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## **6.6. Study VI. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.**

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**Authors:** Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S.

### **Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.**

**Arnau Vidal, Vicente Sanchis, Antonio J. Ramos, Sonia Marín\***

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain

Running title: Kinetics of DON and OTA during baking of wheat products

\* Corresponding author. Food Technology Dept, XaRTA-UTPV, Agrotecnio Center,

University of Lleida, Rovira Roure 191, 25198 Lleida, Spain. Tel.: +34 973 702542;

Fax: +34 973 702596. E-mail address: smarin@tecal.udl.cat (S. Marín).

#### **Abstract**

The stability of deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-glucoside), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), de-epoxy-deoxynivalenol (DOM-1) and ochratoxin A (OTA) during thermal processing has been studied. Baking

temperature, time and initial mycotoxin concentration in the raw materials were assayed as factors. An improved UPLC-MS/MS method to detect DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 in wheat baked products was developed in the present assay. The results highlighted the importance of temperature and time in mycotoxin stability in heat treatments. OTA is more stable than DON in a baking treatment. Interestingly, the DON-3-glucoside concentrations increased (>300 %) under mild baking conditions. On the other hand, it was rapidly reduced under harsh conditions. The 3-ADON decreased during the heat treatment; while DOM-1 increased after the heating process. Finally, the data followed first order kinetics for analysed mycotoxins and thermal constant rates ( $k$ ) were calculated. This parameter can be a useful tool for prediction mycotoxin levels.

**Keywords:** baking, wheat, deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, de-epoxy-deoxynivalenol, ochratoxin A and kinetics.

## 1. Introduction

Mycotoxins are produced by fungi and can contaminate various agricultural commodities either before harvest or under post-harvest conditions. The main mycotoxin-producing fungi in food commodities belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Wheat, such as the majority of cereals, is susceptible to be contaminated with mycotoxins. Different studies show the high presence of mycotoxins, mainly deoxynivalenol (DON), in wheat products (Pacin, Resnik, Neira, Moltó, & Martínez, 1997; Vidal, Marín, Ramos, Cano-Sancho, & Sanchis, 2013). Moreover, cereal products represent one of the main sources of exposure to DON and ochratoxin A (OTA) (Marín, Ramos, Cano-Sancho, & Sanchis, 2013).

DON is not classified as to its carcinogenicity to human by IARC (1993), and it is linked with human gastroenteritis. OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties. This mycotoxin has been classified, as a possible human carcinogen, in the group 2B, by the International Agency for Research on Cancer (IARC, 1993).

Processing of cereals at high temperatures may affect DON and OTA content. However, the extent of DON and OTA reduction during thermal food processing seems to be quite variable and dependent on the processing conditions applied: temperature, time, type of mycotoxin, and size of cereal product. For bakery products, some studies reported a significant decrease in DON levels during baking (Numanoglu, Gökmen, Uygun, & Koksel, 2012; Valle-Algarra, Mateo, Medina, Mateo, Gimeno-Adelantado, & Jiménez, 2009). By contrast, the studies of De Angelis, Monaci, Pascale and Visconti (2013) and Zachariasova, Vaclavikova, Lacina, Vaclavik and

Hajslova (2012) reported that DON is stable in processing steps involving high temperatures. Similarly, OTA seems to be quite more stable at high temperature than DON through baking (Vidal, Morales, Sanchis, Ramos, & Marín, 2014a; Vidal, Marín, Morales, Ramos, & Sanchis, 2014b). Only results on coffee roasting show a clear reduction of OTA, although, the temperature achieved in the product is higher than in bakeries (Castellanos-Onorio et al., 2011, Scudamore, Banks, & MacDonald, 2003; Valle-Algarra et al., 2009). Sometimes, the contradictory published results may be due to the different size of assayed products, which affects heat transfer and favours gradients of temperature inside the products. Some studies have been carried out in aqueous systems in order to avoid the temperature gradient (Jackson, Hlywka, Senthil, Bullerman, & Musser, 1996). All of them showed effective mycotoxin reductions over 150 °C; however their results cannot be extrapolated to solid food products.

On the other hand, unaltered mycotoxins might not be the only source of health hazard for consumers, because exists a group of metabolites called conjugates mycotoxins which cannot be detected in the rutinary mycotoxins analysis. The mycotoxin conjugates are mycotoxins attached with functional groups (masked mycotoxins) such as glycosyl residues or sulfates or attached with polymeric carbohydrate or protein matrices (bound mycotoxins) (Berthiller et al., 2009). The conjugates can be classified in function of their origin, producing 4 differentiate groups: from plants, fungi, mammalian and food processing. The co-occurrence of conjugated DON forms has been documented in wheat, especially deoxynivalenol-3-glucoside (DON-3-glucoside) (Simsek, Burgess, Whitney, Gu, & Qian, 2012), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (Yang, Geng, Yao, Zhang, Zhang, & Ma, 2013). The fate of DON-3-glucoside through breadmaking has been hardly studied. While some authors point out to some reduction (De Angelis et al., 2013), others have seen marked increases (Vidal et al., 2014a). On the other hand, 3-ADON, 15-ADON and de-epoxy-deoxynivalenol (DOM-1) behavior has not been studied before.

HPLC-MS is usually applied for simultaneous detection of DON and its conjugates (Vendl, Berthiller, Crews, & Krska, 2009), specially DON-3-glucoside (Berthiller et al., 2009). Due to the low concentration of DON conjugates found in wheat products, the methods of analysis require low limits of detection. Lately, the ultra-high performance liquid chromatography (UPLC) has demonstrated to be highly effective for the quantification of DON conjugates in cereal products, such as malt and beer (Zachariasova et al., 2012). No previous studies exist on simultaneous analysis of DON, DON-3-glucoside, 3-ADON, 15-ADON, and DOM-1 in bakery products.

The current study aimed to investigate DON and DON conjugates (DON-3-glucoside, 3-ADON, 15-ADON and DOM-1) and OTA kinetics during baking in a short size model bakery product, small enough to avoid temperature gradients in it. Temperature, time and initial mycotoxin concentration were assayed as factors. Moreover an optimised method to quantify DON conjugates in bakery products is presented.

## **2. Materials and methods**

## 2.1 DON and OTA contaminated flours

In order to obtain DON or OTA contaminated flour, one strain each of *Fusarium graminearum* (TA 3.234) and *Aspergillus ochraceus* (TA 3.201) were used. Both of them are kept in the Food Technology Dept. collection, University of Lleida, Spain. They were previously proved to be DON and OTA producers when cultured on wheat flour. The concentration of DON and DON-3-glucoside in the initial uninoculated flour (n=5) was  $250 \pm 44.78$  and  $45.1 \pm 15.3$   $\mu\text{g}/\text{kg}$ , respectively, while OTA could not be detected. The remaining DON conjugates were not analysed in the initial flour.

The strains were inoculated and incubated in MEA (malt extract agar) at 25 °C until strong sporulation. A spore suspension of each strain was made in water and Tween 80 (0.005% v/v). Five millilitres of either *F. graminearum* or *A. ochraceus* spore suspension were inoculated in glass flasks containing 250 g of flour and 50 mL of water. In total, 3 kg of flour were inoculated with one of the two strains. The flasks were stored at 25 °C for 19 days in the case of *F. graminearum* and 8 days in the case of *A. ochraceus*, with periodic shaking. Then, each kind of flour (3 kg) was properly powdered and homogenized and underwent either DON or OTA analysis. The content of DON and OTA was of  $12,500 \pm 1,235$   $\mu\text{g}/\text{kg}$  and  $75.5 \pm 15.2$   $\mu\text{g}/\text{kg}$  respectively (n=3), in each contaminated flour. DON conjugates were not analysed in the flour at this stage.

