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High-performance liquid chromatographic analysis of Romet-30® in salmon following administration of medicated feed

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic assay was developed for the simultaneous quantitation of sulphadimethoxine (SDM) and ormetoprim (OMP) in chinook salmon muscle tissue. SDM and OMP were extracted from tissue samples using a solid-phase extraction technique. Resolution of both drugs was accomplished using an Ultrasphere ion-pair column (250 × 4.6 mm I.D.) and a mobile phase of acetonitrile–methanol–0.1 *M* phosphate buffer, pH 4 (17:10:73) with ultraviolet detection at 280 nm. The calibration curve in salmon muscle tissue was linear over the concentration range 0.2–20 ppm for both SDM (r^2 = 0.9974) and OMP (r^2 = 0.9956). The minimum detectable quantity of SDM and OMP in salmon muscle tissue was 0.2 ppm at a signal-to-noise ratio of 5:1. An *in vivo* feeding experiment was undertaken where chinook salmon were administered Romet-30®-medicated feed for a 10-day period, followed by a 42-day wash-out period. The rate of tissue uptake and decline of SDM and OMP was shown to differ.

INTRODUCTION

The use of antibiotics in the aquaculture industry for the control of bacterial infections in salmon, has led to public concern over antibiotic residues in salmon tissue and the potential health risk to humans. To address these concerns, quality assurance procedures are required for the control of antibiotic residues in farmed salmon. Therefore, there is a need for the development of sensitive, specific assays for the detection and quantitation of antibiotics in salmon tissue and the determination of wash-out times of antibiotics from the tissue.

Romet-30®, an antibiotic approved for use in the aquaculture industry, is a potentiated sulphonamide containing sulphadimethoxine and ormetoprim in a 5:1 ratio. This antibiotic is effective against a wide range of bacterial pathogens including

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Vibrio ordali and *Vibrio anguillarum*, the causal agents of Vibriosis, a disease common to farmed salmon.

Various methods for the analysis of antibiotics in fish tissue have been reported. Microbiological assays are frequently used as screening methods for antibiotics in animal tissues. Two bioassays have been reported for the determination of trimethoprim, an antibiotic potentiator similar to ormetoprim, in the tissues of rainbow trout [1,2]. Microbiological methods often lack the sensitivity required to accurately quantitate antibiotics in fish tissue and are characteristically non-specific and therefore would fail to differentiate between similar drugs in the two products, Romet-30 and Tribissen® (sulphadiazine and trimethoprim).

The Bratton–Marshall colourimetric assay has been used extensively to measure sulphadimethoxine and other sulphonamide levels in the blood and tissues of different trout species [1,3–6]. However, such analyses are also relatively non-specific.

Radio-isotope methods involving liquid scintillation counting have been used to quantitate sulphadimethoxine residue levels. This procedure, when combined with high-performance liquid chromatographic (HPLC) or thin-layer chromatographic analyses of metabolites, has provided information on tissue distribution, pharmacokinetics and metabolism of sulphadimethoxine in rainbow trout (*Salmo gairdneri*) [7] and channel catfish (*Ictalurus punctatus*) [8]. However, these methods are not applicable for the routine analysis of fish treated with non-radioactively labelled drug.

Chromatographic methods offer the advantages of selectivity and increased sensitivity over many other analytical procedures. Trimethoprim concentrations in skin, muscle and blood of rainbow trout, following administration of medicated feed, have been measured by HPLC [9]. One method has been reported for the simultaneous determination of sulphadimethoxine and ormetoprim in catfish tissue [10]. To date, there have not been any methods reported for the simultaneous analysis of sulphadimethoxine and ormetoprim in salmon tissue.

The kinetics of absoprtion, distribution, metabolism and excretion of various sulphonamides, including sulphadimethoxine, following both a single oral dose to rainbow trout [1,3,4,7] and multiple doses to trout [7] and catfish [8] have been investigated. The tissue levels of a number of orally administered sulphonamides in trout, including sulphamerazine [5,6], sulphaguanidine, sulphadiazine, sulphamethazine, sulphanilamide and sulphathiazole [5] and trimethoprim [2] have also been investigated. However, these studies failed to address possible differences in the clearance of the two antibiotics in combination products such as Romet-30.

