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Screening of ergot alkaloids by ELISA test kits available on the market

Veršilovskis A, Pereboom-de Fauw DPKH, Smits N, Mulder PPJ, Mol H, de Nijs M

EU Reference Laboratory for mycotoxins and plant toxins in food and feed

Wageningen Food Safety research (WFSR), part of Wageningen University & Research

Akkermaalsbos 2, 6708 WB, Wageningen, the Netherlands

eurl.mycotoxins-planttoxins@wur.nl

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Abstract

Ergot alkaloids (EAs) are mycotoxins that may occur in cereals (especially rye, wheat, triticale, barley) and cereal products. Harmonised EU legislation is in place for ergot sclerotia in unprocessed food and feed (e.g. cereal grains). Regulation of 12 EAs in processed food is foreseen in the near future which triggered a question on availability and applicability of screening methods. To address this question, an inventory of commercially available tests was made resulting in three ELISA test kits. The kits were not necessarily designed for detection of EAs in food and feed or for the EAs types and levels encountered in food and feed. Nevertheless, the kits were tested with respect to qualitative and quantitative determination of EAs in various naturally contaminated cereals, bread, and complete feed. For this, and also to improve effectiveness and throughput, several protocols in addition to the one prescribed by the supplier were employed. The ELISA results were compared to those obtained by LC-MS/MS for the 12 EA to be included in the regulation.

All three tested commercially available test kits for EAs were capable of detecting EAs in various cereal-based food and feed products, although a limited number of false positives and false negative results occurred. Comparing the test kit results with the LC-MS/MS results, it was noted that the test kit results varied strongly between the test kits, the protocol used for extraction, the matrices, and even within a certain matrix. In part this was attributed to different degrees of cross-reactivity of the ELISA for the different EAs (of which some will not be included in the legislation). At this stage, for one of the test kits a reasonable (semi-)quantitative performance was obtained in a narrow range (100-500 µg/kg). This might be improved once regulatory limits are known and the assays can be tailored to these levels and matrices. However, this will require more efforts from the manufacturers. Following improvement of the screening assays, a validation according to the performance criteria mentioned in Regulation (EC) No 401/2006 is needed to demonstrate whether or not it is fit-for-purpose. Until then, the ELISA screening assays may be suited for qualitative testing (absence/presence).

Contents

Introduction

Ergot alkaloids (EAs) are mycotoxins produced by several members within the fungal orders of *Hypocreales* and *Eurotiales*. Within the *Hypocreales* order, *Claviceps purpurea* is the most widespread ergot alkaloid producing fungal species in Europe. It is known to infect more than 400 plant species, including some economically important cereal grains such as rye, wheat, triticale, barley, millet and oats. Infection of typically rye results in the formation of so-called ergots, purple coloured fungal sclerotia resembling long narrow cereal kernels, in the ears. EAs are present in high concentrations in the ergot sclerotia [1].

EAs are classified as tryptophan-derived alkaloids and the physiological effects of this class of compounds have been known for many centuries. It is believed that in the Middle Ages, the consumption of EAs contaminated grains, flour and bread caused severe epidemics of the condition known as St. Anthony's fire. Today, the cause of the disease, called ergotism is well understood. The increased scientific understanding and improvements in agricultural practices and milling techniques, such as grading, sieving and sorting to eliminate the ergot from the kernels, has eliminated severe epidemic outbreaks of ergotism [1].

The European Food Safety Authority (EFSA) published an opinion in 2012 in which they concluded that chemical analysis should focus on the main *C. purpurea* EAs, namely ergometrine, ergotamine, ergosine, ergocristine, ergocryptine (which is a mixture of α- and β-isomers), ergocornine, as well as on the corresponding "-inine" epimers [1]. Although the "-inine" epimers are described to be biologically inactive, interconversion can occur under various conditions and thus EFSA based its risk assessment on both forms. In 2017 EFSA published un update study on exposure of humans and animals to ergot alkaloids in which they estimated that chronic exposure to EAs is 2-3 times higher in the young population than in the adult population [2]. Highest EAs levels were detected in the processed food categories "Mixed wheat and rye bread and rolls", "Rye bread and rolls" and "Rye flakes".

Harmonised EU legislation is in place for ergot sclerotia in food (0.5 g/kg in unprocessed cereals with the exception of corn and rice) and rye ergot in feed (1.0 g/kg, relative to a feed with a moisture content of 12%, (*Claviceps purpurea*) in feed materials and compound feed containing unground cereals) [3, 4]. A standardized method for visual detection of 'besatz' (impurities), including ergot sclerotia, was published by CEN in 2008 [5]. Subsequently the international association for feeding stuff analyses (IAG) published a method specific for ergot sclerotia and performed a ring trial among European laboratories in 2015 [6, 7].

Once the cereals are cleaned, milled or processed, the presence of EAs can only be established by chemical analysis. As opposed to the ergot sclerotia, the ergot alkaloids are not yet regulated in the EU, but this is foreseen for the near future. At present, high performance liquid chromatography with fluorescence detection (HPLC-FLD) and high-performance liquid chromatography – tandem mass spectrometry (LC-MS/MS) allow the quantification of individual EAs in food and feed commodities at relevant levels. As the epimeric forms of EAs can interconvert, analytical methods should include the determination of both epimeric forms [8].

The methods available for visual inspection of incoming cereals for ergot sclerotia at food and feed industrial premises are quick and cheap but nevertheless do require trained personnel. Also, in exceptional cases the sclerotia present in the product may contain no of low amounts of ergot alkaloids [9]. After cleaning, milling and further processing, the sclerotia are no longer present as such. Therefore, there is a need for easy to use and fast screening methods for on-site detection of EAs.

Enzyme Linked Immunosorbent Assays (ELISA) are well known screening methods that have the advantage of being rapid and inexpensive but they are typically less specific and less accurate than LC-FLD or LC-MS confirmatory methods. ELISA is a rapid technique and especially suited for applications where large numbers of samples need to be screened as multiple samples can be analysed in parallel, and expensive equipment is not required. ELISAs have been developed for EAs and some are available commercially. ELISA methods are not specific for individual EAs. This has the disadvantage that it is not possible to estimate the degree of toxicity from the results, but on the other hand it might detect EAs that are not targeted by confirmatory methods. Antibodies are raised that specifically bind to the lysergic acid ring structure, however many peptide alkaloids (ergocryptine, ergocristine, ergocornine, and ergotamine) have large groups attached to the lysergic acid which may hinder the antibody binding [10]. ELISAs have been used to determine ergovaline in seed and straw, and also in rumen fluid but the concentration measured differed greatly and inconsistently when compared to an HPLC procedure [10]. Excessively high results for contaminated fescue grass analysed by ELISA were attributed to the presence of setoclavine, an alkaloid produced by the oxidation of agroclavine [10]. Immunoassay methods are widely used to measure lysergic acid diethylamide (LSD) in body fluids but are prone to giving false-positive results or substantially higher quantification of LSD, presumably due to cross-reactivity to other compounds [10].