## 2.2 Bakery analogue preparation

The bakery analogue was prepared for each 100 g of mix with 27 g of wheat flour, 26 g of sugar, 26 g of eggs, 21 g of sunflower oil and adding to the 100 g of mix 0.5 g of baking powder (maize starch, sodium bicarbonate and disodium diphosphate). The flour used was previously prepared by mixing the uninoculated flour with the DON contaminated flour and the OTA contaminated flour depending on the desired initial mycotoxin concentration: high mycotoxin concentration (HMC) or low mycotoxin concentration (LMC). The analysed toxin levels in the initial mixed flours (n=3) were: a) HMC,  $1042 \pm 170$   $\mu\text{g}/\text{kg}$  of DON and  $3.01 \pm 0.24$   $\mu\text{g}/\text{kg}$  of OTA; and b) LMC,  $550 \pm 98$   $\mu\text{g}/\text{kg}$  of DON and  $2.11 \pm 0.30$   $\mu\text{g}/\text{kg}$  of OTA. The levels were chosen to be close to real values in food samples.

The mix was manually mixed and 3 g aliquots were poured in small paper moulds. From this point, thermoprobes (Proges Plus, Pluck&Track, Thermo bouton) were always used in some of them to register the baking temperatures; probes were placed in the centre of the moulds. Four oven temperature levels (200, 180, 160 and 140 °C) and 8 baking times (every five minutes starting at minute 5 and finishing at minute 40) were assayed in a full factorial design. These conditions were established on the basis of previous experiments. Thus 2 initial toxin concentrations x 4 baking temperatures x 8 baking times x 3 replicates made 192 different runs (9 equal cakes weighing 3 g each conformed each of the 192 runs). From the 9 cakes, 3 were pooled and used for OTA analysis, other 3 for DON analysis, and the remaining 3 were kept at -

20 °C. All samples were lyophilised for 72 h, and then the samples were stored at –20 °C until analysis.

### 2.3 Chemicals and reagents

Mycotoxin standard solution of OTA was supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). DON, DON-3-glucoside, 3-ADON, 15-ADON, DOM-1 and isotolabeled ( $^{13}\text{C}_{15}$ ) DON were supplied by Biopure (Tulln, Austria). ( $^{13}\text{C}_{15}$ ) DON was used as internal standard for UPLC-MS/MS. Acetonitrile (99.9%), methanol (99.9%) and ethanol (99.5%) were purchased from J.T. Baker (Deventer, The Netherlands). Dichloromethane ( $\geq 99.8\%$ ) and ammonium acetate ( $\geq 98\%$ ) were purchased from Sigma (Sigma–Aldrich, Alcobendas, Spain). All solvents were LC grade. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP<sup>®</sup>) and OTA (OCHRAPREP<sup>®</sup>) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (98-100%, Panreac, Castellar del Vallès, Spain), disodium phosphate anhydrous (1.16 g) (99%, Panreac, Castellar del Vallès, Spain) and sodium chloride (8.0 g) ( $\geq 99.5\%$ , Fisher Bioreagents, New Jersey, USA) in 1 L of milli-Q water; the pH was brought to 7.4 with hydrochloric acid 1 M.

### 2.4 Preparation of standard solutions

The standard solution of OTA was dissolved in methanol at a concentration of 5.0  $\mu\text{g/mL}$  and stored at 4 °C in a sealed vial until use. From this, a stock solution was prepared and checked by UV spectroscopy according to AOAC Official methods of analysis. Working standard solutions (0.5, 0.01, 0.005, 0.001 and 0.0005  $\mu\text{g/mL}$ ) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriated chromatographic system. The standard solutions of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 were dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 °C in a sealed vial until use. DON concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis. Working standard solutions (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05  $\mu\text{g/mL}$ ) were prepared as for OTA, as well as the calibration curves for all the standard solutions.

### 2.5 Sample preparation and analysis

#### 2.5.1 DON and OTA extraction for analysis with HPLC-UV and HPLC-FL.

For DON, 5 g of ground sample was extracted with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 g. Supernatant was filtered through a glass microfiber filter. Five millilitres of filtered sample was loaded on the IAC column and the column washed with 10 mL of distilled water. DON was eluted by applying

1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. The purified extracts were dried under nitrogen stream at 40 °C. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON was determined by HPLC (Waters 2695<sup>®</sup>) coupled with a UV/Visible dual  $\lambda$  absorbance Detector Waters 2487. The column an analytical column Waters Spherisorb<sup>®</sup> 5  $\mu$ m ODS2, 4.6 x 250 mm. Absorption wavelength was set at 220 nm. The HPLC mobile phase flow rate was 1.2 mL/min, the injection volume was 100  $\mu$ L and the column temperature was 40 °C. The retention time for DON was 20 min.

Briefly for OTA, 5 g of ground sample were extracted with 30 mL of extracting solution (60% acetonitrile, 40% water) by magnetically stirring for 10 min and filtered with number 1 filter Whatman. 4 mL of filtered solution was diluted with 44 mL of PBS solution and loaded on the IAC column. After this, the column was washed with 20 mL of PBS and OTA was eluted by applying 1.5 mL of methanol grade HPLC (three times back flushing) and 1.5 mL of milli-Q water, consecutively. The purified extract was dried under nitrogen stream at 40 °C. Each dried sample was resuspended with 0.5 mL of mobile phase (acetonitrile, water and acetic acid, 57:41:2). OTA was determined by HPLC (Waters 2695<sup>®</sup>) coupled with a Multi  $\lambda$  Fluorescence Detector Waters 2475<sup>®</sup>, and an analytical column Waters Spherisorb<sup>®</sup> 5  $\mu$ m ODS2, 4.6 x 250 mm. Excitation and emission wavelengths were set, respectively, at 330 and 463 nm. The mobile phase flow rate was 1 mL/min, column temperature 40 °C, the injection volume was 100  $\mu$ L and the retention time was 15 minutes.

#### 2.5.2 *Extraction of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 for UPLC-MS/MS analysis.*

The samples with the high mycotoxin initial concentration (96 samples) were selected for further UPLC-MS/MS analysis with the objective of quantifying not only DON but its conjugates: DON-3-glucoside, 3-ADON, 15-ADON and DOM-1.

1 g of ground sample was extracted with 7 mL of extracting solution (54 % water, 45 % acetonitrile and 1 % acetic acid) by magnetically stirring for 20 min. Then the sample was centrifuged for 10 min at 1780 g. 2 mL of the supernatant were mixed with 2 mL of dichloromethane and the mixture was stirred for 10 min and then it was centrifuged for 3 min at 500 g for the separation of the two phases. The upper layer was kept in a vial.

Chromatographic separation was performed by a 100 mm x 2.1 mm i.d., 1.8  $\mu$ m, Acquity UPLC HSS T3 column (Waters, Milford, MA, USA). Column temperature was held at 40 °C. The mobile phases consisted of Acetonitrile (A) and 10 mM AcNH<sub>4</sub> in water (B). The gradient was as follows: start with 20 % A, then from 0.5 min a linear increase to 50 % for 2.5 min, then at min 3 it is 50 %. At min 3.01 the mobile phase changes to 100 % A till min 4.5; and at min 4.51 it switches to 20 % A, and then column is equilibrated for 1.5 min before the next injection. The flow rate was 300  $\mu$ L/min, and an injection volume of 2.5  $\mu$ L was enabled.

