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ARTICLE

Degradation of the *Alternaria* Mycotoxins Alternariol, Alternariol Monomethyl Ether, and Altenuene upon Bread Baking

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The stability of the *Alternaria* mycotoxins alternariol, alternariol monomethyl ether, and altenuene upon bread baking was investigated by model experiments using a spiked wholemeal wheat flour matrix. For alternariol and alternariol monomethyl ether, but not for altenuene, degradation products, formed through a sequence of hydrolysis and decarboxylation, could be identified in pilot studies. The simultaneous quantification of alternariol, alternariol monomethyl ether, altenuene, and the degradation products was achieved by a newly developed high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) multimethod. The obtained quantitative data indicate that the *Alternaria* mycotoxins are barely degraded during wet baking, while significant degradation occurs upon dry baking, with the stability decreasing in the order alternariol monomethyl ether > alternariol > altenuene. The novel degradation products could be detected after the wet baking of flour spiked with alternariol and in a sample survey of 24 commercial cereal based baking products.

KEYWORDS: Alternariol; alternariol monomethyl ether; altenuene; degradation; baking; bread; HPLC-MS/MS

INTRODUCTION

Alternariol (**1**, 3,7,9-trihydroxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one), alternariol monomethyl ether (**2**, 3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one), altenuene (**3**, (2*S*, 3*S*,4a*S*)-2,3,7-trihydroxy-9-methoxy-4a-methyl-2,3,4,4a-tetrahydro-6*H*-benzo[*c*]chromen-6-one), and the altenuene diastereomer isoaltenuene (**4**) are structurally related mycotoxins produced by several *Alternaria* strains (*1*) (**Figure 1**). Compounds **1** and **2** were first described in 1953 (*2*) and subsequently shown to occur in a wide variety of agricultural commodities and food products like wheat, barley, apples, tomatoes, or wine (*1*, *3*−*5*). For **3**, which was discovered in 1971 (*6*), less data are available. However, the natural occurrence of this compound on apples, tomatoes, wheat, and barley has been reported as well (*3*, *4*).

While **1**−**3** are very weak acute toxins with an $LD_{50} \gg 400$ mg/kg bw (**1**, **2**) and ≫50 mg/kg bw (**3**) in female mice (*7*), several recent studies have drawn attention to their chronic and subacute effects. It could inter alia be shown that **1** acts as a topoisomerase poison (*8*−*10*), inducing DNA strand breaks. In these studies, **2** showed an activity similar to **1**, while **3** or **4** were not affecting DNA integrity. In competitive assays **1**, but not **3** or **4**, were shown to bind to the minor groove of DNA (*9*) (**2** not tested). **1** also replaced E2 from human estrogen receptors α (EC_{50} = 30 (±20) μM) and β (EC_{50} = 3.1 (±2.9) μM) (*11*) (**2**, **3**, and **4** not tested). In vitro studies with porcine granulosa cells furthermore revealed that **1** and **2** inhibit the synthesis of progesterone (*12*), thus possibly affecting reproductive performance (**3** and **4** not tested). These new toxicological findings have further increased scientific interest in the *Alternaria* mycotoxins and **1** in particular. Hence, a range of analytical methods for the quantification of **1**−**3** has recently been developed and applied to various food and feed matrices (*13*−*16*). However, besides toxicity and occurrence, the chemical behavior of mycotoxins during food processing needs to be understood when assessing risks associated with the consumption of food made from contaminated raw materials. Especially upon thermal treatment, mycotoxins may be subject to a variety of chemical processes. These may be irreversible or reversible and can lead to products being either more or less toxic than the parent compound. Quantitative and qualitative studies on mycotoxin degradation are thus frequently published and reviewed (*17*−*19*). However, for **1** and **2**, we know of only one degradation study (*20*) in which the stability in sunflower flour upon thermal treatment was investigated. While both compounds were stable in flour heated to 100 °C, a significant degradation could be observed at 121 °C (i.e., 75% of **1** and 100% of **2** were degraded after 60 min). Information on the chemical fate of the toxins was not given.

We thus decided to investigate the stability of **1**−**3** upon thermal food processing in more detail. Our efforts were concentrated on baking due to the high relevance of this procedure and its rather harsh conditions (heating to ∼200 °C in an oxidative atmosphere and complex chemical matrix), which make a

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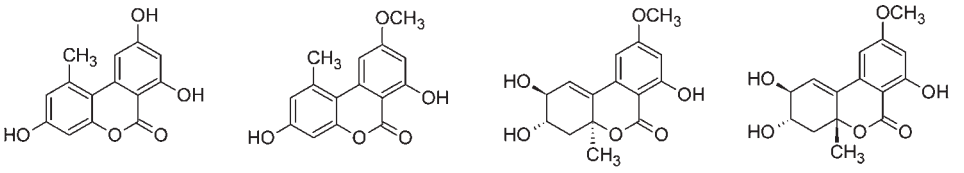

Figure 1. Structures of the dibenzo-α-pyrone based *Alternaria* mycotoxins.

degradation more likely (*17*). Also, the occurrence of **1**−**3** in wheat is known (*21*−*23*). Hence, a series of quantitative model experiments using spiked wheat flour was designed. A newly developed high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) multimethod was used for the simultaneous quantification of all analytes. In the case of **1**, a large supply of standard substance obtained through total synthesis (*24*) allowed further experiments on possible degradation products as well as thermo analysis−mass spectrometry (TA-MS) measurements.

MATERIALS AND METHODS

Chemicals. Alternariol **1** (96%), alternariol monomethyl ether **2** (no purity given), and altenuene **3** (no purity given) were obtained from Sigma-Aldrich (Steinheim, Germany). Crude alternariol for the synthesis of **5** was supplied by Prof. R. Faust, Kassel, by total synthesis according to literature procedures (*24*). Ammonium acetate (pa) was obtained from J. T. Baker (Deventer, Netherlands). The following solvents were used: acetonitrile (MeCN) and methanol (MeOH), all HPLC grade (J. T. Baker, Deventer, The Netherlands), diethyl ether (Et_2O), pa (Th. Geyer, Remmingen, Germany), ethyl acetate (EtOAc), picograde, for residue analysis (LGC Standards, Wesel, Germany) and fuming HCl (37%), pa, (Merck, Darmstadt, Germany). Deionized water was sourced from a Milli-Q Synthesis A10 system equipped with a 15 Quantum EX Ultrapure Organex cartridge (Millipore, Billerica, MA).

