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Comparative Study on the HPLC Determination of Aflatoxins Coupled with Extraction and Clean-up Methods

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Aflatoxin 분석법에 관한 연구; 추출 및 정제방법의 비교

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ABSTRACT－Extraction and Clean-up procedures coupled with quantitation by high performance liquid chromatography(HPLC) was evaluated for the detection of 4 aflatoxins, B_1, B_2, G_1 and G_2, in peanut butter. The Sep-Pak clean-up method showed poorer separation and repeatability than did the modified DeVries' and an immunoaffinity column clean-up methods. No significant difference of detected aflatoxins between the affinity column clean-up and the modified DeVries' method. The coefficients of variation for the 4 aflatoxins were ranging from 6.3～32.3 by the modified DeVries' method and 5.3～9.8 by the affinity column clean-up.

Keywords □ Aflatoxins, extraction and clean-up methods, HPLC

Aflatoxins are carcinogenic secondary metabolites of *Aspergillus flavus* and *Asp. parasiticus* which contaminate and grow in many agricultural products.[1] Because aflatoxins normally occur at residue (ppb) levels, an awareness of the concentrations of aflatoxins in crops and biological samples can only be obtained by developing good analytical methodologies.

There are no uniform methods of analysis collectively or for a specific toxin in various samples. However the analytical procedures for detecting aflatoxins will generally follow the same flow pattern of sampling, extraction, clean-up, separation, detection and quantitation and finally confirmation.[2] Thin layer chromatography(TLC) and high performance liquid chromatography(HPLC) methods for quantifying ppb levels of aflatoxins require that product extracts be cleaned up before analysis. Typical procedures require large volumes of organic solvents and take much time.[3-5] In nowadays the 4 main aflatoxins, B_1, B_2, G_1 and G_2 can be readily identified qualitatively and quantitatively and most current investigations concentrate on increasing sensitivity, accuracy and reproducibility and decreasing time of analysis.

The principle of the immunologically based methods[6-13] is that the formation of bonds between the toxin and the antibody immobilized on a solid phase. Of these methods the immunoaffinity columns are particulary attractive; no specialized apparatus is required and final determinations can be made with high performance liquid chromatograph. In this study, HPLC determination coupled with Sep-Pak C_{18}, an immunoaffinity column and a traditional extraction and clean-up methods for aflatoxins was evaluated and compared.

Materials and Methods

Reagent

All reagent used were analytical grade and

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HPLC grade acetonitrile and methanol were used as HPLC eluents. Aflatoxin standards were purchased from Supelco, Inc.(Bellefonte, PA, U.S.A)

Apparatus

Sep-Pak C_{18}(Millipore, Beaford, MA, U.S.A) and an immunoaffinity column, Aflatest(Vicam, Rhône-Poulenc Diagnostics Ltd., Glasgow G_1, Scotland), was used for clean-up. The affinity column, 1 m*l* syringe-barrel column which contains immobilized monoclonal antibodies to aflatoxins B_1, B_2, G_1 and G_2 is filled with preserved buffer solution.

Chromatographic equipment used was Waters Associates(Milford, Mass., U.S.A) HPLC system. A Waters model 510 solvent delivery system equipped with a Waters U6K injector, a Waters model 470 fluorescence detector with a 360 nm excitation filter and a 440 nm emission filter were utilized for the analysis. A waters Nova-Pak C_{18}(15 cm×3.9 mm ID) column was used for the separations at room temperature. The mobile phase was a mixture of water, methanol, and acetonitrile(60 : 20 : 20, v/v) and eluent combined water, acetonitrile and acetic acid(1 : 100 : 10, v/v). The flow rate for the solvent system was 1 m*l*/min.

Extraction and clean-up

For the traditional extraction and purification of aflatoxins from a contaminated peanut butter sample, the procedure developed by DeVries *et al.*, was followed. In brief, 25 g of sample was extracted with 100 m*l* of methylene chloride and filtered. The amount of final extract which was injected into HPLC was correspond to 30.0 m*l* of methylene chloride extract. For the affinity column clean-up, 25 g of sample and 2.5 g of sodium chloride were placed in a 1 *l* blender jar. 125 m*l* of 60% methonal were added and the mixture was blended at high speed for one minute. The extract was diluted with 125 m*l* of distilled water and filtered through filter paper(Whatman No. 4). 10 m*l* of filtrate was transfered into the glass syringe barrel and pushed through the affinity column at flow rate of ca 1 drops/sec(3 m*l*/min) by using the pump unit. 10 m*l* of distilled water was added to the syring barrel and washed the column by passing the water. 1.0 m*l* of HPLC grade methanol was added into the syringe barrel and eluted through the affinity column. For the Sep-Pak clean-up, 10 m*l* of the above filtrate was eluted through the Sep-Pak C_{18} by using a 10 m*l* syring. The methylene chloride extract, eluate from affinity column and Sep-Pak were evaporated to dryness under the stream of nitrogen at room temperature and used for HPLC analysis.

