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Short communication

Survey of the presence of patulin and ochratoxin A in traditional semi-hard cheeses

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ABSTRACT

Thirty-two traditional hand-made semi-hard cheeses were examined to detect the presence of two mycotoxins – ochratoxin A (OTA) and patulin. OTA was present in both the rind and the inner part of the cheeses, while patulin was mainly detected in the rind and the inner part of only one cheese sample.

The observed patulin concentration ranged from 15 to 460 μg/kg, while the observed OTA concentration ranged from 1 to 262 μg/kg in the rind and from 18 to 146 μg/kg in the cheese interior. The amount of patulin contained in the rind exceeded the maximum level (50 μg/kg) established for fermented apple drinks and other fruit juice by EC Regulation 1881/2006. Therefore, it is crucial to pay close attention to the various production phases of traditional cheeses to provide useful information about the origin of mycotoxin contamination and develop efficient prevention methodologies.

1. Introduction

Mould growth on cheese is common during ripening and storage at low temperatures and is usually accompanied by changes in the texture, smell and taste of the contaminated products due to the production of enzymes and volatile compounds. In some cases, these changes are actively desired (*e.g.,* non-toxigenic strains of *Penicillium rocquefortii* are employed for the production of blue mould cheeses); however, in most other cases, fungal contamination leads to unwanted cheese spoilage in the form of off-flavours, discolouration, rotting and decay of the structure (Filtenborg, Frisvad, & Thrane, 1996).

Furthermore, the development of moulds could lead to the possible formation of mycotoxins, which are well-known to be carcinogenic or genotoxic to both animals and humans and present a severe health hazard (Creppy, 2002). Among the various mycotoxins, patulin and Ochratoxin A (OTA) have attracted increasing interest in recent years due to their potential to act as possible toxic contaminants to many food items.

Patulin is produced by several fungal species belonging to the genera *Aspergillus*, *Penicillium* and *Byssochlamys* as *Aspergillus clavatus*, *Penicillium expansum*, *Penicillium clavigerum*, *Penicillium griseofulvum* and *Byssoclamys nivea*. The production of patulin using strains of *Byssoclamys fulva* remains controversial (Sant'Ana et al., 2010). *B. fulva* is considered neurotoxic, embryotoxic and teratogenic (Dombrink-Kurtzman & Blackburn, 2005) and has been classified by the International Agency for the Research on Cancer (IARC) as category 3 ("unclassifiable as to carcinogenicity in humans"), even though its carcinogenic activity in different animal models is still the subject of some controversy (Schumacher, Müller, Metzler, & Lehman, 2006).

Ochratoxin A is produced by different species of *Aspergillus* (*Aspergillus ochraceus*, *Aspergillus melleus*, *Aspergillus sulphureus*, *Aspergillus nigri*. *Aspergillus lanosus*, *Aspergillus alliaceus*, *Aspergillus carbonarius* and *Aspergillus awamori*) and *Penicillium* (*Penicillium verrucosum*, *Penicillium chrysogenum* and *Penicillium nordicum*) (Clark & Snedecker, 2006; Varga, Frisvad, & Samson, 2011). The hepatotoxic, nephrotoxic and teratogenic effects of OTA in animals are well-documented, and it has been classified by the IARC as a Group 2B carcinogen (Boudra & Morgavi, 2006). In addition, it is apparently involved in Balcan Endemic Nephropathy and Chronic Interstitial Nephropathy (Clark & Snedecker, 2006; Pena, Cerejo, Lino, & Silveira, 2005).

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Few studies on the presence of these mycotoxins in cheese have been published at this point in time. Particularly worthy of mention are those by Spanish (López-Diaz, Román-Blanco, García-Arias, García-Fernandez, & García-López, 1996), Turkish (Erdogan, Gurses, & Sert, 2003) and Italian researchers (Dall'Asta et al., 2008).

The aim of the present paper is to investigate the presence of patulin and ochratoxin A in traditional semi-hard cheeses in which natural mould contamination is likely, particularly in hand-made, traditional cheeses, as the environmental conditions of the cellars where they are stored during ripening are generally not carefully controlled.

