Chapter 4

The Incidence of T-2 and HT-2 Toxins in Cereals and Methods of their Reduction Practice by the Food Industry

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Additional information is available at the end of the chapter

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Abstract

T-2 toxin and its major metabolite HT-2 toxin are type A trichothecene mycotoxins produced by the *Fusarium* moulds, present mainly in cereals and cereal-based products. The studies quoted under this chapter bring data on the incidence of T-2 and HT-2 toxin in unprocessed cereals (n = 285) harvested in Croatia during a two-year period. They also demonstrate the influence of certain thermal food processing methods on the reduction of T-2 and HT-2 toxin in naturally contaminated cereals. In analysed cereals, the highest percentage of T-2- and HT-2-positive samples was determined in oat samples (56.9%), followed by triticale (34.5%), maize (32.1%) and barley samples (22.7%), whereas the highest mean (94.8 ± 63.7 μg/kg) and maximal concentration (420 μg/kg) of the toxins in reference were determined in maize. The summary T-2/HT-2 concentrations found in one maize and one triticale sample were higher than the indicative levels, necessitating further sampling and investigations of conditions under which the production of these toxins takes place. Thermal food processing in terms of roasting (which enabled 28.8–54.4%-toxin reduction) and especially extrusion (which enabled 73.0–92.5%-toxin reduction) efficiently reduces T-2/HT-2 levels, whereas cooking does not significantly aid in their reduction (<10%-toxin reduction achieved).

Keywords: T-2 toxin, HT-2 toxin, cereals, occurrence, thermal reduction, food industry

1. Introduction

Worldwide, cereals are at risk from contamination with mycotoxins, i.e. secondary mould metabolites, both while still in field and during storage. *Fusarium* fungi produce several

trichothecene mycotoxins having a common chemical structure and a similar mode of action [1]. Among them, T-2 toxin (3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutoxy)-12,13-epoxytrichothec-9-ene), and its major metabolite HT-2 toxin (3α,4β-dihydroxy-15-acetoxy-8α-(3-methylbutoxy)-12,13-epoxy-trichothecene), represent not only type A trichothecene mycotoxins produced mainly by the *Fusarium langsethiae*, but also the *Fusarium poae* and the *Fusarium sporotrichioides* [2, 3]. These toxins are produced at temperatures ranging from −2 to 35°C and with water activities above 0.88 [4, 5], and are frequently responsible for the contamination of different grains such as maize, oat, barley, wheat, rice and soya beans. Weather conditions, varieties and the sowing time are the most important factors influencing the T-2 and HT-2 toxin production [6].

Type A trichothecenes, T-2 and HT-2 toxins included, are generally more toxic than type B trichothecenes (e.g. deoxynivalenol and nivalenol). Structure/activity-relationship studies revealed that 12,13-epoxide group and C9-C10-double bond are essential for their toxicity [7]. Toxicological studies show that T-2 toxin is a very potent cytotoxic and immunosuppressive agent, which can cause acute intoxication and chronic diseases in both humans and animals [6]. Given that T-2 toxin is metabolised into HT-2 toxin after ingestion, they are considered to be equally toxic [8]. The symptoms of acute T-2 intoxication of different mammalian species include skin necrosis, asthenia, lack of appetite, panting, vomiting, diarrhoea, anorexia, myocardial damage, lethargy, as well as haemorrhages and necrosis of the epithelium of the stomach and intestines, bone marrow, spleen, testis and ovary [1, 8–10]. The International Agency for Research on Cancer (IARC) classified T-2 toxin into Group 3 carcinogens because of the lack of data on its carcinogenicity in humans and only limited evidence on its carcinogenicity in experimental animals [11].

Data collected in a number of European countries have shown substantial variations in *Fusarium* mycotoxin levels across various cereal types, various countries and various investigated periods [12, 13]. Croatia, as a Central European country, falls into the group of countries in which contamination with *Fusarium* mycotoxins is a frequent occurrence [14, 15]. Since data on the occurrence of T-2 and HT-2 toxins in cereals and cereal by-products are still very limited, the European Commission recommended the member states to gather reliable data on year-to-year variations of these mycotoxins in order to be able to establish their maximal levels (MLs) in different food and feed in the near future [6, 16, 17]. In 2013, the European Commission gave recommendations (Commission Recommendation 2013/165/EU) regarding indicative levels of T-2 and HT-2 toxin in cereals and cereal-based products intended for food and feed. In this document, the Commission also recommended further investigations into the effects of food processing and agronomic factors on the presence of T-2 and HT-2 toxin and different factors favouring high level-contaminations with these toxins, so as to be able to identify measures to be taken to avoid or reduce the above [18]. In view of the evidenced toxicity of T-2 and HT-2 toxin, there exists the need for further collection of data on their presence in different cereals intended for food and feed production. In their recent study, Pleadin et al. [17] stated that further studies shall also be performed in Croatia in order to investigate into the conditions favouring T-2 and HT-2 production and to identify measures that are to

be implemented in order to prevent contamination during cultivation and storage of cereals and their final products.

Literature data on the impact of cereal processing on the levels of T-2 and HT-2 contamination are also very scarce. Data gathered insofar have suggested that toxins in reference, when milled, get to be relocated into various milling fractions, but are not eliminated [19]; in addition, they have been proven resistant to processing. Even more so, due to their hull binding, subsequent processing of cereals leads to significant rise in levels of these toxins in finished products [16]. Scudamore [20] concluded that final processing, such as boiling, fermentation, baking, frying, and extrusion, has no impact on the level of contamination with these mycotoxins. Some studies evidenced that processing of raw cereals greatly reduces T-2 and HT-2 levels in food products, but makes these toxins concentrate in high levels in by-products [6]. In industrial food processing settings, the processing time and temperature combination has been shown to be crucial for the reduction of mycotoxin content in a finished food product. While conventional food preparation at temperatures up to 100°C has a negligible effect on most mycotoxins, higher temperatures used with frying, roasting, toasting and extrusion have been shown to be capable of decreasing mycotoxin contamination [21].

The studies quoted under this chapter bring data on the incidence of T-2 and HT-2 toxin in different unprocessed cereals during a two-year period. They also demonstrate the influence of certain food processing methods, such as cooking, roasting and extrusion, performed under predefined conditions, on the levels of T-2 and HT-2 toxin in contaminated cereals. For this purpose, after application of the quantitative screening method termed the enzyme-linked immunosorbent assay (ELISA), which enabled the determination of summary T-2 and HT-2 toxin concentrations, the concentrations of each mycotoxin in positive samples were determined using a confirmatory method in terms of liquid chromatography tandem-mass spectrometry (LC-MS/MS), also used in the investigations devoted to the possibilities of reduction of concentrations of these mycotoxins.

2. Materials and methods

2.1. Sampling and sample preparation

During the period spanning from May 2015 to April 2017, a total of 285 samples of unprocessed cereals, in specific maize (n = 84), wheat (n = 56), oat (n = 72), barley (n = 44) and triticale (n = 29), were sampled from households situated in the Northern, Central and Eastern part of Croatia. All cereals were grown in the crop season 2015 and 2016 and had not undergone any physical or thermal treatment other than drying, cleaning and sorting prior to sampling. Sampling and sample preparation of unprocessed cereals were performed in full line with the Commission Regulation 401/2006 [22], laying down the methods of sampling to be exercised within the frame of the official control of mycotoxin levels in foodstuffs. The aggregate

cereal samples were combined of three, five or ten incremental samples, depending on the lot weight, each lot thereby weighing at least 1 kg.