General Procedures. Centrifugation was done in an Eppendorf Mini-Spin Plus centrifuge (Eppendorf, Hamburg, Germany) at 14500 rpm. Stock solutions of **1**−**3**, **5**, and **6** were prepared in EtOAc and stored at −20 °C. All uncertainties correspond to standard deviations, which were either obtained directly, or through error propagation, wherever appropriate.

Synthesis of 6-Methylbiphenyl-2,3′,4,5′-tetrol (5) and 5′-Methoxy-6-methylbiphenyl-2,3′,4-triol (6) from 1 and 2, Respectively. First, 104 mg (0.40 mmol) of crude **1** or 30 mg (0.11 mmol) of commercial **2** were suspended in 15 mL of aqueous phosphate buffer (pH 7, 0.1 M, prepared by mixing 10 mL of 0.1 M KH_2PO_4 with 5.8 mL 0.1 M NaOH). The suspension was refluxed until it cleared up (**1**: ∼30 h; **2**: ∼100 h), cooled to room temperature (RT) and extracted three times with 10 mL of Et_2O. The Et_2O was removed by a gentle nitrogen stream, and the residue was taken up in 15 mL of MeCN:water 60:40 (v:v). Cleanup was done by semipreparative HPLC on an Agilent 1200 HPLC tower (Agilent, Böblingen, Germany) using a 250 mm × 7.8 mm i.d., 5 μm, Nucleodur 100−5 C18 column (Macherey-Nagel, Düren, Germany). Eluents were water (A) and MeCN (B). The method was isocratic with 60:40 (v:v) A:B from 0 to 8 min, 0:100 A:B from 8 to 12 and 3 min, 60:40 A:B from 12 to 15 min (re-equilibration). Total runtime: 15 min. The flow rate was 5 mL/min and the injection volume 100 μL. Fractions were collected from t_R = 5 to 6 min (**5**) and t_R = 7 to 8 min (**6**). The collected fractions were kept in the dark under a gentle nitrogen stream and were left to dry under nitrogen after completion. Compounds **5** (yield: 67%, 63 mg, 0.27 mmol) and **6** (yield: 82%, 22 mg, 0.09 mmol) were obtained as white solids. Stock solutions were prepared in EtOAc and stored at −20 °C.

6-Methylbiphenyl-2,3′,4,5′-tetrol (5). Exact mass ((−)electrospray-time-of-flight mass spectrometry: (−)ESI-TOF), 231.063 Da; calculated mass, 231.066 Da; Δ*m*Da, 3.

^{1}H NMR (300 MHz, DMSO-d_6, OH not shown): δ 1.91 (s, 3H, H13), 5.97 (d, 2H, H8 + H12), 6.11 (m, 2H, H2 + H4), 6.19 (d, 1H, H10) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ 20.8 (C13), 100.5 (C10), 100.9 (C2), 108.1 (C4), 109.2 (C8 + C12), 120.9 (C6), 137.4 (C5), 140.2 (C7), 155.6 (C1), 156.8 (C3), 158.2 (C9 + C11) ppm.

Heteronuclear multiple quantum coherence (HMQC, 300 MHz, DMSO-d_6): C2−H2, C4−H4, C8−H8, C10−H10, C12−H12, C13−H13.

HMBC (300 MHz, DMSO-d_6): C2−(H2 + H4), C4−(H2 + H4), C10−H10, (C8 + C12)−(H8 + H12), C13−H13.

5′-Methoxy-6-methylbiphenyl-2,3′,4-triol (6). Exact mass ((−)ESI-TOF): 245.078 Da; calculated mass, 245.082 Da; Δ*m*Da, 4.

^{1}H NMR (600 MHz, DMSO-d_6): δ 1.91 (s, 3H, H13), 3.67 (s, 3H, H14), 6.08 (m, 1H, H8), 6.10 (d, 1H, H4), 6.11 (t, 1H, H12), 6.18 (d, 1H, H2), 6.21 (t, 1H, H10), 8.69 (s, 1H, H15), 8.96 (s, 1H, H16), 9.17 (s, 1H, H17) ppm.

^{13}C NMR (150 MHz, DMSO-d_6): δ 20.3 (C13), 54.6 (C14), 99.0 (C10), 100.0 (C2), 107.0 (C8), 107.6 (C4), 110.2 (C12), 120.1 (C6), 136.8 (C5), 139.8 (C7), 155.0 (C1), 156.5 (C3), 157.7 (C11), 159.8 (C9) ppm.

Heteronuclear single quantum coherence (HSQC, 600 MHz, DMSO-d_6): C2−H2, C4−H4, C8−H8, C10−H10, C12−H12, C13−H13, C14−H14.

HMBC (600 MHz, DMSO-d_6): C1−H2, C2−(H4, H15, H16), C3−(H2, H4, H13), C4−(H2, H13, H16), C5−H13, C6−(H2, H4, H13, H15), C8−(H10, H12), C9−(H8, H10, H14), C11−(H10, H12, H17), C12−(H8, H10, H17), C13−H4, C10−(H8, H12, H17).

