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Research Article

Analytical Quantification of mycotoxin by LC-ESI-MS/MS and MALDI-TOF MS in *Phoma* species - infected sugarcane

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Abstract: *Phoma* is a common fungal plant pathogen that can produce mycotoxins which plays an important role in plant breeding to alleviate disease losses. In this study, the potential metabolites of fungal strain of *phoma sorghina* (BS2-1, BS11-1 and BSQP) was isolated from sugarcane. The analytical method for the simultaneous determination of various mycotoxins, aflatoxins, fumonisins (B1 and B2), zearalenone and B-trichothecene mix (deoxynivalenol, nivalenol, 3-acetyl- deoxynivalenol, 15-acetyl-deoxynivalenol) in sugarcane using LC-MS and MALDI-TOF was developed and validated. These metabolites may influence the functioning of the fungus and are thus important indirectly for its growth and survival. Samples were extracted with acetonitrile-water (80:20, v:v, containing 0.5 % formic acid) were purified with a dispersive SPE method using C18 as a cleaning agent. The final clear extracts were dried by nitrogen blowing and subsequently redissolved in methanol-water (5:5,v:v). The samples were then analyzed by LC-MS and MALDI TOF-MS with 0.1% formic acid in ammonium acetate as mobile phase. However, this result can provide the new insight to explore the virulence on sugarcane and quantify the production of mycotoxin in order to improve the quality and yield of sugarcane.

Keywords: *Phoma sorghina,* LC-ESI-MS/MS, MALDI-TOF MS, Sugarcane.

Introduction

Sugarcane is considered one of the major crop in many part of the world and as well as in China (Zhao, 2015). The production and consumption of sugarcane is constrained by several factors, most imperative among which are fungal species (Jeyakumar, 2018). Mycotoxins are secondary metabolites produced by different fungi that frequently contaminate sugarcane. Aflatoxins, fumonisins, zearalenone, and deoxynivalenol are mycotoxins that are frequently present on sugarcane (Jeyakumar, 2018). Mycotoxins are caused by members of *Fusarium, Aspergillus* and *Phoma* species which possess carcinogenic, teratogenic and mutagenic effects (Tang *et al.*, 2013).

The majority of *Phoma* species are plant pathogens causing leaf and stem spots on land plants (Aveskamp *et al.*, 2010). *Phoma* species on sugarcane leaves has been reported in India, Pakistan, Hawaii, and China (Sanguino, 1980).

Phoma species can be isolated from roots and stems were determined. For example, *P. destructiva* is the causative agent of tomato leaf and stem blight (Boerema *et al.*, 2004), *P. medicaginis* variants and *P. sclerotioides* are the agents of alfalfa black stem and brown root rot, respectively (Boerema *et al.*, 2004 ; Wunsch & Bergstrom, 2011). In Brazil, *P. sorghina* is still related to a maize disease with symptoms similar to *Phaeosphaeria* leaf

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spot (do Amaral *et al.,* 2005). For the detection and quantification of mycotoxins, enzyme-linked immunosorbent assay (ELISA) (Bhattacharya *et al.,* 1999), thin layer chromatography (TLC) (Robb & Norval 1983), high performance liquid chromatography (HPLC) (Peters *et al.,* 2013), gas chromatography (GC) techniques are the common detection methods (Cunha & Fernandes 2010; Spanjer *et al.,* 2008). However, most of the analytical methods are only used for single-target analyses. LC-MS/MS is a method that could analyze multi-mycotoxins simultaneously (Spanjer *et al.,* 2008). Because no derivatization step is required in LC-MS/MS, this method has been commonly used for the determination of multi-mycotoxin contaminations in sugarcane and other crops, and many LC-MS/MS methods have been reported to be used for different types of mycotoxins (Smaoui, 2020). MALDI-TOF-MS is widely used in recent years due to its high resolution, full scan and precise mass number (Feucherolles, 2019).

However, it can provide the new insight to explore the different virulence on sugarcane and to quantify the expression of toxin and correlate the level of transcripts with different mycotoxins production by *P. sorghina*. Therefore, we try to decipher the differences in mycotoxins production of these *phoma sorghina* species by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and MALDI-TOF to reveal high-resolution expressions associated with mycotoxins regulation in *phoma sorghina* species.

Materials and Methods

Fungal strain and culture conditions

Twisted leaf disease affected sugarcane plants were collected randomly from fields. Leaves from the margins of diseased lesions had been surface sterilized by dipping in 70% ethanol. In this study, each strain was grown on ten Petri dishes containing potato dextrose agar (PDA) for 14 days in the dark at 25 ± 1°C, and then extracted with ethyl acetate (Mostert *et al*., 2001). The three selected strains of *Phoma Sorghina* for indepth study were strains *P. sorghina* (BS2-1), *P. sorghina* (BS11-1), *P. sorghina* (BSQP). To get the relative normal level of mycotoxin, the cultures must be avoided conglome-ration until constant mass was achieved in order to determine the dry mycelial weight (Kovac *et al.,* 2018).

