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Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods

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Aflatoxins are among the most toxic mycotoxins. Early detection and quantification of aflatoxin-producing species is crucial to improve food safety. In the present work, two protocols of real-time PCR (qPCR) based on SYBR Green and TaqMan were developed, and their sensitivity and specificity were evaluated. Primers and probes were designed from the o-methyltransferase gene (*omt-1*) involved in aflatoxin biosynthesis. Fifty-three mold strains representing aflatoxin producers and non-producers of different species, usually reported in food products, were used as references. All strains were tested for aflatoxins production by high-performance liquid chromatography–mass spectrometry (HPLC–MS). The functionality of the proposed qPCR method was demonstrated by the strong linear relationship of the standard curves constructed with the *omt-1* gene copy number and Ct values for the different aflatoxin producers tested. The ability of the qPCR protocols to quantify aflatoxin-producing molds was evaluated in different artificially inoculated foods. A good linear correlation was obtained over the range 4 to 1 log cfu/g per reaction for all qPCR assays in the different food matrices (peanuts, spices and dry-fermented sausages). The detection limit in all inoculated foods ranged from 1 to 2 log cfu/g for SYBR Green and TaqMan assays. No significant effect was observed due to the different equipment, operator, and qPCR methodology used in the tests of repeatability and reproducibility for different foods. The proposed methods quantified with high efficiency the fungal load in foods. These qPCR protocols are proposed for use to quantify aflatoxin-producing molds in food products.

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1. Introduction

Aflatoxins are among the most toxic mycotoxins (Molina and Giannuzzi, 2002; Passone et al., 2010; Sardiñas et al., 2011). These toxic compounds are potent carcinogenic and mutagenic secondary metabolites produced by the *Aspergillus* genus, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Mayer et al., 2003; Schmidt-Heydt et al., 2009), but also by other species belonging to this genus such as *Aspergillus ochraceoroseus*, *Aspergillus toxicarius*, *Aspergillus arachidicola* and *Aspergillus minisclerotigenes* (Cary et al., 2005; Pildain et al., 2008; Varga et al., 2009). In addition, some species of *Emericella* genus such as *Emericella astellata*, *Emericella venezuelensis* and *Emericella olivicola* and *Rhizopus* produce also aflatoxins (Erdogan, 2004; Cary et al., 2005; Varga et al., 2009). Although *Penicillium* species are not usually involved in the production of aflatoxins, some particular strains belonging to this genus may produce them. Molds of the above species, some of them aflatoxin producers have been reported as contaminants in different foods such as cereals (Zinedine et al., 2007), peanuts (Pildain et al., 2008; Passone et al., 2010), almonds (Rodrigues et al., 2009) and spices (Manonmani et al., 2005; Hernández et al., 2008). In addition, a relevant fungal population grows on the surface of ripened foods such as dry-cured meat products and dry-ripened cheeses throughout the ripening process when temperatures range from 10 to 25 °C (Núñez et al., 1996; Kure et al., 2004). Thus, in dry-ripened foods presence of aflatoxin-producing molds may be a potential health hazard, especially if it is considered that time of ripening at the temperatures indicated above could take from 3 to 24 months (depending on the product). In fact, production of aflatoxins has been reported in some kind of dry-ripened foods such as dry-cured ham as consequence of fungal growth (Rojas et al., 1991). To prevent aflatoxins production in the above foods, hygienic measures should be taken to avoid contamination and proliferation by producing strains entering the food chain. In addition, rapid and accurate

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methods to quantify aflatoxin-producing molds in raw materials, pre-processed and prepared foods are necessary to take appropriate corrective measures which avoid risk of aflatoxins production. For this purpose, nucleic acid methods could be an appropriate alternative to traditional isolation techniques, as they are rapid, sensitive, easily replaceable, and highly specific allowing accurate detection of fungal species (Shapira et al., 1996; Selma et al., 2008; Gil-Serna et al., 2009). Real-time quantitative PCR (qPCR) moreover provides a tool for accurate and sensitive quantification of target DNA (Mulé et al., 2006; González-Salgado et al., 2009; Rodríguez et al., 2011b), that could be applied to quantify aflatoxins-producing molds. In addition, qPCR has greatly simplified the procedure relative to conventional culturing techniques, with the continuous monitoring of samples through amplification which allows for their easy identification using either the fluorescence of non-specific dyes, such as SYBR Green, which can also give a signal for primer-dimers and non-specific amplified products (Kubista et al., 2006), or a sequence specific hydrolysis probe (TaqMan).

To develop specific qPCR assays to quantify aflatoxins-producing molds, it is essential to confirm from the reference strains tested which of them are really producers. This requires using sensitive methods to measure the aflatoxins production from reference mold strains. High-performance liquid chromatography–mass spectrometry (HPLC–MS) has been reported to be a sensitive method for detecting aflatoxins production (Sosa et al., 2002).

Recently, several qPCR methods for detection of aflatoxin-producing *Aspergillus* spp from different food products have been reported, such as *A. flavus* in maize, pepper and paprika (Mayer et al., 2003), and *A. flavus* and *A. parasiticus* in stored peanuts (Passone et al., 2010) and wheat flour (Sardiñas et al., 2011). However, no qPCR assay has been designed for quantifying aflatoxin producers belonging to different mold species in various commodities. The correct choice of target sequence for the design of primers is essential to develop new qPCR protocols for the detection and quantification of aflatoxin-producing strains belonging to different mold species and genera. The sterigmatocystin O-methyltransferase gene (*omt-1*), that converts sterigmatocystin to O-methylsterigmatocystin (Lee et al., 2004) or dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin (Yu et al., 2004b) depending on the previous expression of the dehydrogenase gene (*aad*), has been reported as a structural gene of the aflatoxin gene cluster together with norsolorinic acid reductase (*nor1*) and versicolorin A dehydrogenase (*ver1*) and is activated by the aflR gene product, AFLR (Sweeney et al., 2000). For this reason, this gene could be useful to detect and quantify every aflatoxin-producing mold regardless of whether it produces aflatoxin B or G. In addition, the *omt-1* gene has successfully been used for the detection of aflatoxin-producing *A. flavus* and *A. parasiticus* by conventional PCR (Shapira et al., 1996; Färber et al., 1997; Richard et al., 2009). Nevertheless, no qPCR protocol has yet been developed to detect and quantify aflatoxin-producing molds regardless of the kind of aflatoxin produced, which would be of great interest to monitor toxigenic molds in food products.

The usefulness of PCR-based detection of microorganisms in complex biological samples, such as food samples, is limited in part by the presence of substances that inhibit the PCR or reduce the amplification efficiency (Al-Soud and Rådström, 1998). PCR inhibitors may act by interference with the cell lysis necessary for DNA extraction, by nucleic acid degradation or capture and by inhibition of the polymerase activity necessary for amplification of the target DNA (Trochimchuk et al., 2003; Elizaquível and Aznar, 2008). The inhibition of the amplification reaction is one of the most limiting factors and can cause complete reaction failure, leading to false negative results or reduced sensitivity of specific detection of the mycotoxigenic producers (Monnet et al., 2006; Mulé et al., 2006). Therefore, testing directly on such foods is required to evaluate the potential application of qPCR-based methods for the detection and quantification of molds. The aim of the present work was to develop sensitive and specific qPCR methods to quantify aflatoxin-producing molds in foods.

