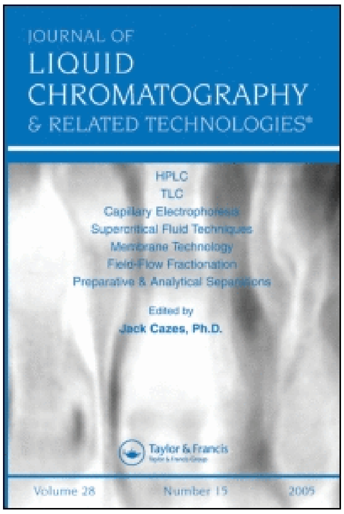

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Pouliquen, H. , Pinault, L. and Bris, H. Le(1994) 'Determination of Oxolinic Acid in Seawater, Marine Sediment, and Japanese Oyster (*Crassostrea Gigas*) by High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 17: 4, 929 — 945

To link to this Article: DOI: 10.1080/10826079408013378

URL: http://dx.doi.org/10.1080/10826079408013378

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(4), 929-945 (1994)

DETERMINATION OF OXOLINIC ACID IN SEAWATER, MARINE SEDIMENT, AND JAPANESE OYSTER *(CRASSOSTREA GIGAS)* BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method was developed to detect oxolinic acid in seawater, marine sediment, and japanese oyster (*Crassostrea gigas*). Seawater was analysed after filtration and centrifugation. Sediment and oyster tissues were respectively analysed after liquid-phase and solid-phase extraction. Linearity and precision were checked over the concentration range 0.05-2.50 µg/mL or µg/g. Limits of detection and determination were respectively 0.01 and 0.04 µg/mL or µg/g. Recoveries of oxolinic acid were 102.1% from seawater, 68.1% from sediment and 88.3% from oyster. Oxolinic acid concentrations in seawater, sediment and oyster spiked at 0.50 µg/mL or µg/g were stable at - 20°C for 60 days.

INTRODUCTION

The quinolone oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid) is a commonly used antibiotic for treatment of infectious diseases in fish farming. The drug is administered to fish as medicated pelleted feed at a dosage of 10-20 mg/kg body weight for 8-10 days.

Since fish suffering from bacterial diseases usually show reduced food intake and oxolinic acid is poorly absorbed through the intestinal tract of fish (1), oxolinic acid may subsequently pass into the environment of the fish farm. On one hand, fragments of the medicated food pellets could be taken up by filter-feeders such as shellfish, or reach sediment (2). Oxolinic acid may also be released from pellets in a dissolved form which can be absorbed by organisms directly from the water. On another hand, much of the drug could pass through the fish and enter the environment in an unchanged and active form via faeces (3). The drug-containing faeces from the farmed fish could, either be eaten by the wild fauna, or reach sediment.

The development of fish farming in ancient salt-marshes along the French Atlantic coastline may be responsible for the pollution of seawater, marine sediment and shellfish by oxytetracycline (4, 5, 6). The current knowledge of the environmental impacts of oxolinic acid used in marine aquaculture is merely poor. Thus, it is necessary to develop adequate methods for kinetic and residues studies of oxolinic acid in seawater, sediment and edible shellfish.

The commonly used microbiological assays for determining oxolinic acid are less sensitive and less specific than the chromatographic methods (7). Several methods using high-performance liquid chromatography (HPLC) with octyl or octadecyl silane columns for analysing oxolinic acid in fish plasma and tissues have been described (1, 8, 9, 10, 11, 12, 13, 14, 15). Only two optimized methods in fish tissues have been adapted to the determination of oxolinic acid in sediment (16) and blue mussel (17).

The purpose of the present work was to develop simple, rapid and accurate HPLC methods for analysing oxolinic acid in seawater, marine sediment and japanese oyster (*Crassostrea gigas*). The need for these methods was the ability to study occurence, persistence and metabolism of oxolinic acid in seawater, sediment and japanese oyster, via experiments carried out both *in situ* and under controled tank conditions.