The European Union Reference Laboratory (EURL) for mycotoxins and plant toxins was asked by the European Commission to assess the applicability of the currently available ELISA test kits for screening of EAs in food and feed.

This report describes the results of the experiments carried out to detect EAs in cereals using three currently available ELISA test kits. Results obtained by ELISA were compared to results obtained by LC-MS/MS. Moreover, the test kits were assessed for user friendliness. To test robustness of the three ELISA test kits, two additional sample extraction methods were included as well as matrices other than cereals such as bread and complete feed samples.

1. Materials and methods

1.1 Samples

In total of 26 samples were collected in 2018 and analysed. Collected samples belong to categories of cereal flour, bread and feed (Table 1).

Table 1. Samples used in the study.

Sample type	Matrix	No. of samples	No of samples tested		
			ELISA test kit 1	ELISA test kit 2	ELISA test kit 3
Cereal flour	Rye	6	4	4	6
Cereal flour	Barley	2	2	2	2
Cereal flour	Buckwheat	1	1	1	1
Cereal flour	Wheat	2	1	2	2
Cereal flour	Rye-wheat	1	-	1	1
Cereal flour	Wheat-barley	1	1	1	1
Bread	Rye	2	-	2	2
Bread	Rye-wheat	2	-	2	2
Bread	Wheat	2	-	2	-
Bread	Multi-grain	2	-	2	2
Animal Feed	Complete Feed	5	3	5	5
Total		26	12	24	24

Eight bread samples were obtained from retail stores in the Netherlands. Ten ground cereal samples were provided by one of the ELISA test kit providers. Five ground feed samples and another three cereal samples were available from a CEN collaborative study. The bread samples were ground under cryogenic conditions and stored at -20 °C until analysis. All 26 samples were first analysed by LC-MS/MS to determine the EAs content.

1.2 Measurement of EAs using LC-MS/MS and ELISA test kits

1.2.1 LC-MS/MS method

The samples were analysed by LC-MS/MS using an in-house validated and accredited method, SOP A1070: Animal feed – Determination of ergot alkaloids and tropane alkaloids – LC-MS/MS. The LC-MS/MS method is also described in Mulder et al. (2015) [11]. The method was used to quantify the following EAs: the "major" EAs – ergocornine, ergocorninine, ergocristine, ergocristinine, α-ergocryptine, β-ergocryptine, α-ergocryptinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine, and the "minor" EAs – agroclavine, chanoclavine-1, elymoclavine, ergine, erginine, festuclavine, lysergol. Samples, for which the amount of individual EAs was exceeding the calibration range (0-1000 µg/kg; LOQ: 5 µg/kg; LOD: 1 µg/kg), were diluted to fit the calibration range of the test method. For each individual EA concentrations were obtained. The total concentration of EAs which are to be included in the foreseen legislation (ergosine, ergocornine, ergocryptine, ergotamine, ergocristine, ergometrine and their "–inine" epimers) was used for comparison with results obtained by the ELISA kits.

1.2.2 ELISA test kits

Three ELISA test kits were identified for the screening of the total sum of EAs through internet searching and contacting known suppliers of ELISA tests kits for mycotoxins in April 2018.

Table 2 shows the ELISA test kits used in the study, as well as the producer's information on the target matrices, the concentration range and specificity.

Table 2. Specifications of ELISA test kits used in the study.

ELISA test kit provider	ELISA test kit's name (qualitative or quantitative)	Target matrix	Test range in matrix & lowest calibration point (LCP), µg/kg	Specificity (as stated by producer)
Agrinostics Ltd. Co. (Test kit 1)	Phytoscreen Ergot Alkaloid Kit for Plant tissue (semi-quantitative)	Plant tissue	0-2000 LCP: 125	Ergoline & ergopeptine types of EAs
Randox Laboratories Ltd. (Test kit 2)	Ergot Alkaloids ELISA (quantitative)	Flour & seed	0-44.4 LCP: 0.6 [ergotamine equivalents] In this study adapted to: 0-2777.5 LCP: 36 [cereal flour & feed] 0-111 LCP: 1.45 [bread]	Ergotamine, ergotaminine, ergosine, ergosinine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, ergocornine, ergocorninine, ergometrine, ergometrinine, ergovaline, agroclavine, lysergic acid, lysergic acid diethylamide (LSD), iso-LSD, lysergol
LCTech GmbH (Test kit 3)	ErgoRead (qualitative)	Wheat, Rye, Triticale	0-5000 LCP: 250 In this study adapted to LCP: 62.5 [bread]	EAs not specifically indicated, but aimed at the detection of EAs present in *C. purpurea*

For the specific ELISA tests the intended target matrix and concentration range did not always match with the intended application in this study (e.g. bread). Therefore, in addition to the producer's protocol also alternative protocols were used (P1 – producer's sample preparation protocol; P2 and P3 – alternative protocols). For practical reasons some deviations had to be made to the method protocols provided by the producers:

- ELISA test kit 1 – The amount of sample recommended by the supplier, 0.1 g, was considered not representative for the application in flour, feed and bread. The sample intake was increased to 0.5 g, thereby increasing buffer volume to 40 mL. A larger sample intake was not possible due to the limited amount of extraction buffer provided.
- ELISA test kit 2 - The provided sample preparation protocol was rather complicated and time consuming and, in addition, the proposed test range was very limited. After consultation with the producer, the method was amended to extend the testing range and an alternative extraction protocol was proposed by the producer. The calibration range was extended to 2800 µg/kg to be able to quantify at higher range. This was achieved by changing sample

intake, extraction solvent volume or by changing dilution factor of final sample extract with the buffer as described in producer's protocol.

- ELISA test kit 3 – The prescribed sample intake was 20 g. Due to the limited amount available of certain samples, the sample intake was reduced to 4 g and the extraction solvent volume was reduced from 50 mL to 10 mL.
- All test kits - Samples, for which the expected amount of EAs would fall outside the calibration range, were diluted to fit the calibration range of the test method.

