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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

XVI[a]. SIMPLE AND RAPID DETERMINATION OF RESIDUAL PYRIDONECARBOXYLIC ACID ANTIBACTERIALS IN FISH USING A PREPACKED AMINO CARTRIDGE

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SUMMARY

A simple and rapid method for the determination of residual pyridonecarboxylic acid antibacterials (PCAs) (oxolinic acid, nalidixic acid and piromidic acid) in fish was developed using a combination of high-performance liquid chromatography (HPLC) and clean-up with an amino-type prepacked cartridge. PCAs were extracted with *n*-hexane–ethyl acetate (1:3) and the extract was applied to a Baker 10 amino cartridge. PCAs were eluted from the cartridge with acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (pH 3.0) (3:1:6) and were determined by HPLC. The separations were performed on Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.) using a mobile phase containing oxalic acid. The recoveries of PCAs from various fishes fortified at the level of 1.0 ppm were 77.1–95.5%, and the detection limits were 0.05 ppm. The analytical time per sample was less than 30 min.

INTRODUCTION

Pyridonecarboxylic acid antibacterials (PCAs) are widely applied to cultured fishes to cure and prevent diseases, and oxolinic acid, nalidixic acid and piromidic acid are permitted in Japan. However, their residues in fish constitute one of the most serious problems for public health agencies. Although some high-performance liquid chromatographic (HPLC) methods[1–8] had been applied to the determination of residual PCAs in fish, we considered them to be unsuitable for routine analysis for the following reasons. For clean-up of PCAs, these methods require a long time because

[a] For Part XV, see *J. Chromatogr.*, 462 (1989) 315.

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they require some time-consuming treatments such as column chromatography[6], concentration in an evaporator[1–8], filtration[1–3,6,8] and partition in a separating funnel[1–7]. For determination, because PCAs appear as tailing peaks in reversed-phase HPLC, ion-pair chromatography[2,5,7,9,10], application of citrate buffer in the mobile phase[11,12] and derivatization of PCAs to methyl esters[1,13] have been attempted. However, ion-pair chromatography and the use of a citrate buffer did not give effective results, and the derivatization is very complicated. Although ion-exchange columns[3,4,14,15] and a wide-pore ODS column[8] were successfully applied to the determination of PCAs, a conventional ODS column is more desirable from considerations of cost and durability of the HPLC column.

In order to establish a suitable method for the routine determination of residual PCAs in fish, we tried to simplify the clean-up of PCAs by using a disposable prepacked cartridge and to control the tailing of PCAs on a conventional ODS column using the techniques we reported previously[16,17]. This paper describes techniques for the sensitive and rapid determination of PCAs using a conventional HPLC column with Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.), and for the simple and rapid clean-up of residual PCAs in fish using a Baker 10 amino cartridge.

EXPERIMENTAL

Materials

Acetic acid, acetonic acid (2-hydroxyisobutyric acid), acetonitrile, anhydrous sodium sulphate, citric acid, ethyl acetate, *n*-hexane, lactic acid, malonic acid, methanol, oxalic acid, phosphoric acid, sodium hydroxide (NaOH) and tartaric acid were analytical-reagent grade materials. Oxolinic acid (OA), nalidixic acid (NA) and piromidic acid (PA) were purchased from Sigma (St. Louis, MO, U.S.A.).

Baker 10 amino (catalogue No. 7088-3), Baker 10 cyano (7021-3), Baker 10 diol (7094-3), Baker 10 primary-secondary amino (7089-3), Baker 10 quaternary amine (7091-3), Baker 10 carboxylic acid (7211-3) and Baker 10 aromatic sulfonic acid (7090-3) cartridges were purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

Preparation of standard solution

Each standard (10 mg) was weighed accurately into a 100-ml volumetric flask and diluted to volume with acetonitrile. Subsequent dilutions were made with the eluent.

Clean-up procedure

A 5-g amount of sample and 10 g of anhydrous sodium sulphate were weighed into a 50-ml centrifuge tube, blended with 20 ml of extraction solvent for 30 s using a high-speed blender (Ultra-Turrax TP 18/2N, IKA WERK, Staufen, F.R.G.), centrifuged (1500 rpm, 300 *g*, for 2 min) and the supernatant was decanted. The above extraction procedure with the solvent was repeated once more and the combined extracts were applied to a Baker 10 amino cartridge pre-washed with 10 ml of methanol. The cartridge was washed with 5 ml of the extraction solvent, *n*-hexane–ethyl acetate (1:3), and air-dried by aspiration for 1 min. PCAs were eluted from the cartridge with 10 ml of acetonitrile–methanol–0.01 *M* aqueous oxalic acid (adjusted to pH 3.0 with NaOH) (3:1:6) and 1–20 μl of the eluate were injected into the HPLC system for the determination of PCAs.