Mycotoxin preparation

To extract the mycotoxin, the supernatant was added 50 mL ethyl acetate and cultured in shaker at the speed of 1200 rpm. After 2 hours, the mixed liquid was separated two layers and the mycotoxins would be got in the filtered liquid (after the superntant filtered by RC 0.2 mm filters) (Jo *et al*., 2021). All the above procedures were repeated three times. The extract mycotoxin sample through a Bond-Elut strong anion-exchange (SAX) clean-up was evaporated to dryness, then dissolved in 5mL 75% methanol. At finally, the dissolved mycotoxins were evaporated and the residue was separated (Ndubea, 2001).

Standard solutions and chemical reagents

The analytical standards of the mycotoxins, fumonisin B1 (FB1), fumonisin B2 (FB2), deoxynivalenol (DON), Nivalenol (NIV), **3**-acetyl-deoxynivalenol (3-AcDON), 15-acetyl -deoxynivalenol (15-AcDON), Aflatoxin G1 (AG1) and zearalenone (ZEA) were prepared. Deionized distilled water was produced using Milli-Q purification system from Millipore, Inc. (Billerica, MA, USA) (Jeyakumar, 2018).

Ethyl acetate, acetone, isopropanol, α-cyano-4-hydroxycinnamic acid solution, ammoni-

um acetate was purchased from Sigma Aldrich. Acetonitrile and MeOH, and acetic acid were obtained from Burdick & Jackson (Ulsan, Korea).

Detection of mycotoxin using LCMS/MS:

A Shimadzu LC/MS-2010 EV system 0EV (Shimadzu, Milan, Italy) with an ESI source and a Discovery HS C18 column (5 µm, 150 mm length × 2.1 mm i.d., Supelco, St. Louis, MO, USA) is used as the spectrometry system, data acquisition and processing with the Microsoft Windows-based software LC/MS Solution *ver.*3.20 (Shimadzu, Milan, Italy). 20 µl extraction was used to quailtative analyze, operating parameters as following: interface voltage 4.5 kV; nebulizer gas flow 1.5 l/min; block heater temperature 250℃; curved desolvation line temperature and voltage 200°C and -20 V, flow rate of 0.2 ml /min. The solvent system used was a gradient of solvent A (water + formic acid 0.05%) and solvent B (ACN+ formic acid 0.05%), with a step gradient from 5% to 55% B (50min).

Liquid chromatography was performed using ESI-MS on the positive ion mode at a vaporizer at 600℃, and Capillary voltage 3.2kV and spectral data were recorded with N2 (CAD=4) as collision gas. In order to optimize the MS parameters, tuning solutions for each compound were in fused continuously to the ESI+ interface which recorded the incoming compound. Tuning solutions (10ng. μL^{-1}) were freshly prepared in mobile phase B/mobile phase A (50:50, v/v). The cone voltage for every compound was optimized. To enhance the sensitivity for all the mycotoxins, product ion scans MS/MS spectra of the selected precursor ion were carried out in 30s using several collision energies. These mass spectra allowed selecting the two most abundant productions and optimizing the corresponding collision energies. Spectra were scanned over a mass range of 100-1500 m/z.

Detection of mycotoxin - MALDI-TOF/MS

For MALDI-TOF MS analysis, samples (1 µL droplets) were spotted directly onto the 384 MTP MALDI polished steel target plate (Bruker-Daltonik GmbH, Bremen, Germany), in triplicate and air-dried. The spotted samples were then coated with 2 µL of alpha -cyano-4-hydroxy-cinnamic acid matrix (α-CHCA) (10 mg mL^{-1}) (Bruker-Daltonik GmbH). The samples were analyzed on the MALDI TOF (Bruker Daltonics) mass spectrometer at the facilities of the Department of Biotechnology, Guangxi University, Nanning. Protein mass spectra were automatically collected in the linear ion, positive mode within a protein mass range from 3 000 to 15 000 Dalton (Da). Spectrum signal acquisition was completed with the Flex-Control (Bruker Daltonics, Germany) in auto-execute mode by pulsating the target spot. The software performs steps of smoothing and baseline correction during peak evaluation, and the peak resolution for the identification of the most significant peaks (Yang, 2009).

Results and Discussion

Mycotoxin analysis with LC-ESI-MS/MS

The strains were collected from a Sugarcane field in Nanning, Guangxi. Even though it is quite impossible to isolate, purify and identify all compounds produced by the fungus. The *Phoma* species produced mycotoxin spectra that allowed for the differentiation between *Phoma* sps. The culturing of *Phoma* sps. on PDA produced mass spectra with more distinct peaks of greater intensities.

