

Research Paper

An Efficient Gas Chromatography–Mass Spectrometry Approach for the Simultaneous Analysis of Deoxynivalenol and Its Bacterial Metabolites 3-keto-DON and 3-*epi*-DON

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ABSTRACT

Deoxynivalenol (DON) is one of the major toxic secondary metabolites produced by *Fusarium* fungi in cereal grains. Among the many promising strategies of DON detoxification are the microbial and enzymatic ones, which transform DON to nontoxic DON metabolites. Thus, proper analytical methods are needed for those DON metabolites. In this study, a robust gas chromatography–mass spectrometry (GC-MS) procedure was developed and validated for the simultaneous analysis of DON and two of its bacterial metabolites, 3-keto-DON and 3-*epi*-DON. The procedure involves a straightforward vacuum drying and derivatization step before the subsequent GC-MS analysis. Following the optimized protocol, DON and these two metabolites were separated on a capillary column within 15 min. The linear ranges for these compounds were 10 to 2,000 ng mL⁻¹ with correlation coefficients >0.99. For DON, 3-*epi*-DON, and 3-keto-DON, the limits of detection were 0.8, 3.0, and 0.05 ng mL⁻¹, and the limits of quantification were 2.6, 10.0, and 1.0 ng mL⁻¹, respectively. For all three compounds, the obtained relative standard deviation was 1.2 to 5.5%, and the recovery rates were 89.5 to 103.6%. The developed method was further validated by analyzing DON metabolites resulting from the biotransformation of DON initiated by cell-free lysates of the bacterium *Devosia mutans* 17-2-E-8. The developed protocol was sensitive, precise, accurate, and robust for the determination of DON, 3-*epi*-DON, and 3-keto-DON in liquid media and potentially other complex matrices without interference from other compounds.

Key words: Deoxynivalenol; 3-*epi*-DON; 3-keto-DON; Gas chromatography–mass spectrometry

Deoxynivalenol (DON) is a type B trichothecene mycotoxin and one of the major toxic secondary metabolites produced by the fungi *Fusarium graminearum* and *Fusarium culmorum* in wheat, corn, rice, oats, barley, and other grains. DON is also known as vomitoxin because of its emetic effect in humans, swine, and other monogastric animals (15, 16). Consumption of food or feed contaminated with DON causes symptoms of nausea, fever, headaches, and vomiting in humans (20) and results in significant economic losses in the productivity of farm animals due to feed refusal, lack of weight gain, and reduced or altered immunity (15, 16). DON-contaminated cereal grains are very common and are encountered in almost every agricultural zone around the globe. According to the latest Biomin Mycotoxin Survey results released in late 2015, DON was found in 56% of all tested samples with concentrations close to the established risk threshold of livestock productivity (11). Climate changes and the spread of resistant *Fusarium* strains are both complicating the situation further and bringing the focus to DON contamination of food and feed commodities worldwide. Among the

many promising strategies for DON detoxification are the microbial and enzymatic ones (7, 9, 13). A plethora of research studies have revealed that DON can be metabolized by many bacterial isolates to less toxic or nontoxic compounds (8). Recently, a bacterial isolate designated *Devosia mutans* 17-2-E-8 was reported to transform DON under aerobic conditions into 3-*epi*-DON and 3-keto-DON (6) (Fig. 1), and the cytotoxicity of these compounds is much lower than that of DON (5, 6).

Methods of determination of mycotoxins, including DON and its plant derivatives or metabolites, have been reviewed thoroughly in the past decade (3, 10, 14). Initially, thin-layer chromatography (19) and enzyme-linked immunosorbent assays (12, 22) were among the most commonly used approaches to screening for DON in food and feed matrices. However, gas chromatography (GC), GC plus mass spectrometry (MS) (3, 10, 14, 17), liquid chromatography (LC), and LC plus MS techniques currently are widely used for the qualitative and quantitative determination of DON (and many of its derivatives) in research and regulatory laboratories around the world (1, 18, 21). Although LC-MS protocols are accurate and robust, GC-MS platforms offer the following advantages: (i) they are cheaper and hence more common in many analytical

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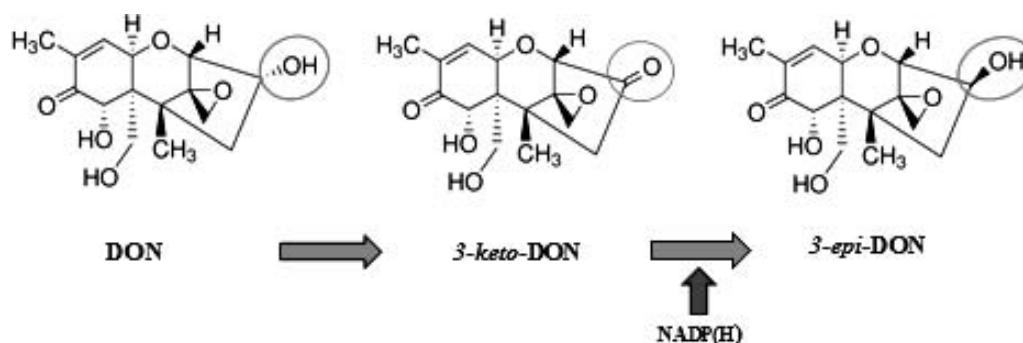


