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FOOD CHEMICAL CONTAMINANTS

Residual Analysis of Aflatoxins in Spice by HPLC Coupled with Solid-Phase Dispersive Extraction and Solid-Phase Fluorescence Derivatization Method

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Abstract

Background: Aflatoxins (AFs) are carcinogenic mycotoxins. A simple, quick, and accurate method for the micro-analysis of AFs in foodstuffs, especially spices, is needed.
Objective: A sophisticated pretreatment method that combines solid-phase dispersive extraction (SPDE) and solid-phase fluorescence derivatization using immunoaffinity (IA) gel as the solid phase was developed to analyze AFs in spices simply, quickly, and sensitively by liquid chromatography with fluorescence detection.
Method: White and black pepper samples were extracted with a mixed solution of methanol/water (4:1) and then diluted with 7% aqueous solution of Triton-X. The solution was subjected to cleanup by SPDE using IA gel. Trifluoroacetic acid was added to the IA gel for on-site solid-phase fluorescence derivatization.
Results: Chromatograms containing well-separated peaks and few interference peaks from contaminants were obtained. The method detection limit of AFs in white and black pepper was 0.15–0.29 ng/g. Repeatability and intermediate precision were $<10\%$ and $<15\%$, respectively, and accuracy was 61.7–87.8%. In addition, inter-laboratory precision was $<29\%$ and mean recovery was 61.5–76.7%. A favorable z-score of $|Z| \leqq 1$ was obtained in seven laboratories, although one laboratory gave $2 < |Z| < 3$.
Conclusions: The validity, reliability, practicality, and robustness of the developed method were verified.
Highlights: By using SPDE and solid-phase fluorescence derivatization in combination for AF analysis, fluorescence derivatization during cleanup was realized, leading to simplification of the pretreatment operation.

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สำเนาเพื่อใช้ในการศึกษาเท่านั้น

Aflatoxins (AFs) are mycotoxins produced by the genus *Aspergillus* and mainly include aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2). AFs in peanut meal used as feed were the causative agent of poisoning (turkey X disease) in more than 1 million turkeys in 1961, and this incident became a food hygiene problem (1). AF toxicity has resulted in such health problems as liver cancer and liver dysfunction (2, 3). AFB_1 is said to be the strongest carcinogen among natural substances, and the International Agency for Research on Cancer of the World Health Organization has classified AFB_1 as a substance that is carcinogenic to humans (Group 1) (4). In Japan, the residual standard for total AFs (AFB_1, AFB_2, AFG_1, and AFG_2) in all foodstuffs is set at 10 µg/kg. However, even if the amount of AFs remaining in foodstuffs is very small, the carcinogenicity cannot be ignored, and therefore a simple, quick, and accurate method for the micro-analysis of AFs in foodstuffs is needed.

Several approaches for the residual analysis of AFs, such as enzyme-linked immunosorbent assay (ELISA) (5, 6), thin-layer chromatography (TLC) (7), liquid chromatography fluorescence detection (LC-FL) (8), and LC tandem mass spectrometry (MS/MS) (9, 10), have been used so far. As LC-FL and LC-MS/MS have high selectivity for the target substance and enable detection with high sensitivity, they are widely used as the microanalytical method for AFs. ELISA and TLC are rapid screening methods that do not require large dedicated instruments, such as LC-MS/MS. However, for samples with many impurities, false positives and false negatives may occur in ELISA, and spotting highly oily samples on thin plates may be difficult in TLC (11). LC-FL has high sensitivity but requires fluorescence derivatization before measurement to enhance the fluorescence intensities of AFB_1 and AFG_1. On the other hand, in LC-MS/MS, ion suppression and ion enhancement due to the matrix effect may occur (12). Therefore, there is a need for a more sophisticated cleanup method that can more effectively remove impurities.

