Determination of Emamectin Residues in the Tissues of Atlantic Salmon (*Salmo salar* L.) Using HPLC with Fluorescence Detection

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An accurate, reliable, and reproducible assay for the determination of residual concentrations of emamectin B_{1a} in muscle, skin, and intact muscle/skin in natural proportions from Atlantic salmon treated with SCH 58854 (emamectin benzoate) is described. The determinative method was developed and validated using fortified control tissues at five levels over a range of 50−800 ng/g as well as tissues containing incurred levels in the same range. Incurred tissues were obtained from a metabolism study of [^{3}H]emamectin benzoate in Atlantic salmon. The assay employs processing of a tissue ethyl acetate extract on a propylsulfonic acid solid phase extraction cartridge, followed by derivatization with trifluoroacetic anhydride in the presence of *N*-methylimidazole. Following separation using reversed phase HPLC, the amount of derivatized emamectin B_{1a} is determined by fluorescence detection. The theoretical limits of detection were determined from the analysis of control tissue matrices to be 2.6, 3.3, and 3.8 ng/g as emamectin B_{1a} for muscle, skin, and intact muscle/skin, respectively. Likewise, the theoretical limits of quantitation (LOQ) were determined to be 6.9, 8.1, and 9.5 ng/g as emamectin B_{1a} for muscle, skin, and intact muscle/skin, respectively. The lowest fortification level used for method validation was 50 ng/g, which served as the effective LOQ for the method. The overall percent recoveries (±% CV) were 94.4 ± 6.89% (n = 25) for muscle, 88.4 ± 5.35% (n = 25) for skin, and 88.0 ± 3.73% for intact muscle/skin (n = 25). Accuracy, precision, linearity, selectivity, and ruggedness were demonstrated. The structure of the final fluorescent derivative of emamectin B_{1a} free base was identified by ESI(+)/LC-MS. The frozen storage stability of [^{3}H]emamectin B_{1a} in tissues with incurred residues was demonstrated for ∼15 months by radiometric analysis and for an additional ∼13 months by fluorometric analysis for a total of ∼28 months.

Keywords: *SCH 58854; avermectin; MK-244; emamectin; emamectin benzoate; emamectin B_{1a}; salmon; analytical method*

INTRODUCTION

Avermectins are a class of natural products consisting of a disaccharide linked to a pentacyclic 16-membered ring, which are produced by the soil microorganism *Streptomyces avermilitis*. Avermectin B_1 (abamectin), consisting of a mixture of the natural avermectins B_{1a} and B_{1b}, is a potent broad spectrum acaricide/insecticide (*1*).

Emamectin benzoate [SCH 58854; 4″-deoxy-4″-(epi-methylamino)avermectin B_1 benzoate salt] is a semi-synthetic avermectin (*2*) registered for use to control lepidopterous pests on vegetable crops in the United States and Japan (*3*). It is also highly effective for the treatment and control of sea lice infestations on Atlantic salmon, *Salmo salar* L. (*4*−*8*) and is registered for use in the United Kingdom, Chile, Ireland, Iceland, Finland, Faroe Islands, Spain, and Norway. The compound, also known as MK-244, is derived through chemical modification of abamectin; an *epi*-aminomethyl (−$NHCH_3$) group is substituted for a hydroxyl (−OH) group at the 4″-position (Figure 1). Similar to abamectin, it is a mixture of two homologues designated B_{1a} and B_{1b}, which differ by one methylene (CH_2) unit on the C-25 side chain, wherein B_{1a} contains a *sec*-butyl group and

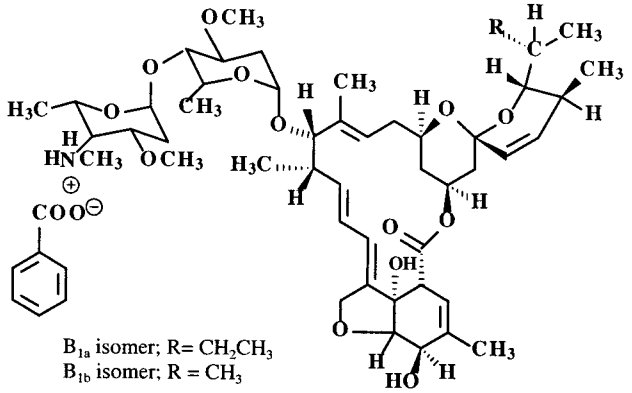

Figure 1. Structure of emamectin benzoate.

B_{1b} contains an isopropyl group. By specification it consists of at least 90% of B_{1a} benzoate and not more than 10% of B_{1b} benzoate. The emamectin homologues are each of ∼900 Da. Emamectin benzoate is formulated in a 0.2% feed premix (SLICE) and administered to fish in medicated feed at a dose rate of 50 μg/kg/day for 7 consecutive days.

A metabolism study, conducted in Atlantic salmon treated by dietary administration of a nominal dose of 50 μg of [^{3}H]SCH 58854/kg/day for 7 consecutive days, indicated that emamectin B_{1a} was the major residue

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component (>80%) in edible tissues (*9*). Due to the extremely low tissue residue levels resulting from the therapeutic dose and the homologue ratio, it was concluded that tissue concentrations of emamectin B_{1b} would be insignificant. Consequently, emamectin B_{1a} (free base) has been established as the marker residue in edible tissues of Atlantic salmon for monitoring of total emamectin benzoate tissue residues in marketed fish. The maximum residue limit (MRL) for emamectin B_{1a} in salmon muscle with skin attached was set at 100 ng/g by the European Medicines Evaluation Agency Committee for Veterinary Medicinal Products (CVMP; *10*). Edible tissues in Atlantic salmon are separate muscle and separate skin (Canada) and intact skin/muscle (all other countries). Quantitative methods for SCH 58854 in lettuce and celery (*11*) or in sea water and fresh water (*12*) using derivatization/fluorescence HPLC have been previously reported.

To determine the emamectin marker residue, emamectin B_{1a}, in Atlantic salmon tissues, a rapid and accurate HPLC method employing fluorescence detection was developed and is presented in this paper.

