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Comparison of different cooking process for the emerging mycotoxins reduction in fresh pasta

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Abstract: Mycotoxins are toxic metabolites from filamentous fungi like *Aspergillus spp., Penicillium spp.,* and *Fusarium spp.* There are emerging mycotoxins (enniatin A, A_1, B, B_1 and beauvericin) which have been discovered recently. The present study evaluates the emerging mycotoxins in fresh pasta and how different culinary treatments affect mycotoxins concentration. The analysis of 19 commercialized samples with an Ultra-Turrax extraction in acetonitrile:methanol and High Performance Liquid Chromatography tandem mass spectrometry of triple quadrupole (HPLC-MS/MS-QqQ) detection. Results show that the 84% of samples were contaminated with at least one analyzed mycotoxin. The most abundant mycotoxins were ENB and ENB_1 with main concentration of 7.03 and 4.43 µg/kg respectively. The study of culinarian treatments shows that pH affects mycotoxins detoxification. Acid pH was the most effective detoxificant process, with depletions from 60% of BEA to 98% of ENA. ENA was the most sensible mycotoxin in most treatments. Easy modification of the traditional cooking process, as adding lemon juice on the boiling water, could improve food safety and decrease human exposition to mycotoxins.

Keywords: Fresh pasta, emerging mycotoxins, cocking process.

Resumen: Comparación del efecto de diferentes tipos de cocción de la pasta fresca en el contenido de micotoxinas emergentes. Las micotoxinas son metabolitos secundarios producidos por hongos filamentosos del género *Aspergillus spp., Penicillium spp.,* y *Fusarium spp.* Las micotoxinas emergentes (Eniatina A, A_1, B, B_1 y beuvericina) han sido descubiertas recientemente. En el presente estudio se analiza la contaminación de las citadas micotoxinas en pasta fresca comercializada en supermercados de la ciudad de Valencia (España), y a continuación la evaluación de la descontaminación tras la cocción de la pasta bajo diferentes condiciones de pH y tiempo. Al análisis de un total de 19 muestras se procede con extracción con Untra-Turrax (acetonitrilo:agua), y posterior detección mediante cromatografía liquida de alta resolución acoplada a espectrómetro de masas en tándem con triple cuádruplo (HPLC-MS/MS-QqQ). Los resultados mostraros que el 84% de las muestras analizadas presentaron contaminación por al menos una de las micotoxinas estudiadas. Las micotoxinas más abundantes fueron ENB y ENB_1 con concentraciones medias de 7.03 y 4.43µg/kg respectivamente. El estudio de los tratamientos culinarios mostro que el pH afectaba a la concentración de micotoxinas. El pH ácido mostro la mayor capacidad para la degradación de micotoxinas con reducciones del 60% de Beauvericina al 98% de ENA. La ENA fue la micotoxina estudiada más sensible al efecto de los tratamientos. Modificaciones sencillas en el tipo de preparación de alimentos en los hogares, como por ejemplo la adición de zumo de limón al agua de cocción, podría mejorar la seguridad alimentaria disminuyendo así la exposición a micotoxinas en la ingesta.

Palabras clave: Pasta fresca, micotoxinas emergentes, tratamientos culinarios.

Introduction

Mycotoxins are toxic metabolites from filamentous fungi like *Aspergillus spp., Penicillium spp., and Fusarium spp*. and they commonly enter into the food chain through contaminated food [1]. FAO consider that about 25% of food plants contain several amounts of mycotoxins [2]. Fungi mainly grow in wheat, barley and maize during the harvest [3], but food products can be also infected by fungi species. Therefore, mycotoxin contamination of food products can be indirect (by ingredients during harvesting) [4], or direct (when the food product becomes infected) [5]. Nowadays are known about 400 mycotoxins, but there are other fungi secondary metabolites which are potentially toxic and whose toxicity or effects in humans have not been evaluated yet [6]. There are emerging mycotoxins which are named like that because they have been discovered recently. In consequence, EFSA has not suggested maximum limits in food products for them. Emerging mycotoxins are enniatins (ENS): enniatin A (ENA), enniatin A_1 (ENA_1), enniatin B (ENB), enniatin B_1 (ENB_1), and beauvericina (BEA). Their structure is shown in Figure 1. They are cyclic depsipeptides, commonly composed of three D-a-hydroxyisovaleric acid (Hiv) residues linked alternatively to three L-configured N-methil. To date are described as molecules with antibiotics and insecticide effects. BEA consists on a d-α-hidroxiisovalina alternated with N-metilfenilalanina. Toxicity has been demonstrated in different human cellular lines. It can induce

