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The effects of the extrusion process used for the production of maize snacks and pasta on the free, bound, and total B fumonisin contents

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ABSTRACT

Mechanical and thermal treatments promote chemical changes in B fumonisins (FBs), mycotoxins that frequently contaminate maize. These changes, in turn, lead to the production of chemically modified or matrix-associated FBs, the so-called "bound" FBs, at the expense of the free forms. The present study has been aimed at evaluating the effects of the maize composition and the technological processes of co-extrusion, used for snack production and pasta-making, on the free and total FB contents, which were released after alkaline hydrolysis, by means of an LC-MS/MS analysis of the hydrolyzed FBs, estimating by difference the content of the bound FBs. Overall, the total content of FBs was always higher (1.1–2.3 times) than the free one, and higher values of bound FBs (2.6–4.3 times) were always reached for the maize-based products than the raw materials, thus confirming that processing promotes the binding of a part of the free FBs to the matrix. The obtained results confirm that the 'masking' phenomenon, which takes place through a matrix-association, is predominant during food processing, and is higher in pasta-making compared to co-extrusion for snack production. Moreover, results to increase in higher particle size flours than finer ones, while no clear effects are related to the starch composition.

1. Introduction

Maize is a versatile, multi-purpose crop of global importance and the basis of many foodstuffs, such as breakfast cereals, porridge/polenta, tortillas, bakery products, and more recently, pasta and different types of snacks (Schaarschmidt & Fauhl-Hassek, 2021). At the global level, 13% of maize (dry grain) is on average used for food purposes, ranging from 5.6% in Europe, 7.5% in the Americas, 11.6% in Asia, up to 54.3% in Africa, contributing in some areas to over 20% of food calories (Erenstein, Jaleta, Sonder, Mottaleb, & Prasanna, 2022). However, maize is often contaminated by B fumonisins (FBs), and among them, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are the most frequent naturally occurring. FB_1 typically accounts for 70%–80% of the total fumonisin produced, while FB_2 usually makes up 15%–25% and FB_3 3%–8% (Zentai et al., 2019). Among fumonisins, FB_1 is the most toxic compound and it was classified by the International Agency for Research on Cancer (IARC) in Group 2B (possibly carcinogenic in humans) (IARC, 2002). The extent of this contamination can vary greatly under certain specific conditions through the world. Indeed, weather might pose a negative influence on the prevention or control of FBs, due to marked environmental variances between regions, and even between crops planted in a same region with different dates of sowing (Ponce-Garcia, Serna-Saldivar, & Garcia-Lara, 2018).

In recent decades, maize has been widely used in gluten-free (GF) formulations, as the market for GF products has significantly increased, due to the growing number of consumers suffering from celiac disease or gluten intolerance (Alfieri et al., 2020; Woomer & Adedeji, 2021).

A large variety of GF food products, are produced by means of an extrusion process (Wang et al., 2016). Unfortunately, mechanical and thermal treatments promote chemical changes in FBs, and this leads to the production of chemically modified or "matrix-associated" FBs, as named by the nomenclature proposed by Rychlik et al. (2014), at the expense of the free forms. According to Rychlik et al. (2014), matrix-associated FBs refer to either complexes with matrix compounds or to complexes that are physically dissolved, trapped, or covalently bound to matrix components, or even a combination of these effects. In the literature, these forms are referred to as both covalently bound FBs (i.e., bound to starch, proteins, or reducing sugars) and non-covalently bound FBs, but they are also referred to as 'bound fumonisins' and 'hidden fumonisins', respectively (Dall'Asta, Mangia, et al., 2009; Scott, 2012), while the 'masked mycotoxins' term is mainly used for conjugated mycotoxins generated by plants. Nevertheless, the generic term 'masking' is still widely used in literature to generically refer to all the

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Abbreviations

ANOVA	analysis of variance
C	conventional hybrid
CH_3COOH	glacial acetic acid
CH_3CN	acetonitrile
CH_3OH	methanol
CID	collision-induced dissociation
EC	European Commission
EL-EM	equine leuko-malacia
EFSA	European Food Safety Authority
ESI	electrospray ionization
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FBs	sum of FB_1 and FB_2, fumonisins B
FB_1 bound	fumonisin B_1 bound
FB_2 bound	fumonisin B_2 bound
FBs bound	sum of FB_1 bound and FB_2 bound, fumonisins B bound
FB_1 free	fumonisin B_1 free
FB_2 free	fumonisin B_2 free
FBs free	sum of FB_1 free and FB_2 free, fumonisins B free
FB_1 total	sum of fumonisin B_1 bound and fumonisin B_1 free
FB_2 total	sum of fumonisin B_2 bound and fumonisin B_2 free
FBs total	sum of FB_1 total and FB_2 total, fumonisins B total
GF	gluten-free
H_2O	water
HA	high-amylose hybrid
HFB_1	hydrolized fumonin B_1
HFB_2	hydrolized fumonin B_2
HFBs	sum of HFB_1 and HFB_2, HFBs
IARC	International Agency for Research on Cancer
KOH	potassium hydroxide
LC	liquid chromatography
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
$NDF\text{-}FB_1$	N-(1-deoxy-d-fructos-1-yl)-fumonisin B_1
$NCM\text{-}FB_1$	N-(carboxymethyl)-fumonisin B_1
PPE	porcine pulmonary edema
RA	apparent recovery
RC	regenerated cellulose
RE	recovery of the extraction
REGW-F	Ryan-Einot-Gabriel-Welsh F post-hoc test
RSDr	relative standard deviation of repeatability
SD	Standard Deviation
S/N	signal-to-noise ratio
SRM	selected reaction monitoring
SSE	signal suppression/enhancement
TQ	triple quadrupole
W	waxy hybrid

covert forms produced by the most disparate mechanisms.

The toxicological role of masked, modified, and matrix-associated mycotoxins has yet to be fully elucidated, but the preliminary results strongly suggest their inclusion in European Food Safety Authority (EFSA) risk assessment studies (EFSA, 2014). Although most of the modified forms are stable under gastric conditions, they are subject to breakdown in the gut due to large intestinal microbial activity (Dall'Erta et al., 2013) and could increase the toxicological burden (Dall'Asta & Battilani, 2016). Moreover, even though the FB derivatives generated when amino acids are coupled with reducing sugars reduce the degree of toxicity of the original FBs (Molina-Pintor et al., 2022), the amino acid group may be released again during digestion.

However, not all these modified forms of FBs are identified in routine analysis, and it has in fact been observed that the alkaline hydrolysis of contaminated maize products often leads to higher amount of released hydrolyzed FBs than those stoichiometrically derived from the conversion of FBs (Dall'Asta, Mangia, et al., 2009; Dall'Asta, Falavigna, Galaverna, Dossena, & Marchelli, 2010; Kim, Scott, & Lau, 2003; Park, Scott, Lau, & Lewis, 2004).

Nevertheless, although routine analysis cannot detect these compounds, it is necessary to evaluate the total FB content to have a clearer picture of the overall risk, especially for consumers who, due to celiac disease or an intolerance to gluten, are more exposed.

In this framework, the present study has been aimed at evaluating the effects of the maize composition, in terms of particle size and amylose content, and of the co-extrusion technological processes used for the production of snacks and pasta-making on the free and total FB contents, just focusing on the FB_1 and FB_2 forms (regulated by the European Commission Regulation (EC) No. 1126/2007), that are released after alkaline hydrolysis, estimating by difference the content of the so-called bound/hidden or matrix-associated FBs, which herein have simply been called bound FBs.

