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Nuts and dried fruits: Natural occurrence of emerging *Fusarium* mycotoxins

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ABSTRACT

The occurrence of enniatins (ENs) and beauvericin (BEA) in nuts, dried fruits and dates available in Valencia (Spain) was surveyed in this study. To do this, seventy-four samples of nuts, dried fruits and dates were analyzed for the determination of mycotoxin contamination. Mycotoxins were identified and quantified using an ultrasonic-C_{18} extraction and LC–MS/MS with a triple quadrupole (QqQ) mass analyzer. The frequencies of contamination of nuts, shell, dried fruits and dates were 50%, 80%, 35.7% and 83.3%, respectively. Enniatin A (ENA) was the most predominant EN found in nuts (45.2%) while ENB was the most common EN found in dates (58.3%). The analytical results of the shell samples showed a protective effect of the shell, avoiding the contamination of the fruit with mycotoxins.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi, mainly *Fusarium* spp., *Aspergillus spp.* and *Penicillium* spp. They are produced in different substrates under certain climatic conditions of temperature and humidity and in presence of large amounts of nutrients. Mycotoxins are common contaminants of different foodstuffs mainly cereals, but its presence is also found in pasta, fruits, honey, eggs, nuts and dried fruits, among others (Bircan, 2009; Heperkan, 2006; Jestoi, Rokka, Järvenpää, & Peltonen, 2009).

Emerging mycotoxins include enniatins (ENs) and beauvericin (BEA) and they can be found in various foodstuffs. They were discovered after other *Fusarium* mycotoxins, such as fumonisins and trichothecenes. They are cyclic hexadepsipeptide (Fig. 1) with insecticidal properties capable of inducing apoptosis in mammalian cells and have antibiotic properties against gram-positive bacteria and mycobacteria (Jestoi, 2008). These emerging mycotoxins act as enzyme inhibitors (inhibiting the enzyme acyl-CoA and cholesterol acyltransferase), antibacterial, antifungal agents and as immunomodulatory substances.

The toxicity studies have been emphasized on toxicodinamia and primary interaction with target cells, but no data is available of its toxicokinetics (absorption, distribution, metabolism and excretion) or its possible interaction with other mycotoxins (Jestoi, 2008), as well as additive or synergistic effects that may occur when interacting with other mycotoxins or between them (Santini, Ferracane, Meca, & Ritieni, 2009).

They can cause a wide range of toxicological effects both in human and animals, ranging from the development of carcinogenic, teratogenic and mutagenic effects to the production of hormonal and immunosuppressive disorders (Köppen et al., 2010).

Some studies about emerging mycotoxins in foodstuffs are available, but most of them are focused on cereals (Mahnine et al., 2011; Meca, Zinedine, Blesa, Font, & Mañes, 2010; Oueslati, Meca, Mliki, Ghorbel, & Mañes, 2011) and Mediterranean crops (Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003). In the published data, the literature consulted was about aflatoxin (AF) and ochratoxin A (OTA) in nuts and dried fruits (Abarca, Accensi, Bragulat, Castella, & Cabañes, 2003; Abdulkadar, Al-Ali, & Al-Jedah, 2000; Bircan, 2009; Cheraghali et al., 2007; Doster and Michailides, 1994; Heperkan, 2006; Yazdanpanah, 2009). However, to our knowledge, there is no data about the occurrence of emerging *Fusarium* mycotoxins in nuts and dried fruits.

Nuts are a matrix susceptible to fungal growth because of their intrinsic characteristics of water activity, moisture and nutrient content, pH and high storage time, which favor the growth of these fungi. In addition, some are collected directly from the soil, such as figs or dates, which favors infection by fungi (Bircan, 2009; De Mello and Scussel, 2007; Doster et al., 1996).

Under the heading of nuts different seeds are included from different botanical families. It is a heterogeneous group with very similar allergenic characteristics and forms of consumption. All of them are oleaginous fruits consumed in dried form. This group

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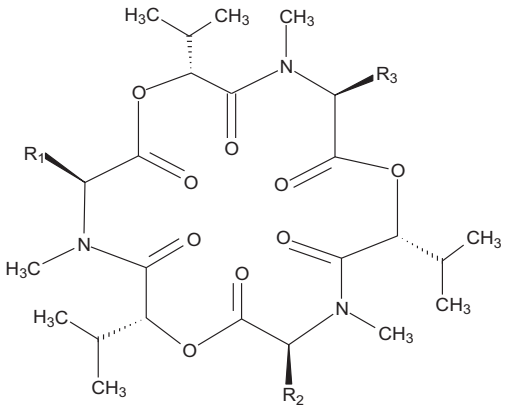

	R_1	R_2	R_3
BEA	Phenyl-methyl	Phenyl-methyl	Phenyl-methyl
ENA	*sec*-butyl	*sec*- butyl	*sec*- butyl
ENA1	*sec*- butyl	*sec*- butyl	*iso*- propyl
ENB	*iso*-propyl	*iso*- propyl	*iso*- propyl
ENB1	*iso*- propyl	*iso*- propyl	*sec*- butyl

Fig. 1. Structure of BEA and ENs.

includes peanuts, almonds, hazelnuts, cashews, walnuts, Brazil nuts, pecan nuts, pistachios, pine nuts and sunflower seeds (AESAN, 2007). In addition, we have analyzed some legume samples (lupines, fried lima beans, chickpeas and pine nuts) because they are consumed in the same way as nuts (such a snack).

