INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

Research Article

POTENTIAL OF FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR) TO DETECTION AND QUANTIFICATION OF OCHRATOXIN A: A COMPARISON BETWEEN REFLECTANCE AND TRANSMITTANCE TECHNIQUES

A. Bozza[1]*, SM. Tralamazza[1], JI. Rodriguez[2], MBS. Scholz[3], DT. Reynaud[4], PR. Dalzoto[1] and IC. Pimentel[1]

[1]Microbiology and Molecular Biology Laboratory (LabMicro) Basic Pathology department, Federal University of Paraná (UFPR), Francisco H. dos Santos s/n, Jardim das Américas, 19031, Curitiba, Paraná, Brazil.

[2]Zoology department, Federal University of Paraná (UFPR), Francisco H. dos Santos s/n, Jardim das Américas, 19031, Curitiba, Paraná, Brazil.

[3]Paraná Agronomic Institute (IAPAR), Rodovia Celso Garcia Cid, km 375, Três Marcos, 481, Londrina, Paraná, Brazil.

[4]Centro Universitário Campos Andrade (UNIANDRADE), João Scuissiato 01, Santa Quitéria, 743, Curitiba, Paraná, Brazil.

ABSTRACT

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, potentially carcinogenic and immunotoxic effects, naturally found in agricultural products including coffee beans. Fourier Transform Infrared Spectroscopy (FT-IR) is a practical procedure for the detection of organic compounds in matter, particularly useful because of its accuracy, rapid response, ease of operation and for being a non-destructive method. The aim of this work was to compare transmittance and reflectance infrared spectroscopy for detection and quantification of ochratoxin A from fungi *of Aspergillus* genera isolated from green coffee beans. The High Performance Liquid Chromatography (CLAE) analysis showed that all strains to the *Circumdati* section are producer of OTA, unlike the representatives *Nigri* section, which did not producer detectable levels of OTA. The FT-IR analysis showed that for detection and quantification of ochratoxin A, both models, incorporating transmittance and reflectance data, showed good results. Thus, the application of FT-IR to the detection and quantification of ochratoxin A in fungal isolates showed itself to be a viable and useful methodology.

Keywords: Mycotoxin, coffee, *Aspergillus*, HPLC, FT-IR

INTRODUCTION

Mycotoxins are a structurally diverse group of mostly small molecular weight compounds produced by the secondary metabolism of fungi. They are ubiquitous in a broad range of commodities and feeds and may cause a toxic response, known as mycotoxicosis, when ingested by humans and animals[1].

The presence of ochratoxin A (OTA) in food is considered a worldwide problem in public health. In countries with tropical climates, species of *Aspergillus* have been found to be the

main agent responsible for the production of ochratoxin A in coffee[2].

Ochratoxin A (OTA) occurs in various foodstuffs and beverages including a variety of cereals, beans, coffee, beer, wine, meat, cocoa, dried fruits, spices and nuts[3]. Furthermore, the presence of OTA in food products such as coffee, wine, beer, grape juice, meat and meat products is due to its relative chemical stability during industrial processing[4]. Other food items, such as milk, animal tissues, muscle, organs and eggs may also be contaminated[5]. Similarly, to other crops, coffee fruits and beans can be contaminated by toxigenic fungi, which besides altering the quality of the coffee may present a serious risk of OTA contamination, compromising the safety of the product[3].

Several toxic effects of OTA have been described, such as inhibition of protein synthesis, oxidative stress and DNA damage[6-1]. Furthermore, this mycotoxin is considered nephrotoxic, cytotoxic and immunosuppressive[7]. OTA has been classified by the International Agency for Research on Cancer as a possible carcinogen in humans (group 2B)[8].

Until now, detection and quantification of ochratoxin A in food and fungi able to produce it have been performed by HPLC (High performance liquid chromatography); however, new techniques have been shown to be more economical and faster. Among these techniques, Fourier Transform Infrared Spectroscopy (FT-IR) has become, according to Hernández-Hierro[9], an important alternative to chromatography for analyses of mycotoxins.

FT-IR is an accurate technique that demonstrates rapid response, ease of operation and is non-destructive[10-11]. In addition, this technique does not require reagents with a high degree of purity and several steps of extraction for acquisition of compounds, as in the case of HPLC[12]. Spectroscopic techniques are based on the identification of functional groups of molecules and the characterization of conformationally distinct structures in biological molecules[13]. Among the various examples of applications of FT-IR are the identification of microorganisms such as bacteria[14], yeast[15] and fungi[12-16], the determination of proteins, lipids and moisture in products[17-18] and the detection of mycotoxins in food[19].

