LC-MS/MS Determination of Alternaria Toxins in Vegetables and Fruit Beverages

Application Note

Authors

Dr Lilly Reinhold and Iris Bartels
LAVES, Lebensmittelinstitut
Braunschweig, Germany

Introduction

Fungi of the genus Alternaria are pathogens of various plants, such as fruits and vegetables. Alternaria alternata is a frequently occuring species of particular interest because it produces a number of harmful mycotoxins, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN) and tenuazonic acid (TEA). The toxins AME and AOH have been found in sorghum, sunflower seeds, barley, wheat, oats, tomatoes, mandarin oranges, pepper and melons[1]. As well as the need to anticipate mycotoxin risks and eliminate risk factors to ensure safe food supplies, reliable alternaria mycotoxin analysis methods must be developed for tracing their source in the food production chain.

Clean-up methods for alternaria toxins from apple juice and other fruit beverages have been established and involve mainly solvent extraction and SPE with C18 or aminopropyl columns[2]. The quantitative determination of silylated toxins in products of stone fruits, berries, citrus fruits, tomatoes and moldy fruits have been performed by GC/MS[3]. LC coupled (mainly) with UV-DAD detection has often been applied[1,4]. More recently, enabled by the development of APCI and electrospray ionization techniques, LC/MS and LC-MS/MS have been used successfully for confirmation and quantification of the toxins in apple juice and other fruit beverages at sub ng/mL levels[2] and in oil seeds, edible oils, fresh grape juice, must and young wine[5]. This application note describes a method for the determination of important alternaria mycotoxins in liquid and solid foodstuff samples of fruit and vegetable origin.

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Method

After the toxins have been extracted from the sample matrix, the extract is diluted with sodium phosphate (NaH_2PO_4) solution and then cleaned up and concentrated by solid phase extraction using Agilent's novel Bond Elut Plexa hydroxylated polymer.

The target analytes are eluted with methanol and acetonitrile, then the eluate is evaporated and reconstituted in the mobile phase. High sensitivity detection and identification of the toxins is performed by electrospray LC-tandem mass spectrometry with positive/negative ion detection in MRM mode. For identification, both the retention times and the transition ions are used. A set of six concentrations (5 µg/kg, 10, 25, 50, 75, 100 µg/kg) of an alternaria toxin mix standard is spiked into blank matrix prior to SPE and used for external matrix calibration. LC is performed on a PFP (pentafluorophenyl) modified Agilent MonoChrom MS silica gel column.

Sample Preparation

Extraction procedure for solid samples eg fruit purées

Weigh 20 g of the homogenized sample. Add 60 mL of acetonitrile-methanol-water (pH 3; 45/10/45 v/v/v) and homogenize for at least 2 min with an ultra-fast mixer. Centrifuge for 10 min at 4000 rpm. Transfer 6 mL of the supernatant to a centrifuge tube, dilute with 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3). Centrifuge again if necessary.

Extraction procedure for liquid samples eg grape and vegetable beverages

Weigh 5 g of juice in a centrifuge tube, add 15 mL of acetonitrile/methanol/water mixture (pH 3; 45/10/45 v/v/v), and shake for 60 sec. Transfer 6 mL of the supernatant to a centrifuge tube and add 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3). Centrifuge again if necessary.

Solid phase extraction

Use a Bond Elut Plexa 200 mg, 6 mL cartridge (part number 12109206). Condition with 5 mL methanol, followed by 5 mL water. Add the sample, rinse the centrifuge tube with water and load rinse. Wash with 5 mL water, dry for 10 min under light vacuum and elute with 5 mL methanol, then 5 mL acetonitrile. Evaporate eluate to dryness and reconstitute in 1 mL water/methanol (7/3, v/v); filter if necessary (0.45 µm).

MS Conditions

Retention Window: 0 to 13.6 min
Ionization Mode: ESI+
Function Type: MRM of 9 channels

Table 1a. MS conditions

Compound	Q1>Q3	Dwell Time (sec)	Cone Voltage	Collision Energy
TEA	198.16 > 125.16	0.10	25.0	15.0
	198.16 > 153.18	0.10	25.0	15.0
AOH	259.03 > 185.22	0.10	25.0	30.0
	259.03 > 213.20	0.10	25.0	25.0
ALT	293.05 > 229.27	0.10	20.0	20.0
	293.05 > 239.41	0.10	20.0	25.0
	293.05 > 257.24	0.10	20.0	15.0
TEN	415.11 > 302.22	0.10	30.0	15.0
	415.11 > 312.25	0.10	30.0	20.0

Retention Window: 13 to 20 min
Ionization Mode: ESI-
Function Type: MRM of 2 channels

Table 1b. MS conditions

Compound	Q1>Q3	Dwell Time (sec)	Cone Voltage	Collision Energy
AME	271.17 > 228.21	0.10	40.0	30.0
	271.17 > 256.19	0.10	40.0	20.0

Source Temperature: 120 °C
Capillary Voltage: 3 KV
Cone Voltage: see MS conditions, Table 2
Extraction Voltage: 8 V
Collision Energy: see MS conditions, Table 2
Desolvation Gas: 630 L/h
Cone Gas: 50 L/h
Collision Cell Pressure: 0.2 mL/min

LC Conditions

Eluent A:	2 mmol (158.12 mg) NH_4HCO_3 + 50 mL water + 950 mL MeOH
Eluent B:	Acetonitrile
Eluent C:	2 mmol (158.12 mg) NH_4HCO_3 + 50 mL MeOH + 950 mL water
Stop Time:	35 min
LC Column:	Agilent MonoChrom MS 5 µm, 150 x 3 mm (p/n A2080150X030)
Column Temperature:	40 °C
Injection Volume:	20 µL