2. Materials and methods

2.1. Samples

32 hand-made semi-hard cheeses, produced with raw cow milk and a cheese-making technique similar to those of "Toma Piemontese PDO", were acquired directly from the cheese producers and examined. In the production of this type of cheese, raw milk is coagulated by adding bovine liquid rennet at a temperature of 32–35 °C. The curd is cut twice to produce granules approximately the size of maize grains. After draining, the curd is put in moulds and pressed for at least 5–6 h. The cheese is then salted in brine for approximately 36 h; dry salting is also common. Ripening takes place in a cellar for 45–60 days.

In the laboratory, the rind was removed, leaving a surface layer of 2–3 mm. The rind and interior of each sample were stored at −20 °C until analysis.

2.2. Reagents

The patulin standard, dissolved in pure acetonitrile, was purchased from Fluka (Buchs, Switzerland). The OTA standard, dissolved in a benzene:acetic acid (99:1) solution, was purchased from Supelco (Bellefonte, PA, USA). The working solutions for both standards were stored at −40.0 °C. All of the solvents were HPLC grade (Merck, Darmstadt, Germany). Distilled water (resistivity value = 18.2 mΩ) was obtained from a MilliQ system using a (Millipore, Billerica, MA, USA).

2.3. Extraction of mycotoxins

All glassware was washed with water (pH of 4.0) to avoid a loss of patulin from the solvents (Baert et al., 2007). The glassware used was also washed with methanol to avoid a loss of OTA through salt formation, precipitation and/or absorption to the glassware (Pena et al., 2005). All analyses were performed twice.

To extract patulin, a modification of the extraction protocol proposed by Kokkonen, Jestoy & Rizzo (2005) was used. A sample (5 g, interior or rind) was homogenised for one min with 10 mL of acetonitrile acidified with 0.1% formic acid. The homogenised sample was then shaken horizontally for 5 min (600 strokes/min) (Ika-Vibramax, Staufen, Germany). An initial centrifugation step at 4677 RCF was performed, and 10 mL of acetonitrile was collected in a separate vial. The centrifugation step was repeated twice under the same conditions. An aliquot (15 mL) of the extract was shaken horizontally at 600 strokes/min for 30 min with 7 mL of hexane to remove any fat. The hexane layer was discharged, and the step was repeated twice with 5 mL of hexane. One mL of the organic extract was evaporated to dryness at 60 °C and stored until analysis, whereupon 1 mL of the mobile phase was added for patulin detection.

To extract OTA, the protocol proposed by Pattono, Gallo, and Civera (2011) was adopted. Cheese samples (2.5 g, interior or rind) were homogenised with 5 mL of acetonitrile after the addition of 20 μL of 18% sulphuric acid (v/v) to obtain a pH of 2.0 ± 0.5. The homogenised sample was then shaken horizontally for 5 min (600 strokes/min). An initial centrifugation step at 4677 RCF was performed, and 5 mL of acetonitrile was collected in a separate vial. The extract was shaken horizontally at 600 strokes/min for 30 min with 5 mL of hexane to remove any fat from the extract. The organic phase was filtered through regenerated cellulose filters (0.45 μm pore size) (Chemtek, Anzola Emilia, Italy). Both the tube and filter were washed once with 1 mL of acetonitrile, which was added to the previously collected samples. A total of 1.5 mL of the extract was evaporated to dryness at 60 °C and stored until analysis, whereupon 300 μL of mobile phase was added for OTA detection.

2.4. Chromatographic conditions

The HPLC apparatus consisted of a Merck-Hitachi L-7100 pump equipped with an L-7614 vacuum membrane degasser (Merck, Darmstadt, Germany) and a Rheodyne 7725i injection valve fitted with a 50 μL loop, which was connected to an end-capped Merck PuroSpher Star RP-18 with dimensions of 250 × 4 mm × 5 μm (Merck, Darmstadt, Germany).

