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Heat-induced reduction of deoxynivalenol and its modified forms during flaking and cooking of oat

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Abstract

Deoxynivalenol (DON) and its modified forms deoxynivalenol-3-glucoside (DON-3G) and 3-acetyl-deoxynivalenol (3-ADON) are common contaminants in Norwegian oats. In order to provide more information about the fate of these mycotoxins during oat processing, the levels of DON, DON-3G, 3-ADON and the sum of them (total DON) were determined using LC-HRMS/MS at different processing steps. Oat groat was softened by either steaming or conditioning, rolled into flakes of two thicknesses, and subsequently cooked to produce flake porridges. Flour of oat groat (untreated or kilned) was cooked to flour porridges. The flaking process had major effect on the mycotoxin levels in resulting flakes, with significant impact for type of softening regime, but not for flake size. Steam-softening caused the largest reduction of DON, DON-3G and total DON in flakes, retaining 41%, 60% and 46% respectively, compared to oat groat. In contrast, 3-ADON in flakes was most reduced by conditioning, to 29% of the levels in oat groat. Cooking to porridge from flakes did not result in any additional mycotoxin reduction, though significant impact of flake size was shown in the final porridges, with highest reduction of total DON in the porridges originating from steamed thick flakes. Cooking porridge from untreated oat flour gave significant reduction in mycotoxin levels, however not for kilned oat flour which had already undergone reduction during kilning. In conclusion, the study shows that processes involving heat-treatment, i.e. kilning, steaming or cooking, efficiently reduced total DON in oats during flaking and porridge cooking, and reduction is dependent on previous processing steps.

Keywords

mycotoxin, oat, processing, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol

Introduction

Cereals are an important part of the human diet with whole grain consumption being associated with lower risk of diseases such as type 2 diabetes, heart disease and certain cancers (Bjorck *et al.* 2012). Due to the World Health Organization (WHO) and European Food Safety Authority (EFSA) recommendations about increased consumption of dietary fibre such as beta-glucan, oat-based products are receiving increased attention. Oat is a popular cereal component in food for infants and young children, and together with rice- and corn-based products oat is an important constituent in the diet for people with celiac disease and gluten intolerance (Gilissen *et al.* 2016).

However, oat and other small grain cereals are often contaminated with *Fusarium* mycotoxins compromising cereal production and food and feed safety worldwide. *Fusarium graminearum* is one of the main producers of deoxynivalenol (DON) and intermediates such as 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON). The 3-ADON *F. graminearum* chemotype is predominant in Norwegian oats (Pasquali *et al.* 2016). As part of the plant resistance machinery DON is converted to the more polar sugar conjugate DON-3-glucoside (DON-3G) (Berthiller *et al.* 2005, Warth *et al.* 2015). DON and its modified forms are among the most common mycotoxin contaminants in Europe, and DON is subject to EU legislation (European Commission 2006). The European Commission has set a maximum limit (ML) of unprocessed wheat for food production at 1250 µg/kg, whereas ML for wheat bran and flour, bread, and processed cereal-based foods for infants is set to 750, 500, and 200 µg/kg, respectively (European Commission 2006). Furthermore, guidelines for tolerable daily intake (TDI) for the sum of DON, acetyl-DON and DON-3G has been established by EFSA (Knutsen *et al.* 2017). According to EFSA, the main contributors to high DON exposure are bakery products and breakfast cereals (European Food Safety Authority 2013). As fungi and their mycotoxins accumulate mostly in the outer part of the grain such as the hulls and bran, there is a particular concern for mycotoxin contamination in whole grain products. Indeed, whole grain products stand at risk of exceeding the fixed ML if the unprocessed grain material is close to the ML (Schaarschmidt and Fauhl-Hassek 2018).

Co-occurrence of DON, DON-3G, and 3-ADON/ 15-ADON has been documented in wheat, oat, barley and other cereal grains (Perkowski *et al.* 2012, Uhlig *et al.* 2013). During processing the modified forms of DON may be cleaved, and DON may be released and contribute to the toxic effects of the contaminated food (Berthiller *et al.* 2011, Dall'Erta *et al.* 2013, Gratz *et al.* 2013, Wu and Wang 2015). Assessment of the influence of processing in cereal food production on DON in wheat has been extensively studied and reviewed (Kaushik 2015, Khaneghah *et al.* 2018, Schaarschmidt and Fauhl-Hassek 2018, Wu *et al.* 2017). Primary processing such as cleaning, sorting and dehulling of cereal grains is known to reduce the content of DON in certain fractions (Schaarschmidt and Fauhl-Hassek 2018). Milling technology may have an impact on DON content as highest amount of DON is found in outer kernel fractions and bran, whereas less is found in the inner starchy endosperm fractions (Kushiro 2008, Sovrani *et al.* 2012, Tibola *et al.* 2015).

Secondary processing (e.g. steaming, extrusion, fermentation and baking) has the potential to degrade, transform, bind or release mycotoxins. Heat seems to be an important factor, however since DON is heat stable, relatively high temperatures are needed to reduce DON (Bretz *et al.* 2006, Schaarschmidt and Fauhl-Hassek 2018). Yumbe-Guevara *et al* found that roasting at 220 ºC for 1 hour reduced DON by 100 % when barley kernels were ground (Yumbe-Guevara *et al.* 2003). However, parameters such as processing time, moisture and many others are known to influence DON reduction. Using superheated steam, Cenkowski *et al* (2007) reduced DON content in naturally contaminated wheat by up to 52% at high temperatures (185 ºC) and a processing time of 6 minutes. Up to 60 % reduction in DON was achieved with extrusion cooking of wheat grits at 170 ºC with high moisture content, but several other physicochemical parameters were shown to influence DON reduction (Wu *et al.* 2011). Other studies have found varying effects on the reduction of DON by steaming and extrusion (Schaarschmidt and Fauhl-Hassek 2018, Scudamore *et al.* 2008, Wu *et al.* 2017). Studies of DON reduction during the complex process of bread baking report variable results (De Angelis *et al.* 2013, Guo *et al.* 2020, Kostelanska *et al.* 2011, Schaarschmidt and Fauhl-Hassek 2018, Wu *et al.* 2017, Zhang and Wang 2015). However, recently it was shown that baking time and temperature, as well as the pH modifying agent $NaHCO_3$ are the main factors determining DON reduction during

baking, and partial degradation products such as isoDON and norDONs have been identified (Stadler *et al.* 2019a, Stadler *et al.* 2019b).

Although many studies have reported DON levels in final oat products (De Boevre *et al.* 2013, Marin *et al.* 2013), knowledge about mycotoxin repartitioning and decontamination during oat processing is still scarce (Ivanova *et al.* 2017, Scudamore *et al.* 2007). Oat is a common ingredient in breakfast cereals, as oat flakes, and in oat porridge. The first steps of oat processing consist of cleaning, grading and dehulling to produce oat groat. After dehulling, oat groats are heat-treated with steam and subsequently dried in a process called kilning, to inactivate fat-hydrolysing enzymes to avoid development of rancid flavor. The kilned oat groats can be milled into oat flour or rolled into flakes. The roll gap size determines the flake thickness and the cooking characteristics of the flaked product (Webster 2002). These processes are poorly studied, but highly relevant in a food safety perspective, not least because the nutritious oat bran is included in the product. The effect of cooking/ boiling on mycotoxin levels in cereal-based products such as noodles and pasta significantly reduce DON as DON is water-soluble and discarded with the cooking water (Cano-Sancho *et al.* 2013, Kushiro 2008). In oat porridge however, water becomes a part of the final serving and DON is not discarded.

In order to provide more information about the fate of DON and its modified forms during processing into oat-based products for human consumption, this study focused on the flaking process and porridge cooking. We aimed to address the influence of the softening regime as a pre-treatment for the flaking process (i.e. conditioning and steaming), flake size, and subsequently the cooking process of flakes, as well as flour, into porridge.

Materials and methods

Chemicals and reagents

Water (Optima, LC/MS) and acetonitrile (Optima, LC/MS) were obtained from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA), whereas MS grade formic acid, acetic acid and ammonium acetate were purchased from Merck KGaA (Darmstadt, Germany). Analytical standards for DON, DON-3G and 3-ADON and ^{13}C-labeled mycotoxins (U-[$_{13}$C^{15}]-DON and U-[$_{13}$C^{17}]-3-ADON) were purchased from Romer Labs (Tulln, Austria). A combined standard solution was prepared in 50% acetonitrile and further diluted to working standard solutions containing DON, DON-3G and 3-ADON in concentrations of 1.3, 6, 12, 60, 125 and 250 ng/ml. A combined internal standard (ISTD) solution for DON and 3-ADON was prepared in 50% acetonitrile containing 100 ng/ml of U-[$_{13}$C^{15}]-DON and 250 ng/ml of U-[$_{13}$C^{17}]-3-ADON. For spiking a mixed spike standard solution of 10 µg/ml DON, DON-3G and 3-ADON was prepared by evaporating the appropriate stock solutions and resuspending in 50% acetonitrile. For all processing experiments involving water we used purified water (pH 6.8) by reverse osmosis (RO) obtained from an Elga Purelab Prima DV35 instrument.

Cereal samples

Naturally DON-contaminated whole grain oat (cv. Ivory) was obtained in 25 kg sacks from Felleskjøpet (Lillestrøm, Norway). A non-contaminated whole grain oat sample (cv. Belinda) was obtained from Lantmännen Cerealia (Stockholm, Sweden) and served as a "blank" control sample for spiking experiments and for matrix-matched calibration (described under chemical analysis by LC-HRMS/MS). Both contaminated and the non-contaminated batches were from the harvest 2014 and were stored at room temperature in the dark under dry conditions. The whole grain oat was dehulled using an oat dehuller of industrial type from Rivakka (NIPERE, Suomi, Finland). Dehulled oat groat from non-contaminated samples showed DON content

lower than the limit of detection (LOD) (**Table 1**). The initial moisture contents were determined using Moisture Analyzer Sartorius Thermo Control YTC 01L (Biovendis Ltd, Mannheim, Germany) and were 10.83% and 9.95%, for blank and DON-contaminated sample, respectively.

