Determination of Ochratoxin A in Roasted Coffee According to DIN EN 14132

Application Note

Food Testing & Agriculture – Pesticides, Mycotoxins & Other Contaminants

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Abstract

This Application Note demonstrates the determination of the mycotoxin ochratoxin A in roasted coffee products according to DIN EN 14132, which is part of a series of quality control measurements of coffee products. The performance of the system is shown for linearity, retention time, and area precision as well as accuracy. The performance is also demonstrated on solvent saver columns with reduced inner diameter. The sample preparation procedure is described and the analysis of a real sample is shown.

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Introduction

Ochratoxin is a mycotoxin produced by *Aspergillus* species and *Penicillium* species. There are three different forms, ochratoxin A, B, and C; ochratoxin A is the most abundant. It is a widespread contaminant of types of foodstuff, such as grain, pork products, coffee, wine grapes, and dried grapes. Ochratoxin A is a potential carcinogen and nephrotoxic[1]. The European Commission Report regulates the Tolerable Weekly Intake (TWI) to a maximum of 120 ng/kg b.w., including the maximum content in specific food products[2a]. The maximum allowed concentration in roasted coffee is 5.0 µg/kg[2b].

To determine the content of ochratoxin A in food, the mycotoxin compound was isolated by an immunoaffinity chromatography step followed by analysis on an HPLC with fluorescence detection.

The measurement of ochratoxin A in roasted coffee products is standardized in the DIN regulations[3]. In addition to the contaminant Ochratoxin A, other important compounds naturally inherent in coffee such as caffeine[4,5], chlorogenic acids[6,7], and cafestol[8,9], have to be controlled.

Experimental

Equipment

Agilent 1260 Infinity LC System:

- Agilent 1260 Infinity Binary Pump (G1312B) with Agilent 1260 Infinity Standard Degasser (G1322A).
- Agilent 1260 Infinity Standard Autosampler (G1329B) with Agilent 1290 Infinity Thermostat (G1330B).
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Fluorescence Detector (G1321B)

Software

Agilent OpenLAB CDS ChemStation Edition Rev. C.01.05

Columns

1. Agilent ZORBAX Eclipse Plus, 4.6 × 150 mm, 5 µm (p/n 959993-902)
2. Agilent Poroshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm (p/n 693975-302)
3. Agilent Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm (p/n 699975-302)

Chemicals

All chemicals were purchased from Sigma-Aldrich, Germany. Acetonitrile was purchased from Merck, 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). Regular roasted coffee was purchased from a local supermarket.

Standards

A standard solution of 5 mg ochratoxin A in 10 mL toluol/acetic acid 1 %, which is equal to 500 ppm, was prepared. An aliquot of 100 µL of this solution was diluted 1/100 to a concentration of 5 ppm. An aliquot of 100 µL of the 5 ppm standard solution was transferred into an empty 10-mL volumetric flask and evaporated to dryness under a nitrogen stream. The residue was dissolved in methanol/water/acetic acid 30/60/1 (v/v/v) to a final concentration of 50 ppb. The first calibration point was created by diluting the 50 ppb concentration to the 20 ppb concentration level. The other calibration levels were created by a dilution pattern of 1:2 down to 156.25 ppt.

HPLC method

Parameter	Value	
Solvents	A) Water + 1 % acetic acid B) Acetonitrile + 1 % acetic acid	
Flow rate	1.0 mL/min with Column 1, 0.43 mL/min with Column 2, and with Column 3 0.86 mL/min and 1.72 mL/min with Column 3	
Elution conditions	Isocratic, 45 % B	
Stop time	20 minutes	
Injection volume	100 µL with Column 1 and 43 µL with Columns 2 and 3	
Sample temperature	8 °C	
Needle wash	In vial with acetonitrile	
Column temperature	25 °C	
Fluorescence detection	Excitation wavelength Emission wavelength Peak width PTM gain	333 nm 460 nm 9.26 Hz 18

Sample preparation

Extraction

A 15 g sample of ground coffee was extracted by shaking it for approximately 30 minutes in methanol/sodium hydrogen carbonate solution 3 % (1/1, v/v). The extract was filtered through a paper filter and centrifuged for 15 minutes at 4 °C and 1,300 g.

