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Bioaccessibility of Enniatins A, A_1, B, and B_1 in Different Commercial Breakfast Cereals, Cookies, and Breads of Spain

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ABSTRACT: Fusarotoxins enniatins (ENs) can represent a potential risk as natural contaminants of cereal commodities. However, only their bioaccessible fraction can exert a toxicity. The purpose of this study was to determine the ENs A, A_1, B, and B_1 bioaccessibility added in 1.5 and 3.0 μmol/g concentrations in breakfast cereals, cookies, and breads using a simulated in vitro gastrointestinal extraction model. Bioaccessibility values ranged between 40.4 ± 1.9 and 79.9 ± 2.8%. The lower values were 50.1, 40.4, 43.9, and 46.3% in wheat bran with fibers, and the higher values were 79.9, 64.2, 69.8, and 73.6% in white loaf bread for the ENs A, A_1, B, and B_1, respectively. Food composition, compounds structure, and presence of natural adsorbing materials can influence the ENs bioaccessibility. Application of a simulated in vitro gastrointestinal method is a good procedure to assess oral ENs bioaccessibility in cookies, breakfast cereals, and bread.

KEYWORDS: *enniatins, bioaccessibility, in vitro gastrointestinal model, HPLC*

INTRODUCTION

The Food and Agriculture Organization (FAO) estimates that as much as 25% of the world's animal feedstuffs is contaminated by some extent by mycotoxins.[1] Any step of the food production chain is susceptible to mold and mycotoxins contamination: before harvesting, between harvesting and drying, and during storage. Furthermore, they are persistent in the final products.[2] The co-occurrence of mycotoxins in a food matrix is also common.[3] The most common pathogen of maize, grain, and small grain in temperate regions of the world is *Fusarium* spp., whose strains may produce cyclic hexadepsipeptidic secondary metabolites such as enniatins (ENs). ENs are composed of three alternating D-α-hydroxyisovaleryl and three *N*-methyl-L-amino acid residues. They possess antimicrobial, insecticidal, phytotoxic, and cytotoxic properties[4] and inhibit cholesterol acyltransferase.[5] The large array of biological activities can be related to their ionophoric properties based on the ability to incorporate into cell membranes forming cation-selective pores with high affinity for K^+, Mg^{2+}, Ca^{2+}, and Na^+.[6] Their occurrences have been amply demonstrated,[7−11] but the potential risk related to the ingestion of contaminated commodities is still not clear. The most important exposure routes for human and animals for ENs are via oral ingestion. It is therefore important to be able to assess the amount of ENs that is potentially available for absorption in the stomach and/or intestines, that is, bioaccessible, or to be excreted. However, the total amount of ENs ingested (intake) does not always reflect the bioaccessible amount of them. The bioaccessibility describes the fraction of a contaminant, that is, ENs, that is mobilized from food matrices during gastrointestinal digestion and theoretically subsequently available to intestinal absorption.[12] So, to study the oral bioaccessibility as part of an overall estimation in assessing the chemical risk coming from food-borne ENs is an important issue.

Because of this, during the past decade, there has been an increasing interest in the use of in vitro methodologies, such as in vitro digestion models that simulate, in a simplified manner, the human digestion process in the mouth, stomach, and small intestine, to enable bioaccessibility investigations of contaminants from their food matrix during transit in the gastrointestinal tract.[12,13] These in vitro models attempt to recreate the aspects of human gastrointestinal physiology, such as chemical composition of digestive fluids, pH, and residence time periods typical for each compartment.[12] Moreover, these in vitro models are simple, rapid, low-cost, and without ethical implications, although a number of comparative studies have suggested that bioaccessibility results are largely dependent on the specific in vitro conditions used, including differences in solid solution ration, the method of mixing, the pH values of the gastric and intestinal juices and their compositions, food contaminants, and food matrices.[14]

Previous studies focused on determining the bioaccessible part of several mycotoxins after simulated human gastrointestinal extraction, confirming the usefulness of these in vitro methodologies to predict intestinal absorption of mycotoxins.[12,13,15] Versanvoort et al.[12] used a simplified digestion process of three steps, where physiologically based conditions of the mouth, stomach, and small intestine were applied.

The aims of the present study were (1) to apply an in vitro gastrointestinal model as it is related to the human digestive system; (2) to determine the bioaccessibility of ENs A, A_1, B, and B_1 from artificially spiked grain-based products (specifically breakfast cereals, cookies, and breads) by using the in vitro gastrointestinal model; and (3) to finally appreciate the role of oral bioaccessibility in assessing EN risks to human.

