IDENTIFICATION OF MYCOTOXINS BY UHPLC-QTOF MS IN AIRBORNE FUNGI AND FUNGI ISOLATED FROM INDUSTRIAL PAPER AND ANTIQUE DOCUMENTS FROM THE ARCHIVE OF BOGOTÁ

Nancy I. Castillo[1], María Ibáñez[2], Eduardo Beltrán[2], Jhon Rivera-Monroy[3], Juan Camilo Ochoa[3], Mónica Páez-Castillo[3], Martha L. Posada-Buitrago[4], Michael Sulyok[5], Félix Hernández[*2]

[1]Facultad de Ciencias Básicas, Universidad Antonio Nariño, Bogotá D.C., Colombia, 111821

[2]Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain, 12071

[3]Laboratorio de Química, Física y Biología. Archivo de Bogotá. Bogotá D.C., Colombia, 111711

[4]Laboratorio de Biofísica. Centro Internacional de Física – CIF, Bogotá D.C., Colombia, 111321

[5]Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Tulln, Austria, 3430

*Author for correspondence: felix.hernandez@uji.es

Abstract

Mold deterioration of historical documents in archives and libraries is a frequent and complex phenomenon that may have important economic and cultural consequences. In addition, exposure to toxic fungal metabolites might produce health problems. In this work, samples of broths of fungal species isolated from the documentary material and from indoor environmental samples of the Archive of Bogotá have been analyzed to investigate the presence of mycotoxins. High resolution mass spectrometry made possible to search for a large number of mycotoxins, even without reference standards available at the laboratory. For this purpose, a screening strategy based on ultra-high pressure liquid chromatography coupled to quadrupole-time of flight mass spectrometry (UHPLC-QTOF MS) under MS^E mode was applied. A customized home-made database containing elemental composition for around 600 mycotoxins was compiled. The presence of the (de)protonated molecule measured at its accurate mass was evaluated in the samples. When a peak was detected, collision induced dissociation fragments and characteristic isotopic ions were also evaluated and used for tentative identification, based on structure compatibility and comparison with literature data (if existing). Up to 44 mycotoxins were tentatively identified by UHPLC-QTOF MS. 34 of these tentative compounds were confirmed by subsequent analysis using a targeted LC-MS/MS method, supporting the strong potential of QTOF MS for identification/elucidation purposes. The presence of mycotoxins in these samples might help to reinforce safety measures for researchers and staff who work on reception, restoration and conservation of archival material, not only at the archive of Bogotá but worldwide.

Keywords: Liquid chromatography, quadrupole time-of-flight mass spectrometry, fungi, mycotoxins, screening

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1. INTRODUCTION

Among the many species of filamentous fungi which exist in nature, some of these are deteriorating agents that may cause bio-deterioration of paper and parchment in ancient books and documents (Gallo, 1985; Zotti, 2008; Zyska, 1997). These molds can be opportunistic fungi or spoilage fungi in documentary material due to their enzymatic activity (Sterflinger, 2012). It has been reported that over 200 fungal species have been isolated from paper items, although only 5-10% of the total fungal species have been identified [Cappitelli, 2010]. Some species have been identified as mycotoxin-producing strains with allergenic potential that might put at risk researchers' or staff's health who work in the reception, restoration, conservation and archival processes at libraries and archives (Bennett, 2003; Bräse, 2009). Exposure to mycotoxins is mostly by ingestion, but may also occur by dermal and inhalation routes. The diseases caused by exposure to mycotoxins are known as mycotoxicoses [Bennett, 2003; Zain, 2011] and, it has been reported that symptoms depend, among other factors, on the type of mycotoxin, the amount and duration of the exposure, and interactions with other toxic insults [Bennett, 2003]. Since its foundation in 2004, the Archive of Bogotá (ADB) has established a strain collection of indoor airborne fungi and fungi isolated from industrial paper and antique documents from all the governmental archive buildings of this city. Among these isolates, some potentially mycotoxigenic species were found by optical microscopy and molecular biology methods. Therefore, it is necessary to investigate whether these species produce mycotoxins and which type in order to guarantee the health of workers and researchers. Modern powerful analytical techniques allow investigating the mycotoxins produced by different fungi. Full spectrum acquisition techniques offer the possibility for screening a huge number of contaminants in post-targeted approaches, *i.e.* without the need of pre-

selecting the analytes for method development. An additional value of high resolution mass spectrometry (HRMS) is that it provides accurate-mass full-spectra data with reasonable sensitivity. By using a hybrid quadrupole-time of flight mass spectrometry (QTOF MS) analyzer, it is feasible to record accurate-mass product ion spectra working in MS/MS mode, which is one of the most valuable tools for confirmatory analysis nowadays. Thus, Kildgaard et al. (Kildgaard, 2014) used ultra-high performance liquid chromatography-diode array detection-QTOF MS (UHPLC-DAD-QTOFMS) providing both accurate mass full-scan MS and MS/HRMS data. The MS/HRMS data were then searched against an in-house MS/HRMS library of ~1300 compounds for unambiguous identification. The methodology was demonstrated on compounds from bioactive marine-derived strains of *Aspergillus, Penicillium*, and *Emericellopsis*.

Another possibility is the MS^E approach, which allows collecting simultaneously information on both (de)protonated molecules and their fragment ions, by acquiring data at low and high collision energy in a single injection. Furthermore, practical parameters, such as isotopic patterns and double bound equivalent (DBE), can be used to facilitate the process of identification/confirmation. With all these possibilities, the tentative identification of the compound detected is commonly feasible, even without reference standards (Ibáñez, 2013; Hernández, 2011; Hernández, 2012).

In this work, ultra-high performance liquid chromatography (UHPLC) coupled to QTOF MS has been applied for identification of mycotoxins in fungal culture broths from ADB. Illustrative examples of the compounds detected and tentatively identified in the samples are presented to demonstrate the potential of the approach applied for investigation of large number of mycotoxins in one single analysis. For this purpose, we used a customized database containing the elemental composition of around 600 mycotoxins previously

reported in the literature. As no reference standards were available in our laboratory for confirmation of the tentatively identified compounds, in a subsequent step the sample extracts were re-analyzed at the Department for Agrobiotechnology, University of Natural Resources and Life Sciences (Vienna, BOKU), using a targeted UHPLC-MS/MS method for more than 500 target analytes (Malachová, 2014).