High-performance liquid chromatography

A high-performance liquid chromatograph equipped with a constant-flow pump (LC-5A, Shimadzu, Kyoto, Japan) was used, with a UV detector (Shimadzu SPD-2AM) operated at 295 nm. The separation was performed on Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.) (Macherey, Nagel & Co., Düren, F.R.G.) with acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (3:1:6) as the mobile phase at a flow-rate of 1.0 ml/min at room temperature.

RESULTS AND DISCUSSION

Establishment of HPLC system

In a previous study[16,17], organic acids such as oxalic acid and acetonic acid were effective in control the tailing of tetracycline antibiotics and dehydroacetic acid, respectively, in reversed-phase (RP) HPLC. We considered that such reagents may also be effective in controlling the tailing of PCAs. In order to find a suitable reagent for the determination of PCAs by RP-HPLC, peak asymmetry factors (A_s) of PCAs were measured using mobile phases containing various acids (acetic, phosphoric, citric, tartaric, malonic, lactic, acetonic and oxalic acid). As shown in Table I, a significant effect was given with oxalic acid, in spite of the low concentration. Therefore, we tried to optimize the HPLC conditions for the determination of PCAs using oxalic acid in the mobile phase, and a satisfactory chromatogram of PCAs was obtained as shown in Fig. 1.

With regard to the stationary phase to be used for the separation of PCAs, Nucleosil C_{18} gave satisfactory separations and A_s values of PCAs using oxalic acid in the mobile phase. In contrast, satisfactory A_s values could not be obtained on LiChrosorb RP-18 (E. Merck, Darmstadt, F.R.G.) using any of the reagents described above in the mobile phase. The difference can be successfully explained by the assumptions that the tailing of PCAs is caused by the influence of residual silanol

TABLE I

COMPARISON OF ASYMMETRY FACTORS OF OA, NA AND PA USING VARIOUS AQUEOUS SOLUTIONS IN THE MOBILE PHASE

Peak asymmetry factors of OA, NA and PA were measured under the following HPLC conditions: column, Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.); mobile phase, acetonitrile–methanol–aqueous solution (3:1:6); flow-rate, 1.0 ml/min; detection, 295 nm.

Aqueous solution	*Asymmetry factor (A_s)*		
	OA	*NA*	*PA*
0.05 *M* acetic acid	9.5	>10.0	>10.0
0.05 *M* phosphoric acid	2.6	7.4	9.0
0.05 *M* citric acid	2.3	3.7	4.3
0.05 *M* tartaric acid	2.1	4.4	5.4
0.05 *M* malonic acid	1.9	4.0	5.5
0.05 *M* lactic acid	1.9	2.0	1.9
0.05 *M* acetonic acid	1.9	1.9	1.9
0.01 *M* oxalic acid	1.8	1.7	1.6

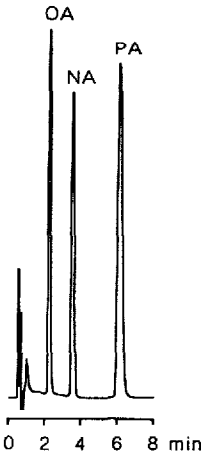

Fig. 1. Typical high-performance liquid chromatogram of OA, NA and PA (100 ng each) under the optimum conditions. Column, Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.); mobile phase, acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (3:1:6); flow-rate, 1.0 ml/min; detection, 295 nm.

groups on the surface of the stationary phase and that oxalic acid has the ability to mask such silanol groups. Nucleosil C_{18} is end-capped and LiChrosorb RP-18 is not. Therefore, Nucleosil C_{18} successfully undergoes such an effect with oxalic acid because of its lower content of silanol groups, but LiChrosorb RP-18 has too many silanol groups to do so. In addition to the satisfactory A_s values of PCAs, a Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.) was advantageous for the sensitive and rapid determination of PCAs because of its small particle size and the short column length, and therefore we used it as an analytical HPLC column.

