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Research Article

MULTIPLE MYCOTOXIN ANALYSIS IN ARECA SEED OIL USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (UPLC-MS/MS)

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ABSTRACT

Analysis of mycotoxins in Areca seed oil is constrained by matrix complexities, such as fats and polar compounds, which affect extraction efficiency and accuracy. This research was performed to propose a method for extraction and analysis of mycotoxins in Areca seed oil using UPLC-MS/MS. Samples were three varieties of *Areca cathecu* seeds with solid phase extraction (SPE) using Carbon/Primary Secondary Amine (carb/PSA), followed by analysis using UPLC-MS/MS. The proposed method went through accuracy validation. The results of UPLC-MS/MS analysis with Carb/PSA extraction were proven optimal for detecting 24 mycotoxins with a high recovery rate (95-102%). The validation results fulfilled the requirements with a test range of 0.5-250 ng/g and a LOQ of 0.5 ng/g. Linearity R^2>0.99 and % RSD <RSD_{max} for intra- and inter-day precision. Accuracy is assessed at LOQ (-50% to 20%), 10LOQ (-30% to 10%), and 100LOQ (-20% to 10%). There was no significant difference ($p<0.05$) for method precision with LC-ID-MS/MS and certified reference materials (CRM). AFB1 dominates in the three varieties of *Areca catechu* in Indonesia with a range of 2.56 - 3.24 ng/g and other potential dangers from mycotoxins<LOQ such as ZEN, DON, T2, HT2, and OTA.

1.Introduction

Mycotoxins are toxic compounds produced by fungi such as Aspergillus, Fusarium, and Penicillium, commonly found in agricultural products, including Areca catechu (Liu et al., 2016). However, information on mycotoxin contamination in Areca, particularly in Indonesia, remains limited. Mycotoxins can lead to serious health issues, including cancer, liver disorders, and immune system suppression (Omotayo et al., 2019), although some mycotoxins have potential applications in drug development (Awuchi et al., 2022). More research is needed to assess mycotoxin contamination in commercial Areca catechu to enhance consumer safety and improve production standards.

Analyzing mycotoxins in Areca seed oil presents several challenges that impact the efficiency and accuracy of the extraction process (Zhang et al., 2018). The complex sample matrix, which includes fat content and polar compounds, can cause mycotoxins to bind strongly to these components (Zhao et al., 2021). Variability in the chemical composition of different Areca varieties also complicates the development of universal extraction methods (Shenoy Heckadka et al., 2022). To enhance method reliability, it is crucial to address matrix interference and other factors through advancements in extraction and analysis techniques.

Current methods for analyzing mycotoxins in Areca catechu exhibit varying technical approaches. Liu et al. (2016) utilized UFLC-ESI-MS/MS with a one-step methanol/water extraction to identify 11 mycotoxins, achieving detection limits between 0.1 - 20 µg/kg. Liang et al. (2022) proposed a centrifugation-forwarded SPE method for detecting 22 mycotoxins, with detection limits ranging from 0.04 - 1.5 µg/kg. Lin et al. (2021) detected 9 mycotoxins with detection limits of 5 - 20 µg/kg. Although Deng et al. (2018) reported only small amounts of analytes (4-Nonylphenol, Bisphenol A, and AFB1) in peanut oil using SPE with carb/PSA, this method remains relevant for multi-mycotoxin analysis in Areca catechu oil.

NH_2, Carb, and PSA sorbents were used individually or in combinationfor improved results. NH_2 is effective for binding polar compounds such as mycotoxins (Chi et al., 2021), while Carb absorbs organic compounds, and PSA helps remove contaminants like free fatty acids and pigments (Munjanja et al., 2023). Based on these principles, a new approach was developed by combining acetonitrile (ACN), hydrogen peroxide (H_2O_2), and formic acid (FA) in a ratio of 84/15.8/0.2 (v/v/v) to enhance mycotoxin extraction from the Areca catechu seed oil matrix.

Recent research highlight the mycotoxin contamination in Indonesian Areca catechu. Asghar et al. (2020) reported that all analyzed Areca catechu samples from Indonesia were contaminated with aflatoxins, with contamination levels ranging from 1.88 to 378.94 µg/kg and an average of 123.76 µg/kg, many exceeding EU and USA maximum limits. Additionally, Asghar et al. (2014) identified AFB1 contamination in Areca catechu fruit imported from Indonesia to Pakistan, ranging from 3.3 to 39.2 µg/kg. These findings underscore the need for rigorous monitoring of Areca with rapid and accurate analysis methods.

This research aims to propose a novel multi-mycotoxin analysis method for Areca catechu seed oil using a combination of UPLC-MS/MS and optimized SPE. The study focuses on three unharvested varieties of Areca catechu in Indonesia to detect early-stage mycotoxin contamination. By utilizing NH2, Carb, and PSA sorbents, this method effectively addresses the challenges of the complex oil matrix, offering greater precision. The validation on local varieties, which have not been extensively studied, adds a new dimension to the development of analytical techniques and food safety standards related to mycotoxins.

2. Materials and methods

2.1. Materials

The seeds of *Areca catechu* var. Betara (BET), *Areca catechu* var. Bulawan (BUL), and *Areca catechu* var. Irian (IRI) were purchased

online from farmers in the form of whole *Areca* fruit (unharvested).

Figure 1. Varieties of *Areca catechu* in Indonesia. BET/ *Areca catechu* var. Betara (a), BUL / *Areca catechu* var. Bulawan (b), IRI/ *Areca catechu* var. Irian (c), sliced *Areca catechu* (d), *Areca catechu* seeds

2.2. Methods

2.2.1. Preparation and Procedure

AFB1, FB2, enniatin B (ENNB), enniatin A (ENNA), enniatin B1 (ENNB1), AOH,15ADON, TeA, 3ADON, DON, and alternariol mono methyl ether (AME) standards were acquired from Merck KGaA, Darmstadt, Germany. Sigma-Aldrich (Jakarta, Indonesia) provided the following: zearalanone, β-zearalenol, and α-zearalanol. Biopure (Tulln, Austria) provided the de-epoxy-deoxynivalenol (DOM-1) and internal standards (IS). The United States Department of Agriculture (USDA) synthesized T-2 toxin-3-glucoside (T2-G). Asam et al. (2011) approach was used to synthesize the internal standard $^{13}C_6{}^{15}N$-TeA, whereas Hu & Rychlik (2012) method was used to synthesise $^{15}N_3$-ENN B.

We bought our methanol, conventional hexane, dichloromethane (UPLC grade), and acetonitrile from Merck Chemicals Co., Ltd. (Darmstadt, Germany). Throughout the entire experiment, ultrapure water (20.0 MΩcm) was utilized. The adsorption cartridge of choice was the 510 mg/10 mL Supelclean Co., USA, PSA (N-propyl ethylenediamine)/carb solid-phase extraction. Typically, 5 mL of methanol–dichloromethane (1.5/8.5, v/v) and an equivalent volume of CH_3OH are used to activate the SPE cartridge. Then, 5 mL of regular hexane is used to stabilize the cartridge.

2.2.2. Instruments

Sample analysis in this research was performed using the Xevo TQD Triple Quadrupole Mass Spectrometry (UPLC-MS/MS, Waters Co., USA). Samples were centrifuged in a CFG-18.5BP high-speed centrifuge (Infitek, China) and mixed using digital vortex mixer (Thermo Fisher Scientific Inc, USA). The extracted liquid was then concentrated with a Turbo Vap LV concentrator (SpectraLab Scientific Inc., Canada). Ultrapure

water for the analysis was provided by the Milli-Q Gradient A10 system from Millipore Corporation (Merck KGaA, Darmstadt, Germany).

