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DETECTION OF MYCOTOXINS USING MALDI-TOF MASS SPECTROMETRY

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ABSTRACT

Mycotoxins are toxic substances produced by some microscopic fungi such as *Aspergillus*, *Fusarium* and *Penicillium* sp. These species commonly contaminate foods and feeds. Therefore, the quick and accurate detection is very necessary. In this study, a six mycotoxins, concretely: aflatoxin B_1, citrinin, deoxynivalenol, zearalenone, T2-toxin, and griseofulvin were detected by Matrix Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS). In addition, sodium adducts were observed in all tested mycotoxins. All mycotoxins were ionizated by HCCA matrix. Our results showed that it is possible to detect selected mycotoxins by MALDI-TOF MS Microflex LT in linear positive ion mode.

Keywords: Mycotoxins, Maldi TOF MS, detection

INTRODUCTION

Mycotoxins are toxic metabolites produced by fungal species that commonly contaminate staple foods and feeds. They represent an unavoidable problem due to their presence in globally consumed cereals such as rice, maize and wheat **(Marroquín-Cardona *et al.*, 2014)**. Furthermore, these compounds have a great financial impact. From an economic point of view, mycotoxins cause money loss to producers, processors and also consumers of food and feed **(Bryden, 2012; Oliveira and Catharino, 2014).** Mycotoxins are thermally stable and demonstrate high levels of bioaccumulation. They are approximately 300-700 Da long and their production is genotypically specific, but is not limited to one species, or one toxin per species **(Turner *et al.*, 2015).** Mycotoxins have been more closely monitored in the past decades due to their harsh effects observed in humans and animals; potent toxic effects in humans and animals have been related to these molecules, such as cytotoxicity, carcinogenicity, mutagenicity, neurotoxicity, hepatotoxicity, immunosuppressive, and estrogenic effects **(Ayed-boussema *et al.*, 2008; Belli *et al.*, 2010)**. Reproductive alterations in animals were induced by mycotoxin addition (**Medvedova *et al.*, 2011; Kolesarova *et al.*, 2012; Maruniakova *et al.*, 2012, 2014, 2015; Halenar *et al.*, 2015**).The main group of moulds and mycotoxins of world – wide concern are *Aspergillus* spp. (producing aflatoxins B1, B2,G1,G2,OTA and patulin), *Fusarium* spp. (producing T-2 toxin, deoxynivalenol, zearalenone and fumonisin B1) and *Penicillium* spp. (producing OTA) (Zain, 2011). The International Agency for Cancer Research (IARC) has classified a number of mycotoxins formally as agents that are proven (Group 1 - AF B1, AF B2, AF G1 and AF G2), probably (Group 2A), and possibly (Group 2B OTA) carcinogenic to humans. With a wide-ranging structural diversity of mycotoxins, severe toxic effects caused by these molecules and their high chemical stability the requirement for robust and effective detection methods is clear **(Bhat *et al.*, 2010; IARC, 2015)**. Mycotoxin toxicity in foodstuff can occur at very low concentrations necessitating early availability of sensitive and reliable methods for their detection. Conventional analytical methods for mycotoxin detection include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV), diode array (DAD), fluorescence (FD) or mass spectrometry (MS) detectors, gas chromatography (GC) coupled with electron capture (ECD), flame ionization (FID) or MS detectors **(Chauhan *et al.*, 2016)** and enzyme-linked immunoassay (ELISA) **(Wang *et al.*, 2011).** Though these methods are well known for their accurate and precise detection of mycotoxin in food or feed samples, they require skilled operators, extensive sample pre-treatment, equipment and may lack accuracy at low analyte concentration and very often the using of classical method is expensive processes **(Cigić and Prosen, 2009; *Goryacheva et al.*, 2007; Blechova *et al.*, 2006)**. Therefore, a rapid, sensitive and specific assay technique is required for the routine analysis of foods, and beverages **(Chauhan *et al.*, 2016)**. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) instrumentation enables highly sensitive and fast analysis and/or detection using a very small sample **(Blechova *et al.*, 2006)**. The Matrix-assisted laser/desorption ionization time of flight mass spectrometry (MALDI-TOF MS) instrumentation is a rather new technique developed two decades ago **(Tanaka *et al.*, 1988)**. It enables highly sensitive and rapid analysis at femtomole or even attomole levels of organic and/or high molecular biomolecules without their destruction. Another advantage of MALDI-TOF MS over conventional techniques is its ability to analyse samples within a few minutes and using an only small sample. Thus, the MALDI-TOF MS method could offer the possibility for fast and sensitive detection of mycotoxins **(Elosta *et al.*, 2007)**.
Therefore, the aim of this study was the detection of selected pure mycotoxins by MALDI-TOF Mass Spectrometry.

