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Analysis of Stilbene Residues in Aquacultured Finfish Using LC-MS/MS

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ABSTRACT: This analytical method was developed for the determination of three stilbene residues, diethylstilbestrol (DES), dienestrol (DEN), and hexestrol (HEX), in edible tissues of finfish including catfish, salmon, trout, and tilapia. Fortified fish samples were extracted with acetonitrile and further cleaned up using silica solid phase extraction columns. Stilbene residues were separated from matrix components by reversed phase high-performance liquid chromatography on a C8 column and analyzed using a tandem mass spectrometer with negative electrospray ionization. The overall average residue recoveries using post-fortified matrix-matched calibrants were 119, 99, and 104% with %RSDs of 18, 11, and 15% for DEN, DES, and HEX, respectively. Method detection limits of DEN, DES, and HEX in each matrix were found to be at or below 0.21 ng/g, and the limit of quantification averaged 0.3 ng/g and ranged from 0.18 to 0.65 ng/g for all analytes in all matrices.

KEYWORDS: *stilbenes, fish, aquaculture, LC-MS/MS*

INTRODUCTION

Diethylstilbestrol (DES), a powerful nonsteroidal synthetic estrogen, is an endocrine disruptor initially used to prevent pregnancy complications between the 1940s and 1970s.[1] It was later shown to have a series of adverse side effects in the mother and/or her offspring including cancer of the breast, vagina, and testicles,[1,2] obesity,[3] and alterations in reproductive tract tissue and function.[4] For these reasons, DES, which had been used in veterinary medicine as a growth promoter in cattle, sheep, and poultry,[5] has been banned for this use in the United States[6,7] and the European Union.[8]

Although the use of DES in veterinary medicine has been well documented,[5,9] its use in aquaculture has been less so. There are many papers describing how DES can be fed to fish to increase weight gain directly[10,11] or to induce sex reversal[12,13] or sterility,[13] both of which indirectly lead to weight gain. The ultimate goal of hormone treatments is to increase growth, which leads to increased profits. No data could be found describing the actual practice of using DES for commercial gain in aquaculture. However, there is indirect evidence of DES use in the aquaculture industry. Studies conducted at water treatment plants near feedlot and aquaculture production areas have found DES in both influent and effluent streams.[14,15] Moreover, although approved uses for DES in human and veterinary medicine are now limited, there are 17 suppliers of DES in the United States alone.[16] The desire for increased growth production and profitability in raising food animals and fish has led to the suspected use and subsequent appearance of DES and other hormones from natural and anthropogenic sources in aquatic systems[7] such as aquaculture farms,[17,18] lakes,[15] rivers,[19] and wastewater treatment plants.[14] DES can enter the food chain through the flora and fauna of contaminated aquatic systems so that the possibility of bioaccumulation must be considered.[20] A "no observable effect concentration" (NOEC) has not been determined for DES. One study found observable effects in fathead minnows (*Pimephales promelas*) in surface water with a DES concentration as low as 1 ng/L.[21] It has been suggested that the potency of DES is at least equal to that of the more thoroughly studied 17α-ethinyl estradiol,[21] which has a predicted NOEC of 0.35 ng/L in surface waters.[22] Whether DES comes from direct dosing of fish for economic gain or through bioaccumulation, the high potency of DES dictates a need for monitoring this hormone in human foods using a rapid and sensitive analytical method of detection.

Analytical methods to determine the use of DES, and the two structurally similar analogues (Figure 1) dienestol (DEN) and hexestrol (HEX), in animal muscle tissue have been described in the literature and include residue detection techniques such as ELISA,[23,24] RIA,[25] GC,[26−28] GC-MS,[29,30] and LC-MS.[31−33] However, none of these methods address all of the following requirements of our laboratory: tested in various types of fish

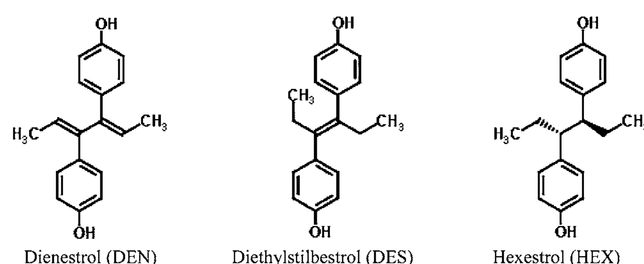

Figure 1. Stilbene structures.

