**Development and validation of a method for detection and quantification of ochratoxin A in green coffee using liquid chromatography coupled to mass spectrometry**

*Desenvolvimento e validação de metodologia na detecção e na quantificação de Ocratoxina A no café verde utilizando cromatografia líquida acoplada à espectrometria de massas*

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**Abstract**

A method using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) with matrix-matched calibration curve was developed and validated for determining ochratoxin A (OTA) in green coffee. Linearity was found between 3.0 and 23.0 ng.g⁻¹. Mean recoveries ranged between 90.45% and 108.81%; the relative standard deviation under repeatability and intermediate precision conditions ranged from 5.39% to 9.94% and from 2.20% to 14.34%, respectively. The limits of detection and quantification were 1.2 ng.g⁻¹ and 3.0 ng.g⁻¹, respectively. The method developed was suitable and contributed to the field of mycotoxin analysis, and it will be used for future production of the Certified Reference Material (CRM) for OTA in coffee.

**Keywords:** coffee; mycotoxin; validation; HPLC; MS/MS.

**Resumo**

Um método utilizando Cromatografia Líquida de Alta Eficiência-Espectrometria de Massas Sequencial (CLAE-EM/EM) com curva de calibração em matriz foi desenvolvido e validado para a determinação de ocratoxina A (OTA) em café verde. A linearidade foi demonstrada entre 3,0 e 23,0 ng.g⁻¹. As recuperações médias variaram entre 90,45% e 108,81%; o desvio padrão relativo sob condições de repetitividade e precisão intermediária foram de 5,39% e 9,94% e de 2,20% e 14,34%, respectivamente. Os limites de detecção e quantificação foram 1,2 ng.g⁻¹ e 3,0 ng.g⁻¹, respectivamente. O método desenvolvido foi adequado, contribuiu para o campo de análises em micotoxinas e será usado para a futura produção de Material de Referência Certificado (MRC) para OTA em café.

**Palavras-chave:** café; micotoxinas; validação; CLAE; EM/EM.

1 Introduction

Coffee is complex food matrix and it has an important role in the economy. Brazil is the third largest consumer of the coffee, according to data from the Brazilian Association of Coffee Industry (ASSOCIAÇÃO..., 2010).

Ochratoxin A (OTA) is classified by the International Agency for Research on Cancer (IARC) as a potent nephrotoxic and nephrocarcinogenic mycotoxin. It is produced by several Aspergillus and Penicillium specie and A. westerdijkiae, which is the most common species found in Brazilian coffee (84.0%) (NOONIM et al., 2008).

This mycotoxin has been found in food commodities such as cereals, oleaginous seeds, wine, meat, cocoa, spices, dried fruits, grapes, beer, green, roasted and instant coffee (ALMEIDA et al., 2007; BECKER et al., 1998; BRESH et al., 2000; BUCHELLI; TANIWAKI, 2002; BULLERMAN, 2003; FUJII et al., 2002; FURLANI; SOARES, 1999; GOLLUCKE et al., 2004; MASOUD; KALTOFT, 2006; MANTLE, 2002; PATEL et al., 1997; PRADO et al., 2000; POHLAND et al., 1992; SFORZA et al., 2006; SFORZA et al., 2006; STUDER-ROHR et al., 1995; TANIWAKI et al., 2003).

Once OTA has been formed, it survives most food-processing stages such as cooking, fermenting and roasting as in the case of coffee beans. It has been reported that 6.0% of the total human OTA intake corresponds to coffee (ASSOCIAÇÃO..., 2010; LOBEAU et al., 2005; NOBA et al., 2009).

No European Union limits have yet been set for green coffee. Therefore, some EU member states such as Czech Republic, Finland, Greece, Hungary, Italy, Portugal, Spain, and Switzerland set limits for OTA in green coffee ranging from 5 to 20 ng.g⁻¹. In Brazil, the limit for OTA in green coffee is under discussion. The European Coffee Federation suggests that OTA contamination...
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and then it was vacuum-dried for 30 seconds. An aliquot of 4.0 mL of methanol HPLC was added and then there was a 3 minutes wait 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).

