

Screening and Quantitation of 191 Mycotoxins and Other Fungal Metabolites in Almonds, Hazelnuts, Peanuts, and Pistachios Using UHPLC/MS/MS

Application Note

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Abstract

This application note describes the development of a multitarget method for the determination of 191 mycotoxins and other fungal metabolites as well as its validation in almonds, hazelnuts, peanuts, and pistachios. In addition to all mycotoxins regulated in the European Union, the method includes many mycotoxins that are frequently found in food. The method comprises a simple extraction with an acidified acetonitrile-water mixture, a subsequent dilution of the raw extract, and measurement by UHPLC/MS/MS. Calibration was performed for all compounds with solvent standards.

Furthermore, this application note presents parts of a validation for the 65 most important mycotoxins in the four tested matrixes. The method showed excellent sensitivity down to 0.04 µg/kg for enniatin B3 in peanuts, and good reproducibility with standard deviations below 10 % for the majority of analytes. Apparent recoveries of 70 to 120 % were obtained in approximately 60 % of the matrix-analyte combinations. In cases with lower recoveries, either extraction recoveries were low (for example, for fumonisins) or signal suppression was observed for several early eluting analytes. There was signal enhancement in a few cases, leading to higher apparent recoveries.

The method was applied to the analysis of naturally contaminated nut samples and overall 40 different fungal metabolites could be identified. Interestingly, the most frequently found toxins were not the compounds currently regulated in the European Union but beauvericin, enniatin B, and macrosporin. The most contaminated hazelnut sample contained 26 different toxins. All results are presented in more detail in Varga *et al.* [1].



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Introduction

Mycotoxins are secondary metabolites of fungi, which can cause acute or chronic toxic effects in humans and animals. As fungal colonization occurs during farming and storage, mycotoxins can be found in a variety of feed and food products including cereals, nuts, fruits, spices, and coffee [2]. Mycotoxins belong to different chemical classes and show very different physicochemical properties. From the several hundreds of mycotoxins identified so far, approximately one dozen are considered a major health risk and are regulated in food and feed. European Commission Regulation (EC) 1881/2006 and its amendments specify maximum levels in food for aflatoxins, deoxynivalenol, fumonisins, ochratoxin A, patulin, and zearalenone [3]. In addition, Commission Recommendation 2013/165/EU provides indicative levels, above which investigations should be performed on the factors leading to the presence of T-2 and HT-2 toxin [4]. These compounds are included in routine monitoring programs (for example, from national food safety authorities) and data has been generated on the occurrence of these mycotoxins in different food commodities. Single target methods have consistently been replaced by LC/MS-based multitarget methods for the simultaneous quantitation of co-occurring mycotoxins. This development has been aided by the increase in performance of modern triple quadrupole instruments, and the development of software tools such as the Agilent Dynamic MRM feature, which allows analysts to more easily develop and evolve fast methods with large numbers of target compounds.

Challenges for multitarget methods are the efficient extraction of analytes with largely different physicochemical properties from a variety of food products and the huge differences in the naturally occurring toxin concentrations. The majority of multitarget methods have been developed for the screening of mycotoxins in raw cereals [5]. Comprehensive information on mycotoxin contamination in other matrixes such as nuts is lacking [1].

Nuts are dry fruits consisting of a hard shell and a seed. While in the botanical context, true nuts are those which have an indehiscent seed. The use of the term nut in the food context is less strict and even involves almonds which, botanically are drupes, or peanuts, actually legumes. For simplicity, all four matrixes investigated in this application note (almonds, hazelnuts, peanuts, and pistachios) are referred to as nuts. While agricultural products on the field are most often infected by fungi of the genera *Fusarium*, *Alternaria*, and *Cladosporium*, fungi of the genera *Aspergillus*, *Penicillium* and *Trichoderma* dominate spoilage during storage [6]. Most data exists on the occurrence of aflatoxins and ochratoxin A in

nuts; information about contamination with other mycotoxins is very limited.

This application note describes a multitarget UHPLC/MS/MS method for the quantitation of 191 mycotoxins and fungal metabolites in nuts. The method comprises a single extraction with an acidified acetonitrile-water mixture and a dilution of the raw extract for subsequent measurement. Method performance parameters are shown exemplarily for the 65 most important contaminants in almonds, hazelnuts, peanuts, and pistachios. The method was applied to a variety of nuts that were bought from Austrian and Turkish markets. Varga *et al.* [1] present the method and obtained results in more detail.

