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Doehlert Matrix Design for Optimization of the Determination of Bound Deoxynivalenol in Barley Grain with Trifluoroacetic Acid (TFA)

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Fusarium head blight (FHB) is an impediment to barley production in many regions of the world. Tricothecene toxins, associated with FHB-infected grain, particularly, deoxynivalenol (DON), pose a serious threat to human and animal health. Recent research has suggested that a portion of the DON present on grain is bound and escapes detection through conventional determination. The objective of this study was to optimize a method for determination of nonextractable DON in barley grain using trifluoroacetic acid (TFA). A Doehlert matrix design was performed to determine the optimal conditions for time, temperature, and TFA concentration. These conditions were treated with 1.25 N TFA in 86:14 acetontrile/water for 54 min at 133 °C. Cleanup, derivatization, and determination of DON by a gas chromatography electron capture detector (GC−ECD) was as normal. Treatment of the test sample resulted in the release of an additional 58% DON under the optimized conditions and an increase of 9−88% in a set of verification samples.

KEYWORDS: Doehlert matrix design; optimization; bound deoxynivalenol; TFA

INTRODUCTION

Serious epidemics of *Fusarium* head blight (FHB) in wheat and barley have been experienced in North America since the early 1990s (*1*), and outbreaks have also been reported in Asia, Europe, and South America (*2*–*9*). Because the species of *Fusarium* that act as FHB pathogens are known to produce trichothecene mycotoxins and zearalenone (ZEA), the widespread occurrence of FHB poses a serious threat to the world's food supply.

Within the upper midwestern U.S.A., *Fusarium graminearum* has been shown to be the primary pathogen in barley (*10*). Deoxynivalenol (DON) has been the predominant mycotoxin detected in barley in this region (*11*), and from 1993 to 2003, DON has been detected (>0.5 μg/g) in 32−81% of barley in North Dakota and Minnesota. Levels in individual samples have ranged from 0.5 to 60.0 μg/g. Testing for DON has become routine within this region, and barley with more than very low levels of DON (>0.5 μg/g) is not accepted for malting and brewing (*1*). The other primary market for barley is livestock feed, and while ruminant cattle can tolerant higher levels of DON, DON contaminated grain is problematic for swine (*7*). Because approximately 25–30% of the barley in the U.S.A. is produced within this region, there has been a significant economic impact upon both regional growers and the national industry (*1*, *12*).

While FHB infection of barley can damage grain quality, the largest problem relates to the presence of mycotoxins. DON present in the malted barely has been shown to be extractable during brewing (*13*), and DON has been detected in commercial beers in several surveys (*14*, *15*). DON levels are often observed to decline during the steeping phase of malting but occasionally are observed to increase during the germination phase and then persist in the finished malt (*13*). The decline is presumably due to solubilization during the steeping process, and the increase is due to the growth of *Fusarium* during germination, with the production of additional toxin. As an alternative, Havlová et al. (*16*) and Váňová et al. (*17*) have attributed the increase to the release of bound mycotoxins from the grain matrix through enzymolysis during malting. We have recently observed that pretreatment of FHB-infected barley samples with papain or a mixture of cell-wall-degrading enzymes lead to the release of up to an additional 28% DON (Zhou, unpublished data).

Bound, conjugated, or masked mycotoxins may be defined as those that escape detection or are not extractable during routine analytical procedures. In the case of masked or conjugated mycotoxins, the toxin is usually covalently bound to a more polar substance, such as glucose (*18*). Conjugation of mycotoxins to form glycosides and glucuronides has been reported as a mechanism

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by which plants metabolize mycotoxins (*19*, *20*). Transformation would represent an alternate metabolic mechanism (*21*). The presence of bound DON or other mycotoxins has a number of interesting ramifications. While these toxins may not be detected in raw materials by routine analytical methodology, they may be released under food-processing conditions or during digestion. It has also been speculated that binding may be a means by which the plant is able to detoxify the toxin and may act as a resistance mechanism (*22*).

ZEA glucosides were produced in maize cell culture (*19*), and it was later demonstrated that zearalenone-4-β-D-glucopyranoside could be decomposed to release ZEA during digestion in swine (*23*). Schneweis et al. (*24*) developed a liquid chromatography−mass spectrometry (LC−MS) method for analysis of zearalenone-4-β-D-glucopyranoside and reported that it was found occurring in naturally infected wheat samples. Savard (*21*) reported the chemical synthesis of DON conjugates, including eight fatty acid esters and two glucosides. In 2005, Berthiller et al. (*18*) first reported the natural occurrence of DON-3-glucoside in *Fusarium*-infected wheat. They used a liquid chromatography−tandem mass spectrometry (LC−MS/MS) method and detected the DON-glucoside in all wheat samples ($n = 5$) and two of three contaminated maize samples analyzed. The amount of DON-glucoside measured represented from 4 to 12% of the free DON.

Detoxification of mycotoxins in plants (by chemical modification) is considered as a possible defense mechanism. Poppenberger et al. (*25*) reported that in *Arabidopsis thaliana* a UDP-glucosyltransferase has the ability to form DON-3-*O*-glucoside for detoxification of *Fusarium* mycotoxins. This compound showed a reduced ability to inhibit protein synthesis *in vitro*. Lemmens et al. (*22*) indicated that the biochemical mechanism(s) of resistance to DON includes the catabolism and conjugation of DON, and in resistant wheat lines, the detoxification process included the conversion of DON to DON-3-glucoside. While this work demonstrates the potential importance of glycosylation, associations between mycotoxins and other plant components, such as phenolics and proteins (*18*, *26*, *27*), have also been suggested. It would seem possible that there are several mechanisms involved in the immobilization of mycotoxins. Noncovalent associations have also been reported in the binding of mycotoxins. The addition of yeast cell walls to contaminated feeds has been shown to reduce the harmful effects of mycotoxins in animals, and Jouany et al. (*28*) showed that weak hydrogen and van der Waals bonds play a role in the binding between ZEA and β-(1,3)- and β-(1,6)-D-glucans in yeast cell walls.