Hernández-Hierro[9], used near infrared spectroscopy (NIRS) to analyze aflatoxin B_1, ochratoxin A and total aflatoxins in red paprika found on the Spanish market. Different spectra were visualized in samples of paprika: (a) non-contaminated, (b) contaminated with all five mycotoxins (ochratoxin A, aflatoxins B_1, B_2, G_1, G_2) and (c) highly contaminated with aflatoxin B_1 and ochratoxin A.

Furthermore, Galvis Sánchez[19], evaluated the application of Fourier infrared spectroscopy-attenuated total reflection (FTIR-ATR) in the determination of ochratoxin A contamination in dried vine fruits, with the authors concluding that the results confirmed that OTA can be detected by this technique.

Thus, the aim of this work was to determine the applicability of FT-IR to the rapid detection and quantification of ochratoxin A in fungi of *Aspergillus* sp. genera isolates from green coffee beans.

MATERIALS AND METHODS

Selection of fungal isolates

Ten fungal isolates were selected, previously identified as *Aspergillus* sp., 3 of which belonged to section *Nigri* and 7 to section *Circumdati,* isolated from green coffee beans from Londrina, Brazil.

Ochratoxin A production

Isolates were grown in triplicate in 150 mL of potato culture medium (potato 200g, dextrose 20g, agar 15 g, distilled water 1000 mL), in 500 mL Erlenmeyer flasks for 14 days at 25°C in the dark. The cultures were homogenized for 30 min and filtered through folded filter paper[20].

High performance liquid chromatography (HPLC) analyses

Samples were analyzed by HPLC according to Perrone[20]. The cultures were first filtered through a 0.45 µm nylon syringe filter. The HPLC system consisted of a Zorbax C18 column (150 mm, 4.6 mm with 5 µm particles), mounted on an Agilent 1100 chromatograph, with quaternary pump degasser and a Rheodyne manual injection system with a 100 µL loop, connected to a fluorescence detector (Agilent 1100) set at 333 nm excitation and 470 nm emission. Stock solution of ochratoxin A with a concentration of 1000 µg/L was prepared in benzene and acetic acid (v/v 99:1) from a standard concentration (50 µg/mL). A calibration curve was constructed using dilutions of the stock solution at 0.5 µg/L, 1.25 µg/L, 5 µg/L and 10 µg/L. The solutions were prepared by evaporating an exact volume of the calibrated solution and redissolving the residue in the mobile phase. We used the following chromatographic conditions: mobile phase of acetonitrile-2% acetic acid (41:59) at 1 mL/min, injection volume of 100 µL, and column temperature at 25°C. Peak areas were quantified with the Agilent ChemStation software, with the detection limit set at 0.5 µg/L. To minimize

sampling error, all the samples were analyzed in triplicate (A, B and C).

Fourier Transform Infrared Spectroscopy

The spectra were captured using a Biorad Excalibur spectrometer with Varian software Varian (Germany). The spectra were obtained in the wavenumber range of 7500 - 400 cm^{-1} and spectral resolution was set at 2 cm^{-1}. Data were analyzed using The Unscrambler 9.1 software. The two methodologies investigated were transmittance and reflectance. In transmittance, samples were mounted between 2 calcium fluoride crystals with a Teflon spacer of 0.05 mm, with approximately 30 µL of sample being injected. For reflectance approximately 100 µL of sample was injected on to a crystal of zinc selenide. To minimize sampling error, all the samples were analyzed in triplicate (A, B and C).

Data analysis

The two methodologies were evaluated for detection and for quantification of ochratoxin A. The partial least squares (PLS) regression method and different pre-treatments were applied to the data obtained for detection and quantification. For ochratoxin A detection a value of 1 was assigned to an OTA producer and 0 to an OTA non producer. For ochratoxin A quantification the samples were assigned a value according to the results of the HPLC analysis.

RESULTS AND DICUSSION

High Performance Liquid Chromatography

Ten samples were evaluated and of those 7 presented a detectable concentration of OTA (above 0.5 µg/L of OTA). In this experiment it was observed that all strains to the *Circumdati* section are producer of OTA, unlike the representatives *Nigri* section, which did not producer detectable levels of OTA. Due to the large differences in OTA concentration among the repetitions, they were analyzed individually. We found that of 30 repetitions, 16 (53.33%) contained OTA in detectable amounts, while 14 (46.66%) did not contain the mycotoxin (TABLE 1).

The presence of OTA in coffee bean has been reported by several authors in wide concentration ranging between 0.2 and 360 µg/kg[21-22-23].