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tissue, have the required sensitivity, are not time-consuming, and can analyze multiple analytes simultaneously. We have developed a fast LC-MS/MS method with a detection level at or below 0.21 ng/g for DES, DEN, and HEX in muscle tissue of catfish, salmon, trout, and tilapia. This procedure is a modification of the Xu et al. method[31] with sufficient sensitivity, improved residue recovery from fatty fish, a significantly shorter sample preparation time, and the monitoring of three product ion transitions for confirmation of analyte identity.

MATERIALS AND METHODS

Reagents and Supplies. LC grade water was purified in-house with a Milli-Q Plus (Millipore, Billerica, MA, USA) water system. Methanol, acetonitrile, *n*-hexane, dichloromethane, and ethyl acetate were of HPLC grade or better and were obtained from EMD Millipore (Billerica, MA, USA). Vetranal analytical standards of DES, DEN, and HEX were obtained from Sigma Aldrich (St. Louis, MO, USA). The internal standard d_8-diethylstilbestrol (d_8-DES) was provided by Cambridge Isotope Laboratories (Andover, MA, USA) and was specified as a 50:50 mixture of cis and trans isomers. Four types of silica SPE columns were tested. All were 500 mg (3 or 6 mL) and were manufactured by J. T. Baker (Center Valley, PA, USA), Varian (Santa Clara, CA, USA), Waters (Milford, MA, USA), or Biotage (Charlotte, NC, USA). Syringe filters were 13 mm Acrodisc 0.2 μm nylon membrane filters from Pall Corp. (Ann Arbor, MI, USA).

Standard Solutions. DEN, DES, and HEX standard stock solutions were prepared individually at a concentration of 1 mg/mL by dissolving 10 mg of each with 10 mL of methanol. Aliquots of individual stock solutions were combined and then serially diluted with methanol to prepare mixed working and calibration solutions at concentrations of 100 and 10 ng/mL. The d_8-DES internal standard stock solution was prepared at a concentration of 1 mg/mL by dissolving 5 mg of d_8-DES in 5 mL of methanol. A 100 ng/mL working internal standard solution was prepared in methanol by serial dilution of the d_8-DES stock solution. All stilbene solutions were stored at 4 °C in 15 mL polypropylene tubes. The DEN, DES, and HEX methanol solutions were stable under these conditions for at least 2 months.

SPE Solutions. Sample extracts were dissolved in a 60:40 mixture (v/v) of *n*-hexane and dichloromethane prior to loading onto SPE cartridges. The SPE wash consisted of a 94:6 mixture (v/v) of *n*-hexane and ethyl acetate. Samples were eluted with a 75:25 mixture (v/v) of *n*-hexane and ethyl acetate.[31]

Sample Preparation. Fish samples obtained from randomly collected in-house samples or purchased from local vendors were tested for suitability as negative control matrix before use. Muscle tissue fillets were homogenized in a food processor with dry ice until homogeneous and then placed in a −20 °C freezer to allow the dry ice to sublime. Homogenized fish tissue (5.0 ± 0.1 g) was weighed into a 50 mL polypropylene centrifuge tube and allowed to thaw. Skin was removed from catfish and tilapia tissue but retained on salmon and trout samples. The appropriate volume of 100 ng/mL mixed working stilbene standard was added to 5 g of fish homogenate to produce tissue fortified at 0.5, 1, or 5 ng/g of DEN, DES, and HEX. Internal standard (250 μL of 100 ng/mL) was also added for a final concentration of 5 ng/g of d_8-DES. Samples fortified only with internal standard were extracted and analyzed alongside stilbene-fortified samples with each batch to serve as method negative controls. All samples were thoroughly mixed by vortexing (2500 rpm) on a multitube vortexer for 15 min. Additional tissue samples without stilbenes or internal standard added were also extracted with each batch to produce negative extract for calibrants as described below.

