Received: 5 July 2019 | Revised: 24 August 2019 | Accepted: 9 September 2019

DOI: 10.1002/rcm.8592

RESEARCH ARTICLE

WILEY Rapid Communications in Mass Spectrometry

In situ analysis of oxytetracycline tablets based on matrix-assisted laser desorption/ionization mass spectrometry imaging

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Funding information
Key Laboratory of Biomedical Effects of Nanomaterials and Nanosafety, CAS, Grant/Award Number: No: NSKF201813; Natural Science Foundation of Shandong Province, Grant/Award Number: ZR2017MB011; Youth Ph.D. Cooperation Funds of Qilu University of Technology (Shandong Academy of Sciences, Grant/Award Number: 2018BSHZ0029; Key Research and Development Program of Shandong Province, Grant/Award Number: 2019GSF111001, 2019GSF111009; Youth Science Funds of Shandong Academy of Sciences, Grant/Award Number: 2019QN009

Rationale: A thorough understanding of the content and distribution of active ingredients in pharmaceuticals is essential for drug efficacy and safety. Technological advancements in mass spectrometry imaging present an opportunity for methodological innovation by providing qualification and quantification analysis, as well as spatial information, in the same assay, which has great potential for applications in the rapid analysis and quality control of drugs.

Methods: Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) was employed to directly analyze oxytetracycline tablets in order to map the distribution of the active constituent within the whole tablet. Quantitative analysis was capable of differentiating tablets containing various doses of the active pharmaceutical ingredient.

Results: To establish the methodology, detailed factors that influence matrix spraying and spatial resolution during sample preparation and the data acquisition process were optimized systematically. Quantitative analysis could differentiate the tablets containing various doses of the active compound. The proposed method was successfully applied to analyze real commercial tablets.

Conclusions: The developed method could successfully achieve the spatial location of oxytetracycline in actual tablet samples. These results could contribute to pharmaceutical tracing technology, especially the formulation process of tablets, which is helpful for monitoring the quality of pharmaceutical products and guaranteeing drug security.

1 | INTRODUCTION

Quality control of pharmaceutical products during the manufacturing process is of vital importance to the pharmaceutical industry for ensuring drug efficacy and human safety. There is an urgent need to develop rapid, real-time and efficient analytical methods to guarantee the quality attributes of drug products, including identification, content uniformity, microbial limits, and water content.[1] Monitoring the content uniformity of the active pharmaceutical ingredients (APIs) should ensure that the treatment effect remains unchanged, especially for solid dosage forms of drugs, *viz.* tablets.[2,3]

Since the US Food and Drug Administration (FDA, Silver Spring, MD, USA) issued its *Guidance for Industry*, which initiated process analytical technology (PAT), various analytical technologies have been developed to guarantee the quality of the finished product. Chromatography/tandem mass spectrometry techniques are widely used for qualitative and quantitative detection, but they may be time-consuming and information limited.[4,5] In addition, imaging techniques have been exploited to examine tablet constitution, and these can provide both spectral and spatial information in the same assay. Raman and near-infrared (NIR) chemical imaging are powerful tools for manufacturing analysis owing to the procedures

being free of sample preparation, minimally time-consuming and noninvasive.[6-9] However, detailed compositional information about unknown compounds cannot be obtained. The limited structure recognition capability and complicated data processing procedures of the techniques have restricted the wide usage of these approaches.

Mass spectrometry imaging (MSI), on the other hand, is a more intuitive and time-saving method that enables component analysis at high spatial and spectral resolution. The key of MSI technology lies in "*in situ*" or quasi-"*in situ*" ionization to ensure accurate spatial distribution of molecules.[10,11] At present, widely investigated ionization techniques used to provide MSI include secondary ion mass spectrometry (SIMS),[12] desorption electrospray ionization (DESI),[13] and matrix-assisted laser desorption/ionization (MALDI).[14] SIMS is a surface-sensitive technique that utilizes a pulsed ion beam to generate secondary ions, and it has been extensively used in materials science and has recently been applied for drug crystal characterization.[15,16] DESI is an ambient soft ionization technique with minimal restrictions on sample preparation. However, the impact of the DESI atomizing gas on the surface of the sample may cause ion sputtering and migration, and the ion collection efficiency and system sensitivity are greatly affected by the structural parameters of the device.[17]

