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Liquid Chromatography Analysis of Erythromycin A in Salmon Tissue by Electrochemical Detection with Confirmation by Electrospray Ionization Mass Spectrometry

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A rapid and sensitive method is described for the quantitation of erythromycin A (EA) in edible salmon tissue by liquid chromatography (LC) analysis using either electrochemical detection (ED) or electrospray ionization mass spectrometric (ESI/MS) detection. The salmon tissue is extracted with 10 mM ammonium formate. The extract is then purified by solid phase extraction using a hydrophilic−lipophilic balanced (HLB) polymeric-based C18 packing, followed by partitioning of EA into methylene chloride at alkaline pH, evaporation, and final dilution. The mean recoveries of EA at 50, 100, 200, and 400 ppb levels in fortified salmon tissue were 63.8 ± 6.0 and 75.5 ± 5.4% by LC-ED and LC-ESI/MS, respectively. There was no evidence of formation of the anhydro-EA (*m*/*z* 716) decomposition product of EA (*m*/*z* 734) that was reported to occur by other published methods.

KEYWORDS: Macrolide antibiotics; erythromycin A; electrochemical detection; mass spectrometry; salmon

INTRODUCTION

Erythromycin A (EA; **Figure 1**) is a macrolide antibiotic produced by strains of *Sacchropolyspora erythrea*. In the aquaculture industry, EA is used to treat various bacterial infections in multiple species of food-fish abroad and nonfood aquarium fish in the United States. For salmon, erythromycin is under U.S. Food and Drug Administration (FDA) review for use to control bacterial kidney disease (BKD). EA is used to manage the transmission of *Renibacterium salmoninarum*, the causative agent of BKD, in salmonids raised in fisheries for enhancement of wild stock supplies (*1*−*3*). Although it is not an approved antibiotic in the United States and Europe, EA has been widely used in aquaculture in a number of fish-producing countries that export to these countries (*4*). Therefore, there is regulatory concern for possible residues of the antibiotic in aquaculture products in these countries. The European Union, Committee for Veterinary Medicinal Products, established a maximum residue limit (MRL) of 200 ppb for EA in food-producing finfish (*5*). EA was added to the FDA's Center for Veterinary Medicine (CVM) Seafood Research Task Force−Aquaculture Subgroup list of priority drugs with a recommended target level of 100 ppb in fish. It was thus deemed important for the FDA to develop analytical methods for the detection of trace levels of the drug in the important food species of salmon and catfish.

Analysis of low concentrations of EA in edible fish tissue was a formidable task due to its polarity, high molecular weight, acid lability, and absence of a chromophore for UV absorptivity. A base-catalyzed dehydration reaction method was recently reported for the addition of an α,β-unsaturated ketone double bond for LC-UV detection of EA at 236 nm in poultry, swine, and cattle (*6*). However, when this method was adapted for EA in salmon, we found that it lacked the sensitivity to achieve the CVM target level of 100 ppb. A recent 9-fluoromethylchloroformate (FMOC) derivatization LC−fluorometric (FLU) method (*7*) was reported to detect EA and other macrolides in meat, fish, eggs, and raw milk at low parts per billion level. Recoveries were low, ranging from 48.6 to 53.2% at fortification levels 100−400 ppb.

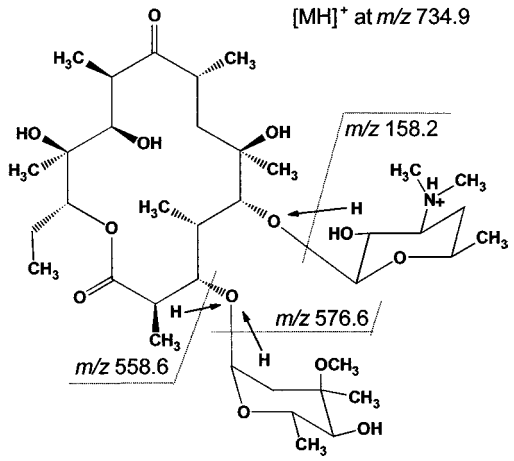

Figure 1. Erythromycin A (EA) and major fragment ions.