2. Materials and methods

2.1. Fungal strains

Fifty-three mold strains, belonging to different species, were obtained from the Spanish Type Culture Collection (CECT), the Centraalbureau voor Schimmelcultures in The Netherlands (CBS), the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark (IBT), and the Culture Collection of Food Hygiene and Safety of the University of Extremadura. Only 10 of them were known as aflatoxin producers, whereas no information on aflatoxins production was available for the remaining strains, as indicated in Table 1.

2.2. Aflatoxins production

Production of aflatoxins was tested after growing the mold strains in Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) for 15 days at 25 °C. Then, the content of three Petri dishes was extracted with chloroform, filtered twice through anhydrous sodium sulfate and evaporated in a rotatory evaporator at 40 °C as described by Sosa et al. (2002). The residue was resuspended in 5 mL of chloroform, filtered through a 0.45-mm pore size nylon membrane, and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4 °C in the dark until required and resuspended in 200 μL of acetonitrile just before analysis. Aflatoxins production was analyzed by HPLC–MS, obtaining the full MS spectra after atmospheric pressure chemical ionization (Sosa et al., 2002).

2.3. DNA extraction

All mold strains were 3-point inoculated on MEA and incubated for 4 days at 25 °C. Grown mycelium was scraped off the agar and about 50 mg of isolated mycelium from each strain were used for genomic DNA extraction following the method described by Sánchez et al. (2008). DNA concentration was quantified spectrophotometrically in a Biophotometer Eppendorf (Eppendorf AG, Hamburg, Germany) and all of the DNA samples extracted from producing and non-producing strains were adjusted to the same concentration for the qPCR assays.

2.4. Primers and probes design

Genomic DNA from both aflatoxin-producing and non-producing strains was used with primers *OMT-forward* and *OMT-reverse*, based upon the *omt-1* gene, for amplification by a conventional PCR method described by Richard et al. (2009). An amplicon of 1254 bp was obtained from most aflatoxin-producing strains of *Penicillium* and *Aspergillus* species. This amplicon was purified and sequenced. The sequences obtained were deposited in the NCBI database. Then, a specific primer pair F/R-omt (Table 2) was designed from conserved regions using Primer Express software (Applied Biosystems, Foster City, CA, USA). With these primer pairs, an amplicon of 123 bp was obtained in the qPCR SYBR Green assay. To test the specificity of the above amplicons, they were purified, sequenced and aligned with the published sequence of the *omt-1* gene (GenBank accession no. L25835.1) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Table 1
Aflatoxins B1 and G1 production and qPCR data with the specific primers (F-omt and R-omt) and *OMT*probe.

Species designation	Strain reference	Information provided by Culture Collections	Aflatoxin production (μg/l) as detected by HPLC–MS	PCR amplification in qPCR reactions (SYBR Green and TaqMan)
Aspergillus awamori	[a]CBS 101702	Unknown	[e]ND	[h]–
Aspergillus flavus	[b]CECT 2684	Unknown	[f]45.64/[g]ND	[i]+
Aspergillus flavus	CECT 2687	Producer	3663.4/ND	+
Aspergillus flavus	[c]IBT 3696	Producer	193.6/ND	+
Aspergillus flavus	CBS 573.65	Producer	16.664.4/ND	+
Aspergillus flavus	CBS 120.62	Producer	528.3/ND	+
Aspergillus foetidus	CBS 101708	Unknown	236,271.9/ND	+
Aspergillus fumigatus	CBS 192.65	Producer	ND	–
Aspergillus niger	CECT 20157	Unknown	ND	–
Aspergillus ochraceoroseus	CBS 101887	Unknown	24.47/ND	+
Aspergillus oryzae(1)	CECT 2095	Unknown	23.21/ND	+
Aspergillus oryzae(2)	CECT 2094	Unknown	393.68/ND	+
Aspergillus parasiticus	CECT 2688	Unknown	1512.2/497.2	+
Aspergillus parasiticus	CECT 2682	Producer	1021.3/590.1	+
Aspergillus parasiticus	CECT 2681	Producer	47,020/ND	+
Aspergillus parasiticus	CBS 571.65	Producer	22.7/ND	+
Aspergillus tamarii	CBS 575.65	Unknown	8.64/ND	+
Aspergillus tamarii	CBS 109.63	Unknown	5.98/ND	+
Aspergillus terreus	CBS 601.65	Unknown	ND	–
Aspergillus toxicarius	CBS 822.72	Producer	ND	–
Aspergillus tubingensis	CECT 20543	Unknown	942.5/ND	+
Aspergillus versicolor	CECT 2664	Unknown	ND	–
Aspergillus versicolor	CECT 2903	Unknown	ND	–
Aspergillus versicolor	CECT 2814	Unknown	24,249.0/ND	+
Emericella heterothallica	CBS 488.65	Unknown	ND	–
Emericella nidulans	CBS 465.65	Unknown	ND	–
Emericella quadrilineata	CBS 235.65	Unknown	ND	–
Emericella rugulosa	CBS 133.60	Unknown	8.8/ND	+
Emericella variecolor var. Astellana	CBS 133.55	Unknown	9.6/ND	+
Penicillium aurantiogriseum	CECT 2918	Unknown	ND	–
Penicillium aurantiogriseum	CBS 112021	Unknown	136.7/ND	+
Penicillium aurantiogriseum	CECT 2264	Unknown	3.9/ND	+
Penicillium camemberti	CECT 2267	Unknown	ND	–
Penicillium carneum	CBS 468.95	Unknown	ND	–
Penicillium commune	CBS 311.48	Unknown	115.2/ND	+
Penicillium commune	CBS 341.59	Unknown	9443.8/ND	+
Penicillium commune	CBS 247.32	Unknown	ND	–
Penicillium dipodomyicola	CBS 110425	Unknown	ND	–
Penicillium dipodomyicola	CBS 110426	Unknown	ND	–
Penicillium expansum	CECT 2278	Unknown	ND	–
Penicillium expansum	CECT 20140	Unknown	ND	–
Penicillium griseofulvum(3)	IBT 14319	Unknown	250.5/ND	±
Penicillium griseofulvum	CBS 485.84	Unknown	ND	–
Penicillium griseofulvum	CECT 2919	Unknown	ND	–
Penicillium melanoconidium	CBS 64195	Unknown	ND	–
Penicillium melanoconidium	CBS 109605	Unknown	ND	–
Penicillium nordicum	CBS 110769	Unknown	ND	–
Penicillium polonicum	Pc 10[d]	Unknown	ND	–
Penicillium polonicum	CBS 112490	Unknown	ND	–
Penicillium verrucosum	Pc 4	Unknown	ND	–
Penicillium verrucosum	CECT 2906	Unknown	ND	–
Penicillium viridicatum	CECT 2320	Unknown	ND	–
Rhizopus oryzae	CBS 607.68	Producer	11.7/ND	+

Strains marked with a number, in the first column (species designation), were identified as *A. flavus* (1), *A. parasiticus* (2) and *P. commune* (3).
No Template Control (NTC) Ct value was established in 35 and 40 by SYBR Green and TaqMan assays, respectively.
All aflatoxin-producing mold strains are shaded.
[a] CBS: Centraalbureau voor Schimmelcultures (The Netherlands).
[b] CECT: Spanish Type Culture Collection.
[c] IBT: Type Culture Collection of the Department of Biotechnology (Technical University of Denmark).
[d] Pc: strains isolated from dry-cured ham belonging to the Culture Collection of Food Hygiene from University of Extremadura.
[e] ND: aflatoxin production is not detected by HPLC–MS.
[f] Aflatoxin B1 concentration (μg/l) detected by HPLC–MS.
[g] Aflatoxin G1 concentration (μg/l) detected by HPLC–MS.
[h] –: PCR amplification was not observed in SYBR Green and TaqMan qPCR reactions.
[i] +: PCR amplification was observed in SYBR Green and TaqMan qPCR reactions.