To clean up AFs in foodstuffs, solid-phase extraction (SPE) using a silica gel column (13), a florisil column (14), or a multifunctional column (15) has been used. Recently, immunoaffinity (IA) chromatography (16–18) utilizing immunological interaction, which has a high cleanup effect, has been employed as well. However, the conventional cartridge-type SPE method has some drawbacks, including clogging of high-viscosity liquid sample, which may result in low recovery. In addition, it is difficult to maintain a constant flow rate for all SPE cartridges when a vacuum-type manifold is used for conventional cartridge-type SPE operation. Furthermore, because most operations in the conventional SPE method are performed in an open system, an experimenter may be exposed to AFs through the operation. We have developed a novel cleanup method called the solid-phase dispersive extraction (SPDE) method, which overcomes the above-described drawbacks for the determination of drugs in serum and urine (19, 20). The SPDE method involves rapidly extracting target compounds by dispersing solid microparticles in a liquid sample. Immediately after the microparticles are dispersed in the liquid sample, the two phases in the suspension seem to reach equilibrium. In conventional liquid–liquid extraction (or solid–liquid extraction), target compound extractability from the phase containing the target compound to another phase usually depends on the distribution coefficient. Clogging of the sample could be suppressed owing to processing by centrifugation in the SPDE method. In addition, sample loading speed adjustment, which is required by the conventional cartridge-type SPE method, is not required in the SPDE method. Further, as the SPDE method is performed in a closed system with a high sealing property compared with a conventional SPE system, exposure to infectious microbes or hazardous chemicals is minimized. Therefore, in this study, the SPDE method using IA gel as the solid phase was examined as the cleanup method.

In AF analysis using highly versatile LC-FL, pre- or post-column fluorescence derivatization is required. The former involves adding trifluoroacetic acid (TFA) to AFs and hydroxylating the double bond of the bisfuran ring to convert it into a single bond, thereby changing the stereo structure (21). This method does not require a dedicated device, unlike the post-column fluorescence derivatization described below, and is simple. However, in the conventional pre-column fluorescence derivatization method (21), it is necessary to remove the solvent from the eluate under a nitrogen stream after cleanup and, therefore, the derivatization is time-consuming and tedious. Furthermore, exposing the experimenter to AFs is a concern during the derivatization operation as well as in the cleanup operation.

On the other hand, post-column fluorescence derivatization involves the derivatization of AFB_1 and AFG_1 into a hydroxide by UV irradiation (22) and by electrochemically generated bromine (23). Post-column fluorescence derivatization by UV irradiation has the advantage that the derivatization is easier than pre-column fluorescence derivatization, whereas that by electrochemically generated bromine has the advantage that impurities can be effectively reduced when analyzing AFs in such foodstuffs as citrus fruits and livestock feed (21). However, for both post-column fluorescence derivatization methods a dedicated apparatus is required.

In this study, to overcome these problems, we devised a solid-phase fluorescence derivatization method using solid-phase IA gel for SPDE as the reaction medium. In addition, in order to evaluate the usefulness and practicality of the developed method, accuracy management for method validation using AF-supplemented spice (white pepper) was performed through intra- and inter-laboratory quality control tests.

Experimental

Chemicals and Materials

AF standards (AFB_1, AFB_2, AFG_1, and AFG_2 mixture solution; each 25 µg/mL), acetonitrile and methanol (both HPLC grade), TFA, polyoxyethylene octyl phenyl ether (Triton X-100), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and sodium hypochlorite (all special grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Sodium chloride (NaCl; special grade) was from Kanto Chemical Co., Inc. (Tokyo, Japan). Polyoxyethylene sorbitan monolaurate (Tween 20) was from Merck Ltd. (Darmstadt, Germany). Water was purified with a Milli-Q Gradient A10 system equipped with an EDS-PAK® polisher (Merck Ltd.). All other chemicals were of special grade. Phosphate-buffered saline (PBS) was prepared as follows: NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.16 g, and KH_2PO_4 0.2 g was dissolved in 1 L of water, and the solution was stored below 4°C.

The IA gels used in the SPDE method and the solid-phase fluorescence derivatization method were AFLAKING (Horiba, Ltd., Tokyo, Japan), AflaStar™R (Romer Labs, Inc., DE, USA), AflaTest WB (Vicam Inc., MA, USA), and EASI-EXTRACT AFLATOXIN (R-Biopharm AG, Darmstadt, Germany). For SPDE, @Roka™ and Captube™ centrifugation filter units were purchased from Frontier Science Co., Ltd. (Hokkaido, Japan).

