Optimization of Electrospray technique coupled to Mass Spectrometry for the Determination of Ochratoxin A in Coffee

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ABSTRACT
Optimization of Electrospray technique coupled to Mass Spectrometry for the Determination of Ochratoxin A in Coffee. Coffee constitutes an extremely complex food matrix and has an important role in the world’s economy, especially in producing and exporting countries like Brazil. High Performance Liquid Chromatography coupled to tandem mass spectrometry with selective monitoring reaction (LC-MS/MS-SMR) was used for the development of a method for the analysis of ochratoxin A (OTA) in roasted coffee samples. The extraction step was based on a liquid-liquid extraction followed by a clean up with immunoaffinity column. This paper shows a method developed for identification and quantification of Ochratoxin A (OTA) in roasted coffee, which will be used for future production of Certified Reference Material (CRM) at Inmetro.

Key words: Coffee; Mycotoxins; HPLC; MS/MS.

RESUMO
Otimização da Técnica de Eletronebulização Acoplada a Espectrometria de Massas para Determinação de Ocratoxina a em Café. O café constitui uma matriz extremamente complexa e tem um importante papel na economia mundial, especialmente nos países produtores e exportadores como o Brasil. A Cromatografia líquida de alta eficiência acoplada à espectrometria
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de massa em série com monitoramento seletivo de reação (CLAE-EM/EM-MSR) foi utilizada para o desenvolvimento de um método para a análise de ocratoxina A (OTA) em amostras de café torrado. A etapa de extração foi baseada em extração líquido-líquido seguida de limpeza em coluna de imunoafinidade. Este trabalho apresenta um método desenvolvido para identificação e quantificação de Ocratoxina A no café torrado, que servirá como subsídio para a futura produção de Material de Referência Certificado (MRC) no Inmetro.

Palavras-chave: Café; Micotoxinas; CLAE; EM/EM.

INTRODUCTION

Ochratoxin A (OTA) is a potent nephrotoxic and nephrocarcinogenic mycotoxin produced by several Aspergillus and Penicillium species, that has been found in several foods, including green, roasted and instant coffee (ALMEIDA et al., 2007; FUJII et al., 2007; SFORZA & DALL’ASTA & MARCHELLI, 2006; MASOUD & KALTOFT, 2006; GOLLUCKE & TANIWAKI & TAVARES, 2004; TANIWAKI et al., 2003; BULLERMAN, 2003; MANTLE, 2002; FUJII & ONO & HIROOKA, 2002; BUCHELLI & TANIWAKI, 2002; PRADO et al., 2000; BRESH & URBANEK & HELL, 2000; FURLANI & SOARES, 1999; BECKER et al., 1998; PATEL et al., 1997; STUDER-ROHR et al., 1995; POHLAND & NESHEIM & FRIEDMAN, 1992).

It is estimated that 12 % of the ochratoxin A consumed by humans corresponds to coffee beverage (CODEX ALIMENTARIUS COMMISSION, 2006).

Coffee is an extremely complex food matrix and constitutes an important role in world’s economy. Brazil is the third consumer of the coffee beverage, according to data from Brazilian Association of Coffee Industry (ABIC-Associação Brasileira da Indústria de Café). In order to analyze OTA in this matrix is necessary an adequate clean up to remove substances like lipids and pigments that could interfere in the analytical techniques. For the extraction of coffee a liquid-liquid extraction with water, organic solvents, mix of salts and acids can be used, taking into account losses of ocratoxin A, followed by solid phase extraction with immunoaffinity columns (FUJII et al., 2007; COMMISSION REGULATION, 2005; VARGAS & SANTOS & PITTET, 2005).

Several techniques have been described for determining OTA in different food matrix using thin-layer chromatography (TLC), gas chromatography (GC), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography with fluorescence detector (HPLC-FLD) and liquid chromatography–mass spectrometry (LC/MS) (ALMEIDA et al., 2007; MASOUD & KALTOFT, 2006; PRADO et al., 2000; GILBERT & ANKLAM, 2002; PARDO et al., 2004; AHMED et al., 2007; SUGITA-KONISH et al., 2006).

