Detection of Residual Chloramphenicol, Florfenicol, and Thiamphenicol in Yellowtail Fish Muscles by Capillary Gas Chromatography−Mass Spectrometry

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Chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) were extracted from yellowtail muscles with ethyl acetate, and the extract was evaporated. The residues was dissolved with sodium chloride solution and partitioned with *n*-hexane to remove lipids, and then the drugs were extracted with ethyl acetate. After evaporation of ethyl acetate extract, the residue was dissolved with *n*-hexane followed by ethyl ether and applied to a Sep-Pak Florisil cartridge successively. The drugs were eluted from the cartridge with methanol−ethyl ether (3:7), and eluate was evaporated to dryness. After acetonitrile and BSA were added to the residues, the drugs were derivatized at 50 °C for 10 min. After the solvent was evaporated, the residue was dissolved with ethyl acetate and applied to gas chromatography−mass spectrometry. The drugs were separated by a capillary column coated with 5% phenyl methyl silicone, and SIM was performed at *m*/*z* 208 and 225 for CAP, at *m*/*z* 257 for FF, and at *m*/*z* 242, 257, and 330 for TAP. Recoveries of each drug from yellowtail muscle fortified at 0.1 ppm were more than 65%, and detection limits were 5 ppb.

Keywords: *Chloramphenicol; thiamphenicol; florfenicol; antibacterials; yellowtail*

INTRODUCTION

Chloramphenicol (CAP) and thiamphenicol (TAP) have been used to treat many kinds of animal diseases as effective broad spectrum antibacterials. Recently, in Japan, florfenicol (FF), the structure of which is quite similar to those of the above two drugs, has been permitted for use on cultured yellowtails. The structures of these three antibacterials are shown in Figure 1.

Many various determination methods of CAP, TAP, and FF by gas chromatography (GC) or high-performance liquid chromatography (HPLC) have been reported so far. Reviews of these three antibacterials have been already published (Allen, 1985; Nagata, 1995). GC and HPLC are useful for the separation and quantitation of residual antibacterials. However, interfering substances from animal tissues render unequivocal identification by GC and HPLC using retention and/or fixed wavelength. Mass spectrometric analysis, which provides molecular weight and characteristic fragmentation patterns, provides confirmation of antibacterials with retention and spectral characteristics. Several determination methods of CAP residues in urine, plasma, meat, egg, and milk by gas chromatography−mass spectrometry (GC−MS) (Gazzaniga *et al*., 1973; Janssen and Vanderhaeghe, 1973; Nakagawa *et al*., 1975; Keukens *et al*., 1992; Kijak, 1994; Borner *et al*., 1995) or liquid chromatography−mass spectrometry (Bories *et al*., 1983; Delepine and Sanders, 1992; Ramsey *et al*., 1989) have been reported so far. The detection limits of these determination methods were between 0.2 ppb and 0.5 ppm. Recently, CAP has been confirmed at very low residual levels. By GC−MS in the electron impact mode, residual CAP in meat was determined as low as 5 ppb and CAP in milk by GC−MS with negative chemical ionization mode was determined at 0.2 μg/L (Keukens *et al*., 1992). By GC−MS in methane negative chemical ionization, CAP in milk was confirmed at a low level of 0.5 ng/mL (Kijak, 1994). By gas chromatography−high-resolution mass spectrometry, CAP residue in egg was confirmed at a low level of 0.5 ppb. However, few reports of the confirmation of residual TAP in tissues have been published. Residual TAP in rat tissues has been determined by GC−MS at a detection limit of 0.1 ppm (Plomp and Maes, 1976).

In this paper, residual CAP, FF, and TAP in yellowtail muscle were determined by GC−MS simultaneously. The drugs were extracted from muscle and cleaned up by liquid−liquid followed by solid−liquid partition as described earlier (Nagata and Saeki, 1992). Then the drugs were derivatized with trimethylsilyl reagent. Selected ion monitoring mass spectrometry (SIM-MS) was employed to quantify and confirm residual CAP, FF, and TAP in yellowtail muscles at low levels of 5 ppb.

