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Comparison of High Performance Liquid Chromatography with Fluorescence Detector and with Tandem Mass Spectrometry Methods for Detection and Quantification of Ochratoxin A in Green and Roasted Coffee Beans

Raquel Duarte da Costa Cunha Bandeira[1*], Thais Matsue Uekane[1], Carolina Passos da Cunha[1], Janaina Marques Rodrigues[1], Marcus Henrique Campino de la Cruz[2], Ronoel Luiz de Oliveira Godoy[3] and Andreia de Lima Fioravante[1]
[1]*Divisão de Metrologia Química; Diretoria de Metrologia Científíca e Industrial; Instituto Nacional de Metrologia, Qualidade e Tecnologia; Duque de Caxias - RJ - Brasil.* [2]*Fundação Osvaldo Cruz; Instituto Nacional de Controle de Qualidade e Saúde;* Manguinhos - *RJ - Brasil.* [3]*Embrapa Agroindustria de Alimentos;* Guaratiba - *RJ - Brasil*

ABSTRACT

Two analytical methods for the determination and confirmation of ochratoxin A (OTA) in green and roasted coffee samples were compared. Sample extraction and clean-up were based on liquid–liquid phase extraction and immunoaffinity column. The detection of OTA was carried out with the high performance liquid chromatography (HPLC) combined either with fluorescence detection (FLD), or positive electrospray ionization (ESI+) coupled with tandem mass spectrometry (MS–MS). The results obtained with the LC-ESI-MS/MS were specific and more sensitive, with the advantages in terms of unambiguous analyte identification, when compared with the HPLC-FLD.

Key words: HPLC-FLD, LC-MS/MS, Green Coffee and Roasted Coffee

INTRODUCTION

Coffee is a complex food matrix and it has an important role in the world's economy. Brazil is the third largest consumer of coffee, according to data from the Brazilian Association of Coffee Industry (ABIC). 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* species, such as by *A. westerdijkiae,* which is most commonly found in Brazilian coffee (84%) (Zollner et al. 2006; 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, as well as in the green, roasted and instant coffee (Pohland et al. 1992; Studer-Rohr et al. 1995; Patel et al. 1997; Becker et al. 1998; Furlani et al. 1999; Bresh et al. 2000; Prado et al. 2000; Buchelli et al. 2002; Mantle 2002; Fujii et al. 2002; Bullerman 2003; Taniwaki et al. 2003; Gollucke et al. 2004; Masoud et al. 2006; Sforza et al. 2006; Almeida et al. 2007; Fujii et al. 2007). Once OTA is formed, it is not destroyed in most food-processing steps, such as fermentation, roasting and cooking, as in the case of coffee beans. Roasted coffee produce the most variable results in terms of OTA stability since losses are in the range of 0 – 100%. It has

[*]Author for correspondence: rdbandeira@inmetro.gov.br

been reported that 6.0% of total human OTA intake corresponds to coffee (Lobeau et al. 2005; Zollner et al. 2006; FAO/WHO 2008; Noba et al. 2009; ABIC 2010).

As the contamination of food and feed with mycotoxins may present a serious hazard to humans and animals, many countries have established limits for these mycotoxins. A regulatory limit of 10.0 ng g^{-1} for the OTA in roasted coffee has been established by Brazil, and monitoring this level is an important step for the health of the consumers (Commission Regulation 2005; RDC 2011). In Brazil, the limit of OTA in green coffee is under discussion. There is no limit set in the European Union for green coffee. However, several countries, such as Czech Republic, Finland, Greece, Hungary, Italy, Portugal, Spain and Switzerland have set the maximum limit of OTA with values ranging between 5.0 to 20.0 ng g^{-1} for green coffee (Commission Regulation 2005).

Several techniques have been described for determining the OTA in 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 spectroscopy* (LC-MS) (Prado et al. 2000; Gilbert et al. 2002; Pardo et al. 2004; Masoud et al. 2006; Sugita-Konishi et al. 2006; Almeida et al. 2007; Ahmed et al. 2007). Method proposed by Ministério da Agricultura, Pecuária e Abastecimento (BRASIL, 2000) is based on HPLC-FLD. Due to its robustness and cost-effective handling *thin-layer chromatography* (TLC) with FLD detection is still routinely used in countries outside Europe and North America. The major disadvantage of this technique is the comparable low sensitivity towards OTA (ppb range) and frequent interferences from the sample matrix. Methods based on the GC have not gain attention since they are time-consuming and require error-prone derivatization protocol (methylation), which is needed to achieve sufficient volatility of the analytes. Nevertheless, CG/MS could be used to confirm the unambiguously positive findings.