In the present study, a sensitive and selective HPLC assay for the simultaneous quantitation of sulphadimethoxine and ormetoprim in salmon tissue was developed for determining withdrawal periods for Romet-30 in chinook salmon (*Oncorhynchus tshawytscha*) following medicated feed administration.

EXPERIMENTAL

Materials

Sulphadimethoxine (SDM) and ormetoprim (OMP) were obtained from Hoffmann-La Roche (Etobicoke, Canada). The internal standard, carbamazepine-diol (*trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine), was obtained from Ciba-Geigy (Mississauga, Canada). HPLC-grade methanol, acetonitrile and disodium hydro-

gen orthophosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) were obtained from BDH (Vancouver, Canada). Purified water was produced using a Milli-Q water purification system (Millipore, Mississauga, Canada). Phosphoric acid (H_3PO_4) 85% was obtained from Mallinckrodt (KY, U.S.A.) and trichloroacetic acid (TCA) from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The HPLC system consisted of a Beckman Model 110A solvent metering system, a Model 210 sample injection valve equipped with a 20-μl loop, a Hitachi Model 155 variable-wavelength detector with a Shimadzu C-R1A Chromatopac data processor (Beckman, Fullerton, CA, U.S.A.). Ultraviolet detection was at 280 nm for all analyses. The HPLC column was an Ultrasphere ion-pair 5-μm column (250 × 4.6 mm I.D.) (Beckman). A NewGuard holder equipped with an RP-18 cartridge (15 × 3.2 mm I.D.) (Brownlee, Santa Clara, CA, U.S.A.) was used as a guard column. A direct-connect column prefilter containing a 0.5-μm filter element (Alltech, Deerfield, IL, U.S.A.) placed between the injector and the guard column was used as an inline filter. The mobile phase, acetonitrile–methanol–0.1 *M* phosphate buffer, pH 4.0 (17:10:73), was delivered isocratically at 1.0 ml/min. Filtration of the mobile phase prior to use was done using a HPLC solvent clean-up assembly (Kontes, Vineland, NJ, U.S.A.) and FP Vericel 47 mm, 0.45-μm membrane filters (Gelman, Ann Arbor, MI, U.S.A.).

Preparation of standard solutions and reagents

Stock solutions of SDM and OMP were prepared by dissolving 10 mg of each compound in 100 ml of acetonitrile to give a final concentration of 100 μg/ml for both SDM and OMP. Serial dilutions of the stock solution were made to give final working concentrations of 50, 30, 10, 5, 3.5, 2.5, 2.0 and 1.0 μg/ml for each antibiotic.

Carbamazepine-diol was used as an internal standard for calibration curve samples, treated fish samples and as an external standard in the recovery study. A stock solution of carbamazepine-diol (100 μg/ml) was prepared in methanol. Each tissue sample, weighing 5 g, was spiked with 1 ml of the internal standard solution to yield a final concentration of 20 μg/g tissue.

Extraction procedure

To each 5-g sample of salmon muscle tissue were added 1 ml of the internal standard solution, 15 ml acetonitrile and 0.5 ml 50% (w/v) TCA. Each sample was homogenized in a 50-ml plastic centrifuge tube for 30 s at medium speed using a Brinkmann Polytron homogenizer Model PT 10/35 (Brinkmann, Rexdale, Canada). The samples were centrifuged for 25 min at 7800 *g* and 4°C (JA-20 rotor) in a Beckman Model J2-21 centrifuge. The supernatant was transferred to a 50-ml tube, and evaporated under nitrogen in a 40°C water bath. The residue was reconstituted in 5 ml of purified water and vortex-mixed for 30 s. The extracts were then filtered in a filtration unit consisting of a Membra-Fil membrane filter disc (8 μm, 13 mm diameter) (Nucleopore , Toronto, Canada) in a Swinnex 13 Millipore filter holder attached to a 5-ml Luer-Lok Multifit B-D glass syringe (Becton Dickenson, Mississauga, Ont., Canada). Following filtration, the extracts were passed directly into a second 5-ml Luer-Lok Multifit B-D glass syringe with an activated Sep-Pak C_{18} cartridge (Wa-

ters, Milford, MA, U.S.A.) attached. Activation of the Sep-Pak cartridge prior to loading the sample was accomplished by passing 5 ml of methanol followed by 5 ml of water. The extracts were then passed through the Sep-Pak cartridge and the eluent discarded. SDM, OMP and the internal standard were subsequently eluted from the cartridge into a 15-ml culture tube with 5 ml of methanol. Samples were dried under nitrogen in a water bath at 40°C and stored at −20°C until required for analyses. Just prior to analysis each sample was reconstituted with 1 ml of the mobile phase and vortex-mixed for 30 s. A 20-μl aliquot was injected onto the HPLC column. Each extract was analyzed in duplicate. The injection valve was flushed with 1 ml mobile phase between each analysis.