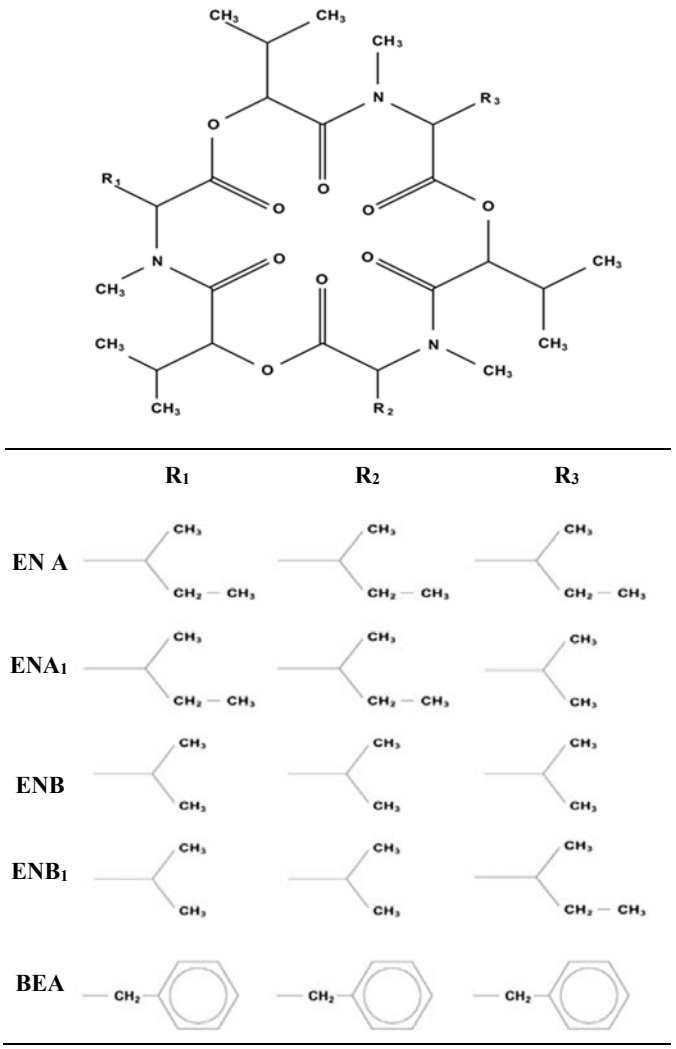

Figure 1. Chemical structures of enniatins (ENs) and beauvericin (BEA).

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apoptosis and DNA fragmentation. Furthermore, BEA could be a specific cholesterol acyltransferase inhibitor [7]. The five molecules have a similar structure to each other [2], all of them are cyclic hexadepsipeptides, and the amino acid residues are aromatic N-methyl-phenylalanines and aliphatic N-methyl-valine or–isoleucine. The amino acids are linked by peptide bond, and there are intramolecular ester bonds. There is a high concentration of negative ions inside of the molecules which makes that the molecules can form metallic bonds [8], and because of the lipophilic properties they can also get into cellular membrane and make channels for K^+, Na^+, Mg^{++}, and Ca^{++}, breaking down the homeostasis of cell like it was said before [9].

ENS and BEA levels vary greatly between different climates and plants. There are even differences among specimens of the same harvest [10,11]. It has been detected DON, OTA, and AFB1 in pasta samples, and Serrano et al. [12] found emerging mycotoxins in pasta products collected in Spain. Different authors study emerging mycotoxins in similar food matrix, for example Oueslati [7] detected emerging mycotoxins in cereals

from Tunisia by LC-DAD, and Vaclavikova [13] found ENS and BEA in cereals by LC-MS.

In many occasions, the intake of food occurs after a culinary process. This culinary treatment could affect emerging mycotoxins levels because of their peptidic structure. Therefore, it is essential to evaluate how culinary treatments affect mycotoxins in order to estimate the intake of population and the health risk. Most mycotoxins are thermo resistance in the range of home treatments temperatures (80-120ºC) [14], but some studies show that emerging mycotoxins can be removed. Vaclavikova et al., [13] detected a decrease of ENS by baking bread, and Serrano et al., [12] detected a 78% of degradation of ENS proportionally to the time of heat treatment. Furthermore, other authors propose that the detoxification depends largely on the pH of the aqueous medium process. For example, the alkaline medium produce a reduction of aflatoxins of the 92% [15], or a reduction of AFB_1 of the 92% with acid medium treatment (pH 3, 7 by citric acid) [16]. Other mycotoxins like OTA are stable under high temperatures, but after boiling those with water Sakuma et al [17] detected a significant decrease of OTA because it leaves the matrix. Maybe this also occurs with emerging mycotoxins.

