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# SCREENING OF FUNGAL METABOLITES IN BRAZIL NUTS USING LC/MS/MS

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**Abstract**

The aim of this study was to evaluate quantitatively the occurrence of fungal metabolites in Brazil nuts. Nuts were collected from Agroforest production areas in Amazon basin region. A total of 235 mycotoxins were investigated/screened by a multi-mycotoxin method based on HPLC-MS/MS. The recovery was between 56 and 136%. Fifteen mycotoxins were detected and quantified, in at least one sample; namely, aflatoxins $B_1$, $B_2$, $G_1$, and $M_1$, kojic acid, sterigmatocystin, methyl-sterigmatocystin, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin. Aflatoxins were detected in just 1 sample (20%), but above its legal limit in Brazil and EU. Ochratoxin A and *Fusarium* toxins were not detected. Alternariol-methylether (from 0.75 to 3.2 $\mu g.kg^{-1}$) was detected in all five samples. This is the first study dealing with the detection of kojic acid, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin in Brazil nuts.

## 1. Introduction

Economically, Brazil nut export market to the EU was greatly reduced because of its high frequency of contamination with Aflatoxins (AF) (Xavier and Scussel, 2008). Recently, the EU revised its limit for aflatoxins in Brazil nuts to 10 $\mu g.kg^{-1}$ (EU, 2010), while the Brazilian regulation establishes maximum limits for AF within 20.0 $\mu g.kg^{-1}$ for AF total ($B_1$+$B_2$+$G_1$+$G_2$) in all human foods (BRASIL, 1966)].Considering that the EU regulation on maximum residue levels (MRLs) has restricted the Brazil nuts export, there is a need for a highly sensitive, self-confirmatory and faster method for measuring mycotoxins, especially AF, to comply with that regulation. The development of multi-mycotoxin methods (Sulyok et al., 2007) enables the simultaneous analysis of a larger fraction of the 300-400 fungal metabolites which are currently recognized as mycotoxins. It was already developed a methodology by LC-MS/MS able to detect and quantify $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ (Xavier and Scussel, 2008) at lower levels than the current methodology (solid phase extraction and quantification by HPLC-FD) for Brazil nuts (AOAC, 2005). Now it is opportune to evaluate other fungi metabolites in Brazil nuts, since many different fungi species have ability to produced others micotoxins than AF. In view of this, the present work aimed to investigate mycotoxin contamination in a Brazil nut matrix by using a HPLC-MS/MS multi-mycotoxin method.

## 2. Materials and methods

Brazil nuts samples 1 to 4 (2008-2009) were obtained from agroforestry while sample 5 (2007) was collected from natural forest. Each sample was thoroughly mixed, and a working sample of 300 g was withdrawn.

After storage, all working samples were milled, homogenized for 15 min, packed under vacuum and frozen stored until being analyzed.

Extraction, dilution and analysis were performed as described by Vishwanath et al. (2009). Detection and quantification (Sulyok et al, 2007) was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionisation (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. 235 metabolites were analyzed by the LC-MS/MS, this protocol was published with 186 metabolites (Vishwanath et al., 2009) and was in the meantime extended to 235 metabolites (201 fungal metabolites and 34 bacterial ones).

The recovery was determined in triplicate by analyzing sample number 2 after spiking with appropriate amounts of a multi-analyte working solution (Vishwanath et al, 2009). Limits of detection (LOD) were calculated from the signal to noise ratios

(LOD = 3 $^{x}$ S/N) of the respective Multiple Reaction Monitoring (MRM) chromatograms deriving from the analysis of the spiked samples.

## 3. Results and discussion

From the 235 analytes tested, only 15 fungal metabolites and two bacterial metabolites were detected in at least one of the Brazil nuts samples. Table 1 reports the detected compounds and the methodology performance data (limit of detection (LOD) and average recoveries). Detected fungal metabolites are mainly produced by *Aspergillus* and *Penicillium* species, and *Fusarium* toxins were not detected. Also, from the main *Aspergillus* and *Penicillium* mycotoxins, only citrinin and aflatoxins and its precursors were detected. Other relevant mycotoxins, such as ochratoxin A and patulin were not detected. Traces of two bacterial metabolites (valinomycin and choramphenicol) were identified as well.