Cleanup on a phenyl silane column

The phenyl silane column was washed with 15 mL methanol and then 5 mL sodium hydrogen carbonate solution (3 %) without applying vacuum.

A 10-mL amount of the prepared coffee extract was mixed with 10 mL sodium hydrogen carbonate solution (3 %) and passed through the previously prepared phenyl silane column. The phenyl silane column was cleaned with 10 mL methanol/sodium hydrogen carbonate solution 3 % (20/80, v/v) and with 5 mL sodium hydrogen carbonate solution (1 %). The bounded material was removed by washing with 10 mL methanol/water (7/93, v/v). The maximum flow rate in the phenyl silane column should not exceed 5 mL/min.

Cleanup on an immunoaffinity column

The eluent obtained from the phenyl silane column was diluted with 30 mL PBS buffer (8 g NaCl, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl in 1 L water) and passed through the immunoaffinity column. The immunoaffinity column was washed with 10 mL water and eluted with 4 × 1 mL methanol. The maximum flow rate in the immunoaffinity column should not exceed 5 mL/min.

Preparation of the injection solution

The methanolic extracts from the immunoaffinity cleanup were combined and evaporated to dryness in a vacuum concentrator at 30 °C. The residue was dissolved in 1 mL methanol/water (30/70 v/v) 1 % acetic acid, and used directly for injection.

Results and Discussion

Method performance

Starting with the 20-ppb standard solution, a calibration curve was created over eight concentration levels using a 1:2 dilution pattern down to 156.25 ppt on the standard ZORBAX Eclipse Plus 4.6 × 150 mm column under standard HPLC conditions at a 1 mL/min flow rate and 100-µL injection (Figure 1). Ochratoxin A eluted at 10.05 minutes. The calibration showed excellent linearity with a coefficient of 1.00000 (Figure 2). The limit of quantification (LOQ) was calculated for a signal-to-noise ratio (S/N) of 10 to be 100 ppt and the limit of detection (LOD) was calculated for a S/N of 3 to be 39 ppt.

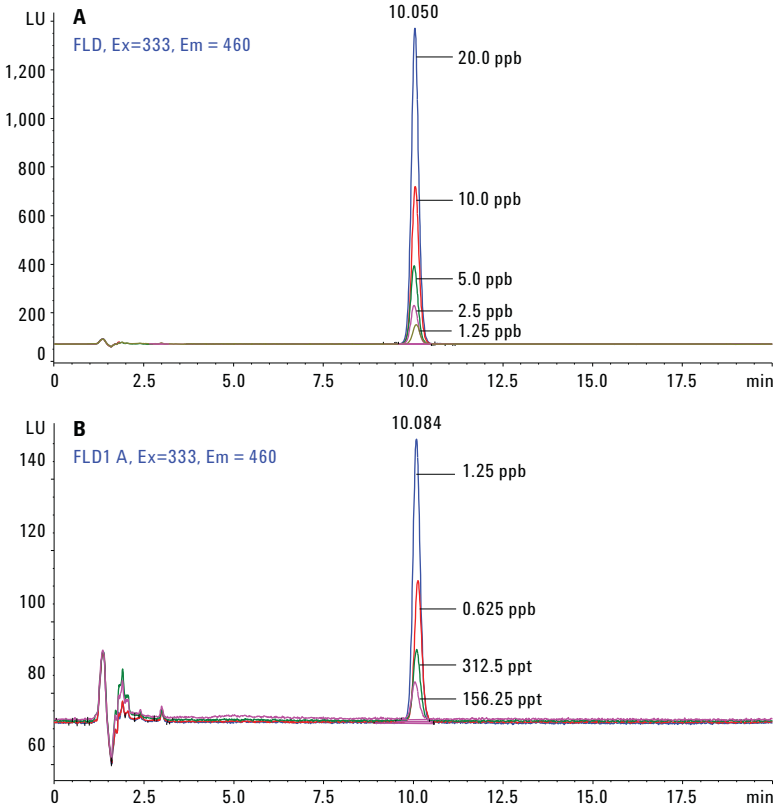

Figure 1. Overlay of ochratoxin A peaks of different concentrations used as calibration levels. A) 1.25 ppb–20 ppb, B) 156.25 ppt–1.25 ppb.