Calibration curve, assay precision and recovery

A calibration curve was prepared from salmon tissue samples (5 g) spiked with 1 ml of the internal standard solution (20 μg/g tissue) and 1 ml of the appropriate SDM/OMP standard solutions to give final concentrations of 0.2, 0.4, 0.5, 0.7, 1, 2, 6, 10 and 20 μg/g tissue. Calibration curves were constructed by plotting the area ratios of SDM and OMP to that of internal standard against the known concentration ratios of SDM and OMP to that of internal standard.

Intra-assay variability was determined by performing multiple injections (n = 7) of a single extracted tissue sample spiked with SDM and OMP at a concentration of 2 μg/g tissue. Inter-assay variability was determined by the duplicate analysis of independent spiked tissue samples (2 μg/g tissue, $n=4$) over a 59-day time period.

The recovery of SDM and OMP from salmon muscle tissue was determined by the addition of 1 ml of each of the prepared standard solutions containing 2.5, 5, 10 and 30 μg each of SDM and OMP to 5-g samples of control salmon tissue. The samples were extracted and analyzed as described above, except that the internal standard was added just prior to the final evaporation. Recovery was determined by calculating the area ratios of SDM and OMP to that of internal standard for extracted tissue samples and comparing these to the area ratios obtained from unextracted standard solutions of identical quantities.

Feeding study

Approximately 80 chinook salmon varying in weight from 141 to 976 g were maintained in a circular (1.2 m deep × 2.3 m diameter) flowing seawater tank at the West Vancouver Laboratory of the Department of Fisheries and Oceans (West Vancouver, Canada). During the 52-day study period the water temperature varied from a minimum of 7.8°C to a maximum of 10.3 °C. Three fish were taken prior to the start of the feeding study and analyzed to confirm the absence of SDM and OMP. These fish also served as control samples for all calibration curve, assay precision and recovery studies. A medicated diet, Extruded New Age Salmon Feed, 3.5 mm pellets (Moore–Clarke, Vancouver, Canada) containing Romet-30 at a concentration of 16.7 kg per tonne of feed was used. Medicated feed was administered twice daily at a rate of 135 mg per kg fish per day for 10 days. After the medication period, the fish were transferred to a non-medicated feed formulation (Moore–Clark). Four fish were removed from the tank prior to the morning feeding on days 2, 4, 6, 8, 11, 12, 14, 17, 20, 24, 27, 31, 34, 38, 41, 45 and 52. The fish were killed immediately by a blow to the head, tagged and stored at −20°C. For analysis three 5-g samples were taken from each fish at evenly spaced sites along the length of the fish.

RESULTS AND DISCUSSION

Representative chromatograms of a blank salmon extract and a salmon extract containing SDM, OMP and internal standard are shown in Fig. 1. Carbamazepine-diol was selected as an internal standard because of its appropriate elution volume

