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Stability Of Aflatoxins In Different Solvent Concentration And Temperature In The Autosampler

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ABSTRACT: *The stability of aflatoxins (B1, B2, G1, and G2) was studied in solutions containing methanol of 30 % and 100% at different temperatures in the ASR. When injecting 5h intervals for 15 h a diluted standard at RT in the ASR, aflatoxins G1, G2, B1, and B2 were stable only in a solution containing 100% methanol. While in 30% methanol, although Aflatoxin B1 and B2 at 15h a little unstable than 100% methanol showed aflatoxin G1 and G2 were significant decreases in the same condition. At 5°C in the ASR, aflatoxins G1, G2, B1, and B2 were stable regardless of solvent concentrations. Our results indicate that aflatoxins B1, B2, G1 and G2 showed a significant stable in only 100% methanol than 30% methanol regardless of the temperature in the ASR. In addition, significant loss of aflatoxins in 30% methanol was higher at 22 ℃ than 5°C in the ASR. Aflatoxins are significantly unstable in aqueous solutions, and this instability can be offset by the presence of at least 30% methanol and keeping the solutions at 5°C in the ASR.*

KEYWORDS: *Aflatoxins, HPLC-FLD, Stability, Temperature, Toxigenic*

I. Introduction

Mycotoxins are natural toxic secondary metabolites produced by various filamentous fungi species (sp) such as *Aspergillus*, *Penicillium*, and *Fusarium* on food commodities in the field and during storage under a variety conditions of moisture, temperature, and nutrients (1, 2). Over 300 mycotoxins are, most chemically stable and not easily eliminated during food processing, commonly present as contaminants in commodities of various kinds including cereals, nuts, herbal teas, spices, vegetables, fruits, and medicinal plants (3, 4).

Mycotoxins are contamination of food and feed, threaten human and animal health, an ongoing global concern to safety. Many national and international public health and governmental authorities such as the US Food and Drug Administration (FDA), World Health Organization (WHO), Food Agriculture Organization (FAO), and the European Food Safety Authority (EFSA), and the Ministry of Good and Drug Safety (MGDS), established guidelines for major mycotoxin classes in food and feed and about 100 countries have regulated a maximum contamination level on the presence of major mycotoxins in food and feed (1, 3).

Aflatoxins (AFs) were the first discovered in the 1960s in the United Kingdom via over 100,000 turkeys died by ground nut meal infected with *A. flavus*, and which are known as the most toxic among the toxic mycotoxins (5, 6). There are more than 20 different types of aflatoxins, only the four major ones are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFBG1), and G2 (AFG2) (7). Chemical structures of naturally occurring four aflatoxins are shown in Fig 1 (1).

AFB1 and AFB2 are produced by *Aspergillus flavus* while AFB1, AFB2, AFG1, AFBG2 are produced by *Aspergillus parasiticus* (8, 9). Aflatoxin M1 (AFM1) is hydroxylated form of AFB1, that is found in milk and dairy products (10). AFB1 is the most potent mycotoxins and leading to hepatocellular carcinoma (HCC) by suppressing system activity of the immune system (11, 12). In 1988, International Agency for Research on Cancer (IARC) classified the aflatoxin B1 as Group 1 carcinogen (carcinogenic to humans) (13, 14).

To qualitative and quantitative determination of AFs used detection techniques of various kinds including high-performance liquid chromatography (HPLC) with fluorescence detection (FLD); enzyme immunoassays (EIAs); liquid chromatography–tandem mass spectrometry (LC-MS/MS); gas chromatography–

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mass spectrometry (GC-MS) techniques (15). Different analytical methods and instruments develop rapidly, sensitive to detect the presence of mycotoxins.

In Korea, aflatoxins analysis in Korean Food Standards Codex (KFSC) and Korean Pharmacopoeia (KP) are based on HPLC separation coupled with FLD. KFSC using the derivatization method by trifluoroacetic acid (TFA) during pretreatment of samples and KP using the pyridinium hydrobromide perbromide (PBPB), photochemical reactor for enhanced detection (PHRED) or Kobra cell (KOBRA) systems of post-column derivatization method for increase fluorescence sensitivity (16, 17).