Extraction Procedure. Samples were extracted by adding 20 mL of acetonitrile to each sample tube, vortexing, or shaking by hand briefly to disperse tissues and then vortexing for 5 min on a multitube vortexer (2500 rpm). Samples were centrifuged at 8000*g* for 5 min at 4 °C. The supernatant was decanted into a 60 mL glass tube (ASE, Thermo/Dionex, Sunnyvale, CA, USA) and evaporated to dryness under nitrogen (Turbo Vap, Hopkinton, MA, USA; 55 °C water bath, 15 psi). Evaporation to dryness under these conditions required about 35−55 min. Polypropylene 50 mL centrifuge tubes were also used for evaporation, but required substantially longer evaporation time (∼1.5 h total). Some fish tissues did not yield complete evaporation, but produced as much as approximately 200 μL of an oily resin-like residue. A 1 mL aliquot of *n*-hexane/dichloromethane (60:40) was added to the residue, and the sample tube was vortexed on a multitube vortexer for 1 min to dissolve the material.

SPE Cleanup. Silica SPE columns were used for sample cleanup.[31] SPE columns from four manufacturers were evaluated and found to have comparable performances. The sample was loaded, washed, and eluted under vacuum at approximately 2 drops/s. Unless stated, the column was prevented from drying out between steps. The column was initially conditioned with 6 mL of *n*-hexane. The dissolved extract was then loaded onto the column, avoiding as much as possible the loading of any undissolved resin-like material. The sample tube was washed twice with 3 mL of *n*-hexane/ethyl acetate (94:6), and each wash was sequentially loaded onto the SPE column. The column was dried under full vacuum, and the eluants from the conditioning, loading, and washing steps were discarded. The compounds of interest were then eluted under vacuum into a clean 15 mL polypropylene centrifuge tube with 6 mL of *n*-hexane/ethyl acetate (75:25). The eluate was evaporated to dryness under nitrogen (40 °C water bath, 15 psi) for 10 min. A 1 mL aliquot of acetonitrile/water (50:50, v/v) was added to each sample and the residue dissolved by vortexing for 1 min (2500 rpm) followed by 1 min of sonication. All samples were filtered through a 0.2 μm nylon syringe filter into LC vials. This method results in a 5 times concentration factor, as the whole extract from 5 g of tissue is brought up to a final volume of 1 mL.

Calibrant Preparation. Seven post-fortified calibrants were prepared in extracted fish matrix. Two negative control fish samples (i.e., previously shown to contain no stilbene residues) not fortified with stilbenes or internal standard were extracted each day to supply the extracted tissue matrix for the calibrants. The calibrants ranged in concentration from 0.156 to 10 ng/g and were prepared by adding the appropriate volumes of 10 ng/mL mixed stilbene calibration solution and 50 μL of working internal standard solution (100 ng/mL) to seven clean 15 mL polypropylene centrifuge tubes. The solutions were evaporated to dryness (40 °C water bath, 15 psi nitrogen, 6−10 min) and reconstituted by adding 200 μL of filtered negative control extract to each tube, vortexing for 1 min, and sonicating for 1 min. Calibrants were transferred without additional filtration to LC vials for injection. As an example, a calibrant prepared using 125 μL of mixed stilbene calibration solution (10 ng/mL), 50 μL of working internal standard solution (100 ng/mL), and 200 μL of fish matrix resulted in concentrations of 1.25 ng/g of DEN, DES, and HEX and 5 ng/g d_8-DES, relative to the tissues extracts (5 times concentration factor).

Liquid Chromatography. The LC system consisted of an Agilent (Santa Clara, CA, USA) 1200 binary pump, degasser, and column heater and a Leap Technologies (Carrboro, NC, USA) HTC Pal autosampler. A Zorbax Eclipse XDB-C8, 4.6 × 150 mm, 5 μm LC column (Agilent) with a Phenomenex (Torrance, CA, USA) Security Guard C8 4 × 2.0 mm guard cartridge was used for the HPLC separation. The mobile phases were water and acetonitrile. The sample injection volume was 50 μL, the column was held at ambient temperature, and the autosampler was set at 15 °C. The LC gradient started at an initial composition of 40% acetonitrile, ramping to 95% acetonitrile over 12 min, and was then brought back to 40% acetonitrile over 1 min and held at 40% for 2 min.

