A method of analysis for determination of aflatoxins in chocolate

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Summary

A method of analysis for determination of aflatoxins (AFs) in chocolate was optimized and evaluated its within laboratory precision. The method uses acetonitrile-methanol-water (60+10+40, v/v/v) as extraction solvent and an immunoaffinity column (IAC) for the cleanup, and HPLC with fluorescence detection for the determination of AFs. The method was validated by testing replicate analyses of the finally ground chocolate samples spiked at 0.1 and 10.0 µg/kg for each AFB1, AFB2, AFG1 and AFG2. The average recovery of AFs at 0.1 and 10.0 µg/kg spiking levels ranged from 90 to 97 % for all AFs. The relative standard deviation (RSD) for within day and between day ranged from 1.7 to 3.3 %, and 0 to 4.1 % for all AFs, respectively. The HorRat for the total RSD at each level was within 0.2 for all AFs. These results show that this method is reliable for the analysis of AFs in chocolate in the range of 0.1 and 10.0 µg/kg.

Key words: aflatoxins, cacao, chocolate, immunoaffinity cleanup, HPLC, within laboratory precision

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Introduction

Aflatoxins (AFs: Aflatoxin B1, B2, G1 and G2) are mycotoxins produced mainly by Aspergillus flavus, A. parasiticus and A. nomius, and known for their carcinogenic and hepatotoxic effects on various animal species1). Because of these high toxicity of AFs, the contamination of AFs in agricultural commodities such as cereals, pulses, nuts, seeds, and spices2) is serious threat to human health.

Cocoa beans (Theobroma cacao) are cropped in warm and humid areas such as Africa, Latin America, and Southeast Asia where AFs contamination in agricultural commodities may be occurred. They are exported throughout the world and processed into cocoa products including chocolate and cocoa powder. In recent years, consumption of cocoa beans amounts to more than 3 million tons per year in the world5). The cocoa production chain includes fermentation step for 2-12 days to remove sugary pulp. Cocoa beans are susceptible to spoilage by fungi during and after fermentation, and A. flavus has been isolated from fermenting cocoa beans6). In fact, cocoa beans imported from Venezuela have been occasionally rejected for contamination of aflatoxin B1 (AFB1) above the regulatory limit (10 µg/kg) in Japan since 20055). Since AFs are generally thought to be stable in most food products5),

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AFs contaminated in cocoa beans could be carried over to cocoa products such as chocolate. There are many analytical methods for AFs in various agricultural products\(^7\), however, there are few reports concerning the analysis for AFs in chocolate until now. These circumstances need developing a reliable method of analysis for AFs in chocolate.

In recent years, the immunoaffinity column (IAC) cleanup-HPLC with fluorescence detection has played an important role in AFs analysis\(^7\), because of its simplicity, high specificity, and no use of carcinogenic solvents such as chloroform and benzene. For the extraction of AFs from a wide variety of matrices, mixtures of methanol-water and acetonitrile-water have been mainly used in the analytical methods introducing the IAC\(^7,8\).

The aim of this study was to develop a reliable method for analysis of AFs in chocolate. Dark chocolate was selected from chocolate samples as a test sample in this study, because dark chocolate contains the highest cocoa ingredients among chocolate samples. The extraction was performed with different composition acetonitrile-water and acetonitrile-methanol-water to establish the optimal extraction solvent for AFs from chocolate. AFs were purified using an IAC and analyzed by HPLC with fluorescence detection after trifluoroacetic acid (TFA) derivatization. The developed method was also evaluated its within laboratory precision.

**Materials and methods**

**Sample preparation**  Commercial dark chocolate was frozen at \(-20^\circ\mathrm{C}\), and ground with a mixer (MX-V100, Matsushita Electric Industrial Co., Ltd., Osaka, Japan) to pass through 1000 µm sieve. The finely ground sample was transferred into plastic bag, and then mixed well to make a homogeneous sample. The sample was stored at \(-20^\circ\mathrm{C}\) until use. For evaluating recovery, 100 µL of each AFs working standard solution (10 ng and 1 µg/mL for each AF) was added to 10.0 g sample (final concentration was 0.1 µg/kg and 10.0 µg/kg, respectively) and left at room temperature for 1 hour prior to analysis.