EXPERIMENTAL PROCEDURES

Chemicals. *N*,*O*-Bis(trimethylsilyl)acetamide (BSA) was obtained from GL Science Inc. (Tokyo, Japan). Chloramphenicol and thiamphenicol were from Sigma Chemical Co. (St. Louis, MO). Florfenicol was a gift from Takeda Yakuhin Kogyo Co. (Tokyo, Japan). Each stock solution was prepared by dissolving 10 mg of each drug in 100 mL of methanol at 100 μg/mL. The working standard solution was prepared at 1 μg/mL in methanol using each 1 mL stock solution. A Sep-Pak Florisil cartridge (Waters Co., Milford, MA) was preconditioned with 5 mL of *n*-hexane followed by 5 mL of ethyl ether prior to use. Other chemicals were the same as described before (Nagata and Saeki, 1992).

Instrumentation. A Hewlett-Packard HP 5972 Series II mass spectrometry system and a Vectra 486/60 XM data system were used. The following operating conditions were used: ionization energy, 70 eV; electron multiplier, 1800 V; emission current, 300 μA; scan range, 150−500. All data for

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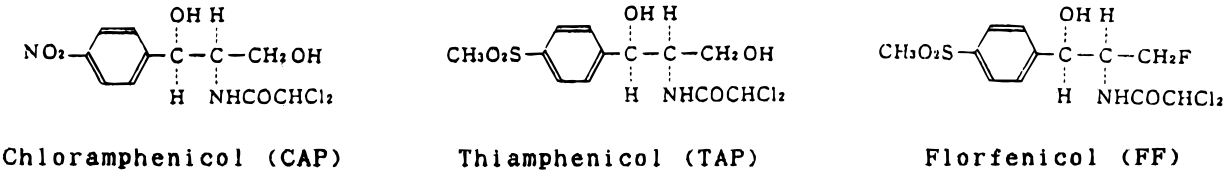

Figure 1. Chemical structures of CAP, FF, and TAP.

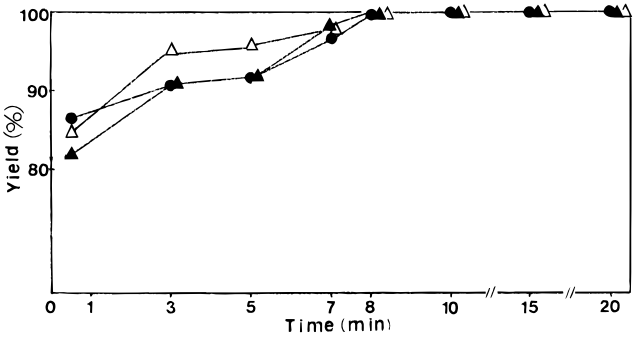

Figure 2. Effect of reaction time on derivatization of CAP (●), FF (▲), and TAP (△).

quantification were collected in the SIM mode at *m/z* 208, 225, 242, 257, and 330 at each dwell time of 100 ms. A Hewlett-Packard HP 5890 Series II gas chromatograph provided with a fused silica capillary column coated with 5% phenyl methyl silicone chemically bonded and cross-linked phase (HP-5MS, 0.25 mm i.d. × 30 m, 0.25 μm film, Hewlett-Packard, Avondale, PA) was used. The following conditions were used: column temperature, 250 °C; injection temperature, 265 °C; detection temperature, 280 °C; carrier gas, He at 1 mL/min; inlet pressure, 40 psi for 1 min, 40 psi to 20 psi, 99 psi/min; injection mode, splitless.

Sample Preparation. The sample preparation was the same as described before (Nagata and Saeki, 1992), with some minor modifications; both evaporation procedures of ethyl acetate extracts were performed at 50 °C. The drugs retained to the Sep-Pak Florisil cartridge were eluted with 5 and 3 mL of methanol−ethyl ether (3:7), successively. Eluate was evaporated to dryness on a rotary evaporator at 35 °C.