For bread samples, sample preparation was the same as for the cereal and feed samples. However, for practical reasons some additional adjustments had to be made:

- ELISA test kit 2: For bread samples a test range of 0-111 µg/kg (0-1.45-3.35-8-19.35-111.1) was used and the following protocols applied:
  1. Protocol No. 1 (P1) – A final dilution of 200 µL of reconstructed extract with 800 µL of provided dilution buffer was used.
  2. Protocol No. 2 (P2) – The final extract was prepared without dilution with buffer.
  3. Protocol No. 3 (P3) – The final extract was prepared without dilution with buffer.
- ELISA test kit 3: for bread samples the following extraction and sample preparation procedures were used:
  1. Protocol No. 1 (P1) – Buffer No. 1 (buffer for wheat samples) was used for all samples.
  2. Protocol No. 2 (P2) – Buffer No. 2 (buffer for rye and triticale) was used for all samples.
  3. Protocol No. 3 (P3) – Extracts were diluted with a mixture of provided buffers 1 and 2 (50/50, v/v). The calibration range was extended down to 62.5 µg/kg (two calibration points added – 62.5 and 125 µg/kg). This was achieved by diluting the lowest calibration standard (250 µg/kg) with the recommended buffer.

Table 3 shows an overview of the extraction methods and sample intakes used for cereal flour, bread and feed samples by LC-MS/MS and each ELISA test kit. ELISA test kit 1 was not evaluated for bread.

Table 3. Extraction solvents and sample intakes used for each method.

Sample preparation procedures	**ELISA test kit 1**	**ELISA test kit 2**	**ELISA test kit 3**
Producer's protocol: extraction solvent & volume used (P1)[#] **In bold**: **modification made to producer's procedure**	Extraction buffer (composition not disclosed) 0.1 g + 8 mL / **0.5 g + 40 mL**	EtOAc/MeOH/ 0.2 M NH_4HCO_3 pH 8.5, (62.5/25/12.5, v/v) 5 g + 40 mL -	MeOH/0.25% H_3PO_4, (60/40, v/v) 20 g + 50 mL / **4 g +10 mL** (+wheat buffer)
Alternative protocol: extraction solvent & volume used (P2)[#]	-	MeCN/MeOH/H_2O* (50/40/10, v/v) 5 g + 25 mL	MeOH/0.25% H_3PO_4, (60/40, v/v) 4 g +10 mL (+rye & triticale buffer)
Alternative protocol: extraction solvent & volume used (P3)[#]	MeOH/H_2O/FA** (60/40/0.4, v/v) 2.5 g + 25 mL -	MeOH/H_2O/FA (60/40/0.4, v/v) 2.5 g + 25 mL **2.5 g + 12.5*** mL**	MeOH/H_2O/FA (60/40/0.4, v/v) 4 g + 10 mL (+mix of 2 buffers)

[#]P1 is the producer's protocol (including modifications), P2 are alternative protocols proposed by the producer and P3 are alternative protocols tested by WFSR. *Proposed by producer; **LC-MS/MS method's original sample intake and extraction solvent volume: 4 g + 40 mL; ***Extraction solvent volume used for bread samples.

For ELISA well plate preparation, the subscribed method by each ELISA test kit supplier was always followed. The calibration line was always prepared as explained in producer's protocol(s). The details are given in Appendices 1-3. EA results obtained by each ELISA kit were compared to the sum of 12 prioritised EAs (ergosine, ergocornine, ergocryptine, ergotamine, ergocristine, ergonovine and their "–inine" epimers) as quantified by the LC-MS/MS method.

1.3 Definition of false negatives and false positives

In screening methods, a "negative sample" means the EAs content in the sample is below a pre-specified value, typically the regulatory limit, with a certainty of 95%. A "false negative sample" means that the EAs content in the sample is above the pre-specified value but the outcome of the screening measurement indicates it as negative (Regulation (EU) No 401/2006)[12]. Similarly, a positive sample (screen positive or suspect sample) means that the EAs content in the sample exceeds a specified value. When the outcome of the screening measurement indicates the sample is positive while the true value is negative, then the outcome is defined as a false positive.

The interpretation in this work slightly deviates from the above, in the sense that at the time the work was done it was not clear what the regulatory limits for EAs in the different products might be. Consequently, the experiments were not done with any specific pre-specified value for EAs in mind. Here, the screening results are compared to the quantitative results (sum of 12 EAs) as obtained by LC-MS/MS analysis. A false negative was assigned when EAs were quantified by LC-MS/MS and not detected by the ELISA. A false positive was assigned when EAs were not quantified by LC-MS/MS, but detected by the ELISA.

2. Results and Discussion

2.1 ELISA test kits

2.1.1 Test range

According to recent literature data, up to 59% of food (up to 95% of rye-based food, up to 86% of wheat-based food) and up to 52% of feed (mainly rye) may be contaminated by measurable amounts of EAs [13, 14]. The contamination has been reported to range from 1 to 12400 µg/kg (contamination by individual EAs could reach up to 3270 µg/kg (ergotamine)). For bread, typical contamination levels between 1 and 500 µg/kg were reported [13, 14].

The working range of ELISA test kit 1 as specified by the supplier (0-2000 µg/kg) was considered fit-for-purpose, and was used as such.

The working range of ELISA test kit 2 (0-44 µg/kg) was very limited. Therefore, after consultation with the producer of the test kit, some adaptions were made to extend the testing range, to 0-111 µg/kg for bread samples and to 0-2800 µg/kg for cereal flour and feed samples.

The working range of ELISA test kit 3 (0-5000 µg/kg) was very wide. However, the lowest calibration point was 250 µg/kg, and experimental evaluation revealed that concentrations lower than 250 µg/kg could not be sufficiently discriminated from the control (blank). In an attempt to extend the calibration range, two calibration points (62.5 and 125 µg/kg) were added. This was done by diluting the standard solution of 250 µg/kg according to the producer's protocol. Unfortunately, this did not help to determine EAs amounts lower than 250 µg/kg.

2.1.2 EAs in test method and cross-reactivity

Individual ergot alkaloids will differ in their relative cross reactivity towards the antibody in the different ELISA tests. Since the relative cross reactivities of the ELISAs for the different EAs are not exactly the same, an overestimation/underestimation of the total content may occur. Furthermore, the tests may be sensitive to EAs not covered by the EU draft legislation (considering only 6 EAs + 6 their epimers). These other EAs may occasionally be present in samples. It is evident, that when the responses/reactivities for individual EAs differ a lot, it will be unlikely that the sum of EAs obtained by ELISA will correspond closely to the sum of EAs obtained by LC-MS/MS. Also, matrix effects must be taken into account as coloured compounds present in the matrix could possibly cross-react to the antibodies of the ELISA tests (or possibly small quantities are not fully washed away from ELISA well plate) affecting final intensity of colour and affecting the final result. It means, that due to the lack of specificity, false-positive or false-negative as well as higher or lower values are always possible.

