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Detection of Aflatoxins in Soybean Food by HPLC

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고속액체 크로마토 그라피에 의한 대두식품중 아플라톡신의 검출

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Abstract

Aflatoxin B_1, B_2, G_1, and G_2 were quantitatively detected by the high pressure liquid chromatography on a Micropak-CN column, with Hexane-THF-IPA-water, using a Lichrosorb-packed flowcell in the fluorometric detector. Under those conditions, the minimum detectable amount of aflatoxin B_1 was 0.2 *ng*. HPLC was used in determining amount of aflatoxins in the commercially manufactured soybean food and home-made *Meju*. Aflatoxin producing abilities of strains used in the industrially fermented soybean food were also studied with the HPLC technique. Although aflatoxin-like substances were detected in a few samples on TLC, they were not identified with the HPLC retention times of standard aflatoxins. The commercial fungal strains used in Korea had no aflatoxin producing abilities.

Introduction

Aflatoxins are known to be produced by *Aspergillus flavus* and *A. parasiticus*. One of these metabolites, aflatoxin B_1, is the most potent liver carcinogen known for rat and has been associated with the high human liver cancer incidence in certain part of Africa.

The *A. flavus* group is a member of the microflora in air and soil, and is found on or in living or dead plants and animals throughout the world. *Aspergillus flavus* appears to compete best in the microbiological world when the water activity is relatively low (0.84~0.86) and the temperature relatively high (25°~40°C).

The presence of extensive mold growth cannot be equated with the presence of aflatoxins since some strains do not produce aflatoxins and producing strains can be affected by the presence of competing microorganisms.[1] Food and feed are maintained after contaminated at a moisture content and temperature that permits rapid growth of storage fungi, however, they may be invaded heavily within a few days, sometimes within a few hours.[2] Food and feed are frequently contaiminated with molds during harvesting, storage and handling before reaching the consumer. A number of labroatories performing the analyses reported interference problems with the methods and the need for simplification for routine work. The recent rapid development of the knowledge concerning the aflatoxins has led to an increasing awareness of the potential chemical environmental contaminants. *Meju* is a raw material of the Korean traditional fermented foods such as soybean paste, soy sauce, and hot sauce. The procedure involves molding of the boiled soybean with common species of fungi. The molds supply necessary enzymes for converting the protein and starch to amino acid, sugar and flavorous materials.

Several studies on the analysis and occurrence of aflatoxins in food and feed have been reported.[3-24] Manabe *et al.*[3] reported that the aflatoxin-producing ability of molds was examined on 136 strains used in fermented food industries in Japan, but no aflatoxin was produced by the molds examined. Manabe and Matsuura[4] reported that the possibility of mycotoxin production by fungus during fermen-

tation of miso and shoyu were examined. However, their result showed that there was not any single case of the mycotoxin contamination during the fermentation. Wei *et al.*[5] reported that the determination of aflatoxins in soy sauce and fermented soybean paste by HPLC with UV detection, but no aflatoxin could be detected in any of the sampls. Lee *et al.*[6] examined the content of aflatoxins in 15 samples of *Meju*. On TLC, uncertain fluorescent spots were appeared in some (6/15) samples. Since the substances giving such spots differed clearly from aflatoxins on their ultraviolet absorption and on Tollen's reaction, they concluded that the fluorescent spots were not aflatoxins, but aflatoxin-like substance. Jung and Kwon[7] examined the content of aflatoxins in 35 samples of bean, *Meju*, soybean paste and soysauce. On TLC, they insisted on the presence of aflatoxin G_1, G_2 in *Meju* and soybean paste. They reported that aflatoxin B_1, G_2, G_1, and G_2 were produced in the strain *(Aspergillus flavus)* from sample. Kim *et al.*[8] reported the detection of alfatoxins in some Korean foodstuffs. They reported that Aflatoxin B_1, B_2, and G_2 were detected in soybean paste and aflatoxin B_1 was identified by TLC and chicken embryo bioassay.