Mass Spectrometry. An Applied Biosystems (Foster City, CA, USA) ABSciex 5500 QTrap mass spectrometer with Analyst software version 1.5.1 was used with electrospray ionization (ESI) in the negative ion mode. The ESI source was operated at 500 °C and −3500 V. Resolution for Q1 and Q3 was set at unit resolution, and scheduled multiple-reaction monitoring (MRM) was not used. The gases had the following pressures: curtain gas (CUR), 40 psi; collision-activated dissociation gas (CAD), medium setting; gas supply 1 (GS1), 45 psi; and gas supply 2 (GS2), 55 psi (CUR and CAD gases are nitrogen,

GS1 and GS2 gases are zero air). Precursor and product ions selected for MRM can be found in Table 1; the dwell time for all transitions was 75 ms.

Table 1. Mass Spectrometer Conditions

analyte	Q1 mass (m/z)	Q3 mass[a] (m/z)	CE[b] (V)	CXP[c] (V)	DP[d] (V)
DEN	265.0	*93	−32	−9	−125
		117	−40	−13	
		235	−36	−27	
DES	267.0	222	−46	−17	−145
		*237	−38	−13	
		251	−50	−11	
HEX	269.0	93	−82	−11	−140
		*119	−48	−13	
		134	−20	−13	
d_8-DES	275.0	227	−44	−19	−105

[a]*, quantitation transition. [b]Collision energy. [c]Collision cell exit potential. [d]Declustering potential.

Validation Design. Analyses were carried out over multiple days for each matrix type by multiple analysts. Residue recovery, or accuracy, was calculated from the respective calibration curve for each analyte as a percentage of the known amount added to each tissue. Precision was calculated as %RSD. Negative control matrix samples were extracted with each set of fortified samples to ensure the absence of matrix interference. Reagent blanks (no tissue) were also extracted and analyzed to test for contamination in the reagents used. A 50 ng/mL solvent standard (10 ng/mL tissue equivalent) was included in the analytical sequences to evaluate any changes in the day-to-day instrument response or potential signal suppression from the fish matrix.

The internal standard corrected calibration curve was constructed by plotting the peak area ratio of DEN, DES, or HEX to d_8-DES versus the concentration of the calibrants. The sample concentrations were determined by linear regression. The method detection limit (MDL) for each residue in each matrix was calculated as the standard deviation in the calculated concentration of 0.5 ng/g fortified samples ($n = 7$) multiplied by the Student's t value at the 99% confidence interval. The limit of quantitation (LOQ) was calculated as 10 times the standard deviation of the 0.5 ng/g fortified samples ($n = 7$).[34] When more than one set of seven 0.5 ng/g samples were extracted and analyzed by different analysts and on different days, the averages of the MDLs and LOQs from individual sets were reported.