ELISA is shown to be extremely suitable for a rapid screening of large sample numbers. It offers sensitivity of OTA comparable to FLD detection. Due to possible cross-reactivity with matrix components, confirmation by other technique is, therefore, highly desirable to avoid false positive results, or inaccurate and overestimated quantitative data. In this context, LC/MS is also an excellent tool to elucidate the structure and predominantly applied to confirm the positive results obtained by the ELISA, or HPLC-FLD. Only few new LC-MS studies have been 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 the doubtful "Ochratoxin A positive" results and its sensitivity is enhanced by operating the MS in the selected reaction monitoring mode (SRM) (Diaz et al. 2004). In general, and whatever the detection technique applied, there is a need for a careful sample clean-up. The extract is usually purified by solid-phase extraction, by immunoaffinity absorbent materials, or by a combination of both. An overview of the process is such that the toxin is usually extracted with water, organic solvents, salt aqueous solution and acids, or the mixtures. Especially for coffee, it is important to have an adequate clean-up to remove the substances such as lipids and pigments that could interfere in the analytical techniques (Ventura et al. 2003; Vargas et al. 2005; Fujii et al. 2007).

Validation procedures of the analytical method are necessary for legislation implementation to show that the method produces reliable results, provide accurate and reprodutibity results for monitoring and risk-assessment studies (Monaci et al. 2004). The absence of available Certified Reference Material (CRM) of roasted coffee is also an issue correlated to the validation of methods since it ensures that reliable data are being generated (Gilbert et al. 2002; Monaci et al. 2004). Due to the possible risk of OTA being a contaminant in coffee (human health problem), allied to the important role of this product in the world´s economy, there is a strong need for a method that could be used by coffee producing and exporting countries to check the compliance of consignments with criteria set by the importing countries. The purpose of this study was to compare the *liquid chromatography with fluorescence detector* method (HLPC-FLD) and the *liquid chromatography coupled with tandem mass spectrometry* (LC-MS/MS) for OTA analysis in green and roasted coffee.

MATERIALS AND METHODS

Material

The solvents and reagents used were 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 dichromate (Merck, Germany, 99.5%), sulfuric acid (Merck, Germany, 97.0%), methanol HPLC grade (Tedia, USA, 99.9%), trifluoroacetic acid HPLC grade (Tedia, USA, 99.8%), acetone pesticide grade (Tedia, USA, 99.8%), 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 HPLC grade (Quimes, Brazil, 95.0%), sodium hypochlorite (Invema, Brazil, 12.0%), alcaline extran (Merck, Germany). Saline phosphate buffer water solution (PBS) at pH 7 was used (0.020% potassium dihydrogen phosphate, 0.110% anhydrous disodium hydrogen phosphate, 0.800% sodium chloride and 0.020% potassium chloride). Crystalline Ochratoxin A standard was obtained from the Sigma-Aldrich (St Louis, 98.0%, USA). The immunoaffinnity columns (IA) were Ochratest™ WB (Vicam Inc.,Watertown, USA).

Standard Solution

Stock standard solution (40 μg mL^{-1}) was prepared in a mixture of toluene and glacial acetic acid (99:1). The nominal concentration was calculated by the UV spectrophotometry at INCQS/FIOCRUZ assuming a molar absorption coefficient of 5440 M^{-1} cm^{-1} (333 nm), according to the recommendation of AOAC (2005). This solution was stored in a freezer at - 18ºC. A working solution of 100.0 ng g^{-1} was prepared by weighting an appropriate mass of the stock standard solution in mobile phase. This working standard solution was used to prepare the calibration curve at concentration levels of 3.0; 8.0; 13.0; 18.0 and 23.0 ng g^{-1} and 3.0; 4.0; 5.0; 6.0 and 7.0 ng g^{-1} for the green and roasted coffee, respectively. Quantification was carried out by comparison to a matrix-matched calibration curve.