For mass spectrometric detection, ultrahigh-resolution orbitrap technology was used. The operation parameters of the orbitrap MS were optimized for heated electrospray interface in both positive and negative ionization modes. Three individual transitions were monitored for each analyte, except for 3-ADON and 15-ADON, where four transitions were measured. One chromatographic run consisted of two MS/MS periods. The first period monitored analytes in positive mode (DON, DON-3-glucoside and DOM-1), whereas in the second period the negative mode was used (ADONs), and both ionization modes were run simultaneously by polarity switching.

All measurements were done with the following settings: source temperature 150 °C, desolvation temperature 350 °C, cone gas flow was 2.5 L/min, desolvation gas flow was 16.7 L/min, collision gas flow 0.17 mL/min and the capillary was 3000 V. The analyte-dependent MS/MS parameters were optimized via direct infusion of reference standard solutions; the resulting parameters are displayed in supplementary material (supplementary material, Table 1).

## 2.6 Methods performance.

The analytical methods used were assessed for linearity, precision and recovery.

### 2.6.1. HPLC-FL and HPLC-UV

Standard curves were generated by linear regression of peak areas against concentration ( $r^2$  were 0.97 and 0.99 for DON and OTA, respectively). Precision was established by determining OTA and DON levels in bakery products at least by triplicate, in those samples fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for DON and OTA are summarized in Table 1.

**Table 1. Method performances for DON, deoxynivalenol-3-glucoside, 3-ADON, 15-ADON and DOM-1 determination in matrix food baked using acetonitrile (79%)/water (19%)/acetic acid (1%) as extraction solution with UPLC MS/MS.**

Mycotoxin	LOD <sup>a</sup> (µg/kg)	LOQ <sup>b</sup> (µg/kg)	n	Spiking level (µg/kg)	Recovery <sup>c</sup> (%)	RSD <sup>d</sup> (%)
DON	0.7	2.1	3	74.53	75.1±10.6	11.1
			3	501.35	88.5±8.7	8.8
			5	1002.70	72.9±8.8	8.9
DON-3-glucoside	1.6	4.8	3	5.61	38.3±5.7	5.9
			3	37.74	54.1±10.1	10.9
			5	75.48	48.5±3.2	3.3
3-ADON	1.5	4.5	3	5.61	87.4±4.7	5.1
			3	37.74	85.4±6.2	6.5
			5	75.48	88.2±8.6	8.9
15-ADON	1.5	4.5	3	5.61	89.0±7.2	7.5
			3	37.74	89.6±9.0	9.6
			5	75.48	78.1±2.3	2.9
DOM-1	0.5	1.5	3	5.56	82.8±4.6	5.8
			3	37.37	84.3±8.4	8.1



	5	74.74	78.4±10.5	10.8
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<sup>a</sup> LOD = Limit of detection.

<sup>b</sup> LOQ = Limit of quantification.

<sup>c</sup> Mean value ± standard deviation.

<sup>d</sup> RSDr = relative standard deviation.

### 2.6.2. UPLC-MS/MS

As in the previous section, standard curves were generated by linear regression of peak areas against concentration (all  $r^2 \geq 0.996$ ). Precision was established by determining mycotoxin levels in bakery products at least by triplicate, in those samples fortified in order to calculate the recovery rates. A calibration curve for external calibration was generated for each analysis based on five concentration levels. For preparation of the standard concentrations, the multi-standard working solution was diluted (v/v) with solvent. Selectivity, sensitivity and stability of the measured product ions were evaluated throughout method development and validation by analysis of the blank food matrixes. The LOD was considered to be three fold the signal of blank noise, and the limit of quantification LOQ was calculated as 3 x LOD. Method performance characteristics for DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 are summarized in Table 1.

### 2.7 Statistical analysis

Multifactorial ANOVA were applied to assess the significance of sample traits in the observed mycotoxin levels. Moreover, multiple linear regressions were applied to assess the temperature/time effect of DON, DON conjugates and OTA reduction during the baking process.

### 2.8 Kinetic calculations.

Reaction order and kinetic constants were calculated by graphic evaluation. Based on the integration of kinetic equations for zero-, first-, and second-order,  $C_A$ ,  $\ln(C_A/C_0)$ , and  $1/C_A$  were plotted against residence time, where  $C_0$  and  $C_A$  refer to the initial and remaining mycotoxin concentration (micrograms per gram), respectively, after time  $t$  (seconds). The reaction rate constant was calculated from the slope of the linearized rate law equation. The half-life was calculated from the rate law equation by allowing  $C_A$  to equal 0.5  $C_0$ .

## 3 Results and discussion

### 3.1. Optimization of the sample preparation for UPLC-MS/MS analysis

For analysis of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1, a simple analytical method allowing low detection limits was used. Previous studies on DON and DON conjugates in bakery products have used acetonitrile/water (different percentages) and in some cases acetonitrile/water/acetic acid ( $\leq 1$  % acetic acid) for extraction (Malachova, Dzuman, Veprikova, Vaclavikova, Zachariasova, & Hajslova, 2011; Zachariasova et al., 2012). Our aim was avoiding the clean-up step, but the high concentration of carbohydrates and fat can interfere.

Therefore, extracting the target compounds from the matrix with weak interference and high recovery was a critical point. Due to the different polarity and solubility of the compounds to be extracted, in the optimization phase of the analytical protocol, spiked bakery products were extracted with mixtures consisting of different acetonitrile/water/acetic acid ratios. First, the extraction phase, acetonitrile (84%)/water (16%) (Malachova et al., 2011) was tested without good recovery results (data not shown). The mixture of acetonitrile (79%)/water (19%)/acetic acid (1%) used in some works for DON and its conjugates (De Boevre et al., 2012), led to low percentage of recovery for DON-3-glucoside (supplementary material, Table 2).

The extraction mixture of water (54%)/acetonitrile (45%)/acetic acid (1%) got an acceptable percentage of recovery for all the analysed mycotoxins (between 74 and 109%, Table 1) and it was used for sample analyses. To reach these recovery levels, an additional step was required after centrifugation, which was not reported in previous studies: 2 mL of dichloromethane were added to 2ml of sample and shaken to obtain a more purified sample in the organic phase. This helped to improve the specificity of the method.

Previous researchers analysed either DON and DON-3-glucoside (De Angelis et al., 2013; Malachova et al., 2011; Vidal et al., 2014a) or DON and 3-ADON and 15-ADON (Yang et al., 2013) in bakery products. However, both DON-3-glucoside and ADONs from DON can be present at significant concentrations in wheat products (Simsek et al., 2012; Yang et al., 2013), thus a suitable method of analysis able to simultaneously detect DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 was required.

### *3.2. Optimisation of UPLC-MS/MS conditions*

The MS parameters (declustering potential, collision energy, and cell exit potential) were optimized by means of syringe infusion on each compound separately. This showed a better response in the ESI+ for DON, DON-3-glucoside, DOM-1 and (<sup>13</sup>C<sub>15</sub>) DON while ADONs had a better response in the ESI-. Once the precursor ions were selected, product scans were recorded to test different values of declustering potential and collision energies. Declustering potential was set according to the sensitivity of precursor ions, whereas collision energies were selected to give the maximum intensity of the obtained fragment ions (supplementary material, Table 1).