MATERIAL AND METHODS

Mycotoxins

A six different type of mycotoxins, aflatoxin B1 ($C_{17}H_{12}O_7$ = 328.27 Da), citrinin ($C_{13}H_{14}O_5$ = 250.25 Da), deoxynivalenol ($C_{15}H_{20}O_6$ = 296.32 Da), zearalenone ($C_{18}H_{22}O_5$ = 318.36 Da), T2-toxin ($C_{24}H_{34}O_9$ = 466.52 Da), and griseofulvin ($C_{17}H_{17}ClO_6$ = 352.77 Da) were used in this study. All mycotoxins were obtained from Sigma Aldrich, Germany.

Preparing of mycotoxins for MALDI-TOF MS

All mycotoxins were dissolved in methanol at concentration 0.1 mg/mL. A 1 µl of mycotoxin solution was replaced by pipetting onto the 96 targets stainless steel plate (Bruker daltonics, Germany). Every second repetition of the sample was mixed with 10 M NaCl solution. After drying, a 1 µl of HCCA (α-cyano-4-hydroxycinnamic acid) dissolved in the solution contained 50 % acetonitrile, 47.5 % water and 2.5 % trifluoracetic acid. This matrix was dried at room temperature.

MALDI-TOF MS mycotoxin detection

After drying, each spectrum was collected by MALDI-TOF MS LT Microflex (Bruker Daltonics, Germany) operating in linear positive mode with following set using flexControl software (Bruker Daltonics, Germany): laser frequency: 60Hz, laser attenuator: 20-30 %, gain detector: 1.0x, spectrum range: 100-700 Da, ion source 1: 18 kV, ion source 2: 15 kV, lens: 6 kV, pulsed ion extraction: 0 ns. Spectra were measured randomly by 500 laser shots. As calibrant HCCA matrix was used.

Spectra analysis

Obtained spectra were observed by flexAnalysis 3.4 software (Bruker Daltonics, Germany). Detection of peaks were done by centroid detection algorithm with a signal-to-noise threshold of 1, a relative intensity threshold of 0 %, a minimum intensity threshold of 0, a peak width of 0.2 m/z, a height of 80 %, a Tophat baseline subtraction, without smoothing algorithm, a width of 0.2 m/z, and cycle of 1. Theoretical peaks of mycotoxins was compared with our detected mass with a ± 0.5 m/z.

RESULTS AND DISCUSSION

Mycotoxins detection

Food and feed may be contaminated by a variety of mycotoxins produced by microscopic filamentous fungi. Therefore, it is necessary to detect these mycotoxins. However, some of the methods actually used for detection of mycotoxins are rely on sample preparation based on volatile organic solvents, often comprising complex multi-step procedures and devoid of clean-up and concentration effects. Here, we used a simple preparation methodology for detection of mycotoxins that offers the advantages of short analysis time and low sample consumption.

Detection of aflatoxin B1 was tested with HCCA matrix. Aflatoxin B1 spectrum was observed between 300 – 350 m/z. The peaks were identified as $[M+H]^+$ 313.109 and $[M+Na]^+$ at 335.059 Da. Peaks in repetition sample with NaCl solution were detected in the same position. Spectrum resolution and purity in higher quality was found in the sample with the addition of NaCl solution but the intensity was lower than sample without NaCl solution. Aflatoxin B1 spectrum obtained by MALDI-TOF MS is presented in figure 1.

