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Quantitative real-time PCR method with internal amplification control to quantify cyclopiazonic acid producing molds in foods

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ABSTRACT

A quantitative TaqMan real-time PCR (qPCR) method that includes an internal amplification control (IAC) to quantify cyclopiazonic acid (CPA)-producing molds in foods has been developed. A specific primer pair (*dmaTF*/*dmaTR*) and a TaqMan probe (*dmaTp*) were designed on the basis of *dmaT* gene which encodes the enzyme dimethylallyl tryptophan synthase involved in the biosynthesis of CPA. The IAC consisted of a 105 bp chimeric DNA fragment containing a region of the *hly* gene of *Listeria monocytogenes*. Thirty-two mold reference strains representing CPA producers and non-producers of different mold species were used in this study. All strains were tested for CPA production by high-performance liquid chromatography-mass spectrometry (HPLC-MS). The functionality of the designed qPCR method was demonstrated by the high linear relationship of the standard curves relating to the *dmaT* gene copy numbers and the Ct values obtained from the different CPA producers tested. The ability of the qPCR protocol to quantify CPA-producing molds was evaluated in different artificially inoculated foods. A good linear correlation was obtained over the range 1–4 log cfu/g in the different food matrices. The detection limit in all inoculated foods ranged from 1 to 2 log cfu/g. This qPCR protocol including an IAC showed good efficiency to quantify CPA-producing molds in naturally contaminated foods avoiding false negative results. This method could be used to monitor the CPA producers in the HACCP programs to prevent the risk of CPA formation throughout the food chain.

1. Introduction

Cyclopiazonic acid (CPA) is a potent mycotoxin that produces focal necrosis in most vertebrate inner organs in high concentrations and affects the ducts or organs originating from ducts (Frisvad et al., 2006). This mycotoxin has also been classified as a neurotoxin (Wannemacher et al., 1991), due to its effects on the central nervous system observed in experimental animals (i.e. ataxia, extensor spasms).

CPA can be produced by different *Penicillium* species including *Penicillium camemberti, Penicillium chrysogenum, Penicillium commune, Penicillium crustosum, Penicillium griseofulvum, Penicillium hirsutum*, *Penicillium melanoconidium,* and *Penicillium viridicatum* (Frisvad et al., 2004; Núñez et al., 2007; Sabater-Vilar et al., 2003). *Aspergillus* species can also produce CPA, including *Aspergillus oryzae*, *Aspergillus tamarii* and *Aspergillus versicolor* and the aflatoxigenic fungus *Aspergillus flavus* (Díaz et al., 2010; Pildain et al., 2008). CPA has been reported in different kind of foods such as corn, peanut, dry-ripened cheese and dry-cured ham as consequence of fungal growth (Baily et al., 2005; Dorner, 2002; Fernández-Pinto et al., 2001; Lee and Hagler, 1991; Taniwaki et al., 2001).

The early detection of CPA in ready-to-eat foods and the elimination of those ones contaminated with this mycotoxin throughout the food chain, could avoid the consumption of hazardous food products. The CPA mycotoxin has been reported as an unstable molecule that can be degraded throughout chemical and physical processes and consequently difficult to detect (Díaz et al., 2010). Thus, the early detection of CPA-producing molds throughout food processing would allow taking corrective actions to avoid production of CPA contaminated foods. This alternative could reduce the cost of elimination of CPA-contaminated foods.

For achieving this purpose, rapid and accurate methods to quantify CPA-producing molds are necessary. Quantitative real-time PCR (qPCR) could be applied to quantify these toxigenic molds. This method has been reported as a specific and highly sensitive technique which allows an accurate quantification of mycotoxin-producing molds (Mulé et al., 2006). Several qPCR assays have been reported for detection of toxigenic molds using SYBR Green and TaqMan technologies (Fredlund et al., 2008; Nicolaisen et al., 2009; Rodríguez et al., 2011a, 2011b, 2012a; Selma et al., 2008). However,

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so far, no qPCR protocols to quantify CPA-producing molds in foods have been developed yet.

The correct choice of the target sequence for the design of primers is essential to develop a new qPCR protocol for the quantification of producing molds. Up to now, little is known about the regulation of the CPA biosynthesis. Only it has been reported that the enzyme dimethylallyl tryptophan synthase (DMAT) is implicated in its production (Chang and Ehrlich, 2011; Chang et al., 2009). Thus, the *dmaT* gene encoding this enzyme could be used as target to design specific primers and probes for quantifying CPA-producing molds by qPCR.

The efficiency of qPCR quantification in food samples may be seriously affected by the presence of inhibitors in the food matrix (Di Pinto et al., 2007). Inhibition may be expressed as false negatives or reduced sensitivity. Organic compounds such as polyphenols, oils, polysaccharides or fatty acids from vegetables, nuts and ripened foods (Makhzami et al., 2008; Passone et al., 2010) can lead to errors in the specific detection of the CPA producers (Monnet et al., 2006; Mulé et al., 2006). Therefore, direct testing in foods is required to evaluate the potential application of the qPCR based method. In addition, secondary metabolites produced by mold may be potential PCR inhibitors or generate mutant fungal strains lacking of genes involved in mycotoxin production, leading in both cases to false negative results in the qPCR (Paterson, 2007; Paterson and Lima, 2009; Sant'Ana et al., 2010).

The use of an internal amplification control (IAC) is an adequate strategy to assess the validity of PCR and avoid false negative results (Hoorfar et al., 2004; Paterson, 2007; Raymaekers et al., 2009). IAC is a non-target DNA sequence present in the same sample reaction, which is coamplified simultaneously with the target sequence. In a qPCR with an IAC, a control signal should always be produced, even though there is no target sequence present.