2.4 Calibration curve

After method’s optimization, two calibration curves were constructed: one with a matrix (fortified samples) and the other without a matrix. Both of them were prepared from gravimetric dilution at five levels (3.0; 8.0; 13.0; 18.0 and 23.0 ng.g⁻¹).

The standard calibration curve was prepared by adding an OTA standard solution (100.0 ng.g⁻¹) to the mobile phase to obtain the five concentration levels. On the other hand, the matrix-matched standard curve was prepared by adding the same standard solution to the green coffee samples (matrix-matched standard curve). Each concentration level was injected in triplicate.

2.5 Liquid Chromatography Tanden Mass Spectrometry (LC-MS/MS)

Chromatographic separations were performed on a Synergi Hydro C₈ column (75 mm × 2.0 mm i.d.; 4 µm, Phenomenex, Torrance, California, USA) with a Security guard cartridge (KJO-4282, AQ C₈, 4 × 2.0 mm) at 25 °C. The mobile phase was obtained by two solvents, solvent A was 0.05% trifluoracetic acid in water and solvent B was 0.05% trifluoracetic acid in methanol at flow rate of 0.3 mL.min⁻¹ using isocratic elution (20:80). The mobile phase was filtered with a LCR PTFE membrane 0.45 µm (Millipore, EUA) and degassed by ultrasonic bath Model USC 1400 (Unique, Brasil). The injection volume was 50 µL. Two mobile phase pumps (Prostar 210), an on-line degasser, an automatic sampler (Pro Star410) and a column oven (Prostar) were used.

The MS/MS was performed on a triple-stage quadrupole 1200 L (Varian, Walnut, CA, USA) equipped with an electrospray interface (ESI) operating in a positive mode. The mass spectrometer was optimized for OTA by direct infusion of standard solution (10.0 µg mL⁻¹). The optimized parameters were: needle voltage 5000 V, capillary voltage 50 V, drying gas temperature 340 °C and pressure 21 psi, nebulizing gas pressure 40 psi, shield voltage 600 V, detector voltage 1600 V and housing temperature 40 °C. High purity nitrogen and argon were used as nebulizer gas and collision gas, respectively.

3 Validation

The parameters selected for method validation were Linearity, Specificity, Accuracy, Limit of Detection and Quantification and Precision. The validation parameters were based on INMETRO’s document (DOC-CGCRE-008) and on the European Union Commission Decision (EC-657/2002) (INSTITUTO..., 2010; EUROPEAN..., 2002) all results were checked for the presence of outliers using the Grubbs test and any value considered outlier was excluded.

Specificity means the ability of a method to distinguish between the analytes measured and other substances, which can change according to the compound class or matrix. The presence of peaks that could interfere in the identification and quantification of OTA was verified by analyzing blank green coffee samples (ROGASTSKY; STEIN, 2005).

Any matrix effects may impair the quantification of target analytes in complex samples. The absolute matrix effect was calculated by comparing the slope of matrix-matched standard curve with the slope of the standard calibration curve.

The linearity of the method was obtained with the linear correlation coefficient (r) from the calibration curve. A reference value higher than 0.90 is recommended, according to the Inmetro’s document (INSTITUTO..., 2010).

For homoscedasticity, the results from the calibration curve were submitted to a Cochran’s test evaluation to verify whether random signal measurement errors are constant and independent of the predictor value (CUADROS-RODRIGUEZ et al., 1998).

The limit of quantification (LOQ) was determined from the lowest concentration of the work range (3.0 ng.g⁻¹). The chromatograms of samples that produced a signal-to-noise ratio equal to ten were considered as the LOQ. For confirmation purposes, three roasted coffee samples were spiked with OTA at the same concentration level and the signal-to-noise ratio was calculated.

The limit of detection (LOD) was calculated from the Limit of Quantification according to Equation 1 shown below.

\[
LOD = \frac{LOQ}{3.3} \tag{1}
\]

Accuracy was expressed by trueness and precision. Trueness means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. When no Certified Reference Material (CRM) is available, it is acceptable that trueness of measurements is assessed through recovery of additions of known amounts of the element to the unknown samples (EUROPEAN..., 2002; AMÉZQUETA et al., 2004).