Concerning the monitoring wavelength, OA and NA were most sensitive at 255 nm and PA at 280 nm; however, simultaneous determination was difficult in the range 230–290 nm because of the difference in peak heights among PCAs. As shown in Fig. 1, PCAs were monitored with similar sensitivity at 295 nm, so this was chosen as a monitoring wavelength.

In order to separate PCAs successfully, the following parameters of the mobile phase were examined; concentration of aqueous oxalic acid solution, pH of aqueous oxalic acid solution and proportions of aqueous solution, acetonitrile and methanol.

The concentration of oxalic acid in the mobile phase hardly influenced the capacity factors (k') of PCAs, whereas the A_s values were improved with increasing concentration. However, they were almost constant above 0.01 *M* and therefore we used 0.01 *M* aqueous oxalic acid solution in the mobile phase.

Whereas, for the pH of aqueous oxalic acid solution in the mobile phase, the k' values of PCAs were almost constant between pH 2.2 and 5.0, the A_s values increased with increasing pH and the best values were given with a pH-unadjusted solution (pH 2.2). We therefore used 0.01 *M* aqueous oxalic acid solution without adjustment of pH in the mobile phase.

In relation to the proportions of acetonitrile and methanol in the mobile phase, a satisfactory separation of PCAs was obtained with any proportion of acetonitrile and methanol, as shown in Fig. 2. However, mobile phases containing acetonitrile

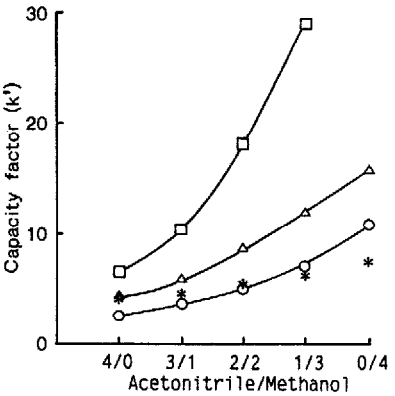

Fig. 2. Influence of ratio of methanol and acetonitrile on k' of PCAs and interfering substance. ○, OA; △, NA; □, PA; ∗, interfering substance from the amino cartridge. Mobile phase: (acetonitrile–methanol)–0.01 *M* aqueous oxalic acid solution (4:6).

and methanol in the ratios 4:0, 2:2 and 1:3 were not applicable in our clean-up system because of interfering peaks originating from the amino cartridge. On the other hand, for a constant ratio of organic solvent and aqueous solution, the k' values increased with increasing proportion of aqueous solution. Considering the retention times of PCAs and the influence of interfering peaks, we used acetonitrile, methanol and 0.01 *M* aqueous oxalic acid solution in the proportions 3:1:6 in the mobile phase.

As a result of these studies, we obtained the optimum HPLC conditions given under Experimental. According to these conditions, PCAs were successfully separated within 7 min. The detection limits of PCAs on the chromatogram were 0.5 ng (signal-to-noise ratio >5) and the calibration graphs were linear between 0.5 and 500 ng.

Establishment of clean-up system

In order to carry out the clean-up of PCAs simply and rapidly, we considered utilizing a disposable prepacked cartridge, and the following summarized clean-up procedure was applied. PCAs are extracted from the sample by blending with an organic solvent, the resulting extract is centrifuged and the supernatant is decanted and applied to a prepacked cartridge. PCAs are eluted from the cartridge with a suitable eluent and determined by HPLC. After the following studies of various aspects of this procedure, the simple and rapid clean-up system described under Experimental was established.

Comparison of prepacked cartridges. In order to select a suitable prepacked cartridge, commercially available normal-phase and ion-exchange type (cyano, diol, primary-secondary amino, amino, quaternary amine, carboxylic acid and aromatic sulphonic acid) cartridges were compared for their ability to retain PCAs. As the solvent to be applied to the cartridges, ethyl acetate was used in this examination, because it has often been used as an extraction solvent for PCAs. PCAs (5 μg each) were dissolved in 40 ml of ethyl acetate and were applied to each cartridge. The ethyl acetate solutions that had passed through the cartridge were collected and evaporated to dryness. PCAs in each residue were dissolved in the mobile phase and were deter-