HPLC-MS/MS Multimethod for 1−3, 5, and 6. All analyses were done using an Agilent 1200 HPLC tower (Agilent, Böblingen, Germany) directly linked to an Applied Biosystems API 4000 QTRAP tandem mass spectrometer (Applied Biosystems, Carlsbad, CA) equipped with a Turbo-Spray ion source. The column used was a 150 mm × 2 mm i.d., 3 μm, Polar Advantage II (Dionex, Idstein, Germany). The chromatographic parameters were as follows: oven temperature, 40 °C; flow rate, 0.4 mL; eluents, water (A) and MeOH (B), both modified with 400 mg/L ammonium acetate. The following linear gradient was used: 0 to 100% B in 10 min followed by 100% B for 6 min and 100% A for 5 min (re-equilibration). The injection volume was 10 μL for the sample survey. To protect the MS ion source from contamination, the injection volume was lowered to 2.5 μL for all baking study injections. Two injections were done for each vial, with the resulting peak areas being averaged. The MS parameters were optimized by using the instruments compound optimization and flow injection analysis functions. The utilized MRM transitions (*m*/*z* mother ion → quantifier/qualifier) were *m*/*z* 259.1 → 185.0/128.1 (**1**, (+)ESI), *m*/*z* 273.1 → 128.1/115.0 (**2**, (+)ESI), *m*/*z* 290.9 → 202.7/247.0 (**3**, (−)ESI), *m*/*z* 231.0 → 187.0/188.8 (**5**, (−)ESI), *m*/*z* 233.1 → 122.9/215.0 (**5**, (+)ESI) and *m*/*z* 244.9 → 229.6/200.9 (**6**, (−)ESI).

Spiking of Wheat Flour. First, 200 g of an uncontaminated wholemeal wheat flour were spiked with **1**−**3** by mixing it with 400 mL of a stock solution containing all three toxins (solvent: Et_2O, $c \sim 1$ mg/L each, exact concentrations known). Then the suspension was ultrasonicated for 5 min and shaken for 30 min. The Et_2O was slowly removed using the rotary evaporator. The spiked flour was allowed to age for one week at RT. A second batch of flour was spiked exclusively with **5** and **6** using the very same procedure. The calculated analyte concentrations in the flour batches were approximately 2 mg/kg (exact concentrations known).

Wet Baking Study. For the wet baking study, three glass bowls were charged with 7 g of spiked flour (exact weight = m_{flour}) and 14 mL of water each. After manual mixing, the open bowls were put into a preheated oven. At t = 30, 45, and 60 min, one bowl was removed from the oven and allowed to cool to RT. This experiment was done in triplicate for three oven temperatures (170, 200, and 230 °C), respectively (for obtaining representative chromatograms, single sample preparations were also done for other times and temperatures). After cooling, the weight of the baked product ($m_{product}$) was recorded and the contents of the glass bowls were homogenized individually in a kitchen mill.

The quantification of **1**−**3** was done by one-point standard addition, whereas both points were determined three times. For each glass bowl, six 20 mL brown glass vessels were charged with $m_{sample} = m_{product}/7$

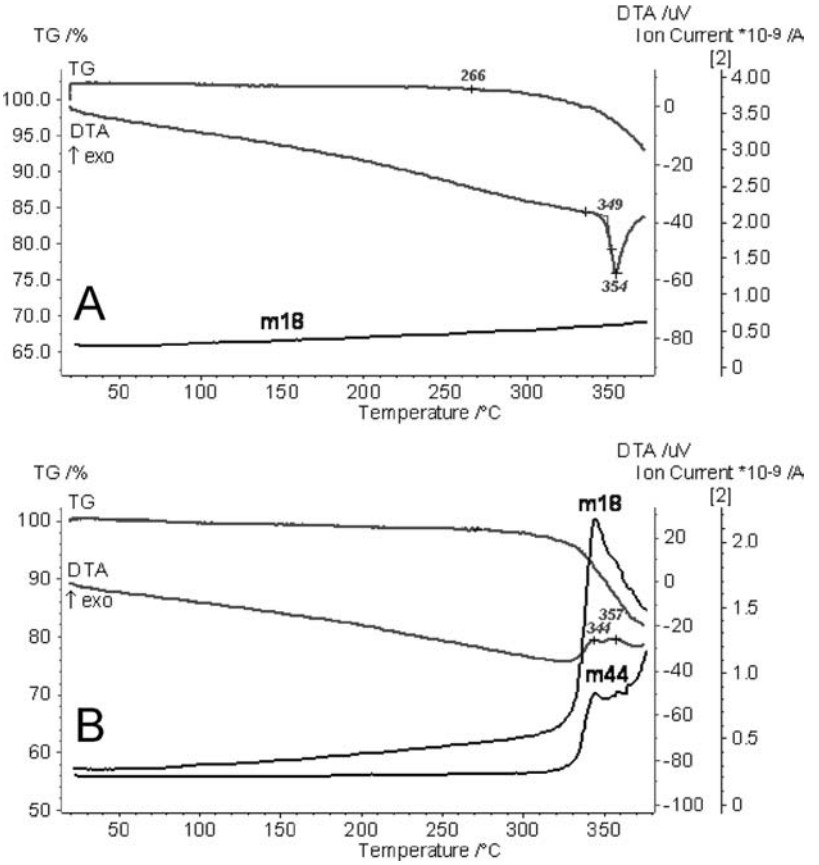

Figure 2. TA-MS curves for bulk **1** in argon (**A**) and air (**B**) with the IC curves for the mass numbers *m*/*z* 18 (H_2O^+) and 44 (CO_2^+).

(the equivalent of 1 g flour) of the homogenized product, respectively. The remaining product was discarded. Depending on baking time and temperature, a varying amount of water was added to each vessel to compensate for the varying amount of residual water in the baked product.

$$m_{water} = 8\ g - m_{sample} \quad (1)$$

Subsequently, 2 mL of 2 M HCl were added. Eventually, three of the six vessels were supplemented with 5 mL of EtOAc, while for the other three, 5 mL of a EtOAc stock solution containing **1**−**3** (c_{stock} = 0.4 mg/L, respectively) were used. The weight of the EtOAc (m_{EtOAc}) was recorded. The resulting ternary phase systems were shaken for 45 min, ultrasonicated for 10 min, and shaken again for 45 min. Then, 2 mL of the upper EtOAc layer were transferred into a 2 mL Eppendorf tube and centrifuged for 10 min to achieve complete phase separation. The EtOAc was eventually transferred to a HPLC vial through a Minisart RC 4 regenerated cellulose syringe filter (Sartorius, Goettingen, Germany) and stored at −20 °C, resulting in a total of six vials per baking time, temperature, and replicate. Subsequent to HPLC-MS/MS analysis, the following additional parameters were obtained for each sample preparation:

