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ScienceDirect
Food Chemistry 106 (2008) 729–734
Food Chemistry
www.elsevier.com/locate/foodchem

The occurrence of ochratoxin A in blue cheese

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Received 15 January 2007; received in revised form 7 May 2007; accepted 27 June 2007

Abstract

Evidence for the occurrence of ochratoxin A in blue cheeses is reported for the first time. The development of an accurate and reliable procedure for the extraction of OTA from cheese, as well as the availability of a new sensitive HPLC-FLD method, has allowed us to determine ochratoxin A in complex matrices such as cheeses, even at very low levels (LOD in cheese: 0.02 μg/kg). A good linearity for the OTA concentration, between 0.2 and 2.5 μg/kg, was obtained and no matrix effect was observed in the same concentration range. The mean recovery for OTA was 97%, while the average RSD was 3%, within a spiking range of 0.5–2.0 μg/kg.

Although the OTA contamination levels found in blue cheeses were very low, occurrence of ochratoxin A in such products opens a new issue for risk assessment and quality control, as far as finding the origin of the OTA contamination and ways to prevent it.

Keywords: *Penicillium roqueforti*; Ochratoxin A; Blue cheese; HPLC; Shelf life; Fluorescence detection

1. Introduction

Moulds are usually considered as undesirable in food since they may produce mycotoxins, which are toxic secondary metabolites affecting a large variety of food commodities. Some of the most common mycotoxins are carcinogenic, genotoxic or may affect the kidneys, the liver and the immune system, thus causing a severe health hazard to the human population (Creppy, 2002).

Nevertheless, moulds are intentionally added to several food products to induce the formation of specific flavours. An example is represented by blue cheeses, which are cow's milk or goat's milk cheeses with an extensively spotted or veined structure due to blue or blue–green moulds. In particular, *Penicillium roqueforti* is used as a secondary starter culture for the ripening of Gorgonzola, Danablu, Roquefort, Blue de Bresse, Blue Stilton and Edelpilzkäse.

The most important toxic metabolite produced by several *Penicillium* moulds is ochratoxin A (OTA), a chlorinated polyketide mycotoxin containing the amino acid L-phenylalanine, which shows nephrotoxic, teratogenic, immunogenic, hepatotoxic and carcinogenic activities and has been classified as a Group 2B carcinogen by the International Agency for Research on Cancer (IARC Monographs, 1993) as reported in Fig. 1.

Ochratoxin A is also produced by several species of the genus *Aspergillus*, such as *A. ochraceus*, *A. carbonarius* and *A. niger*. *Penicillium verrucosum* has been found to be responsible for OTA production in starch-rich foods such as grains and cereal derivatives, whereas *P. nordicum* occurs mainly as a contaminant in protein-rich food, like fermented meats and cheeses (Bogs, Battilani, & Geisen, 2006; Lund & Frisvad, 2003).

Although *P. roqueforti* fungal starters are potentially able to produce different toxic metabolites in culture media, several surveys have restricted the mycotoxin occurrence mainly to cyclopiazonic acid in white mould cheeses and

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doi:10.1016/j.foodchem.2007.06.049

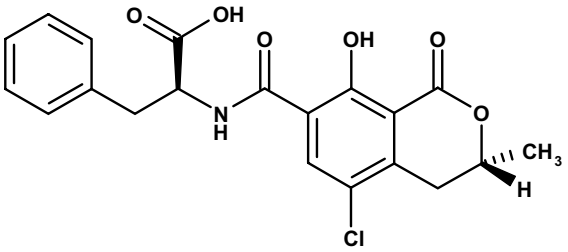

Fig. 1. Chemical structure of ochratoxin A.

to roquefortine C and mycophenolic acid in blue cheeses. Moreover, these studies clearly demonstrate that the low amount of such metabolites in cheese do not represent a hazard for health of the consumer (Finoli, Vecchio, Galli, & Dragoni, 2001).

