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Analysis of Microbial Metabolites of Deoxynivalenol Through an Immuno-Affinity Enrichment Method

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Dear Sir/Madam:

We have prepared a revision of manuscript entitled “A novel approach to analyze microbial metabolites of deoxynivalenol through an immuno-affinity enrichment method” which incorporates/addresses all the points raised by the respected editor and reviewers. The points were covered as requested. The positions of changes were showed in each response to the editor/reviewer’s comments. Please kindly find uploaded supplementary file titled “Rebuttal Letter” for details.

The revision was written by Yan Zhu, Yousef I. Hassan, Chenggang Cai, Xiu-Zhen Li, Suqin Shao, and Ting Zhou. We would like to publish it as a regular research paper within the *JoVE video Journal*. We state that this manuscript has not been published elsewhere and also is not submitted for publication elsewhere.

All of the above authors have contributed substantially to the manuscript and approved the final submission.

P.S. According to the policy of Canada government, I, as an employee of government, will get the authority to sign the Article and Video License Agreement only when the manuscript is accepted. Would you please go through the reviewing procedure? When the manuscript is accepted, I will provide the agreement form. Thank you for your understanding.

With great respect and appreciation,

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TITLE:

Analysis of Microbial Metabolites of Deoxynivalenol Through an Immuno-Affinity Enrichment Method

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KEYWORDS:

immuno-affinity, HPLC, deoxynivalenol, 3-*epi*-DON, 3-keto-DON, DOM-1

SUMMARY:

The presented work highlights the development of a novel and efficient protocol of a high-performance liquid chromatography approach coupled with an immuno-affinity enrichment step to detect and quantify multiple microbial metabolites of deoxynivalenol (DON), namely 3-*epi*-DON, 3-keto-DON, and deepoxy-DON (DOM-1).

ABSTRACT:

Deoxynivalenol (DON), a type B trichothecene mycotoxin, has well-documented short- and long-term adverse effects on human health and animal productivity alike. While effective biological methods of mitigation are being optimized, the need of reliable analytical approaches to analyze and determine the final microbial metabolites is fundamental. In the current study, an immuno-affinity enrichment step coupled with a high-performance liquid chromatography (HPLC) analysis was developed to analyze and detect three bacterial metabolites of DON; namely 3-*epi*-DON, 3-keto-DON, and deepoxy-DON (DOM-1). The finalized approach provides a rapid, accurate, and easy-to-operate protocol for the analytical determination of the aforementioned metabolites (in addition to DON) in milled corn samples. Taking advantage of the noted cross-reactivity of

recently investigated DON monoclonal antibody, the reported immuno-affinity extraction procedure facilitates the detection of such isomers/metabolites simultaneously and robustly.

INTRODUCTION:

Deoxynivalenol (DON) is a type B trichothecene mycotoxin (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one) produced by multiple filamentous fungi that belong mainly to the *Fusarium* genera (*F. graminearum* and *F. culmorum*)[1,2]. This mycotoxin causes great annual economic losses to the global marketing of grains and to the associated livestock production chain[3-5]. Due to the adverse effects of DON on humans and livestock, most countries in addition to world/regional organizations have established maximum tolerated levels of this toxic compound[6,7]. Studies on the most effective strategies of detoxifying DON are peaking[8-10] with increased interests in exploring microbial strains that can catabolize DON to less-toxic derivatives/epimers such as 3-*epi*-DON [(3β,7α)-12,13-epoxy-3,7,15-trihydroxytrichothec-9-en-8-one], 3-keto-DON [(7α)-12,13-Epoxy-7,15-dihydroxytrichothec-9-ene-3,8-dione], and deepoxy-DON [DOM-1, (3α,7α)-3,7,15-trihydroxytrichotheca-9,12-dien-8-one)][8,9,11-14] (**Figure 1)**. This in turn has increased the demand for effective and robust analytical protocols for the determination of such metabolites despite the fact that such metabolites come with altered chemical, structural, and toxicological properties that substantially differ from the parental compound[15-18], thereby forming a core technical challenge from the analytical prospective[12,19-23].

