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Identification of natural toxins and related emerging risks in innovative ingredients for the bakery and meal solutions sectors

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*“*Usate le parole che vi ho insegnato per difendervi
e per difendere chi quelle parole non le ha.
Non siate spettatori ma protagonisti della storia
che vivete oggi: infilatevi dentro, sporcatevi le mani,
mordetela la vita, non "adattatevi", impegnatevi,
non rinunciate mai a perseguire le vostre mete,
anche le più ambiziose,
caricatevi sulle spalle chi non ce la fa:
voi non siete il futuro, siete il presente*”*

— Pietro Carmina,
1953-2021

“*Use the words I have taught you to defend yourselves
and to defend those who do not have those words.
Do not be spectators, but protagonists of the history
you are living today: step into it, get your hands dirty,
bite into life, do not simply adapt — commit yourselves.
Never give up pursuing your goals,
even the most ambitious ones.
Carry on your shoulders those who cannot make it:
you are not the future — you are the present*.”

(Author’s translation)

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Preface

Food safety is a dynamic challenge. Natural toxins, particularly fungal mycotoxins and plant alkaloids, continue to emerge in new and unexpected scenarios as the food system evolves. Mycotoxins may cause genotoxic, hepatotoxic, immunotoxic, neurotoxic or nephrotoxic effects, even at low concentrations, while the growing understanding of emerging and modified forms has further complicated the risk scenario over the last decade.

In parallel, specific plant alkaloids – such as pyrrolizidine alkaloids (PAs) and tropane alkaloids (TAs) – are gaining attention due to contamination cases in cereals, spices and plant-based foods. Consumers often perceive botanical preparations as inherently safe simply because they are "natural"; however, natural origin does not imply absence of toxicity. These compounds are well-known for their hazardous effects, and their occurrence in ingredients for bakery and meal solutions, or even in the increasingly relevant category of legumes, represents an emerging risk that remains insufficiently characterized from both scientific and regulatory perspectives.

This Ph.D. project was carried out within an international food company (Barilla G. & R. Fratelli S.p.A.), where innovation meets industrial feasibility. Ensuring food safety across the entire supply chain – from raw materials to final products – represents a concrete and complex responsibility, particularly in a context of rapidly evolving ingredients, processing technologies, and regulatory frameworks.

Limited data, the fast-growing market of plant-based foods, and increasing regulatory expectations reinforce the need for advanced and fit-for-purpose analytical methodologies. Today's consumers demand transparency, authenticity, and scientifically proven safety. Meeting these expectations requires empowering the food industry with robust monitoring tools and risk assessment strategies capable of keeping pace with innovation.

This doctoral thesis is organized into a general *introduction*, an *aim* section, a core body of *experimental studies*, and a final section dedicated to *general conclusions and future perspectives*. The experimental part is structured into three main sections:

I. Analytical method development for toxic plant alkaloids, including both LC-HRMS and LC-MS/MS approaches and the evaluation of a novel 3D-printed biosensing toolkit under industrial conditions.
II. Investigation of the fate of the most relevant mycotoxins during processing, applying a stable isotope-labelling LC-HRMS under pilot-plant conditions designed to closely mimic industrial production.
III. Assessment of the global risk scenario through a comparative evaluation of legume contamination profiles from Italy and Thailand, integrating multi-mycotoxin and tropane alkaloids occurrence data with dietary exposure assessment.

The overarching purpose of this doctoral project is to provide concrete, science-based solutions for the detection and assessment of natural toxins – from raw materials to processed foods – through screening and mass spectrometry-based analytical techniques. The knowledge generated supports effective risk management strategies and contributes to the broader effort to mitigate mycotoxin-related risks, while ensuring safer innovation in bakery and meal solutions.

Ultimately, this project reflects my commitment to bridging advanced analytical science with real industrial needs, supporting informed decision-making in a food system where risks are continuously evolving.

- *The Shipwreck* by J.M.W. Turner, 1805, Tate Britain, London.

1 | INTRODUCTION

1.1 The Winds of change

Across the global food system, a profound transformation is underway. New raw materials, new products and new processes are entering the market, driven by sustainability, nutrition and consumer expectations. At the same time, natural toxins – especially mycotoxins and plant alkaloids – remain present, often in ways we do not fully understand, particularly, in this work, in innovative ingredients for bakery and meal solutions.

In this scenario, the global food system can be seen as a ship navigating a sea in constant motion: the winds are changing, the routes are new, and some risks are already mapped, while others emerge unexpectedly beneath the surface.

- **The Winds of Change:** Global Challenges

Recent global crises — from pandemic to geopolitical conflicts — have revealed the complexity and fragility of raw-material supply chains. Meanwhile, the current - not really unexpected - climate changes are challenging the global food system: reshaping agro-ecological conditions, altering contaminant profiles in crops and expanding their distribution into previously unaffected regions. With a continuously growing population and finite natural resources, conventional production practices alone cannot meet future food demand. Food industry must therefore set a new course, redefining how raw materials are sourced and processed, implementing approaches to facilitate prioritization of natural toxins for risk management strategies.

- **The Winds of Change:** Innovation in Food Sector

On the other hand, the evolution of the consumers' awareness and common perception, drive the food production chain towards sustainability, transparency, and plant-based options. As a direct consequence, cereals and cereals-based products, gluten free matrices and legumes are under the magnifying glass. This creates new business opportunities — but also new safety uncertainties. Unfamiliar matrices and emerging toxins require updated scientific knowledge and monitoring strategies. analytical priorities shifted, and regulatory frameworks updated.

- **The Ships:** natural toxins and related emerging risks

In this dynamic context, the “ship” is the food matrix and its intrinsic risk. For each ingredient and process, risk depends on which toxins may realistically occur (hazard identification), in which concentration and form (free, emerging, modified, bound) and how processing (milling, baking, extrusion…) may:

  - reduce or redistribute toxins
  - transform them into new modified forms
  - degrade them in specific products

Over the past decade, the mycotoxins scenario has become increasingly complex due to emerging mycotoxins not routinely monitored, matrix-associated mycotoxins which could form complexes with matrix compounds and modified mycotoxins whose toxicological relevance is still unclear.

A clever analysis must be run to avoid investing in screening for toxins that are unlikely to be present in a certain good, as well as missing a potential risk on the other hand. As an outcome of the risk analysis, every industry should update their analytical methods, verify their capacity to stay fit-for-purpose and validating new matrices for an efficient risk assessment.

- **The light of the lighthouse**: Regulations – Guiding but not always present

Regulatory frameworks provide guidance, but their coverage often lags behind this sea in constant motion. Commission Regulation (EU) 2023/915 sets maximum levels for contaminants in food, replacing the older Regulation 1881/2006, covering mycotoxins, some plant toxins and other contaminants to harmonize EU food safety, ensuring stricter, clearer rules while considering agricultural practices and consumer risks. However, many plant-based ingredients, like legumes (soybeans, beans, peanuts...) are poorly regulated for natural toxins or require adaptation of existing legislation. Furthermore, expect the unexpected. A change in consumption habits towards new food trends (like plant-based food), may lead to a significant change in total contaminants exposure.

Blindly relying on incomplete maps may steer the ship into uncharted risks — reinforcing the urgency for proactive research and monitoring programs. Based on the large and progressively growing number of mycotoxins and plant toxins with always new potential concerns, it must be necessary the prioritisation of natural toxins allowing for a rational and systematic approach, to focus monitoring/reduction efforts on those mycotoxins having an impact in terms of exposure.

- **The Compass**: Validation and analytical approach

The development of reliable methods to improve product safety assessment could better preserve the quality of production, with an advantage for the industry and the final consumer, who would have more confidence in the quality and safety of the product.

Within this complex and dynamic scenario, the role of screening test kits validation is crucial, especially in an industrial environment.

Above all, chromatographic applications, based on LC-MS/MS and LC-HRMS, are the compass that allows us to:

- detect low levels of mycotoxins and plant alkaloids
- investigate emerging and modified forms
- follow their fate along the processing chain

Only a balanced monitoring plan — cost-effective yet scientifically robust — can protect the industry from avoidable risks while ensuring consumer confidence in foods.

- **The North Star**

Finally, the North Star to gain knowledge, know-how and to reach the so called “routine scenario” could be a direct connection between the academic and industrial worlds, where industries provide insights, applied scientific knowledge, economical and human resource, while academic research groups have the leading role into the project, transmitting skills and experience. Pilot-scale studies, stable isotope labelling and close interaction make it possible to:

- generate realistic data under industrially relevant conditions
- translate complex analytical information into practical risk management tools
- support safer, innovative ingredients for bakery and meal solutions

In a scenario where the winds of change are intensifying, only a well-equipped ship — guided by regulation, steered by robust analytics, and oriented by strategic collaboration — can continue to navigate safely toward innovation.

1.2 Mycotoxins fate along thermal processing on cereal chain: from pilot testing to analytical understanding and scaling-up confirmation

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Abstract

Thermal processing plays a critical role in shaping mycotoxin occurrence, transformation, and exposure along the cereal chain. Beyond simple concentration changes, processing may induce degradation, conversion, matrix binding, or release of modified and masked forms, making analytical interpretation and risk assessment particularly challenging. This chapter provides a protocol-oriented overview of methodological strategies for studying mycotoxin fate during thermal cereal processing, integrating raw material selection, naturally contaminated matrices, pilot-plant experimentation, and statistically designed processing trials. Special emphasis is placed on the strategic application of Design of Experiments (DoE) to identify critical technological factors and their interactions, and on advanced analytical workflows based on LC-MS/MS and high-resolution mass spectrometry. Stable isotope-labelled (SIL) tracer approaches combined with untargeted HRMS screening are highlighted as powerful tools for detecting and confirming degradation and conversion products without prior structural knowledge. Method validation, reference standard availability and data interpretation challenges are discussed as key limiting factors. The combined use of statistically driven processing design and advanced analytical protocols represents a robust framework for improving the understanding of mycotoxin behavior during processing and for supporting more accurate food safety and risk assessment strategies.

KEYWORDS

Mycotoxins; Thermal food processing; Cereal chain; Mycotoxin degradation; Design of Experiments (DoE); LC-MS/MS; High-resolution mass spectrometry; Stable isotope labelling; Food safety; Risk assessment

1 | INTRODUCTION

One of the most widely accepted definitions of mycotoxins states that they "*are natural products produced by fungi that evoke a toxic response when introduced at low concentration to higher vertebrates and other animals by a natural route*" (1). They may exert a wide range of toxic effects, including genotoxic, immunotoxic, hepatotoxic, neurotoxic, and nephrotoxic activity.

Mycotoxin contamination of food and feed commodities represents a significant challenge for food operators in both developed and developing regions. Climate change is widely recognised as a key factor influencing – and in some cases aggravating – the occurrence, distribution, and concentration of mycotoxins (2).

Plants intended for food and feed production are often the primary hosts for fungal infection, which may lead to mycotoxin contamination. As a result, mycotoxins consitute a global food safety concern, as they may contaminate a wide range of commodities both in the field during plant growth (pre-harvest) and during subsequent harvesting, storage and processing (post-harvest) (3). In addition, mycotoxins or their metabolites may enter the human food chain through the consumption of animal-derived products (e.g., meat, eggs, milk) as a result of carry-over when animals are exposed to contaminated feed (4). In some cases, exposure may also occur through the consumption of mould-ripened foods, such as certain cheeses and fermented meat products, where fungal growth is intentionally involved in the production process.

The production and relative abundance of mycotoxins by mycotoxigenic fungi strongly depend on their growth stage and environmental conditions, particularly temperature and humidity. Food commodities may be contaminated by multiple fungal species, many of which are able to produce more than one mycotoxin, leading to frequent co-occurrence. Post-harvest contamination is especially relevant for cereals and other crops that are improperly stored under conditions favouring mould growth, which may lead to significant mycotoxin contamination (5).

Given the potential risks to human health, the control of mycotoxins must primarily focus on raw materials and ingredients. This creates a need for harvesting, processing, and storage strategies capable of preventing, reducing, and/or eliminating contamination. Food processing under standardized and well-controlled conditions, together with systematic control of each step of the production and storage chain, is therefore essential (6). In this context, appropriate management of processing techniques may represent an additional and effective mitigation step for mycotoxin contamination (7).

Over the past three decades, research studies have investigated whether food processing may have an impact on mycotoxin occurrence and toxicological relevance (3). Operations such as sorting, cleaning, milling, thermal processing (e.g., extrusion, roasting, baking, cooking), fermentation, and enzymatic treatments may potentially alter mycotoxin concentration through mechanical and/or thermal effects, leading to their transformation and/or degradation. In addition, apparent reductions or increases in concentration may arise from dilution or concentration effects associated with ingredient addition and changes in moisture content (8).

These aspects are particularly relevant when assessing compliance with European Union maximum levels, which are generally established for products “as consumed” (Reg (EU) 2023/915).

Within this framework, the present book chapter aims to provide an overview of how to deal with the fate, transformation (including masked and modified forms), and potential mitigation of mycotoxins along the thermal processing of the cereal chain. Particular attention is given to the impact of cereal processing from pilot-scale testing to analytical interpretation and scaling-up confirmation at the industrial level. The chapter is primarily addressed to industry stakeholders, control authorities, and food operators dealing with mycotoxin contamination in cereals and seeking to actively contribute to global mitigation strategies.

2 | BACKGROUND AND MATERIALS

2.1 Mycotoxins of Relevant Occurrence in Cereals

Cereal grains and their derivatives are susceptible to contamination by mycotoxin-producing fungi, mainly belonging to the genera *Aspergillus, Claviceps, Fusarium,* and *Penicillium*. Consequently, several mycotoxins, including aflatoxins (AFs), ochratoxin A (OTA), patulin, and ergot alkaloids (EAs), may occur in these cereals. However, when they are cultivated in cooler temperate regions, mycotoxins produced by *Fusarium* species – such as fumonisins (FBs), trichothecenes (TCT), including deoxynivalenol (DON), nivalenol (NIV), T-2 toxin and HT-2 toxin, as well as zearalenone (ZEN) – represent the main concern.

Among mycotoxins, the term *emerging mycotoxins* refers to compounds that are neither routinely monitored nor legislatively regulated, despite rapidly increasing evidence of their occurrence. This group commonly includes Alternaria toxins (ALT), fusaproliferin (FP), beauvericin (BEA), enniatins (ENNs), and moniliformin (MON) (10).

Over the last decade, the mycotoxin scenario has been further complicated by the growing recognition of modified forms -alongside emerging mycotoxins - in addition to the traditionally known free forms (11). To better clarify this complexity, Rychlik et al. proposed a systematic classification distinguishing free and unmodified mycotoxins from matrix-associated and chemically modified forms. *Matrix-associated mycotoxins* include compounds physically entrapped or covalently bound to matrix components, or both, such as FBs covalently linked to starch or proteins. *Modified mycotoxins* are further subdivided into biologically modified and chemically modified forms, with the latter including thermally and non-thermally formed compounds. To harmonize scientific terminology and future legislation, the authors recommended adopting the term modified mycotoxins, while reserving *masked mycotoxins* for plant-conjugated biologically modified forms (11).

2.2 Analytical Protocols for Mycotoxin Measurement

When investigating the fate of mycotoxins during food processing, it is essential to evaluate contamination at all stages of the production chain, from pre-harvest fungal development to the impact of transformation processes and the occurrence of mycotoxins in finished products (12)

2.2.1 Naturally Contaminated Raw Materials

To reliably assess the effects of thermal food processing on mycotoxin levels and to identify potential degradation products, the use of naturally contaminated samples is of fundamental importance. In naturally contaminated raw materials, masked mycotoxins frequently co-occur with the mycotoxin of interest. During food processing, these masked forms may be partially converted into the parent mycotoxin, potentially leading to biased results if initial contamination is not accurately characterized (13). For this reason, a thorough screening of the initial contamination level of naturally contaminated samples is required before processing. Once the concentration of the mycotoxin of interest is clearly established, product manufacturing can proceed using contaminated flour without artificial spiking. When necessary, contaminated batches may be blended with low- or negligible-contaminated material to achieve the desired starting concentration.

This approach allows the evaluation of mycotoxin behaviour under conditions that more closely reflect real contamination scenarios. Artificial spiking experiments, while useful, do not necessarily reproduce the natural distribution of mycotoxins within the matrix. As a consequence, interactions between mycotoxins and matrix components, as well as matrix-related protection effects, may differ significantly between spiked and naturally contaminated samples (8,14).

Furthermore, it is often unclear in scientific literature whether analytical data have been corrected for analyte recovery, moisture changes, or dilution effects due to ingredient addition. This lack of transparency makes it difficult to assess potential biases arising from incomplete recovery or matrix-related effects (15).

2.2.2 Pilot Plant Testing

Pilot plant testing offers the possibility to study mycotoxin fate in industrially relevant products at a reduced scale, while preserving key technological and organoleptic characteristics. This approach has been successfully applied in several studies reported by our research group (12,15–19).

In case of experimental studies, pilot plant experiments must closely mimic industrial processing conditions and remain technologically feasible. Moreover, when investigating degradation and/or conversion products, it is essential to design marketable products manufactured under realistic processing conditions that respect sensory and quality requirements. In this context, relatively simple recipes and processing conditions may facilitate the attribution of observed degradation effects to specific technological parameters (20).

2.2.3 Experimental Design

Design of Experiment (DoE) represents an efficient statistical tool for maximising the information obtained from experimental studies while minimizing the number of required trials. This approach involves the systematic variation of process input *factors* and measuring their effects on selected *response* variables, allowing the investigation of individual and synergistic effects of processing parameters (the ‘treatments’) to optimise the final process (21).

DoE is widely applied in industrial settings for process screening, optimization, and robustness testing in laboratory, pilot-scale, and full-scale production environments. As the number of factors increases, the number of possible combinations grows exponentially; therefore, fractional factorial designs are commonly employed during screening studies to obtain reliable sensitivity information with a feasible number of experimental runs.

In these studies, technological parameters are varied during the experiment within defined ranges based on processing feasibility and product acceptability. Replicates at the central point are typically included to estimate experimental error, while central conditions represent the optimal combination of ingredients and processing parameters yielding an organoleptically and technologically acceptable product (22).

Bergamini et al. (2010) investigated the influence exerted by bread-making parameters on the DON concentration in bread, starting from a naturally contaminated flour and using both pilot- and industrial-scale equipment. The study was organized on a Screening Design of Experiments (SDoE) where a full-factorial central composite design (CCD) was adopted. The objectives of the SDoE were to explore many factors in order to reveal whether they influence the responses, and to identify their appropriate ranges. Four factors on the final DON level in bread were investigated: fermentation rate, fermentation temperature, baking rate, and baking temperature (13).

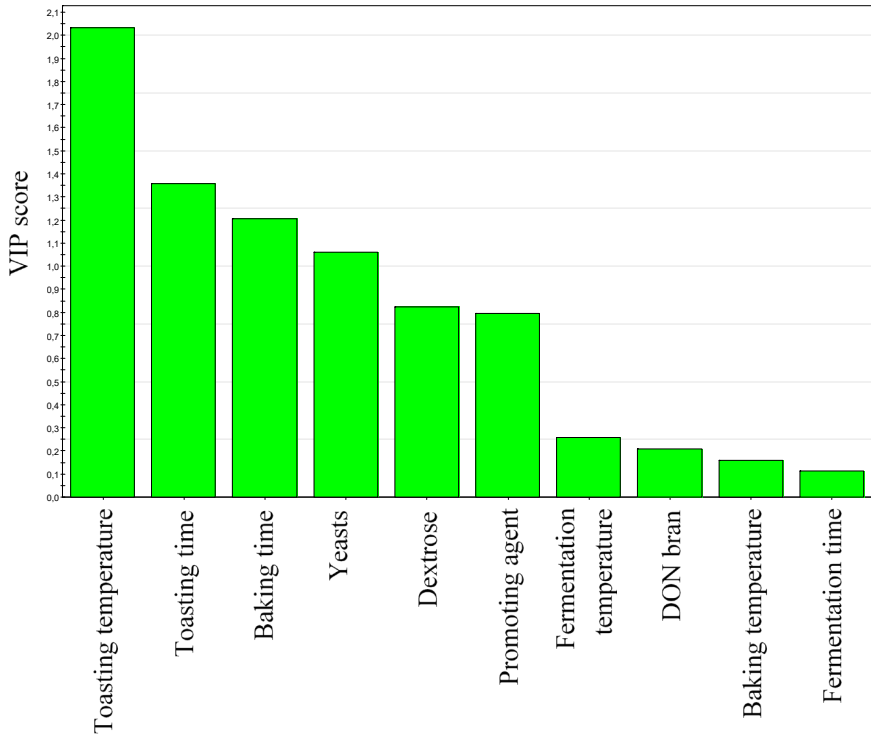
Another study conducted by Generotti et al. (2015) investigated the influence exerted by a set of rusk-making parameters on DON and deoxynivalenol-3-glucoside (DON-3-Glc) level in wholegrain rusks using naturally contaminated bran over an industrial pilot-scale. In this study, ten factors were taken into account: DON contamination level of the starting bran, dextrose, yeast, and enzyme amounts, fermentation time and temperature, baking time and temperature, and toasting time and temperature. For each parameter, a well-defined variation range was assessed (***Table 1***). The statistical model of the DoE required 19 single experiments. In each experiment, the DON and DON-3-Glc levels were measured by liquid chromatography−tandem mass spectrometry (LC-MS/MS) in the mixed flour/bran, before and after fermentation, after baking, and after toasting, as previously explained. After analyzing the sample sets of the chosen screening design, MODDE software was exploited to point out the influence of a particular factor and to calculate the regression coefficient in a corresponding developed polynomial model. All values were collected and computationally combined in a Variable Importance Plot (VIP, ***Figure 1***), illustrating the importance of a variable to the measurements of a process/phenomenon, to better understand how each factor influences mycotoxin response (12).

Table 1. Data set for screening variables effects on mycotoxin levels within the rusk-making process steps (12).

Experiment Number	DON in Bran (µg/kg d.m.) [1]	Dextrose (%)	Yeast (%)	Promoting Agent (%)	Fermentation Stage		Baking Stage		Toasting Stage	
					Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)
1	600 ± 16	2	2	0	40	26	12	180	25	150
2	1500 ± 92	2	2	0	60	26	30	210	15	110
3	600 ± 16	6	2	0	60	46	12	210	15	110
4	1500 ± 92	6	2	0	40	46	30	180	25	150
5	600 ± 16	2	4	0	60	46	30	180	15	150
6	1500 ± 92	2	4	0	40	46	12	210	25	110
7	600 ± 16	6	4	0	40	26	30	210	25	110
8	1500 ± 92	6	4	0	60	26	12	180	15	150
9	600 ± 16	2	2	1	40	46	30	210	15	150
10	1500 ± 92	2	2	1	60	46	12	180	25	110
11	600 ± 16	6	2	1	60	26	30	180	25	110
12	1500 ± 92	6	2	1	40	26	12	210	15	150
13	600 ± 16	2	4	1	60	26	12	210	25	150
14	1500 ± 92	2	4	1	40	26	30	180	15	110
15	600 ± 16	6	4	1	40	46	12	180	15	110
16	1500 ± 92	6	4	1	60	46	30	210	25	150
17	1050 ± 48	4	3	0.5	50	36	21	195	20	130
18	1050 ± 48	4	3	0.5	50	36	21	195	20	130
19	1050 ± 48	4	3	0.5	50	36	21	195	20	130

[1] Data expressed as mean value ± standard deviation on dry matter basis (d.m.) of a final number of four replicates.

(**a**)



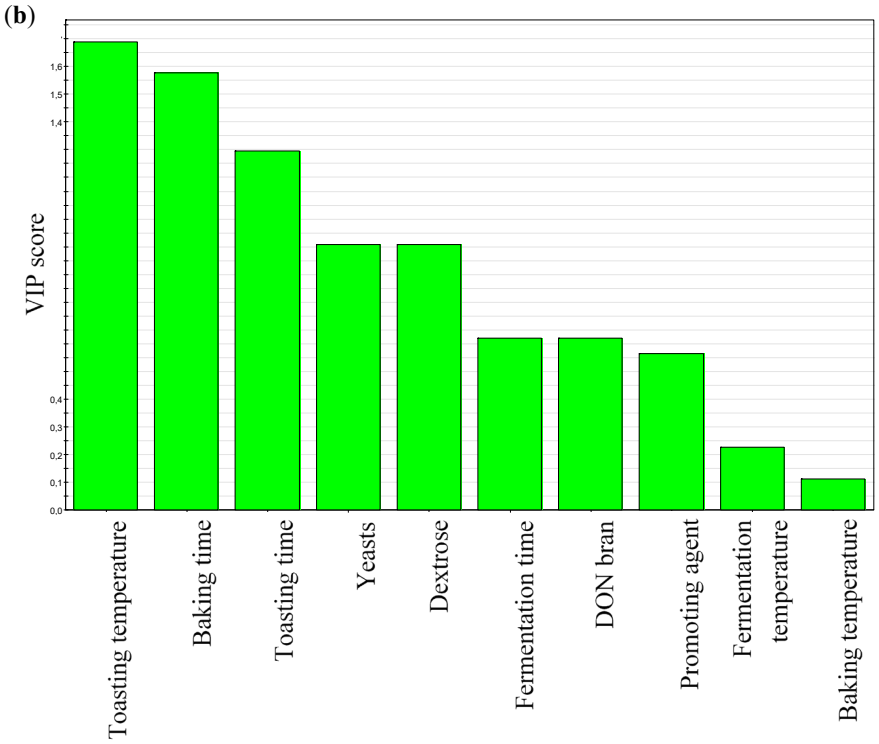


Figure 1. Variable Importance Plot (VIP) obtained from Design of Experiments rusk-making experiments (a) data referred to DON; (b) data referred to DON3Glc levels **(12).**

2.2.4 Analytical Methods and Data Interpretation

Analytical methods play a central role in food safety assessment, and continuous methodological advancements are reported each year (23).

Sample extraction is commonly performed using solvent mixtures designed to recover a broad range of mycotoxins. A frequently adopted approach, according to Sulyok et al. (24,25) involves the extraction of ground samples (5.00 ± 0.01 g) with 20 mL acetonitrile/water/acetic acid (79/20/1, *v/v/v*) for 90 min, followed by centrifugation, dilution, and direct analysis by LC–MS/MS without further clean-up

LC-MS techniques have become increasingly popular due to their ability to simultaneously determine multiple fungal metabolites and detect “masked” or other modified mycotoxins (26). These methods sometimes reveal “surprising” results, such as known mycotoxins in untypical matrices (e.g., fumonisin B2 in grapes) (27) or in unusual geographical regions (e.g., AFs in Europe) (28), for which global warming might partly be responsible (26). Chromatographic separation is typically performed on C18 columns using binary gradient elution, while electrospray ionization tandem mass spectrometry (ESI-MS/MS) is operated in scheduled multiple reaction monitoring (MRM) mode in both positive and negative polarities.

Given the often-limited degradation of mycotoxins under mild processing conditions, analytical methods must be both highly sensitive and accurate. Moreover, as food matrices change throughout processing, validation

must be performed for each relevant stage. For example, ***Figure 2*** represented the increase of DON degradation products isoDON, norDONs A-C, during the production of crackers using different processing conditions. The identification and quantification of degradation products require targeted or untargeted analytical strategies supported by fundamental reference standards (whenever possible). The extra effort and costs involved in obtaining them will be fully rewarded if there is any doubt about the identity, quantity, and toxicity of a formed degradation product, and will certainly give rise to higher quality data (15).

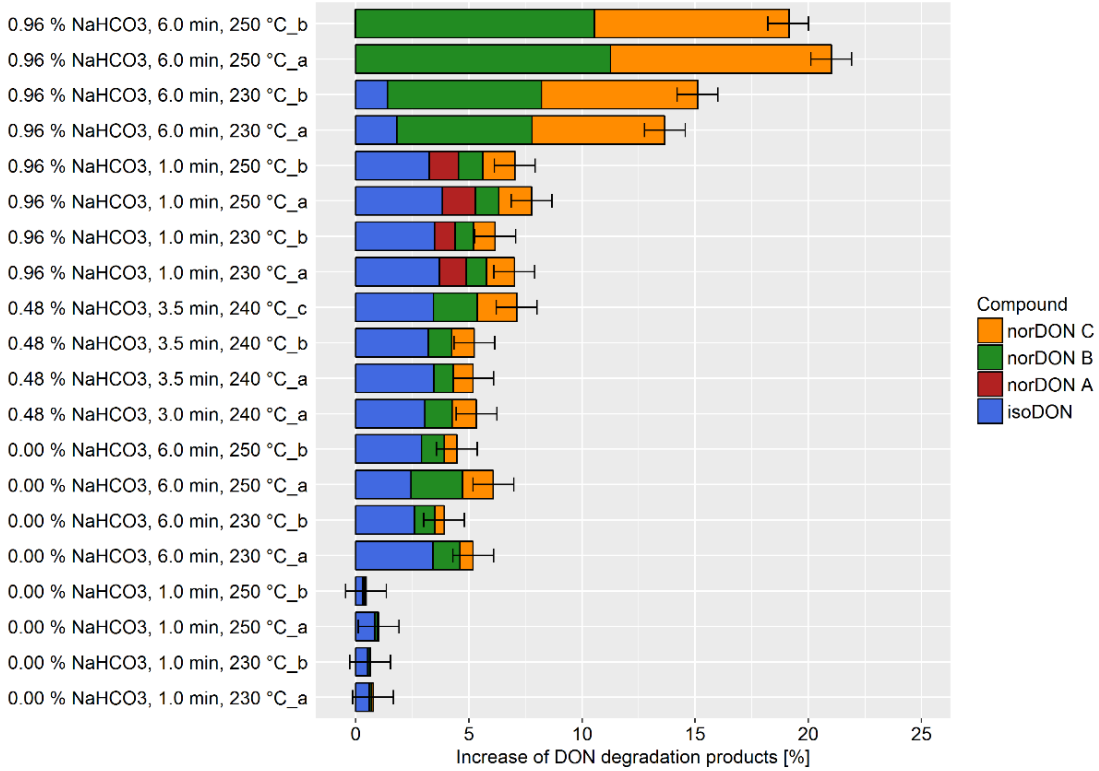


Figure 2. Increase in the deoxynivalenol (DON) degradation products isoDON, norDONs A-C during the production of crackers using different processing conditions. The increase was determined as a ratio of the molal concentration of the DON degradation products to the molal concentration of DON resulting from the natural contamination of the flour. The experimental trials were ordered according to the $NaHCO_3$ concentration, baking time, and baking temperature. Error bars represent the process standard deviation figure **(15)**.

In recent years, high-resolution mass spectrometry (HRMS) instruments, like quadrupole-time-of-flight (TOF) or Orbitrap mass spectrometers, have gained increasing relevance for structural elucidation of mycotoxin degradation products. Accurate mass measurements enable retrospective data analysis (running authentic reference standards and comparing retention times) and semiquantitative assessment of the extent of degradation, particularly when authentic standards are available for confirmation (19).

A further methodological advance, thanks to an increasing production of labelled standards, is represented by the use of stable isotope-labelled (SIL) approach combined with LC–HRMS, enabling unbiased tracking of mycotoxin fate during thermal processing. A SIL-assisted untargeted fate study, which was first used to detect

DON metabolites in wheat (29), was used to study the fate of DON and its degradation products formed during the baking of crackers, biscuits, and bread (19). The flour was treated with a mixture of non-labelled and ^{13}C-labelled standards. The detected pair of signals originating from non-labelled and isotopically labelled compounds is extracted from the full-scan chromatogram. LC-HRMS data were processed using the software MetExtract II developed by Bueschl et al. (30). Following the signals in pairs, the potentiality of this software is represented by the ability to see what happens to the marked molecule and, consequently, confirm it with the unmarked one. Using this untargeted approach, both known and (potentially) unknown degradation products of a tracer can be identified in an unbiased way (***Figure 3***).

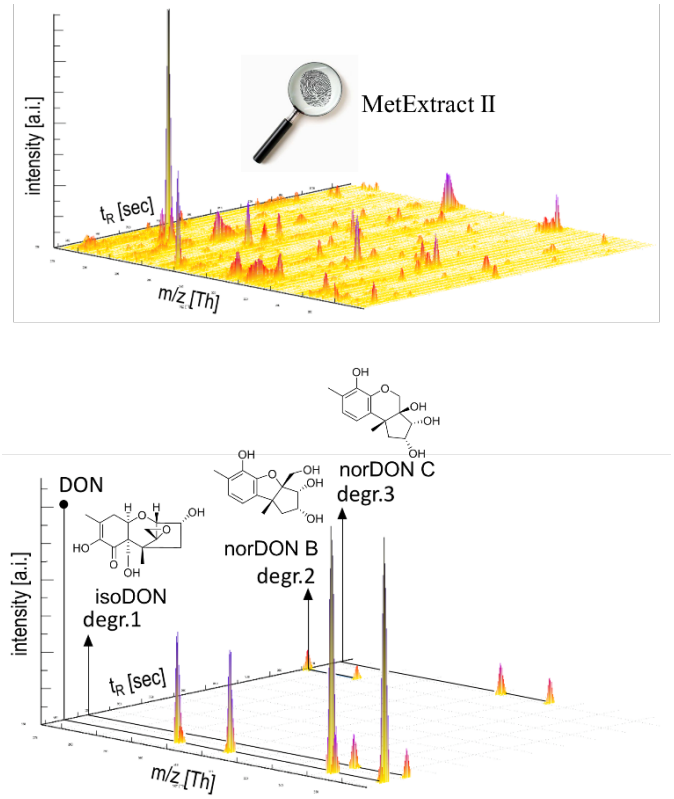


Figure 3. Schematic representation of the untargeted search for the degradation products of deoxynivalenol (DON) that are formed during the production of bakery products. Top: High resolution liquid chromatography - high resolution mass spectrum of a bakery product that was prepared from dough that was fortified with DON and ^{13}C-labelled DON. Bottom: Signals that carried the unique isotopic fingerprint were filtered from the mass spectrum using the software tool MetExtract II. The identification of the putatively annotated degradation products (degr.1-3) was carried out by comparison with reference standards **(19)**.

3 | PROCESSING-RELATED METHODS

3.1 Impact of Thermal Food Processing on Mycotoxin Levels

In recent years, interest in understanding the effects of thermal food processing on mycotoxin levels has increased. An increasing number of studies underline the fact that food processing can cause redistribution, mitigation, degradation, transformation, and/or binding or release of unexpected forms of mycotoxins (8,31).

The behaviour of mycotoxins during processing is strongly influenced by the matrix, their chemical structure, hydrophobicity, and thermal-mechanical susceptibility (32).

It should be noted that analysing and assessing the impact of thermal processing and the fate of mycotoxins is a challenging task for several reasons:

i) characterization and structural elucidation of degradation products in the matrix are difficult,
ii) matrix composition varies over the production process,
iii) the availability of analytical standards is often limited,
iv) preparation of "in-house" analytical standards in larger amounts, needed for structural confirmation and for toxicological assessment, is costly and time-consuming (18).

As highlighted in the first point, it is of utmost importance to effectively demonstrate the outcome of each treatment: whether it leads to total degradation of the mycotoxins or to their mitigation and/or transformation into a molecular structure with proven higher or lower toxicity and carcinogenicity potential. Most published studies have focused on the targeted detection of parent and progeny mycotoxins and the calculation of the rate of decline after production (33). However, a reduction in mycotoxin concentration does not necessarily correspond to a reduction in toxicological risk. Neglecting possible degradation products could lead to an underestimation of the total mycotoxin exposure. The degradation and/or conversion products may have different toxicokinetic and toxicodynamic properties than the parent mycotoxins (34).

A representative example is provided by DON degradation products, in which isoDON, norDON B, and norDON C were found to be less toxic than the respective parent mycotoxin (8). Furthermore, comprehensive toxicological evaluations enabling a robust risk assessment are often still missing (8).

3.1.1 Possible Effects of Thermal Processing Along the Cereal Chain

Growing awareness has emerged regarding the impact of food processing – particularly thermal treatments – on mycotoxin behaviour. Thermal and mechanical energy applied during processing can induce significant changes in mycotoxin concentration by promoting reactions with macromolecules such as sugars, proteins, and lipids, or by triggering the release of parent toxins following the decomposition of conjugated forms. The latter mechanism may explain the increase in DON observed during bread fermentation, likely due to the enzymatic release of bound DON mediated by yeast activity (13).

Many factors must be considered because complex physicochemical modifications (reactions with chemicals or enzymes) occur throughout the processing of raw ingredients into the final product, leading to the formation or disruption of bonds with other food components. Zhang et al. (2020) described the conversion of DON-3-Glc to DON during Chinese steamed bread processing, particularly during the fermentation and steaming steps. Mechanical friction and shear appear to contribute to these mycotoxins' structural changes, but only in combination with other factors, probably related to ingredients and complex physico-chemical modifications that occur, and therefore need further investigation (35).

Humpf et al. (2004) investigated the effects of food matrix components on the degradation of T-2 and HT-2 toxins, as well as the binding of T-2 to starch during thermal food processing (36). In model heating experiments, T-2 was bound to 1-O-methyl-α-D-glucopyranoside, a model compound that was used to simulate starch. The formed reaction products were isolated and identified by Nuclear Magnetic Resonance (NMR), giving detailed insights into a potential binding of T-2 to starch (37).

Thermal reaction products of fumonisins B1 (FB1) formed during the Maillard-type reaction in the presence of reducing sugars include N-(carboxymethyl)-fumonisin B1 (NCM-FB1) and N-(1-deoxy-D-fructos-1-yl)-fumonisin B1 (NDF-FB1) (38). Matsuo et al. (2015) identified NDF-FB2 and NDF-FB3 in addition to NDF-FB1 in corn samples (39). According to the European Food Safety Authority (EFSA), FBs may also covalently bind to macromolecules such as starch and proteins via their two reactive tricarboxylic acid side chains (40). However, their direct quantification in food is not possible, as the covalently bound FBs must first be released by chemical hydrolysis (40). Therefore, these matrix-bound forms are typically determined indirectly by quantifying free FBs and hydrolysed fumonisins (HFBs) before and after chemical hydrolysis or after digestion of the macromolecules (33,41,42). Other modified forms of FBs include fatty acid esters of FB1 (O-fatty acyl FB1) and other FBs with variation in fatty acid chain length and esterification position (3-O-, 5-O- or 10-O-acyl fumonisins) (43).

Furthermore, some studies suggest that food production processes could lead to the reduction of levels of parent mycotoxins in finished food. Schaarschmidt and Fauhl-Hassek, in their review of mycotoxins' changes during the processes of nixtamalization and tortilla production, demonstrated that alkaline cooking is effective in reducing AFs and FBs in cooked maize and tortillas, although acidic conditions may partially reverse this effect (32). However, this reduction phenomenon must be carefully interpreted to ensure that the benefits concerning the formation of low-toxic hydrolysed FBs are not offset by the parallel formation of other toxicologically relevant modified, converted, or degraded products (32). For example, most of the studies have focused on DON reduction rates during processing, while only a limited number have also investigated the formation of degradation products (44).

Stadler et al. (2019) demonstrated for the first time that pilot-plant experiments mimicking large-scale industrial baking can lead to partial detoxification of DON, through optimisation of processing parameters and toxicological evidence showing lower toxicity of the major degradation product, isoDON. During the

production of crackers, biscuits, and bread, 0–21%, 4–16%, and 2–5% DON, respectively, were degraded. The study started from naturally contaminated flour under different processing conditions and showed that higher $NaHCO_3$ concentration, baking time, and baking temperature increased DON degradation, whereas NH_4HCO_3, yeast, vinegar, sucrose concentration, and leavening time did not enhance DON degradation. In vitro cell viability assays confirmed that isoDON is considerably less toxic than DON (15).

Scudamore et al. (2008) reported that, in contrast to kilning, which showed only limited effects on T-2 and HT-2 levels (14), thermal degradation during extrusion cooking accounted for up to 60% and 47% reduction for T2 and HT2, respectively (45). During bread-baking, T-2 mitigation of up to 74% (HT-2 ≤25%) (46) was observed in naturally contaminated flours and up to 60% (HT-2 mitigation 20–30%) in artificially contaminated flours (20). Literature evidence suggests that the high degradation of T-2 during bread preparation may be partially attributed to enzymatic hydrolysis of T-2 to HT-2 during yeast fermentation. This hypothesis was confirmed experimentally (47). Additional studies on non-yeasted baked products and roasting showed matrix- and process-dependent degradation patterns. During biscuit making, up to 45% of T-2 and 20% of HT-2 were degraded, depending on water content, baking time, and temperature. Crunchy muesli showed no significant reduction, while roasting resulted in partial degradation. However, no hydrolysis of T-2 to HT-2 and no known degradation products were detected, suggesting either the formation of other degradation products or more complex matrix binding (20).

Regarding OTA, its thermal stability has been only partially explored. Available studies suggest that OTA is more stable than DON during baking, with maximum reductions reported at high temperatures (around 200 °C). Processing parameters such as time, product size, pH, and fermentation conditions play a critical role during bakery production (48,49)

For AFs, numerous degradation pathways based on physical techniques have been described, like flotation in saturated sodium chloride solution, which removed 3% of kernels but up to 74% of total aflatoxin content in maize (50). However, data on the formation of modified AFs during industrial processing remain very limited.

4 | NOTES

4.1 Raw Material Selection and Initial Mycotoxin Screening

Accurate raw material selection and preliminary mycotoxin screening represent a critical starting point for any study investigating mycotoxin fate during food processing. Naturally contaminated matrices are strongly preferred over artificially spiked samples, as they better reflect the real distribution, binding state, and matrix interactions of mycotoxins. Spiking experiments may alter toxin–matrix interactions and lead to unrealistic accessibility and extractability patterns.

Initial screening of contamination levels should be performed using validated multi-mycotoxin analytical methods before processing trials. The presence of masked or modified forms should also be considered, since processing may convert these forms into the parent toxin and bias apparent concentration changes.

Batch-to-batch variability should be assessed, and when necessary, contaminated and low-contaminated batches may be blended to reach technologically feasible and analytically reliable starting concentrations. Particular attention should be given to moisture correction, analyte recovery correction, and dilution effects due to recipe formulation, as these factors are not always transparently reported in the literature but may significantly affect data interpretation.

Another practical limitation concerns the availability of analytical reference standards, especially for degradation and conversion products. Limited access to standards may restrict structural confirmation and quantitative assessment and should be considered during study design and result interpretation.

4.2 Strategic Use of Design of Experiments in Processing Studies

DoE should be applied strategically when investigating the effect of processing parameters on mycotoxin fate. A clear definition of the study objective is essential before selecting the experimental design. The objective may include screening of influential factors, process optimisation, or robustness assessment.

Relevant technological factors should be identified in advance, and each factor should be varied within a defined range consistent with technological feasibility and product acceptability. Factor ranges that are unrealistic from a processing or sensory perspective should be avoided, as they reduce the practical value of the results.

The choice between screening, fractional factorial, or response surface designs should be aligned with the number of variables and the expected interaction effects. Replicates at the centre point are recommended to estimate experimental error and model stability.

Experimental data should be analysed using appropriate multivariate statistical approaches. Partial least squares (PLS) modelling and related multivariate tools implemented in dedicated statistical software (e.g., MODDE) are particularly suitable for identifying factor importance, interaction effects, and process–response relationships. Proper statistical interpretation is essential to avoid overfitting and misinterpretation of mitigation effects.

4.3 Critical Requirements for Stable Isotope-Labelled (SIL) Untargeted Fate Studies

Stable isotope-labelled tracer approaches combined with high-resolution mass spectrometry represent a powerful strategy for investigating mycotoxin degradation pathways during processing. To elucidate the fate during baking, an untargeted tracer fate study must be carried out to determine all formed extractable degradation products. This study should take into account several commodities (such as differing in ingredients, fermentation agent, and fermentation time) to cover a wide variety of possible degradation and conversion products that could be formed under different processing conditions.

Samples are typically prepared using matrices spiked with both non-labelled and isotopically labelled standards. Detection relies on the unique isotope pattern and co-elution behaviour of labelled/unlabelled signal

pairs. Dedicated software tools such as MetExtract II enable automated filtering of candidate tracer-derived products from HRMS datasets without prior knowledge of their structure.

Untargeted SIL workflows allow unbiased detection of conjugation, degradation, and conversion products formed during processing. However, subsequent targeted validation and quantification using validated LC-MS/MS methods are recommended for confirmed degradation products to ensure quantitative reliability and toxicological relevance assessment.

5 | FINAL OUTLOOK

Thermal processing along the cereal chain could modify mycotoxin exposure by eliminating or degrading mycotoxins, transforming them into differently toxic derivatives, or reducing their bioavailability through chemical binding to food matrix components. However, complete elimination of mycotoxins from food products through processing alone can rarely be achieved.

Most studies on the fate of mycotoxins during thermal food processing have traditionally focused on the reduction of the parent mycotoxin concentration from raw material to finished product. However, this approach is only fully appropriate for processes that do not induce significant degradation or conversion of the parent compounds. So far, studies addressing the identification and structural elucidation of degradation products remain limited to selected mycotoxins and specific cereal processing operations. These investigations clearly demonstrate the analytical and toxicological effort required to identify transformation products and characterize their toxicity, which is essential for drawing sound conclusions on the real impact of thermal processing on final product safety. In both targeted and untargeted workflows, the availability of reliable reference standards remains a decisive limiting factor.

From a methodological perspective, the combined application of statistically planned processing experiments and advanced analytical strategies represents a major step forward. The structured use of DoE enables systematic evaluation of technological factors and their interactions, supporting the identification of processing conditions that most strongly influence mycotoxin mitigation or transformation patterns. In parallel, HRMS and SIL tracer approaches provide powerful tools for the detection and confirmation of degradation and conversion products, including previously unknown forms, through both targeted and untargeted analytical workflows.

Only when a decrease in measured mycotoxin concentration is accompanied by evidence that the resulting degradation and/or conversion products are less toxic than the parent compounds can thermal processing be considered a true mitigation step. For this reason, mycotoxin risk assessment frameworks should progressively integrate processing-derived forms and transformation products. Robust analytical protocols combining DoE-driven process design with HRMS and SIL-based identification strategies will play a central role in supporting this evolution.

Looking forward, the methodological framework described here for cereal thermal processing can be extended to other food processing chains and contaminant classes. Expanding these protocol-oriented approaches will provide food manufacturers and risk assessors with higher-quality mechanistic data, ultimately strengthening science-based decision-making and contributing to improved consumer protection.

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2 | AIM OF THE THESIS

This PhD thesis investigates the identification of natural toxins and related emerging risks in innovative ingredients for bakery and meal solutions, from raw material characterization to the effect of food processing and dietary exposure.

To reach this general goal, three strategic scientific objectives were pursued:

Objective 1 | Development of analytical methods for toxic plant alkaloids

*(*Section I — *Chapters 1–3)*

- To develop and validate an LC-HRMS method for the simultaneous determination of pyrrolizidine alkaloids and tropane alkaloids in cereals and spices (Ch.1)
- To optimize extraction efficiencies and quantification with an LC-MS/MS protocol for ergot alkaloids and their epimers in different cereal matrices (Ch.2)
- To assess the industrial applicability of a rapid 3D-printed biosensing toolkit for atropine and scopolamine detection in buckwheat (Ch.3)

Objective 2 | Tracking mycotoxin fate during food processing

*(*Section II — *Chapters 4–5)*

To exploit a stable isotope labelling LC-HRMS untargeted approach to evaluate the fate (stability, transformation or degradation) of:

- AFB1 and FUM B1/B2 during gluten-free pasta production (Ch.4)
- T-2 and HT-2 toxins during wholemeal cracker production (Ch.5)

Objective 3 | Multi-toxin occurrence and exposure in legumes

*(*Section III — *Chapter 6)*

- To evaluate the occurrence of multi-mycotoxins and tropane alkaloids in legume-based products from Italy and Thailand and
- To assess dietary exposure to highlight geographical differences and regulatory gaps (Ch.6)

Scientific and industrial impact

The proposed strategy strengthens targeted monitoring of natural toxins, improves understanding of how food processing influences toxicological risks, and supports risk-based decision making in the development of innovative bakery and meal solutions.

3 | EXPERIMENTAL STUDIES

SECTION I | Analytical Methods for Monitoring Toxic Plant Alkaloids

1

Chapter

Research Article

QuEChERS method combined to liquid chromatography high resolution mass spectrometry for the accurate and sensitive simultaneous determination of pyrrolizidine and tropane alkaloids in cereals and spices

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Abstract

Within the last decades, in the EU, there has been an increasing interest in toxic plant Alkaloids as food contaminants, especially after the continuous and growing consumption of plant-based foods compared to food of animal origin. In this regard, the once neglected presence of these Tropane and Pyrrolizidine Alkaloids has recently been reconsidered by the European Food Safety Authority, highlighting the lack of data and the need to develop risk assessment strategies. For this reason, the emphasis has been placed on detecting their occurrence in food through the development of accurate and sensitive analytical methods to achieve the determination of these compounds. The present study aims to elaborate and validate an analytical method based on QuEChERS sample preparation approach, exploiting the UHPLC coupled to the HRMS to simultaneously identify and quantify 21 PAs and 2 TAs in different food matrices. For TAs, the obtained Limit of Detection (LOD) is 0.1 μg Kg^{-1} and the Limit of Quantification (LOQ) is 0.4 μg Kg^{-1}, while for PAs, the LODs values ranging between 0.2 to 0.3 μg Kg^{-1} and the LOQ, between 0.4 to 0.8 μg Kg^{-1}, ensuring compliance with the recently established European Regulations. Several commercial samples were analysed to further verify the applicability of this comprehensive analytical approach.

KEYWORDS

Cereals and spices, High-resolution mass spectrometry (HRMS), Isomers, Pyrrolizidine alkaloids, Tropane alkaloids

1 | INTRODUCTION

Alkaloids, which means alkali-like substances, are basic heterocyclic nitrogenous compounds of plant origin that are physiologically active. Since ancient times, Alkaloid-containing plants have been used as poisons and medicines. Consumers attribute the concept of safe to botanical preparation because they often believe that "natural" means "safe". This is not the case. Alkaloids are well-known for their significant toxicity and several cases of poisoning have been reported in the literature [1]. Nowadays, these Alkaloids can be introduced in the food chain (in food or animal feed), originating either from edible plants (e.g. potatoes and aubergine) or when non-edible plants and/or their seeds are co-harvested with the crop, the so called "TAs/PAs-producing plants", or due to horizontal product transfer with other plants, mainly when contamination through co-harvest is excluded (e.g. thorough hand-picking) [2]. For this reason, significant and rising consumption of plant-based products as an alternative to animal products and/or for nutritional and health concerns, caused food research institutions and food research authorities to raise awareness regarding human exposure to these natural toxins in food and feed [3-8]. More than 12000 Alkaloids have been discovered in plants with considerable variety in the chemical structure and pharmacological actions. This group mainly includes approximately 200 Tropane Alkaloids (TAs) produced by plants of several families including *Brassicaceae, Solanaceae* (with species of the genera *Datura*, *Brugmansia*, *Hyoscyamus*, *Scopolia*, *Atropa),* and *Erythroxylaceae,* and 660 Pyrrolizidine Alkaloids (PAs) mainly found in the angiosperm families of the *Boraginaceae*, *Asteraceae,* and *Fabaceae* [9].

TAs are secondary metabolites that naturally occur in plant and present a common structure based on tropane skeleton ($C_8H_{15}N$). The only data available regarding the occurrence in food concern the main compounds atropine, racemic mixture of (-)-hyoscyamine and (+) -hyoscyamine of which only the (-)-enantiomer exhibits anticholinergic activity, and scopolamine. A precise and accurate review on the chemistry, occurrence and regulatory aspects of TAs in food was recently published by González-Gómez et al. [10].

Seeds of plants belonging to the genus *Datura stramonium,* cannot be easily removed from cereals (like linseed, soybean, sunflower and buckwheat) and especially, due to the concurrent fruit maturations and the similar size of the harvested grains, sorting and cleaning methods are not always effective, leading to the presence of these foreign seeds in cereals products and resulting in measurable levels of TAs contamination [11].

The European Food Safety Authority (EFSA) adopted in 2013 an opinion on the risks for human and animal health related to the presence of TAs in food and feed. Since their pharmacological effect occurs within a short time after administration, the Authority established a group acute reference dose (ARfD) of 0.016 µg/kg body weight (b.w.) expressed as the sum of (-)- hyoscyamine and (-)-scopolamine, assuming equivalent potency. In terms of body weight, the tolerable daily intake levels can be derived from the acute reference dose (adult with 70 Kg of body weight: tolerable daily intake of 1,12 µg) [5]. Considering the opinion, to protect the health of the population, maximum levels for atropine and scopolamine were established by the Commission Regulation (EU) No 2021/1408 in cereal-based food for infants and young

children, and from 1st September 2022, new limits entered into force for other cereal-based foods which contain millet, sorghum, buckwheat, maize and herbal infusions [12].

Moreover, it has been demonstrated that untreated matrices, such as buckwheat and millet flour present higher levels of TAs contamination compared to heated products like bread, bakeries, or cookies [13]. However, up to now, few studies investigating the persistence of TAs towards food processing were performed, highlighting the need for further investigation of potential breakdown or transformation products [14].

Nowadays, hazard characterization highlights that atropine and scopolamine do not bioaccumulate and have a short life in humans; they are not genotoxic *in vitro* and do not cause carcinogenicity or progressive toxicity following repeated exposure [7].

PAs are a large group of secondary metabolites occurring in different plants, produced as a defense mechanism against herbivores and insects. The name pyrrolizidine consists of two fused five-membered rings with a nitrogen atom in the bridgehead, which are called necine base. Depending on the mode of esterification of the two available hydroxyl groups with the so-called necic acids (mono-or dicarboxylic acid), a variety of ester derivatives are formed. The necine base can either be saturated or contain a double bond in the 1,2-position [15]. Only the group of PAs which structurally are 1,2-unsaturated are relevant for safety assessment, as the PAs without this structural formula are considered non-toxic but require metabolic activation to exert their toxicities. The CONTAM Panel, in 2011, conclude that 1,2-unsaturated PAs may act as genotoxic and carcinogenic in humans, so the levels in food must be kept as low as possible (ALARA principle) [4,16]. In addition, PAs will form in plants N-oxide (PANO) derivates, but the ratio of the free base and the NO form can vary widely. A complete review on the occurrence, exposure, toxicity and risk assessment of PAs was recently published by Schrenk and co-workers [17].

From the analysis of the available occurrence data, the CONTAM Panel identified a list of 17 PAs of relevance for monitoring in food and feed, and they are: intermedine/lycopsamine, intermedine-N-oxide/lycopsamine-N-oxide, senecionine/senecivernine, senecionine-N-oxide/senecivernine-N-oxide, seneciphylline, seneciphylline-N-oxide, retrorsine, retrorsine-N-oxide, echimidine, echimidine-N-oxide, lasiocarpine, lasiocarpine-N-oxide and senkirkine [6].

Nowadays, PAs can be present in specific plants or parts thereof (PA-producing plant) and the amount depends on many factors (species, plant organ, harvest, storage, extraction procedures) or due to the accidental co-harvesting of the crop of interest with them (adventitious presence) or in food of animal origin, through the ingestion of PA-producing plants by grazing animals or animal feed. This type of contamination is rather unpredictable, highly not homogeneous and can have a variety of causes. In this sense, the application of good agricultural and harvest practices could be the most effective solution for food producers and manufacturing companies, to reduce or prevent weeds infestation and TAs/PAs-producing plants [18].

The recent RASFF notifications (2019 – 2020) have highlighted that PAs can be found in teas as well as in common culinary herbs and spices (oregano, thyme, peppermint and cumin seeds) [19]. For that

reason, the Commission Regulation (EU) 2020/2040 entered into force this 1st July 2022, established the maximum limits for the sum of 35 PAs in tea, herbal infusions, dried herbs and spices [20].

Therefore, the widespread occurrence of PAs/PANOs in different foodstuffs and their potential risk for human health are considered a current food safety issue that needs to be addressed. However, the real exposure levels of the population to these contaminants and their presence in a less studied range of food matrices are still uncertain due to the lack of data. For instance, further investigations of food processing are needed to understand the assessment of the real intake of these alkaloids by the population and to improve the risk management of these natural contaminants [21]. The latest advances in the extraction, separation and identification of this class of Alkaloids was recently summarized in this review by Al-Subaie. et al [22].

The complexity of the matrices of interest (cereals and spices) makes the sample preparation a critical step for this task. For this reason, the emphasis was put on improving the simultaneous extraction and the clean-up of samples, especially in the industrial context, with the QuEChERS sample preparation approach, which allows to minimize the matrix effect since often affects the selectivity and sensitivity of the method [10].

Certainly, the biggest challenge for this method development was to obtain a good chromatographic separation due to the coelution of multiple groups of isomeric alkaloids.

Actually, according to the literature, for a simultaneous determination of TAs and PAs in our matrices of interest, methods exploited the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [22-25] but the majority of them, face separately these two groups of Alkaloids [9, 10, 13, 15, 26-32]. This study proposed the exploitation of Ultra High Performance Liquid Chromatography (UHPLC) coupled to the High Resolution Mass Spectrometry (HRMS) to evaluate in a simultaneous way the most relevant 2 Tropane Alkaloids and 21 Pyrrolizidine Alkaloids, recommended by EFSA to be monitored in food.

To date, only few analytical methods which exploit the HRMS for the simultaneous determination of these Alkaloids, in our matrices, have been published, such as: HRMS workflow for the analysis of food contaminants, plants toxins, mycotoxins and phytoestrogens in plant-based food [2], the development of a QuEChERS method coupled to the HRMS to determine PAs and TAs in honey [33] and the simultaneous determination of 30 TAs and PAs in teas and herbs for infusions by LC-Q-Orbitrap HRMS [34]. To the best of our knowledge, this is the first paper addressing the simultaneous determination of these Alkaloids in cereals and spices by QuEChERS approach, coupled with UHPLC-HRMS.

After the development and in-house validation, the performance of this approach was evaluated using certified reference materials and analysing different commercial samples.

2 | MATERIALS AND METHODS

2.1. Reagents and Standards

Methanol, Acetonitrile, Formic Acid and Ammonium Formate for HPLC – gradient grade ≥ 99.0%, were purchased from VWR International, Ltd (Poole, United Kingdom). For the preparation of the mobile phase and all sample preparations experiments, water was purified using a Milli-Q system (Millipore, Bedford, MA).

Ready-to-use QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) Kit containing 150 mg $MgSO_4$ and 25 mg PSA were from Waters (*DisQuE).*

Solid analytical standard of 2 Tropane Alkaloids (atropine and scopolamine) and 21 Pyrrolizidine Alkaloids (Intermedine, Echimidine, Europine, Heliotrine, Lasiocarpine, Lycopsamine, Retrorsine, Senecionine, Seneciphylline, Senecivernine, Senkirkin with their respective N-oxide) were purchased from Phytolab (Germany) with a chemical purity > 98%.

2.2. Samples

Survey samples for method development and validation:

Wheat. Durum wheat (*n* =3) and soft wheat (*n* =3) samples were obtained from the 2020 harvesting campaign while durum wheat (*n* =6) and soft wheat (*n* =3) samples come from 2021 harvesting campaign.
Maize. Maize samples came from an Italian supplier which produces gluten free products, the following samples were selected: raw yellow corn flour (*n* =6), yellow corn flour flaked (*n* =4), pre-gelatinized flaked yellow corn flour (*n* =2), raw white corn flour (*n* =4) and extrude white maize (*n* =4).
Buckwheat. Buckwheat samples (*n* =12) came from an Italian milling company supplier.
Oregano and Rosemary. Oregano (*n* =2) and rosemary (*n* =1) samples came from different Italian suppliers.

For method's applicability: different commercial samples, maize (*n* =4), buckwheat (*n* =2), oregano (*n* =4) and rosemary (*n* =4) were purchased from the market (in Autumn 2021). Certified reference materials were obtained from FAPAS (FERA Science Ltd. York, UK) and were Tropane Alkaloids in cereals (T22145QC) and Pyrrolizidine Alkaloids in Ispaghula Husk (Psyllium Husk) (T22194PT).

