Application Note

Food and
Beverage Testing

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Determination of Ochratoxin A in Coffee with the Agilent 1290 Infinity III Fluorescence Detector

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Abstract

This application note demonstrates that the Agilent 1290 Infinity III Fluorescence Detector (FLD) can be used to measure fluorescently active mycotoxins like ochratoxin A at high sensitivity, enabling very low limits of detection (LODs) and quantification (LOQs) under UHPLC conditions. An example is shown where the mycotoxin is extracted from a dry sample following enrichment using an immunoaffinity column. We prove that the 1290 Infinity III FLD with its low-dispersion flow cell can be operated under UHPLC conditions, providing sharp peaks at low run time.

Introduction

Ochratoxin A is a mycotoxin produced as a mold byproduct originating from fungi like *Aspergillus* and *Penicillium*. These fungi can grow in green coffee beans during incorrect storage or drying. Ochratoxin is very stable, even during the roasting process, and can therefore be found in coffee products. This toxin can harm the kidneys and act as a carcinogen, and so strict safety limits of 3 µg/kg in roasted coffee and 5 µg/kg in instant coffee have been set through the European Commission Regulation (EU) 2022/1370.[1]

This application note demonstrates the highly sensitive measurement of ochratoxin A by means of the 1290 Infinity III FLD. A calibration curve in the concentration range required by European regulatory authorities is shown, performance parameters are evaluated, and an example of spiked roasted coffee is presented.

Experimental

Instrument setup

- Agilent 1290 Infinity III High-Speed Pump (G7120A)
- Agilent 1290 Infinity III Multisampler (G7167B)
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity III FLD (G7123B)

Software

Agilent OpenLab CDS version 2.8

Method

Method Settings	
Binary Pump	
Mobile Phase A	Water + 1% acetic acid (HOAc)
Mobile Phase B	Acetonitrile (ACN) + 1% HOAc
Stop Time	7 min
Flow Rate	0.5 mL/min
Gradient	Isocratic 45% B
Multisampler	
Injection Volume	50 µL
Needle Wash	3 s ACN
Column Oven	
Column Temperature	35 °C
Fluorescence Detector	
Excitation Wavelength	320 nm
Emission Wavelength	470 nm
PMT	Standard
Peak Width	1 s response time (10 Hz)

Column

Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 100 mm, 1.8 µm (p/n 959758-302)

Standards

Ochratoxin A, 10 µg/mL in acetonitrile

Calibration:

- **Stock solution:** 1 µg/mL ochratoxin A in acetonitrile
- **Dilution series:** 100, 20, 10, 2.0, 1.0, 0.2, 0.1, 0.02 ng/mL (ppb). Dilution in 60:40 ACN:water + 1% HOAc

Sample

Roasted, ground coffee bought from a local store

Immunoaffinity sample preparation

1. Weigh 10 g of ground coffee in a capped centrifuge tube and add 1 g of NaCl, then extract with 20 mL of methanol/water (80/20) for 10 minutes by shaking.
2. Filter the slurry through a paper filter and filter the obtained liquid through a 0.45 µm syringe filter (p/n 5190-5270).
3. Take 2 mL of filtrate and dilute 1:10 with phosphate-buffered sodium chloride (PBS).
4. Wash the immunoaffinity column with 10 mL PBS and let it elute by gravity.
5. Load the immunoaffinity column with the prepared extract (20 mL) and let it elute by gravity.
6. Wash the immunoaffinity column with 10 mL PBS and let it elute by gravity.
7. Remove the residual water from the immunoaffinity column under a slight vacuum.
8. Elute ochratoxin A from the immunoaffinity column with 2 mL of methanol. After approximately 1 mL has eluted, let the column sit in methanol for 5 minutes and allow it to elute by gravity completely.
9. Dilute the eluate 1:1 with water to HPLC starting conditions.

Chemicals

- Sodium chloride
- PBS

Solvents

- Agilent InfinityLab acetonitrile for HPLC (p/n 5191-5100-002)
- Agilent InfinityLab methanol for HPLC (p/n 5191-5110-002)
- Agilent InfinityLab water for HPLC (p/n 5191-5120-002)

Results and discussion

For the determination of the mycotoxin ochratoxin A in coffee, a calibration curve was created using a standard solution of ochratoxin A. The calibration covered the range from the upper concentration of 100 ppb to the lowest used concentration of 0.1 ppb. An overlay of the peaks obtained for the higher and lower concentrations is shown in Figures 1A and 1B, respectively. The obtained linearity was excellent, with an R^2 value of 1.00000 (Figure 1C).

