Development of Method for Ochratoxin A Analysis in Coffee by Liquid Chromatography/Electrospray Tandem Mass Spectrometry

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ABSTRACT

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 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 work shows preliminary results of a developed method which will be used to produce a Certified Reference Material (CRM) for ochratoxin A in food sample such as coffee, here at Inmetro.

1. 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 [2-18]. It is estimated that 12 % of the ochratoxin A consumed by humans corresponds to coffee beverage.

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) [1]. 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 ochratoxin A, followed by solid phase extraction with immunoaffinity columns [12, 21, 22].

Several methods have been described for the determination of ochratoxin A in different food. Most of them are based on column liquid chromatography (LC) using reversed-phase columns with fluorescence detection. For coffee cited immunoenzimatic assays, thin layer chromatography with fluoresce detector, gas chromatography with mass spectrometer detector and high performance liquid chromatography with fluorescence detector or mass spectrometer detector.

A regulatory acceptable level of 5 ng/g in roasted coffee in Europe has been recommended [14-16, 19, 20].

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 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 sample such as coffee at Inmetro.

2. EXPERIMENTAL

2.1. Material

Solvents and standards used were Ochratoxin A (Sigma-Aldrich, St Louis, USA) and Ochratoxin B standards (Sigma-Aldrich, St Louis, USA). Sodium

bicarbonate (Tedia, 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 dicromate (Merck, Germany, 99.5 %), Sulfuric acid (Merck, Germany, 97.0 %), Metanol HPLC grade (Tedia, USA, 99.9 %), Trifluoroacetic acid HPLC grade (Tedia, USA, 99.8 %), Acetone Pesticide grade (Tedia, USA, 99.8%), Water glacial acetic acid (Tedia, USA, 99.9 %), Toluene HPLC grade (Tedia, USA, 99.8 %), Benzene (Merck, Germany, 99.5 %), Acetone A.C.S. grade (Tedia, USA, 99.8 %), Ethyl alcohol (Quimes, Brazil, 95.0 %), Sodium hypoclorite (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 $mScm^{-1}$.

2.2. Solutions

2.2.1. 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 OTB the same dilution was made, but using Benzene and glacial acetic acid (99:1) as solvents. The second stock solution for OTA was prepared to calibrated spectrophotometer at 333 nm, using extinction coefficient 5550 $M^{-1}cm^{-1}$. For OTB was used 320 nm and extinction coefficient 6000 $M^{-1}cm^{-1}$. Like recommendation of AOAC for quantifying the exact concentration for OTA and OTB.

2.2.2. Working Standard Solution
Working standard solution of 0.1 ug/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, carefully inserted the 4 mL flash inside the flask containing the mobile phase and mixed well to obtain a final solution.

2.2.3. 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.

2.2.4. 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 homogenize.

2.2.5. 3 % Aqueous Sodium Bicarbonate Solution
30.0 g of sodium bicarbonate was weighted to 1000 mL of deionized water and homogenize.

2.2.6. 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 ug/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 s. 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.

2.3. Extraction
For sample preparation, an aliquot of twenty-five grams of roasted coffee was weighed in a 250 mL glass flask, and extracted with 100 mL of methanol and 100 mL of 3 % aqueous sodium bicarbonate solution. The suspension was mixed on a blender for 5 minutes and filtered through a folded qualitative paper. Immediately after this filtration, collect the filtrate and re-filter through a fiber glass and cellulose membrane. Both of them using a vacuum system. After that, 4 mL aliquot of filtered extract is transferred to a flask and completed to 100 mL with phosphate-buffered-saline solution and homogenize (Solution 1).

2.3.1. Imunnoaffinity Columns (IA)
Let the immunoaffinity column come room temperature, and connect under vacuum and attach a 60 mL reservoir. Throught the solution 1 for this system at a flow rate of 2-3 mL/min. Wash the column with 10 mL of Milli-Q water at the same flow rate. Dry the column by applying a slight vacuum for 30 s. Apply 4 mL of methanol into the IA and wait 3 minutes to allow the solvent to permeate the gel before elution. The eluate was evaporated to

dryness under a stream of nitrogen gas at 40 ˚C and reconstituted with 1 g of mobile phase and homogeneize in a vortex (Phoenix). Next, add 1 g of internal standard solution, in the same way for preparation of the calibration curve.

