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Detection of new emerging type-A trichothecenes by untargeted mass spectrometry

Jesús M. González-Jartín[a], Amparo Alfonso[a,*], María J. Sainz[b], Mercedes R. Vieytes[c], Luis M. Botana[a,*]

[a] Departamento de Farmacología, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo, Spain
[b] Departamento de Producción Vegetal y Proyectos de Ingeniería, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo, Spain
[c] Departamento de Fisiología, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo, Spain

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ABSTRACT

Mycotoxins occur naturally as agricultural contaminants all over the world. The toxic effects of some of their metabolites are known and their presence regulated in food and feed. This paper describes two methods for the detection of toxins of type-A trichothecenes group, and their modified forms, using mass spectrometry. Ultra-performance liquid chromatography coupled to mass spectrometry-ion trap-time of flight (UPLC-MS-IT-TOF) was employed to characterize the fragmentation pathways of 10 type-A trichothecenes, and characteristic ions were tentatively identified in scan mode through their accurate masses. Unknown signals were detected in a *F. sporotrichioides* extract, which afterwards were identified as seven modified forms of neosolaniol (NEO) and T-2 toxin. Then, UPLC coupled to tandem mass spectrometry (MS/MS) was employed to develop a precursor ion scanning method that can be used as a screening tool to detect any modified type-A trichothecenes.

1. Introduction

Chemical contaminants in food and feed are a potential hazard concerning public health. In this sense, mycotoxins are one of the most prevalent contaminants in the food chain [1]. These compounds are secondary metabolites produced by filamentous fungi, mainly belong to species of *Aspergillus*, *Penicillium*, *Fusarium* and *Claviceps* genera. Among the great variety of metabolites produced by these organisms, just between 300 and 400 compounds are currently classified as mycotoxins because they are toxic to animals [2]. Species of *Fusarium* genus are the most commonly found in temperate regions becoming responsible for worldwide contamination of cereals and feed-stuff with the toxins produced, mainly trichothecenes, zearalenone and fumonisins [3]. Trichothecenes are a group of more than 180 analogs, all of them sesquiterpenes containing an epoxide group. This family of compounds is divided into four groups according to their structure, named types A, B, C and D [4]. Type A trichothecenes are represented by T-2 toxin, which is considered as the most toxic trichothecene. In addition, other relevant mycotoxins like HT-2, diacetoxiscirpenol and neosolaniol (NEO) are included in this group. These toxins are manly produced by strains of *F. sporotrichioides* and *F. Poae* [5]. Type B trichothecenes are represented by deoxynivalenol and its acetyl derivatives 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol. These toxins are mainly produced by strains of *F. culmorum* and *F. graminearum* [6]. In addition, type C and D trichothecenes have different chemical structure and are not produced by *Fusarium* species [7]. Therefore, type A and B compounds are the most relevant trichothecenes, in terms of natural occurrence and high toxicity [8,9]. In addition to these traditional mycotoxins, recently other fungi metabolites have started to be studied since the existing data indicate the toxicity of these compounds. They are called emerging mycotoxins, these metabolites are also mainly produced by species of *Fusarium*, the most representative toxins within this group are fusaproliferin, beauvericin, enniatins, and moniliformin [10].

The presence of mycotoxins in food and feed has been regulated in many countries. However, exposure to fungi toxins is not restricted to regulated mycotoxins since these compounds can be modified by fungi, plant or animal metabolism which leads to products with potential toxicity but not considered in the legislation [11,12]. These products coming from natural metabolizing processes, and also others resulting from food and feed processing, have frequently been called masked, bound, conjugated or hidden mycotoxins, but these terms have been used inconsistently. Thus, modified mycotoxins was a term recently introduced to describe all types of mycotoxin modifications and was adopted by the European Food Safety Authority in a scientific opinion about modified forms of certain mycotoxins [13]. Many modified

* Corresponding authors.
E-mail addresses: amparo.alfonso@usc.es (A. Alfonso), luis.botana@usc.es (L.M. Botana).

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mycotoxins have been recently discovered however many others are still unknown. The lack of toxicological studies for the modified toxins together with the hydrolysis reactions that these compounds can suffer during the digestion process are a real hazard for public health that is extraordinarily difficult to assess [14].

Several analytical techniques have been applied to detect the main groups of mycotoxins. The most currently extended is ultra-high-pressure liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) [15]. Unfortunately, by this technique modified mycotoxins are non-routinely determined, mainly because of the lack of analytical standards to be targeted, although indirect information can be obtained by chemical or enzymatic reactions [16]. To identify unknown molecules, high-resolution mass spectrometers equipped with time of flight or "Orbitrap" analyzers have been used [16,17]. In this way, several modified mycotoxins have been described. The aim of the current work was to analyze the profile of mycotoxins produced by a monosporic culture of *F. sporotrichioides* by using these techniques. From the results, two detection methods for free and/or modified forms of type-A trichothecenes were proposed.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetonitrile and acetic acid (glacial) were obtained from Panreac Quimica S.A. (Barcelona, Spain) and Formic acid was from Merck (Madrid, Spain). All solvents employed in this work were HPLC or analytical grade. Ammonium formate was purchased from Fluka (Buchs, Switzerland). Water was purified in a Millipore Milli-Q Plus system (Millipore, USA). Analytical standards of T-2 toxin, HT-2 toxin and NEO were from Romer Labs (Tulln, Austria).