Thus, guidelines for PCR testing of foodborne pathogens have proposed the presence of an IAC for PCR-based diagnostic tests (OECD, 2007; ISO 22174:2005; ISO 22119:2011). In these assays, it is important to optimize the concentration of the IAC to avoid the interference with the sensitivity of the method (Lund and Madsen, 2006). Several strategies for the design of IAC from housekeeping genes or synthetic plasmid constructs for different microbiological purposes have been previously reported (Khot et al., 2008; Randall et al., 2010; Rodríguez-Lázaro et al., 2005). Some of them could be applied to develop an IAC for accurate detection of CPA-producing molds.

The aim of this work was to develop a sensitive and specific qPCR method for detection and quantification of CPA-producing molds in foods which includes an IAC to avoid false negative results. The efficiency and sensitivity of the developed method to quantify CPA-producing molds in foods were also analyzed.

2. Material and methods

2.1. Fungal strains

Thirty-two mold strains commonly found in foods and belonging to 16 different fungal 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 from the University of Extremadura. Only 9 of them had information about CPA production (Table 1). All reference strains were checked for CPA production by HPLC-MS analyses as it is described below.

2.2. CPA production

Production of CPA was tested after growing each mold strain in 3-points per plate on Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) and incubating for 15 days at 25 °C. The content of three petri dishes was extracted with chloroform, filtered twice through anhydrous sodium sulfate and evaporated in a rotary evaporator at 40 °C as has been previously described by Sosa et al. (2002). The residue was resuspended in 5 mL of chloroform, filtered through a 0.45 μm pore size nylon membrane, and evaporated to dryness under a gentle stream of nitrogen. The extracts were briefly stored at 4 °C in the dark resuspended in 200 μL of acetonitrile just before HPLC-MS analysis. HPLC-MS analysis was performed as previously described (Sosa et al., 2002) in a Hewlett Packard series 1100 equipment (Hewlett Packard, Palo Alto, USA). A Supelcosil LC-18 column (SUPELCO, Bellefonte, USA) was used with mobile phases (A) 100% water and (B) 0.05% trifluoroacetic acid in acetonitrile in a gradient from 10% to 99% B. CPA was identified in a Finnigan LCQ Mass Spectrometer (Finnigan, San Jose, USA) with atmospheric pressure chemical ionization source (APCI), according to its retention time and molecular mass.

2.3. DNA extraction

Every mold strain was 3-point inoculated on MEA and incubated for 4 days at 25 °C. The mycelium of each strain was scraped off the agar and used for genomic DNA extraction. For this, 50 mg of mycelium were digested with proteinase K and lyticase, frozen in liquid nitrogen, and grinded with mortar and pestle, prior to DNA extraction in a semiautomatic vacuum system following the method previously described by Sánchez et al. (2008). DNA concentration was quantified spectrophotometrically in a Biophotometer Eppendorf (Eppendorf AG, Hamburg, Germany) and 0.2 ng/μL of DNA extracted from CPA-producing and non-producing strains was used for qPCR assays.

2.4. Primers and probe design

A specific primer pair (*dmaTF-dmaTR*) and a TaqMan probe (*dmaTp*) were designed on the basis of the *dmaT* gene (GenBank accession no. DQ121455) encoding the enzyme DMAT of a *Penicillium roqueforti* using the Primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 2). The probe *dmaTp* 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) (Table 2).

2.5. Internal amplification control design

The IAC consisted of a 105 bp chimeric DNA fragment containing a region of the *hly* gene of *Listeria monocytogenes* amplified with the *hlyQF-hlyQR* primers previously reported by Rodríguez-Lázaro et al. (2004), flanked by the sequence of the CPA-producing molds specific primers (*dmaTF-dmaTR*). This chimeric DNA fragment was obtained by two-step PCR. In the first PCR reaction, 50 ng of the *L. monocytogenes* DNA were amplified by *dmatFlys-dmatRlys* oligonucleotides (Table 2), which contained the corresponding *hlyQF-hlyQR* primer sequences fused to 5′ tail with the *dmaTF-dmaTR* primer sequences. Reactions were amplified through the following thermal cycling program: 1 cycle of 10 min at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The second PCR round used the first-round PCR product (diluted 1:1000) as template and the *dmaTF-dmaTR* primers. PCR conditions were the same as those described above. The IAC PCR product was purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) following

Table 1
CPA production and qPCR data with the specific primers (*dmaTF-dmaTR*) and *dmaTp* and *IACp* probes of the reference mold strains.