HPLC/MS is also an excellent tool to elucidate the structure and predominantly applied to confirm positive results obtained by ELISA or HPLC-FLD. Only few new LC/MS studies were published for OTA applying different liquid injection. They are focused on the mass spectrometric properties such as ionization efficiency and in-source fragmentation. LC/ESI-MS/MS is especially helpful in confirming doubtful “Ochratoxin A positive” results and the sensitive is enhanced by operating the MS in the selected reaction monitoring mode (SRM).
Coelution problem of interfering compounds and retention time shifts could lead to erroneous positive or negative results can be overcome by structural elucidation provided by coupling of LC and mass spectrometry (MS) (DIAZ & ARIZA, PERILLA, 2004).

The maximum limits for OTA are regulated in many countries but not in Brazil. A regulatory limit of 5.0 ng g\(^{-1}\) for OTA in roasted coffee has been established by European Union (EU, 2005/123/EC). This reference value was followed because main markets for Brazilian coffee are in European countries, like Italy and Germany (ALMEIDA et al., 2007; Pardo et al., 2004; PRADO et al., 2000; MASOUD & KALTOFT, 2006).

Due to the importance of this mycotoxin as a contaminant in coffee, possible risks to human health and very low maximum limit permitted it is necessary to study analytical methods that are more sensitive and will comply with the limits imposed.

The aim of this study is to develop a high performance liquid chromatography method coupled to tandem mass spectrometry with electrospray for the analysis of ochratoxin A (OTA) in roasted coffee samples, using ochratoxin B (OTB) as internal standard, with the extraction step based on a liquid-liquid extraction followed by a clean up with immunoaffinity column. This study is the first step to produce a Certified Reference Material (CRM) for ochratoxin A in food samples such as coffee at Inmetro.

**MATERIAL AND METHOD**

**Material**

Solvents and standards used were Ochratoxin A (Sigma-Aldrich, St Louis, USA) and Ochratoxin B standards (Sigma-Aldrich, St Louis, USA). Sodium bicarbonate (Tedla, USA, 99.7 %), Potassium dihydrogen phosphate (Merck, Germany, 99.0 %), Anhydrous disodium hydrogen phosphate (Merck, Germany, 99.0 %), Sodium chloride (Spectrum, USA, 99.0 %), Potassium chloride (Merck, Germany, 99.5 %), Potassium dichromate (Merck, Germany, 99.5 %), Sulfuric acid (Merck, Germany, 97.0 %), Methanol HPLC grade (Tedla, USA, 99.9 %), Trifluoroacetic acid HPLC grade (Tedla, USA, 99.8 %), Acetone Pesticide grade (Tedla, USA, 99.8%), Water glacial acetic acid (Tedla, USA, 99.9 %), Toluene HPLC grade (Tedla, USA, 99.8 %), Benzene (Merck, Germany, 99.5 %), Acetone A.C.S. grade (Tedla, USA, 99.8%), Ethyl alcohol (Quimes, Brazil, 95.0 %), Sodium hypochlorite (Invema, Brazil, 12.0 %), Alcaline extran (Merck, Germany). Water system Milli-Q (Millipore Inc., Paris, France, type I) was used, ultrapure water with conductivity under 0.056 mS\(\text{cm}^{-1}\).

**Solutions**

**Stock Solution**

The first stock solution of OTA was prepared by dissolving 1 mg OTA in 5 mL of toluene: glacial acetic acid (99:1). For ochratoxin B (OTB) the same dilution was made, but using benzene and glacial acetic acid (99:1) as solvents. The second stock solution for OTA was
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prepared to calibrated spectrophotometer at 333 nm, using extinction coefficient 5550 M⁻¹cm⁻¹. For OTB was used 320 nm and extinction coefficient 6000 M⁻¹cm⁻¹. It follows the recommendation of AOAC for quantifying the exact concentration for OTA and OTB.

**Working Standard Solution**

Working standard solution of 0.1 µg/g for OTA and OTB was prepared as followed. In a 4 mL glass flask, previously tared, a mass of 0.14748 g second stock solution was weighted. In other 250 mL amber glass flask, previously tare discounted, was weighted 100.096 g of mobile phase. Next, it was carefully inserted the 4 mL flask inside the flask containing the mobile phase and mixed well to obtain a final solution.