A 10 g muscle sample was homogenized with 50 mL of ethyl acetate and centrifuged at 2300*g* for 10 min, and the supernatant was transferred to a round-bottom flask. The residue was homogenized with another 50 mL of ethyl acetate and centrifuged, and the supernatant was collected into the flask. The extract was concentrated to 2−3 mL under vacuum on rotary evaporator at 50 °C.

Table 1. Recovery of CAP, FF, and TAP Added to Yellowtail Muscles

	CAP				FF		TAP					
	m/z 208		*m/z* 225		*m/z* 257		*m/z* 242		*m/z* 257		*m/z* 330	
no. of expt	found (μg)	recov %	found (μg)	recov %	found (μg)	recov %	found (μg)	recov %	found (μg)	recov %	found (μg)	recov %
					2 μg Added[a]							
1	1.41	70.5	1.30	65.0	1.28	64.0	1.32	66.0	1.30	65.0	1.29	64.5
2	1.45	72.5	1.64	82.0	1.35	67.5	1.34	67.0	1.35	67.5	1.30	65.0
3	1.43	71.5	1.60	80.0	1.24	62.0	1.40	70.0	1.45	72.5	1.31	65.5
4	1.30	65.0	1.47	73.5	1.29	64.5	1.39	69.5	1.42	71.0	1.32	66.0
5	1.45	72.5	1.53	76.5	1.32	66.0	1.41	70.5	1.44	72.0	1.30	65.0
mean	1.41	70.5	1.51	75.5	1.30	65.0	1.37	68.5	1.39	69.5	1.31	65.5
SD	0.060		0.132		0.042		0.042		0.064		0.016	
CV	4.25		8.73		3.25		3.04		4.57		1.27	
					1 μg Added[a]							
1	0.692	69.2	0.633	63.3	0.622	62.2	0.644	64.4	0.671	67.1	0.644	64.4
2	0.714	71.4	0.661	66.1	0.693	69.3	0.621	62.1	0.642	64.2	0.691	69.1
3	0.603	60.3	0.695	69.5	0.621	62.1	0.662	66.2	0.692	69.2	0.682	68.2
4	0.641	64.1	0.630	63.0	0.621	62.1	0.633	63.3	0.649	64.9	0.659	65.9
5	0.620	62.0	0.690	69.0	0.664	66.4	0.693	69.3	0.608	60.8	0.641	64.1
mean	0.654	65.4	0.662	66.2	0.652	65.2	0.651	65.1	0.652	65.2	0.663	66.3
SD	0.047		0.031		0.031		0.028		0.032		0.022	
CV	7.24		4.62		4.75		4.32		4.85		3.38	

[a] 1 or 2 μg of each drug was added to 10 g of muscles.

The residual solution was transferred with 25 mL of 3% sodium chloride solution to a separatory funnel quantitatively. Twenty-five milliliters of *n*-hexane was added to the separatory funnel, shaken vigorously for 5 min, and allowed to stand until layers were separated. The lower phase was transferred to another separatory funnel, and the drugs were extracted with 40 mL of ethyl acetate twice. Each upper phase was collected