The *Phoma* sps. cultured on PDA media for 1-7 and 8-14 days respectively, produced protein spectra with few peaks and low intensities compared to species cultured on PDA media for 6 days. When *phoma* sp.

strains (BS2-1, BS11-1, BSQP) were cultured on PDA, produced more peaks with higher intensities after 7 and 14 days, respectively. The optimal culture conditions for *phoma* sps. were on PDA at 25°C under constant light for 6 days, while the optimal conditions *phoma* species strains (BS2-1, BS11-1, BSQP) were on PDA under constant light at 25°C for 10 days. LC-MS provides a very powerful method for the separation and detection of metabolites. In this we used various mycotoxins like FB1, FB2, DON, NIV, 3-AcDON, 15-AcDON, ZEA, and AG1. The metabolites that were found in various fungal strain of *phoma* sps. strains (BS2-1,BS11-1,BSQP). This was done by using LC-MS.

Since the nature of the analytes and the separation conditions has a strong influence on which technique provides the best results, ESI interfaces were used. As the ionization mechanisms differ, this allowed for the analysis of a wider range of compounds. The extracts of the strains were obtained in the same manner as for strains BS2-1, BS11-1, BSQP and were dissolved in methanol and filtered prior to injection. Detection of the compounds was based on retention time, mass spectrum and fragmentation. One of the advantages is the detection of groups of compounds belonging to the same families, as biosynthetically related metabolites. Hence, this technique was used for the screening of the eight mycotoxins for further upscale study. The extracts obtained were thus analysed by LC-MS coupled to ESI.

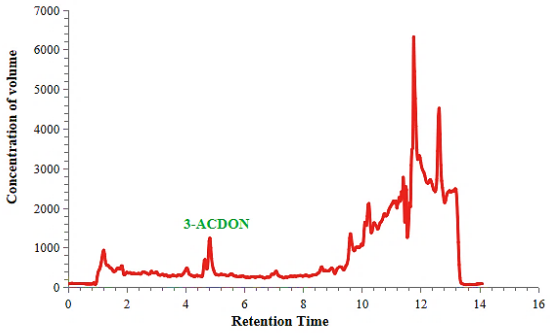
The chromatograms of strains display several peaks but all with very small intensities. For strain *P.Sorghina* (BS2-1), there is one major peak at a retention time of 4.8 minutes but rest of the chromatogram bears barely any peak (Figure 1). ZEA was detected at a lower concentration of 40 and time of 8.9 min. FB1 showed a peak value and retention time of 200 and 12.2 min, Whereas for FB2 550 and 14 min respectively (Table 1). With strain *P.Sorghina* (BS11-1), there are several peaks but all rather in small quantities compare to *P.Sorghina* (BS2-1). The respective peak value of detected toxin such as 3ACDON, ZEA, FB1 and FB2 were 980, 43, 110 and 195 (Figure 1 and Table 2). For strain *P. Sorghina* (BSQP), the chromate-gram shows a vast amount of different compounds, especially in the 4.8-14 minute time range, and also with intensities (Table 3). In *P. Sorghina* (BSQP), there are two major compounds (3ACDON and FB2) that stand out at 4.8 and 14 min and a few other peaks but with smaller intensities (Figure 1).

Strains showed interesting chromatograms with a amount of toxin produced. The chromatogram of strain has peaks standing out, with quite a lot of other compounds distributed all over the range of polarities. The linear regression coefficients of all calibration curves exhibited a high linearity, with equivalent correlation coefficients (r2) better than 0.996. LDs and LQs of the mycotoxins studied revealed a substantial variability. Limits of detection (LDs), limits of quantification (LQs), and linearity represented as a correlation coefficient are all parameters of the LC-MS/MS technology for sugarcane analysis.

This analysis is sensitive, precise, and repeatable when our results for technique validation are taken into consideration. It also has the advantage of using LCMS/MS, which has become the most used method for mycotoxins analysis in recent years. Fig.1 represents the detection chromatograms of mycotoxin by LC/MS in selected *phoma* species of sugarcane.

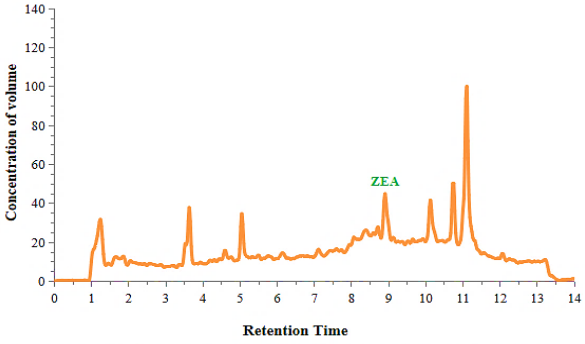
Figure.1: Detection of mycotoxin by LC/MS in *phoma* species of sugarcane

A) *P. Sorghina* (BS2-1) Species Measured 3-ACDON Peak Volume On LC-MS



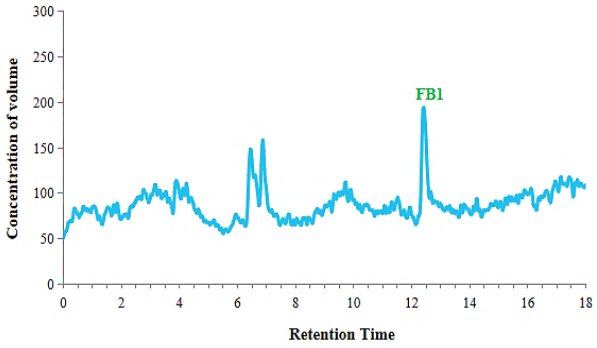
P. Sorghina (BS2-1) species identified 3-ACDON at the retention of time 4.8 minutes.