Several analytical methods are published that utilize LC-ED for EA analysis in fish and other foods. One method (*8*) using 25 g of fish tissue reported detection of EA by LC-ED, +1.1V, oxidation potential at levels to 200 ppb; however, 1 ppm was

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the lowest fortification level shown, and recoveries were not reported. Another LC-ED method (*6*) analyzed EA in cattle, pig, and poultry muscle tissue reporting high recoveries of 97.5-98.4% at a level of 10 ppm in these tissues.

Thus far, mass spectrometric techniques, including electrospray ionization mass spectrometry (ESI/MS) and atmospheric pressure chemical ionization mass spectrometry (APCI/MS), have attained the highest levels of sensitivity for detecting EA and/or its metabolites and breakdown products at low parts per billion levels in various matrices. Pleasance et al. (*9*) with the aid of an alkaline partition cleanup method by Takatsuki (*10*) were able to analyze EA in fortified salmon tissue extracts by full-scan LC−ion spray MS at the 0.5−1.0 ppm level and at the 10−50 ppb level using selected ion monitoring (SIM) and selected reaction monitoring (SRM) MS techniques. In chicken liver, APCI/MS was used to measure EA in chicken fortified at 50 and 100 ppb following a protein precipitation and SPE cleanup procedure (*11*, *12*). These MS techniques are very sensitive for EA detection; however, there are problems associated with multiple sample analyses due to inadequate cleanup that results in LC column deterioration and MS instrumental source contamination. Sample splitting (as much as 1:100 splits to the MS) techniques were used to minimize contamination of the MS source. Moreover, the use of formic acid mobile phases for ionization purposes may cause the breakdown of EA on the LC column prior to MS analysis. By other published LC-MS methods, EA-fortified tissue extracts (*9*) and commercial EA samples (*13*) were reported to contain the breakdown products anhydro-EA (*m*/*z* 716), *N*-demethyl-EA (*m*/*z* 720), and other forms of EA (*m*/*z* 734).

In an intra-agency collaboration between the FDA's Jefferson Regional Laboratories/NCTR, Jefferson, AR, and Center for Veterinary Medicine, Laurel, MD, a method was developed for the analysis of EA in salmon by ED with confirmation by ESI/MS. The EA method presented here utilizes a simple and fast extraction step with a solid phase extraction (SPE) cartridge elution followed by alkaline partition into organic solvent.

EXPERIMENTAL PROCEDURES

Reagents. Erythromycin A was purchased from Fluka Chemicals at 97% stated purity. [*N*-*methyl*-^{14}C-Labeled EA (60 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. Methylene chloride ($MeCl_2$) (Fisher) was pre-extracted with 2% NH_4OH to remove any possible acidic residues or other water soluble contaminants prior to use. The 2% NH_4OH was made by diluting 2 mL of concentrated 15 M NH_4OH to 100 mL with distilled deionized water. The 10 mM ammonium formate (Fluka, 0.63 g dissolved in 1 L of deionized water, without adjustment, pH 6.3−6.6) was used to extract salmon tissue and also as the buffering agent for the LC mobile phase. Methanol (MeOH) and acetonitrile (MeCN) (J. T. Baker, LC grade) were used without further purification.

^{14}C Liquid Scintillation Counting and HPLC Purity Analysis of ^{14}C Erythromycin A. A Packard Tricarb 1900TR liquid scintillation analyzer was used to count ^{14}C-labeled EA recovered from edible salmon tissue using various solvent extraction systems to evaluate extraction efficiences. Also, LC with a Flo-one scintillation detector (Radiomatic) was used to perform a purity assay of the ^{14}C-labeled EA standard. A 5 μm Supelco LC-CN column (250 × 4.6 mm) was used with 50:50 MeCN/50 mM ammonium acetate as the mobile phase at a flow rate of 1 mL/min.

