Article

ELISA and UPLC/FLD as Screening and Confirmatory Techniques for T-2/HT-2 Mycotoxin Determination in Cereals

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Abstract: T-2 and HT-2 toxins are secondary metabolites of various species of *Fusarium*. These molecules can have high potential toxic effects for human and animal health. In this work, ELISA and ultra performance liquid chromatography with fluorescence detection (UPLC/FLD) were implemented and validated as screening and confirmatory tests for the detection of these two toxins in cereal samples. The developed methods were tested by analyzing 100 samples of cereals by ELISA screening for reducing costs and analysis time and then using UPLC/FLD for confirmation purposes. Both methods met the performance criteria for sensitivity, linearity, selectivity, precision, and ruggedness, as reported in the European Decision No. 2002/657/EC and in Regulation (EC) No. 401/2006. The correlation between ELISA and UPLC/FLD approaches showed good results ($r = 0.9056$), confirming that these two techniques should be considered to be complementary in the official control activities of cereal and derived products.

Keywords: cereals; ELISA; food safety; T-2/HT-2 toxins; UPLC-FLD; validation

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1. Introduction

T-2 toxin (T-2) and HT-2 toxin (HT-2) belong to the group of contaminants known as trichothecenes, which are part of the largest group of *Fusarium* mycotoxins, comprising more than 150 compounds. The basic structure of all trichothecenes is a tetracyclic sesquiterpene with a spiro-epoxide group at C-12 and C-13, as well as an olefinic double bond between C-9 and C-10. According to the substituents of the tetracyclic ring system, trichothecenes are grouped into four different types (A–D). T-2 and HT-2 belong to the type A trichothecenes, which are characterized by an esterified or free hydroxyl group at C-8 or an unsubstituted C-8. The structures of T-2 and HT-2 toxins differ only in one functional group; the T-2 toxin is acetylated at C-4, while the HT-2 toxin is not acetylated. The T-2 toxin is readily converted to HT-2 by metabolism without losing its toxicity; therefore, T-2 and HT-2 are usually evaluated together [1].

In 2011, the European Food Safety Authority (EFSA) published a scientific opinion on the risks to human and animal health related to the presence of the T-2 and HT-2 toxins in food and feed [2]. In this opinion, the EFSA Panel on Contaminants in the Food Chain concluded that T-2 is a carcinogen, mutagen, and immunosuppressant. Thus far, no legal limits have been defined in Europe for T-2/HT-2 mycotoxins, and only a recommendation is available (Recommendation 2013/165/EU; Table 1). However, the recommendation specifically asks the member states to monitor for the presence of both T-2 and HT-2 in food and feed. This means that, in the near future, the laboratories in charge of food inspections will need fast and reliable analytical tools for analyzing a large number of samples in a short time.

Table 1. Indicative legal limits for the sum of T-2 and HT-2 in cereals (European Commission Recommendation of 27 March 2013).

Cereal Type		Indicative Level for the Sum of T-2 and HT-2 ($\mu\text{g kg}^{-1}$)
Unprocessed cereals	Barley (including malting barley) and maize	200
	Oats (with husk)	1000
	Wheat, rye and other cereals	100
Cereal grains for direct human consumption	Oats	200
	Maize	100
	Other cereals	50

The main analytical methods used for the T-2 and HT-2 toxin determination in cereal grains are chromatographic methods—mainly gas chromatography coupled with flame ionization detection (FID), electron capture detection (ECD), and mass spectrometry (MS) detection [3–5]. Additionally, liquid chromatography coupled with spectrofluorometric detection or mass spectrometry has been indicated for T-2 and HT-2 analysis [5–9]. Pascale et al. [10] published a sensitive HPLC method for the analysis of T-2 toxin in cereals. This method includes an immunoaffinity column (IAC) clean-up after extraction and a derivatization step with 1-anthrolylnitrile and 4-dimethylaminopyridine before fluorescence detection. Nevertheless, the ELISA approach is more employed due to its simplicity and rapidity [5,11]. However, many authors have reported some selectivity limitations of these approaches [12,13].

Regarding the performances of analytical methods for the research of drug residues and environmental contaminants in food, European legislation requires the validation of both screening and confirmatory methods, according to the European Decision No. 2002/657/EC [14]. This decision defines a list of the definitions and requirements to fulfill through the validation procedure.