For patulin detection, a Merck L-7400 UV detector (Darmstadt, Germany) was employed. The wavelength used was 273 nm. The mobile phase was composed of H_2O:acetonitrile (90:10) at a flow rate of 0.8 mL/min. For OTA detection, a Merck L-7480 fluorescence detector (Darmstadt, Germany) was used. Fluorescence excitation and emission wavelengths were 333 nm and 460 nm, respectively. The mobile phase was composed of H_2O:acetonitrile:acetic acid (49.5:49.5:1) at a flow rate of 1.0 mL/min (Visconti, Pascale, & Centonze, 2000).

2.5. Confirmation of mycotoxins

The protocol proposed by Cunha, Faria, and Fernandes (2009) was applied to confirm the detection of patulin.

For the confirmation test, OTA was converted into its methyl derivative. The cheese extracts were evaporated to dryness and dissolved in a solution of 2.5 mL of methanol and 100 μL of concentrated hydrochloride acid. The mixture was incubated overnight at room temperature and then evaporated. The residue was dissolved in 150 μL of the mobile phase before being injected into the HPLC apparatus (Boudra & Morgavi, 2006).

2.6. Recovery

To calculate the recovery rate, a negative sample of industrial semi-hard cheese was used. This assay was duplicated for each recovery test. Unfortunately, no certified sample was available for the recovery tests. The cheese was not subject to mould growth, and the HPLC chromatogram was negative for both mycotoxins extracted using the chosen protocols. The recovery tests were performed twice, and each instance included 5 replicates. The limit of detection (LOD) was calculated using a 3:1 signal-to-noise ratio by taking the concentration of the analyte that produced a signal equal to the average background (Sblank) plus three times the standard deviation (sblank) of the blank (LOD = Sblank + 3sblank). The limit of quantitation (LOQ) was calculated as LOQ = Sblank + 10sblank (Miller & Miller, 2000).

3. Results and discussion

The results of the recovery tests are summarised in Table 1. The recoveries for patulin were higher than 87% at a spiking level of 100 μg/kg and higher than 89% at a spiking level of 500 μg/kg.

Table 1
Mean values (±standard deviation) of recoveries for ochratoxin A and patulin at two different spiking levels (value obtained by analysing 5 replicates twice).

	Spiking level (μg/Kg)	Recovery (%)	RDS (%)
Ochratoxin A	1	93.86 ± 1.82	1.94
	5	95.02 ± 3.96	4.17
Patulin	100	87.1 ± 6.6	7.7
	500	89.2 ± 5.2	5.9

Legend: RDS – Relative Standard Deviation.

The recoveries for OTA were even higher: at a spiking level of 1 μg/kg, the recovery was 93.9 ± 1.8%, and at 5 μg/kg, the recovery level was 95.0 ± 3.9%.

The standard deviation and relative standard deviation of the two extraction protocols were always less than 10%.

For both mycotoxins, the 6-point calibration curve was linear in the range considered (r^2 = 0.9989). The LOD and LOQ were 2.1 μg/kg and 7.7 μg/kg for patulin, respectively, while for OTA, the LOD and LOQ were 0.17 μg/kg and 0.54 μg/kg, respectively.

Two samples out of 32 produced negative results for both mycotoxins. Patulin was detected in only one sample taken from the inner part of the cheese (26.6 μg/kg), while 8 rind samples tested positive, with amounts ranging between 15.4 and 460.8 μg/kg (Table 2). For 6 of these positive rind samples, the amount of patulin exceeded 50 μg/kg, *i.e.,* the maximum level established by the EC Regulation for fermented apple-derived drinks and other fruit juices (EC Reg. No. 1881/2006).

Previous studies on the subject provided no evidence of the presence of patulin in cheese (Erdogan et al., 2003; Kokkonen et al., 2005; Taniwaki, Hocking, Pitt, & Fleet, 2009). In our opinion, this finding could be due to different contamination conditions in ripening cellars.