FIGURE 1. Anticipated mechanism of biotransformation of DON to 3-keto-DON and 3-epi-DON.

laboratories and (ii) separation is generally more efficient with GC than with LC. In this article, we address the analytical gap that exists for a GC-MS methodology capable of simultaneously detecting and quantifying DON and its 3-keto-DON and 3-*epi*-DON metabolites. Although the finalized and validated protocol can successfully be used to analyze DON, 3-keto-DON, and 3-*epi*-DON in many matrixes, the presented method was validated using cell-free lysates of *D. mutans* 17-2-E-8.

MATERIALS AND METHODS

Chemical and reagents. DON, isotope-labeled DON ($^{13}\text{C}_{15}$ -DON), *N,O*-bis(trimethylsilyl) acetamide (BSA), trimethylchlorosilane (TMCS), *N*-trimethylsilylimidazole (TMSI), sodium dihydrogen phosphate, disodium hydrogen phosphate, and GC-MS-grade hexane were all purchased from Sigma-Aldrich (Oakville, Ontario, Canada). 3-keto-DON was obtained from TripleBond Corp. (Guelph, Ontario, Canada). 3-*epi*-DON (96.8% purity) was purified using high-speed countercurrent chromatography as reported previously (6). High-performance LC-grade solvents (methanol and acetonitrile) were supplied by Caledon Laboratories (Georgetown, Ontario, Canada), and the NADP(H) regeneration system was obtained from Promega (catalog no. V9510, Madison, WI). Ultrapure water (Milli-Q system, EMD Millipore Corp, Billerica, MA) was used for all reported experiments and dilutions.

Standard solutions. Stock solutions of DON, 3-keto-DON, and 3-*epi*-DON were prepared at $200\ \mu\text{g mL}^{-1}$ in a mixture of acetonitrile and water (1:1, v/v). Working solutions containing DON, 3-*epi*-DON, and 3-keto-DON at 10 to $2,000\ \text{ng mL}^{-1}$ were prepared in the same mixture of acetonitrile and water. $^{13}\text{C}_{15}$ -DON ($2.5\ \mu\text{g mL}^{-1}$) dissolved in acetonitrile was used as an internal standard.

Sample preparation and derivatization. A total of $30\ \mu\text{L}$ of standard solution (or sample) was transferred to a 2-mL glass vial and dried in a vacuum oven at 45°C for 2 h, and $50\ \mu\text{L}$ of a mixture of BSA, TMCS, and TMSI (3:2:3) was added to each vial. Vials were properly sealed and vortexed for 20 to 30 s and then heated at 80°C for 20 min in a dry heating block (Boekel Scientific, Feasterville, PA). After they were cooled to room temperature, the vials were stored briefly in a -20°C freezer to prevent the escape of vapor. Derivatized samples were directly subjected to GC-MS analysis or to a further cleaning and extraction step: $200\ \mu\text{L}$ of *n*-hexane was added to the derivatized sample, which was vortexed for 20 to 30 s, and then $200\ \mu\text{L}$ of ultrapure water (or phosphate buffer) was added and vortexed for an additional 10 s. Samples were centrifuged at $2,500 \times g$ for 5 min at room temperature, and

the upper layer was collected and transferred to an autosampler GC-MS vial.

GC-MS analysis and separation conditions and parameters. A gas chromatograph Trace 1310 equipped with an ISO LT single quadrupole mass spectrometer detector (Thermo Scientific, Mississauga, Ontario, Canada) was used in this study. The instrument was equipped with a Triplus RSH series autosampler. Separation was accomplished on a TG-5MS capillary column (60 m by 0.25 mm inside diameter by $0.5\ \mu\text{m}$ film thickness; Thermo Scientific). The carrier gas was helium, and the flow rate was set to $1.5\ \text{mL min}^{-1}$. One microliter of the sample was injected with the splitless mode at 280°C with a programmable temperature vaporization inlet. The heating program of the column oven was 140°C for 0.5 min ramped up to 300°C for 20 min, with a total run time at 20.5 min. The MS transfer line temperature was 280°C . All analytes eluted within 15 min.

