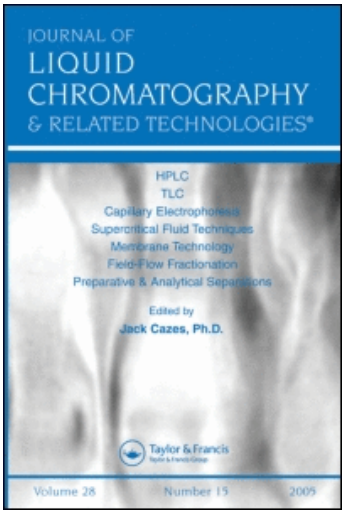

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SIMULTANEOUS HPLC DETERMINATION OF SULFADIAZINE AND TRIMETHOPRIM IN CULTURED GILTHEAD SEA BREAM (*SPARUS AURATA,* L.) TISSUES

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ABSTRACT

An HPLC method is described for the simultaneous determination of sulfadiazine (SDZ) and trimethoprim (TMP) residues in cultured gilthead sea bream tissues (muscle plus skin, liver, gills, fat, and kidney). Tissue samples are extracted with dichloromethane and the analytes are partitioned into hydrochloric acid, to be further analysed isocratically on a Nucleosil 100, C18 reverse-phase column, using a mobile phase of acetonitrile : 10 mM phoshoric acid (16:84, v/v).

The UV detection is performed by using a spectrophotometric detector monitored at 271 nm. The limits of detection of SDZ and TMP in the edible part of fish are 3.1 ng/g and 18.2 ng/g at a signal to noise ratio of 3:1. The average recovery for SDZ is 82.1%, in muscle plus skin, and ranges from 77.8% to 87.4 % in the other tissues. The average recovery for TMP is 66.7% in muscle plus skin, and ranges from 71.7% to 83.1 % in the other tissues.

INTRODUCTION

Potentiated sulfonamides (the combination of sulfonamides and diaminopyrimidines at a 5:1 ratio) are widely used in the salmon and bass and bream aquaculture industry against a variety of fish bacterial pathogens.[1] The only potentiated sulfonamide licensed for use in Greece,[2] like Norway[3] and the United Kingdom,[4] is the sulfadiazine (SDZ) and trimethoprim (TMP) formulation, at a ratio of 5:1, in the form of a premix. The use of SDZ and TMP in aquaculture may lead to residues in farmed fish tissues. The possible hazards associated with the presence of antimicrobial drug residues in edible tissues of products from aquaculture include allergies, toxic effects, changes in colonization patterns in human-gut flora, and acquisition of drug resistance in pathogens in the human body.[5] These potential hazards have resulted in a need for strict control of residue levels in farmed fish after medication, as well as in terrestrial production.

An analytical method suitable for the assessment of SDZ and TMP residue levels has to be available not only for human health aspects but for residue depletion and pharmacokinetic studies as well.

Various methods have been described for the LC determination of SDZ alone, or in combination, with other sulfonamides in fish muscle tissue.[6,7,8,9,10] For the determination of SDZ and TMP a few methods have been published. These cover the determination of SDZ and TMP in human body fluids and urine,[11] in medicated fish feed,[12] and in fish tissues.[3,13] Both methods for fish tissues comprise extraction and clean up procedures more or less laborious, to be used as starting point. Moreover, Gentlemen et al.,[13] developed a procedure for the SDZ and TMP determination in chinook salmon muscle tissue with a separate HPLC assay for each of the drugs.

This paper describes an LC method for the simultaneous determination of SDZ and TMP residues in cultured sea bream tissues. The extraction and clean up procedure is based on the method reported by Simeonidou et al.,[14] for the determination of multiple sulfonamide residues in chicken muscle, with fluorescence detection.

EXPERIMENTAL

Reagents and Chemicals

HPLC-grade water was produced using a Milli-Q (Millipore, Bedford, MO, USA) purification system. HPLC-grade acetonitrile and analytical grade dichloromethane, hydrochloric acid, and phosphoric acid, and sodium acetate anhydrous suprapur-grade were obtained from Merck (Darmstadt, Germany).

