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Determination of Mycotoxins in Bee Pollen by Gas Chromatography−Tandem Mass Spectrometry

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ABSTRACT: Bee pollen, promoted as a natural food supplement, is consumed increasingly by people to maintain a healthy diet. Depending on environmental conditions, pollen can also be an optimum medium for growth of molds such as *Fusarium* and *Penicillium*. A quick, easy, cheap, rapid, and safe (QuEChERS) extraction procedure followed by a gas chromatography−tandem mass spectrometry (GC-MS/MS) determination of eight selected *Fusarium* toxins in bee pollen was developed and optimized. Recovery studies at 20, 80, and 1000 μg/kg showed values between 73 and 95% with relative standard deviations (RSDs) of <15% for all studied mycotoxins. Limits of quantitation (LOQ) ranged from 1 to 4 μg/kg. The proposed method was applied to the analysis of 15 commercial samples. Two of 15 samples showed quantifiable values for neosolaniol and nivalenol.

KEYWORDS: *bee pollen, mycotoxins, QuEChERS, GC-MS/MS, triple quadrupole*

INTRODUCTION

Shifts of structure of global food consumption affect directly eating habits, and people are increasingly looking for healthy and nutritious foods. These diets include natural products such as hive products including honey, bee pollen, and royal jelly.[1] Bee pollen contains, in perfect balance, all essential amino acids that humans require to achieve and maintain optimum vitality. Moreover, it is a storehouse of vitamins, minerals, fats and oils, carbohydrates, and other healthy compounds such as antioxidants.[2,3] For these reasons, its use in the human diet is very highly appreciated, even becoming recognized officially as a medicine by the German Federal Board of Health.[1] In this regard, several papers have appeared in the literature regarding its digestive, antioxidant, and immunostimulation activities.[4−6] These and other properties of bee pollen converted this commodity to a nutrient-rich health food for many centuries, and its benefits have been widely lauded. It is also important, however, to verify the presence of contaminants that are harmful to health.[7]

Mycotoxins are secondary metabolites produced by a wide variety of fungal species such as *Fusarium*, *Aspergillus*, *Alternaria*, *Claviceps*, and *Penicillium*.[8] The ability of molds to produce mycotoxins is influenced by environmental factors, the most important being temperature and relative humidity.[9] There is an increasing concern of mycotoxin contamination in foods and feeds because they can be found in a wide range of commodities including cereals, spices, dried fruits, apple products, wine, and coffee. Human exposure occurs mainly by ingestion of mycotoxin-contaminated products and can lead to serious health problems, including immunosuppression and even carcinogenesis.[10,11] Maximum levels of mycotoxins in foodstuffs and feedstuffs have been established in many countries.[12] For instance, maximum limits for deoxynivalenol in food matrices such as processed cereal-based foods and baby foods for infants and young children have been set at 200 μg/kg. Nevertheless, there is a lack of current legislation with regard to maximum limits of mycotoxins in bee pollen.

Bee pollen is also an adequate substrate for mycotoxin growth when no prompt and adequate drying is performed by the beekeeper after collection by bees.[13,14] The quality of bee pollen is strongly dependent on its preservation.[4,7] Therefore, the water content of the product determines microbiological and organoleptic qualities and also its shelf life. The initial water content of the fresh pollen is 14−18 g/100 g, and pollens should be dried to reduce the moisture content to 6% to keep their nutritional value for a long time.[9,15,16]

Pollen can be infected by different toxigenic molds, which potentially results in the co-occurrence of several mycotoxins. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic.[17,18] In this context, there is a clear need for fast and efficient analytical methods to support the feed and food industry in the management of mycotoxin residues. Multiclass or multiresidue analytical methodologies are becoming the required tools to provide reliable and wider knowledge about the occurrence of mycotoxins. However, the complex sample matrix may contain components that can interfere with good sample analysis. No data for the determination of mycotoxins in bee pollen are found in the scientific literature, with the exception of a screening ELISA test reported for the sum of aflatoxins[9] and a simultaneous chromatographic determination of ochratoxin A and aflatoxins retained by an immunoaffinity column.[19]

For the determination of trichothecenes, nonfluorescent analytes, GC has largely been the method of choice, providing sensitive and accurate results after analyte derivatization, generally based on trimethylsilylation and fluoroacylation.[20] Analytical performance characteristics comparable with those of GC methods can be achieved by application of HPLC methods with pre- or postcolumn derivatization.[21] MS/MS is a highly reliable analyte tool and has become a routine technique in