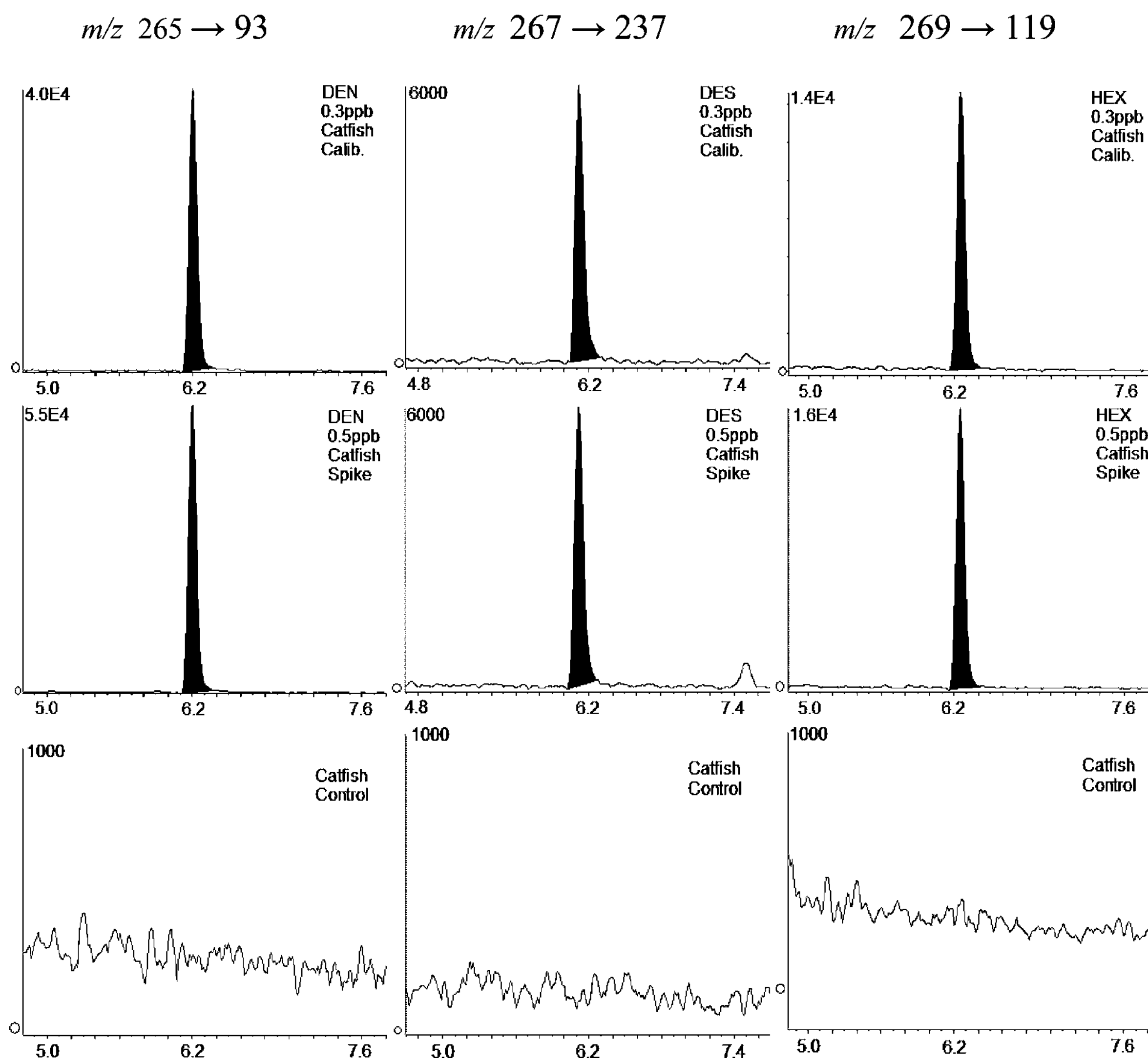

Figure 2. Representative chromatograms of DEN, DES, and HEX in catfish tissue. First and second rows are quantification ions in post-fortified calibrants and in extracted tissue spikes, respectively. The third row shows extracted negative controls.