In this study, we found that all strains to the *Circumdati* section are producer of OTA, unlike the representatives *Nigri* section. Taniwaki[2] examined Brazilian coffee samples and showed that *A. niger* (*Nigri* section) was the species found most commonly, 63 % of potential OTA producers, but only 3 % of them produced OTA. And, *A. ochraceus* also occurred commonly (31 % of isolates), but 75% were capable of OTA production

Fourier Transform Infrared Spectroscopy

Results for the detection of OTA are presented in TABLE 2. The models selected for the prediction were those which resulted in the best values for calibration and validation.

For each model the PLS and prediction values, were NP= non-producer of OTA and P= producer of OTA. The best prediction results for non-producers were closer to 0 and for producers closer to 1. To facilitate further comparison between the models the sum of prediction errors Σ=(expected-observed)^2 was used, where the expected was 1 OTA presence and 0 OTA absence and the observed was the value predicted for OTA by the program.

In this case, note that the best model to detect OTA in the samples was model 2, because the sum of prediction errors had the smallest value (0.11824), and the prediction values were closer to observed values.

The quantification of OTA through FT-IR can be observed in TABLE 3. For quantification analyzes the attributed values, by PLS, were the OTA concentrations quantified by HPLC. The sample used in prediction had a concentration of 3.30 µg/L OTA, thus, the value assigned to the producer sample of OTA was 3.30. Therefore, the best prediction results among non-producers were closer to 0 and among producers closer to 3.30.

The best results were observed in model 1, which used data obtained by reflectance, despite the sum of prediction errors being higher in model 2.

The protocol used for FT-IR, in which liquid medium aliquot was used for the analysis of OTA, we selected because it is considered less laborious, more practical and faster. Samples with high water content have been criticized because of its high absorbance capability[24]. However, some works have shown the benefits of the interaction of water and its IR bands in biological systems, furthermore, protocols are simple, low cost and facilitate its use in routine laboratory[25-26].

Börgesson[27] reported similar results in an evaluation of reflectance and transmittance for the detection of ergosterol in barley. They found that despite the two methodologies producing similar results, transmittance, was easier to perform and produced better results when detecting metabolites that are present in low concentrations, as in the case of ochratoxin A.

In other work, Dowell[28] also found that infrared transmittance presented the best results for the

detection of fumonisin in single corn kernels. According to the authors reflectance or transmittance spectroscopy may have practical applications in the screening of single corn kernel samples to detect fumonisin.

Meanwhile, Pettersson and Aberg[29] used transmittance to determine the level of mycotoxin deoxynivalenol (DON) in wheat kernel samples. Based on their results the authors concluded that it may be possible in the future to develop a calibration model which can be used to routine screen for DON in wheat.

In work by Galvis-Sánchez[30] mid infrared spectroscopy (MID) was used to identify grapes originating from different regions, showing that samples of different origins had different spectral characteristics. Moreover, in this study the authors constructed an OTA calibration curve from 10 to 40 µg/kg. Samples containing OTA at concentrations above 20 µg/kg were regarded as contaminated. According to the authors the methodology demonstrated the potential use of MID as a simple screening procedure for OTA contamination in grapes.

CONCLUSION

This technique should be investigated further with greater numbers for more conclusive results. Meanwhile, The presented results highlight the potential of the reflectance and transmittance spectroscopy be employed as a screening method for the detection and quantification of OTA in fungi samples from food as a fast, reliable and low-cost alternative.

ACKNOWLEDGEMENTS

We thank CEPPA for performing the HPLC analysis; the Chemistry Department of UFPR for the loan of infrared equipment; and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support.

Table 1: Quantification by HPLC of Ochratoxin A production in fungi of the genus *Aspergillus* sp.

Sample	Section	Repetitions	OTA production (µg/L)
38	*Nigri*	A	0
		B	0
		C	0
41	*Nigri*	A	0
		B	0
		C	0
47	*Nigri*	A	0
		B	0
		C	0
13	*Circumdati*	A	1.33443
		B	1.39291
		C	1.95051
18	*Circumdati*	A	0
		B	3.30499
		C	0
10	*Circumdati*	A	1.24648
		B	1.41722
		C	1.60370
11	*Circumdati*	A	0.634607
		B	0
		C	1.88903
31	*Circumdati*	A	2.71227
		B	0.971241
		C	0
16	*Circumdati*	A	1.32428
		B	6.39487
		C	2.50084
20	*Circumdati*	A	2.52742
		B	0
		C	0.559545

Table 2: Pre-treatment (P.T.) used for detection of ochratoxin A, in samples of fungi of the *Aspergillus* sp. genera, with their principals components (PCs) and respective calibration, validation, error, prediction values and prediction errors sum

