FINAL REPORT

Evaluation of Masked Mycotoxins in Food and Their Release and Uptake in the Gut

FS 102101

March 2016

Colin Crews, Susan MacDonald, Silvia Gratz, Simon Jones and Ashlie Butler

Fera Science Ltd.,

York (UK)

FINAL REPORT

EVALUATION OF MASKED MYCOTOXINS IN FOOD AND THEIR RELEASE AND UPTAKE IN THE GUT

Report Number:	**FD 16/01**
Authors:	**Colin Crews, Susan MacDonald, Fera**
	Silvia Gratz, Rowett Institute of Nutrition and Health
	Simon Jones and Ashlie Butler, University of Sheffield
Date:	**January 2016**
Sponsor:	**Food Standards Agency, Aviation House, London**
Sponsor's Project Number:	**FS102101**
Sponsor's Project manager:	**Modupe Ige**
Fera Contract Number:	**A2FY 5040 / FR/000305**
Principal Workers at Fera:	**Irene Leon, Julie Clough, Monika Sehnalova, Susan MacDonald**
Team leader:	**Emma Bradley**

Fera Science Ltd.
Sand Hutton

ACKNOWLEDGEMENTS:

The Prof Rudi Krska and Dr Franz Berthiller of IFA-Tulln, Austria for the kind gift of analytical standards of DON3Glc, ZEN14Glc, ZEN14Sulf, α-ZEL14Glc and β-ZEL14Glc to allow the food survey to take place.

Dr Mark Busman and Dr Susan McCormick of the Bacterial Foodborne Pathogens and Mycology Unit, USDA-ARS-NCAUR in the USA for a generous gift of T23Glc standard for bioassay work.

Dr Simon Jones and Dr Ashlie Butler of Sheffield University Chemistry Department for the preparation of ZEN14Glc, α-ZEL14Glc and β-ZEL14Glc for bioassay work.

GLOSSARY

3Ac-DON	3-Acetyldeoxynivalenol
15Ac-DON	15-Acetyldeoxynivalenol
AR	Acceptable Range
DHZEN	Decarboxylated hydrolysed zearalenone
DON	Deoxynivalenol
DON3Glc	Deoxynivalenol-3-glucoside
EFSA	European Food Safety Authority
ESI	Electrospray Ionisation
FAPAS	Food Analysis Performance Assessment Scheme
FSA	Food Standards Agency
HT2	HT-2 toxin
HRMS	High Resolution Mass Spectrometry
HZEN	Hydrolysed zearalenone
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
Min	minutes
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NIV	Nivalenol
PT	Proficiency Test
QC	Quality Control
Rpm	Revolutions per minute
S/N	Signal to noise ratio
T2	T-2 toxin
T2Glc	T-2 toxin glucoside
ZAN	Zearalanone

α-ZAL	α-Zearalanol
β-ZAL	β-Zearalanol
α-ZEL	α-Zearalenol
β-ZEL	β-Zearalenol
α-ZEL14Glc	α -Zearalenol-14-glucoside
β-ZEL14Glc	β -Zearalenol-14-glucoside
ZEN	Zearalenone
ZEN14Glc	Zearalenone-14-glucoside
ZEN14Sulf	Zearalenone-14-sulphate

1 Contents

1 Contents 1
1.1 TABLE OF TABLES 4
1.2 TABLE OF FIGURES 5
2 Summary 6
3 Introduction 9
4 PART 1 - ANALYSIS OF RETAIL FOOD SURVEY SAMPLES FOR MASKED MYCOTOXINS TO CONTRIBUTE TO THE EFSA DATA SET 12
4.1 Sampling 12
4.2 Sample preparation 13
4.3 Materials and Methods 13
4.3.1 Extraction Methods 14
4.3.2 Calibration series 14
4.4 LC-MS/MS Method - UPLC method 15
4.4.1 MS method 15
4.5 On-going Internal Quality Assurance 16
4.6 Method validation at Rowett Institute and analysis of 20 samples 16
4.6.1 Method setup 16
4.6.2 LC-MS/MS method 17
4.6.3 LOD/LOQ determination 18
4.6.4 Sample extraction 19
4.6.5 Spiking experiments 19
4.7 Rowett sample analysis 20
5 Results and Discussion – Food Survey 20
5.1 Quality assurance – reference material 20
5.2 Quality assurance – recovery experiments 22
5.3 Rowett Results for Method Validation Spiked Samples (wheat) 24
5.4 Rowett LOD/LOQ determination 24
6 RESULTS – Food Survey Mycotoxin Levels 24
6.1 Beers 24
6.2 Breakfast cereals 28
6.3 Cereal products 32
6.4 Cereal based infant foods 34
6.5 Spices 37
6.6 Reporting results 43

6.7 Results - Rowett food sample analysis 44
6.8 Comparison of results 47
7 Summary of Results of PART 1 – Food Survey 47
8 PART 2 – SYNTHESIS OF MASKED MYCOTOXINS AND BIOASSAY WORK 49
8.1 Synthesis of masked metabolites of selected major trichothecenes 49
8.1.1 Introduction 49
8.1.2 Synthesis of zearalenone glucosides 49
8.1.3 Compounds for bioassay studies 51
9 Assessment of hydrolysis and epithelial transport of masked mycotoxins in the human gut in vitro 52
9.1 Introduction 52
9.2 LC-MS/MS analysis 53
9.3 Assessment of hydrolysis of masked mycotoxins by digestive juices of the upper gut 54
9.3.1 Results - Trichothecenes 55
9.3.2 Results - zearalenone compounds 55
9.4 Assessment of hydrolysis of masked mycotoxins by colonic microbiota 56
9.4.1 Results - DON3Glc 57
9.4.2 Results - T2Glc 59
9.4.3 Results - masked zearalenone compounds 61
9.5 Discussion of faecal incubation studies 67
10 Characterisation of novel microbial metabolites of masked mycotoxins (Fera) 69
10.1 Preliminary work 69
10.2 Experimental 69
10.3 Data interpretation 70
10.3.1 Novel microbial metabolites of masked mycotoxins: 72
11 Masked mycotoxins metabolite search protocol using Compound Discoverer 78
11.1 Data sets 78
11.2 Data mining 78
12 Assessment of gut transport of masked mycotoxins and parent mycotoxins 81
12.1 Epithelial transport study using human intestinal cell culture 81
12.2 Results 81
12.3 Discussion - Gut transport studies: 85
12.3.1 DON and DON3Glc 85
12.3.2 T2 and T2Glc toxin 86
12.3.3 ZEN and metabolites 86

13 SUMMARY OF PART 2 – BIOASSAY WORK 87
13.1 Synthesis of masked mycotoxins 87
13.2 (Upper) Small intestinal hydrolysis 87
13.3 Assessment of hydrolysis of masked mycotoxins by colonic microbiota 87
13.3.1 DON3Glc 87
13.3.2 T2Glc 87
13.3.3 ZEN and metabolites 88
13.4 Characterisation of novel microbial metabolites of masked mycotoxins 88
13.5 Gut transport studies 89
14 Conclusions and possible future work 89
14.1 Survey samples 89
14.2 Method comparison 89
14.3 (Upper) Small intestinal hydrolysis 89
14.4 Hydrolysis of masked mycotoxins by colonic microbiota 89
14.5 Gut transport studies 90
14.6 Characterisation of novel microbial metabolites of masked mycotoxins 90
14.7 Suggestions for possible further work 90
15 References 92
Appendix 1 94
16 Appendix 2 – Sheffield University report of synthesis of masked mycotoxin standards 95
17 Appendix 3. Tabulated outputs from Compound Discoverer software – Negative Mode 107
18 Appendix 4 Tabulated outputs from Compound Discoverer software – Positive Mode 110

1.1 TABLE OF TABLES

Table 1 Mycotoxins analysed in the masked mycotoxin food survey 12

Table 2 LC Gradient elution programme. 15

Table 3 MS Conditions for mycotoxins analysis 15

Table 4 Parameters used for the LC-MS/MS. 18

Table 5 LOD and LOQ (µg/kg) 19

Table 6 Spiking levels of wheat couscous (µg/kg food) 19

Table 7 Fera Results for the reference material NRL_02 wheat (µg/kg) 21

Table 8 Rowett Results for the reference material NRL_02 wheat (µg/kg) 22

Table 9 Recovery data (%) for cereals, beers and oil. 23

Table 10 Masked mycotoxin content (µg/kg) of beer category samples. 26

Table 11 Masked mycotoxin content (µg/kg) of breakfast cereal category samples. 29

Table 12 Masked mycotoxin content (µg/kg) of cereal product category samples (flour, rice and oil). 33

Table 13 Masked mycotoxin content (µg/kg) of infant cereal category samples. 35

Table 14 Masked mycotoxin content (µg/kg) of spices category samples. 39

Table 15 Summary of masked mycotoxin forms found in different products. 43

Table 16 Comparison of results from Rowett and Fera for food survey samples (µg/kg) 45

Table 17 Summary of ion transition parameters. 54

Table 18 Results of DON3Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error of the mean (SEM). 58

Table 19 Results of T2Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error or the mean (SEM). 60

Table 20 Results of ZEN14Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error or the mean (SEM). 62

Table 21 Results of α-ZEL14Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented as average of 2 replicate incubations ± SEM. 64

Table 22 Results of β–ZEL14Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error or the mean (SEM). 66

Table 23 Compounds screened for using HRMS (with formula, accurate mass [M-H]-, and retention time where known) 73

Table 24 Peak Area responses for ZEN14Glc faecal incubation experiments from HRMS analysis..... 74

Table 25 Metabolites included in Compound Discoverer workflows. 78

Table 26 Transport of DON and D3Glc through fully differentiated Caco-2 TC7 monolayers. Results are presented as average ± standard deviation of 3 replicates. 83

Table 27 Transport of T2 and T2Glc through fully differentiated Caco-2 TC7 monolayers. Results are presented as average ± standard deviation of 3 replicates. 83

Table 28 Transport of ZEN, ZEN14Glc, α-ZEL, α-ZEL14Glc, β-ZEL and β-ZEL14Glc through fully differentiated Caco-2 TC7 monolayers. Results are presented as average ± standard deviation of 3 replicates. 85

1.2 TABLE OF FIGURES

Figure 1. Results of Rowett spiking experiments in wheat. Results mean ± standard deviation. 24

Figure 2 Hydrolysis experiments of trichothecenes (DON3Glc, DON, T2Glc, T2, HT2) by artificial digestive juices of the upper gut containing digestive enzymes, indicating no hydrolysis of the modified mycotoxin. All results are presented as average of triplicate incubations± SEM. 55

Figure 3 Hydrolysis experiments of zearalenone compounds (ZEN14G, ZEN, α-ZEL14G, α-ZEL, β-ZEL14G, β-ZEL) by artificial digestive juices of the upper gut containing digestive enzymes, indicating no hydrolysis. All results are presented as average of triplicate incubations± SEM. 56

Figure 4 Results of DON3Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM. 59

Figure 5 Results of T2Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM. 61

Figure 6 Results of ZEN14Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM. 63

Figure 7 Results of α-ZEL14Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM. 65

Figure 8 Results of β-ZEL14Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM. 67

Figure 9 T0 Chromatograms ZEN14Glc Volunteer 1 76

Figure 10 T24 Chromatograms ZEN14Glc Volunteer 1 77

Figure 11. Structures of Zearalenone compounds..... 88

2 Summary

Samples of cereals and cereal based products including breakfast cereals (60), beers (30), cereal-based infant foods (30), other cereal products (25), and spices (39) were analysed for nine free (3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, deoxynivalenol, T-2 toxin, HT-2 toxin, nivalenol, α-zearalenol, β-zearalenol and zearalenone) and five bound (deoxynivalenol-3-glucoside, α-zearalenol-14-glucoside, β-zearalenol-14-glucoside, zearalenone-14-glucoside and zearalenone-14-sulphate) mycotoxins using LC-MS/MS.

Deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON3Glc) were the major contaminants of all sample classes. Four of thirty beer samples contained free DON and fifteen samples contained DON3Glc.

Free DON was present in twenty six of sixty breakfast cereal samples (43%) with an average level of 113 µg/kg. DON3Glc was present in only seven breakfast cereal samples (12 %) with an average level of 75 µg/kg with DON being in excess of DON3Glc. 15-acetyl DON was present in one of two corn samples that also contained DON and DON3Glc. HT-2 toxin was found in two samples of oats. Zearalenone (ZEN) was found in sixteen samples (27%) albeit at low level. The occurrence might have been related to the content of bran.

Eight of twenty five other cereal products contained free DON and six contained DON3Glc. The relative abundances of DON and DON3Glc varied widely. Two samples, including a corn oil, contained zearalenone.

Only four of thirty cereal based infant foods contained mycotoxins, Free DON, DON3Glc and ZEN.

In contrast ZEN was the most abundant mycotoxin in the spice samples present in seven of thirty nine samples. Bound forms of zearalenol (ZEL) and ZEN were also present in some samples, and there were isolated occurrences of HT-2 toxin and nivalenol.

There were no clear patterns of occurrence of mycotoxins for any sample type or supplier.

Following from this *in-vitro* studies to determine the fate of masked mycotoxins at different points in the digestive tract were conducted. To be able to do this masked mycotoxin standards were sourced or synthesised to be used in the bioassay experiments. Zearalenone, α-zearalenol and β-zearalenol glucosides were synthesised by Sheffield University. T-2 toxin glucoside (T2Glc) was supplied as a gift by the Bacterial Foodborne Pathogens and Mycology Unit, USDA-ARS-NCAUR in the USA.

Experiments assessed the stability of masked mycotoxins during incubation with artificial gastric, duodenal and bile juices. All masked mycotoxins tested were stable under incubation conditions mimicking the small intestinal digestion and no hydrolysis was observed.

Faecal batch cultures were used to study the hydrolysis and metabolism of masked mycotoxins by the human gut microbiota. These experiments utilise freshly collected faecal samples handled under anaerobic conditions to mimic metabolic conditions in the human colon. Five independent experiments using faecal samples from 2 male and 3 female donors were performed for each masked mycotoxin to assess intra-individual variation. The results showed differences between the rates of hydrolysis for the different compounds and also intra-individual differences between the volunteers as outlined below.

DON3Glc: For all 5 volunteers, hydrolysis of DON3Glc was found to be very rapid with hydrolysis (disappearance of DON3Glc and appearance of DON in culture medium) occurring between 2 – 6 hours, and complete hydrolysis observed after 24 hours. The results show that on entering the lower intestine gut microbiota will release free DON from DON-3Glc.

T2Glc: Hydrolysis was slower for T2Glc compared to DON3Glc, T2Glc hydrolysis started after 2 hours, but was only completed in 2 out of 5 cases by 168 hours. The level of T-2 toxin only reached 30-50% of the total toxin added before further metabolism to HT-2 toxin was observed. This de-acetylation only reached 35% for volunteer 1, whereas 77-101% of T2Glc was transformed to HT-2-toxin by faecal microbiota from the other four volunteers after 168 hours, although this is a much longer time than normal gut transit time.

ZEN and metabolites: Hydrolysis of zearalenone-14-glucoside (ZEN14Glc) was very rapid starting at 30 minutes of incubation, with almost complete disappearance of ZEN14Glc after 4 hours. However, only 40% of the dose was recovered as free ZEN leaving over 50% of ZEN14Glc to be metabolised to unidentified metabolites or bound to cell material. Similarly,

hydrolysis of α-zearalenol-14-glucoside (α-ZEL-Glc) and β-zearalenol-14-glucoside (β-ZEL-Glc) was started at 30 minutes (only 18 and 44% of original dose of α- and β -ZEL recovered) and was almost complete after 4 hours. Only 60 or 30% of the dose were recovered as free α-ZEL and β-ZEL respectively leaving 40 or 70% of the dose unidentified.

Over 300 samples from the faecal incubation experiments were analysed by High Resolution Mass Spectrometry (HRMS). Differences were observed between samples from different volunteers, with some possible metabolites identified. Results also confirmed the almost immediate hydrolysis of ZEN14Glc and ZEL14Glcs after as little as 30 minutes incubation. Peaks were observed in extracted ion chromatograms with masses for zearalanol (ZAL), zearalenol (ZEL), zearalanone (ZAN), hydrolysed zearalenone (HZEN) and decarboxylated hydrolysed zearalenone (DHZEN) although peaks were observed at different retention times in some volunteers. Further data analysis using a software programme 'Compound Discoverer' was also undertaken to assess the data from Volunteer 3. This software can predict metabolites or possible products following known metabolic pathways. A large number of products were found and some putative identifications were assigned, these results are for information only.

None of the modified mycotoxins tested were efficiently hydrolysed by or transported through the intestinal epithelial monolayers, showing that the presence of the glucoside does offer some protection. However, the hydrolysis of the glucoside by gut bacteria to produce free toxin means ingestion of modified mycotoxins will contribute to overall exposure at varying degrees depending on the toxin.

The study has shown some interesting results and also raised some questions. The results of the faecal incubation studies show that through microbial activity modified mycotoxins can be hydrolysed to the parent or free mycotoxins. It is therefore possible that micro-organisms residing in the upper gut could also hydrolyse masked (modified) mycotoxins releasing free mycotoxins that could add further to the mycotoxin exposure of individuals. Further studies using animal models (e.g. pigs) would be required to assess this as no human samples can be obtained.

These results are similar to other published results as they demonstrate the same findings, i.e. that microbial hydrolysis of DON3Glc was complete after 24 hours and 100% of the

DON3Glc dose was recovered as free DON with no further metabolism. In addition hydrolysis of ZEN14Glc was very rapid, and almost complete disappearance of ZEN14Glc was observed after 4 hours. However, only 40% of the dose was recovered as free ZEN leaving up to 50% of ZEN14Glc. This is in agreement with the findings of Dall Erta et al (2013) who reported an apparent loss of 40-60% of ZEN after similar experiments.

Some differences were also observed, although this could be due to differences in the concentration of the compounds tested as well as differences in the microbiota of the volunteers. This is a very active area of research with a large number of recent publications. It may be worth taking time to conduct a full review of all areas of research of modified mycotoxins, including metabolism and toxicity studies before undertaking any future practical research.

3 Introduction

Mycotoxins are a group of naturally occurring chemicals produced by certain moulds.which frequently contaminate cereal crops. There are over 400 known mycotoxins which are fungal secondary metabolites, although these have varying levels of toxicity, and only a small number are significant in terms of their occurrence in food and feed crops. There is no clear consensus on why moulds produce these compounds, although given the fact that many have proven anti-bacterial and cytotoxic activity, and some have been used in medicine as anti-biotics, one theory is that these compounds give the moulds a competitive advantage over other micro-organisms allowing them to colonise and grow on the substrates they infect. In addition, the toxicity of some mycotoxins is used by the mould as part of their pathogenicty when they infect growing plants. This is the case for *Fusarium* species and the trichothecene mycotoxins they produce which are most frequently found in cereals.

Advances in mycotoxin analysis have revealed that in addition to free mycotoxins, cereal samples also contain mycotoxin conjugates which have become known as “masked mycotoxins” as they were not detected using previous methods. The EFSA CONTAM Panel published an Opinion on these compounds in 2014, and due to the fact that masked mycotoxins could include mycotoxins that were structurally altered in different ways, or could be bound to the matrix decided to use the term “modified mycotoxins” to describe this group of compounds (EFSA, 2014). Modified mycotoxins are found in plants and food matrices as a result of plant phase II metabolism as a reaction after fungal infection, i.e. the plant’s defence to the infection of the mould and toxin presence is to detoxify the mycotoxin typically by conjugating it with a polar compound such as a glucose or modified glucose molecule,

although reactions involving addition of glutathione or sulphate also occur. For some mycotoxins there is evidence that the mycotoxin can be covalently or non-covalently bound to the sample matrix, or physically entrapped within the matrix structure. These forms are included in the definition of “modified mycotoxins”. Literature reports make it clear that cereal samples that are contaminated with deoxynivalenol often also contain its glucoside at levels in excess of the parent compound. This suggests that the mycotoxin burden in cereals could be significantly higher if these “masked” mycotoxins are taken into account.

When masked mycotoxins enter the intestinal tract, they are likely to be cleaved by gastric acids, small intestinal enzymes or large intestinal microbial activity. This will lead to the release of free, unbound mycotoxins within the human gut and might add to the toxicological burden. Therefore, the presence of masked metabolites of mycotoxins in foods and their release inside the human gut need to be assessed to add to a more complete assessment of the risk posed by these compounds. EFSA have reviewed the potential effects of these masked mycotoxins on human health, and had a call for analytical data on their occurrence open until October 2014 (EFSA, 2014). EFSA carried out risk characterisation for modified forms of zearalenone, nivalenol, T-2 and HT-2 toxins and fumonisins. A separate assessment for deoxynivalenol and metabolites of deoxynivalenol and masked deoxynivalenol in food and feed is currently in progress (EFSA website, 2015). The CONTAM Panel decided to assess human exposure to modified forms of the various toxins in addition to the parent compounds, because many modified forms are hydrolysed into the parent compounds or released from the matrix during digestion. For modified forms of zearalenone, nivalenol, T-2 and HT-2 toxins and fumonisins, 100 %, 30 %, 10 % and 60 % were added to exposure assessments respectively, based on reports on the relative contribution of modified forms (EFSA, 2014). The EFSA opinion stated that relatively little was known about the bioavailability of modified mycotoxins apart from DON and to a small extent zearalenone (ZEN). However, the literature cited only covers up to 2012 and there has been a large amount of recent work published since then as the research area of ‘modified mycotoxins’ is very active with lots of current research underway, including this study. For example, Frizzel et al (2015) have recently studied the potential of ZEN and its metabolites to cause endocrine disruption through interference with oestrogen receptor transcriptional activity. They confirmed that the formation of glucosides for ZEN and α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) is a detoxification reaction with regard to oestrogenicity, and serves as a potential host defence mechanism against ZEN-induced estrogenic activity (Frizzel et al 2015). In addition, EFSA also had an open call for data, requesting EU Member States submit data on the occurrence of a range of mycotoxins, and

in particular masked or modified forms, in order that it could be used for future evaluations and risk assessments.

The Food Standards Agency (FSA) commissioned a project on masked mycotoxins to answer some of questions about these compounds. The project was split into two main parts to address two key questions. In the first part of this project described in this report a survey of foods on sale in the UK retail market was carried out with the intention of providing data from the UK that would be submitted to EFSA and included in their data set. Therefore 184 samples of cereals and cereal products and herbs and spices were analysed for trichothecenes and zearalenone compounds and masked metabolites using an established and validated LC-MS/MS method (Fera). The mycotoxins analysed in the survey of food samples are listed in Table 1, abbreviations are given for the toxin names (also in the Glossary). These are standardised to the format suggested in the EFSA opinion and are used throughout the report as far as possible. The second part of the project carried out by the Rowett Institute of Nutrition and Health in Aberdeen, assessed the hydrolysis of masked mycotoxins by digestive juices and gut microflora as well as the gut transport of the toxins *in-vitro.* This part of the work required synthesised some masked forms of mycotoxins. This was carried out by the Chemistry Department at the University of Sheffield. Following the hydrolysis experiments Fera conducted analyses of the extracts using HRMS to establish if any novel metabolites had been formed.

Table 1 Mycotoxins analysed in the masked mycotoxin food survey

Mycotoxin	Code
3-Acetyl-deoxynivalenol	3Ac-DON
15-Acetyl-deoxynivalenol	15Ac-DON
Deoxynivalenol	DON
Deoxynivalenol-3-glucoside	DON3Glc
T-2 toxin	T2
HT-2 toxin	HT2
Nivalenol	NIV
α-Zearalenol	α-ZEL
β-Zearalenol	β-ZEL
α-Zearalenol-14-glucoside	α-ZEL14Glc
β-Zearalenol-14-glucoside	β-ZEL14Glc
Zearalenone	ZEN
Zearalenone-14-glucoside	ZEN14Glc
Zearalenone-14-sulphate	ZEN14Sulf

4 PART 1 - ANALYSIS OF RETAIL FOOD SURVEY SAMPLES FOR MASKED MYCOTOXINS TO CONTRIBUTE TO THE EFSA DATA SET

4.1 Sampling

Sampling was organised by the FSA with samples collected from a variety of sources including retail outlets, and sent to Fera for analysis. Details of the individual samples are given in Appendix 1. The resulting data were submitted to EFSA via the Generic Reporting format excel sheet.

All samples received were ambient products with long shelf life and stored under ambient conditions until they were opened for preparation. If not already homogenised prior to

receipt, samples were milled and mixed using either a dry mixing method or a wet slurry technique as agreed and known to be appropriate to ensure homogeneity.

4.2 Sample preparation

Samples were milled and homogenised using protocols previously established to ensure homogeneity.

There are two options, slurry and dry milling. Fera has a long track record of sample homogenisation through its extensive experience of sample preparation for FAPAS so judgment was made on the most appropriate sample preparation method on a case by case basis. For the majority of samples, dry milling was used using a Retsch centrifugal mill and a powder mixer as this produced a homogenous sample of suitable particle size. However, for some samples, particularly breakfast cereals that contained added fruit and other ingredients slurry preparation was used to ensure that the entire sample was adequately homogenised.

For beer samples, the contents of all containers were combined and mixed and the sample degassed before analysis.

In all cases a method that has been shown to produce homogenous samples was used. All samples were divided into 3 aliquots according to the FSA guidelines for carrying out retail surveys. For all slurried samples all 3 aliquots were frozen immediately after homogenisation. For dried samples 2 aliquots were frozen immediately after preparation, the laboratory sub-sample was stored in the freezer immediately after analysis.

4.3 Materials and Methods

Acetonitrile, water and acetic acid were all Analytical grade or better. Zearalenone, α-zearalenol, and β-zearalenol standards were from Sigma Chemicals. T-2 toxin, HT-2 toxin, deoxynivalenol, nivalenol, deoxynivalenol-3-glucoside, 13C-deoxynivalenol and 13C-zearalenone were all from Biopure, supplied by Romer Labs.

Zearalenone-14-sulphate, zearalenone-14-glucoside, α-zearalenol-14-glucoside, and β-zearalenol-14-glucoside (25µg each) were all kindly donated by Franz Berthiller and Rudolf Krska at IFA Tulln, Austria.

Nylon, 0.22µm syringe filters were from Anachem. Solvents for LC-MS analysis were LC-MS grade. The UPLC column was a Raptor Biphenyl 2.7 µm 100 x 2.1 mm Cat # 9309A12.

4.3.1 Extraction Methods

For cereal / cereal product samples 5 ± 0.1 g sample was weighed into a plastic Falcon tube. Extraction solvent (20ml, ACN:H_2O:HOAc, 79 : 20 : 1) was added. This was shaken well by hand to mix and placed on an orbital shaker for ~1.5 - 2 hours (or overnight if convenient). Samples were centrifuged at 4°C, 4000 rpm for 10 minutes and the centrifuged extract decanted into a clean sample tube. 500µl sample extract was transferred to a clean vial. To each sample extract 13C Deoxynivalenol and 13C Zearalenone and 450µl deionised water were added. The diluted extract was filtered through 0.2µm nylon syringe filter, and collected in a clean total recovery autosampler vial ready for analysis.

Spiked samples were prepared in the same way but were spiked at the following concentrations with stock solutions before extraction:

200µg/kg DON, 3Ac-DON, 15Ac-DON, T-2 toxin, HT-2 toxin

400µg/kg NIV

100µg/kg DON3Glc

20µg/kg ZEN, α & β ZEL, ZEN14Sulf, ZEN14Glc and α & β ZEL14Glc

Spices were extracted in the same manner as cereals but a spiked sample was prepared for every sample, as the differences between sample types were too great to allow batch recovery calculations.

Beer samples, an aliquot of 20 ml beer was dispensed into a plastic falcon tube. This was de-gassed in the sonic bath until gas was removed. 1 ml degassed beer was added to 3 ml acetonitrile in a 4 ml vial and mixed well by vortex. Diluted beer extract (500µl) was transferred to a clean vial and 13C Deoxynivalenol, 13C Zearalenone and 450µl deionised water were added. The extract was filtered through 0.2µm nylon syringe filter, and collected in a clean autosampler vial ready for analysis.

4.3.2 Calibration series

Solvent calibration standards containing all the analytes listed above and 13C deoxynivalenol and 13C zearalenone were prepared in ACN : acidified water (45:55), and aliquoted into individual autosampler vials for batch analysis.

4.4 LC-MS/MS Method - UPLC method

A Waters Acquity UPLC system was used, with a Raptor Biphenyl 2.7 µm 100 x 2.1 mm, at a column temperature 40°C. A gradient elution was used, with mobile phase A: 0.1% formic acid (aq) and mobile phase B: 0.1% formic acid (1:1 methanol : acetonitrile). The compounds included in the analytical method are listed in Table 1. The gradient elution programme used is given in Table 2.

Table 2 LC Gradient elution programme.

Time (min)	Flow (ml/min)	% A	% B	Curve
Initial	0.3	95	5	Initial
0.5	0.3	95	5	5
3.5	0.3	70	30	5
7.5	0.3	60	40	9
13.5	0.3	0	100	6
15.5	0.3	0	100	6
15.6	0.4	0	100	6
16.6	0.4	0	100	6
16.7	0.3	95	5	6
20	0.3	95	5	6

4.4.1 MS method

A Waters Xevo TQ-S triple quadrupole mass spectrometer was used. The settings for monitoring the selected ions for the individual analytes are listed in full in Table 3.

Table 3 MS Conditions for mycotoxins analysis

Compound	Expected RT	Q1	Q3	Cone	CE	Ionisation
Nivalenol	2.21	313.13	125	15	12	ES+
		313.13	175	15	19	
Deoxynivalenol	2.76	297.13	231.1	20	12	ES+
		297.13	249.1	20	10	
Deoxynivalenol C13	2.76	312.1	263.0	30	10	ES+
Deoxynivalenol-3-glucoside	2.64	503.18	247	50	25	ES-
		503.18	427.1	50	21	
		503.18	457.1	50	16	
3 / 15 Acetyldeoxynivalenol	4.60 / 4.45	339.14	203	30	16	ES+
		339.14	231	30	12	
β-Zearalenol-14-glucoside	6.04	481.2	275	30	34	ES-
		481.2	319	30	18	
α-Zearalenol-14-glucoside	7.98	481.2	293	30	30	ES-
		481.2	319	30	18	

		481.2	463.2	30	26	
HT-2 toxin	9.17	442.24	215.1	20	12	ES+
		442.24	263.1	20	11	
Zearalenone-14-glucoside	9.40	479.2	175	30	38	ES-
		479.2	317.2	30	18	
β-Zearalenol	9.68	319.16	160	50	33	ES-
		319.16	188	50	28	
Zearalenone-14-sulfate	9.76	397.1	175	40	36	ES-
		397.1	317.1	40	24	
α-Zearalenol	10.26	319.16	160	50	33	ES-
		319.16	188	50	28	
T-2 toxin	10.39	484.25	215.1	20	18	ES+
		484.25	305.1	20	13	
Zearalenone	11.07	317.14	131	40	30	ES-
		317.14	175	40	24	
Zearalenone C13	11.07	335.1	140	30	30	ES-

4.5 On-going Internal Quality Assurance

One reagent blank and a minimum of two fortified samples were analysed with each batch of samples. In addition a proportion of samples were analysed in duplicate. The fortification level for these quality control (QC) samples was at an appropriate level depending on analyte response (as lower limits of quantification could be achieved for some analytes than for others).