The selectivity study demonstrated that interfering peaks at the retention time of analytes only occurred in those samples baked for too long (almost burnt), which were consequently not analysed through this technique. On the other hand, in regular samples, no interfering peaks were observed. The typical Multiple Reaction Monitoring (MRM) chromatogram of spiked bakery product samples is shown in supplementary material (supplementary material, Figure 1).

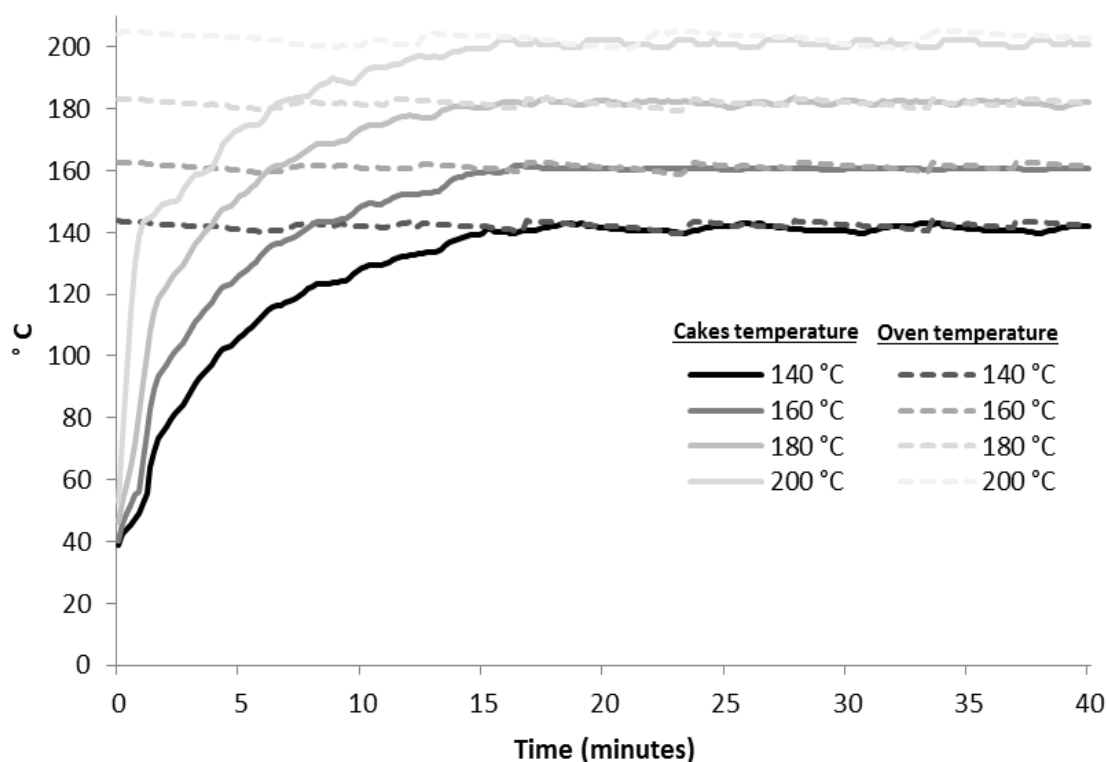
Different mobile phases from previous publications were tested to achieve an optimal separation, including mixtures of water with a volatile organic acid (formic acid, acetic acid) or ammonium acetate with an organic solvent (methanol or acetonitrile) (Yang et al., 2013; Zachariasova et al., 2012). The results showed that the best sensitivity for the mycotoxins analysed was achieved with acetonitrile (mobile phase A) and 10 mM of AcNH<sub>4</sub> in water (mobile phase B). The composition finally chosen is very similar to that in Xu et al. (2014), who analysed

DON, 3-ADON, 15-ADON and DOM-1 using methanol and 0.1 % formic acid in water, or Simsek et al. (2012), who analysed DON and DON-3-glucoside using 0.01 % acetic acid in water and 0.01 % acetic acid in acetonitrile, in bakery products.

Finally, the suitable recovery of DON-3-glucoside (mean 96%), compared to previously reported methods (around 60%, Malachova et al., 2011; Zachariasova et al., 2012), was the most important achievement of the used method. On the other hand, the LOD achieved is low enough (1.6 µg/kg) for cereal DON-3-glucoside analysis. Moreover, the other DON conjugates could be simultaneously analysed. Nowadays, due to the proven presence of DON-3-glucoside in cereals and the suggested transformation to DON during the digestion (Berthiller et al., 2011), a suitable method for analysis is required.

### 3.3. Temperature profiles during baking

Due to their small size, maximum temperature levels reached in the centre of the analogues paralleled those in the oven, after 15 min. Figure 1 shows the evolution of the temperature in the products and in the oven over time. The higher the temperature in the oven, the steeper the initial increase of temperature in the cakes.