The serious disadvantage in the TLC approach is the lack of quantitative accuracy due to the commonly used visual estimation technique. Since fermented soybean foods are complex mixtures, TLC gives a complicated chromatogram. It is extremely difficult to determine aflatoxins in fermented soybean foods on TLC plates by visual estimation. In aflatoxin analyses by TLC procedures, there are certain problems that are posed by the photochemical decomposition of aflatoxin and harmful vapors in the air. These problems have led a number of investigators to examine HPLC for aflatoxin analyses. The major advantages of HPLC include high resolution, speed, sensitivity, automatic operation, and range of applications unequaled by any other technique.[9] HPLC has been widely applied to the separation and determination of aflatoxin. Normal phase HPLC methods for aflatoxin separation have been developed, and applied to the determination of aflatoxins in several agricultural products of plant origin.[11, 14, 16, 18, 20, 21]

Packed cell in fluorometric detection methods has also been developed to increase the sensitivity.[12, 15, 22] But no analytical procedure by HPLC has been described for aflatoxins in *Meju* and fermented soybean food in Korea. Therefore, it was our attempt to study the applicability of the HPLC method to the several Korean soybean food, to check the aflatoxin producing abilities of strains used in the fermented soybean foods in Korea, and to find any suggestions to the still controversial issue of the presence of aflatoxins in Korean soybean food.

Materials and Methods

Materials

Steamed soybean cultured at 30°C for 72 hours with *Aspergillus oryzae* (3 strains) and *Asperigillus sojae* (3 strains) commonly used in industrially fermented soybean food in Korea were obtained from the Department of Food and Nutrition, Sook Myung Women's University, Seoul, Korea.

Commercially manufactured fermented soybean food such as soybean paste, soysauce, hot sauce, special soybean paste, and koji were received from the Sampyo Food Company, Seoul, Korea.

Sixty six samples of domestic *Meju* were received from 21 regional branches of the Sampyo Food Company, and four commercial samples were obtained from a market in Seoul.

Apparatus

(a) Liquid chromatograph-Varian Model 5000; Valco auto injector (10 μl loop)
Fluorescence detector Varian
Excitation filter (360 *nm*) Varian 7-54 and 7-60
Emission filter (430 *nm*) Varian Interference
Flow cell with Lichrosorb 60 silica gel (30 μm)

Recorder;1mV Varian 9176
Integrator;Varian CDS 111L

(b) Column; Varian Micropak CN-10 (4*mm* id × 30*cm* long)
(c) Cleanup column; Glass (2.2*cm* id × 40*cm* long), Teflon stopcock
(d) Development tank; glass tank with cover, 25*cm* × 10*cm* × 25*cm*
(e) Viewing cabinet; Chromato-Vue Model CC-20
(f) Blender; Waring with 1L jar and cover
(g) TLC plastic sheet; Silica gel 60 F254, E. Merck 5735
(h) Sample clarification kit; Varian

Reagents

(a) Aflatoxins B_1, B_2, G_1, and G_2 were obtained from Sigma Chem. Co. They were dissolved in Benzene-Acetonitrile (98+2) to 0.5 *ng* μl
(b) HPLC solvents were obtained from Burdick & Jackson Laboratories. *n*-Hexane, Tetrahydrofuran, Isopropyl alcohol, Benzene, Acetonitrile, and Water.
(c) Silica gel 60 (70-230 mesh), E. Merck 7734 Followed the pretreatment procedure as in the AOAC method.[23]
(d) Silica gel 200 for TLC, E. Merck 7762
(e) Other chemicals not mentioned were the first grade or extra pure reagents.

Extraction and Cleanup

The sample extraction and cleanup procedure in this study is not based on a single literature. The only available cleanup method for the HPLC analysis of aflatoxins in soybean food was proposed by Wei *et al*[5]. It is not clear whether the different result from theirs and our preliminary experiment is caused by the fact that their Chinese soybean paste ingredient is different from the Korean one, but their method could not be reproduced in this laboratory. Therefore, several cleanup methods for TLC (CB method, Tang & Lin, Varian) were used for obtaining relatively clean HPLC chromatogram.

