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Sensitive determination of cyclopiazonic acid in dry-cured ham using a QuEChERS method and UHPLC–MS/MS

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ABSTRACT

An extraction method and an UHPLC–MS/MS method for the quantification of CPA in dry-cured ham were developed and validated. To optimise detection and quantification of CPA, the composition of mobile phase, flow rates, gradient–related factors and solvents used for resuspension of dry extracts were evaluated. Besides, four extraction methods were tested. The best peak shape and resolution were obtained by eluting the mobile phase consisting in acetic acid-ammonium acetate buffer pH 5.75/methanol in gradient mode at a flow rate of 0.2 mL/min. The method 4 relied on the QuEChERS methodology was the most effective one. Almost half of the 61 dry-cured ham samples examined were contaminated with CPA, with values ranging from 36.1 to 540.1 ng/g. The combination of a QuEChERS-based extraction method and analysis by UHPLC–MS/MS allows highly sensitive, fast, reliable and cheap detection and quantification of CPA for routine analysis in ham.

1. Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi when environmental and nutritional conditions are propitious. These compounds have several adverse effects on animal and human health so their presence should be minimised in feed and food to assure the safety of consumers. For this, efficient analytical tools for the qualitative and quantitative analysis of mycotoxins in food and feed are required.

One of the mycotoxins rarely studied is cyclopiazonic acid (CPA) despite its demonstrated cytotoxicity and immunotoxicity on human cells (Hymery, Masson, Barbier & Coton, 2014). In addition, this mycotoxin displays immunosuppressive properties and haematological disorders in humans (Hymery et al., 2014). These adverse effects in human beings together with those observed in animals (Burdock & Flamm, 2000; Hymery, Masson, Barbier, & Coton, 2014) support the optimisation of a reliable analytical method to detect and quantify this toxic compound for minimising the risk associated with its presence in foods.

CPA is synthesised by many fungal species from genera *Aspergillus* and *Penicillium* such as *Aspergillus flavus*, *Aspergillus versicolor*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus tamarii*, *Penicillium griseofulvum*, *Penicillium commune* and *Penicillium chrysogenum* (Burdock & Flamm, 2000; El-Banna, Pitt & Leistner, 1987; Frisvad, 1989). Some of these species have been isolated in dry-cured ham (Alapont, López-Mendoza, Gil & Martínez-Culebras, 2014; Núñez, Rodríguez, Bermúdez, Córdoba & Asensio, 1996). Although CPA is a frequent natural contaminant of vegetal products such as maize and peanuts (Spanjer et al., 2008), and tomato paste (da Motta & Valente Soares, 2001) it can be also found in animal origin food products including cheese (Ansari & Häubl, 2016), milk (Oliveira, Rosmaninho & Rosim, 2006) and dry-cured ham (Alapont et al., 2014; Bailly, Tabuc, Quérin & Guerre, 2005). Regarding dry-cured ham it is remarkable the stability of this mycotoxin comparing to others during ham processing (Bailly et al., 2005) in spite of the chemical instability of CPA under certain experimental conditions (Diaz, Thompson & Martos, 2010). CPA is an indole-hydrindane-tetramic acid mycotoxin that reacts with ambient oxygen, plastic material and even column stationary phase, which may affect chromatographic parameters (Diaz et al., 2010). This supports the necessity of the performance of a good extraction method and a robust analytical technique to detect and quantify this mycotoxin in ham to avoid an underestimation of CPA presence that would provoke an important risk for consumers' health.

Analysis of CPA in food and feed has proven to be challenging and difficult. Normally, the general procedure for the analysis of mycotoxins in foods comprises three steps: extraction, clean-up and quantification. This assures the removal of interferences from food matrix avoiding false results. The utilisation of a rapid and cheap extraction

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method that would avoid extensive clean-up procedures that are time-consuming and complicated before detection of the mycotoxin would be a step forward. Nowadays there is an extraction methodology defined as quick, easy, cheap, rugged and safe (QuEChERS) (Kamala et al., 2015) which include soaking of the sample prior to extraction and the acidification of the solvents. This method was originally developed for analysis of pesticide residues and is also being utilised widely to extract diverse compounds like mycotoxins from cereals or cereal-based food (Annunziata et al., 2017; Juan, Berrada, Mañes & Oueslati, 2017; Kamala et al., 2015) and wine (Pizzuti et al., 2014) but it has not been used to extract CPA from dry-cured meat products yet. In the past, several methods, such as immunoassays (Huang, Dorner & Chu, 1994), capillary electrophoresis (Prasongsidh et al., 1998), thin-layer chromatography (Gqaleni et al., 1996), and high-performance liquid chromatography (HPLC) (Finoli et al., 1999), have been widely used for detection and quantitative determination of CPA in fungal cultures and agricultural commodities. Nowadays, HPLC methods have been optimised to evaluate CPA amounts in some food matrices such as tomato, milk, cornflakes or cheese (Ansari & Häubl, 2016). Besides, an ultra-high-performance liquid chromatography (UHPLC) method to determine the diversity of CPA family of mycotoxins has been also developed (Uka et al., 2017). However, no UHPLC method combined with a sensitive and specific detection technique such as MS/MS spectrometry to detect and quantify CPA in ham has been developed yet, although it has been successfully applied in detection of other mycotoxins (e.g. Manizan et al., 2018). It has been described that the utilisation of UHPLC in comparison to HPLC has numerous benefits, improving resolution, increasing laboratory productivity because of shorter analysis times and drastically reducing solvent consumption and costs (Alves da Silva, Rodrigues Sampaio & Ferraz da Silva Torres, 2017).