MATERIALS AND METHODS

Chemicals, Materials, and Solvents. The analytical reference standard of emamectin benzoate used in the study was provided by Schering-Plough Research Institute (SPRI), Lafayette, NJ. Demethyl (*N*-demethyl)-SCH 58854 (4″-deoxy-4″-epiaminoavermectin B_1 benzoate) and florfenicol were also provided by SPRI. Diflubenzuron, pyrethrum, cypermethrin, dichlorvos, trichlorfon, and azamethiphos were purchased from Chemservice, West Chester, PA. Oxytetracycline dihydrate, oxolinic acid, sulfadiazine, trimethoprim, flumequin, and tetracycline free base were purchased from Sigma, St. Louis, MO. Ivermectin and teflubenzuron were purchased from Sigma-Aldrich, St. Louis, MO. All solvents used were of HPLC or analytical grade. They were obtained from the following suppliers: Acetonitrile (ACN), ethyl acetate (EtOAc), and methanol (MeOH) were obtained from EM Science, Gibbstown, NJ. Ammonium acetate, ammonium hydroxide (28−30%) and phosphoric acid were obtained from J. T. Baker, Phillipsburg, NJ. Trifluoroacetic anhydride (TFAA) (99+% purity) and *N*-methylimidazole (NMIM; 99+% purity) were purchased from Aldrich Chemical Co., Milwaukee, WI. Water was purified on site using a Barnstead NanoPure II water purification system.

^{3}H--Labeled liquid scintillation cocktail for the Harvey Biological Oxidizer was obtained from R. J. Harvey Instrument Corp., Hillsdale, NJ, and Ready Value liquid scintillation cocktail was purchased from Beckman Instruments Inc, Fullerton, CA. Propanesulfonic acid (PRS) cation exchange solid phase extraction (SPE) cartridges were purchased from Varian, Harbor City, CA. Alternate cation exchange cartridges, LC-SCX,were purchased from Supelco Co. Inc., Bellefonte, PA.

Test System. Fish tissue samples from [^{3}H]emamectin benzoate-treated Atlantic salmon containing incurred residues, obtained from a previously conducted metabolism study (*9*), were employed in this study. Atlantic salmon, weighing ∼1000 g and maintained in tanks of artificial sea water, were given emamectin benzoate containing [^{3}H]emamectin B_{1a} formulated in their feed to provide a nominal dose of ∼50 μg/kg of live weight/day for 7 consecutive days. Groups of 10 dosed fish were euthanized at 3 h to 90 days post-withdrawal of medicated feed. Tissues containing incurred residues from 3- and 72-h withdrawal times were used for this study. Control muscle and control intact skin/muscle samples obtained from the metabolism study were also utilized. In addition, control muscle and control intact skin/muscle samples were obtained from five different commercial sources, whereas control skin samples were obtained from six different commercial sources. Each salmon was scaled with a knife and divided into two fillets. From one fillet, the skin was peeled from the muscle and any remaining muscle tissue was removed from the skin; this provided muscle and skin samples. The other fillet provided intact skin/muscle samples. Collected tissue samples (muscle, skin, and intact skin/muscle) were stored frozen until further processing. Frozen muscle and intact skin/muscle samples were partially thawed and diced into smaller pieces. Diced samples were blended in either a Hamilton Beach food processor or a Cuisinart food processor. Separate skin samples were also partially thawed and minced into small pieces by hand using scissors. The freezer storage stability of [^{3}H]-emamectin B_{1a} in tissues with incurred residues was demonstrated for ∼15 months by radiometric analysis and for an additional 13 months by fluorometric analysis for a total of ∼28 months (*13*).

Preparation of Standards. Emamectin benzoate standard solutions were prepared in acetonitrile and stored at or below −10 °C, at which temperature they are known to be stable for at least 6 months. Working standards were prepared by serial dilution of a 100 μg/mL stock solution of emamectin B_{1a} (corrected for content of emamectin B_{1a} free base in emamectin benzoate). The standard curve was prepared using an aliquot of each working standard solution.

Preparation of Untreated Control Samples. For each analysis, unfortified tissue samples from a control (nontreated Atlantic salmon) served as matrix blanks and were processed and analyzed for interference at the retention time of emamectin B_{1a}.

Preparation of Fortified Samples. Fortified tissues were prepared by adding working standards to control tissues at emamectin B_{1a} concentrations of 50, 100, 200, 400, and 800 ng/g. Fortified tissues were extracted along with incurred tissues as described below.

Sample Extraction Procedure. A 2.5 ± 0.05 g of a homogenized fish tissue sample was mixed with 1 mL of ACN, 4 mL of H_2O, and 10 mL of EtOAc in a 50-mL polypropylene centrifuge tube. For the fortified samples, 1 mL of an appropriate working standard solution was added instead of ACN. The tissue mixture was homogenized in each tube using a Polytron homogenizer (Brinkmann Instruments, Littau, Switzerland). The Polytron probe was rinsed by blending in a second centrifuge tube containing 10 mL of EtOAc. For skin samples, a mixture of 1 mL of H_2O and 10 mL of EtOAc was used for the rinsing step. The rinse was added to the first tube containing the homogenized tissue. An additional 10 mL of EtOAc was added to the second 50-mL centrifuge tube, and the probe was further rinsed by blending with EtOAc. The rinse was retained for the second extraction of each sample. Before the next sample was blended, the probe was rinsed by blending in three successive tubes containing water, ACN, and acetone, and the rinses were discarded. The first tubes containing homogenized tissues were capped and shaken on a wrist-cction shaker for 20 min and centrifuged for 5 min at 3000 rpm at a temperature setting of 2 °C. The upper EtOAc phase was transferred from the centrifuged homogenate in the first tube into a third, clean, 50-mL centrifuge tube. The retained EtOAc rinse from the second probe rinse was poured into the first tube, which contained the extracted tissue pellet, and the pellet was resuspended by manual shaking. The tubes were then shaken again for 15 min in a shaker followed by centrifugation for 5 min at 3000 rpm at 2 °C. The EtOAc phase was then transferred into the third tube containing the EtOAc phase from the first extraction. Each tube contained ∼30 mL of EtOAc extract. The entire combined EtOAc extract was loaded onto a preconditioned PRS cartridge at a ∼1−2 mL/min flow rate. The PRS cartridge was preconditioned with 5-mL rinses of each of the following solutions in order: 1% (w/v)NH_4OAc in MeOH, MeOH, 1% (v/v) phosphoric acid in MeOH, Nanopure water, methanol, and EtOAc. A methanol wash was inserted between washes with 1% (w/v) NH_4OAc in MeOH and 1% (v/v) phosphoric acid in MeOH to remove any residual NH_4OAc prior to the wash with 1% (v/v) phosphoric acid in MeOH. Any residual NH_4OAc could form an insoluble salt with phosphoric acid, which would restrict the flow through the cartridge. The above EtOAc extract was then