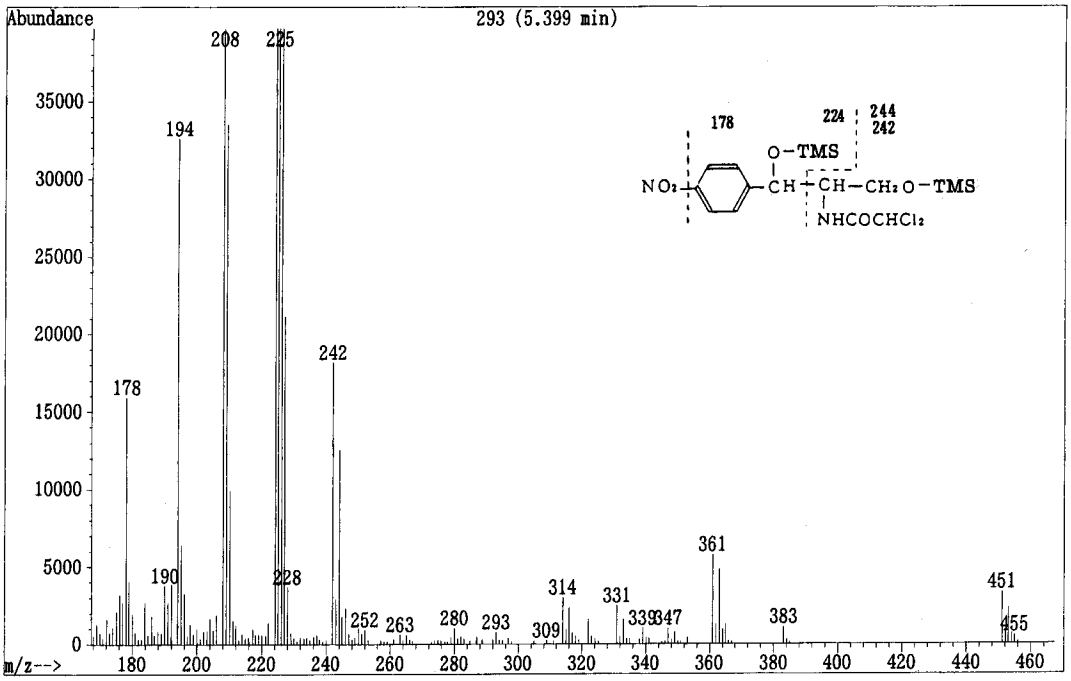

Figure 3. Mass spectrum of TMS derivative of CAP. Mass condition: ionization energy, 70 eV; electron multiplier, 1800 V; emission current, 300 μA; scan range, 150−500.

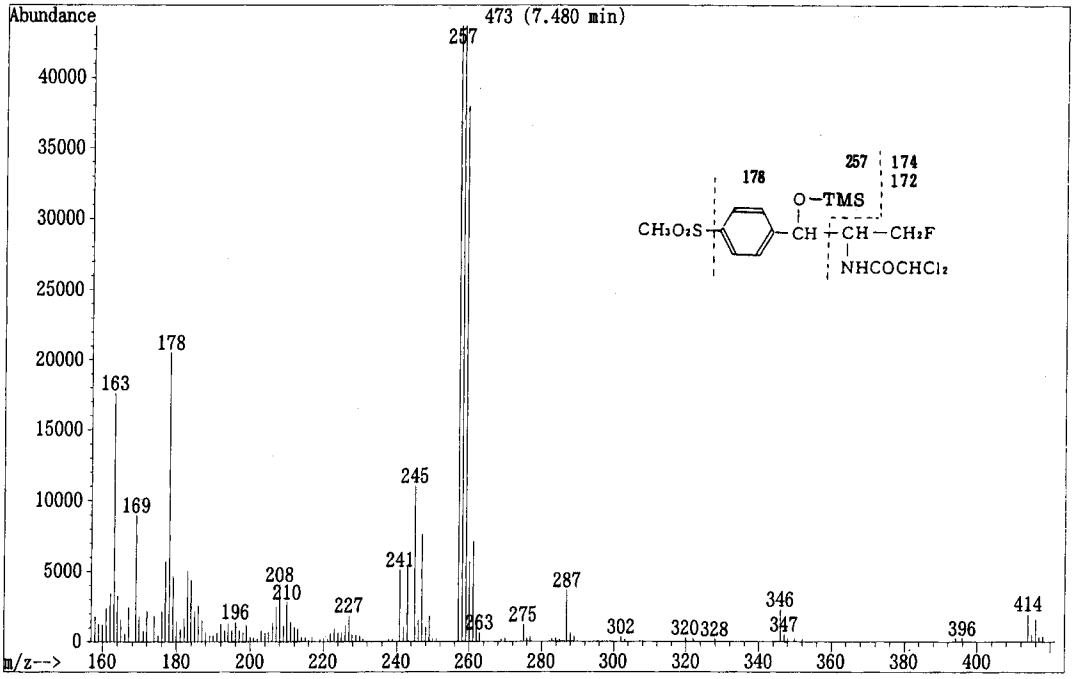

Figure 4. Mass spectrum of TMS derivative of FF. The mass condition was the same as described in Figure 3.

