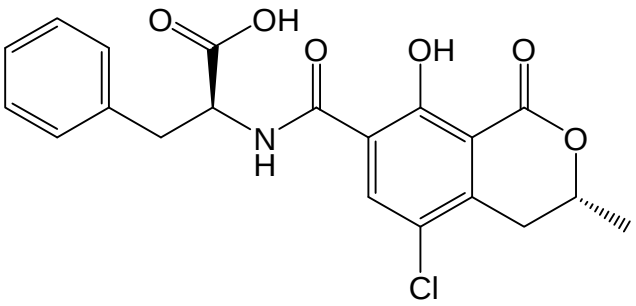
Ochratoxin A



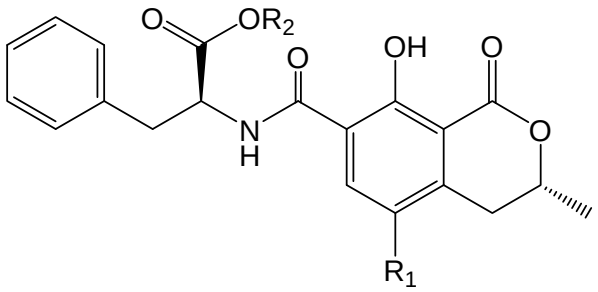
N-{ [(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-
L-phenylalanine
$C_{20}H_{18}ClNO_6$ MW: 403.81 CAS No.: 303-47-9

[Summary of ochratoxin A]

Ochratoxin is a kind of mycotoxin discovered by a research group in South Africa and is a carcinogenic substance.

Reported ochratoxin producers include genus *Aspergillus*, *Aspergillus ochraceus* in particular, and *Penicillium* such as *Penicillium viridicatum*. These ochratoxin producers are widely distributed in foods such as cereals.

Six metabolites as shown in Figure 5.1.18-1 are known to be the toxic components of ochratoxin, and usually ochratoxin A is viewed as the problem because its toxicity in animals is more potent than the other metabolites.



	R_1	R_2		R_1	R_2
Ochratoxin A	Cl	H	Ochratoxin A methyl ester	Cl	CH_3
Ochratoxin B	H	H	Ochratoxin B methyl ester	H	CH_3
Ochratoxin C	Cl	C_2H_5	Ochratoxin B ethyl ester	H	C_2H_5

Figure 5.1.18-1 Structural formulas of ochratoxins

[Methods listed in the Feed Analysis Standards]

1 Liquid chromatography (1) [Feed Analysis Standards, Chapter 5, Section 1 18.1]

Scope of application: Grains

A. Reagent preparation

Ochratoxin A standard solution. Weigh accurately 5 mg of ochratoxin A [$C_{20}H_{18}NO_6Cl$], transfer to a 25-mL amber volumetric flask, dissolve by the addition of methanol, and add the same solvent up to the graduation line to prepare the ochratoxin A standard stock solution (1 mL of this solution contains 0.2 mg as ochratoxin A).

Before use, dilute accurately a certain amount of the standard stock solution with acetonitrile/ water/ acetic acid (300:700:1) to prepare several ochratoxin A standard solutions that contain 0.5-20 ng of ochratoxin A in 1 mL.

B. Quantification

Extraction. Weigh 25.0 g of an analysis sample, transfer it to a 200-mL stoppered Erlenmeyer flask, add 100 mL of acetonitrile/ water/ acetic acid (84:16:1), and extract by shaking for 30 minutes. Transfer the extract to a stoppered centrifuge tube, centrifuge at 1,000×g for 5 minutes, to obtain supernatant as a sample solution to be subjected to column treatment.

Column treatment. Load the sample solution on a multifunctional column (for ochratoxin pretreatment), [Note 1] and discard the first 1 mL of eluate, then collect the following 3 mL of eluate into a 10-mL test tube. Transfer accurately 2 mL of this solution to another 10-mL test tube, evaporate under vacuum in a water bath at 50°C or less to be almost dried up, and then dry up by nitrogen gas flow.