2.4. 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 ^{o}C, with a mobile phase at a flow rate of 0.3 mL/ min. The injection volume was 50 uL. For the MS the following parameters were used: needle 5 000 V, capillary 40 V, drying gas 220 ^{o}C, 19 psi, nebulyzing gas 35 psi, shield 600 V, detector 1700 V. Full-scan spectra were acquired over the range of 50–460 m/z.

3. RESULTS

First, for analytical method development was optimized the ionization conditions for OTA e OTB using electrospray through direct infusion of a working solution (20 ug/mL) into the mass spectrometer.

A full-scan spectrum of these standards, over the range m/z 50-460, was obtained, Figs. 1 and 2. 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).

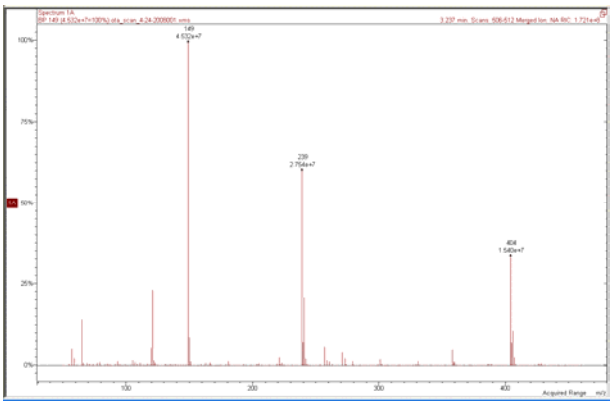

Fig. 1. Full-scan for Ochratoxin A.

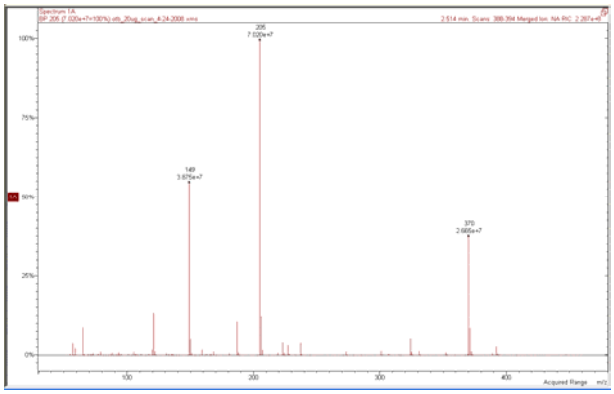

Fig. 2. Full-scan for Ochratoxin B.

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, Figs. 3 and 4.

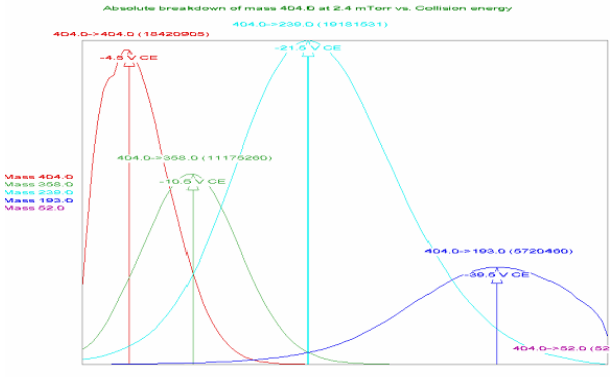

Fig. 3. Breakdown curve for Ochratoxin A.

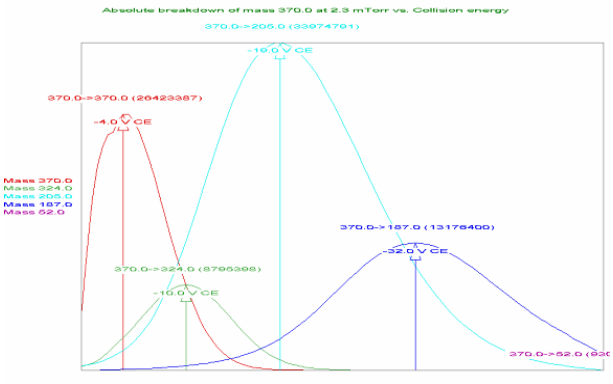

Fig. 4. Breakdown curve for Ochratoxin B.