In this context, the present study raises the following objectives: evaluation of emerging mycotoxins (EN A, EN A_1, EN B, EN B_1 and BEA) in fresh pasta from supermarkets in Valencia, Spain, by extraction with Ultra Turrax and determination by liquid chromatography coupled to mass spectrometry (LC-MS/MS) with triple quadrupole (QqQ). Evaluation of how different culinary treats affect mycotoxins concentration, undergoing fresh pasta to different cooking times and pH.

Material and methods

Sampling

Sampling was conducted on supermarkets in Valencia, Spain. Fresh pasta samples were selected among commercial brands with similar ingredients, like wheat grits and egg. A total of 19 samples were purchased following the legal requirements of the regulation:

commission regulation (EC) No 401/2006 of 23 February 2006 [18], laying down the methods of sampling and analysis for the official control of levels of mycotoxins in foodstuffs. In point B of this rule it is explained the method of sampling for cereals and cereal products for the official control of the maximum levels established for aflatoxin B_1, total aflatoxins, ochratoxin A and Fusarium-toxins in cereals and cereal products.

Culinary process

A total of 6 different culinary processes were applied to a sample mycotoxin free. This sample (PF 19) was spiked with 150µg/g of each studied mycotoxin. In all treatments were used 8 parts of water per part of pasta like manufacturer advise (1:8 pasta:water). The temperature of cooking was always constant, keeping the boiling point. There were two cooking times, 4 and 8 minutes, and different cooking pHs (≤3, 7, ≥9) were also tested. Thereby, from sample PF 19 were taken 6 added aliquots which were undergone like that: 2 aliquots were cooked with acid pH ≤3 (by adding citric acid to the boiling water), 2 with alkaline pH ≥9 (by adding calcic carbonate to the boiling water), and the 2 last samples with neutral pH. Each pH was cooked 4 minutes like the manufacturer advices, and 8 minutes like it is estimated that users do at home. In order to achieve acid pH it was added lemon juice to the cooking water, this addiction was made drop by drop in continuous stirring with a magnetic stirrer, and it was controlled by pH meter; for basic pH it was used the same procedure but it was added calcium carbonate.

Extraction procedure

Samples were crushed to a paste; 5g of the paste were extracted with 50ml of mixture Acetonitrile:H_2O (80:20) for 3 min by an Ultra-turrax (Ika T18 basic, Staufen, Germany). After, the extract was centrifuged (15 min, 5ºC, 4500rpm). The supernatant was decanted and evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The extract was dissolved with 5ml of AcN, and re-evaporated to dryness with nitrogen (N_2) at 35ºC using a Turbovap LV Evaporator (Zymark, Hoptikinton, USA). The extract was reconstituted with 1 ml of AcN/MeOH mix (50/50 v/v), and filtered with 13mm/0.22 µm nylon filter (Membrane Solutions, Texas, USA) before the injection in LC-MS/MS (QqQ).

LC-MS/MS Analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA) consisting on an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Version 4.1, was used for the MS/MS analyses. The separation was achieved by a Gemini-NX C^{18} (150 mm x 4.6 mm I.D., 5 µm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a guard column C^{18} (4 mm x 2 mm I.D.), using a gradient that started at 100% A (AcN) and 0% B (20 mM ammonium formate in methanol), total chromatogram 25 min. The electrospray ionization source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100 ºC; desolvation temperature, 300 ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L/h; cone gas 50 L/h (nitrogen 99.99% purity). Ideal fragmentation conditions were accomplished varying the cone voltage and collision energies for each compound. The cone voltage and the collision energy selected are shown in Table 1. The analyzer settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, -3 and 1; multiplier, 650; collision gas (argon 99.995% purity) pressure, 3.83 x 10-3 mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode

Table 1: *LC-MS/MS (QqQ) optimized parameters.*

	Cone (V)	Collision energy (eV)		Precursor ion (m/z)		Product ion	
		Q[a]	Q[b]			Q	q
ENA	25	25	25	682	$[M+H]^+$	210	228
ENA_1	35	35	35	668	$[M+H]^+$	210	228
ENB	35	35	35	640	$[M+H]^+$	196	214
ENB_1	13	13	12	654	$[M+H]^+$	196	214
BEA	30	30	20	785	$[M+H]^+$	244	262

[a]*Q:* Quantification ion
[b]*q:* Confirmation ion

according with the European Union criteria [18], which establishes that a substance can be identified using LC-MS/MS in MRM mode by at least two transitions.

