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RESEARCH

# Fit-For-Purpose Method Development to Determine Co-Occurring Multiclass Mycotoxins in Apple and Apple Puree Samples

**Laura Carbonell-Rozas[1] · Linde Van der Cruyssen[2] · Chiara Dall'Asta[1] · Marco Camardo Leggieri[3] · Paola Battilani[3]**

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**Abstract**
Due to the impact of the climate change on mycotoxins production and their occurrence in foods, it is important to consider the potential accumulation of unexpected mycotoxins in matrices susceptible to be contaminated such as apples. In this regard, a fit-for-purpose LC–MS/MS method to determine co-occurring mycotoxins in apple purees has been proposed, considering the fungal profile isolated from stored apple fruits. Due to the isolation of *Fusarium* spp., fumonisins were included in the method along with *Alternaria* toxins (alternariol, alternariol monomethyl ether, and tentoxin), aflatoxins, and the only so far regulated patulin. The method was fully characterized in terms of linearity, sensitivity (LODs and LOQs below 0.4 and 1.4 μg $kg^{-1}$, respectively, except for patulin being lower than 1.2 and 4.1 μg $kg^{-1}$), precision, and recovery. The optimized method was then applied to the analysis of stored apples and apple purees from retail market. The preliminary survey brought the first evidence of FB1 occurrence in apple purees and highlighted the need for monitoring mycotoxins co-occurrence in apples and apple-based products.

**Keywords** Multiclass mycotoxins · Apple puree · Food safety · HPLC-MS/MS

## Introduction

The annual apple production in 2020 reached about 87.2 million tons worldwide, of them 11.8 million tons harvested in the European Union (source: FAOSTAT). Processed apple products, e.g., apple juice concentrates, purees, and compotes, represent an important and growing market segment in the fruit industry. Since apples are mainly marketed for fresh consumption, processing is often the destination for those fruits that do not meet quality standards for retailing.

Apples are highly susceptible to fungal infection at both pre- and postharvest, with the most severe diseases leading to fruit spoilage occurring in postharvest (Granado et al. 2008; Tournas and Uppal Memon 2009; Patriarca 2019). Fungal postharvest diseases are responsible for significant economic losses in apple production, which are estimated in a range of 30–40% in developing countries, reaching up to 60% in the worst cases (Naets et al. 2018). In addition to economic losses, fungal infection may give rise to the accumulation of mycotoxins in fruits, entailing therefore a risk for consumers, mainly with incipient or non-visible fungal decay, since fruits may pass the standard quality inspection and reach the process line. Considering that mycotoxins are usually stable during the most common technological treatments (i.e., pasteurization, microfiltration, clarification), these compounds may end up and concentrate in the final product (Aroud et al. 2021).

Infection of fruits by fungi is favored by damaged skin, insect wounds, or splits that can occur along the value crop chain; nevertheless, fungal occurrence is sometimes asymptomatic. Among the most frequent apple pathogens, *Penicillium expansum* is responsible for a soft rot of the whole or part of the fruit, or localized dark spots on the fruit skin and limited tanning of the fruit pulp on the cross-section of the fruit (Karlshøj et al. 2007; Elhariry et al. 2011; Lončarić et al. 2021); it is also responsible for the accumulation of patulin (PAT), a mycotoxin able to exert

✉ Laura Carbonell-Rozas
laura.carbonellrozas@unipr.it

✉ Chiara Dall'Asta
chiara.dallasta@unipr.it

[1] Department of Food and Drug, University of Parma, Viale Delle Scienze 27/A, 43124 Parma, Italy

[2] Centre of Excellence in Mycotoxicology and Public Health, University of Ghent, Ghent, Belgium

[3] Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy

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genotoxic, immunotoxic, and neurotoxic effects (Pal et al. 2017). Based on its widespread occurrence in apple-based products (Piqué et al. 2013; Torović et al. 2017; Zhong et al. 2018; Zheng et al. 2021; Lončarić et al. 2021) and the well-documented adverse effects in humans (Puel et al. 2010), the European Commission has set maximum level of patulin in apple juice and apple cider (50 μg $L^{-1}$), in solid apple products (25 μg $kg^{-1}$), as well as in apple products for infants (10 μg $kg^{-1}$) (European Commission 2006).

Although PAT is so far the only mycotoxin regulated in apple products, the possible occurrence of other mycotoxins of interest has been reported over time. In particular, fungal species from the genus *Alternaria* may colonize the apple outer layers causing fruit spot disease, and cause moldy core, an apple disease which remains often undetected before processing (Patriarca 2019; Pavicich et al. 2020). *Alternaria* spp. may produce a wide range of mycotoxins, among them alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and tentoxin (TEN), with recognized genotoxic and mutagenic effects (Fraeyman et al. 2017; Aichinger et al. 2021). Currently, no regulation has been enforced for *Alternaria* toxins in apple and derived products, although several studies reported a frequent incidence though at low amount of these compounds in juices and purees (Delgado and Gómez-Cordovés 1998; Lau et al. 2003; Ackermann et al. 2011; Broggi et al. 2013; Guo et al. 2021). This data can be explained considering the high incidence of the non-visible moldy core disease in stored fruits destined to apple processing industries (Patriarca 2019).