Samples

Brazilian green coffee samples (*Coffea arabica* cv. Mundo Novo and *Coffea arabica* cv. Catuaí obtained from the East of Minas Gerais, in São Domingos das Dores) were harvested using the technique of striping. The samples were roasted and ground using an electric roaster of 0.3 kg capacity per batch (Cael Ltda) and Leogap grinder (Model M-50), respectively. The roasting temperature was 280°C during 35 min to obtain a medium dark roasting degree according to color disks of SCAA-Agtron of the Specialty Coffee Association of America. The resulting powder was homogenized and stored in a freezer at -18°C until analysis.

Extraction and Clean-up of OTA

The sample preparation procedure was based on Pittet et al. (1996). An aliquot of 25.0 g of coffee samples was weighed into a 250 mL amber glass flask and fortified with a mass of working standard solution and kept at room temperature overnight. Then, it was transferred quantitatively with the 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 homogenized sample was filtered through filter under vacuum using a qualitative paper JP41 28 μm (J.Prolab, Germany), followed by filtrations using a fiberglass (Whatman, EUA) and a cellulose membrane 0.45 μm (Millipore, EUA).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. The *Manifold, Vaccum Elut 20* (Varian, Walnut, USA) was connected to the IA column. A 4.6 g aliquot of the filtrate was weighed into a 100 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 ultrapure 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).

Chromatographic Conditions

HPLC-FLD

An aliquot of 20 μL of coffee samples extract was injected into a Nucleosil RP 18 C_{18} (reversed

phase) liquid chromatography column (250 mm × 4.6 mm i.d., particle size 5 µm; Macherey-Nagel, Germany) on a Waters Alliance HPLC system 2695. The column was eluted with 1.0% acetic acid: water (solvent A); 1.0% acetic acid: acetonitrile (solvent B) and 1.0% acetic acid: methanol (solvent C) at 25°C in a gradient eluition with the following proportions (v/v) [t (min), % solvent]: first, solvent A: solvent C (5.0 min, 30:70). After that, solvent A: solvent C (1.0 min, 25:75). Then, solvent A: solvent B: solvent C (9.0 min, 25:30:45). At the end, solvent A: solvent C (9.0 min, 30:70). At a flow rate of 0.9 mL/min^{-1}. The total run time, including the conditioning of the column to the initial conditions was 20 min. Measurements were performed by fluorescence detection at 333 nm (excitation) and 476 nm (emission) (BRASIL 2000).

LC-ESI-MS/MS

An aliquot of 50 µL of coffee samples extract was injected into a Synergi Hydro C_{18} (reversed phased) *liquid chromatography column* (75 mm x 2.0 mm i.d.; particle size 4 µm, Phenomenex, USA) with a *security guard column* (KJO-4282, AQ C_{18}, 4.0 mm x 2.0 mm) on a Varian 1200L LC-ESI-MS/MS *triple quadrupole*. The column was eluted with 0.05% trifluoracetic acid: water (solvent A) and 0.05% trifluoracetic acid: methanol (solvent B), 20:80 (v/v) at flow rate of 0.3 mL min $^{-1}$ at 25°C in an isocratic elution. The total run time, including the conditioning of the column to the initial conditions was 10 min.

The *mass spectrometer detector* was equipped with an electrospray (ESI) ionization operating in the positive mode. The ESI interface was calibrated using a *polypropyleneglycol solution* (PPG) provided by manufacturer. The *mass spectrometer* was operated in *Selective Reaction Monitoring* (SRM) mode with *positive electrospray ionization* (ESI+) to confirm the identity of OTA. The LC-MS/MS parameters were obtained through *direct infusion* of standard solution of OTA: needle voltage 5000 V, shield voltage 600 V, capillary voltage 50 V, housing temperature at 40ºC and detector voltage 1600 V. High purity nitrogen was used as nebulizer gas (40 psi) and as drying gas (21 psi; 340°C). Standard solution breakdown curves were constructed and through these curves, precursor-to-product ions were selected according to the most abundant intensity, collision energy and high mass. The precursor ion (pseudo-molecular) [M+H]+ *m/z* 404 was selected in the first quadrupole (Q1). This ion was fragmented using ultrapure argon at 2.0 mTorr as collision gas in the second quadrupole (Q2). Products ions [M+H]+ *m/z* 239 and *m/z* 358 were obtained in the third quadrupole (Q3) and were used for SRM mode.