The recovery of OTA in green coffee was obtained from spiked samples of green coffee at three levels of contamination (5.0, 13.0 and 20.0 ng.g⁻¹). The calculated concentration in the green coffee was compared with the theoretical concentration using Equation 2. The acceptance criterion is a recovery between 70 and 110% according to Codex Alimentarius Commission (CODEX..., 2006).

\[
\text{Recovery(%) } = \left[ \frac{C_1 - C_2}{C_3} \right] \times 100 \tag{2}
\]

C₁ = concentration determined on green spiked sample; C₂ = concentration determined on blank sample; C₃ = concentration added to the sample.
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Precise development and validation of a method for detection and quantification of OTA in green coffee using LC-MS/MS.

4 Results and discussions
Electrospray was used because it is considered a mild ionization method that generally produces quasi-molecular ions with low fragmentation. These characteristics agree reasonably well with the LC/Fluorescence detection obtained for complex matrices (LAU et al., 2000).

First, the Selective Ion Monitoring Method (SIM) was tested and good results were obtained. However, in the working range of the calibration curve the ions monitored were not well observed due to noise and poor peak resolution. Thus, the SIM was not used for further development and method validation for OTA analysis (data not shown).

Therefore, the standard solution breakdown curves were obtained by direct infusion of OTA and precursor-to-product ions in the positive electrospray mass spectra (ESI MS). They were observed and used for the Selective Reaction Monitoring (SRM) method using the protonated molecule [M+H]+ m/z 404, presented in Figure 1. After analysis of the breakdown curves, precursor-to-product transitions were obtained for the performance of the SRM method.

The European Union Commission Decision (EC-657/2002) states that confirmatory methods for residue analysis should provide information about the chemical structure of the analyte (EUROPEAN..., 2002). Liquid chromatography coupled with a mass spectrometry detector requires four identification points that can be accomplished by monitoring one precursor ion and two product ions. The method developed and validated in this study monitored two ions according to their collision energies: m/z 404 > 239 (–20.5 V) and m/z 404 > 358 (–10.5 V) for quantification and confirmation of OTA. The product ion showing the highest intensity was used for quantification, m/z 239, which corresponds to the loss of phenylalanine for OTA (BECKER et al., 1998).

4.1 Validation
The experimental results were checked for the presence of outliers using Grubbs test before the validation process, and none of the results were considered an outlier.

4.2 Linearity
The analytical procedure was considered to be linear for the working range of the calibration curve, and the correlation coefficient (r) values were higher than those recommended with reference value of 0.90 according to the Inmetro¿s document (INSTITUTO., 2010). The method presented a correlation coefficient (r) of 0.99845 and 0.98188 for standard solution and

![Figure 1. Breakdown curve of OTA standard solution of 10.0 µg mL⁻¹.](image-url)
matrix-matched calibration of OTA, respectively. Figure 2 shows the results of the regression parameters of the OTA standard solution and matrix-matched calibration curves.

The matrix-matched calibration curve showed a random pattern of residues in the working range (Figure 3), confirming the linearity of the method.

The value of Cochran’s test results was $C_{\text{calc}} (0.54280)$ lower than the value of $C_{\text{tab}} (0.68380)$ for the five levels of matrix-matched calibration curve with three replicates, proving homoscedasticity.

4.3 Specificity

Figure 4 shows a chromatogram of spiked green coffee at 3.0 ng.g$^{-1}$ and an example of blank green coffee. No interference from the matrix around the retention time of OTA was observed; thus the method is considered specific for this analysis.

4.4 Matrix effect

Comparing the slope values of both calibration curves, it can be observed that the values are different indicating significant differences between the calibration curves of matrix and solvent. This means that there is a matrix effect on the response to the linearity. In this case, all subsequent validation parameters were accomplished using a matrix-matched calibration curve.