Fifty grams of sample was placed in the Waring blender jar with 250*ml* methanol-water (55+45 v/v%) and 100*ml* n-hexane. The mixture was blended on low speed for 1 minute and on high speed for 3 minutes. Most of the mixture was transferred to two 250*ml* centrifuge bottles and centrifuged for 5 minutes at 3000g. Fifty *ml* of lower was transferred to a separatory funnel and then 50*ml* n-hexane was added. The separatory funnel was shaken vigorously 2 minutes. The lower layer was transferred to another separatory funnel. Aflatoxin was extracted twice with 50*ml* chloroform. Each extract was transferred to and combined in the third separatory funnel. Fourty *ml* of 25% ammonium sulfate (w/v%) was added to the separatory funnel. The contents were shaken 1 minute and the two layers were allowed to separate. The lower layer was filtered through the funnel packed with anhydrous sodium sulfate. The filtrate was collected in an evaporation flask and was evaporated with a vacuum evaporator. The extract was dissolved with 10*ml* chloroform. The cleanup column was prepared with a small glass wool bed in the bottom of a chromatographic column. Five *g* anhydrous sodium sulfate, 10*g* silica gel 60, and 15*g* anhydrous sodium sulfate were packed with chloroform. The sample extract was trasferred to the column. The column was washed with 100*ml* n-hexane followed by 100*ml* anhydrous ethyl ether. The aflatoxin was eluted with 150*ml* chloroform-methanol (97+3 v/v%). The eluant was concentrated with the vacuum evaporator at 50°C. The concentrate was dissolved with 10*ml* chloroform and filtered through a sample clarification kit. The filtrate was collected in a vial and evaporated to dryness under stream of nitrogen with low heat on a heating block. The vial was closed with a foil-backed screw cap.

TLC for Aflatoxins

The concentrate was diluted with 200*ul* of benzeneacetonitrile (98+2 v/v%). Diluted sample and standard were spotted with 10, 20, 40μl on imaginary line 2*cm* from the bottom edge of the silica gel 60 F254 TLC plate. TLC plate was developed in unlined glass chamber containing

100*ml* chloroform-acetone (9+1 v/v%) for about 45 minutes. Developed plate was removed and air-dried. The plate was examined under the long wave length of UV light. Aflatoxins were quantitatively estimated by comparing fluorescence intensity and the Rf value of sample spots with those of standards.

Preparative TLC for aflatoxin-like substance

TLC plate for cleanup was prepared with silica gel 200 on the 20×20*cm* glass plate (1mm thickness). TLC plate was activated at 105°C before use. Residue from column cleanup was dissolved in 200 μl benzene-acetonitrile (98+2 v/v%). The sample extract was band-applicated on imaginary line 2*cm* from the bottom edge of th plate. The plate was developed in unlined glass chamber with chloroform-acetone (9+1 v/v%). The developed plate was removed and air-dried. Fluorescent bands of sample near the Rf values of aflatoxins were marked off under long wave length of UV light. Each fluoresent band was eluted with 20*ml* acetone and filtered through a sample clarification kit. The filtrate was collected in a vial and evaporated under nitrogen stream with low heat on a heating block. The vial was closed with a foil-backed screw cap.

HPLC for Aflatoxins

The HPLC analysis of aflatoxins followed the Johnson and Abu-Shumays[19] method with slight modification so that the peaks of aflatoxins may well separated from the early eluting peaks. The HPLC was stabilized at the flow rate of 1*ml*/min for 30 minutes before use. Detector attenuator was set at 20. The flowcell was packed with Lichrosorb si-60. Aflatoxin B_1, B_2, G_1, and G_2 were separated by Micro pak-CN column and mobile phase n-hexane-tetra hydrofuran-isopropyl alcohol-water (55+34+10+1 v/v%). Ten μl of aflatoxin standard was injected to the HPLC. The HPLC chromatogram of four aflatoxin peaks was completely recorded in approximately 21 minutes. The concentrate from sample was diluted to 200 *ul* with benzen-acetonitrile (98+2 v/v%). Ten *ul* of sample extract was injected to the HPLC. HPLC chromatogram was recorded. The integrator was used for quantitation. The aflatoxin concentration in a sample was calculated with the following equation.

$$\text{Concentration of mycotoxin } \mu g/kg = \frac{Ax \times Cs \times Vs \times SD}{As \times Vx \times W}$$

Where: Ax = Area, sample
Cs = Concentration of standard, $\mu g/ml$
Vs = Injected standard volume, μl
SD = Dilution of sample extract, μl
As = Area, standard
Vx = Injected samplevolume, μl
W = Sample extract in vial, *g*

Recovery experiment

Spiked samples were prepared by adding approriate amounts of aflatoxins. Aflatoxins B_1, B_2, G_1, and G_2 were dissolved in benzene-acetonitrile (0.5*ng* stardard/μl). Aflatoxins were determined by the same methods as described above.