B) *P. Sorghina* (BS2-1) Species Measured ZEA Peak Volume On LC-MS



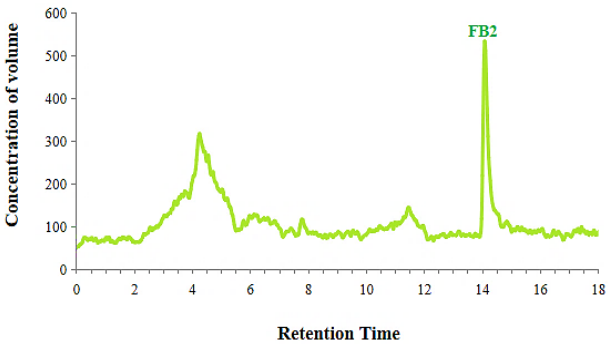
P. Sorghina (BS2-1) species identified ZEA at the retention of time 8.9 minutes.

C) *P. Sorghina* (BS2-1) Species Measured FB1 Peak Volume On LC-MS



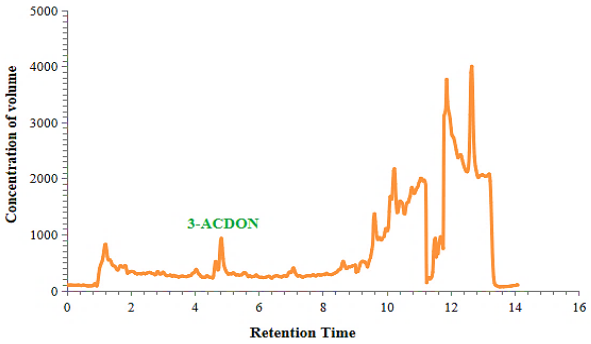
P. Sorghina (BS2-1) species identified FB1 at the retention of time 12.2 minutes.

D) *P. Sorghina* (BS2-1) Species Measured FB2 Peak Volume On LC-MS



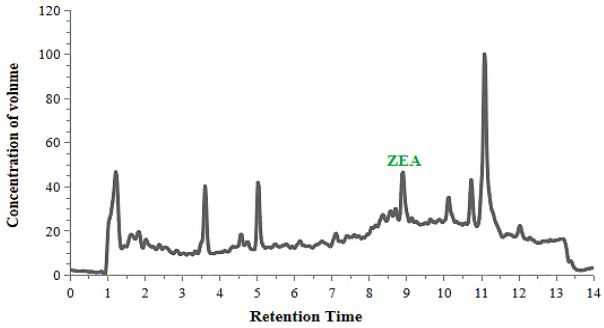
P. Sorghina (BS2-1) species identified FB2 at the retention of time 14 minutes.

E) *P. Sorghina* (BS11-1) Species Measured 3-ACDON Peak Volume On LC-MS



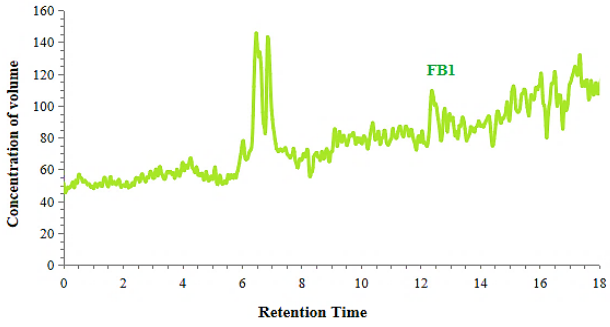
P. Sorghina (BS11-1) species identified 3-ACDON at the retention of time 4.8 minutes.

F) *P. Sorghina* (BS11-1) Species Measured ZEA Peak Volume On LC-MS



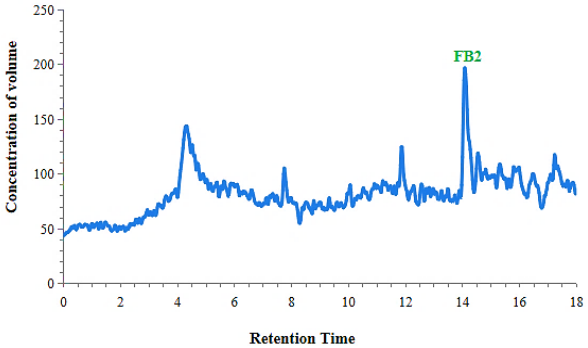
P. Sorghina (BS11-1) species identified ZEA at the retention of time 8.8 minutes.