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Commercially available white pepper and black pepper samples (GABAN; Tokyo, Japan) were used as for the method validation. Only white pepper was used for the inter-laboratory quality control tests.

For the cleaning of glassware used for AF analysis, AFs were sufficiently decomposed by immersing glassware in 1% aqueous sodium hypochlorite solution for 2 h or more. The glassware was subsequently washed with a neutral detergent.

Preparation of Standard Solutions

AF standard stock solution (250 ng/mL) was prepared by diluting the standard mixed solution (1 mL) in 100 mL of acetonitrile. Working solutions were prepared by diluting the stock solution with purified water to the appropriate concentrations. Standard stock solutions and working solutions were stored at −20°C or lower until use.

Extraction

Three grams of sample was introduced into a 50 mL polypropylene centrifuge tube and 0.3 g of sodium chloride and 12 mL of a mixture of purified water and methanol (1:4) were added. The mixture was shake-extracted with a shaker for 30 min. The extraction solution was subjected to suction filtration and 7% aqueous solution of Triton-X was added to 5 mL of the filtrate to make 10 mL. After thorough mixing, the mixture was centrifuged ($2500 \times g$, 5 min) and 8 mL of the supernatant was used as the sample extract.

SPDE

The entire amount of IA gel was removed from the corresponding cartridge and suspended in 1 mL of PBS. This suspension was added to a 15 mL centrifuge tube containing the sample extract prepared as described in the preceding paragraph. After stirring with a vortex mixer for 30 s to disperse the IA gel in the sample solution, the suspension was centrifuged ($2500 \times g$, 20 s) to separate the solid phase and the liquid phase (Figure 1). Next, the supernatant was removed with a micropipette and 6 mL of PBS was added as the washing solution to disperse the solid phase again. The same operation was repeated. Thereafter, 6 mL of water was added as the washing solution and the solid phase was washed again in the same manner. The liquid phase was removed while leaving approximately 0.5 mL of suspension containing the IA gel.

Solid-Phase Fluorescence Derivatization

In the solid-phase fluorescence derivatization method, a Captube™ was mounted on the upper part of @Roka™, and a 2.0 mL micro test tube was mounted on the lower part (Figure 2).

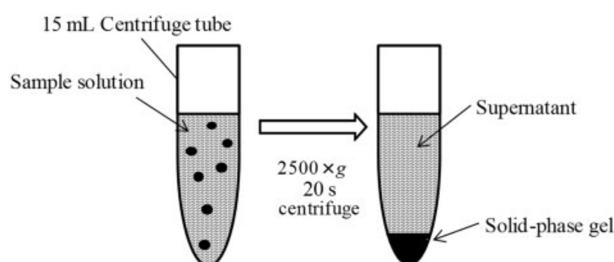

Figure 1. Scheme of the solid-phase dispersive extraction method using a 15 mL centrifuge tube.

The suspension containing the IA gel, which was prepared as previously described, was transferred to the Captube™ and centrifugation ($2500 \times g$, 20 s) was carried out to remove water in the solid phase. After removing the 2.0 mL micro test tube and replacing it with a new one, 0.1 mL of TFA was added to the Captube™ and the SPDE device was inverted and stirred with a vortex mixer for 30 s. The SPDE device was left to stand for 10 min in the dark at room temperature. Thereafter, the SPDE device was returned to its original position and centrifuged ($2500 \times g$, 20 s). The filtrate was collected as TFA eluate. Further, 0.9 mL of purified water was added as the eluent to disperse the solid phase again, and after centrifugation in the same manner as that described above, the total volume was adjusted to 1 mL by combining with 0.1 mL of the TFA eluate (Figure 2).