The samples were stored in a cool and dry place and transported to the laboratory within 48 h. The prepared test portions (500 g per sample) were ground into a fine powder having a particle size of 1.0 mm using an analytical mill (Cylotec 1093, Tecator, Sweden), and then stored at 4°C pending analyses.

2.2. Chemicals, standards and reference materials

T-2 toxin (Art. No. 34071, 100 μg/mL in acetonitrile) and HT-2 toxin (Art. No. 34136, 100 μg/mL in acetonitrile) standards were provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). A RIDASCREEN® T-2/HT-2 toxin kit (Art. No. R3805) was provided by R-Biopharm (Darmstadt, Germany). PuriTox Total Myco-MS solid phase clean-up columns (Art. No. TC-MT3000) were produced by R-BiopharmRône LTD (Glasgow, Scotland). The Certified Reference oat flour Material (CRM) (Art. No. TET039RM) having the reference values of 85.3 ± 13.7 μg/kg for T-2 toxin and 86.9 ± 11.9 μg/kg for HT-2 toxin, was purchased from Fapas, Fera Science Ltd. (York, UK).

All chemicals used for ELISA and LC-MS/MS analyses were of an analytical grade (acetic acid, Art. No. 33209, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or a HPLC grade (acetonitrile, Art. No. 34851, and methanol, Art. No. 34885 Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Ultrapure water was supplied by the Merck system Direct-Q 3 UV (New Jersey, USA).

2.3. Analytical methods

All samples were first analysed using the validated ELISA method. After grinding, to 5 g of a homogenised sample, 25 mL of methanol/distilled water solution (70/30; v/v) were added, except for oat samples, to which 25 mL of the appropriate extraction buffer provided with the ELISA kit were added. The extraction of T-2/HT-2 toxin was performed using a head-over-head shaker for 10 min (100 rpm, room temperature) followed by an additional 10-min centrifugation (4000 rpm, room temperature). When it comes to oat samples, the obtained supernatant was diluted in methanol/distilled water (70/30; v/v) in 1:2 ratio, while the supernatant obtained with all other biological materials under study was diluted in distilled water in 1:2 ratio. The obtained solutions were then transferred into the wells of the ELISA microtitration plate. The ELISA tests were performed using a ChemWell autoanalyser (Awareness Technology Inc. 2910, Palm City, USA), observing thereby the instructions given by the kit provider. Once the stop solution had been injected, the absorbances were determined at 450 nm. In order to calculate the summary T-2/HT-2 concentration in an individual sample, the results provided by the calibration curve were multiplied by the corresponding sample dilution factor. The calculation of the summary toxin concentrations was guided by the average recovery values ascertained by method validation.

Samples, in which T-2/HT-2 concentrations higher than the ELISA's Limit of Detection were established, were further analysed using the LC-MS/MS method. To that effect, to 2.5 g of a test sample, 10 mL of 80%-acetonitrile were added and vortexed for 30 s; afterwards, the samples

were put on a head-over-head shaker for 10 min (100 rpm, room temperature). The samples were then centrifuged (10 min, 4000 rpm, room temperature). Two millilitres of the obtained supernatant were acidified with 20 μL of (glacial) acetic acid; after that, 1.4 mL of the obtained solution was passed through the PuriTox Total Myco-MS columns (R-Biopharm, Glasgow, Scotland). Five hundred microliters were then evaporated under a nitrogen stream at 40°C and reconstituted in 250 μL of 1%-acetic acid in 20% acetonitrile. Fifty microliters were injected onto the LC-MS/MS system. The LC-MS/MS system consisted of a degasser, a binary pump, an auto-sampler and a column compartment (Infinity 1260, Agilent Technologies, Santa Clara, USA) coupled with a triple quadrupole mass detector (6410 QQQ, Agilent Technologies, Santa Clara, USA). The chromatography separation was performed on an XBridge BEH C18 column (particle size 2.5 μm, dimensions 4.6 × 150 mm) (Waters, Milford, Massachusetts, USA). The mobile phase consisted of 0.1%-acetic acid (constituent A) and acetonitrile (constituent B). The flow rate was 0.8 mL/min and the temperature was set at 40°C. A gradient elution program was employed: 0–3 min 90% A, 18 min 10% A, 18.1 min 90% A with a 3-minute post-run time. Mass spectrometry conditions were as follows: electrospray ionisation (ESI), positive polarity, source temperature 350°C, gas flow 9 L/min, nebulizer 45 psi, and capillary voltage 6 kV. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. **Table 1** shows the ions monitored within the frame of LC-MS/MS analyses targeted at T-2 and HT-2 toxin. The final concentrations of T-2 and HT-2 toxin were calculated based on the dilution factor and the average recovery values obtained during the validation process.

2.4. Validation of the analytical methods used

For the ELISA method, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the mean value of 10 control samples of a given cereal (maize, wheat, oat, barley or triticale) plus 3- and 10-fold standard deviation. For each cereal, the recoveries were determined in three replicates per concentration level per day. To that goal, the control samples were supplemented with 50% T-2 and 50% HT-2 toxin standard working solutions (either 500 μg/L or 1000 μg/L).

As for the LC-MS/MS method, the LOD and LOQ values were estimated according to the Guidance document on the estimation of LOD and LOQ for measurements in the field of contaminants in feed and food [23] via paired observations. In brief, 10 pseudo-blank samples of

Mycotoxin	Precursor ion	Fragmentor voltage (V)	Product ions	Collision energy (eV)	Cell accelerator voltage (V)
T-2 toxin	489.2	200	387.1[b]	20	1
			245.1[a]	27	
HT-2 toxin	447.2	100	345.1[a]	18	1
			285.1[b]	20	

[a]A more intense ion—used as a quantifier.

[b]A less intense ion—used as a qualifier.

Table 1. Ions monitored within the frame of LC-MS/MS analyses targeted at T-2 and HT-2 toxin.

different cereals, i.e. maize, oat, wheat, barley and triticale, were chosen based on the results obtained using the ELISA technique (<LOD) and spiked with the T-2 and HT-2 standard at the level of 25 μg/kg. Then, both spiked and pseudo-blank samples were analysed, and the difference in signal abundances and the standard deviation was calculated. The obtained data were used for the calculation of the LOD and the LOQ. Linearity was tested in the range of 5–100 ng/mL, using the standard solution of each mycotoxin. The recovery was determined by virtue of analysing six blank maize samples spiked with T-2 and HT-2 toxin at 50 μg/kg, while the trueness was tested using six replicates, making use of oat flour as the CRM, and later compared with the values assigned for each mycotoxin by the manufacturer.

2.5. Thermal processing

Three samples, which were found to be the most contaminated with T-2 and HT-2 toxins, underwent thermal processing in terms of cooking, roasting and extrusion. These samples and the concentrations of T-2/HT-2 toxins in them were as follows: a maize sample (summary toxin concentration 384 μg/kg that of T-2128 μg/kg and that of HT-2256 μg/kg), an oat sample (summary toxin concentration 267 μg/kg that of T-2107 μg/kg and that of HT-2160 μg/kg) and a triticale sample (summary toxin concentration 151 μg/kg, that of T-2 47.0 μg/kg, and that of HT-2104 μg/kg). From each sample, three parallels were used during processing, and after that three replicates of each were used for the analyses.

The above described contaminated maize, oat and triticale samples were cooked in boiling water (96°C) for 10, 20 and 30 min. After that, cereals were filtered and the samples were left to dry overnight. As for roasting, the contaminated cereals were roasted in an oven (LV9/11/P 320, Nabertherm, Germany) at three different temperatures (180°C, 200°C and 220°C) for 30 min (at each temperature). Once cooked and roasted, the cereals were milled into a fine powder having a particle size of 1.0 mm using an analytical mill (Cyclotec 1093, Tecator, Sweden), intended to be analysed for the levels of T-2 and HT-2 toxin using the LC-MS/MS method.