Species designation	Strain reference	Information provided by culture collections	CPA production detected by HPLC-MS	[g]Ct value ± SD	
				dmaTF-dmaTR	IAC
Aspergillus flavus	[a]CECT 2684	Unknown	[e]ND	40.0 ± 0.01	23.7 ± 0.20
Aspergillus flavus	CECT 2687	Producer	[f]10.66	25.3 ± 0.19	23.6 ± 0.18
Aspergillus flavus	[b]IBT 3696	Producer	36.69	26.1 ± 0.33	23.3 ± 0.27
Aspergillus flavus	[c]CBS 573.65	Unknown	ND	40.0 ± 0.01	23.7 ± 0.67
Aspergillus flavus	CBS 120.62	Unknown	ND	40.0 ± 0.01	23.6 ± 0.21
Aspergillus ochraceus	CBS 589.68	Unknown	17.40	25.1 ± 0.18	23.2 ± 0.30
Aspergillus ochraceus	CECT 2092	Unknown	ND	40.0 ± 0.01	23.2 ± 0.27
Aspergillus oryzae	CECT 2094	Producer	1.42	24.9 ± 0.40	22.9 ± 0.17
Aspergillus parasiticus	CECT 2688	Unknown	ND	40.0 ± 0.01	23.4 ± 0.09
Aspergillus tamarii	CBS 575.65	Producer	1.25	25.9 ± 0.34	23.8 ± 0.20
Aspergillus tamarii	CBS 109.63	Unknown	ND	40.0 ± 0.01	23.7 ± 0.14
Aspergillus versicolor	CECT 2664	Unknown	ND	40.0 ± 0.01	23.4 ± 0.40
Aspergillus versicolor	CECT 2903	Unknown	ND	40.0 ± 0.01	23.5 ± 0.32
Aspergillus versicolor	CECT 2814	Unknown	ND	40.0 ± 0.01	23.6 ± 0.22
Penicillium aurantiogriseum	CECT 2918	Unknown	ND	40.0 ± 0.01	23.5 ± 0.42
Penicillium camemberti	[d]Pc 1	Unknown	396.72	25.1 ± 0.27	23.4 ± 0.10
Penicillium camemberti	CBS 273.97	Producer	49.98	25.2 ± 0.40	23.6 ± 0.38
Penicillium commune	CBS 311.48	Producer	1.68	25.3 ± 0.39	23.4 ± 0.42
Penicillium commune	CBS 341.59	Producer	4.32	25.3 ± 0.21	23.5 ± 0.08
Penicillium commune	CBS 247.32	Unknown	ND	40.0 ± 0.01	23.7 ± 0.15
Penicillium dipodomyicola	IBT 26223	Unknown	ND	40.0 ± 0.01	23.4 ± 0.33
Penicillium dipodomyicola	CBS 110425	Unknown	ND	40.0 ± 0.01	23.3 ± 0.35
Penicillium dipodomyicola	CBS 110426	Unknown	80.10	25.4 ± 0.10	23.6 ± 0.17
Penicillium expansum	CECT 2278	Unknown	ND	40.0 ± 0.01	23.8 ± 0.30
Penicillium griseofulvum	IBT 14319	Unknown	14.15	25.0 ± 0.13	23.5 ± 0.41
Penicillium griseofulvum	CBS 485.84	Producer	0.53	24.8 ± 0.38	23.8 ± 0.79
Penicillium griseofulvum	CBS 110420	Producer	6.21	25.3 ± 0.29	23.9 ± 0.20
Penicillium griseofulvum	CECT 2919	Unknown	6.29	24.9 ± 0.41	23.1 ± 0.32
Penicillium melanoconidium	CBS 64195	Unknown	ND	40.0 ± 0.01	23.3 ± 0.27
Penicillium polonicum	CBS 112490	Unknown	ND	40.0 ± 0.01	23.5 ± 0.32
Penicillium verrucosum	CBS 323.92	Unknown	ND	40.0 ± 0.01	23.6 ± 0.24
Penicillium viridicatum	CECT 2320	Unknown	ND	40.0 ± 0.01	23.7 ± 0.14

All CPA-producing mold strains are shaded.
[a] CECT: Spanish Type Culture Collection.
[b] IBT: Type Culture Collection of the Department of Biotechnology (Technical University of Denmark).
[c] CBS: Centraalbureau voor Schimmelcultures (The Netherlands).
[d] Pc: strains isolated from dry-cured ham belonging to the Culture Collection of Food Hygiene and Safety from University of Extremadura.
[e] ND: CPA production is not detected by HPLC-MS.
[f] CPA concentration (μg/l) detected by HPLC-MS.
[g] Data represent the mean threshold cycle (Ct) ± standard deviation (SD) of the 3 independent experiments each consisting of triplicate samples.

the manufacturer's instructions and was quantified in a Biophotometer Eppendorf (Eppendorf AG). To confirm the correct nucleotide sequence, the IAC amplicon was cloned into pCR2.1TOPO (Invitrogen) and sequenced using M13 Reverse and M13 Forward primers (Invitrogen). After blast analysis in the NCBI database (http://www.ncbi.nlm.nih.gov/) this sequence only showed similarity to the *hly* sequence (nucleotide position 257–321, GenBank accession no. AY174657). The IAC was specifically detected with the previously described *hlyQP* TaqMan probe (Rodríguez-Lázaro et al., 2004), renamed *IACp* in this work (Table 2).

2.6. Optimization of qPCR conditions

The qPCR was carried out in the 7500 Fast Real-Time PCR system (Applied Biosystems). Reactions were prepared in triplicate in MicroAmp optical 96-well reaction plates and sealed with optical

Table 2
The optimized concentrations of oligonucleotide primers, fluorescence probes and chimeric DNA fragment sequences used in the qPCR assay.

Primer/ probe	Nucleotide sequences (5′–3′)	Amplicon length (bp)	Position	Target gene	Concentration (nM/reaction)
C-dmaTF	GAAGGTTGATGGCCAGATT	394	[a]392	*dmaT*	40
C-dmaTR	ATCATCACCAATCCAGCGAG		665		40
dmaTF	TTCACGCTCGTGGAACTTCT	64	598	*dmaT*	300
dmaTR	GGGTCACAAAGATCGCAAGAT		641		300
dmaTp	[HEX]-TACTGCCTCCCCCCGAC-[BHQ1]		621	*dmaT*	200
dmatFlys	TTCACGCTCGTGGAACTTCTCATGGCACCACCAGCATCT	105	–	IAC	150
dmatRlys	GGGTCACAAAGATCGCAAGATATCCGCGTGTTTCTTTTCGA		–		150
[b]*IACp*	[FAM]-CGCCTGCAAGTCCTAAGACGCCA-[TAMRA]		–	IAC	200