MATERIALS AND METHODS

Chemicals

Acetonitrile (BDH Chemicals, Toronto, Canada) and methanol (Carlo-Erba, Milano, Italy) were of HPLC-grade. Chloroform, orthophosphoric acid, potassium dihydrogenphosphate, hydrochloric acid and sodium hydroxide (Merck, Darmstadt, Germany) were analytical-grade reagents. Ethyl acetate (Janssen, Geel, Belgium) was of analytical-grade. The water used in buffers and eluents was distilled and purified with an Elgastat Spectrum RO2 (Elga Ltd, Buckinghamshire, England). Oxolinic acid was purchased as a pure standard from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The HPLC system consisted of a chromatograph Varian 5000 equipped with a Valco injection valve, a Vari-Chrom UV 50 variable-wavelength absorbance detector (Varian, Palo Alto, CA, USA) and a Merck D-2500 integrator (Darmstadt, Germany). The data were handled with a computer Deskpro 386/s Mod 40 3.5 (Compaq, Houston, TX, USA) equipped with the HPLC Manager Software System (Merck). The analytical column, a 5 µm LiChroSpher 100 RP-18E, 125 X 4.6 mm I.D. (Merck), was equipped with a 5 µm LiChroSpher 100 RP-18E guard column, 4 X 4.6 mm I.D.

Chromatographic Conditions

The mobile phase consisted of acetonitrile and 0.02 M orthophosphoric acid solution (24:76 v/v), pH 2.3. The mixture was filtered with a Millipore HPLC solvent filtration system (Millipore, Bedford, MA, USA) and Whatman 47 mm, 0.20 µm nylon filters (Maidstone, England) and then sonicated for 15 min.

The chromatographic experiments were performed at ambient temperature. The operating flow rate was 1.0 mL/min and the UV detector was set at 262 nm and 0.01 a.u.f.s. The sample volume injected on the column was 50 µL. The guard column was removed at intervals of 150 to 200 sample injections.

The new columns were conditioned prior to use by flushing with acetonitrile and water 75:25 v/v (2h), 50:50 v/v (2h), 40:60 v/v (2h), 30:70 v/v (2h) and mobile phase (5h) at a flow rate of 0.2 mL/min. Moreover, the columns were reconditioned for 2 h after each day of operation with acetonitrile and water (24:76 v/v) at a flow rate of 0.2 mL/min.

Standard Solutions

A stock solution of oxolinic acid was prepared in 0.03 M aqueous sodium hydroxide at a concentration of 1 mg/mL and was stable for one month when stored at 4°C. Working standard solutions were prepared by dilution in water immediatly before use. All these solutions were stored in dark.

Extraction Procedures

A 2-mL volume of a seawater sample was filtered through a Minisart NML 26 mm, 0.45 µm cellulose acetate membrane (Sartorius, Göttingen, Germany). A 1.5-mL volume of the filtrate was transferred to a polypropylene tube and centrifugated at 10,000 g for 5 min at 4°C in a Jouan Model MR 1822 centrifuge (Jouan, Saint Herblain, France).

A 1 g-sediment sample was extracted three times with 4 mL of 0.2 M sodium hydroxide. After homogenization for 5 min (Heidolph, Bioblock Scientific, Illkirsch, France) and centrifugation at 10,000 g for 10 min at 4°C (Jouan Model MR 1822 centrifuge), the supernatants were combined. The supernatants, into which 2.5 mL of 1 M hydrochloric acid were added, were extracted with a mixture of 2 mL of chloroform and 2 mL of ethyl acetate. After homogenization for 5 min (Heidolph) and centrifugation at 10,000 g for 10 min at 4°C (Jouan Model MR 1822 centrifuge), the organic phase was transferred in a 5-mL vial and evaporated to dryness under nitrogen stream at 35°C. Prior to analysis, extract was dissolved in 0.5 mL of mobile phase.

Oyster tissues were homogenized using a high-speed blender (Ultra-turrax, Bioblock). A 1 g-homogenate was transferred to a polypropylene tube (8 mL) and extracted three times with 4, 4 and 2 mL of a phosphate buffer (0.1 M aqueous dihydrogenphosphate adjusted with 1 M sodium hydroxide to pH 7.0). After homogenization for 5 min (Heidolph) and centrifugation at 10,000 g for 10 min at 4°C (Jouan Model MR 1822 centrifuge), the combined supernatants were concentrated by passing through a 3 mL-octadecyl solid-phase extraction cartridge (Bond Elut, Analytichem International, Harbor City, CA, USA). Before use, cartridge was activated with methanol (3 mL) and phosphate buffer (3 mL). After the sample had been passed, cartridge was flushed with 3 mL of water and oxolinic acid was eluted with 3 mL of methanol:1 M orthophosphoric acid (90:10 v/v). The eluate was evaporated to dryness under nitrogen at 35°C and reconstituted to 0.5 mL in mobile phase. Prior to analysis, the sample was centrifugated at 10,000g for 10 min at 4°C (Jouan Model MR 1822 centrifuge).