2.2.3. Standard Solution

Standard stock solutions (SS) were prepared in acetonitrile (ACN) at a concentration of 150 µg/mL for FB1, ZEN, ENNs, AZAL, AME, BZAL, AOH, AZEL, AFM1, BZEL, HT2, ZAN, T2-G, T2, and DON. 150 µg/mL methanol solution was used to prepare the TeA standard stock solution, while for OTA, a stock solution was prepared in ACN at 20 µg/mL. The purchased standards included DOM-1 (100 µg/mL in ACN), 3-ADON (150 µg/mL in ACN), and 15-ADON (150 µg/mL in ACN).

DOM-1, the stock solution was prepared at 10 µg/mL in ACN. Solutions for $^{13}C_{15}$-DON (30 µg/mL in ACN), $^{13}C_{17}$-AFB1 (1 µg/mL in ACN), $^{13}C_{34}$-FB1 (10 µg/mL in ACN/H_2O), $^{13}C_{20}$-OTA (25 µg/mL in ACN), $^{13}C_{18}$-ZEN (10 µg/mL in ACN), and $^{13}C_{24}$-T2 (50 µg/mL in ACN) were also obtained as solutions. Additionally, synthesized internal standards $^{13}C_{615}N$-TeA and $^{15}N_3$-ENN B were prepared as stock solutions with concentrations of 100 µg/mL in methanol and 50 µg/mL in ACN, respectively, and stored at -25°C.

To create a workable solution that combines all analytical standards (WSmix, internal standards excepted), 5 µL of stock solutions of DOM-1 (10 µg/mL) and OTA (5 µg/mL) were transferred. This solution was diluted with ACN to a final volume of 0.5 mL, resulting in a working solution with concentrations of 0.5 µg/g. WSmix was further diluted in serial dilutions to achieve concentrations of 5 ng/g and 50 ng/g. Individual working solutions of internal standards (ISs) were prepared at 0.5 µg/mL, except for $^{15}N_3$-ENN B (10 ng/mL) and $^{13}C_{17}$-AFB1 (10 ng/mL). A mixed working solution of all ISs (WSmix_IS) was prepared with a final concentration of 10 ng/mL for each component. All functional solutions were stored at -25°C.

2.2.4. Sample Preparation

1.00 g of Areca seeds, weighed with an accuracy of 0.015 g, was placed into a 20 mL glass centrifuge tube. Following the addition of 20 µL of a mixed isotope internal standard solution, the samples were vortexed for 1 minute.

Oil Extraction Step: Next, 2 mL of hexane was added, and the mixture was vortexed for 40 seconds. Subsequently, 2.5 mL of acetonitrile (ACN) was added, and the solution was mixed again for 2 minutes. The sample was then centrifuged at 3500 rpm for 5 minutes. After centrifugation, 10 µL of a solution containing NaCl, NH_4Cl, CH_3COONa, and NaCOOH was added to the supernatant containing hexane and the oil phase. This supernatant was transferred to a solid-phase extraction (SPE) column packed with Carb/PSA.

The solution extracted with ACN was retained in a glass centrifuge tube for reserve, while the sample solution extracted from the SPE Carb/PSA cartridge was collected. To remove triglycerides, 8 mL of hexane was used to elute the column, followed by the addition of 5 mL of methanol-dichloromethane (1.5/8.5, v/v) to extract the target mycotoxins. The resulting eluent was combined with the ACN extraction solution and almost completely evaporated in a water bath at 55°C under a nitrogen stream. After adding 2.5 mL of methanol and vortexing, the solution was filtered through a 0.22 µm PTFE filter membrane and analyzed using UPLC-MS/MS. The matrix effect was evaluated by preparing a blank matrix from the sample and adding mycotoxins to create a calibrant according to the matrix (Zhou et al., 2017). Matrix effect (ME) was assessed using the UPLC-MS/MS method with Equation (1):

$$\mathrm{ME} = \frac{\mathrm{AM}}{\mathrm{AS}} \quad (1)$$

AM: mycotoxin peak area in the calibrant according to the matrix; AS: mycotoxin peak areas in standard calibrants

2.2.5. UPLC-MS/MS Operation

For chromatographic separation, an Agilent ZORBAX Eclipse Plus C18 column (50 mm x 2.1 mm, 3.5 µm) was utilized. The column

temperature was maintained at 35 °C, and the flow rate was set at 0.5 mL/min. The mobile phase consisted of methanol (B) and ultrapure water (A). A gradient elution method was employed with the following profile: starting from 50% B and reaching 80% B over 0.0–1.9 min, increasing to 100% B from 1.9–2.2 min, holding at 100% B from 2.2–4.2 min, decreasing to 50% B from 4.2–4.5 min, and finally holding at 50% B from 4.5–6.5 min. The injection volume was 5 µL.

Detection was performed using multi-reaction monitoring (MRM) on a triple quadrupole mass spectrometer equipped with a jet stream ESI. Quantification was conducted using ESI+ mode from 0–1.5 min and ESI− mode from 1.5–4.5 min. The capillary voltage was set at 1.5 kV. Optimal temperatures were maintained for the ion source (155 °C) and desolvation (410 °C). Gas flows included desolvation gas (nitrogen) at 810 L/h, cone gas (nitrogen) at 51 L/h, and collision gas (pure argon) at 0.20 mL/min.

2.2.6. Mycotoxin levels in areca seed oil

This research employed a five-point external calibration curve method to quantify mycotoxins. A calibration curve was constructed with mycotoxin concentration on the x-axis and peak area on the y-axis, following the equation y=ax+by. The concentration of mycotoxin (M) in Areca seed oil samples was determined using the following equation:

$$M = \frac{Ax-ba}{AS}\ R. \tag{2}$$

M: mycotoxin concentration in Areca oil samples (ng/g); a: slope of the calibration curve; Ax: peak area of mycotoxins in the sample; b:calibration curve intersection point; AS: Standard peak area; R:dilution factor

2.2.7. Validation Methods

In accordance with previously published procedures by De Baere et al. (2011), the LC-MS/MS method was validated using blank Areca seed oil samples spiked with standard mycotoxins. Validation followed the guidelines and recommendations established by Codex Alimentarius (Alimentarius, 2013), including limits of quantification (LOQ), precision and accuracy (both intra-day and inter-day), linearity, extraction recovery, and matrix effects. Additionally, LC-ID-MS/MS analysis and certified reference materials (CRM) were employed to validate the results of the proposed method.

Linearity: Three matrix-specific calibration curves covering a concentration range of 0.5–250 ng/g were prepared to evaluate linearity. Six concentrations were analyzed: 0, 0.5, 5, 50, 100, and 250 ng/g. The correlation coefficient (r) and goodness-of-fit (GoF) were calculated, with acceptance criteria set at approximately 0.99 for r and 20% for GoF.

Precision and Accuracy: To assess intraday precision and accuracy, six blank samples were spiked with mycotoxins at low (LOQ), medium (5 ng/mL), and high (50 ng/mL) concentrations. Interday precision and accuracy were evaluated using three quality control samples at each concentration level tested on three different days. The acceptance criteria for within-day and inter-day accuracy were: for concentrations ≤0.5 ng/mL, 0.5–5 ng/mL, and ≥5 ng/mL, accuracy should be within -50% to +20%, -30% to +10%, and -20% to +10%, respectively. For concentrations ≥0.5 to <5 ng/mL and ≥5 to <50 ng/mL, the relative standard deviation (RSD%) must be less than the maximum RSD (RSDmax), which is determined using the Horwitz equation (Linsinger & Josephs, 2006). The RSD_{max} values for concentrations of 50 ng/mL, 5 ng/mL, and 0.5 ng/mL were 22.68%, 32.04%, and 45.57%, respectively.

$$RSD = 2^{(1-0.5\ Log\ Concentration\left(\frac{ng}{g}\right))} \tag{3}$$

The limit of quantification (LOQ) was determined following the procedure adapted from De Baere et al. (2011). The LOQ was defined as the lowest concentration of the analyte at which the analytical method met the specified standards for accuracy and precision. This process involved preparing three matrix-

specific calibration curves within the range of 0.5–250 ng/g, where various concentrations of standard mycotoxins were added to blank Areca seed oil samples. Each concentration was analyzed six times on the same day to ensure consistency, with accuracy and precision criteria set based on the corresponding RSD values.

