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Residue Analysis of Ochratoxin A In Coffee Powder Marketed In Buon Ma Thuoc City, Vietnam Using Liquid Chromatography Coupled To Tandem Mass Spectrometer

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Abstract

In this research, we developed and validated a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method to determine Ochratoxin A (OTA) levels in coffee powder samples. Separation of Ochratoxin A was achieved using a Hypersil XDB-C18 column (2.1 mm x 100 mm) with a particle size of 1.8 µm, coupled with tandem mass spectrometry. The mobile phase, composed of acetonitrile: water: acetic acid in a ratio of 80:20:1 (v/v/v), operated at a flow rate of 0.1 mL/min. Each sample underwent a total chromatographic analysis time of approximately 5 minutes, with Ochratoxin A eluting at a retention time of 3.259 minutes. The linear range spanned from 1 to 500 ng/mL, characterized by a linear regression equation (Area = C + 19521) with a high correlation coefficient (r = 0.9991). The limit of detection (LoD) and limit of quantification (LoQ) for Ochratoxin A were established at 0.63 µg/mL and 2.11 µg/mL, respectively. Recovery rates for Ochratoxin A ranged from 84.86% to 94.54%. The Ochra Test WB solid phase extraction column was chosen to purify the extract OTA from a coffee sample. Among the 55 coffee powder samples analyzed, 9 samples (16.36%) were found to be contaminated with OTA. Notably, all Ochratoxin A-contaminated samples fell within the permissible limits set by the Ministry of Health Vietnam, international organizations. While the concentrations of OTA in the studied samples did not surpass the maximum residue limit, but the presence of a positive result indicates pollution of coffee by these toxic substances.

Keywords: Ochratoxin A, coffee powder, SPE, HPLC-MS/MS, Buon Ma Thuoc city, Vietnam.

Introductıon

Coffee is one of the most popular drinks in the world. Buon Ma Thuoc is called the "coffee capital of Vietnam". Coffee is grown in the volcanic regions of the Tay Nguyen which create a unique flavor. Buon Ma Thuot coffee products are present in nearly 80 countries and territories around the world, and the total value of Vietnam's coffee exports from 2018 is more than 3 billion USD [1]. Vietnam is a tropical monsoon country, with hot and humid climate conditions which are favorable for mold growth. Every year, mold causes a significant loss in the quality of coffee and other agricultural products and they are a barrier to agricultural products in general and coffee in particular. The Ochratoxin A (OTA) toxin is produced by the molds Aspergillus ochraceous and Penicillium verrucusum species. OTA is a secondary metabolite capable of causing acute and chronic toxicity to human and animal health [2]. Mold strains Aspergilus ocharaceus and Penicillium verrucusum species can grow on ground coffee, or other agricultural products if storage conditions are not guaranteed, and then OTA toxins are produced. This toxin can affect human and animal health, affects the liver, and kidneys, and can cause cancer [3]. Moss (1996) announced that OTA is a worldwide food safety hazard for food crops [4]. To control the danger of OTA toxins, the European Union has set a maximum allowable limit of TOA is 5.0 µg/kg in coffee; this is also the maximum allowable limit according to Vietnamese Standards [5]. Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA), the acceptable weekly food intake limit is 100 ng/kg of human body weight [6].

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Due to the potential impact on human health when consuming food products containing OTA toxins, many analytical techniques to determine OTA in food matrices have been built and developed, such as gas chromatography (GC) [7, 8], thin layer chromatography (TLC) [9, 10], high-performance liquid chromatography- fluorescence detector (HPLC-FLD) [11-17], enzyme-linked immunosorbent assay (ELISA)[18, 19], liquid chromatography-mass spectrometry (LC/MS)[20, 21]. Many methods for determining OTA in food matrices have been published. Noba et al., (2009) determined the OTA content in drink coffee by immunoaffinity cleanup and liquid chromatography−tandem mass spectrometry Noba, Uyama [13]; Bandeira et al., (2012) developmented a method for the analysis of Ochratoxin A in roasted coffee by liquid chromatography/electrospray-mass spectrometry in Tandem (LC/ESI-MS/MS) [22].

The objective of this study is to develop a method to determine OTA residues in powdered coffee using high-performance liquid chromatography-tandem mass spectrometry, meeting analytical requirements, and this method was applied to evaluate the current status of OTA toxin residue contamination in powdered coffee in Dak Lak province, which can have a significant impact on the coffee supply chain of Vietnam and the world.

Materıals and Methods

Chemicals and equipment

Glacial acetic acid, water for HPLC, pure sodium hydrogen carbonate, pure sodium mono hydrogen phosphate, pure potassium dihydrogen phosphate, methanol, acetonitrile were purchased from Merck, Darmstadt, Germany. Sodium hydrogen carbonate, sodium mono hydrogen phosphate, potassium dihydrogen phosphate were prepared in water solvent and stored at -18 ^{o}C. These solutions were sonicated for 5 min before use. Ochratoxin A standard 10 µg/mL in acetonitrile was purchased from Sigma- Aldrich (10877-HPLC). High-performance liquid chromatography system was used with an Agilent 1200 Module and mass spectrometry tandem (Agilent 6410 Triple quad LC/MS/MS). Hypersil XDB-C18 column (2.1 mm x 100 mm), 1.8 µm particle size, with a 1200 series oven was used for the chromatographic separation. Data recording was integrated with Agilent 1200 Chemstation software.