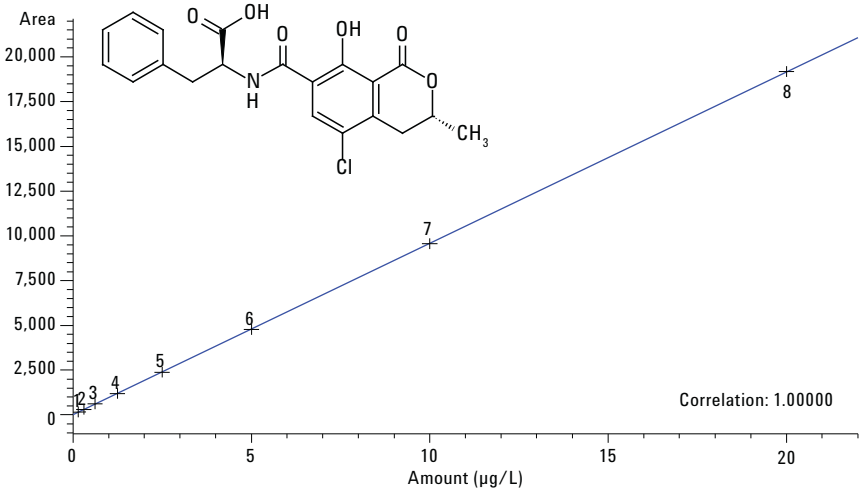

Figure 2. Calibration curve for ochratoxin A for the concentration range 156.25 ng/L–20 µg/L.

A statistical evaluation of the analytical method was done by multiple injections of the 10 ppb concentration level. Table 1A shows that a retention time RSD of 0.27 %, and an area RSD of 0.39 % were found. To determine the method accuracy, a dilution of 8.0 µg/L was used and injected multiple times. For the measured concentrations, a precision RSD of 0.55 % and a concentration accuracy of 96.5 % were found. To determine carryover, the 20 ppb solution was injected, followed by a blank solvent injection. No carryover was detected from the highest concentration level of the calibration to the following blank (Figure 3).

Table 1A. Performance data measured for 10 µg/L (ppb) ochratoxin A with the Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm column as well as concentration precision and accuracy.

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm
Ochratoxin A	10 µg/L
RT	10.05 minutes
RT RSD (%)	0.27
Area RSD (%)	0.39
Calibration	156.25 ng/L–20.0 µg/L
Linearity, R^2	1.00000
LOD	39 ng/L
LOQ	100 ng/L
Carryover	from 20.0 µg/L–n.d.
Concentration precision	0.55 % at 8.0 µg/L
Concentration accuracy	96.5 % at 8.0 µg/L

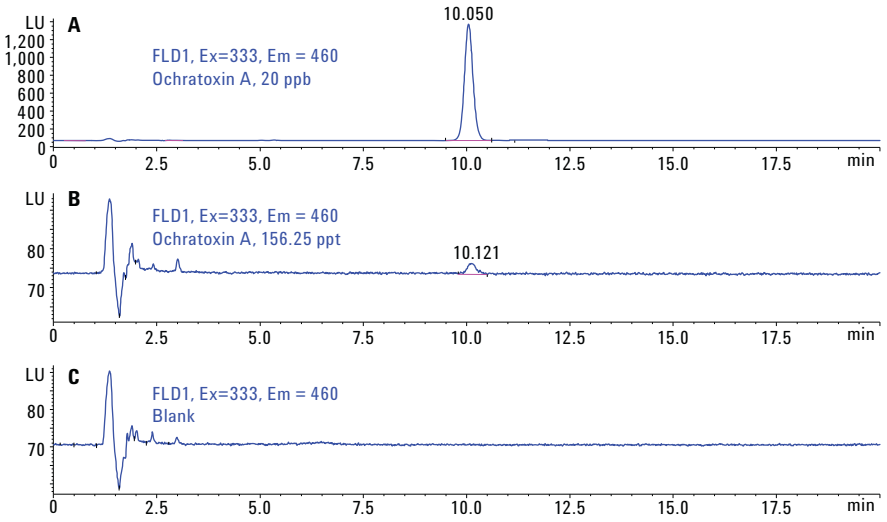

Figure 3. Determination of carry over of ochratoxin A for the maximum concentration used.
A) Maximum concentration of ochratoxin A at 20 µg/L.
B) Ochratoxin A at 156.25 ng/L (LOQ = 100 ng/L), as comparison.
C) Blank injection following maximum ochratoxin A concentration injection showing no carryover.