2. Material and methods

The effect of the maize composition and the technological processes used for the production of snacks and pasta on the contents of free and bound fumonisin B_1(FB_1) and fumonisin B_2 (FB_2) was studied by considering different maize lots and products (co-extruded snacks, uncooked and cooked pasta), and comparing them with their raw materials, i.e. flour or break meal.

2.1. Maize raw materials

Six maize lots, resulting from a factorial combination of two milling fractions with different particle sizes (maize flour, 85% of the particles under 150 μm, and break meal, 77% of the particles between 250 and 500 μm; Vanara, Scarpino, & Blandino, 2018) and three hybrids with different amylose contents were considered in this study, as described in detail by Bresciani, Giordano, Vanara, Blandino, & Marti, 2021: i) a conventional hybrid (Pioneer P1547, amylose = 180 g/kg; C), ii) a high-amylose hybrid (Planta Amylor, amylose = 420 g/kg; HA), and iii) a waxy hybrid (Pioneer P1547E, amylose = 20 g/kg; W). All the hybrids were cultivated in the 2018 growing season in the same area in North West Italy. The raw materials were obtained from multiple stream-roller millings in an industrial mill (Molino Peila, Valperga, Italy).

2.2. Co-extruded snacks

All six maize lots were processed to produce co-extruded snacks, which were produced at an industrial level by Fudex Group S.p.A. (Settimo Torinese, Italy). Dry-extrusion was performed using a co-rotating twin-screw extruder (2FB90HT model, Fudex Group spa; screw speed: 155 rpm; screw temperature: 117 °C; pressure: 7×10^6 Pa). The feed moisture level was adjusted to 100 g/kg. The snacks were milled into fine powder (particle size < 250 μm) using a laboratory mill (IKA Universalmühle M20; IKA Laborteknic, Staufen, Germany), with a water-cooling system (Bresciani et al., 2021).

2.3. Pasta-making and cooking processes

According to the suitability of the technological process, the pasta

was made considering only three lots: the maize flour particles size of C, HA, and W hybrids. Flours were mixed with the mono- and di-glycerides of fatty acids (3 g/kg) and processed into pasta by means of extrusion-cooking. A pre-gelatinization tank (Braibanti, Milan, Italy) was used to treat the flour and water (300 g/kg final moisture content) with steam at 130 °C for 15 min for C, at 130 °C for 30 min for HA, and at 130 °C for 10 min for W, as described in detail by Bresciani, Giordano, Vanara, Blandino, & Marti, 2021. The pre-gelatinized mixture was extruded at 100 °C into small pellets (cylinder shape; 3 mm diameter), and then formed into a macaroni shape using a continuous press for semolina pasta production (Braibanti, Milan, Italy). A jacket with cold water kept the dough temperature at about 50 °C under an extrusion pressure of 10^7 Pa. All the samples were dried in an experimental drying cell (Fava S.p.A., Cento, Italy) using a high-temperature drying cycle (70 °C for 3.5 h) and an aliquot was milled as above.

Pasta (25 g) was cooked in boiling distilled water (pasta:water ratio = 1:10), and then treated with liquid nitrogen, freeze-dried (−80 °C for 72 h; Alpha 1–2 LD plus; Deltek s.r.l., Naples, Italy), ground (particle size <500 μm), using a cyclotec 1093 sample mill (Foss, Padua, Italy), and stored at −25 °C until the analyses (Bresciani et al., 2021).

2.4. Analysis of the free, bound, and total (hydrolized) fumonisins

The moisture content of the raw materials, and of the snack and pasta samples was determined by means of oven-drying the samples at 105 °C for 24 h, in order to express all the results on a dry weight (dw) basis.

2.4.1. Chemicals and reagents

All the reagents were purchased from VWR (Milan, Italy) or Sigma-Aldrich (St. Louis, MO, USA).

FB_1 and FB_2 standards were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria), whereas hydrolyzed fumonisin B_1 (HFB_1) and hydrolyzed fumonisin B_2 (HFB_2) standards were prepared, starting from the analytical standards of FB_1 and FB_2, by means of alkaline hydrolysis, with 2 mol/L KOH, according to Dall'Asta, Galaverna, Aureli, Dossena, & Marchelli, 2008. All the solutions were stored at −20 °C in amber glass vials and were brought to room temperature before use. The chemical structures of the studied mycotoxins are reported in Fig. 1.

2.4.2. Analysis of the free fumonisins (FB1 free and FB2 free)

2.4.2.1. Extraction and sample preparation. The extraction and sample preparation were performed according to the dilute-and-shoot method reported by Scarpino, Reyneri, & Blandino, 2019. Briefly, 5 g of maize sample were extracted, by means of mechanical shaking, with 20 mL of $CH_3CN/H_2O/CH_3COOH$ solution (v: 79 mL : 20 mL : 1 mL). The filtered extract was diluted with the same volume of $CH_3CN/H_2O/CH_3COOH$ solution (v: 20 mL : 79 mL : 1 mL), vortexed and filtered again through 15 mm diameter, 0.2 μm regenerated cellulose (RC) syringe filters (Phenex-RC, Phenomenex, Torrance, CA, USA) and 20 μL of the diluted filtered extract was analyzed without any further pre-treatment.

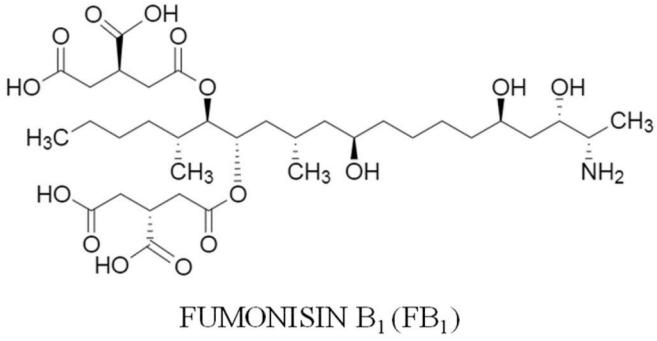
2.4.2.2. LC-MS/MS analysis. The chromatographic and mass spectrometric conditions, and the performance of the method are described in detail in Scarpino et al. (2019) and are summarized in Tables 1 and 2.

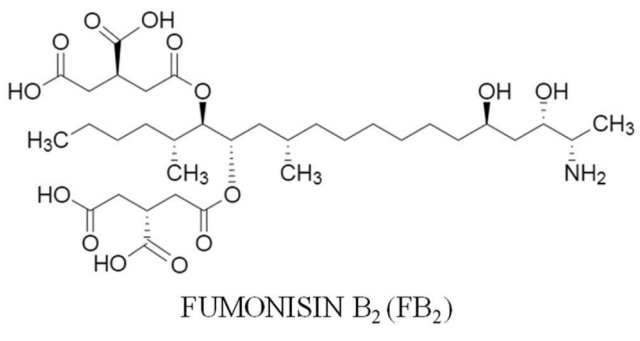
2.4.3. Analysis of the hydrolized (HFB1 and HFB2), total (FB1 total and FB2 total) and bound (FB1 bound and FB2 bound) fumonisins

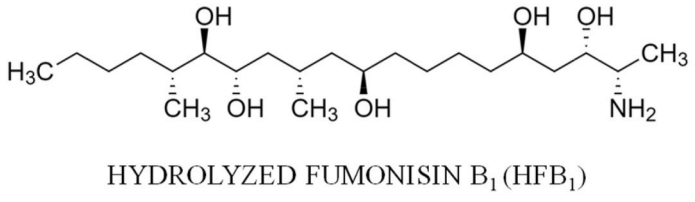
2.4.3.1. Extraction and sample preparation. The extraction and sample preparation were performed according to the method reported in Dall'Asta, Galaverna, Aureli, Dossena, and Marchelli (2008), albeit with some modifications.