The determination of DON is normally carried out using enzyme-linked immunosorbent assay (ELISA), gas chromatography (GC), or high-performance liquid chromatography (HPLC) (*29*–*31*), following solvent extraction. However, such analytical methods are only suitable to determine extractable mycotoxins, and bound mycotoxins would likely escape detection. Berthiller et al. (*18*) used LC−MS/MS for the determination of DON-glucosides. However, this equipment is not available in many laboratories. Liu et al. (*32*) proposed a method to determine bound DON in FHB-infected wheat samples. *Fusarium*-infected wheat samples were extracted with 84:16 acetonitrile/water as per normal analytical procedures for DON. Either the residue remaining after extraction or the extract itself was then hydrolyzed with trichloroacetic acid (TCA, 1 N) at 140 °C for 40 min. After solvolysis, samples were neutralized, passed through a cleanup column, and then derivatized as for their standard GC−MS procedure. They found that an additional 13−63% total DON was released after TCA treatment. DON was found to be released from both the extract and residue, suggesting differences in the solubility/extractability of the bound DON.

Although this method is useful in providing information on the percentage of bound DON, the multiple extraction and solvolysis steps are not amenable to the analysis of a large number of samples. Treatment of a single grain sample may be preferred for routine screening of grain samples. In addition, preliminary results in our laboratory with TCA showed that a higher concentration of TCA (2.5 N) and higher temperatures (155 °C) were needed for optimal solvolysis of bound DON (Zhou, unpublished data). However, these conditions are not safe, leading to occasional leakage of the sample, even from pressure tubes. Resultant chromatograms showed the formation of other compounds under the high-temperature conditions, which co-eluted with DON, making integration problematic.

For these reasons, we suggest a modification of the procedure of Liu et al. (*32*) using TFA. TFA has been found to be useful for the hydrolysis of neutral sugars in glycoproteins and soil carbohydrates (*33*–*35*), the hydrolysis noncellulosic polysaccharides (*36*, *37*), and is used to cleave nitrogen- and oxygen-protecting groups under various conditions, such as Boc and *t*-butyl groups in the synthesis of peptides (*38*). In comparison to TCA ($pK_a = 0.08$), the acidity of TFA ($pK_a = 0.3$) is stronger and its volatility (bp 72 °C) allows it to be easily removed by evaporation without neutralization.

The objective of this study was to optimize the conditions for release of bound DON in FHB-infected barley samples by TFA solvolysis, using response surface methodology (RSM). New methods for extracting and analyzing bound DON in raw grains and finished foods are important for safeguarding the health of humans and domestic animals. RSM is a useful mathematical tool for the development and optimization of processes. The Doehlert matrix design (*39*) was selected for the current study, because it is an effective method, requiring a lower number of experiments than the central composite design (*40*).

MATERIALS AND METHODS

Materials. DON (95%), 3-acetyl-deoxynivalenol (99.9%), 15-acetyl-deoxynivalenol (99.9%), and nivalenol (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO). TFA (99%) was purchased from VWR International (West Chester, PA).

A single sample of barley containing 10 μg/g of DON was selected for the optimization experiments, while 8 samples were used for validation of the method. These samples of DON-contaminated barley were obtained directly from barley producers and NDSU Agricultural Experiment Station Plots at various locations in North Dakota. The samples were of multiple cultivars, from multiple crop years, and were selected to provide a range in DON. Samples were stored at 4 °C until use. All samples were ground with a Udy mill (Udy, Corp., Fort Collins, CO) fitted with a 0.5 mm screen. This yields a particle size distribution of 0.3, 9.5, 11.8, 78.0% through U.S. number 20, 40, 60, and 80 sieves, respectively.

DON Determination. DON was determined according to the method of Tacke and Capser (*41*). Ground samples (5 g) were extracted with 40 mL of acetonitrile/water (84:16) on a reciprocating shaker for 1 h. The extract (4 mL) was applied to an alumina C18 cleanup column (catalog number 229101, Alltech-Grace Davison Discovery Sciences, Deerfield, IL). The eluent (2 mL) was transferred to a fresh test tube and dried at 55 °C under water-free nitrogen until completely dry. DON was then derivatized with 100 μL of trimethylsilylimidazole (TMSI) and trimethylchlorsilane (TMCS) (10:1) for 30 min. Iso-octane (1.0 mL) containing Mirex (0.5 μg/mL), as an internal standard, was added along with 3% sodium carbonate solution (1.0 mL). The solution was

Table 1. Deohlert Experimental Design Matrix with Total DON Response following TFA Solvolysis of Barley Grain[a]

	coded variables			uncoded variables			response
runs	X1 (temperature)	X2 (time)	X3 (concentration)	A (temperature, °C)	B (time, min)	C (concentration, N)	DON (ppm)
1	0.0	0.000	0.000	135	55	1.5	20.56
2	1.0	0.000	0.000	145	55	1.5	15.12
3	0.5	0.866	0.000	140	70	1.5	13.07
4	0.5	0.289	0.817	140	60	2.0	11.54
5	−1.0	0.000	0.000	125	55	1.5	19.40
6	−0.5	−0.866	0.000	130	40	1.5	18.15
7	−0.5	−0.289	−0.817	130	50	1.0	18.53
8	0.5	−0.866	0.000	140	40	1.5	17.78
9	0.5	−0.289	−0.817	140	50	1.0	19.34
10	−0.50	0.866	0.000	130	70	1.5	16.32
11	0.0	0.577	−0.817	135	65	1.0	19.28
12	−0.5	0.289	0.817	130	60	2.0	13.89
13	0.0	−0.577	0.817	135	45	2.0	15.12

[a] Each run was conducted in triplicate, and mean values for total DON are given.

shaken vigorously to break up the carbonate and nonpolar layers and then shaken moderately for 10 min to extract DON into the iso-octane layer. The derivatized sample in iso-octane (1 μL) was then injected into a gas chromatography electron capture detector (GC−ECD).

