

**SPECIAL ISSUE**

# Mycotoxins in Spices

## Validated Method for Simultaneous Determination of Aflatoxins and Ochratoxin A in Paprika and Chilli Powder

Maria Barricelli, Rita Kupfer and Bettina Börner

Since 2006, a maximum level of Aflatoxins B1, B2, G1 and G2 allowable in some spices has been laid down in the EU, and since 1 July 2010 this also applies to Ochratoxin A in paprika and chilli powder. To check these maximum levels, a method has been developed, which enables their simultaneous determination from these matrices.

Spices enrich the taste of our food through their aroma, bitter and spicy substances and have the ability to stimulate our appetite. Physiologically, by activation of saliva secretion and gastrointestinal juices, spices stimulate digestion and are for that reason of nutritional importance. In particular spices stemming from the tropics and subtropics are frequently used [1].

### Spice Import to Germany

Spices have become a permanent part of the food staple in the middle European kitchen. In Germany, for example, ca. 550 g spices are used per head and year [2]. The 2009 statistics show an import of around 85 000 t spices into Germany, and the amounts of pepper and paprika are clearly the

highest ranking, followed by nutmeg, caraway, coriander and ginger [3]. Paprika and chilli, however, are often polluted with Aflatoxins and Ochratoxin A.


### Mycotoxins

Mycotoxins are natural secondary metabolic end products produced by moulds. Moulds belong to the most significant organisms spoiling our food. Aflatoxins and Ochratoxin A are naturally occurring mycotoxins that are highly toxic for humans. Aflatoxins are formed by moulds of the species *Aspergillus* in various foods and animal feed. According to the SCF (Scientific Committee for Food) statement of 23 September 1994, they are to be regarded as genotoxic carcinogens. Aflatoxin B1 is by far the most toxic compound [4]. Ochratoxin A is produced by moulds



Maria Barricelli



State certified food chemist; responsible for the research area of mycotoxins 

**Tab. 1 Parameter settings of the ion source**

Polarity	ES+
Cone Voltage	28 V
Source Temperature	100 °C
Desolvation Temperature	400 °C
Desolvation Gas	450 l/h
Cone Gas	100 l/h

**Tab. 2 MRM-experiments of function 1**

Channel	Reaction	Dwell [s]	Cone [V%]	Collision
1	404 > 239	0.2	28	24
2	404 > 357,9	0.2	28	14

Inter Channel Delay: 0.05 s; RT Window: 3-9 min



**Rita Kupfer**

Bsc Food chemist, holding a scientific placement with LLBB from July 2009 to end of August 2010

of the species *Aspergillus* and *Penicillium* on cereal, coffee, spices and other foods. The scientific committee for contaminants in the food chain of the European Food Safety Authority (EFSA) classed Ochratoxin A, after an updated scientific survey on Ochratoxin A in foods of 4 April 2006, as a mycotoxin with nephrotoxic and genotoxic characteristics. Due to an incomplete set of data, the classification of Ochratoxin A as kidney carcinogen in humans could not be justified [5].

### Examinations

Since 1987, different spice samples are continuously tested for Aflatoxins and Ochratoxin A within the framework of an official monitoring body in Berlin. It came to show that in particular paprika, closely followed by chilli powder, were the most frequent-

ly contaminated with both Aflatoxins and Ochratoxin A. Green, white and black pepper were only little or not at all polluted with these mycotoxins. The same applied to ginger and nutmeg.

### Methodology

Based on our examination results, in order to test for maximum levels of Aflatoxin and Ochratoxin A in paprika or chilli powder, it was deemed necessary to develop a method that would efficiently substitute the individual methods available. Particular desirable was the isolation of both mycotoxins from the matrix in one single step. To achieve this, we used immunoaffinity columns supplied by LCTech GmbH, Dorfen (Afla-OtaClean™) that enable simultaneous isolation of Aflatoxins and Ochratoxin A from a.o. spices. The method was developed on the basis of the user manual for the immunoaffinity columns „Afla-Ota-CLEAN™ immunoaffinity columns for the analysis of Aflatoxins B/G and Ochratoxin A - Perfect Sample Preparation“ [6] and validated for the matrix paprika powder. In the following, the completely validated method will be introduced.

#### Literature

- [1] Franke W: Nutzpflanzenkunde. 3. Aufl., Thieme Verlag, Stuttgart (1985).
- [2] afz-journal: Die Fleischerei, Lebensmittelpraxis. Blick/ff-delikat.
- [3] Source: Official Foreign Trade Statistics, preliminary results.
- [4] Berichte des Wissenschaftlichen Lebensmittelausschusses, fünfund-dreißigste Folge, Stellungnahme des Wissenschaftlichen Lebens-

mittelausschusses zu Aflatoxinen, Ochratoxin A und Patulin, s.45-50. [http://ec.europa.eu/food/fs/sc/scf/reports/scf\\_reports\\_35.pdf](http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_35.pdf)

[5] The EFSA Journal 365, 1-56 (2006).