As seen from Table 2, for test kit 1 the producer has indicated that the specificity of the ELISA covers all ergoline and ergopeptine forms of EAs, thus including the EAs from legislation as well as potentially other EAs not covered by legislation.

The producer of test kit 2 has indicated that its ELISA is specific to ergotamine and 17 other EAs (see Table 2). The test is also sensitive to 4 EAs (ergovaline, LSD, iso-LSD and lysergic acid) which were not included in LC-MS/MS method.

The producer of test kit 3 did not indicate to which EAs the ELISA test is sensitive, other than that it should work for EAs typically encountered in contaminated wheat, rye and triticale. This suggests that the test kit is sensitive towards the EAs produced by *Claviceps purpurea*, which is the most widespread ergot alkaloid producing fungal species in cereal grains.

As can be concluded from information above, the sum of EAs detected by each individual ELISA kit could be significantly different from the sum of the 12 EAs as determined by the LC-MS/MS method.

2.1.3 User-friendliness

The sample preparation protocol of ELISA test kit 1 was straightforward and for trained personal it would not be a problem to produce results within one working day. One drawback of the provided test kit is that the producer indicated that the wells on the perimeter of the well plate could not be used as they were prone to give false readings. So, as a result 36 out of 96 wells could not be used.

The original sample preparation protocol supplied for ELISA test kit 2 was rather complicated and time consuming as it employed several liquid-liquid extraction and evaporation steps. Therefore, even for trained personal to provide a final result would take a throughput time up to two working days. In addition, the working range was very limited (may not be a problem after legislation is in place). Also, the use of extraction solvents such as ethyl acetate and especially *n*-hexane are less desirable. Upon feedback, the producer proposed an alternative, simplified sample preparation procedure. The proposed procedure worked well enough to serve as replacement for the original one, and could reduce the total time to one working day.

The sample preparation protocol of ELISA test kit 3 was simple enough to allow reporting of obtained results within one working day. For this kit, different buffers were to be used for different matrices, which could be experienced as a disadvantage.

2.2 Determination of EAs in cereal flour samples and feed samples

2.2.1 ELISA test kit 1 - Agrinostics "Phytoscreen ELISA kit"

ELISA kit 1 was evaluated for determination of EAs in cereal flour and feed. It is important to remark that ELISA test kit 1 was developed for use in plant tissue, not for food or feed matrices. The assumption that ELISA test kit 1 should be used to identify highly contaminated plant tissue samples, justifies the low sample intake prescribed in the protocol. However, it was therefore necessary to adapt the protocol to accommodate a larger sample intake.

The results obtained for cereal flour samples using ELISA test kit 1 are shown in Table 4.

Table 4. ELISA test kit 1. Determination of EAs in cereal flour samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	ELISA P1# result, µg/kg	ELISA P3# result, µg/kg	LC-MS/MS result, µg/kg
1	Barley	780	958	<LOQ*
2	Rye	869	910	111
3	Buckwheat	753	930	141
4	Wheat+Barley	934	739	302
5	Rye	930	1105	369
6	Barley	933	1022	947
7	Rye	1580	1269	997
8	Wheat	856	770	1038
9	Rye	1300	1718	1133

P1 – producer's protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit highest calibration point (HCP) is 2000 µg/kg. *LOQ of LC-MS/MS method is 5 µg/kg for each of 12 compounds (LOD is 1 µg/kg).

As can be seen from Table 4 there is in general no large difference in the results between the tested extraction protocols P1 and P3. Therefore, both protocols can be equally used. Comparing the results obtained from both protocols with the LC-MS/MS results, ELISA P1 and P3 look similar and all concentrations fall in a range from 750 to 1720 µg/kg. For both protocols much higher indicative values were obtained for buckwheat sample (No 3), rye samples No 2, No 5 and No 7 and for sample No 4 (wheat + barley) than the concentrations estimated by LC-MS/MS. In addition, both protocols produced a false-positive result for barley sample No 1. This may be caused by sensitivity of the test to other structurally related compounds, including matrix compounds or by an interfering signal from the matrix as this test was designed for plant tissue and not for complex matrices like cereals and feed.

The results obtained for feed samples using ELISA test kit 1 are shown in Table 5.

Table 5. ELISA test kit 1. Determination of EAs in compound feed samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	ELISA P1[#] result, µg/kg	ELISA P3[#] result, µg/kg	LC-MS/MS result, µg/kg
1	Broiler Feed	814	0*	179
2	Porcine Feed	1270	888	1243
3	Bovine Feed	741	1040	1386

[#] P1 – producer's protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit HCP is 2000 µg/kg. *Below the lowest calibration point (LCP) 125 µg/kg.

As seen from Table 5, for two samples (porcine feed No 2 and bovine feed No 3) there is almost no difference between LC-MS/MS and the results of both sample preparation protocols (P1 & P3). However for broiler feed No 1 a false-negative result was obtained with protocol P3, while protocol P1 gave an indicative concentration much higher than was obtained by LC-MS/MS.

The false negative result with P3 could be explained by the fact that the LC-MS/MS result is close to the lowest calibration point of the ELISA test. Overall, both protocols (P1 and P3) could be used, but they are not directly comparable to the LC-MS/MS results.

It was noted during the experiments that the chromophore used in ELISA test kit 1 does not give very intense colours, while this is required for reliable measurement. The intensity range used by the ELISA test kit is quite narrow (~0.075 to ~0.225 OD (optical density) corresponding to a range of 0-2000 µg/kg and only ~0.075 to ~0.125 in the range 1000 to 2000 µg/kg. Therefore, a slight change in the colour, which could be caused by matrix components, may potentially influence the final result.

2.2.2 ELISA test kit 2 - Randox "Ergot alkaloids ELISA"

ELISA kit 2 was evaluated for detection of EAs in cereal flour, feed and bread samples. The g results obtained for cereal flour samples are shown in Table 6.

The results in Table 6 show that in most cases the EAs results obtained by ELISA test kit 2 are considerably lower in the low contamination region (<500 µg/kg) and higher in the high contamination region (>500 µg/kg) as compared to the results obtained by LC-MS/MS.

Evaluation of results showed that, even though trends look similar, there is significant difference between the three protocols and LC-MS/MS, but also between protocols P1, P2 and P3. Protocols P2 and P3 have a clear tendency of an overestimation of the EA content in the samples where the EA content in the sample is close to or higher than 1000 µg/kg and of a slight underestimation where the content is between 100 and 300 µg/kg; while protocol P1 tended to underestimate the EA content over the whole concentration range.