Fungal pathogens belonging to *Fusarium* spp. have been described as postharvest rot causing agents in apple, as well (Sever et al. 2012, Kou et al. 2014). Although a range of fungal metabolites, among them moniliformin, enniatins, and aurofusarin, were found in apples affected by wet core rot (Sørensen et al. 2009), no major *Fusarium* toxin has been reported so far in apple-based products. Similarly, reports concerning aflatoxins (AFs) in apples and products thereof are limited to warm regions, which favor the growth of aflatoxin-producing fungi, belonging to *Aspergillus* spp. (Afifi et al. 2003; Hasan 2000).

However, considering the climate change scenario Europe is facing so far, the potential accumulation of unexpected mycotoxins in apples and derived products cannot be ruled out without a careful monitoring. This was recently confirmed by the recall from the Italian market of organic apple purees containing aflatoxins in traces (Italian Ministery of Health 2019), and by the alert set by apple value chain stakeholders about the detection at trace levels of fumonisin B1 in processed organic apples from Northern Italy (Regione Veneto 2021).

Unfortunately, very few methods have been reported in the literature for monitoring mycotoxins other than patulin in apple-based products, and those available so far are based on time- and organic solvent-consuming procedures (Supplementary Material, Table S1). While the determination of Alternaria mycotoxins and AFs has been recently proposed (Tang et al. 2020), there is no method available so far to asses the co-occurrence of PAT, AFs, and Alternaria mycotoxins in apple products as well as no data about potential contamination of apple products by FB1. This is a critical gap, especially under a climate change scenario, hindring regulatory bodies for a careful risk assessment. Another critical point, as mentioned before, is the lack of multi-toxin protocols aligned with principles of green analytical chemistry, whose interest is growing in routine analysis where large numbers of samples are processed over time.

Therefore, our work is aimed at the development of a fit-for-purpose "green" multi-toxin method for the analysis of mycotoxins potentially present in apples and apple-based products, designed moving from the isolation and characterization of the main fungal population naturally infecting stored apples.

## Material and Methods

### Chemicals

Aflatoxin B1, B2, G1, and G2, and patulin (PAT) (5 mg each; purity ≥ 98%) were purchased from Fermentek Ltd (Jerusalem, Israel), while *Alternaria* mycotoxins alternariol, alternariol monomethyl ether, and tenuazoic acid (100 μg each) were obtained from Biopure (Getzersdorf, Austria). Fumonisins B1 (FB1) and FB2 standard solutions (50 μg $mL^{-1}$) were purchased from Romer Laboratories (Tulln, Austria). Ultra-pure water and methanol (MeOH) was purchased from VWR International (Milano, Italy), acetonitrile (MeCN) from Sharlab (Barcelona, Spain), and methyl tert-butyl ether (TMBE) and acetic acid from Sigma-Aldrich (Darmstadt, Germany).

### Sample Collection

Apples from Golden delicious and Imperatore cv. were harvested in Veneto region (Northern Italy) during the 2020 season and stored at 1 °C under conventional commercial condition and delivered to the laboratory in April 2021. Four fruits were promptly collected for each variety (sample code M7-M10). The rest of the fruits was stored at room temperature for 3 weeks; then, sub-samples of 4 fruits were collected and classified based on visual spoiling decay (*mild* versus *strong*) (samples coded M1-M3-M5 and M2-M4-M6, respectively). The apple samples were crushed and mixed using a Moulinex blendforce glass until obtaining homogenized apple puree stored at − 4 °C and dark conditions until their analysis.

For the survey trial, apple puree samples ($n = 20$) were bought from the italian retail market in summer 2022. For each brand, two samples were purchased from the same shop. Each sample was analyzed in duplicate.

## Fungi Isolation, Quantification, and Identification

From each apple cv, 5 pieces of skin, 5 from the flesh, and one in the calyx area were cut, placed on water agar Petri dishes (WA, 15 g $L^{-1}$ agar; 1 L double distilled water) and incubated at 25 °C for 5 days. Developed colonies were transferred on potato dextrose agar (PDA, Biolife, Milano, Italy) and incubated for 7–10 days at 25 °C for identification at genus level.

Colony forming units (CFU $g^{-1}$) were determined for samples M1–M6. Briefly, starting from 1 g of apple puree, serial dilutions were manged ($10^{-2}$–$10^{-6}$), plated on Petri dishes filled with Dichloran Rose Bengal Chloramphenicol Agar (DRBC; Oxoid, Hampshire, UK) and incubated at 25 °C for 7 days. At the end of incubation, the colonies were counted and reported as CFU $g^{-1}$. Furthermore, to identify the fungi at genus level, representative colonies were transferred on potato dextrose agar (PDA: 42 g $L^{-1}$ potato dextrose agar, 1 L double distilled water) and incubated at 25 °C for 7–10 days. Fungi were identified at genus level; macro and microscopic characters were used following taxonomic keys for fungi identification at genus level (Ellis 1976; Leslie and Summerel 2006; Pitt 1979; Raper and Fennell 1965; Rotem 1994; Samson et al. 2014).