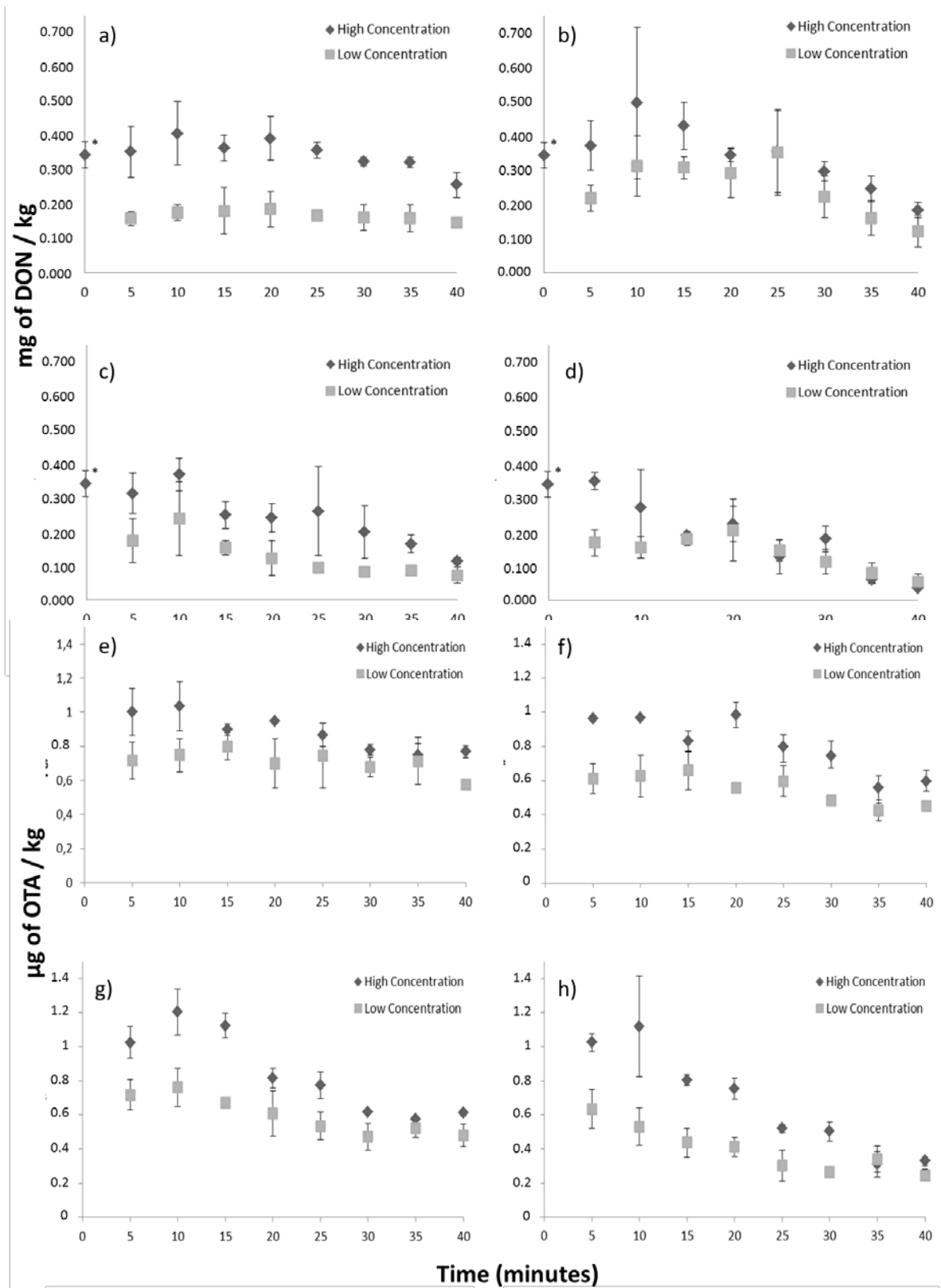


**Figure 1. Recorded temperatures in the oven and in the centre of the baking cakes for 40 minutes at 140, 160, 180 and 200 °C.**

### 3.4. Fate of DON during baking

DON was reduced by the baking process depending on baking temperature. In particular, the cake analogues baked at 160, 180 and 200 °C had a significantly lower DON concentration than at the beginning of the process ( $p < 0.05$ ), while at 140 °C the reduction achieved was not significant. The percentages of reduction at 40 minutes varied from 29 % at 140 °C to 81 % at 200 °C. The higher the temperature the higher the visually observed inactivation rates (Figure 2). For instance, the reduction achieved at 200 °C after 15 min, would require more than 30 min at other temperature levels.

Moreover, the cake analogues with higher initial DON concentration showed highest reduction results. At 200 °C, the percentages of reduction were significantly different ( $p < 0.05$ ), 89% at HMC and 67% at LMC. The differences between treatments with different initial mycotoxin concentration increased with increasing temperature levels.



**Figure 2. Concentration of DON ( $\mu\text{g}/\text{kg}$ ) in cake analogues over time at 140 (a), 160 (b), 180 (c) and 200 (d) °C at two different initial DON concentrations and concentration of OTA ( $\mu\text{g}/\text{kg}$ ) in cake analogues over time at 140 (e), 160 (f), 180 (g) and 200 (h) °C at two different initial OTA concentrations.**

Some studies dealt in the past with the stability of DON during the baking process and, although some trends were pointed out, only a few studies reported statistically significant effects (Lancova et al., 2008; Neira, Pacin, Martínez, Moltó, & Resnik, 1997; Numanoglu et al., 2012; Scudamore, Hazel, Patel, & Scriven, 2009; Valle-Algarra et al., 2009), while in other studies no significant reduction was observed (Kostelanska et al., 2011; Zachariasova et al., 2012). The different size of the bakery product could be the factor which caused such different results. In some cases, the high percentage of reduction can be attributed to the use of mycotoxin spiked products, with lower effects of baking in naturally contaminated products.

In general, the present work presents higher DON reductions than those reported before, due to the easy heat transfer across the products. Analysing the previous studies, the most important reductions were obtained in small bakery pieces where the crust of the product accounted for a high proportion (Scudamore et al., 2009), although still the temperature in the centre of the pieces did not probably reach 100 °C. Our approach, although theoretical due to the size of the cakes, allows assessing DON stability in a solid food matrix, and its reduction kinetics, but still in a much realistic approach than studies on mycotoxin solutions.

A few studies focused on the effect of different baking temperature levels in DON content in bakery products (Bergamini et al., 2010; Valle-Algarra et al., 2009; Vidal et al., 2014a), with not much significant differences among the effect of different temperature levels, and some contradictory results. The big size of the loaves, leading to a homogeneous temperature in the core area could be the reason. In the present study significant differences were found due to the tiny size of the baked product.

Comparing the present results with similar studies, it seems that when the initial concentration of DON is higher, more reduction is observed in the baking step, so the initial concentration is another factor which determines DON reduction, as observed Bergamini et al. (2010).

The time of treatment was the most determinant factor, as shown before (Lancova et al., 2008; Scudamore et al., 2009; Vidal et al., 2014a) the longer the baking time, the higher DON reduction.

Finally, the DON reduction could not produce a totally mycotoxin destruction because the detected DON reduction in the baked products could be caused by the binding of DON to the matrix or by the formation of DON conjugates.

### *3.5. Fate of DON conjugates during baking (further analysed with UPLC-MS/MS)*

Selected samples were analysed by UPLC-MS/MS, in particular HMC samples excluding the harshest treatments, where the method of analysis did not perform optimally. DON concentrations obtained with UPLC-MS/MS confirmed those by HPLC-UV.

The initial flour contained also DON-3-glucoside, and the initial level in the unbaked cakes was  $9.7 \pm 3.0$  µg/kg. DON-3-glucoside is commonly found in cereals, and almost all wheat samples analysed for DON also contain DON-3-glucoside. Simsek et al. (2012) detected levels of 290 µg/kg in wheat flour. The levels of DON-3-glucoside vary among studies, however the ratio

DON-3-glucoside/DON concentration is similar among the assays, from 10 to 30 % (Berthiller et al., 2009). In our study the ratio was 18 %, with an initial concentration of DON-3-glucoside in the flour was 45 µg/kg. Moreover, DON-3-glucoside significantly increased after baking ( $p < 0.05$ ) (Table 2), although significant differences were only observed for certain treatments. The reason was that the levels seemed to increase under lower temperature levels and short time baking periods, while later the toxin was reduced (eg. at 180 °C a significant increase was observed after 10-15 min, while later no toxin was detected). This DON-3-glucoside increase would confirm earlier reports (Vidal et al., 2014a), where a high increase (224 %) of DON-3-glucoside was also observed, although in that case the increase was reported also at high temperature and longer time, suggesting that the bigger size of the bread led to lower temperature levels, which were conducive for DON-3-glucoside release instead of reduction. By contrast, a reduction (40-50 %) in DON-3-glucoside was reported in De Angelis et al. (2013) and Simsek et al. (2012) at 200 and 220 °C baking temperature where >100 g bread loaves were used. The DON-3-glucoside increases observed in breadmaking process have been also observed in brewing process; the increase has been hypothesised to be due to enzyme actuation. Enzymes could degrade the cell walls, membrane bound proteins, and starch depots in kernels, releasing DON-3-glucoside from insoluble forms (Lancova et al., 2008). The enzyme activity could explain the increase of DON-3-glucoside detected in some bakery products. In our case, the increase of DON-3-glucoside could be due to enzymatic release of bound forms from the matrix that would explain that as time increases enzymatic inactivation occurs and the increasing trend turns in a decreasing one.

In the present assay, DON-3-glucoside increased from 30 to 642 %, while a 100% reduction was observed at the higher T/t conditions. There was a positive significant correlation ( $p < 0.05$ ) between DON and DON-3-glucoside concentrations when all samples were included in the analysis, as in both cases degradation occurred at the higher T/t treatments. However, the samples with undetectable values were deleted, the correlation coefficient became negative and also significant, suggesting that in the milder treatments DON still decreased but DON-3-glucoside increased. It is uncertain whether DON-3-glucoside could be formed at expenses of DON in this step or as commented before, the increase of DON-3-glucoside could be due to enzymatic release of bound forms from the matrix.