Sample collection

A random selection of 55 coffee samples was obtained from local markets in Buon Ma Thuoc City, Dak Lak province, Vietnam in March 2023. The treatment and analysis of the samples were conducted at the Tay Nguyen Regional Food Safety and Hygiene Testing Center - Tay Nguyen Institute of Hygiene and Epidemiology.

Preparation of stock solutions

The Ochratoxin A standard solution at 10 µg/mL in acetonitrile was procured from Sigma-Aldrich (10877-HPLC). The OTA (1000 µg/L) stock standard solution was prepared by introducing 100 µL of the standard solution with a concentration of 10000 µg/L into a brown vial. Subsequently, it was evaporated to dryness under a nitrogen stream and dissolution in 1 mL of MeOH. The stock standards are maintained within a temperature range of 2-8 °C for a period of 6 months. This stock standard solution was further diluted with methanol to generate sample solutions with concentrations ranging from 2.5 µg/L to 50 µg/L.

Sample preparation

The extraction solvent is used to extract the OTA from the sample matrix. These solvents can be chloroform, methanol, carbonate solution, etc. Because these solvents often entail many other impurities with similar properties to the analyte being extracted, it is necessary to remove these impurities from the extraction solution. Solid phase extraction is a sample preparation method to enrich and purify an analytical sample from solution by adsorption onto a solid phase extraction column. The analyte is then eluted with a small amount of the appropriate solvent.

- ***The first processing process***: Weigh 25 g of the homogenized powdered coffee sample into a 250 ml Erlenmeyer flask. Add 100 µL of 100 ng/mL standard, add 200 mL of 1% $NaHCO_3$, and shake for 30 minutes. Filter 10 mL of the extract, and add 10 mL of saline phosphate buffer (PBS) pH=7-8. The extracted mixture was passed through a WB Ochra Test solid phase extraction column. Then, the extraction column was washed with 10 mL of distilled water. The analyte was eluted with 3 mL of MeOH. The samples were evaporated to dryness under a nitrogen stream at 40 °C, the residue was then mixed with 1 mL of MeOH, and injected into the HPLC [21]
- ***The second processing process*** [22]: Weigh 25 g of the homogenized powdered coffee sample into a 250 mL Erlenmeyer flask. Add 100 µL of 100 ng/mL standard, add 200 mL of ACN: H_2O (84:16, V/V), and shake for 30 minutes. Filter to get 10 mL of extract. The extracted mixture was passed through a WB Ochra Test solid phase extraction column. Then, the extraction column was washed with 10 mL of distilled water. The analyte was eluted with 3 mL of MeOH. The samples were evaporated to dryness under a nitrogen stream at 40 °C, the residue was then mixed with 1 mL of MeOH, and injected into the HPLC.
- ***The thirst processing process***: Weigh 25 g of the homogenized powdered coffee sample into a 250 mL Erlenmeyer flask. Add 100 µL of 100 ng/mL standard, add 200 mL of methanol: $NaHCO_3$ 3% (50:50, v/v), and shake for 30 minutes. Filter 10 mL of extract, and add 96 mL of saline phosphate buffer (PBS) pH=7.

The extracted mixture was passed through a WB Ochra Test solid phase extraction column. Then, the extraction column was washed with 10 mL of distilled water. The analyte was eluted with 3 mL of MeOH. The samples were evaporated to dryness under a nitrogen stream at 40 °C, the residue was then mixed with 1 mL of MeOH, and injected into the HPLC [25, 26]. Evaluate recovery performance to select optimal sample processing procedures.

Optimization of LC-MS/MS parameters

Investigation of the full scan mode

The quantification ion is selected based on the daughter ion with the highest intensity. The survey process comprises the following steps: The atomic mass range to be scanned is adjusted to acquire the required number of ions within the range of 50 to 400 Da. The standard solution is introduced into the triple quadrupole mass spectrometry probe in Full scan MS mode. The mass spectrum of OTA is obtained from the full scan chromatogram, accurately determining the m/z ratio of the parent ion and some daughter ions. Preliminary fragmentation conditions to generate daughter ions are determined from the precursor ions using computer software.

Investigation of the fragmentation energy of parent ions

Based on the fragmentation energy value, different sensitivities are exhibited by the produced daughter ions. Consequently, to ascertain the optimal fragmentation energy for acquiring quantitative and confirmation ions with the utmost sensitivity, the following steps are undertaken in the survey process:

The m/z of the parent ion is selected, utilizing the software to determine the fragmentation energy and ion tube lens voltage for generating highly sensitive daughter ion fragments (coarse tuning). The pre-ion fragmentation energy is adjusted, either increased or decreased, until the chromatographic intensity no longer increases (fine tuning).

