum range: *m/z* 7,000
Source Parameters	Gas temperature: 350 °C Gas flow: 12 L/min Nebulizer 60 psig Sheath gas temperature: 400 °C Sheath gas flow: 12 L/min
Scan Parameters	Vcap: 4,000 Nozzle voltage: 2,000 V Fragmentor: 320 Skimmer1: 65 Octopole RF Peak: 750

2. Size exclusion method

Parameter	Value
Column	Agilent SEC 300 Å, 50 × 4.6 mm (PL1580-1301)
Solvent	A) PBS buffer pH 7.4
Gradient	Isocratic, 100% A Stop time: 3 min Post time: OFF
Flow Rate	0.35 mL/min
Temperature	30 °C
Detection	280 nm (bandwidth 4 nm; reference OFF) 20 Hz
Injection	Injection volume: 5 µL of a serial dilution of a 10 mg/mL trastuzumab solution Sample temperature: 10 °C

Results and discussion

Photodegradation in high-intensity UV-light detectors

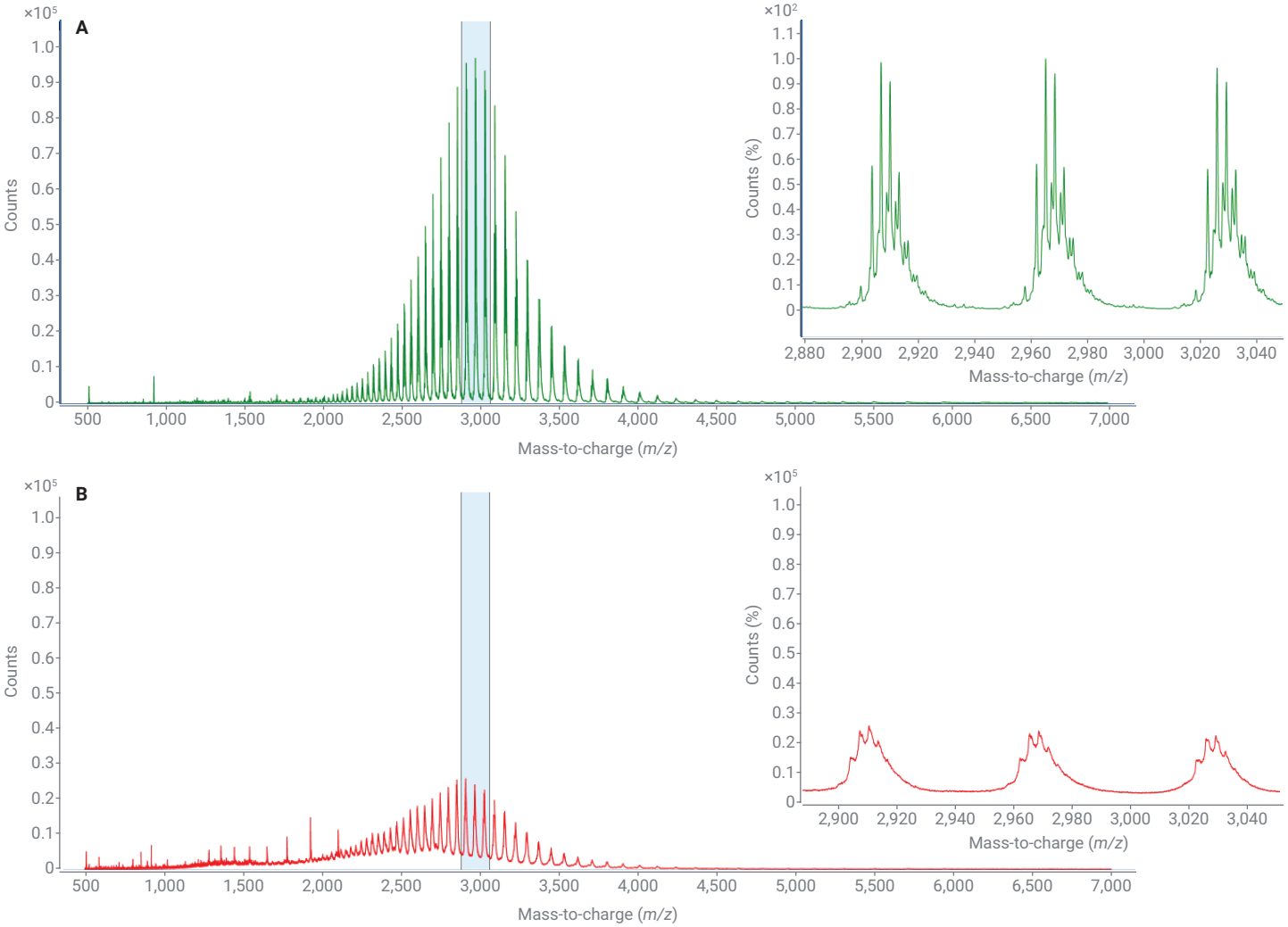
The photodegradation event was recreated with a simple reversed-phase method on a short Agilent PLRP-S 300 Å, 50 × 2.1 mm column and detection on a 1290 Infinity II DAD. The flow then goes directly into a 6545XT AdvanceBio LC/Q-TOF mass spectrometer to observe the degradation products (method 1).