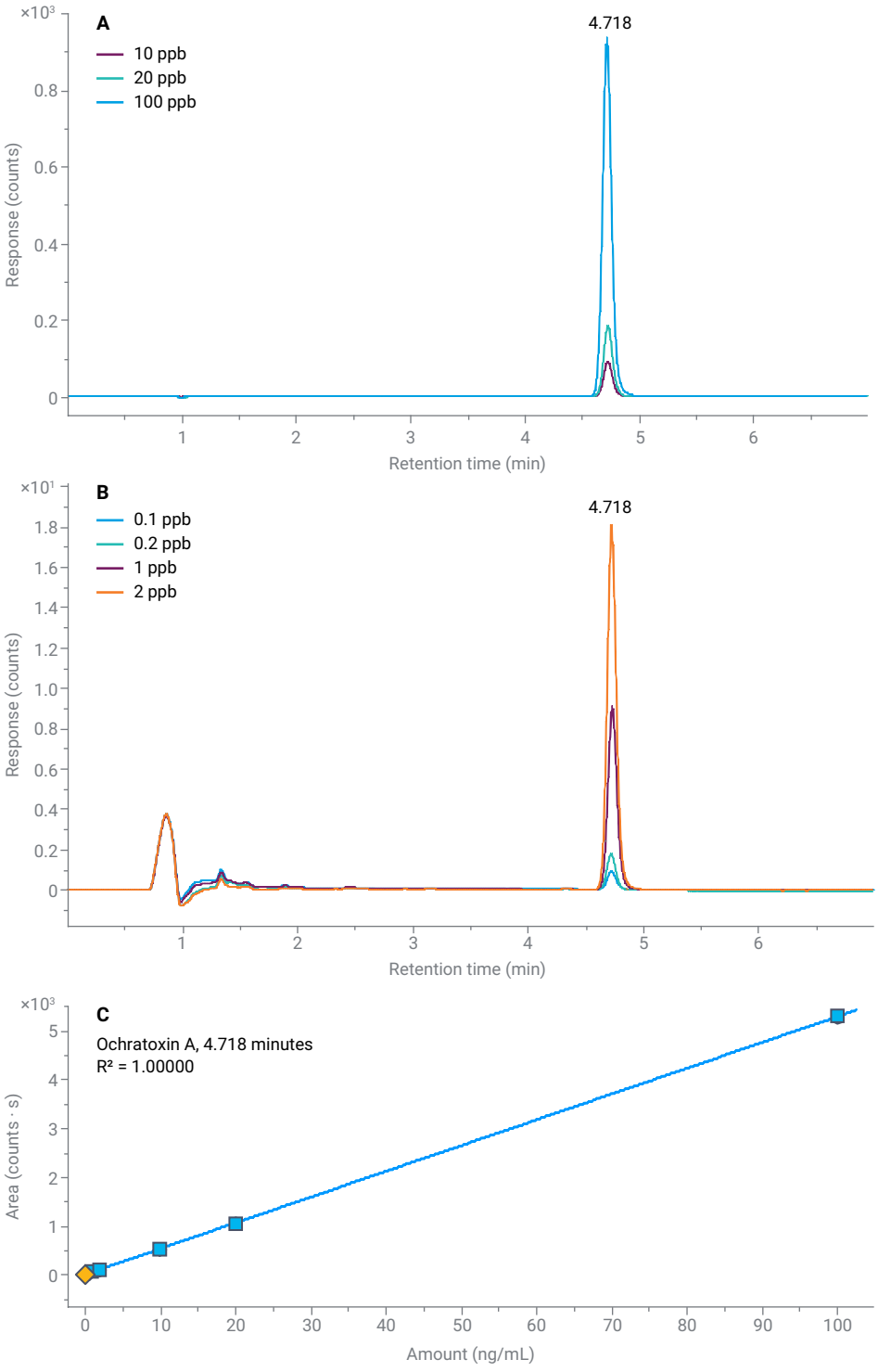

Figure 1. Calibration curve created for the mycotoxin ochratoxin A. (A) Higher concentrations of 100, 20, and 10 ppb. (B) Lower concentrations of 2, 1, 0.2, and 0.1 ppb. (C) Calibration curve of the ochratoxin concentrations used in this application.