Recoveries were determined for the two fortified samples and for the individual samples of spices where the matrices were more variable in nature.

Isotopically (Carbon 13) labelled standards of DON and ZEN were also used in the method to help correct for any matrix suppression / enhancement effects that occurred. Due to the wide variety of sample types analysed it was not possible to use matrix assisted calibration curves therefore, solvent calibration standards were used. The addition of internal standard in each sample allowed for matrix suppression or enhancement to be taken into account when quantifying the analytes in the test samples. Each batch also contained a Reference Material NRL02, that contained DON, HT-2-Toxin, T-2-Toxin, and ZEN.

4.6 Method validation at Rowett Institute and analysis of 20 samples

The method used at Fera, was established and validated at the Rowett Institute with some minor modification. A parallel small scale validation of the LC-MS/MS method was undertaken at the Rowett Institute and a subset of 20 samples were analysed to verify results.***Method setup***

Mycotoxin reference standards in acetonitrile were supplied by Romer labs (DON, 3-Acetyl-DON, NIV, T2, HT2 and ZEN at 100 µg/mL, DON3Glc at 50 µg/mL). C-13 labelled internal standard solutions were supplied by Romer labs: $^{13}C_{15}$ DON, $^{13}C_{22}$ HT-2 toxin, $^{13}C_{18}$ ZEN (all 25 µg/mL).

4.6.2 LC-MS/MS method

The method parameters are summarised in Table 4. Liquid chromatography was performed on an Agilent 1290 Infinity liquid chromatograph (Agilent technology, UK). An Agilent Zorbax C18 150 mm by 4.6 column was used to separate the mycotoxins. Solvents for the mobile phase were 10% ACN in 5 mM Ammonium Acetate (A), 96% ACN in 5 mM Ammonium Acetate (B). The flow rate was constant at 400 µL/min and the injection volume was 20 µL. Each vial was injected in triplicate. The initial gradient was at 75% A and 25% B. For 10 min, the gradient was increased until 5% A and 95% B. It was held for 2 min washing and then returned to the initial conditions to be equilibrated for 4.5 min. The separated mycotoxins were then passed directly into the 6490 triple-quad Mass Spectrometer (Agilent Technologies, UK), in the AJS (Agilent jet stream) ESI source in positive or negative ion mode depending on the mycotoxin. The ion spray voltage was set at 2500 V, with temperature at 350°C. Mycotoxins were quantified using the multiple-reaction monitoring (MRM) parameters below.

Table 4 Parameters used for the LC-MS/MS.

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	Collision Energy (V)	Polarity
T2	489.2	387	50	13	Positive
HT2	483.3	59	50	8	Negative
^{13}C-HT2	469.3	362.2	50	10	Positive
3Ac-DON	397.1	337.1	50	2	Negative
NIV	371.2	281.2	50	9	Negative
$^{13}C_{15}$-DON	370.2	310.1	50	3	Negative
DON	355.2	295.2	50	2	Negative
^{13}C-ZEN	335.2	185.1	50	23	Negative
ZEN	317.2	175.1	50	21	Negative
DON3Glc	517.2	427.2	50	11	Negative

To calibrate the method a 9 point standard curve was used ranging from 1 – 500 ng/ml for DON, 3Ac-DON and HT2 and from 0.1 – 50 for D3Glc, NIV, T2 and ZEN. A triple mix of internal standards was used at final concentrations of 20ng/mL for $^{13}C_{15}$ DON and $^{13}C_{18}$ ZEN and 60ng/mL for $^{13}C_{22}$ HT2.

4.6.3 LOD/LOQ determination

Using the above standard curve, limit of detection (LOD) and limit of quantification (LOQ) were defined as signal/noise ratio (S/N) of 3 and 10, respectively. Values are summarized in Table 5.

Table 5 LOD and LOQ (µg/kg)

	3Ac-DON	DON	DON3Glc	NIV	T2	HT2	ZEN
LOD	<8	8	8	8	0.8	<8	<0.8
LOQ	16	16	16	16	1.6	8	0.8

4.6.4 Sample extraction

4 mL of the solution A (79% ACN, 20% H_2O, 1% acetic acid), were added to 1g of the finely milled food sample. After extraction on a horizontal shaker for 90 min at 200 rpm and a 5 minutes centrifugation at 3500 rpm, supernatant was removed and an equal volume of solution B (20% ACN, 79% H_2O, 1% acetic acid) was added. Aliquots of 400 µL of diluted extract were evaporated at 50°C under a stream of nitrogen, reconstituted in 400 µl of 10% ACN and 80µL aliquots were combined with 20 µL of the internal standard into LC vials with inserts.

4.6.5 Spiking experiments

1g of wheat couscous was spiked in triplicate with a mycotoxin mix at three different levels. The levels used for each mycotoxin are given in Table 6.

Table 6 Spiking levels of wheat couscous (µg/kg food)

	3Ac-DON	DON	DON3Glc	NIV	T2	HT2	ZEN
Level 1	400	400	40	40	40	400	40
Level 2	2000	2000	200	200	200	2000	200
Level 3	4000	4000	400	400	400	4000	400

Sample tubes were left open overnight to allow solvent to evaporate. Samples were then extracted as described above. The spiking experiment was repeated 3 times and results (percentage recovery) are presented as average of 3 independent spiking experiments in Figure 1.

4.7 Rowett sample analysis

20 food samples were received from FERA as finely milled powders and were subjected to extraction as described above. Each sample was extracted in duplicate and 2 independent aliquots of each extract were dried and reconstituted. Extracts were then combined with internal standard mix and injected into LC-MS/MS (triplicate injection). Concentrations were determined against a 9 point standard curve (1 – 500 ng/ml for DON, 3Ac-DON and HT2 and from 0.1 – 50 for D3Glc, NIV, T2 and ZEN), results were multiplied by 8 to correct for solvent dilution and expressed as µg mycotoxin/ kg food sample.

5 Results and Discussion – Food Survey

5.1 Quality assurance – reference material

Reagent blank samples were consistently free of detectable mycotoxins.

An important point to note was that the LC conditions used by Fera were optimised to ensure complete separation and resolution of 3Acetyl and 15Acetyl-DON. The column used (Raptor) allowed these two compounds that have identical atomic mass to be completely separated. Most methods used in literature cannot achieve this and rely solely on monitoring different transitions for what is essentially the same compound to try to quantify both compounds, or would only report the results as a sum of both compounds. However, we were able to separate and quantify both compounds individually which adds value to the data reported to EFSA.

Results for the Reference Material NRL02 are provided in Table 7. The Assigned values for this material were determined by an interlaboratory comparison among UK Official Control Laboratories and are the mean consensus values. Values were assigned for DON, ZEN, T2 and HT2 only. The values for DON and ZEN are very robust as they were the average of a higher number of laboratories, however the results for T2 and HT2 toxin were the consensus of a smaller number of labs (3 or 4) and so there is more uncertainty attached to those values. The acceptable range (AR) was calculated based on the results of the intercomparison and standard Proficiency Test (PT) statistics and is calculated to be the range that would result in a Z-score of -2 to +2 (i.e. satisfactory performance) if the sample was analysed as a PT sample. The results for DON and ZEN all fell within the acceptable range, as did those for T2 with the exception of two failed analyses. Those for HT2 were almost all just above the upper limit of the acceptable range.

Table 7 Fera Results for the reference material NRL_02 wheat (µg/kg)

Batch	Deoxynivalenol	HT-2-Toxin	T-2-Toxin	Zearalenone
1	687	227	98	62
2	696	271	106	75
3	658	268	nd	93
4	516	275	105	64
5	800	216	116	68
6	917	detected	detected	80
7	787	254	101	68
8	877	275	119	61
Assigned	766	235	167	74
AR	510-1021	141-239	97.2-237	41.2-106

Assigned = Assigned value

AR = Acceptable range

Rowett also analysed the same reference material and their results are summarised in Table 8. The results for DON, ZEN and T2 were in good agreement with the assigned values as they were for Fera. However, the HT2 values were higher than expected. This was also observed at Fera, although the values found by the Rowett were higher. In addition Rowett also measured low levels of DON3Glc and NIV in the reference material, although there were no assigned reference values for these analytes. Overall the results show good performance of the analysis between the two laboratories.

Table 8 Rowett Results for the reference material NRL_02 wheat (µg/kg)

	DON	DON3Glc	HT2	NIV	T2	ZEN
NRL_02 Wheat Consensus value	766.0		235		167	73.6
AR	510-1021		141-239		97.2-237	41.2-106
Rowett result Average result ± standard deviation (n=9)	642.3 ± 104.1	80.0 ± 18.7	471.5 ± 119.8	89.6 ± 9.6	140.9 ± 56.4	63.9 ± 16.6

5.2 **Quality assurance – recovery experiments**

Recovery data for cereals, beers and oil are provided in Table 9.

The recovery results for batches 1-4 and 7 are presented as average values for the four different spiked samples. Recoveries for the sample/analyte combinations were mostly good with occasional low values and some consistently high values for ZEL and ZEN metabolites except for ZEN14Sulf for which recoveries were generally lower and more erratic. The most likely cause of this is variable signal suppression from the different matrices that were used for spiking. A recent paper reported good recovery for ZEN14Sulf during method validation, but the signal suppression enhancement ratio was calculated as 75%, and this was for one type of wheat only (Nathanail et al, 2015). It is likely that the variability of the product types tested would result in much bigger differences in signal suppression that could only be controlled completely by the use of isotopically labelled standards for each of the compounds in the analysis. Unfortunately, these are not available and so the best available compromise of using C13 ZEN was used. The recovery results for batch 6 (spices) are presented individually as there were significant differences in the matrix composition of these samples and each sample was spiked individually to assess the recovery for that particular sample.

Average recoveries for the fortified spice samples ranged from 30 to 116%. The highest recoveries (average 100-116 %) were for ZEL14Glc and ZEN14Sulf. Recovery results are provided for individual samples within the results Table 14.

Table 9 Recovery data (%) for cereals, beers and oil.

Batch	Sample	3ADON	15AcDON	DON	D3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
	Spike level (µg/kg)	200	200	200	100	200	400	200	20	20	20	20	20	20	20
Batch 1	Average recovery (n=4)	89	80	62	52	93	44	92	72	93	87	90	95	90	64
Batch 2	Average recovery (n=4)	94	89	71	53	99	58	94	60	82	91	96	90	100	81
Batch 3	Average recovery (n=4)	71	67	73	89	77	49	76	81	73	108	89	78	89	58
Batch 4	Average recovery (n=4)	65	63	55	62	58	34	72	66	68	89	59	70	72	54
Batch 7	Average recovery (n=2)	92	90	68	34	96	40	106	74	96	128	97	95	101	52
Batch 5	Average recovery (beer n=3)	89	81	76	92	101	108	99	90	91	92	88	84	101	77
Batch 6	Spiked choc cereal bar slurry	81	80	90	37	112	50	103	NR	101	125	100	98	110	50
Batch 6	Spiked muesli slurry	100	94	101	43	94	61	106	95	102	125	110	108	107	68
Batch 6	Spiked muesli slurry	99	100	99	39	87	47	98	105	97	120	100	97	110	46
Batch 6	Spiked oil	87	80	87	106	NR	62	NR	NR	80	110	109	60	109	NR
Batch 6	Spiked strawberry cereal bar	105	86	98	37	99	45	106	110	101	120	105	103	114	50
Batch 6	Spiked wet baby food	84	104	77	60	NR	49	NR	NR	80	110	91	70	90	23

NR No result

5.3 Rowett Results for Method Validation Spiked Samples (wheat)

The results of the spiking experiments carried out at the Rowett for method validation are presented in Figure 1**Error! Reference source not found.**. All average recoveries were within the accepted range of 70-110% (from Commission Regulation (EC) 401/2006) except for 3AcDON where average recoveries were close to 125%.

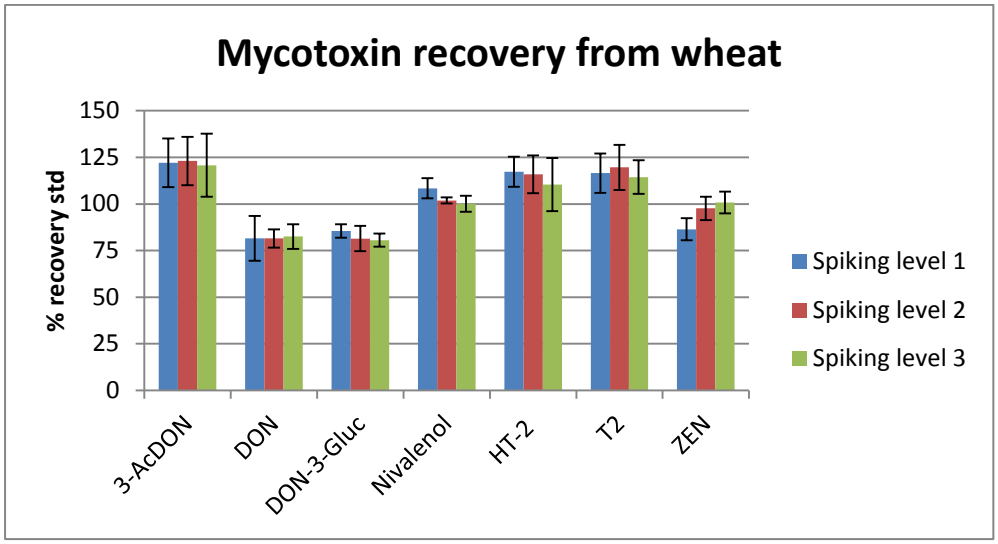


Figure 1. Results of Rowett spiking experiments in wheat. Results mean ± standard deviation.

5.4 Rowett LOD/LOQ determination

The limit of detection (LOD) and limit of quantification (LOQ) were defined as signal/noise ratio (S/N) of 3 and 10, respectively. Values determined by the Rowett are summarized in Table 5**Table 5**. The values determined were lower than those obtained at Fera due to the inclusion of an evaporation and reconstitution step in the procedure.

6 RESULTS – Food Survey Mycotoxin Levels

Results are provided in to below and as an attached Excel file.

6.1 Beers

Results for the beer samples are provided in Table 10. DON and DON3Glc were the only mycotoxins detected in these samples. Only four of thirty samples contained free DON (range 23 to 42 µg/kg, average 30 µg/kg) whereas DON3Glc was present in fifteen samples (range 20 to 78 µg/kg, average 37 µg/kg). Of the four samples containing both forms, in two unrelated samples the ratio of DON to DON3Glc was close to 1:1 and in the other two samples the ratios were 1:1.9 and 1:2.6.

The DON3Glc results in this batch were indicative (not fully quantitative) as there was a high amount of variability in the lower concentration calibration standards for this analyte for this batch of samples.

Table 10 Masked mycotoxin content (µg/kg) of beer category samples.

		3AcDON	15AcDON	DON	DON3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
	LOQ (µg/kg)	20	100	20	20	100	100	20	2.5	2.5	12.5	2.5	2.5	2.5	2.5
Sample	Type														
S14-010982	Bitter	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010983	Pale Ale	-	-	42	78	-	-	-	-	-	-	-	-	-	-
S14-010984	Lager	-	-	-	24	-	-	-	-	-	-	-	-	-	-
S14-010985	Pale Ale	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010989	Bitter	-	-	-	21	-	-	-	-	-	-	-	-	-	-
S14-011001	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011025	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011026	Lager	-	-	-	20	-	-	-	-	-	-	-	-	-	-
S14-011027	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011028	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011029	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011030	Lager	-	-	-	28	-	-	-	-	-	-	-	-	-	-
S14-011031	Lager	-	-	-	28	-	-	-	-	-	-	-	-	-	-
S14-011033	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011034	Pale Lager	-	-	-	27	-	-	-	-	-	-	-	-	-	-
S14-011035	Golden Ale	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011036	Pale Ale	-	-	-	26	-	-	-	-	-	-	-	-	-	-
S14-011038	Pale Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011039	Strong Lager	-	-	23	25	-	-	-	-	-	-	-	-	-	-
S14-011040	Lager	-	-	-	22	-	-	-	-	-	-	-	-	-	-
S14-011041	Lager	-	-	-	24	-	-	-	-	-	-	-	-	-	-
S14-011042	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ

Table 10 (continued). Masked mycotoxin content (µg/kg) of beer category samples.

		3AcDON	15AcDON	DON	DON3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
	LOQ (µg/kg)	20	100	20	20	100	100	20	2.5	2.5	12.5	2.5	2.5	2.5	2.5
Sample	Type														
S14-011046	Pale Ale	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011047	Pale Ale	-	-	23	60	-	-	-	-	-	-	-	-	-	-
S14-011048	Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011094	Pale Lager	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011095	Pale Ale	-	-	-	23	-	-	-	-	-	-	-	-	-	-
S14-011096	Blonde beer	-	-	31	37	-	-	-	-	-	-	-	-	-	-
S14-011097	Wheat beer	-	-	-	27	-	-	-	-	-	-	-	-	-	-
S14-011098	Lager Light	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ

6.2 **Breakfast cereals**

Results for the breakfast cereals category are provided in Table 11. DON and DON3Glc were again the major contaminants. Free DON was present in twenty-six of sixty samples over a wide range (21 to 375 µg/kg) with an average where present of 113 µg/kg. DON3Glc was present in only seven of the sixty samples (range 55 to 121 µg/kg, average 75 µg/kg). Three of these samples were high bran or high fibre cereals. The levels of DON and DON3Glc were similar in two of the six samples that contained both compounds but free DON levels were twice those of DON3Glc in two other (corn) samples (S14-010972 and S14-010973) and even higher in the other two bran samples(S14-010964 and S14-010965) which were unrelated in composition. The two corn samples that contained DON and DON3Glc also contained quite high levels (~100 µg/kg) of 15AcDON but no 3AcDON.

Two samples of oats (S14-010988 and S14-011998) contained HT2 at 53 and 21 µg/kg respectively.

ZEN was detected in sixteen samples (range 3 to 18 µg/kg average 6 µg/kg). These included eight of the nine samples which had bran as a major ingredient.

Table 11 Masked mycotoxin content (µg/kg) of breakfast cereal category samples.

Sample No.	Product	3Ac DON	15Ac DON	DON	DON 3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
LOQ (µg/kg)		20	100	20	50	50	100	20	6.25	2.5	12.5	2.5	2.5	2.5	2.5
S14-010920	Simply Fruity Muesli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010931	Chef's Larder Oats	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010953	Shredded Wheat Honey Nut	-	-	60	-	-	-	-	-	-	-	-	-	-	-
S14-010954	Original Muesli	-	-	21	-	-	-	-	-	-	-	-	-	-	-
S14-010955	Honey Cheerios	-	-	49	-	-	-	-	-	-	-	-	4	-	-
S14-010956	Shreddies	-	-	64	64	-	-	-	-	-	-	-	4	-	-
S14-010957	Cheerios	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010958	Ready Brek Original	-	-	-	62	-	-	-	-	-	-	-	-	-	-
S14-010959	Crunchy Bran	-	-	156	-	-	-	-	-	-	-	-	4	-	-
S14-010960	Crispy Minis Fruit & Nut	-	-	146	-	-	-	-	-	-	-	-	-	-	-
S14-010961	Weetos	-	-	170	-	-	-	-	-	-	-	-	-	-	-
S14-010962	Cornflakes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010963	Honey Nut Cornflakes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010964	Bran Flakes	-	-	246	73	-	-	-	-	-	-	-	4	-	-
S14-010965	Fantastic Fibre	-	-	375	87	-	-	-	-	-	-	-	18	-	-
S14-010966	Bran flakes	-	-	75	64	-	-	-	-	-	-	-	-	-	-
S14-010967	Starting right	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010968	High fibre bran	-	-	345	-	-	-	-	-	-	-	-	16.4	-	-
S14-010969	Crunchy Nut Cranberry & Almonds	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010970	Coco Pops	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010971	Crunchy Nut With Chocolate Curls	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010972	Bran flakes	-	-	89	-	-	-	-	-	-	-	-	3	-	-
S14-010973	Special K Red Berries	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010974	Special K Original	-	-	42	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ

Table 11 (continued). Masked mycotoxin content (µg/kg) of breakfast cereal category samples.

Sample	Product	3Ac DON	15Ac DON	DON	DON 3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
LOQ (µg/kg)		20	100	20	50	50	100	20	6.25	2.5	12.5	2.5	2.5	2.5	2.5
S14-010975	Corn Flakes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010976	Frosties	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010977	Crunchy Nut Cornflakes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010978	Rice Krispies	-	-	36	-	-	-	-	-	-	-	-	-	-	-
S14-010979	Grape Nuts	-	-	50	-	-	-	-	-	-	-	-	-	-	-
S14-010980	Porridge Oats	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010981	Nutty muesli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010986	Bran flakes	-	-	54	-	-	-	-	-	-	-	-	3.8	-	-
S14-010988	Porage oats	-	-	-	-	53	-	-	-	-	-	-	-	-	-
S14-010990	Porridge oats simply value	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-01100	Multigrain Hooplas Cereal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011003	Choco Rice Pops	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011004	Wholegrain Bran Flakes	-	-	61	-	-	-	-	-	-	-	-	2.7	-	-
S14-011005	Sugar puffs	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011006	Multi-grain shapes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011007	Crispy Minis Chocolate Chip	-	-	75	-	-	-	-	-	-	-	-	3.8	-	-
S14-011008	Branflakes	-	-	57	-	-	-	-	-	-	-	-	4.9	-	-
S14-011009	Golden Syrup Oats	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011053	Crunchy Oats Cereal With Tropical Fruits	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011054	Medium Oatmeal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011055	Oat Granola	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011056	Nut & Seed Muesli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011058	Irish Organic Porridge Oats	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011059	Coco Pops Rocks	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ

Table 11 (continued). Masked mycotoxin content (µg/kg) of breakfast cereal category samples.

Sample	Product	3Ac DON	15Ac DON	DON	DON 3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
LOQ (µg/kg)		20	100	20	50	50	100	20	6.25	2.5	12.5	2.5	2.5	2.5	2.5
S14-011060	Instant Hot Oat Cereal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011061	All bran	-	-	156	-	-	-	-	-	-	-	-	12.0	-	-
S14-011062	Milk & Cereals	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011063	Special K Oats & Honey	-	-	83	-	-	-	-	-	-	-	-	5.0	-	-
S14-011064	Chocolate & Coconut Cereal	-	-	27	-	-	-	-	-	-	-	-	-	-	-
S14-011065	White Chocolate And Strawberry Cereal	-	-	26	-	-	-	-	-	-	-	-	-	-	-
S14-011067	Chocolatey Cheerios	-	-	-	-	-	-	-	-	-	-	-	4.0	-	-
S14-011068	Fruit & Fibre	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011069	Crunchy Nut Honey & Nut Clusters	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011072	Cornflakes	-	123	314	121	-	-	-	-	-	-	-	9.7	-	-
S14-011073	Special K Red Berries	-	111	119	55	-	-	-	-	-	-	-	5.1	-	-
S14-011998	Scottish Porridge Oats	-	-	40	-	21	-	-	-	-	-	-	-	-	-

- Below LOQ

6.3 Cereal products

Results for the cereal products category are given in Table 12. This category consisted of flour, rice, crispbreads and oils. Of twenty-five cereal products eight contained free DON over a wide range (36 to 300 µg/kg) with an average where present of 117 µg/kg. DON3Glc was present in six samples (range 56 to 96 µg/kg, average 67 µg/kg). These were a wholemeal flour sample (S14-010941, 56 µg/kg), a brown bread flour sample (S14-010949, 72 µg/kg), three samples of semolina (S14-010946, 96 µg/kg; S14-010991, 67 µg/kg; and S14-010948, 63 µg/kg). Two samples of plain flour contained free DON but no DON3Glc, and one sample of semolina contained DON3Glc but no free DON (S14-010991). One sample of self raising flour contained 300 µg/kg free DON but had no DON3Glc, and a sample of yellow semolina (S14-010946) that contained 267 µg/kg free DON had a low proportion of DON3Glc (ratio was 1:0.36). Otherwise the ratio of DON to DON3Glc varied from 1:1.1 to 1:1.6. Two samples contained ZEN, one being a corn oil. Overall there was no correlation between sample type and mycotoxin content.

Table 12 Masked mycotoxin content (µg/kg) of cereal product category samples (flour, rice and oil).

Sample	Product	3Ac DON	15Ac DON	DON	DON 3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
LOQ (µg/kg)		20	100	20	20	50	100	20	2.5	2.5	12.5	6.25	2.5	6.25	2.5
S14-010921	Organic Long Grain Brown Rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010922	Rye Crispbreads	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010935	Brown Basmati Rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010936	Fine Milled Oatcakes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010937	Gluten Free Cheese Oatcakes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010938	Wholegrain Brown Basmati Rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010939	Premium Plain Flour	-	-	66	-	-	-	-	-	-	-	-	-	-	-
S14-010940	Pearl Barley	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010941	Wholemeal Self Raising Flour	-	-	36	56	-	-	-	-	-	-	-	-	-	-
S14-010942	Basmati Rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010943	Pure Corn Oil	-	-	-	NR	-	-	-	-	-	-	-	53	-	-
S14-010944	Rapeseed Oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010945	Long Grain Rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010946	Yellow Semolina	-	-	267	96	-	-	-	-	-	-	-	-	-	-
S14-010947	White maize meal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010948	Fine Semolina	-	-	55	63	-	-	-	-	-	-	-	-	-	-
S14-010949	Brown bread flour	-	-	65	72	-	-	-	-	-	-	-	-	-	-
S14-010950	Sesame Crispbread	-	-	39	57	-	-	-	-	-	-	-	-	-	-
S14-010951	Semolina	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-010952	Self Raising Flour	-	-	300	-	-	-	-	-	-	-	-	-	-	-
S14-010991	Semolina Fine	-	-	-	67	-	-	-	-	-	-	-	-	-	-
S14-010997	Pearl Barley	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011002	Basics Plain flour	-	-	105	-	-	-	-	-	-	-	-	6	-	-
S14-011057	Dark Rye	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011077	Pearl Barley	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ, NR No result

6.4 **Cereal based infant foods**

Results for the infant food category are given in Table 13. Of thirty cereal based infant foods only four contained mycotoxins. Free DON was present in two samples (S14-011024, 58 µg/kg and S14-011051, 33 µg/kg). DON3Glc was also present in the first sample (72 µg/kg) and in two other samples (at around 50 µg/kg) that did not contain free DON. One sample also contained ZEN, but at a level below the limit of quantification. All of these samples contained mixtures of cereals and fruits.

Table 13 Masked mycotoxin content (µg/kg) of infant cereal category samples.

Sample	Product	3Ac DON	15Ac DON	DON	DON 3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
LOQ (µg/kg)		20	100	20	20	50	100	20	6.25	2.5	12.5	6.25	6.25	6.25	2.5
S14-011010	Pure Baby Rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011011	Fruity Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011012	Multigrain Banana Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011013	Creamy Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011014	Apricot & Apple Rice Pudding	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011015	Very Berry Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011016	Banana & Apple Muesli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011017	Creamed Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011018	Blueberry Porridge	-	-	-	53	-	-	-	-	-	-	-	-	-	-
S14-011019	Summer Fruit Cereal	-	-	-	54	-	-	-	-	-	-	-	-	-	-
S14-011020	Peachy Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011021	Egg custard with rice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011022	Rice Pudding with Apple & Pear	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011023	Creamed Porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011024	Strawberry & banana porridge	-	-	58	72	-	-	-	-	-	-	-	-	-	-
S14-011049	Salmon Risotto	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011050	Banana Baby Brekkie	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011051	Strawberry, mango & banana porridge	-	-	33	-	-	-	-	-	-	-	-	(4.4)	-	-
S14-011052	Gingerbread men	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011066	Creamed porridge	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011070	Rice Pudding	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011071	Banana & Peach Breakfast	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ (value in brackets residue detected but below LOQ so quantification not confirmed)

Table 13 (continued) Masked mycotoxin content (µg/kg) of infant cereal category samples.

Sample	Product	3Ac DON	15Ac DON	DON	DON 3Glc	HT2	NIV	T2	α-ZEL	β-ZEL	α-ZEL 14Glc	β-ZEL 14Glc	ZEN	ZEN 14Glc	ZEN 14Sulf
LOQ (µg/kg)		20	100	20	20	50	100	20	6.25	2.5	12.5	6.25	6.25	6.25	2.5
S14-011074	Tropical Fruit Cereal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011075	Mango, Apple & Banana Breakfast	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011076	Banana & Cookie Crumble	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011078	Garden Vegetable Risotto with Flaky White Fish	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011079	Creamy Rice Breakfast	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011080	Vegetables with Rice & Chicken	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011081	Creamy Porridge breakfast	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14-011082	Tropical Cereal Topped with Yogurt	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Below LOQ

6.5 **Spices**

Results for the spices products category are given in Table 14. Only a few of the thirty nine spices contained mycotoxins. Two samples (S14-010933, mace, and S14-011088, cayenne pepper) both contained free DON at about 40 µg/kg. DON3Glc was not detected in any sample.

During initial analyses of some samples, apparent residues of some toxins were observed, however these were not confirmed as ion ratios did not confirm the residues. In addition recovery for many samples was very poor and large interferences were observed in many chromatograms. For the samples where the results were: (i) not confirmed; (ii) gave very poor or no recovery; (iii) or other issues were observed, modifications were made to the extraction method and the sample re-analysed. The same sample weight was used, but the extraction time was halved and a freeze thaw cycle step was introduced in an attempt to reduce the amount of co-extractives in the sample extracts that were causing the problems in the Mass Spectrometer. The results reported in Table 14 are from the second analysis, and are the data that was submitted to EFSA.

For example, HT2 was found in a nutmeg (S14-010934) during the first analysis when a value of 109 µg/kg was observed (not confirmed), but HT2 was not found in the spiked sample. In the repeated analysis HT2 was not detected although it was found in the spiked equivalent (recovery 50%). In celery seed sample S14-010996 the first analysis gave a value of 168 µg/kg (not confirmed) but HT2 was not found in the spiked sample. In the repeated analysis HT2 was not detected although it was found in the spiked equivalent at a low recovery (<20%).

Contradictory results were also obtained for NIV. It appeared to be present in several samples at the initial analysis, for example curry powder S14-010987 at 212 µg/kg and nigella seed (S14-010992) at 267 µg/kg (for which the spiked sample gave a low calculated recovery but had a high peak area). However there were large interferences in the chromatograms and the data was not considered to be reliable. In the repeated batch NIV was not found in the sample but the nigella seed again gave a high peak area in the spiked sample.

