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Determination of mycotoxins in fruit berry by-products using QuEChERS extraction method

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ABSTRACT

Fruit berries by-products are mainly elaborated with berry but most of them mixed with other fruits, grapes, plums or apples. These present favourable properties for the growth of a great range of mycotoxins mould producers. Consequently, alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), aflatoxins (AFB1, AFB2, AFG1, AFG2) and ochratoxin A (OTA) are the main mycotoxins that should be present in berries by-products. To establish a reliable analytical method of these mycotoxins on two fruit berries by-products (jam and juice), four different QuEChERS extraction method were evaluated. Recoveries obtained were higher than 74% and 76% in berries juice and jam berries, respectively. LDs for berries jam were from 2 to 8 ng/g and for berries juice from 0.1 to 3 ng/ml. The optimized procedure was applied to analyse 52 berries fruit by-products including 32 berries juice and 20 berries jam randomly collected from different markets in Valencia, Spain. 57% and 71% of berries juice samples presented aflatoxins and *Alternaria* mycotoxins, respectively. OTA was detected only in a blueberry juice (5% at 7.8 ng/ml). This work should contribute to EFSA's recommendations to develop sensitive analytical methods and generate more analytical data concerning the occurrence of *Alternaria* toxins in food and feed.

1. Introduction

The most commonly cultivated fruits harvested from the forest are blueberries, blackberries and raspberries. These are small shrub fruits and multiple drupe fruits of *Vaccinium and Rubus* genus. The subtropical Mediterranean climate, as warm and temperate, helps to produce fully flavoured succulent of such fruits. In Spain, two locations have an ideal climate for berries cultivation, Huelva in the South and Jerte Valley in the Center, both locations representing the main cultivation areas for berries in Spain.

Already published studies have shown that wide varieties of fungi (mostly moulds) are capable of growing and spoiling various types of berries (i.e. small berries such as strawberries, blueberries, blackberries, red currants and raspberries). Their soft and fragile skin is susceptible to small lesions, which allow the growth of spoilage fungi both during pre- and post-harvest stages. That is not surprising considering the fact that these fruits contain high levels of sugar as well as water activity, ideal for fungal growth. Additionally, their low pH eliminates the competition from many bacterial species, making it easier for fungi to grow and spoil the fruits (Jackson & Al-Taher, 2008).

As berry fruit crops are highly perishable, their shelf life has been studied to be extended through the application of chemical substances that inhibit micro-organisms growing and careful control of the surrounding temperature, pressure and humidity once the fruit has been picked. Although refrigeration dramatically slows down fungal growth and prolongs the shelf life of fruits; some fruits are sensitive to low temperatures and suffer chilling injuries, becoming very susceptible to microbial spoilage (Bellí, Marín, Sanchis, & Ramos, 2002). On the other hand, some fungi can grow at low temperatures and cause substantial damage especially if the fruits are stored for extended periods of time. Infections may remain latent until the onset of fruit maturation and become evident during storage. *Alternaria* spp. and *Botrytis* spp. behave as latent pathogens on fruit whereas *Aspergillus* spp., and *Penicillium* spp. behave mainly as wound pathogens (Bellí et al., 2002). When harvesting conditions are optimal, *Aspergillus* spp. such as *A. flavus, A. carbonarius* or *A. ochraceus,* can produce aflatoxins (AFB1, AFB2, AFG1 and AFG2) and ochratoxin A (OTA) in fruits as berries (Sage, 2002; Valero, Marín, Ramos, & Sanchis, 2005).

Alternaria fungi (*Alternaria arborescens, A. tenuissima* and *A. Alternate)* are widely distributed in soil and on plant surfaces,

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which are producers of alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), tentoxin (TEN) and altenuene (ALT) (EFSA, 2011). Some studies have been developed focused on fruits which once harvested had been incubated at room temperature for up to 14 days without supplemental media, and subsequently examined for mould and yeast growth (Tournas & Katsoudas, 2005). Results revealed that almost all berry fruits samples (100% of blackberry and raspberry, 97% of strawberry and 95% of blueberry samples) presented fungal contamination. *Alternaria* was found in blueberry (46%) and strawberry samples (8%), whereas *Penicillium* spp. was present in all types of berries tested.

Isolation of *A. arborescens* has been carried out in fruits such as tomato, blueberries, grapevines, apples and cherries (Greco, Patriarca, Terminiello, Fernández-Pinto, & Pose, 2012; Polizzotto et al., 2012; Hartevelda, Akinsanmiab, & Drenth, 2013; Juan, Oueslati, & Mañes, 2016; Somma et al., 2011); while evidences of blueberries containing a high percentage of *Alternaria* spp. strains have been also presented (Greco et al., 2012). Then, if mycotoxins are present in fruits and these commodities are used to elaborate fruit by-products, a co-presence of these varied mycotoxins may occur.

Scientific publications on risk assessment studies and legislation are available on well-established mycotoxins such as aflatoxins (AFs) and OTA for dried fruits, dried grapes and wine (Juan, Zinedine, Moltó, Idrissi, & Mañes, 2008; EC, 2006) with maximum levels (ML) permitted; however, information on *Alternaria* mycotoxins in fresh fruits and by-products are limited. Up to now, there are no specific international regulations for *Alternaria* mycotoxins in food and feed, and with the recent data collected in 2016 it is still an EFSA's objective to assess their presence in food and feed in the near future (EFSA, 2016). EFSA (2011) released the first report on the risks of *Alternaria* toxins for animal and public health, and recently a dietary exposure assessment to Alternaria toxins in the European population, concluding that develop more sensitive analytical methods and more analytical data on *Alternaria* toxins in relevant food commodities (e.g. fruit and fruit products). This assumption means that European countries have undertaken several efforts to gather occurrence data. Moreover, the impact of fruit on the toxin content is still unknown (EFSA, 2011; 2016).