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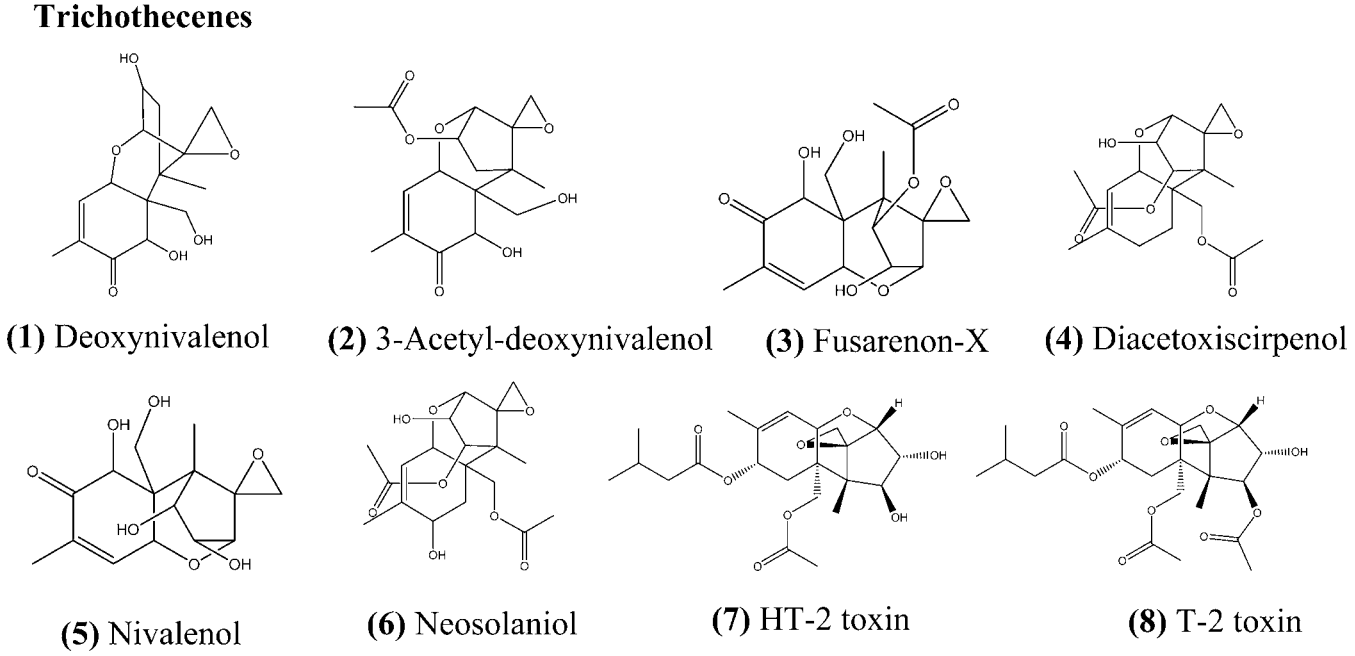

Figure 1. Structures of the trichothecenes studied.

food analysis with high impact on the field of mycotoxin analysis, particularly in the development of multimycotoxin methods. GC-QqQ-MS/MS detection remains a powerful technique for the quantitative determination of lower levels of mycotoxins in complex matrices even in the era of liquid chromatography−tandem mass spectrometry.[22,23]

In this study a multiresidue method for the determination of eight trichothecenes, including type A and type B (deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2, and T-2), in bee pollen using GC-MS/MS was performed. Mycotoxins were extracted from pollen samples using a QuEChERS-based extraction procedure carefully optimized for this food matrix. The method was in-house validated and applied to 15 bee pollen samples commercialized in Spain to evaluate the occurrence of the studied mycotoxins.

MATERIALS AND METHODS

Chemical and Reagents. Solvents (acetonitrile, hexane, and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate ($MgSO_4$) was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck, and C_{18}-E (50 μm, 65 A) was purchased from Phenomenex (Torrance, CA, USA). Bondesil primary−secondary amine (PSA) was acquired from Análisis Vínicos (Tomelloso, Spain).