LC/FL Apparatus and Operating Conditions

An L-6300 Intelligent system (Hitachi, Inc., Ibaraki, Japan) equipped with an FP-2020 (Jasco, Kyoto, Japan) was used. LC separation was performed with an L-column2 ODS (250 × 4.6 mm i.d., 5 µm; CERI, Tokyo, Japan). Column temperature was maintained at 40°C. The mobile phase was a mixture of acetonitrile, methanol, and water in the ratio of 1:3:6 (v/v), and was delivered in the isocratic elution mode at the flow rate of 0.5 mL/min. The FL excitation wavelength was set at 365 nm and emission wavelength was set at 450 nm. A 50 µL aliquot of the sample solution was injected into the system.

Collaborative Study for Inter-Laboratory Precision

We requested the following laboratories to participate as joint research institutions: Saitama Institute of Public Health, Frontier Institute Co., Ltd., Saitama City Institute of Health Science and Research, Yokohama City Institute of Health, Nagoya City Public Health, Kawasaki City Institute for Public Health, and Kanagawa Prefectural Institute of Public Health. We distributed the following items to each institution: analytical samples (high and low concentrations), AF standard solution (20 ng/mL), IA gel (pre-suspended), an IA cartridge (spare), SPDE devices, micro test tubes (2 mL volume), 7% aqueous solution of Triton X-100, an LC column, and the operation manual. As for the other instruments and reagents, we requested that they use the ones in their laboratories. As the samples for analysis, approximately 8 g each of white pepper samples to which AFs had been added in advance (high concentration: 20 ng/g, low concentration: 2 ng/g) were distributed to each laboratory. The prepared concentration was not disclosed to any of the laboratories. The results were analyzed by z-score (24, 25) to evaluate the robustness and practicality of the residual analysis method developed by our laboratory.

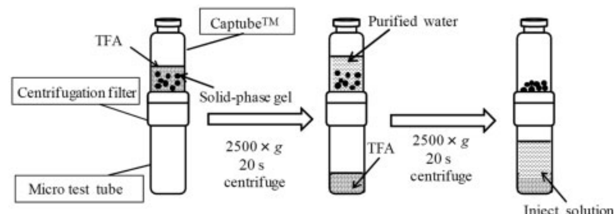

Figure 2. Scheme of the solid-phase fluorescence derivatization method using a centrifugation filter.

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Results and Discussion

Selection of Optimum IA Gel for SPDE

We compared four types of IA gels for SPDE: AFLAKING, AflaStar^{TM}R, AflaTest WB, and EASI-EXTRACT AFLATOXIN. Good recovery (88% or higher) was obtained with AFLAKING and AflaStar^{TM}R, whereas recovery was approximately 70% or lower with AflaTest WB and EASI-EXTRACT AFLATOXIN (Figure 3). This was thought to be due to the fact that AflaTest WB and EASI-EXTRACT AFLATOXIN had low antibody activity or that the amount of antibody introduced into the IA gel was small. Examination of the application range in organic solvents revealed that the antibody used for AFLAKING was stable in 20% acetonitrile and 40% methanol, whereas the antibody used for AflaStar^{TM}R was stable in 5% acetonitrile and 20% methanol, indicating lower stability of AflaStar^{TM}R in organic solvents. Therefore, we employed AFLAKING, which has high stability in organic solvents.

Optimization of Solid-Phase Fluorescence Derivatization

In the conventional pre-column fluorescence derivatization method using TFA, it is necessary to remove the solvent from the eluate from an IA column under a nitrogen stream, which is tedious and time consuming. In addition, the experimenter may be exposed to AFs during the nitrogen purging operation. Therefore, we decided to employ the solid-phase derivatization method (26, 27). In this method, derivatization is performed using a solid phase as the reaction medium while the target substance is retained on the solid phase. The derivatization efficiency is higher than when the reaction is performed in the liquid phase. When the reaction is carried out in the solid phase, solvent removal from the sample extract under a nitrogen stream prior to the reaction can be omitted. As a result, the operation time is reduced. Therefore, we employed the solid-phase fluorescence derivatization method in which AFs are fluorescently derivatized while being retained on the solid phase. We considered that the IA gel used in this study recognizes part of the bisfuran ring and the coumarin skeleton of AFs, that is, the reaction site of AFs is different from the binding site and therefore, the derivatization can proceed without interference from the IA gel.