In this study, a high-performance liquid chromatography (HPLC) protocol with an immuno-affinity purification step was developed to identify and quantify 3-*epi*-DON, 3-keto-DON, and DOM-1 in addition to DON within corn kernels. The approach improves the detection sensitivity by reducing background interface from any grain matrix and provides a reliable analytical method to measure DON and its main microbial metabolites simultaneously.

PROTOCOL:

CAUTION: Please consult all relevant material safety data sheets (MSDS) before implementing this protocol. Mycotoxins [DON and its metabolites including 3-*epi*-DON, 3-keto-DON, and DOM-1] used in this study are toxic compounds despite the fact that some of such metabolites were proved to be less toxic than others. Please use all appropriate safety practices when performing the following analysis.

1. Preparation of standards

NOTE: DON, DOM-1, and 3-keto-DON were purchased from chemical suppliers (**Table of Materials**). 3-*epi*-DON was purified and chemically characterized as previously described[24].

1.1. Prepare stock solutions of DON and 3-*epi*-DON in pure water and stocks of 3-keto-DON and DOM-1 in absolute acetonitrile (ACN).

1.1.1. Accurately weigh the total mass of each toxin powder (except DOM-1 as it comes in a liquid form) within the original commercially provided vial.

1.1.2. Thoroughly dissolve the powder(s) with 5 mL of ACN in the original vial. Transfer the solution to a new clean vial and dry the original vial using a nitrogen evaporator. Weigh the original vial again when all the residual ACN is evaporated.

1.1.3. Calculate the mass of powder by subtracting the mass of original vial (step 1.1.2) from the total mass (step 1.1.1).

1.1.4. Evaporate ACN using a nitrogen evaporator, and re-dissolve the solute with a known amount of stock solvent based on the obtained mass calculations (step 1.1.3).

NOTE: The concentration of DON, 3-*epi*-DON, and 3-keto-DON stock solutions were 10000 µg/mL, 2000 µg/mL, and 1000 µg/mL, respectively, in this study.

1.1.5. For DOM-1, use the commercial product directly as it is provided in a liquid format with a claimed/certified concentration (50 µg/mL).

1.2. Store all stock solutions (1 mL per vial) in -20 °C until use.

2. Sample extraction and immuno-affinity purification

2.1. Sample extraction

2.1.1. Collect corn kernels from local farms. Store the obtained samples in a 4 °C refrigerator until use/analysis.

2.1.2. Grind the corn kernels (approximately 200 g each) for 1 min using a laboratory-grade blender. Weigh 50 g of each ground sample separately and mix samples with 200 mL of sterile deionized water in the blender for 2 min at least.

2.1.3. Pass the resulting slurry through a coarse filter paper (**Table of Materials**) and spike DON, 3-*epi*-DON, 3-keto-DON, or DOM-1 for standard(s) preparations.

NOTE: Final concentrations should contain between 0.05 and 1.0 µg/mL of each tested analyte(s).

2.1.4. Leave samples for 1 h at room temperature before proceeding to the immune-affinity purification step.

2.2. Immuno-affinity purification

2.2.1. Filter the corn powder slurry (step 2.1.4) through a microfiber filter (**Table of Materials**).

2.2.2. Load 1 mL of the collected clear corn powder filtrate directly onto the immune-affinity purification column (**Table of Materials**) while applying mild manual pressure throughout the

column to achieve flow rates at 1−2 drops/second. Wash columns with 5 mL of pure water and elute immediately with 1 mL of HPLC-grade methanol.

2.2.3. Dry elute analytes under nitrogen-streams or possibly using a vacuum concentrator (**Table of Materials**). Re-suspend analytes in 0.5 mL of ACN:water (1:9 by volume for DON and 3-*epi*-DON or 2:8 by volume for 3-keto-DON and DOM-1, respectively) after drying.