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MATERIALS AND METHODS

Materials and Reagents. Methanol, acetonitrile, and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT) ultrasonic bath. Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin, and bile salts were obtained from Sigma-Aldrich (Madrid, Spain).

The ENs (A, A_1, B, and B_1) utilized in this study were produced and purified in our laboratory following the method of Meca et al.[16] for the production of the fusaproliferin. All ENs were >97% purity. They were stored at 4 °C in methanol, protected from light.

Samples. Fourteen samples of grain-based products were collected from Spanish food markets and stored at 4 °C until analysis. Some types of cookies, breads, and breakfast cereals, free from contamination, as determined previously, have been chosen for this study:

- Four types of cookies: cookie with chocolate, cookie with fibers, cookie with oat, and cookie without fibers
- Five types of bread: integral loaf bread, integral loaf bread without sugar, milk bread, multicereals loaf bread, and white loaf bread
- Five types of breakfast cereals: corn flakes, muesli with fruits, wheat bran with fibers, wheat with chocolate, and wheat with honey

Samples (3 g) were spiked with EN A, A_1, B, and B_1 at final concentrations of 1.5 and 3.0 μmol/g and left in darkness overnight to allow methanol evaporation. Detailed types and the nutritional compositions of the above-mentioned samples are shown in Table 1.

Table 1. Nutrients Nutritional Properties of the Analyzed Samples Referred to 100 g of Product (as Reported in Nutritional Labels)

sample	fiber (g)	fat (g)	carbohydrates (g)	protein (g)
cookie with chocolate	5.6	23.0	57.7	5.4
cookie with fibers	3.0	11.5	68.2	9.0
cookie with oats	4.3	19.2	60.3	7.6
cookie without fibers	5.1	12.8	62.5	9.6
corn flakes	3.0	0.7	74.5	8.0
integral loaf bread	5.0	3.5	36.4	10.0
integral loaf bread without sugars	5.0	3.0	37.3	11.0
milk bread	2.0	15.0	22.7	10.0
multicereals loaf bread	6.0	5.5	36.4	11.0
muesli with fruits	6.4	16.3	57.8	8.1
wheat bran with fibers	24.0	3.5	45.5	13.5
wheat with chocolate	6.0	3.0	68.2	9.0
wheat with honey	5.3	1.7	67.3	8.6
white loaf bread	3.0	3.0	41.8	9.0

In Vitro Gastrointestinal Model. An in vitro gastrointestinal procedure was adapted from the method developed previously by Gil-Izquierdo et al.,[17] with slight modifications. The method consists of three sequential steps: first an initial saliva addition to simulate the mouth compartment. The mouth is the point where the process of mechanical grinding of foodstuffs takes place at a pH of 6.5. The pH was adjusted with 0.1 N HCl. In this step, 3 g of sample spiked with ENs (A, A_1, B, and B_1) at 1.5 and 3.0 μmol/g was mixed with 6 mL of artificial saliva (composed of 89.6 g/L KCl, 20.0 g/L KSCN, 88.8 g/L NaH_2PO_4, 57.0 g/L $NaSO_4$, 175.3 g/L NaCl, 84.7 g/L $NaHCO_3$, 25.0 g/L urea, and 290.0 mg of α-amilase). The mixture was put in a plastic bag, containing 40 mL of water, and was homogenized by a Stomacher IUL Instruments (Barcelona, Spain) for 30 s. Larger components were broken down into smaller fragments, thereby increasing the surface area of food particles for swallowing and digestion. The second step consisted of pepsin/HCl digestion to simulate the hydrochloric acid environment of the stomach (pH 1−5). The presence of pepsine acts to breakdown protein, thereby aiding dissolution of the foodstuffs. For this purpose, 0.5 g of pepsin (14,800 U) prepared in 0.1 N HCl was added to the mixture, and the pH was adjusted to 2.0. The mixture was incubated at 37 °C in an orbital shaker (Infors AG CH-4103, Bottmingen, Switzerland) at 250 rpm for 2 h. The last step was the digestion in the small intestine(s) by intestinal juices composed of enzymes (trypsin, pancreatin, and amylase), bile salts, and bicarbonate. The breakdown of food in the small intestine(s) means that the components are more amenable to absorption. The enzymes used in intestinal juice were pancreatin (8 mg/mL) and bile salts (50 mg/mL) in a 1:1 (v/v) solution ratio; the pH of intestinal juice was adjusted to 6.5 with 0.5 N $NaHCO_3$ (0.5 N); the mixture was incubated for 2 h at 37 °C in an orbital shaker (250 rpm). Immediately, aliquots of 30 mL of the mixture were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm and 4 °C for 1 h, and the ENs present in the saliva/pepsin/HCl and pancreatin−bile digestions were extracted and analyzed by high-performance liquid chromatography−diode array detection (HPLC-DAD), as described by Meca et al.[18]