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Glucose, aspartic acid ($C_4H_7NO_4$), KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, KCl, $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ were purchased from (Panreac Química S.L.U., Barcelona, Spain) and the yeast extract was purchased from MP Biomedicals (Santa Ana, CA, USA). *Potato Dextrose Agar* (PDA) medium, Sabouraud agar medium and Sabouraud broth medium were from MP Biomedicals Santa Ana, CA, USA). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), sodium hydroxide (NaOH) and formic acid (HCOOH) were acquired from Scharlau (Barcelona, Spain). Leucine enkephalin, used as the lock mass, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Strains and Media for analysis mycotoxins

Several potentially mycotoxigenic indoor airborne fungi and fungi isolated from industrial paper and antique documents from the Archive of Bogotá were used in this study: *Penicillium chrysogenum* (isolates 5D and DC01), *Penicillium purpurogenum* (isolate 66A), *Aspergillus tamari* (isolate 40A), *A. flavus* (isolates 81D and 79A), *A. niger* (isolate 39A) , *A. fumigatus* (isolates DC08, DC20, 13A and 74D) and *A.versicolor* (isolates 14D

and 52D), *Aspergillus ochraceus* (isolate 78A) *Stachybotrys chartarum* (isolates 12D and 35A) and *Fusarium equiseti* (isolates 50A, 45A and 7D). The isolates named with D correspond to fungi isolated from industrial paper, those with DC to antique documents from the seventeenth century and those with A to indoor airborne fungi.

Stock cultures were maintained on *Potato Dextrose Agar* (PDA) medium. For the secondary metabolites (mycotoxins) induction experiments 20 mL of modified Czapek-Dox medium at pH 4.5 were used: 10 g/L glucose, 0.54 g/L aspartic acid ($C_4H_7NO_4$), 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 0.25 g/L NaCl, 0.25 g/L KCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, and 1 g/L yeast extract. Fungi were cultured at 28°C, 140 rpm, in darkness for 15 days. The culture broths were collected in 50 mL tubes (two replicates per fungal isolate) and kept at -20°C for further analyses at the Research Institute for Pesticides and Water, University Jaume I (Spain).

2.3. Sampling and isolation of filamentous fungi

The filamentous fungi evaluated in the present study belong to the strain collection of the Archive of Bogota, which is composed by several fungi collected from different sources: (i) Industrial paper with an advanced degree of biodeterioration (Rojas et al., 2009), (ii) antique books from the 17^{th}, 18^{th} and 19^{th} century, made of manual paper, parchment and/or leather and (iii) indoor airborne of several public archive buildings of Bogota (Colombia) (Cruz Ramírez et al., 2012). Fungi of the documentary materials were taken carefully with a cotton swab or scalpel and stored in sterile Petri dish until further processing; airborne fungi were collected using a microbial air sampler (Merck MAS100 NT). All fungi were cultured on different media, mainly PDA medium and Sabouraud agar

medium, incubated at 28^{o}C for 1–2 weeks and isolated by the single-spore method (Ho and Ko, 1997).

2.4. Molecular and morphological identification

All the fungal isolates were identified to the genus level by optical microscopy, using lactophenol cotton blue stain (BD, Franklin Lakes, NJ, USA) of reproductive structures (if visible) and then confirmed by molecular identification.

Isolated fungi were cultured in Sabouraud Broth supplemented with glucose 2% per five days at 28°C and 150 rpm. Each biomass produced was filtered through sterile Whatman® paper and washed twice with sterile distilled water, and kept at -80°C until processing. Before DNA isolation, each recovered biomass was pulverized with liquid nitrogen. DNA isolation was performed according to Dellaporta et al., 1983, and DNA integrity and purity were verified by agarose-gel electrophoresis and spectrophotometry A260/280, respectively.

The molecular identification of the fungal isolates was performed by ribosomal DNA loci amplification and sequencing. The PCR amplification was carried out in a C1000 thermal cycler (BioRad, Hercules, CA, USA). For further details, see Supplementary Information

2.5. Instrumentation

Wide-scope screening of mycotoxins was applied using a Waters Acquity UPLC system (Waters, Milford, MA, USA) interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), with an

orthogonal Z-spray-ESI interface operating in both positive and negative ionisation modes. The chromatographic separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size column 100 × 2.1 mm (Waters) at a flow rate of 300 µL/min. The mobile phases used were A = H_2O and B = MeOH, both with 0.01% HCOOH. The initial percentage of B was 10%, which was linearly increased to 90% in 14 min, followed by a 2 min isocratic period and, then, returned to initial conditions during 2 min. Nitrogen was used as drying and nebulizing gas. The gas flow was set at 1000 L/h. TOF-MS resolution was approximately 20,000 at full width half maximum (FWHM) at *m/z* 556. MS data were acquired over an *m/z* range of 50–1000. Capillary voltages of 0.7 and 3.0 kV were used in positive and negative ionisation modes, respectively. A cone voltage of 20 V was selected for both ionisation modes. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The desolvation temperature was set to 600ºC and the source temperature to 130 ºC. The column temperature was set to 40ºC.

Before analysis, broths were thawed at room temperature and then ten-fold diluted with Milli-Q water. Finally, 25 µL of the diluted extract were directly injected in the UPLC-QTOF MS system under MS^E mode.

For MS^E experiments, two acquisition functions with different collision energies were created. The low energy function (LE), selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV in order to obtain a greater range of fragment ions. The LE and HE functions settings were for both a scan time of 0.4 s.

Calibrations were automatically conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.05M NaOH:5% HCOOH, twenty five-fold diluted with ACN:H_2O (80:20). For automated accurate mass measurement, a solution of leucine enkephalin (10 µg/mL) in

ACN:H_2O (50:50) with 0.1% HCOOH was used as lock mass and pumped at a flow rate of 20 µL/min. The (de)protonated molecule of leucine enkephalin (*m/z* 556.2771 in ESI+, *m/z* 554.2615 in ESI-) was used for recalibrating the mass axis and ensuring a robust accurate mass measurement at any time.

For the confirmation of the mycotoxins found in the LC-QTOF MS screening, the samples were reanalyzed by a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboV ESI source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). For further details, see Malachová et al. (Malachová, 2014). Although initially the method included 331 analytes (validation data acquired for 295 analytes), some updates have been performed in the meantime and the method covers at present more than 500 compounds.

3. RESULTS AND DISCUSSION

3.1. Fungi isolation and identification

Work environments which store large amount of documents on paper, such as the municipal archive of Bogotá, could maintain certain amount of fungal particles, both in the air and on the paper. The growth of fungi could be increased by the age of the buildings, the building materials (especially those based on adobe), and climatic conditions. For this reason, the Archive of Bogotá regularly performs analysis of environmental conditions in the municipal buildings, finding a variety of species of filamentous fungi, among which some have been widely reported as producers of mycotoxins in the scientific literature.

Molecular techniques have identified molds in archive and library material, industrial paper, and indoor environment of the archives, which cause biodeterioration. The most frequent genera are *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium*, *Chaetomium*,

Stachybotrys and *Phoma* (Michaelsen, 2009; Abdel-Maksoud, 2011; Manente , 2012; Mesquita , 2009; Rakotonirainy , 2007; Guiamet , 2011). Regarding mycotoxins, the most frequently found in indoor environments on building materials are aflatoxins, fumonisins, ochratoxin, trichothecenes and zearalenones, mainly produced by fungi of *Aspergillus*, *Fusarium*, *Penicillium* and *Stachybotrys* genera (Khan, 2012; Nielsen, 2002). However, the study of the expression of single genes involved in metabolic pathways for the production of these mycotoxins by the potentially fungi found at the Archivo of Bogotá using RT-PCR (ADB, unpublished data) revealed the absence of these compounds in these strains. This was supported by QTOF MS analysis of the corresponding culture broths (as shown below).