To address measurement uncertainty, we applied a procedure that assessed total uncertainty from various sources, including instrument accuracy and measurement variability. Quality control analyses were performed by testing three control samples at each concentration level to evaluate the variability of results. Additionally, certified reference materials (CRM) were used to compare results and reinforce the validation of the method, providing clarity regarding the uncertainty in the analytical outcomes.

3. Results and discussions

The ESI+ and ESI− acquisition modes enhance the detection of mycotoxins with varying polarities, thereby broadening the range of target compounds that can be identified in a single measurement.

MS/MS analysis revealed different results for mycotoxins in Areca seed oil samples. In ESI− mode (Table 1), the detected mycotoxins included ZEN, ZAN, BZEL, BZAL, AZEL, AZAL, TeA, AOH, and AME. Additionally, the labeled isotopes [$^{13}C_{18}$]-zearalenone and [$^{13}C_6$, ^{15}N]-tenuazonic acid were also identified in the samples

Table 1. An overview of the ESI- mode measurements of the MS/MS parameters for *Areca* mycotoxins.

Name	adduction	Precursor ion	Quant. ion (m/z)	Quali. Ion (m/z)	Voltage (V)	CE (eV) (x-y)	Retention Time (min)
[$^{13}C_6{}^{15}N$]-tenuazonic acid	$[M-H]^-$	211.9	112.09	139.4	46	23-28	6.20
[$^{13}C18$]-zearalenone	$[M-H]^-$	341.3	191.9	171.1	23	24-41	8.59
AME	$[M-H]^-$	269.1	261.0	232.0	41	22-33	8.39
AOH	$[M-H]^-$	261.8	221.1	190.2	32	21-23	6.70
TeA	$[M-H]^-$	189.1	109.3	141.0	50	25-21	6.21
AZAL	$[M-H]^-$	319.2	281.3	311.3	32	29-21	7.51
AZEL	$[M-H]^-$	321.2	281.8	312.0	24	29-33	7.58
BZAL	$[M-H]^-$	315.2	273.9	314.3	42	22-32	6.91
BZEL	$[M-H]^-$	321.9	269.2	311.0	37	23-28	6.87
ZAN	$[M-H]^-$	321.8	272.1	212.0	43	21-27	8.52
ZEN	$[M-H]^-$	309.9	169.9	129.8	42	21-33	8.54

Note: **Quant**.: Quantifier Ion; **Quali**.: Qualifier Ion, *m*/*z*=mass-to-charge ratio; **CE**: Collision Energy (x-y): collision energy for the quantifier(x) and qualifier ion (y), respectively.

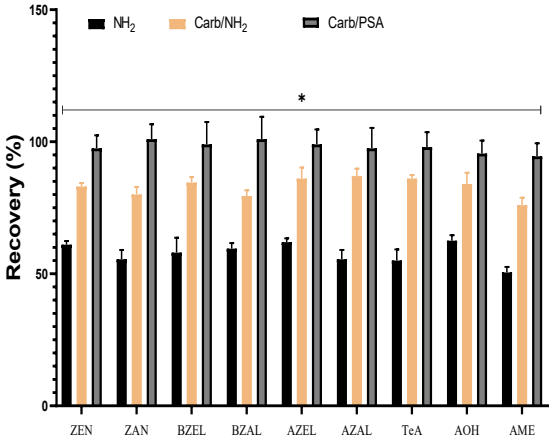
In ESI+ mode (Table 2), the identified compounds include DON, T2, HT2, T2-G, AFB1, AFM1, OTA, ENN A1, ENN A, ENN B, ENN B1, BEA, and FB2, as well as the labeled isotopes [$^{13}C_{15}$]-Deoxynivalenol, [$^{13}C_{17}$]-Aflatoxin B1, [$^{13}C_{20}$]-Ochratoxin A, [$^{13}C_{24}$]-T2-toxin, [$^{13}C_{34}$]-Fumonisin B1, and [$^{15}N_3$]-Enniatin B. These results indicate the presence of various mycotoxins in Areca seed oil samples, each detected under ESI− and ESI+ conditions based on the specific parameters of the compound standards.

Table 2. An overview of the ESI+ mode measurements of the MS/MS parameters for *Areca* mycotoxins.

Name	adduction	Precursor ion	Quant. ion (m/z)	Quali. Ion (m/z)	Voltage (V)	CE (eV) (x-y)	Retention Time (min)
[^{15}N3]-Enniatin-B	$[M+H]^+$	639.21	209.19	221.35	73	21-15	12.69
[^{13}C34]-Fumonisin-B1	$[M+H]^+$	749.46	361.27	369.43	26	42-36	9.67
[^{13}C24]-T2-toxin	$[M+NH_4]^+$	510.91	232.14	210.52	26	23-21	8.44
[^{13}C20]-Ochratoxin-A	$[M+H]^+$	419.91	249.08	381.51	31	30-14	9.43
[13C17]-Aflatoxin-B1	$[M+H]^+$	328.04	261.15	311.09	32	32-24	5.83
[^{13}C15]-Deoxynivalenol	$[M+H]^+$	309.93	259.08	251.08	31	24-18	4.37
FB2	$[M+H]^+$	711.14	323.24	328.42	59	34-41	10.27
AFG2	$[M+H]^+$	329.94	328.15	339.31	79	27-31	5.19
ENNB1	$[M+NH_4]^+$	669.91	205.24	222.43	29	29-27	12.68
ENNB	$[M+H]^+$	638.25	275.83	532.42	79	22-24	12.69
ENNA	$[M+Na]^+$	711.14	348.14	229.32	39	43-41	13.19
ENNA1	$[M+H]^+$	671.82	206.15	231.89	79	25-21	12.43
OTA	$[M+H]^+$	410.92	242.93	218.98	41	24-31	9.46
AFM1	$[M+H[^+$	331.92	269.93	231.09	28	22-357	5.32
AFB1	$[M+H]^+$	309.94	334.18	334.31	28	23-31	5.71
T2-G	$[M+NH_4]^+$	651.23	251.08	221.23	41	23-27	7.19
HT2	$[M+NH_4]^+$	439.12	259.04	239.21	32	21-34	7.09
T2	$[M+NH_4]^+$	493.91	209.26	301.98	31	16-18	8.32
AFG1	$[M+H]^+$	342.92	221.14	228.99	26	24-16	5.09
DOM-1	$[M+H]^+$	279.83	209.04	233.40	31	23-21	4.81
DON	$[M+H]^+$	306.92	252.16	203.44	32	26-29	4.41

Note: **Quant**.: Quantifier Ion; **Quali**.: Qualifier Ion**,** *m*/*z*=mass-to-charge ratio; **CE**: CollisionEnergy (x-y): collision energy for the quantifier(x) and qualifier ion (y), respectively.

This paper presents the recovery capabilities of the extraction process by comparing mycotoxin recovery using three methods: NH_2, Carb/NH_2, and Carb/PSA. The results demonstrate the effectiveness of each method, highlighting variations in efficiency between ESI− and ESI+ modes.



