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Characterization of Three Deoxynivalenol Sulfonates Formed by Reaction of Deoxynivalenol with Sulfur Reagents

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Supporting Information

ABSTRACT: Reduction of the *Fusarium* mycotoxin deoxynivalenol (DON) in animal feed by treatment with sodium bisulfite and sodium metabisulfite has been successfully demonstrated in several studies. All of them reported formation of one DON sulfonate of strongly reduced toxicity compared to DON. The starting point of the present work was investigation of different sulfur reagents for reduction of DON. In the course of these experiments, three different DON sulfonates termed DON sulfonate 1 (**1**), DON sulfonate 2 (**2**), and DON sulfonate 3 (**3**) were identified and structurally elucidated by UHPLC−HRMS/MS as well as NMR spectroscopy. Compound **1** is characterized by loss of the epoxide group, and **2** by formation of a hemiketal. Compound **3** is an equilibrating mixture of two isomers, a ketone and a hemiketal. The MS/MS pattern can be used to differentiate the three DON sulfonates, despite their same mass and molecular formula. Investigation of parameters influencing formation and stability of DON sulfonates revealed that rapid formation of **1** and **2** occurs at alkaline pH, whereas at acidic pH, slow formation of **3** takes place, irrespective of the sulfur reagent used. Whereas **1** and **2** are stable across a broad pH range, **3** decomposes to DON, **1**, and **2** at alkaline pH. In addition, both **2** and **3** are unstable in solid form. The formation, characterization, and stability of three novel DON sulfonates with respect to results from previous studies are discussed, providing insights of relevance for detoxification of DON-containing animal feed.

KEYWORDS: *deoxynivalenol sulfonates, sulfur reagents, sodium sulfite, sodium metabisulfite, ultra high performance liquid chromatography−high resolution tandem mass spectrometry, nuclear magnetic resonance spectroscopy, structure elucidation*

INTRODUCTION

Inactivation of deoxynivalenol (DON) with sodium bisulfite or sodium metabisulfite is a promising postharvest mycotoxin reduction strategy and has been dealt with in several studies. As early as 1986, Young[1] investigated the reaction of various trichothecenones such as DON, nivalenol, 3-acetyl DON, and isoDON with sodium bisulfite and proposed structures of DON sulfonate and 3-acetyl DON sulfonate. The structure of DON sulfonate is identical to that of compound **3A** (Figure 1), albeit with undefined stereochemistry at C9 and C10. In addition, stability tests were performed and rapid decomposition of DON sulfonate and 3-acetyl DON sulfonate to DON and further on to isoDON and norDON products at alkaline pH and elevated temperature was reported. In the same year, Young and co-workers[2,3] treated DON-contaminated wheat and corn with aqueous sodium bisulfite and observed efficient reduction of DON. One year later, Young et al.[4] fed DON-containing corn autoclaved with aqueous sodium bisulfite to pigs and recorded improved performance parameters compared to the group fed with untreated DON-contaminated corn. In addition, pure DON sulfonate administered orally to swine did not provoke toxic effects at levels at which DON caused severe emesis.

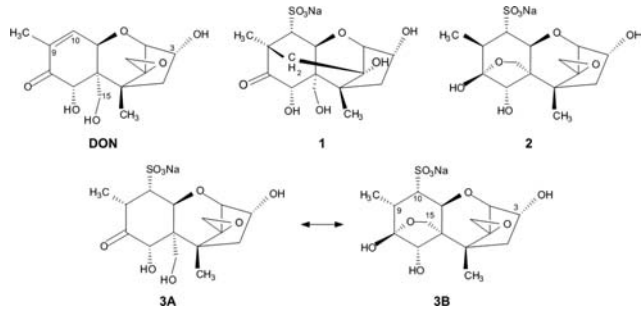

Figure 1. Structures of DON, DON sulfonate 1 (**1**), DON sulfonate 2 (**2**), and DON sulfonate 3 (**3A** and **3B**). Whenever differentiation between **3A** and **3B** was not possible, DON sulfonate 3 is referred to as **3**.

These initial studies were followed by a research break of several years. In 2005, the topic was taken up again by Dänicke and co-workers, who treated DON-contaminated wheat with

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1% sodium metabisulfite at 100 °C for 15 min and fed this treated wheat to growing piglets.[5] Performance parameters were improved, and plasma DON concentrations were reduced compared to the group receiving nontreated DON-contaminated wheat. Between the years 2008 and 2010, several further articles were published,[6−11] dealing with factors influencing DON reduction by sodium metabisulfite, the effects of sodium metabisulfite treated DON-containing *Triticale* on piglets, absorption and blood levels of DON sulfonate in piglets, and the toxicity of DON sulfonate, DON, and sodium metabisulfite for porcine cell lines. An overview of these studies is given in a comprehensive review.[12]

The paper giving the most information on the reaction product, DON sulfonate, and on its determination is that of Beyer et al.[11] in which preparation, preparative isolation, and structural characterization of DON sulfonate is described. DON sulfonate was reported to be a mixture of two diastereomers, the main isomer having the same structure as that proposed by Young,[1] again with undefined stereochemistry at C9 and C10. In addition, a hydrophilic interaction chromatography electrospray ionization mass spectrometric (HILIC-ESI-MS) method for determination of DON sulfonate in wheat samples treated with sodium metabisulfite was developed, and correlation between reduction of DON and increase of DON sulfonate was established.

The original aim of our work was to investigate different sulfur reagents for detoxification of DON. However, in the course of first pilot experiments, formation of three DON sulfonates, termed DON sulfonate 1 (**1**), DON sulfonate 2 (**2**), and DON sulfonate 3 (**3**) according to their order of retention in reversed phase high performance liquid chromatography (RP-HPLC), was observed. This finding triggered a series of experiments with the aim to elucidate the structures of the three sulfonate products and to investigate factors influencing their formation and stability.