In order to avoid a matrix effect on the response to the linearity, an addition of isotopically labelled standard can be used. The use of this substance is useful for the correction of signal deviation because it has the same chemical properties and the same retention times as those of non-labeled substances.

Figure 2. Standard solution and matrix-matched calibration curves of OTA.

Figure 3. Distribution of residues in the working range of 3.0-23.0 ng.g$^{-1}$.

Figure 4. Green coffee chromatograms: (a) Blank and (b) Spiked in 3.0 ng.g$^{-1}$ of OTA.
However, isotopically labeled internal standards may not be available for some analytes due to difficulties associated with synthesis and/or cost, and they were not used in this study.

4.5 Limit of Quantification (LOQ) and Limit of Detection (LOD)

For the green coffee sample, the limit of quantification (LOQ) was 3.0 ng.g\(^{-1}\), which is the first concentration of the calibration curve. The Limit of detection (LOD) of OTA was 1.2 ng.g\(^{-1}\). The LOQ value was higher than that determined using other methods in the literature (VENTURA et al., 2003; DIAZ et al., 2004). The LOD value was lower than that reported by other authors using HPLC-FLD methods (FURLANI; SOARES, 1999; LEONI et al., 2001). Furthermore, LC-ESI-MS/MS is especially helpful in confirming doubtful “OTA positive” results and the coelution problem of interfering compounds can be overcome by structural information provided by these techniques rather than those obtained by LC with Fluorescence detection.

4.6 Accuracy

The trueness values obtained in the present study for different concentrations of OTA ranged between 90.45% and 108.81% and they are in accordance to Codex Alimentarius Commission for a contamination level less than 10.0 ng.g\(^{-1}\) (CODEX..., 2006). Table 1 shows recoveries values, which were considered satisfactory for green coffee samples in all fortification levels and are similar to those obtained with fluorescence detection methods currently in use, such as Enterwile et al. (2001) (65-97%) and Pittet et al. (1996) (89-100%) (PITTET et al., 1996; ENTERWILE et al., 2001). On the other hand, this method presents recovery values higher than those reported by Ahmed et al. (2007), 73-86%; Ventura et al. (2003), 82%; Sibanda et al. (2002), 72-84% and Gilbert and Anklam (2002), 85%.

All RSD\(_r\) values are within acceptable levels for repeatability, ranging between 5.39% and 9.94% and are lower than 20% according to the Codex Alimentarius Commission (CODEX..., 2006). The RSD\(_f\) values for intermediate precision, ranging between 2.20 and 14.34 are lower than 30%; both RSD\(_r\) and RSD\(_f\) were better than the values obtained by Gilbert and Anklam (2002) and by Enterwile et al. (2001). According to F-test and t-test, intermediate precision was equivalent since the F\(_{cal}\) values (1.33175) were lower than the F\(_{table}\) values (19.000)

<table>
<thead>
<tr>
<th>Spiked concentration (ng.g(^{-1}))</th>
<th>Mean recoveries (%)</th>
<th>RSD(_r) (%)</th>
<th>RSD(_f) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>90.45</td>
<td>9.94</td>
<td>14.34</td>
</tr>
<tr>
<td>13.0</td>
<td>108.81</td>
<td>6.27</td>
<td>2.75</td>
</tr>
<tr>
<td>20.0</td>
<td>99.45</td>
<td>5.39</td>
<td>2.20</td>
</tr>
</tbody>
</table>

The developed and validated method for identification and quantification of OTA in green coffee is accurate and sensitive. Linearity was demonstrated for contaminations levels between 3.0 and 23.0 ng.g\(^{-1}\). The validation parameters measured are within acceptable limits and were considered satisfactory. The detection limit of the method (1.2 ng.g\(^{-1}\)) is sufficiently low for surveillance purposes. The method can be used to determine OTA content in soluble and roasted coffee samples.

The method presented shows an extremely useful and rapid clean-up for OTA in green coffee. This green coffee analysis by LC-ESI-MS/MS using the matrix-matched calibration method contributes to the field of mycotoxin analysis.

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References