In addition, the *OMTprobe* TaqMan probe was designed for the TaqMan assay (Table 2). This probe was labeled at the 5′ end with the reporter hexachlorofluorescein (HEX) and at the 3′ end with the quencher Black Hole Quencher 1 (BHQ1) (Sigma Aldrich, Madrid, Spain).

2.5. qPCR reactions

The Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems) was used for qPCR amplification and detection. qPCR was prepared in triplicates of 25 μL reaction mixture in

Table 2
Nucleotide sequence of primers and probe used for either SYBR Green or TaqMan Real-Time PCR assays.

Primer pair	Primer name	Nucleotide sequences (5′-3′)	Product size	[a]Position
F/R-omt	F-omt	GGCCGCCGCTTTGATCTAGG	123 bp	1485
	R-omt	ACCACGACCGCCGCC		1593
OMTprobe		[HEX]-CCACTGGTAGAGGAGATGT-[BHQ1]		1531

[a] Positions are in accordance with the published sequences of *omt-1* gene of *A. flavus* (GenBank accession no. L25835.1).

MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). Three replicates of a control sample without DNA template were also included in the runs.

2.5.1. SYBR Green qPCR conditions

The primer pair F/R-omt was first evaluated in a SYBR Green protocol. For this, the DNA of an aflatoxin-producing strain was used (Fig. 1). To optimize the concentration of primers, different amounts ranging from 700 to 200 nM, were tested. The optimized SYBR Green protocol was carried out in a final volume of 25 μL, containing 5 μL of template DNA, 12.5 μL of 2× SYBR® *Premix Ex Taq*™ (Takara, Otsu, Shiga, Japan), 0.5 μL of 50× ROX™ Reference Dye (Takara) and 200 nM and 400 nM of F-omt and R-omt primers, respectively.

The following thermal cycling conditions were used for the SYBR Green method: a single step of 10 min at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After the final PCR cycle, a melting curve analysis of the PCR products was performed by heating to 60–95 °C and continuous measurement of the fluorescence to verify the PCR product. The threshold cycle (Ct) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated by the 7500 Fast System SDS software (Applied Biosystems). All the above assays were carried out in triplicate. The size of PCR products was verified by electrophoresis in 2.5% agarose gels. These gels were stained with ethidium bromide and visualized with UV transillumination.

2.5.2. TaqMan qPCR conditions

The F/R-omt primer pair and the *OMTprobe* were assayed with the aflatoxin-producing strain *A. flavus* CECT 2687 for the TaqMan-based PCR (Fig. 1). To optimize the reactions, several concentrations ranging from 400 to 125 nM for primers, and 400 nM to 100 nM for the probe were tested. The reaction mixture for these tests consisted of 12.5 μL of *Premix Ex Taq*™ (Takara), 0.5 μL of ROX of 50× ROX™ Reference Dye (Takara), 200 nM of F-omt primer and 400 nM each of R-omt primer and *OMTprobe*, and 5 μL of template DNA in a final volume of 25 μL. The thermal cycling conditions included an incubation of 2 min at 50 °C to allow the uracil-*N*-glycosylase (UNG) enzyme to be activated, an incubation step for 10 min at 95 °C to denature the UNG enzyme and activate AmpliTaq Gold polymerase, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Ct determinations were automatically performed by the instrument using default parameters. All the above assays were carried out in triplicate. The size of PCR products was verified by electrophoresis in 2.5% agarose gels. These gels were stained with ethidium bromide and visualized with UV transillumination.

2.6. Specificity of qPCR reactions

The specificity of the primers F-omt/R-omt was tested on a fixed amount (1.0 ng for SYBR Green and TaqMan assays) of genomic DNA of 53 strains of aflatoxin-producing and non-producing *Aspergillus*, *Emericella*, *Penicillium*, and *Rhizopus* species commonly found on

Assays of qPCR	Aflatoxin producing strains and food tested	Level of inoculation in foods
Development of methods	*A. flavus* CECT 2687	
Specificity of the developed methods	25 aflatoxin producing and 28 non producing strains, belonged to *Penicillium, Aspergillus, Emericella and Rhizopus,* listed in Table 1	
Construction of Standard curves	*A. flavus* CECT 2687, *A. parasiticus* CECT 2688 and *R. oryzae* CBS 607.68	
Sensitivity of developed methods in inoculated foods	Peanut inoculated with *A. parasiticus* CECT 2688 Black pepper inoculated with *P. griseofulvum* IBT14319 Dry fermented sausage "Salchichón" inoculated with *A. flavus* CECT 2687	From 4 to 1 log cfu/g
Conidia quantification in inoculated foods	Wheat, peanut, walnut, almond and raisin inoculated with *A. parasiticus* CECT 2688 Paprika, black pepper and oregano inoculated with *P. griseofulvum* IBT14319 Dry fermented sausages "salchichón" and "chorizo", dry-cured ham, dry-cured pork loin and ripened cheese inoculated with A. flavus CECT 2687	3.47 log cfu/g
Repeatibility and reproducibility of developed methods in inoculated foods	Peanut inoculated with *A. parasiticus* CECT 2688	2.30 log cfu/g
Fungal growth quantification in inoculated foods	Peanut, paprika and dry-cure ham with *A. parasiticus* CECT 2688	3 log cfu/g

Fig. 1. Assays to validate the developed qPCR SYBR Green and TaqMan methods, including aflatoxin-producing strains, foods tested and level of inoculation.

foods (Table 1, Fig. 1). The qPCR reactions were carried out as described in Section 2.5. To evaluate the specificity of the primers designed for the SYBR Green assay, the melting temperature (Tm) was automatically calculated and compared with that deduced from the sequence of the expected fragment. Next, for both SYBR Green and TaqMan assays, the size of amplicons was estimated by electrophoresis in 2.5% agarose gels.

In addition, *β-tubulin* partial sequences and/or the ITS1-5.8S-ITS2 region of the aflatoxin-producing species which had never been described as aflatoxin producers were obtained in order to confirm their identification given by their respective culture collections. PCR reactions for *β-tubulin* were performed using primers Bt2a and Bt2b (Glass and Donaldson, 1995). The amplification program used was: 1 cycle of 5 min at 94 °C, 32 cycles of 1 min at 94 °C, 1 min at 68 °C and 1 min at 72 °C and finally 1 cycle of 5 min at 72 °C. PCR reactions for the ITS1-5.8S-ITS2 region were performed using primers ITS1 and ITS4 (White et al., 1990). The amplification program used was: 1 cycle of 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C and finally 1 cycle of 5 min at 72 °C. Amplification products were purified and sequenced as described in Section 2.4. *β-tubulin* and ITS-5.8S-ITS2 sequences of the aflatoxin-producing strains obtained in this work were deposited in the NCBI Database.