2.2. Fungus culture and extraction

F. sporotrichioides strain (GenBank accession number KF576642) was grown in potato dextrose agar (Sharlau, Barcelona, Spain) for 7 days in the dark at 25 °C. After incubation, three agar plugs (6-mm diameter) were obtained from monosporic cultures. Extraction was carry out in a 4 mL deactivated amber glass vial. Agar plugs were mixed with 0.5 mL of the extraction solvent (acetonitrile/water/acetic acid; 79:20:1, *v/v/v*) and shaking for 3 min using a vortex mixer. Before analysis, the extract was filtered through a 0.45 μm Ultrafree-MC centrifugal filter (Millipore, USA).

2.3. Chromatography and mass spectrometric conditions

2.3.1. UPLC- ion trap - time of flight MS detection

The separation was performed employing an UPLC system (Shimadzu, Kyoto, Japan) coupled with MS-IT-TOF. The UPLC consists of two LC-30AD pumps, SIL-10AC autoinjector with refrigerated rack, DGU-20A degasser, CTO-10AS column oven and a SCL-10Avp system controller. The analytical column Waters ACQUITY HSS T3 (100 mm × 2.1 mm and 1.8 μm particle size) was chosen for perform the separation, and was kept at 40 °C. The binary gradient system consisted of (A) water containing 0.1% formic acid and 5 mM ammonium formate, and (B) methanol. The gradient (14.5 min) was started and held at 0% B for 1 min. Next, the proportion of eluent B was raised linearly to 50% within 3 min and then maintained for 2.5 min. Thereafter, the proportion of eluent B was increased to 100% within 3.5 min and then maintained for 2 min. Finally, the proportion of eluent B was back to 0% in 0.5 min and maintained for 2 min for column equilibration. The flow rate of the mobile phase was kept at 0.3 mL/min and the injection volume was set at 5 μL.

IT-TOF was equipped with an electrospray ionization (ESI) interface (Shimadzu, Kyoto, Japan). The operating conditions were as follows: detector voltage, 1.65 kV; nebulizing gas flow, 1.5 L/min; curved desolvation-line and heat block temperature, 200 °C; drying gas pressure, 105 kPa; pressure of TOF region, 1.4×10^{-4} Pa; ion trap pressure, 1.8×10^{-2} Pa. The nitrogen generator was a NITROMAT N-075 ECO from Worthington Creyssensac (Spain). The MS method was operated in positive full scan MS mode within the mass range 150–850. The event time was set at 300 ms with an ion accumulation time of 20 ms and 3 repetitions. Relevant ions were isolated in MS^1 scan and collision induced dissociation (CID) energy was applied over them obtaining MS^2 product ion spectra (MS^{1-2} experiments). Collision energy parameters were set at 25% and Argon was used as collision gas at 75%. The ion accumulation time was elevated to 30 ms for MS^2 stage, the precursor ion isolation was acquired within a tolerance range of 1 Dalton (Da). The mass range was calibrated prior to data acquisition employing a standard sample from Shimadzu (Kyoto, Japan) as an external reference. Data recorded was processed by Shimatzu LC/MS solution software.

2.3.2. UPLC-triple quadrupole MS detection

An UPLC system 1290 Infinity was coupled to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany), which was equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The ion source parameters were set as follows: sheath gas temperature, 400 °C; sheath gas flow, 12 L/min, gas temperature, 350 °C; gas flow, 8 L/min; nebulizer, 45 psi; capillary voltage, 4000 V; and nozzle voltage 0 V. The nitrogen generator was also NITROMAT N-075 ECO from Worthington Creyssensac (Spain).

2.4. Formula assignments

To assign the elemental composition of ions, the Formula Predictor software (Shimadzu, Kyoto, Japan) was applied to the accurate measured masses of precursor and product ions. Mass accuracy was employed to the calculation of elemental composition allowing errors lower than 10 mDa. Under the described conditions, the software generates a list of candidates taking into account data obtained in MS^{1-2} experiments.

3. Results and discussion

It is known that *F. sporotrichoides* produces type-A trichothecenes, including some modified forms [14]. However, the production of modified compounds is not predictable and therefore these molecules are undetectable by conventional identification methods. Furthermore, since many different compounds can be produced, it is impossible to have standards for all the modified forms. Hence, a strain of *F. sporotrichoides* was chosen to characterize the production of modified toxins and to optimize their detection. For these purposes, a crude extract of *F. sporotrichoides* obtained after 7 days growing was analyzed by UPLC-MS-IT-TOF. To identify the production of mycotoxins, analytical standards of the major type-A trichothecenes, NEO, T-2 toxin and HT-2 toxin were used to set up retention times (RT) and the exact mass of ammonium adducts $[M+NH_4]^+$, usually selected to monitor these toxins [17]. As Fig. 1A shows, when a mixture of standards was injected, NEO, as $[M+NH_4]^+$ adduct at *m/z* 400.1966, was the toxin that eluted first at 6.50 min, the second at 9.90 min was HT-2 toxin, $[M+NH_4]^+$ adduct at *m/z* 442.2435, and finally at 10.55 min it eluted the T-2 toxin, $[M+NH_4]^+$ adduct at *m/z* 484.2541. Then the fungus extract was analyzed, Fig. 1B, and surprisingly five peaks were detected when ammonium adducts of type-A trichothecenes standards were selected. When the presence of NEO was studied, by selecting the $[M+NH_4]^+$ adduct at *m/z* 400.1966, two peaks RT 6.50 min (peak 1) and RT 6.98 min (peak 2), with the same exact mass were obtained. In the case of HT-2 toxin, $[M+NH_4]^+$ (*m/z* 442.2435), two peaks, RT 8.10 min (peak 3) and RT 9.9 min (peak 4) with the same exact mass were shown. For T-2 toxin, $[M+NH_4]^+$ (*m/z* 484.2541), a peak at 10.55 min