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THE DETECTION OF OCHRATOXIN A IN COMMERCIAL 'FUSHI' PRODUCTS, WHICH ARE DRIED BONITO, MACKEREL AND HOUSE MACKEREL, BY THE IMPROVEMENT DIRTHYLAMINOPROPYL SILICA GEL SOLID PHASE EXTRACTION METHOD.

Osamu KAWAMURA, Junko YAMAMOTO, and Asuka SYUTOU

Summary

The improvement diethylaminopropyl silica gel solid phase extraction method, which was simple, low cost and not need toxic organic solvent, good for detection of ochratoxin A (OTA) in foods. This method was partially modified and applied to determine OTA in 'fushi' product. The mean recovery of OTA was 85% in the range 1 - 10 ng/g. The limit of detection was 0.5 ng/g. 'Fushi' products on the market in 2003 – 2005 were collected and analyzed by this method. In 49 samples of powdered 'kezuri-bushi', 7 samples (17%) were positive (mean 4.1 ng/g). In 12 samples of 'dashi-pack', 3 samples (25%) were positive (mean 8.7 ng/g). But 53 samples of 'kezuri-bushi' samples were negative (<0.5 ng/g). The results were suggested that 'fushi' products were not contaminated in inoculated mold steps, probably in neglecting steps after smoking.

Key words: Ochratoxin A, 'Fuchi' products, Dried bonito, Dried mackerel, Solid phase extraction

Introduction

Ochratoxin A (OTA), produced by *Aspergillus ochraceus*, *Penicillium verrucosum*, and other several *Aspergillus* and *Penicillium* spp., was nephrotoxin, teratologenic toxin, and renal carcinogen [1]. OTA was contaminated with cereals, cereal products, coffee products, wine, beer, dried fruit, pork and pork products [1]. 'Fushi' product is a traditional fermented food, made from fish which are bonito, mackerel and horse mackerel, that is used seasoning. To produce 'fushi' product the fish is first boiled and smoked, and next smoked and kept for several times for drying, and then inoculated with Aspergillus strains for completely drying. A. ochraceus was once isolated from 'fushi' product. In about 20 years ago it was reported that 'katsuo-bushi', dried bonito, was contaminated with 1.36 ppm of OTA [2] But there was not generally report about 'katsuo-bushi' in recent 20 years. We developed the improvement diethylaminopropyl (DEA) silica gel solid phase extraction (SPE) method for detection of OTA and B in cereals and green coffee [3]. This DEA SPE method was modified and applied to analysis of OTA in commercial 'fushi' products.

Materials and Methods

Chemicals

OTA was purchased from Sigma-Aldrich Co. (St. Louis, MO). The Bound Elut DEA cartridge column (10 mL/500 MG, Varian) was purchased from GL Sciences Inc. (Tokyo). The mobile phase of HPLC was used HPLC grade from WAKO Pure Chemical Ind. Ltd. (Osaka).

Samples

Total 114 'fushi' products on the market samples in 2003 – 2005 were collected in Tokyo and Kagawa. These 'fushi' products were divided into 53 'Kezuri-bushi' samples which were dried bonito, mackerel and horse mackerel shavings, 49 powdered 'kezuri-bushi' samples included 4 powdered 'kezuri-bushi' sample made from 'honkare-bushi', and 12 'dashi-pack' samples which were packed powdered, shaved or broken 'fushi' products into filter paper for making soup stock. These samples were ground by mill (Millser IFM-700G, Iwatani International Corp. Tokyo) and kept at -20℃ before analysis.

Extraction and clean-up

'Fushi' products were analyzed by the improvement DEA SPE method [3] with the small modification. The ground sample (5 g) was weighed into 110 ml of grass sample tube. 50

ml of acetonitrile : 0.1% phosphoric acid (9 + 1 v/v) was added to this tube and vigorously shaken for 15 min. The extract was filtered through filter paper (Advantec 5C, 12.5 cm). The Bound Elut DEA column was previously washed with 10 ml of water, methanol, and acetonitrile : 0.1% phosphoric acid (9 + 1 v/v) at a flow rate of 3 ml/min before the sample loaded. The filtrate (5 mL) was loaded on the Bound Elut DEA column at a flow rate of 1.5 ml/min. The column was washed with 10 ml of acetonitrile : acetone (1 + 1 v/v), followed by washing with 10 ml of 80% methanol : acetic acid (99.5 + 0.5 v/v) at a flow rate of 3 ml/min. Then OTA was eluted from the column with 10 ml of 80% acetonitrile : trifluoacetic acid (99 + 1 v/v) at a flow rate of 1.5 ml/min. The eluate was evaporated to dryness at 40℃. The residue was dissolved with 0.5 ml of acetonitrile : water (4 + 6 v/v). The solution (20 μl) was injected to HPLC.