While trastuzumab is stable under those conditions, the NIST antibody degrades. Figures 1A and 1B illustrate the difference in the charge envelope with the DAD switched off (A) and the DAD switched on (B). The fine structure representing the different glycoforms also gets heavily distorted if the DAD is switched on.

Although gathering the data from the mass spectrometer while the UV detector is switched off is a possible solution to the degradation problem, it is often preferable to make use of both detection methods in the same run.

On the entrance of the light guide of the Max-Light Cartridge Cell LSS (G7117-60020) is a thread to attach the Light Sensitive Samples (LSS) Aperture (G7117-60101). If this aperture is installed on the flow cell, the light intensity is reduced by a factor of 10, minimizing the photodegradation. Without the aperture installed, the full intensity and therefore full sensitivity of the DAD detector is obtained.

Figure 1C illustrates the effect of the LSS Aperture installed. The charge envelope is completely restored even with the DAD switched on, and the fine structure also becomes visible again.



Figures 1A, 1B. The charge envelope of the NIST standard mAb in a Q-TOF after passing through a DAD. (A) DAD switched off. (B) DAD switched on without aperture. (C) DAD switched on, but with aperture installed. Insets show a zoomed-in view displaying the fine structure and the different glycoforms. In (B), the fine structure is heavily distorted and in (C), this is almost restored. (Continued on the next page).

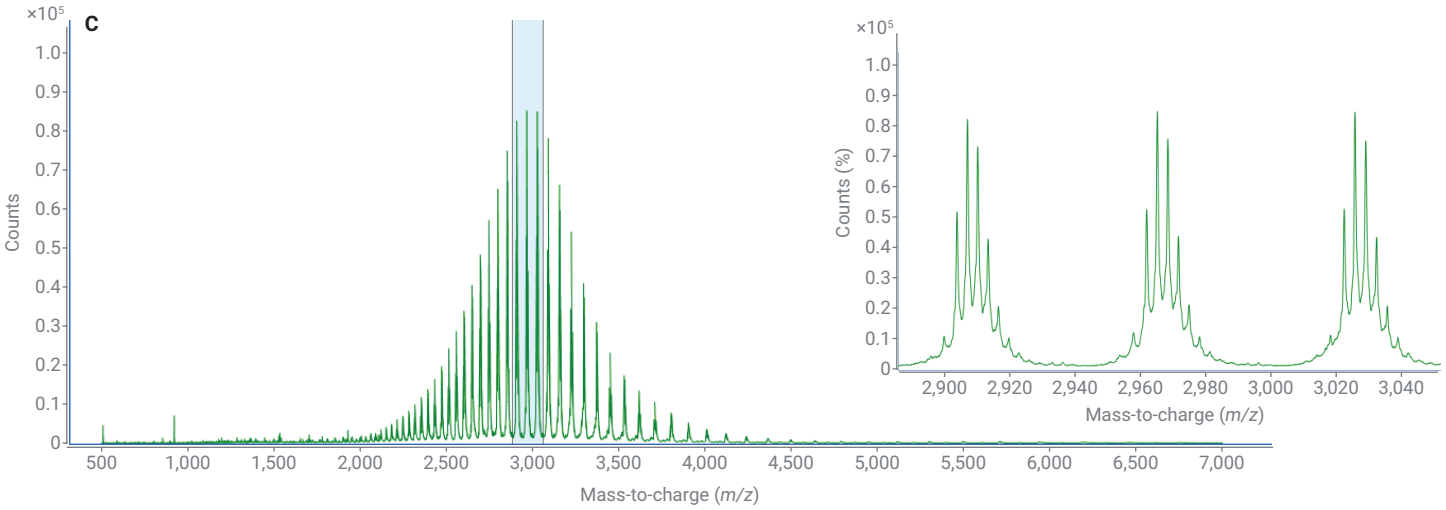


Figure 1C. (Continued from previous page) The charge envelope of the NIST standard mAb in a Q-TOF after passing through a DAD. (A) DAD switched off. (B) DAD switched on without aperture. (C) DAD switched on, but with aperture installed. Insets show a zoomed-in view displaying the fine structure and the different glycoforms. In (B), the fine structure is heavily distorted and in (C), this is almost restored.