The optimal reaction time was examined in the range of 0–15 min. White pepper was used as the sample. After the white pepper sample extract was cleaned up by the SPDE method, 100 µL of TFA was added to the solid phase (IA gel) and the reaction time was examined. As a result, the reaction reached a plateau within 5 min or more, and the reaction time of 10 min was employed.

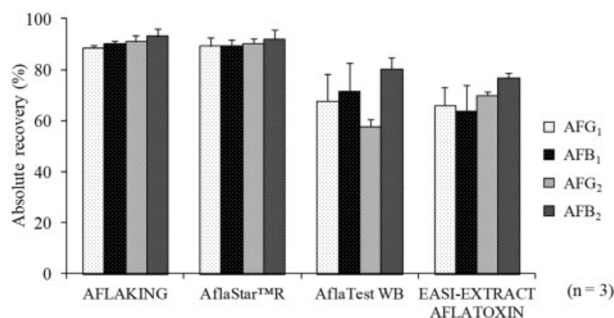

Figure 3. Comparison of absolute recoveries of AFs using four IA gels: AFLAKING, AflaStar^{TM}R, AflaTest WB, and EASI-EXTRACT AFLATOXIN.

The optimal amount of TFA was examined in the range of 25–200 µL. When the amount of TFA added was <50 µL, it was difficult for TFA to uniformly infiltrate the solid phase, and the recovery rate was low. When the amount of TFA added was 25 µL, the recovery rate was approximately 40%. When the amount of TFA added was 50 µL or more, the recovery rate almost plateaued. Therefore, the addition amount of 100 µL was employed.

In the cleanup method using an IA cartridge, an acidic solvent or an organic solvent is usually used as the eluent to denature the antibody. Because AFs easily dissolve in organic solvents, we considered it more effective to use organic solvents. In fact, Horiba, the manufacturer of AFLAKING, recommends the use of 3 mL of acetonitrile for the elution of AFs from the IA gel. On the other hand, in the solid-phase fluorescence derivatization used in this study, TFA was added to make AFB_1 and AFG_1 fluorescent by derivatization. The IA gel, after the addition of TFA, was in the acidic state. The AF eluent after the fluorescence derivatization was a mixed solution of acetonitrile and water and the optimal acetonitrile concentration was examined in the range of 0–100%. When the concentration of acetonitrile was 0–25%, good recovery of approximately 90% was obtained. However, when the concentration of acetonitrile was 50% or higher, the recovery rate of AFs decreased. This was presumed to be due to the fact that higher concentrations of acetonitrile lead to lower conversion rates of AFB_1 and AFG_1 into the corresponding hydroxylated products, resulting in lower recoveries. On the other hand, even if no acetonitrile was added, the IA gel, after the addition of TFA, was in the acidic state, so that the IA gel seemed to be easily denatured and AFs could be eluted and derivatized at the same time. From the above results, purified water was used as the eluent from the IA gel.

Comparison of SPDE Method and Conventional SPE Method for Solid-Phase Fluorescence Derivatization

The solid-phase fluorescence derivatization method was applied to the proposed SPDE method and the conventional cartridge-type SPE method, and the recovery rates were compared. AFLAKING was used for the cartridge-type SPE method. The procedure was as follows: AF standard solution diluted with 7% aqueous solution of Triton X-100 was applied to the SPDE method and the SPE method. In the SPDE method, solid-phase fluorescence derivatization was performed according to the proposed method. In contrast, in the SPE method, the following procedure was carried out: First, 100 µL of TFA was applied to the top of the SPE cartridge. TFA was allowed to flow into the solid phase naturally and the reaction was allowed to proceed for 10 min at room temperature. The TFA eluate was collected. Next, 900 µL of purified water was applied to the top of the SPE cartridge and the eluate was collected again. Finally, the two eluates were combined. The SPDE method showed higher recovery than the SPE method because in the SPE method it was difficult for TFA to uniformly flow into the solid phase and the reaction between TFA and AFs did not proceed sufficiently. In contrast, in the SPDE method, it was speculated that by dispersing the solid phase and TFA using a vortex mixer, TFA could sufficiently penetrate the entire solid phase and reactivity would be improved. From the results, we confirmed that the solid-phase fluorescence derivatization method gave better results when used in combination with the SPDE method rather than the SPE method.