Survey of Mobile Phase Composition

In the research method, a less polar C18 column is utilized as the stationary phase, necessitating the use of a polar solvent system for the mobile phase. Less polar solutes are retained in the column for a longer duration compared to polar solutes. Given the medium polarity of Ochratoxin A, the investigation of the mobile phase was selected as follows:

+ Methanol, CH_3COONH_4 10 mM (80:20, v/v) [27]
+ Acetonitrile: water: acetic acid (80:20:1, v/v/v) [28]
+ Acetonitrile: water: formic acid (80:20:1, v/v/v) [29].

Survey of the flow rate

With MS detectors for ESI mode, it is imperative to avoid excessively large flow rates, as they would impede the ion desolvation process, and the solvent might not evaporate promptly, thereby diminishing sensitivity. Generally, the recommended flow rate for ESI with LC-MS/MS equipment (pressure ≤ 400 bar) is no more than 1 mL/min. In the case of OTA analysis, the flow rate was set to be 0.1 - 0.2 mL/min for conducting device optimizations.

Survey the solid phase extraction column

Due to the weakly acidic nature of Ochratoxin A and the complex composition of the coffee sample matrix influenced by color, 3 types of columns were chosen to investigate: the C18 column, WB Ochra Test column, and Mycosep 228 Romer column. For column activation, 5 mL of MeOH and 5 mL of H_2O were used to activate the C18 column. The Ochra Test WB column required no activation as it already contained an activation solution. In accordance with the manufacturer's instructions, activation was not deemed necessary for the mycosep 228 Romer column. Sample loading into the column involved a slow passage of the sample through the column at a speed of 3 mL/min. Subsequently, the column was washed with a suitable solvent to eliminate impurities while ensuring that the analyte remained on the column. Elution of the analyte was achieved using a MeOH solution at a rate of approximately 1 mL/min. The resulting samples were evaporated to dryness under a nitrogen stream at 40 °C. The residue was then combined with 1 mL of MeOH and injected into the HPLC system.

Validation of analytical methods

Specificity/selectivity

To determine the specificity/selectivity for liquid chromatography-mass spectrometry, confirmation methods were used. For MS technology, it is necessary to meet the requirements for IP points (identification points). The European Council stipulates that the IP scoring method for liquid chromatography-mass spectrometry (LC-MS/MS) is 4, which means that 1 parent ion is required to bombard 2 daughter ions (according to EC657/2002) [30].

2.5 ng/mL standard samples, 25 g of the homogenized powdered blank coffee samples, and 25 g of the homogenized powdered blank coffee samples addition solution at 2.5 ng/mL were analyzed as the optimization of process. The blank sample had no signal at the retention time of the analyte signal. The signal of samples and spiked samples must be at a retention time consistent with the retention time of the standard [31].

Linear range: The linearity of the RP-HPLC method was determined at eight concentration levels ranging from 1 ng/mL to 1000 ng/mL OTA standard. Each of these standard solutions (20 µL) was injected into the chromatographic system (n=3). The peak area and

retention time were recorded, and the mean values of the peak area ratio were plotted against concentrations to obtain the calibration curves. The relative standard deviation (RSD) was determined for each concentration.

Limit of detection & Limit of quantification

The limit of detection (LoD), and limit of quantification (LoQ) were calculated:

LoD = 3SD/b, and LoQ = 10SD/b

Where, SD is the error in calibration line (y-direction)

b- the slope of calibration curve.

The repeatability and accuracy of the method

The repeatability of the method was verified by calculating the % RSD of six replicate injections of 2.5; 5.0, and 10 ng/mL of OTA on the same day and for intermediate precision % RSD was calculated from repeated studies on different days.

To ensure the reliability and accuracy of the method recovery studies were carried out by the sample addition method. A known quantity of pure OTA was added to the pre-analyzed sample contents were reanalyzed by the proposed method and the percent recovery was reported. Calculate the percent recovery of each solution, the mean recovery at each concentration level, and the %RSD of the percent recoveries at each concentration level. The average recovery at each concentration level should be within 80%-120%.

$$\%\ Recovery = \frac{Actual\ amount}{Theoretical\ amount} \times 100$$

Levels of OTA in coffee powder

Weigh 12.5 g of the homogenized powdered coffee sample into a 250 mL Erlenmeyer flask. Add 100 µL of 100 ng/mL standard, add 100 mL of methanol: $NaHCO_3$ 3% (50:50, v/v), and shake for 30 minutes. Filter 4 mL of extract, and add 96 mL of saline phosphate buffer (PBS) pH=7. The extracted mixture was passed through a WB Ochra Test solid phase extraction column. Then, the extraction column was washed with 10 mL of distilled water. The analyte was eluted with 3 mL of MeOH. The samples were evaporated to dryness under a nitrogen stream at 40 °C, the residue was then mixed with 1 mL of MeOH, and injected into the HPLC.