2.3. Sample preparation procedure and optimized clean-up

1.00g ± 0.01 g of finely ground representative sample of cereals (wheat, buckwheat and maize), oregano and rosemary was weighed into 50-mL centrifuge tube in which 10 mL of the extraction solvent (methanol:water:formic acid, 60:40:0.4, v/v/v) was pipetted into the tube, and the sample was shaken (30 min). The suspension was then centrifuged (8 min, 5000 rpm; Rotina 380 R).

The aqueous-methanolic extract (1 ml) was pipetted in the QuEChERS Kit (150 mg MgSO4 + 25 mg PSA), the solution was shaken for 1 min to prevent any lump formation and then centrifuged under the

same condition as above. The mixture was frozen to promote the complete precipitation of the different impurities (1 hour). After thawing, the extract was transferred into centrifuge tube filter (0.22 µm cellulose acetate membrane) and centrifuged (5 min, 5000 rpm; Rotina 380 R).

The clean extract was transferred into a 2-mL amber-glass vial and injected in the UHPLC-HRMS system.

2.4. Standard preparation for quantification

TAs and PAs were quantified using a matrix-matched calibration curve.

2.4.1. Standard preparation for 2 Tropane Alkaloids

TAs were purchased in aliquots of 100 µg and they were dissolved in MeCN (1 ml) to obtain final solutions with a concentration of 102.5 µg/mL (atropine) and 100.9 µg/mL (scopolamine) (stock solutions). Then, the methanol extract of a "blank" buckwheat sample was prepared.

By taking 10 µl of both stock solutions and placing them in a single vial in which 5 ml of methanol extract of a "blank" buckwheat sample was added: a matrix solution was therefore obtained (called Matrix Solution $_{Buckwheat}$ – MS_B) which has a concentration of 200 µg/mL of atropine and 200 µg/mL of scopolamine. Matrix-matched calibrants are obtained by serial dilution of the MS_B solution with different ml of methanol extract of a blank buckwheat sample. Stock solutions were stored at – 20 °C.

2.4.2. Standard preparation for 21 Pyrrolizidine Alkaloids

Fourteen PAs were purchased in aliquots of 10 mg and they were dissolved in MeOH (1ml) to obtain final solutions of 10000 µg/mL (stock solution). By taking 200 µl of the stock solution and diluting it in 10 ml of H_2O:MeOH 90:10 (v/v), a secondary solution$_{(1)}$ with a concentration of 200 µg/mL was obtained.

Seven PAs were purchased in aliquots of 5 mg and they were dissolved in MeOH (1ml) to obtain final stock solutions of 5000 µg/mL (stock solution). By taking 200 µl of the stock solution and diluting it in 10 ml of H_2O:MeOH 90:10 (v/v), a secondary solution $_{(2)}$ with a concentration of 100 µg/mL was obtained.

Furthermore, it was prepared the methanol extract of a "blank" oregano sample.

By taking 200 µl of the 200 µg/mL secondary solution $_{(1)}$ and 400 µl of the 100 µg/mL secondary solution $_{(2)}$ and placing them in a single vial in which 10 ml of methanol extract of a "blank" oregano sample was added, a matrix solution was obtained (called Matrix Solution $_{Oregano}$ – MS_O) which has a concentration of 4000 µg/mL of each PAs. Matrix-matched calibrants are obtained by serial dilution of the MS_O solution with different ml of methanol extract of a blank oregano sample. Stock solutions were stored at – 20 °C.

2.4.3. Six-points overall calibration curve for TAs and PAs

A six-points matrix matched calibration curve of both TAs and PAs was used to quantify the analytes (0.25, 0.5, 2.5, 5, 7.5 and 10 µg/Kg for TAs and 0.5, 2.5, 5, 15, 30 and 100 µg/Kg for PAs). To reach the desired concentrations, the 6 solutions indicated above in the previous paragraphs were mixed 50:50, directly in the vials for HPLC.

2.5. UHPLC – HRMS analysis

The UHPLC - HRMS system consisted of a Thermo Fisher Scientific instrument with an Ultimate 3000 LC system coupled with a Quadrupole-Orbitrap mass spectrometer (Q-exactive).

The separation of TAs and PAs was achieved by using a ACQUITY UPLC BEH^{TM} C_{18} column (2.1 x 150 mm, 1.7 µm; Waters, Milford, MA, USA), heated at 40°C. Mobile phase was 2 mM ammonium formate and 0.2% formic acid in water (eluent A) and in methanol (eluent B), respectively. The autosampler was set to 10°C and the injection volume was 5 µl. The mobile phase flow rate was 0.250 mL/min. The eluent gradient profile was set as follows: 0 – 1.0 min (5% B); 1.0 – 4.0 min (15% B); 4.0 – 12.0 min (35% B); 12.0 – 12.5 min (95% B); 12.5 – 15.5 min (95% B), from 15.5 to 16.0 min return to the initial condition. The system was re-equilibrated with the initial composition for 6 min prior to next injection, yielding a total run analysis time of 22 min.

Ionization was achieved using the Heated electrospray (HESI) source and data were acquired in the positive ionization mode (+). Nitrogen was used as sheath gas (40 a.u.), auxiliary gas (20 a.u.), and as collision gas. Ion spray voltage was set to 3.50 kVand capillary and heater temperature were maintained at 300 °C and 250°C, respectively. The normalized collision energy (NCE) was set to 30. The Data dependent acquisition (DDA) method of the HRMS allow the *full MS* (FS) pre-scan of parent ions followed by isolation and fragmentation of ions present in the *inclusion list* (list of the masses of the target analytes) at the correct retention time. For the method development and data evaluation, Xcalibur ^{TM}, Q-Exactive Tune and TraceFinder ^{TM} software (Thermo Scientific, San Jose, CA, USA) were used. The HRMS parameters are summarized in Table 1.

TABLE 1 Retention times and mass spectrometric conditions for determination of two TAs and 21 PAs.

Pyrrolizidine alkaloids	R_t (min)	Precursor ion $[M + H]^+$	Confirmatory product ions (*m/z*)	Additional product ions (*m/z*)
Europine	6.55	330.19111	138.0914	156.1020 254.1387
Intermedine	6.47	300.18055	138.0915	156.1020 120.0810
Lycopsamine	6.70	300.18055	138.0915	156.1020 120.0810
Europine NO	7.08	346.18603	172.0968	111.0681 328.1754
Scopolamine	7.39	304.15433	138.0914	156.1020 121.0651
Intermedine NO	7.58	316.17546	172.0978	138.0914 111.0683
Lycopsamine NO	7.83	316.17546	172.0978	138.0914 111.0683
Retrorsine	8.54	352.17546	120.0810	324.1805 138.0914
Retrorsine NO	8.62	368.17038	118.0654	120.0811 136.0758
Seneciphylline	9.05	334.16490	120.0810	138.0914 306.1702
Heliotrine	9.30	314.19620	138.0914	156.1020 120.0810
Seneciphylline NO	9.46	350.15981	120.0811	118.0654 136.0758
Atropine	9.92	290.17507	124.1121	260.1644 93.0702
Heliotrine NO	10.02	330.19111	172.0964	111.0681 136.0758
Senecivernine	10.54	336.18055	120.0811	138.0915 308.1860
Senecionine	10.84	336.18055	120.0811	138.0915 308.1860
Senecivernine NO	10.66	352.17546	118.0654	120.0811 136.0758
Senecionine NO	11.12	352.17546	118.0654	120.0811 136.0758
Echimidine NO	12.54	414.21224	254.1387	352.1754 396.2020
Echimidine	12.93	398.21733	120.0811	220.1334 83.0497
Senkirkin	13.26	366.19111	168.1021	150.0915 122.0603
Lasiocarpine	14.62	412.23298	120.0810	336.1806 220.1333
Lasiocarpine NO	14.71	428.22789	254.1386	136.0758 352.1756

2.6 Method Validation

The method was validated in-house determining the matrix effect (ME), linearity of the calibration, recovery rates, repeatability and both limit of detection (LOD) and quantification (LOQ).

Document Nº SANTE/11312/2021 [35] and document CEN/TR 16059:2010 on single laboratory validation of mycotoxin methods [36], were used as references.

Validation was performed on two representative matrix-samples from two commodity groups (cereals and spices).

To evaluate matrix effect, a comparison between the slope of the matrix-matched calibration and the solvent calibration curves was performed. Slope differences were calculated for each analyte applying the following equation (Eq. 1) [37]:

(1)

$$ME\ (\%) = \left(\frac{\text{slope of the matrix} - \text{matched calibration curve}}{\text{slope of the solvent calibration curve}} - 1 \right) x\ 100$$

Negative values represent signal suppression while positive values indicate signal enhancement caused by the matrix interferences.

Peak area was used as analytical signal and linearity, expressed as determination coefficients (R^2), was studied at the concentration range reported in the paragraph 2.4.3.

Recovery data were obtained by spiking blank buckwheat samples (for TAs) and oregano samples (for PAs) at two concentration levels: 2.5 and 10 μg/Kg and 15 and 100 μg/Kg, respectively, in four replicates. These concentrations refer to each individual compounds. Blanks and spiked samples were then extracted and analysed as described in the previous section.

The same dataset was used to calculate the method's repeatability, which was expressed as the relative standard deviation (RSD) of six replicates.

The LOD and the LOQ of each analyte were estimated provided that they present a signal-to-noise ratio (S/N) of 3 and 10, respectively.

To confirm the correct compound identity, identification criteria adopted from SANTE [35] were: (i) a precursor ion detected with a mass accuracy below 5 mg/Kg; (ii) retention time of the analyte in the extract should correspond to that of the calibration standard with a tolerance of ±0.1 min; (iii) minimum number of ions for HRMS are: 2 ions with mass accuracy ≤ 5 mg/Kg, including at least one fragment ion. Compounds were considered "identified" when all criteria were met, "detected" when only one criterion (i) was fulfilled and "not found" when no one of these criteria was met.

2.7 Method's applicability

The method's performance was evaluated using as quality control materials (QC), Tropane Alkaloids in cereals (T22145, FAPAS) and Pyrrolizidine Alkaloids in Ispaghula Husk (Psyllium Husk) (T22194, FAPAS) and was tested with a set of different commercial samples purchased from the supermarket. Maize (*n* =4), buckwheat (*n* =2), oregano (*n* =4), rosemary (*n* =4) were analysed in duplicate.

3 | RESULTS AND DISCUSSION

3.1 Method Development

3.1.1 Optimization of the Chromatographic separation

The biggest challenge encountered during method development was definitely to obtain a good chromatographic separation of all 21 PAs, in particular due to the co-occurence of multiple groups of isomeric compounds, which causes the problem of compound coelution with several identical mass transition [15, 22, 24, 31, 32, 34]. HPLC resolution is crucial for the successful identification and differentiation of isomeric monoesters intermedine and lycopsamine (m/z 300) and their N-oxides (m/z 316) as well as the cyclic diesters senecionine and senecivernine (m/z 336) and their N-oxides (m/z 352), among the PAs/PANOs recommended by EFSA to be monitored in food. To date, according to the literature, many authors present the phenomenon of co-elution (as highlighted above), other, instead of including these 8 isomers, only analyze some of them [33,38] or apply different acidic and alkaline mobile phases [39] or try to separate the highly polar alkaloids using hydrophilic interaction liquid chromatography (HILIC) columns [24, 39, 40]. As far as we know, there are only 3 papers able to obtain the complete separation of all the PAs/PANOs recommended by EFSA including the isomers. Kaltner et al. [29] and Dreolin et al. [32] focused they attention mainly on Pyrrolizidine Alkaloids in teas and herbal teas and, plant-based food and honey, respectively. Klein L. M. et al. [39], in addition to the PAs, also considered Tropane Alkaloids in cow's milk.

Despite the good separation, we can argue that none of them consider the simultaneous quantification of TAs and PAs in our matrices, such as cereals and spices.

However, even though some LC methods may achieve a good separation for the recommended Alkaloids, the bigger challenge is still the full chromatographic separation of critical pairs of isomers, such as intermedine, indicine, lycopsamine, rinderine and echinatine (m/z 300) and also their N-oxides (m/z 316) as well as integerrimine, senecionine and senecivernine (m/z 336) and their N-oxides (m/z 352) [15, 29, 32, 39].

To optimize the chromatographic conditions of these compounds, different stationary phases, composition of mobile phases and elution gradients were tested. In literature, the majority of LC-HRMS method for PAs/PANOs/TAs analysis operate in reverse phase mode with C18 columns combined with alkaline or acidic solvent conditions, with the latter solvent conditions being more common [2, 24, 33, 34].

In order to obtain the best peak shapes and chromatographic separation, the composition of the mobile phase was optimized. Acetonitrile and methanol were tested as organic phases and also different ratio of both ammonium formate and formic acid. An oscillation of retention times occurred using only ammonium formed as an additive solvent, but this was remedied by adding formic acid as an additive in both solvents [29]. The best compromise was discovered by Dzuman Z. et al. [24] who tested seven aqueous methanolic MPs, demonstrating that 2 mM ammonium formate and 0,2% formic acid provided the highest

number of analytes with the increased signal intensities and at the same time the lowest number of analytes with the signal drop.

Two different columns were tested under the same conditions as above: Luna Omega C18 column (2.1 x 150 mm, 1.6 µm particle size; Phenomenex, USA) and BEH C18 column (2.1 x 150 mm, 1.7 µm particle size; Waters, Milford, USA). The choice of a column with small particle size could reduce the matrix load on it and consequently reduced matrix suppression and increased shelf-life of the column.

Initially a slow gradient was used to obtain the separation of the isomeric compounds and achieve sufficient retention of the analyte on the column, starting with low concentration of solvent B (10%) which increased to 30% after 8 min and arrived to 100% in 18 min. An isocratic step was included for 2 min returning to the initial conditions in 0.5 min, yielding a total run time of 25 min (including requilibration). In this case, the critical isomeric groups were not separated and mostly coeluted (intermedine and lycopsamine and their N-oxides) (Figure 1).

Due to medium to rather polarity of PAs/PANOs, the new gradient had to start with higher aqueous proportions in the mobile phase (5% B). After several changes of the gradient, with the aim of understanding the crucial points for a better separation, an isocratic step of 1 min was included at the beginning, increasing the solvent B to 15% after 4 min and to 35% after 12 min. An isocratic step was included for 3 min (12.5 min – 15.5 min), to allows the elution of last PA/PANO compounds. Thus, a steeper increase in the organic concentration was applied to improve the coelution and shorten the total run time, as suggested also by Kaltner F. et al [29]. This optimization of the gradient improved the separation efficiency, resulted in a baseline separation of the coelution isomers intermedine (6.47 min) and lycopsamine (6.70 min) and their No, intermedine No (7.58 min) and lycopsamine No (7.83 min). Senecivernine (10.54 min) and sencionine (10.84 min) with their relative N-oxide, although partially separated with the old gradient, now benefit from better separation and peak shape (Figure 1).

Finally, both columns were evaluated using the new gradient and better separation was achieved with the BEH C18 column (Waters) which led to more sensitive signals and better peak shapes.

Under the final conditions, the 21 PAs/PANOs recommended by EFSA plus 2 TAs were well-separated in a run time of 16 min and an equilibration time of 6 min, with a 4 min decrease respect to the older one (16 min vs 20 min).

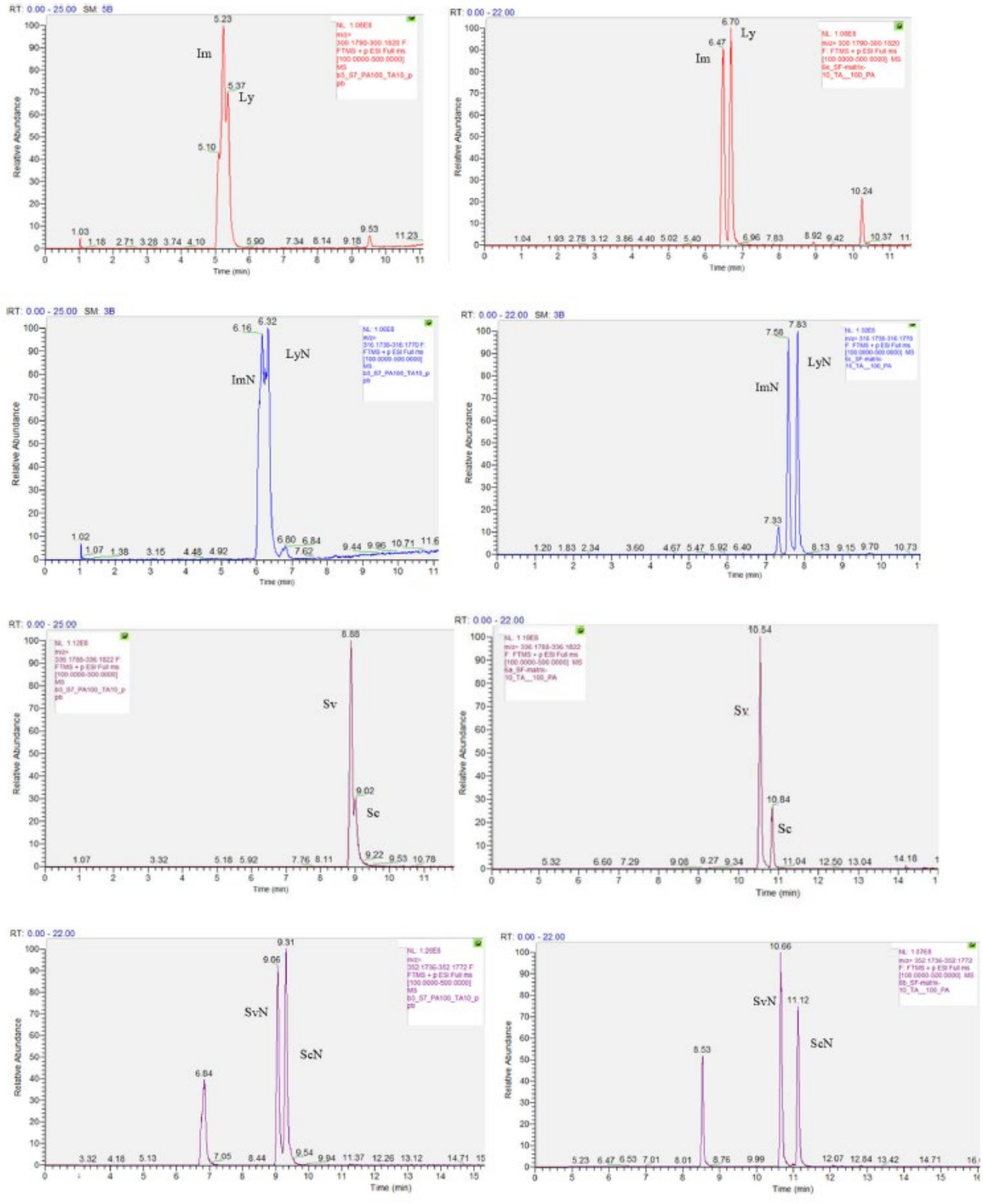

FIGURE 1 Comparison of chromatograms of the matrix-matched calibration curve (10 µg/kg_TA + 100 µg/kg_PAs) for the co-eluting compounds with improved separation from the old (left) to the new (right) gradient for: (Im) intermedine and (Ly) lycopsamine, (ImN) intermedine NO and (LyN) lycopsamine NO, (Sv) senecivernine and (Sc) sencionine, (SvN) senecivernine NO, and (ScN) sencionine NO.

3.1.2 Optimization of the sample extraction and clean-up

Due to the particularity of the target compounds and the complexity of the matrices used, as part of method development, the extraction phase and the dispersive stage of the QuEChERS were tested.

Considering the medium-high polarity of these compounds, according to the literature, they were SLE-extracted (solid-liquid extraction) with polar solvents such as acidified methanol with formic acid or acetic acid, but the use of aqueous solvents, such as dilute acid aqueous solution, was preferred [22,25]. This could be explained by the nitrogen heteroatom of alkaloids that is protonated at low pH, becoming more water-soluble due to solvent acidification [4].

The extraction stage was performed based on previously published protocols. Different ratios of acetonitrile and water were tested by N. Leon et al. [34] and the best results were obtained with a mixture of acetonitrile:water:formic acid (75:25:0.5, v/v/v). This phase was compared with that of Dzuman et al. [24], which exploited a mixture of methanol:water:formic acid (60:40:0.4, v/v/v). In our case, the latter extraction solvent with prolonged extraction time, allows to obtain best results with more sensitive signals.

Once PAs/PANOs/TAs have been extracted by SLE, the acidic aqueous supernatant has been often purified exploiting SPE (solid-phase extraction) to clean-up and analyte enrichment [22,25]. Regarding QuEChERS procedure, a protocol published by M. Martinello et al. [33] with the Q-sep extraction salt EN method (magnesium sulfate 4g, trisodium citrate dehydrate 1g, disodium hydrogen citrate sesquihydrate 0.5g and sodium chloride 1g) and purification Q-sep dSPE (PSA 0.15 g and magnesium sulfate 0.9 g) was compared with a single-step QuEChERS (150 mg MgSO4 + 25 mg PSA). The results were both satisfactory with limited differences in terms of recoveries and removal of contaminants (data not shown), for this reason the faster and easy to use solution was selected.

3.2 Method Validation

The final method was validated using representative matrix-samples (buckwheat and oregano) from two commodity groups (cereals and spices) for the two classes of Alkaloids.

3.2.1 Linearity and Matrix effect

Linearity was studied through matrix-matched calibration curves, spiked at six concentration levels, processed in duplicate and injected in triplicate. The linear range of the method was 0.25 – 10 µg/Kg for TAs and 0.5 – 100 µg/Kg for PAs across the different sample types. Good linear regression for all analytes was achieved, obtaining coefficient of determination (R^2) values ≥ 0.998.

The precise quantitation of all TAs and PAs in these complex matrices can be affected by matrix effects in ESI, responsible of enhancement or suppression on the ion signal intensity. Differences in the degree of matrix effects can be expected between different samples such as buckwheat and oregano as well as between samples of the same type. In case of more than 20% signal suppression or enhancement, ME need to be addressed in calibration [35].

The ME here evaluated ranged between – 44.3 to + 14.9 (see Table 2). As it can be observed, the majority of the analytes are affected by signal suppression despite the addition of the QuEChERS clean-up step, and therefore matrix-matched calibration was used for quantitation purpose.

Alternatively, the quantification of the target analytes could be performed by the standard addition approach [29, 30, 34]. In this case results could be corrected for recovery, but it implies a greater consumption of standards and as Kaltner et al. [29] suggest, isotopically labelled internal standards can safeguard for the induvial losses sustained during sample extraction but generally, they are very expensive and not commercially available for most of the PA/PANOs, therefore not applicable for multi-toxin determination.

3.2.2 **LOD and LOQ**

For TAs, the obtained LOD was 0.1 μg Kg^{-1} and the LOQ was 0.4 μg Kg^{-1} , while for PAs, the LODs values ranging between 0.2 to 0.3 μg Kg^{-1} and the LOQ, between 0.4 to 0.8 μg Kg^{-1} (Table 2). These values are fully in line with the Commission Recommendation (EU) 2015/976 [41] that underlines the LOQ for atropine and scopolamine should be preferably below 5 μg/kg and not higher than 10 μg/kg for agricultural raw materials (buckwheat falls in these categories), ingredients, food supplements and herbal teas.

For comparison with other QuEChERS-HPLC-HRMS, T. Bessaire et al. [2] reported a LOQ of 1 μg Kg^{-1}, N. Leon et al. [34] a LOQ of 5 μg Kg^{-1}, M. Martinello et al. [33] LOQ values from 0.1 to 0.6 μg Kg^{-1}, Z. Dzuman et al. [42] LOQ values ≤ 10 μg Kg^{-1} only for PAs and A. Romera-Torres et al. [40] LOQ value at 20 μg Kg^{-1} only for TAs.

3.2.3 **Recovery and precision**

The recoveries of the analytes were measured by spiking blank samples (buckwheat and oregano), previously checked for the presence of the target analytes, with TAs and PAs standards at two different concentrations, 2.5 and 10 ug/kg and 15 and 100 ug/kg, respectively. Samples were extracted according to the method described above. The percentage of recovery was given by the calculated amount of the spiking minus the calculated amount of the blank sample divided by the theoretical spiked concentration. Results (Table 2), expressed as mean percentage recovery of the four validation sessions, shows good recoveries values for all the analytes ranging from 73 to 111%, in line with the range 70-120% of the recommendation [35].

The precision of the proposed method (expressed as relative standard deviation percentage, RSD %) was evaluated in terms of repeatability (intraday precision) at the same validation levels as the accuracy. Intraday precision was assessed by performing six consecutive injections on the same day of the samples spiked at each concentration level tested. According to the CEN document and SANTE guideline, repeatability RSD_r should be ≤ 20% for each spike level tested. Satisfactory results were obtained at the concentration level evaluated, as the RSD_r were between 1 – 13% (Table 2).

TABLE 2 (a) Validation data for tropane alkaloids in buckwheat samples measured at two spiking levels (2.5 and 10 µg/kg). (b) Validation data for pyrrolizidine alkaloids in oregano samples measured at two spiking levels (15 and 100 µg/kg). Average recovery rates and repeatability measure (expressed as relative standard deviation, RSD_r) at two spiking levels with $n = 6$ replicates each LOD and LOQ, linearity (expressed as coefficient of correlation, R^2) and matrix effect.

(a)

	Level 2.5 µg/kg		Level 10 µg/kg					
Analyte	%Recovery	RSDr %	%Recovery	RSDr %	R^2	LOD µg/kg	LOQ µg/kg	ME %
Atropine	85	7	88	5	0.999	0.1	0.4	−7.4
Scopolamine	87	9	90	9	0.999	0.1	0.4	−10.7

(b)

	Level 15 µg/kg		Level 100 µg/kg					
Analyte	%Recovery	RSDr %	%Recovery	RSDr %	R^2	LOD µg/kg	LOQ µg/kg	ME %
Europine	99	4	99	2	0.999	0.2	0.5	9.4
Intermedine	87	7	88	5	0.999	0.2	0.5	−26.1
Lycopsamine	75	4	77	6	0.999	0.2	0.6	−25.6
Europine NO	90	9	91	2	0.999	0.3	0.7	13.5
Intermedine NO	90	7	95	1	0.999	0.2	0.5	−28.7
Lycopsamine NO	89	8	94	2	0.999	0.2	0.6	−16.1
Retrorsine	90	4	96	6	0.999	0.2	0.4	−20.7
Retrorsine NO	93	4	111	2	0.999	0.2	0.5	14.9
Seneciphylline	92	2	96	1	0.999	0.2	0.5	−28.6
Heliotrine	99	6	101	1	0.999	0.2	0.4	−21.8
Seneciphylline NO	97	3	103	2	0.999	0.2	0.5	−20.7
Heliotrine NO	99	3	99	2	0.999	0.2	0.4	−16.5
Senecivernine	91	5	92	2	0.999	0.2	0.5	−43.6
Senecionine	80	4	85	3	0.999	0.2	0.5	−44.3
Senecivernine NO	80	13	85	1	0.999	0.2	0.5	−21.1
Senecionine NO	88	3	96	1	0.999	0.2	0.5	−26.2
Echimidine NO	92	5	98	2	0.999	0.2	0.4	−8.4
Echimidine	83	6	89	3	0.999	0.2	0.4	−16.8
Senkirkin	85	4	93	3	0.999	0.2	0.4	−23
Lasiocarpine	73	10	78	1	0.999	0.3	0.8	−19.5
Lasiocarpine NO	74	4	84	4	0.999	0.3	0.8	−16.1

3.3 Occurrence data of TAs and PAs in matrices of interest coming from harvesting campaigns and suppliers.

As already mentioned, the strategy of this method aimed to identify in a simultaneous way the presence of both TAs and PAs in cereals and spices, the matrices of interest for the company.

Results underline that the presence of TAs in the survey samples (Paragraph 2.2) such as: wheat (n =15), buckwheat (n =12) and maize (n =20) remain below the limit of quantification (LOQ). Interestingly, for PAs, results showed that oregano samples (n =2) are below the LOQ, while for rosemary (n =1), retrorsine N-oxide, senecionine N-oxide and seneciphylline N-oxide were detected with a concentration of 7.9 µg/Kg, 1.9 µg/Kg and 16.4 µg/Kg, respectively.

3.4 Method's Applicability

To evaluate the applicability of the method, two certified reference materials (FAPAS) and different commercial samples purchased at the supermarket, maize (*n* =3), buckwheat (*n* =2), oregano (*n* =4) and rosemary (*n* =4), were analysed in duplicate for the presence of the target analytes.

For identification of PAs/PANOs/TAs in these samples, the Document SANTE guidelines were followed [35]. So, samples which met all the requirements and with levels of Alkaloids exceeding the LOQ_s, were considered positive. For quantification, the matrix-matched calibration curve obtained during the method development was used. In cereals sample of the QC, two Tropane Alkaloids were successfully identified with 3 product ions detected (Table 3). Moreover, we decided to participate in a proficiency testing (PT) in order to test the quality of our method and increase confidence in the accuracy of our testing regime. In the Ispaghula Husk of the PT, the six Pyrrolizidine Alkaloids were well-identified, although most concentrations are far lower than the legal limit (1000 µg/Kg). We were able to identify a trace of lasiocarpine in the PT, but it was not possible to quantify it, since its concentration resulted comprised between our calculated values of LOD and LOQ (Tables 2 and 3).

Maize and buckwheat were found free of the two targeted TAs (< LOQ). The absence of atropine and scopolamine in these cereals samples is thus aligned with the results obtained from samples collected from suppliers. Atropine and scopolamine were not detected in any of the cereals analysed in this study, except in the FAPAS certified sample. On the contrary, for PAs, oregano and rosemary samples from the supermarket revealed some positive results. The highest concentrations were detected in one sample of oregano for europine and its relative N-oxide, lycopsamine No, lasiocarpine and senkirkin, with an amount of 725.1 and 206 µg/Kg, 437 µg/Kg, 71 µg/Kg and 17 µg/Kg, respectively. Chromatogram of this naturally contaminate sample is shown in Figure 2.

These types of PAs, according to the literature, are the most frequent in oregano samples and their occurrence indicate the possible contamination with plants of the families Heliotropiaceae and Boraginaceae, or possible adulteration [29,31,43].

Moreover, also one sample of rosemary presented positive values for retrorsine 19 µg/Kg, retrosine No 12.8 µg/Kg, seneciphylline No 17 µg/Kg, senecionine 4 µg/Kg and senecivernine No 7.2 µg/Kg. The occurrence of these types of PAs could be associated to the species of the Asteraceae family, such as Senecio vulgaris [29,31]. As we can notice, these PAs are recurrent in rosemary species as they have also been found in rosemary from suppliers (see Paragraph 3.3).

In general, this type of contamination seems to highlight the diversity of botanical species that could contaminate the spices. Moreover, Izacara et al. [31] underlined that the contamination of oregano with PAs/PANOs is not only due to the accidental exposure of PA-producing plants or adulteration but also due to horizontal natural transfer of them through the soil [44]. Considering this and considering that the presence of PAs is a spot-contamination (to a certain extent comparable with the presence of certain mycotoxins), reliable sampling methods can be challenging. The "Guidelines and recommendations to reduce the presence

of Pyrrolizidine Alkaloids in food supplements" [18] underline again that the detection of PA in dried botanicals is hampered by the inhomogeneous nature of the contamination. For that reason, it is interesting to note that during the repeated analyses on the same sample batch, there were different results for Pyrrolizidine Alkaloids, probably due to a not homogeneous distribution of the toxins within the sample matrix, even after an accurate sample homogenization before the extraction. Of course, to achieve a representative test result, suitable sampling is of the utmost importance, but it is not always easy, especially for samples with small packaging from the supermarket.

Due to the small number of samples, the results of our survey could not be considered as representative for the risk evaluation assessment for cereals and spices, as underline also Kaltner F. et al. [29] in its study on tea samples. However, the results obtained and some evidence in literature, allows to confirm the predominant contamination by these types of PAs: europine, heliotrine, lasiocarpine, retrosine, senecionine, seneciphylline and senecivernine with their N-oxides in oregano and rosemary. Therefore, it's extremely importance to monitor their presence in these matrices due to the potential health risk that they can cause and also, try to establish maximum levels for them in order to guarantee the safety of the people.

TABLE 3 (a) Comparison between values for TAs in FAPAS QC material and values obtained with our method. (b) Comparison between values for PAs in FAPAS PT and values obtained with our method.

(a)

Analyte	Assigned value, X_a	Range for $\|z\| \leq 2$[a]	Internal value	Units
Atropine	1.44	0.81–2.08	1.40	µg/kg
Scopolamine	1.98	1.11–2.85	2.01	µg/kg

(b)

Analyte	Assigned value, X_a	Range for $\|z\| \leq 2$[a]	Internal value	Units
Europine	1.37	0.764–1.97	1.39	µg/kg
Europine NO	16.2	9.05–23.3	19.3	µg/kg
Heliotrine	7.42	4.16–10.7	8.10	µg/kg
Heliotrine NO	82.6	46.3–11.9	85.3	µg/kg
Lasiocarpine	1.12	0.627–1.61	<LOQ	µg/kg
Lasiocarpine NO	12.0	6.72–17.3	14.0	µg/kg

[a]The range for $|z| \leq 2$ is the concentration range within the limits of ±2 z scores. The assigned value and its range has been established from the proficiency test data and are suitable for use by laboratories as a fit-for-purpose quality control measure.

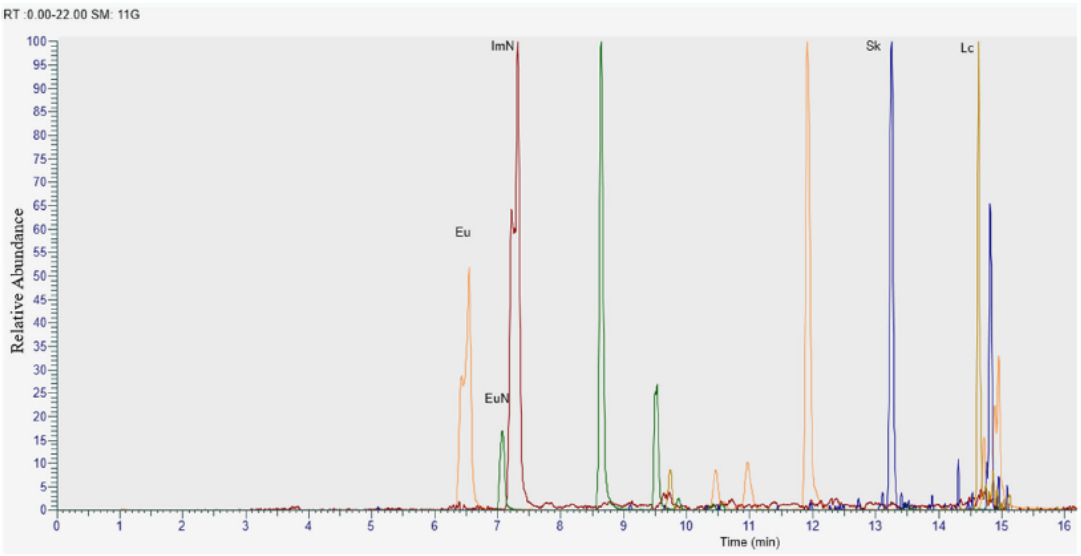

FIGURE 2 UHPLC-HRMS chromatograms of naturally contaminated oregano sample with five PAs: (Eu) europine, (EuN) europine NO, (ImN) intermedine NO, (Sk) senkirkin, (Lc) lasiocarpine.

4 | CONCLUSION

The growing consumption of plant-based products as an alternative to animal products, puts the emphasis on ensuring the food safety of plant-based ingredients before they enter in the food chain, highlighting the need for robust and reliable analytical methodologies for safety analysis.

The present study allows the development of an UHPLC-HRMS method suitable for the simultaneous detection of 2 TAs and 21 PAs, and it represents the first opportunity in scientific literature, to evaluate the applicability on all these relevant matrixes: cereals and spices.

Validation data successfully demonstrated the robustness of the method addressing the industrial needs to comply with the current European legislation regarding Tropane and Pyrrolizidine Alkaloids.

The presence of a verified matrix effect and the uneven distribution of these Alkaloids will still be investigated in further future research works.

In addition, in the near future, more data focusing on their behaviour during processing and/or storage with the possible formation of transformation products, will be needed for a complete and reliable risk assessment.

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2

Chapter

Research Article

Ergot alkaloids: comparison of extraction efficiencies for their monitoring in several cereal-solvent combinations by UPLC-MS/MS

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Abstract

To date, there are more than 80 ergot alkaloids identified, their distribution depends on different factors (e.g. geographic regions, host plants. etc.). These toxins can cause acute and chronic toxic effect on human health and commonly infect cereal crops such as triticale and rye, wheat, barley, oats. Considering the growing consumption of plant-based foods, the European Food Safety Authority has highlighted the need to develop risk assessment strategies.

This work focused on the optimization of extraction efficiency, to quantify the main ergot alkaloids and their epimers, that are available on the market without any legal restriction (ergosine, ergocristine, ergocriptyne, ergocornine, ergosinine, ergocristinine, ergocriptinine and ergocorninine). Considering the quantification of 8 out of 12 regulated compounds by EU (sum of –ine and –inine forms), this approach can be defined as a screening method for a reliable estimation of the risk, specifically devoted to industrial stakeholders that can then possibly outsource to authorized external labs only the samples suspected of significant positivity.

The effectiveness of three different extraction conditions (acidic, alkaline and neutral) followed by a rapid clean-up using dispersive solid-phase extraction with C_{18} sorbent was evaluated by ultra performance liquid chromatography tandem quadrupole mass spectrometry (UPLC-MS/MS), resulting in a short chromatographic run (16 min). The method was developed and validated in five different cereal production chains (rye, oat, wheat, wheat gluten and baby food). The applicability of the method was examined by analysing a set of 54 samples, including also other cereals like spelt, tritordeum and triticale, evaluating also some reference materials.

KEYWORDS

Ergot alkaloids; Epimers; Extraction procedures; Liquid chromatography tandem quadrupole-mass spectrometry; Cereals; Baby food

1 | INTRODUCTION

One of the most notable fungal infections is *Claviceps purpurea* from the *Claviceps* genus. The ascospores from the fungus *Claviceps purpurea* contaminate the seeds heads of living plants at flowering time with the development of a sclerotia, also known as ergot body, which replaces the seed of the crops (Krska and Crews 2008). Ergot sclerotia contain ergot alkaloids (EAs), which are secondary metabolites since they are produced by the fungi but not involved in their normal physiological processes. They are a group of nitrogenous organic compounds (Komarova and Tolkachev 2001), concentrated on the outer edge of the sclerotia, with lower concentrations in the interior (Young et al. 1983).

The most susceptible crops to ergot are cross-pollinators such as triticale and rye, however, also wheat, barley, oats and millet can also become infected. Environmental conditions (e.g. temperature, humidity, fungal concentrations and nutrients) promote ergot infection in crops and grasses, especially seasons with heavy rainfall and wet soils (Agriopoulou 2021). The sclerotia can be harvested together with cereals or grass and thus, they can contaminate cereal-based food and feed products with EAs, whom ingestion can cause ergotism in humans and animals, in addition to acute and chronic effect (Krska and Crews 2008). Ergot alkaloid intoxication is still an issue in developing countries and remains a concern for livestock (Schummer et al. 2020).

The control of EAs in cereals should be based on the pre-harvesting prevention strategies, like the Good Agricultural Practices (GAP) but these are not always feasible, for this reason post-harvesting strategies should be applied, like an integrated system management approach which includes all the individual control strategies that are important to mitigate EAs in cereals and cereal-based foods (Agriopoulou 2021).

EAs are characterized by the presence of tetracyclic ergoline rings methylated on the N-6 nitrogen atom, substituted on C-8 and with a double bond in the C8-C9 or C9-C10 positions. They can be classified into 3 main classes: short chain substituted amides of lysergic acid, clavine alkaloids, and ergopeptines, which are peptide alkaloids with an additional peptide half attached to the basic tetracyclic ring (Fig. 1) (Komarova and Tolkachev 2001; Krska and Crews 2008; EFSA 2012; Crews 2015).

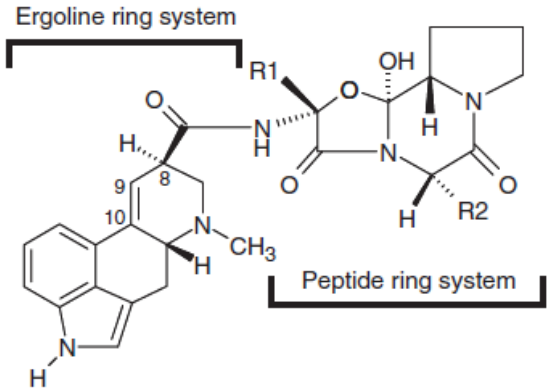


Fig. 1 Structure of ergopeptines with an additional peptide moiety attached to the basic tetracyclic ring (Krska and Crews 2008). The chiral carbon atom C-8 is responsible for epimerization.

There are six main ergot alkaloids produced by *Claviceps species* in the sclerotia, named ergometrine, ergotamine, ergosine, ergocristine, ergocryptine (α- and β-isomers) and ergocornine. These EAs are classified as ergopeptines, apart from ergometrine which is classified as a simple lysergic acid derivative. Each ergot sclerotia can contain a variable concentration of ergot alkaloids (Carbonell-Rozas et al. 2022). The total ergot alkaloid concentrations can vary from 0.01 to 1.04% in a single ergot body (Ruhland and Tischler 2008). The percentages of the six common ergot alkaloids within an ergot body depends on type of crop, fungi, individual ergot bodies, year, and geographical location (Grusie et al. 2017; Kodisch et al. 2020).

Ergot alkaloids have multiple chiral centers, the double bond at C9–C10 allows epimerisation at the carbon 8 chiral center forming C8-(*R*) isomers with right-hand rotation, which are known as ergopept-ines, and C8-(*S*) isomers with left-hand rotation, which are known as ergopept-inines (Komarova and Tolkachev 2001; Krska and Crews 2008). The N6 gives the protonated EA pKa values of 5.0 to 7.4, they have a positive charge in acid solution and are neutral in alkali (Crews 2015). The *R* and *S*-epimers are interconvertible and pass through an intermediate configuration during their energetic conversion, that can occur under various conditions. Cherewyk et al. (2022a) underlined that the *R*-epimer can epimerize to the *S*-epimer under aprotic (e.g., acetonitrile, acetone, chloroform) and protic solvents (e.g., methanol or water:methanol mix), at room temperature over days, at 37 °C in several matrices over hours, and during the extraction process of cereals (Krska et al. 2008a). The *S*-epimer can convert to the *R*-epimer in organic solvent, water, or acidic solutions (Komarova and Tolkachev 2001). The literature reported that *R*-epimers are believed to have biological activity, while *S*-epimers to be inactive, however, recent studies have demonstrated potential bio activity of the -inine epimers which are likely to be harmful similar to the -ine epimers (Crews 2015; Cherewyk 2022a). Thus, the CONTAM panel of the European Food Safety Authority EFSA (2012) recommended to conduct risk assessments on both forms (-ine and -inine), and to consider also the ergot alkaloids concentration, and not the presence of ergot sclerotia alone (EFSA 2012).The European Commission has established a maximum level for ergot sclerotia and alkaloids in food (Reg. EU No 2023/915) considering the sum of the six common *R* and *S*-epimers (EU 2023). The lowest concentration is based on vulnerable populations such as infants and children at 20 µg/kg total ergot alkaloids. From the 1st July 2024, a reduction for some categories of foods (barley, wheat, spelt, oats and rye), should guarantee an higher level of safety. To safeguard humans' health, EFSA reported a total daily intake (TDI) for ergot alkaloids of 0.6 µg/kg body weight per day, and an acute reference dose of 1 µg/kg body weight (EFSA 2012).

Determining EAs is of great importance and for this reason, a stable, efficient, and sensitive analytical method, able to determine and quantify them, should be developed (Chung 2021; Silva 2023; Cherewyk 2024). Considering the medium-high polarity of these compounds, according to the literature, the SLE (solid-liquid extraction) has mainly been used with non-polar organic solvents (like dichloromethane/ethylacetate/methanol/25 % aqueous ammonia) under alkaline conditions (Muller 2009; Bryła 2015) or with polar solvents (like methanol and acetonitrile) under acidic conditions (Mulder 2015; Rubert 2012; Versilovskis 2020). Krska et al. (2008b) compared different extraction solvent protocols and

highlight that acetonitrile mixtures with ammonium carbonate buffer (84:16, v/v) yielded the highest concentrations of EAs. Eight years later, Guo et al. (2016) exploited almost the same extraction solvent acetonitrile with ammonium carbonate buffer (85:15, v/v), comparing the matrix effect after three clean-up procedures: C_{18} sorbent, PSA sorbent and MCX cartridge, discovering that C_{18} sorbent cleaned the cereal matrix slightly better than PSA sorbent, and showed the best performance. Chung (2021) and Silva (2023), in their reviews, concluded that many methods have been developed for the determination and quantification of EAs, and those based on QuEChERS and dispersive-SLE (d-SPE) procedures, are the fastest, cheapest, and most efficient methods developed.

The EU Commission established maximum levels for the sum of the –ine and –inine forms. So far, some ergot alkaloids are available only with a specific permit typically obtained from specific government bodies, which takes time, paperwork and can come with restrictions and government inspections. The present study aims to develop a method able to quantify 4 ergot alkaloids (ergosine, ergocornine, α-ergocryptine and ergocristine) with their corresponding epimers (ergosinine, ergocorninine, α-ergocryptinine and ergocristinine): the ones available without any legal restriction on the market.

Considering the quantification of 8 out of 12 regulated compounds, this approach can be finally defined as a screening method for a reliable estimation of the risk, specifically devoted to industrial stakeholders that can then possibly outsource to authorized external labs only the samples suspected of significant content.

Artificially contaminated samples of rye, oat, wheat, wheat gluten and baby food were extracted comparing three different solvents (acidic, neutral and alkaline) and extraction procedures with different amounts of d-SPE sorbent, to find the best combination of high extraction efficiency and effective clean-up results. To the best of our knowledge, this is the first paper addressing the determination of ergot alkaloids in all these matrices together. After the development and in-house validation, the performance of this approach was evaluated using certified reference materials analysing different naturally contaminated samples, over a range of cereal production chains.

2 | MATERIALS AND METHODS

2.1 Reagents, standards and materials

All solvents were HPLC – MS grade. Acetonitrile was purchased from VWR International, Ltd (Poole, United Kingdom). Formic acid and ammonium carbonate were obtained from Sigma Aldrich (Darmstadt, Germany). For the preparation of the mobile phase and all sample preparations experiments, water was purified using a Milli-Q system (Millipore, Bedford, MA).

Certified reference standards of four ergot alkaloids, ergosine, ergocristine, ergocryptine, ergocornine and the corresponding epimers, ergosinine, ergocristinine, ergocriptinine and ergocorninine were purchased as
dried down films from Romer Labs (Tulln, Austria) with a chemical purity > 98%.

Dispersive Solid Phase Extraction C_{18} sorbent (Bondapak HC18 reference WAT035672), screw-capped amber glass vials (12 x 32 mm Screw Neck Qsert Vial, 300 uL, reference 186002803) and PTFE filters (wwPTFE Acrodisc Mini 0.2um 13mm, reference 186009314) were from Waters (Milford, MA. USA).

2.2 Extraction solvents

Three different extraction protocols were compared: (1) an alkaline mixture of acetonitrile – 3 mM ammonium carbonate buffer 85:15 (*v/v*), (2) an acidic extraction with acetonitrile – water 84:16 (*v/v*) containing 0.5 % formic acid and (3) a neutral aqueous extraction with acetonitrile – water, 84:16 (*v/v*). The pH of the ammonium carbonate in the extraction solvent was investigated in the range pH 7.5 – 10.

2.3 Samples

Samples for method development and validation:
Wheat samples were obtained from the 2023 harvesting campaign. Rye samples came from the Sweden suppliers while oat and wheat gluten samples came from different Italian suppliers. Baby food sample (blended flour of rye, oat and wheat) was purchased at the local supermarket.
Certified reference materials were obtained from FAPAS (FERA Science Ltd. York, UK) and were two proficiency tests (PT) for ergot alkaloids in rye (PT 22190 and PT22203) and a quality control material for ergot alkaloids in baby food (T22196QC).

Survey samples for method applicability:
A set of 54 naturally contaminated samples (from 2020 to 2022) coming from different suppliers, covering rye, wheat (common and durum wheat) and other cereals like spelt, tritordeum and triticale.

2.4 Sample preparation procedure and clean-up

Wheat and Oat

Finely ground representative samples of wheat and oat (2.00g ± 0.01 g) were weighed into 50-mL polypropylene centrifuge tube with 20 mL of the alkaline mixture acetonitrile – ammonium carbonate buffer (200 mg/L, 3 mmol; pH 8.5), 85:15 (*v/v*), followed by short vortex mixing 30s, mild shaking for 20 min and a centrifugation step of 5 min (5000 x *g*) at 4 °C. During the extraction the falcon tubes were covered with an aluminium foil. A total of 5 mL of the supernatant for each sample was transferred to a 50-mL centrifuge tube containing 300 ± 1 mg of C_{18} sorbent for dSPE clean-up and vortex-mixed at high speed for 1 min.

Wheat Gluten, Rye and baby food

Finely ground representative samples of wheat gluten, rye and baby food (2.00g ± 0.01 g) were weighed into 50-mL polypropylene centrifuge tube with 20 mL of the alkaline mixture acetonitrile – ammonium carbonate buffer (200 mg/L, 3 mmol; pH 8.5), 85:15 (*v/v*), followed by short vortex mixing 30s, mild shaking for 20 min and a centrifugation step of 5 min (5000 x *g*) at 4 °C. During the extraction the falcon tubes were covered with an aluminium foil. A total of 5 mL of the supernatant for each sample was transferred to a 50-mL centrifuge tube containing 150 ± 1 mg of C_{18} sorbent for dSPE clean-up and vortex-mixed at high speed for 1 min.

For all the matrices, the mixture of extraction solvent and dSPE was centrifuged for 5 min (5000 x *g*) at 4 °C, and then the upper layer was filtered through the PTFE filter and transferred into a screw-capped amber glass vial prior to injection of 5 µL into the UPLC – MS/MS system. The samples were analysed immediately after extraction otherwise they were evaporated to dryness under a stream of N_2 and reconstituted with the appropriate volumes of each extraction solutions.

2.5 Standard preparation for quantification

Following the indications of the manufacturer, the standards were reconstituted in 5 mL of acetonitrile, to achieve the concentrations of 100 µg mL^{-1} for the -ine epimers and 25 µg mL^{-1} for the inine-epimers. From these stock solutions, three working solutions containing all 8 ergot alkaloids were prepared in pure acetonitrile: (1) A first working solution at a concentration of 1 µg mL^{-1}, and this solution was used to prepare (2) a second working solution of 0.2 µg mL^{-1}, and this was used to prepare (3) a third working solution at 0.05 µg mL^{-1}.

During the analysis of the calibrants for quantification, we found that the best way of working was to prepare the standard freshly each time, or create aliquots in acetonitrile solvent and amber glass vials, evaporated to dryness under a gentle stream of N_2, stored at – 20°C and reconstituted just before use to avoid epimerization or possible degradation during storage.

2.5.1. Calibration

A nine-points matrix matched calibration curve was used to quantify the analytes (0.5, 2, 5, 10, 25, 50, 100, 200 and 500 µg/kg). To reach the desired concentrations, the calibration standards were freshly prepared by serial dilutions using blank sample extract (a multi-matrix blended flour composed of 1/3 rye, 1/3 oat and 1/3 wheat gluten) as diluent. A blank food material was chosen, in which, according to previous analysis, no ergot alkaloids were detected. The blank material was extracted with the three extraction solutions indicated above (see paragraph 2.2) in order to create three different matrix-matched curves and calculate the matrix effect for each of them. Matrix-matched curves were obtained by serial dilution of the calibration standard with different volumes of acetonitrile extract (for each extraction solutions) of a multi-matrix sample.

2.6 Epimerization of EAs during preparation

The stability of ergot alkaloids and their epimers was tested in rye after 30 min or overnight. Waiting 30 min before starting the extraction procedure allowed good recoveries to be obtained without any significant epimerization, while when the sample was left overnight, probably the epimerization occurred for all the 3 extraction procedures tested due to the fact that the decrease of -ines led to a corresponding increase of the -inines form. Sometimes the *S*-epimer may convert to the *R*-epimer in organic solvent, water, or acidic solutions (Komarova and Tolkachev 2001). According to these results, spiking samples should be prepared and analysed on the same day.

Furthermore, the analytical run time should be minimized to avoid possible epimerization during the analysis. In addition, it was better to cover the falcon tube with aluminium foil during the extraction step, avoiding prolonged exposure to light during the other preparation steps and, also centrifuging at 4 °C in order to minimize epimerization during the sample preparation procedure. In general, the stability and epimerization of the *R* and *S*-epimers may depend on the specific ergot alkaloid assessed (Schummer et al. 2020).

2.7 UPLC – MS/MS analysis

The analyses were performed on an ACQUITY I-Class UPLC system coupled to a Xevo-TQS micro tandem quadrupole mass spectrometer (both from Waters Corp., Manchester, UK). The separation column used was an ACQUITY BEH C_{18} column 2.1 x 150 mm, 1.7 µm (Waters Corp., Manchester, UK), heated at 40 °C. Mobile phase was 3 mM ammonium carbonate buffer (200 mg L^{-1} = 3 mmol; pH 8.5±0.3) in water (eluent A), and pure MS grade acetonitrile (eluent B) at a flow rate of 0.4 mL/min. The autosampler was set to 8°C (which was beneficial to control any epimerisation during a sample batch) and the injection volume was 5 µl. As the EAs are medium to rather polar compounds, the run started with higher aqueous proportions in the mobile phase (5% B). An isocratic step of 1 min was included at the beginning, increasing the solvent B to 45% after 2.5 min and to 95% after 9 min. Another isocratic step was included for 3 min (9.0 min – 12.0 min) and then, a steeper increase of the organic concentration was applied. The system was re-equilibrated with the initial composition for 4 minutes prior to the next injection, yielding a total run time of 16 min.

For mass spectrometric acquisition, multiple-reaction monitoring (MRM) was conducted in positive ESI mode. The source temperature was set to 150 °C and the desolvation gas temperature was 500 °C. The desolvation gas flow was held at 700 L/h, and the collision pressure (argon) was 4.3×10^{-3} mbar. The capillary and cone voltages were 2.5 kV and 30 V, respectively.

2.8 Data Management

Data were acquired using MassLynx (v4.2) and processed using TargetLynx XS Application Manager (v4.2). The selection of MRM transitions and optimization of critical parameters was performed by infusion of

individual solutions of each analyte. Table 1 summarizes conditions for all the MRM transitions including the retention times. The dwell time was set to 20 ms (12 points per peak) for each MRM transition.

Table 1 Retention times and mass spectrometric conditions for determination of 8 ergot alkaloids

Analyte	R_t (min)	Precursor ion $[M+H]^+$ (m/z)	Quantifier ion (m/z)	Collision energy 1 (eV)	Qualifier ion (m/z)	Collision energy 2 (eV)
Ergosine	4.57	548.2	223.1	31	268.1	25
Ergocornine	5.11	562.3	223.1	37	268.1	25
α-Ergocryptine	5.40	576.3	223.1	38	268.1	25
Ergocristine	5.48	610.3	223.1	35	268.1	26
Ergosininine	5.73	548.2	223.1	31	268.1	25
Ergocorninine	6.27	562.3	223.1	37	268.1	25
α-Ergocryptinine	6.61	576.3	223.1	38	305.2	30
Ergocristinine	6.74	610.3	223.1	35	268.1	26

2.9 Method Validation

The method was validated in-house determining the matrix effect (ME), linearity of the calibration, recovery rates, repeatability and both limits of detection (LOD) and quantification (LOQ).

Performance criteria from document Nº SANTE/11312/2021 (SANTE 2021) and document CEN/TR 16059:2010 on single laboratory validation of mycotoxin methods (CEN 20210), were used as references.

To evaluate the matrix effect, a comparison between the slope of the matrix-matched and the solvent calibration curves for the three-extraction solvents tested, was performed. Slope differences were calculated for each analyte applying the following equation (Eq. 1) (SANTE 2021):

(1)

$$ME\ (\%) = \left(\frac{\text{slope of the matrix} - \text{matched calibration curve}}{\text{slope of the solvent calibration curve}} - 1 \right) x\ 100$$

Negative values represented signal suppression while positive values indicated signal enhancement caused by the matrix interferences.

Peak area was used as analytical response and linearity, expressed as determination coefficients (R^2), was evaluated at the concentration range reported in paragraph 2.4.1.

Recovery data were performed using blank rye, oat, wheat, wheat gluten and baby food matrices artificially contaminated at three concentration levels: 2, 20 and 100 µg kg^{-1}, with three replicates each. These concentrations refer to each individual analyte in the sample. Blanks and spiked samples were then extracted and analysed as described in the previous section. The percentage of recovery was given by the calculated amount of the spiking minus the calculated amount of the blank sample divided by the theoretical spiked concentration.

The same dataset was used to calculate the method's repeatability, which was expressed as the relative standard deviation (RSD) of six replicates.

The Limits of Detection (LOD) and Quantification (LOQ) were determined as the analyte concentration corresponding to the mean of 3 and 10 standard deviations (SDs), respectively, of the response in a blank sample matrix.

The method's trueness was evaluated by analysing a quality control material for ergot alkaloids in baby food (T22196QC) and two proficiency tests (PT) for ergot alkaloids in rye (PT22190 and PT22203) provided by FAPAS (FERA Science Ltd. York, UK) in 2022-2023. Trueness was expressed as z-scores for the individual EAs compounds considered in the proficiency testing report. Participants' z-scores were calculated as (Eq.2):

(2)

$$z = \frac{(x - x_a)}{\sigma_p}$$

Where:

x	participant's reported results
x_a	assigned value
σ_p	standard deviation for proficiency

The number of z-scores for all the analytes in the range $-2 \leq |z| \leq 2$ were calculated.

2.10 Method applicability

The method's applicability was tested with a set of 54 naturally contaminated samples (harvesting season from 2020 to 2022) covering some validated matrices and other cereals like spelt, tritordeum and triticale. They were analysed in triplicate for the presence of the 8 EAs compounds covered in the current method.

3 | RESULTS AND DISCUSSION

3.1 Method Development

3.1.1 MS optimization

For the determination MRM transitions, single substance solutions of the 8 Ergot alkaloids were directly infused into the MS via a syringe pump and parameters (polarity, collision energy and cone voltage) were optimized for each toxin. Two MRM transitions with the best intensity and signal-to-noise (S/N) ratios in positive electrospray ionization (ESI+) were chosen for each toxin. The optimized mass transitions are summarized in Table 1.