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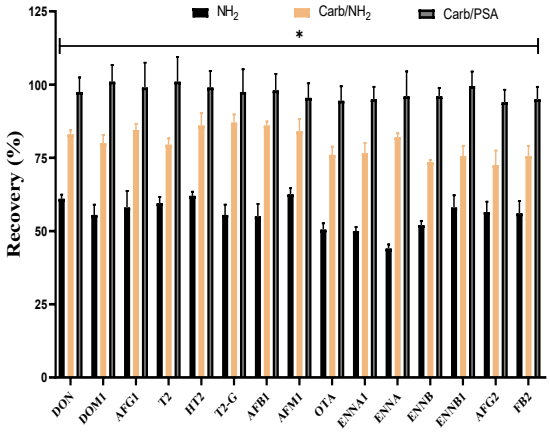

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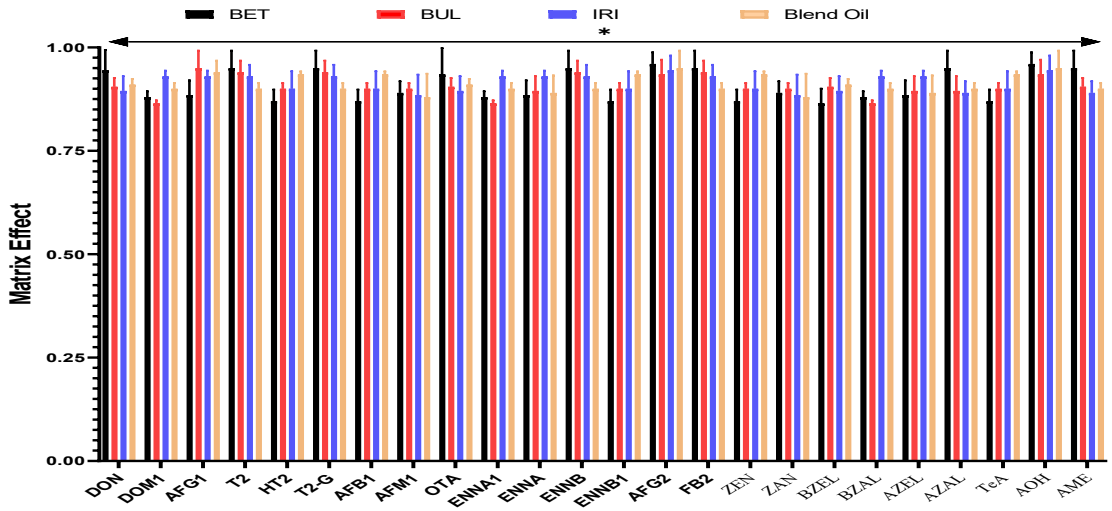
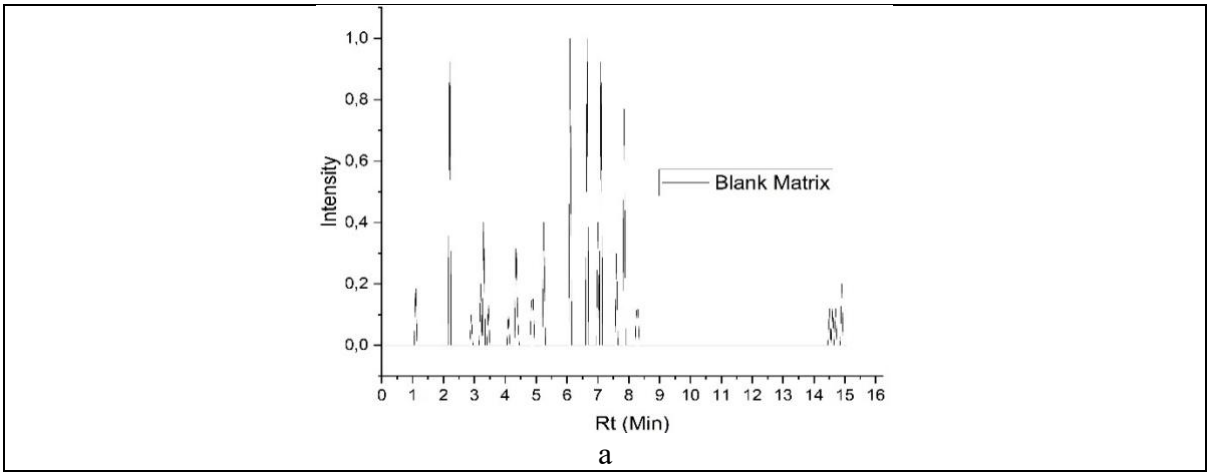


c

Figure 2. Comparison of the extraction process on target analyte recovery and matrix effects on the samples tested. Recovery of compounds in ESI- mode (a), ESI+ mode (b), and matrix effects on areca nut oil samples (c). *Analysis was carried out with a significance level of $p<0.05$

In ESI− mode (Fig. 2a), the extraction method significantly impacted mycotoxin recovery results ($p < 0.05$). The NH_2 extraction method resulted in approximately 60% recovery of mycotoxins with negative ions. The Carb/PSA extraction method exhibited the highest effectiveness, achieving recoveries between 95% and 102%, while the Carb/NH2 method provided recoveries ranging from 80% to 90%. A similar trend was observed for positively charged mycotoxins in ESI+ mode (Fig. 2b). The NH_2 extraction method showed lower recovery rates, between 45% and 60%, particularly for ENNA type mycotoxins. In contrast, the Carb/NH_2 extraction method improved recovery to 75%–82%. The highest recovery, ranging from 95% to 102.25%, was achieved with the Carb/PSA extraction method. The chromatogram depicted is from a test method using a blank matrix spiked with standard mycotoxins to ensure accurate detection. Fig. 3a presents the chromatogram of the blank matrix, which serves as a control for comparing detection results.



a

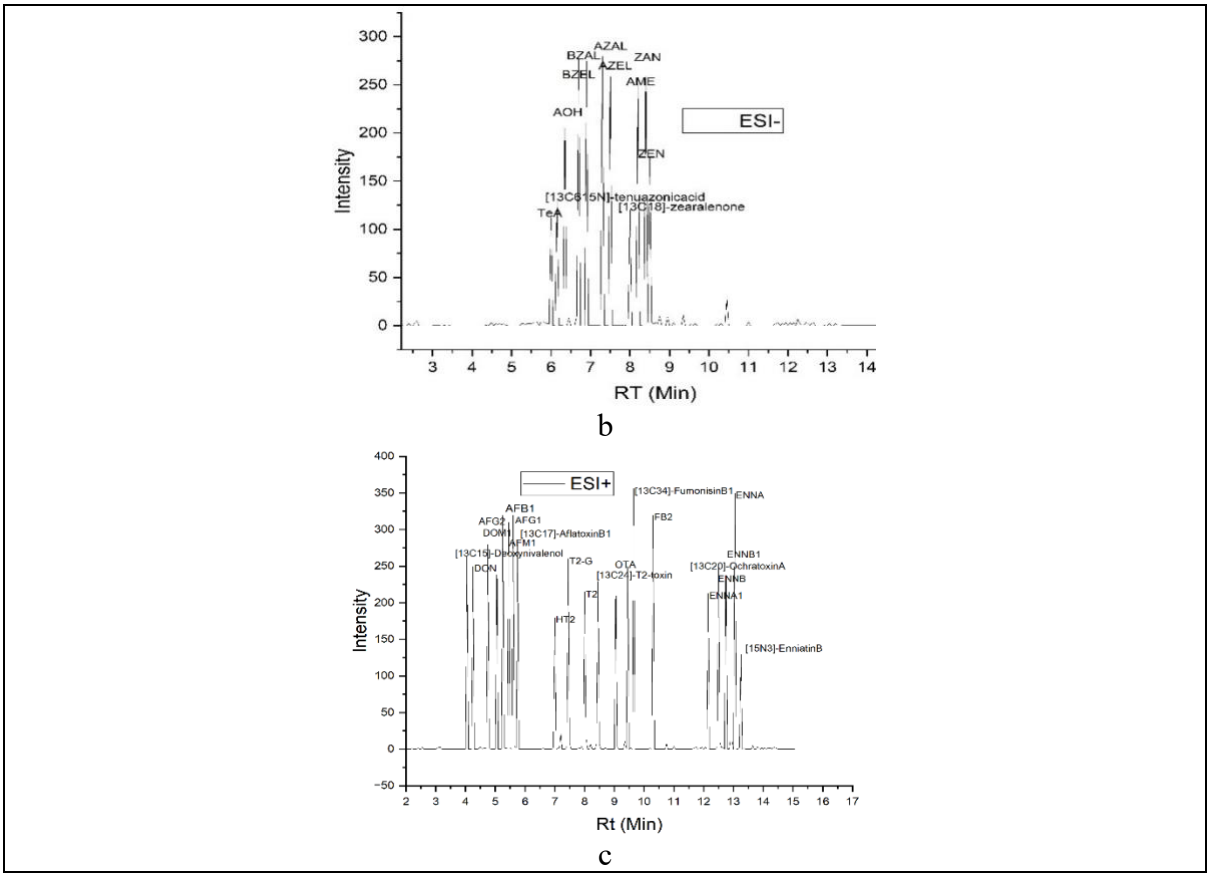


Figure 3. Chromatograms of blank matrix and mycotoxin standards in ESI- and ESI+ modes. Blank matrix (a), ESI- (b), and ESI+ (c).