[6] User Manual Afla-OtaCLEAN™: Immunoaffinity columns for the analysis of Aflatoxin B/G and Ochratoxin A. LCTech GmbH, Dorfen, Germany, as of: October, 2009

[7] Barricelli et al.: Bestimmung von Ochratoxin A in Gewürzen mittels LC-MS/MS nach Reinigung über Immunoaffinitätssäulen. Deut Lebensm-Rundsch 103 (1), 1-3 (2007).

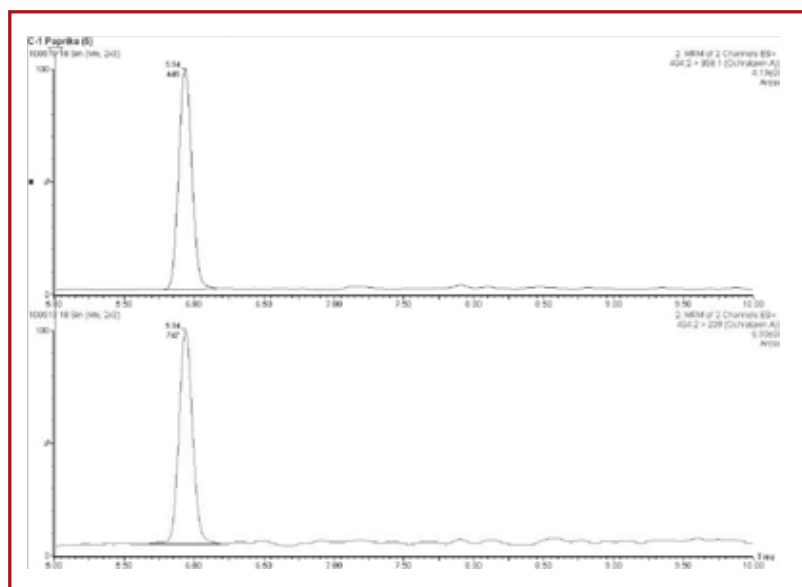
[8] Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

## Sample Preparation

10 g paprika powder is weighed into a 250 ml centrifuge beaker, mixed with 20 ml water and left to soak for at least 10 min. Then, 80 ml methanol and 25 ml hexane are added and homogenised for 2 min using an Ultra-Turrax on a medium setting. This extraction mixture is cool-centrifuged for 10 min at ca. 8 000 rpm. Afterwards, all of the supernatant is filtrated using a fluted filter. Using a volumetric pipette, the hexane phase of the filtrate is penetrated and 5 ml methanol extract are taken up. This is dispensed in a 50-ml measuring cylinder, diluted with 30 ml phosphate buffer solution (PBS, pH 7.3) and the whole mixture is passed through the Afla-Ota-CLEAN™ immunoaffinity column conditioned with 5 ml PBS-buffer. A storage vessel for IAC is to be used. Dropping speed should be at 1–2 ml/min. Subsequently, the column is washed with 10 ml osmosis water at 2 ml/min and then sucked dry for 2 min. The bonded mycotoxins are eluted with 3 x 1 ml methanol (1–2 ml/min). The first ml methanol needs to act on the IAC-column for at least 5 min. The eluate is collected in a 10-ml centrifuge tube, and concentrated to dryness under nitrogen at 55 °C. The residue is resuspended with 1 ml of a mixture of 80 vol% osmotic water/ 0.1 % acetic acid and 20 vol% acetonitrile/ 0.1 % acetic acid (ultrasonic!), and following filtration using a 0.2 µl syringe filter applied to the HPLC-PCD and HPLC-MS/MS, respectively. Quantification is achieved through external calibration.

### Quantification of Ochratoxin A employing HPLC-MS/MS [7]

The HPLC/MS/MS-equipment consists of an HPLC-unit, series Alliance 2695, with quaternary pump, auto-sampler, micro-degasser and column thermostat and a mass spectrometer, Quattro Micro, by Waters (Eschborn). In addition, the HPLC-column Kromasil



**Fig. 1**  
LC-MS/MS-chromatogram of spiked analyte-free paprika powder sample (ca. 5 µg/kg Ochratoxin A)

100 C18 by Phenomenex (Aschaffenburg) was used, which has the following characteristics: length - 75 mm, inner diameter - 3.1 mm, particle size - 4 µm, pore size - 80 µm. Pre-column was the Phenomenex (Aschaffenburg) Security-Cartridge-System, which also contains high purity C-18 material (length - 2 mm, diameter - 2 mm).