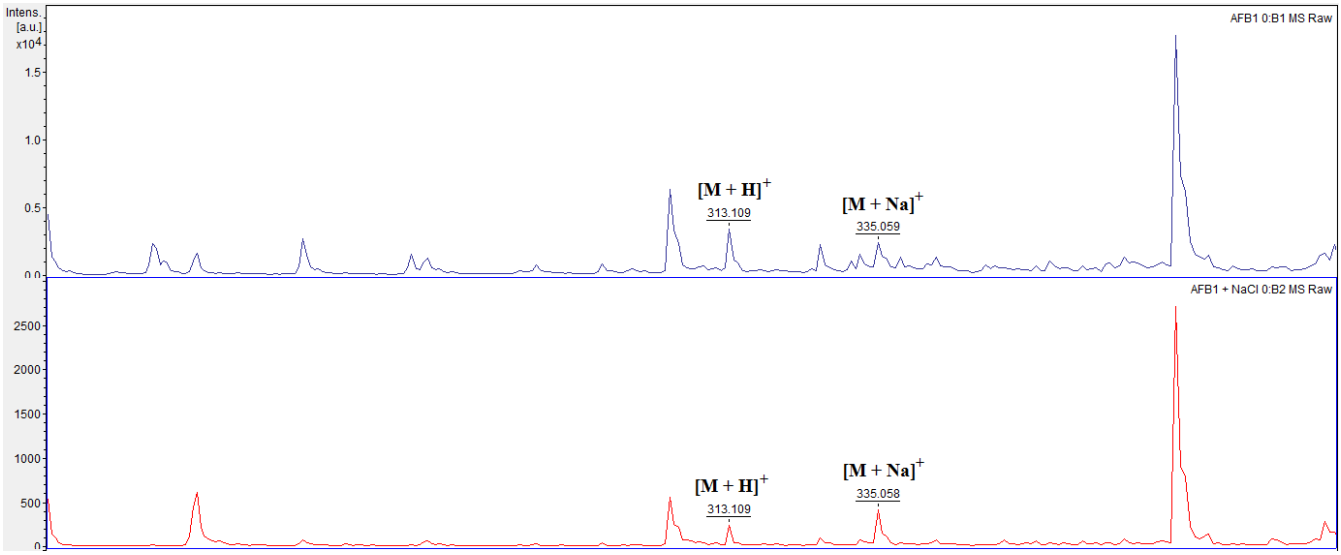

Figure 1 Aflatoxin B1 – spectrum of pure aflatoxin B1 obtained by MALDI-TOF MS (representative peaks are marked)

Authors **Catharino *et al.*, 2005** studied aflatoxins by MALDI-TOF MS in reflectron positive ion modes with using the same matrix (HCCA) with addition of 10 mM NaCl and they detected aflatoxin B1 as $[M+Na]^+$ at 335 Da. In our study we determined that it's possible to detect protonated pure aflatoxin B1 as $[M+H]^+$ at 313 Da. Also **Farzaneh *et al.*, 2012** used ESI triple-quadrupole mass spectrometer for detection of aflatoxin B1 and they identified three peaks in aflatoxin B1 spectrum. Spectrum contains following peaks: $[M+H]^+$ at 313 Da, $[M+Na]^+$ at 335 Da and $[2M+Na]^+$ at 647 Da. In our study peak $[2M+H]^+$ did not detect.

Citrinin spectrum was found as five different peaks. Spectrum was obtained using the same matrix (HCCA) and peaks were identified as $[M-H_2O-CO]^+$ at 205.383 Da, $[M-H_2O]^+$ at 234.393 Da, $[M+H]^+$ at 251.359 Da, $[M+Na]^+$ at 273.218 Da and $[M+K]^+$ at 289.455 Da. Spectrum with addition of NaCl solution was found at the same position. Identified spectrum has the same resolution, purity, and intensity into the both cases. Citrinin spectrum is showed in figure 2.