The accumulation of DON-3-glucoside in bakery products is of concern. Although DON-3-glucoside is far less active as protein biosynthesis inhibitor than DON (Poppenberger et al., 2003), DON-3-glucoside likely will be cleaved in the gastrointestinal tract due to chemical hydrolases or, more important, microbial activity in the intestine as shown *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011), thus its presence is important for food safety.

The concentration of 3-ADON in cake analogues at time 0 was  $8.5 \pm 1.3$  µg/kg, which means that if there was no change in its content during mixing, the concentration in the flour was approximately 30 µg/kg (27 parts of flour in the cake). The 3-ADON is a fungal metabolite which has been previously detected in wheat flour, but its presence is scarce and the contamination

level can vary widely. Yang et al. (2013) found only one sample with 3-ADON from four analysed wheat grain samples. The study of scientific cooperation (SCOOP, 2003) task 3.2.10 pointed out that only 8% of the wheat samples contain 3-ADON, but 27% of the positive samples presented a concentration over 520 µg/kg. In our case 3-ADON could come both from naturally contaminated flour or be produced by the inoculated *F. graminearum*. Due to the lack of information about 3-ADON behaviour during baking, these results are important. The effects of temperature and the interaction temperature x time were significant on the 3-ADON concentration. The levels of 3-ADON tended to decrease with increasing temperature and baking time, although the differences among the levels of treatments were most times not significant (Table 2). The low initial concentration (8.5 µg/kg), close to the LOD, produced contradictory results in some analysed samples. The possible low stability of 3-ADON observed in our results agree with the only existing study on 3-ADON thermal stability, which pointed out a fast transformation of 3-ADON to DON, norDON A, norDON B, and norDON C, when 3-ADON was submitted either to alkali or heat (Young, 1986).

15-ADON was not quantifiable at time 0 (<4.5 µg/kg), and it was only detected in 11 % of the analysed cake analogues, just in some cases in 140 and 160 °C treatments, and never at 180 and 200 °C, thus no significant effect of either temperature or time could be assessed. Similarly to 3-ADON, 15-ADON is another fungal metabolite which has been detected in wheat grain (Yang et al., 2013). The major presence of 3-ADON versus 15-ADON is a phenotypic difference within *Fusarium* species. In southern of Europe the predominant 15-ADON genotype is more predominant (Somma et al., 2014); however, we used a *F. graminearum* strain from Argentina. Castañares et al. (2014) showed predominant presence of 3-ADON phenotypic *Fusarium* species in Argentina than in Europe. No studies exist on 15-ADON thermal stability; however, De Boevre et al. (2013) detected 15-ADON in processed food products, which suggests a certain stability of this mycotoxin.

DOM-1 is known to be a conjugated product from mammalian metabolism, and it is used as a biomarker for the DON exposition (Solfrizzo, Gambacorta, & Visconti, 2014). On the other hand, it has been also identified as a degradation product of DON after a heat process (Mishra, Dixit, Dwivedi, Pandey, & Das, 2014). In our case, at time 0 cakes contained  $1.8 \pm 1.25$  µg/kg of DOM-1, thus the flour could contain DOM-1. No studies analysing the presence of DOM-1 in flour exist, thus the origin of DOM-1 in our samples is unknown. Interestingly, temperature and time treatments had a significant effect on DOM-1 ( $p < 0.05$ ), which in general increased with heat treatments (Table 2). The increase was extremely high (285%) at the higher temperature tested (200 °C, 20 min), which was the only treatment which was significantly different from the initial control, while at 200 °C and 25 min, it was not detected. Moreover, there was a significant negative correlation between DON and DOM-1 concentrations in those samples with quantifiable levels of both metabolites (Pearson correlation coefficient=-0.5884,  $p=0.0000$ ), which confirm DOM-1 as a



degradation product of DON caused by heat. In an aqueous system, Mishra et al. (2014) observed the formation of DOM-1 as a degradation product of DON after heating at 125 – 200 °C.

Finally, more degradation products could be produced after the baking step because still the sum of DON-3-glucoside and DOM-1 increases is lower than the degraded DON. Bretz, Beyer, Cramer, Knecht, & Humpf (2006) detected other degradation products after heating DON, including norDON A, B and C, however the lack of standards for these products avoid the quantification of them in this assay.

**Table 2. Concentration (µg/kg) of DON and DON conjugates (UPLC MS/MS) for the different time and temperature levels assayed.**

Temperature (°C)	DON				DOM-1				DON-3-glucoside				3-ADON			
	140	160	180	200	14	16	18	20	14	16	18	20	14	16	18	20
0 min	347.33 <sup>a</sup>	347.33 <sup>a</sup>	347.33 <sup>a</sup>	347.33 <sup>a</sup>	1.83 <sup>a</sup>	1.83 <sup>a</sup>	1.83 <sup>a</sup>	1.83 <sup>a</sup>	9.67 <sup>a</sup>	9.67 <sup>a</sup>	9.67 <sup>a</sup>	9.67 <sup>a</sup>	8.50 <sup>a</sup>	8.50 <sup>a</sup>	8.50 <sup>a</sup>	8.50 <sup>a</sup>
5 min	339.32 <sup>ab</sup>	348.45 <sup>a</sup>	318.74 <sup>ab</sup>	339.20 <sup>ab</sup>	1.63 <sup>a</sup>	1.74 <sup>a</sup>	1.49 <sup>a</sup>	1.75 <sup>a</sup>	14.27 <sup>a</sup>	12.05 <sup>a</sup>	18.01 <sup>a</sup>	29.23 <sup>a</sup>	8.66 <sup>a</sup>	9.49 <sup>a</sup>	5.39 <sup>ab</sup>	8.07 <sup>ab</sup>
10 min	346.48 <sup>a</sup>	325.49 <sup>ab</sup>	324.62 <sup>ab</sup>	263.47 <sup>ab</sup>	1.74 <sup>a</sup>	1.72 <sup>a</sup>	1.74 <sup>ab</sup>	<L	14.09 <sup>a</sup>	12.62 <sup>a</sup>	67.91 <sup>c</sup>	46.81 <sup>bc</sup>	8.14 <sup>ab</sup>	7.47 <sup>ab</sup>	8.69 <sup>a</sup>	1.08 <sup>a</sup>
15 min	298.86 <sup>ab</sup>	276.71 <sup>ab</sup>	246.92 <sup>abc</sup>	<LO	<L	<L	2.84 <sup>ab</sup>	4.04 <sup>ab</sup>	15.14 <sup>a</sup>	19.65 <sup>a</sup>	63.79 <sup>a</sup>	<L	6.43 <sup>ab</sup>	7.98 <sup>ab</sup>	<L	6.65 <sup>ab</sup>
20 min	307.70 <sup>ab</sup>	260.19 <sup>ab</sup>	144.62 <sup>b</sup>	<LO	1.64 <sup>a</sup>	1.70 <sup>a</sup>	3.37 <sup>ab</sup>	5.12 <sup>b</sup>	13.85 <sup>a</sup>	59.56 <sup>bc</sup>	<L	<L	8.77 <sup>ab</sup>	7.63 <sup>ab</sup>	3.04 <sup>ab</sup>	7.97 <sup>ab</sup>
25 min	291.51 <sup>ab</sup>	227.05 <sup>bc</sup>	<LO	<LO	1.65 <sup>a</sup>	2.74 <sup>ab</sup>	3.92 <sup>ab</sup>	<L	23.30 <sup>a</sup>	34.08 <sup>a</sup>	<L	<L	8.14 <sup>a</sup>	4.85 <sup>ab</sup>	6.74 <sup>ab</sup>	7.88 <sup>ab</sup>
30 min	304.20 <sup>ab</sup>	181.09 <sup>cd</sup>	x	x	<L	3.07 <sup>ab</sup>	x	x	38.98 <sup>a</sup>	41.56 <sup>a</sup>	x	x	8.60 <sup>a</sup>	3.57 <sup>ab</sup>	x	x
35 min	278.70 <sup>ab</sup>	X	x	x	2.02 <sup>ab</sup>	x	x	x	40.88 <sup>a</sup>	X	x	x	4.81 <sup>ab</sup>	X	x	x

