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Evaluation of different RNA extraction methods of filamentous fungi in various food matrices

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ABSTRACT

Aflatoxin producing-moulds may grow on different foods (e.g. cereals, ripened meats) and contaminate such products with aflatoxins. Molecular studies about expression of genes involved in these secondary metabolites biosynthesis require isolation of good quality mould RNA from foods. In this work, six different RNA extraction methods, which combine physical cell disruption protocols and RNA purification techniques, were evaluated in different mouldy foods to obtain intact mould RNA and without these food inhibitors. For this, five food matrices (dry-cured ham, tea, paprika, peanut and wheat) were inoculated with *Aspergillus parasiticus*. Protocols that used bead beating as physical cell disruption and TRIzol yielded RNA of low quality. Differences in RNA yield were found between the two tested commercial kits. The '*mortar-pestle RNeasy*' protocol which consists of freezing the mycelium with liquid nitrogen and grinding it with the aid of a mortar and pestle and the *RNeasy* kit could be utilised to obtain intact RNA from a wide range of aflatoxin-producing fungi of good quantity and quality from contaminated foods.

1. Introduction

Mould contamination of staple foods such as cereals, nuts and ripened meat products has received attention because of their acute and chronic effects in humans and animals (Bernáldez, Rodríguez, Martín, Lozano, & Córdoba, 2014; Pildain et al., 2008; Zinedine et al., 2007). These foods are prone to colonisation by *Aspergillus flavus* and *Aspergillus parasiticus* species due to the climatic conditions occurring either before harvest or under post-harvest as well as in drying/ripening and storage. These species may contaminate such products with aflatoxins (AFs) (Bhatnagar, Cleveland, & Payne, 2000). Aflatoxins are a group of polyketide-derived furanocoumarins possessing hepatocarcinogenic, immunosuppressive, carcinogenic, teratogenic, and mutagenic properties (Peraica, Radic, Lucic & Pavlocic, 1999). Between them, AFB_1 has been classed as 1A carcinogens by the International Agency for Research of Cancer (IARC, 2012).

Early detection in expression of genes associated with AFs before mycotoxin being produced in foods is crucial to take corrective actions for minimising the potential hazard related to mycotoxin production. For this purpose, molecular techniques such as reverse transcription (RT) PCR-based methods and RNA sequencing (RNAseq) may be used. The successful application of this technique depends on an adequate isolation of high-purity mould RNA from foods (Da Silva Messias et al., 2014; Islas-Flores, Peraza-Echeverría, & Canto-Canché, 2006; Leite, Magan, & Medina, 2012).

Thus, mould RNA obtained from foods should be intact and free of contamination from RNAses, proteins, genomic DNA, enzymes and other compounds such as polysaccharides, proteins and polyphenols (Leite et al., 2012) which may compromise the isolation of pure and intact RNA (Coana, Parody, Fernández-Caldas, & Alonso, 2010; Rubio-Piña & Zapata-Pérez, 2011). In addition, the extraction of pure mould RNA *per se* is also quite difficult due to the structure of the mould cell wall that affects the ability to effectively extract RNA (Francesconi et al., 2008). For this reason, standard RNA extraction methods optimised for bacteria and yeasts that contaminate food cannot be utilised.

The most common methodology applied to extract total RNA from cells or tissues includes TRIzol. This reagent improves RNA integrity since it protects RNA from nucleases and prevents its

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degradation (Ali & Alman, 2012). However, this reagent is not advisable for the extraction of a large series of samples in the food industry given that TRIzol is toxic by inhalation or absorption through the skin for the lab workers. Use of RNA commercial extraction kits including purification columns has increased (Ali & Alman, 2012; Al-Saad, Al-Badran, Al-Jumayli, Magan, & Rodríguez, 2016; Ribao, Torrado, Vilariño, & Romalde, 2004; Rodrigues, Venâncio, Kozakiewicz & Lima, 2009; Rodríguez, Medina, Córdoba, & Magan, 2014). Although most of these kits have been proposed by the manufacturer to extract total RNA from plant material, in the last years numerous authors have been utilised such kits for RNA isolation from pure cultures (Al-Saad et al., 2016; Rodrigues et al., 2009; Rodríguez et al., 2014). However, it seems complicated to adequate the commercial kits for extracting total RNA from moulds contaminating foods, the nature of the cell wall together with the abovementioned food inhibitors may block the column resin and avoid the RNA extraction.

However, to date only scarce studies have been performed mould RNA extraction from specific mouldy or contaminated foods including peanuts (Abdel-Hadi, Schmidt-Heydt, Parra, Geisen, & Magan, 2012), cheeses (Bonaïti, Parayre, & Irlinger, 2006) or cereals (Wang, Wang, Zhang, Wang, & Song, 2012). No generalised protocol for RNA extractions from filamentous fungi in different food matrix has been developed. Therefore, mould RNA extraction methods from aflatoxigenic contaminated foods must be evaluated since molecular techniques request a consistent input amount of extracted RNA of good quality (Spornraft et al., 2014).

The aim of this work was to evaluate the efficiency of several mould RNA extraction methods developed by combining different physical grinding methods, commercial extraction kits and use of TRIzol and alcohols to obtain good quality mould RNA from different artificially contaminated foods.