The derivatization reagent composed of BSA (*N*,*O*-bis-(trimethylsilyl)acetamide) + trimethylchlorosilane (TMCS) + N-trimethylsilyimidazole (TMSI) (3:2:3) was purchased from Supelco (Bellefonte, PA, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

Standards of the type A and type B trichothecenes, deoxynivalenol (**1**), 3-acetyldeoxynivalenol (**2**), fusarenon-X (**3**), diacetoxyscirpenol (**4**), nivalenol (**5**), neosolaniol (**6**), HT-2 (**7**), and T-2 (**8**) (Figure 1), were obtained from Sigma-Aldrich (St. Louis, MO, USA). All stock solutions were prepared by dissolving 1.00 mg of the mycotoxin in 1.00 mL of pure methanol, obtaining a 1 mg/mL solution. The stock solutions were diluted with acetonitrile to obtain the appropriate multicompound working standard solutions (50 mg/L). All standards were kept at −20 °C.

GC-QqQ-MS/MS Conditions. One microliter of the extract of mycotoxins was injected in splitless mode at 250 °C in programmable temperature vaporization (PTV) using an Agilent 7890A GC system coupled with an Agilent 7000A triple-quadrupole mass spectrometer with an inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer was operated in electron impact ionization (EI, 70 eV). The transfer line and source temperatures were 280 and 230 °C, respectively. The collision gas for MS/MS experiments was nitrogen, and helium was used as carrier gas at fixed pressure of 20.3 psi, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data were acquired and processed using Agilent MassHunter version B.04.00 software.

Analytes were separated on an HP-5MS 30 m × 0.25 mm × 0.25 μm capillary column. The oven temperature program was initially 80 °C for 2 min, and the temperature was increased to 245 °C at 80 °C/min. After a 5 min hold time, the temperature was increased to 250 °C at 5 °C/min and finally to 270 °C at 10 °C/min and then held for 3 min.

Sampling. A total of 15 pollen samples (100 g) were randomly purchased in supermarkets located in the Valencia metropolitan area (Spain). All samples were homogenized using a laboratory mill and stored in a dark and a dry place in specific plastic food containers and analyzed within 3 days of sampling.

Sample Preparation. *Extraction and Cleanup.* Five grams of milled bee pollen sample, weighed into a 50 mL centrifuge tube, was mixed with 10 mL of distilled water and sonicated for 15 min. To induce phase separation and mycotoxin partitioning, 7.5 mL of acetonitrile, 4 g of $MgSO_4$, and 1 g of NaCl were added to the tube, which was vortexed and centrifuged for 10 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$, 300 mg of C_{18}, and 300 mg of PSA. The tube was vortexed for 30 s and centrifuged for 10 min at 4000 rpm. The extract (2 mL) was then evaporated to dryness under nitrogen flow for derivatization.

Derivatization. The dry extract was treated with 50 μL of BSA + TMCS + TMSI (3:2:3), and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 250 μL with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to an autosampler vial for the chromatographic analysis.

Table 1. Transitions Reactions Monitored by GC-ESI-MS/MS for the Analysis of Mycotoxins and Peak Area Ratio with Their Limits of Acceptance According to Reference[a]

no.	compound	t_R (min)	SRM transition (m/z)	CE (V)	Dt (ms)	ratio Q/q (RSD)
1	deoxynivalenol	8.01	392 → 259	10	25	41.6 (3.2)
			407 → 197	10	25	
2	3-acetyldeoxynivalenol	9.13	392 → 287	5	35	47.5 (12.3)
			467 → 147	10	25	
3	fusarenon-X	9.19	450 → 260	10	35	11.9 (7.0)
			450 → 245	20	35	
4	diacetoxyscirpenol	9.25	350 → 229	15	35	56.9 (10.3)
			378 → 124	10	25	
5	nivalenol	9.57	289 → 73	15	35	29.6 (2.7)
			379 → 73	15	35	
6	neosolaniol	11.02	252 → 195	10	25	40.6 (4.3)
			252 → 167	15	35	
7	HT-2	13.93	347 → 157	10	25	86.7 (7.8)
			347 → 185	10	25	
8	T-2	13.94	350 → 259	10	25	81.9 (5.8)
			350 → 229	15	35	

[a] t_R, retention time; SRM, selected reaction monitoring; CE, collision energy; Dt, dwell time; Q, quantitation transition; q, confirmation transition.

