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Analysis of mycotoxins using laser desorption/ionization mass spectrometry with infrared pulsed fiber laser-produced silver-109-nanoparticles

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ABSTRACT

Mycotoxins are biologically active secondary metabolites produced by specific fungi, known for their structural diversity and high chemical stability. Their synthesis is influenced by environmental factors, leading to regional variability. Mycotoxins, such as citrinin, ochratoxin A, patulin, roquefortine C, and sterigmatocystin, pose significant health risks to humans and animals. Common detection methods include chromatography and ELISA, with liquid chromatography-tandem mass spectrometry (LC-MS/MS) being the gold standard for its sensitivity and selectivity. Alternative methods, like laser desorption/ionization mass spectrometry (LDI-MS) offer unique advantages but face challenges like high background noise and low ionization efficiency for nonpolar compounds. This study concerns the detection and quantification of mycotoxins using silver-109 nanoparticles on a steel plate, produced via a novel pulsed fiber laser method with a 2D galvanometer scanner. Comparative analysis of manual LDI-MS and semi-automatic LDI-MSI for sterigmatocystin quantification highlights their effectiveness, providing a promising alternative for accurate mycotoxin analysis.

Keywords: mycotoxins, low molecular weight compounds, silver-109 nanoparticles, LDI-MS, LDI-MSI

1. Introduction

Mycotoxins are a diverse group of biologically active secondary metabolites synthesized by specific fungi [1]. These low-molecular-weight compounds exhibit significant structural diversity and are characterized by high chemical and thermal stability. Their production is predominantly influenced by environmental factors such as temperature, humidity, and substrate availability leading to geographical variability in their occurrence. In the literature considerable attention is devoted to the presence of toxic mould metabolites in food products and animal feed [1, 2]. Nonetheless, mycotoxin exposure remains a global concern due to the international food trade [3]. Mycotoxins pose a significant threat to both human and animal health with exposure potentially resulting in acute or chronic diseases. The most prominent mycotoxins are produced by fungi of the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria*. Key examples include citrinin, ochratoxin A, patulin, roquefortine C, and sterigmatocystin, each associated with distinct toxicological profiles and considerable health risks [1].

Chandravarnan et al. showed that chromatography and ELISA are the most frequently employed methods in studies. While ELISA is a simple and rapid technique, chromatographic methods are more reliable and sensitive due to their ability to incorporate sample extraction and clean-up steps, such as the use of immunoaffinity columns [3]. Currently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) including multi-mycotoxin approaches is recognized as an accurate, highly selective, and sensitive method for mycotoxin determination [4]. An alternative to LC-MS/MS methods for determining fungal secondary metabolites is the use of techniques based on soft ionization and laser desorption/ionization mass spectrometry (LDI-MS), including matrix-assisted laser desorption/ionization (MALDI) and surface-assisted laser desorption/ionization (SALDI) [2]. MALDI is particularly valuable for analysing medium- to high-molecular-weight compounds (such as proteins

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and lipids) and is useful in identifying clinically relevant fungal species. However, traditional MALDI has certain limitations, such as a high chemical background in the low mass region (*m/z* 1000), the "sweet spot" effect, and low ionization efficiency for low-polarity compounds. These drawbacks are addressed in LDI methods, where a steel target coated with nanoparticles is used instead of a matrix [5-7].

This article presents the detection and quantification results of mycotoxins on a steel plate coated with chemically pure silver-109 nanoparticles, produced using a novel method with a 1064 nm pulsed fiber laser and a 2D galvanometer scanner. It also compares manual LDI-MS and semi-automatic LDI-MSI for sterigmatocystin quantification, along with a discussion of the results and a comparison with various SALDI and MALDI methods previously used.

2. Experimental

2.1. Materials

All mycotoxins analytical standards were purchased from Apollo Scientific. Steel targets were machined from H17 (1.4016) stainless steel. Before the LDI-MS and MS imaging experiments steel targets were cleaned through soaking in boiling solvents: toluene (3×100 mL, each plate for 30 s), chloroform (3×100 mL, each plate for 30 s), acetonitrile (3×100 mL, each plate for 30 s) and deionized water (3×100 mL, each plate for 30 s). Every plate was dried in high vacuum (ca. 0.01 mbar, 24 h). All solvents were of LC-MS quality, except for water (18 MΩ·cm water produced locally).