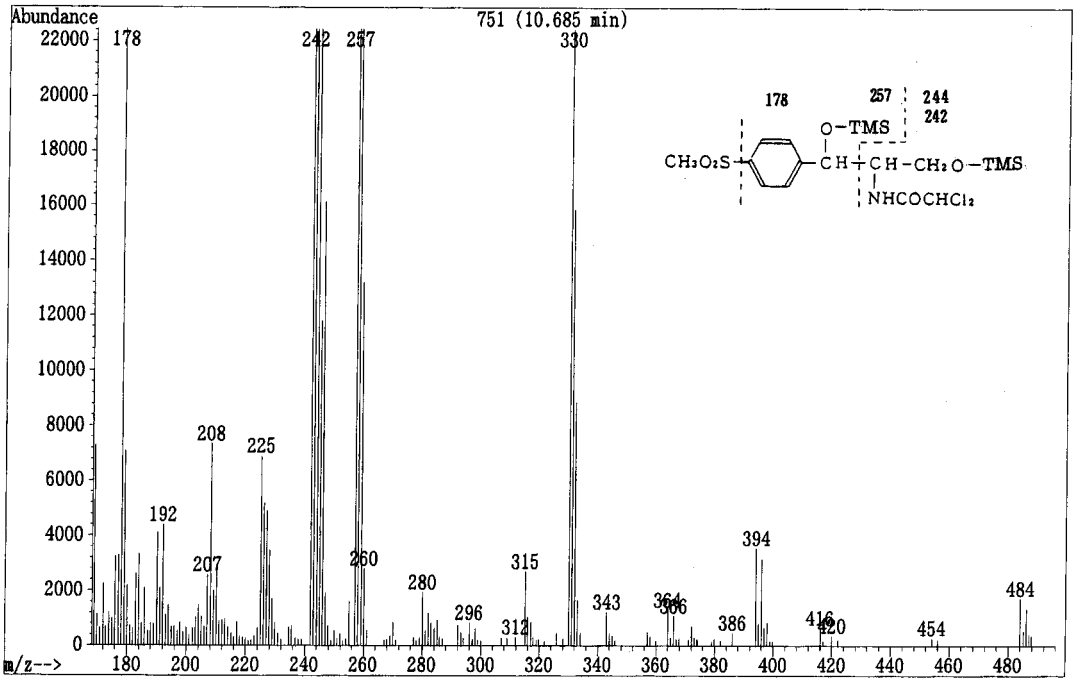

Figure 5. Mass spectrum of TMS derivative of TAP. The mass condition was the same as described in Figure 3.

in a round-bottom flask, and the ethyl acetate layer was evaporated to dryness under vacuum on a rotary evaporator at 50 °C.

The residue was dissolved with 5 mL of *n*-hexane in an ultrasonic bath and poured into a Sep-Pak Florisil cartridge, and the cartridge was washed. The flask was rinsed with 5 mL of ethyl ether in the ultrasonic bath, and the rinsing was poured into the cartridge and then the cartridge was washed. The drugs were eluted with 5 and 3 mL of methanol−ethyl ether (3:7), successively, and both eluates were collected and then evaporated to dryness under vacuum on a rotary evaporator at 35 °C.

Derivatization. The evaporated residue of the sample solution was dissolved with 0.5 mL of acetonitrile and 0.4 mL of BSA. The drugs were derivatized for 10 min at 50 °C and excess solvents were evaporated to dryness using a gentle stream of nitrogen. The derivatives were dissolved with 1 mL of ethyl acetate, and 2 μL of this solution was injected into the GC−MS system.