2.2.4. Pass the re-suspended samples through 0.45 µm filters (**Table of Materials**) before continuing to the HPLC analysis.

3. High performance liquid chromatography analysis

3.1. Analyze both samples and mycotoxin standards using any available HPLC system.

NOTE: In the provided protocol the results were obtained using an HPLC system (**Table of Materials**) equipped with a quaternary pump, an inline degasser, a diode array detector (DAD) with a wavelength set at 218 nm, and a reversed-phase column with a C18-guard column (**Table of Materials**) to achieve optimal separation described here.

3.2. Elute compounds of interest using a binary mobile phase composed of ACN:water (1:9 by volume for DON and 3-*epi*-DON, 2:8 by volume for 3-keto-DON and DOM-1, respectively).

3.3. Set the flow rate at 1.0 mL/min and the sample injection volume at 50 µL.

4. Validation of the protocol

4.1. Instrument linearity

4.1.1. Prepare DON, 3-*epi*-DON, and DOM-1 standard solutions ranging from 0.1 to 5.0 µg/mL. Prepare 3-keto-DON standard solutions that range from 0.2 to 5.0 µg/mL. Directly inject standard solutions into HPLC without any further extraction/dilutions.

4.1.2. Determine instrument linearity by inspecting the correlation between HPLC signals (peak areas) and the known concentrations of analytes.

4.2. Method linearity

4.2.1. Prepare corn extracts spiked with 3-keto-DON with concentrations of 0.05, 0.1, 0.2, 0.5 and 1.0 µg/mL. Prepare corn extracts spiked with DON, 3-*epi*-DON, and DOM-1 with concentrations of 0.1, 0.2, 0.5, 0.75 and 1.0 µg/mL. Extract and purify the spiked corn powders according to the steps described in section 2.2.

4.2.2. Determine method linearity by inspecting the correlation between HPLC signals (peak areas) and spiked concentrations of the analyte standards.

4.3. Method precision

4.3.1. Perform the intra-day analysis by spiking corn extracts with DON (and its three metabolites) in concentrations of 0.1 and 1.0 µg/mL (n = 5).

4.3.2. Perform the inter-day analysis by spiking corn extracts with DON (and its three metabolites) in concentrations of 0.1 and 1.0 µg/mL (n = 3).

4.3.3. Investigate the relative standard deviations (RSD) of intra-day and inter-day analyses.

4.4. Recovery

4.4.1. Calculate each analyte recovery rates of the tested corn extracts: the ratio of measured concentrations within corn extracts, prepared and extracted according to the reported procedure, to the actual spiked concentrations in these samples.

NOTE: Spiking levels of 0.1 and 1.0 µg/mL of DON, 3-*epi*-DON, 3-keto-DON, and DOM-1 (n = 5) are suggested for recovery rates determination.

4.5. Analytical limits

4.5.1. Determine the analytical limits through inspecting the signal-to-noise ratios (S/N).

NOTE: The limits of detection (LOD) are usually estimated at S/N = 3 and the limits of quantification (LOQ) are usually estimated at S/N = 10.

REPRESENTATIVE RESULTS:

Under the currently reported HPLC conditions, DON and its metabolites were completely separated and quantified. The retention times were 13.7 min and 9.4 min for DON and 3-*epi*-DON, respectively, using a mobile phase of 10% ACN in water. When applying 20% ACN in water, 3-keto-DON and DOM-1 appeared at 13.3 min and 6.3 min, respectively. The ultraviolet (UV) spectra of DON, 3-*epi*-DON, and DOM-1 are similar with maximum absorbance at 218 nm. 3-keto-DON displayed a distinct optical property with the maximum absorbance at 225 nm (**Figure 1**).