MALDI is a soft ionization technique widely used for a broad range of analytes from protein to lipids. Matrices with strong absorbance at the laser wavelength, such as 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA), are desorbed and ionized with the analytes under laser irradiation.[18] The application of MALDI-MSI has mainly been focused on biological tissue, while studies on pharmaceutical products using MALDI-MSI are still uncommon. Clench et al demonstrated the first application of MALDI-MSI for imaging drug distribution.[19] Notwithstanding that these authors successfully mapped the drug distribution and provided semiquantitative information, more details could be obtained or improved during MALDI-MSI methodology development including the identification of fragmentation patterns in tandem mass spectrometry (MS/MS) mode, the selection and optimization of methodological parameters and, more importantly, investigating the practicality of rapid detection.

Herein, we describe a fast *in situ* analysis method to map the API distribution of a whole tablet using MALDI-MSI. Tablets of oxytetracycline, a common broad-spectrum antibiotic widely used in food animals, were studied. Detailed factors in the sample preparation and data acquisition process, including matrix selection, delivery solvent, spraying rate and matrix concentration together with laser power, imaging raster and laser profiles, were optimized systematically. Quantitative analysis was feasible and allowed us to differentiate tablets containing various doses of the API. The proposed method could successfully identify the spatial location of oxytetracycline in actual tablet samples. This method could be helpful for monitoring the quality of pharmaceutical products by providing important information on the surface distribution and content uniformity of the API on the tablet.

2 | EXPERIMENTAL

2.1 | Materials and reagents

2,5-Dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-CHCA), 9-aminoacridine (9-AA) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Gillingham, UK). HPLC-grade methanol, acetonitrile and water were purchased from Fisher Scientific (Loughborough, UK). The oxytetracycline hydrochloride (≥96.0%) standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The 0.25 g tablets of oxytetracycline were purchased from regular drug retailers in the local market. Customized tablets containing 0.05, 0.1, 0.15, 0.20, and 0.25 g oxytetracycline were manufactured to imitate the ionization background of the commercial tablets. All chemicals were used without further purification.

2.2 | Sample preparation

Matrix solutions of α-CHCA, DHB and 9-AA were prepared at a concentration of 40 mg mL^{-1} in 50% acetonitrile containing 0.1% TFA. A dilution series of oxytetracycline hydrochloride was prepared from stock solutions in methanol, with concentrations ranging from 5 μg mL^{-1} to 200 μg mL^{-1}. Tablet sections of approximately 1-mm thickness were obtained using a tablet cutter (Rongka, Guangzhou, China). The tablet section was then transferred to an indium tin oxide (ITO) glass surface which was pre-coated with polyacrylate as an adhesive.[20] The flattened surface was then fixed onto ITO glass and delivered to a TM-sprayer for matrix deposition.

2.3 | Instrumentation and conditions

An automated TM-sprayer (HTX Technologies, Chapel Hill, NC, USA) was used for the deposition of matrix and oxytetracycline standards on the tablets. For spraying, a constant flow of N_2 was transported jointly with the matrix solution at a pressure of 10 psi. The temperature of N_2 was set at 80°C for the matrix and 30°C for the standard. An Azura P 4.1S pump (Knauer, Berlin, Germany) was used for solvent delivery at a flow rate of 0.075 mL min^{-1}. A rapifleX MALDI Tissuetyper mass spectrometry system (Bruker Daltonik, Bremen, Germany) was used for the imaging experiments, with a Smartbeam 3D Nd:YAG laser pulsed at 355 nm. The imaging experiments were performed in positive-ion mode for DHB and CHCA and in negative-ion mode for 9-AA over a mass range of *m/z* 100–700. The detailed parameters were as follows: Smartbeam parameter: M5; scan range: 30 μm; resulting field size: 100 × 100 μm; laser shots: 2000; sampling rate: 1.25 GS s^{-1}; acceleration voltage: ±20 kV; vacuum pressure: 3–5 × 10^{-7} mbar; and pulsed ion extraction delay: 100 ns. MS/MS measurements were performed using flexControl 4.0 software (Bruker Daltonik) with the following parameters: ion source voltage: 20 kV; laser shots: 2000; MS/MS pulse voltage: 2.65 kV; lens voltage: 18.3 kV; pulsed ion extraction voltage: 0.55 kV; Reflector 1 voltage: 23.80 kV; Reflector