Analytical standards of SDZ and TMP were obtained from Sigma (St. Louis, MO, U.S.A). Stock solutions of SDZ and TMP (1 mg/mL) were prepared in acetonitrile and were stored in the refrigerator. Aliquots of the stock solutions were diluted with hydrochloric acid 3N to give working standard solutions in the concentration range of 5-150 ng/mL for SDZ and 25-750 ng/mL for TMP. Working solutions were prepared daily. Spiking solutions were prepared from the stock solutions after dilutions in the range of 1000 to 10000 ng/mL for both SDZ and TMP.

Apparatus and Chromatographic Conditions

The instruments used were a Vortex Genie 2 mixer (Scientific Industries, Model G-56OE, Bohemia, NY), a Centrifuge (Hermle 2383K, Germany), an ultrasonic bath (Transsonic 460/H, Elma®, West Germany), an Ultra Turrax T25 (Janke & Kunkel, IKA Labortechnik, Germany) and a magnetic stirrer-hotplate (Gallenkamp, England). Filter paper circles (Rundfilter 100 stuck, ∅110 mm) used were from Schleicher & Schuell (Dassel, Germany).

Liquid Chromatography (LC) was perfomed with a Gilson system (Villiers-le-Bel, France) consisting of a Model 305 piston pump, a Model 805 manometric module, a Model 119 UV/VIS variable wavelength spectrophotometric detector set at 271 nm and a Kipp & Zonnen recorder (Model BD111, Holland).

The injections were made onto a stainless steel (250 x 4.6 mm I.D.) column, packed with Nucleosil 100 RP C18, particle size 5 μm (Rigas, Greece), through a Rheodyne 7125 (Cotati, California, U.S.A.) sample injector equipped with a 100 μL loop. The system was operated at ambient temperature. Recordings were made at a chart speed of 2 mm/min and a detector sensitivity of 0.005 AUFS.

The mobile phase, consisting of acetonitrile:10 mM phosphoric acid (16:84 v/v), was filtered, degassed before use, and delivered at a rate of 1 mL/min.

Sample Preparation

Ground sea bream tissues were accurately weighed (3 g of muscle plus skin, 0.5 g of liver, gills, and fat, and 0.25 g of kidney) in a 50 mL plastic centrifuge tube. Thirty mL of dichloromethane were added and the sample was homogenized with an Ultra Turrax for 1 min and centrifuged at 3000 g for 10 min. The supernatant phase was filtered through filter paper and collected.

Ten mL of this liquid were transferred to a 15 mL glass centrifuge tube, 1 mL of hydrochloric acid 3 N was added, vortexed for 15 sec, and centrifuged for 5 min at 3000 g. From the upper aqueous layer, 250 μL were transferred to a 15 mL glass centrifuge tube and another 250 μL of 3.8 M sodium acetate solution were added and vortexed for 15 sec. Finally, a 100 μL aliquot was injected into the LC system.

Calculations

The calibration curves for SDZ and TMP were obtained by plotting peak height versus concentrations from 100 μL injections of working standard solutions for each compound, respectively. The SDZ and TMP concentrations in the analyzed samples were calculated by reference to calibration curves and multiplication by the appropriate dilution factor.

RESULTS AND DISCUSSION

Chromatography

For the simultaneous determination of SDZ and TMP, literature describes mobile phases delivered under gradient elution conditions and consisting of acetonitrile-0.01M ammonium acetate (pH 5.5),[7] or isocratically under ion-pair conditions using hexane-sulfonate[3] or triethylamine[13] as pairing ions. Even though the pH value of the above isocratic systems (pH 2.8) was compatible with the pH of the samples (a pH value of about 3), the optimum mobile phase composition for our system proved to be a mixture of 10 mM phosphoric acid - acetonitrile (84:16), providing an adequate resolution and good capacity factors k' for the two compounds.