2.7. Standard curves

Standard curves were obtained for three aflatoxin-producing strains (*A. flavus* CECT 2687, *A. parasiticus* CECT 2688 and *Rhizopus oryzae* CBS 607.68) following a procedure described by Mayer et al. (2003) (Fig. 1). For this, the 1254 bp fragment of the *omt-1* gene amplified with the primers *OMT-forward* and *OMT-reverse* (Richard et al., 2009) was obtained as standard stock solution. The concentration of the PCR products in the stock solution was determined in a Biophotometer Eppendorf and the number of copies was calculated. The stock solutions were serially diluted by a factor of 10 and an aliquot of the each dilution was used as a copy number standard during each setup of the qPCR reaction. Each standard curve with the three aflatoxin-producing strains was carried out in triplicate. The concentration of unknown samples was calculated by the 7500 Fast System software (Applied Biosystems).

The criteria considered for reliability of the designed methods were the correlation coefficient and the amplification efficiency calculated from the formula $E = 10^{-1/S} - 1$ (S being the slope of the linear fit).

2.8. Sensitivity of qPCR for aflatoxin-producing molds on artificially inoculated food matrices

The sensitivity of the optimized qPCR methods was tested with DNA extracted from three types of non-sterile commercial food products stored at 4 °C, each inoculated with uninucleate spores of different aflatoxin-producing strains to evaluate the efficiency of the method with different aflatoxin producers. Inoculations were carried out in accordance with the different niches for these producing molds as follows: peanut was inoculated with *A. parasiticus* CECT 2688, black pepper with *Penicillium griseofulvum* IBT 14319, and dry-fermented sausage ("salchichón") with *A. flavus* IBT 3696 (Fig. 1). To test the specificity of the two qPCR methods when applied to food, the three types of foods were also inoculated with the non-producing strain *Aspergillus niger* CECT 20157. For this, the spores were harvested by flooding 3 plates (20 days old) of MEA with 5 mL of sterile nanopure water containing 10% glycerol (Scharlau Chemie S.A., Spain), and rubbing the surface with a glass rod. The conidial suspension was filtered through Whatman paper No 1, diluted in sterile nanopure water as necessary, and quantified by microscopy, using a Neubauer counting chamber. Aliquots of 1 mL from 2 to 6 log conidia/mL of each mold strain were immediately used to inoculate food samples to concentrations of 0, 1, 2, 3, and 4 log cfu per gram of food. Then, 5 g of inoculated foods were treated for DNA extraction following the method described by Rodríguez et al. (2011a). All inoculations and extractions were performed in triplicate for each food. In addition, triplicates of a non-inoculated negative control were included in each experiment.

For qPCR amplification, 3 replicates of 5 μL DNA extracted from inoculated foods and non-inoculated negative controls were assayed per run. Standard curves were generated for each group of food products and the efficiencies for each standard curve were calculated.

To directly estimate the load of the inoculated mold, samples were diluted in 0.1% peptone water, spread-plated on Potato Dextrose Agar (PDA) (Sharlau Chemie S.A., Spain) and incubated at 25 °C for 4 days. The natural fungal contamination of samples was lower than 1 log cfu/g, and characterization of the isolates from non-inoculated food revealed no typical colonies of *A. parasiticus*, *P. griseofulvum* and *A. flavus*. In addition, DNA of these fungal isolates yielded negative results in the developed qPCR with SYBR Green.

To assess the absence of PCR inhibitors in the food matrices, three independent genomic DNA extractions from three aflatoxin-producing molds *A. parasiticus* CECT 2688, *P. griseofulvum* IBT 14319 and *A. flavus* IBT 3696, were inoculated in equal amounts (0.05 ng) to the total DNA extracted from different amounts (10, 5 and 1 g) of non-inoculated peanut, black pepper and dry-fermented sausage ("salchichón"). Amplification plots were compared with those obtained by amplifying the same pure strains diluted in deionized water.

2.9. Conidia quantification by qPCR in artificially inoculated foods

To test the ability of the qPCR methods to quantify aflatoxin-producing molds in different kinds of foods, fungal spores of different aflatoxin-producing strains were inoculated in thirteen different foods (Fig. 1), including cereal and nuts (wheat, raisin, almond, peanut and walnut), spices (black pepper, paprika and oregano) and ripened foods (dry-fermented sausage "salchichón", dry-fermented sausage "chorizo", dry-cured ham, dry-ripened cheese and dry-cured pork loin). Inoculations were carried out as follows: cereal and nuts with *A. parasiticus* CECT 2688, spices with *P. griseofulvum* IBT 14319 and ripened foods with *A. flavus* IBT 3696. Three independent tests were run at one level of fungal spores: 3.47 log cfu/g. The inoculation fungal level was determined by plate count in PDA. DNA extraction was carried out as described above in Section 2.8 for artificially inoculated food matrices. qPCR reactions were carried out as described above in Section 2.5 using 5 μL of DNA in triplicate. The Ct values for inoculated samples were obtained from standard curves obtained from artificially inoculated foods. Triplicates of non-inoculated negative controls were included in each experiment.

2.10. Repeatability and reproducibility of qPCR for aflatoxin-producing molds on artificially inoculated food matrices

To determine the repeatability (defined as the precision determined under conditions where the same methods and equipment are used by the same operator to make measurements on identical specimens) and the reproducibility (defined as the precision determined under conditions where the same methods but different equipment or laboratories are used or different

operators are involved) of proposed qPCR assays, peanut samples were inoculated with spores of an aflatoxin-producing mold (*A. parasiticus* CECT 2688) at a concentration of 2.30 log cfu/g (Fig. 1). For the repeatability tests, three samples were treated by the same operator and analyzed in the same equipment (Applied Biosystems 7500 Fast Real-Time PCR system). In reproducibility assays, five samples were treated by the same operator and analyzed in four laboratories with different Real-Time PCR systems located in the University of Extremadura ("laboratory reproducibility"), and three samples were treated by four different operators and analyzed in the same laboratory and with the same equipment ("*operator reproducibility*").

In both assays, DNA was extracted as described in Section 2.8 and subjected to the qPCR protocols designed as described in Section 2.5. The Ct values for inoculated samples were obtained from standard curves obtained from artificially inoculated peanuts. Triplicates of non-inoculated negative controls were included in each experiment.

2.11. Quantification by qPCR of fungal growth in artificially inoculated foods

Minced peanut, paprika and slices of dry-cured ham cut to a surface area of 25 cm^2 with approximately 5 g of weight were placed separately in pre-sterilized orthogonal receptacles made of methacrylate, where the humidity was kept constant by a saturated KCl solution placed in a reservoir. The samples were inoculated on the surface with spores of the aflatoxin-producing strain *A. parasiticus* CECT 2688 at a concentration of 3 log spores per gram and incubated for 14 days of incubation at 25 °C (Fig. 1). Sampling was carried out in triplicate from each at 0, 3, 5, 7, 10 and 14 days of incubation. Negative controls from non-inoculated peanut, paprika and dry-cured ham samples were also analyzed. DNA was extracted as described in Section 2.8 and subjected to the qPCR methods designed as described in Section 2.5. The Ct values obtained with SYBR Green and TaqMan assays for the inoculated samples were used to estimate both the conidial load and the *omt-1* gene copy number from the corresponding standard curves.

To directly estimate the load of the inoculated mold, the samples were decimally diluted in 0.1% peptone water and spread-plated on PDA. Colonies showing the characteristic morphology of *A. parasiticus* after incubation at 25 °C for 4 days were enumerated.