We were used post-column derivatization method using PHRED of KP for recoveries of AFs in herbal medicine. However, we have been found that the area counts of aflatoxin standards decreased within hours in elution solvent of sample at 22 ℃ in the autosampler (ASR), but the aflatoxin standards diluted in 100% methanol was not decreased. Also, we found that different methanol ratios used elution solvent of sample and external standard. In KP, add 0.5 ml methanol 3 times in the last step of elution on the immunoaffinity column of samples and adjust the final 5 mL volume with pure DW (final con. 30% methanol). Even under the conditions of instrument analysis, there were no precautions such as temperature or amber vial during analysis (17).

In the present study, we were to determine the stability of aflatoxins in solutions containing water used when elution and only 100% methanol at 5°C and 22 ℃ in the ASR.

II. Experimental

2.1. Reagents and materials

Aflatoxin standards B1 (purity 98.8 %), B2 (purity 97.0 %), G1 (purity 97.7 %), G2 (purity 97.8 %) were supplied by Romer Laboratories (MO, USA). HPLC grade methanol was purchased from J.T.Baker (NJ, USA) and water was of Milli-Q quality (Millipore, Bedford, USA).

2.2 Working solutions

All working solutions contained 300 ng/mL and the solution diluted in 30 and 100% methanol to be tested, respectively.

2.3 Stability experiments

The solvent systems and temperatures in the ASR evaluated for aflatoxins stability were 30% methanol, 100% methanol, and 5 ℃, 22 ℃ in the ASR.

For each experimental treatment, a triplicate set of solutions were prepared in amber vials to cut off the light, and each solution was injected time 0, 5, 10 and 15 h intervals after preparation at 5℃ and 22℃. In addition to these experiments, the degradation kinetics of the four aflatoxins were investigated by repeatedly injecting in 25 min intervals for over 20 h a working standard including 30 and 100% methanol at 5℃ and 22 ℃.

2.4 Anayltical condition of HPLC-FLD analysis

Aflatoxins were quantified using an HPLC equipped with a fluorescence detector (Ultimate 3000 UHPLC, Thermofisher Scientific, USA), all controlled by chromeleon software.

Isocratic mobile phase was Water:Methanol:Acetonitrile (6:3:1) and the column was Capcell Pak C18 UG120 (250 mm × 4.6 mm id, 5 μm: Osaka soda, Osaka, Japan). The column temperature was kept at 35 ℃, and the flow rate was 1.0 ml/min. The injection volume was 10 uL. The wavelengths for excitation and emission were 365 nm and 435 nm, respectively.

For enhanced detection, aflatoxins were derivatized postcolumn by means of a PHRED (Aura, New York, USA) consists of a lamp holder, a 254 nm low-pressure mercury lamp, and a holder for the knitted reaction coils (25 m length × 0.5 mm id) and positioned between the column and the detector.

2.5 Statistical Analysis

For 30 and 100% methanol tested, aflatoxins concentration at time 0, 5, 10, and 24 h were compared by means of a student's *t*-test. All statements of significance were based on $P < 0.05$.

III. Results and Discussion

Table 1 summarizes the results of the aflatoxin stability tests in 30% methanol and 100% methanol for 0, 5, 10, and 15h at 22℃ in in the ASR. Table 1 is a significant decrease in AFG1 and AFG2 were observed in all cases where the solutions contained water at 22℃ in the ASR. However, when AFG1 and AFG2 prepared in 100% methanol were did not differ from corresponding control standards at 22℃ in in the ASR.

Table 2 summarizes the aflatoxin stability for 0, 5, 10, and 15 h intervals at 5℃ in the ASR. AFs were no significant decrease regardless of standard concentrations and times in 30% and 100% methanol.