MATERIALS AND METHODS

Reagents. Methanol (LC gradient grade) was purchased from Merck (Darmstadt, Germany), formic acid (p.a.) was from VWR International GmbH (Vienna, Austria), and formic acid (p.a. for HPLC) for ultra high performance liquid chromatography−high resolution mass spectrometry (UHPLC−HRMS) was from Fluka (Sigma-Aldrich, Vienna, Austria). Sulfur reagents (all p.a.) were obtained from Sigma-Aldrich. Reagents for preparation of buffers were of p.a. grade. Water was purified using a Purelab Ultra system (ELGA LabWater, Celle, Germany). Solid deoxynivalenol (DON, purity >95%) was provided by Romer Labs GmbH, Tulln, Austria.

Production of DON Sulfonates. First, two small-scale pilot experiments were carried out. Aliquots (1 mg) of DON were dissolved in 0.5 mL of water containing 10% sodium sulfite (w/v) or in 0.5 mL of phosphate buffer (pH 6.5) containing 15% sodium metabisulfite (w/v). Solutions were shaken at 37 °C for 21 h, and aliquots were taken after 15 min, 1 h, 3 h, and 21 h and measured by UHPLC−HRMS after 1:10^4 dilution. For large-scale production of **1** and **2**, 52 mg of DON was dissolved in 26 mL of water (corresponding to 2000 mg/L). Then 2.6 g of sodium sulfite, corresponding to 10% in solution, was added, and the mixture was shaken at 37 °C for 15 min. For production of **3**, 22 mg of DON was dissolved in 11 mL of phosphate buffer, 1.66 g of sodium metabisulfite (15%) was added, and the solution was shaken at 37 °C for 21 h.

Preparative Isolation of DON Sulfonates. Prior to preparative HPLC, excess sulfur reagents were removed by solid phase extraction. To this end, 2-mL aliquots of the solutions containing **1** plus **2** or **3**, respectively, were applied to preconditioned (5 mL of methanol, 5 mL of water) Strata C18 T cartridges (500 mg, 3 mL) (Phenomenex, Aschaffenburg, Germany). Cartridges were washed with 0.2 mL of water, and DON sulfonates were eluted with 5 mL of methanol/water (80/20, v/v). Finally, the combined SPE eluates were evaporated on a rotary evaporator at 30 °C to about 15 mL (**1** and **2**) and 9 mL (**3**), respectively.

Preparative isolation of **1**, **2**, and **3** was performed on an Agilent 1100 Series preparative HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Sedex LT-ELSD Model 85LT low temperature evaporative light scattering detector (Sedere, Alfortville, France). Compounds were separated at 25 °C on a 150 mm × 21.2 mm i.d., 5 μm, Gemini-NX C18 column (Phenomenex, Aschaffenburg, Germany) with a guard column of the same material using mixtures of methanol/water/formic acid (A: 10/89/1, B: 89/10/1, v/v/v) as mobile phases and gradient elution: 0−1 min, 0% B; 1−5 min, linear increase to 100% B; 5−7 min, isocratic elution at 100% B; 7−7.1 min, return to 0% B; 7.1−10 min, re-equilibration at 0% B. The flow rate was 16 mL/min, and the injection volume was 900 μL. The column effluent was split 1:70, one part moving into the evaporative light scattering detector (ELSD) and the main part to the fraction collector. Compound **1** was collected between 3.4 and 4.4 min and **2** between 4.45 and 5.2 min, as **3** was not present in this reaction mixture. In the case of **3**, two fractions were collected: a mixed fraction containing **2** and **3** (4.75−4.93 min) and a fraction containing pure **3** (4.96−5.55 min). Pooled fractions of **1** were evaporated to dryness on a rotary evaporator, taken up in 1.4 mL of deuterated water, and used for preparation of standard solutions of **1** as well as for structure elucidation by NMR. Due to the instability of **2** and **3** upon evaporation to dryness, the fractions of **2** and **3** were evaporated separately, first at 30 °C on a rotary evaporator and then at 22 °C under nitrogen to a volume of 1.4 mL. Aliquots of 0.4 mL were taken as standards for analytical measurements, and 2 mL of deuterated water was added to each of the remaining solutions. Evaporation was continued until a volume of approximately 1 mL. Addition of deuterated water and evaporation was repeated twice in order to remove water prior to NMR analysis without evaporation to dryness.

NMR Spectroscopy. NMR spectra were obtained from solutions of the corresponding compounds in D_2O using an Avance DRX-400 FT-NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), operating at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C, at 295 K using a 5 mm inverse broadband Z-gradient probehead. Data were recorded and evaluated using TOPSPIN 1.3 software (Bruker Biospin). All pulse programs were taken from the Bruker software library.

UHPLC−HRMS(/MS) Measurements. UHPLC−HRMS/MS analyses were conducted on an Agilent 1290 Infinity LC system coupled to an Agilent 6550 iFunnel Q-TOF mass spectrometer. Separation was achieved at a flow rate of 0.25 mL/min at 20 °C on an Agilent StableBond C18 column (150 mm × 2.1 mm i.d., 1.8 μm) using water and methanol, both containing 0.1% formic acid (v/v) as mobile phases A and B. The gradient was 0−0.1 min, 10% B; 0.1−4 min, linear increase to 35% B; 4−6 min, linear increase to 90% B; 6−8 min, isocratic elution at 90% B; 8−8.1 min, return to 10% B; 8.1−11 min, re-equilibration at 10% B. The injection volume was 2 μL. In routine measurements, the LC stream was directed to MS between 2.6 and 5.2 min. For identification of novel DON sulfonates and of DON sulfonate degradation products, the window was enlarged to 1.7−7.5 min. The Q-TOF instrument was equipped with a dual Agilent Jet Stream electrospray ion source. Electrospray ionization was carried out in negative mode at a gas temperature of 130 °C, drying gas flow of 14 L/min, nebulizer pressure of 30 psig, sheath gas temperature of 350 °C, and sheath gas flow of 10 L/min. The capillary voltage was 4000 V, the nozzle voltage 500 V, and the fragmentor 175 V. Data acquisition was achieved in the 2 GHz extended dynamic range mode. In MS experiments, ions were scanned in the range of m/z 100−1000 (acquisition rate 2 spectra/s, 4111 transients/spectrum), and in MS/MS experiments, data were stored between m/z 50−550 (acquisition rate 3 spectra/s, 2716 transients/spectrum). Fragmentation patterns were recorded in Targeted MS/MS mode, using $[M - H]^-$ of the DON sulfonates as precursors with an isolation width of m/z 1.3. Collision energies were set to 30, 50, and 70 eV. Mass accuracy was ensured by continuous mass calibration using the ions m/z 112.9855

