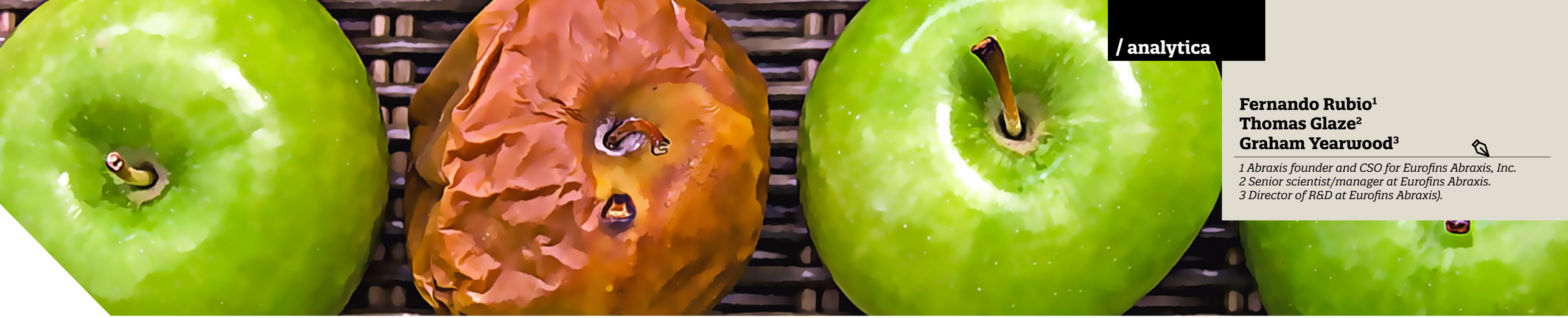

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ELISA test kit for the quantitation of patulin in juices, cider, and purees

Validation of a new and novel Patulin ELISA kit.

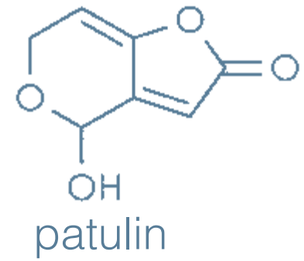

Figure 1:
Patulin, [4-hydroxy-4H-furo[3.2c] pyran-2(6H)-one].

Background

The presence of toxic chemical contaminants in food, particularly those intended for children's consumption, is a matter of great concern to consumers and health authorities. Mycotoxins in food, animal feed, and beverages have been recognized as a potential threat to human and animal health, either caused by direct contamination of plant materials and their byproducts, or by the transfer of said mycotoxins and their metabolites to animal tissues, milk, and eggs as a consequence of the ingestion of contaminated food. Due to the extreme difficulty of avoiding their presence, mycotoxins also pose high economic cost to the agri-food sector that annually represents the loss of millions of dollars worldwide. Mycotoxin contamination is considered an unpredictable problem, even when good agricultural, storage, and processing practices are implemented, posing a difficult challenge to ensure the safety of food and feed. Many mycotoxins are not easily removed during food processing due to their physical and chemical stability. The main mycotoxin-producing fungi belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. So far, more than 400 mycotoxins with great structural diversity have been identified.

The most relevant, due to their incidence and/or toxicity, are aflatoxins, ochratoxins, patulin, fumonisins, deoxynivalenol, zearalenone, T-2, and alternariol. National and international public health authorities have adopted strict regulatory guidelines aimed at controlling the presence of the main classes of mycotoxins in food and feed. Patulin [4-hydroxy-4H-furo[3.2c] pyran-2(6H)-one] (***Figure 1***) is a toxic polyketide metabolite produced mainly by Penicillium expansum. Although it can occur in infected fruits, grains, and other foods, the main route of exposure to patulin is through the ingestion of infected apples and some of its derivatives, such as juices and compotes. The Joint FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization) Committee of Experts on Food Additives (JECFA) established in 1995 a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 µg/kg of body weight/day. Based on this recommendation, the European Union has established maximum allowed levels of patulin of 50 µg/kg for juices, 25 µg/kg for apples, and 10 µg/kg for foodstuffs intended for children consumption (European Commission Regulation 1881/2006). Other national bodies, such as the United States Food and Drug Administration (FDA 2004), the Ministry of Health of the People's Republic of China (CFDA 2011), the Ministry of Health of Canada (Health Canada 2014), have adopted similar criteria. Brazil only regulates apple juice (Ministerio da Saude do Brazil 2011). Various international studies on the incidence of patulin in apples and apple-derived products show that a very high percentage of the samples analyzed contained detectable concentrations of patulin. In many cases, those levels are close to the established limit, and occasionally exceedances are reported (Erdoğan et al. 2018; Pallarés et al. 2019). More importantly, there have been a