2.12. Statistical analysis

All the statistical analyses were performed with the SPSS v.15.0. One-way analysis of variance (ANOVA) was carried out to determine significant differences within and between groups. Tukey's test was applied to compare the mean values. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Detection of aflatoxins by HPLC–MS

HPLC–MS analysis confirmed aflatoxins production in 8 strains identified as aflatoxin producers by the Culture Collections (Table 1). Nevertheless, 2 strains (*Aspergillus fumigatus* CBS 192.65 and *A. toxicarius* CBS 822.72) reported as aflatoxin producers by the above Collections did not produce detectable amounts of this mycotoxin. In addition, 17 strains belonging to different species of the genera *Aspergillus*, *Penicillium* and *Emericella* with no information on aflatoxins production in the Culture Collections produced this mycotoxin. The concentration levels of aflatoxins produced by all of the producing strains tested ranged from 3.9 μg/l to 236 mg/l (Table 1).

In addition, several strains which showed aflatoxin production by HPLC–MS and had previously never been described as aflatoxin producers were identified by partial sequencing the ITS region or the *β-tubulin* gene. These sequences were analyzed and deposited in GenBank (GenBank accession no JN217227, JN217228, JN217229, JN217230, JN217231, JN217232, JN217233, JN217234, JN217235, JN217236, JN217237, JN217238, JN217239, JN217240, JN217241, JN217242). When the sequences obtained by the two genes were compared with the NCBI database the identification agreed with that previously reported by the Culture Collection in all cases, except *Aspergillus oryzae* CECT 2094, CECT 2095 and *P. griseofulvum* IBT 14319. These strains showed the greatest similarities (99%) with *A. flavus*, *A. parasiticus* and *Penicillium commune*, respectively.

3.2. Analysis of omt-1 gene sequences of aflatoxin-producing species

The DNA fragment of 1254 bp from the *omt-1* gene of the different species targeted was amplified and sequenced. These sequences were analyzed and deposited in GenBank matching with *omt-1* genes (GenBank accession no JN217219, JN217220, JN217221, JN217222, JN217223, JN217224, JN217225, JN217226). All these sequences were compared with the published sequence of the *omt-1* gene of *A. flavus* (GenBank accession no. L25835.1), showing a similarity greater than 99% in all cases.

3.3. Optimization of qPCR conditions

The F/R-omt primer pair gave one PCR product of the expected size with a Tm value of 86.4 ± 0.4 °C when using the SYBR Green method. In addition, non-specific amplification was observed, including primer-dimers.

The best primers and probe concentrations giving the lowest Ct value with an adequate fluorescence for a given target concentration were selected for further analyses. Therefore the F-omt and R-omt concentrations used for the SYBR Green reaction were 200 nM and 400 nM, respectively. The lowest Ct value for the TaqMan assay was obtained with 200 nM of forward primer and 400 nM of both reverse primer and probe.

3.4. Specificity of the qPCR reactions using reference strains

The primers and probe specificity was tested on genomic DNA from selected producing and non-producing species of *Aspergillus*, *Emericella*, *Penicillium*, and *Rhizopus* (Table 1). All aflatoxin-producing mold reference strains detected by HPLC–MS analysis showed Ct values of 21.3 ± 1.32 and 25.1 ± 1.43 in the SYBR Green and TaqMan assays, respectively, while no PCR amplification (Ct = 40) was detected in non-producing strains (Table 1). In addition, all aflatoxin producers showed Tm values ranging from 86.0 to 86.8 °C in the SYBR Green method, confirming the specificity of the two qPCR methods.

3.5. Standard curves constructed to calculate the number of copies of the omt-1 gene

The 1254-bp PCR product obtained by the conventional PCR method was used to build the standard curve to calculate the number of copies of the *omt-1* gene in the SYBR Green and TaqMan assays.

An example of standard curves which relate Ct values and the logarithm of template copies for each optimized method are shown in Fig. 2. No significant differences ($P \leq 0.05$) were found between

standard curves obtained by the different aflatoxin producers since the slopes and R^2 were nearly identical. A good linear relationship between Ct values and the *omt-1* gene copy number was obtained for both qPCR methods, with R^2 values close to 0.99 for SYBR Green and 0.98 for TaqMan assays. The slopes of the linear regression curves were −3.35 and −3.46 for SYBR Green and TaqMan assays, respectively (Fig. 2). The efficiency values were 98.8% for SYBR Green and 94.5% for TaqMan assays.

3.6. Sensitivity, repeatability and reproducibility of the qPCR assays on artificially inoculated food matrices

The ability of the optimized qPCR protocols to quantify aflatoxin-producing molds was evaluated in different artificially inoculated foods. Standard curves using DNA extracted from inoculated foods were generated for each food matrix. The slopes of the linear regression equations in the SYBR Green assays for peanut, black pepper and dry-fermented sausage ("salchichón") were respectively −3.12, −3.45 and −3.51, while with TaqMan assays the slope values for those products were −3.01, −3.50 and −3.62, respectively. A good linear correlation (R^2) was also obtained over the range 4 to 1 log cfu/g per reaction for all the food matrices (Table 3). The efficiencies ranged from 89 to 116% (Table 3). The detection limit was 1 log cfu/g for the SYBR Green in all the above inoculated foods, as well as for the TaqMan assay in peanut and dry-fermented sausage "salchichón" (data not shown). In black pepper, the detection limit for the TaqMan assay was 2 log cfu/g.

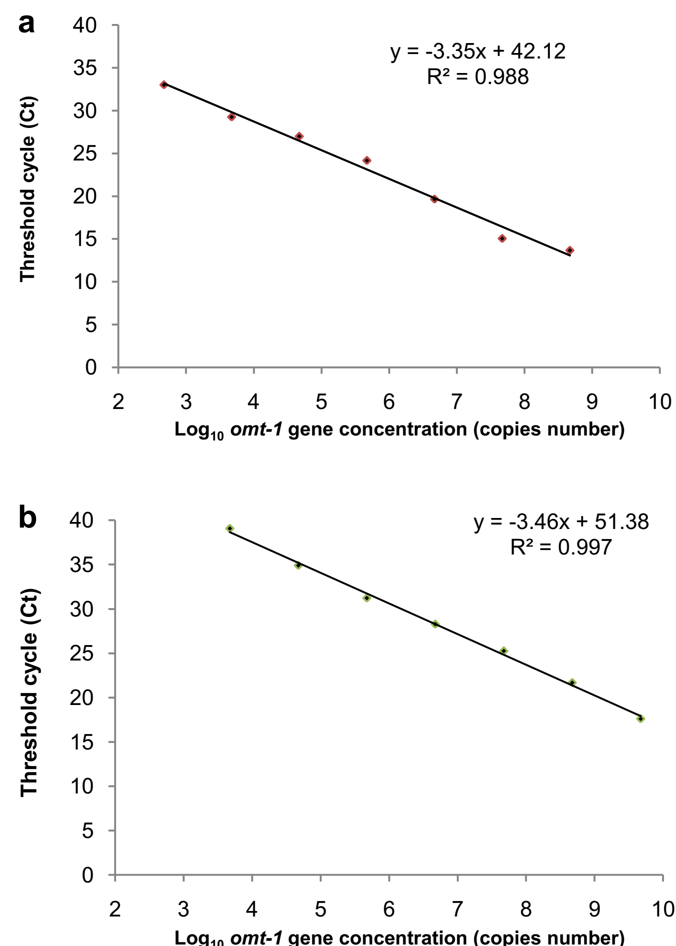

Fig. 2. Representative standard curves showing the correlation between the initial copy number of the *omt-1* gene (obtained with dilutions of the 1254-bp *omt-1* fragment) and threshold cycle (Ct) values of the SYBR Green (a) and TaqMan (b) Real-Time PCR methods for different concentrations of the *omt-1* gene copies. These curves were generated with three independent reactions.