and m/z 966.0007 of the Agilent ES TOF reference mass solution. Instrument control and data evaluation were achieved by the Agilent Technologies software MassHunter (Acquisition: version B.05.01, Build 5.01.5125; Qualitative Analysis: version B.05.00, Build 5.0.519.13; Quantitative Analysis: version B.05.00, Build 5.0.291.0).

Preparation of Standard Solutions and Determination of the Concentrations of DON Sulfonates. Stock solutions of **1** were prepared by evaporation of aliquots of the original solution (obtained by preparative HPLC, evaporation, and uptake in 1.4 mL of D_2O) in preweighed weighing boats, reweighing, and dissolving in water. As **2** and **3** partly decompose upon evaporation to dryness, their concentration in solution was calculated on the basis of molar ELSD calibration functions recorded for **1** and DON. Measurements were carried out on an Agilent 1100 HPLC system coupled to a Sedex LT-ELSD Model 85LT low temperature evaporative light scattering detector. DON sulfonates and DON were separated isocratically at a flow rate of 0.25 mL/min on a 50 mm × 2.1 mm i.d., 2.6 μm, C18 Kinetex column (Phenomenex, Aschaffenburg, Germany) using methanol/water/formic acid (10/89/1, v/v/v) as mobile phase. Quadratic ELSD (50 °C, gain 9) calibration functions were established for DON and for **1** in the range between 10 and 100 mg/L and concentrations of **2** and **3** in diluted stock solutions were calculated on the basis of the calibration curve recorded for **1**. Finally, calibration functions ranging between 1 and 600 ng/mL of **1**, **2**, **3**, and DON were prepared in methanol/water/formic acid (10/89.9/0.1, v/v/v).

Test of Different Sulfur Reagents for Conversion of DON. Solutions containing 0.5 mg/L DON in water, phosphate buffer (pH 6.5), sodium acetate buffer (pH 5), or citrate buffer (pH 3) were incubated separately with 0.5% sodium metabisulfite, sodium sulfite, sodium sulfate, or sodium dithionite by shaking at 37 °C for 1 h. pH values of individual solutions were measured, and product mixtures were analyzed by UHPLC–HRMS. The turnover of DON was determined on the basis of molar neat solvent calibration functions for **1**, **2**, **3**, and DON, and included calculation of the percentage of formed metabolites on a molar basis.

Formation of DON Sulfonates at Different pH Values. For this evaluation 0.5% (w/v) sodium metabisulfite and sodium sulfite, respectively, was dissolved in Teorell Stenhagen buffer solutions of pH values between 2 and 10.[13] The pH was readjusted to the original value by addition of 1 M hydrochloric acid or 1 M sodium hydroxide solution. The reaction was started by mixing 20 μL of DON stock into 980 μL of sulfur reagent buffer solution (final concentration 0.5 mg/L DON) in an HPLC vial and stopped after 1 and 24 h, respectively, at 22 °C by holding the reaction vessel at −20 °C until 20 min before measurement. Evaluation was carried out as described above.

Stability of DON Sulfonates at Different pH Values and Different Temperatures. Stability of pure **1**, **2**, and **3** was tested by adding 20-μL aliquots of separate solutions of **1**, **2**, and **3** (7.5 mg/L) to 480 μL of Teorell Stenhagen buffer solutions[13] of integer pH values (2–10), resulting in concentrations of **1**, **2**, and **3** of 0.3 mg/L. Samples were incubated for 0, 1, 2.5, 5, 8, and 24 h at 37 °C and for 0 and 3 h as well as for 1, 4, and 7 d at room temperature and stored frozen until UHPLC–HRMS analysis. DON sulfonates and formed DON were quantitated on the basis of molar neat standard calibration functions.

RESULTS AND DISCUSSION

Production of DON Sulfonates. First experiments on conversion of DON by different sulfur reagents revealed that **1** and **2** are formed rapidly at alkaline pH, whereas **3** is formed more slowly at slightly acidic to neutral pH values. These experiments were later repeated for quantitative analysis. This finding was advantageous, as conditions for production of **1**, **2**, and **3** should allow for separate production of **1** plus **2** and **3** because of close retention times of **2** and **3** even upon UHPLC separation (Figure 2). On the basis of results of DON sulfonate formation experiments with different sulfur reagents and literature reports recommending high percentages of sodium

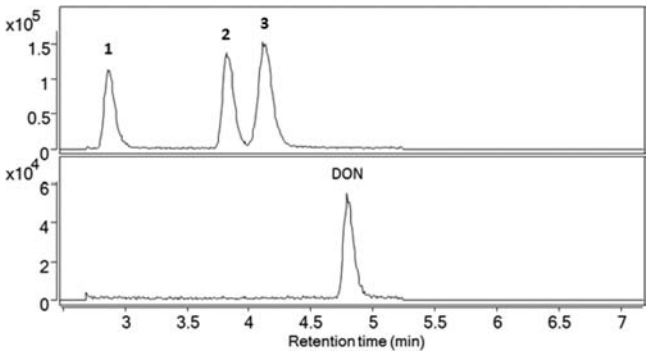

Figure 2. UHPLC–HRMS extracted ion chromatograms of a standard solution of **1**, **2**, **3**, and DON.