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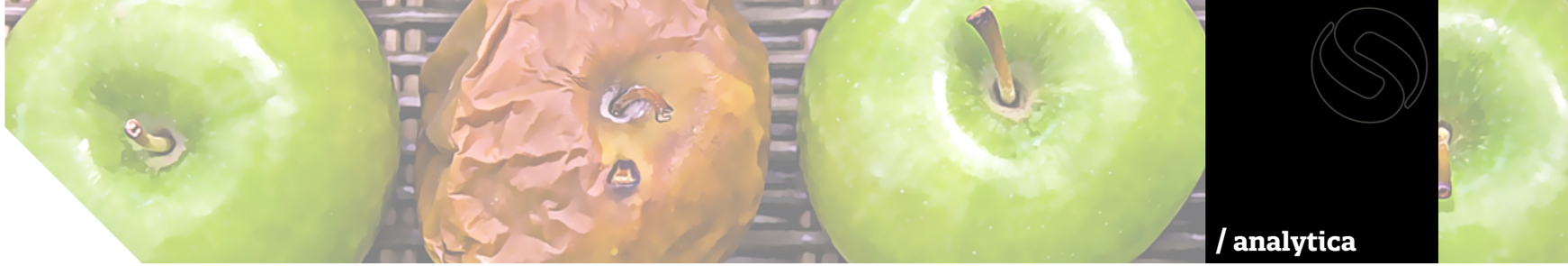
ELISA test kit for the quantitation of patulin in juices, cider, and purees



series of alerts and product recalls in recent years due to the presence of excessive levels of patulin in apple-derived products in Europe, the United States, Canada, Australia, Hong Kong, and others. Therefore, the control of patulin in food is essential for both food safety reasons and quality control of raw materials.

Analytical methods are essential tools for regulatory bodies to ensure food and feed safety. The procedures for the determination of mycotoxins are essentially of two types: chromatographic methods and molecular recognition techniques. Chromatographic methods are considered to be reference techniques, since they are capable of simultaneously determining several mycotoxins with great sensitivity, selectivity, and reproducibility. Concerning patulin, the method adopted by the AOAC (Association of Official Analytical Chemists) is a high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV, official methods 995.10 and 2000.02).

The main disadvantage of this method is the lack of selectivity, since compounds such as 5-hydroxymethylfurfural and phenolic derivatives, both present in apple-based products, can interfere with the measurement. Other instrumental techniques that have also been used to determine patulin are capillary electrophoresis (CE), micellar electro-kinetic capillary chromatography (MEKC), gas chromatography (GC) with derivatization, and HPLC coupled to mass spectrometry detection (HPLC-MS) (Li et al. 2017).

Despite their high reliability and precision, instrumental techniques have some drawbacks, the most notable of which is the need to perform the analysis in well-equipped facilities with highly qualified personnel able to handle sophisticated instrumentation. Other drawbacks are associated with the complexity of the matrices to be analyzed and the need to implement cumbersome steps for sample preparation, purification, and concentration.