3.2. Screening by QTOF MS

In this work, a customized database containing around 600 mycotoxins was compiled at the Research Institute for Pesticides and Water for QTOF MS analysis. It included aflatoxins, ochratoxins, fumonisins, trichotecenes, zearalenons, roquefortines, ergot alkaloids, atranones, cytohalasins, tremorgens and sphinganin mycotoxins, and other related compounds. No reference standards were available at our laboratory (except for 20 compounds relevant for food analysis included in a previous work (Nácher-Mestre, 2015); therefore, the elemental composition of the compounds was the only information included in the database. Information about adducts and fragmentation of certain compounds (either in exact or in nominal mass) was also included in the target list, when this information was available in the literature.

In the LC-QTOF MS target screening applied, the presence of the (de)protonated molecule was searched in the samples by performing automated narrow-mass window extracted ion

chromatograms (nw-XICs) for all compounds included in the database. Due to the narrow mass window employed (150 ppm), only one chromatographic peak was commonly observed. After that, characteristic isotopic ions and fragment ions, typically in the HE function, were further evaluated. UHPLC was a valuable tool for selecting perfectly co-eluting fragment ions that might correspond to the same precursor, minimizing in this way spectrum interferences that would complicate the identification process.

Some examples are discussed below to illustrate the strategy applied and the different cases under study.

Figure 1 shows the detection and tentative identification of Nigragillin by UHPLC-QTOF MS. An abundant chromatographic peak at 3.24 min was observed in the LE function at *m/z* 223.1812 (**Figure 1A, bottom**). This seemed to correspond to the protonated molecule of Nigragillin ($C_{13}H_{23}N_2O$, expressed as protonated molecule), with a mass error of 0.9 ppm in relation with the theoretical exact mass of this ion. The HE spectrum showed ions at *m/z* 129.1390 ($C_7H_{17}N_2$) and 95.0496 (C_6H_7O), both with mass errors below 1.5 ppm (**Figure 1A, top**). The structure of the 2 fragment ions could be justified on the basis of their accurate masses. These fragment ions perfectly fitted with the structure of this mycotoxin, corresponding to both sides of the molecule after the nitrogen-carbonyl group bond cleavage. Moreover, the fragment ion at *m/z* 129 is in accordance with reported data (Nielsen, 2003). This compound could not been confirmed by additional LC-MS/MS analysis as was not included in the list of target analytes of the method. However, there was reliable information supporting this compound to be Nigragillin, based on acquired accurate-mass data and on the literature data (Nielsen, 2003). The same situation occurred for Pyranogrinin in sample 35A, or Tryprostatin B in 13A, amongst others (see **Table 1**).

Another example is shown in **Figure 2**, which illustrates the detection and tentative identification of Fumagillin. The LE spectrum of a chromatographic peak at 12.59 min in sample DC08 showed an intense signal at m/z 459.2374 (**Figure 2A**, bottom). The LE spectrum also showed the corresponding sodium and potassium adducts, as well as two fragment ions at m/z 441.2271 (corresponding to H_2O loss), m/z 427.2113 (CH_3OH loss) and m/z 409.2010 (corresponding to loss of water from 427). The HE spectrum (**Figure 2A**, top) showed the ions m/z 233.1545, 215.1433, 177.0549 (obtained after the ester group cleavage) and 131.0493 (explained as the loss of formic acid from 177), all with mass errors far below 5 ppm. The structure of all fragment ions was justified on the basis of their accurate masses. The two latter fragment ions were in accordance with previous literature (Vishwanath, 2009). After careful evaluation (including testing for other metabolites produced by Aspergillus fumigatus) and the well-supported tentative identification by QTOF MS, this mycotoxin was confirmed in the samples DC08, DC20 and 13A by additional target analysis by LC-MS/MS (Malachová, 2014). The same approach was applied for the detection of Roquefortine C in samples 5D and DC01, Penicillic acid (in sample 78A), Fumiquinazoline D (DC08, DC20 and 13A), Fumiquinazoline F (13A), Meleagrin (5D and DC 01), Kojic acid (81D,79A and 40A), and Strachybotramide (35A and 12D), among others (see **Table 1**).

Regarding data obtained by ESI under negative mode, Sydonic acid (52D and 14D), Methylsulochrin (13A), Helvolic acid (13A, DC08 and DC20), Demethylsulochrin (DC08) and Pseurotin A (DC08, DC20 and 13A) were detected. Andrastin A (5D), Fumiquinazoline A (DC08, DC20, 13A), Cyclopiazonic acid (81D, 79A, 40A) and Fumifungin were detected in both positive and negative ionization modes.

In most cases, tentative identifications were supported by the information reported in literature for these compounds (Malachová, 2014, Nielsen, 2003; Sulyok, 2007) and were afterwards confirmed by LC-MS/MS analysis. In three cases (Fumiquinazoline D in sample 13A, and Cyclopiazonic acid in 81D and 79A), positive findings were only found by LC-MS/MS method, while the first screening by QTOF MS was not able to detect these compounds. However, after a manual reviewing of the QTOF MS data (retrospective analysis, without the need of new injections), the presence of these mycotoxins was confirmed although with low signals obtained. Surely, the lower sensitivity in QTOF screening in comparison with the target LC-MS/MS analysis, together with the low concentrations of these analytes in samples, was the reason of the non-detection in the first general screening.

A more complicated situation occurred when more than one candidate was feasible for a given detected peak, as some isomeric/isobaric compounds were included in the database. Obviously, this fact complicated the elucidation process. In these cases, justifying the fragment ions observed was helpful and allowed reducing the number of potential compounds. The tentative identification of the suspect compounds could be additionally supported by MS/MS product ions reported in the literature (either in exact or nominal mass) (Malachová, 2014, Nielsen, 2003; Rasmussen, 2010; Sulyok, 2007; Vishwanath. 2009).