3.1.2 Optimization of the chromatographic separation

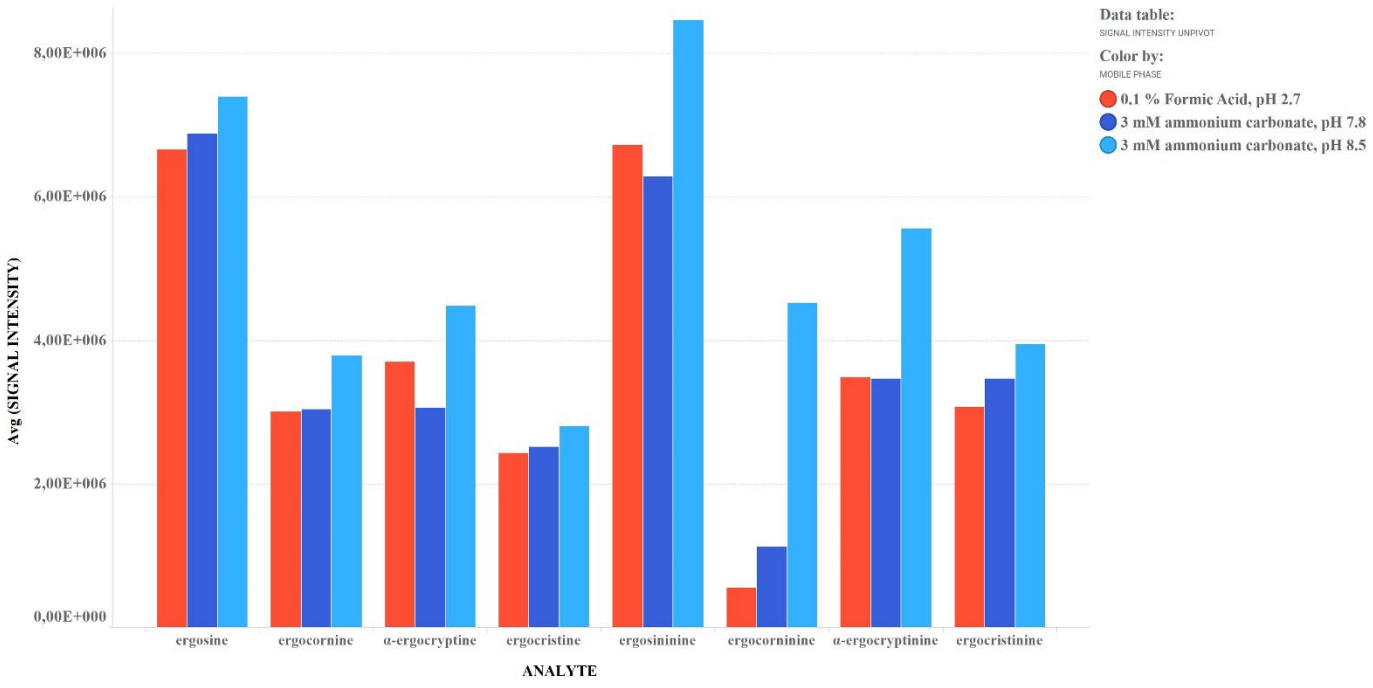
Different mobile phases and elution gradients were tested and optimised.

In literature, some studies underlined that for ergot alkaloids analysis on C_{18} columns, high pH conditions are required to achieve full separation. At the same time, there is considerable concern that using high pH could cause a decrease in sensitivity of mass-spectrometric detection under conditions that could suppress analyte ionization in solution. For that reason, acidic mobile phases are increasingly being used, such as the addition of volatile weak acids for enhancing the ionization of basic compounds in ESI+ (Rubert 2012; Carbonell- Rozas 2022; Liang 2022). Nevertheless, the methods exploiting acidic phases often are less suitable for the detection of both epimers (ines and inines), on the other hand several studies have reported the successful detection of EAs in electrospray positive mode when using high pH buffers in the mobile phase (Mulder 2015; Guo 2016; Veršilovskis 2020).

To optimize the chromatographic conditions and find the best solution for this study, we compared the analyte signal intensity of ergot alkaloids and their epimers, within a range of pKa values (4.8 ~ 6.2) (Krska et al. 2008b), in different pH mobile phases. For separation under acidic conditions, 0.1 % formic acid was added to water (Solvent A) and methanol (Solvent B). In the case of alkaline conditions, 3 mM ammonium carbonate buffers at two different pH (7.8; 8.5) (Solvent A) with acetonitrile (Solvent B) were tested. The pH was adjusted to 8.5 by adding 25% ammonia.

As shown in Figure 2, high pH mobile phases (blue) did not suppress the ionization of the compounds in ESI+; positive ions are formed abundantly, and analyte responses are often enhanced in high pH compared to acidic mobile phases (red).

Fig. 2 Comparison of LC-MS/MS responses of the standard at 10 µg/kg in acidic (red) and alkaline (blue) mobile phases, at different pH values

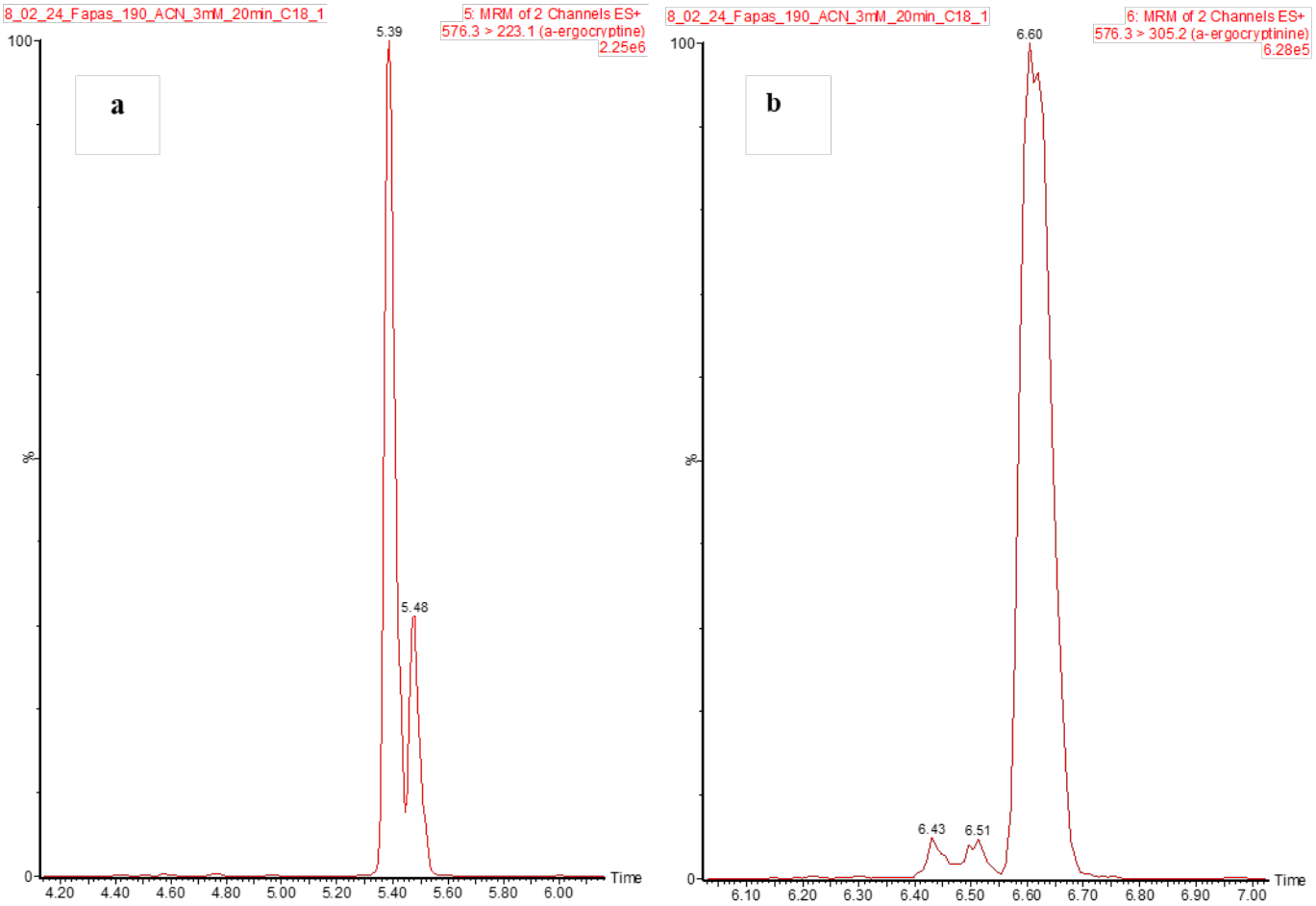


Waters BEH C_{18} column showed good stability for the alkaline conditions, 3 mM ammonium carbonate buffer, at the chosen pH 8.5, delivering the best compromise for more sensitive signals and better peak shapes (i.e., less tailing and higher S/N).

During the analysis of naturally contaminated samples (FAPAS) with the transition *m/z* 576 → 223 for α-ergocryptine (Rt = 5.40 min) an additional peak was detected at Rt = 5.48 min. We ascribed this peak to the presence of β-ergocryptine. On the other hand, α- and β-ergocryptinine isomers (m/z 576 → 305) cannot be separated by traditional liquid chromatography columns, they are isobaric by nature and cannot be distinguished by mass spectrometry unless ion mobility mass spectrometry is employed (Krska and Crews 2008; Chang 2021, Carbonell-Rozas 2022).

Figure 3 shows a chromatogram of this transition obtained from naturally contaminated rye flour, where, despite the close elution, there is a clear separation between α- and β-ergocryptine compounds, while the related isomers cannot be separated. Thus, the sum of α- and β-ergocryptinine is commonly monitored.

Fig. 3 Chromatogram of naturally contaminated rye flour (FAPAS PT 22190) with (a) good separation between α- and β-ergocryptine compounds and (b) no separation between α- and β-ergocryptinine isomers



3.1.3 Optimization of the sample extraction and clean-up

The selection of the right extraction procedure is critical to obtain satisfactory recovery of EAs. The main problem is that solvent, pH, temperature but also light exposure could induce epimerization at the C-8 position of these alkaloids (Komarova and Tolkachev 2001; Hafner et al. 2008; Krska et al. 2008a; Schummer et al. 2020), leading to different relative intensities of the -ine and -inine compounds and thus an incorrect relative quantification and risk assessment of them. The parameters influencing the recovery (extraction efficiency and matrix effect), were optimized.

Here, three different extraction protocols were compared: (1) an alkaline mixture of acetonitrile – ammonium carbonate buffer 85:15 (*v/v*), (2) an acidic extraction with acetonitrile – water 84:16 (*v/v*) containing 0.5 % formic acid and (3) a neutral aqueous extraction with acetonitrile – water, 84:16 (*v/v*). The pH of the ammonium carbonate in the extraction solvent was investigated in the range pH 7.5 – 10, finding that an increase in the extraction solvent buffer pH increased the overall extraction efficiency. However, increasing the pH also increased the epimerisation rate, for this reason, a pH of 8.5 was established as the best candidate.

Although fast extraction is commonly the goal for any separation procedure, we decided to compare two different extraction times (5 and 20 min) for each matrix. 5 minutes extraction time didn't allow proper recoveries for all the matrices tested, recoveries were very high, while, a combination of short vortex mixing 30s, mild shaking for 20 min and 5 min centrifugation at -4°C, was found to increase the efficiency of separation in our study.

In literature some of the methods exploited only SLE procedures leading to successful results, but co-extracted substances could induce matrix enhancement or suppression effects and shorten the lifetime of the column. For that reason, once EAs have been extracted by SLE, the aqueous supernatants of each different matrix, were purified through the dispersive SPE using C_{18} sorbent as a rapid one-step clean-up.

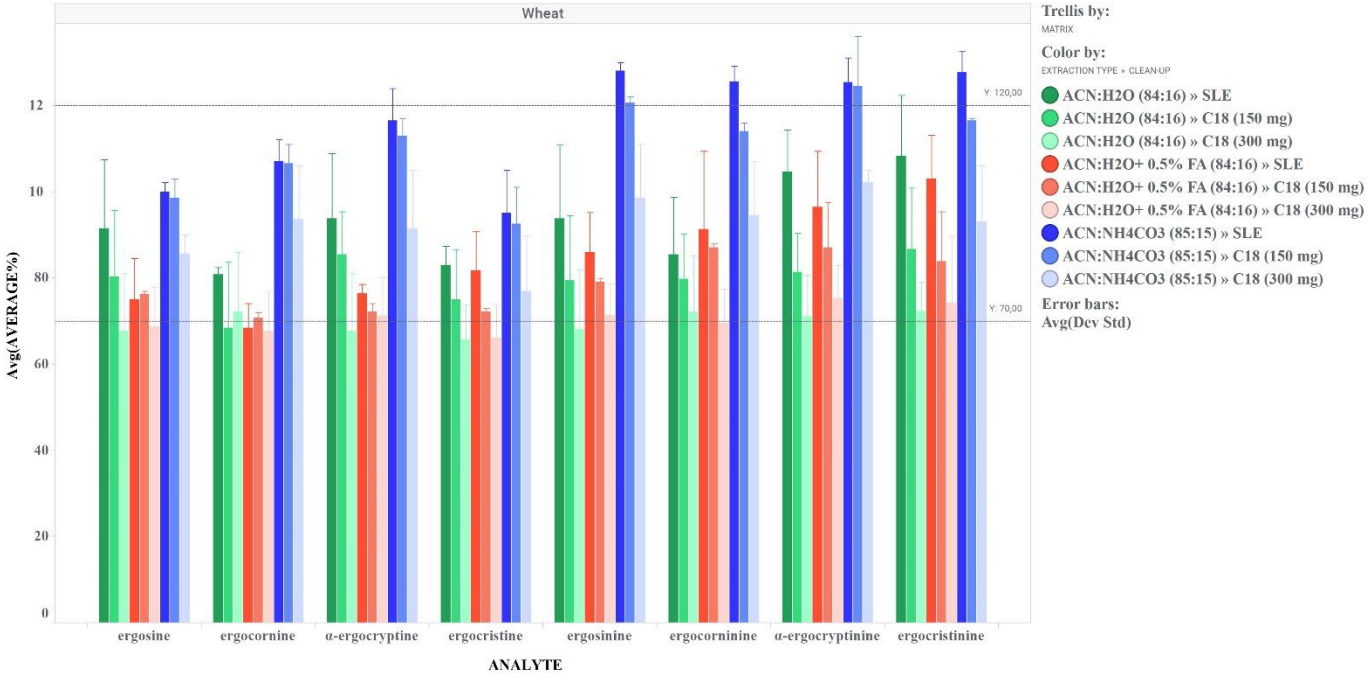
Guo et al. (2016) compared the spiked recovery of EAs in rye using purification by dispersive SPE with C_{18} sorbent, PSA sorbent and MycoSep 150 cartridge ergot column supplied by Romer Laboratories. Amongst these purification procedures, C_{18} cleaned the cereal matrix slightly better than the PSA sorbent and had also the best performance in terms of matrix effect.

Fig. (**4 – 8**) display the average recoveries (Y-axis) for each ergot alkaloid in every matrix tested, analysing the spiked samples at a medium-low level (20 µg/kg) in triplicates, using three different extraction solvents (*green* = neutral, *red* = acidic, *blue* = alkaline) and two different amounts of C_{18} sorbents (150 mg and 300 mg).

For wheat samples (**Fig. 4**), the acidic and neutral extraction with the SLE and the dSPE with 150mg of C_{18} sorbent, present acceptable recoveries (except for *ergocornine*), with an increasing trend for the epimers. Both extractions with the dSPE adding 300mg of C_{18}, showed worse recoveries.

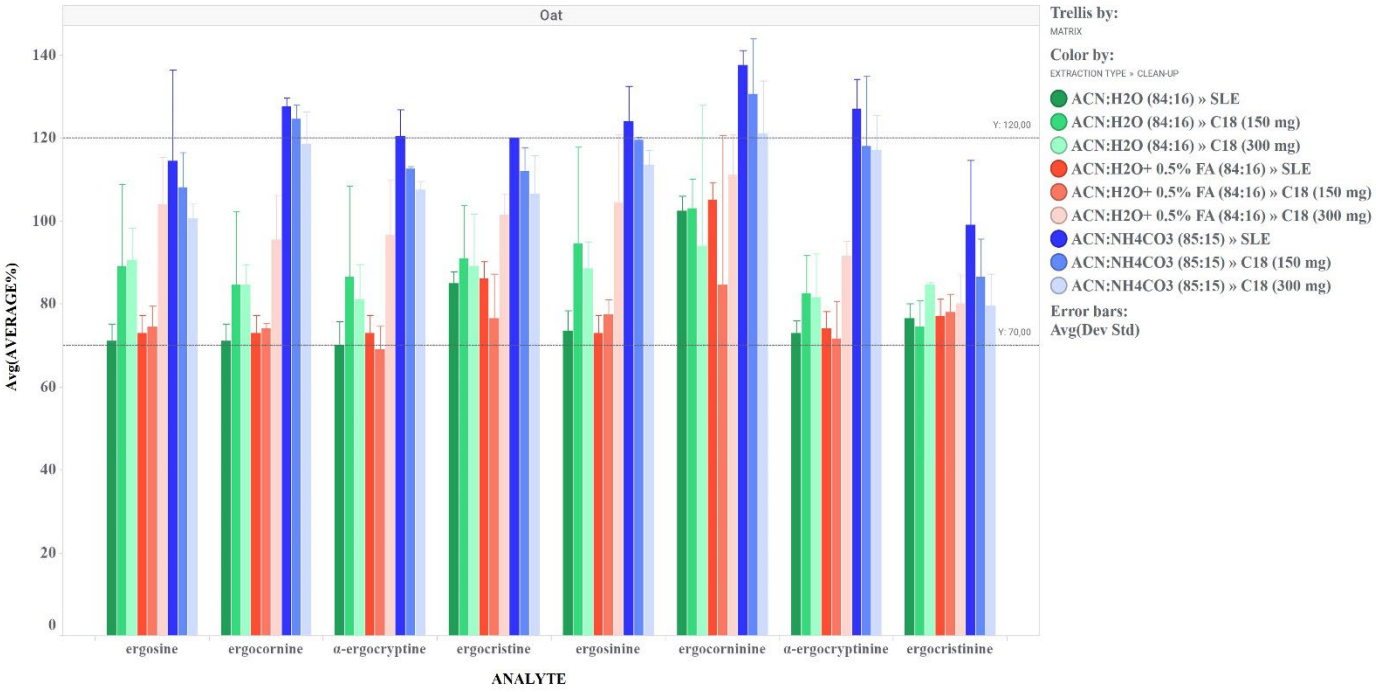
With the alkaline extraction, the SLE procedure leads to higher recoveries for the epimers; the dSPE, with 150mg of C_{18} sorbent, cleans the extract and improves the trend but it still has high recovery values for the analytes *ergosinine* and *a-ergocryptine* (> 120%). The best performance in terms of reproducibility and stability was obtained adding 300mg of C_{18} sorbent, yielding recoveries between 74 to 102%.

Fig. 4 Comparison of the three different extraction conditions (*green* = neutral, *red* = acidic, *blue* = alkaline) and two different amounts of C_{18} sorbents (150 mg and 300 mg), for each ergot alkaloids at the spiking level of 20 µg/kg in wheat sample



For oat samples (**Fig. 5**), good results (70 – 120 %) were obtained for all three-extraction procedures (except for the neutral SLE for some analytes) and this represents the versatility of the matrix. However, the extraction procedure with the overall best performance in terms of signal abundance and highest recoveries is the alkaline extraction, without SLE, which still shows too high recoveries, while cleaning the sample with 300 mg of C_{18} sorbent yields recoveries between 80 to 120%.

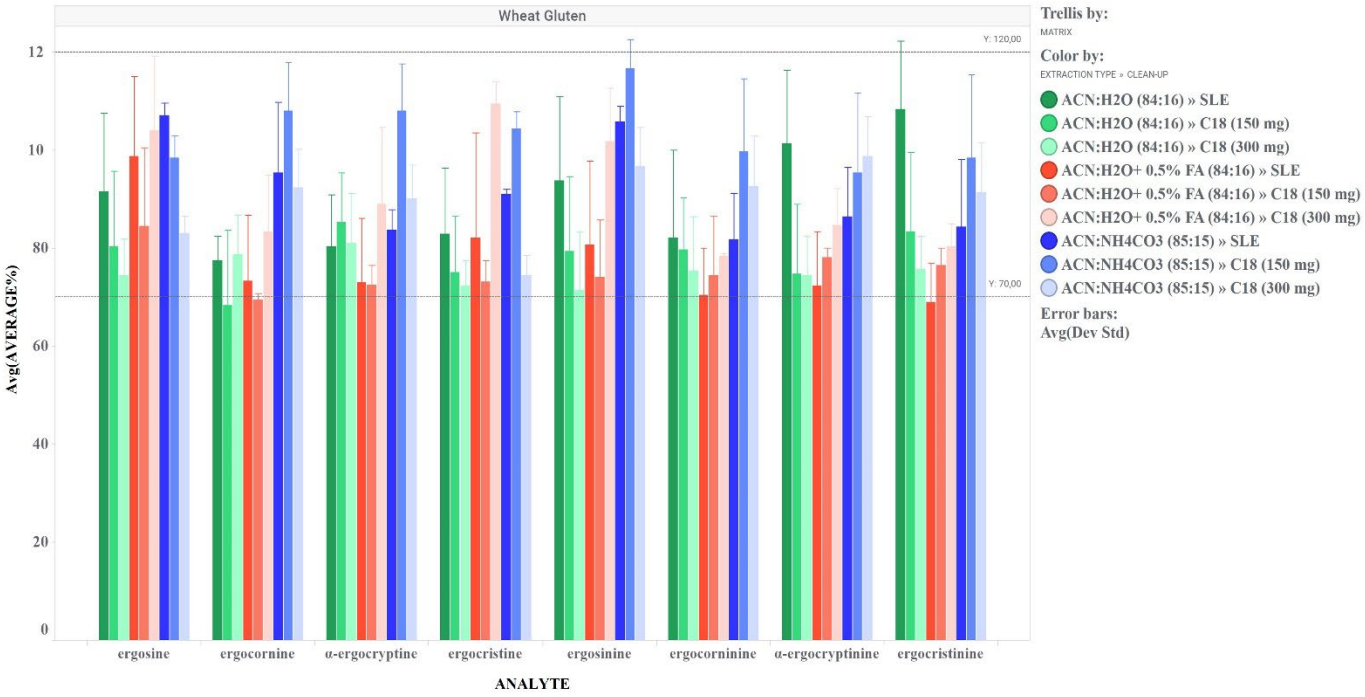
Fig. 5 Comparison of the three different extraction conditions (*green* = neutral, *red* = acidic, *blue* = alkaline) and two different amounts of C_{18} sorbents (150 mg and 300 mg), for each ergot alkaloids at the spiking level of 20 µg/kg in oat sample



Wheat gluten (**Fig. 6**) is a more complex matrix. The neutral procedure showed acceptable recoveries mainly with the SLE extraction, while cleaning the extract, recoveries decreased especially for the epimers. The acidic extraction with 300 mg of C_{18} sorbent yields good values and shows how the analytes suffer from ionic suppression caused by the matrix effect when the extract (SLE) is not cleaned.

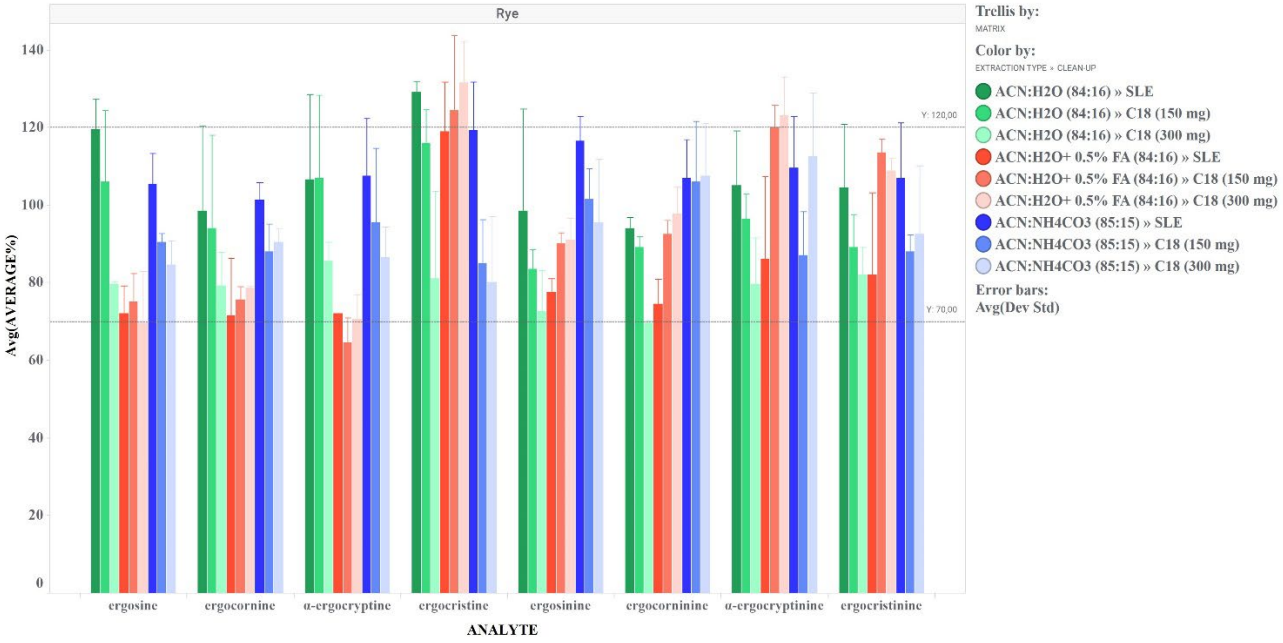
Comparing the acidic with the alkaline extraction, recoveries are even higher with basic extraction (150 mg) of C_{18} sorbent, while when using 300 mg of C_{18}, the values dropped slightly, especially for *ergocristine*. In general, the alkaline extraction adding 150 mg of C_{18} led to greater overall recoveries, however, it is fair to point out that even an acid extraction with 300 mg C18 yielded good values, especially for *ergosine* and *ergocristine*.

Fig. 6 Comparison of the three different extraction conditions (*green* = neutral, *red* = acidic, *blue* = alkaline) and two different amounts of C_{18} sorbents (150 mg and 300 mg), for each ergot alkaloids at the spiking level of 20 µg/kg in wheat gluten sample



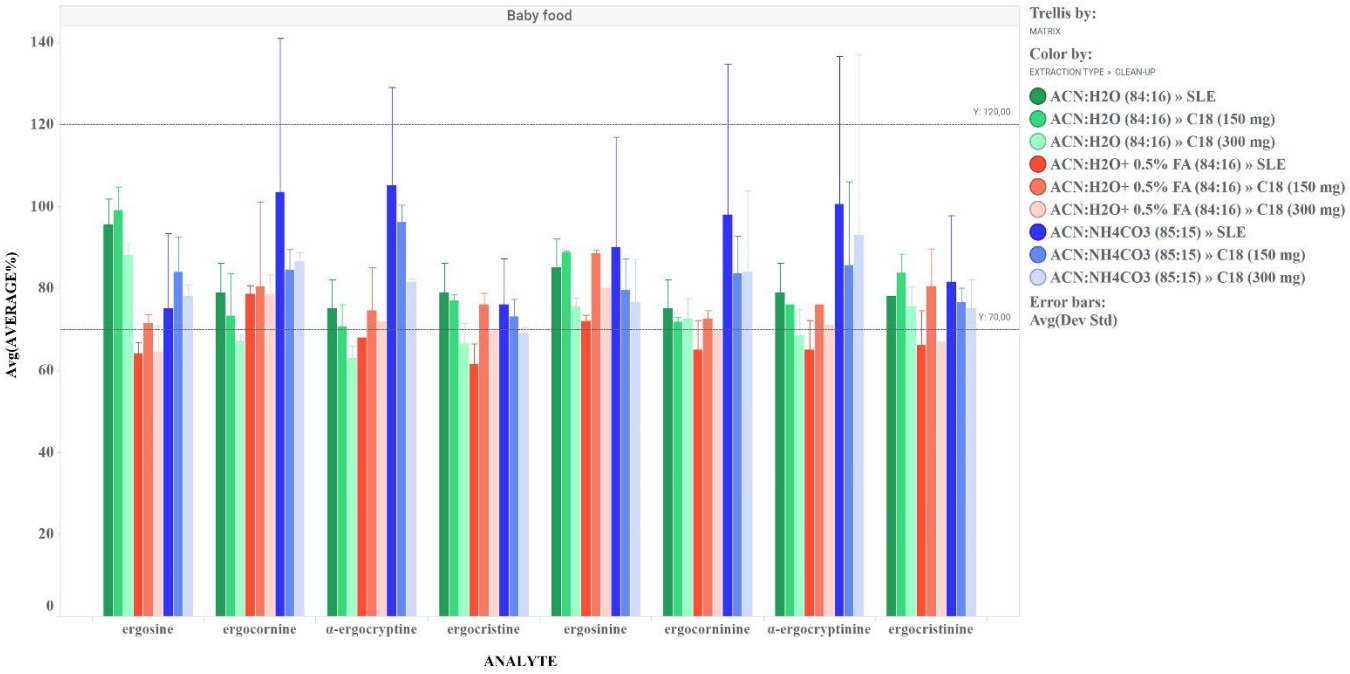
For rye samples (**Fig. 7**), the neutral extraction with the SLE and the dSPE with 150 mg of C_{18} sorbent, presented higher recoveries compared to 300 mg, that cleaned the samples more by lowering the recoveries, especially for ergocornine. The acidic extraction is critical for the main EAs, while for *ergocristine* and α-*ergocryptinine* it yielded the highest recoveries (> 120%). With the alkaline extraction, the cleaning of the extract again showed better recoveries as can be observed from the differences between the SLE and the dSPE procedure. Comparing the latter's performance at 150 or 300 mg, the trend is quite similar, and both led to good results, which is why it was decided to continue with the 150mg approach (85 – 106 %) without increasing to 300mg.

Fig. 7 Comparison of the three different extraction conditions (*green* = neutral, *red* = acidic, *blue* = alkaline) and two different amounts of C_{18} sorbents (150 mg and 300 mg), for each ergot alkaloids at the spiking level of 20 µg/kg in rye sample



For the baby food sample (**Fig. 8**), acid extraction did not perform very well, and of the three procedures tested, the one with 150 mg of C_{18} sorbent delivered acceptable recoveries, but slightly above 70%. Similar but slightly higher results, especially for *ergosine* and its epimer, and for *ergocristinine*, were obtained with the neutral extraction with 150 mg of C_{18} sorbent. With alkaline extraction, the SLE procedure showed good recoveries, but the standard deviation trend was rather high, which is probably due to the multi-matrix composition (mixed rye, oat and wheat flour) of the baby food, which didn't allow reproducibility in the measurement without cleaning the sample from the matrix. Comparing the performance of the C_{18} sorbent, in this case 150 mg, compared to 300 mg, seems to be sufficient for a good cleaning of the extract (as also observed in wheat gluten and rye) and produced recoveries between 73 and 96%.

Fig 8. Comparison of the three different extraction conditions (*green* = neutral, *red* = acidic, *blue* = alkaline) and two different amounts of C_{18} sorbents (150 mg and 300 mg), for each ergot alkaloids at the spiking level of 20 µg/kg in the baby food sample.



In general, acceptable results were obtained with all three extraction solvents, also in terms of signal intensity, however, overall better recoveries for all the matrices tested, were achieved with the alkaline solution. This was also confirmed by Huybrechts (2021) when he compared a modified QuEChERS approach using an alkaline acetonitrile/water mixture with acidic aqueous methanol (MeOH/water/formic acid, 60/39/1, v/v/v). The alkaloids are deprotonated under alkaline conditions, and they tend to migrate more easily to the upper organic layer, leading the analytes to greater separation form the matrix and higher recoveries.

In addition, a cleaner extract with 300 mg of C_{18} sorbent seemed to be the best solution for wheat and oat, while for more complex matrices, such as gluten, rye and baby food, a lower amount of C_{18} sorbent, 150 mg, showed excellent recoveries and good clean extracts.

3.2 Method Validation

3.2.1 Linearity and Matrix effect

Linearity was studied through matrix-matched calibration curves, spiked at nine concentration levels (see Paragraph 2.5.1), processed in duplicate and injected in triplicate. The linear range of the method was 0.5 – 500 µg/kg for all the different sample types. Good linear regression for all analytes was achieved, obtaining coefficient of determination (R^2) values ≥ 0.997.

The precise quantitation of all ergot alkaloids in these complex matrices can be affected by matrix effects in ESI, responsible for enhancement or suppression of the ion abundance. Differences in the degree of matrix effects can be expected between different sample types as well as between the different extraction

protocols. In cases where more than 20% signal suppression or enhancement is observed, ME need to be addressed in calibration (SANTE 2021).

The matrix effect here has been evaluated in a multi-matrix blended flour composed of rye, oat and wheat, extracted with the three different extraction protocols proposed (**Table 2**). As can be observed, with the acidic extraction (red) the majority of the analytes are affected by signal suppression except for three epimers *ergosinine, ercocorninine* and *ergocristinine*, for which slight ion enhancement is present. While with the neutral (green) and alkaline (blue) extraction, the analytes are affected by signal enhancement, and therefore matrix-matched calibration was used for quantitation purposes.

With the selected alkaline extraction, for all the matrices, the ME ranged between + 9.2 to +31.3%.

Table 2 Matrix effect of 8 ergot alkaloids in a multi-matrix blended flour with three different extraction procedures and their relative coefficient of correlation, R^2 (linearity) values

Analytes	ME % neutral extraction	R^2	ME % acidic extraction	R^2	ME % alkaline extrcation	R^2
Ergosine	25.5	0.999	− 6.8	0.997	23.3	0.997
Ergocornine	9.9	0.999	− 7.5	0.997	10.8	0.999
α-Ergocryptine	15.4	0.999	− 3.7	0.998	16.3	0.999
Ergocristine	8.3	0.999	− 5.5	0.997	9.2	0.999
Ergosinine	21.5	0.999	17.2	0.999	28.9	0.999
Ergocorninine	22.1	0.999	19.6	0.998	31.3	0.999
α-Ergocryptinine	0.0	0.999	− 0.7	0.998	9.3	0.999
Ergocristinine	14.8	0.999	11.0	0.999	26.8	0.999

3.2.2 Recovery and precision

The recoveries of the analytes were measured by spiking blank samples (wheat, oat, wheat gluten, rye and baby food), previously checked for the presence of the target analytes, with EA standards at three different concentrations, 2, 20 and 100 µg/kg. Baby food samples was spiked at two concentrations (2 and 20 µg/kg) in line with the European Commission's MRL for ergot alkaloids of 20 µg/kg in processed cereal-based baby food (EU 2021). Samples were extracted, according to the procedure described above, with an alkaline extraction solvent combined with 300 mg of C_{18} sorbent for wheat and oat, and 150 mg of C_{18} sorbent for wheat gluten, rye and baby food.

Results (**Tables 3, 4, 5, 6 and 7**), expressed as mean percentage recovery of the three concentrations, showed good recovery values for all the analytes ranging from 70 to 105 % for wheat, 79 – 120% for oat, 70 – 118% for wheat gluten, 73 – 106% for rye and 71 – 96% for baby food. Overall, recovery rates in all validated matrices are in line with the recommendation (SANTE 2021) and in the range 70 – 120%.

The precision of the proposed method (expressed as percentage relative standard deviation, RSD %) was evaluated in terms of repeatability (intraday precision), assessed by performing six consecutive injections on the same day of the samples spiked at each concentration level tested. According to the CEN (2010) document and SANTE (2021) guideline, repeatability RSD_r should be ≤ 20% for each spike level tested. Satisfactory results were obtained at the concentration level evaluated, as the RSD_r were between 1 – 12% (**Tables 3, 4, 5, 6 and 7**).

Table 3 Validation data for ergot alkaloids in wheat samples measured at three spiking levels (2, 20 and 100 µg/kg). Average recovery rates and repeatability measure (expressed as relative standard deviation, RSD_r) with $n = 6$ replicate each, LOD and LOQ

Analyte	Level 2 µg/Kg		Level 20 µg/Kg		Level 100 µg/Kg		LOD µg/kg	LOQ µg/kg
	% Recovery	**RSD*r* %**	**% Recovery**	**RSD*r* %**	**% Recovery**	**RSD*r* %**		
Ergosine	80	3.7	86	4.0	71	3.1	0.04	0.1
Ergocornine	90	3.5	94	3.9	80	2.7	0.03	0.1
α-Ergocryptine	85	3.7	92	3.2	83	2.4	0.03	0.1
Ergocristine	70	4.9	74	4.7	86	4.9	0.07	0.2
Ergosinine	82	3.8	99	3.8	91	2.8	0.03	0.1
Ergocorninine	82	3.5	95	2.6	81	3.4	0.03	0.1
α-Ergocryptinine	105	3.0	102	2.8	79	2.9	0.03	0.1
Ergocristinine	82	3.9	93	4.0	87	4.5	0.04	0.1

Table 4 Validation data for ergot alkaloids in oat sample measured at three spiking levels (2, 20 and 100 µg/kg). Average recovery rates and repeatability measure (expressed as relative standard deviation, RSD_r) with $n = 6$ replicate each, LOD and LOQ

Analyte	Level 2 µg/Kg		Level 20 µg/Kg		Level 100 µg/Kg		LOD µg/kg	LOQ µg/kg
	% Recovery	**RSD_r %**	**% Recovery**	**RSD_r %**	**% Recovery**	**RSD_r %**		
Ergosine	96	3.4	101	3.5	102	3.9	0.04	0.1
Ergocornine	88	6.1	119	4.7	109	3.8	0.03	0.1
α-Ergocryptine	87	5.4	108	4.2	106	4.1	0.03	0.1
Ergocristine	88	7.2	97	6.1	82	6.4	0.07	0.2
Ergosinine	93	4.3	114	4.8	97	4.3	0.03	0.1
Ergocorninine	79	4.0	117	5.2	115	4.6	0.03	0.1
α-Ergocryptinine	93	5.7	120	4.7	118	5.2	0.03	0.1
Ergocristinine	85	4.6	80	5.1	93	5.3	0.04	0.1

Table 5 Validation data for ergot alkaloids in wheat gluten sample measured at three spiking levels (2, 20 and 100 µg/kg). Average recovery rates and repeatability measure (expressed as relative standard deviation, RSD_r) with $n = 6$ replicate each, LOD and LOQ

Analyte	Level 2 µg/Kg		Level 20 µg/Kg		Level 100 µg/Kg		LOD µg/kg	LOQ µg/kg
	%Recovery	**RSD_r %**	**%Recovery**	**RSD_r %**	**%Recovery**	**RSD_r %**		
Ergosine	115	4.7	83	3.0	118	3.1	0.04	0.1
Ergocornine	84	4.5	92	3.9	90	2.7	0.03	0.1
α-Ergocryptine	81	4.7	90	3.2	98	2.4	0.03	0.1
Ergocristine	70	4.9	73	3.7	87	2.9	0.07	0.2
Ergosinine	90	4.8	97	4.8	108	2.8	0.03	0.1
Ergocorninine	67	4.5	93	3.6	78	3.4	0.03	0.1
α-Ergocryptinine	93	4.0	99	3.8	89	2.9	0.03	0.1
Ergocristinine	79	4.9	91	4.0	83	4.5	0.04	0.1

Table 6 Validation data for ergot alkaloids in rye sample measured at three spiking levels (2, 20 and 100 µg/kg). Average recovery rates and repeatability measure (expressed as relative standard deviation, RSD_r) with $n = 6$ replicate each, LOD and LOQ

Analyte	Level 2 µg/Kg		Level 20 µg/Kg		Level 100 µg/Kg		LOD µg/kg	LOQ µg/kg
	%Recovery	**RSD_r %**	**%Recovery**	**RSD_r %**	**%Recovery**	**RSD_r %**		
Ergosine	74	5.1	91	2.1	84	11.3	0.04	0.1
Ergocornine	73	3.6	88	9.9	82	5.8	0.03	0.1
α-Ergocryptine	76	8.7	105	4.4	99	12.4	0.03	0.1
Ergocristine	74	11.9	85	10.2	87	10.7	0.07	0.2
Ergosinine	73	8.9	102	4.8	89	11.5	0.03	0.1
Ergocorninine	79	12.0	106	6.0	97	9.8	0.03	0.1
α-Ergocryptinine	73	11.9	87	10.2	91	10.7	0.03	0.1
Ergocristinine	80	13	88	6.3	94	10.9	0.04	0.1

Table 7 Validation data (mean recoveries and relative standard deviation) for ergot alkaloids in baby food sample measured at two spiking levels (2 and 20 µg/kg). Average recovery rates and repeatability measure (expressed as relative standard deviation, RSD_r) with $n = 6$ replicate each, LOD and LOQ

Analyte	Level 2 µg/Kg		Level 20 µg/Kg		LOD µg/kg	LOQ µg/kg
	% Recovery	**RSD_r %**	**% Recovery**	**RSD_r %**		
Ergosine	80	5.6	91	3.2	0.04	0.1
Ergocornine	78	4.0	85	3.6	0.03	0.1
α-Ergocryptine	87	3.4	96	4.2	0.03	0.1
Ergocristine	71	6.3	73	5.4	0.07	0.2
Ergosinine	77	4.4	88	3.6	0.03	0.1
Ergocorninine	79	4.1	94	3.8	0.03	0.1
α-Ergocryptinine	83	3.4	96	3.6	0.03	0.1
Ergocristinine	86	5.5	79	5.7	0.04	0.1

3.2.3 LOD and LOQ

Assessed limits of detection (LOD) ranged from 0.03 to 0.07 µg kg^{-1} and estimated limits of quantification (LOQ) were 0.1 µg kg^{-1}, except for *ergocristine*, at 0.2 µg kg^{-1} (**Tables 3, 4, 5, 6 and 7**). The low LOQ values were additionally confirmed with the recovery and repeatability values obtained from the lowest tested level of the calibration curve, 0.5 µg/Kg.

The results presented here show that the method is very sensitive and yields favourable performance characteristics. It is fast and simple, without needing the time consuming SPE approach exploiting only dispersive sorbent and it also enables results in compliance with the new values proposed by the European Commission that will come into force 1.07.2024 (EU 2021). Table 8 represents a comparison with other method presented in the literature.

Table 8 Comparison of the performance characteristics of the proposed method with other published methods in literature

Sample	Extraction + clean-up	Analytical technique	LOQ **(µg/kg)**	Reference
Rye	ACN:2 mM $(NH_4)_2CO_3$ (84:16, v/v) + neutral alumina based	LC-IT-MS/MS	1.0–3.0	Bryla et al. (2015)
Rye flour, wheat flour, bread and noodles	ACN:$(NH_4)_2CO_3$ (85:15, v/v) + C_{18} sorbent	UPLC-MS/MS	0.2–0.5	Guo et al. (2016)
Bread samples (wheat, multi-grain, rye and wheat rye)	MeOH:H_2O:FA (60:40:0.4, *v/v/v*)	LC–MS/MS	0.3–1.2	Versilovskiks et al. (2020)
Wheat Barley	ACN:$(NH_4)_2CO_3$ (85:15, v/v) + C_{18}/Z-SEP +	UHPLC-MS/MS	0.49–3.33 0.50–3.92	Carbonell-Rozas et al. (2021)
Oat	ACN:$(NH_4)_2CO_3$ (85:15, v/v) + C_{18}/Z-SEP +	UHPLC-MS/MS	0.2–3.2	Carbonell-Rozas (2022)
Baby food (breakfast cereals and cookies)	MeOH:H_2O:FA (60:40:0.4, *v/v/v*)	LC–MS/MS	4	Mulder et al. (2015)
Dry cereal-based baby food	ACN:3 mM $(NH_4)_2CO_3$ (84:16, v/v) + 6 g MgSO4/1.5 g NaCl	UHPLC-MS/MS	0.1–0.3	Huybrechts et al. (2021)
Oat Barley Wheat	H_2O:ACN + 5% FA + 4 g $MgSO_4$ and 1 g NaCl	HPLC–MS/MS	0.16–0.36 0.10–0.39 0.19–0.36	Kim et al. (2022)
Wheat, oat, rye, wheat gluten and baby food	ACN:3 mM $(NH_4)_2CO_3$ (84:16, v/v) + C_{18} sorbent	UPLC-MS/MS	0.1–0.2	Present study

3.2.4 Method's trueness

To evaluate the trueness of the method, two proficiency test samples FAPAS (ergot alkaloids in rye flour, PT 22190 and PT22203) were purchased, extracted with the 3 different extraction procedures, and the z-scores were calculated for the EA compounds (**Tables 9** and **10**). Here, we sum the results of *α-ergocryptine* and *β-ergocryptine* to meet the request of the FAPAS test. Z scores between +2 and −2 are considered a satisfactory performance, Z scores between +2 and +3 or between −2 and −3 are considered questionable performance, and anything outside this range (> +3 or < −3) are considered unsatisfactory. In our analysis, the diverse extraction procedures delivered satisfactory results, except for *ergocristine* (**Table 10**), which had a questionable performance with the dispersive SLE (300 mg) approach. In general, better performances were obtained with the alkaline extraction with 150 mg of C_{18} sorbent, as confirmed also in the Paragraph 3.1.3, optimization of sample extraction and clean-up.

In addition to the PT samples, baby food quality control (QC) material (FAPAS) was analyzed, with the three extraction protocols presented above. The results are summarized in Table **11**.
In totally, 5 -ine and -inine ergot alkaloids were present in the sample. *Ergotamine* and its epimer are present in the QC material but they are not quantified in our study, but to calculate the total EAs content we have considered their value, for all three extraction solutions, as the optimal value given by the test sample (21.8 µg/kg for ergotamine and 5 µg/kg for ergotaminine).

In this case, the acidic extraction yields poor results for *ergosine* and *ergocornine* and quantify a total number of ergot alkaloids below the range proposed by the QC. The neutral extraction presents better results for *ergosine* but lower results for *ergocornine*. In this case, the total amount of EAs obtained with SLE is centred in the proposed range but, as the sample is not being cleaned, it may have a higher value due to the ME. In fact, we see a lower result when the sample is cleaned by the dispersive C_{18} clean-up. With the alkaline extraction, the SLE procedure delivers good results, but as the sample is not being cleaned, the performance of 150 mg of C_{18} sorbent seems to be sufficient for cleaning the extract and yields suitable values within the proposed range.

In conclusion, the achievement of the required z-scores benchmark was confirmation of the trueness of the final method.

Table 9 Z-scores of ergot alkaloids in rye flour with the three extraction protocols from the 2023 FAPAS proficiency test

Analyte	SLE 84:16	84:16 + 150 mg C_{18}	84:16 + 300 mg C_{18}	SLE 84:16 + 05FA%	84:16 + 05FA% + 150 mg C18	84:16 + 05FA% + 300 mg C_{18}	SLE 85:15	85:15 + 150 mg C_{18}	85:15 + 300 mg C_{18}
Ergosine	0.9	0.2	0.4	−0.9	−0.3	−0.9	−0.5	−0.3	−1.0
Ergocornine	−0.3	−0.8	−1.1	−0.6	−0.5	−0.2	−0.3	0.0	−1.4
α + β Ergocryptine (sum)	0.1	−0.5	−0.4	0.9	0.7	0.5	1.1	0.6	0.1
Ergocristine	1.3	0.7	1.5	−1.2	+1.3	+1.4	1.2	0.1	−1.5
Ergosinine	−1.0	−1.5	−1.4	−1.2	−0.5	−1.9	−1.3	−0.8	−1.6
Ergocorninine	−1.6	−0.7	−1.7	−1.6	−1.5	−1.2	−1.4	−1.0	−1.5
α + β Ergocryptinine	−1.1	−1.5	−1.8	−0.9	−0.9	−1.0	−0.6	−0.8	−0.5
Ergocristinine	−0.5	−1.1	−1.5	−0.5	−0.3	−1.3	−0.3	−0.3	−0.2

Table 10 Z-scores of ergot alkaloids in rye flour with the three extraction protocols from the 2022 FAPAS proficiency test

Analyte	SLE 84:16	84:16 + 150 mg C_{18}	84:16 + 300 mg C_{18}	SLE 84:16 + 05FA%	84:16 + 05FA% + 150 mg C_{18}	84:16 + 05FA% + 300 mg C_{18}	SLE 85:15	85:15 + 150 mg C_{18}	85:15 + 300 mg C_{18}
Ergosine	−0.6	−1.2	−1.7	−0.9	−0.2	0.2	−0.1	−0.4	−1.3
Ergocornine	1.6	−0.2	−1.8	−1.0	−1.2	−1.6	0.1	0.8	−0.4
α + β Ergocryptine (sum)	−0.5	−0.5	−1.0	−0.4	−0.2	−0.5	1.0	0.5	−0.9
Ergocristine	1.2	−0.5	−2.7*	1.0	1.1	+2.4*	1.1	−0.1	−2.5
Ergosinine	−0.2	−0.2	−0.3	−1.0	−0.8	−0.7	−0.2	−0.1	−0.7
Ergocorninine	−0.4	−0.4	−0.5	−0.6	−0.5	−0.6	−0.4	−0.2	−0.6
α + β Ergocryptinine	−1.5	−1.7	−2.1*	−1.9	−1.2	−1.5	−0.7	−0.6	−1.6
Ergocristinine	−0.9	−1.3	−1.2	−0.2	0.2	−0.4	−1.2	−0.3	−0.4

*Questionable performance

Table 11 Comparison between values for ergot alkaloids in FAPAS QC baby food cereals and values obtained with the three extraction protocols tested

Analyte	Assigned value µg/kg	[a]Range for $z \leq 2$	SLE 85:15	85:15 + 150 mg C_{18}	85:15 +300 mg C_{18}	SLE 84:16	84:16 + 150 mg C_{18}	84:16 + 300 mg C_{18}	SLE 84:16 + 05FA%	84:16 + 05 FA% + 150 mg C_{18}	84:16 + 05 FA% + 300 mg C_{18}
Ergosine	**8.9**	**4.98–12.82**	7.9	7.8	6.5	7.7	7.0	6.0	0.1	0.4	2.8
Ergocornine	**14.4**	**8.0–20.7**	15.3	15.6	15.7	6.6	5.2	4.3	9.8	9.0	6.0
α + β Ergocryptine (sum)	**21.7**	**12.1–31.2**	25.7	24.2	23.5	18.0	16.0	13.4	19.0	18.1	13.3
Ergocristine	//		34.1	36.7	34.6	50.0	46.0	42.0	26.4	26.4	24.5
Ergosinine	//		0.9	0.5	0.4	0.9	0.7	0.5	<	<	<
Ergocorninine	//		5.2	4.5	3.2	0.7	0.3	0.2	1.8	1.7	0.9
α + β Ergocryptinine	//		5.6	6.6	5.7	3.4	3.0	2.6	3.6	3.7	2.5
Ergocristinine	//		5.8	6.8	4.9	8.0	6.6	6.0	6.0	5.9	4.6
Total	**161**	**93–228**	101	103	95	95	85	75	67	65	55
Total (considering the optimal value for ergotamine and ergotaminine)	**161**	**93–228**	127	130	121	122	112	102	94	92	81

[a]The range for $|z| \leq 2$ is the concentration range within the limits of ± 2 *z*-scores. The assigned value and its range have been established from the proficiency test data and are suitable for use by laboratories as a fit-for-purpose quality control measure

3.3 Method's applicability: occurrence of ergot alkaloids

To evaluate the applicability of the validated method, a set of 54 samples (from 2020 to 2022) covering some validated matrices and other cereals like spelt, tritordeum and triticale, were analysed to monitor the natural occurrence of EAs. Here, we report only the positive cereals tested and results showed that 27 of the 54 samples contain EA compounds, some of which are above the legal maximum levels (**Table 12**).

For tritordeum and triticale, no regulatory limit for EAs content has been set, so we have decided 'by convention' to consider them to be like other cereals, which have a maximum legal level of 100 μg/kg that became of 50 μg/kg on 1st July 2024.

Rye samples from 2020 are highly contaminated, with one sample that largely exceed the maximum legal level (1302 μg/kg) (EU 2023). In the same year, also common wheat, spelt and tritordeum (Fig. **9**) present EAs above the MRLs, especially if we consider the level that came into force on 1st July 2024.

Considering this small survey, rye was confirmed to be the crop more susceptible to the fungal infection (EAs content up to 1302 μg/kg) but also tritordeum, in the following two years, presents high values.

Ergocryptine and *ergocristine* (sometimes also *ergosine*) compounds are the toxins with the highest occurrence in the cereals tested. These results are in line with other previous studies that also reported they predominance in the literature (Malysheva 2014; Agriopoulou 2021). Therefore, it is extremely important to monitor their presence in these matrices due to the potential health risk that they can cause and try to establish maximum levels for them in order to guarantee the safety of the consumers.

To conclude, the developed analytical method was found to be applicable to a wide variety of different cereals, including tritordeum, triticale and spelt, highlighting the possibility of its use in different cereal chains, in addition to those tested.

Table 12 Occurrence of ergot alkaloids in different cereal samples and the total amount per sample

Cereals	Years	Analytes (µg/kg)								Total (µg/kg)
		Ergosine	Ergocornine	Ergocryptine	Ergocristine	Ergosinine	Ergocorninine	Ergocryptinine	Ergocristinine	
Rye	2020	53.8	35.8	63.8	33.5	18.3	6.5	15.5	8.6	236
Rye	2020	185.3	133.0	551.5	136.0	85.9	36.6	138.3	35.5	1302
Common wheat	2020	3.3	3.6	5.3	55.8	3.7	4.3	3.2	14.7	94
Common wheat	2020	72.1	5.6	6.3	125.3	36.4	4.9	6.8	21.2	279
Common wheat	2020	8.5	3.8	3.8	32.0	4.8	3.8	3.2	4.3	64
Common wheat	2020	5.3	4.5	3.5	4.6	2.8	3.2	3.8	2.5	30
Durum wheat	2020	5.3	< LOQ	1.9	5.3	3.6	< LOQ	< LOQ	3.3	19
Durum wheat	2020	3.6	< LOQ	1.5	3.3	1.9	< LOQ	< LOQ	1.8	12
Spelt	2020	5.1	< LOQ	3.9	131.9	3.6	< LOQ	< LOQ	36.8	181
Tritordeum	2020	33.3	13.6	15.3	66.8	15.9	6.3	8.8	33.5	194
Tritordeum	2020	8.5	13.3	30.5	< LOQ	6.6	6.9	16.8	< LOQ	83
Tritordeum	2020	11.9	3.8	5.8	56.3	8.3	3.1	3.8	18.5	112
Tritordeum	2020	58.5	58.5	83.5	50.6	35.3	15.3	31.8	8.5	342
Rye	2021	36.1	3.8	3.8	139.3	10.9	3.5	5.3	15.6	218
Rye	2021	3.1	5.0	3.0	8.3	3.5	3.6	5.3	3.1	35
Rye	2021	8.0	55.9	33.0	3.0	3.9	8.8	9.6	3.3	126
Common wheat	2021	3.8	3.0	3.3	3.1	< LOQ	< LOQ	< LOQ	0.1	13
Tritordeum	2021	31.6	3.0	3.5	86.9	15.0	3.3	3.3	33.3	180
Tritordeum	2021	3.6	3.6	3.5	6.8	3.3	3.1	3.3	3.5	31
Triticale	2021	3.9	3.8	3.6	3.3	3.5	3.3	3.3	0.8	26
Triticale	2021	5.1	< LOQ	1.8	5.1	3.8	< LOQ	0.1	0.9	17
Rye	2022	5.5	18.8	15.3	10.6	6.5	8.6	8.5	5.3	79
Rye	2022	3.5	5.6	5.9	5.3	5.9	3.1	3.3	3.0	36
Rye	2022	15.6	3.3	3.0	55.1	16.8	3.6	3.6	18.5	120
Spelt	2022	3.8	3.0	1.6	< LOQ	1.5	3.3	1.5	< LOQ	15
Tritordeum	2022	15.1	3.1	3.9.	109.8	13.0	3.6	3.6	38.5	191
Tritordeum	2022	3.1	6.5	6.8	3.3	5.8	5.3	6.3	1.8	39

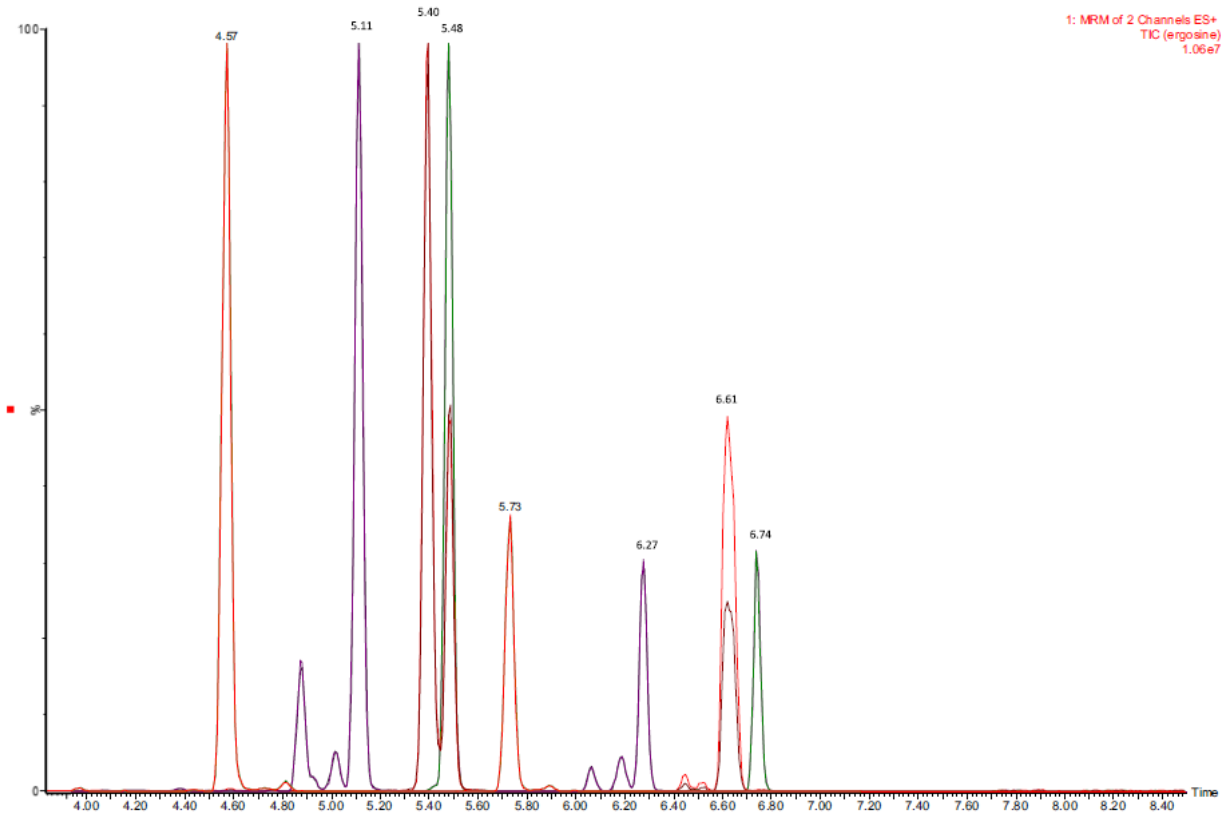


Fig. 9 Chromatogram of naturally contaminated tritordeum sample (2020) with eight ergot alkaloids. For retention times of the analytes, see Table 1

4 | CONCLUSION

The present study developed and validated an accurate and detailed UPLC-MS/MS method for the quantification of 8 ergot alkaloids (-ine and -inine isomers) in five different matrices, wheat, oat, durum wheat, rye and baby food.

The MRLs in the European Union are based on the sum of the –ine and –inine form, implying that epimerisation is not so impactful. In our method it was chosen to quantify only those ergot alkaloids that are now available on the market without any legislative permission. This was done in order to guarantee the applicability of the method to all entities involved in monitoring, giving special emphasis in this sense also to small-medium enterprises and in general to industrial stakeholders for permitting a screening approach valuable in several matrices and able to select, for further confirmatory determination of the sum of the 12 epimers, only possible suspected samples. The critical parameters that could induce epimerization (solvent, pH, temperature and light) were evaluated and the conditions chosen for both the preparation of standards and the extraction, i.e., alkaline conditions combined with acetonitrile, minimizing epimerization as much as possible. Data presented on extraction performance of various protocols gave valuable information for future applications and method developments. Dispersive SPE, using C_{18} sorbent as clean-up step, alleviated the problem of matrix effect. It allowed proper recoveries addressing the needs of the industry in each category

of matrix tested, especially for cereal-based food for infants in which the maximum legal level is set to 20 µg/kg.

Satisfactory results came from participation in the proficiency tests and analysis of a quality control material. Applicability was also confirmed by analysing a set of several samples covering some validated matrices and other cereals like spelt, tritordeum and triticale. Here, results showed that 27 of the 54 samples contain EA compounds, some of which are above the legal levels.