Experimental

Reagents, reference compounds, and nut samples

All reagents and solvents were HPLC or LC/MS grade. Acetonitrile, methanol, and acetic acid were purchased from VWR International (Vienna, Austria); ammonium acetate was purchased from Sigma-Aldrich (Vienna, Austria). Ultrapure water was produced using a MilliQ Plus system (Molsheim, France). Mycotoxin analytical standards were either purchased from Alexis Austria (Vienna, Austria), Alfarma (Prague, Czech Republic), Axxora Europe (Lausanne, Switzerland), Bioaustralis (distributed by Tebu-Bio, Germany), Iris Biotech GmbH (Marktredwitz, Germany), LGC Promochem GmbH (Wesel, Germany), Romer Labs (Tulln, Austria), and Sigma-Aldrich, or were provided as isolates from research groups around the world.

Stock standard solutions were prepared by dissolving the reference compounds in acetonitrile, methanol, water, or mixtures thereof, depending on the physicochemical properties of the substance. The individual standard solutions were combined to intermediate dilutions containing up to 13 target compounds. Immediately before use, these intermediate dilutions were combined to a multi-analyte working solution, which was used for calibration and for spiking of blank nut matrixes. Stock standard solutions, as well as intermediate dilutions, were stored until use at $-20\text{ }^{\circ}\text{C}$. Calibration samples were prepared by stepwise dilution of the working solution with a mixture of acetonitrile/water/acetic acid (20:79:1, v/v/v).

Nut samples were purchased from various stores in different regions of Austria and Turkey. All samples were stored at $-20\text{ }^{\circ}\text{C}$ until use. Samples were peeled and ground using an electric blender. A 5.00 g (± 0.01 g) portion of the samples was weighed in 50-mL polypropylene tubes and 20 mL of extraction solvent (acetonitrile/water/acetic acid, 79/20/1, v/v/v) was added. The samples were extracted at room

temperature for 90 minutes on a rotary shaker (200 rpm). After the solid residue settled, 500 μ L of the raw extract was diluted with the same volume of a dilution solvent (acetonitrile/water/acetic acid, 20/79/1, v/v/v) resulting in an 8-fold dilution.

One blank sample of each of the four analyzed nuts was selected and thoroughly homogenized. Three portions of each sample were spiked before extraction at one medium concentration level with the multi-analyte working solution. After evaporation of the solvent, the spiked samples as well as the blank samples were extracted as described above. The raw extract of the blank samples was spiked with the multi-analyte working solution at different levels to evaluate matrix effects in electrospray ionization as well as extraction recoveries by comparing apparent recoveries of samples spiked before and after extraction.

Equipment

Separation was carried out using an Agilent 1290 Infinity UHPLC system consisting of:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

The UHPLC system was coupled to an Agilent G6460A Triple Quadrupole Mass Spectrometer equipped with an Agilent Jet Stream electrospray ionization source. Agilent MassHunter Workstation B.06.00 Software was used for data acquisition and analysis.

Method

The 1290 Infinity UHPLC conditions are summarized in Table 1, and a summary of the 6460 Triple Quadrupole parameters are shown in Table 2. Identification of polarity, precursor, and product ions, as well as optimization of fragmentor voltages and collision energies was done using the MassHunter Optimizer Software with flow injection of reference standard solutions. For most analytes, two mass transitions were monitored to comply with identification criteria specified in Commission Decision 2002/657/EC [7]. Analysis was carried out with positive and negative electrospray ionization in dynamic multiple reaction monitoring (DMRM) in two consecutive runs. A complete listing of all transitions and conditions, as well as retention times for all analytes can be found in [1].

Table 1. Agilent 1290 UHPLC Parameters

UHPLC column	Agilent ZORBAX RRHD Eclipse Plus C18 2.1 \times 150 mm, 1.8 μ m (p/n 959759-902) at 25 $^{\circ}$ C	
Mobile phase	A: 5 mM ammonium acetate in methanol/water/acetic acid (10/89/1, v/v/v) B: 5 mM ammonium acetate in methanol/water/acetic acid (97/2/1, v/v/v)	
Gradient program	Minutes	% B
	0	0
	2	0
	5	50
	14	100
	18.5	100
	18.6	0
Stop time	21 minutes	
Flow rate	0.25 mL/min	
Injection volume	5 μ L	
Needle wash	5 seconds with acetonitrile/water (50/50; v/v)	