Results and discussion

Method Validation and analytical parameters

The analytical method was validated for fresh pasta samples (Table 2). Figure 2 shows a chromatogram of a spiked sample with the optimized conditions. Matrix effects were corrected by matrix-assisted calibration curves with a mycotoxins-free sample. For the evaluation of the linearity, calibration curves were constructed at six concentration levels: 0.025-25 µg/kg for the 5 mycotoxins. The results showed good correlation coefficients ($R^2 > 0.9965$). Limits of detection (LODs) were calculated using a signal-to-noise ratio of 3 (from 0.05 to 0.10 µg/kg). Limits of quantification (LOQs) were calculated using a signal-to-noise ratio of 10 (from 0.16 to 0.30 µg/kg). The accuracy was evaluated through recovery studies at two concentration levels (LOQ and 100 x LOQ). Intra-day precision was assessed by three determinations at each addition level in the same day, while inter-day precision was assessed by one determination at each addition level during three days. Recovery values for the low spiked level (LOQ) and the high spiked level (100 x LOQ) ranged from 85 to 110% and from 86 to 112%, respectively. Therefore, the results were in accordance to the limits set in European Commission [18]: a mean recovery (n=3) between 70% and 120%, and a RSD lower than 20%.

Table 2: *Analytical parameters of the validated method*

	SS [a]	LOD (µg/kg)	LOQ (µg/kg)	Linearity (R^2)	Recovery	
					intra-day (n =6)	inter-day (n = 6)
ENA	40	0.15	0.50	0.999	91 ± 4	90 ± 6
ENA_1	43	0.08	0.25	0.997	86 ± 9	88 ± 8
ENB	33	0.15	0.50	0.999	109 ± 9	112 ± 5
ENB_1	40	0.02	0.50	0.998	97 ± 11	95 ± 15
BEA	46	0.03	0.10	0.998	94 ± 4	94 ± 5

[a]SS: Suppression of Signal (Matrix Effect) = (slope matrix-matched/slope standard in solvent)*100

Natural occurrence of BEA y ENS

A total of 19 different samples of fresh pasta were evaluated. The results of the natural occurrence are shown in table 3. The 84% of the total of samples were contaminated with one or more studied mycotoxins. Talking about the co-occurrence of ENS, half of the total samples were positive for the four mycotoxins simultaneously (ENA, ENA_1, ENB, ENB_1), while only in the 12% of the samples were detected the five evaluated mycotoxins (ENA, ENA_1, ENB, ENB_1 and BEA) in agreement with Serrano et al., [19] that found similar percentages of occurrence in 114 pasta samples. On an overall assessment, the results showed that the most abundant mycotoxins were ENB and ENB_1 (main concentration 7.03 and 4.43 µg/kg respectively) in accordance with Juan et al., [20] that found ENB as the most concentrated enniatin in multicereal baby food and pasta. Nevertheless, rang of concentrations from Juan et al [20] (1100 and 106 µg/kg) were not suitable with the results of the present study (0.08 and 33.13 µg/kg). Concentrations of ENA and ENA_1 were 1.61 and 3.30 µg/kg respectively with occurrences of 63% and 53%, in disagree with Oueslati et al., [7] that found ENA and ENA1 the most concentrated mycotoxins in wheat products. BEA was the less abundant mycotoxin in concentrations ranging from 0.06 to 2.36 µg/kg.

Reduction of ENS and BEA in spiked samples

Degradation of mycotoxins in pasta after different heat treatments (different times and different pH) are shown in Figure 3. According to

Table 3: *Occurrence, main concentration and range of emerging mycotoxins in 19 commercial samples.*

	OCCURENCE[a] (%)	Mean[b] (µg/kg)	RANGE	
			MAX	MIN
BEA	21%	0.64	2.36±0.03	0.06±0.02
ENA	63%	1.61	5.09±0.04	0.07±0.01
ENA1	53%	3.30	9.28±0.03	0.11±0.03
ENB	84%	7.03	33.13±0.07	0.08±0.05
ENB1	63%	4.43	13.41±0.05	0.15±0.01

[a]Occurrence of mycotoxins in the total analyzed samples.
[b]Mean concentration in positive samples.