Fig 2(a, b) shows the degradation profiles of AFs standard during an over 21 h period when kept at 22℃ in in the ASR. AFB1, AFB2, AFG1, and AFG2 remained constant for up to an hour. However, AFB1 and AFB2 were remained modest decrease from 1h to 21h, while especially AFG1 and AFG2 were significantly showed loss of peak areas (Fig 2a).

After 21 h, the rate of loss AFB1, AFB2, AFG1, and AFG2 in 30% methanol were 7.8, 11.4, 48.6 and 56.3%, respectively. But, AFs were not decreased in 100% methanol as Fig 2(b).

At 5℃, Fig 2(c) shows the rate of loss AFB1, AFB2, AFG1, and AFG2 in 30% methanol were 4.1, 2.2, 9.8 and 9.6%, respectively. However, AFB1, AFB2, AFG1, and AFG2 in 100% methanol were no significant difference in concentration (Fig 2d). Although AFs were a little decreased in 30% methanol at 5℃, which was stable than AFs in 30 % methanol at 22℃.

Fig 3(a) shows the first and last chromatograms obtained the same concentration in 30 % methanol at 22℃ in in the ASR, which the drop-in area can be seen as well as the development of an unknown peak that appeared at 8.7 ~ 11.25 min. Fig 3(b, d) shows the same concentration in 100% methanol at 22℃ and 100% methanol at 5℃ in in the ASR which could not show any difference between the peaks and were not found unknown peaks. While Fig 3(c) observed the slightly decreased the peaks areas for AFG1 and AFG2 by 30% methanol containing water or room temperature (RT) as Fig 2(c), but till 21 h the remaining the aflatoxin standards of 90%. When aflatoxins were 100% methanol or cold temperature (CT) in the ASR, no significant difference in aflatoxin concentration without degradation, only the rate of loss of aflatoxins were observed significant instability within hours in water solutions containing 30 % methanol at 22℃ in the ASR.

Aflatoxins are a group of difurocoumarolactones (difurocoumarin derivatives) that are produced by many strains of *Aspergillus* species, which chemistry structure consists of a coumarin nucleus fused to a bifuran and a pentanone ring in aflatoxins B, and aflatoxins G is the presence of a six-membered lactone ring instead of a pentanone ring (18). Diaz et al. reported that when stored at 20℃ for 24 h, aflatoxins G1 and G2 were unstable in solutions containing aqueous solutions, only stable in 100% organic solvent. At 5°C, aflatoxins G1 and G2 showed a significant decrease in concentration only when kept in less than 20% aqueous organic solvent (19). Elizalde-González et al. who reported that AFG1 in methanol–water (1:1, v/v) solution is electrochemically oxidized at a higher rate than AFB2. It might be possible that the six-membered lactone ring is more susceptible to oxidation and degradation, which could explain the higher rate of loss of G aflatoxins compared with B aflatoxins (20).

The rate of degradation observed for all aflatoxins at RT, and especially for AFG1 and AFG2, has important implications for the accurate quantification of these analytes in contaminated food or feed. Beaver reported that no loss of peak area was observed over for 3100 minutes for solutions stored in the freezer (-18℃) in MeCN:H_2O (50: 50). However, aflatoxin G1 and G2 showed substantial degradation within 290 minutes when stored in the freezer in 50:50 MeOH:H_2O. At 22°C, aflatoxin B1, B2, G1, and G2 was extensively degraded in both 50:50 MeCN:H_2O and 50:50 MeOH:H_2O. Especially, G toxins were rapidly and extensively degraded where the MeCN and MeOH contained 50 % water (21).

Interestingly, a significant degraded in G aflatoxins concentration observed when used solution containing water and RT, but 30% methanol was slightly decreased in CT. However, only when no different from their corresponding control standard of G aflatoxins used 100% methanol as a solution regardless of CT and RT.

IV. Conclusions

In this study, indicated that the G aflatoxins are much more unstable than the B aflatoxins in 30% methanol at 22℃ than 5℃. While AFs are more stable in 100% methanol regardless of temperature in the ASR. Therefore, if the samples are elution by the KP method, it becomes 30% methanol, so the aflatoxin decrease will be if the ASR is used in a CT rather than RT in the ASR.