Table 2. Agilent 6460 Triple Quadrupole Parameters

Ionization mode	Positive or negative ESI with Agilent Jet Stream
Scan type	Dynamic MRM
Gas temperature	200 $^{\circ}$ C
Gas flow	8 L/min
Nebulizer pressure	40 psi
Sheath gas temperature	350 $^{\circ}$ C
Sheath gas flow	11 L/min
Capillary voltage	3,500 V
Nozzle voltage	500 V (pos); 0 V (neg)
Cycle time	750 ms
Total number of MRMs	304 (pos); 70 (neg)
Maximum number of concurrent MRMs	36 (pos); 8 (neg)
Minimum dwell time	17.3 ms (pos); 90.2 (neg)
Maximum dwell time	371.5 ms (pos); 750 (neg)
Resolution	Unit

Data were evaluated using the MassHunter Quantitative Analysis Software. Calibration was done using neat standard solutions and linear, 1/x weighted calibration curves. The limits of quantitation (LOQs) were calculated based on a signal-to-noise (S/N) ratio of > 10 (peak-to-peak, based on signal height) for the less abundant qualifier transition, and taking into account the dilution factor and apparent recovery for each matrix. Positive identifications of mycotoxins in the real samples were reported if the concentrations were above the specified LOQs, retention times were within \pm 2.5 % of the expected retention times, and the qualifier ratios were within the defined target range specified by Commission Decision 2002/657/EC [7].

Results and Discussion

Development of the UHPLC/MS/MS method

A new multi-target UHPLC/MS/MS method for the screening and quantitation of mycotoxins and fungal metabolites was developed using the 1290 Infinity UHPLC System coupled to the 6460 Triple Quadrupole Mass Spectrometer. Due to the large number of analytes and the expected matrix load caused by the crude solvent extraction, a column length of 150 mm and a total runtime of 21 minutes was chosen. By having a steep gradient in the beginning and a flattened profile later in the run, a better separation of the analytes was achieved. Figure 1 shows the chromatograms of all target compounds spiked into a hazelnut sample at an intermediate level (0.04 to 250 µg/kg, depending on the analyte) and acquired in positive (A) or negative mode (B).

It is important to separate aflatoxin M₂ and aflatoxin G₂, cytochalasin C and D, enniatin B₂ and K₁, or fumonisin B₃, and fumonisin B₂ as they are isomeric, and even share some MRM transitions. For those compounds, baseline separation was achieved, however, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol could not be separated based on the chromatography. Peak shapes in general were very good with an average peak width of 0.11 minutes (full width at half maximum). Very few compounds, such as cyclosporin A, C, and D and HC-toxin had a peak width of > 0.3 minutes under the chosen chromatographic conditions. For those compounds and for compounds with closely eluting isomers, the window width in the dynamic MRM acquisition of typically 1 minute was extended.

Each analyte was detected in the polarity that resulted in the most abundant signal and highest S/N ratio. The singly charged, protonated ion species was most often used as the precursor in positive mode. As some mycotoxins readily form sodium adducts, which typically show weak fragmentation in

collision-induced dissociation, ammonium acetate was added to the mobile phase to promote and stabilize the ammonium adduct formation. For a few compounds, the doubly protonated ion species or in-source fragments were used as precursors, as these ions were predominantly formed in the electrospray ionization. The predominant precursor ion in negative mode was the deprotonated ion species, and for approximately 20 % of all compounds, the acetate adduct led to the most abundant signals when chosen as the precursor ion.

Two mass transitions were selected per compound, and the MassHunter data analysis software automatically chose the more abundant transition as the quantifier and the other as the qualifier. In cases the S/N ratio for the lower abundant transition was significantly higher, this transition was chosen as the quantifier. Qualifier-to-quantifier ratios were calculated based on the peak areas, and noncompliant values, according to the limits specified in Commission Decision 2002/657/EC [7] were automatically flagged.

Method performance characterization

The method was developed and validated for nuts because mycotoxins are the major source of complaints for this commodity in the Rapid Alert System for Food and Feed (RASFF) in the European Union, and since there is only limited data available for the occurrence of mycotoxins other than aflatoxins and ochratoxin A. Method performance parameters were obtained by spiking blank samples of almond, hazelnut, pistachio, and peanut before extraction. Matrix effects were evaluated by spiking the raw extract of blank samples after extraction. By comparing those two sample sets, the recovery of the extraction step could be calculated. Performance parameters for the 65 most important analytes (regulated mycotoxins, mycotoxins found in the naturally contaminated nuts, and mycotoxins often found in other commodities) and all tested matrixes are described in detail in [1].