As an example, the compounds detected at *m/z* 257.1646 at 2.85 and 3.85 min in sample DC20 might correspond to the isomers Chanoclavine and Fumigaclavine B ($C_{16}H_{21}N_2O$, expressed as protonated molecule) (**Figure 3**). Regarding the peak at 3.85 min (**Figure 3A)**, the HE spectrum showed a first loss of CH_3NH_2 (fragment ion at *m/z* 226.1226) followed by water loss (*m/z* 208.1120). An abundant fragment ion was also observed at *m/z*

168.0806 ($C_{12}H_{10}N$). For the peak at 2.85 (**Figure 3B)**, low-abundant fragment ions at *m/z* 239.1539 (corresponding to an initial water loss), 208.1123 (corresponding to the loss of CH_3NH_2 from 239) and 168.0808 ($C_{12}H_{10}N$) were observed in the HE spectrum. After studying the MS fragmentation (mainly the most relevant fragments at *m/z* 239 and 226) and considering the structures of the two candidates, the peak at 3.85 was assigned to Chanoclavine whereas that at 2.85 min was assigned to Fumigaclavine B. It seemed more plausible that Fumigaclavine B lost initially a water molecule, whereas this loss seemed more complicated for Chanoclavine. Moreover, the initial CH_3NH_2 loss seemed more favored for Chanoclavine. Product ions at nominal *m/z* 226, 208 and 168 have been reported for Chanoclavine, which supports our hypothesis (Malachová, 2014; Nielsen, 2003; Sulyok, 2007). For Fumigaclavine B, the only information found in literature reported the ions at *m/z* 192 and 167 (Rasmussen, 2010) , which however did not fit with our observed fragment ions. Nevertheless, considering the fragments observed for Fumigaclavine A and Fumigaclavine C (also tentatively identified in the same sample), and the similarities/differences between their structures (**Figure 4**), fragment ions at *m/z* 239 and 208 could be predicted for Fumigaclavine B, which perfectly matched with the experimental accurate-mass spectra. The LC-MS/MS method confirmed the presence of all these 4 compounds in samples DC08, DC20 and 13A.

Only in one of the cases under study (two peaks at 4.82 and 5.17 min for *m/z* 241.1696, corresponding to $C_{16}H_{21}N_2$ expressed as protonated molecule), it was not possible to distinguish between 4 possible compounds This occurred for C/D-trans-fused pair Festuclavine and Pyroclavine, and the C/D-cis-fused Costaclavine and Epicostaclavine. They are all stereoisomers compounds, and therefore, fragmentation did not allow discarding any possibility. For both chromatographic peaks, fragments at *m/z* 154, 168,

210, 84 and 182 were observed. According to the literature (Nielsen, 2003), Festuclavine presented fragments at *m/z* 154 and 168, and Secoclavine at *m/z* 210. For Pyroclavine and Epicostaclavine, no information was found. Therefore, additional techniques or the injection of all 4 reference standards would be necessary to confirm their identity. After applying the LC-MS/MS method, one of the peaks was confirmed to be Festuclavine. The remaining 3 compounds were not included in the scope of the multi- method, and therefore could not be confirmed.

There was only one case of disagreement between QTOF and LC-MS/MS analysis. It corresponded to Stachybotrylactam, which tentative identification by QTOF MS seemed to fail, as it was not confirmed by the LC-MS/MS method. However, the main fragment ion observed at *m/z* 178 was in accordance with previous data reported in literature (Malachová, 2014; Phenomenex Technical Note-1119, 2014) and the remaining two ions (*m/z* 368.2222 and 178.0510) were compatible with its chemical structure. In addition, its retention time was in good agreement with that predicted for this mycotoxin from the retention times of seven close compounds under the chromatographic gradient conditions applied in both LC-MS/MS and QTOF. The poor sensitivity observed for this compound in the LC-MS/MS system under the experimental conditions applied might be the reason of the non-detection. The confirmation of this compound by QTOF MS would require the injection of the reference standard, which at the time of writing this paper was not available to our laboratory.

Table 1 shows the mycotoxins detected and tentatively identified in the samples under study following the strategy described above, except for *F. equiseti* (50A, 7D, 45A), *P. purpurogenum* (66A) and *A. fumigatus* (only sample 74D was not included) where only

tryptophan, cinnamic acid and p-hydroxycinnamic acid were found. The positives found by QTOF MS (marked as ×) were classified as a function of the information available for their identification. Several possibilities were feasible:1) the observed fragment ions were in agreement with data reported in the literature; 2) no information was found in the scientific literature, but fragment ions were compatible with the chemical structure of the candidate; 3) with the information available was not possible to distinguish between isomeric compounds; 4) only one peak was observed for the exact-mass monitored (commonly the protonated molecule), but information available on fragment ions was not sufficient for a confident identification. This table also shows the compounds that were confirmed by additional analysis in BOKU laboratory using a target LC-MS/MS method with reference standards (marked as •).

To sum up, 44 mycotoxins were tentatively identified by QTOF MS without the use of reference standards, and 34 of them were afterwards confirmed by LC-MS/MS. The only compound included in the LC-MS/MS method which was not confirmed was Stachybotrylactam. The remaining 11 compounds could not been confirmed as they were not included in the LC-MS/MS method. Four compounds (3-Nitropropionic acid, Orsellinic acid, Chrysogine, and Averufin) were detected by LC-MS/MS but could not be found by QTOF MS. This was surely due to the low levels of these compounds in the samples, as revealed by the low signal intensities obtained.

A brief discussion on our findings, which are in general consistent with data available in the literature, is included below.

Discussion on results obtained and consistency with literature

In general, our findings are consistent with data available in the literature. Thus, in *Aspergillus fumigatus,* our data revealed the presence of Fumagillin, Helvolic acid, Pseurotin A, Chanoclavine, Fumigaclavine A, B, C, Demethylsulochrin, Fumiquinazoline A, D, F, Tryprostatin B (tentative identification), Methylsulochrin and Cyclopiazonic acid. Among these, Fumigaclavines, Pseurotin A, Fumiquinazoline D, Fumagillin and Helvolic acid (Khan, 2012; Frisvad, 2009) as well as Tryprostatins A and B (Cui, 1996; Yamakawa , 2011), have been described as antimitotic agents produced by *A. fumigatus*. The production of Cyclopiazonic acid has been reported mainly for *Penicillium* species, though it has been also reported for a few species of *Aspergillus*, such as *A. flavus*, *A. oryzae*, *A. fumigatus*, *A. versicolor*, and *A. tamarii* (Ohmomo, 1973; Dorner, 1983; Dorner, 1984, Vinokurova, 2007; Hymery, 2014; Chang, 2009). Other compounds found in our analysis, as Chanoclavine, have been reported for *Aspergillus* species such as *A. nidulans* (Ryan, 2013); and Demethylsulochrin, involved in geodin biosynthetic pathway, is produced by *A. terreus* (Fujii, 1988; Chen, 1995; Boruta, 2014). In *A. flavus*, we found Kojic acid and Cyclopiazonic acid, which is consistent with the literature (Hedayati, 2007; Chang, 2009; Mohamad, 2010). Kojic acid was also detected in *A. tamarii*, as previously reported by Gould (Gould, 1938); and Fumifungin was also detected, although it has been reported as produced by *A. fumigatus* (Mukhopadhyay, 1987). Penicillic acid was detected in *A. flavus*, while some papers reported this compound as produced by *A.ochraceus* (Lindenfelser, 1977). Sydonic acid, detected in *A. versicolor*, has been reported in *A. sydowii* and other *Aspergillus* sp. (Hamasaki, 1978; Wei, 2010).