2.2. Methods

2.2.1 PFL 2D GS Laser Generated Nanomaterial (LGN) of silver-109 nanoparticles and nebulization of ^{109}AgNPs suspension

Silver-109 nanoparticles (^{109}AgNPs) were generated with pulsed fiber laser (PFL) 2D galvoscanner (2D GS) laser synthesis in solution (LASiS) as described in our previous publication [6]. The description of the nebulization step of the generated nanoparticle suspension was also described in previous publications [5, 6].

2.2.2 Sample preparation and handling

All mycotoxin standards (citrinin, patulin, sterigmatocystin, roquefortine C, and ochratoxin A) were dissolved in acetonitrile to prepare a mixture. The concentrations of the individual compounds in the mixture were 100 μg/mL for citrinin and patulin, and 20 μg/mL for sterigmatocystin, roquefortine C, and ochratoxin A. Lower concentrations were obtained by tenfold dilution of the higher concentrations. Aliquots of 0.5 μL of the mycotoxin mixtures were directly placed on the target plate. After air drying, the target was sprayed with a suspension of ^{109}AgNPs.

2.2.3 LDI mass spectrometry

LDI-ToF mass spectrometry experiments were performed in reflectron mode using Bruker Autoflex Speed time-of-flight mass spectrometer equipped with a SmartBeam II laser (352 nm). Laser impulse energy was approx. 90-140 μJ, laser repetition rate 1000 Hz. The total number of laser shots was 4000 for each spot. This amount of laser shots was divided into four symmetrically positioned points laying in distance of ca. 1/3 of spot radius from its center. At each point 1000 laser shots were made with default random walk applied (random points with 50 laser shots). Measurement range was *m/z* 80-1500. Suppression was turned on typically for ions of *m/z* lower than 80. Reflector voltages used were 21 kV (the first) and 9.55 kV (the second). The data was calibrated and analysed with FlexAnalysis (version 3.3) using centroid calibration model. Mass calibration (enhanced cubic calibration based on 8-9 calibration points) was performed using internal standards (silver-109 ions and clusters from $^{109}Ag^+$ to $^{109}Ag_9^+$).

2.2.4 LDI mass spectrometry imaging

Measurements were performed using a Bruker Autoflex Speed time-of-flight mass spectrometer in reflectron positive mode. The apparatus was equipped with a SmartBeam II 1000 Hz 352 nm laser. Laser impulse energy was approximately 100−120 μJ, laser repetition rate was 1000 Hz, and deflection was set on *m/z* lower than 80 Da. The *m/z* range was 80−1500 and spatial resolution 600 × 600 μm. The imaging experiments were made with 2000 laser shots per individual spot with a default random walk applied (FlexImaging 4.0). All spectra were pre-calibrated (cubic calibration function) with the use of silver-109 ions ($^{109}Ag^+$ to $^{109}Ag_9^+$) as internal standard. The first accelerating voltage was held at 19 kV, and the second ion source voltage was held at 16.7 kV. Reflector voltages used were 21 kV (the first) and 9.55 kV (the second). All of the shown imaging pictures are for ± 0.05% *m/z* window. MSI experiments were performed on all spots of mycotoxin.

3. Results and discussion

Monoisotopic silver-109 nanoparticles (^{109}AgNPs) were synthesized using the pulsed fiber laser (PFL) laser-generated nanomaterial (LGN) technique with a 2D galvanometer scanner (2D GS) and nebulization. Our recent publications have detailed the properties and applications of ^{109}AgNPs in LDI-MS and MSI analyses [6]. These studies include results from UV-Vis spectrophotometry, dynamic light scattering (DLS), and high-resolution scanning electron microscopy (HR SEM) analyses, which were used to characterize the suspensions of ^{109}AgNPs. These analyses confirmed the shape and size of the nanoparticles produced by the PFL 2D LGN method. This innovative technique significantly reduces the preparation time for targets from 48 hours (as required for chemical synthesis) to just a few minutes. Additionally, it is cost-effective, requiring no reducing or stabilizing agents, and allows the reuse of metal foil.

The LGN method generates suspensions with high chemical purity, enabling simplified mass spectra with low chemical background. Recent publications have also demonstrated the quantitative analysis capabilities of ^{109}AgNPs synthesized *via* LGN for amino acids, carboxylic acids, 3-hydroxycarboxylic acids, and steroid hormones, highlighting their versatility and efficacy in various analytical applications [8-11].

3.1. Detection mycotoxins using ^{109}AgNPs LGN LDI MS

LDI-MS measurements using monoisotopic silver-109 nanoparticles produced *via* the PFL 2D GS LGN method were performed on a mycotoxin mixture. The mixture contained individual compound concentrations of 100 µg/mL for citrinin and patulin, and 20 µg/mL for sterigmatocystin, roquefortine C, and ochratoxin A.