On the other hand, analytical techniques based on antibodies are deemed the best option when the analysis of large number of samples is required in a short time, in situ, and/or in low-resource settings, and when the selective purification, concentration, and cleaning of samples is required before analysis by instrumental methods. Immunoanalytical methods are based on the high affinity, selective, reversible, and non-covalent binding between a target substance (analyte) and an antibody. Depending on the analytical requirements, antibodies are implemented in different assay formats, such as enzyme-linked immunosorbent assays (ELISA), lateral-flow immunochromatography, biosensors, microarrays, and flow injection systems, as well as in immunoaffinity columns. In this regard, among chemical contaminants potentially present in food and animal feed, mycotoxins are the group of compounds for which immunoanalytical techniques enjoy the highest degree of implementation and acceptance in analytical laboratories worldwide, in some cases even endorsed and recommended by regulatory agencies. In fact, a large number of immunodiagnostic companies are currently marketing rapid methods for the control and detection of several mycotoxins.

However, until now, none of the companies have commercialized rapid kits for the determination of patulin. This remarkable deficiency is certainly due to the lack of antibodies capable of recognizing this mycotoxin with the affinity and specificity required for robust immuno-analysis.

This study validates the newly developed Eurofins Abraxis Patulin ELISA test kit for the rapid quantitative analysis of patulin in apple and orange juice, apple cider, and apple sauce, as a means of obtaining reliable results (up to 41 samples) in 3 hours without the need of expensive analytical instrumentation and with minimal operator training.

There are some examples in the scientific literature reporting approaches to obtain anti-patulin antibodies

State of the art

There are some examples in the scientific literature reporting approaches to obtain anti-patulin antibodies.

Most of these articles use protein conjugates of patulin-hemiglutarate as immunogens (Kwon et al. 2016; Xu et al. 2011; Mhadbhi et al. 2005; McElroy and Weiss 1993), or patulin-hemisuccinate (Mehl et al. 1986; Sheu et al. 1999).

In all those examples, unsatisfactory results were obtained, most likely due to the small size and low structural complexity of the patulin molecule (MW=154.12), and especially to its high electrophilic reactivity which allows the mycotoxin to easily react with thiolated nucleophiles, such as glutathione, and with proteins containing free residues of cysteine (Fliege and Werner 2000;

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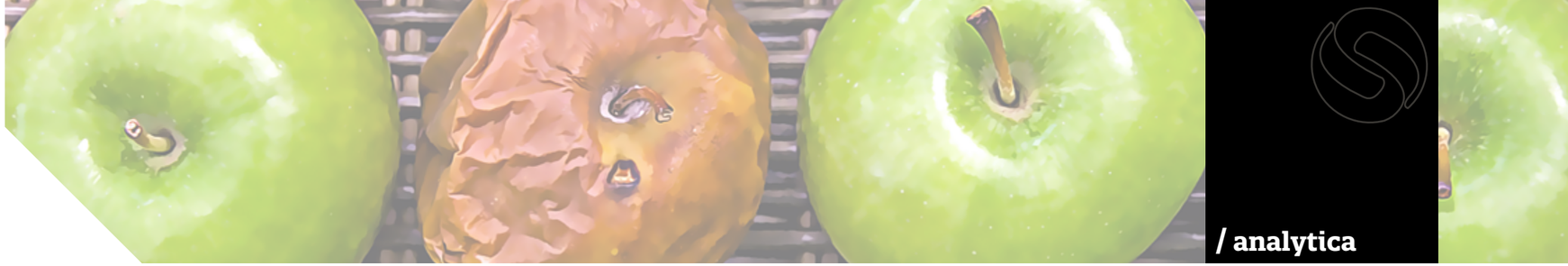

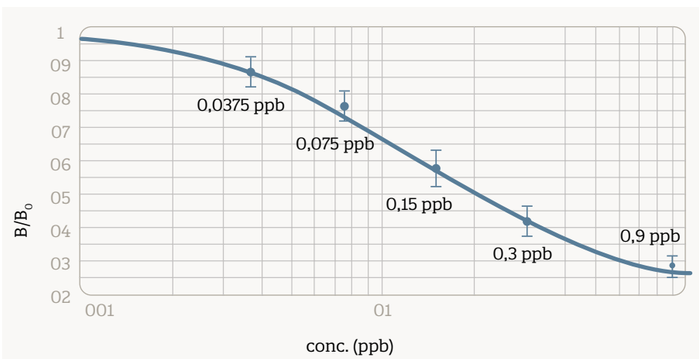

Figure 2:
Representative patulin standard curve. Error bars represents +/- 1 SD.