Statistical analysis

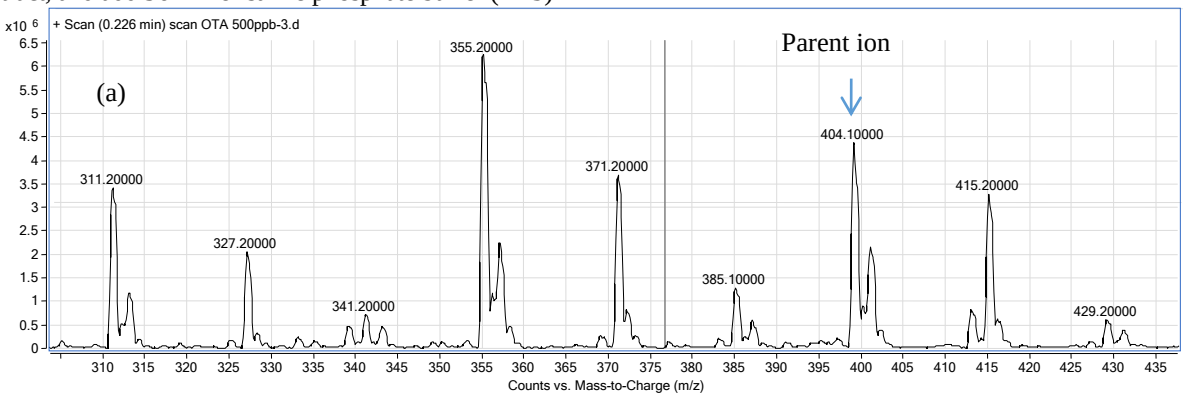
Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 22.0. Data were expressed by mean ±SD. Differences between means were evaluated by one-way analysis of variance (ANOVA). Statistical significance of the differences between mean was assessed by Duncan's test, $p < 0.05$ was considered significance.

Results and Dıscussıon

The optimized parameters of LC-MS/MS

Parent ion survey results (Full Scan mode)

According to European decision 2002/657/EC [31], for the validation of each analyte compound, there must be a parent ion and two daughter ion fragments. OTA is a substance with a low molecular weight and medium polarity. OTA was determined by ESI electrospray ionization technique with positive ion bombardment mode. The standard was injected directly into the LC to go to the mass spectrometry detector. An automatic survey mode was selected for each substance to select parent and daughter ions for quantification and identification. The results of the parent ion and two daughter ion fragments survey of OTA are obtained in Figure 1. The selected parameters, which were shown in Table 1, were the Dwell, fragment, and collision energy.



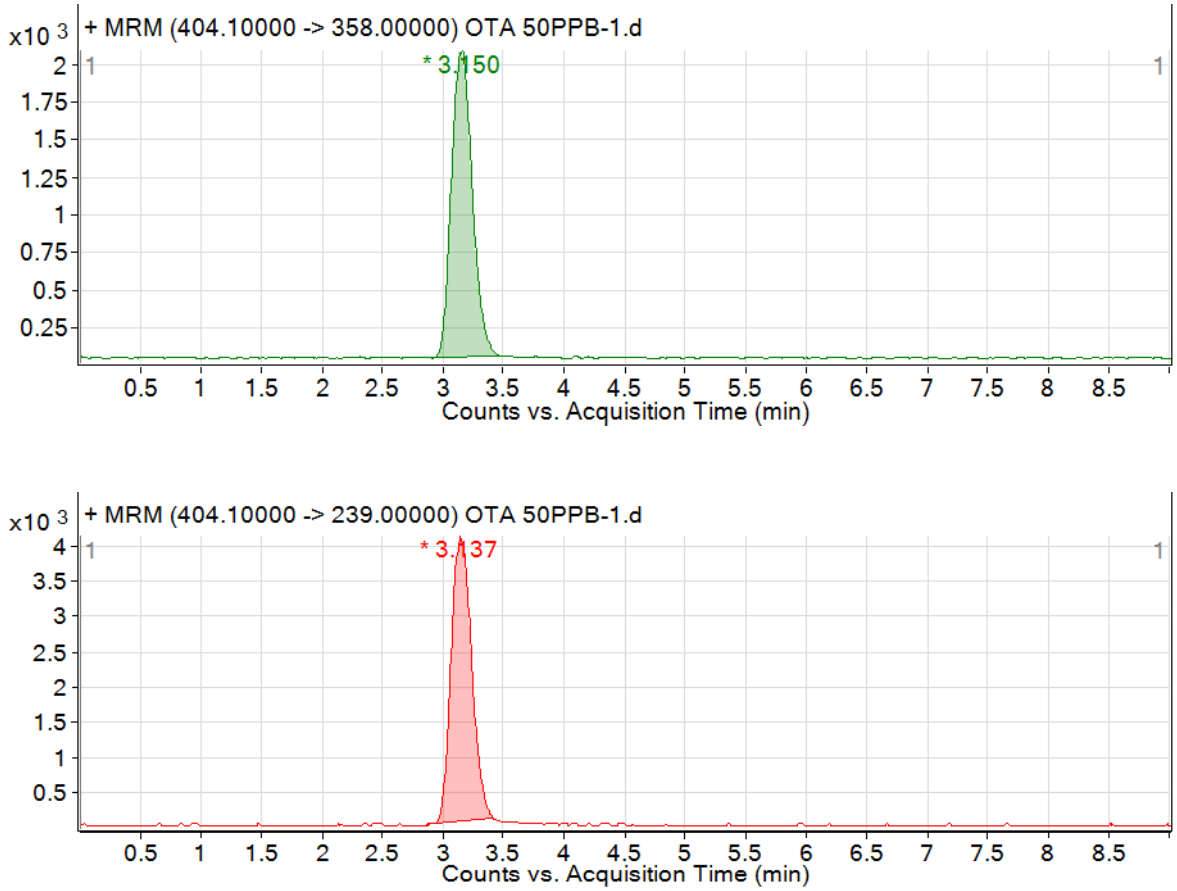


Figure 1. a) OTA standard chromatogram in SRM mode; b) Daughter ion at m/z 358; c) Daughter ion at m/z 239.