Processing of oat samples

All processing was done in laboratory scale and the processing steps and sampling regime are schematically shown in **Figure 1.** Six replicate batches of dehulled oat groat were thoroughly mixed, and each batch was used in four main processes. In two of the processes oat groat was subject to softening by steaming or conditioning, followed by flaking and cooking to produce flake porridge. The softening was necessary to obtain a moisture content of 20% in the oat groat facilitating rolling into flakes. In the other two processes, oat groat was left untreated or kilned, and subjected to milling to produce flour and flour porridge. Untreated milled oat groat was designated S1 and served as the reference sample in the study (see below). All processing pathways were done in replicates of six (n=6). In order to facilitate comparison, all processing steps to flake porridge had identical amounts of input material. All sampling for chemical analysis (indicated with an "S") was done using 2.5 g of freeze-dried material, except for S1 and S2 where 2.5 g flour was extracted directly (**Figure 1**). Residual water content was measured after freeze drying and used to re-calculate dry weight matter (DM).

- *Preparation of flour by kilning and milling:* Two portions of oat groat were taken through either kilning and milling (S2), or milling alone (S1; **Figure 1**). The S1 sample was used as the reference oat groat sample for comparison in this study. Kilning (sample S2) was done by steaming with RO water at 99 °C (at 1 bar) for 20 min until 20% moisture content using a Steamcooker HD9140 (Philips, Oslo, Norway), and followed by drying at 35 °C for 5 hours in a Termaks drying cabinet (Heigar, Oslo, Norway), bringing moisture content back to 10%. Milling was done using a hammer mill (Retsch RM100) with a 0.5 µm sieve (Retsch, Dale, Norway).
- *Softening of oat groat to 20% moisture level: Conditioning* was done by adding 20 g RO water to 160 g of oat groat, mixing thoroughly and leaving it for 18 hours at room temperature. *Steaming* was done similarly to kilning, but without drying, using 160 g of oat groat and steaming at 99 °C (at 1 bar) for 20 min using a Steamcooker HD9140 (Philips, Oslo, Norway) to the desired moisture level. Final weight after softening was in both cases 180 g. Moisture content was determined using a Moisture Analyzer Sartorius Thermo Control YTC 01L (Biovendis Ltd). The softened oat groat was rolled directly into flakes.
- *Flaking*: Oat groat batches softened by conditioning or steaming were thoroughly mixed and each was divided further in two (each 90 g), for flaking in two thicknesses (0.25 mm (S3, S5) and 1.27 mm (S4, S6); **Figure 1**). Flaking was done using a Laboratory Flaking Mill (Ferrell Ross Inc., Hereford, TX, USA) equipped with Syntron® Volumetric Feeder (Syntron Material Handling South Saltillo, MS, USA). The resulting flakes were thoroughly mixed, and 66 g of each batch were used further for porridge cooking. The rest of the material was freeze-dried, and 2.5 g was used for chemical analysis (S3-6).
- *Preparation of porridge*: In order to obtain similar consistency in flake and flour porridges, standard in house recipes were used as follows: *Porridge from flakes* was prepared by mixing 66 g flakes (0.25 mm or 1.27 mm) with 544 ml boiling RO water and cooking for 10 min with continuous stirring (S7 – S10). pH after boiling was found to be approximately 6.2 (data not shown). *Porridge from flour* was prepared by mixing 60 g oat flour (S1) or kilned oat flour (S2) with 300 ml RO water. The mixture was

stirred thoroughly for 20 min until boiling and was boiled for 1 min (S11, S12). Porridges were freeze-dried and 2.5 g were used for chemical analysis (S7-12).

Testing of enzymatic activity

Enzymatic activities were determined in heat-treated and non-heat-treated flour using standard methods and according to the manufacturer's descriptions. Alpha-amylase activity was tested using the Ceralpha method K-CERA 01/12 (Megazyme International, Kildare, Ireland). Xylanase activity was determined using a Megazyme tablet test kit according to the method K-XYLS 10/05 (Megazyme International, Kildare, Ireland). Protease activity was measured according to the method of Ichinose *et al* using the protezyme test tablets from Megazyme International (Ichinose *et al.* 2001). Acetyl esterase activity was determined according to the method of Hou *et al* where 1-naphthyl acetate was used as substrate for plant-esterases (Hou *et al.* 2012). Absorbance was measured spectrophotometrically at 400 nm for the amylase assay and 590 nm for the xylanase, protease and acetyl-esterase assays.

Extraction of mycotoxins for chemical analysis

All samples (i.e. 2.5 g of oat groat, freeze-dried flakes and porridges) were homogenised before extraction. To extract DON, DON-3G and 3-ADON from samples (S1-S12, **Figure 1**), we used a two-step extraction method described by Ivanova *et al (Ivanova et al. 2017)*. Briefly, 10 ml of extraction solvent N1 (acetonitrile/water/formic acid; 80:19.9:0.1, v/v/v) was added to 2.5 g of homogenized sample in a 50 ml centrifuge tube, vortexed for 30 s and extracted for 30 min using an Innova40 horizontal shaker at 250 rpm (New Brunsvick Scientific, Edison, NJ). The samples were then centrifuged at 4000 g for 10 min at 4 °C (Multifuge 4 KR Heraeus, Thermo Fisher Scientific, Waltham, MA), and the liquid phase was transferred into a new 50 ml centrifuge tube. The residue was subjected to a second extraction with 10 ml of extraction solvent N2 (acetonitrile/water/formic acid; 20:79.9:0.1, v/v/v) and shaken for 30 min (250 rpm) prior to centrifugation for 10 min at 4000 g and 4°C (Multifuge 4 KR Heraeus, Thermo Fisher Scientific, Waltham, MA). In order to facilitate precipitation and removal of residue material both supernatants were combined and kept at 4 °C for 16 – 18 hours prior to a final centrifugation at 4000 g for 10 min (4°C). Combined supernatant (0.5 ml) was further centrifuged for 1 min at 15000 g (Multifuge 4 KR Heraeus, Thermo Fisher Scientific, Waltham, MA) through 0.22 µm nylon filters (Costar Spin-X 0.22 Nylon filter; Corning Inc., Corning, NY). Each filtered sample extract (0.040 ml) was mixed with 0.010 ml ISTD-solution in chromatographic vials prior to LC-HRMS/MS analysis.

Chemical analysis using LC-HRMS/MS

Identification and quantification of mycotoxins was performed using an LC-HRMS/MS multiplex method previously developed in our group and the Xcalibur 2.2 software (Thermo Fisher Scientific) (Ivanova *et al.* 2017). The method was validated for mycotoxin analysis in flakes, flour and porridge from oats by evaluation of mycotoxin recovery in "blank" sample (control oat sample with levels of DON below LOD) spiked to 100 or 250 µg/kg with DON, DON-3G and 3-ADON, respectively. The method performance characteristics obtained during the validation are presented in **Table 1**. Recoveries were satisfactory for all three compounds ranging from 89% to 115% with relative standard deviation (RSD) < 10%. The LOD and limit of quantification (LOQ) were estimated using standard deviation of response and the slope of the calibration curves, and were in the range of 13.8 – 31.3 µg/kg and 46.9 – 95.9 µg/kg, respectively, for all compounds tested as shown in detail in **Table 1**. Standard calibration curves were acquired with standards prepared in "blank" oat matrix as follows: 0.010 ml of ISTD solution was added to 0.040 ml of working standard solutions, evaporated to dryness under a

stream of nitrogen, and re-dissolved in 0.050 ml of extract from blank oat sample. Matrix-matched internal standard calibration was used for quantification of DON and 3-ADON, while external matrix-matched calibration was used for quantification of DON-3G. Quantification of mycotoxins was always normalized to dry weight matter (DM) by measuring water content after freeze drying and re-calculating according to DM.

Table 1. Method performance characteristics and validation parameters determined in processed oat.

Matrix	Mycotoxin	% recovery (RSD)		LOD µg/kg	LOQ µg/kg	R^2
		Spiking level 1[a] 100 µg/kg	Spiking level 2[b] 250 µg/kg			
Flour	DON	94 (9)	99 (10)	17.2	56.8	0.9976
	DON-3G	97 (8)	102 (9)	21.6	71.3	0.9979
	3-ADON	89 (10)	89 (3)	26.9	89.5	0.9962
Flakes	DON	-	96 (8)	13.8	46.9	0.9937
	DON-3G	-	115 (9)	31.3	95.9	0.9926
	3-ADON	-	91 (10)	30.8	92.4	0.9981
Flake porridge	DON	-	92 (4)	28.1	90.8	0.9987
	DON-3G	-	111 (9)	28.6	91.3	0.9954
	3-ADON	-	94 (10)	31.0	93.6	0.9983
Flour porridge	DON	95 (5)	91 (10)	27.9	90.6	0.9993
	DON-3G	93 (8)	96 (6)	30.2	92.0	0.9981
	3-ADON	96 (10)	92 (7)	27.9	90.7	0.9966

[a] Number of replicates, n=3
[b] Number of replicates, n=4

Statistical analysis

The effect of softening and flake size on the content of DON, DON-3G and 3-ADON in flakes and flake porridges were tested using two-way analysis of variance (ANOVA) in Minitab 19.2 software (Minitab Inc., State College, PA, USA), with the following parameters: softening (α, conditioning vs steaming), flake thickness (β, 0.25 mm vs 1.27 mm) and their interaction (αβ) according to the model:

$$y_{ijk}=\mu+\alpha_i+\beta_j+\alpha\beta_{ij}+s_k+e_{ijk}$$

where y is the mycotoxin content measured in flakes (S3 – S6) and porridge (S7 – S10), s is subject (replicate samples) 1, 2, …, 6 (random), and e is random error. To test for any differences in mycotoxin content between flakes vs flake porridge with the processing factors softening and flake size, the same model was applied with the following changes: y is the difference in mycotoxin content between flake vs flake porridge per subject, and without subject s in the model. Significantly different was set if $p<0.05$. These data are presented in Table S2.