Add accurately 1 mL of acetonitrile/ water/ acetic acid (300:700:1) to dissolve the

residue. Transfer this solution to a plastic centrifuge tube (volume: 1.5 mL), centrifuge at 5,000×*g* for 5 minutes, to obtain supernatant as a sample solution to be subjected to liquid chromatography.

Liquid chromatography. Inject 50 µL each of the sample solution and the respective standard solutions to a liquid chromatograph to obtain chromatograms.

Example of measurement conditions

Detector: Fluorescence detector (excitation wavelength 385 nm; fluorescence wavelength 444 nm)

Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 µm) Note 2

Eluent: Acetonitrile/ water/ acetic acid (500:500:1)

Alkalizing solution Note 3: Sodium hydroxide solution (0.1 mol/L)

Flow rate: Eluent, 1.0 mL/min; alkalizing solution, 0.3 mL/min

Column oven temperature: 40°C

Calculation. Obtain peak heights or areas from the resulting chromatograms[1] to prepare a calibration curve, and calculate the amount of ochratoxin A in a sample.

Note 1 MultiSep 229 Ochra (Romer Labs) or equivalents

2 Inertsil ODS-2 (GL Sciences) or equivalents

3 Using a PEEK mixing tee etc., mix the eluate eluted from the column and the alkalizing solution, and then send to a fluorescence detector.

<<Summary of analysis method>>

In this method, ochratoxin A in grains is extracted with acetonitrile/ water/ acetic acid (84:16:1), purified with a multifunctional cleanup (MFC) column, and quantitated by a liquid chromatograph with post-column alkalization and a fluorescence detector.

Ochratoxin A can be detected sensitively because its fluorescence intensity increases under alkaline conditions.

The flow sheet of the analysis method is shown in Figure 5.1.18-2.

25.0 g sample
- 100 mL Acetonitrile/ water/ acetic acid (84:16:1)
- Shake for 30 minutes
- Centrifuge (1,000x g (2,000 rpm), 5 minutes)

MultiSep 229 Ochra
- Load the supernatant
- Discard the first 1 mL of eluate

2 mL eluate
- Evaporate under vacuum, dry up
- 1 mL Acetonitrile/ water/ acetic acid (300:700:1)
- Centrifuge (5,000x g (10,000 rpm), 5 minutes)

LC-postcolumn alkalization-FL (Ex. 385 nm; Em. 444 nm)

Figure 5.1.18-2 Flow sheet of the analysis method for ochratoxin A by liquid chromatography

References: Kouji Aoyama, Eiichi Ishiguro, Naoki Kanemaru, and Masatoshi Minamisawa: Research Report of Animal Feed, 31, 109 (2006)

History in the Feed Analysis Standards [28] New

<<Analysis method validation>>

- Spike recovery and repeatability

Sample type	**Spike concentration (µg/kg)**	**Repeat**	**Spike recovery (%)**	**Repeatability RSD (% or less)**
Corn	1~20	3	95.8~121.3	0.4
Barley	1~20	3	96.4~118.0	2.5
Rye	1~20	3	95.9~106.2	1.6

- Collaborative study

Sample type	Number of laboratories	Spike concentration (µg/kg)	Spike recovery (%) (measured value (µg/kg))	Intra-laboratory repeatability RSDr (%)	Inter-laboratory reproducibility RSDR (%)	HorRat
Flour	7	5	98.2	3.7	8.8	0.40
Wheat	7	Natural contamination	(3.83)	7.1	11.0	0.50

- Lower limit of quantification: 1 µg/kg in samples (Spike recovery 106-121 %, repeatability 2.5 % or less)

<<Notes and precautions>>

[1] Examples of chromatograms are shown in Figure 5.1.18-3.