Chimeric sequence	Nucleotide sequence (5′–3′)	Amplicon length (bp)	Concentration (copies)
IAC	TTCACGCTCGTGGAACTTCTCATGGCACCACCAGCATCTCCGCCTGCAAGTC CTAAGACGCCAATCGAAAAGAAACACGCGGATATCTTGCGATCTTTGTGACCC	105	100

Underlined text is equivalent to *Listeria monocytogenes hly* gene (corresponding to primers *hlyQF* and *hlyQR* reported previously by Rodríguez-Lázaro et al., 2004).
[a] Positions are in accordance with the published sequences of *dmaT* gene of *P. roqueforti* (GenBank accession no. DQ121455).
[b] The FAM-labeled *IACp* has been previously described by Rodríguez-Lázaro et al. (2004) previously named as *hlyQP*.

adhesive covers (Applied Biosystems). Three replicates of a control sample without DNA template were also included in all the runs. To optimize the qPCR method, one CPA-producing strain, *P. commune* CBS 341.59, was used. For testing the concentration of specific primers and both probes, several concentrations ranging from 50 to 600 nM were assayed. To determinate the optimal IAC concentration, various IAC amounts (1000, 300, 100, 10 copies per reaction) were also tested. The optimized TaqMan protocol was carried out in a final volume of 25 μL, containing 5 μL of DNA template and 100 copies of IAC DNA (1 μL), 12.5 μL of 2× *Premix Ex Taq*™ (Takara, Otsu, Shiga, Japan), 0.5 μL of 50× ROX™ Reference Dye, 300 nM of both *dmaTF-dmaTR* primers and 200 nM of both TaqMan probes (*dmaTp* and *IACp*). The thermal cycling conditions included two holding periods, one at 50 °C for 2 min and other one at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (Ct) which is the intersection between each fluorescence curve and a threshold line, was automatically calculated by the instrument using default parameters. 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.

2.7. Specificity of qPCR reaction

The specificity of the *dmaTF-dmaTR* primers was tested using 0.2 ng/μL of genomic DNA from the 32 tested mold strains (Table 1). In addition, to test the absence of cross-amplification from *IACp* probe when *L. monocytogenes* DNA was present in the reaction, 10-fold DNA dilutions of the above pathogen ranging from 10 ng to 0.01 pg were also prepared. The qPCR reactions were carried out in triplicate as it is described in Section 2.6.

2.8. Standard curves

Standard curves were obtained for three CPA-producing strains (*P. commune* CBS 341.59, *Penicillium cammemberti* CBS 273.97 and *A. flavus* CECT 2687) following a procedure previously described by Mayer et al. (2003). For this, genomic DNA from the above mold strains was used with *C-dmaTF* and *C-dmaTR* primers (Table 2), based upon the *dmaT* gene, for amplification by a conventional PCR method (Casado et al., 2008). Reactions were performed in a programmable thermal cycler, Mastercycler Gradient (Eppendorf AG) in volumes of 50 μL containing 2 μL of DNA template, 2 μL each primer (10 μM), 5 μL of 10× PCR buffer, 3 μL of $MgCl_2$ (50 mM), 1 μL of dNTPs (10 mM) and 1 μL of Taq DNA polymerase (2 U/μL, Finnzymes, Espoo, Finland). The amplification program used was: 1 cycle of 5 min at 95 °C, 38 cycles of 30 s at 95 °C, 1 min at 41 °C and 1.5 min at 72 °C and finally 1 cycle of 7 min at 72 °C. The obtained 394 bp fragment of the *dmaT* gene was used as a standard stock solution. Concentration of PCR products in the stock solution was determined in a Biophotometer Eppendorf (Eppendorf AG) and the number of copies was then calculated. Stock solutions were diluted serially by a factor of 10 and an aliquot of dilutions was used as a copy number standard during each setup of the qPCR. Each standard curve with the three CPA-producing fungal 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 designed method 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).

2.9. Sensitivity of qPCR on artificially inoculated food matrices

The sensitivity of the optimized qPCR method was assayed with DNA extracted from four kinds of inoculated non-sterile commercial without protective mold (dry-cured ham, dry-ripened cheese, paprika and peanut). These products were inoculated with spores of different CPA-producing fungal strains to know the efficiency of the developed method. Thus, dry-cured ham was inoculated with *P. commune* CBS 341.59, dry-ripened cheese with *P. camemberti* CBS 273.97 and paprika and peanut with *A. flavus* CECT 2687. To test the specificity of the qPCR method in foods, a CPA-non producing strain *P. commune* CBS 247.32 was also used. The preparation of spores for inoculation purposes and foods inoculation were performed as it was previously reported by Rodríguez et al. (2011b). Next, the inoculated food samples were then treated for DNA extraction according to the "CTAB-EZNA" method previously optimized by Rodríguez et al. (2012b).

All inoculations and DNA extractions were performed in triplicate for each food. In addition, triplicates of non-inoculated negative control were included in each experiment.

For qPCR amplification, 3 replicates of 5 μL of DNA extracted from the inoculated and non-inoculated foods were assayed per run. Standard curves were then generated for each group of food products and the efficiency for the standard curves was calculated as it was indicated above.

To directly estimate the load of the inoculated mold, artificially inoculated samples were diluted in 0.1% peptone water, spread-plated on Potato Dextrose Agar (PDA, Scharlau Chemie S.A.) and incubated at 25 °C for 4 days. The natural fungal contamination of samples was lower than 1 log cfu/g. After characterizing the isolates from non-inoculated foods, no typical colonies of *P. commune, P. camemberti* and *A. flavus* were found. In addition, DNA of these fungal isolates yielded negative results in the developed qPCR.