Table 1. The results of the parent ion and two daughter ion fragments survey of Ochratoxin A

Compound		Molecular mass (g/mol)	Parent ion (m/z)	Daughter ion (m/z)	Dwell	Fragment ion	Collision Energy
Ochratoxin A		403	404 [M-H]$^+$	239	200	135	20
				358	200	135	10

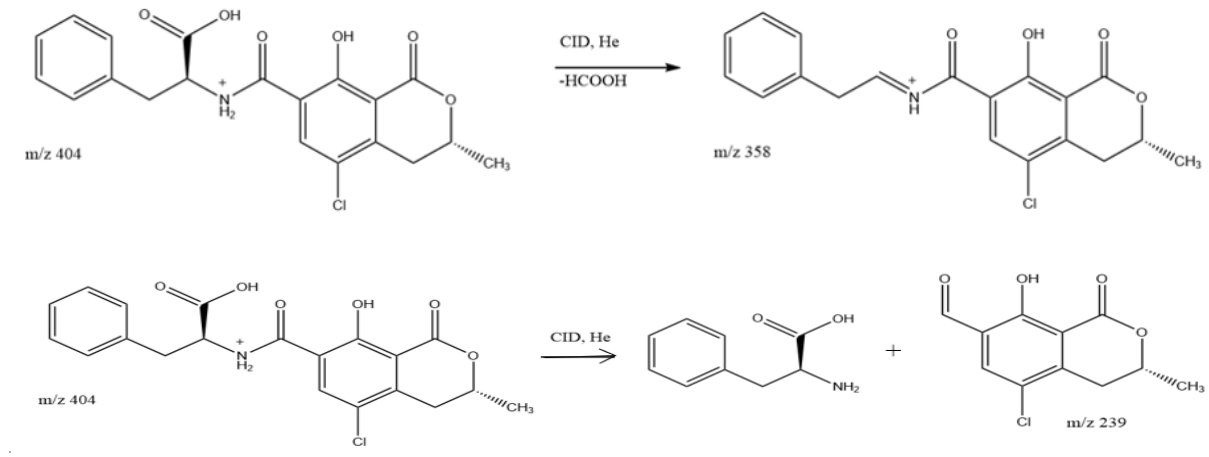


Figure 2. Formation and structure of probable ion with m/z 358 and m/z 239 resulted through fragmentation (CID – collision-induced dissociations in the presence of helium) in the MS/MS reactive spectrum of OTA in positive mode.

The m/z 404 → 239 (OTA) precursor-to-fragment transitions were used for quantification and the m/z 404 → 358 (OTA) precursor-to-fragment transitions were used for qualitative analysis.

Optimize analytical conditions on high-performance liquid chromatography (HPLC)

Results of mobile phase composition survey

The mobile phase not only affects the separation of substances but also affects the ionization process and signal of the analyte. With the electrospray ionization technique, the ionization process increases when adding substances such as acetic acid, formic acid, ammonium acetate, ... In this study, the C18 column was used as the stationary phase, which was less polar, so the mobile phase must be a polar solvent system. Because Ochratoxin has a medium polarity, the mobile phase was studied as follows: Methanol: ammonium acetate, acetonitrile: water: acetic acid, acetonitrile: water: acid formic. The results of the mobile phase composition survey of Ochratoxin OTA were obtained in Table 2.

Table 2. Results of mobile phase composition survey

Mobile phase	Signal of OTA
Methanol: ammonium acetate 10 mM (80:20, v/v)	Not detected
Acetonitrile: water: acetic acid (80:20:1, v/v/v)	Retention time: 3.137 min
	Area: 6585
Acetonitrile: water: acid formic (80:20:1, v/v/v)	Not detected

In the case of acetonitrile: water: acetic acid (80:20:1, v/v/v) mobile phase, the best chromatographic signal was obtained with shape, not splitting or dilution peak. In these conditions, the retention time of OTA was 3.137 min, and the run time of 5 min.

Results of flow rate survey

The flow rate of the mobile phase affects elution time and the amount of used solvent. In this study, the stationary phase is a 125 mm column, so the flow rate of the mobile phase investigated was 0.1 mL/min; and 0.2 mL/min. The results of the flow rate survey of OTA are obtained in Table 3.

Table 3. Results of flow rate survey

Retention time (min)	Flow rate (mL/min)	Peak area
3.136	0.1	1397538
1.607	0.2	542919

When increasing the flow rate, the chromatographic peaks tend to be sharp and clear. At a flow rate of 0.2 mL/min, the peaks are very sharp but the peak area was decreased. At this speed, the pressure of the system was high, which greatly affected the input pressure of the column and reduced the life of the column. Therefore, a flow rate of 0.1 mL/min was selected and the retention time of OTA was about 3.136 min. The peaks were completely separated and sharp.