(Reference) Ochratoxin B Ochratoxin A

Retention time/ min

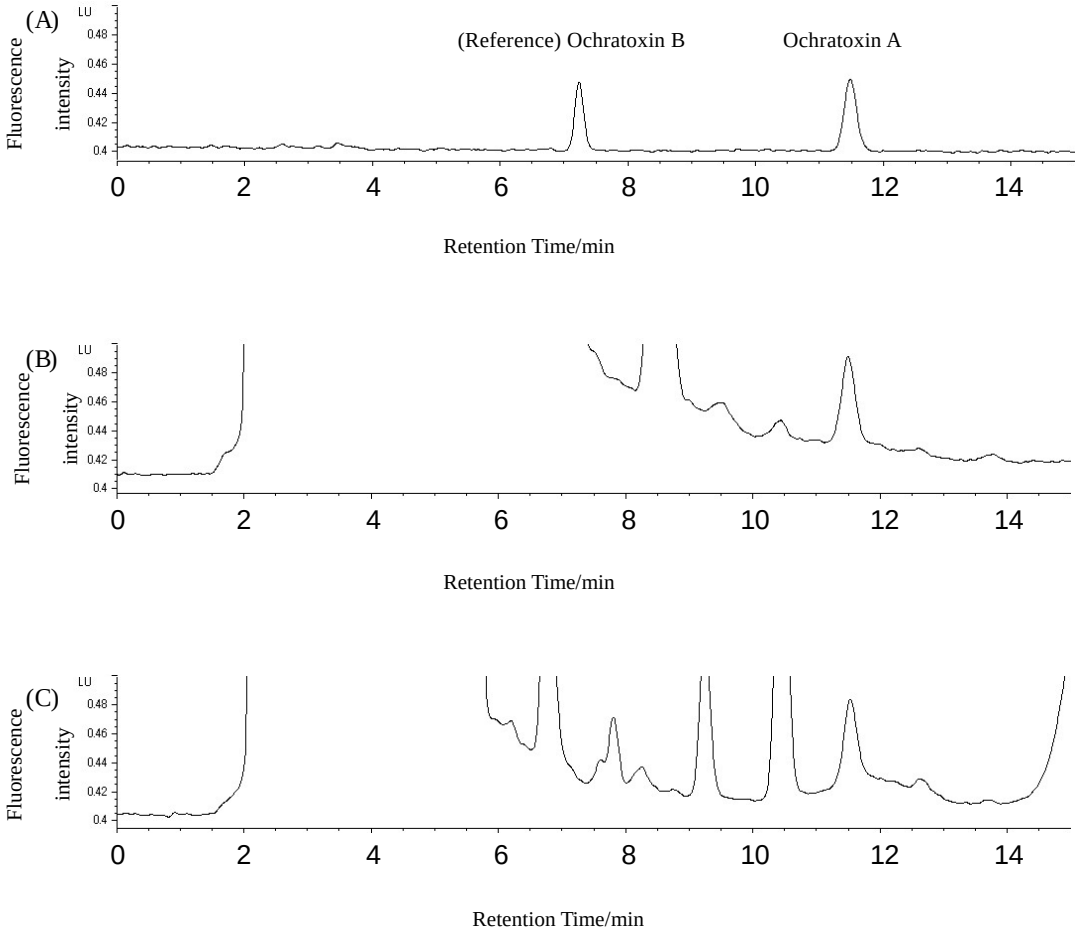


Figure 5.1.18-3 Chromatograms of ochratoxin A

Measurement conditions were according to Example of measurement condition. Column used was GL Sciences Inertsil ODS-2.

(A) Standard solution (0.5 ng/mL)

(B) Corn (spiked with 1 µg/kg equivalent)

(C) Barley (spiked with 1 µg/kg equivalent)

2 Simultaneous analysis of ochratoxin A and citrinin by liquid chromatography [Feed Analysis Standards, Chapter 5, Section 1 18.2]

Analyte compounds: Ochratoxin A and citrinin (2 components)

Scope of application: Grains and formula feeds (except formula feeds for citrinin)