Analysis of ENs. The extraction of ENs (A, A_1, B, and B_1) contained in gastroduodenal fluids were carried out as Meca et al.[19] Briefly, 5 mL of each mixture obtained as previously described was put in a 20 mL test tube and extracted three times with 5 mL of ethyl acetate utilizing a vortex VWR international (Barcelona, Spain) for 1 min and centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm and 4 °C for 10 min. After the organic phase was completely evaporated in a rotary evaporator (Buchi, Switzerland), the residues were dissolved in 1 mL of methanol and analyzed by LC-DAD. All samples were filtered through a 0.22 μm syringe filter Phenomenex prior to injection (20 μL) into the column.

LC-10AD pumps and a DAD detector Shimadzu (Japan) were used to perform HPLC analysis of ENs. LC separation was carried out on a Gemini (150 mm × 4.6 mm, 5 μm) analytical column Phenomenex (Madrid, Spain). The analytical separation was performed using gradient elution with water as mobile phase A and acetonitrile as mobile phase B. After an isocratic step of 70% B for 5 min, the gradient was linearly modified to 90% B in 10 min. After 1 min, the mobile phase was taken back to the starting conditions in 4 min. The flow rate was maintained at 1.0 mL/min. ENs were detected at 205 nm. EN identification was performed by comparing retention times and UV spectra of purified samples to pure standards. A further confirmation was performed by coinjecting pure standards together with each sample. Quantification of ENs was carried out by comparing peak areas of investigated samples to the calibration curve of the standards. Recovery (%) studies in intestinal fluid were performed during routine analysis by spiking the samples with standard solutions of each ENs at 1.5 and 3.0 μmol/g concentrations.

Method Validation. The analytical method was validated according to the European Directive 2002/26/EC for methods of analysis of mycotoxins in foodstuffs.[20] Recovery experiments were carried out on fortified intestinal fluid (free from contamination) (n = 5) by spiking ENs A, A_1, B, and B_1 at a level ranging from 0.3 to 50 μg/g. Mean recoveries (%) were as follows: 88.6 ± 2.4, 84.2 ± 4.3, 86.6 ± 2.7, and 89.5 ± 3.1% for EN A, A_1, B, and B_1, respectively. Interday variation values (through five different days) ranged from 1.8 to 3.1%. Intraday variation values were in the range of 6.4−10.1%. These values did not exceed 15%, which is the maximum variation for certification exercises for several mycotoxins. The detection limits (LOD) and the limit of quantification (LOQ) values were calculated according to s/n = 3 and s/n = 10, respectively. The LODs obtained for EN A, A_1, B, and B_1 were 215, 140, 145, and 165 μg/kg, respectively, whereas the LOQs were 600 μg/kg for EN A, 400 μg/kg for ENs A_1 and B, and 500 μg/kg for EN B_1.

Statistical Analysis. Statistical analysis of data was carried out using the PSAW Statistic 19.0 (SPSS, Chicago, IL) statistical software package. Data were expressed as means ± SDs of three independent experiments.

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Table 2. Bioaccessibility (%) of the ENs A, A_1, B, and B_1, Spiked with 1.5 and 3.0 μM Concentrations in Different Breakfast Cereals, Cookies, and Breads Digested by an in Vitro-Simulated Gastrointestinal Model[a]