So far, few information is known on the alternaria mycotoxins presence as well as its co-presence with other mycotoxins in berries fruits and derived products (Van de Perre et al., 2014); which makes important to develop multi-mycotoxins determination. In recent years, the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method is a widely used sample preparation methodology, known for its simplicity and applicability in mycotoxins simultaneous analysis (Juan et al., 2016; Rodríguez-Carrasco, Berrada, Font, & Mañes, 2012). Thereby, the main aim of this work was to develop a robust analytical method for the simultaneous extraction and determination of AFB1, AFB2, AFG1, AFG2, OTA, AOH, AME and TEN in two main berry by-products: juice and jam. Afterward, different berries by-product samples collected from Valencian local markets (Spain) were analyzed to assess these mycotoxins incidence. For this, several QuEChERS extraction methods were assayed, compared and evaluated and its validation was performed by following the EU guidelines (EC, 2002); Analysis was carried out using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2. Material and methods

2.1. Chemical and reagents

Methanol UHPLC Supergradient (purity 99.9%) and LC-MS grade (purity 99.9%); and acetonitrile UHPLC Hipergradient (purity 99.9%) were supplied by PanReac AppliChem (Castellar del Vallés, Spain). Ammonium formate (assay 98.4% VWR Chemicals Prolabo, Leuven-Belgium); sodium chlorure (assay 99.9%, VWR Chemicals Prolabo, Leuven-Belgium); formic acid (purity 98% Fischer-Chemical) was used for the mobile phase and the extraction procedure.

Pure standards of the target compounds, including AFB1 (purity HPLC $\geq$ 98.00%), AFB2 (purity HPLC $\geq$ 98.00%), AFG1 (purity HPLC $\geq$ 98.00%), AFG2 (purity HPLC $\geq$ 98.00%), OTA (purity HPLC $\geq$ 98.00%), AOH (purity TLC $\geq$ 94.00%), AME (purity TLC $\geq$ 98.00%), and TEN (purity HPLC $\geq$ 95.00%) were purchased from Sigma Aldrich (Madrid, Spain). The individual stock solutions of each mycotoxin at 500 μg/ml were prepared in methanol. On the other hand, a working mixed standard solution at 5 and 10 μg/ml were prepared immediately before use by diluting the individual stock solution in methanol and stored at −20 °C in amber glass vials and darkness before use. This solution was used to prepare the calibration curves and matrix matched calibration curves.

2.2. Sample collection

A total of 52 berry product samples (juices and jams) were purchased from different local markets from Valencia in Spain during 2015. Of these, 20 samples were jams and 32 juices. Both berry by-products mainly contained fruit berries (strawberries, blackberries, blueberries, cranberries and raspberries) and mixed in minor percentage with: grape, pomegranate, cherry, apple and plum.

Commercial jams contained different percentage of fruits ranging from 50 to 75%, but most of them had 60% of fruit and were constituted by a maximum of two mixed fruits. However, berry juices contained different combination of fruits with a percentage of berries around 60% (ranging from 55 to 100%) and a maximum of six different mixed fruits. Before analysis, all the samples were stored in polyethylene tubes maintained at refrigeration temperature (4 °C) during a maximum of 48 h. For the mycotoxins extraction, samples were previously homogenized, juice with a vortex (Genie1 touch mixer, Scientific Industries, USA) and jam fruit with Ultra-Turrax (IKA T18-basic, Staufen Germany).

2.3. Optimization of QuEChERS extraction procedure

QuEChERS procedure from Rodríguez-Carrasco et al. (2012) was modified and employed to extract mycotoxins from juice and jam berries. To maintain constant the variability of fruit type, the blanks used were selected according to its similar composition and proportion. In fact, the blank juice and jam used were a mixed of four berries (blackberries, blueberries, cranberries and raspberries), so that raw berries blank prepared were also a mixed of these four fruits. It was also carried out proves with apple sauce to broaden the scope of applicability of the extraction procedure. Previously four different QuEChERS procedure were tested to choose the best one on berry fruit by-products. So that, different steps and different proportions of salt and solvent were used. “Method 1” (M1) consisted in a first step of putting into 50 ml PTFE centrifuge tube, 10 ml of juice or 5 g of jam, 10 ml of acetonitrile solvent and 1.5 g sodium citrate, and then centrifuged for 5 min at 4500 rpm. Afterwards, two phases were obtained and the upper layer (6.5 ml) was transferred in other PTFE centrifuge tube where it was added and mixture with 4 g $MgSO_4$ and 1 g NaCl, and consecutively the mixture was centrifuged for 5 min at 4500 rpm (final upper layer 5 ml). However, in “Method 2” (M2) the initial step consisted in mixing and centrifuging 10 ml (juice) or 5 g (jam) of sample with 10 ml of acetonitrile without sodium citrate; and after this, the same procedure as described for M1 was followed (first upper layer 8.5 ml and the second 7.5 ml).