Table 1:
Specificity and sensitivity study. Orange juice, apple juice, apple sauce samples were fortified with Patulin at 12.5 ppb (false negative study), and without fortification (false positive study), n = 20.

	ORANGE JUICE		APPLE JUICE		APPLE SAUCE	
	Blank	12.5 ppb spike	Blank	12.5 ppb spike	Blank	12.5 ppb spike
Avg. ppb	6,16	15,04	6,86	14,70	5,86	13,80
SD	1,527	3,150	1,472	1,555	1,422	2,451
% CV	24,8	21,0	21,4	10,6	24,3	17,8
% Recovery		120,3		117,6		110,4

Pfenning et al. 2016). Accordingly, the epitopes of the patulin-functionalized analogues are probably destroyed or modified after in vivo exposure of the bioconjugate to the immune system during immunization. Other authors have tried obtaining anti-patulin antibodies using for immunization structural analogues that maintain the original skeleton of the natural mycotoxin, but in which some of the functional groups have been modified to reduce the hapten reactivity, thus increasing its chemical stability (D'Auria et al. 2007; de Champdore et al. 2007).
However, not even this strategy has led to anti-patulin antibodies suitable for their incorporation into commercial analytical systems and rapid tests. Nevertheless, biosensors incorporating anti-patulin antibodies produced according to the above-mentioned strategies have been reported in the scientific literature (Starodub et al. 2013; Pennachio et al. 2014; Funari et al. 2015). Regardless of whether these analytical systems are actually suitable for the determination of patulin at regulated levels, their use also has most of the drawbacks previously mentioned for instrumental methods based on chromatographic techniques.
According to the state of the art, it is clear that there was an outstanding need for immunoreagents (bioconjugates and antibodies) that enable the development of simple, fast, reliable, and high-throughput methodologies for point-of-need detection of patulin that satisfies the demands of the agri-food sector, and the regulatory institutions for patulin analysis.

Immuno-reagents and assay procedure

It is possible to obtain patulin adducts (patent pending), as well as the bioconjugate compounds derived from said adducts, from which it has been possible to generate antibodies capable of specifically recognizing stable patulin adducts. This approach allows overcoming the aforementioned problems related to the small size, low structural complexity, and high reactivity of patulin, which greatly hinders the induction of an adequate immune response against this mycotoxin.
The antibodies developed are useful for the development of rapid, robust and sensitive immuno-analytical methods for the determination of patulin.
The immunoassay method, based on monoclonal antibodies, includes a simple and rapid step to quantitatively convert patulin in the kit standards and in the samples into a predefined derivative, which is then analyzed in the ELISA (***Figure 2***).

ELISA kit performances

The developed competitive ELISA allows the detection and quantification of patulin in the range of 0.0375 to 0.9 ng/ml (***Figure 2***). Considering the dilution of samples in the sample preparation procedure, the Limit of Detection (LOD) is 9.375 µg/l (ppb) and the Limit of Quantification (LOQ) is 10 µg/l (ppb).

Sample preparation

- Apple Sauce/Puree

1. Weigh 0.50 ± 0.05 grams of sample into a 15 mL plastic centrifuge tube.
2. Add 5.0 mL of Standard/Sample Diluent, vortex thoroughly for 10 seconds. Mix using a rotator for 10 minutes.
3. Let the sample settle for >2 minutes.
4. Remove 2 mL of sample and add to 2.0 mL microcentrifuge vial. Centrifuge for 5 min at 8.100 X g or 10.000 rpm.
5. Dilute supernatant 25-fold by adding 40 µL of supernatant to 960 µL Standard/Sample Diluent in a 4 mL glass vial. Vortex to mix.
6. Derivatize and analyze by ELISA procedure. The Patulin concentration contained in sample is then determined by multiplying the ELISA result by the dilution factor of 250.