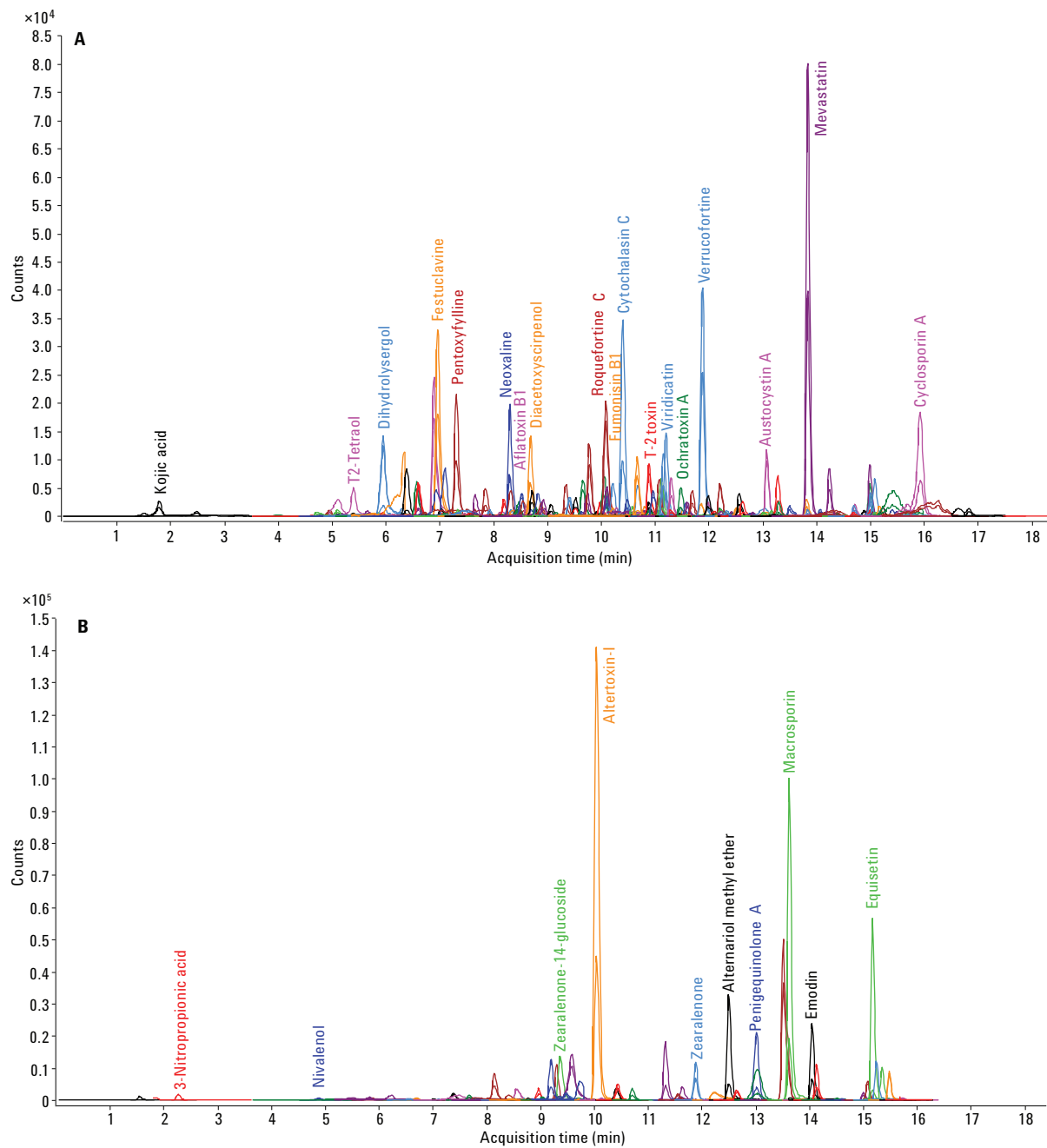


Figure 1. Chromatograms of all targeted mycotoxins and fungal metabolites spiked into a blank hazelnut sample and acquired in positive (A) and negative electrospray (B). For the sake of readability, only selected peaks are labeled.