## Sample Preparation for Mycotoxin Analysis

One gram portion of apple puree sample was placed in a centrifuge 15-mL falcon tube and 4 mL of MeOH was added and vortexed for 10 s. Subsequently, the tube was shaken in a horizontal shaker (Ika-werke, Staufen im Breisgau, Germany) for 10 min at 230 strokes per minute. Afterwards, the mixture was centrifuged for 10 min at 10,000 rpm. Then, 1 mL of the organic upper layer was transferred to a glass vial, and the solvent was evaporated to dryness under a gentle nitrogen stream. The residue was reconstituted with 250 µL of MeOH/water (75:25 v/v) and vortexed for 10 s before its injection into the HPLC system. Figure 1 shows the schematic overview of the optimized procedure.

## LC–MS/MS Analysis

UHPLC Dionex Ultimate 3000 separation system coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electrospray source (ESI) was employed for mycotoxins analysis. For the chromatographic separation, SunShell C18 Column (Chromanik Technologies Inc. Osaka, Japan) with 2.1 × 100 mm and a particle size of 2.6 µm was used. The mobile phase consisted of ultrapure water with 0.2% acetic acid and 5 mM ammonium acetate as aqueous solvent A and MeOH with 0.2% acetic acid as organic solvent B. The following multi-step elution gradient was used: 0–1 min 5% B; 1–8 min 5–90% B; 8–11 min 90% B;

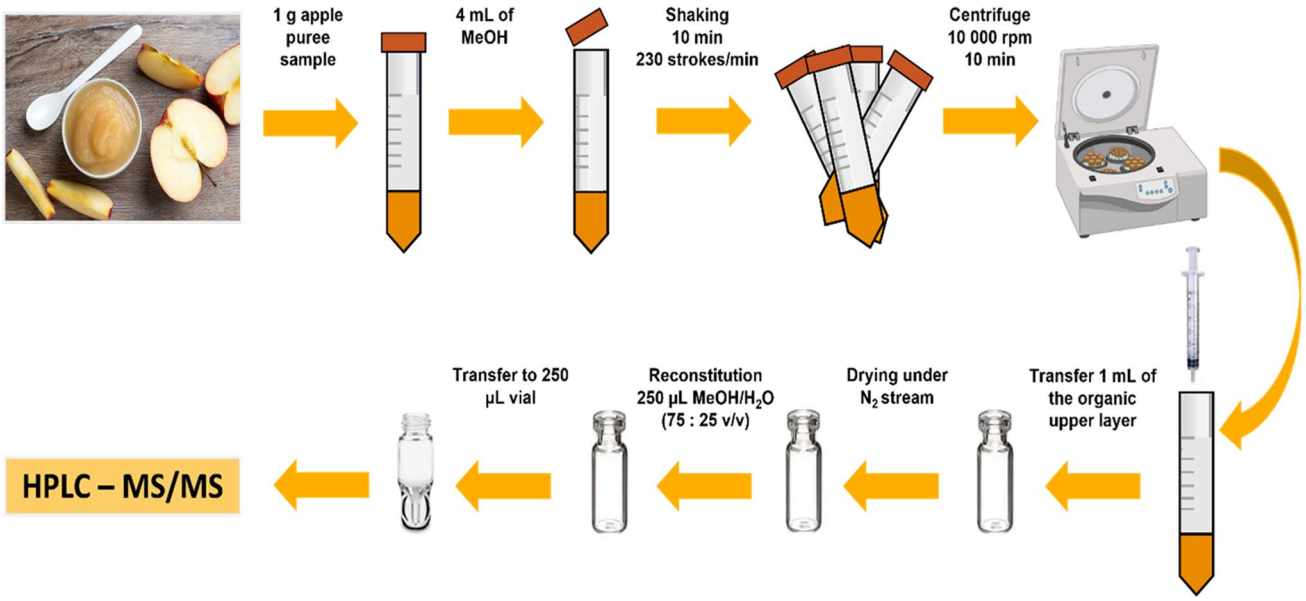


**Fig. 1** Scheme of the proposed SLE method

11–13 min 90–5% B and 13–18 min 5% using a flow rated of 4 mL $min^{-1}$. The system was re-equilibrated to the initial conditions for 5 min to ensure the reproducibility between injections. The column temperature was set at 40 °C and the injection volume was 3 µL. During the analysis, samples were permanently kept at 10 °C.

The mass spectrometer operated in positive electrospray ionization (ESI+) mode for AFB1, AFB2, AFG1, AFG2, FB1, FB2, and TEN, whereas to ionize PAT, AOH, and AME, the negative electrospray ionization (ESI-) was employed. All the compounds were analyzed under selected reaction monitoring (SRM) conditions. Thus, once the precursor ion was fixed for each compound, the main fragments were investigated by collision-induced dissociations selecting the optimum collision energy to be applied in order to obtain the highest signal in each case. Collision energies (V) were set between 11 and 42, depending on the analyte, and product ions were analyzed in the range of 53–354 m/z. MS parameters for the analysis such as the voltage were set at 3500 V, the capillary and vaporizer temperature at 270 °C at 200 °C, respectively. The sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. Optimized MS/MS parameters are summarized in Table 1.

## Method Validation

The proposed SLE-HPLC–MS/MS method was evaluated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), extraction recovery, and precision (i.e., repeatability and intermediate precision) in apple puree blank samples in which the absence of target mycotoxins was previously checked.

In order to compensate possible matrix effects, matrix-matched calibration curves were calculated for each compound. For that, blank sample extracts were spiked with analytes (range: 0.1–160 µg/kg, 10 concentration levels) before analysis. The samples were processed in duplicate and injected in triplicate. Peak area was selected as analytical response and considered as a function of the analyte concentration on the sample. LODs and LOQs were calculated as the minimum analyte concentrations with a signal-to-noise ratio equal of 3 and 10, respectively.