To conclude, in the future, more data focusing on the behaviour of ergot alkaloids during processing and/or storage with the possible formation of modified and/or transformation products, will be needed for a complete and reliable risk assessment.

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3

Chapter

Research Article

Development and methodological user-validation in industry of a 3D-printed biosensing toolkit for tropane alkaloid detection

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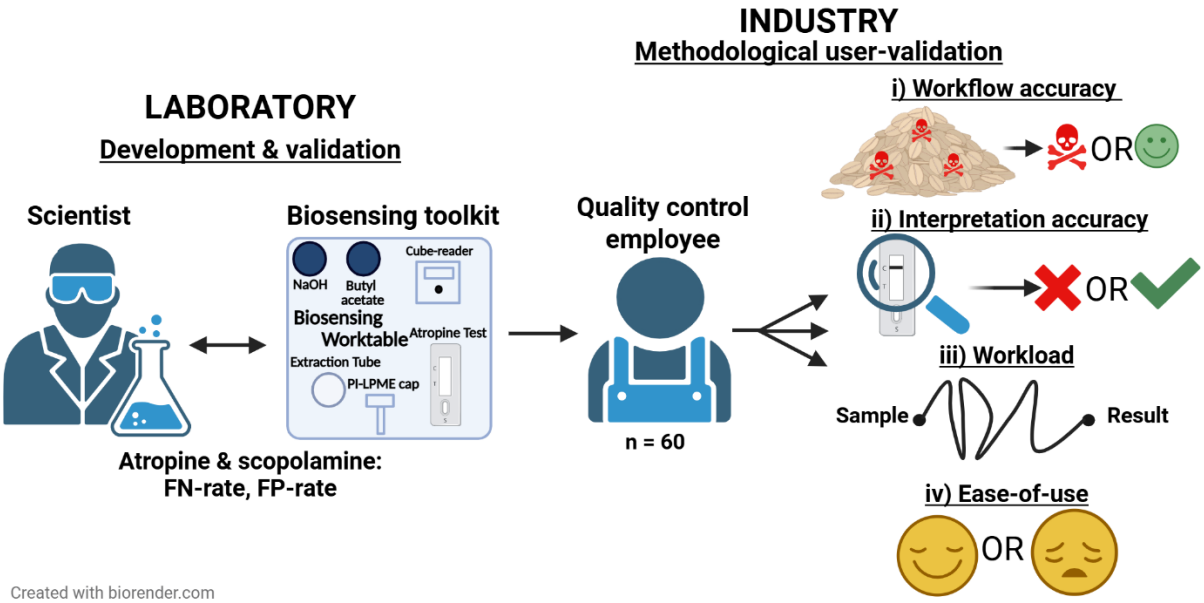
HIGHLIGHTS

- Development of a user-friendly 3D-printed biosensing toolkit for atropine detection in buckwheat
- Methodological and quantitative user-validation of the biosensing toolkit in industry
- Detailed ease-of-use and workload evaluation using post-test questionnaires
- Assessment of indirect competitive lateral flow immunoassay (icLFIA) interpretation by end-users
- Expansion and validation with an anti-scopolamine icLFIA (IC_{50}-value: 0.86 ng mL^{-1})

GRAPHICAL ABSTACT



Abstract

The number of biosensors for food contaminants are growing, yet few are tested in real-world settings. Here, we developed and validated a biosensor for detecting tropane alkaloids in buckwheat, emphasizing in-field accuracy and user-experience. Sixty participants from a major food producer tested the biosensor in a pre-structured manner. All quality control employees (n=31) correctly identified blank or contaminated buckwheat. However, non-analytical-users (n=29) showed lower accuracy when visually interpreting icLFIA-results (Kappa-score=0.5/1), which improved substantially when using a digital reader (Kappa-score=0.9/1). Post-test questionnaires yielded an excellent usability score (88/100±8). A difficulty score, based on how many participants struggled and the corresponding perceived difficulty, allowed identification of the challenging workflow steps. Notably, most challenging steps could be resolved by adjusting the instructions. This user-validation represents pioneering effort in food safety analysis, especially point-of-need detection, to offer quantitative evidence for claims related to stakeholder usability and provides a framework for other innovative (bio)sensing approaches.

KEYWORDS

User-validation, Immunoassay, 3D-printing, Tropane alkaloids, Food Safety

1. INTRODUCTION

A range of drivers, such as climate change, green transition, and geopolitics affect the risk of food contaminations (e.g. with natural toxins), reinforcing the need for the food industry to adapt to these realities in their product safety assessment.[1–3] In response, a growing number of point-of-need tools are being developed for rapid and cost-effective screening of such contaminants.[3] These devices are designed to enable end-users, such as food safety inspectors and quality control (QC) personnel, to perform fast, qualitative screening directly at the point-of-need.[4] Although many point-of-need tools have been developed, only a few have undergone a validation to determine their analytical performance.[5,6] Even more critically, point-of-need tests that are validated are often evaluated only by (i) scientists in (ii) controlled laboratory settings, rather than by (i) the intended end-users in (ii) real-world settings (e.g., slaughterhouses, silos, or food production facilities).[5,6] This disconnect presents a major hurdle towards understanding the performance of these tools during actual use.

A screening method for food safety should in principle be validated in a laboratory to demonstrate that, when properly used under specific conditions, the uncertainty associated with the outcome is within the internationally accepted criteria for enforcement purposes. For example, for plant toxins (such as atropine and scopolamine), screening methods should be validated to be fit-for-purpose by analyzing at least 20 blank and contaminated samples in the food commodity of interest. After the samples are measured, a cut-off value with an estimated FN-rate of 5% should be established and the corresponding estimated false positive (FP) rate reported (EU regulation 2023/2783).[7]

Still, a conventional laboratory validation does not account for uncertainty associated with taking the test out of the lab and to the point-of-need. Moreover, critical assessment of the usability and in-field accuracy of such tests is not provided. Often, the starting point for point-of-need testing is based on the World Health Organization (WHO) Real-time connectivity, Affordable, Sensitive, Specific, User-friendly, Rapid & Robust, Equipment-free, and Deliverable to end-users (REASSURED) criteria.[8] While, it is typically accepted that most screening tests are or can be cost-effective, the user-experience of these tests are mostly not evaluated, and instead assumed as 'REASSURED by design' – which is not necessarily reassuring.[3,6,9] User-experience is defined by ISO (9241-11) as "A person's perceptions and responses that result from the use and/or anticipated use of a product, system or service".[10] User-experience is generally considered as an holistic extension of usability in which the interactions of the end-user with the tool before, during, and after use are included.[11] Several health-monitoring tests have been assessed on their user-experience during a user-validation.[12–16] In an elaborate user-validation of a SARS-CoV-2 test, the user-experience was assessed on five different criteria's: i) ease-of-use, ii) effectiveness, iii) efficiency, iv) accuracy, and v) satisfaction.[16] Although health-monitoring and food safety tests are both focused on rapid analysis, food safety tests generally require a more elaborate sample preparation workflow. This typically includes extraction, analyte enrichment, and matrix clean-up steps, due to the high complexity, solidity, and heterogeneity of many food products.[3] During such sample preparation, the main challenge is that the presence of matrix should be minimized while still extracting and preferably concentrating the analytes-of-interest.

In previous work, we have successfully developed and validated strategies for the point-of-need detection of tropane alkaloids, which are strictly regulated poisonous plant toxins that may contaminate grains through mechanical co-harvesting of toxin-producing weeds.[17,18] Since the tropane alkaloids atropine and scopolamine must not exceed 10 μg kg^{-1} in cereals (EU regulation 2023/915), it is critical for both food safety authorities and food producers to be able to monitor their ingredients and products effectively and efficiently. This was the reason that the fast tests were developed in the first place. In this work, we challenged the assumption underlying the previous laboratory test development and validation, and performed a quantitative investigation of the in-field accuracy and user-experience through a methodological user-validation. The primary objective was to develop a broadly applicable framework for generating evidence to support claims regarding ease-of-use and practical applicability of (bio)sensing technologies at the point-of-need. Prior to the user-validation, we hypothesized that: (i) when expert end-users analyze blank or highly contaminated buckwheat samples, errors will arise more frequently from complex sample handling than from interpretation of the icLFIA results, (ii) automated icLFIA read-out will be more accurate then visual read-out, and (iii) end-users with at least moderate laboratory-experience will interpret icLFIA results more accurately than end-users without such background. In addition, as a secondary objective, we aimed to identify which steps in the sample-handling workflow caused difficulties, to understand why these difficulties occurred, and to propose potential solutions, highlighting the importance of iterative improvements.

2. MATERIALS AND METHODS

2.1 Experimental strategy

A 3D-printed biosensing toolkit for tropane alkaloid detection in buckwheat cereals was evaluated through an elaborate user-validation at a major food producing company. The overall workflow of this research is summarized in Figure 1. First, the previously developed and validated modular workflow for atropine detection was integrated in a 3D-printed biosensing toolkit, aiming to enhance user-friendliness by reducing the number of handling steps and providing structure to the test kit (see Figure 1I).[17] Then, the biosensing toolkit was tested by 60 employees of Barilla company across several QC-laboratories in Italy (see Figure 1II). During this user-validation, the biosensing toolkit was assessed based on its in-field accuracy and user-experience. The in-field accuracy was assessed based on the test results and the interpretation of those results by the end-users, while user-experience was measured through post-test questionnaires. Based on the feedback received during the user-validation, a rapid icLFIA was developed to allow for the rapid monitoring of scopolamine with the biosensing toolkit (see Figure 1III).

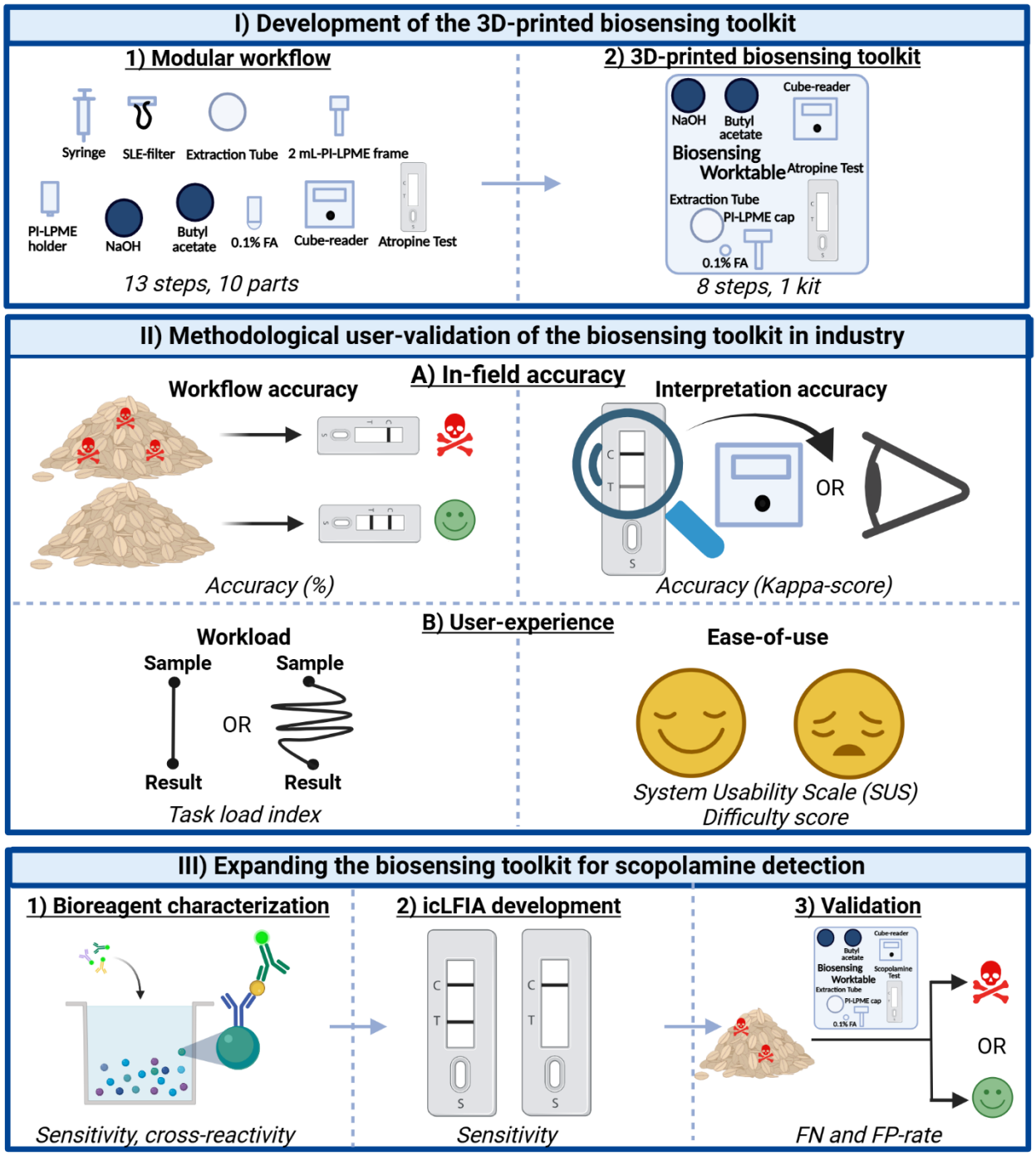


Figure 1. Schematic overview of (**I**) the development of the 3D-printed biosensing toolkit, (**II**) methodological user-validation in industry, and (**III**) expansion of the biosensing toolkit for scopolamine detection (created with biorender.com).

2.2 Chemicals and consumables

2.2.1 Chemicals

Scopolamine (>99%), tropine (97%), homatropine hydrobromide (>99%), aposcopolamine (>99%), atropine (>99%), and butyl acetate (>99%) were purchased from TCI Europe (Zwijndrecht, Belgium) and formic acid (FA, LC-MS grade), Tween 20, and bovine serum albumin (BSA, ≥98%) from Sigma Aldrich Co. (Saint Louis, USA). Anisodamine (>98%), and anisodine hydrobromide (>99%) were purchased from Phytolab (Vestenbergsgreuth, Germany). Methanol (MeOH, LC-MS grade) and acetonitrile (ACN, LC-MS grade) were obtained from Actu-all Chemicals (Oss, The Netherlands) and sodium hydroxide (NaOH, pellets), and phosphate-buffered saline (PBS) tablets from Merck KGaA (Darmstadt, Germany). 'Spezial Schwartz 4' carbon nanoparticles were purchased from Degussa AG (Frankfurt, Germany) and goat anti-mouse IgG in PBS (pH 7.6) (1.2 mg mL^{-1}; AffiniPure F(ab')2 Fragment GAM IgG Fcγ) from Jackson Immunoresearch Laboratories Inc. (West Grove, USA). A monoclonal atropine-specific antibody and an atropine-BSA conjugate were obtained from Jiangnan University (Wuxi, China). A polyclonal anti-scopolamine antibody was obtained from CER Groupe (Marloie, Belgium).[19] Deionized water was obtained from a Milli-Q direct ultrapure water system (Millipore, USA). The running buffer (RB) for the icLFIAs as applied in this work was 0.01 M PBS in water containing 1% BSA and 0.05% Tween 20. PLA+ filament (diameter = 1.75 mm) was obtained from 123-3D.nl (Almere, The Netherlands), and proprietary clear resin Type V4 from Formlabs (Summerville, USA).

2.2.2 Consumables

UniSart 95 CN nitrocellulose membranes were purchased from Sartorius (Gottinghem, Germany) and 30-mL plastic vials from DaklaPack Europe (Lelystad, The Netherlands). Plastic backing cards (30 cm × 6 cm), glass fiber (25.4 cm × 30.48 cm), and sample pads (21 cm × 29.7 cm) were obtained from Kenosha (Amstelveen, The Netherlands) and Whatman 1 CHR chromatography paper (200 mm × 200 mm) from Sigma-Aldrich corporation (Saint Louis, USA). Protein LoBind safe-lock tubes 1.5-mL and 2.0-mL (PCR clean) were purchased from Eppendorf Corporate (Hamburg, Germany). Plastic stopcock valves were purchased from Nanjing life store (Nanjing, China) and ceramic homogenizers from Thermo Fisher scientific (Hampton, USA).

2.2.3 Preparation of (user-)validation samples

Due to the route of contamination (i.e., by cross-crop contamination), certified reference materials for tropane alkaloids are not commercially available. As a result, most analytical methods have been validated using materials spiked in-house.[20–24] In this study, milled buckwheat cereals were purchased from local grocery stores. Buckwheat was selected as food commodity of interest as it has been reported to be relatively frequently contaminated with tropane alkaloids.[25] In addition, buckwheat is experiencing a renewed commercial interest due to its valuable nutritional composition.[26] Subsequently, all buckwheat cereals were analyzed with a

validated LC-MS/MS method to verify these did not contain tropane alkaloids above the limit of detection (0.3 µg kg^{-1}).[21] Then, two grams of blank samples were precisely weighed in the extraction tube and then spiked with different concentrations of tropane alkaloid in 200µL of ACN (0, 50, 100, or 1000 ng mL^{-1}) to achieve contamination levels of 0, 5, 10, 100 µg kg^{-1}. Subsequently, the buckwheat cereals were dried for at least 24 hours and each cereal aliquot was thoroughly mixed prior to further use. For information on how to obtain the required number of 2 g replicate samples from bulk lots of milled buckwheat cereals for analysis with the biosensing toolkit in accordance with EU Regulations 2023/2782 and 2023/2783 (see SI, Protocol S1).

2.3 Instrumental setups

2.3.1 3D-design, development, and 3D-printing of sample preparation and detection modules

Computer-aided design software SOLIDWORKS Education Edition 2021-2022 (SOLIDWORKS Corp; Waltham, USA) was used for designing the 3D-printable parts and converting them to printable STL. files. All 3D-printed components that come in contact with butyl acetate (e.g. the paper-immoblized liquid phase microextraction cap and holder) were printed with a stereolithography (SLA) printer Form3 (FormLabs; Summerville, USA) at a layer resolution of 100 µM using proprietary clear resin V5, because it has a high compatibility with butyl acetate.[27] After printing, the resin parts were washed with isopropyl alcohol:H_2O (70/30, v/v) for 15 min, dried under a nitrogen flow, and subsequently cured using 405 nm light for 1h at RT with a Form Cure V1 (FormLabs; Summerville, USA). The components were then ready for use. A fused deposition modeling (FDM) Original Prusa i3 MK3S+ printer (Prusa Research; Praque, Czech Republic) was used to print the biosensing worktable and the icLFIA cassette (see SI, Table S1 for printer settings). After printing, the components were directly ready for use. As both during the SLA and FDM printing standard settings were used, the 3D-printed components of the biosensing toolkit can be easily printed using a wide-variety of different 3D-printers.[28,29] All STL. Files can be found in the supplementary information (SI, see Print-files).

2.3.2 Microsphere-based immunoassay

A microsphere-based immunoassay was used to characterize the sensitivity and cross reactivity of the antibody raised against scopolamine. For this, a MAGPIX planar array analyzer (Luminex; Austin, USA) equipped with xPONENT 4.3 software (Luminex; Austin, USA) was used for read-out of the fluorescence of the microspheres. The acquisition volume was set at 50 µL. GraphPad Prism version 10 (Domatics; Boston, USA) was used for data processing and for five-parameter logistic curve fitting.

2.4. Development of the biosensing toolkit

The biosensing toolkit was constructed from readily available laboratory consumables and 3D-printed components. For recording the intensity of the test line (T-line) and control line (C-line), i.e. grey color intensity on the icLFIA, a digital reader was used (Cubereader; Chembio Diagnostics GmbH; Berlin, Germany). After development of the 3D-printed biosensing toolkit, it was used to analyze 5 blank and 5 contaminated

buckwheat cereal samples containing 10 µg kg^{-1} atropine, following SI, Protocol S2. In addition, a pilot study was conducted with five first-time users with laboratory experience similar to the QC-employees of Barilla, to evaluate usability and test performance by first-time users at regulatory levels. This panel analyzed in total 10× blank, 10× 5 µg kg^{-1}, and 10× 10 µg kg^{-1} atropine containing buckwheat cereal samples. The experimental set-up was blind as each participant started by first analyzing one buckwheat cereal sample contaminated with 10 µg kg^{-1}, without knowing this sample was contaminated. Subsequently, each participant analyzed 5 samples (2× blank, 2× 5 µg kg^{-1}, and 1× 5 µg kg^{-1}) simultaneously, without knowing which sample was blank or contaminated. The experimental workflow was similar as during the user-validation in industry, and can therefore be found in Section 2.5.2.

2.5 Methodological user-validation in industry

2.5.1 User-validation: study-design

The user-validation of the biosensing toolkit was completed over 14 days (13-Jan-2025 to 24-Jan-2025) at multiple QC-laboratories of Barilla G.R. F.lli S.p.A company distributed throughout Italy. Participants were recruited during two (online) information sessions held on October 5 and November 10, 2024 with QC and research and development (R&D) managers from various production plants, with the aim of recruiting as many participants as possible. In total, 60 Barilla employees expressed interest in participating in the user-validation (see SI, Figure S1 for a flow diagram showing the participation inclusion, allocation, and distribution of the samples). Only employees with no prior experience with operating icLFIAs - apart for personal medical use (e.g. SARS-CoV-2 or pregnancy test) - were eligible to participate in the user-validation. These participants were then distributed in an expert (n = 31) and non-expert (n = 29) group based on their laboratory experience. Participants were subsequently divided into expert (n = 31) and non-expert (n = 29) groups based on their laboratory experience. Those with regular laboratory experience, either current or past, were classified as experts, whereas participants with no or very limited laboratory experience were classified as non-experts. Detailed information about the participants' job description, level of education, and age distribution, can be found in the SI, Table S2. For both groups, sample distribution was performed in a blinded and randomized manner, as participants selected their own samples for analysis without prior knowledge of whether the sample was negative or positive. Informed consent from all participants was obtained prior to commencing the study.

2.5.2 User-validation: experiments

The user-validation was conducted in two phases: expert testing, in which employees with laboratory experience (QC and R&D employees) tested the complete biosensing workflow, and non-expert testing, in which employees without laboratory experience interpreted the icLFIA (see Figure 2).

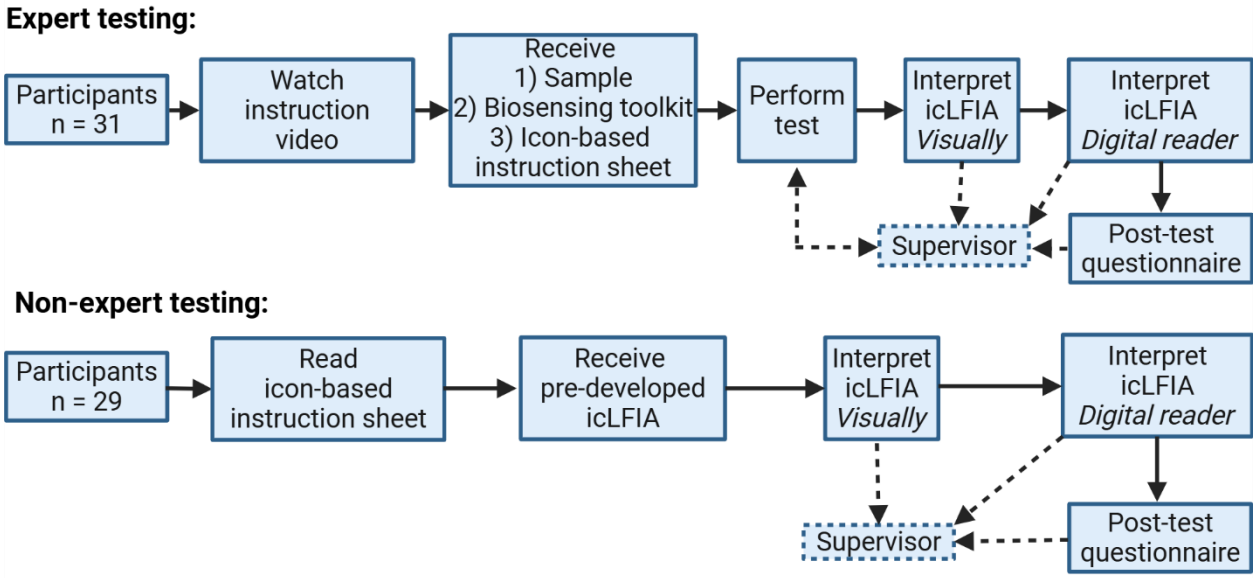


Figure 2. Schematic overview of the steps taken by the participants during the expert and non-expert testing of the user-validation (created with biorender.com). All steps taken by the participants are indicated with regular arrows, interactions between the participant and the test supervisor are indicated with dashed arrows. If the dashed arrow is one-sided this indicates that the experimental/questionnaire results of the participants are collected by the test supervisor. In case the dashed arrow is two-sided this indicates (the possibility for) questions of the participant to the test supervisor.

During the expert testing, the in-field accuracy and user-experience of the participants operating the biosensing toolkit was assessed. Once the participants arrived at the test location, they were instructed to watch an instructional video (see SI, Video). Subsequently, they received a falcon tube with 2 grams of blank (sample A) or spiked buckwheat cereals with 100 µg kg^{-1} atropine (sample B), the biosensing toolkit, and an icon-based instruction sheet (see SI, Figure S2). At that point, the participants could start to analyze their buckwheat cereal samples using the biosensing toolkit. During testing, the participants were allowed to ask any questions to the test supervisors in case they could not solve this with the icon-based instruction sheet. However, the test-supervisors noted all the questions asked. For analysis, the participants interpreted their icLFIA results, first visually and then with a digital reader. As reference, the test supervisors subsequently also measured the same icLFIAs using the digital reader. At the end of the assessment, the participants were asked to fill out a questionnaire (see SI, Questionnaire S1).

During the non-expert testing, the participants received a positive, negative, or invalid icLFIA and an icon-based instruction sheet on how to interpret these icLFIAs (see SI, Figure S3). Subsequently, the participants interpreted the icLFIA (first visually and then with a digital reader) and filled out their result in the questionnaire (see SI, Questionnaire S2). In case, a participant forgot to fill in a question in the questionnaire, this question was discarded.

2.5.3 User-validation: data evaluation

During the expert testing, the biosensing toolkit was evaluated on in-field accuracy and user-experience. In-field accuracy was defined as "the accuracy and completeness with which specified end-users can achieve specified goals in particular environments".[16] In the present study, in-field accuracy was further divided into two components: workflow accuracy and interpretation accuracy. Workflow accuracy was evaluated by checking the ability of the expert participants to obtain a correct icLFIA result when analyzing blank or contaminated buckwheat samples. Interpretation accuracy was evaluated by checking the ability of the expert participant to correctly interpret the icLFIA results. Interpretation accuracy was also assessed during the non-expert testing.

For workflow accuracy, after analysis of the buckwheat with the biosensing toolkit, the test supervisor also measured the intensities of the test-line (T-line) and control-line (C-line) with the digital reader. The software of the digital reader was used to obtain grey scale intensity values for each line, across the length of the icLFIA. The grey scale intensities were normalized by dividing the T-line by the C-line intensity, to obtain the T/C-ratio.[30] In the previously published laboratory validation of the icLFIA-based modular workflow for atropine detection, a T/C-ratio of 0.69 was established as the cut-off value between blank and contaminated buckwheat cereal samples with 5 μg kg^{-1} atropine (estimated FN-rate of 5% and estimated FP-rate of 2.71%).[17] Therefore, the workflow accuracy was quantified, following:

$$\text{Workflow accuracy} = \frac{(TP+NP)}{N} * 100\% \quad (1)$$

Where TP = true positive: total number of contaminated buckwheat cereal samples that obtained a T/C-ratio ≤0.69 (i.e. correctly identified as suspect), TN = true negative: total number of negative buckwheat cereal samples that obtained a T/C-ratio >0.69 (i.e. correctly identified as negative), and N = total number of blank and contaminated buckwheat cereal samples.

In addition, the obtained T/C-ratios were grouped by day to assess inter-day repeatability using a one-way ANOVA. Furthermore, stability was evaluated by comparing the T/C-ratios obtained for blank buckwheat cereal samples analyzed during the user-validation with those obtained during prior in-house testing approximately one month earlier using a T-test.

The interpretation accuracy was evaluated with a Kappa-score, in which the agreement between the interpretation of the expert and non-expert participants and the interpretation by the test supervisors with the digital reader (ground truth) were assessed, following:[31–35]

$$\text{Kappa-score} = \frac{p_o - p_e}{1 - p_e} \quad (2)$$

Where p_o = the observed agreement between the participants and the test supervisors, and p_e = the expected agreement between the participants and the test supervisors as a result of chance (see SI, Protocol S3 for the exact calculation of p_e and p_o).

The Kappa-scores were interpretated following: < 0 = poor agreement, 0.00–0.20 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and > 0.8 = almost perfect agreement.[35]

After the sample handling and interpretation was performed, the interpretation accuracy was calculated following Equation 1, only now using the interpretation results of the end-users.

User-experience was also divided into two components: ease-of-use and workload. Ease-of-use referred to how easily expert participants could operate the biosensing toolkit, while the perceived workload described the (e.g. mental and physical) resources required to do so.[16] The ease-of-use of the biosensing toolkit was evaluated using a system usability scale (SUS, see SI, Questionnaire S1, section Q5).[16,36] Following previous user-experience studies, to obtain more detailed information about the difficulty of the steps within the workflow, the participants were asked to answer 21 questions (3 open questions and 18 questions on a five-point Likert scale, see SI, Questionnaire S1, section Q1-Q4).[16,37] The 18 questions on a five-point Likert scale were evaluated using a difficulty score following:

$$\text{Difficulty score} = \sum_{i=1}^{5} (n_i \times 2^{5-i}) - n_t \quad (3)$$

Where n_i = the number of expert participants who selected response option *i*, based on how easy they found a step; *i* = 1: strongly disagree (i.e. not easy at all), *i* = 2: disagree, *i* = 3: neutral, *i* = 4: agree, *i* = 5: strongly agree (i.e., very easy), n_t = total number of participants.

This scoring method assigns higher weights to responses indicating greater perceived difficulty. Thus, the difficulty score reflects both the frequency and the severity of experienced difficulty as reported by expert participants.

The perceived workload was estimated using the raw NASA's task load index (TLX) score (see Questionnaire S1, section Q6). The raw TLX (i.e. without weighting the contribution of each factor in a predefined manner) was used as it has a high correlation with the weighted TLX, but is simpler and more efficient to apply.[16]

A priori acceptance criteria for the 3D-printed biosensing toolkit before the user-validation were defined as: (i) an in-field accuracy (workflow accuracy + interpretation accuracy) with a false-negative rate ≤5%, in line with EU Regulation 2023/2783; (ii) a SUS-score of ≥80/100, indicating good to best imageable usability and being consistent with industrial practice;[38] and (iii) a raw TLX-score of ≤30/100, reflecting a medium to low perceived workload.[39]

2.6.3 Biosensing toolkit expansion with a scopolamine-specific icLFIA: development and validation

2.6.1 Bioreagent characterization

Based on the feedback gathered during the user-validation, the biosensing toolkit for atropine detection was expanded for scopolamine by the development and a laboratory validation of a scopolamine-specific icLFIA.

For this reason, first, an antibody raised against scopolamine and synthesized scopolamine-BSA conjugate were evaluated for their sensitivity and specificity for seven different, but structurally-related tropane alkaloids in an indirect competitive microsphere-based immunoassay. For the synthesis protocol of the scopolamine-BSA conjugate see SI, Figure S4. The protocol for the indirect competitive microsphere-based immunoassay measurements has been described in the SI (see Protocol S4).[40] The median fluorescence intensity (MFI) of approximately 100 microspheres was measured with the MAGPIX™ system. After five-parameter logistic curve fitting, half-maximum inhibitory concentration (IC_{50}) for each of the seven different tropane alkaloids were estimated with GraphPad Prism version 10 (Domatics: Boston, USA). The specificity was expressed in terms of cross-reactivity (CR):

$$CR = \frac{IC_{50}[\text{target TA}]}{IC_{50}[\text{competing TA}]} \times 100\% \quad (4)$$ Where

CR = cross reactivity (%), IC_{50}[target TA] = half-maximum inhibitory concentration of the target tropane alkaloid (ng mL^{-1}), and IC_{50}[competitor TA] = half-maximum inhibitory concentration of another tropane alkaloid (ng mL^{-1}).[41]

2.6.2 Indirect competitive lateral flow immunoassay development

After the sensitivity and specificity of the scopolamine-specific antibody and scopolamine-BSA conjugate were determined, an icLFIA was developed, by optimizing the type of NC-membrane, RB-composition, drying buffer composition, and primary scopolamine-specific antibody, secondary goat anti-rabbit carbon-labelled antibody, scopolamine-BSA conjugate, and donkey anti-goat antibody concentrations. For a schematic overview of the final icLFIA, see SI (Figure S5). Scopolamine calibration standards of 0, 0.1, 0.5, 1, 5, 10, and 30 ng mL^{-1} in RB were prepared to test the sensitivity of the developed icLFIA in a 3D-printed icLFIA cassette. The icLFIAs were wetted with 100 µL of calibration standard solution and allowed to develop for 15 min. After 15 min, the intensities of the test-line (T-line) and control-line (C-line) were measured with a digital reader.

2.6.3 Laboratory validation

When the scopolamine-specific icLFIA was developed and integrated in the biosensing toolkit, it was validated in the lab for the detection of scopolamine in milled buckwheat cereals by measuring 24 blank and contaminated buckwheat samples spiked at 0.5× maximum level (5 µg kg^{-1}) and maximum level (10 µg kg^{-1}), distributed over three consecutive days. Each sample was analyzed following protocol S2 and the instruction video (see SI).[17] After analyzing the samples with the immunoassay, a cut-off value with a 5%-FN rate (β = 0.05) between blank and contaminated buckwheat cereal samples with 5 µg kg^{-1} scopolamine was determined, following EU regulation 2023/2783 (see SI, Protocol S5).[7]

3. RESULTS AND DISCUSSION

3.1 3D-printed biosensing toolkit development

To reduce the number of samples that need to be transported to high-end laboratories for LC-MS/MS analysis, assays enabling cost-effective screening at the point-of-need can be implemented.[4] Such screening assays for tropane alkaloids often rely on indirect competitive lateral flow immunoassays (icLFIAs), owing to the superior selectivity achieved through the use of antibodies.[3] However, icLFIAs already developed for tropane alkaloid detection have three major drawbacks: (i) they focus on relatively simple (viscous) liquid matrices (e.g. saliva, urine, or honey), (ii) have LODs above the European regulatory limits, or (iii) require a workflow with extensive laboratory-oriented sample preparation.[19,42–45] Only recently, we reported the first modular workflow for atropine detection in buckwheat cereals at regulatory levels.[17] To enhance user-friendliness, this modular workflow is now upgraded into a fully integrated 3D-printed toolkit (see Figure 3A for an overview, and SI, Video for a demonstration of usage of the biosensing toolkit). Although the main steps during operation of the biosensing toolkit are the same as in the modular workflow, the new PI-LPME cap (see Figure 3B) and the new PI-LPME holder (see Figure 3C) have been designed to enhance operational simplicity. For example, the PI-LPME cap allows the PI-LPME to be directly performed inside the SLE-tube, removing the necessity of transferring a part of the SLE-extract to an 2-mL Eppendorf tube. In addition, the new PI-LPME holder is directly connected to the icLFIA cassette via a plastic stopcock valve. This allows the running buffer, in which atropine is eluted, to flow directly onto the icLFIA in the 3D-printed cassette instead of manually removing a stopper. A cost and greenness assessment of the 3D-printed biosensing toolkit can be found in the SI (Table S3 and Figure S6).

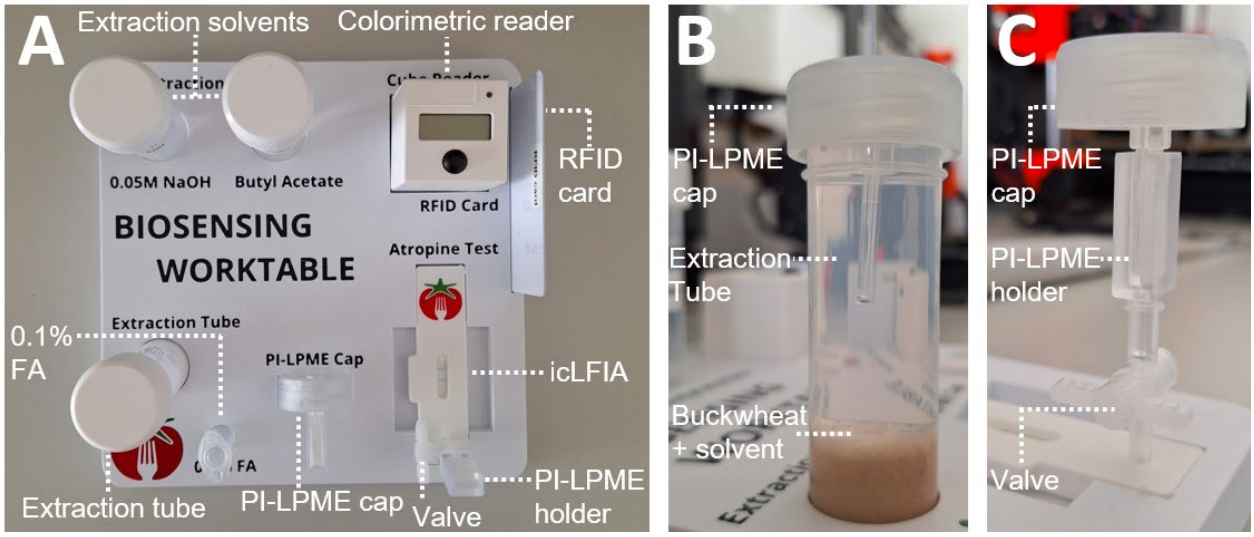


Figure 3. Overview of the 3D-printed biosensing toolkit with, (**A**) top view of the complete 3D-printed biosensing toolkit, (**B**) side view of the PI-LPME cap screwed on the extraction tube for a PI-LPME, and (**C**) side view of the PI-LPME cap being inserted in the PI-LPME holder which is connected to a plastic valve.

To evaluate the performance of the new biosensing toolkit and to assess whether the cut-off value (T/C ratio = 0.69) established during a previous laboratory validation of the modular workflow remained applicable, blank and contaminated buckwheat cereal samples containing 10 µg kg^{-1} atropine (the regulatory limit) were analyzed (see SI, Figure S7).[17] Using this threshold, all samples were correctly classified. Therefore, this cut-off was also used during the user-validation. In addition, during a pilot study at regulatory levels, five first-time users could clearly discriminate blank and contaminated buckwheat cereal samples containing 5 or 10 µg kg^{-1} atropine (see SI, Figure S8).

3.2 User-validation in industry

After the development of the biosensing toolkit, it was methodologically evaluated in a user-validation study at Barilla company (see SI, Figure S9 for photos taken during the user-validation). During this user-validation, the in-field accuracy and user-experience were assessed. The in-field accuracy was divided into two components: workflow accuracy (Section 3.2.1) and interpretation accuracy (Section 3.2.2), and user-experience was divided into: perceived workload (Section: 3.2.3) and ease-of-use (Section 3.2.4).

3.2.1 Workflow accuracy

To assess workflow accuracy, all icLFIA strips resulting from the analysis of the blank and contaminated buckwheat cereal samples by the expert participants were presented to a test supervisors, who subsequently recorded the T/C ratios (see Figure 4). In this manner, the user-induced uncertainty originating from the readout-step was excluded, allowing it to be studied separately for the assessment of the read-out accuracy (see Section 3.2.2).[17] When applying the previously established T/C threshold of 0.69, all blank samples had a T/C-ratio >0.69, while all contaminated samples had a T/C-ratio ≤0.69, ensuring a 100% classification accuracy (n = 31, see Figure 4). Despite this 100% classification accuracy, 3 out of 31 participants noted errors in there sample handling that potentially affected analytical performance. Specifically, one participant indicated that the cereals were not fully pre-wetted with NaOH, potentially resulting in a lower extraction recovery. Another participant reported that the PI-LPME paper was partially displaced from the PI-LPME frame during wetting, while a third participant experienced both issues. To prevent such sample-handling steps from occurring in the future, iterative improvements are further discussed in Section 3.2.4 (Ease of use and end-user feedback for iterative improvement). In addition, to prevent unnoticed sample-handling errors from leading to false-negative results, it is recommended to always include a positive reference sample contaminated at or near the regulatory limit, when implementing the biosensing toolkit.

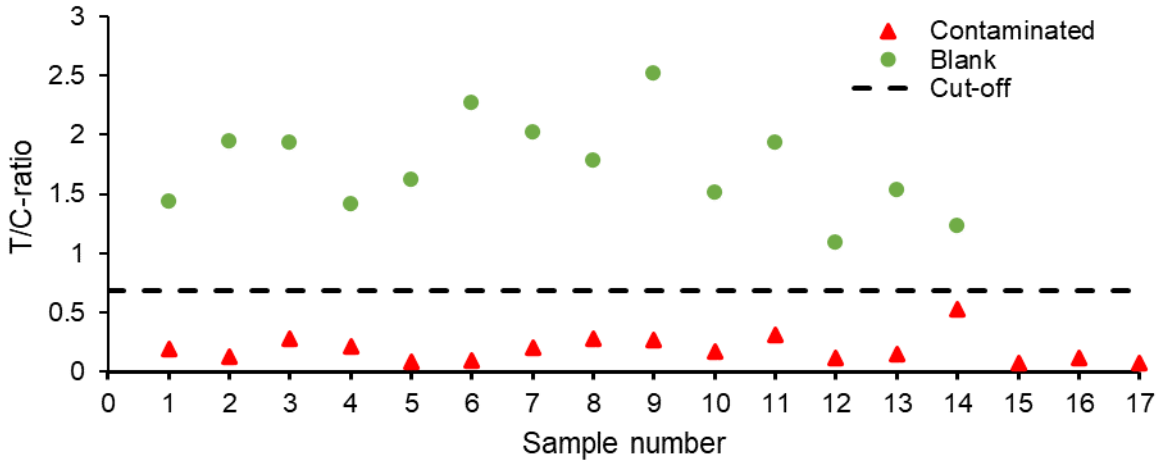


Figure 4. T/C-ratios of the icLFIA's measured by the test supervisors using the digital reader, after 31 R&D/QC-employers analyzed 14 blank and 17 spiked buckwheat samples with 100 µg kg^{-1}: T/C-ratio obtained for the blank buckwheat (green circles), T/C-ratio obtained for the contaminated buckwheat (red triangles), and cut-off (dashed black line).

In terms of repeatability, the biosensing toolkit had a high inter-day repeatability, as there were no significant differences in the icLFIA results over different days of the user-validation ($p > 0.05$). In addition, no significant differences were found between the T/C-ratios obtained after analyzing blank samples during the user-validation and those measured approximately one-month earlier ($p > 0.05$, see SI, Figure S7), which indicates good stability under storage conditions during transport and at the point-of-need.

3.2.2 Interpretation accuracy

Interpretation accuracy was defined as the ability of the end-user to interpret the corresponding icLFIA results both visually and with a digital reader. Digital read-out requires approximately 20 seconds (see SI, Video) while the time needed for visual interpretation can vary substantially depending on the perceived difficulty of the icLFIA result and the end-user. Given the interpretation results, the in-field accuracy of the expert end-users (i.e. participants with laboratory experience) was 97% with visual read-out and 100% with automated read-out (see Table 1). With this in-field accuracy, the first a priori criteria – (i) FN-rate <5% - has been accomplished, which indicates that with the biosensing toolkit its possible for the intended end-users to distinguish between blank and contaminated buckwheat cereal samples.

To assess our first hypothesis – (i) when analyzing blank or highly contaminated buckwheat samples, errors will arise more frequently from complex sample handling than from interpretation of the icLFIA result – the expert end-user workflow accuracy obtained was compared to their interpretation accuracy. For these end-users, the workflow accuracy was 100% (see Section 3.2.1) and the interpretation was in almost perfect agreement with the test supervisor when interpreting the icLFIA visually (Kappa-score = 0.94, 95CI = 0.81 - 1.0) or with a digital reader (Kappa-score = 1.00, see Table 1A). Therefore, hypothesis (i) was falsified, which

indicates that with the 3D-printed tools the complex sample handling required for the atropine-test was substantially simplified, leading to better performance than anticipated.

For our second hypothesis - automated icLFIA read-out will be more accurate then visual read-out - the results for the non-experts supported this hypothesis. Visual interpretation was only in moderate agreement (Kappa-score: 0.46, 95CI = 0.21 - 0.70) with the test supervisor, while the interpretation of the non-expert end-users significantly improved to almost perfect agreement (Kappa-score: 0.89, 95CI = 0.74 – 1.0), when using a digital reader (see Table 1B). These results indicate that especially for the non-expert end-user, interpretation by a digital reader is required. The invalid results from the non-expert group involved icLFIAs where the C-line was missing (intentional, during production). When the expert end-users operated the 3D-printed biosensing toolkit, these intentionally invalid icLFIA were not used. However, if such invalid tests had occurred, the digital reader is programmed to immediately detect the absence of the C-line and therefore would have classified the icLFIA as invalid.

For our third hypothesis – (iii) expert end-users will interpret icLFIA results more accurately than non-expert end-users - when interpreting icLFIA visually this hypothesis was supported, whereas when digital icLFIA-readout was used this hypothesis was falsified. Given that the non-expert end-users using digital read-out were able to interpret icLFIAs correctly, it may be feasible to train these regular employees (e.g. factory workers) to perform straightforward food safety tests in the future.

These results indicate that especially for the non-expert end-user, interpretation by a digital reader is required. This result is in line with prior user-validations with sandwich-format LFIAs, where the researchers reported that interpretation of a sandwich-format LFIA is often challenging especially when only a weak-test line is present.[16] It is important to note that with a sandwich-format LFIA, both strong and weak test lines indicate a positive result. In contrast, in a competitive-format LFIA, a strong test line indicates a negative sample, while a weak test line suggests a positive sample – potentially causing mis-classifications. As a result, the misinterpretation of a weak test line in competitive-format LFIAs may have more serious implications than in a sandwich-format LFIA, further highlighting the need for digital read-out.

Table 1. Comparison of interpretation of icLFIA results by (**A**) expert end-users and (**B**) non-expert end-users, using either visual inspection or a digital reader. Cells indicating correct identifications are highlighted in green, while those indicating incorrect identifications are highlighted in red.

A) Expert end-user

		Visual interpretation		
		Positive	Negative	Invalid
Ground truth	Positive (17)	16	1	0
	Negative (14)	0	14	0
	Invalid (0)	0	0	0

		Digital reader		
		Positive	Negative	Invalid
Ground truth	Positive (17)	17	0	0
	Negative (14)	0	14	0
	Invalid (0)	0	0	0

B) Non-expert end-user

		Visual interpretation		
		Positive	Negative	Invalid
Ground truth	Positive (12)	11	0	1
	Negative (12)	9	3	0
	Invalid (5)	0	0	5

		Digital reader		
		Positive	Negative	Invalid
Ground truth	Positive (12)	10	0	1
	Negative (12)	0	11	1
	Invalid (5)	0	0	5

3.2.3 Workload

Workload was defined as the amount of resources used to achieve the required goals, and was estimated by using a normalized NASA's TLX.[16] Several studies have found the TLX to be a valid measure to assess a subjective workload, consisting of: mental demand, physical demand, temporal demand, performance, effort, and frustration.[16,46] It has been used widely to measure workload in a broad range of applications in e.g. technology or healthcare domains.[16,47–49] Raw TLX (without weighting the contribution of each factor in a predefined manner) has a high correlation with the weighted TLX, but is simpler and more efficient to apply.[16] Therefore, in this study no weighting was applied for the TLX-score. The results of the normalized TLX-scores are 31 (±6) for mental demand, 21 (±6) for physical demand, 15 (±5) for performance, 24 (±5) for temporal load, and 18 (±3) for frustration (see SI, Figure S10). With an average TLX score of 24 (±8), the biosensing toolkit is classified to have only a medium subjective workload (see SI, Table S4 for a distribution of the TLX score per subgroup).[39] Furthermore, with this TLX, the second a priori acceptance criteria - (ii) TLX-score ≤ 30 - was successfully accomplished. Given this low workload, in the future, it is likely possible to let quality control personnel operate multiple biosensing toolkits at the same time.

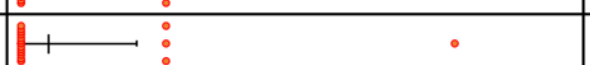
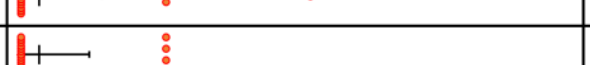
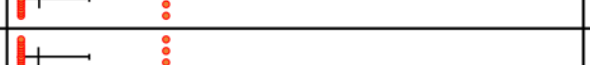
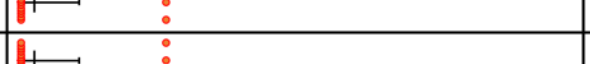
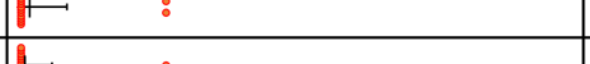
Moreover, compared to a previously reported AbC-19 LFIA (LFIA to check if a person has IgG antibodies against SARS-CoV-2), which analyzed a drop of blood from the finger, our test obtained a comparable TLX score of 24 (±8) vs 27 (±19).[16] It must be stated that our envisioned end-users were predominantly QC-personnel with laboratory experience, where in case of the AbC-19 LFIA it were regular citizens who frequently have no laboratory experience. On the other hand, in our method substantial sample pretreatment

was preformed – a step missing in many rapid (bio)sensing tools – yet this did not appear to increase the perceived workload.

3.2.4 Ease-of-use and end-user feedback for iterative improvement

The ease-of-use of the biosensing toolkit was assessed using a SUS. A SUS score of 88 (±8, n = 31) out of 100 was obtained, indicating an excellent (B) rating (see SI, Table S4 for a distribution of the SUS per subgroup).[38] With this SUS the third a priori acceptance (SUS > 80) was successfully accomplished, and above the generally acknowledged cut-off for industrial application, which indicates that in terms of ease-of-use, our biosensing toolkit is ready for actual implementation.[50] To further evaluate the ease-of-use of the individual steps within the workflow, participants responded to 3 open-questions (see SI, Table S5-7), and 18 questions on a five-point Likert scale, which were converted into a difficulty score (see Table 2). The newly developed difficulty score (observed range: 1 – 21) was used in addition to the traditional mean Likert score (observed range: 4.5 – 5.0), as it amplifies differences in perceived difficulty between workflow steps, thereby facilitating identification of steps that were perceived as more difficult by some participants. In addition, workflow steps that obtained a similar mean Likert score, could be differentiated using the new difficulty score (see SI, Figure S11A). For example, the 3 workflow steps that obtained a mean-Likert score of 4.7 obtained difficulty scores ranging between 12-18, due to differences in the individual participant responses (see SI, Figure S11B).

Table 2. Participant responses to the questionnaire items, along with the corresponding difficulty scores (see Materials and Methods, Equation 3). Individual responses are marked with red circles, the vertical bar indicates the mean response, and the error bars represent the standard deviation (n = 31).

Question	Response: (5 = strongly agree, 1 = strongly disagree) 5 4 3 2 1	Difficulty score
Q1e: The extraction tube was easily shaken manually		21
Q2e: The PI-LPME cap was easily inserted in the icLFIA holder		18
Q3c: The tap on the PI-LPME holder was easily opened		17
Q3f: The measurement on the colorimetric reader was easily started		17
Q2b: The 2 mL Eppendorf tube with 0.1% FA was easily identified		15
Q2c: The paper inside the PI-LPME cap was easily wetted with 10 µL 0.1% FA		15
Q3e: The colorimetric reader was easily connected to the icLFIA		12
Q3g: The output of the colorimetric reader was easily interpreted		10
Q3a: The colorimetric reader was easily identified		8
Q3d: The atropine-test was easily interpreted visually		7
Q2a: The PI-LPME cap was easily identified		6
Q1d: The 4 mL 0.05M NaOH solution was easily added to the extraction tube		5
Q1a: The tube with 4 mL 0.05M NaOH solution was easily identified		4
Q1b: The tube with 20 mL butyl acetate was easily identified		4
Q3b: The RFID card was easily identified		3
Q1f: The 20 mL butyl acetate was easily added to the extraction tube		3
Q1c: The extraction tube with 2 gram of cereals was easily identified		2
Q2d: The PI-LPME cap was easily screwed upon the extraction tube		1

In the first section of the workflow (SLE), only one step – "shaking the extraction tube manually" (Question 1e, difficulty score = 21) – received a difficulty score above the average of 9. In the corresponding open-ended question, 4 out of 31 participants mentioned difficulty in determining the appropriate shaking speed (see SI, Table S5: Response 1-4). For this reason, 2 out of 31 participants noted that their cereal samples were not fully pre-wetted with NaOH solution before adding the butyl acetate (See SI, Table S5: Response 2 and 3). To address these challenges, the instructions in the icon-based instruction sheet have been improved by modifying the phrase "Shake for five seconds" to "Shake vigorously for five seconds, to fully wet the cereals" (see SI, Figure S12; step 2, for the adjusted version of the icon-based instruction sheet).

In the second section of the workflow (PI-LPME), three steps received a difficulty score above the average. Two of these steps – "wetting the PI-LPME paper with 10 µL 0.1% FA" (difficulty score = 15, Question Q2c) and "inserting the PI-LPME cap into the icLFIA holder" (difficulty score = 18, Question Q2e) – were related. In the open-ended responses, 2 out of 31 participants reported that while pipetting, the PI-LPME paper was partially pushed out of the PI-LPME frame (see SI, Table S6: Response 1 and 2). As a result, inserting the frame with the displaced paper into the icLFIA holder became a challenge. A potential improvement could be

to implement a slightly modified 3D-printed design with a fixed paper to prevent this issue. The other step that obtained a difficulty score above the average was – "identifying the 2 mL Eppendorf tube containing 0.1% FA" (difficulty score = 15, Question Q2b). Four participants mistakenly pipetted butyl acetate from the SLE section onto the PI-LPME paper instead of formic acid. However, all of them recognized their mistake themselves directly, when in the subsequent step they should place the PI-LPME frame into the SLE-holder. After asking the test supervisors, they were provided with a new PI-LPME cap to repeat this step. In order to avoid this mistake in the future, the use of color-coded labels on the tubes was suggested multiple times by the expert participants (See SI, Table S5: Response 5 and 6, and Table S6: Response 3). Notably, the four requests for a new PI-LPME cap were the only four questions asked to the supervisors during the user-validation. This indicates that the biosensing toolkit demonstrates high operational robustness, as it can be operated using only provided instruction material (instruction video and icon-based instruction sheet), and additional equipment was only required during 4 out of 31 operations. Although the use of an additional PI-LPME cap has resulted in a small amount of extra waste, participants independently recognized their mistake and requested a replacement. Thus, providing an additional cap did not result in an overestimation of field-performance. In real-world settings a QC-lab would have access to sufficient biosensing toolkit components to perform multiple tests. Consequently, operators would simply retrieve a replacement item from the available supply when needed, rather than requesting one.

In the final section of the workflow (icLFIA analysis), four steps received a difficulty score above the average (>9). The two most challenging steps – "opening the tap on the PI-LPME holder" (Question Q3c) and "starting the measurement using the digital reader" (Question Q3f) – both obtained a difficulty score of 17. For opening the tap, 2 participants noted difficulty in determining whether the tap was open or closed (see SI, Table S7: Response 1 and 2). A possible reason for this confusion is that the wicking of the solution through the icLFIA takes time, and the liquid front only becomes visible approximately one minute after opening the tap. Providing more clear instructions on the open and closed positions of the icLFIA tap can overcome this unclarity (see SI, Figure S12; step 10, for an adjusted version of the instructions). In addition an evaluation of the development of the T/C-ratio when analyzing blank or contaminated buckwheat samples has been performed, and highlights the importance to only analyze the atropine-test 15 minutes after opening the tap (see Figure S13). To make this more clear for the end-user the instructions in step 11 has been more specified (see SI, Figure S12; step 11). For starting the measurement with the digital reader, currently, it requires two manual presses and one press with the RFID card, limiting current user-experience. The other two steps with difficulty scores >9 were "positioning the digital reader over the icLFIA" (difficulty score = 12, Question Q3e) and "interpreting the output of the digital reader" (difficulty score = 10, Question Q3g). If the digital reader was positioned incorrectly over the icLFIA, it generated an error response, ensuring that incorrect placement did not affect the identification process. For the interpretation of the output of the digital reader the results in Table 1A show that this did not affect the final identification as all the identifications were correct. To guide future operators of the 3D-printed biosensing toolkit in dealing with potential sample-handling errors and their consequences, a risk

assessment has been integrated in the icon-based instruction sheet (see SI, Figure S12). If any of these sample-handling errors are observed and the analysis result is negative, the analysis should be repeated.

One additional point raised during the industrial user-validation was that tropane alkaloids are regulated as the sum of atropine and scopolamine with a limit of 10 µg kg^{-1} in milled buckwheat cereals (EU regulation 2023/915). Reviewing all reported cases of tropane alkaloid contamination in buckwheat cereals within the European Union from 2010 to 2023, atropine was consistently the tropane alkaloid present at the highest concentration.[17,25] Therefore, if atropine levels are below 5 µg kg^{-1} it is likely that scopolamine levels are also below 5 µg kg^{-1}, meaning the legal limit is not exceeded. However, companies would likely prefer to be on the safe side and therefore screen their food commodities for both atropine and scopolamine. In response to this, a scopolamine-specific icLFIA was developed enabling scopolamine detection with the 3D-printed biosensing toolkit (see Section 3.3).

3.3 Expanding the biosensing toolkit with a scopolamine-specific icLFIA: development and validation

3.3.1 Bioreagent characterization

In order to develop a scopolamine-specific icLFIA, first the sensitivity and specificity of the antibody raised against scopolamine was determined for seven different tropane alkaloids using an indirect competitive microsphere-based immunoassay (see SI, Figure S14 and Table S8). The lowest IC50-value was obtained for scopolamine (0.22 ng mL^{-1}), followed by anisodine (1.38 ng mL^{-1}, CR = 15%) and aposcopolamine (6.68 ng mL^{-1}, CR = 3.4%). Based on these CR-values, the presence of anisodine at ~66 µg kg^{-1} or aposcopolamine at ~294 µg kg^{-1} in buckwheat would be expected to produce an assay response comparable to that of scopolamine at ~10 µg kg^{-1} (EU-regulatory limit). However, the risk on false positive results due to anisodine or aposcopolamine is low, as these compounds are typically present at substantially lower concentrations than atropine or scopolamine.[25,51] For example, in a recent occurrence study in which 268 single component flours (including buckwheat) and 578 cereal-based products were analyzed, anisodine and aposcopolamine were only present above LOD in ≤1% of samples, compared to ≥5% for scopolamine and ≥10% for atropine.[51] Moreover, in the positive samples, average concentrations of anisodine and aposcopolamine were approximately tenfold lower than those of scopolamine and atropine.

3.3.2 icLFIA development

The scopolamine-specific antibody and in-house synthesized scopolamine-BSA conjugate were integrated in an icLFIA (see SI, Figure S5), and its sensitivity for scopolamine was tested in running buffer. Here, an IC_{50}-value of 0.86 ng mL^{-1} (see SI, Figure S15) was obtained, making its sensitivity comparable to previously reported icLFIAs for scopolamine detection (IC_{50}-values: 0.51 – 1.03 ng mL^{-1}).[44,52] For a comparison between the atropine-specific and scopolamine-specific icLFIA see SI (Table S9).

3.3.3 Laboratory validation of the 3D-printed biosensing toolkit with a scopolamine-specific icLFIA

Subsequently, the scopolamine-specific icLFIA was integrated in the 3D-printed biosensing toolkit, and validated by analyzing blank and contaminated buckwheat cereal samples spiked with scopolamine (see Figure 5). A T/C-ratio of 0.70 was established as cut-off value between blank and contaminated buckwheat cereal samples with 5 µg kg^{-1} scopolamine (estimated FN-rate of 5% and estimated FP-rate of 1.6%).[17] Therefore, our biosensing toolkit with the scopolamine-specific icLFIA can detect scopolamine at regulatory levels in buckwheat cereals and, together with the previously validated icLFIA for atropine, help companies to screen their food commodities for both atropine or scopolamine.