Salmon Sample Preparation. A 5 g homogenized salmon tissue (muscle with attached skin) sample was weighed into a 50 mL polypropylene tube. Using a tissue homogenizer (Ultra-Turrax T25, Janke and Kunkel) with a 1.5 cm diameter rotating circular blade, the salmon was extracted with 10 mL of 10 mM ammonium formate for ∼1 min. The sample tube was centrifuged at 1100*g* for 10 min. The supernatant was filtered through a 6 cm glass filter funnel with a plug of silanized glasswool into a clean 50 mL polypropylene tube to remove small floating particles of salmon skin. The compressed tissue plug was homogenized a second time with 10 mL of 10 mM formate buffer for 1 min as before. The homogenate was centrifuged and filtered, and the supernatant was combined in the 50 mL tube (total extract volume ∼20 mL). A 60 mg Oasis HLB 3 cm^3 cartridge was activated by eluting with 3 mL MeOH followed by 3 mL of water under gravity to prepare for cleanup of the aqueous-salmon extract containing EA. The final adjusted pH of the extract was measured at 7.0−7.2 with an Orion pH meter. Salmon extract was added to the 3 cm^3 HLB cartridge using a vacuum-adapted Phenomenex SPE apparatus. The SPE elution rate was regulated to 2 mL/min or less by adjusting the vacuum. Following elution of the 20 mL of aqueous extract, the HLB cartridge was washed with 2 mL of water. A subsequent wash was performed with 3 mL of 20% MeOH/water to elute loosely bound salmon coextractives while retaining EA. The HLB cartridge was dried by vacuum prior to final elution. A 1 mL aliquot of 100% MeOH was eluted at ∼1 mL/min with slight pressure into a 12 mL separatory funnel containing 6 mL of aqueous 2% NH_4OH (pH 10.5). The aqueous/slightly alkaline extract was partitioned with 1 mL of $MeCl_2$ three times. The $MeCl_2$ layer was dried through an Na_2SO_4 column (3 cm long in a silanized glasswool plugged disposable pipet) and collected in a 4 mL glass sample vial. After combination of the $MeCl_2$ fractions, the vial was capped tightly and refrigerated at 4 °C until evaporation with N_2. The dried salmon residue was dissolved in 200 μL of mobile phase for subsequent analyses via LC-ED or LC-ESI/MS.

LC-ED Analysis. A Shimadzu LC600 low-pulse LC pumping system, equipped with a 20 μL injector and an Ansys 5 μm Polaris C18-A column (250 × 4.6 mm), was used for the separation of EA from the salmon sample extract. The mobile phase used was 45:55 MeCN/10 mM ammonium formate flowing at 1 mL/min under isocratic conditions. A Coulochem II (ESA) coulometric electrochemical detector (ED) was used in the oxidative mode, $E_1 = +0.75$ and $E_2 = +0.9$ V detection potential. An electrochemical "polishing" or guard cell was used at +1.0 V prior to the LC injector to reduce the mobile phase ED background signal to ∼1.0 μA, which allowed detection of EA down to the 50 ppb level (25 ng injected on column).