The objective of this study was to develop and optimise an extraction method based on the QuEChERS-based approach and a reliable UHPLC–MS/MS method for the detection and quantification of CPA in dry-cured ham. Validation and applicability of both the extraction and analytical methods were also determined.

2. Materials and methods

2.1. Reagents, solvents and materials

Acetonitrile and methanol were purchased from Fisher Scientific (Fisher Scientific UK Ltd., UK); ammonium acetate Optima® LC/MS and acetic acid Optima® LC/MS from Fisher Scientific (USA). The components of culture media belonged to Scharlab S.L. (Spain), and Pronadisa (Conda Laboratory, Spain). Sodium chloride and anhydrous magnesium sulphate were supplied by Scharlab S.L. and Fisher Scientific, respectively. All solvents were HPLC grade. Pure water was obtained from a Milli-Q® Integral 5 water system (Millipore, USA). Vials, inserts, closures and 0.22 µm filters used for mycotoxins were provided by Cosela S.L. (Spain).

2.2. Standards preparation

CPA standard (Sigma–Aldrich, Germany) was dissolved in acetonitrile at a concentration of 1 mg/mL and stored at −20 °C in a sealed vial until use. Different amounts of the CPA stock standard were placed in amber 1.5 mL vials and were evaporated to dryness under a gentle stream of N_2. Working standards (10–1000 ng/mL of CPA in methanol) were prepared by appropriate dilution of known volumes of the stock solution with methanol.

2.3. Culture media preparation

Malt Extract Agar (MEA) was prepared by dissolving 20 g/L of malt extract, 20 g/L of D(+)-glucose monohydrate, 20 g/L of Bacto agar and 1 g/L of bactopeptone (Rodríguez, Werning, Rodríguez, Bermúdez & Córdoba, 2012). The culture media were prepared by autoclaving for 20 min at 121 °C (103 kPa). Then, the media were vigorously shaken prior to pouring into 9-cm diameter Petri plates.

2.4. Instrumentation

Initially, two chromatographic equipment were evaluated: (1) an HPLC system coupled to an Agilent 1260 Infinity Diode Array Detector (Agilent Technologies, USA) set at 284 nm using a column Phenomenex® Luna C_{18}, 250 mm × 4.6 mm, 5 µm particle size (Phenomenex, UK) preceded by a pre-column (security guard, 4 mm × 3 mm cartridge, Phenomenex® Luna) and (2) a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC UHPLC system with an autosampler thermostat (UltiMate® 3000 Rapid Separation Autosampler, Thermo Scientific, USA) coupled to an Ion Trap Mass (MS) Spectrometer System amaZon SL (Bruker Daltonics Inc., Germany) using the column Agilent Poroshell 120 EC-C_{18}, 150 mm × 2.1 mm, 2.6 µm particle size (Agilent Technologies). Once the UHPLC equipped with the MS/MS spectrometer detector was selected as analytical technique, two columns were evaluated: (1) Agilent Poroshell 120 EC-C_{18} (Agilent Technologies) and (2) Bruker Intensity Solo C_{18}, 100 mm × 2.1 mm, 1.8 µm particle size (Bruker, USA). Once, the Agilent Poroshell 120 EC-C_{18} column was chosen as the best column for CPA analysis by UHPLC, the utilisation of the appropriate guard column (Phenomenex SecurityGuard) was tested.

Finally, the chromatographic system used for CPA analysis was the Thermo Scientific UHPLC. The chromatographic separation was performed using the Agilent Poroshell 120 EC-C_{18} column without the guard column. The injection volume was 10 µL and the run time was 15 min.

Next, CPA by UHPLC were detected and quantified by using the MS detector equipped with an electrospray ionisation (ESI) source. Experiments were conducted in both positive and negative ionisation modes and with a duel ion funnel to increase the ionic transmission to the trap. The operating conditions of the ionisation source were those recommended by the manufacturer for a mobile phase flow rate of 0.2 mL/min: capillary voltage: 4500 V, nebulizer pressure: 15 psi, nitrogen drying gas flow rate: 8 L/min, at a temperature of 200 °C. The *m/z* range was from 50 u to 400 u and working in ultrascan (32,000 u) mode. Other trap parameters were set in ion charge control with a target of 30,000 and a maximum accumulation time of 50 ms, to avoid the oversaturation of the trap. Signals were processed by Hystar 3.2 software (Bruker Daltonics Inc.)

2.5. Optimisation of the analytical method

To optimise detection and quantification of CPA, the composition of mobile phase, flow rates, gradient–related factors and solvents used for dry extracts resuspension were evaluated as detailed below.

a) Comparison of the composition of mobile phases and flow rates

Two mobile phases consisting of formic acid-ammonium formate buffer pH 4.70 (eluent A)/acetonitrile (eluent B) and acetic acid-ammonium acetate buffer pH 5.75 (eluent A)/methanol (eluent B) were used. A binary gradient was applied with flow rate of 0.2 or 0.3 mL/min: 0–0.2 min 2% B, 0.2–0.4 min 40% B, 0.4–11 min linear increase from 40 to 95% B and kept until 12.9 min, followed by re-equilibration of the column until 15 min.

b) Comparison of mobile phase gradients

Once the composition of the mobile phase and the flow rate were selected, different gradients of mobile phases were evaluated. For this, different percentages of the eluent B (methanol) when linear increase starts (from 50% to 70%), and the time period of linear increase of the