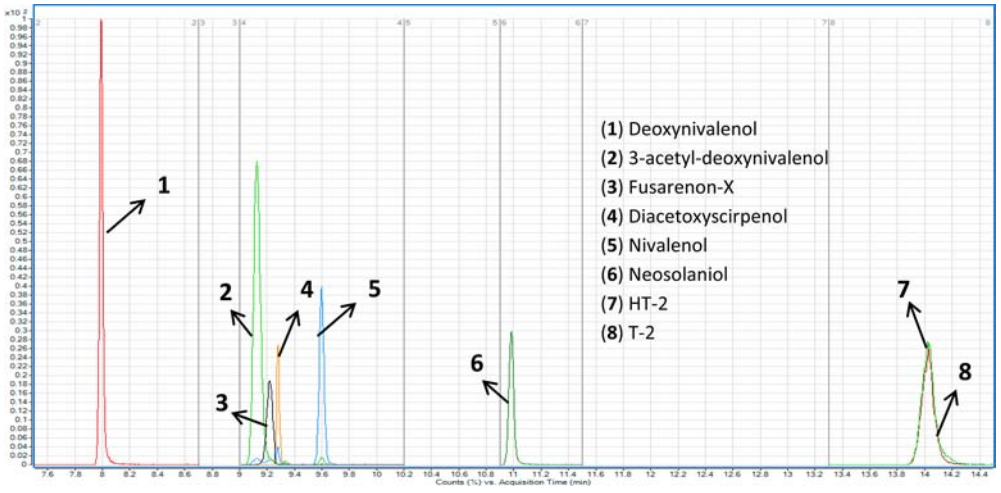

Figure 2. GC-MS/MS chromatogram obtained from a blank bee pollen sample spiked at 80 μg/kg.

Method Validation. The developed method was validated following the SANCO 12495/2011 document.[24] Linearity was first evaluated by a triplicate of standard calibration curves at seven concentration levels (5, 20, 80, 150, 500, 750, and 1000 μg/kg). Matrix-matched calibration curves were built by spiking blank sample extracts with selected mycotoxins at the same concentration levels as the standard calibration curves. The accuracy was verified by measuring the recoveries from spiked blank samples at three concentration levels (20, 80, and 1000 μg/kg), six replicates at each fortification level.

Precision (expressed as %RSD) of the method was determined by repeatability (intraday precision) and reproducibility (interday precision). Intraday variation was evaluated in six determinations per concentration in a single day, whereas interday variation was tested on six different working days within 20 days. Sensitivity was evaluated by LOD and LOQ values. LOD was determined as the analyte concentration that produced a peak signal of 3 times the background noise from the chromatogram regarding SRM2, confirmation transition. LOQ was determined as the analyte concentration that produced a peak signal of 10 times the background noise from the chromatogram regarding SRM1, quantitation transition. Matrix effect is used to describe the analyte ionization efficiency. Recovery describes the efficiency of separating analyte from the sample. Process efficiency (PE) summarizes the efficiency of sample preparation (recovery) and analyte ionization (matrix effect). Therefore, process efficiency is suitable for assessing the overall performance of an analysis method. To assess PE the peak areas of pre-extraction addition extracts (A) with peak areas of calibration solutions prepared in solvent (B) were compared. Thus, the ratio ($A/B \times 100$) was defined as the PE (%) as described Kruve et al.[25]

Two MS/MS transitions were acquired for each mycotoxin, giving four identification points with a defined SRM transitions ratio for the developed method as indicated in the requirements for mass

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spectrometry.[24,26] The most abundant SRM transition was used for quntitation (Q) and the second one for confirmation (q). In addition, the intensities ratio of the different transitions monitored was used as a confirmatory parameter. The ion ratio was calculated as the quotient between Q/q areas. Relevant MS/MS data are reported in Table 1. A GC-MS/MS chromatogram obtained from a blank bee pollen sample spiked at 80 μg/kg is also provided in Figure 2.

RESULTS AND DISCUSSION

The extraction of the selected mycotoxins in bee pollen was carried out according to the modified QuEChERS method previously developed for the determination of several mycotoxins in wheat semolina as a starting point.[23] In this previous study, the effect of the pH on extraction medium, the effect of solvent volume, and the influence of the cleanup step were carefully evaluated. Nonetheless, due to the complexity of the bee pollen as food matrix, some critical parameters such as volume of solvent and cleanup strategy required reappraisal.