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Table 1. Validation of proposed HPLC method for AF analysis in white and black pepper samples

AFs	LOD[a], ng/mL	LOQ[b], ng/mL	MDL[c], ng/g	MQL[d], ng/g	Linear range, ng/mL	Correlation coefficient, *r*
AFG_1	0.08	0.28	0.20 (0.20)[e]	0.70 (0.71)	0.28–10	0.999
AFB_1	0.08	0.26	0.21 (0.21)	0.66 (0.69)	0.26–10	0.999
AFG_2	0.06	0.21	0.15 (0.15)	0.54 (0.54)	0.21–10	0.999
AFB_2	0.11	0.36	0.29 (0.29)	0.95 (0.96)	0.36–10	0.970

[a] Limit of detection ($S/N = 3$).
[b] Limit of quantification ($S/N > 10$).
[c] Method detection limit ($S/N = 3$).
[d] Method quantification limit ($S/N > 10$).
[e] Data in parentheses are for black pepper samples.

Table 2. Accuracy, repeatability, and intermediate precision of AFs added to white and black pepper samples as measured by proposed method ($n = 2 \times 5$ days)

AFs	Added, ng/g	Accuracy, %	Repeatability, %	Intermediate precision, %
AFG_1	(1)[a]	(78.4)	(4.0)	(5.0)
	1	80.0	4.8	5.0
	2	81.5	8.1	14.5
	20	87.8	4.0	9.2
AFB_1	(1)	(75.7)	(3.7)	(3.9)
	1	78.4	5.7	6.6
	2	83.0	9.9	13.8
	20	87.1	4.1	9.8
AFG_2	(1)	(77.6)	(2.5)	(4.7)
	1	78.4	3.6	5.1
	2	61.7	9.1	10.7
	20	80.1	3.1	12.2
AFB_2	(1)	(75.2)	(3.7)	(4.1)
	1	75.8	4.4	5.0
	2	61.9	7.6	7.0
	20	80.0	2.3	14.0

[a] Data in parentheses are for the black pepper sample.

Method Validation

The limit of detection (LOD, $S/N = 3$) and the limit of quantification (LOQ, $S/N > 10$) of AFs were in the range of 0.06–0.11 ng/mL and 0.21–0.36 ng/mL, respectively. Each calibration curve showed good linearity ($r > 0.9997$) over the range of each LOQ to 10 ng/mL. The method detection limit (MDL) and the method quantification limit (MQL) were 0.15–0.29 ng/g and 0.54–0.95 ng/g, respectively (Table 1).

Accuracy, repeatability, and intermediate precision tests were conducted using commercially available white and black pepper as spice samples. AF standard solution was added to white pepper and AF concentration was adjusted to the low concentration level (1 or 2 ng/g) or the high concentration level (20 ng/g). Two replicate determinations at each concentration were carried out for 5 days each. Statistical analyses were performed using one-way analysis of variance. Accuracy (average recovery) at the low concentration level was 61.7–83.0% and that at the high concentration level was 80.0–87.8%. Repeatability and intermediate precision values at the low concentration level were 2.5–9.9% and 3.9–14.5%, respectively (Table 2). Those at the high concentration level were 2.3–4.1% and 9.2–14.0%, respectively. It was confirmed that the proposed method has sufficient sensitivity and precision for the residual analysis of AFs in spices. The representative chromatograms of AF standards and those of samples are shown in Figure 4.

External Accuracy Control

Validation of inter-laboratory reproducibility and data analysis by z-score were performed against data from eight laboratories. Inter-laboratory precision for the high-concentration sample was 24.8–25.9% and mean recovery was 66.6–71.6%, and inter-laboratory precision for the low-concentration sample was 26.9–28.9% and mean recovery was 61.5–76.7% (Table 3).

