BIPM Capacity Building & Knowledge Transfer Program

2022 BIPM - TÜBİTAK UME Project Placement

REPORT

Project Name	Characterization of an aflatoxin`s hazelnut certified reference material by Isotope Dilution Mass Spectrometry-IDMS and High-Performance Liquid Chromatography with fluorescence detection (HPLC-FLD)
Description	This project aims to transfer knowledge on pathways for the characterization and value assignment of a hazelnut aflatoxins-certified reference material by mass spectrometry isotope dilution and High-Performance Liquid Chromatography with fluorescence detection methodologies.
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Motivation & Introduction

Mycotoxins are secondary fungal metabolites produced by a variety of mold and can be found in a wide range of food commodities, either before harvest or after harvest, during storage, on/in the food itself often under warm, damp, and humid conditions. These molecules represent a big concern in food safety, affecting human and animal health, and even leading to economic losses (1–3). The most common occurring mycotoxins include aflatoxins, ochratoxins, Fusarium toxins [including trichothecenes, zearalenone (ZEN), and fumonisins, and patulin (2). In fact, aflatoxin B1 (AFB1) is considered the most potent naturally occurring carcinogen whose exposure impairs the function of liver cells which facilitates cancer development, creating hepatocellular carcinoma (2, 4).

On the other hand, Colombia is one of the main exporters of coffee and agricultural products, many of which are susceptible to mycotoxin contamination. This can lead to non-compliance with the phytosanitary requirements of the destination countries, many of which have already imposed regulations that establish the maximum permitted limits of these highly toxic compounds (1). Additionally, the diet of the Colombian population is based mainly on commodities such as corn, wheat, and other foods that may also be prone to contamination by mycotoxins, with AFB1 being the most prevalent in contamination of corn due to factors said above (2, 4, 5). To face and limit the mycotoxins exposure and avoid technical barriers to trade in food and feed a strong measurement infrastructure for mycotoxin analysis is required (5). To demonstrate compliance with national and international regulations, testing laboratories as well as official laboratories must have adequate measurement methods and guarantee the reliability of their analytical results. However, the availability of metrological tools in Colombia as in other developed economies is scarce, so the comparability of measurement results can be seriously compromised (6).

To generate metrological tools like Certified Reference Materials (CRM) that allow supporting SI traceability, measurement methods with high metrological quality are required for characterization and value assignment of these. For mycotoxins in foods, Isotope Dilution Mass Spectrometry-IDMS, which is a potential primary measurement method (5, 6), and HPLC-FLD based methods are common to accomplish this purpose. In this sense, the National Institute of Metrology of Colombia -INM- is currently interested in developing the capability of measuring organic contaminants in food-based reference materials by these kinds of methods.

In this way, the experience gained by TUBITAK UME through the development of the certified reference material for dried figs (UME-CRM 1302), its participation in the key comparison CCQM-K138 with satisfactory results, in addition to the CMCs published in the KCDB related to the measurement of mycotoxins using the aforementioned methods (7), make TÜBİTAK UME a suitable metrology center to carry out training in the method to allow the transfer of knowledge to the INM.

The project to be developed at TUBITAK UME focuses on the training and transfer of knowledge in the characterization of a CRM of aflatoxins in hazelnut by mean using two methods of demonstrable accuracy in one competent laboratory in compliance with ISO 17034. These methods are Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS) with isotopic dilution mass spectrometry (IDMS) and HPLC-FLD. The knowledge acquired will allow the implementation of the measurement method

in the INM while other metrologists can be trained which will allow it to strengthen its technical capacity. Finally, it is expected to generate new metrological tools and/or services to allow for improving the measurement capability and competency of the network of national laboratories.