Method precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision), by spiking blank samples at two concentration levels in the linear range of each analyte. For repeatability, three blank samples were spiked at given concentration levels, and independently extracted (experimental replicates); each extract was then injected in triplicate (instrumental replicates) the same day under the same conditions ($n=9$). For intermediate precision, a blank sample was spiked at a given concentration level, then extracted on three consecutive days; each extract was injected in triplicate ($n=9$). The concentration levels were as follows: for AFB1, AFB2, AME, and TEN, level 1 was set at 10 µg $kg^{-1}$, while level 2 at 20 µg $kg^{-1}$. For AFG1, AFG2, AOH, FB1, and FB2 level 1 was established at 5 µg $kg^{-1}$ and level 2 at 40 µg $kg^{-1}$. For PAT, level 1 was set at 20 µg $kg^{-1}$ and level 2 at 80 µg $kg^{-1}$.

Recovery experiments were carried out at two different concentration levels. Three blank samples were spiked at the proper concentration, extracted as previously described, and analyzed in triplicate. In parallel, three blank samples underwent extraction, and the extracts were spiked before injection at the same concentration levels. The percentage of recovery was calculated comparing the data obtained in both cases in terms of peak area. For each mycotoxin, the spiking levels were the same already applied in the precision study.

**Table 1** MS/MS conditions for the determination of the target mycotoxins

| Mycotoxin | Retention time (min) | Molecular ion | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|
| PAT | 1.93 | $[M-H]^-$ | 152.9 | 53.0; 81.0 |
| AFG2 | 7.19 | $[M+H]^+$ | 331.3 | 245.3; 270.0 |
| AFG1 | 7.41 | $[M+H]^+$ | 329.0 | 243.0; 311.0 |
| AFB2 | 7.62 | $[M+H]^+$ | 315.0 | 259.0; 287.0 |
| AFB1 | 7.80 | $[M+H]^+$ | 313.0 | 241.2; 271.1 |
| AOH | 8.58 | $[M-H]^-$ | 257.0 | 147.0; 213.0 |
| FB1 | 8.77 | $[M-H]^+$ | 722.4 | 334.0; 352.0 |
| TEN | 8.83 | $[M+H]^+$ | 415.0 | 302.0; 312.1 |
| FB2 | 9.56 | $[M-H]^+$ | 706.4 | 318.0; 354.0 |
| AME | 9.78 | $[M-H]^-$ | 271.0 | 213.0; 228.0 |

## Results and Discussion

The current climate change scenario may lead to the unexpected occurrence of mycotoxins in food products, due to a different distribution and growth of fungal pathogens on crops. Therefore, analytical methods targeting only regulated mycotoxins in their respective matrices could be blind to such unexpected occurrence. Large multi-mycotoxins LC-HRMS methods are surely a comprehensive tool, but their adoption in small control laboratories is often challenging due to instrumental and training constrains. A less powerful but still effective alternative is represented by more focused multi-toxin methods including only those analytes that are likely to occur in the matrix of interest, based on the realistic evaluation of the potential pathogens present in the field.

### Characterization of the Natural Fungal Population in Stored Apples

With the aim to develop a fit-for-purpose multi-toxin method and include all those mycotoxins which could be

present in apple products, a small batch of apples was collected from storehouses and analyzed for fungal spoilage. Fungi were isolated from skin, flesh, and calyx area of each apple orchard, irrespective of the cv considered. The fungal profile returned the presence of isolates belonging to *Fusarium* spp., only with direct isolation from apple pieces, irrespective of the position (skin, flesh, or calyx). *Penicillium* spp. were isolated from apple pieces and found as colonies from puree ($10^3$ CFU/g), mainly from strongly degraded apples. *Alternaria* spp. were instead isolated only in apple puree from mildly degraded fruits (10 CFU/g). *Penicillium* spp. are probably the best adapted fungus to apple and it produces many small conidia; it is therefore easy to isolate this fungus irrespective of the method used. On the contrary, *Fusarium* spp. are not commonly reported in apples and due to its interaction with the host crop, it is mainly detected directly from fruit pieces (Sever et al. 2012).

Therefore, taking into consideration the fungal profile and the data from the literature, we decided to include in the proposed workflow PAT, AOH, AME and TEN, FB1, and FB2. Then, taking into consideration a recent withdrawn from the market due to AFB1 in apple puree (Italian Ministry of Health 2019), the four main aflatoxins were included as well in the analysis.

## Optimization of the Sample Treatment Procedure

The solid–liquid extraction (SLE) was optimized based on the ten principles for green sample preparation (GSP) (López-Lorente et al. 2022). As far as the extraction solvent, MeOH, MeCN, and MTBE were selected based on their relative polarity (0.762, 0.460, and 0.124 respectively, according to Reichardt and Welton 2003) and compared for their ability to extract the target analytes from apple puree. Poor recoveries were provided by MTBE, particularly for PAT and FBs, with values below 40 and 10%, respectively. On the other hand, MeOH offered recoveries higher than 80% for all the target mycotoxins, and therefore, it was selected for further experiments (Fig. 2a). The MeOH volume was then optimized for exctraction in the range 1–5 mL and the extraction time (horizontal sjaker, 320 strokes/min) in the range 5–15 min (Fig. 2b and c). The optimum was set at 4 mL and 10 min, respectively. After evaporation to driness, the sample was reconstituted in 250 µL MeOH, to increase method sensitivity.