A 2 g aliquot of the ground maize samples (raw materials, snacks, and pasta) was weighed in a 50 mL Falcon tubes and 20 mL of a 2 mol/L KOH solution was added, mixed for 5 min at 4000 rpm using a highspeed mixer (Ultraturrax T25, IKA, Stauffen, Germany) and subsequently mechanically stirred at 300 rpm for 50 min. Each sample was then extracted with 20 mL of CH_3CN, and after stirring for 5 min, the two layers formed were separated by centrifugation at 2054×*g* for 15 min. A 4 mL portion of the upper layer richer in CH_3CN was dried under N_2 flow, and the residue was dissolved in 2 mL of $CH_3CN/H_2O/CH_3COOH$ solution (v: 49.5 mL: 49.5 mL: 1 mL) and filtered directly into vials, using nylon disc filters (0.45 μm) (J.T.Baker®, Avantor®, VWR Milan, Italy), and 20 μL of filtrate was injected for the LC-MS/MS analysis.

The HFB_1 and HFB_2 obtained after alkaline hydrolysis of the samples were expressed as FB_1 and FB_2 equivalents, considering a conversion factor based on the difference in molecular weight of the free fumonisins and the hydrolyzed ones and this amount represented the total







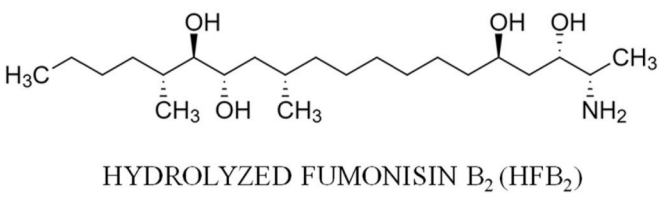


Fig. 1. Chemical structure of Fumonisins B_1 (FB_1), Fumonisin B_2 (FB_2), hydrolized Fumonisins B_1 (HFB_1) and Fumonisin B_2 (HFB_2).

Table 1
Optimized ESI-MS and ESI-MS/MS parameters and the monitored transition reactions for the analyzed mycotoxins.

Mycotoxin	Retention time (min)	Precursor ion (*m/z*)	Adduct ion	Declustering potential (V)	Product ions[a] (*m/z*)	Collision energy (V)
FB_1	3.79	722.5	$[M+H]^+$	80	334.3/352.3	39/35
FB_2	4.03	706.5	$[M+H]^+$	80	336.4/318.4	37/39
HFB_1	3.59	406.3	$[M+H]^+$	70	352.3/334.3	19.5/18
HFB_2	3.87	390.3	$[M+H]^+$	74	336.4/354.4	25/25

[a] Numerical values are given in the quantifier/qualifier order (/2nd qualifier).

Table 2
Performance parameters of the LC-MS/MS method (linearity range, LOD = limit of detection, LOQ = limit of quantification, spike levels, $R_A \pm RSD_r$ = apparent recovery ± relative standard deviation of repeatability, SSE ± RSD_r = suppression/enhancement ± relative standard deviation of repeatability, $R_E \pm RSD_r$ = recovery of the extraction ± relative standard deviation of repeatability) considered for the maize samples (n = 3).

Mycotoxin	Method	Linearity range	LOD	LOQ	Spike level	$R_A \pm RSD_r$	SSE ± RSD_r	$R_E \pm RSD_r$
		(μg/kg)	(μg/kg)	(μg/kg)	(μg/kg)	(%)	(%)	(%)
FB_1	Scarpino et al. (2019)	5–10000	1.5	5	5, 50, 200, 500, 1000, 5000	64 ± 1	84 ± 10	76 ± 8
FB_2	Scarpino et al. (2019)	2–2000	0.6	2	2, 10, 40, 100, 200, 1000	69 ± 3	64 ± 2	109 ± 5
HFB_1	*Present*	6.3–2520	1.9	6.3	10.1, 40.4, 101, 404, 1010, 2020	71 ± 15	85 ± 11	83 ± 5
HFB_2	*Present*	1.25–500	0.4	1.25	2.2, 11, 44, 110, 220, 440	59 ± 8	82 ± 15	72 ± 7

fumonisins ($FB_{1\ total} + FB_{2\ total} = FBs_{total}$) present in the sample after hydrolysis. The bound fumonisins ($FB_{1\ bound}$ and $FB_{2\ bound}$) were subsequently calculated as the difference between the total and the free fumonisins.

2.4.3.2. LC-MS/MS analysis. Liquid chromatography, coupled with tandem mass spectrometry (LC-MS/MS) analysis, was carried out on a Varian 310 triple quadrupole (TQ) mass spectrometer (Varian, Turin, Italy), equipped with an electrospray ionization (ESI) source, a 212 LC pump, a ProStar 410 AutoSampler, and dedicated software. Liquid chromatography (LC) separation was performed on a Gemini-NX C18 (100 × 2.0 mm i.d., 3 μm particle size, 110 Å) equipped with a C_{18} 4 × 2 mm security guard cartridge column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of two eluents: H_2O (eluent A) and CH_3OH (eluent B), both of which were acidified with 1 mL/L CH_3COOH, delivered at 200 μL/min. CH_3OH was preferred to CH_3CN for the chromatographic separation, for sensitivity reasons. The addition of acetic acid to both mobile phases increased the overall sensitivity and led to a better peak shape of the acidic compounds, i.e., FB_1 and FB_2, due to the presence of four carboxylic groups in their molecular structure.

The chromatographic run took 13 min and consisted of the following gradient: the elution was started with 90% A, and the proportion of B was increased linearly over 2 min to 100%; this condition was kept for 2 min, and B was then decreased linearly to the initial condition of 10% over 5 min and kept at this level for 4 min in order to re-equilibrate the column.

Mass spectrometry (MS) analysis was performed according to the multi-mycotoxin method described in Scarpino et al. (2019), with updated parameters for the acquisition of the hydrolized fumonisins (HFB_1 and HFB_2) considered in the present study. The detection and acquisition were performed in positive ionization mode, and in selected reaction monitoring (SRM) mode, by monitoring two transition reactions for each compound (HFB_1 and HFB_2) with the following settings: the nebulizing gas was N_2 (137,895 Pa); the drying gas was air (300 °C, 172,369 Pa); the needle voltage was +5000 V, the shield voltage was +600 V, the detector voltage was +1950 V, and the collision gas was Ar (267 Pa).

MS tuning and the optimization of the analyte-dependent MS/MS parameter were performed by means of direct syringe-infusion of a separate standard solution of each analyte into the triple quadrupole (TQ) using an 11 Plus syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 10 μL/min. Tuning experiments were conducted to choose the best ionization mode and to select the most intense adduct ions as both precursors and product ions. A better ionization yield was recorded using the positive ESI mode (ESI^+, $[M+H]^+$). Collision-induced dissociation (CID) experiments were then conducted to select at least two SRMs per analyte. The more intense of these two fragmentation pathways was used for quantification purposes (quantifier product ion), whereas the less intense one was selected for identification purposes (qualifier product ion).