In addition, the evaluation of z-scores for high and low concentrations, inter-laboratory z-score (ZB), and intra-laboratory z-score (ZW) was carried out. In the high-concentration addition test, all four AFs were within $|Z| < 2$ in all the laboratories, whereas in the low-concentration addition test, the data from one laboratory showed $2 < |Z| < 3$. Regarding ZW, one laboratory gave $2 < |Z| < 3$ for AFB_2. Generally, these results can be used to examine bias and variability. However, in this study, the differences between high and low concentrations were so large that it was difficult to verify them by only comparing ZB and ZW values. Therefore, we created a z-score scatter plot (Figure 5) and a ZB/ZW scatter plot, the so-called Youden plot, to verify why one laboratory gave $2 < |Z| < 3$ (Figure 6). As can be seen from Figures 5 and 6, the AFB_1, AFB_2, AFG_1, and AFG_2 plots of the laboratory that gave $2 < |Z| < 3$ are concentrated mostly in one area. As only AFB_1 and AFG_1 underwent fluorescence derivatization,

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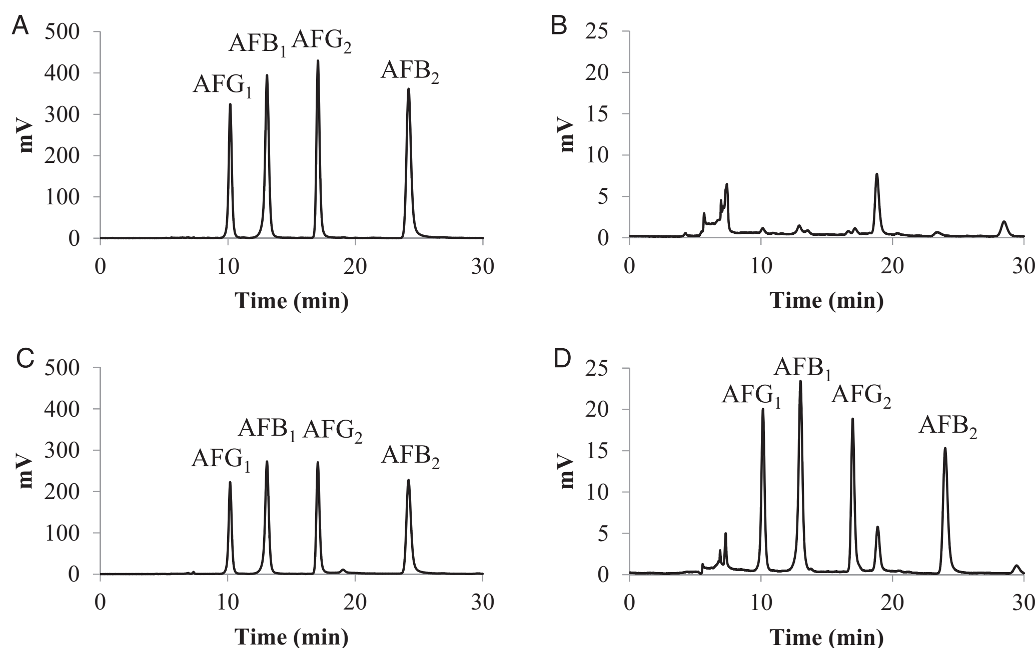

Figure 4. Representative chromatograms of AFs: (A) AF standards (20 ng/mL), (B) blank from white pepper, (C) AFs added at a high concentration (20 ng/g) to the white pepper sample, (D) AFs added at a low concentration (2 ng/g) to the white pepper sample.

Table 3. Mean recovery and inter-laboratory precision for white pepper samples to which AFs were added at low and high concentrations (2 ng/g and 20 ng/g, respectively) as determined by the proposed method among eight laboratories

AFs	Added concentration, ng/g	Mean recovery, %	Inter-laboratory precision, %
AFG_1	2	75.3	27.5
	20	69.2	25.9
AFB_1	2	76.7	26.9
	20	71.6	25.2
AFG_2	2	61.5	28.8
	20	66.6	25.7
AFB_2	2	65.5	28.9
	20	71.2	24.8