Comparison of sensitivity and limits of detection and quantification of different detectors and G7117B with and without aperture

Reduced light intensity also carries the risk of reducing the sensitivity of the detector, mostly due to increased noise.

To measure the noise increase that results from installing the apertures, we determined the noise produced with and without installed LSS apertures.

For the noise tests, pure water was pumped with 0.5 mL/min through the system without column, but with a restriction capillary to get a system pressure of 60 to 80 bar. The noise and drift was measured at 230 nm with 4 nm bandwidth and a reference at 360 nm with 100 nm bandwidth at 20 Hz. The detector was changed to an alternative with very low, but still acceptable intensity, to increase the impact of a strongly reduced intensity. If newly assembled or moved, the system can take up to 24 hours to get into equilibrium until stable noise and drift levels are reached.

Figure 2. Drift and noise results without aperture and with different apertures installed. Red lines indicate the specifications for this parameter.

Figure 2 shows the noise and drift without aperture and with several apertures installed.

The drift is not affected significantly by the aperture and stays well below the limit of 500 µAU/h for this setup. The noise, on the other hand, is increased roughly by a factor of 2 and is slightly above the limit of 6 µAU for the specification without an aperture. However, the noise lies well below the noise specification limit with applied aperture (12 µAU).

To test how the aperture influences the sensitivity under real-world conditions, an isocratic method was used to minimize the influence of other factors. The analyte was also changed to trastuzumab, which shows no degradation in detectors with high light intensity, to eliminate the influence that degraded protein might have on the noise of the system. A short size exclusion method was used and a serial dilution from 10 to 0.001 mg/mL of trastuzumab in elution buffer was prepared.

To classify the influence of the aperture, the 1290 Infinity II DAD detector was compared with the 1260 Infinity II DAD WR and the 1290 Infinity II Variable Wavelength Detector (VWD) with 14 and 2 µL cells under the same conditions.

All detectors showed good linearity in this range and were not affected by the aperture in any way. Figure 3 shows the calibration graph for the serial dilution with the 1290 Infinity II DAD with applied aperture. The correlation coefficient of the linear regression $R^2 = 0.99999$ shows a good fit. The analytical sensitivity, represented by the slope of the calibration graph and indicating the capacity of the method to differentiate between two very close concentrations of analyte[2], is similar to the detector without aperture, as seen in Table 3.

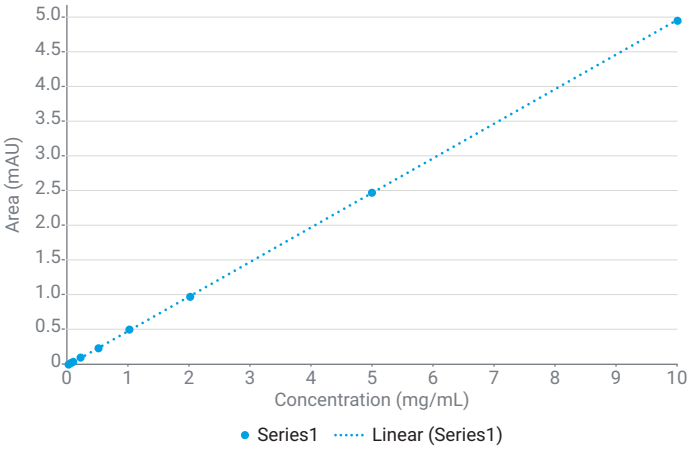


Figure 3. Calibration graph of a serial dilution of trastuzumab on an Agilent 1290 Infinity II Diode Array Detector with applied aperture.

Table 3. Comparison of the different analytical sensitivities of the detectors for this method.

Detector	Slope [mAU/(mg/mL)]
Agilent 1290 Infinity II DAD (G7117B)	504
Agilent 1290 Infinity II DAD (G717B) with aperture	495
Agilent 1260 Infinity II WR (G7115A)	506
Agilent 1290 Infinity II VWD 2 µL (G7114B)	155
Agilent 1290 Infinity II VWD 14 µL (G7114B)	509

For the determination of the limits of detection and quantification (LOD/LOQ), several procedures were used. We took the most common method for determining these limits by measuring the signal-to-noise ratio (S/N) of a peak with a concentration expected to be in the area of the detection/quantification limits. For determining the noise, the area 20 times the peak width in a blank injection could be used, or an area of baseline in an actual run could be defined. We took the time intervals in the runs between 0.5 and 1.0 minutes and 2.3 and 2.8 minutes where no signal is expected to be. The LOD should be 3.3 × S/N and the LOQ 10 × S/N. The measurement was performed 10 times for each setup and the average taken.