loaded onto the cartridge. The PRS cartridge was rinsed with 2 mL of EtOAc followed by 5 mL of MeOH. After the MeOH rinse, the PRS cartridge was dried using vacuum prior to elution. The retained residues were then eluted with 5 mL of 1% NH_4OAc in MeOH into a graduated 15-mL polypropylene centrifuge tube. When the eluate flow stopped, the residual solvent was collected by increasing the vacuum. The total volume was adjusted to 5 or 5.5 mL with MeOH using the mark on the centrifuge tube. The adjusted total volume was recorded. The tube was then stoppered and vortexed for 1 min using a multitube vortexer (VWR, West Chester, PA). One-fifth of the methanolic eluate (1 mL from a total of 5 mL or 1.1 mL from a total of 5.5 mL) was transferred into another 15-mL centrifuge tube, and the remaining sample was stored in a freezer. This was a stopping point in the method. If the next step was not done immediately, the subsample was stored in a freezer as well.

Four milliliters of H_2O was added to each tube containing the subsample of the methanolic eluate followed by the addition of 2 drops of concentrated NH_4OH and 5 mL of EtOAc. The mixture was vortexed for 1 min using a multitube vortexer and centrifuged for 3 min at 2 °C at 3000 rpm. The EtOAC phase was transferred into a second tube; and an additional 5 mL of EtOAc was added to the first tube, and the tubes were vortexed for 1 min and centrifuged for 3 min at 2 °C at 3000 rpm. The EtOAc phase was transferred into a second tube containing the first EtOAc extract. The pooled EtOAc extract was evaporated to complete dryness under nitrogen using a turbo evaporator (Zymark, Hopkinton, MA) at 15 psi for ~10 min and then at 25 psi for ~5−10 min in a water bath set at 70 °C. One milliliter of ACN was added to each tube, and the tubes were capped and vortexed for 1 min using a multitube vortexer. Tubes were then placed in a beaker containing hot tap water (~50 °C) and sonicated for 10 min. A flow diagram of the sample extraction procedure is presented in Figure 2.

Derivatization. Following sonication of the sample in 1 mL of ACN, 0.1 mL of NMIM was added to each tube and vortexed for 1 min using a multitube vortexer. The contents were again sonicated in hot tap water for 5 min. Then 0.3 mL of a reagent mixture, prepared by mixing TFAA and ACN in a ratio of 1:2 (v/v) and vortexing the mixture immediately before use, was added to each tube. The tubes were vortexed for 1 min and sonicated for 20 min in hot tap water. Upon derivatization, the contents were diluted to 5 mL by adding 0.6 mL of H_2O and 3 mL of ACN and vortexed using a multitube vortexer. A subsample (~200 μL) was transferred into an HPLC vial, and the remainder was stored in a freezer. The sample was analyzed by reversed-phase HPLC in conjunction with fluorescence detection.

Preparation of Calibration Curve (Absolute). An absolute calibration curve was prepared containing six points in the concentration range of 2−100 ng/mL emamectin B_{1a} (equivalent to 20−1000 ng/g in tissue). The calibration curve standard solutions were prepared by volumetrically transferring 1 mL each of the working standard solutions to separate 15-mL tubes followed by the addition of 0.1 mL of NMIM. The mixture was then subjected to the same reaction conditions as used for the sample extracts (refer to Derivatization).

HPLC Conditions. Two HPLC conditions, designated I and II, were used for the study. The HPLC system for condition I was used for fluorometric analysis and consisted of the following instruments: Waters 600E (Milford, MA) solvent delivery system, Waters 474 fluorescence detector, Waters 717 autosampler, and Waters Millennium 2020 Chromatography Manager. Zorbax Rx-C8 (4.6 mm i.d. × 250 mm, 5-μm particle size) from MacMod, Chadds Ford, PA, was used as an analytical column preceded by a Brownlee RP-18 Newguard guard column (3.2 mm i.d. × 15 mm) (Perkin-Elmer, Norwalk, CT). The mobile phase used was Nanopure water/methanol (5:95) at a flow rate of 1 mL/min. The fluorescence detector parameters were set as follows: excitation wavelength at 365 nm and emission wavelength at 470 nm; gain at 100×; bandwidth at 18 nm; response time at standard; filter at RC filter; and flow cell size, 16 μL. An injection volume of 10 μL and a total run time of 15 min were maintained for all analyses. HPLC condition II was used for the radiometric analyses. The HPLC systems for condition II consisted of the following conditions: Waters 616 solvent delivery system, Waters 490 multiwavelength UV detector set at 246 nm, Waters 717 autosampler. A Primesphere 5 C_{18}-HC 110A, 4.6 mm i.d. × 150 mm, 5 μm, from Phenomenex was used as an analytical column preceded by a Phenomenex Primesphere guard column (4.6 mm i.d. × 30 mm, 5 μm). The gradient condition used was as follows: 60% A, 25 min to 30% A, 1 min to 100% B (10 min), 2 min to initial. The mobile phases used were (A) 0.1% H_3PO_4 (v/v) and (B) acetonitrile at a flow rate of 1 mL/min. The column temperature was maintained at 35 °C using a column heater (Eppendorf, Westbury, NY) throughout the analyses. Radioactivity in the tissue extracts was monitored by a combined technique consisting of fraction collection followed by LSC. HPLC eluate fractions from each sample analysis were collected every 30 s, and the radioactivity in the entire 30-s fraction was determined by liquid scintillation counting (LSC). Liquid scintillation counters from Beckman Instruments, Inc. (models LS 5000 and LS 3801), were used for LSC assay.