The aim of our work was to validate and then compare the ELISA, as a screening method, and ultra performance liquid chromatography with fluorescence detection (UPLC/FLD), as a confirmatory method, for the quantitative determination of the T-2 and HT-2 toxins in cereals. The comparison was carried out while considering both the obtained validation parameters (selectivity, precision, recovery, robustness, and measurement uncertainty) and the results coming from the analysis of real samples.

2. Materials and Methods

2.1. Samples

A total of 100 cereal samples for animal and human use were randomly collected from the Puglia region (Italy) during the period from May to July 2018, according to Regulation No. 2017/625/EC [15]. These samples were represented by: 11 common wheat, 20 durum wheat, 31 barley, 15 maize, and 23 oat samples, starting from an initial quantity of 1 kg. Bulk lots were sampled and analyzed according to Regulations EC No. 401/2006 [16], and EU No. 519/2014 [17], which define the methods of sampling and analysis for the control of the levels of mycotoxins in foodstuffs according to the national official programs in order to guarantee satisfactory representativeness of samples. All samples of cereals were ground by a high-capacity mill (Mill Series II, Romer Labs, Newark, DE, USA), homogenized, and stored at room temperature until analysis (within 24 h to avoid sample deterioration).

2.2. Chemicals

Standards of T-2 ($\geq 98\%$) and HT-2 ($\geq 98\%$) were supplied by Trilogy Analytical Laboratory (Washington, MO, USA). Sodium chloride ($\geq 99.9\%$) was purchased from VWR International (Milan, Italy). Methanol of HPLC grade ($\geq 99.9\%$) was purchased from Honeywell (Raleigh, NC, USA). The derivatization reagents 1-anthrolyl cyanide was

purchased from Wako Chemicals (Neuss, Germany). Water of HPLC grade was supplied by Baker (Deventer, The Netherlands).

2.3. Screening Analysis

2.3.1. Sample Preparation for Enzyme Immunosorbent Assay

A competitive ELISA (Ridascreen T-2/HT-2 R3805, R-Biopharma, Darmstadt, Germany) was used for the quantitative analysis of the T-2 and HT-2 toxins. Sample preparation was carried out following the instructions given by the kit manufacturers, with slight modification: 5 g of each representative ground sample were weighed, added to 1 g of NaCl, and thoroughly mixed with 25 mL of the extraction buffer supplied in the kit for 3 min on a horizontal shaker at room temperature. The suspension was centrifuged at $400\times g$ for 10 min at 4 °C, and then the supernatant was collected.

2.3.2. Immunoassay Procedure

The determination of the T-2 and HT-2 mycotoxins was performed following the procedure described by the kit manufacturers. All reagents were brought to room temperature before use. Fifty microliters of standard/sample, T-2 toxin enzyme conjugate, and anti-T-2/HT-2 toxin antibodies were added into the wells. After incubation for 30 min at room temperature, the liquid was poured from the wells. The wells were then washed three times with a washing buffer using a “squeeze bottle.” Subsequently, 100 µL of the “developing solution” were added into each well. The reactions were stopped by adding 100 µL of the “stop solution” after 15-min of incubation at room temperature. The absorbance was read at 450 nm within 30 min using a Multiskan™ FC microplate photometer microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Confirmation Analysis

2.4.1. Sample Preparation for UPLC-FLD Analysis

Ten grams of finely ground cereal sample (durum wheat, common wheat, barley, oats, and corn) were added with 1 g of NaCl and extracted with 40 mL of methanol–water (90–10 *v/v*) for 30 min on a horizontal shaker. After centrifugation (10 min at $4000\times g$ and 4 °C), the supernatant was filtered through tissue paper. The filtrate was diluted to 1:5 with deionized water and subsequently filtered using a glass microfiber filter (Whatman GF/A, Fisher Scientific Italia, Milan, Italy). Ten milliliters of filtered extract were passed through an immunoaffinity column (IAC; Vicam, Watertown, MA, USA) containing antibodies specific for T-2 and HT-2 at a flow rate of one drop per second. The IAC was then washed with 15 mL of distilled water. The T-2 and HT-2 toxins were then eluted with 1.5 mL of methanol collected in a glass test tube. The solution was dried under nitrogen flow in a heater block set at 50 °C (± 3 °C). The derivatization was performed according to the procedure of Visconti et al. [6]. Briefly, 50 µL of a 4-dimethylaminopyridine (DMAP) solution and 50 µL of 1-anthroylcyanide (1-AN) reagents were added to the dried residue (Wako Chemicals, Neuss, Germany). The reaction mixture was left to react for 15 min at 50 °C in the heater block and then cooled in ice for 10 min. The reaction mixture was dried under nitrogen flow in the heater block at 50 °C, and then the residue was dissolved in 700 µL of acetonitrile. After 5 min, 300 µL of HPLC-grade water were added for the subsequent UPLC analysis.