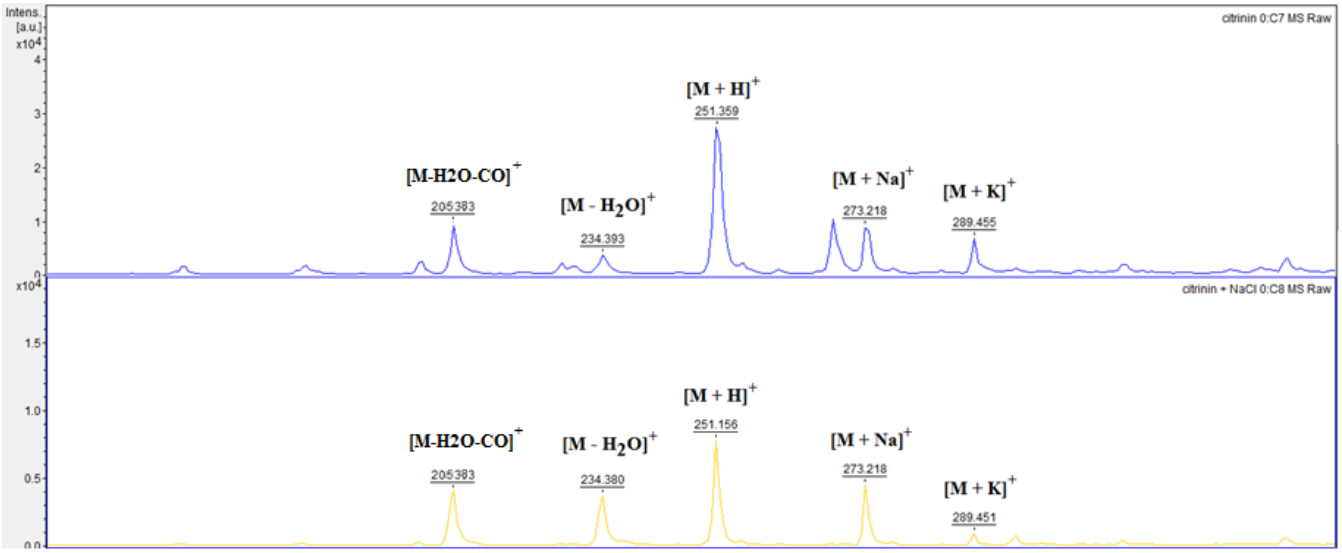

Figure 2 Citrinin – spectrum of pure citrinin obtained by MALDI-TOF MS (representative peaks are marked)

Citrinin was detected by **Blanc *et al.*, 1995** using tandem 6F LSIMS (Liquid Secondary Ionization Mass Spectrometry). The main peak was detected as [M+H]+ at 251 Da. Expect this one, other adducts were detected as $[M+Na]^+$ at 273 Da, $[M+K]^+$ at 289 Da and fragment with water loss $[M-H_2O]^+$ at 233 Da. On the other hand, **Li *et al.*, 2012** used LC-ESI-MS/MS for citrinin detection and they found also fragment with water and carbon oxid loss $[M-H_2O-CO]^+$. Our

study, where the Microflex LT in linear positive ion modes was used, showed the same results.

Deoxynivalenol spectrum was found as three peaks. In comparing with theoretical peaks of deoxynivalenol were found at the same position. Deoxynivalenol contain following peaks: $[M+H]^+$ at 296.207 Da, $[M+Na]^+$ at 319.486 Da and $[M+K]^+$ at 335.764 Da. The intensity of identified peaks was higher in without addition of NaCl solution to sample and purity and resolution were at the same level into the both cases. Spectrum for deoxynivalenol obtained by MALDI-TOF MS is presented in figure 3.