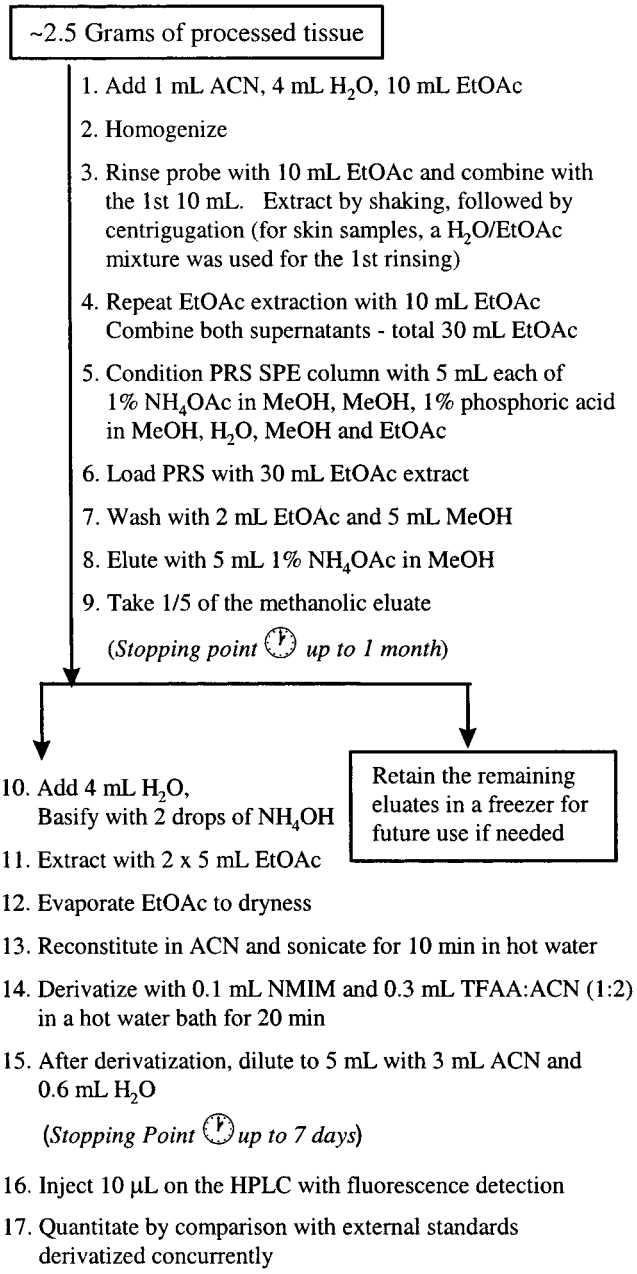

Figure 2. Flow diagram of the procedure to determine emamectin benzoate marker residue in fish tissues.

LC-MS Conditions. The LC-MS system used consisted of the following instruments: Finnigan MAT LCQ ion trap mass spectrometer, Varian 9012 solvent pump, Waters 474 fluorescence detector, Waters 717 autosampler, and Waters Explore data system. The same columns and HPLC conditions (condition I) as described under HPLC Conditions were used but at a flow rate of 0.8 mL/min. Electrospray ionization (ESI) in the positive ion mode was used. A daughter ion scan was obtained at 20−40% of 35 eV with collision gas of N_2. The structure of the fluorescent derivative of emamectin B_{1a} was confirmed by ESI(+)/LC-MS in conjunction with fluorescence detection.

Quantitation. A linear regression calibration curve was constructed using external standard calibration [emamectin B_{1a} peak response as *y* coordinate and emamectin B_{1a} standard concentration (ng/mL) as the *x* coordinate] for the fluorometric analysis. Six external standards were injected before and after the samples to ensure the system suitability and sample stability. A typical analysis set during the method validation was 18 samples plus 3 controls and 15 method recovery (fortified) samples. Duplicate injections were made from each sample extract, and the system stability was maintained throughout the 36 injections made between the analyses of six external standards. Using the slope (*S*) and intercept (*I*) from the linear regression equation, the unknown sample concentration of emamectin B_{1a} (derivative) in the final sample extract was calculated as follows:

$$\text{concentration (ng/mL) in the final sample extract } (x) = [\text{peak response } (y) - I]/S$$

The resulting sample extract concentration was converted to nanograms per gram (nanograms of emamectin B_{1a} per gram of tissue) by the following step calculation; it was first multiplied by the final milliliters of take-up volume by 5 to take into account that only one-fifth of the methanolic elute was used for the further workup. The resulting value was then divided by the sample weight in grams. The formula is expressed in the following way:

$$\text{concentration (ng/g) in tissue} = (X \times V \times 5)/W$$

where X = concentration in the final sample extract (ng/mL), V = total volume of the final sample extract = 5 mL, 5 = correction for the subsampling of one-fifth of the methanolic eluate, and W = total tissue weight (g) = 2.5 g.

Recovery was determined by dividing the calculated nanograms per gram in the fortified samples by the theoretical concentration (nanograms of emamectin B_{1a} added per gram of control tissue) and expressing the result as a percentage (× 100%).

Notes to Analysts and Safety Considerations. Care should be taken to wear proper eye and hand protection, and work with TFAA should be done in a properly vented laboratory hood. The use of a respirator during the weighing of emamectin benzoate standard is recommended.

RESULTS AND DISCUSSION

Accuracy (Recovery). Percent recoveries generated during the validation of the method are presented in Table 1. The method was successfully validated for the three fish tissues as measured by average recoveries ranging from 86.2 to 96.4% for fortification levels ranging from 50 to 800 ng/g. The average recoveries of emamectin B_{1a} for muscle, intact skin/muscle, and skin tissue over all fortification levels were 94.4 ± 6.89% (N = 25), 88.0 ± 3.73% (N = 25), and 88.4 ± 5.35% (N = 25), respectively. The results of the intraday assay (N = 15) were very similar to the interday assay results for each tissue, further demonstrating the accuracy of the method (Table 2).

Precision. The interday (intermediate) precision for the method for all fortification levels ranged between 4 and 10% CV in muscle, between 2 and 5% CV in intact skin/muscle, and between 1 and 4% CV in skin (Table 1). The intraday precision (repeatability) for the method for all fortification levels ranged between 1 and 5% CV in muscle, between 0 and 3% CV in intact skin/muscle, and between 1 and 4% CV in skin (Table 2).