LC-ESI/MS Analysis. Components were resolved using a 5 μm Phenomenex Prodigy ODS (3) LC column (250 × 2.0 mm) with a SecurityGuard cartridge. The mobile phase, delivered at 0.2 mL/min, was a linear 45 min gradient from 20:80 MeCN/water to 80:20 MeCN/water (with constant 3 mM ammonium formate) with a final hold for 10 min. Individual samples were kept frozen and were thawed just prior to manual injection of 10 μL. An HP 5989B mass spectrometer was operated in positive-ion electrospray mode with the capillary exit voltage variable. Full-scan spectra were acquired from *m*/*z* 150 to 750. For EA the protonated molecule was the base peak, and minor ions were seen at *m*/*z* 716.6 (0.5%) and 576.6 (2.0%). Analyses of the 400 ppb fortified samples and a control salmon extract indicated that there were extra peaks from the salmon matrix eluting between 30 and 50 min. Subsequent analyses of the EA standard indicated that the MeCN gradient with the 10 min hold was adequate for eliminating the late-eluting salmon matrix peaks. UV absorption at 230 nm was monitored on-line during all of the analyses to check for extraneous peaks from the matrix, and none of the samples showed a significant UV response. For EA quantitation and to look for degradation products, limited scans were acquired from *m*/*z* 710 to 740 and the mass chromatograms for *m*/*z* 734.9 were integrated. For LC-ESI/MS the response of EA was linear in the range of 12.5−400 ppb. Quantitation was by external standard. For confirmation, the capillary exit voltage was set at +200 V for in-source collision-induced dissociation (CID), and acquisition was selected ion monitoring. Mass chromatograms for *m*/*z* 734.9 $[MH]^+$, 576.6, 558.6, and 158.2 were integrated for the EA peak in each sample (see **Figure 1** for proposed structures of the fragment ions).

RESULTS AND DISCUSSION

Purity Analysis of ^{14}C-Labeled and Unlabeled Erythromycin Standards. LC−scintillation detection analysis was performed on the ^{14}C-labeled EA standard used for the determination of extraction efficiencies from edible salmon tissue

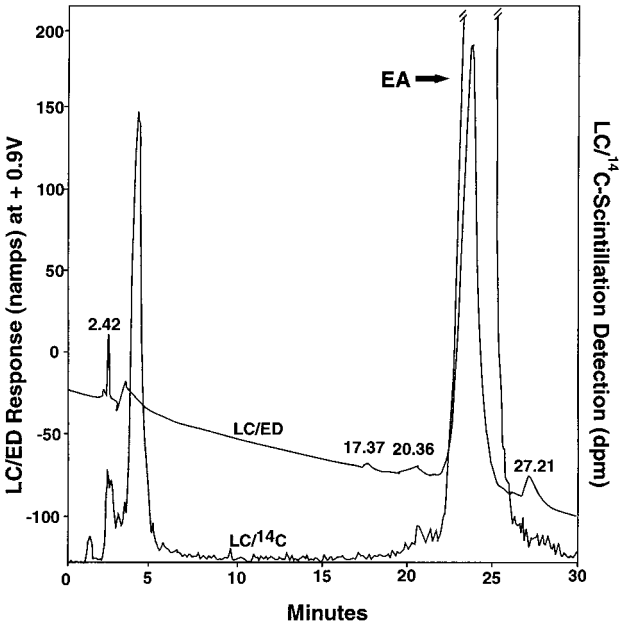

Figure 2. LC purity analysis of 0.075 μCi of ^{14}C-labeled erythromycin A and LC-ED of 3.3 μg of unlabeled erythromycin A.

samples. An LC chromatogram is shown (**Figure 2**) of 0.075 μCi of ^{14}C-labeled erythromycin and 3.3 μg of unlabeled EA, each injected on the same cyanopropyl column with similar conditions. Unlabeled EA was also assayed for purity by LC-ESI/MS using similar conditions (MS data not shown). A purity of 88.9% was indicated for ^{14}C-labeled EA with two unknown peaks detected at 2.4 and 4.2 min related to 1.4 and 9.7%, respectively. The purities of EA by LC-ED and ESI-MS were 91.6 and 98.1%, respectively. It was noted that by LC-ED, impurity peaks were detected at 17.4, 20.4, and 27.2 min, which were not seen in the ^{14}C-labeled EA standard. These three minor impurities were also detected by ESI-MS.