In the analysis of cayenne pepper S14-011088 a peak assigned as nivalenol was measured in the first batch at 1500 µg/kg (with a corresponding very high peak area for the spiked sample). Again this sample suffered from interferences and the ion ratio did not confirm the peak was NIV. The toxin was not detected in either sample or spike in the repeated batch, however this sample also contained DON and ZEN.

T2 and α-ZEL were not detected in any sample and only one sample (curry powder S14-010987) contained β-ZEL at the low level of 3.8 µg/kg. No α-ZEL14Glc was detected in any sample and only one sample (cumin S14-0109300) contained β-ZEL14Glc, at 13 µg/kg.

The most abundant mycotoxin was ZEN, present in seven samples. The highest level was in the cayenne pepper sample S14-011088 at 30 and 47 µg/kg on duplicate analyses. The remaining samples contained less than 10 µg/kg. ZEN14Glc and ZEN14Sulf were each detected in two (different) samples at 10-20 µg/kg. One of these, turmeric S14-010999 contained free zearalenone in one analysis.

Table 14 Masked mycotoxin content (µg/kg) of spices category samples.

	Type	3AcDON		15AcDON		DON		DON3Glc		HT2		NIV		T2	
	Level (µg/kg)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)
Sample No.	LOQ (µg/kg)	20		100		20		20		50		200		20	
S14-010923	Ginger Ground	-	75	-	NR	-	42	-	72	-	NR	-	NR	-	15
S14-010924	White pepper ground	-	95	-	113	-	53	-	42	-	100	-	51	-	99
S14-010925	Black pepper cracked	-	73	-	50	-	59	-	19	-	22	-	40	-	22
S14-010926	Marjoram	-	37	-	30	-	33	-	NR	-	27	-	NR	-	33
S14-010927	Coriander leaf	-	62	-	34	-	47	-	NR	-	55	-	NR	-	63
S14-010928	Chilli powder	-	58	-	67	-	61	-	14	-	38	-	NR	-	69
S14-010929	Paprika powder	-	52	-	67	-	43	-	NR	-	31	-	NR	-	60
S14-010930	Cumin ground	-	35	-	NR	-	34	-	8	-	70	-	NR	-	44
S14-010932	Mustard powder	-	65	-	57	-	15	-	13	-	78	-	NR	-	103
S14-010933	Mace ground	-	52	-	41	41	68	-	9	-	30	-	34	-	25
S14-010934	Nutmeg ground	-	64	-	65	-	45	-	22	-	49	-	36	-	54
S14-010987	Curry powder	-	55	-	52	-	47	-	14	-	NR	212	19	-	14
S14-010992	Nigella seed	-	78	-	59	-	57	-	29	-	80	-	56	-	100
S14-010993	Allspice ground	-	39	-	44	-	25	-	NR	-	47	-	NR	-	62
S14-010994	Star anise	-	NR	-	NR	-	33	-	NR	-	NR	-	NR	-	22
S14-010995	Caraway seed	-	58	-	45	-	57	-	20	-	65	-	36	-	87
S14-010996	Celery seed	-	13	-	43	-	53	-	13	-	18	-	23	-	48
S14-010999	Turmeric	-	52	-	NR	-	67	-	30	-	37	-	49	-	26

- Below LOQ NR No recovery result – recovery could not be determined due to interference or other matrix problem in sample

Table 14 (continued) Masked mycotoxin content of spices category samples.

	Type	3AcDON		15AcDON		DON		DON3Glc		HT2		NIV		T2	
	Level (µg/kg)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)
Sample No.	LOQ (µg/kg)	20		100		20		20		50		200		20	
S14-011000	Garlic granules	-	106	-	131	-	63	-	57	-	99	-	NR	-	105
S14-011083	Basil	-	36	-	67	-	37	-	34	-	22	-	NR	-	45
S14-011084	Cinnamon ground	-	40	-	34	-	44	-	23	-	21	-	NR	-	52
S14-011085	Mixed spice	-	58	-	60	-	46	-	40	-	33	-	NR	-	48
S14-011086	Mustard seeds	-	74	-	71	-	14	-	11	-	84	-	NR	-	113
S14-011087	Rosemary	-	41	-	NR	-	25	-	NR	-	NR	-	27	-	30
S14-011088	Cayenne pepper	-	71	-	90	49	50	-	34	-	39	-	NR	-	64
S14-011089	Cardamom whole	-	63	-	65	-	49	-	8	-	59	-	NR	-	70
S14-011090	Cloves whole	-	25	-	44	-	3.3	-	NR	-	21	-	NR	-	29
S14-011091	Oregano	-	29	-	29	-	43	-	9	-	29	-	NR	-	31
S14-011092	Coriander seed	-	61	-	NR	-	52	-	20	-	67	-	36	-	95
S14-011093	Cardamom	-	63	-	49	-	41	-	15	-	65	-	NR	-	69

- Below LOQ NR No recovery result – recovery could not be determined due to interference or other matrix problem in sample

Table 14 (continued) Masked mycotoxin content (µg/kg) of spices category samples.

		α-ZEL		β-ZEL		α-ZEL14Glc		β-ZEL14Glc		ZEN		ZEN14Glc		ZEN14Sulf	
	Level (µg/kg)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)	level	rec (%)
Sample No.	LOQ (µg/kg)	2.5		2.5		2.5		2.5		2.5		2.5		2.5	
S14-010923	Ginger Ground	-	13	-	23	-	90	-	75	-	34	-	70	-	160
S14-010924	White pepper ground	-	70	-	96	-	135	-	95	-	89	-	104	-	86
S14-010925	Black pepper cracked	-	27	-	70	-	130	-	73	-	70	-	131	-	111
S14-010926	Marjoram	-	NR	-	47	-	98	-	89	-	51	-	54	-	155
S14-010927	Coriander leaf	-	7	-	59	-	125	-	105	-	68	-	-	-	110
S14-010928	Chilli powder	-	54	-	80	-	107	-	74	6.1	50	-	97	-	114
S14-010929	Paprika powder	-	28	-	50	-	127	-	73	3.0	51	-	21	-	96
S14-010930	Cumin ground	-	37	-	73	-	114	13	42	-	61	-	26	-	133
S14-010932	Mustard powder	-	81	-	65	-	114	-	76	-	75	-	132	-	97
S14-010933	Mace ground	-	NR	-	55	-	125	-	93	8.2	67	-	26	-	37
S14-010934	Nutmeg ground	-	78	-	50	-	120	-	74	-	115	-	102	-	59
S14-010987	Curry powder	-	34	3.8	49	-	141	-	100	6.4	11	-	33	11.0	245
S14-010992	Nigella seed	-	79	-	65	-	110	-	74	-	62	-	126	-	82
S14-010993	Allspice ground	-	34	-	64	-	116	-	48	-	52	-	31	-	116
S14-010994	Star anise	-	47	-	43	-	99	-	75	-	46	-	-	-	118q
S14-010995	Caraway seed	-	23	-	100	-	125	-	81	5.2	69	-	38	-	89
S14-010996	Celery seed	-	80	-	55	-	109	-	75	-	37	-	90	-	109
S14-010999	Turmeric	-	57	-	33	-	127	-	92	10.0	5.2	-	89	13.4	135

- Below LOQ NR No recovery result – recovery could not be determined due to interference or other matrix problem in sample

Table 14 (continued) Masked mycotoxin content (µg/kg) of spices category samples.

		α-ZEL		β-ZEL		α-ZEL14Glc		β-ZEL14Glc		ZEN		ZEN14Glc		ZEN14Sulf	
	Level (µg/kg)	level	Rec (%)	level	Rec (%)	level	Rec (%)	level	Rec (%)	level	Rec (%)	level	Rec (%)	level	Rec (%)
Sample No.	LOQ (µg/kg)	2.5		2.5		2.5		2.5		2.5		2.5		2.5	
S14-011000	Garlic granules	-	70	-	30	-	135	-	108	2.5	111	-	122	-	123
S14-011083	Basil	-	45	-	49	-	105	-	80	-	38	-	-	-	122
S14-011084	Cinnamon ground	-	41	-	49	-	102	-	61	-	38	-	30	-	153
S14-011085	Mixed spice	-	50	-	38	-	120	-	90	-	50	-	135	-	129
S14-011086	Mustard seeds	-	63	-	80	-	116	-	71	-	85	-	96	-	92
S14-011087	Rosemary	-	27	-	50	-	89	-	85	-	24	18.0	83	-	153
S14-011088	Cayenne pepper	-	85	-	50	-	110	-	80	46.5	64	-	90	-	111
S14-011089	Cardamom whole	-	7	-	90	-	130	-	115	-	60	-	22	-	189
S14-011090	Cloves whole	-	30	-	26	-	100	-	67	-	21	-	NR	-	119
S14-011091	Oregano	-	31	-	45	-	102	-	114	-	44	10.0	29	-	159
S14-011092	Coriander seed	-	51	-	91	-	129	-	125	-	82	-	40	-	113
S14-011093	Cardamom	-	21	-	81	-	138	-	110	-	67	-	31	-	117

- Below LOQ NR No recovery result – recovery could not be determined due to interference or other matrix problem in sample

6.6 Reporting results

The data set was submitted to FSA and EFSA in July 2014 in the form of an Excel spreadsheet. EFSA contacted Fera with requests for clarifications and additional information. On the whole this was information about the ingredients in processed and mixed products such as the infant foods. This information was provided, and its receipt confirmed by EFSA. Formal confirmation has been received that the data set containing 2449 has been accepted and uploaded to the EFSA data collection framework (DCF) and will be used in current evaluations. A summary of the masked mycotoxins found and their percentage with respect to the parent compound is given in Table 15.

Table 15 Summary of masked mycotoxin forms found in different products.

Matrix		**n (samples)**	**Conjugates**	**n (pos)**	**% with respect to parent**
Beverage	Bitter / Ale	9	DON3Glc	5 (56%)	186 and 261% (3 with no parent DON detected)
	Lager	21	DON3Glc	9 (43%)	109 and 119% (7 with no parent DON detected)
Herbs & Spices		30	β-ZEL, β-ZEL14Glc, ZEN14Glc, ZEN14Sulf	5 (17%)	134 and 231% (sum) (3 with no parent ZEN detected)
Cereal products	Infant food	30	DON3Glc	3 (10%)	124% (2 with no parent DON detected)
	Cereal products	25	DON3Glc	6 (24%)	36-156% (1 with no parent DON detected)
	Breakfast cereals	60	DON3Glc	7 (12%)	23-100% (1 with no parent DON detected)

.

6.7 Results - Rowett food sample analysis

Twenty food samples were received from Fera as finely milled powders and were subjected to extraction as described above. Each sample was extracted in duplicate and two independent aliquots of each extract were dried and reconstituted. Extracts were then combined with internal standard mix and injected into LC-MS/MS (triplicate injection). Concentrations were determined against a 9 point standard curve (1 – 500 ng/ml for DON, 3AcDON and HT2 and from 0.1 – 50 for DON3Glc, NIV, T2 and ZEN), results were multiplied by 8 to correct for solvent dilution and expressed as µg mycotoxin/ kg food sample.

The results of the Rowett analysis are presented in Table 16.

Table 16 Comparison of results from Rowett and Fera for food survey samples (µg/kg)

		Rowett results							Fera results						
Sample no.	Sample	Average result ± standard deviation (µg/kg) (n=9)							Result (µg/kg)						
		3AcDON	**DON**	**DON 3Glc**	**HT2**	**NIV**	**T2**	**ZEN**	**3AcDON 15AcDON**	**DON**	**DON 3Glc**	**HT2**	**NIV**	**T2**	**ZEN**
LOQ	**(µg/kg)**	**16**	**16**	**16**	**8**	**16**	**1.6**	**0.8**	**20 / 100**	**20**	**50**	**20**	**100**	**20**	**2.5**
S14-010948	Fine Semolina	<LOQ	172.2 ± 70.6	53.6 ± 22.8	<LOQ	40.6 ± 11.2	<LOQ	<LOQ	<LOQ	54.9	63.4i	<LOQ	<LOQ	<LOQ	<LOQ
S14-010950	Sesame Crispbread	<LOQ	171.8 ± 21.6	107.1 ± 12.7	<LOQ	92.4 ± 13.4	<LOQ	<LOQ	<LOQ	38.8i	56.8i	<LOQ	<LOQ	<LOQ	<LOQ
S14-010964	Bran Flakes	<LOQ	418.0 ± 28.3	90.1 ± 20.9	<LOQ	158.9 ± 9.2	<LOQ	<LOQ	<LOQ	246.1	73.4i	<LOQ	<LOQ	<LOQ	3.5
S14-010959	Crunchy Bran	<LOQ	305.0± 54.0	128.6 ± 22.0	15.4 ± 2.1	130.8 ± 13.7	<LOQ	<LOQ	<LOQ	155.9	<LOQ	<LOQ	<LOQ	<LOQ	3.5
S14-010956	Shreddies	<LOQ	214.9 ± 10.6	97.8 ± 32.8	<LOQ	139.8 ± 7.3	<LOQ	11.0 ± 7.2	<LOQ	64.2	63.8i	<LOQ	<LOQ	<LOQ	4
S14-010965	Fibre Cereal	<LOQ	624.1 ± 38.0	320.6 ± 48.5	<LOQ	238.8 ± 15.1	<LOQ	14.7 ± 7.7	<LOQ	374.5	86.6i	<LOQ	<LOQ	<LOQ	17.9
S14-011072	Value Corn Flakes	<LOQ	567.0 ± 23.6	432.4 ± 129.1	<LOQ	135.7 ± 14.7	<LOQ	9.3 ± 4.3	122.5 (15AcDON	314.1	121.4	<LOQ	<LOQ	<LOQ	9.7
S14-011073	Smart Price Corn Flakes	<LOQ	410.4 ± 43.8	232.1 ± 89.2	<LOQ	137.9 ± 13.8	<LOQ	<LOQ	111.1 (15AcDON	119.4	55.1	<LOQ	<LOQ	<LOQ	5.1
S14-010998	Porridge Oats	<LOQ	132.1 ± 13.4	109.3 ± 30.7	37.3 ± 9.2	<LOQ	10.6 ± 2.1	<LOQ	<LOQ	40.0	<LOQ	20.8i	<LOQ	<LOQ	<LOQ
S14-010966	Smart Price bran Flakes	<LOQ	257.8 ± 23.4	116.4 ± 22.5	<LOQ	151.2 ± 47.0	<LOQ	<LOQ	<LOQ	75.0	64.2i	<LOQ	<LOQ	<LOQ	<LOQ
S14-010939	Premium Plain Flour	<LOQ	99.5 ± 10.4	22.2 ± 3.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	66.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
S14-010949	Brown Bread Flour	<LOQ	131.6 ± 7.8	66.6 ± 18.5	<LOQ	26.8 ± 8.7	<LOQ	<LOQ	<LOQ	64.5	71.5i	<LOQ	<LOQ	<LOQ	<LOQ
S14-010955	Honey Cheerios	<LOQ	130.3 ± 30.0	<LOQ	<LOQ	52.8 ± 2.5	4.7 ± 1.6	<LOQ	<LOQ	49.3	<LOQ	<LOQ	<LOQ	<LOQ	4
S14-010946	Oxford Whole Foods	<LOQ	334.2 ± 19.5	220.0 ± 80.5	<LOQ	104.2 ± 9.7	<LOQ	<LOQ	<LOQ	267.5	96.2i	<LOQ	<LOQ	<LOQ	<LOQ

Table 16 (contd) Comparison of results from Rowett Institute and Fera for food survey samples (µg/kg)

		3Ac DON	DON	DON 3Glc	HT2	NIV	T2	ZEN	3AcDON 15AcDON	DON	DON 3Glc	HT2	NIV	T2	ZEN
		Rowett results							**Fera results**						
Sample no.	Sample	**Average result ± standard deviation (µg/kg) (n=9)**								**Result (µg/kg**					
S14-010953	Shredded Wheat Honey	<LOQ	128.9 ± 15.1	<LOQ	<LOQ	58.1 ± 5.2	<LOQ	<LOQ	<LOQ	60.2	<LOQ	<LOQ	<LOQ	<LOQ	<2.5
S14-010968	High Bran	<LOQ	655.2 ± 39.2	276.8 ± 53.0	<LOQ	279.9 ± 36.1	<LOQ	20.0 ± 18.9	<LOQ	345.0	<LOQ	<LOQ	<LOQ	<LOQ	16.4
S14-010952	Self Raising flour	<LOQ	256.9 ± 6.2	159.3 ± 58.5	<LOQ	63.7 ± 24.2	<LOQ	<LOQ	<LOQ	299.7	<LOQ	<LOQ	<LOQ	<LOQ	<2.5
S14-010961	Weetos	<LOQ	267.3 ± 20.3	163.9 ± 91.7	<LOQ	50.8 ± 21.3	<LOQ	<LOQ	<LOQ	169.9	<LOQ	<LOQ	<LOQ	<LOQ	<2.5

6.8 Comparison of results

On the whole the results of the two laboratories are in good agreement. Rowett analysis of the NRL 02 reference sample found values within the acceptable range for all analytes except HT2 where the result was higher than the expected range.

The major differences in results the survey samples were for NIV. Rowett used a sample concentration step and had a lower LOQ for nivalenol than Fera and so found more positive samples. Many of these were at or around the Fera LOQ at a level that it was possible for positive confirmation of the analyte. One major difference was for sample S14-010968 a bran sample where the Rowett found much higher levels overall (all below EU maximum levels). This is most likely to be a function of the sample heterogeneity and the smaller sample size taken by Rowett. Mycotoxins can be unevenly distributed and using a very small size can increase the chance of hotspots being picked up or missed leading to differences in results.

The agreement between the labs is reasonable as the methods are not standardised or fully formally validated by full interlaboratory method validation study. This is because it is a relatively new area of analysis and there are issues around lack of availability and sources of standards, and there are no true reference materials. The results here are acceptable given that it would be expected to find variation of up 30% for long established, thoroughly validated methods (like aflatoxins) and even up to 60% for fumonisins. Therefore, the results here are not unexpected. It will be difficult to improve this analysis in the short term until better standards, including isotopically labelled standards become widely available, and methods are validated by multi-laboratory validation study, which will give a better understanding of the true method variability.

Rowett did not include analysis of the masked ZEN and ZEL mycotoxins and masked T2 in the food sample comparison as they did not have analytical standards for these compounds when this part of the study was conducted. Fera did not receive enough standard to allow it to be shared (only 25 µg of each compound was available). Modification of the method to include the additional masked compounds was carried out when Rowett received these compounds after Sheffield supplied larger amounts, further details of this are given in Part 2.

7 Summary of Results of PART 1 – Food Survey

The results of the food survey are in agreement with those of surveys published elsewhere with DON and DON3Glc being the major contaminants of all sample categories at levels close to those reported (Crews and MacDonald, 2015). Contamination levels were much lower in cereal based infant foods than in other products. In many cases, masked forms

were found where no parent was present, or at a higher level than the parent compound, the levels found are summarised in Table 15. As more data is published it has been observed there is considerable variability in the relative proportions of DON and DON3Glc found, with reports of DON3Glc being measured in samples where no DON is detected (Crews and MacDonald, 2015). This has been reported in beer and lager so was not unexpected in these samples. For breakfast cereals higher fibre or bran samples were found to contain DON3Glc, the sample numbers and levels were low but again this is in agreement with other studies.

ZEN was the most abundant mycotoxin in the spice samples with masked forms also being present in some samples.

There was no clear correlation between the mycotoxin content and the sample type or the supplier. There was similarly no clear correlation between the mycotoxin content and the ingredients other than a possible relationship between ZEN and the bran content of breakfast cereals.

The analysis for many of the analytes in this study was difficult due the lack of any available reference materials and in particular isotopic labelled standards that can be used as internal standards to control matrix effects. It would be extremely beneficial to all the analytical community and to anyone who uses the data produced if these standards were available.

No sample in this study exceeded any maximum permitted level for any mycotoxin. The data was submitted to FSA and EFSA in July 2014 in the form of an Excel spreadsheet (using the EFSA GenericDataReporting spreadsheet). EFSA have included this UK data in the DCF and it will be used to inform scientific opinions and risk assessments.

8 PART 2 – SYNTHESIS OF MASKED MYCOTOXINS AND BIOASSAY WORK

8.1 Synthesis of masked metabolites of selected major trichothecenes

8.1.1 Introduction

In order to carry out the bioassay studies at the Rowett Institute, pure standards of the masked forms of the mycotoxins of interest were required. Only one is available commercially (DON3Glc). Therefore, another source for these compounds was sought.

A range of masked mycotoxins have been reported in literature, including zearalenone-14-sulphate, zearalenone-14-glucoside, zearalenone-16-glucoside as well as glucosides of nivalenol, T2 and HT2 toxin, and fusarenon X. Attempts were made to obtain some of these compounds from contacts and collaborators. Although IFA-Tulln kindly gifted standards of glucosides of zearalenone, and α- and β- zearalenol and zearalenone sulphate to allow the food survey to be undertaken, they did not want to supply further standards to support the hydrolysis work as they were carrying similar studies in their institute.

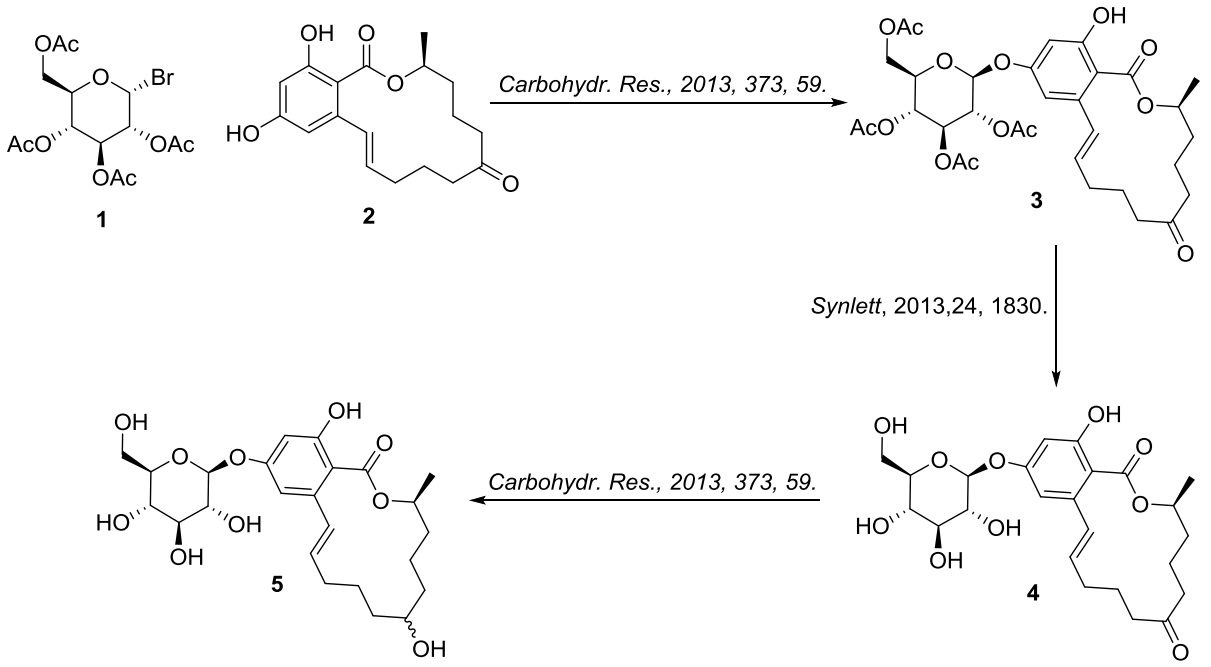
T2 toxin glucoside however was obtained as a kind gift from the Bacterial Foodborne Pathogens and Mycology Unit, USDA-ARS-NCAUR in the USA, following discussions with a colleague from there at the JIFSAN conference. It was not included in the food survey as it was received after the survey samples had been analysed.

Where it was not possible to obtain standards from contacts it was decided that chemical synthesis would be attempted. Several teams were contacted and asked if they could produce specific glucosides of zearalenone, α- and β- zearalenol, HT-2 toxin and nivalenol. All laboratories said it would be too difficult and time consuming to attempt the synthesis of nivalenol and HT-2 toxin. Dr Simon Jones and Dr Ashlie Butler at the Chemistry Department of Sheffield University conducted the synthesis of the zearalenone glucoside compounds as a subcontract.

8.1.2 Synthesis of zearalenone glucosides

Several groups have reported the use of a borate buffer to facilitate the reaction of acetobromoglucose (**1**) and ZEN (**2**) to form the ZEN-glucoside (**3**). Initially, the reaction was performed using a cheap and readily available ZEN analogue, resorcinol, but only the starting material was recovered despite several attempts to optimise the reaction conditions.

The reaction was repeated using a Lewis acid mediated approach. Once again the reactions were performed with resorcinol as a model before any ZEN was committed. Both iron(III) chloride and silver(I) oxide were tested and only the silver(I) oxide method (laid out by Thiem *et al*. *Synthesis*, 1992, 1078) was found to be successful.



The reaction using silver(I) oxide was then repeated using ZEN and the product ZEN-glucoside **3** was obtained in a low yield. The removal of the acetyl protecting groups was then performed under the conditions laid out by Weber *et al*. and the product **4** was successfully obtained and purified by preparative HPLC.

The reaction to synthesise **3** was repeated under the same conditions in order to perform the further reactions described in the scheme above. ^{1}H NMR spectroscopy suggested the product had been formed, although the product was not pure after flash column chromatography. According to the report by Mikula *et al*. the reaction sequence was performed using the crude materials from each previous reaction and so it was decided to continue with the sequence described in the scheme above. After performing the deprotection reaction conditions previously demonstrated to form the product **4**, the crude reaction mixture was subjected to the reduction conditions laid out by Mikula *et al*. in an attempt to form the mixture of isomers shown by product **5**. Purification of the reaction mixture by preparative HPLC showed that the desired products were not formed.

Once again it was required to synthesise the ZEN-glucoside **3**. As the previously used method had not produced a high yield the reaction was tried under the conditions laid out by Mikula *et al*. which, though utilising the same reagents, described the use of a different

solvent. The reaction provided a higher yield of product, however, it was not pure after flash column chromatography. The deprotection reaction to obtain **4** was performed but after attempts at purification it was determined that the product was not formed and starting material was also not recovered.

Having encountered problems following the literature protocols with impure intermediates, purification of each intermediate was conducted to ensure that no side products were interfering with the sequential reactions. Therefore, the formation of **3** under the conditions laid out by Mikula *et al*. was repeated and the product purified by both flash column chromatography and preparative HPLC to obtain **3** as a pure product.

The deprotection method laid out by Weber *et al*. was followed by analytical HPLC on **3**. After 28 h there was no conversion to the deprotected product **4**. Following this, an alternative deprotection method laid out by Morales *et al*. (Tetrahedron, 2011, 67, 7268.) was employed that afforded the product **4**. The crude reaction mixture was subjected to the reduction laid out by Mikula *et al*. and the reaction mixture was purified by preparative HPLC to afford the isolated isomers of product **5**.

Three products were produced:

Zearalenone-14-β,D-glucoside – 5mg

α-zearalenol-14-β,D-glucoside – 1.5mg

β-zearalenol-14-β,D-glucoside – 1mg

Full elemental analysis and spectroscopic data to demonstrate the products identity and purity were provided.

A report describing the experimental approach taken and the results of the identity and purity checks was produced and is included here as Appendix 2.

8.1.3 Compounds for bioassay studies

Aliquots of the compounds in acetonitrile obtained were supplied to the Rowett institute to allow the bioassay work to take place. In total 5 compounds will be assessed:

Deoxynivalenol – 3 – glucoside - (a Type B trichothecene)

T2 3-α glucoside - (a Type A trichothecene)

Zearalenone-14-β,D-glucoside –	an oestrogenic mycotoxin, chemically described as a phenolic resorcyclic acid lactone
α-zearalenol-14-β,D-glucoside –	α-zearalenol is a major metabolite of zearalenone
β-zearalenol-14-β,D-glucoside -	β-zearalenol is a major metabolite of zearalenone

Therefore, a range of modes of action / toxicity across the compounds would be evaluated in the bioassay work.

9 Assessment of hydrolysis and epithelial transport of masked mycotoxins in the human gut in vitro

9.1 Introduction

Upon ingestion of contaminated food, mycotoxins and masked mycotoxins are exposed to the varying digestive processes in the human intestinal tract but little is known about the bioavailability of modified mycotoxins. The specific questions arising are i) are masked mycotoxins hydrolysed in the small intestine; ii) do human gut microbiota hydrolyse or metabolise masked mycotoxins and iii) are masked mycotoxins and their hydrolysed parent mycotoxins transported through the intestinal epithelium to add to systemic mycotoxin exposure.

The aim of the second phase of the project was to assess the fate of important masked trichothecenes (deoxynivalenol-3-glucoside DON3Glc; T2-3-glucoside T2Glc) and masked zearalenone compounds (zearalenone-14-glucoside, ZEN14Glc; α and β zearalenol-14-glucoside, α-ZEL14Glc and β-ZEL14Glc) and their respective parent mycotoxins under gastrointestinal conditions in vitro.

The first part of this study assessed the hydrolysis of masked mycotoxins under small intestinal conditions by exposing them to artificial saliva, artificial gastric and duodenal juices including all digestive enzymes and to artificial bile juice.

The second part of the work assessed the hydrolysis of masked mycotoxins and metabolism of parent mycotoxins via microbial metabolism by human gut bacteria. Kinetic experiments were performed in faecal batch cultures to monitor microbial mycotoxin metabolism for up to 168 hours.

The third part examined the transport of masked mycotoxins through intestinal epithelial cell layers and compared them to parent compounds. Caco-2 TC7 cells were exposed to mycotoxins on the apical (luminal) side of a 2-compartment model and transport to the basolateral (systemic) chamber was monitored up to 24 hours.

For all three parts, samples were analysed for masked mycotoxins and parent mycotoxins using LC-MS/MS technology.

9.2 **LC-MS/MS analysis**

The liquid chromatography separation of the mycotoxin metabolites was performed on an Agilent 1200 HPLC system (Agilent Technologies, Wokingham, UK) with the metabolites separated on an Agilent Zorbax 5µm, 150mm × 4.6mm C18 column. Mobile phase solvents were (A) 0.1% acetic acid and (B) methanol for faecal incubation experiments. The starting gradient was 30% B rising linearly to 100% B over 15 min, held at 100% B for 2 min then returned to 30% B and re-equilibrated for 8mins. The flow rate was 400 µL/min and the injection volume was 20 µL. The LC eluent was directed into, without splitting, a Q-Trap 4000 triple quadrupole mass spectrometer (AB Sciex, Warrington, UK) fitted with a TurboV Ion Spray™ (TIS) source. The mass spec was run in both negative and positive ion mode with the following source settings: ion spray voltage -4000, temperature 200°C, Gases 1 & 2 set at 24 and 40 respectively and the Curtain Gas set at 10 in negative ion mode, for positive ions the conditions were the same except for the spray voltage which was set at 4500. A summary of the ion transition parameters used is given in Table 17. Mycotoxins were quantified using the multiple reaction monitoring (MRM) technique. Standard solutions of 500 ng/mL were pumped directly into the TIS via a syringe and their transition values optimized. Calibration curves were prepared for each metabolite by preparation of a series of standards ranging from to 2 – 2 000 pmoles/ml. No internal standard was used and the mobile phase contained acetic acid, hence the precursor ions were used as [M+Ac].