(meta)bisulfite for production of DON sulfonate,[1,11] two pilot experiments were carried out at small scale. Incubation of a 2000 mg/L solution of DON in water with 10% sodium sulfite (pH 9.6) at 37 °C resulted in quantitative formation of **1** and **2** (69% and 31%, respectively, of used DON) within 15 min. Conversion of DON into **3** (97%) and to a small extent into **2** (3%) was quantitative after 21 h when a 2000 mg/L solution of DON was shaken in phosphate buffer with 15% sodium metabisulfite (pH 4.2) at 37 °C. Hence, these conditions were used for separate large-scale production of **1** plus **2** and **3**.

Preparative Isolation of DON Sulfonates. In order to remove excess sulfur reagents, solutions containing DON sulfonates and sulfur reagents were subjected to solid-phase extraction (SPE). As also optimized in pilot experiments, application and wash volumes were kept small (2 and 0.2 mL, respectively) to avoid loss of analytes. HPLC parameters for separation of **1**, **2**, and **3** were optimized on an Agilent 1290 Infinity LC System using a C18 Gemini HPLC column and afterward scaled-up for preparative HPLC. Addition of 1% formic acid turned out to be required for good chromatographic behavior of all DON sulfonates on the used RP-C18 HPLC columns. Baseline separation was achieved at analytical scale, the critical pair being **2** and **3**. At preparative scale, **2** and **3** partly coeluted, so that during production of **3**, a small mixed fraction of **2** and **3** was collected in addition to the large fraction containing pure **3**.

First preparative isolations of **1**, **2**, and **3** included evaporation of methanol from the pooled fractions of the individual compounds and subsequent lyophilization. However, lyophilization and storage of the crystals at 4 °C for 2 d resulted in partial degradation of **2** (ca. 25% of **2** remaining) and almost complete decomposition of **3** (<1% remaining). Mass spectrometric characterization of the degradation products is described in the Supporting Information. Therefore, production of **2** and **3** was repeated, and evaporation to dryness was avoided throughout the total production and isolation process. The yields of pure **1**, **2**, and **3** were 62%, 28%, and 88%, respectively, of the used DON.

NMR Spectroscopy. For structure elucidation and signal assignment, 1D (^1H and ^{13}C) as well as 2D spectra ($^1H^1H$ COSY, $^1H^{13}C$ HSQC, and $^1H^{13}C$ HMBC) of **1**, **2**, and **3** were recorded. The stereochemistry of the centers involved in the reaction (C9, C10) was established on the basis of NOE measurements (1D-NOE difference, 2D-NOESY) and analysis of coupling patterns. The resulting structures of these sulfonates are shown in Figure 1, and complete assignments for H and C signals are given in Tables 1 and 2, respectively.

Table 1. ^{1}H NMR Assignments of 1, 2, 3A, and 3B (δ, ppm; J, Hz)[a]

position	1	2	3A	3B
2	3.79 (d, 2.8)	3.51 (d, 4.4)	3.67 (d, 4.2)	3.56 (d, 4.4)
3	4.23 (m)	4.32 (dt, 11.2, 4.4)	4.38 (m)	4.37 (m)
4	2.05−1.90 (m)	1.94 (dd, 14.7, 11.2)	2.73 (m)	1.98 (m)
		1.73 (dd, 14.7, 4.5)	1.99 (m)	1.69 (m)
7	3.95 (s)	3.99 (s)	4.78 ([b])	3.96 (s)
9		2.46 (dq, 2.6, 7.3)	3.37 (quint, 6.9)	2.55 (quint, 7.2)
10	3.50 (d)	2.79 (t, ∼2.5)	3.88 (m)	3.26 (m)
11	4.08 (d)	4.77 ([b])	5.11 (d)	4.71 ([b])
13	2.22 (d, 15.0)	3.19 (d, 3.5)	3.25 (d, 3.3)	3.20 (d, 3.4)
	1.78 (d, 15.0)	3.13 (d, 3.5)	3.12 (d, 3.3)	3.16 (d, 3.4)
14	0.80 (s)	0.97 (s)	1.02 (s)	1.02 (s)
15	4.04 (d, 11.7)	4.05 (d, 8.5)	3.95 (d, 10.4)	4.08 (d, 9.4)
	3.80 (d, 11.7)	3.71 (d, 8.5)	3.89 (d, 10.4)	3.50 (d, 9.4)
16	1.24 (s)	1.07 (d, 7.3)	1.24 (d, 6.9)	1.18 (d, 7.2)

[a]s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. [b]Covered by HDO signal.

Table 2. ^{13}C NMR Assignments of 1, 2, 3A, and 3B (δ, ppm)

position	1	2	3A[a]	3B[a]
2	81.1	80.9	80.6	80.2
3	70.9	68.1	68.2*	68.4*
4	43.0	43.3	43.0	43.3
5	48.8	43.0	46.3	43.3
6	50.2	55.3	54.7	54.9
7	70.3	70.6	76.7	73.9
8	215.0	107.1	212.7	106.0
9	46.4	40.5	39.1	37.9
10	72.4	67.0	67.6	61.8
11	71.1	77.3	72.2	78.0
12	79.9	67.0	66.8	67.3
13	46.7	47.6	48.4	47.4
14	17.4	14.3	13.7**	14.5**
15	63.4	66.5	59.8	66.9
16	21.6	19.6	10.5	10.5

[a]Asterisks (*,**) indicate assignments may be interchanged between the ketone and the hemiketal structure.