- Apple Juice/Apple Cider/Orange Juice

1. Add 0.5 mL of sample into a 15 mL plastic centrifuge tube.
2. Add 4.5 mL of Standard/Sample Diluent, vortex thoroughly for 10 seconds.
3. Remove 2 mL of sample and add to 2.0 mL microcentrifuge vial. Centrifuge for 5 min at 8.100 X g or 10.000 rpm.
4. Dilute supernatant 25-fold by adding 40 µL of supernatant to 960 µL Standard/Sample Diluent in a 4 mL glass vial. Vortex to mix.
5. Derivatize and analyze by ELISA procedure. The Patulin concentration contained in sample is then determined by multiplying the ELISA result by the dilution factor of 250.

Specificity and sensitivity study

Apple juice, orange juice, and apple sauce samples previously screened to be below the assay LOQ (10 µg/kg) were assayed 20 times in a single assay (true negative) and after spiking with patulin at a concentration of 12.5 µg/l (ppb) for juices and 12.5 µg/kg (ppb) for apple sauce (true positive). Standards and samples were analyzed in duplicate after derivatization, following the sample treatment and assay procedure described above. All samples were correctly identified (***Table 1***), indicating a 0 false positive rate and 0 false negative risk or 100% test specificity and sensitivity at the stated LOQ.

Spike recovery

Apple juice, orange juice, and apple cider samples were spiked with various amounts of patulin, 12.5 µg/l, 37.5 µg/l, 125 µg/l. Apple sauce was spiked at 12.5 µg/kg, 37.5 µg/kg, and 125 µg/kg.
Each sample was analyzed 5 times in duplicate. As indicated in ***Table 2***, the mean sample recoveries for three apple juice spike concentrations was determined to be 118%, 101%, 102%; orange juice 105%, 125%, 112%; apple cider 94%,

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Specificity and sensitivity study

	Orange Juice		Apple Juice		Apple Sauce	
	Blank	12.5 ppb spike	Blank	12.5 ppb spike	Blank	12.5 ppb spike
avg. ppb	6,16	15,04	6,86	14,70	5,86	13,80
SD	1,527	3,150	1,472	1,555	1,422	2,451
% CV	24,8	21,0	21,4	10,6	24,3	17,8
% Recovery		120,3		117,6		110,4

Table 2:
Patulin Recoveries. Orange juice, apple juice, apple sauce, and apple cider sample were fortified with patulin at 12.5, 37.5, 125 ppb. n = 5.

120%, 113%; apple sauce 100%, 103%, 113%.

FAPAS certified samples

FAPAS apple juice certified sample T1674QC, and an apple puree certified sample T1673QC were analyzed using the ELISA kit by an outside laboratory following the ELISA procedure after performing the extraction and dilution procedures listed above. The results obtained (***Table 3***) were accurately determined and fell in the middle of the acceptable range (z-score).

FAPAS Patulin Sample	Target µg/kg	Acceptable Range (+/- 2 z-score)	Value µg/kg
T1673QC Apple Juice	18.6	10.4 - 26.8	21.8
T1674QC Apple Puree	15.3	8.6 - 22.1	17.2

Table 3:
FAPAS sample study.

Conclusions

The Patulin ELISA kit is capable of accurately analyzing apple and orange juice, cider and sauce samples. The assay demonstrated good recovery and reproducibility, allowing for the rapid detection of this mycotoxin on a variety of samples. Evaluation of other sample matrices such as mango, pear, plum, and others will be performed and reported in the future. The availability of this rapid and cost-effective analytical method for patulin will allow the easy and fast screening of many samples and sample types, and allow consumers and food manufacturers the benefit of a safer food supply.

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