	bioaccesibility (%)							
	EN A (μM)		EN A_1 (μM)		EN B (μM)		EN B_1 (μM)	
sample	1.5	3.0	1.5	3.0	1.5	3.0	1.5	3.0
cookie with chocolate	59.2 ± 2.7 a,1	55.3 ± 2.7 a,1	47.8 ± 2.9 b,1	44.6 ± 2.4 b,1	52.0 ± 2.9 b,1	48.5 ± 2.5 b,1	54.7 ± 2.6 a,1	51.1 ± 2.5 a,b,1
cookie with fibbers	64.4 ± 3.1 a,2	66.6 ± 2.2 a,2	52.0 ± 2.1 b,1	51.9 ± 2.8 b,2	56.5 ± 2.8 c,1,2	56.4 ± 2.9 c,2	59.5 ± 2.8 c,1	59.4 ± 2.7 c,2
cookie with oats	55.2 ± 3.5 a,1,5	52.2 ± 2.6 a,1	44.6 ± 3.3 b,2	46.8 ± 3.1 b,1,2	48.4 ± 3.1 a,b,1	51.1 ± 2.2 a,b,1,3	51.0 ± 3.3 a,b,1	53.8 ± 3.3 a,1,2
cookie without fibers	72.0 ± 3.3 a,3	70.7 ± 2.5 a,2	58.2 ± 3.1 b,3	57.1 ± 2.4 b,3	63.2 ± 2.9 c,2	62.1 ± 2.6 b,c,4	66.6 ± 3.1 a,c,2	65.4 ± 3.1 c,3
corn flakes	72.1 ± 3.4 a,3	70.7 ± 3.3 a,2,3	58.2 ± 2.2 b,3	57.1 ± 3.1 b,3	63.3 ± 2.1 c,2	62.0 ± 3.2 b,c,4	66.6 ± 2.6 a,c,2	65.4 ± 3.4 c,3
integral loaf bread	72.2 ± 2.3 a,3	71.6 ± 2.9 a,2,3	58.3 ± 2.2 b,3	57.5 ± 3.4 b,3	63.4 ± 3.2 c,2	62.5 ± 2.1 c,4	66.7 ± 3.5 c,2	65.8 ± 3.4 c,3
integral loaf bread without sugars	72.2 ± 2.1 a,3	71.8 ± 3.3 a,2,3	58.3 ± 2.6 b,3	58.0 ± 2.4 b,3	63.3 ± 3.3 c,2	63.0 ± 2.7 c,4	66.7 ± 3.7 c,2	66.4 ± 3.1 c,3,4
milk bread	62.1 ± 3.1 a,2	57.6 ± 2.1 b,1	50.6 ± 2.3 c,1	46.1 ± 2.1 d,1,2	55.0 ± 2.2 b,1	50.2 ± 3.3 c,d,1	57.9 ± 2.1 b,1	52.8 ± 3.2 b,c,d,1
multicereals loaf bread	75.0 ± 2.8 a,3,4	70.0 ± 3.1 b,2	60.3 ± 2.7 c,3	56.9 ± 2.1 c,3	65.1 ± 3.5 d,2	61.9 ± 2.4 c,4	69.1 ± 2.8 b,2,3	65.2 ± 2.7 b,c,d,3
muesli with fruits	64.2 ± 2.6 a,2	66.6 ± 2.2 a,2	62.9 ± 1.7 a,3	62.1 ± 2.5 a,4	68.3 ± 3.3 a,2	67.6 ± 3.2 a,4	72.0 ± 3.2 a,2,3	71.1 ± 2.2 a,3
wheat bran with fibers	50.1 ± 3.1 a,5	52.2 ± 3.1 a,1	40.4 ± 1.9 b,2	47.0 ± 2.7 a,1	43.9 ± 3.4 a,b,3	51.1 ± 3.4 a,1	46.3 ± 3.1 a,4	53.8 ± 3.1 a,1,2
wheat with chocolate	70.2 ± 2.4 a,3	67.1 ± 1.9 a,2	62.0 ± 1.8 b,3	57.2 ± 2.2 c,3	67.4 ± 3.1 a,2	62.1 ± 2.1 b,4	71.0 ± 3.3 a,2,3	65.5 ± 3.2 a,b,3
wheat with honey	76.0 ± 2.1 a,3,4	70.0 ± 3.8 b,2,3	61.3 ± 2.4 c,3	56.3 ± 3.3 d,3	66.6 ± 2.7 b,2	61.5 ± 2.3 c,4	70.2 ± 2.4 b,2,3	64.8 ± 2.2 b,3
white loaf bread	79.9 ± 2.8 a,4	76.9 ± 3.4 a,3	64.2 ± 2.4 b,3	58.1 ± 2.2 c,3	69.8 ± 2.9 d,2	64.1 ± 3.1 b,3,4	73.6 ± 2.2 d,3	67.5 ± 3.3 b,d,3,4
mean value	67.5 ± 2.7 a	65.6 ± 2.8 a	55.6 ± 6.2 b	54.0 ± 2.7 b,c	60.4 ± 2.6 b	58.9 ± 2.9 b	67.3 ± 2.7 a	62.0 ± 2.5 b