PA: peak area of the analyte

c_{stock}: concentration of the analyte in the EtOAc stock solution [mg/kg]

SF: scaling factor = $7 \times m_{EtOAc}/m_{flour} \approx 4.51$

The standard addition curves were constructed by plotting the terms PA (*y*-axis) against $c_{stock} \times SF$ (*x*-axis) for all six sample preparations and conducting linear least-squares regression (the latter term equals zero for the sample preparations without addition of standard substance). From the resulting regression curve, the parameters $a \pm s_a$ (slope) and $b \pm s_b$ (*y*-axis intercept) were obtained. The concentration of the analyte in the flour equivalent then computes as

$$c_{flour} = -b/a \quad (2)$$

and its RSD as

$$s_{c\,rel}{}^2 = (s_a/a)^2 + (s_b/b)^2 \quad (3)$$

The semiquantitative determination of the **5** formed during the baking study was done by external calibration.

Dry Baking Study. For the dry baking study, 18 brown glass vessels (20 mL) were charged with 1 g of spiked flour, respectively. The open vessels were put into a preheated oven. At t = 30, 45, and 60 min, six vessels were removed from the oven and allowed to cool to RT. The sample preparation was done directly inside the brown glass vessels exactly as described above. The amount of water added was fixed to 7 g for all samples.

Quantification of Water. The water content of the spiked flour was determined by automated Karl Fischer titration using a KF 756 coulometer, a KF 728 stirrer, and a KF oven (METROHM, Filderstadt, Germany). Water was extracted from the flour for 10 min at 165 °C and a nitrogen gas flow of 60 mL/min. A flour sample of 50 mg was used for each determination. The resulting water content was 8.43 ± 0.3%. The water content in the baking study products was approximated by assuming the weight loss (m_{loss}) due to baking to be caused by water evaporation only. The total amount of water in the baking product (m_{water}) was then calculated according to

$$m_{water} = \text{water added to the flour} + \text{water naturally present in the flour} - m_{loss} = 14.0\ g + 1.2\ g - m_{loss} \quad (4)$$

All measurements were done in triplicate.

Calibration, Determination of Matrix Effects, and Recoveries. Calibration curves were constructed on the day of the analysis by weighing variable portions of an EtOAc stock solution into HPLC vials. After removal of the EtOAc by a gentle nitrogen stream, the respective solvent was added gravimetrically and the vials were ultrasonicated briefly. For the calculation of matrix effects, two five-point curves in the concentration range 0.2−3.2 mg/kg were constructed. For curve 1, the solvent was pure EtOAc. For curve 2, the solvent was EtOAc originating from a sample preparation of the nonspiked, noncontaminated wholemeal wheat flour used throughout this study. The matrix effect (ME) then calculates as

$$ME = 100 \times (\text{slope curve 2}/\text{slope curve 1}) \quad (5)$$

For the determination of the overall recoveries, curve 3 was constructed from the standard addition data obtained during the baking study. The *x*-values of curve 3 corresponded to the concentration of the analyte in the EtOAc added during the sample preparation (either 0 or 0.4 mg/L) and the *y*-values to the obtained peak area. The overall recovery then computes as

$$\text{recovery} = (\text{slope curve 3}/\text{slope curve 1}) \times 100 \quad (6)$$

The sample preparation recovery was calculated by dividing the overall recovery by the matrix effect. The limits of detection (MLD) were defined as the concentrations at which the signal-to-noise ratio was 3:1. The limit of quantification (MLQ) was defined as the MLD × 3.

Sample Survey. Baking product samples were purchased at a local supermarket and homogenized in a kitchen mill. The sample preparation was done in triplicate as described under "Dry Baking Study" with the amounts of sample and added water being fixed to 2 and 6 g, respectively. The results were evaluated semiquantitatively using calibration curves. The MLDs were 20 (**1**), 5 (**2**), 10 (**3**), 15 (**5**), and 15 (**6**) μg/kg.

Thermo Analysis−Mass Spectrometry (TA-MS). A STA 409 C *Skimmer* system (Netzsch, Selb, Germany) equipped with a QMG 421 (Balzers, Balzers, Liechtenstein) quadrupole mass spectrometer was used to record the thermoanalytical curves (temperature (T), differential thermo analysis (DTA), thermogravimetry (TG), and differential thermogravimetry (DTG)) together with the ionic current (IC) curves in the multiple ion detection (MID) mode (*25*, *26*). Further experimental details were as follows: DTA-TG sample carrier system; Pt/PtRh10 thermocouples; platinum crucibles (0.8 mL); sample mass 9−15 mg (measured versus empty reference crucible); constant purge gas flow of 70 mL/min argon 4.8 or synthetic air; constant heating rate 10 K/min; raw data evaluation with manufacturer's software PROTEUS (v. 4.3) and QUADSTAR 422 (v. 6.02). The determination of the initial (T_i), extrapolated onset (T_{on}^{ex}), and peak (T_P) temperatures was done following international recommendations (*27*).

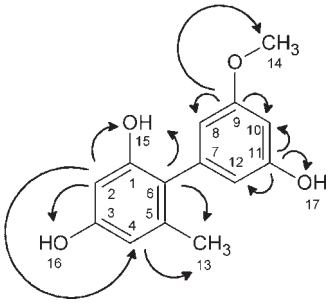

Figure 3. Selected HMBC correlations for **6**.