A. Reagent preparation

1) Ochratoxin A standard solution. Weigh accurately 5 mg of ochratoxin A [$C_{20}H_{18}NO_6Cl$], put in a 25- mL amber volumetric flask, dissolve by the addition of methanol, and further add the same solvent up to the graduation line (1 mL of this solution contains 0.2 mg as ochratoxin A.). Moreover, dilute accurately a certain amount of this solution with methanol, to prepare the ochratoxin A standard stock solution that contains 1 μg as ochratoxin A in 1 mL.
2) Citrinin standard solution. Weigh accurately 5 mg of citrinin [$C_{13}H_{14}O_5$], put in a 25- mL amber volumetric flask, dissolve by the addition of methanol, and further add the same solvent up to the graduation line (1 mL of this solution contains 0.2 mg as citrinin.). Moreover, dilute accurately a certain amount of this solution with methanol, to prepare the citrinin standard stock solution that contains 1 μg as citrinin in 1 mL.
3) Mixture standard solution. Before use, mix a certain amount of ochratoxin A and citrinin standard stock solutions, dilute accurately with acetonitrile- water (1:1), to prepare several mixture standard solutions that contain 1-50 ng as ochratoxin A and citrinin, respectively, in 1 mL.

B. Quantification

Extraction. Weigh 25.0 g of an analysis sample, transfer it to a 200- mL stoppered Erlenmeyer flask, moisten by the addition of 100 mL of acetonitrile - hydrochloric acid - water (8:1:1), then leave at rest for 5 minutes, [Note 1] and further extract by shaking for 30 minutes. Filter the extract with filter paper (No. 5A), transfer accurately 5 mL of the filtrate to a 50- mL recovery flask, and concentrate under vacuum to be 1 mL or less at 40°C or less. Further, remove acetonitrile by a mild flow of nitrogen gas, [1] to be a sample solution to be subjected to purification.

Purification. Add about 0.5 g of sodium chloride to the sample solution, then add accurately 10 mL of ethyl acetate, mix well, transfer to a 10- mL test tube, and centrifuge at 1,500×*g* for 5 minutes. Transfer accurately 5 mL of the ethyl acetate layer (upper layer) to a 50- mL recovery flask, concentrate under vacuum at 40°C or less to be almost dried up, and then dry up by nitrogen gas flow.

Dissolve the residue by the addition of accurately 2 mL of acetonitrile- water (1:1). Transfer this solution to a filter cup with ultrafiltration membrane (molecular weight cutoff: 30,000) [Note 2] that is attached to a plastic centrifuge tube (capacity: 1.5 mL) in advance, centrifuge at 5,000×*g* for 15 minutes, to obtain filtrate to be a

sample solution to be subjected to column treatment.

Liquid chromatography. [2] Inject 20 μL each of the sample solution and respective mixture standard solutions to a liquid chromatograph to obtain chromatograms.

Example of measurement conditions

Detector: Fluorescence detector (excitation wavelength, 335 nm; emission wavelength, 480 nm)

Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 3[3][4]

Eluent: Acetonitrile- water/ 1 v/v% phosphoric acid (230:230:1)

Flow rate: 1.0 mL/min

Column oven temperature: 40°C

Calculation. Obtain peak heights or areas from the resulting chromatograms [5][6]to prepare a calibration curve, and calculate the amounts of ochratoxin A and citrinin in the sample.

Note 1 Leave at rest until it stops foaming.

2 Use Microcon YM-30 (Millipore) or equivalents.

3 Inertsil ODS-2 (GL Sciences) or equivalents.

<<Summary of analysis method>>

This is an analysis for ochratoxin A and citrinin in the case of grains, and for ochratoxin A in the case of formula feeds.

Mycotoxins in a sample are extracted with acetonitrile acidified with hydrochloric acid. After the solvent is replaced, the solution is passed through ultrafiltration filter (molecular weight cutoff: 30,000), and quantitated by a liquid chromatograph with a fluorescence detector.

The flow sheet of the analysis method is shown in Figure 5.3.9-1.