HPLC

The HPLC system consisted a Shimazu LC-10Advp pump, SIL-10Advp autoinjector, a CTO-10ADvp column oven, and a RF-10ADXL fluoresce detector. The mobile phase was a mixture of acetonitrile : water : acetic acid (40 + 58 + 2 v/v/v). A Capcell Pak C18 MG (4.6 mm i.d. x 250 mm, Shiseido) was used as the separative column. It was maintained at 40℃ and at a flow rate of 1.0 ml/min. The fluorescence detector was operated at 335 nm (excitation) and 465 nm (emission).

Recovery tests

The ground sample (5 g) in 110 ml of grass sample tube was added 100 μl of OTA in methanol (50, 100, 250, and 500 μg/ml) and kept into draft chamber in the darkness for 12-16 hours. After methanol was removed, the spiked samples were analyzed by above method.

Chemical Confirmation of OTA

OTA was derivetized to its methyl ester derivative by using boron trifluoride-methanol complex (BF_3-CH_3OH, Kanto Chemical Co. Inc., Tokyo)[4].

Results and Discussion

At first, a recovery test from powdered 'kezuri-bushi' spiked 5 ng OTA/g was carried out accordingly the original improvement DEA SPE method[3]. Its recovery was only 65.9%. Therefore we inspected for leakage of OTA at every steps in this method. We found that a small amount of OTA was lost at the step of the EDA column washing with 80% methanol : acetic acid (99 + 1 v/v). We modified this solvent to 80% methanol : acetic acid (99.5 + 0.5 v/v). As a result, its recovery has increased about 20% to 86.5% (Table 1). The results of recovery tests from 1, 2, 5 and 10 g/g were shown in Table 1. In case of 1 ng/g, its recovery was 79.3%. But the other recoveries were 85.6-90.7%.

Table 1 Recovey of ochratoxin A from powdered 'kazuri-bushi'

OTA added (ng/g)	Recovery ± SD (%, n = 3)
1	78.3 ± 4.4
2	85.6 ± 5.2
5	86.8 ± 3.8
10	90.7 ± 2.9
Mean	85.4

We analyzed OTA in commercial 'fuchi' products, were collected in 2003-5 by this modified improvement DEA SPE method. These results were shown in Table 2 and 3. OTA positive samples were 10 (9%) in total 114 samples. Seven out of 49 powdered 'kazuri-bushi' were positive for OTA with the average of 4.1 ng/g (min. 0.5 – max. 11.3 ng/g). Three out of 12 'dashi-pack' were positive with the average of 8.7 ng/g (min. 0.5 – max. 18.5 ng/g). The chromatograms of standard OTA and B and the highest contaminated sample ('dashi-pack' #5104) were shown in Fig. 1. All positive samples were confirmed by chemical methylation of OTA. In Fig. 2, the chromatograms of methylated standard OTA and highest positive sample were shown. Any 'kazuri-bushi' samples were not determined. Fore powdered 'kazuri-bushi' samples made from 'honkare-bushi' were negative for OTA. The contamination of OTA in 'katsuo-bushi', dried bonito, were reported in 1983[2]. It was reported that one out of 23 samples was found 1.36 μg of OTA. This concentration was about 100 times higher than the highest contaminated 'dashi-pack' sample. Because there was barely the report about the contamination of OTA in 'katsuo-bushi' and 'fushi' products n the last 20 years, our data are important.