Results

In this study, the concentrations of DON and its modified forms DON-3G and 3-ADON were determined in naturally contaminated oat groat subjected to different types of processing in laboratory scale, with the aim to simulate conditions relevant to industry and private

households. Upon dehulling six batches of oat groat were used as starting material for four main processes as shown in **Figure 1**.

Figure 1. Schematic diagram of processing and sampling regime.

The first two parts of the study involved processing to flake and subsequent flake porridge. The second two parts involved processing to flour and flour porridge. Average concentrations of DON, DON-3G and 3-ADON in untreated, milled oat groat (S1) were measured to 5431 ±999 µg/kg, 2233 ±268 µg/kg and 629 ±102 µg/kg, respectively, and were used as reference (100%) for all downstream samples. All absolute and relative concentrations of DON, DON-3G, 3-ADON and total DON are summarized in **Table 2**.

Table 2. Levels of DON, DON-3G, 3-ADON and total DON in oat groat and processed oat products, µg/kg dry matter (DM)[a].

Product, sample code[b]	Process and treatments	Analyte, in µg/kg ± std dev, (% relative to oat groat, S1)			
		DON	DON-3G	3-ADON	Total DON
Oat groat	**Milling**				
S1	Untreated	5431 ± 999 (100)	2233 ± 268 (100)	629 ± 102 (100)	8293 ± 1197 (100)
S2	Kilning	3603 ± 476 (66)	1582 ± 192 (71)	391 ± 89 (62)	5577 ± 639 (67)
Flakes	**Flaking**	2823 ± 774 (52)	1477 ± 316 (66)	221 ± 57 (35)	4521 ± 970 (55)
	Conditioning				
S3	0.25 mm	3459 ± 336 (64)	1690 ± 273 (76)	173 ± 19 (27)	5321 ± 483 (64)
S4	1.27 mm	3409 ± 572 (63)	1543 ± 203 (69)	188 ± 52 (30)	5140 ± 664 (62)
	Steaming				
S5	0.25 mm	2484 ± 402 (46)	1490 ± 360 (67)	290 ± 34 (46)	4264 ± 579 (51)
S6	1.27 mm	1942 ± 429 (36)	1184 ± 227 (53)	234 ± 31 (37)	3360 ± 625 (41)
Flake porridge	**Flake cooking**	2877 ± 625 (53)	1588 ± 191 (71)	253 ± 65 (40)	4718 ± 742 (57)
	Conditioning				
S7	0.25 mm	3508 ± 464 (65)	1677 ± 164 (75)	234 ± 53 (37)	5419 ± 483 (65)
S8	1.27 mm	3093 ± 497 (57)	1740 ± 137 (78)	188 ± 35 (30)	5021 ± 603 (61)
	Steaming				
S9	0.25 mm	2688 ± 481 (49)	1533 ± 189 (69)	325 ± 41 (52)	4546 ± 595 (55)
S10	1.27 mm	2219 ± 84 (41)	1402 ± 72 (63)	266 ± 45 (42)	3887± 139 (47)
Flour porridge	**Flour cooking**	3267 ± 190 (60)	1776 ± 105 (80)	364 ± 42 (58)	5407 ± 219 (65)
S11	Kilned flour	3148 ± 200 (58)	1782 ± 112 (80)	353 ± 55 (56)	5284 ± 203 (64)
S12	Untreated flour	3385 ± 80 (62)	1769 ± 108 (79)	376 ± 23 (60)	5530 ± 167 (67)

[a] Quantification of the mycotoxins was normalized to dry weight matter (DM).
[b] Sample codes are shown in Figure 1.

Effect of different flaking treatments and flake porridge cooking on DON, DON-3G and 3-ADON

As means to increase the moisture content in oat groat before rolling to flakes moisture was brought to 20% by either steaming or conditioning, and softened oat groat was rolled into flakes of two thicknesses, 0.25 mm and 1.27 mm (**Figure 1**). The effect of the two softening regimes on mycotoxin level was tested. Concentrations of DON, DON-3G and 3-ADON were measured in the final flakes. Regardless of the different flaking treatments, i.e. softening regime or flake size, the concentrations of all three mycotoxins were significantly reduced in the final flakes.

Flakes retained an overall mean of 52%, 66% and 35% of DON, DON-3G and 3-ADON, respectively, with an overall retention in total DON of 55% relative to oat groat (**Table 2**). The impact of softening regime, flake size and flake cooking are presented in **Figure 2** and statistical data in **Table S2**.

Interestingly, type of softening regime had significant impact on all the analysed mycotoxins in the flakes (**Figure 2A**), where steaming resulted in the largest reduction in the content of DON, DON-3G and total DON, flakes retaining 41%, 60% and 46%, respectively, relative to oat groat. In contrast, conditioning resulted in the largest reduction of 3-ADON to 29% of oat groat. This had a dramatic impact on the DON/3-ADON ratio, which increased from approximately 9 in oat groat to 20 in conditioned flakes (**Table S1**). Such increase was not seen after steaming. The flaking process of softened kernels into two flake sizes (0.25 mm and 1.27 mm) did not yield significant differences in mycotoxin levels in the final flakes. However, significant interaction effect between softening regimes and flake sizes was found for 3-ADON, with more 3-ADON found in thin steamed flakes (**Figure 2A**).

Further cooking of flakes to porridge showed that the reduced mycotoxin levels in the conditioned and steamed flakes persisted in the flake porridges, with lowest total DON in porridges originating from steamed flakes (**Figure 2B**). Indeed, comparison between the flakes and corresponding flake porridges, showed no statistical difference in any of the mycotoxins (**Table S2**). Impact of flake size in porridges was, however, found, where significantly lower levels of DON, 3-ADON and total DON were found in the porridges originating from thick flakes as opposed to thin flakes (**Figure 2B**).

Figure 2. Effect of softening regime, flake size and their interaction effect on mycotoxin levels in flakes (A) and flake porridge (B).

Effect of kilning and flour porridge cooking on DON, DON-3G and 3-ADON

The impact of kilning on the content of DON, DON-3G and 3-ADON was investigated by comparing their levels in flour from kilned and untreated oat groat, and in final porridges (**Figure 1**). Indeed, kilning significantly reduced the levels of DON, DON-3G and 3-ADON in flour to an average retention of 66%, 71% and 62%, respectively, with an overall retention in total DON of 67% (**Table 2)**. The ratios of DON/DON-3G and DON/3-ADON remained largely unchanged after the kilning process (**Table S1**). Subsequent cooking of kilned oat flour yielded flour porridge with an overall retention of 64% in total DON, indicating that cooking did not yield additional reduction in mycotoxin concentrations in already kilned flour. Cooking of untreated oat flour, however, did reduce the total DON content to 67% in the final flour porridge (**Table 2**).

Discussion

In the present study we have followed the fate of DON, DON-3G, 3-ADON, and their sum (total DON) through laboratory scale processing of oat groat to common oat products; flakes, flake porridge, flour and flour porridge. As part of the flaking process we investigated the impact of two softening regimes and two flake thicknesses. For the processing of flour we compared untreated and kilned oat groat. It is important to note that, although laboratory scale mimics industrial scale production it is still only an estimate of real-life conditions. In order to

compensate for the uncertainties in collecting representative samples and other biases due to the small scale, all batches were mixed thoroughly and six parallels were used for all experiments. Also, naturally contaminated oat was used rather than spiking of mycotoxins, and process parameters were careful approximates of industrial conditions, e.g. dehulling, kilning, milling, and flaking, as to maintain authentic particle size and volume to surface ratios.

During the flaking process, both softening regimes reduced the mycotoxin content. The highest reduction was, however, achieved with steam-softening, which reduced the content of DON and total DON to less than 50% relative to oat groat. It is well known that mycotoxins are heat stable and DON is no exception (Bullerman and Bianchini 2007). Several studies have reported degradation of DON using heat, and often in combination with other factors. Farahany and Jinap achieved more than 40% reduction in processing of noodles using heat in combination with alkaline pH (Farahany and Jinap 2011). Wu *et al*. (2011) achieved up to 60% reduction rate during wheat extrusion and showed that several parameters in addition to temperature (i.e. moisture, compression, residence time, as well as alkaline pH) are influencing DON degradation. Stadler *et al*. (2019a) showed that temperature, time and alkaline conditions are important factors for DON degradation during baking. In the present study DON reduction is comparable to that achieved in wheat using superheated steam and extrusion cooking (Cenkowski *et al.* 2007, Wu *et al.* 2011). Both Cenkowski *et al.* and Wu *et al.* achieved the highest reductions at high temperatures (185 and 170°C) and 4-6 minutes of treatments. In our study steaming was done for a longer period (20 min) and might have compensated for the lower temperature (99°C). Under our processing conditions no pH altering additives were used and pH went down slightly upon mixing of oat products with water (from pH 6.8 in only water to 6.2 in porridge). Hence, pH is not an enhancing factor for the degradation. The high reduction of DON could be due to a washing effect of water-soluble DON, however, we found negligible amounts (below LOD) of DON in the remaining steam water (data not shown). As exemplified by the cited literature DON degradation is a result of a complexity of factors in addition to temperature, making further comparison too speculative. It may be hypothesized that the differences in the matrix of oat versus wheat can partly explain the differences in results with regards to mycotoxin reduction.

Studies reporting on mycotoxins and processing of oat are still scarce. Reduction in levels of DON and 3-ADON were shown with processing of oat flakes from whole grain, however, the studies are not directly comparable to this study due to differences in processing, low mycotoxin levels (below LOD) and comparisons to whole grains (Scudamore *et al.* 2007, Stuper-Szablewska *et al.* 2016). In this study we have focused on reduction in mycotoxin content relative to oat groat, as we in a previous study already described the reduction of DON and its modified forms during dehulling (Ivanova *et al.* 2017).