In the last few years, increased efforts have been made to develop analytical methods for the detection of very low concentrations of mycotoxins in different food samples by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Furthermore, some authors have developed a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for the quantification of various mycotoxins simultaneously, including emerging mycotoxins such as ENs and BEA (Berthiller, Sulyok, Krska, & Schuhmacher, 2007; Monbaliu et al., 2009, Monbaliu, Van Poucke, Heungens, Van Peteghem, & De Saeger, 2010; Sulyok, Berthiller, Krska, & Schuhmacher, 2006; Van Pamel, Verbeken, Vlaemynck, De Boever, & Daeseleire, 2011). However, these studies dealt with cereal samples, except Sulyok, Krska, and Schuhmacher (2007) and Spanjer, Rensen, and Scholten (2008), that applied the method to chestnuts (and other foodstuffs) and peanuts, pistachios, raisins and figs (among cereals). Recently, in our laboratory a new rapid, sensitive and reproducible analytical strategy has been developed to determinate the emerging mycotoxins in nuts and dried fruits using an ultrasonic-C_{18} extraction and LC–MS/MS with a triple quadrupole (QqQ) mass analyzer.

Because of the susceptibility to mycotoxin contamination in nuts and the increased consumption of them (AESAN, 2007), currently, the aim of this study is to determine emerging mycotoxins in nuts and dried fruits by ultrasonic extraction and determination by liquid chromatography mass spectrometry (LC–MS/MS) with a triple quadrupole (QqQ).

2. Material and methods

2.1. Chemical and reagents

All solvents (acetonitrile and methanol) were purchased from Merck (Darmstadt, Germany). Deionized water (<8 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) was supplied by Sigma–Aldrich (Madrid, Spain). All solvents were filtered through a 0.22 μm cellulose filter from Membrane Solutions, Texas, USA, before use.

The stock standard of BEA was purchased from Sigma–Aldrich (St. Louis, USA). ENs toxin solutions were provided by Biopure (Tulln, Austria). Individual stock solutions of BEA, ENA, ENB and ENB1 with concentration of 500 μg/ml were prepared in methanol and ENA1 with concentration of 250 μg/ml was prepared in methanol. They were stored in glass-stoppered bottles and darkness in security conditions at −20 °C. These stock solutions were then diluted with pure methanol in order to obtain the appropriate working solutions and were stored in darkness at 4 °C until the LC–MS/MS analysis.

2.2. Sampling

Seventy-four samples of nuts, including crude peanuts ($n = 6$), toasted peanuts ($n = 3$), fried peanuts ($n = 2$), crude almonds ($n = 8$), toasted almonds ($n = 2$), pistachios ($n = 3$), walnuts ($n = 5$), hazelnuts ($n = 4$) and sunflower seeds ($n = 5$), and dried fruits, including dried apricots ($n = 2$), dried figs ($n = 2$), raisins ($n = 3$), dried plums ($n = 4$), blueberries ($n = 1$), Goji berries ($n = 2$) and dates ($n = 12$) were purchased from Valencian supermarkets and local markets. Besides, we decided to analyze 6 samples of legumes (lupines, fried lima beans, chickpeas and pine nuts) and 4 samples of fried maize because of their treatment (roasted) and their way of consumption (as snacks) we considered interesting to include them in the study.

Its composition includes less than 50% of water and a low quantity of carbohydrates (except walnuts), but they are rich in protein (10–30%) and fat (30–60%), specially mono and polyunsaturated fatty acids (AESAN, 2004).

All samples were stored in a dark and dry place until analysis. After their packages had been opened they were put into specific glass food containers and analyzed within 3 days.

Shell samples were separated by analyzing the fruit on one side and the shell on the other. Previously, according to European Regulation 401/2006, the percentage of shell and fruit was calculated for each sample to estimate the exact content of each mycotoxin in them.

2.3. Mycotoxin extraction procedure

The method used for mycotoxins analysis (ENs and BEA) was previously optimized in our laboratory. The optimization results are showed in Section 3.1. A 10 g aliquot of each nut samples was homogenized with 50 ml of acetonitrile for 30 min and 35 °C using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). The extract was centrifuged at 3544.4 g for 15 min and 5 °C. The supernatant was filtered and purified using C_{18} columns (Waters, Milford, Massachusetts) by applying a slight vacuum. The extract was transferred to a 15 ml conical tube and evaporated to dryness at 35 °C using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the solution was reconstituted with 1 ml of AcN–MeOH 50:50 v/v and placed again in the ultrasonic bath (30 min, 35 °C). Then, the solution was