The optimisation of the sample preparation allowed to reduce solvent volume as well as decrease the extraction steps and time in comparison with other procedures from the literature (Walravens et al. 2016; Tang et al. 2020). Offering a simple protocol is a key factor in increasing efficiency and sample throughput in routine analysis.

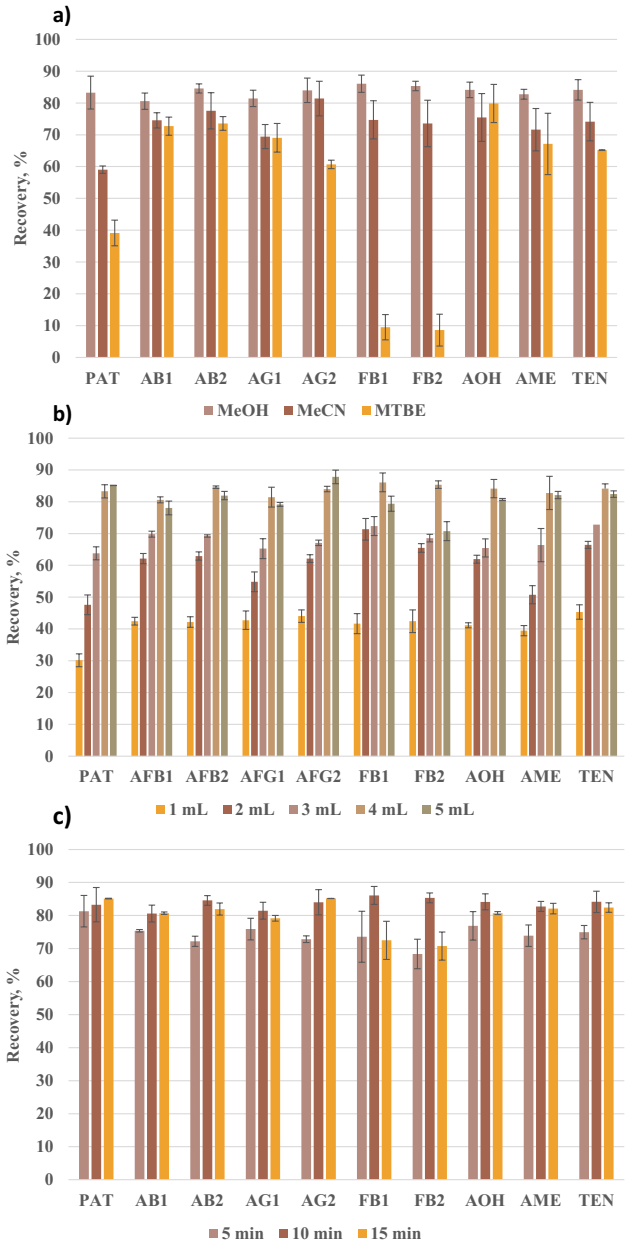


**Fig. 2** Optimization of the extraction parameters, **a** extraction solvent, **b** extraction volume, and **c** extraction time. Error bars represent standard error ($n = 4$)

## Method Validation

The linearity range and associated determination coefficient ($R^2$), LODs, and LOQs (expressed as S/N 3 and 10, respectively) are shown in Table 2. All the compounds presented a good linearity ($R^2 > 0.99$) over the studied range. The chromatographic separation of the target mycotoxins as well as the extracted ion chromatograms (EICs) resulted from the analysis of a spiked apple puree sample using their corresponding quantification transition can be seen in

**Table 2** Performance characteristics of the proposed method for determination of mycotoxins in apple puree

| Mycotoxin | Linearity ($R^2$) | LOD (μg kg$^{-1}$) | LOQ (μg kg$^{-1}$) |
|---|---|---|---|
| PAT | 0.9900 | 1.22 | 4.05 |
| AFG2 | 0.9970 | 0.14 | 0.45 |
| AFG1 | 0.9910 | 0.15 | 0.51 |
| AFB2 | 0.9970 | 0.01 | 0.02 |
| AFB1 | 0.9960 | 0.02 | 0.05 |
| FB1 | 0.9989 | 0.19 | 0.63 |
| FB2 | 0.9905 | 0.43 | 1.42 |
| AOH | 0.9960 | 0.26 | 0.88 |
| TEN | 0.9950 | 0.02 | 0.05 |
| AME | 0.9980 | 0.01 | 0.04 |

supplementary Material, Figure S1. Concerning PAT, the only regulated mycotoxin in apple products, the LOQ was well-below the maximum permitted limit in apple-based products marketed in Europe, and comparable with those previously reported methods in similar samples using HPLC (Walravens et al. 2016; Dias et al. 2019). For the other analytes, LOQs were in the range 0.02–0.09 μg kg$^{-1}$, and thus considerably lower than those previously reported for *Alternaria alternata* mycotoxins (Lopez-Lorente et al. 2022).

Precision was expressed as relative standard deviation (% RSD) of peak areas (Table 3). In both cases, the RSD values were under 17% fulfilling the recommendation of the guidance for analytical quality control and method validation (Regulation EC No 401/2006; Guidance SANTE 11312/2021).