All the aforementioned steps of the extraction procedures were conducted in subdued light.

Method of Validation

An overall validation of the method was performed using seawater, sediment and japanese oyster. A daily calibration curve was obtained by spiking samples at six levels of oxolinic acid (0.05, 0.10, 0.25, 0.50, 1.00 and 2.50 μg/mL or μg/g) and analysing two replicates for three consecutive days. The precision of the method was checked by spiking samples at a concentration of 0.50 μg/mL or μg/g with oxolinic acid and analysing five replicates for two consecutive days. The extraction recoveries of oxolinic acid were determined for three consecutive days by comparing peak heights obtained by chromatographing spiked and extracted samples with peak heights obtained by chromatographing the pure drug standard. A 60 days-study of stability was performed by analysing samples spiked at a concentration of 0.50 µg/mL or µg/g with oxolinic acid and stored at - 20°C.

RESULTS AND DISCUSSION

Chromatographic Conditions

Reversed phase-HPLC has often been used for determining quinolones antibiotics in fish plasma and tissues and gave good performance in terms of plate numbers (11). In our experiment, a LiChroSpher 100-RP 18E prepacked column was used as a reversed phase column in order to separate, with high efficiency, oxolinic acid in seawater, marine sediment and oyster tissues. The importance of using "endcapped" materials which contain a negligible proportion of accessible hydroxyl groups had yet been demonstrated.

All the previous workers using reversed-phase systems added an organic modifier to the predominantly aqueous eluent. Comparisons of the three modifiers - methanol, tetrahydrofuran and acetonitrile - indicated that more symmetrical peaks were given by acetonitrile (data not shown). Different aqueous buffers - disodium hydrogenphosphate, potassium dihydrogenphosphate and dipotassium hydrogenphosphate

- were tested in order to optimize the chromatographic conditions. When using them, baseline drift and severe peak tailing were consistently noted, and the buffer precipitated inside the tubing that fed organic solvent from the pump to the column. When using 0.02 M orthophosphoric acid solution as aqueous eluent, the chromatograms were free of interfering peaks. Neither tailing nor baseline drift were evident and no precipitation occured inside the tubing.

Different proportions of acetonitrile and 0.02 M orthophosphoric acid solution, giving a scale of pH from 2.0 to 5.0, were tested. The highest efficiency was obtained with acetonitrile and 0.02 M orthophosphoric acid solution (24:76 v/v), pH 2.3. Lower proportions of acetonitrile and higher pH values of the mobile phase gave tailing peaks or lower capacity factors and showed lower correlation between peak height and drug concentration (data not shown).

Under the operating conditions, the capacity and asymmetry factors were respectively 3.8 and 1.3. Ending each day of operation, recycling with acetonitrile and water (24:76 v/v), pH 7.4 through the HPLC column for 2h allowed the column life to increase, because reversed phases are unstable at low pH values. Under these conditions, there was no loss of bonded layer and column was stable for a long period.

Oxolinic acid was eluted in 6.5 min. No changes in retention times were noted with continual column use. No additionnal peaks, possibly resulting from impurities or degradation products, that could interfere with the oxolinic acid peaks, were noted on the chromatograms. The samples did not contain these related compounds in significant amount for the HPLC assay. Typical chromatograms are shown in Figure 1.

After 150-200 injections, precolumn was saturated with seawater, sediment and shellfish tissues components and gave a drifting baseline. Analytical column was used for more than 1,500 injections of samples without any change in its performances. The final number of theoretical plates was 2,500 compared with the initial value of 2,700.

Extractions and Recoveries

The mean recovery of oxolinic acid from spiked seawater was 102.1% [relative standard deviation (RSD) = 4.5%, number of samples (n) = 47] over the concentration range 0.05-2.50 µg/mL (Table 1). Thus, prior to analysis, a simple clean-up procedure of seawater samples was enough for obtaining a high recovery.