Analysis of an actual live sample

To show an actual example with enriched content of ochratoxin A, a commercially available roasted coffee was treated as described in the sample preparation section. This sample was measured on both Columns 1 and 2 as described in the method section. The content of ochratoxin A in the roasted coffee analyzed on Column 1 was determined using the previously created calibration. The injected sample contained approximately 130 ppt ochratoxin A (Figure 4A). This was approximately the LOQ, and yielded a total content of approximately 0.130 µg/kg, which was far below the recommended limit of 5.0 µg/kg. The calculation of the content of ochratoxin A (µg/kg) from the total injected amount (ng on column), considering the total amount of coffee and dilutions used, is described in DIN EN 14132[3]. The concentration limit given by the European Commission Report is reached at a concentration of 5.0 ppb in the injection solution obtained from the described sample preparation. The achieved sensitivity was sufficient to detect the concentration for the required limit. The measurement with the solvent saver Column 2, containing a comparable stationary phase with the 2.7-µm superficially porous particles, delivered better separation performance with higher and narrower peaks at less than half of the solvent consumption (Figure 4B).

Optimizing sample throughput

The above described experiments were repeated with a Poroshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm solvent saver column. The flow rate and the injection volume were adjusted according to the narrower id of this column to 0.43 mL/min and 43 µL, respectively. For the calibration, similar linearity was found, but the LOQ and LOD were lower on the 2.7-µm solvent saver column. This was due to the better separation performance, showing narrower and sharper peaks with improved S/N enabled by the 2.7-µm superficially porous particles used in this column (Table 1B).