[a]Values are expressed as means ± SDs (n = 4). The significance of the differences between each EN and each food sample was assayed by one-way ANOVA followed by Tukey's test for multiple comparisons. Within a line, values with different letters (a−d) are significantly different ($p \leq 0.05$). Within a column, values with different numbers (1−5) are significantly different ($p \leq 0.05$). Control values (%) obtained from the experiments of the fortified intestinal fluid: EN A (88.6 ± 2.4%), EN A_1 (84.2 ± 4.3%), EN B (86.6 ± 2.7%), and EN B_1 (89.5 ± 3.1%). All ENs extracted from food samples were statistically different ($p \leq 0.05$) from the respective control.

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The statistical analysis of the results was performed by Student's *t* test for paired samples. Differences between mycotoxins were analyzed statistically with analysis of variance (ANOVA) followed by the Tukey's HDS posthoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

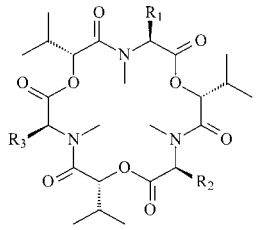
Results obtained after the application of the in vitro gastrointestinal method for determining ENs bioaccessibility in different breakfast cereals, cookies, and breads are shown in Table 2. Bioaccessibility values were from 50.1 ± 3.1 to 79.9 ± 2.8% for EN A, from 40.4 ± 1.9 to 64.2 ± 2.4% for EN A_1, from 43.9 ± 3.4 to 69.8 ± 2.9% for EN B, and from 46.3 ± 3.1 to 73.6 ± 2.2% for EN B_1. As shown in Table 2, the bioaccessibility of ENs was statistically significant ($p < 0.05$) from their respective controls.

Concerning EN A, the mean bioaccessibility value added at 1.5 and 3.0 μmol/g concentrations in the commercial samples was similar, that is, 67.5 ± 2.7 and 65.7 ± 2.8%, respectively (Table 2). Regarding EN A_1 spiked at 1.5 and 3.0 μmol/g, the mean bioaccessibility obtained was 55.7 ± 2.2 and 54.1 ± 2.7%, respectively (Table 2). As can be observed in Table 2, the ENs of the B group, the mean bioaccessibility values obtained for the EN B were also comparable between 1.5 μmol/g of EN B added (60.4 ± 2.6%) and 3.0 μmol/g of EN B added (58.9 ± 2.9%). However, when samples were spiked with EN B_1 at 1.5 and 3.0 μmol/g, the mean bioaccessibility value that resulted was significantly different. These values were 67.3 ± 2.7 (1.5 μmol/g of EN B_1) and 62.0 ± 2.5% (3.0 μmol/g of EN B_1). The order of mean values obtained when mycotoxins had been spiked at 1.5 μmol/g was as follows: 67.5 ± 2.7% (EN A) = 67.3 ± 2.7% (EN B_1) > 60.4 ± 2.6% (EN B) > 55.6 ± 2.2% (EN A_1). When mycotoxins had been spiked at 3.0 μmol/g, the order of mean values obtained was 65.6 ± 2.8% (EN A) = 62.0 ± 2.5% (EN B_1) = 58.9 ± 2.9% (EN B) > 54.0 ± 2.7% (EN A_1).

The wheat bran with fibers was the type of sample that showed the minimum bioaccessibility values for all of the ENs tested, ranging from 40.4 ± 1.9 for EN A_1 to 50.1 ± 3.1% for EN A (Table 2). In the same way, the wheat loaf bread showed high bioaccessibility values for all ENs tested (Table 2). For this type of sample, the higher values were observed for EN A.

Results show that not the total amount of mycotoxins ingested is available to intestinal absorption. The reduction in recoveries could be related to the digestion process and the use of different pH values that can reduce mycotoxins levels as stated for aflatoxin.[20] Moreover, according to Versanvoort et al.,[12] it is possible that bioaccessibility may be underestimated because of a compound saturation that can occur in the chyme, a situation not possible in vivo where a compound, when it is released from the food matrix, is transported across the intestinal epithelium into the body, keeping the compound concentration low in the chyme.