The MS and MS/MS optimized parameters (selection of the most abundant SRM transitions and the adduct ions used as precursor ions, the declustering potentials, and the collision energy) are summarized in Table 1. The performance of the updated method was evaluated for HFB_1 and HFB_2, as described in Scarpino et al. (2019). Briefly, a set of three different calibration curves and six different concentration levels was prepared in triplicate as follows: (i) an external calibration in a neat solvent; (ii) a matrix-matched calibration in a blank extract; (iii) a matrix-matched calibration in sample extracts. The following parameters were then assessed using this set of calibration curves: the linearity range; the limit of detection (LOD); the limit of quantification (LOQ); the matrix effect, considering the signal suppression/enhancement, SSE (%); and the recovery of the extraction, R_E (%), through the evaluation of the apparent recovery, R_A (%). The analyses were conducted in triplicate for all the parameters to evaluate their repeatability (Relative Standard Deviation of Repeatability, RSD_r).

The LOD and LOQ were defined for each mycotoxin as the concentrations that yielded measure peaks with a signal-to-noise ratio (S/N) of 3 and 10, respectively, and were calculated by injecting (n = 5) neat solvent standard solutions at different concentration levels. The LOQ was then verified and validated using the calculated value as the lowest validated level in the spiking experiment.

The R_A, SSE due to the matrix effects, and R_E were calculated from the previously described six-point calibration curves, as explained in detail in Scarpino et al. (2019). The results pertaining to the linearity range, the LOD, the LOQ, the R_A (%), the SSE (%), and the R_E (%) are reported for all the analyzed mycotoxins in Table 2. The R_E of all the targeted mycotoxins was in the 72–109% range, while the RSDr was always ≤15%. Since most target compounds had different levels of matrix effects, matrix-matched calibration was adopted for quantification.

2.5. Statistical analysis

The normal distribution and homogeneity of variances were verified applying the Kolmogorov–Smirnov normality test and the Levene test, respectively.

An analysis of variance (ANOVA) was used to compare the

mycotoxin contaminations, only taking into account the raw materials common to both technological processes (e.g., maize flour of C, HA and W hybrids), using a completely randomized block design, in which product and lot were the independent variables. An in-depth study was carried out for snack production, considering all the six lots, and ANOVA was run considering the effect of co-extrusion and lots with different particle sizes or amylose contents. Multiple Ryan-Einot-Gabriel-Welsh F (REGW-F) post-hoc tests were carried out on the FB contaminations from different lots and products. Moreover, ANOVA was run separately for each lot to compare the effect of the process (raw materials, snack, uncooked and cooked pasta) on the FB contaminations. The effect of lots on the ratio between the free/bound FB_1 and FB_2 was investigated for the snacks, by means of ANOVA. Finally, an ANOVA was carried out to study the effect of products (snack, uncooked and cooked pasta) and lots, only taking into account the raw materials common to both technological processes (e.g., maize flour of C, HA and W hybrids), on the ratio between the free/bound FB_1 and FB_2 and compare the observed ration in the FB forms. SPSS Version 24.0 of the Windows statistical package, (SPSS Inc., 2017) was used for the statistical analysis.

3. Results and discussion

3.1. Free, bound, and total FB_1, FB_2, and FBs contents in maize raw materials

The contents of free, bound and total FB_1, FB_2 and FBs in the six maize lots, obtained from the combination of three hybrids (C, HA, and W) and two different particle sizes (for maize flour and break meal), are reported in Table 3.

Overall, the maize flour lots (n = 3) with the lower particle sizes on average showed higher contents of free (+157%), bound (+88%), and total FBs (+138%) than the break meal from the same lots, thus confirming an inverse relationship with the milling fraction particle size, as previously reported, albeit only for free FBs, by Vanara, Reyneri, & Blandino, 2009 (+133%); Vanara et al., 2018 (+188%); and by Scarpino, Vanara, Reyneri, & Blandino, 2021 (+177%).

The W hybrid was the most contaminated, for both particle sizes, and on average showed higher contents of free (+164% and +126%), bound (+136% and +151%), and total FBs (+148% and +129%) than the C and HA hybrids.

Only the W maize flour showed a contamination level for the free FBs that was above the EU legal limit for the milling fractions of maize with a particle size ≤500 μm (sum of FB_1 and FB_2, 2000 mg/kg - European Commission Regulation (EC) No. 1126/2007).

Bound FBs have already been detected in the maize raw materials sampled in an industrial mill and they on average accounted for ca. 20% of the free FBs, with values ranging from 13% to 44%, for the different hybrids and particle sizes. These values were on average higher in break meal lots than in maize flour ones, and the highest value was reached in the break meal lot of the C hybrid (44%). The percentage of bound FBs in the raw materials increased, compared to the free ones, as the concentration of the free forms decreased, although it did not appear to be affected to a great extent by the amylose content of the different hybrids considered in the study. Similarly, Dall'Asta, Galaverna, et al. (2009); Dall'Asta, Falavigna, Galaverna, & Battilani (2012) and Oliveira et al. (2015) reported the occurrence of bound FBs in raw maize and suggested that non-covalent interactions were responsible for the phenomenon. Dall'Asta et al. (2012) also reported that maize genotypes may support the 'masking' phenomenon to a different extent, particularly under poorly conducive conditions for FB accumulation, whereas the hybrid-related response ability and 'masking' rate decrease when there are more conducive conditions for FB production. Moreover, although the type of maize hybrid, the water activity, and the amylose/amylopectin ratio in the grains could all affect the extent of 'masking' (Dall'Asta & Battilani, 2016), this phenomenon is mostly related to lipid compounds, such as fatty acids and, in particular, the oleic/linoleic acid ratio, in plants (Dall'Asta et al., 2012).

3.2. Free, bound, and total FB_1, FB_2, and FBs in maize flour snacks and pasta

The contents of free, bound, and total FB_1, FB_2, and FBs in products made from the three considered hybrids maize flours, were compared with those present in the respective raw materials (Table 4). Overall, a significant decrease in the content of free FBs was recorded, from the maize flour to the uncooked pasta, and the lowest content was reached in the cooked pasta, probably because FBs are water-soluble (Humpf & Voss, 2004). The trend recorded for the free FBs was reversed for the bound FBs. Indeed, the uncooked pasta exhibited a significantly higher contamination of bound FBs than the cooked pasta (+1.1 times), the snacks (+1.8 times), and the raw materials (+3.2 times), thus underlining a greater increase during the pasta making process than during the co-extrusion of snacks (Table 4). As a result, the free/bound ratio of FB_1, FB_2, and the FBs significantly decreased from the maize flour to the snacks, to the uncooked pasta, and to the cooked pasta, as shown in Table 5. Indeed, on average, the bound FBs accounted for 15%, 51%, 105%, and 125% of the free FBs in the flours, in the snacks, in the uncooked pasta, and in the cooked pasta, respectively. These results confirmed that, although bound FBs are also found in maize and unprocessed food, the 'masking' phenomenon is predominant during food processing, where complexes of FBs are generally produced with other food macromolecules, such as proteins, carbohydrates, and starch (Bryła et al., 2013). The W maize lot was confirmed to be more contaminated, by all the FB forms (free, bound, and total), than the C and HA lots (Table 4). On the other hand, the free/bound FB_1, FB_2, and FB ratios of the different lots did not differ significantly from each other. A significant interaction between product and lot was reported for the free and total FB_1, FB_2, and FBs, while the interaction was never significant for the bound FB_1, FB_2, and FBs or for the ratios of free/bound FB_1, FB_2, and FBs.