25.0 g sample
- 100 mL Acetonitrile - hydrochloric acid - water (8:1:1)
- Leave at rest for 5 minutes.
- Shake for 30 minutes.
- Filter with filter paper (5A)

5 mL Filtrate
- Concentrate to be 1 mL or less
- Mild nitrogen gas flow
- Add 0.5 g sodium chloride and 10 mL ethyl acetate, and mix
- Centrifuge (1,500x g (3,000 rpm), 5 minutes)

5 mL Upper layer
- Concentrate under vacuum, and dry up
- 2 mL Acetonitrile- water (1:1)

Ultrafiltration membrane (molecular weight cutoff: 30,000),
- Centrifuge (5,000x g (10,000 rpm), 15 minutes)

LC-FL (Ex, 335 nm; Em, 480 nm)

Figure 5.3.9-1 Flow sheet of the simultaneous analysis method for ochratoxin A and citrinin

References: Koji Aoyama, Kaori Morifuji, Eiichi Ishiguro: Research Report of Animal Feed, **29**, 11 (2004)

History in the Feed Analysis Standards [27] New

<<Analysis method validation>>

- Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Ochratoxin A	Formula feed for adult chickens	10~100	3	96.3~97.1	12.6
	Formula feed for pork pig fattening	10~100	3	98.6~103.4	13.6
	Corn	10~100	3	105.6~106.5	8.9
	Barley	10~100	3	98.2~103.8	10.2
Citrinin	Formula feed for adult chickens	100~400	3	85.0~86.2	3.0
	Formula feed for pork pig fattening	100~400	3	87.5~88.7	3.0
	Corn	100~400	3	77.2~90.8	12.5
	Barley	100~400	3	83.0~84.0	7.4

- Collaborative study

Name of analyzed component	Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%) (measured value (μg/kg))	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Ochratoxin A	Pig formula feed	11	20	100.4	3.3	7.9	0.36
	Milo	11	Natural contamination	(18.6)	20.6	23.5	1.07
Citrinin	Pig formula feed	11	200	83.6	6.1	11.8	0.58

- Lower limit of quantification: 5 μg/kg for ochratoxin A (Spike recovery 93.9~100.7 %, relative standard deviation 23.3 % or less, *SN* ratio 10), citrinin 20 μg/kg (Spike recovery 90.0~94.9 %, relative standard deviation 19.2 % or less, *SN* ratio 10)

<<Notes and precautions>>

[1] Recovery of citrinin becomes poorer if it is dried up in this step. When it happens to be dried up, add about 1 mL of 6 mol/L hydrochloric acid before analysis.

[2] Conduct measurement after removing methanol by sufficiently flowing the eluent, etc., when methanol remains in the column. The citrinin peak may become broad if methanol remains.

[3] In addition to Inertsil ODS-2, Shodex C18M 4E (Showa Denko) can be used.

[4] The order of elution of ochratoxin A and citrinin may vary even if the same column is used.

[5] Examples of chromatograms are shown in Figure 5.3.9-2.