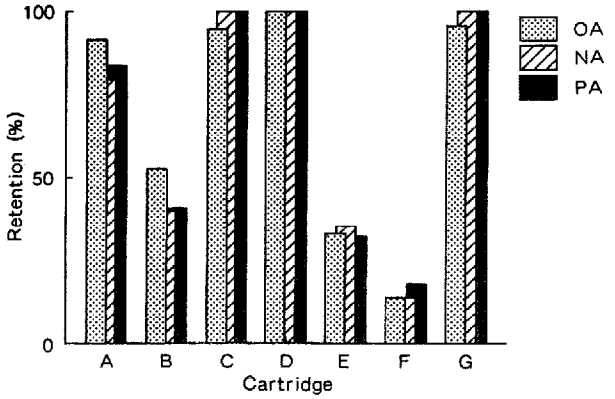

Fig. 3. Comparison of the abilities of prepacked cartridges to retain PCAs. Cartridges: A, Baker 10 cyano; B, Baker 10 diol; C, Baker 10 primary-secondary amino; D, Baker 10 amino; E, Baker 10 quaternary amine; F, Baker 10 carboxylic acid; G, Baker 10 aromatic sulfonic acid. HPLC conditions as in Fig. 1.

mined by HPLC. From the results, the percentages of PCAs retained on each cartridge were calculated. As shown in Fig. 3, PCAs were completely retained on the amino cartridge, so this was used in subsequent work.

Elution from the cartridge. Because the mobile phase is generally suitable for preparing sample and standard solutions to be injected into an HPLC system, we wished to elute PCAs from the cartridge with the mobile phase. However, satisfactory recoveries of PCAs from the cartridge could not be obtained in an attempt to use the mobile phase solution (acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution, 3:1:6) as the eluent. It was considered that the pH of the eluent is one of the most important factors for the elution of PCAs from the cartridge and that the elution of PCAs is expected to be improved by increasing the pH. In order to examine the influence of the pH of the eluent on the elution of PCAs from the cartridge, acetonitrile, methanol and 0.01 *M* aqueous oxalic acid solution (adjusted to various pH values with NaOH) were mixed in the same proportions as in the mobile phase and the recoveries of PCAs retained on the cartridge were investigated. The sample (eel, 5 g) and anhydrous sodium sulphate (10 g) were blended with 40 ml of *n*-hexane–ethyl acetate (1:3) and an extract of the sample was obtained. After addition of PCAs (5 μg each) to the extract, they were applied to the cartridge. PCAs were eluted with 10 ml of each eluent and were determined by HPLC. Satisfactory recoveries were obtained when the pH of 0.01 *M* aqueous oxalic acid solution was adjusted above 2.5. The elution pattern of PCAs from the cartridge was investigated using acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (pH 3.0) (3:1:6) as the eluent. After application of PCAs to the cartridge in the same manner as described above, the PCAs were eluted with the eluent. The eluate was fractionated into 2-ml fractions and PCAs in each fraction were determined by HPLC. As shown in Fig. 4, PCAs were satisfactorily eluted in 8 ml of eluate. Therefore, we used 10 ml of acetonitrile–methanol–0.01 *M* aqueous oxalic acid solution (pH 3.0) (3:1:6) as the eluent for the amino cartridge in subsequent work.

Comparison of extraction solvents. PCAs are slightly soluble in polar solvents such as water and insoluble in non-polar solvents such as benzene and hexane, so

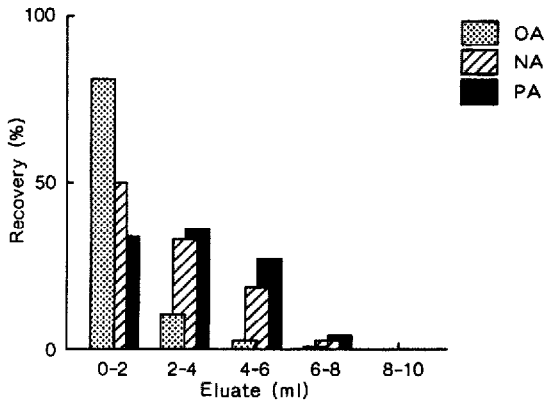

Fig. 4. Influence of elution volume on recoveries of PCAs from the amino cartridge. HPLC conditions as in Fig. 1.