Before the extrusion cooking, all samples were milled using an IKA MF10 laboratory mill (IKA Werke GmbH, Staufen, Germany) having a 2-mm sieve. The blend preparation was performed based on 1 kg d. m. The samples were conditioned at 25% moisture by spraying an adequate amount of distilled water, while continuously mixed using a laboratory mixer (Kenwood KMM020, JVC Kenwood, Uithoorn, The Netherlands). The prepared mixtures were then put into plastic bags (one bag per sample) and stored overnight in the refrigerator at 4°C in order to equilibrate the moisture. Before the extrusion, the samples were brought to room temperature. The prepared samples were extruded in a single-screw laboratory extruder (Brabender GmbH, Model 19/20DN, Duisburg, Germany) at three different temperature profiles: 135/150/150°C; 135/170/170°C and 135/190/190°C (extruder's dosing/compression/ejection zone). Other constant extrusion parameters were as follows: screw: 4:1; die: 4 mm; screw speed: 100 rpm; and dosing speed: 40 rpm. After the extrusion, the samples were air-dried overnight.

In all cereal samples, the moisture content was measured before and after thermal processing by taking a 5-g sample and heating it in an oven at 105 ± 2°C. Moisture was determined according to the 712:2009 ISO standard [24].

2.6. Data analysis

Concentrations (μg/kg) obtained by the ELISA assay are expressed as mean summary values (T-2/HT-2) ± standard deviation (SD), while in toxin-positive samples, the concentrations are given as individual concentrations of each mycotoxin (T-2 or HT-2) obtained by the LC-MS/MS. Statistical analysis was performed using the Statistica ver. 10.0 software (StatSoft Inc. 1984-2011, Tulsa, OK, USA) and made use of the analysis of variance (ANOVA), the statistical significance thereby being set at 95% ($p = 0.05$).

3. Results and discussion

3.1. Validation of analytical methods used

The ELISA assay, used as a quantitative screening method for the determination of summary concentrations of T-2/HT-2 toxins, was first validated and then applied for the analyses of the sampled cereals. Its cross-reactivity declared by the kit manufacturer is approximately 85% for T-2 toxin and 100% for the HT-2 toxin. Validation of the ELISA method resulted in LODs ranging from 20.6 to 30.1 μg/kg and LOQs ranging from 26.7 to 37.4 μg/kg, depending on the type of cereal under consideration. The mean recovery value equalled to 90.1%, with the mean coefficient of variation (CV) of 7.8% (**Table 2**).

Validation of the LC-MS/MS method resulted in LODs spanning from 5.5 to 8.3 μg/kg, and LOQs ranging from 18.2 to 27.5 μg/kg for T-2 and HT-2 toxin, respectively. The mean recovery values were 89.6 and 77.0%, with the mean CV of 5.1% and 8.9%, respectively. The analyses of oat flour sample (CRM) used for the determination of trueness, resulted in concentrations of 77.9 μg/kg for T-2 (91.3% of the mean certified value) and 75.6 μg/kg for HT-2 (86.9% of the mean certified value). **Figure 1** presents a typical LC-MS/MS-MRM chromatogram of the analysed CRM. The determination of linearity resulted in correlation coefficients higher than 0.99 for both analytes (**Table 3**).

Given the obtained validation results, both analytical methods were recognised as suitable for analyses of different cereals, the ELISA assay thereby being employed as a screening method used for the determination of summary concentrations of T-2/HT-2 toxins, and the LC-MS/MS thereby being used as a confirmatory method exploited to the effect of determination of individual concentrations of mycotoxins under study.

3.2. The occurrence of T-2/HT-2 toxin in cereals

Data published insofar have revealed that the exposure to T-2 and HT-2 toxins primarily comes as a result of the consumption of cereal grains and cereal by-products, wherein the levels of these toxins found in forages and oilseed meals are generally low. It has been established that T-2 and HT-2 toxins occur together, HT-2 thereby representing approximately two-thirds of the summary T-2/HT-2 concentration. The highest mean T-2/HT-2 concentrations were determined in grains and milled grain products, in particular in oat and oat-based

Cereals	LOD (μg/kg)	LOQ (μg/kg)	Level of fortification (μg/kg)	Mean recovery[a] (%)	CV (%)
Maize	20.6	26.7	50	94.3	6.3
			100	97.8	5.8
			200	100.5	9.2
Wheat	27.3	33.6	50	90.7	7.2
			100	89.3	8.3
			200	95.8	6.4
Oat	25.8	31.9	50	72.2	6.1
			100	75.3	7.6
			200	78.5	7.9
Barley	30.1	37.4	50	88.3	7.4
			100	92.6	8.9
			200	94.8	10.3
Triticale	26.2	34.7	50	90.7	8.6
			100	94.1	7.7
			200	96.2	9.3

[a]Three replicates per concentration level per day; analyses were performed by spiking the negative material (before validation, confirmed by the LC-MS/MS).

LOD: limit of detection; LOQ: limit of quantification; CV: coefficient of variation.

Table 2. Validation of the ELISA method used for the determination of summary concentrations of T-2/HT-2 toxins in cereals.

products, the aforementioned applying equally for food, feed and raw cereals. Higher toxin concentrations established in unprocessed vs. processed grains indicate that grain processing succeeds in lowering, at least to some point, both T-2 and HT-2 concentrations [16].

In this study, the levels of T-2 and HT-2 were analysed in different unprocessed cereals (maize, wheat, oat, barley and triticale) sampled from Croatian households during a two-year period. In the first study step, summary concentrations of these toxins were established using the ELISA method. The results obtained in unprocessed cereal samples are shown in **Table 4**.

The study results show a significantly higher ($p < 0.05$) incidence of T-2/HT-2 toxins in oats (56.9%) in comparison to other unprocessed cereals. However, the maximal summary concentration was determined in maize sample (420 μg/kg), but the maximal mean value was observed in oat (136 ± 55.6 μg/kg). The results also show that in two samples, one maize (mentioned above) and one triticale (169 μg/kg), summary concentrations of T-2/HT-2 were higher than stipulated indicative levels from which onwards/above investigations should be performed in case of repetitive findings according to the Commission Recommendation [18]. Such a finding imposes the need for further sampling and investigations of the causes of such a high level of mycotoxin contamination. The lowest percentage of positive samples was