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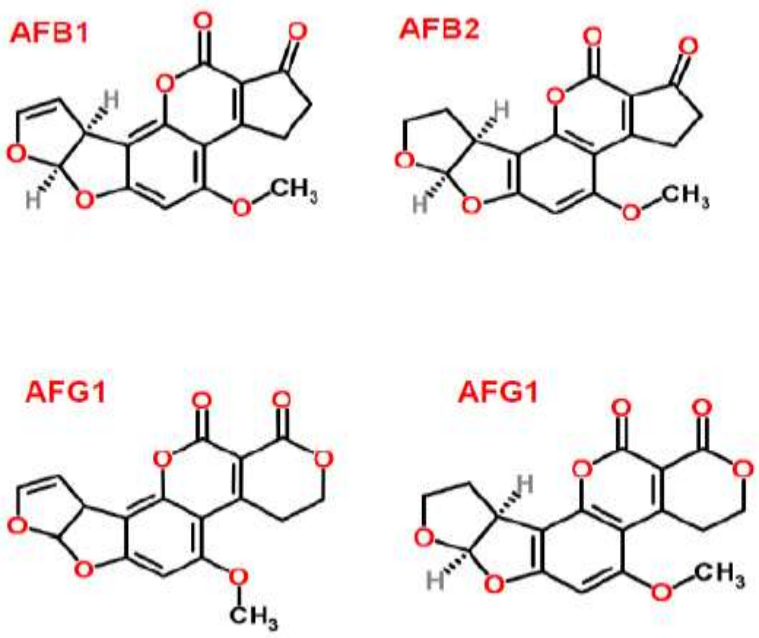

Figure 1. Chemical structures of four major aflatoxins.

Table 1. Effect of 30 % methanol and 100% methanol on AFB1, AFB2, AFG1, and AFG2 stability during 0, 5, 10, and 15 h at 22 ℃ in ASR

Methanol %	Aflatoxins	ng/mL RT						
		0h	5 h	%[a]	10 h	%[a]	15 h	%[a]
30	AFB1	0.988	0.910 ± 0.05[b]	92	0.907 ± 0.05[b]	92	0.862 ± 0.02[b]	87
		4.94	4.669 ± 0.13[b]	95	4.548 ± 0.24[b]	92	4.422 ± 0.21[b]	90
		49.4	48.971 ± 0.64[b]	99	49.251 ± 0.51[b]	100	49.156 ± 0.86[b]	100
	AFB2	0.97	0.927 ± 0.04[b]	96	0.890 ± 0.02[b]	92	0.876 ± 0.02[b]	90
		4.85	4.623 ± 0.10[b]	95	4.509 ± 0.19[b]	93	4.438 ± 0.17[b]	92
		48.5	47.920 ± 0.33[b]	99	48.202 ± 0.36[b]	99	48.254 ± 0.49[b]	99
	AFG1	1.954	1.374 ± 0.16[b]	70	0.988 ± 0.26[b]	51	0.790 ± 0.23[b]	40
		9.77	6.846 ± 1.49[b]	70	4.688 ± 1.64[b]	48	3.456 ± 1.40[b]	35
		97.7	86.147 ± 7.68[b]	88	79.271 ± 9.03[b]	81	72.618±9.98[b]	74
	AFG2	1.956	1.423 ± 0.18[b]	73	1.134 ± 0.25[b]	58	0.876 ± 0.14[b]	45
		9.78	7.267 ± 1.16[b]	74	5.343 ± 1.62[b]	55	4.119 ± 1.28[b]	42
		97.8	88.010 ± 6.55[b]	90	82.233 ± 6.76[b]	84	76.872 ± 6.12[b]	79
100	AFB1	0.988	1.005 ± 0.04[b]	102	1.019 ± 0.03[b]	103	1.008 ± 0.01[b]	102
		4.94	5.057 ± 0.09[b]	102	5.082 ± 0.10[b]	103	5.164 ± 0.05[b]	105
		49.4	50.949 ± 0.31[b]	103	51.658 ± 0.27[b]	105	51.912 ± 0.89[b]	105
	AFB2	0.97	0.969 ± 0.01[b]	100	0.986 ± 0.01[b]	102	0.988 ± 0.01[b]	102
		4.85	4.899 ± 0.03[b]	101	4.952 ± 0.03[b]	102	4.978 ± 0.03[b]	103
		48.5	49.395 ± 0.32[b]	102	49.999 ± 0.29[b]	103	50.105 ± 0.92[b]	103
	AFG1	1.954	1.997 ± 0.05[b]	102	1.966 ± 0.07[b]	101	1.981 ± 0.06[b]	101
		9.77	9.818 ± 0.07[b]	100	9.888 ± 0.17[b]	101	9.827 ± 0.22[b]	101
		97.7	98.904 ± 1.06[b]	101	100.255 ± 1.34[b]	103	99.711 ± 1.12[b]	102
	AFG2	1.956	1.960 ± 0.01[b]	100	1.977 ± 0.01[b]	101	2.004 ± 0.02[b]	102
		9.78	9.855 ± 0.02[b]	101	9.922 ± 0.07[b]	101	10.019 ± 0.12[b]	102
		97.8	99.240 ± 1.07[b]	101	100.909 ± 0.85[b]	103	101.188 ± 2.00[b]	103