The correlations between method linearity and instrument linearity with the HPLC peak(s) areas and the standard/spiked concentration(s) of DON, 3-*epi*-DON, 3-keto-DON, and DOM-1 were excellent. For all analytes, the correlation coefficients (R^2) of method and instrument linearity were greater than 0.999 (**Table 1**).

The developed method showed high sensitivities towards DON, 3-*epi*-DON, and DOM-1. The LODs of these compounds were determined to be as low as 15.0, 7.5 and 5.0 ng/mL, respectively. Furthermore, the LOQs of these metabolites were determined to be 49.5, 24.8 and 16.5 ng/mL,

respectively. In contrast, 3-keto-DON displayed a higher LOD and LOQ, which were estimated at 25.0 ng/mL and 82.5 ng/mL, respectively.

The RSD of all compounds at the levels of 0.1 µg/mL and 1.0 µg/mL ranged from 1.2% to 6.8% (**Table 1**), which attested to the precisions of the developed approach.

Recoveries of 3-*epi*-DON, 3-keto-DON, and DON in corn extracts at the tested concentrations (0.1 µg/mL and 1.0 µg/mL) ranged from 74.1% to 94.0% (**Table 1**). However, DOM-1 showed a significantly lower recovery (60.8% of 1.0 µg/mL spiked DOM-1) after the immuno-affinity column purification.

FIGURE AND TABLE LEGENDS:

Figure 1: HPLC chromatograms of (A) DON, (B) 3-*epi*-DON, (C) 3-keto-DON and (D) DOM-1. All analytes (1.0 µg/mL) were spiked individually in corn extract and purified through the immune-affinity column with two folds concentration.

Table 1: The linearity, analytical limits (LOD and LOQ), precisions, and recovery rates of DON (and metabolites) using the developed method.

DISCUSSION:

Although the provided protocol focuses on robustness and ease-of-use, understanding the immuno-affinity column purification step is critical and should be carefully evaluated. In this protocol, the utilized affinity column contained a conjugated monoclonal antibody originally designed to specifically adsorb and enrich DON. The fact that DON and its three bacterial metabolites (DOM-1, 3-keto-DON, and 3-*epi*-DON) were able to bind to the purification column in our study (**Figure 1**) highlights the potential of this antibody/column to be implemented in applications intended for the detection of DON and its microbial metabolites when studying DON natural detoxifications. A recently established molecular modeling of DON (and its metabolites)[25] helped presumptively narrow down the shared epitope of these chemicals. The core sesquiterpene region is possibly the one responsible for the ability of this antibody to recognize and specifically bind DON and its three microbial metabolites[25], which in turn can empirically contribute to the simultaneous purification and determination of such analytes.

In practice, at least three consecutive preparation steps during the above column purification should be maintained to ensure the highest quality of analysis and best outcomes. First, the sample should be extracted and prepared in aqueous solution (water preferably) before the immuno-affinity column loading given the interference that organic solvents can introduce to antibody-antigen interactions. Samples extracted with organic solvents (e.g., methanol, acetonitrile, etc.) should be dried first and resolved in water. Second, the column maximum binding capacity of DON and its metabolites should be determined prior to any routine and regular utilization. A dilution step could be incorporated if the estimated concentration(s) approach or exceed the maximum theoretical binding capacity of the column. Finally, the flow rate of this immuno-affinity column should be kept constant as much as possible. If it is feasible,

a vacuum manifold system (commercially available) may be used to harmonize passage times among samples.

The observed outstanding instrument/method linearity, high sensitivity, and precise determination of DON and its metabolites are among the many strengths of the developed approach, despite its noticeable slightly lower sensitivity towards 3-keto-DON due to the lower UV absorption coefficient of this compound as previously reported[25]. Significantly lower recoveries of DOM-1 were also noticed after the immuno-affinity column purification. These are possibly related to the extreme conformational changes in the three-dimensional (3D) structure of DON resulting from the epoxy ring opening. These structural changes could influence interactions of DOM-1 with the investigated antibody, although such an affinity does not completely abrogate[25]. The two other metabolites (3-keto-DON and 3-*epi*-DON) that share a closer conformation to DON did not show any detectable decreases in recovery rates.