As a result of the significant interactions between products and lots, the effects of the pasta-making and the co-extrusion on the content of

Table 3
Free, bound and total fumonisin content in maize lots processed in the industrial mill, expressed as μg/kg ± standard deviation (SD).

Maize Lot[a]		Free			Bound			Total		
Particle size	Hybrid	FB_1	FB_2	FBs	FB_1	FB_2	FBs	FB_1	FB_2	FBs
Maize flour	C	1249 ± 42	372 ± 22	1621 ± 39	147 ± 68	60 ± 32	207 ± 52	1396 ± 33	432 ± 10	1828 ± 25
(<150 μm)	HA	1377 ± 76	368 ± 28	1745 ± 100	158 ± 31	69 ± 28	227 ± 59	1535 ± 73	436 ± 21	1971 ± 94
	W	4067 ± 195	996 ± 158	5062 ± 37	540 ± 31	277 ± 112	817 ± 82	4607 ± 180	1273 ± 119	5880 ± 105
Break meal	C	547 ± 79	135 ± 3	683 ± 82	199 ± 65	99 ± 39	298 ± 33	746 ± 35	234 ± 40	981 ± 49
(250–500 μm)	HA	691 ± 45	220 ± 9	911 ± 44	109 ± 39	53 ± 8	162 ± 41	800 ± 26	274 ± 17	1074 ± 9
	W	1258 ± 24	219 ± 19	1477 ± 42	68 ± 30	162 ± 31	230 ± 18	1326 ± 54	381 ± 14	1707 ± 47

Data are expressed on a dw basis. The reported fumonisin contamination values are the average of 3 replications ± standard deviation.
Fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and total fumonisins (FBs). Total free FBs (sum of free FB_1 and FB_2); total bound FBs (sum of bound FB_1 and FB_2); total FB1 (sum of free FB1 and bound FB_1), total FB_2 (sum of free FB_2 and bound FB_2); total FBs (sum of total FB_1 and FB_2).
[a] Maize lot: combination of 2 particle sizes (maize flour or break meal), and 3 hybrids (C, conventional; HA, high-amylose; W, waxy).

Table 4
Free, bound, total, and the ratio of the free/bound fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and total fumonisin (FBs) in different maize products and lots.

Factor	Source of variation	Free			Bound			Total			Free/Bound Ratio		
		FB_1	FB_2	FBs	FB_1	FB_2	FBs	FB_1	FB_2	FBs	FB_1	FB_2	FBs
Product	Maize flour	2231 ± 1382a	579 ± 323a	2810 ± 1692a	282 ± 198d	135 ± 122c	417 ± 306d	2513 ± 1575a	714 ± 424a	3226 ± 1992a	8.7 ± 2.3a	6.0 ± 3.0a	7.5 ± 1.9a
	Snack	1154 ± 739b	277 ± 162b	1432 ± 899b	523 ± 255c	213 ± 151b	736 ± 391c	1677 ± 972c	490 ± 304c	2168 ± 1271c	2.2 ± 0.7b	1.5 ± 0.6b	1.9 ± 0.6b
	Uncooked pasta	1014 ± 470c	256 ± 109bc	1270 ± 578c	1025 ± 314a	312 ± 197a	1338 ± 496a	2040 ± 763b	568 ± 301b	2608 ± 1054b	1.0 ± 0.2b	0.9 ± 0.3b	0.9 ± 0.2b
	Cooked pasta	742 ± 312d	202 ± 84c	943 ± 394d	885 ± 329b	296 ± 154a	1181 ± 468b	1627 ± 629c	497 ± 230c	2124 ± 852c	0.8 ± 0.1b	0.7 ± 0.2b	0.8 ± 0.1b
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lot [a]	C	744 ± 324c	208 ± 103b	952 ± 426c	459 ± 272c	145 ± 71b	603 ± 333b	1203 ± 218c	353 ± 57b	1555 ± 263c	3.4 ± 4.2a	2.7 ± 3.3a	3.0 ± 3.4a
	HA	874 ± 324b	231 ± 88b	1105 ± 409b	565 ± 320b	138 ± 53b	703 ± 365b	1439 ± 283b	370 ± 59b	1808 ± 327b	3.1 ± 3.6a	2.4 ± 2.6a	2.9 ± 3.3a
	W	2238 ± 1167a	546 ± 288a	2784 ± 1443a	1013 ± 379a	434 ± 139a	1447 ± 493a	3251 ± 863a	980 ± 206a	4231 ± 1050a	3.1 ± 2.8a	1.7 ± 2.0a	2.6 ± 2.3a
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.804	0.325	0.573
Product × Lot	*p*-value	<0.001	<0.001	<0.001	0.248	0.177	0.122	<0.001	<0.001	<0.001	0.522	0.637	0.289

Within each factor, means followed by different letters are significantly different, according to the REGW-F test (the significance level, *p*-value, is shown in the table). Data of FB concentrations are expressed as μg/kg on a dw basis ±standard deviation. The reported mycotoxin contamination values are the average of 9 replications (3 lots × 3 repetitions) of the different products, and the average of 12 replications (4 products × 3 repetitions) for the different lots.
Total free FBs (sum of free FB_1 and FB_2); total bound FBs (sum of bound FB_1 and FB_2); total FB_1 (sum of free FB_1 and bound FB_1), total FB_2 (sum of free FB_2 and bound FB_2); total FBs (sum of total FB_1 and FB_2).
[a] Maize lot: maize flour (<150 μm) of 3 hybrids (C, conventional; HA, high-amylose; W, waxy).

Table 5
Effect of the co-extrusion and pasta-making process on content of free and bound fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), and fumonisins $B_1 + B_2$ (FBs) for each maize lot, starting with maize flour (<150 μm) as the raw material.

Lot [a]	Product	Free		Bound		Total [b]		
		FB_1	FB_2	FB_1	FB_2	FB_1	FB_2	FBs
C	Maize flour	1249 ± 42a	372 ± 22a	147 ± 68c	60 ± 32b	1396 ± 33a	432 ± 10a	1828 ± 25a
	Snack	708 ± 62b	186 ± 24b	329 ± 81bc	108 ± 52b	1037 ± 75b	294 ± 46c	1330 ± 121b
	Uncooked pasta	568 ± 79bc	156 ± 10bc	804 ± 171a	201 ± 13a	1372 ± 221a	357±6b	1729 ± 225a
	Cooked pasta	452 ± 82c	117±7c	555 ± 42b	210±5a	1007 ± 90b	328 ± 10bc	1334 ± 99b
	p-value	<0.001	<0.001	<0.001	0.001	0.008	0.001	0.003
HA	Maize flour	1377 ± 76a	368 ± 28a	158 ± 31c	69 ± 28b	1535 ± 73ab	436 ± 21a	1971 ± 94ab
	Snack	619 ± 21c	158 ± 18c	410 ± 88b	134 ± 43 ab	1029 ± 68c	292 ± 29c	1320 ± 87c
	Uncooked pasta	862 ± 52b	218 ± 21b	875 ± 114a	167 ± 30a	1737 ± 163a	385 ± 29 ab	2122 ± 137a
	Cooked pasta	638 ± 80c	183 ± 26bc	815 ± 134a	184 ± 19a	1453 ± 74b	367 ± 24b	1820 ± 97b
	p-value	<0.001	<0.001	<0.001	0.009	<0.001	0.001	<0.001
W	Maize flour	4067 ± 195a	996 ± 158a	540 ± 31c	277 ± 112b	4607 ± 180a	1273 ± 119a	5880 ± 105a
	Snack	2137 ± 35b	489 ± 43b	829 ± 174b	397 ± 100ab	2966 ± 174b	886 ± 116b	3852 ± 232b
	Uncooked pasta	1614 ± 35c	395 ± 25b	1397 ± 196a	569 ± 71a	3010 ± 180b	964 ± 91b	3974 ± 240b
	Cooked pasta	1135 ± 42d	305±9b	1287±9a	493 ± 78ab	2421 ± 45c	798 ± 78b	3219 ± 105c
	p-value	<0.001	<0.001	<0.001	0.022	<0.001	0.002	<0.001