The highest recoveries of oxolinic acid from sediment were achieved with three extraction cycles, each with 4 mL of 0.2 M sodium hydroxide, followed by another extraction with a mixture of chloroform and ethyl acetate at an acid pH. The mean recovery of oxolinic acid from spiked sediment was 68.1% [RSD = 1.7%, n = 30] over the concentration range 0.10-2.50 µg/g (Table 1). This mean recovery was similar to the one obtained by Björklund *et al.* (16) applying their method described for fish muscle tissues to sediment (mean = 70.9%, RSD = 5.1%). The relatively low recovery obtained could be explained by the formation of complexes between the drug and some components of the sediment, such as divalent cations (Mg^{2+}, Fe^{2+}, Cu^{2+}). Formation of such complexes could modify hydrophilic and

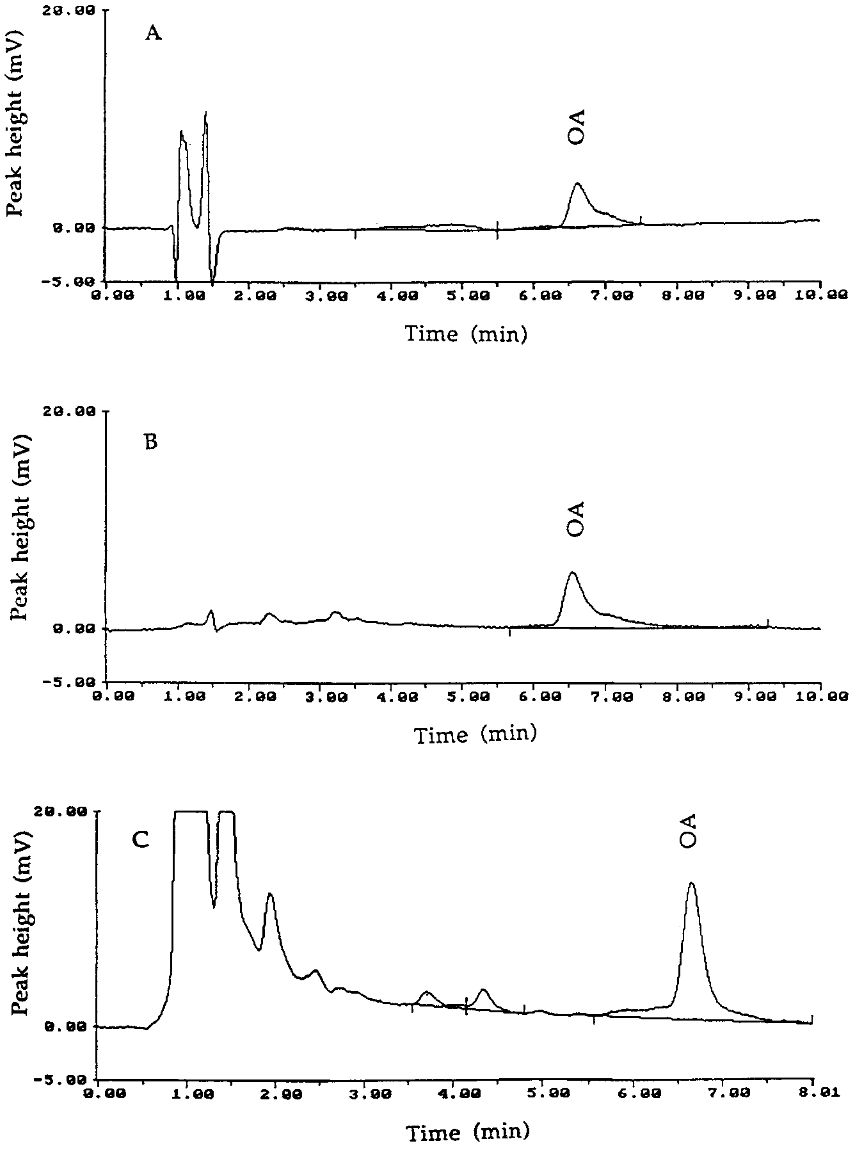

FIGURE 1

HPLC Chromatograms **A**, Seawater Sample Containing Oxolinic Acid (OA) at a Concentration of 0.50 µg/mL; **B**, Sediment Sample Containing OA at a Concentration of 0.40 µg/g; **C**, *Crassostrea gigas* Sample Containing OA at a Concentration of 1.40 µg/g. Conditions: Mobile Phase, Acetonitrile and 0.02 M Orthophosphoric Acid Solution, pH 2.3 (24:76 v/v); Column 125 X 4 mm, C_{18} (5 µm); Flow Rate, 1.0 mL/min; Wavelength, 262 nm; Detector Sensitivity 0.01 a.u.f.s.; Injection Volume, 50 µL.

TABLE 1

Recoveries of Oxolinic Acid from Seawater, Sediment and Japanese Oyster Tissues Spiked with Various Amounts of Oxolinic Acid. Mean in % (Coefficient of Variation in %) [Number of Samples].

	Seawater	Sediment	Oyster
0.05 µg	98.3 (9.0) [6]		86.3 (3.5) [6]
0.10 µg	102.5 (7.7) [6]	68.5 (3.0) [6]	87.9 (2.3) [6]
0.25 µg	104.6 (4.2) [9]	68.9 (1.7) [6]	88.8 (4.2) [6]
0.50 µg	102.7 (3.5) [10]	67.8 (1.7) [6]	87.2 (1.7) [6]
1.00 µg	103.6 (0.7) [8]	68.2 (1.1) [6]	88.5 (2.1) [6]
2.50 µg	100.9 (2.4) [8]	68.5 (1.0) [6]	90.9 (0.6) [6]

hydrophobic properties of free oxolinic acid. Thus, they could not be quantitatively extracted and consequently detected by the extraction and HPLC method developed for free oxolinic acid.

In a preliminary study, recoveries of oxolinic acid from spiked oyster tissues were compared by using the liquid-phase extraction procedure above described for sediment and the solid-phase extraction procedure for fish tissues described by Björklund (11). Since better recoveries were obtained with the second procedure, the method of Björklund (11) was optimized in order to adapt it to oyster