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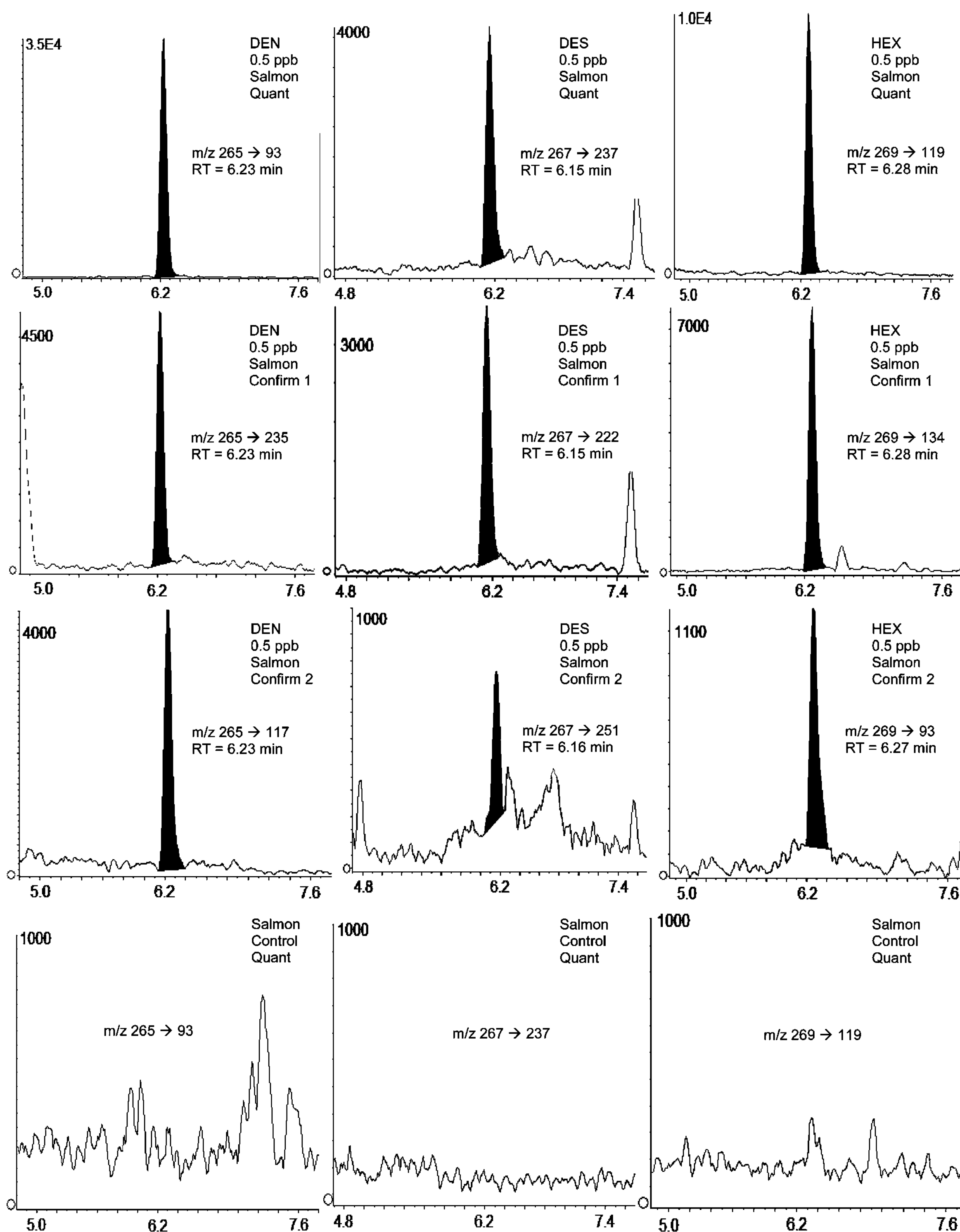

Figure 3. Representative chromatograms of the three product ion transitions used for DEN, DES, and HEX in salmon tissue fortified at 0.5 ng/g. The bottom row shows the three quantification transitions for negative control salmon.

Confirmation of identity was based on the U.S. Food and Drug Administration's Guidance to Industry 118 "Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues",[35] which requires that the retention times of the sample and standards are within 5% of each other, that the peak area ion ratios of the sample and standards are within ±20% absolute of each other, and that the signal-to-noise ratios of the confirmation ions are greater than 3:1. An additional requirement of the ion transition used for quantification having a signal-to-noise ratio of 10:1 or greater was imposed by our laboratory.[34]

RESULTS AND DISCUSSION

Our original objective was to validate the Xu et al. method[31] for use in our laboratory for stilbene residue analysis. When we applied that method to analyze fish with a high fat content such as salmon and catfish, we obtained uncorrected recoveries (no internal standard correction) of only 35% for the three stilbene compounds. Therefore, several changes were made to improve the sensitivity of the method for these types of fish. Replacing