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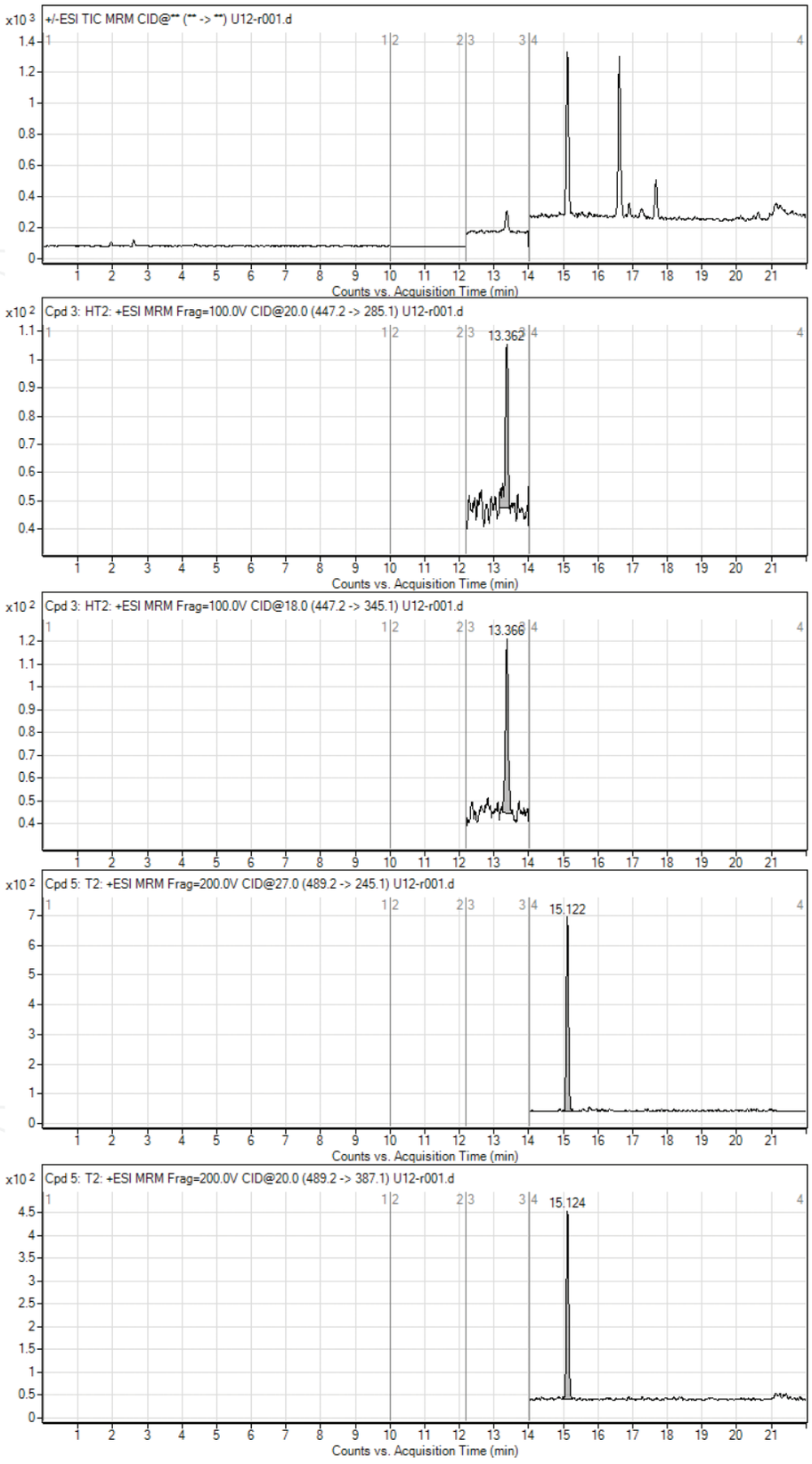

Figure 1. A typical LC-MS/MS-MRM chromatogram of the certified reference material (CRM, oat flour) with values of 77.9 μg/kg determined for T-2 and 75.6 μg/kg determined for HT-2; TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

Analyte	LOD (μg/kg)	LOQ (μg/kg)	Correlation coefficient	Mean recovery[b] (%)	CV (%)	Trueness[c] (%)
T-2 toxin	5.5	18.2	0.998	89.6	5.1	91.3
HT-2 toxin	8.3	27.5	0.995	77.0	8.9	86.9

[a]Samples in which T-2/HT-2 summary concentrations were higher than the LOD of the ELISA method.

[b]Six blank maize samples spiked at 50 μg/kg per day.

[c]Six oat flour CRM replicates with the assigned reference values of 85.3 ± 13.7 μg/kg for T-2 and 86.9 ± 11.9 μg/kg for HT-2 toxin.

LOD: limit of detection; LOQ: limit of quantification; CV: coefficient of variation.

Table 3. Validation of the LC-MS/MS method used for the determination of T-2 and HT-2 toxin in positive cereal samples[a].

established in the wheat (21.4%) and barley sample pool (22.7%), in which the lowest mean T-2/HT-2 concentrations were determined, as well.

Literature data mainly address individual T-2 toxin concentrations rather than the summary T-2/HT-2 concentrations. Of note, the level of contamination greatly varies depending on the geographical region (country), the period of investigation and the type of cereal and/or final product investigated [25–27]. It is known that T-2/HT-2 toxins are produced by moulds of the *Fusarium* genus in high-temperature environments, with the maximum productivity at temperatures lower than 15°C accompanied by high relative humidity (of 60–95%) [3, 5]. Higher T-2/HT-2 concentrations ascertained in certain samples (although below the indicative levels) come, first and foremost, as a consequence of the plenitude of rain that facilitated the production of *Fusarium* moulds and therefore also the production of T-2/HT-2 toxins. The occurrence of these toxins in higher concentrations is also linked to other factors that could cause their formation independent of climate conditions.

T-2 toxin levels in different food components retrieved from different parts of the world were found to range from 6 to 2406 μg/kg [28–33]. Investigations performed in eight European countries on over 3000 samples showed 20% of T-2-positive and 14% of HT-2-positive samples. In the United Kingdom, T-2 was found in 16% of wheat and 12% of barley samples, the LOD thereby being 10 μg/kg. When it comes to oat, T-2 was identified in 84% of samples, with the mean and

Cereals	Indicative level[a] (μg/kg)	Positive (%)	Mean[b] (μg/kg)	SD (μg/kg)	Min (μg/kg)	Max (μg/kg)
Maize (n = 84)	200	32.1	94.8	63.7	26.9	420
Wheat (n = 56)	100	21.4	65.6	25.2	29.4	50.6
Oat (n = 72)	1000	56.9	136	55.6	32.4	273
Barley (n = 44)	200	22.7	61.3	20.6	31.6	70.2
Triticale (n = 29)	100	34.5	76.6	30.4	36.0	169

[a]Indicative levels of the summary T-2/HT-2 concentrations from which onwards/above investigations should be performed in case of repetitive findings according to the Commission Recommendation 2013/165/EU [18].

[b]Mean concentration found in positive samples (>LOD).

Table 4. Summary concentrations of T-2/HT-2 toxins determined in unprocessed cereal samples using the ELISA method.

the maximal concentration of 84 and 2406 μg/kg, respectively [31–33]. In a study performed in Germany, toxin concentrations found in oats varied from 14 to 214 μg/kg [34]. Within the 2005–2008 timeframe, T-2 toxin was detected in 50% of different barley cultivars coming from the Czech Republic, in the mean concentration of 30 μg/kg [35]. In Serbia, the toxin concentrations determined in 54 analysed samples were lower than the LOD of 0.3 μg/kg [36]. The most frequently *Fusarium*-contaminated T-2-positive food items are maize (28%), wheat (21%) and oats (21%); when it comes to the HT-2 toxin, the most frequently contaminated foodstuffs are oat (41%), corn (24%) and rye (17%) [37]. In a study by the JECFA [8], the T-2 contamination rate was found to be 11% on the overall, with annual variations dependent on the cereal type. In the large-scale study by Pettersson et al. [38] on 243 raw oat samples, 529 oat flake samples and 105 oat meal samples, T-2 contamination was corroborated in 73%, 24% and 17% of samples, respectively, the mean concentrations of the toxin thereby being 32 μg/kg, 5 μg/kg and 4 μg/kg, respectively.