For barley sample No 1, rye sample No 3 and wheat sample No 4 protocol P1 gave "negative" results, which is in agreement with the LCP of the ELISA test kit (36 µg/kg) (see Table 2). Since the levels found by LC-MS/MS were very low, these negatives were not considered false negatives here. For samples No 1 (barley) and No 2 (rye) both – protocol P1 and P2 showed false positive results (above the LOQ by LC-MS/MS), but these results could not be considered as false positives as they are still below LCP of the ELISA test kit (36 µg/kg).

Table 6. ELISA test kit 2. Determination of EAs in cereal flour samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	ELISA P1# result, µg/kg	ELISA P2# result, µg/kg	ELISA P3# result, µg/kg	LC-MS/MS result, µg/kg
1	Barley	0*	25*	15*	<LOQ**
2	Rye	4*	33*	24*	<LOQ**
3	Rye	0*	26*	22*	5
4	Wheat	0*	37	24*	14
5	Rye+Wheat	8*	48	25*	40
6	Rye	41	45	13*	111
7	Buckwheat	83	123	88	141
8	Wheat+Barley	104	121	98	302
9	Rye	116	NT	NT	369
10	Barley	655	2916***	2916***	947
11	Rye	239	NT	NT	997
12	Wheat	426	2916***	2916***	1038
13	Rye	381	2916***	2916***	1133

#P1 – producer's original protocol; P2 – producer's alternative protocol; P3 – WFSR alternative protocol (LC-MS/MS method). Test kit HCP is 2800 µg/kg; *Below the lowest calibration point (LCP) of 36 µg/kg; **LOQ of LC-MS/MS method is 5 µg/kg for each of 12 compounds (LOD is 1 µg/kg); ***Above the highest calibration point (HCP) of the method (2800 µg/kg); NT – not tested.

The results obtained for different feed samples are shown in Table 7.

Table 7. ELISA test kit 2. Determination of EAs in compound feed samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	P1# result, µg/kg	P2# result, µg/kg	P3# result, µg/kg	LC-MS/MS result, µg/kg
1	Feed	0*	38	30*	13
2	Feed	25*	51	58	58
3	Feed-Broiler	108	87	61	179
4	Feed-Porcine	122	2916**	2916**	1243
5	Feed-Bovine	126	2916**	2916**	1386

P1 – producer's original protocol; P2 – producer's alternative protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit HCP is 2800 µg/kg.; *Below the lowest calibration point (LCP) of 36 µg/kg; **Above the highest calibration point (HCP) of the method (2800 µg/kg); LOQ of LC-MS/MS method is 5 µg/kg for each of 12 compounds (LOD is 1 µg/kg).

One "negative" result was obtained using protocol P1 for feed sample No 1 (containing 13 µg/kg according to LC-MS/MS). As mentioned above, since the level found by LC-MS/MS was very low, this negative was not considered false negative here. However, the variation in the results using various extraction methods is considerable. Protocol P1 strongly underestimated the EA content present in feed samples No 4 and No 5, while protocol P2 and P3 underestimated the content only

in sample No 3 and overestimated in the samples No 4 and 5. In general, the trends obtained for protocols P2 and P3 are similar to what was seen for the cereal samples (overestimation for samples with content above 1000 µg/kg, tendency to underestimation in the lower concentration range).

Although, ELISA test kit 2 was not developed and validated for bread, its performance was tested using different bread samples. The results obtained for 8 different bread samples are shown in Table 8.

Table 8. ELISA test kit 2. Determination of EAs in bread samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	P1# result, µg/kg	P2# result, µg/kg	P3# result, µg/kg	LC-MS/MS* result, µg/kg
1	Wheat	8	36	7	3
2	Wheat	18	21	5	4
3	Multi-grain	24	117	20	7
4	Multi-grain	20	39	9	9
5	Rye	22	18	10	10
6	Rye-wheat	38	25	11	17
7	Rye-wheat	5	44	15	22
8	Rye	117	19	3	35

#P1 – producer's original protocol; P2 – producer's alternative protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit HCP is 111 µg/kg. *LOQ of LC-MS/MS method varied from **0.3 up to 1 µg/kg** (LODs 0.1-0.3 µg/kg) for each of 12 compounds; lowest calibration point (LCP) of the ELISA test kit was 1.45 µg/kg.

As the expected EAs concentrations in the bread samples were lower than the calibration range indicated by the producer, the sample extracts were prepared without final dilution with buffer in all preparation protocols (P1, P2 and P3), in an attempt to lower the detection limit of the ELISA.

As can be seen from Table 8, except for a few cases, there is no large difference between results obtained by the tested protocols and the results obtained by LC-MS/MS. Different to what was found for the cereal and feed samples, protocols P1 and P2 gave higher indicative concentrations than protocol P3. For one result with protocol 2 (multi-grain bread sample 3) and one result with protocol 1 (rye bread sample 8) the estimated concentration was substantially higher than the LC-MS/MS result, while one result with protocol 3 (rye bread sample 8) gave a lower estimated EA content than LC-MS/MS. Therefore, protocols 1, 2 and 3 (all without dilution with buffer at the end) can be used as alternative to the producer's protocol, giving results which in most cases are relatively close to ones obtained by LC-MS/MS.

As mentioned before, deviations may be explained by differences in specificity between ELISA and LC-MS/MS. Comparing results obtained for cereal flour, compound feed and bread, ELISA test kit 2 worked reasonably well for bread samples. For a number of bread samples, the indicative concentrations were close to those obtained by LC-MS/MS. Due to the sensitivity of the test kit "negative" results may be obtained if in the sample the total EAs content in cereal flour samples is lower than 15 µg/kg and lower than 13 µg/kg in feed samples. Optimisation of the calibration range of the test kits in relation to the extraction procedure used, could further improve its applicability for cereal and feed samples. It may be interesting to try the adapted protocol for

bread for the feed samples with low contamination levels. Furthermore, for a more reliable evaluation, bread samples covering a wider concentration range should be tested.

2.2.3 ELISA test kit 3 - LC Tech "ErgoRead ELISA"

ELISA kit 3 was evaluated for detection of EAs in cereal flour, feed and bread samples. The results obtained for cereal flour samples are shown in Table 9.