MS parameters were electron impact ionization with 70 eV energy and ion source temperature at 230°C . The MS quadrupole temperature was 150°C , and solvent delay was 8.0 min. The MS system detection was at full scan from 35 to 500 amu and selective ion monitoring (SIM) mode with 211, 235, and 245 amu. The SIM parameters and retention times of each analyte are shown in Table 1. DON and its derivatives were identified by comparing retention time(s) and mass spectra of obtained fragment ions to those of standards. The quantification was based on the peak area of each analyte and the internal standard. The data were obtained and processed using the Xcalibur software package (version 3.1.66.10, Thermo Scientific).

Method validation. Under the previously optimized experimental conditions, the linearity, precision, recovery rates, and limits of detection (LODs) for the developed method were established. To obtain the linearity, standard solutions of DON (and its metabolites) from 10 to $2,000\ \text{ng mL}^{-1}$ were analyzed following the GC-MS protocol. The linear range and coefficient of determination (R^2) were both calculated for each compound separately. To evaluate reproducibility, five separate measurements at two analyte concentrations (10 and $100\ \mu\text{g kg}^{-1}$) were carried

TABLE 1. Mass spectrometry parameters for the analysis of DON, 3-*epi*-DON, and 3-keto-DON

Analyte	t_R (min)	SIM ions (m/z)
DON-3TMS	13.65	235, 259, 295, 422
3- <i>epi</i> -DON-3TMS	14.24	235, 259, 295, 429
3-keto-DON-3TMS	14.12	211, 269, 298, 429
$^{13}\text{C}_{15}$ -DON-3TMS	13.65	216, 245, 304, 437

out, and precision was expressed as the relative standard deviation (RSD). To determine the LODs and limits of quantification (LOQs), DON, 3-keto-DON, and 3-*epi*-DON standard solutions were subjected to GC-MS analysis at decreasing concentrations. The signal-to-noise ratio (S/N) was used to calculate the analytical limits of the method. Concentrations with S/N = 3 were considered the LOD, and concentrations with S/N = 10 were considered the LOQ. Recovery rates were determined by following the standard method described by Burns et al. (2). The spiked concentrations were similar to those used during the precision determination (10 and 100 ng mL⁻¹ for each metabolite individually).

Bacterial lysates and enzymatic biotransformations of DON. *D. mutans* 17-2-E-8 cells from our laboratory stock were grown in Luria-Bertani broth (1 L contains 10 g of peptone 140, 5 g of yeast extract, and 5 g of sodium chloride) with shaking (120 to 150 rpm) at 28°C for 5 to 6 days. Bacterial pellets were collected by centrifugation at 10,000 × *g* for 30 min and stored frozen (−20°C) until used. Pellets were resuspended in Tris-HCl buffer (pH 8.5), and a protease cocktail (100×; catalog no. 1861278, Halt protease inhibitor, Thermo Fisher Scientific, Waltham, MA) was added before sonication. Lysates were cleared by centrifugation at 10,000 × *g* for 1 h at 4°C and then filter sterilized (0.2-μm pore size, 25 mm; GD/X sterile syringe filter, Whatman, Clifton, NJ). Lysates were stored frozen at −20°C until incorporated into the final enzymatic reactions.

Cell-free DON enzymatic biotransformation proceeded by including 10 μL of 10% glycerol, 60 μL of bacterial cell lysates, 5 μL of a source of NADP(H) at 100 μM (Promega), and 20 μL of 200 μg/mL DON in a final volume of 100 μL. The negative control had the same composition as the treatment but the NADP(H) regeneration system was replaced with Tris-HCl buffer. Reaction mixes were incubated for 2 h at 37°C and terminated by adding an equal volume of high-performance LC-grade methanol (final volume of 200 μL). Samples were then centrifuged at 18,000 × *g* for 5 min at room temperature, and supernatants were collected for GC-MS analysis as described below.

RESULTS AND DISCUSSION

Chemical derivatization of DON, 3-*epi*-DON, and 3-keto-DON. The compound of interest must be volatile and thermally stable to be suitable for GC analysis. Only when the compound is gaseous at 400 to 450°C or below without showing any major degradation at such temperatures can it be directly analyzed with GC techniques. The boiling point of DON is 543.88°C. Although no boiling points have been reported for 3-*epi*-DON or 3-keto-DON but considering the similarity between their structures and that of DON, the boiling points of these two bacterial metabolites probably are close to that of DON. Thus, a derivatization step is needed to render DON, 3-keto-DON, and 3-*epi*-DON suitable for GC analyses (Fig. 1).