Compared to DON, the most significant difference in the spectra of **1** is the absence of the unsaturated element (C9═C10) as well as of the epoxide moiety. Neither the methyl protons (H16) nor the CH_2 (H13) show any additional proton coupling, and thus both groups are connected to a quaternary carbon atom. The pattern of H10 (doublet with H11) has not changed either. This led to the proposed structure presented in Figure 1, which was further verified by several long-range couplings in the HMBC spectrum (e.g., ^3J(H13−C16) and ^3J(H13−C8)): the sulfonate has been added at C10, and C13 (the former epoxide CH_2) has formed a bridge between C9 and C12. A similar case of bridge formation between C12 and the A ring of a trichothecene had been observed for a scirpenol derivative.[14] The stereochemistry at C9 is defined by the new bridge above the A ring (the side of the A ring *cis* to the B ring of the skeleton is arbitrarily referred to as the "upper" side for simplicity reasons in the further text), and the observation of a NOE interaction between H10 and one of the protons at C13 shows the sulfonate group to be oriented downward.

The spectra of **2** also lack olefinic signals. Here, H16 shows the coupling to a neighboring proton. A sequence of vicinal couplings (H16 ↔ H9 ↔ H10 ↔ H11) can be deduced from the COSY, which indicates that addition of $NaHSO_3$ to the C═C double bond has occurred. Furthermore, the ketone signal at >200 ppm in the ^{13}C spectrum has disappeared. Instead, a peak at 107 ppm is observed for C8, which shows three-bond long-range correlations to the H15 protons. Thus, a hemiketal has formed between the 15-OH group and the ketone of the primary addition product, a phenomenon that has already been observed for DON and nivalenol.[15] Upon NOE irradiation of the methyl group, H7 as well as H10 show signal enhancement, and therefore the CH_3 is located above and the sulfonate below the A ring.

From both the ^{1}H and the ^{13}C spectrum of **3** it becomes immediately obvious that this product exists as a mixture of two compounds at roughly equal ratio. One of these compounds (**3B**) exhibits spectra strikingly similar to those of **2**, including the long-range correlation proving the intramolecular hemiketal formation. Thus this compound has the same skeleton; however, it differs in stereochemistry in the C9/C10 region: Overhauser effects between H7, H9, and H10 indicate that both the methyl and the sulfonate group are arranged below the A ring.

The major difference between **3B** and the second constituent of the mixture (**3A**) is the occurrence of a ketone moiety instead of the ketal, as seen by a peak at 212 ppm in the ^{13}C spectrum of the latter. The carbonyl group also causes some changes in the spectroscopic features of neighboring atoms, but apart from that the spectra of **3A** and **3B** appear to be very similar. Thus, **3A** is the parent C8 ketone from which **3B** is derived by hemiketal formation. In the aqueous medium used for chromatography and spectroscopy, **3A** and **3B** exist in equilibrium, which also explains the inseparability of the two components, at least under the used RP-UHPLC conditions.

UHPLC−HRMS(/MS) Measurements. UHPLC separation coupled with high resolution mass spectrometry in the full scan mode was used for qualitative and quantitative analysis to determine the accurate masses of DON sulfonates and to investigate factors influencing their formation and stability. Fragmentation spectra for characterization of DON sulfonates and their degradation products were recorded by UHPLC−HR-targeted MS/MS measurements. A UHPLC−HRMS chromatogram of **1**, **2**, **3**, and DON (all 100 ng/mL) is shown in Figure 2.

Beyer et al.[11] stated that classical reversed phase separation of DON sulfonate is not possible. They performed gradient RP-HPLC with decreasing proportion of organic solvent in the mobile phase for preparative isolation of DON sulfonate and HILIC chromatography for determination of DON sulfonate in cereal samples. HILIC−MS run times were 36 min (including 15 min of re-equilibration). We discovered that RP-chromatography is well suited for analysis of DON sulfonates, both at preparative and at analytical scale, provided that the mobile phases are acidified. For UHPLC on Agilent StableBond C18 columns, 0.1% formic acid is sufficient, whereas HPLC separation on Phenomenex C18 Gemini columns (as for preparative isolation of **1**, **2**, and **3**) requires 1% formic acid in the mobile phases. The runtime of our preparative HPLC

method was 10 min, that of our UHPLC−HRMS method, 11 min.

Determination of the Accurate Mass and Fragmentation Pattern of DON Sulfonates. All *m/z* ratios and molecular masses given in the text are the theoretical exact values. In most cases, the mass accuracy was <5 ppm. Mass accuracies between 5 and 10 ppm were observed only for ions of relative intensities <10%.

UHPLC−HRMS measurements in negative ion mode yielded the same accurate mass (378.0985 Da) and sum formula ($C_{15}H_{22}O_9S$) for all three DON sulfonates. These values are in line with data published for the one DON sulfonate characterized in the literature.[1,11] Spectra recorded at fixed collision energies (CE) of 30, 50, and 70 eV showed different fragmentation behavior of the three individual DON sulfonates. At CE 30, **1** remained mostly intact, whereas **2** was largely fragmented to one main fragment of *m/z* 80.9652 $[HSO_3]^-$. Unlike **1** and **2**, **3** fragmented to *m/z* 347.0806 ($C_{14}H_{19}O_8S$, loss of CH_2O), 80.9652, and 79.9574 $[SO_3]^-$ at relative intensities of 64%, 74%, and 40%. Increase of the CE to 70 eV resulted in fragmentation of all DON sulfonates solely to $[HSO_3]^-$ and $[SO_3]^-$, albeit at greatly different ratios: whereas **1** formed predominantly $[SO_3]^-$, **2** fragmented to mainly $[HSO_3]^-$, and **3** showed both fragments $[SO_3]^-/[HSO_3]^-$ at relative intensities of 100/40. In the case of **3**, the ratio of $[SO_3]^-/[HSO_3]^-$ changed from 0.54 at CE 30 to 2.37 at CE 70. All other fragments formed at CE 30, 50, and 70 eV were ≤6%: For **1**, *m/z* 359.0806 ($C_{15}H_{19}O_8S$, loss of water), 347.0806 ($C_{14}H_{19}O_8S$), and 331.0857 ($C_{14}H_{19}O_7S$) were observed at CE 50 eV, all fragments still containing the sulfonate group. For **2**, the fragment of *m/z* 347.0806 (formed by loss of CH_2O) and the DON specific fragments of *m/z* 295.1187 ($C_{15}H_{19}O_6$, $[DON - H]^-$), 265.1081 ($C_{14}H_{17}O_5$, $[DON - H - CH_2O]^-$), and 247.0976 ($C_{14}H_{15}O_4$, $[DON - H - CH_2O - H_2O]^-$) were detected at CE 30 eV. Similarly, for **3**, DON based fragments of 265.1081, 247.0976, 229.0858 ($C_{14}H_{13}O_3$), and 163.0764 ($C_{10}H_{11}O_2$) and the $[HSO_4]^-$ ion of *m/z* 96.9601 were measured.