2.4.2. UPLC-FLD Analysis

Chromatographic analysis was performed using an Acquity UPLC H-Class Bio System (Waters Chromatography B.V., Etten-Leur, The Netherlands) including a quaternary pump, a thermostated autosampler, a thermostated Acquity UPLC BEH-C18 column (50 $\times$ 2.1 mm, 1.7 µm), and a fluorescence detector. The excitation and emission wavelengths adopted for the fluorescence detection were 370 and 481 nm, respectively. The chromatographic separation was carried out at a flow rate of 0.4 mL min^{−1} using a binary gradient based on water (solvent A) and acetonitrile (solvent B). The temperatures of the column and

the autosampler were set at 40 and 10 °C, respectively. The gradient was set as follows: the initial condition was 70% solvent A, solvent A increased to 85% after one minute and then was kept constant up to 5 min. Solvent A was brought to 100% in 3 min, and then the initial conditions were restored followed by a re-equilibration time of 2 min. The total run time was 11 min. The injection volume was 5 µL. Under these conditions, the retention times were 1.9 and 4.0 min for the T-2 and HT-2 toxins, respectively.

2.5. Validation of Screening and Confirmatory Methods

The screening and confirmatory methods were validated in accordance with ISO/IEC 17025:2017 [18], taking the indications reported in Decision No. 2002/657/EC and Regulation EC No. 2017/625 as reference and some updated guidelines for analytical method validation [19,20]. The full validation of two methods assured the compliance and conformity with the criteria established in Regulation EC No. 401/2006. Since 2016, these two methods have also been accredited by ACCREDIA, the Italian Organization for laboratory accreditation. The parameters considered during the validation of ELISA and UPLC-FLD analytical procedures were: selectivity, sensitivity, precision, recovery, and ruggedness. The measurement uncertainty was only estimated for UPLC-FLD, since the evaluation of this parameter is only compulsory for confirmatory methods. On the basis of the propagation of uncertainty law, the measurement uncertainty was calculated for each toxin by using the following equation:

$$v = \sqrt{(v(C))^2 + v(V_f)^2 + v(w)^2}$$

where u indicates the relative uncertainty, C is the analyte concentration in the sample, V_f is the volume of the final extract, and w is the sample weight. The determination of v was performed by considering four sources of uncertainty: (a) standard preparation, (b) method reproducibility, (c) method recovery, and (d) calibration curve.

For the selectivity and accuracy tests, the samples were spiked, prior to the extraction, with the proper amounts of the T-2/HT-2 toxin standard solutions for obtaining the required concentrations.

Within the ELISA validation, the trust semi-interval was calculated by using the following equation:

$$\pm \frac{t \cdot s_0}{\sqrt{n}}$$

where s_0 is the standard deviation of the signal obtained from the 20 sample blank tests, t is the Student's t value tabulated for 19 degrees of freedom (one-tail, $p = 0.05$), and n is the number of performed tests.

Regarding UPLC/FLD, the limits of detection and quantification (LOD and LOQ, respectively) were calculated according to the following equations:

$$LOD = 3.9 \times S_b/b \text{ and } LOQ = 3.3 \times X_{LOD}$$

where S_b is the standard deviation of the pseudo-blank difference and b is the slope of the mean regression line. For selectivity evaluation, 20 blank samples (common wheat, durum wheat, oats, maize, and barley—4 each) were tested based on an immunoenzymatic assay to verify the absence of the T-2/HT-2 toxins.

Method ruggedness was tested for both techniques by introducing seven minor changes in the analytical procedure (e.g., different centrifugation speeds and manual shaking time) and then assessing their influence on the final result. The effect of each factor was calculated by subtracting the mean result obtained using the variable at a high level (reference value) to the mean result at a low level (investigated factor).