Although the chromatogram of the blank sample showed several peaks with very low abundance, the presence of these peaks could be explained by background signal fluctuations that are common in mass spectrometry analysis, which could have been caused by environmental contamination or interactions with the complex matrix. These minor peaks were far below the detection limits for mycotoxins and were not identified as target analytes. Therefore, despite the presence of small peaks, the blank chromatogram served as an important control, ensuring that the observed mycotoxin detections originated from the tested samples and did not affect the accuracy of the analysis results.

Fig. 3b displays the chromatogram for ESI− mode, highlighting the detection of certain mycotoxins that are more effectively ionized in this mode. Meanwhile, Fig. 3c shows the chromatogram for ESI+ mode, illustrating the detection of mycotoxins that are ionized under these conditions.

The effectiveness of using Solid Phase Extraction (SPE) with carb/PSA has been demonstrated as optimal for detecting 24 mycotoxins, achieving high recovery rates. In comparison, extraction with carb/NH2 yielded moderate recoveries, while NH2 alone resulted in the lowest recovery performance. Liang et al. (2022) achieved similar results with SPE, detecting 22 mycotoxins using an ACN/H_2O_2/FA solvent (84/15.8/0.2, v/v/v), centrifugation, and a MycoSpin™ 400 column for UPLC-MS/MS analysis. Our research utilized a different approach, involving internal standards, hexane, and ACN for triglyceride separation, followed by an SPE (carb/PSA) column.

Matrix effects (MEs)

Matrix effects (MEs) significantly influence detection sensitivity and accuracy, making their assessment crucial in this research. Four types of

oil were evaluated: **BUL, IRI, BET,** and a blend of these three oils. The MEs for BET oils ranged from 0.89 to 0.99, for BUL oils from 0.87 to 1.02, for IRI oils from 0.88 to 1.02, and for blended oils from 0.92 to 0.98 (Fig. 2c). These results indicate that for the 24 mycotoxins tested across the various oil matrices, there was no significant ionization effect, whether increasing or decreasing.

The SPE method demonstrated a consistent ionization profile (Fig.1a), with no significant signal amplification or suppression from co-eluting matrix components in the LC pathway.

Matrix effects (MEs), which include interference from non-target components such as lipids, proteins, and pigments, can alter detection signals and cause quantification errors (Kunzelmann et al. 2018; Mao et al. 2018). To mitigate these effects, calibration standards were matched to the sample matrix and sorbents like NH_2, carb, and PSA to remove non-target components, enhancing accuracy and precision. Although carb/PSA has also been used by Deng et al. (2018) for analyzing Aflatoxin B1 and other compounds, our research reveals different results for the matrix effects in *Areca catechu* oil.

The Validation of the Proposed Method

Linearity: The analytical method's ability to produce results directly proportional to the BET analyte concentration in the range of 0.5-250 ng/g was evaluated. Linearity, which indicates the accuracy of the measured value, met the acceptance criteria for all methods, with a correlation coefficient (r) greater than 0.99 and a goodness-of-fit (GoF) coefficient of ≤20, and a limit of quantification (LOQ) of 0.5 ng/g (Table 3).

Table 3 Validation of linearity (linear range, correlation coefficient (cr), goodness-of-fit coefficient (GoF)) and limit of quantification (LOQ) for mycotoxins in *Areca catechu* seed oil.

Analyte	Linear Range (ng/mL)	Cr ± SD	BET		BUL		IRI	
			GoF(%)±SD	LOQ (ng/mL)	GoF(%)±SD	LOQ (ng/mL)	GoF(%)±SD	LOQ (ng/mL)
AFG2	0.5–150	0.9988±0.0001	13.16±2.15	0.5	14.12 ±2.04	0.5	9.32 ±4.28	0.5
ENN B1	0.5–150	0.9987±0.0012	14.19±2.43	0.5	12.71±4.31	0.5	12.82±7.03	0.5
ENN B	0.5–150	0.9989±0.0021	10,32±1.32	0.5	8.81±2.21	0.5	12.28±6.16	0.5
ENNA	0.5–60	0.9978±0.0032	12.19±3.22	0.5	10.02±3.25	0.5	7.43 ±3.05	0.5
ENNA1	0.5–250	0.9978±0.0011	8.09±0.19	0.5	11.71±2.22	0.5	11.32±2.41	0.5
OTA	0.5–250	0.9988±0.0012	9.36±2.19	0.5	10.51±4.22	0.5	11.21±5.12	0.5
AFM1	0.5–250	0.9972±0.0021	11.28±5.16	0.5	11.71±0.21	0.5	13.04±4.16	0.5
AFB1	0.5–250	0.9962±0.0021	13.37±2.28	0.5	9.92 ±3.21	0.5	11.82±3.12	0.5
T2-G	0.5–250	0.9971±0.0031	11.24±1.22	0.5	10.23±1.42	0.5	12.24±2.13	0.5
HT2	0.5–150	0.9984±0.0021	16.15±2.18	0.5	11.12±4.24	0.5	8.32 ±1.21	0.5
T2	0.5–250	0.9981±0.0011	10.29±1.26	0.5	9.81 ±3.11	0.5	7.32 ±5.18	0.5
AFG1	0.5–250	0.9982±0.0012	7.32±3.41	0.5	11.14±4.12	0.5	13.29±5.03	0.5
AFB2	0.5–250	0.9983±0.0031	11.39±2.15	0.5	1.16 ±0.13	0.5	10.71 ±3.27	0.5
DON	0.5–250	0.9981±0.0021	12.92±4.24	0.5	9.61±4.11	0.5	11.12±5.42	0.5
AME	0.5–250	0.9967±0.0012	12.29 ±5.18	0.5	5.12±5.13	0.5	13.04±1.29	0.5
AOH	0.5–250	0.9971±0.0021	13.92±5.12	0.5	9.51±4.16	0.5	9.13 ±4.32	0.5
TEA	0.5–250	0.9982±0.0012	12.08±4.19	0.5	9.91 ±4.12	0.5	7.35±7.18	0.5
ZAN	0.5–250	0.9972±0.0011	13.38±3.18	0.5	7.91 ±2.15	0.5	6.27 ±1.22	0.5
BZEL	0.5–250	0.9986±0.0021	14.91±3.72	0.5	5.42±2.21	0.5	7.22±1.39	0.5
BZAL	0.5–250	0.9977±0.0012	11.72±2.08	0.5	10.62±2.20	0.5	10.26 ±4.16	0.5
BZAL	0.5–250	0.9967±0.0012	16.72±2.08	0.5	12.29±3.24	0.5	3.15 ±2.17	0.5
AZAL	0.5–250	0.9972±0.0031	12.22±3.36	0.5	10.12±3.21	0.5	7.19 ±3.01	0.5
AZEL	0.5–250	0.9957±0.0021	11.05±1.22	0.5	10.23±1.14	0.5	12.22±5.13	0.5
ZEN	0.5–250	0.9974±0.0031	11.93±3.81	0.5	14.12 ±2.04	0.5	9.32 ±4.28	0.5

Linearity (n=3 Different Day); SD: standard deviation; *acceptance criteria*: cr ≥ 0.99 and GoF ≤ 20.