A producing process of 'fushi' products was shown in Fig. 3. 'namari-bushi' was made from raw fish after boiling and smoking. 'ara-bushi' was made from 'namari-bushi' after repeatedly smoking and keeping for a reduction of water. 'hadaka-bushi' was made from 'ara-bushi' after shaving and remodeling. The refuses from 'ara-bushi' were the ingredients of powdered 'kazuri-bushi' and 'dashi-pack' which was pack

Table 2 Summary of ochratoxin A contaminated samples

Samples	No. of samples	No. of positives	% of positives
Powdered 'kezuri-bushi'	49*	7	14
(Powdered 'kezuri-bushi' made from 'honkare-bushi'	4	0	0)
'Dashi-pack'	12	3	25
'Kezuri-bushi'	53	0	0
Total	114	10	9

* Included powdered 'kezuri-bushi' made from 'honkare-bushi'

Table 3 Ochratoxin A positive samples

Samples	Sample No.	Ochratoxin A (ng/g)
Powdered 'kezuri-bushi'	1102	11.2
	1013	1.6
	2106	11.3
	2108	0.5
	3101	1.2
	5105	1.2
	5118	1.6
'Dashi-pack'	(Mean	4.1)
	1106	7.1
	2112	0.5
	5104	18.5
	(Mean	8.7)

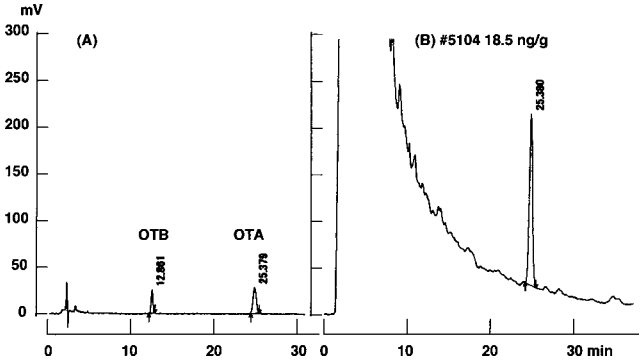

Fig. 1 The chromatograms of standard OTA and B (A), and the highest contaminated sample (#5104, 18.5 ng/g, B).

'fushi' products and other foods with filer paper for brewing stock. 'hadaka-bushi' was inoculated with fungi for a reduction of water and addition of flavor. After once inoculated with fungi, it is called 'jyoukare-bushi' . 'honkare-bushi' were completely dried after more 1-4 times inoculated with fungi. 'kazuri-bushi' was made from 'jyoukare-bushi' or 'honkare-bushi' after shaving. 'jyoukare-bushi' or 'honkare-bushi' was ground into some powdered 'kazuri-bushi' and an ingredient of 'dashi-pack' . *Aspergillus* strains, included *A. ochraceus*, had been used to produce 'fushi' products before. But now non-mycotoxigenic stains of *Eurotium ruber* and *E. repens* were

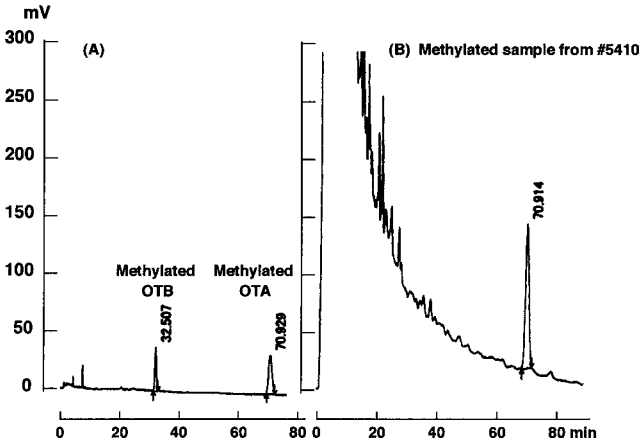

Fig. 2 The chromatograms of methylated standard OTA and B (A), and sample from the highest contaminated sample (#5104, 18.5 ng/g, B).

selected for producing 'fushi' products and used in Japan [5]. In our data, the powdered 'kazuri-bushi' and 'dashi-pack' samples made from 'ara-bushi' were contaminated with OTA, but any powdered 'kazuri-bushi' and 'kazuri-bushi' made from 'Jyoukare-bushi' or 'Honkare-bushi' were not contaminated. These matters suggested that OTA-producing fungi were probably grown at kept for soaking water to out side steps after smoking, and 'ara-bushi' was contaminated with OTA. OTA was removed from 'ara-bushi' by shaving steps into powdered 'kazuri-bushi' and 'dashi-pack'.