**LC Mobile Phase**

The mobile phase consisted of mixture of trifluoracetic acid in water (0.05 %; solvent A) and trifluoracetic acid in methanol (0.05 %; solvent B) at flow 0.3 mL/min in an isocratic system (20:80). The mobile phase should be filtered with membrane and degassed by ultrasonic system.

**Phosphate-Buffer-Saline Solution (PBS)**

0.20 g potassium dihydrogen phosphate, 1.10 g anhydrous disodium hydrogen phosphate, 8.00 g sodium chloride and 0.20 g potassium chloride were weighted to 1000 mL of deionized water and homogenized.

**Aqueous Sodium Bicarbonate Solution (3 %)**

30.0 g of sodium bicarbonate was weighted to 1000 mL of deionized water and homogenized.

**Calibration Curve**

The calibration curve (1.0 ng/g; 3.0 ng/g; 5.0 ng/g; 7.0 ng/g and 9.0 ng/g) was prepared from gravimetric dilution of OTA working solution (0.1 µg/g) in mobile phase. In a previously tare discounted 10 mL glass flask, weighted the desired amount of OTA stock solution. Next, added mobile phase up to relative mass to obtain the desired concentration and record the final mass. Mix and vortex (Phoenix) during 30 seconds. This procedure was repeated for each point of calibration curve and calculate the final concentration of them.

Added the internal standard (IS), OTB, to the curve points weighting 1.00 g of IS solution to 1.00 g of each point of the calibration curve solution. Mix in vortex (Phoenix) during 30 seconds and transfer 1.00 mL to a 1.5 mL vial amber flask.

**Extraction**

The sample preparation procedure was based on Pittet’s work. An aliquot of 25.0 g of roasted coffee samples was weighted into a 250 mL amber glass flask and fortified with standard solution and kept at room temperature overnight. Then, it was transferred quantitatively, in addition of 190.0 g of a mixture of methanol and an aqueous sodium bicarbonate solution...
3.0 % (1:1), and mixed in a blender for 5 min at low speed. The homogenated sample was filtered through three filters under vacuum using a qualitative paper JP41 28 μm (J.Prolab, Germany), followed by a fiberglass (Whatman, EUA) and a cellulose membrane 0.45 μm (Millipore, EUA). After that, 4 mL aliquot of filtered extract was transferred to a flask and completed to 100 mL with phosphate-buffered-saline solution and homogenized (Solution 1).

Imunnoaffinity Columns (IA)

For further purification, the ochratest immunoaffinity column (IA) was placed at room temperature. On the top of this column a 60.0 mL syringe was attached. A vacuum Manifold Vac Elut 20 (Varian, Walnut, USA) was connect to the IA column as well. A 4.6 g aliquot of the filtrate was weighted into a 100.0 mL volumetric flask, diluted with a saline phosphate buffer and homogenized. This extract was eluted into the IA column at a flow rate of 2.0 - 3.0 mL min⁻¹. After that, the IA column was flushed with 10.0 mL of Milli-Q water at the same flow rate and then slightly dried by vacuum for 30 s. An aliquot of 4.0 mL of methanol HPLC was added and then a period of 3 min was waited to allow the solvent to permeate the gel before elution step. The OTA was collected in a test tube. The solvent was removed under nitrogen stream at 37 °C. Finally, the extract was reconstituted with 1.0 g of mobile phase and homogenized in a vortex (Phoenix, USA).

Next, add 1 g of internal standard solution, in the same way for preparation of the calibration curve.

Determination of OTA

The MS was performed on a triple-stage quadrupole 1200L (Varian, Walnut, CA, USA) equipped with electrospray interface (ESI). For HPLC it was used an auto injector, a column thermostat and two mobile phase pumps. Chromatographic separations were performed on a Synergi Hydro column (100 mm x 2.0 mm i.d: 4 μm, Phenomenex, Torrance, California, USA) at 25 °C, with a mobile phase at a flow rate of 0.3 mL/min. The injection volume was 50 μL. For the MS the following parameters were used: needle 5000 V, capillary 40 V, drying gas 220°C, 19 psi, nebulizing gas 35 psi, shield 600 V, detector 1700 V. Full-scan spectra were acquired over the range of 50–460 m/z.