The cleanup procedure used gave rise to samples with a pH value of about 3, this fact being important for the composition of the mobile phase as the compounds for separation are ionizable. Under the conditions that were selected, SDZ and TMP were eluted in 6.5 and 9 min, respectively.

A typical chromatogram of a standard solution of SDZ and TMP using this developed system is shown in Figure 1a.

Sample Preparation

In the method reported by Simeonidou et al.,[14] chloroform was used as an organic extractant. However, for SDZ and TMP extraction from fish tissues, chloroform had to be replaced since this solvent has been considered a car-

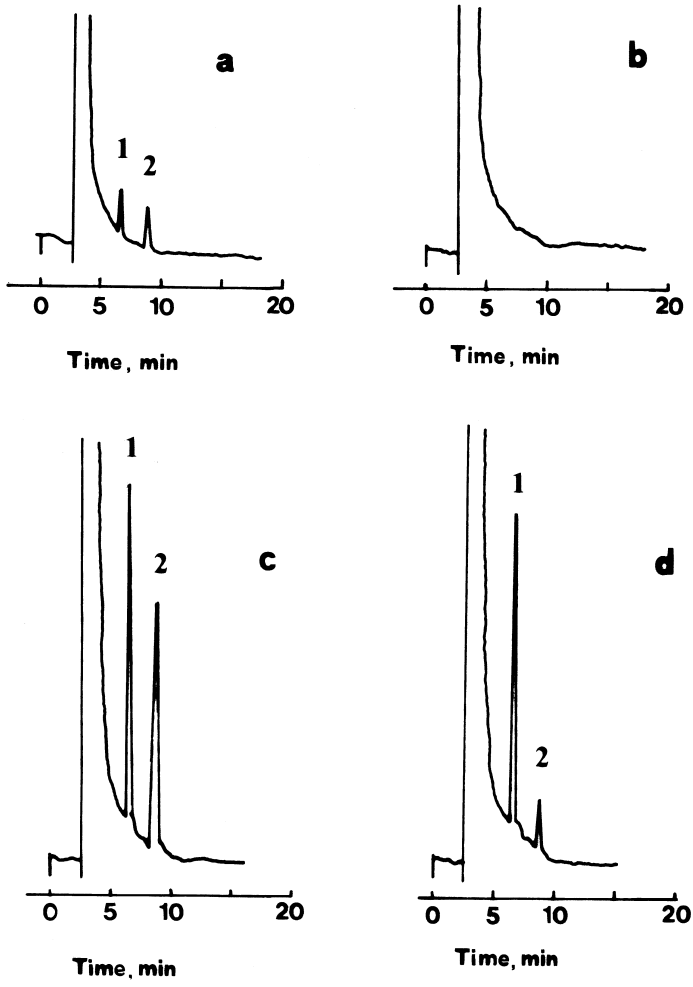

Figure 1. Chromatograms of (a) a standard solution of 10 ng/mL of SDZ(1), and 50 ng/mL of TMP (2); (b) a control gilthead sea bream (*Sparus aurata*) muscle plus skin tissue extract; (c) a gilthead sea bream muscle plus skin tissue extract spiked with 200 ng/g of SDZ (1) and 1000 ng/g of TMP(2); (d) a real muscle plus skin sample containing 185.5 ng/g of SDZ and 200.1 ng/g of TMP. Chromatographic conditions: Column: Nucleosil 100, C18 RP (250 x 4.6 mm); ambient column temperature; mobile phase: acetonitrile: 10 mM phosphoric acid (16:84, v/v); flow rate 1.0 mL/min; wavelength, 271 nm; detector sensitivity, 0.005 AUFS; chart speed 2 mm/min; injection volume 100 μL.

cinogen.[15] Therefore, dichloromethane was tried, leading to a satisfactory extraction yield for both SDZ and TMP. Finally, the derivatization procedure was omitted and aliquots of the test samples were injected into the LC system with UV detection for both drugs.