Results and Discussion

Aflatoxins have been commonly determined by thin layer chromatographic (TLC) procedure. A limit of detection with the TLC procedure in this study was about 5 *ppb*. Aflatoxin B_1, B_2, G_1, and G_2 were separated fairly well by silica gel 60F254 plate and chloroform-acetone (9+1) and their Rf values were 0.2-0.4. Sample analyses were carried out in duplicate. Aflatoxin-producing abilities of molds were examined on the strains in fermented soybean food industries in Korea, and the quantities of aflatoxin in the commercially manufactured fermented soybean foods and *Meju* were determined by TLC and HPLC. On TLC, fluorescent spot near the Rf values of aflatoxins was not detected in the extract of soybean culture with each strain and the

extract of a koji culture during the culture time. Fluorescent spot near the Rf value of aflatoxin did not appear in the extract of soy sauce during the fermentation time. Some uncertain fluorescent spots near the Rf of aflatoxins appeared in the extract of soybean paste, hot sauce and special soybean paste. Soybean paste samples contained fluorescent spots near those of aflatoxin G_1 and G_2. Hot sauce samples showed fluorescent spots near the spot of aflatoxin B_2. In special soybean paste, fluorescent spots were observed near those of the aflatoxin B_2, G_1, and G_2. Fluorescent spots were separated from those of aflatoxins in chloroform-acetone (3+1) but its intensities of fluorescence were weak (Fig. 2). In some *Meju* (14 out of 70 samples), uncertain strong fluorescent spots were seen near the Rf values of aflatoxins on TLC (Fig. 3). Table 1 shows the regional occurrence of aflatoxin-like substances as detected by TLC.

The *Meju* which showed fluorescence spot on TLC were further purified with the preparative TLC in order to check the identity with HPLC. However, no peak was seen near the retention time of aflatoxins in the fermented soybean food and *Meju* with HPLC as shown in Figure 4 and 5. This implies that the HPLC technique could be one of the powerful tool for the identification of aflatoxins with the amounts less than those

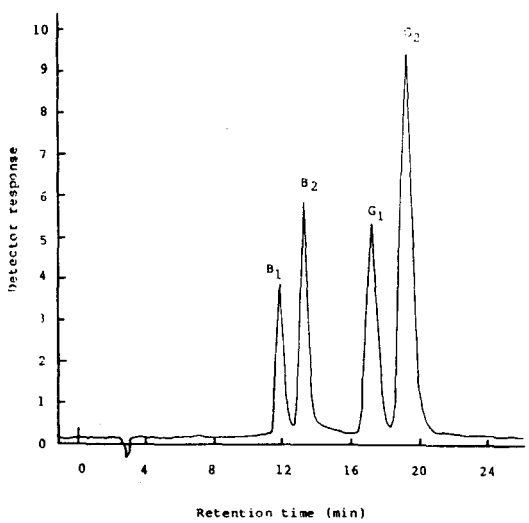

Fig. 1. HPLC chromatogram of aflatoxins B_1, B_2, G_1, and G_2

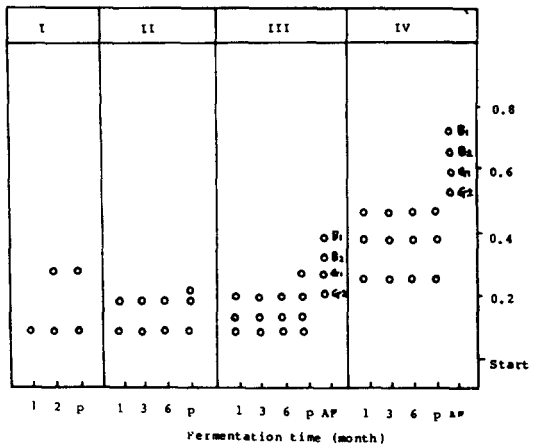

Fig. 2. TLC Chromatograms of hot sauce (I), soybean paste (II), and special soybean paste (III, IV) during the fermetation time

I, II, III : Developing solvent : Chloroform Acetone (9+1)
IV : Developing solvent : Chloroform Acetone (3+1)
P : Marketing product AF : Aflatoxin

Table 1. Regional occurrence of aflatoxin-like substances in home-made *Meju* as detected by TLC

Province	Detection frquency of Aflatoxin-like substances
Kyung-ki Do	1/8
Kang-won Do	1/8
Chung Bug	0/1
Chung Nam	1/6
Chun Bug	1/4
Chun Nam	2/17
Kyung Bug	4/21
Kyung Nam	4/5

needed for IR, NMR, and mass spectroscopy. The previous researchers used UV, derivative formation and chicken embryo bioassy for the identification of aflatoxins. Still, none of them could have been definitive in the elucidation of the presence of aflatoxin in Korean soybean food although Kim *et al's.*[8] effort was the most intensive and reasonable. The result of this study confirmed that the aflatoxin from manufactured soybean food could hardly be found and the TLC result might mislead the presence of aflatoxin in