methanol, acetonitrile, dichloromethane, chloroform and ethyl acetate have generally been used as extraction solvents from various samples[1–5,7–12]. However, chloroform and dichloromethane were unsuitable as extraction solvents for our clean-up system, because their high specific gravity prevented the decantation of the extracts. The applicability of methanol, acetonitrile and ethyl acetate was investigated by means of the following examination. Extracts of sample (eel, 5 g) with 40 ml of each extraction solvent were obtained in the same manner as described above. After addition of PCAs (5 μg each) to each extract, they were applied to the amino cartridge. PCAs were eluted from the cartridge with the eluent and were determined by HPLC. When methanol and acetonitrile were used as extraction solvents, the recoveries were very poor. Ethyl acetate gave satisfactory recoveries of NA and PA, but the recovery of OA was slightly lower than those of NA and PA. These results suggested that the retention power of the cartridge increases with decreasing polarity of the solvent and that the polarities of methanol and acetonitrile are too high to give sufficient retention of PCAs on the cartridge. As described above under *Comparison of prepacked cartridges*, because PCAs were completely retained on the cartridge using ethyl acetate alone as the solvent, the different retention behaviour was attributed to the influence of the sample matrix and OA was the most susceptible to such an influence. We considered that the use of a less polar extraction solvent than ethyl acetate would be effective for improving the retention of OA on the cartridge.

On the basis of the above considerations, several solvent systems were prepared by combination of *n*-hexane and ethyl acetate (1:3, 2:2, 3:1 and 4:0), and the recoveries of PCAs were investigated in the same manner as described above. Although satisfactory results were obtained in every instance with these mixtures, the efficiency of extraction of PCAs from the sample decreased with increasing proportion of *n*-hexane in ethyl acetate. In order to select a suitable mixing ratio of *n*-hexane and ethyl acetate, the overall recoveries of PCAs were investigated using various mixtures of *n*-hexane and ethyl acetate. After addition of PCAs (5 μg each) and anhydrous sodium sulphate (10 g) to the sample (eel, 5 g), they were extracted with 40 ml (2 × 20 ml) of *n*-hexane–ethyl acetate (0:4, 1:3, 2:2, 3:1 and 4:0) and applied to the cartridge. As

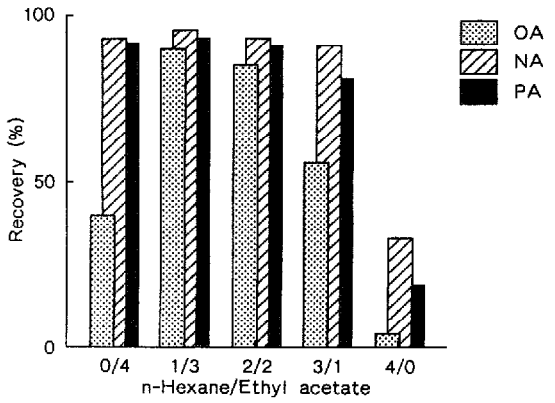

Fig. 5. Effect of ratio of *n*-hexane and ethyl acetate in the extraction solvent on recoveries of PCAs from fortified eel. HPLC conditions as in Fig. 1.

shown in Fig. 5, the best recoveries were obtained when *n*-hexane–ethyl acetate (1:3) was used as the extraction solvent. Next, the effect of extraction volume was examined using *n*-hexane–ethyl acetate (1:3). After addition of PCAs (5 μg each) and anhydrous sodium sulphate (10 g) to the sample (eel, 5 g), they were extracted with 20 ml, 40 ml (2 × 20 ml), 60 ml (3 × 20 ml) and 80 ml (4 × 20 ml) of the extraction solvent and each extract was applied to the cartridge. The best recoveries were obtained when 40 ml of extraction solvent were used. When more than 40 ml of extraction solvent was used, the recoveries of OA and NA became poorer because of weak retention on the cartridge. Therefore, we used 40 ml (2 × 20 ml) of *n*-hexane–ethyl acetate (1:3) as the extraction solvent in our clean-up system.

Effect of anhydrous sodium sulphate. In order to improve the efficiency of extraction of PCAs from the sample, anhydrous sodium sulphate was used, otherwise the sample formed a gummy mass at the bottom of the centrifuge tube during blending with the extraction solvent and satisfactory recoveries of PCAs could not be obtained. In addition, the use of anhydrous sodium sulphate was advantageous for the retention of PCAs on the cartridge, because the retention power of the cartridge was weakened by the influence of water in the extract. It was sufficient to use 10 g of anhydrous sodium sulphate for extraction of PCAs from 5 g of sample.

