Novel Multiresidue Method for the Determination of Eight Trichothecene Mycotoxins in Pollen Samples Using QuEChERS-Based GC-MS/MS

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A simple multi-residue method based on QuEChERS-sample preparation was developed for the determination of eight trichothecene mycotoxins (nivalenol, fusarenon-x, diacetoxyscirpenol, 3-acetyl-deoxynivalenol, neosolaniol, deoxynivalenol, T-2 and HT-2 toxins) in pollen, using gas chromatography coupled with mass spectrometry. The developed method was validated by evaluating the following parameters: linearity, limit of quantification, matrix effect and precision of measurements. All mycotoxins showed good linearity. The method gave good recoveries (91-104%) with RSDs > 11% for most of the analites (6 out of 8). The limits of quantification varied from 1 to 4μg·kg^{-1}.

Keywords: mycotoxins, bee pollen, GS-MS/MS, QuEChERS, triple quadrupole

Bee pollen been used for its high nutritional value for many centuries and its multiple benefits have been widely praised [1-3]. The presence of mycotoxins in pollen poses a high risk to human health. Human exposure occurs mainly after ingestion of mycotoxin-contaminated products and it can lead to serious health problems, including immunosuppression and even carcinogenesis [4].

The trichothecene mycotoxins are the largest group of mycotoxins produced as secondary metabolites by some species in the fungal genera *Fusarium, Myrothecium, Trichothecium* and *Cephalosporium*. Their molecules are based on the same basic chemical structure, a 12, 13-epoxytrichothec-9-ene ring system [1, 5].

In this study a new multiresidue method for the determination of eight trichothecene mycotoxins (nivalenol, fusarenon-x, diacetoxyscirpenol, 3-acetyl-deoxynivalenol, neosolaniol, deoxynivalenol, T-2 and HT-2 toxins) has been developed using gas chromatography coupled with mass spectrometry. The mycotoxins had been extracted from pollen samples using a QuEChERS-based extraction procedure.

Quick, Easy, Cheap, Effective, Rugged, and Safe, the QuEChERS ("catchers") method is a fast, simple, and effective alternative to conventional sample prep for multiresidue analysis of various types of substances [6-14].

Experimental part

Materials and methods

Reagents

Acetonitrile, hexane, methanol and sodium chloride were purchased from Merck, Germany. Anhydrous magnesium sulphate was provided by Alfa Aesar GmbH & Co, Germany. The primary secondary amine sorbent was purchased from Vinicos Spain and octadecyl silica-bonded sorbent was purchased from Phenomenex, USA. Sodium dihydrogen phosphate and disodium hydrogen phosphate were acquired from Panreac Quimica S.L.U. Spain. Nitrogen and helium gases (99.999% purity) were supplied by Carburos Metálicos S.L., Spain.

The eight trichothecenes standards (deoxynivalenol, 3-acetyldeoxynivalenol, diacetoxyscirpenol, nivalenol, fusarenon-X, neosolaniol, T-2 and HT-2 toxins) were obtained from Sigma-Aldrich, USA.

The derivatization reagent composed of N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and N-trimethylsilyimidazole in a 3:2:3 ratio was purchased from Supelco Bellefonte PA.

The eight trichothecenes stock solutions were prepared by dissolving 1mg of standard mycotoxin in 1mL methanol, obtaining a 1mg×mL^{-1} solution. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions. All standards were stored at -20°C.

Sample preparation

Mycotoxins were extracted from spiked pollen samples using a QuEChERS-based extraction procedure without applying any further clean-up step. 10mL of distilled water were added to 5g of each homogenized sample and the mixture was sonicated for 15 minutes. The main extraction involved the addition of 7.5mL acetonitrile, 4g $MgSO_4$ and 1g NaCl. To induce phase separation and mycotoxins partitioning, the test tube was shaken on a vortex for 30 s and then it was centrifuged for 10 min at 4000 rpm. Then the upper layer was submitted to a dispersive Solid-Phase Extraction clean up with a mixture of 900mg $MgSO_4$, 300mg octadecyl silica-bonded sorbent and 300 mg primary secondary amine sorbent. The mixture was vortexed for 30 s and centrifuged for 10 min at 4000 rpm. After purification, the extract was transferred into a vial and evaporated under a nitrogen flow to dryness.