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

Five grams of minced peanut and 25 cm^2 slices of dry-ripened ham were placed separately in presterilized orthogonal receptacles made of methacrylate, where the humidity was kept constant by a saturated K_2SO_4 solution (water activity 0.97) placed at the bottom of the receptacles. The samples were inoculated on the surface with spores of the CPA-producing strain *P. commune* CBS 341.59 at a concentration of 3 log cfu/g. Sampling was carried out by triplicate from each at 0, 3, 5, 7, 10 and 14 days of incubation at 25 °C. Negative controls from non-inoculated dry-cured ham and peanut samples were also analyzed. DNA was extracted as previously described in the Section 2.9 and subjected to the designed qPCR method as described in the Section 2.6. The Ct values obtained with the qPCR assay for the inoculated samples were used to estimate the fungal load from the corresponding standard curves. In addition, the Ct values obtained with the 100 IAC copies were used in order to calculate the log IAC copies in the reactions.

The estimation of the load of the inoculated mold was obtained as described in Section 2.9.

2.11. Quantification of CPA-producing strains in naturally infected samples

Thirty dry-cured Iberian hams, 15 paprika and 15 durum wheat semolina samples were analyzed for testing the capacity of qPCR methods to quantify CPA-producing molds in naturally infected samples. For this, samples of dry-cured Iberian hams with visible grown mycelium on the surface were collected at 6 months of ripening, by scraping onto a surface of 25 cm^2 of mycelium of the hams. The 15 paprika samples were collected in 15 different industries at the end of processing, that included smoke-drying phase for 15 days in a traditional dryer. Finally, the durum wheat

semolina samples from the type commonly employed for pasta production were collected from various mills.

DNA was extracted as previously described in the Section 2.9 and subjected to the designed qPCR method as described in the Section 2.6. The Ct values obtained with the qPCR assay for naturally infected samples were used to estimate the fungal load from the corresponding standard curves. The presence of fungal DNA was also determined in all samples by qPCR based on the *β-tubulin* gene as previously described Rodríguez et al. (2012b).

The estimation of the load of the inoculated mold was obtained as described in Section 2.9.

2.12. Statistical analysis

All statistical analyses were performed with the SPSS v.15.0. One way analysis of variance (ANOVA) was carried out to determinate 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 CPA by HPLC-MS

HPLC-MS analyses confirmed CPA production in the 9 fungal strains identified as CPA-producers by their respective Culture Collections (Table 1). In addition, 5 strains belonged to different species of *Aspergillus* and *Penicillium* genera which did not have information on CPA production in their Culture Collections produced CPA, whereas the remaining strains did not produce detectable amounts of this mycotoxin. The concentration levels of CPA produced by the CPA-producing strains in PDA medium ranged from 0.53 to 396.72 μg/mL (Table 1).

3.2. Optimization of qPCR conditions

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. Thus, the optimized reaction conditions for qPCR were obtained by using 300 nM of *dmaTF-dmaTR* primers, 200 nM of *dmaTp* and *IACp* probes and 100 IAC copies. The DNA amplification of CPA-producing molds and IAC was monitored by hybridation probes labeled with two different fluorophores: FAM and HEX, respectively (Table 2). These fluorophores were selected according to their maximum emission wavelengths (FAM, 520; HEX, 556 nm) to reduce spectral overlapping.

3.3. Evaluation of the specificity of the qPCR assay using reference strains

Primers and probes specificity was tested on genomic DNA from the CPA-producing and non-producing species of *Aspergillus* and *Penicillium* (Table 1). All CPA-producing strains verified by HPLC-MS analysis as CPA producers showed mean Ct values of 25.3 ± 0.75, while no qPCR amplification (Ct ≥ 40) was detected in any of the non-producing strains (Table 1). In addition, IAC amplification in all the producing and non-producing reference mold strains was found showing mean Ct values of 23.5 ± 0.42 (Table 1). No cross-amplification was observed when different amounts of *L. monocytogenes* genomic DNA were tested.

3.4. Standard curves for the dmaT gene

The 394 bp PCR product obtained by the conventional PCR method was used as a reference to make the standard curves for calculation of the number of copies of the *dmaT* gene in the TaqMan assay.

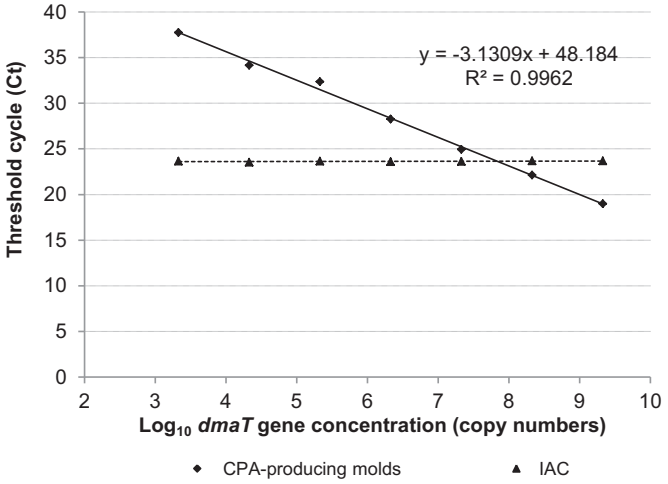

Fig. 1. Representative standard curve showing the correlation between the initial copy number of the *dmaT* gene and threshold cycle (Ct) values of the TaqMan Real-Time PCR assay including an IAC. This curve was generated with the mean values obtained in three different assays.

An example of standard curve constructed using Ct values and the log of the template copies for the optimized method is shown in Fig. 1. The log of the IAC copies was also shown in Fig. 1. No significant differences ($P \leq 0.05$) were found between standard curves obtained by different CPA-producers since slopes and linear correlations (R^2) were similar. Good linear relationship between Ct values and the *dmaT* gene copy numbers was obtained for the qPCR method, with R^2 values greater than 0.99. The slope of the linear regression curve was −3.13 (Fig. 1). The efficiency value was then 108.6%. IAC amplification was detected in all cases (mean Ct values = 23.5 ± 0.42).