Research

According with the aim of this project, the following steps were carried to out to achieve the mycotoxin in hazelnut CRM characterization:

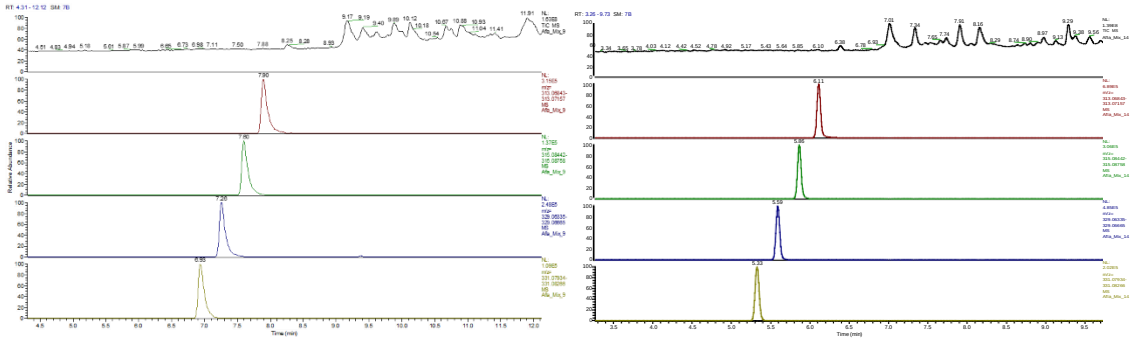
- Sample under study selection
- LC-HRMS method development
- LC-HRMS method validation
- HPLC-FLD method optimization

Sample under study

A candidate for Certified Reference Material (CRM) UME CRM 1321 of aflatoxins: Aflatoxin-B1 (AfB1), Aflatoxin-B2 (AfB2), Aflatoxin-G1 (AfG1), and Aflatoxin-B2 (AfG2) in hazelnut were produced previously, as briefly described at next. Raw material was homogenized by mixing with an Ultra-Turrax (UTC 115kt) for 8 h. The hazelnut homogenate was bottled in aluminum mylar bags and vacuumed. 860 units with a net content of 60 g were obtained. Finally, bags were labeled using and automated labeling machine in strict order of filling. HPLC -FLD method validation (BS EN 14123:2007), Homogeneity and short-term stability studies were performed last year in the framework of the "BIPM–TÜBİTAK UME project placements" (8).

LC-HRMS method development

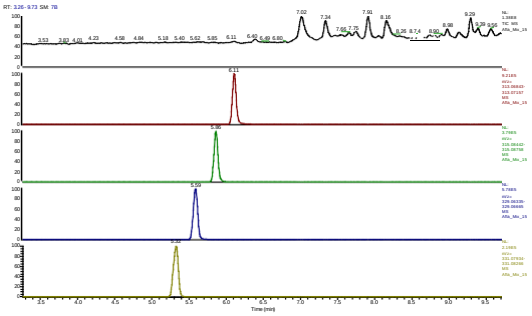
Measurement for the characterization started with establishing the optimal conditions for the instrument (LC-HRMS) through analysis of calibrant solutions. In first place, two columns were assessed: Hypersil Gold 100 mm x 2.1 mm x 1.9 µm and Kinetex C18 100 mm x 2.1 mm x 2.6 µm. As seen from Figure 1, both columns give good chromatographic separation but with Hypersil column back tailings were observed whereas better peak shapes were observed with Kinetex column.



a) b)

Figure 1. Assessed columns. A) Hypersil (5µL injection), b) Kinetex (15µL injection).

Likewise, different volume injections were assessed for the Kinetex column. As is shown in Figure 2, an injection volume greater than 15 µL worsens the peak symmetry.



a) b)

Figure 2. Volume injections assessing. a) 20 µL, b) 25 µL

In this project, characterization would be carried out through two independent methods of demonstrable accuracy in one competent laboratory in compliance with ISO 17034:2016. These two methods consist in: i) In-house validated method based on immunoaffinity column (IAC) clean-up and reversed-phase HPLC with post-column derivatization and fluorescence (FLD) detection modified from BS EN 14123:2007 and, ii) In-house validated method for the determination of aflatoxins by ID-LC-MS.