Table 1. Emamectin B_{1a} Salmon Tissue Method Validation Results

fortification level (ng/g)	av % recovery	% CV	*N*
	Muscle		
50	96.4	9.71	5
100	95.4	8.01	5
200	93.4	6.14	5
400	94.3	6.41	5
800	92.3	4.19	5
overall	94.4	6.89	25
	Intact Skin/Muscle		
50	89.8	4.16	5
100	88.1	4.78	5
200	89.6	2.14	5
400	86.5	2.79	5
800	86.2	4.76	5
overall	88.0	3.73	25
	Skin		
50	87.9	4.95	5
100	88.2	6.37	5
200	88.5	4.50	5
400	90.6	5.56	5
800	86.8	5.36	5
overall	88.4	5.35	25

Table 2. Emamectin B_{1a} Salmon Tissue Method Validation Results; Summary of Intraday Assay Results

fortification level (ng/g)	av % recovery	% CV	*N*
	Muscle		
50	102.4	4.25	3
100	100.6	2.83	3
200	97.5	1.52	3
400	98.2	3.09	3
800	94.6	1.18	3
overall	98.7	2.57	15
	Intact Skin/Muscle		
50	89.2	2.71	3
100	89.3	1.75	3
200	90.7	1.83	3
400	86.3	2.46	3
800	84.0	0.81	3
overall	87.9	1.91	15
	Skin		
50	87.7	2.31	3
100	90.1	2.78	3
200	89.0	3.57	3
400	91.0	1.72	3
800	87.0	3.04	3
overall	89.0	2.68	15

Linearity and Range. All coefficients of determination of the calibration curve linear regression equations were 0.98 or greater in the range of 2−100 ng/mL (equivalent to 20−1000 ng/g in tissue), demonstrating good linearity of the derivatization and detection system (data not shown).

Ruggedness. Ruggedness tests were performed on the SPE cartridges and the analytical column. A PRS SPE cartridge from a different manufacturer, LC SCX from Supelco, yielded comparable recoveries. Use of a C8 analytical column from another manufacturer, Su-

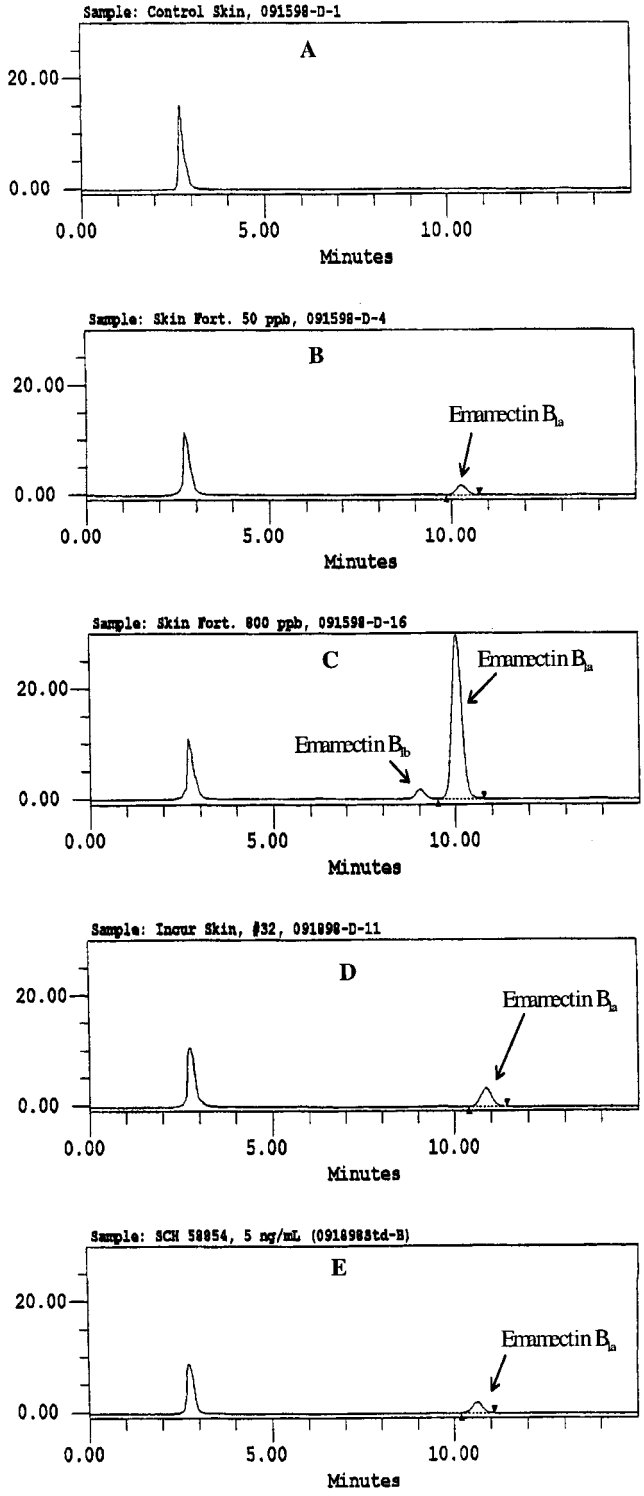

Figure 3. Representative chromatograms—condition I: (A) control skin; (B) fortified skin at 50 ppb; (C) fortified skin at 800 ppb; (D) incurred residue skin at 90 ppb; (E) calibration standard at 5 ng/mL (equivalent to 50 ppb).

pelcosil LC-8-DB by Supelco, also yielded comparable results (data not shown).