A gas chromatograph (Agilent model 6890) equipped with dual ^{63}Ni ECDs, dual HP samplers, dual on-column injectors, and dual capillary columns was controlled by ChemStation chromatography software (Agilent, Palo Alto, CA). The columns were a Rtx-5 column (30 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA) equipped with a 2 m × 0.53 mm i.d. phenylmethyl-deactivated guard column (Restek, Bellefonte, PA). The GC oven temperature was initially set at 70 °C, increased to 170 °C at a rate of 25 °C/min, raised to 300 °C at a rate of 5 °C/min, and then held at 300 °C for 10 min. With a cool on-column inlet, the temperature of injectors was programmed as starting at 90 °C and increasing to 300 °C at a rate of 20 °C/min. The temperature of the detectors was 300 °C. The carrier gas was helium, with a constant pressure of 20 psi. The makeup gas for ECDs was argon/methane (95:5), with a combined flow rate of 60.0 mL/min. For DON derivatized with TMSI/TMCS (10:1), the limit of quantification (LOQ) was 0.4 μg/g and the range for detection was up to 120 μg/g, while LOQ of both 3- and 15-ADON was 0.04 μg/g based on the standard curve used on our GC−ECD.

Solvolysis of Bound DON with TFA. The solvolysis method was modified from that described by Liu et al. (*32*) using TCA. Acetonitrile/water (84:16, v/v, 40 mL) was added to the barley samples (5 g) in a 50 mL screw-cap glass test tube (Teflon rubber liner). A total of 1 mL of TFA (1.0−2.0 N) was added into the tube, and the tube was tightly capped and heated in an oven at 125−145 °C for 40−70 min, as specified in the experimental design (**Table 1**). After solvolysis, the tube was cooled to room temperature, 4 mL of the upper extract was passed through the alumina C18 cleanup column, and the normal procedure for the determination of DON was followed. An adjustment was made to the calculations to account for the additional 1.0 mL of volume.

The recovery of DON under the final optimized conditions of solvolysis was also determined. The stock solution of DON (5 mg) and 3- and 15-ADON (1 mg) (Sigma-Aldrich, St Louis, MO) was dissolved in acetonitrile. The final concentration of the DON standard was determined by measuring A_{219} in methanol according to Bennett and Shotwell (*42*) (coefficient of extinction = 5913). Different amounts (1.25−5.00 mL) of the DON and (0.20−1.00 mL) 3- and 15-ADON standard solutions were then spiked into ground samples (5.0 g) of trichothecene-free (clean) barley (**Table 4**). The samples were then subjected to extraction with acetonitrile/water (84:16) and TFA solvolysis (1.25 N TFA) for 54 min at 133 °C. The reported recovery rate is the ratio of the observed value to the theoretical value.

Experimental Design. Preliminary experiments with TFA solvolysis (data not shown) indicated that the treatment factors, time, temperature, and acid concentration, had significant effects on the release of DON and helped to define the range of factors used in the current study.

The Doehlert design (*43*) can be employed to generate response surfaces with a good estimation of the parameters of the quadratic mathematical model, allowing the study of three independent variables at a different number of levels. The Doehlert design shows great flexibility compared to more classical designs used in process optimization. Points of the network can be reused to build and explore adjacent domains, and new variables can be added during a study, without quality loss for the model.

The whole experimental domain is explored with a minimum number of experiments, following a sequential approach, first studying only two factors, then three factors, and so on. According to the Doehlert design, the total number of experimental combinations is $k^2 + k + n_0$, where k is the number of independent variables and n_0 is the number of repetitions of the experiments at the center point. The experimental domain is designed by its center and the variations of each factor around the center.

For statistical calculations, the experimental variables X_i have been coded as x_i according to the following transformation equation (eq 1):

$$x_i = \frac{X_i - X_i'}{\Delta X_i} \times \alpha \quad (1)$$

where X_i' is the real value at the center of the experimental domain, ΔX_i is the step of variation of the real value, and α is equal to 1, 0.866, and 0.816 for the first, second, and third factor, respectively. The design generated 13 combinations of factors (runs), and the entire experiment was replicated 3 times. The experimental design factors of time, temperature, and acid concentration for TFA solvolysis, as well as the corresponding mean values (total DON), are shown in **Table 1**.

Statistical Analyses. The results of the experiments were tested using the Lagrange criteria (*44*) for three-variable functions. The second-order model for three variables (X_1, X_2, and X_3) is given by eq 2

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}^2 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 \quad (2)$$

where Y (total DON) is the experimental response to be optimized, A_0 is the constant term, A_1, A_2, and A_3 are coefficients of the linear terms, A_{11}, A_{22}, and A_{33} are coefficients of the quadratic terms, and A_{12}, A_{13}, and A_{23} are coefficients of the interaction between the three factors. The fitness of the above polynomial model can be evaluated by the coefficient of determination R^2. Independent variables that are found significant at $p < 0.05$ in the full model are retained in the reduced models. Those variables that were significant ($p < 0.05$) with models that had $R^2 > 0.75$ were used to generate response surface plots for total DON as a function of two variables, while the other variable was held constant.