Table 9. ELISA test kit 3. Determination of EAs in cereal flour samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	ELISA P1# result, µg/kg	ELISA P2# result, µg/kg	ELISA P3# result, µg/kg	LC-MS/MS result, µg/kg
1	Barley	1031	3181	706	<LOQ*
2	Rye	256	359	336	<LOQ*
3	Rye	250	268	300	5
4	Wheat	374	332	514	14
5	Rye+Wheat	520	654	471	40
6	Rye	5247***	5247***	2946	111
7	Buckwheat	5247***	5247***	1667	141
8	Wheat+Barley	4032	1177	831	302
9	Rye	844	600	586	369
10	Barley	318	330	188**	947
11	Rye	5247***	5247***	5247***	997
12	Wheat	5247***	5247***	3911	1038
13	Rye	722	764	352	1133

#P1 – producer's original protocol; P2 – producer's alternative protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit HCP is 5000 µg/kg. *LOQ of LC-MS/MS method is 5 µg/kg for each of 12 compounds (LOD 1 µg/kg); **Below the lowest calibration point was 250 µg/kg; ***Above the highest calibration point (HCP) of the method (5000 µg/kg).

As can be seen from Table 9, in most cases, results obtained by ELISA test kit 3 were higher as compared to the results obtained by LC-MS/MS. For samples No 6 (rye) and No 7 (buckwheat) all three protocols gave a very large overestimation (10- to 50-fold) of the EA content. In addition, for the barley sample No 1, all sample preparation protocols gave a false-positive result. Also for samples No 2 (rye), No 3 (rye), No 4 (wheat) and No 5 (rye + wheat) the ELISA test indicated a substantial EA content while according to LC-MS/MS analysis the EA content was very low. In contrast, for samples No 8 (wheat + barley), No 9 (rye), No 10 (barley), No 12 (wheat) and No 13 (rye), a reasonable correlation was found between the estimated concentrations with the three protocols and the LC-MS/MS results.

No significantly different results were obtained with respect to the application of buffer 1 (according to producer this buffer needs to be used for wheat samples, protocol P1) and buffer 2 (this buffer needs to be used for rye and triticale, protocol P2). In fact, in combination with our extraction solvent, a mixture of both buffers in most cases worked better (P3), than each of them separately.

Although ELISA test kit 3 was not developed for feed, its performance was tested using different compound feed samples. The results obtained for feed are shown in Table 10.

Table 10. ELISA test kit 3. Determination of EAs in compound feed samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	ELISA P1# result, µg/kg	ELISA P2# result, µg/kg	ELISA P3# result, µg/kg	LC-MS/MS result, µg/kg
1	Feed	5247*	5247*	2975	13
2	Feed	347	1270	249**	58
3	Feed-Broiler	203**	206**	228**	179
4	Feed-Porcine	1361	5247*	987	1243
5	Feed-Bovine	229**	323	270	1386

#P1 – producer's original protocol; P2 – producer's alternative protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit HCP is 5000 µg/kg. *Above the highest calibration point (HCP) of the method (5000 µg/kg); **Below the lowest calibration point was 250 µg/kg; (LOQ of LC-MS/MS method is 5 µg/kg for each of 12 compounds (LOD 1 µg/kg).

As can be seen from Table 10, for some samples reasonable results were obtained with ELISA test kit 3 (e.g. broiler feed No 3 and porcine feed No 4), while for other samples the outcome was (much) less satisfying. For feed sample No 1 all ELISA protocols gave very high indicative concentrations, while according to LC-MS/MS the sample contained only trace levels of EAs. This indicates that matrix type and nature can significantly affect the final result. It also shows that for this particular matrix all applied protocols could be used equally.

Although, ELISA test kit 3 was not developed for bread, its performance was tested using different bread samples. Results obtained for bread are shown in Table 11.

Table 11. ELISA test kit 3. Determination of EAs in bread samples. Results sorted by the amount of EA in the sample as determined by LC-MS/MS.

Sample No	Matrix	ELISA P1# result*, µg/kg	ELISA P2# result*, µg/kg	ELISA P3# result*, µg/kg	LC-MS/MS** result, µg/kg
1	Multi-grain	200	218	110	7
2	Multi-grain	120	371	116	9
3	Rye	347	432	199	10
4	Rye-wheat	99	393	171	17
5	Rye-wheat	102	328	92	22
6	Rye	162	1601	139	35

#P1 – producer's original protocol; P2 – producer's alternative protocol; P3 – WFSR alternative protocol (LC-MS-MS method). Test kit HCP is 5000 µg/kg. *The lowest calibration point of ELISA test kit 3 was 62.5 µg/kg; **LOQ of LC-MS/MS method varied from 0.3 up to 1 µg/kg (LODs 0.1-0.3 µg/kg) for each of 12 compounds.

As can be seen from Table 11, for all ELISA protocols the indicative values obtained for bread samples were significantly higher than the ones obtained by LC-MS/MS. Protocol 2 produced the

highest results, while there was not much difference between P1 and P3. The reason for this discrepancy is unclear. It should be noted that the current working range of test kit 3 (LCP 250 µg/kg to HCP 5000 µg/kg) is not matching well with the concentrations present in the bread samples.

There were no false-negative or false-positive results and all used protocols could be equally used for screening of EAs presence in different bread samples, provided that the calibration of the ELISA test could be brought more in line with the actual concentrations in the bread samples.

2.3 Overall

The results obtained by the different ELISA test kits, using different sample preparation protocols, show that the ELISA test kits can detect EAs in a qualitative way (in some cases semi-quantitative) in a broad range of samples, although false positives and false negatives do occur in some cases.

The working range of the tests is somewhat limited and this may need improvement, especially when the tests are used for a wider range of matrices, containing different levels of EAs. The optimal working range is often smaller than the working range covered by the calibration standards. Optimisation of the calibration range of the test kits in relation to the extraction procedure used, may in some cases further improve the applicability of the test kits.

The tested ELISA kits may not be specific enough. There is always a chance of cross-reactivity with other EAs, not included in the scope of proposed legislation.

In terms of overall analysis time (more or equal to 1 working day) required to obtain a result, the ELISAs are not much faster than LC-MS/MS analysis. This is mainly due to the fact that the required sample preparation procedures are similar as for LC-MS/MS analysis, or even more laborious. Obviously, the ELISA test does not need a sophisticated laboratory environment equipped with costly LC-MS/MS equipment and staff to operate such instrumentation.

All results obtained for determination of EAs by the three ELISA test kits using the different extraction protocols tested are summarised in Table 12 (quantitative performance) and Table 13 (qualitative performance).

Table 12. Quantitative performance of ELISA tests for the 3 matrices investigated.