3.5. Sensitivity of the qPCR assay on artificially inoculated food matrices

Standard curves for each food matrix were generated by using DNA extracted from inoculated foods. The slopes of linear regression equations in the qPCR assay for dry-cured ham, dry-ripened cheese, paprika and peanut, were −3.08, −3.10, −3.80 and −3.73, respectively (Table 3). A good R^2 was also obtained over the range from 1 to 4 log cfu/g per reaction for all the food matrices (Table 3). Efficiency values ranged from 82.3 to 110.8%. The detection limit was 2 log cfu/g in paprika and peanut and 1 log cfu/g in dry-cured ham and dry-ripened cheese.

No amplification (Ct ≥ 40) with *dmaTF-dmaTR* primers was obtained in the above food matrices inoculated with a CPA-non producing strain (*P. commune* CBS 247.32). In all cases, the PCR amplification of the IAC was observed with Ct values between 23.2 and 25.1.

Table 3
Efficiencies of amplification and linear correlations (R^2) obtained from standard curves constructed with log of cfu/g and the Ct values from CPA-producing molds in artificially inoculated foods by qPCR.

Food sample	CPA-producing species	Efficiency (%)	R^2
Paprika	*A. flavus* CECT 2687	[a]83 ± 0.7	0.98
Peanut	*A. flavus* CECT 2687	85 ± 1.0	0.99
Dry-cured ham	*P. commune* CBS 341.59	110 ± 0.4	0.98
Dry-ripened cheese	*P. camemberti* CBS 273.93	110 ± 0.8	0.98

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

3.6. Quantification of the fungal load in artificially inoculated foods

The ability of the designed qPCR method to quantify the fungal load in inoculated food samples after different incubation times is shown in Fig. 2. In addition, the fungal count by plating was determined (Fig. 2). The growth of *P. commune* CBS 341.59 differed in the tested foods. In peanut, this strain started to grow after 3 days of incubation, whereas in dry-cured ham showed 5 days lag phase approximately. No natural fungal contamination was observed in all the non-inoculated food controls. Besides, no amplification by the qPCR assay was observed when the extracted DNA from these control samples was used, while IAC amplification could be observed between 1.95 and 2.10 log IAC copy numbers.

In general, the fungal load obtained by qPCR correlated well with that obtained by counting plate yielding R^2 values around 0.97. In both peanut and dry-cured ham, qPCR was able to quantify the fungal load just after inoculation and before mycelium growth. In most of the cases, the *dmaT* gene copy numbers determined by qPCR was higher than the cfu data obtained by plate. Nevertheless, no significant differences ($P \leq 0.05$) were observed between the fungal load determined by qPCR and that obtained by counting in PDA throughout the 14 days of incubation time. The standard deviation was lower than 0.33 in all phases of growth.

3.7. Naturally infected samples analyses

To evaluate the applicability of qPCR method to quantify CPA-producing molds in naturally infected samples of diverse origin, 30 dry-cured hams, 15 durum wheat semolina and 15 paprika samples were analyzed by using of the designed qPCR assay.

The results obtained with the qPCR assay showed that all 30 dry-cured Iberian ham samples contained fungal DNA and 5 of them were contaminated with CPA-producing molds (Table 4). The CPA-producer counts found in the ham samples ranged from 1.2 to 2.9 log cfu/cm^2 approximately. For durum wheat semolina samples, only 2 of the 15 tested samples showed positive results with the primers based on the *β-tubulin* gene, and, 1 of them had CPA-producer counts up to 1.5 log cfu/g (Table 4). Finally, 11 of 15 of paprika samples had fungal DNA, and, 2 of the 11 samples, showed amplification in the designed qPCR assay to quantify CPA-producing molds. The counts found in paprika samples ranged between 1.7 and 2.3 log cfu/g of CPA producers (Table 4).

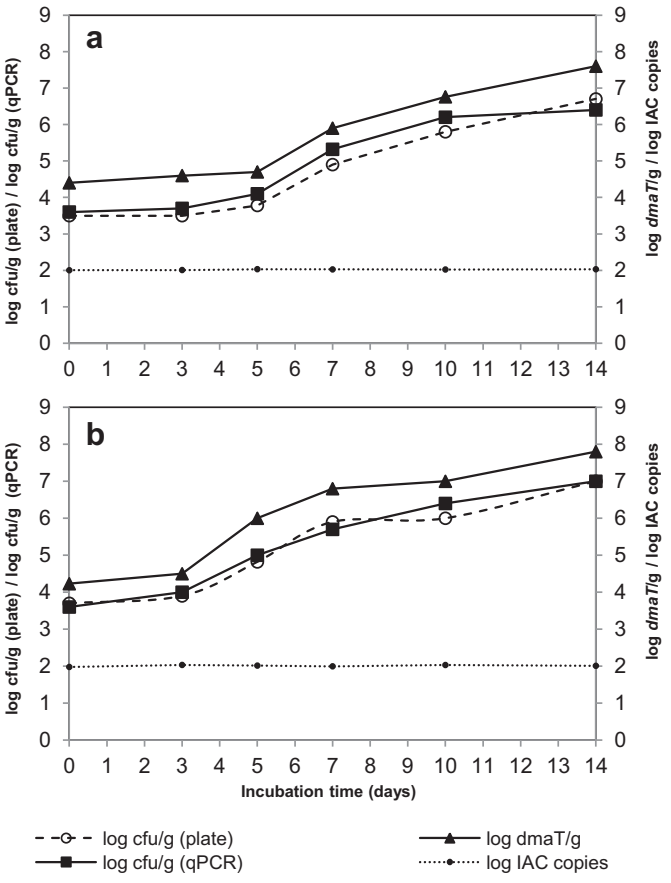

Fig. 2. Fungal count obtained by plating, the *dmaT* gene copy numbers and the fungal load found by qPCR with an IAC, in dry-cured ham (a) and peanut (b) inoculated with *P. commune* CBS 341.59, throughout 14 days of incubation.