Recovery experiments were carried out in order to asses the efficiency of the proposed sample preparation at different concentration levels. In all cases, the recoveries ranged between 70 and 106%, as shown in Table 3. These results demonstrated that the proposed SLE pre-treatment could be satisfactorily applied for the extraction and isolation of the target mycotoxins in apple puree samples.

## Analysis of Stored Apples for Puree Production

The proposed method was applied to stored apples intended for processing, as a preliminary study. The same batches (Golden delicious and Imperatore cv.) already considered for fungal isolation were sampled at the delivery time (t0) and after 3-week storage at room temperature (t1). Spoilage decay degree, when visible, was qualitatively assigned based on visual inspection (*mild* versus *strong*). All the samples were analyzed for mycotoxins, and the concentrations found as well as their corresponding standard deviation (SD) are reported in Table 4. Overall, AOH, AME, and PAT were found in most samples, with PAT in very high concentrations reaching 3200 μg kg$^{-1}$. In all cases, the concentration of PAT was far above the maximum levels established for apple puree products intended for adult consumption (25 μg kg$^{-1}$) and for those destined for infants and young children (10 μg kg$^{-1}$). Comparing the results for PAT in mildly and strongly degraded apples, it can be noticed that the PAT concentration in the latter group is 27 times higher. This agrees with fungi quantification, being *Penicillium* dominant in strongly rotten apples. These results are in accordance with Zhong et al., 2018, who reported that the level of degradation of apples is related with the *P. expansum* decay, leading therefore to a higher PAT accumulation.

The widespread occurrence of *Alternaria* toxins, although at low amounts, is consistent with all the reports in the literature, highlighting the need of a proper monitoring plan for this class of compounds in apple fruit and products thereof (Patriarca 2019; Puntscher et al. 2020). It is also justified by the occurrence of *Alternaria*, mainly low degraded fruits.

Surprisingly, sample M3 showed the presence of AFB1, AFB2, and AFG2, with an AFB1 concentration of 29 μg kg$^{-1}$, without *Aspergillus* detection. *Aspergillus* spp. commonly occur with very low incidence and it is not easy to be detected (Arciuolo et al. 2020; Leite et al. 2014; Yobo et al. 2017), especially in degraded fruits where other fungi

**Table 3** Precision and recovery studies of the proposed method for the determination of mycotoxins in apple puree

| Mycotoxin | Repeatability, % RSD ($n=9$) | | Intermediate precision, % RSD ($n=9$) | | Recovery, % RSD ($n=9$) | |
|---|---|---|---|---|---|---|
| | Level 1 | Level 2 | Level 1 | Level 2 | Level 1 | Level 2 |
| PAT | 4.9 | 3.8 | 12.6 | 11.6 | 75 | 82 |
| AFG2 | 4.3 | 4.2 | 16.8 | 6.9 | 77 | 84 |
| AFG1 | 7.7 | 3.6 | 15.3 | 5.5 | 78 | 100 |
| AFB2 | 3.2 | 5.0 | 11.3 | 10.1 | 88 | 106 |
| AFB1 | 7.6 | 4.9 | 16.7 | 11.1 | 70 | 90 |
| FB1 | 5.1 | 5.8 | 13.4 | 9.1 | 80 | 83 |
| FB2 | 9.8 | 10.1 | 9.6 | 7.0 | 75 | 80 |
| AOH | 6.7 | 3.2 | 9.7 | 9.8 | 78 | 81 |
| TEN | 8.0 | 4.5 | 4.9 | 3.4 | 76 | 91 |
| AME | 4.0 | 3.7 | 11.6 | 7.7 | 74 | 99 |

**Table 4** Occurrence and concentration (μg $kg^{-1}$ d.w.) ± STD of the mycotoxins found in the apple samples. (#Field management O = organic; C = conventional)

| Sample | Cultivar | Field management# | Visual decay | Aflatoxins | | | | Fumonisins | | Alternaria toxins | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | AFB1 | AFB2 | AFG1 | AFG2 | FB1 | FB2 | AOH | AME | TEN | PAT |
| M1 | Golden | C | Mild | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| M2 | Golden | O | Strong | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | 81.2 ± 9.2 | 57.7 ± 3.5 | 0.2 ± 0.0 | 2403.7 ± 35.6 |
| M3 | Golden | O | Mild | 29.0 ± 1.2 | 0.4 ± 0.0 | <LOD | 1.5 ± 0.1 | <LOD | <LOD | 599.1 ± 12.5 | 379.8 ± 11.0 | <LOD | 54.4 ± 17.2 |
| M4 | Imperatore | O | Strong | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | 11.4 ± 3.1 | 0.9 ± 0.2 | 0.1 ± 0.0 | 3206.5 ± 47.9 |
| M5 | Imperatore | C | Mild | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | 178.8 ± 9.7 | 217.6 ± 13.4 | 0.2 ± 0.0 | 2704.6 ± 33.5 |
| M6 | Imperatore | C | Strong | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | 88.3 ± 7.8 | 29.5 ± 4.2 | <LOD | 153.9 ± 21.8 |
| M7 | Golden | C | No | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| M8 | Golden | O | No | 1.9 ± 0.2 | <LOD | <LOD | <LOD | <LOD | <LOD | 0.8 ± 0.1 | 13.8 ± 0.9 | <LOD | 11.7 ± 0.9 |
| M9 | Imperatore | C | No | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | 6.8 ± 3.1 |
| M10 | Imperatore | O | No | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |

are largely dominant (Grantina-Ievina 2015). This lack of detection can anyway be associated with AFs contamination; sporadic occurrence can result in highly contaminated products. Although AFB1 is not regulated in apple-based products, the found concentration is clearly above all the maximum permitted levels for AFB1 in other food categories (range: 0.1–8 μg $kg^{-1}$), and therefore, potential AFB1 occurrence in apples should be taken into consideration for further studies. Considering that recently apple puree batches were withdrawn from the Italian market due to AFB1 occurrence (Italian Ministery of Health 2019), the presence of aflatoxins in apple from the field surely deserves attention and further studies in order to provide a large base of data for risk assessment.

## Analysis of Apple Puree Samples from the Market

The method was then applied to the determination of mycotoxins in apple puree products from the market. As a preliminary screening, a small batch of purees was purchased from the local market, aiming at understanding mycotoxin co-occurrence in retail products. Samples ($n = 20$) were selected among conventional and organic brands from the Italian market. The samples that meet the following criteria were identified as positives: (i) S/N ratio of the peak of at least 3, (ii) the relative ion intensities for detection and quantification ions must correspond to those of the ions in standards solutions, (iii) the calculated concentration of the mycotoxins should exceed the corresponding LOQs.

As can be seen in Table 5, surprisingly, PAT and FB1 were found in all the considered samples, with PAT at concentration levels close or even higher than the maximum permitted limit. AFB1 was found in 5 out of 10 samples, at concentration levels closed to the regulatory limits in force for other food commodities. *Alternaria* toxins, mainly AOH, were also widespread in the considered samples. In general, at least 2 mycotoxins were found in all the considered samples. Although the co-occurrence of aflatoxins, Alternaria mycotoxins, fumonisins, and patulin has never been reported in apple puree so far, it should be noticed that our data are in line with those previously reported in the literature and consistent with the recent product recall from the Italian market due to aflatoxins (Italian Ministry of Health 2019) and fumonisins (Regione Veneto 2021) contamination. In addition, they are consistent with the fungal population isolated within this study from stored apples intended for processing. Although very preliminary, this data shade a light on the urgency of collecting large co-occurrence data for mycotoxins in apple purees as a base for risk assessment, especially considering that such products are often consumed by children and elderly. As a representative example of the naturally contaminated samples, the EICs of a highly contaminated sample from brand 6, in which PAT, AFB2, AFB1, AOH, FB1, TEN, FB2, and AME where co-occurring, are shown in Fig. 3.

**Table 5** Mycotoxin co-occurrence in retail apple puree samples. Data are presented as the average of two samples per brand, each analyzed in duplicate

| Product | Concentration (μg $kg^{-1}$) ± STD | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AFB1 | AFB2 | AFG1 | AFG2 | FB1 | FB2 | AOH | AME | TEN | PAT |
| Brand1 | 0.8 ± 0.3 | 0.9 ± 0.1 | < LOD | 1.6 ± 2.3 | 37.5 ± 4.3 | < LOD | < LOD | < LOD | 0.9 ± 0.1 | 92.4 ± 5.7 |
| Brand2 | 0.2 ± 0.1 | 0.1 ± 0.2 | < LOD | < LOD | 39.5 ± 0.3 | < LOD | 45.6 ± 6.5 | 46.6 ± 0.8 | 49.8 ± 1.3 | 101.8 ± 4.7 |
| Brand3 | < LOD | < LOD | < LOD | < LOD | 41.2 ± 1.7 | < LOD | < LOQ | < LOD | < LOD | 94.2 ± 0.2 |
| Brand4 | 0.22 ± 0.03 | < LOD | < LOD | < LOD | 90.5 ± 1.1 | < LOD | 2.7 ± 0.3 | < LOD | < LOD | 62.0 ± 2.8 |
| Brand5 | 0.1 ± 0.08 | < LOD | < LOD | < LOD | 39.7 ± 3.6 | < LOD | 2.1 ± 0.6 | < LOD | < LOD | 18.6 ± 1.1 |
| Brand6 | 0.9 ± 0.1 | 1.05 ± 0.2 | < LOD | < LOD | 95.2 ± 14.0 | 4.3 ± 2.5 | 2.6 ± 0.8 | 0.8 ± 0.2 | 0.5 ± 0.1 | 34.5 ± 11 |
| Brand7 | 0.3 ± 0.1 | 0.27 ± 0.1 | < LOD | < LOD | 46.3 ± 14.0 | < LOD | 3.2 ± 0.3 | 1.85 ± 0.2 | 2.2 ± 0.3 | 480.2 ± 13.4 |
| Brand8 | < LOD | < LOD | < LOD | < LOD | 38.8 ± 1.8 | < LOD | < LOD | < LOD | < LOD | 321.4 ± 13.4 |
| Brand9 | < LOD | < LOD | < LOD | < LOD | 40.9 ± 7.7 | < LOD | 5.8 ± 2.0 | < LOD | < LOD | 165 ± 9.8 |
| Brand 10 | < LOD | < LOD | < LOD | < LOD | 82 ± 14.4 | < LOQ | < LOD | < LOD | < LOD | 87.5 ± 10.1 |