Optimization of the d-SPE. QuEChERS involves mainly acetonitrile extraction and extract purification using d-SPE. Sorbent type is an important parameter in the QuEChERS method. $MgSO_4$ is employed to separate water from the organic solvent. C_{18} is the most popular hydrophobic silica-based sorbent with high affinity for nonpolar compounds. On the other hand, PSA, originally employed in the QuEChERS analytical methodology developed by Anasstasiades et al.,[27] is commonly used to remove sugars and fatty and organic acids as well as some pigments. When PSA is used in combination with C_{18}, additional lipids and sterols can be removed, offering the best performance in terms of cleanup efficiency, removing the greatest amount of interfering substances.[28] Therefore, different combinations of d-SPE were tested to check the best sorbent combinations regarding recovery results in spiked pollen samples at 80 μg/kg. Specifically, (a) 900 mg of $MgSO_4$ + 300 mg of C_{18}, (b) 900 mg of $MgSO_4$ + 300 mg of C_{18} + 150 mg of activated carbon, and (c) 900 mg of $MgSO_4$ + 300 mg of C_{18} + 300 mg of PSA were employed to purify the extracts. The selection of the sorbents was carried out according to the QuEChERS application review.[29] The chromatograms of a spiked sample analyzed using $MgSO_4$ + C_{18} + PSA provided better cleanup than the other sorbents tried. Activated carbon retained apolar compounds; however, a thin fatty acid film was formed, leading to poor recoveries (<30%) (Figure 3). Similar results were previously reported for pesticide and veterinary drug extraction from honey and pollen.[30] Recoveries between 40 and 80% were obtained with a single use of C_{18} (Figure 3). The combination of PSA and C_{18} provided better cleanup than single-use of C_{18}. With PSA, the solution after cleanup was colorless and transparent, whereas the color of the solution cleaned without PSA was light yellow. Indeed, results showed that $MgSO_4$ in combination with PSA + C_{18} assured recoveries >70% for all of the mycotoxins studied (Figure 3).

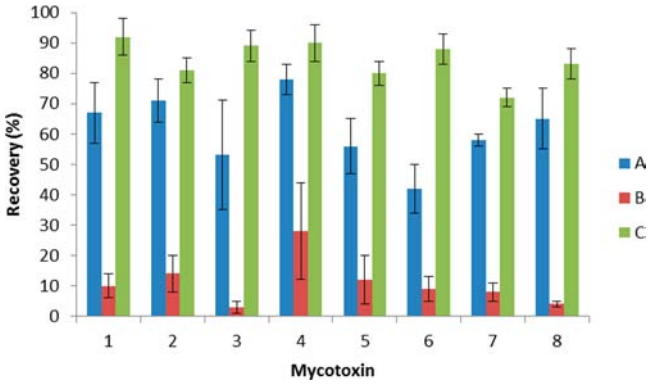

Figure 3. Effect of different mixtures of d-SPE sorbents on cleanup of fortified bee pollen samples (error bars are ± SD; n = 6) at a level of 80 μg/kg of studied mycotoxins.

Optimization of the Volume of Solvent. The main objective of this step was to minimize the use of solvent employedm ensuring acceptable recovery results. Three different volumes were assayed (5, 7.5, and 10 mL). Statistical analysis (Student's t test) of repeated measures (n = 6) was applied to analyze the results. No significant statistical differences for a confidence interval of 95% were found in terms of recovery results when 7.5 and 10 mL of acetonitrile were employed, obtaining with both volumes tested recoveries up to 70% in all analytes evaluated (Figure 4). However, 5 mL of solvent was not enough to create an upper layer after centrifugation to be submitted to d-SPE. Finally, 7.5 mL of acetonitrile was chosen due to its being less time-consuming when the subsequent extract was dried.

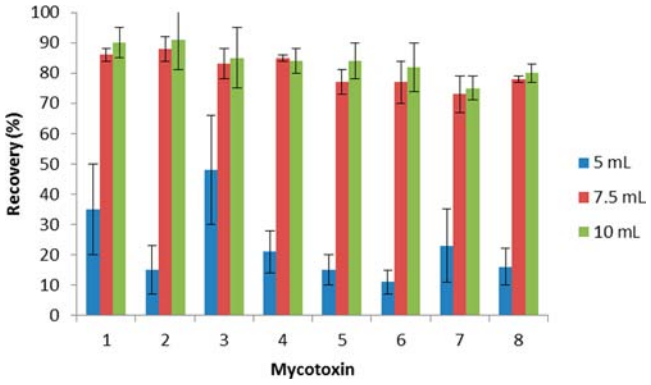

Figure 4. Effect of different acetonitrile volumes on extraction of fortified bee pollen samples (error bars are ± SD; n = 6) at a level of 80 μg/kg of studied mycotoxins.