Validation results were statistically evaluated using of an ANOVA, Fisher's exact test and Student's t test ($p > 0.05$). In 2019, the ELISA was submitted to a proficiency test round (Progetto Trieste, Mycotoxins 2019, round of October, test Veritas s.r.l., Trieste, Italy), and it

obtained good results in quantifying the T-2 and HT-2 toxins in naturally contaminated common wheat (result: $185.5 \mu\text{g kg}^{-1}$; acceptability range: $89.5\text{--}220.9 \mu\text{g kg}^{-1}$).

3. Results and Discussion

An explicative workflow reporting the complete scheme of validation of both approaches, together with related time of execution, is shown in Figure 1. The workflow was optimized in terms of time required for the completion of each step of validation. Obviously, due to the longer times required for the sample-by-sample UPLC injections, the confirmatory technique required more time to complete the validation (~44 days) than the ELISA (~21 days).

Figure 1. Validation procedure of ELISA and ultra performance liquid chromatography with fluorescence detection (UPLC/FLD) methods: step-by-step optimized workflow.

3.1. Screening Analysis (ELISA) Results

3.1.1. Selectivity and Sensitivity

The data reported by the kit producer indicated that the T-2/HT-2 kit presented a high selectivity for the target toxins, with specificities corresponding to 100% and ~85% for the HT-2 and T-2 toxins, respectively. For the estimation of method selectivity, a total of 20 cereal samples (wheat, maize, composed feed, barley, and oat; four samples each) was analyzed, and the absence of two toxins was verified (concentration lower than limit of detection indicated by the kit producer: $3.0 \mu\text{g kg}^{-1}$, corresponding to $75 \mu\text{g kg}^{-1}$ in matrix). Though four different types of samples were characterized by different compositions in terms of fat, anthocyanin, soluble and non-soluble fiber, as well as other grain constituents that may have interfered with the extraction of mycotoxins, the same procedure of sample preparation could be used. This was in accordance with the procedure declared by the kit manufacturer in a related technical sheet. Thus, these samples were considered “blank”. The same samples were then fortified at a concentration of $250 \mu\text{g kg}^{-1}$ for the HT-2 toxin and then analyzed in a short time within two analytical sessions. Each sample was analyzed twice, and the mean value of two measurements was used in data processing to report the relative absorbance, B/B_0 (%). The resulting data are presented in Table 2. The statistical evaluation was assessed by variance analysis (one-way ANOVA), Fisher’s exact test, and Student’s *t* test ($p < 0.05$) for paired data between the signals of ten blank samples and related fortified samples. For both sessions, the *t* critical value (Stat *t*) was higher than the experimental (one-tail *t* critical), so the difference between the blank samples and the corresponding fortified samples was verified as significant ($p < 0.05$). For both sessions, the fortified samples showed B/B_0 (%) values below the trust semi-interval (65.10 and 65.40 for the first and second session, respectively). Thus, all 20 fortified samples were recognized as “contaminated.” Hence, the β error was satisfied, and the detection limit was assessed at $75 \mu\text{g kg}^{-1}$.

Table 2. Selectivity tests of ELISA validation. Results obtained by analyzing 20 cereal blank samples and samples spiked with $250 \mu\text{g kg}^{-1}$ of the HT-2 toxin.

Sample	First Analytical Session				Sample	Second Analytical Session			
	Matrix	B/B ₀ (%) Blank Sample	B/B ₀ (%) Spiked Sample	Spiked Samples Concentration ($\mu\text{g kg}^{-1}$)		Matrix	B/B ₀ (%) Blank Sample	B/B ₀ (%) Spiked Sample	Spiked Samples Concentration ($\mu\text{g kg}^{-1}$)
1	Wheat	0.75	0.30	261.3	11	Wheat	0.75	0.30	261.3
2	Wheat	0.75	0.34	218.0	12	Wheat	0.81	0.35	212.8
3	Maize	0.78	0.31	249.0	13	Maize	0.79	0.31	248.5
4	Maize	0.62	0.30	261.5	14	Maize	0.62	0.30	261.8
5	Composed feed	0.63	0.28	298.0	15	Comp. feed	0.63	0.26	323.0
6	Composed feed	0.65	0.31	253.5	16	Comp. feed	0.65	0.32	249.0
7	Barley	0.77	0.30	263.5	17	Barley	0.77	0.30	262.5
8	Barley	0.67	0.33	230.3	18	Barley	0.67	0.34	222.3
9	Oat	0.65	0.29	285.0	19	Oat	0.65	0.26	346.8
10	Oat	0.62	0.29	280.5	20	Oat	0.63	0.26	340.0
Average		0.69	0.30	260.1	Average		0.70	0.30	272.8
SD		0.0655	0.0184		SD		0.0742	0.0324	

SD: standard deviation.