Derivatization

The dry extract was mixed with 50μL of N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and N-trimethylsilyimidazole (3:2:3) mixture and the sample was kept for 30 min at room temperature. The derivatized sample was diluted with hexane to 250μL. The hexane solution was washed with 1mL of phosphate buffer (60mM, *p*H 7.2). In the end, the hexane layer was filtered and transferred into an autosampler vial to be analyzed through GC–MS/MS.

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All authors have equal contributions in the experiments and editing the article.

GC-MS/MS parameters

Chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler. The mass spectrometer was operated in electron impact ionization (EI, 70 eV). The transfer line and source temperatures were 280 and 230ºC, respectively. Nitrogen gas was used as collision gas for MS/MS, and helium was used as quenching gas. Data was acquired and processed using the Agilent Masshunter version B.04.00 software.

Analytes were separated on a HP-5MS 30m x 0.25mm x 0.25µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode at 250ºC in programmable temperature vaporization inlet employing helium as carrier gas at 20.3psi pressure. The oven temperature program was initially 80ºC, and then the temperature was increased to 245ºC at a rate of 60ºC·min^{-1}. After 3 min spent at 245ºC, the temperature was increased to 260ºC at a rate of 3ºC·min^{-1} and finally to 270ºC at a rate of 10ºC· min^{-1} and then the temperature was maintained constant for 10 more min.

Results and discussions

General set up of mass tandem identification as ions production, ions processing and ions detection were performed to build a multiple reaction monitoring (MRM) mode for the selected mycotoxins. Two transitions per compound were used, fulfilling the European Council Directives regarding mass spectrometric detection [15-17]. For each compound, the most abundant MRM transition was used for quantification while the other transition was used for confirmation (table 1).

In figures 1 and 3 the chromatographic peak of neosolaniol and its mass spectrum and the characteristic transitions viewed in MRM mode are presented, while figure 2 illustrates the MS/MS spectrum of a spiked pollen sample obtained in full scan mode in which neosolaniol has a mass/charge ratio of 252.0.

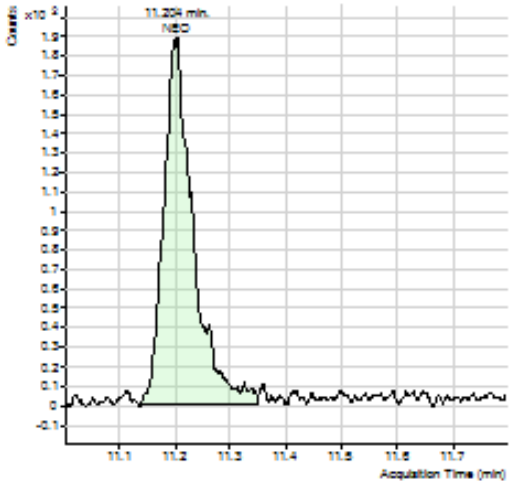

Fig. 1. GC chromatogram peak of neosolaniol

Compound	Retention time (min)	MRM transitions (m/z)		Ratio Q/q ± RSDs (% RSD)
deoxynivalenol	8.38	392>259	407>197	41.6 ± 3.2
3-acetyl-deoxynivalenol	9.42	392>287	467>147	47.5 ± 12.3
fusarenon X	9.48	450>260	450>245	11.9 ± 7.0
diacetoxyscirpenol	9.53	350>229	378>124	56.9 ± 10.3
nivalenol	9.89	289>73	379>73	29.6 ± 2.7
neosolaniol	11.24	252>195	252>167	40.6 ± 4.3
HT-2	14.66	347>157	347>185	86.7 ± 7.8
T-2	14.71	350>259	350>229	81.9 ± 5.8