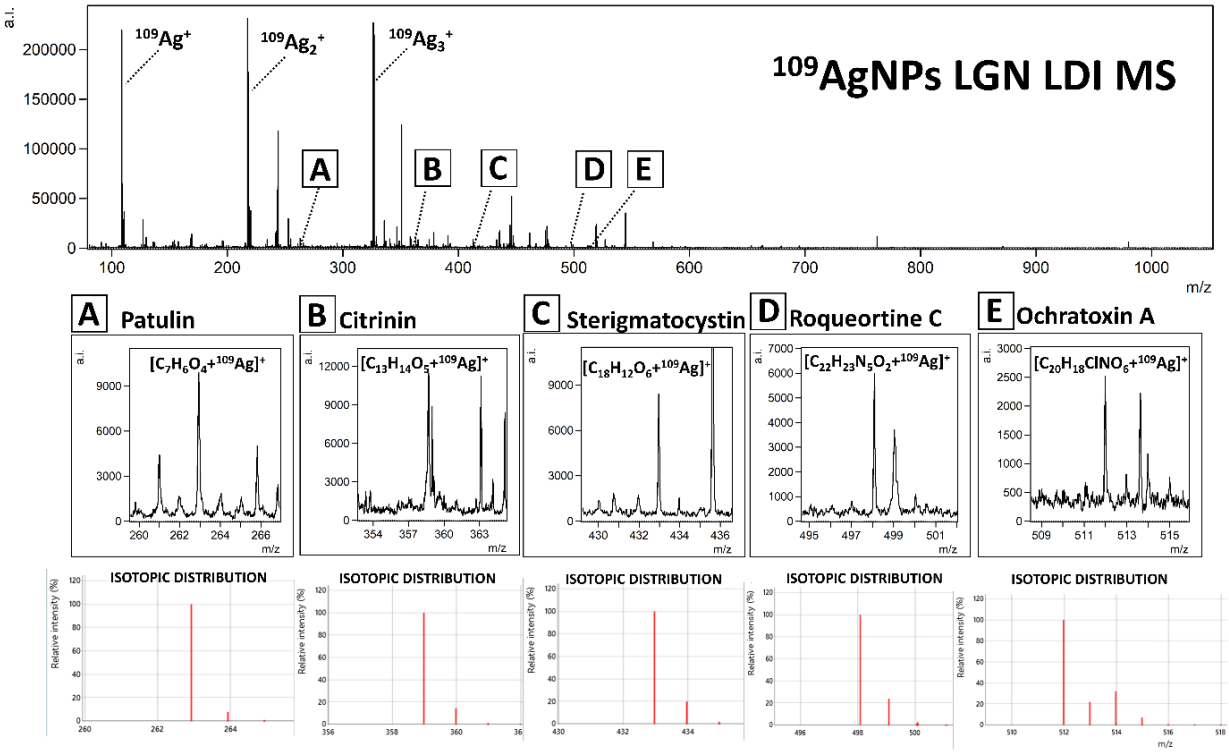

Figure 1. The mass spectrum of the tested mycotoxin mixture obtained *via* manual LDI-MS measurements using ^{109}AgNPs generated by the PFL 2D GS LGN method. The sample contained individual compound concentrations of 100 µg/mL for patulin (A) and citrinin (B), and 20 µg/mL for sterigmatocystin (C), roquefortine C (D), and ochratoxin A (E). The isotopic distributions of the identified ions are shown at the bottom.

The LDI-MS spectrum of ^{109}AgNPs produced by PFL 2D GS LGN and deposited onto the surface of a stainless-steel target plate using nebulization is presented in Fig. 1. The mass spectrum, recorded within the *m/z* range of 80–1500, includes both analyte signals and silver-109 ion peaks corresponding to compositions from $^{109}Ag^+$ to $^{109}Ag_{10}^+$. The analysed compounds included patulin, citrinin, sterigmatocystin, roquefortine C, and ochratoxin A (Fig. 1). All these compounds were observed as ^{109}Ag-adducts. Notably, no signals of $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ adducts were detected, as illustrated in Fig. 1. Additionally, matching theoretical isotopic patterns for each ion is presented to the corresponding spectrum. The analysis revealed the detection of analytes as silver adducts