From the lowest concentration acquired for the generation of the calibration curve from standard solutions, the LOQ was determined using the peak-to-peak (ptp) noise calculated in OpenLab. The peak at 0.1 ppb showed a ptp signal-to-noise ratio (S/N) of 17. This equated to an LOQ of 0.06 ppb at an S/N of 10 (Figure 2). For the determination of the LOD, a concentration of 0.02 ppb was measured, and the ptp S/N was determined to be 3.3, resulting in a calculated LOD of 0.018 ppb.

For the determination of peak area and retention time precision, RSD values were calculated. For this purpose, the calibration concentration of a standard solution at 1 ppb was used and injected six times (Figure 3). The average retention time was 4.727 minutes. The RSDs of retention time and peak area were 0.03% and 0.274%, respectively. This demonstrates the excellent repeatability of the injection, enabling precise measurement at low concentrations.

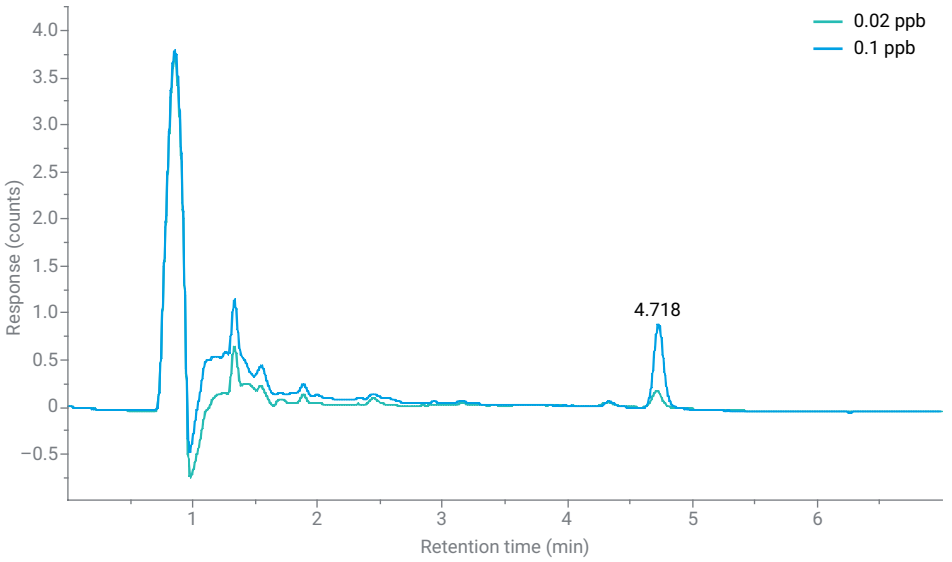

Figure 2. Peaks measured at 0.1 and 0.02 ppb used for the calculation of LOQ and LOD. The peak at 0.1 ppb showed an S/N of 17, giving an LOQ of 0.06 ppb. The peak at 0.02 ppb showed an S/N of 3.3, giving a calculated LOD of 0.18 ppb.

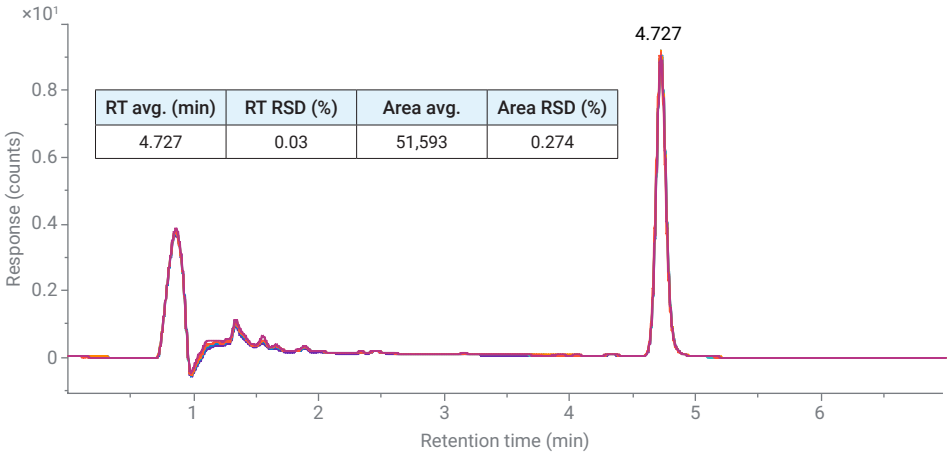

Figure 3. Determination of peak area and retention time precision at calibration concentration from the standard solution of 1 ppb (n = 6).