Table 1
MASS SPECTROMETRY PARAMETERS FOR THE OPTIMIZED GC–MS/MS METHOD

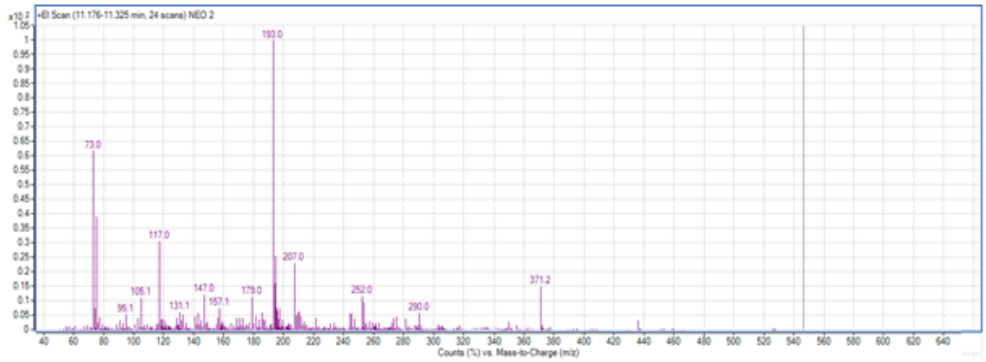

Fig. 2. MS/MS spectrum of a spiked pollen sample

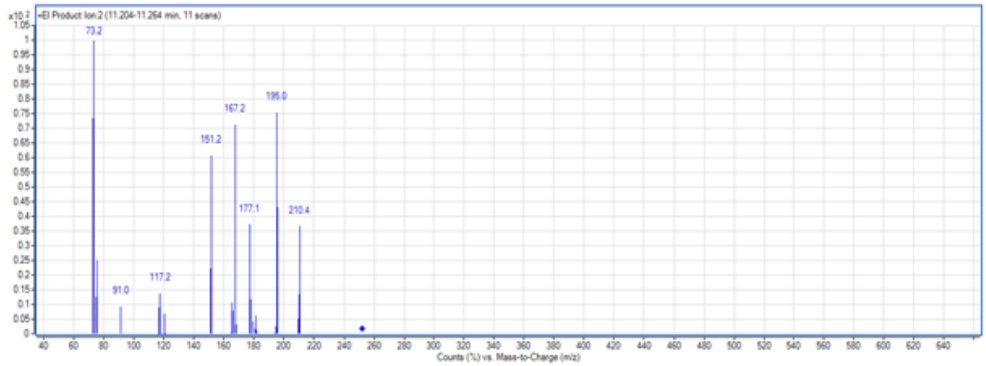

Fig. 3. The mass spectrum of neosolaniol

Table 2
ANALYTICAL PERFORMANCE OF THE PROPOSED METHOD

Analyte	LOQ ($\mu g \cdot Kg^{-1}$)	Recovery (%)			Precision (%RSDs)	Linearity r^2	Matrix effect (%)
		80µg·Kg^{-1}	250µg·Kg^{-1}	1000µg·Kg^{-1}			
deoxynivalenol	1	86.2	119.5	82.9	17.5	0.9307	68
3-acetyl-deoxynivalenol	1	88.1	118.9	88	5.7	0.9447	70
fusarenon X	4	83.4	119.6	87.4	10.6	0.9317	63
diacetoxyscirpenol	4	85.2	119.4	83.2	3.1	0.9265	86
nivalenol	1	77.5	119.4	83.5	26.3	0.9111	30
neosolaniol	2	76.8	118.7	90.1	1.0	0.9230	141
HT-2	1	72.8	141.6	86.9	6.4	0.9110	115
T-2	4	77.9	138.7	85.8	2.8	0.9001	122

Linearity and matrix effects were studied during the validation of the method using standard solutions and matrix matched calibrations (table 2). Matrix matched calibration curves were built by spiking blank samples with selected mycotoxins before extraction. The limit of quantification was defined as the concentration with a signal-to-noise ratio (S/N) of 10:1. That parameter was determined by analysis decreasing concentrations of mycotoxins in spiked pollen samples. To assess matrix effect the slope of pollen matrix matched (A) and the slope of external calibration (B) were calculated. Thus, the ratio (A/B x 100) is defined as the matrix effect (%). A value of 100% indicated that there was no matrix effect. There was signal enhancement if the value was higher than 100% and signal suppression if the value was lower than 100%.

All limits of quantification for the analytes were in between 1 and 4µg·Kg^{-1}. In term of the matrix effect the optimized method demonstrated a good linearity for all mycotoxins except for neosolaniol, HT-2 and T-2 which showed a slight enhancement in the analytical response.

The method's recovery and precision were calculated by analyzing spiked pollen samples at three concentration levels: 80µg·Kg^{-1}, 250µg·Kg^{-1} and 1000µg·Kg^{-1}, repeatedly.

In general, recoveries were satisfactory, with values in between 91 and 104% with RSDs > 11% for the analytes except for deoxynivalenol and nivalenol.

The obtained results from table 2 showed a good linearity for all analytes within the tested range.

Conclusions

Eight mycotoxins were extracted from pollen samples using a QuEChERS-based extraction procedure without applying any further clean-up step. Extraction, chromatographic and detection conditions were optimized in order to increase sample throughput and sensitivity. The method gives quantitative results for the assayed mycotoxins, providing good validation parameters in terms of linearity, precision and LOQ.

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