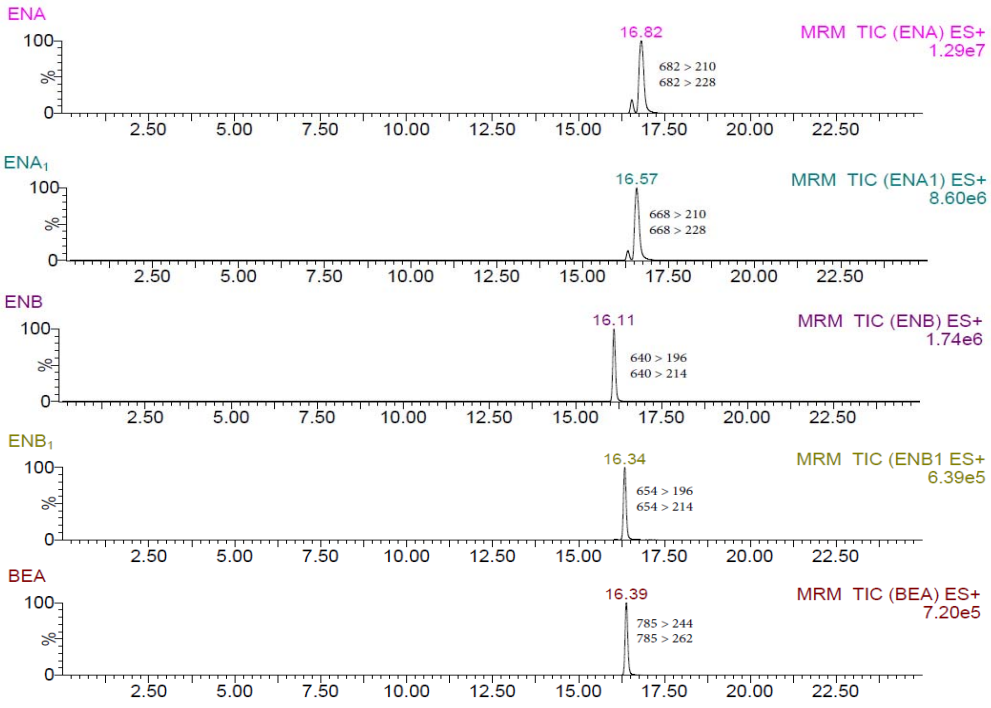

Figure 2: Chromatogram of a spiked sample of each mycotoxin (ENA, ENA1, ENB, ENB1 and BEA)

other authors [16], pH affects mycotoxins removal. The most significant decrease was with acid heat treatment (from 60% of BEA to 98% of ENA). It was tested that the organoleptic properties were not modify. ENA was the most sensible mycotoxin; it was hardly removed in all treatments (≥95%). The rest of mycotoxins showed percentages of degradation in all treatments but not as high as ENA. ENB presents degradations ranging from 45% to 69% (with alkaline 4 minutes treatment); BEA from 24% to 67% (alkaline pH); ENB1 from 17% to 67% (Acid pH), and the most resistant mycotoxin was ENA_1 ranging from 3% of degradation (neutral pH) to 64% (acid pH). Mendez-Albores et al., [16] detected a degradation of aflatoxis significantly affected by acid medium in sorghum. Cano-Sancho et al., [21] detected the loose of DON during the boiling of pasta, and Brera et al., [22] showed a significant reduction of DON (78%) after cooking pasta.

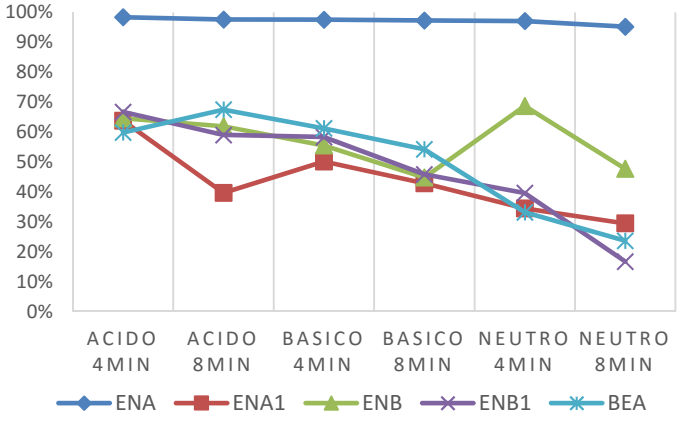

Figure 3: graphic representation of degradation percentage of each mycotoxin at different treatments conditions.

Conclusions

The analysis of emerging mycotoxins in real samples of fresh pasta collected in Valencia (Spain) has been performed. The results show that fresh pasta present contamination levels in order of µg/Kg. After the culinarian process, mycotoxins concentration was lower in acid cooking, and then cooking process at acid pH performed the higher decontamination in fresh pasta samples. Easy modification of the traditional cooking process, as adding lemon juice on the boiling water, could improve food safety and decrease human exposition to mycotoxins.

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