Matrix type	ELISA test kit 1			ELISA test kit 2			ELISA test kit 3		
	P1	P2	P3	P1	P2	P3	P1	P2	P3
Cereals	-	NT	-	-	+/-	+/-	-	-	-
Feed	+	NT	+	-	-	-	-	-	-
Bread	NT	NT	NT	-	+/-	+/-	-	-	-

(+): reasonable (deviation ≤ 30%) agreement between LC-MS/MS and ELISA results for more than a half of tested samples; (+/-): variable (deviation between 30 and 50%) agreement between LC-MS/MS and ELISA results for at least half of tested samples; (-): poor (deviation > 50%) agreement between LC-MS/MS and ELISA results for more than a half of tested samples; NT: not tested.

Table 13. Qualitative performance of ELISA tests for the 3 matrices investigated.

Matrix type	ELISA test kit 1			ELISA test kit 2			ELISA test kit 3		
	P1	P2	P3	P1	P2	P3	P1	P2	P3
Cereals	+/-	NT	+/-	+	+	+	+/-	+/-	+/-
Feed	+	NT	-	+	+	+	+/-	+/-	+/-
Bread	NT	NT	NT	+	+	+	+	+	+

(+): good performance: ELISA showed positive/negative result for samples positive/negative by LC-MS/MS; no false-positive or false-negative; (+/-): medium performance: ELISA showed one or more false-positive results (>LOQ by LC-MS/MS); (-): variable performance: ELISA showed at least one false-negative result for samples where EA content by LC-MS/MS is higher than ELISA's lowest calibration point; NT: not tested.

3. Conclusions

Three commercially available ELISA test kits for EAs were tested for their capability of detection/quantification. This was done by comparison of the ELISA result with that obtained by quantitative LC-MS/MS analysis (sum of 12 EAs to be regulated).

All three tested commercially available test kits for EAs were capable of detecting EAs in various cereal-based food and feed products, although modification of the extraction protocols was needed in some cases, and a limited number of false positives and false negatives occurred.

In terms of (semi-)quantitative performance, the comparability of the result from the ELISAs with those obtained by LC-MS/MS varied between the test kits, the protocol used for extraction, the matrices, and even within a certain matrix.

Alternative sample preparation protocols could be used instead of the producer's ones giving equal or better results, and in addition, could result in shorter overall analysis times.

The tested ELISA kits could be used, after some modifications, for matrices beyond those indicated by the producers.

Taking into account that many food/feed products are contaminated with varying levels of EAs, screening method(s) providing only an EA presence/absence answer are not good enough, because (if they are sensitive enough) the outcome could be that a high number of samples will test positive and need to be re-analysed by a confirmatory method. Once regulatory limits are known, it may be possible to tailor the ELISA protocols to perform optimal around these limits to facilitate testing whether or not the contamination is below or above the limit.

ELISA test kits are not necessarily faster than LC-MS/MS based methods, especially not when the number of samples to be simultaneously analysed is limited, but they do potentially offer the possibility of testing in a more basic laboratory environment such as production site QC labs.

4. Recommendations

Based on this first evaluation of three ELISA test kits the following recommendation can be made:

- The amount of sample to be extracted needs to representative for the lot tested, in practice even after rigorous homogenisation/milling, the amount to be extracted will be >1 gram).
- The extraction as currently provided by the suppliers should (and can) often be simplified to lower overall analysis time and make analysis more efficient
- The applicability with respect to matrices should (and can) be extended since EAs can end up in a large variety of cereal-based products.
- The ELISA protocols should be tailored to the (intended) regulatory limits for EAs in the various food products to allow compliance classification.
- Before use of the screening assays for compliance testing, the assay should be validated according to CR (EU) 519/2014, with specific attention to validity to different matrices.
- Validation of the screening assays should be done with naturally contaminated samples, with known content of the 12 regulated EAs.

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Appendix 1

ELISA test kit 1 (Agrinostics "Phytoscreen ELISA kit")

Sample extraction procedure according to producer's protocol (P1)

1. Weigh exactly 0.1* g of ground plant tissue and place it into 10 mL disposable tubes
2. Add 8* mL of the diluted extraction buffer to each tube and invert to min
3. Let the extraction process continue for 1 hour, inverting the tubes every 15 minutes to mix
4. Let the samples stand for 15 minutes after the final mix to permit the solid and liquid phases to separate
5. Using a pipette, transfer 1 mL of the liquid fraction to a micro centrifuge tube and cap
6. Centrifuge the sample at 5000 g for 3 minutes
7. Withdraw 50 µL of liquid from the centrifuged samples
8. Dispense the liquid into ELISA microplate well

***Modifications made: 0.5 g** of sample + **40 mL** of extraction buffer

Alternative extraction protocol tested (P3)

1. Weight 2.5 g of homogenised sample into 50 mL PP tube
2. Add 25 mL of MeOH/H_2O/FA, 60/40/0.4, v/v
3. Extract on overhead extraction shaker for 30 min
4. Using a pipette, transfer 1 mL of the liquid fraction to a micro centrifuge tube and cap
5. Centrifuge the sample at 5000 g for 3 minutes
6. Withdraw 100 µL of liquid from the centrifuged samples
7. Dilute with 700 µL of extraction buffer
8. Dispense 50 µL the liquid into a ELISA microplate well

Figure 1. Example pictures of used ELISA kit (test kit 1)
Lot No: #ENDO899-96p
Expiration date: Aug 2018

Appendix 2

ELISA test kit 2 (Randox "Ergot alkaloids ELISA")

Sample preparation procedures used for cereal flour and feed samples

Sample extraction procedure according to producer's protocol (P1)

1. To 5 g of homogenised sample (powder form) add 40 mL of ethyl acetate-methanol-0.2M ammonium bicarbonate pH 8.5 (62.5/25/12.5; v/v)
2. Vortex then roll for 30 minutes
3. Centrifuge at 3000 rpm for 10 minutes
4. Transfer 15 mL of the supernatant into a new tube and add 5 mL of ammonium bicarbonate buffer pH10 and 5 mL ammonium sulphate (saturated solution) to induce phase separation
5. Vortex for 1 minute and then leave to stand for 10 min
6. Transfer 5 mL of the ethyl acetate layer (top layer) into a test tube and evaporate at 40°C until dry
7. Reconstitute in: 200 µL of MeOH/MeCN/H_2O (20/40/40, v/v/v) and add 200 µL of n-hexane
8. Vortex for 1 minute
9. Transfer content to Eppendorf vial before centrifuging at 13000 rpm for 10 minutes using micro-centrifuge
10. Discard the n-hexane fraction
11. Dilute 1:9 using working strength wash buffer (200 µL + 1800 µL) *
12. Dispense 50 µL of the liquid into a microplate well

***Modifications made to extend range:**

a) Dilute **40 µL** with **960 µL of buffer** (test range for cereals and feed extended from 0-44.5 µg/kg to 0-2777.5 µg/kg)

b) Dilute **200 µL + 800 µL for bread samples** (test range for bread 0-111 µg/kg)