For sensibility improvement it was tested a MRM method with segment analysis. The method was divided into two segments in time, one from 0 min to 1.90 min, monitoring only OTB transitions and other 1.91 min to 5.0 min for OTA transitions. The retention time was 1.6 min for OTB and 2.0 min for OTA. Fig. 5 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.

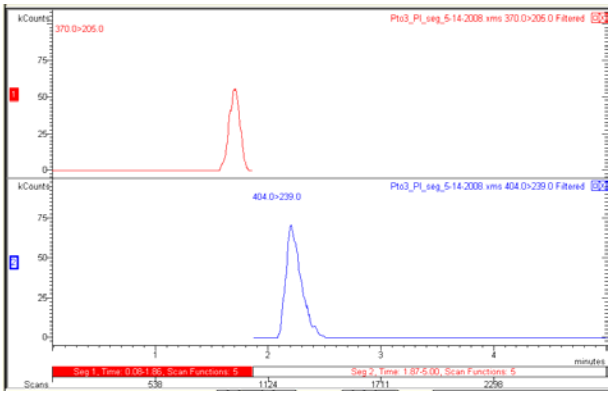

Fig. 5. Mixed standard solution at 5 ng/g of MRM with segment.

Fig. 6 shows a commercial coffee chromatogram, obtained at local market, contaminated with 5 ng/g of standard solution of OTA using MSR with segment.

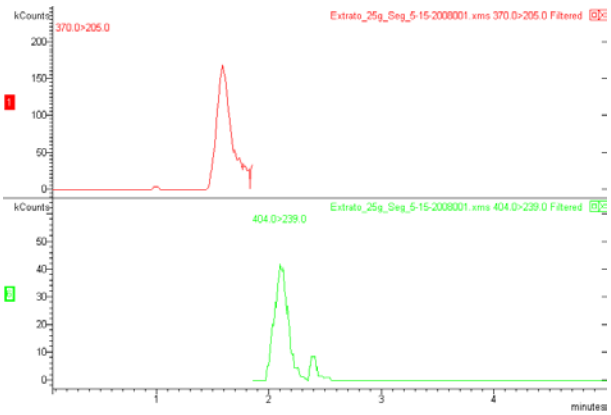

Fig. 6. MSR with segment chromatogram of a roasted coffee extract.

4. DISCUSSION

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 [23].

As shown in Figs. 1 and 2, 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 [18].

SIM method was tested for the analysis of OTA and OTB in solution, 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 calibration curve (2.0 ng/g to 9.0 ng/g) were not observed.

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. Fig. 5 shows that MRM is better with segment, it increases sensibility by monitoring only three ions *per* time.

Fig. 6 shows a chromatogram of a coffee sample contaminated with 5 ng/g of ochratoxin A and extracted as described above. As can be seen, at the maximum limit permitted for roasted coffee, good chromatograms can be obtained and quantification is possible. Validation parameters will be under investigation.

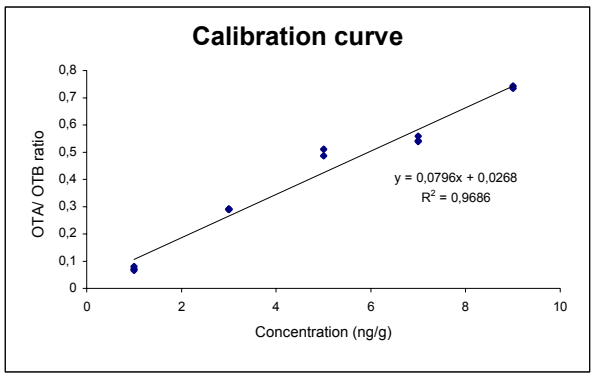

Fig. 7. Calibration curve of standard solution.

After optimizations of method, calibration curve was prepared in the range of concentration of 2.0 ng/g to

9.0 ng/g to determine the linearity of the method, resulting in a linear correlation coefficient (r) equal to r=0.968 6, above the reference value (0.90) established by the document of Inmetro DOQ-CGCRE 008, indicating the linearity of the method, Fig. 7 [24].

5. CONCLUSION

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 obtained peaks resolved for quantification in range of curve calibration. 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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