<sup>abcd</sup> Different letters next to the value indicate significant differences among the treatments, same letter, no significant difference (Tukey's test  $p < 0.05$ ).  
x, not analysed samples

### 3.6. Fate of OTA during baking

OTA was significantly affected by both baking temperature and time ( $p < 0.05$ ). The maximum reduction was at 200 °C for 40 minutes (64%), while at 140 °C for the same time it was 21%. No significant differences in the reduction profiles were observed between the two different initial concentrations, although the inactivation was slightly higher at the higher concentration (Figure 2). Valle-Algarra et al. (2009) detected different level of reduction as a function of the initial OTA concentration (5 and 10 µg/kg flour), however, our initial concentrations were much closer (2 and 3 µg/kg flour).

Few works exist on OTA stability in bakery products and they showed conflicting results. Our results agree with those of Ferraz et al. (2010), Subirade (1996) and Valle-Algarra et al. (2009) who reported certain reduction in baking and roasting processes. On the other hand, some

authors reported no changes or little changes in OTA content during baking (Scudamore et al., 2003; Vidal et al., 2014a); however, they used bigger size pieces of bread which probably led to lower temperatures in the inner part. In particular, Subirade (1996) showed a reduction of 66% in OTA in biscuits. Moreover, Valle-Algarra et al. (2009) found in the crust of the bread a reduction of 40% after 50 minutes at 190 °C, which is very close to our results (37-68% reduction). Significant effects of temperature levels were reported by Boudra, Le Bars, & Le Bars (1995), however, other similar works observed none (Scudamore et al., 2003; Valle-Algarra et al., 2009). Although the effect of baking temperatures has been previously assayed, this is the first report on the effect of time.

As in DON, the possible reduction of OTA after baking does not mean a totally safer product if not OTA could be bound to the matrix or it could be transformed to degradation products. Although in our work OTA degradation products have not been studied, the literature points out to a partial isomerization of OTA in position C3 into a diastereomer (Studer-Rohr, Dietrich, Schlatter, & Schlatter, 1995), which results in the formation of 14-(R)-ochratoxin A and 14-decarboxy-ochratoxin A. These degradation products are less toxic than original OTA (Cramer, Königs, & Humpf, 2008), so it is important because the final baked products may be safer. They have not been analysed because no standards exist and moreover they have not been detected in cereals products.

### 3.7. Kinetic study

Kinetic data were studied for DON, DON-3-glucoside, DOM-1 and OTA.

#### 3.7.1 Determination of DON inactivation kinetics

DON concentrations from HPLC-UV analysis were taken, as more results were available.

First of all, the kinetic order was determined. As the log-transformed concentrations showed a linear trend versus time (1), the degradation reaction was assumed to be of first-order ( $dC / dt = k \cdot C$ ).

$$\ln C = \ln C_0 - k \cdot t \quad (1)$$

$C$  = concentration of DON at time  $t$  ( $\mu\text{g}/\text{kg}$ ).

$C_0$  = initial concentration of DON at time 0 ( $\mu\text{g}/\text{kg}$ ).

$k$  = thermal degradation rate constant ( $\text{min}^{-1}$ ).

$t$  = processing time (min).

The equation (1) is the result from the integration of the differential equation  $dC / dt = k \cdot C$ . The slope of the line is the thermal degradation rate constant ( $k$ ). Both first order degradation constants and half-lives are presented in Table 3. The half-life ( $t_{1/2}$ ) is a timescale by which the initial concentration is decreased by half of its original value; when the half-life is smaller it means the reaction is faster. It is calculated from the rate law equation by allowing  $C$  to equal  $0.5 C_0$ .

**Table 3. Estimated reaction rate constants ( $k$ ) and half-lives ( $t_{1/2}$ ) for DON, 3-ADON, DON-3-glucoside, DOM-1 and OTA.**

Mycotoxin	Temperature (°C)	Initial concentration	$k$ ( $\text{min}^{-1}$ )	$t_{1/2}$ (min)	r
DON	140	High	0.009	77.02	0.777
		Low	0.003	231.05	0.547
	160	High	0.023	30.14	0.880
		Low	0.019	36.48	0.649
	180	High	0.028	24.75	0.919
		Low	0.032	21.66	0.929
	200	High	0.057	12.16	0.910
		Low	0.031	22.36	0.835
DON-3-glucoside	140	-	-0.042	16.23	0.890
	160	-	-0.058	11.81	0.834
	180	-	-0.126	5.50	0.844
	200	-	-0.094	7.37	1.000
DOM-1	140	-	-0.016	42.78	0.847
	160	-	-0.023	30.01	0.961
	180	-	-0.063	11.00	0.979
	200	-	-0.110	6.26	0.921
OTA	140	High	0.010	69.31	0.936
		Low	0.005	138.63	0.660
	160	High	0.016	43.32	0.888
		Low	0.012	57.76	0.874
	180	High	0.022	31.51	0.918
		Low	0.014	49.51	0.932
	200	High	0.038	18.24	0.967
		Low	0.025	27.72	0.928

The half-lives and first order reaction constants confirmed the faster reduction for the high mycotoxin initial concentration and at 200 °C ( $t_{1/2} = 12.16$  s and  $k = 0.057 \text{ min}^{-1}$ ). The slowest was at 140 °C with the low mycotoxin concentration ( $t_{1/2} = 231.05$  s and  $k = 0.003 \text{ min}^{-1}$ ).

Other studies about kinetics of mycotoxin inactivation also used the first order kinetic model (Castells, Pardo, Ramos, Sanchis, & Marín, 2006; Jackson et al., 1996; Numanoglu et al., 2012). There is only a previous work on kinetics of inactivation of DON in a solid matrix, where authors used maize bread disks containing over 4500  $\mu\text{g}/\text{kg}$  (Numanoglu et al., 2012). The estimated  $k$  at 150 °C was  $0.026 \text{ min}^{-1}$ , while our  $k$  at 160 °C was  $0.023 \text{ min}^{-1}$ , confirming the trend for faster inactivation when DON concentration is higher.

Knowing the reaction order of the system and  $k$ , it is possible to find the energy of activation for the degradation of DON from the Arrhenius equation (2). Logarithmic transformation of equation (2) results in equation (3).

$$k = A e^{-E_a/RT} \quad (2)$$

$k$  = thermal degradation rate constant ( $\text{min}^{-1}$ ).

$A$  = frequency factor.

$E_a$  = degradation activation energy (kJ/mol).

$R$  = gas constant 8.31 kJ/(mol·K).

$T$  = absolute temperature (K).

$$\ln k = - (E_a/RT) + \ln A \quad (3)$$

Based on equation (3) Arrhenius graphs were built with  $\ln k$  versus  $1/T$  (Figure 3). The  $r^2$  obtained shows a high correlation between temperature and thermal degradation rate constant (Fig. 5a and b for the high and low mycotoxin initial concentration respectively). From the slope of the line it is possible to estimate the  $E_a$  which resulted to be 46.29 and 62.39 kJ/mol for the high and low concentration respectively, which means that activation of the degradation reaction is easier when the toxin concentration is higher.