If the quadratic function only shows one stationary point, four situations are possible: (1) there is no information, $\Delta_2 = 0$; (2) relative maximum, $\Delta_1 < 0$, $\Delta 2 > 0$, and $\Delta_3 < 0$; (3) relative minimum, $\Delta_1 > 0$, $\Delta_2 > 0$, and $\Delta_3 > 0$; and (4) saddle point, none of the above situations applies, where Δ_3 is the Hessian determinant of the function $H(X_1, X_2,$ and $X_3)$ and Δ_2 and Δ_1 are calculated using the following equations:

$$\Delta_1 = \frac{\partial^2 Y}{\partial X_1^2} \quad (3)$$

$$\Delta_2 = \begin{pmatrix} \frac{\partial^2 Y}{\partial X_1^2} & \frac{\partial^2 Y}{\partial X_1 \partial X_2} \\ \frac{\partial^2 Y}{\partial X_2 \partial X_1} & \frac{\partial^2 Y}{\partial X_2^2} \end{pmatrix} \quad (4)$$

$$\Delta_3 = \begin{pmatrix} \frac{\partial^2 Y}{\partial X_1^2} & \frac{\partial^2 Y}{\partial X_1 \partial X_2} & \frac{\partial^2 Y}{\partial X_1 \partial X_3} \\ \frac{\partial^2 Y}{\partial X_2 \partial X_1} & \frac{\partial^2 Y}{\partial X_2^2} & \frac{\partial^2 Y}{\partial X_2 \partial X_3} \\ \frac{\partial^2 Y}{\partial X_3 \partial X_1} & \frac{\partial^2 Y}{\partial X_3 \partial X_2} & \frac{\partial^2 Y}{\partial X_3^2} \end{pmatrix} \quad (5)$$

Initial information about of the geometrical nature of the surface can be obtained from the signs and magnitudes of the quadratic coefficients in the polynomial functions. If all of these coefficients are negative, the function can show a maximum; if all of these coefficients are positive, the function can show a minimum; and when some are positive and others are negative, the stationary point can be a saddle point with a relative maximum for the variables with a negative term and with a relative minimum for the ones with a positive coefficient.

Data from the Doehlert design shown in **Table 1** were used to determine the regression coefficients of a second-order multiple regression model. The Design-Expert package (version 7.0.2; Stat-Ease, Minneapolis, MN) was employed for regression analysis of the data and estimation of the coefficients of the regression equation. The statistical significance of the model was determined by the application of Fisher's *F* test. The canonical analysis was also carried out to predict the shape of the curve generated by the model. The two-dimensional graphical representation of the system behavior, called the response surface, was used to describe the individual and cumulative effects of the variables as well as the mutual interactions between the independent and dependent variables.

RESULTS AND DISCUSSION

Regression Models of the Response for the Optimization of TFA Solvolysis Conditions for Total DON. The objective of this work was to optimize the solvolysis and extraction of DON using TFA. The resultant values for DON are assumed to consist of both initially free DON and DON that was liberated by solvolysis. The barley sample selected in the optimization experiment contained 12.99 μg/g DON ($\pm$0.72 μg/g) when analyzed by the standard procedure (no solvolysis). Thus, subtraction of 12.99 μg/g from the resultant total DON value (TFA solvolysis) provides an estimate of bound DON. The sample also contained 0.44 μg/g 15-ADON, 0.59 μg/g 3-ADON, and 0.32 μg/g nivalenol.

Table 1 shows the maximum and minimum levels of the experimental variables, time, and temperature, and TFA concentration, chosen for trials in Doehlert design. The variables used for the factorial analysis were designated as X_1, X_2, and X_3 in this design, respectively. There was considerable variation in the amount of DON following solvolysis. The maximum DON (20.56 μg/g) value was achieved in run number 1 (135 °C, 55 min, and 1.5 N TFA) while the minimum DON (11.54 μg/g) was observed in run number 4 (140 °C, 60 min, and 2.0 N TFA). Increasing the temperature, TFA concentration, and time beyond the maximal conditions did not help to increase the release of bound DON. Although DON is stable under the high-temperature conditions (*45*), the exact mode by which DON is bound is not clear. Chemical conjugation has been demonstrated (*18*), but weaker associations and matrix effects are also possible. The association between ZEA and the β-(1,3)- and β-(1,6)-D-glucans of yeast cell walls was related to weak hydrogen and van der Waals bonds (*28*). Determination of the mode of binding will require additional experiments and may be aided by the use of model compounds.

Table 2. ANOVA for the Response of Total DON following TFA Solvolysis[a]

sources	degree of freedom	sums of square	R^2	*F* value	$p > F$
linear	3	69.43		42.61	0.0059
quadratic	3	16.16		9.92	0.0457
cross-product	3	7.97		4.89	0.1125
total model	9	93.56		19.14	0.0168
total error	3	1.63	0.54		

[a] Total model: CV = 4.39, R^2 = 0.9829, and R = 0.9914.

Table 3. ANOVA and Regression Coefficients from the Polynomial Model for Total DON following TFA Solvolysis

parameter	degree of freedom	estimate	standard error	*t* value	$p > \|t\|$
intercept	1	20.56	0.74	27.90	0.0001[a]
X1	1	−1.72	0.37	−4.65	0.0187[a]
X2	1	−1.71	0.37	−4.65	0.0188[a]
X3	1	−3.39	0.37	−9.19	0.0027[a]
X1 × X1	1	−3.30	0.90	−3.66	0.0353[a]
X1 × X2	1	−1.66	0.85	−1.95	0.1457
X2 × X2	1	−4.54	0.90	−5.03	0.0151[a]
X2 × X3	1	−1.35	0.95	−1.42	0.2520
X1 × X3	1	−2.38	0.95	−2.50	0.0876
X3 × X3	1	−4.45	0.88	−5.08	0.0148[a]

[a] Significant at a 5% level ($p < 0.05$).

Testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA), and the results are presented in **Table 2**. It is evident that the total model is highly significant, as judged from the *F* and probability values. ANOVA (*F* test) showed that the quadratic model is well-adjusted to the experimental data. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared, and in the current study, the low CV (4.39) indicates better precision and reliability of the experiments. The fitness of the model can be checked by the determination coefficient (R^2) and correlation coefficient (R). In the current study, the R^2 implies that the sample variation of 98.29% for total DON (including free DON and bound DON) is attributed to the independent variables, and only about 1.71% of the total variation cannot be explained by the model. Linear and quadratic terms were both at a significant ($p \leq 0.05$) level. Therefore, the second-order model was employed in this optimization study.

The Student *t* distribution and the corresponding *p* values, along with the parameter estimates, are given in **Table 3**. The *p* values are used as a tool to check the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the *p* values, the greater the significance of the corresponding coefficient (*46*). This implied that the first- and second-order main effects of time, temperature, and TFA concentration are highly significant. Negative coefficients for all of the three variables indicated that the total DON content generally decreased with an increase in the value of the variables. The interactions between the three variables were found to be insignificant ($p > 0.05$) to the model.

Comparison of the Observed and Predicted Total DON following TFA Solvolysis. Figure 1 compares observed values

Table 4. Recovery of Trichothecenes Spiked in Barley and Subjected to TFA Solvolysis[a]

amount of trichothecene standard stock spiked in 5 g of clean barley (mL)	theoretical concentration of trichothecene in clean barley (μg/g)	trichothecene measured in spiked samples (μg/g)[b]	recovery rate (%)
	DON		
1.25	6.61	5.82	88.0
2.50	13.22	11.45	86.6
3.75	19.82	18.36	92.6
5.00	26.42	22.39	84.7
	15-ADON		
0.25	0.20	0.33	165.0
0.50	0.40	0.70	175.0
0.75	0.60	0.83	138.3
1.00	0.80	1.02	127.5
	3-ADON		
0.25	0.20	0.38	190.0
0.50	0.40	0.68	170.0
0.75	0.60	0.89	148.3
1.00	0.80	0.92	115.0
	nivalenol		
0.25	1.35	1.58	117.0
0.50	2.71	2.48	91.5
0.75	4.06	3.71	91.4
1.00	5.41	5.22	96.5

[a] Solvolysis with 1.25 N TFA at 133 °C for 54 min. [b] Values are the mean of three treatments.

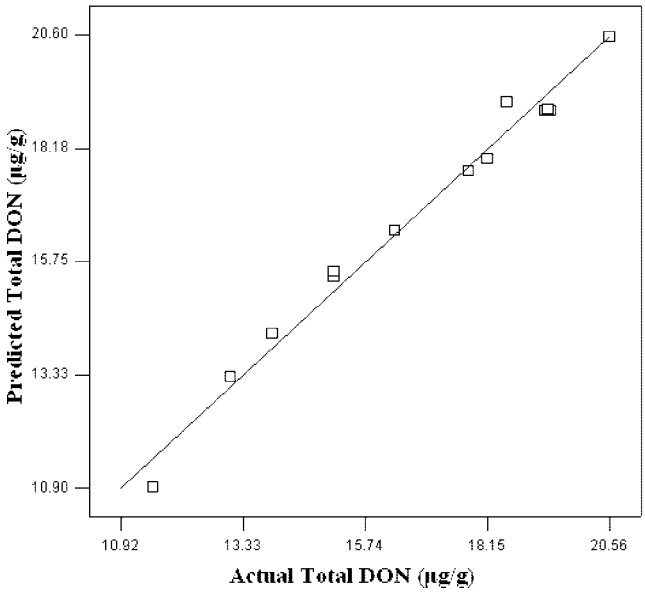

Figure 1. Observed DON response following TFA solvolysis versus DON predicted by the polynomial model.

for DON (the response) with values predicted from the empirical model (eq 3). The results strongly suggest that the predicted data for the total DON from the empirical model are in agreement with the observed values, within the range of the operating variables used in this study. However, it must be considered that, while a model fits well within the region of the original data, it will no longer fit outside the region.

Localization of the Optimal Condition for DON Determination with TFA Solvolysis. Figure 2A depicts the 3D plot and its corresponding contour plot, showing the effects of temperature and time on total DON, while the TFA concentration was fixed at the middle level (1.5 N). **Figure 2B** indicates that the total DON increased from the time of 40 to approximately 55 min and then decreased with additional time. It was the same situation with temperature. The DON response increases when the temperature increased from 125 to less than 135 °C and then fell as temperatures were above 135 °C. This suggests that increasing the temperature from 125 to 135 °C and the time from 40 to 55 min contributed to the release of bound DON in this barley sample. This could be explained by the fact that the bound DON in the sample was easier to be cleaved and released by acid solvolysis of TFA under such a test range. The analysis of **Figure 2** shows that the optimal ranges of the temperature and time for total DON content were 131−134 °C and 50−55 min, respectively. The maximum predicted value of total DON was obtained as 20.9 μg/g (time, 53 min; temperature, 133 °C) using Design-Expert software.