Similar results were observed for BUL and IRI samples, with GoF values ranging from 7.12 to 18.71 for BUL and 3.15 to 17.04 for IRI, both achieving cr> 0.99. Detailed results are provided in Tables 3. All three samples were analyzed with LOQ at 0.5 ng/g for T2-G and a linear range of 0.5-50 ng/g for ENNA, while other mycotoxins were analyzed within the range of 1-250 ng/g.

Precision: Precision was assessed by measuring *Areca catechu* oil both within-day and between-days. Within-day precision showed relative standard deviation (RSD%) values below the maximum RSD (RSD_{max}), with RSD_{max} set at <25% for concentrations ≥0.5 to <5 ng/mL and <15% for concentrations ≥5 to <50 ng/mL. Between-day precision also demonstrated RSD% values lower than RSD_{max}, with RSD_{max} values of 22.60% for 50 ng/g, 32% for 5 ng/mL, and 45% for 0.5 ng/mL, respectively. These results confirm the consistency and reliability of the BET, BUL and IRI analysis methods (Table 4).

Within-day precision for BET oil ranged from 7.92% to 21.91% (LOQ), 2.26% to 13.47% (10LOQ), and 1.32% to 9.23% (100LOQ). Intra-day precision, using samples in triplicate (3x3), ranged from 4.91% to 39.21% (LOQ), 5.22% to 17.08% (10LOQ), and 2.82% to 15.45% (100LOQ). Complete results for BUL and IRI can be seen in Table 4.

***Accuracy*:** The accuracy of *Areca catechu* seed oil analysis was assessed to ensure that results met the specified criteria. For concentrations ≤0.5 ng/mL, accuracy ranged from −50% to +20%; for 0.5–5 ng/mL, accuracy ranged from −30% to +10%; and for ≥5 ng/mL, accuracy ranged from −20% to +10%. These results demonstrate that the BET, BUL, and IRI seed oil analysis methods provide accurate and reliable mycotoxin detection.

Validation with LC-ID-MS/MS: The results of the proposed method were validated using a liquid chromatography-isotope embedding tandem mass spectrometry (LC-ID-MS/MS) approach. Table 5 shows that the measured results from both approaches were in close agreement. Independent sample t-tests confirmed that the t-values for each mycotoxin were below the critical value of $t_{0.05}$ (2.042), indicating that the precision of the proposed method aligns well with the LC-ID-MS/MS method.

CRM Analysis: The proposed method was also used to analyze mycotoxins in certified reference materials (CRM), with results showing good agreement with certified values, reflecting the high accuracy of the approach (Table 6).

Our validation process met the required standards. Testing across a range of 0.5-250 ng/g and with an LOQ of 0.5 ng/g yielded $R^2 > 0.99$. Precision assessments showed % RSD values within the acceptable range of RSD_{max}, and accuracy at LOQ (-50% to +20%), 10LOQ (-30% to +10%), and 100LOQ (-20% to +10%). Comparisons with LC-ID-MS/MS showed t-values below the critical value of $t_{0.05}$ (2.042) and certified reference materials (CRM) indicated no significant differences ($p < 0.05$). Despite varying LOQs reported in other studies 0.5 ng/g (Lin et al. 2021), 0.1 ng/g (Liang et al. 2022) and LOQ < 50 ng/g (Liu et al., 2016), our method with carb/PSA demonstrated superior matrix effects. Unlike previous studies, our method included additional recovery validation with instruments and CRM.

Analysis of mycotoxins in *Areca catechu* varieties using the proposed method.

The research included 10 samples from each variety of *Areca catechu*, sourced from different regions, except for *Areca catechu* var. Irian **(IRI),** which is only found in Irian Jaya (Papua). Three varieties of *Areca catechu* in Indonesia that had not been harvested were analyzed. AFB1 contamination was found to be predominant in all three varieties, with varying concentrations: BET (2.56 ± 0.34 ng/g), BUL (3.24 ± 0.23 ng/g), and IRI (2.97 ± 0.38 ng/g). AFG1 ranked second in contamination levels for BET, BUL, and IRI, with concentrations of 2.56 ± 0.34 ng/g, 2.98 ± 0.52 ng/g, and 1.98 ± 0.25 ng/g, respectively. Complete results for other mycotoxins are detailed in Table 7.

Table 4 Results of precision and accuracy tests conducted both within and between days for mycotoxins in the seed oil of *Areca catechu* seeds.

Analyte	Within-Day (*n*=6)						Between-Day (*n*=6)						sample
	LOQ (0.5 ng/g)		10LOQ (5 ng/g)		100LOQ (50 ng/g)		LOQ (0.5 ng/g)		10LOQ (5 ng/g)		100LOQ (50 ng/g)		
	a(RSD %)	b(%)	a(RSD %)	b(%)	a(RSD %)	b(%)	a(RSD %)	b(%)	a(RSD %)	b(%)	a(RSD %)	b(%)	
AFG2	12.12	-2.22	5.72	-10.43	9.23	-3.23	21.24	-11.22	5.22	-10.42	9.71	-4.22	
ENN B1	7.92	1.32	6.22	-0.83	8.23	-1.71	28.08	4.08	6.17	1.33	6.82	-2.91	
ENN B	14.33	9.02	5.52	2.53	1.32	-0.63	4.91	6.81	6.17	0.15	2.82	-2.46	
ENNA	5.32	-7.62	4.93	-0.84	5.08	1.03	22.42	-10.16	12.18	-5.33	9.53	-2.05	
ENNA1	12.42	-9.02	3.81	-5.63	6.72	-4.72	37.92	-3.61	9.09	-0.53	5.34	-5.04	
OTA	11.61	-8.14	6.01	3.91	7.06	1.13	28.54	-19.56	9.06	0.63	7.82	2.92	
AFM1	11.031	12.23	6.81	-4.07	4.16	-7.62	27.72	12.55	8.52	-3.06	8.81	-3.62	
AFB1	12.13	-1.52	11.06	-3.42	5.91	-1.18	19.37	-5.39	11.91	-7.31	6.32	1.62	
T2-G	11.42	-2.43	3.91	-9.28	8.42	4.62	39.21	-18.91	7.84	-8.52	8.32	4.06	
HT2	9.01	-5.72	5.62	-19.08	6.08	-18.91	7.71	-5.63	11.71	-12.41	12.71	-12.52	
T2	12.61	-6.03	9.72	-7.37	5.56	-2.81	10.54	-0.83	10.26	-7.06	7.32	-1.91	
AFG1	21.91	5.63	8.61	-13.62	7.91	-14.43	22.65	-0.85	7.52	-10.52	9.93	-8.61	BEL
AFB2	21.72	7.43	6.53	3.38	3.91	0.91	24.18	-0.52	6.25	2.82	4.13	3.15	
DON	8.73	3.13	7.08	1.07	7.28	-5.62	6.19	1.47	10.15	4.32	8.03	-2.04	
AME	12.22	-4.43	7.91	1.91	7.54	2.09	17.81	-0.15	8.61	-3.53	8.24	4.81	
AOH	13.61	-5.03	3.54	3.33	5.37	1.41	22.82	-9.91	5.61	1.63	4.14	1.81	
TEA	11.73	-2.65	2.26	-1.73	6.18	-4.07	29.91	6.08	1.93	-1.53	7.64	0.53	
ZAN	10.64	-4.84	4.46	-5.32	3.81	-3.82	18.82	-12.06	6.51	-4.13	5.33	-0.44	
BZEL	21.31	-1.74	6.62	9.91	7.08	6.54	25.38	0.56	11.42	3.22	10.92	-1.74	
BZAL	21.51	-11.63	13.47	-5.81	5.72	-3.91	29.71	-22.81	15.71	0.86	13.01	1.55	
BZAL	17.41	-6.22	6.63	6.08	4.37	2.38	21.73	4.56	7.31	5.44	15.45	-7.93	
AZAL	20.22	-5.11	5.73	-2.71	9.08	-8.62	30.81	-20.91	13.13	4.92	10.86	-6.11	
AZEL	21.83	-0.71	13.32	-7.91	8.91	1.08	25.61	15.81	17.08	1.41	12.41	-0.72	
AFG2	11.12	-4.22	4.72	-13.43	7.23	-11.23	13.24	-12.22	4.22	-5.42	8.71	-5.22	
ENN B1	6.92	4.32	5.22	-0.73	6.23	-2.71	23.08	6.08	8.17	3.33	9.82	-6.91	
ENN B	12.33	9.02	7.52	3.53	3.32	-0.73	7.91	7.81	9.17	6.15	12.82	-7.46	
ENNA	3.32	-13.62	3.93	-0.94	2.08	1.13	21.42	-11.16	11.18	-3.33	7.53	-6.05	
ENNA1	1.42	-11.02	4.81	-7.63	4.72	-54.72	27.92	-5.61	5.09	-1.53	9.34	-7.04	
OTA	18.61	-18.14	7.01	4.91	3.06	1.53	24.54	-13.56	6.06	3.63	4.82	6.92	
AFM1	18.031	12.23	7.81	-7.07	2.16	-9.62	23.72	11.55	7.52	-4.06	6.81	-8.62	
AFB1	9.13	-12.52	9.06	-4.42	7.91	-1.19	17.37	-6.39	13.91	-4.31	5.32	4.62	
T2-G	8.42	-21.43	4.91	-10.28	6.42	6.62	32.21	-17.91	9.84	-4.52	5.32	5.06	
HT2	17.01	-15.72	4.62	-13.08	7.08	-13.91	9.71	-7.63	10.71	-10.41	10.71	-10.52	BUL
T2	11.61	-12.03	6.72	-5.37	9.56	-5.81	11.54	-1.83	12.26	-4.06	8.32	-10.91	
AFG1	17.91	3.63	7.61	-9.62	4.91	-13.43	21.65	-1.85	5.52	-1.52	6.93	-5.61	
AFB2	17.72	6.43	3.53	4.38	3.91	10.91	21.18	-2.52	9.25	3.82	5.13	4.15	
DON	6.73	3.13	6.08	1.09	5.28	-7.62	9.19	4.47	14.15	7.32	6.03	-6.04	
AME	10.22	-5.43	9.91	3.91	6.54	3.09	13.81	-2.15	12.61	-5.53	4.24	7.81	
AOH	9.61	-6.03	5.54	5.33	6.37	4.41	21.82	-9.71	15.61	6.63	7.14	3.81	
TEA	9.73	-5.65	2.46	-3.73	5.18	-5.07	22.91	8.08	11.93	-10.53	8.64	4.53	
ZAN	13.64	-8.84	3.46	-2.32	8.81	-5.82	15.82	-14.06	16.51	-9.13	7.33	-2.44	
BZEL	8.31	-8.74	9.62	8.91	3.08	7.54	21.38	5.56	10.42	8.22	11.92	-1.74	
BZAL	11.71	-	11.47	-7.81	2.72	-4.91	22.71	-20.81	12.71	10.86	11.01	1.55	