Data	P. T.			PCs				Prediction	Σ=(e-o)²
Reflectance	1	4	error	5*	error	6	Error		
		C: 0.99	C:0.04	C: 0.99	C: 0.01	C:0.99	C: 0.01	NP:0.168	0.53965
		V:0.93	V: 0.19	V: 0.95	V: 0.17	V: 0.96	V: 0.16	P:1.168	
Transmittance	2	1	error	2*	error	3	Error		
		C: 0.98	C: 0.09	C: 0.99	C: 0.04	C:0.99	C: 0.01	NP:0.296	0.11824
		V: 0.97	V: 0.22	V: 0.97	V: 0.19	V: 0.97	V: 0.19	P:0.825	

Note: Pre-treatment: 1 – Model function MSC; 2 – Model function MSC. Prediction: NP – non producer (assigned value 0); P – producer (assigned value 1). * optimal number of principal component, e= observed value (0 or 1), o= value obtained in prediction.

Table 3: Pre-treatment (P.T.) used for quantification of ochratoxin A in samples of fungi of *Aspergillus* sp. genera, with their principals components (PCs) and respective calibration, validation, error and prediction values

Data	P. T.			PCs				Prediction	Σ=(e-o)²
Reflectance	1	2	error	3*	error	4	Error		
		C: 0.95	C: 0.41	C: 0.96	C: 0.33	C: 0.97	C: 0.31	NP:-0.06	0.03262
		V: 0.88	V: 0.66	V: 0.91	V: 0.56	V: 0.91	V: 0.55	P:3.131	
Transmittance	2	1	error	2*	error	3	Error		
		C: 0.94	C: 0.29	C: 0.98	C: 0.01	C: 0.99	C: 0.11	NP:0.44	0.77296
		V: 0.79	V: 0.62	V: 0.89	V: 0.46	V: 0.89	V: 0.45	P:2.54	

NOTE: Pre-treatment: 1 - Model without pre-treatment; 2 - Model without pre-treatment. Prediction: NP- non producer (assigned value 0); P- producer (assigned value 3.30). * optimal number of principal component. e= observed value, in other words, values obtained by HPLC, o= value obtained in prediction.

REFERENCES

1. Meca G and Ritieni A. Production and analysis of ochratoxin A produced by Aspergillus ochraceus ITEM 5137 in submerged culture. Food Chem. 2009;117:470-472.
2. Taniwaki MH, Pitt JI, Teixeira AA and Lamanaka BT. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. Int J Food Microbiol. 2003;82:173-179.
3. Batista LR, Chalfoun SM, Silva CF, Cirillo M, Varga EA and Schwan RF. Ochratoxin A in coffee beans (Coffea arabica L) processed by dry and wet methods. Food Control. 2009;20:784-790.
4. Al-Anati L and Petzinger E. Immunotoxic activity of ochratoxin A. Vet Pharmacol Th. 2006;29:79-90.
5. Dinis AM, Lino CM and Pena AS. Ochratoxin A in nephropathic patients from two cities of central zone in Portugal. J Pharm Biomed Anal. 2007;44:553-557.
6. Almela L, Robe V, Sanchez B, Torella F, Perez-Lopez J and Gabaldon JA. Ochratoxin A in red paprika: relationship with the origin of the raw material. Food Microbiol. 2007;24:319-327.
7. Rinaldi G, Mancini E, Ferruzza S, Sambuy Y and Peruzzi G. Effect of red wine on ochratoxin A toxicity in intestinal Caco-2/TC7 cells. Toxicol in Vitro. 2007;21:204-210.
8. IARC - International Agency for Research on Cancer. Ochratoxin A. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins,. Monographs on the Evaluation of Carcinogenic Risk to Humans. 1993;56:489-452.
9. Hernández-Hierro JM, García-Villanova RJ and González-Martín I. Potential of near infrared spectroscopy for the analysis of mycotoxins applied to naturally contaminated red paprika found in the Spanish market. Anal Chim Acta. 2008;622:189-194.
10. Berardo N, Pisacane V, Battilani P, Scandolara A, Pietri A and Marocco A. Rapid detection of kernel rots and mycotoxins in maize by near-infrared reflectance spectroscopy. J Agr Food Chem. 2005;53:8128-8134.
11. Gaspardo B, Del Zotto S, Torelli E, Cividino SR, Firrao G, Della Riccia G, and Stefanon B. A rapid method for detection of fumonisins B1 and B2 in corn meal using Fourier transform near infrared (FT-NIR) spectroscopy implemented with integrating sphere. Food Chem. 2012;135:1608-1612.