3.1.2. Precision and Recovery

Since the screening procedure may also be used as quantitative method, precision tests were performed. The validation of the kit allowed for the determination of the concentration of the sum of the T-2 and HT-2 toxins in the range between 75 and $900 \mu\text{g kg}^{-1}$. Precision was calculated either in terms of repeatability (or within-laboratory reproducibility) and reproducibility (or between-laboratory reproducibility). These analyses were carried out

on two sets of blank feed samples (a mix of composed feed and raw materials) fortified at two different concentrations of the T-2 and HT-2 toxins (100 and 250 $\mu\text{g kg}^{-1}$) using the T-2/HT-2 standard solution. Good performances were obtained in terms of the coefficient of variation (CV; %) under repeatability conditions (6.5%). As reported in the European Commission Decision No. 657/2002, this parameter has to be lower than 23% and 16% for the fortification levels equal to 100 and 250 $\mu\text{g kg}^{-1}$, respectively. This parameter was comparable with the precision of ELISA obtained by Pleadin et al. [21], who analyzed the same types of cereals samples investigated in the present study, and higher than that reported by Oplatowska-Stachowiak et al. [22] for rye and baby porridge analysis. The recovery percentage was satisfactory as well, since both fortification levels gave a mean value of 102%, whereas the required range set in Decision No. 657/2002 is 80–110%.

3.1.3. Ruggedness

The samples were spiked with 62.5 μL of a standard solution of HT-2 at a concentration of 20 mg L^{-1} to obtain the fortified products at 250 $\mu\text{g kg}^{-1}$. The variables were modified using the experimental plan according to the procedure developed by Youden and Steiner [23]. The Youden fractional factorial design adopted in this part of method validation and related results are reported in Supplementary Tables S1 and S2. Method ruggedness was demonstrated under minor changes conditions, since the SD of difference was lower than the SD of the intra-laboratory reproducibility.

3.2. Confirmatory Analysis Results

3.2.1. Selectivity and Sensitivity

The samples showing signals below the ELISA detection limit (3 $\mu\text{g L}^{-1}$ and 75 $\mu\text{g kg}^{-1}$ in matrix) were considered to be “blank” and then analyzed to verify the selectivity of the UPLC/FLD method. After UPLC analysis, no interfering compound was found in the retention time windows of interest: $\pm 2.5\%$ of T-2 and HT-2 chromatographic peak retention times. Thus, all samples were characterized by the absence of interfering peaks, demonstrating method selectivity. The chromatograms reported in Figure 2B,C show an example of a blank maize sample and the same spiked sample with the T-2 and HT-2 toxins at 250 $\mu\text{g kg}^{-1}$.

Method sensitivity, linearity, and limits of detection were evaluated by using the regression line approach [24]. The calibration curve determination coefficient (R^2) was higher than 0.995 for the T-2 toxin and 0.999 for the HT-2 toxin. The linear regressions were obtained by injecting six standard solutions of the T-2 and HT-2 toxins at concentrations of 15, 50, 100, 250, 500, and 1000 $\mu\text{g L}^{-1}$. By means of the least squares method, the pairs of values were interpolated (concentration of the standard and area of the chromatographic peak). The limits of detection and quantification were 9.0 and 29 $\mu\text{g kg}^{-1}$ for the T-2 toxin, respectively, and 5.7 and 19 $\mu\text{g kg}^{-1}$ for the HT-2 toxin, respectively (Table 4). The obtained LODs were comparable with those reported by Pascale et al. [25] in their improved method by ultra-performance liquid chromatography with photodiode array detection. Though these sensitivity levels were obviously lower than those achievable by using mass spectrometry [26], they can be considered satisfactory with respect to the indicative legal limits reported for the sum of the T-2 and HT-2 toxins in the European Commission Recommendation of 27 March 2013 [27]. Thus, given its higher simplicity and cheapness compared to mass spectrometry, the UPLC/FLD approach may represent a valid alternative for reliable confirmatory analysis.