Results of the extraction solvent

The accuracy of the sample processing process was verified by performing recovery studies by blank sample addition method. The percent recovery of the sample added to the pre-analyzed sample was calculated, and it was found to be 44% to 82% (Table 4).

Table 4. Recovery of the sample processing process survey

Sample processing process	Amount added (ng/mL)	Ochratoxin A	
		Amount found (ng/mL)	Recovery (%)
1	5	2.2	44
2	5	3.1	62
3	5	4.1	82

In sample processing process 1, OTA in the sample was extracted with $NaHCO_3$, the resulting extract was very turbid, affecting the process through the column and the extract could not remove all the color after going through the column. This not only takes a lot of extraction time but also reduces the efficiency of the

extraction column. Meanwhile, in process 2, OTA extracted with ACN: H_2O also did not give high extraction efficiency. From the results obtained in Table 4, process 3 gives the best recovery efficiency. Process 3 was chosen to process the coffee sample.

Results of the solid phase extraction column

Solid phase extraction columns are intended to remove background interference from the coffee sample and increase the analyte concentration in the analytical sample. C18 column, Ochra Test WB column, and Mycosep 228 Romer column were chosen to test the ability to extract OTA from coffee samples. The accuracy of the method was verified by performing recovery studies by blank coffee addition method. The results of the solid phase extraction column of Ochratoxin A are obtained in Table 5. The percent recovery of the solid phase extraction column to the pre-analyzed sample was calculated, and it was found to be 84.5% (Table 5).

Table 5. Results of the solid phase extraction column

Column	Amount added (ng/mL)	Ochratoxin A	
		Amount found (ng/mL)	Recovery (%)
C18	10	Not detected	-
Ochra Test WB	10	8.45	84.5
Mycosep 228 Romer	10	Not detected	-

From the results in Table 5, the Ochra Test WB solid phase extraction column is better than the C18 and Mycosep 228 Romer columns. The Ochra Test WB extraction column was chosen to purify the extract OTA from a coffee sample.

Validation of the RP-HPLC-MS/MS method

The selected parameters for method validation were specificity/selectivity, linearity, limit of detection (LOD) and quantification (LOQ), accuracy, and precision. The validation parameters were based on the European Union Commission Decision (EC-657/2002) [31].

Specificity/selectivity

For MS/MS techniques, it is necessary to meet the identification point (IP) requirements. The European Council stipulates that the IP scoring method for liquid chromatography-tandem mass spectrometry (LC-MS/MS) is 4, meaning it requires 1 parent ion to bombard 2 daughter ions (EC657/2002) [31].

Table 6. Parent ion and daughter ion of Ochratoxin *A*

Compound	Parent ion	Daughter ion	Identification point
Ochratoxin A	404	358	4
		239	

The results in Figure 3 show that the blank sample solution did not have a peak of OTA. The standard and blank sample addition solution appeared peak of OTA at the retention time from 3.109 to 3.259 min and the total time required for chromatographic analysis was 5.0 min. The research method has a specificity that meets the requirements.

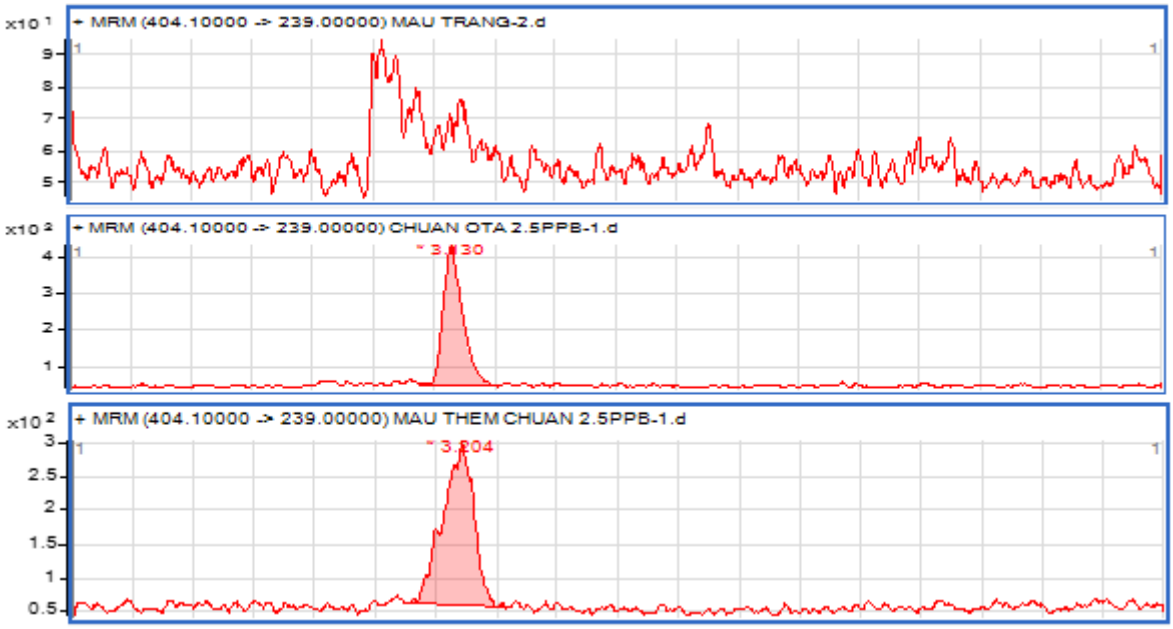


Figure 3. Chromatograms of OTA in blank sample, standard and blank sample addition solution