Data published earlier in Croatia showed the T-2 toxin presence in 57% of maize, 25% of wheat, 32% of barley and 18% of oat samples [15]. The highest detected concentration of T-2 toxin in maize was 210 μg/kg, with the pertaining mean value of 110 μg/kg pointing towards a *Fusarium*-induced maize contamination that occurred after heavy rainy periods [14]. In a study performed on different cereals harvested in 2011, the maximal level of T-2 toxin was 42 μg/kg, with the mean value of 24 ± 27 μg/kg found in maize the maximal and the mean T-2 level found in oat on the same occasion was 10 μg/kg and 7 ± 2 μg/kg, respectively [15]. In the study by Pleadin et al. [17], the highest mean summary concentration of T-2/HT-2 toxins was, as also in this study, found in oats (102.2 ± 73.6 μg/kg), followed by maize (63.1 ± 36.7 μg/kg) and barley (51.7 ± 18.6 μg/kg), while the lowest concentration was found in wheat (34.3 ± 11.2 μg/kg). The maximal toxin level was determined in oats (304.1 μg/kg), but all of the obtained summary T-2/HT-2 concentrations were below the indicative levels necessitating further investigation as advised under the Commission Recommendation 2013/165/EU [18].

In this study, after the implementation of the ELISA method, which was used for the determination of summary values of T-2/HT-2 toxins in all samples under investigation, the LC-MS/MS method was implemented for the determination and confirmation of each mycotoxin in positive samples (>LOD). **Table 5** shows individual levels of T-2 and HT-2 toxin determined using the LC-MS/MS method.

Cereals	T-2 toxin (μg/kg)				HT-2 toxin (μg/kg)				Share T-2:HT-2[a]
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Maize (n = 25)	27.6	10.7	23.1	128	66.3	21.9	30.9	256	1:2.4
Wheat (n = 12)	23.1	6.8	18.2	42.4	39.0	16.1	28.4	89.4	1:1.7
Oat (n = 44)	45.2	21.1	32.4	107	89.1	38.3	31.6	160	1:2.0
Barley (n = 10)	22.4	8.7	18.5	50.3	37.6	15.9	28.0	80.4	1:1.7
Triticale (n = 11)	26.4	6.8	21.0	47.0	47.6	24.9	27.7	104	1:1.8

[a]the share of mean values of T-2 and HT-2.

Table 5. Levels of T-2 and HT-2 toxin determined in positive unprocessed cereal samples using the LC-MS/MS method.

When the individual concentrations of T-2 and HT-2 toxin obtained by the LC-MS/MS method were summed up, it was established that the latter sum slightly differs from, and is mainly lower than, the summary concentrations of these mycotoxins determined using the ELISA method. This can be explained by the cross-reactivity and a lower specificity of the ELISA method. It is known that the ELISA represents an easy-to-use purification technique with a lesser need for an extensive clean-up, but it may suffer from undesired cross-reactivity with other trichothecenes that give rise to metric uncertainty [16].

Figure 2 presents the LC-MS/MS-MRM chromatogram of the most contaminated sample (maize), in which the concentration of T-2 toxin of 128 μg/kg and the concentration of HT-2 toxin of 256 μg/kg was determined (summary T-2/HT-2 toxins concentration, 384 μg/kg). Together with the most contaminated oat and the most contaminated triticale sample, this sample was further subjected to thermal processing.

Based on the comparison of the mean T-2 and HT-2 toxin concentrations, the shares of T-2:HT-2 were established to be in the range from 1:1.7 in wheat and barley to 1:2.4 in maize, with the mean share value of 1:1.9 in all investigated cereal samples. The determined share values are comparable to those stated by other literature sources, which show that HT-2 is present in cereals and their products in levels higher than those of T-2 toxin, representing approximately two-thirds (1:2) of the summary T-2/HT-2 concentrations [16].

3.3. The reduction of T-2 and HT-2 toxin

In general, different cereal treatments implemented by the food industry are known to decrease mycotoxin concentrations, but mostly do not eliminate these toxins completely. These food processing operations include sorting, trimming, cleaning, cooking, baking, frying, roasting, flaking and extrusion, and have variable effects on the level of contamination. In their recently published study, Schmidt et al. [39] stated that in comparison to other mycotoxins, thermal degradation of T-2 and HT-2 has not been the subject of many studies. In the last decades, some research on the effects of thermal degradation has mainly been performed on oats, known to be the cereal most contaminated with these mycotoxins [34, 39–41]. Scudamore [20] concluded that final processing, such as boiling, fermentation, baking, frying, and extrusion, has no impact on T-2 and HT-2 contamination. A greater extent of thermal degradation of T-2 as compared to HT-2 has been established, as well [34, 39, 41].

Nevertheless, the efficiency of T-2 and HT-2 toxin reduction using thermal processing techniques is still under-established, mostly because of the fairly small data pool on the subject-matter provided insofar, obtained under various, mutually different thermal degradation conditions, which, in turn, yields inconsistent study outcomes and study conclusions. In light of the foregoing, in order to establish the degree of thermal degradation and reduction of T-2 and HT-2 toxin in naturally contaminated cereals, this study made use of three thermal processing methods, that is to say, cooking, roasting and extrusion, each of them running at three different temperatures for different lengths of time.

Cooking is the preparation of a meal using heat [42]. Several studies have reported about the effect of cooking on the reduction of *Fusarium* mycotoxins in contaminated cereals [34, 43, 44].