The kilning process is particular to oat in order to prevent rancidity. Interestingly, kilning of oat groat resulted in reduction of DON and its modified forms by approximately 30%. These results are in agreement with a recent study by Tittlemier *et al* using approximately the same parameters and achieving 27% and 20% reduction in DON and DON-3G, respectively (Tittlemier *et al.* 2020). The slightly higher reduction in our study may be due to a prolonged cooling and drying period for 5 hours as opposed to 90 minutes. An older study compared kilning of oat groat with whole untreated oats and found reduction in DON, however the study did not show how much of the reduction was caused by kilning as opposed to dehulling (Scudamore *et al.* 2007).

Contrary to DON and DON-3G, 3-ADON was most effectively reduced during conditioning. A plausible explanation for this is that intact enzymes such as esterases may hydrolyse the ester 3-ADON to DON. We showed that heat-treatment during kilning deactivates most of the

enzymatic activity in flour from kilned oat groat (**Table S3)**. During conditioning we can assume that the enzymatic activity is intact. This hypothesis is supported by the doubled DON/3-ADON ratio in conditioned flakes, indicating that 3-ADON is hydrolysed to DON. Wu and Wang demonstrated that ADONs were converted to DON during the fermentation and proofing stage of bread making (Wu and Wang 2016).

Collectively, our results with heat-treatment indicate that maximum reduction of the mycotoxins was achieved within the first heating period and subsequent heating during cooking did not give further reduction. In general, cooking to produce porridge did not significantly add to the reduction of DON, DON-3G, or 3-ADON. Notably, the exception from this was for oat porridge cooked from non-treated oat flour. In this case a reduction in DON and the modified forms was achieved to a level similar to that for kilned flour porridge. These results rise an important point in that any processing step has to be considered within the context of the whole process and that it is dependent on preceding treatments. This has been pointed out in other studies as well (Kostelanska *et al.* 2011, Wu and Wang 2016, Wu *et al.* 2017).

One additional interesting aspect of porridge cooking was the effect of particle size. We found significantly less DON and 3-ADON in porridge made from thick flakes relative to thin flakes, indicating that flake size influences the mycotoxin extractability and the amount of toxin freed during cooking. The importance of food structure has been highlighted in relation to glycemic index, comparing flake and flour porridge (Mackie *et al.* 2017, Tosh and Chu 2015), however there has been little attention directed to the importance of particle size on the bioaccessibility of contaminants such as mycotoxins in oat flakes and porridges. This needs to be investigated in further detail.

One aspect of studying the degradation of DON and its conjugated forms is the identification of partial degradation products, as they may represent toxic forms that should be considered in a food safety perspective. Degradation products such as isoDON, norDONs and others have been described for wheat and mostly in association with bread baking and similar processes (Bretz *et al.* 2006, Greenhalgh *et al.* 1984, Kostelanska *et al.* 2011, Stadler *et al.* 2019a, Stadler *et al.* 2019b, Zhang and Wang 2015). Due to the lack of standards these analyses were not included in the present study. It is also unsure whether isoDON or norDONs would be formed as previous reports during bread baking used temperatures much higher than those applied in the present study. Regarding food safety it has been shown that both isoDON and norDONs are less toxic than DON by at least 50-fold (Bretz *et al.* 2006, Pierron *et al.* 2016, Stadler *et al.* 2019a), thus we anticipate that potential degradation products would not increase the toxicity of the final oat products. To our knowledge there are no reports available on DON degradation products in oat. Good practice would be to include such compounds in future studies.

Conclusion

Our study has shown that the levels of mycotoxins such as DON, DON-3G and 3-ADON can be greatly reduced during processing of oats to flakes and porridge. Heat-treatments, i.e. kilning, steaming and cooking, can be effective in reducing total DON. In this study, steaming showed the largest potential for mycotoxin reduction. Particle size also seems to play a role in final porridge, where larger particles contribute to higher reduction than smaller particles. In agreement with others, our study also indicates that the expected impact of each process on mycotoxin reduction is not constant, but needs to be considered in context of previous treatments.

Oat is a preferred ingredient in the diet for infants and young children as well as for people with celiac disease and gluten intolerance, yet also one of the small grain crops most haunted by

Fusarium head blight infections. The Norwegian Scientific Committee for Food and Environment (VKM) reported in 2013 that oat-based infant porridges contained at least twice the levels of mycotoxins compared to infant porridges based on other grains (The Norwegian Scientific Committee for Food and Environment 2013). In light of this our study advocates a close monitoring and strengthened research on oat-based products for food.

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Conflict of Interest

The authors declare no conflict of interest and that the research meets ethical guidelines.

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Heat-induced reduction of deoxynivalenol and its modified forms during flaking and cooking of oat

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Abstract

Deoxynivalenol (DON) and its modified forms deoxynivalenol-3-glucoside (DON-3G) and 3-acetyl-deoxynivalenol (3-ADON) are common contaminants in Norwegian oats. In order to provide more information about the fate of these mycotoxins during oat processing, the levels of DON, DON-3G, 3-ADON and the sum of them (total DON) were determined using LC-HRMS/MS at different processing steps. Oat groat was softened by either steaming or conditioning, rolled into flakes of two thicknesses, and subsequently cooked to produce flake porridges. Flour of oat groat (untreated or kilned) was cooked to flour porridges. The flaking process had major effect on the mycotoxin levels in resulting flakes, with significant impact for type of softening regime, but not for flake size. Steam-softening caused the largest reduction of DON, DON-3G and total DON in flakes, retaining 41%, 60% and 46% respectively, compared to oat groat. In contrast, 3-ADON in flakes was most reduced by conditioning, to 29% of the levels in oat groat. Cooking to porridge from flakes did not result in any additional mycotoxin reduction, though significant impact of flake size was shown in the final porridges, with highest reduction of total DON in the porridges originating from steamed thick flakes. Cooking porridge from untreated oat flour gave significant reduction in mycotoxin levels, however not for kilned oat flour which had already undergone reduction during kilning. In conclusion, the study shows that processes involving heat-treatment, i.e. kilning, steaming or cooking, efficiently reduced total DON in oats during flaking and porridge cooking, and reduction is dependent on previous processing steps.

Keywords

mycotoxin, oat, processing, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol

Introduction

Cereals are an important part of the human diet with whole grain consumption being associated with lower risk of diseases such as type 2 diabetes, heart disease and certain cancers (Bjorck *et al.* 2012). Due to the World Health Organization (WHO) and European Food Safety Authority (EFSA) recommendations about increased consumption of dietary fibre such as beta-glucan, oat-based products are receiving increased attention. Oat is a popular cereal component in food for infants and young children, and together with rice- and corn-based products oat is an important constituent in the diet for people with celiac disease and gluten intolerance (Gilissen *et al.* 2016).

However, oat and other small grain cereals are often contaminated with *Fusarium* mycotoxins compromising cereal production and food and feed safety worldwide. *Fusarium graminearum* is one of the main producers of deoxynivalenol (DON) and intermediates such as 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON). The 3-ADON *F. graminearum* chemotype is predominant in Norwegian oats (Pasquali *et al.* 2016). As part of the plant resistance machinery DON is converted to the more polar sugar conjugate DON-3-glucoside (DON-3G) (Berthiller *et al.* 2005, Warth *et al.* 2015). DON and its modified forms are among the most common mycotoxin contaminants in Europe, and DON is subject to EU legislation (European Commission 2006). The European Commission has set a maximum limit (ML) of unprocessed wheat for food production at 1250 µg/kg, whereas ML for wheat bran and flour, bread, and processed cereal-based foods for infants is set to 750, 500, and 200 µg/kg, respectively (European Commission 2006). Furthermore, guidelines for tolerable daily intake (TDI) for the sum of DON, acetyl-DON and DON-3G has been established by EFSA (Knutsen *et al.* 2017). According to EFSA, the main contributors to high DON exposure are bakery products and breakfast cereals (European Food Safety Authority 2013). As fungi and their mycotoxins accumulate mostly in the outer part of the grain such as the hulls and bran, there is a particular concern for mycotoxin contamination in whole grain products. Indeed, whole grain products stand at risk of exceeding the fixed ML if the unprocessed grain material is close to the ML (Schaarschmidt and Fauhl-Hassek 2018).

Co-occurrence of DON, DON-3G, and 3-ADON/ 15-ADON has been documented in wheat, oat, barley and other cereal grains (Perkowski *et al.* 2012, Uhlig *et al.* 2013). During processing the modified forms of DON may be cleaved, and DON may be released and contribute to the toxic effects of the contaminated food (Berthiller *et al.* 2011, Dall'Erta *et al.* 2013, Gratz *et al.* 2013, Wu and Wang 2015). Assessment of the influence of processing in cereal food production on DON in wheat has been extensively studied and reviewed (Kaushik 2015, Khaneghah *et al.* 2018, Schaarschmidt and Fauhl-Hassek 2018, Wu *et al.* 2017). Primary processing such as cleaning, sorting and dehulling of cereal grains is known to reduce the content of DON in certain fractions (Schaarschmidt and Fauhl-Hassek 2018). Milling technology may have an impact on DON content as highest amount of DON is found in outer kernel fractions and bran, whereas less is found in the inner starchy endosperm fractions (Kushiro 2008, Sovrani *et al.* 2012, Tibola *et al.* 2015).