tissues. Octyl and octadecyl solid-phase extraction cartridges from Analytichem were compared. The effects of the nature and volume of solvents for conditioning, washing and elution of the columns were also tested. The best recovery was obtained with octadecyl solid-phase extraction cartridges, methanol and phosphate buffer as conditioning solvents, water as washing solvent and a mixture of methanol and 1 M orthophosphoric acid as elution solvent (data not shown). The carrying out of an elution gradient showed the optimal pourcentage of methanol in the elution solvent to be 70%. Since the evaporation to dryness should be time and money-saving, an elution solvent containing 90% of methanol was choosen. Under these conditions, the mean recovery of oxolinic acid from oyster was 88.3% [RSD = 2.4%, n = 36], over the concentration range 0.05-2.50 µg/g (Table 1). This mean recovery was near-by the one obtained by Samuelsen *et al.* (17) by applying their method described for fish muscle tissue to blue mussel (85.4-93.5%).

Linearity, Sensitivity, Precision and Stability

A statistical test of linearity was performed for each calibration curve separately, using a weighted analysis of variance ANOVA (18, 19). All the calibration curves showed linearity in the range examined (0.05-2.50 µg/mL or µg/g) at the 0.05 level (Table 2). A good correlation was obtained between concentrations and peak heights : all the correlation coefficients were between 0.970 and 0.999 (Table 2).

The limits of detection and determination of oxolinic acid in seawater, sediment and oyster tissues were respectively 0.01 and

TABLE 2

Linearity and Regression Data for the Calibration Graphs Obtained from Seawater, Sediment and Japanese Oyster Spiked with Oxolinic Acid from 0.05 to 2.50 μg/mL or μg/g
$y = ax + b$; y = Peak Height (μV) ; x = Oxolinic Acid Concentration (μg/mL or μg/g) ; a = Slope ; b = Intercept

		Seawater	Sediment	Oyster
Slope	Day 1	1.173 10^{-4}	9.513 10^{-5}	6.193 10^{-5}
	Day 2	1.139 10^{-4}	9.114 10^{-5}	5.970 10^{-5}
	Day 3	1.261 10^{-4}	9.258 10^{-5}	6.086 10^{-5}
Intercept	Day 1	0.002	0.037	- 0.125
	Day 2	0.012	0.014	- 0.080
	Day 3	0.007	0.008	- 0.081
Correlation Coefficient	Day 1	0.999	0.992	0.981
	Day 2	0.999	0.970	0.980
	Day 3	0.998	0.998	0.980
F Linearity	Day 1	15600.94*	1755.69*	306.93*
	Day 2	12121.68*	325.55*	541.07*
	Day 3	5606.87*	7253.07*	295.05*

* No Significative Difference Between the Calculated Slope and 0 at the 0.001 Level.