G) *P. Sorghina* (BS11-1) Species Measured FB1 Peak Volume On LC-MS



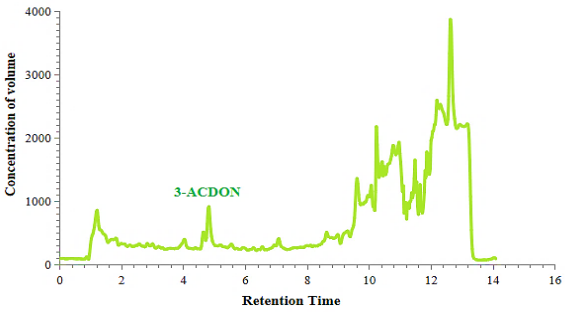
P. Sorghina (BS11-1) species identified FB1 at the retention of time 12.2 minutes.

H) *P. Sorghina* (BS11-1) Species Measured FB2 Peak Volume On LC-MS



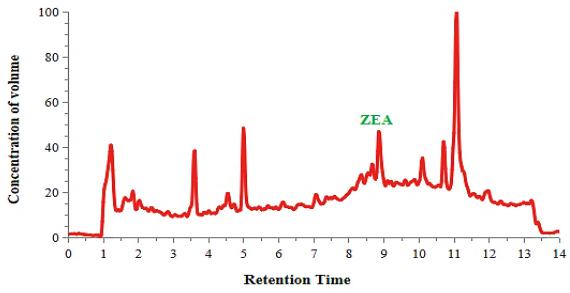
P. Sorghina (BS11-1) species identified FB2 at the retention of time 14 minutes.

I) *P. Sorghina* (BSQP) Species Measured 3-ACDON Peak Volume On LC-MS



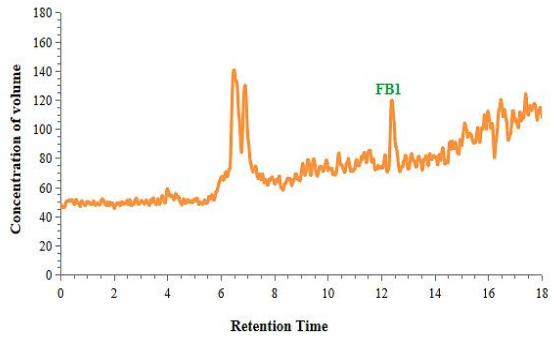
P. Sorghina (BSQP) species identified 3-ACDON at the retention of time 4.8 minutes.

J) *P. Sorghina* (BSQP) Species Measured ZEA Peak Volume On LC-MS



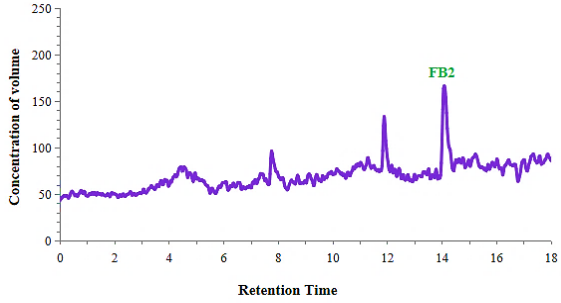
P. Sorghina (BSQP) species identified ZEA at the retention of time 8.8 minutes.

K) *P. Sorghina* (BSQP) Species Measured FB1 Peak Volume On LC-MS



P. Sorghina (BSQP) species identified FB1 at the retention of time 12.2 minutes.

L) *P. Sorghina* (BSQP) Species Measured FB2 Peak Volume On LC-MS



P. Sorghina (BSQP) species identified FB2 at the retention of time 14 minutes.

Table 1: LC-MS/MS parameters for detection and quantification of the measured *P.Sorghina* (BS2-1) species

Analytes of Toxin	Retention Time (Min)	Peak Area Value	LOD	LOQ
3-ACDON	4.8	1200	1.6	5.0
ZEA	8.8	40	4.5	13.7
FB1	12.2	200	4.1	12.6
FB2	14	550	12.4	37.7

The LC-MS/MS parameters for detection and quantification of *P.Sorghina* (BS2-1) species were identified with 3-ACDON, ZEA, FB1 and FB2. Liquid extract separations of toxins were attempted using 0.1% formic acid and acetonitrile solution in water as mobile phases. The various standard sample analyzed the concentration of volume (0.05, 0.1, 0.2 and 0.5 µg/ml) are observed in the calibration plot.

Table 2: LC-MS/MS parameters for detection and quantification of the measured *P.Sorghina* (BS11-1) species

Analytes of Toxin	Retention Time (Min)	Peak Area Value	LOD	LOQ
3-ACDON	4.8	980	1.7	5.2
ZEA	8.8	43	4.1	12.6
FB1	12.2	110	1.0	3.0
FB2	14	195	2.4	7.3

The LC-MS/MS parameters for detection and quantification of *P.Sorghina* (BS11-1) species were identified with 3-ACDON, ZEA, FB1 and FB2. Liquid extract separation of toxins were attempted using 0.1% formic acid and acetonitrile solution in water as mobile phases. The various standard sample analyzed the concentration of volume (0.05, 0.1, 0.2 and 0.5 µg/ml) are observed in the calibration plot.