Secondary processing (e.g. steaming, extrusion, fermentation and baking) has the potential to degrade, transform, bind or release mycotoxins. Heat seems to be an important factor, however since DON is heat stable, relatively high temperatures are needed to reduce DON (Bretz *et al.* 2006, Schaarschmidt and Fauhl-Hassek 2018). Yumbe-Guevara *et al* found that roasting at 220 ºC for 1 hour reduced DON by 100 % when barley kernels were ground (Yumbe-Guevara *et al.* 2003). However, parameters such as processing time, moisture and many others are known to influence DON reduction. Using superheated steam, Cenkowski *et al* (2007) reduced DON content in naturally contaminated wheat by up to 52% at high temperatures (185 ºC) and a processing time of 6 minutes. Up to 60 % reduction in DON was achieved with extrusion cooking of wheat grits at 170 ºC with high moisture content, but several other physicochemical parameters were shown to influence DON reduction (Wu *et al.* 2011). Other studies have found varying effects on the reduction of DON by steaming and extrusion (Schaarschmidt and Fauhl-Hassek 2018, Scudamore *et al.* 2008, Wu *et al.* 2017). Studies of DON reduction during the complex process of bread baking report variable results (De Angelis *et al.* 2013, Guo *et al.* 2020, Kostelanska *et al.* 2011, Schaarschmidt and Fauhl-Hassek 2018, Wu *et al.* 2017, Zhang and Wang 2015). However, recently it was shown that baking time and temperature, as well as the pH modifying agent $NaHCO_3$ are the main factors determining DON reduction during

baking, and partial degradation products such as isoDON and norDONs have been identified (Stadler *et al.* 2019a, Stadler *et al.* 2019b).

Although many studies have reported DON levels in final oat products (De Boevre *et al.* 2013, Marin *et al.* 2013), knowledge about mycotoxin repartitioning and decontamination during oat processing is still scarce (Ivanova *et al.* 2017, Scudamore *et al.* 2007). Oat is a common ingredient in breakfast cereals, as oat flakes, and in oat porridge. The first steps of oat processing consist of cleaning, grading and dehulling to produce oat groat. After dehulling, oat groats are heat-treated with steam and subsequently dried in a process called kilning, to inactivate fat-hydrolysing enzymes to avoid development of rancid flavor. The kilned oat groats can be milled into oat flour or rolled into flakes. The roll gap size determines the flake thickness and the cooking characteristics of the flaked product (Webster 2002). These processes are poorly studied, but highly relevant in a food safety perspective, not least because the nutritious oat bran is included in the product. The effect of cooking/ boiling on mycotoxin levels in cereal-based products such as noodles and pasta significantly reduce DON as DON is water-soluble and discarded with the cooking water (Cano-Sancho *et al.* 2013, Kushiro 2008). In oat porridge however, water becomes a part of the final serving and DON is not discarded.

In order to provide more information about the fate of DON and its modified forms during processing into oat-based products for human consumption, this study focused on the flaking process and porridge cooking. We aimed to address the influence of the softening regime as a pre-treatment for the flaking process (i.e. conditioning and steaming), flake size, and subsequently the cooking process of flakes, as well as flour, into porridge.

Materials and methods

Chemicals and reagents

Water (Optima, LC/MS) and acetonitrile (Optima, LC/MS) were obtained from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA), whereas MS grade formic acid, acetic acid and ammonium acetate were purchased from Merck KGaA (Darmstadt, Germany). Analytical standards for DON, DON-3G and 3-ADON and ^{13}C-labeled mycotoxins (U-[$_{13}C^{15}$]-DON and U-[$_{13}C^{17}$]-3-ADON) were purchased from Romer Labs (Tulln, Austria). A combined standard solution was prepared in 50% acetonitrile and further diluted to working standard solutions containing DON, DON-3G and 3-ADON in concentrations of 1.3, 6, 12, 60, 125 and 250 ng/ml. A combined internal standard (ISTD) solution for DON and 3-ADON was prepared in 50% acetonitrile containing 100 ng/ml of U-[$_{13}C^{15}$]-DON and 250 ng/ml of U-[$_{13}C^{17}$]-3-ADON. For spiking a mixed spike standard solution of 10 µg/ml DON, DON-3G and 3-ADON was prepared by evaporating the appropriate stock solutions and resuspending in 50% acetonitrile. For all processing experiments involving water we used purified water (pH 6.8) by reverse osmosis (RO) obtained from an Elga Purelab Prima DV35 instrument.

Cereal samples

Naturally DON-contaminated whole grain oat (cv. Ivory) was obtained in 25 kg sacks from Felleskjøpet (Lillestrøm, Norway). A non-contaminated whole grain oat sample (cv. Belinda) was obtained from Lantmännen Cerealia (Stockholm, Sweden) and served as a "blank" control sample for spiking experiments and for matrix-matched calibration (described under chemical analysis by LC-HRMS/MS). Both contaminated and the non-contaminated batches were from the harvest 2014 and were stored at room temperature in the dark under dry conditions. The whole grain oat was dehulled using an oat dehuller of industrial type from Rivakka (NIPERE, Suomi, Finland). Dehulled oat groat from non-contaminated samples showed DON content

lower than the limit of detection (LOD) (**Table 1**). The initial moisture contents were determined using Moisture Analyzer Sartorius Thermo Control YTC 01L (Biovendis Ltd, Mannheim, Germany) and were 10.83% and 9.95%, for blank and DON-contaminated sample, respectively.

Processing of oat samples

All processing was done in laboratory scale and the processing steps and sampling regime are schematically shown in **Figure 1.** Six replicate batches of dehulled oat groat were thoroughly mixed, and each batch was used in four main processes. In two of the processes oat groat was subject to softening by steaming or conditioning, followed by flaking and cooking to produce flake porridge. The softening was necessary to obtain a moisture content of 20% in the oat groat facilitating rolling into flakes. In the other two processes, oat groat was left untreated or kilned, and subjected to milling to produce flour and flour porridge. Untreated milled oat groat was designated S1 and served as the reference sample in the study (see below). All processing pathways were done in replicates of six (n=6). In order to facilitate comparison, all processing steps to flake porridge had identical amounts of input material. All sampling for chemical analysis (indicated with an "S") was done using 2.5 g of freeze-dried material, except for S1 and S2 where 2.5 g flour was extracted directly (**Figure 1**). Residual water content was measured after freeze drying and used to re-calculate dry weight matter (DM).

- *Preparation of flour by kilning and milling:* Two portions of oat groat were taken through either kilning and milling (S2), or milling alone (S1; **Figure 1**). The S1 sample was used as the reference oat groat sample for comparison in this study. Kilning (sample S2) was done by steaming with RO water at 99 °C (at 1 bar) for 20 min until 20% moisture content using a Steamcooker HD9140 (Philips, Oslo, Norway), and followed by drying at 35 °C for 5 hours in a Termaks drying cabinet (Heigar, Oslo, Norway), bringing moisture content back to 10%. Milling was done using a hammer mill (Retsch RM100) with a 0.5 µm sieve (Retsch, Dale, Norway).
- *Softening of oat groat to 20% moisture level: Conditioning* was done by adding 20 g RO water to 160 g of oat groat, mixing thoroughly and leaving it for 18 hours at room temperature. *Steaming* was done similarly to kilning, but without drying, using 160 g of oat groat and steaming at 99 °C (at 1 bar) for 20 min using a Steamcooker HD9140 (Philips, Oslo, Norway) to the desired moisture level. Final weight after softening was in both cases 180 g. Moisture content was determined using a Moisture Analyzer Sartorius Thermo Control YTC 01L (Biovendis Ltd). The softened oat groat was rolled directly into flakes.
- *Flaking*: Oat groat batches softened by conditioning or steaming were thoroughly mixed and each was divided further in two (each 90 g), for flaking in two thicknesses (0.25 mm (S3, S5) and 1.27 mm (S4, S6); **Figure 1**). Flaking was done using a Laboratory Flaking Mill (Ferrell Ross Inc., Hereford, TX, USA) equipped with Syntron® Volumetric Feeder (Syntron Material Handling South Saltillo, MS, USA). The resulting flakes were thoroughly mixed, and 66 g of each batch were used further for porridge cooking. The rest of the material was freeze-dried, and 2.5 g was used for chemical analysis (S3-6).
- *Preparation of porridge*: In order to obtain similar consistency in flake and flour porridges, standard in house recipes were used as follows: *Porridge from flakes* was prepared by mixing 66 g flakes (0.25 mm or 1.27 mm) with 544 ml boiling RO water and cooking for 10 min with continuous stirring (S7 – S10). pH after boiling was found to be approximately 6.2 (data not shown). *Porridge from flour* was prepared by mixing 60 g oat flour (S1) or kilned oat flour (S2) with 300 ml RO water. The mixture was

stirred thoroughly for 20 min until boiling and was boiled for 1 min (S11, S12). Porridges were freeze-dried and 2.5 g were used for chemical analysis (S7-12).

Testing of enzymatic activity

Enzymatic activities were determined in heat-treated and non-heat-treated flour using standard methods and according to the manufacturer's descriptions. Alpha-amylase activity was tested using the Ceralpha method K-CERA 01/12 (Megazyme International, Kildare, Ireland). Xylanase activity was determined using a Megazyme tablet test kit according to the method K-XYLS 10/05 (Megazyme International, Kildare, Ireland). Protease activity was measured according to the method of Ichinose *et al* using the protezyme test tablets from Megazyme International (Ichinose *et al.* 2001). Acetyl esterase activity was determined according to the method of Hou *et al* where 1-naphthyl acetate was used as substrate for plant-esterases (Hou *et al.* 2012). Absorbance was measured spectrophotometrically at 400 nm for the amylase assay and 590 nm for the xylanase, protease and acetyl-esterase assays.