Analytical Performance. The linear ranges of determination were from LOQ to 1000 μg/L with coefficients of determination >0.990. LODs were from 0.3 to 1.2 μg/kg, and LOQs obtained were in a range from 1 to 4 μg/kg (Table 2). Matrix-matched calibration standards were used to compensate matrix effects. The recoveries obtained ranged from 78 to 95% for the 20 μg/kg spiking level, from 73 to 88% for the 80 μg/kg spiking level, and from 83 to 90% for the 1000 μg/kg spiking level. In all spiking levels the values of intraday precision (n = 6) were <15% (Table 2), whereas those for interday precision were <20%. Following the EU guideline[24] (recovery of 70–110%, RSD ≤ 20%), the proposed method was found to be accurate, with satisfactory recoveries at three fortification levels. Process efficiency is the overall performance characteristic of the method. Percent PE values near 100% generally indicate that both the percentage of matrix effect and the percentage of recovery are near 100%. Table 2 shows the PE results obtained for the selected mycotoxins in bee pollen samples analyzed. For the majority of compounds, PE values of <100% were obtained. Ion suppression may be due to matrix effects and compound losses during the sample preparation process. A few compounds showed PE > 100%, which was probably due to matrix effects resulting in ionization enhancement.

Table 2. Performance Characteristics of the Proposed Method for Determining Mycotoxins in Bee Pollen[a]

					recovery (RSD) (%)			
no.	mycotoxin	LOD (μg/kg)	LOQ (μg/kg)	coefficient of determination (R^2)	20 μg/kg	80 μg/kg	1000 μg/kg	PE (%)
1	deoxynivalenol	0.3	1	0.998	78 (7)	86 (2)	83 (7)	68
2	3-acetyldeoxynivalenol	0.3	1	0.992	81 (8)	88 (11)	88 (10)	70
3	fusarenon-X	1.2	4	0.990	93 (11)	83 (14)	87 (13)	63
4	diacetoxyscirpenol	1.2	4	0.991	79 (5)	85 (1)	83 (7)	86
5	nivalenol	0.3	1	0.997	87 (9)	77 (4)	83 (10)	30
6	neosolaniol	0.7	2	0.995	87 (12)	77 (7)	90 (15)	141
7	HT-2	0.3	1	0.999	95 (10)	73 (6)	87 (7)	115
8	T-2	1.2	4	0.991	92 (12)	78 (1)	86 (4)	122

[a]PE, process efficiency.