No amplification (Ct = 40) was obtained in the above food matrices inoculated with a non-producing strain (*A. niger* CECT 20157).

Furthermore, absence of PCR inhibitors was studied for both qPCR assays in all of the inoculated foods by the comparison of the amplification plots of pure aflatoxin producer DNA and that added of DNA of non-inoculated foods. No significant differences were found between the Ct values obtained from aflatoxin-producing mold DNA, pure and pure added with food DNA (data not shown).

The ability of the designed qPCR methods to quantify a known conidial suspension inoculated on several foods is shown in Table 4. The levels of conidia assessed by both SYBR Green and TaqMan qPCR methods were very close to the inoculated amount for most foods. There were no significant differences between the levels of cfu per gram obtained by counting in PDA and those found in the SYBR Green and TaqMan qPCR. In addition, no significant effect was observed due to the different food matrices used ($P \leq 0.05$).

The repeatability of the two developed qPCR assays is shown in Table 5. The amount of conidia quantified by both qPCR methods was close to the inoculated amount in each peanut sample. There were no significant differences between methods and repetitions. The results of the two reproducibility assays carried out by the same operator in different laboratories and by different operators in the same laboratory are given in Tables 6 and 7, respectively. No significant effect was observed due to the different equipment and qPCR methods used ($P \leq 0.05$). The results obtained from the reproducibility assay of different operators showed the quantity of conidia determined by the developed qPCR assays to be very similar to than that obtained by counting in PDA and the standard deviations were very low. In addition, no significant differences were obtained due to the different operator or qPCR method used.

3.7. Quantification of the fungal load and omt-1 gene copies in inoculated foods

The ability of the designed qPCR methods to quantify the fungal load in inoculated food samples was evaluated after different incubation times. The number of *omt-1* gene copies and the cfu values were also determined. The results are shown in Fig. 3. The growth behavior of *A. parasiticus* CECT 2688 differed in the foods tested. In paprika, this *Aspergillus* strain started to grow after 1 days of incubation, whereas in dry-cured ham and peanut it showed a 3-day lag phase. The natural fungal contamination of the samples was lower than 1 log cfu/g, so that it was easy to identify and count the characteristic *A. parasiticus* colonies. The characterization of the isolates from non-inoculated food revealed no typical colonies of *A. parasiticus*. In addition, the DNA extracted from non-inoculated

Table 3
Efficiencies of amplification and R^2 obtained from standard curves of cfu from aflatoxin-producing molds in artificially inoculated food by qPCR.

Food sample	Aflatoxin-producing species	qPCR method	Efficiency (%)	R^2
Peanut	*A. parasiticus* CECT 2688	SYBR Green	[a]109 ± 0.3	0.99
		TaqMan	116 ± 1.8	0.98
Black pepper	*P. griseofulvum* IBT 14319	SYBR Green	95 ± 0.9	0.99
		TaqMan	93 ± 0.7	0.98
Dry-fermented sausage "salchichón"	*A. flavus* IBT 3696	SYBR Green	93 ± 1.5	0.98
		TaqMan	89 ± 1.3	0.98

[a] Data represent the mean efficiency ± standard deviation of the 3 independent assays each consisting of triplicate samples.

Table 4
Quantification of conidia (log cfu/g) from aflatoxin-producing molds in artificially inoculated foods by qPCR (inoculum level for all tested foods was 3.47 log cfu/g).

Food products	Foods	Plate count in PDA (log cfu/g)	Quantification by qPCR	
			SYBR Green (log cfu/g)	TaqMan (log cfu/g)
Cereal and nuts	Wheat	3.4 ± 0.21	[a]3.4 ± 0.15	[a]3.4 ± 0.03
	Raisin	3.4 ± 0.19	[a]3.5 ± 0.02	[a]3.4 ± 0.09
	Almond	3.5 ± 0.09	[a]3.5 ± 0.16	[a]3.4 ± 0.14
	Peanut	3.5 ± 0.09	[a]3.5 ± 0.06	[a]3.4 ± 0.05
	Walnut	3.4 ± 0.13	[a]3.4 ± 0.04	[a]3.4 ± 0.12
Spices	Black pepper	3.5 ± 0.16	[b]3.5 ± 0.10	[b]3.5 ± 0.09
	Paprika	3.4 ± 0.10	[b]3.4 ± 0.19	[b]3.4 ± 0.12
	Oregano	3.4 ± 0.14	[b]3.4 ± 0.07	[b]3.4 ± 0.14
Ripened foods	Dry-fermented sausage "salchichón"	3.5 ± 0.08	[c]3.5 ± 0.05	[c]3.5 ± 0.13
	Dry-fermented sausage "chorizo"	3.5 ± 0.11	[c]3.5 ± 0.17	[c]3.5 ± 0.21
	Dry-cured ham	3.5 ± 0.12	[c]3.4 ± 0.01	[c]3.5 ± 0.14
	Ripened cheese	3.5 ± 0.09	[c]3.5 ± 0.13	[c]3.5 ± 0.10
	Dry-cured pork loin	3.5 ± 0.19	[c]3.4 ± 0.08	[c]3.5 ± 0.11

All data represent the mean amount of conidia quantified ± standard deviation of the 3 independent assays, each consisting of triplicate samples.
[a] Data obtained from standard curve for peanut inoculated with *A. parasiticus* CECT 2688.
[b] Data obtained from standard curve for black pepper inoculated with *P. griseofulvum* IBT 14319.
[c] Data obtained from standard curve for dry-fermented sausage "salchichón" inoculated with *A. flavus* IBT 3696.

foods was tested by SYBR Green qPCR assays and no amplification (Ct = 40) in the above reactions was observed.

In general, the *omt-1* gene copies and the fungal load obtained by qPCR correlates well with the cfu data always obtaining R^2 values around 0.98. In all foods, both qPCR methods were able to quantify *omt-1* gene copies just after inoculation and before mycelium growth. In the most of the cases, the number of *omt-1* gene copies determined by qPCR was higher than the cfu data. Nevertheless there were no significant differences between fungal load determined by qPCR and by counting in PDA throughout the 14 days of incubation time. The standard deviation was lower than 0.30 in all phases of growth for both qPCR methods, except that at the beginning of the growth for dry-cured ham it was 0.41.

4. Discussion

In the present work, the *omt-1* gene was target for the development of specific qPCR assays for detecting and quantifying aflatoxin-producing molds. The primer pair F/R-omt designed from the *omt-1* gene was appropriate for qPCR, since non-specific amplifications were detected. The small divergence in the Tm value (86.0–86.8 °C) could be due to small differences in the sequence of the target *omt-1* gene of the different species and genera analyzed, given that the DNA melting curves are a function of the GC/AT radio, length, and sequences (Ririe et al., 1997).

Table 5
Repeatability assay carried out by the same operator on the same equipment in inoculated peanuts (inoculum level was 2.30 log cfu/g).

Repetition	Plate count in PDA (log cfu/g)[a]	Quantification from qPCR standard curves[a]	
		SYBR Green (log cfu/g)	TaqMan (log cfu/g)
1	2.4 ± 0.18[a]	2.4 ± 0.12	2.4 ± 0.07
2	2.3 ± 0.03	2.3 ± 0.12	2.2 ± 0.05
3	2.3 ± 0.04	2.4 ± 0.20	2.4 ± 0.16

[a] Data represent the mean log cfu/g ± standard deviation (SD).