Instability of Compounds 2 and 3 in Solid Form. During preparative isolation of DON sulfonates, compounds **2** and **3** proved to be unstable upon freeze-drying. Several degradation products were observed, the most prominent of molecular mass 396.1090 Da and sum formula $C_{15}H_{24}O_{10}S$. Evaporation of **2** to dryness under nitrogen and storage for up to 3 h at room temperature did not affect its stability. However, when **3** was subjected to the same procedure, reduction of the concentration by 15% and formation of **2** was observed. In the literature, evaporation steps were common, but no information on storage time of the solid substances was provided. Therefore, it is difficult to assess whether and to what extent degradation took place. Similar to our findings, Beyer et al.[11] reported conversion of DON sulfonate isomers into each other during solvent evaporation.

Preparation of Standard Solutions and Determination of the Concentrations of DON Sulfonates. Due to instability of **2** and **3** upon evaporation to dryness, their concentration in stock solutions cannot be determined by weighing. In addition, quantitation on the basis of LC−MS calibration functions for **1** is not acceptable because of different ionization and fragmentation behavior. A versatile alternative proved to be nonselective evaporative light scattering detection (ELSD), the signal of which depends mainly on the molecular mass of the compounds. In order to evaluate the applicability of ELSD measurements and to assess the quality of the standard of **1**, quadratic molar ELSD calibration functions were established both for DON and for **1**, and concentrations of diluted stocks of **2** and **3** were determined on the basis of both calibration functions. The obtained concentrations differed by only 5%, which is well within the estimated precision of the method. Isocratic elution on a Phenomenex Kinetex column allowed acceptable separation of all compounds. Finally, stock solutions containing 1696 mg/L of **1**, 690 mg/L of **2**, and 757 mg/L of **3** were prepared in water/formic acid (99.9/0.1, v/v) and used for establishment of calibration functions and for stability tests. Standard stock solutions stored at 4 °C were stable for at least 1 year.

Test of Different Sulfur Reagents for Conversion of DON. In order to obtain closer insight into transformation of DON by sulfur reagents and to investigate alternatives for sodium metabisulfite, sodium sulfite, sodium sulfate, and sodium dithionite were tested. Like sodium metabisulfite, sodium sulfite is generally recognized as safe and may be used as feed additive in the U.S. As the pH value was likely to have influence on formation of DON sulfonates, tests were carried out in buffers of different pH values and in water. Whereas the pH of citrate and phosphate buffer (original pH values 3 and 6.5, respectively) was not strongly changed by addition of 0.5% of sulfur reagent, dissolution of sulfur reagent in water resulted in acidic pH values for sodium metabisulfite and sodium dithionite and in alkaline pH of almost 9 for sodium sulfite and sodium sulfate (Table 3). At acidic pH, reduction of DON was low, and **3** was the only reaction product. The extent of conversion of DON into **3** increased with increasing pH value. In the pH range 6−7, conversion of DON took place to >95%, the main reaction product being **3**, but **1** and **2** were also formed. At alkaline pH, reduction of DON was quantitative, and **1** and **2** were the predominant products. The results of these experiments were the first strong indication that pH, rather than the sulfur reagent used, is the key factor governing reaction speed and the type of DON sulfonate formed.

Table 3. Conversion of DON by Sulfur Reagents in Water and Citrate and Phosphate Buffer

			percentage of used DON			
buffer	sulfur reagent	pH	**1**	**2**	**3**	DON
citrate	sodium dithionite	2.80	0.0	0.0	4.2	96
	sodium metabisulfite	2.83	0.0	0.0	6.4	94
	sodium sulfate	3.39	0.0	0.0	17	83
	sodium sulfite	3.48	0.0	0.0	22	78
phosphate	sodium dithionite	5.92	5.0	8.0	83	4.4
	sodium metabisulfite	5.82	5.2	8.9	83	3.0
	sodium sulfate	6.71	13	18	69	1.1
	sodium sulfite	6.72	12	17	71	0.9
water	sodium dithionite	3.39	0.0	0.7	9.8	89
	sodium metabisulfite	3.78	0.0	2.4	12	86
	sodium sulfate	8.99	39	46	14	0.0
	sodium sulfite	8.92	42	48	11	0.0

Formation of DON Sulfonates at Different pH Values. The hypothesis presenting the pH value as key factor in formation of DON sulfonates was investigated further in a series of experiments where 0.5% sodium metabisulfite or sodium sulfite was dissolved in DON-containing buffer

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solutions of pH 2−10, including readjustment of the pH to the original values after addition of sulfur reagent. The curves obtained by plotting the percentage of formed DON sulfonates and residual DON against the pH value were almost identical for sodium sulfite and sodium metabisulfite (shown for sodium sulfite in Figure 3), which proves that the pH dictates the type