General Aspects of Solvent Extraction and Cleanup of Edible Salmon Tissue. In our laboratory, previous tissue analysis was centered on the recovery of EA from chicken liver followed by LC-ED or LC-APCI/MS (*11*, *12*). The extraction system used was 20:80 MeCN/aqueous ion pair solution (0.02 M KH_2PO_4, 0.02 M sodium octanesulfonate, pH 4.5). From this previous experience and results of several other researchers (*12*, *14*), the use of an aqueous-based buffer system was chosen as the extractant of choice and had several advantages over those procedures using neat solvents and/or mixtures, including the following: (1) fewer lipids were extracted from the tissue than with MeOH and MeCN mixtures; (2) tissue samples contained fewer coextractants that may interfere or coelute with the EA peak on reversed phase LC; and (3) slow evaporation steps were not required to remove volatile solvents prior to SPE or partition cleanup steps. In the present method, an aqueous 10 mM ammonium formate buffer was used in place of the 10.5 pH Tris buffer (*14*) due to EA breakdown products that occurred in buffers other than those near neutral pH (*15*). The method developed by Dubois et al. (*14*) is well recognized for introducing the use of the Waters Oasis HLB polymeric-based reversed phase SPE cartridge for cleanup of macrolide antibiotics in biological matrices (i.e., muscle, kidney, liver, milk, and eggs). We have found that a 60 mg HLB cartridge, rather than 200 mg of solid support, was sufficient for EA purification. Additionally, the amount of MeOH (100%) was reduced from 5 to 1 mL for quantitative elution of the EA. Experiments were performed with [^{14}C]EA-fortified salmon to show that successive elution with 2 mL of water and 3 mL of 20% MeOH/water removed none of the EA from the HLB SPE cartridge. Addition of the 1 mL of MeOH quantitatively eluted [^{14}C]EA. To reduce the probability of breakdown of EA, reported by others (*9*, *12*), the salmon extract was eluted from the HLB cartridge directly into a 12 mL separatory funnel containing 6 mL of 2% NH_4OH/water and partitioned immediately with (alkaline preextracted) $MeCl_2$ (3 × 1 mL). This is the most critical step of the cleanup procedure. The 2% NH_4OH was used in place of pH-adjusted phosphate buffers and hydroxide salts, which could cause breakdown of EA and to reduce problems associated with salts and their impurities contaminating the LC columns and MS equipment. A major advantage of the combined aqueous formate buffer extraction, HLB elution, and 2% NH_4OH partition cleanup is the elimination of the need for protein precipitation and lipid removal steps, which are time-consuming and labor intensive and can lead to lower EA recovery from salmon tissue.

Table 1. Erythromycin A Recoveries in Fortified Salmon Tissue (5 g) by LC-ED and LC-ESI/MS

	LC-ED		LC-ESI/MS	
amount of EA added (ng)	amount of EA found (ng)	EA % recovery, mean ± SD (CV)	amount of EA found (ng)	EA % recovery, mean ± SD (CV)
control[a] (0)	15.7 ± 8.1		3.65 ± 1.85	
250	132.3 152.0 120.3	53.9 ± 6.4 (11.8)	191.3 206.8 119.5	69.0 ± 18.6 (27.0)
500	369.0 332.5 318.5	68.0 ± 5.2 (7.6)	368.5 392.0 315.0	71.7 ± 7.9 (11.0)
1000	661.0 769.0 626.0	68.5 ± 7.5 (10.9)	778.0 806.0 759.0	78.1 ± 2.4 (3.1)
2000 (day 1)	1198 1240 1284	62.0 ± 2.2 (3.5)	1580 1728 1666	82.9 ± 3.7 (4.5)
2000 (day 13)	1456 1328 1170	65.9 ± 7.2 (10.9)	1492 1612 1454	75.8 ± 3.9 (5.1)

[a] Control salmon data are reported as background levels in ppb ± SD (n = 5).

Salmon Analysis for Erythromycin A. For LC-ED analysis, isocratic elution was necessary to accommodate the electrochemical detector (+0.9 V) need for constant buffer flow to maintain stable detection potential and responses. Interferences from salmon matrix components were minimal, and a 10−15 min analysis was sufficient. LC-ED analyses of EA standards at 50, 100, 200, and 400 ppb were subjected to linear regression, producing a linear curve with r^2 = 0.9993. The MS analysis was significantly longer due to the LC solvent program used.