Among the common derivatization approaches used for GC analysis is silylation, in which silylating reagents are used to react with active hydrogen groups. Because DON and 3-*epi*-DON contain three hydroxyl groups and 3-keto-DON contains two (Fig. 1), silylation was chosen for derivatization. Several silylating reagents have been described, including BSA, TMCS, TMSI, and pentafluoropropionic anhydride (3, 10, 14). In this work, a mixture of BSA, TMCS, and TMSI (3:2:3) was used.

The active hydrogens of the DON, 3-*epi*-DON, and 3-keto-DON hydroxyl groups were replaced into trimethylsilyl (TMS) ethers (Fig. 2), and the presence of representative molecular ions in the obtained electron ionization mass spectra confirmed that the derivatized products of DON, 3-*epi*-DON, and 3-keto-DON were indeed DON-3TMS, 3-*epi*-DON-3TMS, and 3-keto-DON-2TMS, respectively (Fig. 2). Scrutinization of the full-scan mass spectra of DON suggested that the C6–C11 bond was broken to obtain mass fragments 147 and 235, respectively, and the C5 and the attached O bond was ionized to yield mass fragments 295 and 217, respectively (Fig. 2a).

Sample preparation before derivatization. Nonpolar organic solvents (e.g., hexane, ether, benzene, and toluene) are considered excellent derivatization media or solvents in analytical chemistry applications despite the widespread use of polar solvents (e.g., pyridine, dimethylformamide, dimethylsulfoxide, tetrahydrofuran, and acetonitrile) for facilitating derivatization reactions. Under the present experimental conditions and when the silylation reactions of DON, 3-*epi*-DON, and 3-keto-DON were conducted in acetonitrile, the GC-MS signal was weak, almost half of the signal recovered from vacuum dried samples (data not shown). Therefore, a vacuum drying step was used before derivatization.

Although DON is stable at high temperatures, the stability of 3-keto-DON and 3-*epi*-DON are not known at such temperatures; hence, we examined the effect of diverse drying conditions (temperature and time combinations) on the GC-MS results. Two temperatures (45 and 65°C) coupled with two vacuum treatments (1 and 2 h) were explored. The results revealed no significant difference between the peak areas under the tested temperatures and times (data not shown). Therefore, in subsequent experiments all standards and samples were dried under vacuum at 45°C for 2 h before derivatization.

Sample cleanup and extraction after derivatization. In this study, we compared the GC-MS chromatograms of DON standards, derivatized as mentioned above, with and without further cleaning and extraction steps. Two solutions, ultrapure water or a phosphate buffer (pH 7.0), were utilized to remove impurities (3). A 200-μL volume of water or phosphate buffer and then 200 μL of hexane was added to 50 μL of the derivatized sample or standard to extract derivatized DON, 3-*epi*-DON, and 3-keto-DON.

The introduced extraction and cleaning step removed most of the water-soluble impurities plus any carried over silylation agents (silylation reagents were often used in excess concentrations to ensure complete derivatization reactions). Although such a cleaning step is considered advantageous when working with complex sample matrices (e.g., cellular lysates), the retrieved peak areas revealed a significant numerical difference between samples with and without further cleaning(s) or extraction(s). The mean (±SD) integrated peak areas of DON standards with the same concentrations analyzed without extractions were 77,368 ± 6.12 and were 1.85 times higher than those of