Table 17 Summary of ion transition parameters.

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	Collision Energy (V)	Polarity
DON3Glc	517.3	457.3	20	-20.5	negative
DON	355.1	265.1	20	-21.0	negative
T2Glc	646.4	305.4	20	29.0	positive
T2	489.1	387.4	20	31	positive
HT2	447.3	345.4	20	27.5	positive
ZEN14Glc	479.4	317.3	20	-19	negative
ZEN	317.2	131.1	20	-41	negative
α–ZEL14Glc	481.4	319.2	20	-16	negative
α-ZEL	319.0	160.0	20	-44.5	negative
β-ZEL14Glc	481.4	319.2	20	16	negative
β-ZEL	319.0	160.0	20	-44.5	negative

9.3 Assessment of hydrolysis of masked mycotoxins by digestive juices of the upper gut

Hydrolysis of masked trichothecenes (DON3Glc and T2Glc) and zearalenone compounds (ZEN14Glc, α-ZEL14Glc, β-ZEL14Glc) were studied by exposing them to artificial digestive juices to mimic small intestinal digestion (Dall'Erta et al. 2013). The respective parent mycotoxins (DON, T2, HT2, ZEN, α-ZEL and β-ZEL) were also assessed for their stability towards digestive juices.

Each mycotoxin or masked mycotoxin (0.2nmol) was individually incubated with saliva for 5 minutes followed by the addition of gastric juice for 2 hours and the addition of duodenal juice and bile juice for another 2 hours. Reactions were terminated by adding acetonitrile, and samples were centrifuged and supernatant directly injected into LC-MS/MS. Enzyme-free control incubations were performed simultaneously for all mycotoxins and masked mycotoxins.

9.3.1 Results - Trichothecenes

DON3Glc and T2Glc were stable during incubations with artificial digestive juices (Figure 2) and enzyme free controls (data not shown). There was no evidence of hydrolysis or release of parent mycotoxins DON or T2 toxin from their masked forms. When incubating the parent mycotoxins with artificial digestive juices, DON and HT2 toxin were also stable. T2 toxin was partly converted to HT2 toxin upon incubation with artificial digestive juices (13%) and enzyme free controls (19%), indicating spontaneous conversion rather than enzymatic hydrolysis. The results in the present study are in agreement with findings in the literature indicating that DON3Glc and T2Glc are stable during upper GI conditions *in vitro* (De Nijs 2012, Dall'Erta 2013, McCormick 2015).

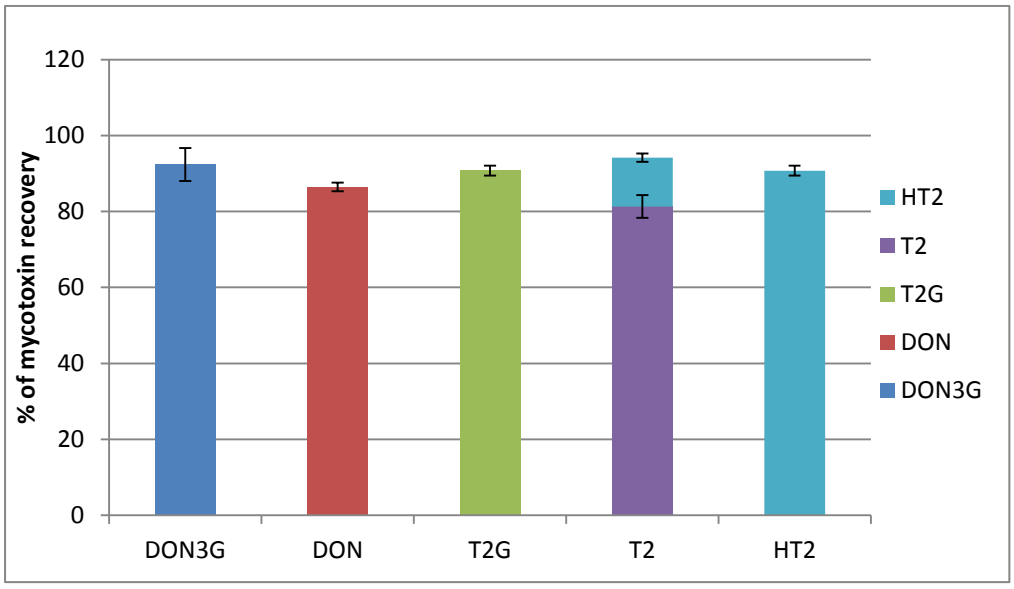


Figure 2 Hydrolysis experiments of trichothecenes (DON3Glc, DON, T2Glc, T2, HT2) by artificial digestive juices of the upper gut containing digestive enzymes, indicating no hydrolysis of the modified mycotoxin. All results are presented as average of triplicate incubations± SEM.

9.3.2 Results - zearalenone compounds

All masked zearalenone compounds (ZEN14Glc, α-ZEL14Glc, β-ZEL-Glc) and parent mycotoxins (ZEN, α-ZEL, β-ZEL) were stable during incubations with artificial digestive juices (Figure 3) and enzyme free controls (data not shown) indicating no hydrolysis in the upper GI-tract.

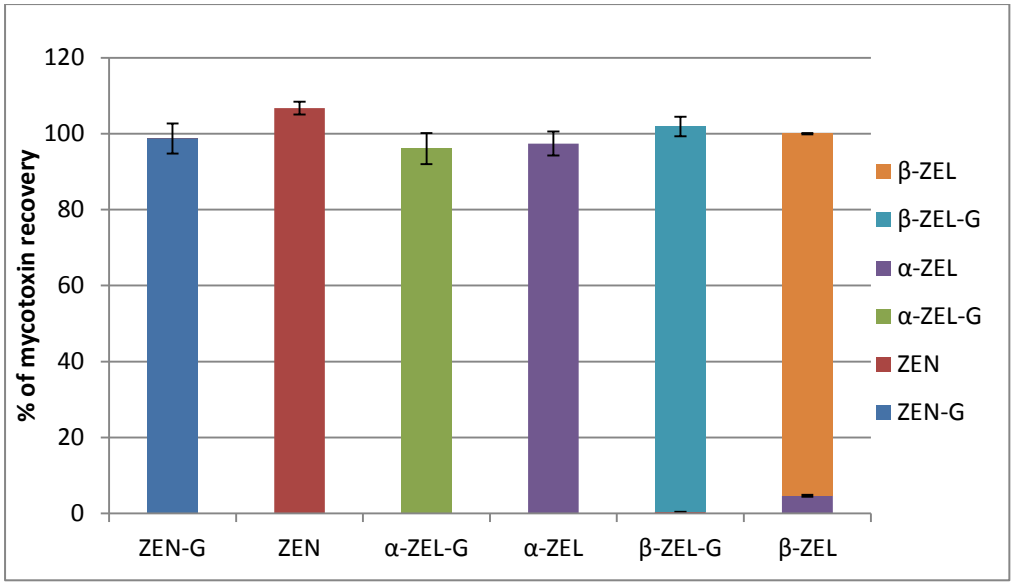


Figure 3 Hydrolysis experiments of zearalenone compounds (ZEN14Glc, ZEN, α-ZEL14Glc, α-ZEL, β-ZEL14Glc, β-ZEL) by artificial digestive juices of the upper gut containing digestive enzymes, indicating no hydrolysis. All results are presented as average of triplicate incubations± SEM.

In summary all masked mycotoxins tested were stable with no hydrolysis being observed under incubation conditions mimicking the small intestinal digestion.

9.4 Assessment of hydrolysis of masked mycotoxins by colonic microbiota

Faecal batch cultures were used to study the hydrolysis and metabolism of masked mycotoxins by the human gut microbiota (Gratz et al 2013). These experiments utilise freshly collected faecal samples handled under anaerobic conditions to mimic metabolic conditions in the human colon. Five independent experiments using faecal samples from 6 donors (3 female, male, age 27-64) were performed for each masked mycotoxin to assess intra-individual variation.

Masked trichothecene compounds (DON3Glc and T2Glc) were studied in faecal batch culture experiments. Freshly collected faecal samples from donors 1-5 were diluted 1/10th under CO_2 with anaerobic M2 culture medium. Duplicate aliquots of 1ml faecal slurry were spiked with 2 nmol of DON3Glc or T2Glc in acetonitrile and incubated anaerobically at 37°C for various periods (2, 4, 6, 24, 48, 72 hours or 7 days). Slurry was then extracted with 3mL of acetonitrile, centrifuged (2000 x g, 5 min) and supernatant was evaporated under nitrogen stream and reconstituted in 1mL water. Additional aliquots of faecal slurry were spiked with

DON, DON3Glc, T2, T2Glc or HT2 toxins in duplicates and extracted immediately (time 0) to determine the toxin extraction from faecal slurry. All samples were then cleaned through C18 columns, evaporated and reconstituted in 50% methanol for LC-MS/MS analysis. Blank faecal incubations omitting spiking with mycotoxins were included in all experiments.

Masked zearalenone compounds (ZEN14Glc, α-ZEL14Glc, β-ZEL14Glc) and their respective parent mycotoxins (ZEN, α-ZEL, β-ZEL) were studied individually in similar faecal batch culture experiments. Fresh faecal samples from donors 1-4 and 6 were diluted and anaerobic incubations set up as described above. Donor 5 dropped out of the study due to antibiotic use. Following incubation (0.5, 1, 2, 4, 6, 24, 48, 72 hours or 7 days) faecal slurry was extracted with 3 mL acetonitrile, centrifuged and clear supernatant was injected directly into LC-MS/MS omitting C18 clean-up. This minimal processing of extracts was necessary to allow further characterisation of potential novel microbial zearalenone metabolites by Fera as clean-up could have resulted in potential compounds of interest being removed.

9.4.1 Results - DON3Glc

Hydrolysis of DON3Glc was found to be a rapid process with hydrolysis (disappearance of DON3Glc and appearance of DON in culture medium) occurring between 2 – 6 hours and complete hydrolysis being observed after 48 hours for all 5 donors. Results of individual time points are given in Table 18 and are summarised in Figure 4.

Table 18 Results of DON3Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error of the mean (SEM).

		Experiment 1		**Experiment 2**		**Experiment 3**	
Toxin added	**Time**	**DON3Glc**	**DON**	**DON3Glc**	**DON**	**DON3Glc**	**DON**
DON3Glc	0	100±4	0±0	99±5	0±0	98±3	0±0
DON3Glc	2	87±1	4±0	76±1	23±1	116±2	1±2
DON3Glc	4	77±1	11±0	0	79±2	118±7	0±0
DON3Glc	6	69±1	18±1	0	87±4	100±4	0±0
DON3Glc	24	0	95±2	0	97±6	0	100±11
DON3Glc	48	0	105±10	0	114±4	0	110±7
DON3Glc	72	0	100±3	0	102±3	0	101±3
DON3Glc	168	0	100±3	0	114±1	0	120±11
DON	0	0	100±3	0	100±10	0	100±7
		Experiment 4		**Experiment 5**			
Toxin added	**Time**	**DON3Glc**	**DON**	**DON3Glc**	**DON**		
DON3Glc	0	100±2	0±0	100±2	0±0		
DON3Glc	2	84±3	8±0	84±3	8±0		
DON3Glc	4	60±1	32±1	60±1	32±1		
DON3Glc	6	0±0	80±3	0±0	80±3		
DON3Glc	24	8±6	107±4	8±6	107±4		
DON3Glc	48	0	111±1	0	111±1		
DON3Glc	72	0	126±4	0	126±4		
DON3Glc	168	0	120±2	0	120±2		
DON	0	0	100±2	0	100±2		

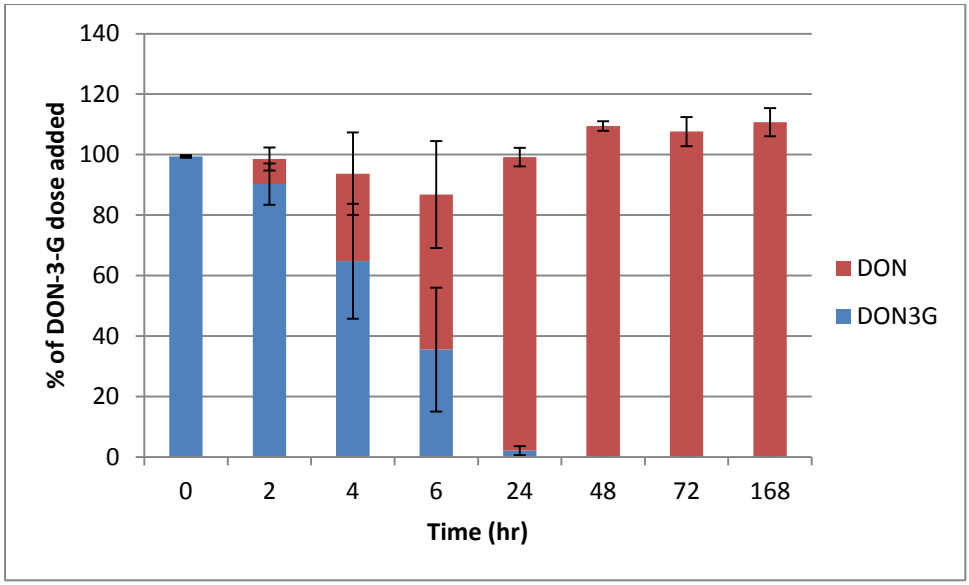


Figure 4 Results of DON3Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM.

9.4.2 Results - T2Glc

Hydrolysis of T2Glc by microbiota from 5 donors resulted in T2 toxin appearing in culture medium followed by further de-acetylation to form HT2 toxin. T2Glc hydrolysis started after 4 hours, but was only completed in 3 out of 5 cases by 72 hours. Hydrolysis to T2 toxin only reached 30-50% of the total toxin added before further metabolism to HT2 toxin was observed. This de-acetylation only reached 40% for donor 1, whereas 100% of T2Glc was transformed to HT2-toxin by faecal microbiota from donor 2 and 4 after 72 hours. Results of individual time points are given in Table 19 and are presented in Figure 5.

Table 19 Results of T2Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error or the mean (SEM).

		Experiment 1			**Experiment 2**			**Experiment 3**		
Toxin added	Time	**T2Glc**	**T2**	**HT2**	**T2Glc**	**T2**	**HT2**	**T2G**	**T2**	**HT2**
T2Glc	0	98±1	0	0	100±5	0	0	100±2	0	0
T2Glc	2	122±1	1±0	0	105±5	6±0	0	108±2	0	0
T2Glc	4	109±2	8±0	0	98±1	20±0	2±0	108±2	0	0
T2Glc	6	109±3	14±0	0	73±4	29±0	6±0	94±2	0	0
T2Glc	24	65±2	40±1	3±0	19±2	20±1	49±2	47±0	50±8	38±11
T2Glc	48	34±6	46±3	10±1	5±0	6±0	78±1	43±11	13±1	66±12
T2Glc	72	25±1	47±0	17±0	2±0	2±0	74±3	10±1	14±1	96±3
T2Glc	168	16±1	30±1	35±2	3±1	0±0	79±8	0	0±0	82±3
T2	0	0	100±2	2±0	0	100±3	3±0	0	100±4	0
HT2	0	0	0	100±5	0	0	100±7	0	0	100±3
		Experiment 4			**Experiment 5**					
Toxin added	Time	**T2Glc**	**T2**	**HT2**	**T2-Glc**	**T2**	**HT2**			
T2Glc	0	100±3	0	0	100±4	0	0			
T2Glc	2	105±1	2±0	0	105±2	2±0	0			
T2Glc	4	102±3	8±0	0	97±7	6±0	0			
T2Glc	6	91±3	16±0	1±0	86±2	10±0	0			
T2Glc	24	1±0	32±1	67±3	46±2	41±1	19±1			
T2Glc	48	2±0	5±0	89±2	24±1	35±0	50±1			
T2Glc	72	1±1	2±0	89±3	15±3	17±1	77±3			
T2Glc	168	0±0	0±0	77±2	5±0	3±0	101±3			
T2	0	2±0	100±5	1±0	2±0	100±3	0			
HT2	0	0	0	100±6	0	0	100±15			

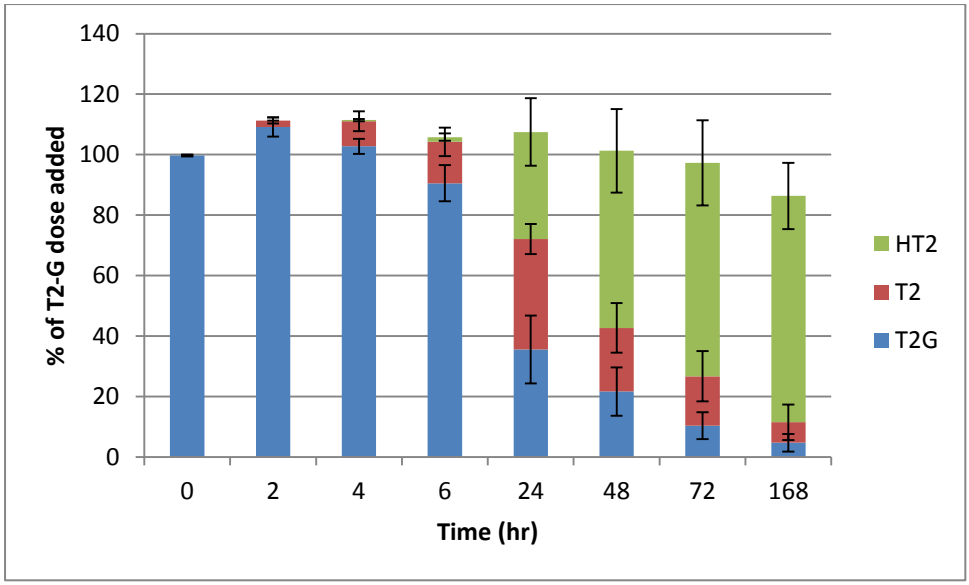


Figure 5 Results of T2Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM.

9.4.3 Results - masked zearalenone compounds

Hydrolysis of masked zearalenone compounds by faecal microbiota of 5 donors occurred more rapidly as compared to trichothecenes with cleavage of ZEN14Glc, α-ZEL14Glc and β-ZEL14Glc being detectable after 0.5 hours of incubation. Results for ZEN14Glc are presented in Table 20 and Figure 6, α-ZEL14Glc results are presented in Table 21 and Figure 7 and β-ZEL14Glc results in Table 22 and Figure 8.

Table 20 Results of ZEN14Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error or the mean (SEM).

Exp. 1	**Toxin added**	Time	**ZEN 14Glc**	**ZEN**	**α-ZEL 14Glc**	**α-ZEL**	**β -ZEL 14Glc**	**β -ZEL**
	ZEN14Glc	0	100±11	2±0	0	0	0	0
	ZEN14Glc	0.5	15±1	38±1	0	0	0	0
	ZEN14Glc	1	8±3	46±0	0	0	0	0
	ZEN14Glc	2	13±1	46±1	0	0	0	0
	ZEN14Glc	4	0±0	46±0	0	0	0	0
	ZEN14Glc	6	3±3	45±1	0	1±0	0	0
	ZEN14Glc	24	3±3	38±2	0	4±0	0	1±0
	ZEN14Glc	48	0	36±1	0	6±0	0	1±0
	ZEN14Glc	72	0	37±1	0	8±0	0	2±0
	ZEN14Glc	168	0	38±1	0	11±1	0	2±0
	ZEN*	0	ND*	ND*	ND*	ND*	ND*	ND*
Exp. 2	**ZEN14Glc**	0	100±10	1±1	0	0	0	0
	ZEN14Glc	0.5	43±0	26±0	0	0	0	0
	ZEN14Glc	1	13±1	39±0	0	0	0	0
	ZEN14Glc	2	3±1	40±3	0	0	0	0
	ZEN14Glc	4	3±2	42±1	0	0	0	0
	ZEN14Glc	6	1±0	42±1	0	0	0	1±0
	ZEN14Glc	24	0±0	41±0	0	4±0	0	3±0
	ZEN14Glc	48	1±1	35±1	0	7±1	0	4±1
	ZEN14Glc	72	0	36±2	0	8±0	0	4±0
	ZEN14Glc	168	0	36±0	0	11±1	0	8±1
	ZEN	0	0	100±2	0	0	0	0
Exp. 3	**ZEN14Glc**	0	100±0	2±0	0	1±0	0	0
	ZEN14Glc	0.5	14±3	34±0	0	11±0	0	0
	ZEN14Glc	1	2±1	40±1	0	12±0	0	0
	ZEN14Glc	2	5±2	40±1	0	12±0	0	0
	ZEN14Glc	4	3±3	41±1	0	13±0	0	0
	ZEN14Glc	6	4±2	38±1	0	12±0	0	0
	ZEN14Glc	24	1±1	39±0	0	11±0	0	2±0
	ZEN14Glc	48	1±1	37±1	0	9±0	0	8±0
	ZEN14Glc	72	0	34±3	0	7±0	0	11±0
	ZEN14Glc	168	0	39±1	0	5±0	0	14±1
	ZEN	0	0	100±1	0	2±0	0	0
Exp. 4	**ZEN14Glc**	0	100±6	3±0	0	1±0	0	0
	ZEN14Glc	0.5	12±2	34±5	0	11±3	0	0
	ZEN14Glc	1	4±1	40±2	0	14±1	0	0
	ZEN14Glc	2	2±0	39±2	0	13±0	0	0
	ZEN14Glc	4	5±1	32±0	0	11±0	0	0
	ZEN14Glc	6	0	36±1	0	12±0	0	1±1
	ZEN14Glc	24	0	33±3	0	10±2	0	6±3
	ZEN14Glc	48	0	27±0	0	8±0	0	15±0
	ZEN14Glc	72	0	28±1	0	6±0	0	19±0
	ZEN14Glc	168	0	30±0	0	4±0	0	24±1
	ZEN	0	0	100±1	0	2±0	0	0

Exp. 5	**Toxin added**	Time	**ZEN 14Glc**	**ZEN**	**α-ZEL 14Glc**	**α-ZEL**	**β -ZEL 14Glc**	**β -ZEL**
	ZEN14Glc	0	100±2	2±0	0	0	0	0
	ZEN14Glc	0.5	19±2	31±0	0	0	0	0
	ZEN14Glc	1	4±0	34±1	0	0	0	0
	ZEN14Glc	2	4±0	36±0	0	0	0	0
	ZEN14Glc	4	4±2	38±0	0	0	0	0
	ZEN14Glc	6	7±0	37±1	0	0	0	0
	ZEN14Glc	24	7±1	36±1	0	1±0	0	0
	ZEN14Glc	48	2±2	38±2	0	2±0	0	0
	ZEN14Glc	72	0	37±2	0	6±1	0	0
	ZEN14Glc	168	0	42±0	0	10±0	0	1±0
	ZEN	0	0	100±0	0	2±0	0	0

* ZEN time 0 was not determined (ND) in this experiment

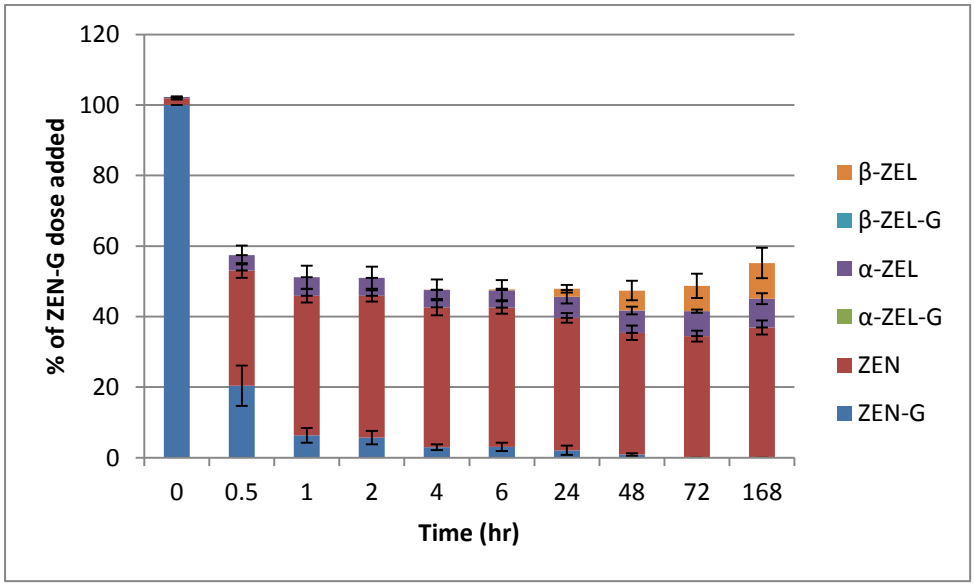


Figure 6 Results of ZEN14Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM.

Table 21 Results of α-ZEL14Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented as average of 2 replicate incubations ± SEM.

Exp. 1	**Toxin added**	Time	**ZEN-14Glc**	**ZEN**	**α-ZEL-14Glc**	**α-ZEL**	**β-ZEL-14Glc**	**β-ZEL**
	α-ZEL14Glc	0	0	0	100±2	6±0	0	0
	α-ZEL14Glc	0.5	0	0	14±2	56±0	0	0
	α-ZEL14Glc	1	0	0	3±3	64±2	0	0
	α-ZEL14Glc	2	0	1±0	4±4	70±3	0	0
	α-ZEL14Glc	4	0	2±0	0±0	68±1	0	0
	α-ZEL14Glc	6	0	1±0	2±2	64±1	0	0
	α-ZEL14Glc	24	0	2±0	4±2	58±3	0	0
	α-ZEL14Glc	48	0	4±0	2±2	55±1	0	0
	α-ZEL14Glc	72	0	4±0	0	52±1	0	0
	α-ZEL14Glc	168	0	8±0	0	49±3	0	0
	α-ZEL	0	0	0	0	100±0	0	0
Exp. 2	**α-ZEL14Glc**	0	0	0	100±0	4±1	0	0
	α-ZEL14Glc	0.5	0	0	37±1	53±2	0	0
	α-ZEL14Glc	1	0	0	10±3	82±4	0	0
	α-ZEL14Glc	2	0	1±0	8±5	79±1	0	0
	α-ZEL14Glc	4	0	1±0	0±0	85±1	0	0
	α-ZEL14Glc	6	0	1±0	1±1	89±2	0	0
	α-ZEL14Glc	24	0	1±0	2±2	82±1	0	0
	α-ZEL14Glc	48	0	2±0	0	78±0	0	0
	α-ZEL14Glc	72	0	2±0	0	78±1	0	0
	α-ZEL14Glc	168	0	3±0	0	72±2	0	0
	α-ZEL	0	0	0	0	100±2	0	0
Exp. 3	**α-ZEL14Glc**	0	0	0	100±1	4±0	0	0
	α-ZEL14Glc	0.5	0	0	11±1	54±4	0	0
	α-ZEL14Glc	1	0	0	4±1	58±0	0	0
	α-ZEL14Glc	2	0	1±0	4±4	54±6	0	0
	α-ZEL14Glc	4	0	1±0	4±1	55±2	0	0
	α-ZEL14Glc	6	0	1±0	1±1	58±0	0	0
	α-ZEL14Glc	24	0	1±0	0±0	56±1	0	0
	α-ZEL14Glc	48	0	2±0	1±1	53±3	0	0
	α-ZEL14Glc	72	0	2±0	0	50±0	0	0
	α-ZEL14Glc	168	0	5±0	0	45±3	0	0
	α-ZEL	0	0	0	0	100±0	0	0
Exp. 4	**α-ZEL14Glc**	0	0	0	100±5	6±1	0	0
	α-ZEL14Glc	0.5	0	0	11±0	66±2	0	0
	α-ZEL14Glc	1	0	0	7±2	69±0	0	0
	α-ZEL14Glc	2	0	1±0	4±4	70±4	0	0
	α-ZEL14Glc	4	0	1±0	5±2	60±2	0	0
	α-ZEL14Glc	6	0	1±0	0±0	67±3	0	0
	α-ZEL14Glc	24	0	2±0	0±0	58±2	0	0
	α-ZEL14Glc	48	0	2±0	2±0	56±2	0	0
	α-ZEL14Glc	72	0	3±0	0±0	54±1	0	0
	α-ZEL14Glc	168	0	4±0	1±1	53±1	0	0
	α-ZEL	0	0	0	0	100±6	0	0

Exp. 5	Toxin added	Time	ZEN 14Glc	ZEN	α-ZEL 14Glc	α-ZEL	β-ZEL 14Glc	β -ZEL
	α-ZEL14Glc	0	0	0	100±7	2±0	0	0
	α-ZEL14Glc	0.5	0	0	15±1	56±1	0	0
	α-ZEL14Glc	1	0	1±0	2±2	58±2	0	0
	α-ZEL14Glc	2	0	1±0	5±1	61±1	0	0
	α-ZEL14Glc	4	0	1±0	4±4	62±2	0	0
	α-ZEL14Glc	6	0	1±0	6±1	59±1	0	0
	α-ZEL14Glc	24	0	1±0	1±1	62±2	0	0
	α-ZEL14Glc	48	0	1±0	0±0	60±2	0	0
	α-ZEL14Glc	72	0	2±0	2±2	56±2	0	0
	α-ZEL14Glc	168	0	4±0	0	60±2	0	0
	α-ZEL	0	0	0	0	100±6	0	0

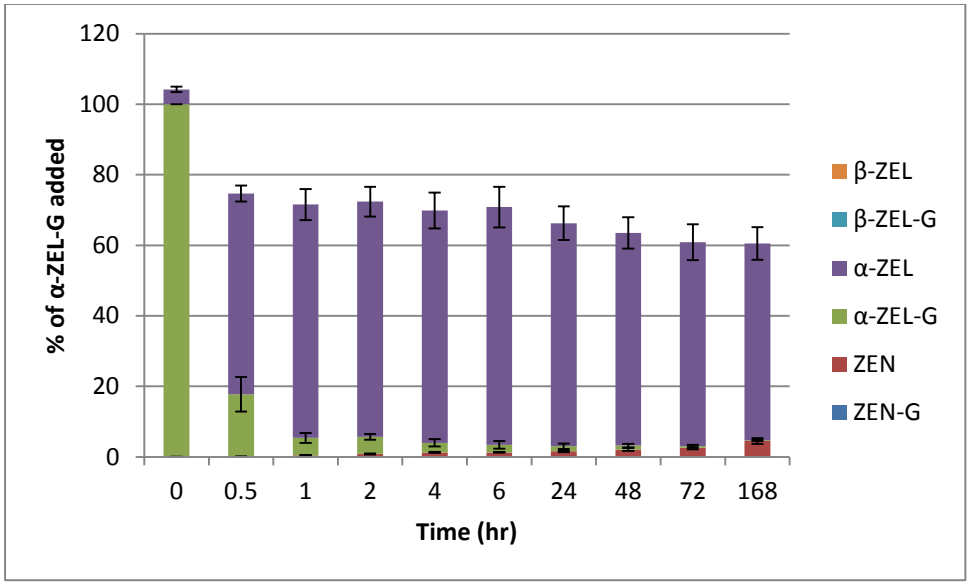


Figure 7 Results of α-ZEL14Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM.