Figure 2 shows the calibration curves for aflatoxin B₁ (A), beauvericin (B), and mycophenolic acid (C), which were acquired in positive mode, as well as alternariol (D), emodin (E), and zearalenone (F) acquired with negative ionization.

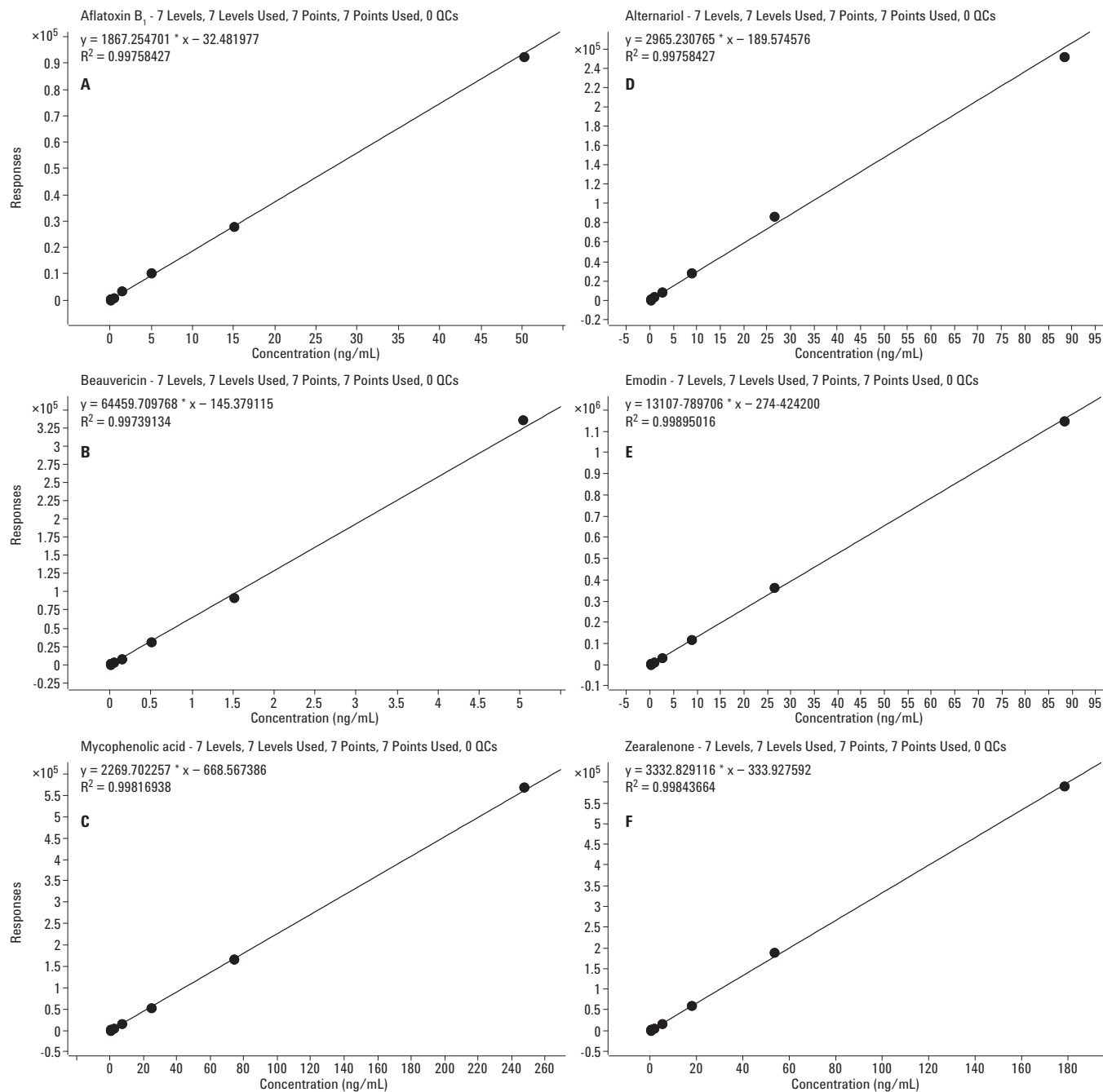


Figure 2. Calibration curves for aflatoxin B₁ (A), beauvericin (B), and mycophenolic acid (C), acquired in positive mode, alternariol (D), emodin (E), and zearalenone (F), acquired in negative mode.

