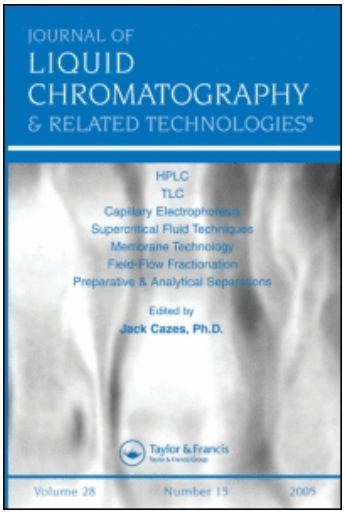

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SIMULTANEOUS EXTRACTION AND DETERMINATION OF OXOLINIC ACID AND FLUMEQUINE IN FISH SILAGE BY HPLC

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ABSTRACT

A simple and rapid method for the simultaneous extraction and determination of residues of oxolinic acid (OX) and flumequine (FQ) in fish silage, is presented. The samples were extracted with acetone and ammonia. When applying traditional liquid-liquid extraction, clean extracts were obtained, the recovery being 95.3 - 96.2 % for OX and 96.9 - 99.2 % for FQ. The detection limits were 40 ng/g for OX and 50 ng/g for FQ.

INTRODUCTION

In recent years, the quinolones oxolinic acid (OX) and flumequine (FQ) have been the most used drugs for treatment of infectious diseases in Norwegian fish farming.

In 1992 for instance, the total consumption of OX and FQ was 7687 kg and 9833 kg, respectively (Statistics provided by the Norwegian Medicinal Depot, Oslo).

The annual production of farmed fish in Norway is about 160.000 tons, a substantial proportion of the fish being treated with drugs during the growing period.

The slaughter of fish for human consumption is not allowed until the fish have been screened for residues of drugs in liver and muscle.

Residues of OX and FQ have appeared to be especially bound to bone and skin (1) and can be detected in these organs even though edible tissue like muscle and liver from the same fish are free of drugs.

A lot of fish waste, such as guts, skin and bone arise from the fish processing industry. In Norway, in order to utilize this waste, it is ensilaged. This silage can then further be mixed into animal feed.

According to Norwegian regulations, the addition of antibiotics to feed for food-producing animals is not allowed . An official method of analysis for OX and FQ in silage is therefore needed.

Several analytical methods have been developed for the determination of OX in biological materials using microbiological and high performance liquid chromatographic procedures (2, 3, 4, 5, 6). Analytical methods have also been developed for the determination of FQ in plasma, urine and tissues (7, 8, 9, 10). Tao et al. (11) published a microbiological method for residue analysis of FQ in fish tissues.

Rogstad et al. (12), Steffenak et al. (13) and Rasmussen et al. (14) have published methods for the simultaneous determination of OX and FQ in fish tissues.

However, none of the published methods appeared to be applicable to such a complex material as fish silage, because of problems with interfering peaks in the HPLC chromatograms, or unspecific inhibition zones in microbiological test-systems.

The purpose of the present study was thus to develop a rapid and efficient HPLC method for routine analysis of OX acid and FQ in fish silage.

MATERIALS AND METHODS

Materials and Reagents

The fish silage which served as sample material, was supplied by Stormøllen A/S (Vaksdal, Norway).

All chemicals were of analytical or HPLC grade. OX was supplied by Sigma Co. (St. Louis, MO, USA.), and FQ by Solchem Italiana s.p.a. (Mulazzano, Italy).

Solvents were of analytical and HPLC grade. Stock solutions (1 mg/ml) of OX and FQ were prepared in 0.03 M sodium hydroxide, and working standards were prepared by dilution with 0.002 M phosphoric acid-acetonitrile-tetrahydrofurane (68:17:15).

The solutions were stored in the refrigerator in dark stoppered flasks.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a cooler (14°C) Lauda RMT6 from Messgeräte Werk Lauda, (Lauda-Königshafen, Germany), and a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 325 nm and emission wavelength of 360 nm, with a Resp. 5 and a Factr. 256. The analytical column (stainless steel, 150 X 4.6 mm I.D.) and guard column (stainless steel, 5.0 X 3.0 mm I.D.) were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA). The mobile phase was 0.02 M phosphoric acid-acetonitrile-tetrahydrofurane (68:17:15). The flow rate was 0.7 ml/min. for 6 min. followed by 0.8 ml/min. for 3 min. The samples were injected at intervals of 10 min.