2 voltage: 1.79 kV; and Reflector 3 voltage: 9.85 kV. A calibration using the matrix peaks was performed before each MALDI experiment. A QuanTOF instrument (Intellibio, Qingdao, China) was also employed to test the conductivity effect with a grounded target plate. The experiments were performed in positive-ion mode over a mass range of *m/z* 100–1000. The detailed parameters were as follows: acceleration voltage: 19 kV; laser pulse energy: 5.0 μJ; laser pulse frequency: 4000 Hz; and spatial resolution: 50 × 50 μm. The morphology of the matrix film on the tablets was observed using a Smartzoom 5 digital microscope (Zeiss, Oberkochen, Germany). To compare the results of the quantification analysis, the standard and tablet samples were also analyzed using a 1200 high-performance liquid chromatography (HPLC) tandem 6410B triple quadrupole mass spectrometer system (Agilent, Santa Clara, CA, USA). An Accucore C18 column (2.1 × 100 mm, 2.6 μm; Thermo, Madison, WI, USA) was employed, with water containing 0.1% formic acid as solvent A and acetonitrile as solvent B. The flow gradient was set as follows: 0–0.5 min: 10% B; 0.5–4 min: 40% B; 4.1–5.5 min: 10% B. The flow rate was set to 0.4 mL min^{-1}. Mass spectrometry was performed in the positive-ion mode.

2.4 | Data analysis

The MS/MS data were analyzed by FlexAnalysis 4.0 software and the obtained images were analyzed by FlexImaging 5.0 software (both from Bruker Daltonik). The total ion count (TIC) was used to normalize all images. Masses were selected with a mass-selection window width corresponding to a mass ratio of ±0.025%.

3 | RESULTS AND DISCUSSION

3.1 | Workflow and model tablet

MALDI-MSI has been primarily used for tissue-based analysis in biological and clinical research.[21] The principal process for MALDI-MSI usually contains four steps: (1) tissue preparation, which represents specimen cutting, optional staining, on-tissue digestion and derivatization; (2) matrix coating by a manual or automated sprayer or sublimation; (3) image acquisition under irradiation on matrix crystals with the pulsed laser; and (4) data analysis using software programs.[22] In our MALDI-MSI workflow for tablet analysis similar procedures were performed, as shown in Figure 1. A two-step spray procedure was employed for the model tablet preparation and matrix spray stages. To establish the analytical method applicable for the actual tablet of oxytetracycline, we first built a model tablet by spraying the oxytetracycline standard onto a blank tablet to ensure the homogeneous distribution of the API. The standard sprayed on the blank tablet should serve as the indicator of any imaging effect influenced by the sample preparation. First, the blank tablets were sliced into sections approximately 1 mm thick using a tablet cutter. The obtained sections were then mounted on ITO glass with polyacrylate as an adhesive. After the deposition of oxytetracycline, the model tablet was sprayed with the matrix. Shortly afterwards, the ITO glass loaded with the tablet section was evacuated and was then ready for MALDI-MSI analysis.

3.2 | Selection of matrix

To achieve optimal image acquisition, several details should be investigated during the above preparation process. Since the tablet analysis avoids specimen cutting, matrix selection becomes the primary problem for the co-crystallization of target molecules. 2,5-Dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) are the most commonly used matrices for small molecules. In addition, 9-aminoacryidine (9-AA) has been developed as a matrix for low molecular weight compounds in negative-ion mode MALDI.[23] Thus, DHB and CHCA in positive ion mode and 9-AA in negative ion mode were selected as matrices to be investigated in this study, with the model oxytetracycline-containing tablets being sprayed with each of the selected matrices. We did not obtain a spectrum for oxytetracycline in negative-ion mode with 9-AA or for CHCA in positive-ion mode (data not shown); thus, these matrices were not considered further.