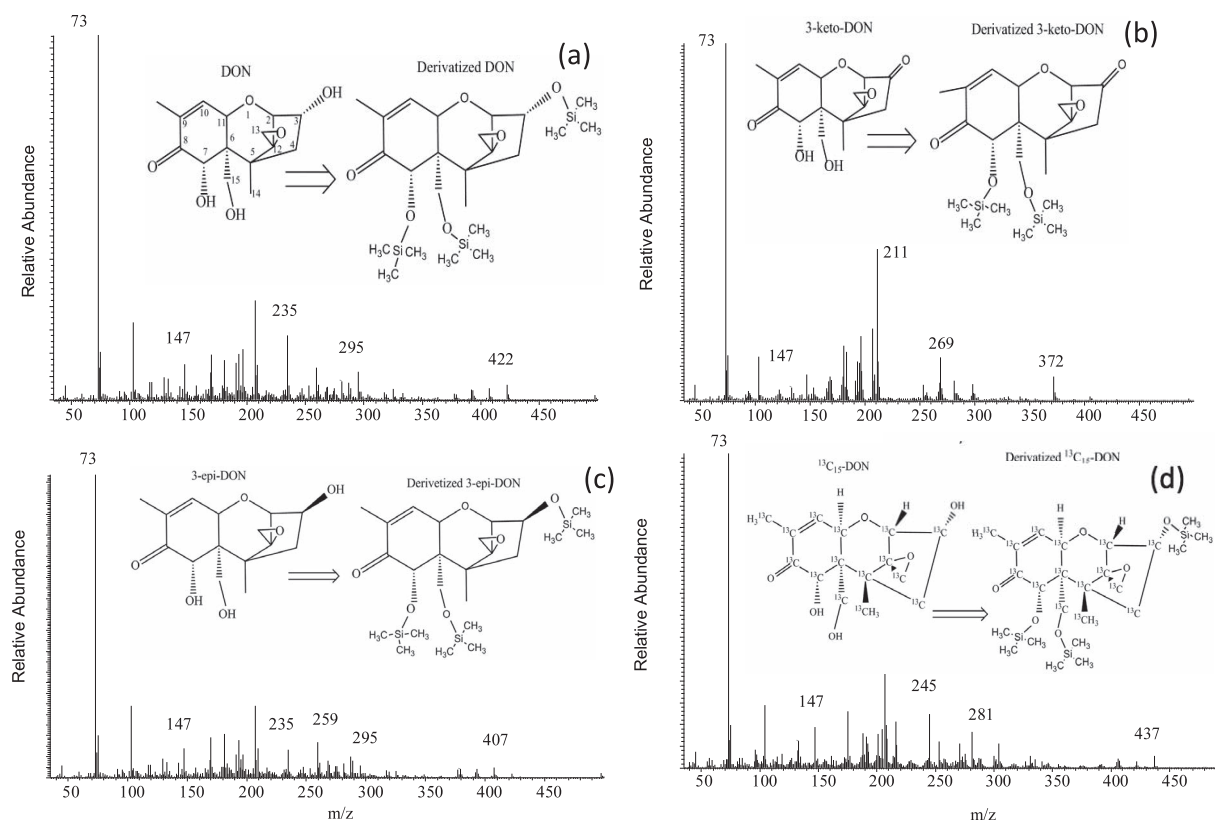


FIGURE 2. Chemical structures and mass spectra of the TMS conjugates of DON, 3-keto-DON, 3-epi-DON, and $^{13}\text{C}_{15}$ -DON. (a) DON and DON-3TMS and the mass spectrum of DON-3TMS; (b) 3-keto-DON and 3-keto-DON-2TMS and the mass spectrum of 3-keto-DON-2TMS; (c) 3-epi-DON and 3-epi-DON-3TMS and the mass spectrum of 3-epi-DON-3TMS; (d) $^{13}\text{C}_{15}$ -DON and $^{13}\text{C}_{15}$ -DON-3TMS and the mass spectrum of $^{13}\text{C}_{15}$ -DON-3TMS.

the samples that were analyzed after further cleaning ($14,3195 \pm 5.03$), indicating that a major loss in sample recovery accompanied this extra cleaning step. Therefore, an internal standard, $^{13}\text{C}_{15}$ -DON, was used to correct for such losses.

No significant differences were observed between samples cleaned with water (as diluents) or with phosphate buffer; hence, ultrapure water was used as the preferred cleaning medium in all subsequent trials and analyses.

GC analysis and condition optimization. GC conditions, including the choice of capillary column, oven temperature, and heating program, were scrutinized to optimize the separation of DON, 3-keto-DON, and 3-epi-DON. Because the polarity indexes of silylated DON, 3-keto-DON, and 3-epi-DON were predicted to be low, the TG-5MS (5% phenyl phase) capillary column was chosen for this separation owing to its proven applicability in analyzing compounds of low polarities. By combining this column with a heating program that started at 140°C for 0.5 min and then ramped up to 300°C for 20 min, the three metabolites were separated within the following retention times: 13.65 min for DON, 14.12 min for 3-keto-DON, and 14.24 min for 3-epi-DON (Fig. 3a). A further investigation of the effect of the splitless injection mode and other oven temperature(s) on resolutions or shape(s) of peaks indicated that the above heating program was the most suitable for this separation.

Mass analyzer mode and parameters. A full scan from 35 to 500 m/z was useful in our case for obtaining all possible mass fragments of the tested analytes (Fig. 2a through 2d). However, the sensitivity of each compound at this mode was not satisfactory, i.e., the LODs were 20, 50, and 10 ng mL^{-1} for DON, 3-epi-DON, and 3-keto-DON, respectively. Therefore, the SIM mode was used instead, and a selected ion was monitored to track each analyte; the 235 m/z fragments were used for DON and 3-epi-DON, and the 211 and 245 m/z fragments were used for 3-keto-DON and $^{13}\text{C}_{15}$ -DON, respectively (Table 1).

Under the SIM mode, the detection sensitivity of each compound was significantly increased and a sharp single peak was obtained for each of the metabolites (Fig. 3a and 3b). The selective monitoring was advantageous also in comparisons with previous observations (4), in which double peaks for DON were reported when it was derivatized with TMSI or Tri-Sil-Z because of the presence of the C-7 hydroxyl group. We found only one peak for 3-epi-DON and 3-keto-DON in standard solutions and cell lysates when analyzed using the presented protocol.