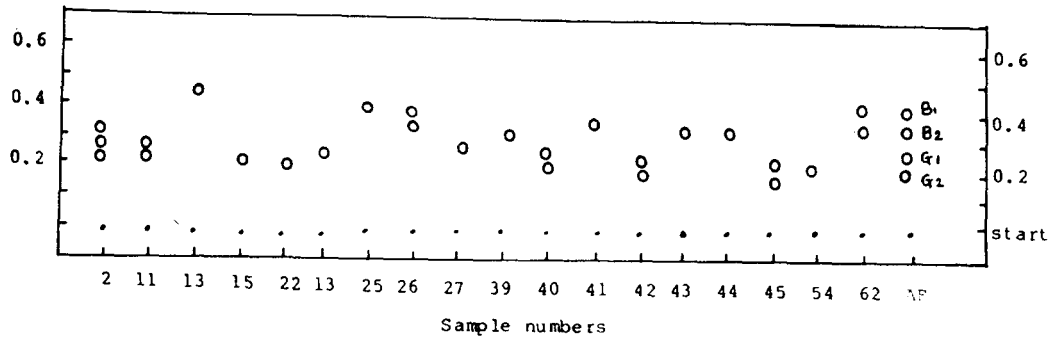

Fig. 3. TLC chromatogram of Fluorescent substances in *Mejus*
Developing solvent : Chloroform-Acetone (9+1) Af : Aflatoxin