**Chemicals**  AFB\(_1\), AFB\(_2\), AFG\(_1\), and AFG\(_2\) crystalline standards were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Original standard solutions of each AF were prepared in toluene-acetonitrile (90+10, v/v) and each concentration was determined according to the Official Method of Analysis of AOAC International\(^7\). The AFs working standard solutions (1 µg, 100 ng, and 10 ng/mL for each AF) were prepared by dilution with toluene-acetonitrile (90+10, v/v) for spiking and standard curves. Easi-Extract AFLATOXIN IAC was purchased from R-Biopharm Rhône Ltd. (Glasgow, Scotland). Phosphate buffered saline (PBS) tablet was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). PBS-0.01 % Tween 20 solution was prepared by dissolving 5 PBS tablets and 100 µl of Tween 20 in 1000 mL of purified water. Acetonitrile and methanol were of HPLC grade. All the other reagents were of reagent grade.

**Extraction and cleanup**  Ten grams of the finely ground sample was homogenized with 40 mL of extraction solvent at 10,000 rpm for 5 min using the homogenizer (Ace Homogenizer, Nihonseiki Kaisha Ltd., Tokyo, Japan). The extract was filtered through glass microfiber filter (Whatman 934AH, Whatman International Ltd., Maidstone, England). Four milliliters of filtrate (1 g sample equivalent) was diluted to 100 mL with PBS-0.01 % Tween 20, and filtered through glass microfiber filter. Fifty
milliliters of diluted filtrate (0.5 g sample equivalent) was applied onto an IAC at a flow rate of 1 drop/sec. After washing with PBS-0.01 % Tween 20 (3.3 mL x 4 times) followed by purified water (3.3 mL x 3 times), the column was dried by passing the air into the column with a syringe. One milliliter of acetonitrile was applied onto the IAC and kept for 5 min, and then AFs were eluted into a silanized amber vial (4 mL, Supelco, Bellefonte, PA, USA) by gravity. The above eluting procedure was repeated another 3 times (total 4 mL elution). The eluate was combined and evaporated to dryness under a gentle stream of nitrogen gas at 45 °C. The residue was resolved in 0.1 mL of TFA and kept for 15 min at room temperature, and 0.9 mL of injection solvent (acetonitrile-water, 10+90, v/v) was added to the mixture.

**HPLC analysis** The HPLC system consisted of a LC-20AD pump, a SIL-20AC auto sampler, a CTO-20AC column oven, a RF-10AXL fluorescence detector (excitation 360 nm, emission 450 nm), a DGU-20A3 degasser, a CBM-20A communication bus module and a LCsolution data system (Shimadzu Co., Kyoto, Japan). The analytical column (Inertsil ODS-3V, 4.6 mm x 250 mm, 5 µm, GL Sciences Inc., Tokyo, Japan) was kept at 45 °C with a mobile phase of acetonitrile-methanol-0.2 mol/L ammonium acetate buffer (pH5.0)-purified water (10+30+5+55, v/v/v/v) and a flow rate of 1.0 mL/min. 100 µL of AFs standard solutions (0.05, 0.1, 1.0, 5.0, 10.0, 20.0 ng/mL for each AF) or test sample solutions was injected to HPLC system. The standard curves were prepared by plotting the peak height against the concentration of AF standards. Quantification of each AF in the test sample solution was performed by measuring peak height at retention time of each AF and comparing them with the relevant standard curves.

**Statistical analysis** The within laboratory precision (recovery, relative standard deviations (RSD) for within day and between day, HorRat) was deduced using the one-way analysis of variance (ANOVA) according to the AOAC guideline and the others.