Several analytical methods have been published in the last decade for the determination of *Penicillium* mycotoxins in food commodities. These methods are usually based on liquid chromatography (LC) with fluorescence detection (FLD), after a solid-phase extraction (SPE) clean-up step with a reversed-phase C_{18}, with silica gel 60 cartridge or with immunoaffinity columns (IAC), which allow for a good sensitivity. However, they are usually troublesome for complex matrices such as cheese (Rundberget & Wilkins, 2002). Recently Kokkonen, Jestoi, and Rizzo (2005) proposed a feasible LC-MS/MS method for quantifying several *Penicillium* and *Aspergillus* mycotoxins in blue and white mould cheeses and the method allowed for the detection of nine mycotoxins (aflatoxins B1, B2, G1, G2 and M1, mycophenolic acid, ochratoxin A, roquefortin C, penicillic acid) produced by *Aspergillus* and *Penicillium* species. The method was applied to blue and white moulded cheeses from the Finnish market: only roquefortine C was detected in blue cheese, whereas the other investigated mycotoxins were absent in all the samples. However, analytical methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) are mostly employed as a confirmatory tool, since they require highly trained staff and expensive instrumentation.

A few years ago, we proposed an improved HPLC-FLD method which allowed us to obtain an eightfold enhancement of the ochratoxin A chromatographic signal (Dall'Asta, Galaverna, Dossena, & Marchelli, 2004). This method, based on an alkaline elution, was successfully applied to the OTA determination in wine, without any previous clean up procedures, allowing it to meet the more recent EU legislation for OTA (EC No. 123/2005; EC No. 856/2005). Occurrence of ochratoxin A in mould-ripened cheese has not been observed so far and no specific guidelines have been set.

By using this reliable and sensitive method, we report for the first time that ochratoxin A was occasionally found in blue cheeses, in particular in several typical manufactures from EU Countries: Gorgonzola from Italy and Roquefort from France.

2. Materials and methods

2.1. Reagents

Ochratoxin A (1 mg) was obtained from Sigma–Aldrich (Steinheim, Germany). All solvents used (LC grade) were obtained from Carlo Erba (Milan, Italy); bidistilled water was produced in our laboratory utilising an Alpha-Q system (Millipore, Marlborough, MA, USA). Ammonia solution (33%) was from Riedel-de Haen (Seelze, Germany). Immunoaffinity columns OCHRAPREP™ were obtained from Rhone Diagnostic (Glasgow, UK). Phosphate buffered saline (PBS) buffer was prepared dissolving 8 g NaCl, 1.2 g Na_2HPO_4, 0.2 g K_2HPO_4 and 0.2 g KCl in 1 l bidistilled water and adjusting the pH to 7.6 with 2 N HCl.

2.2. Cheese sample preparation

A whole serving of blue cheese (150 g), after addition of bidistilled water (15 ml), was homogenised by using a high-speed blender (Ultraturrax T25, IKA, Stauffen, Germany) until a creamy and homogeneous mixture (slurry) was obtained. An amount of 5 g was then taken from the slurry and mixed with 50 ml of $CHCl_3$ and 1 ml of saturated NaCl solution; after acidification with 0.75 ml of 85% H_3PO_4 (pH 3), the sample was blended for 2 min at 2000 rpm in a high-speed blender. The extract was filtered through filter paper (Whatman no. 4), then the filtrate (25 ml) was extracted with a saturated $NaHCO_3$ aqueous solution (2×10 ml); finally, the aqueous phase was purified on immunoaffinity columns (Ochraprep, Rhone-Diagnostic). After conditioning with 10 ml of PBS solution, 10 ml of the extract was applied on the IAC column at a flow rate of about 1 drop/s. The column was washed first with 5 ml of the PBS solution and then with distilled water (5 ml) at a flow rate of 1–2 drops/s. OTA was eluted with methanol (2 ml) and collected in a glass vial. The eluted extract was evaporated under nitrogen and redissolved in 0.5 ml of acetonitrile.