Figure 3A presents the 3D plot and its corresponding contour plot showing the effects of the reaction time and acid concentration on the determination of total DON when the temperature was fixed at the middle level (135 °C). **Figure 3B** indicates that the total DON increased from the time of 40 to approximately 55 min and then decreased with time. The effect of the acid concentration was the same in the fact that total DON increased with the TFA concentration from 1.00 to 1.35 N and then decreased at concentrations higher than 1.35 N. This also suggests that increasing the acid concentration from 1.00 to 1.35 N and the time from 40 to 58 min contributed to the release of bound DON. The analysis of **Figure 3** shows that the optimal ranges of the acid concentration and time for total DON content were 1.22−1.34 N and 52−55 min and the maximum predicted value was obtained as 21 μg/g (time, 53 min; concentration, 1.28 N).

Figure 4A shows the effects of the acid concentration and temperature on the determination of total DON when the reaction time was fixed at the middle level (55 min). **Figure 4B** indicates the total DON increased starting from the temperature of 125 to 137 °C and acid concentration from 1.00 to 1.40 N and then decreased with the temperature and acid concentration out of the range mentioned above. It suggests that increasing the acid concentration from 1.00 to 1.40 N and the temperature from 125 to 137 °C contributed to the release of bound DON. Then, with the additional increase of the acid concentration and reaction temperature, it might be that the bound DON is destroyed or the following procedure to determine DON is affected. It is also possible that the products produced under the high-temperature acid conditions, such as Maillard reaction products, might affect the derivatization of DON and other mycotoxins and decrease the total DON value analyzed on GC−ECD. The analysis of **Figure 4** shows that the optimal ranges of the acid concentration and temperature for total DON content were 1.24−1.34 N and 132−134 °C, respectively, and the maximum predicted value was obtained as 21.3 μg/g (concentration, 1.26 N; temperature, 133 °C).

Adequacy of the Model. Figure 5 shows a normal probability plot of the residuals. This is needed to check the normality assumption in the fitted model, to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface will likely give poor or misleading results. As seen in **Figure 5**, the normality assumption was satisfied as the residual plot approximated a straight line. The general impression is that the residuals scatter randomly on the display, suggesting that the variance of the original observation is constant for all values of total DON. Both of the plots (**Figures 1** and **5**) are satisfactory; therefore, we conclude that the empirical model is adequate to describe total DON (including free and bound DON) by the response surface.

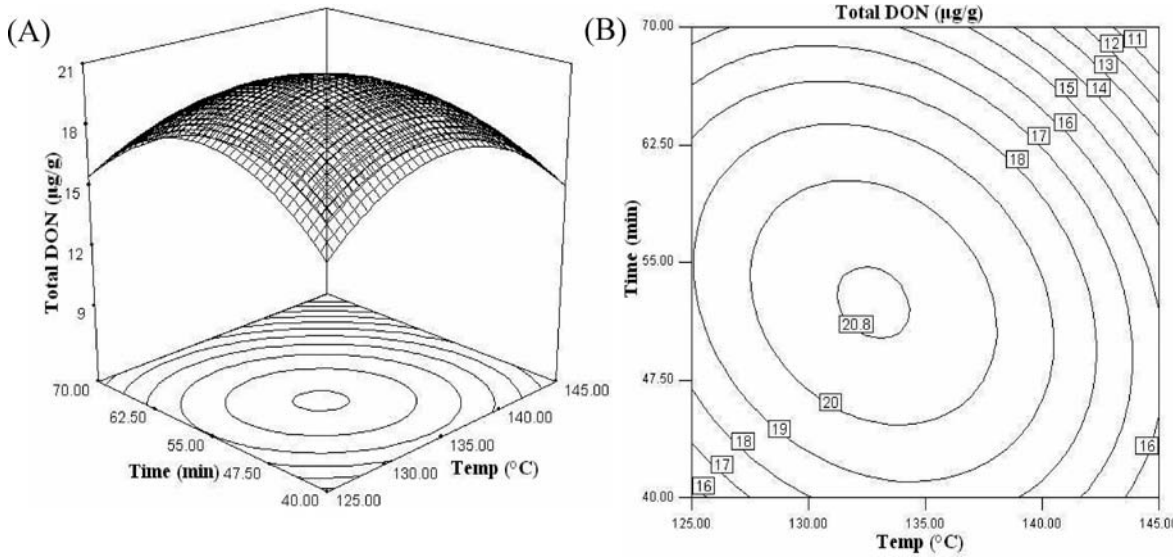

Figure 2. Response surface plot (**A**) and contour plot (**B**) of the combined effects of time and temperature on the determination of total DON under a constant concentration of 1.5 N TFA.