Fortified controls were analyzed at 0, 50, 100, 200, and 400 ppb levels. EA recoveries by LC-ED and LC-ESI/MS were obtained and are presented in **Table 1**. Data from LC-ED analysis of 5 g of homogenized control salmon tissue (n = 5) were also used to calculate background response levels of 3.14 ± 1.61 ppb. The limit of detection (LOD) (3σ) and limit of quantitation (LOQ) (10σ) were calculated to be 5 and 16 ppb, respectively. The effects of lability of EA in aqueous buffers and to some extent in methanol solutions lead to some losses of the compound during the cartridge cleanup, alkaline partition, and evaporation steps. EA standards at 50−400 ppb were subjected to the cleanup procedure, and recoveries were 69.1 ± 8.2% by LC-ED (n = 5). These method standards were analyzed concurrently with each set of salmon extracts and were

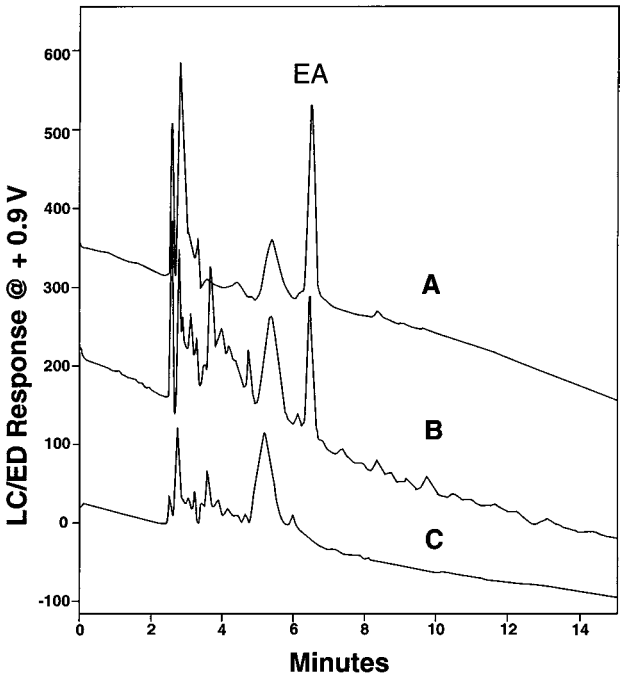

Figure 3. HPLC-ED chromatograms of 20 μL injections (25 mg of equiv/μL of salmon) of (A) a 50 ppb EA standard, (B) a 50 ppb fortified salmon, and (C) control salmon (5 g) extract.

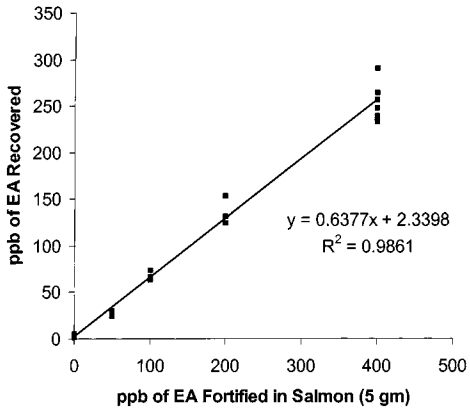

Figure 4. Linear regression plot of parts per billion of EA recovered from fortified edible salmon tissue at 0, 50, 100, 200, and 400 ppb analyzed by LC-ED.

used to calculate the extraction efficiencies of EA from salmon tissue shown in **Table 1**.