Extraction of mycotoxins for chemical analysis

All samples (i.e. 2.5 g of oat groat, freeze-dried flakes and porridges) were homogenised before extraction. To extract DON, DON-3G and 3-ADON from samples (S1-S12, **Figure 1**), we used a two-step extraction method described by Ivanova *et al (Ivanova et al. 2017)*. Briefly, 10 ml of extraction solvent N1 (acetonitrile/water/formic acid; 80:19.9:0.1, v/v/v) was added to 2.5 g of homogenized sample in a 50 ml centrifuge tube, vortexed for 30 s and extracted for 30 min using an Innova40 horizontal shaker at 250 rpm (New Brunsvick Scientific, Edison, NJ). The samples were then centrifuged at 4000 g for 10 min at 4 °C (Multifuge 4 KR Heraeus, Thermo Fisher Scientific, Waltham, MA), and the liquid phase was transferred into a new 50 ml centrifuge tube. The residue was subjected to a second extraction with 10 ml of extraction solvent N2 (acetonitrile/water/formic acid; 20:79.9:0.1, v/v/v) and shaken for 30 min (250 rpm) prior to centrifugation for 10 min at 4000 g and 4°C (Multifuge 4 KR Heraeus, Thermo Fisher Scientific, Waltham, MA). In order to facilitate precipitation and removal of residue material both supernatants were combined and kept at 4 °C for 16 – 18 hours prior to a final centrifugation at 4000 g for 10 min (4°C). Combined supernatant (0.5 ml) was further centrifuged for 1 min at 15000 g (Multifuge 4 KR Heraeus, Thermo Fisher Scientific, Waltham, MA) through 0.22 µm nylon filters (Costar Spin-X 0.22 Nylon filter; Corning Inc., Corning, NY). Each filtered sample extract (0.040 ml) was mixed with 0.010 ml ISTD-solution in chromatographic vials prior to LC-HRMS/MS analysis.

Chemical analysis using LC-HRMS/MS

Identification and quantification of mycotoxins was performed using an LC-HRMS/MS multiplex method previously developed in our group and the Xcalibur 2.2 software (Thermo Fisher Scientific) (Ivanova *et al.* 2017). The method was validated for mycotoxin analysis in flakes, flour and porridge from oats by evaluation of mycotoxin recovery in "blank" sample (control oat sample with levels of DON below LOD) spiked to 100 or 250 µg/kg with DON, DON-3G and 3-ADON, respectively. The method performance characteristics obtained during the validation are presented in **Table 1**. Recoveries were satisfactory for all three compounds ranging from 89% to 115% with relative standard deviation (RSD) < 10%. The LOD and limit of quantification (LOQ) were estimated using standard deviation of response and the slope of the calibration curves, and were in the range of 13.8 – 31.3 µg/kg and 46.9 – 95.9 µg/kg, respectively, for all compounds tested as shown in detail in **Table 1**. Standard calibration curves were acquired with standards prepared in "blank" oat matrix as follows: 0.010 ml of ISTD solution was added to 0.040 ml of working standard solutions, evaporated to dryness under a

stream of nitrogen, and re-dissolved in 0.050 ml of extract from blank oat sample. Matrix-matched internal standard calibration was used for quantification of DON and 3-ADON, while external matrix-matched calibration was used for quantification of DON-3G. Quantification of mycotoxins was always normalized to dry weight matter (DM) by measuring water content after freeze drying and re-calculating according to DM.

Table 1. Method performance characteristics and validation parameters determined in processed oat.

Matrix	Mycotoxin	% recovery (RSD)		LOD µg/kg	LOQ µg/kg	R^2
		Spiking level 1[a] 100 µg/kg	Spiking level 2[b] 250 µg/kg			
Flour	DON	94 (9)	99 (10)	17.2	56.8	0.9976
	DON-3G	97 (8)	102 (9)	21.6	71.3	0.9979
	3-ADON	89 (10)	89 (3)	26.9	89.5	0.9962
Flakes	DON	-	96 (8)	13.8	46.9	0.9937
	DON-3G	-	115 (9)	31.3	95.9	0.9926
	3-ADON	-	91 (10)	30.8	92.4	0.9981
Flake porridge	DON	-	92 (4)	28.1	90.8	0.9987
	DON-3G	-	111 (9)	28.6	91.3	0.9954
	3-ADON	-	94 (10)	31.0	93.6	0.9983
Flour porridge	DON	95 (5)	91 (10)	27.9	90.6	0.9993
	DON-3G	93 (8)	96 (6)	30.2	92.0	0.9981
	3-ADON	96 (10)	92 (7)	27.9	90.7	0.9966

[a] Number of replicates, n=3
[b] Number of replicates, n=4

Statistical analysis

The effect of softening and flake size on the content of DON, DON-3G and 3-ADON in flakes and flake porridges were tested using two-way analysis of variance (ANOVA) in Minitab 19.2 software (Minitab Inc., State College, PA, USA), with the following parameters: softening (α, conditioning vs steaming), flake thickness (β, 0.25 mm vs 1.27 mm) and their interaction (αβ) according to the model:

$$y_{ijk}=\mu+\alpha_i+\beta_j+\alpha\beta_{ij}+s_k+e_{ijk}$$

where y is the mycotoxin content measured in flakes (S3 – S6) and porridge (S7 – S10), s is subject (replicate samples) 1, 2, …, 6 (random), and e is random error. To test for any differences in mycotoxin content between flakes vs flake porridge with the processing factors softening and flake size, the same model was applied with the following changes: y is the difference in mycotoxin content between flake vs flake porridge per subject, and without subject s in the model. Significantly different was set if $p<0.05$. These data are presented in Table S2.

Results

In this study, the concentrations of DON and its modified forms DON-3G and 3-ADON were determined in naturally contaminated oat groat subjected to different types of processing in laboratory scale, with the aim to simulate conditions relevant to industry and private

households. Upon dehulling six batches of oat groat were used as starting material for four main processes as shown in **Figure 1**.

Figure 1. Schematic diagram of processing and sampling regime.

The first two parts of the study involved processing to flake and subsequent flake porridge. The second two parts involved processing to flour and flour porridge. Average concentrations of DON, DON-3G and 3-ADON in untreated, milled oat groat (S1) were measured to 5431 ±999 µg/kg, 2233 ±268 µg/kg and 629 ±102 µg/kg, respectively, and were used as reference (100%) for all downstream samples. All absolute and relative concentrations of DON, DON-3G, 3-ADON and total DON are summarized in **Table 2**.

Table 2. Levels of DON, DON-3G, 3-ADON and total DON in oat groat and processed oat products, µg/kg dry matter (DM)[a].

Product, sample code[b]	Process and treatments	Analyte, in µg/kg ± std dev, (% relative to oat groat, S1)			
		DON	DON-3G	3-ADON	Total DON
Oat groat	**Milling**				
S1	Untreated	5431 ± 999 (100)	2233 ± 268 (100)	629 ± 102 (100)	8293 ± 1197 (100)
S2	Kilning	3603 ± 476 (66)	1582 ± 192 (71)	391 ± 89 (62)	5577 ± 639 (67)
Flakes	**Flaking**	2823 ± 774 (52)	1477 ± 316 (66)	221 ± 57 (35)	4521 ± 970 (55)
	Conditioning				
S3	0.25 mm	3459 ± 336 (64)	1690 ± 273 (76)	173 ± 19 (27)	5321 ± 483 (64)
S4	1.27 mm	3409 ± 572 (63)	1543 ± 203 (69)	188 ± 52 (30)	5140 ± 664 (62)
	Steaming				
S5	0.25 mm	2484 ± 402 (46)	1490 ± 360 (67)	290 ± 34 (46)	4264 ± 579 (51)
S6	1.27 mm	1942 ± 429 (36)	1184 ± 227 (53)	234 ± 31 (37)	3360 ± 625 (41)
Flake porridge	**Flake cooking**	2877 ± 625 (53)	1588 ± 191 (71)	253 ± 65 (40)	4718 ± 742 (57)
	Conditioning				
S7	0.25 mm	3508 ± 464 (65)	1677 ± 164 (75)	234 ± 53 (37)	5419 ± 483 (65)
S8	1.27 mm	3093 ± 497 (57)	1740 ± 137 (78)	188 ± 35 (30)	5021 ± 603 (61)
	Steaming				
S9	0.25 mm	2688 ± 481 (49)	1533 ± 189 (69)	325 ± 41 (52)	4546 ± 595 (55)
S10	1.27 mm	2219 ± 84 (41)	1402 ± 72 (63)	266 ± 45 (42)	3887± 139 (47)
Flour porridge	**Flour cooking**	3267 ± 190 (60)	1776 ± 105 (80)	364 ± 42 (58)	5407 ± 219 (65)
S11	Kilned flour	3148 ± 200 (58)	1782 ± 112 (80)	353 ± 55 (56)	5284 ± 203 (64)
S12	Untreated flour	3385 ± 80 (62)	1769 ± 108 (79)	376 ± 23 (60)	5530 ± 167 (67)

[a] Quantification of the mycotoxins was normalized to dry weight matter (DM).
[b] Sample codes are shown in Figure 1.

Effect of different flaking treatments and flake porridge cooking on DON, DON-3G and 3-ADON

As means to increase the moisture content in oat groat before rolling to flakes moisture was brought to 20% by either steaming or conditioning, and softened oat groat was rolled into flakes of two thicknesses, 0.25 mm and 1.27 mm (**Figure 1**). The effect of the two softening regimes on mycotoxin level was tested. Concentrations of DON, DON-3G and 3-ADON were measured in the final flakes. Regardless of the different flaking treatments, i.e. softening regime or flake size, the concentrations of all three mycotoxins were significantly reduced in the final flakes.

Flakes retained an overall mean of 52%, 66% and 35% of DON, DON-3G and 3-ADON, respectively, with an overall retention in total DON of 55% relative to oat groat (**Table 2**). The impact of softening regime, flake size and flake cooking are presented in **Figure 2** and statistical data in **Table S2**.

Interestingly, type of softening regime had significant impact on all the analysed mycotoxins in the flakes (**Figure 2A**), where steaming resulted in the largest reduction in the content of DON, DON-3G and total DON, flakes retaining 41%, 60% and 46%, respectively, relative to oat groat. In contrast, conditioning resulted in the largest reduction of 3-ADON to 29% of oat groat. This had a dramatic impact on the DON/3-ADON ratio, which increased from approximately 9 in oat groat to 20 in conditioned flakes (**Table S1**). Such increase was not seen after steaming. The flaking process of softened kernels into two flake sizes (0.25 mm and 1.27 mm) did not yield significant differences in mycotoxin levels in the final flakes. However, significant interaction effect between softening regimes and flake sizes was found for 3-ADON, with more 3-ADON found in thin steamed flakes (**Figure 2A**).