The sample preparation scheme used is simple and easier to perform than the method previously reported,[3,13] as it involves a three step scheme for sample preparation with two extractions only.

Spiking Studies and Real Samples

Quantitation was carried out by using the external standard calibration technique. The calibration curve was linear throughout the range 5-150 ng/mL for SDZ ($y=7.9926x+0.5749$, $r^2=0.9999$) and 25-750 ng/mL for TMP ($y=1.644x+0.8345$, $r^2=0.9991$). The limits of detection for SDZ and TMP in the edible part of the fish (muscle plus skin in natural proportion) were 3.1 and 18.2 ng/g, respectively, at a signal to noise ratio of 3:1.

Levels of 10 and 80 ng/g for SDZ and TMP could be easily quantitated in these tissues. No interference from endogenous compounds from the tissues were found at the retention times of both substances (6.5 min for SDZ and 9 min for TMP).

Recovery experiments were carried out on various sea bream tissues, by spiking blank samples at three fortification levels and analysing six replicates at each level. Blank gilthead sea bream muscle plus skin tissues were spiked at 20, 200, and 300 ng/g for SDZ and 100, 1000, and 1500 ng/g for TMP. For liver, gill, and fat samples the spiking levels ranged from 120 to 1800 ng/g for SDZ and from 600 to 9000 ng/g for TMP.

Finally, the spiking levels for kidney samples ranged from 240 to 3600 ng/g for SDZ and from 1200 to 18000 ng/g for TMP. The results are presented in Tables 1 and 2. Good average recoveries and an acceptable standard deviation were attained for both compounds.

The day-to-day variation was studied as well. Blank sea bream muscle plus skin samples were spiked at 200 ng/g of SDZ and 1000 ng/g of TMP; liver, gills, and fat samples were spiked at 1200 ng/g and 6000 ng/g levels, respectively, while kidney samples were spiked at 2400 ng/g and 12000 ng/g, respectively. The spiked test portions were analysed in 6 replicates on three different days, following the procedure outlined above.

Table 1

Recovery Data for SDZ in Tissues of Gilthead Sea Bream (*Sparus aurata,* L.)

Tissue	Concentration Added (ng/g)	Mean[a] Concentration Found ± SD (ng/g)	Rel SD, %	Recovery, %
Muscle plus skin	20	16.1 ± 0.6	3.7	80.3
	200	167.8±6.0	3.6	83.9
	300	246.1±8.2	3.3	82.0
Liver	120	88.9±6.7	7.5	74.1
	1200	969.5±40.5	4.2	80.8
	1800	1414.7±49.1	3.4	78.6
Gills	120	103.9±4.4	4.2	86.6
	1200	1046.5±4.5	4.7	87.2
	1800	1517.4±59.2	3.9	84.3
Fat	120	97.1±5.12	5.3	80.9
	1200	1036.3±54.5	5.2	86.3
	1800	1454.9±82.3	5.6	80.8
Kidney	240	202.2±13.4	6.6	84.3
	2400	2162.7±144.5	6.7	90.1
	3600	3163.8±250.7	7.9	87.9

[a] 6 replicates

The results are shown in Tables 3 and 4. A reasonable SD of the mean for both compounds was attained; the RSD being better than 4.9% for SDZ in all tissues. The RSD for TMP was better than 5.4% for TMP in all tissues.

Typical chromatograms of blank and spiked muscle plus skin samples are shown in Figure 1b,c.

An experiment was undertaken to obtain real samples and test the applicability of the described method. The potentiated sulfonamide was incorporated into feed and orally administered to a few gilthead sea bream with a mean weight of 210 g, for seven days at the therapeutic dose of 30 mg active ingredients/kg biomass/day, at a water temperature 22±0.5 °C. Immediately after the

Table 2

Recovery Data for TMP in Tissues of Gilthead Sea Bream (*Sparus aurata*, L.)