Selectivity. Control samples were free from significant interferences. Figure 3 presents typical chromatograms obtained from the use of the method. Specificity of the method was confirmed by assessing the potential for interference of the assay by 16 drugs commonly used in salmon and the major emamectin benzoate metabolite, that is, 4″-deoxy-4″-epiaminoavermectin B_{1a} (*N*-demethylated emamectin B_{1a}; *14*), known in fish. Following 7 consecutive days of dosing of [^{3}H]emamectin benzoate in Atlantic salmon, the main residue component was [^{3}H]emamectin B_{1a} and the major metabolite was *N*-demethylated emamectin B_{1a} (*9*), which also has been observed in rats orally administered radiolabeled emamectin benzoate (*15*). Products analyzed for demonstration of method selectivity were azamethiphos, diflubenzuron, teflubenzuron, pyrethrum, cypermethrin, dichlorvos, trichlorfon, flumequin, tetracycline, oxytetracycline dihydrate, oxolinic acid, sulfadiazine, trimethoprim, florfenicol, ivermectin, emamectin B_{1b}, and desmethyl (*N*-demethyl)-SCH 58854. Excluding emamectin B_{1b}, all compounds evaluated were prepared in solvents at a concentration equivalent to ~1000 ng/g in tissues. Emamectin B_{1b} was a constituent of the emamectin B_1 benzoate standard that would be present at a level of ~50 ng/g when emamectin B_{1a} is at a level of 1000 ng/g, given the approximate molar ratio of ~0.05× emamectin B_{1a}. None of the drugs except for ivermectin, emamectin B_{1b}, and desmethylemamectin benzoate yielded a detectable response by fluorescence detection under the conditions used. Although these three compounds (ivermectin, emamectin B_{1b}, and desmethylemamectin benzoate) formed fluorescent derivatives and yielded detectable responses, none of the derivatives cochromatographed with derivatized emamectin B_{1a}, thus confirming the specificity of the method. A representative chromatogram obtained from the analysis of a mixture of ivermectin B_{1a} and emamectin B_{1a} derivatives is presented in Figure 4. Furthermore, the neutral ivermectin compound would be expected to be separated from emamectin B_{1a} at the PRS SPE cartridge step (Figure 2) and therefore would not be present during derivatization and subsequent HPLC analysis of emamectin B_{1a}. The resolution of the derivatized emamectin homologues is demonstrated in Figure 3C. The derivatized *N*-demethyl metabolite of emamectin B_{1a} eluted ~3 min before derivatized emamectin B_{1a} (data not shown).

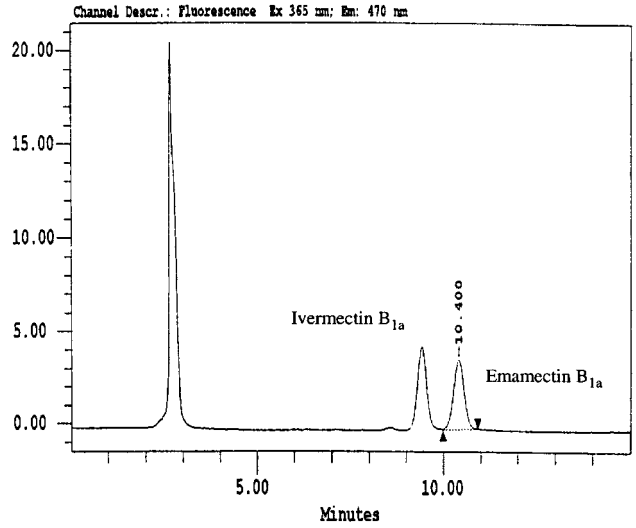

Figure 4. HPLC chromatogram of a mixture of derivatized emamectin B_{1a} and ivermectin—condition I.

Extraction Efficiency. This procedure has been used to analyze muscle, skin, and intact skin/muscle from a metabolism study of [^{3}H]emamectin benzoate in salmon for incurred emamectin B_{1a} residues (*9*). Tissue levels of emamectin B_{1a} in incurred fish samples, obtained by using this determinative HPLC fluorometric method, were comparable (84−98%) to those obtained

Table 3. Overall Summary of Emamectin B_{1a} Free Base Residue Data Determined in Incurred Tissues by Fluorometric versus Radiometric Method

	incurred sample/sacrifice interval[a]							
	muscle		intact skin/muscle		skin			
	3 h	72 h	3 h	72 h	3 h		72 h	
	fish 3	fish 32	fish 5	fish 32	fish 4	fish 9	fish 32	fish 38
emamectin B_{1a}[b] (ng/g)								
mean	49.6	53.8	44.0	57.2	48.0	48.5	89.0	86.3
N	5	5	5	5	3	2	2	3
% CV	5.26	3.33	2.78	2.28	9.08	1.46	1.58	3.72
[^{3}H]emamectin B_{1a}[c] (ng/g)	NA[e]	58	NA	68	NA	NA	91	NA
N	NA	1	NA	1	NA	NA	1	NA
% recovery[d]	NA	92.76	NA	84.12	NA	NA	97.80	NA

[a] Sacrifice interval: 3 or 72 h post-dose. [b] Values were obtained by the determinative method (HPLC/fluorometry). [c] Values were obtained by HPLC/radiometry. [d] Recovery based on the mean ng/g (ppb) by HPLC/fluorometry vs HPLC/radiometry. % Recovery = (ng/g by HPLC/fluorometry)/(ng/g by HPLC/radiometry). [e] NA, not analyzed.

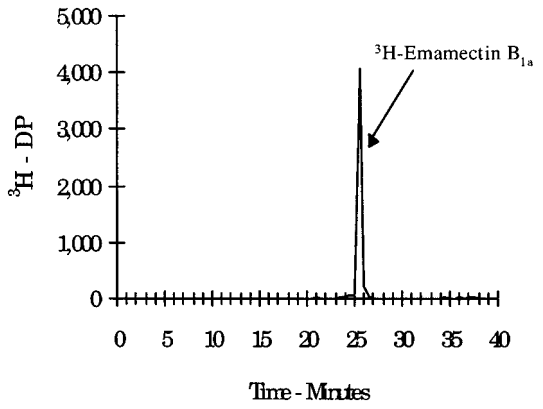

Figure 5. Reconstructed HPLC chromatogram of the EtOAc fraction of skin sample with incurred marker residue—condition II.

by the HPLC radiometric assay (Table 3). A representative reconstructed HPLC chromatogram obtained from the analysis of the EtOAc fraction of a skin sample containing incurred marker residue is presented in Figure 5. Radiovalidation results verified that the extraction procedure outlined here can adequately extract incurred emamectin B_{1a} residues from fish tissues with high efficiency.

Limits of Detection (LOD and Quantitation (LOQ). The theoretical LOD were determined from the analysis of control tissue matrices. Duplicate extractions of each tissue type from six control samples and duplicate injections per each sample extract were made. Residues were quantitated on the basis of manual integration of the baseline for six control samples at the retention time for emamectin B_{1a} quantified against the average response of the lowest concentration calibration standard. The average apparent residue levels found for each control tissue type were 0.8 ng/g with a standard deviation (SD) of 0.61 ng/g for muscle, 1.3 ng/g with an SD of 0.68 ng/g for skin, and 1.4 ng/g with an SD of 0.81 ng/g for intact skin/muscle. The theoretical LOD was calculated as LOD = average + 3 × SD = 2.6, 3.3, and 3.8 ng/g for muscle, skin, and intact skin/muscle, respectively. The theoretical LOQ was calculated as LOQ = average + 10 × SD = 6.9, 8.1, and 9.5 ng/g for muscle, skin, and intact skin/muscle, respectively. The lowest level used for validation of the method was 50 ng/g, which served as the effective LOQ for the method. The validated upper limit of quantitation was 800 ng/g for all three fish tissues.