*LODs (μg $kg^{-1}$): AFB1 (0.02); AFB2(0.01); AFG1(0.2); AFG2(0.1); FB1 (0.2); FB2 (0.4); AOH (0.3); AME(0.01); TEN (0.02); PAT (1.2)*

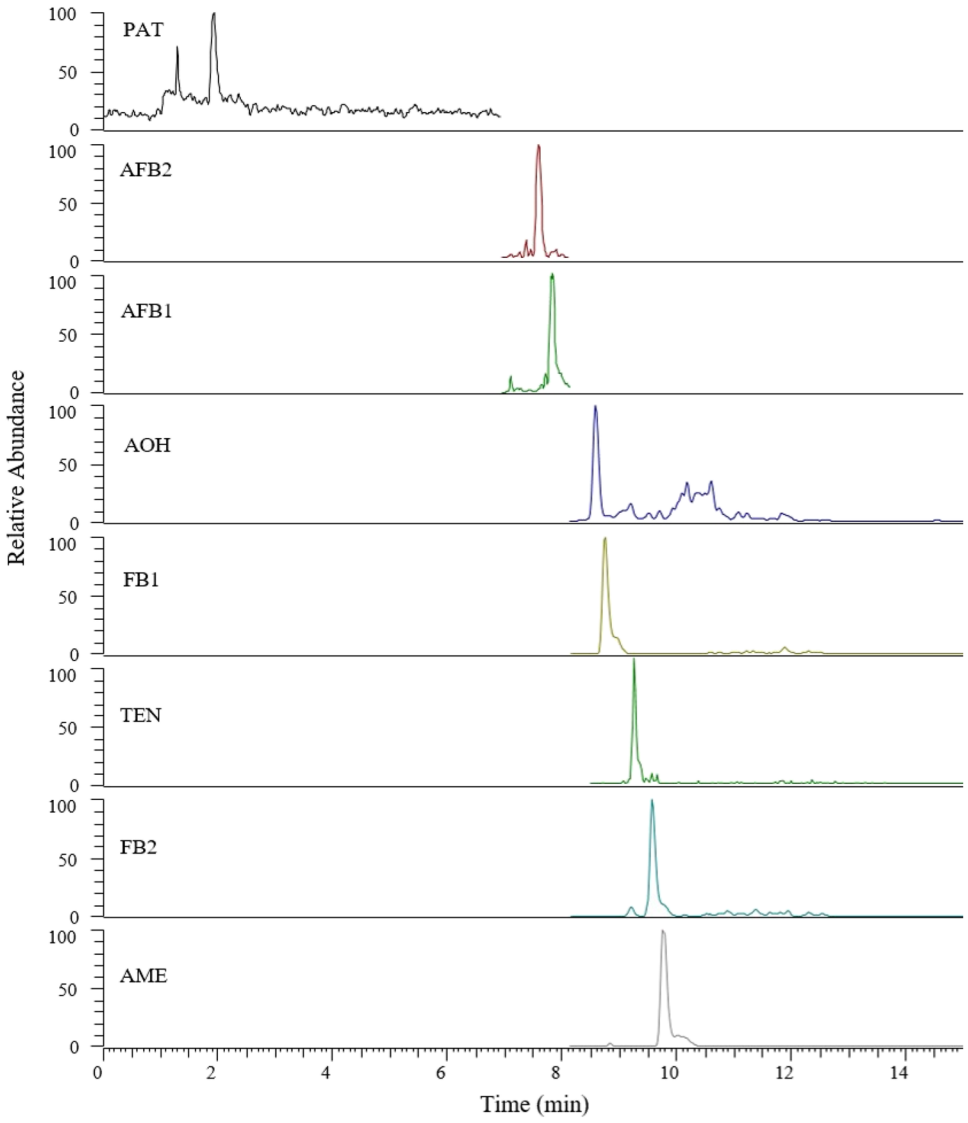


**Fig. 3** EICs of a naturally contaminated sample from comercial apple puree destined for children consumption (brand 6)

## Conclusions

To the best of our knowledge, this is the first work that reports the simultaneous determination of AFs, FBs, *Alternaria* toxins, FBs, and PAT in apple puree samples.

The optimized sample treatment procedure was an easy, fast, and effective SLE in which sample dilution was avoided leading to an improvement in sensitivity and reducing organic solvents consumption. The proposed method was successfully characterized reaching LOQs in the low μg $kg^{-1}$ for all studied mycotoxins. The method was then applied for the analysis of apple puree obtained from apples intended for processing, with different spoilage decay degree, and further applied to the analysis of apple puree samples from the italian retail market. Stored apples showed a large co-occurrence of mycotoxins and a complex fungal profile. The preliminary survey from the market returned the common co-occurrence of mycotoxins in retailed products, among them FB1 and AFB1. Due to the potential accumulation of unexpected mycotoxins in these samples, the determination of a higher number of mycotoxins should be considered. The observed fungal profile and the occurrence of other mycotoxins apart from PAT in apple puree samples highlight the need of including them in next studies and future legislation.

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**Data Availability** The data available in this study are available in Tables 1, 2, 3, 4, and 5; Figs. 1, 2, and 3; and Supplementary materials.

## Declarations

**Conflict of Interest** The authors declare no competing interests.



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