[a] % corresponds to the percentage of aflatoxin remaining after 5, 10, and 15 h.
[b] Values are mean ± SD of three replicate observations/treatment. For each comparison (0 versus 5, 10, and 15 h), means with different superscripts are significantly different (P <0.05).

Table 2. Effect of 30 % methanol and pure methanol on AFB1, AFB2, AFG1, and AFG2 stability during 0, 5, 10 and 15 h at 5°C in ASR

Solvent %	Aflatoxins	ng/mL CT						
		0h	5 h	%[a]	10 h	%[a]	15 h	%[a]
30	AFB1	0.988	0.992 ± 0.04[b]	100	0.978 ± 0.04[b]	99	0.997 ± 0.02[b]	101
		4.94	4.928 ± 0.00[b]	100	4.920 ± 0.02[b]	100	4.912 ± 0.04[b]	99
		49.4	49.365 ± 0.20[b]	100	49.376 ± 0.44[b]	100	49.512 ± 0.14[b]	100
	AFB2	0.97	0.962 ± 0.02[b]	99	0.953 ± 0.02[b]	98	0.962 ± 0.01[b]	99
		4.85	4.820 ± 0.05[b]	100	4.846 ± 0.01[b]	100	4.825 ± 0.03[b]	99
		48.5	48.417 ± 0.13[b]	100	48.549 ± 0.07[b]	100	48.439 ± 0.13[b]	100
	AFG1	1.954	1.883 ± 0.08[b]	96	1.820 ± 0.07[b]	93	1.849 ± 0.10[b]	95
		9.77	9.557 ± 0.37[b]	98	9.468 ± 0.51[b]	97	9.349 ± 0.56[b]	96
		97.7	97.368 ± 0.26[b]	100	97.266 ± 0.48[b]	100	96.903 ± 1.12[b]	99
	AFG2	1.956	1.944 ± 0.02[b]	99	1.919 ± 0.06[b]	98	1.873 ± 0.05[b]	96
		9.78	9.668 ± 0.16[b]	99	9.585 ± 0.28[b]	98	9.504 ± 0.40[b]	97
		97.8	97.535 ± 0.45[b]	100	97.259 ± 1.14[b]	99	97.141 ± 1.51[b]	99
100	AFB1	0.988	0.987 ± 0.01[b]	100	0.991 ± 0.04[b]	100	1.024 ± 0.02[b]	104
		4.94	4.943 ± 0.02[b]	100	4.884 ± 0.06[b]	99	4.885 ± 0.11[b]	99
		49.4	49.620 ± 0.16[b]	100	49.790 ± 0.18[b]	101	49.439 ± 0.53[b]	100
	AFB2	0.97	0.953 ± 0.01[b]	98	0.953 ± 0.01[b]	98	0.954 ± 0.00[b]	98
		4.85	4.853 ± 0.01[b]	100	4.845 ± 0.04[b]	100	4.827 ± 0.06[b]	100
		48.5	48.521 ± 0.12[b]	100	48.498 ± 0.17[b]	100	48.226 ± 0.66[b]	99
	AFG1	1.954	1.944 ± 0.05[b]	99	1.948 ± 0.03[b]	100	1.954 ± 0.06[b]	100
		9.77	9.770 ± 0.05[b]	100	9.754 ± 0.03[b]	100	9.814 ± 0.01[b]	100
		97.7	97.410 ± 0.82[b]	100	97.172 ± 1.06[b]	99	96.971 ± 1.32[b]	99
	AFG2	1.956	1.943 ± 0.02[b]	99	1.925 ± 0.01[b]	98	1.955 ± 0.05[b]	100
		9.78	9.959 ± 0.42[b]	102	9.937 ± 0.46[b]	102	9.900 ± 0.47[b]	101
		97.8	97.796 ± 0.45[b]	100	97.888 ± 0.41[b]	100	97.048 ± 1.65[b]	99