Validation

The selected parameters for validation were Linearity, Specificity (Matrix Effect), Accuracy (Recovery, Repeatability and Intermediate Precision), Limit of Detection (LOD) and Quantification (LOQ). The validation parameters were based on the National Institute of Metrology, Quality and Technology (INMETRO, the Brazilian Nacional Metrology Institute - NMI) document and the European Union Commission Decision (Comission Decision, 2002; Brasil, 2011). Validation procedure was carried out at five different concentrations of 3.0; 8.0; 13.0; 18.0 and 23.0 ng g^{-1} for green coffee, and 3.0, 4.0, 5.0, 6.0 and 7.0 ng g^{-1} for roasted coffee, respectively. Each level was injected in three replicates. For each matrix, two calibration curves were developed: one with the matrix (fortified samples) and another without the matrix. These experiments included the spiking of blank matrices with concentration levels of calibration curve to evaluate linearity and specificity the spiking of blank matrices on three concentrations levels in order to evaluate accuracy (Recovery, Repeatability and Intermediate Precision) and the spiking of blank matrices with the lowest concentration of the working range to assess limit of quantification.

Statistical Analysis

All the calculations were performed using an Excel sheet®. The results were checked for the presence of outliers using Grubbs test and the value considered an outlier was excluded. The arithmetic mean, relative standard deviation (RSD %) and the variance were calculated. The F and *t*-test for double-sided paired data was used to determine the significance statistic between the treatments.

RESULTS AND DISCUSSION

The results from the quantification method developed and validated for the green and roasted coffee using LC-ESI-MS/MS are summarized in

Table 1. None of the experimental results were considered outlier through Grubbs test, thus were not excluded.

Table 1 - Validation data for LC-ESI-MS/MS method.

	Green coffee[a]	Roasted coffee[b]
Linearity		
Determination coefficient (r)	0.98	0.92
Slope	69,817.9	23,9065.9
Cochran's test (C_{calc})	0.543	0.58
Mean recovery	90.45-108.81	89.02-108.85
RSD_r	5.39-9.94	2.43-13.73
RSD_R	2.75-14.34	12.57-17.84

[a]working range of 3.0 to 23.0 ng g^{-1}. [b]working range of 3.0 to 7.0 ng g^{-1}. RSD_r= relative standard deviation for repeatability. RSD_R= relative standard deviation for intermediate precision.

LC-MS/MS

Linearity

Blank green and roasted coffee samples were spiked with OTA standard solution at five different concentration levels and the determination coefficient (r) was calculated for each concentration. The Selective Reaction Monitoring (SRM) experiment used in this study monitored the protonated molecule $[M+H]^+$ *m/z* 404 and two products ions, 404>239 (quantification transition) and 404>358 (confirmation transition), increasing sensitivity of the LC-MS/MS validated method. Linearity was assessed by determining the coefficient value (r), which was in agreement with the reference value (0.90) established by the document guide from National Institute of Metrology, Quality and Technology (INMETRO, The Brazilian NMI) (Table 1). The parameters of the calibration curves showed a good linear adjust between the peak area (y) and OTA concentration (x). Matrix-matched calibration curves showed a random pattern of residues in the working range, confirming the linearity of the methods for the green and roasted coffee. Also, results of *Cochran*'s test (C_{calc}) were lower than the value tabled (C_{tab}=0.683) for the five levels of concentration of matrix-matched calibration curve either for the spiked green and roasted coffee, proving the homocedasticity for both the methods (Cuadros-Rodiguez et al. 1998; Rogastsky et al. 2005).

Selectivity

The selectivity/ specificity of the method were tested by the analysis of blank samples of green and roasted coffee. For the confirmation of this result, OTA (3.0 ng g^{-1}) was added to the blank coffee samples and it was reanalyzed under the same chromatographic conditions. The result of selectivity/specificity of the method was confirmed, since no peak appeared on the blank samples at the same retention time of the OTA standard added to the coffee samples, without interfering the peaks (Fig. 1).