Table 3: LC-MS/MS parameters for detection and quantification of the measured *P.Sorghina* (BSQP) species

Analytes of Toxin	Retention Time (Min)	Peak Area Value	LOD	LOQ
3-ACDON	4.8	960	1.6	4.9
ZEA	8.8	45	4.2	12.8
FB1	12.2	120	0.9	3.0
FB2	14	160	1.6	4.9

The LC-MS/MS parameters for detection and quantification of *P.Sorghina* (BSQP) species were identified with 3-ACDON, ZEA, FB1 and FB2. Liquid extract separations of toxins were attempted using 0.1% formic acid and acetonitrile solution in water as mobile phases. The various standard sample analyzed the concentration of volume (0.05, 0.1, 0.2 and 0.5 µg/ml) are observed in the calibration plot.

Table 4: Identified the toxin metabolites of *P.Sorghina* species analyzed by MALDI –TOF

Standard Sample	Molecular Weight (M/Z)	*P.Sorghina* (BS2- 1) Sp. sample Peak Area response of value	*P.Sorghina* (BS11-1) Sp. sample Peak Area response of value	*P.Sorghina* (BSQP) Sp. sample Peak Area response of value
DON	294.05	3247.82	87238.46	138585.84
NIV	317.11	-	7614.60	9449.13
ZEA	318.36	539684.69	734881.06	733512.69
AG1	329.07	-	71124.73	-
FB1	722.41	1089.22	-	5418.74
FB2	706.43	1422.77	-	-

The standard sample of matrix solution CHCA were achieved with 50% acetonitrile, 50% water and 0.1% TFA (trifloroacetic acid) was spotted over the dried sample. The calibration curve are observed using the concentration of volume (0.1, 0.2, 0.5 and 1.0 µl).