RESULTS AND DISCUSSION

The study on sample preparation has been described in detail elsewhere (Nagata, 1995). Evaporation of ethyl acetate extracts and eluate from the Sep-Pak Florisil cartridge was performed at 50 and 35 °C, respectively, resulting in satisfactory evaporating speed. Elution of the three drugs was performed with 5 mL followed by 3 mL of methanol−ethyl ether (3:7) solution, resulting in a minor improvement of the recoveries of the drugs, especially TAP.

It was found that more than 8 min was required to complete silylation of the three drugs, as shown in Figure 2. On derivatization solvents, such as acetonitrile, ethyl acetate, and pyridine, which were used to dissolve the drug residues, did not show any significant difference.

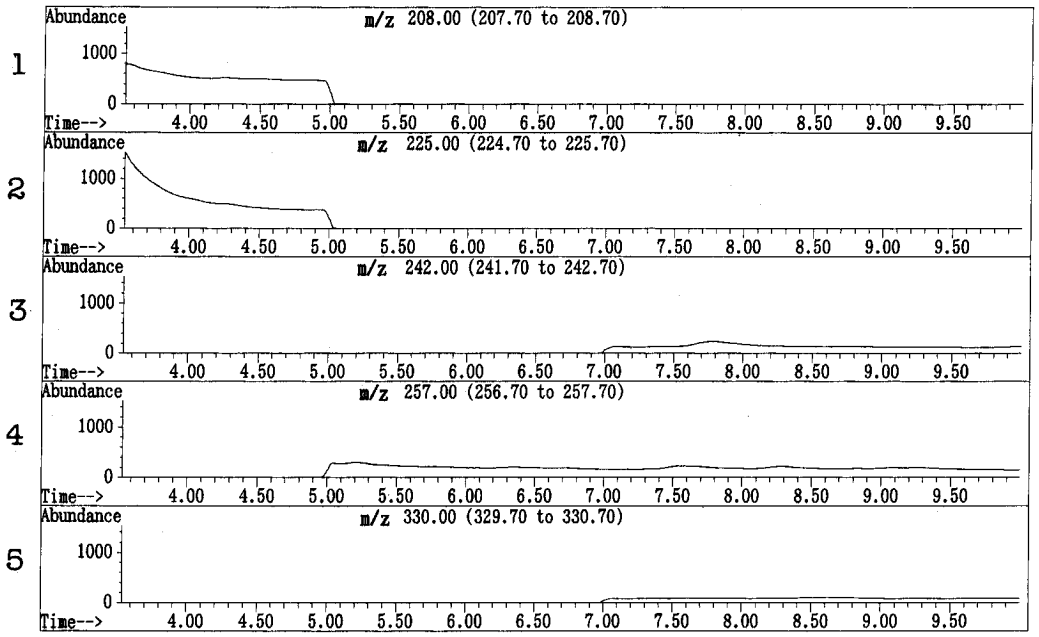

Figure 6. SIM of blank yellowtail muscle extract. CAP at *m/z* 208 and 225 was scanned from 3.5 to 5 min (1, 2). FF and TAP at *m/z* 257 were scanned from 5 to 10 min (4). TAP at *m/z* 242 and 330 was scanned from 7 to 10 min (3, 5).