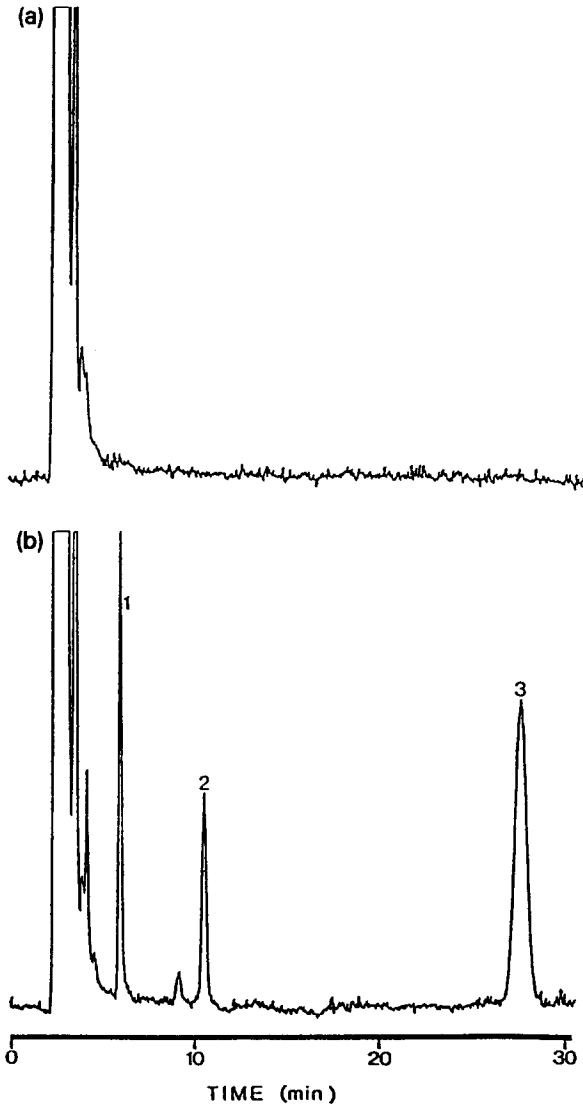

Fig. 1. Chromatogram of (a) a control salmon muscle tissue extract and (b) a salmon muscle tissue extract spiked with 2.0 ppm of ormetoprim and 2.0 ppm of sulphadimethoxine. Chromatographic conditions: column: Ultrasphere I.P. 5 μm (25 cm × 4.6 mm I.D.); mobile phase: acetonitrile–methanol–0.1 *M* phosphate buffer pH 4.0 (17:10:73); HPLC flow-rate: 1.0 ml/min; ultraviolet detection wavelength: 280 nm. Peaks: 1 = ormetoprim; 2 = carbamazepine diol; 3 = sulphadimethoxine.

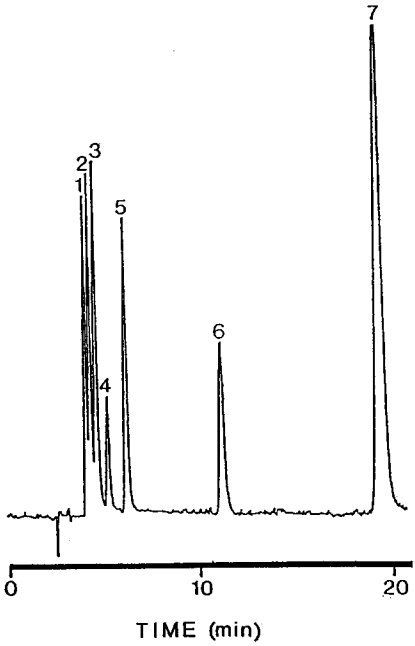

Fig. 2. Chromatogram of various sulphonamides and ormetoprim. Chromatographic conditions as for Fig. 1 except mobile phase: acetonitrile–methanol–0.1 *M* phosphate buffer pH 4.0 (20:10:70). Peaks: 1 = sulphacetamide; 2 = sulphadiazine; 3 = ormetoprim; 4 = sulphamerazine; 5 = sulphamethazine; 6 = sulphisoxazole; 7 = sulphadimethoxine.

and ultraviolet absorption characteristics. The assay developed in the present study has the capacity to separate other sulphonamides in addition to SDM and OMP (Fig. 2). However, their potential use as antimicrobial agents in the aquaculture industry negated their use as internal standards.

Calibration curves for SDM and OMP from extracted salmon muscle tissue are presented in Fig. 3. The area ratio measurements showed a linear relationship between SDM and the internal standard ($r^2 = 0.9974$) and OMP with the internal standard ($r^2 = 0.9956$) over the concentration range 0.2–20 μg/g tissue.

The intra-assay coefficient of variation was 4.8% for SDM and 4.3% for OMP (Table I). The precision of the extraction procedure was determined over a 59-day period and the resultant inter-assay coeffient of variation was found to be 4.6% and 4.4% for SDM and OMP respectively (Table II).