### 3.7.2. Determination of DON conjugates inactivation/release kinetics

The kinetics for DON conjugates were calculated as for DON (see section 3.7.1), but for DON-3-glucoside and DOM-1 the values for which inactivation was reported after increasing concentrations over time were omitted. In this case, the reactions (degradation or increase) were also assumed to be of first-order.

The obtained  $r$  values suggested that the release of DON-3-glucoside and DOM-1 followed a first order reaction (Table 3). Firstly, the  $k$  values for DON-3-glucoside and DOM-1 are negative which indicates that these mycotoxins increased during baking. The increase of DON-3-glucoside was faster than in DOM-1, and both of them in general faster than DON degradation. The  $k$  absolute values in DON-3-glucoside increased with the temperature, so the increase is higher with the temperature, however the  $k$  at 180 °C is bigger than  $k$  value at 200 °C, this situation is produced because in our results the DON-3-glucoside at 200 °C only was detectable until minute 10, so few data were available to build the kinetics. For DOM-1 absolute  $k$  always increased with the temperature.

Estimated  $E_a$  were 25.87 and 49.95 kJ/mol for DON-3-glucoside and DOM-1, respectively (Fig. 5c and d), which suggests that more energy is required to trigger the release of DOM-1 from the matrix or from DON transformation than to release DON-3-glucoside from the matrix or from DON reaction.

This is the first report on kinetics of these conjugated forms through a thermal treatment. Knowing their kinetics can allow for an approximate estimation of their concentration in the final products. For further studies, it would be of interest to check the kinetics under different initial DON concentrations.

### 3.7.3. Determination of OTA inactivation kinetics

The degradation reaction followed a first-order kinetic model. The estimated  $k$  values increased with the temperature (Table 3), with higher  $k$  values with higher initial OTA level. Half-lives and first order reaction constants at 200 °C were  $t_{1/2} = 18.24$  min and  $k = 0.038$  min<sup>-1</sup>, at the high mycotoxin concentration. The high  $r^2$  showed in Fig. 5e,f confirm a high correlation between temperature and thermal degradation rate constant (estimated  $E_a$ , 34.99 and 40.62 kJ/mol).

Kinetics of OTA degradation in solid substrates have only been studied in coffee (Castellanos-Onorio et al., 2011; Ferraz et al., 2010). The kinetic constants ( $k$ ) in coffee works are higher,  $0.11 \text{ min}^{-1}$  at  $200 \text{ }^\circ\text{C}$  (Ferraz et al., 2010) and  $0.38 \text{ min}^{-1}$  at  $230 \text{ }^\circ\text{C}$  (Castellanos-Onorio et al., 2011) because the reduction is clearly faster as a result of more extreme roasting conditions in the coffee case.

Differences between the two parent mycotoxins studied are detected, mainly in the kinetics, where DON is less thermostable than OTA.  $k$  was higher for DON than for OTA, however,  $E_a$  for OTA was lower, leading to a higher increase in  $k$  with temperature. Despite this, the  $k$  value at high temperature was still higher for DON than for OTA. The bigger extent of reduction of DON than OTA in the baking step agrees with other works (Vidal et al., 2014a). The higher thermo stability of OTA than DON may be due to the structure of the molecules. While OTA has many double bonds between carbons, DON only has one. Maybe for this reason the main OTA degradation products known are made by isomerization at the C3 position (Cramer et al., 2008).

Kinetic studies on degradation of mycotoxins are essential to provide baking factories with applied knowledge about mycotoxins degradation, but nowadays scarce works exist about this (Castells et al., 2006; Ferraz et al., 2010; Numanoglu et al., 2012). The Arrhenius equations obtained in the present work allow for easy estimation of DON and OTA concentration after the baking process as a function of temperature and time used if the initial concentration is known.

Thus the knowledge of the mycotoxin kinetics can be crucial in the control of mycotoxins. For instance, bakeries could set their maximum allowable toxin levels in the flour (always under the legal limit) depending on the final product to be manufactured and its known reduction factor. Moreover, the study of conjugated mycotoxins is of much interest. The possible increases for some of them through food processing increases the importance of their monitoring and their control.

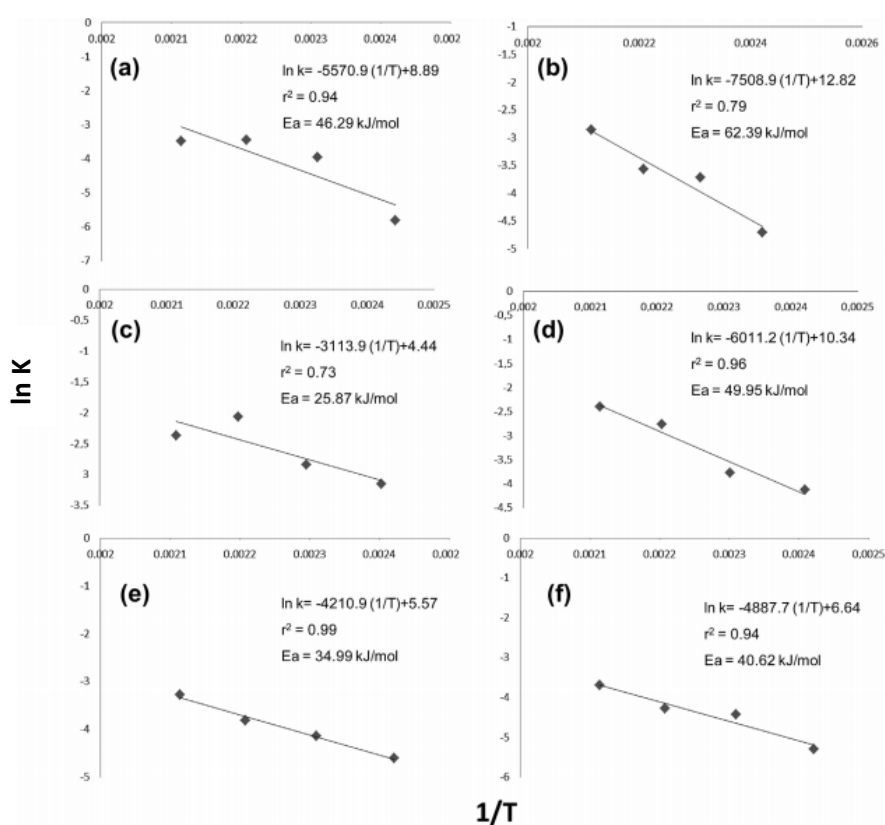


Figure 3. Arrhenius equations for DON in high (a) and low (b) initial mycotoxin concentration, DON-3-glucoside (c), DOM-1 (d) and OTA in high (e) and low (f) initial mycotoxin concentration.

#### 4. Conclusions

DON and OTA can be highly reduced in baking processes, however, temperature, time and size of the product are critical factors. Moreover, OTA is more stable than DON in front of a baking treatment. The analysis of DON conjugates revealed that DON-3-glucoside, under mild baking conditions may be released, while it is erased fast in harsh conditions. The different behaviour as a function of the baking conditions might explain the contradictory existing reports. Moreover, DOM-1 increased during the heating process.

The use of kinetics developed in the work can be a useful tool for the control of DON, DON conjugates and OTA levels in the bakery industry, through the prediction of expected final levels once the mycotoxins concentration in the raw materials are known.

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