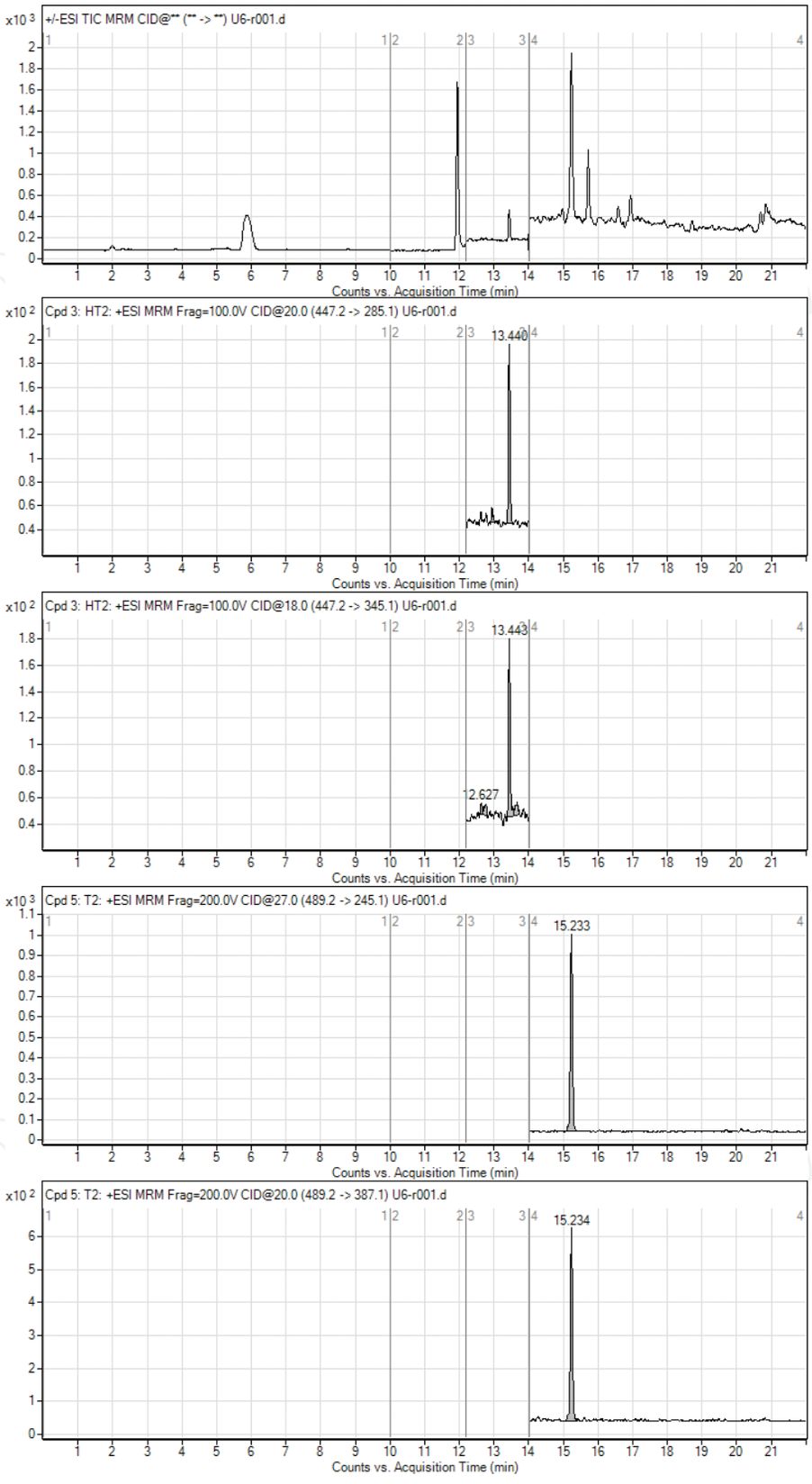

Figure 2. A typical LC-MS/MS-MRM chromatogram of the contaminated maize sample subsequently subjected to thermal reduction processing (128 μg/kg of T-2 and 256 μg/kg of HT-2); TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

The results descriptive of T-2 and HT-2 reduction achieved by various cooking times (10:30 min) are presented in **Table 6**.

The maximal reduction of T-2 toxin was observed in oat (14.7%) cooked for 30 min, whereas the greatest HT-2 (7.5%) and summary T-2/HT-2 concentration reduction (9.3%) were obtained in oat cooked for 20 min. A slightly greater reduction was observed for T-2 toxin (9.3%) in comparison to the HT-2 toxin, which was mostly unreduced in all three types of cereals despite cooking. A prolonged cooking time (30 min) achieved no significantly greater reduction of summary T-2/HT-2 concentrations or individual toxin levels. The results show that the reduction of T-2/HT-2 summary concentrations achieved by cooking can be considered negligible (R < 10%), suggesting that cooking as a thermal processing method does not represent a valuable tool when it comes to the decontamination of cereals contaminated with T-2 and HT-2. After cooking of noodles, Kamimura et al. [43] determined the residual rate

Cereals[a]	T-2/HT-2 after cooking	Cooking time (96°C)		
		10 min	20 min	30 min
Maize	T-2/HT-2 (μg/kg)	375	366	372
	T-2/HT-2 R (%)	2.3	4.7	3.1
	T-2 (μg/kg)	117	123	110
	T-2 R (%)	8.6	3.9	14.1
	HT-2 (μg/kg)	258	243	270
	HT-2 R (%)	NR	5.1	NR
Triticale	T-2/HT-2 (μg/kg)	252	271	262
	T-2/HT-2 R (%)	5.6	NR	1.9
	T-2 (μg/kg)	104	99.5	94.8
	T-2 R (%)	2.8	7.0	11.4
	HT-2 (μg/kg)	172	151	167
	HT-2 R (%)	NR	5.6	NR
Oat	T-2/HT-2 (μg/kg)	152	137	144
	T-2/HT-2 R (%)	NR	9.3	4.6
	T-2 (μg/kg)	43.2	40.8	40.1
	T-2 R (%)	8.1	13.2	14.7
	HT-2 (μg/kg)	108	96.2	104
	HT-2 R (%)	NR	7.5	NR

[a]Concentrations in cereals before cooking: 384 μg/kg (128 μg/kg of T-2 and 256 μg/kg of HT-2) in maize, 267 μg/kg (107 μg/kg of T-2 and 160 μg/kg of HT-2) in oat and 151 μg/kg (47.0 μg/kg of T-2 and 104 μg/kg of HT-2) in triticale.

T-2 and HT-2 toxin concentrations are presented as the mean value of three replicates; R: reduction; NR: not reduced.

Table 6. Reduction of T-2/HT-2 toxins achieved by various cooking times.

of T-2 toxin in fortified samples of up to 76%. Schwake-Anduschus et al. [34] stated that T-2/HT-2 toxin levels are relatively stable during short-time cooking. This was also confirmed by this study, as it resulted in a very small reduction of both mycotoxins despite the prolonged cooking time (30 min).

Roasting is a cooking method that uses dry heat in form of an open flame, oven, or other heat sources. Roasting can enhance flavour through caramelisation and Maillard browning of the food surface [42]. The results of T-2 and HT-2 reduction via roasting, obtained in this study at three different temperatures (180–220°C), are presented in **Table 7**. **Figure 3** presents a typical LC-MS/MS-MRM chromatogram of a contaminated maize sample obtained after roasting at the temperature of 220°C during 30 min, in which a significant reduction of both T-2 toxin (60.7%) and HT-2 toxin (46.1%) can be seen.

Cereals[a]	**T-2/HT-2 after roasting**	**Temperature of roasting**[b]		
		180°C	**200°C**	**220°C**
Maize	T-2/HT-2 (μg/kg)	270	225	188
	T-2/HT-2 R (%)	29.7	41.4	51.0
	T-2 (μg/kg)	80.1	65.3	50.3
	T-2 R (%)	37.4	49.0	60.7
	HT-2 (μg/kg)	190	160	138
	HT-2 R (%)	25.8	37.5	46.1
Triticale	T-2/HT-2 (μg/kg)	190	173	151
	T-2/HT-2 R (%)	28.8	35.2	43.4
	T-2 (μg/kg)	56.2	43.3	38.2
	T-2 R (%)	47.5	59.5	64.3
	HT-2 (μg/kg)	138	130	113
	HT-2 R (%)	13.8	18.8	29.4
Oat	T-2/HT-2 (μg/kg)	101	84.3	68.9
	T-2/HT-2 R (%)	33.1	44.2	54.4
	T-2 (μg/kg)	28.1	22.7	ND
	T-2 R (%)	40.2	51.7	CR
	HT-2 (μg/kg)	72.9	61.6	59.3
	HT-2 R (%)	29.9	40.8	43.0

[a]Concentrations in cereals before reduction: 384 μg/kg (128 μg/kg of T-2 and 256 μg/kg of HT-2) in maize, 267 μg/kg (107 μg/kg of T-2 and 160 μg/kg of HT-2) in oat and 151 μg/kg (47.0 μg/kg of T-2 and 104 μg/kg of HT-2) in triticale.

[b]Roasting was carried out for 30 minutes at the default temperatures.

T-2 and HT-2 toxin concentrations are presented as the mean value of three replicates; R: reduction; ND: not detected; CR: completely reduced.

Table 7. Reduction of T-2/HT-2 toxins achieved by roasting of contaminated cereals at different temperatures.