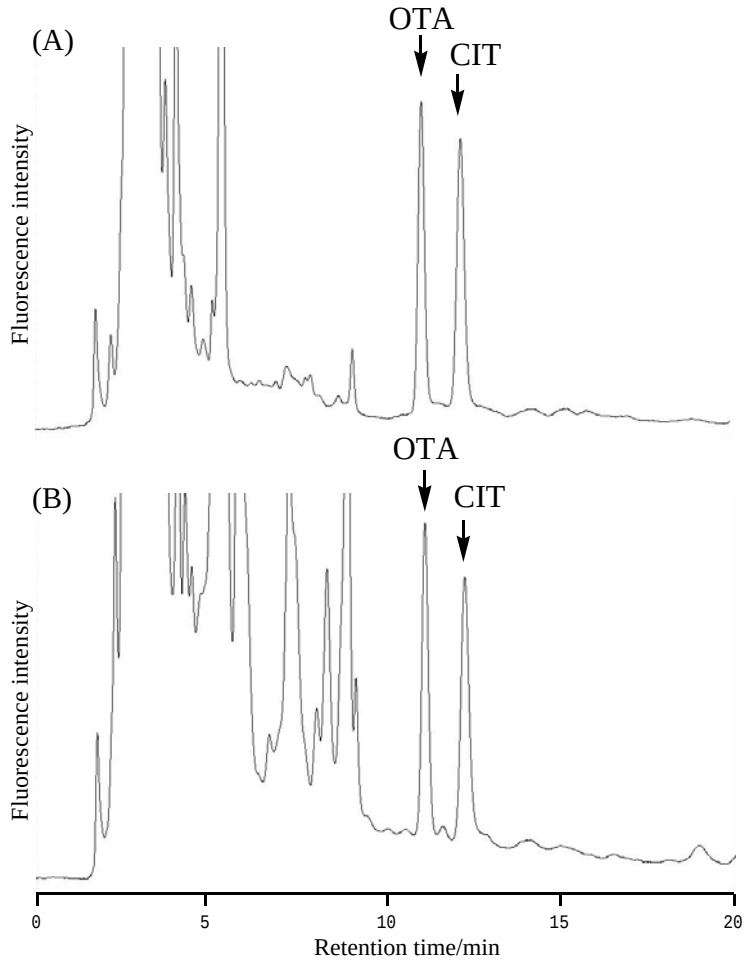


Figure 5.3.9-2 Chromatograms of ochratoxin A and citrinin
(OTA, peak of ochratoxin A; CIT, peak of citrinin.)

LC conditions are according to the Example of measurement conditions. The column is Inertsil ODS-2 (GL Sciences).

(A) Barley (spiked with amounts equivalent to 400 µg/kg as citrinin, and 100 µg/kg as ochratoxin A)

(B) Formula feeds for pork pig fattening (spiked with amounts equivalent to 400 µg/kg as citrinin, and 100 µg/kg as ochratoxin A)

[6] Peaks can be identified by changing the acid concentration in the eluent, emission wavelength, etc.

3 Liquid chromatography (2) [Feed Analysis Standards, Chapter 5, Section 1 18.3]

Scope of application: Grains and formula feeds

A. Reagent preparation

Ochratoxin A standard solution.[1] Weigh accurately 5 mg of ochratoxin A [$C_{20}H_{10}ClNO_6$], [2] put in a 250-mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the ochratoxin A standard stock solution (1 mL of this solution contains 20 μg as ochratoxin A).

Before use, dilute accurately a certain amount of the standard stock solution with acetonitrile/ dilute phosphoric acid Note 1 (11:9) to prepare several ochratoxin A standard solutions that contain 0.01-0.05 μg of ochratoxin A in 1 mL.

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 500-mL separatory funnel, moisten the sample by the addition of 25 mL of acetic acid (1:19), add 250 mL of chloroform 250 mL, and extract by shaking for 30 minutes.

Transfer the extract to a stoppered centrifuge tube, and centrifuge at 1,500×*g* for 5 minutes. Transfer the chloroform layer (lower layer) to an Erlenmeyer flask, dehydrate with a suitable amount of sodium sulfate (anhydrous), and filter with filter paper (2 types).

Transfer 50 mL of the filtrate[3] to a 100-mL recovery flask, evaporate under vacuum in a water bath at 40°C or less to be almost dried up, and then dry up by nitrogen gas flow. [4] Add 2 mL of toluene to dissolve the residue, to be a sample solution to be subjected to column treatment.

Column treatment.[5] Wash a silica gel mini column (690 mg) with 10 mL of toluene.

Load the sample solution to the mini column, and wash the recovery flask that contained the sample solution twice with 2 mL each of toluene, and add the washing to the mini column, and elute by pressurized flow. Note 2 Moreover, add 10 mL of toluene and 6 mL of chloroform/ methanol (97:3) sequentially to the mini column, and elute by pressurized flow. Note 2

Place a 50-mL recovery flask under the mini column. Add 10 mL of toluene/ acetic acid (9:1) to the mini column, and pressurize Note 2 to elute ochratoxin A. Evaporate the eluate under vacuum in the water bath at 40°C or less to be almost dried up,

and further dry up by nitrogen gas flow. Add accurately 2 mL of acetonitrile/ dilute phosphoric acid [Note 1] (11:9) to dissolve the residue, filter with membrane filter (pore size 0.5 μm or less), to obtain a sample solution to be subjected to liquid chromatography.

Liquid chromatography. Inject 20 μL each of the sample solution and respective ochratoxin A standard solutions to a liquid chromatograph to obtain chromatograms.