with varying signal intensities and signal-to-noise (S/N) ratios. Patulin was identified as the adduct $[C_7H_6O_4+^{109}Ag]^+$ with a signal intensity of $9.5\cdot10^3$ and an S/N ratio of 18.3. Citrinin was observed in the spectrum as $[C_{13}H_{14}O_5+^{109}Ag]^+$, showing a signal intensity of $1.1\cdot10^4$ and an S/N ratio of 15.8. Sterigmatocystin was detected as the adduct $[C_{18}H_{12}O_6+^{109}Ag]^+$ at *m/z* 432.9676, with a signal intensity of $7.9\cdot10^3$ and an S/N ratio of 18.8. Roquefortine C was observed as $[C_{22}H_{23}N_5O_2+^{109}Ag]^+$ at *m/z* 498.0880, with a mass error of 2.8 ppm, a signal intensity of $5.7\cdot10^3$, and an S/N value of 19.0. Lastly, ochratoxin A was identified as $[C_{20}H_{18}ClNO_6+^{109}Ag]^+$, with a signal intensity of $2.2\cdot10^3$ and an S/N ratio of 7.9.

In 2021, Szulc et al. used silver-109 nanoparticle-assisted laser desorption/ionization mass spectrometry (^{109}AgNPET SALDI MS) to detect mycotoxins on building materials. The method enabled the detection of, among others: patulin, citrinin, sterigmatocystin and roquefortine C [2].

3.2. ^{109}AgNPs LGN LDI MS and ^{109}AgNPs LGN LDI MSI for sterigmatocystin quantification

LDI-MS and LDI-MSI measurements using monoisotopic silver-109 nanoparticles produced *via* PFL 2D GS LGN method were conducted on a mycotoxin mixture. Within the mixture, sterigmatocystin was analysed across a wide concentration range from 20 µg/mL to 0.2 ng/mL, representing a 100,000-fold variation in concentration. The limit of detection (LOD) was determined using signal-to-noise (S/N) ratios obtained from spectra of the lowest concentration samples containing detectable signals.

Each sample was deposited on the target plate in a 0.5-µL volume, corresponding to 10 ng to 1000 fg of sterigmatocystin per measurement spot. A regression analysis of signal intensity versus concentration was performed, yielding a high correlation with an R^2 value exceeding 0.99. This demonstrates the effectiveness of MSI for quantitative analysis. Notably, these regressions were achieved over an exceptionally broad concentration range, spanning six tested concentrations. These findings demonstrate the capability of MSI for accurate and reliable mycotoxin quantification, even at extremely low concentrations, underscoring its potential as a powerful tool for analytical applications.