A solid phase extraction method was developed to simultaneously recover SDM and OMP from salmon muscle tissue. The percent recovery of SDM and OMP over the concentration range 0.5–6.0 ppm is presented in Table III. A mean recovery of 54.6% for SDM and 67.1% for OMP was computed from the various levels of antibiotic-spiked salmon tissue. The combination of acetonitrile and TCA as the chosen extraction solvent was found to provide the most efficient recovery for SDM and OMP. Repeated extractions were not found to significantly enhance recovery of either antibiotic. The extraction efficiencies for both SDM and OMP were lower in this study using salmon muscle tissue, than previous reports with other domestic

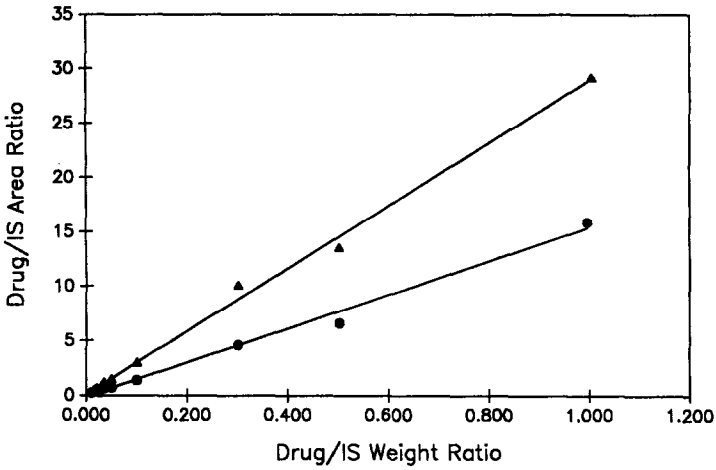

Fig. 3. Calibration curve for sulphadimethoxine (▲) and ormetoprim (●) from extracted salmon tissue over the concentration range 0.2–20 ppm. Sulphadimethoxine: $r^2 = 0.9974$; y intercept = 0.1027; slope = 28.8409. Ormetoprim: $r^2 = 0.9956$; y intercept = −0.0748; slope = 15.4948. IS = internal standard.

animal species including catfish, cattle and chicken [10]. This difference may be due to the presence of relatively high cholesterol levels in salmon muscle tissue. Sheridan [11] has reported that coho salmon (*Oncorhynchus kisutch*) have cholesterol levels in dark muscle tissue that are approximately twice that of other salmonids such as rainbow trout. The high cholesterol level, in addition to the carotenoid content of salmon muscle, may increase the total lipid content of the tissue and may adversely affect antibiotic extraction efficiencies. Despite the relatively low recoveries, the minimum quantifiable amount of both SDM and OMP detected in salmon muscle tissue was 0.2

TABLE I

INTRA-ASSAY VARIABILITY OF SULPHADIMETHOXINE AND ORMETOPRIM IN CHINOOK SALMON MUSCLE TISSUE

Injection No.	SDM/IS Area ratio	OMP/IS Area ratio
1	1.723	0.823
2	1.596	0.777
3	1.504	0.836
4	1.555	0.818
5	1.640	0.737
6	1.588	0.799
7	1.700	0.788
Mean area ratio	1.615	0.797
S.D.[a]	0.078	0.033
C.V.[b]	4.8%	4.2%

[a] Standard deviation.
[b] Coefficient of variation.

TABLE II

INTER-ASSAY VARIABILITY OF SULPHADIMETHOXINE AND ORMETOPRIM IN CHINOOK SALMON MUSCLE TISSUE

Sample Concentration (ppm)	SDM/IS Area ratio	OMP/IS area ratio	Analysis day
2.0	2.720	1.280	1
2.0	2.506	1.277	45
2.0	2.453	1.278	45
2.0	2.599	1.393	59
Mean area ratio	2.570	1.307	
S.D.[a]	0.117	0.057	
C.V.[b]	4.6%	4.4%	

[a] Standard deviation.
[b] Coefficient of variation.

ppm, when taken at a signal-to-noise ratio of 5:1. In addition, this method is advantageous in that it allows a single extraction and analysis for both drugs. The sensitivity of this assay could be improved by increasing the amount of fish tissue extracted and/or by increasing the amount of sample injected onto the HPLC column; however, this would increase the quantity of endogenous substances injected and shorten column lifetime.