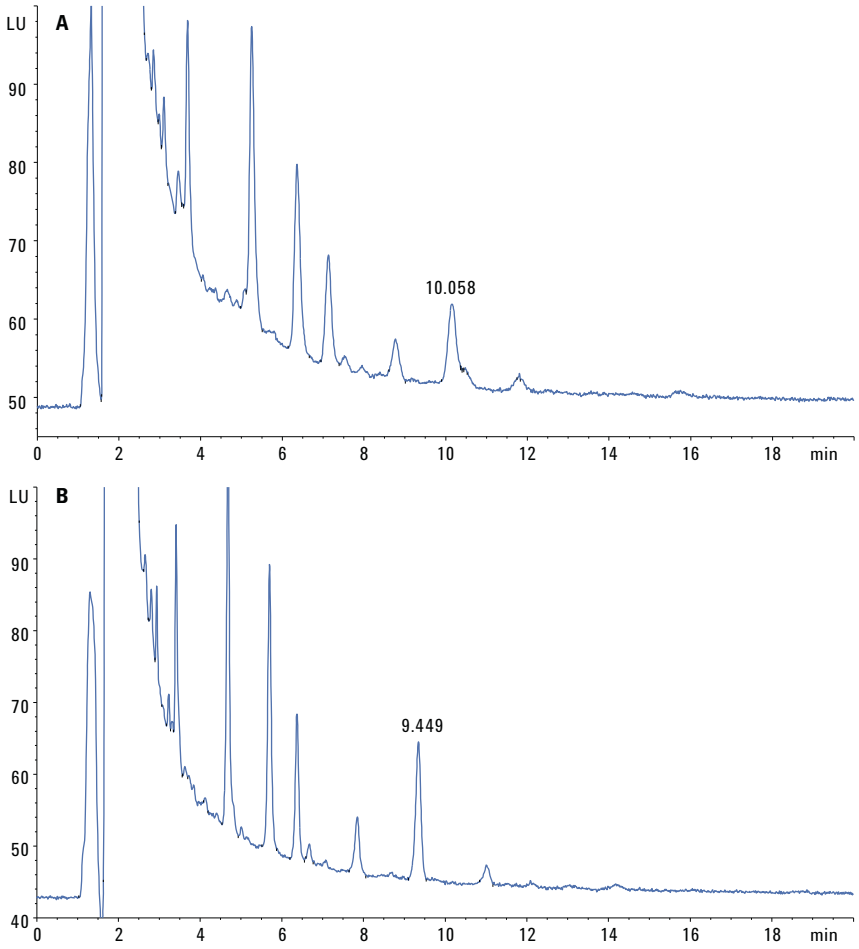

Figure 4. Determination of ochratoxin A in roasted coffee. A) Column 1: 4.6 × 150 mm, 5 µm. B) Column 2: 3.0 × 150, 2.7 µm.

Table 1B) Performance data measured for 10 µg/L (ppb) ochratoxin A with the Agilent Poroshell 3.0 × 150 mm, 2.7 µm column as well as concentration precision and accuracy.

Parameter	Value
Column	Agilent Poroshell EC 120, 3.0 × 150 mm, 2.7 µm
Ochratoxin A	10 µg/L
RT	9.45 minutes
RT RSD (%)	0.25
Area RSD (%)	0.19
Calibration	78.125 ng/L–10 µg/L
Linearity, R^2	1.00000
LOD	14 ng/L
LOQ	70 ng/L
Carryover	from 20.0 µg/L–n.d.
Concentration precision	0.35 % at 8.0 µg/L
Concentration accuracy	99.3 % at 8.0 µg/L

Other statistical performance parameters such as retention time and area RSDs, as well as concentration precision and accuracy, were in the same order for both columns. The advantage of Column 2, with the lower id, was the solvent consumption, which was 57 % lower than Column 1.

To improve the analysis efficiency, the 150-mm column was exchanged with a 3.0 × 50 mm column with the identical stationary phase. This immediately allowed a reduction of the run time to approximately one third, and improved the throughput by a factor of three (Figure 5A). Further improvement was achieved by doubling the flow rate to 0.86 mL/min, which reduced the run time to 3 minutes, and the elution time of ochratoxin A to 1.59 minutes (Figure 5B). With a flow rate of 1.72 mL/min, the total run time was reduced to 1.5 minutes and the elution time of ochratoxin A to 0.84 minutes (Figure 5C).

Conclusion

This Application Note demonstrates the use of a standard HPLC in combination with fluorescence detection to determine the mycotoxin compound ochratoxin A in roasted coffee according to the DIN EN 14132. The linearity of the calibration curve is excellent as well as the RSD values for retention time and area. It shows that comparable results with even lower LOD and LOQ can be achieved by means of solvent a saver column on the same instrument with 57 % less solvent consumed.