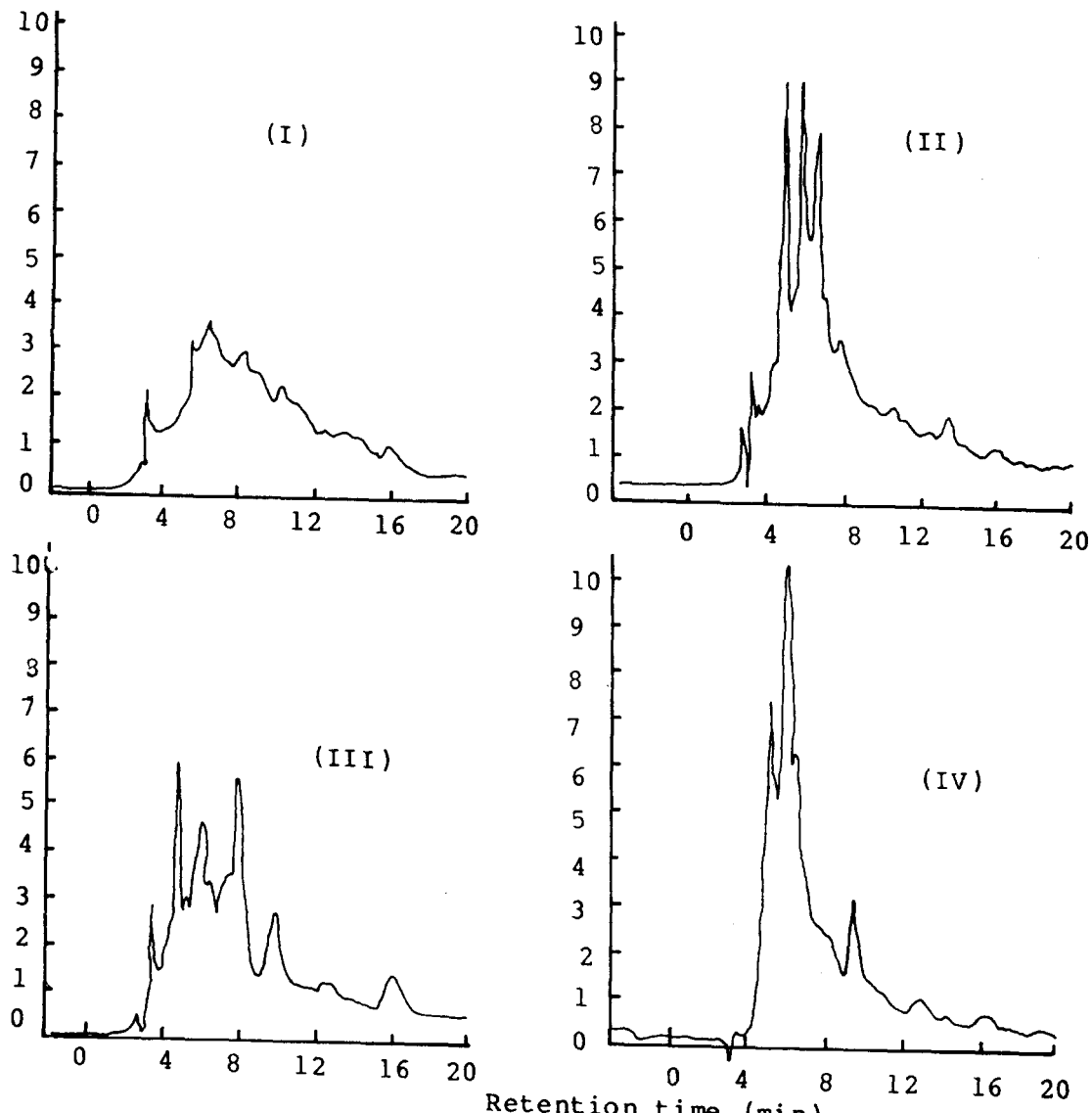

Fig. 4. Typical HPLC chromatograms of koji(I), soy sauce(II), special soybean paste(III) and soybean paste(IV)