Aliquots of 20 µl and 30 µl were injected onto the column for the determination of OX and FQ respectively.

Sample Preparation and Clean-up (Figure 1)

The tissue sample, 0.5 g of ground fish silage, was weighed into a 50 ml centrifuge tube with screw cap (NUNC); 1.5 ml H_2O,

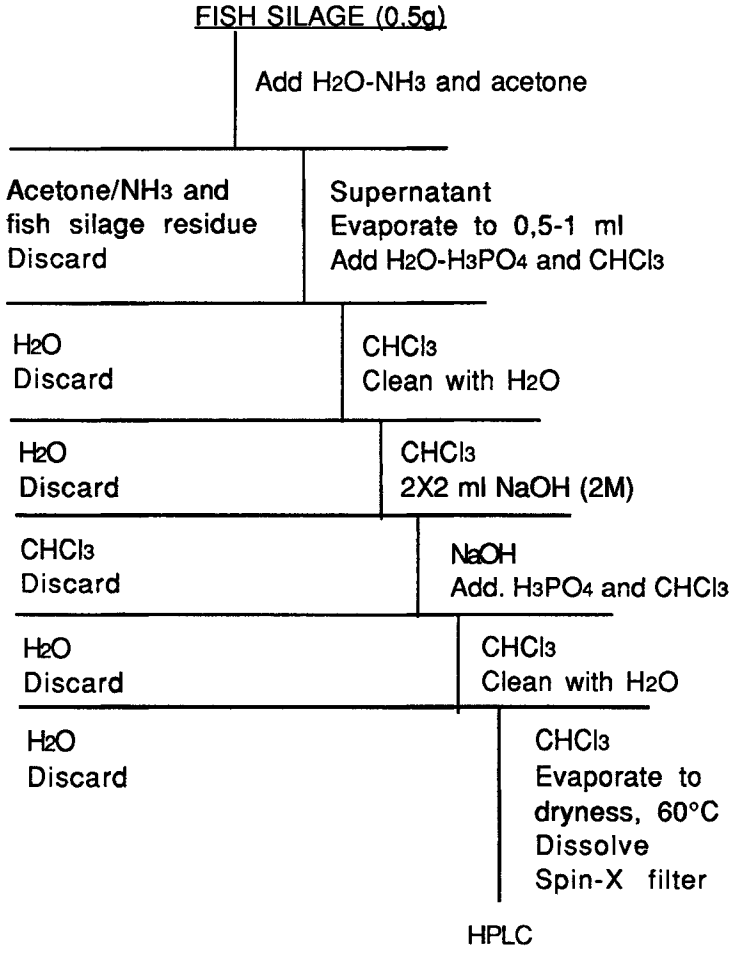

FIGURE 1

Extraction and Clean-up procedure for OX and FQ from Fish Silage

1 ml NH_3 (25%), and 7 ml acetone were added. The sample was mixed for 5 s., and then left with the extraction fluid for 5 min. before again being whirlimixed for 5 s. The homogenate was then centrifuged for 3 min. at 5000 rpm.

Five ml of the supernatant was pipetted into a graduated glass-stoppered centrifuge tube, (Rep. 0.250 g). The acetone phase was evaporated to 0.5 -1 ml under a stream of nitrogen (60°C), and 0.5 ml 85% H_3PO_4, 3 ml H_2O and 4 ml $CHCl_3$ were added. The sample was shaken vigorously for 10 s. followed by centrifugation for approximately 1 min. (3000 rpm). The upper aqueous layer and all solid residues between the two phases were discarded. Two ml H_2O were added (no mixing). The water layer was discarded. Two ml of sodium hydroxide (2M) were added. The sample was then mixed for 10 s. After centrifugation (4000 rpm, 5 min.), the water phase was collected. The chloroform was blended twice more with 2 ml sodium hydroxide and centrifuged. The collected water phases were acidified (1 ml 85% H_3PO_4) and extracted once more with 4 ml chloroform. The water layer was discarded, 2 ml H_2O were added (no mixing), and the water layer was again discarded. The chloroform was evaporated to dryness under a stream of nitrogen (60°C), after which the residue was dissolved in 2 ml 0.002 M phosphoric acid-acetonitrile-tetrahydrofurane (68:17:15), and approximately 0.5 ml filtered through a Costar Spin-X centrifuge filter (lowtype) with 0.22 µm cellulose acetate binding by centrifugation for 5 min. at 10.000 rpm. Aliquots of the filtrate were injected onto the HPLC.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for OX and FQ were determined by spiking silage samples with standard solutions to yield 40, 50, 75, 100, 150, 200, 500 and 1000 ng OX and FQ per gram of sample, respectively. The samples were extracted using the above procedures. Duplicate samples were used. The recovery rates were determined by comparing the results of analysis of spiked fish silage to those of pure standard solutions.