Table 7. Results for a standard, and blank sample addition solution

Sample	No.	Retention time (min)	Area
Standard at 2.5 ng/mL	1	3.130	4731
	2	3.137	4524
	3	3.123	4669
	4	3.109	4940
	5	3.130	4432
	%RSD	0.34%	4.21%
Blank sample addition solution at 2.5 ng/mL	1	3.204	4350
	2	3.204	4325
	3	3.171	4474
	4	3.225	4902
	5	3.259	4694
	6	3.245	4349
	%RSD	1.08%	5.40%

Linearity and Ochratoxin A standard curve

Linearity was evaluated by using different concentrations in the range from 1 to 1000 ng/mL with a determination coefficient (r^2) not less than 0.995. The results were given in Table 8. The standard curve was evaluated by repeated five times using different concentrations in the range from 1 to 50 ng/mL with a determination coefficient (r) of 0.9991 (Figure 4). The relative standard deviation (%RSD) was determined for each concentration.

Table 8. Results for linearity

No	*Concentrations range (ng/mL)*	*Correlation coefficient (r^2)*	*Result*
1	*1 - 1000*	*0.9143*	
2	*1 - 500*	0.9991	*Linearity range:* Area = x + 19521

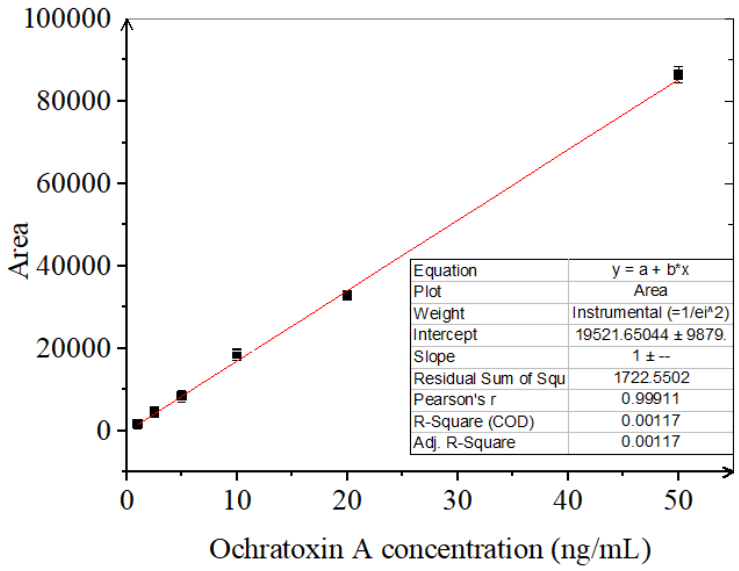


Figure 4. *OTA Standard curve.*

Limit of detection and Limit of quantification

The LoQ was defined as the lowest concentration with RSD ≤ 2%. The limit of detection was determined based on the standard deviation of 10 replicate analyses of a blank solution which was spiked at a concentration of 5 ng/mL. The LoD and the LoQ were 0.63 ng/mL and 2.11 ng/mL, respectively. The results were given in Table 9.

Table 9. Results for LOD, LOQ

Parameters	The results
The average of concentration (n=10) (ng/mL)	4.28
The standard deviation (SD)	0.21
LOD (ng/mL)	0.63
LOQ (ng/mL)	2.11
F value	6.65

The results in Table 9 show that the F value meets the requirements ($4 \leq F \leq 10$) [32]. The blank sample addition at 5 ng/mL was compatible with the requirements and LOD, and LOQ are reliable.

Results of accuracy (precision and trueness)

According to the regulations of the European Council for safety criteria, adding a standard to the blank sample is at three concentration levels which are 0.5, 1.0, and 2 times the maximum residue limit (MRL). In this study, the determination of OTA concentration in the blank solution which was spiked at a concentration of 2.5; 5.0, and 10 ng/mL. The results were given in Table 10.

Table 10. Results for precision (%RSD) and trueness (H%)

Parameters	Amount added (ng/mL)		
	2.5	5.0	10.0
The average amount found (n=6) (ng/ml)	2.36	4.33	8.49
The standard deviation (SD)	0.12	0.24	0.31
The relative standard deviation (%RSD)	5.03	5.64	3.70
The percent recovery (H%)	94.54	86.57	84.86

According to the EC, at concentrations in the range from 10 – to 100 µg/kg, the %RSD is less than 20%; The percent recovery (H%) of the concentration in the range from 1 to 10 µg/kg is between 70 and 110% [8]. This indicates that the method was accurate and the values obtained were given in Table 10.

Results of repeatability (RSD_r), reproductibility (RSD_R)

In this study, the determination of OTA concentration in the blank solution which was spiked at a concentration of 2.5; 5.0, and 10 ng/mL. Each concentration level was analyzed by three testers in six replicates.