RESULTS AND DISCUSSION

Thermal Stability of Bulk 1. Initially, the behavior of bulk **1** upon dry heating was investigated. To do so, a TA-MS coupling device (*25*, *26*) was employed. In the present case, this device allows for the detection of endo- or exothermic processes (e.g., isomerization) by DTA while the TG curves show mass loss due to evaporation or chemical split off reactions. Furthermore, IC curves recorded by the coupled quadrupole mass spectrometer allow for a qualification of liberated gaseous species. TA-MS experiments were performed in argon and air (**Figure 2**). In argon, the constant sample mass indicates absence of any dynamics up to 270 °C. The subsequent mass loss is attributed mainly to the evaporation of **1**. The endothermic effect with T_{on}^{ex} 349 °C is the melting peak (literature value: 350 °C (*2*)) followed by stronger evaporation. Even when sublimed at 380 °C (ambient pressure, argon atmosphere), **1** deposits in the form of crystalline needles (*28*), thus further confirming the high thermal stability of the compound. In air, the mass loss above 300 °C is due to a burning process (as indicated by *m*/*z* 18 (H_2O^+) and *m*/*z* 44 (CO_2^+)). Both in argon and air the temperatures required for a substantial mass loss exceed typical food processing conditions. Hence, the conclusion which can be drawn at this stage is that bulk **1** is thermally stable at common baking temperatures both in oxidizing and nonoxidizing atmospheres.

Formation of 5/6 from 1/2. In pilot studies, **1**−**3** were refluxed separately in (i) aqueous phosphate/citrate buffer (0.15 M, pH 5), (ii) 0.1 M KOH, and (iii) aqueous phosphate/citrate buffer (0.18 M, pH 7) for 5 h with the solutions being analyzed by HPLC-diode array detection (DAD). While all three compounds were stable in (i), complete degradation accompanied by browning of the solution occurred in (ii), however, no well-defined degradation products could be identified by HPLC-DAD. In (iii), **3** was stable, but for **1** and **2**, degradation took place. For both compounds, a novel DAD peak, corresponding to the degradation products **5** (6-methylbiphenyl-2,3′,4,5′-tetrol) and **6** (5′-methoxy-6-methylbiphenyl-2,3′,4-triol), could be observed. Subsequently, **5** and **6** were synthesized and purified as described above. The unambiguous structural assignment was possible on the basis of the ^{1}H, ^{13}C, HSQC, and HMBC NMR spectra as well as exact mass measurements (**Figure 3**).

The mechanism suggested for the formation of **5** and **6** from **1** and **2** is shown in **Figure 4**. It is initiated by hydrolysis of the lactone group and thus favored by an elevated pH. The resulting

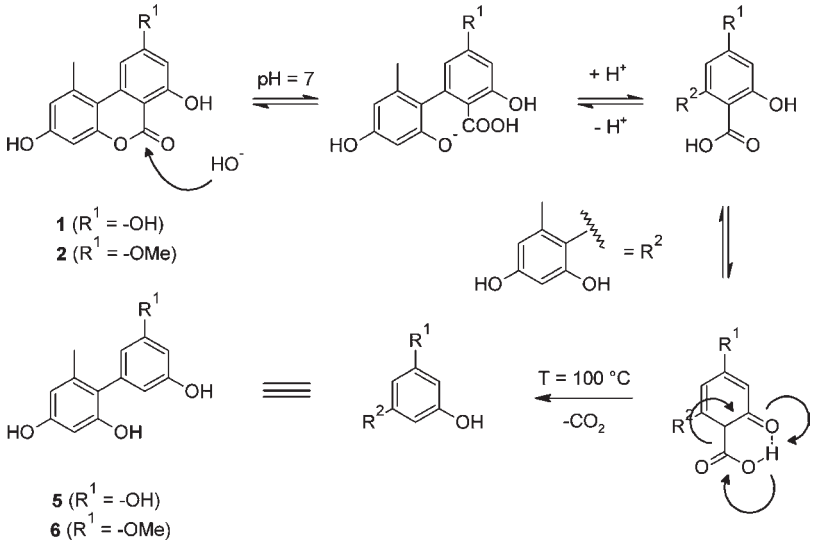

Figure 4. Postulated mechanism for the formation of **5**/**6** from **1**/**2** upon refluxing in aqueous citrate/phosphate buffer (pH 7).