The *in vivo* feeding experiment was intended to evaluate the applicability of our HPLC method and to establish a wash-out period for Romet-30 in salmon muscle tissue. The feeding study was designed to model the situation on a fish farm with respect to method and time course of treatment. The data in Fig. 4 show the tissue uptake for SDM and OMP during the first 10 days of treatment followed by the decline of tissue levels from days 11 to 52 after cessation of medicated feed administration. It is apparent from these graphs that there is a large variation in tissue antibiotic concentration between the fish sampled on a given day; however, there was no observable difference in tissue antibiotic concentration between sites sampled on

TABLE III

RECOVERY OF SULPHADIMETHOXINE AND ORMETOPRIM FROM CHINOOK MUSCLE TISSUE

Sample concentration (ppm)	Sulphadimethoxine recovery (%)[a]	Ormetoprim recovery (%)[a]
0.5	54.4	57.1
1.0	68.8	76.4
2.0	46.0	66.7
6.0	49.3	68.3
Mean recovery	54.6	67.1

[a] Each value represents a single determination at each sample concentration.

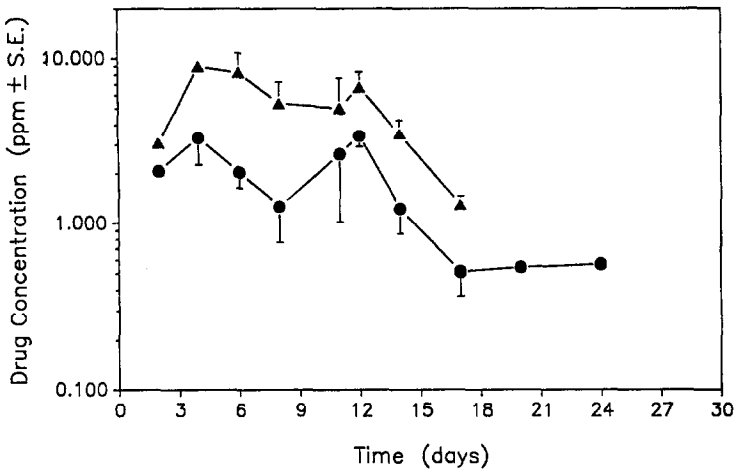

Fig. 4. Tissue uptake (day 1–10) and decline of sulphadimethoxine (▲) and ormetoprim (●) in muscle tissue samples from chinook salmon administered medicated feed. Each point represents an average of four individuals sampled on each day ± standard error.

the same fish. The variation between individual fish is likely the consequence of the hierarchical feeding dominance which results in certain individuals feeding more aggressively than others. In the present study, the fish size varied considerably from 141 to 976 g which may have exacerbated this feeding pattern contributing significantly to the large range of tissue antibiotic concentrations at a given sampling time.

During the 10-day medicated feed administration period, the tissue antibiotic concentration in individual fish varied from below quantifiable limits to a peak concentration of 13.86 ppm for SDM on day 6 and 4.28 ppm for OMP on day 11. The ratios of tissue levels of SDM to OMP found in the present study were not the same as the 5:1 SDM to OMP ratio in Romet-30 and suggests a difference in the rate of maximal uptake or excretion of these two drugs in salmon tissue. Moreover, SDM was detectable in salmon tissue for up to 7 days, while OMP could be detected for as much as 14 days after cessation of treatment. These results show that there are differences in the rate of tissue uptake and decline of SDM and OMP in salmon. Due to the variability in individual fish antibiotic concentrations, a wash-out period could not accurately be determined; however, in this study OMP residues appeared to remain in the tissues approximately twice a long as SDM residues.

In summary, the HPLC method developed herein allows for the simultaneous quantitation of SDM and OMP in farmed chinook salmon treated with Romet-30. This assay has the advantage of requiring only a single extraction for the determination of both SDM and OMP. The HPLC analysis of SDM and OMP reported in this study was reliable and allowed for the detection of the antibiotics contained in Romet-30 at levels down to 0.2 ppm. The *in vivo* feeding experiment illustrated the differences in the muscle tissue antibiotic concentration between individual fish after administration of medicated feed. In addition, the rate of tissue uptake and decline of SDM and OMP was shown to differ pointing to the necessity for measuring residues of both SDM and OMP in salmon treated with Romet-30.

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