Tissue	Concentration Added (ng/g)	Mean[a] Concentration Found ± SD (ng/g)	Rel SD, %	Recovery, %
Muscle plus skin	100	68.3±3.9	5.7	68.3
	1000	664.4±28.5	4.3	66.4
	1500	979.4±51.6	5.3	65.3
Liver	600	380.5±20.4	5.3	63.4
	6000	45959±258.0	5.6	76.6
	9000	6758.9±198.5	2.9	75.1
Gills	600	463.0±37.4	8.1	77.2
	6000	4338.1±319.9	7.4	72.3
	9000	6366.1±304.2	4.8	70.7
Fat	600	482.0±222	4.6	80.3
	6000	4473.4±159.2	3.5	74.5
	9000	6645.6±225.6	3.4	73.8
Kidney	1200	952.0±60.7	6.4	79.3
	12000	10389.1±878.1	8.4	86.6
	18000	15007.9±1354.2	9.0	83.4

[a] 6 replicates

Table 3

The Recovered SDZ Content of Spiked (200 ng/g for Muscle plus Skin, 1200 ng/g for Liver, Gills and Fat, and 2400 ng/g for Kidney) Gilthead Sea Bream Tissue Test Portions Analysed on Different Days

	Mean Concentration Found ± SD (ng/g)				
Day	Muscle plus Skin	Liver	Gills	Fat	Kidney
1 (n=6)	170.3±8.4	991±46.9	1047.7±29.9	1044.9±30.5	2013.1±60.3
2 (n=6)	168.3±4.6	941.4±28.6	1095.3±22.7	974.5±29.3	2152±85.3
3 (n=6)	164.9±3.6	975.9±32	996.4±32.3	1089.3±17	2323±46.9
Total (n=18)	167.8±6.04	969.4±40.5	1046.4±49.5	1036.2±54.5	2162.6±144.4

Table 4

The Recovered TMP Content of Spiked (1000 ng/g for Muscle plus Skin, 6000 ng/g for Liver, Gills and Fat, and 12000 ng/g for Kidney) Gilthead Sea Bream Tissue Test Portions Analysed on Different Days

	Mean Concentration Found ± SD (ng/g)				
Day	Muscle plus Skin	Liver	Gills	Fat	Kidney
1 (n=6)	665.9±17.8	4501.5±242	4415.6±75.4	4555.9±85.4	9525.4±258.7
2 (n=6)	646.7±23.9	4433.4±148.1	4641±136.1	4319.4±101.5	10170.1±257
3 (n=6)	680.5±34.4	4852.7±155.9	3958.3±191.5	4544.9±160.8	11471.8±358.8
Total (n=18)	664.3±28.5	4595.8±257.9	4338.4±319.9	4473.3±159.2	10389.1±878.1

end of the treatment, three fish were sacrificed and muscle plus skin, liver, gills, fat, and kidney samples were collected and analyzed as described above. The levels of the incurred SDZ and TMP residues in muscle plus skin, liver, gill, fat (pooled), and kidney (pooled) samples of the three treated sea bream are presented in Table 5. A chromatogram of a real sample is shown in Figure 1d.

In conclusion, the results of the present study show that the described method for the simultaneous determination of SDZ and TMP in sea bream tis-

Table 5

Tissue Levels (ng/g) of SDZ and TMP Residues[a] in Three Individual Sea Breams (*Sparus aurata,* L.) After Oral Administration*

	Muscle plus Skin		Liver		Gills		Kidney[b]	
Fish	SDZ	TMP	SDZ	TMP	SDZ	TMP	SDZ	TMP
1	582.9	377.9	314.4	1158.9	359.4	839.5		
2	185.5	200.1	298.6	1338.1	187.1	359.5	305.5	18155.1
3	301.1	222.3	1500.4	7613.7	661.2	1319.5		

* 30 mg Act. Ingred./kg biomass/day for 7 days (22±0.5 °C). [a]Fat concentrations were below the detection limit. [b]Pooled sample

sues is sensitive, simple, fast, cost-effective, and should prove to be useful for routine analysis.

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