BZAL	146.41	10.63 - 11.22	8.63	9.08	6.37	6.38	11.73	14.56	11.31	7.44	12.45	-7.93
AZAL	19.22	- 12.11	7.73	-6.71	7.08	-5.62	23.81	-2.91	15.13	8.92	11.86	-6.11
AZEL	17.83	- 11.71	11.32	-9.91	6.91	2.08	21.61	12.81	11.08	11.41	8.41	-0.72
AFG2	12.18	7.12	14.71	-4.54	6.08	-3.73	12.32	7.08	8.43	-7.22	7.91	-7.34
ENN B1	12.22	-11.3	12.92	-5.72	4.51	-7.74	19.63	-3.91	12.62	- 10.81	5.91	-8.18
ENN B	13.43	-14.3	15.52	-9.74	2.81	-6.47	26.02	-15.42	10.93	- 11.32	5.08	-9.41
ENNA	11.65	- 19.09	12.31	-9.51	5.62	-7.55	15.03	-10.28	11.82	- 11.12	10.43	- 11.28
ENNA1	11.19	17.81	13.12	-11.08	6.83	-9.56	12.93	1.18	10.81	-7.91	11.63	-4.81
OTA	11.28	- 12.16	7.21	-18.18	3.18	-6.37	11.13	-18.28	8.52	-8.42	9.72	-6.18
AFM1	21.34	-5.54	12.52	-11.7	7.06	-9.61	27.84	10.47	11.63	- 11.34	10.42	-8.32
AFB1	11.15	- 13.36	6.64	-1.29	11.63	7.73	28.23	10.27	11.13	-8.45	6.72	1.51
T2-G	7.26	- 19.37	7.55	7.72	7.91	8.82	16.73	-18.29	15.24	9.61	11.62	3.62
HT2	12.74	11.08	6.46	2.81	5.54	-10.08	16.64	18.91	7.08	-9.64	7.71	-6.52
T2	22.82	- 19.45	4.91	5.91	8.47	-8.71	25.76	-11.52	4.18	12.74	8.83	-7.71
AFG1	13.91	12.61	3.27	4.74	9.44	-17.18	22.61	11.73	11.82	- 12.91	11.22	-1.72
AFB2	13.57	12.28	4.72	-7.91	9.37	-12.08	16.72	-21.42	9.81	- 13.91	4.94	-7.08
DON	12.91	- 16.08	3.07	-11.62	9.08	-19.81	29.47	-13.42	11.81	- 11.54	11.15	- 10.25
AME	14.54	15.65	13.61	4.74	9.45	4.09	1.56	15.63	6.34	7.15	5.15	8.61
AOH	14.08	17.09	13.81	-2.28	5.38	-13.26	12.18	10.07	14.42	-6.92	14.78	-3.81
TEA	12.41	- 18.43	8.18	-19.14	6.36	-18.91	13.47	-18.43	11.21	- 11.45	11.09	-2.51
ZAN	14.26	- 17.28	13.82	-3.72	3.62	-15.08	13.81	-13.61	14.42	- 14.82	13.15	-2.72
BZEL	12.28	- 10.72	15.72	1.81	5.91	-8.42	14.28	-12.34	12.61	- 11.75	10.58	- 97.54
BZAL	10.46	- 12.18	8.72	4.08	6.09	- 121.37	24.27	-11.14	6.18	11.27	8.81	-4.18
BZAL	12.18	15.62	13.57	-7.09	3.41	-12.62	17.71	12.16	13.28	-5.61	9.21	10.09
AZAL	16.18	11.18	6.08	-5.91	5.93	10.52	14.62	-12.82	8.67	10.71	11.08	-7.91
AZEL	13.81	13.32	11.91	-12.91	4.08	-13.14	26.09	-12.18	10.91	- 10.92	8.61	-4.62

IRI

[a]: **Precision; [b] Accuracy;** The acceptance criteria: Accuracy:−50% to +20% **(≤0.5ng/g)**;−30% to +10% **(0.5–5ng/g)**;−20% to +10% **(≥5ng/g)**.**Within-day precision**: RSD%<RSD_{max} with RSD_{max} for ≥0.5 to <5 ng/mL: <25% and ≥5 to <50 ng/mL: <15%. **Between-day precision**:the RSD% <RSD_{max} with RSD_{max} 22.60%, 32% and 45% for the respective concentrations of 50 ng/g,5 ng/g and 0.5 ng/g.