In order to compare the OTA content in the green and in the white area of the cheese samples, a 5 g sample was taken alternatively from moulded spots or from creamy white areas. A slurry was obtained, by mixing each sample with 1 ml of bidistilled water and then treated as reported above.

In order to evaluate the variation of OTA contamination during the shelf life, three contaminated and two blank samples were studied over one month. As the contaminated samples, three Gorgonzola cheeses (G1, G3, G14) have been considered, while two previously analysed Roquefort cheeses have been chosen as blank samples (F4, F23). In particular, each serving (450 g) was cut into three aliquots (150 g): the first portion was analysed immediately, the other two sub-samples were conserved in sterilised plastic wrap at 4 °C and analysed after 15 and 30 days, respectively.

2.3. HPLC analysis and method optimisation

Chromatographic analyses were performed with an Alliance 2695 chromatographic system (Waters, Milford, MA, USA) equipped with a Model 474 fluorescence detector ($\lambda_{ex} = 380$ nm and $\lambda_{em} = 440$ nm). A X-Terra™ C_{18} Waters column (250 mm × 2.1 mm, 3 μm particles) was used, on account of its stability in a wide pH range (2–12). The eluent was a mixture of acetonitrile-ammonia buffer (NH_4Cl/NH_3, 20 mM, pH 9.8) 15:85 v/v; the flow rate was set at 0.2 ml/min. The injection volume was 5 μl. The linearity was tested by injecting OTA standard solutions at six concentration levels (range: 0.2–2.5 ng/ml; levels: 0.2, 0.5, 1.0, 1.5, 2.0 and 2.5 ng/ml; three determinations were performed at each level). The significance of linear regression and intercept (not significantly different from 0) were calculated by SPSS 8.0 statistical software with the ANOVA model and the Student's t-test, respectively (regression: $Y = (53402 \pm 434)X$, $r^2 = 0.995$). Recovery experiments and calibration curves were obtained in the same concentration range. The recovery experiments were performed at three concentration levels (0.5, 1.0, 2.0 μg/kg) by adding a proper amount of freshly prepared OTA solution (100 μg/ml in water) to the slurry. After spiking, the slurry was mixed and left overnight at −8 °C. The limit of quantification (LOQ) and the limit of detection (LOD) in blue cheese were 0.1 and 0.02 μg/kg, respectively. Inter-day repeatability was evaluated at two concentration levels (0.5 and 1.5 μg/kg) giving 11% as average CV%.

2.4. Identity assessment of OTA by hydrolysis with carboxypeptidase A

Hydrolysis of OTA to its hydrolysed form ochratoxin α (OTα) and phenylalanine was performed according to the protocol of Hult et al. (1979). To the dried OTA residue, 200 μl of bovine carboxipeptidase A and 2 ml of PBS solution were added. The mixture was incubated for 20 h at 38 °C (nominal), then 5 μl were used for the HPLC determination. The reaction yield was quantitative. This procedure was used both for the OTA standard solution and for the cheese extracts.

2.5. Confirmation of OTA identity by LC-ESI-MS/MS

The identity of the OTA was also confirmed by LC-ESI-MS/MS analysis, using a LC-MS system which consisted of a 2695 Alliance and a Quattro™ triple quadrupole mass spectrometer (all from Waters). The chromatographic conditions involved a narrowbore X-Terra™ C18 Waters column (150 × 2.1 mm, 3 μm particles) and H_2O–CH_3CN 1:1 v/v as mobile phase, acidified with HCOOH 0.2%. The flow was set at 0.2 ml/min. The electrospray parameters, optimised for the OTA analysis in the positive ion mode, are the following: the capillary and the cone voltages were 3.0 kV and 35 V, respectively; the source and the desolvation temperatures were 120 °C and 250 °C, respectively; the collision energy was set at 22 eV. The acquisition was performed in multiple reaction monitoring (MRM), using the specific transitions m/z 404 → 239 and m/z 404 → 358 (Zoellner & Mayer-Helm, 2006).