Assay Timing. The procedure takes ~7 h to prepare 15−18 samples for HPLC analysis, and assay results can be obtained the morning after an overnight HPLC run. A typical sample set for using this method would contain a minimum of 10 unknown samples for residue quantitation, an untreated matrix control, four fortified matrix controls (duplicate samples at low and high levels) for method recovery, and a set of six standards injected before and after the samples. The remaining (four-fifths of the total) samples of methanolic eluates from PRS SPE cartridges can be retained for potential reanalysis. The methanolic eluates were stable for at least 1 month stored at or below −10 °C.

Freeze—Thaw Stability of Emamectin Residues in Tissues. Freeze−thaw stability was evaluated after three freeze/thaw cycles. One sample from each tissue type containing incurred residues was also subjected to three freeze−thaw cycles and analyzed by the determinative method. The results demonstrated the stability of the samples after three freeze−thaw cycles; average recoveries for 50 and 800 ng/g fortified samples were 93.3% (n = 6) and 91.9% (n = 6), respectively. Values obtained from the extraction of three tissues containing incurred residues after three freeze−thaw cycles were compared to the values obtained from the extraction of corresponding incurred residue tissues before the three freeze−thaw cycles; an average recovery of 99.2% (n = 3) was obtained from all three tissues containing incurred residues after three freeze−thaw cycles.

In-Process Stability. The derivatized emamectin B_{1a} residues in the tissue sample extracts prepared for HPLC analyses (HPLC injection solutions) were analyzed both on the day of extraction and after 24 h of storage at room temperature. The stored HPLC injection solutions gave emamectin B_{1a} residue levels ranging from approximately 89 to 100% of the initial assay values for all tissues and fortification levels with overall means of 91.4% for the 50 ng/g samples (n = 6) and 96.9% for the 800 ng/g samples (n = 6). The underivatized emamectin B_{1a} residues in the methanolic eluates from the PRS cartridges from all three tissues fortified at 50 and 800 ng/g were shown to be stable for at least 29 days when stored at or below −10 °C. The stored methanolic eluates gave emamectin B_{1a} residue levels ranging from 94.7 to 105.6% of the initial assay values for all tissues and fortification levels with overall means of 102.5% for the 50 ng/g samples (n = 6) and 98.5% for the 800 ng/g samples (n = 6).

Stock Solution Stability. Stock solutions of underivatized emamectin B_{1a} were freshly prepared, and

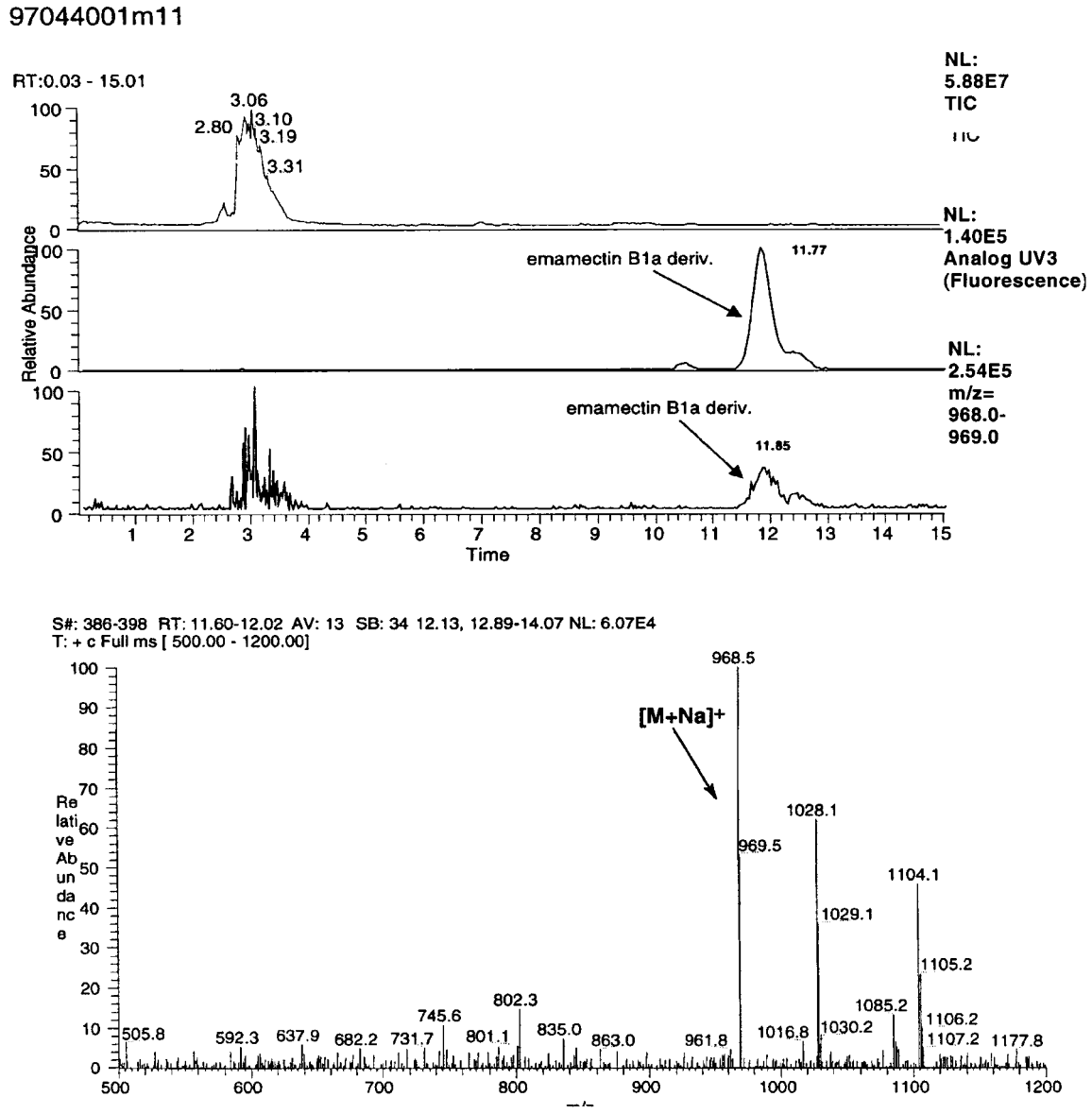

Figure 6. Ion chromatogram and mass spectrum of the derivative of emamectin B_{1a} free base standard.