Table 2. Accuracy, Precision, and Identity Confirmation

			recovery (%) (accuracy)			%RSD (precision)			residues confirmed (%)		
matrix	fortification level (ng/g)	n	DEN	DES	HEX	DEN	DES	HEX	DEN	DES	HEX
catfish	0.5	28	132	102	107	15	14	15	100	100	100
catfish	1	12	110	101	107	15	5	16	100	100	100
catfish	5	7	99	97	115	8	4	10	100	100	100
salmon	0.5	19	136	94	111	14	9	12	100	89	95
salmon	1	14	125	96	102	14	8	16	100	100	100
tilapia	0.5	27	105	99	96	17	11	13	100	100	100
tilapia	1	7	114	104	97	7	2	10	100	100	100
trout	0.5	7	122	116	121	6	4	5	100	100	100
trout	1	7	96	87	87	3	4	4	100	100	100

tert-butyl methyl ether with acetonitrile as the extraction solvent reduced the amount of extracted fat and improved the extraction efficiency. Adding an LC gradient better separated the stilbenes from the fish matrix components, thus decreasing matrix effects. Finally, the use of electrospray ionization instead of atmospheric pressure chemical ionization increased the instrument response. Overall, these changes resulted in uncorrected average recoveries (no internal standard correction) of 96, 78, and 81% for DEN, DES, and HEX, respectively. The improvement in sensitivity also allowed for the monitoring of a third product ion transition for identity confirmation. The three product ion transitions (Table 1) for each of the stilbene residues have previously been observed using electrospray ionization.[31,32,36]

Representative post-fortified calibrant and fortified sample chromatograms of DEN, DES, and HEX in catfish can be seen in Figure 2. Individual product ion chromatograms for the three compounds in salmon are shown in Figure 3. Both cis and trans isomer peaks can be observed in the DES chromatograms in Figures 2 and 3,[37] yet all quantification and confirmation in this study was based solely on the peak areas of the *trans*-DES and *trans*-d_8-DES isomers. The *trans*-DES isomer is the first eluting and larger of the two isomers using this method. The coefficient of determination (R^2) values obtained for the calibration curves ranged from 0.993 to 1.000 and averaged 0.998 for all analytes in all matrices. All validation and instrument performance verification requirements were met throughout the validation process.[34] The accuracy, precision, and percent of samples with confirmed residue identity are given in Table 2 for each fish matrix fortified at 0.5 and 1.0 ng/g (and 5.0 ng/g for catfish). The overall internal standard corrected average residue recoveries using post-fortified matrix matched calibrants were 119, 99, and 104% for DEN, DES, and HEX, respectively. The overall %RSD values were 18, 11, and 15% for DEN, DES, and HEX, respectively. The precision calculation was based on the calculated recovery for all samples at a given fortification level regardless of day or analyst; therefore, this measure of precision represents a wide range of method performance variability. Intraday precision can be observed from the data in Table 2 for the sets of fortified samples with only $n = 7$ replicates, namely, catfish fortified at 5.0 ng/g, tilapia fortified at 1.0 ng/g, and trout fortified at 0.5 and 1.0 ng/g. These sets of seven replicates were extracted and analyzed by a single analyst on a single day, and the resulting precision is correspondingly lower, ranging from 2 to 10% RSD for the three residues.

As shown in Table 2, the d_8-DES corrected recovery for DEN was typically well above 100%, particularly for salmon and catfish. This is likely an indication that d_8-DES was not the most appropriate internal standard to correct for DEN; however, at the time this study was undertaken, isotopically labeled compounds were not available to us for DEN or HEX. The data were alternately evaluated for DEN without internal standard correction and were found to have excellent recovery and %RSD for catfish, salmon, and trout: 102 ± 24, 96 ± 13, and 101 ± 21%, respectively (all concentration levels averaged). Tilapia had a somewhat lower uncorrected average recovery of 85% and a larger variability of 31% RSD. At the 0.5 ng/g fortification level for tilapia, data collected on four days by three different analysts resulted in average DEN recovery and %RSD of 79 ± 32% without internal standard correction and 105 ± 17% with internal standard correction. Therefore, to better normalize daily variations in method performance among different analysts, we accepted higher than ideal recoveries (96−136%) for d_8-DES corrected DEN as a characteristic of the performance of this method.