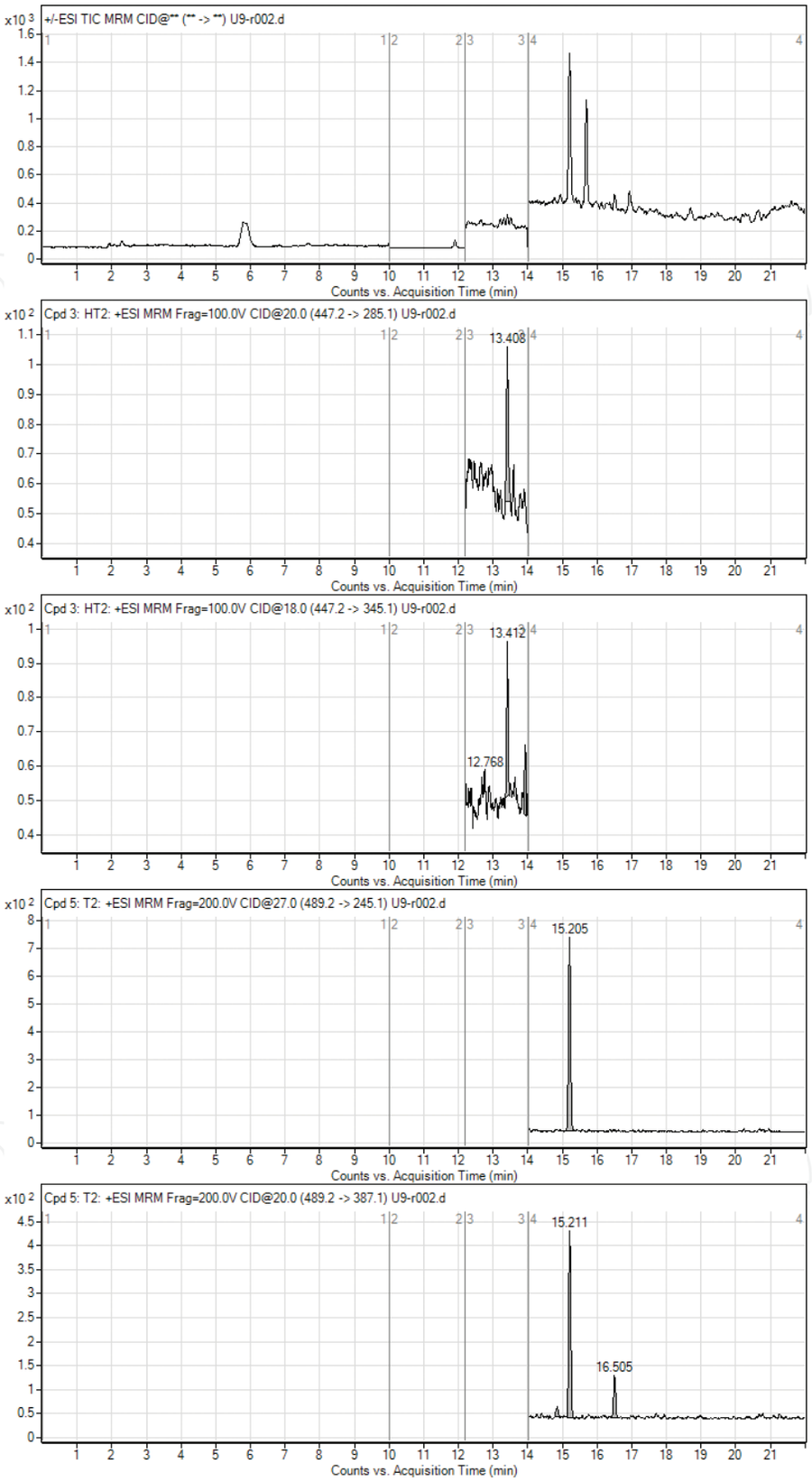

Figure 3. A typical LC-MS/MS-MRM chromatogram of a contaminated maize sample after roasting at the temperature of 220°C for 30 min (50.3 μg/kg of T-2 and 138 μg/kg of HT-2); TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

Published data on the influence of roasting on the reduction of mycotoxins are mostly linked to aflatoxins, ochratoxins or some *Fusarium* mycotoxins other than T-2/HT-2 [45–48] and refer to baking [34, 49]. In this study, the reduction of summary T-2/HT-2 toxin levels, achieved with roasting at 180°C, ranged from 28.8 to 33.1%; at 200°C, the achieved reduction ranged from 35.2 to 44.2%, while at 220°C, a 43.4 to 54.4% reduction was witnessed. A complete T-2 toxin reduction was achieved when roasting oats; after roasting at 220°C, no detectable concentration of this mycotoxin was determined. The results show that in comparison to the HT-2 toxin, a significantly higher (nearly 2-fold) reduction of T-2 was achieved, which is in line with an earlier observation that thermal processing reduces T-2 concentrations to a greater extent as compared to those of HT-2 [39]. Roasting can be considered as an effective thermal processing method suitable for cereal decontamination, as it resulted in a roughly 40% T-2/HT-2 toxins reduction in contaminated samples.

Extrusion is used in the production of cereal products such as breakfast foods, snacks and animal feedstuffs, and represents one of the fastest-growing food-processing operations in the recent years due to its many advantages. Extrusion cooking of cereals combines pumping, kneading, mixing, shearing, cooking and forming, all in one processing session. As several operations are carried out simultaneously, they interact with each other [39]. Cereals are passed through an extruder under pressure, undergo mechanical shearing stresses at elevated temperature, and rapidly expand when forced through the outlet die [50]. Apart from its main goal in terms of improving the product quality, extrusion may also significantly improve the product safety because of its potential to reduce mycotoxin levels in cereals [51]. The effectiveness of mycotoxin reduction also depends on the presence of minor ingredients or additives. Scudamore et al. [20] explained that during extrusion, contaminants are subjected to both high temperatures and chemical reactions mediated by free radical mechanisms so that they might be susceptible to some degree of breakdown, the effects on mycotoxins thereby generally being variable.

The reduction of T-2/HT-2 toxins achieved by extrusion cooking of contaminated cereals in this study using three different regimes of extrusion is shown in **Table 8**. **Figure 4** presents a typical LC-MS/MS-MRM chromatogram obtained after extrusion cooking of a contaminated maize sample at the defined temperatures of 135-190-190°C.

By virtue of extrusion cooking under three temperature regimes and with the same moisture content (25%), an almost complete reduction of T-2 and HT-2 toxin was achieved. The results show a similar effect of extrusion independent of the type of cereal and the applied temperature regime, based on which this method can be considered as the most effective and most valuable when it comes to the reduction of mycotoxins. As the LC-MS/MS method failed to determine any of the two mycotoxins in any of the extruded samples, the summary T-2/HT-2 concentrations were analysed using the ELISA method. The results showed T-2/HT-2 concentrations slightly higher than the method's LOQs, except for the oat subjected to the extrusion temperature regime of 135-190-190°C, in which the presence of the above toxins was not detected at all. The reduction of T-2/HT-2 achieved under the 135-150-150°C extrusion regime ranged from 73.0% in oats to 92.5% in maize. However, it should be taken into account that the presence of individual mycotoxins was not confirmed by the LC-MS/MS method and that the ELISA method showed higher T-2/HT-2 summary concentrations.

Cereals[a]	T-2/HT-2 after extrusion	Regime of extrusion[b]		
		135-150-150°C	135-170-170°C	135-190-190°C
Maize	T-2/HT-2 (μg/kg)	28.9	49.7	32.2
	T-2/HT-2 R (%)	92.5	87.1	91.6
	T-2 (μg/kg)	ND	ND	ND
	T-2 R (%)	CR	CR	CR
	HT-2 (μg/kg)	ND	ND	ND
	HT-2 R (%)	CR	CR	CR
Triticale	T-2/HT-2 (μg/kg)	37.7	45.9	41.3
	T-2/HT-2 R (%)	85.9	82.8	84.5
	T-2 (μg/kg)	ND	ND	ND
	T-2 R (%)	CR	CR	CR
	HT-2 (μg/kg)	ND	ND	ND
	HT-2 R (%)	CR	CR	CR
Oat	T-2/HT-2 (μg/kg)	40.7	38.1	ND
	T-2/HT-2 R (%)	73.0	74.8	CR
	T-2 (μg/kg)	ND	ND	ND
	T-2 R (%)	CR	CR	CR
	HT-2 (μg/kg)	ND	ND	ND
	HT-2 R (%)	CR	CR	CR

[a]Concentrations in cereals before reduction: 384 μg/kg (128 μg/kg of T-2 and 256 μg/kg of HT-2) in maize, 267 μg/kg (107 μg/kg of T-2 and 160 μg/kg of HT-2) in oat and 151 μg/kg (47 μg/kg of T-2 and 104 μg/kg of HT-2) in triticale.