### HPLC-Conditions

Eluent A: 50 % 0.1 % acetic acid, eluent B: 50 % acetonitrile, isocratic, flow rate: 0.2 ml/min, column temperature: 30 °C, injection volume: 25 µl

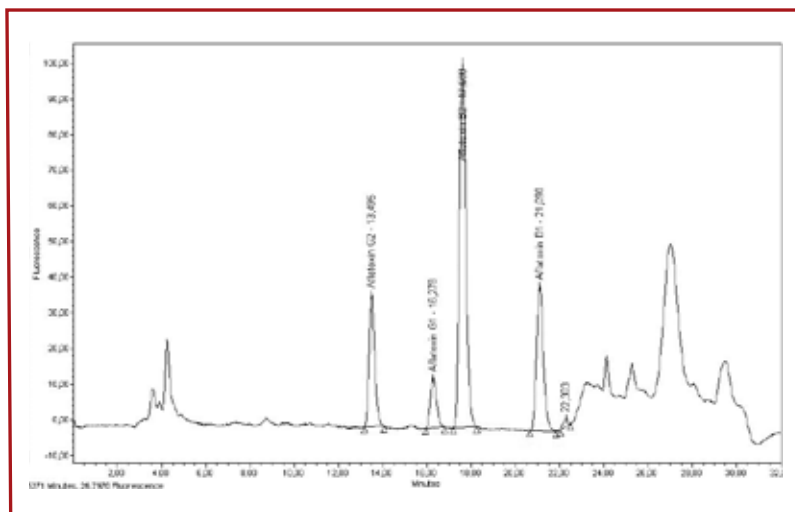
### MS/MS-Parameter

The mass spectrometer was equipped with an electrospray-ion source (ESI+) to ionise the analyte and operated in the multiple reaction monitoring mode (MRM). As quantification trace for Ochratoxin A, the ion mass pair 404/239 m/z was referred to and for validation trace, the ion mass pair 404/357.9 m/z.

## Contact Details

**Maria Barricelli**  
Maria.Barricelli@Landeslabor-BBB.de

**Rita, Kupfer**  
**Bettina Börner**  
Landeslabor  
Berlin-Brandenburg  
Fb II-3  
Invalidenstr. 60  
10557 Berlin



**Fig. 2**  
Chromatogram of a spiked  
analyte-free sample of  
paprika powder (ca. 5 µg/kg  
for each aflatoxin)

### Quantification of Aflatoxins using HPLC-FLD following photochemical PCD

For chromatography, an HPLC-equipment by Waters was used, which comprised the Waters autosampler 717 plus, a Waters HPLC-pump 510, a Waters multi  $\lambda$ -fluorescence-detector 2475 and a column thermostat. In addition, the HPLC-column Kromasil-100, RP 18 was used with the following characteristics: length - 250 mm, inner diameter - 4.6 mm, particle size - 5 µm. A pre-column was filled with the same mate-

rial (20 x 4.6 mm). Detection was performed with the fluorescence-detector following photochemical post-column derivatisation using a photochemical reactor UVE™ by LCTech GmbH, Dorfen.

### HPLC-Parameters

Flow rate: 0.96 ml/min, eluent A: osmosis water, eluent B: acetonitrile/methanol 1:1, injection volume: 50 µl, column temperature: 35°C

### Validation

The applicability of this method was tested by processing and analysis of an analyte-free sample of paprika powder spiked with Aflatoxins and Ochratoxin A in the range of 1–25 µg/kg per mycotoxin. Detection and quantification limits according to DIN 32645 and average recovery rate were assessed.

### Investigation of Certified Reference Material

The applicability of this method has been validated through processing of certified materials (paprika powder FA-PAS test material T1753, chilli powder T0475). The detected level for both lay within the certified range. This is now routinely used to determine Ochratoxin A in paprika and chilli powder.

### Conclusion

By employment of the above described combined immunoaffinity column, Aflatoxins B1, B2, G1, G2 and Ochratoxin A can be isolated from the matrix paprika and chilli powder in one single processing step. Thereby, the laboratory efficiency can be increased.

The determined quantification limits are adequate to test for the maximum values stipulated by the EU. Furthermore, the described analysis method achieves recovery rates as required in appendix II EU-regulation no. 401/2006 of the Commission for the specification of sampling procedures and analysis methods for the official control of mycotoxin contents in food [8].

Gradient Elution			
Time [min]	Flow [ml/min]	%A	%B
0	0.96	65	35
17	0.96	60	40
18	0.96	20	80
25	0.96	20	80
26	0.96	65	35
32	0.96	65	35

Parameter of the Fluorescence Detector  
 $\lambda_{Ex}$ : 365 nm,  $\lambda_{Em}$ : 435 nm, PMT Gain: 100, Attenuation: 64

**Tab. 3** Detection and quantification limits and recovery rates

Analyte	Detection Limit [µg/kg]	Quantification Limit [µg/kg]	Average Recovery Rate [%]
Aflatoxin B1	1	4	93
Aflatoxin B2	1	4	95
Aflatoxin G1	1	3	93
Aflatoxin G2	2	7	74
Ochratoxin A	2	4	99