Typical LC-ED chromatograms of a control salmon (5 g) extract, a 50 ppb fortified salmon, and a 50 ppb EA standard are shown in **Figure 3**. The intraday 50, 100, 200, and 400 ppb recoveries of EA ($n = 3$) were consistent at 53.9 ± 6.4, 68.0 ± 5.2, 68.5 ± 7.5, and 62.0 ± 2.2%, respectively. A plot (**Figure 4**) of EA recovered from fortified edible salmon tissue at 0, 50, 100, 200, and 400 ppb indicates a linear relationship for EA recoveries at the five levels with $r^2 = 0.9861$ and a slope of 0.6377, indicating a mean recovery of 63.8% for all levels. The 400 ppb between-day relative percent difference in mean recoveries by LC-ED and LC-ESI/MS were 3.9 and 7.1%, respectively. This indicates acceptable interday variability by either method.

The 400 ppb interday mean recoveries were 64.0 ± 5.2% by LC-ED and 79.4 ± 5.2% by LC-ESI/MS for $n = 6$. An indication of increased recovery by LC-MS compared to LC-ED was also noted by Blasco et al. (*16*) in their analysis of various fungicides in fruits and vegetables. They attributed this

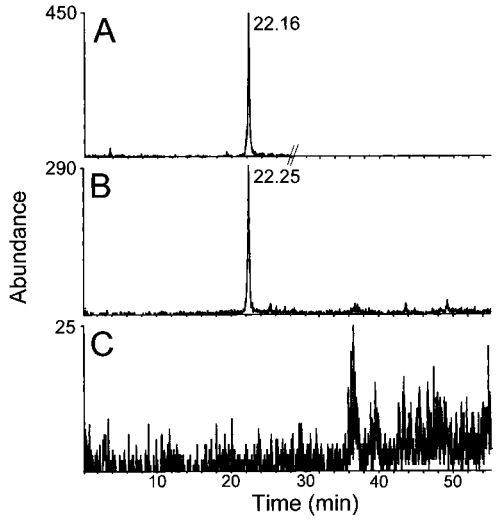

Figure 5. LC-ESI/MS chromatograms showing the TIC and mass spectra scanned from *m/z* 710 to 740 for (A) a 50 ppb EA standard, (B) a 50 ppb fortified salmon tissue (5 g), and (C) a salmon control tissue (5 g).

increase to MS signal enhancement due to the presence of sample matrix by LC-ESI/MS. In the present study, the MS signal enhancement may be due to less ion suppression in the presence of the matrix during LC-MS analysis than by LC-ED, where there may be signal suppression due to fouling of the electrode.

The LC-ESI/MS chromatograms in **Figure 5** show the total ion chromatograms (TIC) of limited scan range from *m/z* 710 to 740 for a 50 ppb EA standard, which was stopped at 28 min (A), for a salmon tissue (5 gm) fortified at 50 ppb (B), and for a salmon control tissue (5 g) (C). Also in **Figure 5**, (B) shows that no major EA degradation products were generated in the preparatino of the 50 ppb fortified salmon sample and (C) indicates no interfering ions evident in the 5 g salmon control sample. To further illustrate that EA degradation had not occurred during the sample preparation, **Figure 6** shows the TIC and mass chromatograms of *m/z* 734.9, 720.8, and 716.6 for a 400 ppb fortified salmon sample. Minor impurities that were seen at the same level for the EA standard probably include erythromycin C at 18.12 min, demethyl-EA at 19.63 min, and anhydro-EA at 23.40 min; they represent 1.03, 0.5, and 0.15% of the sample, respectively. The minor ion for EA at *m/z* 716.6 appears at 20.40 min. The only peak that could have been due to EA degradation during sample preparation was probably pseudo-EA enol ether at 26.21 min, which was only 0.2% of the sample.

The four major CID ions detected by LC-ESI/MS were at *m/z* 734.9, 576.6, 558.6, and 158.2 (base peak) having respective relative ion abundances (percent) of 29.5 ± 1.8, 32.6 ± 2.0, 8.0 ± 0.7, and 100.0 ± 0 (by definition) for EA in salmon fortified at 50, 100, 200, and 400 ppb. For LC-ESI/MS confirmation, **Table 2** shows the ion abundance ratios calculated from the CID data for three fragment ions relative to the protonated molecule. The relative percent differences between the EA standards and fortified samples of the ion abundance ratios of *m/z* 576.6, 558.6, and 158.2 relative to the protonated molecule at *m/z* 734.9 were 1.8, 12.5, and 3.3%. Therefore, confirmation for all four ions was possible for EA at all fortification levels (50−400 ppb).