**Results and discussion**

**Linearity of standard curves** As shown in Fig. 1, the standard curves and the linear regression equations for each AF in the range of 0.05–20.0 ng/mL (equivalent to 0.1 to 40 µg/kg of each AF in a sample) showed R² values greater than 0.9999, indicating good linearity.

**Optimization of extraction** A preliminary experiment was carried out to establish the optimal extraction solvent for AFB₁ from spiked chocolate samples. Since acetonitrile-water has been previously reported to give better mycotoxins recovery than methanol-water in mycotoxins analysis, acetonitrile-water (60+40, v/v) was firstly selected as extraction solvent for AFB₁ from chocolate samples. However, this solvent mixture was not applicable for chocolate samples, because the use of this solvent mixture resulted in phase separation showing the two separated layers by the salting out effect derived from sample ingredients as shown in Fig. 2A. Our data showed that most AFB₁ was concentrated in the upper layer (acetonitrile layer) and the recovery rate of AFB₁ was nearly to 150 % when the upper layer was used as a sample extract. In conclusion, this solvent mixture seemed to be inappropriate for analysis of AFs in chocolate samples because AFs accumulation in the upper layer can give unrealistic high recovery. The mixture of acetonitrile-water (90+10, v/v) adopted by the Japanese official method of analysis for AFB₁ was also tested as an extraction solvent, however, the
recovery rate of AFB₁ from a chocolate sample was only about 70 % due to hardly mix the sample with solvent.

Scott et al.⁵⁰ reported that the problem of phase separation was avoided by using a mixture of acetonitrile-methanol-water in the fumonisins analysis. According to this information, the extraction
was performed with different composition acetonitrile-methanol-water to get optimized recovery of AFB₁ from chocolate samples. Optimization was based on the following criteria: 1. Whether the extract after homogenization showed the homogeneous or the heterogeneous state. 2. Whether the filtered extract showed a single layer or two separated layers. 3. Whether the recovery rate of AFB₁ was acceptable or unacceptable. As shown in Table 1, with acetonitrile-methanol-water (70+10+30, v/v/v) and (80+10+20, v/v/v), both extracts after homogenization showed the heterogeneous state, and both extracts after filtration were separated into two liquid layers. With acetonitrile-methanol-water (60+10+40, v/v/v) and (70+15+30, v/v/v), both extracts showed the homogeneous state and a single layer (Table 1 and Fig. 2B), however, the recovery rate of the latter extraction mixture exceeded 100 %. Taking into account these results, acetonitrile-methanol-water (60+10+40, v/v/v) was adopted as extraction solvent in the method of analysis for AFs in dark chocolate.

**Chromatograms of chocolate samples** The chromatograms of chocolate samples spiked at 0.1 and 10.0 µg/kg for each AF were shown in Fig. 3. Each AF at 0.1 µg/kg spiking level was fully detected (Fig. 3A), and all AFs were well separated under the given conditions, and no interfering peaks were detected in the chromatograms.

<table>
<thead>
<tr>
<th>Composition of solvent</th>
<th>Condition after homogenization</th>
<th>The number of liquid layer</th>
<th>Recovery of aflatoxin B₁ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile Methanol Water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 10 40</td>
<td>homogeneous</td>
<td>1 layer</td>
<td>94</td>
</tr>
<tr>
<td>70 10 30</td>
<td>heterogeneous</td>
<td>2 layers</td>
<td>116</td>
</tr>
<tr>
<td>70 15 30</td>
<td>homogeneous</td>
<td>1 layer</td>
<td>109</td>
</tr>
<tr>
<td>80 10 20</td>
<td>heterogeneous</td>
<td>2 layers</td>
<td>113</td>
</tr>
</tbody>
</table>