Method validation. The stable DON isotope $^{13}\text{C}_{15}$ -DON was used as an internal standard to correct for losses in DON, 3-epi-DON, and 3-keto-DON concentrations during the extraction and cleaning steps. DON, 3-keto-DON, and 3-epi-DON concentrations were determined by GC-MS under the SIM mode using ratios of peak areas of these three

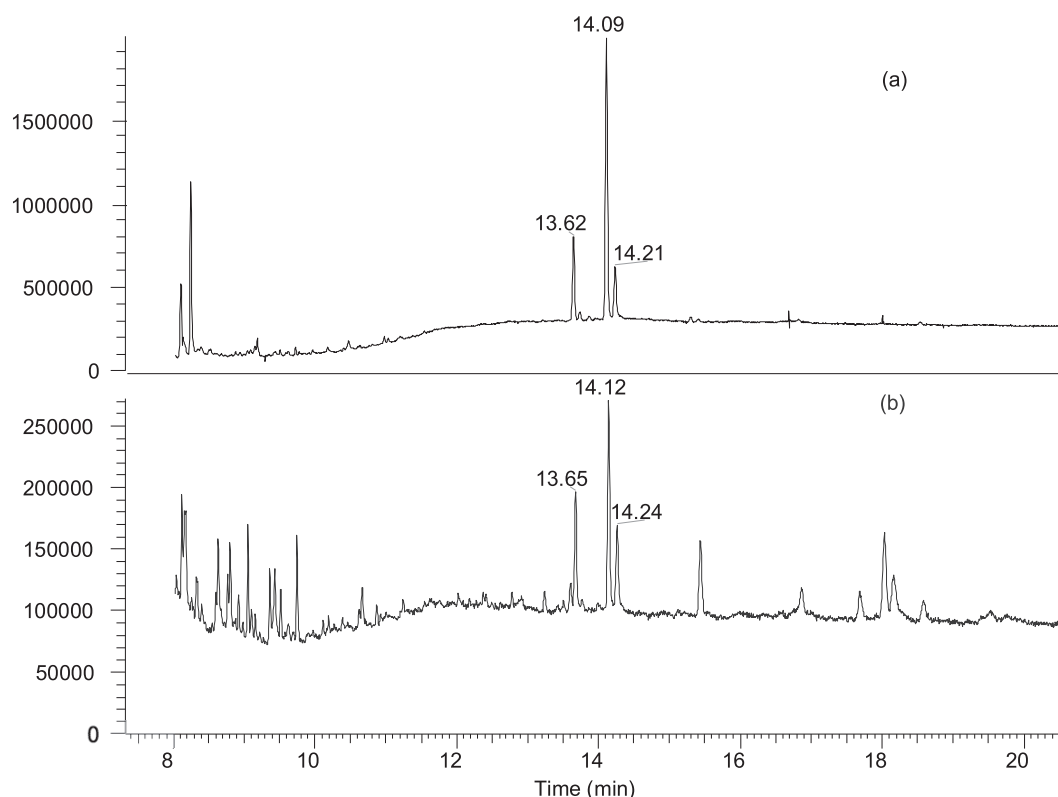


FIGURE 3. Collected chromatograms of the standard mixture solution of DON, 3-*epi*-DON, and 3-keto-DON under the selective ion monitoring mode (a) and the total ion current scan mode (b).

analytes to the internal standard at m/z 245. An excellent linear correlation between the concentrations of these three metabolites and the calculated peak area/internal standard ratios was observed with calculated $R^2 \geq 0.99$ and linear ranges of 10 to 2,000 ng/mL (Table 2), reflecting the suitability of the developed method for the analysis of actual samples. The LODs with concentrations that gave $S/N = 3$ were 0.8, 3.0, and 0.05 ng mL⁻¹ for DON, 3-*epi*-DON, and 3-keto-DON, respectively, and the LOQs with $S/N = 10$ were 2.6, 10.0, and 1.0 ng mL⁻¹ for DON, 3-*epi*-DON, and 3-keto-DON, respectively. These values (both LOQ and LOD) are lower than those previously reported for DON obtained through LC with tandem MS (18). For all three metabolites, the obtained RSD was 1.2 to 5.5%, supporting the precision and robustness of the method (Table 2). The recovery of each metabolite at two concentrations (10 and 100 $\mu\text{g kg}^{-1}$) in *D. mutans* 17-2-E-8 cell lysates was investigated (Table 3). This protocol resulted in high recovery rates of 89.5 to 103.6% among the three

compounds. The use of the stable ¹³C₁₅-DON as an internal standard and the simple sample preparation procedure were among the major factors allowing the high recovery rates.