Sample preparation was assessed through of the standard procedure based in the BS EN 17641:2022, where two strategies were studied, with IAC clean-up (S1) and without IAC clean-up (S2). Briefly, for both S1 and S2, 2.0 g of independent sub-samples of CRM were weighted in a 50 mL conic tube. Samples were extracted with pure water (10 mL) and acetonitrile/acetic acid 0.5% (10 mL). A partitioning salts mixture (4 g MgSO4 + 1 g NaCl) was added, and vigorous shaking was applied. The mixes were centrifuged (4000 *g, 10 min), and 5 mL of supernatant was defatted with 5 mL of n-hexane and shaken. Two phases were separated through centrifugation (4000 *g, 1 min).

For S1, a 2 ml aliquot of the defatted acetonitrile phase (lower) was transferred into another 50 ml polypropylene tube. PBS solution was added up to the 25 mL mark of the polypropylene tube followed by appropriate mixing. The entire PBS-diluted extract was subjected to an IAC cleaning (AflaTest-VICAM), through 20 mL water cleaning and 3 x 750 µL methanol aliquots to elute aflatoxins. The final extract was evaporated to dryness under N_2 stream at about 40 °C. The dry residue was reconstituted with 30 µl of methanol and mixed for about 1 min to re-suspend the residue. 170 µl of water was added and mixed for about 5 s using a laboratory shaker. Finally, the resulting mixture was transferred into a 1,5 ml polypropylene microcentrifuge tube and centrifuged at 8500 *g at room temperature for 10 min. The supernatant was transferred into an HPLC glass vial for LC-MS analysis.

For S2, 1 mL of acetonitrile defatted phase (lower) was transferred into another 15 ml polypropylene tube and evaporated to dryness under N_2 stream at about 40 °C. The dry residue was reconstituted with 75 µl of methanol and mixed for about 1 min to re-suspend the residue. 425 µl of water was added and mixed for about 5 s using a laboratory shaker. Finally, the resulting mixture was transferred into a 1,5 ml polypropylene microcentrifuge tube and centrifuged at 8500 *g at room temperature for 10 min. The supernatant was transferred into an HPLC glass vial for LC-MS analysis.

At the same time, a sub-sample was treated according with the method based in BS-EN 14123:2007 (S3), and measurement was done by LC-HRMS.

The different extracts obtained were measured by LC-HRMS according to the conditions shown in Table 1.

Table 1. Instrumental conditions for LC-HRMS

Parameter	LC-MS
Instrument/detector	UHPLC Thermo Ultimate 3000 coupled to Q Exactive Quadrupole-Orbitrap Mass Spectrometer
Detector operating conditions	Polarity: positive Mode: Full scan Mass range: 150 Da to 500 m/z Resolution: 70,000
Column	Phenomenex Kinetex 100 x 2.1 mm, 2.6 µm Temperature: 40 °C
Flow	0.400 mL/min

Mobile phases and gradient program	Mobil Phase A: Water/Methanol/Formic acid/Ammonium acetate 98:2:0.1%/5mM Mobil Phase B: Methanol/Formic acid/Ammonium acetate 0.1%/5mM Program: 0 min (5%B), 0.5 min (5%B), 10 min (95%B), 15 min (95%B), 15.1 min (5%B), 18 min(5%B)
Injection volume	15 µL

According to Figure 3, the method based in the BS EN 14123 and BS EN 17641 with clean-up got similar results in terms of interferences and signal ratio for aflatoxins.