Table 11. Results of repeatability (r), reproductibility (R)

No	2.5 ng/ml			5.0 ng/ml			10 ng/ml		
	1st tester	2nd tester	3rd tester	1st tester	2nd tester	3rd tester	1st tester	2nd tester	3rd tester
1	2.27	2.34	2.49	4.23	4.34	4.05	8.04	8.35	8.53
2	2.46	2.24	2.38	4.30	4.48	4.16	8.46	8.73	8.97
3	2.39	2.22	2.30	4.37	4.48	4.25	8.13	8.30	8.52
4	2.29	2.17	2.56	4.10	4.17	3.93	8.69	8.97	9.21
6	2.28	2.15	2.51	4.58	4.72	4.47	8.13	8.39	8.68
Concentration (ng/mL)	2.37	2.25	2.44	4.33	4.43	4.19	8.21	8.48	8.73
The percent recovery (H%)	94.63	90.03	97.68	86.56	88.61	83.78	82.10	84.76	87.28
The standard deviation (SD_r)	0.094	0.096	0.097	0.16	0.18	0.19	0.32	0.31	0.30
The relative standard deviation (%RSD_r)	3.99	4.29	3.97	3.70	4.12	4.43	3.88	3.71	3.46
The standard deviation (SD_R)	0.09			0.12			0.26		
The relative standard deviation (%RSD_R)	4.09			2.81			3.06		

According to the AOAC, at 10 ng/mL, the %RSD_R is less than 21%; in this study, the %RSD_r and %RSD_R met the requirements [32]. This indicates that the method was repeatability, reproductibility and the values obtained were given in Table 11.

Content of OTA in a coffee sample from the local market in Buon Ma Thuot city

In this research, the LC-MS/MS method has been developed and validated for determination of OTA residues level in coffee powder in Buon Ma Thuoc City. The situation of Ochratoxin A residues in coffee powder in Buon Ma Thuoc City was conducted assessment with a total sample of 55 samples. The samples were random taken from local markets. The samples were homogenized, then weighed and analyzed. Each sample conducted 3 repetitions, taking the average result and calculating the standard deviation. OTA were found in 9 samples, but its were below the safe limit value set by the Ministry of Health of Vietnam. The obtained results confirm that the quality of coffee powder base on the OTA residues in within the prescribed safe limits.

Table 12. Results of HPLC-MS/MS measurements of OTA in coffee samples from local market in Buon Ma Thuot city (n=6)

No	Sample	m (g)	Observed (ng/g)	The ML value of OTA (ng/g of coffee)*
1	Sample -01	12.5967	2.24	5
3	Sample -03	12.5535	0.92	
6	Sample -06	12.5065	0.88	
9	Sample -09	12.5934	2.45	
10	Sample -10	12.5006	1.26	
13	Sample -13	12.5907	0.80	
19	Sample -19	12.5042	0.74	
20	Sample -20	12.5135	0.72	
37	Sample -04	12.5645	0.68	

** The safe limit value set by the Ministry of Health of Vietnam [26]*

OTA infection is still present in some coffee powder samples despite warnings about the potential for contamination and high toxicity of this toxin. Our investigation indicated that in Table 12 showed that 9 samples were contaminated with OTA out of a total of 55 samples (16.36%). Ochratoxin A contaminated samples are all within the allowable limits of the Ministry of Health Vietnam. Consequently, there is no risk in consuming the coffee from the local market site of Buon Ma Thuoc City [26]. The findings are consistent with previous publication by Kelecha et al. (2023), who analyzed 20 samples of coffee beans and brewed coffee in Ethiopia using the high-performance liquid chromatography–fluorescent detector method [33]. Their study revealed the presence of OTA toxin, albeit within permissible limits. Furthermore, the investigation conducted by Studer-Rohr et al. (1995) identified OTA toxin in 13 out of 25 commercial green coffee bean samples [34]. Conversely, Ventura et al. (2004) did not detect OTA in 20 coffee samples from various countries and different manufacturers. They utilized Solid-Phase Cleanup and Narrow-Bore Liquid Chromatography–Fluorescence Detector–Mass Spectrometry methods for their analysis [35].

Conclussion

The results presented in this research have introduced modern analytical methods with high reliability in determining OTA residues in coffee powder and achieved some significant results as follows:

The method of determination of OTA residues in coffee powder using the LC/MS/MS technique was developed and validated. This is the new point of the topic because we have developed a method of analyzing liquid chromatography coupled mass spectrometry in a complex sample is coffee powder. At this time, there are no Vietnam Standard in Vietnam about OTA determination in coffee powder by LC/MS/MS technique has been published.

The analysis process is relatively not too complicated, but meets the requirements of European regulations (according to decision 2002/657/EC). The results indicate that the recovery efficiency of the method is over 70%, the repeatability is less than 15%.

The detection of limit (LOD) of ochratoxin A is 0.63 ng/mL, the quantitative of limit (LOQ) is 2.11 ng/mL. The LOD value (LOD ≤ 1/5 of the maximum residue limit) is consistent with the maximum permissible the maximum residue limit value according to both European and Codex standards.

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