Although AFs are the most relevant mycotoxins in Brazil nuts only one sample presented contamination (180.0 $\mu g.kg^{-1}$ $AFB_1$, 21 $\mu g.kg^{-1}$ $AFB_2$, 320.0 $\mu g.kg^{-1}$ $AFG_1$, 5.8 $\mu g.kg^{-1}$ $AFM_1$) and no $AFG_2$ was found. These levels are high, considering the maximum limits established in Brazil and in the EU. The high AF value found in this sample could be related to either the condition in the field in the natural forest at the collection site or to the stored period time of more than two years in our facilities.

**Table 1:** Results of mycotoxins concentrations in Brazil nuts samples

| **Analyte** | **Sample number (µg/kg)** | | | | | **LOD** | Recovery **(%)** |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | (µg/kg) | |
| Aflatoxin $B_1$ ($AFB_1$) | < LOD | < LOD | < LOD | < LOD | 180 | 0.8 | 56 |
| Aflatoxin $B_2$ ($AFB_2$) | < LOD | < LOD | < LOD | < LOD | 21 | 4 | 56 |
| Aflatoxin $G_1$ ($AFG_1$) | < LOD | < LOD | < LOD | < LOD | 320 | 24 | 60 |
| Aflatoxin $M_1$ ($AFM_1$) | < LOD | < LOD | < LOD | < LOD | 5.8 | 4.8 | 65 |
| Kojic Acid. (KA) | 2200 | < LOD | < LOD | < LOD | < LOD | 60 | 113 |
| Sterigmatocystin (SMC) | < LOD | < LOD | < LOD | < LOD | 5.9 | 1.5 | 57 |
| Methyl-Sterigmatocystin (MSMC) | < LOD | < LOD | < LOD | < LOD | 84 | 1.5 | 69 |
| Citrinin (CTN) | 135 | < LOD | < LOD | < LOD | < LOD | 8 | 106 |
| Cyclosporin A | < LOD | < LOD | 2.3 | 650 | 130 | 1 | 136 |
| Cyclosporin C | < LOD | < LOD | < LOD | 76 | < LOD | 6 | 88 |
| Cyclosporin D | < LOD | < LOD | < LOD | 180 | 27 | 15 | 82 |
| Cyclosporin H | < LOD | < LOD | < LOD | 1100 | 260 | 6 | 72 |
| Rugolusin | < LOD | < LOD | < LOD | 830 | < LOD | 10 | 90 |
| Alternariolmonomethyl-ether (AME) | 0.75 | 2.7 | 3.2 | 1.4 | 1.6 | 0.3 | 91 |
| Emodin | 40 | < LOD | < LOD | 230 | < LOD | 0.3 | 80 |
| Valinomycin | 0.41 | < LOD | < LOD | 0.19 | 0.22 | 0.2 | 86 |
| Choramphenicol | < LOD | < LOD | < LOD | < LOD | 20 | 0.3 | 136 |

nd = not detected; nd < LOD (Limit of detection)

## 4. Conclusions

The performance of the present method was compared with some of the methods already reported in the literature. The LC–MS/MS method and the sample pre-treatment procedures employed in the present work can be regarded as a selective, accurate, precise, and robust quantification method successfully applied to the simultaneous quantification of secondary metabolites in Brazil nuts.

As concerns aflatoxins, only one sample was contaminated and the contamination levels exceeded the maximum levels established by the EU, also other mycotoxin as Kojic acid appeared in high quantities. This would lead to increase an additional concern mycotoxins risk to the Brazil nuts consumers.

This is the first study detecting kojic acid, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin in Brazil nuts. Although some mycotoxins content in Brazil nut were low, samples presented one to 10 fungal metabolites.

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