Fig. 3. Chromatograms of chocolate samples spiked at 0.1 µg/kg (A), and 10.0 µg/kg (B) for each aflatoxin.
**Within laboratory precision**  The optimized method of analysis for AFs in chocolate was evaluated its within laboratory precision by spiked samples. Chocolate samples were spiked with AFs working standard solution at 0.1 and 10.0 µg/kg for each AF, and 5 replicates were analyzed for 4 days at each level. Table 2 and 3 show the results of the recovery and the within laboratory precision. The average recovery of AFs at 0.1 and 10.0 µg/kg spiking levels was 91 % and 91 % (B1), 94 % and 92 % (B2), 90 % and 92 % (G1), 97 % and 93 % (G2), respectively. The within day RSD at each level was 2.9 % and 2.4 % (B1), 1.9 % and 2.2 % (B2), 3.3 % and 2.6 % (G1), 1.7 % and 3.0 % (G2), respectively. The between day RSD at each level was 1.2 % and 0.6 % (B1), 4.1 % and 0.6 % (B2), 2.0 % and 1.2 % (G1), 2.1 % and 0 % (G2), respectively. The total RSD at each level was 3.1 % and 2.5 % (B1), 4.5 % and 2.3 % (B2), 3.8 % and 2.8 % (G1), 2.7 % and 3.0 % (G2), respectively. The HorRat for the total RSD at each level was 0.1 and 0.2 (B1), 0.1 and 0.1 (B2), 0.1 and 0.2 (G1), 0.1 and 0.2 (G2), respectively. In conclusion, it was proved that the developed method in this study was successfully applied to analysis for AFs in dark chocolate in the range of 0.1 and 10.0 µg/kg.

<table>
<thead>
<tr>
<th></th>
<th>Average recovery (%)</th>
<th>RSD (%)</th>
<th>HorRat</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Within day</td>
<td>Between day</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>91</td>
<td>2.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Aflatoxin B2</td>
<td>94</td>
<td>1.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Aflatoxin G1</td>
<td>90</td>
<td>3.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Aflatoxin G2</td>
<td>97</td>
<td>1.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*1: Relative standard deviation

<table>
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<tr>
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<th>RSD (%)</th>
<th>HorRat</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Within day</td>
<td>Between day</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>91</td>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Aflatoxin B2</td>
<td>92</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Aflatoxin G1</td>
<td>92</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Aflatoxin G2</td>
<td>93</td>
<td>3.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*1: Relative standard deviation

References

5 ) http://www.mhlw.go.jp/topics/yunyu/tp0130-1.html, Ministry of Health, Labor and Welfare, Japan  

8) Application Note: Aflatoxin extraction method (2001), R-Biopharm Rhône, Glasgow, Scotland


10) http://www.aoac.org/method_validation/Addenda/, AOAC International, MD, USA


15) Notification No. 326001 (March 26, 2002), Director of Inspection and Safety Division, Department of Food Safety, Ministry of Health, Labor and Welfare, Japan


チョコレート中のアフラトキシン分析法の検討

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チョコレート中のアフラトキシン（アフラトキシン B\textsubscript{1}, B\textsubscript{2}, G\textsubscript{1}, G\textsubscript{2} ; AFs）分析法を確立し，室内再現精度の確認を実施した。チョコレート中のAFsは，アセトニトリル - メタノール - 水（60+10+40, v/v/v）で抽出し，イムノアフィニティカラムにより精製後，蛍光検出HPLCにて定量を行った。試料にAFs標準液を0.1および10.0 µg/kgの濃度となるように添加し，日内および日間における繰り返し試験を行った結果，全てのAFsにおける回収率は90 - 97 ％，併行再現性の相対標準偏差は1.7 - 3.3 ％，日間再現性の相対標準偏差は0 - 4.1 ％，異日分析における室内再現性の相対標準偏差に対するHorRatは，全て0.2以内であった。以上の結果から，チョコレート中のAFsは本法により精度良好分析できる事が確認された。

キーワード：アフラトキシン，カカオ，チョコレート，イムノアフィニティクリーンアップカラム，高速液体クロマトグラフィー，室内再現精度