Table 22 Results of β–ZEL14Glc incubation with faecal microbiota from 5 volunteers (experiments 1-5) during 0 – 168 hours. Results are presented % of the mycotoxin dose added and calculated as average of 2 replicate incubations ± standard error or the mean (SEM).

Exp. 1	Toxin added	Time	ZEN 14Glc	ZEN	α-ZEL 14Glc	α-ZEL	β-ZEL 14Glc	β-ZEL
	β-ZEL14Glc	0	0	0	0	0	100±6	1±0
	β-ZEL14Glc	0.5	0	0	0	0	31±2	24±0
	β-ZEL14Glc	1	0	0	0	0	8±2	37±0
	β-ZEL14Glc	2	0	0	0	0	0	40±0
	β-ZEL14Glc	4	0	1±0	0	0	0	39±0
	β-ZEL14Glc	6	0	1±0	0	0	0	36±0
	β-ZEL14Glc	24	0	3±0	0	0	0	26±1
	β-ZEL14Glc	48	0	5±0	0	1±0	0	22±0
	β-ZEL14Glc	72	0	5±0	0	1±0	0	22±0
	β-ZEL14Glc	168	0	7±0	0	1±0	0	22±1
	β-ZEL	0	0	0	0	6±2	0	100±3
Exp. 2	**β-ZEL14Glc**	0	0	0	0	0	100±1	2±0
	β-ZEL14Glc	0.5	0	0	0	0	69±4	19±1
	β-ZEL14Glc	1	0	0	0	0	26±2	33±1
	β-ZEL14Glc	2	0	0	0	0	7±1	36±1
	β-ZEL14Glc	4	0	1±0	0	0	6±1	41±2
	β-ZEL14Glc	6	0	1±0	1±0	0	0±0	44±2
	β-ZEL14Glc	24	0	2±0	0	0	1±1	30±1
	β-ZEL14Glc	48	0	4±0	0	0	0	29±0
	β-ZEL14Glc	72	0	5±0	0	0	0	29±1
	β-ZEL14Glc	168	0	7±0	0	1±0	0	28±2
	β-ZEL	0	0	0	0	7±0	0	100±1
Exp. 3	**β-ZEL14Glc**	0	0	0	0	0	100±2	1±0
	β-ZEL14Glc	0.5	0	0	0	1±0	39±1	23±1
	β-ZEL14Glc	1	0	0	0	1±0	9±0	33±0
	β-ZEL14Glc	2	0	0	0	1±0	2±2	35±2
	β-ZEL14Glc	4	0	0	0	1±0	6±2	38±0
	β-ZEL14Glc	6	0	1±0	1±0	1±0	0±0	41±1
	β-ZEL14Glc	24	0	1±0	0	1±0	2±2	36±2
	β-ZEL14Glc	48	0	1±0	0	1±0	1±1	34±1
	β-ZEL14Glc	72	0	1±0	0	0	0	34±0
	β-ZEL14Glc	168	0	1±0	0	0	0	28±1
	β-ZEL	0	0	0	0	7±0	0	100±3
Exp. 4	**β-ZEL14Glc**	0	0	0	0	0	100±2	2±0
	β-ZEL14Glc	0.5	0	0	0	1±0	32±5	32±0
	β-ZEL14Glc	1	0	0	0	1±0	13±4	39±2
	β-ZEL14Glc	2	0	0	0	1±0	0	45±3
	β-ZEL14Glc	4	0	0	0	1±0	0	41±2
	β-ZEL14Glc	6	0	1±0	0	1±0	0	42±1
	β-ZEL14Glc	24	0	1±0	0	1±0	0	37±1
	β-ZEL14Glc	48	0	1±0	0	1±0	0	38±0
	β-ZEL14Glc	72	0	1±0	0	1±0	0	31±1
	β-ZEL14Glc	168	0	2±0	0	1±0	0	28±0
	β-ZEL	0	0	0	0	6±0	0	100±1

Exp. 5	Toxin added	Time	ZEN 14Glc	ZEN	α-ZEL 14Glc	α-ZEL	β-ZEL 14Glc	β-ZEL
	β-ZEL14Glc	0	0	0	2±0	0	100±12	1±0
	β-ZEL14Glc	0.5	0	0	0	0	50±1	18±1
	β-ZEL14Glc	1	0	0	0	0	15±1	26±0
	β-ZEL14Glc	2	0	0	0	0	3±3	29±6
	β-ZEL14Glc	4	0	0	0	0	4±4	30±0
	β-ZEL14Glc	6	0	0	0	1±0	0±0	28±0
	β-ZEL14Glc	24	0	1±0	0	1±0	4±4	30±0
	β-ZEL14Glc	48	0	1±0	0	1±0	0	30±1
	β-ZEL14Glc	72	0	1±0	0	1±0	0	29±1
	β-ZEL14Glc	168	0	1±0	0	1±0	0	30±1
	β-ZEL	0	0	0	0	8±0	0	100±3

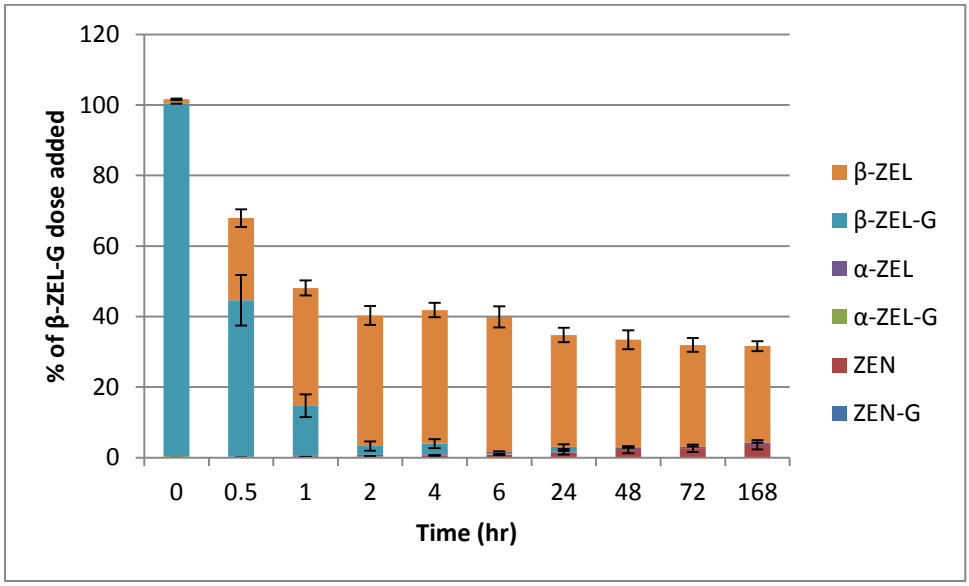


Figure 8 Results of β-ZEL14Glc incubation with faecal microbiota from 5 volunteers during 0 – 168 hours. Results are presented as average of 5 independent experiments ± SEM.

9.5 Discussion of faecal incubation studies

Microbial hydrolysis of DON3Glc peaked at 4 – 6 hours of incubation for all 5 donors tested and was complete after 24 hours. 100% of the DON3Glc dose was recovered as free DON with no further metabolism.

Hydrolysis of T2Glc was slower compared to DON3Glc, starting after 4 hours and continuing up to 168 hours of incubation. T2 only reached 30-50% of the total toxin added before further metabolism to HT2 toxin was observed.

Hydrolysis of ZEN14Glc was very rapid starting at 0.5 hours of incubation and over 97% of all three masked compounds disappearing after 4 hours. However, only 40% of the dose was recovered as free ZEN and small amounts of α- and β-ZEL (up to 10% each) leaving up to 50% of ZEN14Glc to be metabolised to unidentified metabolites. This is in agreement with the findings of Dall Erta et al (2013) who reported an apparent loss of 40-60% of ZEN after similar experiments.

Similarly, hydrolysis of α-ZEL14Glc and β-ZEL14Glc was started at 0.5 hours (only 18 and 44% of original dose of α- and β-ZEL recovered) and was almost complete after 4 hours. Only 60 or 30% of the dose were recovered as free α- and β-ZEL respectively and small amounts of ZEN (up to 5%) leaving up to 40 or 70% of the dose to be unidentified. Dall Erta et al (2013) reported approximately 10 other unidentified catabolic products from ZEN14Glc breakdown were found. It is not known if the 'lost' compounds were bound or adsorbed to the cell material as this was separated from the extracts before analysis.

The identity of the undetected metabolites of ZEN, α-ZEL and β-ZEL and their respective glucosides was investigated and is reported in the following section.

10 Characterisation of novel microbial metabolites of masked mycotoxins (Fera)

10.1 Preliminary work

Blank faecal extracts from different volunteers were supplied by Rowett to Fera. Extracts were supplied both in a crude form and also following clean-up by C18 SPE.

Samples and standards of most of the mycotoxins of interest, including glucosides were analysed by LC-TOF-MS to establish suitable instrumental analytical conditions. Initial results showed differences between all volunteers studied.

There were distinct differences in the chemical constituents from each volunteer. This makes sense and differences in response to other mycotoxins have been observed between volunteers in previous studies. Therefore, blank or background extracts for all volunteers from the study were prepared at the next stage of the project.

In addition, from the initial set-up analyses, there was an indication that one volunteer may have a natural background exposure to ZEN in the sample tested.

10.2 Experimental

The extracts from the ZEN, α-ZEL and β-ZEL experiments from Section 9.4 were received at Fera and stored in a fridge until analysis. In total approximately 340 sample vials were received. Blank faecal extracts were not supplied for 2 volunteers', but replacements were supplied later. For each volunteer and analyte 'pooled' extracts were prepared by transferring 50µl from each vial in the sample series (20 in total for each analyte/volunteer) and combining them in an LC autosampler vial.

The extracts were analysed by LC-HRMS using a LC-Exactive (Thermo Scientific, Waltham Massachusetts) consisting of an Accela U-HPLC and an Exactive mass spectrometer. Chromatographic separation was achieved on a Raptor biphenyl 100 x 2.1 mm, 2.7 µm column (Restek, Bellefonte Pennsylvania) kept at 40^{o}C. The mobile phase consisted of 1 mM ammonium formate in water (channel A) and 50:50 acetonitrile: methanol (channel D). The mobile phase gradient was a starting mixture of 98% A and 2% B that changed to 98% B over 20 minutes. This was held for 5 minutes before returning to the original mobile phase

composition. The column was then equilibrated for 6 minutes prior to the next injection. The flow rate was 0.3 mL/min with an injection volume of 3 µL.

TOF-MS detection was carried out in positive and negative mode electrospray with sheath gas 60 and aux 10 L/min, Spray voltage 4200V (positive ionisation) or 3500 V (negative ionisation), capillary temperature 375 ^{o}C, capillary voltage 65 V (positive ionisation) or -60 V (negative ionisation), tube lens 120 V (positive ionisation) or -110 V (negative ionisation), and skimmer 22 V (positive ionisation) or -22 V (negative ionisation). The mass range measured was 50 – 1000 m/z. The Exactive MS data produced was processed using Thermo Xcalibur software.

16 batches of extracts were run from 20/5/15 to 15/06/15.

10.3 **Data interpretation**

Some recent literature was reviewed to identify possible target compounds to search for during the analysis of the samples at Fera. Without carrying out a comprehensive literature search, several publications were found that identified products produced as a result of biotransformation of ZEN by mould (Kakeya et al 2002) and yeast (Vekiru et al 2010). The latter authors were investigating the potential of *Trichosporon mycotoxinivorans* to be used as a feed additive to reduce toxicity of ZEN *in vivo*.

As a result of searching the literature several possible breakdown products were identified (Hahn et al, 2015; Kakeya et al. 2002; Vekiru et al 2010 and Dall'Erta et al, 2013), and these as well as known metabolites and sulphate derivatives were searched for during data processing, as even without standards it is possible to search for compounds of interest of known accurate mass.

Table 23 summarises the compounds for which chromatograms were extracted from the data. These included glucosides and sulphates of ZEN and α and β-ZEL, as well as free zearalanol, zearalenol, zearalanone (ZAN), hydrolysed zearalenone (HZEN), decarboxylated hydrolysed zearalenone (DHZEN) and (5S)-5-({2,4-dihydroxy-6-[(1E)-5-hydroxypent-1-en-1-yl]benzoyl}oxy)hexanoic acid (ZOM-1). The peak area responses for the compounds identified are given in Table 24. For all of the ZEN14Glc incubations it was apparent that ZEN14Glc and parent ZEN were present at T0, ZEN14Glc was seen in the chromatograms

for ZEN14Glc, but also in the ZEN chromatograms. This effect is observed because although the extract contained ZEN14Glc, when this compound enters the mass spectrometer source the glucose conjugate is cleaved from the ZEN. As a result the mass that is detected is that of parent ZEN. However, the key difference that determines the true identity of the ZEN observed is the chromatographic retention time. ZEN and ZEN14Glc have different chemistry and therefore interact with the HPLC column differently, meaning they have different retention times. So although the m/z response is the same the retention time is different allowing the individual compounds to be identified. This effect was observed during the initial method set-up work when the individual standard compounds were analysed. It is not clear where the parent ZEN present at T0 comes from, it may be a minor component of the ZEN14Glc standard, but it is also possible that the volunteers had been exposed to ZEN in their normal diet as ZEN was observed in 'blank' faecal culture during the initial method set up.

At 30 minutes (T0.5) the majority of ZEN14Glc had been hydrolysed to ZEN. For 3 volunteers (Vol 2, 4 and 7) hydrolysis was complete by 1 hour (T1) with no glucoside detected at this time. For the other 2 volunteers only a small proportion (<5%) of the glucoside remained at T1, and none was detected in any volunteer at T6. Peaks were observed in extracted ion chromatograms with masses for zearalanol, zearalenol, zearalanone (ZAN), hydrolysed zearalenone (HZEN) and decarboxylated hydrolysed zearalenone (DHZEN) although peaks were observed at different retention times in some volunteers. However based on the peak areas of these responses it is unlikely the amount of these compounds present accounts for all of the apparent loss of zearalenone measured by LC-MS/MS in Section 9.4.3. Figure 9 shows the extracted ion chromatogram for Volunteer 1 for ZEN14G at T0. The peak for ZEN14G can be clearly seen in the trace for [M-H]- 480.20010, with smaller peaks present in the trace for [M-H]- 317.13943, these correspond to ZEN14Glc and ZEN. The chromatograms shown in Figure 10 are for the same volunteer after 24 hours incubation. It can be clearly seen the ZEN14Glc peak has completely disappeared and the ZEN peak is visible. In addition peaks are present in other traces showing the potential presence α/β-ZALs, α/β-ZELs and HZEN.

For α/β-ZEL14Glcs, in both cases the glucoside was not observed in the trace for the [M-H]- 482.21570, but was seen in the trace for the parent form. This is due to the cleavage of the glucoside in the MS. For nearly all the subjects, for both α/β-ZEL14Glc, by T6 ZEN was detected, and this increased up to T24. This is perhaps an unexpected observation as ZELs

are usually metabolic products of ZEN, but in these cases the transformation seems to have been reversed.

Further data processing using a Biotransformation software package (Compound Discoverer) that calculates probable breakdown products based on the structure of a starting compound was carried out in an attempt to identify further novel metabolites. The starting compounds ZEN14Glc and α/β-ZEL14Glcs were used to predict probable metabolites and breakdown products. Also further processing to look for other postulated products from literature will be undertaken for the samples already reviewed. Dall'Erta et al (2013) reported the presence of up to 60% unknown catabolic products from similar experiments and stated they would do further work to try to identify them, although at the time of this report this work had not been published so no further information was available to inform these investigations.

10.3.1 Novel microbial metabolites of masked mycotoxins:

Work to establish LC-TOF-MS conditions to be used to look for new metabolites in faecal extract samples was carried out. Over 300 samples from the faecal incubation experiments were analysed by HRMS. Differences were observed between samples from different volunteers, with some possible metabolites identified. Results also confirmed the almost immediate hydrolysis of ZEN and ZEL glucosides after as little 30 minutes of incubation time.

Peaks were observed in extracted ion chromatograms with masses for ZAL, ZEL, ZAN, HZEN and DHZEN although peaks were observed at different retention times in some volunteers. However, based on the peak areas of these responses it is unlikely the amount of these compounds present accounts for all of the apparent loss of zearalenone measured by LC-MS/MS in Section 9.4.3.

Further data analysis using a software programme 'Compound Discoverer' was also undertaken. This software can predict metabolites or possible products following known metabolic pathways. Using this software to assess the data from Volunteer 3 a large number of products were found. Some putative identifications were assigned, but at best these results are for information only. Without standards it is not possible to conclusively identify any of the possible metabolites seen.

Table 23 Compounds screened for using HRMS (with formula, accurate mass [M-H]-, and retention time where known)

Compound	Formula	[M-H]-	RT
Zearalenone-glucoside (ZEN14Glc)	C24H33O10	480.20010	11.4
Zearalenone (ZEN)	C18H22O5	317.13943	14.2
α/β-Zearalenol-glucoside (α/β-ZEL14Glc)	C24H35O10	482.21570	β 9.3 / α 10.3
α/β-Zearalenol (α/β-ZEL) and Zearalanone (ZAN)	C18H24O5	319.15508	β 11.9 / α 12.9 / ZAN 14.1
α/β-Zearalanol (α/β-ZAL)	C18H26O5	321.17073	ZALs elute shortly before ZELs
Zearalenone-4-sulphate (ZEN14Sulf)	C18H22O8S	397.09625	
α/β-Zearalenol-4-sulphate (α/β-ZEL14Sulf) and Zearalanone-14-sulphate (ZAN14Sulf)	C18H24O8S	399.11190	
α/β-Zearalanol-4-sulphate (α/β-ZAL-4S)	C18H26O8S	401.12760	
Hydrolysed zearalenone (HZEN)	C18H24O6	335.15000	
Decarboxylated hydrolysed zearalenone (DHZEN)	C17H24O4	291.16020	
ZOM-1	C18H24O7	351.14490	

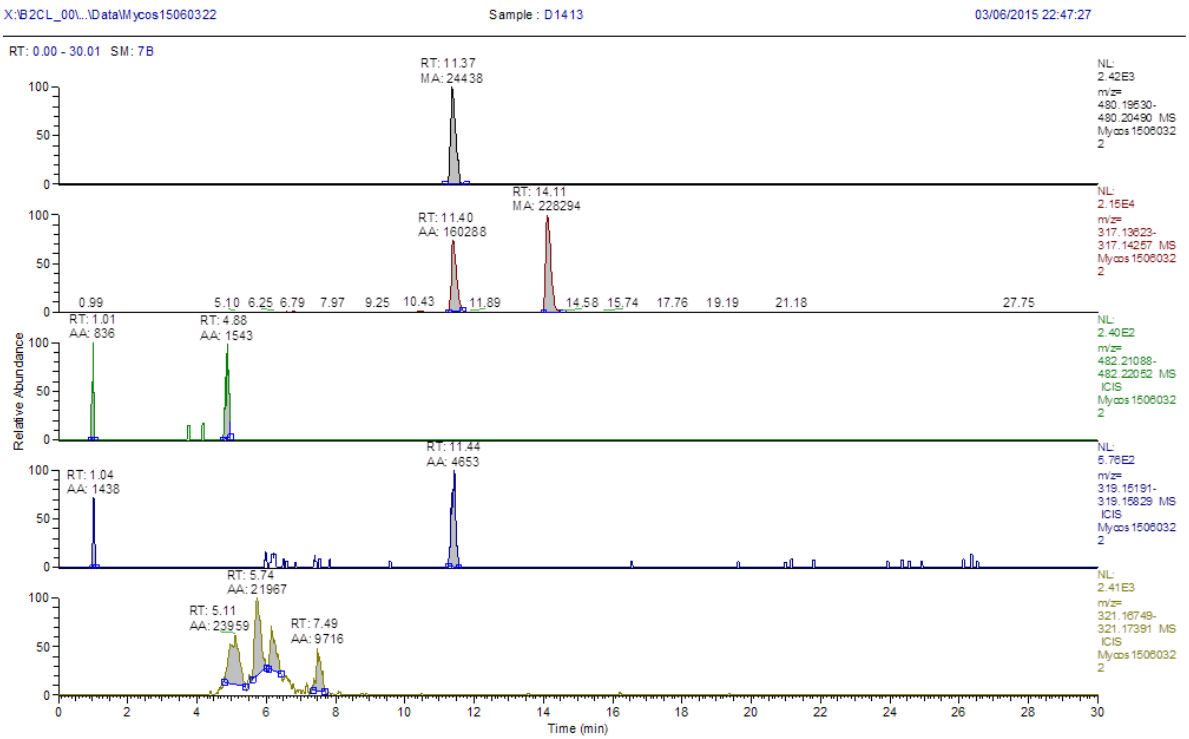
Table 24 Peak Area responses for ZEN14Glc faecal incubation experiments from HRMS analysis

Vol 1	ZEN 14Glc	ZEN	αZEL 14Glc	βZEL 14Glc	αZEL	βZEL	ZAN	αZAL / βZAL	ZEN14 Sulf	αZELSulf / βZELSulf / ZANSulf	HZEN	DHZEN	ZOM-1
T0	24438 + 160288	228924				4653							
T0.5	1714 + 20063	794505						6623 4462 / 26556				8474	
T1	5642	958662						7937 / 22640				7567	
T6		927036			4156	487	4549	14636 / 32087				5393	
T24		652284			27472	9974	1651	5834 / 35282				17563	
Vol 2													
T0	20979 + 178400											14514	
T0.5	2456	10056004					2489	47314				11205 + 4165	
T1		808922					4385	9419				6013 + 1269	
T6		786350			2480		3883	22972				2015 + 631	
T24		771653			22235		2810	22514 / 1454				4651 + 1811	
Vol 4	**ZEN 14Glc**	**ZEN**	**αZEL 14Glc**	**βZEL 14Glc**	**αZEL**	**βZEL**	**ZAN**	**αZAL / βZAL**	**ZEN14 Sulf**	**αZELSulf / βZELSulf / ZANSulf**	**HZEN**	**DHZEN**	**ZOM-1**
T0	19094 + 359835	34182	6527					11089					
T0.5	46025	711086						10299			3966		
T1		847214		7542				14500				12880	
T6		971085					5579	15699					
T24		716955						12583					

Vol 5	ZEN 14Glc	ZEN	αZEL 14Glc	βZEL 14Glc	αZEL	βZEL	ZAN	αZAL / βZAL	ZEN14 Sulf	αZELSulf / βZELSulf / ZANSulf	HZEN	DHZEN	ZOM-1
T0	33523 + 259054	91591										9511	
T0.5	11737 + 103903	541764				2530	8464					9948	
T1	2287	959951					4506					14503	
T6		658062					4213	2687			387	10059	
T24		1045409			31644	peak	2055				1559	5783 + 1849	
Vol 7	**ZEN 14Glc**	**ZEN**	**αZEL 14Glc**	**βZEL 14Glc**	**αZEL**	**βZEL**	**ZAN**	**αZAL / βZAL**	**ZEN14 Sulf**	**αZELSulf / βZELSulf / ZANSulf**	**HZEN**	**DHZEN**	**ZOM-1**
T0	29933 + 217846	37965		3943				14611 + 7258				4274	
T0.5	1975 + 26202	762450					3334+8468	9477				4327 + 1049 + 550	
T1		968927					7275	7881 + 4207				1651 + 1267	
T6		922836					4193	9165				2942	
T24		910518			4998		3174	16342				3133	

Figure 9 T0 Chromatograms ZEN14Glc Volunteer 1

Peaks from top to bottom: 1. ZEN14Glc, 2. ZEN, 3. α/βZEL14Glc, 4. α/βZEL and ZAN, 5. α/βZAL



Peaks from top to bottom: 1. ZEN-4-Sulf, 2. α/βZEL-Sulf and ZAN-Sulf, 3. α/βZAL-Sulf, 4. H-ZEN, 5. DHZEN, 6. ZOM-1

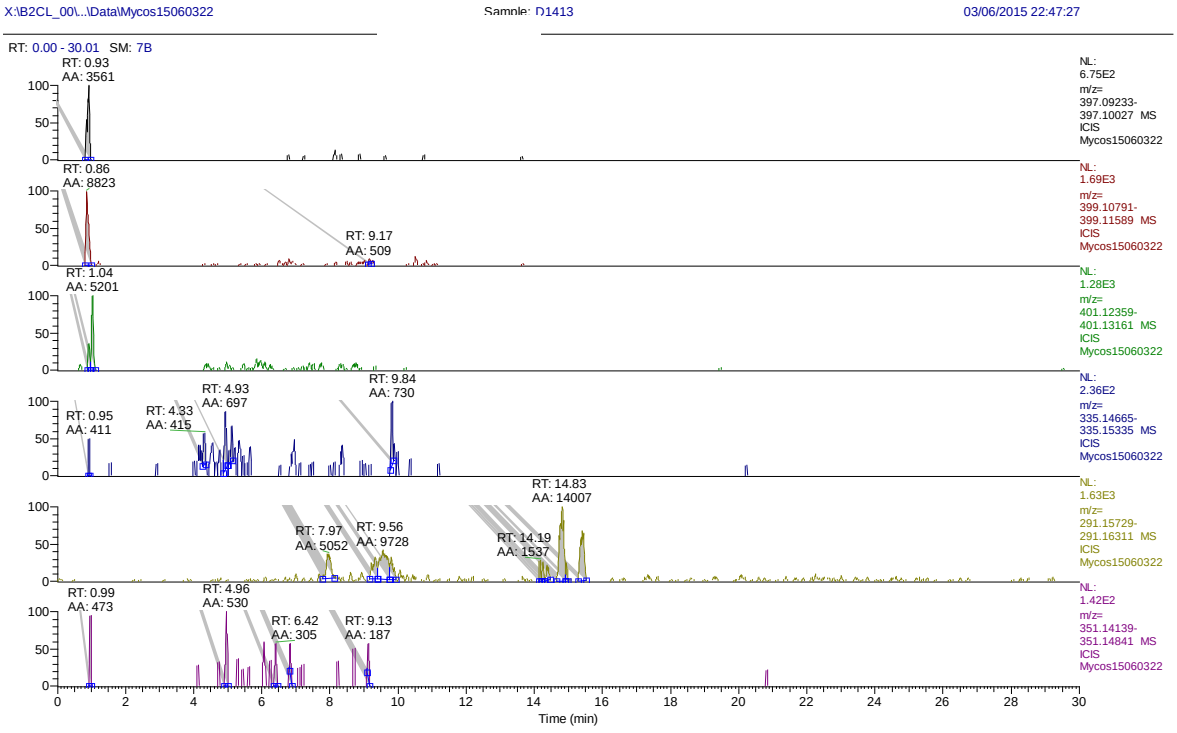
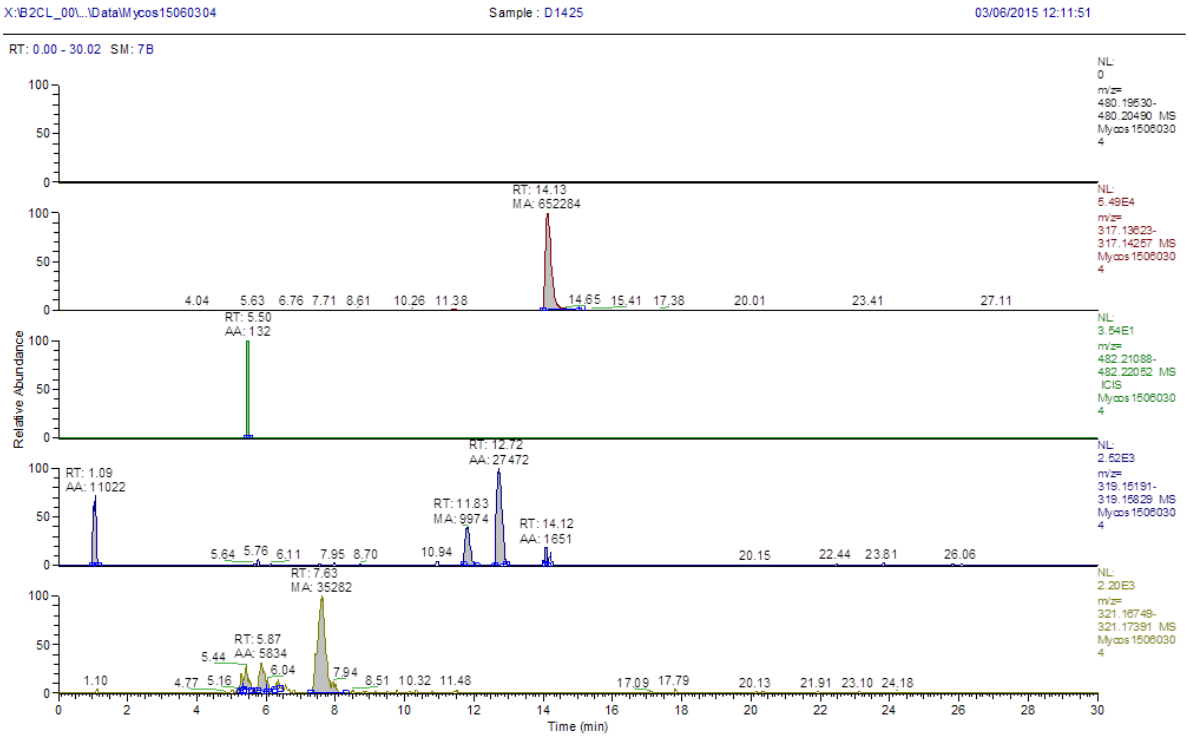
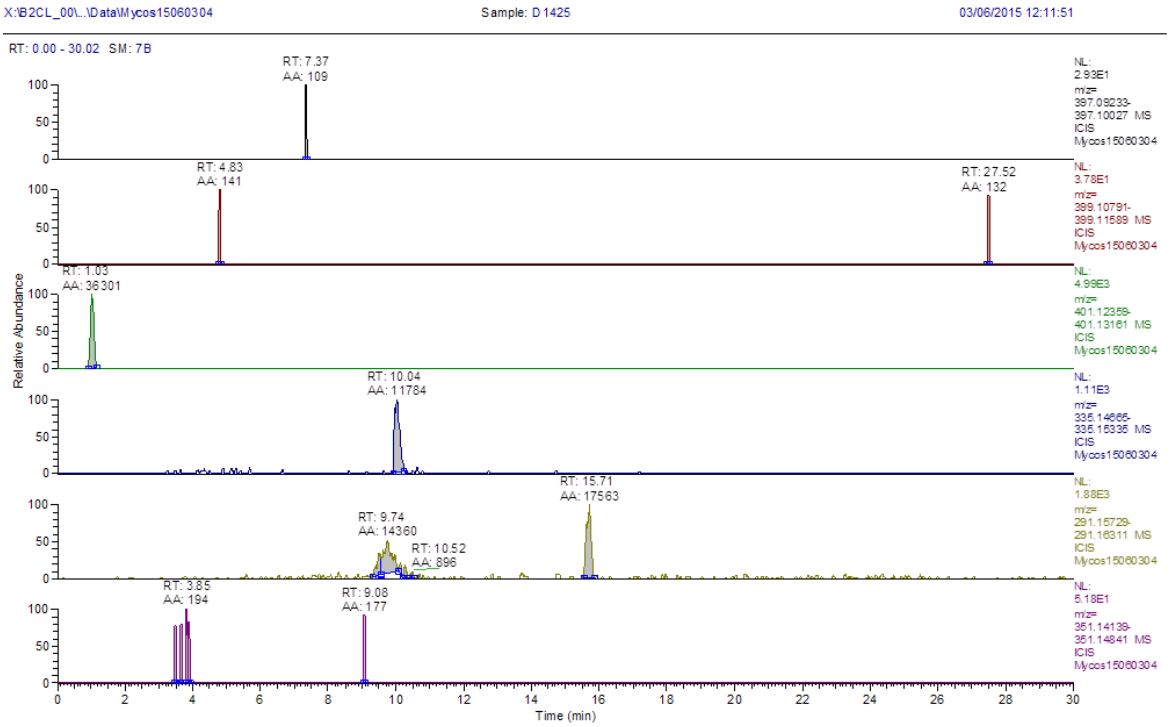


Figure 10 T24 Chromatograms ZEN14Glc Volunteer 1

Peaks from top to bottom: 1. ZEN14Glc, 2. ZEN, 3. α/βZEL14Glc, 4. α/βZEL and ZAN, 5. α/βZAL



Peaks from top to bottom: 1. ZEN14SulfS, 2. α/βZEL14Sulf and ZAN-Sulf, 3. α/βZAL-Sulf, 4. H-ZEN, 5. DHZEN, 6. ZOM-1



11 Masked mycotoxins metabolite search protocol using Compound Discoverer

11.1 Data sets

Because time and resource constraints meant that it would not be possible to investigate in detail all of the large volume of data available, it was decided to focus on data from a single experiment, Volunteer 3.