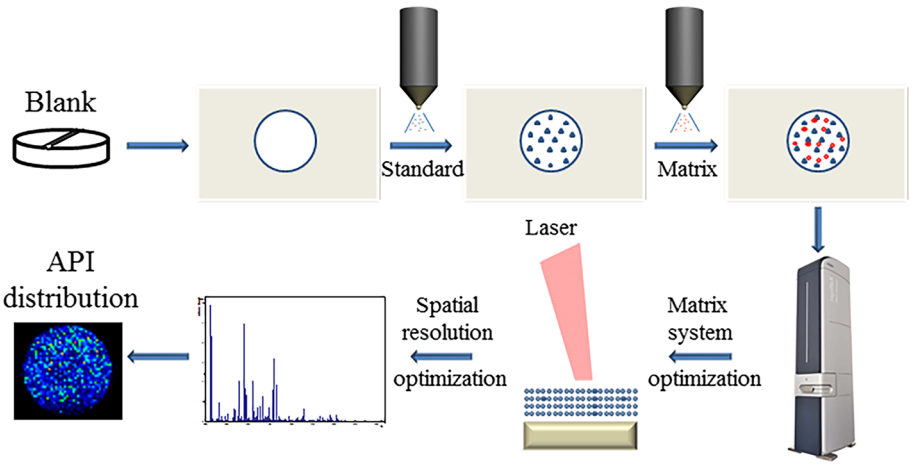

FIGURE 1 Scheme of MALDI-MSI workflow for tablet analysis [Color figure can be viewed at wileyonlinelibrary.com]

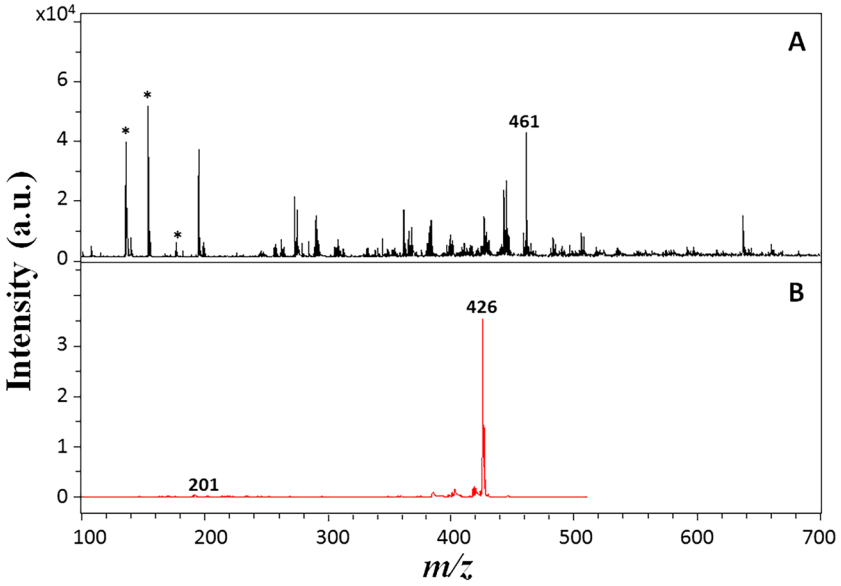

FIGURE 2 A, Positive ion MALDI MS spectrum of oxytetracycline tablet using DHB matrix. The peaks for DHB ($[M + H - H_2O]^+$ at *m/z* 137.24, $[M + H]^+$ at *m/z* 155.24 and $[M + Na]^+$ at *m/z* 177.26) are labelled with asterisks. B, MS/MS spectrum of protonated oxytetracycline at *m/z* 461 [Color figure can be viewed at wileyonlinelibrary.com]