The linearity of the standard curves for OX and FQ in fish silage was calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean fish silage samples, spiked samples and real samples are shown in Figure 2.

The standard curves were linear in the investigated area 40-1000 ng/g for both OX and FQ in fish silage, while the corresponding correlation coefficients were r=0.9994 and r=0.9987, respectively.

The recovery of OX and FQ varied from 95.3 - 96.2 % and from 96.9 - 99.2 %, respectively, with a standard deviation ranging from 1.56 to 6.70 and from 1.58 to 8.10, respectively (Table 1).

The limit of determination for OX and FQ was 40 ng/g and 75 ng/g tissue, respectively, when aliquots of 20 µl were injected onto the column. The limit of determination for FQ was 50 ng/g when aliquots of 30 µl were injected. However, when injecting 30 µl aliquots of OX, an interfering peak was sometimes observed close to the OX peak (Figure 2B). When 20 µl was injected onto the HPLC, for the determination of OX, no such interference was seen (Figure 2A).

The method presented in this paper should be useful for most work on residues of OX and FQ in fish silage. The method is

TABLE 1

Recovery of Oxolinic Acid and Flumequine from Spiked Samples of Fish Silage.

Sample	No. of samples	Amount (µg/g)	Recovery % OX		FQ	
			Mean	SD*	Mean	SD*
Silage 0.5 g	8	0.15	95.3	6.70		
	8	0.50	96.2	1.56		
	8	0.15			96.9	8.10
	8	0.50			99.2	1.58

SD* = standard deviation

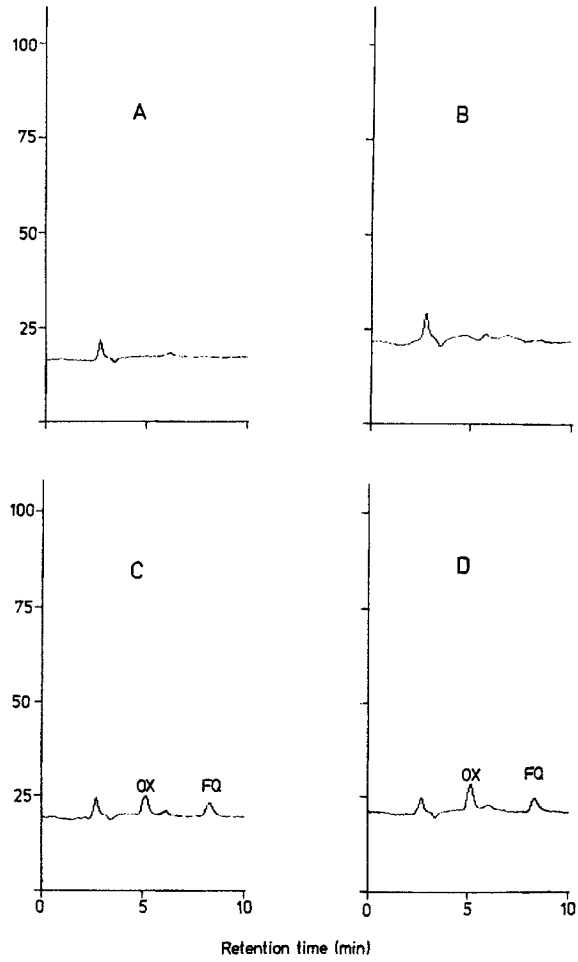

FIGURE 2

Chromatograms of extracts from 0,5 g fish silage for the determination of oxolinic acid and flumequine.

A - Unspiked sample (20 µl injected onto the HPLC)

B - Unspiked sample (30 µl injected onto the HPLC)

C - Fish silage spiked with 200 ng OX and 200 ng FQ per gram sample (20 µl injected onto the HPLC)

D - Chromatograms of real samples. The samples contained 259 ng/g OX and 190 ng/g FQ (20 µl injected onto the HPLC).

selective, and robust, and should be generally applicable for monitoring drug levels in fish silage. The method is also very rapid, a technician easily managing to analyse 18 samples a day. Moreover, the consumption of reagents is low.

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