11.2 Data mining

Workflows (positive and negative) specific to the detection of metabolites of four compounds (ZEN α/ß-ZEL, ZEN14Glc and α/ß-ZEL14Glc were constructed and used to analyse data obtained from the supplied samples. The metabolites investigated are listed in Table 25.

Table 25 Metabolites included in Compound Discoverer workflows.

Phase I	Phase II
Dehydration (-H2O)	Acetylation (-H, +C2H3O)
Desaturation (-H2)	Arginine conjugation (-OH,+C6H13N4O2)
Hydration (+H2O)	Glucoside conjugation (-H,+C6H11O5)
Oxidation (+O)	Glucuronide conjugation (-H, +C6H9O6)
Oxidative deamination to alcohol (-NH2, +OH)	Glutamine conjugation (-OH, +C5H9H2O3)
Oxidative deamination to ketone (-NH3, +O)	Glycine conjugation (-OH, +C2H4NO2)
Reduction (+H2)	GSH conjugation 1 (+C10H15N3O6S)
	GSH conjugation 2 (+C10H17N3O6S)
	Methylation (-H, +CH3)
	Ornithine conjugation (-OH, +C5H11N2O2)
	Palmitoyl conjugation (-H, +C16H31O)
	Stearyl conjugation (-H, +C18H35O)
	Sulfation (-H, +HO3S)
	Taurine conjugation (-OH, +C2H6NO3S)

Transformations are additive, to maximum of 4 steps (maximum of 2 phase II transformation). This means that combinations of transformations are searched for within the software, up to a maximum 4 (and a maximum of 2 phase II transformation). Ionization was set to $[M-H]^-$ (negative mode) and $[M+H]^+ + [M+Na]^+ + [M+NH_4]^+$ (positive mode).

An 'unknowns detection' step was also included with a peak threshold set at 5000. Inclusion of this step means that following deconvolution of the data, peaks which cannot be assigned to metabolites as above are listed and could be investigated manually.

Data was analysed in 3 sets corresponding to the analyte added. As part of the workflow, data was aligned against the control sample.

Data was filtered to features that had a maximum area >5000, were not found in the control, but were found in both replicates at any time-point. The feature list produced by this means was then used to reverse search the control data file using a +/- 10 ppm window to eliminate spurious results.

Using this method for negative mode 22 results were found for ZEN, of these one possible transformation production that was putatively identified as Parent compound (or stereoisomer or ZEN reduction product) was observed in all sample extracts analysed. For α-ZEL over 20 products were found, and for β-ZEL 11 major products were observed. In both cases the only possible identification was of Parent compound (or stereoisomer or ZEN reduction) as for ZEN. The table of the peaks identified and their masses is given in Appendix 3. The process does not allow for quantification of the products found, their relative amounts can be assessed by direct comparison of their peak areas.

For positive mode many more products were observed. For ZEN over 80 products were observed at different time points. It should be noted however that these time points were mostly for 48, 72 hours and 7 days, so may not be relevant for identifying compounds formed in-vivo as this is much longer than normal gut transit time. A peak with m/z 534.29257 was observed in time points 6, 24, 48, 72 hours and seven days had several possible products. These were $[M+H]^+$ products: ZEL - arginine conjugation, glycine conjugation (+C8H15N5O2) or ZEN - reduction - arginine conjugation, glycine conjugation (+C8H17N5O2). Other possible identifications could be $[M+NH4]^+$ products ZEL14Glc - hydration, reduction, methylation (+CH6O); ZEL - hydration, reduction glucoside conjugation,

methylation (+C7H16O6); ZEL - desaturation, acetylation, arginine conjugation (+C8H12N4O2); ZEN - acetylation, arginine conjugation (+C8H14N4O2). These same products were also seen in the data for β-ZEL but not for α-ZEL. Another product at m/z 523.25475 was observed for β-ZEL, this had several possible identities as either $[M+Na]^+$ (ZEL - dehydration, acetylation, arginine conjugation (+C8H12N4O), ZEN - dehydration, reduction, acetylation, arginine conjugation (+C8H14N4O), $[M+H]^+$ ZEL14Glc - dehydration, reduction, acetylation, methylation (+C3H4) or $[M+NH4]^+$ ZEL - dehydration, dehydration, ornithine conjugation, taurine conjugation (+C7H11N3OS). However, the peak area response of this peak was very small so it would be a minor product. Over 80 products were seen for α-ZEL and over 100 were seen for β-ZEL. A full list of the products and some putative identification are given in Appendix 4, where the varying peak areas can also be seen. Although many compounds were seen it was not possible to positively identify any products, however it can be seen that the compounds are present in different amounts by comparing peak area responses.

Further data analysis could be conducted of the saved data to determine if all volunteers' extracts produced similar products. This would be very time consuming and is not possible within the budget and time constraints of the project, but would provide more information about similarities or differences between the way the faecal cultures from different volunteers metabolise the mycotoxins.

Further metabolomic studies could be undertaken that would allow more information to be determined about the changes that have taken place. Typically metabolomic studies of this kind require a specific study design, usually involving large numbers of replicate experiments (min n=6 for all samples) to allow differences between control and test samples to be determined.

In conclusion the Data mining exercise was useful as it showed a wide range of additional compounds that could be related to ZEN metabolism were present in the extracts. A larger number of possible metabolites were observed from the analysis carried out using negative ionisation mode, while the only compounds found from positive mode analysis seemed to be parent compounds or stereoisomers of these. Many more potential metabolites were observed for negative mode analysis, but concentrations were low and it was not possible to

unequivocally identify them. Further data mining could be carried out on the data that has already been accumulated for the other volunteers, although the results here may indicate the concentrations used are perhaps too low to provide informative results. It may be more beneficial to carry out further incubation experiments at higher concentrations, and within experiments designed for metabolic profiling, to further exploit and gain maximum benefit from the potential of this current work.

12 Assessment of gut transport of masked mycotoxins and parent mycotoxins

12.1 Epithelial transport study using human intestinal cell culture

Gut epithelial transport of masked and unmasked trichothecenes (DON, DON3Glc, T2, T2-Glc) and ZEN compounds (ZEN, ZEN14Glc, α-ZEL, α-ZEL14Glc, β-ZEL, β-ZEL14Glc) was studied in vitro using the human intestinal epithelial cell line Caco-2 clone TC7. Cell cultures were maintained in complete Dulbecco's Modified Essential Medium (Lonza, Biowhittaker®, Belgium) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, UK) and 1% penicillin/streptomycin (Sigma-Aldrich, UK) under 10% CO_2. Cells were seeded on semi-permeable filters at a density of $3x10^5$ cells per filter and maintained over 21 days to fully differentiate into functional epithelial cells. Cells were then exposed to 2 nmoles/mL of trichothecenes (DON, DON3Glc, T2, T2-Glc) or zearalenone compounds (ZEN, ZEN14Glc, α-ZEL, α-ZEL14Glc, β-ZEL, β-ZEL14Glc) from the luminal (apical) side for 0, 1, 6 or 24 hours and transport to the systemic (basolateral) side was studied. All experiments are performed in triplicate and mycotoxins were measured in cell culture medium using LC-MS/MS. Stability of all mycotoxins in culture medium was assessed in cell-free controls.

12.2 Results

DON was transported through the epithelial cell layer with up to 38% of the added dose recovered from the basolateral compartment after 24 hours of incubation. DON3Glc was not transported or metabolised in the cell culture system with 98 – 100% of the dose recovered from the apical compartment (Table 26). T2 was spontaneously converted to HT2 in cell free culture medium as well as in all cell cultures. Both T2 and HT2 toxin were efficiently transported through the epithelial cell layer with 24 and 32% of the added dose recovered from the basolateral side as T2 and HT2 toxin, respectively. T2Glc was not transported and no evidence of hydrolysis or conversion to HT2 was found (Table 27). De Nijs et al. (2012) conducted similar experiments with human Caco-2 cells. They found the cells could not hydrolyse the DON3Glc to parent DON. They also observed an obvious difference in

absorption between DON and DON3Glc by intestinal Caco-2 cells. About 23% of the spiked amount of DON was detected at the basolateral side in the *in vitro* model after the 24 h period, while this was less than one per cent for DON3Glc. These authors concluded that this lack of hydrolysis and transport means that the bioavailability of DON3Glc is much less than DON (De Nijs et al 2012).

Table 26 Transport of DON and D3Glc through fully differentiated Caco-2 TC7 monolayers. Results are presented as average ± standard deviation of 3 replicates.

		% of dose added		
Toxin added	Time (hr)	Apical	Basolateral	Total
DON	0	100.8±4.6	0.0±0.0	100.8
DON	1	97.6±6.2	1.1±0.2	100.5
DON	6	90.1±6.9	10.6±2.1	100.7
DON	24	63.3±3.3	37.7±3.0	101.0
DON3Glc	0	100.1±0.0	0.0±0.0	100.1
DON3Glc	1	98.2±0.6	0.0±0.0	98.2
DON3Glc	6	99.2±4.0	0.0±0.0	99.2
DON3Glc	24	98.4±1.4	0.0±0.0	98.4

Table 27 Transport of T2 and T2Glc through fully differentiated Caco-2 TC7 monolayers. Results are presented as average ± standard deviation of 3 replicates.

		% of dose added				
Toxin added	**Time (hr)**	**Apical T2**	**Apical HT2**	**Basolateral T2**	**Basolateral HT2**	**Total**
T2	0	70.0±3.8	13.7±2.2	0.0±0.0	0.0±0.0	83.6
T2	1	53.7±4.2	22.0±2.1	8.0±1.0	3.9±0.8	87.5
T2	6	16.1±1.3	36.8±3.2	23.5±1.3	13.8±2.3	90.2
T2	24	2.4±0.1	35.3±3.6	10.2±0.3	31.7±0.1	79.6
T2Glc	0	91.0±3.5	0.0±0.0	0.0±0.0	0.0±0.0	91.0
T2Glc	1	91.3±1.0	0.0±0.0	0.0±0.0	0.0±0.0	91.3
T2Glc	6	98.6±2.7	0.0±0.0	0.0±0.0	0.0±0.0	98.6
T2Glc	24	95.5±6.4	0.0±0.0	0.0±0.0	0.0±0.0	95.5

Free zearalenone compounds ZEN, α-ZEL, and β-ZEL were completely metabolised by epithelial cell monolayers after 24 hours into undetected metabolites (Table 28). Transport of the toxins to the basolateral compartments reached a maximum of 5.3 % of the dose for ZEN after 6 hours, 1.6% for α-ZEL after 2 hours and 9.4% for β-ZEL after 6 hours of incubation. The masked metabolites ZEN14Glc, α-ZEL14Glc and β-ZEL14Glc were almost fully recovered from the apical compartment without evident hydrolysis or metabolism up to

24 hours. For ZEN14Glc, α-ZEL14Glc and β-ZEL14Glc, 91-93% of the starting amounts? were recovered after 24 hours incubation. For the α-ZEL14Glc this looked like a real drop as the amount decreased at each time point, whereas for β-ZEL14Glc the results fluctuated at the different time points and >95% (mean + sd) of the starting amount remained after 24 hours. These results will require further verification. No evidence of transport of ZEN14Glc, α-ZEL14Glc and β-ZEL14Glc to the basolateral compartment was found.

A similar study was carried out by Videmann et al (2008). They studied transport from apical-basolateral and basolateral-apical sides, and tested for presence of metabolites in the cells too. They found the Caco-2 cells metabolised ZEN and produced metabolites including, α–ZEL, β-ZEL as well as ZEN14Glc and to a lesser amount α–and β-ZEL14Glc. They found that at 10 µM ZEN concentration, α-ZEL was concentrated in the basolateral compartment, whereas β-ZEL and glucosides were highly concentrated at the apical side of the cells. α-ZEL was also the main metabolite in the cells. β-ZEL was strongly excreted in the apical compartment and only small amounts were found in the cell (<10%). The ZEN glucosides and α-ZEL glucosides were undetectable in the cells. They postulated this differential transport of metabolites suggested the involvement of active mechanism(s) of membrane transport for ZEN metabolites. They did also note that distribution of metabolites was modified at higher ZEN concentrations (Videmann et al 2008). A second study by Videmann et al (2009) showed that ZEN and its metabolites were substrates for ABCC1–3 proteins. They showed that extrusion in intestinal cells was mostly due to ABCC2 at the apical side, and that ABCC3 was able to transport β-ZEL at the basolateral side. Considering that α-ZEL and ZEN have the strongest biological effect, extrusion at the apical side by ABCC2 might contribute to protection from these compounds (Videmann et al 2009).

Table 28 Transport of ZEN, ZEN14Glc, α-ZEL, α-ZEL14Glc, β-ZEL and β-ZEL14Glc through fully differentiated Caco-2 TC7 monolayers. Results are presented as average ± standard deviation of 3 replicates.

		% of dose added		
Toxin added	**Time (hr)**	**Apical**	**Basolateral**	**Total**
ZEN	0	81.1±1.8	0.3±0.1	81.5
ZEN	1	37.9±3.0	2.8±0.2	40.7
ZEN	6	0.7±0.8	5.3±5.5	6.0
ZEN	24	0.0±0.0	0.1±0.1	0.1
ZEN14Glc	0	97.8±0.7	0.0±0.0	97.8
ZEN14Glc	1	99.3±2.0	0.0±0.0	99.3
ZEN14Glc	6	97.2±2.6	0.0±0.0	97.3
ZEN14Glc	24	93.0±6.5	0.0±0.0	93.0
α-ZEL	0	80.1±1.8	0.0±0.0	80.1
α-ZEL	1	42.8±4.6	1.6±0.1	44.4
α-ZEL	6	0.0±0.0	0.0±0.0	0.0
α-ZEL	24	0.0±0.0	0.0±0.0	0.0
α-ZEL14Glc	0	99.8±1.8	0.0±0.0	99.8
α-ZEL14Glc	1	94.6±1.3	0.0±0.0	94.6
α-ZEL14Glc	6	91.8±5.5	0.0±0.0	91.8
α-ZEL14Glc	24	90.6±1.3	0.0±0.0	90.6
β-ZEL	0	88.8±6.4	1.0±1.7	89.8
β-ZEL	1	52.0±4.2	7.7±0.9	59.7
β-ZEL	6	1.6±1.6	9.4±2.5	10.9
β-ZEL	24	0.0±0.0	0.7±1.3	0.7
β-ZEL14Glc	0	96.5±6.8	0.0±0.0	96.5
β-ZEL14Glc	1	101.7±5.6	0.0±0.0	101.7
β-ZEL14Glc	6	97.7±1.8	0.0±0.0	97.7
β-ZEL14Glc	24	93.3±2.4	0.0±0.0	93.3

12.3 **Discussion - Gut transport studies:**

12.3.1 DON and DON3Glc

Unmodified DON (up to 38%) was transported through the epithelial cell layers by 24 hours of incubation. The DON3Glc was not transported and was fully recovered after 24 hours incubation showing no loss or breakdown. This would mean the DON3Glc would not be bioavailable in the intestine. However, the results from Section 9.3.1 show that DON3Glc is hydrolysed by gut bacteria to release free DON. Since approximately 38% of free DON was

transferred across the gut epithelial cells, intake of DON3Glc could result in some absorption of DON.

12.3.2 T2 and T2Glc toxin

T-2 toxin was spontaneously converted to HT-2 toxin. By 24 hours incubation, 24 and 30% of the added dose were recovered from the basolateral side as T2 and HT2 respectively showing they were both efficiently transported through the epithelial cell layer. By the end point (24 hours) 80% of the added dose was recovered in total, with ~40% in the basolateral side showing a low level of accumulation or metabolism by Caco-2 cells. The situation of bioavailability is less clear cut than DON, as less T2 was hydrolysed by gut bacteria (Section 9.3.1), however the proportion that is hydrolysed in the gut will be converted to HT2 and a proportion of that and free T2 (up to 30-40% in total) could be transferred across gut epithelial cells.

T2Glc was not transported and no evidence of hydrolysis or conversion to HT2 by the Caco-2 cells was found as over 95% of the administered dose was recovered after 24 hours incubation.

12.3.3 ZEN and metabolites

The results for zearalenone compounds were different to the trichothecene compounds. For parent zearalenone after 1 hour only 41% of ZEN was recovered, ~3% of it had been transported through the epithelial layer. By 24 hours no ZEN was recovered, so it must have undergone complete metabolism by the cells or else been accumulated or absorbed by the cells. For α-ZEL 43% was found in the apical compartment and 44% was detected in total after 1 hour, for β-ZEL ~60% was recovered after 1 hour, and for both ZEL derivatives <1% was recovered after 24 hours.

For ZEN14Glc, α- and β-ZEL14Glc 91-93% of the starting material was found after 24 hours apically with no evidence of transport. For the α-ZEL14Glc this looked like a real drop as the amount decreased at each time point, whereas for β-ZEL14Glc the results fluctuated at the different time points and >93% (mean + sd) of the starting amount remained after 24 hours.

In summary, one of the tested masked mycotoxins were efficiently hydrolysed or transported through the intestinal epithelial monolayers. Free trichothecenes were partially metabolised

and free zearalenone compounds were completely metabolised by intestinal epithelial cells to undetected metabolites.

13 SUMMARY OF PART 2 – BIOASSAY WORK

13.1 Synthesis of masked mycotoxins

Masked mycotoxin standards were sourced or synthesised to be used in the bioassay experiments. Zearalenone, α-zearalenol and β-zearalenol glucosides were synthesised by Sheffield University. T2 toxin glucoside was supplied as gift by the Bacterial Foodborne Pathogens and Mycology Unit, USDA-ARS-NCAUR in the USA.

13.2 (Upper) Small intestinal hydrolysis

Experiments assessed the stability of masked mycotoxins towards artificial gastric, duodenal and bile juices. All masked mycotoxins tested were stable under incubation conditions mimicking the small intestinal digestion and no hydrolysis was observed.

13.3 Assessment of hydrolysis of masked mycotoxins by colonic microbiota

Faecal incubation experiments showed differences between the rates of hydrolysis for the different compounds and also intra-individual differences between the volunteers.

13.3.1 DON3Glc

For all 5 volunteers, hydrolysis of DON3Glc was found to be very rapid with hydrolysis (disappearance of DON3Glc and appearance of DON in culture medium) occurring between 2 – 6 hours, and complete hydrolysis was observed after 24 hours. The results show that on entering the lower intestine gut microbiota will release free DON from DON3Glc.

13.3.2 T2Glc

Hydrolysis was slower for T2 glucoside compared to DON3Glc, T2Glc hydrolysis started after 2 hours, but was only completed in 2 out of 5 cases by 168 hours. T-2 toxin only reached 30-50% of the total toxin added before further metabolism to HT-2 toxin was observed. This de-acetylation only reached 35% for volunteer 1, whereas 77-101% of T2Glc

was transformed to HT-2-toxin by faecal microbiota from the other four volunteers after 168 hours.

13.3.3 ZEN and metabolites

Hydrolysis of ZEN14Glc was very rapid starting at 0.5 hours of incubation and almost complete disappearance of ZEN14Glc after 4 hours. However, only 40% of the dose was recovered as free ZEN leaving over 50% of ZEN14Glc to be metabolised to unidentified metabolites or bound to cell material. Similarly, hydrolysis of α-ZEL14Glc and β–ZEL14Glc was started at 0.5 hours (only 18 and 44% of original dose of α- and β-ZEL recovered) and was almost complete after 4 hours. Only 60 or 30% of the dose were recovered as free α-ZEL and β-ZEL respectively leaving 40 or 70% of the dose unidentified.

13.4 **Characterisation of novel microbial metabolites of masked mycotoxins**

Work to establish LC-TOF-MS conditions to be used to look for new metabolites in faecal extract samples was carried out. Over 300 samples from the faecal incubation experiments were analysed by HRMS. Differences were observed between samples from different volunteers, with some possible metabolites identified. Results also confirmed the almost immediate hydrolysis of ZEN and ZEL glucosides after as little 30 minutes of incubation time.

Peaks were observed in extracted ion chromatograms with masses for ZAL, ZEL, ZAN, HZEN and DHZEN although peaks were observed at different retention times in some volunteers.

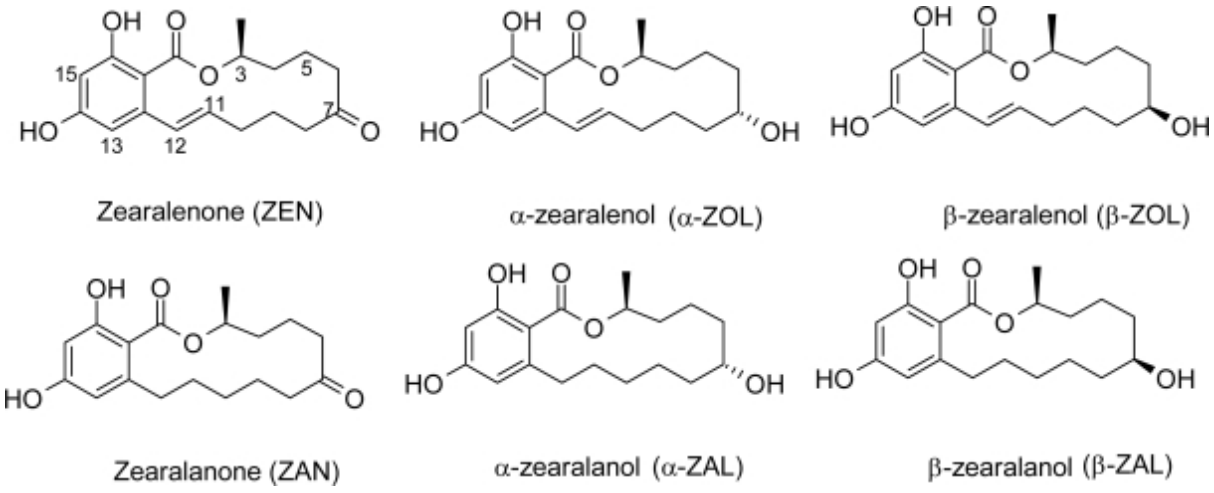


Figure 11. Structures of Zearalenone compounds

13.5 Gut transport studies

None of the modified mycotoxins tested were efficiently hydrolysed by or transported through the intestinal epithelial monolayers, showing that the presence of the glucoside does offer some protection. However the hydrolysis of the glucoside by gut bacteria to produce free toxin means ingestion of modified mycotoxins will contribute to overall exposure at varying degrees depending on the toxin.

14 Conclusions and possible future work

14.1 Survey samples

Levels of masked mycotoxins in cereal based products, beers and spices were measured and UK data (over 2400 results) were submitted to the EFSA database. No samples collected from the UK market contained any of the mycotoxins analysed at or above the maximum permitted levels (Commission Regulation (EC) No 1881/2006).

14.2 Method comparison

The LC-MS method for mycotoxins was validated and demonstrated acceptable performance, and although there were some differences in results between Fera and Rowett results were in good agreement for most analytes.

14.3 (Upper) Small intestinal hydrolysis

There was no evidence of hydrolysis or release of parent mycotoxins DON or T-2 toxin from their masked forms. When incubating the parent mycotoxins with artificial digestive juices, DON and HT-2 toxin were also stable.

14.4 Hydrolysis of masked mycotoxins by colonic microbiota

Faecal incubation experiments showed differences between the rates of hydrolysis for the different compounds and also intra-individual differences between the volunteers.

14.5 Gut transport studies

Different results were seen for the trichothecene compounds and the zearalenone compounds. However none of the masked mycotoxins were transported through the intestinal epithelial monolayers. However a proportion of the free mycotoxins was transported, therefore combined with the hydrolysis in the upper GI tract, intake of masked mycotoxins could lead to increased uptake of mycotoxins.

14.6 Characterisation of novel microbial metabolites of masked mycotoxins

Further data analysis using a software programme 'Compound Discoverer' was also undertaken to assess the data from Volunteer 3. This software can predict metabolites or possible products following known metabolic pathways. A large number of products were found and some putative identifications were assigned, these results are for information only.

14.7 Suggestions for possible further work

The study has shown some interesting results and also raised some questions. For instance it would be interesting to understand the differences in gut microbiota that may cause some of the differences observed between volunteers. The use of Next Generation Sequencing could perhaps give some insight into the makeup of the different microbiota samples. In this project, only faecal microbiota populations were assessed for their capacity to hydrolyse masked mycotoxins and release parent compounds. It is, however, likely that microbes residing in the upper gut could also hydrolyse masked mycotoxins and add further to the mycotoxin body burden. This will require further studies in using animal models (e.g. pigs) as no human samples can be obtained.

Some results appear similar to published results and some appear different. Although this could be due to differences in the concentration of the compounds tested. This is a very active area of research with a large number of recent publications that were not available at the start of this project, or indeed when the EFSA evaluation of 'modified mycotoxins' was carried out. It may be worth taking time to conduct a full review of all areas of research of modified mycotoxins, including metabolism and toxicity studies before undertaking any future practical research.

Further data interrogation of the HRMS data could be conducted to establish differences between the volunteers. It may also be possible to ’reverse search’ to look for the possible metabolites suggested by the ‘Compound Discoverer’ software. Further experiments, better designed to specifically suit metabolomics study protocols and using higher concentrations of mycotoxins, could also be undertaken. However it would be better to do this after a thorough review of the recent and current literature to fully understand the state of the art of the knowledge on the metabolism of modified mycotoxins.

15 References

Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

Crews, C., and MacDonald, S., (2015). Natural Occurrence of Masked Mycotoxins. In: Masked Mycotoxins in Food: Formation, Occurrence and Toxicological Relevance, edited by Chiara Dall'Asta, Franz Berthiller. Royal Society of Chemistry.

Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G., and Dall'Asta, C. (2013): Masked mycotoxin are efficiently hydrolysed by human colonic microbiota releasing their aglycones. Chem Res Toxicol 26,305-12.

De Nijs, M., Van den Top, H. J., Portier, L., Oegema, G., Kramer, E., Van Egmond, H. P., & Hoogenboom, L. A. P. (2012). Digestibility and absorption of deoxynivalenol-3-ß-glucoside in in vitro models. World Mycotoxin Journal, 5(3), 319-324.

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), (2014). Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA Journal 2014;12 (12):3916, 107 pp. doi:10.2903/j.efsa.2014.3916

EFSA website, August 2015. -

http://registerofquestions.efsa.europa.eu/roqFrontend/ListOfQuestionsNoLogin?0&unit=BIOCONTAM

Frizzell, C., Uhlig, S., Miles, C.O., Steven Verhaegen, S., Elliott, C.T., Eriksen, G.S., Sørlie, M., Ropstad, E., and Connolly, L. (2015) Biotransformation of zearalenone and zearalenols to their major glucuronide metabolites reduces estrogenic activity. Toxicology in Vitro 29, no. 3 (2015): 575-581.

Gratz, S.W., Duncan, G. & Richardson, A. (2013): Human fecal microbiota metabolize deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary de-epoxy deoxynivalenol. Appl Environ Microb 79(6): 1821-5.

Hahn, I., Kunz-Vekiru, E., Twarużek, M., Jan Grajewski, Krska, R. & Berthiller, F., (2015) Aerobic and anaerobic in vitro testing of feed additives claiming to detoxify deoxynivalenol and zearalenone, Food Additives & Contaminants: Part A, 32:6, 922-933, DOI: 10.1080/19440049.2015.1023741

Kakeya, H., Takahashi-Ando, N., Kimura, M., Onose, R., Yamaguchi I., & Osada, H., (2002). Biotransformation of the Mycotoxin, Zearalenone, to a Non-estrogenic Compound by a Fungal Strain of Clonostachys sp., Bioscience, Biotechnology, and Biochemistry, 66:12, 2723-2726, DOI: 10.1271/bbb.66.2723

McCormick S.P., Kato T., Maragos C.M., Busman M., Lattanzio V.M., Galaverna G., Dall-Asta C., Crich D., Price N.P., Kurtzman C.P. (2015), Anomericity of T-2 toxin-glucoside: masked mycotoxin in cereal crops. J Agric Food Chem. 2015 Jan 21;63(2):731-8. doi: 10.1021/jf504737f. Epub 2015 Jan 6.

Mikula, H., Weber, J., Svatunek, D., Skrinjar, P., Adam, G., Krska, R., Hametner, C., and Fröhlich, J., (2014). Synthesis of zearalenone-16-β,D-glucoside and zearalenone-16-sulfate: A tale of protecting resorcylic acid lactones for regiocontrolled conjugation. Beilstein J. Org. Chem. 2014, 10, 1129–1134.