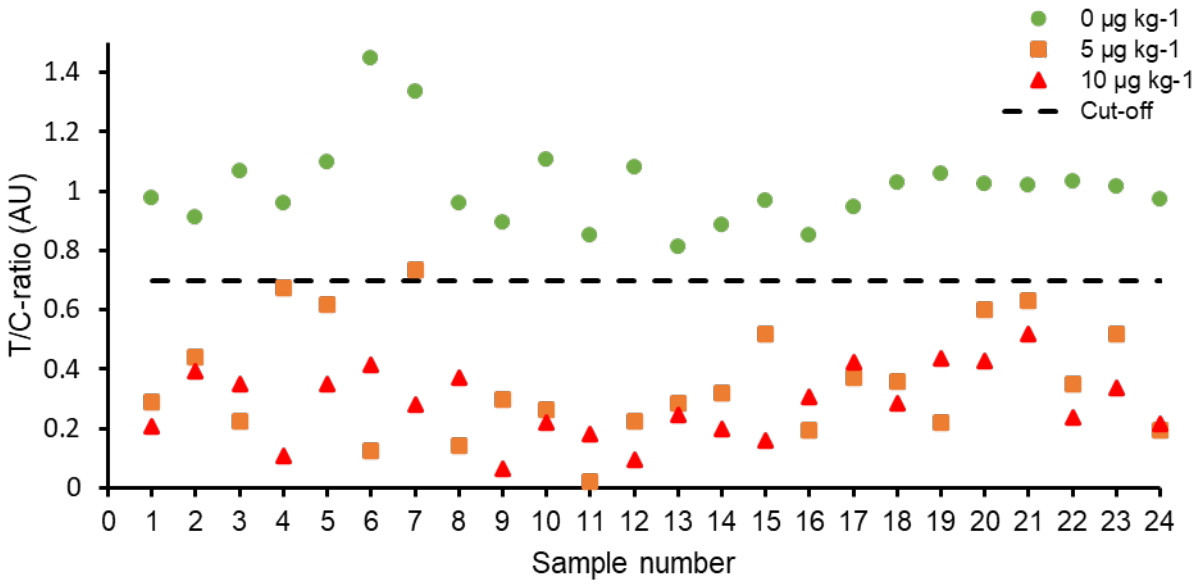


Figure 5. T/C ratios of the icLFIA after analyzing 24 blank and spiked buckwheat samples at 5 µg kg^{-1} (0.5× maximum level) and 10 µg kg^{-1} (1.0× maximum level) scopolamine with the modular workflow: T/C ratio for the blank samples (green circles), 5 µg kg^{-1} samples (orange squares), or 10 µg kg^{-1} samples (red triangles), and cut-off with an estimated false negative rate (FN) of 1% (dashed black line).

4. OUTLOOK

Here, a 3D-printed biosensing toolkit that allows for either the detection of atropine or scopolamine in buckwheat cereals at regulatory levels is reported. During the user-validation in industry, the biosensing toolkit was well-received by end-users and – given that a digital reader is used – the in-field accuracy was good. Therefore, our biosensing toolkit represents a promising tool for food companies and food safety institutions to screen buckwheat cereals on the presence of atropine or scopolamine, especially considering that a rapid screening workflow

is often more cost-effective than traditional LC-MS/MS workflows.[9] Further research will focus on expanding the biosensing toolkit workflow with other (potentially aptamer-based) LFAs for strictly regulated food contaminants in the field of natural toxins. Perhaps most importantly, the methodological user-validation underscores the importance of evaluating point-of-need tools for food safety screening in terms of their user-experience and in-field accuracy, ensuring a better understanding of their eventual performance in the field. This study thus provides a framework that can adopted more broadly for providing evidence for claims about ease-of-use and application potential at the point-of-need for innovative (bio)sensing approach in and beyond food safety.

5. DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in this paper.

6. DECLARATION OF AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During preparation of this work the authors used ChatGPT to evaluate the readability of several sentences. After using this tool/service, the authors reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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SUPPLEMENTARY INFORMATION

Table of Contents

Table S1. Prusa Original i3 MK3S+ slicer settings used to print the SLE-filter attachment and indirect competitive lateral flow immunoassay cassette.

Settings	
Layer height	0.1 mm
Solid layers top	9 layers
Solid layers bottom	7 layers
Infill density	15%
Infill pattern	Gyroid
Filament	PolyLite PLA Pro
Perimeter print speed	35 mm/s
Supports print speed	50 mm/s
Nozzle temperature first layer	200°C
Nozzle temperature	190°C
Bed temperature	60°C
Filament cooling	100%
Nozzle diameter	0.4 mm
Retraction	0.4 mm
Z-lift	0.2 mm

Protocol S1. How to obtain the required number of 2-gram replicate samples from bulk lots of milled buckwheat cereals for analysis with the biosensing toolkit in accordance with EU Regulations 2023/2782 and 2023/2783.

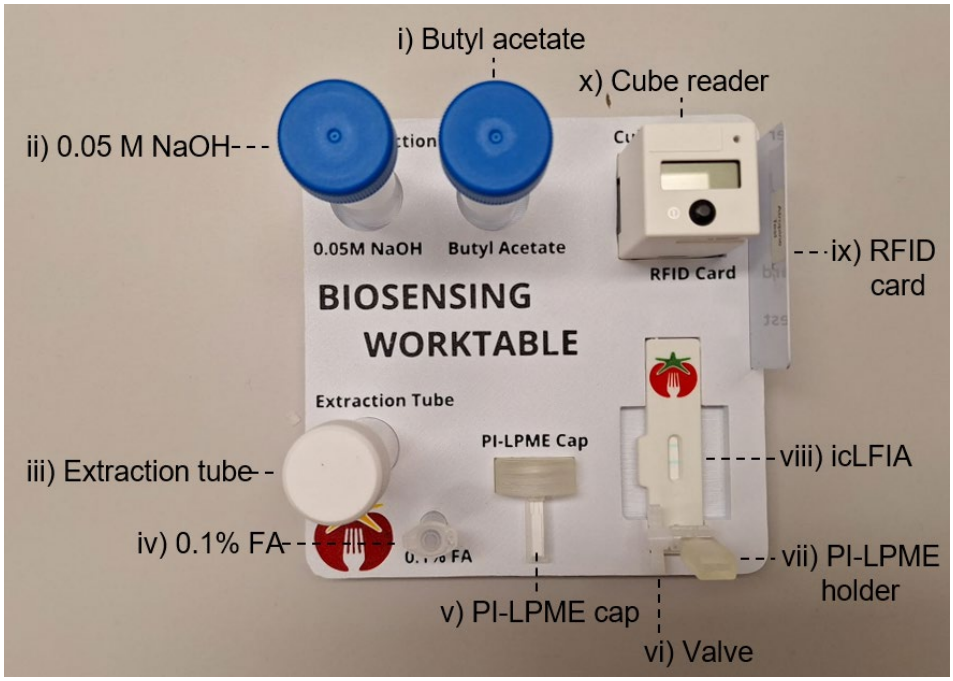
The buckwheat cereals arriving at Barilla SPA are already pre-milled (particle size < 0.2 mm). Subsequently, EU-regulation 2023/2782 specifies that the number of incremental samples that should be sampled and combined in one aggregate sample are:

Lot weight (tonnes)	**Number of incremental samples**	**Aggregate sample weight (kg)**
≤0.05	3	0.25
>0.05 - ≤0.5	5	0.25
>0.5 - ≤1	10	0.25
>1 - ≤3	20	0.5
>3 - ≤10	40	1.0
>10 - ≤20	60	1.5
>0.05 - ≤100	100	2.5

Following Bouzembrak *et al.*[1], particularly for low-level contaminations, the best way to obtain these incremental samples is by using a systematic sampling strategy, rather than simple random sampling or stratified random sampling.

After combining the incremental samples to one aggregate sample, this aggregated sample should be thoroughly mixed to ensure it is well homogenized. After which, samples of 2 gram of the aggregated sample can be analyzed with the biosensing toolkit.

Protocol S2. Step-by-step operating procedure for the 3D-printed biosensing toolkit equipped with the indirect competitive lateral flow immunoassay



Overview of all components of the 3D-printed biosensing toolkit, and their corresponding identification number (as used throughout the protocol): i) 20 mL butyl acetate, ii) 4 mL 0.05M NaOH, iii) Extraction tube, iv) 0.1% FA, v) PI-LPME cap, vi) Valve, vii) PI-LPME holder, viii) icLFIA, ix) RFID card, x) Cube reader

Workflow step i) Solid liquid extraction (SLE) – Duration: 30 min

1) Add 4 mL of 0.05M NaOH (ii) to the extraction tube (iii) containing 2 gram of buckwheat cereals
2) Shake the extraction tube (iii) vigorously for 5 seconds – until all cereals are wetted with NaOH
3) Add 20 mL butyl acetate (i) to the extraction tube (iii)
4) Shake the extraction tube for 30 minutes in an overhead-shaker

Workflow step ii) Paper-immobilized (SLE) – Duration: 20 min

5. Pipette 10 µL 0.1% FA (iv) on the paper in the PI-LPME cap (v)
6. Screw the PI-LPME cap (v) on the extraction tube (iii)
7. Wait for 20 minutes, while the scopolamine is concentrating in the acidic solution immobilized in the paper

Workflow step iii) Indirect competitive lateral flow immunoassay (icLFIA) – Duration 25 min

8. Unscrew the PI-LPME cap (v) and insert it in the PI-LPME holder (vii)
9. Wait for 10 minutes, to elute the tropane alkaloids from paper
10. Open the valve (vi) on the icLFIA (viii)
11. Wait for 15 minutes, while the icLFIA (viii) develops
12. Connect the cube reader (x) to the icLFIA
13. Press the black button of the Cube reader (x) twice manually
14. Press the black button of the Cube reader (x) with the RFID card (ix) to start the measurement
15. Read-out the results

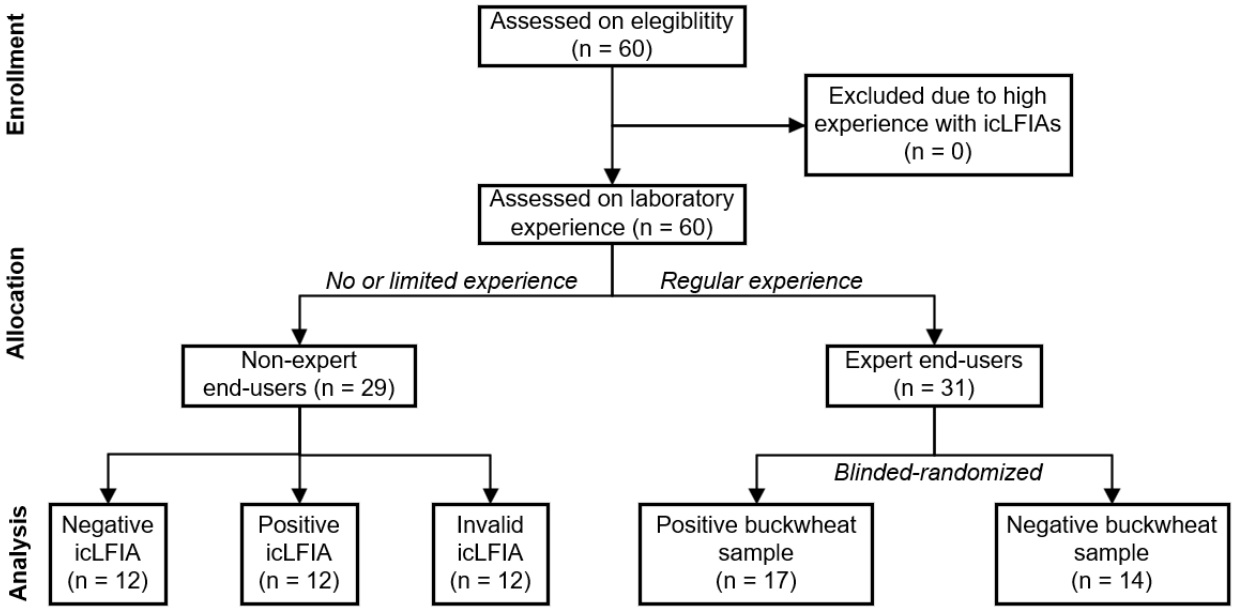


Figure S1. Flow diagram showing the participant inclusion/exclusion, allocation to expert or non-expert group, and distribution of the samples.

Table S2. Detailed information about the participants of the user-validation regarding their (**A**) education level, (**B**) job description, (**C**) age distribution. The participants of the user-validation were split into two groups: 1) experts, participants with regular laboratory experience, either current or past, and 2) non-experts, participants without or with very limited laboratory experience.

A

Education level		
	Expert (n = 31)	**Non-expert (n = 29)**
High school	16	12
Graduate (e.g. Bachelor/Master)	12	17
Post-graduate (e.g. PhD)	3	0

B

Job description		
	Expert (n = 31)	**Non-expert (n = 29)**
Quality Control	24	7
Research and Development	7	3
Factory employee	0	13
Desk-worker	0	6

C

Age distribution		
	Expert (n = 31)	**Non-expert (n = 29)**
20 – 35	14	21
36 – 50	7	6
51 – 68	10	2

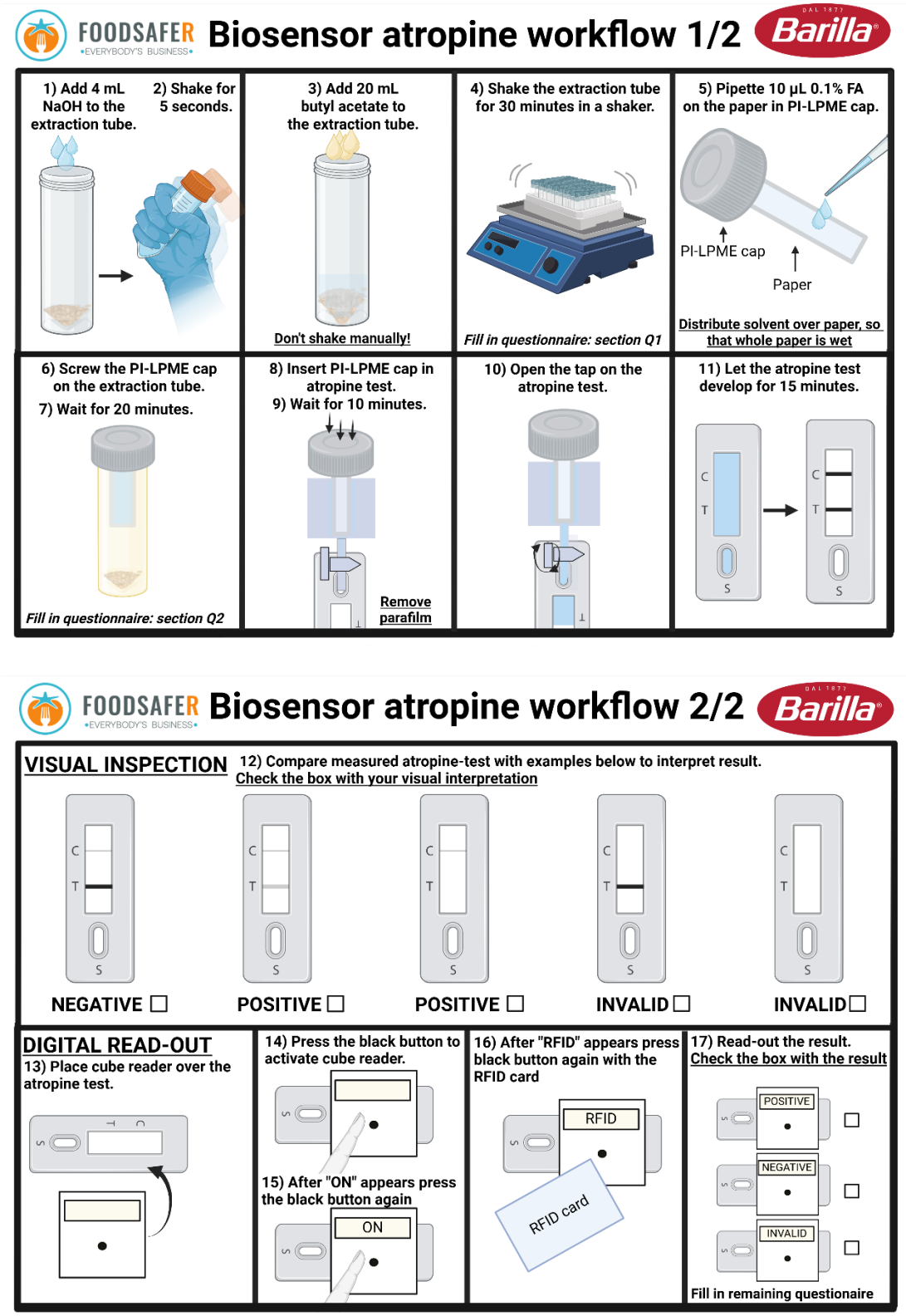


Figure S2. Icon-based instruction sheet for operating the 3D-printed biosensing toolkit, received by participants during the expert testing phase of the user-validation.

Questionnaire S1. Post-test questionnaire received by the expert participants after they analyzed a buckwheat cereal sample with the biosensing toolkit on the presence of atropine.

This questionnaire has been compiled to establish your user-experience of the rapid atropine test in buckwheat cereals. Please complete the details below and complete the questionnaire

Age .....
Job description .....
Highest education level .....
Laboratory experience None / Minor / a lot

Which buckwheat cereals was received: A or B

Results

Was the test completed and a control line obtained	□ Yes □ No
If the test was not completed, please provide details on reason for failure .... …….. ……..	
The result of the test after visual inspection was	□ Negative □ Positive □ Invalid □ Not sure
The result of the test after inspection with digital read-out was	□ Negative □ Positive □ Invalid □ Not sure

Questionnaire

Section Q1: Workflow step I) Solid liquid extraction **1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree**					
	1	**2**	**3**	**4**	**5**
Q1a: The tube with 4 mL 0.05M NaOH solution was easily identified					
Q1b: The tube with 20 mL butyl acetate was easily identified					
Q1c: The extraction tube with 2 gram of cereals was easily identified					
Q1d: The 4 mL 0.05M NaOH solution was easily added to the extraction tube					
Q1e: The extraction tube was easily shaken manually					
Q1f: The 20 mL butyl acetate was easily added to the extraction tube					
Q1g: Were there any other things that caused difficulties during this step of the biosensor Yes □ No □ Explain ……... …… …… …… ……					

Section Q2: Workflow step II) Paper-immobilized liquid phase micro extraction (PI-LPME) **1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree**					
	1	**2**	**3**	**4**	**5**
Q2a: The PI-LPME cap was easily identified					
Q2b: The 2 mL Eppendorf tube with 0.1% FA was easily identified					

Q2c: The paper inside the PI-LPME cap was easily wetted with 10 µL of 0.1% FA					
Q2d: The PI-LPME cap was easily screwed upon the extraction tube					
Q2e: After the extraction, the PI-LPME cap was easily inserted in the icLFIA holder					
Q2f: Were there any other things that caused difficulties during this step of the biosensor Yes □ No □ Explain ……... …… …… ……					

Section Q3: Workflow step III) icLFIA analysis **1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree**					
	1	**2**	**3**	**4**	**5**
Q3a: The digital reader was easily identified					
Q3b: The RFID card was easily identified					
Q3c: The tap on the PI-LPME holder was easily opened					
Q3d: The atropine-test was easily interpreted visually by comparing it with the examples in the instruction sheet					
Q3e: The digital reader was easily connected to the icLFIA					
Q3f: The measurement on the digital reader was easily started					
Q3g: The output of the digital reader was easily interpreted					
Q3h: Were there any other things that causes difficulty during this step of the biosensor Yes □ No □ Explain ……... …… …… ……					

Section Q4: Instructions before use **1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree**					
	1	**2**	**3**	**4**	**5**
Q4a: The provided video instructions were easy to follow					
How many times during the workflow did you consult the video instructions 1-3 □ 4-6 □ 7-9 □ 10+ □					
Q4b: The provided written instructions were easy to follow					
How many times during the workflow did you consult the written instructions 1-3 □ 4-6 □ 7-9 □ 10+ □					
Q4c: The provided icon-based instructions were easy to follow					
How many times during the workflow did you consult the icon-based instructions 1-3 □ 4-6 □ 7-9 □ 10+ □					

Section Q5: System Usability Scale **1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree**					
	1	**2**	**3**	**4**	**5**
Q5a: If I need to analyze buckwheat cereals for atropine contamination, I think that I would like to use this system more frequently					
Q5b: I found the system unnecessarily complex					
Q5c: I thought the system was easy-to-use					
Q5d: I think that I would need the support of a technical person to be able to use this system					
Q5e: I found the various components in this system well integrated					
Q5f: I thought there was too much inconsistency in this system					
Q5g: I would imagine that most people would learn to use this system very quickly					
Q5h: I found the system very awkward to use					

Q5i: I felt very confident using the system					
Q5j: I needed to learn a lot of things before I could get going with this product					

Section Q6: Nasa TLX score **1 = low, 7 = High**							
	1	**2**	**3**	**4**	**5**	**6**	**7**
Q6a: How mentally demanding was the task?							
Q6b: How physically demanding was the task?							
Q6c: How hurried or rushed was the pace of the task							
Q6d: How successful were you in accomplishing what you were asked to do?							
Q6e: How hard did you have to work to complete the task and get a result							
Q6f: How insecure, discouraged, irritated, stressed, and annoyed were you?							

Additional feedback

Is there any additional feedback covered above that you would like to provide regarding your experience when operating the biosensing toolkit

.....

.....

……..

.....

.....

……..

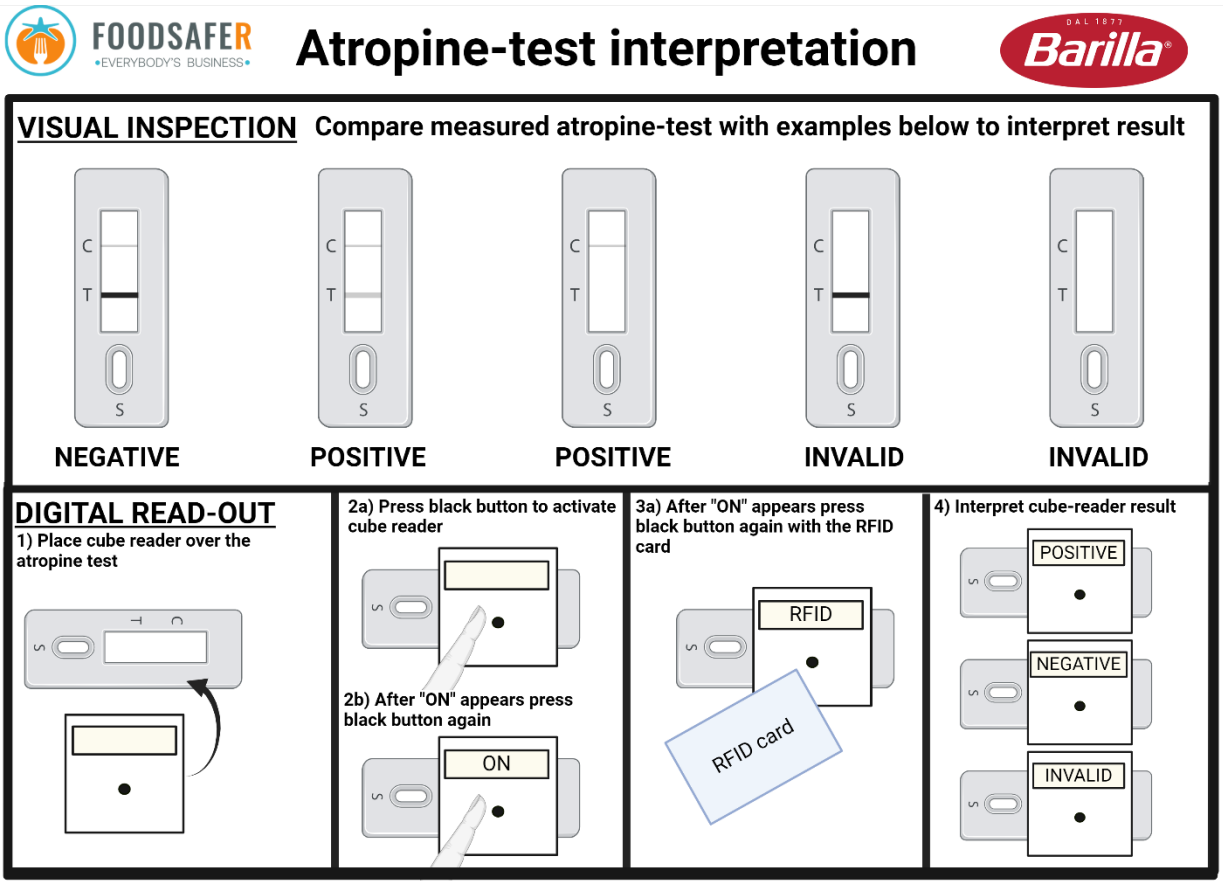


Figure S3. Icon-based instruction sheet for interpreting the indirect competitive lateral flow immunoassay, received by the participants during the non-expert testing phase of the user-validation.

Questionnaire S2. Questionnaire received by the non-expert participants of the user-validation

This questionnaire has been compiled to establish your user-experience of the rapid atropine test in buckwheat cereals. Please complete the details below and complete the questionnaire

Age ……
Job description .....
Highest education level .....
Laboratory experience None / Minor / a lot

Which predeveloped atropine-test was received: A, B, C, D, E

Results

The result of the test after visual inspection was	□ Negative □ Positive □ Invalid □ Not sure
The result of the test after inspection with digital read-out was	□ Negative □ Positive □ Invalid □ Not sure
If the atropine test could not be interpretated, please provide a reason …… ……..... …… …….. …… ……	

Protocol S3. Calculation for the observed agreement (p_o) and the expected agreement as a result of chance (p_e) between the participants and test supervisors interpreting an indirect competitive lateral flow immunoassay (icLFIA).

The observed agreement between the participants and the test supervisor (p_o) was calculated following:

(1) $$p_o = \frac{n_a}{n}$$

Where n_a = total number of icLFIA's which were classified identically by the participants and the test supervisor, n = total number of icLFIA's that were classified by the participants and the test supervisor.

The expected agreement between the participants and the test supervisors as a result of chance (p_e) was calculated following:

(2) $$p_e = \frac{PP * PS}{n^2} + \frac{NP * NS}{n^2} + \frac{IP * IS}{n^2}$$

Where, PP = total number of icLFIAs that were identified as positive by the participant, PS = total number of icLFIAs that were identified as positive by the test supervisor, NP = total number of icLFIAs that were identified as negative by the participant, NS = total number of icLFIAs that were identified as negative by the test supervisor, IP = total number of icLFIAs that were identified as invalid by the participant, IS = total number of icLFIAs that were identified as invalid by the test supervisor.

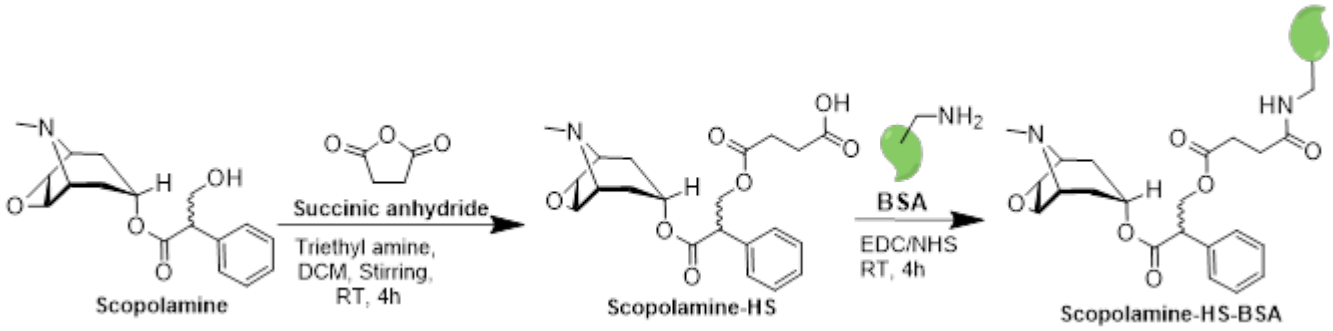


Figure S4. Synthesis of the scopolamine-bovine serum albumin (BSA) conjugate.

The pathway for conjugate synthesis is shown in Figure S4. In the first step scopolamine-succinic anhydride is synthesized by mixing 524 mg scopolamine (SCO), 259 mg succinic anhydride (HS), and 260 mg Triethylamine in 10 mL dichloromethane (DCM). Subsequently, the mixture was stirred for 4 hours at RT. After 4 hours, 10 mL 0.1% formic acid in H_2O was added. Approximately, 8 mL of the aqueous solution was recovered and the presence of HS-SCO checked using a high pressure liquid chromatography-high resolution mass spectrometry. Subsequently, SCO-HS was purified using preparative HPLC after which the fractions with SCO-HS were evaporated using freeze drying.

HS-SCO was subsequently, coupled to bovine serum albumin (BSA) by dissolving 1.7 mg HS-SCO with 1.5 mg N-hydroxysuccinimide (NHS), and 2.5 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) 300 µL DMF (solution A). The solution was stirred for 8 hours using a micro stirrer. In the meantime, 4 mg of BSA was dissolved in 0.01M phosphate-buffered saline (PBS) buffer (solution B). Subsequently, solution A was dropwise added to solution B and stirred overnight. The next day, the reaction solution was pipetted into a dialysis bag, where it was kept for 3 days. Every day, the PBS buffer was replaced. After the synthesis, the SCO-BSA conjugate was tested in an indirect competitive microsphere immunoassay using a polyclonal anti-scopolamine antibody obtained from CER Groupe (Marloi, Belgium).

Protocol S4. Protocol for the indirect competitive microsphere-based immunoassay (icMI) measurements.[2]

The icMI measurements were performed in a polystyrene 96-well microplate with flat bottom. The scopolamine-bovine serum albumin (BSA) conjugates were immobilized on paramagnetic MagPlex microspheres (No. 55) with a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysulfosuccinimide (Sulfo-NHS) coupling as described by Angeloni et al.[3] Ten-fold serial dilutions of standards from atropine, scopolamine, homatropine, aposcopolamine, anisidine, anisodamine, and tropine (ranging from 0.01 ng mL^{-1} to 1000 ng mL^{-1}) were added to individual wells (100 µL per well). Subsequently, the icMI was performed by adding 10 µL scopolamine-BSA coated microspheres (~100 per well) and 10 µL of anti-scopolamine antibody (4.5 µg mL^{-1}) obtained from CER Groupe (Marloie, Belgium). The plate was incubated at room temperature for 20 min with moderate shaking (400 rpm), and then washed with PBST buffer (0.01M phosphate-buffered saline, 0.1% BSA, 0.02% tween 20; 100 µL/well) twice using a magnetic plate separator (Sigma-Aldrich Co.; St. Louis, USA). The fluorescently labeled secondary antibody, R-phycoerythrin coupled goat-anti-rabbit IgG, was then added to the plate (100 µL per well) to a final concentration of 2 mg L^{-1}. After another 20 min incubation, followed by two washing steps with PBST, the microspheres were resuspended in 100 µL of PBST. After which they were measured with the MAGPIX™ system.

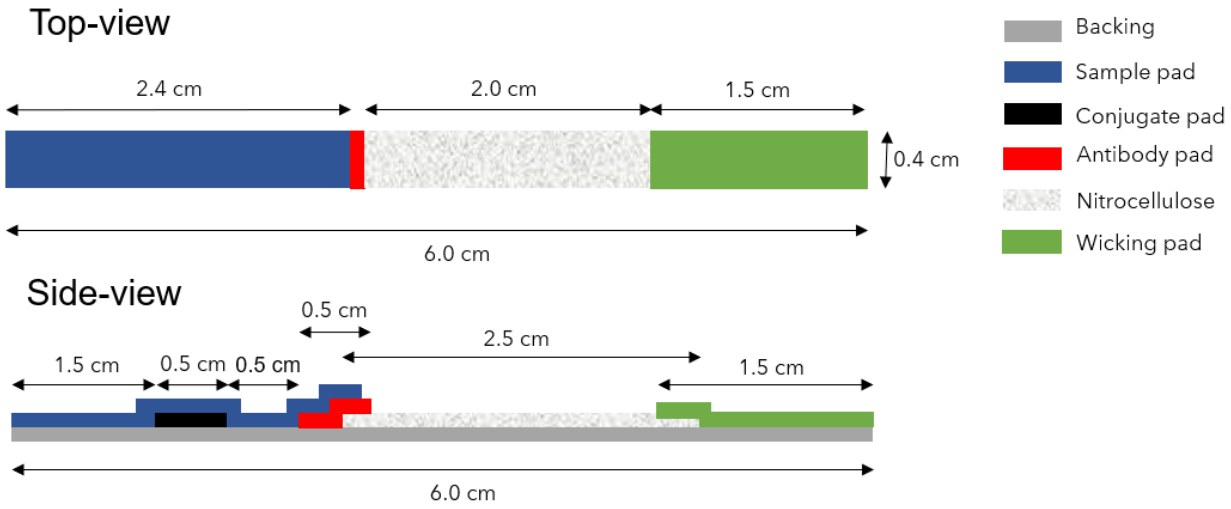


Figure S5. Schematic overview of the developed indirect competitive lateral flow immunoassays.

On the nitrocellulose (NC) scopolamine-bovine serum albumin (BSA) conjugate (0.5 mg mL^{-1}) for use on the test line and donkey anti-goat IgG antibody (0.15 mg mL^{-1}) for use on the control line were dissolved in 0.01 M PBS buffer, and subsequently sprayed onto NC membranes (1 µL per strip). Polyclonal anti-scopolamine antibody (32 µg mL^{-1}) for the antibody pad and goat anti-rabbit IgG antibody coupled to carbon nanoparticles for the conjugate pad were dissolved in 0.005 M PBS buffer with 1% BSA and 0.01% Tween 20, and subsequently sprayed onto glass fiber (12 µL per strip). Subsequently, the antibody pad (red, 4 mm × 5 mm), sample pad (blue, 4 mm × 20 mm), conjugate pad (black, 4 mm × 5 mm), nitrocellulose (NC) membrane (white, 4 mm × 25 mm), and absorbent pad (green, 4 mm × 15 mm) were laminated on top of the backing card (gray, 4 mm × 60 mm). The absorbent pad (green) and antibody pad (red) partially overlay the NC-membrane (white). The sample pad (blue) overlays the conjugate pad (black) and antibody pad (red).

Protocol S5. Calculation of the cut-off value, estimated false positive rate, and estimated false negative rate following EU-regulation (2023/2783)

After analyzing the samples with the immunoassay, a cut-off value with a 5%-FN rate ($\beta = 0.05$) between blank and contaminated buckwheat cereal samples with 5 µg kg^{-1} scopolamine was determined. The cut-off value was calculated following:

(3) $$\text{Cut-off value} = \bar{x}_5 + \text{t-value}_{0.05} \times SD_5$$

Where $\bar{x}_5$ = average T/C ratio after analysis of the buckwheat samples spiked at 5 µg kg^{-1} scopolamine, $\text{t-value}_{0.01}$ = t-value for a one tailed t-test with FN rate of 5% with 23 degrees of freedom, and SD_5 = standard deviation of T/C ratios after analysis of the buckwheat samples spiked at 5 µg kg^{-1} scopolamine.

With the calculated cut-off value, the t-value corresponding to the event that a T/C-ratio of a blank buckwheat sample is below the cut-off value and thus erroneously classified as positive was calculated. The t-value on a FP result was calculated following:

(4) $$\text{t-value false positive} = \frac{\bar{x}_{blank} - \text{cut-off value}}{SD_{blank}}$$

Where $\bar{x}_{blank}$ = average T/C ratio after the analysis of blank buckwheat samples, cut-off value = cut-off value as calculated in equation 1, and SD_{blank} = standard deviation in T/C-ratio's after the analysis of blank buckwheat samples.

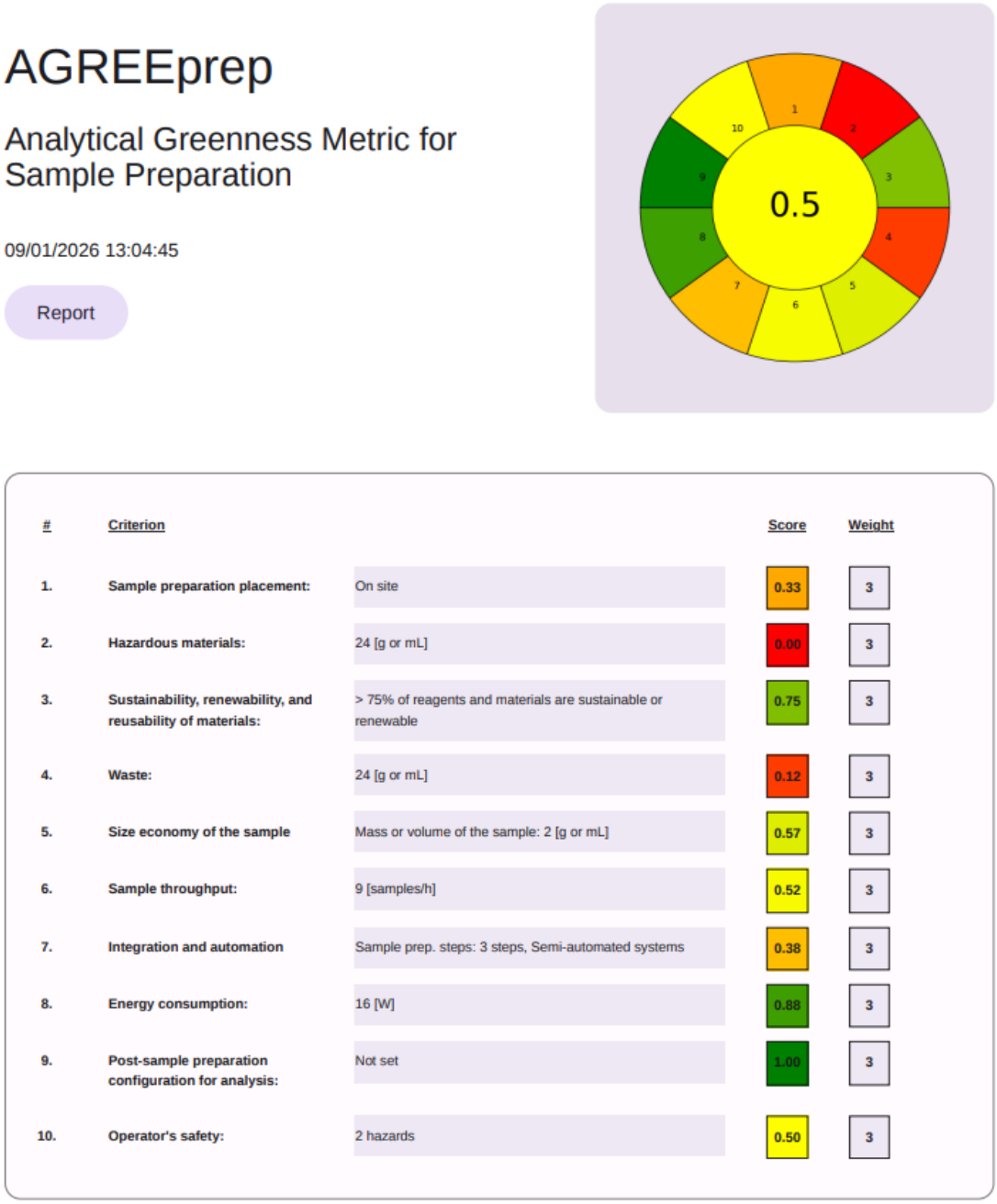
With the t-value, the probability on a FP result was calculated for a one-tailed t-distribution with 23 degrees of freedom.

Table S3. Cost-assessment of the 3D-printed biosensing toolkit

Disposables			
Description	**Supplier**	**Bulk price (€), amount**	**Price per test (€)**
Laboratory consumable, amount			
Extraction tube, 3×	Daklapack	25.81, pack of 100	0.71
Eppendorf tube, 1×	Merck	71.10, pack of 1000	0.07
Ceramic homogenizers, 2×	Fisher	38.46, pack of 100	0.38
Plastic stopcock, 1×	Nanjing life store	0.97, sold individually	0.97
Whatman filter paper (23 mm × 5 mm), 1×	Merck	88.30, 70 mm × 90 mm pack of 100	0.01
Chemicals, volume (mL)			
Butyl acetate, 20 mL	TCI	29.00, 500 mL	1.16
Formic acid, 1 µL	FA	67.00, 500 mL	0.01
0.05M NaOH, 4 mL	Sigma Aldrich	30.10, 500 gram NaOH	0.01
Running buffer, 100 µL	Made in-house	-	0.04
Indirect competitive lateral flow immunoassay, amount			
Atropine/scopolamine-test, 1×	Made in-house	-	5.00
3D-print, weight			
PI-LPME cap, 5.21 mL clear resin	Formlabs	119.79, 1L	0.63
PI-LPME holder, 2.19 mL clear resin	Formlabs	119.79, 1L	0.30
Casing of atropine test, 7.3 g PLA+	123-3D.nl	24.95, 1 kg	0.18
		Total price single test (€)	9.47

One-time investments			
Description	**Supplier**	**Bulk price (€), amount**	**One-unit**
Biosensing worktable, 166 g PLA+	123-3D.nl	24.95, 1 kg	4.14
Cube + RFID card	Chembio	330.00, sold individually	330.00
		One-time investment (€)	334.14

The total price of disposables per test was estimated to be ~€9. Of this cost, the SLE accounted for ~€2, the PI-LPME for ~€1, and the icLFIA analysis for ~€5. For some of the components in the biosensing toolkit, it is important to mention that 3D-printed parts are typically used in prototyping stages. There are more cost-effective ways to make such devices from materials with well-described properties and durability, should it come to mass production . Compared to commercially available strong cation-exchange SPE columns (~€6 per column including required washing and elution solvents) or in-house synthesized molecularly imprinted polymers used to extract atropine, the PI-LPME is both low-cost and constructed from widely available materials (paper, water, and acid).[4,5] When comparing the icLFIA analysis with traditional LC-MS/MS analysis - where new instrumentation typically costs more than €100,000 and requires highly trained personnel - our icLFIA can provide a low-cost screening solution at the point-of-need, reducing the pressure on regular laboratories.[6,7]



#	Criterion		Score	Weight
1.	Sample preparation placement:	On site	0.33	3
2.	Hazardous materials:	24 [g or mL]	0.00	3
3.	Sustainability, renewability, and reusability of materials:	> 75% of reagents and materials are sustainable or renewable	0.75	3
4.	Waste:	24 [g or mL]	0.12	3
5.	Size economy of the sample	Mass or volume of the sample: 2 [g or mL]	0.57	3
6.	Sample throughput:	9 [samples/h]	0.52	3
7.	Integration and automation	Sample prep. steps: 3 steps, Semi-automated systems	0.38	3
8.	Energy consumption:	16 [W]	0.88	3
9.	Post-sample preparation configuration for analysis:	Not set	1.00	3
10.	Operator's safety:	2 hazards	0.50	3

Figure S6. AGREEprep assessment of the 3D-printed biosensing toolkit. Each criteria has been assigned equal weights.[8]

The AGREEprep report indicates that the sample-handling workflow of the biosensing toolkit performs strongly with respect to renewable material (criterium 3), sample size (criterium 5), energy consumption (criterium 8), and analysis method (criterium 9). However, further improvements could be achieved by reducing the use of hazardous solvents (criterium 2) and by decreasing or automating certain steps in the workflow (criterium 7).

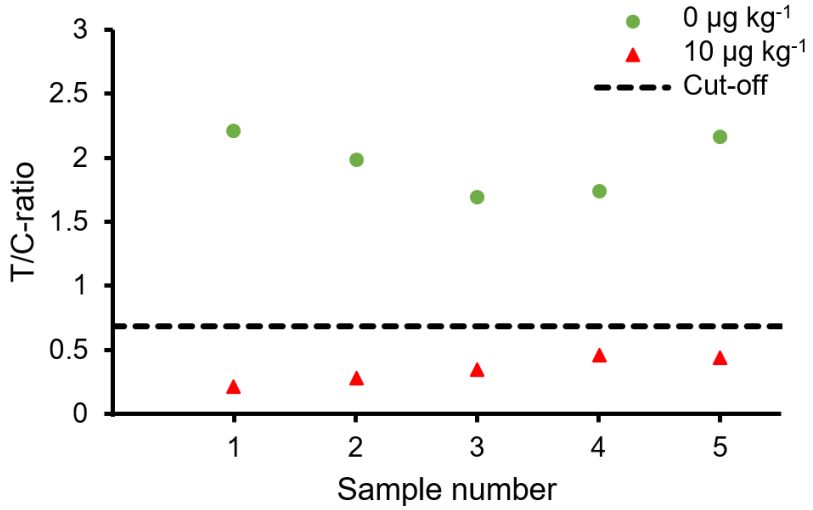


Figure S7. T/C-ratios of the indirect competitive lateral flow immunoassays after in-house analysis of 5 blank and 5 spiked buckwheat cereal samples containing 10 µg kg^{-1} atropine using the 3D-printed biosensing toolkit.

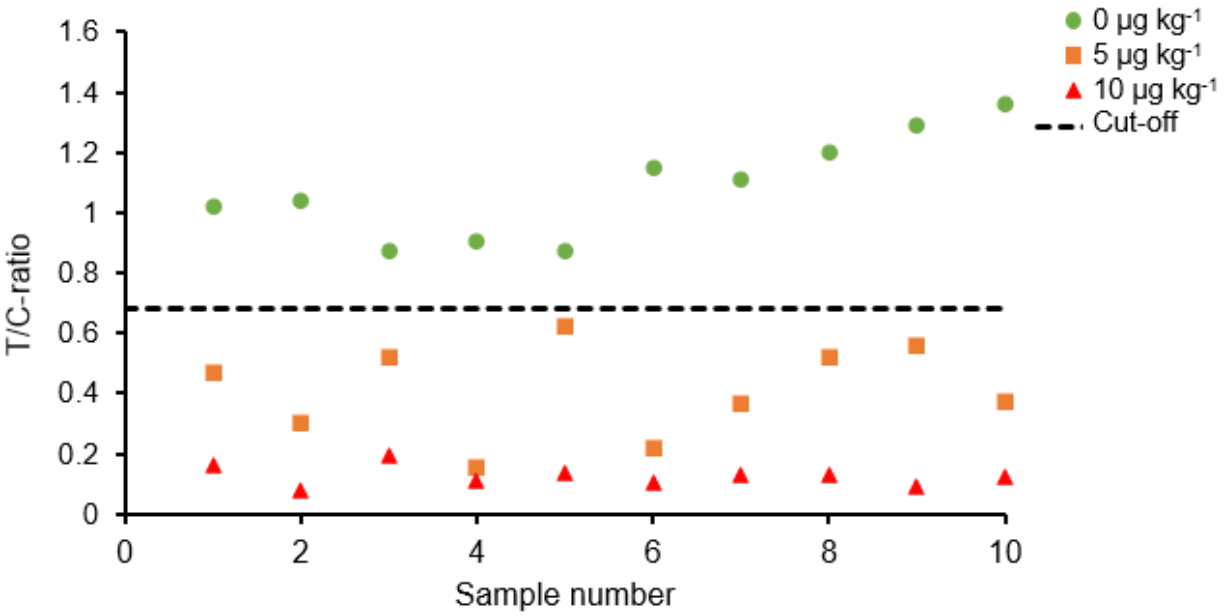


Figure S8. T/C-ratios of the icLFIAs measured by the test supervisors using the digital reader, after 1 MS technician (age: 54, level of education: bachelor of applied science) and 4 chemistry/(micro)biology students (age range: 21-26, level of education: high school/bachelor of applied science) analyzed 10× blank, 10× 5 µg kg^{-1} and 10× 10 µg kg^{-1} atropine containing buckwheat cereals.

Figure S9. Photos of the user-validation at Barilla company showing (**A**) the introduction video being shown to a group of participants, (**B**) expert testing in the quality control laboratory of the bakery product production plant in Castello, and (**C**) expert testing in a quality control laboratory of the pasta production plant in Parma. Permission for use of these photos was obtained before submission of the manuscript.

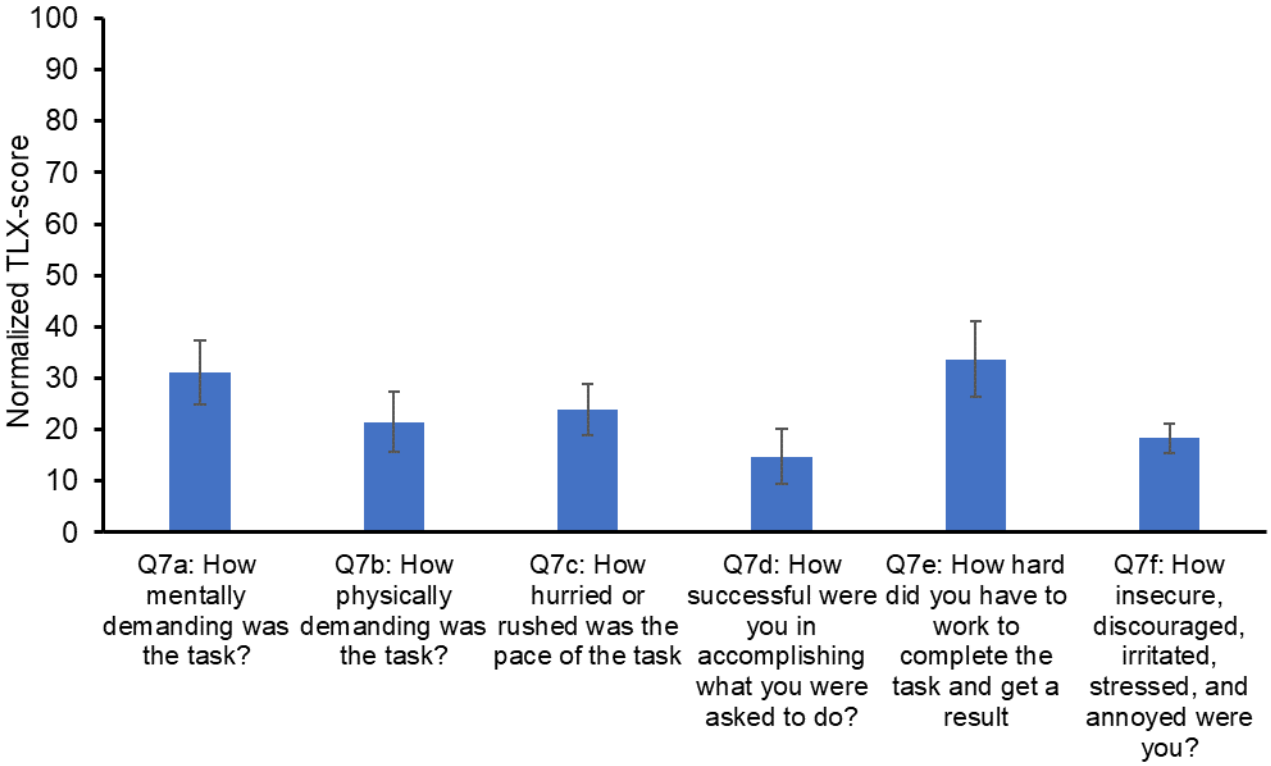


Figure S10. Normalized NASA-TLX score for subjective workload distributed in mental demand (Q7a), physical demand (Q7b), temporal demand (Q7c), performance (Q7d), effort (Q7e), and frustration (Q7f) of the biosensing toolkit assessed by the expert testing phase during the user-validation. The error bars represent the 95% confidence intervals (n = 31).

Table S4. Average and standard deviation of the system usability score (SUS) and NASA-TLX score per subgroup of the expert end-user participants.

A

Education level		
	SUS	**NASA-TLX**
High school (n = 16)	88 (±6)	28 (±9)
Graduate (e.g. Bachelor/Master, n = 12)	83 (±13)	25 (±9)
Post-graduate (e.g. PhD, n = 3)	95 (±5)	24 (±12)

B

Job description		
	SUS	**NASA-TLX**
Quality Control (n = 24)	87 (±7)	26 (±9)
Research and Development (n = 7)	91 (±7)	26 (±9)

C

Age group		
	SUS	**NASA-TLX**
19 – 35 (n = 14)	89 (±9)	26 (±9)
36 – 50 (n = 7)	85 (±6)	28 (±10)
51 – 68 (n = 10)	88 (±6)	26 (±11)

Table S5. Responses on the open question for section Q1 (Solid liquid extraction). The responses were translated from Italian into English.

Section Q1	**Solid liquid extraction**
Question Q1g	Were there any other things that caused difficulties during this step of the biosensor Yes ☒ No ☐ Explain
Response 1	During SLE, determining the shaking speed was difficult
Response 2	Difficult to fully homogenize with sodium hydroxide
Response 3	Doubts about shaking manually was sufficient
Response 4	Not all cereals were wetted when shaking. NaOH solution was lost when opening falcon (partly).
Response 5	Maybe a suggestion different color tubes/caps
Response 6	A color code on the cap could help a more clear identification. Put pictograms on butyl acetate (flammable, do not breath) close vail after use
Response 7	Very good!!!

Table S6. Responses on the open question for section Q2 (Paper-immobilized liquid phase microextraction). The responses were translated from Italian into English.

Section Q2	**Paper-immobilized liquid phase microextraction**
Question Q2	Were there any other things that caused difficulties during this step of the biosensor Yes ☒ No ☐ Explain
Response 1	Hard to pipet on paper without moving or pushing it through. Touched with tip the paper
Response 2	The paper inside screw cap was moved and not in the correct position it could be better to have it fixed
Response 3	Color the tube of formic acid
Response 4	Don't know if I should remove butyl acetate droplet from PI-LPME holder
Response 5	In worktable it's called atropine test and not icLFIA like in questionnaire

Table S7. Responses on the open question for section Q3 (icLFIA analysis). The responses were translated from Italian into English.

Section Q3	**icLFIA analysis**
Question Q2	Were there any other things that caused difficulties during this step of the biosensor Yes ☒ No ☐ Explain
Response 1	Make it clearer that the tap on the PI-LPME holder needs to be vertical to be open
Response 2	Not easy to open the tap
Response 3	Please attention to stability of kit when insert cap
Response 4	The wetting front could be confused with T-line. Not clear how to turn off the cube reader
Response 5	Use same name for the atropine test in the icon sheet as in questionnaire

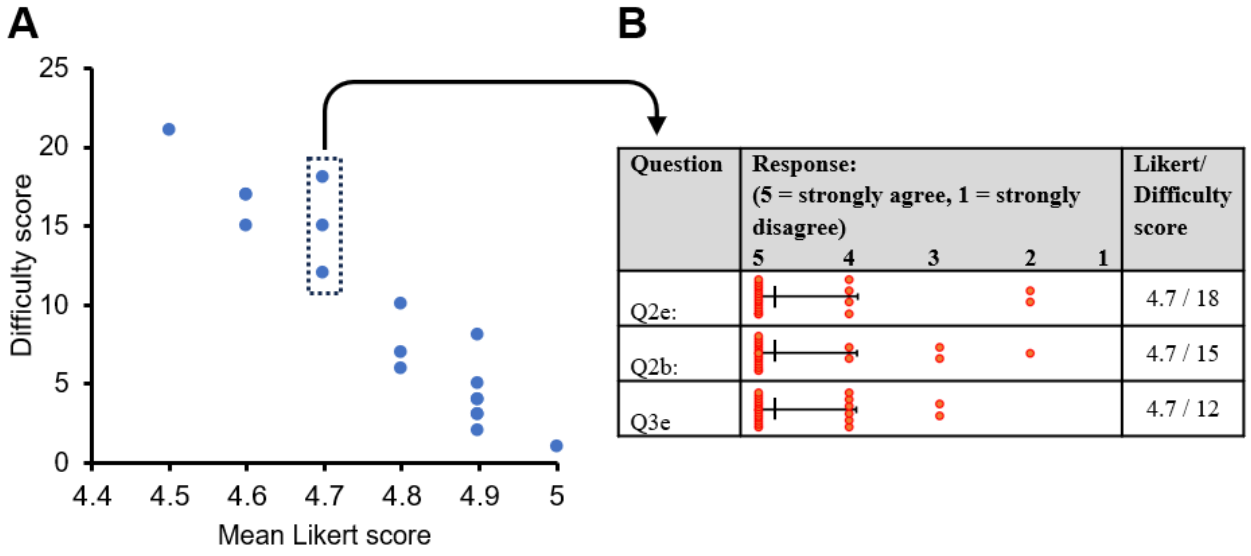
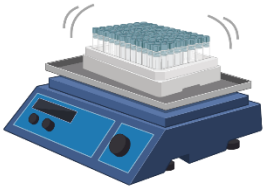
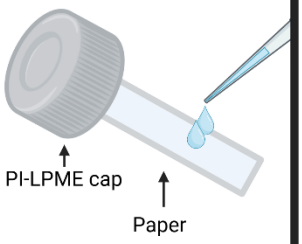
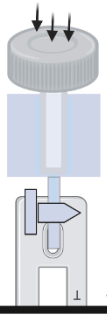
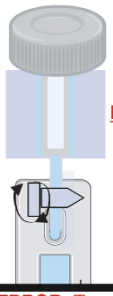
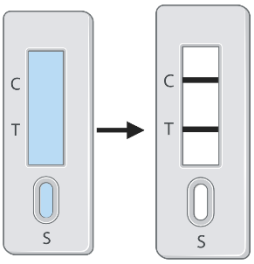


Figure S11. Comparison between the mean Likert score and new difficulty score with **(A)** the relationship between both score systems, and **(B)** participant responses to the questionnaire items that obtained a mean Likert score of 4.7. The mean Likert scores were rounded to one decimal.

Biosensor atropine workflow 1/2

1) Add 4 mL NaOH to the extraction tube. 2) Shake vigorously for 5 seconds, to fully pre-wet the cereals	3) Add 20 mL butyl acetate to the extraction tube.	4) Shake the extraction tube for 30 minutes in a shaker.	5) Pipette 10 µL 0.1% FA on the paper in PI-LPME cap.
		 Don't shake manually! *Fill in questionnaire: section Q1*	 Distribute solvent over paper, so that whole paper is wet
ERROR: NaOH is spilled or the cereals are not completely prewetted. **Consequence:** Lower extraction recovery. **Follow-up**: If the analysis results is negative, analyse the sample again.	**ERROR:** Buytl acetate is spilled. **Consequence:** Lower extraction recovery. **Follow-up**: If the analysis results is negative, analyse the sample again.	**ERROR:** Sample is shaken for less then 30 minutes or shaken manually. **Consequence:** Lower extraction recovery. **Follow-up**: If the analysis results is negative, analyse the sample again.	**ERROR:** PI-LPME paper is not completely wetted or wetted with butyl acetate. **Consequence:** Lower extraction recovery. **Follow-up**: If the analysis results is negative, analyse the sample again.
6) Screw the PI-LPME cap on the extraction tube. **7) Wait for 20 minutes.**  *Fill in questionnaire: section Q2*	**8) Insert PI-LPME cap in atropine test.** **9) Wait for 10 minutes.**  Remove parafilm	**10) Open the tap on the atropine test.**  TAP POSITION Horizontal = closed Vertical = open 	**11) Let the atropine test develop for 15 minutes after opening the tap.** 
ERROR: PI-LPME is performed for less then 20 minutes. **Consequence:** Lower extraction recovery. **Follow-up**: If the analysis results is negative, analyse the sample again.	**ERROR:** Paper is pressed out of the PI-LPME holder. **Consequence:** Lower atropine elution of the paper. **Follow-up**: If the analysis results is negative, analyse the sample again.	**ERROR:** Tap is opened before 10 min elution. **Consequence:** Lower atropine elution of the paper. **Follow-up**: If the analysis result is negative, analyse the sample again.	**ERROR:** Atropine-test is interpreted before 15 minutes or after 25 minutes after opening the tap. **Consequence:** Potentially incorrect interpretation results are obtained. **Follow-up**: Repeat the interpretation of the icLFIA 15 minutes after opening the tap or re-do the analysis in case of a negative result.