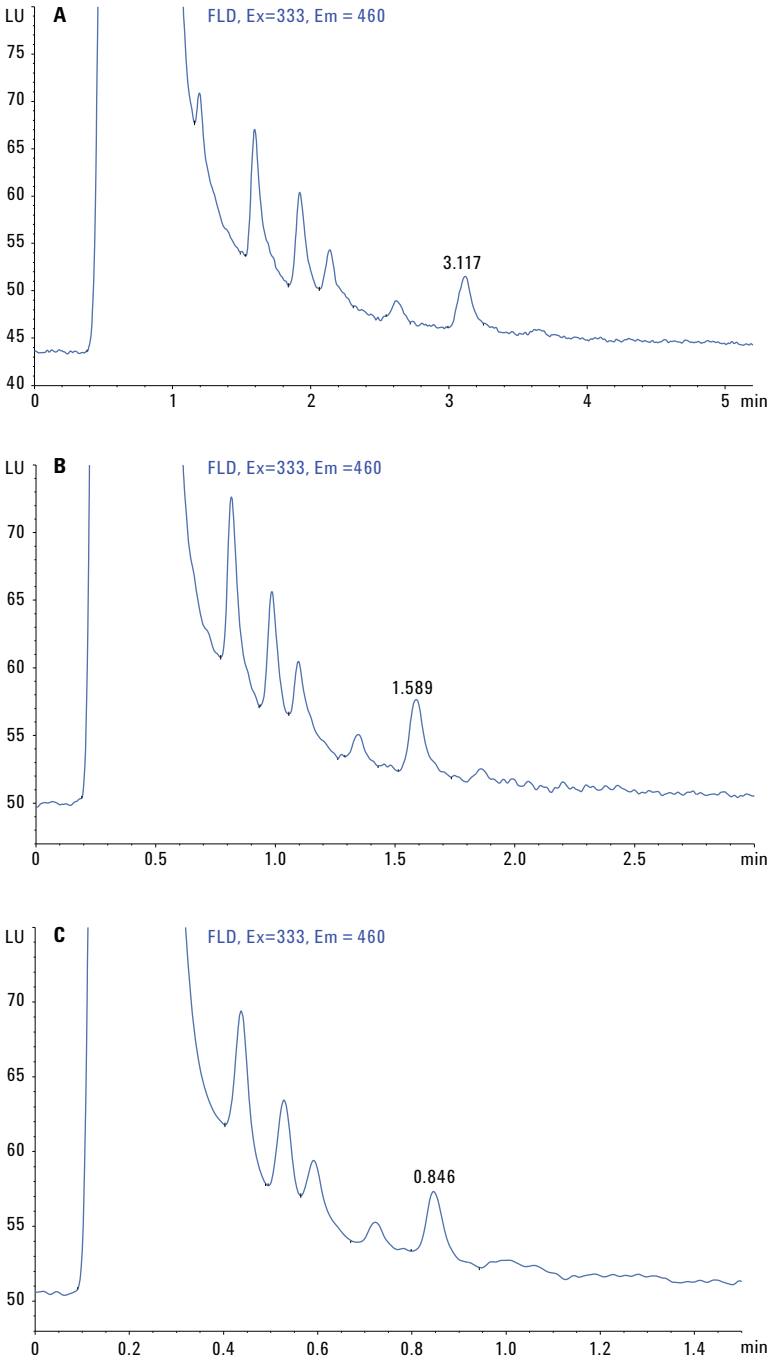

Figure 5. Improved efficiency by means of a shorter column (3.0 × 50 mm, 2.7 µm) at 0.43 mL/min and higher flow rates. Reduction of column length to one third reduces the elution time of ochratoxin A to 3.11 minutes, run time to 6 minutes, and increases sample throughput three times. B) Doubling the flow rate to 0.86 mL/min reduces the run time to 3 minutes and the elution time of ochratoxin A to 1.59 minutes. C) A four times higher flow rate of 1.72 mL/min reduces the run time to 1.5 minutes, and the elution time of ochratoxin A to 0.84 minutes.

References

1. www.wikipedia.org

2a. Commission Regulation (EU) No 594/2012 of 5 July 2012 amending Regulation (EC) 1881/**2006** as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs.

2b. Commission Regulation (EC) No 1881/2006 of 19 December **2006** setting maximum levels for certain contaminants in foodstuffs.

3. DIN EN 14132, Foodstuff – Determination of ochratoxin A in barley roasted coffee – HPLC method with immunoaffinity column clean-up, (EN 14132:2009) Sept. **2009**.

4. DIN ISO 20481, Coffee and coffee products – Determination of caffeine content by HPLC, (ISO 20481:2008) Jan. **2011**.

5. Naegele, E., Determination of Caffeine in Coffee Products According to DIN 20481, *Agilent Technologies Application Note*, publication number 5991-2851EN, **2013**.

6. DIN 10767, Coffee and coffee products – Determination of chlorogenic acids by HPLC, **1992**.

7. Naegele, E, Determination of Chlorogenic Acid in Coffee Products According to DIN 10767, *Agilent Technologies Application Note*, publication number 5991-2852EN, **2013**.

8. DIN 10779, Coffee and coffee products – Determination of 16-O-methyl cafestol content in roasted coffee by HPLC, March **2011**.

9. Naegele, E., Determination of Methylcafestol in Roasted Coffee Products According to DIN 10779, *Agilent Technologies Application Note,* publication number 5991-2853EN, **2013**.

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