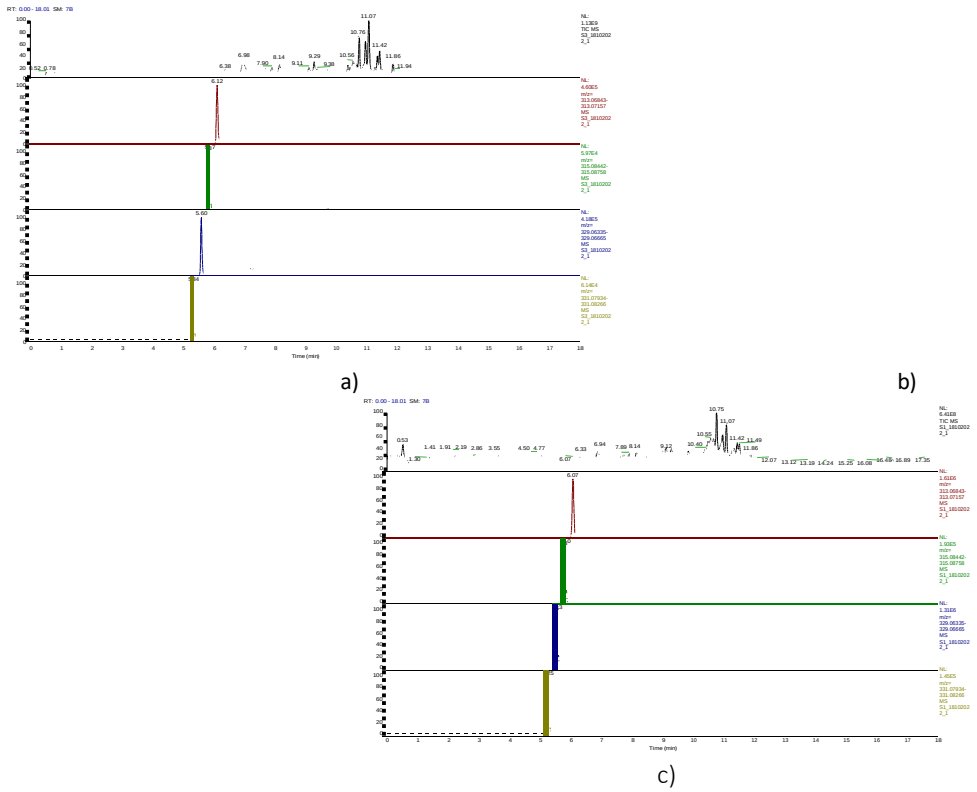


Figure 3. Different sample treatments assessed. a) S1, b) S2, c) S3

ID-LC-HRMS method validation

As established by quality system based in ISO 17034:2016, methods used for CRM characterization activities should be validated. In this sense, LC-HRMS method based in BS EN 17641:2022 was validated in the parameters of precision for 1 sample and three sub-samples, intermediate precision (inter-days) for 2 days and three sub-samples each, linearity, and bias (NIST SRM 2387). Table 2 shows results for each assessed parameter. In this case, samples were spiked with the internal standard stock solution (AfB_1-$^{13}C_{17}$, AfB_2-$^{13}C_{17}$, AfG_1-$^{13}C_{17}$, AfG_2-$^{13}C_{17}$).

Table 2. LC-HRMS method validation outcomes

Parameter	Result			
	AFB_1	AFB_2	AFG_1	AFG_2
Mass fraction (ng/g)	3.28	0.44	3.91	0.66
Precision (CV%)	0.86	1.00	0.52	0.87
Intermediate precision	NC	NC	NC	NC
Linearity (R^2)	0.9996	0.9996	0.9998	0.9993
Bias as recovery (%) *	129	124	NA	NA

*Reference values for AfB1 and AfB2; NC: not completed

HPLC-FLD method optimization

Sample preparation for HPLC-FLD determination of Aflatoxin-B1 (AfB1), Aflatoxin-B2 (AfB2), Aflatoxin-G1 (AfG1), and Aflatoxin-B2 (AfG2) was done following the BS EN 14123:2007 with some modifications. Briefly, a test portion of 6 g of candidate Reference Material was weighted and extracted with 12 mL of water, 18 ml of methanol, and 0.6 g of NaCl using a high-speed mixer. Then, the slurry was filtered, and an aliquot was diluted 4-fold with PBS. The diluted extract was cleaned up by immunoaffinity columns (AflaTest. VICAM). Finally, aflatoxins were eluted in two steps with methanol (1.5 mL) and water (1.5 mL).

The cleaned-up extracts were measured by HPLC-FLD according to the conditions shown in Table 3.