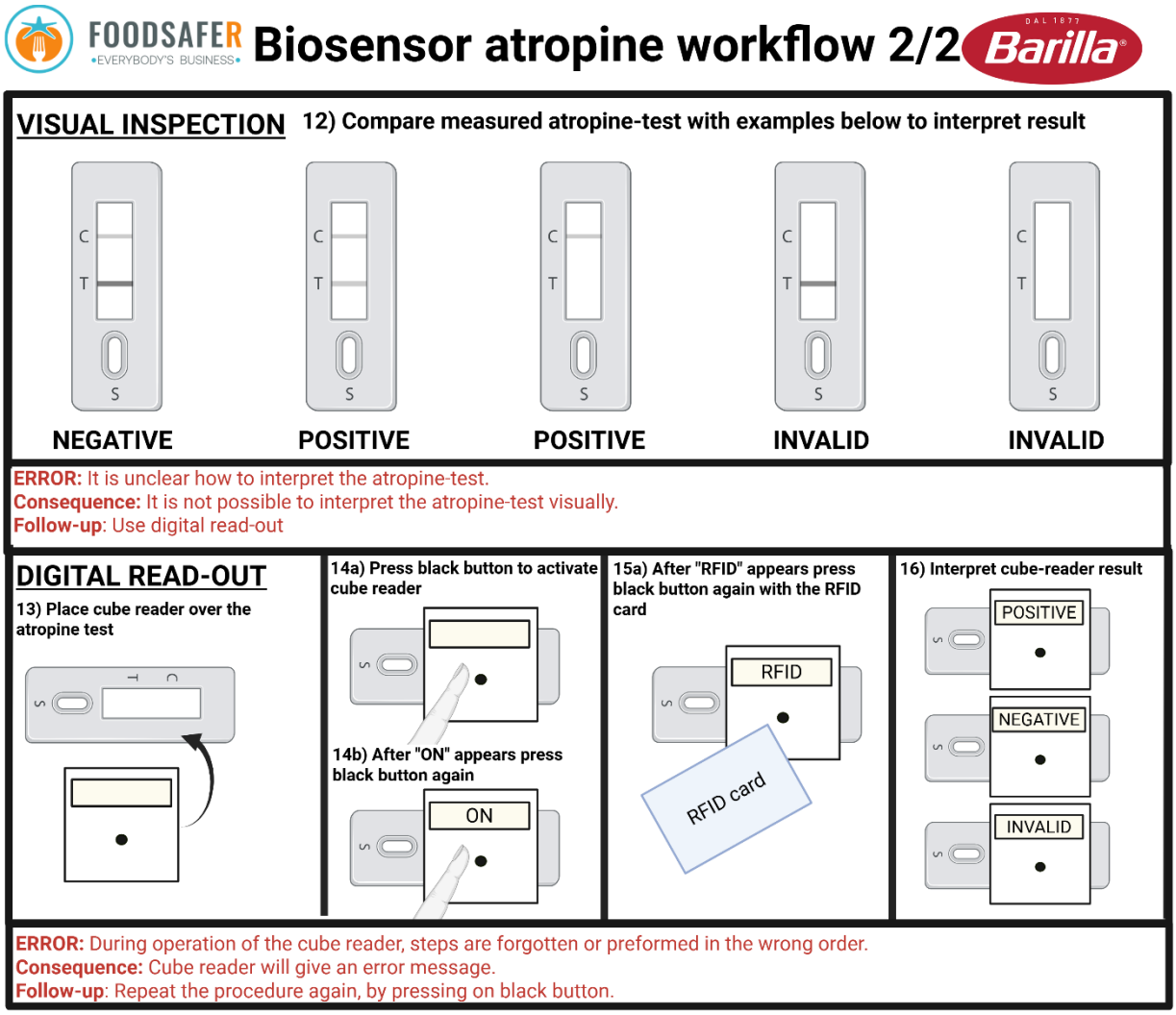


Figure S12. Updated icon-based instruction sheet for operating the 3D-printed biosensing toolkit, adjusted with the feedback received by the expert participants and containing for each step a risk assessment, describing potential sample handling errors, their consequences, and required follow-up actions.

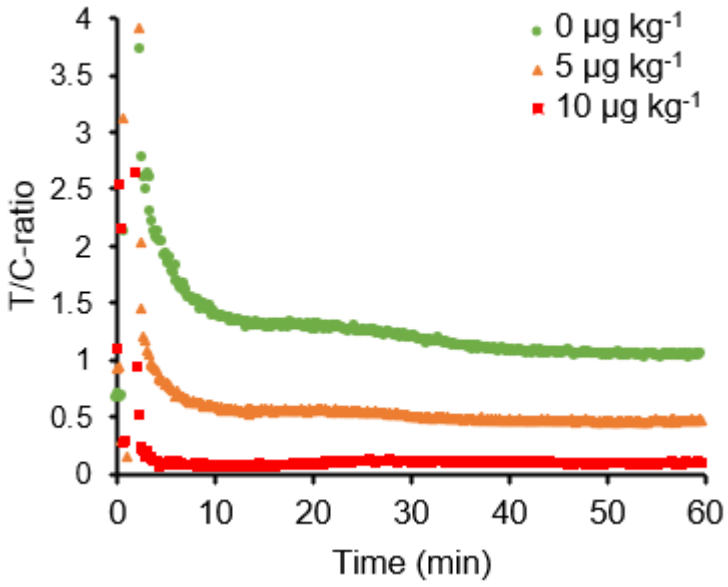


Figure S13. T/C-ratio of the developing atropine-icLFIA after opening the tap when analyzing a blank or contaminated buckwheat cereal sample with 5 or 10 µg kg^{-1} atropine. The intensity of the T-line and C-line were measured every 10 seconds for 1 hour.

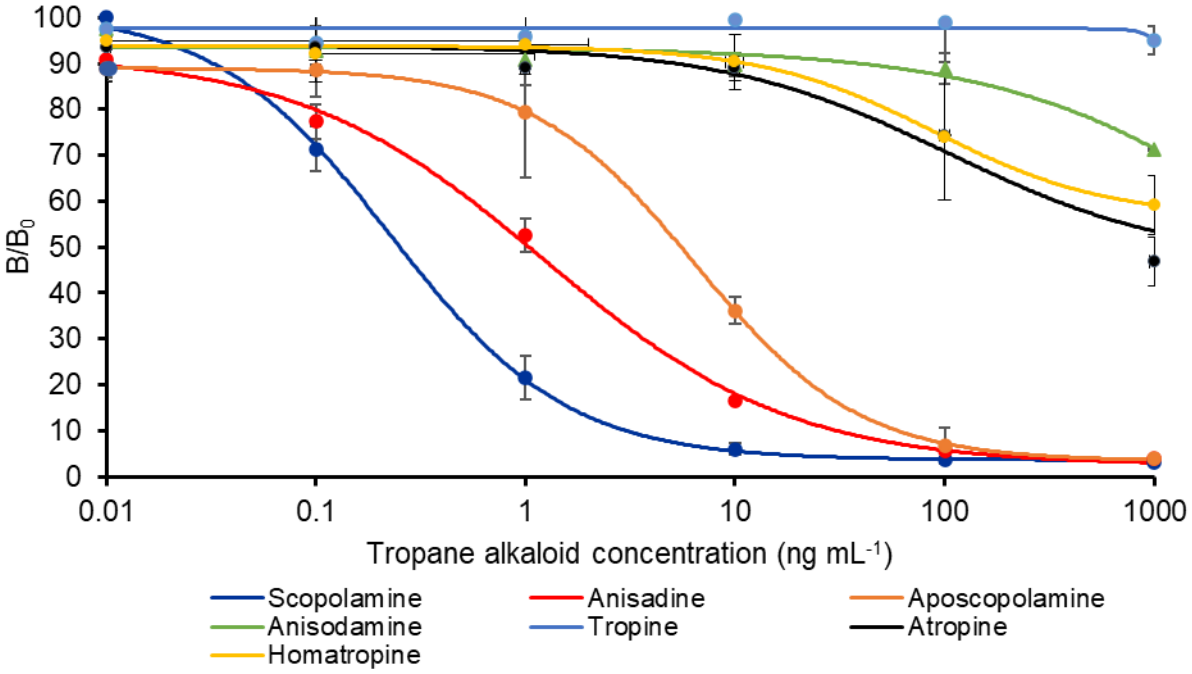


Figure S14. Dose-response curves of the scopolamine-specific antibody combined with the scopolamine-bovine serum albumin conjugate for scopolamine (dark blue), anisodine (red), aposcopolamine (orange), atropine (black), homatropine (yellow), anisodamine (green) and tropine (light blue) in the single plex indirect competitive microsphere-based immunoassay. Error bars represent the standard deviation (n = 2).

Table S8. Sensitivity and specificity of the scopolamine-specific antibody combined with the scopolamine-bovine serum albumin conjugate for scopolamine, anisodine, aposcopolamine, atropine, homatropine, anisodamine, tropine

Alkaloid	IC_{50} (ng mL^{-1})	Cross-reactivity (%)
Scopolamine	0.22	100%
Anisodine	1.38	15.0%
Aposcopolamine	6.68	3.4%
Atropine	>100	<0.22%
Homatropine	>100	<0.22%
Anisodamine	>100	<0.22%
Tropine	>100	<0.22%

The IC_{50} was computed by fitting a five parameter logistic curve through the microsphere-based immunoassay results with GraphPad Prism version 10 (Domatics; Boston, USA). The cross-reactivity (CR) was calculated following:

(5) $$CR = \frac{IC_{50}[\text{scopolamine}]}{IC_{50}[\text{competitor}]} \times 100\%$$

Where IC_{50}[scopolamine] = half-maximum inhibitory concentration of scopolamine (ng mL^{-1}),and IC_{50}[competitor] = half-maximum inhibitory concentration of another TA (ng mL^{-1}).[9]

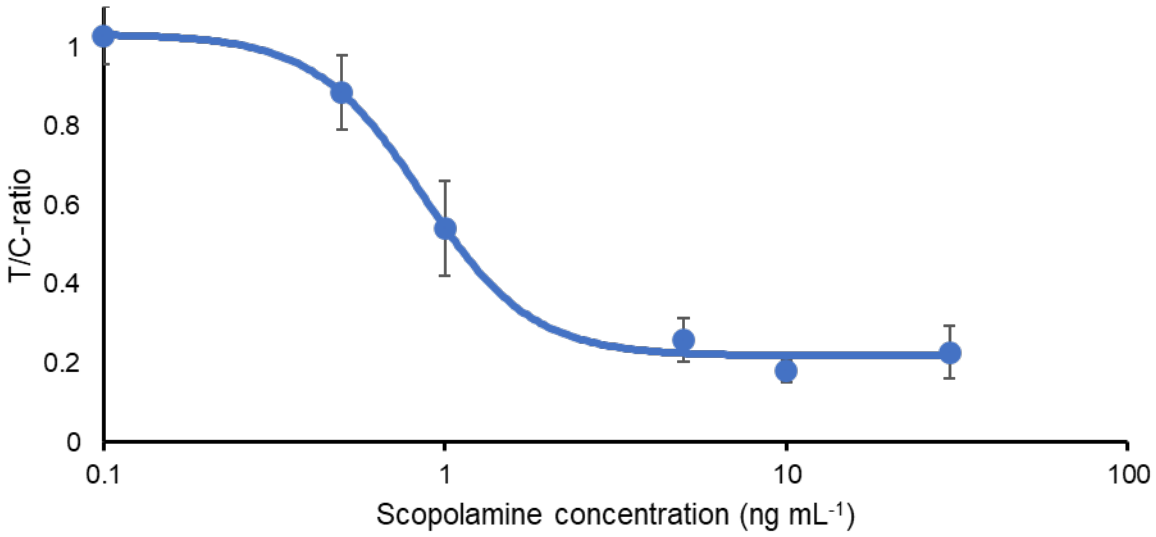


Figure S15. Determined T/C-rations after testing scopolamine dissolved in running buffer at different concentrations with the scopolamine-specific indirect competitive lateral flow immunoassay. Colorimetric read-out was performed after 20 min. Error bars represent the standard deviation (n=3).

Table S9. Comparison between the atropine-specific and scopolamine-specific indirect competitive lateral flow immunoassay (icLFIA).

	Atropine-specific icLFIA	**Scopolamine-specific icLFIA**
Test-duration	15 min	15 min
IC_{50}-value for target analyte (ng mL^{-1})	0.64 (95% confidence interval: 0.47-0.87)	0.85 (95% confidence interval:: 0.78-0.93)
Dynamic range (IC_{20}-IC_{80})	0.04 ng mL^{-1} - 4.2 ng mL^{-1}	0.52 ng mL^{-1} - 1.7 ng mL^{-1}
Cross reactivity of the antibody*	Atropine: 100% Homatropine: 4.71% Other tropane alkaloids: <0.80%	Scopolamine: 100% Anisodine: 15% Aposcopolamine: 3.4% Other tropane alkaloids: <0.80%
Nitrocellulose membrane	CN95	CN95
Running buffer composition	0.01M PBS in H_2O containing 1% BSA, 0.05% Tween	5 mM BB in H_2O containing 1% BSA, 0.05% Tween

*The cross-reactivity of the antibody was determined using an indirect competitive microsphere-based immunoassay (see Table S8)

Both icLFIAs have similar IC_{50}-values for their respective target-analytes. In addition, both assays used CN95 (estimated capillary speed: ~0.5 mm/s) as nitrocellulose membrane and had a run time of 15 min. The only difference between the two assays was the composition of the running buffer. The atropine-specific icLFIA used 0.01 M PBS (pH 7.3) in H_2O containing 1% BSA and 0.05% Tween, whereas the scopolamine-specific icLFIA used 5 mM BB (pH 8.8) in H_2O with 1% BSA and 0.05% Tween.

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SECTION II | Fate of Mycotoxins during Food Processing

4

Chapter

Research Article

Fate of aflatoxins and fumonisins during gluten-free pasta processing: untargeted ^{13}C-labelling LC-HRMS based approach

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Submitted – *Journal of Mass Spectrometry*

Abstract

Based on previous experience and current knowledge, it is assumed that mycotoxins contaminating raw materials could be degraded or modified due to exposure to thermal and mechanical conditions during food processing, potentially resulting in degradation and/or conversion products. Nowadays, most of the information on the novel degradation products of mycotoxins is still missing, identification and characterization of their structure and their toxicity potential is of a high interest for risk assessment strategies. In the present study the fate of aflatoxins and fumonisins during the production of gluten-free pasta made from maize flour was investigated. Gluten-free pasta was produced from both, spiked dough and naturally contaminated flour, processed under pilot plant conditions that mimic the industrial conditions, resulting in a technologically feasible product. Possible degradation products of the spiked mycotoxins were identified using an untargeted stable isotope labelling liquid chromatography high-resolution mass spectrometry approach. No degradation product of aflatoxin B_1 was detected and only one possible degradation product of fumonisin B_1 or B_2 at very low levels was found. The results suggest the stability of these mycotoxins under the production conditions of gluten-free pasta.

KEYWORDS

Aflatoxin B1, Fumonisins, Gluten-free pasta, Food processing, Degradation products

1 | INTRODUCTION

Pasta is produced and consumed worldwide, with Italy being the leading producer in the European Union, manufacturing 4.2 million tons in 2023 [1]. Traditionally, pasta is made from durum wheat and thus contains gluten. However, in recent years, there has been a growing trend of people avoiding gluten in their diets due to celiac disease or gluten intolerance either because of fashion or the belief that gluten free products are healthier [2]. This shift is reflected in the expanding global market for gluten-free products, which was valued at $7.70 billion in 2024 and is projected to reach $11.48 billion by 2029 [3]. Gluten-free pasta is typically made from maize or rice [4], both of which are often contaminated with mycotoxins such as aflatoxins (AFs) and B-group fumonisins (FBs), making strict quality control essential. Due to the harmful effects of mycotoxins, many countries have implemented regulations to limit exposure. In the European Union, the maximum allowable levels in maize flour are set at 2 µg/kg for aflatoxin B_1 (AFB1) and 1000 µg/kg for the sum of fumonisins B_1 (FB1) and B_2 (FB2) [5].

Mycotoxin levels in food are primarily monitored using liquid chromatography mass spectrometry (LC-MS) [6]. Typically, food samples are tested only for the intact forms of mycotoxins [7]. However, these toxins can undergo degradation or modification due to non-enzymatic reactions, such as exposure to heat during food processing. As a result, modified, matrix-associated, or masked mycotoxins may form at the expense of their free forms [8-11]. In these altered forms, mycotoxins often go undetected by routine quality control methods [12]. However, a reduction in mycotoxin levels does not necessarily mean a decrease in toxicological risk. In some cases, degradation products can be even more toxic than the original mycotoxins and may have different toxicokinetic and toxicodynamic properties [13]. While numerous studies have examined mycotoxin reduction during food processing such as thermal treatment, information on novel degradation products remains scarce [14, 11, 15]. Therefore, identifying and characterizing these compounds, as well as assessing their potential toxicity, is crucial for improving risk assessment strategies [16,17].

For FB1, thermal reaction products that form in the presence of reducing sugars include N-(carboxymethyl)-fumonisin B_1 (NCM-FB1) and N-(1-deoxy-D-fructos-1-yl)-fumonisin B_1 (NDF-FB1) [18]. Matsuo et al. [19] further identified NDF-FB2 and NDF-FB3, in addition to NDF-FB1, in corn samples. In a scientific opinion on the risks associated with fumonisins, the European Food Safety Authority highlighted that these toxins can also covalently bind to macromolecules such as starch and proteins via their reactive tricarboxylic acid side chains [20]. However, direct quantification of these bound fumonisins in food is not feasible, as they must first be released through chemical hydrolysis [21]. Consequently, matrix-bound fumonisins are typically assessed indirectly by measuring free fumonisins and hydrolyzed fumonisins before and after chemical hydrolysis or following macromolecule digestion [22,23]. Other modified forms of fumonisins include fatty acid esters of FB1 (O-fatty acyl FB1, EFB1) and fumonisins with variations in fatty acid chain length and esterification positions (3-O-, 5-O-, or 10-O-acyl fumonisins) [20]. While numerous degradation pathways have been described in the literature for AFs using various physical techniques, there is still very little research available on the formation of modified AFs due to industrial processing.

This study aims to investigate the fate of AFB1 and FBs during the production of gluten-free pasta made from maize flour in a pilot plant experiment that replicates industrial processing to obtain a technologically feasible product. To identify all possible degradation or conversion products of the mycotoxins, an untargeted stable isotope labelling (SIL) liquid chromatography high-resolution mass spectrometry (LC-HRMS) approach was followed. This method has been successfully applied in a previous study to track the fate of deoxynivalenol during the production of crackers, biscuits and bread [13, 24]. The SIL approach leverages the use of stable isotopes, such as ^{13}C, which naturally occur at low abundance [25]. The approach is based on the spiking of raw material, in this study maize flour, with both, the native (non-labelled) and uniformly ^{13}C-labelled forms of the target mycotoxins. The native mycotoxin standard contains carbon in its natural isotopic distribution (98.93% ^{12}C and 1.07% ^{13}C), whereas the uniformly labelled standard contains usually 95–99% ^{13}C and 1–5% ^{12}C, depending on its purity. In HRMS, the native mycotoxin standard primarily exhibits a signal for its monoisotopic ^{12}C isotopologue (*M*) alongside minor signals corresponding to naturally occurring ^{13}C isotopologues (*M*+1, *M*+2). In contrast, the uniformly labelled standard predominantly produces a signal for the fully ^{13}C-labelled isotopologue (*M*'), with additional minor signals for partially back-exchanged isotopologues (*M*'-1, *M*'-2), which contain one or two ^{12}C atoms. In addition to the mycotoxin standards, also possible degradation products formed during pasta production show these isotope patterns. Software detects pairs of isotopologues in HRMS data and thus identifies mycotoxin-derived transformation products [26].

2 | MATERIALS AND METHODS

2.1 Chemicals and reagents

HiPerSolv Chromanorm HPLC gradient grade acetonitrile was obtained from VWR International GmbH (Vienna, Austria), acetic acid (LC-MS gradient grade) and LiChropur ammonium acetate from Sigma Aldrich (Vienna, Austria), LC-MS Chromasolv methanol from Fisher Scientific (Illkirch, France), and NaCl from Merck KGaA (Darmstadt, Germany). Ultra-pure water purified in-house by a Purelab Ultra system (ELGA LabWater, Celle, Germany) was used. For the spiking of maize flour, the aflatoxin standards aflatoxin B_1 (2.0 µg/mL) in acetonitrile and U-[$^{13}C_{17}$]-aflatoxin B_1 (0.5 µg/mL) in acetonitrile (both biopure, Romer Labs, Tulln, Austria) and the fumonisin standards Biopure Mix 3 (fumonisin B_1 and B_2 in acetronitrile/water, Romer Labs, Tulln, Austria) and LIB'UP® U-[$^{13}C_{34}$]-fumonisins B_1 & B_2 (each 10.0 µg/mL) mixture in acetonitrile/water (Libios, Vindry Sur Turdine, France) were used.

2.2 Samples

Compliant maize flour, sourced from an Italian supplier, was used for the spiking experiments. Naturally contaminated maize kernels, harvested from a highly contaminated field during a previous harvesting campaign, were used for the production of gluten-free maize flour at the pilot plant scale.

2.3 Untargeted tracer fate study of AFB1 and FB1 & FB2

An untargeted metabolomics workflow, which was first used to detect metabolites of deoxynivalenol in wheat [27], was used to study the fate of AFB1 as well as FB1 and FB2 during pasta making.

To assess the degradation of AFB1 during lasagne production, 20 g of compliant maize flour was spiked with:

- either 65 µg/kg AFB1 (non-labelled) or 65 µg/kg AFB1, plus an equivalent amount of U-[$^{13}C_{17}$]-AFB1.

For the analysis of the degradation of fumonisins, 20 g of compliant maize flour was spiked with:

- either 400 µg/kg FB1 and FB2 (non-labelled)or 400 µg/kg FB1 and FB2, plus an equivalent amount of U-[$^{13}C_{34}$]-FB1 and U-[$^{13}C_{34}$]-FB2.

After pipetting the mycotoxin standards onto the flour, the bowls were sealed with a porous parafilm and left under the fume hood for approximately 60 min to allow solvent evaporation before proceeding with lasagne production. In addition to the spiked maize flour, lasagne was also made from 20 g compliant non-spiked maize flour for comparison.

The workflow of the gluten free pasta production is illustrated in Figure 1. To achieve the desired initial moisture content for the lasagne production, 65% water was added to 20 g spiked or non-spiked maize flour in a small bowl and mixed by stirring. Then, the dough was transferred into vacuum-sealed bags and heated in a thermostatic bath at 85°C for 12 minutes, ensuring the dough temperature reaching 75°C (Fig. 1). Next, the dough was rolled out manually using a rolling pin to form a thin lasagne sheet. The sheet was then placed in a drying chamber, where a drying cycle (360 min) was applied, regulating humidity, air speed, time, and temperature (max. 80°C) (Italpast, Parma, IT). The water content of the dried pasta was checked (Sartorius, Göttingen, DE). After drying, the pasta was cooked for 7 minutes in 200 mL of boiling water containing 7 g/L NaCl. Samples (approximately 4 g each) were collected at multiple stages of production for subsequent analysis.

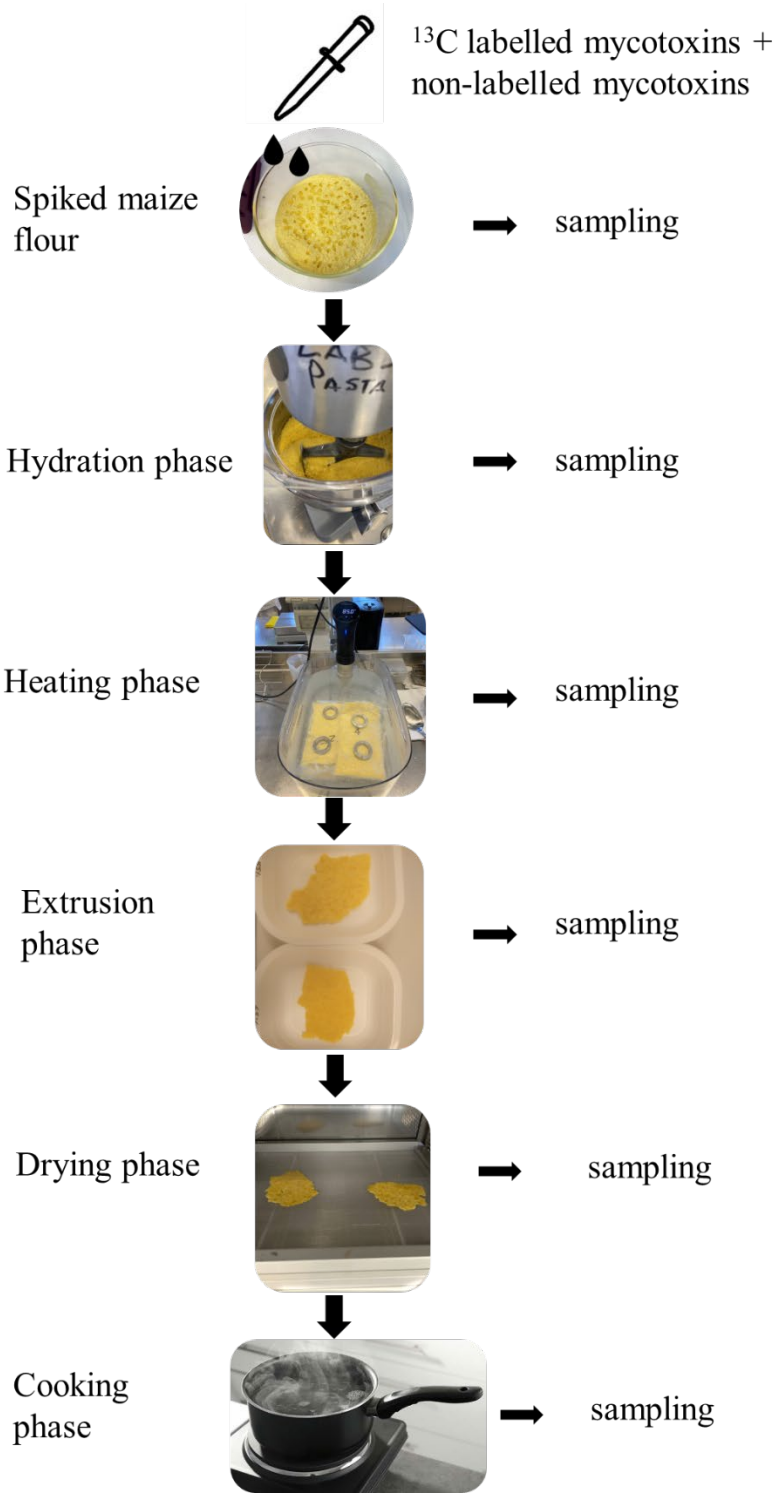

Fig. 1. Workflow of gluten-free pasta production and sampling (4g) for subsequent analysis.

2.1.1 Extraction and sample preparation

The samples “dried pasta” and “cooked pasta” were homogenised and ground for a few seconds using a blender (Osterizer). This step was not required for the remaining samples, as these were already sufficiently fine for extraction. For each sample, 0.75 g was weighed in duplicate (two technical replicates). Mycotoxins were extracted using 3 mL of extraction solvent (acetonitrile/water/acetic acid, 79/20/1, v/v/v) by shaking the samples horizontally for 90 min on a rotary shaker (GFL 3017, Burgwedel, DE). After centrifugation at 4,000 rpm for 10 min (Sigma 3-18 KS, Linder Labortechnik, Vienna, AUT), 0.5 mL of the supernatant was transferred into deactivated amber glass HPLC vials (Waters, Vienna, Austria), and an equal volume (0.5 mL) of acetonitrile/water/acetic acid (20/79/1, v/v/v) was added. Then, the samples were vortexed and analysed by LC-HRMS and the AFB1 samples additionally by LC-MS/MS (see also Paragraph 3.1.2). In addition, the spiking solutions of the non-labelled standards (AFB1 at 0.1 µg/mL; FB1 and FB2 at 0.5 µg/mL) and the mixture of non-labelled and ^{13}C-labelled standards (at the same respective concentrations) were analysed by LC-HRMS.

2.1.2 LC-HRMS measurement

The sample extracts were analysed in a randomized order on an UHPLC system coupled to an Orbitrap Q Exactive HF mass spectrometer (Thermo Scientific, Bremen, DE) applying the method described in detail by Doppler et al. [26]. Chromatographic separation was achieved on a RP C18 column (3.5 µm; 2.1 × 150 mm; XBridge; Waters; Milford; MA; USA) using a 20-minute gradient method. The temperature in the autosampler was set to 10°C and in the column oven to 25°C. For each sample, a volume of 2 µL was injected. Full scan mass spectra were recorded from *m/z* 100 to 1000 in fast polarity switching mode and with a resolution of 120 000 (FWHM at *m/z* 200). Quality control standards were injected regularly throughout the whole sequence.

2.1.3 Automated data processing

The software MetExtract II was used for LC-HRMS data evaluation, which is described in detail by Bueschl et al. [26]. The datasets corresponding to the aflatoxin- and fumonisin-spiked samples were analysed separately. Briefly, data-processing was performed as follows: Full-scan mass spectrometer data were checked for characteristic isotopologue pairs of mass peaks of *M*, which represents a possible degradation or transformation product derived from the unlabelled standard, and *M’*, which represents the corresponding product derived from the uniformly ^{13}C-labelled standard (Table 1). These pairs have a mass difference (Δm/z), which corresponds to the number of tracer-derived ^{13}C atoms incorporated in *M’*. Further, co-elution of *M* and *M’* as well as the conformity of their concentrations, as labelled and non-labelled standards were spiked at the same concentrations, were verified. To eliminate potential false positives, peaks detected in control samples (without spiked mycotoxins) were subtracted from those in spiked samples. This ensured

that only signals specifically associated with mycotoxin transformation or degradation products were considered for further analysis.

	Parameter	AFB1	FB1/FB2
	Module	TracExtract	TracExtract
Single file processing	Isotopic enrichment % (N / L)	98.9 / 99.0	98.9 / 97.8
	Atom count	17	34
	Δ m/z	1,00335	
	Min - max Cn atoms	10-17; 34	6-34; 65-69
	Scan range start - stop (minutes)	1-13	
	Intensity threshold (≥)	10000	1000
	Intensity cutoff (≤)	0	
	Consider isotopolog abundance	No	
	Mass deviation (± ppm)	6	
	Isotopologs verified (N / L)	2 / 2	
	Max. isotopolog ratio error % (N / L)	15 / 15	30 / 30
	Clustering (ppm)	8	
	Min. number of spectra	3	
	EIC width (± ppm)	5	
	Peak width (min / max scans)	3 / 11	
	Peak matching (scans)	0	
	Min. peak correlation	0.85	
	Min. convolution correlation	0,85	
Bracketing	Max. m/z width (± ppm)	12	
	Max. time window (minutes)	0.1	
	Integration max. time difference (minutes)	0.1	

Table 1. Parameter settings for data processing using MetExtract II.

2.1.4 LC–MS/MS measurement

Sample extracts were additionally analysed by LC-MS/MS to quantify AFB1 and FBs. LC-MS/MS measurement was performed as described by Sulyok et al. [28]. Briefly, metabolite analysis of the extracts was performed on a 1290 Series HPLC System (Agilent, Waldbronn, DE) connected to a QTrap 5500 LC-

MS/MS System (Applied Biosystems SCIEX, Framingham, MA) equipped with Turbo Ion Spray electrospray ionization source. The injection volume was 5 µL. The chromatographic separation was achieved on a Gemini® C_{18}-column, (150 x 4.6 mm i.d., 5 µm), connected with a C_{18} 4 x 3mm i.d. security guard cartridge (Phenomenex, Torrance, CA, US) using methanol/water/acetic acid 10:89:1 (v/v/v) and 97:2:1 (v/v/v), both with 5 mM ammonium acetate, as eluents A and B at a flow rate of 1,000 µL/min. After 2 min at 100% eluent A, eluent B was linearly increased to 50% within 3 min. Then, eluent B was further linearly increased to 100% within 9 min, followed by 4 min at 100% eluent B and 2.5 min for column equilibration at 100% eluent A. Results were corrected for apparent recoveries and the humidity levels of the sample taken at the different production steps (e.g., raw material, dough).

2.4 Verification of the presence of degradation products in pasta made from naturally contaminated maize flour

To determine whether the degradation products detected in samples from the spiked lasagne were also present in pasta made from naturally contaminated maize flour, the pasta production process was repeated under comparable conditions as described in paragraph 2.3, but with an increased maize flour quantity (200 g). The heated dough was then divided into two equal portions: one was used to produce lasagne sheets as described above, while the other was used to produce maccheroni (Italian shape of pasta, referred to as macaroni in English). For the maccheroni production, the dough was extruded using an extruder machine (Philips pasta maker, Amsterdam, NL) equipped with a die for maccheroni (Fig. 2). Extracts of samples collected at the processing steps outlined in paragraph 2.3. were analysed as a single replicate using LC-HRMS.

Fig. 2. Extrusion phase with the extruder machine equipped with a die for maccheroni.

3 | RESULTS

3.4 Untargeted tracer fate study of AFB1 and FB1 & FB2

3.4.1 Control experiment

The first step was the production of gluten-free pasta with compliant maize to optimize the production process (hydration, heating, extrusion, drying and cooking). A key challenge was to minimize raw material amount (20 g) while ensuring a technologically feasible and marketable product. This approach aimed to limit the amount of costly ^{13}C-labelled standard required.

The humidity of the dried lasagne sheets (n = 4) was 10.8 %, which aligns with the company's specified range (10 –11.5%) and complies with the legal limit of less than 12.5% (Presidential Decree No. 187). Naturally occurring mycotoxin levels of the compliant maize flour were 1.95 µg/kg AFB1, 625 µg/kg FB1 and 148 µg/kg FB2.

3.4.2 Fate of AFB1

Lasagne sheets were produced from maize flour spiked with either non-labelled AFB1 or a combination of non-labelled and ^{13}C-labelled AFB1, and samples taken at five different production steps (listed in the section 2.3) were analyzed. The unique isotope patterns of the labelled and the non-labelled mycotoxin standards were used by the software MetExtract II to filter possible degradation or conversion products from the mass spectrum of toxin extracts of all these samples. No such products of AFB1 were detected.
To assess the stability of AFB1 during the lasagne production, the mycotoxin was additionally quantified in the sample extracts by LC-MS/MS. As shown in Fig. 3, AFB1 levels varied only slightly across samples taken at different production stages. The remaining variations can be attributed to differences in matrix effects, moisture content of the various materials, such as dough, dried, or cooked pasta, and the small sample volumes analysed.

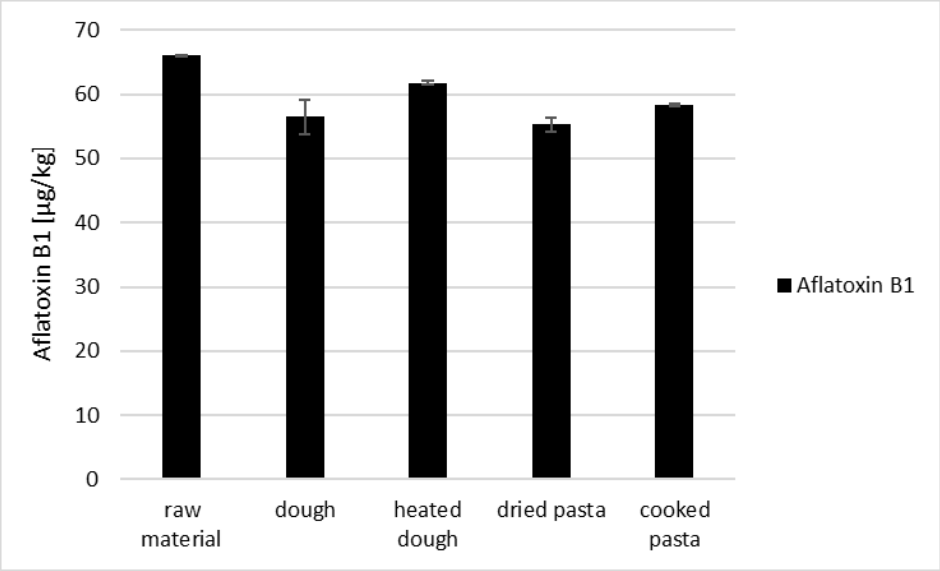

Fig. 3: Aflatoxin B_1 (AFB1) levels [µg/kg] in samples collected during lasagne production using maize flour spiked with AFB1 at a concentration of 65µg/kg.

3.1.3 Fate of FB1 and FB2

Lasagne sheets were also produced using maize flour spiked with non-labelled FB1 and FB2, as well as maize flour spiked with both, non-labelled and ^{13}C-labelled FB1 and FB2. Again, samples were collected at five production steps to monitor potential degradation or conversion products. Initially, three fumonisin-derived compounds were detected (Table 1). However, two of these metabolites were also found in the labelled and the non-labelled standards, as well as in the raw maize flour samples. This suggests that they were likely contaminants present in both standards and the raw maize flour. Metabolite 2 was most likely a partially hydrolyzed FB2. The third metabolite was present as a contaminant in the non-labelled standard but was absent in the labelled standard and raw maize flour. At very low intensities, the metabolite was detected in both replicates of the dried pasta samples and in one replicate of the cooked sample,

appearing as a metabolite of the labelled standard. However, as shown in Fig. 4, the isotope pattern of the samples revealed on the non-labelled side only the first isotopologue (*M*+1), containing a single ^{13}C atom, along with the monoisotopic ^{12}C isotopologue (*M*). On the labelled side two isotopologues (*M'*-2 and *M'*-1), incorporating one or two ^{13}C atoms, were detected in addition to the monoisotopic ^{13}C isotopologue (*M'*). Optimally, the isotope pattern should display a mirror-image pattern with two isotopologues (*M*+1 and *M*+2, *M'*-2 and *M'*-1) of the monoisotopic ^{12}C or ^{13}C isotopologue (*M, M'*). Therefore, the metabolite did not meet the quality criteria required for confirmation as a relevant fumonisin-derived compound.

Metabolite number	*m/z* (in positive ionation mode)	Retention time [min]	Detected in non-labelled fumonisin standard	Detected in ^{13}C-labelled fumonisin standard
1	564.3746 $[M+H]^+$	8.97	yes	yes
2	548.3795 $[M+H]^+$	9.70	yes	yes
3	868.4533	9.82	yes	no

Table 1. List of fumonisin-derived metabolites detected using the software MetExtract II.

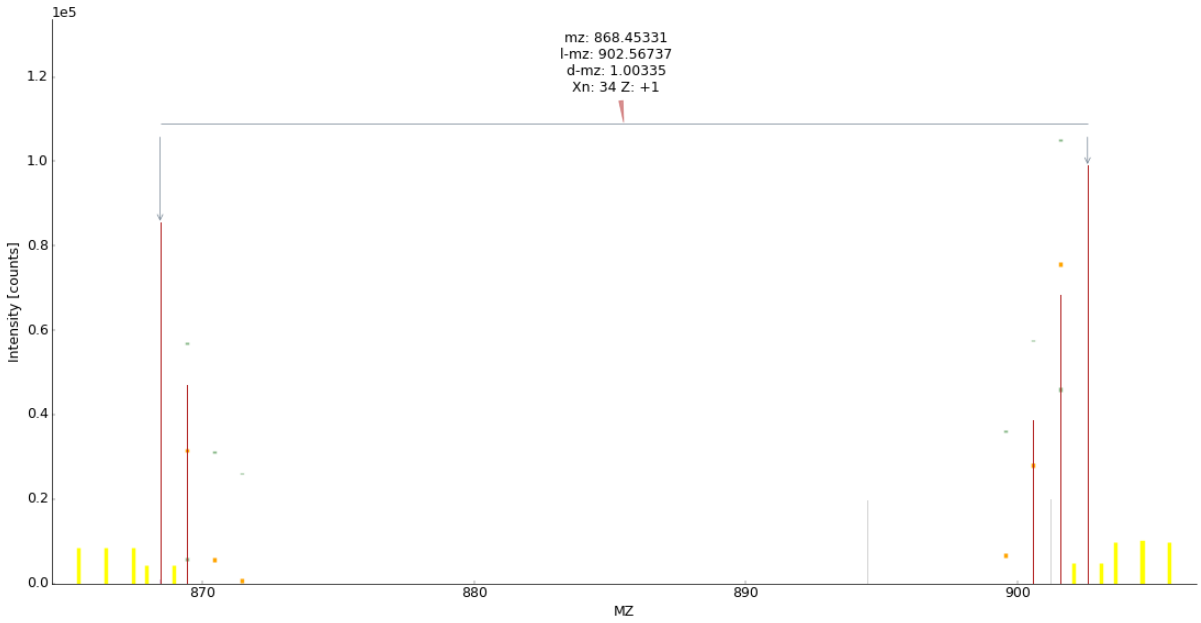

Fig. 4. Mass spectrum displaying the isotope pattern of a fumonisin-derived metabolite (*m/z* 868.4533) in the extract of a dried pasta sample produced using maize flour spiked with labelled and non-labelled fumonisin standards. On the left side of the spectrum, the signal of the monoisotopic ^{12}C isotopologue (*M*) is visible along with a single isotopologue (*M*+1), while on the right side, the monoisotopic ^{13}C isotopologue (*M'*) appears alongside two isotopologues (*M'*-2 and *M'*-1).

3.5 Control of the formation of fumonisin-derived metabolites in pasta produced using naturally contaminated maize flour

Lasagne sheets and maccheroni were produced from naturally contaminated maize. The mycotoxin levels in the contaminated maize determined by LC-MS/MS were as follows: AFB1, 74.9 µg/kg; FB1, 708 µg/kg and FB2, 146 µg/kg. Samples of the maize flour and five further pasta production steps were controlled for the above-mentioned degradation or conversion products. The metabolites 1 and 2 of the FBs were detected in the naturally contaminated raw maize flour as well as in all the following production steps. The third metabolite was only detected in the dried maccheroni sample at very low intensity but not in the raw maize flour, the dried lasagne sample or any other sample of the pasta production.

4 | DISCUSSION

In the current study, pasta was produced from maize flour spiked with the mycotoxins AFB1 or FB1 and FB2. Samples taken after each production step were analysed for the presence of degradation or conversion products.

The aim of the study was to detect degradation products formed at low percentages of the spiked mycotoxin standard. The spiking level of AFB1 (65 µg/kg) was significantly higher than the legal limit of 2 µg/kg in maize in the EU [5]. No degradation products of AFB1 were detected, suggesting that AFB1 remains stable during the production of gluten-free pasta under the specified conditions, which included heating at 75 °C, drying at a maximum of 80 °C, and boiling for 7 min. This stability was further supported by the relatively consistent AFB1 levels observed in samples across the different production steps. The variations seen in Fig. 4 should not be overinterpreted, as the study was designed primarily for qualitative analysis rather than quantitative measurement. To prevent the potential formation of degradation products due to an additional drying step, which is not part of the pasta production process, samples were not dried before extraction. Instead, the results were adjusted based on the average humidity levels of each sample type (e.g., dough, dried pasta). However, variations in water content and matrix effects of the different sample types likely still influenced the results. Additionally, the sample volume taken at each production step was very small due to the high cost of ^{13}C-labelled mycotoxin standards. Consequently, the quality of the measured data was affected. We cannot rule out the possibility that degradation products were formed at concentrations below their respective detection limits less than 1% of the spiked AFB1 level.

The results of this study are aligned with the observation that aflatoxins generally exhibit high stability under various conditions [29]. Stoloff and Trucksess [30] demonstrated that the degradation of AFB1 is strongly dependent on the processing conditions of contaminated maize. Experiments employing more challenging thermal production conditions (e.g., higher temperatures or longer treatment times) than in the current experiment resulted in losses of AFB1. For example, boiling maize grit for 30 min resulted in a recovery of 72 ± 9% of the original AFB1, while baking maize flour at 218 °C for 20 to 25 min into muffins resulted in an AFB1 recovery of 87 ± 4%. However, these publications did not investigate the formation of degradation products of AFB1.

Only one fumonisin-derived product was detected in the current study, suggesting also a high stability of the spiked fumonisins under the tested conditions for the production of gluten-free pasta. In general, FBs are considered to be quite heat stable and survive thus many food production processes [14]. The stability seems to be significantly influenced by the type of processing condition, as baking maize muffins spiked or naturally contaminated with fumonisins at 204 °C for 20 min did not significantly reduce mycotoxin levels, while roasting (dry heating) of maize meal samples at 218 °C for 15 min reduced fumonisins nearly completely [31].

The one fumonisin-derived product (*m/z* 868.4533) detected in the spiked fumonisin samples was also detected in the dried maccheroni sample made from naturally contaminated maize at very low intensities. Thus, based on the results gained, it can be assumed that this unknown metabolite is formed during the drying step of the pasta production. However, it was not detected in all dried pasta samples. Thus, either it is not constantly produced or its concentration in the other dried pasta samples was simply too low that it could have been detected. As the isotope pattern of the metabolite did not fulfil the quality criteria and as the concentration was very low, we did not further focus on this metabolite.

5 | CONCLUSION

The fate of aflatoxin (AFB1) and fumonisins (FB1 & FB2) was investigated during the production of gluten-free pasta. The pasta was made from maize flour that was either spiked with these mycotoxins or naturally contaminated, and all processing steps were conducted under pilot plant conditions. Under these conditions, no degradation or conversion products of AFB1 were detected, highlighting its stability during the production of gluten-free pasta. For FB1 & FB2, a metabolite (*m/z* 868.4533) was detected at very low intensities. However, due to isotope pattern of this metabolite in the mass spectrum, which did not meet our quality criteria, it could not be confirmed as a degradation or conversion product of the spiked fumonisins. Thus, also FB1 and FB2 can be considered stable under the conditions used, likely due to the relatively low temperatures applied during pasta production.

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5

Chapter

Research Article

Tracking the fate of T-2 and HT-2 mycotoxins during wholemeal cracker production using a combined targeted and untargeted ^{13}C-labelling LC-HRMS based approach

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Highlights

- Fate of T-2 and HT-2 toxins investigated during wholemeal cracker production.
- Search for previously unknown degradation or conversion products.
- Targeted and untargeted ^{13}C -labelled LC-HRMS approach applied.
- Baking at 195 °C did not induce degradation but promoted T-2 hydrolysis to HT-2.

Abstract

T-2 and HT-2 toxins remain among the most challenging Fusarium mycotoxins in cereal-based foods, yet their stability and degradation during processing is still insufficiently understood. To track the fate of both toxins during wholemeal cracker production, a combined targeted and untargeted ^{13}C-labelled LC-HRMS approach was utilized. Crackers were produced from both dough spiked with the mycotoxins and naturally contaminated flour under pilot-plant conditions that closely mimic industrial processing, yielding a technologically realistic product. Despite the baking step, HT-2 toxin remained remarkably stable, while T-2 toxin underwent only partial conversion to HT-2 during dough mixing—driven by enzymatic or matrix-mediated reactions rather than thermal degradation. No additional transformation or degradation products were detected at any stage. Since T-2 and HT-2 share comparable toxic potency, this conversion does not constitute a meaningful detoxification step. Overall, these findings emphasize the need to reinforce pre-processing control measures, refine risk-assessment strategies, and explore alternative processing technologies capable of achieving toxin reduction without compromising product quality.

KEYWORDS

T-2 toxin, HT-2 toxin, Wholemeal cracker, Food processing, Thermal degradation, Isotope-labelled LC-HRMS.

1 | INTRODUCTION

The *Fusarium* genus is a renowned producer of trichothecenes, considered a highly toxic class of mycotoxins that is subdivided into four groups (A–D). Among them, trichothecenes A and B are the most common, with the former comprising T-2 and HT-2 toxins, primarily formed by *F. langsethiae* and *F. sporotrichioides* (Meneely et al., 2023). Type A trichothecenes commonly cause inhibition of protein synthesis and mitochondrial function, thereby triggering toxic effects and potentially causing severe economic impact to the cereal industry (De Angelis et al., 2013).

T-2 and HT-2 toxins are mainly contaminants in cereals such as oats (Scudamore et al. 2007; Pettersson et al., 2011), barley (Hietaniemi et al. 2016), maize and wheat (Schollenberger et al. 2008), demonstrating that these toxins undergo redistribution during cleaning and milling rather than being eliminated. While the final food products (e.g., oat flakes, bread) may have lower toxin levels, by-products (such as bran or hulls) can become highly contaminated with T-2 and HT-2 toxins (Bullerman & Bianchini, 2007; Pettersson et al., 2011; Pleadin et al., 2018).

The fate of these mycotoxins during food processing is influenced by factors such as their chemical structure, the food matrix, and processing parameters (like time and temperature) (Kuchenbuch et al., 2018). However, the effectiveness of thermal processing in reducing T-2 and HT-2 toxins is still poorly understood. This is mainly due to the limited and heterogeneous data currently available, which were obtained under differing thermal degradation conditions and therefore lead to inconsistent study results and conclusions. T-2 and HT-2 toxins are notably stable under typical food processing conditions. Studies show that standard processes like cooking, baking, and fermentation have minimal impact on their levels, with reductions often less than 10% (Meneely et al., 2023; Pleadin et al., 2018). However, more intense thermal treatments such as roasting and especially extrusion can achieve greater reductions. Roasting naturally contaminated maize, triticale, and oats for 30 min at three different temperatures (180°C, 200°C and 220°C) reduced T-2 and HT-2 toxin levels by 28.8 to 54.4%, while extrusion at three different temperature profiles (135-150°C; 135-170°C and 135-190°C) achieved 73–92.5% until an almost complete reduction of T-2 and HT-2 especially in oat (Pleadin et al., 2018). Despite this mitigation effect, the degradation or transformation of T-2 and HT-2 toxins during processing is poorly understood and not always guaranteed. Schmidt et al. (2017) stated that, due to the strong interference of various parameters during extrusion, it is not possible to attribute toxin degradation to a single factor (Schmidt et al., 2017) .

Since data on the presence of modified forms of T-2 and HT-2 toxins are very limited, and no routine analytical methods are currently available for their determination, maximum levels (MLs) have been established in the European Union (EU) only for the combined sum of T-2 and HT-2 toxins. At present, the ML is set at 20 µg/kg for bakery wares (Reg. EU No. 2024/1038). In most other regions, such as the United States, Canada, or the United Kingdom, no MLs for T-2 and HT-2 toxins have been established for these products to date.

The lack of knowledge about changes in T-2 and HT-2 toxin levels during baking has prompted us to investigate their metabolic fate during cracker production. The aim of the current study was thus to find

currently unknown degradation or conversion products of these mycotoxins formed during this process. Wholemeal crackers were produced using wholemeal durum wheat flour in addition to common wheat flour, as wholemeal durum wheat flour contains more bran and, thus, usually higher initial mycotoxin levels than refined flour. The production was carried out in a pilot plant experiment that replicates industrial processing to obtain a technologically feasible product. To identify all possible metabolites of the mycotoxins, an untargeted stable isotope-assisted (SIA) liquid chromatography high-resolution mass spectrometry (LC-HRMS) approach was utilized. This method was successfully applied in a previous study to track the fate of deoxynivalenol during the production of crackers, biscuits, and bread (Stadler, Lambertini, Bueschl, et al., 2019b).

Continued research with the aim of collecting more information on the influence of food processing while identifying and characterizing possible degradation and/or transformation compounds, as well as assessing their potential toxicity, is crucial for improving risk assessment strategies in cereal-based food production (Schaarschmidt & Fauhl-Hassek, 2018; Stadler, Lambertini, Bueschl, et al., 2019b).

2 | MATERIALS AND METHODS

2.1 Chemical and reagents

HiPerSolv Chromanorm HPLC gradient grade acetonitrile was obtained from VWR International GmbH (Vienna, Austria), acetic acid (LC-MS gradient grade) and LiChropur ammonium acetate from Sigma Aldrich (Vienna, Austria), and LC-MS Chromasolv methanol from Fisher Scientific (Illkirch, France). Ultra-pure water purified in-house by a Purelab Ultra system (ELGA LabWater, Celle, Germany) was used. For the spiking of durum wheat flour, T-2 and HT-2 toxin (100.1 and 100.2 µg/mL in acetonitrile) and the ^{13}C labelled analogues U-[$^{13}C_{24}$]- T-2 and U-[$^{13}C_{22}$]-HT-2 toxin (25.2 and 25.1 µg/mL in acetonitrile) were used (Romer Labs, Tulln, Austria).

2.2 Crackers preparation

Compliant wholemeal durum wheat flour (coming from Barilla mill) was used for the spiking experiments. Additionally, durum wheat kernels naturally contaminated with T-2 and HT-2 toxins were milled using a BONA machine (BONA, Monza, Italy) to produce naturally contaminated wholemeal durum wheat flour, which was then sifted in order to obtain the same granulometry as the previously mentioned flour. Both flours used were first screened for a multitude of different mycotoxins and other fungal secondary metabolites using a multi-toxin method by applying liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described in the paragraph (2.5), and the metabolites were quantified.

The preparation of the wholemeal wheat crackers, which is illustrated in **Figure 1**, involved two steps. The first step was the production of the sponge, followed by the preparation of the final dough. To prepare the sponge, common wholemeal wheat flour, water, yeast, malt solution, and enzyme were mixed for 5 min using a pilot planetary kneader (company). The sponge was then left to ferment at 25 °C for 30 min at 85%

relative humidity (RH). This sponge was used for the production of all crackers. It was frozen in jars and defrosted on the day of use. The pH value of the sponge was checked each time before use. The dough was prepared by adding the spiked wholemeal durum wheat flour and wholemeal common wheat flour, potato flakes, water, glucose syrup, sugar, sodium bicarbonate, salt, and oil to the sponge, and by kneading all ingredients for 3 min. This dough was then left to rise in a specific leavening cell at 25 °C (FermaLievita Alaska, Bologna, Italy) for 2 hours at 85% RH. After leavening, the dough was rolled out to a thin sheet (1.7 mm), which was then shaped with a specific mold with holes (cracker cutter). Finally, the crackers (approximately 2.6 g each) were baked in an electric oven (Whirlpool, Michigan, USA) at 195 °C for 6 min. The moisture content of the dried crackers was measured (Sartorius, Göttingen, Germany). Samples for subsequent analysis (approximately 6 g each) were collected at multiple stages of the production, from the wholemeal durum wheat flour (after spiking), after mixing and after leavening the dough, and after baking the crackers. All samples were stored at -18 °C.

2.3 Untargeted tracer fate study of T-2 and HT-2 toxins

The formation of degradation or conversion products was investigated by applying an untargeted metabolomics workflow, which was already successfully used to detect metabolites of various mycotoxins (Bueschl et al., 2017; Stadler, Lambertini, Bueschl, et al., 2019b).

To assess the degradation of T-2 toxin during cracker production, 14.5 g of compliant wholemeal durum wheat flour was spiked with:

- either 3000 µg/kg of T-2 toxin (non-labelled, fully ^{12}C) or
- 3000 µg/kg of non-labelled ^{12}C T-2 toxin, plus an equivalent amount of U-[$^{13}C_{24}$]-T-2 toxin.

For the analysis of the degradation of HT-2 toxin, 14.5 g of compliant wholemeal durum wheat flour was spiked with:

- either 3000 µg/kg of HT-2 toxin (non-labelled, fully ^{12}C) or
- 3000 µg/kg of non-labelled ^{12}C HT-2 toxin, plus an equivalent amount of U-[$^{13}C_{22}$]-HT-2 toxin.

After the mycotoxin standards were pipetted onto the flour, the bowls were covered with porous parafilm and placed under the fume hood for about 60 min to allow the solvent to evaporate before starting cracker production. For comparison, crackers were also prepared from compliant non-spiked wholemeal durum wheat flour.

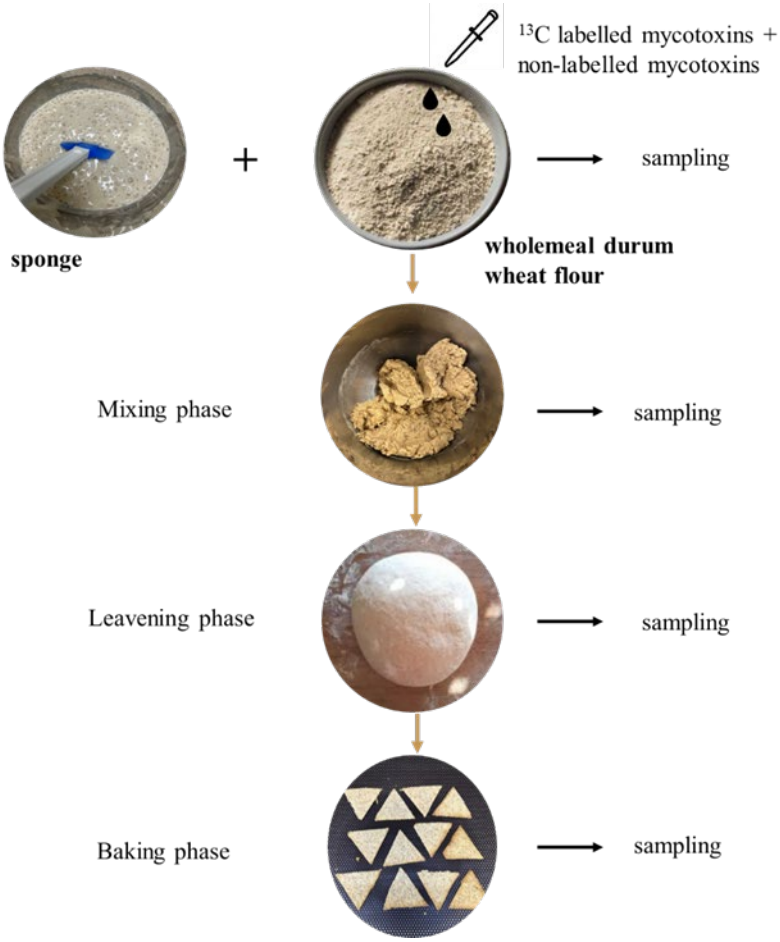

Fig. 1 Workflow for cracker production and sampling for subsequent analysis.

2.3.1 Sample extraction

The samples taken after mixing, leavening, and baking were first homogenised and ground for a few seconds using a blender (Osterizer). This step was not necessary for the flour, which was already fine enough for the subsequent extraction.

For each sample, 1.00 g was weighed in duplicate or triplicate to obtain two technical replicates for LC-HRMS analysis and three technical replicates for liquid chromatography- tandem mass spectrometry (LC-MS/MS) analysis. Mycotoxins were extracted by adding 4 mL of an extraction solvent composed of acetonitrile, water, and acetic acid (79:20:1, v/v/v). The samples were horizontally shaken on a rotary shaker (GFL 3017, Burgwedel, Germany) for 90 min. Then, the supernatant was centrifuged for 10 min at 15,000×g (Sigma 3-18 KS, Linder Labortechnik, Vienna, Austria) to remove any possible impurities. After centrifugation, a dilute and shoot approach was applied. A volume of 0.5 mL from the supernatant was transferred to HPLC vials and mixed with an equal volume (0.5 mL) of a dilution solvent (acetonitrile/water/acetic acid, 20:79:1, v/v/v). After vortexing, the samples were subjected to LC-HRMS analysis. For LC-MS/MS analysis, the samples were appropriately diluted using the dilution and extraction solvents (1:1, v/v) to get the target analytes T-2 and HT-2 toxins into the calibrated range (0.1-35 ppb).

2.3.2 LC-HRMS measurement

The extracts of the samples were analysed in randomized order using an ultra-high-performance liquid chromatography (UHPLC) system coupled to an IQ-X Orbitrap mass spectrometer (Thermo Scientific) 2 µL per sample was used. Chromatographic separation was carried out on a reversed-phase C18 column (XBridge, 3.5 µm, 2.1 × 150 mm; Waters, Milford, Massachusetts, USA) as described in detail by (Doppler et al., 2022) . Full-scan spectra were acquired across a mass range of *m/z* 80–1000, using fast polarity switching and a resolution setting of 120,000 (FWHM at *m/z* 200). Quality control standards were injected at regular intervals throughout the whole sequence.