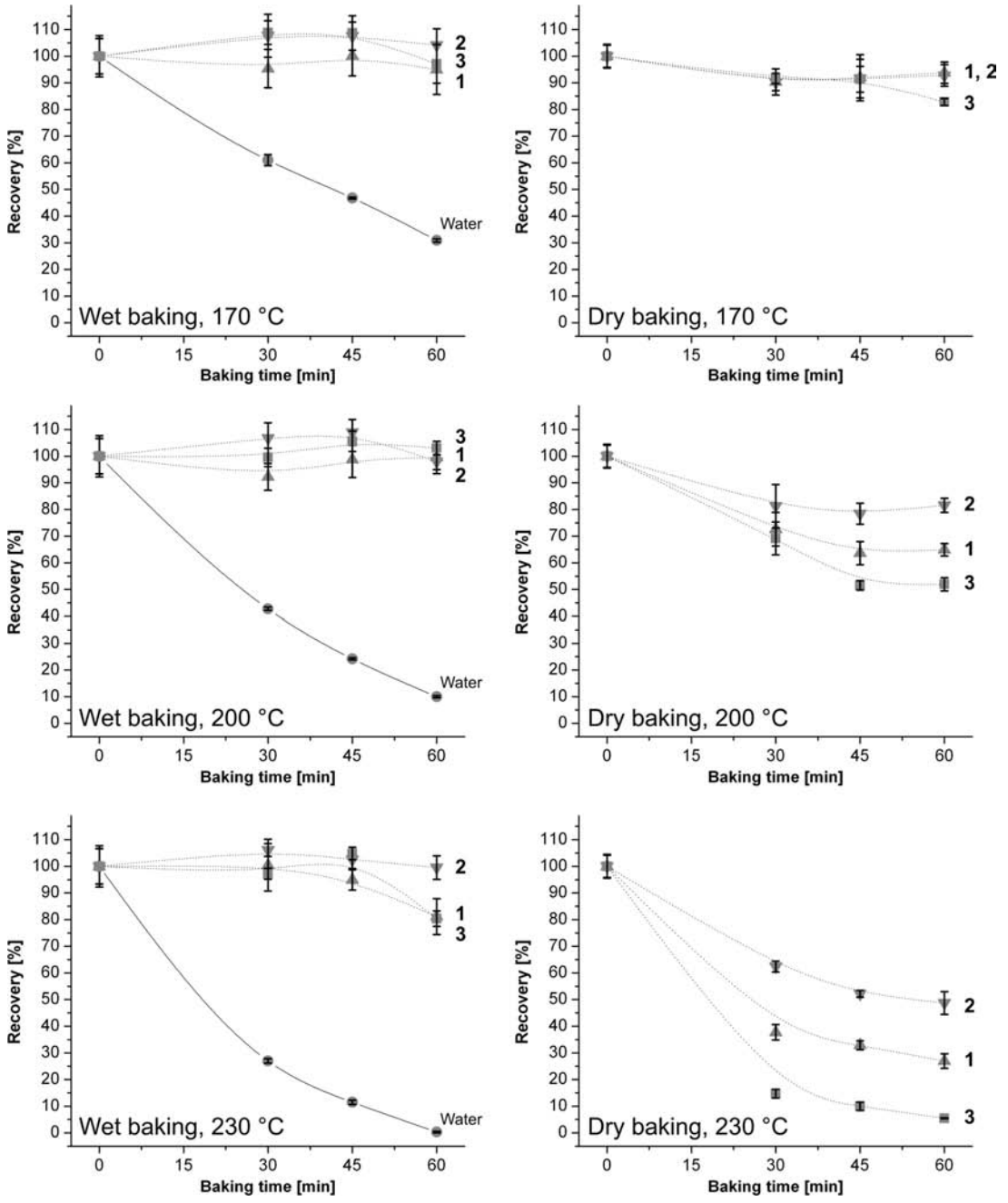

Figure 5. Graphical representation of the baking study results, analytes: solid circle, water; upward-pointing triangle, **1**; downward-pointing triangle, **2**; solid square, **3**.

hydrolyzed intermediate is finally decarboxylated. Decarboxylation is favored by the presence of a hydroxyl group in an *ortho* position of the carboxylic acid moiety, allowing for a cyclic decarboxylation intermediate (*29*). This may explain why decarboxylation takes place at the relatively low temperature dictated by the boiling point of the aqueous solvent. That **3** is not susceptible to the shown mechanism is possibly due to the lack of aromaticity in the C1−C6 ring (**Figure 3**). If this ring is not aromatic, the charged, hydrolyzed intermediate (**Figure 4**) lacks resonance stabilization, thus disfavoring hydrolysis. Furthermore, the steric shielding of the **3** lactone group by the diastereotopic methyl moiety, which is not present in **1** and **2**, might reduce reactivity. The same arguments apply to the structurally comparable *Fusarium* mycotoxin zearalenone, which was also unreactive under the conditions that led to the formation of **5** and **6**.

It can furthermore be noted that the reaction rates of **1** and **2** are limited by their low solubility in water. In the employed phosphate/citrate buffer (pH 7), the approximate solubilities were 1.1 mg/kg (**1**) and 0.1 mg/kg (**2**). This explains the significantly longer reaction time needed for quantitative conversion of **2**.

Because the degradation of **1** and **2** by the mechanism shown in **Figure 4** requires water, heat, and a pH of 7, no **5** was formed during the TA-MS experiments. Also, **5** and **6** could not be detected in preliminary boiling experiments (results not shown) with tomato soup (pH 4.3) and apple puree (pH 3.6) spiked with **1** and **2**.

Design of the Baking Experiments. Temperature is an essential parameter in the degradation of chemical substances. However, the temperature inside an oven differs from the temperature inside the dough being baked. A steep temperature gradient of 200 → 120 °C is observed only in the outermost layer of the

dough, whereas the inside (i.e., the bulk of the material) does not exceed 106 °C even at the end of the baking process (*30*). This should be considered when evaluating baking studies.

During our study, two experimental designs were employed: one using spiked flour only (dry baking) and one using spiked flour and water (wet baking). Oven temperatures (170−230 °C), baking times (0−60 min) as well as the amounts of flour and water were chosen in a way that all stages of the baking process could be reproduced (i.e., whereas baking for 15 min at 170 °C afforded a moist sponge, baking for 60 min at 230 °C caused the complete evaporation of all added water, yielding a rusk-like product). The average water content of most bread types lies between 37 and 42% (*30*). To obtain such a water content with the recipe used in our baking study, baking for 45−60 min at 200 °C or baking for 30−45 min at 230 °C are most suited and may thus be considered most realistic.

In this context, the experiments done with flour only are less realistic, as the absence of water causes the bulk flour to attain the actual oven temperature, which is substantially higher than the 106 °C usually reached. However, these experiments have some relevance for the processes occurring inside the outer layer of the dough and can show some general tendencies in compound stabilities.

Method Development. During baking, a range of chemical processes occur, e.g. the denaturation of proteins, starch gelatinization, the release of dextrins, mono- and disaccharides as well as caramelization and nonenzymatic browning reactions (*30*). This implies that a baked product will have chemical and physical properties significantly different from the dough. This is also the case for the different products to be analyzed during a quantitative time-resolved baking study. If the same quantitative analytical method is used on all baking products, whether they were obtained after 30 min at 170 °C or 60 min at 230 °C, it is important to ensure that the chemical changes during baking do not falsify the quantitative data through varying matrix effects. Ultimately this means that either a reliable internal standard (e.g., an isotope standard when using mass spectrometry) has to be used or that the recovery of the employed method needs to be established for each baking time and temperature. As isotope standards are not yet commercially available for **1**−**3**, we decided to employ the method of standard additions. Standard additions were done for each baking time and temperature.

The MRM transitions for the quantification of **1**−**3**, **5**, and **6** were chosen on the basis of both intensity and selectivity, e.g. for **2** the most intense (−)ESI transitions were: *m*/*z* 270.9 → 255.9 (100%) and 270.9 → 228.0 (24%) as opposed to *m*/*z* 273.1 → 128.1 (70%) and 273.1 → 115.0 (47%) for (+)ESI. However, as the most intense transition corresponds to a mere demethylation, (+)ESI was chosen for the sake of selectivity.