12. Garon D, El Kaddoumi A, Carayon A and Amiel C. FT-IR spectroscopy for rapid differentiation of Aspergillus flavus, Aspergillus fumigatus, Aspergillus parasiticus and characterization of aflatoxigenic isolates collected from agricultural environments. Mycopathologia. 2010;170:131-142.
13. Naumann D. Infrared spectroscopy in microbiology. In: Meyers RA, editor. Encyclopedia of Analytical Chemistry. Chichester: Wiley. 2000;102-131.
14. Helm D, Labischinski H, Schallehn G and Naumann D. Classification and identification of bacteria by Fourier-transform infrared spectroscopy. J Gen Microbiol. 1991;137:69-79.
15. Wanning M, Seiler H and Scherer S. Fourier transform infrared spectroscopy, a novel and rapid tool for identification of yeasts. Appl Environ Microbiol. 2002;68:4717-4721.
16. Tralamazza SM, Bozza A, Destro JGR, Rodríguez JI, Dalzoto PR and Pimentel IC. Potencial of Fourier Transform Infrared Spectroscopy (FT-IR) to differentiate environmental Aspergillus fungi species A. niger, A. ochraceus and A. westerdijkiae using two different methodologies. Appl Spectrosc. 2013;67(3):274-278.
17. Togersen G, Isaksson T, Nilsen BN, Bakker EA and Hildrum KI. On-line NIR analysis of fat, water and protein in industrial scale ground meat batches. Meat Sci. 1999; 51: 97-102.
18. Biswal PK and Bhadouriya AS. Use of Fourier Transform Infrared Spectroscopy to cooking oils. Int J Pharm Chem Biol Sci. 2012;2(4):670-680.
19. Galvis-Sánchez A, Barros and Delgadillo I. Method for analysis dried vine fruits contaminated with ochratoxin A. Anal Chim Acta. 2008;617:59-63.
20. Perrone G, Mule G, Susca A, Battilani P, Pietri A and Logrieco A. Ochratoxin A production and amplified fragment length polymorphism analysis of Aspergillus cabonarius, Aspergillus tubingensis and Aspergillus niger strains isolated from grapes in Italy. Appl Environ Microbiol. 2006;72:680-685.
21. Levi CP, Trenk HL and Mohr HK. Study of the occurrence of ochratoxin A in green coffee beans. J Assoc Official Anal Chem. 1974;57:866–870.
22. Taniwaki MH. An update on ochratoxigenic fungi and ochratoxin A in coffee. Adv Exp Med Biol. 2006;571:189–202
23. Perrone G, Susca A, Cozzi G, Erlich K, Varga J, Frisvad JC, Meijer M, Noonim P, Mahakarnchanakul W and Samson RA. Biodiversity of Aspergillus species in some important agricultural products. Stud Mycol. 2007;59:53-66.
24. Essendoubi M, Toubas D, Lepouse C, Leon A, Bougeade F, Pinon J.M, Manfait M and Sockalingum GD. Epidemiological investigation and typing of Candida glabrata clinical isolates by FTIR spectroscopy. J. Microbiol. Methods. 2007;71(3):325-331.
25. Jinendra B, Tamaki K, Kuroki S, Vassileva M, Yoshida S, and Tsenkova R. Near-Infrared Spectroscopy and Aquaphotomics: Novel Approach for Rapid In Vivo Diagnosis of Virus-Infected Soybean. Biochem. Biophys. Res. Commun. 2010;397(4):685-690.
26. Tsenkova R. Introduction: Aquaphotomics: Dynamic Spectroscopy of Aqueous and Biological Systems Describes Peculiarities of Water. J. Near Infrared Spec. 2009;17(6):303-313.
27. Börjesson T, Stenberg B and Schnürer J. Near-Infrared Spectroscopy for estimation of ergosterol content in barley: a comparison between reflectance and transmittance techniques. Cereal Chem. 2007;84:231-236.
28. Dowell FE, Pearson TC, Maghorang EB, Xie F and Wicklow DT. Reflectance and transmittance spectroscopy applied to detecting fumonisin in single corn kernels infected with Fusarium verticillioides. Cereal Chem. 2002;79:222-226.
29. Petterson H. and Aberg L. Near infrared spectroscopy for determination of mycotoxins in cereals. Food Control. 2003;14:229-232.
30. Galvis-Sánchez A, Barros A and Delgadillo I. FTIR-ATR infrared spectroscopy for the detection of ochratoxin A in dried vine fruit. Food Addit Contam. 2007;24:1299-1305.