0.04 μg/mL or μg/g (precision ±10%). This concentration of 0.04 μg/mL or μg/g was accepted as the limit of determination because a t-test with four degrees of freedom showed this is significantly different from 0 and the mean response was greater than three standard deviations (18, 19). The expression of the later experimental results will be subordinated to these two limits (Figure 2).

The relative standard deviations of within-day precision for samples spiked with oxolinic acid at 0.500 μg/mL or μg/g were between

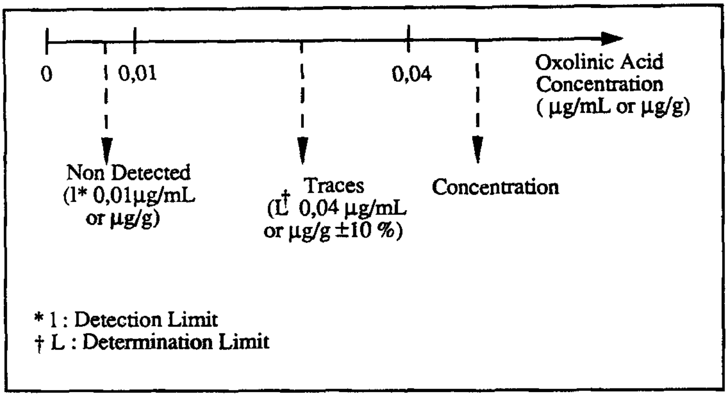

FIGURE 2

Limits of Detection and Determination : Consequences for the Expression of the Oxolinic Acid Concentration.

TABLE 3

Precision Data Obtained from Seawater, Sediment and Japanese Oyster Tissues Spiked with Oxolinic Acid at 0.50 µg/mL or µg/g

	Mean (µg/mL or µg/g)	Standard Deviation (µg/mL or µg/g)	Variation Coefficient (%)	F Scheffé
Seawater				
Day 1	0.509	0008	1.58	0.202*
Day 2	0.508	0.006	1.25	
Sediment				
Day 1	0.339	0.007	2.00	0.095*
Day 2	0.340	0.006	1.83	
Oyster				
Day 1	0.400	0.014	3.14	0.020*
Day 2	0.442	0.015	3.36	

* No Significative Difference Between Means at the Level 95 %.

1.58 and 3.14% (Table 3). The relative standard deviations of between-day precision for samples spiked with oxolinic acid at 0.500 µg/mL or µg/g were between 1.25 and 3.36% (Table 3). An analysis of variance (ANOVA) showed there was no significant difference between the within- and between-day precision at the 0.05 level.

A study of the stability of oxolinic acid in seawater, sediment and oyster samples spiked at a concentration of 0.500 µg/mL or µg/g and stored at - 20°C was performed. All the recoveries of oxolinic acid from the samples stored at - 20°C fall within the average limits calculated using the recoveries results from the validation (18, 19). Moreover, there was no significant decrease of the oxolinic acid recoveries from samples maintained at - 20°C during 60 days.

CONCLUSION

The described methods provide a selective, reliable and precise mean for the rapid determination of oxolinic acid in seawater, marine sediment and japanese oyster. They do not require time-consuming, complex extraction or derivatization techniques. An analyst familiar with the methods could easily process fifteen samples a day. They are suitable for pharmacokinetics and residues studies on oxolinic acid in seawater, sediment and japanese oyster.

ACKNOWLEDGEMENTS

We thank Ms. Michaëlle Larhantec and Christelle Moriceau for their technical assistance.

This research was supported by a grant from the IFREMER Laboratory (Brest, France).

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Received: July 30, 1993
Accepted: August 11, 1993