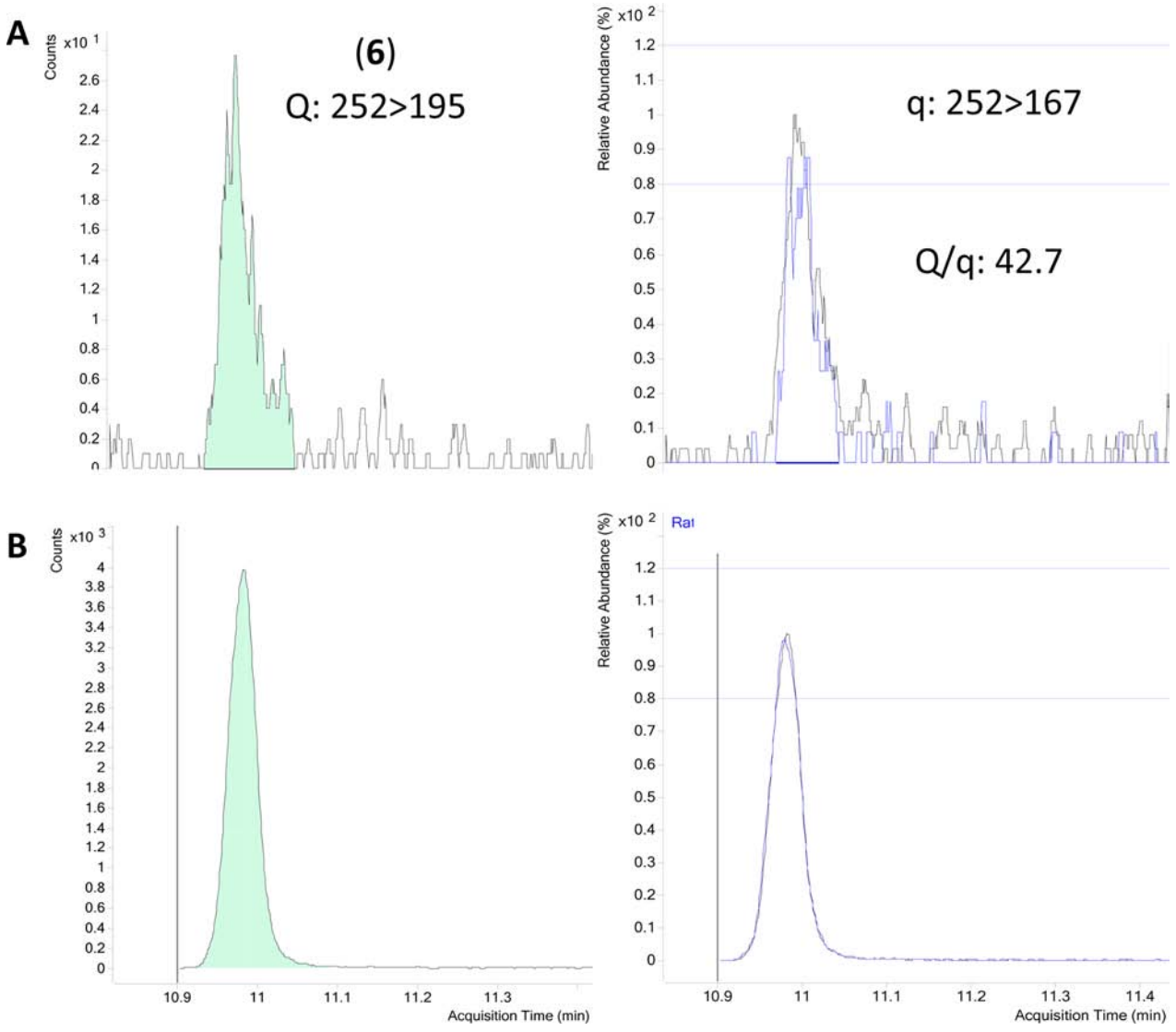

Figure 5. GC-MS/MS chromatograms for (A) blank bee pollen sample spiked at LOQ (2 μg/kg) of neosolaniol and (B) bee pollen sample naturally contaminated with neosolaniol at 29 μg/kg.

Analysis of Samples. Very little literature is reported regarding mycotoxin-producing fungi in bee pollen and their capability to produce mycotoxins under some circumstances. A survey carried out by González et al.[13] highlighted the occurrence of fungi in bee pollen and reported the isolation of *Penicilium* spp. *Aspergillus* spp., and *Fusarium* spp., which are producers of ochratoxin A, aflatoxins, and trichothecenes. Nevertheless, neither aflatoxins nor ochratoxin A was detected in the 20 bee pollen samples analyzed by Garcia-Villanova et al.[19] In the present work, 15 bee pollen samples were analyzed with the previously described methodology. Two of 15 bee pollen samples presented mycotoxin contamination. Neosolaniol (**6**) was detected in both naturally contaminated samples at 30 ± 5 and 22 ± 3 μg/kg (mean ± SD; n = 3),

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respectively. Nivalenol (**5**) was also found in these samples with values close to the limit of quantitation (1 μg/kg). Figure 5 shows the GC-MS/MS chromatogram of one naturally contaminated bee pollen sample with neosolaniol as well as a GC-MS/MS chromatogram of blank bee pollen sample spiked with neosolaniol at the LOQ value (2 μg/kg). None of the other studied mycotoxins were detected. GC-QqQ-MS/MS proposed in this paper allows the single QuEChERS extraction and simultaneous determination of eight *Fusarium* mycotoxins in bee pollen with a chromatographic run of 14 min. The method performance fulfilled the EU guideline standardized in the SANCO/12495/2011 document, offering reliable results in terms of sensitivity, mean recovery, precision, and limit of quantitation. The results reported in the present work show for the first time the presence and co-occurrence of *Fusarium* toxins in two bee pollen samples collected in Spain.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GC-MS/MS, gas chromatography−tandem mass spectrometry; QqQ, triple quadrupole; PE, process efficieny; d-SPE, dispersive solid phase extraction; SRM, selected reaction monitoring; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation

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