The specificity of the designed F/R-omt primers pair and *OMTprobe* probe was confirmed in this study since all the strains detected by both qPCR methods showed production of aflatoxin (B and/or G) in the HPLC–MS analysis. No amplification by qPCR was observed for any of the strains that did not produce aflatoxins (B and/or G) in the HPLC–MS assay, even in the cases of *A. fumigatus* CBS 192.65 and *A. toxicarius* CBS 822.72, considered to be producers by their respective Culture Collections. Probably the information provided by their respective Culture Collection about the production of aflatoxin of these two strains should be revised, since no production was found when they were tested by HPLC–MS and negative results were obtained with both the proposed qPCR SYBR Green and TaqMan assays. In addition, several species of *Penicillium* (*Penicillium aurantiogriseum*, *P. griseofulvum* and *P. commune*), *Aspergillus* (*A. oryzae*, *Aspergillus tamarii* and *Aspergillus tubingensis*) and *Rhizopus* (*R. oryzae*), which previously had never been described as aflatoxin producers, showed production of this mycotoxin under HPLC–MS. To discard this fact could be due to species misidentifications, the former strains were tested for identifying by partial sequencing of ITS region or *β-tubulin* gene. In all of the above strains, the identification agrees with that reported by the respective Culture Collection, except for *A. oryzae* CECT 2094 and CECT 2095 and *P. griseofulvum* IBT 14319. *A. oryzae* CECT 2094 and CECT 2095 always showed a similarity greater than 99% with *A. flavus* and *A. parasiticus*, respectively, by both amplification partial sequencing of the ITS region and the *β-tubulin* gene. These results are in consonance with a more properly identification according to production of aflatoxins, since *A. flavus* and *A. parasiticus* are mold species usually reported as aflatoxin producers (Mayer et al., 2003; Schmidt-Heydt et al., 2009), but not *A. oryzae* which genetic basis for the non-ability to produce aflatoxins have been reported (Van den Broek et al., 2001; Chang and Ehrlich, 2010). Thus, the identification of these two strains as *A. oryzae* provided by their Culture Collection should be revised. In the present work these strains were tentatively renamed as *A. flavus* CECT 2094 and *A. parasiticus* CECT 2095 respectively. With regard to *P. griseofulvum* IBT 14319, the identification does not agree with that reported by its Culture Collection since it showed a similarity of around of 99% with the *P. commune* species. Thus, this strain was tentatively renamed as *P. commune* IBT 14319.

The specificity of the two developed SYBR Green and TaqMan qPCR assays was demonstrated, since both of them provided a good discrimination between aflatoxin-producing and non-producing strains across species and genera. The functionality of the two methods was also demonstrated by the strong linear relationship of the standard curves constructed with the *omt-1* gene copy number and Ct values for the different aflatoxin producers tested. In addition, there was no significant difference in the slope and R^2 between standard curves generated with different producing strains. This allows one to use whichever of the standard curve constructed for the quantification of *omt-1* gene copies in the different producing mold strains. Although no guidelines have been established for standard curves used in qPCR assays that measure fungi, Fredlund et al. (2008) and Suanthie et al. (2009) suggested the use of criteria established for Genetically Modified Organism analysis of foods where the slope of the standard curve should range between −3.1 and −3.6, corresponding to a PCR efficiency of 80 and 110% and the R^2 value should be ≥0.98. These guidelines should also be valid for the analysis of fungal DNA in foods. In the present work, the two optimized methods had R^2 values greater than 0.98. Both the SYBR Green and the TaqMan assays gave slope values in the acceptable range and a good efficiency close to 100%, which indicates good optimization of the PCR reaction and that no errors occurred in this process. Similar values of efficiency have

Table 6
Reproducibility assay carried out by the same operator on different equipment in inoculated peanuts (inoculum level was 2.30 log cfu/g).

Repetition	Plate count in PDA (log cfu/g)	Laboratories							
		1		2		3		4	
		SYBR Green (log cfu/g)	TaqMan (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)
1	2.4 ± 0.02	2.2 ± 0.01	2.3 ± 0.12	2.2 ± 0.09	2.2 ± 0.27	2.1 ± 0.10	2.1 ± 0.08	2.4 ± 0.09	2.1 ± 0.07
2	2.5 ± 0.08	2.4 ± 0.01	2.4 ± 0.09	2.3 ± 0.06	2.3 ± 0.22	2.2 ± 0.14	2.2 ± 0.18	2.4 ± 0.05	2.3 ± 0.10
3	2.3 ± 0.03	2.4 ± 0.11	2.2 ± 0.03	2.5 ± 0.23	2.2 ± 0.07	2.4 ± 0.07	2.4 ± 0.15	2.4 ± 0.07	2.3 ± 0.02
4	2.4 ± 0.05	2.4 ± 0.18	2.4 ± 0.12	2.3 ± 0.15	2.3 ± 0.05	2.3 ± 0.17	2.3 ± 0.06	2.4 ± 0.09	2.3 ± 0.11
5	2.4 ± 0.04	2.4 ± 0.09	2.3 ± 0.09	2.5 ± 0.18	2.4 ± 0.09	2.4 ± 0.24	2.4 ± 0.09	2.3 ± 0.09	2.2 ± 0.08

All data represent the mean log cfu/g ± standard deviation (SD) of the 3 independent assays.

been reported for qPCR protocols to detect mycotoxin-producing molds (Mulé et al., 2006; Fredlund et al., 2008).

When the sensitivity of the qPCR assays was evaluated in different food matrices all the standard curves showed suitable linearity ($R^2 > 0.98$) and also the slopes were within the recommended range suggested by Fredlund et al. (2008), except for the value of −3.01 for the TaqMan method to detect *A. parasiticus* CECT 2688 in peanut. This fact could be because concentration of peanut oils can reduce the purity of the extracted DNA, and because of the presence of PCR-inhibiting substances in this kind of food, such as phenolic and polysaccharide compounds (Yu et al., 2004a; Passone et al., 2010). In addition, the detection limit in all inoculated foods ranged from 2 to 1 log cfu/g for both optimized qPCR methods, and it was at the level of those previously reported (Selma et al., 2008; Rodríguez et al., 2011a, 2011b).

To test the ability of the qPCR methods to quantify aflatoxin-producing molds in foods, a known amount of fungal conidia was added to different foods and quantified by the qPCR methods. There were no significant differences in the quantification of conidia by the two proposed qPCR methods and direct counting by plating on PDA agar. In addition, no differences in the quantification of aflatoxin-producing molds between the food matrices were found. The reproducibility and repeatability of the quantification by the two developed methods was very good, since there were no significant differences between assays with different operators and equipment, and also the standard deviation was, in all cases, very low. Comparison with previous studies on mycotoxin-producing mold qPCR assays was not possible since these studies did not test the reproducibility and repeatability in the same way. Nevertheless, the standard deviations were in the same range as that reported for *Fusarium* species by Nicolaisen et al. (2009). This means that the two methods are very repetitive and may be applied in any laboratory to quantify aflatoxin-producing molds.

The quantification of the fungal contamination in foods is a difficult task due to the nature of the fungal colony, which consists of filamentous mycelia cells and single celled spores. For this reason, in this work we also evaluated the efficiency of the developed methods to quantify aflatoxin producers in complex matrices, where just spores are not representative of fungal biomass due to hyphal development. Results of quantification throughout the 14 days incubation time of different inoculated food (in minced peanut, in paprika and in dry-cured ham slices), showed that the calculated *omt-1* copy number and the fungal load obtained with the qPCR methods were nearly identical to the fungal load determined by plating. The mean values of fungal load, including conidia and mycelium, quantified with the SYBR Green and TaqMan methods were close to the inoculated amount. Similar results have been reported for other qPCR protocols developed to quantify the copy number of the *nor-1* gene of *A. flavus* in foods (Mayer et al., 2003).