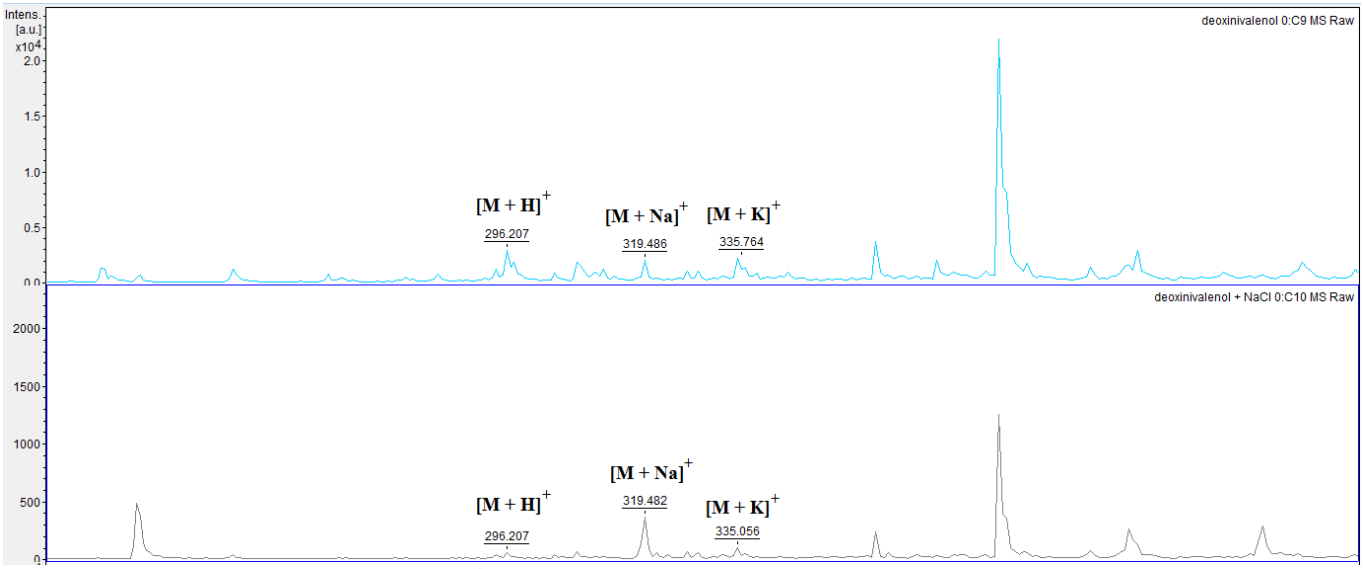

Figure 3 Deoxynivalenol - spectrum of pure deoxynivalenol obtained by MALDI-TOF MS (representative peaks are marked)

Blechova *et al.*, 2006 used Axima-CFR instrument, which uses laser with 337 nm and reflectron linear positive mode was used. They detected typical peaks for deoxynivalenol as $[M+Na]^+$ at 319.11 Da and $[M+K]^+$ at 335.09 Da. Expect these adducts, results from our study showed the peak of pure deoxynivalenol as [M+H]+ at 295.207 Da. Other authors, **Berthiller *et al.*, 2009** detected deoxynivalenol using LC-MS/MS as acetate adduct $[M+CH_3COO]^-$ at 355.1 Da.

Zearalenone spectrum was found as complex of three peaks into the both samples. The both spectrum showed the same position, purity and resolution of peaks. The sample where NaCl was added showed the lower intensity as without solution. The peaks were identified as $[M-H_2O]^+$ at 301.165 Da, $[M+H]^+$ at 319.483 Da and $[M+Na]^+$ at 341.648 Da. Zearalenone spectrum is presented in figure 4.