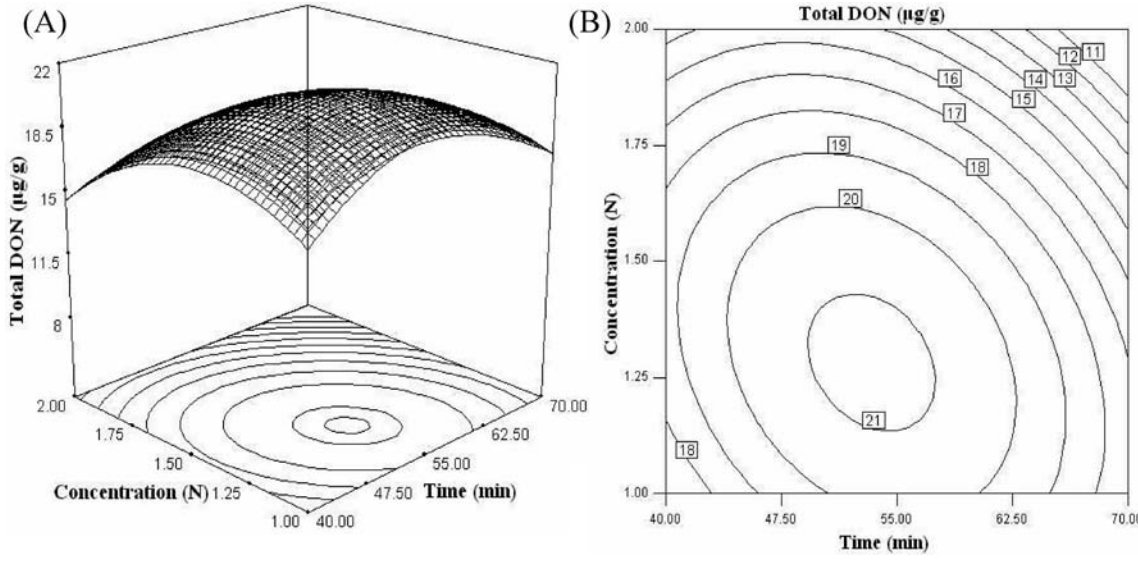

Figure 3. Response surface plot (**A**) and contour plot (**B**) of the combined effects of the TFA concentration and time on the determination of total DON with a constant temperature (135 °C).

Application of Lagrange's Criterion to the Model. Multiple regression analysis showed that the following second-order polynomial equation was able to explain the response of total DON following TFA solvolysis regardless of the significance of coefficients:

total DON = 20.56 − 1.72(temperature) − 1.71(time) − 3.39(concentration) − 3.66(temperature)^2 − 4.54(time)^2 − 4.45(concentration)^2 − 1.66(temperature)(time) − 2.38(temperature)(concentration) − 1.35(time)(concentration).

Application of the Lagrange criterion aims to test whether the response surface is a minimum, maximum, or saddle point in the whole statistical model.

$$\Delta_1 = -6.6 < 0$$

$$\Delta_1 = 57.2 > 0$$

$$\Delta_3 = -456.0 < 0$$

The values of $\Delta_1 < 0$, $\Delta_2 > 0$, and $\Delta_3 < 0$ demonstrated that there is a maximum point. The canonical analysis from SAS analysis (SAS software, version 9.13) also confirmed that the maximum point is a stationary point. The stationary point presenting the maximum total DON had the following critical values: 20.89 μg/g under these conditions.

Validation of the Model. The stationary point in this model was obtained for the maximum response of the total DON content (20.89 μg/g) under the following critical values of the three variables: temperature, 133 °C; reaction time, 54 min; and TFA concentration, 1.25 N.

Two repeat determinations of total DON in the same barley sample under optimal conditions were carried out for verification. The resultant total DON was 18.65 and 19.17 μg/g, respectively. These values were found to be 6–10% less than the predicted value but fall within the expected analytical variation of DON in grain samples. A CV of 5–15% for this analysis by GC−ECD is normal (*47*) and may be due to the variation in sampling and experimental conditions, such as derivatization and GC−ECD analysis conditions.

Recovery of Mycotoxin Standards following TFA Solvolysis. The recovery of a DON standard spiked onto trichothecene-free barley was investigated under the optimal solvolysis

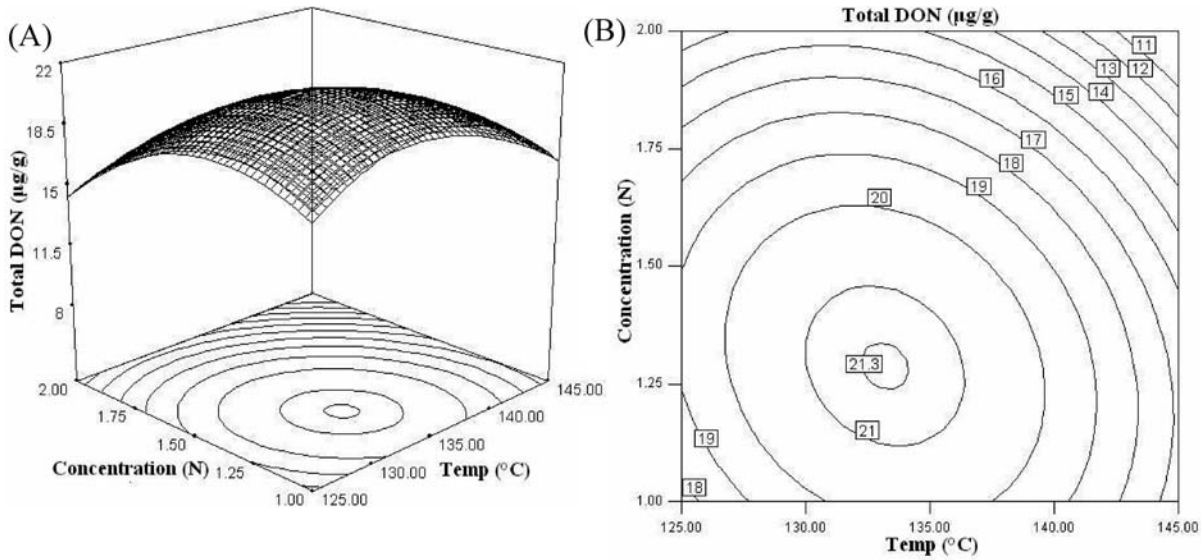

Figure 4. Response surface plot (**A**) and contour plot (**B**) of the combined effects of the TFA concentration and temperature on the determination of total DON with a constant time (55 min).