Analysis of the enzymatic biotransformations of DON by *D. mutans* 17-2-E-8 cell-free lysates. The procedure developed in this study was validated through tracking the final metabolites of DON when incubated with *D. mutans* 17-2-E-8 cell-free lysates (Fig. 4). This procedure also was used to further investigate the mechanism of the reported enzymatic detoxification of DON by *D. mutans* 17-2-E-8 (Table 4).

When the cell-free lysates of *D. mutans* 17-2-E-8 were incubated with DON and a source of NADP(H) for 2 h at 37°C, only 3-*epi*-DON (not DON or 3-keto-DON) was detected in these reactions (Fig. 4e), indicating that DON was completely biotransformed to 3-*epi*-DON by the assembled enzymatic reactions in an NADP(H)-dependent fashion (Table 4). These results are in agreement with data

TABLE 2. Data of method validation of the proposed GC-MS method for the simultaneous analysis of DON, 3-*epi*-DON, and 3-keto-DON^a

Analyte	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)	Linear range (ng mL ⁻¹)	R^2	RSD (%; $n = 5$)	
					10 ng mL ⁻¹	100 ng mL ⁻¹
DON	0.8	2.6	10–2,000	0.9987	3.28	5.49
3- <i>epi</i> -DON	3.0	10.0	10–2,000	0.9968	5.02	5.14
3-keto-DON	0.05	1.0	10–2,000	0.9999	1.24	4.25

^a LOD, limit of detection; LOQ, limit of quantification; R^2 , coefficient of determination; RSD, relative standard deviation.

TABLE 3. Recovery rates of DON, 3-*epi*-DON, and 3-keto-DON in spiked bacterial cell lysates

Analyte	Concn (ng mL ⁻¹)	Avg recovery (%) ^a
DON	10	103.6
	100	98.2
3- <i>epi</i> -DON	10	90.3
	100	89.5
3-keto-DON	10	101.2
	100	97.8

^a *n* = 5.

reported earlier by He et al. (7). However, both 3-keto-DON (major product) and 3-*epi*-DON (minor product) were detected in the enzymatic reactions that were not supplemented with exogenous NADP(H) (Fig. 4d), with 3-keto-DON concentrations of 1.89 ± 0.59 $\mu\text{g mL}^{-1}$ and 3-*epi*-DON concentrations of 0.25 ± 0.12 $\mu\text{g mL}^{-1}$ (Table 4). The amount of 3-*epi*-DON in the lysates supplemented with exogenous NADP(H) was 8.2 times that in samples with only endogenous NADP(H), clearly demonstrating the reliance of this enzymatic biotransformation mechanism on

TABLE 4. Concentrations of DON, 3-*epi*-DON, and 3-keto-DON in biotransformation reactions using *D. mutans* 17-2-E-8 lysates^a

Sample treatment	DON ($\mu\text{g mL}^{-1}$)	3- <i>epi</i> -DON ($\mu\text{g mL}^{-1}$)	3-keto-DON ($\mu\text{g mL}^{-1}$)
DON standard	2.15 ± 0.78	0	0
Reactions without NADP(H)	ND	0.25 ± 0.12	1.89 ± 0.59
Reactions with NADP(H)	ND	2.04 ± 0.86	ND

^a Values are means \pm SD, *n* = 5. NADP(H) reactions included 10 μL of 10% glycerol, 60 μL of bacterial cell-free lysates, 5 μL of a source of NADP(H) at 100 μM , and 20 μL of 200 $\mu\text{g/mL}$ DON. ND, not detected.

this cofactor. The observed kinetics of DON biotransformations and 3-keto-DON and 3-*epi*-DON accumulations in the tested enzymatic mixtures support the previously hypothesized two-step enzymatic epimerization of DON (Fig. 1).

In conclusion, increasing interest in biological means of mycotoxin detoxification has led to a demand for optimized methods that can simultaneously analyze samples for mycotoxins and their end metabolites. In this study, an