The non-labelled standard spiking solutions (T-2 toxin/HT-2 toxin at 1 ppm) and mixed solutions containing the non-labelled and the uniformly ^{13}C-labelled standards (at the same respective concentrations) used for the spiking of flour were additionally analysed by LC-HRMS to confirm the absence of degradation products in the standard solutions.

2.3.3 Automated data processing

LC-HRMS data were processed using the in-house developed software MetExtract II (Bueschl et al., 2017). In the first step, the full-scan mass spectrometer data were screened for isotopolog patterns originating from the native (denoted M) and ^{13}C-labeled (denoted M') mycotoxin forms or their putative degradation products (variable number of 13C-atoms: 4-24, 13C-isotopic enrichment: 99.4%, M:M' ratio: 0.82, allowed M:M' ratio: 0.33 – 1.6, Isotopolog pattern checked: 2 on native and 13C-side). Chromatographic peaks of such pairs were extracted for the native and uniformly 13C-labeled form (intensity threshold: 1e4, minimum scans detected: 2, EIC width: 3-11 scans, peak correlation: ≥0.85) and convoluted into feature groups (minimum peak similarity: 0.85). Subsequently, detected metabolites were grouped across samples (maximum RT deviation: 0.1 minutes, maximum m/z deviation: 10 ppm). All results were manually inspected and false positives removed.

2.4 Investigation of the presence of degradation products in crackers made from naturally contaminated wheat

Crackers were also produced using naturally contaminated wholemeal durum wheat flour, following the same procedure described in paragraph 2.3. The mycotoxin levels in the contaminated wholemeal durum wheat flour were determined by LC-MS/MS (see next paragraph). Extracts from contaminated wholemeal durum wheat flour, the dough after mixing and after leavening, and baked crackers were analysed as one replicate using LC-HR-MS.

2.5 LC-MS/MS analysis

In addition to untargeted LC-HRMS analysis, targeted LC-MS/MS analysis was used to quantify T-2 and HT-2 toxins in the unspiked wholemeal durum wheat flour and the samples spiked with unlabelled mycotoxins following a published method (Sulyok et al. 2020). Chromatographic separation of the 5 µL

injection volume per sample was achieved using a 1290 Series HPLC System (Agilent, Waldbronn, Germany) on a Gemini® C18 column (150 × 4.6 mm, 5 µm) with a C18 security guard cartridge (4 × 3 mm; Phenomenex, Torrance, CA, USA). The HPLC system was coupled to a QTrap 5500 mass spectrometer (Applied Biosystems SCIEX, Framingham, Massachusetts, USA) equipped with a Turbo Ion Spray electrospray ionization source. The mobile phases were methanol/water/acetic acid (10:89:1, v/v/v) and methanol/water/acetic acid (97:2:1, v/v/v), both containing 5 mM ammonium acetate and delivered at a flow rate of 1,000 µL/min. The gradient started with 100% eluent A for 2 minutes, followed by a linear increase to 50% eluent B over 3 minutes, and then to 100% eluent B over the next 9 minutes. This was followed by a 4-minutes hold at 100% eluent B and a 2.5-minutes re-equilibration with 100% eluent A. The data was evaluated using the software MultiQuant (version 3.0.3, Applied Biosystems SCIEX, Framingham, Massachusetts, USA).

The toxin levels in the spiked raw material (wholemeal durum wheat flour) were divided by the factor of 5.65 to account for the other ingredients of the cracker dough, since the wholemeal durum wheat flour was only one component. Additionally, the concentrations of HT-2 toxin formed from T-2 toxin were corrected by a factor of 1.099 to reflect the difference in molecular weight between T-2 and HT-2 toxins.

3 | RESULTS

3.1 Cracker optimization

The first step was the production of crackers using compliant, non-spiked wholemeal durum wheat flour to optimize the processing parameters, including mixing, leavening, extrusion, and baking. A major challenge was to minimize the amount of raw material (to 9.5 g of wholemeal durum wheat) while ensuring a technologically feasible and marketable product. This strategy was designed to reduce the use of the costly ^{13}C-labelled standards. Another important aspect was the optimization of the baking conditions, with the optimal baking time determined to be 3 min per side, resulting in a total of 6 min. The moisture content of the wholemeal durum wheat flour was 11.78%, while that of the baked crackers (n = 4) ranged from 2.83 to 3.05%. These values were consistent with the company's specified range.

3.2 Untargeted tracer fate study

To analyze the fate of the mycotoxins T-2 and HT-2 during the production of wholemeal crackers, an untargeted tracer fate study was performed. This study aimed to identify all degradation and transformation products of the toxins formed during the production process. Prior to cracker production, the wholemeal flour used for the spiking experiment was analyzed for the presence of mycotoxins and further fungal secondary metabolites. The concentrations of T-2 and HT-2 toxins as well as of HT-2 glucoside were below the limit of detection (LOD of T-2, HT-2, and HT-2 glucoside: 0.141, 0.642, and 1.446 µg/kg), whereas low levels of type B trichothecenes such as deoxynivalenol and nivalenol as well as of enniatins were detected. Next, the flour was spiked with ^{13}C labelled and non-labelled T-2 or HT-2 toxins and crackers were produced from it. Potential degradation or transformation products, indicated by mass peaks of the labelled and

unlabelled standards that were absent in the unspiked controls, were extracted from the LC-HRMS data. The analysis of the samples collected during cracker production using HT-2 toxin-spiked wholemeal durum wheat flour showed that HT-2 toxin remained stable under the tested cracker production conditions, and the 13C-labeling assisted workflow did not report any conversion or degradation products of the tested mycotoxin in either the dough or the final cracker samples. In contrast, untargeted analysis of extracts from the T-2 toxin spiking experiment identified one conversion product. Specifically, T-2 toxin was rapidly converted into HT-2 toxin during dough preparation. This deacetylation occurred immediately after the addition of ingredients such as water, yeast, sunflower oil, and potato flakes to the wholemeal wheat flour and the mixing step. HT-2 toxin was detected in dough samples both before and after leavening, as well as in the final cracker products. However, it was not present in the wholemeal wheat flour or in the spiking solution, clearly indicating that the degradation of T-2 toxin to HT-2 toxin took place during processing. No additional degradation or transformation products of T-2 toxin were detected. However, it should be noted that small levels of such T-2 or HT-2 toxin products present below their detection limits may have formed.

Finally, also no conversion or degradation products were found in the extracts of the naturally contaminated crackers.

3.3 Targeted analysis for T-2 and HT-2 toxins

To verify the results, the experiment was repeated in a slightly modified way by spiking only unlabelled T-2 or HT-2 toxins at the same level as in the first experiment (Paragraph 2.3), and samples taken at the same production steps were analyzed. In parallel, unspiked wholemeal flour, dough, and cracker samples were also analyzed. Neither T-2 nor HT-2 toxin was detected in any of these unspiked samples.

In the HT-2 toxin spiking experiment, the toxin remained at comparable levels throughout all processing stages—from the spiked wholemeal flour to the dough (both before and after leavening), and into the final cracker samples (Fig. 2). This indicates clearly that HT-2 toxin was not degraded during production, thereby confirming the results obtained from the LC-HRMS analysis.

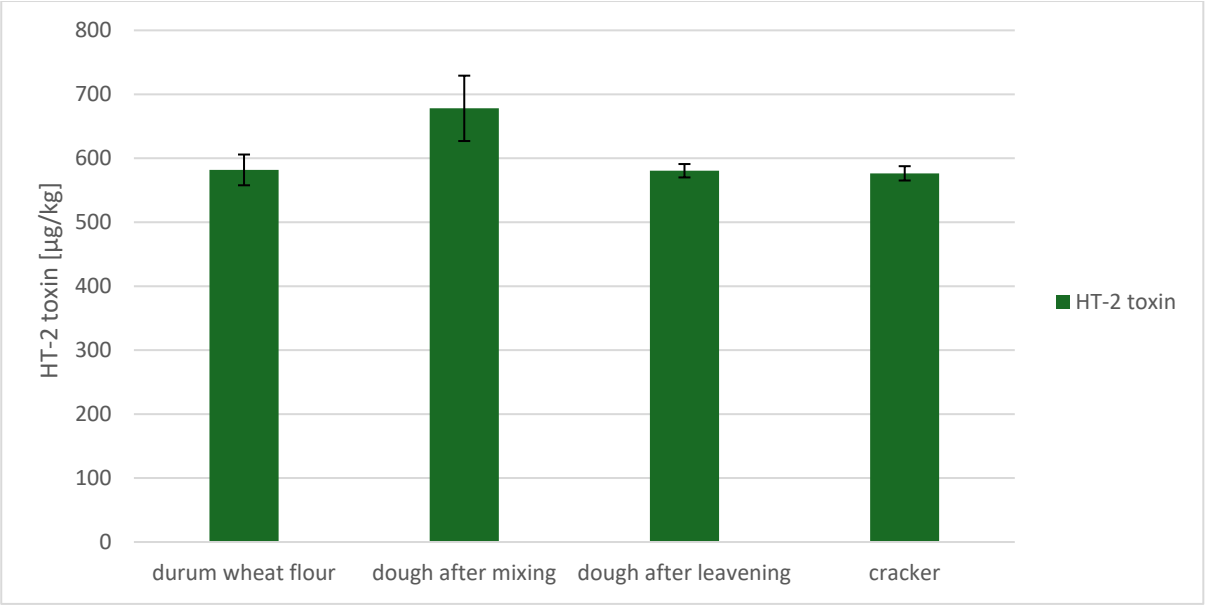

Fig. 2 HT-2 toxin concentrations (µg/kg) monitored across sequential steps during the production of wholemeal crackers in the HT-2 toxin spiking experiment.

Targeted analysis of extracts from the T-2 toxin spiking experiment confirmed that T-2 toxin was partly converted to HT-2 toxin as soon as other ingredients were added to the wholemeal wheat flour and the dough was mixed. No HT-2 toxin was detected in the extract of the spiked flour itself. However, more than one quarter of the spiked T-2 toxin was already present as HT-2 toxin in the dough before leavening. This proportion increased to over 80% after two hours of leavening. In the final cracker samples, approximately 68% of the sum of T-2 and HT-2 toxins was present as HT-2 toxin (Fig. 3). Since the reverse conversion of HT-2 toxin back to T-2 toxin is chemically impossible, the slight decrease in HT-2 levels in the final product (compared to the dough samples after the leavening step) is likely due to the cessation of T-2 toxin to HT-2 toxin conversion during baking. In contrast, in the samples taken after leavening, some chemical transformation may have continued during sample handling, despite efforts to halt the reaction by drying the samples as quickly as possible, which might explain the high HT-2 toxin levels in the dough samples.

In general, variations in the toxin levels across different sample types can be attributed to the small amount of material used for extraction per sample. In the case of the spiked durum wheat flour, these variations may also be due to the difficulty of achieving a homogeneous distribution of the toxins in the flour after spiking it with the toxin standard.

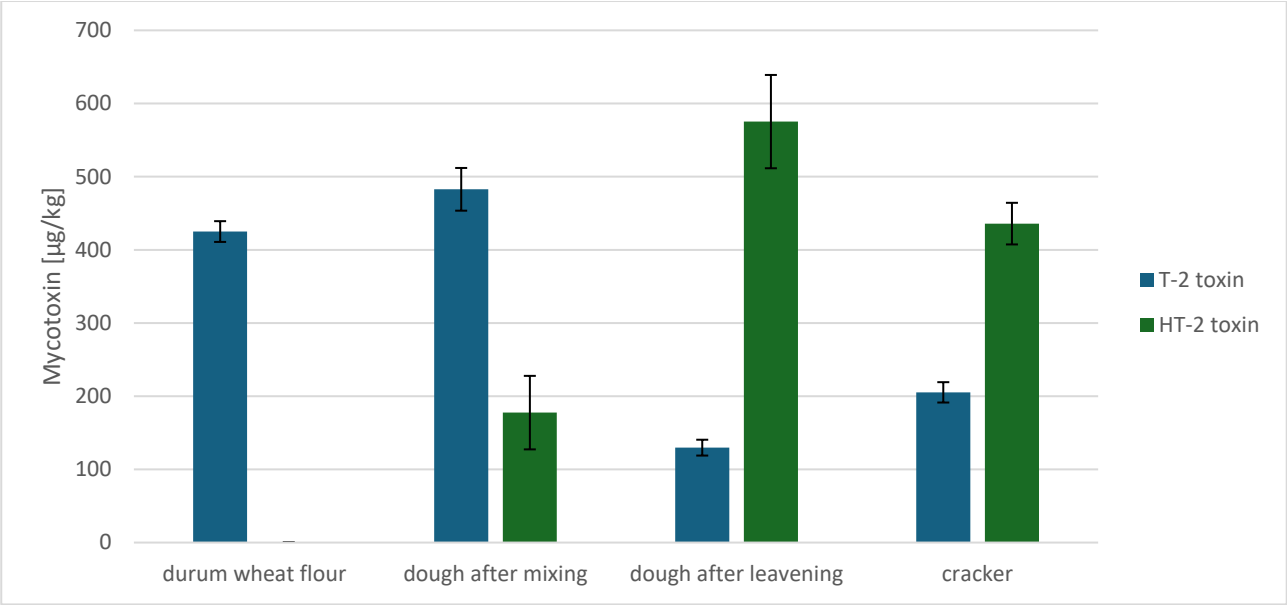

Fig. 3 T-2 and HT-2 toxin concentrations (µg/kg) monitored across sequential steps during the production of wholemeal crackers in the T-2 toxin spiking experiment.

4 | DISCUSSION

The present study investigated the fate of T-2 and HT-2 toxins during wholemeal cracker production using a combined targeted and untargeted ^{13}C-labelled LC-HRMS approach.

The results demonstrate a high chemical stability of HT-2 toxin throughout all processing steps, while T-2 toxin exhibited partial conversion to HT-2 toxin during dough preparation. The difference in the stability of T-2 and HT-2 toxins observed confirms the results from previous studies showing T-2 toxin reductions of up to 74% during bread baking (compared to ≤25% for HT-2) in naturally contaminated flours (De Angelis et al., 2013), and around 60% (HT-2: 20–30%) in artificially contaminated flours (Monaci et al., 2011; Pleadin et al., 2018). Such thermal mitigation effects are generally attributed to chemical reactions induced by heat, leading to partial decomposition or structural modifications of the parent compound (Karlovsky et al., 2016b). However, our findings indicate that the observed T-2 toxin reductions are not caused by thermal degradation but occurred during the early stages of dough formation, most likely driven by the presence of water, yeast or other dough components. In fact, Kuchenbuch et al., (2018), who applied temperatures up to 170 °C during muesli production, also did not observe any hydrolysis of T-2 toxin to form HT-2.

Specifically, in our study, targeted analyses from the T-2 toxin spiking experiment, conducted in the absence of HT-2 toxin, showed that more than one third of T-2 toxin was converted to HT-2 toxin immediately after mixing with other ingredients. This supports earlier hypotheses that enzymatic activities in dough systems, particularly in the presence of yeast, promote the conversion of T-2 to HT-2 toxin (Kuchenbuch et al., 2018). Similar observations were reported by Monaci et al., (2011), who found up to 60% conversion of T-2 to HT-2 toxin during proofing in artificially contaminated flours. Since, the toxicological potency of T-2 and HT-2 toxin is described to be comparable (Schuhmacher-Wolz et al., 2010),

this transformation does however not represent a meaningful detoxification process. Importantly, no additional transformation products, neither from T-2 nor from HT-2 toxin, were detected at any processing stage in comparison to the model heating experiment studied by Beyer et al., (2009), which identified three degradation products of T-2 toxin (Beyer et al., 2009). From a technological point of view, these findings indicates that conventional cracker baking does not provide an effective mitigation strategy for T-2 and HT-2 toxins. It also implies that inactivation strategies focusing on thermal destruction alone are unlikely to achieve substantial reductions in finished products of this type.

5 | CONCLUSION

Given the limited knowledge on the behaviour of T-2 and HT-2 toxins during food processing, an LC-HRMS based untargeted tracer fate study was successfully performed to investigate the formation of all extractable degradation products throughout the production of wholemeal crackers. Accurate quantification of T-2 and HT-2 toxins in both unspiked wholemeal durum wheat flour and samples spiked with unlabelled mycotoxins was achieved by a targeted LC-MS/MS based method. In summary, our small-scale cracker production experiment demonstrated that baking at 195 °C did not lead to the formation of prominent degradation products but mainly promoted the hydrolysis of T-2 toxin into HT-2 toxin. From a toxicological perspective, this transformation does not substantially reduce overall toxicity, since HT-2 toxin retains comparable toxic potency to its parent compound. The results emphasize that mitigation of T-2 and HT-2 toxin contamination must primarily rely on pre-processing measures such as improved grain quality management. Future studies should explore alternative processing conditions or technologies that may enhance degradation while ensuring product safety and quality.

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SECTION III | Multi-Toxin Occurrence and Dietary Exposure in Legumes

6

Chapter

Research Article

Multi-mycotoxins and tropane alkaloids occurrence in legumes: comparative exposure assessment between Italy and Thailand

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Abstract

Legumes are one of important sources of sustainable proteins, yet their safety remains a growing concern. Contamination with mycotoxins and plant alkaloids can compromise both health and market confidence, with risk profiles influenced by dietary habits and production systems. Italy is experiencing a rise in organic, pasta- and snack-based legume products, while Thailand relies heavily on soybeans, mung beans, and peanuts. Regulatory frameworks mirror this division. This study compared the occurrence of 42 multiple mycotoxins and tropane alkaloids in 132 legume-based samples collected from Italy and Thailand, including raw legumes, flours and pasta, and snacks purchased from supermarkets, online shops, and local markets. Contamination was detected in 43% of raw legumes from Italy and 33% of those from Thailand, 48% of flours and pasta from both countries, and in 75% and 63% of snacks, respectively. Distinct contaminant profiles were observed. Italian samples showed a higher occurrence of regulated mycotoxins and tropane alkaloids (atropine, scopolamine), even in organic products, whereas Thailand samples were characterized by a predominance of emerging mycotoxins (beauvericin, fusaric acid, alternariol monomethyl ether), with peanut products exceeding EU limits. Dietary exposure assessment highlighted snacks as the most critical category, due to frequent multi-toxin co-occurrence and exposure-driving events. Overall, these findings demonstrate that emerging toxins contribute substantially to the contamination profile of legume-based foods and are insufficiently addressed by current regulatory frameworks. Expanding surveillance and risk assessment strategies to include emerging mycotoxins and plant toxins are therefore essential to safeguard consumer health and support the sustainable growth of legume-based food systems.

KEYWORDS

Legumes; Legumes-based food; Multi-Mycotoxins; Emerging mycotoxins; Tropane alkaloids; Dietary exposure assessment

1 | INTRODUCTION

Global food accessibility is an increasing concern due to the projected world population growth, expected to reach over 9 billion by 2050, potentially leading to a food insecurity crisis (Ehrlich & Harte, 2015). Among sustainable strategies, legumes represent a promising alternative protein source able to provide proper nutrition, environmental benefits (sustainable food production), and health-promoting effects (Jafarzadeh et al., 2024; Malila et al., 2024; Pavicich et al., 2025). While these alternatives offer many benefits, they can also pose potential safety concerns due to the natural occurrence of toxic secondary metabolites, such as mycotoxins and plant toxins.

Mycotoxins, produced by fungi belonging mainly to the genera *Aspergillus*, *Fusarium*, and *Penicillium*, are frequently found in legumes such as peanuts, soybeans, beans, and chickpeas. Among them, aflatoxins, ochratoxin A, deoxynivalenol, fumonisins, and zearalenone are the most prevalent (Acuña-Gutiérrez et al., 2022; Augustin Mihalache et al., 2023). Recent research has highlighted the co-occurrence of multiple mycotoxins in legume-based products, occasionally at levels exceeding regulatory limits, posing potential health risks—especially for vulnerable populations such as children and those in developing countries (Kunz et al., 2020; Leslie et al., 2025).

Tropane Alkaloids are secondary metabolites that naturally occur in plant (Solanaceae species) or part thereof and the amount depends on many factors (species, plant organ, harvest, storage, extraction procedures) or due to the accidental co-harvesting of the crop of interest with them (adventitious presence) or in food of animal origin, through the ingestion of TA-producing plants by grazing animals or animal feed (González-Gómez et al., 2022; 'Scientific Opinion on Tropane Alkaloids in Food and Feed', 2013). The European Food Safety Authority (EFSA), in a large-scale monitoring project conducted in 2014, analyzed over 1700 plant-derived products—including legumes—and reported sporadic but notable detections (Mulder et al., 2016). Although data are still limited, more recent studies suggest that TAs can persist through food processing (Kaltner, 2022).

The occurrence of mycotoxins and plant toxins in food may therefore derive from their natural presence in the plant-derived food, as cross-contamination during harvesting or as contaminated raw material used for food production. Furthermore, the simultaneous presence of multiple toxins, including regulated, emerging, and masked mycotoxins, may aggravate their properties, resulting in synergistic toxic effects (Siri-anusornsak et al., 2022).

Climate change plays an additional role in shaping the global contamination scenario. Ongoing global warming promotes fungal growth and toxin production, leading to increasing contamination both at pre- and post-harvest stages. This phenomenon is particularly relevant in tropical and subtropical regions, where high temperature and humidity favor fungal proliferation (Casu et al., 2024). According to the Climate Risk Index (Germanwatch), Thailand and Myanmar were among the countries most affected by extreme weather events between 2000–2019, followed by Cambodia and Laos (GLOBAL CLIMATE RISK INDEX 2021, n.d.; Siri-

anusornsak et al., 2022). Such climatic vulnerability makes Thailand an interesting benchmark country to investigate toxin correspondent impacting scenario.

Given this context, continuous surveillance and updated risk management strategies are crucial to ensure food safety compliance and protect consumers. However, the regulatory frameworks governing toxin limits differ substantially between regions. In Europe, Regulation (EU) 2023/915 establishes maximum limits for several regulated mycotoxins, and Regulation (EU) 2016/239 also includes tropane alkaloids in specific food categories—although no limits are currently defined for legumes ('COMMISSION REGULATION (EU) 2023/915', 2023) . In contrast, Thai regulations (*Notification of the Ministry of Public Health No. 414 B.E. 2563*, n.d.) focus primarily on major mycotoxins such as aflatoxins, deoxynivalenol, fumonisins, and ochratoxin A, without establishing limits for emerging mycotoxins or plant toxins. Again, no maximum limits are currently set for legumes.

Considering these differences and the limited occurrence data available, this research aimed to investigate the prevalence and co-occurrence of mycotoxins and plant toxins in legumes collected from several markets in Italy and Thailand. A total of 132 samples were analyzed for 42 compounds, including two *tropane alkaloids*, atropine and scopolamine, and *regulated mycotoxins*, aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1), ochratoxin (OTA, OTB), deoxynivalenol (DON), fumonisin (FB1, FB2), HT-2, T-2 toxin, nivalenol (NIV), zearalenone (ZEN), *emerging mycotoxins*, alternariol (AOH), alternariolmethylether (AME), α-Zearalanol (α-ZAL), α-Zearalenol (α-ZEL), beauvericin (BEA), β-Zearalanol (β-ZAL), β-Zearalenol (β-ZEL), citrinin (CIT), Cyclopiazonic acid (CPA), diacetoxyscirpenol (DAS), emodin (EMO), enniatin type A (ENN A and A1), enniatin type B (ENN B and B1), fusaric acid (FA), monoliformin (MON), mycophenolic (MPA), neosolaniol (NEO), paxilline (PAX), Penitrem A (PEN-A), sterigmatocystin (STC), tentoxin (TEN), tenuazonic acid (TeA) and, *masked mycotoxins*, 3-acetyl deoxynivalenol (3-Acetyl-DON) and 15-acetyl deoxynivalenol (15-Acetyl-DON).

Despite advancements in detection technologies, systematic studies addressing tropane alkaloids and multi-mycotoxin co-occurrence in legumes remain scarce (Augustin Mihalache et al., 2022). Additionally, limited data exist on dietary exposure and the effect of processing on toxin stability in legume-based foods. These knowledge gaps underline the importance of updated risk assessments and harmonized monitoring systems. In this context, the present work aimed to:
(i) evaluate the occurrence of multiple mycotoxins and tropane alkaloids in legume-based products from Italy and Thailand; (ii) compare the contamination profiles across two countries and food categories (raw legumes, flours/pasta, and snacks); (iii) assess dietary exposure based on national consumption data.

Given the growing market for legumes' products, plant-based food made from 100% legumes (*raw beans, flours, pasta*) and processed *snacks* containing at least 40% legume flour were selected. This approach ensures that the detected toxins can be attributed primarily to legume ingredients.

At a glance, this study does not want to "condemn" the use of plant-based products, on the contrary contributes to filling current research gaps by benchmarking two different geographical and regulatory contexts—Italy and Thailand—providing new data on toxin occurrence, co-occurrence, and exposure in

legumes. Future research could help to understand the risks that come along with the shift to alternative diets, overall suggesting the need for a reconsideration of the exposure assessment to natural toxins within new dietary patterns (Pavicich et al., 2025).

The results are intended to support the development of more effective surveillance strategies, improved analytical capabilities, and evidence-based regulatory frameworks for safer and more sustainable food systems.

2 | MATERIALS AND METHODS

2.1 Sample collection

A total of 72 legume samples and their processed products (33 raw pulses, 8 flour, 15 pasta, and 16 snacks) were purchased in 2025 from supermarkets, online shops, and local markets in Thailand, while 60 similar samples (*21* raw pulses*, 11* flour, *12* pasta, *16* snacks) were purchased from corresponding sources in Italy (supplementary material S1 – S4). These products were reported to be originated and/or produced from the corresponding countries. Samples (40 g – 500 g) are milled with Retsch mixer mill MM400 (Haan, Deutschland) at a frequency of 30 Hz for 30 seconds, to increase homogeneity and surface area for extraction. These are then subsampled into 3 falcon tube (50 ml), with one used for analysis and the other stored (-80°C).

2.2 Chemicals and standard

Mycotoxin standards (15-AcDON, 3-AcDON, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, BEA, CPA, DAS, DON, EMO, FB_1, FB_2, FA, HT2, MON, MPA, NIV, OTA, OTB, T-2, TeA, TEN, ZEN, α-ZAL, α-ZEL, β-ZAL, β-ZEL), atropine and scopolamine were purchased from Romer Labs (Tulln, Austria) (Biopure™, certified reference materials). AOH, AME, BEA, ENNA, ENN A_1, ENN B, ENN B_1, STG, were from Sigma-Aldrich (Diegm, Belgium). PEN-A, PAX, NEO were purchased from Fermentek Ltd. (Jerusalem, Israel). Working standard mixtures diluted in acetonitrile (ACN) and stored at -20°C. LC/MS-grade ACN, methanol (MeOH) from RCI Labscan (Ireland), and acetic acid (glacial ≥99.7%) from (Diegem, Belgium) were used for sample preparation and analysis. Ammonium acetate ($C_2H_7NO_2$) was obtained from Merck (Germany). Ultra-pure-grade water (18MΩcm) was obtained from a Milli-Q® ultrapure water (type 1).

2.3 Sample extraction

Sample extraction was optimized using a dilute-and-shoot (DnS) approach (Fig. 1) (Malachová et al., 2014). Each sample (1 g) was extracted with 5 mL of acetonitrile/water/acetic acid (79:20:1, v/v/v) by vortex (90 min, 2,500 rpm), followed by centrifuged for 15 min at 5,000 rpm at 8°C and 500 µL of supernatants were transferred into 1.5 mL tubes containing 500 µL of dilution solvent acetonitrile/water/acetic acid (20/79/1, v/v/v) filtered through a 0.22 µm PTFE syringe filter. The tubes were centrifuged at 12,000 rpm at 8°C for 10 minutes and finally injected into the LC-MS/MS apparatus.

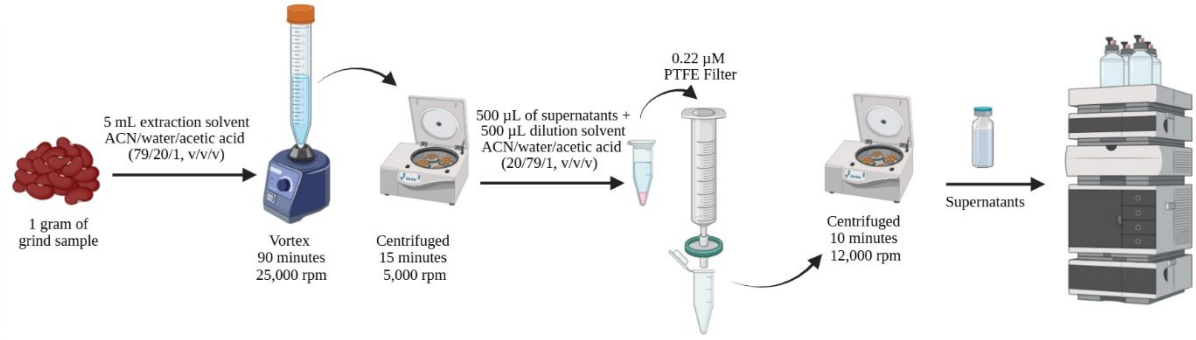


Fig. 1| Schematic illustration of the sample preparation procedure.

2.4 Working Standard and calibration curve preparation:

A working standard (*WS*) of the 40 mycotoxins plus 2 tropane alkaloids at various concentrations was prepared to enable preparation of the calibration curve (c/c), quality control and spike for the recovery check during extraction. For the WS preparation, the multi-analyte standard was mixed with the dilution solvent, (acetonitrile/water/acetic acid 20/79/1, v/v/v). A solvent (external) c/c was obtained by serial dilution of the WS with different volumes using acetonitrile/water/acetic acid (49.5:49.5:1, v/v/v) to obtain dilution levels of 1:2, 1:4, 1:10, 1:20, 1:40, 1:100, 1:200, and 1:1000, respectively. An eight-points matrix matched c/c was used to quantify the analytes. The matrix-matched c/c was obtained by serial dilution of the WS with the blank sample extract (a multi-matrix blended flour composed of ¼ pea flour, ¼ chickpea flour, ¼ lentils flour and ¼ mung bean flour) as diluent. A blank food material was chosen, in which, according to previous analysis, the levels of regulated toxins were below the EU maximum limits (MLs). The blank material was extracted with the extraction protocol indicated above (see paragraph 2.3).

2.5 UHPLC-MS/MS condition

The determination of mycotoxins was carried out on an UHPLC-MS/MS system consisting of an Agilent Infinity 1260 series Infinity II UHPLC system coupled to an Ultivo triple quadrupole mass spectrometry equipped with an Agilent Jet Stream electrospray ionization source (ESI) (Agilent Technologies, Singapore). Chromatographic separation was carried out on a Gemini C18 110 Å 5.0µm (100 × 4.6 mm, Milford, MA, USA) at 30 °C. The injection volume was 5 µL with a flow rate of 0.9 mL/min. Gradient conditions were established to achieve complete separation for 40 mycotoxins plus 2 TAs. Elution was achieved within 14 min using the following gradient of solvent A (5 mM ammonium acetate in 89% water, 10% methanol, 1 % acetic acid, v/v/v) and solvent B (5 mM ammonium acetate in 97 % MeOH, 2% water, 1 % acetic acid, v/v/v): 1-3 min, 99 % A, 1% B; 9 min, 50 % A 50 % B; 11.5–12.0 min, 1 % A, 99 % B; 14 min, 99 % A, 1 % B. PH: 3.61 for solvent A and 5.6 for solvent B.

The MS parameters were as follows: gas temperature, 250 ◦C; gas flow, 10 L/min; nebulizer, 35 psi; capillary voltage, – 4,000 V in both ESI-negative and ESI-positive mode; sheath gas temperature, 350 °C and sheath gas flow, 10 L/min. The MS parameters, such as fragmentor voltage (V) for each compound and ion transitions, were optimized by combining infusion (UHPLC+MS) of individual solutions of each analyte. For

the optimized multiple reaction monitoring (MRM) conditions of the analyte, according to the European Commission (EC) criteria, one precursor ion and two product ions were applied for each mycotoxin ('COMMISSION REGULATION (EC) No 401/2006', 2006). For quantification, the transition of the precursor ion with the highest signal-to-noise (S/N) ratio was chosen, while the transition of the second most abundant ion was monitored for identification. The retention time (RT), most abundant m/z ions and optimized MS parameters are summarized in Supplementary Material – S5.

2.6 Method Validation

Performance criteria from document No SANTE/11312/2021 and document CEN/TR 16059:2010 on single laboratory validation of mycotoxin methods (CEN 2010), were used as references. The method was validated in-house determining the matrix effect (ME), linearity of the calibration, recovery rates, intra-day precision and both limits of detection (LOD) and quantification

(LOQ).

To calculate the matrix effect, a comparison between the slope of the matrix-matched and the solvent calibration curves was performed. The linearity was evaluated by the coefficient of determination (R^2) of eight-point matrix-matched calibration curves generated by peak area (signal intensity) against the analyte concentration. Recoveries of the analytes were measured by spiking at three different concentrations, low concentration level (LL), medium concentration level (ML), and high concentration level (HL), a multi-matrix blended flour composed of 4 different beans: (1/4) pea, (1/4) chickpea, (1/4) lentils and (1/4) mung beans, previously checked for the presence of the target analytes, in six replicates. The precision of the proposed method (expressed as percentage relative standard deviation, RSD %) was evaluated in terms of repeatability (intraday precision), assessed by performing six consecutive injections on the same day of the medium spinking level. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the slope of the matrix-matched calibration curve (S) and the standard deviation (SD) of the peak area from the lowest concentration where the molecular ion can be identified (mass error value) in the matrix-matched calibration curve. A positive sample is confirmed by retention length within ±0.5% compared to the analyte in a pure solvent, provided the ion ratio of the quantifier and the qualifier transitions within ±25%, and if both the qualifier and the quantifier show transitions above the S/N ratio of 10:1.

The following proficiency testing samples were used for the verification of the method trueness for the 2 tropane alkaloids: (i) FAPAS® Proficiency Testing – Tropane Alkaloids in cereals (22220, 22236). *Z*-scores between + 2 and − 2 are considered a satisfactory performance, *Z*-scores between + 2 and + 3 or between − 2 and − 3 are considered questionable performance, and anything outside this range (> + 3 or < − 3) are considered unsatisfactory.

2.7 Data analysis

Data analysis and quantification were performed with the Agilent Mass Hunter Acquisition software (version 10.0). Statistical analysis was performed to calculate the means, standard deviations, and RSD% using Microsoft Excel 2019.

2.8 Legumes consumption scenario

2.8.1 Italy

Legumes are a traditional part of the Mediterranean diet in Italy, valued for their health benefits and role in preventing chronic diseases (Ferreira et al., 2021). In the last years, Italy has seen a sharp increase in the availability and consumption of legume-based products, especially pasta and snacks. This is driven by health, sustainability, and gluten-free trends (Foschia et al., 2017; Petrontino et al., 2023).
Legume pasta (made from pea, lentil, chickpea flours) is popular for its high protein, fiber, and low glycemic index. These products have significantly higher polyphenol and flavonoid content, and lower glycemic index compared to traditional wheat pasta, making them attractive for health-conscious consumers (Foschia et al., 2017).The market for legume-based snacks (extruded products, puffs, crackers) is expanding. Italian consumers value origin certification, recyclable packaging and are willing to pay a premium for these attributes (Petrontino et al., 2023). Legume flours are increasingly used in gluten-free bakery and pasta products, providing improved nutritional profiles and functional properties (Foschia et al., 2017).

2.8.2 Thailand

Thailand cultivates a wide variety of beans, including Glycine (soybean), Phaseolus (kidney, lima beans), and Vigna (mung, black beans) (Sahasakul et al., 2022). There is a little growing interest in developing some legume-based products for health and sustainability, but the market for legume pasta and snacks is less mature than in Italy. Most legumes are consumed in traditional forms (tofu/soy-based products, mung bean (noodles or desserts), peanuts) rather than as processed flour or pasta, even if some snacks are present in the supermarket. There is ongoing research and breeding efforts to enhance the nutritional value and health potential of local bean varieties, which could support future growth in this sector (Amoah et al., 2023; Sahasakul et al., 2022). The main challenge is the limited knowledge and consumer familiarity with legume-based processed foods (Amoah et al., 2023).

2.9 Exposure assessment and risk characterization

For the dietary exposure assessment, Italian food consumption data on legumes were collected from the latest Italian Food Consumption Surveys (2017–2020). Food items were categorized using FoodEx2, a hierarchical classification system developed by EFSA(‘The Food Classification and Description System FoodEx 2 (Revision 2)’, 2015). This framework organizes a wide array of individual foods into structured groups and broader categories. In order to cover all the food categories analysed in this study, an average of the chronic food consumption data (Exposure hierarchy L5) that includes data from borlotti and other common beans, broad beans, soybeans, lentils, chickpeas and peas, was calculated.

For Thailand, data was gathered by the Database of Food Consumption of Thai people (last update 2017), based on the same principle and calculation of the Italian database.

Legumes consumption data were retrieved for the average and high (97.5th percentile) daily intakes for adult consumer groups, Italy (18–64 years old) and Thailand (18–65 years old).

The estimated daily intake (EDI) of each mycotoxin was calculated using Equation (1):

EDI (μg/kg bw/day) = (C x CON *bw*) / 1000 (1)

where C = the average concentration of the mycotoxins found in the food items (μg/kg), CON = food consumption in g/day, and bw = bodyweight 70 kg (adults and elderly) (Valanou et al., 2024).

The most common method used to treat left-censored data (LCD) is the application of the lower bound (LB) scenario and the upper bound (UB) scenario. In the LB the values < LOD and/or LOQ are replaced by zero, while in the UB the values below < LOD and or/LOQ are replaced by the values reported for the LOD and LOQ (*Principles and Methods for the Risk Assessment of Chemicals in Food*, 2009)' ('Management of Left-censored Data in Dietary Exposure Assessment of Chemical Substances', 2010).

EFSA established a tolerable daily intake (TDI) of 1 μg/kg bw/day for FUMs based on increased incidence of megalocytic hepatocytes in mice (H. Knutsen et al., 2018) and the same TDI for DON based on reduced body weight gain of mice(H. K. Knutsen et al., 2017). An *in vivo* toxicity study with rats showed a reduction of total leukocyte count due to T-2 and HT-2 and was identified as the critical effect for establishing a group TDI (H. K. Knutsen et al., 2017). Based on this effect, EFSA established a group TDI of 0.02 μg/kg bw/day. For ZEN, considering its oestrogenic effects in pigs, EFSA established a TDI of 0.25 μg/kg bw/day ('Scientific Opinion on the Risks for Public Health Related to the Presence of Zearalenone in Food', 2011).

For substances whose chemical structure is known, and the dietary exposure can be estimated but there are few specific toxicity data, the threshold of toxicological concern (TTC) can be applied as tool for providing scientific advice about possible human health risks from low level exposures(More, Bampidis, Benford, Bragard, et al., 2019). For *Alternaria* toxins, EFSA set a TTC = 0.025 μg/kg bw/day for genotoxic Alternaria: AOH, AME; TTC = 1.5 μg/kg bw/day for non-genotoxic Alternaria: TEN and TeA) (Arcella et al., 2016). Considering that there are only few or no relevant toxicity data available for BEA and ENNs and that their chemical structures are known, a TCC = 1.5 μg/kg bw/day was established('Scientific Opinion on the Risks to Human and Animal Health Related to the Presence of Beauvericin and Enniatins in Food and Feed', 2014).

For the risk characterization of mycotoxins that are both carcinogenic and genotoxic such as AFs, OTA and STC there are no toxicological thresholds established. Therefore, the margin of exposure (MOE) approach is used. The MOE is a key tool used in chemical risk assessment to consider potential health concerns especially for substances like genotoxic carcinogens in food for which a health-based guidance value is inappropriate to establish (*Margin of Exposure*, 2023). The MOE is equal to the ratio of the benchmark dose lower limit (BMDL) of the mycotoxin and the exposure of the consumer to the mycotoxin as shown in Equation (2):

MOE = BMDL10 / *EDI* (2)

The BMDL10 values were 0.4 μg/kg bw/day for AFs for the incidence of hepatocellular carcinoma in rats(Schrenk, Bignami, et al., 2020), 14.5 μg/kg bw/ day for OTA (Schrenk, Bodin, et al., 2020) and 16.0 μg/kg

bw/ day for STC ('Scientific Opinion on the Risk for Public and Animal Health Related to the Presence of Sterigmatocystin in Food and Feed', 2013). An MOE of 10,000 or higher is of low concern, while an MOE lower than 10,000 raises a health concern from a public health point of view and should be a priority for risk managers (*Margin of Exposure*, 2023).

EMO and FA could not be risk-characterized due to the absence of toxicological reference values.

For tropane alkaloids (atropine and scopolamine), risk characterization was performed on an acute basis, as the EFSA CONTAM Panel established a group acute reference dose (ARfD) of 0.016 µg/kg body weight(Binaglia, 2022). Acute exposure estimates were calculated using acute food consumption data when available and compared to the ARfD to derive a screening acute risk characterization, known as Hazard Quotient (HQ). Consistent with EFSA guidelines, an HQ exceeding 1 indicates that potential adverse health effects cannot be excluded(More, Bampidis, Benford, Bennekou, et al., 2019). Acute consumption data were available only for Italy from EFSA databases. For Thailand, where acute intake data were not available, chronic consumption values were used as a surrogate to provide a proxy acute risk characterization, acknowledging the associated uncertainty.

For the cumulative risk assessment of AFs, we used the total margin of exposure (MOET) (More, Bampidis, Benford, Bennekou, et al., 2019). The MOET was calculated as the sum of the MOEs and it was considered to indicate a potential health risk if it was lower than 10,000 (Equation (3).

$$MOET = 1 / (1/MOEAFB1 + 1/MOEAFB2 + 1/MOEAFG1 + 1/MOEAFG2) \quad (3)$$

3 | RESULTS

The complex composition of legume matrices results in matrix effects and isobaric interferences (poor selectivity), making an effective clean-up one of the most critical steps in multi-toxin analysis. Previous studies have demonstrated that the dilute-and-shoot (DnS) method provided reliable results for determining mycotoxins and their metabolites in food (Malachová et al., 2014; Sulyok et al., 2020). Unlike conventional clean-up techniques which removes unwanted co-extracted matrix components, DnS simply decreases matrix effect by dilution, enabling rapid, cost-effective analysis while still covering a wide range of toxins with high chemical diversity.

3.1 Method validation

Matrix-matched calibration curves were constructed by measuring eight concentration levels of spiked samples (see Paragraph 2.4). The range of detection was established based on the EU Maximum Limits ('COMMISSION REGULATION (EU) 2023/915', 2023). For the non-regulated analytes, the EU Reg. was the reference starting point for the establishment of a detection range. LOD and LOQ of the method and all the performance characteristics are present in the supplementary material – S6. Good linear regression for all analytes is achieved, obtaining coefficient of determination (R^2) values ≥ 0.998. Only moniliform present a R^2 of 0.994. After the calculation of the matrix effect, 3-AcDON and EMO show higher ion suppression effect while MON presents an ion enhancement. Average recovery across the three concentrations were

within the recommendation range of 70-120% (Pihlström et al., n.d.), indicating satisfactory method performance. Exceptions included citrinin, which showed recovery levels below the recommendations at all levels; MON at the low level (54%); and NIV at the medium and high levels (61% and 62 %, respectively). The suboptimal recovery and peak efficiency for NIV may be due to a non-ideal mobile phase which might not be best elution condition for this toxin. According to SANTE (2021) guideline, intraday precision should not exceed 20% (Pihlström et al., n.d.). Satisfactory results were obtained for all the analytes, except for α-ZAL (22%).

To evaluate the trueness for tropane alkaloids, two proficiency test samples (FAPAS) were extracted and the *z*-scores were calculated. Satisfactory results with the *z*-scores between + 2 and – 2 were obtained for atropine and scopolamine.

3.2 Occurrence of multi-mycotoxins and plant toxins

A total of 132 samples produced in and purchased from Italy and Thailand were analyzed for contamination of multi-mycotoxins and plant toxins. Overall, 64 samples (48%) were positive for at least one compound (Fig. 2). Contamination was detected in both countries and across all food categories, with the highest prevalence in snacks (75% in Italy and 63% in Thailand), followed by flour/pasta (48% in both countries) and raw legumes (43% in Italy and 33% in Thailand). Supplementary Material (S7a-S7b to S9a-S9b) summarized the occurrence of multi-toxins in each category of products.

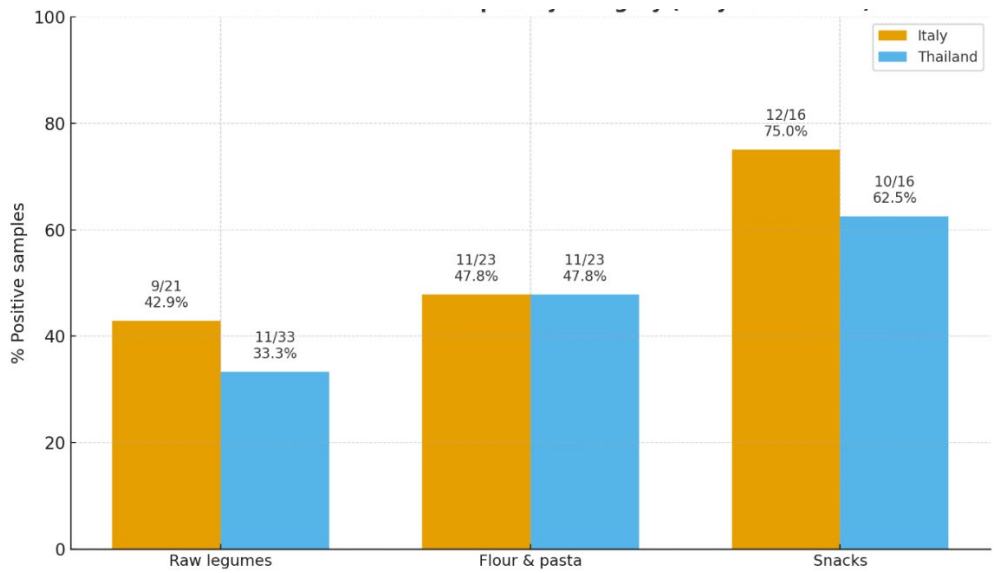


Fig. 2 | Percentage (%) of positive samples by category (Italy vs Thailand)

In raw legumes, Italian samples (Table S7a) showed that 9 out of 21 (43%) were positive for at least one contaminant, with a broader occurrence of emerging mycotoxins, BEA, STG, TEN and EMO, generally at low levels, with only sporadic detection of regulated mycotoxins (AFB2 and ZEN). Interestingly, the plant toxin atropine was found in organic mung beans (0.1 µg/kg), confirming the potential occurrence of tropane alkaloids in legumes. Among the 21 Italian raw samples, 7 were organic and 4 of these (57%) were

contaminated. In contrast, Thailand raw legumes (Table S7b) showed a slightly lower overall prevalence (33%; 11 out of 33 samples) but included cases of higher contamination levels and broader toxin profiles. AFB1 was detected in peanuts at 6 µg/kg, exceeding the EU maximum levels (Reg. EU 2023/915), indicating a potential food safety concern. Among emerging mycotoxins, BEA was the most frequent, detected in chickpeas, soybeans, black and red beans, often at higher levels than in Italian counterparts. Notably, FA reached 418.3 µg/kg in chickpeas, clearly standing out compared with other matrices. Similar to Italy, atropine was also detected in Thailand mung beans (0.5 µg/kg). No organic samples were found for this category.

As shown in **Fig. 3**, emerging mycotoxins represented the most prevalent toxin class in raw legumes from both countries, with BEA detected in 10% of Italian and 21% of Thailand samples.

Regulated mycotoxins, including aflatoxins (AFB1, AFB2) and ZEN, were sporadically detected, with a slightly higher occurrence in Thai samples (up to 12% for ZEN). Other emerging toxins, such as EMO and TEN, occurred at comparable frequencies in both countries (5–10%).

Legume-based flour and pasta from both countries showed the same proportion of positives (48%; 11 out of 23 each; Tables S8a–S8b), although with different toxin patterns. In Italy, regulated mycotoxins were detected, especially with AFB2 found at concentrations ranging from 0.9 to 1.10 µg/kg and, in one case, DON at 44.3 µg/kg in a durum wheat flour sample. Overall, emerging mycotoxins—particularly BEA and enniatins—were more frequently detected, even if at low levels. Atropine and scopolamine were also identified in green pea pasta and chickpea flour, respectively. Among Italian pasta products, 6 out of 12 were organic, and 3 of these (50%) were contaminated.

In Thailand, flour and pasta samples showed a comparable number of positives (11/23), but contamination was dominated by emerging mycotoxins, like BEA and FA. FA reached its highest concentration in a mixed organic pasta containing 21.7% organic chickpea flour, mirroring the elevated FA levels observed in raw chickpeas, which emerged as the most critical matrix for this toxin. Only atropine was detected in Thai flour and pasta products, while scopolamine was not observed. Of the 15 Thailand pasta samples, 7 were organic and 4 (57%) of these were positive.

In **Fig. 4**, regulated mycotoxins were found more in Italian samples, with AFB2 detected in 22% of products and DON and OTA each occurring in 4.3% of samples, whereas in Thailand only a single positive case of ZEN (4.3%) was observed. Overall, emerging mycotoxins represented the most prevalent toxin class in both countries, with BEA (13%) and STG (8.7%) predominating in Italy, while FA (34%), BEA (30%) and AME (17%) were most frequent in Thailand.

Legume-based snacks represented the most critical product category in both countries.

In Italy (Table S9a), 12 out of 16 products (75%) were positive for at least one contaminant. Regulated mycotoxins were frequently detected, with one sample exceeding the EU MLs for AFB1 (3.8 µg/kg), and the presence of fumonisins (FB1 and FB2), confirming that these toxins may also occur in legume-based matrices. Emerging mycotoxins were widespread, particularly BEA, which was frequently detected in lentil-

and chickpea-based snacks. Atropine was the only plant toxin identified. Notably, most contaminated products were organic snacks (7 out of 8, i.e., 88%).

In Thailand (Table S9b), 10 out of 16 snack samples (63%) were positive. The contamination profile was dominated by emerging mycotoxins, particularly AME and FA, whereas regulated mycotoxins were less frequently detected. A peanut-based snack made from unpeeled peanuts showed a very high FA concentration (287 µg/kg), representing the second critical case after raw chickpea, further confirming the relevance of fusaric acid as a key contaminant in legume-based products. DON was mainly found in Italian snacks based on 100% toasted broad beans and soybeans. Interestingly, it was detected in unpeeled broad beans from Thailand but not in peeled ones, suggesting a possible contribution of the outer coat to toxin persistence, while all Italian broad-bean snacks were peeled. No plant toxins were detected in Thailand snacks, and all products were conventional, as no organic formulations were available in this survey. In **Fig. 5**, snacks exhibited the highest prevalence of contamination among all product categories, with emerging mycotoxins representing the dominant toxin class in both countries, while regulated mycotoxins and plant toxins occurred less frequently but contributed to the overall multi-toxin exposure scenario.

Fig. 3 | Prevalence of toxin classes in raw legumes: Italy vs Thailand.

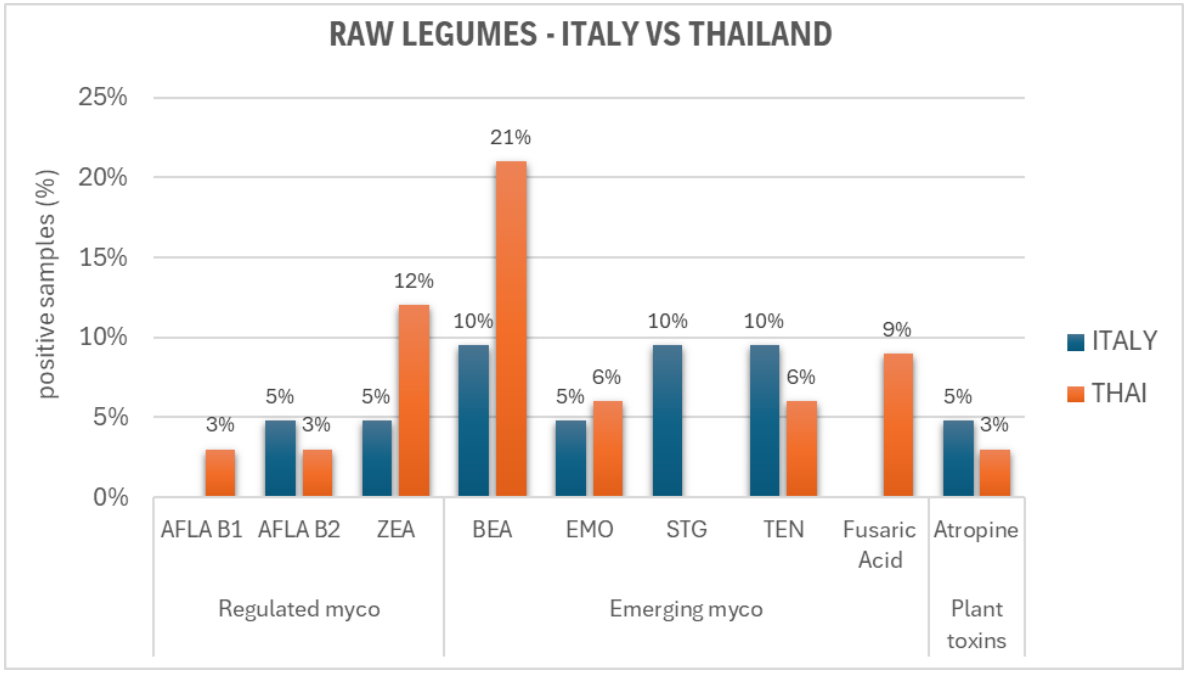


Fig. 4 | Prevalence of toxin classes in legume-based flour and pasta: Italy vs Thailand.

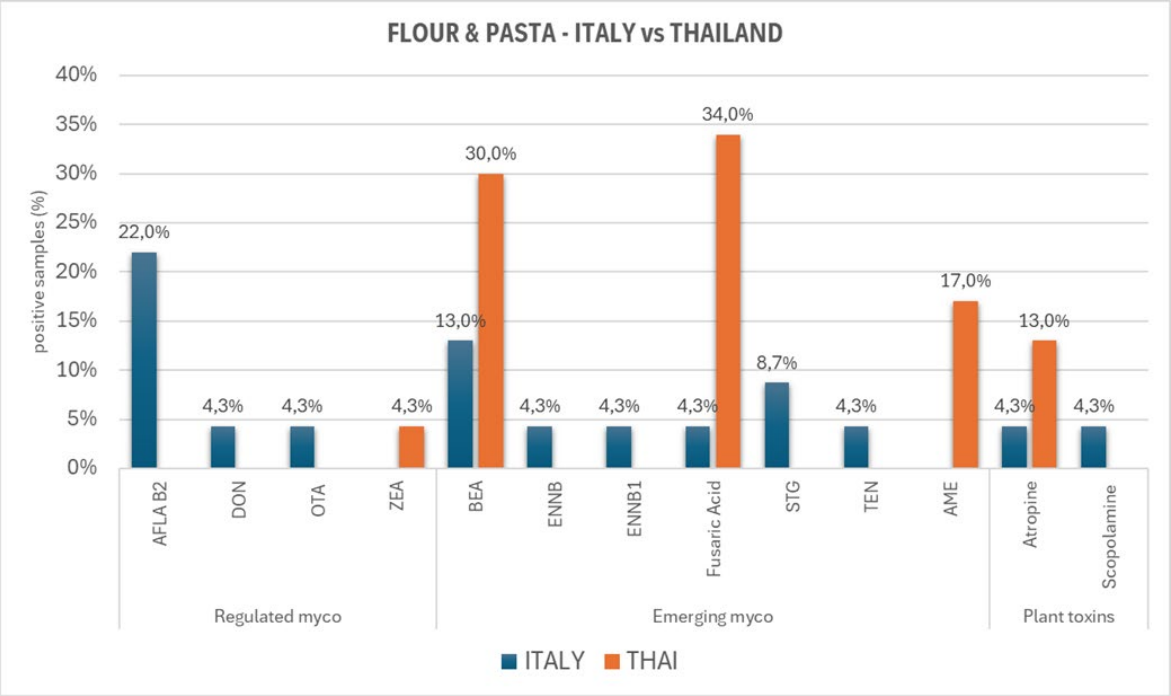
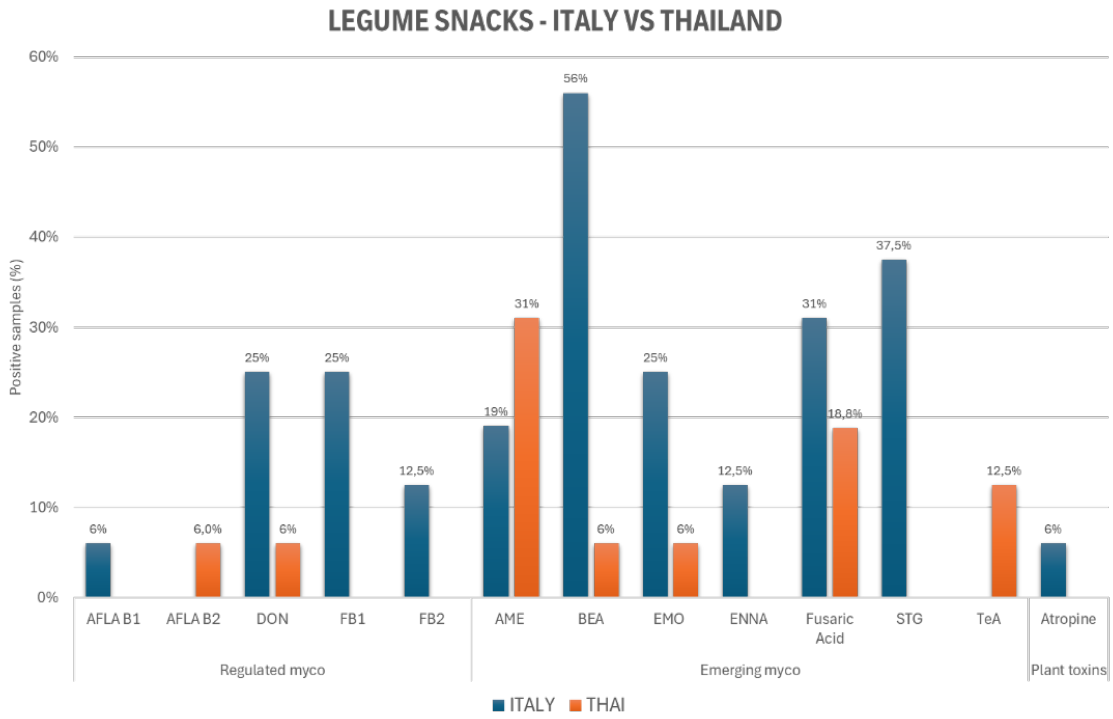


Fig. 5 | Prevalence of toxin classes in legume-based snacks: Italy vs Thailand.



3.3 Co-occurrence of toxins in legumes

The co-occurrence of multiple toxins was observed in both Italian and Thai legume-based products, although with different frequencies and contamination patterns across raw materials and processed formats (**Fig. 6**). A systematic review of mycotoxin co-occurrence in plant-based meat alternatives highlighted the potential for combined toxic effects, particularly under realistic exposure scenarios (Augustin Mihalache et al., 2022).