Morales et al. 2011. Tetrahedron, 67, 7268

Nathanail, A.V., Syvähuoko, J., Malachová, A., Jestoi, M., Varga, E., Michlmayr, H., Adam, G., Sieviläinen, E., Berthiller, F., and Peltonen, K., (2015). Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. Anal Bioanal Chem. 2015; 407(16): 4745–4755. Published online 2015 May 3. doi: 10.1007/s00216-015-8676-4. PMCID: PMC4446524

Thiem, J., Grabley, S., Gareis, M., and Böckers, W., (1992). Glycosylation of Mycotoxins. *Synthesis*, 1992, 1078-1080. DOI: 10.1055/s-1992-26306

Vekiru, E., Hametner, C., Mitterbauer, R., Rechthaler, J., Adam, G., Schtzmayr, G., Krska, R., and Schumacher, R., (2010). Cleavage of Zearalenone by Trichosporon mycotoxinivorans to a Novel nonestrogenic Metabolite. Applied and Environmental Microbiology, 76, 7, 2353-2359.

Videmann, B., Mazallon, M., Tep, J., Lecoeur, S., (2008). Metabolism and transfer of the mycotoxin zearalenone in human intestinal caco-2 cells. Food Chem. Toxicol. 46, (10), 3279–3286.

Appendix 1 – Full descriptions of samples analysed in the masked mycotoxin retail food survey

A2FY 5040 Masked Mycotoxins_Fera San

16 Appendix 2 – Sheffield University report of synthesis of masked mycotoxin standards

Synthesis of β-D-glucosides of α and β-ZOL and ZEN

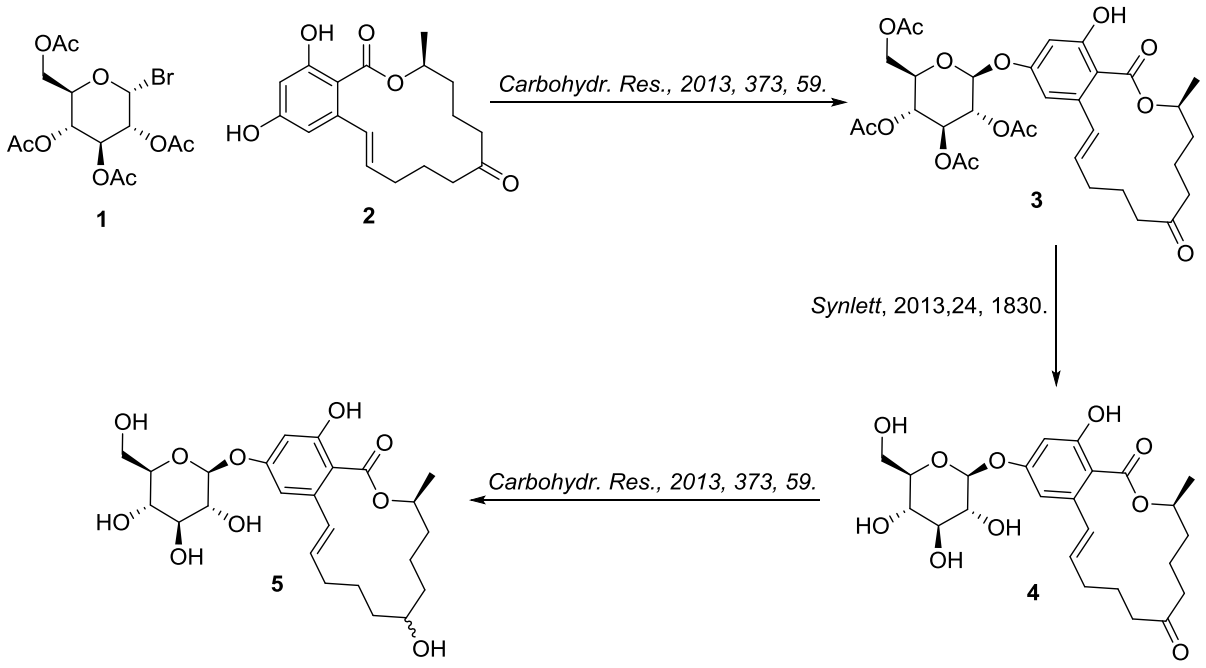
University Reference TD/141833

Dr Simon Jones & Dr Ashlie Butler

University of Sheffield

Several groups have reported the use of a borate buffer to facilitate the reaction of acetobromoglucose (**1**) and ZEN (**2**) to form the ZEN-glucoside (**3**). Initially, the reaction was performed using a cheap and readily available ZEN analogue, resorcinol, but only the starting material was recovered despite several attempts to optimise the reaction conditions.

The reaction was repeated using a Lewis acid mediated approach. Once again the reactions were performed with resorcinol as a model before any ZEN was committed. Both iron(III) chloride and silver(I) oxide were tested and only the silver(I) oxide method (laid out by Thiem *et al*. *Synthesis*, 1992, 1078) was found to be successful.



The reaction using silver(I) oxide was then repeated using ZEN and the product ZEN-glucoside **3** was obtained in a low yield. The removal of the acetyl protecting groups was then performed under the conditions laid out by Weber *et al*. and the product **4** was successfully obtained and purified by preparative HPLC.

The reaction to synthesise **3** was repeated under the same conditions in order to perform the further reactions described in the scheme above. ^{1}H NMR spectroscopy suggested the product had been formed, although the product was not pure after flash column chromatography. According to the report by Mikula *et al*. the reaction

sequence was performed using the crude materials from each previous reaction and so it was decided to continue with the sequence described in the scheme above. After performing the deprotection reaction conditions previously demonstrated to form the product **4**, the crude reaction mixture was subjected to the reduction conditions laid out by Mikula *et al*. in an attempt to form the mixture of isomers shown by product **5**. Purification of the reaction mixture by preparative HPLC showed that the desired products were not formed.

Once again it was required to synthesise the ZEN-glucoside **3**. As the previously used method had not produced a high yield the reaction was tried under the conditions laid out by Mikula *et al*. which, though utilising the same reagents, described the use of a different solvent. The reaction provided a higher yield of product, however, it was not pure after flash column chromatography. The deprotection reaction to obtain **4** was performed but after attempts at purification it was determined that the product was not formed and starting material was also not recovered.

Having encountered problems following the literature protocols with impure intermediates, purification of each intermediate was conducted to ensure that no side products were interfering with the sequential reactions. Therefore, the formation of **3** under the conditions laid out by Mikula *et al*. was repeated and the product purified by both flash column chromatography and preparative HPLC to obtain **3** as a pure product.

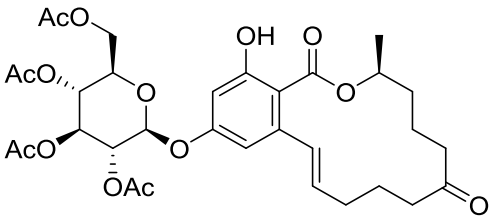
The deprotection method laid out by Weber *et al*. was followed by analytical HPLC on **3**. After 28 h there was no conversion to the deprotected product **4**. Following this, an alternative deprotection method laid out by Morales *et al*. (Tetrahedron, 2011, 67, 7268.) was employed that afforded the product **4**. The crude reaction mixture was subjected to the reduction laid out by Mikula *et al*. and the reaction mixture was purified by preparative HPLC to afford the isolated isomers of product **5**.

Experimental Data

General methods:

Dry solvents were obtained from the university Grubbs system. Reagents were used as supplied without further purification unless otherwise stated. Thin layer chromatography was performed on aluminium backed plates pre-coated with silica (0.2 mm, Merck DC-alufolien Kieselgel 60 F_{254}). Plates were visualised using UV light or by dipping in $KMnO_4$ solution, followed by exposure to heat. Flash column chromatography was performed on silica gel (Merck Kieselgel 60 F_{254}230–400 mesh), unless otherwise stated. ^{1}H NMR spectra were measured using $CDCl_3$ as solvent unless otherwise stated, on a Bruker AV-400 MHz machine with an automated sample changer. Chemical shifts for hydrogen are given, on the δ scale. Coupling constants were measured in Hertz (Hz). High resolution mass spectra (HRMS) recorded for accurate mass analysis, were performed on a MicroMass LCT operating in Electrospray mode (TOF ES). HPLC was performed on Water Separation Module with a ZORBAX® Bonus-RP analytical column (4.6 mm x 150 mm, 5-Micron, 5-95 % MeCN in water over 20 mins at 1 mL/min, 15 μL injection, UV detection at 254 nm).

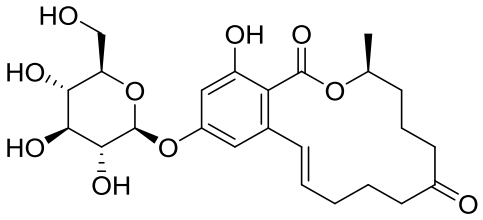
(*S*)-Zearalenone 4-*O*-(2,3,4,6-tetra-*O*-acetyl-β,D-glucopyranoside) (3)



To a solution 1,2,3,4-tetra-*O*-acetyl-β,D-glucose (0.257 g, 0.625 mmol) and ZEN (0.079 g, 0.25 mmol) in dry acetonitrile (5 mL) powered molecular sieves (3 Å, 0.100 g) were added. The reaction mixture was stirred at room temperature for 1.5 h. Ag_2O (0.087 g, 0.375 mmol) was added and stirring was continued in the dark for 24 h. The reaction mixture was then diluted with dichloromethane and filtered through a Celite pad. The mixture was concentrated under reduced pressure and purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate 8/1 → 1/1). Further purification was performed by preparative HPLC utilising the conditions describes under general methods to yield **3** as a white solid (0.035 g, 0.054 mmol).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.40 (3H, d, *J* = 6.0), 1.48-1.57 (1H, m), 1.61-1.81 (5H, m), 2.02-2.13 (12H, m), 2.13-2.28 (3H, m), 2.33- 2.42 (1H, m), 2.52 (1H, m), 2.74-2.89 (1H, m), 3.88-3.95 (1H, m), 4.12-4.20 (1H, m), 4.23-4.30 (1H, m), 4.97- 5.06 (1H, m), 5.11-5.19 (2H, m), 5.23-5.36 (2H, m), 5.61-5.72 (1H, m), 6.49 (1H, d, *J* = 2.5), 6.51 (1H, d, *J* = 2.5), 7.00 (1H, d, *J* = 15.5), 12.01 (1H, s).

(*S*)-Zearalenone-4-*O*-β,D-glucopyranoside (4)

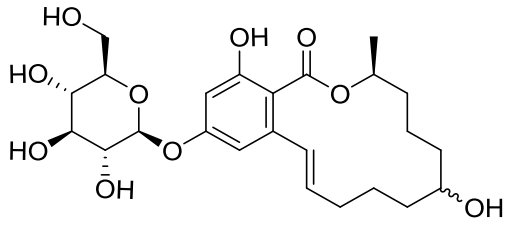


First method: Compound **3** (0.022 g, 0.034 mmol) was dissolved in 20 mL THF-H_2O (4:1) and KOH was added (0.019 g, 0.340 mmol). The reaction was stirred at room temperature for 2.5 h and the mixture was neutralised by the addition of 0.1 M HCl and diluted with H_2O. The mixture was immediately extracted with ethyl acetate, dried over $MgSO_4$ and concentrated at reduced pressure. The product was purified by preparative HPLC under the conditions described in the general methods section to yield **4** as a white solid (0.010 g, 0.02 mmol).

Second method: Compound **3** (0.035 g, 0.054 mmol) was dissolved in 2 mL methanol and Na_2CO_3 (0.002 g, 0.016 mmol) was added. The mixture was stirred at room temperature and the reaction progress was monitored by analytic HPLC under the conditions described in the general methods section. After 3.5 h 0.1 M HCl was added to pH 7 and the reaction mixture was filtered and concentrated at reduced pressure.

^{1}H NMR (d^3-MeOD, 400 MHz): δ 1.41 (3H, d, *J* = 6.0), 1.53-1.83 (5H, m), 1.99-2.10 (1H, m), 2.11- 2.26 (2H, m), 2.29-2.39 (2H, m), 2.60-2.69 (1H, m), 2.76-2.88 (1H, m), 3.38-3.91 (1H, m), 3.48-3.52 (3H, m), 3.67-3.72 (1H, m), 3.90-3.98 (1H, m), 4.99-5.01 (1H, m), 5.02-5.11 (1H, m), 5.70-5.81 (1H, m), 6.55 (1H, d, *J* = 2.5), 6.71 (1H, d, *J* = 2.5), 6.99 (1H, d, *J* = 15.5). HRMS (ES^+) calcd for $C_{24}H_{33}O_{10}$ ($[M+H]^+$) 481.2074, found 481.2088.

α- and β-Zearalenol-14-β,D-glucoside (5)



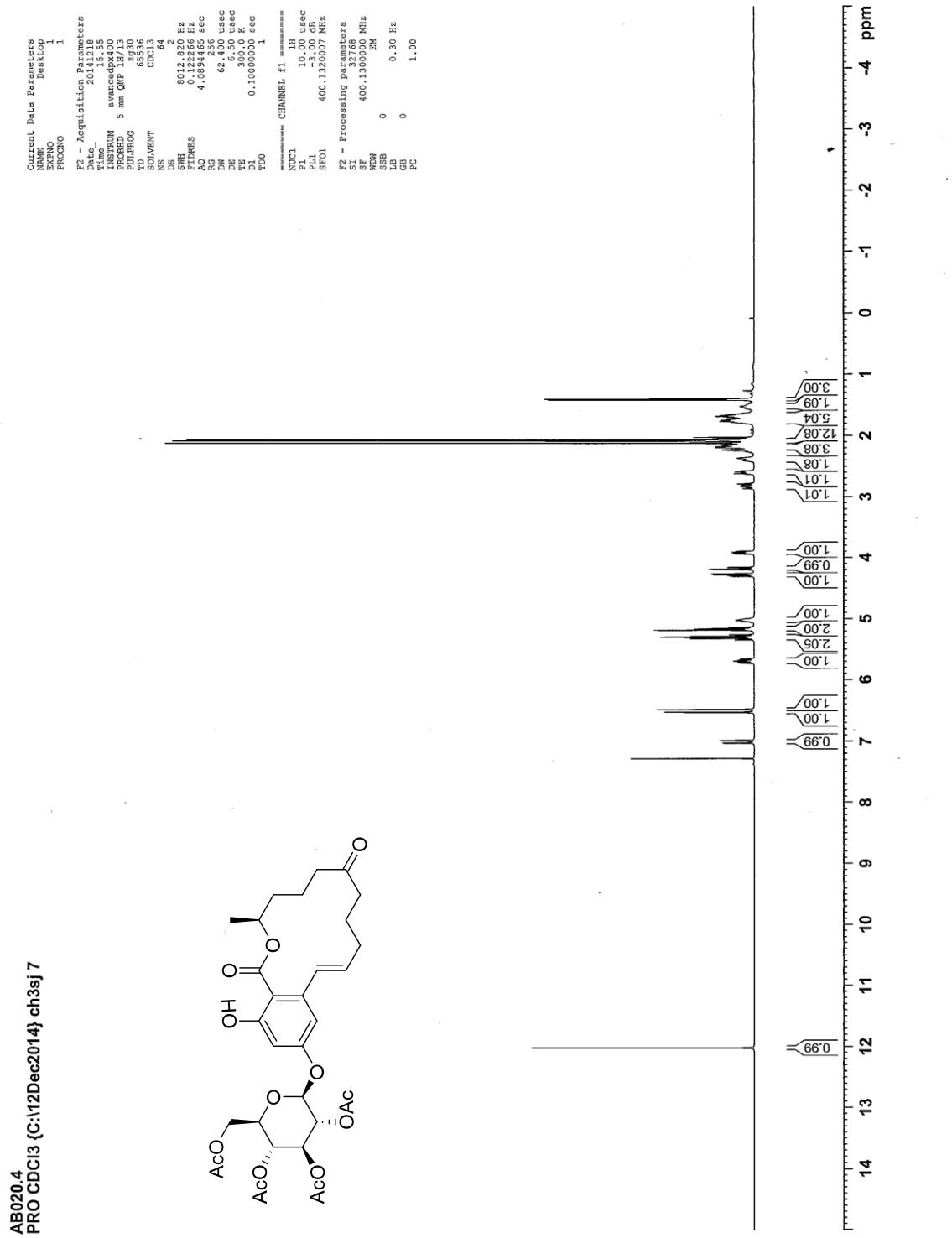
The crude product (0.105 g, assumed as 0.162 mmol) from the second method described above was dissolved in 5 mL methanol and $NaBH_4$ (0.061 g, 1.62 mmol) was added. The mixture was stirred at room temperature for 1 h then quenched with 0.1 M HCl followed by immediate extraction with ethyl acetate. The organic layer was washed with sat. Na_2CO_3 solution, dried over $MgSO_4$ and concentrated at reduced pressure. The product was purified and isomers separated by preparative HPLC under the conditions described in the general methods section to yield β-Zearalenol-14-β,D-glucoside (0.001 g) and α-Zearalenol-14-β,D-glucoside (0.001 g).

α-Zearalenol-14-β,D-glucoside: ^{1}H NMR (d^3-MeOD, 400 MHz): 1.12-1.22 (1H, m), 1.41 (3H, d, *J* = 6.0), 1.43-1.77 (7H, m), 1.83-1.98 (2H, m), 2.29-2.41 (2H, m), 3.36-3.41 (1H, m), 3.48-3.52 (3H, m), 3.67-3.73 (1H, m), 3.74-3.82 (1H, m), 3.89-4.98 (1H, m), 4.99-5.07 (2H, m), 5.80-5.89 (1H, m), 6.55 (1H, d, *J* = 2.5), 6.70 (1H, d, *J* = 2.5), 7.15 (1H, d, *J* = 15.5). HRMS calcd for $C_{24}H_{35}O_{10}$ ($[M+H]^+$) 483.2230, found 483.2208.

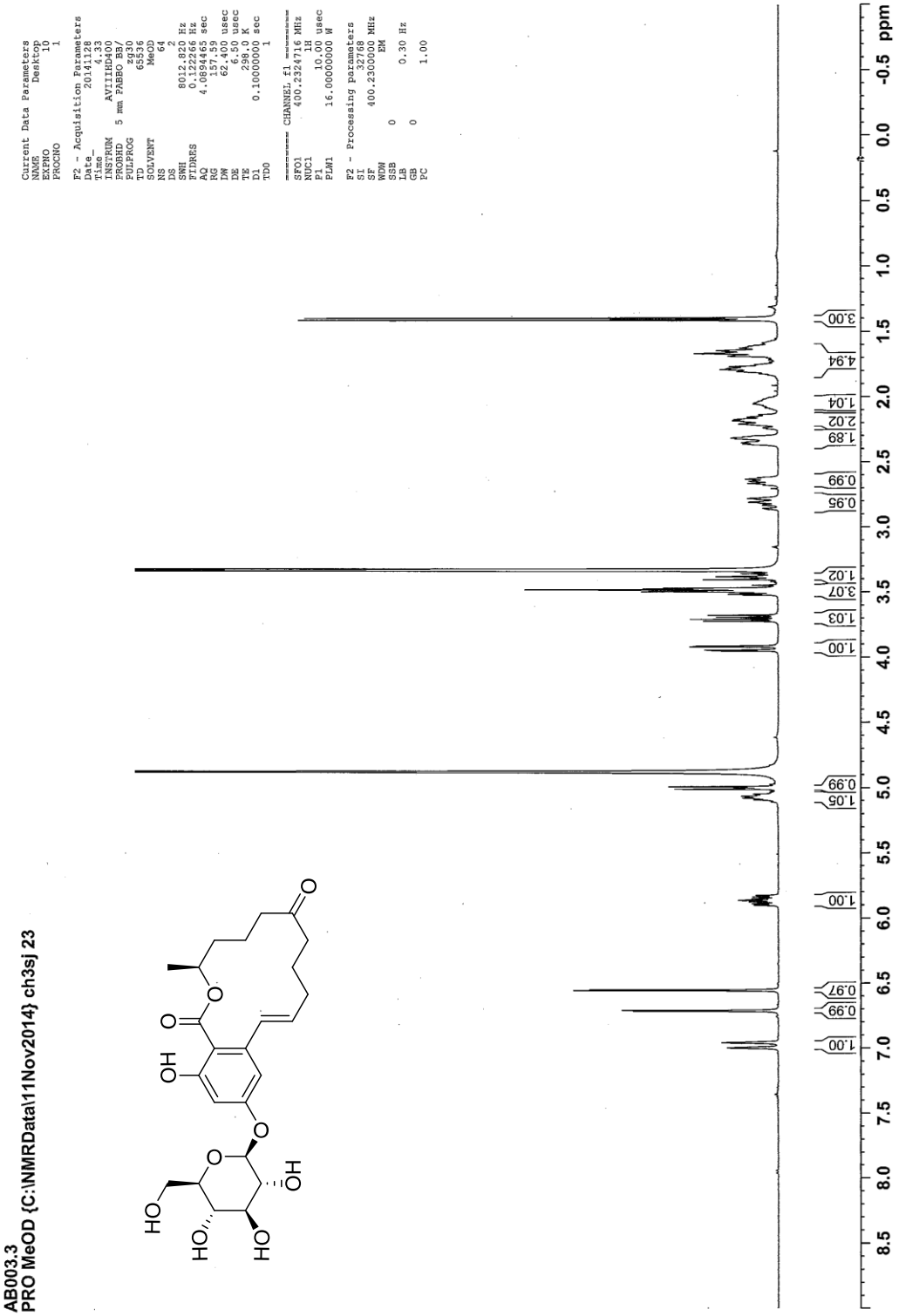
β-Zearalenol-14-β,D-glucoside: HRMS (ES^+) calcd for $C_{24}H_{35}O_{10}$ ($[M+H]^+$) 483.2230, found 483.2253.

Spectroscopic Data

(*S*)-Zearalenone 4-*O*-(2,3,4,6-tetra-*O*-acetyl-β,D-glucopyranoside) (**3**):



(*S*)-Zearalenone-4-*O*-β,D-glucopyranoside (**4**):



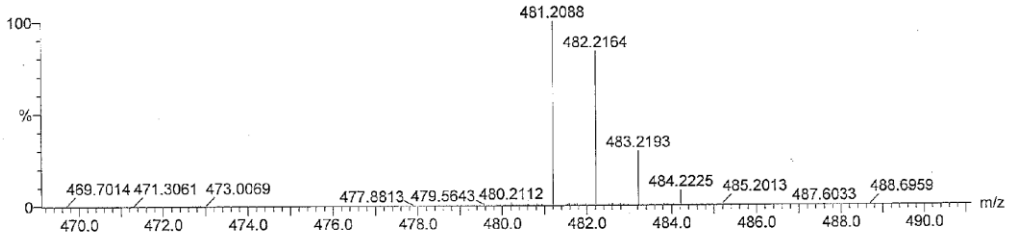
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

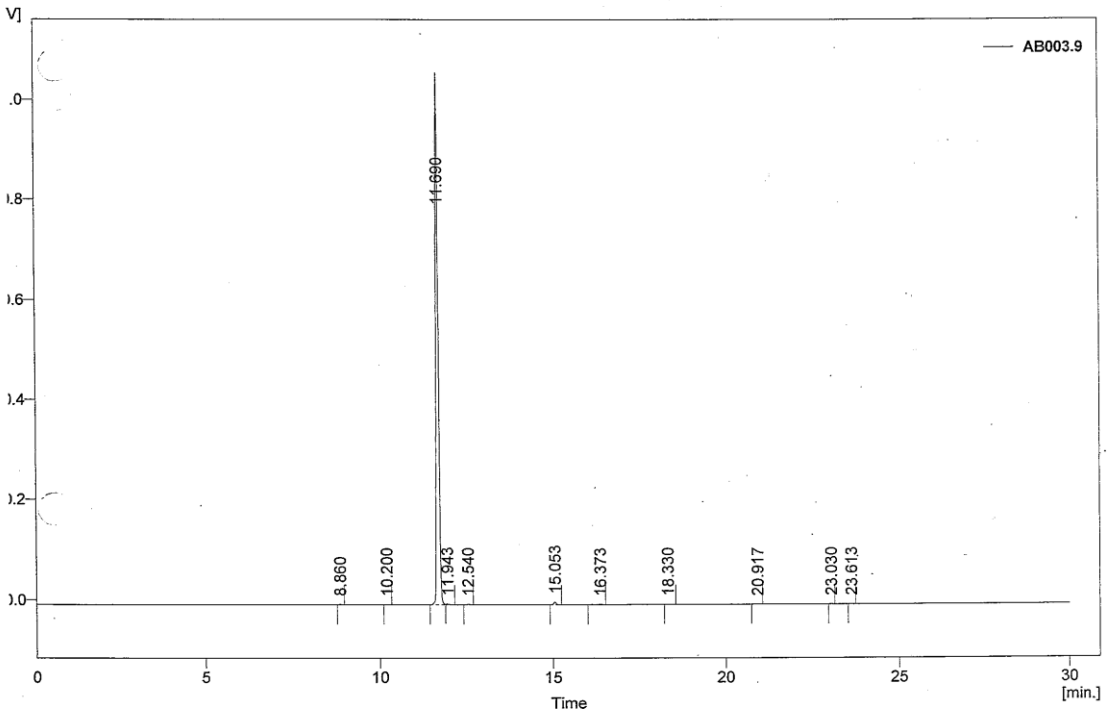
Monoisotopic Mass, Even Electron Ions
4 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-46 H: 0-80 O: 10-10

A. Butler AB003.2
49jones06 451 (2.666) Cm (451:455-431:436)

05-Dec-2014
1: TOF MS ES+
1.19e+004



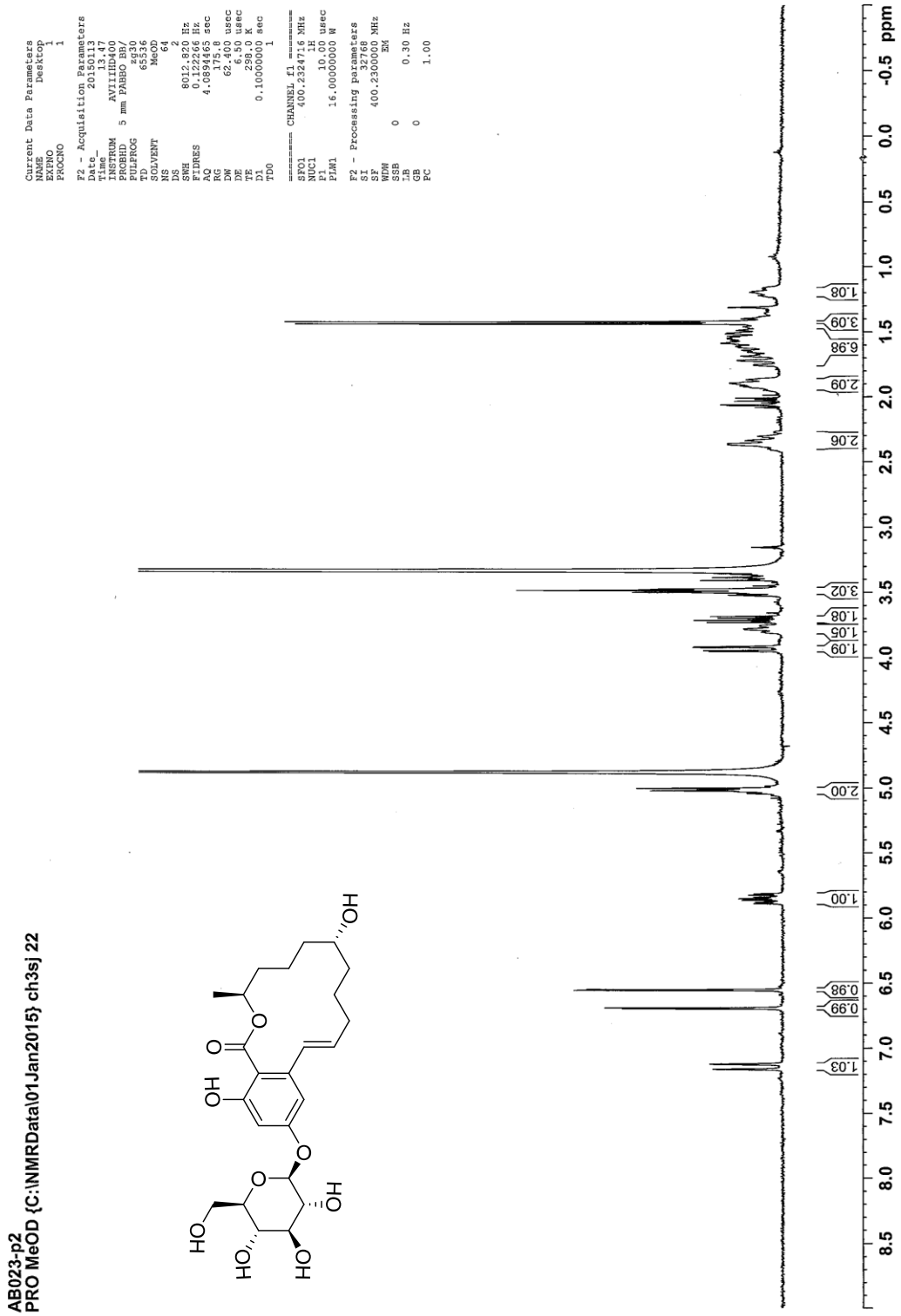
Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
481.2088	481.2074	1.4	2.9	8.5	165.7	0.0	C24 H33 O10



Result Table - Calculation Method Uncal

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	8.860	1.634	0.380	0.032	3.5e-02	0.07
2	10.200	2.482	0.566	0.049	0.1	0.07
3	11.690	4974.844	1063.406	98.845	99.0	0.08
4	11.943	10.665	1.865	0.212	0.2	0.10
5	12.540	4.315	0.770	0.086	0.1	0.09
6	15.053	27.492	5.610	0.546	0.5	0.08
7	16.373	2.363	0.322	0.047	3.0e-02	0.08
8	18.330	4.640	0.697	0.092	0.1	0.08
9	20.917	2.020	0.262	0.040	2.4e-02	0.11
10	23.030	0.955	0.217	0.019	2.0e-02	0.07
11	23.613	1.568	0.324	0.031	3.0e-02	0.08
	Total	5032.977	1074.420	100.000	100.0	

α-Zearalenol-14-β,D-glucoside (**5**):



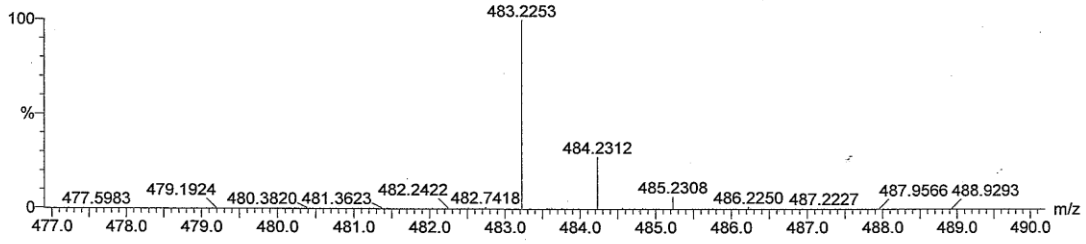
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