Example of measurement conditions

Detector: Fluorescence detector (excitation wavelength 337 nm, fluorescence wavelength 467 nm)

Column Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) [Note 3] [6]

Eluent: Acetonitrile/ dilute phosphoric acid [Note 1] (11:9)

Flow rate: 1.0 mL/min

Calculation. Obtain peak heights or areas from the resulting chromatograms[7] to prepare a calibration curve, and calculate the amount of ochratoxin A in a sample.

Note 1 Phosphoric acid (1:1,000)

2 Adjust the flow rate to be 2-3 mL/min.

3 UNISIL PACK 5C_{18} (GL Sciences) or equivalents

<<Summary of analysis method>>

In this method, ochratoxin A in a sample is extracted with chloroform, purified with a silica gel mini column, and quantitated by a liquid chromatograph with a fluorescence detector.

References: Masayuki Shimomura and Eiichi Ishiguro: Research Report of Animal Feed, 14, 1(1989)

History in the Feed Analysis Standards [11] New

<<Notes and precautions>>

[1] Accurate concentration testing for the ochratoxin A standard stock solution is stipulated in Section 4 in this chapter.

[2] 10 μg/mL Ochratoxin A standard solution is commercially available from Kanto Chemical, etc.

[3] Pressurizing the sample solution to flow in column treatment may be difficult in

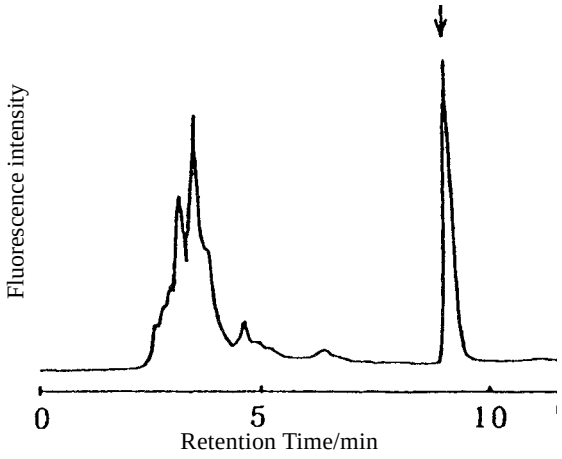
analysis of formula feeds etc. In that case, adjust the solution volume to about 25 mL.

[4] Eliminate the odor of acetic acid completely.

[5] The flow rate and elution rate is sufficient at around 5 mL per minute. In addition, make sure that there is no bubble getting inside during column treatment.

[6] An example of chromatograms for ochratoxin A is shown in Figure 5.1.18-4.

[7] The column to be used only needs to be one that uses packing treated by corresponding endcapping. The column used in the development of this analysis method was UNISIL PACK $5C_{18}$.



Measurement conditions

Column: UNISIL PACK $5C_{18}$

Column temperature: 40°C

Eluent: Acetonitrile -phosphoric acid solution (0.1 v/v%) (11:9)

Flow: 1.0 mL/min

Detector: Hitachi spectrofluorometer 650-10LC Ex: 337 nm, Em: 467 nm

Figure 5.1.18-4 Chromatogram of ochratoxin A

[Other analysis methods]

4 ELISA

Products manufactured by Neogen (available from AR Brown, Kikkoman, etc.), R-Biopharm Rhône (available from AZmax, etc.), etc. are available in Japan from several distributors .

Table 5.1.18-1 shows major kits commercially available now in Japan, and their summaries.

Table 5.1.18-1 ELISA kits for ochratoxin A analysis commercially available in Japan

Item		Lower limit of detection	Analysis time	Shelf life of the kit	Applicable samples	Notes
Quantitation kit	Veratox® for Ochratoxin	1 ppb	20 minutes	6 months	Corn, coffee beans, bran	Neogen
	RIDASCREEN® FAST Ochratoxin A	5 ppb	25 minutes	6-9 months	Grains, feeds, foods	R-Biopharm Rhône
	AgraQuant® Och	2 ppb	25 minutes	6-9 months	Grains	Romer Lab
	Charm ROSA® Ochratoxin	2 ppb	12- 25 minutes	6-9 months	Grains, wine, grape juice	Charm Science
	Max Signal™	0.15 ppb	25 minutes	6-9 months	Grains, feeds, nuts and seeds, milk	BIOO Scientific