Further cooking of flakes to porridge showed that the reduced mycotoxin levels in the conditioned and steamed flakes persisted in the flake porridges, with lowest total DON in porridges originating from steamed flakes (**Figure 2B**). Indeed, comparison between the flakes and corresponding flake porridges, showed no statistical difference in any of the mycotoxins (**Table S2**). Impact of flake size in porridges was, however, found, where significantly lower levels of DON, 3-ADON and total DON were found in the porridges originating from thick flakes as opposed to thin flakes (**Figure 2B**).

Figure 2. Effect of softening regime, flake size and their interaction effect on mycotoxin levels in flakes (A) and flake porridge (B).

Effect of kilning and flour porridge cooking on DON, DON-3G and 3-ADON

The impact of kilning on the content of DON, DON-3G and 3-ADON was investigated by comparing their levels in flour from kilned and untreated oat groat, and in final porridges (**Figure 1**). Indeed, kilning significantly reduced the levels of DON, DON-3G and 3-ADON in flour to an average retention of 66%, 71% and 62%, respectively, with an overall retention in total DON of 67% (**Table 2)**. The ratios of DON/DON-3G and DON/3-ADON remained largely unchanged after the kilning process (**Table S1**). Subsequent cooking of kilned oat flour yielded flour porridge with an overall retention of 64% in total DON, indicating that cooking did not yield additional reduction in mycotoxin concentrations in already kilned flour. Cooking of untreated oat flour, however, did reduce the total DON content to 67% in the final flour porridge (**Table 2**).

Discussion

In the present study we have followed the fate of DON, DON-3G, 3-ADON, and their sum (total DON) through laboratory scale processing of oat groat to common oat products; flakes, flake porridge, flour and flour porridge. As part of the flaking process we investigated the impact of two softening regimes and two flake thicknesses. For the processing of flour we compared untreated and kilned oat groat. It is important to note that, although laboratory scale mimics industrial scale production it is still only an estimate of real-life conditions. In order to

compensate for the uncertainties in collecting representative samples and other biases due to the small scale, all batches were mixed thoroughly and six parallels were used for all experiments. Also, naturally contaminated oat was used rather than spiking of mycotoxins, and process parameters were careful approximates of industrial conditions, e.g. dehulling, kilning, milling, and flaking, as to maintain authentic particle size and volume to surface ratios.

During the flaking process, both softening regimes reduced the mycotoxin content. The highest reduction was, however, achieved with steam-softening, which reduced the content of DON and total DON to less than 50% relative to oat groat. It is well known that mycotoxins are heat stable and DON is no exception (Bullerman and Bianchini 2007). Several studies have reported degradation of DON using heat, and often in combination with other factors. Farahany and Jinap achieved more than 40% reduction in processing of noodles using heat in combination with alkaline pH (Farahany and Jinap 2011). Wu *et al*. (2011) achieved up to 60% reduction rate during wheat extrusion and showed that several parameters in addition to temperature (i.e. moisture, compression, residence time, as well as alkaline pH) are influencing DON degradation. Stadler *et al*. (2019a) showed that temperature, time and alkaline conditions are important factors for DON degradation during baking. In the present study DON reduction is comparable to that achieved in wheat using superheated steam and extrusion cooking (Cenkowski *et al.* 2007, Wu *et al.* 2011). Both Cenkowski *et al.* and Wu *et al.* achieved the highest reductions at high temperatures (185 and 170°C) and 4-6 minutes of treatments. In our study steaming was done for a longer period (20 min) and might have compensated for the lower temperature (99°C). Under our processing conditions no pH altering additives were used and pH went down slightly upon mixing of oat products with water (from pH 6.8 in only water to 6.2 in porridge). Hence, pH is not an enhancing factor for the degradation. The high reduction of DON could be due to a washing effect of water-soluble DON, however, we found negligible amounts (below LOD) of DON in the remaining steam water (data not shown). As exemplified by the cited literature DON degradation is a result of a complexity of factors in addition to temperature, making further comparison too speculative. It may be hypothesized that the differences in the matrix of oat versus wheat can partly explain the differences in results with regards to mycotoxin reduction.

Studies reporting on mycotoxins and processing of oat are still scarce. Reduction in levels of DON and 3-ADON were shown with processing of oat flakes from whole grain, however, the studies are not directly comparable to this study due to differences in processing, low mycotoxin levels (below LOD) and comparisons to whole grains (Scudamore *et al.* 2007, Stuper-Szablewska *et al.* 2016). In this study we have focused on reduction in mycotoxin content relative to oat groat, as we in a previous study already described the reduction of DON and its modified forms during dehulling (Ivanova *et al.* 2017).

The kilning process is particular to oat in order to prevent rancidity. Interestingly, kilning of oat groat resulted in reduction of DON and its modified forms by approximately 30%. These results are in agreement with a recent study by Tittlemier *et al* using approximately the same parameters and achieving 27% and 20% reduction in DON and DON-3G, respectively (Tittlemier *et al.* 2020). The slightly higher reduction in our study may be due to a prolonged cooling and drying period for 5 hours as opposed to 90 minutes. An older study compared kilning of oat groat with whole untreated oats and found reduction in DON, however the study did not show how much of the reduction was caused by kilning as opposed to dehulling (Scudamore *et al.* 2007).

Contrary to DON and DON-3G, 3-ADON was most effectively reduced during conditioning. A plausible explanation for this is that intact enzymes such as esterases may hydrolyse the ester 3-ADON to DON. We showed that heat-treatment during kilning deactivates most of the

enzymatic activity in flour from kilned oat groat (**Table S3**). During conditioning we can assume that the enzymatic activity is intact. This hypothesis is supported by the doubled DON/3-ADON ratio in conditioned flakes, indicating that 3-ADON is hydrolysed to DON. Wu and Wang demonstrated that ADONs were converted to DON during the fermentation and proofing stage of bread making (Wu and Wang 2016).

Collectively, our results with heat-treatment indicate that maximum reduction of the mycotoxins was achieved within the first heating period and subsequent heating during cooking did not give further reduction. In general, cooking to produce porridge did not significantly add to the reduction of DON, DON-3G, or 3-ADON. Notably, the exception from this was for oat porridge cooked from non-treated oat flour. In this case a reduction in DON and the modified forms was achieved to a level similar to that for kilned flour porridge. These results rise an important point in that any processing step has to be considered within the context of the whole process and that it is dependent on preceding treatments. This has been pointed out in other studies as well (Kostelanska *et al.* 2011, Wu and Wang 2016, Wu *et al.* 2017).

One additional interesting aspect of porridge cooking was the effect of particle size. We found significantly less DON and 3-ADON in porridge made from thick flakes relative to thin flakes, indicating that flake size influences the mycotoxin extractability and the amount of toxin freed during cooking. The importance of food structure has been highlighted in relation to glycemic index, comparing flake and flour porridge (Mackie *et al.* 2017, Tosh and Chu 2015), however there has been little attention directed to the importance of particle size on the bioaccessibility of contaminants such as mycotoxins in oat flakes and porridges. This needs to be investigated in further detail.

One aspect of studying the degradation of DON and its conjugated forms is the identification of partial degradation products, as they may represent toxic forms that should be considered in a food safety perspective. Degradation products such as isoDON, norDONs and others have been described for wheat and mostly in association with bread baking and similar processes (Bretz *et al.* 2006, Greenhalgh *et al.* 1984, Kostelanska *et al.* 2011, Stadler *et al.* 2019a, Stadler *et al.* 2019b, Zhang and Wang 2015). Due to the lack of standards these analyses were not included in the present study. It is also unsure whether isoDON or norDONs would be formed as previous reports during bread baking used temperatures much higher than those applied in the present study. Regarding food safety it has been shown that both isoDON and norDONs are less toxic than DON by at least 50-fold (Bretz *et al.* 2006, Pierron *et al.* 2016, Stadler *et al.* 2019a), thus we anticipate that potential degradation products would not increase the toxicity of the final oat products. To our knowledge there are no reports available on DON degradation products in oat. Good practice would be to include such compounds in future studies.

Conclusion

Our study has shown that the levels of mycotoxins such as DON, DON-3G and 3-ADON can be greatly reduced during processing of oats to flakes and porridge. Heat-treatments, i.e. kilning, steaming and cooking, can be effective in reducing total DON. In this study, steaming showed the largest potential for mycotoxin reduction. Particle size also seems to play a role in final porridge, where larger particles contribute to higher reduction than smaller particles. In agreement with others, our study also indicates that the expected impact of each process on mycotoxin reduction is not constant, but needs to be considered in context of previous treatments.

Oat is a preferred ingredient in the diet for infants and young children as well as for people with celiac disease and gluten intolerance, yet also one of the small grain crops most haunted by

Fusarium head blight infections. The Norwegian Scientific Committee for Food and Environment (VKM) reported in 2013 that oat-based infant porridges contained at least twice the levels of mycotoxins compared to infant porridges based on other grains (The Norwegian Scientific Committee for Food and Environment 2013). In light of this our study advocates a close monitoring and strengthened research on oat-based products for food.

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Conflict of Interest

The authors declare no conflict of interest and that the research meets ethical guidelines.