When DHB was employed as the matrix, however, we obtained an abundant $[M + H]^+$ ion for oxytetracycline at *m/z* 461. Figure 2A shows the MS image of the oxytetracycline tablet using DHB as the matrix, where the DHB ions ($[M + H - H_2O]^+$ at *m/z* 137.24, $[M + H]^+$ at *m/z* 155.24 and $[M + Na]^+$ at *m/z* 177.26) are labeled with asterisks. To further confirm the presence of the target compound, the formation of the MS/MS product ions of the $[M + H]^+$ ion at *m/z* 461 was studied using DHB as matrix. As shown in Figure 2B, the main product ions were at *m/z* 426 ($[M + H - H_2O - NH_3]^+$) and 201 ($[C_{12}H_9O_3]^+$), in agreement with a previous report on the analysis of oxytetracycline.[24] Therefore, DHB was selected as the matrix in our subsequent experiments.

3.3 | Matrix system composition

Once the preferred matrix has been identified, the solvent used for matrix application has a significant influence on the performance of

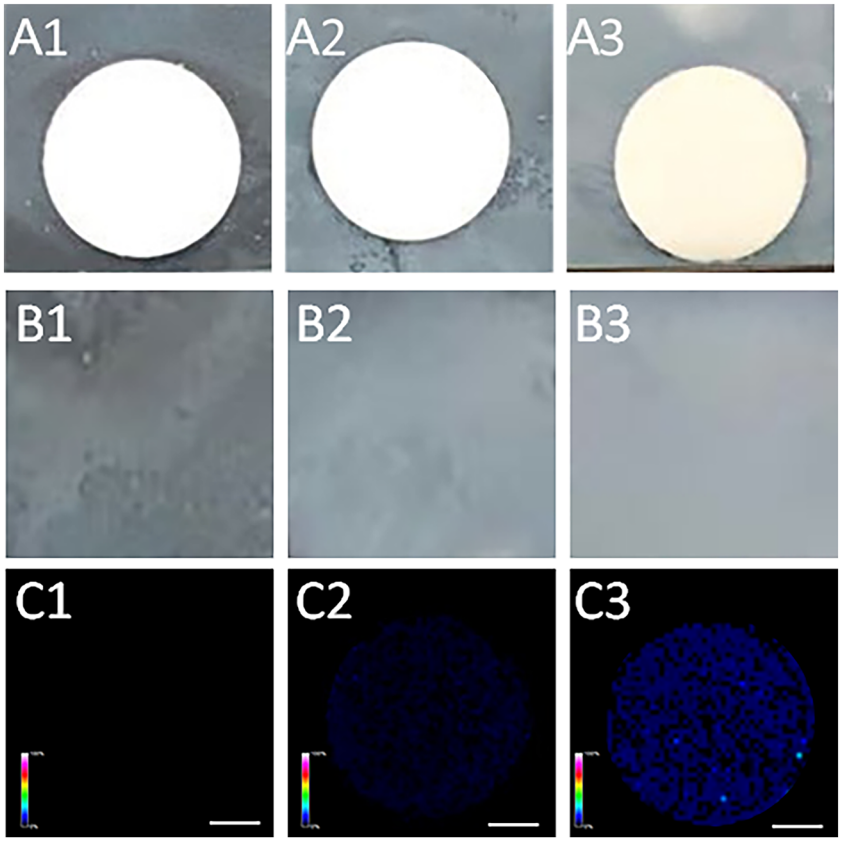

FIGURE 3 A1–A3, Pictures of model tablet section after spraying with DHB of 10 mg mL^{-1} (A1), 20 mg mL^{-1} (A2) and 40 mg mL^{-1} (A3); B1–B3, zoomed picture of matrix film on ITO glass of 10 mg mL^{-1} (B1), 20 mg mL^{-1} (B2) and 40 mg mL^{-1} (B3); C1–C3. MALDI-MS images at *m/z* 461 ($[M + H]^+$) of model tablets after spraying with DHB of 10 mg mL^{-1} (C1), 20 mg mL^{-1} (C2) and 40 mg mL^{-1} (C3). The scale bar represents 2 mm [Color figure can be viewed at wileyonlinelibrary.com]