Linear calibration curves were obtained for all targeted mycotoxins over at least three orders of magnitude. Lower limits of quantitation (LLOQs) were compound-dependent and ranged from sub- μg per kilogram levels up to several hundred μg per kilogram. These values already include the 8-fold dilution during sample preparation. Due to the dilution, and since LLOQs were calculated based on the S/N ratio of the lower abundant qualifier transition, the LLOQs for aflatoxins were slightly higher than the maximum levels set in Commission Regulation 2006/1881/EC [3]. In contrast, lower LLOQs were observed for zearalenone and T-2 toxin compared to other published multitarget methods. As method parameters were optimized to represent a good compromise for all target analytes, additional sensitivity for specific compounds can be gained by adjusting source parameters to more ideal values. In terms of the chosen extraction solvent and chromatographic conditions, a compromise was made to cover the huge number of chemically diverse analytes, and more dedicated methods will increase the sensitivity for specific analytes. Other alternatives to gain sensitivity would be a sample cleanup as described in [8], which most likely will not work equally well for all 191 analytes, enrichment strategies, or the use of a more sensitive detector such as the Agilent 6490 Triple Quadrupole MS [9].

Apparent recoveries were calculated for the results obtained for nut samples spiked before extraction. Figure 3 shows a histogram summarizing the apparent recoveries for all validated compounds in the four matrixes.

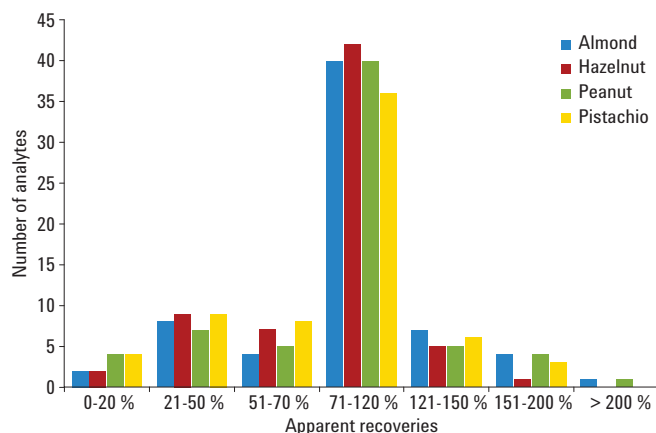


Figure 3. Histogram summarizing the apparent recoveries for all 65 validated compounds in the four matrixes.

Apparent recoveries ranged between 70 to 120 % for approximately 60 % of the validated analytes. Larger observed deviations in the apparent recoveries were either caused by matrix effects or insufficient extraction. This was expected for a multitarget method covering a large number of chemically diverse analytes as both extraction solvent and chromatographic separation need to be a compromise. Most apparent recoveries were very similar for the four different matrixes, and very repeatable, with standard deviations below 10 % for almost all analytes.

The extraction recoveries were calculated by comparing samples spiked before and after extraction. In general, for half of the compounds, the used extraction procedure resulted in extraction recoveries of 50 % and higher. Lower extraction recoveries were observed for fumonisins.

The signal suppression or enhancement was calculated for the samples spiked after extraction in comparison to the neat standard solutions. For 57 % of the analytes, signal suppression or enhancement resulted in a signal decrease or increase of less than 20 %. Suppression of the early eluting compounds such as 3-nitropropionic acid, kojic acid, or deoxynivalenol was more severe. In contrast, several compounds showed significant signal enhancement. The highest values were observed for physcion (up to 295 %) and equisetin (up to 285 %). It has been shown that stable isotope dilution or matrix matched calibration can be effective strategies to compensate for signal suppression or enhancement [9]. For a limited number of target compounds within the method, such an approach could be beneficial. However, the main attribute of this method is a simple and fast screening of 191 compounds.

Screening for mycotoxins in naturally contaminated nut samples

The method was applied for the screening of mycotoxins in more than 50 nut samples. Figure 4 shows the chromatograms of several mycotoxins that were found in a naturally contaminated hazelnut sample including aflatoxins, *Alternaria* toxins, mycophenolic acid, and T-2 toxin, which was found for the first time in hazelnuts.