Table 2. LC conditions

Time	A%	B%	C%	Flow Rate (mL/min)
0.00	30	0	70	0.3
3.00	30	0	70	0.3
3.10	30	0	70	0.4
7.00	90	0	10	0.4
9.90	90	0	10	0.4
10.00	90	0	10	0.3
11.90	90	0	10	0.3
12.00	100	0	0	0.3
16.00	50	50	0	0.3
26.00	50	50	0	0.3
30.00	100	0	0	0.3
30.10	30	0	70	0.3

Results and Discussion

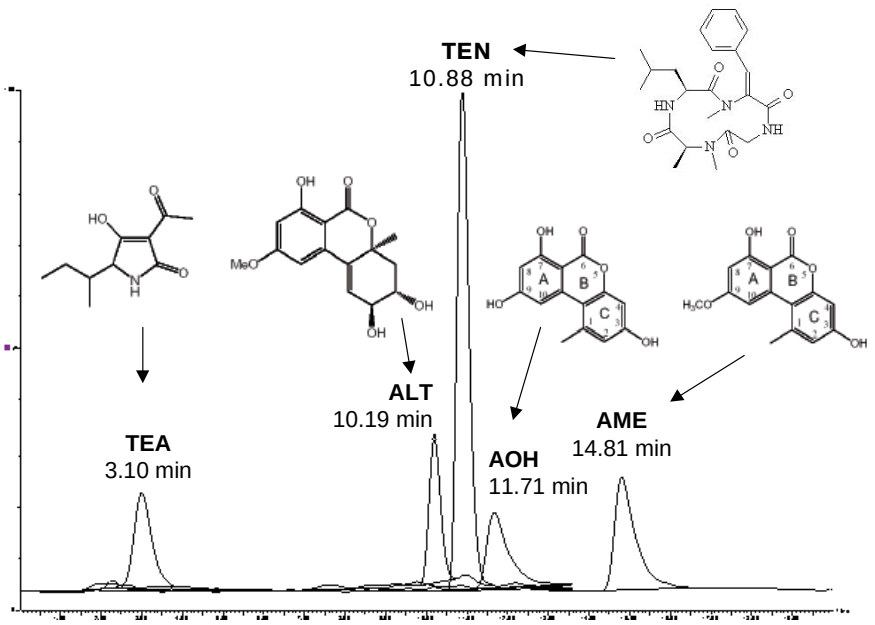
The LC-MS/MS method describes the qualitative and quantitative determination of five important alternaria toxins in different food matrices. Extraction of the toxins from beverage samples was done efficiently by solvent extraction at pH 3 (Table 3).

Table 3. Validation parameters for the determination of alternaria toxins in vegetables and grape juices.

Compound	Juice	Concentration (µg/kg or µg/L)	SD%	CV% (Horwitz)	Repeatability (N=5)	Recovery %
ALT	grape	50	2.95	10.2	8.35	100
ALT	veg.	50	1.04	4.3	2.94	105
AME	grape	50	3.05	6.7	8.62	103
AME	veg.	50	0.89	4.1	2.51	105
AOH	grape	50	2.33	9.9	6.61	105
AOH	veg.	50	0.41	6.9	1.15	106
TEA	grape	50	3.57	11.4	10.1	97
TEA	veg.	50	2.76	11.1	7.82	103
TEN	grape	50	2.38	9.0	6.74	102
TEN	veg.	50	1.01	5.5	2.86	111

Further dilution with buffer solution delivered a raw extract that was additionally cleaned by solid phase extraction. The Bond Elut Plexa polymer, which provides improved analytical performance for a wide range of acidic, neutral and basic analytes, was used as the stationary phase for SPE. Due to its advanced polymer architecture and narrow particle size distribution, the flow characteristics of the Bond Elut Plexa polymer are excellent. As there is no amide functionality, which might contribute to the retention of polar matrix interferences, these will be washed away whereas more lipophilic analytes are extracted into the non polar pores of the polymer. Overall, ion suppression and/or enhancement in the mass spectrometry was reduced. Typical recoveries for the alternaria toxins from grape juice ranged from 97 to 105% (Table 3).

For the method, the limit of detection as signal to noise ratio was 5 µg/L; the limit of determination was >10 µg/L. Liquid chromatography was performed using a pentafluorophenyl column with a unique selectivity for compounds with aromatic properties. In general, fluorinated or perfluorinated LC columns are a good alternative to traditional C8 and C18 phases. Their retention behavior includes some π-π interactions as well as other mechanisms, such as charge transfer and electrostatic interactions. In this case, tenuazonic acid had the shortest retention time and alternariol monomethyl ether was eluted the last, both as sharp peaks (Figure 1).



KEY
AOH alternariol
AME alternariol monomethyl ether
ALT altenuene
TEN tentoxin
TEA tenuazonic acid

Figure 1. Chromatogram of the separation of five alternaria toxins (blank grape juice matrix spiked at a concentration of 25 µg/kg)

Conclusion

With this multi analyte method, it is possible to confirm and quantify relevant toxins from alternaria contaminated organic foodstuffs at the ng/mL level with good validation parameters. Solid phase extraction is an excellent technique for clean up of the raw extracts and enrichment of the toxins. Bond Elut Plexa solid-phase extraction in combination with LC tandem mass spectrometry allows for the determination of trace levels of mycotoxins with lower background and higher sensitivity.

References

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