3.2.2. Precision and Recovery

A one-way ANOVA was performed on repeated analyses in order to verify the homoscedasticity of the data obtained on different days and at different levels of fortification. Table 3 shows the results of intra-day (relative standard deviation of repeatability (RSD_r)) and inter-day (relative standard deviation of reproducibility (RSD_R)) repeatability for the T-2 and HT-2 toxins using durum wheat samples spiked at three different contamination

levels. While also considering the accuracy parameters reported in Table 4, it was possible to confirm the compliance of the validated method with the performance criteria reported for T-2 and HT-2 toxins in Regulation (EC) No. 519/2014 [17] and, more generally, in Decision No. 657/2002/EC [14].

3.2.3. Ruggedness

As described above for the immunoenzymatic analysis, the HPLC/FLD method's ruggedness was evaluated by using the Youden experimental design under conditions of minor changes [23]. Youden tests were performed for maize and barley samples spiked at $500 \mu\text{g kg}^{-1}$ of the T-2 and HT-2 toxins. An analysis of samples gave a calculated standard deviation of difference (SDi) of $43 \mu\text{g kg}^{-1}$ for T-2 and $45 \mu\text{g kg}^{-1}$ for HT-2. At a confidence level of $p = 0.05$, SDi values were not significantly different from the reproducibility deviation (S_R) registered at the same level of fortification (46 for T-2 and 41 for HT-2). Consequently, method ruggedness was demonstrated (Supplementary Tables S1 and S2).

Figure 2. Chromatogram examples: T-2 and HT-2 at concentration of $500 \mu\text{g L}^{-1}$ (A); corn “blank” sample with no residues of T-2 and HT-2 toxins (B); corn samples fortified with $250 \mu\text{g kg}^{-1}$ of T-2 and HT-2 toxins (C); and corn sample naturally contaminated: $315.9 \mu\text{g kg}^{-1}$ of HT-2 toxin (D).

Table 3. UPLC/FLD intra-day and inter-day precision studies on durum wheat samples spiked with the T-2 and HT-2 toxins.

Toxin	Analytical Session	Contamination Level ($\mu\text{g kg}^{-1}$)	RSD _r (%)	RSD _R (%)
T-2	1	100	13	18
	2		13	
	1	250	11	13
	2		8	
	1	500	11	8
	2		5	
HT-2	1	100	5	7
	2		8	
	1	250	10	19
	2		7	
	1	500	9	7
	2		5	

RSD_r: relative standard deviation of repeatability (intra-day, $n = 6$); RSD_R: relative standard deviation of reproducibility (inter-day, $n = 12$).

Table 4. Comparison of method performances of the ELISA and UPLC for the determination of the T-2 and HT-2 toxins in cereals.

VALIDATION PARAMETER	ELISA		UPLC/FLD	
	T-2	HT-2	T-2	HT-2
SPECIFICITY (%) / SELECTIVITY	~85 *	100 *	No interfering compounds	No interfering compounds
MEAN RECOVERY (%) ($n = 12$)	102	102	106	100
PRECISION (CV %) ($n = 12$)	6.5	6.5	13	11
LIMIT OF DETECTION (instrumental, $\mu\text{g kg}^{-1}$)	3.0	3.0	9.0	5.7
MEASUREMENT RANGE ($\mu\text{g kg}^{-1}$ in matrix)	75–900	75–900	29–100	19–100
ANALYSIS TIME / SAMPLE	about 1 h		about 4 h	

* Sum of T-2 and HT-2. CV: coefficient of variation.

3.2.4. Measurement Uncertainty

The evaluation of the uncertainty of analytical results is compulsory for laboratories accredited according to ISO/IEC 17025 [18], and several methods for the determination of this parameter have been proposed [28–30]. In this work, we used the “bottom-up” method on validation data obtained from each step of the analytical procedure [30].

A relative expanded measurement uncertainty was calculated by using a coverage factor (k) of 2, corresponding to a 95% confidence level [30]. This parameter was calculated for three fortification levels: 100, 250, and 500 $\mu\text{g kg}^{-1}$. Values in the range of 6–16.5% were obtained, assuring a good level of accuracy of the final result.