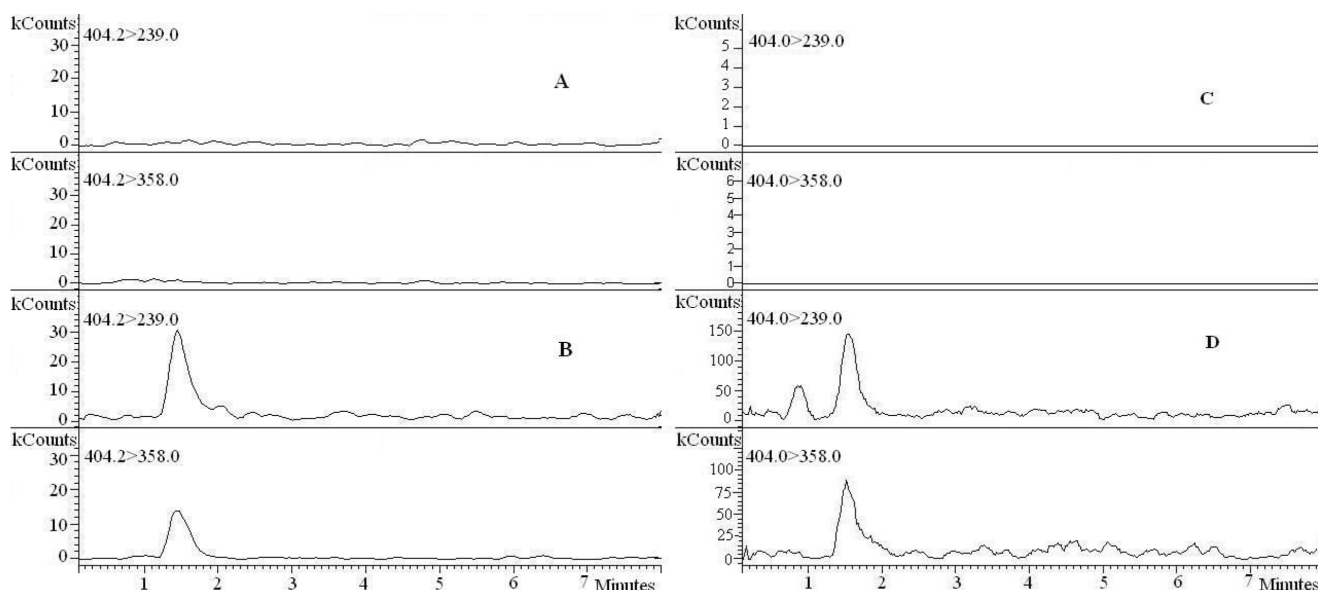

Figure 1 - SRM mass chromatograms from coffee samples Blank (A); Spiked (B) for green coffee and Blank (C); Spiked (D) for roasted coffee both of them with 3.0 ng g^{-1} of OTA.

Matrix Effect

Matrix effect was assessed by comparing the slopes of standard solution calibration curves and matrix-matched calibration curves for the green and roasted coffee samples. Slope values from the standard solution calibration curve were 1.6×10^5 and 1.1×10^7 for the green and roasted coffee, respectively. Comparing the slope values from the standard and matrix-matched calibration curves, it was observed that the values were different, indicating significant differences between the calibration curves of matrix and solvent. For the confirmation of these results, the F and *t*-test were performed to compare the variances and means, respectively (Table 2).

The results from both tests confirmed that there was a matrix effect on the response to the linearity. In this case, all subsequent validation parameters were accomplished using a matrix-matched calibration curve.

Table 2 - Results for F and *t*-test from standard and matrix-matched calibration curves.

	Green coffee	Roasted coffee	Tabled value
F test	7.786	25.234	1.984
t-test	5.021	10.200	2.042

Accuracy (recovery, repeatability and intermediate precision)

The recovery means the amount of substance obtained in the quantification step (after extraction) in relation to the amount added to the material before the extraction and is expressed as a percentage. The recovery of OTA was obtained from the spiked samples at three levels of contamination (5.0, 13.0 and 20.0 ng g^{-1} for the green coffee and 3.5, 5.0 and 6.5 ng g^{-1} for the roasted coffee. Recovery values for both coffee samples were in accordance with the established values by the *Codex Alimentarius Commission* (70 to 110% for the concentration level between 1 to 10 ng g-1,Table 1) (*Codex Alimentarius Commission* 2007). The repeatability and intermediate precision were assessed from the relative standard deviation (R.S.D.) calculated from the spiking of blank matrices on three concentrations levels as for the recovery. Spiked coffee samples analyzed under the same conditions (same day by the same analyst) were considered R.S.D_r for repeatability and spiked coffee samples analyzed under independent conditions (different analyst) were considered R.S.$D._R$ for intermediate precision. R.S.D_r and R.S.$D._R$ values for both coffee samples were in accordance with the established values by the *Codex Alimentarius Commission* (< 20% and < 30%, respectively for the concentration level between 1.0 to 10.0 ng g-1 Table 1) (*Codex Alimentarius Commission* 2007).