In *Penicillium chrysogenum* we found Roquefortin C and Meleagrin, which are part of the metabolic pathway Roquefortine/Meleagrin of *P. chrysogenum* (García-Estrada, 2011; Ries, 2013). Vansteelandt et al.. (Vansteelandt, 2012) reported Maculosin in *P.*

chrysogenum for the first time. Andrastin A, a protein farnesyl transferase inhibitor and antitumoral compound, has been reported for *P. chrysogenum* (Matsuda, 2013). Stachybotramide was detected in *S. chartarum*, which is in agreement with data obtained by Takahashi et al. (Takahashi, 1995) and Wu (Wu, 2014).

The health effects of indoor mycotoxins have only been studied recently (Mendell et. al, 2011 Knutsen, et. al., 2012; Haleen Khan and Mohan Karuppayil, 2012; Kim, et. al. 2013; Nevalainen, et. al., 2015, Baldacci, et. al., 2015). Mendell, et. al., 2011, conducted epidemiological studies and meta-analyses about publications on the online database PubMed (National Library of Medicine 2010) and concluded that there is sufficient evidence to relate the indoor molds with increased asthma development and exacerbation, current and ever diagnosis of dyspnea, wheeze, cough, respiratory infections, bronchitis, allergic rhinitis, eczema, and upper respiratory tract symptoms. On the contrary, other studies resolved that fungal concentrations in indoor environments do not correlate with respiratory symptoms, asthma or atopy (Nevalainen, 2014; Kirjavainen, 2015). Most authors agreed that is possible to establish a relationship between the presence of molds in the environment and human health, but the relationship between chemical agents or biological agents that lead to the manifestation of diseases is not clear. Therefore, it is essential to continue research within this field to better understand the possible effect of mycotoxin production and molds themselves on the people who live often in mold contaminated environments.

CONCLUSIONS

This work shows the excellent capabilities of the LC-QTOF MS for efficient and rapid wide-scope screening of a large number of mycotoxins without having *a priori* reference standards. This approach is highly useful when facing situations as in this work, where many different compounds might be present but no information on the identity of potential mycotoxins in samples was available. Obviously, reference standards are always necessary at a final stage for the unequivocal confirmation of the findings, but they can be acquired specifically for only those compounds tentatively identified in previous analysis avoiding problems of costs and expiring dates associated to the acquisition of a large number of mycotoxins standards.

Following the screening strategy applied in this work to samples of broths of fungal species isolated from the documentary material and some species isolated from indoor environmental samples of the Archive of Bogotá, up to 44 mycotoxins were tentatively identified by QTOF MS and 34 of them were afterwards confirmed by LC-MS/MS. It is thought that the presence of potentially mycotoxigenic fungi in documentary material and airborne environments from archives and libraries might cause health problems among their staff and researchers. These fungi are usually analyzed for the production of aflatoxins, fumonisins, ochratoxin, trichothecenes and zearalenones, which are the most common and well-studied mycotoxins because of their presence in contaminated foods and feeds, and whose exposure is mostly by ingestion. However, this study showed that the analyzed fungal strains from the Archive of Bogota did not produce these mycotoxins. Instead, the analytical methodology used in this study led to the detection of compounds whose risk to human health has not yet been determined. Further research is required to

determine the level of toxicity of those emerging mycotoxigenic compounds on human beings and to propose early diagnosis strategies for archives and libraries. Future monitoring to quantify the concentration levels of the mycotoxins identified in this work (e.g. by LC-MS/MS) would be recommendable in selected samples collected from the Archive of Bogotá. In the meantime, caution measurements are suggested to be reinforced in archives and libraries for appropriate workers protection until more data are available in the near future.

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COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests to declare

DISCLOSURE STATEMENT

The authors report they have no conflicts of interest or financial relationships to disclose.

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FIGURE CAPTIONS

Figure 1. Tentative identification of of mycotoxin Nigragillin in sample 39A by UHPLC-QTOF MS. (A) Low energy (bottom) and high energy (top) TOF mass spectra for the sample; (B) From bottom to top, Extracted Ion Chromatograms (XICs) at 150 ppm mass window for $(M+H)^+$ in LE function and main fragments in HE function.

Figure 2. Tentative identification of mycotoxin Fumagillin in sample DC08 by UHPLC-QTOF MS. (A) Low energy (bottom) and high energy (top) TOF mass spectra for the sample; (B) Extracted Ion Chromatograms (XICs) at 150 ppm mass window for $(M+H)^+$ in LE function, and main fragments in both LE and HE function.

Figure 3. Tentative identification of mycotoxins Fumigaclavine B and Chanoclavine in sample DC20 by UHPLC-QTOF MS. (A) High energy TOF mass spectra for the peak at 3.85 min; (B) High energy TOF mass spectra for the peak at 2.85 min; (C) From bottom to top, Extracted Ion Chromatograms (XICs) at 150 ppm mass window for $(M+H)^+$ in LE function, and main fragments in HE function.

Figure 4. High energy TOF mass spectra for (A) Fumigaclavine A and (B) Fumigaclavine C, both tentatively identified in sample DC20; (C) High energy TOF mass spectra for presumably Fumigaclavine B. The distinctive functional groups are marked in the structures.

Table 1. Summary of the results obtained in the analysis of broths by UHPLC-QTOF MS. Confirmation performed by LC-MS/MS.

	P. Chrysogenum		*A. Fumigatus*			*A. Flavus*		*A. Ochraceus*	*A. Niger*	*A. Tamarii*	*A. Versicolor*	*S. Chartarum*
Compound	5D	DC01	DC08	DC20	13A	81D	79A	78A	39A	40A	52D 14D	35A 12D
Andrastin A[2]	×●											
Asperlactone[1]								×●				
Asperloxine A[1]								×●				
Aspyrone[1]								×●				
Aurasperone B[2]									×●			
Aurasperone C[2]									×●			
Brevianamid F[1]		×●	×●	×●	×●			×●				
Chanoclavine[1]			×●	×●	×●							
Cyclopiazonic acid[1]						×●	×●			×●		
Demethylsulochrin[1]			×●									
Dihydrolysergol[4]			×●	×●								
Festuclavine[3]			×●	×●	×●							
Fumagillin[1]			×●	×●	×●							
Fumifungin[2]										×●		
Fumigaclavine A[1]			×●	×●	×●							
Fumigaclavine B[2]			×●	×●	×●							
Fumigaclavine C[1]			×●	×●	×●							
Fumiquinazoline A[1]			×●	×●	×●							
Fumiquinazoline D[1]			×●	×●	×●							
Fumiquinazoline F[2]					×●							
Helvolic acid[1]			×●	×●	×●							
Kojic acid[1]						×●	×●			×●		
Maculosin[1]	×●	×●										
Meleagrin[1]	×●	×●										
Methylsulochrin[1]					×●							
Penicillic acid[1]								×●				
Pseurotin A[1]			×●	×●	×●							
Pyripyropene A[4]			×●	×●								
Pseurotin D[1*]			×●	×●	×●							
Roquefortine C[1]	×●	×●										
Stachybotramide[2]												×●
Stachybotrylactam[1]												×
Sydonic acid[4]											×●	
Trichodimerol[4]	×●											
Tryptoquivaline F[1]			×●	×●	×●							
Compounds not included in the LC-MS/MS method												
Aspergillic acid[2]								×				
Barnol[2]							×					
Cinnamic acid[2]							×	×		×	×	×
Cinnamic acid, hydroxy[2]											×	×
Isofumigaclavine A[2]			×	×	×							
Nigerazine B[2]									×			
Nigragillin[1]									×			
Pyranonigrin[1]												×
Tryprostatin B[1]			×	×	×							