Sample extraction procedure according to alternative protocol proposed by the producer (P2)

1. To 5 g of homogenised sample add 25 mL of MeCN/MeOH/H_2O (50/40/10, v/v)
2. Shake for 1 min and then roll for extra 10 minutes (overhead shaker)
3. Centrifuge at 3000 rmp for 2 min
4. Dilute 40 µL with 960 µL of working strength wash buffer (**no dilution for bread samples**)
5. Apply 50 µL to ELISA plate

Alternative sample extraction protocol tested (P3)

1. Extract 2.5 g of homogenised sample with 25 mL of MeOH/H_2O/FA (60/40/0.4, v/v) for 30 min
2. Centrifuge at 3000 rmp for 15 min
3. Dilute 80 µL with 920 µL of working strength wash buffer (**no dilution for bread samples**)
4. Apply 50 µL to ELISA plate

Appendix 2 continued

ELISA test kit 2 (Randox "Ergot alkaloids ELISA")

Figure 2. Example pictures of used ELISA kit (test kit 2)

Batch number: 450540

Kit expiry date: 2019-04

Catalogue Number: EA3491

Appendix 3

ELISA test kit 3 (LC Tech "ErgoRead ELISA")

Sample extraction procedure according to producer's protocol (P1)

1. 20* g of homogenized sample are transferred into a 200 mL beaker and 50* mL of MeOH/0.25 % phosphoric acid (40/60, v/v) are added
2. The sample is extracted by mixing for 20 min
3. The sample is filtered using a plaited filter
4. An aliquot (5 mL)* is diluted using the 1 x concentrated ready to use "sample dilution buffer" (5 mL)*
5. The diluted sample should be filtered through a syringe filter or a glass fibre filter immediate prior use to remove turbidity
6. Dispense 100 µL of the liquid into a ELISA microplate well

***Modifications made**:

a) 4 g of sample was extracted with 10 mL of 40/60 (MeOH/0.25 % phosphoric acid; v/v)
b) An aliquot of 500 µL (instead of 5 mL) was diluted with 500 µL (instead of 5 mL) of corresponding buffer

Alternative sample extraction procedure according to producer's protocol (P2)

The same as above, but instead of "sample dilution buffer" the second – "sample dilution buffer for rye and triticale samples" was used

Alternative sample extraction protocol tested (P3)

1. Extract 4 g of homogenised sample with 10 mL of MeOH/H_2O/FA, 60/40/0.4, v/v for 20 min
2. The sample is filtered using a plaited filter
3. An aliquot (500 µL) is diluted using the mixture of 1 x concentrated ready to use "sample dilution buffer" diluted 1:1 with "sample dilution buffer for rye and triticale samples" (500 µL)
4. The diluted sample should be filtered through a syringe filter or a glass fibre filter immediate prior use to remove turbidity
5. Dispense 100 µL of the liquid into a ELISA microplate well

In addition, the calibration range was extended down 62.5 µg/kg (two calibration points added – 62.5 and 125 µg/kg) to be able to measure at lower range (extension was obtained by diluting the lowest calibration standard (250 µg/kg) with recommended buffer as described in producer's procedure.

Appendix 3 continued

ELISA test kit 3 (LC Tech "ErgoRead ELISA")

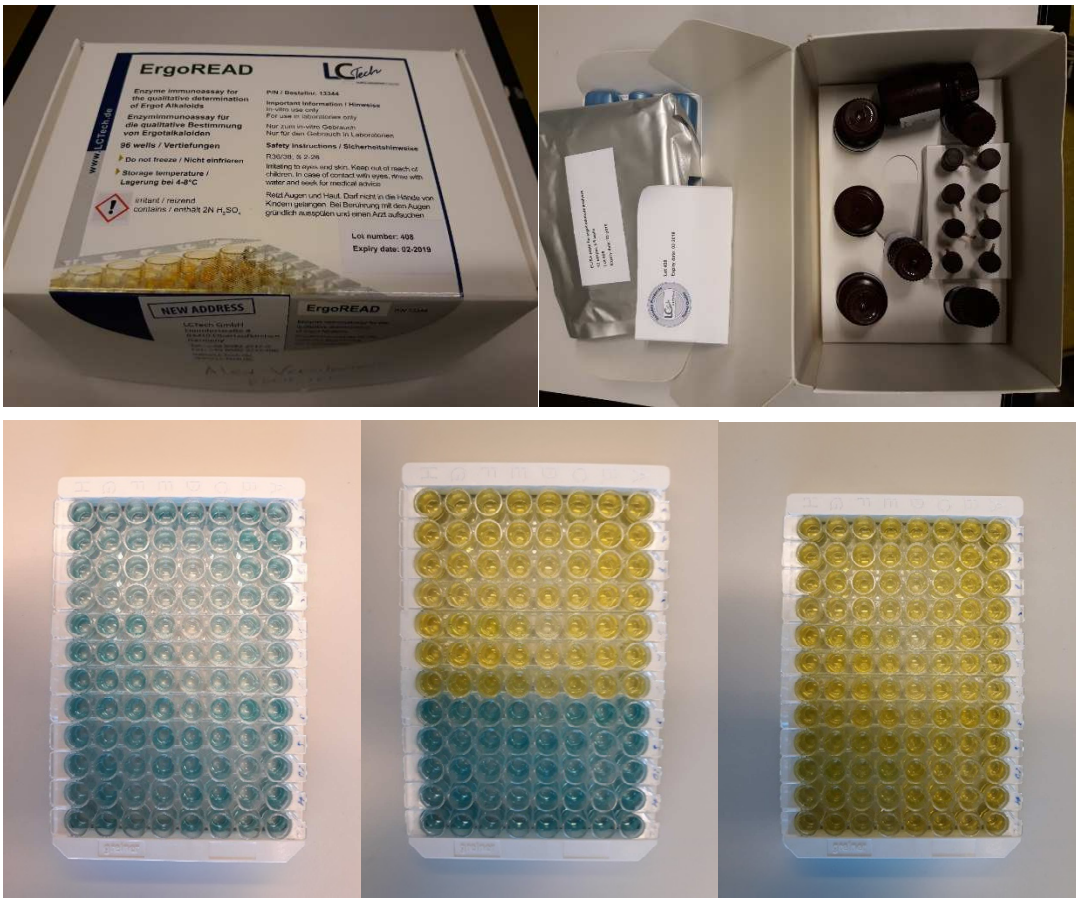

Figure 3. Example pictures of used ELISA kit (test kit 3)

Lot No: 408

Expiry date: 02-2019