For the confirmation of R.S.$D._R$ results, F and *t*-tests were calculated by comparing the variances and means, respectively. The results presented in Table 3 showed that for both the tests, the F and t calculated were lower than tabled value confirming the equivalence of the two analysts for the analysis of OTA in the green and roasted coffee samples.

Table 3 - Results for F and *t*-test for intermediate precision.

	Green coffee[a]	Roasted coffee[b]	Tabled value
F test	1.332	6.986	19.000
t-test	0.021	0.914	2.776

[a]working range of 3.0 to 23.0 ng g^{-1}.
[b]working range of 3.0 to 7.0 ng g^{-1}

Limit of quantification (LOQ) and Limit of Detection (LOD)

The LOQ was verified by the signal-to-noise (S/N) ratio approach, which was obtained by measuring the chromatographic response of the OTA and the chromatographic noise. This value should be equal to 10. The LOQ was determined from the lowest concentration of the working range. Green and roasted coffee samples were spiked with 3.0 ng g^{-1} OTA and the signal-to-noise ratio was calculated. The LOQ was 1.2 ng g^{-1} for the green coffee samples and 3.0 ng g^{-1} for the roasted coffee samples, the first point of matrix-matched calibration curve. The LOD was 0.36 and 1.0 ng g^{-1} for the green and roasted coffee samples, respectively. These values were higher than the ones determined by other reported techniques. However, this method was especially helpful in confirming the doubtful “Ochratoxin A positive” results. Coelution problem of interfering compounds and retention time shifts could lead to erroneous positive, or negative results, which could be overcome by the structural elucidation provided by coupling of the LC and mass spectrometry (MS) (Diaz et al. 2004).

Comparison of two techniques HPLC-FLD and LC-MS/MS

A comparison performance was performed between the LC-MS/MS method developed in the present work and the HLPC-FLD used in routine analysis. The same coffee extracts were used in both the techniques. The results for the HPLC-FLD method for the green and roasted coffee are summarized in Table 4.

Table 4 - Performance data for HPLC-FLD method.

	Green coffee[a]	Roasted coffee[b]
Linearity:		
Determination coefficient (r)	0.99	0.98
Slope	12,592	6,355.2
Mean recovery	77.82-115.67	79.54-99.51
RSD_r	0.30-3.19	0.49-12.82

[a]working range of 3.0 to 23.0 ng g^{-1}. [b]working range of 3.0 to 7.0 ng g^{-1}. RSD_r= relative standard deviation for repeatability.

Linearity for the HPLC-FLD method was in agreement with the reference value (0.90) and the parameters of the calibration curves showed a good linear adjust between the peak area (y) and OTA concentration (x). Sensitivity is a parameter that shows the variation of the response versus analyte concentration. This parameter can be expressed by the slope of the regression calibration and it is determined simultaneously with the linearity tests (Table 4). Both the techniques showed good sensitivity for both coffee samples. HPLC-FLD slope values were lower than the LC-MS/MS, thus the latter technique was more sensitive, since small variations on concentration resulted in higher responses.

Recovery range values for the green coffee samples by HPLC-FLD were above the established values by the *Codex Alimentarius Commission* (70 to 110%) for the concentration level between 1 to 10 ng g-1. However, the method proposed for this matrix was applied for a working range above the recommended range and an interfering compound could be coeluting with OTA peak, thus this result was considered adequate for this analysis. The roasted coffee samples recovery values by the HPLC-FLD were within the established by the *Codex Alimentarius* Commission (*Codex Alimentarius Commission* 2007). R.S.D.$_r$ values for both coffee samples were in accordance to the established by *Codex Alimentarius* Commmission < 20% (*Codex Alimentarius Commission* 2007). For both coffee samples, LC-MS/MS recoveries values were slightly higher than HPLC-FLD method and similar R.S.D.$_r$ values were presented.