Conclusions. A rugged and reliable method for the analysis of EA in salmon has been developed. Using the present method, LC-ED analysis of EA standards from 50 to 400 ppb resulted in a linear response. The LC-ED calculated LOD (3σ) and LOQ

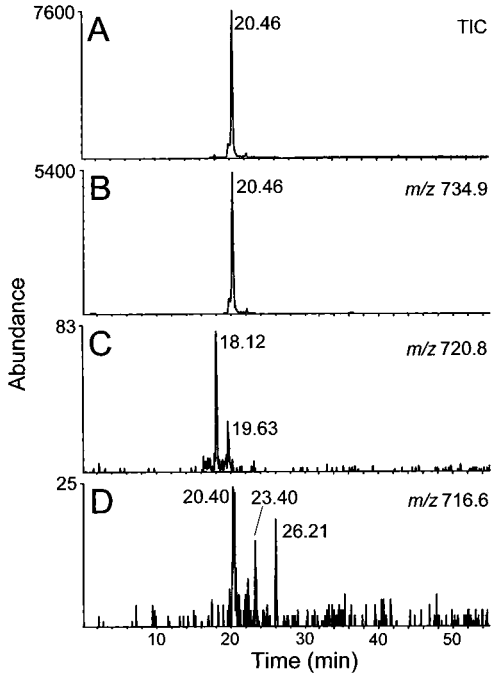

Figure 6. LC-ESI/MS chromatograms for a 400 ppb EA-fortified salmon tissue showing (A) the TIC and mass chromatograms of (B) *m/z* 734.9, (C) *m/z* 720.8, and (D) *m/z* 716.6.

Table 2. Ion Abundance Ratios[a] for Authentic Standards and Salmon Tissue Fortified with 50, 100, 200, and 400 ppb Levels of Erythromycin A Using LC-ESI/MS

	ion abundance ratios			
sample	*m/z* 734.9	*m/z* 576.6	*m/z* 558.6	*m/z* 158.2
Erythromycin Standards				
50 ppb	1.00	1.13	0.23	3.75
100 ppb	1.00	1.03	0.24	3.18
200 ppb	1.00	1.08	0.24	3.23
400 ppb	1.00	1.13	0.26	3.05
mean ± SD	1.00 ± 0.00	1.09 ± 0.05	0.24 ± 0.01	3.30 ± 0.31
Fortified Salmon Tissue				
50 ppb	1.00	1.07	0.31	3.56
100 ppb	1.00	1.12	0.26	3.36
200 ppb	1.00	1.15	0.26	3.56
400 ppb	1.00	1.09	0.25	3.14
mean ± SD	1.00 ± 0.00	1.11 ± 0.04	0.27 ± 0.03	3.41 ± 0.20
% difference[b]	0.0	1.8	12.5	3.3

[a] The abundance ratio data for the four major confirmatory ions were ratioed with respect to the 734.9 *m/z* peak (the MH^+ parent ion of EA). [b] Percent difference between the ion abundance ratios of the EA standard and the fortified salmon tissue samples.

(10σ) were 5 and 16 ppb, respectively. When differences in background interferences for a variety of salmonids are taken into account, the LOQ for EA by LC-ED should remain lower than 50 ppb or at less than half the anticipated CVM tolerance for EA residues in fish. LC-ESI/MS is more sensitive, and the response of EA was linear in the range of 12.5−400 ppb. However, for laboratories not equipped with expensive LC-MS instrumentation, the LC-ED analysis is a rapid, accurate, and low-cost alternative procedure for quantitation of EA in salmon and may have application to other fish species.

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