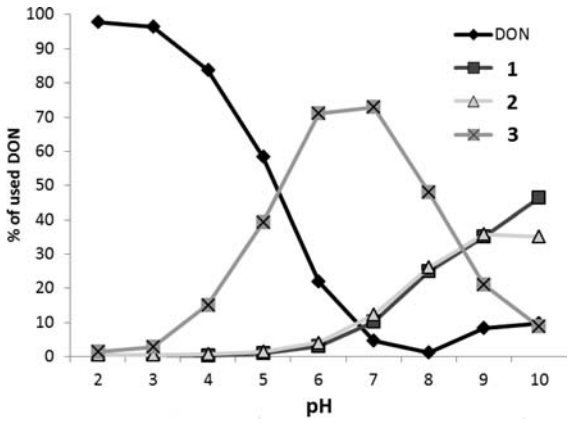

Figure 3. Decrease of DON and formation of **1**, **2**, and **3** by incubation of 0.5 mg/L DON with 0.5% sodium sulfite at different pH values for 1 h.

of DON sulfonate formed and the speed of reaction. The explanation lies in the different chemical forms in which the active components of sulfur reagents occur in solution. As shown by Rose[16] and summarized by Dänicke et al.,[12] formation of SO_2 occurs at acidic pH and results in loss of total sulfites and therefore in slow formation of DON sulfonates. In the pH range 4−5, the HSO_3^- ion prevails, whereas the SO_3^{2-} ion dominates at pH > 8.

In our experiments, the concentration of DON decreased steadily from pH 2 to 8 and increased again slightly at pH 9 and 10, probably due to instability of **3**. The concentration of **1** and **2** increased until pH 10 and 9, respectively. Slight decrease of the concentration of **2** at pH 10 is also explained by instability of **2** under strongly alkaline conditions. The shape of the curve of **3** is explained by both a pH optimum of formation at pH 6−7 and instability at alkaline pH. The curves recorded for incubation for 24 h were similar to those obtained for an incubation period of 1 h with some exceptions: The formation curve of **3** had a clear maximum at pH 6 after incubation for 24 h. At pH 5 and 7, the same percentage of **3** was measured (57%), whereas at pH 8, 9, and 10, only 19%, 3%, and 0% of **3** was left. The formation of **1** and **2** was slightly enhanced (by ca. 10% compared to incubation for 1 h) from pH ≥ 6 onward. In addition, the decrease of DON was slightly greater (by 10−20%) at pH 4−6. DON remained below the limit of detection (3 ng/mL) at pH 9 and 10, possibly due to decomposition of DON itself under strongly alkaline conditions.

Stability of DON Sulfonates at Different pH Values and Different Temperatures. Stability tests carried out at 37 °C for 0−24 h and at 22 °C for 0−7 d revealed stability of **1** in the pH range 2−10. Compound **2** was stable at pH 2−7 for 24 h. However, at pH 8−10, back formation to DON was observed, albeit only from 2.5 h onward and to a very small extent. Greatest release of DON occurred at pH 10, the extent increasing with incubation time (1%, 3%, 65%, and 17% after 2.5, 5, 8, and 24 h). After 24 h, minor formation (1%) of **1** was also detected. At pH 8 and 9, formation of DON from **2** was 2% and 6%, respectively, after 24 h. Incubation of **2** at 22 °C for 0−7 d yielded similar results on a different time scale. After 1, 4, and 7 d at pH 10, DON formation was 1%, 7%, and 10%. At pH 8 and 9, respectively, incubation for 7 d resulted in DON release of 2% and 3%. Hence, **2** is partly unstable at alkaline pH, elevated temperature favoring the back conversion to DON.

Compound **3** was completely stable at pH 2−4 when incubated at 37 °C for 24 h or at 22 °C for 7 d. At pH 5 or higher, reduction of **3** and formation of DON, **1**, and **2** was observed. Degradation of **3** ranged from marginal at pH 5 to complete at pH 9 and 10 after 24 h at 37 °C and after 4 d at 22 °C. The time dependence of degradation of **3** upon incubation at 37 and 22 °C is depicted in Figure 4. The main degradation product was DON (up to 80% at 37 °C, up to 70% at 22 °C), followed by **1** (up to 13% at 37 °C, up to 20% at 22 °C) and **2** (up to 5% and 13% at 37 and 22 °C, respectively). The ratios of all DON sulfonates and DON upon incubation of **3** at 37 °C for 24 h are shown in Figure 5. A very similar curve was

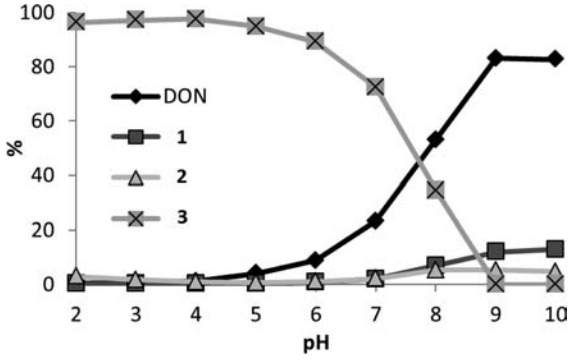

Figure 5. Stability of **3** upon incubation in buffer solutions of pH 2−10 at 37 °C for 24 h.

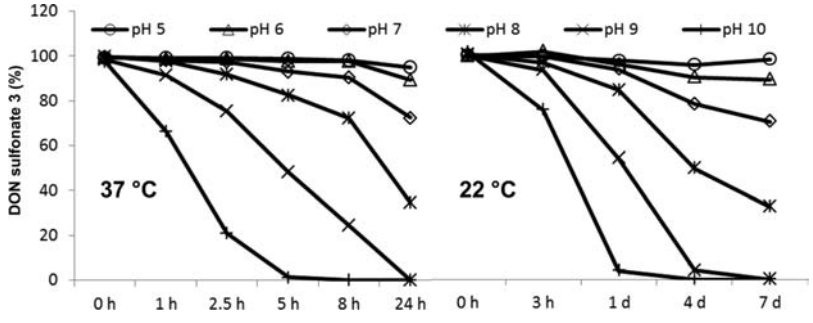

Figure 4. Stability of **3** upon incubation in buffer solutions of pH 5−10 at 22 and 37 °C for 0−24 h.