In contrast to the standard solution used for the determination of injection precision, for the determination of the recovery of ochratoxin A from roasted coffee, coffee samples were spiked with a concentration of 10 and 3 ppb ochratoxin A. The ochratoxin A was extracted from the ground roasted coffee samples and removed by an immunoaffinity reaction (see the Experimental section). As a control sample, a matrix blank was generated from an unspiked coffee sample. In the resulting FLD chromatogram, it can be seen that remaining matrix compounds were clearly separated from the ochratoxin A peak at 4.718 minutes and the matrix blank showed no interference at that time point.

Due to the applied sample preparation protocol, a dilution of 1 to 4 could be expected for the measured concentrations. The 10 ppb spike of ground roasted coffee led to a measured concentration of 2.578 ppb, which corresponds to a recovery of 103.12%. The sample spiked with 3 ppb showed a measured concentration of 0.736 ppb, which is a recovery of 98.13%.

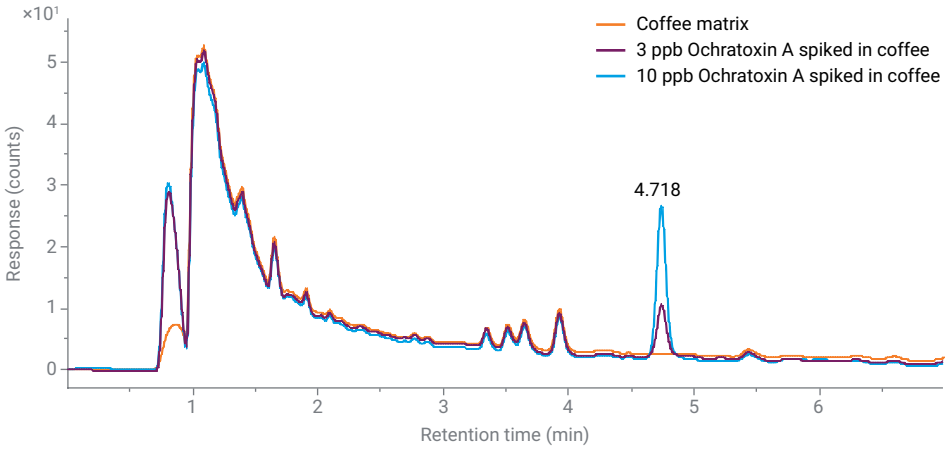

Figure 4. Overlay of chromatograms obtained from roasted coffee samples spiked at 10 and 3 ppb and a matrix blank.

Conclusion

This application note demonstrated the highly sensitive determination of ochratoxin A by means of the Agilent 1290 Infinity III Fluorescence Detector. The calibration curve generated between 0.1 and 100 ppb showed excellent linearity, with an R^2 value at 1.00000. The achieved injection precision was 0.274% area RSD. The calculated sample equivalent LOQ achieved from the measurement of standard solutions of 0.06 ppb is sufficient for sensitive determination of ochratoxin A at the lowest level required by European regulations. Taking into consideration that the EU regulation requires a maximum level of 3 ppb of ochratoxin A in roasted coffee, the applied sample preparation would allow a detection of approximately 0.24 ppb of ochratoxin A in a sample preparation from roasted coffee. In addition, the highly sensitive detection and repeatability achieved here is fully compatible with application using an Agilent 1290 Infinity III LC System.

Reference

1. European Commission. Commission Regulation (EU) 2022/1370 of 5 August 2022 Amending Regulation (EC) No 1881/2006 as Regards Maximum Levels of Ochratoxin A in Certain Foodstuffs. *Off. J. Eur. Union* **2022**, *L 206/211*, 19–25. https://eur-lex.europa.eu/eli/reg/2022/1370/oj (accessed 2026-11-02).

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