3. Results and discussion

3.1. Method development and performance

Cheese and dairy products are well-known complex matrices and, in particular, the mould-ripened cheese is a non homogenous material usually difficult to sample. Therefore, a "slurry" preparation was chosen as sampling strategy, following Spanjer et al. (2006): although this approach is more time-consuming than the commonly randomised sampling, it assures a representative and reproducible analysis even for non homogenous media.

The extraction procedure was developed taking into account the protonation equilibria of ochratoxin A, which is soluble either in alkaline solutions or in organic solvents according to pH. In particular, a crude extract was obtained from cheese by a solid–liquid extraction with chloroform after acidification of the sample (pH 3), in order to keep OTA in its neutral form. Then, OTA was extracted from the organic phase by liquid–liquid partition, using a bicarbonate saturated solution (pH 8.5). The slightly alkaline pH allowed for the back-extraction of the dianionic form of OTA, allowing purification of the analyte from most of the interferents. The purified extract was then applied to an immunoaffinity column, achieving a better clean up and an enrichment of the analyte concentration. In comparison with the method recently proposed by Kokkonen et al. (2005), which requires a solid-liquid extraction followed by a liquid–liquid separation step, our method is more suitable for HPLC-FLD analysis since the immunoaffinity step provides a better purification of the extract.

The chromatographic separation was then performed by HPLC with fluorescence detection, using the method previously proposed by us (Dall'Asta, Galaverna, Dossena, & Marchelli, 2004). The use of an alkaline eluent allowed very good sensitivity, reaching a limit of detection (LOD) in blue cheese of 0.02 μg/kg, expressed as signal to noise ratio 3/1. The limit of detection obtained here is similar to those usually obtained for OTA detection in grains and beverages by using IAC clean up and HPLC-FLD detection (Castellari, Fabbri, Fabiani, Amati, & Galassi, 2002) and it is more then one order of magnitude lower then the LOD obtained by Kokkonen et al. (2005) for ochratoxin A (0.3 μg/kg), using a tandem mass spectrometry technique.

The method showed a good linearity for OTA between 0.2 and 2.5 μg/kg and no matrix effect was observed in the same concentration range. Repeatability at both concentration levels was satisfactory, the RSDs being lower than 12.1%.

Recovery experiments were performed on OTA free samples (three determinations at each concentration level were performed), as described in the Section 2. With the

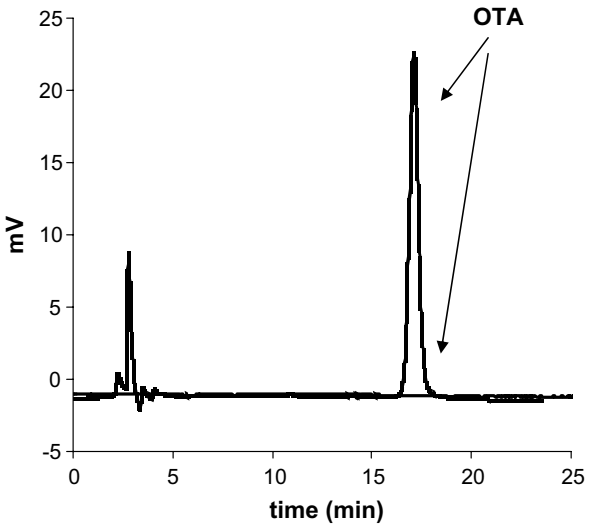

Fig. 2. Chromatogram of an OTA contaminated cheese sample (0.5 μg/kg) before (grey line) and after (black line) enzymatic reaction by carboxypeptidase A.

present method, within the spiking range of 0.5–2.0 μg/kg, an average mean recovery of 97 ± 1% was obtained for the OTA, while the average RSD was 3%. Thus, the procedure was proven to be simple and suitable for detecting ochratoxin A in cheese.

3.2. Ochratoxin A in blue cheese samples

The method developed here was applied to 92 blue cheeses purchased from the market: 54 samples of Gorgonzola cheese from Italy, 20 samples of Bleau and 14 samples of Roquefort cheeses from France and 4 samples of Bergader cheese from Germany.