The utilized sample preparation routine is based on the extraction of the homogenized sample with a binary phase system of aqueous hydrochloric acid and EtOAc. This extraction methodology has been applied previously for the analysis of a derivative of the *Alternaria* mycotoxin tenuazonic acid (*31*, *32*) and provides basic cleanup through liquid−liquid partitioning. The recoveries obtained for the spiked wholemeal wheat flour used during this study are summarized in **Table 1**. In the case of **2**, the recovery was elevated to 141%. The reason for this could be identified as signal enhancement in the MS/MS ion-source due to a matrix effect (**Table 1**). Such matrix effects are commonly observed. Lattanzio et al. (*33*) communicated, for example, that upon analyzing mycotoxins in cereals by HPLC-MS/MS a statistically significant increase in the signal due to matrix effects occurred for 20 out of 32 analyte/cereal sample combinations. Although recoveries >120% are generally not desirable for quantitative analysis, the elevated recovery for **2** was judged to be acceptable for the frame of this study, as it was consistently compensated for by standard additions.

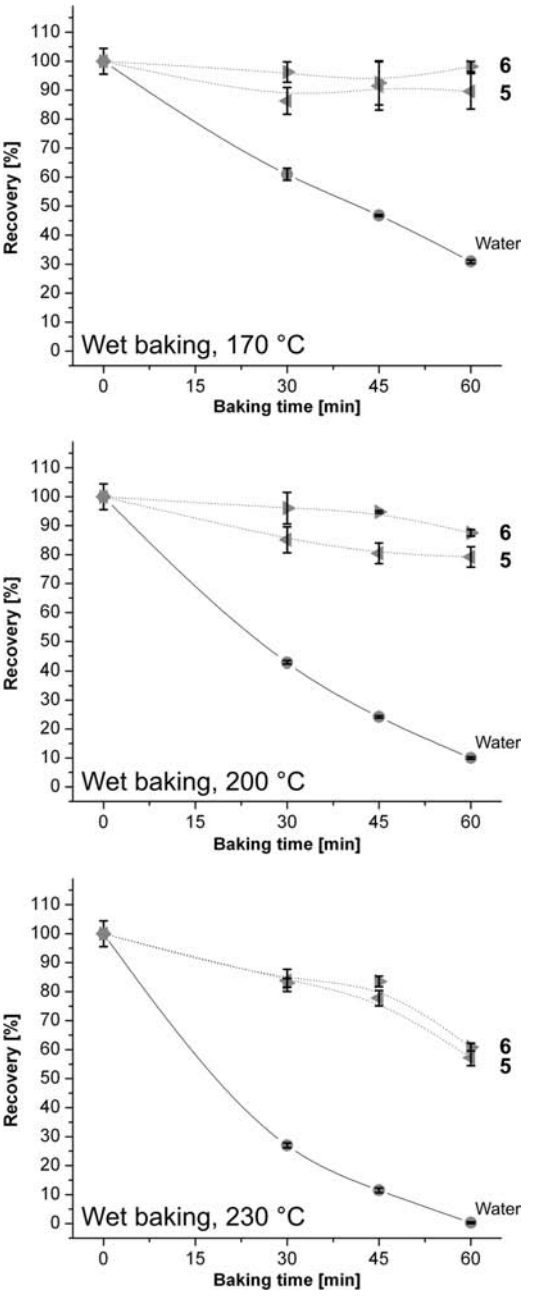

Figure 6. Graphical representation of the baking study results. Analytes: solid circle, water; left-pointing triangle, **5**; right-pointing triangle, **6**.

Table 1. Recovery Data of the Employed Analytical Method (Determined for the Untreated, Spiked Wholemeal Flour Used in the Baking Studies)

analyte	overall method recovery [%]	matrix effect [%]	sample preparation recovery [%]
1	99 ± 9	113 ± 3	88 ± 8
2	141 ± 3	160 ± 5	88 ± 3
3	102 ± 3	120 ± 2	85 ± 3
5	95 ± 4	98 ± 4	97 ± 6
6	92 ± 3	106 ± 4	87 ± 4

Stability of 1−3 upon Wet and Dry Baking. The wet baking results are summarized in **Figure 5**. Under the most realistic baking conditions (45−60 min at 200 °C or 30−45 min at 230 °C), no degradation was observed and **1**−**3** were recovered quantitatively.

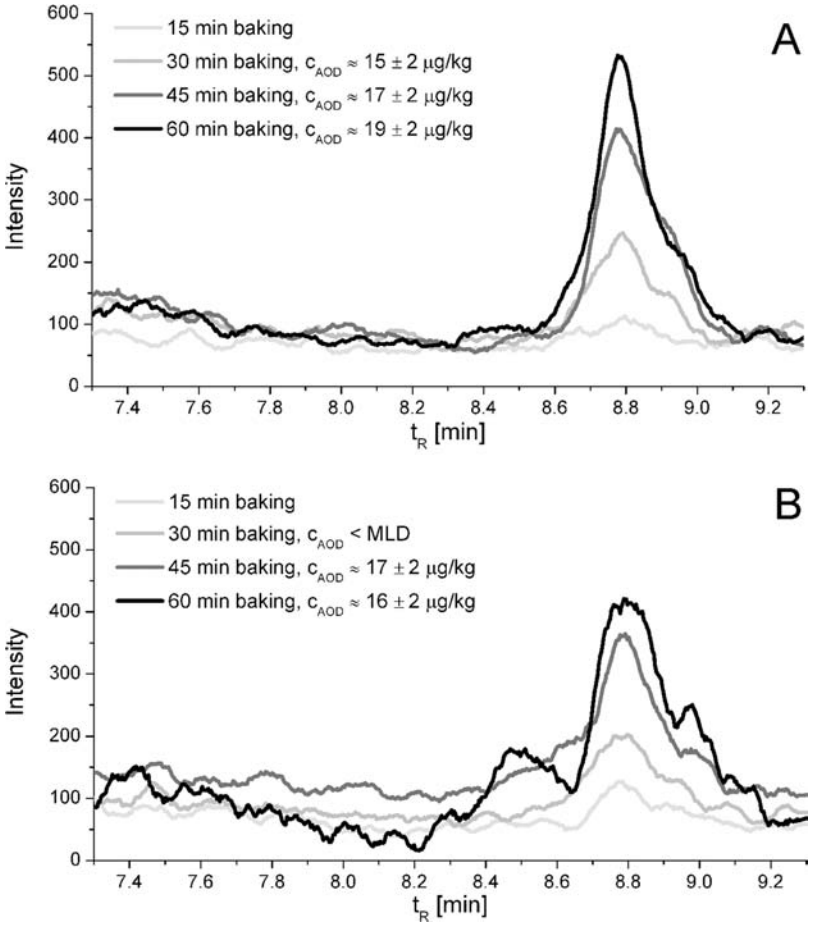

Figure 7. Excerpts of HPLC-MS/MS chromatograms showing the formation of **5** upon wet baking at 170 °C (**A**) and 200 °C (**B**).