Considering the mean recoveries (%) of ENs tested (Table 2) and the recoveries (%) obtained from method validation (section 2.5), it is possible to observe that with the in vitro-simulated gastrointestinal extraction method, the initial bioactive EN concentrations present in the 14 sample analysis have been reduced statistically significant ($F_{3,92} = 13.98$, $p \leq 0.001$, Table 2) of 35% for EN A_1, followed by EN B_1 with a reduction of 25−31%, EN B of 31%, and EN A of 25% ($p < 0.05$) as compared to the extraction of ENs in the control. Differences in bioaccessibility (%) can be related to the structure of ENs. ENs are compounds with a cyclic aminoacidic structure, differentiated by the presence in the lateral chain of methyl, ethyl, propyl, and butyl groups (Figure 1). The presence of these groups can be responsible for the difference in the absorption of these compounds.[22]



Enniatin	R1	R2	R3
A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
A_1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
B1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$

Figure 1. ENs chemical structures.

It has been reported that mycotoxins bioaccessibility could depend on several factors, such as chemical structure, food composition, pH used in the in vitro digestion process, and food matrices.[21,23] Differences in bioaccessibility (%) shown in Table 2 also could be explained by the food composition of samples. Previous studies had demonstrated that the bioaccessibility of nutrients, as well as toxic compounds, can be affected by the food matrix.[24,25] In this case, the amount and type of fibers (Table 1) could be responsible as natural absorbing materials. In cereal-based foods, there are normally some natural compounds, such as dietary fibers, that could combine some bioactive compounds as mycotoxins or polyphenols, reducing their percent of bioaccessibility.[26,27] In Table 1 is shown fiber, fat, carbohydrates, and protein compositions of each product. The sample white loaf bread, where higher bioaccessibility values were obtained (Table 2), is one of the lowest fiber contents (3 g/100 g of product), whereas wheat bran with fibers, where the lower bioaccessibility values were observed, is the highest fiber contents (24 g/100 g of product) sample. As can be observed, the bioaccesibility from wheat bran with fibers was statistically significantly ($F_{3,92} = 14.30$, $p \leq 0.001$, Table 2) for ENs A, A_1, B, and B_1 with respect to those in the white loaf bread. For wheat bran with fibers, the bioaccessibility of all ENs spiked at 1.5 μmol/g concentration is 1.6-fold higher than those obtained in white loaf bread. In the case of mycotoxins, the inclusion of dietary fibers has been demonstrated to protect against toxicoses resulting from numerous xenobiotic compounds and can be applied in food and feed as a cost-effective method to detoxify them from mycotoxins.[28,29]

Nevertheless, the bioaccessibility of mycotoxins can be affected by interactions with other food components. Mycotoxins can bind food matrix components, mainly proteins and lipids, and be released by in vitro digestion as previously determined for other mycotoxins.[30] As can be observed in Table 2, for all ENs at both concentrations tested, low recovery values can be found in cookies with chocolate and cookies with oats that are both nutritionally rich in lipid components with the highest percentages of fat, with 23.0 g/100 g of sample and 19.2 g/100

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g of sample, respectively. When comparing the sample with a lower proportion of fat (corn flakes) to the samples with the highest (cookies with chocolate or with oats), the bioaccesibility for those samples with high fat content was statistically significantly ($F_{3,22}$ = 3.92, $p \leq 0.01$) for ENs A, A_1, B, and B_1 with respect to those with a low fat content. The bioaccessibility in corn flakes spiked at 1.5 μmol/g concentration of all ENs was 1.3-fold higher than those obtained in cookies with chocolate or with oats. It could be assumed that ENs interacting with the fat components of food are not released totally since the in vitro gastrointestinal digestion characteristics (such as the time period that food may spend in each step and the chemical composition of saliva, gastric juice, duodenal juice, and bile juice) are kept constant for all samples analyzed, even if the fat content is not the same.[31] Moreover, considering their physical−chemical properties, it could be possible to hypothesize that ENs are retained in lipid/bile micelles.[32]

The same ENs bioaccessibility was evaluated by Meca et al.[19] in artificially contaminated (1.5 and 3.0 μmol/g) wheat crispy bread after applying an in vitro-simulated gastric and duodenal digestion. The amount (%) of all mycotoxins tested contained ranged from 69.0 ± 2.1 to 91 ± 1.1% in gastric fluid and from 68.6 ± 2.9 to 87.3 ± 2.9% in duodenal fluid, considering both spiking concentrations.