RESULTS AND DISCUSSIONS

Electrospray was used for this analysis in spite of being considered to be a mild ionization method and generally produces quasi-molecular ions with little fragmentation, since it results agree reasonably well with LC/ Fluorescence detection for complexes matrices (LAU & SCOTT & LEWIS & KANHERE, 2000).

The LC/MS/MS parameters were optimazed through direct infusion of the standard solution (20 ug/mL) of Ochratoxin A (OTA) and Ochratoxin B (OTB).
First, a full-scan spectra of these standards, over the range $m/z$ 50-460, was obtained (Figures 01 and 02). Ions of the scan were selected for Selective Ion Monitoring (SIM) and were monitored (239 m/z, 241 m/z, 371 m/z and 404 m/z for OTA; 187 m/z, 205 m/z, 223 m/z, 324 m/z, 370 m/z and 392 m/z for OTB).

![Figure 01. Full-scan for Ochratoxin A.](image1)

![Figure 02. Full-scan for Ochratoxin B.](image2)
As shown in Figures 01 and 02, the fragmentation ions were 149, 239 and 404 m/z for OTA and m/z 205, 149, 370, the ion 239 and 205 m/z corresponded to the loss of phenylalanine for OTA and OTB, respectively (BECKER et al., 1998).

After the optimization of LC-MS-MS parameters for OTA and OTB, a collision using Argon gas at a pressure of approximately 2.0 mTorr was performed for Multiple Reaction Monitoring experiment (MRM) in the positive mode using the protonated molecule [M+H]+ at 404 m/z for OTA and [M+H]+ 370 m/z for OTB obtaining breakdown curves for ochratoxins. (Figure 03 and 04).

**Figure 03.** Breakdown curve for Ochratoxin A.

**Figure 04.** Breakdown curve for Ochratoxin B.
For sensibility improvement it was tested a MRM method with segment analysis. The method was divided into two segments in time, one from 0-1.90 minutes, monitoring only OTB transitions and other 1.91-5.0 minutes for OTA transitions. The retentions time were 1.6 minutes for OTB and 2.0 minutes for OTA. Figure 05 shows a transition 370>205 and 404>239 of a mixed standard solution (OTB and OTA) at 5 ng/g injected on a method of MRM with segment. MRM is better with segment, it increases sensibility by monitoring only three ions per time.

Through the analysis of the breakdown curves precursor-to-product transitions was obtained for the performance of MRM method, these ions were chosen according to their collisions energies: m/z 404>404 (-4.5 V), 404>358 (-10.5 V), 404>239 (-21.5 V) for OTA and for OTB, 370>370 (-4.0 V), 370>324 (-10.0 V), 370>205 (-19.0 V), thus the most intensity product ion was used for quantification, m/z 239 for OTA and m/z 205 for OTB.

Figure 06 shows a transition 370>205 and 404>239 (OTB and OTA) of a commercial coffee chromatogram, obtained at local market, contaminated with 5 ng/g of standard solution of OTA, extracted as described in 2.3, using MSR with segment. As can be seen, at the maximum limit permitted for roasted coffee, good chromatograms can be obtained and quantification is possible. Validation parameters are under investigation.
SIM method was tested for the analysis of OTA and OTB in solution each in the concentration level of 100 ng/g, monitoring 121, 149, 239, 241, 404 m/z for OTA and 149, 187, 205, 206 and 370 m/z for OTB. However, concentrations in the range of the calibration curve (2.0-9.0 ng/g) were not observed well with high noise and poor peak resolution.

A calibration curve was prepared in the range of concentration of 2.0-9.0 ng/g. The linearity of the method was obtained by linear correlation coefficient (r) equal to r=0.98417, above the reference value (0.90) established by Inmetro’s document, indicating the linearity (Figure 07) (DOQ-CGCREE-008, 2007).
CONCLUSIONS

This work enabled the development of the technique of liquid chromatography coupled with mass spectrometry triple quadrupole for the analysis of ochratoxin A. Method with SIM showed to be worse than MRM because of the difficulty of obtaining peaks resolved for quantification in range of calibration curve. The analytical method is under validation and will be used for quantifying the OTA in complex matrix such as coffee in the Chemical Metrology Division (DQUIM) at Inmetro.

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