Within each lot, means followed by different letters are significantly different, according to the REGW-F test (the significance level, *p*-value, is shown in the table). Data of FB concentrations are expressed as μg/kg on a dw basis ±standard deviation. The reported fumonisin contamination values are the average of 3 replications.
[a] Maize lot: maize flour (<150 μm) of 3 hybrids (C, conventional; HA, high-amylose; W, waxy).
[b] Total FB_1 (sum of free FB_1 and bound FB_1), total FB_2 (sum of free FB_2 and bound FB_2); total FBs (sum of total FB_1 and FB_2).

total FBs is reported separately in Table 5 for each maize flour lot. The maize flour from the W lot shows a significantly higher content of total FBs than its food products, with significant reductions of 34%, 32%, and 42% for the snacks, uncooked pasta, and cooked pasta, respectively. This trend was not fully confirmed in the other lots (C and HA), in which the uncooked pasta never differed significantly from the maize flour. The bound FB_1 content was higher in all the products and for all the lots than in the respective flours. Indeed, the snacks recorded from 1.5 to 2.6 times higher bound FB_1 contents than the maize flour, the uncooked pasta recorded from 2.6 to 5.5 times higher values and the cooked pasta from 2.4 to 5.2 times higher ones, thus confirming the crucial role of the transformation process in 'masking' and binding FBs to the matrix.

For pasta products the contamination of total FBs, after hydrolysis, (in the 1334–3974 μg/kg range) was always clearly higher than the EU legal limit (sum of FB_1 and FB_2, 1000 μg/kg - European Commission Regulation (EC) No. 1126/2007) for all the pasta samples (Table 5). On the other hand, the contamination of free FBs (range: 570–821 μg/kg) was compliant with the EU legal limit, with the exception for the W (2008 μg/kg) and HA (1079 μg/kg) uncooked pasta and the W (1439 μg/kg) cooked pasta. The effect of processing on free FB_1 and FB_2 content was similar, while the bounding effect of the compared extrusion process led to more consistent and significant differences for FB_1 than FB_2. In this framework, as reported by Bolger et al. (2001) FB_1 is the most significant of the fumonisins in terms of toxicity and occurrence.

3.3. Free, bound, and total FB1, FB2, and FBs in the maize flour and break meal snacks

The co-extruded snacks on average showed significantly lower content of free and total FB_1, FB_2, and FBs than the raw materials (Table 6). However, as far as the content of the bound FB_1, FB_2, and FBs are concerned, the snacks exhibited a significantly higher contamination than the raw materials, thus confirming their increase during the technological co-extrusion process performed to obtain snacks. As a result, the free/bound ratio of FB_1, FB_2, and FBs significantly decreased in the snacks, in comparison to the raw materials, as shown in Table 6. Indeed, on average, the bound FBs in the raw materials and in the snacks accounted for 17% and 63% of the free FBs, respectively. Therefore, taking into account the free and total FBs, their contents on average decreased after the co-extrusion process by 49% and 25%, respectively, and this trend was closely linked to a significant increase in the bound forms, on average of 119%. Reductions in FB levels were mostly reported in the 30–90% range, and were often accompanied by HFB_1, by elevated levels of the glucose reaction product N-(1-deoxy-d-fructos-1-yl)-fumonisin B_1 (NDF-FB_1) and by the presence of the sugar-FB_1 adduct N-(carboxymethyl)-fumonisin B_1 (NCM-FB_1) (Yang et al., 2022). The most marked reductions in FBs were observed in extrudates processed at higher temperatures (160 °C) and in the presence of glucose (Ponce-Garcia et al., 2018). Moreover, Kamle et al. (2019) reported that the reduction of FBs in extruded products is related to the extrusion temperature, the screw speed, the moisture content, and to the extrusion time.

Overall, the maize flour from all the different hybrids (C, HA, and W) used to produce the snacks showed significantly higher contents of free, bound, and total FBs than those recorded in their respective break meal maize fractions (Table 6). Although the bound FBs were significantly higher in the W maize flour lot, the other maize lots did not differ significantly from each other. Interestingly, overall, the C maize break meal showed the highest ratio of free/bound FBs (Table 6). Moreover, because of the significant interactions between products and lots, the effect of the co-extrusion on the content of total FBs is reported separately in Table 7 for each maize hybrid and each particle size of the starting raw material. Although the flour showed a significantly higher content of total FBs than the snacks for almost all the lots, but not for the HA lots, the bound FB_1 content was higher (from +1.4 to +6.5 times) in the snacks from the flour from all the lots than in the respective raw materials (Table 7). The effect of extrusion in reducing the content of free FB_1 and FB_2 was similar and always significant. Furthermore, the increase of bound FB_2 in snack compared to raw material was never significant, while, with the exception of C break meal, was always significant for FB_1.

As observed for the pasta, the contamination of the free FBs in the snacks (range: 403–776 μg/kg) was always compliant with the EU legal limit (sum of FB_1 and FB_2, 800 μg/kg - European Commission Regulation (EC) No. 1126/2007), except for the snacks made with the C (893 μg/kg) and W (2626 μg/kg) maize flours. However, after hydrolysis, the contamination of the total FBs (in the 963–3852 μg/kg range) was significantly higher than the EU limit for almost all the snacks, but not for the C break meal ones (782 μg/kg).

3.4. Free/bound ratio of FBs in the maize products: the role of the process and raw material composition

The data reported so far in the literature on this topic and on the 'masking' mechanisms of FBs (Dall'Asta et al., 2008; Dall'Asta, Galaverna, et al., 2009; Falavigna, Cirlini, Galaverna, & Dall'Asta, 2012; Bryła, Roszko, Szymczyk, Jędrzejczak, & Obiedziński, 2016) only refer to maize products (maize pasta and/or snacks) purchased from the retail market, thus they are not comparable with each other because they were not derived from the same starting raw material and, consequently, the processes used for their production are not comparable either. The present study has compared different processes (pasta making and co-extrusion) and different maize lots with different amylose contents, in a full experimental factorial design approach, and this has allowed a direct comparison to be made of the role of the different components.

For all the hybrids, with different amylose contents, the free/bound ratio of FBs in the snacks produced from the break meal was on average 44% lower than those produced with the maize flours (Table 8), although the difference between maize flour and break meal within each

Table 6
Free, bound, total, and the ratio of the free/bound fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisins $B_1 + B_2$ (FBs) in the maize raw materials and in the snacks from different lots.