HPLC determination

The amount of aflatoxins in extracts was determined by HPLC. A working standard solutions of aflatoxin and the extracts were treated with trifluoracetic acid(TFA) to aid detection of aflatoxin B_1 and G_1 by converting them to the more intensely fluorescence B_{2a} and G_{2a}. 50 μ*l* of TFA was added to the dried extract and stirred with a vortex mixer. After 15 minutes, 4.0 m*l* of injection solvent was added and mixed again and 10 μ*l* of the mixture was injected into HPLC.

Results and Discussion

Tables 1, 2, 3 and 4 show the aflatoxin B_1, B_2, G_1 and G_2 concentrations found for the peanut butter sample. HPLC coupled with the three extraction and clean-up procedures provided information on all four aflatoxins.

The Sep-Pak C_{18} clean-up showed poor repeatability and separation as shown in Table 3 and Fig. 1. Comparative analyses between immunoaffinity column clean-up and the modified DeVries' method, the affinity column clean-up gave more con-

Table 1. Determination of aflatoxins in peanut butter by HPLC with modified DeVries' method

Trial	Aflatoxin, ng/g			
	B_1	B_2	G_1	G_2
1	6.33	2.06	5.94	2.46
2	5.80	2.03	6.18	2.02
3	7.08	3.44	5.46	2.74
Mean± S.D.	6.40± 0.64	2.51± 0.81	5.86± 0.37	2.41± 0.36
CV, %	10.0	32.3	6.3	15.0

Table 2. Determination of aflatoxins in peanut butter by HPLC with immunoaffinity column

Trial	Aflatoxin, ng/g			
	B_1	B_2	G_1	G_2
1	6.33	2.87	6.92	2.49
2	6.11	2.99	5.72	2.47
3	6.70	2.70	6.11	2.23
Mean± S.D.	6.48± 0.46	2.85± 0.15	6.25± 0.61	2.40± 0.15
CV, %	7.1	5.3	9.8	6.3

Table 3. Determination of aflatoxins in peanut butter by HPLC with Sep-Pak C_{18}

Trial	Aflatoxin, ng/g			
	B_1	B_2	G_1	G_2
1	6.99	3.39	4.62	1.39
2	7.70	1.27	4.24	0.70
3	8.32	2.04	3.43	1.58
Mean± S.D.	7.67± 0.67	2.23± 1.07	4.10± 0.61	1.23± 0.46
CV, %	8.7	48.0	14.9	37.4

stant data though there were no significant difference between the results by the two methods. In the statistical comparison of precision, the affinity column clean-up showed relatively lower standard deviation and coefficient variation than did the modified DeVries' method and required smaller amount of analytical time and labor.

Results of this study indicate that the immunoaffinity column clean-up method provides results equivalent to the traditional method described. In order to apply this procedure, it is better for us to check the recovery rates of each aflatoxin in spiked samples. Trucksess *et al.*[14] recommended that the recoveries for a standard aflatoxin

Table 4. Comparative analyses of aflatoxins in peanut butter by extraction and clean-up method

Method	Aflatoxin, ng/g			
	B_1	B_2	G_1	G_2
Modified DeVries' method	6.40^{NS}	2.51^{NS}	5.86^{NS}	2.41^{a}
Immunoaffinity column	6.48	2.85	6.25	2.40^{a}
Sep-Pak C_{18}	7.67	2.23	4.10	1.23^{b}

NS: No significant difference was found among groups. Values in the same column followed by different superscript letters are significantly different ($p<0.05$).

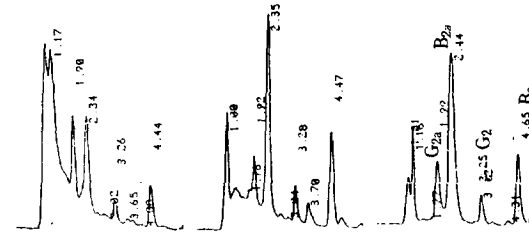

Fig. 1. HPLC chromatogram of aflatoxins of derivatized extract from peanut butter. Sample analysis with #1; Sep-Pak C_{18}, #2; modified DeVries' *et al.* and #3; Immunoaffinity column.

solution of 15 ml CH_3OH-H_2O(3+1) containing 25 ng B_1, 5 ng B_2, 15 ng G_1 and 5 ng G_2 should be at least 90, 80, 90 and 60%, respectively.

The combination of the highly specific monoclonal antibodies in the affinity column and HPLC could bring successful identification for aflatoxins in wide variety of foods and feeds. We can see this from the fact many laboratories are actively pursuing this approach for shorter assay time and minimum possibility of hazard.

국문요약

HPLC를 이용하여 aflatoxin B_1, B_2, G_1 및 G_2를 분석함에 있어서 Sep-Pak C_{18}, immunoaffinity column 및 DeVries 등의 변법에 의한 추출 및 정제방법을 비교하였다. DeVries 등의 변법 및 immunoaffinity column에 의한 땅콩 버터에서 aflatoxins 분석결과 사이에는 유의한 차이가 없었으며 aflatoxin B_1, B_2, G_1 및 G_2 측정치의 변이계수가 각각 6.3~32.3 및 5.3~9.8로 나타났다. Sep-Pak C_{18}에 의한 추출 및 정제 결과는 분리도 및 반복 측정치가 두가지 방법보다 불량하였다.

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