Although the blue cheese classes considered here should be inoculated only with *Penicillium roqueforti* (known as a non OTA producer), surprisingly OTA was found in several samples: in 23 out of 54 Gorgonzola samples and 7 out of 14 Roquefort samples OTA was present at a variable level (0.25–3.0 μg/kg), moreover, the contamination was under 0.25 μg/kg but still detectable in seven samples. No contamination was found in the Bleau and Bergader cheeses examined. However, the small number of Bergader cheese ($n = 4$) considered in this study cannot actually be believed to be significative, to exclude the possible occurrence of ochratoxin A also in this cheese typology.

Since the ochratoxin A occurrence was surprising, a confirmation test with carboxypeptidase A was performed for all the positive samples, in order to unambiguously identify the analyte. The test unequivocally recognised the chromatographic peak at a retention time of 18 min as ochratoxin A (Fig. 2), since it disappeared after incubation with the enzyme.

The identity of the analyte was furtherly confirmed by LC-ESI-MS/MS analysis, by monitoring the specific OTA transitions 404 → 358 and 404 → 259, which arise from the loss of CO_2 and from the cleavage of the amidic bond from the protonated molecular ion, respectively. Also in this case the analysis unambiguously confirmed the identity of the peak as ochratoxin A, as shown in Fig. 3.

The OTA concentrations found in blue cheeses are reported in Table 1, where both media and median values are also reported for each category.

Since the occurrence of OTA in blue cheeses had not been detected previously, the origin of the contamination was investigated by separately analysing the white and the green portions of the cheese. If OTA was produced by toxinogenic-strains, its occurrence should be localised around the moulded spots, whereas if it is related rather

Table 1
Occurrence of OTA in mould-ripened blue cheeses (each determination was performed in triplicate)

	N° positive /N° total	Media (μg/kg)	Median (μg/kg)	Concentration range (μg/kg)
Gorgonzola (I)	23/54	0.71	0.30	0.2– 3.0
Bleau (F)	0/20	–	–	–
Roquefort (F)	7/14	0.63	0.44	0.1–1.4
Bergader (D)	0/4	–	–	–
Total samples	30/92	0.69	0.35	0.1–3.0

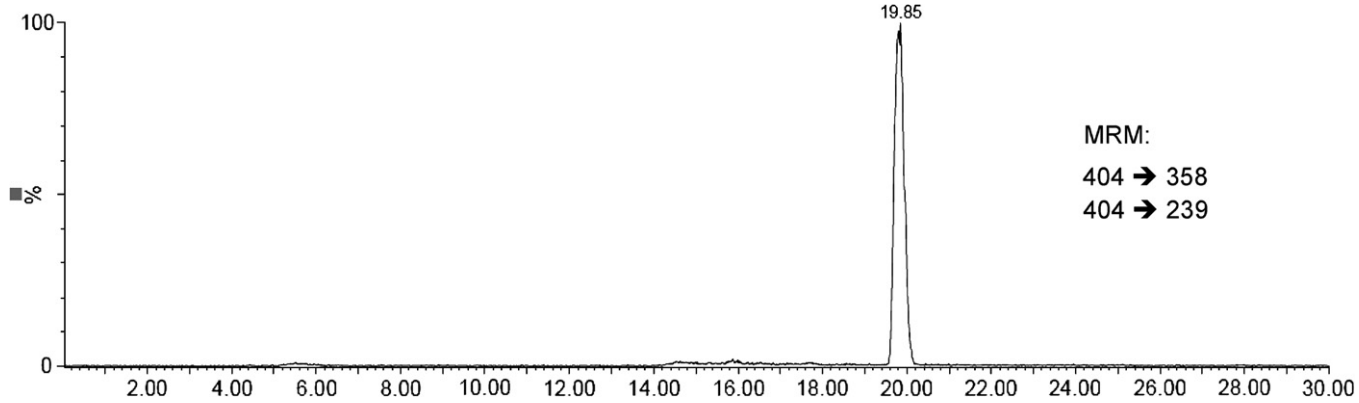

Fig. 3. Confirmation of the OTA identity by LC-ESI-MS/MS analysis: chromatogram of an OTA contaminated blue cheese (1.2 μg/kg), obtained using MRM acquisition mode and reported by adding together the acquired transitions (404 → 358; 404 → 239).