4. Discussion

In this study, a qPCR assay for detecting and quantifying CPA-producing molds in foods has been developed. For this, the *dmaT* gene encoding the enzyme DMAT was used as target for the design of specific primers and probe. This enzyme is critical for the CPA biosynthesis since it has been demonstrated that the disruption of the *dmaT* gene avoid the production of this mycotoxin (Chang and Ehrlich, 2011; Chang et al., 2009).

The designed qPCR includes a competitive IAC. Nowadays, the use of an IAC in diagnostic PCR is becoming mandatory to exclude the possibility of false negative results caused by the presence of inhibitors substances, incorrect PCR mixture, poor DNA polymerase activity, or a malfunction of the thermal cycler (Hoorfar et al., 2004; Radström et al., 2003). In the present work was designed a competitive IAC, which it contains flanking nucleic acid sequences with the same *dmaT* gene specific primer recognition sites (*dmaTF-dmaTR*) as the target and a non-target internal sequence of the *hly* gene of *L. monocytogenes*. Thus, the target and the IAC are co-amplified in a single reaction tube with the same primer set. The different fluorophores used for labeling *dmaTp* and *IACp* probes and the differences in the amplicon size between *dmaT*-specific amplicon (64 bp) and the IAC (105 bp) allowed easy discrimination between these two PCR products by qPCR. This strategy has been used to design competitive IAC protocols in qPCR to quantify several foodborne pathogens (Hyeon et al., 2010; Jofré et al., 2005; Rodríguez-Lázaro et al., 2004), but it has not been reported to quantify toxigenic molds.

The most critical parameter to consider in the optimization of a qPCR assay with IAC is the optimal initial number of IAC copies (Abdulmawjood et al., 2002), since small amounts of them lead to substantial variations in the amplification and high amounts could negatively affect the PCR sensitivity (Rodríguez-Lázaro et al., 2007). Thus, initial IAC copies number in the reaction must be determined empirically, to allow its reproducible detection without out-competing the target. Hundred IAC molecules for qPCR reaction were used in this work without significantly affecting the amplification of the target DNA. This initial number of IAC molecules is similar to that used in other previously reported PCR methods (Hyeon et al., 2010; Jofré et al., 2005; Rodríguez-Lázaro et al., 2005).

The specificity of the designed *dmaTF-dmaTR* primers pair and *dmaTp* probe was confirmed in this study, since only was detected amplification in those strains showing CPA production in the HPLC-MS analysis. In addition, the IAC Ct values ranged from 22.9 to 23.9 by using producing and non-producing strains, indicating the absence of false negative results in the qPCR reactions.

The functionality of the developed method was also demonstrated by the high linear relationship of the standard curves which relating the *dmaT* gene copy numbers and the Ct values obtained by the different tested CPA producers. In addition, there were no

Table 4
Quantification of the CPA-producing molds (log cfu/g) in 30 dry-cured ham, 15 durum wheat semolina, and 15 paprika naturally infected samples by the developed qPCR protocol.

Naturally infected food products									
Number of sample	Dry-cured Iberian ham			Durum wheat semolina			Paprika		
	qPCR based on *β-tubulin* gene	qPCR based on *dmaT* gene		qPCR based on *β-tubulin* gene	qPCR based on *dmaT* gene		qPCR based on *β-tubulin* gene	qPCR based on *dmaT* gene	
		CPA-producing mold counts (log cfu/cm^2 ± SD)	IAC (Ct value ± SD)		CPA-producing mold counts (log cfu/g ± SD)	IAC (Ct value ± SD)		CPA-producing mold counts (log cfu/g ± SD)	IAC (Ct value ± SD)
1	[a]+	<1.0	[c]22.7 ± 0.40	[a]+	[b]1.5 ± 0.09	[c]23.1 ± 0.23	[a]+	<1.0	[c]23.0 ± 0.19
2	+	<1.0	23.0 ± 0.11	−	<1.0	22.3 ± 0.09	+	<1.0	23.1 ± 0.10
3	+	<1.0	23.2 ± 0.31	−	<1.0	23.1 ± 0.31	+	<1.0	23.1 ± 0.12
4	+	[b]1.2 ± 0.23	23.3 ± 0.38	−	<1.0	22.2 ± 0.02	+	<1.0	22.8 ± 0.30
5	+	1.7 ± 0.29	23.1 ± 0.34	−	<1.0	22.9 ± 0.28	+	[b]1.7 ± 0.09	23.3 ± 0.34
6	+	<1.0	23.4 ± 0.19	−	<1.0	23.1 ± 0.07	+	<1.0	23.4 ± 0.48
7	+	<1.0	23.1 ± 0.21	+	<1.0	23.4 ± 0.37	−	<1.0	23.6 ± 0.42
8	+	<1.0	22.9 ± 0.27	−	<1.0	23.0 ± 0.19	+	<1.0	23.4 ± 0.18
9	+	<1.0	22.8 ± 0.38	−	<1.0	23.0 ± 0.13	+	<1.0	23.4 ± 0.28
10	+	<1.0	23.1 ± 0.07	−	<1.0	23.1 ± 0.33	−	<1.0	23.5 ± 0.35
11	+	<1.0	23.1 ± 0.11	−	<1.0	23.2 ± 0.23	+	<1.0	23.6 ± 0.39
12	+	<1.0	23.3 ± 0.18	−	<1.0	23.1 ± 0.40	+	2.3 ± 0.32	23.2 ± 0.16
13	+	1.8 ± 0.10	22.7 ± 0.51	−	<1.0	23.2 ± 0.33	+	<1.0	23.1 ± 0.36
14	+	<1.0	22.6 ± 0.31	−	<1.0	23.3 ± 0.41	−	<1.0	22.7 ± 0.34
15	+	<1.0	22.9 ± 0.30	−	<1.0	23.4 ± 0.18	−	<1.0	22.9 ± 0.23
16	+	<1.0	23.0 ± 0.22						
17	+	<1.0	23.3 ± 0.14						
18	+	<1.0	23.2 ± 0.18						
19	+	2.1 ± 0.06	22.9 ± 0.16						
20	+	2.9 ± 0.30	22.8 ± 0.13						
21	+	<1.0	23.0 ± 0.22						
22	+	<1.0	23.2 ± 0.32						
23	+	<1.0	23.1 ± 0.10						
24	+	<1.0	22.8 ± 0.13						
25	+	<1.0	22.2 ± 0.43						
26	+	<1.0	22.5 ± 0.30						
27	+	<1.0	23.0 ± 0.17						
28	+	<1.0	23.2 ± 0.16						
29	+	<1.0	23.1 ± 0.14						
30	+	<1.0	23.4 ± 0.19						