Still, **1** and **3** were degraded slightly after 1 h at 230 °C. **2** was stable at all times and temperatures.

Upon dry baking, degradation was much more pronounced. Here, a clear graduation in compound stabilities could be observed, with **2** being the most stable, followed by **1** and **3**. In fact, **3** was almost fully degraded after dry baking at 230 °C for 1 h, while in the case of **2** the recovery was still about 50%.

The dry baking results show that degradation mechanisms different from the one given in **Figure 4** exist because no free water is available for solubilization and hydrolysis during dry baking (also, no **5** and **6** were detected). These additional mechanisms can be expected to involve compounds originating from the flour matrix, as the TA-MS results for bulk **1** (no matrix present) indicate stability up to 270 °C.

It can also be noted that, while dry baking at 200 and 230 °C caused a degradation of the considered toxins, it was (quite naturally) accompanied by changes in color, consistency, and smell of the heated flour. Hence, dry heat is not suitable as a detoxifying pretreatment for flour.

Formation and Stability of 5 and 6 upon Wet Baking. 5 was present in concentrations < 20 µg/kg in those baking products obtained from flour spiked with **1**−**3** after wet baking at 170 and 200 °C (**Figure 7**). It was not found for 230 °C. **6** was not detected at all. The detected quantities of **5** are low and correspond merely to approximately 1% conversion of **1**. Therefore, no significant decrease of the **1** concentration was detectable.

To understand why **5** and **6** were not found in the baking products prepared at 230 °C, the wet baking study was repeated with a wholemeal flour batch spiked exclusively with **5** and **6**. The results of this second study are summarized in **Figure 6**. While both **5** and **6** were stable at 170 °C, significant degradation occurred at 200 and 230 °C, with **6** being more stable than **5**.

Table 2. Results of the Sample Survey (24 Baking Product Samples Analyzed)

analyte	samples above MLD	samples above MLQ	detected in (concentration)
1	0	0	
2	1	0	buckwheat cookies (<15 µg/kg)
3	2	0	wholemeal bread roll (<30 µg/kg), rusk (<30 µg/kg)
5	2	0	rye crisp bread (<45 µg/kg), wholemeal rye bread roll (<45 µg/kg)
6	2	0	spelt rusk (<45 µg/kg), wholemeal crisp bread (<45 µg/kg)

Upon interpreting the results further, it can be seen that the degradation rates of **5** and **6** at 200 and 230 °C are higher than the degradation rates of **1** and **2** under the same conditions. This means that **5** and **6** are degraded faster than they are formed and explains why no **5** and **6** can be detected at 230 °C. That no **6** is formed even at 170 °C may be attributed to the low water solubility of **2**, causing a lower hydrolysis rate (**Figure 4**).

In summary, **5** is the stable end product of **1** degradation upon wet baking at 170 °C, while it should be considered a mere degradation intermediate at higher temperatures. The further chemical fate of **5** is yet unclear and will be subject to further studies.

Sample Survey. A total of 24 baking product samples was obtained from a local supermarket. The samples were: wholemeal bread rolls (6×), wholemeal bread (3×), rusk (3×), wholemeal

rusk (3×), crispbread (2×), wholemeal crispread (2×), rye crispbread, spelt rusk, wholemeal rye bread roll, pumpernickel bread, and buckwheat cookies. Compounds **2**, **3**, **5**, and **6** were above the LOD in 7 samples (**Table 2**, confirmed by the MS quantifier and qualifier transitions).

The encountered levels of **5** and **6** are low, which is probably due to low levels of the parent compounds **1** and **2**. That **5** and **6** could be detected at all might be attributed to more favorable formation conditions compared to the ones of the baking study. In this context, it is noteworthy that the majority of **5**/**6** positive samples are rusks or crispbreads. The latter can be produced by extrusion, a process which relies on high temperatures and the application of pressure (*30*). Extrusion has been shown to promote the degradation of a range of *Fusarium* mycotoxins (*34*−*36*) and might thus also favor **5**/**6** formation. The toxicological properties of **5** and **6** are yet unknown, as these compounds are described for the first time. In this respect, further studies are necessary. However, in view of the low acute toxicity of **1**−**3** and the low levels encountered in the sample survey, a risk of acute intoxications is not indicated. To quantify the risk of chronic and subacute effects caused by the repeated ingestion of low quantities of **1**−**6**, food monitoring data and further toxicological studies (particularly concerning bioavailability) are required, not the least because our data indicate that consumers of bakery products will be exposed to the full quantity of **1**−**3** originally present in the flour.

ABBREVIATIONS USED

DAD, diode array detection; DTA, differential thermo analysis; DTG, differential thermo gravimetry; ESI, electrospray ionization; Et_2O, diethyl ether; EtOAc, ethyl acetate; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum coherence; IC, ionic current; MeCN, acetonitrile; MeOH, methanol; MLD, limit of detection; MLQ, limit of quantification; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tTandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; RT, room temperature; TA, thermo analysis; TG, thermo gravimetry; TOF, time-of-flight mass spectrometry.

Supporting Information Available: Analyte specific parameters of the QTRAP 4000 MS instrument as well as data on the working ranges of the employed analytical method. This material is available free of charge via the Internet at http://pubs.acs.org.

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