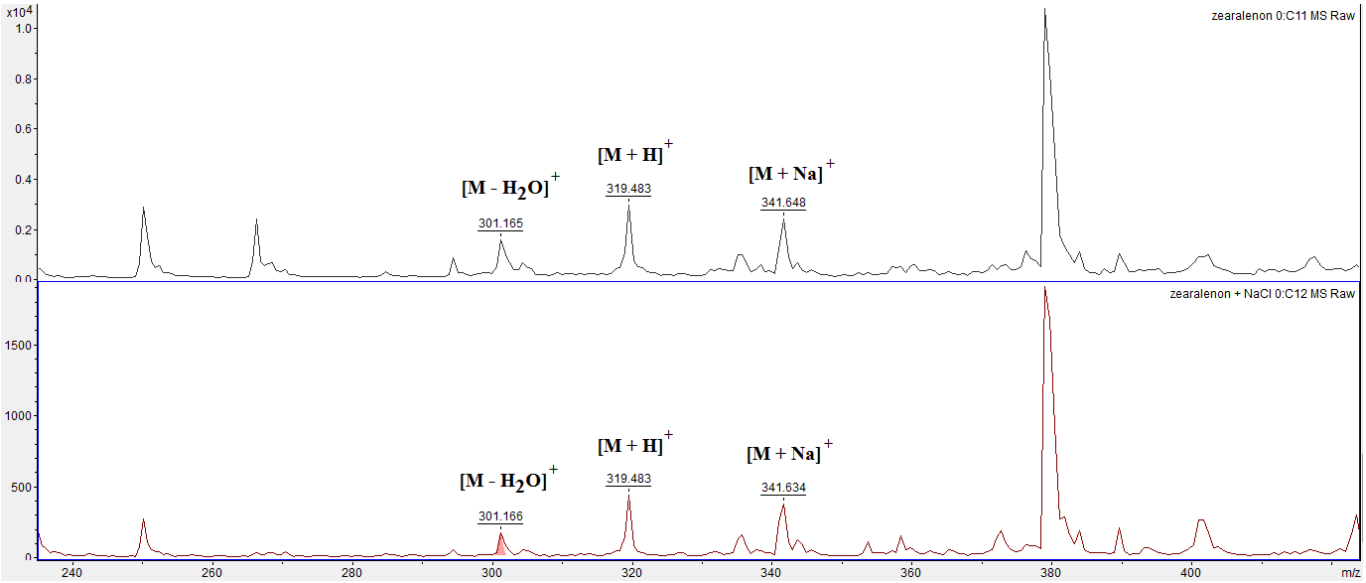

Figure 4 Zearalenone – spectrum of pure zearalenone obtained by MALDI-TOF MS (representative peaks are marked)

Engelhardt *et al.*, 1988 researched transformation of the *Fusarium* mycotoxin zearalenone in maize cell suspension cultures using DCI-mass spectrometry after TLC and HPLC purification and they detected zearalenone as $[M+H]^+$ at 319 Da. The intensity of spectrum for T2-toxin was found on the same level into the both samples. Resolution and purity of peaks too. The pure zearalenone spectrum was found as two peaks. Peak with 489.501 Da was identified as $[M+Na]^+$ and peak with 505.487 Da as $[M+K]^+$. The protonated peak of pure zearalenone $[M+H]^+$ was not found. In the case of NaCl solution one peak with 489.511 Da was identified as $[M+Na]^+$. Other peaks were not found. T2-toxin spectrum obtained by MALDI-TOF MS is showed in figure 5.

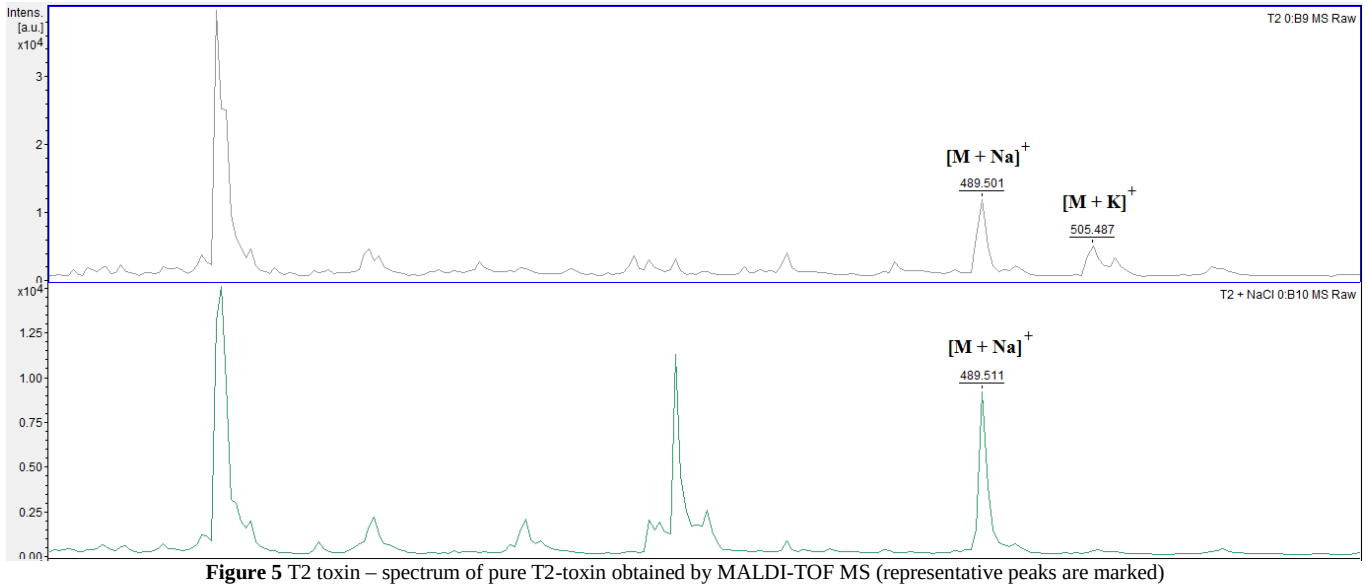

Figure 5 T2 toxin – spectrum of pure T2-toxin obtained by MALDI-TOF MS (representative peaks are marked)

T2-toxin-d3was detected by **Binder *et al*., 2007** using HPLC as spectrum of two peaks: 487 Da and 492 Da.