In summary, the developed protocol above provides an accurate, reliable, and reproducible method to quantitatively determine concentrations of 3-*epi*-DON, 3-keto-DON, and DOM-1 (in addition to DON) with minimum background interference emerging from the analyzed corn samples. The simultaneous analysis of these metabolites without the need for sophisticated instrumentation (such as liquid chromatography tandem mass spectrometry platforms) is another clear advantage of this protocol. These features will facilitate and boost future research efforts aiming to track and determine microbial biotransformation(s) and detoxification of DON through biological strategies. Further optimization and enhancement efforts of the above protocol can be extended to include other common DON derivatives (e.g., deoxynivalenol 3-glucoside) that might share the same 3D configuration/epitopes with DON to address many issues in the analysis and characterization of detoxification products and/or masked mycotoxins.

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DISCLOSURES:
Authors do not have any conflict of interests to disclose nor do they endorse the use of any product/technology/service over the other.

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Figure-1

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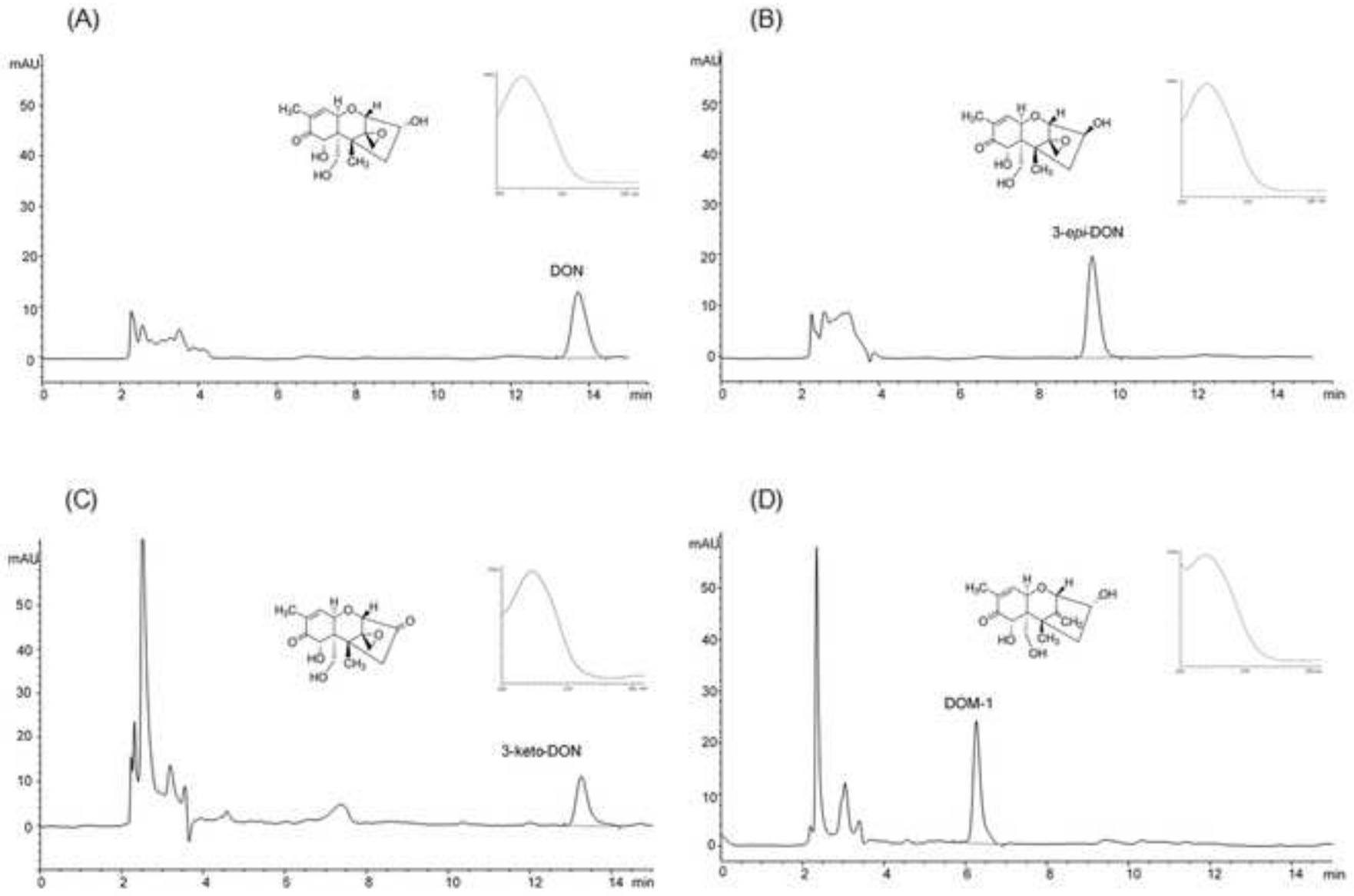