Table 3. Instrumental conditions for HPLC-FLD

Parameter	HPLC-FLD
Instrument/detector	HPLC Agilent 1290 coupled to FLD with post-column derivatization using Kobra cell System at 100 µA. λ_{Exc}: 362 nm λ_{Emi}: 425 nm
Column	Phenomenex Luna C18 250 mm x 4.6 mm x 5 µm
Flow	1.0 mL/min
Mobile phases and gradient program	Water/methanol/acetonitrile/nitric acid/potassium bromide 600 mL:300 mL:200 mL: 4 N 385 µL:132 mg- Isocratic
Injection volume	100 µL

Because in the validation of the LC-HRMS method a difference of close to 35% was found for aflatoxins concentration regarding previous property measurement of the CRM candidate by HPLC-FLD, it was necessary to carry out a series of trials to investigate the cause of the difference in the HPLC-FLD method, otherwise the characterization could not be carried out.

The first trial consisted of adapting the isotopic dilution method to the method based on the BS EN 14123:2007 standard, for which a 6 g subsample of the candidate was spiked with the internal standard stock solution. Subsequently, the method established for the treatment of the sample in the BS EN 14123:2007 standard for hazelnut was followed without further changes. Finally, the cleaned-up sample obtained was measured by LC-MS under the conditions shown in Table 1. The concentration results obtained were close to those obtained when applying the methodology based on the BS EN 17641: 2022 standard. This result suggests firstly that any problems associated with low recovery can be overcome using the internal standard regardless of the sample treatment that is applied.

In a different experiment, two independent sub-samples of the CRM were spiked with an aliquot of a calibrant mix stock solution of native aflatoxins. The procedure was followed as normal with the addition of a step of centrifugation (3000 *g, 5 min) before filtration of the extraction mix was made. The concentration gotten for each aflatoxin allowed to confirm a recovery issue (70-80 %) for the HPLC method.

The next step was to investigate the VICAM AflaTest performance, for which an aliquot of a calibrant mix stock solution of native aflatoxins was diluted with 10 mL of PBS and passed through the IAC. Finally, aflatoxins were eluted as above mentioned and HPLC-FLD measurement was done for concentration estimation. The results showed that VICAM IACs have a low recovery of about 80-90%, which would worsen when the matrix is present.

In another experiment, the HPLC-FLD sample preparation protocol was modified as follows: two independent subsamples of 6.0 g of the CRM were extracted as previously mentioned HPLC method; after, the first extract was diluted 1:7 with PBS, second extract was defatted with n-hexane in a ratio of 1:1 and then hexane layer discarded by the help of centrifugation for clear separation of the two layers then defatted extract was diluted with PBS (1:7). Clean up with VICAM-AflaTest IAF column was applied to both diluted extracts and a conditioning step of cartridges with 10 mL PBS added to the method. The results shown in Table 4 suggests a little loss of aflatoxins by using the n-haxane to defat the extract. In general, recovery was not improved with these modifications.

Table 4. Mass fraction for HPLC-FLD method

Experiment	Mass fraction (ng/g)			
	AFB_1	AFB_2	AFG_1	AFG_2
Without n-hexane (original method)	2.37	0.33	3.06	0.78
With n-hexane	2.17	0.31	2.97	0.71

Conclusions and Future Work

At the end of my participation in this project on the characterization of the candidate for CRM of mycotoxins in hazelnut puree (UME 1321), I can conclude that all expectations were met, since it was possible to receive training in two important techniques to characterize materials of reference of this nature, such as HPLC-FLD and ID-LC-HRMS. It was possible to carry out the optimization and preliminary validation of the method based on Isotope Dilution with Liquid Chromatography coupled to High-Resolution Mass Spectrometry with results that are within the established criteria. Therefore, it can be considered that this methodology is about to be used as one of the techniques to carry out the characterization of CRM. Regarding the methodology based on HPLC-FLD, the preliminary results showed that the method required a more in-depth study to set it up. In this regard, this was important for me since it allowed me to deepen into strategies to overcome the challenges that usually arise in chemical metrology thanks to the high competence of my tutors.

Therefore, I hope to implement this type of methodology with the purpose of expanding the CRM portfolio in Colombia, in such a way that support can be provided for the metrological traceability and reliability of the measurements made in Colombia to respond to the demanding phytosanitary barriers in international trade and protect the health of consumers of foods prone to contamination by mycotoxins, always under the criteria of the CIPM MRA.

Acknowledgments

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