Application to various fish

The recoveries of PCAs from fortified eel, rainbow trout, sweetfish, red sea bream and yellowtail were investigated at the levels of 1.0 and 0.1 ppm. PCAs (5 and 0.5 μg) were spiked with 50 μl of acetonitrile to 5 g of each sample and determined in accordance with the present method.

As shown in Table II, satisfactory recoveries and coefficients of variations were obtained at the low concentration level of PCAs. The detection limits were 0.05 ppm and the time required for the analysis of one sample was less than 30 min. Typical high-performance liquid chromatograms of these fish are shown in Fig. 6. No interfering peaks appeared on the chromatograms.

In conclusion, a method for the determination of residual PCAs in fish was established using a combination of HPLC and clean-up with a Baker 10 amino car-

TABLE II

RECOVERIES OF OA, NA AND PA FROM FORTIFIED FISH

Recoveries of OA, NA and PA from 5 g of various fish fortified at the levels of 1.0 and 0.1 ppm according to the present method. Results for four replicates (1.0 ppm) and three replicates (0.1 ppm).

Sample	*Addition level (ppm)*	*Recovery (%)*[a]		
		OA	*NA*	*PA*
Eel	1.0	90.3 (2.4)	95.5 (1.3)	92.6 (1.7)
	0.1	92.4 (4.0)	95.2 (1.0)	89.8 (2.0)
Rainbow trout	1.0	87.5 (3.9)	93.2 (3.8)	87.3 (3.9)
	0.1	76.1 (9.8)	81.4 (4.8)	79.9 (8.3)
Sweetfish	1.0	88.3 (3.4)	93.2 (1.1)	90.0 (0.6)
	0.1	84.9 (2.2)	87.3 (4.5)	87.4 (1.5)
Red sea bream	1.0	85.8 (0.9)	88.4 (3.5)	82.0 (3.3)
	0.1	80.7 (2.8)	87.5 (3.7)	73.9 (3.5)
Yellowtail	1.0	77.1 (1.2)	90.4 (1.5)	83.9 (3.2)
	0.1	75.5 (6.8)	88.9 (6.8)	90.9 (7.8)

[a] Coefficients of variation (%) in parentheses.

tridge. For the determination of PCAs by HPLC, oxalic acid was effectively used to control the tailing of PCAs on a conventional RP-HPLC column of Nucleosil $3C_{18}$ (3 μm, 75 × 4.6 mm I.D.). In the clean-up procedure, PCAs in fish were successfully extracted with *n*-hexane–ethyl acetate (1:3) and a Baker 10 amino cartridge was effective for the simple and rapid clean-up of PCAs. Using this method, PCAs spiked in various fishes were rapidly determined with good recoveries, coefficients of variation and sensitivity. Further, this method does not require any special HPLC columns and any time-consuming treatments. Therefore, we recommend it for the routine determination of residual PCAs in fish.

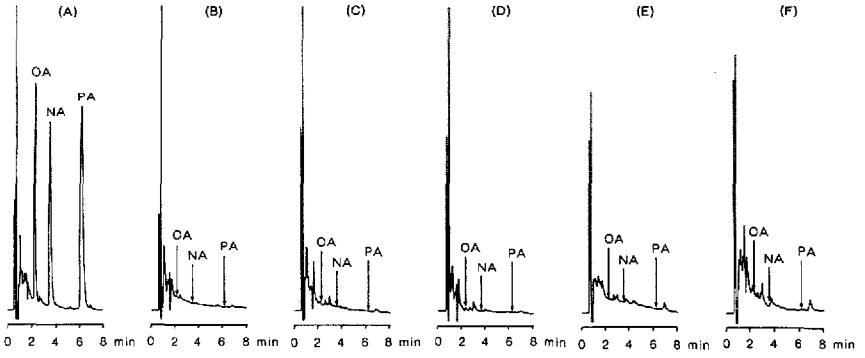

Fig. 6. Typical high-performance liquid chromatograms of different fish. (A) Fortified eel (1.0 ppm); (B) eel; (C) rainbow trout; (D) sweetfish; (E) red sea bream; (F) yellowtail. HPLC conditions as in Fig. 1.

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