Table

Compounds	Instrumental linearity		Method linearity		LOD	LOQ	Intra-day RS
	Linear range (µg/mL)	R^2	Linear range (µg/mL)	R^2	(µg/mL)	(µg/mL)	0.1 µg/mL
DON	0.1-5.0	1.0000	0.05-1.0	0.9999	0.0150	0.0495	4.39
3-*epi*-DON	0.1-5.0	0.9999	0.05-1.0	0.9994	0.0075	0.0248	4.24
3-keto-DON	0.2-5.0	0.9999	0.1-1.0	0.9991	0.0250	0.0825	3.26
DOM-1	0.1-5.0	0.9997	0.05-1.0	0.9990	0.0050	0.0165	5.10

* The presented values represent the calculated means ± standard deviation (SD).

SD (%, n = 5)		Inter-day RSD (%, n = 3)		Recovery rates (%) within corn-extract samples (n = 5) at different concentrations (μg/mL)
1.0 μg/mL	**0.1 μg/mL**	**1.0 μg/mL**	**0.1 μg/mL**	**1.0 μg/mL**
1.24	4.02	2.92	76.0 ± 6.9*	74.1 ± 5.9
3.16	4.95	3.67	89.1 ± 6.2	94.0 ± 7.2
2.50	4.26	2.96	77.3 ± 3.8	75.2 ± 5.7
2.06	6.82	5.73	72.5 ± 3.0	60.8 ± 2.4

Name of Material/ Equipment	Company	Catalog Number
3-*epi* -DON	N/A	N/A
3-keto-DON	TripleBond	N/A
Acetonitrile	EMD	AX0145P1
Coarse filter paper (VICAM, MA, USA; Cat. #31242)	VICAM	31242
DOM-1	Sigma-Aldrich	34135
DON	Sigma-Aldrich	D0156
DONtest HPLC column	VICAM	G1005
Filter (0.45 µm) (Whatman, Florham Park, NJ, USA; Cat. #6765-1304)	Whatman	6765-1304
HPLC system	Agilent	1200
Laboratory-grade blender	Waring	N/A
Methanol	EMD	106035
Milled corn	N/A	N/A
Microfiber filter (VICAM, MA, USA; Cat. #31955)	VICAM	31955
Phenomenex 4µ Jupiter Proteo 90A (250 × 4.6 mm)	Phenomenex	00G-4396-E0
SpeedVac concentrator (SPD2010; Thermo Scientific, Waltham, MA, USA).	Thermo	SPD2010

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Yes, we double checked the manuscript and corrected spelling or grammar errors what we found.