OTA was present in 6 samples, with amounts ranging between 18.4 and 146.0 μg/kg in the interior and between 1.0 and 262.2 μg/kg in the rind. These values are higher than those reported by other authors for blue cheese (Dall'Asta et al., 2008; Kokkonen et al., 2005). However, it must be noted that our investigation concerned hand-made cheeses processed in uncontrolled environmental conditions. It has been reported that the industrial starter moulds used in controlled conditions for the production of blue cheese may exhibit a lesser ability to produce mycotoxins than the strains isolated from traditional products (Fernández-Bodega, Mauriz, Gómez, & Martín, 2009).

With regard to mycotoxin distribution in the cheese, patulin was detected in the interior of only one sample, while OTA was found in both the cheese interior and rind. The likely presence of mycotoxins on the cheese surfaces could suggest that the environmental conditions of the cellars (*e.g.*, temperature, Water activity – A_w) during cheese ripening can be favourable to the metabolic activity of some mycotoxin-producing strains (Erdogan et al., 2003; Morales, Marín, Ramos, & Sanchis, 2007; Pardo, Marín, Ramos, & Sanchis, 2006; Taniwaki et al., 2009).

However, it has also been reported (Pardo et al., 2006; Sulyok, Krska, & Schuhmacher, 2010; Zhang, Cudjoe, Vuckovic, & Pawliszin, 2009) that toxin concentrations and visible mould colonies may not always correlate.

Table 2
OTA and patulin contents in the analysed samples.

	OTA (μg/Kg)		Patulin (μg/Kg)	
	Interior	Rind	Interior	Rind
Maximum	146.0	262.2	–	460.8
Minimum	18.4	1.0	–	15.4
Average	83.1	109.6	26.6	154.6

Mould strains able to produce patulin and OTA can be part of the environmental microflora of various food items and cheese-making factories(Montagna et al., 2004; Skaug, Wijnand, & Størmer, 2000; Sørensen, Jacobsen, Nielsen, Frisvad, & Koch, 2008). Thus, cheese-making, ripening and storage should be strictly monitored to prevent contamination. It is well known that moulds developing on cheese rinds generally come from the environment; thus, the wooden shelves on which traditional cheeses are placed during ripening could also be a source of mycotoxin-producing moulds (López-Diaz, Santos, García-López, & Otero, 2001; Sørensen et al., 2008).

Furthermore, mycotoxins on the rind surface can diffuse into the inner part of the cheese. This phenomenon has been observed in apples, and it seems to be favoured by the softening of the tissues (Sulyok et al., 2010). The same process could also be hypothesised for cheeses.

The UE Regulations have not yet established the maximum levels of OTA and patulin allowable in cheese (either with or without the rind in sampling stage), but the amounts of the two mycotoxins found in the samples examined in this study exceed the levels indicated for wine (OTA 2.0 μg/kg) and fruits, (Patulin 50 μg/kg) as detailed by EC Reg. No. 1881/2006. Furthermore, although rind is not usually considered to be an edible part of the cheese, we cannot consider the rind to be "inedible" if it is not indicated as such on the cheese product label. Furthermore, rind is often eaten by consumers even when it is covered with mould, as they may consider such products to be more "typical and wholesome" (Sulyok et al., 2010).

4. Conclusions

Mycotoxins are becoming an issue of great concern for public health. However, for some items, such as cheese, there is a lack of precise information available about mycotoxin contents (Jørgensen, 2005). To perform a complete and adequate "risk analysis", a first step could be the investigation of the occurrence of these mycotoxins and other mycotoxins (*e.g.,* Roquefortine C) in different types of cheese.

The present paper aimed to provide information about the presence of OTA and patulin in hand-made cheeses, *i.e.*, in food not usually considered in control tests. Taking into account the amounts found, it cannot be denied that eating this type of cheese could increase the total amount of mycotoxins ingested.

Further investigations must now be performed to isolate toxigenic strains from samples and the environment to better understand how environmental conditions (during the various steps of cheese making and ripening) can influence the development of such strains. All of this information will be essential for developing scientific tools capable of matching consumer expectations regarding the complete safety of food.

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