The MDLs of DEN, DES, and HEX in each matrix were determined to range from 0.06 to 0.21 ng/g and the LOQs averaged 0.32 ng/g and ranged from 0.18 to 0.65 ng/g for all analytes in all matrices. Individual MDL and LOQ values are reported in Table 3.

Table 3. MDL/LOQ

	DEN (ng/g)		DES (ng/g)		HEX (ng/g)	
	MDL	LOQ	MDL	LOQ	MDL	LOQ
catfish	0.14	0.43	0.08	0.24	0.21	0.65
salmon	0.08	0.26	0.06	0.18	0.11	0.33
tilapia	0.10	0.31	0.07	0.23	0.10	0.31
trout	0.12	0.38	0.07	0.23	0.10	0.32

The identity of the three stilbenes was positively confirmed for all fortified samples with the exception of two salmon samples. In these, DES and HEX residues could not be confirmed in one sample and DES could not be confirmed in the other. These confirmation failures occurred at the 0.5 ng/g fortification level on the same day by a single analyst; however, the two fortified samples came from two different salmon matrices. Samples from both of these salmon matrices were observed to produce a red oily substance in the final extract, prior to filtering. A similar observation was previously reported[38] and was suggested to originate from a carotenoid-

type compound such as astaxanthin. In our experiments, red particulates could be observed in some of the LC vials several days after the original analysis, suggesting that oil may have separated out of solution in the chilled autosampler tray. Background interference was not observed in the chromatograms of these samples.

The stability of the sample extracts was not directly studied, but in addition to possible oil separation in salmon extracts, an indication of instability of HEX in catfish extract arose during the validation process. A set of catfish extracts were reinjected three days after their initial injection due to an incorrectly prepared standard curve. Two of the three product ion transitions had the expected responses, but the m/z 269 → 93 transition (the least abundant) displayed interference, which caused the ion ratio to fall well out of the acceptable confirmation range in all (n = 7) of the samples. The original injection of these samples showed no interference, and all of the samples passed the confirmation criteria.

In addition to the detection of parent stilbene residues, the presence of steroid and hormone glucuronide metabolites, including DES-glucuronide, has been well documented in animal excretory system organs (e.g., kidney) and their products (e.g., urine).[5,25,33] There are conflicting data on the extent to which stilbene glucuronide compounds are present in muscle tissue in treated animals. Some research suggests that conjugation can range from 5 to 50% in beef tissue depending on the type of estrogenic steroid administered.[30] Another study reported no detection of DES or DES-glucuronide in the lean muscle of treated cattle.[26] Xu et al. reported a 30% increase in chromatograph peak areas for stilbenes when an overnight enzymatic deconjugation step with glucuronidase was made part of the analytical method,[31] yet it is unclear if these measurements were made on tissues from treated animals or on stilbene-fortified tissue samples. We were unable to find conclusive data on what amount of DEN, DES, and HEX would undergo conjugation and be present in fish muscle. For this reason, we omitted a deconjugation step from the method to avoid a lengthy overnight process and allow tissue residue analysis to be conducted in one day instead of two. Without enzymatic deconjugation, conjugated residues, if present, would not be detected, yet the sensitivity of the method for the unconjugated stilbenes makes it likely that illegal stilbene use would still be determined. Studies are underway in this laboratory to determine what additional quantity of stilbene residues can be extracted after enzymatic deconjugation from fish that have been dosed with DEN, DES, and HEX.

In conclusion, the method presented here has excellent sensitivity and provides the required performance for the analysis of edible fish muscle with both high and low fat content. The use of three product ion transitions for residue identification gives increased confidence in the confirmation of three stilbene compounds in muscle tissue of catfish, salmon, tilapia, and trout.

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Notes

The authors declare no competing financial interest.

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