Figure. 2,3,4 :Detection of mycotoxin by MALDI-TOF in *phoma* sps. of sugarcane

Figure. 2: *Phoma Sorghina*(BS2-1) sample

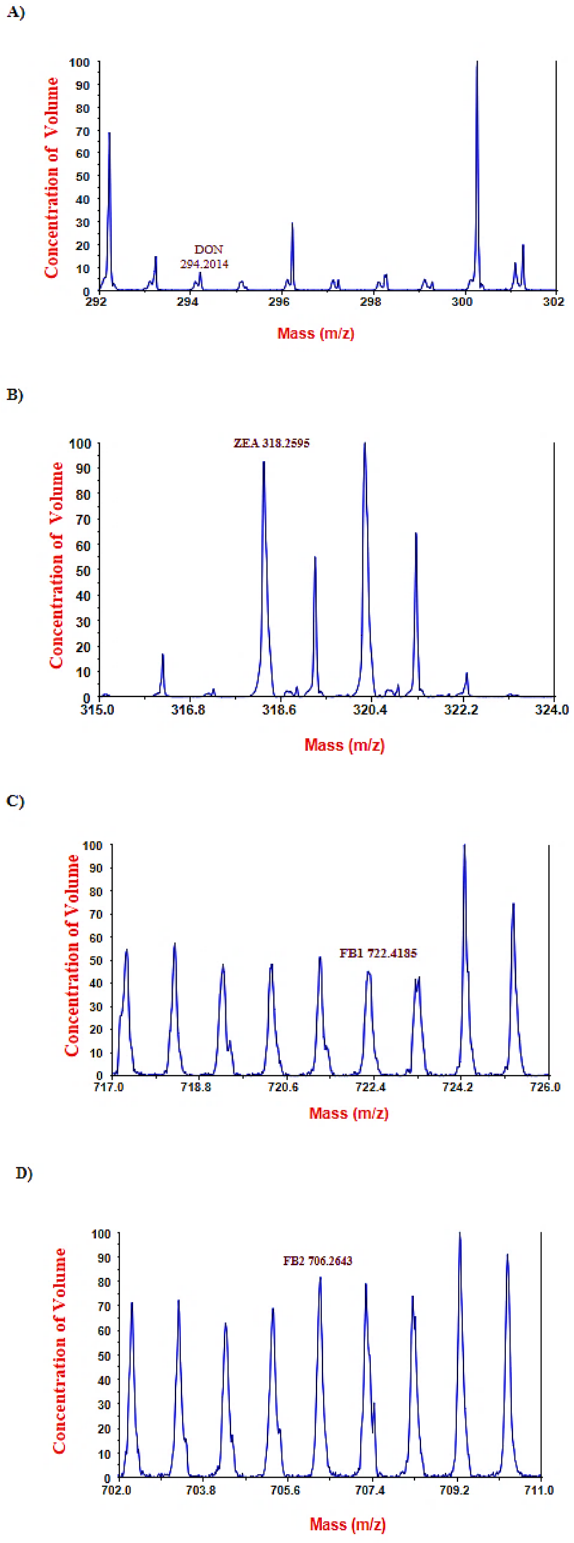


Figure 3: *Phoma Sorghina* (BS11-1) sample

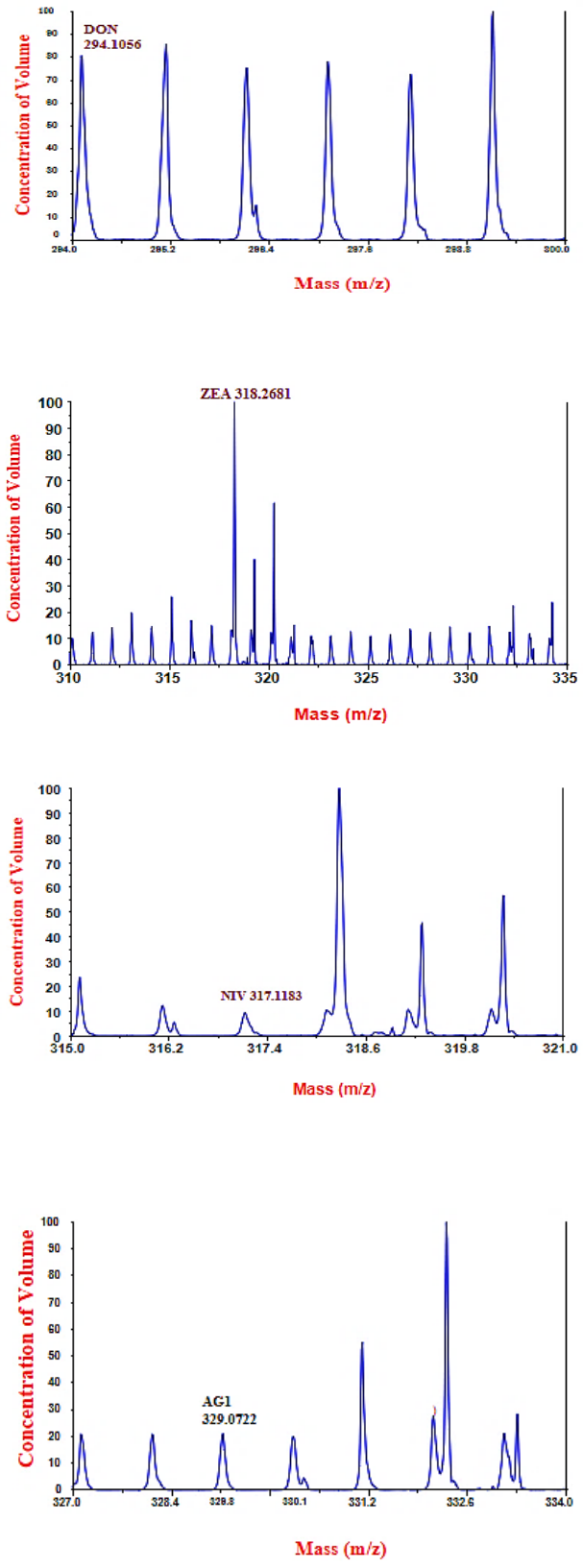
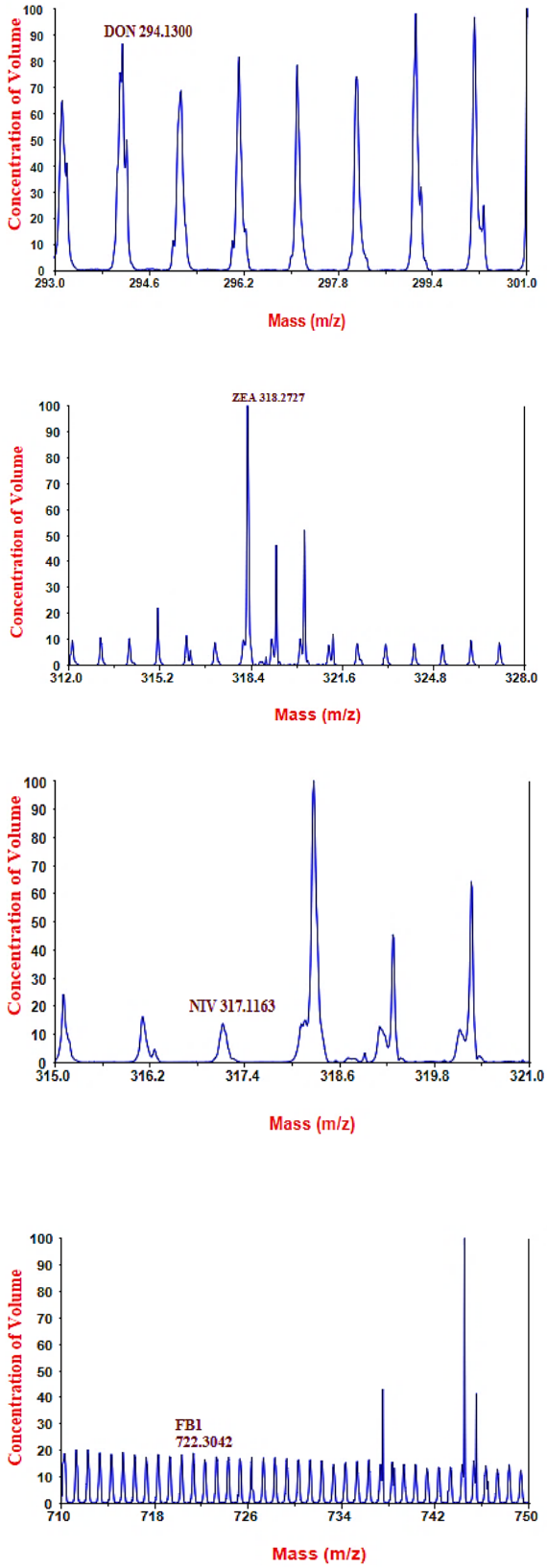