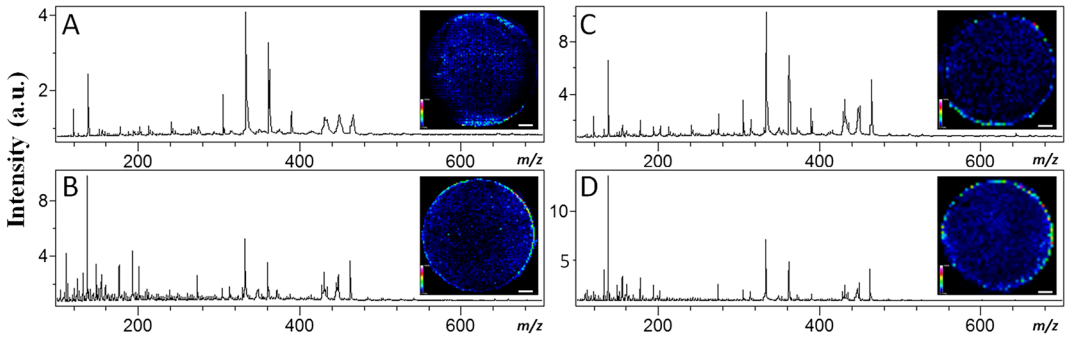

FIGURE 4 Overall spectra and images at *m/z* 461 of model tablet with different spatial resolution: A, raster at 100 µm, Smartbeam parameter: Single; B, raster at 100 µm, Smartbeam parameter: M5; C, raster at 200 µm, Smartbeam parameter: Single; D, raster at 200 µm, Smartbeam parameter: M5. The scale bar represents 1 mm [Color figure can be viewed at wileyonlinelibrary.com]

crystallization.[25] Based on previous work, a matrix solution concentration of 10–20 mg mL^{-1} using 50–70% of a volatile solvent, such as methanol and acetonitrile, which contains 0.1% TFA, could be efficacious.[26] In our pre-experiment, 20 mg mL^{-1} DHB in 50% methanol or 50% acetonitrile was tested for spraying with the automated TM-sprayer, with both solvents containing 0.1% TFA.

As parameters such as the solvent delivery flow rate also have a considerable effect on the spray outcome, a pre-experiment comparison of flow rates at 0.075 and 0.2 mL min^{-1} was carried out (the detailed results are shown in Figure S1, supporting information). As can be seen in Figures S1A and S1B (supporting information) the matrix layers were more homogeneous after spraying at the lower flow rate of 0.075 mL min^{-1}, while, at the higher flow rate at 0.2 mL min^{-1}, the delivered rate was so fast that the matrix did not yield a uniform distribution (Figures S1C and S1D, supporting information). Although relatively uniform films were obtained using 50% acetonitrile and 50% methanol at 0.075 mL min^{-1}, the matrix spray outcome using 50% acetonitrile was considered to be better (see Figure S1B, supporting information). Thus, the 50% acetonitrile solvent at a flow rate of 0.075 mL min^{-1} was used in the following experiments.

Once 50% acetonitrile had been chosen as the matrix solvent, we further evaluated the concentration of DHB in 50% acetonitrile that was suitable for imaging, and DHB solutions at 10, 20 and 40 mg mL^{-1} were studied. The detailed results are shown in Figure 3. Figures 3A and 3B show pictures of the model tablet and the matrix film on ITO glass after spraying. All the MALDI-MS images shown in Figure 3C indicate the distribution of oxytetracycline at *m/z* 461 ($[M+H]^+$). It was found that the 10 mg mL^{-1} DHB solution concentration was too low for complete imaging with the non-dense matrix film, as shown in Figures 3B1 and 3C1. Both the matrix film and the MALDI-MS image improved at the increased concentration of 20 mg mL^{-1} while, as shown in Figure 3C3, a dense and uniform film of matrix with a clear and a complete MALDI-MS image was obtained on spraying with DHB solutions at 40 mg mL^{-1}. Based on the above results, the optimum concentration of DHB in 50% acetonitrile was determined to be 40 mg mL^{-1}.