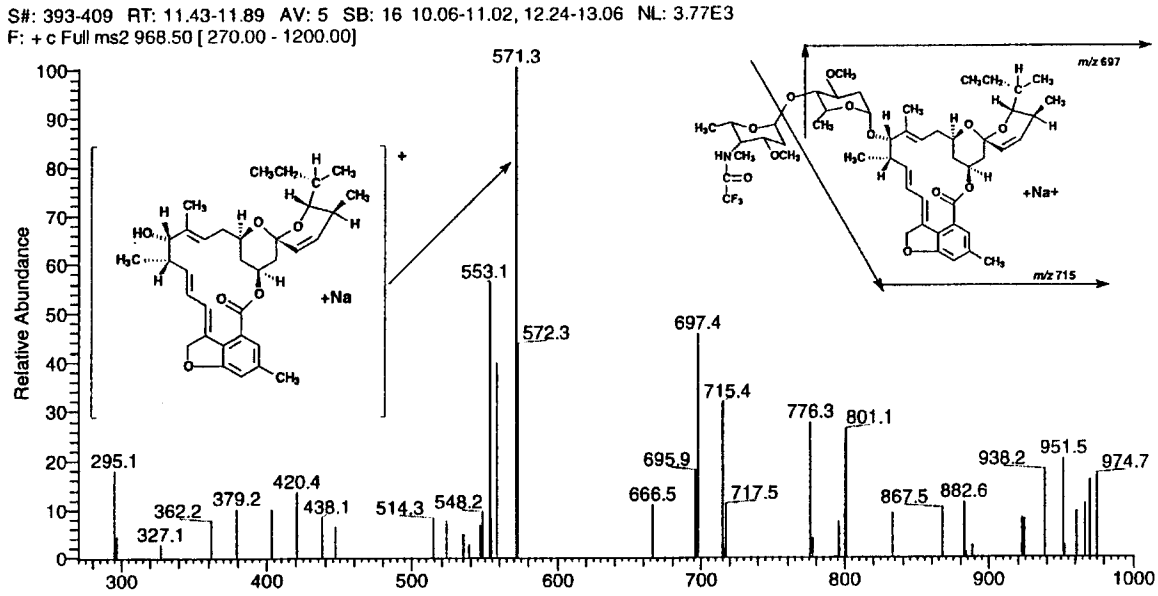

Figure 7. LC-MS/MS analysis of the derivative of emamectin B_{1a} free base standard.

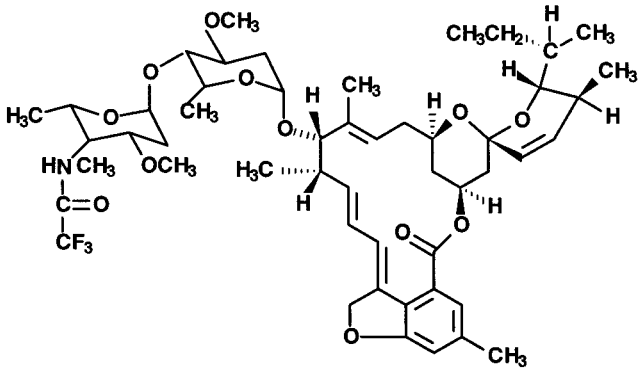

Figure 8. Structure of emamectin B_{1a} fluorescent derivative.

calibration standard solutions were directly prepared with them. The responses obtained from the analyses of these solutions were compared to those of the calibration standards prepared from the stock solutions prepared 6 months previously and stored in a freezer at or below −10 °C. The results, an average recovery of 95% (n = 6), demonstrated that the stock solutions were stable for at least 6 months.

Tissue Freezer Storage Stability. The freezer storage stability of [^{3}H]emamectin B_{1a} in all three tissues containing incurred residues was demonstrated for ∼15 months by radiometric analysis and an additional 13 months by fluorometric analysis for a total of ∼28 months (data not shown).

Identification of the Final Derivative by ESI(+)/LC-MS/MS. The structure of the derivative of emamectin B_{1a} was positively identified as a dehydrated *N*-trifluoroacetylated derivative by LC-MS and LC-MS/MS using electrospray ionization in the positive ion mode. Figure 6 shows the ion chromatogram and mass spectrum of the peak at $t_R \sim 11.8$ min. The peak at *m/z* 968.5 corresponded to $(M + Na)^+$. The peaks at *m/z* 1028 and 1104 were considered to correspond to solvent adducts of the derivative: $[M + Na + HOAc]^+$ for the ion at *m/z* 1028 and $[M + K + 2HOAc]^+$ for the ion at *m/z* 1104. The precursor ion at *m/z* 968.5 was subjected to ESI-(+)/LC-MS/MS, and the mass spectrum is shown in Figure 7. The proposed structures of the major product ions are shown in the same figure. From the LC-MS/MS analysis data, the final derivative was considered to be formed from the loss of two water molecules, and the structure is presented in Figure 8.

Conclusion. The method reported herein is a rapid (24 h) and sensitive procedure for the determination of the marker residue, emamectin B_{1a} (free base), in edible fish tissue: muscle, skin, and intact skin/muscle. The method has theoretical LODs of 2.6, 3.3, and 3.8 ng/g for muscle, skin, and intact skin/muscle, respectively. It has theoretical LOQs of 6.9, 8.1, and 9.5 ng/g for muscle, skin, and intact skin/muscle, respectively. Potential interferences from other drugs used in salmon aquaculture and a major emamectin metabolite were investigated, and no interferences were found. The method was evaluated for ruggedness and found to be robust. The method adequately determined the emamectin benzoate marker residue, emamectin B_{1a} free base, over a concentration range of 50−800 ng/g. Overall fortification recoveries for each tissue over the five fortification levels were 94.4% for muscle, 88.4% for skin, and 88.0% for intact skin/muscle. Emamectin B_{1a} was stable in tissues containing incurred residues stored frozen for at least 28 months and in fortified tissues for at least 13 months.

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