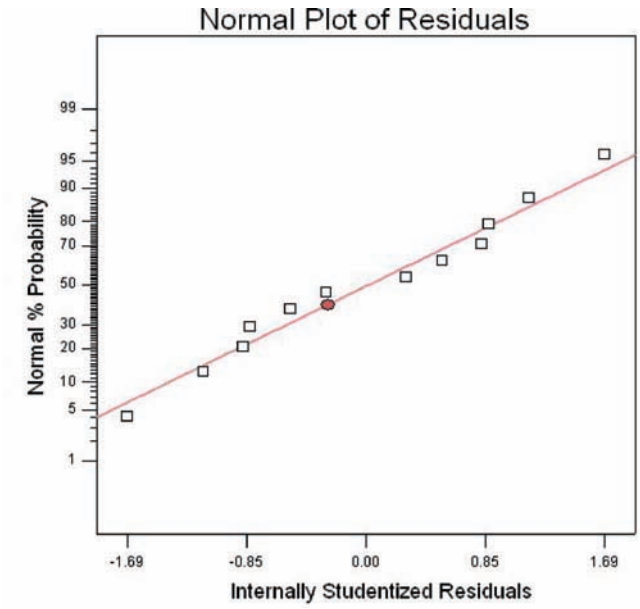

Figure 5. Normal probability of internally studentized residuals.

conditions (133 °C, 54 min, and 1.25 N TFA). Four samples were spiked to yield theoretical concentrations of 6.21−24.83 μg/g DON. These concentrations were based on the absorbance at 219 nm (*42*). Observed recoveries following TFA solvolysis ranged from 85 to 93% (**Table 4**). Tacke and Casper (*41*) also reported that the recovery of DON was 100–105% in barley samples. This suggests little to no degradation of DON under the optimized solvolysis conditions. However, it can be seen from the Doehlert matrix in **Table 1** that increases in the TFA concentration, reaction time, or temperature did lead to reductions in DON recovered. Trace amounts of 3- and 15-ADON were detected in the DON recovery study. However, because these were detected in both the control and hydrolyzed samples, they were assumed to be contaminants from the chemical standard.

Similar recovery experiments were performed with nivalenol and 3- and 15-ADON. The concentration of these compounds is generally found to be much lower than that of DON, and for this reason, a lower range of concentrations was selected for the recovery study. Theoretical concentrations were based on weight only. Recovery of NIV was 91–117%. The recovery of 3- and 15-ADON following TFA solvolysis ranged from 115 to 190%, suggesting little degradation (**Table 4**). The large values for recovery probably related to low mean values and proportionally larger variances for these compounds, as well as the fact that the theoretical concentration was based on weight only. After TFA solvolysis, about 0.02–0.10 μg/g of DON was observed in the 3- and 15-ADON samples. If this DON was formed from 3- and 15-ADON, it only accounted for approximately <10% of the acetylated DON detected. On the contrary, Liu et al. (*32*) reported significant degradation of 15-ADON to DON under conditions of TCA solvolysis. In fact, DON was reported as a direct product of the TCA solvolysis of 15-ADON. The reasons for the difference in the recovery of 15-ADON between the current study and that of Liu and coworkers are not readily apparent. However, they evaluated the degradation of a 15-ADON standard in acetonitrile/water only, while in the current study, the chemical standard was first spiked onto clean barley flour. Further, the initial concentration of 15-ADON in the experiments of Liu et al. (*32*) was not defined.

Table 5. Determination of Free, Total, and Bound DON in Barley Samples[a]

barley sample	standard method (free DON)	DON (μg/g)[b] TFA solvolysis (total DON)	increase with TFA (bound DON)	percent increase
1	26.57 A	30.53 B	3.96 BCD	14.90
2	7.54 CD	12.82 CA	5.28 B	70.03
3	22.45 B	36.68 A	14.23 A	63.39
4	5.17 D	9.71 D	4.54 BC	87.81
5	8.65 C	14.26 C	5.61 B	64.86
6	8.11 CD	10.08 D	1.97 DE	24.29
7	5.88 CD	6.43 E	0.55 E	9.35
8	6.91 CD	9.02 D	2.12 CDE	30.68

[a] Values within a column with the same letter are not significantly different at $p \leq 0.05$, as determined by one-way ANOVA. [b] Values are the means of three treatments.

Verification of Bound DON in Other Barley Samples. Eight DON-contaminated barley samples were selected to verify the TFA solvolysis method and confirm the presence of bound DON. **Table 5** shows the comparison of the standard procedure for DON (free DON) with the TFA solvolysis method under optimized conditions (total DON). Subtraction of free DON from the total DON is assumed to indicate bound DON. The results

suggest that these eight barley samples all contained bound DON, which could be released after TFA solvolysis. An additional 9–88% of DON was released. The coefficients of variance ranged from 3.0 to 13.6% and from 5.4 to 21% for the standard method and TFA solvolysis procedures, respectively.

These results are similar to those of Liu et al. (*32*), who reported an additional 13−63% DON released from *Fusarium*-infected wheat grain following solvolysis with TCA. While they indicated that 3- and 15-ADON could be degraded to DON, their results still indicated that a considerable amount of DON existed in bound or conjugated forms. This also seems to be the case in the current study. In the optimization experiment, up to an additional 7.57 μg/g DON was released. As previously stated, this sample contained 0.44 and 0.59 μg/g of 15- and 3-ADON, respectively. Both compounds were observed to be relatively stable in the recovery study. However, even if there had been complete degradation of these compounds to DON, it would still only account for <15% of the observed increase. For the eight verification samples, the concentration of 3-ADON ranged from 0.12 to 0.64 μg/g and no significant changes in the concentration were observed following solvolysis. The content of 15-ADON in this sample set ranged from 0.16 to 1.72 μg/g. After solvolysis, the content of 15-ADON decreased in five of the eight samples. The average decrease was approximately 30%, and because this had only a very limited impact on the percent increase calculations shown in **Table 5**, the 15-ADON data are not presented.

We feel that this data suggest that the optimized TFA solvolysis method is useful to determine total DON in *Fusarium*-infected grain samples and can be used to acquire additional information on the prevalence of bound DON. This information is needed before the necessity of measuring bound DON can be determined.

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