Figure 4: *Phoma Sorghina* (BSQP) sample



Mycotoxin analysis with MALDI-TOF

MALDI-TOF MS analysis of Fungi is based on specific molecular masses, which are mostly provided by ribosomal proteins. When compared to electrospray ionization, this approach has a high sensitivity but requires cleanup processes and produces less powerful ionization effects. Protonated peptides have a single charge and mass of MH+, with M indicating peptide mass and H+ indicating the proton. The analyzer identifies peptides based on their mass to charge ratio (m/z). The separated molecules are detected and data are obtained in mass spectra that visualize the relative the presence of molecules at different values for m/z. Ionization of molecules can achieved by various techniques, one of which is MALDI, which is characterized by the fact that macromolecules into a greater extent remain intact. This technique is often used in conjunction with TOF, which well suited for analysis of molecules with larger mass. The sample is transferred to an assay plate and is covered by a matrix solution, whereby a crystallline structure is formed. Then it is irradiated with pulsed laser which leads to it being vaporized and above all the matrix is excited and ionized in turn the molecules in the sample. These are then accelerated in vacuum tubes by means of an electric field.

The spectra exhibited peaks ranging between 1000 and 7,50,000 molecular mass to charge (m/z) ratio (Table 4). As shown in the Figure (2) the mass spectra of *Phoma* (BS2-1) showed distinguishing toxin peaks (3247.82, 539684.69, 1089.22, 1422.77 m/z). Four mycotoxin peaks were produced by *Phoma* (BS11-1) species with peaks 87238.46, 7614.60, 734881.06, 71124.73 m/z exclusively identified for *P. sorghina* (Figure 3). The mass spectra produced for *Phoma* (BSQP) species 138585.84 (DON), 9449.13(NIV), 733512.69 (Zea), 5418.74 (FB1) uniquely associated with *P. sorghina* (Figure 4). The extraction protocol resulted in the generation of spectra to distinguish among *Phoma* species.

Overall, a downward trend was observed in the peak ion count as the aqueous component increased. This was expected, as the vapor pressure of water is considerably higher than that of methanol. However, introducing an aqueous component greatly increased the number of signals observed in subsequent mass spectra.

Conclusion

This work addresses many analytical method challenges. A sensitive and accurate LC-ESI-MS /MS method for the simultaneous detection and quantification of mycotoxins types with a LOD and LOQ was developed and reported. In addition to, a quick, easy and low cost extraction method was investigated, and was employed with different matrices. Presented data showed that it is possible to detect mycotoxins with using MALDI-TOF operating in linear ion positive mode in very short time. The methodlogy 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 mycotoxin. A new method for the detection of mycotoxins by MALDI-TOF MS was developed. Different solid phase extraction (SPE) cleanup methods were tried to optimize the purification of sugarcane matrix, and an optimal extraction method was designed to recover the mycotoxin toxins. In addition, various MALDI matrices were examined and α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix provide good reproducibility for all mycotoxin toxins. The extraction emphasis a novel extraction process, that reduced the cost, time and effort consumed in the extraction process of mycotoxins. This may allow ease of analysis in order to obtain additional data about the occurrence of mycotoxins in sugarcane. This extraction method could be used with broader range of mycotoxins and matrices without the need for specialized extraction columns.

Moreover, the data generated in this study provide valuable information concerning mycotoxin levels in sugarcane. The ionization techniques were shown to be useful for the identification of closely related *phoma* species. Some *phoma* strains generate high levels of mycotoxin, indicating that these toxigenic strains can produce many toxins such as fumonisins, 3- CDON, 15- ACDON, DON, NIV, AG1 and ZEA. As a result, *Phoma sorghina* are frequently detected as contaminants of sugarcane. Therefore accurate and rapid identification of mycotoxicogenic fungi contaminating economically important crops is vital, and could contribute to the application of appropriate disease management strategies.

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