1) fragment ions were in accordance with those reported in the literature;

2) no information was found in the scientific literature but fragment ions were compatible with the structure of the candidate;
3) it was not possible to distinguish between the different isomeric compounds;
4) a peak was observed for the exact-mass monitored but no sufficient information on fragment ions was available to assure, with more or less confidence, the identity of the candidate.

(×) Compounds detected and tentatively identified by UHPLC-QTOF MS.
(●) Compounds confirmed by additional analysis in another laboratory using LC-MS/MS method with reference standards.

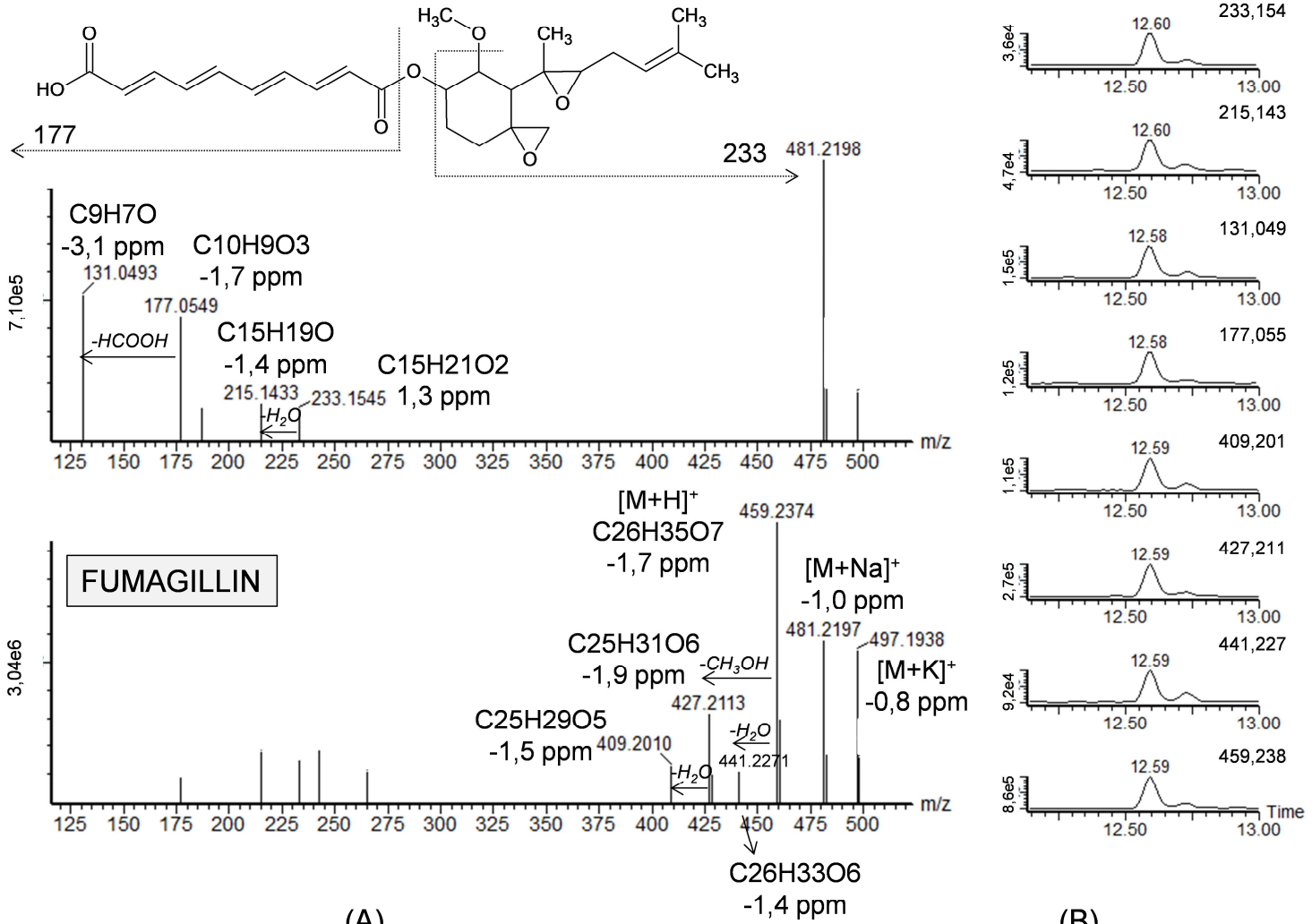

177
233
481.2198
C9H7O
-3,1 ppm
C10H9O3
-1,7 ppm
131.0493
177.0549
-HCOOH
C15H19O
-1,4 ppm
C15H21O2
1,3 ppm
215.1433
233.1545
-H2O
7,10e5
m/z
125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500
FUMAGILLIN
[M+H]+
C26H35O7
-1,7 ppm
459.2374
[M+Na]+
-1,0 ppm
481.2197
497.1938
[M+K]+
-0,8 ppm
C25H31O6
-1,9 ppm
-CH3OH
427.2113
C25H29O5
-1,5 ppm
409.2010
-H2O
-H2O
441.2271
3,04e6
m/z
125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500
C26H33O6
-1,4 ppm
(A)
233,154
215,143
131,049
177,055
409,201
427,211
441,227
459,238
12.60
12.58
12.59
3,6e4
4,7e4
1,5e5
1,2e5
1,1e5
2,7e5
9,2e4
8,6e5
12.50
13.00
Time
(B)