2. Please complete and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

According to the policy of Canada government, I, as an employee of government, will get the authority to sign the agreement form only when the manuscript is accepted. Would you please go through the reviewing procedure? When the manuscript is accepted, I will provide the agreement form. Thank you for your understanding.

3. Please revise lines 220-225 to avoid textual overlap with previously published work.

We revised the description of Figure 1 (line 217-219).

4. Please ensure that the manuscript is formatted according to JoVE guidelines—letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and single-line spaces between all paragraphs and protocol steps/substeps. Do not underline any text in the protocol.

We modified the format accordingly.

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We made modifications accordingly.

6. Please list approximate volumes for all buffers and stock solutions to be set up.

We made modifications accordingly (line 120).

7. Please use imperative tense throughout the protocol as if directing someone how to do your experiment. Please be as specific as you can with respect to your experiment providing all necessary details.

We made modifications accordingly (line 124-132).

8. 1.2.2: What volume of acetoacetate is needed? Please specify. What container is used?

Please see the revision (line 108). We use acetonitrile (ACN) instead of acetoacetate.

9. 1.2.3: How to dry the vial?

Please see the revision (line 109)

10. 1.2.6: How to evaporate acetoacetate?

Please see the revision (line 113)

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We made modifications accordingly.

12. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

We highlight in yellow for protocol section.

14. JoVE is a methods-based journal. Thus, the discussion section of the article should be focused on the protocol and not on the representative results. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps in the protocol
b) Modifications and troubleshooting of the method
c) Limitations of the method

d) The significance of the method with respect to existing/alternative methods
e) Future applications or directions of the method

Please see the revision (line 224-274)

15. JoVE article does not have a Conclusion section. Please move information in the Conclusion section to Results or Discussion (as appropriate).

The conclusion was moved to the discussion section.

16. References: Please do not abbreviate journal titles; use full journal name. If there are six or more authors, list the first author and then "et al.".

We checked and modified accordingly.

17. Figure 1: Please submit multipanel figures (A, B, C, etc.) as a single image file that contains the entire figure.

We will submit the multipanel figure accordingly.

18. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

We checked and sorted the materials alphabetically.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript described a novel approach for the detection of deoxynivalenol and its derivatives by immuno-affinity enrichment and HPLC analysis. The method mentioned hereby was efficient and reliable without the need for the costly LC-MS/MS platforms.
Despite the paper is well prepared, some revisions should be done before it is published which is listed as follows:

Major Concerns:
1- Line 133, the authors used deionized water for mycotoxin extraction to adapt the following immuno-affinity clean-up procedure, however, in most previously studies, the trichothecenes were extracted by methanol or ACN solutions. So please clarify whether the change of extracting solvent would affect the recovery ratio.

DON and its metabolites studied in this work are well resolved in water although most previous studies suggested using 20-50% ACN or methanol solution. There is no significant difference in recovery based on our pre-test. Choosing water instead of organic solvents facilitated the immune-affinity column purification because the organic solvent in sample should be removed before the operation.

2- The reproducibility of the method was elaborated in the manuscript, nevertheless, all the results were obtained from one single HPLC. So the authors should add the results acquired from other instrument(s) if possible.

We thank for the suggestion from the reviewer. The term of “reproducibility” using in the description of Table 1 was not corrected since no inter-laboratory analysis were performed. We changed the “reproducibility” to “precision” in order to reflect our work.

Reviewer #2:
Manuscript Summary:
This manuscript will be reconsidered after minor revision.

Minor Concerns:
Abstract Line 73 "detect three of DON's bacterial metabolites", why „bacterial metaboltes"? There is a mistake.

Although DON is the secondary metabolite of fungi (mainly *Fusarium* spp.), the 3-*epi*-DON, 3-keto-DON, and DOM-1 mentioned in this manuscript were reported as biotransformation products with less/none toxicity, which were produced by various bacteria. That is the reason we used the term of “bacterial metabolites”.