These results were also compared to the uncertainty function (U_f), as reported in Regulation (EC) No. 401/2006 [16], useful for evaluating the “fitness-for-purpose” of an analytical method. This function, which gives the acceptable maximum uncertainty level of the method, was calculated by using the following equation:

$$U_f = \sqrt{\left[\left(\frac{LOD}{2}\right)^2 + (\alpha C)^2\right]}$$

where C is the target concentration and α is a constant depending on C value. The evaluated measurement uncertainties were considerably lower than corresponding U_f values, namely

18%, for each concentration level (100, 250, and 500 $\mu\text{g kg}^{-1}$). These results confirmed that the method is “fit-for-purpose.”

3.3. Comparison between the ELISA and UPLC/FLD Method

The comparison among different approaches for a specific analytical determination is essential when a laboratory aims at implementing a new procedure. Point-to-point comparisons, mainly based on the available validation parameters and the application to commercial/real samples, are extremely useful for evaluating the pros/cons of each approach, as well as relating to the specific need, ability, equipment, and expertise of a laboratory [13,31,32]. However, to date, there is no available comparison regarding screening and confirmatory approaches in mycotoxin determination.

Concerning the performances of analytical methods for the research of drug residues and environmental contaminants, European legislation (Decision No. 2002/657/EC) provides indications about both screening and confirmatory methods. Technically, confirmatory methods are generally more sophisticated and expensive with respect to screening. They can be used as a first analytical approach; however, where possible, it is extremely cheaper and faster to use screening methods. Indeed, screening methods allow for greater productivity when the analysis of a large amount of samples in a brief time is required. In this study, the determination of the T-2 and HT-2 toxins by using the ELISA method required less than 60 min for a single analysis, while UPLC/FLD required a longer time (about 240 min). Thus, it is possible to suggest that the scheme illustrated in Supplementary Figure S1 as an effective approach for a laboratory. After having arrived in a laboratory, the sample should be “screened” by using ELISA, and only in case of suspect positivity it should be analyzed by a confirmatory method. The screening validation, in fact, aims to eliminate “false negative” responses through the determination of specific $\text{CC}\beta$, which is the smallest substance content that can be detected, identified, and/or quantified in a sample with the probability of a β -error. The β error, that should be $< 5\%$, is “the probability that the sample is truly non-compliant even though a compliant measurement has been obtained” [20]. It should be noted that no false positive or false negative results were observed in this study. The β -error calculated during this study was $< 5\%$. It resulted in compliance with the European requirements, since the maximum percentage of β -error permitted by Decision No. 2002/657/EC is 5%. The samples were considered suspected positive if their concentrations were $\geq \text{MRL}$ (maximum residue limit, as defined by a related regulation) subtracted from two times the repeatability SD of the method; in this case: $250 - 24.32 = 225.68 \mu\text{g kg}^{-1}$. This approach was developed by considering the main focus of this study, which was the comparison between two analytical methods. Thus, the coefficient of 1.64, usually adopted for evaluating $\text{CC}\alpha$ and $\text{CC}\beta$, was rounded up to 2 in order to increase the number of samples useful for comparison. Regarding “suspected positive” samples, the UPLC/FLD approach, after screening analysis, was characterized by an absolute reliability thanks to its high selectivity, high accuracy, and low measurement uncertainty.

From Table 4, it is possible to appraise a point-to-point comparison between two approaches developed in this study. As stated above, this type of comparison is considered very useful for laboratories involved in food control activities, since it shows the most significant pros and cons of each approach, thus simplifying the choice of a lab manager.

Some characteristics, such as method selectivity and sensitivity, are obviously better for instrumental approaches, especially regarding fluorometric detection coupled to solid phase immunoaffinity extraction, which guarantees the absence of interfering signals and very low limits of quantification. Regarding accuracy performance, method trueness (expressed as recovery %) was comparable, while method precision (CV%) was slightly higher for the ELISA due to the sample preparation procedure of UPLC/FLD being composed of more steps and thus having a decrease in repeatability.