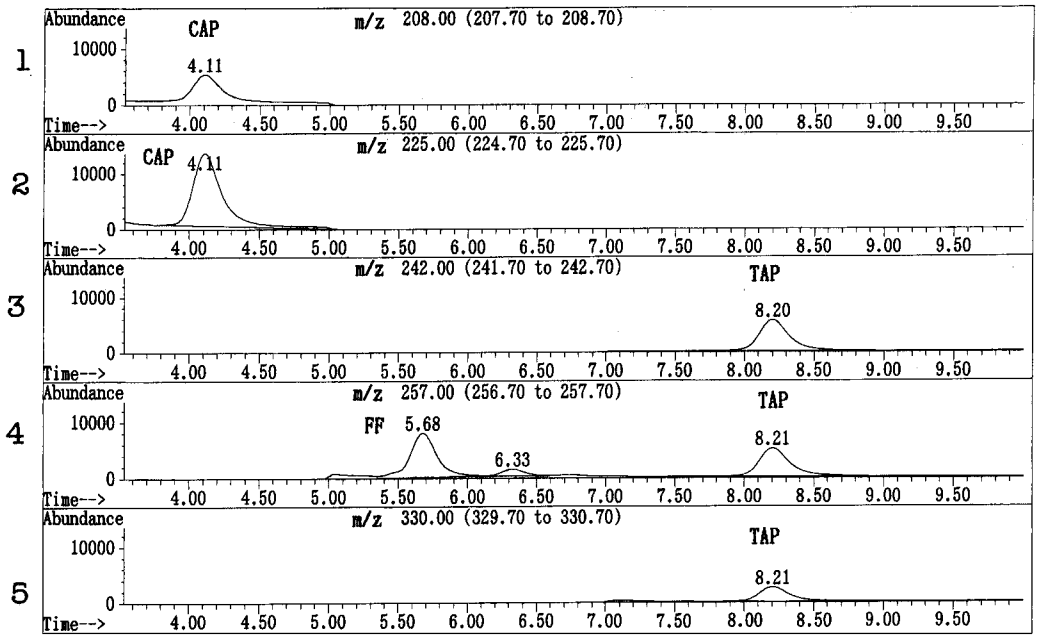

Figure 7. SIM of drug-fortified yellowtail muscle extract. Two micrograms of drug was added to 10 g of yellowtail muscle. Scan conditions were the same as described in Figure 6.

Mass spectra of derivatives of CAP, FF, and TAP standards are shown in Figures 3−5. The silylated molecular ion of each drug was not observed. With BSA derivatization, two positions of CAP and TAP, as described before (Nakagawa *et al.*, 1975; Plomp and Maes, 1976), and one position of FF were considered to be trimethylsilylated. In CAP, *m/z* 453 and 451, in FF, *m/z* 416 and 414, and in TAP, *m/z* 396 and 394, contributed to M − CH_3 (15) in each drug. In CAP, *m/z* 363 and 361, and in TAP, *m/z* 396 and 394, were considered to be M − (CH_3 + TMS − OH) (105) in both drugs. For SIM analysis, the base peak *m/z* 225 and additional peak *m/z* 208 for CAP, the base peak *m/z* 257 for FF, and the base peak *m/z* 242 and additional peaks *m/z* 257 and 330 for TAP were selected. SIM chromatograms of blank and fortified yellowtail muscle extracts are shown in Figures 6 and 7. As shown in Figure 6, there were no interfering peaks observed in the blank yellowtail muscle extract chromatogram. CAP, FF, and TAP peaks were observed at 4.11, 5.68, and 8.21 min, respectively, as shown in Figure 7.

The standard curves of CAP at *m/z* 225, of FF at *m/z* 257, and of TAP at *m/z* 242 and 257 were linear over the range 0.05−2 μg/mL with correlation coefficients of 0.9987, 0.9987, 0.9982, and 0.9965, respectively. The standard curves of CAP at *m/z* 208 and of TAP at *m/z* 330 were both linear at 1−2 μg/mL with correlation coefficients of 0.9975 and 0.9979, respectively. The detection limit, defined as the level of each drug in animal tissue that produces a signal-to-noise ratio of 3, was 5 ppb for CAP, FF, and TAP.

Recovery studies were performed by adding 1.0 or 2.0 mL of standard solution containing 1 μg/mL of CAP, FF, or TAP to 10 g of minced yellowtail muscles. The average recoveries of 0.1 and 0.2 ppm were more than 65%, as shown in Table 1. There were no significant differences statistically between the recoveries at *m/z*

208 and 225 in CAP or among *m*/*z* 242, 257, and 330 in TAP at the 95% confidence level.

This determination method is useful for the accurate and specific detection of residual drugs.

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