OTA retention time was 7.5 and 1.4 min for both the matrices using HPLC-FLD and LC-MS/MS, respectively. No interference from the matrix on the retention time of OTA was observed for both the methods (HPLC-FLD and LC-MS/MS), thus the methods were specific for both the matrices (Figs. 1 and 2).

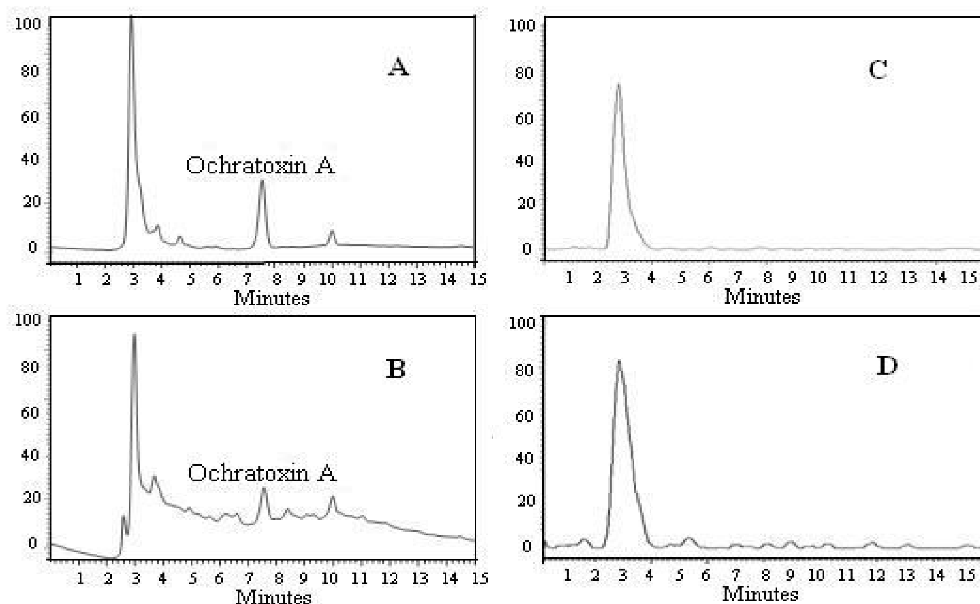

Figure 2 - Chromatogram of green (A) and roasted (B) coffee using HPLC-FLD method and green (C) and roasted (D) coffee using LC-MS/MS method.

HPLC-FLD chromatograms showed several peaks for the green and roasted coffee-samples demonstrating the non-selectivity of this technique. The peaks could interfere in quantification and produce false positive results and the fluorescence detector might not provide a sufficient degree of certainty and a confirmation of the positive results would necessary. The problem of *coelution* with interfering compounds could be overcome by the structural information provided by the tandem mass spectrometry. LC-MS/MS provided the identification of OTA with high sensitivity when working with SRM, thus, best suited to comply with the strict permitted residue levels regulated for several toxic compounds in different food matrices (Milicevic et al. 2010). The HPLC-FLD and LC-MS/MS methods for both the matrices were compared using the F and *t*-test (Table 5).

Table 5 - Results for F and *t*-test for comparing HPLC-FLD and LC-MS/MS methods.

	Green coffee[a]	Roasted coffee[b]	Tabled value
F test	1.016	1.317	19.000
t-test	0.077	1.112	2.776

[a]working range of 3.0 to 23.0 ng g^{-1}.
[b]working range of 3.0 to 7.0 ng g^{-1}.

Results presented in Table 5 showed that $F_{calculated}$ values were lower than F_{tabled} for both the matrices, therefore variances were considered statistically similar. Then, a *t*-test was performed considering the equivalence of means; values of $t_{calculated}$ were lower than t_{tabled} for both the matrices. It was concluded that both the methods (HPLC-FLD and LC-MS/MS) were equivalent for the green and roasted coffee.

CONCLUSION

Validation parameters measured for both the techniques were within acceptable limits and were considered statistically satisfactory. Both the methods (HPLC-FLD and LC-MS/MS) were equivalent for the green and roasted coffee samples. The use of two independent measurement methods was useful in the quantitative determination and confirmation of the OTA in coffee samples at low levels. In addition, LC-MS/MS showed the advantage of unambiguous analyte identification without further time-consuming and error-prone confirmation steps and it demonstrated excellent sensitivity and specificity. This method could also be used for other matrices such as spice, cereal, cocoa, beer and wine.

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