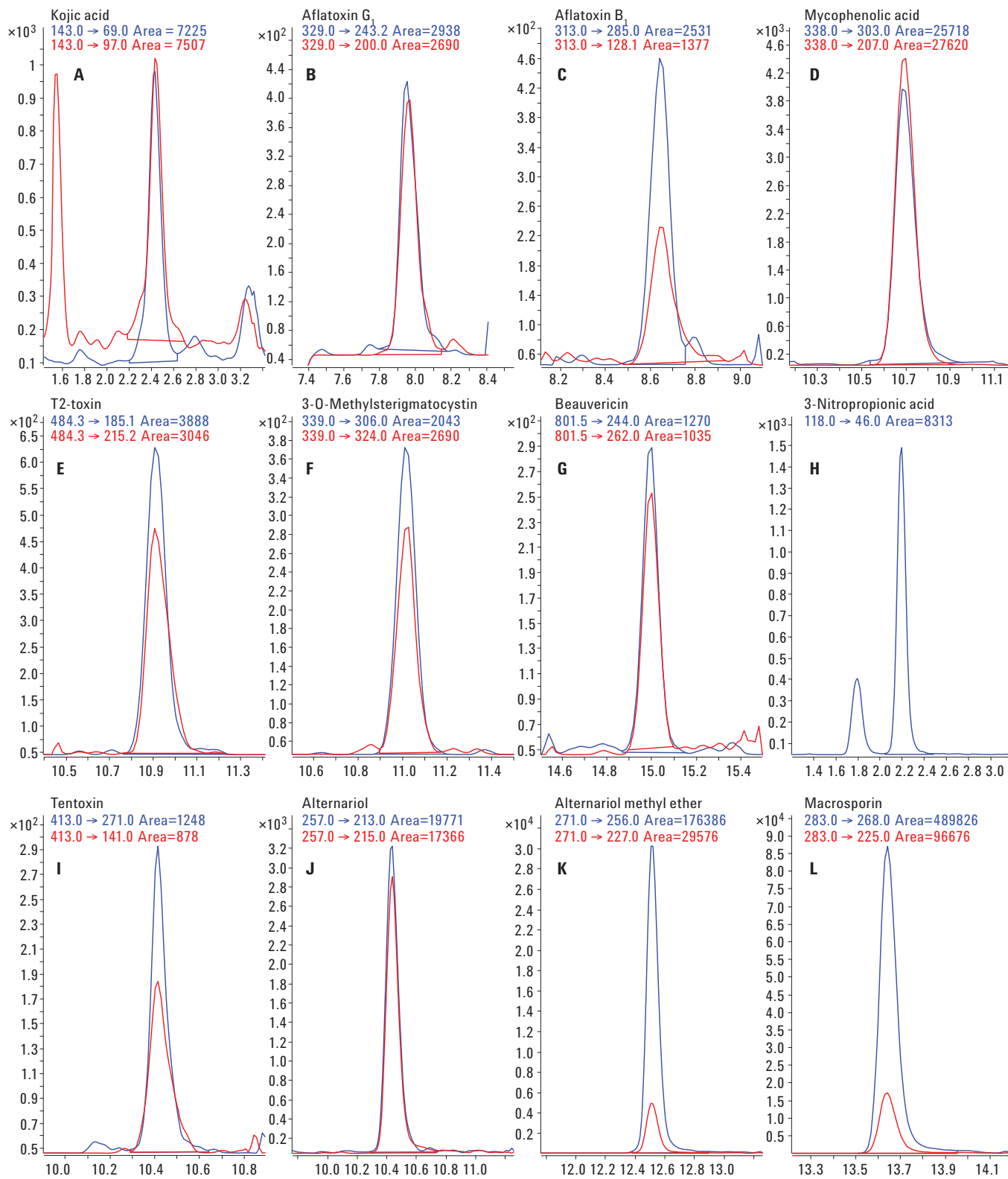


Figure 4. Chromatograms for mycotoxins detected in one naturally contaminated hazelnut sample. (A) kojic acid (1,300 µg/kg), (B) aflatoxin G₁ (22 µg/kg), (C) aflatoxin B₁ (15 µg/kg), (D) mycophenolic acid (570 µg/kg), (E) T-2 toxin (41 µg/kg), (F) 3-O-methylsterigmatocystin (3.9 µg/kg), (G) beauvericin (1.3 µg/kg), (H) 3-nitropropionic acid (850 µg/kg), (I) tentoxin (2.6 µg/kg), (J) alternariol (49 µg/kg), (K) alternariol methyl ether (79 µg/kg), and (L) macrosporin (340 µg/kg).