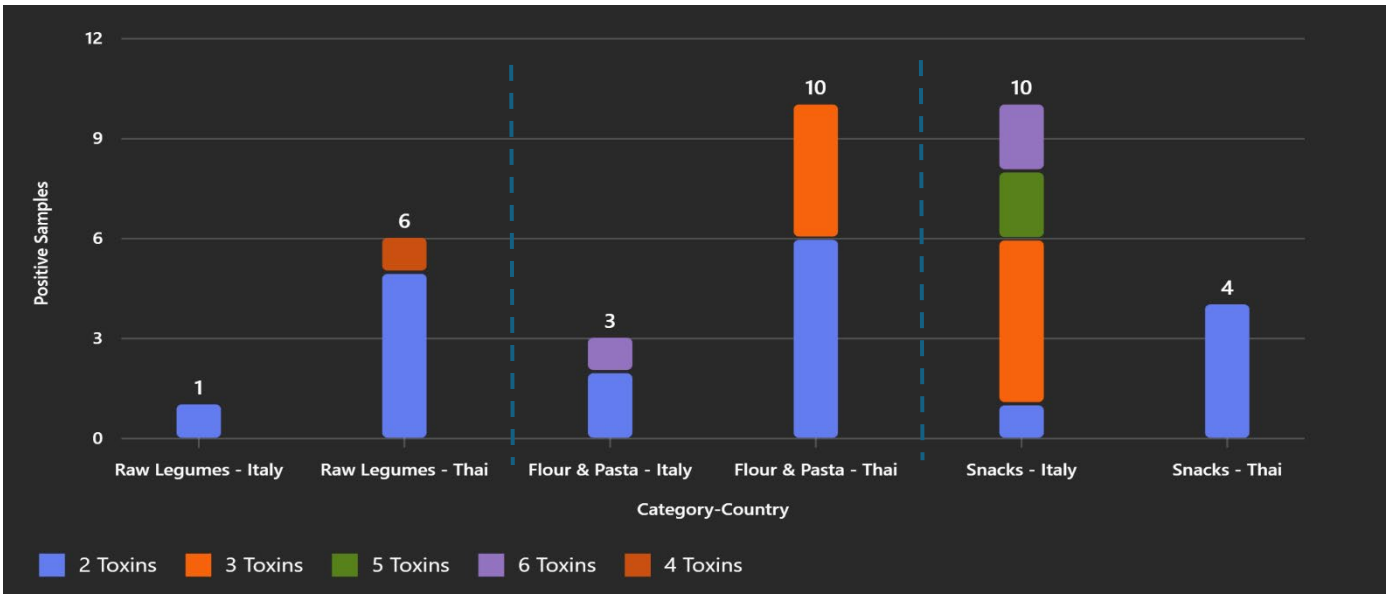
In Italian raw legumes, co-occurrence was detected in a limited number of samples (≈11%)**,** specifically in organic mung beans, where both atropine and ZEN were present. Although the detected concentrations were low, this finding confirms that even single-ingredient raw materials can act as carriers of multiple toxins, particularly in organic production systems where natural contamination sources are more likely. In contrast, Thai raw legumes exhibited a higher frequency and diversity of co-contamination. 6 out of 11 positive samples (≈55%) showed multi-toxin co-occurrence. The most common combinations included emerging mycotoxins such as BEA**,** FA, and EMO. Mung beans, chickpeas, and black beans were the most affected matrices, often containing both fungal and plant-derived toxins (e.g. atropine), suggesting a broader contamination profile.

Among Italian flour & pasta, 3 out of 11 positive samples (27%) showed co-occurrence. The most complex contamination was found in green pea pasta, where six different toxins – including both regulated and emerging compounds – were detected. Two other samples, wholemeal chickpea pasta and chickpea flour, showed co-contamination with two toxins each. Overall, AFB2 and BEA were the most frequently detected toxins. In contrast, Thai flour & pasta showed a significantly higher rate of co-occurrence: 10 out of 11 positive samples (91%) contained multiple toxins (Table 8b). The most common patterns involved two or three toxins, with organic mung bean flour and mixed pea flour showing the highest diversity. The most prevalent compounds were BEA and FA, consistently found across several samples. These findings highlight that even processed matrices, such as flours and pasta, which typically undergo thermal treatments, can still carry traces of both regulated and emerging mycotoxins, suggesting their relative stability during processing.

In Italian legume snacks, 10 out of 12 positive samples (83%) showed co-occurrence of multiple toxins. Most of these samples contained three or more toxins, with combinations involving both regulated and emerging mycotoxins. The most frequently detected compounds were BEA, FA and STG, suggesting a recurring pattern of contamination across various legume-based matrices such as chickpeas, lentils, and broad beans. In Thai legume snacks, 4 out of 10 positive samples (40%) exhibited co-occurrence primarily involving two toxins. While the toxin profiles were less complex than those observed in Italian samples, the contamination was more evenly distributed. The most prevalent toxins were AME, and notably, TeA appeared for the first time in salted green peas and mung beans, marking a new concern in Thai snack products.

These findings underscore the need for region-specific monitoring strategies and given the toxicological potential of additive or synergistic effects between mycotoxins and plant alkaloids, reinforce the importance of a multi-analyte surveillance approach.

Fig. 6 | Distribution of the co-occurrence between raw legumes, flour & pasta and snacks in Italy vs Thailand.



3.4 Dietary exposure and risk characterization

Dietary exposure and risk characterization to mycotoxins and plant toxins through the consumption of legume-based products were performed using both lower bound (LB) and upper bound (UB) scenarios, following EFSA recommendations for the treatment of left-censored data. Exposure was estimated for average consumers and high consumers (97.5th percentile) and expressed as estimated daily intake (EDI). In the lower bound (optimistic) scenario, left-censored data (LCD), i.e. results below the LOD or LOQ, were replaced by zero, while in the upper bound (pessimistic) scenario LCD values were replaced by the corresponding LOD or LOQ. MOET was computed only when more than one aflatoxin contributed to exposure.

EDIs for individual toxins are reported in **Tables 1, 2 and 3**, while risk characterization for each legume-based products category is presented in the Supplementary material S10 – S12.

For raw legumes, the dietary exposure assessment indicated an overall low chronic risk for both Italian and Thai consumers under LB and UB scenarios. In Italian samples, ZEN exposure remained below the TDI in all exposure scenarios. For aflatoxins, AFB2 showed MOE values >10,000 for average consumers under the optimistic scenario, indicating low concern; however, under the pessimistic scenario (UB), MOE values <10,000 were observed for high consumers (97.5th percentile), while MOE values remained >10,000 for average consumers, indicating a potential health concern limited to high consumers only. Emerging mycotoxins, such as BEA and TEN, resulted in EDIs well below the TTC values in all exposure scenarios. In Thai raw legumes, higher EDIs were observed for aflatoxins and fusaric acid (FA) compared with Italy. Both AFB1 and AFB2 resulted in MOE values <10,000 under LB and UB scenarios, indicating a potential health

concern. ZEN exposure remained below the TDI, while BEA EDIs were below the TTC. Comparable exposure patterns to aflatoxins have been reported in legume-based meat alternatives, where AFB1 EDIs ranged from 0.007 to 0.02 μg/kg bw/day under substitution scenarios (Augustin Mihalache et al., 2023, 2024), confirming aflatoxins as key drivers of dietary risk in legume-derived foods.
For atropine, the proxy acute risk characterization showed HQs well below 1 in both countries, indicating no acute health concern.

For legume-based flour and pasta products, Italian samples showed DON exposure below the TDI in all exposure scenarios. Regarding aflatoxins, AFB1 resulted in MOE values >10,000 under the UB scenario, indicating low concern, whereas AFB2 showed MOE values <10,000 under both LB and UB scenarios, indicating a potential health concern. OTA and STC resulted in MOE values >10,000, indicating low concern. Emerging mycotoxins (BEA, ENNs and tentoxin) showed EDIs below the TTC values in all scenarios. In Thai flour and pasta products, regulated mycotoxins were largely absent. ZEN exposure remained below the TDI in all scenarios. Emerging mycotoxins, including AME and BEA, resulted in EDIs below the TTC values, while FA could not be assessed due to the lack of toxicological reference values.
For plant toxins, the proxy acute risk characterization showed no acute health concern under the applied screening approach.

Legume-based snacks represented the most critical category in terms of dietary exposure. In Italian products, DON and fumonisins (FB1 and FB2) showed EDIs below their respective TDIs, while STC resulted in MOE values >10,000, indicating low concern. In contrast, AFB1 showed MOE values <10,000 under both LB and UB scenarios, indicating a potential health concern. Emerging mycotoxins were the main contributors to exposure, but with BEA, AME and ENNA showing EDIs below the TTC values. In Thai snacks, dietary exposure was dominated by emerging mycotoxins. EDIs for AME and BEA were below the TTC values. AFB2 resulted in MOE values <10,000, indicating a potential health concern, while DON exposure remained below the TDI.

MOET was calculated only for aflatoxins in Thailand raw legumes, where AFB1 and AFB2 co-occurred, and resulted in values <10,000 under both LB and UB scenarios for average and 97.5th, indicating a potential health concern. In the remaining categories, where only one aflatoxin was detected, MOET equaled the corresponding individual MOE.

Table 1. Estimated daily intake (EDI) of toxins in adult consumers, calculated under LB and UB scenarios for raw legumes from Italy and Thailand, based on mean dietary intake data.

toxins	LB				UB			
	Italy raw legumes		**Thai raw legumes**		**Italy raw legumes**		**Thai raw legumes**	
	Average	97.5th	Average	97.5th	Average	97.5th	Average	97.5th
	Chronic mean dietary intake (g/Kg bw/day)		*Mean Dietary Intake (g/Kg bw/day)*		*Chronic mean dietary intake (g/Kg bw/day)*		*Mean Dietary Intake (g/Kg bw/day)*	
	0,33	**0,55**	**0,38**	**0,84**	**0,33**	**0,55**	**0,38**	**0,84**
AFB1			0,000228	0,000501			0,000209	0,000460
AFB2	0,000018	0,000029	0,000077	0,000170	0,000035	0,000058	0,000052	0,000115
BEA	0,000010	0,000017	0,000712	0,001566	0,000015	0,000025	0,000705	0,001553
EMO	0,000013	0,000022	0,000077	0,000169	0,000043	0,000072	0,000045	0,000099
FA			0,014844	0,032666			0,014756	0,032473
STC	0,000027	0,000045			0,000034	0,000057		
TEN	0,000089	0,000148			0,000091	0,000152		
ZEA	0,000046	0,000077	0,001926	0,004239	0,000100	0,000167	0,001882	0,004141
	Acute mean dietary intake (g/Kg bw/day)				*Acute mean dietary intake (g/Kg bw/day)*			
	0,78	**1,22**			**0,78**	**1,22**		
Atropine	0,000011	0,000018	0,000042	0,000092	0,000063	0,000099	0,000016	0,000034

Table 2. Estimated daily intake (EDI) of toxins in adult consumers, calculated under LB and UB scenarios for legume-based flour and pasta from Italy and Thailand, based on mean dietary intake data.

	LB				UB			
	Italy legume flour and pasta		**Thai legume flour and pasta**		**Italy legume flour and pasta**		**Thai legume flour and pasta**	
toxins	Average	97.5th	Average	97.5th	Average	97.5th	Average	97.5th
	Chronic mean dietary intake (g/Kg bw/day)		*Mean Dietary Intake (g/Kg bw/day)*		*Chronic mean dietary intake (g/Kg bw/day)*		*Mean Dietary Intake (g/Kg bw/day)*	
	0,33	**0,55**	**0,38**	**0,84**	**0,33**	**0,55**	**0,38**	**0,84**
AFB1					0,000015	0,000025		
AFB2	0,000145	0,000243			0,000160	0,000590		
AME			0,000066	0,000146			0,000074	0,000162
BEA	0,000013	0,000022	0,000132	0,000291	0,000019	0,000031	0,000138	0,000305
DON	0,001332	0,002227			0,001441	0,002409		
ENNA1	0,000000	0,000000			0,000006	0,000011		
ENNAB	0,000065	0,000109			0,000077	0,000129		
ENNAB1	0,000015	0,000025			0,000021	0,000035		
FA	0,000038	0,000063	0,001181	0,002598	0,000133	0,000222	0,001217	0,002679
OTA	0,000009	0,000015			0,000033	0,000056		
STC	0,000024	0,000040			0,000033	0,000055		
TEN	0,000030	0,000051			0,000033	0,000055		
ZEA			0,000881	0,001939			0,000945	0,002079
	Acute mean dietary intake (g/Kg bw/day)				*Acute mean dietary intake (g/Kg bw/day)*			
	0,78	**1,22**			**0,78**	**1,22**		
Atropine	0,000011	0,000017	0,000022	0,000048	0,000063	0,000045	0,000055	0,000121
Scopolamine	0,000127	0,000200			0,000188	0,000296		

Table 3. Estimated daily intake (EDI) of toxins in adult consumers, calculated under LB and UB scenarios for legume snacks from Italy and Thailand, based on mean dietary intake data.

toxins	LB				UB			
	Italy legume snacks		Thai legume snacks		Italy legume snacks		Thai legume snacks	
	Average	97.5th	Average	97.5th	Average	97.5th	Average	97.5th
	Chronic mean dietary intake (g/Kg bw/day)		*Mean Dietary Intake (g/Kg bw/day)*		*Chronic mean dietary intake (g/Kg bw/day)*		*Mean Dietary Intake (g/Kg bw/day)*	
	0,33	**0,55**	**0,38**	**0,84**	**0,33**	**0,55**	**0,38**	**0,84**
AFB1	0,000105	0,000175			0,000116	0,000194		
AFB2			0,000050	0,000110			0,000070	0,000154
AME	0,000039	0,000064	0,000150	0,000329	0,000046	0,000077	0,000156	0,000342
BEA	0,000173	0,000289	0,000025	0,000055	0,000174	0,000291	0,000031	0,000068
DON	0,006037	0,010095	0,001080	0,002378	0,006117	0,010228	0,001205	0,002652
EMO	0,000174	0,000290	0,000031	0,000067	0,000198	0,000331	0,000066	0,000144
ENNA	0,000014	0,000023			0,000017	0,000028		
FB1	0,004047	0,006767			0,004091	0,006841		
FB2	0,000518	0,000866			0,000559	0,000935		
FA	0,002235	0,003738	0,011254	0,024767	0,002296	0,003839	0,011339	0,024953
STC	0,000158	0,000264			0,000163	0,000272		
TeA			0,000338	0,000743			0,000529	0,001165
	Acute mean dietary intake (g/Kg bw/day)				*Acute mean dietary intake (g/Kg bw/day)*			
	0,78	**1,22**			**0,78**	**1,22**		
Atropine	0,000039	0,000061			0,000092	0,000145		

4 | DISCUSSION: Italy vs Thai

This comparative survey shows that legume-based products from both Italy and Thailand are contaminated by multiple mycotoxins and plant toxins, although with different patterns across matrices and countries. Overall, nearly half of the samples analyzed contained at least one toxin, and snacks emerged as the most affected category in both regions. As illustrated in the Results (**Fig. 3, 4 and 5**), emerging mycotoxins were the predominant group, while regulated mycotoxins and tropane alkaloids occurred less frequently but still contributed to the overall risk profile.

In raw legumes, emerging mycotoxins were the most prevalent toxin group in both countries, whereas regulated mycotoxins occurred less frequently. Overall, this pattern reflects two distinct but complementary risk scenarios: a widespread low-level exposure to emerging mycotoxins in Italy, and sporadic contamination events at high concentration in Thailand, particularly involving the detection of AFB1 in peanuts and FA in chickpeas.

Data on mycotoxin occurrence in legumes remain limited at the international level, and only a few validated multi-mycotoxin analytical approaches have been reported for this commodity (Pavicich et al., 2024). In a non-representative market screening conducted in the Netherlands and Germany, OTA was detected at high levels in white beans and green peas, while T-2 toxin was found in black lentils; other

regulated mycotoxins were generally present at low or undetectable levels (Kunz et al., 2020). A study conducted in Nigeria on peanuts reported a high prevalence of aflatoxins (64% of samples), with mean concentrations of 1281 µg/kg (Leslie et al., 2025). Current research (Hamida et al., 2023; Iqbal et al., 2024) shows FA is important in Fusarium wilt of legumes (chickpea, broad bean), but there is little systematic data on its natural occurrence as a contaminant in harvested legume grains. FA is known to exert cytotoxic effects and to potentiate the toxicity of other *Fusarium* mycotoxins, potentially acting synergistically (e.g. with DON or fumonisins), thereby raising concern for combined exposure scenarios (Gruber-Dorninger et al., 2017). However, most occurrence data concern cereals; targeted residue surveys in legume commodities remain a clear gap.

In flour and pasta products, contamination patterns differed markedly between Italy and Thailand, despite a comparable overall prevalence of positive samples in both countries (48%). Similar to what was observed for raw legumes, Italian samples were characterized by a broader occurrence of low-level toxin classes, whereas Thai samples showed a profile dominated by emerging mycotoxins, particularly fusaric acid and beauvericin, with fewer but more prominent contamination events. Overall, these findings illustrate how the presence of toxins in flour and then legume-based ingredients may indicate their persistence even after processing, thereby contributing to potential dietary exposure. From a future perspective, tracing the entire supply chain—from raw materials to final products originating from the same batch—would be of great interest to better elucidate the fate of individual natural toxins during processing.

Legume-based snacks represented the most critical product category in both countries, showing the highest prevalence of contamination and the most complex toxin profiles. In Italian samples, the detection of aflatoxins above regulatory limits, together with the occurrence of fumonisins, underscores that legume-based snacks may accumulate mycotoxins typically associated with other commodity groups. This pattern likely reflects shared processing environments or cross-contamination along the production chain. The frequent co-occurrence of emerging mycotoxins, particularly beauvericin (BEA), further increases the toxicological relevance of these products. In Thailand, snack contamination was largely driven by emerging mycotoxins, with alternariol monomethyl ether (AME) and fusaric acid predominating. Peanut-based snacks, in particular, stood out for their elevated fusaric acid concentrations, confirming the role of specific raw materials in shaping the contamination profile of finished products. Compared with Italy, regulated mycotoxins and plant alkaloids were less prominent, suggesting different exposure scenarios influenced by dietary habits, ingredient selection and local production practices. Overall, Figure 4 highlights legume-based snacks as a key contributor to cumulative dietary exposure, especially in the context of frequent consumption and multi-toxin co-occurrence.

Comparison with existing literature remains challenging due to the limited availability of data on legume-based snacks, as most studies have focused on meat alternatives, such as soy-based burgers and processed soy ingredients (Augustin Mihalache et al., 2024; Rodríguez-Carrasco et al., 2019; Schollenberger et al., 2007). Unlike Pavicich et al. (Pavicich et al., 2024), who reported the absence of sterigmatocystin (STG) in processed legume proteins except for dust residues, STG was detected in Italian matrices across all

product categories in the present study. Given its genotoxic and carcinogenic potential (IARC Group 2B) ('Scientific Opinion on the Risk for Public and Animal Health Related to the Presence of Sterigmatocystin in Food and Feed', 2013; World Health Organization (WHO), n.d.), this finding deserves particular attention.

In line with previous studies, AME was detected in final snack products from both countries, supporting evidence of its stability during processing and its relevance for dietary exposure, as also indicated by human biomonitoring data (Jamnik et al., 2022). Recent investigations on plant-based and legume-based meat alternatives further corroborate these findings, showing that legume-based products can represent a relevant source of exposure to both regulated and emerging mycotoxins (Augustin Mihalache et al., 2023, 2024). These studies highlighted that cumulative exposure and MOET values above the level of low concern may occur for *Alternaria* toxins, aflatoxins and ochratoxin A, particularly under scenarios of frequent consumption or partial to full substitution of conventional foods with legume-based products.

Although the present study was based on real-market products rather than substitution models, the convergence of evidence underscores the role of legume-based processed foods, particularly snacks, as potential drivers of cumulative exposure to natural toxins. This comparison reinforces the need for continuous monitoring and risk-based prioritization strategies, especially in the context of the rapidly expanding market for plant-based and legume-derived foods.

5 | CONCLUSION

In Italy, legumes are a cornerstone of the Mediterranean diet, and the market is rapidly expanding toward innovative formats such as legume-based pasta, flours, and snacks. These products undergo diverse processing steps that may alter contamination patterns and introduce new exposure pathways for mycotoxins and plant toxins. In this context, analytical approaches must be able to cope with complex and highly processed legume matrices to ensure reliable detection and risk assessment.

In Thailand, legume consumption is still largely linked to traditional products such as tofu, soy-based foods, mung bean noodles and peanuts. However, the warm and humid climate favours fungal growth during storage, while data on emerging and non-regulated toxins remain scarce. This underlines the need to broaden surveillance strategies for tropical food systems beyond regulated natural toxins.

This comparative study highlights the value of cross-country collaboration in food safety monitoring. While regulated mycotoxins were only sporadically detected—mainly in Italian products—emerging mycotoxins and plant toxins accounted for most positive findings, particularly in flours and legume-based snacks. These results suggest that the current regulatory focus on a limited set of compounds may not fully capture the real contamination profile of legume-derived foods. Notably, fusaric acid and beauvericin showed high occurrence, and the detection of tropane alkaloids in both raw and processed products further supports the need to extend monitoring to non-regulated toxins.

Among the investigated categories, legume-based snacks emerged as the most critical in terms of dietary exposure, due to their high prevalence of contamination, frequent multi-toxin co-occurrence, and the presence of exposure-driving events involving both regulated and emerging mycotoxins. The recurrent co-

occurrence of multiple toxins within the same samples raises additional concerns regarding potential additive or synergistic effects, which are not addressed by current single-compound risk assessment frameworks.

In conclusion, strengthening risk-based monitoring strategies – together with regulatory updates that consider emerging toxins – is essential to ensure the safety of the rapidly growing legume-based food sector. Alongside regulatory actions, consumer education remains a key complementary measure, particularly in regions where food safety awareness is still developing. Promoting diversified diets, appropriate storage practices and informed food choices can contribute to reducing exposure risks while supporting the transition toward more sustainable, plant-based diets.

SUPPLEMENTARY MATERIALS

Supplementary material 1. Raw legumes (n= 51) used in this study.

Legume	Italy	Thailand
Lentils (n=9)	Green lentils Organic red lentils Yellow lentils Black lentils Red lentils (n = 2)	Orange lentils (n =2) Brown lentils
Chickpea (n =8)	Organic chickpea White chickpea - reale Chickpea	Brown chickpea Chickpea (n=3) Organic Chickpea
Red Kidney beans (n=4)	Red Kidney beans	Red Kidney beans (n=3)
Red beans (n=4)	Red beans - Azuki Rossi	Red beans (n=2) Dried red beans
Navy beans (n=4)	Organic Navy beans Navy beans	Navy beans (n=2)
Borlotti Beans (n=2)	Oganic Borlotti beans Borlotti beans	
Peanuts (n =6)	Organic peanuts toasted with shell Peanuts toasted with shell	Pelled peanuts (n=2) Peanuts (n=2)
Mung beans (n=4)	Organic Mung beans	Mung beans (n=2) Peeled split mung bean (n=2)
Soy (n=4)		Peeled split soy bean Soy bean (n=2) Split Soy bean
Black beans (n=4)	Black beans	Black beans (n=3)
Pea (n=2)	Organic Pelled Pea Green Pea	

Supplementary material 2. Legumes-based flour (n= 20) used in this study.

Legume	Italy	Thailand
Lentils (n=2)	red lentils flour (n=2)	
Chickpea (n=6)	chickpea flour (n =3) whole chickpea flour (n =2)	chickpea flour
Red bean (n=1)		Red bean flour
Pea (n=3)	Pea flour Pea protein isolate	Yellow pea flour
Mung bean (n=3)		Mung bean flour Peeled mung bean flour Mung bean starch
Soy (n=3)	Soy grits (n=2)	Soybean flour
Black bean (n=2)		Black bean flour (n=2)

Supplementary material 3. Legume-based pasta (n= 26) used in this study.

Legume	Italy	Thailand
Lentils (n=6)	Red lentils pasta (n=2) Organic red lentils pasta	Red lentils pasta Organic red lentils pasta (n=2)
Chickpea (n=5)	Organic chickpea pasta Chickpea pasta (n=2)	Organic chickpea pasta Chickpea pasta
Green pea (n=3)	Green pea pasta (n=2) Organic green pea pasta	
Mung beans (n=6)		Organic mung bean pasta (n=2) Glass noodles (n=4)
Mixed beans (n=6)	chickpea & red lentils & pea	Pea Flour (25%), Chickpea Flour (25%), Borlotti Bean Flour (25%), Lentil Flour (25%).
	chickpea & red lentils	Organic Mung Bean Flour 28.33%, Organic Brown Rice Flour 25.67%, Organic red lentils 24.33%; Organic Chickpea Flour 21.67% - Fusilli
	red lentils & pea	Organic Multi-Color Beans Pasta: mung beans, red kidney beans

Supplementary material 4. Legume-based snacks (n= 27) used in this study.

Legume	Italy	Thailand
red lentils (n=3)	Organic red lentils balls (51%) Red lentils - toasted Organic red lentils snack (96%)	
chickpea (n=4)	Chickpea Maremma chips (60%) Chickpea - toasted Chickpea salted snack	Chickpea 65%
Broad bean (n=3)	Broad beans - toasted	Salted peeled broad beans Salted **not** peeled broad beans
Soybean (n=2)	Edamame - toasted Organic soybeans - toasted	
Green Pea (n=4)	Organic Green Pea Snack Salted chips (91%)	Salted green pees Green pea snack - roasted (76%) Green Pea snack (70%)
Mung bean (n=1)		Salted mung beans
Black bean (n=1)		Black bean (60%)
Red bean (n=2)		Red bean (60%) Red bean (60%)
White Bean (n=1)		white beans (50%)
Mixed Snacks (n=6)	Organic snack with green pea (76%) + red lentils (20%) Snack with green lentils (50%) + chick pea flour Organic snack chickpea (47%) + lentils (37%) Crackers of chickpea flour + bean flour + lentils flour (40%) Organic snack chickpea 66% and rice 30%	Green mung bean chips (61%) + soy beans (34%)

Supplementary Material 5. Retention time (RT), the most abundant m/z ions, and optimal collision energy (CE)

Compound name	Precursor ion (m/z)	Product ions (m/z)	RT (min)	Fragmentor (V)	CE (eV)	Polarity
15-AcDON	356.1	321.1/137.0	5.5	70/70	12/6	Positive
AFB_1	313.3	285.1/241/1	6.5	190/190	20/40	Positive
AFB_2	315.1	287.1/259.1	6.3	154/154	26/30	Positive
AFG_1	329.1	243.1/200.0	6.0	139/139	26/46	Positive
AFG_2	331.1	245.0/189.1	5.8	150/150	12/30	Positive
AFM_1	329.1	273.2/259.0	5.9	180/180	33/33	Positive
Atropine	290.1	93.0/124.0		95/95	35/25	Positive
BEA	801.5	133.9/119.0	10.3	138/138	78/146	Positive
CPA	337.1	196.1/140.1	8.7	90/90	20/30	Positive
DAS	384.2	105.0/107.1	6.4	60/90	40/25	Positive
ENA	699.5	682.5/209.9	10.1	138/138	14/34	Positive
ENA_1	686.3	669.4/210	10.4	120/140	30/30	Positive
ENB	658.5	87.1/55.0	10.0	138/138	82/170	Positive
ENB_1	672.5	655.2/99.8	10.2	138/138	14/74	Positive
FB_1	722.4	352.5/334.4	7.2	180/120	40/50	Positive
FB_2	706.4	336.3/74.1	8.3	220/170	36/45	Positive
FUS	180.0	134.1/92.0	5.2	65/65	15/30	Positive
HT2	442.4	262.9/197.1	7.2	40/100	45940	Positive
MPA	321.1	207.0/159.0	7.7	110/100	25/20	Positive
NEO	400.3	305.2/215.2	4.7	70/70	10/20	Positive
OTA	404.1	239.0/221.0	8.6	135/135	20/36	Positive
OTB	370.1	187.1/159.0	7.7	100/80	40/40	Positive
PAX	436.2	182.2/129.9	9.7	70/70	40/60	Positive
PEN-A	634.3	558/332	9.5	135/100	20/40	Positive
Scopolamine	304.1	138.0/145.0		95/90	29/20	Positive
STG	325.1	310.0/281.0	8.8	130/100	25/37	Positive
T-2	484.2	215.2/185.1	7.8	140/140	4/14	Positive
TEN	415.2	91/58	7.1	100/100	75/50	Positive
3-AcDON	397.1	307.2/59.0	5.5	82/80	22/86	Negative
AOH	257.0	242.0/215.0	7.8	100/100	30/30	Negative
AME	271.1	228.0/256.0	9.1	100/100	25/25	Negative
CIT	281.1	249/205.1	8.1	85/85	20/25	Negative
DON	355.1	294.8/59.0	4.6	75/75	4/20	Negative
EMO	269.0	224.9/182.1	9.9	125/125	30/40	Negative
MON	97.0	41.1/41.0	2.4	60/65	15/15	Negative

NIV	371.2	205.0/281/.2	4.1	80/80	20/20	Negative
TeA	319.1	212.9/227.0	9.1	161/161	38/33	Negative
ZEN	317.1	273.2/175.0	8.6	150/180	20/25	Negative
α-ZAL	322.1	62.8/40.9	8.0	100/100	50/90	Negative
α-ZEL	319.1	144.1/41.1	8.2	90/100	40/90	Negative
β-ZAL	321.1	132.0/107.1	7.4	130/135	35/40	Negative
β-ZEL	319.1	159.9/130.1	7.6	100/100	30/40	Negative

Supplementary Material 6. Performance characteristics of the method for the validated analytes at 3 spiking levels, calculated based on the detection range.

Analyte	Multi-legumes matrix								
	Range of detection (µg/kg)	Linearity (R^2)	Recovery (%)			ME (%)	LOD (µg/kg)	LOQ (µg/kg)	Intra day (%) (n=6)
			LL	ML	HL				
15-AcDON	5 - 125	0.998	107	100	100	-26,72	4,79	16,0	7
3-AcDON	2.5 - 125	0.999	114	95	89	-37,22	2,41	8,0	14
AFB1	0.125 - 12.5	0.999	101	99	88	-10,23	0,04	0,1	4
AFB2	0.125 - 12.5	0.999	106	115	100	-10,46	0,06	0,2	3
AFG1	0.025 - 6.25	0.999	117	108	101	-14,19	0,01	0,0	2
AFG2	0.025 - 12.5	0.999	101	104	101	-15,38	0,05	0,2	4
AFM1	0.125 - 12.5	0.999	114	108	103	7,01	0,08	0,3	1
AOH	0.25 - 125	0.999	78	86	82	-10,22	0,08	0,3	2
AME	0.1 - 50	0.999	108	105	100	-11,84	0,03	0,1	2
Atropine	0.07 - 12.5	0.999	114	110	101	-47,99	0,07	0,2	3
α-ZAL	5 - 125	0.996	95	75	86	-8,69	3,51	11,7	22
α-ZEL	2.5 - 125	0.999	117	105	100	-14,33	0,38	1,3	1
BEA	0.5 - 50	0.999	101	106	101	-5,93	0,02	0,1	3
β-ZAL	1.25 - 125	0.999	98	101	94	-15,89	1,21	4,0	10
β-ZEL	0.25 - 125	0.999	104	109	103	-19,55	0,21	0,7	7
CIT	0.2 - 100	0.999	43	51	59	22,67	0,01	0,0	3
CPA	1 - 100	0.999	95	100	97	-14,49	0,37	1,2	6
DAS	5 - 250	0.999	93	108	111	-3,54	0,59	2,0	4
DON	0.5 - 250	0.999	97	95	86	-19,19	0,36	1,2	10
EMO	0.2 - 100	0.999	93	105	97	-62,25	0,10	0,3	7
ENNA	0.1 - 50	0.999	105	111	106	-11,45	0,01	0,0	2
ENNA1	0.1 - 50	0.999	105	111	106	-11,16	0,02	0,1	3
ENNB	0.1 - 50	0.999	104	110	106	-29,49	0,03	0,1	3
ENNB1	0.5 - 50	0.999	97	102	98	-6,72	0,00	0,0	2
FB1	0.7 - 100	0.999	72	78	73	3,59	0,20	0,7	10
FB2	0.5 - 100	0.999	79	87	82	5,79	0,15	0,5	7
FA	0.5 - 250	0.999	73	97	101	-16,41	0,32	1,1	4
HT-2	0.5 - 250	0.999	112	115	119	-7,47	0,02	0,1	3
MON	2.5 - 50	0.994	54	81	86	60,91	0,04	0,1	3
MPA	5 - 250	0.999	93	113	113	-4,15	0,29	1,0	4
NEO	2.5 - 250	0.999	99	114	119	2,70	0,19	0,6	3
NIV	7 - 125	0.990	91	61	62	-27,27	6,16	20,5	15
OTA	0.1 - 50	0.999	98	109	107	-5,56	0,08	0,3	3
OTB	0.1 - 50	0.999	102	112	106	-10,94	0,04	0,1	2
PAX	2.5 - 250	0.999	102	100	102	-19,75	0,24	0,8	4
PEN- A	1 - 100	0.999	109	116	104	-14,15	0,08	0,3	3
Scopolamine	0.09 - 12.5	0.999	102	103	98	-6,04	0,09	0,3	2
STG	0.2 - 100	0.999	115	112	106	-27,82	0,03	0,1	3
T2	0.1 - 50	0.999	105	103	105	-7,48	0,07	0,2	3
TEN	0.1 - 50	0.999	111	115	110	-5,62	0,01	0,0	2
TeA	1 - 101	0.999	106	98	97	-11,62	0,63	2,1	8
ZEN	1.25 - 125	0.999	111	107	103	-6,71	0,18	0,6	2

Supplementary Material 7a. Occurrence of multi-toxins raw legumes in Italy

ITALY RAW LEGUMES			AFB2 Results	Atropine Results	BEA Results	EMO Results	STG Results	TEN Results	ZEA Results
N° positive samples	Name	Type of Legume	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)
1	IR_LCR6	Red lentils	< LOD	< LOD	< LOD	**0,4**	< LOD	< LOD	< LOD
2	IR**_Og_L2**	Organic Red lentils	**0,5**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3	IR_YL3	Yellow lentils	< LOD	< LOD	< LOD	< LOD	**0,37**	< LOD	< LOD
4	IR_BB1	Black Beans	< LOD	< LOD	< LOD	< LOD	< LOD	**1,4**	< LOD
5	IR_**Og**_MB1	Organic Mung Beans	< LOD	**0,1**	< LOD	< LOD	< LOD	< LOD	**1,3**
6	IR_AZ1	Red Beans	< LOD	< LOD	**0,1**	< LOD	< LOD	< LOD	< LOD
7	IR_NB1	White Beans	< LOD	< LOD	**0,2**	< LOD	< LOD	< LOD	< LOD
8	IR_**Og**_BoB1	Organic Borlotti Beans	< LOD	< LOD	< LOD	< LOD	< LOD	**1,0**	< LOD
9	IR_**Og**_C3	Organic Chickpea	< LOD	< LOD	< LOD	< LOD	**0,4**	< LOD	< LOD

*Og: organic

Supplementary Material 7b. Occurrence of multi-toxins raw legumes in Thailand

THAI RAW LEGUMES			AFB1 Results	AFB2 Results	Atropine Results	BEA Results	EMO Results	FA Results	ZEA Results
N° positive samples	Name	Type of Legume	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)
1	TR_MB36	Mung beans	**< LOQ**	< LOD	**0,5**	**< LOQ**	< LOD	< LOD	**26,8**
2	TR_PMB2	Peeled split mung bean	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**5,8**
3	TR_MB1	Mung beans	**< LOQ**	< LOD	< LOD	< LOD	< LOD	< LOD	**20,2**
4	TR_BC1	Brown chickpea	< LOD	**1,5**	< LOD	< LOD	< LOD	< LOD	< LOD
5	TR_C4	Chickpea	< LOD	< LOD	< LOD	**0,7**	< LOD	**418,3**	< LOD
6	TR_P2	Peanuts	**6,0**	**< LOQ**	< LOD	**0,1**	< LOD	< LOD	< LOD
7	TR_S4	Soybean	< LOD	< LOD	< LOD	**2,0**	< LOD	**4,4**	< LOD
8	TR_BB4	Black beans	< LOD	< LOD	< LOD	**7,9**	< LOD	< LOD	< LOD
9	TR_BB21	Black beans	< LOD	< LOD	< LOD	**7,5**	**0,6**	< LOD	< LOD
10	TR_RB1	Red beans	< LOD	< LOD	< LOD	**0,5**	< LOD	< LOD	< LOD
11	TR_dRB61	Dried red beans	< LOD	< LOD	< LOD	**1,5**	**0,7**	**1,0**	**1,2**

Supplementary Material 8a. Occurrence of multi-toxins in legumes' flour and pasta in Italy

ITALY FLOUR AND PASTA			AFB1 Results	AFB2 Results	Atropine Results	BEA Results	DON Results	ENNA1 Results	ENNB Results	ENNB1 Results	FA Results	OTA Results	Scopolamine Results	STG Results	Tentoxin Results
N° positive samples	Name	Ingredients	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)
1	IP_**Og**_C2	Organic chickpea pasta	< LOD	**1,10**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2	IP_L1	Red lentils pasta	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**0,4**	< LOD
3	IP_**Og**_L3	Organic red lentils pasta	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**0,4**	< LOD
4	IP_**Og**_P3	Organic green pea pasta	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**0,3**	< LOD	< LOD	< LOD
5	IP_P1 +	Green Pea Pasta (+ durum wheat)	< LOD	**< LOQ**	**0,2**	**0,2**	**44,3**	**< LOQ**	**2,2**	**0,5**	< LOD	< LOD	< LOD	< LOD	**1,0**
6	IF_C25	Wholemeal chickpea Flour	< LOD	< LOD	< LOD	**0,2**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
7	IF_C24	Wholemeal chickpea Flour	**< LOQ**	**1,0**	< LOD	**< LOQ**	< LOD	< LOD	**< LOQ**	**< LOQ**	**1,3**	< LOD	< LOD	< LOD	< LOD
8	IF_Cnov	Chickpea Flour	< LOD	**1,0**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**1,8**	< LOD	< LOD
9	IF_L51	Red lentils flour	< LOD	< LOD	< LOD	**0,1**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
10	IF_P48	Pea flour	< LOD	**0,9**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
11	IF_plus+	Pea flour	< LOD	**0,9**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**	< LOD

*Og: organic

Supplementary Material 8b. Occurrence of multi-toxins legumes' flour and pasta in Thailand

THAI FLOUR AND PASTA			AME Results	Atropine Results	BEA Results	FA Results	ZEA Results
N° positive samples	Name	Ingredients	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)
1	TP_**Og**_MB1	Organic Mung beans Pasta	< LOD	**0.3**	**0.4**	**5.0**	< LOD
2	TP_**Og**_MB2	Organic Mung bean pasta (85%), organic brown rice (15%)	< LOD	**0.1**	< LOD	**2.4**	< LOD
3	TP_Mix1	Pea Flour (25%), Chickpea Flour (25%), Borlotti Bean Flour (25%), Lentil Flour (25%).	< LOD	< LOD	**0.2**	**1.9**	< LOD
4	TP_**Og**_Mix2	Organic Mung Bean Flour 28.33%, Organic Brown Rice Flour 25.67%, Organic red lentils 24.33%; Organic Chickpea Flour 21.67%	**0.2**	**< LOQ**	**2.0**	**10.4**	< LOD
5	TP__**Og**_Mix3	Organic mung bean flour, brown rice flour, red lentil flour, chickpea flour	< LOD	**< LOQ**	**0.2**	**1.8**	< LOD
6	TP_L1	100% red lentils	**0.2**	< LOD	**0.2**	**2.6**	< LOD
7	TF_116	Mung bean flour	< LOD	**0.23**	< LOD	**2.5**	< LOD
8	TF_117	Peeled mung bean flour	**0.2**	< LOD	**< LOQ**	**< LOQ**	**25.3**
9	TF_122	Mung bean starch flour	**1.3**	< LOD	**0.4**	**2.6**	< LOD
10	TF_123	Black bean flour	< LOD	< LOD	< LOD	**1.8**	< LOD
11	TF_124	Red bean flour	< LOD	< LOD	**0.4**	**2.9**	< LOD

*Og: organic

Supplementary Material 9a. Occurrence of multi-toxins in legume snacks in Italy

ITALY LEGUME SNACKS			AFB1 Results	AME Results	Atropine Results	BEA Results	DON Results	EMO Results	ENNA Results	FB1 Results	FB2 Results	FA Results	STG Results
N° positive samples	Name	ingredients	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)
1	IS_BB1	100% broad Beans	0,0	< LOD	< LOD	**0,3**	**68,8**	< LOD	< LOD	< LOD	< LOD	< LOD	**1,2**
2	IS_C1	60% chickpeas	**3,8**	**0,2**	**0,6**	**1,1**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**0,4**
3	IS_Og_C3	66% organic chickpea	< LOD	< LOD	< LOD	0,0	< LOD	< LOD	< LOD	< LOD	< LOD	**4,3**	< LOD
4	IS_Og_C5	37% organic chickpea flour + 37% organic lentils	< LOD	< LOD	< LOD	**0,6**	< LOD	< LOD	< LOD	**5,2**	< LOD	< LOD	**1,1**
5	IS_GL1	50 % green lentils + chickpea flour	< LOD	< LOD	< LOD	**1,4**	< LOD	**0,9**	< LOD	**64,1**	**12,0**	**20,2**	**1,0**
6	IS_Og_L2	51% organic red lentils + mais 48%	< LOD	< LOD	< LOD	**0,7**	**10,7**	< LOD	< LOD	**53,1**	**6,8**	**47,4**	< LOD
7	IS_L3	100% red lentils	< LOD	**0,5**	< LOD	**0,7**	< LOD	**1.07**	**0,3**	< LOD	< LOD	**3,4**	**0,9**
8	IS_S1	100% soyabeans	< LOD	< LOD	< LOD	**0,3**	**68,7**	< LOD	< LOD	< LOD	< LOD	**5,8**	< LOD
9	IS_Og_S2	100% organic soy beans	< LOD	< LOD	< LOD	**0,3**	**71,0**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
10	IS_Og_P1	91% organic Green Pea	< LOD	< LOD	< LOD	0,0	< LOD	**3,3**	**0,2**	< LOD	< LOD	< LOD	**1,1**
11	IS_Og_P2	76% green pea + red lentils 20%	< LOD	< LOD	< LOD	0,0	< LOD	**1,6**	< LOD	< LOD	< LOD	< LOD	< LOD
12	IS_Og_MIX4	40% chickpea flour + bean flour + lentils flour	< LOD	**0,7**	< LOD	**0,8**	< LOD	< LOD	< LOD	**24,5**	< LOD	< LOD	< LOD

*Og: organic

Supplementary Material 9b. Occurrence of multi-toxins in legume snacks in Thailand

THAI LEGUME SNACKS			AFB2 Results	AME Results	BEA Results	DON Results	EMO Results	FA Results	TeA Results
N° positive samples	Name	Ingredients	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)	Final Conc. (µg/Kg)
1	TS_SGP1	100% salt green pees	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**4,2**
2	TS_GP2	76% green pea	< LOD	**1,4**	< LOD	< LOD	< LOD	< LOD	< LOD
3	TS_GP3	70% green peas	**1,3**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
4	TS_CMB1	61% Green mung beans + soy beans 34.3%	< LOD	**0,4**	< LOD	< LOD	< LOD	< LOD	< LOD
5	TS_SMB2	100% mung beans	< LOD	**1,1**	< LOD	< LOD	< LOD	< LOD	**4,6**
6	TS_SP1	100% salted not peeled peanuts	< LOD	< LOD	< LOD	< LOD	< LOD	**287,4**	< LOD
7	TS_SBB1	100% salted peeled broad beans	< LOD	**0,6**	< LOD	< LOD	**0,8**	< LOD	< LOD
8	TS_SBB2	100% salted not peeled broad beans	< LOD	**0,4**	< LOD	**28,2**	< LOD	< LOD	< LOD
9	TS_149	50% white beans	< LOD	< LOD	**0,7**	< LOD	< LOD	**1,8**	< LOD
10	TS_150	60% Red bean	< LOD	< LOD	< LOD	< LOD	< LOD	**4,6**	< LOD

*Og: organic

Supplementary Material 10: Risk characterization of toxins in adult consumers under LB and UB exposure scenarios for raw legumes from Italy (a) and Thailand (b).

a

ITALY RAW LEGUMES				EDI (µg/kg bw/day)						
Category	Mean Dietary Intake (g/Kg bw/day)	Scenario		AFB2	Atropine	BEA	EMO	STC	TEN	ZEA
Adults	0,33	**LB**	Average	0,000018	0,000011	0,000010	0,000013	0,000027	0,000089	0,000046
Adults	0,55	**LB**	97.5	0,000029	0,000018	0,000017	0,000022	0,000045	0,000148	0,000077
Adults	0,33	**UB**	Average	0,000035	0,000063	0,000015	0,000043	0,000034	0,000091	0,000100
Adults	0,55	**UB**	97.5	0,000058	0,000099	0,000025	0,000072	0,000057	0,000152	0,000167
	Risk Characterization			**MOE**	ARfD of 0.016 µg/kg bw	**TTC**		**MOE**	**TTC**	**TDI**
		LB		>10,000 low concern	HQ<1	EDI lower than TTC	no Data	>10,000 low concern	EDI lower than TTC	EDI <0.25
		UB		low concern for average / **health concern for 97.5**	HQ<1	EDI lower than TTC	no Data	>10,000 low concern	EDI lower than TTC	EDI <0.25

b

THAI RAW LEGUMES				EDI (µg/kg bw/day)						
Category	Mean Dietary Intake (g/Kg bw/day)	Scenario		AFB1	AFB2	Atropine	BEA	EMO	FA	ZEA
Adults	0,38	**LB**	Average	0,000228	0,000077	0,000042	0,000712	0,000077	0,014844	0,001926
Adults	0,84	**LB**	97.5	0,000501	0,000170	0,000092	0,001566	0,000169	0,032666	0,004239
Adults	0,38	**UB**	Average	0,000209	0,000052	0,000016	0,000705	0,000045	0,014756	0,001882
Adults	0,84	**UB**	97.5	0,000460	0,000115	0,000034	0,001553	0,000099	0,032473	0,004141
	Risk Characterization			**MOE**	**MOE**	ARfD of 0.016 µg/kg bw	**TTC**			**TDI**
		LB		**<10,000 health concern**	**<10,000 health concern**	HQ<1	EDI < TTC	no Data	no Data	EDI < TDI
		UB		**<10,000 health concern**	**<10,000 health concern**	HQ<1	EDI < TTC	no Data	no Data	EDI < TDI

Supplementary Material 11: Risk characterization of toxins in adult consumers under LB and UB exposure scenarios for legume-based flour & pasta from Italy (a) and Thailand (b).

b

THAI FLOUR AND PASTA										
Category	Mean Dietary Intake (g/Kg bw/day)	Scenario	AME Results	Atropine	BEA	FA	ZEA
Adults	0,38	LB	Average	0,000066	0,000022	0,000132	0,001181	0,000881
Adults	0,84	LB	97.5	0,000146	0,000048	0,000291	0,002598	0,001939
Adults	0,38	UB	Average	0,000074	0,000055	0,000138	0,001217	0,000945
Adults	0,84	UB	97.5	0,000162	0,000121	0,000305	0,002679	0,002079
Risk Characterization			**TTC**		**TTC**		**TDI**	
		LB	EDI < TTC	HQ <1	EDI < TTC	no Data	EDI < TDI	
		UB	EDI < TTC	HQ <1	EDI < TTC	no Data	EDI < TDI	

a

ITALY FLOUR AND PASTA																				
Category	Mean Dietary Intake (g/Kg bw/day)	Scenario	AFB1	AFB2	Atropine	BEA	DON	ENNA1	ENNB	ENNB1	FA	OTA	Scopolamine	STC	TEN
Adults	0,33	LB	Average	0	0,000145	0,000011	0,000013	0,001332	0	0,000065	0,000015	0,000038	0,000009	0,000127	0,000024	0,000030
Adults	0,55	LB	97.5	0	0,000243	0,000017	0,000022	0,002227	0	0,000109	0,000025	0,000063	0,000015	0,000200	0,000040	0,000051
Adults	0,33	UB	Average	0,000015	0,000160	0,000063	0,000019	0,001441	0,000006	0,000077	0,000021	0,000133	0,000033	0,000188	0,000033	0,000033
Adults	0,55	UB	97.5	0,000025	0,000590	0,000045	0,000031	0,002409	0,000011	0,000129	0,000035	0,000222	0,000056	0,000296	0,000055	0,000055
Risk Characterization			**MOE**	**MOE**	ARfD of 0,016 µg/kg bw	**TTC**	**TDI**	**TTC**	**TTC**	**TTC**	**TTC**	**MOE**	Acute Exposure / ARfD	**MOE**	**TTC**
		LB	0	**<10,000 health concern**	HQ <1	EDI < TTC	EDI <1	0	EDI < TTC	EDI < TTC	no Data	>10,000 low concern	ARfD of 0,016 µg/kg bw	>10,000 low concern	EDI < TTC
		UB	0	**<10,000 health concern**	HQ <1	EDI < TTC	EDI <1	EDI < TTC	EDI < TTC	EDI < TTC	no Data	>10,000 low concern	Acute Exposure / ARfD	>10,000 low concern	EDI < TTC

Supplementary Material 12: Risk characterization of toxins in adult consumers under LB and UB exposure scenarios for legume-based snacks from Italy (a) and Thailand (b).

a

ITALY LEGUME SNACKS				EDI (µg/kg bw/day)										
Category	Mean Dietary Intake (g/Kg bw/day)	Scenario		AFB1	AME	Atropine	BEA	DON	Emodin	ENNA	FB1	FB2	FA	STC
Adults	0,33	**LB**	Average	0,000105	0,000039	0,000039	0,000173	0,006037	0,000174	0,000014	0,004047	0,000518	0,002235	0,000158
Adults	0,55	**LB**	97.5	0,000175	0,000064	0,000061	0,000289	0,010095	0,000290	0,000023	0,006767	0,000866	0,003738	0,000264
Adults	0,33	**UB**	Average	0,000116	0,000046	0,000092	0,000174	0,006117	0,000198	0,000017	0,004091	0,000559	0,002296	0,000163
Adults	0,55	**UB**	97.5	0,000194	0,000077	0,000145	0,000291	0,010228	0,000331	0,000028	0,006841	0,000935	0,003839	0,000272
	Risk Characterization			**MOE**	**TTC**	ARfD of 0.016 µg/kg	**TTC**	**TDI**		**TTC**	**TDI**	**TDI**		**MOE**
		LB	Average / 97.5	**<10,000 health**	EDI lower than TTC	HQ<1	EDI lower than TTC	EDI <1	no Data	EDI lower than TTC	EDI <1	EDI <1	no Data	>10,000 low concern
		UB	Average / 97.5	**<10,000 health concern**	EDI lower than TTC	HQ<1	EDI lower than TTC	EDI <1	no Data	EDI lower than TTC	EDI <1	EDI <1	no Data	>10,000 low concern

b

THAI LEGUME SNACKS				EDI (µg/kg bw/day)						
Category	Mean Dietary Intake (g/Kg bw/day)	Scenario		AFB2	AME	BEA	DON	EMO	FA	TeA
Adults	0,38	**LB**	Average	0,000050	0,000150	0,000025	0,001080	0,000031	0,011254	0,000338
Adults	0,84	**LB**	97.5	0,000110	0,000329	0,000055	0,002378	0,000067	0,024767	0,000743
Adults	0,38	**UB**	Average	0,000070	0,000156	0,000031	0,001205	0,000066	0,011339	0,000529
Adults	0,84	**UB**	97.5	0,000154	0,000342	0,000068	0,002652	0,000144	0,024953	0,001165
	Risk Characterization			**MOE**	**TTC**	**TTC**	**TDI**			**TTC**
		LB	Average / 97.5	**<10,000 health concern**	EDI < TTC	EDI < TTC	EDI < 1	no Data	no Data	EDI < TTC
		UB	Average / 97.5	**<10,000 health concern**	EDI < TTC	EDI < TTC	EDI < 1	no Data	no Data	EDI < TTC

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4 | GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Taken together, the results of this doctoral project demonstrate that effective management of natural toxin risks in plant-based foods cannot rely on isolated measures. Robust analytical tools for the identification of plant alkaloids, realistic assessment of processing effects tracking the fate of known and emerging mycotoxins, and comprehensive exposure evaluation must be integrated within a coherent, risk-based strategy.

The combined evidence shows that while food processing often modifies the food matrix, it does not necessarily lead to meaningful toxin degradation. Consequently, risk mitigation must primarily focus on upstream measures, including raw material quality control, targeted screening at critical control points, and informed selection of samples for confirmatory analysis. In this context, the integration of point-of-need screening tools with mass spectrometry methods emerges as a pragmatic and efficient approach to support decision-making along the food supply chain.

Furthermore, the occurrence and exposure data obtained for legume-based foods reveal that emerging toxins, not yet routinely monitored or legislatively regulated, can substantially contribute to overall dietary exposure. Currently available data remain insufficient to provide a fully reliable and representative picture of mycotoxin occurrence in legume-based foods, particularly in light of the large variability of raw materials, processing conditions, and consumption patterns. This highlights the need to move beyond single-compound perspectives and toward more integrated monitoring and risk assessment frameworks that reflect real consumption scenarios and complex contamination profiles.

This doctoral work provides an integrated framework for understanding and managing natural toxin risks in cereals and plant-based foods, bridging advanced analytical science with real industrial needs. Rather than proposing definitive solutions, the findings offer practical tools and evidence-based insights that support informed risk management in a rapidly evolving food system. In this context, there is a clear need for fit-for-purpose analytical methods, the availability of (certified) reference materials, and harmonized interlaboratory testing schemes to ensure data comparability and robustness across different matrices and monitoring settings.

Future research should further explore innovative processing technologies capable of achieving toxin reduction without compromising product quality, while expanding surveillance efforts to include emerging and modified toxins. At the same time, combined and coordinated efforts from the scientific community, regulatory bodies, and international initiatives are essential to keep up with the rapid shift toward plant-based dietary patterns and the continuous evolution of the global food system. Alongside analytical and regulatory advances continued efforts in risk communication through consumer education and awareness remain essential complementary measures to reduce exposure risks.

Ultimately, ensuring the safety of the food supply chain requires not a single intervention, but a holistic and coordinated strategy. A future direction should include the development of a system-based approach in which artificial intelligence and big data are deliberately integrated to support early warning systems and the prediction of emerging risks, for both known and unknown hazards. Within this framework, analytical methodologies and approaches for emerging and re-emerging risks must be developed, both targeted and untargeted. This enables the development of holistic risk assessment method in which food safety is included within a comprehensive evaluation of the entire food system, accounting not only for health risks, but also for environmental and economic dimensions.

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STUDIES

Eleonora Rollo obtained her Bachelor's degree in Food Science and Technology (*110/110*) from the University of Parma, Italy, in September 2019. She subsequently earned her Master's degree in Food Safety and Food Risk Management (*110/110 with honors*) from the same institution in December 2021. During her Master's program, she conducted an experimental thesis at Barilla Group (Parma, Italy) entitled *"QuEChERS method combined with LC-HRMS for the accurate and sensitive simultaneous determination of pyrrolizidine and tropane alkaloids in cereals, spices and herbs."*

Following one year of professional experience as an R&D Specialist in the Analytical Food Science laboratory at Barilla Group, she was awarded an industrial Ph.D. scholarship in Food Science in January 2023 within the European project OnFoods (Spoke 3 – *Food safety of traditional and novel foods*). The Ph.D. programme was carried out at Barilla Group in collaboration with the University of Parma, under the supervision of Prof. Michele Suman and Prof. Chiara Dall'Asta. The project aimed to bridge advanced analytical science with real industrial needs, focusing on the identification of natural toxins and related emerging risks in innovative ingredients for bakery and meal solutions.

During her Ph.D. program, she was a visiting researcher at the BIOTEC–NSTDA Research Center (Pathum Thani, Thailand), within the framework of the MycoBeans project *"Exploring (emerging) mycotoxin risks in beans: a global alliance for climate change resilience"*.

SCIENTIFIC ACTIVITY

Participation in Research Projects

- My Ph.D. Project was founded by **Research and innovation network on food and nutrition Sustainability, Safety and Security (ONFoods)**. Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3 - Call for tender No. 341 of 15/03/2022 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU
  Award Number: Project code PE0000003, Concession Decree No. 1550 of 11/10/2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001, Project title "Research and innovation network on food and nutrition Sustainability, Safety and Security – Working ON Foods" (ONFoods).

- The European Union's Horizon Europe research and innovation program under the Marie Sklodowska-Curie grant agreement No 101131125 - **MycoBeans**.

- **FoodSafeR –** European Union's Horizon Europe research and innovation programme with grant agreement no. 101060698.

Publications arising from this thesis

Published articles

- **QuEChERS method combined to liquid chromatography high-resolution mass spectrometry for the accurate and sensitive simultaneous determination of pyrrolizidine and tropane alkaloids in cereals and spices**

  Eleonora Rollo, Dante Catellani, Chiara Dall'Asta, Michele Suman

  J Mass Spectrom. **2023**; e4969 https://doi.org/10.1002/jms.4969

- **Determination of Ergot Alkaloids: Comparison of extraction efficiencies in several cereal production chains by UPLC-MS/MS**

  Eleonora Rollo, Dante Catellani, Chiara Dall'Asta, Nicola Dreolin, Michele Suman

  Mycotoxin Res. **2024** Nov 11. https://doi.org/10.1007/s12550-024-00569-8

Accepted manuscript

- **Mycotoxins fate along thermal processing on cereal chain: from pilot testing to analytical understanding and scaling-up confirmation**
  Michele Suman, **Eleonora Rollo**

  Section "Food Contaminants", book series "Methods and Protocol in Food Science" published by Springer Nature 2026.

- **Methodological user-validation in industry of a 3D-printed biosensing toolkit for tropane alkaloid detection**
  Ids B. Lemmink; **Eleonora Rollo**; Erik Beij; Anne-Catherine Huet; Toine F.H. Bovee; Han Zuilhof; Michele Suman; Gert IJ. Salentijn.

  Food Chemistry (2025)

Submitted manuscripts

- **Fate of aflatoxins and fumonisins during gluten free pasta processing: untargeted ^{13}C-labelling LC-HRMS based approach**
  Eleonora Rollo, Alexandra Schamann, Maria Doppler, Alexandra Malachová, Rudolf Krska, Michele Suman (2025) – *J Mass Spectrom* – Special Issue (submitted)

- **Tracking the fate of T-2 and HT-2 mycotoxins during wholemeal cracker production using a combined targeted and untargeted ^{13}C-labelling LC-HRMS based approach**
  Eleonora Rollo, Alexandra Schamann, Maria Doppler[4], Christoph Bueschl[5], Alexandra Malachová[3], Rudolf Krska, Michele Suman (2025) – Applied Food Research (submitted)

- **Multi-Mycotoxins and tropane alkaloids occurrence in legumes: comparative survey between Italy and Thailand**
  Eleonora Rollo, Kawisara Siwarak, Nazmi Waesoh, Umaporn Uawisetwathana, Octavian Augustin Mihalache, Chiara Dall'Asta, Michele Suman, Nitsara Karoonuthaisiri (2025) – npj Science of Food (submitted)

Participation to Conferences

- 14th World Mycotoxin Forum 2023
  Bruxelles | WMF*meets*Belgium (Poster)

- VII CONGRESSO MICOTOSSINE, 2024
  Istituto Superiore di Sanità, *Roma* | Italy (Oral communication)

- 28° Workshop on the Developments in the Italian PhD Research on Food Science Technology, 2024
  Catania | Italy (Poster)

- 11th International Symposium on RECENT ADVANCES IN FOOD ANALYSIS, 2024
  Prague | Czech Republic (Poster & Oral communication)

- 15th World Mycotoxin Forum 2025
  WMFmeetsSalzburg | Austria (Oral communication)

Doctorate Schools

- Food Authenticity PhD Excellence Academy, 2023
  | University of Parma

- Mass Spectrometry School, 2024
  | University of Siena

- Winter School – Packaging, 2025
  "Packaging Challenges for the Circular Economy" | University of Parma