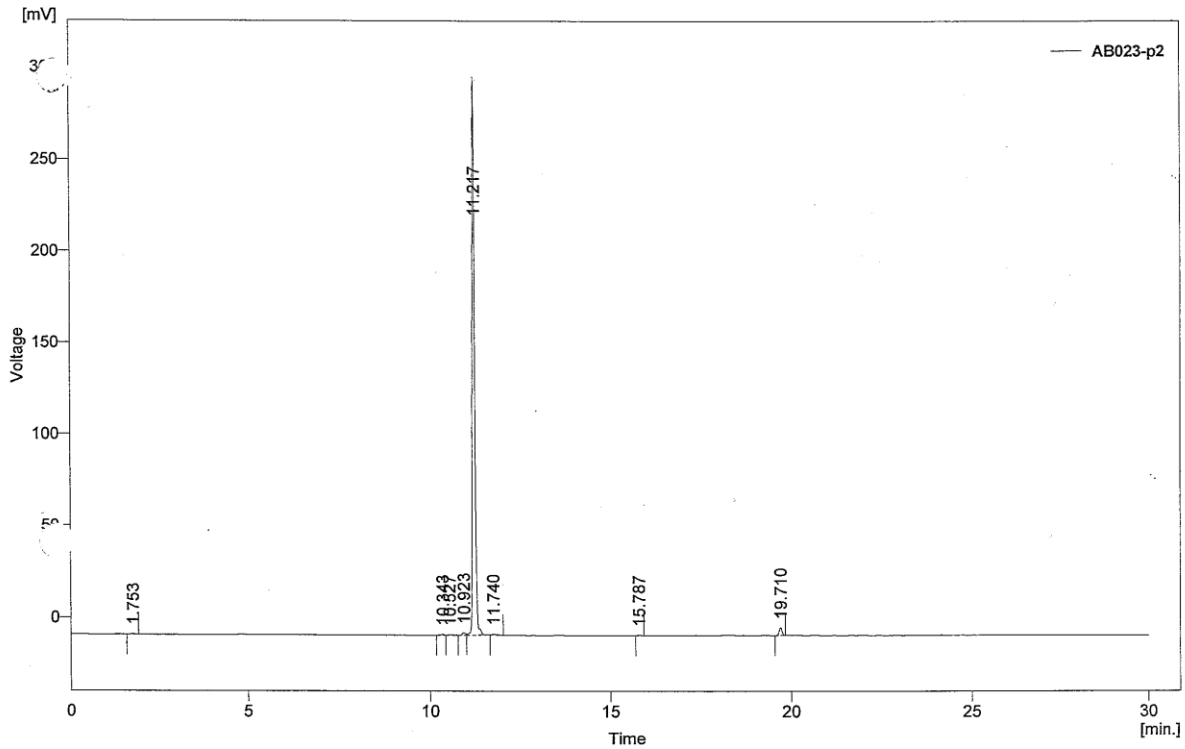
Monoisotopic Mass, Even Electron Ions
11 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-25 H: 0-40 O: 0-10

A. Butler AB023-p2
02jones02 414 (2.412) Cm (411:426-364:380)

12-Jan-2015
1: TOF MS ES+
1.04e+005



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
483.2253	483.2230	2.3	4.8	7.5	280.1	0.0	C24 H35 O10



Result Table - Calculation Method Uncal

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.753	2.297	0.315	0.156	0.1	0.11
2	10.343	2.581	0.530	0.175	0.2	0.08
3	10.527	3.005	0.349	0.204	0.1	0.15
4	10.923	8.097	1.368	0.551	0.4	0.11
5	11.217	1423.324	304.451	96.767	97.5	0.07
6	11.740	6.955	0.672	0.473	0.2	0.20
7	15.787	1.652	0.301	0.112	0.1	0.09
8	19.710	22.964	4.203	1.561	1.3	0.09
	Total	1470.874	312.189	100.000	100.0	

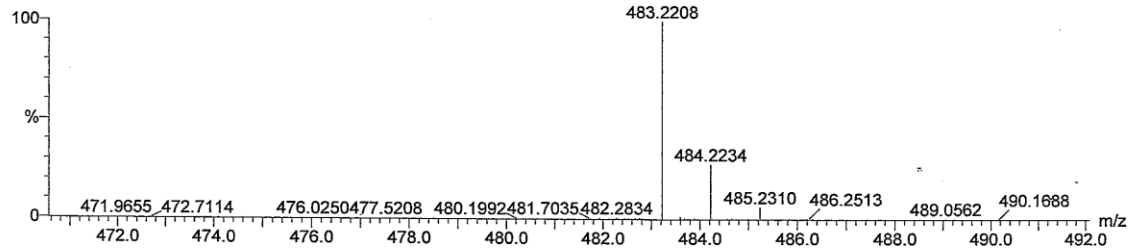
β-Zearalenol-14-β,D-glucoside (**5**):

Elemental Composition Report **Page 1**

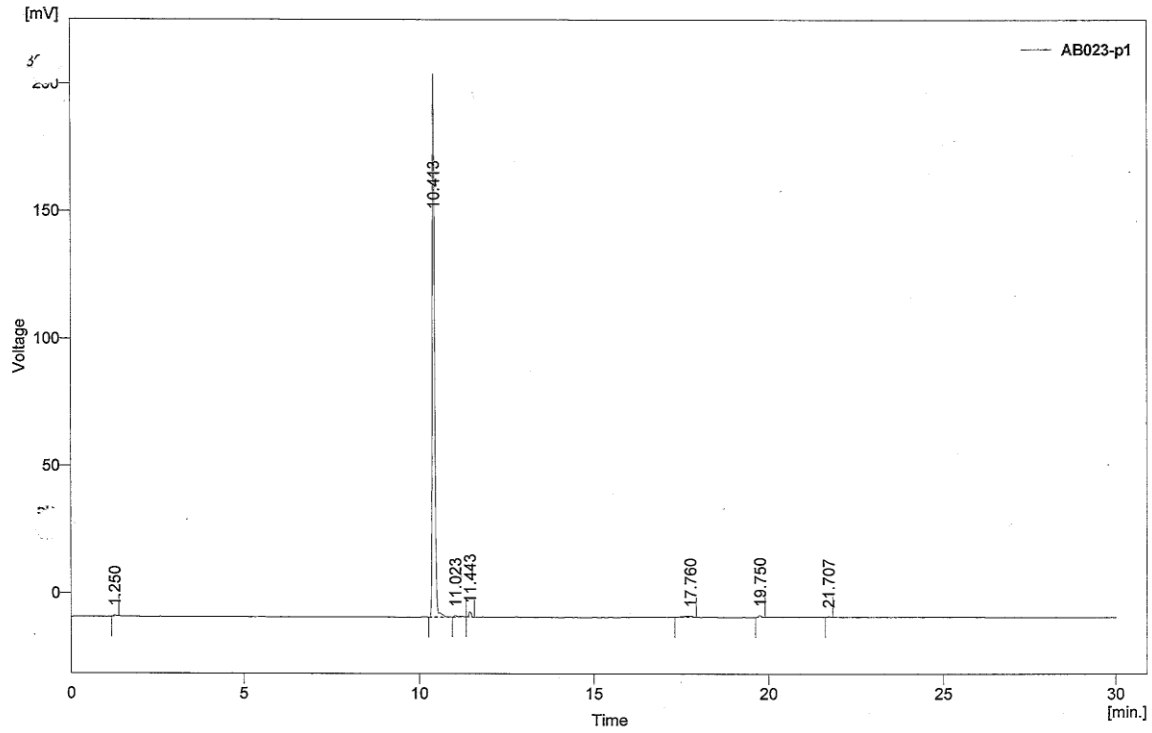
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
11 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-25 H: 0-40 O: 0-10
A. Butler AB023-p1
02jones01 365 (2.133) Cm (365:368-345:348)

12-Jan-2015
1: TOF MS ES+
8.86e+003



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
483.2208	483.2230	-2.2	-4.6	7.5	93.9	0.0	C24 H35 O10



Result Table - Calculation Method Uncal

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.250	2.292	0.499	0.224	0.2	0.08
2	10.413	990.317	213.125	96.759	97.8	0.07
3	11.023	6.662	0.630	0.651	0.3	0.09
4	11.443	9.951	2.165	0.972	1.0	0.08
5	17.760	8.728	0.472	0.853	0.2	0.32
6	19.750	4.148	0.753	0.405	0.3	0.09
7	21.707	1.390	0.261	0.136	0.1	0.09
	Total	1023.487	217.905	100.000	100.0	

17 Appendix 3. Tabulated outputs from Compound Discoverer software – Negative Mode

1) Zearalenone results

Apex m/z					
184.95787	0.73	11825	M-H	48, 72 hours, 7 days	
216.93013	0.74	5632	M-H	72 hours	
165.02215	0.77	6021	M-H	48, 72 hours, 7 days	
439.08444	0.86	17357	M-H	2 hours	
197.07911	0.92	5409	M-H	48 hours	
184.05868	0.96	33711	M-H	48, 72 hours, 7 days	
169.04765	1.01	17326	M-H	48 hours	
145.08638	1.03	18002	M-H	7 days	
144.10234	1.08	15603	M-H	7 days	
219.03296	1.08	12336	M-H	7 days	
152.08230	1.38	9934	M-H	7 days	
125.03496	1.80	6824	M-H	48 hours	
253.13046	3.94	7334	M-H	72 hours	
116.04976	9.94	11855	M-H	7 days	
317.13933	14.11	22773	M-H	7 days	Parent compound (or zearalenol desaturation)
147.01409	15.22	18388	M-H	72 hours	
434.26740	15.38	5269	M-H	72 hours	
178.98630	16.24	21495	M-H	72 hours	
147.01410	16.25	16741	M-H	72 hours, 7 days	

2) α-zearalenol results

Apex m/z	RT [min]	Max. Area	Ion Type	Time points	Possible transformation(s)
216.93021	0.74	5564	M-H	48, 72 hours	
184.95797	0.74	11914	M-H	48, 72 hours	
230.94598	0.76	10093	M-H	72 hours	
165.02214	0.77	6158	M-H	48, 72 hours, 7 days	
197.98966	0.90	13377	M-H	48 hours	
201.03754	0.91	29166	M-H	24 hours	
232.02584	0.92	6657	M-H	48 hours	
263.07332	0.96	5635	M-H	24 hours	
184.05867	0.96	26611	M-H	48 hours, 7 days	
288.12175	1.04	6873	M-H	7 days	
268.15307	1.06	6825	M-H	7 days	
219.03301	1.10	15006	M-H	72 hours, 7 days	
144.10233	1.10	15658	M-H	7 days	
253.13056	3.91	7702	M-H	24 hours	
358.17723	5.68	16586	M-H	24 hours	
146.02410	6.24	5576	M-H	72 hours	
319.15505	12.67	16546	M-H	All time points	Parent compound (or steroisomer or zearalenone reduction)
147.01416	13.87	9503	M-H	72 hours, 7 days	
147.01414	15.19	19120	M-H	72 hours, 7 days	
147.01417	16.22	22584	M-H	72 hours, 7 days	
178.98639	16.23	30678	M-H	72 hours, 7 days	
177.97857	17.23	7027	M-H	72 hours	

3) ß-zearalenol results

Apex m/z	RT [min]	Max. Area	Ion Type	Time points	Possible transformation(s)
184.95780	0.70	7462	M-H	48, 72 hours	
228.09225	0.86	7200	M-H	24 hours	
184.05850	1.03	16743	M-H	7 days	
214.14434	4.21	15068	M-H	7 days	
116.04971	10.02	15341	M-H	7 days	
319.15497	11.81	7617	M-H	All time points	Parent compound (or steroisomer or zearalenone reduction)
147.01404	13.94	6003	M-H	72 hours, 7 days	
147.01406	15.24	22728	M-H	72 hours, 7 days	

178.98625	16.27	20414	M-H	48, 72 hours, 7 days
147.01406	16.28	15847	M-H	48, 72 hours, 7 days
177.97841	17.29	7577	M-H	72 hours, 7 days

18 Appendix 4 Tabulated outputs from Compound Discoverer software – Positive Mode

1) Zearalenone results

Apex m/z	RT [min]	Max. Area	Ion Type	Time points	Possible transformation(s)	Comment
148.03436	0.87	21221	M+H	48, 72 hours, 7 days		
241.00329	0.91	9987	M+H	72 hours		
243.05778	0.92	22277	M+H	48, 72 hours, 7 days		
257.07332	0.96	10939	M+H	7 days		
224.12555	1.01	10077	M+H	7 days		
227.00788	1.01	10504	M+H	7 days		
232.09180	1.03	12046	M+H	7 days		
92.01676	1.03	10717	M+H	7 days		
190.08131	1.03	7471	M+H	7 days		
277.12693	1.04	56528	M+H	24, 48 72 hours, 7 days		
238.11847	1.06	13352	M+H	24, 48 72 hours, 7 days		
293.10080	1.06	73770	M+H	24 hours		
176.07942	1.15	5564	M+H	7 days		
89.10771	1.45	8279	M+H	24 hours		
159.14911	2.92	48135	M+H	48, 72 hours		
238.11848	3.49	16602	M+H	6, 24,48 72 hours		
255.14499	3.50	11038	M+NH4	24,48 72 hours		M+NH4 of previous ion
159.14913	3.66	45311	M+H or M+NH4	7 days		
312.19179	3.68	11895	M+H	24 hours		
255.14497	3.90	19558	M+NH4	24, 48 72 hours, 7 days		M+NH4 of following ion
238.11847	3.90	36276	M+H	24, 48 72 hours, 7 days		
265.11167	4.28	13239	M+H	6, 24 hours		
173.16481	4.38	150795	M+H	48, 72 hours, 7 days		
216.15937	4.61	9446	M+H	7 days		
534.29257	4.90	6700	M+H	6, 24,48 72 hours, 7 days	Zearalenol - arginine conjugation, glycine conjugation (+C8H15N5O2) Zearalenone - reduction - arginine conjugation, glycine conjugation (+C8H17N5O2)	
			M+NH4		Zearalenol glucoside - hydration, reduction, methylation (+CH6O) Zearalenol - hydration, reduction glucoside conjugation, methylation (+C7H16O6)	

					Zearalenol - desaturation, acetylation, arginine conjugation (+C8H12N4O2)
					Zearalenone - acetylation, arginine conjugation (+C8H14N4O2)
270.19234	4.98	27983	M+H	24 hours	
173.16480	4.99	130905	M+H/M+NH4	48, 72 hours, 7 days	
180.14941	5.10	6950	M+H	48, 72 hours, 7 days	
196.14443	5.95	39092	M+H	48, 72 hours, 7 days	
118.03220	6.56	11464	M+H	72 hours	
265.11807	6.56	43732	M+H	48 hours	
187.18038	6.73	40625	M+H/M+NH4	48 hours, 7 days	
210.16009	7.70	80620	M+H	72 hours, 7 days	
287.11376	7.83	6246	M+H	72 hours, 7 days	
187.18037	7.92	31139	M+H/M+NH4	7 days	
270.11231	8.01	7509	M+H	24, 48 hours	
279.13387	8.16	8816	M+H	48, 72 hours	
321.15965	8.21	5646	M+H	48 hours	
355.17642	8.24	6412	M+H	48 hours	Zearalenol - hydration, oxidation (+H2O2)
					Zearalenone - hydration, oxidation, reduction (+H4O2)
205.13357	8.43	24137	M+H	72 hours, 7 days	
201.19610	8.81	100551	M+H	72 hours, 7 days	
150.00416	8.97	8350	M+H	48, 72 hours, 7 days	
198.00751	8.97	11947	M+H	48, 72 hours, 7 days	
146.06340	9.18	7517	M+H	72 hours, 7 days	
283.11892	9.39	14114	M+H	48 hours	
232.14440	9.65	8313	M+H	7 days	
187.18051	10.40	5869	M+H	7 days	
151.01199	10.90	5454	M+H	48, 72 hours, 7 days	
274.15488	10.98	20332	M+H	48 hours	
185.10731	11.09	6835	M+H	48, 72 hours, 7 days	
226.03877	11.20	15022	M+H	48, 72 hours, 7 days	
178.03545	11.20	6457	M+H	48, 72 hours, 7 days	
234.09133	11.30	9282	M+H	48, 72 hours	
249.10215	12.03	5294	M+H	48, 72 hours	
245.16477	12.26	15178	M+H/M+NH4	48, 72 hours	
329.18588	12.35	7313	M+H	48, 72 hours, 7 days	
321.15967	12.41	12514	M+H	48, 72 hours	
446.32640	12.85	6967	M+H	48, 72 hours, 7 days	
227.11781	12.91	11664	M+H	48 hours, 7 days	
305.16468	12.92	34609	M+H	48, 72 hours	
310.19119	13.04	14353	M+H	48, 72 hours	

335.17523	13.51	6960	M+H	48, 72 hours
382.33148	13.76	6674	M+H	48, 72 hours
261.05138	14.55	8314	M+H	48, 72 hours, 7 days
218.09973	15.16	8937	M+H	72 hours
252.08407	15.92	21138	M+H	48, 72 hours
292.18067	15.93	9768	M+H	48, 72 hours, 7 days
319.18038	16.47	7305	M+H	48, 72 hours
268.18064	16.74	34113	M+H	48, 72 hours, 7 days
305.16469	16.92	12068	M+H	48, 72 hours
350.31653	17.21	12152	M+H	48, 72 hours, 7 days
319.18039	17.81	12420	M+H	48, 72 hours
355.32056	18.84	5074	M+H	48, 72 hours, 7 days
447.39398	20.33	5598	M+H	7 days
461.40986	20.66	9583	M+H	48 hours, 7 days
359.29423	21.13	7689	M+H	48, 72 hours, 7 days

2) α-zearalenol results

Apex m/z	RT [min]	Max. Area	Ion Type	Time points	Possible transformation(s)	Comment
170.04248	0.79	5156	M+H	6, 24 hours		
142.93864	0.80	9341	M+H	24, 48 hours, 7 days		
164.92057	0.81	6090	M+H/M+Na	24, 48 hours, 7 days		
169.05847	0.83	6356	M+H/M+NH4	1, 2 hours		
304.97514	0.85	10422	M+H	24 hours		
220.00882	0.85	6760	M+H	48 hours, 7 days		
398.04124	0.86	5071	M+H	24 hours		
375.00414	0.87	5143	M+H	24 hours		
148.03450	0.88	16687	M+H	48 hours, 7 days		
243.05801	0.93	17237	M+H	48 hours, 7 days		
327.01939	0.93	5161	M+H	48 hours		
257.07349	0.94	10522	M+H	7 days		
224.12583	1.02	14887	M+H/M+Na	48 hours		
92.01684	1.04	11828	M+H	7 days		
190.08143	1.06	10181	M+H	7 days		
238.11875	1.07	13577	M+H	48 hours, 7 days		
229.04232	1.09	13253	M+H	7 days		
293.10100	1.10	85143	M+H	30 min, 7 days		
176.07948	1.18	6243	M+H	7 days		
132.11321	1.60	126742	M+H	6, 24, 48 hours, 7 days		

238.11877	3.51	22696	M+H	24, 48, 72 hours, 7 days	
255.14528	3.51	13753	M+NH4	24, 48, 72 hours, 7 days	M+NH4 of previous ion
195.11294	3.57	36704	M+H	24, 72 hours, 7 days	
312.19189	3.69	6693	M+H	30 min, 6, 24 hours	
255.14528	3.92	21748	M+H/M+NH4	30 min, 24, 48, 72 hours, 7 days	M+NH4 of following ion
238.11876	3.92	51687	M+H	30 min, 24, 48, 72 hours, 7 days	
277.12738	3.92	6864	M+H/M+Na	72 hours, 7 days	
173.16493	4.54	175707	M+H/M+NH4	48 hours, 7 days	
78.96755	4.74	5692	M+H	7 days	
106.99870	4.75	6964	M+H	72 hours, 7 days	
255.17026	4.90	5407	M+H	4 hours	
173.16493	5.10	216508	M+NH4	48, 72 hours, 7 days	
498.30664	5.16	5228	M+H/M+NH4	30 min, 6, 24 hours	
180.14961	5.21	7487	M+H	48 hours, 7 days	
170.07468	5.88	6122	M+H	7 days	
196.14469	6.02	37324	M+H	48, 72 hours	
118.03231	6.56	36646	M+H	72 hours, 7 days	
265.11841	6.60	42875	M+H	48 hours	
187.18054	6.88	42857	M+H/M+NH4	48, 72 hours, 7 days	
226.09754	7.61	5761	M+H	7 days	
202.04686	7.75	5299	M+H	7 days	
210.16023	7.76	76002	M+H	72 hours, 7 days	
287.11406	7.85	5470	M+H	72 hours	
187.18070	8.15	5013	M+H	72 hours	
279.13414	8.18	8394	M+H	48 hours	
355.17677	8.27	5720	M+H	24 hours	Zearalenol - hydration, oxidation (+H2O2) Zearalenone - hydration, oxidation, reduction (+H4O2)
205.13376	8.66	14889	M+H	48 hours	
221.12865	8.73	23789	M+H	48 hours	
150.00432	8.99	10965	M+H	48, 72 hours, 7 days	
198.00776	9.00	14612	M+H	48, 72 hours, 7 days	
201.19625	9.08	110719	M+H/M+NH4	48 hours, 7 days	
146.06351	9.20	17248	M+H	72 hours, 7 days	
187.12306	9.40	23338	M+H/M+NH4	7 days	
283.11915	9.41	20805	M+H	24, 48 hours	
170.09647	9.41	15392	M+H	7 days	
232.14468	9.92	15105	M+H	48, 72 hours, 7 days	
435.23935	10.76	7177	M+H	7 days	

151.01215	10.91	6728	M+H	48, 72 hours, 7 days
229.97983	10.92	5824	M+H	7 days
185.10752	11.12	7141	M+H	48 hours
274.15518	11.15	17017	M+H	24, 48 hours, 7 days
201.13875	11.18	6638	M+NH4	7 days
226.03904	11.21	17191	M+H	48, 72 hours, 7 days
178.03564	11.21	6865	M+H	48 hours, 7 days
234.09151	11.31	10362	M+H	48, 72 hours, 7 days
237.13874	12.02	6636	M+H/M+NH4	48 hours
321.15997	12.41	7414	M+H	48 hours
245.16505	12.46	15651	M+H/M+NH4	48 hours, 7 days
329.18614	12.58	5622	M+H	48, 72 hours, 7 days
258.01116	12.79	5124	M+H	48, 72 hours, 7 days
179.04344	12.79	5239	M+H	48 hours, 7 days
227.11810	12.92	9142	M+H	48 hours
305.16503	12.93	23773	M+H	48, 72 hours
310.19163	13.25	9024	M+H	48, 72 hours
335.17560	13.53	5598	M+H	48, 72 hours
218.09995	15.17	10205	M+H	48, 72 hours
250.08640	15.57	5697	M+H	48, 72 hours, 7 days
252.08429	15.92	20573	M+H	48, 72 hours
292.18102	16.16	5883	M+H	48 hours
319.18076	16.71	6022	M+H	48, 72 hours
268.18097	17.04	38928	M+H	48, 72 hours
305.16501	17.15	8416	M+H	48 hours
350.31685	17.18	7479	M+H	48 hours, 7 days
319.18069	18.09	9015	M+H	48 hours, 7 days
355.32095	18.84	5349	M+H	48 hours, 7 days
360.14941	19.19	19173	M+H	7 days
339.28963	20.68	6102	M+H	6, 24, 48 hours
461.41044	20.95	8902	M+H	48 hours, 7 days

3) ß-zearalenol results

Apex m/z	RT [min]	Max. Area	Ion Type	Time points	Possible transformation(s)	Comment
220.00840	0.85	5856	M+H	48, 72 hours, 7 days		
163.04746	0.87	5508	M+H	24 hours		
148.03424	0.89	16392	M+H	48, 72 hours, 7 days		
182.07857	0.90	7793	M+H	7 days		

241.00302	0.93	11616	M+H	48 hours, 7 days		
243.05752	0.93	19165	M+H	48, 72 hours, 7 days		
257.07317	0.97	10790	M+H	7 days		
291.05754	1.00	5143	M+H	7 days		
227.00778	1.00	12370	M+H	7 days		
323.09714	1.01	5153	M+H	1, 24 hours		
210.10982	1.02	12235	M+H	7 days		
224.12544	1.03	9444	M+H	7 days		
238.11821	1.05	11685	M+H	48 hours, 7 days		
293.10041	1.09	110272	M+H	24, 72 hours, 7 days		
263.02633	1.10	5788	M+H	7 days		
195.03899	1.16	8343	M+H	7 days		
176.07925	1.19	6800	M+H	7 days		
132.11287	1.67	140488	M+H	24, 48 ,72 hours		
159.14899	3.21	97351	M+H/M+NH4	48 hours, 7 days		
277.12668	3.53	5283	M+H	48 hours, 7 days		
238.11821	3.53	20465	M+H	6, 24, 48, 72 hours, 7 days		
255.14468	3.54	12399	M+NH4	6, 48, 72 hours, 7 days	M+NH4 of previous ion	
312.19114	3.70	7698	M+H	6, 24 hours		
255.14469	3.94	20467	M+NH4	24, 48 ,72 hours, 7 days	M+NH4 of following ion	
238.11821	3.94	48082	M+H	6, 24, 48 ,72 hours, 7 days		
159.14896	3.95	70960	M+NH4	48 hours, 7 days		
277.12664	3.95	5107	M+H	48, 72 hours, 7 days		
382.17893	3.95	8264	M+H	48 hours, 7 days		
173.16465	4.64	189643	M+H/M+NH4	24, 48 ,72 hours, 7 days		
216.15917	4.71	12482	M+H	7 days		
534.29176	4.86	5387	M+H	6, 24,48 hours, 7 days	Zearalenol - arginine conjugation, glycine conjugation (+C8H15N5O2) Zearalenone - reduction - arginine conjugation, glycine conjugation (+C8H17N5O2)	
			M+NH4		Zearalenol glucoside - hydration, reduction, methylation (+CH6O) Zearalenol - hydration, reduction glucoside conjugation, methylation (+C7H16O6) Zearalenol - desaturation, acetylation, arginine conjugation (+C8H12N4O2) Zearalenone - acetylation, arginine conjugation (+C8H14N4O2)	
78.96742	4.97	7414	M+H	48 hours, 7 days		
106.99851	4.97	9948	M+H	48 hours, 7 days		
173.16465	5.23	285019	M+H/M+NH4	48, 72 hours, 7 days		
216.20661	5.35	5107	M+H	48 hours		

170.07447	5.93	6985	M+H	7 days	
196.14427	6.09	45908	M+H	48 hours, 7 days	
118.03219	6.58	23905	M+H	72 hours	
265.11783	6.63	61552	M+H	24, 48 hours	
187.18028	7.01	46123	M+H/M+NH4	48, 72 hours, 7 days	
523.25475	7.33	5187	M+Na	2, 4, 6 hours	Zearalenol - dehydration, acetylation, arginine conjugation (+C8H12N4O)
			M+H		Zearalenone - dehydration, reduction, acetylation, arginine conjugation (+C8H14N4O)
			M+NH4		Zearalenol glucoside - dehydration, reduction, acetylation, methylation (+C3H4)
					Zearalenol - dehydration, dehydration, ornithine conjugation, taurine conjugation (+C7H11N3OS)
123.07913	7.77	7103	M+H	72 hours, 7 days	
202.04653	7.78	5775	M+H	72 hours, 7 days	
210.15990	7.79	38364	M+H	72 hours, 7 days	
287.11360	7.85	6256	M+H	72 hours, 7 days	
132.04771	7.85	5432	M+H	72 hours	
270.11211	8.02	9052	M+H	48, 72 hours	
244.10771	8.12	42793	M+H	24 hours	
279.13350	8.17	10301	M+H	48, 72 hours	
187.18025	8.27	27859	M+H/M+NH4	48, 72 hours	
355.17597	8.28	5475	M+H	24, 48 hours	Zearalenol - hydration, oxidation (+H2O2)
					Zearalenone - hydration, oxidation, reduction (+H4O2)
203.11776	8.30	10038	M+H	7 days	
205.13336	8.83	23236	M+H	48, 72 hours	
221.12809	8.92	30361	M+H	48 hours	
150.00405	8.99	14776	M+H	48, 72 hours, 7 days	
118.03216	8.99	6933	M+H	48, 72 hours, 7 days	
198.00735	8.99	20852	M+H	48, 72 hours, 7 days	
146.06325	9.18	12323	M+H	48, 72 hours, 7 days	
201.19596	9.21	81628	M+H/M+NH4	48, 72 hours, 7 days	
283.11842	9.42	22569	M+H	24, 48 hours	
170.09631	9.55	6721	M+H	7 days	
187.12286	9.55	9880	M+H	7 days	
335.15980	9.56	5740	M+H	7 days	
234.01857	9.63	7977	M+H	48, 72 hours, 7 days	
232.14424	10.04	27815	M+H/M+NH4	48, 72 hours, 7 days	
212.02290	10.11	7154	M+H	48 hours	
260.13893	10.43	6417	M+H	24 hours	
187.18034	10.87	6735	M+H	7 days	

151.01192	10.91	10557	M+H	72 hours, 7 days
229.97948	10.91	7873	M+H	72 hours, 7 days
185.10711	11.13	7671	M+H	48 hours
226.03855	11.20	19314	M+H	48, 72 hours, 7 days
178.03528	11.20	7048	M+H	48, 72 hours, 7 days
234.09105	11.30	8974	M+H	48, 72 hours
274.15448	11.31	23853	M+H	24, 48 hours, 7 days
249.10193	12.01	5567	M+H	48, 72 hours, 7 days
321.15928	12.40	10517	M+H	48, 72 hours
245.16446	12.62	15412	M+H/M+NH4	24, 48 ,72 hours
329.18551	12.71	6690	M+H	24, 48, 72 hours, 7 days
446.32590	12.72	6054	M+H	48, 72 hours, 7 days
179.04311	12.76	5343	M+H	48, 72 hours, 7 days
258.01065	12.76	5574	M+H	48, 72 hours
227.11748	12.90	11504	M+H	48, 72 hours
305.16437	12.91	23015	M+H	48, 72 hours
310.19086	13.42	12772	M+H	48, 72 hours
335.17491	13.51	7039	M+H	48, 72 hours
218.09959	15.13	9108	M+H	48, 72 hours
250.08600	15.54	5803	M+H	48, 72 hours
254.01235	15.61	5383	M+H	72 hours
292.18038	16.39	8277	M+H	48, 72 hours
319.18014	16.85	7085	M+H	48, 72 hours
350.31635	17.14	14494	M+H	48 hours, 7 days
268.18035	17.35	32076	M+H	48, 72 hours
305.16439	17.38	11585	M+H	48, 72 hours
340.93607	18.01	6617	M+H	72 hours, 7 days
319.18003	18.31	9804	M+H	48, 72 hours
355.32022	18.84	5336	M+H	48, 72 hours, 7 days
359.29393	21.05	8501	M+H	48, 72 hours, 7 days
461.40970	21.14	9203	M+H	48, 72 hours, 7 days
369.38354	24.40	5278	M+H	72 hours, 7 days