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obtained for incubation of **3** at 22 °C for 7 d. The results obtained for **3** correspond to those published by Young,[1] who reported stability of DON sulfonate in acidic solution and hydrolysis back to DON under basic conditions, the reaction rate increasing with pH and temperature.

The excellent stability of **1** and **2** in a pH range between 2 and 8 at 37 °C suggests stability of **1** and **2** under physiological conditions such as during digestion and in plasma of pigs. Compound **3** is likely to survive the acidic environment of the stomach but might partly degrade to DON, **1**, and **2** in the neutral to slightly alkaline ambience of small intestine and plasma. The extent of degradation will depend on the actual pH value and on the retention time and may vary between animals.

Considering the slightly acidic pH of animal feed, formation of **3** seems more likely than formation of **1** or **2**. Compound **3** is probably stable in slightly acidic nonthermally treated animal feed but, as discussed above, might degrade in the animal body. Hence, from the stability point of view, detoxification of DON-containing animal feed should aim at formation of **1** and **2** rather than **3**. Hydrothermal treatment in the presence of additives raising the pH value might favor formation of **1** and **2** over **3**.

Comparison with Structures Reported in the Literature. In the pioneering work by Young,[1] DON sulfonate and 3-acetyl DON sulfonate were produced by incubation of DON and 3-acetyl DON, respectively, with 20% sodium bisulfite in water overnight at room temperature. After SPE on C18 cartridges, the eluate was evaporated to dryness and dissolved in methanol (DON sulfonate) or stored as a white solid after purification by preparative thin layer chromatography (3-acetyl DON sulfonate). The structure of DON sulfonate proposed by Young was **3A**, the keto form of **3** (Figure 1), even though the stereochemistry at C9 was not defined and NMR data of DON sulfonate were not provided. As the conditions applied for production of DON sulfonate were similar to those that were used for production of **3**, and as the resulting product was probably dissolved directly after evaporation of solvent and therefore not significantly degraded, the compound reported by Young was most probably **3**. However, 3-acetyl DON sulfonate, which was produced under the same conditions as DON sulfonate and is likely to differ from DON sulfonate only by the acetyl group at C3, was stored as a white powder. Taking into account that crystalline **3** decomposes completely into a series of degradation products, decomposition of 3-acetyl DON sulfonate is also possible. Despite that, NMR data matched those of **3A**, the keto form of **3** obtained in our work, except for some differences that might be due to NMR measurement in different solvents (DMSO versus D_2O) and due to effects exerted by the acetyl group at C3.

In the article published by Beyer et al.,[11] DON sulfonate was prepared by stirring a 2000 mg/L solution of DON in water with 24% of sodium metabisulfite at room temperature for 48 h. This reaction mixture was prepurified by SPE and evaporated to dryness prior to further purification. HPLC−ELSD chromatograms of the reaction mixture showed, in order of increasing retention time, sodium metabisulfite, residual DON, and one minor and one major peak of DON sulfonate isomers. Purification of the reaction mixture was carried out by preparative HPLC on a C8 column in gradient elution mode with decreasing percentage of organic solvent in the mobile phase, upon which a mixture of two diastereomers of similar peak areas in LC−MS chromatograms was obtained. Interconversion of isomers during solvent evaporation even under mild conditions was reported, which explains the increased percentage of the earlier eluting isomer compared to the original reaction mixture. This observation is in line with our findings that **3** converts into **2** upon evaporation.

Beyer et al.[11] provided spectroscopic data only for the main DON sulfonate isomer and assigned the structure **3A** (Figure 1), albeit with undefined stereochemistry at C9 and C10, to that isomer. The percentage of the other isomer in the solution investigated by NMR was not stated. HPLC−ELSD and LC−MS chromatograms given in the paper suggest that a mixture of a ratio of main isomer to minor isomer between 90:10 and 60:40 was analyzed by NMR. NMR data provided by Beyer et al. were similar to those obtained for **3B**, the hemiketal form of **3** in our work, except for a signal at 208.3 ppm, which is characteristic for a keto group. Hence, from the data provided, it is not possible to distinguish between keto and hemiketal form at C8 of the DON sulfonate produced by Beyer et al. In addition, the stereochemistry at C9 and C10 cannot be elucidated. Similarly, the relative intensities of fragment ions in MS/MS spectra recorded at CE 36 eV did not match with our data either for **2** or for **3** but were intermediate. However, for serious comparison of mass spectra, the same instruments and the same settings should have been used. Interestingly, the fragmentation spectra were reported to be the same for both DON sulfonate isomers, which is not the case for **2** and **3**. Considering that **3** is a mixture of two compounds in equilibrium (ketone and hemiketal at C8), which could not be separated by RP-UHPLC under the tested conditions, it would theoretically be possible that Beyer et al. succeeded in separation of these two compounds. However, there are two arguments against this hypothesis: Upon evaporation of **3**, there is partial formation of **2**, so that three compounds would have been detected in the chromatograms. In addition, considering the differences in MS/MS spectra of **2** and **3**, it is unlikely that keto and hemiketal forms show the same MS/MS fragmentation pattern.

In conclusion, both Young and Beyer et al. proposed one structure for DON sulfonate that represents the product of SO_3Na addition to C10 of the DON skeleton, formulated in its keto form, **3A** (Figure 1). No hints concerning the stereochemistry of the isolated compound(s) at C9 and C10 were given. In addition, hemiketal formation was not considered. Isomeric forms were detected but not characterized.

In summary, our research group discovered that three DON sulfonates are formed upon incubation of DON with sulfur reagents. By elucidating the structures and providing information on formation and stability, existing literature data were complemented. Treatment of DON-contaminated cereals with sodium metabisulfite and sodium sulfite will give insight into the formation pattern of the individual DON sulfonates.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of the mass spectrometric characterization of degradation products of DON sulfonates **2** and **3** upon lyophilization and evaporation under nitrogen; NMR spectra of **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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