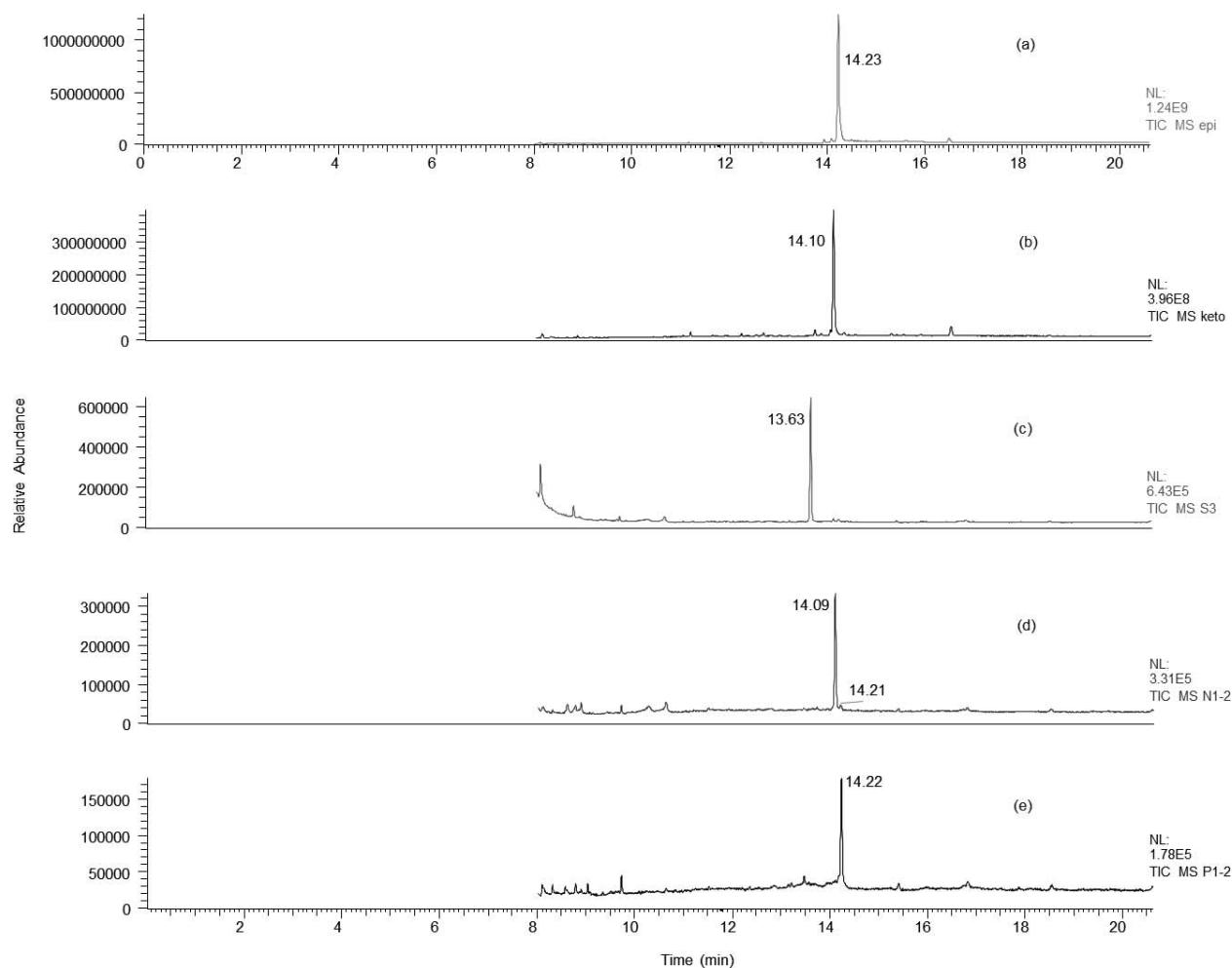


FIGURE 4. GC-MS chromatograms of the enzymatic biotransformations of DON utilizing cell-free lysates of *Devosia* mutans 17-2-E-8 analyzed using the analytical protocol reported in this article. (a) 3-*epi*-DON standard in acetonitrile-water (1:1, v/v); (b) 3-keto-DON standard in acetonitrile-water (1:1, v/v); (c) DON standard in acetonitrile-water (1:1, v/v); (d) enzymatic reaction without the inclusion of NADP(H) cofactor; (e) enzymatic reaction with the NADP(H) cofactor at 100 μM .

efficient procedure was developed for the analysis of DON, 3-keto-DON, and 3-*epi*-DON in complex liquid matrices or cellular lysates. The analytical procedure included a vacuum drying step followed by silylation using a mixture of BSA, TMCS, and TMSI (3:2:3) and a subsequent sample extraction and cleaning step with ultrapure water before the actual GC-MS analysis. With the use of C¹³-labelled DON as internal standard, the provided protocol was a simple, accurate, and robust alternative for analyzing DON bacterial metabolites. With this procedure, very small volumes of sample, reagents, and solvents were required, and a large number of samples can be prepared and analyzed within a short time (less than 18 to 20 min for each run). The GC-MS protocol presented here for the regular analysis of DON, 3-keto-DON, and 3-*epi*-DON is a more affordable method than previously reported LC-MS/MS protocols, especially in small to medium laboratory settings with limited needs for analyzing liquid samples. The developed procedure also can be easily modified for the analysis of DON bacterial metabolites in other food or feed samples.

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