[a] PCR amplification was observed in qPCR reaction based on the *β-tubulin* gene.
[b] Data represent the mean amount of fungal load quantified (log cfu/g) ± standard deviation (SD) of 3 independent experiments each consisting of triplicate samples.
[c] Data represent the mean threshold cycle (Ct) ± standard deviation (SD) of 3 independent experiments each consisting of triplicate samples.

significant differences within the slope and R^2 values from the standard curves generated with different producing strains. This allowed using whatever of the constructed standard curves by the different producing mold strains for the quantification of *dmaT* gene copy numbers. Although no guidelines have been established for standard curves used in qPCR assays to quantify fungi, it was suggested (Fredlund et al., 2008) 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 a R^2 value $\geq$ 0.98. These guidelines should also be valid for the analysis of fungal DNA in foods. In the present work, the optimized method had R^2 value higher than 0.99. The qPCR showed slope values in the acceptable range and a good efficiency close to 100%, which indicates a good optimization of the qPCR method and no errors occurred in this process. Similar values of efficiency have been reported for qPCR protocols to detect mycotoxin-producing molds (Fredlund et al., 2008; Mulé et al., 2006; Rodríguez et al., 2011a, 2011b, 2012a).

When the sensitivity of the qPCR assay was evaluated in different food matrices all standard curves showed a suitable linearity ($R^2 > 0.98$) and all slopes were within the recommended range by Fredlund et al. (2008). In addition, the limit of detection in all inoculated foods ranged from 1 to 2 log cfu/g for the optimized qPCR method, and was in the level of those previously reported (Rodríguez et al., 2011a, 2011b, 2012a; Selma et al., 2008). In addition, the IAC amplification could be detected with Ct values between 23.2 and 25.1. Therefore, the sensitivity of the qPCR assay for detection and quantification of CPA-producing molds was not influenced by food components, and the results obtained from IAC amplification excluded successfully false negative results.

The quantification of the fungal contamination in inoculated foods (spores and mycelium) was also tested. The developed qPCR assay in this study seems appropriate for monitoring the growth of CPA-producing molds in foods, since the *dmaT* gene copy numbers and the fungal load determined by the qPCR correlated rather well with those cfu data obtained by plating for the toxigenic molds throughout the 14 days of the incubation time. In addition, no significant differences ($P \leq 0.05$) were found between fungal load determined by qPCR and that one obtained by counting in PDA. Similar results have been obtained for other qPCR protocols developed to quantify the copy number of the *idh* gene of patulin-producing molds or the *nor-1* gene of *A. flavus* in foods (Mayer et al., 2003; Rodríguez et al., 2011a). Furthermore, the number of log IAC copies obtained from Ct values was similar to that number included in each reaction. This fact seems to demonstrate that the qPCR reactions were carried out appropriately.

Finally the ability of the developed method to detect CPA-producing molds was tested in naturally infected food samples where total fungal contamination was also evaluated by qPCR based on the *β-tubulin* gene. Up to 17% of the tested dry-cured ham, 18% of paprika and 50% of durum wheat samples, which containing fungal DNA showed CPA-producing molds. Although in all cases

counts of CPA producers were lower than 3 log cfu per gram or cm^2, presence of these toxigenic molds may pose a hazard of accumulation of CPA in those ready-to-eat foods. This could be a great problem, especially in abuse conditions of relative humidity and temperature storage, mainly in dry-cured Iberian ham, mainly in dry-cured Iberian ham, where samples were taken at 6 months of ripening and fully ripened hams take up to 24 months. In all of the naturally infected samples the IAC Ct values ranged from 22.2 to 23.6, indicating the absence of false negative results in the qPCR reaction. In addition, the fact that only in those foods samples showing fungal DNA were detected CPA-producing molds ratifies that the designed method based on *dmaT* gene did not give false results. These results show the efficiency of the developed qPCR to detect CPA-producing molds and its applicability to monitor these toxigenic molds in raw materials/ingredients in HACCP programs. This would allow taking rapid corrective actions to avoid CPA accumulation in prepared foods.

In conclusion, the designed qPCR with IAC allows a reliable and rapid quantification of CPA-producers in foods, avoiding false negative results. This method could be proposed to quantify these toxigenic molds throughout the processing of ready to eat foods.

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