Both the SYBR Green and the TaqMan qPCR procedures developed in the present study to quantify aflatoxin-producing molds in foods, can be performed within a relatively short time (5–6 h for DNA extraction and 2–3 h for qPCR). This is considerably shorter than what is needed to quantify aflatoxin-producing mold strains by conventional culturing techniques (3–5 days).

Hence, both methods allow reliable rapid estimation of contamination with aflatoxin-producing molds for evaluating the quality of raw materials or ingredients, and monitoring hygiene conditions of mold-ripened foods. Thus, the proposed methods would be used to quantify contamination by aflatoxin-producing molds, even before fungal development, which would be very useful to prevent toxin production during nut, cereal or spice storage and dry-ripening or fermenting in foods. Furthermore, the high sensitivity and quantification capacity of the qPCR methods designed in this study would allow to monitor the target molds in HACCP programs and to minimize the health hazard due to aflatoxin formation in foods.

In conclusion, two specific qPCR methods were developed for detecting from 1 to 2 log cfu/g of aflatoxin-producing molds in different food matrices. No significant effect was observed due to different equipment, operator or qPCR methodology used in the tests of repeatability and reproducibility for different foods. The application of the qPCR methods designed in this study would contribute to food safety by improving the prediction of the different sources of aflatoxins contamination in nuts, cereals, spices and ripened foods.

Table 7
Reproducibility assay carried out by different operators on the same equipment in inoculated peanuts (inoculum level was 2.30 log cfu/g).

Repetition	Operators											
	1			2			3			4		
	[a]Plate count in PDA (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)	[a]Plate count in PDA (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)	[a]Plate count in PDA (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)	[a]Plate count in PDA (log cfu/g)	SYBR Green (log cfu/g)	TaqMan (log cfu/g)
1	2.4 ± 0.05	2.2 ± 0.01	2.3 ± 0.20	2.3 ± 0.08	2.2 ± 0.13	2.2 ± 0.01	2.3 ± 0.16	2.4 ± 0.06	2.3 ± 0.05	2.3 ± 0.08	2.3 ± 0.05	2.3 ± 0.04
2	2.4 ± 0.18	2.3 ± 0.02	2.3 ± 0.01	2.3 ± 0.03	2.3 ± 0.02	2.4 ± 0.10	2.3 ± 0.07	2.3 ± 0.18	2.4 ± 0.05	2.3 ± 0.05	2.3 ± 0.02	2.3 ± 0.01
3	2.5 ± 0.06	2.3 ± 0.08	2.4 ± 0.02	2.2 ± 0.05	2.3 ± 0.10	2.3 ± 0.05	2.3 ± 0.03	2.2 ± 0.02	2.4 ± 0.12	2.3 ± 0.03	2.2 ± 0.08	2.3 ± 0.17

[a] Data represent the mean log cfu/g +/– standard deviation (SD) of the 3 independent assays.

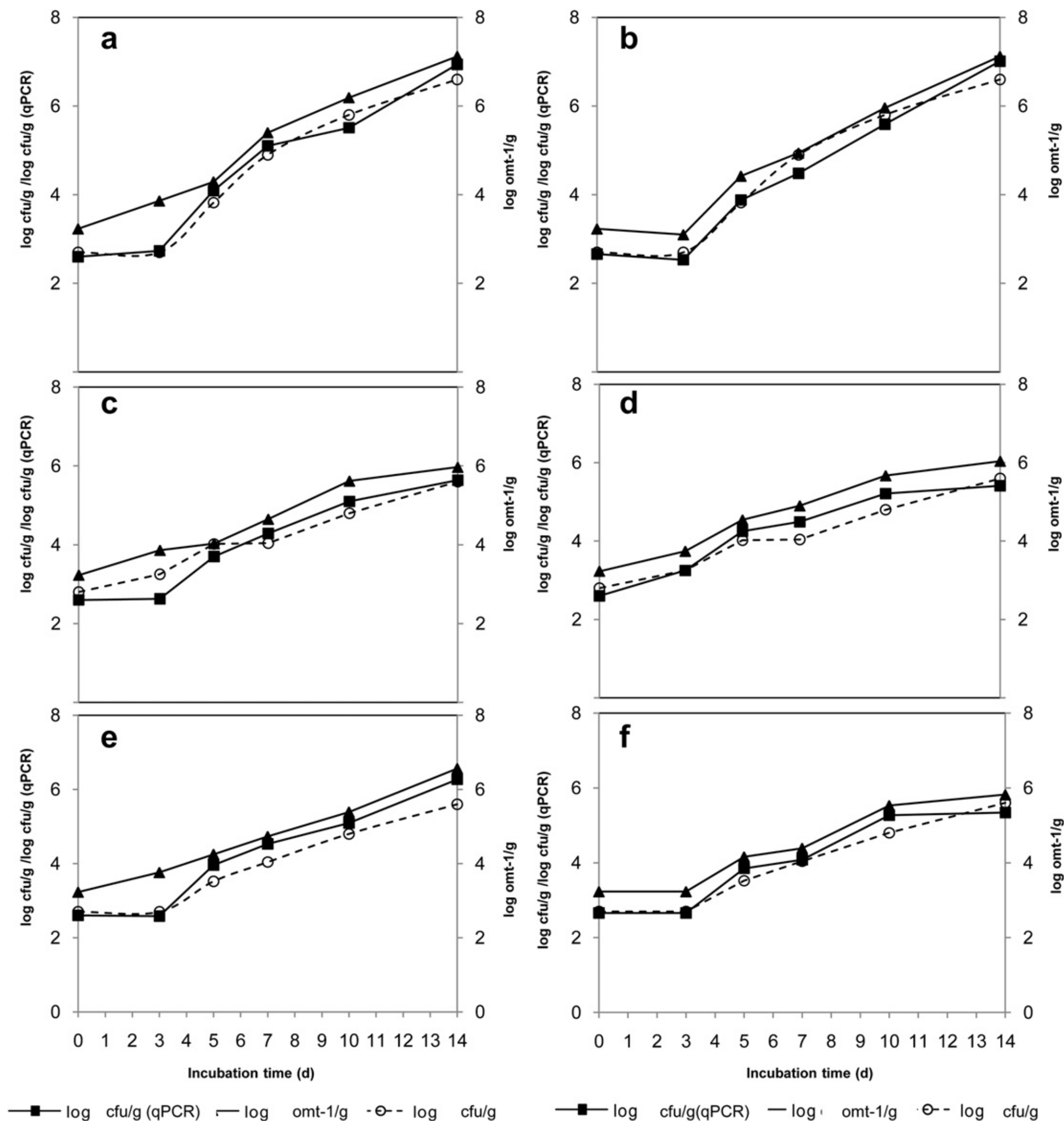

Fig. 3. Comparison of the fungal count (cfu data) with *omt-1* gene copy number and fungal load obtained by SYBR Green (a, c, e) and TaqMan (b, d, f) qPCR of *A. parasiticus* CECT 2688 in inoculated minced peanut (a, b), paprika, (c, d) and dry-cured ham (e, f) during 14 days of incubation.

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