[b]Moisture in extruded samples ranges from 11.0 to 12.0%.

T-2 and HT-2 toxin concentrations are presented as the mean value of three replicates; R: reduction; ND: not detected; CR: completely reduced.

Table 8. Reduction of T-2/HT-2 toxins achieved by extrusion cooking of contaminated cereals at different temperatures.

When comparing the degradation rates of T-2 against those of HT-2 toxin, it was revealed that T-2 shows a higher mitigation in the extrusion cooking process [39, 41]. Some investigations showed that T-2 and HT-2 degradation during extrusion are not influenced by the heating temperature to the same extent and that other variables present during processing are responsible for a more complex degradation pattern. This observation can be linked to the results of this study, in which a significant influence of the extrusion temperature regime was not determined. Schmidt et al. [39] stated that due to the strong interference of various parameters during extrusion, it is not possible to attribute toxin degradation to just one of them. Among the factors of influence, the water content plays an important role in

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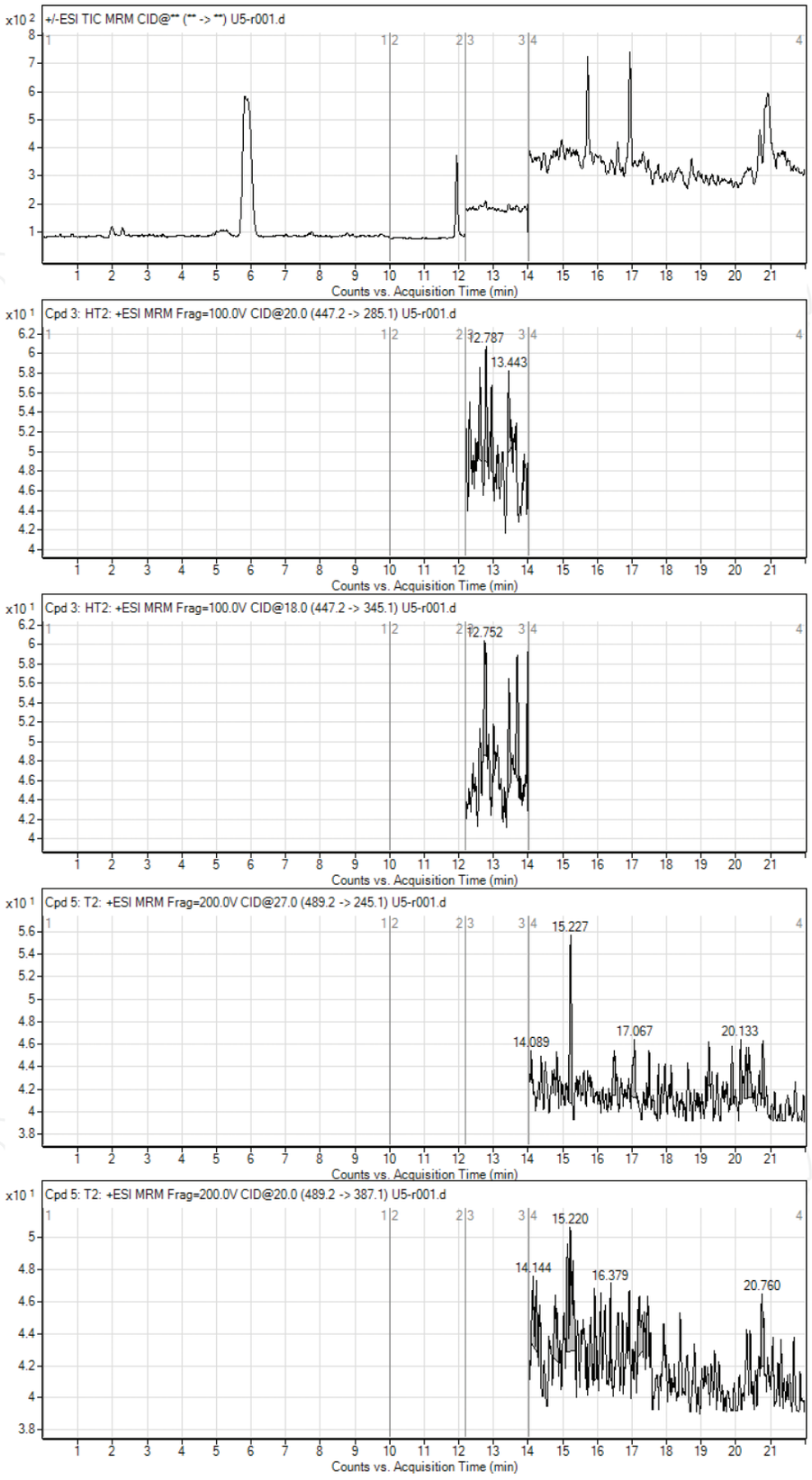

Figure 4. A typical LC-MS/MS-MRM chromatogram of a contaminated maize sample after extrusion at 135-190-190°C (T-2 and HT-2 toxin not detected); TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

extrusion cooking, because it is essential for starch gelatinization and strongly affects fluid viscosity and the expansion ratio. Extrusion cooking was shown to decrease the mycotoxin content at rates depending on the moisture level, screw centrifugation, extruder geometry, die temperature, die size, screw speed and additives [51], while the extrusion temperature was found to be a minor factor of influence. As opposed to that, high moisture levels and high shear rates substantially contribute to the toxin degradation [39, 52]. The authors elaborated that since the fate of T-2 and HT-2 and the formation of so far unknown degradation products or bound forms remains unclear, it cannot be concluded that extrusion cooking of contaminated oats is accompanied by a detoxification process. Scudamore et al. [52] pointed out that the inconsistency of the results presented in the literature may be a consequence of failure to control or report all conditions under which the extrusion process was taking place. For example, chemical breakdown taking place during an extrusion process is related to the duration of the process, so that the loss of mycotoxin will depend on the residence time of the material in the extruder. Differences in parameters implemented during extrusion cooking carried out in this study may also explain an almost complete reduction of T-2 and HT-2 toxin achieved, as opposed to other studies quoted above, in which only partial or smaller toxin reduction has been witnessed when using this thermal processing method.

4. Conclusions

Among the analysed cereals, the highest percentage of T-2- and HT-2-positive samples were determined in oats, followed by maize, triticale and barley, whereas the highest mean and maximal concentration of the toxins was determined in maize. The summary T-2/HT-2 concentrations found in one maize and one triticale sample were higher than the indicative levels stipulated by the European Commission, suggesting that further sampling should be performed and that the production conditions should be investigated more thoroughly. As for the thermal methods of toxin reduction, roasting appears to efficiently reduce T-2 and HT-2 concentrations, whereas cooking does not significantly reduces these mycotoxins. Extrusion cooking seems to be far more efficient since it resulted in an almost complete T-2/HT-2 elimination in all cereals, independent of the temperature regime applied during the extrusion process.

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