Factor	Source of variation		Free			Bound			Total			Free/Bound Ratio		
			FB_1	FB_2	FBs	FB_1	FB_2	FBs	FB_1	FB_2	FBs	FB_1	FB_2	FBs
Product		Raw material	1531 ± 1212a	385 ± 299a	1917 ± 1500a	204 ± 165b	120 ± 93b	323 ± 235b	1735 ± 1358a	505 ± 364a	2240 ± 1719a	9.7 ± 3.9a	4.2 ± 2.9a	6.2 ± 2.4a
		Snack	793 ± 635b	184 ± 148b	977 ± 782b	456 ± 205a	163 ± 119a	619 ± 310a	1249 ± 810b	347 ± 259b	1596 ± 1066b	1.7 ± 0.8b	1.2 ± 0.6b	1.5 ± 0.6b
		p-value	<0.001	<0.001	<0.001	<0.001	0.021	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lot [a]	Maize flour (<150 μm)	C	978 ± 300b	279 ± 104b	1257 ± 403b	238 ± 120b	84 ± 47b	322 ± 154b	1216 ± 204b	363 ± 82b	1579 ± 284b	6.0 ± 3.7a	4.7 ± 3.8a	5.2 ± 3.6a
		HA	998 ± 418b	263 ± 117b	1261 ± 535b	284 ± 150b	101 ± 49b	385 ± 195b	1282 ± 284b	364 ± 82b	1646 ± 366b	5.3 ± 4.2b	3.7 ± 3.2ab	4.8 ± 3.9a
		W	3102 ± 1064a	743 ± 296a	3844 ± 1335a	685 ± 194a	337 ± 116a	1021 ± 254a	3786 ± 913a	1079 ± 236a	4866 ± 1122a	5.1 ± 2.7b	2.8 ± 2.4abc	4.2 ± 2.3a
	Break meal (250–500 μm)	C	442 ± 128c	101 ± 38c	543 ± 164e	244 ±8b	95 ± 25b	338 ± 80b	685 ± 80c	196 ± 49c	881 ± 120c	2.1 ± 1.3b	1.2 ± 0.7bc	1.7 ± 0.8b
		HA	525 ± 185c	149 ± 79c	673 ± 263d	274 ± 193b	71 ± 30b	345 ± 218b	799 ± 60c	220 ± 63c	1018 ± 96c	3.9 ± 3.7b	2.6 ± 1.8abc	3.4 ± 3.0ab
		W	927 ± 363b	174 ± 52c	1102 ± 414c	255 ± 219b	160 ± 22b	416 ± 221b	1183 ± 171b	335 ± 53b	1517 ± 218b	11.7 ± 7.5a	1.1 ± 0.4c	3.9 ± 2.9a
		p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	0.004	<0.001
Product × Lot		*p*-value	<0.001	<0.001	<0.001	0.099	0.329	0.087	<0.001	<0.001	<0.001	0.005	0.076	0.006

Within each factor, means followed by different letters are significantly different, according to the REGW-F test (the significance level, *p*-value, is shown in the table). Data of FB concentrations are expressed as μg/kg on a dw basis ±standard deviation. The reported mycotoxin contamination values are the average of 30 replications (10 lots × 3 repetitions) for the different products, and the average of 6 replications (2 products × 3 repetitions) for the different lots.
Total free FBs (sum of free FB_1 and FB_2); total bound FBs (sum of bound FB_1 and FB_2); total FB_1 (sum of free FB_1 and bound FB_1), total FB_2 (sum of free FB_2 and bound FB_2); total FBs (sum of total FB_1 and FB_2).
[a] Maize lot: combination of 2 particle sizes (maize flour or break meal), and 3 hybrids (C, conventional; HA, high-amylose; W, waxy).

Table 7
Effect of the co-extrusion on content of free and bound fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), and fumonisins $B_1 + B_2$ (FBs) for maize from different lot (content of amylose and particle size).

Lot		Product	Free		Bound		Total [b]		
			FB_1	FB_2	FB_1	FB_2	FB_1	FB_2	FBs
C	Maize flour	Raw material	1249 ± 42	372 ± 22	147 ± 68	60 ± 32	1396 ± 33	432 ± 10	1828 ± 25
		Snack	708 ± 62	186 ± 24	329 ± 81	108 ± 52	1037 ± 75	294 ± 46	1330 ± 121
		p-value	<0.001	<0.001	0.041	0.246	0.002	<0.001	0.002
	Break meal	Raw material	547 ± 79	135 ± 3	199 ± 65	99 ± 39	746 ± 35	234 ± 40	981 ± 49
		Snack	337 ± 38	66 ± 6	288 ± 95	91 ± 5	625 ± 60	157 ± 5	782 ± 64
		p-value	0.014	<0.001	0.253	0.731	<0.001	0.028	0.013
HA	Maize flour	Raw material	1377 ± 76	368 ± 28	158 ± 31	69 ± 28	1535 ± 73	436 ± 21	1971 ± 94
		Snack	619 ± 21	158 ± 18	410 ± 88	134 ± 43	1029 ± 68	292 ± 29	1321 ± 87
		p-value	<0.001	<0.001	0.010	0.092	0.001	0.002	0.001
	Break meal	Raw material	691 ± 45	220 ± 9	109 ± 39	53 ± 8	800 ± 26	274 ± 17	1074 ± 35
		Snack	359 ± 27	77 ± 4	439 ± 103	89 ± 35	798 ± 92	166 ± 32	963 ± 58
		p-value	<0.001	<0.001	0.007	0.161	0.969	0.007	0.182
W	Maize flour	Raw material	4067 ± 195	996 ± 158	540 ± 31	277 ± 112	4607 ± 180	1273 ± 119	5880 ± 105
		Snack	2137 ± 35	489 ± 43	829 ± 174	397 ± 100	2966 ± 174	886 ± 116	3852 ± 232
		p-value	<0.001	0.005	0.048	0.239	<0.001	0.016	<0.001
	Break meal	Raw material	1258 ± 24	219 ± 19	68 ± 30	162 ± 31	1326 ± 54	381 ± 14	1707 ± 47
		Snack	597 ± 31	129 ± 20	442 ± 119	159 ± 15	1039 ± 92	288 ± 18	1327 ± 94
		p-value	<0.001	0.006	0.006	0.911	0.010	0.002	0.003

Within each lot, the significance level (*p*-value) is shown in the table.
Data of FB concentrations are expressed as μg/kg on a dw basis ±standard deviation. The reported fumonisin contamination values are the average of 3 replications.
[a] Maize lot: combination of 2 particle sizes (maize flour, <150 μm or break meal, 250–500 μm), and 3 hybrids (C, conventional; HA, high-amylose; W, waxy).
[b] Total FB_1 (sum of free FB_1 and bound FB_1), total FB_2 (sum of free FB_2 and bound FB_2); total FBs (sum of total FB_1 and FB_2).

hybrid was never significant for both FB_1 and FB_2 ratio. On average, the free/bound ratio in snack was 1.7 and 1.2 for FB_1 and FB_2, respectively, although the ratio between the compared fumonisin forms was significant only for waxy maize flour.