[a] % corresponds to the percentage of aflatoxin remaining after 5, 10, and 15 h.

[b] Values are mean ± SD of three replicate observations/treatment. For each comparison (0 versus 5, 10, and 15 h), means with different superscripts are significantly different (P <0.05).

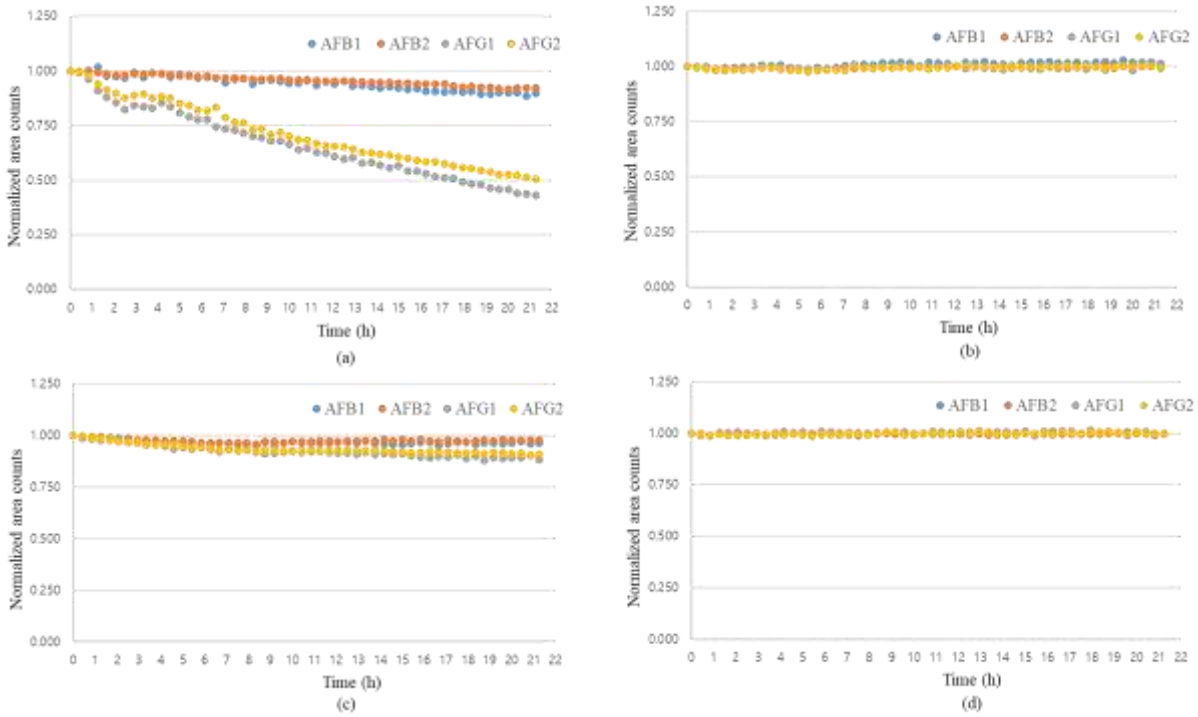

Figure 2. Comparison of degradation kinetics of aflatoxins. AFB1 4.94 ng/mL, AFB2 4.85 ng/mL, AFG1 9.77 ng/mL and AFG2 9.78 ng/mL in 30% methanol and 