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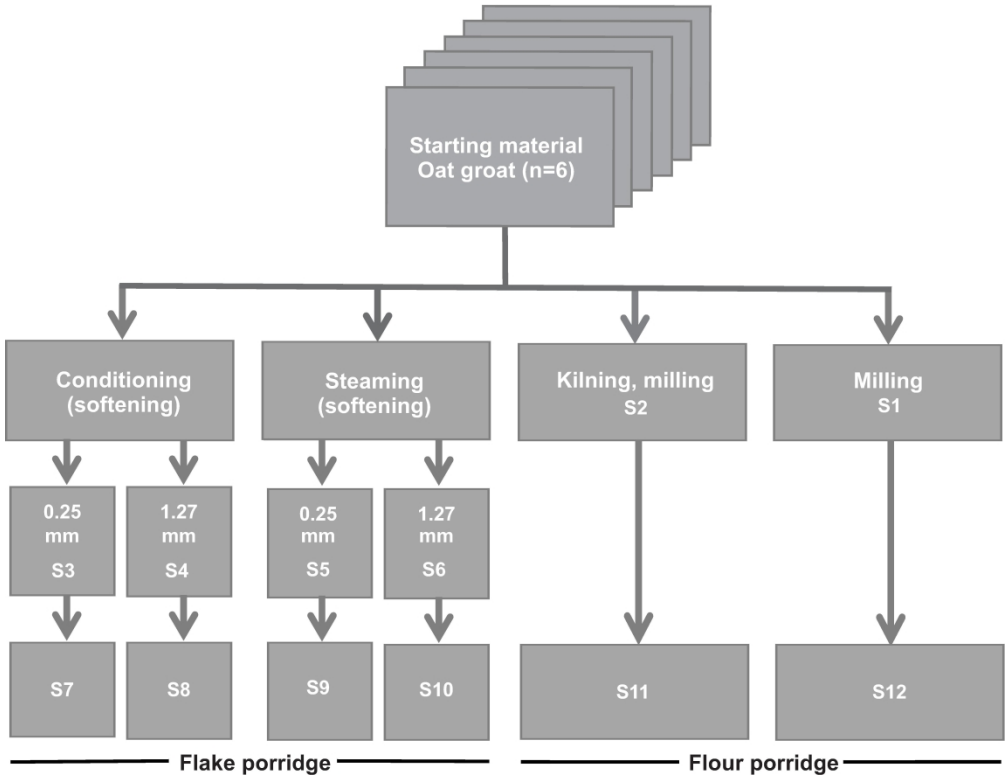

Figure 1. Schematic diagram of processing and sampling regime. Six batches of oat groat were used as starting material and followed through four main processes; i) softening by conditioning, flaking in two thicknesses (S3-4) and porridge cooking (S7-8), ii) softening by steaming, flaking in two thicknesses (S5-6) and porridge cooking (S9-10), iii) kilning and milling with hammer mill (S2), and iv) milling alone with hammer mill without heat treatment of any kind (S1). S1 – S12 indicate sampling steps for chemical analysis.

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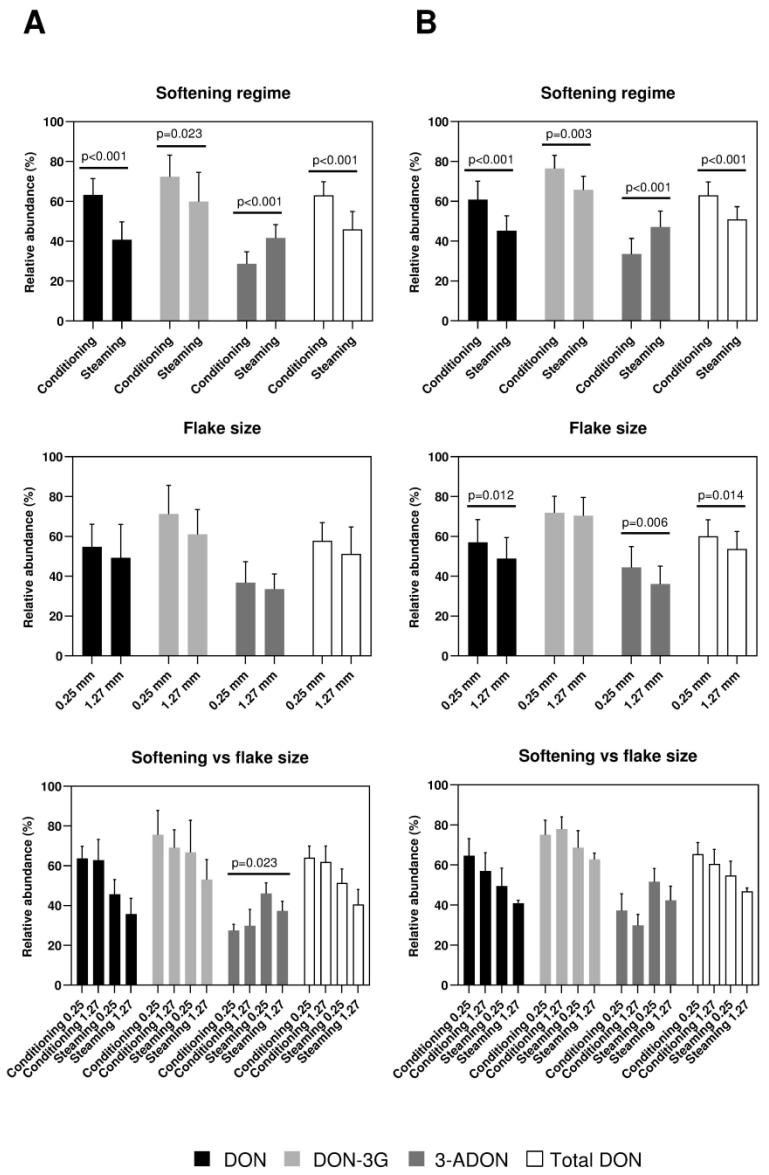

Figure 2. Effect of softening regime, flake size and their interaction effect on mycotoxin levels in flakes (A) and flake porridge (B). Abundance of DON, DON-3G, and 3-ADON and total DON in flakes and porridges are shown in % relative to oat groat pr DM (S1), and colour coded as indicated. Each bar in Softening regime and Flake size represent the mean and standard deviation of n=12, and bars in Softening vs flake size (interaction effect) represent the mean of n=6. Significant p-values ($p<0.05$) of the process treatments using two-way ANOVA are indicated in the figure, where each sample is compared to its respective reference sample S1.

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Electronic supplementary material

Table S1. Ratio of concentrations of free to modified DON in oat groat and the processed oat products.

Product, sample code[a]	Process and treatments	Ratio		
		DON/DON-3G	DON/3-ADON	DON/(DON-3G + 3-ADON)
Oat groat	**Milling**			
S1	Untreated	2.43 ± 0.30	8.86 ± 2.34	1.89 ± 0.27
S2	Kilning	2.28 ± 0.22	9.63 ± 2.37	1.83 ± 0.17
Flakes	**Flaking**			
	Conditioning			
S3	0.25 mm	2.09 ± 0.36	20.31 ± 3.62	1.89 ± 0.28
S4	1.27 mm	2.23 ± 0.40	19.69 ± 7.57	1.98 ± 0.33
	Steaming			
S5	0.25 mm	1.75 ± 0.51	8.70 ± 1.88	1.45 ± 0.39
S6	1.27 mm	1.65 ± 0.26	8.35 ± 1.79	1.38± 0.21
Flake porridge	**Flake cooking**			
	Conditioning			
S7	0.25 mm	2.12 ± 0.41	15.33 ± 2.07	1.85 ± 0.30
S8	1.27 mm	1.78 ± 0.24	16.60 ± 1.48	1.60 ± 0.19
	Steaming			
S9	0.25 mm	1.76 ± 0.26	8.41 ± 2.06	1.44± 0.20
S10	1.27 mm	1.59 ± 0.09	8.52± 1.39	1.33 ± 0.09
Flour porridge	**Flour cooking**			
S11	Kilned flour	1.77 ± 0.17	9.09 ± 1.49	1.48 ± 0.14
S12	Untreated flour	1.92 ± 0.10	9.03 ± 0.56	1.58 ± 0.07

[a] sample code as shown in Figure 1.

Table S2. Statistical overview of the impact of different processing treatments on mycotoxin content in flakes and flake porridge. The table summarizes *p*-values from two-way ANOVA comparing the impact of softening regime (conditioning vs. steaming), flake size (thin vs. thick flakes), and their interactions on mycotoxin content in flakes, porridges and between those products.

Product	Processing factor	Mycotoxin			
		DON	DON-3G	3-ADON	Total DON
Flakes	Softening regime (conditioning vs steaming)	p<0.001	p=0.023	p<0.001	p<0.001
	Flake size (0.25 mm vs 1.27 mm)	p=0.124	p=0.057	p=0.164	p=0.058
	Interaction softening regime and flake size	p=0.196	p=0.478	p=0.023	p=0.193
	Subject (1-6)	p=0.470	p=0.407	p=0.230	p=0.889
Porridge	Softening regime (conditioning vs steaming)	p<0.001	p=0.003	p<0.001	p<0.001
	Flake size (0.25 mm vs 1.27 mm)	p=0.012	p=0.625	p=0.006	p=0.014
	Interaction softening regime and flake size	p=0.864	p=0.175	p=0.715	p=0.500
	Subject (1-6)	p=0.163	p=0.994	p=0.187	p=0.240
Flakes vs porridge	Softening regime (conditioning vs steaming)	p=0.107	p=0.720	p=0.960	p=0.154
	Flake size (0.25 mm vs 1.27 mm)	p=0.516	p=0.084	p=0.236	p=0.960
	Interaction softening regime and flake size	p=0.334	p=0.872	p=0.285	p=0.421

Table S3. Effect of kilning on enzyme activity in oat flour. Enzyme activity of α-amylase, xylanase, protease, and esterase was measured in flour of kilned versus untreated oat groat. All measured enzymatic activities were nearly abolished by kilning, with the exception of xylanase, which was reduced by approximately 80%.

Oat groat flour	Enzymatic activity[a]			
	α-amylase (CU/g)	Xylanase (mU/g)	Protease (U/h/g)	Esterase (µmol/min/g)
Untreated (S1)	0.383 ± 0.053	5.679 ± 0.108	5.922 ± 0.009	44.265 ± 0.142
Kilned (S2)	0.002 ± 0.001	1.169 ± 0.073	ND	0.488 ± 0.019

[a] Enzymatic activity is given in relevant units as indicated ± std dev, (n= 3).