Fig 1

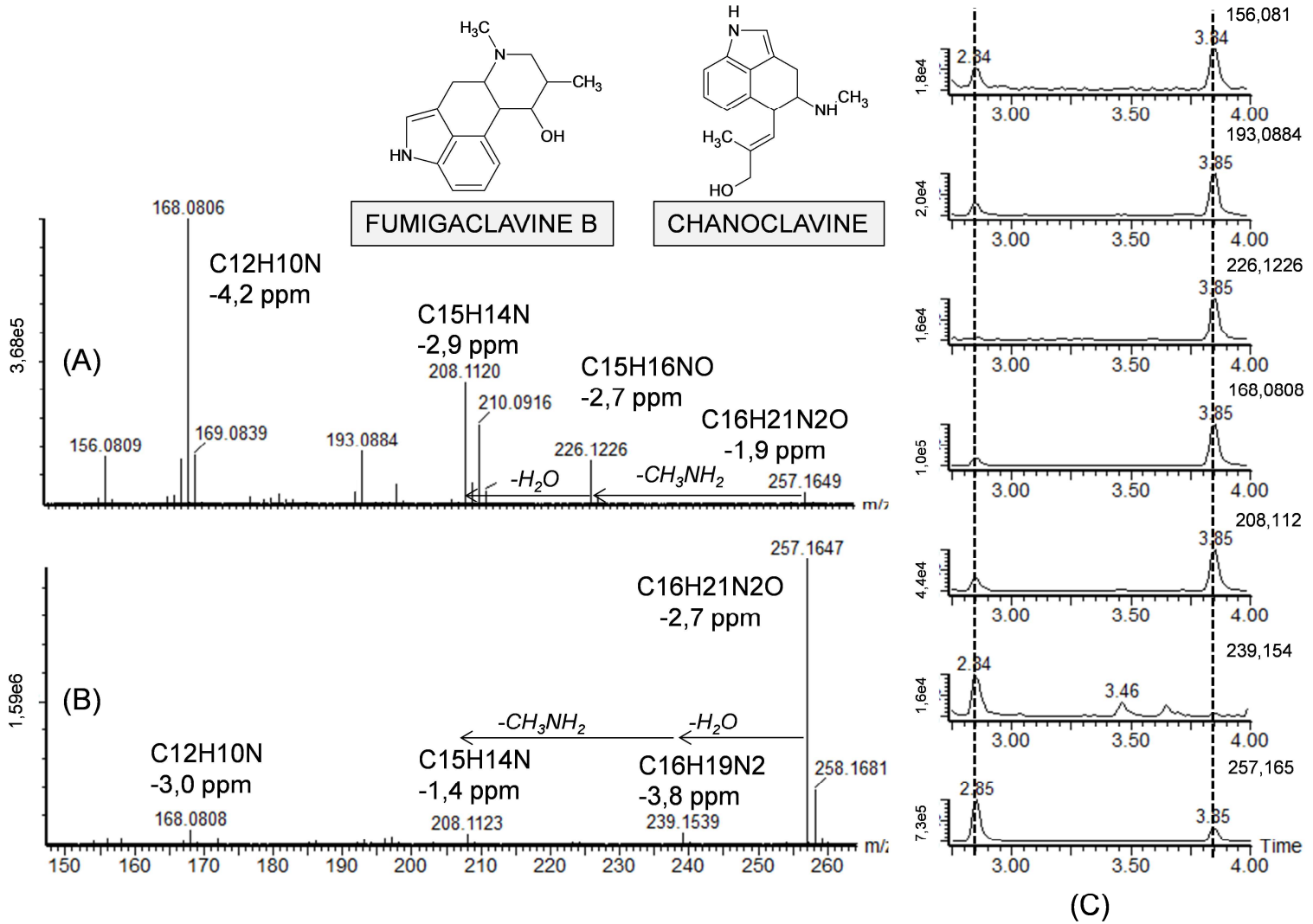
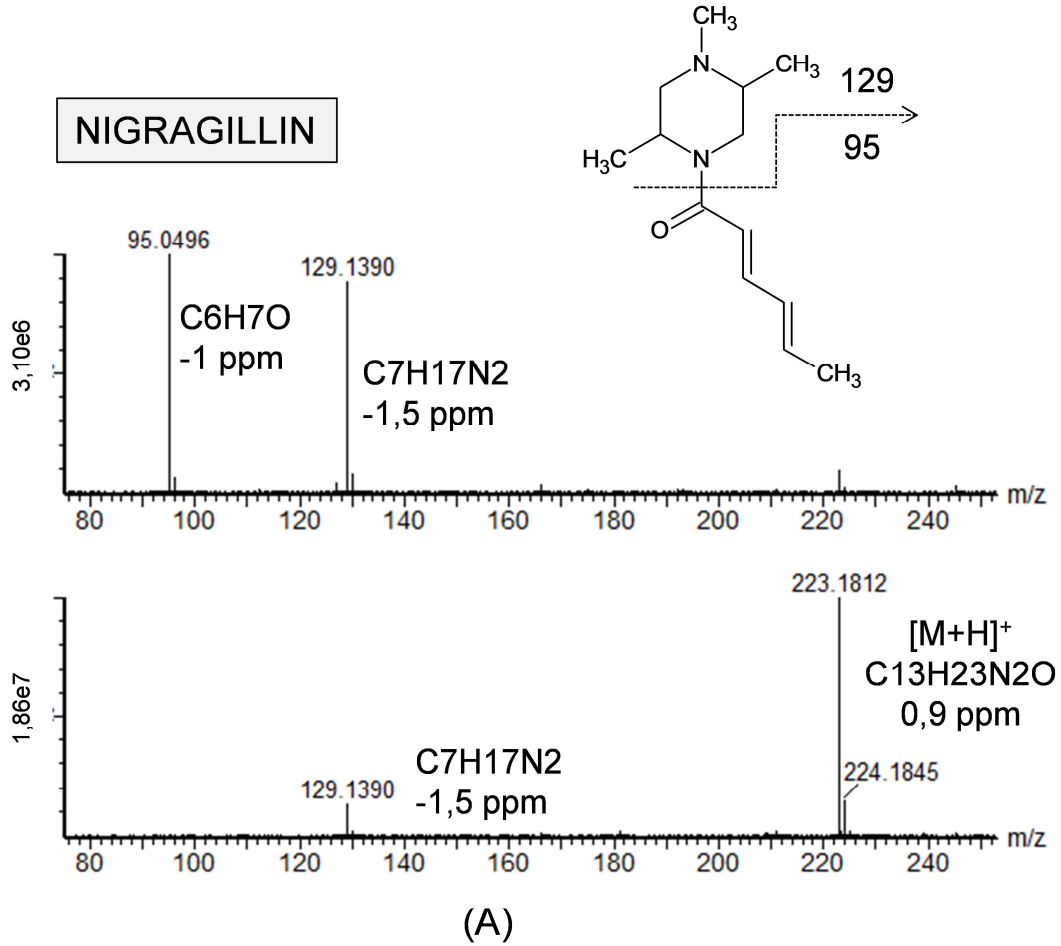
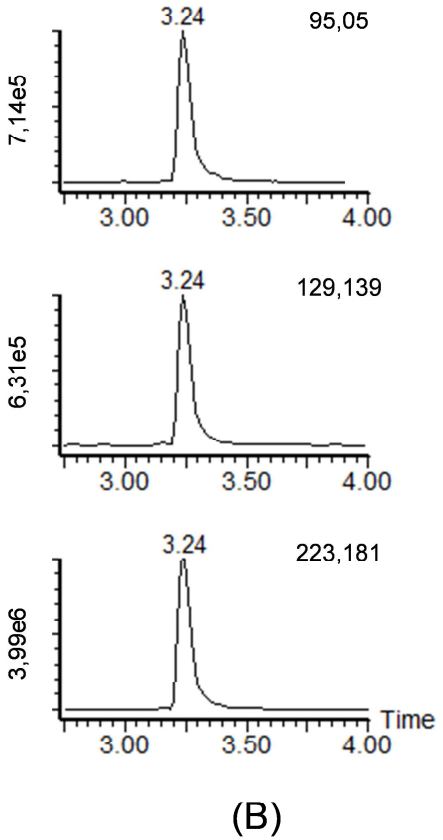