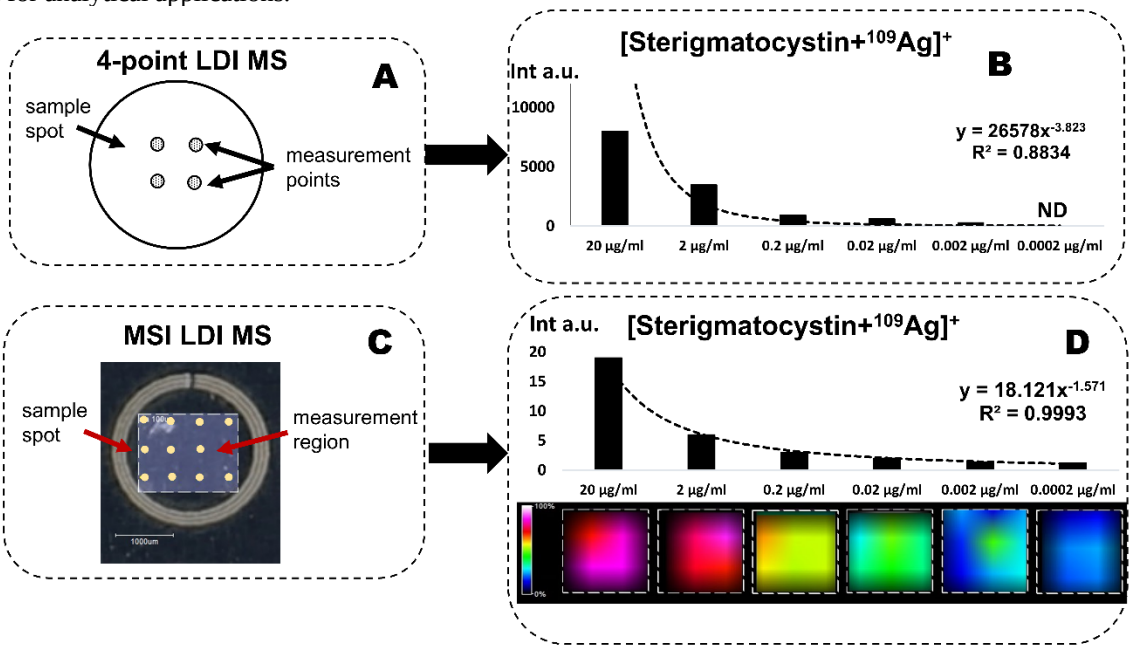

Figure 2. Panel A shows scheme of manual LDI-MS measurement with 4 random measure points. Column charts (B and D for MS and MSI, respectively) present quantitative results for [Sterigmatocystin + ^{109}Ag]$^+$ ion as signal intensity versus concentration. Panel C presents photograph of sample spot on target plate and measurement region for LDI-MSI with grid of measurement points. ND – not detected.

The Fig. 2 presents the results of LDI MS and MSI analysis for sterigmatocystin with ^{109}AgNPs PFL 2D GS laser generated nanomaterial. Manually made measurements were taken at four random locations, which is presented in Fig. 2A. Sterigmatocystin was found in spectra mainly as silver-109 adduct of $[C_{18}H_{12}O_6+ {}^{109}Ag]^+$ ion formula. Intensities of sterigmatocistine-silver-109 adduct peaks were of $7.9\cdot10^3$ for the highest concentration, $3.5\cdot10^3$ for 2 µg/mL, 921 for 0.2 µg/mL, 600 for 20 ng/mL and 275 for 2 ng/mL. At the lowest concentration, sterigmatocystin was not detected, which is presented in Fig. 2B.

Table 1. Comparison of regression function and R^2 values for manual MS and semi-automatic MSI measurements in the quantification of sterigmatocystin.

Regression function	LDI-MS		LDI-MSI	
	Regression equation	R^2	Regression equation	R^2
Exponential	Int = 80165 $e^{-1.513\ c}$	0.9595	Int = 20.752$e^{-0.531\ c}$	0.9104
Linear	Int = -1424.1 c + 7193.3	0.7411	Int = -2.9657c + 15.813	0.6515
Power	Int = 26578 $c^{-3.823}$	0.8834	**Int = 18.121 $c^{-1.571}$**	**0.9993**
Polynomial	Int = 538.36 c^2 – 5192.6 c + 12218	0.9671	Int = 1.3143 c^2 – 12.166 c + 28.08	0.9244

A comparison of the results from fitting the regression function to experimental data for manual MS and semi-automatic MSI measurements is presented in Table 1. Regression analysis of this data provided trendlines with R^2 values of 0.7411 for LDI-MS and 0.6515 for LDI-MSI. However, the best fit for LDI-MS was obtained using polynomial trendline, yielding an R^2 value of 0.9671. Mass spectrometry imaging utilizes a grid or raster of measurement points with specified resolution, as shown in Fig. 2C. The application of MSI for analysis the same samples allowed detection of sterigmatocystine-silver-109 adduct signals in all concentrations (Fig. 2D). The best results were obtained using power trendline (R^2 equals 0.9993), which outperformed the manual measurements. As shown in Fig. 2D, ion images reveal that the studied sterigmatocystin is nonuniformly deposited across all the analysed sample spots. The optimal fit for sterigmatocystin analysis by MSI was obtained for the data set covering concentrations from 20 µg/ml to 0.2 ng/ml. The limit of detection, based on S/N ratio of 3 was found to be 0.68 ng (2.09 pmol, 4.2 µM) of sterigmatocytin per measured spot for both LDI-MS and LDI-MSI.

In 2021, Szulc et al. used ^{109}AgNPET SALDI MS and MALDI MS methods to determine mycotoxin concentrations on building materials. The lower limit of detection for sterigmatocystin with ^{109}AgNPET was shown to be 47.1 µM, whereas MALDI measurements did not detect sterigmatocystin [2].

3.4. Conclusions

The use of monoisotopic silver nanoparticles generated by the PFL 2D GS LGN method for manual LDI-MS and semi-automatic LDI-MSI enabled the detection and quantification of mycotoxins. The R^2 values obtained indicate that ^{109}AgNPs, when used in MSI measurement mode, provide significantly better quantification compared to LDI-MS manual measurements. Ion images obtained from MSI experiments revealed highly non-uniform analyte deposition, highlighting the necessity of semi-automatic, multi-pixel MSI as a modern requirement rather than a mere improvement.

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