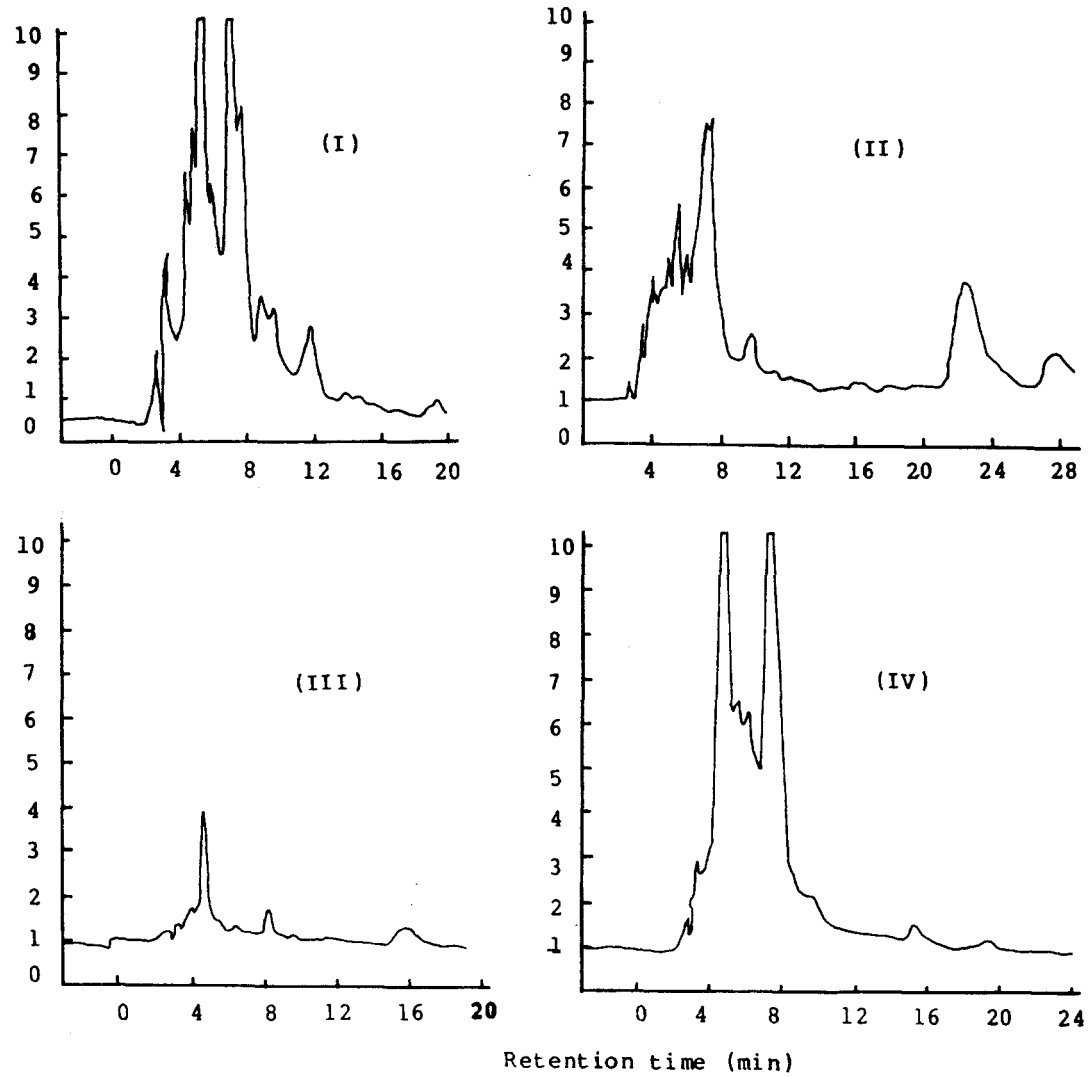

Fig. 5. Typical HPLC chromatograms of hot sauce (I), *Meju* (II), *A. oryzae* culture (III) and aflatoxin suspected substance purified by TLC (IV)

Korean soybean food, especially home-made *Meju*.

HPLC is thought to be an excellent technique for improving the accuracy and precision of TLC technique, as well as the qualitative analysis. Various kinds of columns and mobile phase were tested for fine separation of aflatoxins. Four aflatoxins were easily separated with Micropak-CN column and n-hexane-tetrahydrofuran-isopropyl alcohol-water (55+34+10+1 v/v%) as shown in Fig 1. This result is even better than the resolution achieved by Johnson and Abu-Shumays[19] by modifying solvent system.

Sensitivities in the nanogram region are typical using UV absorbance detectors. Fluorescence detectors could achieve detection of aflatoxins at the picogram level with ease. This means that HPLC detection of aflatoxin with fluorescence with packed cell aid is approximately 20 times as sensitive as with UV. Under those conditions, the minimum detectable amount of aflatoxin B_1 was 0.2*ng*. Mean sensitivity of detection, mm peak height/5 *ng* of aflatoxins were 86(B_1), 134(B_2), 125(G_1), and 225(G_2) (Fig. 1).

Each 250*ng* of aflatoxin B_1, B_2, G_1, and G_2

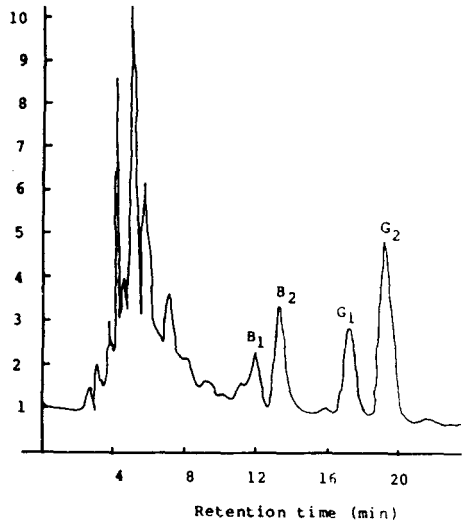

Fig. 6. HPLC chromatogram of aflatoxins (each250 *ng*) added to *Meju* and analyzed

was added to the aflatoxin free *Meju* and analyzed by the procedure above. A detection limit of the HPLC procedure was about 0.5 *ppb*. Recoveries of aflatoxin B_1 (70%), B_2(85%), G_1(70%), and G_2(82%) were obtained in ±5% range (Fig. 6). This recoveries are not as good as the ones for single a agricultural products, such as peanut butter,[16, 17] corn[22] cottonseed products,[11, 14] but are much better than for some crops[24] and animal tissues.[21] It is suggested that the chemical complexity of the Korean soybean products due to the fermentation process in the production might have decreased the recovery. In comparing with the recoveries from TLC method of other researchers, the recovery in this study is far better than that of Kim *et al*'.*s*[8] study on soybean food and foodstuff. Their recovery ranged 30 to 75% at 25 *ppb* level and 30 to 70% at 50 *ppb* level. This might be one of the main reasons why we should resort to the HPLC method in the quantitative analysis of aflatoxins from complex food or feed samples, even if the HPLC need a few more steps in cleanup procedure.

It has been established in this study that the HPLC analysis of aflatoxin in Korean soybean foodstuff is more effective in identification and quantitation than the conventional TLC method. It was also found that the commercial soybean foods in Korea and house-hold soybean foodstuff are free from aflatoxins with a detection limit of 0.5 *ppb*. All samples contained less than 0.5 *ppb*, which is far less than the regulation level of 15 *ppb* in the United State of America.