ENs bioaccessibility values obtained from our study were slightly lower to those obtained by Meca et al.[19] Moreover, in our study, only ENs recovered in gastrointestinal fluids obtained after simulated digestion have been taken into account since in vivo food digestion and absorption mainly take place in the small intestine.[33] However, as obtained in our study, EN A, spiked at 3.0 μmol/g, was the most bioaccessible mycotoxins as in the gastric fluid (91.0 ± 1.2%), as in duodenal fluid (87.3 ± 2.9%). EN A_1, considering both spiking concentrations, was the lowest bioaccessible mycotoxins concerning the gastric fluid (69.0 ± 2.1 and 73.0 ± 2.3% for 1.5 and 3.0 μmol/g spiking, respectively), whereas concerning duodenal fluid, the lowest values were obtained for EN B spiked at 1.5 μmol/g (68.6 ± 2.9%) and EN A_1 spiked at 3.0 μmol/g (70.0 ± 1.7%).

Concerning other *Fusarium* toxins, it was shown that higher values of 92.6 ± 1.2 and 90 ± 1.3% of cyclic hexadepsipeptidic beauvericin (BEA) intake through artificially contaminated wheat (5 and 25 mg/L, respectively) were released from the food matrix to the bioaccessible fraction by the same in vitro digestion method as used in our study.[34]

The bioaccessibility of the trichothecenes deoxynivalenol (DON) was determined from dried pasta samples applying an in vitro digestion model both for adults and for children. The children's digestion model was basically the same as that of adults with slight modifications (the pH of the stomach was 3.0, the quantity of pepsin used for the gastric digestion was 0.02 g, and the amounts of pancreatin and bile salts were 0.0005 and 0.03 g, respectively). Referring to values obtained from our study, lower bioaccessibility values were obtained since the DON percentages in the gastric fluid ranged from 2.12 to 41.5%, while after the duodenal process, they ranged from 1.1 to 24.1%.[35]

Avantaggiato et al.[13,15] studied the intestinal absorption of zearalenone (ZEA), Nivalenol (NIV), and DON using an in vitro gastrointestinal model that simulates the metabolic processes of the gastrointestinal tract of healthy pigs. This model avoided the use of animals simulating in vivo experiments by its multicompartimental dynamic computer-controlled system. Approximately 32% of ZEA intake through artificially contaminated wheat (4.1 mg/kg) was released from the food matrix to the bioaccessible fraction during 6 h of digestion, and it was rapidly absorbed at the intestinal level. The intestinal absorptions recorded using the same model were 51 and 21% for DON and NIV ingested through spiked wheat samples, respectively. Later intestinal absorption of 105% for FB_1, 89% for FB_2, 87% for OTA, 74% for DON, 44% for AFB_1, and 25% for ZEA were determined by the same laboratory model.[36]

Similarly, Versanvoort et al.[12] demostrated the bioaccessibility obtained for AFB_1 (94%) and OTA (100%) from peanuts and buckwheat. They evidenced that these mycotoxins were released from the food matrix during the simulated in vitro model toward the intestinal fluid. [12] However, these results were partly in contrast with Kabak et al.,[37] who studied the bioaccessibility by the in vitro digestion model of AFB_1 and OTA from different food products. They found similar bioaccessibility values for AFB_1 (90%), but different values for OTA (30%).

The results obtained from this study and in the literature concerning in vitro digestion methods are unlikely comparable. Differences in the types of in vitro methods, operating procedures, pH used, mycotoxins structures, and food compositions could contribute to differences in bioaccessibility.

The last factor aforementioned can affect the bioaccessibility of ENs since they could build up complexes with food components.[38−40] However, evidence of a strong correlation between in vitro bioaccessibility and in vivo bioavailability data has been observed previously for different mycotoxins,[12] although no in vivo data are still available about ENs bioaccessibility. Therefore, for this purpose, more quantitative data are required. Further research should be performed to ensure that in vitro data are in agreement with in vivo methods for ENs present in food samples. In this way, bioaccessibility data obtained by the in vitro models proposed in this study can be incorporated to the whole data related to the cytotoxicity of mycotoxins in the literature and contribute to the risk assessment for *Fusarium* mycotoxins present in food and feed ENs A, A_1, B, and B_1.

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Notes

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