Other than validation parameters, the two approaches were also compared by analyzing 100 samples of cereals (20 durum wheat, 11 common wheat, 15 corn, 31 barley,

and 23 oats) by using both methods. All samples were collected from a specific area located in the north-east of the Puglia region (Italy) named “Capitanata.” All samples of cereals were harvested in the field after reaching the optimal ripeness in the period between May and July 2018. The ELISA method allowed for the determination of the sum of T-2 and HT-2 toxins contents, with an LOD of $75 \mu\text{g kg}^{-1}$, while the UPLC/FLD method, which was more sensitive, allowed for the determination of T-2 and HT-2 toxins with LOQs equal to 29 and $19 \mu\text{g kg}^{-1}$, respectively. No samples with T-2 or HT-2 concentrations greater than the reference values reported in European Recommendation No. 165/2013 were verified. Sixty-six samples were not contaminated (concentration lower than quantification limit) for both the ELISA and UPLC/FLD.

The results obtained by analyzing other 27 samples were substantially in accordance between two methods. These results were elaborated by using linear regression. A good correlation ($r = 0.9056$; $n = 27$) was found (Figure 3). Seven samples were characterized by different results if analyzed through two approaches. Five samples were found to not be contaminated by using ELISA, but quantifiable amounts were detected by using the chromatographic method (in the range of $32.6\text{--}56.9 \mu\text{g kg}^{-1}$). In Figure 2D, a chromatogram example related to the analysis of a corn sample that was contaminated with $315.9 \mu\text{g kg}^{-1}$ of the HT-2 toxin is shown. From this figure, it is possible to appreciate the good level of selectivity and sensitivity that characterizes the analytical method proposed in this study. This result confirmed the higher sensitivity of UPLC/FLD, as already stated above. Finally, two samples gave quantifiable amounts of toxins after ELISA analysis, but these levels were not confirmed by using UPLC/FLD. Additionally in this case, the result confirmed the possibility of obtaining “false positive” responses using the ELISA, which was, in this case, equal to 2%. This last characteristic was also confirmed by considering the overall concentrations detected, since the ELISA overestimated the content of the sum of the T-2 and HT-2 toxins compared to UPLC/FLD (mean over estimation: 17%).

Figure 3. Correlation between results obtained by analyzing 27 samples using the ELISA and UPLC/FLD.

Regarding the overall monitoring on the presence of these two mycotoxins in cereals, the 32 confirmed quantifiable levels of two toxins (concentration $> \text{LOQ}$: 9.0 and $5.7 \mu\text{g kg}^{-1}$ for the T-2 and HT-2 toxins, respectively) were detected in barley, oat, durum wheat, maize, and common wheat (12, 9, 6, 3, and 2 samples, respectively). The highest percentage of positive samples was verified in the barley and oat samples (39%), and the lowest was verified in common wheat (18%). However, the levels detected in different types of cereals samples were compared by using a one-way ANOVA ($p < 0.05$), verifying not statistically significant differences among groups. The global area of sampling was not particularly wide ($\sim 7000 \text{ km}^2$), so no evidence of different levels of contamination was verified. Regarding the specific occurrence of two mycotoxins, HT-2 and T-2 were

detected alone in 50% and 25% of samples, respectively, with mean concentrations equal to 203.0 and 125.0 $\mu\text{g kg}^{-1}$, respectively.

If compared to other similar surveys completed in Italy in the last few years, the prevalence of the T-2 and HT-2 toxins in cereals (samples > LOQ: 32%) was comparable to the value obtained by Morcia et al. [33], who analyzed barley samples during the years of 2011–2014 (31.5%). The same authors detected levels of the T-2 and HT-2 toxins (as the sum) in the range of 69–264 $\mu\text{g kg}^{-1}$. Both these levels and those reported by Aureli et al. [34] in their 2011–2013 survey on durum wheat, in which the highest concentration of the detected T-2 and HT-2 toxins (as the sum) was equal to 212 $\mu\text{g kg}^{-1}$, were slightly lower than that registered during the presented monitoring (315.9 $\mu\text{g kg}^{-1}$ of HT-2 toxin; Figure 2D).

4. Conclusions

In this study, two analytical methods based on ELISA and UPLC/FLD were fully validated and then compared based on validation parameters and the results obtained by analyzing 100 samples of animal feed.

The study confirmed that ELISA and UPLC/FLD should be considered to be complementary within official controls, especially when a laboratory needs to analyze high amounts of samples with a high level of reliability in a brief time. The samples should be “screened” by using ELISA, and all samples with concentrations higher than the limit of detection should be confirmed by UPLC/FLD.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2076-3417/11/4/1688/s1>, Figure S1: laboratory approach in screening/confirmation analysis, Table S1: variables chosen for robustness studies, Table S2: results of robustness studies.

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