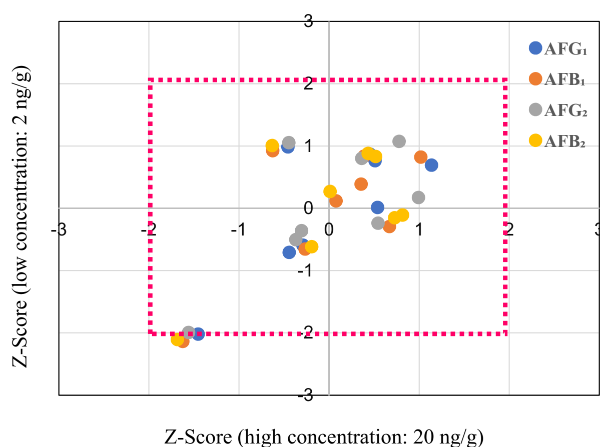

Figure 5. Scatter plot of z-scores for inter-laboratory precision. The horizontal axis shows the z-score of the sample with high concentration (20 ng/g) and the vertical axis shows the z-score of the sample with low concentration (2 ng/g). The dotted line in the scatter plot shows the range of "|Z| = 2".

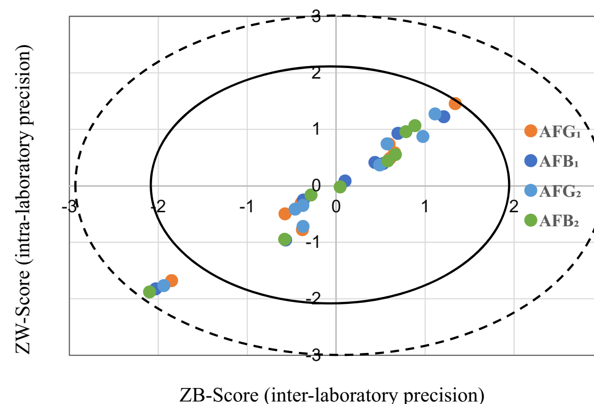

Figure 6. Youden plot of z-scores for intra- and inter-laboratory precisions. The horizontal axis shows the inter-laboratory z-score (ZB) and the vertical axis shows the intra-laboratory z-score (ZW). Solid ellipses indicates a ZB of "|Z| = 2" and dotted ellipses a ZW of "|Z| = 3".

if the derivatization reaction was inadequate, or if there was a problem with the extraction or cleanup operation, the four plots would be more widely scattered. Therefore, we speculated that the data deviated from $|Z| < 2$ not because of the analysis method or technical problems, but because of other factors, such as an error in dilution when creating the calibration curve. The other seven laboratories yielded data in the range of $|Z| \leqq 1$, which was an extremely favorable result. These results suggest that the analytical method developed in this study is a satisfactory tool for testing both robustness and practicality.

Conclusions

The SPDE method using the IA gel and the solid-phase fluorescence derivatization method were developed as pretreatment methods for AFs in spice (white pepper) and evaluated for potential applicability. The solid-phase fluorescence derivatization

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method allowed TFA to flow into the entire solid phase when used in combination with the SPDE method, in contrast to the case where it was used in combination with the conventional cartridge-type SPE method. Thus, the reactivity of the fluorescence derivatization was improved and high accuracy was obtained. After cleanup by SPDE, it became possible to perform fluorescence derivatization in the SPDE device without a nitrogen purging operation prior to derivatization, greatly simplifying the operation. Furthermore, as the method can be performed in a closed system, exposure of the experimenter to AFs is reduced.

Method validation was performed to evaluate the applicability of the developed pretreatment method of white pepper. As regards internal quality control, repeatability was $<10\%$, intermediate precision was $<15\%$, and accuracy was 61.7–87.8% for the high- and low-concentration samples.

Regarding external accuracy control, inter-laboratory precision was 24.8–28.9% and mean recovery was 61.5–76.7% for the low- and high-concentration samples. In addition, the intra- and inter-laboratory Z-scores were approximately $|Z| < 2$. These data suggest that the developed method is applicable and has high reliability and robustness.

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Conflict of Interest

There are no financial or other relations that could lead to a conflict of interest.

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