to milk contamination, then the analyte should be present also in the white areas. Indeed, although the well-known ability of cow's rumen microflora to cleave the amidic bond of ochratoxin A to the less toxic metabolite ochratoxin α, an OTA occurrence in milk was reported for animals fed with strongly contaminated feed (Breitholtz-Emanuelsson, Olsen, Oskarsson, Palminger, & Hult, 1993; Skaug, 1999).

In order to perform this investigation, three OTA-containing cheeses were analysed by sampling separately the green and white portions in triplicate for each sample. The results are reported in Fig. 4.

The data clearly demonstrates that the contamination did not derive from contaminated milk, but it was strictly associated with the moulded spots, as suggested by the absence of OTA in the creamy white portion of cheese. Moreover, the non homogenous occurrence of high levels of OTA in different areas of the cheese strongly support the hypothesis that OTA-producing strains are present,

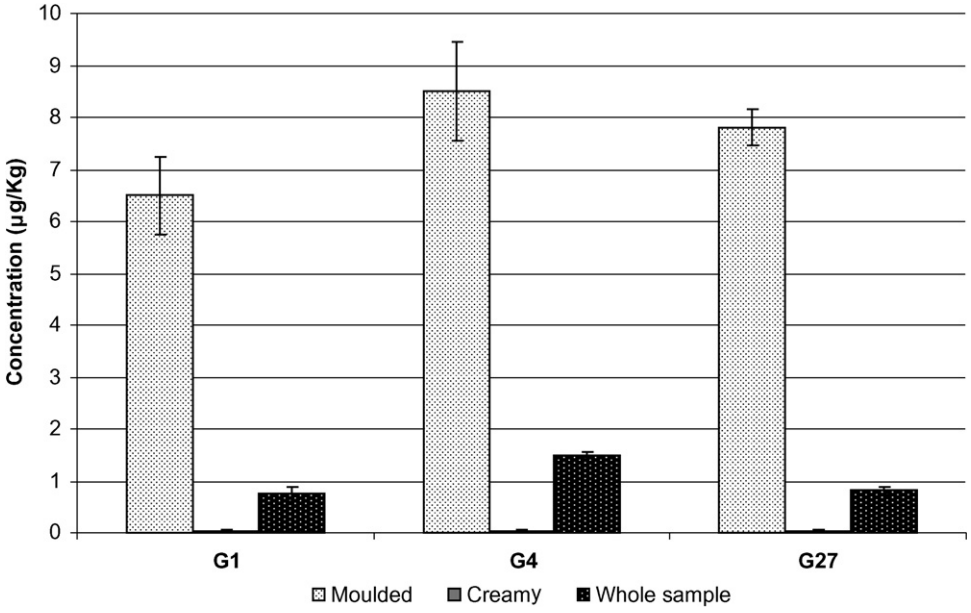

Fig. 4. Comparison of the OTA content in the whole cheese, in the green spots and in the white cream in three Gorgonzola cheese samples (G1, G4, G27). Each determination was performed in triplicate.