2. Materials and methods

2.1. Mould strain and inoculum preparation

A strain of *A. parasiticus* (CECT 2688) producer of aflatoxin B_1 and G_1, was used in this study. This mould strain was initially inoculated by spreading on Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone and 2% agar; Panreac Química S.L.U., Spain) and incubated at 25 ± 0.2 °C for 7 days. Spores were collected in 10 mL sterile water containing 10% glycerol (Scharlau Chemie S.A., Spain) by rubbing the surface with a glass rod in order to release conidia. The spore suspensions were maintained in glycerol solutions at −80 °C ± 1 °C and new starter cultures were used for each experiment.

The spore suspensions were counted using a Thoma counting chamber and adjusted to 10^7spores/mL and used as an inoculum.

2.2. Experimental settings: inoculation of food matrices and controls

Five different non-sterile commercial foods (slices of dry-cured ham, leaves of black tea, paprika powder, peanut seeds and wheat grains) were aseptically prepared and placed separately in pre-sterilised oblong receptacles made of methacrylate, where the humidity was kept by a saturated K_2SO_4 solution (0.97 water activity) put at the bottom of the receptacles to promote mould growth. Each food matrix (2–3 g) was centrally inoculated with 100 μL of the inoculum which was spread on its surface with a sterile glass rod and then the inoculated food was incubated at 25 ± 0.2 °C for 4 days. After the incubation period, similar fungal growth was observed in all the tested food matrices. In addition, no differences between mycelium weights of the different food matrices evaluated were detected. For RNA extraction, whole samples were aseptically collected and quickly snap-frozen in liquid nitrogen. Next samples were stored in a freezer (Thermo Scientific, Spain) at −80 ± 1 °C until RNA extraction. Only, in the case of *A. parasiticus*-contaminated dry-cured ham, fungal biomass was harvested from its surface by scraping for further RNA extraction in order to simulate the usual non-destructive sampling process to take mycelium from the dry-cured ham surface in the meat industry.

Positive controls to ensure that each RNA extraction method used in this study is adequately performed were obtained as follows: A 100 μL aliquot of *A. parasiticus* CECT 2688 (10^7 spores/mL) was used to inoculate a yeast extract sucrose peptone [YESP; 1% yeast extract (Biomol S.L., Spain), 2% sucrose (Scharlab S.L., Spain) and 2% bacteriological peptone (Biomol S.L., Spain)] based liquid medium modified with addition of 1% tryptophan (YESP-T medium; Sigma Aldrich Química S.A., Spain) and incubated with shaking (100 rpm) for 4 days at 25 ± 0.2 °C, as previously described by Lozano-Ojalvo, Rodríguez, Bernáldez, Córdoba, and Rodríguez (2013).

2.3. RNA extraction methods

The effect of different steps on the RNA extraction procedures such as the type of extraction buffer, method for breakage of fungal cell walls, type of commercial RNA extraction kit and RNA extraction solvents were evaluated (Table 1). Thus, six RNA extraction methods depending on cell lysis method and purification method were assayed on inoculated food matrices and on inoculated YESP-T medium (positive controls, *see* Section 2.2). An overview of these methods is given in Table 1. All methods were tested on each food with three replicates per treatment and repeated twice.

2.3.1. Cell lysis methods

2.3.1.1. Mortar-pestle methods. In these methods, food samples of above 1 g were ground in 20 mL of liquid nitrogen to a fine powder in a −80 ± 1 °C pre-frozen mortar and pestle for 2 min. 50 mg of this powder was transferred to a 2 mL Safe-Lock tube (Eppendorf, Germany). Then, 1 mL of TRIzol (Life Technologies Ltd, Spain), 750 μL RB buffer (provided by E.Z.N.A.™ Fungal RNA Kit, Omega Bio-Teck, USA) or 750 μL RLT buffer (provided by RNeasy® Plant Mini Kit, QIAGEN, Spain) supplemented with 0.1% of β-mercaptoethanol (Table 1) was added. After a quick vortex (10 s) the samples were immediately frozen using liquid nitrogen (5 min) and stored at −80 ± 1 °C (at least 20 min) until required. Next, samples were thawed on ice and processed in two different ways: (a) they were treated using the '*TRIzol*' protocol and, (b) they were processed according to instructions of commercial RNA extraction kits ('*EZNA*' and '*RNeasy*' protocols). The extraction methods are described in Section 2.3.2.

Protocols are identified as '*Mortar-pestle TRIzol*', '*Mortar-pestle EZNA' and 'Mortar-pestle RNeasy'* (*see* Table 1).

2.3.1.2. Bead-beating methods. In these methods, above 50 mg of frozen food samples was placed into a 2 mL Safe-lock tube (Eppendorf) containing 425–600 μm sized acid-washed glass beads (Sigma-Aldrich, St. Louis, USA). Then, 1 mL of TRIzol (Life Technologies Ltd), 750 μL RB buffer (provided by E.Z.N.A.™ Fungal RNA Kit, Omega Bio-Teck) or 750 μL RLT buffer (provided by RNeasy® Plant Mini Kit, QIAGEN) supplemented with 0.1% of β-mercaptoethanol (Table 1) was added. After a quick vortex (10 s) the samples were immediately frozen using liquid nitrogen (5 min) and stored at −80 ± 1 °C (at least 20 min) until required. The extraction was carried out in a mixer mill MM 400 bead beater (Retsch, Germany). Samples were agitated for 25 s at 30 Hz which