Commercial 'fushi' products in 2003 – 2005 were contaminated with OTA, but its frequency and concentration were no so high. An output of 'fushi' products per one Japanese person per year was only about 2.4 g. This level of OTA contamination in 'fushi' products was not probably caused human' s health breaks down. However, the regulation level of OTA in dried fruits was 10 ng/g in EU and dried fruits were intaken about 2.3 g per day per person [6]. This amount was the almost same amount of 'fushi' products in Japan. We found that same 'fushi' products were contaminated with over 10 ng/g of OTA. Therefore. we think that an inspection of OTA in 'fushi' products is need in future

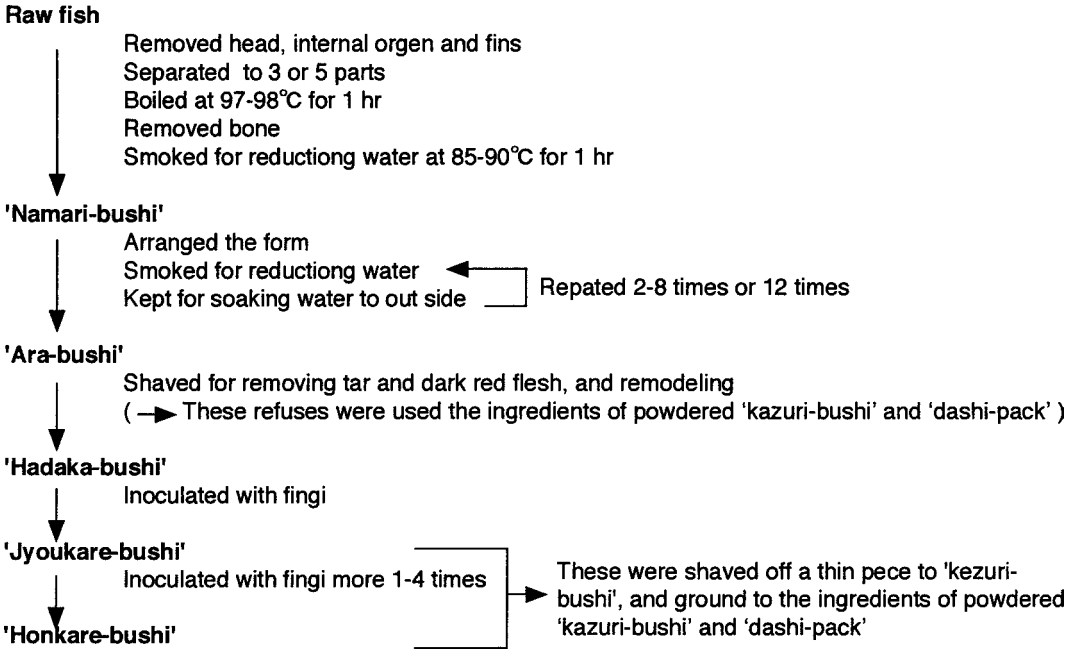

Fig. 3 The processing process of 'fushi' products.

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改良ジエチルアミノプロピルシリル化シリカゲル固相抽出法による市販節製品（鰹節，さば節，アジ節）中のオクラトキシンAの検出

川村 理，山本純子，首藤明日香

改良ジエチルアミノプロピルシリル化シリカゲル固相抽出法によるオクラトキシンA（OTA）の分析法は，簡便かつ低コストで毒性の強い有機溶媒を使わない優れた方法である．しかし，この方法をそのまま節製品に適用させた場合，回収率が約65％と低かった．そこで，固相抽出での洗浄を80％メタノール：酢酸（99＋1）から（99.5＋0.5）に変更したところ，85％以上の高回収率であった．本法で，2003－05年の市販節製品114検体を分析した結果，粉末削り節49検体中7検体（17％）平均4.1 ng/gとだしパック12検体中3検体（25％）平均8.7 ng/gのOTAを検出した．しかし，削り節53検体からはOTAは検出されなかった．以上の結果から，節製品の製造工程からOTA汚染は，カビ付け工程以前で起こっている可能性が示唆された．