To further validate the applicability of the method for determining the model tablet and matrix composition, we also tested the effect caused by conductivity using the QuanTOF instrument under the optimized conditions. As the target plate was grounded in this instrument, there was no electrical conductivity difference across the whole tablet surface. The built model was shown to have consistent signal intensity across the tablet.

3.4 | Spatial resolution selection

Based on the factors optimized for sample preparation, parameters influencing the spatial resolution during data acquisition were further investigated in the following experiments. For MALDI-MSI, the spatial resolution was determined by sampling the pitch and the area ablated by the laser. The former was set as the FlexImaging raster in µm, and the latter was defined in the Smartbeam parameter with different scan ranges and resulting field sizes and was also significantly affected by laser power. Therefore, the laser power was optimized and fixed for imaging under different Smartbeam parameters. To achieve a rapid measurement, rasters at 100 µm and 200 µm were selected for optimization. In addition, two distinct laser profiles, Smartbeam Single and M5, were compared for the image outcome. The corresponding results are shown in Figure 4. Figures 4A and 4B exhibit obviously clearer images with higher pixels

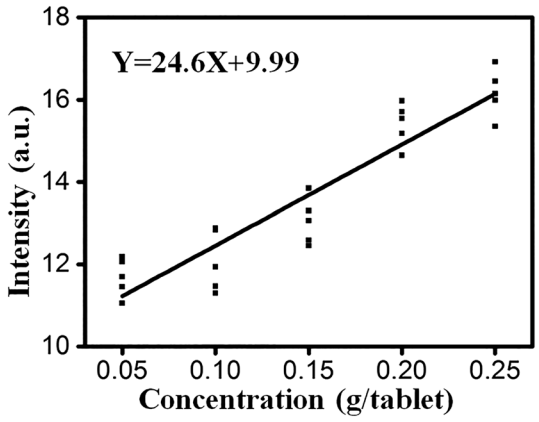

FIGURE 5 Analytical curve of oxytetracycline based on simulated tablets using MALDI-MSI

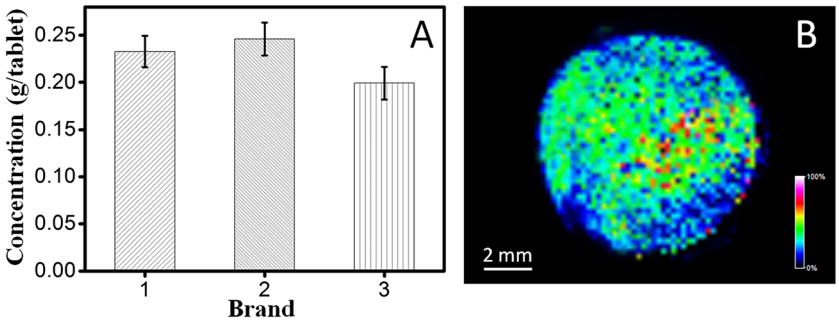

FIGURE 6 A, Content of oxytetracycline in commercial tablets from different brands and B, MALDI-MS image of brand 3 sample at *m/z* 461 ($[M + H]^+$) [Color figure can be viewed at wileyonlinelibrary.com]

than those in Figures 4C and 4D. However, the tests whose results are shown in Figures 4A and 4B with a raster at 100 µm lasted approximately 30 min, while the acquisition time for those displayed in Figures 4C and 4D was just less than 15 min. On the other hand, as shown in Figures 4A and 4C, as the laser ablation area under the Smartbeam set to Single mode (50 µm) was smaller than the desired pixel dimension (100 µm), random walk patterns were exhibited rather than a square profile. Although the spatial resolution was better with the raster at 100 µm, images acquired with the raster at 200 µm were sufficient to reflect the distribution of the target compound, with the further benefit of better sensitivity and lower time consumption. As a result, a raster at 200 µm and a Smartbeam parameter of M5 were employed in the following experiments.