More than 40 different mycotoxins were found in all types of nuts. Most contaminants were found in the hazelnut samples (36), followed by peanuts (30), almonds (13), and pistachios (5). This might be due to the larger number of hazelnut samples analyzed compared to the other commodities. However, one single hazelnut sample was contaminated with 26 mycotoxins, and in eight other hazelnut samples 20 or more mycotoxins were found. In peanut, almond, and pistachio samples up to 17, 13, and 5 analytes were detected in single samples, respectively. Beauvericin was found most often, with 42 positive detects, followed by enniatin B (33), macrosporin (30), and 3-nitropropionic acid (29). In addition, several *Alternaria* mycotoxins were found, with alternariol methyl ether in 27 samples, followed by alternariol (24), tentoxin (22), and tenuazonic acid (21).

The mycotoxins identified in hazelnut and peanut samples also contained aflatoxins for which maximum levels are set in the European Union. For eight hazelnut and eight peanut samples, the determined aflatoxin B₁ concentrations were above the regulatory limits of 5 µg/kg for hazelnuts and 2 µg/kg for peanuts, with maximum concentrations of up to 15 µg/kg. In addition, sterigmatocystin, the most toxic aflatoxin B₁ precursor was found in 21 of the 22 analyzed hazelnut samples in concentrations up to 5.5 µg/kg. For the first time, T-2 and HT-2 toxin was identified in 15 hazelnut samples, with average concentrations of 39 µg/kg for HT-2 and 32 µg/kg for T-2 toxin.

Some hazelnut samples were significantly contaminated with *Alternaria* mycotoxins in concentrations up to 650 µg/kg for alternariol, and up to 220 µg/kg for alternariol methyl ether. The most abundant mycotoxin in hazelnut samples was 3-nitropropionic acid, which was detected in all of the hazelnut samples in concentrations of up to 980 µg/kg, followed by enniatin B, which was detected in 17 samples in concentrations up to 540 µg/kg. The highest concentrations were observed for mycophenolic acid, a potent immunosuppressive compound, which was detected in one sample at a concentration of 6,100 µg/kg. In addition, macrosporin was found up to 2,200 µg/kg.

In all peanut samples, beauvericin was detected in concentrations up to 12 µg/kg. Peanuts also had the highest contamination in a single sample, with 40,000 µg/kg kojic acid. *Alternaria* toxins and enniatins were found in the almond samples. The highest contamination was observed for cyclopiazonic acid, with concentrations up to 130 µg/kg, and secalonic acid D, with concentrations up to 51 µg/kg.

In pistachios, only five analytes could be detected, and only one sample was contaminated with macrosporin above the LLOQ.

Conclusions

A UHPLC/MS/MS-based multitarget method for the determination of 191 mycotoxins and other fungal metabolites including all mycotoxins regulated in Europe was developed. It takes full advantage of the low delay volumes of the Agilent 1290 Infinity LC System and its ability to handle high backpressures in UHPLC separations to increase the chromatographic resolution for a better separation of the analytes from the matrix. The method comprises a fast, easy, and cheap solvent extraction, as well as the subsequent injection of the diluted raw extract into the UHPLC/MS/MS system. The method benefits from the sensitivity and robustness of the Agilent 6460 Triple Quadrupole MS and from the versatile ionization capabilities of the Agilent Jet Stream ionization source. Dynamic MRM acquisition was used to maximize dwell times for each individual compound and source parameters were optimized for a good compromise across an array of target compounds.

The method was validated for all regulated and most frequently found mycotoxins in almonds, hazelnuts, peanuts, and pistachios and, thus, quantitation was possible for those 65 compounds. For the other analytes, the method can still provide semiquantitative information. Although the method has been validated for nuts, it can be used to screen mycotoxins in several other food and feed matrixes. The presented method is an appropriate supplement to single-analyte or analyte-group detection methods to increase knowledge on the occurrence of mycotoxins in various food commodities.

The method has been applied to 53 different nut samples during which 40 different mycotoxins and fungal metabolites were detected. In addition to aflatoxins, which are the only mycotoxins currently regulated in nuts in the European Union, other toxins are relevant contaminants in nuts. In more than 50 % of all samples, beauvericin, enniatin B, macrosporin, 3-nitropropionic acid, emodin, and alternariol methyl ether were found, partially in extremely high concentrations. For the first time, the presence of HT-2 and T-2 toxin in hazelnuts was confirmed. Many of the detected mycotoxins are not yet fully toxicologically evaluated and moreover information on additive or even synergistic effects of co-occurring toxins is missing.

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© Agilent Technologies, Inc., 2014
Published in the USA
August 8, 2014
5991-4991EN



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