However, this result does not necessarily exclude the possibility of the presence of aflatoxin in Korean soybean food because of the limited number of samples in this study.

It was noticed that the present cleanup procedure is rather extensive in order to get clean chromatograms. The cleanup step should further be pursued. That will be a great help in performing fast precise, and accurate analysis of aflatoxins in other kinds of food and feed which has become an important class of the active international trade.

요 약

고속액체 크로마토 그라피에 CN컬럼과 분리용매로 핵산-테트라 하이드로퓨란-아이소프로필알콜-물을 사용하고 형광 검출기의 검출관에 리클로솔브로 채워 아플라톡신 B_1, B_2, G_1, G_2를 분리 정량하였다. 이 조건에서 아플라톡신 B_1의 최저 검출한계는 0.2*ng* 이었다. 대량생산 대두발효 식품과 메주에서 아플라톡신 함량을 조사하였고, 대두 발효식품에서 사용되는 균주의 아플라톡신 생성능을 알아보았다. TLC에서는 아플라톡신 유사물질이 검출되었지만 HPLC에서 아플라톡신이 아닌 것으로 나타났다. 시험한 균주의 아플라톡신의 생성능은 없었다.

References

1. Wyllie, D.T. and Morehouse, L.G: *Mycotoxic fungi, Mycotoxins, Mycotoxicoses.* Marcel Dekker. Inc, New York (1977)
2. Rodricks, J.V., Hesseltine, C.W. and Mehlman, M.A.: *Mycotoxins in Human and Animal health,* Pathotox Publishers Inc. Illinois (1977)
3. Manabe, M., Matsuura, S. and Nanano, M.: *Japan J. of Food Eng.,* 15, 7 (1968)
4. Manabe, M. and Matsuura, S.: *Japan Food Research Institute Report,* 12, 206 (1979)

5. Wei, R.D., Chang, S.C. and Lee, S.S.: *J. Assoc. Off. Anal. Chem.*, **63**, 1269 (1980)
6 Lee, T.Y. and Lee, S.K.: *J. Korean Food Sci. Technol.*, **1**, 78 (1969)
7. Jung, Y. and Kwon, S.P.: *KIST Report*, BS-E-47 (1)-1344-5, Roh, J.K. (ed) (1977)
8. Kim, Y.H., Hwang bo, J.S. and Lee, S.R.: *Korean J. Food Sci. Technol.*, **9**, 73 (1977)
9. Garner, R.C.: *J. Chromatog.*, **103**, 186 (1975)
10. Seitz, L.M.: *J. Chromatog.*, **104**, 81 (1975)
11. Pons, W.A.Jr.: *J. Assoc. Off. Anal. Chem.*, **58** , 746 (1975)
12. Pons, W.A. Jr.: *J. Assoc. Off. Anal. Chem.*, **59** , 101 (1976)
13. Takahashi, D.M.: *J. Chromatog.*, **131**, 147 (1977)
14. Pons, W.A. Jr. and Franz, A.O. Jr.: *J. Assoc. Off. Anal. Chem.*, **60** , 89 (1977)
15. Panalaks, T. and Scott, P.M.: *J. Assoc. Off. Anal. Chem.*, **60** , 583 (1977)
16. Pons, W.A. Jr. and Franz, A.O. Jr.: *J. Assoc. Off. Anal. Chem.*, **61** 793 (1978)
17. Hurst, W.J. and Toomey, P.B.: *J. Chromatog Sci.*, **16**, 372 (1978)
18. Colley, P.J. and Neal, G.E.: *Anal. Biochem.*, **93**, 409 (1979)
19. Johnson, E.L., and Stevenson, R.: *Basic Liquid Chromatography*, Varian Associates, Inc., California (1977)
20. Friesen, M.D., Walker, E.A. and Castegnayo, M.: *J. Assoc. Off. Anal. Chem.*, **63** 1057 (1980)
21. Gregory, J.F. III. and Manley, D.: *J. Assoc. Off. Anal. Chem.*, **64** , 144 (1981)
22. Cohen, H. and Lapointe, M.: *J. Assoc. Off. Anal. Chem.*, **64**, 1372 (1981)
23. A.O.A.C.: *Official Mthods of Analysis*, 13th ed., Association of Official Analytical Chemists, Washington, D.C., p. 414 (1980)
24. Shotwell, O.L. and Holaday, C.E.: *J. Assoc. Off. Anal. Chem.*, **64**, 674 (1981)

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