Griseofulvin was identified as two peaks with following positions and peaks: $[M+H]^+$ at 353.023 Da and $[M+Na]^+$ at 375.613 Da. Peaks was found in high quality of resolution and purity in the both samples, expect intensity. Intensity was higher in the sample without NaCl solution. Second peak $[M+Na]^+$ with 375.613 Da can be difficult to detect because it has very close to the matrix $[2M+H]^+$ with 379 Da. Griseofulvin spectrum is presented in figure 6.

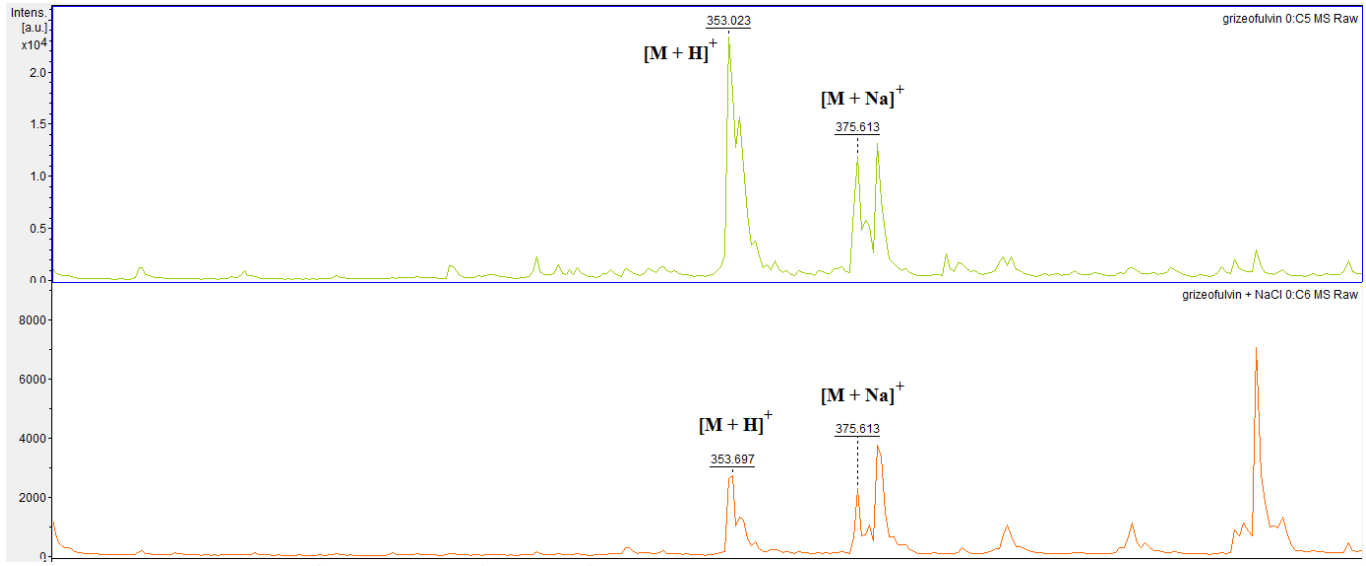

Figure 6 Griseofulvin - spectrum of pure griseofulvin obtained by MALDI-TOF MS (representative peaks are marked)

In study of **Petit *et al*., 2004** was used ESI-IT Mass Spectrometry for griseofulvin detection. They detected griseofulvin as [M+H]+ at 353 Da. Authors **Rundberget and Wilkins, 2002** determinated some types of mycotoxins, produced by *Penicillium* spp., including the griseofulvin in food and feeds using LC-MS and LC–MS–MS. They obtained griseofulvin as [M+H]+ at 355 Da.

CONCLUSION

Presented data showed that it is possible to detect mycotoxins with using MALDI-TOF Mass Spectrometer Microflex LT operating in linear ion positive mode in very short time. The methodology is very fast and cheap, and it can be used for various mycotoxins which is produced by microscopic fungi. This analytical method is useful and powerful tool for qualitative identification different kind of small organic molecules such as mycotoxins.

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