100% methanol were injected at 25 min intervals over 21 h; (a) 30% methanol at 22°C (b) 100% methanol at 22°C (c) 30% methanol at 5°C (d) 100% methanol at 5°C in ASR.

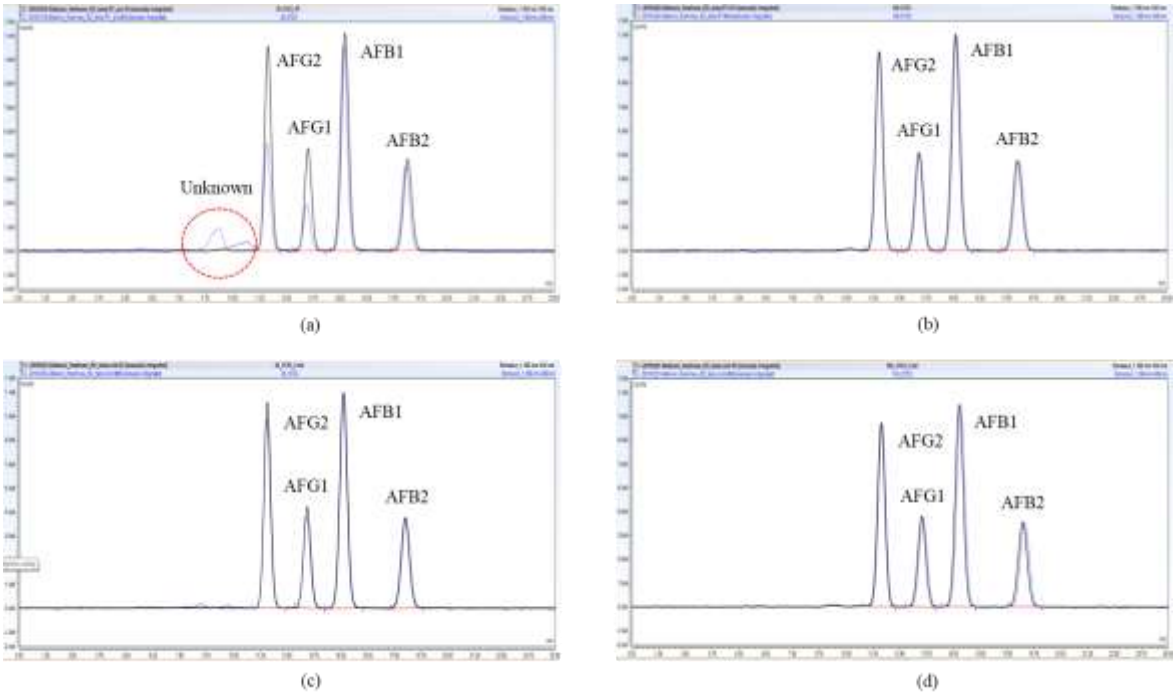

Figure 3. Comparison of chromatograms first and last injection of aflatoxins used for degradation kinetics. 30% methanol at 22°C (b) 100% methanol at 22°C (c) 30% methanol at 5°C (d) 100% methanol at 5°C in ASR. Unknown peaks appeared at 8.7 ~ 11.25 min.