The diagram in Figure 4 shows the comparison of the LOD/LOQ values of the different setups. The LOD/LOQ of the 1290 Infinity II DAD is increased by roughly a factor of 3 when applying the aperture, but it is still below the 1260 Infinity II Diode Array Detector WR and the 1290 Infinity II Variable Wavelength Detector with the micro flow cell.

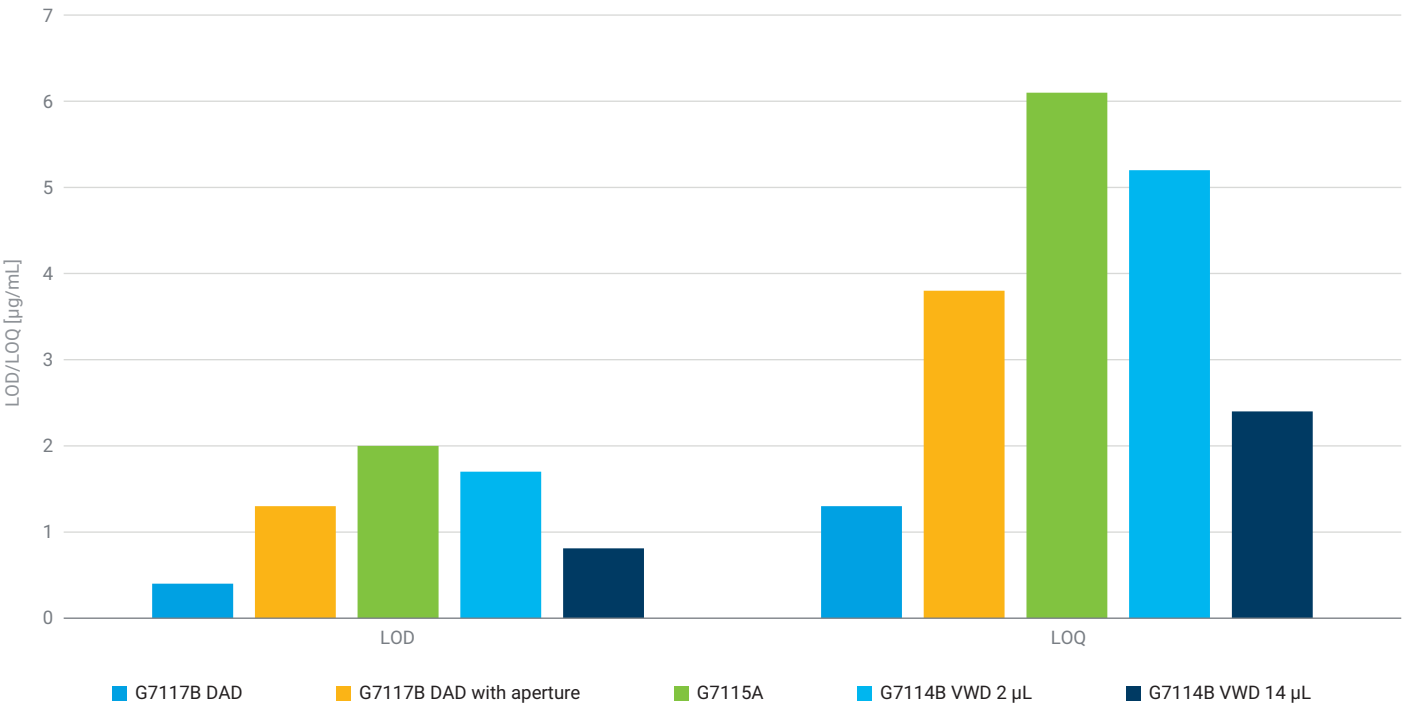


Figure 4 . LOD/LOQ comparison of the different setups used.

Conclusion

The influence of the light intensity of the detector on a light sensitive sample was shown. The photodegradation was clearly visible in a connected mass spectrometer. To reduce the photodegradation, the new Agilent Max-Light Cartridge Cell LSS can be applied easily without compromising too much of the sensitivity and detection limit of the UV detector. With aperture and a reduction of the detector intensity by a factor of 10, the photodegradation in a light-sensitive sample could be significantly reduced. The analytical sensitivity was mostly unaffected and the detection/quantification limit raised by a factor of about three.

There are a lot of different analytes and for some, the light intensity might be still too high to stay intact under these conditions. The only possible option here would be to switch off the UV detector completely or to switch to a single wavelength detector.

References

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2. Koch, D. D.; Peters, T. Selection and Evaluation of Methods. In: Tietz Textbook of Clinical Chemistry; Burtis, C. A., Ashwood, E. R., eds.; Saunders: Philadelphia, **1994**, 510.

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