Several authors have reported the possibility of the formation of covalent bonds between the tricarboxylic moiety and hydroxyl groups of carbohydrates or the amino groups of amino acids of the food matrix upon heating or extrusion processes (Berthiller et al., 2013; Humpf & Voss, 2004; Seefelder, Knecht, & Humpf, 2003). However, another 'masking' phenomenon may occur as a result of a probable physical entrapment of the mycotoxins in the structure of macromolecular components, such as starch (Berthiller et al., 2013; Kim et al., 2003; Park et al., 2004). Taking into consideration the chemical composition of the raw materials considered in the present studied (Table S1), this decrease might mainly be related to a protein content in the C hybrid break meal that is approximately 50% higher than that present in maize flour. Moreover, starch, including the amylose/amylopectin ratio, and fats appeared to play marginal roles in the FB 'masking' mechanism. The presence of a higher protein content in the break meal could therefore have increased the amount of FBs that bound to the matrix, through protein binding, and consequently decreased the ratio. Isotope labeling assays have demonstrated that half of FB_1 may be covalently bound to the protein matrix in thermally processed maize foods via the tricarboxylic acid side chain (Freire & Sant'Ana, 2018). Seefelder et al. (2003) used two amino acid derivatives, that is, N-α-acetyl-L-lysine methyl ester and N-(tert-butoxycarbonyl)-L-cysteine methyl ester, as protein reaction models to react with FBs. These authors also verified the covalent binding of FBs to proteins.

Considering the free/bound ratio of FBs for the different maize products (snacks, uncooked pasta, and cooked pasta) made from maize flour (Table 9), it was possible to directly compare the role of the extrusion on the formation of bound FBs. The uncooked and cooked pasta overall presented an average 54% lower ratio than the snacks, which resulted in a higher capacity to bind FBs. The C and W uncooked pasta (−67% and −52% for the C and W uncooked pasta, respectively)

Table 8
The distribution ratio of free/bound Fumonisin B_1 (FB_1) and Fumonisin B_2 (FB_2) in the maize extruded snacks.

Lot[a]		Free/Bound ratio	
		FB_1	FB_2
maize flour	C	2.3 ± 0.5 ab	2.0 ± 0.3 abc
(<150 μm)	HA	1.6 ± 0.3 abc	1.3 ± 0.4 bc
	W	2.6 ± 0.5 a	1.3 ± 0.2 bc
break meal	C	1.3 ± 0.7 bc	0.7 ± 0.6 c
(250–500 μm)	HA	0.9 ± 0.4 c	1.0 ± 0.5 bc
	W	1.4 ± 0.5 abc	0.8 ± 0.3 c

Different letters indicate significant differences between maize lot and/or fumonisin form, according to the REGW-F test (*p*-value <0.01).
Data are expressed on a dw basis ±standard deviation. The reported fumonisin contamination values are the average of 3 replications.
[a] Maize lot: combination of 2 particle sizes (maize flour, <150 μm or break meal, 250–500 μm), and 3 hybrids (C, conventional; HA, high-amylose; W, waxy).

Table 9
Comparison of the free/bound FBs ratio in the different maize products (snacks, uncooked pasta, and cooked pasta) starting with maize flour (<150 μm) as the raw material.

Product	Lot[a]	Free/Bound ratio	
		FB_1	FB_2
Snack	C	2.3 ± 0.6 ab	2.0 ± 0.7 abc
	HA	1.6 ± 0.4 bcd	1.3 ± 0.5 cd
	W	2.6 ± 0.5 a	1.3 ± 0.3 cd
Uncooked pasta	C	0.7 ± 0.1 d	0.8 ± 0.1 d
	HA	1.0 ± 0.1 cd	1.3 ± 0.3 bcd
	W	1.2 ± 0.2 cd	0.7 ± 0.1 d
Cooked pasta	C	0.8 ± 0.2 d	0.6 ± 0.1 d
	HA	0.8 ± 0.2 d	1.0 ± 0.2 cd
	W	0.9 ± 0.1 d	0.6 ± 0.1 d

Different letters indicate significant differences between maize lot and/or fumonisin form, according to the REGW-F test (*p*-value <0.001).
Data are expressed on a dw basis ±standard deviation. The reported fumonisin contamination values are the average of 3 replications.
[a] Maize lot: C, conventional hybrid; HA, high-amylose hybrid; W, waxy hybrid.

and the cooked pasta (−66% and −63% for the C and W uncooked pasta, respectively) presented significant lower ratios considering the sum of FB_1 and FB_2 than that present in the respective snacks.

However, although the ratio decreased with respect to the corresponding snacks for the HA uncooked pasta (−31%) and the cooked pasta (−44%), this decrease was not significant. Moreover, no significant differences were found between the free/bound ratio of FBs for the uncooked pasta and that of the cooked pasta. Within each maize lot and products combination, the free/bound ration was slightly higher for FB_1 compared to FB_2, although a significant difference was noticed only for snack made with waxy flour.

During extrusion, a step that is common to both processes, the product is forced through metal tubes by rotating screws, and is subjected to high temperatures, high pressures, and severe shear. Extrusion usually causes decreases in the free concentrations of FBs and favors a matrix-association and the formation of bound forms (Humpf & Voss, 2004).

The lower free/bound ratio of FBs observed in pasta than in snacks could be due to a longer duration, as well as to higher temperatures, pressures, and screw speeds of the pasta making process than the snack production process. Moreover, a pre-gelatinization step was applied before extrusion in the pasta-making process, and this may have resulted in a higher FB binding to the matrix. Further studies are needed to understand whether a pre-gelatinization process affects the formation of bound FBs more than an extrusion process, or vice versa. As reported by Humpf and Voss (2004) and by Kamle et al. (2019), the degree by which FB concentrations are reduced during processing, which favors the formation of bound FBs, not only depends on the temperature, pressure, and processing time, but also on the moisture content. Indeed, they recorded a decrease in the free FB_1 levels as the moisture content increased, as observed in the present study, where going from 100 g/kg moisture content for snack extrusion to 300 g/kg moisture content for pasta-making, the free FB levels decreased. It will therefore be necessary to investigate the role of the moisture content, as well as of the other process parameters mentioned so far, in the 'masking' phenomenon.

4. Conclusions

In conclusion, for most of the products considered in the present study, if the contribution of bound FBs are considered, the EU legal limit for the total FBs in maize-based products for human consumption (800 μg/kg or 1000 μg/kg - European Commission Regulation (EC) No. 1126/2007) was exceeded to a great extent, thereby potentially exposing those people who suffer from celiac disease or allergies, whose diets are mainly based on such products (gluten-free maize products), to higher risks than those consumers who follow normal diets.

Many factors can influence the production and accumulation of FBs and their modified forms, and they can cause changes in their ratio (i.e., the climate, agronomic factors, maize hybrid composition, storage conditions, and further postharvest technological processing), starting from the plant, during the transformation process, and up to the finished product. For this reason, further studies are needed to experimentally investigate these multiple interactions, that is, to study the entire food-chain in depth by comparing different transformation processes that start from the same raw material. This study has highlighted a greater involvement of proteins than starch and fats in the formation of FB bound forms and has clearly indicated the crucial role of the technological extrusion process in influencing the 'masking' phenomenon.

CRediT authorship contribution statement

Valentina Scarpino: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Andrea Bresciani:** Writing – review & editing, Methodology, Investigation. **Massimo Blandino:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Massimo Blandino reports financial support was provided by Piedmont Region. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.115977.

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