Table 5. Comparison of precision of the proposed method with LC-ID-MS/MS method

Methods	Parameter	AFB1	AFB2	AFG1	AFG2	DON	OTA	ZEN	FB1	T-2
LC-ID-MS/MS	Mean ±SD (ppb)	7.42±0.29	5.29±0.18	7.53±0.13	15.26±0.26	665.57±23.53	24.58±2.09	208.49±5.32	47.84±11.09	205.39±14.02
	RSD (%)[a]	3.42	3.2	2.44	2.2	2.73	6.30	3.09	11.75	11.05
Proposed approach	Mean ±SD (ppb)	7.42±0.19	5.36±0.15	7.53±0.15	15.58±0.30	667.72±21.27	24.45±2.84	209.09±5.93	45.31±8.17	208.26±13.46
	RSD (%)	1.92	2.21	2.50	1.82	2.43	7.93	4.38	7.48	11.23
$\|t\|$		0	0.78	1.59	1.65	1.76	0.09	0.77	0.47	0.23
$t_{0.05}$ (df 30)					2.042					

Table 6. Comparative analysis of mycotoxins in certified reference materials

CRMs	Analyte	Matrix	Certified value (ppb)	Measured value (ppb)
CRM-03-OTA[a]	OTA	*Areca catechu* oil	13.1 ± 0.6	12.84±0.3
CRM-00-ZON[a]	ZEN	*Areca catechu* oil	65 ± 5	63.34±3.25
CRM-03-AFG2[a]	AFG2	*Areca catechu* oil	2.69 ± 0.18	2.59± 0.21
CRM-03-AFG1[a]	AFG1	*Areca catechu* oil	2.82 ± 0.13	2.78± 0.16
CRM-03-AFB2[a]	AFB2	*Areca catechu* oil	2.74 ± 0.15	2.69±0.23
CRM-03-AFB1[a]	AFB1	*Areca catechu* oil	2.91 ± 0.13	2.81± 0.11

[a] Materials listed in the Technical Annex of ISO 17034 accreditation (Cifga Laboratory. accredited by ENAC under accreditation number 2/PMR003. produces reference materials).

Although certain mycotoxins such as AZEL, AZAL, BZAL, BZEL, ZAN, TEA, AOH, AME, T2-G, ENNA, ENNA1, ENNB, and ENNB1 were not detected, other potential risks were identified for mycotoxins with values below the LOQ, including ZEN, DON, T2, HT2, and OTA. It is estimated that the levels of these mycotoxins could increase during the post-harvest process.

Table 7. Mycotoxin levels in three *Areca catechu* varieties in Indonesia

Mycotoxin	BET (ng/g)	BUL (ng/g)	IRI (ng/g)
ZEN	<LOQ *a, c, d, f, h,*	<LOQ *a, b, d, f, g, h,i*	<LOQ *j*
AZEL	ND	ND	ND
AZAL	ND	ND	ND
BZAL	ND	ND	ND
BZEL	ND	ND	ND
ZAN	ND	ND	ND
TEA	ND	ND	ND
AOH	ND	ND	ND
AME	ND	ND	ND
DON	<LOQ *b, d, e, f, h*	<LOQ	<LOQ
AFB2	2,32 ± 0,08 (1.98 – 2.98)	1.98 ± 0.12 (1.54 – 2.32)	<LOQ *j*
AFG1	2.56 ± 0.34 (1.98 – 3.21) *a, b, c, d, e, f, g, h,i*	2.98 ± 0.52 (1.57 – 3.41)	1.98 ± 0.25 (1.04 – 2.32) *j*
T2	<LOQ *a, b, c, d, e, f, g, h,i*	<LOQ *a, b, c, d, e, f, g, h,i*	<LOQ *j*
HT2	<LOQ *a, b, c, d, e, f, g, h,i*	<LOQ *a, b, c, e, f, g, h,i*	ND
T2-G	ND	ND	ND

AFB1	4.98 ±0.15 (5.3 - 3.87) *a, b, c, d, e, f, g, h,i*	3.24 ± 0.23 (4.32 – 2.97) *a, b, c, d, e, f, g, h,i*	2.97 ± 0.38 (3.28 – 2.16) *j*
AFM1	1.01 ± 0.04 (ND – 1.5) *a, b, c, d, e, f, g, h,i*	<LOQ *a, b, c, d, e, f, g, h,i*	<LOQ *j*
OTA	<LOQ *a, b, c, d, e, f, g, h,i*	<LOQ *a, b, c, d, e, f, h,i*	<LOQ *j*
ENNA1	ND	ND	ND
ENNA	ND	ND	ND
ENN B	ND	ND	ND
ENN B1	ND	ND	ND
AFG2	1.89 ± 0.15 (1.65 – 2.04)	1.34 ± 0.21 (1.01 – 1.82)	1.41 ± 0.25 (1.03 – 2.07)

Testing was carried out with a significant level at $p<0.05$ with 5 repetitions. **BET**/ *Areca catechu* var. Betara, **BUL** / *Areca catechu* var. Bulawan, **IRI**/ *Areca catechu* var. Irian, ND: Not detected; *a*, East java; *b*, Central Java; *c*, West java; *d*, West Nusa Tenggara; *e*, East Nusa Tenggara; *f*, South Sulawesi; *g*, east Sulawesi; *h*, East Kalimantan; *I,* Riau; *j*, Papua.

Our UPLC-MS/MS method effectively controls mycotoxin contamination. AFB1 was the predominant mycotoxin with concentrations of 2.56 ± 0.34 ng/g in BET, 3.24 ± 0.23 ng/g in BUL, and 2.97 ± 0.38 ng/g in IRI. Other potential mycotoxins with values below LOQ, such as ZEN, DON, T2, HT2, and OTA, might increase during post-harvest. Previous research reported AFB1 contamination in *Areca catechu* at 5.43 ng/g for all fruit parts and 7.55 ng/g for seeds in China (Liang et al. 2022).

Regulatory standards for mycotoxins are stringent. The EU sets maximum limits for aflatoxins in nuts at 2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins (Contam et al. 2018; Juan et al. 2008), while the FDA limits aflatoxins in foods to 20 ppb, with stricter limits of 0.5 ppb for milk (Jallow et al. 2021; Keener 2019). Asghar et al. (2014) reported average AFB1 contamination of 92.5 µg/kg, with ranges from 11.7 to 262.0 µg/kg, and high contamination rates in Indonesia and Sri Lanka. Further research by Asghar et al. (2020) found all Indonesian samples contaminated with AFB1, with an average of 123.76 µg/kg and ranges from 1.88 to 378.94 µg/kg, exceeding EU and FDA limits by over 94.9% and 71.8%, respectively.

Given these concerns, a fast and reliable analysis method is crucial. Our proposed method addresses the complexity of *Areca catechu* oil matrices, providing accurate and reliable mycotoxin detection. This approach could enhance quality control for *Areca catechu* on the global market, reducing health risks associated with mycotoxin contamination.

4. Conclusions

The use of Solid Phase Extraction (SPE) with carb/PSA was demonstrated to be optimal for detecting 24 mycotoxins, achieving high recovery rates of 95-102%. This method outperformed carb/NH_2 (75-82%) and NH_2 (45-60%) methods. To address matrix effects, calibration standards matching the sample matrix and sorbents such as NH_2, carb, and PSA were employed to eliminate non-target components, thereby enhancing accuracy and precision.

The method validation included testing across a range of 0.5-250 ng/g and an LOQ of 0.5 ng/g, resulting in $R^2 > 0.99$. Precision assessments showed % RSD values within acceptable limits (RSDmax), and accuracy ranged from -50% to +20% at LOQ, -30% to +10% at 10LOQ, and -20% to +10% at 100LOQ. Comparisons with LC-ID-MS/MS and certified reference materials (CRM) indicated no significant differences ($p < 0.05$).

Our research revealed that AFB1 contamination was predominant in three *Areca catechu* varieties from Indonesia, with values of

2.56 ± 0.34 ng/g for BET, 3.24 ± 0.23 ng/g for BUL, and 2.97 ± 0.38 ng/g for IRI. Potential hazards from mycotoxins below the LOQ, such as ZEN, DON, T2, HT2, and OTA, were also noted and could increase post-harvest. Implementing a reliable mycotoxin analysis strategy is crucial for ensuring product safety in the global market. The accurate UPLC-MS/MS methods used in this research facilitate effective control of mycotoxin contamination.

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