There is a distinct edge effect in the images shown in Figure 4. Since the model tablets are produced by spraying the oxytetracycline standard on the blank tablet, the ITO glass is also covered with the oxytetracycline standard. We re-checked the MS image of the oxytetracycline obtained on the glass and found that the intensity obtained on the glass is much higher than that on the model tablet. Since the tablet is 1 mm higher than the glass, the signal intensity obtained on the glass around the tablet is stronger than that on the center of the tablet, thus resulting in the generation of edge effects when we outline the imaging area at the outer edge of the tablet shown in Figure 4.

3.5 | Quantification of oxytetracycline

After matrix identification and parameter optimization based on the model tablet, a further issue was whether we could establish an analytical curve for the quantification of oxytetracycline using the linear relation between the concentration and the MALDI-MSI signal intensity. Customized tablets containing 0.05, 0.1, 0.15, 0.20 and 0.25 g oxytetracycline were employed to imitate the ionization background of the commercial tablets. All the tablet images were normalized by the total ion count (TIC) method, which normalized every spectrum separately by dividing each spectrum's intensity by the sum of all its intensities. Average oxytetracycline peak intensities at *m/z* 461($[M + H]^+$) obtained from the spectra of five parallel tablet samples were plotted against the concentration of tablets. The error bars (Figure 5) for the standard error are marked at each point (R^2 = 0.8596).

3.6 | Application to real tablets

To demonstrate the application of the developed method, real samples of commercial tablets from three brands were obtained, and the oxytetracycline content was determined. As shown in Figure 6A, samples of two brands within the warranty period and one brand out of the warranty period were sliced and subjected to MALDI-MSI. Statistical analyses of five samples collected from each brand were run in quintuplicate. For the samples of brands 1 and 2, which were within the warranty period, the average content of oxytetracycline was determined to be 93.1% and 98.4%, respectively, while for samples of brand 3, which were out of warranty, the average content was measured to be 79.7%. The error bars for samples of each brand are also marked in Figure 6A. The MALDI-MS image of the brand 3 sample at *m/z* 461($[M + H]^+$) is shown in Figure 6B. According to the Chinese Pharmacopoeia 2015, the amount of oxytetracycline should be 90.0–110.0% of the labeled amount in each tablet. The results obtained with MALDI-MSI were further compared with the data acquired by the traditional HPLC/MS method. Figure S2 (supporting information) presents the HPLC result of the analyses of commercial oxytetracycline tablets from sample 1. No significant differences were found between the MALDI-MSI results and the HPLC data (P <0.05), which verified the reliability of the proposed MALDI-MSI method.

4 | CONCLUSIONS

In this work, we demonstrated a novel method based on MALDI-MSI for directly mapping the API distribution within a whole oxytetracycline tablet. The methodological parameters were optimized for sample preparation and data acquisition. Quantitative analysis was feasible for differentiating tablets containing various doses of the active compound. The merits of the proposed method, such as rapid acquisition time, simple operation, minimized pretreatment, and an extraction-free and separation-free procedure, make it a desirable approach for tracking tablet manufacturing. This method could be helpful for monitoring the quality of pharmaceutical products and is promising and competitive for actual use in the pharmaceutical industry.

ACKNOWLEDGEMENTS

Financial support from the Youth Science Funds of Shandong Academy of Sciences (2019QN009), the Key Research and Development Program of Shandong Province (2019GSF111001, 2019GSF111009), Youth Ph.D. Cooperation Funds of Qilu University of Technology (Shandong Academy of Sciences) (2018BSHZ0029), Natural Science Foundation of Shandong Province (ZR2017MB011) and the Key Laboratory of Biomedical Effects of Nanomaterials and Nanosafety, CAS (No: NSKF201813) is gratefully acknowledged.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Xie H, He Q, Zhao Y, et al. *In situ* analysis of oxytetracycline tablets based on matrix-assisted laser desorption/ionization mass spectrometry imaging. *Rapid Commun Mass Spectrom*. 2020;34:e8592. https://doi.org/10.1002/rcm.8592