FUMIGACLAVINE B
CHANOCLAVINE
(A)
168.0806
C12H10N
-4,2 ppm
C15H14N
-2,9 ppm
208.1120
210.0916
C15H16NO
-2,7 ppm
C16H21N2O
-1,9 ppm
156.0809
169.0839
193.0884
226.1226
257.1649
-H2O
-CH3NH2
3,68e5
(B)
257.1647
C16H21N2O
-2,7 ppm
-CH3NH2
-H2O
C12H10N
-3,0 ppm
C15H14N
-1,4 ppm
C16H19N2
-3,8 ppm
258.1681
168.0808
208.1123
239.1539
1,59e6
150 160 170 180 190 200 210 220 230 240 250 260
156,081
193,0884
226,1226
168,0808
208,112
239,154
257,165
2.34
3.34
3.85
3.46
1,8e4
2,0e4
1,6e4
1,0e5
4,4e4
7,3e5
3.00 3.50 4.00
Time
(C)

Fig 2


NIGRAGILLIN
CH3
N
CH3
H3C
N
O
CH3
129
95
95.0496
129.1390
C6H7O
-1 ppm
C7H17N2
-1,5 ppm
3,10e6
m/z
80 100 120 140 160 180 200 220 240
223.1812
[M+H]+
C13H23N2O
0,9 ppm
C7H17N2
-1,5 ppm
129.1390
224.1845
1,86e7
m/z
80 100 120 140 160 180 200 220 240

(A)


3.24
95,05
7,14e5
3.00 3.50 4.00
3.24
129,139
6,31e5
3.00 3.50 4.00
3.24
223,181
3,99e6
Time
3.00 3.50 4.00

(B)

Fig 3

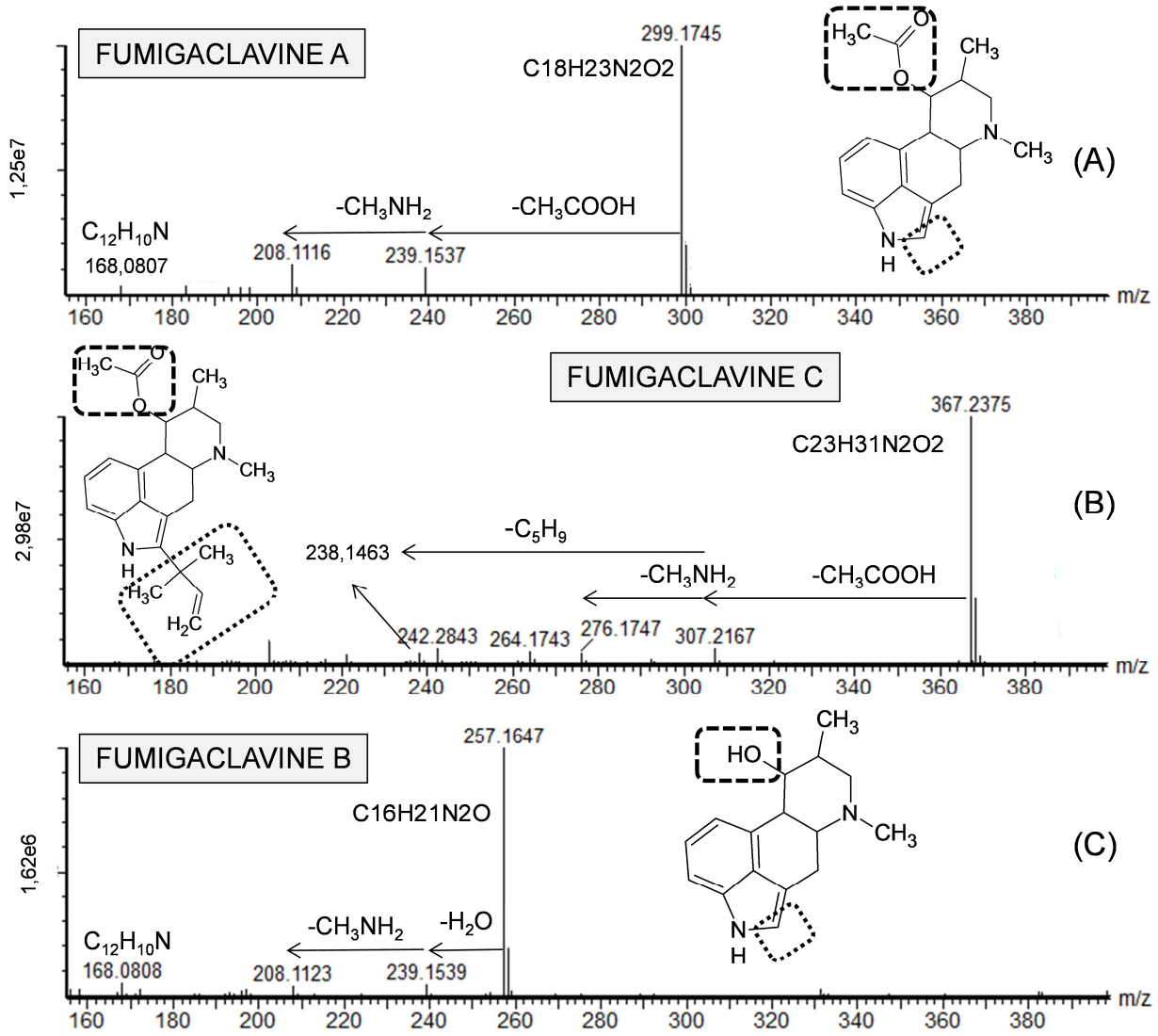

FUMIGACLAVINE A
299.1745
C18H23N2O2
1,25e7
-CH3NH2
-CH3COOH
C12H10N
168,0807
208.1116
239.1537
m/z
(A)
FUMIGACLAVINE C
367.2375
C23H31N2O2
2,98e7
-C5H9
238,1463
-CH3NH2
-CH3COOH
242.2843
264.1743
276.1747
307.2167
m/z
(B)
FUMIGACLAVINE B
257.1647
C16H21N2O
1,62e6
-CH3NH2
-H2O
C12H10N
168.0808
208.1123
239.1539
m/z
(C)

Fig 4