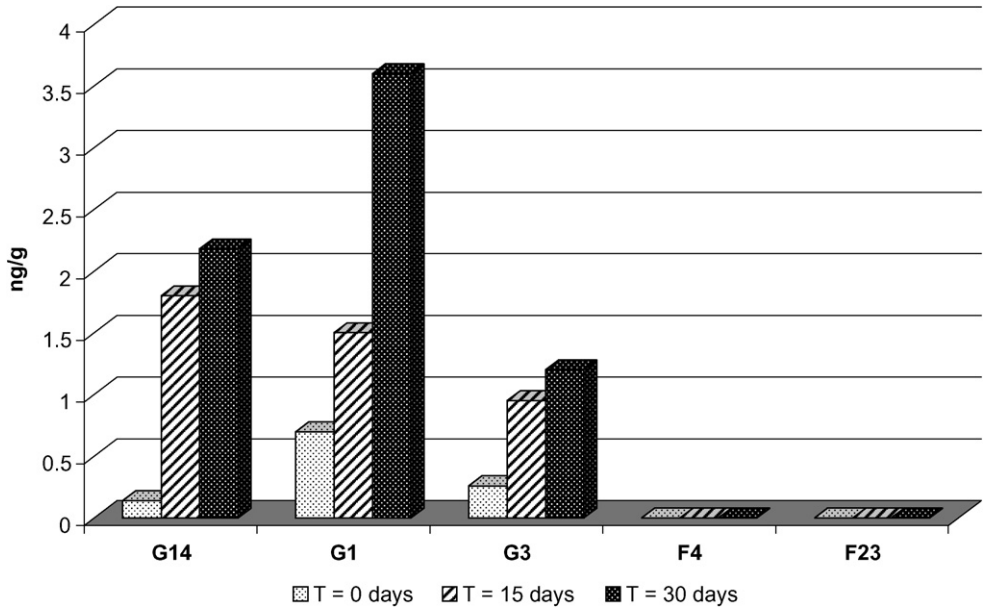

Fig. 5. Variation of OTA content during the shelf life in three Gorgonzola cheese samples (G14, G1, G3; contaminated samples at T0) and in two Roquefort cheese samples (F4, F23; blank samples at T0).

which contaminated the cheese during the manufacturing procedure.

In order to define if the contamination level increases during the shelf life, three contaminated and two blank samples were studied over one month. From the data reported in Fig. 5, the OTA level increased during shelf life for all the samples contaminated, whereas no traces of OTA were detected, when the contamination was not initially present.

The fact that the OTA level increased during the shelf life suggests that some ochratoxinogenic strains might be present and active in cheese over that period, excluding the occurrence of accidental contamination during storage. On the other hand, the storage temperature was not suitable for a significative OTA production: the high increase in OTA concentration could be due to a better extraction of the analyte from the matrix, induced by the proteolysis of the system. Whether OTA is produced by the actual industrial *Penicillium* strains or by an accidental concomitant microorganism is yet to be studied. The identification of the contamination source is actually a question of concern for food safety and risk assessment, although the OTA level in blue cheeses found in the present study does not appear to be hazardous for the consumers.

4. Conclusions

In this paper, we have shown for the first time that, by using an optimised procedure of sampling, extraction, purification and detection by HPLC-FLD, ochratoxin A may be present in different commercial samples of blue-mould ripened cheeses (Gorgonzola and Roquefort), although at very low levels.

The sampling procedure, based on a slurry strategy, which requires the homogenisation of the whole portion of cheese bought at the supermarket, is reliable and representative for the whole sample and it is fundamental for an accurate determination of low amounts of OTA in moulded cheese, since the risk of false negative results is serious. Indeed, high concentrations of OTA in a relatively small area (the green moulded spots) are averaged with the broadly extended and not OTA-contaminated white portion, acting as a kind of "dilution effect". For this reason, the sensitivity of the analytical procedure is a crucial point for OTA detection in moulded cheese.

The immunoaffinity clean up, which gives a good purification and an enrichment of the analyte, associated to the use of an alkaline mobile phase in HPLC, which brings about a fluorescence enhancement of the OTA signal, allowed very low detection limits. Therefore, the procedure proposed here is a reliable control tool for risk assessment in blue cheese.

Although the ochratoxin A occurrence in blue cheese should be considered as not hazardous for the consumers, since the detected levels are similar to those usually accepted for other food commodities, the origin of the contamination has not yet been clarified. Further studies will be necessary to evaluate the critical control points in blue-cheese manufacturing and to adopt, when required, a preventive strategy to avoid OTA contamination.

Acknowledgements

The present work has been performed within a project (SIQUAL) partially financed by the Emilia-Romagna Region (Italy).

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